Atheroprotective vaccination with MHC-II restricted peptides from ApoB-100

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INTRODUCTION

Improvements in the treatment and prevention of cardiovascular disease (CVD) resulted in a 30% reduction in its mortality rate between 1998 and 2008. Despite this, a recent summary statement from the American Heart Association estimates that each year 785,000 Americans will have a new heart attack, and that someone dies from a coronary event every 60 s (1). These statistics underscore the importance of research that seeks to develop novel therapies in the fight against atherosclerosis.

Recent discoveries suggest that local arterial tissue inflammation is likely a major instigator in the development of atherosclerosis (2–6). This inflammation is mediated, in part, by local immunologic processes at the site of plaque lesions which involve both innate and adaptive immunity (2, 6–10). The antigens in atherosclerosis driving both pro-atherosclerotic and atheroprotective immune responses are not known with certainty. Oxidized low-density lipoprotein (LDL) (ox-LDL), and the lipoprotein portion of LDL (ApoB-100) have been proposed as candidate antigens (10–13). Recent evidence suggests that autoantigens are presented to antigen-experienced CD4+ T-cells (14). In particular, the presence of CD4+ NKT-cells (15, 16) and CD8+ T-cells (17–19) indicates that inflammation within the plaque is a regulated process, offering hope...
that therapeutics can be designed targeting T-cell function and differentiation.

Regulatory T-cells are the focus of many studies in atherosclerosis because of their atheroprotective potential. T<sub>REGS</sub> are reduced in atherosclerotic plaques (18) and in circulating peripheral blood of human subjects with CVD (20) compared to healthy subjects. In murine studies, adoptive transfer of a subset of T<sub>REGS</sub> (Tr1 cells, CD4<sup>+</sup>FoxP3<sup>+</sup>IL-10<sup>+</sup>) administered to Apoe<sup>−/−</sup> mice showed a significant decrease in pro-atherosclerotic IFN-γ production, increased IL-10 production, and significant reduction in atherosclerotic lesion size when compared with control mice (21). IL-10, secreted by T<sub>REGS</sub>, is atheroprotective and protects both from fatty streak formation and atherosclerotic plaque formation (15, 22).

Several studies have reported a protective effect of vaccination with LDL, or ox-LDL, on the development of atherosclerosis (15, 22). The mechanism by which these immunizations convey atheroprotection is an ongoing area of research. One line of investigation suggests that protective autoantibodies generated during immunization with ox-LDL might be the source of atheroprotection (24, 26, 27). This was initially a promising hypothesis which subsequently led to the search for atheroprotective B-cell (antibody) epitopes. The discovery of such epitopes was first reported by Fredrikson et al. (28) in 2003. These researchers assessed the binding of endogenous antibodies (from pooled human plasma in a case-control cohort of patients with a history of acute coronary heart events) and identified several epitope sequences from human ApoB-100 that bound to endogenous human antibodies. Since then several of these peptides have been used to vaccinate mice, including P2 (TRFKHLRKYTYNYEAESSH) (29), P143 (IALDDAKNFNEKLSQLQTY) (30), and P210 (KTTKQSDLVSVKAYKKNKH) (30) each conferring ~40–60% atheroprotection. While the original premise of immunizing with B-cell epitopes was atheroprotection via an increase in peptide-specific antibody levels, this was eventually determined to not be the case (31).

More recently, researchers have sought to describe the changes in cellular (rather than humoral) immunity that may result from immunization with B-cell epitopes from ApoB-100. Several recent papers have reported an increase in FoxP3<sup>+</sup> expressing T<sub>REGS</sub> in secondary lymphoid organs [spleens (32) and lymph nodes (33)] after immunization of mice with P210. While these findings are consistent with reports that suggest T<sub>REGS</sub> might be protective in atherosclerosis (17–19), it is unclear how T-cells can be directly affected by vaccination with peptide sequences originally recognized as B-cell epitopes. This is especially peculiar in the case of P210, which has been shown not to bind to I-Ab (the MHC Class II allele expressed by both ApoB<sup>−/−</sup> and LDL-R<sup>−/−</sup> mice) (34), which is a prerequisite for any direct interaction with T-cell receptors. Subsequent studies have further described the changes to the T-cell compartment after immunization with B-cell epitopes (in particular the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>REGS</sub> population) (35). However, there are currently no T-cell epitopes related to atherosclerosis that have been described. Knowledge of such epitopes would help to better characterize T-cell phenotypes after peptide immunization in the treatment of atherosclerosis.

The existence of atherosclerosis-related CD4<sup>+</sup> T-cell epitopes is suggested by several experiments. First, human CD4<sup>+</sup> T-cells clones derived from atherosclerotic plaques responded to incubation with ox-LDL and autologous monocytes (acting as APCs) by proliferation and production of cytokine (IFNγ) in an antigen-specific, HLA-DR-restricted manner (11). More recently, live-cell imaging of explanted aortas from CD11c<sup>+</sup>FAP Apoe<sup>−/−</sup> mice after 12 weeks of western diet (WD) showed that activated CD4<sup>+</sup>CD62L<sup>+</sup> Apo<sup>−/−</sup> CD<sup>+</sup> T-cells isolated from Apo<sup>−/−</sup> mice, but not wild-type mice, productively interacted with CD11c<sup>+</sup>FAP<sup>+</sup> APCs in the aortic wall (14). These T-cells were effector-memory CD4<sup>+</sup> T-cells, had long interactions with APCs in the vessel wall and slowed migration speeds compared to T-cells isolated from naive wild-type C57BL/6 mice. Furthermore, the “productiveness” of these interactions was demonstrated by increased T-cell proliferation and induction of the pro-inflammatory TNFα cytokines (IFNγ and TNF). These data suggest that endogenous T-cell antigens are presented in the aortic wall. It is therefore reasonable to suspect that these interactions may be promoting, or inhibiting, atherosclerosis by driving effector T-cell or regulatory T-cell responses, respectively. Manipulation of either these antigens or the T-cells that respond to these antigens would be of great therapeutic value.

Here, we report the discovery of two novel MHC-II restricted peptides identified in the murine ApoB-100 molecule. These peptides have high affinity for I-A<sup>b</sup> (the MHC class II molecule in C57BL/6 mice), and injection of these two peptides into ApoB<sup>−/−</sup> mice in complete Freund’s adjuvant (CFA), followed by four boosts in incomplete Freund’s adjuvant (IFA), reduces atherosclerosis. These data suggest that atheroprotective CD4<sup>+</sup> T-cell vaccines can be developed.

**MATERIALS AND METHODS**

**MICE**

Eight-week-old female ApoB<sup>−/−</sup> mice on C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in a specific pathogen free environment and fed chow diet until 10 weeks of age. At 10 weeks of age, mice were started on WD (adjusted calories diet with 42% from fat, Harlan Labs Cat #: TD.88137, CA, USA) and remained on WD until sacrifice at 23 weeks old.

**PEPTIDES**

Using previously described algorithms (36–38) for predicting I-A<sup>b</sup> (MHC Class II) peptide binding motifs in a protein molecule, we scanned the entire 4505 amino acid sequence of murine ApoB-100 and identified candidate sequences based solely on matches for I-A<sup>b</sup> binding. Six of these were selected based on favorable anchor residue and hydrophobicity profiles. These sequences are listed in Table 1. Although the minimal sequence to bind to the MHC Class II peptide groove is a 9-mer, all peptides were custom synthesized (Genemed Synthesis, Inc., San Antonio, TX, USA) as 15-mers to build “ends” on to the peptides for better binding. Peptides were synthesized in 0.1% trifluoroacetic acid (TFA) for stability.

**I-A<sup>b</sup> BINDING AFFINITY ASSAY**

We measured I-A<sup>b</sup> binding affinity using a classical competitive inhibition assay utilizing purified MHC and high affinity
radio-labeled I-A\(^b\) ligands. Their ability to displace the radio-labeled I-A\(^b\) ligand was measured, and an inhibitory concentration required to displace 50% of the radio-labeled ligand (IC\(_{50}\)) was determined. Under the conditions utilized, where (label) < (MHC) and IC\(_{50}\) ≥ (MHC), the measured IC\(_{50}\) values are reasonable approximations of the true K\(_d\) values.

**ANTIGEN-SPECIFIC PROLIFERATION**

Peptides were emulsified in CFA and mice were immunized with 200\(\mu\)g of peptide subcutaneously. Ten days later, draining lymph node cells were harvested and single cell suspensions were made. 5 \(\times\) 10\(^3\) viable cells per microtiter well were cultured with 5 \(\mu\)g/mL of relevant (or irrelevant) peptide for 4 days. Purified protein derivative (PPD) served as a positive control. Sixteen hours before harvesting, 1 mCi of tritiated thymidine (\(^3\)H) was added to each well. Cells were harvested and incorporation of \(^3\)H was determined in a scintillation counter. Results are expressed as stimulation index using the following formula:

\[
\text{Stimulation index (SI)} = \frac{\text{cpm experimental} - \text{cpm media control}}{\text{cpm media control}}
\]

**ATHEROPOREDOCTIVE IMMUNIZATION**

Previous work (25) has shown that atheroprotection through immunization could be achieved by injecting mice with LDL (or ox-LDL) with a combination of CFA initially, followed by antigen in IFA for booster immunizations. Using the same immunization model, 50 \(\mu\)g of ApoB\(_{3501-3516}\) or ApoB\(_{978-993}\) (diluted in PBS) was emulsified in equal volumes of CFA (BD Difco, Sparks, MD, USA) and injected into the subcutaneous inguinal area at 8 weeks of age. Repeated boosters with 25 \(\mu\)g of ApoB\(_{3501-3516}\) or ApoB\(_{978-993}\) emulsified in IFA (BD Difco, Sparks, MD, USA) were administered intraperitoneally at age 12, 16, 20, and 22 weeks. This immunization scheme will be referred to as 1× CFA + 4× IFA for the remainder of this manuscript. Mice were sacrificed at age 23 weeks and organs were harvested for analysis. Control immunizations with PBS emulsified in CFA and IFA were also performed. MOG\(_{35-55}\) [MEGVWYRSPFSRVHLYRNKG, (40)] immunizations were also done under identical conditions with the same adjuvants.

**ATHEROSCLEROSIS QUANTIFICATION**

Aortic root sections were examined as follows. Hearts were harvested, placed in Optimal Cutting Temperature medium (OCT, Electron Microscopy Sciences, Hatfield, PA, USA), and frozen at −80°C. Beginning at the first appearance of the tri-leaflet aortic valve, successive 5 \(\mu\)m transverse sections were made for a distance of 100 \(\mu\)m. From these, we analyzed every other section, for a total of 10 sections per root. Sections were then stained with Oil Red O and counter-stained with hematoxylin. Extent of atherosclerosis was then determined as the area involved on each section. To measure en face lesion formation, the whole aorta was carefully cleaned in situ and then the whole aorta pinned out after paraformaldehyde incubation at RT for at least 2 h. Staining for atherosclerotic plaque was performed by incubating samples in Sudan IV. Quantification was performed using ImagePro software (Media Cybernetics, Rockville, MD, USA).

**LIPOP ANALYSIS**

Mouse whole blood was collected by cardiac heart puncture during organ harvest. No anticoagulant was used. The blood was placed on ice for at least 3–6 h, and then spun at 6300 rpm for 15 min at 4°C. The supernatant was collected and frozen at −80°C until analysis to reduce multiple freeze/thaw cycles. Individual samples were then analyzed by Roche COBAS 8000 Analyzer (Roche Diagnostics, Indianapolis, IN, USA).

**MEASUREMENT OF ANTIBODY TITERS TO ApoB\(_{3501-3516}\) AND ApoB\(_{978-993}\)**

Antibody titers in plasma were determined by chemiluminescent enzyme immunoassay as previously described (41). In brief, white "U" bottom plates (Thermo Lab systems, USA) were coated with various antigens at 5 \(\mu\)g/mL in PBS for overnight incubation. Following blocking with 1% BSA-TBS serum was added in increasing dilutions and incubated at RT for 90 min. Bound antibodies levels were detected using appropriate alkaline phosphatase-conjugated secondary antibodies and a 50% aqueous solution of LumiPhos 530 (Lumigen, USA). Data are expressed as relative light units counted per 100 ms (RLU/100 ms).

**FLOW CYTOMETRY**

Aortas, lymph nodes, and spleens were digested as previously described (42). Aortic cell suspensions, spleens, and lymph nodes were individually pressed through a 70-\(\mu\)m filter and incubated for 30–60 min in complete RPMI to encourage CD4 re-expression. Approximately (1–2) \(\times\) 10\(^6\) cells were then placed into 96-well round bottom plates and incubated for 5 min with FC block (1:200), and subsequently stained with primary antibody [CD45-PerCP 1:50 (BioLegend, San Diego, CA, USA, Cat#130310), CD4-PE-Cy7 1:50 (eBioscience, San Diego, CA, USA, Cat#25-0041-82), TRC8-AF700 1:50 (BioLegend, Cat#109224), and Live/Dead Aqua 1:200 (Invitrogen, Grand Island, NY, USA, Cat# L34957)] for 45–60 min. Plates were washed twice and then incubated with Fix/Perm buffer solution (eBioscience, Cat# 00-5523-00) for additional 30 min. Plates were again washed twice with permeabilization buffer prior to intracellular staining with intracellular transcription factor staining [FoxP3-eFluor450 1:50 (eBioscience, Cat# 48-5773-82)]. Intracellular staining was performed for 30–45 min and plates were washed twice with permeabilization buffer.
solution. Samples were analyzed by LSR-II (BD Biosciences, San Jose, CA, USA). Data was acquired on FACSDiva software (BD Biosciences) and analyzed by FlowJo (Ashland, OH, USA).

**QUANTITATIVE RT-PCR**

Spleens, lymph nodes, and aortas were placed in 50 μL of RNA later from Qiagen (Valencia, CA, USA) immediately after harvest. QIAshredder kit (Valencia, CA, USA) was used to homogenize each sample after Trizol treatment. RNA extraction performed using RNeasy Mini kit (Valencia, CA, USA). RNA was converted to cDNA using iScript Reverse Transcription kit for RT-qPCR (BioRad, Hercules, CA, USA). Primers were commercially obtained as part of a Taqman PCR kit (Life Technologies, New York, NY, USA), and included CD4, IFNγ, TNFα, IL-2, IL-4, IL-10, IL-17A, Tbx21 (Tbet), GATA3, RORγT, and FoxP3. Housekeeping genes used in the analysis were one of two ribosomal proteins, Rpl32 or Rpl13A.

**STATISTICAL ANALYSIS**

Between groups analysis was performed by one-way ANOVA. Data are expressed as mean ± SEM. P-values < 0.05 were considered significant.

**RESULTS**

**IDENTIFYING CANDIDATE EPITOPES ApoB3501–3516 AND ApoB978–993**

A peptide can only be recognized by a CD4+ helper T-cell if it is bound to MHC class II. Because Apoc4+/− mice are on a C57B/6 background, candidate epitopes must be able to bind to the Class II allele, I-Aβ. Previously, it has been shown that I-Aβ binding motifs can be predicted using algorithms based on the number of anchor residues and hydrophobicity (36–38). We measured I-Aβ binding affinity of six candidate peptides predicted by these algorithms. The binding affinity is reflected by the amount of peptide, in nanomoles, needed to inhibit binding of a standardized radio-labeled peptide by 50% (the IC50) (39). Of the six peptides synthesized, only ApoB3501–3516 and ApoB978–993 bound I-Aβ with significant affinity (Table 1, ApoB3501–3516 IC50 = 4.3 nM, ApoB978–993 IC50 = 7.3 nM). Peptides containing only parts of these sequences showed reduced binding affinities (Table 2). By comparison, the known T-cell epitopes OVA224–337 and MOG18–51 (truncated form of MOG35–55, i.e., myelin oligodendrocyte glycoprotein) have an IC50 of 400 nM (43) and 354 nM (44), respectively (Table 1). Based on these binding affinities, the remainder of this work was performed using ApoB3501–3516 and ApoB978–993.

**ANTIGEN-SPECIFIC T-CELL PROLIFERATIVE RESPONSES**

Two hundred micromolars (μM) of either ApoB3501–3516 or ApoB978–993 (emulsified in CFA) were administered intraperitoneally at age 12, 16, 20, and 22 weeks (Figure 1A). Mice were sacrificed at age 23 weeks of age (13 weeks WD) and organs were harvested for analysis. Control immunizations with adjuvant only (1 μCFA + 4 μIFA) and an irrelevant peptide (MOG35–55) were done under identical conditions.

**CHOLESTEROL LEVELS**

Western diet-fed Apoc4+/− mice exposed to the PBS plus 1 × CFA + 4 × IFA regimen had significantly decreased total plasma cholesterol from 1292 to 960 mg/dL, HDL from 246 to 168 mg/dL, and non-HDL from 1046 to 797 mg/dL, and triglycerides from 203 to 183 mg/dL compared to WD-fed Apoc4+/− mice not exposed to adjuvant. Similar observations have been reported in other studies (45). However, none of the mice immunized with ApoB3501–3516 or ApoB978–993 had plasma lipid levels different than the PBS plus 1 × CFA + 4 × IFA controls (Table 3). Therefore, all subsequent statistical analyses were performed without the untreated group.

**IMMUNIZATION WITH ApoB3501–3516 AND ApoB978–993**

ApoB3501–3516 or ApoB978–993 were each used to vaccinate 10–14 female Apoc4+/− mice. Fifty micrograms of ApoB3501–3516 or ApoB978–993 emulsified in CFA were subcutaneously injected above the inguinal LN at 8 weeks of age. A WD was then started at 10 weeks of age. Repeated boosters with 25 μg of ApoB3501–3516 or ApoB978–993 emulsified in IFA were administered intraperitoneally at age 12, 16, 20, and 22 weeks (Figure 1A). Mice were sacrificed at age 23 weeks of age (13 weeks WD) and organs were harvested for analysis. Control immunizations with adjuvant only (1 × CFA + 4 × IFA) and an irrelevant peptide (MOG35–55) were done under identical conditions.

**IMMUNIZATION WITH EITHER ApoB3501–3516 OR ApoB978–993 RESULTS IN LESS ATHEROSCLEROTIC PLAQUE WHEN USED IN AN IMMUNIZATION SCHEME USING BOTH CFA AND IFA**

Both ApoB3501–3516 and ApoB978–993–treated mice showed ~40% reduction in en face lesion size by Sudan IV staining of whole aortas when compared with PBS and MOG35–55 (Figures 1B,C) immunized mice. Aortic root lesions were also examined for plaque burden by oil red O (ORO) staining. ApoB3501–3516 immunized mice had >60% reduction (p < 0.01) in overall aortic sinus plaque development compared to 1 × CFA + 4 × IFA (adjuvant-only treated) mice (Figures 1D,E). ApoB978–993 immunized mice showed no significant reduction in aortic root plaque burden. MOG35–55 immunized mice showed no significant decrease. These data demonstrate that immunization with I-Aβ restricted peptide

| Peptide | Sequence | Len | Pos | H-2 I-Ab (IC50 nM) |
|---------|----------|-----|-----|------------------|
| ApoB3501–3516 | SFTKGNIKSSFLSQEY 16 3489 1169 | SFSLQGEYSVGSAVNEA VY 16 3497 6.8 |
| ApoB978–993 | SQEYSVGSAVNEAY 15 3501 4.3 | SGSVANEVNYLNKG 3505 172 |
| ApoB978–993 | NLYVNLNSGTRSSVRLQ 16 3513 907 |
| ApoB978–993 | TGFNYCTTGAYNSA 16 969 655 |
| ApoB978–993 | TTGAYNSATSEASY 15 977 17 |
| ApoB978–993 | TSTESASYPLGTGDT 16 985 1258 |

Sequence of ApoB3501–3516 and ApoB978–993 indicated in boldface.
Atherosclerosis is decreased in ApoB<sub>3501–3516</sub> and ApoB<sub>978–993</sub>-treated mice compared to controls. (A) Vaccination schedule: 8-week-old female Apoe<sup>−/−</sup> mice were immunized once with either PBS or peptide in CFA, then boosted four more times with PBS or peptide in IFA. WD was maintained for 13 weeks. Mice were sacrificed and organs harvested at 23 weeks of age. (B,C) Results of aortic pinning analysis after Sudan IV staining are shown with representative photographs. N = 12–15 in each group, *p < 0.05 when compared to 1 × CFA + 4 × IFA group. (D) Representative aortic root staining sections after ORO staining, counter-stained with hematoxylin. (E) Plaque area from aortic roots stained from each group. Lesion sizes from 30 to 40 µm distal to start of the aortic valve were averaged per group. N = 5 in each group, *p < 0.05 when compared to 1 × CFA + 1 × IFA control group.

Table 3 | Lipid profile of mice.

|                  | Untreated | PBS          | ApoB<sub>3501–3516</sub> | ApoB<sub>978–993</sub> | MOG<sub>35–55</sub> |
|------------------|-----------|--------------|---------------------------|-------------------------|----------------------|
| TC (mg/dL)       | 1292.0 ± 145.9* | 960.3 ± 100.5 | 870.4 ± 103.6             | 835.2 ± 108.3           | 1014.7 ± 76.4        |
| HDL (mg/dL)      | 245.7 ± 90.9*  | 1677 ± 34.6  | 202.1 ± 23.8              | 170.3 ± 6.9             | 213 ± 75             |
| Non-HDL (mg/dL)  | 1046.3 ± 105.8* | 796.6 ± 119.3 | 663.7 ± 124.7             | 592.2 ± 187.1           | 801.7 ± 72.4         |
| TG (mg/dL)       | 203.3 ± 21.0*  | 182.6 ± 21.9 | 158.1 ± 32.5              | 144.7 ± 37.3            | 142.3 ± 33.2         |
| Weight (g)       | 26.8 ± 2.2    | 25.4 ± 1.9   | 26.3 ± 1.8                | 25.9 ± 2.1              | 25.3 ± 1.1           |

N = 6–7 per group, presented as mean ± SD.
*p < 0.05 when untreated group is compared to groups exposed to adjuvant.

Fragments from murine ApoB-100 can reduce plaque burden in Apoe<sup>−/−</sup> mice.

**IMMUNIZATION WITH EITHER ApoB<sub>3501–3516</sub> OR ApoB<sub>978–993</sub> RESULTS IN PEPTIDE-SPECIFIC IgG TITERS**

One possible mechanism of atheroprotection is the development of protective antibodies (24). The production of IgG requires antigen-specific T-cell help and gives insight into antigen-specific T-cell activation and lineage bias. IgG1 is a marker of T-helper type 2 (Th2) activity and IgG2c of T-helper type 1 (Th1) activity in C57BL/6 mice, which do not express IgG2a (25). Pooled plasma from each group was analyzed for immunoglobulin titers by formal antibody dilution curves using chemiluminescent ELISA (Figure S1 in Supplementary Material). As shown in Figures 2A,B for the 1:250 dilution, as expected IgG responses to ApoB<sub>3501–3516</sub> and ApoB<sub>978–993</sub> peptides were detected in...
ApoB3501–3516 and ApoB978–993 immunized mice, respectively. Their responses showed complete peptide specificity, with strong responses in both the Th1 and Th2 helper T-cell compartments. Total IgG1 and IgG2c antibody levels (not antigen-specific) were similar across all groups (data not shown). MOG35–55 immunization produced a predominantly IgG2c response to MOG35–55 (Figure 2C), but no antibody titers to ApoB3501–3516 or ApoB978–993 were detected (Figures 2A,B). 1× CFA + 4× IFA immunized mice did not have detectable levels of IgG1 or IgG2c against ApoB3501–3516 or ApoB978–993 (Figures 2A,B). None of the immunized mice had elevated IgG titers against native LDL or MDA-LDL compared to adjuvant only (Figures 2D,E).

REDUCED ATHEROSCLEROTIC PLAQUE BURDEN DOES NOT CORRELATE WITH AN INCREASE IN THE NUMBER OF FoxP3-EXPRESSING CELLS

Another possible mechanism of atheroprotection conveyed by immunization with ApoB3501–3516 or ApoB978–993 could be related to increased numbers of FoxP3-expressing regulatory T-cells.
Whole aortas, along with spleens and lymph nodes (para-aortic, inguinal, axillary, mesenteric) were harvested from immunized mice at the time of sacrifice. There were no significant differences in FoxP3+ cells within the CD4+/TCRβ+ cell population in the aorta (Figures 3A,B), para-aortic lymph nodes (Figure 3C), spleens (Figure 3D), or non-draining lymph nodes (inguinal, axillary, mesenteric; Figure 3E) when ApoB3501–3516 and ApoB978–993 were compared to the 1× CFA + 4× IFA control group.

**IMMUNIZATION WITH ApoB3501–3516 OR ApoB978–993 INCREASES mRNA EXPRESSION OF THE AHEROPROTECTIVE CYTOKINE, IL-10**

RT-PCR analysis of spleens, lymph nodes, and aortas of mice from each group were analyzed for mRNA expression levels of the Th1 cytokines IFNγ, TNFα, and the Th1 transcription factor Tbx21 (Tbet), the Th2 cytokines IL-4, IL-10, and the Th2 transcription factor GATA3, the Th17 cytokine IL-17A, and the Th17 transcription factor RORγT, and the regulatory T-cell transcription factor
FIGURE 4 | Real time RT-PCR analysis of IL-10 mRNA expression. Organs were harvested and immediately placed into RNA stabilization reagent, and frozen at −80°C. IL-10 mRNA expression from (A) aortas, (B) para-aortic lymph nodes, (C) spleens, and (D) non-draining lymph nodes are shown. *p < 0.05 compared to 1× CFA + 4× IFA. N = 3–5 in each group.

DISCUSSION

Our results show that MHC Class II restricted CD4+ T-cell peptides from the ApoB-100 are effective at reducing atherosclerotic plaque burden in Apoe−/− mice. ApoB3501–3516 or ApoB978–993 are high affinity binders for MHC Class II (I-Aβ). This is the first publication to identify such peptides within an atherosclerosis-relevant protein (ApoB-100).

T-cell responses to vaccination with either (ox)LDL or peptide epitopes from ApoB-100 have become an area of great interest because they may enable the development of a vaccine for clinical use. Recent reports have suggested that immunization with an epitope from ApoB-100 that binds to serum antibodies, P210, results in atheroprotection via an increase in TREGs (32, 33), but the mechanism by which a B-cell epitope could induce TREGs was not elucidated. It is not known whether these TREGs are causally related to the atheroprotection that is observed. In fact, P210 does not bind to I-Aβ (34), suggesting that any actions immunizing with P210 might have are highly unlikely to be related to CD4+ helper T-cells. One publication has instead suggested that the observed atheroprotection is a result of enhanced CD8+ cytotoxic T-cell activity against dendritic cells leading to a decreased number of CD11c+ cells within the aorta, and thus reduced atherosclerosis (46). These researchers demonstrated that adoptive transfer of P210 primed CD8+ T-cells recapitulated the atheroprotective response in naive mice. These contrasting views of how immunization may result in atheroprotection highlight the need for systematic studies into MHC-restricted peptide vaccinations.

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Mice immunized with ApoB3501–3516 or ApoB378–993 in the context of CFA and IFI show increased IL-10 mRNA expression levels in the aortas, significantly above control mice. Since this increased expression is not associated with an increased in the percent of aortic FoxP3+ regulatory T-cells, we speculate that the IL-10 mRNA may be derived from FoxP3+ T Tr cells (47, 48) (i.e., one subset of inducible Tregs) or from myeloid cells (49). While both IFNy and IL-17A have been implicated as pro-atherosclerotic cytokines, no changes in mRNA expression of either cytokine was observed in the aortas, lymph nodes, or spleens of immunized mice compared to controls.

We did not detect IgG1 or IgG2c antibody titers to ApoB3501–3516 or ApoB378–993 in non-immunized mice. This is despite highly elevated plasma cholesterol levels in Apoe−/− mice (50). One possibility is that ApoB3501–3516 and ApoB378–993 may not be naturally processed products of APCs. Another possibility is that the endogenous forms of ApoB3501–3516 and ApoB378–993 are not presented efficiently or in high enough quantities by APCs. Further investigations into the mechanism of atheroprotection will provide new targets for therapy and prevention of atherosclerosis. It is our hope that these two peptides, and future peptides that can be discovered by the described immunologic methods, will lead to a new frontier in atherosclerosis research and ultimately provide a treatment for this worldwide epidemic.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at SUPPLEMENTARY MATERIAL.

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