SUPPLEMENTAL MATERIAL

EXPERIMENTAL PROCEDURES

Animal models

Animal care was provided in accordance with the Laboratory Animal Welfare Act, and all experimental protocols were approved by the University of Michigan Medical School Committee on the Use and Care of Animals (UCUCA). All mice were housed in a standard temperature controlled facility with a 12-hour light/dark cycle, and were provided with a standard chow diet and water ad libitum. Bcl10 deficient mice (*Bcl10*$^{-/-}$) were generated as described and backcrossed onto the C57BL/6J background for more than 10 generations (1). ApoE deficient mice (*ApoE*$^{-/-}$) were purchased from The Jackson Laboratory and have also been bred onto the C57BL/6J background. To generate double-knockout mice, the two strains were cross-bred using a standard approach.

Atherosclerosis studies

Male *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice between the ages of 48 and 103 days of age were implanted subcutaneously with osmotic minipumps (Alzet model 2004) designed to deliver Ang II at a continuous rate of 500 ng/kg/min. After 28 days, mice were sacrificed and aortic trees were dissected, opened longitudinally, stained with Oil-Red-O, and pinned on black wax, as previously described (2). For each aorta, the percent of total surface area (ascending, arch, and descending to point of the femoral artery bifurcation) involved by fatty streaks or frank atherosclerosis was quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD) after obtaining images with a Spot Insight color camera system (Diagnostic Instruments). Positive Oil-Red-O staining was used as a guide in the analysis, but care was taken to use standard procedures, as outlined by Daugherty *et al* (3), for identification of all lesions regardless of whether or not they reacted with the stain. Specifically, lesions seen under the dissecting scope as areas of thickening, often due to smooth muscle proliferation or accumulation of non-neutral lipids, were included in the calculation of lesional area. Abdominal aortic aneurysms were defined as an increase in aortic diameter of >50%. At the time of sacrificing, serum was also prepared and subjected to multiplex immunoassay for 58 separate antigens (RodentMAP v2.0; Rules Based Medicine).

Blood pressure monitoring

Male *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice between the ages of 48 and 103 days of age were trained to undergo conscious blood pressure monitoring using a non-invasive tail cuff method, through the core laboratory of the University of Michigan Center for Integrative Genomics. This method utilizes cuffs equipped with photoelectric sensors, capable of obtaining accurate measurements that have been validated when compared to both radiotelemetry and direct arterial blood pressure monitoring (4). Baseline systolic pressures were obtained and mice were then implanted subcutaneously with osmotic minipumps (Alzet model 2004) designed to deliver Ang II at a continuous rate of 500 ng/kg/min. Repeat systolic pressures were obtained weekly following implantation for the course of the 28 day infusion.

Quantitative RT-PCR

Wild-type C57BL/6J mice were sacrificed and various tissues were immediately harvested and snap-frozen in liquid nitrogen. Total RNA was subsequently prepared using the RNeasy Mini Kit (Qiagen) and equivalent amounts of RNA (500-1000 ng) were used for cDNA synthesis with the Superscript First-Strand Synthesis System (Invitrogen) using oligo dT primers. Quantitative PCR was performed using TaqMan gene expression primers (Applied Biosystems) specific for mouse Bcl10, CARMA1, CARMA3, or MALT1 on an Applied Biosystems 7900HT apparatus supplied by the University of Michigan Microarray Core Facility. Cycle thresholds were determined and normalized with those for reactions

performed with β-Actin specific TaqMan primers. For each gene, relative expression across tissues was determined, setting a value of 100 for that tissue with the highest level of expression.
In separate studies, male *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*Bcl10*$^{-/-}$ mice were implanted with osmotic minipumps (Alzet model 2004) designed to deliver Ang II at a continuous rate of 500 ng/kg/min. After 3 days, aortas were excised and RNA prepared for cDNA synthesis and quantitative RT-PCR analysis using Taqman primers for IL-6, CXCL1, and MCP-1. Similar analyses were performed for IL-6 using cDNA obtained from the aortas of wild-type or *Bcl10*$^{-/-}$ mice following 3 day infusions with either PBS or Ang II. Transcript levels were normalized to either GAPDH, β-actin, or TFIID transcription factor.

Finally, quantitative RT-PCR was performed using TaqMan primer sets to analyze efficiency of CARMA3 knockdown in PAC1-AR cells, and to assess induction of IL-6 and LOX-1 in primary ECs. For analysis of cell lines, the process for RNA isolation, cDNA preparation, and quantitative PCR was the same as that described for tissue samples.

Cell culture
PAC1 vascular smooth muscle cells (5) (L. Li; Wayne State University) were stably infected with a retrovirus expressing HA-tagged AT_{1A} cDNA, using an approach described previously (6). A pure population of cells with stable retroviral vector integration were then isolated by FACS. These cells, which constitutively express the AT_{1A} receptor, are referred to as the PAC1-AR cell line. The PAC1-AR cell line, in addition to the FLTR human fetal aortic vascular smooth muscle cell line (7) (T.S. Elton, Ohio State University) and EA.hy926 human endothelial cell line (8) (C.-J. S. Edgell, University of North Carolina) were maintained in DMEM with 10% FBS.

Primary ECs and VSMCs were isolated from adult male Sprague-Dawley rat aorta using a method of sequential collagenase digestion (9). Briefly, rats were anesthetized and aortas were exposed through a midline incision. Aortas were then briefly perfused *in vivo* with PBS before being excised, cleaned of adventitia, and transferred to 10 cm plates. Following additional flushing, the lumen of each aorta was filled with a collagenase solution using a syringe (2 mg/ml type II collagenase; Worthington). Digestion of the intimal layer was allowed to proceed for 15 minutes at 37^{o}C, after which time digestion was halted by flushing aortas three times with DMEM containing 20% FBS. Released ECs were collected by centrifugation at 170xg and washed prior to plating in EGM-2 endothelial growth media (Cambrex). To isolate VSMCs, the remaining aortic tissue was minced and digested at 37^{o}C for up to 60 minutes with fresh collagenase solution, supplemented with elastase (2.5 U/ml; Worthington). Digestion was halted with the addition of DMEM containing 20% FBS, and cells were passed through 100 µM filters (BD Falcon), centrifuged, washed, and plated in SmGM-2 smooth muscle growth media (Cambrex). To evaluate for contamination of EC preps by VSMCs, cells were immunostained using antibody against α-smooth muscle actin (anti-SMA; Sigma). Preps of ECs were only used if <5% of cells stained positively for SMA. Primary ECs and VSMCs were used for no more than 5 passages.

Western analysis
Immunoblotting was performed as described (6). Antibodies used included monoclonal anti-Bcl10 (clone 151; Zymed), polyclonal anti-Bcl10 (Santa Cruz Biotechnology), polyclonal anti-MALT1 (Santa Cruz Biotechnology and Cell Signaling Technology), anti-GAPDH (Chemicon), monoclonal and polyclonal anti-myc (Santa Cruz Biotechnology), monoclonal anti-Flag (M2; Sigma), monoclonal anti-HA (Roche), anti-pERK and anti-pIκBα (Ser-32/34) (Cell Signaling Technology). When analyzing transient induction of pERK and pIκBα, cells were treated for the indicated periods of time with either 500-1000 nM Ang II (Sigma), 1-5 ng/ml IL-1β (R&D Systems), or 10 ng/ml TNFα (BD Biosciences) in serum-free DMEM before harvesting for western analysis.

Transfection and luciferase reporter assay

Cells were transfected with an NF-κB-responsive luciferase reporter plasmid, pBVIx-Luc (10), and the phRL-TK control renilla plasmid (Promega) using either Lipofectamine 2000 (Invitrogen) or FuGene6 (Roche). The NF-κB reporter contains a minimal promoter with 6 tandem repeats of the NF-κB canonical consensus sequence (GGGGACTTTCC) driving the coding region of luciferase. This consensus sequence recognizes p50/p65 heterodimers and p65 homodimers, both of which can be induced by the canonical pathway of NF-κB activation. 24 hours later, cells were treated for an additional 16 hours with or without medium containing either 1 μM Ang II, 10 ng/ml TNFα, or 1 μM IL-1β. Cells were harvested and luciferase activity in lysates was measured as described (6).

RNA interference

OnTarget *Plus* siRNA smartpools (Dharmacon) were transiently transfected into PAC1-AR cells using Lipofectamine RNAiMAX reagent (Invitrogen). Concentrations of siRNA used for transfection were: Bcl10, 10 nM; CARMA3, 50 nM; MALT1, 50 nM. For the experiments shown in Fig. 2 *E* and *F*, a single siRNA oligonucleotide duplex (Dharmacon) specific for rat Bcl10 (sense strand; 5'-GAAAUUUCUUGCCGAACUUUU-3') was utilized, transfected at a concentration of 5 nM. In all cases, control cells were similarly transfected with non-targeting, scrambled siRNAs. For the experiment shown in Fig. 2*E*, PAC1-AR cells were allowed to recover for 24 hours, at which time they were transfected with NF-κB-responsive luciferase and renilla reporter plasmids, as described above. Following treatment with cytokines, cells were harvested for parallel luciferase assays and for western blotting, to analyze the efficiency of Bcl10 knock-down. In the case of primary ECs, cells were allowed to recover from the siRNA transfection for 72 hours, at which time they underwent treatment with Ang II or TNFα to assay for transient induction of pIκBα.

Ubiquitination assay

PAC1-AR or primary rat ECs were transfected with expression vectors encoding myc-tagged IKKγ and HA-tagged ubiquitin. Cells were then treated with Ang II or TNFα and ubiquitination of IKKγ was assayed as described in detail previously (6).

Statistics

Data are expressed as mean ± SEM. Differences between groups were compared for significance using paired or unpaired 2-tailed Student's *t* tests, as appropriate, with the assistance of GraphPad InStat software. *P* values of less than 0.05 were considered statistically significant.

SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIGURE 1. **Bcl10 and MALT1 are required for Ang II-dependent NF-κB activation in VSMCs, but not for activation of the ERK pathway.** *A* and *B*, PAC1-AR VSMCs were transiently transfected with either control siRNA, or siRNA targeting Bcl10 (*A*) or MALT1 (*B*). After 48 hours, cells were treated as indicated and lysates were prepared for immunoblot analyses. While knockdown of either Bcl10 or MALT1 abrogated Ang II-dependent phosphorylation of IκB, there was no effect on p-IκB generation stimulated in response to treatment with IL-1β or TNFα. In addition, Ang II-dependent stimulation of phospho-ERK was completely unaffected by the absence of either Bcl10 or MALT1, indicating that the CBM signalosome is not critical for all signaling pathways emanating from the Ang II receptor. Rather, the CBM signalosome specifically mediates Ang II-dependent NF-κB activation but not activation of the ERK pathway.

SUPPLEMENTAL FIGURE 2. **Primary rat aortic endothelial cells respond to Ang II with NF-κB activation.** *A*, ECs and VSMCs were isolated from fresh rat aorta. Purity was confirmed using immunoreactivity for smooth muscle actin (SMA) as shown in the low-power micrographs (*insets* show detail of a single representative cell for each preparation). Preparations were not used for further analysis unless judged to be >95% pure by this method. Cells were then transfected with an NF-κB luciferase reporter plasmid, along with a renilla control plasmid, and NF-κB induction following overnight Ang II treatment was determined using a dual-luciferase assay system; *$P<0.05$ *B*, Primary ECs were prepared and treated with Ang II or TNFα as in panel *A*, but with increasing concentrations of telmisartan, an AT_1R-specific antagonist. Data (mean ± SEM) are from at least 3 separate determinations. *C*, Quantitative RT-PCR was used to demonstrate that Ang II treatment of primary ECs results in the induction of the indicated NF-κB-responsive genes. Transcript levels were normalized to those for β-actin. Data (mean ± SEM) are from at least 3 separate determinations; *$P< 0.05$.

SUPPLEMENTAL FIGURE 3. **Identical blood pressure responses in *ApoE$^{-/-}$* and *ApoE$^{-/-}$Bcl10$^{-/-}$* mice chronically infused with Ang II.** *A*, Baseline systolic blood pressures (BP) were determined for male mice of each strain prior to implantation with osmotic minipumps, designed to deliver Ang II at a dose of 500 ng/kg/min for 28 days. Systolic pressures were obtained on a weekly basis thereafter. While Ang II infusion produced a moderate pressor effect, there was no difference in the response between the two strains (n=5). *B*, A representative aortic aneurysm from the group of *ApoE$^{-/-}$* mice is shown. Only a single small aneurysm was observed in the group of *ApoE$^{-/-}$Bcl10$^{-/-}$* mice, and is shown at right.

SUPPLEMENTAL FIGURE 4. **Analysis of pro-inflammatory mediators from the serum of *ApoE$^{-/-}$* and *ApoE$^{-/-}$Bcl10$^{-/-}$* mice chronically infused with Ang II.** Levels of 58 serum pro-inflammatory mediators were evaluated by multiplex ELISA following 4 weeks of Ang II treatment. Levels observed in *ApoE$^{-/-}$* mice were arbitrarily given a value of 100 and the relative levels observed in *ApoE$^{-/-}$Bcl10$^{-/-}$* mice were then plotted in comparison. While there was no difference in the levels for most mediators between the two strains, a select group of NF-κB responsive factors were seen to be reduced in *ApoE$^{-/-}$Bcl10$^{-/-}$* mice, and are shown at top. Statistical significance was achieved for one of these mediators (CD40 ligand; $p<0.05$), while the others showed a strong trend toward reduction. Data (mean ± SEM) are from 5 mice/group.

SUPPLEMENTAL FIGURE 5. **Bcl10 deficiency does not abrogate Ang II-mediated induction of IL-6 in mouse aorta.** *A*, Wild-type (WT) and *Bcl10$^{-/-}$* mice were implanted with osmotic minipumps delivering vehicle (PBS) or Ang II. After 3 days, aortas were harvested and the levels of IL-6 mRNA determined by quantitative RT-PCR, normalized to GAPDH. Results are expressed as fold induction of IL-6 mRNA, relative to vehicle treatment (n=4-6). *B*, Male *ApoE$^{-/-}$* and *ApoE$^{-/-}$Bcl10$^{-/-}$* mice (n=14,13; respectively) were similarly infused with Ang II for 3 days, after which IL-6 mRNA levels were quantified from excised aortas. The level of IL-6 mRNA in aortas from *ApoE*$^{-/-}$ mice, normalized to GAPDH and TFIID mRNA, was set arbitrarily at 100.

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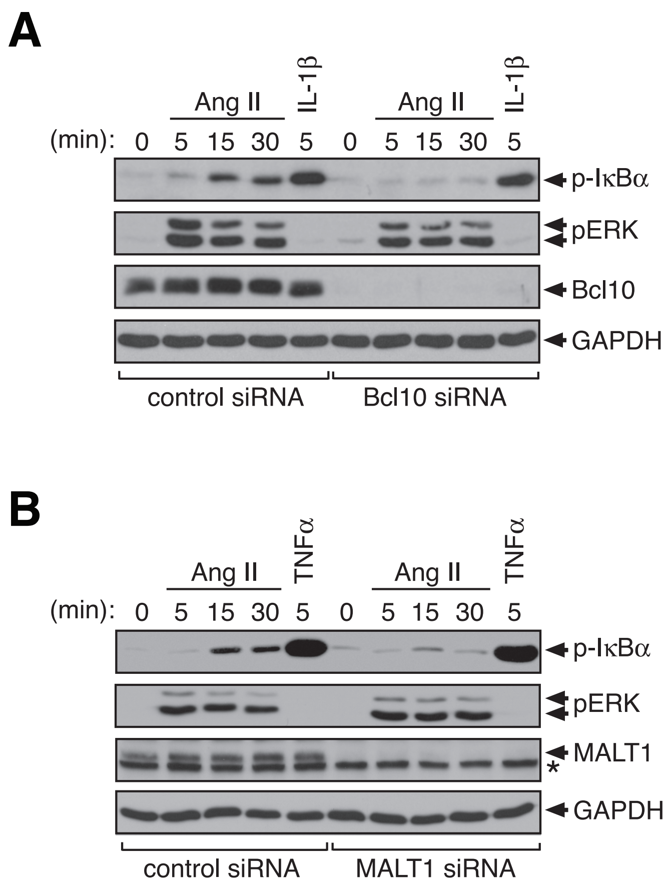

Supplemental Figure 1
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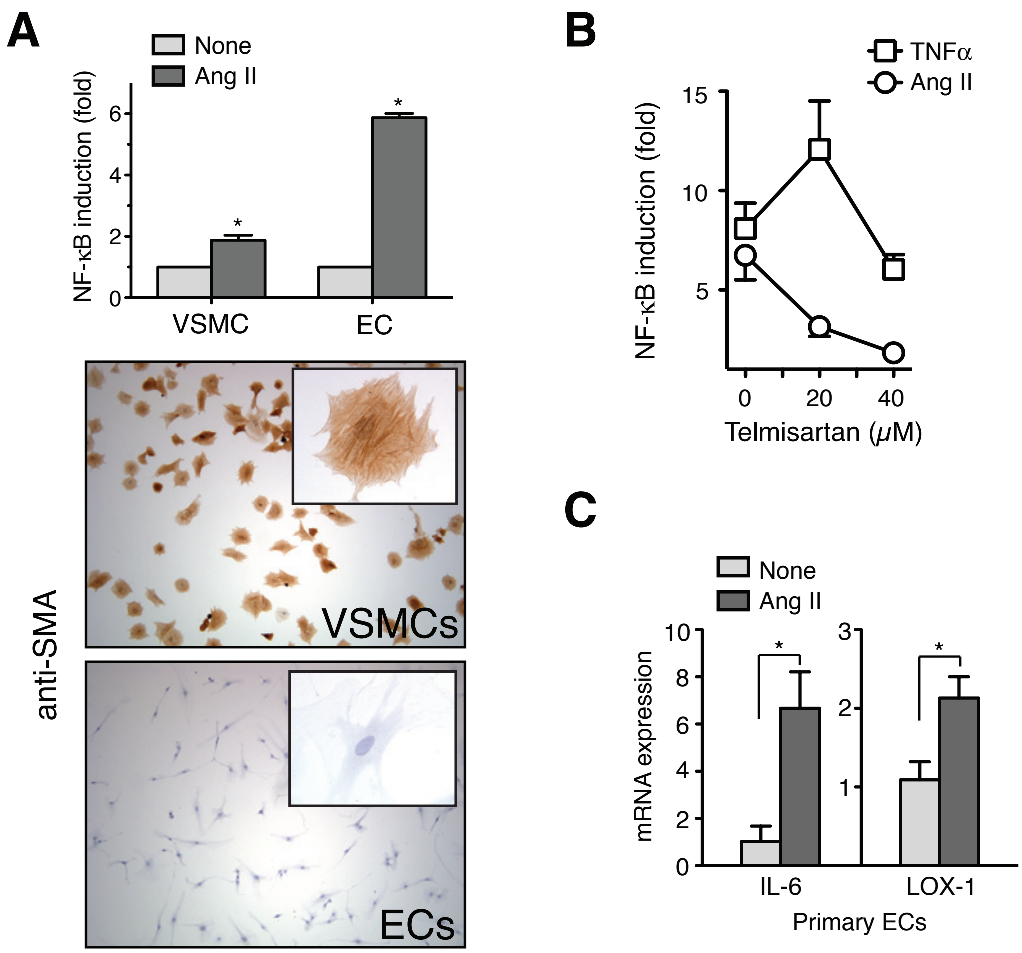

Supplemental Figure 2
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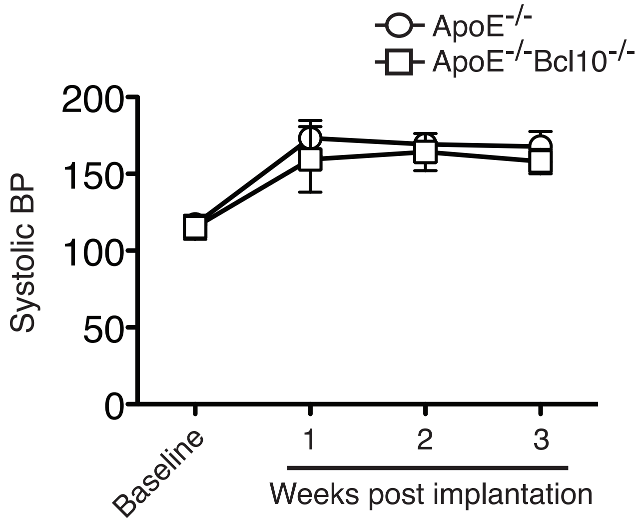
A



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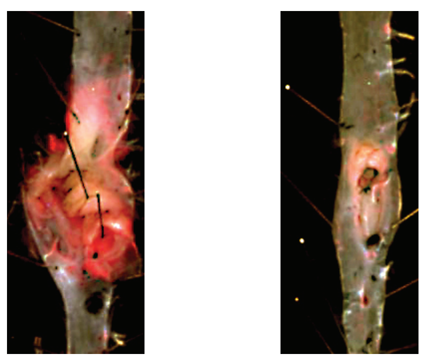

Representative aneurysm; ApoE$^{-/-}$ strain

Single aneurysm; ApoE$^{-/-}$Bcl10$^{-/-}$ strain

Supplemental Figure 3
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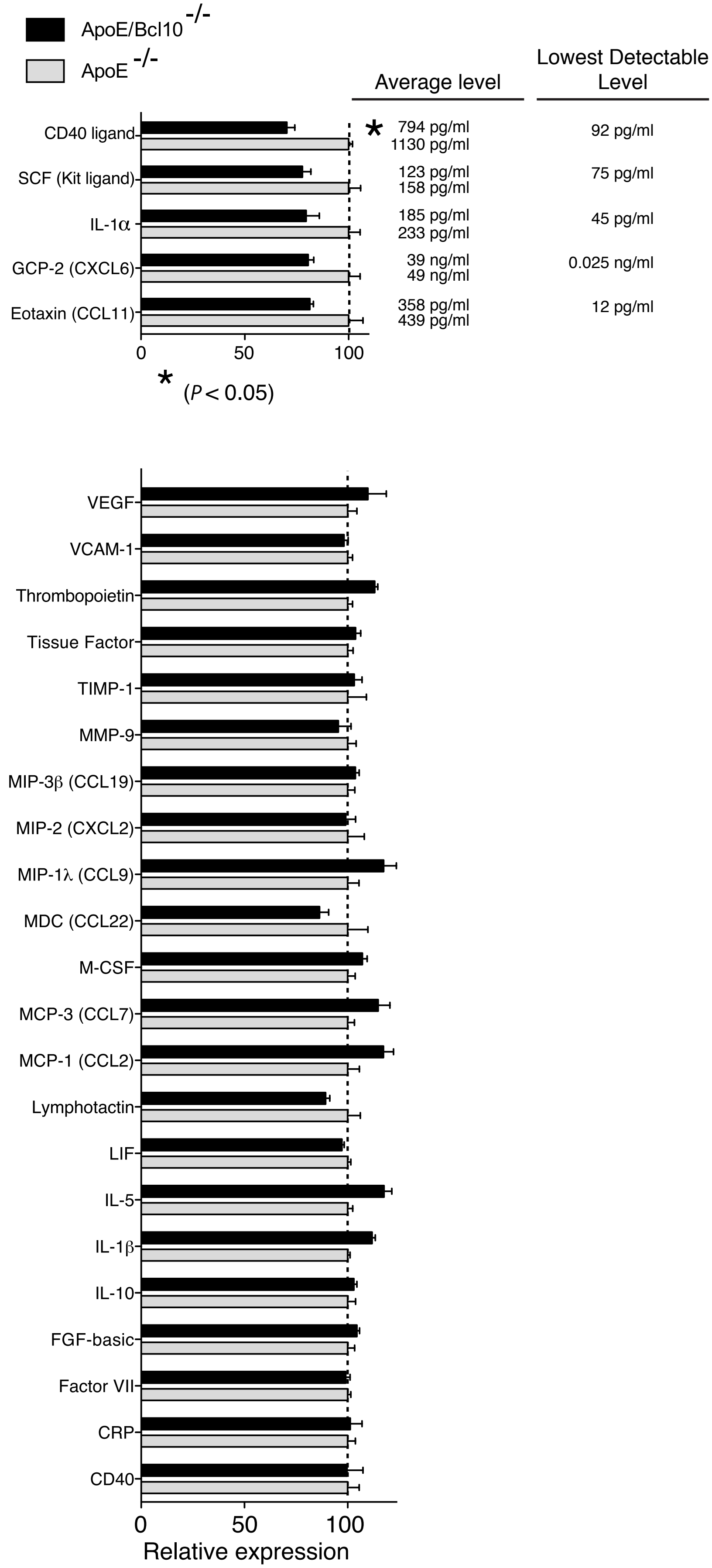

Supplemental Figure 4
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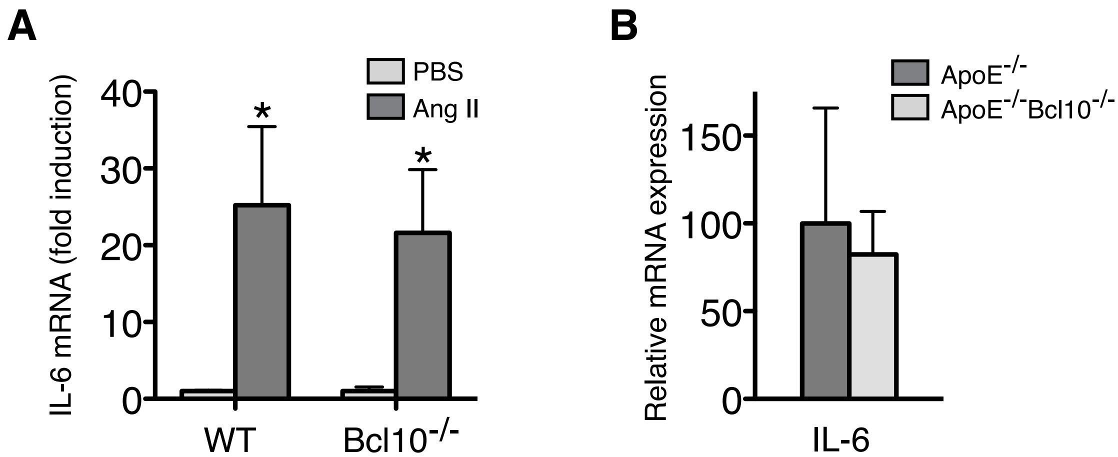

Supplemental Figure 5
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