Supplementary Data

a **Codon-optimized genomic *APOAI* sequence**

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GACCCCCCTCCCACATCCCAACCCCTGGCACTCTGGCTCAGAGCTGGCCTAACCCGGGGC
TGCCTTCTCACGCCCAG**GATCTCAGGCCAGACACTTTTGGCAGCGCGACGAACCAAGGTC**
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GTGCCTGGAAGGGTCCCCCCTCAGCGCCTTGTGTCTGCCTCCAG**CCTGAAACTGCTGGACA**
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CAGCCCTGGAGGACCTGAGACAGGGCCTGCTGCCTGTGCTGGAGAGCTTCAAAGCCAGCG
TCCAGAACGTCCTGGATGAAGCCACAAAGAAACTGAACACACAGTGA

b **Codon-optimized *APOAI* cDNA sequence**

CCACCATGAAGGCCGTGGTGCTGACCCTGGCCGTGCTGTTCCTGACCGGCAGCCAGGCCA
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CCGTGTCCAAGCTGCAGGAACAGCTGGGCCCCGTCACCCAGGAATTCTGGGACAACCTGG
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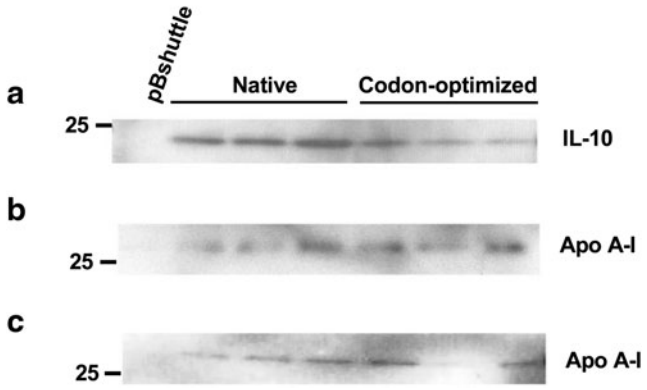
CCAGGGAGCACCTGAGCGTGCTGAGCGAGAAGGCCAGACCCGCCCTGGAGGACCTGAGAC
AGGGCCTGCTGCCCGTGCTGGAGTCCTTCAAGGCCAGCGTGCAGAACGTGCTGGACGAGG
CCACCAAGAAGCTGAACACCCAGTGA

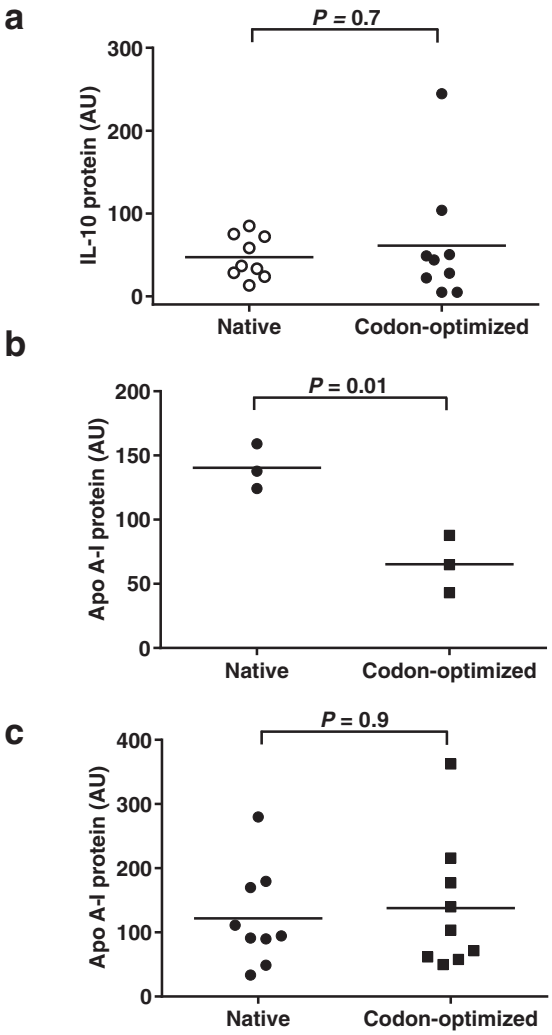
c **Codon-optimized *IL10* cDNA sequence**

CCACCATG**CTGTCTAGCGCCCTGCTGTGTTGTCTGGTGTTCCTGGGAGGCACAGGCGCCA**
GCCGCGGACAGGATACACCCGCCGAAAATAGCTGCATCCACTTCCCCGGCGGGCTGCCTC
ACATGCTGAGAGAGCTGAGAGCCGCCTTTGGCCGGGTGAAGACCTTCTTTCAGTCTAAAG
ACCAGCTGAACTCCATGCTGCTGACAGAGTCCCTGCTGGAGGACCTGAAGGGATACCTGG
GCTGTCAGGCCCTGAGCGAGATGATCCAGTTCTATCTGAAAGACGTGATGCCACAGGCCG
AGAACCACTCTCCCGCCATCCGGGAGCACGTCAACTCCCTGGGGGAAAATCTGAAGACCC
TGAGACTGAGACTGAGGCAGTGCCACAGGTTCCTGCCTTGTGAGAATAAGAGCAAAGCCG
TGGAACAGGTCAAGTCCGCCTTTAGCAAGCTCCAGGAGGAAGGAGTCTACAAGGCCATGT
CTGAGTTCGACATCTTCATCAACTACATCGAGACCTATATGACCATGAAGATCAAGTCCT
GA

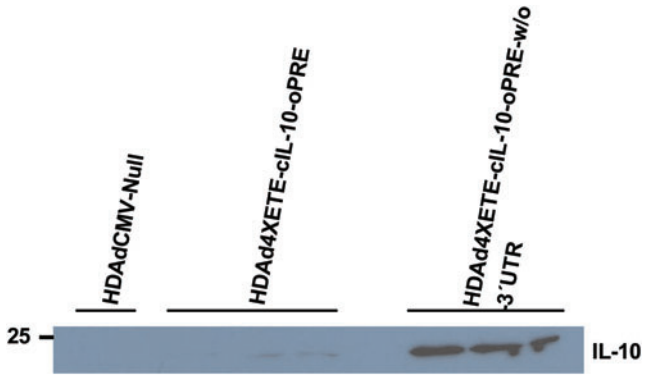
Supplementary Figure S1. Codon-optimized sequences. *Oryctolagus cuniculus*–specific codon optimization was done by commercial laboratories, using proprietary algorithms (see "Methods"). A Kozak sequence "CCACC" (in blue) was inserted upstream of the start codons "ATG" (in green). The stop codons ("TGA") are red. Coding sequences are bolded; intronic sequences are not. **(a)** APOAI gene; **(b)** APOAI cDNA); and **(c)** *IL10* cDNA. APOAI, apolipoprotein A-I; IL10, interleukin-10.



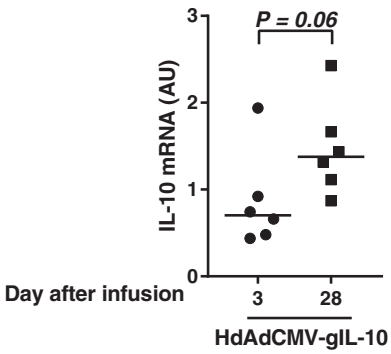
Supplementary Figure S2. Codon optimization does not increase expression of transgene protein. Rabbit skin fibroblasts were transfected with plasmids expressing native or codon-optimized versions of **(a)** IL10 cDNA; **(b)** APOAI genomic DNA; or **(c)** APOAI cDNA. The pBshuttle plasmid (lacks a transgene) was transfected as a negative control. Conditioned medium was analyzed by western blotting; each lane is from a separate well of cells. Size markers are in kDa. Quantitative results from this gel and others are in Fig. S3.



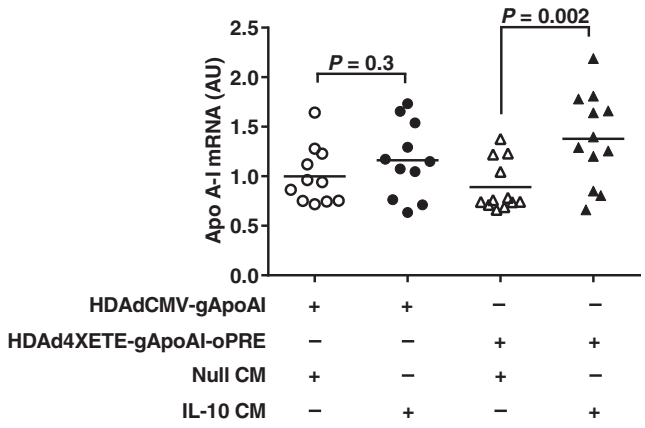
Supplementary Figure S3. No improvement of protein expression after codon optimization. Rabbit skin fibroblasts were transfected with plasmids containing either native or codon-optimized versions of **(a)** IL10 cDNA, **(b)** APOAI gene, or **(c)** APOAI cDNA. Conditioned medium was collected after 24 hours, and IL-10 or apo A-I protein was measured by western blot (see Fig. S2). The western blot bands were scanned, quantified by densitometry, and normalized to the amount vector DNA measured by quantitative PCR of DNA isolated from the same cells that generated the conditioned medium. Bars are group means; *P*-values are from Mann–Whitney rank-sum tests **(a**, **c)** and *t*-test **(b)**.



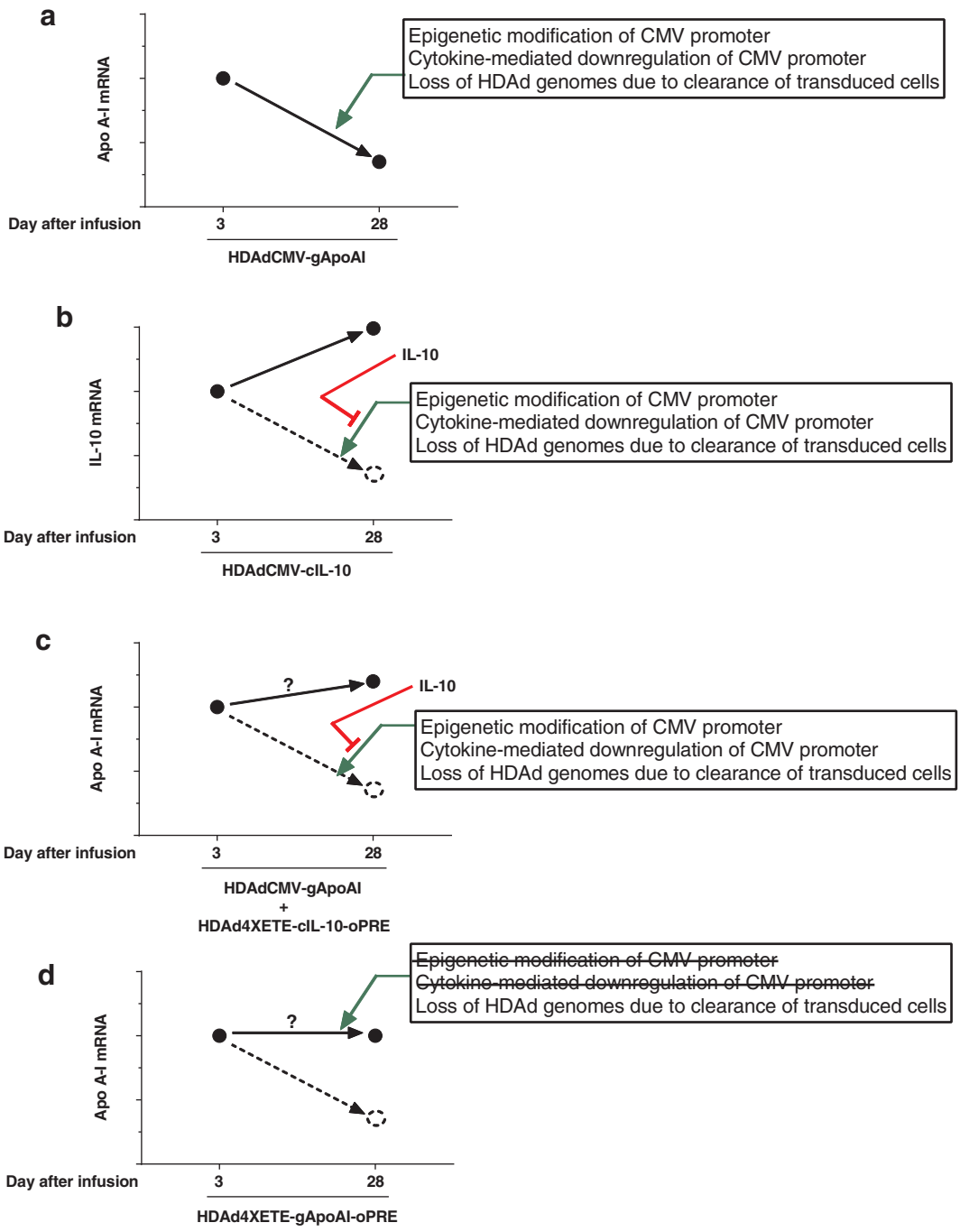
Supplementary Figure S4. Removal of 3′ untranslated (3′ UTR) sequences from the IL10 cDNA increases expression of IL-10 protein. Bovine aortic endothelial cells were transduced with HDAd4XETE-cIL-10-oPRE (includes 3′ UTR), HDAd4XETE-cIL-10-oPRE-w/o3′ UTR, or HDAdCMV-Null (as a negative control). Conditioned media were collected and analyzed for IL-10 protein by western blotting; each lane is from a separate well of cells. Size markers are in kDa. HDAd4XETE-cIL-10-oPRE, helper-dependent adenoviral vector using 4XETE promoter to express IL-10 cDNA with optimal post-translational regulatory element; HDAdCMV-Null, helper-dependent adenoviral vector containing CMV promoter and empty (Null) expression cassette.



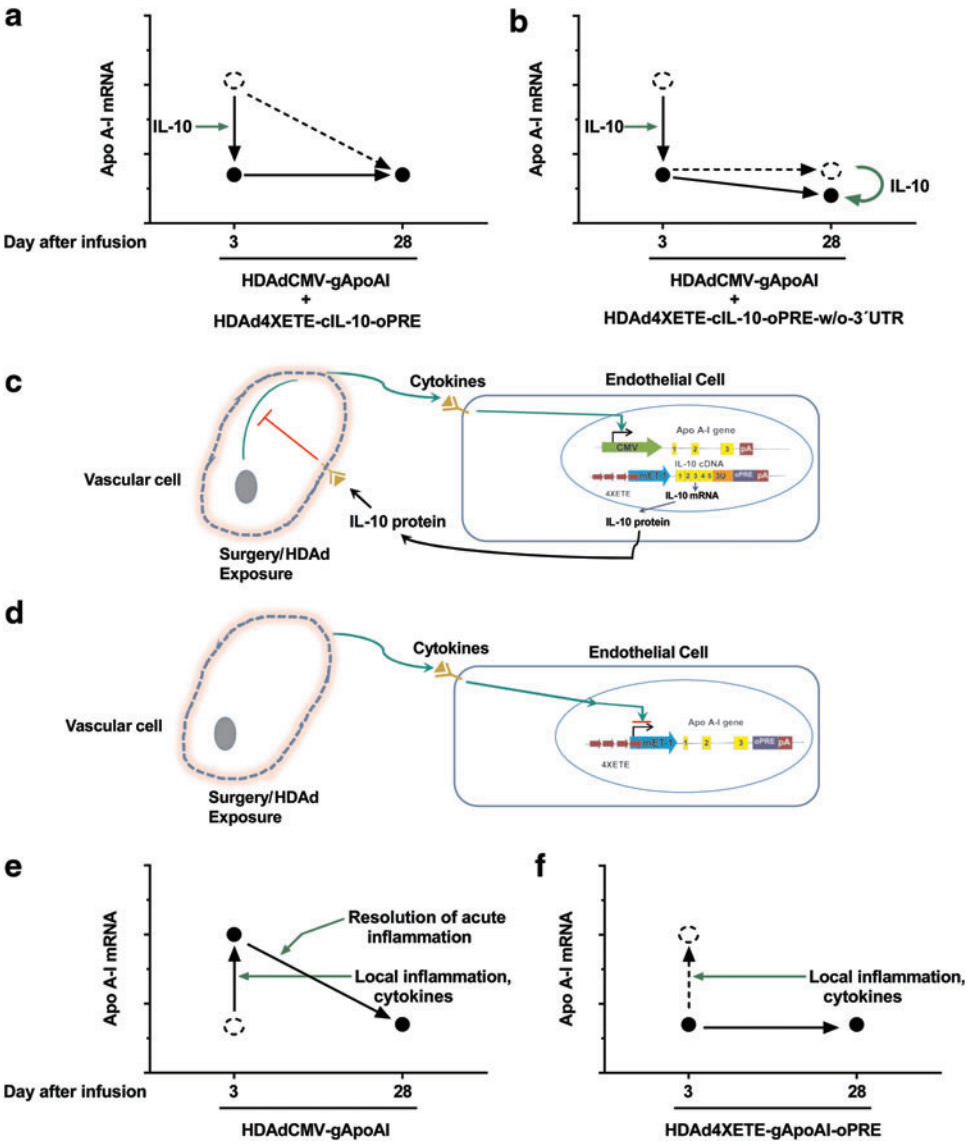
Supplementary Figure S5. Expression of IL-10 mRNA increases between 3 and 28 days after *in vivo* transduction with HDAdCMVgIL-10. Rabbit carotid arteries were infused with HDAdCMV-gIL-10 and harvested 3 or 28 days later. IL-10 mRNA was measured by qRT-PCR and normalized to GAPDH mRNA in the same samples. Data points are individual arteries; bars are group medians. *P*-value from Mann–Whitney rank-sum test.



Supplementary Figure S6. IL-10 protein does not suppress expression from HDAdCMV-gApoAI-transduced endothelial cells *in vitro*. Bovine aortic endothelial cells were transduced with either HDAdCMV-gApoAI or HDAd4XETE-gApoAI-oPRE. The transduced cells were then treated with conditioned medium (CM) harvested from other bovine aortic endothelial cells that had been transduced with either HDAdCMV-Null (Null CM) or HDAd4XETE-cIL-10-oPRE (IL-10 CM). Apo A-I mRNA, measured by qRT-PCR, and normalized to GAPDH, is measured in arbitrary units (AU). Data points are from individual wells, obtained in two independent experiments; bars are group means. *P*-values are from two-way analysis of variance with *post-hoc* correction for pairwise comparisons.



Supplementary Figure S7. Initial models of promoter- and IL-10–dependent regulation of transgene expression after *in vivo* gene transfer to endothelial cells. **(a)** The decline in apo A-I expression from HDAdCMV-gApoAI[1] is attributed to effects of one or more of three processes. **(b)** The stability of IL-10 expression from HDAdCMV-gIL-10[2] is attributed to actions of IL-10 that block the three processes. Increased IL-10 expression between 3 and 28 days was observed[2] but is not explained by this model. **(c)** The model portrayed in **(a)** and **(b)** predicts that co-expression of IL-10 in HDAdCMV-gApoAI-transduced arteries would prevent loss of apo A-I expression through anti-inflammatory activities of IL-10 that block the 3 processes. **(d)** The model in **(a)** predicts that replacement of the cytomegalovirus (CMV) promoter with the 4XETE promoter would mitigate loss of apo A-I expression because the 4XETE promoter is not susceptible to epigenetic modification and cytokine-mediated downregulation. **(a–d)** Solid lines represent observed effects and values. Solid lines with question marks represent predicted effects and values. Dotted lines represent effects and values that are avoided (or are predicted to be avoided) by the illustrated experimental manipulations. Positive actions are green; negative actions are red.



Supplementary Figure S8. Current models of promoter- and IL-10-dependent regulation of transgene expression after *in vivo* gene transfer to endothelial cells, revised according to the results of experiments reported here. **(a)** Expression of apo A-I in arteries co-transduced with HDAdCMV-gApoAI and HDAd4XETE-cIL-10-oPRE is stable because vector-produced IL-10 lowers 3-day apo A-I expression (see **(c)** for mechanism). **(b)** Expression of apo A-I in arteries co-transduced with HDAdCMV-gApoAI and HDAd4XETE-cIL-10-oPRE-w/o-3′UTR have lower expression of apo A-I at 28 days than at 3 days because increased IL-10 production at 28 versus 3 days from HDAd4XETE-cIL-10-oPRE-w/o-3′UTR lowers apo A-I expression below 3-day levels. **(c)** Hypothetical pathways through which the gene-transfer protocol upregulates apo A-I expression from the CMV promoter and through which IL-10 blocks this upregulation. Host inflammatory responses to surgery or infusion of HDAd include local release of cytokines by vascular cells. These cytokines bind to endothelial cells and acutely (3 days) upregulate expression from the CMV promoter. When an IL-10-expressing vector is also present, vector-expressed IL-10 binds to vascular cells and downregulates their production of cytokines. Consequently, the CMV promoter is less active and apo A-I expression decreases. **(d)** Hypothetical mechanism to explain low and stable apo A-I expression observed with HDAd4XETE-gApoAI-oPRE. Local cytokine production occurs as in **(c)**; however, the 4XETE promoter is not responsive to these signals and therefore expresses lower levels of apo A-I at 3 days than the CMV promoter. **(e)** New mechanism to explain decline in apo A-I expression from HDAdCMV-gApoAI between 3 and 28 days (compare with Fig. S7a). Apo A-I expression is elevated at 3 days via inflammatory processes, as in **(c)**. By 28 days this inflammation has largely resolved, and apo A-I expression from the CMV promoter declines. **(f)** New mechanism to explain stability of apo A-I expression from HDAd4XETE-gApoAI-oPRE between 3 and 28 days (compare with Fig. S7d). Because the 4XETE promoter is not responsive to signals generated from local inflammatory processes, apo A-I expression from HDAd4XETE-gApoAI-oPRE is not upregulated at 3 days. Consequently, apo A-I expression at 28 days is at the same level as at 3 days. Solid lines represent observed effects and values; dotted lines represent effects and values that are avoided by the illustrated experimental manipulations, including co-expression of IL-10 or replacement of the CMV promoter with 4XETE. Positive actions are green; negative actions are red.

REFERENCES

1. Flynn R, Qian K, Tang C, et al. Expression of apolipoprotein A-I in rabbit carotid endothelium protects against atherosclerosis. Mol Ther 2011;19:1833–1841.
2. Du L, Dronadula N, Tanaka S, Dichek DA. Helper-dependent adenoviral vector achieves prolonged, stable expression of interleukin-10 in rabbit carotid arteries but does not limit early atherogenesis. Hum Gene Ther 2011;22:959–968.