Peptide-siRNA nanoparticles targeting NF-κB p50 mitigate experimental abdominal aortic aneurysm progression and rupture

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Peptide-siRNA nanoparticles targeting NF-κB p50 mitigate experimental abdominal aortic aneurysm progression and rupture

Huimin Yan, Ying Hu, Antonina Akk, Samuel A. Wickline, Hua Pan, Christine T.N. Pham

Abstract

Abdominal aortic aneurysm (AAA) is a progressive vascular condition associated with high risk of mortality if left untreated. AAA is an inflammatory process with excessive local production of extracellular matrix degrading enzymes, leading to dilatation and rupture of the abdominal aorta. We posit that targeting NF-κB, a signaling pathway that controls inflammation, will halt AAA progression and prevent rupture. In an elastase-induced AAA model we observed that NF-κB activation increased progressively post-elastase perfusion. Unexpectedly, we found that AAA progression was marked by predominant nuclear accumulation of the NF-κB p50 subunit at the exclusion of p65. Using the amphipathic peptide p5RHH to form nanocomplexes with siRNA, we sought to mitigate AAA progression by knocking down the expression of different NF-κB subunits. We found that the administration of NF-κB p65 siRNA was only beneficial when given early (day 3 post-elastase perfusion) while p50 siRNA was still effective in mitigating elastase-induced AAA even when delivery was delayed until day 5.

1. Introduction

Abdominal aortic aneurysm (AAA) is a progressive vascular condition that accounts for approximately 15,000 deaths per year in the United States due to rupture. AAA is a complex disease associated with male gender, advanced age, hypertension, hypercholesterolemia, coronary artery disease, atherosclerosis, and cigarette smoking. Open surgical repair of AAA is an effective option in preventing death from rupture. Although effective, open surgical repair is recommended only for large aneurysms (>5.5 cm in diameter) and can be associated with high post-operative mortality. Given the high mortality rate associated with surgical treatment, the advanced age of patients with AAA, which may preclude them from undergoing major surgery, medical treatment represents an attractive alternative approach.

It is generally accepted that AAA is an inflammatory condition. Key components of the inflammatory process characterizes AAA include production of matrix metalloproteinases (MMPs) that facilitate the recruitment of inflammatory cells to the site of tissue injury and help degrade the extracellular matrix (ECM). Recruited leukocytes release pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-12/23, interferon gamma (IFN-γ) that accelerate experimental AAA. Despite their proven role in experimental models, clinical trials aimed at reducing the activity of MMPs to mitigate AAA progression have so far met with mixed results.

NF-κB is a signaling pathway that controls gene expression involved in a myriad of cellular responses, including immunity and inflammation. The role of NF-κB in the promotion of MMP expression and AAA...
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2

2.2. p50 siRNA specificity

ON-TARGET plus mouse Nkhl siRNA smart pools were ordered from Dharmacon and the p50 siRNA sequences were: 1) 3’-CUG-CAAAGGGUAUCUGUAU-5’; 2) 3’-GGAACCCGACAGCUUU-5’; 3) 3’-GGAUUUGUACUGCUUAU-5’; 4) 3’-UAUAGGGAACCGUAUA-5’. Raw 264.7 cells at 0.5 × 10^6 cells/well were seeded in 6-well plates in 10 % FBS DMEM culture medium. The cells were transfected with p5RHH-p50 siRNA NP mixed with Opti-MEM (final concentration of p50 siRNA was 50 nM or 100 nM) at 1 mL/well for 5–6 h at 37 °C. The cells were washed and fresh DMEM culture medium added. After 48 h incubation in culture medium, the cells were lysed in Pierce™ RIPA buffer (Cat #: 89900 Thermo Fisher Scientific) with HalTM Protease and Phosphatase Inhibitor Cocktail (Cat #:78442, Thermo Fisher Scientific), sonicated in lysis buffer, and centrifuged at 18,000 g for 15 min at 4 °C. Protein content was quantified and equivalent amount fractionated by SDS-PAGE under reducing condition. Membranes were blotted with anti-NF-κB p65 rabbit mAb (D14E12 Cat #: 8242, 1:1000, Cell Signaling Technology, Danvers, MA), anti-NF-κB p50 rabbit mAb (D4P4D, Cat #13586, 1:1000, Cell Signaling Technology, Danvers, MA) or anti-mouse β-actin (1:500 dilution; Cat # sc-1615, Santa Cruz Biotechnology). The blots were washed and incubated with HRP-conjugated anti-rabbit IgG (1:2000, Jackson ImmunoResearch Laboratories). Bands were visualized using a SuperSignal Chemiluminescent substrate Kit (Cat # 34080, Thermo Scientific).

2.3. p5RHH-siRNA nanoparticle (NP) preparation and characterization

p5RHH peptide (provided by Genscript) was dissolved at 20 mM in DNAse-, RNase-, and protease-free sterile purified water (Cellgro) and stored in 5 μL aliquots at −80 °C before use. The scrambled (eGFP siRNA), p65, or p50 siRNAs were ordered from Sigma-Aldrich, dissolved at 100 μM in 1 × siRNA dilution buffer (Thermo Scientific), and stored in 5 μL aliquots at −80 °C before use. 10 μg Sodium Hyaluronate (Cat # HA1M-1, Lifecore Biomedical) was dissolved in 1 mL HBSS with Ca^{2+} and Mg^{2+} (Gibco, Life Technologies) by sonification for 1 h and then ultracentrifuged at 90,000 g for 40 min. The supernatant was aliquoted at 50 μL and stored at −80 °C until use. The p5RHH-siRNA NPs were prepared by mixing p5RHH and siRNA at a peptide:siRNA ratio of 100:1 in HBSS with Ca^{2+} and Mg^{2+}: 1 μL (10 μmol) of p55 (5’-GGA-GUACCGGAAUGUA-3’), p50 (5’-CAUGGGGAGUAGAAGAAC-3’) or scrambled siRNA (5’-GAAACCGUGGCGGCUUUGACGC-3’) was added to 98.5 μL of HBSS with Ca^{2+} and Mg^{2+} and mixed well, then 0.5 μL p5RHH was added to the mixture and incubated at 37 °C for 40 min. After incubation, 5 μL of hyaluronic acid (HA) solution (10 mg/mL, Cat # FI177, Sigma-Aldrich) was added to the assembled NPs and incubated on ice for 5 min. The HA-coated p5RHH-siRNA NPs were then ready for injection. Non-coated p5RHH-siRNA NPs were prepared by mixing the same amount of scrambled siRNA, p65 or p50 siRNA with 0.5 μL of p5RHH, placed on ice for 10 min without the addition of HA.

Nano particle size was measured by transmission electron microscopy (TEM, performed by the Washington University Center for Cellular Imaging, https://wucci.wustl.edu/) and by dynamic light scattering (DLS) using NanoBrook Omni (Brookhaven) and reported on a number basis for comparison with TEM imaging. Zeta potential was measured by Phase Analysis Light Scattering (PALS) by using NanoBrook Omni (Brookhaven). The TEM images were loaded onto ImageJ and the average size was acquired based on 3 independent NP samples and ~500–1500 particles were measured per sample.

2.4. Murine elastase-induced AAA

Only male mice were used in these studies, as AAA is a disease with strong male predominance. Briefly, 8- to 10-week-old male wild-type (WT) C57BL/6J mice were anesthetized with an i.p. injection of ketamine (87 mg kg^-1), xylazine (13 mg kg^-1), and acepromazine (2 mg kg^-1) KXA cocktail. For post-op pain control mice were administered Buprenorphine-SR (0.5 mg kg^-1) 1 h prior to surgery and the effect was expected to last 72 h. Immediately prior to surgery, lidocaine (0.5 %) was injected into the subcutaneous space below the planned incision line. A laparotomy was then performed under sterile conditions. The infrarenal aorta, from the left renal vein to the aortic bifurcation was isolated, ligated and perfused for 5 min and 30 s with a solution containing 0.145 U/mL type 1 porcine pancreatic elastase (Cat # E-1250, Sigma-Aldrich, St Louis, MO) via infusion pump. The tie was released and the maximal post-perfusion aortic diameter was measured with a calibrated ocular grid and the aortotomy closed. On day 14, unless otherwise indicated, a second laparotomy was performed following anesthesia with KXA cocktail and the aortic diameter measured prior to euthanasia and tissue procurement. In some studies WT mice were injected i.v. with peptide-siRNA NP (at 0.1 μmol siRNA per injection) on the indicated days. Five mmol/L Spermine NONOate, a nitric oxide (NO) donor (ab144522) was administered i.p. on day 4–12. The number of mice per genotype per treatment is indicated in the figure legends.

2.5. TGF-β blockade model of AAA rupture

C57BL/6J male WT mice (8- to 10-week-old) were anesthetized and analgesic described as above. The solution of pancreatic porcine elastase (Cat # E-1250, Type I, ≥4.0 units/mg protein, Sigma-Aldrich, St Louis, MO) was filtered through a 0.22 μm filter. A laparotomy was performed under sterile conditions and the abdominal aorta from below the left renal vein to the iliac bifurcation was isolated in situ circumferentially after careful dissection. Ten μL of heat inactivated or active pancreatic porcine elastase was topically applied onto the exposed segment of abdominal aorta for 10 min. The diameter was measured in situ after a 10 min-application of elastase. Where indicated, mice were injected i.p. 5 times/week with 250 μg of the anti-mouse TGF-β (BioXcell, clone 1D11.16.8) starting on the day of the surgery and continued until day 13 after surgery. On day 3, 5, 7 and 9 after surgery, HA-coated-p50 or p50 siRNA NP was administered i.v. Control mice received heat-inactivated or active elastase application without TGF-β antibody administration. The aortic diameter of mice that survived was re-measured in situ on day 8.
14. The entire infrarenal aorta was harvested on day 14 and analyzed by histology.

2.6. Aortic morphometry

After aortic diameter measurement, mice were euthanized and perfused with 8% formaldehyde in PBS for 5 min. The abdominal aorta was dissected and excised. After photography, all the aortas were frozen in –80 °C for histological analysis. Mice were monitored closely for physical signs of rupture (sudden death) and immediately laparotomized and rupture recorded photographically. All aortic morphometry was performed by an investigator blinded to the experimental groups.

2.7. Verhoeff–van Gieson (VVG) staining

VVG staining was performed on OCT-embedded 9 μm frozen cross-sections of mouse AAA tissue using the Elastic Stain Kit (Cat#: HT25, Sigma-Aldrich). Briefly, the cross-sections were fixed with 4% PFA and stained in Working Elastic Stain Solution for 10 min and differentiated in Working Ferric Chloride Solution. Van Gieson Solution was used to counterstain collagen fibers red. The sections were dehydrated with Xylene and mounted. The images were acquired with a Leica Digital Microscope 2000 with a Leica DMC 4500 camera using Leica Application Suite (LAS) X software or ZEISS AXIO Imager M2 attached with ORCA-Flash4.0 LT PLUS Digital CMOS camera C11440.

2.8. Immunohistochemistry

Immunohistochemical staining was performed on OCT-embedded 9 μm frozen cross-sections of mouse AAA tissue. Rabbit anti-mouse TNF-α (1:100 dilution; Cat# ab34674, Abcam, Cambridge, MA), biotin-IL-12p40 (1:100 dilution; Cat# 505302, Biolegend, San Diego, CA), rat anti-mouse IL-10 (1:100 dilution; Cat# 11-7101-82, eBioscience, San Diego, CA), rat anti-mouse MMP-9 (1:200 dilution; Cat# 116103, R&D, Minneapolis, MN), rabbit anti-mouse iNOS (1:100 dilution; Cat# ab3523, Abcam, Cambridge, MA), rat anti-mouse MOMA-2 (1:200 dilution; Cat# ab33451, Abcam, Cambridge, MA), rabbit anti-mouse pp65 (1:100 dilution; Cat# ab28856, Abcam, Cambridge, MA) or pp50 (1:100 dilution; Cat# NB100-82074, Novus Biologicals, Littleton, CO) primary antibodies were applied to the frozen cross-sections for 1 h at room temperature (RT) followed by the appropriate HRP-conjugated secondary antibodies. Data presented was derived from 6 to 9 serial cross-sections that spanned the entire abdominal aorta, with 5–8 aortas per treatment.

2.9. IVIS imaging

Mice were injected i.v. with HA-coated or Naked p5RHH-Cy5-p50 siRNA NP on day 9 post elastase perfusion. The mice were sacrificed at 4 or 20 h after injection and perfused with 0.9% saline. The aorta, heart, liver, spleen, lung, kidney and bladder were harvested and fluorescent images of the organs were acquired with IVIS® Spectrum CT In Vivo Imaging System (Perkin Elmer). The settings (excitation, 640 nm; emission, 700 nm; exposure time, 1 s) were used for image acquisitions at various time points after i.v. injection of the NP. The total luminescence density for different organs (n = 3/each organ) were analyzed with Living Image software (PerkinElmer) and calibrated as radiant efficiency. The aortas were frozen and embedded in OCT. The aortic 9 μm frozen cross-sections were stained with F-actin (Cat# T7471, 1:200 dilution, Invitrogen at Thermo Fischer Scientific) or FITC-conjugated anti-CD44 (Pgp-1) mAb (Cat# 01224D, 1:200 dilution, PharMingen). The slides were mounted with VECTASHEILD containing 4', 6-diamino-2-phenylindole (DAPI) (Cat# D3571 Molecular Probes at Thermo Fisher Scientific). The images were visualized and acquired with ZEISS LSM 880 Confocal Laser Scanning Microscope.

2.10. Confocal microscopy of AAA tissue

After fixation and permeabilization with 0.05% Triton X-100/PBS the frozen aortic cross-sections were blocked in 8% BSA and incubated with rat anti-mouse MOMA-2 (1:200 dilution; Cat# ab33451, Abcam, Cambridge, MA) and rabbit anti-mouse pp65 (1:100 dilution; Cat# ab28856, Abcam, Cambridge, MA) or pp50 (1:100 dilution; Cat# NB100-82074, Novus Biologicals, Littleton, CO). The FITC-conjugated donkey anti-rabbit (1:100 dilution; Cat# 711-095-152, Jackson Immunoresearch Laboratories) or Rhodamine Red-X-conjugated donkey anti-rat secondary antibody (1:100 dilution; Cat# 712-295-153, Jackson Immunoresearch Laboratories) was applied to sections. Nuclei were counterstained with DAPI. All images were visualized and acquired with ZEISS LSM 880 Confocal Laser Scanning Microscope. Images were loaded onto ImageJ (http://rsb.info.nih.gov/ij) for analysis. The number of cells that stained positively for pp65 or pp50 in the nucleus or cytoplasm were enumerated. The data was obtained from 4 to 6 non-overlapping fields per aortic section and 3–5 sections per aorta, 5–6 aortas per treatment.

2.11. In vitro NP uptake by bone marrow-derived macrophages (BMDM)

Bone marrow from C57BL/6 WT mice (Cat# 000664, Jackson Laboratory, Bar Harbor, ME, USA) was cultured in complete RPMI-1640 medium with 10% fetal bovine serum (FBS) containing recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF) (10 ng/mL, Cat# PMC2015, Thermo Fisher Scientific, Waltham, MA, USA) for 7 days at 37 °C. Cultured cells were plated in 12-well plates at 0.5 × 10^6 cells/well overnight. The cells were starved for 30 min prior to culture in Opti-Minimum Essential Medium (MEM) (Thermo Fisher Scientific) containing HA-coated Cy3-labeled p5RHH-siRNA NP or Naked Cy3-labeled p5RHH-siRNA NP at 37 °C for 4 h. The cells were collected with ethylenediaminetetraacetic acid (EDTA) solution (1:10 dilution with phosphate buffered saline (PBS)), spun down, resuspended in Flow Cytometry Staining (FACS) buffer, and analyzed by flow cytometry. For confocal analysis, cells were seeded at 1.0 × 10^6/well in 8-well Nunc™ Lab-Tek™ II Chamber Slide™ Glass slide system (Cat#:177402 Thermo Fisher) and transfected with HA-coated Cy3-labeled NP or naked Cy3-labeled NPs at 37 °C for 4 h. Then the cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100/PBS, and blocked with 8% BSA. F-actin (Cat# T7471, 1:200 dilution, Invitrogen at Thermo Fisher Scientific) was added and the cells mounted with DAPI. The images were captured by a ZEISS LSM 880 confocal laser scanning microscope.

2.12. In vitro NP uptake by human monocytic cell line (THP1)

The THP1 cell line was maintained in RPMI 1640 medium supplemented with 10% FBS and 2 mmol/L L-glutamine. Cells were seeded 0.5 × 10^6 per chamber of Nunc™ Lab-Tek™ II Chamber Slide™ System (Cat# 154534, Thermo Fisher Scientific). THP1 cells were differentiated using 200 nM phorbol 12-myristate 13-acetate (Cat# 1585, Sigma-Aldrich) for 2 days. After the initial 2 days, cells were washed with PBS and cultured in fresh RPMI 1640 (10% FBS, 1% L-glutamine) for one additional day. On the day of experiment, cells were washed with PBS and CD44 antibody (Cat# MU310, BioGenex Laboratories) was added at 1:100 dilution in Opti-Minimum Essential Medium (MEM) (Thermo Fisher Scientific) for 30 min at 4 °C. HA-coated NP was added to the cells at 1:100 ratio (v/v) and the cells were cultured for 26 h at 37 °C, washed with PBS and fixed with 2% PFA on 1 h. The cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100/PBS, and blocked with 8% BSA. F-actin (Cat# T7471, 1:200 dilution, Invitrogen at Thermo Fisher Scientific) was added and the cells mounted with DAPI. The images were captured by a Leica DM 2000 microscope with a Leica DMC 4500 color camera and analyzed with Leica Application Suite (LAS) X software.
2.13. Western blotting

Organs were collected on day 14 and homogenized with Pierce™ RIPPA buffer (Cat#: 89900 Thermo Fisher Scientific) with Halt™ Protease and Phosphatase Inhibitor Cocktail (Cat#: 78442, Thermo Fisher Scientific) and then sonicated in the lysis buffer, and centrifuged at 18,000g for 15 min at 4 °C. Protein content was quantified and equivalent amount fractionated by SDS-PAGE under reducing conditions. Membranes were blotted with anti-NF-κB p65 rabbit mAb (D14E12 Cat#: 8242, 1:1000, Cell Signaling Technology, Danvers, MA), anti-NF-κB p50 rabbit mAb (D4P4D, Cat#: 13586, 1:1000, Cell Signaling Technology, Danvers, MA) or anti-mouse β-actin (1:500 dilution; catalog no. sc-1615, Santa Cruz Biotechnology). The blots were washed and incubated with HRP-conjugated anti-rabbit IgG (1:2000, Jackson ImmunoResearch Laboratories). Bands were visualized using a Super Signal Chemiluminescent substrate Kit (Cat#: 34080, Thermo Scientific).

2.14. Hematologic parameters and serum chemistries

On day 7 following the last NP administration, mice were euthanized and blood was collected from the inferior vena cava and cell counts, differentials and serum chemistries (hepatic and renal function) were assessed. Analysis was performed by the Washington University Department of Comparative Medicine.

2.15. In situ zymography

Gelatinase activity was assessed by incubating 9 μm unfixed frozen sections in DQ Gelatin (25 μg/mL; Cat# 12054; Invitrogen Molecular Probes) for 1 h at RT; 25 mmol/L EDTA was set for negative control. All zymographic images were acquired on a LEICA DM 2000 microscope attached with Digital Microscope Camera Leica DMC4500 and acquired with Leica Application Suite (LAS) X software. The quantification of the gelatinase activity was performed with ImageJ and presented as mean integrated density (IntDen). Data were analyzed from 6 to 9 cross sections per aorta, with 5–8 aortas per treatment.

2.16. TUNEL assay

DNA fragmentation associated with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was used to detect apoptotic cells (TUNEL⁺). Detection of apoptotic cells was performed on day 14 non-fixed frozen aortic sections using an In Situ Cell Death Detection Kit (Cat: 11–684-795-910, Roche). Briefly, the aortic sections were rinsed with PBS, then permeabilized with 0.5 % TWEEN-20/PBS for 15 min and blocked with 8 % BSA solution. Freshly prepared TUNEL reaction mixture, according to the manufacturer’s protocol, was applied to aortic sections for 1 h at 37 °C, rinsed 3 to 5 times with PBS, and mounted with VECTASHIELD mounting medium with DAPI (Cat: H-1200, Vector Laboratories). The TUNEL⁺ cell number cells were enumerated across the entire aortic section. Data represent 5–8 sections per aorta, 6–8 aortas per treatment.

2.17. Statistical analysis

Comparisons between two groups were performed by two-tailed, unpaired t-test without correction. Comparisons between multiple groups (≥ 3) were performed by one-way ANOVA followed by Bonferroni’s post-test to compare all groups of data. F test was used to compare variances within each group of data and the difference in variances was found to be not significant between groups. The Log Rank (Mantel-Cox) test was used for survival analysis. Data are presented as the mean ± SEM or SD. A P value <0.05 was considered significant.

3. Results

3.1. Characterization of p5RHH-siRNA nanoparticles

We have previously reported that the cationic amphiphatic peptide designated as “p5RHH” (VLTGGLPALISWIRRRHHRC) is capable of siRNA transfection with an IC₅₀ as low as 25 nM without significant cytotoxicity at all tested doses [20]. The mixing of p5RHH with siRNA at 100:1 ratio (mol:mol; peptide:siRNA) in HBSS followed by incubation at 37 °C for 40 min led to a non-covalent coupling, self-assembling NP [20,21]. We further functionalized the NP with a coating of hyaluronic acid (HA, MW 750 kDa-1.0 MDa), a synthetic polysaccharide that binds to CD44 expressed on a wide range of cells, including macrophages (Fig. 1A) [22,23]. Our protocol yielded an HA-coated p5RHH-siRNA NP of 72.20 ± 2.48 nm in diameter as measured by transmission electron microscopy (TEM), 81.81 ± 3.19 nm in diameter as measured by dynamic light scattering (DLS), with a polydispersity of 0.26 ± 0.09, and a zeta potential of −31.08 ± 4.54 mV (Fig. 1B–C). The slightly larger size measured by DLS may be attributed to interparticle interaction, leading to aggregates, which DLS could not differentiate [24]. TEM on the other hand allows for direct visualization of the particles and exclusion of aggregates from calculation. Addition of the highly negative HA to the self-assembled NP led to an overall negatively charged polyplex (zeta potential of approximately −31 mV) suggesting that coating was complete. In vitro, we showed that HA-coated p5RHH-siRNA NP was taken up more efficiently by bone marrow-derived macrophages than naked, non-coated NP (Fig. 2A–B) [25]. CD44 blockade significantly decreased the cell’s mean fluorescent intensity, confirming that less fluorescein-labeled, HA-coated siRNA NPs were taken up inside the cells in the presence of blocking anti-CD44 antibody (Fig. 2C–D).

3.1.1. HA-coated p5RHH-siRNA NP uptake in vivo in an experimental AAA model

We used the well-established elastase-induced AAA model in which transient porcine elastase perfusion of the (intraluminal) infrarenal abdominal aorta on day 0 leads to aneurysmal dilatation at day 14 [26]. AAA is defined as an increase in the aortic diameter (AD) of >100 % over the pre-elastase perfusion measurements [26]. In this model, elastase perfusion led to an immediate increase in AD of ~70 % on day 0 (Fig. 3A) [27]. On day 14, WT C57BL/6 mice uniformly developed AAA, which was accompanied by aortic dilatation and fragmentation of the internal elastic lamellae (Fig. 3B). We injected Cy5-labeled, p5RHH-siRNA NP on day 9 post-elastase perfusion and performed semi-quantitative in vivo imaging studies (IVIS) at 4 and 20 h (Fig. 3C–D). We found accumulation of Cy5-labeled NP in the aortic wall, especially at 4 h, but no significant difference between naked and HA-coated NP by IVIS (Fig. 3C–D). We also examined the distribution of NP in major organs (Suppl Fig. 1). We found the highest NP accumulation in the kidney followed by liver at 4 h, confirming our previous report that the NP is renally cleared [28]. Of note, immunofluorescence in the kidney was lower in the HA-coated NP at both time points (Suppl Fig. 1). To examine the in vivo localization of the NP at the cellular level, we used confocal microscopy to visualize the distribution/uptake of naked and HA-coated NP in the abdominal aortic wall tissue. We observed naked NP accumulating mainly around small venules while HA-coated NP was widely taken up by individual cells in the adventitia, many of which were CD44⁺ (Fig. 3E–F). Taken together these results strongly support the notion that the HA coating enhances the cellular uptake of NP in vivo in this model.

3.2. NF-κB knockdown in the mitigation of elastase-induced AAA

To begin to explore the role of NF-κB in the progression of elastase-induced AAA, we examined the pattern of NF-κB phosphorylation over time. We observed a marked increase in NF-κB activation from day 3 to day 14 post-elastase perfusion (Fig. 4A), as evidenced by the high
number of phosphorylated-p65-positive (pp65+) and pp50+ cells populating the abdominal aortic wall at day 14 (Fig. 4B). Of note, the number of pp50+ cells was significantly higher on day 14 (Fig. 4A–B). We also observed that on day 14 most of pp65 was excluded from the cell nucleus in AAA tissue (Fig. 4C–D). In contrast, pp50 was found almost exclusively in the nuclei (Fig. 4C–D). These results suggest that p65 and p50 may contribute differentially to the progression of experimental AAA at different stages of disease.

To examine the contribution of each NF-κB subunit in elastase-induced AAA, we used the p5RHH peptide to deliver p50 or p65 siRNA in vivo. We chose sequences of p50 siRNA (Suppl. Fig. 2) or p65 siRNA [18,28] that have been shown to specifically knock down the expression of their intended targets. Administration of the HA-coated p5RHH-p50 siRNA NP (0.1 μmol siRNA per treatment per day) on days 5 and 8 post-elastase perfusion (Fig. 5A) led to significant reduction in the AD by day 14 (AD = 0.79 ± 0.08 mm in HBSS controls vs. AD = 0.51 ± 0.08 mm in p50 siRNA NP, P < 0.001) (Fig. 5B). Mice receiving HA-coated scrambled siRNA NP or non-coated (naked) p5RHH-p50 siRNA NP (at the same siRNA dose) were not protected against aneurysm development and progression (AD = 0.77 ± 0.09 mm in scrambled siRNA NP and AD = 0.76 ± 0.07 mm in naked p50 siRNA NP on day 14) (Fig. 5B). The administration of p50 siRNA led to a reduction in both pp50+ and pp65+ cells (Fig. 5C). We reasoned that the decrease in pp65+ cells following p50 siRNA delivery can be explained by the marked suppression of inflammation, as evidenced by the significant decrease in inflammatory cell recruitment/expansion and overall MMP activity (Fig. 5D–E). Moreover, on day 14, we observed marked suppression of inducible nitric oxide synthase (iNOS) expression (Fig. 5F) along with significantly reduced apoptotic index, as evidenced by a marked decrease in the number of TUNEL+ cells (Fig. 5G). HA-coated p5RHH-p50 siRNA NP treatment also largely abrogated cytokine responses (TNF-α and IL-12p40, IL-10) in abdominal aortas (Suppl. Fig. 3). While HA-coated p5RHH-siRNA NP delivery suppressed the expression of p50 in the abdominal aorta, it did not affect p50 (or p65) protein expression in off-target organs such as liver, spleen, brain, kidney and heart (Suppl. Fig. 4A). In addition, repeated NP administration did not affect hematologic parameters or liver/kidney function (Suppl. Fig. 4B).

On the other hand, p5RHH-p65 siRNA NP administration started on day 5 following elastase perfusion did not mitigate AAA progression (Suppl. Fig. 5A–C), suppress macrophage accumulation, MMP and iNOS upregulation, or cell death (Suppl. Fig. 5D–G). Given that p565 was largely excluded from the nucleus on day 14, we posit that this subunit likely contributes to the inflammatory process at an earlier stage of the disease and knockdown of p65 prior to day 5 might have an effect on AAA progression. Indeed, HA-coated p5RHH-p65 siRNA NP administration starting on day 3 and continued on days 5 and 8 post-elastase perfusion (Fig. 6A) led to significant mitigation of AAA on day 14 (Fig. 6B). Mitigation of disease was accompanied by a decrease in the number of pp65+ and pp50+ cells (Fig. 6C), although not as profoundly as observed with p50 knockdown (Fig. 5C). The decrease in pp65+ and pp50+ cells was likely the result of reduced inflammatory responses (Fig. 6D–G).

Additionally, we posited that HA-coated p5RHH-p50 siRNA NP mitigated AAA progression via suppression of iNOS activity and nitric oxide (NO) production. To further determine whether exogenous NO will reverse the effects of p50 siRNA NP, we administered an NO donor, spermine NONOate to mice that received p50 siRNA NP. Spermine NONOate administration reversed the mitigating effects following HA-coated p5RHH-p50 siRNA NP delivery (AD = 0.49 ± 0.06 mm in p50 siRNA NP vs AD = 0.68 ± 0.10 in mice receiving NO donor + p50 siRNA NP, P < 0.01) (Suppl. Fig. 6A). Histological analysis showed that NONOate administration also increased the number of iNOS+ cells, MMP activity and apoptotic index (Suppl. Fig. 6B–D). Taken together these results suggest that p50 modulates AAA progression via the production of NO. The results also place NF-κB upstream of iNOS induction in AAA genesis.

Table 1. Characteristics of HA-NP

<table>
<thead>
<tr>
<th>Particle composition</th>
<th>Peptide : siRNA (mol:mol)</th>
<th>100:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average size (nm)</td>
<td>TEM</td>
<td>72.20 ± 2.48</td>
</tr>
<tr>
<td>Average size (nm)</td>
<td>DLS</td>
<td>81.81 ± 3.19</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
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<td>-31.08 ± 4.54</td>
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<tr>
<td>Polydispersity</td>
<td></td>
<td>0.26 ± 0.09</td>
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Fig. 1. Characterization of hyaluronic acid (HA)-coated p5RHH siRNA nanoparticle (NP). (A) NP was prepared by mixing p5RHH and siRNA at a peptide:siRNA ratio of 100:1 in HBSS with Ca2+ and Mg2+ and incubated at 37°C for 40 min. The self-assembled NP was then incubated with HA for 10 min on ice. (B) TEM of HA-coated p5RHH-siRNA NP. Scale bar = 50 nm. (C) Average NP size distribution by TEM and DLS, zeta potential, and polydispersity. Average size (nm) ± SD obtained from 3 separate NP samples prepared simultaneously.
3.3. NF-κB knockdown in the prevention of AAA rupture

Since the elastase-induced AAA does not progress to rupture, we turned to a model developed by Lareyre and colleagues in which peri-aortic application of elastase combined with TGF-β inhibition leads to rapid aortic diameter expansion and rupture in 50% of experimental animals within 14 days [29]. The model preserves several features of human AAA, including elastin degradation, inflammatory cell infiltrates/responses, and intraluminal thrombus.

Elastase applied peri-adventitially to the abdominal aorta followed by systemic administration of TGF-β antibody on days 0, 3, 5, 7, 10, 13 (Fig. 7A) led to steady and dramatic enlargement of the abdominal aorta and rupture in some cases while periaortic elastase application alone only led to mild dilatation and heat-inactivated elastase was ineffective at inducing AAA (Fig. 7B–C). Staining of aortic sections with Verhoeff-Van Gieson (VVG) revealed significant elastin degradation in mice treated with elastase and anti-TGF-β antibody (Fig. 7D). AAA rupture occurred between days 7 and 14 after elastase application with approximately 47% of the mice exhibiting sudden death due to aortic rupture (Fig. 7E).

Micrometry measurements of the external aortic diameter were performed in situ on day 14 on the surviving mice (Fig. 7F). The peri-adventitial elastase application led to a modest increase in the aortic diameter (AD) in 100% of the mice compared with heat-inactivated (inactive) elastase application (1.128 ± 0.118 mm vs 0.110 ± 0.024 mm, P < 0.05). Blockade of TGF-β activity further enhanced AD dilation to mean increase in AD of 2.594 ± 0.202 mm (P < 0.001 compared with elastase application alone) (Fig. 7F). These findings suggest that TGF-β blockade after periaortic elastase application strongly promotes aneurysmal expansion.

To examine the role of p50 in AAA rupture, we administered HA-coated p5RHH-p50 siRNA NP i.v. to mice on days 3, 5, 7 and 9 after elastase application. Our study showed that HA-coated p5RHH-p50 siRNA NP administration delayed the onset of rupture (until day 12) and significantly protected mice from sudden death (85% survival rate in treated mice vs 53% survival rate in untreated mice, P < 0.05) (Fig. 7E). On the other hand, administration of p65 siRNA did not significantly alter the risk of sudden death (36% sudden death in p65 siRNA-treated vs 47% sudden death in untreated animals). NF-κB block of p50 and p65 knockdown did not significantly affect AAA expansion (increase in mean AD of 2.009 ± 0.198 mm in p50 siRNA-treated vs 1.861 ± 0.234 mm in p65 siRNA-treated vs 2.594 ± 0.202 mm in untreated animals, P > 0.05) (Fig. 7F) but significantly suppressed the inflammatory responses and oxidative stress, as evidenced by a marked reduction in TNF-α and MMP-9 expression, iNOS+ and apoptotic (TUNEL+) cells (Fig. 7G–J). These results suggest that there are additional pathways modulated by p50 that contribute to AAA rupture.
Fig. 3. In vivo uptake of HA-coated pSRRH siRNA NP. (A) Mice were transiently perfused with elastase on day 0. There was an immediate increase in aortic diameter (AD) post-perfusion after which the AD increased progressively over time. (B) Representative VVG stain on days 0 and 14 post-elastase. Scale bar = 200 μm (low magnification); 50 μm (high magnification) (C) Elastase-perfused mice were injected with naked or HA-coated Cy5-labeled pSRRH siRNA NP on day 9, IVIS of aortas were obtained 4 and 20 h after injection, and intensity of fluorescence quantified (D). Values represent mean radiant efficiency ± SEM (n = 3 aortas per treatment per time point). Aortas were harvested 4 h after Cy5-NP (magenta) injection and stained with F-actin (green) in (E) and CD44 (green) in (F). DAPI (blue). L = lumen. Scale bar = 100 μm (E, F); inserts 10 μm (E) 20 μm (F). ***P < 0.001, n.s. not significant.

Fig. 4. NF-κB activation in elastase-induced AAA. (A) Aortic sections were stained for phospho-p65 (pp65) or pp50 on days 3 and 14 and positive cells were enumerated. (B) Representative IHC of pp50 and pp65 on day 14. Scale bar = 200 μm (C) Day 14 aortic sections were stained for MOMA-2 (red) and pp65 or pp50 (green) and analyzed by confocal microscopy. DAPI stained nuclei blue. Co-localization appeared orange/yellow. Note the predominant nuclear localization of pp50 (arrows) versus the nuclear exclusion (cytoplasmic localization) of pp65. Scale bar = 20 μm. (D) Enumeration of nuclear versus cytoplasmic pp65+ and pp50+ cells on day 14. Values represent mean ± SEM derived from 4 to 6 non-overlapping fields per aortic section and 3–5 sections per aorta, n = 5–6 day 14 aortas. L = lumen. *P < 0.05, ***P < 0.001, n.s. not significant.
4. Discussion

Herein we establish that, in the AAA inflammatory microenvironment, continued NF-κB activation led to the accumulation of p50 in the nucleus at the exclusion of p65. We also establish that knockdown of p50 mitigated AAA progression even when initiation was delayed to a later-stage disease suggesting that p50 may be a potential target for small AAA treatment. Moreover, the administration of p50 siRNA, but not p65 siRNA in the TGF-β blockade model also significantly decreased the risk of AAA rupture and sudden death. These results altogether suggest that the timing of intervention is important and understanding the kinetics of the different signaling pathways involved in AAA development is paramount to the successful development of medical treatment approaches.

The NF-κB family comprises five proteins containing a Rel-homology domain: p65 (RelA), RelB, c-Rel, p50, and p52. Canonical activation of NF-κB leads to the heterodimerization of p65 and p50 subunits and their translocation to the nucleus where the p65 binds to inflammatory gene promoters, leading to pro-inflammatory gene transcription. With continued activation, the NF-κB p50 subunit can also homodimerize to give rise to a p50/p50 homodimer that has inhibitory activity since it lacks a transactivating domain [30]. Reports, however, have shown that p50/p50 homodimers can synergistically cooperate with IFN-γ to promote iNOS expression and functionalities [31]. The induction of iNOS leads to the production of reactive oxygen species such as NO that increases the expression of MMPs in aortic endothelial cells and the progression of experimental AAA [32,33]. That fact that p50 knockdown decreases iNOS expression and delivery of NO donor reverses the mitigating effects of p50 siRNA further support the notion that NF-κB functions upstream of iNOS activity.

While the canonical NF-κB p65/p50 heterodimer is the predominant form of functionally active NF-κB with pro-inflammatory activity, the p50/p50 homodimer regulation and function in inflammation are less well understood. Recent studies suggest that an adaptive cellular response to pro-inflammatory insult is the production of pro-resolving endogenous mediators, which are essential for the resolution of inflammation and restoration of homeostasis [34]. Resolvin D1 (RvD1), endogenously generated during the resolution of inflammation, has been shown to decrease AAA formation by inhibiting NETosis in a mouse model [35]. Moreover, RvD1 has been shown to stimulate the conversion of p105 to p50, promoting the production and nuclear localization of p50/p50 homodimer that competes with p65/p50 binding [36]. This scenario is reminiscent of the picture observed at day 14 in the elastase-induced AAA model. And although it is well appreciated that p50/p50 homodimer suppresses inflammatory cytokine production [37], studies have also demonstrated that p50/p50 homodimer can bind to IL-10 promoter, activating transcription of the cytokine [38]. And while IL-10 is considered anti-inflammatory in providing negative feedback in the control and resolution of inflammation and limiting local tissue injury [39] its effects are pleiotrophic and sometimes conflicting, depending on organ system and disease state [40]. The paradoxical role of IL-10 is exemplified in studies examining cardiac fibrosis wherein
human patients and mouse models of the disease demonstrated excess cardiac macrophages secreting IL-10, leading to an autocrine loop that further promoted recruitment of fibrogenic macrophages [41]. Deletion of IL-10 secreting macrophages improved organ dysfunction [41]. Therefore, suppression of IL-10 by p50 siRNA administration in the elastase-induced AAA model may have added benefits via its indirect effects on vascular remodeling.

TGF-β plays an essential role in extracellular matrix (ECM) remodeling and is believed to control tissue homeostasis in the injury/repair process. It is generally accepted that TGF-β modulates the induction of an anti-inflammatory phenotype and promotes the resolution of inflammation. The mechanism implicated in the protective role of TGF-β includes maintenance of homeostasis between ECM synthesis and degradation, activation of regulatory T cells, inhibition of inflammation, and abrogation of vascular smooth muscle cell dysfunction [42,43]. Knockdown of p50 and 65 partially interrupted the pro-inflammatory effects of TGF-β inhibition in AAA, including significantly suppressing macrophage expansion, overall MMP activity, iNOS expression and apoptotic index (TUNEL+ cells). However, suppression of MMP and iNOS activities was insufficient to mitigate aneurysm expansion and, in the case of p65 knockdown, alter the risk of rupture in this aggressive model. Thus, the cross-talk between TGF-β and NF-κB pathway signaling that promotes AAA expansion and rupture awaits further investigation.

To mitigate AAA progression and rupture we employed a peptide-based siRNA NP structure that represents the culmination of a number of specific sequence modifications to the amphipathic cationic peptide, melittin [20,21,44]. This 21 amino acid version of the peptide, called p5RHH, exhibits <40 % homology to melittin and has been proven safe and stable in vivo after systemic administration for delivery of both siRNA [28] and mRNA [45]. The mechanism by which the modified melittin-derived p5RHH peptide forms self-assembled nanostructures has been previously described [20,21]. The p5RHH peptide is amphipathic with a hydrophobic N-terminal segment and hydrophilic cationic C-terminal segment. Initial electrostatic interactions between the cationic p5RHH and anionic nucleotides facilitate the formation of strong noncovalent hydrogen bonds and hydrophobic forces [46]. The hydrogen bonding between acceptor histidine imidazole side chains and nucleotides is extinguished by pH-dependent protonation within acidifying endosomal structures to disassemble the peptide-nucleotide complex and release the siRNA and peptide. The stable tripartite complex protects the RNA from degradation in circulation, but upon pH-dependent disassembly occurring exclusively in the endosome, endosomal escape is complete due to endosomal membrane permeabilization by the free p5RHH peptide [21].

For this study, we functionalized the p5RHH-siRNA NP with a HA coating with the goal of enhancing macrophage uptake [25]. The highly negative HA coating is added in a terminal step to the cationic nanoparticle complex, which results in an overall negatively charged polyplex (zeta potential: approximately ~30 mV) that forestalls further nanoparticle growth. HA coating is completed by the incubation of self...

Fig. 6. In vivo delivery of HA-coated p5RHH-p65 siRNA NP in elastase-induced AAA. (A) Mice were perfused with elastase on day 0 and administered HBSS, HA-coated p5RHH-scrambled siRNA NP (Scram HA-NP), HA-coated p5RHH-p65 siRNA NP (p65 HA-NP), or non-coated p5RHH-p65 siRNA NP (Naked p65 NP) i.v. on days 3, 5, and 8 post-elastase perfusion (siRNA = 0.1 μmol per treatment per day). Increase in (AD), measured on day 14 as mm increase or % increase (B). Day 14 aortic sections were examined for pp50 or p65 (C), MOMA-2 (D), in situ MMP activity (E), iNOS (F), and TUNEL+ cells (G). Values represent mean ± SEM derived from 4 to 6 non-overlapping fields per aortic section and 3–5 sections per aorta, n = 5–6 aortas per treatment. L = lumen. Scale bars = 200 μm (D, F), 100 μm (E, G). *P < 0.05, **P < 0.01, ***P < 0.001, n.s. not significant.
assembled NP with HA solution on ice for 10 min, where HA is integrated into the nanoparticle through hydrophobic forces. HA coating is confirmed with a decrease in zeta potential. Enhanced cellular uptake is further demonstrated with lowering of the effective therapeutic dose of HA-coated p5RHH-p50 siRNA NP by 10-fold compared to the dose required to mitigate arthritis in an RA model previously reported by our lab [28]. Moreover, we showed that non-coated peptide-p50 siRNA NP at the same siRNA dose did not suppress AAA, confirming the enhanced therapeutic effect of the HA-coating.

5. Conclusions

In summary, we have identified the NF-κB p50 and p65 subunits as key modulators of the inflammatory process contributing to the development of AAA. Knockdown of NF-κB subunits using p5RHH-siRNA NP mitigates aneurysm progression and rupture in two different experimental models. However, our results also demonstrate that the p50 and p65 subunits contribute differentially to the aneurysmal process at different stages of disease. Since p50 knockdown was more effective at preventing rupture, we argue that p50 represents a preferable target for rational drug design in AAA treatment since it lacks a transactivating domain and its knockdown may carry less risk of adverse effects.

Data availability

The raw data in this present study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Huimin Yan: Methodology, Investigation, Formal analysis, Writing -original draft, review and editing Ying Hu Resources, Investigation Antonina Akk Investigation Samuel Wickline Resources, Conceptualization, Writing-review and editing Hua Pan Resources,
Conceptualization, Writing-review and editing Christine Pham Conceptualization, Validation, Writing-original draft, review and editing, Supervision, Funding acquisition.

Declaration of competing interest

SAW declares ownership interests in Altamira Therapeutics, Inc. HP declares stock options in Altamira Therapeutics. The rest of the authors declare no competing interests. Patents: Universal Anchor peptide for nanoparticles (April 2011; USPTA #12/910,385); Compositions and Methods for Polynucleotide Transfection (granted 22.08.2018) pct/ us2014/010212, wo 2014/107596, ep 2 941 273 b1; Peptide-Polynucleotide-Hyaluronic Acid Nanoparticles and Methods for Polynucleotide Transfection (May 10, 2019; USPTA #62/845,974). SAW and HP’s competing interests did not influence the work reported in this study, which was conducted independently in CTNP’s laboratory.

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Appendix A. Supplementary data

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References


