Cellular Biology

Differential Role of an NF-κB Transcriptional Response Element in Endothelial Versus Intimal Cell VCAM-1 Expression


Rationale: Human and murine Vcam1 promoters contain 2 adjacent nuclear factor-κB (NF-κB)–binding elements. Both are essential for cytokine-induced transcription of transiently transfected promoter–reporter constructs. However, the relevance of these insights to regulation of the endogenous Vcam1 gene and to pathophysiological processes in vivo remained unknown.

Objective: Determine the role of the 5′ NF-κB–binding element in expression of the endogenous Vcam1 gene.

Methods and Results: Homologous recombination in embryonic stem cells was used to inactivate the 5′ NF-κB element in the Vcam1 promoter and alter 3 nucleotides in the 5′ untranslated region to allow direct comparison of wild-type versus mutant allele RNA expression and chromatin configuration in heterozygous mice. Systemic treatment with inflammatory cytokines or endotoxin (lipopolysaccharide) induced lower expression of the mutant allele relative to wild-type by endothelial cells in the aorta, heart, and lungs. The mutant allele also showed lower endothelial expression in 2-week atherosclerotic lesions in Vcam1 heterozygous/low-density lipoprotein receptor-deficient mice fed a cholesterol-rich diet. In vivo chromatin immunoprecipitation assays of heart showed diminished lipopolysaccharide-induced association of RNA polymerase 2 and NF-κB p65 with the mutant promoter. In contrast, expression of mutant and wild-type alleles was comparable in intimal cells of wire-injured carotid artery and 4- to 12-week atherosclerotic lesions.

Conclusions: This study highlights differences between in vivo and in vitro promoter analyses, and reveals a differential role for a NF-κB transcriptional response element in endothelial vascular cell adhesion molecule-1 expression induced by inflammatory cytokines or a cholesterol-rich diet versus intimal cell expression in atherosclerotic lesions and injured arteries. (Circ Res. 2015;117:166-177. DOI: 10.1161/CIRCRESAHA.117.306666.)

Key Words: atherosclerosis ■ chromatin immunoprecipitation ■ endothelial cells ■ gene expression ■ NF-κB ■ tunica intima ■ vascular cell adhesion molecule-1

Vascular cell adhesion molecule-1 (VCAM-1), a 110-kD type 1 transmembrane cell surface glycoprotein encoded entirely by type 1 exons,1,2 was identified in cytokine-activated human umbilical vein endothelial cells (ECs) by monoclonal antibody and expression cloning approaches3–5 and found to recognize the primary ligand α5β1 integrin (very late antigen 4 [VLA-4], CD 49d/CD29) plus several secondary ligands, including αvβ3, (LPAM-1),6 αvβ5, αvβ3, and α5β1 integrins2–9 and secreted protein acidic and rich in cysteine (a 32 kDa matricellular glycoprotein also known as osteonectin or BM-40).10 VCAM-1 mediates leukocyte migration in adults where its expression is induced in chronic inflammatory conditions, including atherosclerosis, rheumatoid arthritis, multiple sclerosis, organ allografts, and other pathological processes in vivo.11–13 VCAM-1 may also participate in lymphocyte homing, hematopoiesis, and retention of...

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progenitor cells in bone marrow. Unexpectedly, VCAM-1 (via binding to \( \alpha_\beta_1 \) integrin) also plays a critical role during placentation by mediating fusion of allantois to chorion and may also participate in myogenesis, pericardial development and later trophoblast differentiation.

Placental failure leading to embryonic lethality in VCAM-1 null gestations complicates genetic loss-of-function analysis of VCAM-1 in adults. Therefore, to better define the physiological and pathophysiological roles of VCAM-1, we used targeted mutagenesis in embryonic stem (ES) cells to develop a hypomorphic VCAM-1 allele (\( Vcam1^{DAD} \)). Decreased VCAM-1 expression in \( Vcam1^{DAD/ DAD} \) mice partially circumvented embryonic lethality and revealed a predominant role for VCAM-1 in atherosclerotic lesion formation in adults in vivo. Other investigators have also used conditional gene inactivation to reveal a role for \( Vcam1 \) in lymphocyte migration to bone marrow.

The precise temporal and spatial expression patterns of VCAM-1 are critical for its functions in multiple settings. VCAM-1 expression is low on unactivated endothelium and is rapidly but transiently induced after stimulation by interleukin-1\( \beta \) (IL-1\( \beta \)), tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)), or bacterial lipopolysaccharide. Other stimuli inducing endothelial expression of VCAM-1 include ligands of receptor for advanced glycation end products and binding of CD154, the CD40 ligand on activated T cells, to endothelial CD40. However, VCAM-1 expression in vivo may also be prolonged or constitutive on ECs and other cell types, such as follicular dendritic cells in secondary lymphoid tissues, and in hepatic Kupffer cells.

Nuclear factor-\( \kappa B \) (NF-\( \kappa B \)) plays a prominent role in rapid induction of VCAM-1 by mediators of acute inflammation and systemic endothelial activation. Such mediators bind to cell surface receptors and induce complex intracellular signaling leading to phosphorylation, polyubiquitination, and proteasomal degradation of NF-\( \kappa B \) inhibitors (I\( \kappa B \)s) that otherwise sequester NF-\( \kappa B \) in the cytoplasm. Degradation of I\( \kappa B \)s enables NF-\( \kappa B \) translocation across the nuclear membrane and binding to recognition sites in promoters of responsive genes leading to assembly of multiprotein complexes, including basal transcription factors and coactivators that initiate transcription.

In vitro studies using primary and transformed cell lines demonstrated that the 258 bp 5′ flanking sequence of the \( Vcam1 \) gene, when placed upstream of reporter genes in episomal vectors, directed full cytokine-induced expression of VCAM-1 in ECs. Two adjacent NF-\( \kappa B \) cis elements (−73 and −58 bp 5′ from the transcription initiation site), which are conserved in the highly homologous human and murine \( Vcam1 \) promoters, are required for this response. In TNF-\( \alpha \)-activated ECs, NF-\( \kappa B \) cis elements in the \( Vcam1 \) promoter, acting in unison, bound primarily p50/p65 heterodimers that then interacted with other transcription factors, including interferon regulatory factor-1 and Sp1 to upregulate \( Vcam1 \) transcription. Interferon regulatory factor-1 modulates TNF-\( \alpha \)-induced VCAM-1 expression in response to stimulation by interferon-\( \alpha \) and interferon-\( \gamma \) and hyperosmotic stimuli. Members of the GATA (GATA binding protein 1) family of transcription factors also enhance both constitutive and inducible \( Vcam1 \) transcription. TNF-\( \alpha \) induces expression of c-Fos and c-Jun, which together bind to an activating protein-1 consensus element to enhance \( Vcam1 \) transcription. These observations suggested that altering NF-\( \kappa B \)’s interaction with either of its cis-acting recognition elements might drastically disrupt normal regulatory mechanisms and significantly modulate VCAM-1 expression in vivo.

Although these are valuable insights, their relevance to regulation of the endogenous \( Vcam1 \) gene and their significance in physiological and pathophysiological processes remained unknown because these experiments were performed devoid of \( Vcam1 \)’s normal chromosomal context. As such, they did not evaluate the potential effects of many factors that modulate gene expression in vivo, including distant regulatory elements that are located several kilobases up or downstream of transcription initiation sites and enhance or suppress transcription. For example, promoter-bound NF-\( \kappa B \) and associated coactivators together ultimately regulate local chromatin remodeling: activating stimuli such as TNF-\( \alpha \) induce histone deacetylation and trichostatin A treatment, which inhibits histone deacetylation suppresses TNF-induced VCAM-1 expression. Thus, given these contributions of multiple transcription factors, cognate cis-acting elements, and local chromatin configuration, a full understanding of \( Vcam1 \) transcriptional activation requires studying the function of individual regulatory mechanisms in the normal \( Vcam1 \) chromosomal context. This insight will help predict how intentionally altering such mechanisms will modulate VCAM-1 expression in vivo. In addition, the role of NF-\( \kappa B \) in VCAM-1 expression by endothelial and other cells during inflammation and atherosclerosis in vivo has not been determined.

To further probe the function of VCAM-1 in adults, we optimized the tag and exchange approach for gene targeting in ES cells to generate mice in which the 5′ NF-\( \kappa B \) element in the \( Vcam1 \) promoter was selectively inactivated without other functional or primary structural changes to the endogenous gene except for a 3 nucleotide substitution in the 5′ untranslated region (UTR) to enable simultaneous differential
quantification and direct comparison of expression from the mutant and wild-type alleles. Unexpectedly, we found that inducible VCAM-1 expression was partially preserved in some pathological contexts in vivo and that the Vcam1

NF-κB geno-

type conferred functional deficits in a model of immunization-

induced leukocyte emigration. We also gained novel insights about the nature of inducible chromatin-associated regulatory proteins present at the Vcam1 promoter in vivo and about how NF-κB regulates transcription of the endogenous Vcam1 gene during acute and chronic inflammation.

**Methods**

Additional Methods are available in the Online Data Supplement.

**Selective Inactivation of the Vcam1 5′ NF-κB Transcriptional Response Element**

An optimized sequential tag and exchange strategy was used in ES cells to selectively mutate the Vcam1 5′ NF-κB–binding site. This approach was adopted in preference to recombinase-mediated approaches because the latter introduce residual recombinase recognition sequences in the resulting mutant allele, and these extraneous genetic changes may affect promoter function. The GGTTTCC to TGAATTCAA mutation (73 to 64 nucleotides 5′ of the transcription start site) disrupted the 5′ NF-κB contact site and created an Eco RI restriction enzyme site (Figure 1B), which was used for genotyping by Southern blot and polymerase chain reaction (PCR; Online Figure I). The 3′ NF-κB cis element was not altered. A second mutation (GAAGCAGAGACT to GAACTCGAGACT) was introduced 11 to 13 nucleotides 5′ from the translation start site in the 5′ UTR of Vcam1 in exon 1 (Figure 1B), and allowed differential assay of wild-type and mutant allele expression and chromatin configuration by chromatin immunoprecipitation (ChIP).

Mice were backcrossed 10 generations into the C57BL/6 background.

**Acute Induction of VCAM-1 Expression by Inflammatory Mediators Lipopolysaccharide, IL-1β, and TNF-α**

Lipopolysaccharide (Escherichia coli serotype O55:B5) was purchased from Sigma–Aldrich (St. Louis, MO), and murine recombinant

![Diagram](http://circres.ahajournals.org/ by guest on August 13, 2017)

Figure 1. A, Targeting constructs designed to produce the Vcam1

mutant allele by homologous recombination in embryonic stem cells. This illustrates the Tag and Exchange targeting constructs as well as the starting genomic structures and the desired products of both homologous recombination steps. The scale indicates genomic sequence length in kilobases (kb). Dashed lines indicate 5′ and 3′ flanking regions of the Vcam1 gene. NEO, neomycin resistance gene; TK, selection marker containing a herpes simplex virus thymidine kinase gene; pSK, Bluescript SK+phagemid; SP, exon 1 encoding the signal peptide; D1, D2, and D3, exons 2, 3, and 4, encoding immunoglobulin-like domains 1, 2, and 3, respectively; PI, exon 5 unique to murine Vcam1 when used by alternative splicing results in a truncated 3 immunoglobulin domain form that is bound to the cell surface by a glycosyl-phosphatidylinositol linkage; arrowheads, locations of mutations; Eco RI, Xmn I, Msc I, Nhe I, restriction enzyme sites. B, The murine Vcam1 gene transcription start site and 5′ flanking region illustrating the mutations introduced in the Vcam1

mutant allele. Nucleotides altered in the mutant allele are shown under the wild-type sequence, as are the first 5 amino acids (International Union of Pure and Applied Chemistry abbreviations[5]) encoded by vascular cell adhesion molecule-1 (VCAM-1) mRNA. Double overlines, 5′ and 3′ nuclear factor-κB (NF-κB)–binding sites defined by in vitro experiments; oversquare, TATA box; triple overline, IRF–binding site; thin arrow, transcription start site and direction; dark underline, Bgl I restriction enzyme site; gray arrowheads, sites of RNase cleavage of RPA probe protected by wild-type and Vcam1xB mutant RNA, respectively.
IL-1β and TNF-α were from R&D Systems (Minneapolis, MN). VCAM-1 expression in the heart was induced by injection of lipopolysaccharide (0.1–100 μg/mouse or 0.004–4 μg/g body weight intraperitoneal). In experiments measuring VCAM-1 expression in different tissues, IL-1β (120 ng), TNF-α (500 ng), or lipopolysaccharide (10 μg) were injected intravenously in 200-μL sterile phosphate-buffered saline. Phosphate-buffered saline–injected littermates served as controls. RNA was harvested from tissues after 2 hours.

### Ovalbumin peritonitis
Mice were immunized by a single intraperitoneal injection of ovalbumin (100 μg in 0.1 mL phosphate-buffered saline, filter sterilized). After 14 days, mice were challenged with ovalbumin (100 μg IP) and a peritoneal lavage was performed at 48 hours.

### Atherosclerosis and Carotid Wire Injury
Heterozygous Vcam1+/− mice (backcrossed 10 generations onto the C57BL/6 background) were mated with Ldlr−/− mice (C57BL/6J background) to produce Vcam1+/−, Ldlr−/−, Vcam1+/−, Ldlr+/− and Vcam1+/−; Ldlr−/− animals. These mice were placed on a semipurified 1.25% cholesterol diet for 2, 4, 8, or 12 weeks as described previously.22,38 At which time aortas were harvested for RNA isolation or immunostaining. Heterozygous Vcam1+/− mice (C57BL/6J background) were subjected to right carotid artery wire injury or sham injury, as described previously.38 RNA was isolated from both carotid arteries after 4 days.

### Northern Blotting
Total RNA was isolated from heart tissues using TRIzol reagent (Invitrogen). Northern blots were performed as described1 using mouse cDNA probes for VCAM-1 (from Biogen Inc, Boston, MA), intercellular adhesion molecule-1 (from T. Springer, Harvard University), and β-actin (Ambion Inc, Austin, TX).

### Western Blotting
Total protein was isolated from heart tissues, centrifuged to remove nuclei and insoluble cytoskeleton and 40 μg of protein was loaded into each lane. After electrophoresis and electrotransfer, membranes were probed with anti-VCAM-1 (R&D Systems, AF647) and antiactin (Sigma, A2066) antibodies followed by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection system (Amersham).

### Reverse Transcription Quantitative PCR
Gene expression was assessed in mouse tissues (heart, lung, aorta, and carotid arteries) using reverse transcription quantitative PCR (RT-qPCR). Total RNA was isolated using the RNaseasy Mini Plus Isolation Kit (Qiagen). RT into single-stranded cDNA was performed using the high capacity random primer reverse-transcription kit (Applied Biosystems, Foster City, CA or PowerScript, Clontech, Palo Alto, CA). Primers to detect mRNA and heterogeneous nuclear (hn) RNA of wild-type and mutant Vcam1 alleles and housekeeping genes (CD31 and hypoxanthine guanine phosphoribosyltransferase) were designed using the Primer Express software (Applied Biosystems). Primer sequences are in Online Table I.

### Immunohistochemistry
Segments of heart tissues from Vcam1+/+ and Vcam1+/− mice were immersed in OCT compound and snap frozen in liquid nitrogen-cooled 2-methylbutane. Serial cross sections were cut using a cryostat and immunostained with antimouse VCAM-1 and intercellular adhesion molecule-1 antibodies (M/K-2.7 and Y11/1.7.4, respectively, American Type Culture Collection), as was described previously.21

### Chromatin Immunoprecipitation
ChIP was performed on hearts from heterozygous Vcam1+/− mice injected 2 hours previously with lipopolysaccharide or control carrier. Allele-specific association of RNA Polymerase 2, NF-kB p65, NF-kB p50, and p300 was determined by qPCR using specificity-validated primers. Methods for primer identification and validation, ChIP and analysis by qPCR are detailed in the Online Data Supplement.

### Statistical Analyses
Statistical differences between 2 groups were determined using a paired Student t test. In experiments consisting of multiple groups, ANOVA and Dunnett multiple comparisons test were used. All values are expressed as mean±SEM.

### Results

#### Production and Characterization of Mice Bearing a Vcam-1 Allele With a Mutant NF-κB–Binding Element (Vcam1κB)
Given that previous transfection studies using promoter–reporter constructs demonstrated that the integrity of both NF-κB–binding elements in the Vcam1 promoter were essential for cytokine-induced transcription, we inactivated the 5′ NF-κB element without introducing other primary structure changes in the endogenous gene except for a 3 nucleotide substitution in the 5′ UTR (Figure 1). Initially, bovine aortic ECs were transiently transfected with either the wild-type or the mutated Vcam1 gene (the Exchange targeting construct in Figure 1B) with the lacZ coding region inserted into the 5′ UTR (Online Figure II) and β-galactosidase expression was assayed 4 hours after activation by TNF-α. This experiment confirmed previously published data, and demonstrated that the introduced mutation inactivated the 5′ NF-κB element and entirely prevented cytokine-induced activation of transcription.

Transfection of ES cells12–15 with the Tag construct produced a single correctly targeted clone that was transected with the Exchange construct. Clones bearing the desired mutation were derived using a pool/subpool strategy (because the overall efficiency of the Tag and Exchange process was relatively low) and were used for blastocyst-mediated transgenesis to produce mice transmitting the Vcam1+ allele in the germline (see Online Data Supplement for details of Tag and Exchange transfection and selection).

Vcam1κB mice derived from heterozygous intercrosses were born at the expected Mendelian frequency (Online Table II; Online Figure I), developed normally as adults and were fertile. In contrast, gestations entirely lacking VCAM-119 show absence of chorioallantoic fusion and umbilical cord formation leading to failed placentation and embryonic lethality around E8.5–E9.5. Thus, Vcam1κB expression allowed sufficient chorioallantoic fusion to avoid embryonic lethality.

Systemic stimulation of adult Vcam1κB mice with lipopolysaccharide readily induced the expression of VCAM-1 mRNA (Northern blot, Figure 2A) and protein (Western blot, Figure 2B and 2C) in the heart, predominantly by ECs (Figure 2D); however, Vcam1κB induction was blunted relative to wild-type mice. In subsequent experiments, the 3-nucleotide mutation introduced into the 5′ UTR of VCAM-1 was exploited for RT-qPCR because allele-specific primers successfully distinguished wild-type from mutant VCAM-1 mRNA and hnRNA in homozygous mice (Online Figure III). This enabled independent quantification of expression from wild-type and mutant VCAM 1 alleles in complex settings in vivo.
The Role of the 5′ NF-κB Element in Acute Induction of VCAM-1 Expression

Previous work demonstrated that both the 5′ and 3′ NF-κB cis elements in the Vcam1 promoter are required for cytokine-induced transcription from episomes and experiments with the Vcam1$\kappa B$ mutation generated here (Online Figure II) confirmed the importance of the 5′ element. To determine if the 5′ NF-κB–binding site is also critical for inducible expression of the endogenous Vcam1 gene in vivo, the expression of wild-type and mutant Vcam1 alleles was evaluated simultaneously in heterozygous Vcam1$+/\kappa B$ mice (backcrossed 10 generations into the C57BL/6 background). Systemic administration of lipopolysaccharide induced cardiac expression of both wild-type (Vcam1*) and mutant (Vcam1$\kappa B$) mRNA in a dose-dependent manner (Online Figure IV). However, although wild-type mRNA increased as much as 35-fold in mice receiving lipopolysaccharide (100 µg or 4 µg/g body weight) compared with unstimulated controls, mutant mRNA increased less than half of this amount (to a maximum of ≈10-fold) at all lipopolysaccharide doses tested. Levels of hnRNA, which more directly reflect the rate of transcription, closely paralleled mRNA expression (Online Figure IV). Similar mRNA expression patterns were also observed on administration of IL-1β or TNF-α not only in heart but also in the lung and the aorta (Figure 3).
In general, the ratio of $Vcam1^\beta$ to $Vcam1^\alpha$ expression induced by each of the inflammatory mediators was lower in the heart (range, 0.39–0.54) than in either the lung (0.59–0.88) or the aorta (0.50–0.76). RNase protection assay confirmed the qPCR findings (Online Figure V). Taken together, these results reveal that the 5′ NF-κB–binding site of the $Vcam1$ gene is required for full and rapidly induced expression of the endogenous $Vcam1$ gene in different tissues in response to potent inflammatory stimuli. In spite of this, substantial induction still occurs when only the 3′ element is available for NF-κB binding and recruitment of active transcription factor complexes. This observation is in stark contrast to the results of the many in vitro promoter–reporter transfection studies cited above (and confirmed here in Online Figure II), which revealed that both tandem NF-κB elements must be functional for any increase in $Vcam1$ transcription in response to potent inflammatory mediators.

The $Vcam1$ 5′ NF-κB Element Is Required for Normal Leukocyte Recruitment in Immune-Mediated Peritonitis

The functional significance of decreased VCAM-1 expression in $Vcam1^{\alpha/\beta}$ mice relative to $Vcam1^{+/+}$ mice was tested in a model of peritoneal inflammation. Mononuclear leukocyte and to a lesser extent polymorphonuclear leukocyte (predominantly eosinophil) recruitment into the peritoneal cavity were both reduced after an intraperitoneal challenge of immunized mice with ovalbumin (Figure 4). Experiments in mice with hypomorphic VCAM-1 expression ($Vcam1^{\alpha/\beta}$ mice;22,23) revealed that VCAM-1 is essential for full leukocyte recruitment in this model, in contrast to the thioglycollate peritonitis model, in which intercellular adhesion molecule-1 fully compensated for VCAM-1 deficiency (Online Figures VI and VII).

VCAM-1 Expression During Atherogenesis in Ldlr<sup>−/−</sup> Mice

To determine if the 5′ NF-κB–binding site in the $Vcam1$ promoter participates in expression during atherogenesis, heterozygous $Vcam1^{+/a}$ mice were bred into the Ldlr<sup>−/−</sup> background and fed an atherogenic cholesterol-rich diet for 2, 4, 8, or 12 weeks. The expression of $Vcam1^+$ and $Vcam1^{a/β}$ alleles was determined in 3 regions of the aorta by RT-qPCR. Relative to control aortas, the abundance of $Vcam1^+$ mRNA increased progressively with the duration of hypercholesterolemia and extent of lesion formation: $Vcam1^+$ mRNA increased >70-fold in the ascending aortic arch of mice with 12-week lesions (Figure 5C). In early (2-week) lesions, VCAM-1 was
Expressed predominantly in endothelium (Online Figure VIII A) and was diminished by the \( Vcam1^{+/+} \) mutation (Figure 5A and 5B). In contrast, in later (4-, 8-, and 12-week) lesion expression of \( Vcam1^{+/+} \) mRNA was only marginally lower than the wild-type allele with significant differences in only occasional samples (Figure 5D). To determine if the 5' NF-κB-binding site in the \( Vcam1 \) promoter participates in lesion formation the extent of atherosclerosis was determined in homozygous \( Vcam1^{+/+} \) and \( Vcam1^{+/+} \) mice on the \( Ldlr^{−/−} \) background that were maintained for 12 weeks on an atherogenic cholesterol-rich diet. The percent lesion area in the ascending arch, descending thoracic aorta (DTA), and abdominal aorta was similar in \( Vcam1^{+/+} \), \( Ldlr^{−/−} \) and \( Vcam1^{+/+} \) mice (Figure 5E). Thus, in contrast to expression induced acutely by systemic inflammatory mediators in vivo (Figure 3; Online Figures IV and V), expression early during atherogenesis (Online Figure VIII A; Figure 5A and 5B), and function in acute immune-mediated peritonitis (Figure 4) the 5' NF-κB-binding site of the endogenous \( Vcam1 \) promoter is largely dispensable for full expression and lesion formation in advanced atherosclerotic lesions (Figure 5D and 5E). Laser capture microdissection of advanced lesions showed that \( Vcam1 \) is expressed primarily by intimal cells (Online Figure VIII B).

**VCAM-1 Expression Induced by Carotid Wire Injury**

Activation of NF-κB is also thought to mediate responses to endothelial denudation injury resulting from many other vascular insults, including mechanical trauma during vascular surgery and invasive intracoronary procedures and therapeutic interventions. To determine if the \( Vcam1 \) promoter 5' NF-κB-binding site participates in VCAM-1 expression induced by vascular injury heterozygous \( Vcam1^{+/+} \) mice were subjected to carotid wire injury. Expression of \( Vcam1^{+} \) and \( Vcam1^{−/−} \) alleles was determined by RT-qPCR 4 days after injury. \( Vcam1^{+} \) expression increased >15-fold in the injured relative to the uninjured contralateral artery (Figure 6A). The abundance of \( Vcam1^{+} \) and \( Vcam1^{−/−} \) allele mRNA was comparable in the injured artery, whereas the contralateral artery showed a small, but statistically significant decrease in expression of the \( Vcam1^{+} \) allele compared with the \( Vcam1^{−/−} \) allele (Figure 6B). Thus, in contrast to acutely induced expression by systemic inflammatory mediators in vivo and during early atherogenesis, and similar to expression during advanced atherosclerosis (Figure 5), the function of the 5' NF-κB-binding site of the endogenous \( Vcam1 \) promoter is dispensable for full induction of expression after traumatic carotid wire injury.
Vcam1<sup>+</sup> and NF-κB p65 with the Vcam1 Promoter Region

Vcam1 transcription is regulated by NF-κB–dependent formation of complex, multimeric super enhancers at several sites, including the Vcam1 promoter region. To determine if the diminished mRNA and protein expression conferred by the Vcam1<sup>+</sup> mutation was accompanied by an altered chromatin configuration state, we performed allele-specific ChIP in heterozygous mice injected intraperitoneally with lipopolysaccharide to evaluate proteins associated with the wild-type and mutant Vcam1 loci. We first assayed lipopolysaccharide-enhanced association of Pol II (Online Figure IXB) at several locations spanning the 5′ NF-κB–binding site and the site of the 5′ UTR mutation (Online Table IC; Online Figure IXA): the amplimer generated with oligonucleotides 2F and 2R+ was the most sensitive. Substituting a Vcam1<sup>+</sup> allele-specific primer (2R5′κB) demonstrated that qPCR detection was allele-specific (Online Figure IXC) and equivalently sensitive (Online Figure IXD). Taken together, these results validate the qPCR approach used to quantify Vcam1 alleles in ChIP DNA.

We extended existing ChIP protocols<sup>47,48</sup> by preparing chromatin from individual 100 mg hearts (instead of pooling two) and by analyzing heterozygous mice to minimize allele-specific variability for what we anticipated would be quantitatively small differences. These modifications reduced by 4-fold the amount of Vcam1<sup>+</sup> and Vcam1<sup>+</sup> DNA in each individual chromatin preparation but we were still able to assay chromatin association of 4 proteins in each preparation. Allele-specific ChIP/qPCR demonstrated that Pol II and NF-κB p50 at both alleles the Pol II lipopolysaccharide response was absent and NF-κB p65 association was diminished compared with the Vcam1<sup>+</sup> allele (Figure 7A and 7B). Association of NF-κB p50 and p300 were equivalent at both alleles (Figure 7A and 7B). Thus, the Vcam1<sup>+</sup> allele shows diminished lipopolysaccharide-induced association with Pol II and NF-κB p65 consistent with the hypothesis that an altered chromatin state is causally linked to the decreased Vcam1<sup>+</sup> hnRNA, mRNA and protein abundance observed during acute inflammation.

We also determined association of Pol II, NF-κB p65, NF-κB p50, and p300 with 2 regions of the murine E-selectin (Sele) gene that are homologous to regions of the human gene that were previously examined by ChIP in cultured human umbilical vein ECs.<sup>50</sup> Region Sele A includes the NF-κB–binding sites within the cytokine regulatory region of the human promoter, whereas region Sele B covers +65 to +139 nucleotides 3′ from the transcription start site. The results (Online Figure IXE) indicate that Pol II, NF-κB p50, and p300 (but not NF-κB p65) associate with Sele A and Sele B in the basal state, whereas lipopolysaccharide induces association of Pol II and NF-κB p65 at both Sele A and Sele B. Thus, Pol II, NF-κB p65, NF-κB p50, and p300 show similar basal and absence of lipopolysaccharide administration (Figure 7A). Basal Pol II association is consistent with results at ENCODE (Encyclopedia of DNA Elements).<sup>49</sup> However, basal association of only NF-κB p50 was diminished at the Vcam1<sup>+</sup> allele relative to the Vcam1<sup>+</sup> allele. Lipopolysaccharide administered intraperitoneally enhanced Pol II and NF-κB p65 association with Vcam1<sup>+</sup> while NF-κB p50 association was reduced and p300 did not change (Figure 7A). In contrast, at the Vcam1<sup>+</sup> allele the Pol II lipopolysaccharide response was absent and the NF-κB p65 response was diminished compared with the Vcam1<sup>+</sup> allele (Figure 7A and 7B).

*Figure 7. Lipopolysaccharide (LPS) induced chromatin association of transcriptional regulatory factors with Vcam1<sup>+</sup> and Vcam1<sup>+</sup> genomic DNA. Mice were injected with phosphate-buffered saline (PBS) or LPS IP and hearts were analyzed 2 hours later by chromatin immunoprecipitation for binding of Pol II, nuclear factor-κB (NF-κB) p65, NF-κB p50, and p300. Legends show antibodies used for immunoprecipitation. Values are means±SD from 3 independent biological replicate experiments. A and B, Vcam1<sup>+</sup> shows decreased association of Pol II and NF-κB p65 but not NF-κB p50 or p300, *P<0.05. C, Lines indicate paired values from PBS- and LPS-injected mice (the same data are presented in aggregate form in B) analyzed together in 3 independent biological replicate experiments. Diamonds indicate paired male mice. Circles indicate paired female mice. WCE indicates whole-cell extract.
lipopolysaccharide-induced changes in association with both the Vcam1 and Sele loci.

### Discussion

NF-κB activation and inducible Vcam1 expression are central mediators of inflammation. However, specific contributions of the tandem NF-κB sites in the Vcam1 promoter remain unclear. Understanding how VCAM-1 expression is regulated will illuminate physiological and pathological processes and will facilitate the rational design of diagnostic and therapeutic approaches to target acute and chronic inflammation in diseases, including atherosclerosis, rheumatoid arthritis, multiple sclerosis, and organ allografts. Using targeted mutagenesis by Tag and Exchange in ES cells to develop mice bearing an inactivating mutation of the 5′ NF-κB recognition element in an otherwise intact Vcam1 core promoter and genomic locus, we now report the first analysis of NF-κB regulation of the endogenous Vcam1 gene. A 3-nucleotide substitution, without deletions or insertions, was also introduced into the 5′ UTR of the Vcam1 allele to allow differential quantification of mutant and wild-type mRNA and hnRNA. Thus, the transcriptional and translational efficiency of the mutant allele should not be altered allowing Vcam1 and Vcam1 expression and function to be assessed in models of acute inflammation, atherosclerosis, and traumatic vascular injury.

We anticipated that NF-κB–independent expression of Vcam1 in the allantois would rescue Vcam1-deficient placental failure and embryonic lethality and that Vcam1 expression would be normal in mature tissues but would not be induced by inflammatory mediators or in models of chronic inflammatory disease and injury, in which NF-κB–mediated VCAM-1 expression was presumed to play a prominent role. However, although Vcam1 embryos were viable acute inflammation/cytokine injection in adults revealed only a 30% to 60% decrease in Vcam1 mRNA expression compared with Vcam1. Two aspects of this observation are significant. First, maintenance of robust, although diminished expression in the absence of a functional 5′ NF-κB element is unexpected in light of the abundant in vitro experiments, using diverse experimental cell models (as well as our results in Online Figure II), indicating that both tandem functional elements are required for even minimal inducible expression. Several possible mechanisms might explain this discrepancy between in vivo and in vitro results. Disrupting either NF-κB element prevents binding of NF-κB dimers to Vcam1 promoter sequences in cell-free systems. Therefore, mechanisms that operate only in the normal chromosomal context may preserve NF-κB binding to the mutant 5′ recognition element in the intact Vcam1 locus. Alternatively, binding of NF-κB to the single intact 3′ element may be sufficient for significant though reduced inflammatory induction of endogenous expression. NF-κB elements of other promoters can function in this manner. For example, on thrombin (but not TNF-α) stimulation, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc) binds not only to the tandem NF-κB elements in the Vcam1 promoter but also to 1 of the tandem NF-κB elements in (intron 1) of the Icam1 gene. Such NFATc binding to tandem elements, which is required for full thrombin–induced expression, may not be disrupted by mutating a single element as done here. NFATc binding also may play a more important role in nonendothelial expression of VCAM-1 during the later stages of atherosclerosis and in traumatic wire-induced arterial injury, as studied here. Our results are less likely to reflect primarily differences between the in vitro and in vivo properties of cells, rather than effects of episomal versus chromosomal context, given that ChIP analysis of Sele gene activation in vitro and in vivo (Online Figure I) suggests strongly that similar epigenetic interactions at chromosomal loci operate in both settings. However, the extent to which the intracellular milieu can modulate NF-κB–dependent gene activation remains to be determined.

Despite this partial preservation of inducible VCAM-1 expression ovalbumin-immunized Vcam1 mice showed diminished recruitment of VCAM-1–dependent mononuclear and polymorphonuclear leukocytes into the peritoneal cavity 24 hours after ovalbumin-induced inflammation. The polymorphonuclear leukocytes consisted primarily of eosinophils in which, unlike neutrophils, VLA-4 is known to mediate inflammatory recruitment. This establishes that dysfunction of the single 5′ NF-κB element of the Vcam1 promoter is sufficient to alter inflammatory leukocyte trafficking in vivo and suggests that alterations in NF-κB signaling, whether because of genetic, epigenetic, endocrinologic, pharmacologic, or other causes likely contribute to human disease.

NF-κB recognition elements occur in the promoters of many genes that are induced in atherosclerosis and that mediate lesion formation. In addition, the NF-κB system is preconfigured in ECs located specifically at regions of the vasculature that are prone to developing atherosclerosis; endothelial NF-κB expression is elevated at such sites even in the absence of lesions, and atherogenic stimuli lead to 1κB degradation with nuclear translocation of NF-κB dimers in such regions. Furthermore, endothelial expression of microRNA-10a, which inhibits expression of mitogen-activated protein kinase 7 (TAK1 [TGF-beta activating kinase 1]) and β-transducin repeat-containing gene that promote 1κBα degradation, is also reduced in regions predisposed to atherosclerosis. These observations predict that expression of pathophysiologically relevant genes, and the development of atherosclerosis itself, is mediated by NF-κB–dependent transcription. Surprisingly, we show here in cholesterol-fed Ldlr−/− mice that a mutation that completely prevents NF-κB–dependent VCAM-1 expression in vitro and diminishes expression in acute inflammation in vivo has virtually no effect on VCAM-1 expression in atherosclerotic lesions; Vcam1 is expressed throughout the diseased aorta with normal kinetics and at nearly normal levels.

Compared with reduced expression of Vcam1 observed during acute inflammation (discussed above) and early in atherogenesis, equivalent expression of Vcam1 and Vcam1 during advanced atherosclerosis may be related to the fact that VCAM-1 is expressed predominantly by endothelium in early lesions (Online Figure VIII), whereas our previous reports show that expression in advanced lesions is by intim al macrophage and smooth muscle cells located below the endothelium. Collectively, these data imply that the Vcam1
mutation has different effects in ECs versus macrophages and smooth muscle cells.

Consistent with these results in atherosclerosis, expression of the Vcam1\(^{−/−}\) allele was not altered compared with the wild-type allele 4 days after wire-induced mechanical injury of the carotid artery (Figure 6) that denudes the endothelium and induces VCAM-1 expression primarily in smooth muscle cells near the site of injury. VCAM-1 expression in a similar wire-induced injury model (2 weeks after carotid wire injury in mice on a high cholesterol diet) occurs primarily in medially smooth muscle cells with minimal expression in endothelium.58 Although the models differ in important ways, the results reported here in atherosclerosis and wire injury support the hypothesis that the NF-κB recognition elements in the Vcam1 promoter function differently in acutely inflamed endothelium compared with mural cells in chronic atherosclerosis, and these requirements may diverge even further in acute compared with chronic inflammation. If so, then interfering with NF-κB interactions at the genomic level, as done here, might best be viewed as creating qualitatively imbalanced VCAM-1 expression in different cell types and with different kinetics relative to the inflammatory response rather than uniformly upregulating or downregulating expression in all cells. In contrast, in vivo treatment with decoy oligonucleotides, designed to globally inhibit NF-κB binding to recognition elements in responsive genes, decreases VCAM-1 expression and intimal hyperplasia after balloon injury in rats.59 However, the cell type(s) and molecular mechanism(s) responsible for this effect are not clear, in part because of the systemic route of administration used but also because apoptosis is strongly induced and intercellular adhesion molecule-1 expression is decreased in these protected lesions.

Activation of Vcam1 transcription in endothelium during inflammation is accompanied by NF-κB–dependent formation of complex, multimeric super enhancers, and decommissioning of basal complexes at several genomic sites including the Vcam1 promoter region.47 Allele-specific ChIP revealed absent and diminished enhancement of Pol II and NF-κB p65 association, respectively, with the Vcam1\(^{−/−}\) promoter region compared with Vcam1\(^{+/+}\) during acute inflammation. This is consistent with an epigenetic basis for the differences observed in hnRNA, mRNA, and protein expression from the mutant and wild-type alleles in acute inflammation. Lack of enhanced Pol II association with the Vcam1\(^{−/−}\) promoter region after lipopolysaccharide induction despite upregulated (but blunted) mRNA expression probably reflects the fact that this static snap shot ChIP data are relatively insensitive to Pol II processivity, which better reflects the rate of transcription: a topic for future investigation stimulated by these findings. ChIP of the Sele locus also confirmed that analysis of chromatin interactions in cultured ECs30 and murine endothelium in vivo can give comparable results and that ChIP/qPCR can distinguish patterns of chromatin remodeling in response to inflammatory stimuli at different loci in intact tissues in vivo. This supports the conclusion that the differences in Vcam1 expression observed after altering NF-κB recognition sites in vivo and in transient transfection studies may also result at least, in part, from differences in epigenetic regulation in chromosomal compared with episomal DNA.

Taken together, our results suggest that interfering with the 2 tandem NF-κB recognition elements of the Vcam1 promoter may have distinct effects depending on the specific functions of these elements individually, together and in association with other regulatory factors. Given that such factors vary in different cell types, responses to stimuli and disease states such context-specific effects on NF-κB regulation of Vcam1 will determine if and how experimental results obtained in vitro might be extended to patient care settings. Further studies of NF-κB–dependent Vcam1 expression and function in vivo should thus help to determine the potential of such approaches for more effective and specific therapeutic interventions in inflammatory disorders.

Acknowledgments

We acknowledge the Transgenic Facility at Brigham and Women’s Hospital for performing blastocyst-mediated transgenesis, and Haiyan Xiao for maintaining the mouse colony at University Health Network.

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Disclosures

None.

References


A new mouse model was created in which the 5′ NF-κB element of the Vcam1 promoter was mutated by tag and exchange to produce a predicted noninducible allele (Vcam1′ allele). A 3-nucleotide substitution in the 5′ untranslated region enabled simultaneous differential quantification and direct comparison of expression and chromatin configuration of mutant and wild-type alleles.

- Inflammation increased endothelial Vcam1 expression, chromatin binding by RNA poly merase II and NF-κB p65 and function in leukocyte recruitment although all these parameters were diminished in comparison with the wild-type Vcam1 allele.

- Vcam1′ allele induction and function were equivalent to wild-type in neointimal leukocytes and smooth muscle cells during atherosclerosis and after vascular mechanical injury.

- NF-κB signal transduction is critical for the induction of multiple proinflammatory genes. Tandem NF-κB recognition sites conserved in the mouse and human Vcam1 promoters are considered to be essential for inflammatory cytokine-inducible expression. However, little is known about how NF-κB regulates the endogenous Vcam1 gene in vivo. We created mice in which the 5′ NF-κB element in the Vcam1 promoter was selectively inactivated (Vcam1′ allele) and demonstrated lower expression in endothelium in response to systemic inflammatory stimulation and in 2-week atherosclerotic lesions. In contrast, the expression of Vcam1′ and wild-type alleles was comparable in intimal cells of 4- to 12-week atherosclerotic lesions and wire-injured carotid artery. We also gained novel insights about inducible chromatin-associated regulatory proteins present in the normal chromosomal context at the Vcam1 promoter. Our results illustrate why genes with sequence polymorphisms in cis-acting regulatory elements may respond differently to the same inflammatory stimulus. Many genetic determinants of common inflammatory diseases may exert their influence by conferring subtle effects similar to those we report here for the Vcam1′ allele.

**What Is Known?**
- Nuclear factor-κB (NF-κB) regulates induction of gene expression during inflammation by binding to sequence-specific cis-acting DNA elements of many genes.
- When 2 such cis-acting elements occur close together, as in the Vcam1 gene, both are often required for inflammatory cytokine induction of ep iso mal promoter-reporter constructs.
- The importance of these regulatory elements acting alone or together and in the normal chromosomal context in vivo is not known.

**What New Information Does This Article Contribute?**
- A new mouse model was created in which the 5′ NF-κB element of the Vcam1 promoter was mutated by tag and exchange to produce a predicted noninducible allele (Vcam1′ allele). A 3-nucleotide substitution in the 5′ untranslated region enabled simultaneous differential quantification and direct comparison of expression and chromatin configuration of mutant and wild-type alleles.

**Novelty and Significance**


Differential Role of an NF-κB Transcriptional Response Element in Endothelial Versus Intimal Cell VCAM-1 Expression


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Detailed Methods

Production of tag and exchange targeting constructs

In the first (Tag) targeting construct, a 1.2 kb Xmn I/Nhe I fragment of the Vcam1 gene beginning ~100 bp 5' of the transcription start site and ending within exon 2 was substituted with tandem neomycin resistance/herpes simplex virus thymidine kinase (NEO/TK) selection cassettes (the murine phosphoglycerate kinase-1 promoter driving NEO, and a mutant polyoma virus enhancer driving TK) (Figure 1A). The substituted region contained the sequences to be mutated. The NEO/TK selection cassette was flanked by the 1.2 kb and 4.3 kb Vcam1 genomic regions lying immediately 5' and 3', respectively, to the substituted region. DNA sequencing confirmed the fidelity of the ligated sites in this construct.

To generate the “exchange” construct PCR was used to introduce mutations into the 0.8 kb Hinc II/Bgl II fragment of the Vcam1 gene (beginning 5' of the NF-κB cis recognition elements and ending in intron 1), which was then ligated between the 1.0 kb and 4.7 kb genomic regions lying immediately 5' and 3', respectively, to the mutated region (Figure 1A). DNA sequencing confirmed the fidelity of the ligated sites, the promoter region and exon 1 as well as the presence of both mutations in the “exchange” construct.

We avoided recombinase-facilitated mutagenesis in order to obviate residual extraneous alterations (including selection markers and recombinase recognition sequences) that might have unpredictable effects on Vcam1 transcription. For example, it is not clear if the diminished basal VEGF expression in neural tissues of mice bearing a Cre-mediated deletion of the hypoxia response element of the VEGF promoter reflects solely the absence of the hypoxia response element itself or, alternatively, whether the 14 bp Cre recognition site that was also inserted into the VEGF gene might also contribute to the observed phenotype. We also optimized the tag and exchange transfection and selection conditions, which will facilitate using this approach to introduce other subtle mutations at the Vcam1 and other endogenous loci.

The Vcam1 3' NF-κB cis element was not mutated for two reasons: (a) in vitro experiments indicate that mutation of a single NF-κB element is sufficient to entirely abolish the induction of VCAM-1 expression by inflammatory cytokines and (b) to test the functional consequences of minimally disrupting the Vcam1 promoter. A three-nucleotide substitution in the 5' untranslated region is downstream of several critical regulatory elements (the 3' NF-κB element, the TATAA element, an interferon regulatory factor-1 (IRF-1) binding site downstream of the TATAA element, and the Kozak sequence of the translational start site) that were included in previous transient transfection experiments. These features, along with other portions of the Vcam1 gene and its normal genomic context on chromosome 1, remained intact in the Vcam1 allele created here whose transcriptional and translational efficiency should thus not be altered.

Transfection and selection of embryonic stem cells.

J1 embryonic stem cells (provided by Dr. R. Jaenisch, Massachusetts Institute of Technology, Cambridge, MA) were transfected with the Vcam1 “Tag” construct essentially as described previously, followed by simultaneous selection with both G418 (200 μg/ml active) and 5-FC (5-fluorocytosine, 125 μg/ml). Homologous recombination was identified by Southern blotting (Eco RI-digested DNA hybridized with a Vcam1 promoter probe to the targeting constructs) and by PCR and was confirmed by hybridization with a Vcam1 probe 3' to the targeting construct. Additional random integration sites were excluded by Southern blot using a probe from the second Ig-like domain of VCAM-1. A single correctly targeted clone was identified among ~150 G418/5FC-resistant clones. Subsequent transfection of this clone with the “Exchange” construct followed by selection with FIAU (1-[2'-deoxy-2'-fluoro-ß-D-arabinofuranosyl]-5-iodouracil) under standard conditions did not yield any subclones bearing
the desired mutation. Two modifications of the selection conditions led to a successful outcome. The first was prolonged culture with G418 beyond the time of transfection with the "Exchange" construct. This presumably selected against cells undergoing spontaneous loss of heterozygosity to the homozygous wild type Vcam1 genotype (such cells are G418 sensitive and FIAU resistant). The second modification was delaying FIAU addition to allow degradation of HSVTK protein in “tagged” cells that subsequently underwent homologous recombination with the “Exchange” construct to delete HSVTK and introduce the desired mutation (such cells remain FIAU-sensitive until HSVTK enzymatic activity declines sufficiently to prevent FIAU toxicity).

The successful protocol consisted of expanding the single “tagged” clone in G418 alone, transfecting the expanded clone by electroporation with the “Exchange” construct (1 x 10^7 cells, 25 µg DNA) and culturing the resulting cells in ten 10 cm dishes of murine embryo feeder (MEF) cells. G418 remained in the culture medium for the first 24 h after the “Exchange” transfection and was omitted thereafter. FIAU, 0.2 µM) was added after 4 days and maintained for a total of 12 days.

A sequential pooling/subpooling strategy was then used to identify clones bearing the final desired Vcam1^κB mutation. Thirteen days after the “Exchange” transfection each dish contained an average of 65 FIAU-resistant colonies that were trypsinized together and cryopreserved as “pools” (one dish per pool). DNA was also prepared from each pool after expanding without additional MEF. PCR revealed the desired mutation in 6 of these 10 pools. One pool was then plated at 13, 39 and 117 viable cells per well in 12-well dishes. When these colonies were mature the contents of each well were passaged together and PCR was performed in parallel on DNA prepared from a small number of cells. The desired mutation was detected in two of these sub-pools. Positive sub-pools were cultured at 1 x 10^3, 3 x 10^3 and 1 x 10^4 cells per 10 cm dish and 24 individual colonies were isolated from each sub-pool. These clones were passaged to individual wells of 24-well plates and genotyped in parallel by PCR. Three and 17 clones bearing the Vcam1^κB mutation were identified from the two positive sub-pools, respectively. These clones were cryopreserved and their genotypes were confirmed by Southern blot analysis. Two such heterozygous clones from each initial positive sub-pool were injected into C57BL/6J and BALB/cJ blastocysts.

Production of chimeric Vcam1^κB mice and backcrossing into the C57BL/6J strain.

Blastocyst-mediated transgenesis was performed by the Transgenic Facility at Brigham and Women’s Hospital. Male chimeric mice were used to initiate backcross of the mutation for 10 generations to C57BL/6J mice (Jackson Laboratories stock number 000664).

Animals:

All procedures involving animals were approved by and conducted according to the guidelines of the Institutional Animal Care and Use Committee at Brigham and Women's Hospital, or the Animal Care Committee at the University Health Network in accordance with guidelines of the Canadian Council on Animal Care. Mice were fed a standard laboratory chow diet, bred and housed in microisolation cages.

Vcam1^D4D/D4D 13 and Icam1^-^- 14 mice were used in ovalbumin and thioglycollate peritonitis experiments.

Genotyping.

DNA was isolated from tail biopsies, as described by Laird et al 15, and genotyped by PCR. PCR primers corresponded to the Vcam1 promoter (FP: GGAGCTGAAGGTCAGGAAAAG) and the first intron (RP: GTGGAAAGAAAATCCGTCCC). The PCR products were digested with Eco RI and electrophoresed through agarose gels (Supplemental Figure I).
Atherosclerosis experiments.

Vcam1+/-kB mice backcrossed 10 generations onto the C57BL/6J background were bred with Ldlr-/- mice (Jackson Laboratories, stock number 002207) to generate Vcam1+/-kB: Ldlr-/-, Vcam1+/-: Ldlr-/- and Vcam1+/-kB: Ldlr-/- mice. At 10 to 12 weeks of age, these mice were fed a semi-purified cholate-free cholesterol-rich diet (1.25% cholesterol, 40% kcal lipid, Research Diets, Inc., D12108) for 2, 4, 8 or 12 weeks. Age and sex-matched control mice were fed standard chow.

Lipid accumulation in lesions of Vcam1+/-: Ldlr-/- and Vcam1+/-kB: Ldlr-/- mice was quantified by Oil-Red-O (Sigma) staining, as described previously 11.

RNA was obtained from Vcam1+/-kB: Ldlr-/- mice. At 2 weeks of cholesterol-rich diet, RNA was isolated just from intimal lesions in the lesser curvature of the ascending aortic arch. Aortae were pinned en face in ice-cold PBS containing 1 mM aurintricarboxylic acid (Sigma). Tissues were treated with 5U DNase I (Fermentas) and Liberase TM (1:100 in Ca2+/Mg2+-containing PBS, Roche) for 8 min at 37°C. Intimal cells were visualized by overlaying 0.1 μM fluoresbrite polystyrene microspheres (Polysciences) and were scraped gently with a 30G needle prior to harvesting directly into RNA extraction buffer (RNeasy Micro Kit, Qiagen).

At 4, 8 and 12 weeks of cholesterol-rich diet, RNA was isolated with the RNeasy Mini plus Isolation Kit (Qiagen) from the intima, media, and adventitial tissue that remained after dissection and removal of periaortic fat. Segments of the aortic arch, descending thoracic aorta and abdominal aorta were harvested independently from each mouse.

Immunofluorescent staining was performed on formalin-fixed paraffin-embedded longitudinal sections of aortic arches following antigen retrieval. Slides were immersed in citrate buffer (Boster Biologics, Pleasanton, CA) and microwaved for 30 min in a pressure cooker. Sections were permeabilized in 0.1% Triton X-100 for 10 min, quenched with 3% H2O2 for 45 min, blocked for 1 h, stained with primary antibodies: VCAM-1, (EPR5047, Abcam, Cambridge, MA) and Mac-2 (M3/38, BioLegend, San Diego, CA). Primary antibodies were visualized with fluorescein-tyramide amplification (Perkin Elmer, Waltham, MA) and fluorochrome-conjugated secondary antibody.

Laser capture microdissection was performed by the Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, ON) using an Arcturus PixCell IIe system (Life Technologies, Burlington, ON). Aortic roots were harvested from Vcam1+/-kB: Ldlr-/- mice fed a cholesterol-rich diet for 12 weeks, snap frozen in OCT Compound (Sakura Finetek, Torrance, CA). Cryosections were stained with HistoGene LCM Frozen Section Staining Kit (Life Technologies), and subendothelial cells in lesions were identified. Captured tissue was harvested directly into lysis buffer (RNeasy Micro Kit, Qiagen, Valencia, CA). RNA was isolated and sample integrity was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA); RNA Integrity Number (RIN) for all samples was ≥7.0. Reverse transcription and quantitative real-time PCR was performed as described below.

Carotid wire injury:

Homozygous Vcam1+/-kB mice (backcrossed for 10 generations onto the C57BL/6J background) were subjected to right carotid artery wire injury or sham injury, as described previously 16. Mice were anesthetized using ketamine-HCl (100 mg/kg i.p.) xylazine-HCl (10 mg/kg, i.p.), and placed on a warming pad. The bifurcation of the right common carotid artery was isolated via a midline neck incision, and 2 ligatures were placed around the external branch. The distal ligature was tied, and flow through the common carotid artery was temporarily occluded with a vascular clamp. An incision was made in the external carotid artery between the
two ligatures, and a curved 350 µm polished copper wire was introduced into the lumen. The wire was advanced past the primary bifurcation into the common carotid artery, and the vessel systematically injured by simultaneously rotating the curved copper wire while passing along the vessel four times. The wire was removed, and the external carotid artery tied off proximal to the incision with the second ligature. The vascular clamp was removed restoring flow through the common carotid artery. The skin was closed with a single suture, and animals were allowed to recover on a warming pad. Sham injury involved the same procedure except the copper wire was not introduced. Injured (or sham injured) and contralateral (left) common carotid arteries were harvested 4 days after surgery and RNA was isolated.

Ovalbumin (OA) peritonitis.

Mice were immunized by a single i.p. injection of OA (100 µg in 0.1 ml PBS, filter-sterilized). After 14 days, mice were challenged with OA (100 µg i.p.) and a peritoneal lavage was performed 48 h later. Mononuclear and polymorphonuclear leukocytes in the lavage were quantified. Total leukocytes were determined by hemocytometer counts and differential counts were performed on Wright-Giemsa-stained cytospins.

Thioglycollate peritonitis.

Mice were injected i.p. with 1.0 ml of 4% thioglycollate medium and peritoneal lavage was performed and analyzed as above 48 h later.

RNase protection assays (RPA).

Total RNA was isolated using guanidinium thiocyanate and cesium chloride gradients. A 277 nt [32P]-labeled riboprobe was synthesized from a genomic 5' NF-κB mutant VCAM-1 fragment (produced by PCR and cloned into the Bluescript phagemid (Stratagene, San Diego, CA) spanning the transcription initiation site (Figure 1B). Wild type and 5' NF-κB mutant VCAM-1 mRNA protected 80 nt and 163 nt fragments of this probe, respectively (Supplemental Figure IV). Between 1 and 100 µg of total cellular RNA per sample were analyzed by RPA using a commercially available kit (Ambion) essentially as described. A β-actin probe served as loading control. Protected fragments were quantified using a Molecular Dynamics PhosphorImager model 425E and ImageQuant software. Abundance of VCAM-1 mRNA is expressed relative to that of β-actin determined simultaneously by including all probes in a single hybridization reaction. Probe specific activity was adjusted to facilitate this analysis.

Reverse transcription (RT) quantitative PCR (qPCR).

Gene expression was assessed in mouse tissues (heart, lung, aorta and carotid arteries) using RT-qPCR. Total RNA was isolated using the RNeasy Mini plus Isolation Kit (Qiagen). RT into single stranded cDNA was performed using the high capacity random primer reverse-transcription kit (Applied Biosystems or PowerScript, Clontech, Palo Alto, CA). Primers to detect mRNA and hnRNA of wild type and mutant Vcam1 alleles and housekeeping genes (CD31 and HPRT) were designed using the Primer Express software (Applied Biosystems). Primer sequences are in Supplemental Table I.

Real-time PCR reactions to assess mRNA expression were performed using an ABI PRISM ® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) or a Light Cycler®480 (Roche Applied Science). PCR reactions to assess hnRNA expression were performed using a Light Cycler®480. Amplified DNA was detected by SYBR Green (Applied Biosystems or Roche) incorporation. A standard curve was generated for each primer pair using a mixture of normal and inflamed tissues. Dissociation curve analyses were performed to confirm specificity of the SYBR green signals in each experiment, and controls without reverse transcriptase were used when primers targeting hnRNA were used. Quantification was performed by ΔΔCt or standard curve methods (the comparative standard curve method, Sequence Systems Software 2.0, Applied Biosystems or the advanced relative quantification
method, Light Cycler®480 software, Roche).

Chromatin immunoprecipitation

Age and sex-matched pairs of Vcam1\(^{kb}\) mice were injected i.p with 2 µg LPS per gram body weight in PBS (10 µL per gram body weight of 200 µg LPS/ml PBS) or with an equivalent volume of PBS alone. Two hours later mice were euthanized, chromatin was prepared from heart and chromatin immunoprecipitation (ChIP) was performed as previously described except that each sample was prepared from one individual heart (hearts were not pooled or split) and approximately one fifth of each single heart preparation was immunoprecipitated separately with 5 µg purified rabbit IgG (Cell Signaling Technology 2729S) or 5 µg affinity purified rabbit IgG antibody (each from Santa Cruz Biotechnology) recognizing RNA polymerase II (Pol II) (sc-899 N-20), NF-κB p65 (sc-372X C-20), NF-kB p50 (sc-114 X NLS) or histone acetyltransferase p300 (sc-585 X C-20).

qPCR analysis of ChIP DNA:

Testing and validating Vcam1\(^{+}\) and Vcam1\(^{kb}\) allele-specific primers: Several primers terminating within the wild type sequence that is mutated in Vcam1\(^{kb}\) (Supplemental Figure IXA) were tested by qPCR for detection of LPS-induced Pol II association with the Vcam1\(^{+}\) gene. The specificity of each primer pair was screened by BLAST while agarose gel electrophoresis and thermal dissociation curves confirmed amplification of single products of appropriate size and melting temperature. As shown in Supplemental Figure IXB, all tested primer pairs detected enhanced Pol II association after LPS stimulation of wild type mice while primer pair 2F/2R showed the greatest sensitivity. Therefore, primer 2R5'kB, identical to 2R+ except that the 3' terminal nucleotide of 2R5'kB was complementary to the Vcam1\(^{kb}\) mutant allele, was tested along with 2R+ in allele-specific qPCR paired with the common primer 2F. Primer pairs 2F/2R+ and 2F/2R5'kB specifically detected the Vcam1\(^{+}\) and Vcam1\(^{kb}\) alleles, respectively, in homozygous Vcam1\(^{+/+}\), Vcam1\(^{+/kb}\) and Vcam1\(^{kb/kb}\) genomic DNA (Supplemental Figure IXC) and amplified the corresponding alleles with identical sensitivities in heterozygous Vcam1\(^{+/kb}\) genomic DNA (Supplemental Figure IXD). Minimal but detectable amplification of non-complementary alleles (<1% compared to complementary alleles, IXC) likely represents inefficient extension during early PCR cycles from 2R+ and 2R5'kB primers mismatched with non-complementary 3' nucleotides in the Vcam1\(^{kb}\) and Vcam1\(^{+}\) alleles, respectively. Such “off-target” amplification does not compromise the utility of these primer pairs for allele-specific quantification of ChIP DNA from heterozygous Vcam1\(^{+/kb}\) mice.

ChIP and whole cell extract (from non-immunoprecipitated chromatin) DNA were analyzed using SYBR Select Master Mix (Invitrogen) and primer pairs 2F/2R+ and 2F/2RkB in a StepOnePlus real-time quantitative PCR thermal cycler (Applied Biosystems). After 10 min at 95°C, 40 one-step cycles were performed of 15 s at 95°C and 1 min at 60°C. Cycling was followed by melt curve determination. Relative allele abundance in ChIP DNA was determined by the \(\Delta\Delta CT\) method and normalized to input whole cell extract DNA. Binding of each tested protein to the Vcam1\(^{+}\) and Vcam1\(^{kb}\) alleles was determined by comparing the fraction of input DNA recovered in each ChIP DNA preparation. Results are mean ± SD of the values from three independent experiments (biologic replicates) each using one mouse injected with LPS and another receiving PBS alone. Vcam1\(^{kb}\) binding was compared to Vcam1\(^{+}\) binding in the same Vcam1\(^{+/kb}\) heterozygous mouse as an internally normalized control.
### Supplemental Tables and supporting information

#### A) mRNA primer pairs

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>Vcam1⁺</td>
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#### B) hnRNA primer pairs

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<th>Reverse primer</th>
</tr>
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<tr>
<td>Vcam1⁺</td>
<td>GCACAAAGAAGGCTTTGAAGCA</td>
<td>CACCAGAAGCTGTAGATGTGTGA</td>
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<tr>
<td>Vcam1kB</td>
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<td>CACCAGAAGCTGTAGATGTGTGA</td>
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#### C) ChIP primers

<table>
<thead>
<tr>
<th>Gene or Allele</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
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<td>AF 1710</td>
<td>TTTTAACTCAGTGATATTCCC</td>
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<tr>
<td>Sele A</td>
<td>AR 1711</td>
<td>TGAGTCATAGGACATTATATCAAA</td>
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<tr>
<td>Sele B</td>
<td>BF 1712</td>
<td>TACACCTAAGGGATCCAACGCC</td>
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<tr>
<td>Sele B</td>
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<td>2F 1694</td>
<td>GGACTTGGCTGGCTGTCA</td>
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<td>Vcam1kB</td>
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<td>Vcam1</td>
<td>3R 1695</td>
<td>GGCTGCTGGAGTGAATAGA</td>
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Supplemental Table I: Real-time RT-PCR primer sequences.

A. mRNA primer pairs are located in adjacent exons or span exon boundaries. B. hnRNA primer pairs. Vcam1 allele-specific mRNA forward primers are paired with a common reverse intronic
primer. C. ChIP primers. Superscript + and κB indicate allele-specific ChIP primers that amplify only $Vcam1^+$ and $Vcam1^{κB}$ alleles, respectively. Primers without superscript are common to and amplify both alleles.
<table>
<thead>
<tr>
<th>Genotype</th>
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<th>+/κB</th>
<th>κB/κB</th>
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<tr>
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<tr>
<td>Percent</td>
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<td>46</td>
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**Supplemental Table II:** Live born progeny of Vcam1^{+/-κB} heterozygous intercrosses. Mixed 129/C57BL/6 background
Supplemental References


Supplemental Figure I: Genotyping the progeny of chimeric Vcam1^κB mutant mice crossed to wild type mice. PCR primers corresponding to the Vcam1 promoter (FP: GGAGCTGAAGGTCAGGAAAAG) and the first intron (RP: GTGGAAAGAAAATCCGTCCC) were used. The PCR products were digested with Eco RI and electrophoresed through agarose gels. Homozygous wild type progeny (lanes 1, 3, 4, 5 and 7) do not contain an Eco R1 restriction enzyme site in the 481 bp PCR product (arrow), whereas the mutant allele in heterozygous mice (lanes 2 and 6) is digested into 332 and 149 bp fragments (arrowheads).
**Supplemental Figure II:** Expression of Vcam1-lacz and Vcam1κB-lacz promoter-reporter constructs in bovine aortic endothelial cells. Constructs were generated by inserting the lacZ gene into an Xho I restriction enzyme site engineered into the 5′-UTR of wild type and NF-κB mutant mouse Vcam1 genes (Figure 1B). Bovine aortic endothelial cells were transiently transfected by the calcium phosphate precipitation method. Cells were stimulated with buffer or TNFα (100 ng/ml, 4 h), and the expression of β-galactosidase was measured. Data from a representative experiment is shown.
Supplemental Figure III: Specificity of qPCR primers for VCAM-1 mRNA and hnRNA transcribed from wild type (Vcam1\(^{+/+}\)) and NF-κB mutant (Vcam1\(^{\kappa B}\)) alleles. RNA was isolated from hearts 2 h after injection of LPS (4 µg/g) into wild type (+/+) and Vcam1\(^{\kappa B/\kappa B}\) (κB/κB) mice. Vcam1\(^{+/+}\) and Vcam1\(^{\kappa B}\) mRNA and hnRNA levels were normalized to CD31 mRNA or HPRT hnRNA, and are expressed relative to levels detected in wild type or Vcam1\(^{\kappa B/\kappa B}\) hearts, respectively (mean ± SEM, n=3 for +/+ and n=4 for κB/κB; ND, not detected). Primers specific for Vcam1\(^{+/+}\) amplified transcripts only from wild type mice, whereas primers for Vcam1\(^{\kappa B}\) amplified transcripts only from Vcam1\(^{\kappa B/\kappa B}\) mice. The efficiency of real-time PCR amplification using Vcam1\(^{+/+}\) and Vcam1\(^{\kappa B}\) allele primers was comparable.
Supplemental Figure IV: LPS dose-response experiments. Sex-matched heterozygous Vcam1<sup>+/k<sub>B</sub></sup> littermates received an i.p. injection of LPS and RNA was isolated after 2 h. qPCR was performed with primers specific for the wild type and the Vcam1<sup>kB</sup> alleles. (A) Induction of wild type VCAM-1 mRNA in response to increasing doses of LPS (mean ± SEM; n=3; **, p < 0.01; ANOVA/Dunnett). (B,C) Ratio of mutant to wild type Vcam1 allele mRNA (B) and hnRNA (C) expression at different doses of LPS (mean ± SEM; n=3; **, p < 0.01; ANOVA/Dunnett). Note that significant difference was found at all LPS doses and were highest at 0.4 and 4 µg/ml. (D,E) mRNA (D) and hnRNA (E) expression data from 3 independent experiments that were used to calculate the means in A-C. In experiment C, mice weighed approximately 25 g, thus the doses correspond to those used in experiments B and C. Each pair of solid and open bars represents wild type and mutant allele values, respectively, from a single heterozygous mouse.
**Supplemental Figure V:** Expression of Vcam1\(^+\) and Vcam1\(^{\kappa B}\) mutant alleles and ICAM-1 in heart and lung of LPS- or IL-1-stimulated mice analyzed by RPA. Age-matched wild type and Vcam1\(^{\kappa B}\) homozygous mutant mice received single intraperitoneal injections of 100 µg LPS or 120 ng IL-1\(\beta\). RNA was isolated from hearts and lungs 2 h later and analyzed by RPA (Supplemental Methods). Expression (mean ± SD of triplicate determinations of Vcam1\(^+\) and Vcam1\(^{\kappa B}\) mutant allele mRNA and of ICAM-1 mRNA, normalized to actin mRNA in each individual animal and then normalized to the value observed in unstimulated wild type mice, is shown. Values for IL-1\(\beta\) are singlecate determinations. Statistical comparison of Vcam1\(^+\), Vcam1\(^{\kappa B}\) mutant and ICAM-1 mRNA: *; \(p = 4.7 \times 10^{-7}\); †; \(p = 0.034\). Other value pairs are not statistically significantly different (\(p > 0.05\)).
Supplemental Figure VI: VCAM-1 but not ICAM-1 mediates mononuclear leukocyte recruitment in response to ovalbumin challenge. Data are presented in graphical (A) and table (B) formats. Leukocytes (x10^6) were recovered from peritoneal cavities of immunized mice that were challenged with ovalbumin, as was described in the methods (mean ± SD, n = number of mice). The number of lymphocytes and monocytes was significantly reduced in Vcam1^{D4D/D4D} mice compared to wild type littermates. In contrast, leukocyte infiltrates were comparable in Icam1^{-/-} and Icam1^{+/+} littermates. Asterisks indicate significant differences between groups (p < 0.05). When 3 groups were compared, significant differences were found between Vcam1^{+/+} vs. Vcam1^{D4D/D4D}, Vcam1^{+/+} vs. Vcam1^{D4D/D4D} and Vcam1^{+/+} vs. Vcam1^{D4D/D4D} groups in the Icam1^{-/-} background. Mast cells ranged from 0.04 to 0.2 x 10^6, and significant differences were not found...
between groups. Mφ, macrophages; Mono, monocytes; Lymph, lymphocytes; Neut, neutrophils; Eos, eosinophils.
Supplemental Figure VII: Neither VCAM-1 nor ICAM-1 deficiency influences mononuclear leukocyte recruitment in thioglycollate peritonitis. The same data is presented in graphical (A) and table (B) format. Intraperitoneal injection of thioglycollate induces a robust acute inflammatory response in the first 24 h followed by a progressive accumulation of mononuclear leukocytes. Leukocytes recovered from peritoneal cavities of mice 48 h after thioglycollate injection (mean ± SD, n = number of mice) were elevated 4- to 5-fold above control levels and consisted of monocyte/macrophages and to a lesser extent granulocytes (neutrophils and eosinophils). Inflammation was unaffected by deficiency of either VCAM-1 or ICAM-1. In contrast, monocyte/macrophages were diminished by 70% in mice with combined VCAM-1 and ICAM-1 deficiency, but lymphocytes and granulocytes were comparable. Asterisks indicate significant differences between groups (p < 0.01). Mast cells ranged from 0 to 0.3%, and significant differences in mast cells were not found between groups. Mφ, macrophages; Mono, monocytes; Lymph, lymphocytes; Neut, neutrophils; Eos, eosinophils.
Supplemental Figure VIII: VCAM-1 expression in atherosclerotic lesions. (A) Longitudinal formalin-fixed paraffin-embedded sections of 2-week lesions in the ascending aortic arch of \textit{Ldlr}/\textsuperscript{-} mice were stained for VCAM-1 (green), Mac2 (red) and DNA (blue). Only VCAM-1 and DNA staining is shown in the upper panel. Note that VCAM-1 is expressed by endothelial cells overlying early lesions. Mac2-positive foam cells in early lesions did not express VCAM-1. (B) Laser capture microdissection of intimal cells below the endothelium from 12-week atherosclerotic lesions in the aortic root of \textit{Ldlr}/\textsuperscript{-} mice heterozygous for mutant and wild type \textit{Vcam1} alleles. Since endothelium was not microdissected, qPCR detected very low expression of endothelial-specific genes VE-cadherin and CD31 relative to CD68, a myeloid cell marker (upper panel). In contrast, the expression of the wild type \textit{Vcam1} allele was comparable to CD68. The expression levels of mutant and wild type \textit{Vcam1} alleles were similar (lower panel, mean ± SEM; n = 3; ***, p < 0.001).
Supplemental Figure IX: Testing and validating Vcam1* and Vcam1κB allele-specific primers:

(A) Seven tested wild type amplimers using the indicated primers in Supplementary Table IC are shown below the Vcam1 genomic locus. Arrowheads represent the 3' termini of wild type-specific primers that each overlap by one base pair with the position corresponding to the mutant sequences in either the 5' NF-κB site (5' NF-κB mut) or the 5' untranslated region (5'UT mut). Each amplimer that uses one or more arrowed primers should therefore be produced only from the wild type allele but not from the 5'κB mutant allele. Amplimer 2F/2R+ best detected Pol II association with wild type Vcam1 (see B below) and is thereby highlighted in A by the thicker black line. Based on this evaluation wild type amplimer 2F/2R+ and the corresponding mutant amplimer 2F/2R5'κB (produced by substituting mutant primer 2R5'κB from Supplementary Table 1C for wild type primer 2R+, and which should be produced only from the Vcam1κB allele) were chosen for further analysis and for allele-specific detection in ChIP. Ticks represent 100 bp intervals. Dropped arrows indicate transcriptional and translational start sites. (B) Pol II
association after LPS stimulation of wild type mice detected by the primer pairs in A. Values are the ratio of whole cell extract (WCE) DNA recovered by Pol II ChIP from hearts of mice receiving LPS in PBS compared to mice receiving PBS alone. (C) Allele-specific DNA amplification using primer pairs 2F/2R+ and 2F/2R5′kB (see Supplementary Table 1C). (D) Vcam1⁺ and Vcam1⁺kB primer pairs show identical allele-specific amplification efficiency of genomic DNA from Vcam1⁺/kB heterozygous mice. (E) ChIP analysis of Sele from hearts of LPS-stimulated and control Vcam1⁺/kB mice. Sele shows LPS-induced association of Pol II and NF-κB p65 at both the cytokine regulatory region (Sele A) and at a region 3′ of the transcription start site (Sele B). Sele A and Sele B are homologous to regions of human Sele previously analyzed by ChIP in HUVEC 23. The abscissa in E shows antibodies used for immunoprecipitation. Values are mean +/- range from two independent biological replicate experiments.