Andrographolide Ameliorates Abdominal Aortic Aneurysm Progression by Inhibiting Inflammatory Cell Infiltration through Downregulation of Cytokine and Integrin Expression

Jun Ren, Zhenjie Liu, Qiwei Wang, Jasmine Giles, Jason Greenberg, Nader Sheibani, K. Craig Kent, and Bo Liu

Division of Vascular Surgery, Department of Surgery (J. R., Z. L., Q. W., J. G., J. G., K. K., and B. L.) and Department of Ophthalmology and Visual Sciences (N. S.), University of Wisconsin – Madison, USA. Department of Vascular Surgery (Z. L.), 2nd Affiliated Hospital School of Medicine, Zhejiang University, Zhejiang, China.
a) *Running title*: Andrographolide Ameliorates Abdominal Aortic Aneurysm

b) Address correspondence to:
Bo Liu, Ph. D.
Department of Surgery, University of Wisconsin Madison
1111 Highland Avenue, WIMR 5137, Madison, WI 53705
Tel: 608-263-5931, Fax: 608-262-3330
E-mail: liub@surgery.wisc.edu

c) Numbers
Number of text pages: 33
Number of tables: 0
Number of figures: 7
Number of supplement figures: 5
Number of references: 49
Number of words in Abstract: 222 words
Number of words in Introduction: 507 words
Number of words in Discussion: 1470 words

d) Abbreviations:
AAA, abdominal aortic aneurysm; Andro, Andrographolide; CCL2, chemokine (C-C motif) ligand 2; CCL5, chemokine (C-C motif) ligand 5; CCL7, chemokine (C-C motif) ligand 7; CXCL10, chemokine (C-X-C motif) ligand 10; CXCL16, chemokine (C-X-C motif) ligand 16; ECs, endothelial cells; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; LFA-1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SMα-actin, smooth muscle alpha actin; SMCs, smooth muscle cells; SM MHC 11, smooth muscle
myosin heavy chain 11; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion protein 1; VLA-4, very late antigen 4.

e) A recommended section assignment: Cardiovascular
Abstract:

Abdominal aortic aneurysm (AAA), characterized by exuberant inflammation and tissue deterioration, is a common aortic disease associated with a high mortality rate. There is currently no established pharmacological therapy to treat this progressive disease. Andrographolide (Andro), a major bioactive component of herbaceous plant *Andrographis paniculata*, has been found to exhibit potent anti-inflammatory property by inhibiting NF-κB activity in several disease models. In this study, we investigated the ability of Andro to suppress inflammation associated with aneurysms and whether it may be used to block the progression of AAA. While diseased aortae continued to expand in the solvent-treated group, daily administration of Andro to mice with small aneurysms significantly attenuated aneurysm growth, as measured by the progressive expansion of aortic diameter (165.68 ± 15.85% vs 90.62 ± 22.91%, p<0.05). Immunohistochemistry analyses revealed that Andro decreased infiltration of monocytes/macrophages and T cells. Mechanistically, Andro inhibited arterial NF-κB activation and reduced the production of proinflammatory cytokines (CCL2, CXCL10, TNFα and IFNγ) in the treated aortae. Furthermore, Andro suppressed α4 integrin expression and attenuated the ability of monocytes/macrophages to adhere to activated endothelial cells. These results indicate that Andro suppresses progression of AAA, likely through inhibition of inflammatory cell infiltration via downregulation of NF-κB-mediated cytokine production and α4 integrin expression. Thus, Andro may offer a pharmacological therapy to slow disease progression in patients with small aneurysms.
Introduction:

Abdominal aortic aneurysm (AAA), the progressive weakening and dilation of the abdominal aorta, is a major aortic disease associated with a high mortality rate. It occurs most commonly in those over 50 years old, in men, and among those with a family history (Kent, 2014). As the elderly population increases, AAA becomes a more serious health problem, especially in Western nations. In the United States, the total number of AAAs remains stable at 45,000 cases per year (Dua et al., 2014). The majority of these new cases are small AAAs (defined as less than 5.5 cm and 5 cm aortic diameter in men and women, respectively) and largely asymptomatic. If left untreated, however, the continuing extension and thinning of the vessel wall may eventually result in a sudden unpredictable rupture. Ruptured AAAs are responsible for at least 13,000 deaths in the United States and 152,000 deaths worldwide annually (Kent, 2014; Collaborators, 2015). Unfortunately, an effective pharmacological treatment for AAAs is not currently available. Moreover, surgical treatment for small aneurysm is not usually recommended due to a low benefit to risk ratio. As a result, patients with small aneurysms are left untreated and in a “watchful waiting” program to monitor AAA progression (Filardo et al., 2012; Collaborators et al., 2013).

The clinical need for effective and safe drugs to slow aneurysm progression in patients with small AAAs has motivated active studies on aneurysm pathophysiology. Intense inflammation, implied by excessive cytokines and massive infiltrated inflammatory cells, is a major pathological feature of AAAs, which has been replicated in various animal models of AAA (Daugherty and Cassis, 2004; Shimizu et al., 2006). Therefore, we focused on inflammation in search of a potent anti-aneurysm strategy.

Andrographis paniculata, also known as Kalmegh (Hindi), Sambiloto (Malay) and Chuanxin Lian (Chinese), is a herbaceous plant in the family Acanthaceae. It has been widely used for treating sore throat, flu, and upper respiratory tract infections in India, Thailand, Malaysia and China for centuries. Andrographolide (Andro), a major bioactive chemical constituent of the plant, has been demonstrated for
its potent anti-inflammatory effects and well tolerance in various disease models including asthma, stroke, arthritis, restenosis and myocardial infarction (Wang et al., 2007; Woo et al., 2008; Jayakumar et al., 2013). Data largely from in vitro studies reveal that Andro suppresses inflammation by inhibiting NF-κB activity without showing cytotoxicity (Xia et al., 2004; Hsieh et al., 2011).

Since NF-κB activities are elevated in human aneurysmal tissues as well as experimental AAA models (Nakashima et al., 2004), we posit that Andro, through its putative anti-NF-κB properties, may serve as a candidate drug to halt disease progression of small AAAs. Using an elastase-induced murine AAA model, we demonstrate that Andro effectively blocks further aortic expansion and tissue damage of existing aneurysms. Data generated in vivo and in vitro indicate that Andro potently inhibits NF-κB activation and decreases expression of chemokines and cytokines in smooth muscle cells (SMCs) and monocytes/macrophages. Additionally, Andro treatment significantly downregulates α4 integrin expression and subsequently reduces the ability of monocytes/macrophages to adhere to activated endothelial cells.
Materials and Methods:

Reagents

Dulbecco’s Modified Eagles Medium (DMEM) and cell culture reagents were purchased from Gibco Life Technologies (Carlsbad, CA). Andrographolide (Andro) (≥ 98% purity, Cat. No. 365645, lot No. MKBL4120V and lot No. MKBS0063V), Lipopolysaccharide (LPS) (E.coli 0111:B4) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO). Recombinant mouse TNF-alpha was purchased from R&D Systems (Minneapolis, MN). Primary antibodies used include anti-phosphorylated p65, anti-p65, anti-IκB (Cell Signaling Technology, Danvers, MA), anti-β-actin (Sigma-Aldrich, St. Louis, MO), anti-NF-κB antibody (p65 subunit, active subunit, clone 12H11) (Millipore, Boston, MA), anti-CD3 gamma (Epitomics, Burlingame, CA), anti-monocyte + macrophage (MOMA-2), anti-smooth muscle myosin heavy chain 11 (SM MHC 11), anti-alpha smooth muscle actin (SM α-actin) (Abcam, Cambridge, MA), and anti-Ly6G (eBioscience, San Diego, CA). Horseradish Peroxidase (HRP)-conjugated Antibodies were purchased from Bio-Rad (Hercules, CA). Elastin was stained by using Richard-Allan Scientific™ Elastic Stain kit (Thermo Fisher Scientific, Rockford, IL). CellTiter-Glo® Reagent was purchased from Promega (Madison, WI). Other chemicals and reagents, if not specified, were purchased from Sigma-Aldrich.

Mouse Model of AAA and Administration of Andrographolide

Male C57BL/6J mice aged 8-12 weeks underwent AAA induction by elastase-perfusion as described previously (Pyo et al., 2000; Liu et al., 2015; Wang et al., 2015). Briefly, after anesthesia, the abdominal aorta was isolated and the external diameter of the largest portion of abdominal aorta was measured with a digital caliber. The abdominal aorta was ligated temporarily then perfused with 0.45 U/mL type I porcine pancreatic elastase (Sigma) or equal concentration of heat-inactivated (100°C for 15 min) elastase (control) for 5 min at a constant pressure of 100 mm Hg. After perfusion, the aortostomy and abdominal incision were closed. The mouse was kept on a warming pad until fully recovered from anesthesia. 7 days
post aneurysm induction, mice were randomly assigned to two groups: (1) solvent (DMSO) control and
(2) Andro. Immediately prior to intraperitoneal (IP) injection, 3 μl of Andro (dissolved in DMSO at 50
mg/ml) was diluted with 200 μl saline. An equal volume of DMSO was diluted and injected accordingly.
We chose postoperative day 7 to begin treatment because aneurysmal dilations at this time are small but
significant (Suppl. Fig.1 A&B) (Miyake et al., 2007; Liu et al., 2014). Daily IP injection of DMSO to
mice produced insignificant effect on aneurysmal dilation compared to mice not receiving DMSO (165.68
± 15.85% vs. 166.67 ± 15.46%, p>0.05 in elastase-treated arteries postoperative at day 14; and 43.54 ±
7.24% vs. 43.62 ± 3.56%, p>0.05 in inactive elastase-treated arteries postoperative at day 14). The in
vivo dosage of Andro in the literature ranges from 1 to 100mg/kg body weight, with 5 mg/kg body weight
(used in the current study) as a commonly used dosage and is considerably lower than LD50 for
intraperitoneally administered andrographolide (11.6 g/kg body weight) (Handa and Sharma, 1990; Wang
et al., 2007; Hsieh et al., 2011; Zhu et al., 2013). At the selected time points, mice were euthanized by
CO2 inhalation. The external diameter of the largest portion of abdominal aortas was measured and used
to calculate the percentage increase in maximal external aortic diameter comparing to the diameter
recorded prior to the elastase perfusion. Tissues meant for RNA isolation were harvested and stored in
RNAlater RNA Stabilization Reagent (Qiagen Valencia, CA). Tissues meant for immunohistochemistry
were imbedded in O.C.T. Compound (Sakura Tissue Tek, Netherlands). Frozen sections were cut to 6μm
thick using a Leica CM3050S cryostat (Buffalo Grove, Ill). All animal experiments in this study were
approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison
(Protocol M02284) and performed in accordance with the Guide for the Care and Use of Laboratory
Animals published by the United States National Institutes of Health.

**Cell culture and In Vitro Treatment**

Primary mouse aortic smooth muscle cells (SMCs) were isolated from arteries of C57BL/6J mice as
previously described (Clowes et al., 1989; Lengfeld et al., 2012). Primary SMCs were grown at 37°C in
5% CO2 in DMEM modified to contain 4mM L-Glutamine, 1g/L D-Glucose, and 110mg/L Sodium
Pyruvate (Life Technologies) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Cells between passages three and seven were used. The mouse monocyte/macrophage cell line RAW264.7 (RAWs) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and was maintained as recommended in DMEM modified containing 4.5g/L D-Glucose (Life Technologies) supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Mouse aortic endothelial cells (ECs) were isolated from male C57BL/6J mice, as described previously (Su et al., 2003). ECs were grown on 1% gelatin-coated dishes in DMEM containing 20% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% non-essential amino acids, 100 μg/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma), endothelial growth supplement 100 μg/ml (Sigma), and murine recombinant interferon-γ (R&D, Minneapolis, MN) at 44 units/ml (Su et al., 2003).

For in vitro treatment, a stock solution of 15 mM was prepared by dissolving Andro in DMSO then diluted to desired concentrations with medium immediately prior to experiments. The final concentration of DMSO was 0.1%, which showed no effects by itself (Suppl. Fig. 2A-G, Suppl. Fig.3A-G, and Fig.5B-E). Andro at the final concentration of 15 μM has been reported to effectively inhibit NF-κB in ECs and SMCs (Xia et al., 2004; Ren et al., 2014), while the dosage of Andro for treating macrophages is less established. To determine the in vitro dosage, we conducted dose-response studies using RAWs. Andro did not significantly decrease cell viability below a dose of 30 μM (Suppl. Fig. 4A). Pre-incubation of RAWs with 15 or 20 μM of Andro reduced adhesion to fibronectin-coated surface by 70% and 80%, respectively. However, difference in adhesion inhibition caused by 15 and 20 μM did not reach significance (Suppl. Fig. 4B), suggesting 15 μM of Andro caused close-to-maximal inhibition on adhesion of monocytes/macrophages without affecting viability. Therefore, we conducted the rest of in vitro studies with 15 μM of Andro.

**RNA Isolation and Quantitative real-time PCR (qPCR)**

Total RNA was extracted from cultured cells or mouse aortic arteries using Trizol reagent (Life Technologies) according to manufacturer's protocols. Two micrograms of RNA were used for the first-
strand cDNA synthesis (Applied Biosystems, Carlsbad, CA). A no-RT (reverse transcriptase) control was included in the same PCR mixtures without reverse transcriptase to confirm the absence of DNA contamination in RNA samples. The qPCR primers for Ccl2, Ccl5, Ccl7, Ccl10, Ccl16, Tnf, Ifng and Gapdh were purchased from Qiagen. Primers for amplification of integrins were Itga4 (forward: 5’ GAATCCAAACCAGACCTGCGA 3’; reverse: 5’ TGACGTAGCAAATGCCAGTGG 3’), ItgaL (forward: 5’ ATGCACCAAGTACAAAGTCAGC 3’; reverse: 5’ TTGGTCGAACCTCAGGATTGC 3’), Itgb1 (forward: 5’ TTCAGACTTCCGCATGGCTTTGG 3’; reverse: 5’ TGGGCTGTGGTCAGTTTTGTTCAC 3’), and Itgb2 (forward: 5’ CAGGAATGCACCAAGTACAAAGT 3’; reverse: 5’ CCTGGTCCAGTGAAGTTCAGC 3’) (Ip et al., 2007). Reactions were carried out in 96-well optical reaction plates using SYBR® Green PCR Master Mix (Applied Biosystems) with gene specific primers in 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification of each sample was analyzed by melting curve analysis, relative differences in each PCR sample were corrected using GAPDH mRNA as an endogenous control, and normalized to the level of control by using 2^ΔΔCt method (Si et al., 2012).

**CellTiter-Glo® Luminescent Cell Viability Assay**

RAWs were seeded in 96-well plates at a density of 3×10^4 cells per well. 24h later, medium was replaced with fresh growth media containing Andro or DMSO. After culturing for 48h, CellTiter-Glo® Reagent was added. Luminescence was recorded 10 minutes after reagent addition using a FlexStation 3 Microplate Reader (Molecular Devices, CA).

**Cell Adhesion Assay**

Adhesion to fibronectin-coated surface was evaluated in 96-well plates as described previously (Liu et al., 2015). Briefly, RAWs pretreated with Andro or DMSO were seeded in fibronectin-coated wells and incubated at 37ºC for 45 minutes. After 3 washes with PBS, cells were fixed in 4% paraformaldehyde (PFA) then stained with Crystal Violet (5mg/ml in 2% Ethanol) for 10min. Wells were washed 3 times
with PBS then turned upside down to dry completely. After adding 2% SDS to each well and incubating for 30 min, plates were read at 550 nm using a FlexStation 3 Microplate Reader (Molecular Devices, CA).

**Monocyte-endothelial cell interaction**

A monolayer of ECs was grown on 1% gelatin coated 24-well plate. To visualize cell adhesion, RAWs were fluorescently labeled with the CellTracker Green CMFDA Dye (Life Technologies) prior to adding the cells to ECs. ECs were activated with TNFα (10ng/ml) for 12h with or without Andro (15μM) pretreatment, whereas RAWs were only pre-incubated with Andro or DMSO for 12h. After incubating RAWs with ECs for 20 min, loosely adherent cells were shaken off followed by three washes with PBS. Six fields were randomly chosen and adherent cells were photographed by fluorescence microscopy (Nikon DS-Ri1). The number of adherent cells was counted using imageJ (National Institutes of Health, Bethesda, MD), and data are expressed as mean number of counted cells/field ±SEM.

**Immunoblotting**

Cells were lysed in RIPA buffer (Sigma) and total protein was extracted. Equal amounts of protein extract were separated by SDS–PAGE and transferred to PVDF membranes. The membranes were then incubated with primary antibody followed by horseradish peroxidase (HRP)–labeled goat anti–rabbit or anti–mouse immunoglobulin G (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (Thermo Fisher Scientific). For quantification, optical density of secreted proteins, determined by ImageJ (National Institutes of Health), was normalized to the loading control density.

**Immunohistochemistry**

Tissue sections were permeabilized with 0.1% Triton X-100 in Tris-buffered saline (TBS) for 10 minutes at room temperature. Non-specific sites were blocked using 5% bovine serum albumin (BSA), 10% normal donkey serum in TBS for 2 hours at room temperature. Primary antibodies diluted in TBS with
5% BSA were then applied onto arterial sections and incubated overnight at 4°C. The next day, arterial sections were rinsed with TBS plus 0.025% Triton X-100, followed by incubating with horseradish peroxidase (HRP)- or fluorophore-conjugated secondary antibodies diluted in TBS with 1% BSA for 1 hour at room temperature. After DAB development and counterstaining with hematoxylin or DAPI for immunofluorescence staining, stained tissue sections were visualized with a Nikon Eclipse Ti inverted microscope system and digital images were acquired using a Nikon DS-Ri1 digital camera. Quantification of stains was performed with ImageJ software (National Institutes of Health) as previously described.

**Statistical analysis**

Student’s *t* test or one-way ANOVA with Bonferroni’s post-hoc test was used to evaluate the statistical differences. All experiments were repeated at least three times. Data are presented as mean ± SEM. Differences with *p* < 0.05 were considered significant.
Results:

**Andrographolide inhibits progression of abdominal aortic aneurysm**

We tested Andro in a murine model of AAA, which utilized a brief (5 minutes) intraluminal perfusion of abdominal aorta with porcine pancreas elastase. The control mice were subjected to the identical surgical manipulation, but were perfused with heat-inactivated elastase. With time, the elastase-treated group exhibited continuous aortic expansion (Suppl. Fig. 1A&B), which was accompanied by the appearance of typical aneurysmal pathological features including inflammation infiltration, SMC depletion and elastin degradation (Liu et al., 2014; Liu et al., 2015; Wang et al., 2015). In contrast, aorta treated with inactivated elastase showed neither continuous aortic expansion nor tissue deterioration (Suppl. Fig. 1A&B).

To evaluate the therapeutic potential of Andro, we selected day 7 post elastase-perfusion as the starting point for drug treatment. At this time point, the aortic expansion was small but significantly larger than arteries perfused with inactivated elastase (Suppl. Fig. 1A&B). Furthermore, inflammation as measured by monocyte and macrophage accumulation in the aortic tissue had clearly developed (Liu et al., 2015). The elastase-treated mice were randomly divided into two groups, one treated daily with solvent (DMSO) and the other with Andro (5 mg/kg body weight) (Fig. 1A). A separate group of mice that were perfused with inactivated elastase and then treated with solvent were used as a baseline control. Mice were sacrificed 14 days post aneurysm induction. Grossly, elastase-perfused solvent-treated aortae appeared dilated and inflamed, while elastase-perfused Andro-treated aortae appeared normal, which resembled arteries perfused with inactivated elastase (Fig. 1B). We quantified the inhibitory effects of Andro on aortic dilation by calculating the percentage increase in maximum aortic diameter. As shown in Fig. 1B, Andro markedly decreased the aortic expansion compared to the solvent treated mice (90.62 ± 22.91% vs 165.68 ± 15.85%, p<0.05). Histologically, administration of Andro preserved vascular tissue integrity. It reduced the tissue damage, elastin degradation as well as loss of SMCs (Fig. 1C).
Andrographolide inhibits NF-κB activation during AAA pathogenesis

Since Andro has been implicated to act on the NF-κB pathway, we investigated whether Andro affects NF-κB activation in aneurysm using an antibody that recognizes the DNA-binding or activated form of p65 (Aoki et al., 2007). Prior to Andro treatment, activated p65 was readily detectable in aortic wall 7 days after elastase perfusion, mostly in cells that were positive for SM α-actin (a SMC marker) as well as in cells positive for MOMA-2 (a monocyte and macrophage marker) (Fig. 2A&B). Activation of NF-κB persisted in the aortic wall to at least 14 days post aneurysm induction (Fig. 2C). The one-week Andro treatment (day 7-13) nearly eliminated NF-κB activation (Fig. 2C).

Andrographolide attenuates AAA-associated inflammatory cytokine expression

To further investigate how Andro halts aneurysm progression, we turned to cultured SMCs and macrophages, two primary sources of inflammatory cytokine during AAA pathogenesis. To mimic inflammation associated with AAA, we treated SMCs isolated from the mouse aorta with TNFα (10 ng/ml), which caused a rapid and sustained phosphorylation of p65 at Serine536 as well as degradation of IκB (Fig. 3A&B). Pretreating SMCs with Andro (15μM) greatly attenuated p65 phosphorylation but did not significantly alter IκB degradation (Fig. 3A&B). Next, we examined whether Andro inhibits the expression of NF-κB target genes, in this case, chemokines and cytokines found in aneurysmal tissues. As shown in Fig. 3C-H, TNFα stimulated expression of Ccl2, Ccl5, Ccl7, Cxcl10, Cxcl16, and Tnf in SMCs, ranging from 10- to 160-fold. Compared to DMSO, Andro significantly decreased production of these inflammatory cytokines (Fig. 3C-H). Interestingly, expression of Ifng in SMCs was not affected by TNFα or Andro treatment (Fig. 3I).

Although Andro effectively blocked basal levels of cytokine and chemokine expression in RAWs, this monocyte/macrophage cell line was not as responsive to TNFα (10 ng/ml) as SMCs (Suppl. Fig. 5A-G). Since toll-like receptor (TLR) signaling plays a crucial role in pathogenesis of aneurysms (Vorkapic et al.,...
2015), we used the well-characterized TLR ligand LPS (100 ng/ml) as a stimulus. Andro decreased LPS-induced p65 phosphorylation at serine 536 without affecting IκB in RAWs (Fig. 4A&B). Moreover, Andro reduced the LPS-induced expression of Ccl2, Ccl5, Ccl7, Cxcl10, and Tnf by 40-80% (Fig. 4C-F&H). Of note, expression of Cxcl16 and Ifng in RAWs was not sensitive to LPS, even through Andro decreased basal level of these inflammatory cytokines (Fig. 4G&I).

**Andrographolide decreases monocytes/macrophages adhesion**

Firm adhesion of monocytes/macrophages to the endothelium is another crucial event leading to vascular inflammation. To simulate this early inflammatory step, we used an in vitro adhesion assay in which fluorescence-labeled RAWs were allowed to adhere to a monolayer of ECs for 20 min. After washing vigorously, adhered RAWs were imaged and counted. Activation of ECs with TNFα (10 ng/ml) increased the number of adhered RAWs by 130% (Fig. 5A). Pretreating ECs or RAWs or both cell types with Andro resulted in significantly reduced adhesion of RAWs. However, treating RAWs or both cell types with Andro produced more profound inhibition than treating EC alone (Fig. 5A).

NF-κB is known to regulate endothelial expression of adhesion molecules including vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Wang et al., 2007). In comparison, the underlying mechanism of Andro on macrophage adhesion is less understood. Very late antigen 4 (VLA-4; also known as α4β1 integrin) and lymphocyte function-associated antigen 1 (LFA-1; also known as αLβ2 integrin) are major integrins on the monocyte/macrophage surface that interact with VCAM-1 and ICAM-1 during adhesion. Therefore, we tested whether Andro affects integrin expression in RAWs. As shown in Fig. 5B, treatment of RAWs with Andro for 6 or 12h reduced α4 (Itga4) integrin expression by 57% and 58%. The expression of β1 (Itgb1), αL (Itgal) and β2 (Itgb2) integrins was not significantly affected (Fig. 5C-E).

**Andrographolide inhibits inflammatory cytokine production and inflammatory cell infiltration in**
aneurysm

Having established the inhibitory role of Andro in cultured SMCs and macrophages, we re-examined mouse aneurysm tissues. Consistent with the literature, expression of AAA-associated inflammatory cytokines, including Tnf, Ifng, Ccl2, Ccl5, Ccl7, Cxcl10, and Cxcl16, was greatly increased in elastase-perfused arterial wall as compared to heat-inactivated elastase treated arteries (Fig. 6A-G). Daily administration of Andro 7 days after aneurysm induction significantly inhibited expression of Ccl2, Cxcl10, Tnf, and Ifng as compared with solvent treatment (Fig. 6A-D). Interestingly, Ccl5, Ccl7 and Cxcl16 mRNA expression was not significantly affected by Andro treatment (Fig. 6E-G).

We next examined vascular infiltration of inflammatory cells. Immunohistochemistry with antibodies against MOMA-2 (a monocyte and macrophage marker), CD3 (a T cell marker), and Ly6G (a neutrophil marker) revealed that infiltration of monocytes/macrophages as well as T cells was significantly decreased by Andro treatment (Fig. 7A&B). The number of Ly6G+ cells was comparable between Andro and solvent treatment groups. Of note, at day 14 after aneurysm induction, Ly6G+ cells constituted less than 1% of arterial wall cells (Fig. 7A&B).
Discussion:

Chronic inflammation has long been recognized as a crucial pathological event in AAAs. However, its underlying molecular and cellular mechanisms remain incompletely understood. Experimental evidence published by several investigative groups including our own suggests active interplays between infiltrating macrophages and resident SMCs (Curci and Thompson, 2004; Liu et al., 2015). On one hand, vascular SMCs are a major source of extra cellular matrix proteins that render elastic property and tensile strength to the aortic wall. On the other hand, injured SMCs actively recruit inflammatory cells by producing chemokines. If SMCs are considered as the soil of inflammation, the recruited inflammatory cells, particularly M1 macrophages, are inflammatory amplifiers and tissue destroyers. They amplify inflammation by releasing cytokines (Curci and Thompson, 2004), destroy tissues by producing matrix-degrading enzymes such as matrix mateloproteases (MMPs) (Thompson et al., 1995; Longo et al., 2002), and eliminate SMCs through pro-apoptotic or necrotic signals including reactive oxygen species (ROS), TNFα as well as Fas ligand (Li et al., 1997; Wang et al., 2014). As such, anti-inflammatory strategies including those that deplete neutrophils or neutralize cytokines, such as TNFα, preserve smooth muscle and reduce matrix proteolysis (Eliason et al., 2005; Jayaraman et al., 2008). Evidence presented here proved that inhibition of inflammation through systematic administration of Andro blocked aneurysm progression in mice, evidenced by halted aortic expansion, reduced elastin degradation, preserved medial SMCs, and reduced the number of infiltrating monocytes, macrophages as well as T lymphocytes.

Among the Andro-sensitive chemokines/cytokines, CCL2, also called monocyte chemoattractant protein-1 (MCP1), is well known for its role in inflammatory recruitment in aneurysm. Targeted gene deletion of Ccl2 or its receptor Ccr2 confers aneurysm resistance (MacTaggart et al., 2007; Aoki et al., 2009; Moehle et al., 2011). In this study, Andro potently reduced the Ccl2 mRNA accumulation in the aortic wall by 93.18% compared to the solvent control. Andro also decreased the local expression of other established pro-inflammatory cytokines including Tnf, Ifng, and Cxcl10. Inhibition of cytokine expression by Andro
was replicated to a large extent in cultured aortic SMCs as well as in a monocyte/macrophage cell line RAWs. Of note, inhibition of certain chemokines such as Ccl2, Ccl7, and Cxcl10 was more profound in RAWs than in SMCs (80-95% vs 25-30%). Since Andro produced a similar reduction in p65 phosphorylation in these two cell types, we speculate that the differential response reflects a cell-type dependent transcription regulation of chemokines. Regulation of gene expression is a complex process requiring coordination of multiple transcription factors and co-activators, which is frequently controlled by epigenetic modifications. It is possible that NF-κB plays a more dominant regulatory role in chemokine transcription in monocytes/macrophages than in SMCs. However, this potential mechanism needs to be experimentally tested.

Our data revealed a novel function of Andro, i.e. inhibition of leukocyte adhesion. Aside from the reported inhibitory effect of Andro on expression of ICAM-1, VCAM-1 and E-selectin in ECs (Jiang et al., 2007; Wang et al., 2007; Lu et al., 2014), the present study demonstrated that Andro inhibited the expression of α4 integrin in monocytes/macrophages. To the best of our knowledge, this is the first demonstration that Andro inhibited the α4 integrin expression and endothelium adhesion of monocytes/macrophages. Interestingly, in the in vitro monocyte-endothelial cell interaction study, we found that pre-treating RAWs with Andro (15 μM) produced a far more inhibitory effect on adhesion than pre-treating endothelial cells.

In dose-response studies, Andro did not significantly decrease the viability of RAWs until it reached 30 μM. Pre-incubation of RAWs with 15 and 20 μM of Andro reduced adhesion ability, but not viability, to fibronectin-coated surface by 70% and 80%, respectively. However, differences in adhesion inhibition caused by these two doses of Andro did not reach significance, suggesting that Andro at 15 μM caused close-to-maximal inhibition on adhesion of monocytes/macrophages. This observation is somewhat different from a prior study, which showed that 20 and 50 μM of Andro dose-dependently suppressed NF-κB activity by 16.4% and 55.6% respectively. However, neither concentration diminished cell viability,
as measured by MTT assay (Hsieh et al., 2011). It is possible that monocytes and macrophages are more sensitive to Ando or NF-κB inhibition. However, further experimental evidence is needed to confirm this possibility.

At the molecular level, we showed that Andro significantly decreased aortic activity of NF-κB. While IκB degradation constitutes a canonical regulatory step for NF-κB activation (Oeckinghaus and Ghosh, 2009; Ren et al., 2014), post-translational modifications of p65, such as phosphorylation, influence its dimerization, DNA binding and transcriptional activity, and are recognized as an alternative mechanism leading to NF-κB activation (Oeckinghaus and Ghosh, 2009). The effect of Andro on NF-κB activation appears to depend on the cell type and stimulus. In collagen-stimulated platelets, Andro affects the NF-κB at the step of IκB degradation (Lu et al., 2012). In SMCs, Cheng et al. reported that Andro suppressed LPS/IFNγ-induced p65 nuclear translocation, p65 phosphorylation and DNA binding activity, but not degradation of IκB (Hsieh et al., 2011). Consistent with their findings, we found that Andro decreased p65 phosphorylation at serine 536 in both TNFα treated SMCs and LPS treated RAWs. However, Andro did not significantly alter IκB degradation in either cell types. We speculate that this IκB-independent effect of Andro allow cells to maintain certain NF-κB-mediated anti-apoptotic proteins including Bcl-2, A1, and IAP. This notion may explain the low cytotoxicity of Andro that we and other groups observed (Xia et al., 2004; Wang et al., 2007; Hsieh et al., 2011).

The involvement of NF-κB in aneurysm pathophysiology is implicated by a markedly elevated NF-κB activity in biopsies of human aneurysmal tissues and a mouse model of AAA (Miyake et al., 2006; Miyake et al., 2007). Using an antibody specific to the activated p65, we confirmed the prior reports and identified SMCs and macrophages as major cell types that harbor activated NF-κB. At 14 days post aneurysm induction, CD31-positive ECs remained sporadic in the elastase-treated aortic sections, likely due to endothelial damage during the perfusion procedure. The contribution of endothelial specific NF-κB activation in aneurysm has been previously highlighted. Using a transgenic mouse line expressing
dominant-negative IkBα selectively in ECs, Saito et al demonstrated that selective NF-κB inhibition decreased macrophage infiltration and aneurysmal dilatation in the angiotensin II AAA model (Saito et al., 2013). The involvement of NF-κB in aneurysm development was also demonstrated by Nakushima who inhibited the signaling pathway with an oligodeoxynucleotide decoy delivered to the aortic wall during the elastase perfusion procedure in rats (Nakashima et al., 2004). Given the established effects of Andro on the NF-κB pathway and the involvement of NF-κB in inflammation and aneurysm pathophysiology, it is plausible to attribute the anti-aneurysm effect of Andro to NF-κB inhibition. However, our data do not exclude other potential targets.

Beneficial effects of Andro have also been observed in several other experimental cardiovascular disease models, including restenosis, myocardial infarction, and hyperglycemia (Yu et al., 2003; Wang et al., 2007; Woo et al., 2008). While these prior studies and our current work puts Andro in a strong position as a drug candidate, caution should be taken when translating mouse work into clinical treatments. One concern relates to aneurysm modeling. Several murine AAA models have been created with different strengths and weaknesses (Daugherty and Cassis, 2004). In this study, we used an elastase-induced AAA model, which recapitulates important pathological features of human AAA including SMC depletion, inflammation, and elastin degradation (Daugherty and Cassis, 2004; Thompson et al., 2006). However, it does not lead to AAA rupture, a major complication in human AAA (Nordon et al., 2011). Another concern in translation is drug toxicity. In this study, the mortality rate was 0 in both Andro- (n=7) and solvent- (n=9) treated groups. Consistent with prior studies, no differences in body weight and appearance were noticed between Andro- and solvent-treated mice. The safety of Andro is supported by a phase II clinical study in rheumatoid arthritis patients which revealed that *A. paniculata* tablets containing 30 mg of andrographolide (3 times a day for 14 weeks) was well tolerated and significantly more effective in reducing symptoms and serological parameters of the disease (Burgos et al., 2009). However, higher doses of Andro (4-6mg/kg body weight, oral administration) may cause isolated cases of allergic reactions, tiredness, headache, pruritus/rash, diarrhea, nausea, metallic taste, bitter taste, dry tongue, and
etc. (Coon and Ernst, 2004). Three additional clinical trials are currently being conducted to evaluate efficacy, safety and tolerability of Andro in patients with multiple sclerosis and colorectal neoplasms.

In closing, our results indicate that Andro suppresses small aneurysm progression in an elastase induced AAA mouse model. The therapeutic effect of Andro on AAA progression is achieved at least in part through inhibition of NF-κB activity, which subsequently diminishes production of inflammatory cytokines and chemokines as well as integrins. Thus, Andro may offer a safe and effective therapeutic strategy to slow disease progression in patients with small AAAs.
Acknowledgments

We thank Carmel Assa and Noel Phan for their intellectual inputs.
Authorship Contributions.

Participated in research design: Ren, Z. Liu, Wang, and B. Liu.

Conducted experiments: Ren, Z. Liu, Wang, Giles, and Greenberg.

Contributed new reagents or analytic tools: Sheibani.

Performed data analysis: Ren, Z. Liu, Giles, and Wang.

Wrote or contributed to the writing of the manuscript: Ren, Sheibani, Kent, and B. Liu.
References:


This study was supported by National Institutes of Health (NIH) National Heart, Lung, and Blood Institute (NHLBI) [Grant R01HL088447 to B. L.]; and American Heart Association [15PRE25670074 to J. R.].
Figure Legends

Figure 1. Andro inhibits progression of small AAAs. (A) Schematic diagram of Andro treatment in mice. (B) Representative photos and aortic expansion of Andro- and solvent-treated mice, taken 14 days after surgery. IE: inactivated elastase; E: elastase. An AAA is defined as a percentage increase in aortic diameter that is equal or greater than 100% (red dashed line). All values represent mean±SEM. n=3-9; * p<0.05, one-way ANOVA. (C) Representative images of arterial sections stained with Haematoxylin and Eosin (H&E), Verhoeff-Van Gieson (VVG), and smooth muscle myosin heavy chain 11 (SM MHC 11; green) overlaid with DAPI (blue). Arteries were harvested 14 days after surgery. Scale bar = 200 μm.

Figure 2. Andro attenuates NF-κB activation in aortic tissues. (A&B) Representative images of arterial sections co-immunostained for activated p65 (green) and smooth muscle α-actin (SM α-actin; red) (A) or activated p65 (red) and Monocyte + Macrophage (MOMA-2; green) (B) overlaid with DAPI (blue). Areas highlighted by white dashed boxes are shown at higher magnification on the right. Arteries were harvested 7 days after elastase perfusion. Scale bar = 50 μm. (C) Representative images of arterial sections immunohistochemical (IHC) stained for activated p65. IE: inactivated elastase; E: elastase. Areas highlighted by black dashed boxes are shown at higher magnification at the bottom. Arteries were harvested 14 days after elastase perfusion. Scale bar = 100 μm.

Figures 3. Andro inhibits NF-κB activation and cytokine expression in TNFα-treated SMCs. (A&B) SMCs were pretreated with solvent (DMSO) or Andro (15 μM) for 1 h before incubation with TNFα (10 ng/ml) for indicated times. Whole-cell lysates were subjected to immunoblotting analysis with indicated antibodies. (C-I) SMCs were pretreated with solvent (DMSO) or Andro (15 μM) for 1 h before incubation with TNFα (10 ng/ml) for 6 h. mRNA expression of inflammatory chemokines and cytokines was analyzed by qPCR. All values represent mean±SEM. n=3-6; * p<0.05, one-way ANOVA (A-I).
Figures 4. Andro inhibits NF-κB activation and cytokine expression in LPS-treated monocytes/macrophages. (A&B) RAW264.7 cells were pretreated with solvent (DMSO) or Andro (15μM) for 1 h before incubation with LPS (100 ng/ml) for indicated time. Whole-cell lysates were subjected to immunoblotting analysis with indicated antibodies. (C-I) RAW264.7 cells were pretreated with solvent (DMSO) or Andro (15 μM) for 1 h before incubation with LPS (100 ng/ml) for 6 h. mRNA expression of inflammatory chemokines and cytokines was analyzed by qPCR. All values represent mean±SEM. n=4-5; * p<0.05, one-way ANOVA (A-F&H) and two-tailed Student’s t test (G&I).

Figures 5. Andro reduces the adherence of monocytes/macrophages to endothelial cells (ECs). (A) Fluorescently labeled RAW264.7 cells, pretreated with Andro (15μM) or DMSO, were incubated with TNFα- (10 ng/ml) treated ECs. After washing vigorously, adhered RAW264.7 cells were imaged (upper panel) and quantified (lower panel). Data are expressed as mean number of adhered cells/field±SEM. n=4; * p<0.05, one-way ANOVA. (B-E) RAW264.7 cells were treated with or without Andro (15 μM) or solvent for indicated time. mRNA expression of α4 (B), β1 (C), αL (D), and β2 (E) integrins was analyzed by qPCR. All values represent mean±SEM. n=4-6; * p<0.05, one-way ANOVA.

Figures 6. Andro inhibits inflammatory cytokine expression in elastase perfused aortic tissues. mRNA expression of Ccl2 (A), Cxcl10 (B), Tnf (C), Ifng (D), Ccl5 (E), Ccl7 (F), and Cxcl16 (G) in aorta of solvent or Andro treated animals 14 days after surgery was analyzed by qPCR. All values represent mean±SEM. n=3-8; * p<0.05, one-way ANOVA.

Figures 7. Andro decreases infiltration of inflammatory cells in elastase perfused aortic tissues. (A) Representative images of arterial sections immunohistochemical (IHC) stained for MOMA-2 (a monocyte
and macrophage marker), CD3 (a T cell marker), and Ly6G (a neutrophil marker). Higher-magnification views of boxed areas are shown at the lower left corner. E: elastase. Scale bar=200μm. (B) Quantification of inflammatory cell infiltration in aortic tissue is expressed as positive cells/nuclei. All values represent mean±SEM. n=6-10; * p<0.05, two-tailed Student’s t test.
Figure 1

A

Solvent or Andro i.p. Injection
Day 7~13

Surgery
Day 0

Sacrifice
Day 14

B

IE

Solvent

Solvent

Andro

E

C

IE

Solvent

Solvent

Andro

E

H&E

VVG

SM MHC 11

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7