Ezrin-Radixin-Moesin-binding Phosphoprotein 50 (EBP50) and Nuclear Factor-κB (NF-κB)

A FEED-FORWARD LOOP FOR SYSTEMIC AND VASCULAR INFLAMMATION*

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Background: EBP50 is a scaffolding protein that is critical during vascular remodeling.

Results: EBP50 expression is induced by inflammatory stimuli and potentiates NF-κB activation and inflammation.

Conclusion: EBP50 and NF-κB participate in a feed-forward loop leading to increased macrophage activation and enhanced response of vascular cells to inflammation.

Significance: This work reveals a novel mechanism regulating systemic and vascular inflammation.

The interaction between vascular cells and macrophages is critical during vascular remodeling. Here we report that the scaffolding protein, ezrin-binding phosphoprotein 50 (EBP50), is a central regulator of macrophage and vascular smooth muscle cells (VSMC) function. EBP50 is up-regulated in intimal VSMC following endoluminal injury and promotes neointima formation. However, the mechanisms underlying these effects are not fully understood. Because of the fundamental role that inflammation plays in vascular diseases, we hypothesized that EBP50 mediates macrophage activation and the response of vessels to inflammation. Indeed, EBP50 expression increased in primary macrophages and VSMC, and in the aorta of mice, upon treatment with LPS or TNFα. This increase was nuclear factor-κB (NF-κB)-dependent. Conversely, activation of NF-κB was impaired in EBP50-null VSMC and macrophages. We found that inflammatory stimuli promote the formation of an EBP50-PKCζ complex at the cell membrane that induces NF-κB signaling. Macrophage activation and vascular inflammation after acute LPS treatment were reduced in EBP50-null cells and mice as compared with WT. Furthermore, macrophage recruitment to vascular lesions was significantly reduced in EBP50 knock-out mice. Thus, EBP50 and NF-κB participate in a feed-forward loop leading to increased macrophage activation and enhanced response of vascular cells to inflammation.

Inflammation is a necessary biological response to injury and infections. However, overactivation and chronic inflammatory status can lead to diseases such as cancer, asthma, rheumatoid arthritis, and cardiovascular disease. Over the past two decades, the critical role of inflammation in the etiology of neointimal hyperplasia and atherosclerosis has emerged (1–5). Compelling evidence correlates risk factors for vascular disease (such as dyslipidemia, obesity, hypertension) to inflammation (3, 6, 7). Moreover, a strong link between inflammation and the progression and adverse prognosis of atherosclerosis has been documented in humans (8, 9). Vascular inflammation is a major cause of the increased growth and migration of VSMC (5, 10) and of the expression of adhesion molecules (such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)) that are critical for the further recruitment of inflammatory cells to the lesion site (11). The local production of cytokines and growth factors stimulates phenotypic changes in VSMC, leading to increased proliferation, motility, and matrix production ultimately resulting in plaque formation and neointimal hyperplasia.

Many of the genes implicated in vascular dysfunction are regulated by the transcription factor NF-κB (12, 13). NF-κB is rapidly induced by the cytokines IL-1β and TNFα and infectious agents such as LPS. NF-κB is sequestered in the cytoplasm under basal conditions by IkBα (14). Activation of the IkB kinase (IKK) complex results in the phosphorylation and subsequent degradation of IkBα (15), allowing translocation of NF-κB into the nucleus. NF-κB activity is normally low in vessels but is rapidly activated during vascular injury. Degradation of IkBα and increased NF-κB-dependent gene expression was observed after balloon injury (16). Similarly, administration of an IkBα adenovirus during angioplasty decreased ICAM-1 and monocyte chemotactic protein 1 (MCP-1) expression as well as macrophage recruitment and lumen narrowing (17).

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The abbreviations used are: VSMC, vascular smooth muscle cells; EBP50, ezrin-binding phosphoprotein 50; NF-κB, nuclear factor-κB; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; IKK, IkB kinase; MCP-1, monocyte chemotactic protein 1; PDZ, PSD-95, Discs-large, and ZO1; TIRF, total internal reflection fluorescence; TLR4, toll-like receptor 4; EBD, ezrin-binding domain; iNOS, inducible nitric-oxide synthase; HUVEC, human umbilical vein endothelial cells.
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The physical and functional interaction between vascular cells and macrophages is therefore critical during vascular remodeling, and the identification of mediators of this interaction is significant for understanding the molecular mechanisms leading to vascular disease. Here we report that the PSD-95, Discs-large, and ZO1 (PDZ)-scaffolding protein EBP50 is a central mediator of this interaction.

EBP50, also known as Na\(^+\)/H\(^+\) exchange regulatory factor (NHERF1), is a scaffolding protein that assembles signal transduction complexes through its two PDZ domains (18, 19). This adaptor protein also contains an ezrin-binding domain (EBD) that tethers binding partners to the cytoskeleton. In healthy vessels, EBP50 is predominantly expressed by endothelial cells and, at lower levels, by VSMC. EBP50 is significantly up-regulated in the neointima formed after endoluminal injury (20). EBP50 increases VSMC proliferation and motility, and consequently neointima formation following wire injury is greatly reduced in EBP50\(^{-/-}\) mice (20–22). However, the molecular mediators that increase EBP50 expression in injured vessels are not known. Similarly, the effects of EBP50 on inflammation and on the response of vascular cells to inflammatory stimuli have not been established. In this study, we used a combination of in vitro and in vivo approaches to determine the mechanisms controlling EBP50 expression and the role of EBP50 on NF-\(\kappa\)B signaling and inflammation. Our observations indicate that EBP50 and NF-\(\kappa\)B participate in a feed-forward loop propagating macrophage activation and enhancing vascular inflammation.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—The N-terminal FLAG-tagged EBP50 mutants were constructed as described previously (22). The PKC\(\zeta\)-EESA mutant construct was made from wild-type PKC\(\zeta\) (a generous gift from Peter Parker, King’s College London) by using the QuikChange site-directed mutagenesis kit from Stratagene. Mutagenic primers were designed based on rat PKC\(\zeta\) sequence (5’-GTCCTGAGAGTCCGCGTGACTCTAGAG-3’). N-terminally CFP-tagged PKC\(\zeta\) was constructed by inserting WT PKC\(\zeta\) into a pcDNA3-CFP vector (Addgene plasmid 13030, a generous gift from Doug Golenbock). DNA sequences were confirmed by sequence analysis (GENEWIZ).

Experimental Animals and Surgeries—Animal surgeries were performed in 10-week-old WT C57BL/6 mice and EBP50\(^{-/-}\) littersmates. Mice were anesthetized using Ketamine (100 mg/kg) and Xylazine (5 mg/kg) intramuscularly. A 0.015-inch diameter fixed core wire guide (Cook Medical Inc.) was inserted into the left femoral artery and passed within the artery three times. The right femoral artery was used as uninjured control artery. Femoral arteries were harvested 1 week after surgery.

For the LPS studies, mice were injected with 10 mg/kg of LPS intraperitoneally for the indicated times before sacrifice. Blood was collected by cardiac puncture, and the serum was used in a TNF\(\alpha\) ELISA (R&D Systems) according to the manufacturer’s instructions. The aorta and femoral arteries were also harvested and analyzed as described later. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Cell Culture, Treatments, and Transfections—To isolate peritoneal macrophages, mice were sacrificed by CO\(_2\) inhalation and cervical dislocation, and warm RPMI medium containing 10% fetal bovine serum (FBS) was injected into the abdominal cavity. After gentle shaking for 1–2 min, the lavage was collected, and cells (3 \times 10\(^6\)) were plated in 6-well plates and incubated at 37 °C for 2 h. Cultures were vigorously washed with cold PBS to remove nonadherent cells. Macrophages were incubated overnight in 5% CO\(_2\) at 37 °C before treatment to allow for quiescence. Primary VSMC were isolated from abdominal aortic explants and grown in DMEM containing 10% FBS in 5% CO\(_2\) as described previously (22). All experiments were performed with cells between passages 5 and 15. Monolayers of clonal mouse RAW 264.7 macrophages (ATCC) were grown in DMEM containing 10% FBS. Human umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 medium containing 10% FBS and EGM-2 SingleQuot kits (Clonetics).

p65-FLAG (Addgene plasmid 20012, a generous gift from Dr. Stephen Smale) (23) and I\(\kappa\)B\(\alpha\) S32A/S44A (a generous gift from Dr. Lawrence Kane, University of Pittsburgh School of Medicine) were transfected in RAW 264.7 cells using HP X-tremeGENE (Roche Applied Science) according to the manufacturer’s instructions and used for experiments 1–2 days later. Various constructs including pcDNA3.1, p65-FLAG, PKC\(\zeta\), PKC\(\zeta\)-EESA, FLAG-EBP50, FLAG-EBP50-S1S2, and FLAG-EBP50-\(\Delta\)EBD were introduced into VSMC by electroporation using an AMAXA electroporator and the basic Nucleofector kit for primary smooth muscle cells (Lonza) as described previously (22). For expression of different PKC\(\zeta\) constructs, VSMC were infected with adenoviruses encoding LacZ, WT PKC\(\zeta\), myristoylated PKC\(\zeta\) (containing the NH\(_2\)-terminal e-Src myristoylation signal (24)), or kinase-dead PKC\(\zeta\) (K281W), all generous gifts from Dr. Adolfo García-Ocaña (Mount Sinai Medical Center). Cells were incubated with adenovirus in serum-free medium for 1 h and incubated with 10% FBS-supplemented medium overnight. Experiments were performed at 48 h after infection. CHO cells were cultured in Ham’s F-12 medium supplemented with 10% FBS and transfected using X-tremeGENE HP (Roche Applied Science) according to the manufacturer’s instructions.

Small interfering RNA (siRNA) for human EBP50 knockdown was generated by Thermo Scientific as follows: 5’-GGAGAACA-GUGCUGGAGCCCUU-3’ (sense) and 5’-GGCGUCAGCGU-GUUCUCCUU-3’ (antisense). Accell nontargeting siRNA (Thermo Scientific) was used as control siRNA. HUVEC were transfected with siRNA using RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer’s instructions and used for experiments 2–3 days later.

VSMC and macrophages were treated with 1 \(\mu\)g/ml and 100 ng/ml LPS (Sigma, catalogue number L4516, source Escherichia coli 0127:B8), respectively. Recombinant mouse TNF\(\alpha\) and IL-4 (R&D Systems) were used at a concentration of 10 and 20 ng/ml, respectively, for all cell treatments unless otherwise indicated. Cells were preincubated with inhibitors as indicated for 1 h, including 60 \(\mu\)M IKK inhibitor II (Calbiochem) and 25 \(\mu\)M PKC\(\zeta\) pseudosubstrate (Calbiochem). VSMC and HUVEC
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were serum-starved overnight with 0.1% FBS in DMEM and EBM-2, respectively, prior to experiments.

**Immunoblot Analysis**—Cells were solubilized in ice-cold lysis buffer (4 M urea, 62.5 mM Tris-HCl, 2% SDS, 1 mM EDTA) containing a protease inhibitor mixture. Proteins were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Blots were incubated with antibodies (1:500 dilution for all except p65, FLAG (both 1:1000), and actin (1:2000)) to EBP50, VACAM-1, IκBα, PKCζ (Santa Cruz Biotechnology), phospho-IKKα/β (Ser-176/180), IKKβ, phospho-IκBα (Ser-32), phospho-p65 (Ser-536) (Cell Signaling Technology), p65, IL-1β (Abcam), iNOS (BD Biosciences), β-actin, and FLAG (Sigma) with secondary mouse or rabbit antibodies (Cell Signaling Technology, 1:2000 dilution).

**Real-time RT-PCR**—RNA was isolated from cells and tissue using the RNeasy mini kit (Qiagen), and cDNA was generated using Im-Prom II reverse transcription system (Promega). Mouse primers to EBP50, VCA M-1, ICAM-1, IL-10, and GAPDH and human primers to VCAM-1 and ICAM-1 were designed using Primer 3 software and synthesized by Invitrogen. The specific primers were as follows: EBP50, forward 5'-GGTGCTGCAAGTCAATGAGA-3', reverse 5'-ATGTAGGGTGGTGACTGCACTC-3'; IKKα, forward 5'-GCATGAGGTCCAC-3', reverse 5'-ACTGTGGGGTTCAACCTCTG-3'; phospho-IKKα/β, forward 5'-GATTGATCTG-3', reverse 5'-GAAACTTG-3'; GATA, forward 5'-GTTC-3', reverse 5'-GTCCACTCTCGAGCTCATTCCATC-3'; IκBα, forward 5'-GGTGTGCTGCAAGTCAATGAGA-3', reverse 5'-GGTGCTGCAAGTCAATGAGA-3'; IKKβ, forward 5'-GGTGCTGCAAGTCAATGAGA-3', reverse 5'-GGTGCTGCAAGTCAATGAGA-3'. PCR reactions were run using the Power SYBR Green (Applied Biosystems) reagent. The amplification program was as follows: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C (or 55 °C for ICAM-1), and 60 s at 72 °C. A melting curve was run for 60 s at 60 °C and 15 s at 95 °C. TaqMan primers to murine TNFα, IL-1β, IL-6, MCP-1, iNOS, IL-10, arginase 1 (Arg1), mannose receptor 1 (Mrcl), and actin were purchased from Applied Biosystems. The amplification program was as follows: 20 s at 90 °C, 40 cycles of 1 s at 90 °C and 20 s at 60 °C.

**Total Internal Reflection Fluorescence (TIRF) Microscopy**—CHO cells expressing CFP-PKCζ with or without YFP-EBP50 were analyzed by TIRF at room temperature. After a 5-min stabilization, 10 ng/ml TNFα was added, and images were collected every 20 s for 20 min. YFP-EBP50 was used to determine which cells were positive or negative for EBP50. The translocation of CFP-PKCζ to the cell membrane was calculated as a slope = ΔCFP fluorescence/time after TNFα stimulation.

**Immunofluorescence**—Femoral arteries were fixed with 4% paraformaldehyde and embedded in OCT Tissue-Tek (Sakura Finetek). Sections (10 μm) were incubated in boiled Tris-EDTA buffer (10 mM Tris base, 5 μM EDTA, 0.05% Tween 20) for 10 min, blocked in 4% normal goat serum, 1% BSA, 0.5% Triton X-100 for 20 min at room temperature, and incubated with antibodies to CD68 (AbD Serotec, 1:100 dilution), α-smooth muscle actin (FITC-conjugated, Sigma, 1:1000), VCA M-1 (Santa Cruz Biotechnology, 1:100), or ICAM-1 (Santa Cruz Biotechnology, 1:100). As a secondary antibody, anti-rabbit Alexa Fluor 546- or Alexa Fluor 488-conjugated IgG (Invitrogen, 1:250) was used before DAPI staining. Fluorescence intensities were measured with ImageJ software (National Institutes of Health).

**RESULTS**

**Inflammatory Cytokines Increase EBP50 Expression via NF-κB**—EBP50 expression increases in VSMC after endoluminal arterial injury (20, 21), but the mechanisms for this up-regulation are unknown. In the first series of experiments, we tested whether inflammatory stimuli mediate EBP50 expression in VSMC and macrophages. Stimulation of primary peritoneal macrophages and VSMC with LPS for 16 h resulted in a significant increase in EBP50 expression (Fig. 1, A and B). Additional experiments in mice treated with LPS confirmed that inflammatory stimuli increase EBP50 transcripts in the aorta (Fig. 1C). Similar to LPS, EBP50 mRNA expression in VSMC and RAW 264.7 increased with TNFα (Fig. 1, D and E), indicating that the effect was not receptor-specific.

LPS signaling and TNFα signaling converge on the activation of NF-κB, suggesting that this transcription factor mediates EBP50 expression in inflammatory conditions. We tested this hypothesis by using the irreversible IKK inhibitor II (wedelolactone) and a dominant negative form of IκBα (S32A/S36A) that is unable to be phosphorylated and prevents NF-κB activation. We found that both IKK inhibitor II and IκBα (S32A/S36A) abrogated LPS-induced EBP50 expression in peritoneal macrophages (Fig. 1F) and RAW 264.7 cells (Fig. 1G). Conversely, overexpression of the p65 subunit of NF-κB, which overwhelms the basal inhibitory machinery and mimics activation, resulted in a 2-fold increase in EBP50 expression (Fig. 1H). Comparable results were obtained in primary VSMC (Fig. 1I). These studies show that NF-κB regulates the expression of EBP50 in macrophages and VSMC under inflammatory conditions.

**EBP50 Promotes NF-κB Activation**—Because of the critical role that inflammation plays in vascular disease and the importance of NF-κB in the transduction of inflammatory signals, we hypothesized that EBP50 regulates NF-κB signaling. Indeed, LPS-induced activation of IKKβ was decreased in EBP50-/- VSMC as compared with WT VSMC (Fig. 2A). EBP50-null cells also exhibited decreased IκBα phosphorylation and degradation and p65 phosphorylation at serine 536 (Fig. 2B). Importantly, LPS stimulated phosphorylation of p38 and c-Jun in both WT and EBP50-/- VSMC (Fig. 2C), indicating that the effect of EBP50 on NF-κB signaling is specific and not related to defects in Toll-like receptor 4 (TLR4) signaling.

To further verify the role of EBP50 in NF-κB activation, we performed a rescue experiment using EBP50-/- VSMC. Intro-
duction of EBP50 into these cells restored LPS-induced IKK phosphorylation (Fig. 2D). EBP50 contains two PDZ domains, which are important for scaffolding binding partners, and a C-terminal EBD that interacts with cytoskeletal components. To identify the domains of EBP50 that are necessary for NF-κB activation, we utilized EBP50 constructs containing inactivating mutations in both PDZ domains (S1S2) or lacking the EBD domain (EBD). Expression of either mutant was not sufficient to rescue IKK activation in EBP50+/− VSMC (Fig. 2D).

EBP50 Interacts with PKC and Regulates NF-κB Activation—The previous experiments show that the effect of EBP50 occurs between TLR4 and the IKK complex. Examination of potential PDZ ligands in the C termini of TLR4-mediated signaling components suggested PKCζ as a potential target for EBP50. Indeed, PKCζ contains a typical type III consensus motif for PDZ binding (-EESV) and participates in NF-κB activation (25–27). We determined the interaction between PKCζ and EBP50 in CHO cells that do not constitutively express EBP50. As shown in the co-immunoprecipitation experiment in Fig. 3A, TNFα promoted the association between PKCζ and EBP50. This was dependent on the PDZ-binding motif in PKCζ because mutation of the C-terminal valine to alanine abrogated the interaction with EBP50 (Fig. 3A).
We next determined the role of the interaction between EBP50 and PKCζ on NF-κB activation. Consistent with previous studies (27–29), adenoviral expression of a kinase-dead PKCζ or a PKCζ pseudosubstrate inhibitor abrogated LPS-induced IKK phosphorylation in WT VSMC (Fig. 3, B and C). Conversely, adenoviral expression of a membrane-targeted myristoylated PKCζ (30), but not of WT PKCζ, was sufficient to restore LPS-induced IKK phosphorylation in EBP50−/− VSMC (Fig. 3D). These results suggested that EBP50 regulates membrane localization of PKCζ in response to inflammatory stimuli. We therefore recorded PKCζ translocation by TIRF microscopy. TNFα stimulated CFP-PKCζ translocation only in CHO cells expressing EBP50 (Fig. 3E). This translocation began promptly after TNFα application and continued for at least 20 min. The slope of the membrane-delimited CFP fluorescence over time after TNFα stimulation was significantly greater in EBP50-positive cells than in naive CHO cells (0.57 ± 0.14 versus 0.14 ± 0.13 fluorescence × min−1 for EBP50-positive and -negative cells, respectively; p = 0.031, n = 10) (Fig. 3F). Collectively, these experiments demonstrate that EBP50 potentiates NF-κB activity in response to inflammatory stimuli by a mechanism involving the formation of an EBP50-PKCζ complex at the cell membrane. Interestingly, the PKCζ-EESA mutant that does not interact with EBP50 significantly inhibited LPS-induced IKKβ phosphorylation (Fig. 3G), suggesting that this mutant functions as a partial dominant negative PKCζ for NF-κB activation.

**EBP50 Increases Macrophage Activation**—Macrophage activity and the secretion of cytokines are important factors for the induction of proliferation, migration, and adhesion molecule expression in VSMC during vascular remodeling (4). Many of these cytokines are regulated by NF-κB, and because we have demonstrated the importance of EBP50 in the activation of this pathway, we next sought to determine the effect of EBP50 on downstream gene expression. To determine the role of EBP50 on the classical M1 activation, we treated peritoneal macrophages isolated from WT and EBP50−/− mice with LPS for 3 h and assessed expression of pro-inflammatory mediators by quantitative RT-PCR. We found that the induction of IL-1β, iNOS, and TNFα was significantly decreased in EBP50−/− macrophages as compared with WT (Fig. 4A). In contrast, we observed no differences for IL-6 and IL-10. We then assessed
macrophage activation in WT and EBP50−/− mice treated with 10 mg/kg of LPS for 2 or 16 h. Serum TNFα concentrations and the expression of IL-1β in macrophages were both significantly decreased in EBP50−/− as compared with WT mice (Fig. 4, B and C). To determine whether EBP50 regulates the alternative M2 polarization, peritoneal macrophages from WT and EBP50−/− mice were treated with IL-4 for 3 h. The induction of arginase1 (Arg1) and mannose receptor 1 was determined by quantitative RT-PCR. As shown in Fig. 4D, we detected no differences in these markers, indicating that EBP50 does not affect M2 polarization of macrophages. These experiments show that EBP50 increases macrophage activation and the production of inflammatory cytokines both in vitro and in vivo.

**EBP50 Increases Inflammatory Responses in VSMC**—The expression of adhesion molecules by vascular cells in response to inflammation is critical for the homing of macrophages to lesion sites (11, 31). We therefore tested whether EBP50 regulates the response of VSMC to inflammatory stimuli. We found that LPS-induced expression of the adhesion molecules ICAM-1 and VCAM-1, and of iNOS, was significantly reduced in EBP50−/− VSMC as compared with WT cells (Fig. 5). In contrast, no differences in the expression of VCAM-1 (Fig. 5A), determined by immunoblotting, was also decreased in EBP50−/− VSMC treated with TNFα for 16 h.

**FIGURE 3.** EBP50 interacts with PKCζ to activate NF-κB. A, CHO cells were transfected with vector (pcDNA3.1), WT PKCζ, or PKCζ-EESA mutant in conjunction with or without FLAG-EBP50 as indicated and treated with TNFα for 2 min. Cell lysates were immunoprecipitated with anti-FLAG antibody. The immunoprecipitate (IP) and lysates (Input) were immunoblotted with anti-PKCζ antibody. The immunoprecipitate (IP) and lysates (Input) were immunoblotted with anti-PKCζ antibody, as indicated. B, WT VSMC were infected with LacZ control or kinase-dead (KD) PKCζ (K281W) adenoviruses as indicated for 72 h before incubation with LPS for 15 min. Lysates were immunoblotted as shown. p, phosphorylated. C, WT VSMC were pretreated with 25 μM PKCζ pseudosubstrate (c-PS) as indicated for 1 h before 15 min of LPS treatment. Lysates were immunoblotted as shown. D, EBP50−/− VSMC were infected with LacZ control, WT PKCζ, or myristoylated (Myr) PKCζ adenoviruses for 72 h before incubation with LPS for 0, 5, or 15 min as shown. Lysates were immunoblotted as shown. E, time-resolved changes in CFP fluorescence were measured using TIRF in single CHO cells expressing CFP-PKCζ with or without YFP-EBP50. Shown are the changes in membrane-delimited CFP fluorescence over 20 min after TNFα treatment. Error bars denote S.E. (n = 10). F, data were quantified as the slope of the CFP fluorescence over time during the first 5 min of TNFα treatment and are expressed as the mean ± S.E. (*, p < 0.05, n = 10). G, WT VSMC were electroporated with vector, WT PKCζ, or PKCζ-EESA as indicated. After 24 h, cells were treated with or without LPS for 15 min, lysed, and immunoblotted as indicated. Results are quantified as the mean ± S.E. (*, p < 0.05, n = 3).
Because endothelial cell activation is important for macrophage recruitment and infiltration in various vascular pathologies, we performed similar studies in HUVEC. The expression of EBP50 in these cells was efficiently inhibited by a designed siRNA specific for human EBP50 (Fig. 5C). Similar to VSMC, we found that TNFα-induced expression of ICAM-1, VCAM-1, and iNOS was significantly reduced in siEBP50-treated HUVEC as compared with control cells (Fig. 5D). Thus, EBP50 promotes the response of VSMC and endothelial cells to inflammatory stimuli.

**EBP50 Increases Vascular Inflammation**—To determine the role for EBP50 on vascular inflammation in vivo, we used two distinct experimental systems: an acute LPS-induced inflammatory model and an arterial injury model. In the first setting, WT and EBP50<sup>−/−</sup> mice were injected with 10 mg/kg of LPS for 16 h before excision of the femoral arteries. VCAM-1 and ICAM-1 expression was low in control (PBS-injected) femoral arteries (data not shown). In WT mice, LPS induced robust expression of VCAM-1 in both endothelial and VSM cells, which was reduced by ~60% in EBP50<sup>−/−</sup> mice (Fig. 6A). LPS-induced ICAM-1 expression occurred predominantly in the endothelium and was also significantly reduced in EBP50<sup>−/−</sup> mice (Fig. 6A).

To determine whether EBP50 contributes to macrophage homing to sites of vascular injury, we performed endoluminal denudation in the femoral arteries of 10-week-old WT and EBP50<sup>−/−</sup> male mice. One week after injury, macrophages in the lesion site were detected by CD68 staining. We chose this marker because its expression, determined by quantitative RT-PCR, is equivalent in primary WT and EBP50<sup>−/−</sup> macrophages (data not shown). As shown in Fig. 6B, abundant macrophages populated the intimal region of the injured vessel in WT mice. In contrast, we detected significantly fewer macrophages at the lesion site in EBP50<sup>−/−</sup> mice.

The expression of EBP50 in the injured vessels was determined by immunofluorescence (Fig. 6C). Consistent with our previous observations (20, 21), the expression of EBP50 in intimal α-smooth muscle actin-positive cells and in the adventitial fibroblasts was higher than in medial VSMC. Importantly, CD68-positive macrophages in the neointima expressed high levels of EBP50.

Collectively, these experiments show that EBP50 increases the expression of adhesion molecules under inflammatory conditions and promotes the homing of macrophages to injured vessels.

**DISCUSSION**

In this study, we show the reciprocal relationship between EBP50 and NF-κB, whereby NF-κB increases EBP50 expression under inflammatory conditions and EBP50 propagates NF-κB signaling. Consequently, EBP50 potentiates systemic inflammation and promotes the expression of adhesion molecules and macrophage recruitment at sites of vascular injury. A schematic representation of the action of EBP50 under inflammatory conditions is shown in Fig. 7.

The up-regulation of EBP50 has been reported in multiple disease states including hepatocellular carcinomas, cholangiopathies, glioblastoma, breast cancer, psoriasis, and vascular injury (21, 32–37). However, little is known about the transcriptional regulation of EBP50 expression in normal and disease states. Estrogen is the best characterized inducer of EBP50 expression (38–40), and we reported a modest increase in EBP50 expression in VSMC overexpressing parathyroid hormone-related protein (20). However, these mechanisms do not account for the remarkable EBP50 up-regulation upon vascular injury in male rats and mice (20, 21). Confirming an earlier study from Davis et al. (41), we observed a significant increase in EBP50 expression upon treatment with either LPS or TNFα in macrophages and VSMC. Furthermore, we found that this effect was transcriptional and fully dependent on NF-κB activity. The regulation of EBP50 by NF-κB is relevant in animal models of vascular injury; NF-κB activity is normally low in uninjured arteries, but IkBα degradation and up-regulation of NF-κB-responsive genes (such as VCAM-1) are observed upon angioplasty (16). We recently reported that EBP50 expression is four times higher in intimal versus medial VSMC following endoluminal denudation in rat and mouse arteries (20, 21).
addition, we show that macrophages within the vascular lesion express abundant EBP50. Thus, both NF-κB activity and EBP50 expression increase during vascular remodeling, and our studies provide a mechanistic basis for these effects.

Interestingly, we found that EBP50-null VSMC and macrophages were impaired in their ability to activate NF-κB in response to inflammatory stimuli. This is consistent with a study from Estell et al. (42) showing that NF-κB binding to DNA in bronchial epithelial cells was dependent on EBP50. In our studies, the reduction in IKK phosphorylation in EBP50-null cells indicates that EBP50 exerts its effects early in the activation of NF-κB, upstream of the IKK complex. Moreover, the effect of EBP50 is not receptor-specific because it is observed with both LPS and TNFα. We reasoned that a common effector of TLR4 and TNF receptor could be the site of action of EBP50 and focused on PKCζ because it possesses a C-terminal PDZ-binding motif and is essential for NF-κB activation. Indeed, our experiments show that inflammatory stimuli induce the formation of an EBP50-PKCζ complex at the cell membrane. The magnitude of this effect is consistent with that observed in kidney-derived opossum kidney cells (CHO) in response to dopamine (43). PKCζ translocation to the membrane appears to be the critical step for EBP50 action because NF-κB activity in EBP50-null cells was rescued by a membrane-targeted myristoylated form of PKCζ but not by overexpression of WT PKCζ. Consistent with this mechanism, we found that at least a functional PDZ domain and the ezrin-radixin-moesin (ERM) domain in EBP50 are required for cytokine-induced IKK phosphorylation. Therefore, both the ability to bind PKCζ and the ability to interact with the cytoskeleton are crucial for the effect of EBP50 on NF-κB activation.

The regulation of NF-κB signaling by EBP50 has important consequences in inflammatory states. We show that the expression of the classical M1 activation genes IL-1β, TNFα, and iNOS is decreased in EBP50−/− macrophages in vitro and in vivo. In contrast, there was no effect on IL-6 or IL-10. IL-10 is an anti-inflammatory cytokine that can inhibit NF-κB activation, enhance macrophage efferocytosis, and promote the conversion of M1 pro-inflammatory macrophages to an M2 regulatory phenotype (44–47). Moreover, M2 polarization induced by IL-4 was unaffected by EBP50. Thus, an overall decrease in pro-inflammatory cytokines and a lack of effect on IL-10 and M2 polarization suggest that the balance between pro- and anti-inflammatory events could be altered in the absence of EBP50, leading to a more efficient resolution of inflammation.

Our experiments focus on the role of EBP50 in macrophages and VSMC. However, other cell types are involved in the establishment and maintenance of inflammatory conditions and the overall response of vessels to injury (restenosis) and metabolic imbalance (atherosclerosis). Indeed, recent studies highlight the emerging role for EBP50 in leukocyte function. Mañes et al. (48) showed that the interaction between EBP50 and PIP5 kinase β (PIP5K1β) is necessary for chemoattractant-induced neutrophil polarization. Wu et al. (49) determined that EBP50 is essential for neutrophil migration by scaffolding CXCR2 and PLCβ2, and in this study, we found that EBP50 is important for
Macrophage activation both in vitro and in vivo. It is becoming increasingly clear that EBP50 plays a central role in the activation and function of inflammatory cells. Further work is required to fully define the cell-specific effects of EBP50 during vascular remodeling.

The effect of EBP50 on NF-κB-dependent gene expression was evident in VSMC and endothelial cells as well. Expression of the adhesion molecules ICAM-1 and VCAM-1 (and of iNOS) was decreased in EBP50-null VSMC and HUVEC and in EBP50−/− mice. Consequently, homing of macrophages to the site of arterial injury was significantly reduced in EBP50−/− mice. These observations provide further insight into the critical role of EBP50 during vascular remodeling. As we reported previously, EBP50 potentiates VSMC proliferation and motility (20–22). Here we show that EBP50 regulates the response of vascular cells to inflammatory stimuli and the recruitment of macrophages to injured vessels. Therefore, EBP50 promotes multiple critical events, leading to aberrant vascular remodeling, and genetic ablation of EBP50 confers remarkable protection from injury-induced restenosis.

In summary, we have identified EBP50 as a positive regulator of NF-κB activation. Conversely, we have also demonstrated a role for NF-κB in the up-regulation of EBP50 expression. We propose that NF-κB and EBP50 participate in a positive feed-
forward loop leading to increased macrophage activation and enhanced responses of vascular cells to inflammation.

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