

## **Prolonged shear stress and KLF2 suppress constitutive pro-inflammatory transcription through inhibition of ATF2**

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## **Abstract**

Absence of shear stress due to disturbed blood flow at arterial bifurcations and curvatures leads to endothelial dysfunction and pro-inflammatory gene expression, ultimately resulting in atherogenesis. KLF2 has recently been implicated as a transcription factor involved in mediating the anti-inflammatory effects of flow. We investigated the effect of shear on basal and TNF- $\alpha$ -induced genome-wide expression profiles of human umbilical vein endothelial cells (HUVEC). Cluster analysis confirmed that shear stress induces expression of protective genes including KLF2, eNOS and thrombomodulin, whereas basal expression of TNF- $\alpha$ -responsive genes was moderately decreased. Promoter analysis of these genes showed enrichment of binding sites for ATF transcription factors, whereas TNF- $\alpha$ -induced gene expression was mostly NF- $\kappa$ B-dependent. Furthermore, human endothelial cells overlying atherosclerotic plaques had increased amounts of phosphorylated nuclear ATF2 compared to endothelium at unaffected sites. In HUVEC, a dramatic reduction of nuclear binding activity of ATF2 was observed under shear and appeared to be KLF2-dependent. Reduction of ATF2 with siRNA potently suppressed basal pro-inflammatory gene expression under no-flow conditions. In conclusion, we demonstrate that shear stress and KLF2 inhibit nuclear activity of ATF2, providing a potential mechanism by which endothelial cells exposed to laminar flow are protected from basal pro-inflammatory, atherogenic gene-expression.

## **Introduction**

Atherosclerosis is a vascular disease with a clear focality, which has been shown to correlate with shear stress levels on the endothelium, resulting from specific blood flow patterns<sup>1</sup>. Absence of shear stress due to oscillatory blood flow at arterial bifurcations and curvatures leads to endothelial dysfunction as characterized by a diminished barrier function and pro-inflammatory gene expression. These conditions facilitate the entry of lipids and inflammatory cells in the vascular wall, ultimately leading to the formation of an atherosclerotic plaque. On the other hand, endothelial cells exposed to high levels of shear stress maintain an athero-protective gene expression profile and have a differentiated, quiescent phenotype.<sup>2</sup> Transcription factors, being the integrators of various mechanical and biological stimuli, play a pivotal role in the regulation of gene expression and determine the resulting biological effect. A possible candidate that could be critical in the protection from atherogenesis is the shear-inducible transcription factor Krüppel-like factor 2 (KLF2). It has become clear in recent publications that KLF2 plays a significant role in maintaining an athero-protective, quiescent endothelial phenotype.<sup>3-5</sup> Protective genes like endothelial nitric oxide synthase (NOS3) and thrombomodulin (TM) are induced by KLF2, whereas expression of the pro-atherogenic monocyte chemoattractant protein 1 (CCL2) and endothelin (EDN1) is reduced.

The inflammatory component in atherosclerotic pathology suggests that inflammatory gene expression is activated in the atherosclerotic vascular wall, which is mediated by transcription factors associated with inflammation. Inflammatory gene expression has

been detected at sites predilected for plaque formation.<sup>6</sup> Furthermore, the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) and its inhibitor I $\kappa$ B were shown to be present at elevated levels in the cytoplasm of endothelial cells at sites exposed to disturbed blood flow.<sup>7</sup> These cells, however, do not show increased nuclear levels of NF- $\kappa$ B, which only becomes transcriptionally active when translocated to the nucleus after liberation from its inhibitor I $\kappa$ B. This translocation was only observed after inflammatory activation by a secondary stimulus like LPS or atherogenic diet, which then indeed occurred much more prominently at the low-flow regions.<sup>7</sup> Thus, endothelial cells at sites with disturbed blood flow should not exhibit inflammatory gene expression to the same magnitude in the absence of induction by cytokines. Indeed, a moderate induction of pro-inflammatory gene expression was observed at the disturbed flow regions of the porcine carotid artery bifurcation.<sup>8</sup> A potent induction of inflammatory gene expression by inflammatory cytokines depends on the actively promoted formation of a transcriptional complex, usually composed of nuclear factor kappa-B (NF- $\kappa$ B), activator protein-1 (AP-1) and co-activators like CREB-binding protein CBP/p300.<sup>9</sup> The transcriptional effects of TNF- $\alpha$  on HUVEC are considered a physiological representative of atherogenesis<sup>10</sup> and are indeed mediated by both NF- $\kappa$ B and the AP-1 activating p38-MAPKinase.<sup>11</sup> Pharmacological interventions and the protective effects of short-term flow preconditioning *in vitro* suggested a dominant role of the former transcription factor.<sup>11,12</sup> The anti-inflammatory action of shear-induced KLF2, however, was shown to be mainly dependent on co-factor modulation, rather than on directly affecting NF- $\kappa$ B activation and nuclear translocation<sup>13</sup>, consistent with the documented effects of flow *in vivo*.

Given the absence of translocated, nuclear NF- $\kappa$ B at non-inflamed atheroprone sites *in vivo* and the complex effects of long versus short-term shear and KLF2 on NF- $\kappa$ B activity, we decided to reevaluate prolonged shear and KLF2 modulation of pro-inflammatory gene-expression. In the present study, we demonstrate that shear stress and KLF2 can modulate transcription factor activity and basal inflammatory gene expression of human umbilical vein endothelial cells (HUVEC). We show that shear stress inhibits, in a KLF2-dependent manner, the nuclear activation of activating transcription factor 2 (ATF2), one of the heterodimeric components of AP-1. Moreover, elevated levels of phosphorylated ATF2 protein are shown in endothelial cells overlying early atherosclerotic plaques, compared to healthy endothelium. This provides a novel mechanism by which shear stress might protect endothelial cells from a pro-atherogenic phenotype.

## Methods

### Cell culture and shear stress experiments

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured in Medium-199 (M199, Invitrogen, Carlsbad, California, USA), supplemented with 20% (vol/vol) fetal bovine serum (FBS), 50  $\mu\text{g}/\text{mL}$  heparin (Sigma, St Louis, Missouri, USA), 6-25  $\mu\text{g}/\text{mL}$  endothelial cell growth supplement (ECGS; Sigma), and 100 U/mL penicillin/streptomycin (Invitrogen) as described.<sup>10</sup> 24-hour shear stress experiments were performed in a parallel plate-type flow chamber with pulsatile flow ( $12 \pm 7$  dynes/cm<sup>2</sup>) as described<sup>4,14</sup>, using a CellMax Quad positive-displacement pump (Cellco, Germantown, MD). Long term shear stress exposure (6 days) was as described.<sup>4,14</sup> In brief: HUVEC were seeded in fibronectin-coated artificial capillary cartridges (Polypropylene 70, Cat No. 400-025, Cellco; DIV-BBB cartridge, Flocel Inc, Cleveland, Ohio, USA) in medium containing 10  $\mu\text{g}/\text{mL}$  ECGS. Cells were allowed to adhere and reach confluency overnight, with medium flowing through the extra-capillary space using the CellMax Quad pump system to provide oxygen and nutrients. Next, flow was guided through the capillaries and gradually increased to correspond to a pulsatile shear stress of  $19 \pm 12$  dynes/cm<sup>2</sup>, which was maintained over the next 6 days with intermediary medium changes. For static controls, HUVEC from the same isolate were seeded in fibronectin-coated cell culture flasks and grown to confluency. After indicated treatments, either total RNA was obtained using Trizol reagent (Invitrogen) or nuclear extracts were made.

### **Inflammatory cytokine stimulation during shear stress experiments**

TNF- $\alpha$  (R&D systems, Abingdon, UK) was reconstituted in PBS supplemented with 1% (w/v) BSA and used at a final concentration of 25 ng/ml during the final 6 hours of shear stress exposure, KLF2 overexpression or controls.

### **Real-time RT-PCR**

cDNA from 0.5-1  $\mu$ g of total RNA was synthesized according to the manufacturers protocol (Invitrogen) and diluted 10x for gene-specific analysis with real-time RT-PCR. All RT-PCR reactions were performed in a 15  $\mu$ l reaction on an iCycler thermal cycler system (Biorad Laboratories, Veenendaal, Netherlands). Measured mRNA level were expressed as normalized ratios compared to ribosomal phosphoprotein P0 expression levels. Gene-specific primers were designed using Beacon Designer 3 software (Premier Biosoft International, Palo Alto, CA) and optimal melting temperature was obtained using a temperature gradient reaction.

### **Microarray probe synthesis and hybridization**

A human oligonucleotide library containing 18.659 gene specific 65-mer sequences was purchased from Sigma/Compugen and spotted on glass slides by the Microarray Department of the University of Amsterdam. Microarrays and coverslips were pretreated for 1 hour at 40°C in a buffer containing 25% formamide (v/v), 5X SSC, 0.2% (w/v) SDS and 0.1% (w/v) BSA. All microarray experiments were performed using a common reference RNA, comprised of a pool of RNA from HUVEC, the monocytic cell-line THP-1, and wholemount human carotid and aortic arteries. Up to 1  $\mu$ g of total RNA from

samples or common reference was amplified a single round using the T7-based Ambion MessageAmp kit (Cat No. 1750, Ambion, Huntingdon, UK), with 50% of rUTP ribonucleotides replaced by aminoallyl-rUTP (Cat No. A5660, Sigma). Aminoallyl-modified amplified RNA (aRNA) was labeled with either Cy3 (common reference) or Cy5 (samples) monoreactive dyes (GE Healthcare, Uppsala, Sweden). Next, labeled probes were fragmented followed by purification using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration as well as dye incorporation was measured using the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). Equivalent amounts of labeled aRNA were applied to pretreated oligonucleotide microarrays in duplo and hybridized for 16 hours at 40°C. After hybridization slides were washed and subsequently scanned using an Agilent-II Scanner (Agilent Technologies Palo Alto, CA). Feature extraction was done using Arrayvision 8.0 software (GE Healthcare Europe, Diegem) and background subtracted intensities were subsequently LOESS-normalized in R (LIMMA package, Bioconductor software, <http://www.bioconductor.org>). Normalized data was imported into Rosetta Resolver (Rosetta Biosoftware, Seattle, WA).

### **Microarray data analysis**

Re-ratio based experiment definitions were constructed in Rosetta Resolver, followed by initial analysis, consisting of marker gene verification and hierarchical clustering. For promoter analysis, data was exported from Rosetta Resolver and analyzed using whole genome rVista software (<http://genome.lbl.gov/vista/index.shtml>). These calculation used a database of all transcription factor binding sites (TFBS) conserved in the human to

mouse whole genome alignment of May 2004. Locus link IDs of the genes from each group (listed in Supplemental Table 1) were used as input. Calculated were the TFBS over-represented in 500 basepair upstream regions of these genes using all upstream regions of human refseq3 genes as outgroup (Supplemental Table 2).

### **Immunohistochemistry**

Human vascular tissue specimens were collected from organ donors after obtaining informed consent with approval of the AMC Medical Ethical Committee, and procedures conformed to the Declaration of Helsinki. Paraffin sections were deparaffinized and dried, followed by antigen retrieval by boiling the slides for 10 minutes in a 10 mM citrate buffer at pH 6.0. Primary antibody incubation with Thr71-phosphorylated ATF2 antibody was performed overnight at 4°C. Next, biotinylated secondary antibodies were used for 1 hour at room temperature, followed by incubation with streptavidin-biotin complexes conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). Peroxidase substrate coloring with the VECTOR NovaRED substrate kit (Vector Laboratories, Burlingame, CA) was allowed to proceed for 10 minutes. Sections were examined using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany) and photographed using a Sony DXC-950P digital camera (Sony Corp., Tokyo, Japan) operated with the Leica QWin software (Leica Imaging Systems Ltd., Cambridge, UK). Overview images of entire vessels were obtained by scanning the slides on an Epson EU-35 flatbed scanner (Seiko Epson Corp., Nagano, Japan) with a resolution of 6400 dpi and importing the images into Adobe Photoshop CS2 9.0 (Adobe Systems, San Jose, CA) using Epson TWAIN Pro software (Seiko Epson Corp.).

### **Lenti-viral KLF2 overexpression and knock-down**

Stable lenti-viral KLF2 overexpression or knock-down experiments were performed by transducing HUVEC with lentiviral vectors expressing KLF2 cDNA or specific short hairpin RNA sequences directed against KLF2 and FLUC, as described.<sup>4,5</sup>

### **Nuclear extract preparation and transcription factor ELISA**

Nuclear proteins were prepared with the nuclear extract kit in accordance with the manufacturer's protocol (Active Motif Europe, Rixensart, Belgium). Transcription factor activity was determined with TransAM MAPK family kit (Active Motif). In brief, 2-20 µg of nuclear extract was added to each microtiter plate well into which an oligonucleotide with an ATF2 or NF-κB consensus binding site had been immobilized. Transcription factors bound to their cognate DNA binding site were detected using specific horseradish peroxidase-conjugated antibodies for p65 or Thr71-phosphorylated ATF2 supplied in the kit. After substrate coloring, absorption at 450nm was measured on an EL808 microplate reader (BioTek, Winooski, VT).

### **Immunofluorescence**

For immunofluorescence, mock- and KLF2-transduced HUVEC were grown on gelatin-coated glass coverslips and fixed with 4% (v/v) formaldehyde. Primary antibody incubation using Thr71-phosphorylated ATF2 antibody (Cell Signaling Technology, Danvers, MA) was performed overnight at 4°C. Alexa488-labeled secondary antibodies were used for 1 hour at room temperature, followed by Hoechst nuclear staining,

mounting and fluorescence microscopy analysis using a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany).

### **RNA interference with duplex siRNA and Western blotting**

HUVEC from 3 different isolates were grown to 80% confluency according to cell culture methods described above. Cells were then changed to 1 ml/well of Optimem reduced serum medium (Invitrogen) and transfected with 325 pmol of non-specific (5'-CAGUCGCGUUUGCGACUGG-3' synthesized siRNA, Ambion) or ATF2 siRNA (Silencer pre-designed siRNA #16704, Ambion) using the Oligofectamine reagent (Invitrogen) according to manufacturer's protocol. After 4 hours, 2 ml of M199 supplemented with 20% (vol/vol) FBS, 50 µg/mL heparin, 12.5 µg/mL ECGS and 100 U/mL of penicillin/streptomycin were added to the Optimem medium in each well. After 24 hours, medium was changed to 2 ml of full M199 containing 12.5 µg/mL ECGS and RNA or protein was harvested after 48 hours. Where applicable, cells were stimulated with 10 ng/ml of TNF- $\alpha$  or vehicle (PBS + 1% BSA) during the final 6 hours of the experiment. Western blotting was performed as described.<sup>15</sup> Total ATF2 protein levels were detected using a monoclonal ATF2 antibody (Cell Signaling Technology) and an  $\alpha$ -tubulin staining was performed as a control for equal loading. Densitometric quantification of the Western blot was performed using cyQuant software version 2003.03 (Amersham, Piscataway, NJ).

### **Statistical analysis**

Experimental data are shown as mean of normalized ratios  $\pm$  SEM for the indicated number of experiments. The paired or unpaired Student's T-test was used to calculate statistical significance of expression ratios or optical densities versus controls. P-values of less than 0.05 were considered statistically significant.

## Results

### **Shear stress inhibits basal but not TNF- $\alpha$ -induced expression of inflammatory genes**

The artificial capillary system<sup>14</sup> was used to obtain gene-expression profiles of endothelial cells stimulated by inflammatory cytokines, comparing their inflammatory response under prolonged, unidirectional pulsatile laminar flow to static conditions. We studied the effects of these conditions using genome-wide expression profiling. Figure 1A shows a hierarchical clustering of a selection of genes modulated more than 2-fold by shear, TNF- $\alpha$ , or shear and TNF- $\alpha$  combined, with all 3 treatments relative to static control conditions. Three main clustergroups can be discriminated: group A contains genes whose expression is downregulated by shear, but that are unaffected by TNF- $\alpha$  (Supplemental Table 1A); group B contains genes whose expression is upregulated by TNF- $\alpha$  under both static and shear conditions (Supplemental Table 1B); group C contains genes that are upregulated by shear stress and are unaffected or downregulated by TNF- $\alpha$  (Supplemental Table 1C). Validation of microarray expression data by real-time PCR was performed for a selection of genes from each group and showed that the expression of 8 out of 9 genes agreed with the microarray data (Figure 1B-D, Supplemental Tables 1A-C). In endothelial cells under prolonged pulsatile flow compared to static conditions, endothelin (EDN1) and plasminogen activator inhibitor 1 (PAI-1) from group A were downregulated or unchanged, respectively, whereas PAI-1 was only slightly induced after 6 hours of TNF- $\alpha$ , which did not match the microarray expression data (Figure 1B, Supplemental Table 1A). In contrast, several adhesion molecules and chemokines were potently induced by TNF- $\alpha$  and moderately suppressed by shear stress (Figure 1C). In

line with our previous reports, mRNA levels of KLF2, NOS3 and TM were found to be highly upregulated by prolonged shear stress and to be inhibited by TNF- $\alpha$  (Figure 1D).

To gain insight in the coordinate regulation of genes within a specific group, a search for transcription factor binding elements in upstream regions of constituent genes was performed using whole genome rVISTA. This softwarepackage evaluates which transcription factor binding sites (TFBS), conserved between pairs of species, are statistically significantly over-represented in upstream regions in a group of genes.<sup>16</sup> Using the human-mouse alignment of May 2004, and setting a 500 bp upstream search region, we found over-representation of distinct transcription factor binding sites in each of the 3 main groups (Supplemental Table 2). Importantly, the TNF- $\alpha$ -responsive group B is enriched in binding sites for NF- $\kappa$ B and the AP-1 family of transcription factors (indicated in Supplemental Table 2 as AP1FJ). Group B also shows enrichment of an ATF4 binding site. Furthermore, ATF3 and ATF4 binding sites are over-represented in group A, comprised of genes downregulated by shear stress but not modulated by TNF- $\alpha$ . The latter is also evident from a lack of enrichment of NF- $\kappa$ B binding sites in group A. Thus, it seems that group A and B share ATF binding site enrichment as well as downregulation of unstimulated basal expression by shear stress. In group C, comprised predominantly of shear induced genes, enrichment for AP-1 and NRF2 binding sites is detected.

Evidently, there seems to be a clear distinction in identity of different members of the AP1/ATF families in the different clusters. Detailed inspection showed this to be based on subtle sequence differences as homo- or heterodimers of the ATF family bind the cAMP response element 5'-TGACGTCA-3' which differs by one nucleotide from the

consensus AP-1 binding site 5'-TGAC/GTCA-3'.<sup>17,18</sup> One of the most studied members of the ATF transcription factor family, ATF2, is crucial for cytokine-induced expression of E-Selectin in endothelial cells.<sup>19,20</sup> Furthermore, the ATF2 transcription factor has been described to be constitutively expressed,<sup>17</sup> including in HUVEC,<sup>21</sup> which makes it a prime target through which downregulation of unstimulated basal expression of group A and B genes by shear stress could be mediated. In contrast to this, key members of the AP-1 family, c-Jun and c-Fos, are known to be inducible transcription factors at the expression level in response to growth factors, cytokines and stress.<sup>22</sup> ATF3 and ATF4 also are inducible transcription factors, acting mostly through increased expression in response to ER stress.<sup>23</sup> with ATF3 having almost no detectable levels in unstimulated endothelial cells.<sup>21</sup> We validated this in our microarray expression profiles from unstimulated endothelial cells. Signal intensity levels for ATF3, ATF4 are around or below reliable detection levels, whereas the ATF2 signal is well above this threshold. The constitutive expression of ATF2 in unstimulated endothelial cells and its key role in pro-inflammatory signaling prompted us to further investigate its role in the atheroprotective effect of shear stress.

### **Human lesional endothelial cells are positive for phosphorylated ATF2**

To our knowledge, ATF2 has not been described in the context of shear stress and/or atherosclerosis, therefore its potential physiological relevance was first assessed. The presence of active, phosphorylated ATF2 in endothelial cells from healthy or early atherosclerotic lesions was probed by immunohistochemistry (Figure 2). After determining the presence of a continuous layer of endothelial cells by CD31 staining in

human iliac and carotid arteries, a clear and consistent positive signal for phosphorylated ATF2 could be seen in endothelial cells overlying early atherosclerotic lesions. In contrast, endothelium overlying morphologically healthy vessel wall is completely devoid of phosphorylated ATF2, although a strong positive signal for phosphorylated ATF2 was present in the media, presumably in smooth muscle cells.

### **Shear stress suppresses nuclear levels of activated ATF2 via KLF2**

Based on the promoter analysis and immunohistochemical data described above, we investigated the role of ATF2 in the modulation of gene expression by shear stress in more detail. For this purpose, we used an ELISA-based assay which measures nuclear levels of activated transcription factors that are able to bind to oligonucleotides containing their cognate DNA-binding sites. Nuclear extracts from HUVEC exposed to pulsatile flow show a clear reduction in levels of phosphorylated ATF2, most prominently after 5 days of shear (Figure 3A). Stable lentiviral overexpression of KLF2 for 7 days resulted in a suppression of nuclear activated ATF2 to a level similar to that reached by prolonged shear (Figure 3B). The reduced nuclear levels of phosphorylated ATF2 were not due to a change in total ATF2 levels, since neither shear stress, nor KLF2 overexpression altered ATF2 mRNA expression compared to static or mock controls, as measured by RT-PCR (Supplemental Figure 1). Knock-down of KLF2 using lentivirally delivered small interfering RNA (siRNA) abrogates the shear-stress mediated inhibition of ATF2, implicating a direct dependence on KLF2 in this observation (Figure 3C). Even under static conditions, KLF2 siRNA increases ATF2 levels significantly compared to a control siRNA against firefly luciferase (FLUC). The latter observation correlates with an

increase in expression levels of several pro-inflammatory genes in static cells transduced with KLF2 siRNA, compared to mock siRNA (Supplemental Figure 2).

### **KLF2 overexpression inhibits TNF- $\alpha$ -induced nuclear activation of ATF2**

To further investigate the mechanism by which shear stress mediates its effects on ATF2, we measured nuclear activation of ATF2 in cells overexpressing lentiviral KLF2. KLF2 has been shown to be the causal factor in shear-mediated inhibition of several genes involved in inflammation and vascular tone.<sup>3-5,13</sup> Figure 4A shows that nuclear ATF2 was potently suppressed by KLF2 compared to mock transduced cells in the unstimulated control. Furthermore, TNF- $\alpha$ -induced activation of nuclear ATF2, as seen in mock transduced cells, was completely abolished by KLF2. Conversely, it appeared that TNF- $\alpha$ -induced activation of NF- $\kappa$ B component p65 was only partly inhibited by KLF2, whereas basal levels showed no difference between mock and KLF2-transduced cells (Figure 4B).

### **Phosphorylated ATF2 is excluded from the nucleus by KLF2**

Since the activated transcription factor assay cannot discern between the degree of nuclear localization and the degree of phosphorylation, immunofluorescence was used to clarify this issue. Fluorescent staining of phosphorylated ATF2 in HUVEC showed a clear and predominant cytoplasmic localization of phosphorylated ATF2 in KLF2-overexpressing cells (Figure 5B), whereas mock transduction resulted in exclusive nuclear staining of phosphorylated ATF2 (Figure 5A). These observations were confirmed by an additional nuclear staining with Hoechst (Figure 5C, D), which led to

merged pictures (Figure 5E, F), clearly identifying the major effect of KLF2 on the localization of phosphorylated ATF2. Quantification of the immunofluorescence data from 2 independent experiments show a clear reduction in p-ATF2 positive nuclei from 85% in mock cells to 25% in KLF2 transduced cells (Figure 5G).

### **ATF2 knock-down leads to reduction of basal and TNF-induced pro-inflammatory gene expression levels**

Having shown that ATF2 is inhibited by shear stress via KLF2, we next investigated the effect of direct inhibition of ATF2 on pro-inflammatory gene expression employing duplex siRNA against ATF2. A solid knock-down of ATF2 mRNA and protein levels by ~80% was achieved in HUVEC 48 hours after addition of ATF2 siRNA (Figure 6A-C). The siRNA-mediated inhibition of ATF2 was equally potent in vehicle- and TNF- $\alpha$  treated cells, even though TNF- $\alpha$  caused a minor 1.5-fold increase in ATF2 expression in the non-specific siRNA control (Figure 6Di). Knock-down of ATF2 caused a reduction in both basal and TNF- $\alpha$ -induced expression levels of pro-inflammatory genes from cluster groups A (EDN1) and B (SELE, CCL2, VCAM1, IL8) could be confirmed by real-time PCR (Figure 6Dii-vii). The expression of PAI-1 was not significantly reduced by siATF2 by either vehicle or TNF- $\alpha$  treatment. Interestingly, the inducibility of pro-inflammatory genes by TNF- $\alpha$  as seen in cells treated with the non-specific control, was preserved when ATF2 was knocked down.

## Discussion

There is still no definitive explanation for the cause of the moderate pro-inflammatory status of endothelium in the absence of flow *in vitro* or at disturbed flow regions of the vasculature *in vivo*. In this study we focused on non-cytokine induced gene expression as modulated by prolonged shear stress. The promoters of genes that are downregulated by prolonged exposure to shear stress, show a clear enrichment for ATF binding sites (Figure 1A-D). Our present results show that both shear stress and the shear-induced transcription factor KLF2 inhibit the activity of the constitutively expressed pro-inflammatory transcription factor ATF2, by inhibiting its nuclear translocation in HUVEC (Figures 3, 5). Furthermore, constitutive and cytokine-induced expression of the panel of inflammatory genes, several adhesion molecules and chemokines<sup>5</sup> is indeed sensitive to ATF2 activity as shown by siRNA mediated knockdown (Figure 6C) and their expression is suppressed by both shear stress and KLF2 (Figures 1, 3). Interestingly, knockdown of residual KLF2 results in a further increase of constitutive expression of these genes under static conditions (Supplemental figure 2). Together, these data strongly suggest that blunting of ATF2 activity is one prominent mechanism by which shear stress and KLF2 inhibit the constitutive pro-inflammatory gene expression observed in the absence of biomechanical stimulation of endothelium. The physiological relevance of these *in vitro* data are supported by our novel observation that endothelial cells overlying human early atherosclerotic plaques have vastly increased levels of phosphorylated ATF2, compared to healthy endothelium (Figure 2). Interestingly, we consistently observed a much stronger staining for phosphorylated ATF2 in medial smooth muscle

cells, located directly underneath the endothelial layer in plaque-free areas, compared to neointimal smooth muscle cells. This finding demonstrates for the first time a differential expression of activated, phosphorylated transcription factor ATF2 between medial and intimal smooth muscle cells, suggesting that it would be one of the molecular mediators of the phenotypic change undergone by smooth muscle cells during plaque formation. Together, these findings warrant further detailed investigation into the role of ATF2 during *in vivo* atherosclerosis.

The effect of shear stress on basal and TNF- $\alpha$ -induced gene expression was investigated by genome-wide expression profiling and cluster-based promoter analysis showing three general cluster groups. Promoters of genes in cluster group B, comprised of TNF- $\alpha$ -induced genes, showed enrichment in transcription factor binding sites for NF- $\kappa$ B and AP-1 (Figure 1A), confirming that both transcription factors are crucial for a potent transcriptional response to cytokine stimulation.<sup>11</sup> It is well-documented that potent induction of inflammatory gene expression depends on formation of a transcriptional complex, the enhanceosome, composed of NF- $\kappa$ B, AP-1 and co-factor CBP/p300.<sup>9</sup> ATF2, together with Jun and Fos subfamilies, collectively constitute the family of AP-1 transcription factors, which are homo- and heterodimers composed of basic-region leucine zipper (bZIP) proteins.<sup>17</sup> Jun proteins form stable homodimers or heterodimers with Fos that bind the AP-1 DNA recognition element 5'-TGAG/CTCA-3'. However, ATF2 also forms homodimers or heterodimers with Jun that bind preferentially to the slightly different sequence of the cAMP response element (CREB), 5'-TGACGTCA-3'. The latter response element is also the preferential binding site for other members of the

CREB family, including ATF3 and ATF4. However, involvement of ATF3 and ATF4 in shear stress-mediated inhibition of basal pro-inflammatory transcription is less likely, because, like c-Jun and c-Fos, they are normally expressed at low levels in resting cells and are usually induced at the expression level.<sup>9,20,24</sup> Still, a role for other ATF members in prolonged inflammatory signaling is evident as Gargalovic and co-workers very recently showed that both basal and ox-PAPC-induced expression of CCL2, IL6 and IL8 was partly dependent on ATF4.<sup>25,26</sup> Furthermore, TNF- $\alpha$  apparently overrides the suppressive effects on ATF2 by highly inducing NF- $\kappa$ B, as shown by induction of genes containing NF- $\kappa$ B binding sites under stimulated conditions in both static and sheared endothelial cells (Figure 1) and by increased nuclear NF- $\kappa$ B protein even during overexpression of KLF2 (Figure 4). Indeed several reports have directly shown that NF- $\kappa$ B prevails over p38/AP-1 driven expression after TNF- $\alpha$  activation.<sup>11,12</sup> In line with this, the actual induction of pro-inflammatory genes by TNF- $\alpha$  was preserved in cells in which specifically ATF2 had been knocked down by siRNA, even though ATF2 knock-down did attenuate both basal and TNF- $\alpha$ -stimulated pro-inflammatory transcription levels (Figure 6). This finding seemingly contradicts previous reports that show a decrease in cytokine-induced inflammatory gene expression in endothelial cells after up to 24 hours of pre-exposure to laminar flow.<sup>27,28</sup> It has become increasingly evident, however, that these time points may still be regarded as preconditioning and are not sufficient for termination of transient shear effects or for establishing the full KLF2 effect.<sup>5,14</sup> We now indeed show that a full suppression of activated ATF2 in nuclear extracts from HUVEC requires more than 24 hours of arterial-level shear exposure to reach the same suppression level as caused by over-expressing KLF2 (Figure 3).

KLF2 has been shown to control multiple processes that maintain a healthy, functional endothelium and confer protection from initiation of atherosclerosis.<sup>3,5,14</sup> For cluster group C, real-time PCR validated that shear stress increased KLF2, NOS3 and TM mRNA expression compared to static conditions, and that these genes were inhibited by TNF- $\alpha$  (Figure 1D). Both observations are in accordance with previous results.<sup>3,14</sup> KLF2 was also shown to dampen IL-1 $\beta$  induced pro-inflammatory gene expression.<sup>3,13</sup> Paradoxically, we and others report that TNF- $\alpha$  and IL-1 inhibit KLF2 expression<sup>14,29</sup> and KLF2 moderately inhibits NF- $\kappa$ B activation<sup>13</sup> (Figure 4B). It is likely that prolonged cytokine stimulation through NF- $\kappa$ B and AP-1 will therefore lift KLF2-mediated inhibition of pro-inflammatory genes, allowing a transient but potent inflammatory response. Along these lines, we argued that the protective effects of shear stress and KLF2 are more likely due to suppression of basal inflammatory gene expression depending on ATF2. In support of this view, the absence of shear stress indeed elevates basal expression levels of adhesion molecules and chemokines, an effect that is KLF2 dependent<sup>3,4</sup> (Figure 1, 3). Additionally, knock-down of KLF2 in static cells elevated basal transcription of these pro-inflammatory genes (Supplemental Figure 2).

Recruitment of CBP/p300 has recently been implicated in the KLF2 mediated inhibition of cytokine-induced gene expression via NF- $\kappa$ B.<sup>13,29</sup> Another recent study in monocytes has shown that KLF2 can also recruit the PCAF co-factor away from the NF- $\kappa$ B complex.<sup>30</sup> Interestingly, ATF2 has intrinsic histone acetyltransferase (HAT) activity and might recruit other HATs, like co-factors CBP/p300 and PCAF.<sup>31</sup> Suppression of

phosphorylated ATF2 levels in the nucleus by shear stress, would result in decreased HAT-activity, reduced co-factor recruitment, and ultimately decreased constitutive transcription of genes (partially) dependent on ATF2. Thus, a straightforward explanation is supplied for the observed decreased CBP/p300 and PCAF activities and the effects on NF- $\kappa$ B-dependent gene expression reported previously.<sup>13,29</sup> The next challenge is to explain the mechanism by which shear stress and KLF2 modulate nuclear ATF2 levels as in HUVEC overexpressing KLF2, total ATF2 phosphorylation does not seem to be affected (Figure 5). It could be that the nuclear import and export machinery is directly involved, possibly through c-Jun as a prerequisite nuclear anchor for ATF2<sup>32</sup> or that modulation of upstream MAP kinase pathways is affected.<sup>27</sup> Further investigation is currently undertaken to find the detailed mechanism underlying the crucial observation of suppression of ATF2 nuclear localization by KLF2.

In conclusion, our *in vitro* results show a prominent role for the activated transcription factor ATF2 in basal but also inducible inflammatory transcription in endothelial cells, with endothelial activated ATF2 being present *in vivo* only at lesional areas of the human vasculature. This pro-inflammatory status is repressed by both shear stress and KLF2, which are also able to inhibit the nuclear activity of the transcription factor ATF2 *in vitro*. This strongly suggests that shear stress through KLF2, inhibits pro-inflammatory gene expression in regions of the vasculature that appear to be protected against the focal initiation of atherosclerosis.

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## Figure legends

### **Figure 1. Analysis of the effect of shear stress on basal and TNF- $\alpha$ -induced gene expression in endothelial cells.**

(A) Hierarchical clustering of a selection of genes modulated more than 2-fold by shear, TNF- $\alpha$ , or shear and TNF- $\alpha$  combined, all three treatments relative to static control conditions. Three main cluster groups can be discriminated, which were analyzed for the presence of overrepresented transcription factor binding sites in the promoters of the genes in each group compared to the whole genome in conserved 500 basepair upstream regions in the human-mouse alignment of May 2004. A selection of transcription factors with overrepresented binding sites is shown for each cluster group and highlighted in orange are transcription factors that are well-described to be involved in inflammatory signaling. (B-D) Real-time PCR validation of gene expression as obtained by microarray. PCR analysis was done for a selection of genes from each of the three cluster groups as identified by microarray data analysis. Real-time PCR was performed in duplicate on HUVEC cDNA from 3 independent isolates, comparing static conditions to either 7 days of pulsatile shear stress (19 +/- 12 dynes/cm<sup>2</sup>), a 6 hour treatment of TNF- $\alpha$  (25 ng/ml), or both. Expression levels relative to P0 housekeeping gene were obtained for a selection of genes from each cluster group and represented relative to static controls. Single asterisks (p<0.05) or double asterisks (p<0.01) indicate significant difference versus static control conditions; ns: not significant.

### **Figure 2. Phosphorylated ATF2 is expressed specifically in lesional endothelium.**

(A) Human donor tissue (iliac artery, male, age 43, died from subdural hematoma after fall) was probed for the presence of a continuous layer of endothelial cells by immunohistochemical CD31 staining. After confirmation of CD31 positivity, adjacent sections were stained for Thr71-phosphorylated ATF2 (pATF2). The left part of the panel shows an overview of the entire vessel, indicating the

lesional and lesion-free areas that are enlarged in the composite images on the right, which show pATF2 or CD31 staining as red coloration. (B-E) Enlarged parts of lesional and lesion-free areas stained for pATF2 or CD31, taken from panel A as indicated by the red-lined boxes. Similar enlargements are shown for two other vascular specimens, obtained from human donor (panels F-I; iliac artery, female, age 35, died in falling accident), or human obduction tissue (panels J-M; internal carotid artery, male, age 85, died from liver failure). All sections were counterstained for nuclei with hematoxylin. The enlargements in panels A and B were obtained using a 63x magnification.

**Figure 3. Functional analysis of nuclear ATF2 activity in endothelial cells exposed to prolonged shear and its dependence on KLF2.** Nuclear extracts from HUVEC exposed to 5 days of pulsatile flow (A) or HUVEC overexpressing KLF2 (B) were assayed for the presence of functional Thr71-phosphorylated ATF2 protein. Data from 3 independent isolates are expressed relative to static or mock controls. (C) HUVEC containing lentiviral delivered double-stranded siRNA directed against KLF2 or a control siRNA against firefly luciferase (FLUC) were exposed for 5 days to shear stress or to static conditions and assayed for nuclear activated ATF2. The means of 3 different isolates are expressed relative to static FLUC. Single asterisks ( $p < 0.05$ ) or double asterisks ( $p < 0.01$ ) indicate significant difference versus static control conditions; ns: not significant.

**Figure 4. Effect of TNF- $\alpha$  on nuclear transcription factor activity in mock- and KLF2-transduced HUVEC.** Nuclear activation of ATF2 (A) or p65 (B) was determined in mock- and KLF2-overexpressing HUVEC activated for 0-3 hours with TNF- $\alpha$  (10 ng/ml). Basal and TNF- $\alpha$ -induced ATF2 levels were strongly suppressed by KLF2, whereas basal p65 levels were not changed and TNF- $\alpha$ -induced p65 levels were only partially inhibited. Data are represented as mean ratios of 2-3 different isolates relative to the unstimulated mock condition. Single asterisks

( $p < 0.05$ ) or double asterisks ( $p < 0.01$ ) indicate significant difference between mock and KLF2 values, double daggers ( $\ddagger$ ) indicate significant difference ( $p < 0.01$ ) between TNF- $\alpha$ -stimulated and unstimulated control values.

**Figure 5. KLF2 suppresses nuclear localization of phosphorylated ATF2.** HUVEC transduced with mock (A,C,E) or KLF2 (B,D,F) lentivirus were fixed with paraformaldehyde and stained for Thr71-phosphorylated ATF2 protein by immunofluorescence (A,B). Nuclei were made visible with a Hoechst nuclear staining (C,D) and picture were merged (E,F). Photographs representative for 2 independent experiments were obtained by fluorescence microscopy using a 63x magnification. (G) Quantification was performed by counting nuclei with strong nuclear pATF2 positivity in 3 representative microscopic fields from mock and KLF2 transduced cells taken from the 2 independent experiments, each field containing on average ~350 cells. Double asterisks ( $p < 0.01$ ) indicate significant difference in pATF2-positive nuclei between mock and KLF2 values.

**Figure 6. ATF2 knockdown reduces basal and TNF- $\alpha$ -induced expression of pro-atherogenic genes.** Total cell mRNA and protein were harvested from HUVEC that were untransfected (control) or transfected with non-specific (siNS) or ATF2 siRNA (siATF2). (A) ATF2 mRNA expression levels represented relative to ribosomal protein P0, as measured by real-time PCR. (B) Total ATF2 protein levels were measured using Western blot and equal loading was verified with  $\alpha$ -tubulin. Protein levels were quantified and shown in (C) as ratios versus siNS, corrected for  $\alpha$ -tubulin. (D) Inhibition of basal and TNF- $\alpha$ -induced expression of genes from cluster groups A and B by knock-down of ATF2 compared to a non-specific control. ATF2 expression was inhibited by siATF2 during both vehicle and TNF- $\alpha$  treatment (i), resulting in reduction of basal and TNF- $\alpha$ -induced expression of SELE (ii), CCL2 (iii), IL8 (iv), VCAM1 (v) and EDN1 (vii), but not of PAI-1 (vi). Represented are the mean P0-corrected, relative mRNA

expression levels from 3 different HUVEC isolates measured in duplo by RT-PCR. Single asterisks ( $p < 0.05$ ) or double asterisks ( $p < 0.01$ ) indicate significant difference versus non-specific siRNA.

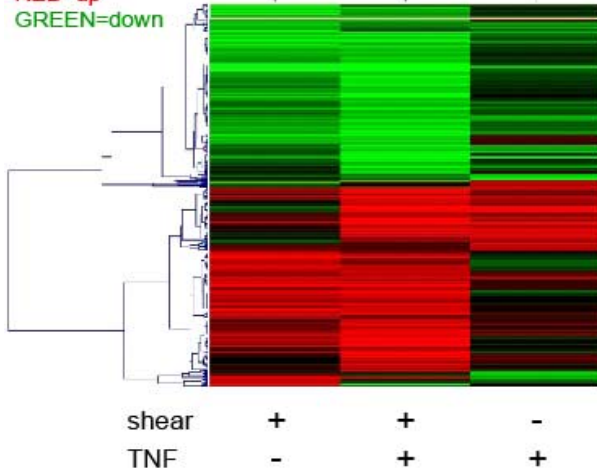
**Figure 1**

**A**

Fold Change vs static control > 2  
p<0.01



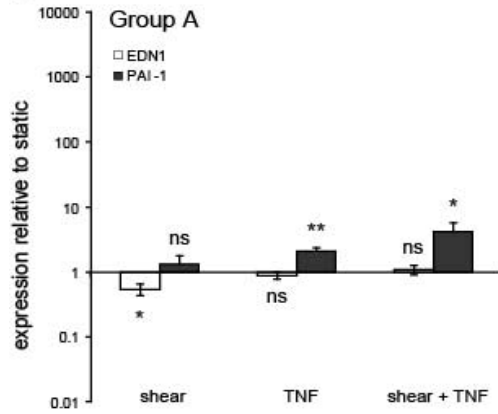
RED=up  
GREEN=down



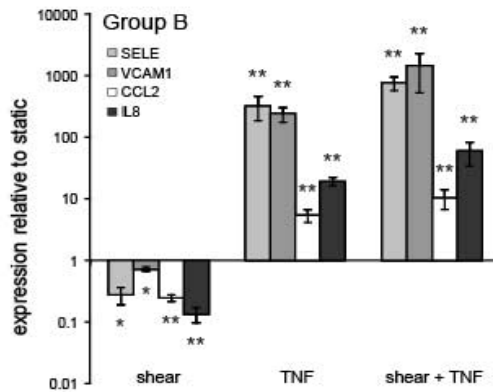
Group	Description	Over-represented TFBS in 500bp upstream region
A	Shear inhibited	ATF / SMAD / SOX
B	TNF induced	NF-kB / AP-1 / ATF / NRF2 / ETS / SOX
C	Shear induced	AP-1 / NRF2 / FXR / PR

shear + + -  
TNF - + +

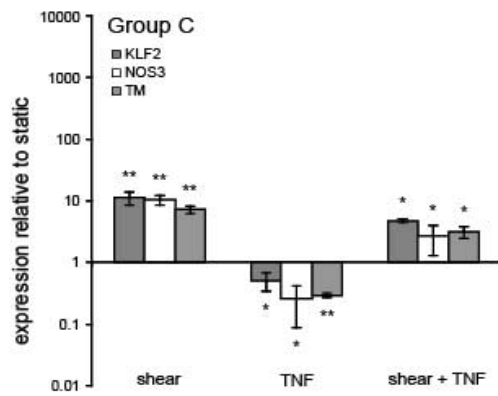
**B**



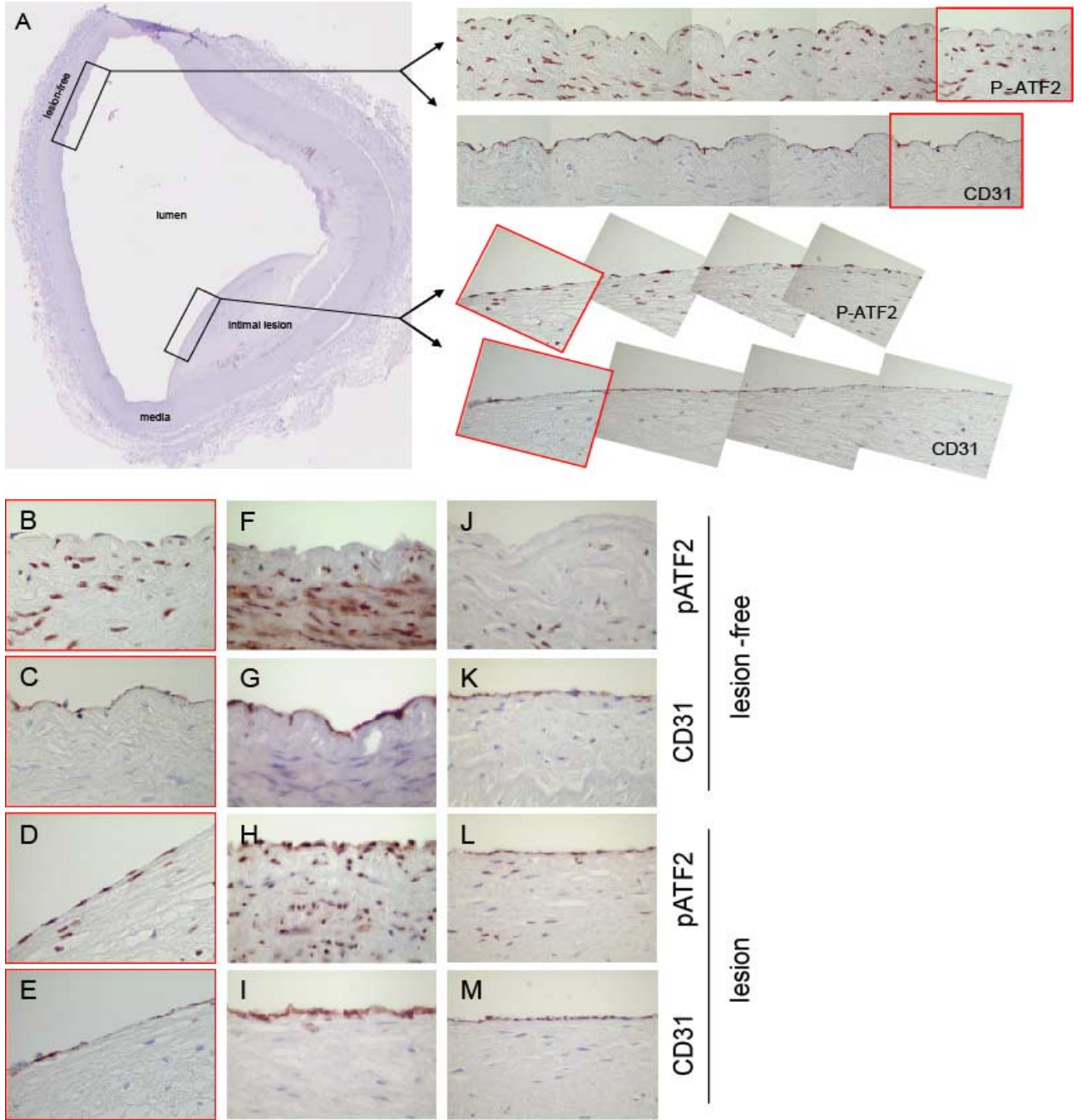
**C**



**D**

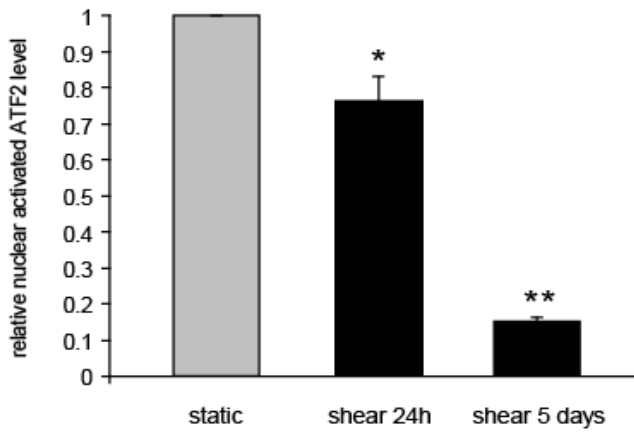


**Figure 2**

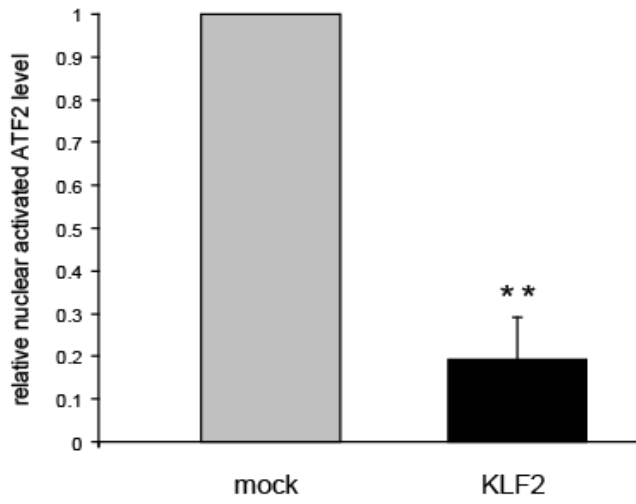


**Figure 3**

**A**



**B**



**C**

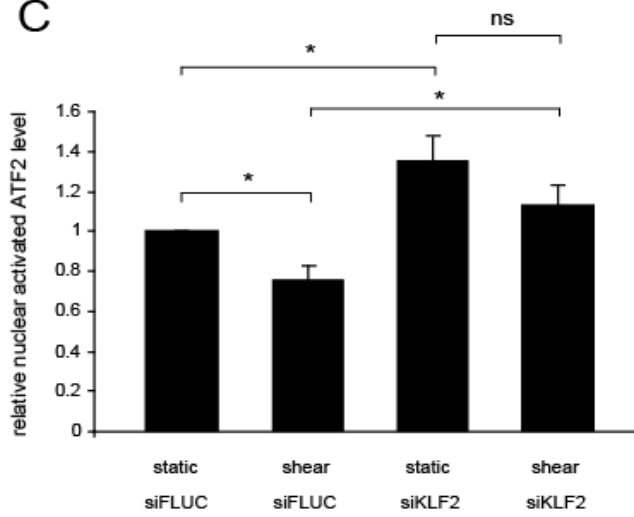
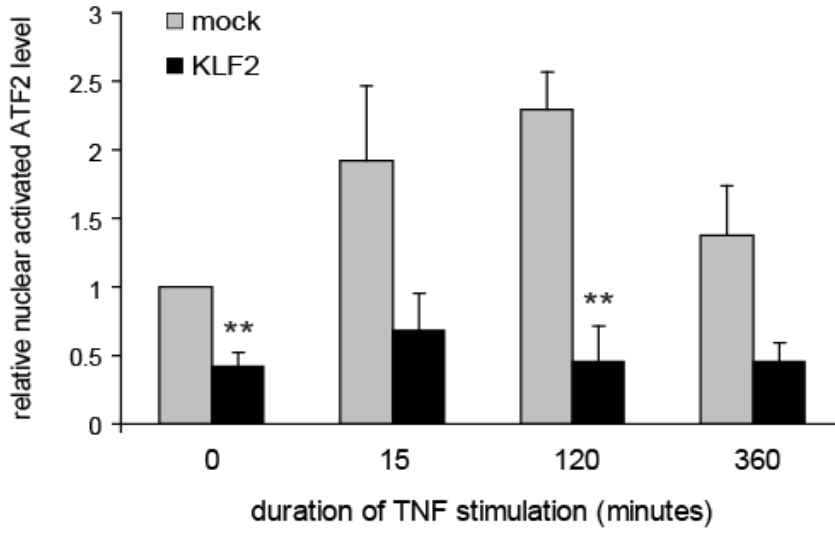


Figure 4

A



B

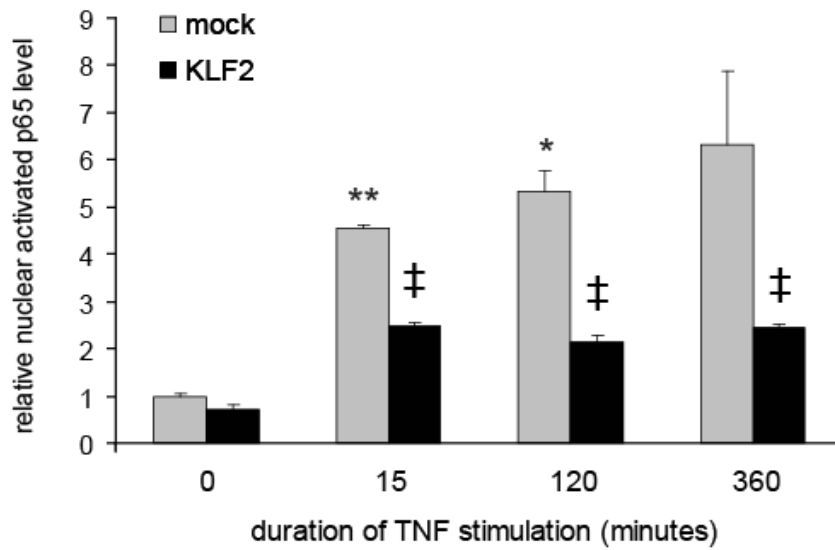
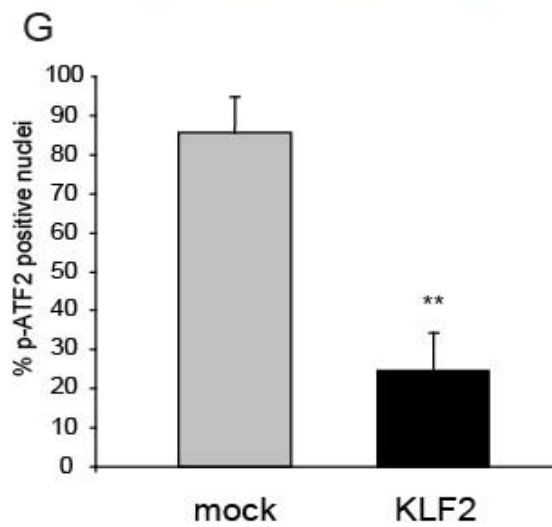
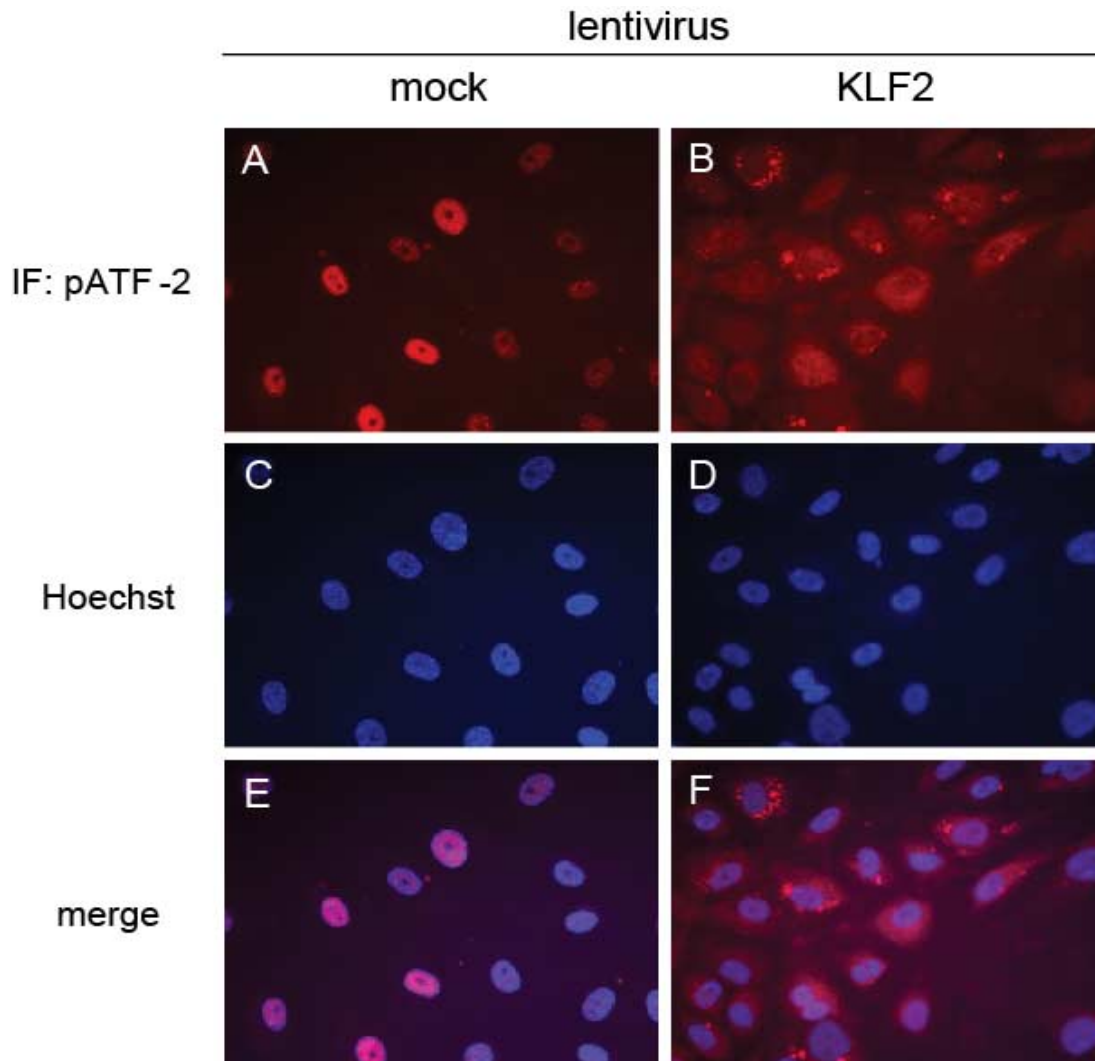


Figure 5



**Figure 6**

