

# Mucosal Administration of Collagen V Ameliorates the Atherosclerotic Plaque Burden by Inducing Interleukin 35-dependent Tolerance\*

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We have shown previously that collagen V (col(V)) autoimmunity is a consistent feature of atherosclerosis in human coronary artery disease and in the *Apoe*<sup>-/-</sup> mouse model. We have also shown sensitization of *Apoe*<sup>-/-</sup> mice with col(V) to markedly increase the atherosclerotic burden, providing evidence of a causative role for col(V) autoimmunity in atherosclerotic pathogenesis. Here we sought to determine whether induction of immune tolerance to col(V) might ameliorate atherosclerosis, providing further evidence for a causal role for col(V) autoimmunity in atherogenesis and providing insights into the potential for immunomodulatory therapeutic interventions. Mucosal inoculation successfully induced immune tolerance to col(V) with an accompanying reduction in plaque burden in *Ldlr*<sup>-/-</sup> mice on a high-cholesterol diet. The results therefore demonstrate that inoculation with col(V) can successfully ameliorate the atherosclerotic burden, suggesting novel approaches for therapeutic interventions. Surprisingly, tolerance and reduced atherosclerotic burden were both dependent on the recently described IL-35 and not on IL-10, the immunosuppressive cytokine usually studied in the context of induced tolerance and amelioration of atherosclerotic symptoms. In addition to the above, using recombinant protein fragments, we were able to localize two epitopes of the  $\alpha 1(V)$  chain involved in col(V) autoimmunity in atherosclerotic *Ldlr*<sup>-/-</sup> mice, suggesting future courses of experimentation for the characterization of such epitopes.

Atherosclerosis underlies coronary artery disease (CAD)<sup>3</sup> and stroke, major global causes of death (1), and is a chronic

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<sup>3</sup> The abbreviations used are: CAD, coronary artery disease; col(V), collagen type V; TV-DTH, *trans vivo* delayed-type hypersensitivity; TT/DT, tetanus and diphtheria toxoid; ORO, Oil Red O; T<sub>R</sub>, T regulatory; T<sub>E</sub>, T effector.

inflammatory disease that is modulated by both innate and adaptive immune pathways. It is increasingly accepted that autoimmunity constitutes some portion of the pathological processes underlying atherosclerosis, with oxidized LDLs, native lipoproteins, and heat shock proteins identified as autoantigens involved in atherogenesis (1–8). Recognition of the autoimmune aspects of atherosclerosis has suggested the possibility of employing immunomodulatory approaches to ameliorate symptoms. That such an approach is feasible has been borne out in studies in which blockage of T cells reactive to native lipoprotein ApoB100 (4) or mucosal or subcutaneous immunization with HSP65 (9, 10), oxidized LDL (11), native  $\beta 2$ -glycoprotein I (12), or apolipoprotein B-100 peptide (13, 14) have been shown to be atheroprotective.

Collagens can compose up to 60% of the total protein content in atherosclerotic plaques (15) and stimulate the growth and inflammation of atheromas (16). We have shown previously that interleukin 17-dependent autoimmunity to type V collagen col(V) is a consistent feature of atherosclerosis in advanced CAD in humans and in *Apoe*<sup>-/-</sup> mice on a high-fat diet (17). Moreover, the same study provided evidence of a causative role for col(V) autoimmunity in the pathogenesis of atherosclerosis because sensitization of *Apoe*<sup>-/-</sup> mice on normal chow to col(V) has been shown to markedly increase the atherosclerotic burden (17). Col(V) is broadly expressed as  $\alpha 1(V)_2\alpha 2(V)$  heterotrimers that are sequestered within the interiors of fibrils of the abundant type I collagen (col(I)) (18). However, excessive amounts of  $\alpha 1(V)$  chains have been shown to be deposited in atheromas (19), and when  $\alpha 1(V)$  chains are in excess compared with available  $\alpha 2(V)$  chains with which to complex, the former can form aberrant  $\alpha 1(V)_3$  homotrimers (20) that may be excluded from col(I) fibrils (21). These considerations, and the finding of immune reactivity to  $\alpha 1(V)$  but not  $\alpha 2(V)$  chains in atherosclerotic humans and mice, led us previously to suggest a model in which  $\alpha 1(V)_3$  homotrimers, in the inflammatory environment of atherosclerotic plaques, present normally cryptic epitopes that initiate autoimmunity (17).

Here we present new evidence of a causative role for col(V) autoimmunity in the pathogenesis of atherosclerosis and for possible novel immunomodulatory approaches for ameliorating atherosclerosis by demonstrating that mucosal col(V)

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administration induces immune tolerance and an accompanying reduction in plaque burden in *Ldlr*<sup>-/-</sup> mice on a high-fat, high-cholesterol diet. Interestingly, we show both induction of immune tolerance to col(V) and reduction of the plaque burden to be dependent on the relatively recently described immunosuppressive cytokine IL-35 (22–24). In addition, use of recombinant fragments localizes two autoimmune epitopes in the N-terminal half of the  $\alpha 1(V)$  triple-helical domain.

### Experimental Procedures

**Mice**—*Ldlr*<sup>-/-</sup> mice (strain B6.129S7-*Ldlr*<sup>tm1Her1</sup>) were obtained from The Jackson Laboratory, and TV-DTH recipient C57BL/6 mice were obtained from Harlan Sprague-Dawley. Five-week-old mice were inoculated intranasally with 4  $\mu$ g of bovine col(V) (a gift from ImmuneWorks Inc.), 4  $\mu$ g of col(I) (PureCol, Advanced BioMatrix), or PBS every other day for 2 weeks while on normal chow (Harlan Teklad 8604) and then placed on a “Western” high-fat (20 gram percent), 1.25% cholesterol diet (D12108C, Research Diets) for 14 or 24 weeks and inoculated two times per week for the duration of the experiment. A subset of the col(V)-treated mice for the 24-week study was subjected to intraperitoneal injection with 100  $\mu$ g of murine anti-mouse Ebi3 antibody (antibody V1.4C4.22 (25)) or with isotype-matched control murine IgG2b at 12 weeks subsequent to the start of the Western diet and then with 50  $\mu$ g of anti-Ebi3 or IgG2b once per week until sacrifice. Two weeks prior to sacrifice, all mice were immunized subcutaneously in the inguinal region with 1.5 limits of flocculation tetanus toxoid and diphtheria (TT/DT) pediatric vaccine (Sanofi-Aventis Pasteur). Upon sacrifice, spleens and aortas were collected and processed. The experiments were performed in accordance with National Institutes of Health and United States Department of Agriculture guidelines after approval by the University of Wisconsin Institutional Animal Care and Use Committee.

**Trans Vivo Delayed-type Hypersensitivity (TV-DTH) Assay**—TV-DTH assays were performed by cotransfer of 8–10  $\times 10^6$  mouse splenocytes into the footpads of naïve C57BL/6 recipients, as described previously (26). TT/DT (0.25 limits of flocculation, Sanofi-Aventis Pasteur) was used as a positive control recall antigen. Bovine col(V) (ImmuneWorks Inc.) and col(I) (PureCol, Advanced BioMatrix) were tested at 20  $\mu$ g each. Cytokines were neutralized by coinjection of 1  $\mu$ g of antibodies against mouse IFN- $\gamma$ , IL-17A, TGF- $\beta$  (BD Biosciences), IL-10, IL-27 p28, IL-12/IL-35 p35 (R&D Systems), and Ebi3 (antibody V1.4C4.22) (25).

**Atherosclerotic Plaque Burden**—After euthanasia, aortas were perfused to remove residual blood by puncturing the left ventricle with a 25-gauge needle, followed by slow perfusion with PBS. Fat was dissected away, and a midline incision was created along the length of the aorta from the diaphragm through the lesser curvature of the arch. Aortic branches were removed, and an incision was made along the greater curvature of the arch from the heart to the left subclavian artery. The aorta was then removed from the body and pinned open on black wax for fixation and Oil Red O (ORO) staining, as described previously (27). Briefly, aortas were rinsed with 78% methanol, incubated for 50 min in ORO stock solution (0.2% weight/volume ORO, 78% methanol mixed with 22% volume 1 M NaOH and

filtered), destained for 5 min in 78% methanol, and then placed in PBS. Aortas were photographed under a dissecting microscope at  $\times 7$  magnification. Plaque areas in the descending thoracic aortas, from the left subclavian artery to the diaphragm, were quantified using Photoshop, with the Color Range function used to identify areas of positive ORO staining. To prevent bias, the same Color Range settings were used for all analyses. Percent plaque area was calculated by the number of pixels selected by the Color Range function divided by the total number of pixels of the aorta.

**Immunoblots**—For blots of aortas, to compensate for variations in the extent of plaque burden between the aortas of col(V)-, PBS-, and col(I)-treated *Ldlr*<sup>-/-</sup> mice, plaques were removed from *en face* prepared descending thoracic aortas by forceps prior to extraction of the aortas in SDS-PAGE sample buffer. For blots of inguinal lymph nodes, the nodes were dissected out and extracted in SDS-PAGE sample buffer. Aorta blots were probed with mouse monoclonal anti-MCP-1 primary antibodies (eBioscience) and secondary goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, 1:6000). The antibodies for lymph node blots were as described above, except for the anti-p40 antibody, which was from BD Biosciences. Blots on PVDF membranes were repeated twice for aorta blots with similar results. Lymph node blots were repeated three times with samples from three different mice, with similar results. All blots were visualized using the ECL kit (Pierce). Anti-tubulin antibodies for loading controls were from Millipore. All primary antibodies were diluted 1:1000. Secondary goat anti-rabbit IgG was diluted 1:4000 (Bio-Rad).

**Flow Cytometry**—Aortic single-cell suspensions were prepared and stained for lineage markers (B220(RA3-6B2), CD8 (53-6.7), CD4 (RM4-5), NK1.1 (PK136), Ter-119 (TER-119), Ly6G (1A8), and CD90.2 (53-2.1)) and with antibodies to determine monocyte populations, including CD11c (N418), CD11b (M1/70), and F4/80 (BM8), essentially as described by Dutta *et al.* (28). Myeloid cells were defined as lineage-negative/CD11b<sup>+</sup> populations. Inflammatory monocytes were further discriminated by myeloid cells that were F4/80-negative/Ly-6C positive. All data were acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using Flowjo data analysis software (TreeStar).

**Production and Purification of Col(V) Recombinant Protein Fragments**—Recombinant DNA expression constructs for producing six fragments of similar lengths that, together, constitute the sequences of the major triple-helical (COL1) domain of the human  $\alpha 1(V)$  collagen chain were produced by PCR amplification from a full-length human pro- $\alpha 1(V)$  cDNA clone (29) using the following oligonucleotide primers: fragment 1, 5'-CTAGCTAGCTGGACCAGCTGGCCCCGATG-3' (forward) and 5'-CCCTTCGAACCTGGGACCCACATTTCCCTT-3' (reverse); fragment 2, 5'-CTAGCTAGCTGGAGAGCCTGGCCCC-3' (forward) and 5'-CCCTTCGAAACCTCCGCGACCTTTGG-3' (reverse); fragment 3, 5'-CTAGCTAGCTAATGGTGACCCCGGTCTCT-3' (forward) and 5'-CCCTTCGAACGGAAGCCCCGTGTTACC-3' (reverse); fragment 4, 5'-CTAGCTAGCTGGCCTTGCTGGAAAAGAAGGG-3' (forward) and 5'-CCCTTCGAAGGGACCTTCATCACCTTCTGTC-3' (reverse); fragment 5, 5'-CTAGCTAGCTAGAG-

GCTTTCTGGACCCC-3' (forward) and 5'-CCCTTCGAA-CGATGGACCTGGTTCACCACT-3' (reverse); and fragment 6, 5'-CTAGCTAGCTGGGCCCTCCAGGAAAAAGGGG-3' (forward) and 5'-CCCTTCGAAGATTGGCAGGGGCTGGATGA-3' (reverse). In each case, NheI and BstBI restriction sites were added to the 5' and 3' ends of each fragment, respectively. The PCR products were then ligated between NheI and BstBI sites of a modified pcDNA4 vector (Life Technologies), containing sequences encoding a BM40 signal peptide (to optimize secretion) directly 5' of the NheI restriction site and a His<sub>6</sub> tag directly 3' of the BstBI restriction site. Additionally, sequences encoding the pro- $\alpha$ 1(V) C-propeptide were added 3' to each of the fragments to enable chain association and the formation of triple-helical molecules. The primer set 5'-CCCTTCGAAA-CATCGACGC-3' (forward) and 5'-CCCTTCGAAGCCCATGAAGCA-3' (reverse) was used to amplify C-propeptide sequences from the full-length human pro- $\alpha$ 1(V) clone described above, adding BstBI sites to both the 5' and 3' ends. The PCR product was then ligated into each of the previously constructed vectors at the single BstBI site. Several clones of each fragment construct were sequenced to ensure proper orientation of the C-propeptide insert.

Purified constructs were transfected with TransIT-LT1 (MirusBio, Madison, WI) into T-REx HEK293 cells, followed by selection for zeocin resistance. Cells were maintained in DMEM (Cellgro, Manassas, VA) supplemented with 10% FBS (MidSci, Valley Park, MO) in 5% CO<sub>2</sub>. To obtain conditioned media for harvesting, cells were first rinsed twice with PBS and then serum-starved in DMEM supplemented with 75  $\mu$ g/ml ascorbic acid, 40  $\mu$ g/ml soybean trypsin inhibitor (Sigma), and 1  $\mu$ g/ml tetracycline. Conditioned media were collected every 24 h for 3–5 consecutive days and supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 1 mM *N*-ethylmaleimide, and 0.1 mM *p*-aminobenzoic acid. The medium was then purified on Co<sup>2+</sup> columns (Thermo Scientific, Waltham, MA). Eluents were dialyzed against PBS. Purity and quantification were determined by SDS/PAGE gel stained with Coomassie Brilliant Blue.

**Statistical Analysis**—Data were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Unpaired Student's *t* tests were used for all other analyses.

## Results

**Mucosal Administration of ColV Induces Tolerance in *Ldlr*<sup>-/-</sup> Mice on a Western Diet**—In initial experiments to determine whether mucosal administration of colV might induce tolerance to this autoantigen in atherosclerotic mice, 5-week-old *Ldlr*<sup>-/-</sup> mice on normal chow were treated with small-volume nasal infusions of colV or, as negative controls, PBS or col(I). After 2 weeks, the mice were shifted to a high-fat, high-cholesterol Western diet (Fig. 1A) to induce atherosclerosis. All mice were also immunized with TT/DT to provide a positive control for immunity. Preliminary studies employing TV-DTH assays of splenocytes indicated that col(V) autoimmunity becomes consistently detectable in *Ldlr*<sup>-/-</sup> mice after ~12 weeks on the Western diet (data not shown). Therefore, after 14 weeks on the Western diet, col(V)-, col(I)-, and PBS-treated *Ldlr*<sup>-/-</sup> mice were sacrificed, and their splenocytes were tested for anti-col(V) cellular immunity via TV-DTH

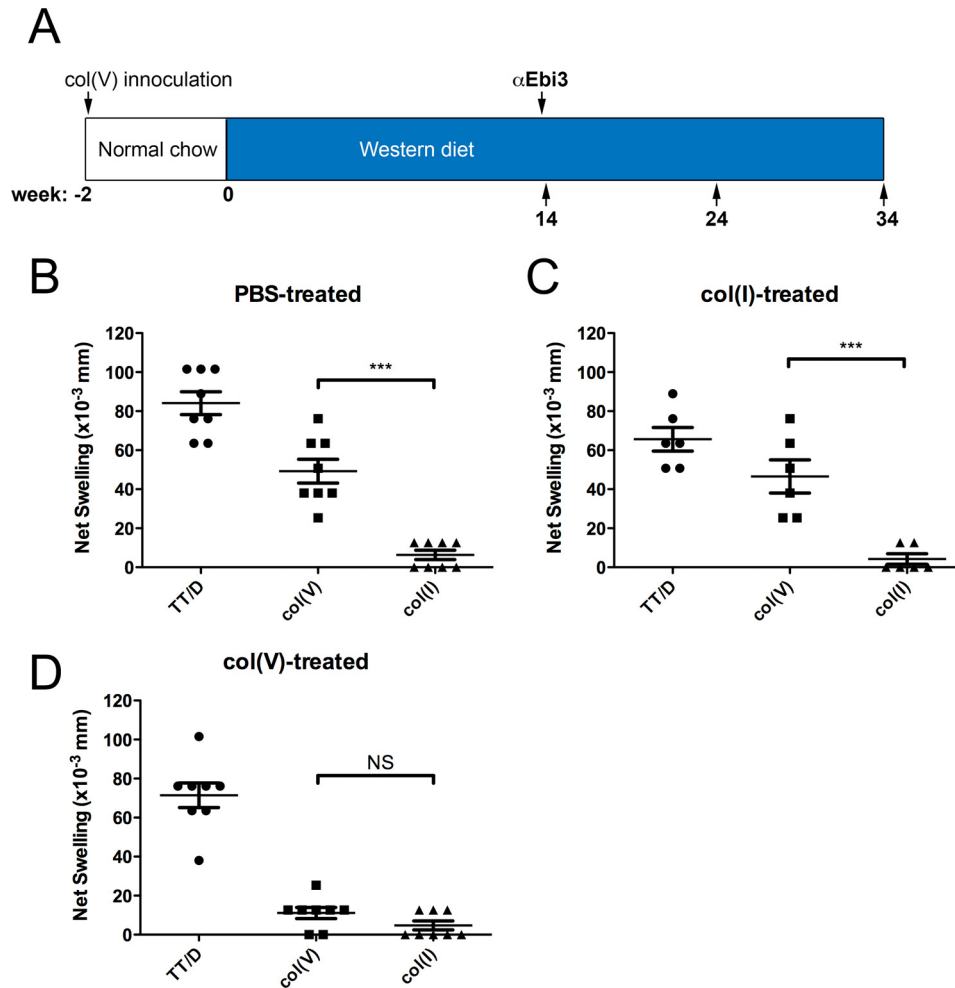
assay. Splenocytes obtained from all differently treated *Ldlr*<sup>-/-</sup> mice exhibited a high immunoreactivity to the TT/DT control antigen, indicative of intact immune systems (Fig. 1, B–D). In addition, *Ldlr*<sup>-/-</sup> mice that were treated with PBS or col(I) showed substantial immune reactivity to col(V) but no reactivity to col(I) (Fig. 1, B and C), a collagen that, although present abundantly in atherosclerotic lesions (30), does not appear to act as an autoantigen (17). In contrast, *Ldlr*<sup>-/-</sup> mice that were administered col(V) showed a lack of immune reactivity to col(V) (Fig. 1D), consistent with induced immune tolerance.

When splenocytes from the PBS- or col(I)-treated *Ldlr*<sup>-/-</sup> mice were co-injected with neutralizing antibodies to IFN- $\gamma$  in TV-DTH assays, immune responses to col(V) were blocked significantly for splenocytes from the PBS-treated mice (Fig. 2A), with a trend toward reduced immunity to col(V) for splenocytes from col(I)-treated mice (Fig. 2B). Immune responses to col(V) were blocked more effectively and consistently in TV-DTH assays via co-injection of splenocytes from either PBS- or col(I)-treated *Ldlr*<sup>-/-</sup> mice with neutralizing antibodies to IL-17 (Fig. 2, A and B). These results suggest a variable dependence of the anti-col(V) response on the Th1 pathway and a more consistent, stronger dependence on the Th17 pathway. These results are consistent with those obtained previously using atherosclerotic *ApoE*<sup>-/-</sup> mice, which also demonstrated a col(V) autoimmunity with consistent dependence on the Th17 pathway and more variable involvement of the Th1 pathway (17). Therefore, the immune pathways involved in atherosclerosis-associated anti-col(V) immunity seem to be similar in these two mouse models.

**Induced Tolerance to Col(V) Is Regulated by IL-35 but Not by IL-10 or TGF- $\beta$ 1**—We have shown previously that *ApoE*<sup>-/-</sup> mice on normal chow have an IL-10-suppressed anti-col(V) autoimmune response associated with incipient atherosclerosis (17). To characterize the nature of the tolerance induced in the col(V)-treated *Ldlr*<sup>-/-</sup> mice and determine the extent to which it might be mediated by IL-10-producing cells, splenocytes from the col(V)-treated *Ldlr*<sup>-/-</sup> mice were co-injected in the TV-DTH assay with col(V) with or without neutralizing antibodies to IL-10 or to another immunosuppressive cytokine, TGF- $\beta$ 1. Surprisingly, neutralization of IL-10 or TGF- $\beta$ 1 did not have any effect on the TV-DTH swelling response (Fig. 2C) and, therefore, no effect on tolerance. We then sought to determine the extent to which this tolerance might be mediated by the more recently characterized immunosuppressive cytokine IL-35 (22–24). IL-35 is a unique heterodimer comprising subunits p35 and Ebi3. However, p35 is also found, along with the subunit p40, in the heterodimeric pro-inflammatory cytokine IL-12, whereas Ebi3 is also found, along with the subunit p28, in the heterodimeric cytokine IL-27, which can both promote and suppress effector T cell responses, depending upon the microenvironment (24). Therefore, to test for a possible role for IL-35 in the tolerance induced in col(V)-treated *Ldlr*<sup>-/-</sup> mice, we co-injected splenocytes from these mice with neutralizing antibodies to p35 or Ebi3. We also co-injected splenocytes with antibodies to p28 to demonstrate that effects on anti-col(V) responsiveness from co-injection of splenocytes with anti-Ebi3 were due to neutralization of Ebi3-containing IL-35 and not



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**FIGURE 1. Mucosal administration induces tolerance to col(V) in  $Ldlr^{-/-}$  mice on a Western diet.** *A*, schematic relating the beginning of col(V) inoculation of  $Ldlr^{-/-}$  mice on normal chow with placing the mice on a Western diet, injections with anti-Ebi3 antibodies, and times of sacrifice. *B–D*,  $Ldlr^{-/-}$  mice were fed a Western diet for 14 weeks before experimentation. All mice were immunized with TT/DT 2 weeks before the assays. TV-DTH measured cellular responses to TT/DT, col(V), and col(I) by splenocytes from mice treated nasally with PBS (*B*,  $n = 8$  mice/assay), col(I) (*C*,  $n = 6$  mice/assay), and col(V) (*D*,  $n = 8$  mice/assay). Data are shown as mean  $\pm$  S.E. \*\*\*,  $p \leq 0.0005$ ; NS, not significant.

due to neutralization of Ebi3-containing IL-27. Neutralization of either p35 or Ebi3 was sufficient to break the tolerance (Fig. 2D), whereas neutralization of both p35 and Ebi3 together did not produce a greater effect on the TV-DTH swelling response than neutralization of either subunit separately. This lack of an additive effect is consistent with the conclusion that effects on TV-DTH swelling were solely via neutralization of p35 and Ebi3 subunits found in the same population of p35/Ebi3 heterodimers (*i.e.* IL-35) and did not include effects of neutralization of p35 or Ebi3 bound to other types of subunits in other cytokines. This conclusion was bolstered by the finding that neutralization of p28, bound to Ebi3 in IL-27 heterodimers but not found in IL-35 heterodimers, had no effect on TV-DTH swelling responses (Fig. 2D). Moreover, the anti-Ebi3 monoclonal antibodies used for this study have been shown previously to neutralize only IL35 and not IL-27 (22), even though both contain Ebi3 chains. Therefore, the induced tolerance to col(V) in col(V)-treated  $Ldlr^{-/-}$  mice is mediated by IL-35.

In support of the possibility that the induced tolerance to col(V) in col(V)-treated mice is mediated by IL-35, immunoblotting of inguinal lymph nodes showed increased levels of

both IL-35 subunits, Ebi3 and p35, in the lymphocytes of col(V)-inoculated mice compared with levels in the lymphocytes of PBS- and col(I)-inoculated mice (Fig. 3, A–C). That this increase is specific for IL-35 and does not also reflect increases in IL-12 or IL-27, which also contain p35 and Ebi3 subunits, respectively, was demonstrated by showing that neither IL-12 subunit p40 nor IL-27 subunit p28 was elevated in the lymph nodes of col(V)-inoculated mice (Fig. 3, A–C).

*Induced Tolerance to Col(V) Can Ameliorate the Atherosclerotic Burden*—We have shown previously that sensitization of  $ApoE^{-/-}$  mice on normal chow to col(V) breaks the tolerance to col(V), resulting in exacerbated atherosclerosis (17). This provided evidence that col(V) autoimmunity can play a causal role in the pathogenesis of atherosclerosis. To determine whether inducing tolerance to col(V) in  $Ldlr^{-/-}$  mice on a Western diet might ameliorate atherosclerosis, the plaque burden was compared in the descending thoracic aortas of PBS-, col(I)-, and col(V)-treated  $Ldlr^{-/-}$  mice after 14 weeks on a Western diet. ORO staining showed a significant reduction in plaque burden in col(V)-treated  $Ldlr^{-/-}$  mice, which had a percent plaque

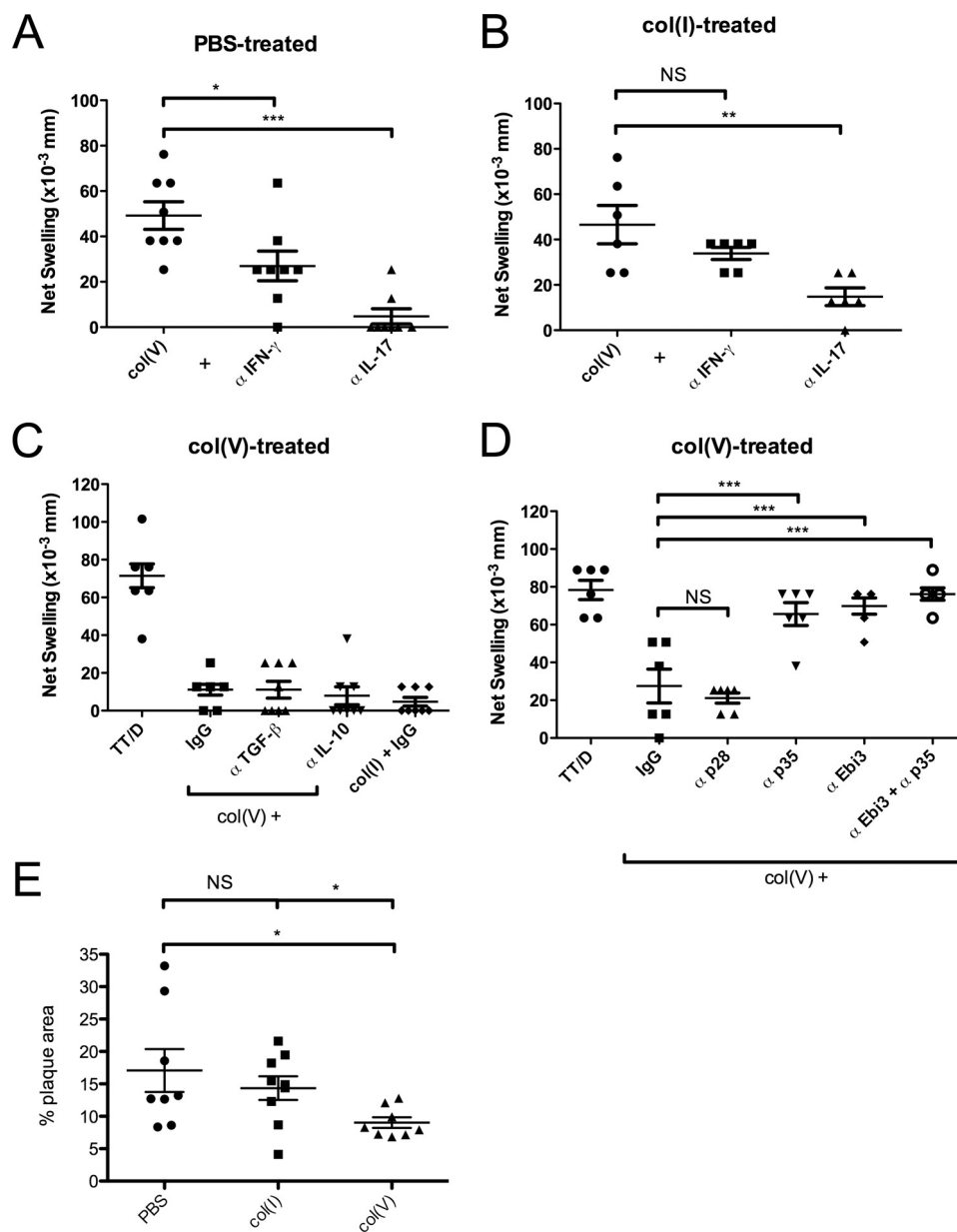


FIGURE 2. **Induced col(V) tolerance is regulated by IL-35 but not by IL-10 or TGF- $\beta$ 1.** *Ldlr*<sup>-/-</sup> mice were fed a Western diet for 14 weeks and were immunized with TT/DT 2 weeks before the assays. TV-DTH assays compared T cell responses between splenocytes injected with col(V) alone or in the presence of neutralizing antibodies to cytokines. A and B, TV-DTH responses were compared for splenocytes from PBS-treated mice (A, *n* = 8 mice/group) and from col(I)-treated mice (B, *n* = 6 mice/group) injected with col(V) alone or together with neutralizing antibodies to IFN- $\gamma$  or IL-17. C, splenocytes from col(V)-treated *Ldlr*<sup>-/-</sup> mice kept on a Western diet for 14 weeks were injected with TT/DT, with col(V) or col(I) alone or together with control IgG, or with neutralizing antibodies to TGF- $\beta$  or IL-10 (*n* = 8 mice/group). D, splenocytes from col(V)-treated *Ldlr*<sup>-/-</sup> mice kept on a Western diet for 14 weeks were injected with TT/DT, or with col(V) alone (*n* = 6) or together with neutralizing antibodies to p28 (a subunit of IL-27 but not IL-35), p35 (a subunit of both IL-35 and IL-12), or Ebi3 (a subunit of both IL-35 and IL-27), or together with both p35 and Ebi3 (*n* = 6 mice/group). E, quantitative analysis of the percent of plaque area in ORO-stained *en face* preparations of the descending aortas of *Ldlr*<sup>-/-</sup> mice treated nasally treated with PBS, col(I), or col(V) and maintained on a Western diet for 14 weeks (for each group, *n* = 8 mice). Data are shown as mean  $\pm$  S.E. \*, *p*  $\leq$  0.05; \*\*, *p*  $\leq$  0.005; \*\*\*, *p*  $\leq$  0.0005; NS, not significant.

area reduced by 47% and 37% compared with PBS- and col(I)-treated *Ldlr*<sup>-/-</sup> mice, respectively (Fig. 2E). This provides further evidence of a causal role for col(V) autoimmunity in the pathogenesis of atherosclerosis.

**Amelioration of the Atherosclerotic Burden by Induced Tolerance to Col(V) Is IL-35-dependent**—Because neutralizing antibodies to IL-35 can break immune tolerance to col(V) in TV-DTH assays (Fig. 2D), we tested whether IL-35-neutralizing antibodies might also be able to break *in vivo* tolerance to col(V) autoimmunity in *Ldlr*<sup>-/-</sup> mice on a Western diet and, if so, how

this breaking of tolerance might affect the plaque burden. Toward this end, *Ldlr*<sup>-/-</sup> mice were nasally administered PBS, col(I), or col(V) and kept on a Western diet for 24 weeks. One cohort of the col(V)-treated *Ldlr*<sup>-/-</sup> mice was subjected to a regimen of injections with monoclonal anti-Ebi3 antibody, shown previously to neutralize IL-35 (22), and another was subjected to injections with isotype-matched control murine IgG. As with mice kept on the Western diet for 14 weeks (above), PBS- and col(I)-treated mice on the Western diet for 24 weeks showed IL-17-dependent and, to a lesser degree, IFN- $\gamma$ -depen-

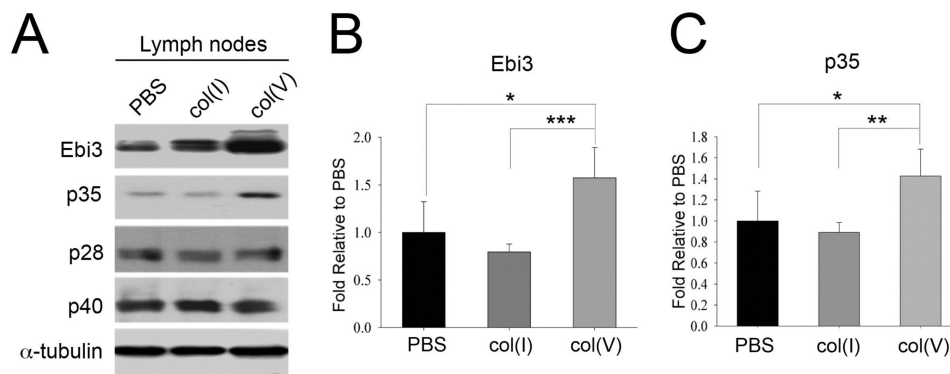


FIGURE 3. Levels of the IL-35 subunits Ebi3 and p35 are increased in the inguinal lymph nodes of col(V)-treated mice. A, representative immunoblots of inguinal lymph nodes probed with antibodies to Ebi3, p35, p28, p40, and tubulin, the latter as a loading control. B and C, quantification of the intensity of bands for Ebi3 (B) and p35 (C) for three immunoblots of inguinal lymph nodes from three different mice. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ; \*\*\*,  $p \leq 0.0005$ .

dent immune reactivity to col(V) in TV-DTH assays (Fig. 4). All mice in the col(V)-treated group subjected to injections with control IgG were tolerized to col(V), with tolerance shown to be IL-35-dependent via use of TV-DTH assays (Fig. 5A). In contrast, none of the col(V)-treated *Ldlr*<sup>-/-</sup> mice subjected to injections with anti-Ebi3 antibodies demonstrated evidence of col(V) tolerance, showing instead strong col(V) immunity with significant IL-17 dependence and a trend suggestive of some dependence on IFN- $\gamma$  (Fig. 5B). These results strongly support the conclusion that *in vivo* col(V) immune tolerance, induced by mucosal col(V) administration, is IL-35-dependent.

Importantly, col(V)-treated *Ldlr*<sup>-/-</sup> mice injected with anti-Ebi3 antibodies did not show the reduction in atherosclerotic plaque burden seen in col(V)-treated *Ldlr*<sup>-/-</sup> mice injected with IgG (Fig. 5, C and D) or in col(V)-treated *Ldlr*<sup>-/-</sup> mice in the experiments in which mice were kept on the Western diet for 14 weeks (above). In fact, the percent of plaque area was increased significantly in the aortas of col(V)-treated atherosclerotic *Ldlr*<sup>-/-</sup> mice injected with anti-Ebi3 antibodies compared with the percent of plaque area found in the aortas of PBS- and col(I)-treated atherosclerotic *Ldlr*<sup>-/-</sup> mice (Fig. 5, C and D). This suggests that neutralizing antibody to Ebi3 not only blocked col(V) tolerance, but likely other immunosuppressive effects of IL-35 as well. It should be noted again that although signaling via the IL-27 receptor appears capable of playing an anti-atherosclerotic role (31), the anti-Ebi3 monoclonal antibody used in the present study does not appear capable of blocking IL-27 (22), even though the latter, like IL-35, contains the Ebi3 subunit.

To gain further insights into the effects of induced col(V) tolerance on atherosclerotic pathology, we analyzed the levels of MCP-1, a chemokine (expressed by vascular endothelial cells, smooth muscle cells, and macrophages) that is increased in atherosclerotic lesions and is key in initiating and propagating the inflammatory process underlying atherogenesis (32). Interestingly, MCP-1 levels were reduced greatly in the aortas of col(V)-treated atherosclerotic *Ldlr*<sup>-/-</sup> mice subjected to injections with IgG (Fig. 5E). In contrast, MCP-1 levels were high in the aortas of atherosclerotic *Ldlr*<sup>-/-</sup> mice subjected to anti-Ebi3 antibody injections, with levels similar to those found in the aortas of atherosclerotic *Ldlr*<sup>-/-</sup> mice treated with PBS or col(I) (Fig. 5E). Therefore, the *in vivo* col(V) immune toler-

ance induced by mucosal administration of col(V) induces reductions in atherosclerotic plaque burden and in levels of a cytokine that is key in vessel inflammation, and, in both cases, these reductions seem to be IL-35-dependent.

When total numbers of CD11b<sup>+</sup> monocytes/macrophages were ascertained in the aortas of mice treated with PBS or treated with col(V) and then subjected to injections with IgG or with anti-Ebi3 antibodies, the col(V)-treated mice injected with IgG were found to have a significant decrease in CD11b<sup>+</sup> cells compared with the PBS-treated controls whereas, in contrast, col(V)-treated mice injected with anti-Ebi3 antibody did not (Fig. 5F). However, although there was a trend toward more CD11b<sup>+</sup> cells in the aortas of anti-Ebi3-injected mice than in the aortas of IgG-injected mice, the difference did not reach significance. In contrast, aortas of the anti-Ebi3-injected mice had markedly and significantly increased numbers of Ly-6C<sup>high</sup> inflammatory (28) CD11b<sup>+</sup> monocytes/macrophages compared with the aortas of IgG-injected mice (Fig. 5F). Therefore, MCP-1 levels (Fig. 5E) correlated with levels of Ly-6C<sup>high</sup> inflammatory CD11b<sup>+</sup> monocytes/macrophages (Fig. 5F), and both were reduced by induction of IL-35-dependent tolerance to col(V).

*Epitopes Underlying Col(V) Autoimmunity in Ldlr*<sup>-/-</sup> *Mice Are Localized to the N-terminal Half of the  $\alpha$ 1(V) Triple-helical Domain*—We have shown previously that the epitopes underlying col(V) autoimmunity in both human CAD and bronchiolitis obliterans syndrome map to the  $\alpha$ 1(V) chain triple-helical domain (17, 33). Toward further locating and characterizing such epitopes, recombinant fragments representing essentially the entire human  $\alpha$ 1(V) chain triple-helical domain divided into six fragments of similar length (Fig. 6, A and B) were subjected to TV-DTH assays with splenocytes from *Col5a2*<sup>loxP/loxP</sup>:*Ldlr*<sup>-/-</sup> mice that had been on a Western diet for 24 weeks. As can be seen in Fig. 6C, only fragments 1 and 3 garnered immune responses, and these were ~67% that of intact col(V). Fragments 1 and 3 comprise amino acids 41–156 and 294–471, respectively, of the 1014-amino acid triple-helical domain (Fig. 7). Previously, Keller *et al.* (34) have screened a library of 101 overlapping 15-mer peptides spanning the human  $\alpha$ 1(V) chain triple-helical domain, employing an assay that identifies conformational changes induced in MHC class II molecules upon binding to peptide epitopes. Only two of the

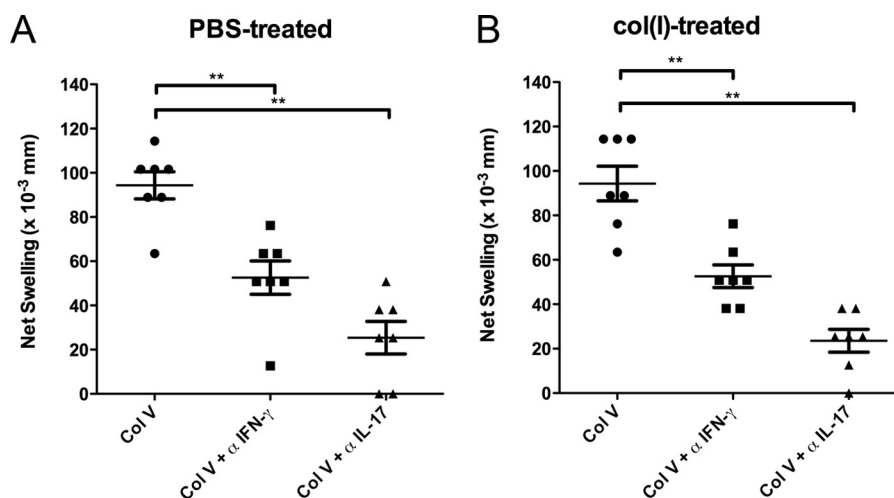


FIGURE 4. *Ldlr*<sup>-/-</sup> mice on a Western diet for 24 weeks have IL-17-dependent immune reactivity to col(V). A and B, TV-DTH assays of splenocytes from PBS-treated (A) and col(I)-treated (B) mice on a Western diet for 24 weeks and then injected into the footpads with col(V) alone or in the presence of neutralizing antibodies to cytokines showed IL-17-dependent and, to a lesser degree, IFN- $\gamma$ -dependent immune reactivity to col(V). Data are shown as mean  $\pm$  S.E.

101 peptides of that assay bound human DQ molecules and H2-I-A<sup>b</sup>, the DQ-like MHC class II equivalent present in C57BL/6 mice, the background employed this study. Interestingly, one of these peptides (p599) is located within recombinant fragment 1 and the other (p909) within fragment 3, consistent with the possibility that these two fragments contain immunologically relevant epitopes recognized by DQ-like MHC class II molecules. Peptides p599 and p909 from the previous study (34) are shown, like recombinant fragments 1 and 3 from this study, to garner immune responses in splenocytes from *Col5a2*<sup>loxP/loxP</sup>:*Ldlr*<sup>-/-</sup> mice that had been on a Western diet for 24 weeks (Fig. 6C). Because the experiments described here employ bovine and human  $\alpha$ 1(V) fragments and peptides from the  $\alpha$ 1(V) major triple-helical domain in a mouse model, we note that sequences of this domain are highly conserved across mammalian species, with 98% identity between mouse and either bovine or human COL1 sequences. All three species are identical for sequences corresponding to peptide p909, whereas human and bovine are identical for sequences corresponding to peptide p599, and mouse sequences differ at one residue (PPGPTIGKPGRRG for mouse, PPGPAGKPGRRG for human and bovine) for this peptide.

**Discussion**

It is increasingly apparent that proinflammatory processes that affect atherosclerotic plaque growth and destabilization and that perpetuate/amplify the inflammatory state in this disease involve autoimmune responses that develop in the lesion against local self-antigens. Documented local self-antigens include heat shock proteins, oxidized LDL, and native lipoproteins (1–8). Most recently, we have demonstrated autoimmunity to the  $\alpha$ 1(V) chain of col(V) to be a consistent feature of atherosclerosis in human CAD and in the *ApoE*<sup>-/-</sup> mouse model (17). Moreover, sensitization of *ApoE*<sup>-/-</sup> mice to col(V) via intravenous injection, which intensified anti-col(V) immunity, also exacerbated the plaque burden. This result was consistent with the conclusion that col(V) autoimmunity can play a causal role in the pathogenesis of atherosclerosis (17). Here we extend previous findings by showing that col(V) autoimmunity

is also a feature of atherosclerosis in the *Ldlr*<sup>-/-</sup> mouse. More importantly, we show that induction of immune tolerance via mucosal vaccination with col(V) results in a reduced atherosclerotic burden in *Ldlr*<sup>-/-</sup> mice on a Western diet, providing further evidence of causality for col(V) autoimmunity in atherosclerosis.

In previous studies, intranasal inoculation with HSP65 or an apolipoprotein B-100 peptide or oral feeding with  $\beta$ 2-glycoprotein I resulted in some degree of amelioration of atherosclerosis that correlated with indications of increased IL-10 expression (9, 12–14). However, none of these studies demonstrated a causative role for IL-10 in the induction of immune tolerance. In contrast, we show here that induction of col(V) immune tolerance via nasal inoculation of *Ldlr*<sup>-/-</sup> mice is IL-10-independent and is, instead, dependent on the more recently described immunosuppressive cytokine IL-35 (22–24). Moreover, reductions in aorta MCP-1 levels and in recruitment to aortas of Ly-6C<sup>high</sup> inflammatory monocytes/macrophages (key mediators of the chronic inflammatory response in experimental atherosclerosis (28, 35)) and reductions in plaque burden, all of which were concomitant with induced col(V) immune tolerance, are also shown to be IL-35-dependent.

Because the percent of plaque area was greater in aortas of col(V)-inoculated mice subsequently injected with neutralizing anti-Ebi3 antibodies than in aortas of PBS- or col(I)-treated controls (Fig. 5C), it is likely that the neutralizing antibody to Ebi3 not only blocked col(V) tolerance but also blocked other immunosuppressive effects of IL-35 normally in play in atherosclerosis and unrelated to col(V) tolerance.

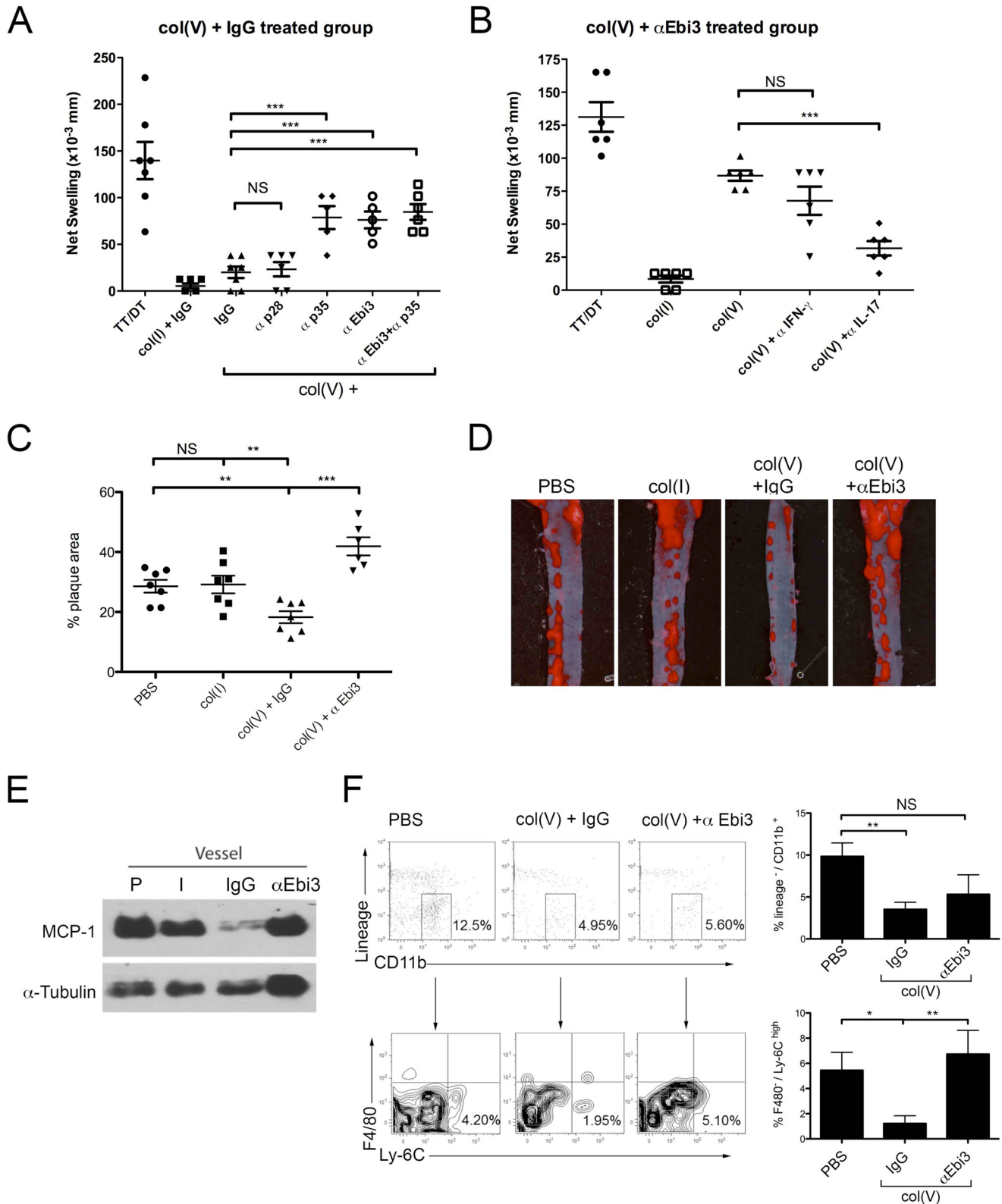
The *in vivo* experiments that demonstrated the inoculation-induced col(V) tolerance and reduction of the atherosclerotic burden to be IL-35-dependent employed an antibody to Ebi3 that, in addition to being a subunit of IL-35, is also, along with p28, a subunit of IL-27. However, the anti-Ebi3 monoclonal antibody used for this study has been shown to neutralize only IL-35 and not IL-27 (22). Moreover, TV-DTH assays showed that, although the anti-Ebi3 antibody was sufficient to break the tolerance to col(V), anti-p28 antibodies had no effect on col(V)-



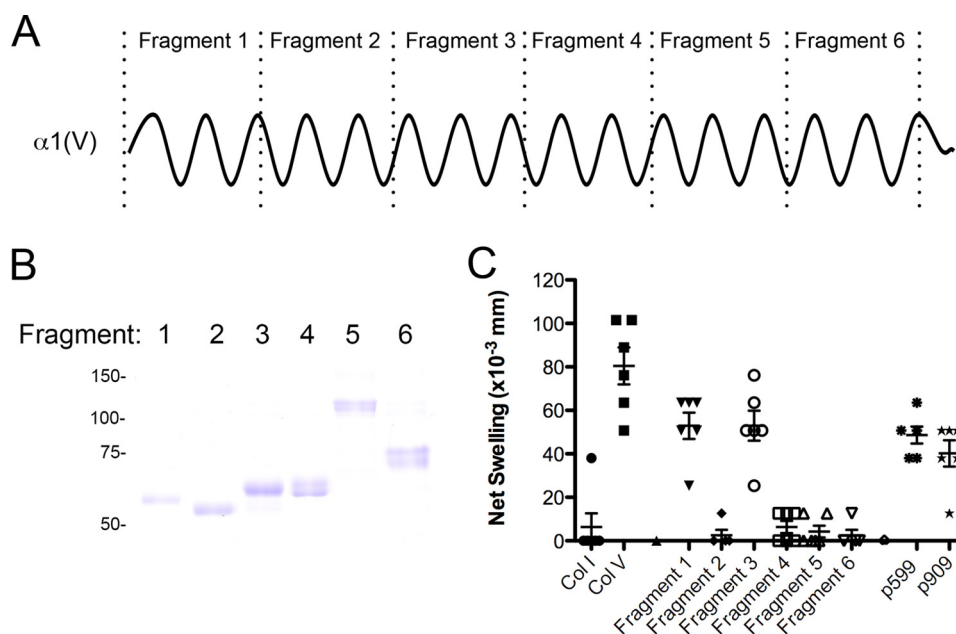
## IL-35-based Collagen V Immune Tolerance and Atherosclerosis

induced tolerance (Fig. 2D). Therefore, it may be inferred from the various data that IL-35 is an important factor involved not only in the induction of col(V) tolerance demonstrated here but also in the attempts of the body to counteract the chronic inflammatory and autoimmune responses associated with pronounced atherosclerosis.

It is clear that an imbalance in the ratio of T regulatory ( $T_R$ ) and T effector ( $T_E$ ) cells can be an important cause of autoimmunity (36) and that  $CD4^+$   $T_R$  cells play a protective role in the progression of atherosclerosis (37, 38). In addition to direct suppressive activity, IL-35 is able, like the other immunosuppressive cytokines TGF- $\beta$ 1 and IL-10, to expand the breadth of







**FIGURE 6. Epitopes underlying Col(V) autoimmunity in *Ldlr*<sup>-/-</sup> mice are localized to the N-terminal half of the  $\alpha 1(V)$  triple-helical domain.** *A*, schematic aligning the six recombinant fragments with the triple-helical region of the  $\alpha 1(V)$  chain. *B*, Coomassie-stained SDS-PAGE gel of purified  $\alpha 1(V)$  recombinant fragments. Fragments 1–4 ran with the expected mobilities, whereas fragments 5 and 6 had delayed mobilities, presumably because of extensive posttranslational modifications. Molecular masses (in kilodalton) are indicated for protein standards. *C*, TV-DTH measured T cell responses to  $\alpha 1(V)$  recombinant fragments 1–6 and 15-mer peptides p599 and p909 (34) using splenocytes from *Col5a2*<sup>loxP/loxP</sup>:*Ldlr*<sup>-/-</sup> mice that had been on a Western diet for 24 weeks. Data are shown as mean  $\pm$  S.E.

suppressive immunity by inducing and mobilizing additional regulatory cells in a process known as infectious tolerance (24, 39). In the case of IL-35, this includes the induction of naïve and effector T cells into a distinct population of CD4<sup>+</sup> T<sub>R</sub> cells (iTr35 cells) that mediate immune suppression via IL-35, and not via either IL-10 or TGF $\beta$ , and that appear to have increased stability and efficiency relative to IL-10- or TGF $\beta$ -induced T<sub>R</sub> cells (25, 39). Because we have shown that the immune tolerance induced by mucosal inoculation of col(V) is not only IL-35-dependent but also IL-10- and TGF $\beta$ -independent, the data suggest iTr35 cells to be of major importance in establishing/maintaining the immune tolerance to col(V) that leads to amelioration of atherosclerosis in *Ldlr*<sup>-/-</sup> mice. However, we do not exclude possible roles for other IL-35-secreting cells, including regulatory B cells and CD8<sup>+</sup> T<sub>R</sub> cells, which may both mediate immune suppression directly and contribute to the induction/expansion of iTr35 cells via secretion of IL-35 (24, 39). Although iTr35 cells do not mediate immune suppression via IL-10, initial induction of iTr35 cells appears to be via the concerted actions of IL-35 and IL-10 (24, 39). We have previously provided data that suggested a role for IL-10 in the sup-

pression of col(V) autoimmunity in nascent atherosclerosis (17). Therefore, IL-10-based suppressive mechanisms may play a substantive role in incipient atherosclerotic disease, whereas IL-35-based suppressive mechanisms appear to predominate in pronounced atherosclerosis and in the type of therapeutic induction of tolerance demonstrated here. However, the presence of IL-10 even in incipient atherosclerotic disease suggests that IL-10 from activated T<sub>R</sub> cells associated with atherogenesis even at its earliest stages would be available for the induction/expansion of iTr35 cells. Nevertheless, our results, in which col(V) immune tolerance is shown to be IL-10-independent, support the conclusion that this IL-10 is not involved in the maintenance or efficacy of iTr35 populations responsible for induced col(V) immune tolerance or the resulting amelioration of atherosclerotic disease.

Previous reports have provided evidence for IL-35 roles in ameliorating inflammation in various autoimmune and chronic inflammatory settings. These include mouse models of inflammatory bowel disease, experimental autoimmune encephalomyelitis, and collagen II-induced arthritis (39). In

**FIGURE 5. Amelioration of the atherosclerotic burden by induced col(V) tolerance is dependent on IL-35.** *A*, in TV-DTH assays, splenocytes from col(V)-treated *Ldlr*<sup>-/-</sup> mice subjected to injections with IgG and kept for 24 weeks on a Western diet were injected with TT/DT ( $n = 7$ ), col(I) plus IgG ( $n = 7$ ), or col(V) plus IgG ( $n = 7$ ) or plus neutralizing antibodies to p28 (a subunit of IL-27 but not IL-35) ( $n = 6$ ), p35 (a subunit of both IL-35 and IL-12,  $n = 5$ ), or Ebi3 (a subunit of both IL-35 and IL-27,  $n = 5$ ), or plus both p35 and Ebi3 ( $n = 6$ ). *B*, in TV-DTH assays, splenocytes from col(V)-treated *Ldlr*<sup>-/-</sup> mice subjected to injections with neutralizing antibodies to Ebi3 and kept for 24 weeks on a Western diet were injected with TT/DT, with col(I) or with col(V) alone, or plus neutralizing antibodies to IFN- $\gamma$  or IL-17 ( $n = 6$  in each case). *C*, quantitative analysis of the percent plaque area in ORO-stained *en face* preparations of descending thoracic aortas of *Ldlr*<sup>-/-</sup> mice treated nasally with PBS or col(I) or treated nasally with col(V) and then subjected to injections with IgG or neutralizing antibody to Ebi3. *D*, representative ORO-stained *en face* preparations of descending thoracic aortas. *E*, immunoblotting with anti-MCP-1 antibody or with antibody to  $\alpha$ -tubulin (as a loading control) of descending thoracic aortic extracts from mice treated nasally with PBS (*P*) or col(I) (*I*) or treated nasally with col(V) and then subjected to injections with IgG or neutralizing antibody to Ebi3. *F*, representative flow plots (*left panels*) and flow cytometric quantification (*right panels*) of the percentage of CD11b<sup>+</sup> monocytes/macrophages (*top panels*) or of Ly-6C<sup>high</sup> inflammatory CD11b<sup>+</sup> monocytes/macrophages (*bottom panels*) in the aortas of *Ldlr*<sup>-/-</sup> mice treated nasally with PBS ( $n = 9$ ) or with col(V) and the latter subjected to injections with IgG ( $n = 8$ ) or neutralizing antibodies to Ebi3 ( $n = 5$ ) and maintained for 24 weeks on a Western diet. Data are shown as mean  $\pm$  S.E. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ; \*\*\*,  $p \leq 0.0005$ ; NS, not significant.

### Fragment 1

GPAGPMGLTGRPGFVGPVPPGSGGLKGEVPGDVGPPQGRGVQGP**PPGPAGKPGRRG**RAGSDGARGMPGQ  
 TGPVKDRGFDGLAGLPGEKGRGDPGSPGPPGDDGERGDDGEVGPGRGLPGEVGPGRGLLGPVK  
 PGPVPPGVTGMDGQPGPKGNVGPQ **/C-propeptide**-HHHHHH

### Fragment 2

GEPGPPGQQGNPGAQGLPGPQGAIGPPGEKGLKPLGKPLGMPGADGPPGHPGKEGPPGEKGGQGP  
 PGPQGPVIGYVGPGRVKGADGIRGLKGTKEKGEDGFPVGFKGMGIKGRGEIGPPGPRGEDGPEP  
 PKGRGG **/C-propeptide**-HHHHHH

### Fragment 3

NGDPGPLGPPGEKGLGVPGLPGYPGRQPKGSIGFPGFPGANGEKGGRTGPKPGP**RGQRGPTG**  
**PRGERGPR**GITGKPGPKNSGGDPAGPPGERGPNPQGPPTGFPKGPVPPGKDGLPVHPGQR  
 GETGFQKGTGPPGPPGVVGPQGPPTGETGPMGERGHPGPPGPPGEQGLP **/C-propeptide**-  
 HHHHHH

### Fragment 4

GLAGKEGTDGPPAGLPGKDGPPGLRGFPGDRGLPGVVGALGLKNEGPPGPPGAGSPGERGP  
 AGAAGPIGIPGRPGPQPPGAGEKGAPEKGPQGPAGRDGLQGPVGLPGPAGVPPGDDGDKG  
 EIGEPGQKSGDKGEQPPGPTGPGPIGQPGPSGADGEPGRGQGLFGQKGDGEP  
**/C-propeptide**-HHHHHH

### Fragment 5

RGFPGPPGVPGLQGLPFPPEKGETGDVGMGPPGPPGPRGSGAPGADGPPGPGIGNPGAVG  
 EKGEPEAGEPGLPGEVGGPPGKGERGEKESGSPGAAGPPGPKGPPGDDGPKGSPGVPVGGDP  
 GPPGEPGAGQDGPVGDGDDGEPGQTGSPGPTGEPGPS **/C-propeptide**-HHHHHH

### Fragment 6

GPPGKRGPPGAPGPEGRQGEKAGKEAGLEGPPGKTGPIGPGQAPGKPGPDGLRGIPGVGEQGL  
 PPSFPDGPVPMGPPGLPGLKGDSPGKGEKHPGLIGLIGPPGEQGEKDRGLPGPQSSGPKG  
 EQGITGSPGPIGPPGPPGLPVPVGGKAGSSGPTGPKGEAGHPGPPGPPGPEVIQPLPI  
**/C-propeptide**-HHHHHH

FIGURE 7. **Amino acid sequences of the six recombinant fragments spanning the  $\alpha 1(V)$  COL1 domain.** Not shown is the amino acid sequence (MRAWIF-FLCLAGRALAAPLA) of the signal peptide at the N terminus of each fragment. Each fragment had at its C terminus the pro- $\alpha 1(V)$  C-propeptide to enable chain association and the formation of triple-helical molecules (necessary for efficient secretion), followed by a His tag (HHHHHH) for purification.

addition, strong co-expression of Ebi3 and p35 in smooth muscle cells, endothelial cells, and macrophages of advanced lesions in patients with symptomatic carotid plaques (40) and positive correlation of plasma IL-35 levels with the left ventricular ejection fraction in a large number of CAD patients (41) has led to the hypothesis that IL-35 may be involved in amelioration of atherosclerosis and improved prognosis in CAD (42). Here we provide strong evidence that IL-35 can play a key role in the amelioration of plaque burden in atherosclerosis.

The use of recombinant fragments in this study localizes two  $\alpha 1(V)$  chain epitopes involved in col(V) autoimmunity in atherosclerotic *Ldlr*<sup>-/-</sup> mice to between amino acid residues 41–156 and 294–471 of the triple-helical domain. The locations of these two epitopes correspond to the positions of two peptides shown previously to bind to human DQ-like MHC class II molecules and to the C57BL/6 DQ-like murine equivalent H2-I-A<sup>b</sup> (34) and shown here to garner immune responses in splenocytes from atherosclerotic *Ldlr*<sup>-/-</sup> mice. Localization via two independent approaches of these two epitopes involved in col(V) autoimmunity and the progression of atherosclerosis will enable their further characterization.

*Author Contributions*—A. C. P. and G. H. were involved in the design and performance of most experiments. D. M. performed key work on aorta evaluation. E. J. G. performed and mentored A. P. in performing TV-DTH assays. J. F. K. assisted with setting up and performing portions of a number of the experiments. D. A. V. provided and helped design experiments for use of anti-Ebi3 antibodies. J. A. S. designed and performed the flow cytometry experiments. D. S. W. oversaw the production of and provided the bovine col(V) used in the experiments. W. J. B. provided intellectual input into the design of and oversaw some experiments. D. S. G. conceived the study and wrote the paper

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