The American Journal of Pathology, Vol. 182, No. 5, May 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Peptide Inhibitor of NF-κB Translocation Ameliorates Experimental Atherosclerosis

Beñat Mallavia,* Carlota Recio,* Ainhoa Oguiza,* Guadalupe Ortiz-Muñoz,† Iolanda Lazaro,* Virginia Lopez-Parra,* Oscar Lopez-Franco,‡ Susann Schindler,§ Reinhard Depping,§ Jesus Egido,* and Carmen Gomez-Guerrero*

From the Renal and Vascular Inflammation Laboratory, Department of Nephrology, IIS-Fundación Jiménez Díaz, Autonoma University, Madrid, Spain; the Cardiovascular Research Institute,† University of California, San Francisco, San Francisco, California; the Institute of Infection, Immunity and Inflammation,‡ University of Glasgow, Glasgow, United Kingdom; and the Institute of Physiology,§ Centre for Structural and Cell Biology in Medicine, University of Lübeck, Lübeck, Germany*

Accepted for publication January 10, 2013.

Address correspondence to Carmen Gomez-Guerrero, Ph.D., Renal and Vascular Inflammation Laboratory, IIS-Fundación Jiménez Díaz, Autonoma University, Avda Reyes Católicos 2, 28040 Madrid, Spain. E-mail: cgomez@fjd.es.

Atherosclerosis is a chronic inflammatory disease of the arterial wall. NF-κB is a major regulator of inflammation that controls the expression of many genes involved in atherogenesis. Activated NF-κB was detected in human atherosclerotic plaques, and modulation of NF-κB inflammatory activity limits disease progression in mice. Herein, we investigate the anti-inflammatory and atheroprotective effects of a cell-permeable peptide containing the NF-κB nuclear localization sequence (NLS). In vascular smooth muscle cells and macrophages, NLS peptide specifically blocked the importin α—mediated nuclear import of NF-κB and prevented lipopolysaccharide-induced pro-inflammatory gene expression, cell migration, and oxidative stress. In experimental atherosclerosis (apolipoprotein E—knockout mice fed a high-fat diet), i.p., 0.13 μmol/day NLS peptide administration for 5 weeks attenuated NF-κB activation in atherosclerotic plaques. NLS peptide significantly inhibited lesion development at both early (age 10 weeks) and advanced (age 28 weeks) stages of atherosclerosis in mice, without affecting serum lipid levels. Plaques from NLS-treated mice contained fewer macrophages of pro-inflammatory M1 subtype than those from respective untreated controls. By contrast, the relative smooth muscle cell and collagen content was increased, indicating a more stable plaque phenotype. NLS peptide also attenuated pro-inflammatory gene expression and oxidative stress in aortic lesions. Our study demonstrates that targeting NF-κB nuclear translocation hampers inflammation and atherosclerosis development and identifies cell-permeable NLS peptide as a potential anti-atherosclerotic agent. *(Am J Pathol 2013, 182: 1910—1921; http://dx.doi.org/10.1016/j.ajpath.2013.01.022)*

Atherosclerosis is a multifactorial disease of the vascular wall and is one of the leading causes of death in Western countries.[1] The atherosclerotic plaque is characterized by lipid accumulation, leukocyte infiltration, and a continuous inflammatory stage that leads from the development of plaques to destabilization and thrombus formation.[2] Modulation of the different steps in plaque formation is the objective of many anti-inflammatory and immunomodulatory compounds.[3] Numerous cellular and molecular inflammatory components participate in the disease process, including release of pro-inflammatory cytokines, leukocyte infiltration, and vascular smooth muscle cell (VSMC) activation and migration.[2] The atherosclerotic process is also mediated by uncontrolled activation of pro-inflammatory transcription factors, among which NF-κB plays a significant role.[4,5]

NF-κB belongs to the Rel family; its members share the Rel homology domain involved in protein dimerization and DNA binding activity. Many different stimuli activate NF-κB via canonical and noncanonical pathways.[6] In resting cells, inactive homodimers and heterodimers are sequestered in the cytoplasm by either the inhibitory subunit IκB or the precursor proteins p100 and p105. The canonical pathway drives to IκB kinase activation and IκB phosphorylation with further ubiquitination and degradation. This allows the unmasking of the nuclear localization sequence (NLS) of NF-κB members[7] and the activation of p65-p50 NF-κB

Supported by Spanish Ministry of Science grants SAF2009/11794 and SAF2012-38830, Ministry of Health grant FIS-RECAVA RD06/0014/0035, the Lilly Foundation, and Comunidad de Madrid grant S2006/GEN-0247.

http://dx.doi.org/10.1016/j.ajpath.2013.01.022

complex, followed by its nuclear translocation. Once in the nucleus, NF-κB binds to functional κB sites in the promoter region of target genes, including cytokines, chemokines, cell adhesion molecules, apoptotic proteins, and cell surface receptors, most of them also involved in atherogenesis and vascular inflammation.[4]

The import of macromolecules into the nucleus occurs through nuclear-pore complexes, large multiprotein assemblies that penetrate and fuse the nuclear envelope, and this process is mediated by specific soluble carrier proteins belonging to the importin superfamily.[8] Importin α functions as an adaptor that links classic NLS-containing proteins to importin β, which, in turn, docks the ternary complex at the nuclear-pore complex.[9] In humans, only one importin β is known to interact with importin α, whereas six importin α molecules have been described, with variable binding affinity and cell/stimulus specificity.[10] The nuclear import of NF-κB members is a highly regulated process mediated by a subset of importin α molecules.[7,11]

Because of its critical role in inflammatory diseases, there are many NF-κB modulators targeting upstream activators, the IκB kinase complex, IκB degradation, DNA binding, and gene transactivation.[12–14] However, little interest has been paid to the potential role of nuclear localization inhibition in cardiovascular diseases. Herein, we investigate whether selective inhibition of NF-κB nuclear import has a beneficial effect on initiation and progression of atherosclerosis. We, therefore, analyze the anti-inflammatory and atheroprotective properties of a cell-permeable peptide containing the NLS of NF-κB both *in vitro* (VSMCs and macrophages under inflammatory conditions) and *in vivo* (atherosclerosis mouse model). We also identify the importin α/NF-κB interactions that NLS peptide specifically inhibited in vascular cells.

Materials and Methods

Reagents

The cell-permeable peptide containing the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor (AAVALLPAVLLALLAP)[15] and a single NLS from NF-κB (VQRKRQKLMP)[16] was synthesized and cyclized (intrachain disulfide bond between cysteines inserted in the NLS motif) by Creative Biolabs (Shirley, NY). A mutated NLS peptide (mNLS; KR→NG) was used as the structural control. Peptides were dissolved (2.5% acetonitrile in saline solution) and filter sterilized. Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies (Abs) were as follows: Moma-2 (Serotec, Oxford, UK); α-smooth muscle actin (α-SMA; Sigma-Aldrich); chemokine ligand (CCL) 2 (Peprotech, Rocky Hill, NJ); CCL5 (Antibodies-Online, Aachen, Germany); intracellular adhesion molecule-1 (ICAM-1; R&D Systems, Minneapolis, MN); STAT1 (Invitrogen, Carlsbad, CA); STAT3 (Cell Signaling, Beverly, MA); and p65, glutathione-S-transferase (GST), lamin B, tumor necrosis factor α (TNF-α), and arginase (Arg) I/II (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary Abs (peroxidase, biotin, and fluorescein isothiocyanate conjugated) were provided by Amersham Biosciences (Buckinghamshire, England) and Sigma-Aldrich.

Cell Cultures

VSMCs from mouse aorta were isolated by enzymatic digestion, cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Rockville, MD) and used between the second and seventh passages.[17] Mouse macrophage cell line RAW 264.7 (TIB-71; ATCC, Manassas, VA) was maintained in Dulbecco's modified Eagle's medium with 10% FBS. Quiescent cells were treated for 90 minutes with 4 to 54 μmol/L NLS and control peptide before stimulation with 1 μg/mL LPS.

Electrophoretic Mobility Shift Assay

Cells were homogenized [10 mmol/L HEPES (pH 7.8), 15 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail], and 10-μg nuclear proteins were incubated with 0.035 pmol [^{32}P]-consensus oligonucleotides of NF-κB, activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT), and STAT3. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels and exposed for autoradiography. A competition experiment was performed with 100-fold excess of unlabeled oligonucleotides.[18]

Western Blot Analysis

Nuclear proteins (20 μg) were developed by polyacrylamide gel electrophoresis, transferred to membranes, and then immunoblotted with p65 and lamin B Abs.

Immunofluorescence

NF-κB localization in fixed, permeabilized cells was detected with p65 Ab, followed by fluorescein isothiocyanate—secondary Ab and nuclear counterstaining (DAPI).

Pull-Down Assay

Importin proteins (α3, α4, and α5) were expressed in *Escherichia coli* JM101 cells as GST-tagged fusion proteins under 1 mmol/L isopropyl-1-thio-β-D-galactopyranoside and 2% ethanol induction.[19] Bacteria were lysed and clarified by centrifugation. Fusion proteins were bound to 2 mL of glutathione—Sepharose 4B beads (Amersham Biosciences), then eluted with 10 mmol/L reduced glutathione (Sigma-Aldrich) in 50 mmol/L Tris-HCl buffer and dialyzed against 50 mmol/L HEPES buffer containing 200 mmol/L NaCl and 5%

glycerol. For the pull-down assay, 15 μg of importin-GST fusion proteins was incubated for 12 hours at 4°C with glutathione−Sepharose 4B beads in L-buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L Na_3VO_4, and protease inhibitor cocktail; modified from Fagerlund et al[7]]. Then, total cell lysates (30 μg of protein) were added and rotated at 4°C for 2 hours. After washing, beads were boiled in Laemmli's buffer. The eluted proteins were electrophoresed, transferred, and immunoblotted for p65, STAT1, STAT3, and GST.

ELISA

CCL2 levels in cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA; BD Biosciences, Erembodegem, Belgium).

Cell Migration Assays

In the wound-healing assay, VSMCs in 24-well plates were serum depleted, followed by a wound injury using a plastic pipette tip, and then treated with NLS peptide before LPS stimulation. Wound closure was measured at 4 to 24 hours of incubation. Six to eight images from each well along the wound were captured, and remaining wound areas were quantified and normalized to time 0 values.

In the transwell chemotaxis assay, 3×10^5 VSMCs in Dulbecco's modified Eagle's medium with 0.5% FBS were placed in the upper chamber of an 8.0-μm−pore size 24-well transwell plate (Corning Inc., Corning, NY). Medium containing NLS peptide was added to the lower chamber and incubated for 90 minutes at 37°C before LPS stimulation. After 12 hours, nonmigratory cells were removed from the upper surface of the membrane, and migrated cells were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet. Each experimental condition was performed in triplicate, and the number of migrated cells was determined from three random 200× fields per membrane. For transwell co-culture system studies, 5×10^5 RAW 264.7 macrophages were seeded on the upper transwell inserts and then placed onto a 24-well plate containing 2×10^5 per well VSMCs in the lower chamber. VSMCs were previously stimulated with LPS in the presence or absence of NLS and mNLS peptides. Co-cultures were incubated for a further 8 hours at 37°C, and then migrated macrophages were counted.

Cell viability was assessed with the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan thiazolyl blue formazan method. Cell proliferation was quantified by the methylene blue assay.[18]

Atherosclerotic Mouse Model and Treatments

Apolipoprotein E (ApoE) knockout mice (Jackson Laboratory, Bar Harbor, ME) were randomly distributed into treatment (NLS peptide) and control (vehicle) groups and were fed a high-fat Western diet (21% fat and 0.15% cholesterol; Harlan Labs, Madison, WI) for 5 weeks. During this time, animals received i.p. injections of either NLS peptide (0.13 μmol/day in 200 μL) or vehicle every second day. The early treatment study started at 10 weeks of age (NLS group, $n = 10$; control group, $n = 10$) when hardly any atherosclerotic lesions were present. The delayed treatment study (NLS group, $n = 7$; control group, $n = 5$) started at 28 weeks of age, the time point at which advanced atherosclerotic plaques had developed. At the study end point, mice were anesthetized using 100 mg/kg ketamine and 15 mg/kg xylazine, then saline perfused and euthanized. Aortas were removed and divided into two parts: the upper aortic root was embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Flemingweg, The Netherlands) for histological analysis, and the abdominal/thoracic aorta was processed for mRNA analysis.[17] Serum cholesterol levels were measured using the Amplex Red Cholesterol assay kit (Invitrogen). The studies conform to the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committees Autonoma University and IIS-Fundacion Jimenez Diaz).

Histological Analysis and Quantification

Serial aortic sections (8 μm thick, covering approximately 1000 μm from valve leaflets) were quantified by morphometry after oil-red-O/hematoxylin staining. The individual lesion area was determined by averaging the maximal values (three to four sections). Activated NF-κB was assessed by Southwestern histochemistry using digoxigenin-labeled probes, as previously described.[20] Collagen content with picrosirius red staining was measured under polarized light microscopy. VSMCs (α-SMA), total macrophages (Moma-2), macrophage phenotypes (M1 marker Arg II; M2 marker Arg I), and protein expression levels (CCL2, CCL5, ICAM-1, and TNF-α) were detected by indirect immunoperoxidase/immunofluorescence.[21] Positive staining was quantified using Image Pro-Plus software version 4.5.0.29 (Media Cybernetics, Bethesda, MD) and expressed as percentage of total plaque area or number of positive cells per lesion area.

mRNA Expression

Total RNA extracted from cells and aortic tissues[17,21] was analyzed by real-time quantitative PCR using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The expression of target genes (*Ccl2*, *Ccl5*, *Icam1*, *Tnfα*, and *Il6*) was normalized to the housekeeping gene *Gapdh*.

Oxidative Stress

Intracellular superoxide in tissues was assessed by fluorescence microscopy with 2 μmol/L superoxide-sensitive fluorescent dye dihydroethidium (DHE; Invitrogen), and expressed as number of DHE-positive cells versus total cells (DAPI staining). NADPH-dependent oxidase activity in cell

homogenates was measured by chemiluminescence using 5 μmol/L lucigenin and 100 μmol/L NADPH.[22]

Statistical Analysis

Results are given as means ± SEM. Differences across groups were considered significant at $P < 0.05$ using an unpaired Student's *t*-test and a one-tailed analysis of variance, followed by a post hoc Bonferroni pairwise comparison test (Prism version 5; GraphPad Software Inc., La Jolla, CA).

Results

NLS Peptide Blocks the Nuclear Import of Activated NF-κB

Activated NF-κB is transported into the nucleus via different importin α isoforms that directly interact with the NLS region on NF-κB subunits.[7,11] To study whether NLS peptide affects the nuclear traffic of activated NF-κB under inflammatory conditions in vessel cells, primary mouse VSMCs were treated with NLS peptide before stimulation with bacterial endotoxin LPS, and then protein pull-down assays with bacterially expressed GST-importins were performed. In LPS-stimulated cells, p65 proteins bound strongly to importin α isoforms (α3 >> α4 > α5), whereas small amounts of p65 were detected in NLS pretreated cells (% inhibition versus LPS: α3, 84% ± 5%; α4, 60% ± 19%; α5, 73% ± 6%; $P < 0.03$) (Figure 1A). By contrast, NLS did not affect the importin α binding to STAT1 and STAT3 transcription factors (Figure 1B). These results suggest that NLS peptide specifically inhibits the direct binding of activated NF-κB to importin α isoforms and the subsequent nuclear translocation in VSMCs.

NLS Peptide Inhibits NF-κB Activation in Vascular Cells

The ability of NLS peptide to inhibit NF-κB activation in several cells involved in atherogenesis was measured by the gel shift assay. As shown in Figure 2A, NLS pretreatment drastically reduced the DNA-binding activity of NF-κB in LPS-stimulated VSMCs without inhibiting other transcription factors with a similar translocation sequence, such as AP-1, NFAT, and STAT3. In RAW 264.7 macrophages (Figure 2B), NLS peptide dose-dependently inhibited the activation of NF-κB induced by LPS, whereas the structural control (mNLS peptide, 36 μmol/L) had no significant effect. Nuclear translocation of the NF-κB complex was further assessed by immunodetection of the p65 subunit. Western blot data revealed low levels of p65 protein in the nuclear fractions of VSMCs and RAW 264.7 macrophages preincubated with NLS peptide (Figure 2C). Furthermore, immunofluorescence studies in VSMCs showed that nuclear p65 localization was prevented in cells treated with NLS peptide before LPS stimulation (Figure 2D).

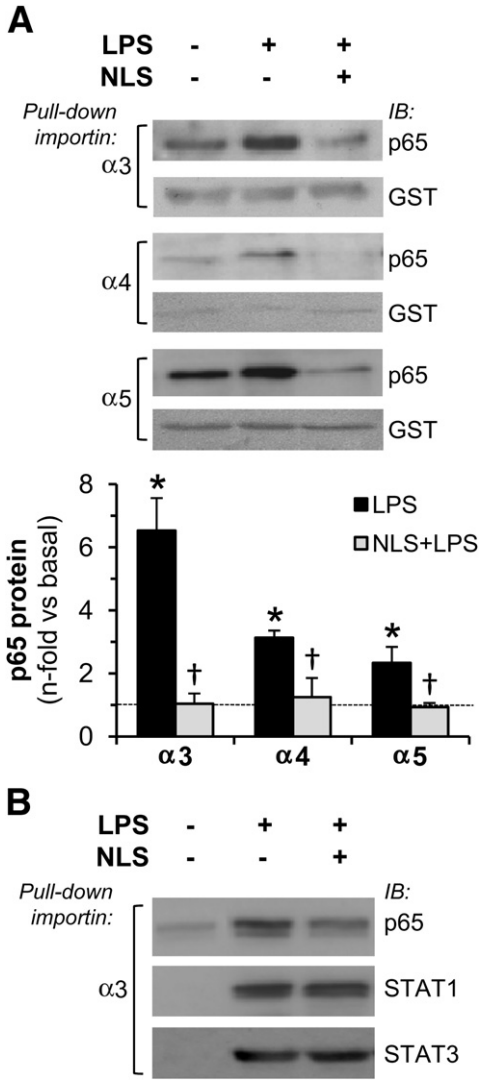

Figure 1 NLS peptide inhibits NF-κB—importin interactions in vascular cells. Pull-down assays in protein extracts from VSMCs pretreated with 36 μmol/L NLS peptide for 90 minutes before stimulation with 1 μg/mL LPS for 30 minutes. Proteins bound to glutathione-Sepharose—immobilized GST—importin α isoforms were electrophoresed and immunoblotted for p65/GST (**A**) and p65/STAT1/STAT3 (**B**). Representative blots and summary of quantitative analysis are shown. Data are expressed as *n*-fold versus basal and are means ± SEM of three to five experiments. $^{*}P < 0.03$ versus basal; $^{\dagger}P < 0.03$ versus LPS. The **dashed line** represents basal conditions.

Anti-Inflammatory Effects of NLS Peptide *in Vitro*

Among the different inflammatory molecules implicated in cell recruitment and activation into the vascular wall, we studied *in vitro* the expression of those genes regulated by NF-κB (namely, the chemokines CCL2 and CCL5, the adhesion molecule ICAM-1, and the pro-inflammatory cytokine TNF-α). Real-time quantitative PCR analysis revealed that NLS peptide drastically decreased the expression of NF-κB target genes induced by LPS both in VSMCs (Figure 3A) and RAW 264.7 macrophages (Figure 3B). This effect was concentration dependent, with a half-maximal inhibition reached at 30 μmol/L (Figure 3C).

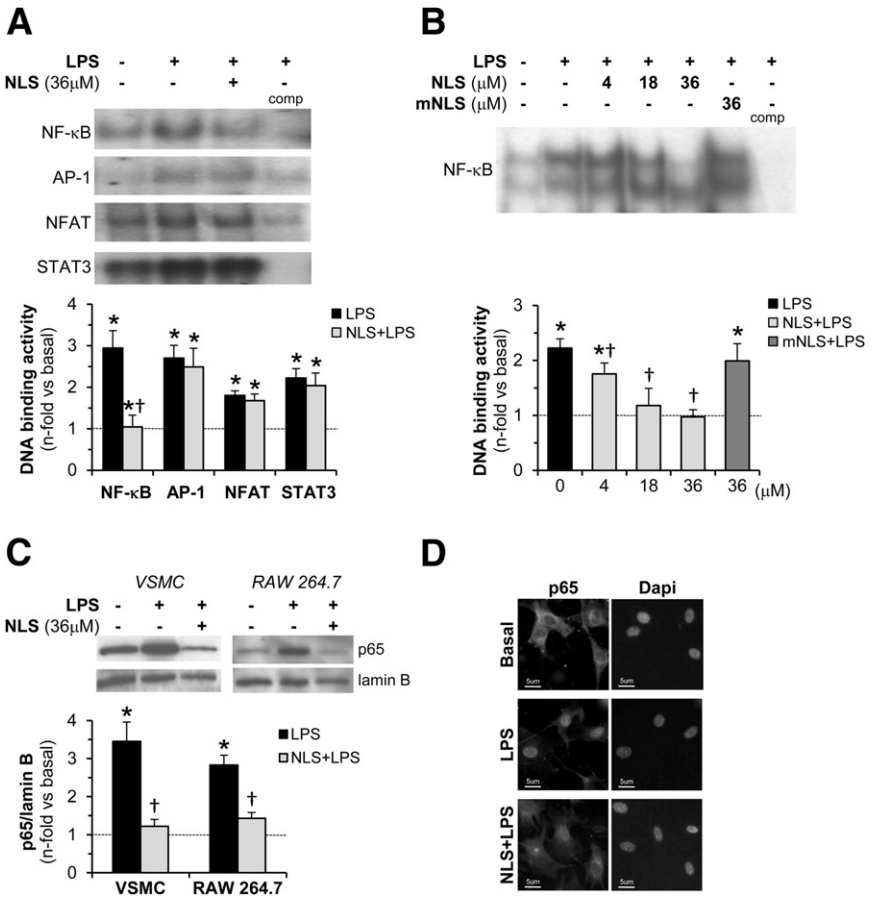

Figure 2 NLS peptide inhibits NF-κB activation in cultured cells. Primary VSMCs (**A**, **C**, and **D**) and RAW 264.7 macrophages (**B** and **C**) were pre-incubated for 90 minutes with the indicated concentrations of inhibitory NLS peptide or its structural control (mNLS) before 1 μg/mL LPS stimulation for 30 minutes. **A** and **B**: DNA-binding activity of NF-κB, AP-1, NFAT or STAT3 transcription factors was analyzed by gel shift assay in nuclear proteins from VSMCs (**A**) and RAW 264.7 macrophages (**B**). **C**: Western blot analysis for p65 protein in cell nuclear extracts. Representative blots (comp, competition assay with unlabeled probe) and summary of densitometric analysis are shown. Results are expressed as the relative increase (*n*-fold) over basal conditions and represent means ± SEM of three to five independent experiments. $^{*}P < 0.03$ versus basal; $^{\dagger}P < 0.03$ versus LPS. The **dashed line** represents basal conditions. **D**: Subcellular distribution of the p65 subunit in VSMCs was assessed by immunofluorescence. Representative images of four independent experiments. Original magnification, ×200.

NLS peptide also prevented the secretion of CCL2 protein (LPS, 1.94 ± 0.35 ng/mL; NLS + LPS, 0.53 ± 0.28 ng/mL; $n = 4$; $P = 0.02$), as measured by ELISA in cell supernatants.

Because NF-κB is a key factor in controlling NADPH oxidase expression and function, we also assessed the potential antioxidant activity of NLS peptide. A lucigenin chemiluminescence assay revealed that NLS peptide, but not control mNLS, dose-dependently inhibited the generation of superoxide anion by the NADPH oxidase in LPS-stimulated macrophages (Figure 3D).

To understand the functional significance of the reduced inflammatory gene expression, we further studied the effects of NLS peptide on cell migration and growth, two important

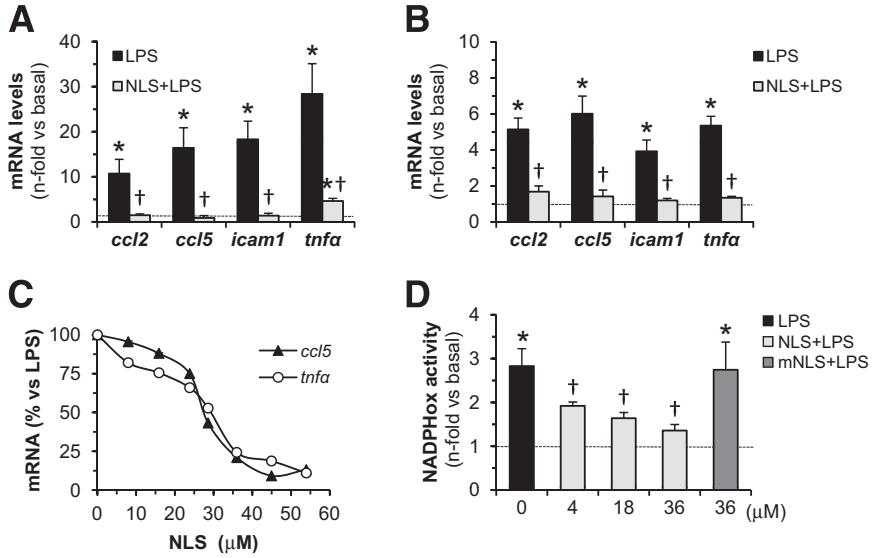

Figure 3 Anti-inflammatory effects of NLS peptide *in vitro*. **A** and **B**: Real-time quantitative PCR analysis of different inflammatory genes in VSMCs (**A**) and RAW 264.7 macrophages (**B**) pretreated with 36 μmol/L NLS peptide for 90 minutes and then stimulated with 1 μg/mL LPS for 3 hours. Values normalized by GAPDH endogenous control are expressed as fold increases versus basal. The **dashed lines** represent basal conditions. **C**: Dose-response curves of the effect of NLS peptide on CCL5 and TNF-α expression in LPS-stimulated VSMCs. Representative curves from triplicate experiments. **D**: NADPH oxidase–dependent superoxide production in RAW 264.7 macrophages was detected by lucigenin assay. Cells were preincubated with the indicated doses of NLS and control mNLS peptide before stimulation with 1 μg/mL LPS for 3 hours. Values represent means ± SEM of four to six independent experiments. $^{*}P < 0.01$ versus basal; $^{\dagger}P < 0.05$ versus LPS.

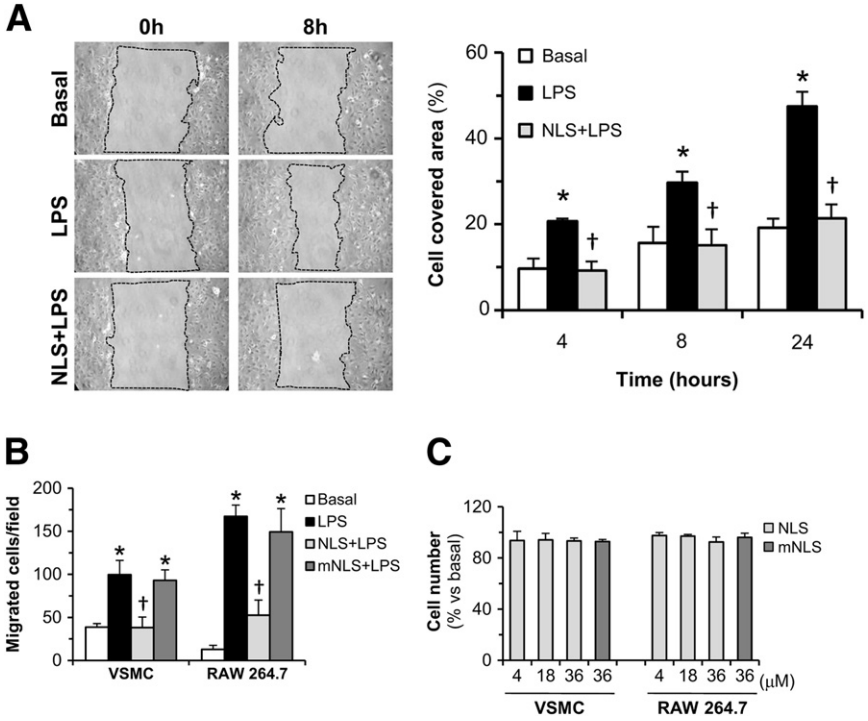

Figure 4 Effects of NLS peptide on cell migration and viability. **A**: *In vitro* assay of scratch wound healing with VSMCs under LPS stimulation in the absence or presence of 36 μmol/L NLS peptide. Cell migration to the wound surface was monitored from 0 to 24 hours. **Left panel**: Representative phase-contrast images of cells migrating into the wounded area at 8 hours after wounding are displayed. **Dotted lines** delimit the wound surface. **Right panel**: The covered healing regions were measured and expressed as percentage of the initial wound area. **B**: Chemotaxis assay in LPS-activated VSMCs (transwell migration) and macrophages (transwell co-culture migration of RAW 264.7 cells toward LPS-activated VSMCs) in the absence or presence of NLS peptide and its structural control, mNLS (36 μmol/L each). Migrated cells were counted on the lower side of the membrane after 8 hours (RAW 264.7 cells) and 12 hours (VSMCs). **C**: Methylene blue assay in VSMCs and RAW 264.7 macrophages maintained for 24 hours in growth medium (10% FBS) containing the indicated concentrations of NLS and mNLS peptide. Each condition was performed in duplicate (**B**) or quadruplicate (**A** and **C**). Values represent means ± SEM of three to five independent experiments. $^{*}P < 0.04$ versus basal; $^{\dagger}P < 0.02$ versus LPS.

steps involved in neointima formation. An *in vitro* wound-healing assay with VSMCs revealed that LPS caused a time-dependent increase in directed cell migration, and also demonstrated the antimigratory effect of NLS peptide (% reduction versus LPS at 24 hours, 55% ± 7%; $P = 0.004$) (Figure 4A). NLS peptide also significantly inhibited the transwell migration of VSMCs induced by LPS (Figure 4B). Moreover, in a co-culture transwell system, the migration of RAW 264.7 macrophages toward LPS-stimulated VSMCs was effectively prevented by NLS peptide (% inhibition versus LPS, 69% ± 10%; $P = 0.018$) (Figure 4B). By contrast, control mNLS peptide had no significant effect on cell migration (Figure 4B). Cell viability remained >90% under all experimental conditions, thus discarding any possible cytotoxic effect of NLS peptide (data not shown). Furthermore, cell proliferation was not altered by either NLS or mNLS peptide, even after 24 hours of continuous exposure to higher concentrations (Figure 4C).

NLS Peptide Attenuates NF-κB Activity in Atherosclerotic Mice

To confirm the *in vitro* data of NF-κB inhibition by NLS peptide, we further studied *in vivo* the potential benefits of NLS administration in ApoE knockout mice, an experimental model relevant to human atherosclerosis. In these experiments, ApoE knockout mice were fed a high-fat diet and treated with either NLS peptide or vehicle (control group). By using Southwestern histochemistry, a technique developed in our laboratory for *in situ* nuclear detection of activated transcription factors,[17,18,20] we observed intense NF-κB activation within the aorta of ApoE knockout mice (Figure 5A), in both the intima and the media. Double staining with Moma-2 and α-SMA, respective markers for macrophages and VSMCs, indicated the presence of activated NF-κB within both cell types in atherosclerotic lesions (Figure 5A). Furthermore, quantification of NF-κB–positive staining in the NLS and control groups revealed that NLS peptide treatment significantly attenuated NF-κB activation within the aortic plaques of atherosclerotic mice (% reduction versus control group, 58% ± 5%; $P = 0.007$) (Figure 5, B and C).

NLS Peptide Reduces Plaque Size at Both Early and Advanced Stages of Atherosclerosis in Mice

Because our *in vitro* data suggested anti-inflammatory properties of NLS peptide, we next examined whether NF-κB inhibition by NLS therapy influences development and progression of atherosclerotic plaques. In these experiments, ApoE knockout mice were fed a high-fat diet and treated with NLS peptide or vehicle for 5 weeks. The effect of NLS peptide was analyzed either at the onset of atherosclerosis (early treatment, age 10 weeks) or after the development of advanced plaques (delayed treatment, age 28 weeks). In the early study, NLS-treated mice showed reduced lesion size at the cross section of the aortic root compared with vehicle-treated mice (mean lesion area: $2.74 \pm 0.54 \times 10^4$ μm^2 versus $5.70 \pm 0.39 \times 10^4$ μm^2; $P = 0.002$) (Figure 6, A and B), and regression analysis showed significant correlation of lesion area with NF-κB staining in the

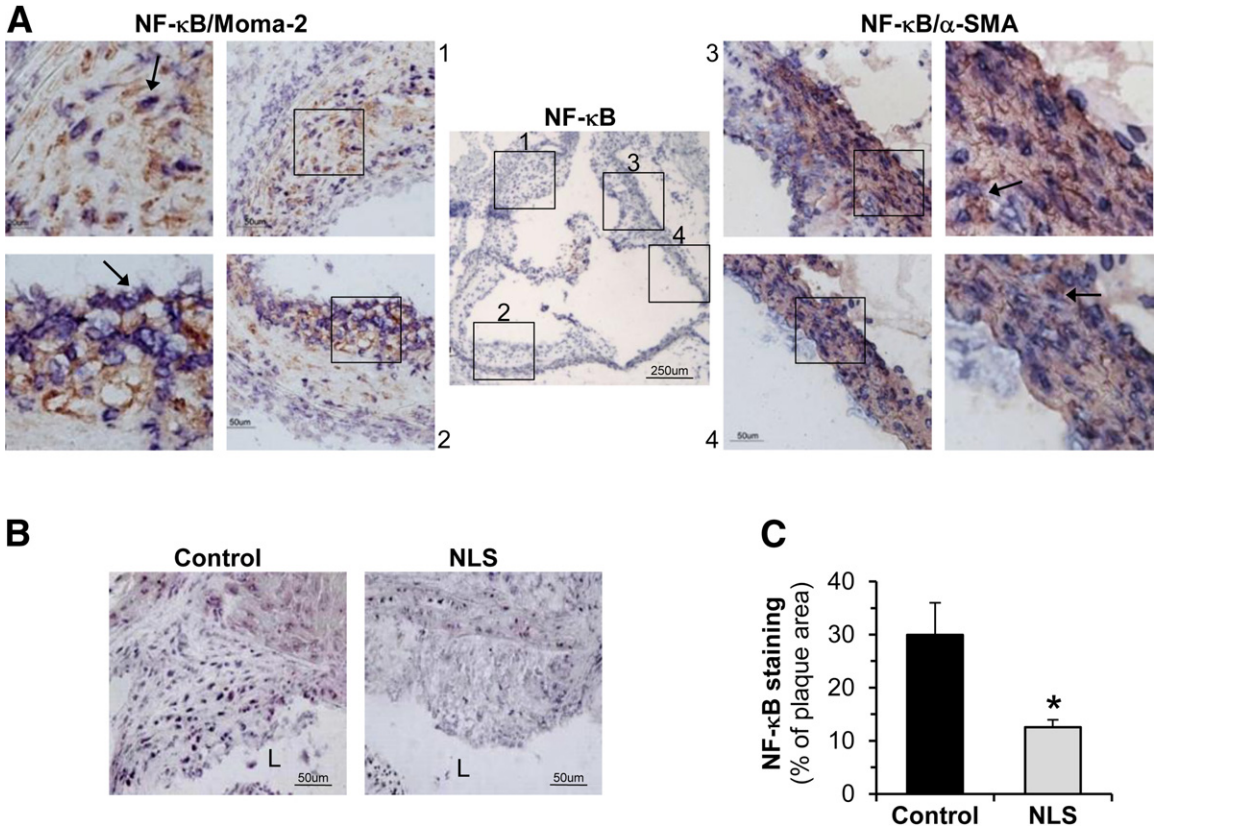

Figure 5 NLS peptide inhibits NF-κB activation *in vivo*. **A:** *In situ* detection of activated NF-κB by Southwestern histochemistry in aortic sections of 10-week-old ApoE knockout mice fed a high-fat diet for 5 weeks. Representative micrographs (original magnification, ×40) and higher-magnification images (original magnification, ×200 and ×400) of four different aortic regions (**boxed**) showing colocalization (Southwestern/immunoperoxidase) of NF-κB with macrophages (Moma-2) and VSMCs (α-SMA). **Arrows** indicate double staining (blue, nuclear NF-κB; brown, cell type marker). **B:** Histological examination of NF-κB in atherosclerotic plaques from ApoE knockout mice after 5 weeks of treatment with NLS peptide. Representative images are shown. Original magnification, ×200. L, lumen. **C:** Quantitative analysis of the area with positive staining in the control and NLS groups. Values are expressed as means ± SEM of 10 animals per group. *$P = 0.007$ versus control.

experimental groups (Figure 6C). In the delayed study, aortic lesions of NLS-treated mice were twofold smaller by area ($P = 0.005$) than those of control mice (Figure 6, D and F). Interestingly, NLS peptide therapy diminished not only the mean lesion area, but also the extension along the aorta (Figure 6, B and E). Groups at both early and delayed treatment had comparable body weights (23.8 ± 1.0 versus 23.8 ± 0.7 g and 33.4 ± 2.9 versus 29.9 ± 3.0 g; $P > 0.05$) and total cholesterol levels (265 ± 11 versus 296 ± 3 mg/dL and 876 ± 53 versus 821 ± 103 mg/dL; $P > 0.05$) at the end of the study, thus discarding a potential effect of NLS peptide on lipid levels.

Attenuated Inflammation and Oxidative Stress and Enhanced Stability in Atherosclerotic Plaques of NLS-Treated Mice

The reduced lesion size by early treatment with NLS peptide was associated with a significant decrease in the relative number of macrophages (Moma-2 immunostaining) within the atherosclerotic plaques (% decrease versus control, 55% ± 5%; $P = 0.0039$) (Figures 7A and 8A). Regression analysis showed correlation of macrophage content with lesion area ($r = 0.566$, $P = 0.018$) and NF-κB staining ($r = 0.755$, $P = 0.0001$) in the experimental model, thus indicating a more inflammatory component in larger lesions. Furthermore, distribution of macrophage phenotypes (pro-inflammatory classic M1 and anti-inflammatory alternative M2) in atherosclerotic lesions was analyzed by immunofluorescence, observing a predominance of M2 marker (Arg I) versus M1 marker (Arg II) in plaques of NLS-treated mice (Figures 7B and 8B).

Aortic lesions were also analyzed for collagen (picrosirius red staining) and VSMC (α-SMA immunofluorescence) content, which are important stabilizing components of the plaque. Atherosclerotic plaques of NLS-treated mice exhibited a modest increase in total collagen and VSMC content compared with those of control mice (Figure 7C). Interestingly, the ratio of collagen/lipids (picrosirius red/oil-red-O) and VSMCs/macrophages (α-SMA/Moma-2) did significantly increase by NLS treatment (Figure 8C), which is consistent with a more stable plaque phenotype.

We also tested, by immunohistochemistry and real-time quantitative PCR, the expression of a panel of pro-inflammatory

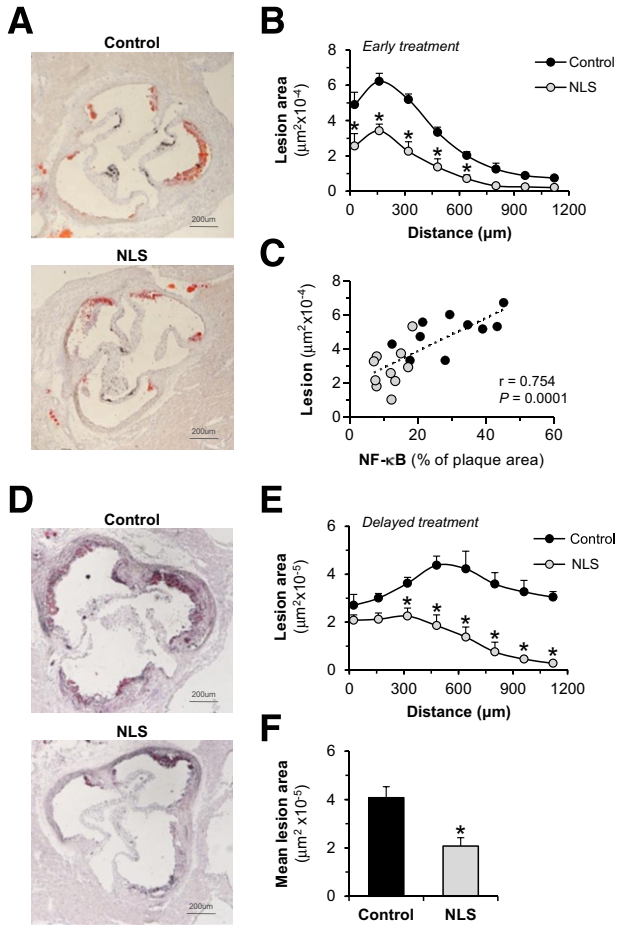

Figure 6 NLS treatment reduces atherosclerosis lesions in ApoE knockout mice. NLS peptide was administered to ApoE knockout mice for 5 weeks either at the onset of atherosclerosis (age, 10 weeks, early treatment; **A**–**C**) or after the development of advanced plaques (age, 28 weeks, delayed treatment; **D**–**F**). **A**: Representative photomicrographs of aortic root sections stained with oil-red-O/hematoxylin in the early treatment study. Original magnification, ×40. **B**: Quantification of lesion area throughout the studied region in control and NLS treated groups ($n = 10$ animals per group). **C**: Regression analysis of individual lesion area versus NF-κB staining in plaques from control and NLS-treated mice. Pearson r and two-tailed P values are indicated. **D**: Representative images of oil-red-O/hematoxylin–stained plaques from control and NLS groups of the delayed treatment study. **E**: Quantification of lesion area along the aorta (control group, $n = 5$; NLS group, $n = 7$). **F**: Average of maximal lesion area in each group. Measurements are expressed as means ± SEM. *$P <$ 0.05 versus the control group.

genes that are known to be regulated by NF-κB. As illustrated by representative images (Figure 7D) and quantitative analysis (Figure 8D), lesions of NLS-treated mice showed less expression of chemokines (CCL2 and CCL5), adhesion molecule ICAM-1, and pro-inflammatory cytokine TNF-α when compared with the control group (% decrease versus control, 71% ± 1%, 71% ± 3%, 49% ± 6%, 47% ± 6%, and 47% ± 8%, respectively; $P < 0.05$). Accordingly, the mRNA expression levels of those pro-inflammatory genes in aortic tissues were strongly reduced by NLS peptide administration (Figure 8E). Finally, DHE staining was used to reflect the extents of superoxide generation occurring in aortic root sections of atherosclerotic mice. As shown in Figures 7E and 8F, the number of DHE-labeled nuclei in plaques from NLS-treated mice was reduced by 50% compared with the control group, suggesting an inhibition of oxidative stress by NLS peptide treatment.

Together, our results indicate that inhibition of NF-κB nuclear import by NLS peptide hampers inflammation, oxidative stress, and atherosclerosis development in mice.

Discussion

The NF-κB system is a crucial factor regulating the expression of genes in different steps of the atherosclerotic process, from early phases characterized by lipid modification, chemotaxis, adhesion of leukocytes, monocyte differentiation, foam cell formation, and inflammatory cytokine expression to more advanced lesions involving cell death, migration and proliferation of VSMCs, and fibrous cap formation.[4,5] Aberrant activation of NF-κB has been detected in blood cells and in atherosclerotic plaques of patients.[23–25] The importance of this activation has been previously studied in animal models[18,26,27] and confirmed by either macrophage or endothelial cell–specific ablation of the NF-κB pathway.[28,29] Accordingly, modulation of NF-κB inflammatory activity has been proposed as a feasible approach to inhibit disease progression.[3,14] Although several NF-κB inhibitors are in phase 2/3 clinical development against various inflammatory diseases,[12–14] most of cardiovascular research is in the preliminary laboratory experimental phase. In fact, studies in rodent models of atherosclerosis have reported the efficacy of natural and synthetic compounds that mainly affect different steps upstream the formation of NF-κB protein dimer, from IκB kinase activation and IκB phosphorylation to the ubiquitin-proteasome system.[3,14,18,30,31] To our knowledge, the present study is the first to describe that a peptide suppressor of NF-κB nuclear import restrains key mechanisms of atherogenesis, such as pro-inflammatory gene expression, oxidative stress, leukocyte infiltration, and vascular cell activation and migration.

Herein, we used a cyclized peptide sequence containing the NLS region of the Rel family protein p50 that is able to inhibit nuclear import of the classic NF-κB complex.[16] Furthermore, the inhibitory sequence was coupled to a membrane-permeable sequence designed from the signal sequence of Kaposi fibroblast growth factor, which has been shown to translocate covalently attached cargo peptides across the cell membrane.[15] Several different cell-penetrating peptides have been developed to directly inhibit the NF-κB signaling pathway.[32] Among them, peptides designed to mimic the NLS sequence of p50 have been proved to block NF-κB activity and target gene expression in cultured cells.[16,33] Further evidence supporting the efficacy of nuclear import inhibitors *in vivo* is derived from animal models of

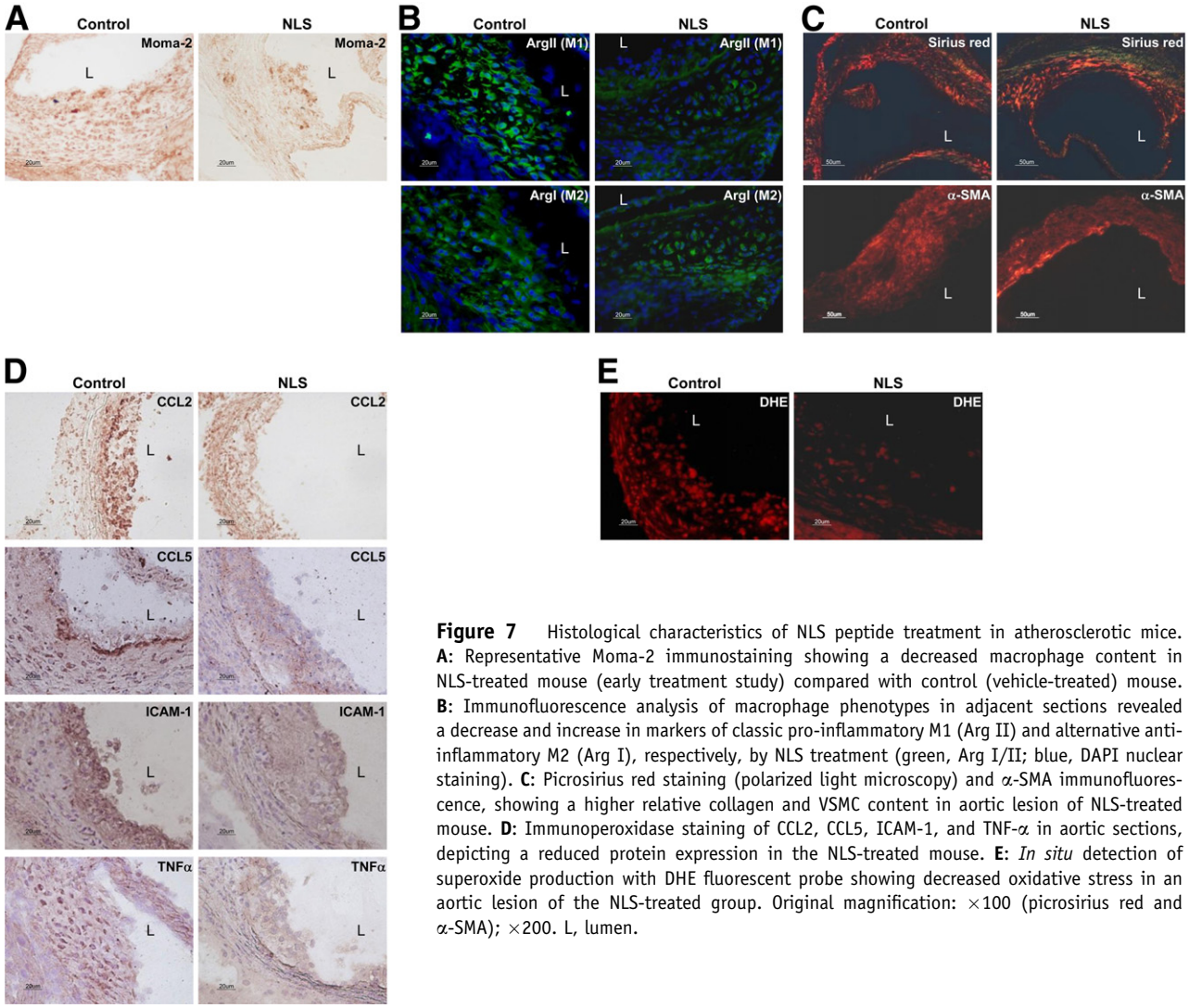

Figure 7 Histological characteristics of NLS peptide treatment in atherosclerotic mice. **A**: Representative Moma-2 immunostaining showing a decreased macrophage content in NLS-treated mouse (early treatment study) compared with control (vehicle-treated) mouse. **B**: Immunofluorescence analysis of macrophage phenotypes in adjacent sections revealed a decrease and increase in markers of classic pro-inflammatory M1 (Arg II) and alternative anti-inflammatory M2 (Arg I), respectively, by NLS treatment (green, Arg I/II; blue, DAPI nuclear staining). **C**: Picrosirius red staining (polarized light microscopy) and α-SMA immunofluorescence, showing a higher relative collagen and VSMC content in aortic lesion of NLS-treated mouse. **D**: Immunoperoxidase staining of CCL2, CCL5, ICAM-1, and TNF-α in aortic sections, depicting a reduced protein expression in the NLS-treated mouse. **E**: *In situ* detection of superoxide production with DHE fluorescent probe showing decreased oxidative stress in an aortic lesion of the NLS-treated group. Original magnification: ×100 (picrosirius red and α-SMA); ×200. L, lumen.

acute inflammation.[32,34,35] However, cardiovascular studies have not yet been reported. This article directly addresses the functional consequences of targeting NF-κB nuclear translocation during atherosclerosis development in ApoE knockout mice. Systemic administration of NLS peptide reduced the nuclear NF-κB activity in VSMCs and macrophages of aortic plaques of mice. More important, NLS peptide inhibited lesion development either at the onset of atherosclerosis (early treatment) or after the development of advanced plaques (delayed treatment), without affecting serum cholesterol levels. Furthermore, NLS peptide alters plaque composition and inflammation in atherosclerotic lesions. In fact, the decreased number of infiltrating macrophages, the phenotypic shift from pro-inflammatory M1 to anti-inflammatory M2 macrophages, and the high relative content of collagen and VSMCs observed in lesions of NLS-treated mice are all suggestive of a less inflamed, more stable plaque phenotype.

The anti-inflammatory action of NLS peptide *in vivo* was corroborated *in vitro* in the cellular constituents of the atherosclerotic lesion that are thought to participate actively in the propagation of inflammation and plaque development. In cultured VSMCs and macrophages under inflammatory stimulation (bacterial endotoxin), non-cytotoxic doses of NLS peptide specifically prevented the nuclear translocation of NF-κB and the subsequent binding to a consensus DNA sequence, thus indicating that both cell types in the atherosclerotic plaque are susceptible to the NLS peptide action. Our results with vascular cells are in line with previous studies on leukocytes, endothelial cells, and hepatic cells that have also reported an attenuated inflammatory response by different peptide[33,35] and nonpeptide[36] nuclear import inhibitors.

The nuclear import pathway of NF-κB family members is mediated by a heterodimeric receptor consisting of importin β, which mediates interactions with the nuclear-pore complex, and the adaptor protein importin α, which directly binds the NLS motif.[9] The specificity of NF-κB proteins to importin α molecules is different and changes

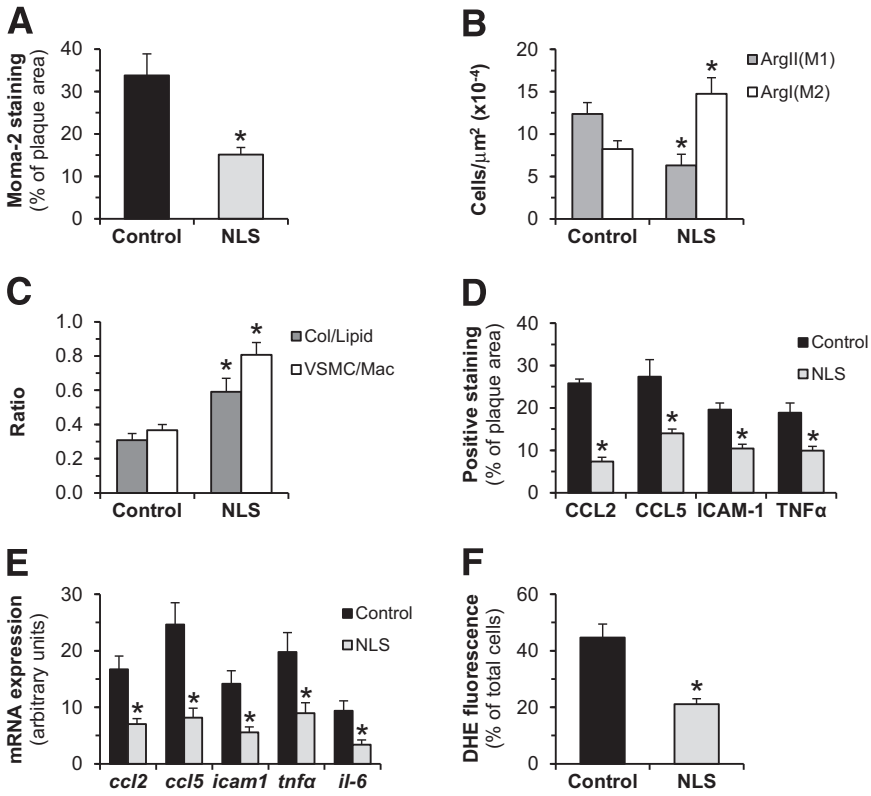

Figure 8 Quantification of atherosclerotic plaque characteristics in control and NLS-treated mice. **A:** Macrophage content (Moma-2—positive area). **B:** Arg II (M1 marker) and Arg I (M2 marker) positive cell counts per lesion area. **C:** Plaque stability features are expressed as the ratio of collagen (Col)/lipid (picrosirius red/oil-red-O area) and VSMCs/macrophages (Mac; α-SMA/Moma-2 area). **D:** Chemokine, adhesion molecule and cytokine content (immunoperoxidase staining). **E:** Inflammatory gene expression in aortic tissue was analyzed by real-time PCR, normalized by GAPDH, and expressed in arbitrary units. **F:** Superoxide production in aortic sections expressed as percentage of red DHE fluorescence versus total cell nuclei. Bars represent means ± SEM of 10 animals per group. $*P < 0.05$ versus control.

with the composition of the imported dimer.[7,11] To our knowledge, our study is the first to demonstrate in VSMCs that the classic NF-κB complex (p65-p50) is preferentially transported by importin α3 and, to a lesser extent, by α4 and α5 molecules. Furthermore, NLS peptide specifically impeded the nuclear import of NF-κB mediated by importin α isoforms in vascular cells, thus resulting in reduced transcription of NF-κB—dependent genes. These data are in agreement with a recent study,[35] in which peptide inhibition of NF-κB/importin α3 interaction decreased cytokine expression in hepatoma cells.

Leukocytes are involved in each step of atherosclerosis lesion development. Leukocyte adhesion is a starting point of fatty streak formation and is mediated by different cell adhesion molecules, such as ICAM-1, in which gene deletion results in atherosclerotic plaque reduction in mice.[37] Monocyte recruitment, particularly at the periphery of the lesion, also contributes to lesion expansion and involves the local expression of chemotactic proteins, including the NF-κB—regulated chemokines CCL2 and CCL5.[38,39] In fact, chemokine antagonists and NF-κB inhibitors are able to reduce monocyte migration and adhesion *in vitro*.[39,40] Within lesions, leukocytes produce cytokines that promote the migration of VSMCs into the intima and stimulate cell replication, and also secrete reactive oxygen species and matrix metalloproteases involved in plaque remodeling and destabilization.[37,38] Our work demonstrates that NLS peptide inhibits the expression of genes involved in leukocyte infiltration (ICAM-1, CCL2, and CCL5) in LPS-stimulated VSMCs and macrophages and in atherosclerotic plaques, which is consistent with the reduced macrophage accumulation in aortic lesions from our NLS-treated mice. Furthermore, the prevention of vascular inflammation by NLS peptide was associated with a reduced expression of TNF-α and IL-6 cytokines, two important autocrine/paracrine factors in vascular cells that amplify the local inflammatory response in the lesion during atherogenesis.[38]

Previous studies have demonstrated that cytokines and bacterial endotoxins increase VSMC migration and accelerate neointimal formation and atherosclerosis,[41] and that the NF-κB pathway mediates the vascular cell migration and activation under inflammatory conditions.[42,43] Furthermore, regulation of NADPH oxidase by NF-κB also represents a mechanism by which pro-inflammatory factors induce oxidative stress in atherosclerosis.[44] Our results show that NLS peptide significantly inhibits migration and NADPH oxidase—dependent superoxide production induced by LPS in VSMCs and macrophages. These antimigratory and antioxidant effects, in conjunction with the reduced expression of inflammatory genes *in vitro* and *in vivo*, prompted us to speculate that NLS peptide could be clinically beneficial as an anti-atherosclerotic agent.

Collectively, these results demonstrate that peptide inhibition of the NF-κB nuclear translocation reduces inflammatory response and oxidative stress in vascular cells and macrophages and has an atheroprotective effect in mice. These properties make cell-permeable NLS peptide a

potential prevention/intervention strategy to inhibit inflammation in cardiovascular diseases.

References

1. Ross R: Atherosclerosis: an inflammatory disease. N Engl J Med 1999, 340:115−126
2. Libby P, Ridker PM, Hansson GK: Progress and challenges in translating the biology of atherosclerosis. Nature 2011, 473:317−325
3. Gomez-Guerrero C, Mallavia B, Egido J: Targeting inflammation in cardiovascular diseases: still a neglected field? Cardiovasc Ther 2012, 30:e189−e197
4. Collins T, Cybulsky MI: NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J Clin Invest 2001, 107:255−264
5. Baker RG, Hayden MS, Ghosh S: NF-kappaB, inflammation, and metabolic disease. Cell Metab 2011, 13:11−22
6. Shih VF, Tsui R, Caldwell A, Hoffmann A: A single NFkappaB system for both canonical and non-canonical signaling. Cell Res 2011, 21:86−102
7. Fagerlund R, Kinnunen L, Kohler M, Julkunen I, Melen K: NF-{kappa}B is transported into the nucleus by importin {alpha}3 and importin {alpha}4. J Biol Chem 2005, 280:15942−15951
8. Goldfarb DS, Corbett AH, Mason DA, Harreman MT, Adam SA: Importin alpha: a multipurpose nuclear-transport receptor. Trends Cell Biol 2004, 14:505−514
9. Lange A, Mills RE, Lange CJ, Stewart M, Devine SE, Corbett AH: Classical nuclear localization signals: definition, function, and interaction with importin alpha. J Biol Chem 2007, 282:5101−5105
10. Friedrich B, Quensel C, Sommer T, Hartmann E, Kohler M: Nuclear localization signal and protein context both mediate importin alpha specificity of nuclear import substrates. Mol Cell Biol 2006, 26:8697−8709
11. Fagerlund R, Melen K, Cao X, Julkunen I: NF-kappaB p52, RelB and c-Rel are transported into the nucleus via a subset of importin alpha molecules. Cell Signal 2008, 20:1442−1451
12. Gilmore TD, Herscovitch M: Inhibitors of NF-kappaB signaling: 785 and counting. Oncogene 2006, 25:6887−6899
13. Gupta SC, Sundaram C, Reuter S, Aggarwal BB: Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy. Biochim Biophys Acta 2010, 1799:775−787
14. Pamukcu B, Lip GY, Shantsila E: The nuclear factor−kappa B pathway in atherosclerosis: a potential therapeutic target for atherothrombotic vascular disease. Thromb Res 2011, 128:117−123
15. Chang MS, Tam JP, Sanders-Bush E: Dissecting intracellular signaling pathways with membrane-permeable peptides. Sci STKE 2000, 2000:pl1
16. Yan LX, Robinson D, Veach RA, Liu D, Timmons S, Collins RD, Hawiger J: Peptide-directed suppression of a pro-inflammatory cytokine response. J Biol Chem 2000, 275:16774−16778
17. Hernandez-Vargas P, Ortiz-Munoz G, Lopez-Franco O, Suzuki Y, Gallego-Delgado J, Sanjuan G, Lazaro A, Lopez-Parra V, Ortega L, Egido J, Gomez-Guerrero C: Fcgamma receptor deficiency confers protection against atherosclerosis in apolipoprotein E knockout mice. Circ Res 2006, 99:1188−1196
18. Lopez-Franco O, Hernandez-Vargas P, Ortiz-Munoz G, Sanjuan G, Suzuki Y, Ortega L, Blanco J, Egido J, Gomez-Guerrero C: Parthenolide modulates the NF-kappaB-mediated inflammatory responses in experimental atherosclerosis. Arterioscler Thromb Vasc Biol 2006, 26:1864−1870
19. Depping R, Steinhoff A, Schindler SG, Friedrich B, Fagerlund R, Metzen E, Hartmann E, Kohler M: Nuclear translocation of hypoxia-inducible factors (HIFs): involvement of the classical importin alpha/beta pathway. Biochim Biophys Acta 2008, 1783:394−404
20. Hernandez-Presa MA, Gomez-Guerrero C, Egido J: In situ non-radioactive detection of nuclear factors in paraffin sections by Southwestern histochemistry. Kidney Int 1999, 55:209−214
21. Ortiz-Munoz G, Martin-Ventura JL, Hernandez-Vargas P, Mallavia B, Lopez-Parra V, Lopez-Franco O, Munoz-Garcia B, Fernandez-Vizarra P, Ortega L, Egido J, Gomez-Guerrero C: Suppressors of cytokine signaling modulate JAK/STAT-mediated cell responses during atherosclerosis. Arterioscler Thromb Vasc Biol 2009, 29:525−531
22. Lopez-Parra V, Mallavia B, Lopez-Franco O, Ortiz-Munoz G, Oguiza A, Recio C, Blanco J, Nimmerjahn F, Egido J, Gomez-Guerrero C: Fcgamma receptor deficiency attenuates diabetic nephropathy. J Am Soc Nephrol 2012, 23:1518−1527
23. Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltschmidt C, Baeuerle PA, Neumeier D: Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. J Clin Invest 1996, 97:1715−1722
24. Wilson SH, Best PJ, Edwards WD, Holmes DR Jr, Carlson PJ, Celermajer DS, Lerman A: Nuclear factor-kappaB immunoreactivity is present in human coronary plaque and enhanced in patients with unstable angina pectoris. Atherosclerosis 2002, 160:147−153
25. Martin-Ventura JL, Blanco-Colio LM, Munoz-Garcia B, Gomez-Hernandez A, Arribas A, Ortega L, Tunon J, Egido J: NF-kappaB activation and Fas ligand overexpression in blood and plaques of patients with carotid atherosclerosis: potential implication in plaque instability. Stroke 2004, 35:458−463
26. Bustos C, Hernandez-Presa MA, Ortego M, Tunon J, Ortega L, Perez F, Diaz C, Hernandez G, Egido J: HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in a rabbit model of atherosclerosis. J Am Coll Cardiol 1998, 32:2057−2064
27. Wolfrum S, Teupser D, Tan M, Chen KY, Breslow JL: The protective effect of A20 on atherosclerosis in apolipoprotein E-deficient mice is associated with reduced expression of NF-kappaB target genes. Proc Natl Acad Sci U S A 2007, 104:18601−18606
28. Gareus R, Kotsaki E, Xanthoulea S, van der Made I, Gijbels MJ, Kardakaris R, Polykratis A, Kollias G, de Winther MP, Pasparakis M: Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. Cell Metab 2008, 8:372−383
29. Goossens P, Vergouwe MN, Gijbels MJ, Curfs DM, van Woezik JH, Hoeksema MA, Xanthoulea S, Leenen PJ, Rupec RA, Hofker MH, de Winther MP: Myeloid IkappaBalpha deficiency promotes atherogenesis by enhancing leukocyte recruitment to the plaques. PLoS One 2011, 6:e22327
30. Norata GD, Marchesi P, Passamonti S, Pirillo A, Violi F, Catapano AL: Anti-inflammatory and anti-atherogenic effects of cathechin, caffeic acid and trans-resveratrol in apolipoprotein E deficient mice. Atherosclerosis 2007, 191:265−271
31. Chew P, Yuen DY, Koh P, Stefanovic N, Febbraio MA, Kola I, Cooper ME, de Haan JB: Site-specific antiatherogenic effect of the antioxidant ebselen in the diabetic apolipoprotein E-deficient mouse. Arterioscler Thromb Vasc Biol 2009, 29:823−830
32. Orange JS, May MJ: Cell penetrating peptide inhibitors of nuclear factor-kappa B. Cell Mol Life Sci 2008, 65:3564−3591
33. Liu D, Liu XY, Robinson D, Burnett C, Jackson C, Seele L, Veach RA, Downs S, Collins RD, Ballard DW, Hawiger J: Suppression of staphylococcal enterotoxin B-induced toxicity by a nuclear import inhibitor. J Biol Chem 2004, 279:19239−19246
34. Letoha T, Somlai C, Takacs T, Szabolcs A, Jarmay K, Rakonczay Z Jr, Hegyi P, Varga I, Kaszaki J, Krizbai I, Boros I, Duda E, Kusz E, Penke B: A nuclear import inhibitory peptide ameliorates the severity of cholecystokinin-induced acute pancreatitis. World J Gastroenterol 2005, 11:990−999
35. Xue H, Chen B, Fan Y, Palikhe M, Li Y: The inhibitory effect of polypeptide cSN50 on alcoholic hepatic injuries through blocking the binding of NF-kappaB to importin alpha. Scand J Gastroenterol 2011, 46:931−940
36. Funakoshi T, Yamashita K, Ichikawa N, Fukai M, Suzuki T, Goto R, Oura T, Kobayashi N, Katsurada T, Ichihara S, Ozaki M, Umezawa K, Todo S: A novel NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin, ameliorates inflammatory colonic injury in mice. J Crohns Colitis 2012, 6:215−225

37. Cybulsky MI, Lichtman AH, Hajra L, Iiyama K: Leukocyte adhesion molecules in atherogenesis. Clin Chim Acta 1999, 286: 207–218
38. Tedgui A, Mallat Z: Cytokines in atherosclerosis: pathogenic and regulatory pathways. Physiol Rev 2006, 86:515–581
39. Braunersreuther V, Mach F, Steffens S: The specific role of chemokines in atherosclerosis. Thromb Haemost 2007, 97:714–721
40. Riou S, Mees B, Esposito B, Merval R, Vilar J, Stengel D, Ninio E, van Haperen R, de Crom R, Tedgui A, Lehoux S: High pressure promotes monocyte adhesion to the vascular wall. Circ Res 2007, 100: 1226–1233
41. Lee SO, Jeong YJ, Yu MH, Lee JW, Hwangbo MH, Kim CH, Lee IS: Wogonin suppresses TNF-alpha-induced MMP-9 expression by blocking the NF-kappaB activation via MAPK signaling pathways in human aortic smooth muscle cells. Biochem Biophys Res Commun 2006, 351:118–125
42. Zahradka P, Werner JP, Buhay S, Litchie B, Helwer G, Thomas S: NF-kappaB activation is essential for angiotensin II-dependent proliferation and migration of vascular smooth muscle cells. J Mol Cell Cardiol 2002, 34:1609–1621
43. Jiang P, Xu J, Zheng S, Huang J, Xiang Q, Fu X, Wang T: 17beta-Estradiol down-regulates lipopolysaccharide-induced MCP-1 production and cell migration in vascular smooth muscle cells. J Mol Endocrinol 2010, 45:87–97
44. Manea A, Tanase LI, Raicu M, Simionescu M: Transcriptional regulation of NADPH oxidase isoforms, Nox1 and Nox4, by nuclear factor-kappaB in human aortic smooth muscle cells. Biochem Biophys Res Commun 2010, 396:901–907