Inhibition of T cell response to native low-density lipoprotein reduces atherosclerosis

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Atherosclerosis is a chronic inflammatory disease in which lipoproteins accumulate, eliciting an inflammatory response in the arterial wall (Hansson, 2005). Adaptive immune responses that engage clonally expanded T cell populations contribute to this process, as do innate immune responses that are mounted by macrophages and other cells. Several studies have suggested that components of low-density lipoprotein (LDL) particles trigger vascular inflammation (Tabas et al., 2007; Hartvigsen et al., 2009).

When LDL particles infiltrate the intima, they are prone to oxidative modification, caused by products of enzymatic activity of myeloperoxidase and lipoxygenases, as well as nonenzymatic oxidative reactions (Hevonoja et al., 2000). As a consequence of oxidation, the double bonds of fatty acid residues in phospholipids, cholesteryl esters, and triglycerides are cleaved, thus generating reactive aldehydes and truncated lipids (Esterbauer et al., 1990). Among the latter, modified phospholipids, such as lyso-phosphatidylcholine and oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (ox-PAPC), induce endothelial cells, macrophages, and B1-type B cells to initiate innate immune responses, effecting adhesion molecule expression, chemokine production, and secretion of natural antibodies containing germline IgM sequences (Leitinger et al., 1997; Binder et al., 2004; Gharavi et al., 2007).

The protein moiety of LDL can also undergo oxidative modification, whereby, for example, malondialdehyde (MDA) adducts, 4-hydroxy-2-nonenal, and other molecular species form on lysyl residues of apolipoprotein B-100 (ApoB100) (Esterbauer et al., 1992). Antibodies are formed against MDA-lysine (Palinski et al., 1990) and

Immune responses to oxidized low-density lipoprotein (oxLDL) are proposed to be important in atherosclerosis. To identify the mechanisms of recognition that govern T cell responses to LDL particles, we generated T cell hybridomas from human ApoB100 transgenic (huB100tg) mice that were immunized with human oxLDL. Surprisingly, none of the hybridomas responded to oxidized LDL, only to native LDL and the purified LDL apolipoprotein ApoB100. However, sera from immunized mice contained IgG antibodies to oxLDL, suggesting that T cell responses to native ApoB100 help B cells making antibodies to oxLDL. ApoB100 responding CD4+ T cell hybridomas were MHC class II–restricted and expressed a single T cell receptor (TCR) variable (V) chain, TRBV31, with different Vα chains. Immunization of huB100tg×Ldlr−/− mice with a TRBV31–derived peptide induced anti–TRBV31 antibodies that blocked T cell recognition of ApoB100. This treatment significantly reduced atherosclerosis by 65%, with a concomitant reduction of macrophage infiltration and MHC class II expression in lesions. In conclusion, CD4+ T cells recognize epitopes on native ApoB100 protein, this response is associated with a limited set of clonotypic TCRs, and blocking TCR-dependent antigen recognition by these T cells protects against atherosclerosis.

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other oxidatively generated epitopes of LDL particles (Ketelhuth et al., 2008). Such antibodies circulate in peripheral blood and are found in atherosclerotic lesions (Ylä-Herttuala et al., 1989; Zhou et al., 1998). In addition, circulating antibodies have been identified that recognize a large number of peptide sequences in native ApoB100 (Fredrikson et al., 2003; Sjögren et al., 2008).

In contrast to the B1 cell–produced natural antibodies against oxidized phospholipids, antibodies to native and MDA-modified ApoB100 are largely IgG molecules (Ylä-Herttuala et al., 1994; Fredrikson et al., 2007; Sjögren et al., 2008), implying that T cells activate isotype switching in the B cell. Although T cells recognizing components of oxidized LDL (oxLDL) have been identified both in hypercholesterolemic mice and among clones from human atherosclerotic lesions (Stemme et al., 1995; Nicoletti et al., 2000; Zhou et al., 2006), the molecular properties of the T cell epitopes are poorly understood because of the biochemical complexity of the LDL particle and the oxidative process. This lack of knowledge has hampered progress in our understanding of the mechanisms that regulate atherosclerosis and, as a result, in the development of immunotherapies.

To study T cell recognition of oxLDL, we created T cell hybridomas from human ApoB100 (huB100tg) transgenic mice that were immunized with oxLDL. These mice generate high levels of ApoB100 that is packaged into humanlike LDL particles (Linton et al., 1993). Similar to other transgenic mice that produce nonmurine proteins, huB100tg mice tolerate the orthologous transgene, neither producing antibodies to it nor developing spontaneous autoimmune disease. Based on previous studies, we had expected that they would respond to immunization by mounting cellular and humoral immune responses toward oxidation–induced epitopes on the LDL particles.

Unexpectedly, oxLDL–immunized huB100tg mice developed T cell responses against native LDL and purified ApoB100. The responding T cells were MHC class II–restricted CD4+ cells and expressed TCRs that contained the variable β domain TRBV31. Abrogation of TRBV31+ T cells attenuated the cellular immune response to ApoB100, and immunization of hyperlipidemic mice against TCR TRBV31 inhibited the development of atherosclerosis. These results strongly suggest that autoimmune T cells that recognize protein epitopes from native ApoB100 promote atherosclerosis.

RESULTS

T cell hybridomas generated after immunization with oxidized LDL recognize native LDL and ApoB100

We used huB100tg mice to characterize the T cell response to oxLDL. These mice express full-length human ApoB100 in the liver and gut and harbor humanized lipoprotein profiles. The huB100tg model permits the use as antigen of human oxLDL, the immunogenic epitopes of which have been carefully characterized. This model makes it possible to study cellular immune responses to human LDL–derived epitopes in a controlled model system and with easy access to large amounts of antigen for purification and characterization.

After subcutaneous primary immunization with human oxLDL in CFA, followed by a booster injection in IFA, LN cells were collected and fused with thymoma cells to generate hybridomas. Of 268 growing hybridoma cultures, 117 expressed CD3 and CD4.

Monoclonal hybridomas were screened for their response to human oxLDL, native LDL, and purified unmodified ApoB100. T cell hybridoma activation was examined, as measured by IL-2 production after exposure to the putative antigen in the presence of syngeneic, irradiated APCs. 48% of the hybridomas responded to native human LDL and ApoB100, but none recognized oxLDL (Fig. 1 A).

Based on TCR genotyping, 3 subgroups were identified among the clones that responded to native LDL and ApoB100 (Table I and Table S1); a representative clone from each subgroup (15–2, 45–1, and 48–5) is shown. There was a clear dose–dependent response to unmodified ApoB100 (Fig. 1, B–D). Data from a clone with a different TCR genotype is shown for comparison in the online supplemental material; it recognized neither purified human ApoB100 nor recombinant ApoB100 from transgenic mice (Fig. S1 A). To ensure that the response was not caused by a human-specific modification of ApoB100, hybridomas were also exposed to ApoB100 isolated from LDL of huB100tg x Ldlr−/− mice. This recombinant ApoB100 also was recognized by the T cell hybridomas (Fig. 1, B–D). Therefore, native LDL and ApoB100 contain epitopes that are recognized by these T cells.

T cells that react to native LDL and ApoB100 express TRBV31

We characterized the TCRs of our T cell hybridomas by RT-PCR of the rearranged variable domains. The fusion partner thymoma BW5147 expressed rearranged TRAV20 and TRBV12.1 variable chains, and all hybridomas expressed mRNA for these TCR chains. In addition, native human LDL– and ApoB100–specific T cell hybridomas uniformly expressed TCR TRBV31 (previously called TCR–VB14; Table I and Fig. S2); no other Vβ segment was identified (Table S1). In contrast, the reactive hybridomas used several families of Vα chains, including TRAV3 (hybridoma 15–2), 4 (45–1), and 13 (48–5) (Table I). In unreactive hybridomas, Vβ and Vα TCR chains were expressed in a nonrestricted fashion, not including TRBV31 (Table S1). For each LDL–responsive hybridoma, the surface expression of TRBV31 was confirmed by flow cytometry (Table I and Fig. S2 B). Interestingly, all TCR chains identified in these ApoB100 reactive hybridomas were previously identified in the aorta of atherosclerotic Apoe−/− mice (Paulsson et al., 2000).

Oxidation of LDL extinguishes its recognition by T cells

We further analyzed the relationship between oxidation and the antigenicity of LDL particles by exposing T cell hybridomas to LDL particles that were oxidized by copper for various times, resulting in a range of oxidation (Fig. 1, E–G). For all T cell hybridomas, there was an inverse relationship between the degree of oxidation and the amplitude of activation.
MDA exposure of the lipoprotein particles, similar to the finding for copper oxidized LDL (Fig. 2 A). Of note, a dose dependent response could be detected against MDA modified LDL particles at any given concentration but its amplitude was inversely proportional to the extent of modification of the particles (Fig. 2, A and B). Similar findings were made for copper-oxidized LDL (Fig. 2 B).

To exclude an antigen-independent, detrimental effect of oxLDL on T cell activation, we examined its effect on T cells activated by ApoB100, native LDL, OVA, or the polyclonal T cell mitogen, Concanavalin A. The T cell hybridoma (Figs. 1, E–G, and Fig. 2 A). Thus, native LDL induced the strongest IL-2 response, whereas heavily oxidized LDL did not trigger activation.

In another set of experiments, LDL was modified by MDA adduct formation. MDA is formed during lipid peroxidation and reacts with free amino groups in ApoB100 to generate MDA-lysine and other modified residues (Fogelman et al., 1980, Haberland et al., 1988, Hamberg et al., 1974). Therefore, it can be considered as a chemically defined oxidative modification of LDL. MDA modification reduced T cell recognition of LDL proportionally to the extent of

| Clone  | TRAV gene | TRBV gene | Anti-TRAV14 | Anti-TRAV12 | Anti-TRBV31 | Resulting phenotype |
|--------|-----------|-----------|------------|------------|------------|---------------------|
| 15-2   | TRAV14, 3 and 20 | TRBV31 and 12.1 | negative | – | positive | TRAV3/TRBV31 |
| 45-1   | TRAV4 and 20 | TRBV31 and 12.1 | – | – | positive | TRAV4/TRBV31 |
| 48-5   | TRAV12, 13 and 20 | TRBV31 and 12.1 | – | negative | positive | TRAV13/TRBV31 |

The cDNA from reactive clones was amplified by PCR using appropriate Vβ-specific 5’ primers with a constant region Cβ 3’ primer, or relevant Vα-specific 5’ primers with a constant region Cα 3’ primer (Tables S1 and S2). Further phenotype of Vα and Vβ chain expression was analyzed by flow cytometry using antibodies to TRAV14, TRAV12, and TRBV31.
recognizing native LDL and ApoB100 was less activated when increasing concentrations of oxLDL were added to the culture, but showed unchanged activation levels when co-incubated with Concanavalin A (Fig. 2 C). Unchanged activation of T cells was also seen when using cells from OT-II mice that carry a transgenic TCR recognizing OVA and are therefore activated by OVA in the context of MHC class II (Fig. 2, A and C). T cell viability was unaffected by oxLDL at the concentrations that were used in this study (Fig. S3). Collectively, these data suggest that T cell recognition of LDL is gradually extinguished by LDL oxidation.

T cell responses to native LDL and ApoB100 are I-A<sup>b</sup> restricted

Because purified ApoB100 induced activation of the CD4<sup>+</sup> T cell hybridomas, we hypothesized that the epitopes are peptides that are presented by MHC class II—in our mice, the I-A<sup>b</sup> haplotype. When we added an antibody to block I-A<sup>b</sup> on APC, T cell activation was suppressed in all clones. Mismatched, I-A<sup>d</sup>-expressing APCs from BALB/c mice failed to present ApoB100 to antigen-specific T cell hybridomas (Fig. 3).

Cellular immune responses can also be triggered by lipid antigens after presentation by CD1. Because ApoB100 associates with lipids in the LDL particle, we measured T cell responses to ApoB100 that was presented by I-A<sup>b</sup>-bearing APCs that lacked CD1d, the only CD1 molecule that is expressed in mice. CD1d-deficient APCs, however, did not impair the response (Fig. S1). Similarly, APCs from I-A<sup>d</sup> mice that lacked MHC class I molecules presented ApoB100 antigens to T cell hybridomas (Fig. S1). These results show that ApoB100 antigen is recognized by MHC class II-restricted CD4<sup>+</sup> T cells.

The cellular immune response to native ApoB100 is preserved in polyclonal T cell populations

Having established that atherosclerotic lesions contain oligoclonsal T cells (Paulsson et al., 2000) and observing that hybridomas from mice that are immunized with oxLDL recognize native ApoB100, we asked whether such autoimmune responses occur in polyclonal T cell populations. This hypothesis was tested in huB100<sup>tg</sup>xLdlr<sup>−/−</sup> mice, which develop significant hypercholesterolemia with high plasma levels of LDL containing human ApoB100, even on a chow diet. These mice were immunized with oxLDL or native ApoB100, followed by in vitro challenge of splenocytes from these mice with oxLDL or native ApoB100.

As before, native ApoB100 elicited the highest proliferative T cell response, whereas highly oxidized LDL did not trigger activation, as registered by DNA synthesis. This activation pattern was similar in oxLDL- and ApoB100-immunized mice (Fig. 4). In contrast, antibody responses were detected against oxLDL, native LDL, and ApoB100 (Fig. 4 B). Consequently, T cell responses to native ApoB100 may help B cells produce antibodies to a variety of epitopes on oxidized as well as native LDL epitopes. T cell responses to native ApoB100 were not detectable in spleen cell preparations from nonimmunized mice, possibly because of limited sensitivity of the assay, thus expansion of autoreactive T cell clones by immunization with ApoB100 was necessary to detect a response (Fig. S4).

Similar to the findings in huB100<sup>tg</sup>xLdlr<sup>−/−</sup> mice, immunization of Apoe<sup>−/−</sup> mice with native murine LDL elicited a T cell response to the native LDL particles, indicating that

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**Figure 2. T cell recognition of LDL is decreased by oxidative modification.** 10<sup>5</sup> hybridoma cells (4B–5) were coincubated with 4 × 10<sup>5</sup> irradiated APCs. (A) 20 µg/ml of LDL or LDL modified to different extents (malondialdehyde [MDA] or copper oxidation [20 µM CuSO<sub>4</sub>] for varying lengths of time) was added to the coincubation, and 24 h later IL-2 secretion was measured in the supernatant. Filled columns show response to native LDL and striped columns show response to medium control. (B) Dose-response curves for the coincubation with different concentrations of LDL-modified either with MDA or copper. (C) Co-incubation with fixed concentrations of ApoB100, native LDL, or concanavalin A together with increasing concentration of copper-oxidized LDL. For OVA, co-incubations were done with splenocytes from OT-II mice and activation was measured as CPM from [<sup>3</sup>H]thymidine incorporation, as described in the Materials and methods section. Data show means ± SEM. Results are representative of three independent experiments.
such responses were not limited to the huB100tg model (unpublished data).

Depletion of TRBV31+ T cells reduces the proliferative response to ApoB100
Because all LDL responding T cell hybridomas expressed TRBV31, we surmised that this TCR Vβ chain is required for the recognition of ApoB100. To test this hypothesis, we immunized mice with ApoB100, isolated splenocytes, and removed TRBV31+ T cells by FACS. These steps blunted the response against ApoB100 on in vitro challenge. No such effect was observed when T cells that expressed a different variable chain, TRBV19, were depleted from splenocytes (Fig. 5 A). Therefore, a significant portion of the cellular immune response to ApoB100 in this model is fulfilled by TRBV31+ T cells.

Immunization against TRBV31 peptide inhibits T cell recognition of ApoB100
To inhibit T cell responses to LDL protein, we synthesized a peptide from TRBV31 including its CDR2 domain, fused it to KLH carrier protein, and used the preparation for immunization of huB100% x Ldlr−/− mice. This treatment induced the production of antibodies specific for the TRBV31 sequence (Fig. 5 B). Circulating IgG antibodies from immunized mice bound to LDL-reactive TRBV31+ hybridomas (Fig. 5 C), but not to nonreactive TRBV31 negative hybridomas (Fig. 5 D), and the addition of IgG from TRBV31 peptide-immunized mice inhibited T cell hybridoma activation in response to ApoB100 (Fig. 5 E). Thus, immunization of huB100% x Ldlr−/− mice with TRBV31 peptide induced the production of blocking antibodies that prevented TCR TRBV31 from recognizing LDL protein. We observed significantly reduced levels of TRBV31 mRNA in aorta and spleen at sacrifice (Fig. 5 F), possibly because antibodies binding to their TCR interfered with the expansion of TRBV31+ T cells.

Immunization against TRBV31+ peptide reduces atherosclerosis
Finally, we examined the role of TRBV31+ T cells in atherosclerosis. HuB100% x Ldlr−/− mice, which develop spontaneous atherosclerosis, were immunized against TRBV31. This led to a 65% reduction in lesion size in the aortic root compared to controls.

Figure 3. T cell responses to ApoB100 are I-Ab–restricted. 10^5 hybridoma cells were incubated with 4 x 10^5 irradiated APCs from mice of either the I-A^b or I-A^d haplotype and different concentrations of human ApoB100. Hybridoma cells were also challenged with ApoB100 in the presence of a blocking antibody to MHC class II (I-A^b). After 24 h of incubation, IL-2 secretion was measured in the supernatant. A, 15–2 clone; B, 48–5 clone; C, 45–1 clone. Data show means ± SEM. Results are from one experiment.

Figure 4. Immunization with oxLDL or ApoB100 expands T cell populations that recognize native epitopes of LDL and induces antibodies to oxLDL, native LDL, and ApoB100. huB100tg x Ldlr−/− mice were immunized with oxLDL (A) or ApoB100 (B). 5 x 10^5 splenocytes were challenged in vitro with 20 μg/mL of human oxLDL or native human ApoB100. Error bars represent the mean ± SEM of the stimulation index from [3H]thymidine incorporation, as described in the Materials and methods. Sera were assayed for antibodies to oxLDL, native LDL, and ApoB100. Values are expressed as mean ± SEM of the OD values for each dilution. Data are representative of two independent experiments (n = 4 mice per group). **, P < 0.01.
We observed substantially reduced inflammation, accompanied by decreased expression of MHC class II protein I-A (Fig. 7 B). Furthermore, aortic mRNA for the chemokine, CCL2 (monocyte chemoattractant protein–1) was significantly reduced in peptide immunized mice, whereas CCL5 (RANTES) mRNA was unchanged (Fig. 7, D and E). In summary, abrogation of TCR TRBV31 recognition of native ApoB100 reduces vascular inflammation and inhibits the development of atherosclerosis.

DISCUSSION
In this study, we have identified a cellular immune response to ApoB100 protein of native LDL, and show that this response plays an important role in atherosclerosis. T cells from control mice that were immunized with carrier protein (Fig. 6 A). Lesion size was uniformly reduced in cross sections of the proximal aorta of TRBV31-immunized mice, without any detectable change in lesion distribution (Fig. 6 A). Lesions were also analyzed after Sudan IV staining of lipid lesion area in en face preparations of the aortic arch (Fig. 6 B). Here, TRBV31 immunization led to a 57% reduction in lesion area.

Plasma cholesterol, triglycerides, ApoB100 levels, and lipoprotein profiles were unchanged (Fig. S5), as well as antibody titers to LDL and oxLDL (unpublished data).

Immunohistochemistry of the lesions showed that macrophage levels were reduced by 50% (Fig. 7 A), whereas no significant effect was registered on T cell infiltration (Fig. 7 C).

Figure 5. TRBV31+ T cells recognize ApoB100. (A) Depletion of TRBV31+ cells reduces T cell responses to ApoB100. HuB100tg x Ldlr−/− mice were immunized with ApoB100. Splenocytes, harvested after a booster injection, were depleted of TRBV31+ or TRBV19+ cells by fluorescence-activated cell sorting. 5 × 10^5 TRBV31+/TRBV19− or TRBV19+/TRBV31− cells were challenged in vitro with different concentrations of human ApoB100. T cell activation was measured by [3H]thymidine incorporation and stimulation index, calculated as described in the Materials and methods section. Data show mean ± SEM and are representative of two experiments. *, P < 0.05. (B) Induction of IgG antibodies to TRBV31 peptide after immunization with TRBV31. HuB100tg x Ldlr−/− mice were immunized and boosted with TRBV31 peptide conjugated to KLH, or with KLH alone as control. Serum IgG antibodies to TRBV31 peptide were measured by ELISA. Results are presented as mean ± SEM and representative of two independent experiments. **, P < 0.01; ***, P < 0.001. (C) Anti-TRBV31 IgG binds to LDL-reactive T cell hybridoma. Affinity-purified IgG antibodies from TRBV31-immunized mice were incubated with an LDL-reactive TRBV31+ hybridoma (48–5 clone), followed by incubation with FITC-labeled anti–mouse IgG and flow cytometric analysis. The graph shows representative histograms from 48–5 cells in the FITC channel (% of Max) after labeling with IgG from mice immunized with the TRBV31-KLH conjugate (gray profile) or KLH alone. Total IgG from untreated mice was used as negative control (black line). Results are representative of three independent experiments. (D) Anti-TRBV31 IgG does not bind to a non–LDL-reactive T cell hybridoma. Affinity-purified IgG antibodies from TRBV31-immunized mice were incubated with a non–LDL-reactive hybridoma (97–3 clone), followed by incubation with FITC-labeled anti–mouse IgG and flow cytometric analysis. The graph shows representative histograms from 97–3 cells in the FITC channel (% of Max) after labeling with IgG from mice immunized with the TRBV31-KLH conjugate (gray profile) or KLH alone (dashed line). Total IgG from untreated mice was used as negative control (black line). Results are representative of three independent experiments. (E) Anti-TRBV31 IgG inhibits T cell recognition of ApoB100. 10^4 48–5 hybridoma cells were challenged in vitro with 20 µg/ml ApoB100 in the presence of 4 × 10^5 irradiated APCs with IgG from mice immunized with TRBV31-KLH or KLH alone. After 24 h of incubation, IL-2 secretion was measured in the supernatant. Results show mean ± SEM and are representative of three independent experiments. *, P < 0.05. (F) Immunization against TRBV31 reduces TRBV31 TCR mRNA in spleen and aorta. mRNA transcript levels were evaluated in aorta and spleen from huB100tg x Ldlr−/− mice immunized with TRBV31-KLH conjugate or KLH alone. Bar graphs represent the mean ± SEM of TRBV31 mRNA relative to HPRT mRNA/CD3 mRNA relative to HPRT mRNA (n = 9 mice per group). *, P < 0.05. Results were pooled from two experiments.
oxLDL-immunized mice recognize unmodified ApoB100 from native LDL rather than oxLDL. This response is clonotypic and involves TRBV31+ TCR, and immunization against this T cell receptor significantly reduces atherosclerosis.

OxLDL triggers a strong humoral immune response that generates antibodies to oxidation-induced epitopes on LDL particles (Palinski et al., 1989, 1996). Because T cell–dependent antibodies form against aldehyde adducts on ApoB100 (Steinbrecher et al., 1984; Fredrikson et al., 2003) and exposure of APC–T cell cultures to oxLDL activates CD4+ T cells (Frostegård et al., 1992; Stemme et al., 1995), T cells have been assumed to selectively recognize epitopes on ApoB100 that are generated by oxidation of the native apolipoprotein.

To the contrary, we have found that T cells from oxLDL-immunized mice preferentially recognize motifs from native LDL. These epitopes are components of native ApoB100 and the T cell reactivity toward them is extinguished rather than induced by oxidative modification of LDL. Although we cannot rule out that nominally native LDL preparations may contain oxidized structures even in the presence of antioxidants, the finding that purified, delipidated ApoB100 protein induced similar T cell responses as native LDL supports the notion that the epitopes inducing these responses were present in the native ApoB100 protein of LDL.

When reanalyzing data published previously, we have noticed that T cell reactivity to native LDL was registered for plaque clones as well as peripheral blood (Frostegård et al., 1992; Stemme et al., 1995). These responses have clearly been underestimated by us and others. Our present data do not rule out the existence of oxLDL–reactive T cells, but demonstrate the presence and pathogenetic role of T cells recognizing native ApoB100 epitopes.

The cellular immune response to LDL that we observed was mounted by CD4+ T cells and showed MHC class II restriction. In conjunction with data that demonstrate that purified ApoB100 protein elicits an identical response to that of intact LDL, our results strongly suggest that intracellular processing of ApoB100 in the APC generates oligopeptide epitopes that are recognized by the T cells as peptide-MHC complexes. The finding that I-Ab is required for the T cell response and cannot be substituted by another MHC class II molecule or by CD1d further supports the model that specific oligopeptides that are bound to MHC class II constitute the ligand with which clonotypic TCRs interact.

Interestingly, among the peptides that have been eluted from I-Ab on B cells and macrophages, several are derived from unmodified ApoB100 (Dongre et al., 2001). Moreover, among HLA-DR molecules isolated from human APCs grown under standard cell culture conditions, a high proportion carry ApoB100 oligopeptides in their antigen-presenting grooves (Urban et al., 1994). These observations suggest that ApoB100 oligopeptides associate with certain MHC class II molecules. Because APCs can present ApoB100 epitopes, there should be a significant risk for autoimmune reactions.

Figure 6. Immunization against TRBV31 reduces atherosclerosis. HuB100/9 x Ldlr−/− mice were immunized with the TRBV31 peptide-KLH conjugate or with KLH alone. The mice were sacrificed after 10 wk on a Western diet. (A) Atherosclerotic lesion size in the proximal aorta. Lesions were analyzed by microscopic morphometry of serial sections at 100–800 µm from the aortic valves. Data show cross-sectional lesion size (mm²). Inset shows means of the eight cross-sectional lesion sizes for each animal, and the line indicates mean value per group of mice. n = 9 mice per group. Pooled data are presented from two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Representative micrographs from immunized mice. Hematoxylin-Oil red O staining, original magnification 20x. White arrows indicate lesion areas. Bars, 250 µm. (B) Atherosclerotic lesion size in the aortic arch. Dissected arches were stained with Sudan IV en face and the percentage lesion area of total vessel area was calculated using ImageJ image analysis software. The additive area of all the plaques in a given aortic arch was calculated as a percentage of the total surface area of the arch. n = 8 mice per group. Two representative stained samples from each group are shown. *, P < 0.05.
to LDL. Systemic reactions such as these present an obvious threat, because LDL is present throughout the circulatory system and in all organs. We have assumed that all ApoB100-reactive T cell clones are deleted in the thymus during development, i.e., that autoimmunity is avoided by central tolerance.

Our current data, however, rule out this possibility, as we identified a T cell population that reacts to native ApoB100. Consistent with these results, it has been suggested that the immune system is not tolerized to all peripheral autoantigens and that the existence of autoreactive T cells, per se, does not place the healthy individual at risk for autoimmunity (Kyewski and Klein, 2006). Consequently, ApoB100-reactive T lymphocytes most likely constitute part of the peripheral cell repertoire.

If autoreactivity is not eliminated by central tolerance during development, autoimmune reactions must be avoided by peripheral tolerance mechanisms, which could rely on active inhibition of autoreactivity; e.g., by cells that secrete immunoregulatory cytokines, such as M2 macrophages (Gordon, 2003).

These cells internalize modified LDL particles through scavenger receptors and can secrete the immunosuppressive cytokines IL-10 and TGF-β (Mills et al., 2000; Bouhlel et al., 2007). Furthermore, LDL that enters the scavenger receptor pathway carries ligands for antiinflammatory PPAR transcription factors (Li and Glass, 2007). Therefore, the milieu at the sites of antigen accumulation can inhibit activation of LDL-reactive T cell clones.

In addition, regulatory T cells regulate atherosclerosis (Ait-Oufella et al., 2006) by dampening disease progression and by inhibiting LDL-reactive Th1 cells (Klingenberg et al., 2010). If regulatory T cells are preferentially induced when endogenous LDL is presented to T cells, peripheral tolerance would be maintained. Interestingly, expression of proteins in the liver, where LDL is produced, promotes induction of T reg cells and peripheral tolerance to autoantigens (Lüth et al., 2008).

Parenteral administration of LDL in a proinflammatory adjuvant can break such tolerance, because oxidized particles are amenable to scavenger receptor-mediated uptake into APCs.

**Figure 7.** Immunization against TRBV31 reduces inflammation in atherosclerotic lesions. (A) Reduced accumulation of macrophages in lesions of TRBV31-immunized mice. Images show CD68 immunostaining of representative aortic root sections from mice immunized with KLH alone (left) or the TRBV31-KLH conjugate (right). The dot plot shows proportion of total lesion area stained by CD68 antibody in the two groups. Data show values for individual mice and mean value for each group (line; n = 8 mice per group). (B) Reduced MHC class II (I-Ab) expression in lesions of TRBV31-immunized mice. Images show I-Ab immunostaining of representative sections from the aortic root. The dot plot shows the number of I-Ab+ cells per mm² lesion area in the two groups (n = 8 mice for KLH and 9 mice for TRBV31). (C) No change in CD3 expression in lesions of TRBV31-immunized mice. Images show CD3 immunostaining of representative sections from the aortic root. The dot plot shows the number of CD3+ cells per square millimeter of lesion area in the two groups (n = 7 mice for KLH and 8 mice for TRBV31), n.s., not significant. Immunoperoxidase labeling, original magnification 100×. Bars: (A–C) 100 µm. (D–E) Immunization with TRBV31 reduces CCL2 mRNA. mRNA levels of CCL2 and CCL5 were measured by real-time RT-PCR in aorta from huB100/x Ldlr−/− mice immunized with TRBV31-KLH conjugate or KLH alone. Error bars represent the mean ± SEM for mRNA under study/mRNA for the housekeeping gene, HPRT (n = 9 mice per group). *, P < 0.05 in A–D. Results in A–E show pooled data from two experiments.
tributions to LDL immunoreactivity may arise from NKT MHC class II–restricted pathway. Additional important con to CD4+

Finally, our strategy focused on antigens that were presented represented in the hybridoma repertoire that was analyzed. A subset of cells, and certain reactivities may not have been Heinecke, 1996), which may contribute to T cell recognition.

Our data show that T cells recognize this type of native ApoB100 epitopes, but also provide help to B cells that produce antibodies to native and oxidatively induced structures on LDL. In this scenario, modest oxidation would increase antigen uptake; survival of native, i.e., nonmodified peptide epitopes would be necessary for T cell recognition; and extensive oxidation would eliminate T cell–dependent immune reactions to the LDL particle.

Our data implicate TCR TRBV31-expressing CD4+ T cells that recognize native ApoB100 as proatherogenic. Our results, however, do not rule out the possibility that the oxidative changes to LDL in vivo may differ from those that are induced by copper or MDA. Among the defined adducts to the polypeptide chain that are formed during lipid peroxidation, we examined only carbonyls (such as MDA), therefore we cannot rule out that other more physiological modification pathways, i.e., by HOCl, chloramines, phenoxy radical intermedias, peroxynitrite (ONOO–), and myeloperoxidase-dependent nitrogen dioxide (NO2) production (Berliner and Heinecke, 1996), which may contribute to T cell recognition.

Furthermore, our hybridoma strategy detailed a small subset of cells, and certain reactivities may not have been represented in the hybridoma repertoire that was analyzed. Finally, our strategy focused on antigens that were presented to CD4+ T cells by professional APCs through the endocytic, MHC class II–restricted pathway. Additional important contributions to LDL immunoreactivity may arise from NKT cells that recognize lipid antigens that are presented via CD1, CD8+ T cells that recognize MHC class I–restricted antigens, and B cells.

Immunization of atherosclerosis-prone huB100% x Ldlr−/− mice with TRBV31 peptide provided important insights into the immunopathology of atherosclerosis. Antibodies that were isolated from hyperimmune sera blocked activation of T cells in response to ApoB100, and elimination of TRBV31+ cells by flow cytometry reduced T cell responses to ApoB100 in splenocyte cultures.

The induction of anti-TRBV31 antibodies in huApoB100% x Ldlr−/− mice reduced vascular inflammation and was accompanied by a prominent, 65% reduction in atherosclerosis in the aortic root, and 57% in the arch. Thus, our data strongly suggest that a subset of T cells that recognize ApoB100 epitopes regulate the development of atherosclerosis. Reduction of lesion size was accompanied by reduced MHC class II, CCL2 chemokine, and macrophages in lesions, therefore inhibition of the T cell response to ApoB100 likely reduced inflammation in the lesions. Our findings thus confirm and extend previous reports showing an important role for CD4+ T cells and their restriction element, MHC class II, in atherosclerosis (Huber et al., 2001; Zhou et al., 2006). In line with this notion, mice carrying different MHC haplotypes differ in their susceptibility to atherosclerosis (Schulte et al., 2008).

The conditions during activation of naïve T cells, including the phenotype of antigen-presenting cells, presence of co-stimulatory factors, local cytokines, and, when immunization protocols are used, type of adjuvant, determine the differentiation of activated T cells into effector phenotypes (Lu and Rudensky, 2009). Therefore, preexisting, ApoB100-reactive CD4+ T cells may differentiate into proatherogenic Th1 cells or atheroprotective regulatory T cells depending on local conditions in the plaque and draining LNs during immunization (Andersson et al., 2010; Wigren et al., 2009).

The use of the huB100% x Ldlr−/− model permitted us to study human LDL-derived epitopes to dissect the cellular autoimmune response in atherosclerosis. Our data

![Figure 8. Proposed hypothesis for CD4+ T cell recognition of LDL. LDL particles are taken up by LDL receptors and, if modified, by scavenger receptors on the APC. Uptake could also be mediated by Fc receptors ligating opsonized LDL particles. After endosomal degradation, peptides derived from ApoB100 are transferred into the antigen-presenting pathway, where they bind to nascent MHC class II molecules. The peptide–MHC complexes are transported to the cell surface and recognized by CD4+ T cells equipped with TRBV31+ TCR. The ensuing T cell activation leads to cytokine secretion that can promote macrophage activation and inflammation. Once T cells are activated toward native ApoB100 epitopes, they may provide help for B cells recognizing native LDL, ApoB100, or lipids such as phosphocholine (PC), but also oxidatively modified epitopes such as MDA–ApoB100 or oxPC. T, T cells; B, B cells; blue symbol, Scavenger receptors or Fc receptors; red symbol, LDL receptors.](image-url)
demonstrate that clonotypic T cells that recognize ApoB100 are needed for the development of advanced atherosclerosis. Future work should address whether the immunopathology of human atherosclerosis is similar to that of our model and evaluate a TCR-directed immunomodulatory strategy for the treatment or prevention of atherosclerotic cardiovascular disease.

MATERIALS AND METHODS

Animals and immunization. We used 7-wk-old male human ApoB100 transgenic mice, huB1000"tm2Sgy; DNX Transgenics), to generate T cell hybridomas. These mice carry the full-length human APOB100 gene, in which codon 2153 has been converted from leucine to glutamine to prevent the formation of ApoB48, generating only ApoB100 (Linton et al., 1993; Boren et al., 1998). They were first immunized s.c. with 50 µg of copper-oxidized human LDL in CFA, and were boosted after 2 wk with 50 µg oxLDL in IFA.

To evaluate the role of TRBV31" T cells in disease development, we used same strains crossed with the Ldlr"/" (C57BL/6;129-Apob"tm2Sgy; DNX Transgenics) to develop large atherosclerotic lesions, even on a normal chow diet, permitting quantitative studies of disease. Both strains have the same I-A" MHC haplotype and both strains react to native LDL and ApoB100. 11-wk-old male huB1000"tm2Sgy; DNX Transgenics mice were immunized s.c. with 100 µg TRBV31 peptide conjugated to KLH and emulsified in CFA and boosted 4 wk later with the same antigen emulsified with IFA. Control mice were immunized s.c. with 100 µg KLH.

The mice were fed a high-fat diet (maize starch, cocoa butter, casein, glucose, sucrose, cellulose flour, minerals, and vitamins; 17.2% protein, 21% fat [62.9% saturated, 33.9% polyunsaturated; 0.15% cholesterol, 43% carbohydrates, 10% H2O, and 3.9% cellulose; R638] starting 5 d after the immunization until sacrifice 10 wk later with CO2. The TRBV31 peptide includes the CDR2 variable region of the TCR-β chain (amu acid residues 45-62, "ATGTLQQLQFSITVGVQ," synthesized and conjugated to KLH by AnaSpec). In addition, irradiated splenocytes from C57BL/6 mice were used as APCs in the hybridoma experiments. All experiments were approved by the Stockholm North Committee for Experimental Animal Ethics.

T cell hybridomas. T cell hybridomas were generated by polyethylene glycol-induced fusion of 5 × 10^6 LNs with 5 × 10^6 BWS147 thymoma cells, as previously described (Kappler et al., 1981). In brief, LN cells from the immunized mice were stimulated with 3 µg/ml oxLDL for 3 d before fusion. After fusion, 10^6 thymocytes were added as feeder cells, and the cell suspensions were plated in 96-well plates and incubated at 37°C and 7.5% CO2. The extent of oxidation was evaluated by TBARS, as previously described (Havel et al., 1955). 2 mM benzamide, 0.5 mM PMSF, and 0.1 U/ml aprotinin were added immediately after the plasma was prepared. After isolation, LDL was dialyzed extensively against PBS. 1 mM EDTA was added to an aliquot of LDL to generate unmodified LDL. Using the same procedure, recombinant LDL was prepared from plasma of huB1000"tm2Sgy; DNX Transgenics mice.

Highly oxidized LDL was obtained by incubating 1 ml of LDL (1 mg/ml protein content, determined by Bradford assay) Bio-Rad Laboratories) in the presence of 20 µM CuSO4 for 18 h at 37°C; different degrees of oxidation were obtained by incubating LDL with 20 µM CuSO4 for 1, 2, 4, or 8 h. The extent of oxidation was evaluated by TBARS, as previously described (Puhl et al., 1994).

Preparation of soluble ApoB100. Soluble ApoB100 was isolated by using a modification of previously described methods (Steele and Reynolds, 1979; Wessel and Flugge, 1984). In brief, 0.4 ml methanol, 0.1 ml chloroform, and 0.3 ml water were added to 0.1 ml of LDL (1 mg/ml); the suspension was then vortexed and centrifuged at 9,000 g for 1 min. The upper phase was removed and 0.3 ml of methanol added to the lower phase and interphase with precipitated protein, which was mixed again and centrifuged at 9,000 g for 2 min to pellet the protein.

To obtain soluble and pure ApoB100, the protein pellet was resuspended in a minimum volume of 10% SDS (Bio-Rad Laboratories) until it solubilized. These preparations first were filtered on a PD-10 column (GE Healthcare) to remove excess SDS. They were then purified on a Superdex-200 size-exclusion column (0.5 ml/min, in Tris-HCl, pH 7.4). ApoB100 preparations were greater than 90% pure, as evaluated in a second injection into a Superdex-200 column (GE Healthcare) and analyzed on SDS-PAGE (Fig. S6). Finally, protein concentration was determined by Bradford assay (Bio-Rad Laboratories).

Flow cytometric analysis of TCR V domain expression. Commercially available anti-μ mouse TCR-Vα and TCR-Vβ mAb (BD) were used to detect TCR-Vα and TCR-Vβ. They were combined with anti-CD3-Pacific Blue and anti-CD4-APC to stain T cell hybridomas. Spleenocytes from unimmunized mice were used as positive controls for all antibodies. The cells were analyzed on a CyAn ADP flow cytometer (Dako).
In vitro proliferation assay. Splenocytes from immunized mice were isolated and resuspended. In 96-well plates, 5 × 10^5 splenocytes were incubated in duplicate with different antigens, as described in the figure legends, in 200 µl of serum-free medium, 1:100 BD ITS’ Premix (BD), 1 mg/ml BSA (Sigma-Aldrich), 10 mmol/liter Hepes (Invitrogen), 1 mmol/l Na pyruvate (Invitrogen), 1 mmol/l nonessential amino acids (Sigma-Aldrich), and 50 µg/ml gentamicin sulfate (Sigma-Aldrich) for 72 h, at 37°C in a humid 5% CO_2_ atmosphere. One microrange [H^3]thyminidine (Sigma-Aldrich) was added after 60 h and DNA replication was measured with a scintillation counter (Wallac). Results are expressed as stimulation index = (s – c)/c, where s is the cpm of the sample with antigen and c is the cpm of the sample without antigen.

β′ T cell depletion by fluorescence activated cell sorting. Splenocytes were isolated from huApoB100 LDL−/− mice that were immunized with 100 µg ApoB100. 30 million splenocytes were stained with anti-TRBV31 or anti-TRBV19 (BD). TRBV19 was used as a control for the sort, because none of the hybridomas that recognized ApoB100 expressed this TCR. Two clones that expressed the TRBV19/TRAV13.2 TCR (96.7 and 97.3) did not recognize LDL or ApoB100 (Table S1). After staining, the cells were sorted on a MoFlo Cytomation cell sorter (Cytomation Biosystems) to deplete positive events. Negative cells were collected and used in the proliferation assay in response to ApoB100.

Plasma analyses. The titers of specific antibodies to TRBV31 peptide, LDL, oxLDL and ApoB100 were measured by ELISA. In brief, 50 µl of the different antigens (10 µg/ml in PBS pH 7.4) was added to 96-well ELISA plates and incubated overnight at 4°C. Coated plates were washed with PBS and blocked with 1% gelatin (Invitrogen) in PBS for 1 h at room temperature. Next, plates were washed and incubated for two additional hours with mouse plasma, diluted in TBS/gelatin 0.1%. After washing, total IgG levels were measured using enzyme-conjugated anti-mouse antibodies (BD). The plates were washed, and colorimetric reactions were developed using TMB (BD). The absorbance was measured using a microplate reader (VersaMax; MDS Analytical Devices). Plasma cholesterol and triglycerides were measured using enzymatic colorimetric kits (Random Laboratory, Ltd.) according to the manufacturer’s protocol. ApoB100 levels were measured by commercial ELISA following the manufacturer’s instructions (ALerCHEK, Inc.).

Lipoprotein lipid profiles. Plasma cholesterol lipoprotein profiles were determined using a modification of the method of Okazaki et al. (1981). In brief, plasma samples (50 µl) from mice immunized with TRBV31-peptide or KLH were fractionated using an HR10/30 Superose 6 column (GE Healthcare) and a Discovery BIO GFC-500 as precolumn (5 cm × 7.8 i.d.; MDS Analytical Devices). Plasma cholesterol and triglycerides were measured using enzymatic colorimetric kits (Random Laboratory, Ltd.) according to the manufacturer’s protocol. ApoB100 levels were measured by commercial ELISA following the manufacturer’s instructions (ALerCHEK, Inc.).

Tissue processing, immunohistochemistry, and lesion analysis. Blood from sacrificed mice was collected by cardiac puncture and vascular perfusion performed with sterile RNase-free PBS. Abdominal aorta, one-third of the spleen, and draining LNs were dissected and snap-frozen for later RNA isolation. Hearts and aortic arch were dissected and preserved for immunohistochemistry and lesion analysis. Lesion analysis was performed as described (Nicolletti et al., 1998). In brief, hearts were serially sectioned from the proximal 1 mm of the aortic root to a cryostat. Hematoxylin- and Oil red O-stained sections were used to evaluate lesion size. Lesion size was determined by measuring 8 hematoxylin- and Oil red O-stained sections, collected at every 100 µm over a 1 mm segment of the aortic root. For each section, images were captured with a DM-LB2 microscope (Leica) equipped with a 20×/0.9 objective and a DC300 camera (Leica), and the surface areas of the lesion(s) and of the entire vessel were measured. Primary antibodies to CD3, CD68, and I-A^k (all rat anti-mouse from BD) were applied to acetone-fixed cryosections followed by detection with the ABC alkaline phosphatase kit (Vector Laboratories). En face lipid accumulation was determined in the aortic arch from immunized mice using Sudan IV staining. In brief, dissected arches were fixed in 4% neutral buffer formalin. Samples were then cut longitudinally, splayed, pinned, and subjected to Sudan IV staining (red color). Images were captured using a DC480 camera connected to a MZ6 stereo microscope (both from Leica). The additive area of all the plaques in a given aortic arch was calculated as a percent of the total surface area of the arch (not including branching vessels). Quantitation of plaques was performed using ImageJ software (NIH). Immunohistochemical data were obtained using Qwin computerized analysis (Leica) of stained sections.

RNA isolation, cDNA synthesis, and real-time PCR. RNA was isolated from the indicated tissues or cells using the RNeasy kit (QIAGEN). Total RNA was analyzed on a BioAnalyzer (Agilent Technologies). Reverse transcription was performed with Superscript-II and random hexamers (both from Life technologies) and amplified by real time-PCR using Assay-on-demand primers and probes for Ccl2, Ccl5, CD3, and hypoxanthine guanidine ribonucleosyl transferase (HPRT) (Applied Biosystems) in an ABI 7700 Sequence Detector (Applied Biosystems). For the TRBV31 expression analysis, the primers used in the genotyping (Table S2) were combined with a probe designed based on the nucleotide sequences of the constant region of TCR β chain (5′-TTCACCCCAAGGTCT-3′). The probe was designed using ABI Primer Express software (Applied Biosystems) and it was synthesized with a 6-carboxy-fluorescein (FAM) reporter molecule attached at the 5′ end (Applied Biosystems). Data were analyzed on the basis of the relative expression method with the formula 2^ΔΔCT, where ΔΔCT = ΔCT (sample) – ΔCT (calibrator = mean CT values of all samples within each group), and ΔCT is the CT of the housekeeping gene (HPRT) subtracted from the CT of the target gene. For TRBV31, values of the mean ± SEM of TRBV31 expression/C3D3 expression are shown.

Antibody-mediated blocking of TRBV31′ cell responses to LDL in vitro. Total IgG plasma antibodies from KLH- or TRBV31-immunized mice were affinity-purified using a protein G column (GE Healthcare). 10,000 hybridoma cells (clone 48–5) were cultured with 20 µg/ml LDL in the presence of 4 × 10^3 irradiated APCs. To block hybridoma activation, various antibodies were added at the beginning of culture at the concentrations indicated in the figure and were present throughout the culture. After 24 h of incubation, IL-2 was measured in the supernatant.

Statistical analysis. Values are expressed as mean ± SEM unless otherwise indicated. The nonparametric Mann–Whitney U test was used for pairwise comparisons and the Kruskal–Wallis test for multiple comparisons. Correlations were calculated using the Spearman test. Differences between groups were considered significant at P values below 0.05.

Online supplemental material. Fig. S1 shows negative controls for hybridomaspecific responses and MHC restriction assay. Fig. S2 shows the PCR-amplified fragments from the T cell receptor genotyping, visualized on agarose gels and staining of T cell receptors from ApoB100-responding hybridomas. Fig. S3 shows that the antigen preparations used in experiments were not toxic to the cells. Fig. S4 shows that immunization is necessary for priming of T cells and detection of proliferation in vitro. Fig. S5 shows that immunization with TRBV31 peptide or KLH do not influence ApoB100 or lipid levels in plasma. Fig. S6 shows the biochemical evaluation of soluble ApoB100 preparations. Table S1 provides information about the T cell receptor genotyping, visualized on agarose gels and staining of T cell receptors from ApoB100-responding hybridomas. Table S2 lists the primers that were used to genotype the hybridoma T cell receptors. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092243/DC1.

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