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Premature senescence of endothelial cells upon chronic exposure to $\text{TNF}\alpha$ can be prevented by N-acetyl cysteine and plumericin

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Cellular senescence is characterized by a permanent cell-cycle arrest and a pro-inflammatory secretory phenotype, and can be induced by a variety of stimuli, including ionizing radiation, oxidative stress, and inflammation. In endothelial cells, this phenomenon might contribute to vascular disease. Plasma levels of the inflammatory cytokine tumor necrosis factor alpha ($\text{TNF}\alpha$) are increased in age-related and chronic conditions such as atherosclerosis, rheumatoid arthritis, psoriasis, and Crohn's disease. Although $\text{TNF}\alpha$ is a known activator of the central inflammatory mediator NF- κ B, and can induce the intracellular generation of reactive oxygen species (ROS), the question whether $\text{TNF}\alpha$ can induce senescence has not been answered conclusively. Here, we investigated the effect of prolonged $\text{TNF}\alpha$ exposure on the fate of endothelial cells and found that such treatment induced premature senescence. Induction of endothelial senescence was prevented by the anti-oxidant N-acetyl cysteine, as well as by plumericin and PHA-408, inhibitors of the NF- κ B pathway. Our results indicated that prolonged $\text{TNF}\alpha$ exposure could have detrimental consequences to endothelial cells by causing senescence and, therefore, chronically increased $\text{TNF}\alpha$ levels might possibly contribute to the pathology of chronic inflammatory diseases by driving premature endothelial senescence.

Cardiovascular diseases are the leading cause of death in the elderly population of western countries¹. Endothelial cells form the inner lining of the vasculature and regulate vascular tone and hemostasis, thus playing a pivotal role in vascular function². Evidence indicates that cellular senescence, characterized by a cell-cycle arrest and pro-inflammatory changes in gene expression³, occurs in endothelial cells *in vivo* and may play a role in age-related vascular pathology such as atherosclerosis, e.g. by reducing important vasodilatory factors such as nitric oxide and prostacyclin and promoting a pro-adhesive and pro-thrombotic phenotype³⁻⁸.

Senescence can be induced by a plethora of stimuli, including ionizing radiation^{9,10} telomere dysfunction^{4,11}, reactive oxygen species (ROS)^{12,13}, high glucose concentrations^{14,15} or inflammatory cytokines^{16,17}. It has been established that the underlying cell-cycle arrest is mediated by p21 and p16, two cyclin-dependent kinase inhibitors¹⁸⁻²⁰, and that persistent DNA damage signaling drives the hallmark - inflammatory and tumorigenic - phenotype of senescent cells, termed the senescence-associated secretory phenotype (SASP)^{21,22}. This SASP, which prominently involves NF- κ B signaling^{23,24}, comprises adhesion molecules, metalloproteinases, and many cytokines^{3,25-27}. Some of these, such as IL-1, IL-6, and $\text{TNF}\alpha$, have been implicated in atherosclerosis^{28,29} and diabetes³⁰.

Although $\text{TNF}\alpha$ is a known activator of NF- κ B, and can induce the intracellular generation of ROS³¹, the question whether prolonged exposure to $\text{TNF}\alpha$ can induce senescence in endothelial cells has not been answered.

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Since many SASP genes are responsive to TNF α stimulation within a short time and play an essential role in acute inflammation³², it could be important to discriminate between short- and long-term effects of TNF α on endothelial senescence.

In the present study, we investigated whether prolonged stimulation with TNF α might induce a senescence phenotype in human umbilical vein endothelial cells (HUVECs) *in vitro*. We addressed this by assessing the proliferative marker Ki-67, the cyclin-dependent kinase inhibitors p16 and p21, as well as components of the aforementioned SASP, namely E-selectin, intracellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), insulin like growth factor binding protein 5 (IGFBP-5) as well as the cytokines IL-6 and IL-8. In addition, we examined the involvement of NF- κ B activity and ROS generation in this process, by assessing nuclear levels of the p65 NF- κ B subunit, and employing the commercially available ROS probe H2-DCF. Furthermore, we studied the effect of two IKK2- targeting inhibitors of NF- κ B signaling - the synthetic PHA-408³³ and the plant-derived plumericin³⁴ - as well as the anti-oxidant N-acetyl cysteine (NAC)^{35,36}, on the induction of senescence features induced by TNF α in HUVECs.

Results

Chronic TNF α exposure induces cell-cycle arrest in HUVECs. To test the hypothesis that chronic stimulation of endothelial cells with TNF α might induce premature cellular senescence, we exposed HUVECs propagated in full growth medium to 10 ng/ml TNF α for six days. This induction period was followed by an additional recovery period of three days in full growth medium only, in order to determine the persistence of the growth arrest after six days of TNF α stimulation (Fig. 1a). As a control, HUVECs were exposed solely to the solvent (0.01% DMSO). The acquisition of features associated with senescence was tested using published markers, including the proliferation marker Ki-67 and the cyclin-dependent kinase inhibitors p16 and p21. Standard staining controls were applied.

We found that the growth rate of cells brought into contact with TNF α for six days was lower than that of control cells, exposed to full growth medium only (Fig. 1b). Following treatment, we observed an increase in size and flattening of cells, a common feature of senescence (Fig. 1c). Staining of treated and control HUVECs with Ki-67 revealed that only half as many cells ($p < 0.001$) expressed this proliferation marker at the end of six days of TNF α stimulation (data not shown). After the recovery phase of three days, the percentage of Ki-67 positive cells decreased further in the previously treated cells (Fig. 1d). Staining with p21 or p16 revealed that TNF α treatment of HUVECs yielded significant increases (both $p < 0.001$) in cyclin-dependent kinase inhibitor expression (Fig. 1e,f) as compared to the controls.

We found a similar increase in p16 positivity in human dermal microvascular endothelial cells (HDMECs) after their exposure to TNF α for six days (Supplemental Fig. 1a). These cells also exhibited an increase in size and cell flattening, similar to TNF α -treated HUVECs (Supplemental Fig. 1b).

Furthermore, we analyzed effects of prolonged cultivation in TNF α (10 ng/ml) in HUVECs that were exposed as previously for six days to TNF α , but cultivated for a longer recovery period (7–10 days) without TNF α . We show that this treatment resulted in progressive retardation of growth rate during the analyzed period (Supplemental Fig. 2a). The growth curve did not, though, reach a complete plateau, likely due to heterogeneity in senescence induction, which left a portion of cells still able to proliferate. Nevertheless, the portion of p16 positive cells was much higher in TNF α -treated cells in comparison to controls, as detected at days 13 and 16 (Supplemental Fig. 2b).

Hence, these results showed a decrease in proliferative capacity in HUVECs after their prolonged TNF α exposure, and we investigated whether this treatment might evoke further features of senescence.

Chronic TNF α exposure promotes the senescence-associated secretory phenotype. In previous studies, senescence was shown to confer characteristic changes in gene expression, i.e. of inflammatory adhesion molecules, cytokines, and matrix metalloproteinases, collectively referred to as SASP^{3,25}. To determine if our treatment with TNF α would also induce components of the SASP, we assessed the endothelial adhesion proteins ICAM-1 and E-selectin, the prothrombotic PAI-1, IGFBP-5, as well as two cytokines IL-6 and IL-8, shown previously to be elevated in endothelial cells at replicative senescence^{7,37–40}. Levels of ICAM-1, E-selectin, IL-6, and IL-8 in TNF α -treated cells were determined using cell ELISA⁴¹ and levels of PAI-1 and IGFBP-5 mRNA were measured using qPCR. As a comparison, cells not treated with TNF α were similarly assessed.

The ELISA results showed that in the cells pretreated with TNF α , adhesion molecules E-selectin and ICAM-1 were significantly increased at all time points compared to control HUVECs (Fig. 2a,b). QPCR analysis at day six demonstrated a statistically significant increase in the abundance of the respective PAI-1 and IGFBP-5 mRNA levels (Fig. 2c,d). Importantly, levels of cytokines IL-6 and IL-8 were significantly increased at day six of TNF α treatment (Fig. 2e,f), pointing to the induction of these cytokines following TNF α exposure. Similarly, levels of IL-6 and IL-8 were also increased in HDMECs that were exposed for six days to TNF α (Supplemental Fig. 1c,d).

In addition, we analyzed IL-6 and IL-8 levels in an experiment of prolonged cultivation in TNF α (10 ng) in HUVECs that were exposed as previously for six days to TNF α , but cultivated further for additional 10 days. The levels of both these cytokines were high, albeit declining, throughout the six-day period of direct TNF α stimulation. In spite of their tendency to further decline, they remained significantly elevated also during the entire 10 days period after withdrawal of TNF α treatment (Supplemental Fig. 2c,d).

Hence, these data demonstrated the induction of several SASP-related proteins upon prolonged TNF α exposure, further supporting the idea that inflammatory stress might promote cellular senescence.

NF- κ B translocation and ROS production are enhanced in TNF α treated cells. Emerging hallmarks in cellular senescence are increased activity of the NF- κ B pathway and increased generation of ROS^{23,42,43}. To investigate the time course of NF- κ B activation in HUVECs in response to TNF α , we assessed the

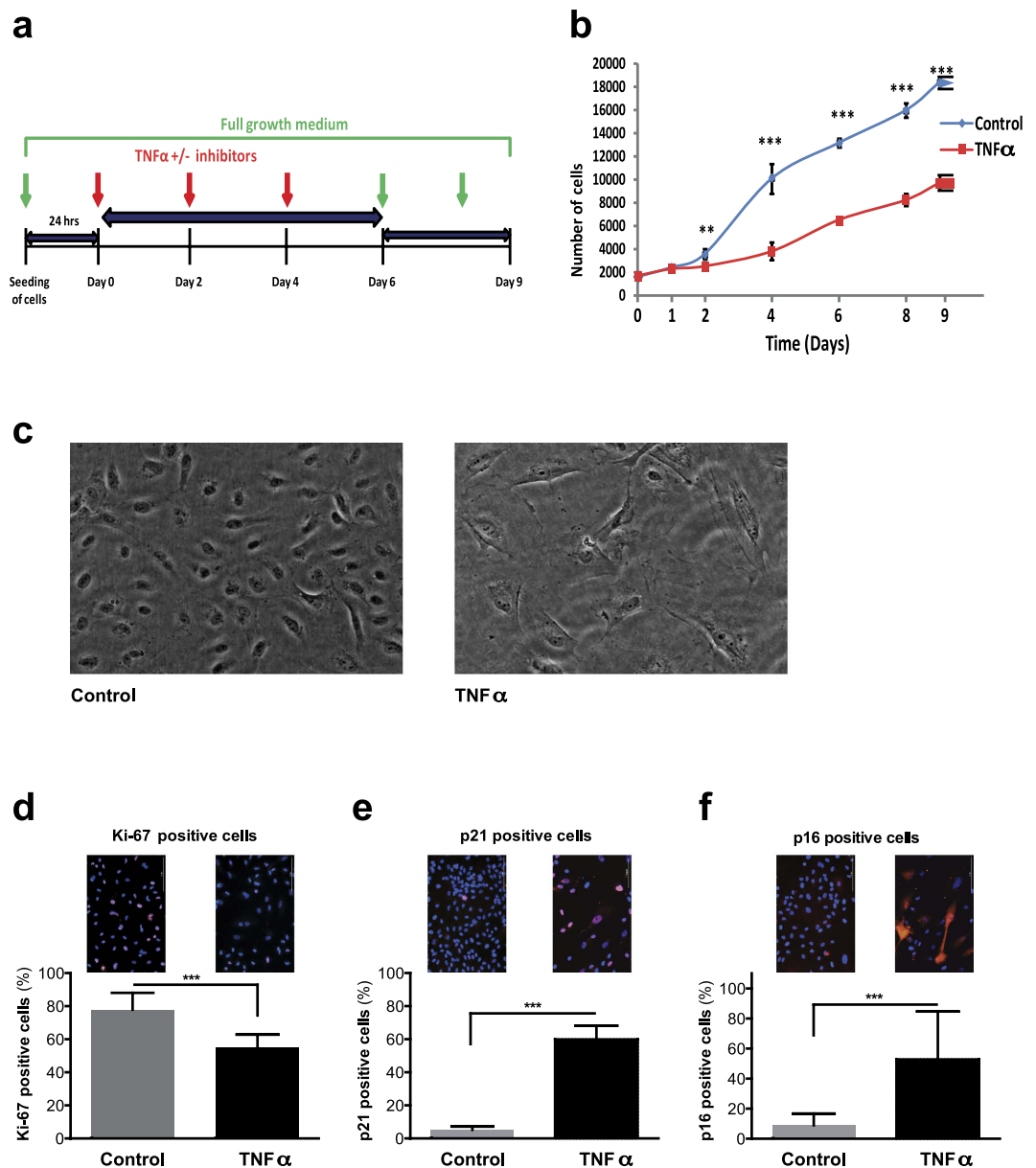


Figure 1. TNF α -induces inhibition of cell proliferation and premature senescence in HUVECs.

(a) Experimental design: Upon propagation in full growth medium for 24 hours, cells were exposed to TNF α (10 ng/ml) \pm inhibitors (NF- κ B inhibitors plumericin (1.5 μ M), PHA-408 (2 μ M) and antioxidant NAC (2 mM)) for a period of six days, followed by a three days recovery period in full growth medium. Control HUVECs were grown in full growth medium only during the whole period. (b) Growth curves of HUVECs treated with or without TNF α for the indicated time points. (c) Increase in size and flattening of TNF α -treated cells. (d–f) Representative fluorescent images of HUVECs (day nine) grown in presence or absence of TNF α and their quantification: (d) Ki-67, (e) p21, and (f) p16. Values are presented as mean \pm SD of technical triplicates (** $p < 0.01$, *** $p < 0.001$). The results shown are representative of three independent experiments.

translocation of the NF- κ B subunit p65 over the course of two days using a commercially available antibody and fluorescence microscopy. ROS levels were evaluated based on a fluorescence method applying H2-DCF⁴⁴ and in comparison to control cells.

We found a statistically significant increase in the nuclear levels of p65 in TNF α -treated HUVECs at 20 min (Fig. 3a,b) compared to the controls, which were less pronounced but still significant at 40 min and at two hours (Fig. 3a). At day one and day two time points, NF- κ B translocation was also significantly increased, and higher than at two hours (Fig. 3a), suggesting a biphasic response. Similarly, ROS production increased over the first two hours, thereafter dropped to basal levels at day one, and was increased again between days two and six (Fig. 3c).

In addition, we analyzed ROS production in an experiment of prolonged cultivation where HUVECs were exposed, as previously, for six days to TNF α , but cultivated further for additional 7 days. We found that ROS levels remained significantly elevated after withdrawal of TNF α treatment (Supplemental Fig. 2e).

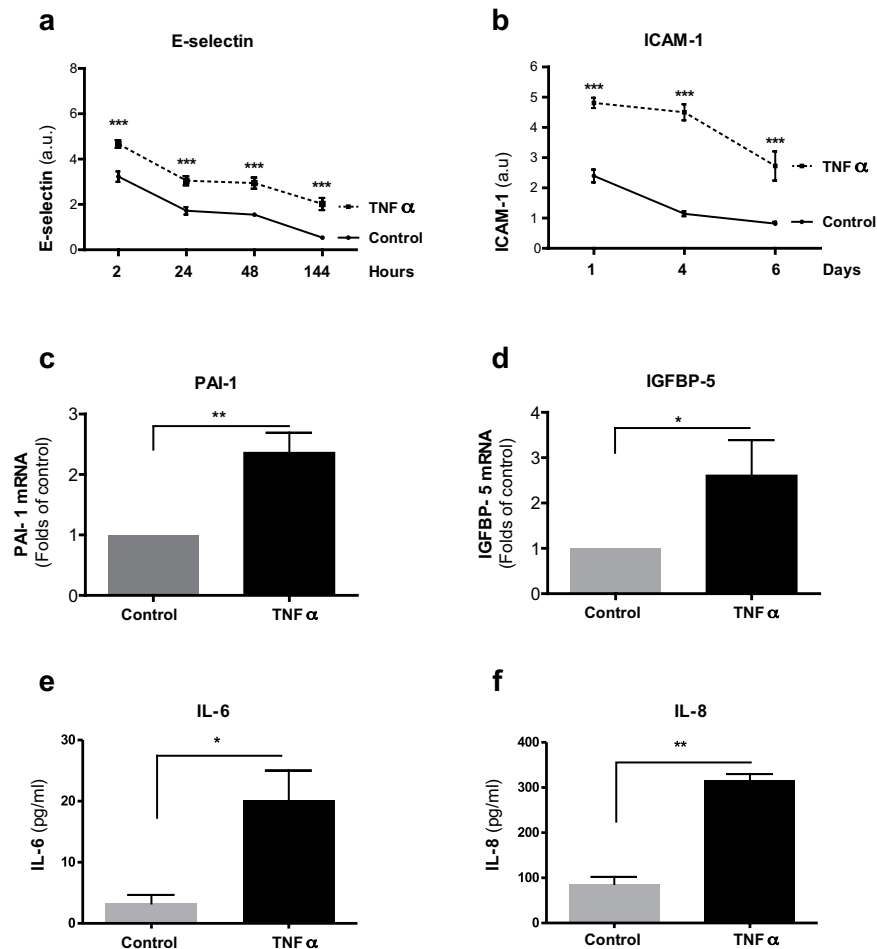


Figure 2. Prolonged TNF α -exposure promotes SASP in HUVECs. (a,b) Surface expression of E-selectin (a) and ICAM-1 (b) of HUVECs grown in the presence of TNF α (10 ng/ml) in comparison to control HUVECs for indicated time points, quantified by cell ELISA. (c,d) mRNA levels of PAI-1 (c) and IGFBP-5 (d) of HUVECs grown in the presence of TNF α (10 ng/ml) in comparison to control HUVECs at day six, quantified by qPCR. Levels of (e) IL-6 and (f) IL-8 in supernatants of HUVECs grown in the presence of TNF α (10 ng/ml) in comparison to control HUVECs at day six, quantified by ELISA. Values are presented as mean \pm SD of technical triplicates (a–d) or technical duplicates (e,f). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). a.u.: arbitrary units. The results shown are representative of three independent experiments.

Finally, we also investigated if the above regimen of TNF α treatment might induce apoptosis. To this, we used combination of annexin V and propidium iodide staining. The levels of apoptosis were similarly low in control as well as in TNF α -stimulated HUVECs at all analyzed time points (Supplemental Fig. 2f).

Therefore, by demonstrating increased NF- κ B translocation and ROS production, as well as showing that TNF α treatment at the used concentration of 10 ng/ml did not induce apoptosis, we could support the notion that prolonged TNF α treatment of HUVECs would cause them to senesce.

Inhibition of ROS and NF- κ B translocation prevents TNF α -induced cell-cycle arrest. We next questioned if the inhibition of NF- κ B and/or ROS production would prevent the effects we observed after prolonged TNF α stimulation in HUVECs. We employed inhibitors of NF- κ B signaling, including PHA-408³³ and plumericin³⁴ that target IKK2 (a kinase targeting the inhibitors of NF- κ B), and other inhibitors that act against ROS, including the anti-oxidant NAC^{35,36} and diphenyleneiodonium chloride (DPI), a known inhibitor of NADPH oxidase⁴⁵. We contemporaneously applied TNF α and each of these inhibitors to HUVECs, and measured their effects on translocation of the NF- κ B subunit p65 and generation of ROS, using the fluorescent methods described previously⁴⁴. TNF α -non treated HUVECs incubated with inhibitors were considered as controls.

The results showed that each of the inhibitors attenuated TNF α -induced NF- κ B translocation as well as ROS generation (Fig. 4). In the absence of inhibition, TNF α caused an approximately onefold increase in nuclear translocation of p65 of NF- κ B (Fig. 4a, column 5). However, when plumericin, PHA-408, or NAC were present, the increased p65 translocation was largely prevented. Application of inhibitors to cells not treated with TNF α (Fig. 4a, columns 2–4) did not alter the nuclear p65 levels. In reference to ROS production, plumericin was able to limit TNF α -induced ROS production to basal levels following 20 min (Fig. 4b, column 7), while the other

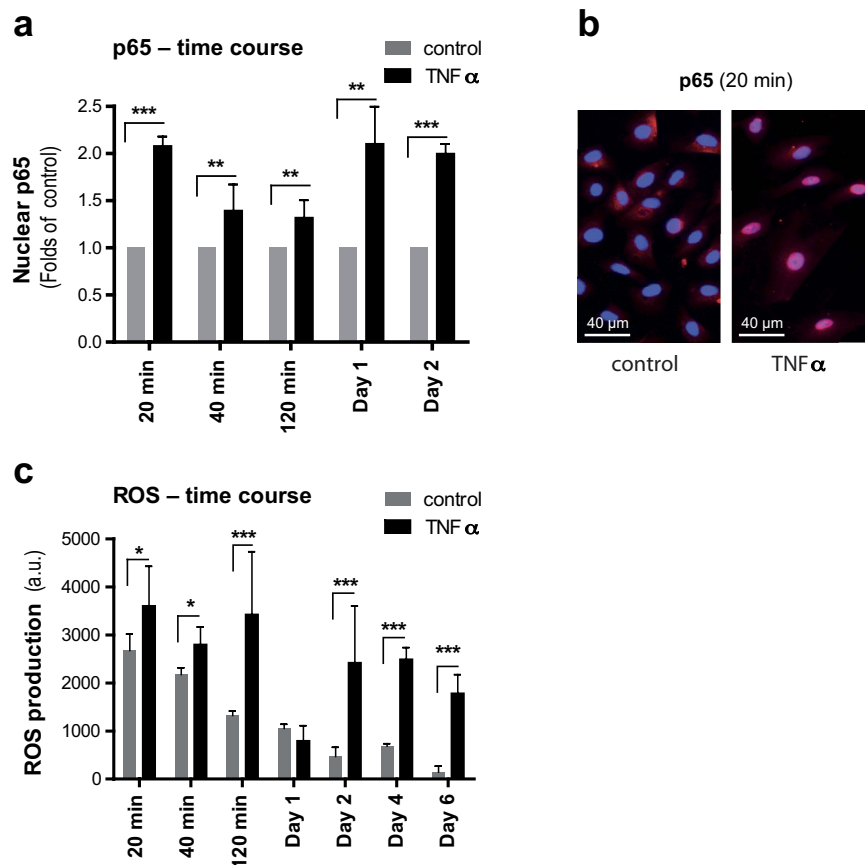


Figure 3. TNF α increases the nuclear translocation of NF- κ B and induces ROS generation in HUVECs.

(a) Quantification of nuclear translocation of NF- κ B in HUVECs grown in the presence of TNF α (10 ng/ml) for indicated time points, in comparison to control HUVECs, by integrated mean intensity of p65 in nucleus using CellProfiler software. (b) Representative fluorescent images of nuclear translocation of NF- κ B in HUVECs (20 min) grown in presence or absence of TNF α . (c) Determination of ROS production in HUVECs grown in the presence of TNF α (10 ng/ml) for indicated time points, in comparison to control HUVECs, by using fluorophore H2-DCF (10 μ M). Values are presented as mean \pm SD of technical triplicates (* p < 0.05, ** p < 0.01, *** p < 0.001). a.u.: arbitrary units. The results shown are representative of three independent experiments.

inhibitors tested took longer to negate ROS production (Fig. 4b–d). The effect of these inhibitors on control cells not treated with TNF α was minor. DPI, when co-applied to the TNF α treated cells, blocked ROS generation, albeit only after 40 min (Fig. 4b–d). In non-treated cells, it did not significantly alter this process.

Since the used inhibitors were efficient in blocking both p65 translocation and ROS production, we further tested if they might also affect features of endothelial senescence.

We first analyzed the ability of these inhibitors to prevent the associated cell-cycle arrest. HUVECs were propagated in full growth medium and treated with 10 ng/ml TNF α for six days in the presence of the aforementioned inhibitors. As before, this induction period was followed by an additional recovery time of three days in full growth medium. HUVECs growth was quantified until day nine, and expression levels of Ki-67, p21, or p16 were assessed by standard immunocytochemistry techniques in combination with commercially available antibodies at day nine. Similar steps were undertaken using control HUVECs that were not pretreated with TNF α .

In these control cells, the addition of inhibitors changed growth dynamics only minimally compared to control cells cultured without them (Supplemental Fig. 3a). In contrast, all three inhibitors significantly mitigated the TNF α -induced growth impairment of HUVECs over the whole analyzed period of nine days (Fig. 5a) and attenuated the decrease in proliferating cells, as evidenced by immunofluorescent detection of Ki-67, as well as the increase in cells positive for p21 and p16 (Fig. 5b–d). These data suggest that NAC, plumericin, and PHA-408 were able to prevent TNF α -induced cell-cycle arrest. Therefore, as further evidence for the prevention of senescence, we intended to evaluate further the phenotype of these cells.

Inhibition of ROS and NF- κ B signaling prevents induction of SASP. Having shown that inhibitors of ROS and NF- κ B attenuated the induction of a cell cycle arrest by TNF α , we next wished to examine whether their use would also affect the induction of SASP. To this end, we assessed the expression of six endothelial SASP proteins (E-selectin, ICAM-1, IGFBP-5, PAI-1, IL-6, and IL-8) upon utilization of our inhibitors. Untreated HUVECs were used as controls.

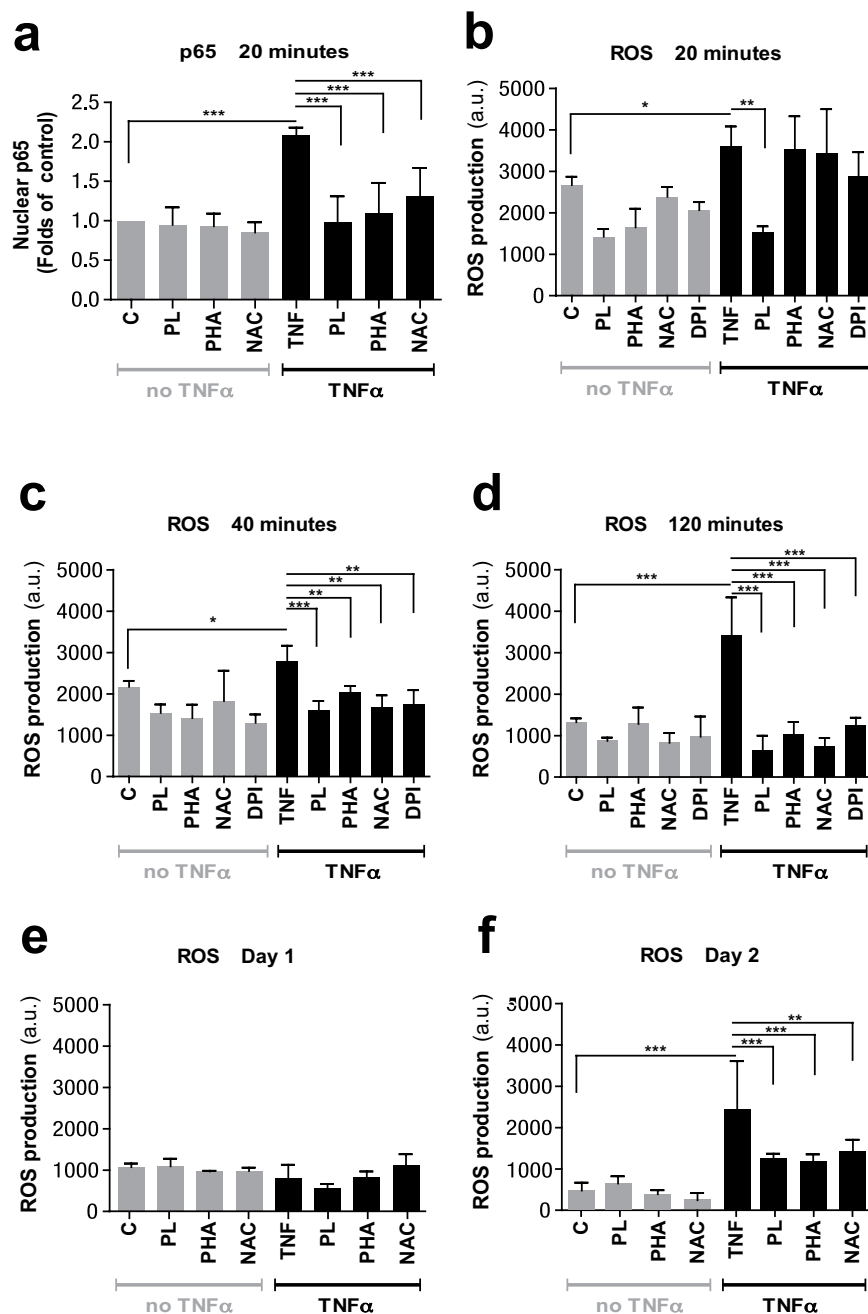


Figure 4. Plumericin, PHA-408, and NAC prevent TNF α -induced nuclear translocation of NF- κ B and ROS generation in HUVECs. (a) Quantification of nuclear translocation of NF- κ B in HUVECs grown in the presence of TNF α (10 ng/ml) \pm inhibitors in comparison to control HUVECs \pm inhibitors at 20 min, by integrated mean intensity of p65 in nucleus using CellProfiler software. (b–f) Determination of ROS production in HUVECs grown in the presence of TNF α (10 ng/ml) \pm inhibitors in comparison to control HUVECs \pm inhibitors for indicated time points, by using fluorophore H2-DCF (10 μ M). Values are presented as mean \pm SD of technical triplicates (* p < 0.05, ** p < 0.01, *** p < 0.001). The results shown are representative of three independent experiments. C: control, PL: plumericin, PHA: PHA-408, NAC: N-acetyl cysteine, a.u.: arbitrary units.

The results showed that addition of the three inhibitors (NAC, plumericin, and PHA-408) on their own hardly affected E-selectin levels (Fig. 6a). However, TNF α treatment led to an approximately fivefold higher level of E-selectin, but this increase was reduced by about 20% when the three inhibitors had been applied (Fig. 6a). The results for IGFBP-5 yielded essentially the same pattern (Fig. 6b). Investigation of the expression of ICAM-1 and PAI-1 showed less marked changes (not shown). Importantly, levels of the cytokines IL-6 and IL-8, which were significantly increased upon prolonged TNF α exposure, were reduced by employing the above inhibitors, as

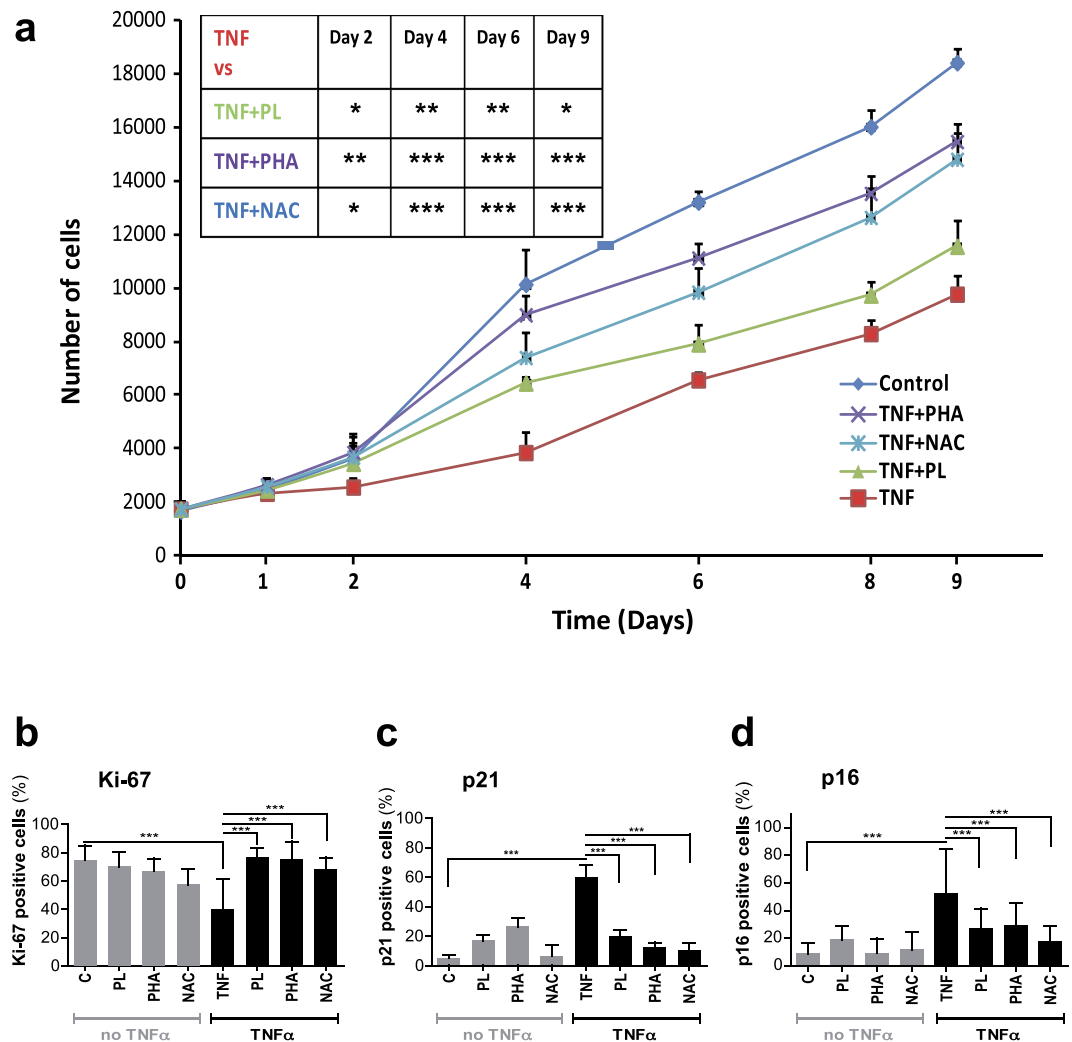


Figure 5. Plumericin, PHA-408, and NAC inhibit TNF α -induced cell-cycle arrest in HUVECs. (a) Growth curves of HUVECs treated with TNF α \pm inhibitors in comparison to control HUVECs for indicated time points. (b,d) Quantification of staining for (b) Ki-67, (c) p21 and (d) p16 of HUVECs treated with TNF α \pm inhibitors in comparison to control HUVECs \pm inhibitors at day nine. Values are presented as mean \pm SD of technical triplicates. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The results shown are representative of three independent experiments. C: control, PL: plumericin, PHA: PHA-408, NAC: N-acetyl cysteine.

determined by analyzing conditioned media (Fig. 6c,d). The levels of IL-6 and IL-8 in the presence of inhibitors and in control cells are presented in Supplemental Fig. 3b,c.

Hence, taking into account a representative cell surface protein (E-selectin) and the secreted factors IGFBP-5, IL-6 and IL-8, we were able to underscore that the prevention of the growth arrest coincided with a reduction of the senescence associated secretory phenotype in HUVECs.

Discussion

Senescence of endothelial cells, encompassing an irreversible growth arrest and secretion of pro-inflammatory mediators, may contribute to age-related endothelial dysfunction and vascular pathology⁶. In the present work, we showed that long-term application of TNF α to HUVECs induced features of senescence, such as a cell-cycle arrest and secretion of selected SASP components. In correspondence with previous studies, our results suggest the involvement of NF- κ B signaling and ROS generation in evoking senescence, as both inhibitors PHA-408 and plumericin, as well as the antioxidant NAC, were able to attenuate the induction of features of senescence.

TNF α has been shown previously to induce senescence in certain cell types^{46,47}, including fibroblasts¹⁶, but it remained hitherto unclear whether this is also true for endothelial cells, exemplified by HUVECs, a routinely used endothelial culture model. Although it was reported that TNF α may reduce proliferative capacity in these cells⁴⁸, it was not determined if this was due to senescence. A further study demonstrated that this cytokine may induce certain markers of dysfunction and possibly initiate senescence in HUVECs within 20 hours⁴⁹, but it was not investigated if such changes persisted after the end of treatment.

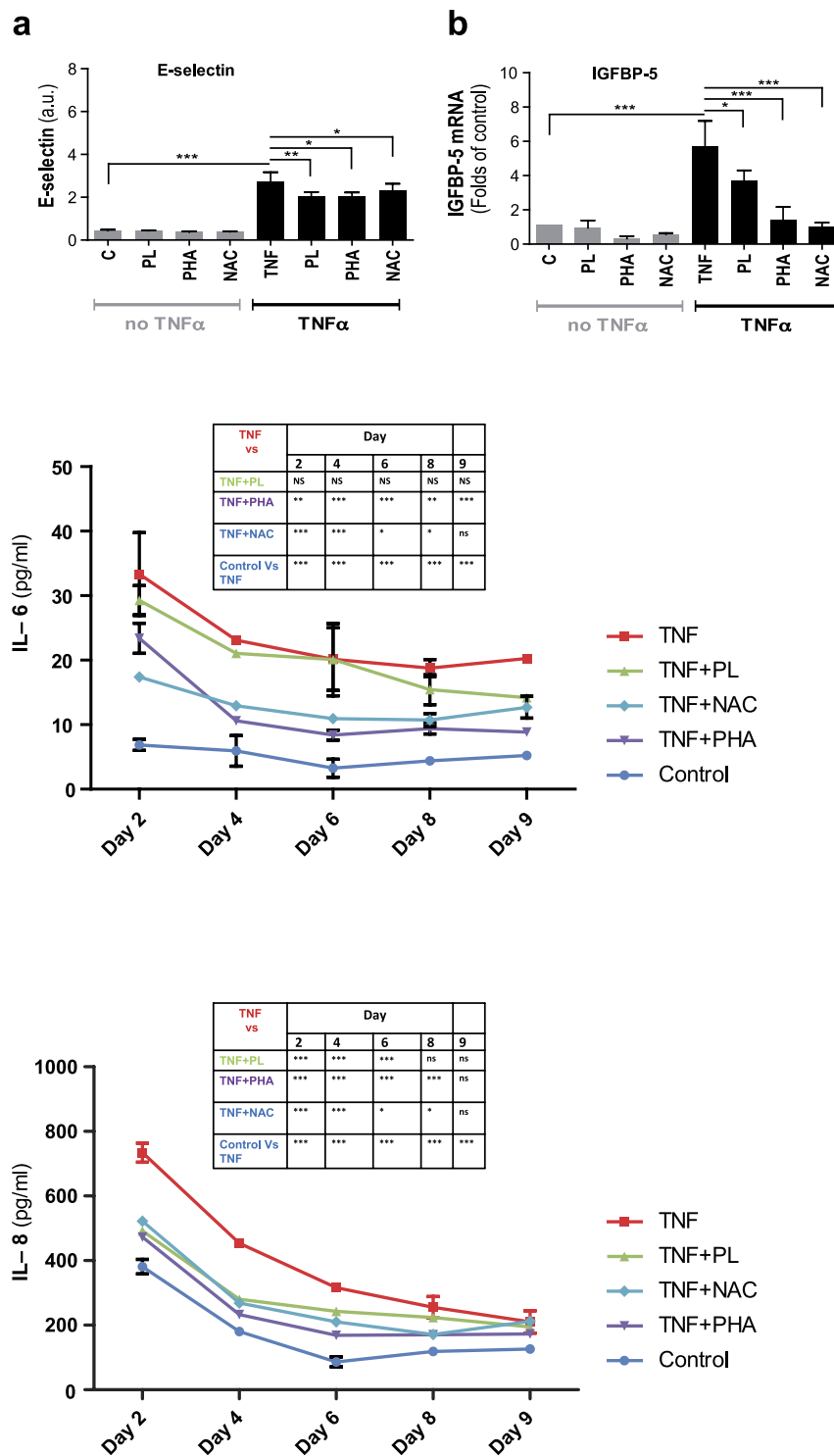


Figure 6. Plumericin, PHA-408, and NAC prevent induction of SASP in HUVECs. (a) Surface expression of E-selectin quantified by cell ELISA at day nine and (b) mRNA level of IGFBP-5 quantified by qPCR of HUVECs grown in the presence of TNF α (10 ng/ml) \pm inhibitors in comparison to control HUVECs \pm inhibitors. (c) IL-6 levels and (d) IL-8 levels in HUVECs grown in the presence of TNF α (10 ng/ml) \pm inhibitors in comparison to control HUVECs. Values are presented as mean \pm SD of technical triplicates (a,b) or duplicates (c,d). (*p < 0.05, **p < 0.01, ***p < 0.001). The results shown are representative of three independent experiments. C: control, PL: plumericin, PHA: PHA-408, NAC: N-acetyl cysteine.

In our study, we found that six days of treatment with TNF α were sufficient to induce features of senescence that persisted after removal of TNF α . Moreover, we found that the concomitant addition of IKK2 inhibitors or antioxidants with TNF α treatment mitigated the TNF α -induced growth impairment. Our data collectively

suggest that although certain features of senescence may be observed shortly after treatment with $\text{TNF}\alpha$, prolonged stimulation may promote the transition from a potentially reversible response towards manifestation of senescence.

A characteristic feature of cellular senescence is the SASP^{25–27,50} involving increased surface expression and release of inflammatory molecules⁵¹. In the present work, we chose to assess the expression of the adhesion molecules E-selectin and ICAM-1, as well as of PAI-1, IGFBP-5, IL-6, and IL-8, previously reported to be upregulated in senescence^{7,37–40,52,53}. We found that the expression levels of all of these proteins were elevated after six days of treatment, and with the exception of PAI-1, remained high for at least three further days of cell culturing in normal growth medium, thereby supporting the notion of their senescence. The induction of these changes was attenuated by concomitant application of inhibitors of ROS production and NF- κ B during $\text{TNF}\alpha$ treatment. In the case of IL-6 and IL-8, we observed a decline of expression over the course of stimulation. However, their levels remained significantly elevated over control cells at all investigated time points. While these findings support the notion of senescence in our cells, they also suggest that the effect of $\text{TNF}\alpha$ on the expression of certain cytokines may be greater than that elicited by the induction of senescence.

ROS generation in response to $\text{TNF}\alpha$ is known to occur both in the mitochondria and at the plasma membrane^{31,54–58} and is believed to contribute to endothelial dysfunction, in part by decreasing the availability of vasodilatory nitric oxide⁵⁴. ROS also influence - both positively and negatively, at multiple signaling levels - the transcriptional activity of NF- κ B^{59,60}. We explored the involvement of NF- κ B and ROS in our model of $\text{TNF}\alpha$ -induced senescence by employing immunocytochemical staining of the NF- κ B subunit p65 and utilizing the commercially available ROS probe H2-DCF. We found that both, the antioxidant NAC, as well as the IKK2 inhibitors PHA-408 and plumericin, suppressed the $\text{TNF}\alpha$ -induced translocation of p65 to the nucleus at 20 min time-point (Fig. 4a). However, our results indicate that only plumericin blocked the $\text{TNF}\alpha$ -induced increase in ROS at this early time point. In addition, this early increase in ROS appeared to be independent of the NADPH oxidase or the mitochondrial NADH-ubiquinone oxidoreductase, which were reported to be susceptible to inhibition by DPI^{45,61}.

We further found that $\text{TNF}\alpha$ -induced ROS generation, although significant already at 20 min, continued to show a relative increase over the next 120 min. At these later time points, all tested inhibitors, including DPI, potentially mitigated ROS generation, thus indicating a likely involvement of NADPH oxidase. Although after 24 hours of treatment, levels of ROS in $\text{TNF}\alpha$ -treated cells appeared to be similar to untreated and concomitantly inhibited cells, we observed a second stage of increased ROS levels from day two to six. During this stage, our inhibitors appeared to mitigate potentially the increase in ROS observed under $\text{TNF}\alpha$ stimulation. Intriguingly, the mean nuclear levels of p65 followed a similar biphasic response in $\text{TNF}\alpha$ treated cells, although they declined after a first peak at 20 min, and again after a second peak at 24 hours. Such biphasic response under continuous $\text{TNF}\alpha$ stimulation has been previously observed and was correlated with transient degradation of I κ B α at early time-points and persistent downregulation of I κ B α at late time points⁶². We observed, at the (early) 20 min and 40 min time points, elevated ROS levels also in control cells, possibly due to cell culture stress and persisting effects of harvesting and re-seeding.

Plumericin was originally identified as an inhibitor of IKK2³⁴, but has recently been demonstrated to also deplete glutathione levels in smooth muscle cells, presumably due to its high thiol reactivity⁶³. Depletion of glutathione has been shown to augment $\text{TNF}\alpha$ -induced ROS production in HUVECs^{64,65}. However, our results indicate that the overall effect of plumericin on ROS production is suppressive. Collectively, the results indicate that plumericin, while having a more pronounced effect on ROS production (especially at early time points), had a lesser effect on rescuing $\text{TNF}\alpha$ -impaired cell proliferation and preventing expression of SASP proteins than the specific NF- κ B inhibitor PHA-408 or the ROS inhibitor NAC.

In conclusion, we showed that prolonged stimulation of HUVECs by $\text{TNF}\alpha$ induced a senescent phenotype. Therefore, premature endothelial senescence may contribute to the pathology of chronic inflammatory diseases in which $\text{TNF}\alpha$ is presumed to play a critical role. We believe that our findings may motivate other researchers to explore in more detail the roles of $\text{TNF}\alpha$ and endothelial senescence in chronic disease and aging.

Materials and Methods

Chemicals and reagents. Plumericin was isolated from bark material of *Himatanthus sucuuba*, as described earlier⁶⁶. PHA-408 was obtained from Axon Medchem BV (Groningen, Netherlands) and NAC, DPI, Triton X-100, medium M199, fetal bovine serum (FBS), goat serum, gelatin, and paraformaldehyde (PFA) were purchased from Sigma Aldrich (Saint Louis, USA). Penicillin, streptomycin, fungizone, and trypsin-EDTA were bought from LONZA (Visp, Switzerland), endothelial cell growth supplement (ECGS) with heparin from PromoCell (Heidelberg, Germany), and human recombinant $\text{TNF}\alpha$ from PeproTech (Vienna, Austria). The products 2',7'-dichlorodihydrofluorescein diacetate (H2-DCF) and TMB ELISA substrate were obtained from ThermoFischer Scientific (Vienna, Austria). Hoechst 33342 and Alexa 555 were obtained from Life Technologies (Columbus, USA), mouse IgG HRP-linked antibody from GE Health care (Little Chalfont, UK), anti-CD31 and anti-p16 from BioLegend (San Diego, USA), anti-p65 from Santa Cruz (Santa Cruz, USA), anti-Ki-67 from ThermoFischer Scientific, anti-p21 from BD Bioscience (Franklin Lakes, USA), anti-E selectin and anti-ICAM-1 from R&D Systems (Abingdon, UK). Trizol, MuLV-reverse transcriptase, RNase inhibitor and, oligo dT primers were obtained from Life Technologies, and FastStart SYBR Green Master Mix from ThermoFischer Scientific.

Isolation and culturing of primary endothelial cells. HUVECs were isolated from umbilical cords as described earlier⁶⁷. Subconfluent cultures of HUVECs were cultured at 37 °C in a CO₂ incubator with 95% humidity in M199 medium containing 20% FBS, supplemented with 3 mg/ml ECGS and 22.5 mg/ml heparin, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.25 µg/ml fungizone (full growth medium). Cells used for experiments were at passages three to five.

Human dermal microvascular endothelial cells (HDMECs) were isolated in the laboratory of Dr. Peter Petzelbauer (Medical University of Vienna, Austria) from foreskins as previously described⁶⁸. Cells were cultured in endothelial cell growth medium EGM-2 supplemented with EGM-2 BulletKit (LONZA) and FBS of final concentration 15% and used for experiments at passage five.

Induction of senescence by chronic exposure to TNF α . For the induction of senescence, cells were seeded into 1% gelatin-coated 96 well plates in full growth medium and incubated for 24 h. Thereafter, cells were exposed to TNF α (10 ng/ml) alone or TNF α in combination with plumericin (1.5 μ M), PHA-408 (2 μ M) or NAC (2 mM) for the indicated time points. Fresh full growth medium containing TNF α (10 ng/ml) with or without inhibitors was supplied every other day, i.e. at day two and day four. On day six, cells were switched back into full growth medium without TNF α or inhibitors and kept cultivated for additional three to 10 days.

Assessment of cellular proliferation of HUVECs by absolute cell count. The proliferation of HUVECs was assessed by absolute cell counting at the indicated time points. For this purpose, 1.5×10^3 cells were seeded into 1% gelatin-coated 96-well plates and treated with TNF α (10 ng/ml) alone or in combination with plumericin (1.5 μ M), PHA-408 (2 μ M), and NAC (2 mM). The cells were fixed for 10 min in 4% PFA, washed with PBS and stained with Hoechst 33342 for nuclear staining at the indicated time points. Cells were imaged with the multiplex reader Cytation 5 and counted using Gene 5 software version 2.07, both from BioTek (Winooski, USA).

Immunostaining for Ki-67, p21 and p16. HUVECs were exposed to TNF α (10 ng/ml) with or without plumericin, PHA-408, and NAC for the indicated time points. Cells were then fixed for 10 min in 4% PFA, washed with PBS, permeabilized with 0.2% Triton-X 100, blocked with 10% goat serum, and stained with primary antibody for proliferation marker Ki-67 or cyclin-dependent kinase inhibitors p21 and p16, followed by washing with PBS and staining with secondary antibody Alexa 555. Thirty fluorescent images of cells were taken randomly with the multiplex reader Cytation 5, following staining. The images were assessed using the software Gene 5 version 2.07.

Assessment of apoptosis. Apoptosis was detected using a combination of propidium iodide staining and Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, CA, USA). In order to induce apoptosis as a positive control, HUVECs were treated with 500 μ mol H₂O₂ for 90 min.

Determination of NF- κ B nuclear translocation in HUVECs. Nuclear localization of NF- κ B subunit p65 was evaluated in HUVECs grown in 1% gelatin coated 96 well plates, in the presence or absence of TNF α (10 ng/ml), or in combination with plumericin (1.5 μ M), PHA-408 (2 μ M) or NAC (2 mM) for the indicated time points. Subsequently, the cell were fixed and stained with anti-p65 subunit of NF- κ B, as previously described⁶⁹. Fluorescent images were taken on an IX-50 Olympus microscope using a 10x lens and a cooled CCD camera, F-View II (Olympus, Münster, Germany). The mean integrated intensity of the p65 in nucleus was evaluated by using the cell image analysis software CellProfiler 2.1.

Measurement of intracellular ROS in HUVECs. Intracellular ROS was measured in HUVECs as previously described⁴⁴ by using the fluorescent probe H2-DCF with slight modifications. Briefly, HUVECs grown in 1% gelatin-coated 96-well plates overnight were treated either with TNF α (10 ng/ml) alone or in the presence of inhibitors for the indicated periods. At the end of the treatment period, cells were incubated at 37 °C with 10 μ M H2-DCF in phenol red-free medium M199, supplemented with 10% FBS for 30 min in dark and the fluorescence was measured at an excitation wave length of 490 nm and an emission wavelength of 525 nm by a 96 well fluorescent plate reader Synergy 3 (BioTek).

Detection of E-selectin and ICAM-1 by cell ELISA. Surface expression of E-selectin and ICAM-1 in HUVECs exposed to TNF α (10 ng/ml) or TNF α with NF- κ B inhibitors plumericin (1.5 μ M), PHA-408 (2 μ M) or NAC (2 mM) for the indicated times was measured by cell ELISA as described before⁴¹.

Detection of IL-6 and IL-8. Conditioned media were collected from HUVECs and HDMECs and analyzed for IL-6 and IL-8 using an ELISA kit (eBioscience, San Diego, CA, USA).

qPCR analysis. Gene expression of IGFBP-5 and PAI-1 was determined by RNA isolation and subsequent qPCR. Shortly, total RNA from HUVECs grown in 6 well plates was isolated and reverse-transcribed. The following primers designed with the Primer 3 software were used: for IGFBP-5: 5'-GAG CTG AAG GCT GAA GCA GT-3' (fwd.) and 5'-GAA TCC TTT GCG GTC ACA AT-3' (rev.) and for PAI-1: 5'-CAG ACC AAG AGC CTC TCC AC-3' (fwd.) and 5'-ATC ACT TGG CCC ATG AAA AG-3' (rev.). The expression levels of these genes were normalized to the expression of β 2-microglobulin using 5'-GAT GAG TAT GCC TGC CGT GTG-3' (fwd.) and 5'-CAA TCC AAA TGC GGC ATC T-3' (rev.) primers.

Statistical analysis. All experiments were performed at least in triplicate if not stated otherwise. Statistical analysis was carried out by using one way ANOVA/Newman Keuls post-test or unpaired two tailed t-test (when comparing just two experimental groups) with the help of GraphPad Prism version 5 (La Jolla, CA, USA). P values < 0.05 were considered as significant.

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Author Contributions

J.M.B., E.M.A., and S.Y.K., conceived and designed the study. S.Y.K., E.M.A., A.O., B.W., M.L., X.Y., and P.U., performed experiments and analyzed data. J.M.B., E.M.A., H.S., and M.M., supervised the execution of experiments and their interpretation. J.M.B., S.Y.K., P.U., A.O., E.M.A., and M.M. drafted the manuscript. All authors proofread and agreed on the manuscript.

Additional Information

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