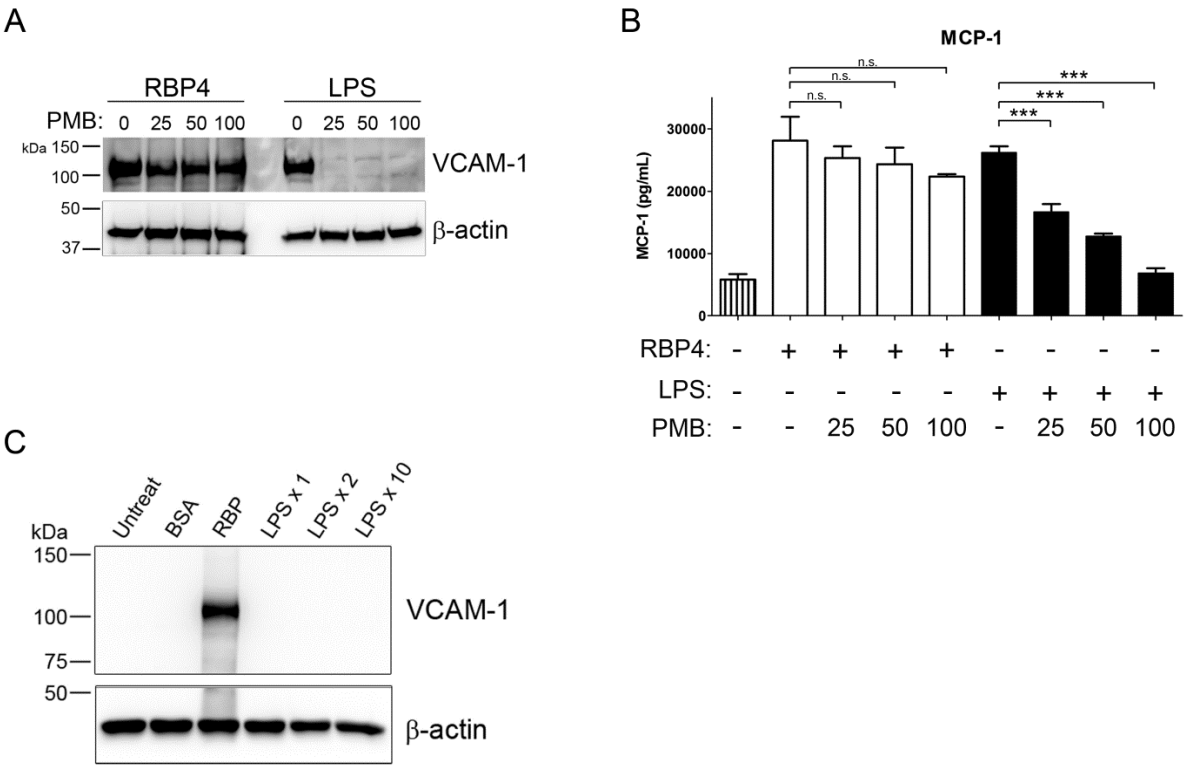
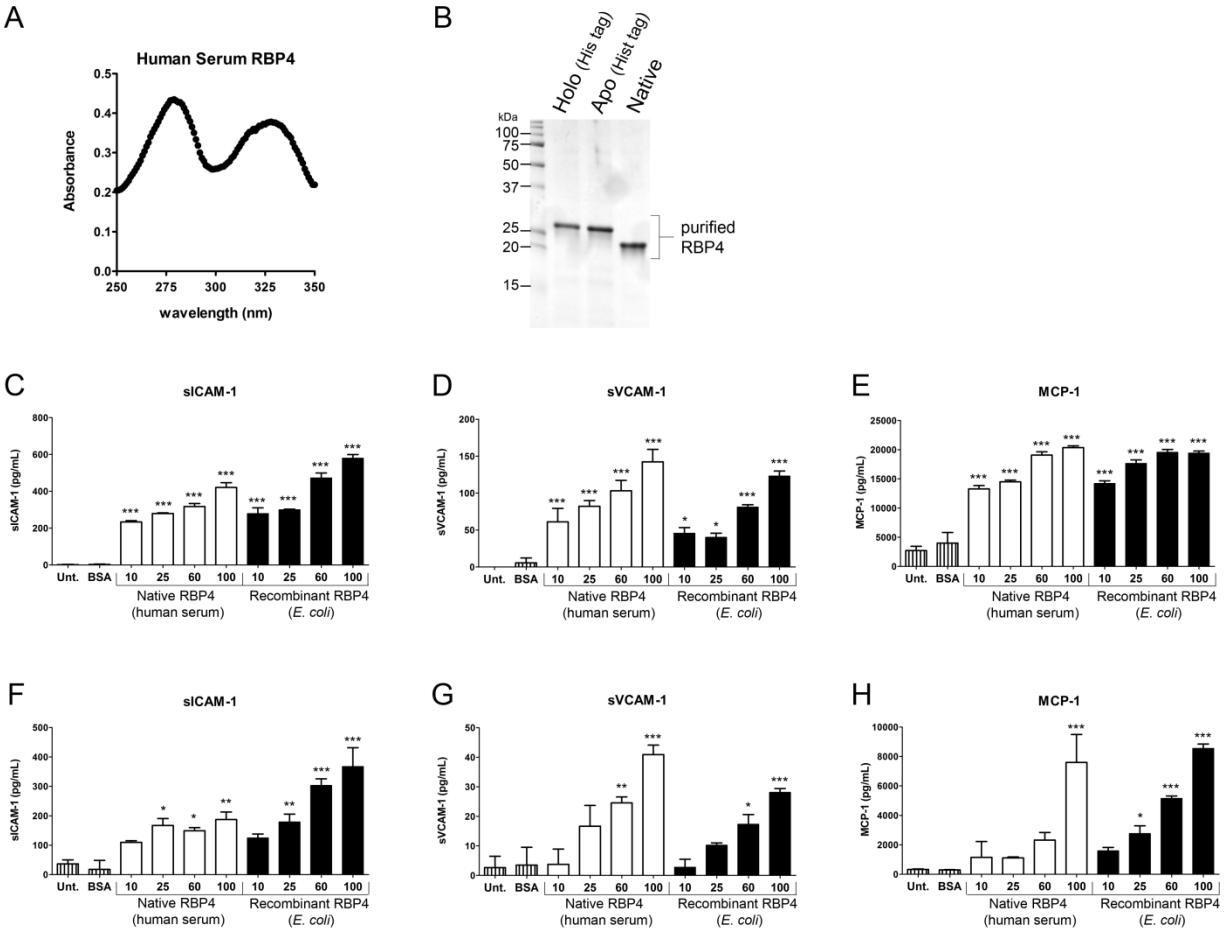
SUPPLEMENTAL MATERIAL

Gene	Forward Primer	Reverse Primer
HPRT	GGGAGGCCATCACATTGTAG	CTTGCGACCTTGACCATCTT
VCAM1	TCCGTCTCATTGACTTGCAG	CCAGCCTGTCAAATGGGTAT
ICAM1	GGCTGGAGCTGTTTGAGAAC	CCTCTGGCTTCGTCAGAATC
MCP1	GCCTCCAGCATGAAAGTCTC	GTCTTCGGAGTTTGGGTTTG
E-Selectin	AGCCCAGAGCCTTCAGTGTA	AACTGGGATTTGCTGTGTCC
TLR4	ACAACCTCCCCTTCTCAACC	AAACTCTGGATGGGGTTTCC
TLR2	GATGCCTACTGGGTGGAGAA	CGCAGCTCTCAGATTTACCC
LRP2	CTGCAAAGTGATGGCAAGAA	ACAGTGCGGTTAGACCCATC
STRA6	TGTTGGATGAGCTTCAGTGC	CGTTGTAGAGGGCAGAGAGG

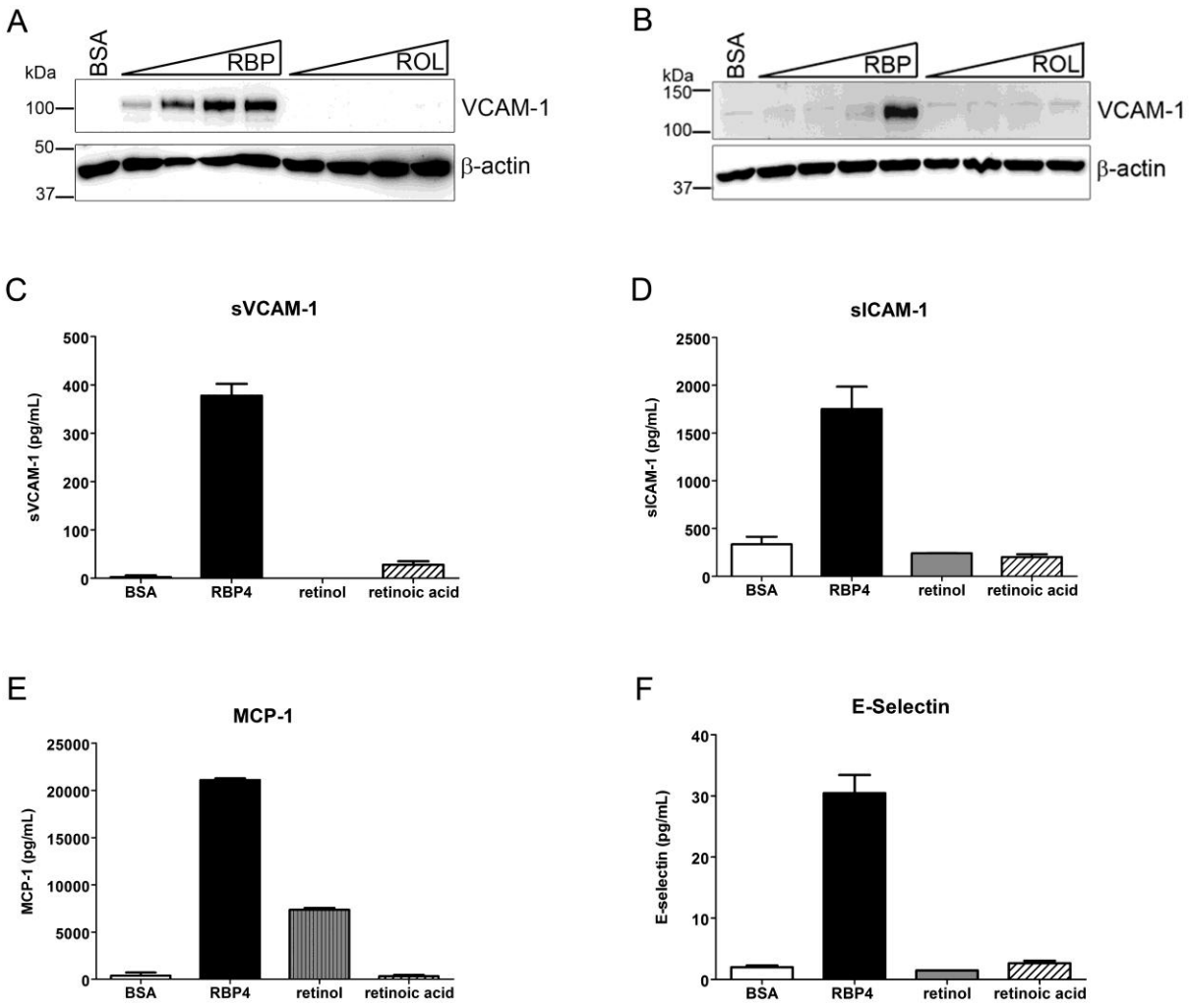
Supplemental TABLE 1 Primer pairs for qRT-PCR.



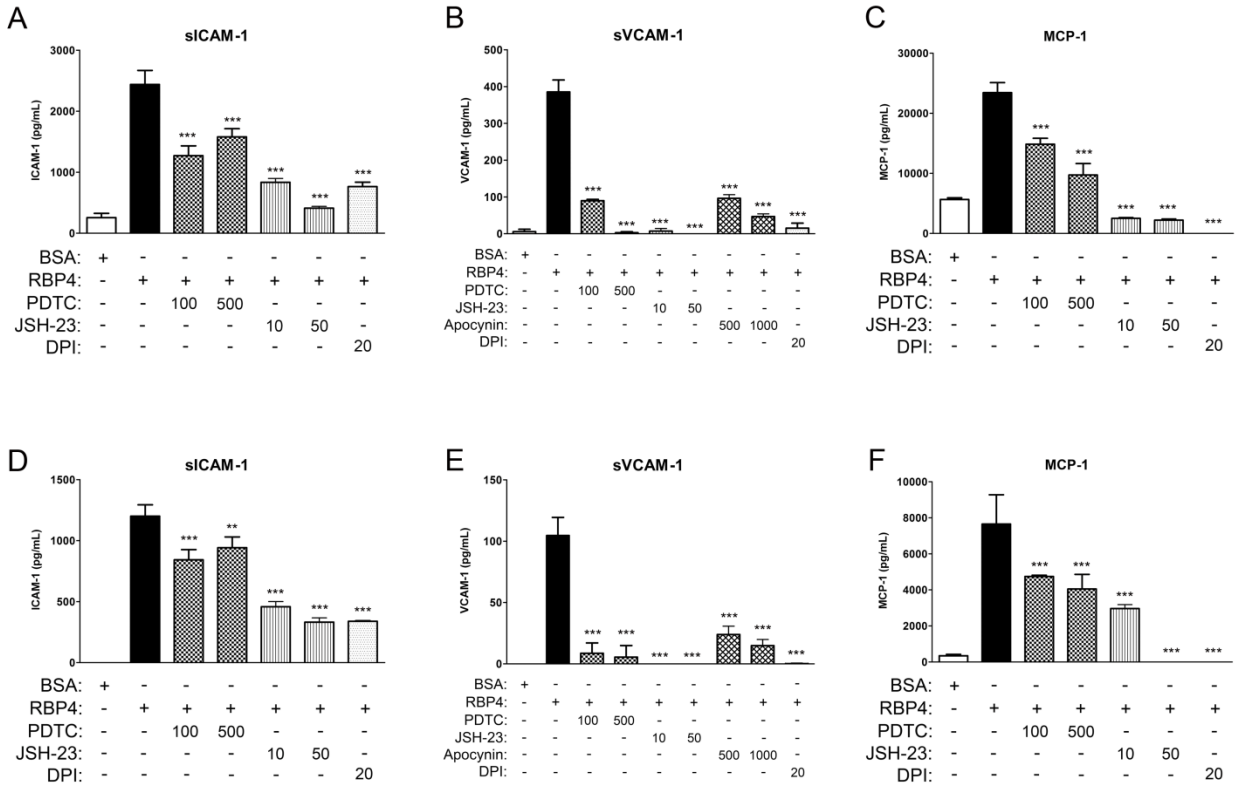
Supplemental FIG 1 RBP4-mediated induction of proinflammatory protein expression is not due to LPS contamination. (A and B) HRCEC cells were treated with either holo-RBP4 (100 μg/mL) or lipopolysaccharide (LPS) (100 ng/mL) alone or in the presence of increasing concentrations of polymixin B sulfate (PMB) (25-100 μg/mL) for 24 hr. (A) Western blot of VCAM-1 in HRCEC shows that polymixin B blocked LPS-mediated induction of VCAM-1, but did not reduce RBP4-mediated induction of VCAM-1. (B) ELISA-based quantification of secreted MCP-1 shows that polymixin B significantly reduced LPS-mediated induction of MCP-1, but did not reduce RBP4-mediated induction of MCP-1. (C) Measurement of endotoxin/LPS levels determined that a dose of 100 μg/mL purified RBP4 contained <0.05 EU/mL of endotoxin. HUVEC cells were treated with BSA, RBP4 (100 μg/mL), or LPS at a dose equivalent to 1x (0.043 EU/mL), 2X (0.086 EU/mL), or 10X (0.43 EU/mL) the endotoxin level measured in RBP4 for 24 hr. Western blot of VCAM-1 in HRCEC shows that a dose of up to 10X the amount of LPS present in purified RBP4 was insufficient to induce VCAM-1 protein expression. These results demonstrate that RBP4-mediated proinflammatory protein expression is LPS-independent. ***, $P < 0.001$ by one-way ANOVA with Tukey's post-hoc test.



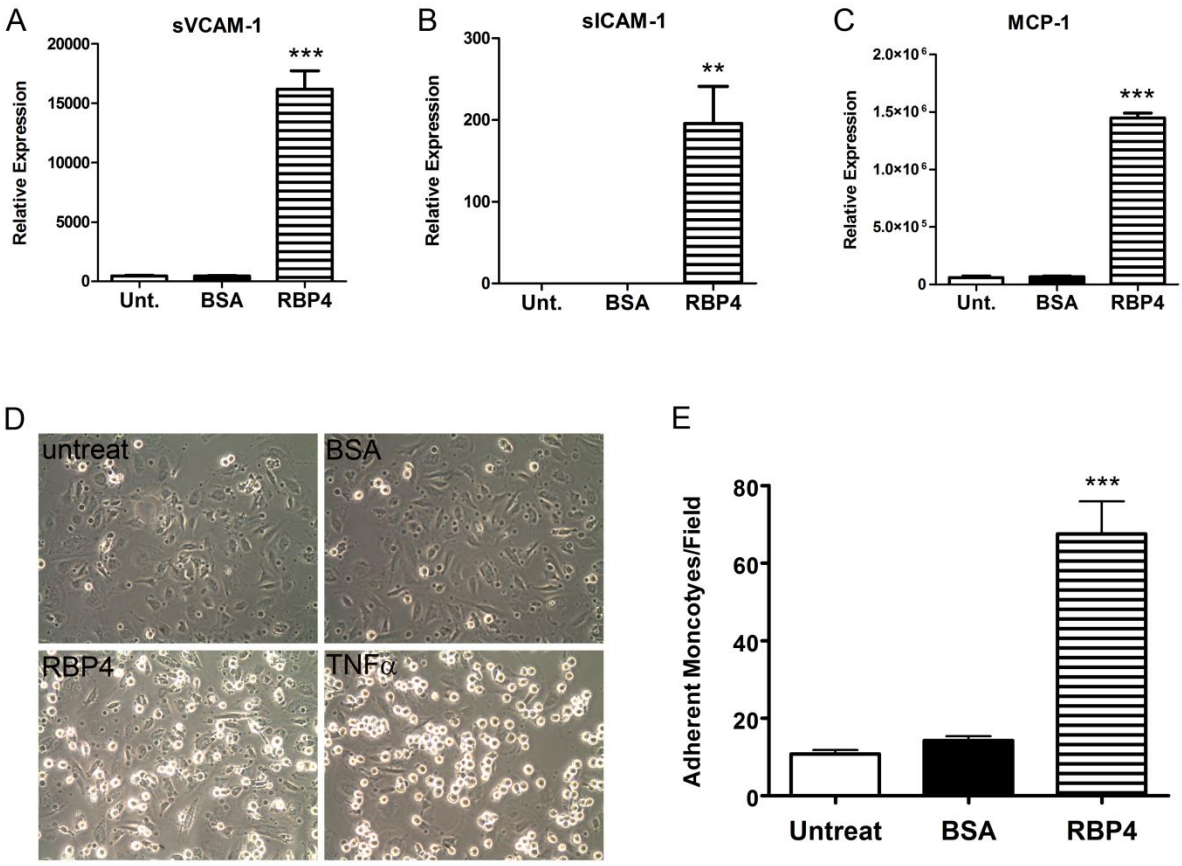
Supplemental FIG 2 Native RBP4 purified from human serum has similar proinflammatory effect to recombinant holo-RBP4. (A) UV spectrum of native human RBP4 purified from human serum. The 330/280 nm peak ratio near 0.9 shows that purified native RBP4 is primarily in the form of holo-RBP4. (B) Representative Coomassie blue staining of purified native human RBP4 compared to purified recombinant human holo-RBP4 and apo-RBP4 (1 μg protein/lane). The his-tag on recombinant RBP4 causes a noticeable shift in band migration. (C-E) HRCEC were treated with increasing concentrations of either native RBP4 or recombinant holo-RBP4 for 24 hr as indicated, and graphs represent ELISA-based quantification of soluble extracellular protein levels of (C) sICAM-1, (D) sVCAM-1, and (E) MCP-1 in HRCEC media. (F-H) HUVEC were treated with increasing concentrations of either native RBP4 or recombinant holo-RBP4 for 24 hr as indicated, and graphs represent ELISA-based quantification of soluble extracellular protein levels of (F) sICAM-1, (G) sVCAM-1, and (H) MCP-1 in HUVEC media. Significant differences compared to BSA treatment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with Tukey's post-hoc test.



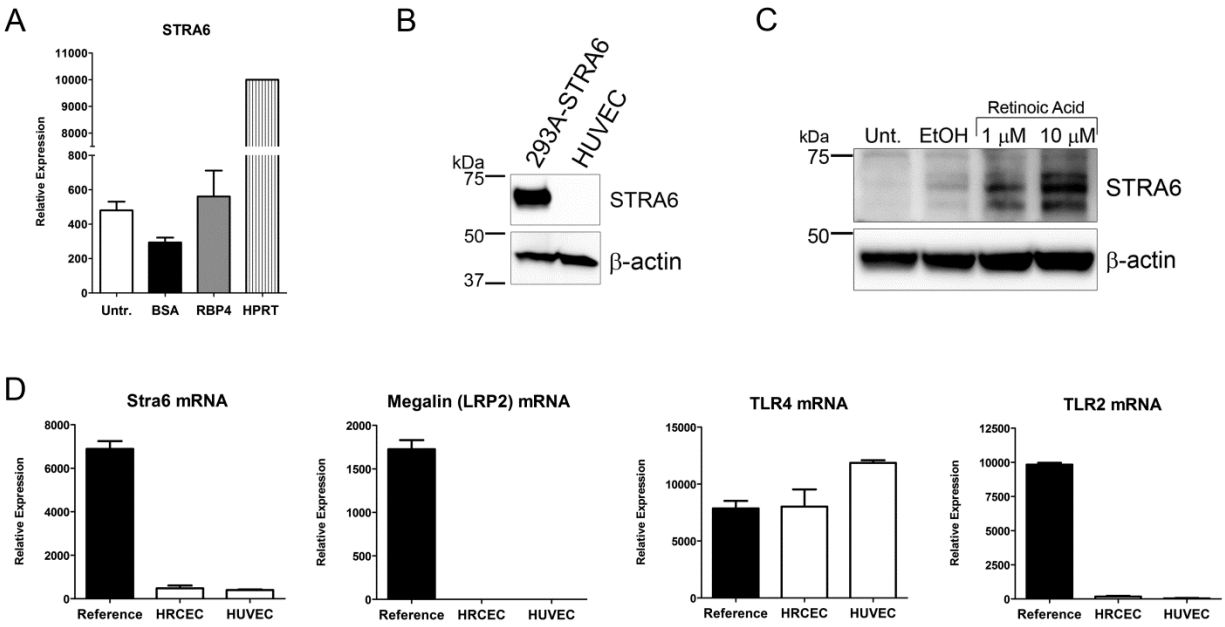
Supplemental Fig 3 RBP4-mediated induction of proinflammatory molecules in HRCEC and HUVEC is retinoid-independent. (A and B) HRCEC and HUVEC were treated with increasing concentrations of holo-RBP4 (10-100 μg/mL) or an equimolar dosing regimen of retinol alone (0.475 – 4.75 μM) for 24 hr. Western blot of VCAM-1 in (A) HRCEC and (B) HUVEC shows that even the highest equimolar dose of retinol was unable to induce VCAM-1 protein expression. (C-D) HRCEC were treated with RBP4 (100 μg/mL), or an equimolar concentration (4.75 μM) of BSA, retinol, or retinoic acid for 24 hr. ELISA-based quantification of soluble extracellular levels of (C) sVCAM-1, (D) sICAM-1, (E) MCP-1, and (F) E-selectin from HRCEC media shows that neither retinol nor retinoic acid increased the level of soluble proinflammatory molecules. These results demonstrate that RBP4-mediated induction of proinflammatory molecules is retinoid-independent.



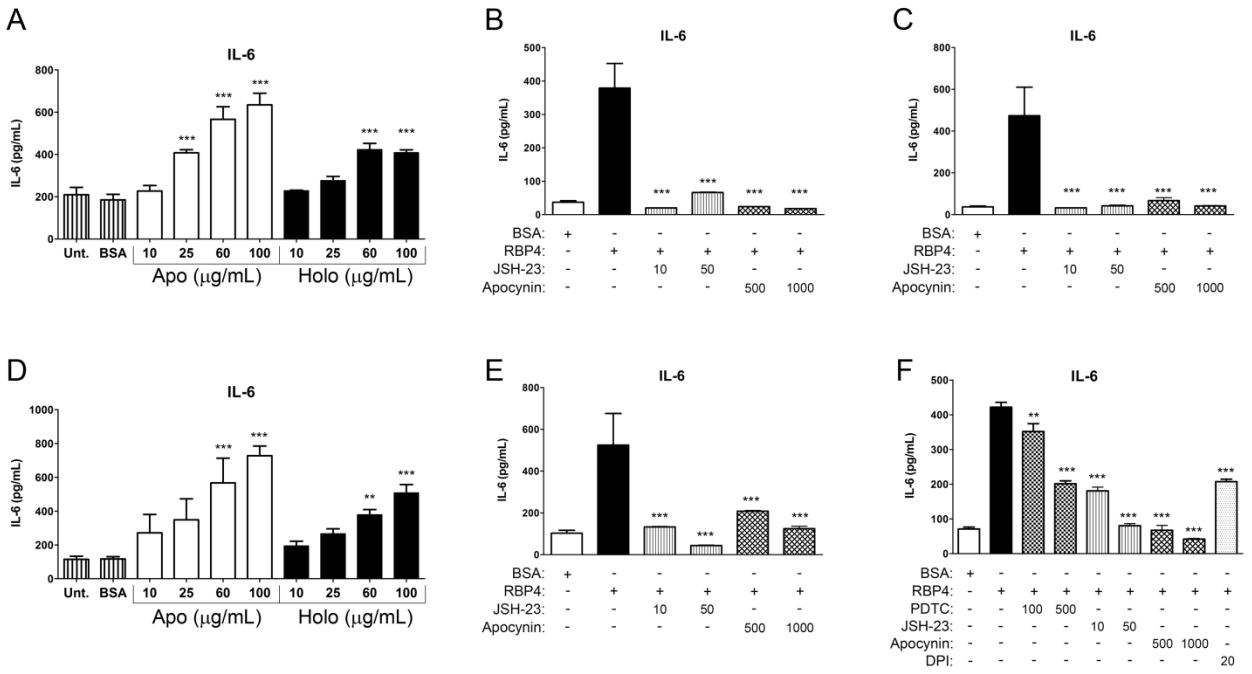
Supplemental FIG 4 Apo-RBP4-mediated increase of proinflammatory factors in HRCEC and HUVEC is NF-κB and NADPH oxidase-dependent. (A-C) HRCEC were pre-treated were pre-treated with NF-κB inhibitors PDTC (100 or 500 μM) or JSH-23 (10 or 50 μM), or NADPH oxidase inhibitors DPI (20 μM) or apocynin (500 or 1000 μM) for 2 hr prior to the addition of apo-RBP4 (100 μg/mL) for 24 hr. Graphs display ELISA-based quantification of the level of soluble extracellular (A) sICAM-1, (B) sVCAM-1, and (C) MCP-1. (D-F) HUVEC were pre-treated were pre-treated with NF-κB inhibitors PDTC (100 or 500 μM) or JSH-23 (10 or 50 μM), or NADPH oxidase inhibitors DPI (20 μM) or apocynin (500 or 1000 μM) for 2 hr prior to the addition of apo-RBP4 (100 μg/mL) for 24 hr. Graphs display ELISA-based quantification of the level of soluble extracellular (D) sICAM-1, (E) sVCAM-1, and (F) MCP-1. Significant differences compared to RBP4 treatment alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with Tukey's post-hoc test.



Supplemental FIG 5 RBP4 induces expression of proinflammatory molecules and increases leukocyte adherence in human umbilical vein endothelial cells. Quantitative PCR analysis for mRNA expression of VCAM-1, ICAM-1, and MCP-1 in human umbilical vein endothelial cells (HUVEC) treated with holo-RBP4 (100 μg/mL), BSA, or untreated (Unt.). (D-E) Confluent monolayers of HUVEC were treated with either RBP4 (100 μg/mL), BSA, or TNF-alpha (100 ng/mL) for 18 hr. Then THP-1 monocytes were added and co-cultured for 3 hr. (A) Representative phase contrast images (20X magnification) of monocyte adherence to HRCEC after the indicated treatment. (B) Adherent monocytes were counted per visual field at 20X magnification. The graph represents the mean +/- s.d. from 4 different visual fields for each treatment group (adherent leukocytes in TNF-a positive control were 161+/-64 per visual field). Significant differences compared to BSA treatment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with Tukey's post-hoc test.



Supplemental FIG 6 Expression of candidate RBP4 receptors in HRCEC and HUVEC. (A) Quantitative RT-PCR analysis of STRA6 mRNA expression in HUVEC following 24 hr of treatment with RBP4 (100 μg/mL), BSA or no treatment (Untr.). STRA6 mRNA expression is shown relative to HPRT housekeeping gene expression, and is 20-fold less abundant than HPRT. (B) STRA6 protein expression was undetectable by western blotting of HUVEC versus 293A cells stably expressing STRA6. (C) STRA6 expression is inducible in HRCEC in response to retinoic acid treatment as shown by western blotting. (D) Quantitative RT-PCR analysis of candidate endothelial RBP4 receptors mRNA expression in HRCEC and HUVEC. Stratagene human reference total RNA was used as a positive control to validate qRT-PCR primer pairs for each receptor.



Supplemental FIG 7 Apo-RBP4 and Holo-RBP4 treatment increases secretion of IL-6 in HRCEC and HUVEC in a NF-kB- and NADPH oxidase-dependent manner. (A) ELISA-based quantification of secreted IL-6 protein in HRCEC media following 24 hr of treatment with increasing concentrations of either apo- or holo-RBP4 as indicated. (B-C) HRCEC were pre-treated with NF-κB inhibitor JSH-23 (10 or 50 μM) or NADPH oxidase inhibitor apocynin (500 or 1000 μM) for 2 hr prior to the addition of holo-RBP4 (B) or apo-RBP4 (C) for 24 hr. Graphs display the level of secreted IL-6 protein. (D) ELISA-based quantification of secreted IL-6 protein in HUVEC media following 24 hr of treatment with increasing concentrations of either apo- or holo-RBP4 as indicated. (E-F) HUVEC were pre-treated with NF-κB inhibitors PDTC (100 or 500 μM) or JSH-23 (10 or 50 μM), or NADPH oxidase inhibitors DPI (20 μM) or apocynin (500 or 1000 μM) for 2 hr prior to the addition of holo-RBP4 (E) or apo-RBP4 (F) for 24 hr. Graphs display the level of secreted IL-6 protein. In panels A/D, significant differences compared to BSA treatment, but in panels B/C/E/F, significant differences compared to RBP4 treatment alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with Tukey's post-hoc test.