“Pathogenic autoimmunity in atherosclerosis evolves from initially protective ApoB-reactive CD4+ T-regulatory cells”

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SUPPLEMENTAL METHODS

**Data availability:** Raw and processed sequencing data are available on the NCBI Gene Expression Omnibus repository (GSE149070) at [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149070](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149070). All other data and methods used in the analysis, and materials used to conduct the research will be made available to any researcher for the purpose of reproducing the results or replicating the procedures on personal request (klaus@lji.org).

**Mouse experiments:** All mice were female. If not otherwise indicated, the standard age for all experiments at start was 8 weeks. All mice consumed a standard chow diet (CD) if not otherwise indicated. WT (wild-type, stock number #000664) and *Apoe*<sup>−/−</sup> [B6.129P2-*Apoe*<sup>em1Unc/J</sup>]<sup>30</sup> mice on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME, USA) for experiments in which mouse strains were directly compared. *Foxp3*-YFP-Cre [B6.129(Cg)-*Foxp3*<sup>3tm4(YFP/cre)Ayr/J</sup>]<sup>31</sup> crossed to a cre-activated RFP-reporter [B6.Cg-*Gt(ROSA)26Sor<sup>tm9(CAG-tdTomato)Hze/J</sup>]<sup>32</sup>. All mice were CD45.2, alternatively CD45.1<sup>+</sup> mice on a C57BL/6J background were obtained from Jackson Laboratories (B6.SJL-<sup>Ptprc<sup>a</sup>Pepc<sup>b</sup>BoyJ) and cross-bred to *Apoe*<sup>−/−</sup> mice in-house. The genetic background of mouse strains bred in-house was confirmed regularly by a 1440-SNP panel to avoid a genetic drift (Dartmouse, NH, USA). All mice were housed in a specific pathogen free environment. *Apoe*<sup>−/−</sup> mice were fed a standard chow diet or, to induce atherosclerosis, with a western diet (WD, 42 % kcal from fat, 0.2 % cholesterol, Envigo, # TD.88137) when indicated. Feeding with a WD was started at the age of 8 weeks and continued for the indicated duration. All mouse experiments were carried out according to institutional guidelines. The number of animals included in each experiment has been adjusted to the minimum level required for sufficient statistical power according to local institutional animal welfare guidelines.

**Experimental atherosclerosis:** After feeding of *Apoe*<sup>−/−</sup> mice with a WD for the indicated duration, the whole aorta (thoracic and abdominal) was excised, cleaned in situ, and pinned out after
paraformaldehyde incubation at room temperature for at least 2 hours. Atherosclerotic lesions were visualized by Sudan-IV staining and quantified as the percentage Sudan-IV-positive area of the size of the whole aorta. For the quantification of aortic root lesion size, hearts were embedded in TissueTek, snap frozen, and sections of the aortic sinus were stained with lipid-specific stain Oil-red-O (ORO) as previously described\textsuperscript{33}. The vertical location of sections was chosen according to accepted protocols\textsuperscript{34}. Quantification was performed using ImagePro software (Media Cybernetics, Rockville, MD, USA).

**Adoptive transfer studies:** For the adoptive transfer of antigen-specific T cell subsets into immunocompetent female, 8-week-old Apoe\textsuperscript{-/} mice, transferred ApoB-specific CD4\textsuperscript{+} T cell subsets were flow sorted from pooled lymph nodes from 8-week-old female CD45.1 Apoe\textsuperscript{-/} mice. T cells pooled from 2 to 3 donor mice were used for one recipient. T\textsubscript{regs} were identified as FR4\textsuperscript{+}CD25\textsuperscript{+}CD4\textsuperscript{+}TCR-\textbeta\textsuperscript{-}Lin\textsuperscript{-}L/D\textsuperscript{-} cells followed by the incubation with the ApoB:MHC multimer. ApoB\textsuperscript{+} and ApoB\textsuperscript{-/} T\textsubscript{regs} were injected retro-orbitally (2 x 10\textsuperscript{5}) into female, 8-week-old CD45.2 Apoe\textsuperscript{-/} mice. Feeding with a WD started simultaneously and was continued for 12 weeks. Engraftment of the transferred cells was checked by the presence of CD45.1-expressing T cells.

**Fate mapping of T\textsubscript{regs}:** Foxp3\textsuperscript{-}YFP-Cre crossed to Rosa26-fl-STOP-fl-RFP, both on a C57BL/6J Apoe\textsuperscript{-/} background (T\textsubscript{reg} lineage tracker mice), were used to display historic/current FoxP3 expression (RFP\textsuperscript{+}) in conjunction with current expression of FoxP3, indicated by YFP-expression. To validate current FoxP3-protein expression in YFP-expressing cells, YFP\textsuperscript{+}CD4\textsuperscript{+} T cells from T\textsubscript{reg} lineage tracker mice were flow sorted and stained for FoxP3-protein expression by an anti-mouse FoxP3 antibody (anti-FoxP3-eF450) (data not shown). As indicated, T\textsubscript{reg} lineage tracker mice consumed a chow diet or a WD for 20 weeks. YFP/RFP expression was quantified in CD4\textsuperscript{+}TCR-\textbeta\textsuperscript{-}Lin\textsuperscript{-}L/D\textsuperscript{-} T cells in pooled lymph nodes or aortic T cells. For the in vivo T\textsubscript{reg} conversion assay, 2 x 10\textsuperscript{5} bulk T\textsubscript{regs} from female, 8-week-old CD45.2 Apoe\textsuperscript{-/} mice were flow sorted and transferred into atherosclerotic Apoe\textsuperscript{-/} mice (CD45.1), pre-fed with a western diet for 16 weeks, by retro-orbital injection. Western diet (WD) feeding was continued for 6 weeks before aortic
CD4⁺ T cells were stained for CD45.1, CD45.2, and the indicated transcription factors in an intracellular flow cytometry staining.

**Infection with Murine Cytomegalovirus (MCMV):** Female, 8-week-old WT mice on a C57BL/6J background were infected with mouse CMV (MCMV, smith strain at an i.v. inoculation dose of 1x10⁶ viral particles as performed previously 35). After 21 days, MCMV-specific CD4⁺ T cells were identified in isolated CD4⁺ T cells from pooled lymph nodes by the MCMV:MHC tetramer M25₄₀₉–₄₂₃ (M25) or the MCMV:MHC tetramer M09. Tetramers were obtained from the NIH tetramer core facility (Emory University, Atlanta, GA, USA).

**Immunization:** 8-week-old, female wildtype C57BL/6J, BALBc, or Nur77-GFP reporter mice received 250 µg of the peptide ApoB₉₇₈–₉₉₃ or the peptide peptide MOG₃₅–₅₅ emulsified in equal volumes of complete Freund’s adjuvant (CFA, BD Difco, Sparks, MD, USA), or the adjuvant alone (CFA), by subcutaneous injection into the base of the tail as previously described 22. Mice were sacrificed after 10 days. CD4⁺ T cells were isolated from pooled lymph nodes (inguinal, para-aortic, mesenteric, axillary, cervical) and stained with the indicated antibodies and ApoB:MHC tetramers.

**Peptides:** The peptide ApoB₉₇₈–₉₉₃ (TGAYSNASSTESASY, alternatively termed ‘p6’) was custom synthesized (DBA Synthetic Biomolecules, USA) as 15-mers. Human ApoB-peptides were synthesized by Peptide Speciality Laboratories (Heidelberg, Germany). All peptides were synthesized in 0.1 % trifluoroacetic acid (TFA) for sterility and stored in DMSO at a concentration of 20 µg/ml. Several control peptide pools were used: “CEFX3”: Positive control pool of 68 known MHC-II restricted epitopes for a broad range of HLA sub-types and different infectious agents for T-cell stimulation of populations with a diverse ethnic background; “ACTS”: Negative control pool of 92 peptides derived from a peptide scan through human Actin, alpha skeletal muscle; “MOG”: Negative control pool of 29 peptides derived from a peptide scan through human Myelin-oligodendrocyte glycoprotein (MOG); “CEFX4”: positive control pool
of 80 known MHC-I restricted epitopes for a broad range of HLA sub-types and different infectious agents for T-cell stimulation of populations with a diverse ethnic background. Peptide Pools were bought from JPT Peptide Technologies (Berlin, Germany). The peptide MOG\textsubscript{35-55} was obtained from SigmaAldrich.

**ELISPot assay:** CD4\textsuperscript{+} T-helper cells were isolated from the pooled lymph nodes of untreated 8-week-old, female CD45.2 Apoe\textsuperscript{-/-} mice (from 5-10 mice per replicate), sorted for ApoB\textsuperscript{+} T cells as described above and cultured for 24 hours with irradiated, LPS-stimulated CD45.1\textsuperscript{+} splenocytes and the peptide ApoB\textsubscript{978-993} in 1:100 DMSO in RPMI (200 ng/ml) or 1:100 DMSO in RPMI as control in ELISPOT 96-well plates. Each well contained 2 x 10\textsuperscript{5} CD4\textsuperscript{+} T cells and 1 x 10\textsuperscript{5} splenocytes. IL-17 producing T cells were detected by ELISPOT as previously described\textsuperscript{36}.

**In vitro-re-stimulation:** 8-week-old, female wildtype C57BL/6J reporter mice received 250 \(\mu\)g of the peptide ApoB\textsubscript{978-993} emulsified in equal volumes of complete Freund’s adjuvant (CFA, BD Difco, Sparks, MD, USA) by subcutaneous injection into the base of the tail and one booster injection of ApoB\textsubscript{978-993} emulsified in incomplete Freund’s adjuvant (IFA, 100 \(\mu\)g, BD Difco, Sparks, MD, USA). 10 days later, a suspension of cells from draining lymph nodes (inguinal, para-aortic, mesenteric) was incubated *in vitro* with the peptide ApoB\textsubscript{978-993} (alternatively termed ‘P6’) or ApoB\textsubscript{3501-3516} (alternatively termed ‘P3’) in complete RPMI-1640 cell culture media (concentration of peptides was 200 ng/ml). Cell suspensions were stimulated for 2 hours with the indicated peptides or Concanavalin A (ConA) as unspecific stimulator, followed by another 4 hours in the presence of a transport port inhibitor cocktail. IL-17 was then detected in ApoB\textsuperscript{+} specific T cells by intracellular flow cytometry after staining with the ApoB:MHC tetramer.

**Flow Cytometry:** Cell suspensions were incubated with fluorochrome-coupled antibodies against the indicated antigens for 15 minutes at RT in RPMI-1640 containing 10% rat serum and 10 \(\mu\)g/ml anti-CD16/CD32 antibodies to block unspecific F\textsubscript{c}-receptor interactions. Cells were washed in PBS and fixed in 2 \% Paraformaldehyde (PFA) for 10 minutes. If not otherwise indicated, T-helper cells were identified as
CD4^+ TCR-β^+ Lin^-/D^ (live/dead dye). Lin contained antibodies against CD11b, F4/80, CD19, B220, CD11c, Nk1.1, TER-119, and CD8. Intracellular phosphorylation and transcription factors were stained with a combined permeabilization/fixation protocol according to the manufacture’s recommendations (eBioscience). T\textsubscript{reg} were identified as follows: CD4^+CD25^+FR4^+ or alternatively as FoxP3^+CD25^+ in FoxP3 reporter mice as indicated. T-effector memory subsets were identified as T\textsubscript{EM} (CD44^+CD62L^-), T-central memory cells as T\textsubscript{CM} (CD44^+, CD62L^+), and naïve T cells as T\textsubscript{naïve} (CD44^-CD62L^+). All antibody stainings have been validated before the panel design with fluorescence-minus-one (FMO) controls. Data were acquired on FACS Canto-II, FACS LSR-II, FACS Aria, or FACS Fortessa (BD Biosciences). All anti-mouse antibodies were from eBioscience (San Diego, CA, USA) and were used in a final dilution of 1:50 (cytokines/transcription factors/cytoplasmatic proteins) and 1:200 (extracellular markers). A list of the used antibody clones is available from the authors upon request. If not otherwise indicated, the term “T cell” refers to a CD45^+L/D dump TCR-β^+CD8^-CD4^+ T cell not gated for CD44.

**ApoB:MHC-II multimers:** ApoB:MHC monomers were expressed as previously described\(^{19}\). Briefly, sequences encoding the antigenic peptide ApoB\textsubscript{978-993} were fused to the N-terminus of the mouse MHC-II (I-A\textsuperscript{b}) beta chain by a flexible polyglycine linker in the pRMHa-3 expression vector and co-expressed in Drosophila melanogaster S2 cells with the mouse MHC-II (I-A\textsuperscript{b}) alpha chain and BirA ligase. In vivo biotinylated ApoB:MHC monomers were purified from culture supernatants using nickel affinity chromatography, followed by an additional purification on a Pierce Monomeric Avidin UltraLink Resin column (Thermo Scientific, Asheville, NC, USA). Biotinylated ApoB:MHC monomers were coupled to streptavidin-phycoerythrin (PE) or streptavidin-allophycocyanin (APC) (Prozyme, Hayward, CA, USA) to generate tetramers. Alternatively, biotinylated monomers were coupled to streptavidin-phycoerythrin (PE) or streptavidin-allophycocyanin (APC) labeled dextran backbones by Immudex (Copenhagen, Denmark) for in vivo adoptive transfer studies. Briefly, ApoB:MHC dextramers were generated as described previously\(^{37}\) and had the following specifications: ApoB:MHC dextramer-PE carried ~20 ApoB:MHC monomers and ~4 PE fluorochromes per dextran, while ApoB:MHC dextramer-APC carried ~12
**ApoB:MHC monomers** and ~9 APC fluorochromes per dextran.

**Peptide:MHC multimer staining and quantification:** If not otherwise indicated, cell suspensions were prepared from pooled lymph nodes (axillary, cervical, inguinal, para-aortic, mesenteric). Before incubation with multimers, CD4\(^+\) T cells were enriched by a negative magnetic bead separation with biotinylated anti-CD11b, -CD11c, -TER119, -CD8, -F4/80, -Nk1.1, -B220, -CD19 antibodies (Tonbo, San Diego, USA) and streptavidin-coupled magnetic microbeads (MagniSort, eBioscience, San Diego, USA). Enriched CD4\(^+\) T cells were > 90 % pure, based on surface expression of TCR-\(\beta\) and CD4 as measured in flow cytometry. ApoB:MHC-streptavidin-PE and ApoB:MHC-streptavidin-APC tetramers or –dextramers were incubated for 1 hour at room temperature in the dark in RPMI supplemented with 10 % rat serum and 10 \(\mu\)g/ml anti-CD16/32 (Tonbo, San Diego, CA, USA). Final concentration was 10 nM (for ApoB-MHC as tetramer) or 12.5 nM (for ApoB-MHC as dextramer). Cells were subsequently stained for the indicated surface markers and live-dead dye (eF450 viability dye, eBioscience, San Diego, USA). Non-T cells were identified by fluorochrome-labelled antibodies against CD11b, CD11c, F4/80, CD19, CD8, B220 (Lineage, all from eBioscience, San Diego, CA, USA). For extracellular staining, cell suspensions were fixed in 2 % PFA. As indicated, intracellular staining for transcription factors was performed according to the manufacturer’s instructions (eBioscience, San Diego, USA). Data were analyzed with FlowJo software (Treestar, San Diego, CA, USA). For calculation of the absolute numbers of ApoB\(^+\) T cells, leukocyte numbers of enriched CD4\(^+\) lymph node T cells were quantified (Hemavet, DrewScientific, Miami Lakes, USA) before multimer staining.

**Cell sorting of antigen-specific T cells:** Lymph node CD4\(^+\) T cells from untreated female, 8-week-old C57BL/6J mice were stained with ApoB:MHC and with fluorochrome-labelled anti-CD4, TCR-\(\beta\), and live-dead eF450 and sorted into the indicated cell populations on a Aria2 cytometer (BD, San Diego, USA). Nozzle size was 100nM. Cells were collected in serum-free T cell media (TexMACS, Miltenyi, Germany). For confocal microscopy, cells were immobilized on cover slips coated with Poly-L-Lysine, fixed with 2 %
PFA, and imaged with a SP5 confocal microscope (Leica, Wetzlar, Germany). Alternatively, cells were cultured in RPMI-1640 with 10% FCS for 24 hours in the presence of PMA/Ionomycin, and supernatant was collected for quantification of cytokines by the high-sensitivity murine cytometric bead assay (CBA) according to the manufacturer’s instructions (BD Bioscience, San Diego, CA, USA).

**RNA-sequencing:** Cell sorting, library preparation, and sequencing: RNA-sequencing of CD4$^+$ T cell subsets was performed after flow sorting of the indicated CD4$^+$ T cell subpopulations from pooled lymph nodes from mice at the indicated age/diet. Cells were sorted, washed, pelleted and resuspended in Trizol LS. Total RNA was isolated (Zymo Direct-zol Kit) and analyzed on an Agilent Bioanalyzer RNA Pico chip to test for RNA integrity. Input cell number varied on the T cell population. Number for rare T cell subsets (ApoB$^{\text{high}}$) from lymph nodes of 10 pooled mice were < 1000. Average RNA-concentration was 0.256 ng/µl, average RIN number was 9.04. Library preparation was done with a SMART-Seq v4 Ultra Low Input RNA Kit by Clontech and the Nextera XT Kit (Illumina) with an input of 0.5 ng RNA. The resulting average concentration was 6.5 ng/µl of DNA library. DNA Library was additionally checked for integrity on an Agilent Tapestation using the High Sensitivity D1000 tapes. Samples were sequenced in pools of 7-8 samples on a HiSeq 4000 (UCSD Genomics Center, La Jolla) on the 50 base pairs single read protocol. Mapping and post-sequencing quality controls: The single-end reads that passed Illumina filters were filtered for reads aligning to tRNA, rRNA, adapter sequences, and spike-in controls. The reads were then aligned to UCSC mm10 reference genome using TopHat (v 1.4.1)$^{38}$ DUST scores were calculated with PRINSEQ Lite (v 0.20.3)$^{39}$ and low-complexity reads (DUST > 4) were removed from the BAM files. The alignment results were parsed via the SAMtools to generate SAM files$^{40}$. The sequencing performance was assessed by calculating mapping statistics based on the total number of mapped reads, total number of uniquely mapped reads, and read distribution over known gene models was computed by gene body coverage in RSeQC$^{41}$. Read counts of each genomic feature were obtained with the htseq-count program (v 0.6.0) 70 using the ‘union’ option. Average percentage of successfully mapped reads to the reference genome was 92.3 %, of unmapped reads was 5.2 %. Differentially expressed genes: Expression values
retrieved as above were expressed as Reads Per Kilobase of transcript per Million mapped read (RPKM).
The complete table of expression values across all experiments showing RNAseq data is provided in Online-only Data File III. Differentially expressed (DE) genes were calculated by the comparative marker selection tool (GenePattern, Broad Institute). Only a statistical significance of \( p < 0.05 \) (T-test) and FDR \( q < 0.05 \) (false discovery rate) was considered significant. Heatmaps of gene expression values normalized as \( \log_{10} \) (RPKM) were generated by Morpheus (Broad Institute). By default, values were presented as minimum-to-maximum heatmaps for columns or rows as indicated. In addition, heatmaps were clustered in one or both dimensions (genes, samples) by the hierarchical clustering tool (one minus Pearson correlation, ‘average’ linkage method). Cluster analyses including principal component analysis (PCA) and hierarchical clustering were performed using standard algorithms and metrics in the software R. Functional pathway analysis: Gene expression data from RNA-sequencing were used to identify differentially expressed genes between the indicated groups. These genes and their fold change of regulation served as input for pathway analysis by Ingenuity Pathway Analysis (IPA, Qiagen, Germany) using the Core Analysis function. Up- and down-regulated genes were used as input to determine the enrichment of pathways in the tested comparisons (functional annotations or master regulators). Significantly enriched \( (p < 0.05) \) pathways were filtered. Gene set enrichment analysis (GSEA). GenePattern 2.0 (Broad Institute) was used to calculate an enrichment score of a gene signature within a comparison of two testing data sets with the GSEA-module ran with the default settings (100 iterations, weighted). Normalized gene expression (testing data set) and gene signatures served as input. A p-value \(< 0.05\) and a FDR q-value \(< 0.2\) were considered significant. Gene signatures were either calculated based on DE-genes or by a combination of DE-genes after pre-filtering the datasets for non-hematopoietic genes (according to) when building a gene signature for T cell subsets described in this study, or by published gene signatures of the Molecular Signatures Database v6.1 (MSigDB, http://software.broadinstitute.org/gsea/msigdb/index.jsp). GSEA-signatures were used as indicated. Alternatively, GSEA was performed on published BIOCARTA pathways/signature genes.

**TCR sequencing:** Parallel DNA-sequencing (Adaptive Biotechnology, Seattle, WA, USA) of the
T-cell receptor β-chain was carried out in DNA isolated from sorted T cells from pooled lymph nodes after staining with the ApoB:MHC-II tetramer. Clonal proportion was expressed by the percentage of the indicated top number of clones of all productive clonotypes identified. Entropy was calculated by summing the frequency of each clone times the log (base 2) of the same frequency over all productive reads in a sample. This value was normalized based on the total number of productive unique sequences and subtracted from 1 (clonality index). Values range from 0 to 1. Alternatively, frequencies of clones or usage of specific V, D, J gene segments was calculated. TCR-sequencing results with frequencies and gene segment usage is shown in Online-only Data File1-2.

**Single Cell RNA-sequencing (scRNAseq).** Suspensions of sorted ApoB+ T-helper cells were loaded on the Chromium Single Cell Controller (10xGenomics) to generate a single cell and gel bead emulsion (GEM). ScRNAseq libraries were prepared using the Single Cell 3’ Solution v2 Reagent Kit (10xGenomics). GEM reverse transcription was performed by the SimpliAmp thermal cycler (Thermo Fisher Scientific) running the following program: 53°C for 45 minutes and 85°C for 5 minutes; held at 4°C. Following reverse transcription, GEMs were broken, and single-strand cDNA was purified with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific). cDNA was amplified with the SimpliAmp thermal cycler: 98°C for 3 minutes, 12 cycles of 98°C for 15 seconds, 67°C for 20 seconds, 72°C for 60 seconds; 72°C for 1 minute, and held at 4°C. Subsequently, cDNA was cleaned up using the SPRSelect reagent kit (Beckman Coulter), quantified, and quality controlled with the Agilent Bioanalyzer High Sensitivity Kit. Indexed sequencing libraries were prepared by Single Cell 3’ Solution v2 Reagent Kit's components: (1) enzymatic fragmentation, (2) end repair, (3) A-tailing, (4) adaptor ligation, (5) post ligation cleanup with the SPRSelect reagent kit, and (6) sample index PCR using the SimpliAmp thermal cycler at 98°C for 45 seconds, 10 cycles of 98°C for 20 seconds, 54°C for 30 seconds, 72°C for 20 seconds; 72°C for 1 minute, and held at 4°C. The SPRSelect reagent kit was used for post sample index double-sided size selection, and the library was quality controlled post construction using the Agilent Bioanalyzer High Sensitivity Kit and Agilent Bioanalyzer high sensitivity chip. The library was quantified post construction.
by quantitative PCR (Kapa DNA Quantification Kit for Illumina platforms). The constructed libraries were loaded at 13 pM on a HiSeq2500 Rapid Run using a HiSeq Rapid Cluster Kit V2- Paired End (PE), and a HiSeq Rapid SBS Kit V2. A 26-base-pair Read 1 was used to sequence the cell barcode and UMI, an 8 bp i7 index read was used to sequence the sample index, and a 98 bp Read 2 was used to sequence the transcript on an Illumina HiSeq4000 using paired-end sequencing with dual indexing. ScRNAseq data analysis: 10x genomics single cell transcriptome sequencing data was processed using the Cell Ranger Single Cell software suite Version 1.3 (https://support.10xgenomics.com) as described previously45. Briefly, the FASTQ files were processed with the CellRanger count pipeline, which uses STAR46 to align the reads to mm10 mouse reference transcriptome. Raw data were processed with the R/Bioconductor single cell RNA-sequencing analysis package SEURAT47. Raw sequencing reads from individual samples (n=3 for ApoBneg cells, one pooled sample from 10 donor mice for ApoB+ T cells) were merged, the gene space was log-normalized, and filtered for the following parameters: unique gene count per cell >50 and <2500, percent of mitochondrial genes of all genes <0.05. A PCA-reduction (up to 12 dimensions) was performed and a Stochastic Neighbor Embedding (t-SNE) and automated cluster detection algorithm was performed on the gene sets defining the PCA variability. Only significant PCA-dimensions were taken into account. Cluster detection was run with resolution of 0.8 to 0.9. Average gene expression per cluster was displayed in column minimum-maximum value heatmaps generated by Morpheus (GenePattern, Broad Institute), followed by free hierarchal clustering (one-minus-Pearson with average linkage). The expression data matrix with scaled values that served as input for tSNE-clustering is available on the NCBI Gene Expression Omnibus repository (GSE149070). Alternatively, we re-analyzed a previously published data set20: leukocytes from aortas from CD and WD (‘hfd’) fed Apoec- mice were selected for Cd3e-Cd4-Cd8a- and tSNE clustering was performed on this subset (data sets available as part of GSE149070).

**Identification of human MHC-II restricted ApoB-peptides:** To identify binding of peptides to human MHC-II, we employed previously described algorithms48-50 for predicting MHC-II peptide binding motifs. Briefly, the human ApoB sequence was screened for all possible, including overlapping, peptides in
silico. The best candidate sequences were tested in affinity measurements between purified MHC-II molecules and peptides using a classical competitive inhibition assay with high affinity radio-labeled MHC-II ligands as previously performed\textsuperscript{41}. The ability of the tested peptides to displace radio-labeled known MHC-II ligands was measured. An inhibitory concentration required to displace 50\% of the radio-labeled ligand (IC\textsubscript{50}) was determined. Under the experimental conditions employed, IC\textsubscript{50} values are approximations of the $K_d$ values (affinity).

**Clinical Study:** Patients undergoing coronary angiography at the University Heart Center in Freiburg, Germany, were included in the Adaptive Immunity in Human Atherosclerosis (ANIMATE) trial approved by the local Institutional Review Board at the University of Freiburg, Germany. Patients at an age $> 43$ - 90 years were included. Exclusion criteria included: Acute inflammation, acute infection, any other chronic inflammatory disease, known immune disease, pregnancy, immunosuppression by disease or drugs, antibody treatment, cancer. Based on coronary angiography, all patients were divided into those with signs of stenotic coronary artery disease (CAD) in at least one coronary artery and those without signs indicative of coronary atherosclerosis (no CAD). Clinical characteristics of the patient cohort are listed in main Table 1. Blood collection: 40 ml of arterial blood was withdrawn from the sheath before coronary angiography by cardiac catheterization.

Two experiments were performed to enumerate ApoB-reactive CD4$^+$ T cells and cytokine production as response to ApoB-re-stimulation: A short re-stimulation over 6 hours for intracellular expression of CD40L, TNF-$\alpha$, and IFN-$\gamma$, and a long re-stimulation over 10 days for intracellular expression of IL-$17$ and IL-$10$.

**Short re-stimulation:** Whole blood samples were split into 1 ml samples in tissue culture tubes (Greiner Bio one) and stimulated with a mix of 30 human ApoB-peptides (Online-only Table V, concentration for the pool was 100 $\mu$g / ml and for each peptide in the pool 3.3 $\mu$g / ml ), in the presence of a stimulating anti-CD28 (1 $\mu$g / ml) antibody at 37\° C in a cell culture incubator. After 2 hours, a protein transport inhibitor cocktail at a dilution of 1:500 (eBioscience, San Diego, USA) was added and incubated
at 37° C for another 4 hours. After the incubation, red blood cells from whole blood were lysed with a cell Lysis buffer for 10 minutes at room temperature (eBioscience, San Diego, USA). Leukocytes were washed with FACS washing buffer (PBS w/o Ca/Mg, 10 % FCS) and kept in FACS buffer (complete RPMI, 10 µg / ml anti-human Fc-Block, eBioscience, San Diego, USA) until antibody staining for extra- and intracellular cytokine detection according to standard protocols (eBioscience) was performed.

**Long re-stimulation:** Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient in BD Vacutainer CP Mononuclear Cell Preparation Tube tubes according to the manufacturer’s protocol, washed, and cultured in 24-well tissue culture plates at a concentration of 10⁶ cells / ml in AIM-V serum-free cell culture medium (ThermoFisher) containing the ApoB-peptide pools in the same concentration as for the short re-stimulation protocol. After 5 days in culture, 0.5 ml of a total of 1 ml cell culture media was carefully removed and replaced by 0.5 ml fresh AIM-V containing 40 U / ml human recombinant IL-2 (PeproTech). After an additional 5 days in culture, cells were isolated, washed, resuspended in 1 ml AIM-V containing a protein transport inhibitor (1:500, eBioscience), and transferred to FACS tubes. After 5 hours, cells were washed with FACS washing buffer (PBS w/o Ca/Mg, 10 % FCS) and kept in FACS buffer (complete RPMI, 10 µg / ml anti-human Fc-Block, eBioscience, San Diego, USA) until antibody staining for extra- and intracellular cytokine detection according to standard protocols (eBioscience) was performed.

In both experiments, as indicated, additional controls were included during the entire duration of re-stimulation: no addition of peptide pools, only carrier substance for the peptides in the same concentration as in the peptide re-stimulations (DMSO), termed “control”; addition of Staphylococcal enterotoxin B (SEB, SigmaAldrich) at a concentration of 1 µg/ml, or addition of PMA/Ionomycin (Cell stimulation cocktail, eBioscience) at a dilution of 1:500. As indicated, different control peptide pools (listed under “Peptides”) were incubated at the same concentration as the ApoB-peptide pool. In addition, in some experiments, an MHC-II-neutralizing, anti-human anti-HLA-DR/DP/DQ antibody (clone Tu39, BD Bioscience) was added to the ApoB-peptide pool in a concentration of 10 µg / ml.

Cytokines were identified by FACS using a master mix containing fixable viability dye and
antibodies against CD4 (clone SK3), CD3 (clone SK7), IL-17 (clone N49-653), TNF-α (clone Mab11), IFN-γ (clone 4S.B3), IL-10 (clone JES3-9D7), and CD40L (clone TRAP1). In some experiments, antibodies against CD25 (clone BC96), CD69 (clone FN50), CD38 (clone HB-7, eBioscience), and HLA-DR (clone L243, eBioscience) were used for the quantification of alternative T cell activation markers by extracellular staining. All anti-human antibodies were from Biolegend (San Diego, CA, USA) and were used in a final dilution of 1:50 (cytokines) and 1:200 (extracellular markers). To quantify ApoB-antigen-specificity in T cells, intracellular expression of the immediate activation marker CD40L was measured in CD4+ cells after ApoB-re-stimulation. Expression of CD40L and other cytokines were expressed either as absolute value (% of cytokine+ CD4+ T cells, main Figure 7, online-only Figure XVI / XVII) or as relative increase of CD40L+ cells compared to unstimulated samples (% untreated) in Online-only Figure XVI.

To quantify transcription factors and activation markers (Online-only Table VIII), PBMCs from study participants were stained with antibodies against CD4, CD3, CD45RA (clone UCHL1, Biolegend), CCR7 (clone 150503, BD Bioscience), FoxP3 (clone 259D/7, eBioscience), and ROR-γT (clone Q21-559, BD Bioscience) according to standard protocols for extracellular and intracellular transcription factor staining (eBioscience).

**Statistical analyses:** The number of mice in the experimental groups was defined based on power analysis. Data are expressed as mean ± SEM throughout the manuscript. p-values < 0.05 were considered significant. Statistical test employed are indicated in each figure.
### SUPPLEMENTAL TABLES

**Supplemental Table I: Atherosclerosis-relevant mouse MHC-II (I-A<sup>b</sup>) restricted ApoB-100 peptides**

| Peptide (Name) | Peptide Sequence (amino acids) | Measured I-A<sup>b</sup> affinity (IC<sub>50</sub> nM) | Position (mouse ApoB) | Predicted I-A<sup>b</sup> affinity (IC<sub>50</sub> nM) | Predicted I-A<sup>b</sup> affinity (rank*) |
|----------------|---------------------------------|-----------------------------------------------|------------------------|-----------------------------------------------|-------------------------------------------|
| P3             | SQEYSGSVANEANVY                 | 4.3                                           | 3501                   | 13-133                                        | 0.62                                      |
| P6             | TGAYSNASSTESASY                 | 7.3                                           | 978                    | 300-505                                       | 4.95                                      |
| P18            | SLFFSAQPFEITAST                 | 53                                            | 3030                   | 125-588                                       | 4.02                                      |
| P101           | FGKQGFPDSVKNKLY                 | 5.5                                           | 705                    | 748-981                                       | 9.63                                      |
| P102           | TLYALSHAVNSYFVDVD               | 6.8                                           | 441                    | 202-430                                       | 3.87                                      |
| P103           | LYYKEDKTSLSASAAS                | 95                                            | 3953                   | 921-949                                       | 10.27                                     |

* percentile rank of the predicted affinity (high to low) among all mouse ApoB-peptides
Supplemental Table II: scRNAseq cluster assignment of ApoB\textsuperscript{neg} and ApoB\textsuperscript{+} T cells

|   | ApoB\textsuperscript{neg} (cells) | ApoB\textsuperscript{neg} (%) | ApoB\textsuperscript{+} (cells) | ApoB\textsuperscript{+} (%) | ApoB\textsuperscript{+} (%) / ApoB\textsuperscript{neg} (%) | Cluster occupancy (%) with ApoB\textsuperscript{+} |
|---|---------------------------------|-------------------------------|-------------------------------|----------------|---------------------------------|-----------------------------------------------|
| 1 | 269                             | 26.6                          | 12                            | 1.6           | 0.1                             | 4                                             |
| 2 | 42                              | 4.2                           | 198                           | 27.1          | 6.5                             | 83                                            |
| 3 | 62                              | 6.1                           | 123                           | 16.8          | 2.7                             | 66                                            |
| 4 | 149                             | 14.7                          | 20                            | 2.7           | 0.2                             | 12                                            |
| 5 | 159                             | 15.7                          | 7                             | 1.0           | 0.1                             | 4                                             |
| 6 | 18                              | 1.8                           | 116                           | 15.9          | 8.9                             | 87                                            |
| 7 | 28                              | 2.8                           | 96                            | 13.1          | 4.7                             | 77                                            |
| 8 | 109                             | 10.8                          | 9                             | 1.2           | 0.1                             | 8                                             |
| 9 | 59                              | 5.8                           | 54                            | 7.4           | 1.3                             | 48                                            |
| 10| 57                              | 5.6                           | 33                            | 4.5           | 0.8                             | 37                                            |
| 11| 43                              | 4.2                           | 26                            | 3.6           | 0.8                             | 38                                            |
| 12| 17                              | 1.7                           | 37                            | 5.1           | 3.0                             | 69                                            |
| total | 1012                          | 100                           | 731                           | 100           |                                  |                                               |
|    | CD (cells) | CD (%) | WD (cells) | WD (%) | CD (%) / WD (%) |
|----|------------|--------|------------|--------|-----------------|
| 1  | 7          | 5.4    | 35         | 11.9   | 2.2             |
| 2  | 55         | 42.6   | 120        | 40.7   | 1.0             |
| 3  | 45         | 34.9   | 115        | 39.0   | 1.1             |
| 4  | 22         | 17.1   | 25         | 8.5    | 0.5             |
| total | 129      | 100    | 295        | 100    |                 |
### Supplemental Table IV: Core genes in the gene signature of ApoB⁺ CD4⁺ T cells

| Gene   | Gene   | Gene   | Gene   | Gene   | Gene   |
|--------|--------|--------|--------|--------|--------|
| CPE    | SERPINA3G | SYTL3 | CYB5D1 | TMEM170B | ACSBG1 |
| IL17F  | EMP1   | POLK   | NPDC1  | SH3GL3  | ARMCX3 |
| IL23R  | WDFY2  | BHLHE40 | MX2    | ZFP41   | PAPR14 |
| GP49A  | 2010002N04 | EIF5A2 | MTHFR  | PALB2   | C230081A13 |
| IL1R1  | RIK    | DENND4C | H2-Q4  | PPFIBP1 | RIK    |
| IL17A  | CASP1  | TIGIT   | INPPL1 | ANGPTL2 | SH3BGRL |
| LILRB4 | KLRB1F | 2210039B01 | XDH | GBP6   | CEP76 |
| IL17E  | ANKR6D | RIK      | CXC46 | CPEP61 | ZXDB |
| ABCB1A | STOM   | TST     | USP6NL | RAB8B  | NEK4   |
| IL2    | KCNK5  | MIR3112 | B3GN5  | CEP170  | DENND2D |
| IFI44  | PLCB4  | P2RX7   | HEG1   | ZBTB41  | ACSL4  |
| FANCD2 | ZFP365 | ARHGAP5 | GM4636 | KDELCl  | PLXNC1 |
| TMEM176A | TNFSF8 | 1110067D22 | ZFP300 | PTPN11  | ZFP292 |
| ADAM8  | ANXA1  | RIK      | ZFP445 | PISD-P52 | ZFP74 |
| H2-Q1  | GM7609 | FRMD4B   | ATAD2  | TNFAIP3 | MIB1   |
| H2-Q2  | TOX2   | NCAPG2  | 261008E11 | PEG13  | UHM1   |
| 5830411N06 | GM9766 | TUFT1   | RIK    | MYO1F   | DNAJA4 |
| RIK    | MATN2  | ITGB1   | SLC4A7 | B4GALT4 | VWA5A |
| F830016B08 | GTG1A | NR1D2   | PON3   | HIF1A   | PLEKHO1 |
| RIK    | GRPRN3 | GNPTAB  | SORL1  | NR1D1   | MFSD6  |
| TMEM176B | GM5595 | RSAD2   | IG5P1  | XRN1    | IL12RB1 |
| CYSLTR1 | GPR155 | SLC25A24 | SNX10  | SEPSecs  | ARHGAP26 |
| GM4951 | SLC35E4 | CAR5B  | SNX29  | GM14446 | BIVM   |
| UNC5A  | P2RY1  | MPP2    | HELL5  | CMPK2   | IFI11  |
| GZMK   | MIR704 | RPS6KC1 | DAAM1  | SLFN3    | RNF43  |
| ASB2   | GM20199 | IL18RAP | ACVR2A | OAS3    | ZFP935 |
| SERPINB1A | CORO2B | NAIP2   | ARC    | PLEKHA8 | KLF9   |
| SLFN9  | H2-Q6  | APOBEC1 | APAF1  | GBP9    | HSPA13 |
| CXC5R5 | 42797  | PRDM10  | GCNT1  | ANKRd28 | DGKE   |
| APOL10B | IRAK3  | OAS1A   | IPO7   | SLCO4A1  | ZFP947 |
| LTBR4  | CYFIP1 | 2210403K04 | SAMD9L | ZFP95A  | ZFP14  |
| ABCA1  | TMEM2  | RIK      | CCR2   | A130022J15 | NEAT1 |
| CDP    | MMP9   | FAM57A  | PIK3C2A | RIK    | ABCB1B |
| C030034L19 | ETV6 | HLF     | SDC4   | TCC39C  | TRPS1  |
| RIK    | REPIN1 | SLFN8   | USP11  | GM14718  | HIVEP2 |
| SLC43A1 | FARP1  | ATF6    | ZFP760 | PLEK    | CPEB2  |
| PLTP   | CYBB   | ZIK1    | DPP8   | SERINC5  | OCR1   |
| ROBC   | 993011LJ21 | CYP4F16 | NLRP1A | ABI2    | ICOSL  |
| IFI204 | RIK1   | TRIM30D | SUSD2  | ARHGFL6  | AKAP11 |
| CES2D-PS | ELL2   | SLC45A4 | 4933427D14 | AHNAXK | GOLM1 |
| CCR6   | SLC26A11 | TBC1D4 | RIK    | ZFP738  | FAR1   |
| LRRN3  | HPGDS  | GM4759  | DTL    | ZFP160  | VPS8   |
| 5430427O19 | CASP4 | BAIAP3  | ZFP518A | SYT11   | RABGAP1 |
| RIK    | NEBL   | RUNCDC3B | STAG2  | KDM6A   | 1190002N15 |
| B4GALNT4 | MX1    | DDX60   | ARHGFL2 | TNKS    | RIK    |
| PBX3   | GRH1L  | ERO1LB  | RASL11A | IL1RAP  | ZCAC6  |
| IL12RB2 | ZFP442 | HIP1R   | JAK2   | RBL2    | PNPLA6 |
| PLEKHF1 | SLC9A7 | FBX17   | SOS2   | GM16386  | NTSE   |
| PGCP   | 6330503K22 | EEA1 | GTPBP10 | AHCTF1  | PPISP52 |
| GPM6B  | RIK    | MALT1   | RAB20  | MAP3K5  | MOBI   |
| KSR1   | SLC9A9 | PROS1   | RTTN   | ZFP867  | METT7A1 |
| CYP4V3 | CD200R4 | POLE    | KIFAP3 | 4922501C03 |
Supplemental Table V: Pool of human MHC-II restricted ApoB-100 peptides

| Peptide | Sequence                  | Length (aa) | Position (human ApoB) |
|---------|---------------------------|-------------|-----------------------|
| 1       | HFSNVFRSVMAPFTM           | 15          | 1891                  |
| 2       | GKIDFLNNYALFLSP           | 15          | 3066                  |
| 3       | IKHIYAISSAALSAS           | 15          | 1836                  |
| 4       | DKRLAAYLMLMRSPS           | 15          | 556                   |
| 5       | EGHLRFLKNIILPVY           | 15          | 3666                  |
| 6       | QEVFKAIQSLKTTEV           | 15          | 4281                  |
| 7       | QIHQYIMALREEEYFD          | 15          | 4376                  |
| 8       | QLYSKFLLKAEPFLA           | 15          | 1926                  |
| 9       | FLHYIFMENAFELPT           | 15          | 826                   |
| 10      | RGLKLATALSLSNKFI          | 15          | 3391                  |
| 11      | SLFFSAQPFEITAST           | 15          | 3036                  |
| 12      | VEFVTNMGIIPDF            | 15          | 881                   |
| 13      | VGSKLIVAMSSSLQK           | 15          | 1226                  |
| 14      | KFTYLINIQDEINT            | 15          | 4321                  |
| 15      | LHDLKIAIANIIIDEI          | 15          | 2191                  |
| 16      | LINWLQEALESASL            | 15          | 2491                  |
| 17      | GKLYSILKIQSPLFT           | 15          | 2756                  |
| 18      | LEVNLDFQANAQLS           | 15          | 2801                  |
| 19      | KIVSLIKNLLVALKD           | 15          | 4406                  |
| 20      | TLTAJGFASADLIEI          | 15          | 676                   |
| 21      | PALLALLALPALLL           | 15          | 6                     |
| 22      | YKLRLTSFALNLPT           | 15          | 3771                  |
| 23      | LSQLQTYMIQFDQYI          | 15          | 2171                  |
| 24      | HVKHFVINLIGDFEV           | 15          | 2316                  |
| 25      | DEINTIFSDYIPYVF          | 15          | 4331                  |
| 26      | ILFSYFQDLVTLPF           | 15          | 4241                  |
| 27      | QELLDIANYLMEIQ            | 15          | 461                   |
| 28      | FLIYITEELLKQLQST          | 15          | 4531                  |
| 29      | LLIDVVTYLVALIPE          | 15          | 406                   |
| 30      | IDLSIQNYHTFLIYI         | 15          | 4521                  |
Supplemental Table VI: MHC II affinities (IC50, nM) of the human ApoB peptides

| Peptide | DRB1*04:01 | DRB1*04 | DRB1*08:02 | DRB1*1101 | DRB1*13:02 | DRB1*15:01 | DRB3*02:02 | DRB5*01:01 |
|---------|-------------|---------|-------------|------------|-------------|------------|-------------|------------|
| DRB1*04:01 | 13209     | 5090    | 391         | 7          | 572         | 13         | 58          | 1          |
| DRB1*04 | 7          | 1        | 16          | 13209      | 5090        | 391        | 7           | 572        |
| DRB1*08:02 | 5         | 9        | 16          | 7          | 13209       | 5090       | 391         | 572        |
| DRB1*1101 | 13         | 5        | 572         | 13209      | 5090        | 391        | 7           | 572        |
| DRB1*13:02 | 5         | 9        | 13209       | 5090       | 391         | 7          | 572         | 13         |
| DRB1*15:01 | 572       | 13       | 7           | 572        | 13       | 5        | 13209       | 5090       |
| DRB3*02:02 | 13         | 5        | 572         | 13209      | 5090        | 391        | 7           | 572        |
| DRB5*01:01 | 572       | 13       | 7           | 572        | 13       | 5        | 13209       | 5090       |
| MHC Class I Allele | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM | nM |
|-------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| DRB1*04:05       | 497| 479| 664| 703| 211| 69 | 843| 789| 159| 664| 576| 803| 664| 722| 693| 919|
| DRB1*07:01       | 4106| 2035| 2447| 3729| 6580| 7731| 1185| 1911| 4111| 3831| 865| 644| 925| 1284| 1591| 1591|
| DRB1*08:02       | 9175| 248| 1451| 3627| 2657| 28| 416| 1284| 5251| 1802| 1| 1802| 1907| 1802| 1802| 1802|
| DRB3*02:01      | 6465| 9942| 96| 7171| 7205| 25| 92| 926| 96| 96| 35| 4558| 4184| 1990| 1990| 4923|
| DPB1*02:01       | 6465| 23235| 6560| 9158| 925| 955| 1185| 1497| 2737| 2467| 4923| 3979| 2141| 2141| 2141| 2141|
| DPB1*05:01       | 4124| 1337| 671| 955| 966| 6465| 1497| 2737| 3358| 2737| 2737| 2141| 2141| 2141| 2141| 2141|
| DQB1*03:02       | 2884| 3050| 1241| 1667| 2141| 2141| 2141| 2141| 2141| 2141| 2141| 2141| 2141| 2141| 2141| 2141|
| DQB1*05:01       | 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642| 642|
Supplemental Table VIII: Frequency of immune cell subsets (% CD4\(^+\)) in the human population

|                | No CAD     | CAD        | p-value   |
|----------------|------------|------------|-----------|
| FoxP3\(^+\) CD25\(^+\) | 3.0 ± 0.55 | 6.5 ± 0.52 | 0.0002    |
| FoxP3\(^+\) RORγT\(^+\) | 0.018 ± 0.007 | 0.050 ± 0.014 | 0.044    |
| FoxP3\(^+\) RORγT\(^-\) | 2.02 ± 0.64 | 6.59 ± 0.57 | < 0.0001  |
| FoxP3\(^-\) RORγT\(^+\) | 0.046 ± 0.017 | 0.128 ± 0.036 | 0.036    |
| T\(_{naive}\) (CD45RO\(^-\) CCR7\(^-\)) | 23.5 ± 2.9 | 21.9 ± 1.7 | 0.79     |
| T\(_{CM}\) (CD45RO\(^+\) CCR7\(^-\)) | 54.0 ± 3.2 | 49.0 ± 1.6 | 0.24     |
| T\(_{EM}\) (CD45RO\(^+\) CCR7\(^-\)) | 10.4 ± 1.5 | 15.9 ± 1.3 | 0.014    |

Statistical significance was tested with a Mann-Whitney U test between the two groups ‘No CAD’ and ‘CAD’.
Supplemental Figure I: Tissue distribution of ApoB$^+$ CD4$^+$ T cells. Total numbers of ApoB$^+$ CD4$^+$ T cells in individual animals were quantified in 4-8-week old Apoe$^{-/}$ mice on a C57BL/6J background in pooled lymph nodes and the spleen separately. Total cell numbers were quantified, normalized to 100% per animals, and the origin was displayed as percentage of all ApoB$^+$ T cells. **** P <0.0001. All bars indicate mean values per group. Significance was assessed by an unpaired Student’s T-test. N = 4 animals per group. LN indicates pooled lymph nodes.
Supplemental Figure II: ApoB+ T cells specifically respond to a re-stimulation with the specific ApoB-peptide ApoB<sub>978–993</sub>. (a) ELISPOT IL-17 secretion assay. CD45.2 ApoB<sup>+</sup> or CD45.2 ApoB<sup>neg</sup> T cells from female, 8-week-old Apo<sup>e</sup><sup>−/−</sup> mice were isolated by flow sorting after incubation with the ApoB:MHC dextramer, and incubated with irradiated, LPS-stimulated splenocytes from CD45.1 Apo<sup>e</sup><sup>−/−</sup> mice in the presence of ApoB<sub>978–993</sub> or the peptide-carrier DMSO alone (DMSO). After 24 hours, secretion of IL-17 was visualized on the cell culture membrane and IL-17-positive spots were counted. 3 donor mice were included per group. (b) Intracellular cytokine expression after in vitro stimulation of whole lymph node cell suspensions with the peptides ApoB<sub>978–993</sub>, ApoB<sub>3501–3516</sub>, or ConA. Lymph nodes were harvested from Apo<sup>e</sup><sup>−/−</sup> mice 10 days after a single prime of the ApoB-peptide p6 (ApoB<sub>978–993</sub>) s.c. in the base of the tail and one booster immunization in IFA 10 days later. Cell suspensions were stimulated for 2 hours with the indicated peptides or ConA, followed by another 4 hours in the presence of a transport inhibitor cocktail. Unstimulated cells (none) were used as a negative control. IL-17 was detected by intracellular flow cytometry. All bars indicate mean values per group. Statistical significance was calculated by an unpaired Student’s T-test (b) or 2-way ANOVA with Dunnett’s multiple comparison test (c). * p < 0.05, ** p < 0.01
Supplemental Figure III: Clonality index analysis of the TCR-restriction of ApoB$^\text{neg}$ and ApoB$^+\; T$ cells.

Parallel DNA-sequencing of the T cell receptor (TCR) $\beta$-chain was performed on the indicated, FACS-sorted cell populations described in Figure 1G, H. The clonality index was calculated as follows: Sum of the frequency of each clone times the log (base 2) of the same frequency over all productive reads in a sample. This value is normalized to the total number of productive unique sequences and subtracted from 1 (clonality index).
Supplemental Figure IV: Fractions of effector subsets within tetramer-positive antigen-specific CD4\(^+\) T cells. MHC-II tetramers specific for M09, originating from MCMV, MOG (Myelin oligodendrocyte glycoprotein) and 2W1S (E\(\alpha\) protein) were used to stain CD4\(^+\) T cells from pooled lymph nodes in 4-8-week-old female Apoe\(^{-/-}\) mice. The fraction of antigen-unexperienced T\(\text{naïve}\) (CD44\(^-\)CD62L\(^+\), white), antigen-experienced T effector memory (T\(\text{EM}\), CD44\(^+\)CD62L\(^-\), black), and T central memory (T\(\text{CM}\), CD44\(^+\)CD62L\(^+\), grey) cells among all antigen-specific T-helper cells of the shown specificity was quantified in flow cytometry and expressed as % of all cells. Representative data of 5-6 animals were pooled in one pie chart expressing average values.
Supplemental Figure V: ApoE-deficiency does not increase numbers of murine CMV-specific T cells.

Female, 8-week-old WT or Apoe<sup>-/-</sup> mice on a C57BL/6J background were used to identify MCMV-specific CD4<sup>+</sup> T cells by the CMV:MHC-II tetramer M25<sub>409-423</sub> (M25). Total numbers of M25<sup>+</sup> CD4<sup>+</sup> T cells in pooled lymph nodes were quantified per animal. All bars indicate mean values per group. Statistical significance was assessed by an unpaired Student’s T-test between both groups. Numbers of M25<sup>+</sup> T-helper cells in infected wt or Apoe<sup>-/-</sup> mice did not differ significantly (p > 0.05, b). N = 5 mice per group.
Supplemental Figure VI: Gating strategy to determine T cell transcription factors in ApoB+ CD4+ T cells. Representative data are shown for lymph node CD4+ T cells after a magnetic bead negative isolation of enriched CD4+ T cells and staining with the ApoB:MHC-II tetramer followed by an intracellular staining for transcription factors. A concatenated file containing all individual replicates shown in Figure 3A-C served as input data.
Supplemental Figure VII: Expression of the T_\text{H}-lineage-defining transcription factors in naïve CD4^{neg} CD4^{+} T cell subsets. Expression of FoxP3 (T_{reg}), ROR\gamma T (T_{H17}), GATA3 (T_{H2}), T-bet (T_{H1}), and Bcl6 (T_{FH}) was determined by intracellular flow cytometry. Lymph node CD4^{+} T cells were from 8-week-old Apoe^{-/-} mice (n=5 per group) and stained with the ApoB:MHC-II tetramer. Analysis was performed on CD4^{neg} CD4^{+} T cells in the indicated populations and fractions of cells expressing the indicated transcription factors were expressed as % of all cells in the parent population. All bars indicate mean values per group. ****p<0.0001. Statistical significance was calculated by an unpaired, two-sided Student’s T-test between the indicated groups.
Supplemental Figure VIII: Expression of the T\textsubscript{H}-lineage-defining transcription factors in CD4\textsuperscript{+} T cell subsets from 28-week-old WD-fed \textit{Apoe}\textsuperscript{-/-} mice. Expression of FoxP3 (T\textsubscript{reg}), ROR\textgamma T (T\textsubscript{H}17), GATA3 (T\textsubscript{H}2), T-bet (T\textsubscript{H}1), and Bcl6 (T\textsubscript{FH}) was determined by intracellular flow cytometry. Lymph node CD4\textsuperscript{+} T cells were from 28-week-old Apoe\textsuperscript{-/-} mice (n=5 per group) after consuming a WD for 20 weeks and stained with the ApoB:MHC-II tetramer. Analysis was performed on all CD4\textsuperscript{+} T cells without pre-gating for CD44\textsuperscript{+} in the indicated populations. Fractions of cells expressing the indicated transcription factors were expressed as % of all cells in the parent population. All bars indicate mean values per group.
Supplemental Figure IX: Transcription factor expression in MCMV-specific CD4+ T cells in murine CMV-infection.

Female, 8-week-old wild-type mice on a C57BL/6J background were infected with mouse CMV (MCMV, smith strain at an inoculation dose of 1x10^6 viral particles as performed previously) or left untreated. After 21 days, MCMV-specific CD4+ T cells from pooled lymph nodes were identified by the CMV:MHC tetramer M25 (a) or -M09 (b). Percentage of CD4+ T cells positive for the indicated transcription factor in intracellular flow cytometry among all cells in the parental population. No pre-gating for CD44 was done. All bars indicate mean values per group. Statistical significance was assessed by an unpaired Student’s T-test between the corresponding value in the group of tetramer-negative cell populations and tetramer-positive cell population in naïve mice or between tetramer-positive cell population in naïve and infected mice. *p<0.05, **p<0.01 *** p < 0.001, **** p < 0.0001, non-significant p-values are not displayed. N = 5 mice per group.
Supplemental Figure X: Comparison of transcriptomes of atherosclerosis relevant CD4⁺ T cell populations, ApoB⁺ T cells, and in vitro differentiated lineages.

Integrated analysis of gene expression from Li et al.¹⁶ and Abadier et al.⁵⁵ with the transcriptome of ApoB⁺ T cells from 4-week-old ApoE⁻/⁻ mice (a) Principal-component analysis (PCA) of gene expression assessed by RNA-sequencing of the indicated, flow-sorted T-helper cell subsets: ApoB⁺, and ApoB⁻reg Tregs from lymph nodes (Treg LN) and spleen (Treg SPL) from 4 week old ApoE⁻/⁻ mice were compared to atherosclerosis-specific CCR5-Teff, conventional CD4⁺ T cells (Tconv), and bulk Tregs from old, western-diet fed mice (Treg old). In vitro differentiated T cells served as additional control. (b) Heatmap of T cell lineage signature genes in the indicated populations. Heatmap in (b) based on row expression score. Data points in (k) pooled from 3-5 mice and experiments (in vitro differentiation) per group.
Supplemental Figure XI: ApoB⁺ T cells share a pro-atherogenic CCR5⁺ T_{eff} like phenotype.

To interrogate the transcriptional relationship of the ApoB⁺ CD4⁺ T cell population with the previously described pro-atherogenic CCR5⁺ T_{eff} population, an ApoB⁺ T cell gene signatures was tested in an unsupervised gene set enrichment analysis (GSEA, GenePattern, Broad Institute) against the published CCR5⁺ T_{eff} transcriptome. The top 16 gene matches that are part of the tested signatures and upregulated in CCR5⁺ T_{eff} are displayed in the heatmaps on the right. Blue color indicates the minimal, red the highest gene expression value. p-values and GSEA enrichment scores (ES) are displayed within the enrichment curve plot. The red, dotted line indicates an ES = 0.
Supplemental Figure XII: Feeding with a western diet (WD) does not further increase the effector/memory phenotype in ApoB\(^+\) CD4\(^+\) T cells.

8-week-old, female Apoe\(^-\) mice were fed a western diet (WD) or a standard chow diet (chow) for 8 weeks starting at the age of 8 weeks (baseline). Relative proportions of T\(_{EM}\) (CD44\(^+\) CD62L\(^-\)), T\(_{CM}\) (CD44\(^+\), CD62L\(^+\)), or T\(_{naive}\) (CD44\(^+\) CD62L\(^+\)) cells were quantified in peripheral lymph nodes in ApoB\(^+\) CD4\(^+\) T cells. Sum of T\(_{EM}\), T\(_{CM}\), and T\(_{naive}\) was adjusted to 100\%. All bars indicate mean values per group. Significance was assessed by an unpaired Student’s T-test between chow- and WD-consuming animals within the same subpopulation. No statistical differences were detected. \(n = 3-5\) mice per group were included per group.
Supplemental Figure XIII: exT_{reg} cells show enhanced expression of proinflammatory cytokines.

exTregs were identified by using a Foxp3-YFP-Cre/ROSA26-RFP mouse on an Apoe^{-/-} background to display historic/current FoxP3 expression (RFP^{+}) in conjunction with current expression of FoxP3, indicated by YFP-expression. CD4^{+} T cells were isolated from peripheral lymph nodes and stimulated with PMA/ionomycin for 5 hours. Intracellular flow cytometry was performed in current T_{regs} (YFP^{+}RFP^{+}), exT_{regs} (YFP^{+}RFP^{+}) and conventional T cells to quantify IFN-\gamma (a) and IL-17 (b) expression. All bars indicate mean values per group. Significance was assessed by a one-way ANOVA with Dunnett's multiple comparisons test. N=8 per group. **p<0.01 *** p < 0.001.
Supplemental Figure XIV: T\textsubscript{regs} lose FoxP3-expression after an adoptive transfer into \textit{Apoe}\textsuperscript{−/−} recipients after 6 weeks.

Adoptive transfer of 6x10\textsuperscript{4} bulk T\textsubscript{regs} (CD45.2) from 8-week-old \textit{Apoe}\textsuperscript{−/−} mice into \textit{Apoe}\textsuperscript{−/−} recipients (CD45.1) that had consumed western diet for 16 weeks. 6 weeks later, expression of TH-lineage markers FoxP3, ROR\textgreek{t}T, and T-bet was quantified by intracellular flow cytometry at the indicated locations in cells from the donor and the recipient, quantified as % of transcription factor positive cells of all CD4\textsuperscript{+} T cells. Averages were expressed as pie charts.
Supplemental Figure XV: Differentially expressed genes in the 4 aortic cell clusters in scRNAseq.

Differentially expressed genes were displayed as cluster average for the clusters 1-4 and split into cells originating from CD- and WD-consuming animals.
Supplemental Figure XVI: Validation of the human ApoB re-stimulation assay.

Human whole blood samples (n=6) were co-incubated with the pool of the 30 MHC-II-restricted human ApoB-peptides in vitro (ApoB), the carrier DMSO alone (carrier), PMA/Ionomycin as positive control, or the following control peptide pools: CEFX3: Pool of 68 known MHC-II restricted epitopes for a broad range of HLA sub-types and different infectious agents for T-cell stimulation of populations with a diverse ethnic background, ACTS: Pool of 92 peptides derived from a peptide scan through Actin, alpha skeletal muscle of Homo sapiens (Human) for T cell assays, MOG: Pool of 29 peptides derived from a peptide scan through Myelin-oligodendrocyte glycoprotein (MOG) of Homo sapiens (Human), or CEFX4: Pool of 80 known MHC-I restricted epitopes for a broad range of HLA sub-types and different infectious agents for T-cell stimulation of populations with a diverse ethnic background. An additional control received the ApoB-peptide pool in addition to a blocking anti-human HLA-DR antibody as negative control (10 µg/ml). ApoB-
reactive CD4+ T cells (CD3+CD4+CD8−L/D−) were identified by the intracellular expression of CD40L after 6 hours in intracellular flow cytometry. In addition, expression of the activation markers CD25 and CD69 is shown. (b) Readouts were expressed as % positive cells of all CD4+ T cells or (c) as the percentage of positive cells normalized to the unstimulated control (carrier). Statistical significance was calculated by a Mann-Whitney U test between the indicated groups. p-values are indicated in the figures. Representative dot plots are shown in (a). All bars indicate mean values per group.
Supplemental Figure XVII: Expression of cytokines in human CD4\(^+\) T cells after re-stimulation with a human ApoB-peptide pool.

Human whole blood samples (a, b) or density-gradient isolated PBMCs (c) from individuals without (n=11) or with (n=23) stenotic coronary artery disease (CAD) were co-incubated with the pool of the 30 MHC-II-restricted human ApoB-peptides \textit{in vitro} (ApoB), the carrier DMSO alone (control), or staphylococcal enterotoxin B (SEB) as positive control for 6 hours (a, b) or 10 days (c). (a) ApoB-reactive CD4\(^+\) T cells (CD3\(^+\)CD4\(^+\)CD8\(^-\)L/D\(^-\)) were identified by the intracellular expression of CD40L after 6 hours in intracellular flow cytometry. (b, c) Intracellular cytokine expression after re-stimulation with ApoB or the controls was quantified as % of cytokine\(^+\) CD4\(^+\) T cells. All bars indicate mean values per group. Statistical significance was calculated by a Mann-Whitney U test between the indicated groups (a-c). *p<0.05, **p<0.01, *** p < 0.001
Supplemental Figure XVIII: ApoB-reactive T cells increase across age- and gender matched CAD patients.

CD4⁺ T cells recognizing a mix of 30 human ApoB-peptides in PBMCs from humans with (n=23) or without (n=11) stenotic coronary artery disease (CAD), as diagnosed in coronary angiography. ApoB⁺ CD4⁺ T cells were identified by intracellular expression of the activation marker CD40L after stimulation with a human ApoB-peptide mix in whole blood samples for 6 hours. ApoB-reactive CD4⁺ T-helper cells (CD3⁺CD4⁺CD8⁻L/D⁺) were identified by the intracellular expression of CD40L over baseline (CD40L⁺ expressed as % of unstimulated cells). (a) Patients were divided into groups of different ages (40-49, 50-59, 60-69, 70-79, 80-90). (b) Patients were divided into male and female patients. Statistical significance was tested by an unpaired Mann-Whitney test for non-normal distributed samples. Normal distribution was tested by the D’Agostino & Pearson normality test. P-values are displayed in the graph (b). Bars indicate mean values per group.
Supplemental Figure XIX: Expression of CD4\(^+\) T cell activation markers after re-stimulation of human CD4\(^+\) T cells from patients with and without CAD with human ApoB-peptides.

Human whole blood samples from individuals without (n=11) or with (n=23) stenotic coronary artery disease (CAD) were co-incubated with the pool of the 30 MHC-II-restricted human ApoB-peptides in vitro (ApoB) or the carrier DMSO alone (control) for 6 hours. Expression of the extracellular activation markers CD38 (a), HLA-DR (b), and CD25 (c) was quantified as percentage of activation marker-positive cells (% of all CD4\(^+\) T cells). Statistical significance was tested by an unpaired Mann-Whitney test for non-normal distributed samples. Normal distribution was tested by the D'Agostino & Pearson normality test. No significant regulation was observed. All bars indicate mean values per group.
SUPPLEMENTAL DATA FILES

Supplemental Data File I: Sequencing of TCR-β chain of ApoBneg T cells after FACS cell sorting. A total of 23,488 unique, productive clonotypes was detected in 411,397 overall clonotypes. Nucleotide sequence, number of detected clonotypes with the same nucleotide sequence (count reads), the fraction of this TCR-β chains with the same nucleotide sequence among all clonotypes (frequency %) and the V,D,J-chain usage is indicated in the table.

Supplemental Data File II: Sequencing of TCR-β chain of ApoB+ T cells after FACS cell sorting. A total of 85 unique, productive clonotypes was detected in 5,168 overall clonotypes. Nucleotide sequence, number of detected clonotypes with the same nucleotide sequence (count reads), the fraction of this TCR-β chains with the same nucleotide sequence among all clonotypes (frequency %) and the V,D,J-chain usage is indicated in the table.

Supplemental Data File III: Complete expression table with RPKMs of all detected genes in the indicated FACS-sorted cell populations after RNA-sequencing. Cells were sorted from different ages (4-, 8-, or 20-week-old mice) and genotypes (WT, Apoe+). Cells were either located in the ApoBneg or ApoB+ gate after tetramer staining. Tregs were defined as CD25+FR4+ CD4+ T cells. This Online-only Tableerved for generating heatmaps, GSEA, and IPA between the indicated groups in Figure 3 D-E and Figure 4 B-D.

Supplemental Data File IV: List of 1,044 differentially expressed (DE) genes between the tSNE-defined clusters in Figure 5B. DE-genes were calculated with the non-parameteric Wilcoxon rank sum test between the indicated clusters (‘cluster’) and all other clusters. P-value for statistical testing is indicated as ‘p_val’. ‘avg_logFC’ indicates log fold-change of the average expression between the two groups (cluster vs all others). ‘pct_1’ indicates the percentage of cells positive for tested gene in the tested cluster vs. percentages in all other clusters (‘pct_2’).