Title

Regulatory CD4+ T Cells Recognize Major Histocompatibility Complex Class II Molecule–Restricted Peptide Epitopes of Apolipoprotein B

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Regulatory CD4+ T cells recognize MHC-II-restricted peptide epitopes of apolipoprotein B

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Abstract

Background—CD4^{+} T cells play an important role in atherosclerosis, but their antigen specificity is poorly understood. Immunization with apolipoprotein B (ApoB, core protein of low density lipoprotein) is known to be atheroprotective in animal models. Here, we report on a human APOB peptide, p18, that is sequence-identical in mouse ApoB and binds to both mouse and human MHC-II.

Methods—We constructed p18-tetramers to detect human and mouse APOB-specific T cells and assayed their phenotype by flow cytometry including CD4 lineage transcription factors, intracellular cytokines, and TCR activation. $Apoe^{-/-}$ mice were vaccinated with p18 peptide or adjuvants alone and atherosclerotic burden in the aorta was determined.

Results—In human peripheral blood mononuclear cells from donors without cardiovascular disease (CVD), p18 specific CD4^{+} T cells detected by a new HLA-DR-p18 tetramers were mostly Foxp3^{+} regulatory T cells (Tregs). Donors with subclinical CVD as detected by carotid artery ultrasound had Tregs co-expressing RORγt or T-bet which were both almost absent in donors without CVD. In $Apoe^{-/-}$ mice, immunization with p18 induced Tregs and reduced atherosclerotic lesions. After peptide restimulation, responding CD4^{+} T cells identified by Nur77-GFP were highly enriched in Tregs. A new mouse I-A[b]-p18 tetramer identified the expansion of p18-specific CD4^{+} T cells upon vaccination, which were enriched for IL-10-producing Tregs.

Conclusion—These findings show that APOB p18 specific CD4^{+} T cells are mainly Tregs in healthy donors, but co-express other CD4 lineage transcription factors in donors with subclinical CVD. This study identifies ApoB peptide 18 as the first Treg epitope in human and mouse atherosclerosis.

Keywords

Introduction

About 10-15% of human blood CD4^{+} T cells are regulatory T cells (Tregs), characterized by expression of the transcription factor FoxP3, the IL-2 receptor subunit CD25, and other markers[1]. These cells mediate peripheral tolerance, an antigen-specific protection from autoimmunity. Patients with congenital null mutations in the *FOXP3* gene suffer from severe autoimmune symptoms (IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome).

Atherosclerosis is a chronic inflammatory disease of the arterial wall. Atherosclerosis is driven by low density lipoprotein (LDL), which accumulates in the arterial wall. There, it is modified by oxidation, which leads to recognition by various scavenger receptors on antigen presenting cells[2-4]. Atherosclerotic plaques contain T cells, most of which are CD4^{+} T cells in humans[5] and mice[6]. Apolipoprotein B (ApoB) is the core protein in LDL and is a known atherosclerosis antigen[7]. Recently, we identified peptide epitopes from ApoB that bind to MHC class II molecule (MHC-II) and are presented to CD4^{+} T cells[8, 9]. That such antigen

presentation can occur in the arterial wall has been demonstrated in mice[10]. Recent studies have shown that immunization with ApoB peptides reduces atherosclerotic plaque[8, 9].

Tregs are known to be protective against atherosclerosis. Adoptive transfer of Tregs has been shown to reduce atherosclerosis[11] whilst depletion of Tregs exacerbated atherosclerosis[12]. The atheroprotective action of Tregs is, at least in part, mediated by secretion of anti-inflammatory cytokines such as IL-10[13] and TGF-β[14]. Regulatory T cells type 1 (Tr1: IL-10$^+$FoxP3$^-$) are also atheroprotective[15]. Tregs in human peripheral blood decline in patients with coronary artery disease[16], suggesting that a loss of Tregs is associated with disease progression. Similarly, the abundance of aortic Tregs decreased in progression of atherosclerosis in *Ldlr*$^{-/-}$ mice fed a cholesterol-rich diet. Recently, antigen-specific regulatory T cell were reported to be protective in Goodpasture syndrome, a rare lung and kidney disease[17]. Atherosclerosis antigen-specific Treg epitopes have not been identified.

Other CD4$^+$ T cell subsets can be pro-atherogenic, especially T-helper-1 cells (Th1)[18]. Th1 cells express the lineage-defining transcription factor T-Bet (*TBX21*). Studies of autoimmune disease suggest that antigen-specific autoreactive Th1 and Th17 play a role in disease progression of rheumatoid arthritis[19] and type 1 diabetes[20]. The role of Th17 cells, defined by the transcription factor RORγt (*RORC*), in atherosclerosis is controversial[21, 22].

CD4$^+$ T cells recognize antigenic peptides only when presented in the groove of MHC-II. In previous work, we showed that MHC-II binding peptides from mouse ApoB are atheroprotective when mice are vaccinated using a suitable protocol and adjuvant[8, 9]. Interestingly, vaccinating with these same peptides conjugated to splenocytes had no protective effects[23]. Also, immunization with the same peptide in CFA and following subcutaneous injection of peptide with CFA (CFA/CFA) has been shown to exacerbate atherosclerosis[24].

Here, we constructed tetramers[25] to identify ApoB-specific autoreactive CD4$^+$ T cells in humans and mice to study the mechanism by which atheroprotective vaccination works. We screened human APOB for epitopes binding to the 28 most common human HLA-DR alleles[26], first by a prediction algorithm (NN-align)[27] and then by experimental competitive peptide binding[28]. Among the binding peptides, we identified peptide 18 (p18) that binds DRB1*01:01, DRB1*04:01, DRB1*07:01, DRB1*09:01, DRB1*13:02, and also mouse I-A^b. The p18 sequence, SLFFSAQPFEITAST, is found in both human APOB and mouse ApoB (Figure 1A, B). We constructed human (DRB1*0701) and mouse (I-A^b) tetramers and used them to interrogate the antigen specificity and phenotype of CD4$^+$ T cells. We tested p18-specific CD4$^+$ T cells isolated from peripheral blood mononuclear cells (PBMCs) from women without and with subclinical cardiovascular disease (CVD). We interrogated samples from the women's interagency HIV study (WIHS), which is a long-running, NIH-funded large clinical/epidemiological study with exceptionally well-phenotyped participants who have been followed with standardized examinations and specimen collections every six months. HIV-infected subjects have a higher risk of developing cardiovascular disease[29]. We built on almost 2 decades of experience, starting from a longitudinal study of carotid artery ultrasound along with standard CVD risk assessments (blood pressure, lipids, glycemic status, smoking) which has been of proven value for characterizing the excess burden of

vascular disease associated with HIV infection and its comorbidities. The subclinical cardiovascular disease measure used in this study is assessed in a highly standardized fashion by an experienced imaging reading center (both intima media thickness and lesion count by ultrasound)[30]. We have shown that carotid ultrasound measurements bear the expected relationships with known cardiovascular risk factors[31]. In fact, long term follow-up shows that the presence of carotid artery ultrasound-defined lesions is an independent predictor of future development of cardiovascular events[32]. Many of the participants have been HLA-typed, which is essential for the present study, because we have a very limited set of reagents (currently DRB1*01:01 and *07:01). To our knowledge, this is the first study to successfully use tetramers to any atherosclerosis autoantigen.

In healthy humans, we find that most p18-specific CD4 T cells are Tregs. In mice, vaccination induces p18-specific CD4$^+$ Tregs. Thus, our data identify the first natural Treg epitope in human and mouse atherosclerosis.

Methods

The data, analytic methods, and study materials will be/have been made available to other researchers for purposes of reproducing the results or replicating the procedure. All materials will be made available to other investigators. The human tetramers described are produced on a fee-for-service basis by Dr. William W. Kwok (Tetramer Core Laboratory, Benaroya Research Institute at Virginia Mason, Seattle, Washington, USA). The mouse tetramers were made by Dr. Marc K. Jenkins as part of a subcontract of NIH R01 HL 121697, which supported this work. Dr. Klaus Ley will make small amounts (up to 10 tests per investigator) available. Larger amounts will require an agreement with Dr. Jenkins. All other reagents are commercially available and sourced throughout the material and method section.

Mice

Eight-week-old female *Apoe*$^{-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *FoxP3*^{YFPcre} mice[33] which were provided by Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology) and crossbred with *Apoe*$^{-/-}$ mice to obtain *FoxP3*^{YFPcre} *Apoe*$^{-/-}$ mice. *Nur77*^{GFP} mice[34] were purchased from Jackson Laboratories and crossed into *Apoe*$^{-/-}$ mice to obtain *Nur77*^{GFP} *Apoe*$^{-/-}$ mice. *IL-10*^{GFP} mice[35] were provided by Mitchell Kronenberg and crossbred with *Apoe*$^{-/-}$ mice. 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 western-type diet (WD), adjusted calories diet with 42% from fat (Harlan Labs Cat #: TD.88137, CA, USA) and remained on WD until sacrifice. All animal experiments were conducted in accordance with the institutional guideline for the La Jolla Institute for Allergy and Immunology animal facility.

Peptide

p18 peptide (Figure 1A) was custom synthesized as purified material by A&A Labs (San Diego, CA, USA). Predicted MHC class II binding nonamer was determined using the

Immune Epitope Database and Analysis Resource (IEDB, http://www.iedb.org/). The predicted binding (IC_{50}) to I-A^b, and DRB1*07:01 is 124.9, and 11.2 nM, respectively (based on NN-align[27]). Their MHC class II binding affinity was measured using a competitive inhibition assay utilizing purified MHC and high affinity radio-labeled MHC class II ligand (Figure 1B), as previously described[28]. Under the conditions utilized, the measured IC_{50} values are reasonable approximations of the true K_d values. Synthesized peptides were dissolved in DMSO at 20 mg/ml and stored at −80°C.

Immunization

For mouse immunization, 50 μg of peptide was emulsified in CFA (BD Difco, Sparks, MD, USA) and injected into the subcutaneous (s.c.) inguinal area at 8 weeks of age. Repeated boosters with 50 μg of the same peptide emulsified in IFA (BD Difco) were administered intraperitoneally (i.p.) at age 12, 16, 20, and 22 weeks. Western diet was started at 10 weeks of age. This immunization scheme will be referred to as 1xCFA + 4xIFA. Mice were sacrificed at age 23 weeks and organs were harvested for analysis. Control immunizations were with DMSO emulsified in CFA and IFA. Some experiments were done by subcutaneous injection of peptide with CFA at 8 weeks and intraperitoneal injection of peptide with IFA at 10 weeks, which is referred to as 1xCFA + 1xIFA.

Atherosclerosis Quantification

To measure en face lesion formation, each aorta was carefully cleaned in situ and then the whole aorta pinned out after 4% paraformaldehyde incubation at RT for at least 2 hours. Staining for atherosclerotic plaque was performed by incubating samples in Sudan IV. The aortic surface area was determined using Photoshop CS (Adobe Systems, CA, USA). The quantification of plaque lesion was performed using Image-Pro Premier software (Media Cybernetics, Rockville, MD, USA). The atherosclerotic lesions were determined independently by two blinded investigators.

Lipid Analysis

Mouse whole blood was collected in EDTA tubes by cardiac heart puncture during organ harvest. The blood was spun at $3000 \times g$ for 15 min at 4°C. The supernatant was collected and frozen at −80°C until analysis to reduce multiple freeze/thaw cycles. Lipid analysis was performed by IDEXX BioResearch (MO, USA).

I-A^b Tetramer

Biotin-labeled I-A^b monomers containing ApoB peptide covalently linked to the I-A^b beta chain were grown in *Drosophila melanogaster* S2 cells also expressing the I-A^b alpha chain. Monomers were purified and combined with PE- or APC-conjugated streptavidin (Prozyme, Hayward, CA) to produce fluorescence-labeled I-A^b tetramers as described[36, 37].

HLA-DRB1*07:01 tetramer

Recombinant HLA-DR proteins were generated as described[38]. Briefly, HLA-DR was purified from the supernatants of transfected insect cells, biotinylated, and dialyzed into 0.1M phosphate buffer. Biotinylated monomer was loaded with 0.2 mg/ml of peptide by incubating at 37°C for 72 hours in the presence of 2.5 mg/ml n-octyl-β-D-glucopyranoside and 1 mM Pefabloc SC protease inhibitor (Sigma-Aldrich, St. Louis, MO) and then conjugated using PE- or APC-conjugated streptavidin (Biosource International, Camarillo, CA) at a molar ratio of 8 to 1.

Human PBMC

Blood samples were collected in the Women's Interagency HIV Study (WIHS)[39]. The samples used in this study were collected between 2003 and 2005. Briefly, patients enrolled in the WIHS undergo semi-annual follow up visits including detailed examinations, specimen collection, and structured interviews about health behaviors, medical history, and medication use. All participants provided informed consent at each site participating in WIHS which obtained institutional review committee approval. Peripheral blood mononuclear cells (PBMCs) were processed and stored in liquid nitrogen at 6 million cells per aliquot or more[39]. Subclinical atherosclerosis was measured by carotid artery intima-media thickness using high-resolution B-mode carotid artery ultrasound[30]. Presence of lesions was defined as a focal intima-media thickness (IMT) more than 1.5 mm in any of the imaged segments[31]. The study was approved by an institutional review committee and.

HLA genotyping of human PBMCs

Human PBMCs were assessed for HLA genotype as previously described[40]. In brief, lymphoblastic B cells were generated from PBMCs of respective individuals. Genomic DNA was prepared and genotyped for HLA by standardized protocols provided by the International Histocompatibility Working Group (www.ihwg.org). Specifically, HLA class II typing was performed by applying sequence-specific oligonucleotide (SSO) probes for HLA-DQA, HLA-DQB, and HLA-DRB1 loci using the polymorphic exon 2. For DRB genotyping, the broad serological DR types were determined using a pair of DRB generic primers and a panel of SSO probes. Subsequently, allele-level DRB typing was exerted with group-specific primers amplifying the DRB alleles determined in the previous genotyping reaction followed by SSO hybridization. DQA and DQB were genotyped by locus-specific PCR followed by SSO hybridization.

Detailed methods about flow cytometry, ELISPOT, and ELISA are provided in the online-only Data Supplement.

Statistics

Between groups analysis was performed by Mann-Whitney U-test for 2 groups and Kruskal-Wallis test with Dunn's multiple comparison test for 3 groups. Where appropriate, statistical significance was tested with unpaired t-Test. For the analysis of mice vaccinated and fed chow diet or western-type diet ordinary one-way ANOVA was performed and followed by

Dunnett's test for multiple comparisons. The mean of each column was compared to the mean of the CD PBS group. The respective statistical tests are indicated in each figure legend. Data are expressed as mean ± SEM. *P*-values less than 0.05 were considered significant.

Study approval

All animal protocols were approved by The Animal Care Committee of La Jolla Institute for Allergy and Immunology. All human experiments of PBMC have been approved by the WIHS Executive Committee.

Results

The human tetramer reagent consisting of recombinant DRA and DRB1*07:01 carrying the p18 peptide (Figure 1C) was biotinylated, and tetramerized with streptavidin labeled with PE (phycoerythin) or APC (allophycocyanin). We used these two differently labeled tetramers in order to reduce non-specific events and considered only PE[+]APC^{+} cells p18-specific. We screened human PBMCs collected from participants in the Women's Interagency HIV Study for expression of the HLA-DRB1*07:01 allele[39]. Some participants had subclinical atherosclerosis as detected by B mode carotid artery ultrasound whereas most participants were HIV infected (Supplementary Table 1)[30, 31]. Subclinical CVD (sCVD) was defined as at least one detectable lesion. We selected HLA-DRB1*07:01 because p18 peptide has a high affinity for DRB1*07:01 (Figure 1B), and the DRB1*07:01 allele frequency is relatively high (8-14% dependent on race[26]). Characteristics of all donor participants are shown in Supplementary Table 1. We consistently and reproducibly detected p18-specific CD4^{+} T cells in human PBMC using this novel DRB1*07:01-p18 tetramer (Figure 1D-F) at a frequency of 0.17% (1700 cells per million CD4^{+} T cells). In mice the natural repertoire is 10 to 100 per million CD4^{+} T cells[36], and a similar frequency has been reported in humans[41]. Thus, this number suggests that CD4^{+} T cells with T cell receptors (TCRs) recognizing p18 (a self antigen) are already expanded even in subjects without CVD. Specificity was tested by staining PBMCs from mismatched controls who do not express the DRB1*07:01 allele, and although the background staining was found to be less than 0.04% (Figure 1E,F), significantly lower than in subjects expressing DRB1*07:01, not all DRB1*07:01 subjects had detectable p18^{+} CD4^{+} T cells above background. Back-gating was used to monitor non-specific staining of non-CD4 T cells, which was found to be very low or absent (Supplementary Figure 1). These findings show that APOB p18-specific CD4^{+} T cells exist in human blood.

Next, we tested the phenotype of the human p18-specific CD4^{+} T cells by intracellular staining for transcription factors. Staining DRB1*07:01-p18^{+}CD4^{+} T cells for RORγt, Tbet, GATA3, and FoxP3 classified more than half of DRB1*07:01-p18^{+}CD4^{+} T cells both in the sCVD group and the non-CVD group into Th17, Th1, Th2 and Treg subsets, respectively. A large fraction of Tregs (8.8±3.7%) of the DRB1*07:01-p18^{+}CD4^{+} T cells from sCVD donors co-expressed RORγt, a combination that was almost undetectable in sCVD^{−} participants. Also, the frequency of Tregs co-expressing T-bet increased among DRB1*07:01-p18^{+}CD4^{+} T cells from sCVD patients (4.1±1.4% vs. 1.2±0.5%). Conversely,

fewer cells expressed FoxP3; the ratio of FoxP3 and RORγt double expressing to FoxP3 single p18-specific CD4+ T cells significantly increased in sCVD donors (Figure 1G,H,I; Supplementary Figure2). These effects were specific to DRB1*07:01-p18+CD4+ T cells and not observable in p18-negative, bulk CD4+ T cells (Supplementary Figure 3). These findings suggest that the phenotype of p18-specific T cells may change during the development of atherosclerosis.

For mechanistic experiments, we interrogated ApoB p18-specific CD4+ T cells in *Apoe*−/− mice, a commonly used model of atherosclerosis. Previously, we had shown that vaccination with ApoB peptides was atheroprotective and induced IL-10-producing CD4+ T cells, many of which were found to reside in the peritoneal cavity[8, 9]. We measured FoxP3 expression (by YFP in *FoxP3*YFPcre*Apoe*−/− mice[33], Figure 2A). The fraction of CD25+FoxP3+CD4+ T cells was significantly doubled from ~6% to ~12% of all CD4+ T cells in the peritoneal cavity 2 weeks after immunization with p18 (1xCFA s.c. and 1xIFA i.p., Figure 2B). Intracellular staining of CD4+ T cells for FoxP3 in *Apoe*−/− mice gave a similar result (Figure 2C).

This data was corroborated in a second model of vaccination in which *Apoe*−/− mice were administered chow diet (CD) or western diet (WD) and peritoneal CD4+ T cells were probed for CD4 T cell lineage defining transcription factor expression. Vaccination with p18 (1x in CFA and 1x in IFA) significantly increased the abundance of Tregs in the peritoneal cavity (Figure 2D). Besides classical Tregs, also FoxP3+Tbet+ (Figure 2E) and FoxP3+GATA3+ cells (Figure 2F) were increased. Interestingly, the expansion of the FoxP3+GATA3+ cells was only observed in mice fed CD and not in mice fed WD.

Most of the classical Tregs were inducible Tregs (iTregs), which do not express the natural Treg marker Neuropilin-1 (Nrp-1, Figure 2G). We also tested expression of IL-10, a known atheroprotective cytokine[16]. Its anti-inflammatory effect is mediated, in part, by preventing activation of the mTOR pathway in macrophages[42]. We found a significant expansion of IL-10-producing cells among all Tregs (FoxP3+CD4+ cells, Figure 2H). IL-10-producing iTregs (Nrp-1−FoxP3+CD4+) significantly expanded from ~12% to ~25% (Figure 2I). These data show that vaccination with p18 induces IL-10-producing Tregs.

To more directly monitor T cell activation, we made use of the *Nur77*GFP reporter mouse[34] crossed into the *Apoe*−/− background. Nur77, an orphan nuclear receptor, is an immediate early gene up-regulated by T cell receptor stimulation in T cells. In *Nur77*GFP mice, GFP is transiently expressed in T cells after antigen receptor stimulation, but not by inflammatory stimuli[34]. 24 hours after intraperitoneal injection of p18 in IFA (two weeks after boost immunization of p18 in IFA) to restimulate vaccination-induced T cells, we observed Nur77GFP+CD4+ T cells in the peritoneal cavity (Figure 3A, B). The percentage of Nur77GFP+ cells significantly increased from ~2% to ~ 7% among CD4+ T cells (Figure 3C), indicating antigen-specific activation of their T cell receptors. Among CD25+FoxP3+ Tregs, the frequency of Nur77GFP+ cells was higher than among all CD4+ T cells, and this difference was further elevated in mice immunized with p18 (Figure 3D, E). These data show that Tregs respond to immunization with p18 by expanding and by expressing the activation marker Nur77. CD25+FoxP3+ Tregs were significantly enriched in the Nur77GFP+

activated cell population in p18 immunized mice (Figure 3F). However, mice treated with adjuvant only showed a similar fraction of Tregs among the Nur77^{GFP+} cells (data not shown), which likely reflects the known propensity of Tregs to express NUR77[34].

To directly test antigen-dependent CD4$^+$ T cell responses, we developed a mouse MHC-II tetramer to detect p18-specific CD4$^+$ T cells in mice. Recombinant I-A^b with p18 tethered by a short sequence tetramerized and labeled as described[37]. CD4$^+$ T cells were harvested from the peritoneal cavity of *Nur77^{GFP}Apoe$^{-/-}$* mice immunized with p18 (1x in CFA and 1x in IFA), enriched for CD4$^+$ T cells by negative selection with magnetic beads and stained with p18:I-A^b-PE and p18:I-A^b-APC tetramer (Figure 4A). PE- and APC- double positive CD4$^+$ T cells were considered ApoB p18-specific T cells and significantly increased ~3-fold after immunization with p18 compared to adjuvant only control (Figure 4B). Among the p18:I-A^{b+}CD4$^+$ T cells, CD25$^+$FoxP3$^+$ Tregs were greatly enriched, reaching 40% of all p18:I-A^{b+}CD4$^+$ T cells, compared to 9% in p18:I-A^{b-}CD4$^+$ T cells (Figure 4C). The p18:I-A^{b+}CD4$^+$ T cells were also highly enriched in Nur77^{GFP+} cells (Figure 4D). Almost no Nur77^{GFP+} cells were found among the p18:I-A^{b-} population (data not shown).

To evaluate IL-10 production of antigen-specific CD4$^+$ T cells, we immunized *IL-10^{GFP}* mice[35] crossed into the *Apoe$^{-/-}$* background with p18. We confirmed that the intensity of IL-10-GFP is well correlated with IL-10 expression determined by intracellular staining (Supplementary Figure 4). p18:I-A^{b+}CD4$^+$ T cells were also increased in spleens harvested from mice immunized with p18 (Figure 5A). A large fraction of p18:I-A^{b+}FoxP3$^+$CD4$^+$ T cells produced IL-10 in immunized mice significantly more than in adjuvant-only controls (Figure 5B, C). p18:I-A^{b-}FoxP3$^+$CD4$^+$ did not increase IL-10 production after immunization (Figure 5C).

Since p18-specific T cells are exposed to endogenous antigen from ApoB, we expected that they would have an antigen-experienced memory phenotype. Most of p18:I-A^{b+}CD4$^+$ T cells from vaccinated *Apoe$^{-/-}$* mice indeed showed a memory phenotype (CD44$^+$). Among those, 80% were effector memory cells defined as CD62L$^-$CD44$^+$ (Figure 5D), and 20% were central memory cells (CD62L$^+$CD44$^+$). We did not find a significant difference in the fraction of splenic bulk FoxP3$^+$CD4$^+$ T cells between p18-immunized mice and adjuvant only controls (data not shown). However, significantly more p18:I-A^{b+}CD4$^+$ T cells harvested from spleens of p18-immunized mice showed expression of FoxP3 compared to adjuvant controls (Figure 5E). A similar expansion of p18:I-A^{b+}Tregs was also observed in the spleen of p18 immunized *Apoe$^{-/-}$* mice fed CD (Figure 5F). We did not observe any significant differences among peritoneal p18:I-A^{b+}CD4$^+$ T cells from p18-immunized or adjuvant-injected *Apoe$^{-/-}$* mice fed CD or WD (Supplementary Figure 5).

Next, we tested cytokine production in response to peptide restimulation using ELISPOT. We isolated CD4$^+$ T cells from spleens of *Apoe$^{-/-}$* mice vaccinated with p18 or adjuvant only (1xCFA and 1xIFA). CD4$^+$ T cells were cultured with antigen presenting cells and p18 for 24 hours on ELISPOT plates coated with IL-10 capturing antibody. CD4$^+$ T cells from *Apoe$^{-/-}$* mice immunized with p18 showed significantly more IL-10$^+$ spots compared to adjuvant only when p18 peptide was present (Figure 5G).

Then, we tested the clinical efficacy of immunizing $Apoe^{-/-}$ mice with p18 according to the known atheroprotective vaccination protocol (1x in CFA and 4x in IFA, Figure 6A)[8]. Harvested aortas were pinned, fixed and stained with Sudan IV for assessment of *en face* lesions (Figure 6B). Lesion sizes were reduced by ~35% both in the whole aorta (Figure 6C) and in the aortic arch (Figure 6D), the area with the highest lesion burden. Plasma lipids were unchanged by vaccination with p18 (Supplementary Figure 6). To assess the humoral adaptive immune response, we measured antibody titers. Both IgG1 and IgG2c antibodies to p18 were detected in plasma from vaccinated $Apoe^{-/-}$ mice (Supplementary Figure 7), suggesting that vaccination induces sufficient T cell help to B cells to undergo isotype switching and produce peptide-specific antibodies.

Discussion

In this study, we assessed ApoB specific CD4^{+} T cells in humans and mice and analyzed their phenotype. In women with no sCVD, most APOB p18-specific CD4^{+} T cells were FoxP3^{+} Tregs. Patients with sCVD showed a significant increase in FoxP3^{+}Rorγt^{+} cells among p18-specific CD4^{+} T cells, a phenotype that was almost undetectable in women without sCVD.

These cells have not been described in atherosclerosis yet, but can be found in the mouse intestine where these cells inhibit intestinal inflammation mediated by Th1, Th2, and Th17 cells[43, 44]. A recent report demonstrated FoxP3^{+}RORγt[+] cell induction upon immunization in lymph nodes which were transcriptomically distinct from intestinal cells and capable of suppressing Th17-mediated pathologies[45]. However, the role of FoxP3^{+}RORγt^{+} cells in atherosclerosis is currently unknown. Mouse experiments demonstrated that vaccination with a human APOB peptide induces antigen-specific Tregs, some of which produce IL-10. These findings show the existence of p18-specific CD4^{+} T cells in the development of atherosclerosis and indicate that p18-specific Tregs can be induced by peptide immunization.

We identified APOB p18 as the first endogenous Treg epitope in atherosclerosis in humans and mice. Self-epitope specific Tregs have previously been shown in Goodpasture syndrome[17], but not in atherosclerosis. p18 tetramer staining revealed the existence of APOB-specific CD4 T cells in humans. Nur77 expression assays in GFP reporter mice show that the CD4^{+} T cells from p18 peptide vaccinated mice specifically respond to vaccine antigen and are enriched for Tregs. These findings transcend our recent report describing IL-10 and FoxP3 expression on CD4^{+} T cells after vaccination[11] in that TCR activation is directly shown by Nur77-GFP expression, IL-10 production from antigen-specific CD4^{+} T cells was measured at the single cell level by Elispot assays, and a newly developed p18:I-A[b] tetramer directly confirmed the existence of p18-specific CD4^{+} T cells allowing to assess their phenotype. These findings indicate the existence of self-epitope specific Tregs, which can be boosted by immunization with this ApoB peptide.

Moreover, our findings show that atherosclerosis is modulated by an immunization protocol that induces ApoB-specific CD4^{+} T cells, suggesting that it may be possible to vaccinate for atherosclerosis using this approach. Previous studies reported that subcutaneous

immunization with oxidized LDL reduced aortic atherosclerosis in rabbits[46, 47] and $Ldlr^{-/-}$ mice[48]. Another study showed that immunization with peptides derived from human APOB reduced atherosclerosis in the descending aorta of $Apoe^{-/-}$ mice[49]. However, the antigenic peptides of human APOB were not clearly identified, and no antigen-specific T cells were shown. Here, we demonstrate one such epitope and show that vaccination with p18 induces IL-10 producing Tregs, which are known to be atheroprotective[11]. ApoB antigen-specific CD4^{+} T cells reside both in the peritoneal cavity and the spleen. This indicates that vaccination-induced CD4^{+} T cells might translocate from the injection site to other organs. The endogenous trafficking patterns of these cells remain to be explored. Taken together, our data suggest APOB p18 as the first candidate to be considered as an immunogen in the development of a vaccine to prevent atherosclerosis in humans.

To develop an atherosclerosis vaccine effective in humans, more animal work is needed. First, we need to learn whether the atheroprotection seen after vaccination is long-lasting. Second, it may be necessary to optimize the administration protocol of several booster injections. Third, better adjuvants are needed, because CFA and IFA adjuvants cannot be used in humans. Fourth, we identified p18 by *in silico* screening and competitive binding assays as a potential candidate studying ApoB-specific immune responses, but this is not the only or most important immunogenic epitope of ApoB. However, with the p18-tetramer, we are now able to monitor the antigen-specific CD4^{+} T cell response in atherosclerosis and enables us to address the stability of p18-specific Tregs over time. The present study advances the field by identifying the first human APOB peptide that induces atheroprotection, by defining its mechanism of action, and by introducing antigen-specific reagents to detect p18-specific CD4^{+} T cells in mice and humans.

Vaccinated mice produced IgG1 and IgG2c antibodies specific to p18 in this study (Supplementary Figure 6) as we have observed in experiments applying other ApoB peptides[8, 11] Though antibody production against oxLDL has been thought to be related to atherosclerosis[50], there is currently no direct evidence that antibodies against ApoB are atheroprotective. A previous study showed that injection of recombinant human antibodies against modified LDL suppressed the progression of atherosclerosis in mouse models[51]. However, a clinical trial infusing recombinant IgG1 antibody to oxLDL failed to show efficacy of treatment in imaging studies (GLACIER trial)[52]. Our findings of anti-inflammatory CD4^{+} T cells suggest that this failure of inhibition may have been due to the lack of a protective CD4^{+} T cell response.

Our study has several limitations. First, almost all individuals with (N=11) or without sCVD (N=11) were infected with HIV. HIV-infected people often go through a significant reduction of their CD4 T cell pool and have phenotype skewing of T helper cells. Indeed, individuals with HIV infections have a higher frequency of Tregs, despite an overall decline in circulating CD4^{+} T cells. These changes were normalized and returned to baseline upon antiviral therapies[53, 54]. All HIV-infected WIHS participants in our study were treated with antiretrovirals leading to superior control of virus replication and had normal CD4 T cell counts at the time of the blood draw at least 2.5-fold above the AIDS defining threshold below 200/mm^{3} (Supplementary Table 1)[55]. Future studies are needed to address the effect the substantial loss of their CD4^{+} T cell pool may have on the overall CD4^{+} T cell

repertoire. The change in the frequency of Tregs and the gain of other CD4^{+} T cell lineage transcription factors was limited to APOB-specific CD4^{+} T cells isolated from patients with sCVD and was not detectable in the bulk population (Figure 1, Supplementary Figure 3). However, we cannot exclude confounding effects of HIV infection on APOB-specific CD4^{+} T cells. Second, this study is owing to the limited availability of DRB1*07:01^{+} blood samples. Thus, although we observe a trend towards more p18-specific CD4^{+} T cells in sCVD patients, this did not reach statistical significance.

In this study, we identified human APOB-specific CD4^{+} T cells and a natural Treg epitope in human APOB. Our findings make p18 a promising candidate for vaccine development for atherosclerosis by modulating the inflammatory immune response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Clinical Perspective

What is New?

- This is the first study to detect ApolipoproteinB-peptide18-specific CD4 T cells in mice and humans with a newly designed tetramer.

- These p18-specific T cells are mainly anti-inflammatory regulatory T cells in healthy donors, but co-express other CD4 lineage transcription factors in patients with subclinical cardiovascular disease.

- Immunization with p18 reduced atherosclerotic burden in Apoe-deficient mice and induced antigen-specific Tregs.

- This study identifies the ApoB peptide 18 as the first Treg epitope in human and atherosclerosis.

What Are the Clinical Implications?

- We identify the ApoB-peptide 18 as an atherosclerosis related autoantigen, which induces specific T cell responses.

- This study confirms the self-reactive component of the immune system in atherosclerosis.

- The successful protective vaccination of atherosclerotic mice with p18 and simultaneous expansion of antigen-specific Tregs advances the field in designing a new therapeutic strategy to prevent atherosclerosis in humans.

Figure 1. Apolipoprotein B (APOB) peptide specific CD4^{+} T cells in human peripheral blood mononuclear cells (PBMCs)

(**A**) APOB peptide p18 is sequence-identical in mouse ApoB and human APOB; residue numbers of mature protein. The MHC-II binding core nonamers are shown in bold. (**B**) Binding affinity of p18 to mouse (I-A[b]) and human MHC-II (DRB1*07:01). (**C**) Structure of tetramer reagent which was made from HLA-DRA and HLA-DRB1*07:01 loaded with p18 for detecting p18-specific T cell receptors and conjugated separately with PE or APC. (**D**) Human PBMCs were collected from Women's Interagency HIV Study participants

expressing DRB1*07:01 or not (mismatch). PBMCs were analyzed by multi-color flow cytometry after staining with DRB1*07:01-p18 tetramer (PE- and APC-labeled) and cell surface markers. The analyzed populations were gated on singlet, dump channel (CD8, CD14, CD19, CD56, and viability dye) negative, CD3$^+$CD4$^+$ T cells. (**E**) Representative plots of PE- and APC-labeled tetramer staining gated as shown above. Tetramer$^+$ (tet$^+$) cells were defined as PE- and APC-positive. (**F**) Percentage of tet$^+$ cells among CD3$^+$CD4$^+$ T cells of individuals expressing DRB1*07:01 (*N*=29) or not (mismatch) (*N*=11) (left) or between individuals with (*N*=14) or without sCVD (*N*=15). (**G**) Lineage-defining transcription factors were analyzed by intracellular staining of tet$^+$CD4$^+$ T cells. Average frequencies among tet$^+$CD4$^+$ T cells isolated from participants with (*N*=10) or without sCVD (*N*=11) are displayed. (**H**) Percentage of FoxP3$^+$RORgt$^+$ and (**I**) ratio of FoxP3$^+$RORgt$^+$ to FoxP3$^+$ cells among tet$^+$CD4$^+$ T cells. (**F, H, I**) Data is presented as mean ± SEM. *p<0.05 by unpaired t-test.
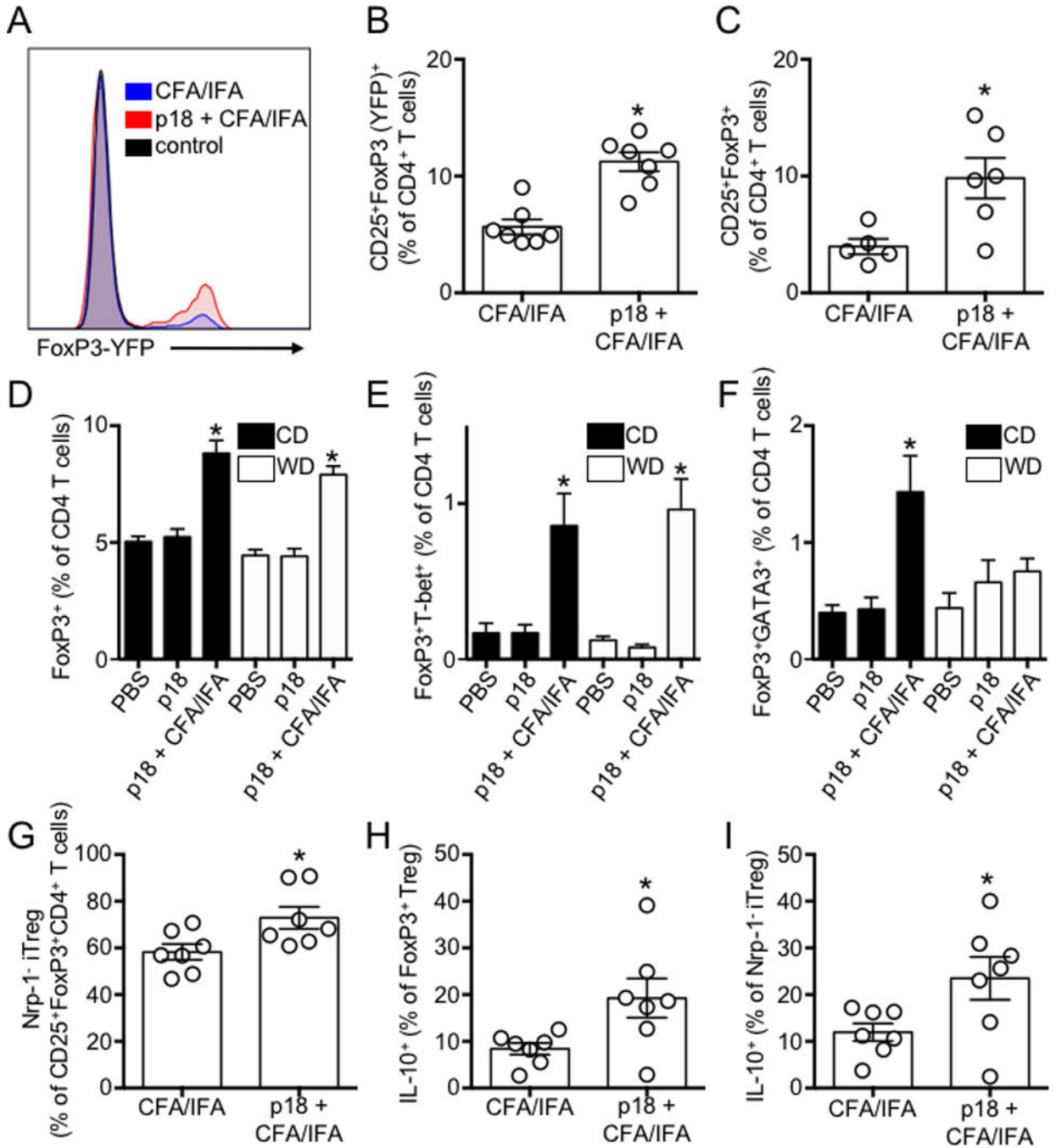
Figure 2. Increased Tregs in peritoneal cavity after immunization with p18

(**A**) Representative histograms of FoxP3-YFP expression in peritoneal CD4+ T cells of western diet (WD) fed *FoxP3^{YFPcre} Apoe$^{-/-}$* mice immunized with p18 (one prime in CFA followed by one boost in IFA) (red) or adjuvant (CFA/IFA) only (blue). CD8+ T cells from the same mouse as negative control (black). (**B**) Percentage of CD25+FoxP3+ by YFP in *FoxP3^{YFPcre} Apoe$^{-/-}$* mice and (**C**) by intracellular staining of FoxP3 in *Apoe$^{-/-}$* mice. (**D-F**) Percentage of FoxP3+ (**D**), FoxP3+T-bet+ (**E**), and FoxP3+GATA3+ cells among peritoneal CD4+ T cells in control (PBS or p18) or p18 immunized (p18 + CFA/IFA) *Apoe*

−/− mice fed chow diet (CD, black) or western diet (WD, white) for 4 weeks. N=9-10 per group. Data is presented as mean ± SEM. *p<0.05 by one-way ANOVA followed by Dunnett's test for multiple comparisons. The mean of each column was compared to the mean of the CD PBS group. **(G)** Percentage of neuropilin-1 (Nrp-1) negative inducible Tregs (iTregs) among all Tregs (CD25$^+$FoxP3$^+$). **(H)** Percentage of IL-10 producing cells among Tregs and **(I)** Nrp-1$^-$ iTregs. N=7 mice (**B, G, H, I**) and N=6 mice (**C**) per group, *p<0.05, by Mann-Whitney U-test.
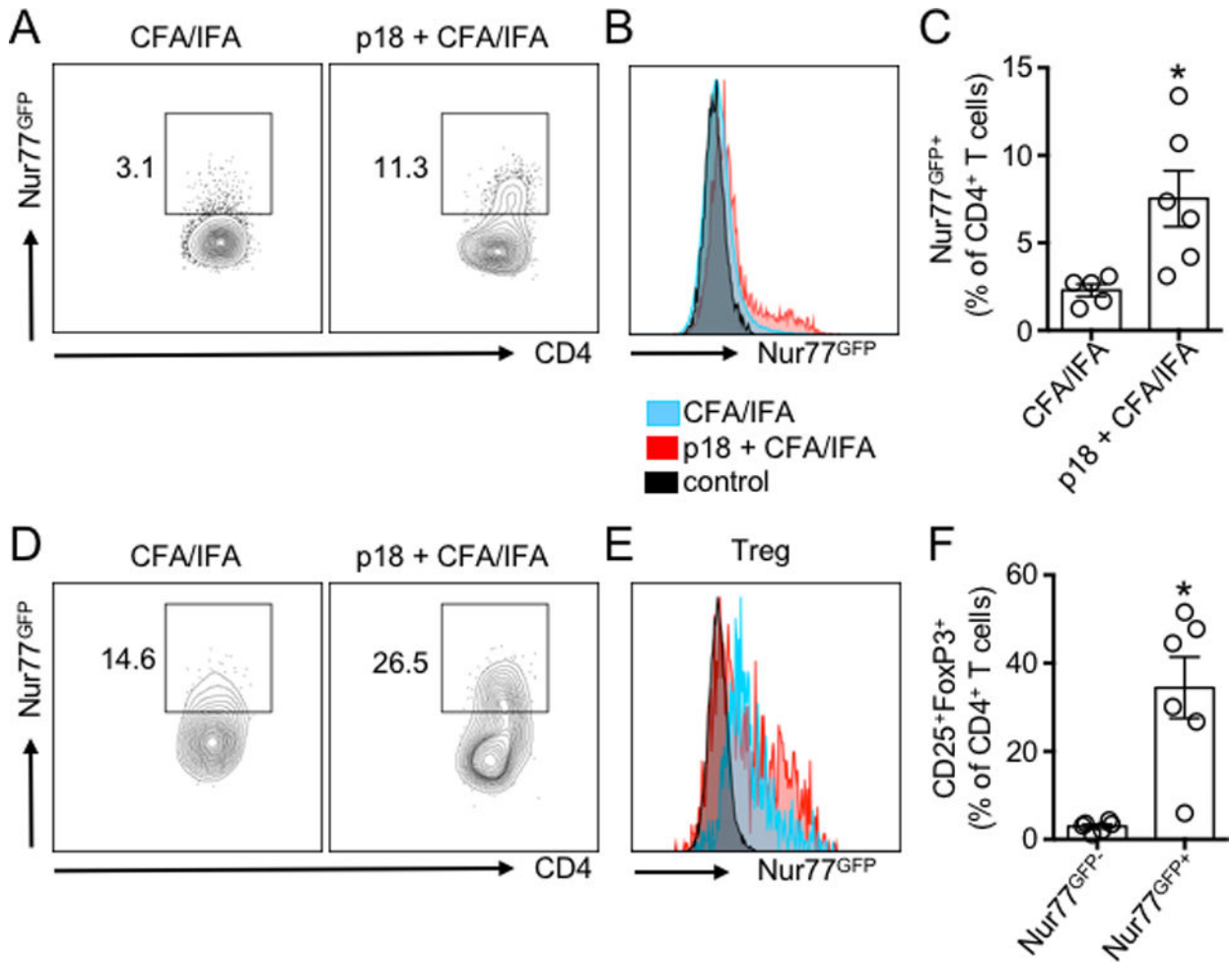
Figure 3. Activation of Tregs in mice vaccinated with p18

Peritoneal cells from *Nur77GFP Apoe*⁻/⁻ mice immunized with p18 (one prime in CFA and one boost in IFA) or adjuvant (CFA/IFA) only were gated on live singlet TCRβ+CD4+ T cells and analyzed by multi-color flow cytometry 24 hours after peptide restimulation. (**A**) Representative plots of Nur77GFP expression in CD4+ T cells. (**B**) Histogram of Nur77GFP expression among CD4+ T cells after p18 in CFA/IFA (red) or CFA/IFA only (blue). (**C**) Percentage of Nur77GFP+ cells among CD4+ T cells. (**D**) Representative plots of Nur77-GFP expression in CD25+FoxP3+ Tregs. (**E**) Histogram of Nur77GFP expression on CD25+FoxP3+ Tregs from mice immunized with p18 or adjuvant only. (**F**) Percentage of CD25+FoxP3+ Tregs among Nur77GFP+ or Nur77GFP− CD4+ T cells (immunized with p18). *N*=5-6 (**C, F**) per group. Mean ± SEM, *p<0.05 by Mann-Whitney U-test.
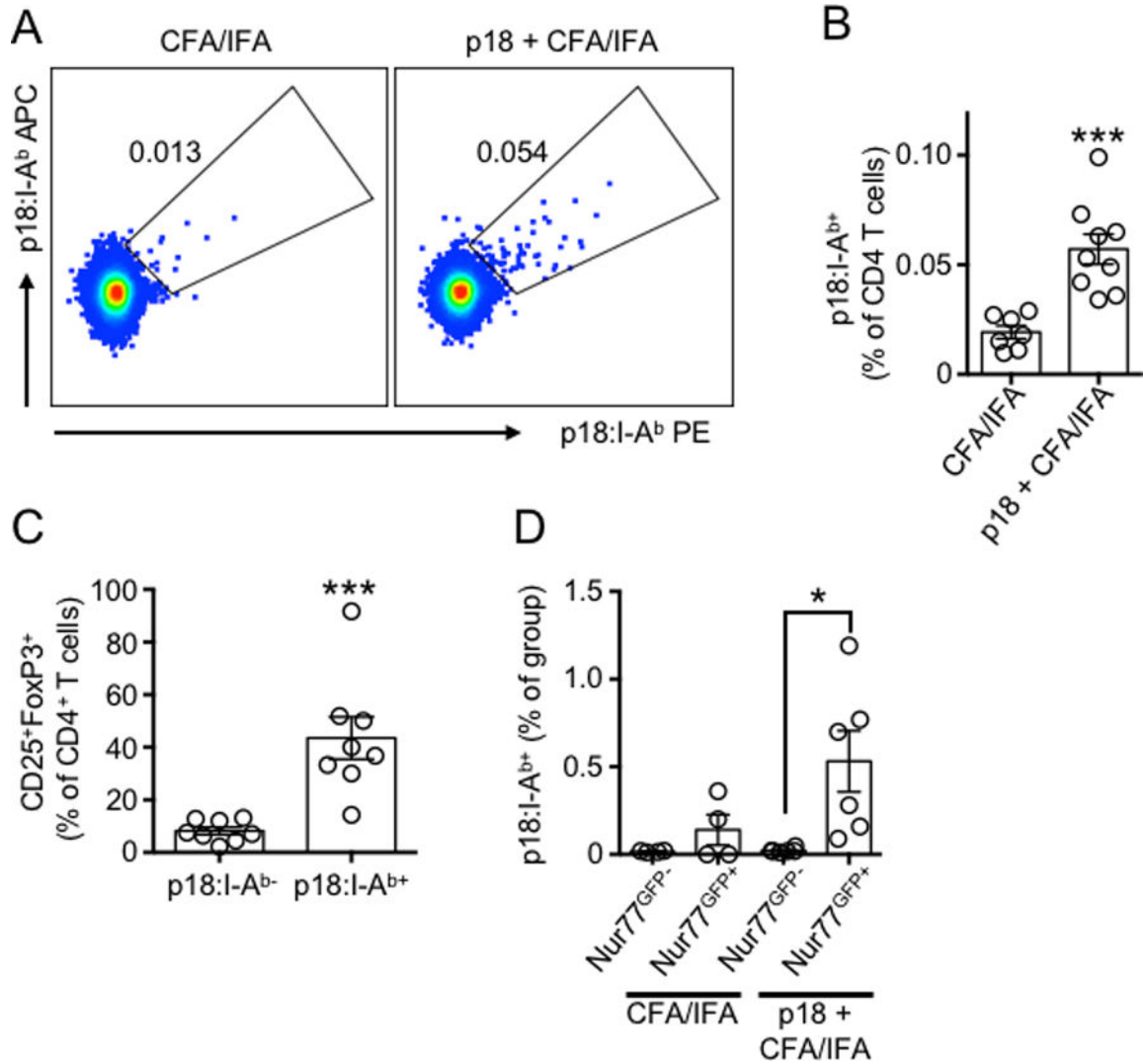
Figure 4. p18-specific peritoneal CD4+ T cells in mice immunized with p18
(**A**) Peritoneal cells were harvested from western diet (WD) fed *Nur77GFPApoe−/−* mice immunized with p18 (one prime in CFA and one boost in IFA) or adjuvant only (CFA/IFA) and analyzed by multi-color flow cytometry after staining with I-A^b-p18 tetramer (PE and APC-labeled). All gated on live singlet CD45+TCRβ+CD4+ T cells. Representative plots of PE-labeled and APC-labeled tetramer staining of CD4+ T cells. Tetramer+ cells were defined as PE- and APC-double positive population. (**B**) Percentage of p18:I-A^b tetramer-labeled cells among all peritoneal CD4+ T cells. (**C**) Percentage of CD25+FoxP3+ Tregs among p18:I-A^b tetramer+ and p18:I-A^b tetramer− peritoneal CD4+ T cells. (**D**) Percentage of CD4+ T cells among Nur77GFP+ and Nur77GFP− peritoneal CD4+ T cells. *N*=5-6 per group. Means ± SEM. *$p<0.05$ and ***$p<0.001$ by Mann-Whitney U-test.
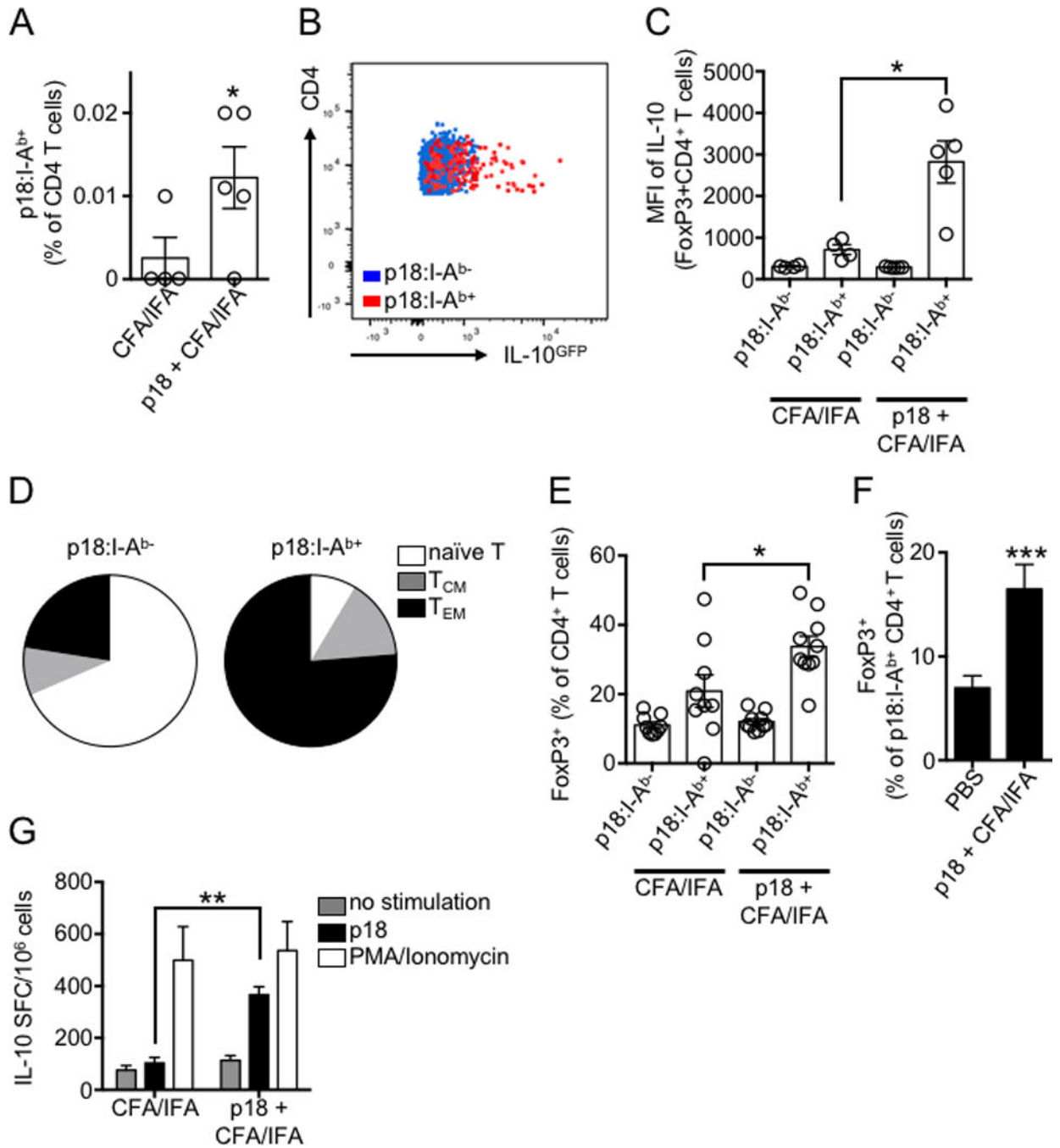
Figure 5. p18-specific CD4+ splenocytes produce more IL-10 after immunization with p18
(**A**) Splenic CD4+ T cells from *IL-10^GFP Apoe^−/−* mice immunized with p18 or adjuvant only were analyzed by multi-color flow cytometry after staining with I-A^b-p18 tetramer. Percentage of p18:I-A^b+ CD4+ T cells (gated on live singlet CD45+TCRβ+CD4+). *N*=4-5 mice per group. (**B**) Representative scatter plot of IL-10 expression (GFP) on p18:I-A^b+ (red) and p18:I-A^b− (blue) CD4+ T cells. (**C**) Mean fluorescence intensity (MFI) of IL-10 (GFP) in splenic FoxP3+CD4+ T cells. (**D**) Phenotype of p18:I-A^b+ and p18:I-A^b− CD4+ T cells in spleen. Naïve T cells, central memory T cells (T_CM), and effector memory T cells

(T_{EM}) are defined as CD62L$^+$CD44$^-$, CD62L$^+$CD44$^+$, and CD62L$^-$CD44$^+$, respectively. (**E**) Frequency of FoxP3$^+$ cells among splenic p18:I-A^{b+} and p18:I-A^{b-} CD4$^+$ T cells after immunization with p18 or adjuvant only. (**F**) Percentage of splenic p18:I-A^{b+}FoxP3$^+$ among CD4$^+$ T cells from control (PBS injected) or p18 immunized (p18 + CFA/IFA: one prime, one boost) *Apoe*$^{-/-}$ mice fed chow diet (CD). (**G**) Splenic CD4$^+$ T cells from *Apoe*$^{-/-}$ mice immunized with p18 were co-cultured with antigen-presenting cells and p18 peptide or PMA/ionomycin as positive control. IL-10 production was evaluated with ELISPOT and quantified by spot forming colonies (SFC) per 1 million cells. *N*=4-5 (**A, C**), *N*=9-10 (**E-G**) mice per group. Means ± SEM. *p<0.05, **p<0.01, and ***p<0.001 by Mann-Whitney U-test.
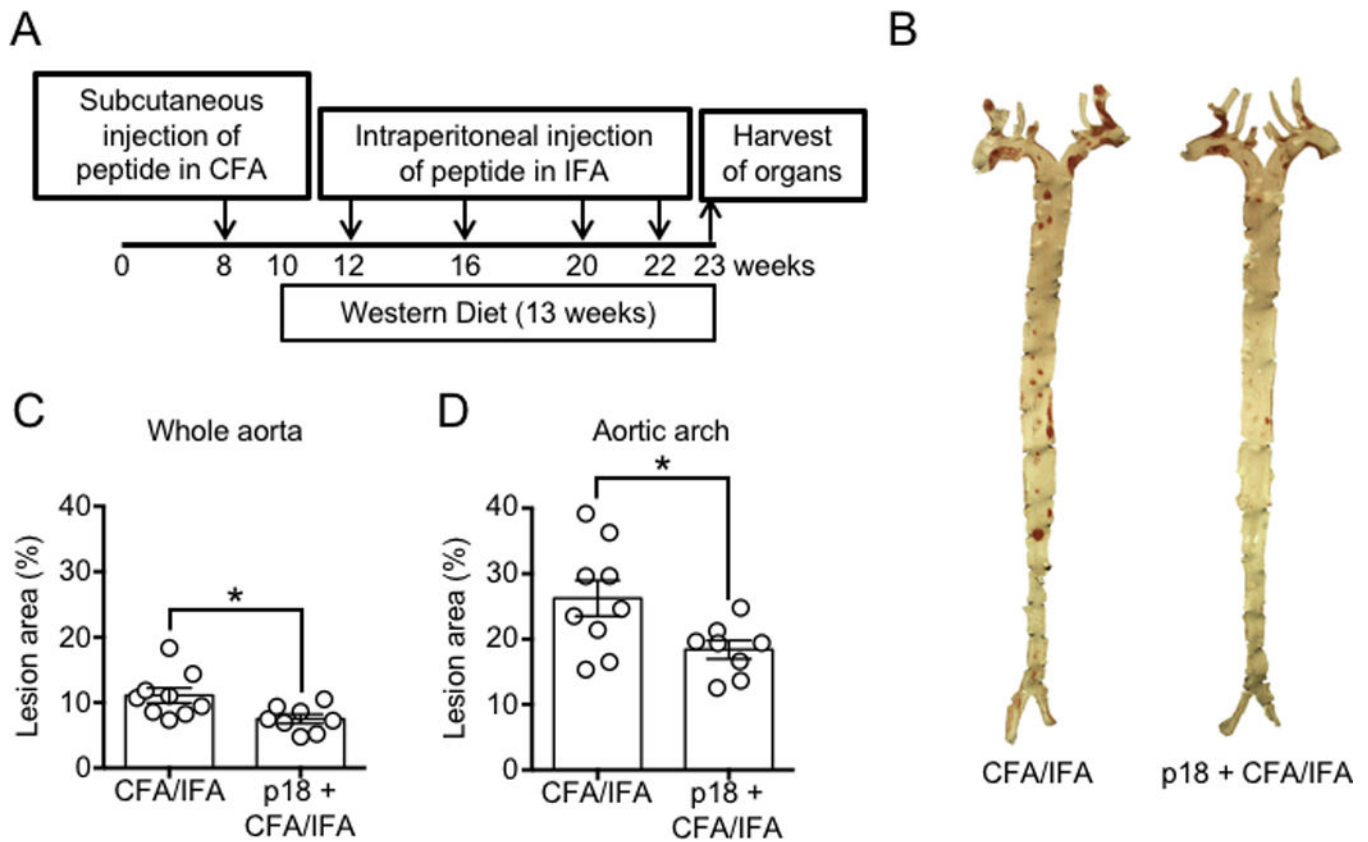
Figure 6. Aortic atherosclerosis in *Apoe*$^{-/-}$ mice vaccinated with p18

(**A**) Female *Apoe*$^{-/-}$ mice were fed at 10 weeks of age for 13 weeks western diet and immunized once with either p18 or adjuvant only in CFA, then boosted four times with p18 or adjuvant only in IFA. (**B**) Representative images of whole aorta stained with Sudan IV. Atherosclerotic plaque was quantified as percentage of whole aorta (**C**) and aortic arch (**D**). *N*=8-9 mice per group, means ± SEM. *p<0.05 by Mann-Whitney U-test.