Restoring vascular endothelial autophagic flux reduces atherosclerotic lesions

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Abstract:

Atherosclerotic lesions preferentially develop in arterial areas exposed to low shear stress, where endothelial cells express a pro-inflammatory, apoptotic, and senescent phenotype. Autophagy is a lysosomal mechanism that recycles damaged organelles and protein aggregates to maintain cellular homeostasis. Stimulation of autophagy in high shear stress conditions is an atheroprotective process. Conversely, endothelial cells exposed to atheroprone low shear stress present a defective autophagic flux, which favors a pro-inflammatory phenotype and the formation of atherosclerotic lesions. Since an efficient autophagic flux is dependent on α -tubulin acetylation, which is reduced under low shear stress, we hypothesized that increasing α -tubulin acetylation could restore adequate levels of autophagy in endothelial cells exposed to low shear stress. We found that blocking Histone Deacetylase 6 (HDAC6) activity, either by pharmacological inhibition (Tubastatin-A) or genetic approaches (shHDAC6), raised levels of acetylated α -tubulin, as well as LC3-II/I ratio, LC3 punctae area and autophagic flux in cultured endothelial cells exposed to low shear stress. This effect was associated with a reduced expression of inflammatory markers (Intercellular adhesion molecule-1 (ICAM-1), Vascular cell Adhesion Protein-1 (VCAM-1) and Monocyte Chemoattractant Protein-1 (MCP-1)) in Tumor Necrosis Factor-alpha (TNF- α)-stimulated cells. We observed increased endothelial autophagic flux in the aortic arch of the *HDAC6*^{-/-}/*ApoE*^{-/-} mice. Subsequently, atherosclerotic plaque size was significantly reduced in the atheroprone areas of chimeric *HDAC6*^{-/-}/*ApoE*^{-/-} mice, transplanted with *HDAC6*^{+/+}/*ApoE*^{-/-} bone marrow, when compared to *HDAC6*^{+/+}/*ApoE*^{-/-} littermate controls. Taken together, these results indicate that targeting α -tubulin acetylation, via HDAC6-inhibition, may be an interesting strategy to restore endothelial autophagic flux and to promote an atheroprotective endothelial phenotype despite unfavorable shear stress conditions.

Keywords: Endothelial cells, Autophagy, Atherosclerosis, HDAC6, Tubastatin-A, inflammation, ICAM-1, VCAM-1, MCP-1

Abbreviations: CQ: Chloroquine; ICAM-1: Intercellular adhesion molecule 1; HDAC6: Histone Deacetylase 6; HUVEC: Human umbilical vein endothelial cell; LC3: microtubule-associated protein 1 light chain 3 β ; MCP-1: Monocyte chemoattractant protein 1; SS: Shear stress; TNF- α : Tumor necrosis factor alpha; TUBA: Tubastatin-A; VCAM-1: Vascular cell adhesion protein 1

Introduction

Over the years, several studies have shown that atherosclerotic plaques form in predisposed areas such as arterial bifurcations, or the inner curvature of aortic bends. In these regions, endothelial cells are exposed to disturbed blood flow, which exerts low levels of shear stress (SS) on the vessel wall (Souilhol et al. 2020; Lee and Chiu 2019; Baeyens et al. 2016). Conversely, cells exposed to laminar flow, which generates high SS, exhibit an anti-inflammatory, anti-senescent and anti-apoptotic phenotype, resulting in reduced plaque formation. Low SS triggers the expression of various inflammatory markers, such as adhesion proteins and chemokines (Chiu and Chien 2011; Souilhol et al. 2020). This in turn favors the recruitment of leukocytes to the lesion site and contributes to the initial stages of plaque formation. While the mechanisms by which SS regulates the endothelial phenotype have yet to be fully elucidated, our group has previously demonstrated that a modulation of endothelial macroautophagy (hereafter referred to as autophagy) is a determining factor (Vion et al. 2017).

Autophagy is an evolutionarily conserved, cytoprotective mechanism, which helps degrade and recycle damaged organelles and proteins, to maintain cellular homeostasis (Feng et al. 2014). As such, autophagy occurs at a basal rate under physiological conditions, and is amplified under stress conditions, such as nutrient starvation, to promote cell survival (Wen and Klionsky 2016). Defects in the autophagic process have been reported in multiple pathologies, including cancer, neurodegenerative, auto-immune and cardiovascular diseases (Mizushima et al. 2008; Mizushima 2018; Levy, Towers, and Thorburn 2017; Mialet-Perez and Vindis 2017). Often, the defect lies in the crucial autolysosome formation step, wherein autophagosomes fuse with lysosomes to allow the degradation of their content (Levy, Towers, and Thorburn 2017; Wong, Cheung, and Ip 2011). Low SS has been shown to inhibit autophagic flux in endothelial cells, which then contributes to the onset of a pro-inflammatory and senescent phenotype (Guo et al. 2017; Chiu et al. 2004; Vion et al. 2017).

While numerous factors can regulate autophagy, studies have highlighted the importance of several post-translational tubulin modifications and their effects on microtubule dynamics and cellular trafficking (Mackeh et al. 2013). In addition to concentrating signaling pathways that stimulate autophagosome formation, microtubules are integral to the movement of pre-autophagosomal structures, as well as the transport of mature autophagosomes towards lysosomes (Mackeh et al. 2013; Ravikumar et al. 2005; Xie et al. 2010). In that sense, microtubules stabilized through α -tubulin acetylation favor the recruitment and walking of motor

proteins, resulting in enhanced cellular trafficking (Xie et al. 2010). Interestingly, endothelial cells exposed to high SS have been shown to express elevated levels of acetylated α -tubulin when compared to cells cultured under static conditions (McCue et al. 2006). This may, in part, explain why endothelial cells exposed to high SS have an increased autophagic activity. The level of endothelial α -tubulin acetylation is regulated by the cytosolic protein deacetylase Histone Deacetylase 6 (HDAC6) (Birdsey et al. 2012; Eshun-Wilson et al. 2019). The phosphorylated active form of HDAC6 in particular, is known to remain active mainly in the cytosol (Ustinova et al. 2020; S. Chen et al. 2010).

Based on these data, we hypothesized that increasing α -tubulin acetylation, through HDAC6 inhibition, could restore adequate levels of autophagy in endothelial cells exposed to atheroprone low SS. In this study, we found that targeting HDAC6 with a selective inhibitor, Tubastatin-A, or an shRNA, led to hyperacetylation of α -tubulin in HUVECs exposed to low SS. This then resulted in an increased autophagic flux and reduced expression of inflammatory markers ICAM-1, VCAM-1 and MCP-1. *In vivo* experiments demonstrated that knocking out *HDAC6* in hypercholesteremic mice reduces the formation of atherosclerotic lesions in otherwise predisposed areas in the aorta.

Results

Shear stress regulates levels of acetylated α -tubulin in endothelial cells. As previously described by McCue *et.al*, shear stress regulates elongation and polarity of endothelial cells via reorganization of the cytoskeleton (McCue et al. 2006). Therefore, we first sought to verify if this was also the case in our experimental model, by measuring acetylated α -tubulin expression in low passage cultured HUVECs exposed to either physiological high or low SS. Western blot data indicated higher levels of acetylated α -tubulin in HUVECs exposed to high SS for 24 hours, when compared to HUVECs exposed to low SS (Suppl. Fig. 1).

HDAC6 activity is increased in endothelial cells exposed to low SS. α -tubulin modifications result from the balance between acetyltransferases and deacetylases such as HDAC6. We hypothesized that the effects of shear stress on levels of acetylated α -tubulin could be relayed by a modulation of HDAC6 activity. Previous data indicated that HDAC6 interacts with α -tubulin when it is phosphorylated at Ser22 by GSK-3 β (S. Chen et al. 2010). The expression and localization of both the canonical and phosphorylated forms of HDAC6 were analyzed by immunofluorescence (Fig.1A). While no difference in the staining intensity of total-HDAC6 was observed, we found that HUVECs exposed to low SS exhibited higher levels of phosphorylated-HDAC6, compared to cells exposed to high SS conditions (Fig.1B). Furthermore, using a fluorometric assay (Lemon et al. 2011), we found that HDAC6 activity was significantly higher in cells exposed to low SS, when compared to high SS (Fig. 1C). Taken all together, these results indicate that endothelial cells exposed to atheroprone low SS have a higher HDAC6 activity than cells exposed to atheroprotective high SS.

HDAC6 inhibition increases levels of acetylated α -tubulin. To evaluate the consequences of inhibiting HDAC6 activity in endothelial cells, we chose to utilize a known HDAC6 inhibitor, Tubastatin-A (Butler et al. 2010). HUVECs were exposed to low SS for 24h, under Tubastatin-A treatment (3 μ M) or vehicle (DMSO). Acetylated α -tubulin expression levels were measured by Western blot and normalized to GAPDH and total α -tubulin. As expected, Tubastatin-A treatment markedly increased levels of acetylated α -tubulin in HUVECs exposed to low SS (Suppl. Fig. 2A and 2B). We further characterized the effects of Tubastatin-A on acetylated α -tubulin by immunofluorescence staining. In accordance with our Western blot data, we found that cells treated with Tubastatin-A presented a more intense acetylated α -tubulin signal. Additionally, we observed that acetylated α -tubulin displayed a more organized pattern in

Tubastatin-A-treated cells (Suppl. Fig. 2C and 2D). This indicates that inhibition of HDAC6 leads to stabilization of a polymerized α -tubulin network.

HDAC6 inhibition restores autophagic activity in endothelial cells exposed to low SS.

As previously described by Mackeh et al. (Mackeh et al. 2013) stable acetylated microtubules are required for the efficient formation of autophagosomes, as well as for their fusion with lysosomes. This prompted us to evaluate whether blocking α -tubulin deacetylation, via HDAC6 inhibition, would stimulate autophagic activity in cells exposed to low SS. We first assessed autophagic activity by measuring LC3-II/I ratio in HUVECs. In accordance with our previous findings, cells exposed to low SS for 24h displayed a lower LC3-II/I ratio than cells exposed to high SS (Fig.2). Treatment with Tubastatin-A, in low SS conditions, led to an elevation of the LC3-II/I ratio to approximately twice that of control conditions (Fig. 2A and 2B). Similar results were obtained when expressing LC3 as a ratio of LC3-II to GAPDH. Interestingly, treating cells with Tubastatin-A did not modify levels of other essential autophagy proteins, such as ATG5 (Suppl. Fig. 3A and 3B) and p62 (Suppl. Fig. 3C and 3D). We obtained comparable data when measuring the area of LC3-positive puncta per cell by immunostaining. LC3 staining was consistently less abundant in HUVECs exposed to low SS. However, treating cells with Tubastatin-A elevated the area of LC3-positive structures per cell to levels (Fig. 2C and 2D).

To confirm that HDAC6 inhibition led to autophagy stimulation, we used a tandem LC3-B fluorescently tagged with mRFP and GFP. In this assay, autophagosomes emit a yellow signal (mRFP-GFP-LC3), and their maturation into autolysosomes is attested by a red signal due to quenching of the GFP fluorescence in low pH environments (Fig. 3A). We therefore evaluated autophagic flux by quantifying the ratio of autolysosomes/autophagosomes per cell (Fig. 3B). As previously demonstrated, HUVECs exposed to high SS conditions had approximately twice the number of autolysosomes than those exposed to low SS conditions, while the number of autophagosomes remained similar (Fig. 3C and 3D). This indicated that maturation of autophagosomes into autolysosomes was impaired under low SS conditions. Treatment with Rapamycin (1 μ M), a known autophagy inducer, resulted in a slight decrease in the number of autophagosomes, and an increase in the number of autolysosomes. A similar trend was observed in HUVECs treated with Tubastatin-A under low SS conditions, suggesting that HDAC6 inhibition stimulates autophagic flux in endothelial cells exposed to low SS. However, these results could also be explained by an increase in autophagosome biogenesis. To test this hypothesis, we examined the effects of Tubastatin-A in combination with chloroquine, a lysosomotropic agent that neutralizes the acidic pH of lysosomes, thereby blocking the fusion

between autophagosomes and lysosomes (Mauthe et al. 2018). As expected, treating cells with chloroquine (300 μ M) resulted in an increase in the number of autophagosomes (Fig. 3E and 3F). In the presence of chloroquine, Tubastatin-A failed to elevate the number of autolysosomes, but increased the number of autophagosomes, which suggests an acceleration of autophagosome formation (Fig. 3G). Taken together these data indicate that HDAC6 inhibition may stimulate autophagic flux, by increasing autophagosome biogenesis and maturation, in endothelial cells exposed to low SS.

HDAC6 inhibition reduces the endothelial inflammation caused by exposure to low SS

conditions. Our group has previously demonstrated that the defect in endothelial autophagy, which occurs in low SS areas, causes inflammation and favors the development of atherosclerotic lesions (Vion et al. 2017). Since Tubastatin-A treatment restored autophagic flux in endothelial cells, we wondered if this would also hamper the pro-inflammatory consequences of exposure to low SS conditions. To induce inflammation, cells were treated with TNF- α , at 1ng/mL, for the last 12 hours of the shear stress experiment. By western blot, we assessed the expression levels of inflammatory markers ICAM-1 and VCAM-1. Firstly, a pronounced elevation of ICAM-1 (Fig. 4A and 4C) and VCAM-1 (Fig. 4B and 4D) expression was observed in response to TNF- α treatment. HUVECs exposed to low SS expressed higher levels of both proteins, as compared to cells exposed to high SS. However, treating cells with Tubastatin-A significantly reduced levels of ICAM-1 and VCAM-1 in cells exposed to low SS. Interestingly, Tubastatin-A also suppressed the TNF- α -induced elevation in phospho-NF- κ B levels (Suppl. Fig. 4). We next measured the concentration of MCP-1 found in the HUVEC supernatant, by ELISA. We found that cells exposed to low SS released approximately 5 times more MCP-1 than cells exposed to high SS conditions. Here also, adding Tubastatin-A to cells, markedly reduced levels of MCP-1 released by cells exposed to low SS (Fig. 4E). These results show that HDAC6 inhibition hampers low SS-induced endothelial inflammation.

To confirm these data, we knocked down HDAC6 in HUVECs using a shRNA-carrying lentivirus. With this strategy, we observed a 30% reduction in the expression of HDAC6 (Fig. 5A and 5B). This was sufficient to increase levels of acetylated α -tubulin by 50% (Fig. 5C). HDAC6-knockdown also led to an increase in the LC3-II/I ratio (Fig. 5D), while reducing ICAM-1 (Fig. 5E) and VCAM-1 (Fig. 5F) expression as well as MCP-1 release (Fig. 5G) in TNF α -treated cells. Taken together, these data show that blocking HDAC6, by a pharmacological or a genetic approach, increases the level of acetylated α -tubulin, restores endothelial autophagy, and reduces endothelial inflammation in cells exposed to low SS.

The anti-inflammatory effects of HDAC6 inhibition are relayed by autophagy. To verify whether the anti-inflammatory effects of HDAC6-inhibition were linked to a restored autophagic flux, we assessed the effects of Tubastatin-A in cells deficient for autophagosome formation (i.e., HUVECs infected with a lentivirus carrying an ATG5 shRNA). Knocking down ATG5 has indeed been shown to reduce autophagosome biogenesis. As expected, this approach resulted in lowered ATG5 protein levels (Fig. 6A) and LC3-II/I ratio (Fig. 6B). We verified that the HDAC6 protein was still present under shATG5 conditions (Suppl. Fig. 6A and 6B), and that Tubastatin-A was able to substantially increase levels of acetylated α -tubulin (Suppl. Fig. 6A and 6C). In these ATG5-deficient endothelial cells, the anti-inflammatory effect of Tubastatin-A treatment was significantly reduced, as indicated by the lower expression of ICAM-1 (Fig. 6C and Suppl. Fig. 5A), VCAM-1 (Fig. 6D and Suppl. Fig. 5B) and the impaired MCP-1 release (Fig. 6E and Suppl. Fig. 5C). These data suggest that the anti-inflammatory effects of HDAC6-inhibition rely on a functional autophagic activity.

Deletion of HDAC6 enhances autophagic flux in murine aortic arch. To observe the effect of HDAC6 deletion on autophagic activity *in vivo*, we performed *en face* staining of murine aorta for LC3 in HDAC6^{-/-} / ApoE^{-/-} and littermate controls (HDAC6^{+/+} / ApoE^{-/-}). As expected, we observed higher LC3 staining in high SS regions such the descending aorta, when compared to the low SS region that is the aortic arch (Fig. 7A and 7B). Interestingly, LC3 staining was higher in the arch of HDAC6^{-/-} animals compared to HDAC6^{+/+} controls (Fig. 7A and 7B). Following this, we evaluated autophagic flux by measuring the colocalization of the LC3 with the lysosomal marker LAMP2A. Similarly, colocalization of LC3 and LAMP2A was significantly higher in the aortic arch of HDAC6^{-/-} animals compared to HDAC6^{+/+}, which led us to infer that the absence of HDAC6 may favor promotion of autophagic flux in endothelial cells (Fig. 7C and 7D).

Deletion of HDAC6 reduces atherosclerotic lesions in hypercholesterolemic mice. To evaluate the effect of HDAC6 deletion on atherosclerotic plaque development, chimeric HDAC6^{-/-} / ApoE^{-/-} and their littermate controls (HDAC6^{+/+} / ApoE^{-/-}) mice were transplanted with HDAC6^{+/+} / ApoE^{-/-} bone marrow and fed a high fat diet for 10 weeks. This regimen resulted in a doubling of plasma cholesterol levels (Suppl. Fig. 7A). HDAC6 knockout had no effect on cholesterol levels (Suppl. Fig. 7A), body and organ weight, nor did it alter cell count for different populations in the blood (Suppl. Fig. 7B), or the immune cell profile in the spleen (Suppl. Fig.

7C) and bone marrow (Suppl. Fig. 7D). As previously, described, atherosclerotic plaques preferentially formed low SS areas such as the inner curvature of the aortic arch and around carotids, whereas high SS areas, such as the descending aorta, remained relatively protected (Fig. 8A). We found that plaque size was reduced by approximately 30% in the aortic arch of *HDAC6*-knockout animals when compared to littermate controls (Fig.8B). Altogether, these results demonstrate that HDAC6 inhibition stimulates autophagic flux, reduces inflammation, and limits the development of atherosclerotic lesions in atheroprone low SS conditions.

DISCUSSION

Defect in endothelial autophagy occurs in low SS areas of the vasculature, therefore favoring inflammation and the development of atherosclerotic lesions (Vion et al. 2017). Data presented in this study show that increasing α -tubulin acetylation, via HDAC6 inhibition, can restore adequate levels of endothelial autophagic flux, thereby reducing inflammation and plaque development.

Shear stress influences the endothelial cell's ability to polarize and align, by redistribution of the microtubule network and reorganization of the microtubule organizing center (McCue et al. 2006). Microtubule stability can be attributed to several types of post-translational modifications, such as acetylation, which are regulated by shear stress (Bailey et al. 2017; McCue et al. 2006). In line with these previous studies, we found that α -tubulin acetylation on lysine 40 is increased in endothelial cells exposed to high SS. HDAC6 inhibition and knockdown, significantly increased α -tubulin acetylation in HUVECs. HDAC6 phosphorylation and activity was higher in cells exposed to atheroprone low SS when compared to cells exposed to high SS. This difference may, in part, explain the higher microtubule stability observed in atheroprotective SS conditions. Interestingly other pro-atherogenic stimuli, such as cigarette smoke, were also found to increase HDAC6-phosphorylation (Borgas et al. 2016). At this point, little is known about the regulation of HDAC6 by shear stress. Several upstream effectors have been identified, however. HDAC6 can be phosphorylated by different kinases, including glycogen synthetase kinase 3 β (GSK-3 β). Studies performed in HUVECs have demonstrated flow-induced activation of GSK-3 β through a G $\alpha_{q/11}$ -Akt-1-dependent pathway (McCue et al. 2006). Moreover, HDAC6 activation may also be relayed through the zeta isoform of Protein Kinase C, which has been shown to be activated by oscillatory shear stress (Du et al. 2015). In addition to post-translational modifications, recent work by Manea et al. has revealed that multiple HDACs, including HDAC6, are upregulated in human carotid-derived atherosclerotic lesions and in atherosclerotic aortas of *ApoE*^{-/-} mice (Manea et al. 2020). More data would be needed to fully understand how different shear stress intensities modulate these pathways.

Tubastatin-A has a higher specificity for HDAC6, compared to other members of the HDAC family (Butler et al. 2010). We found that Tubastatin-A treatment had a marked effect on α -tubulin acetylation, and restored autophagic flux in HUVECs cultured in low SS conditions. Similarly, we observed a greater colocalization of LC3 and the lysosomal marker LAMP-2, in the aortic arch of *HDAC6*-knockout mice, indicating a more robust autophagic flux in endothelial cells lacking HDAC6 and exposed to low SS. Interestingly, removing HDAC6 resulted in aortic arch endothelial cells displaying similar autophagic flux levels to those observed in high SS

areas. Furthermore, our *in vitro* experiments performed in the presence of chloroquine revealed that HDAC6 inhibition also increased the rate of autophagosome biogenesis, without modifying the expression levels of essential autophagy proteins such as ATG5. While we cannot exclude that other mechanisms may be relaying the effects of HDAC6-inhibition, we suspect that they are mediated by increased trafficking of autophagic structures along acetylated microtubules. Hyperacetylation of tubulin enhances the recruitment of motor proteins to microtubules, which subsequently contribute to the concentration of signaling pathways involved in autophagosome biogenesis and to the transport of mature autophagosomes towards lysosomes (Geeraert et al. 2010; Bánréti, Sass, and Graba 2013). Several studies have indeed described impaired autophagosome and autolysosome formation in conditions where microtubules were disassembled using taxol or nocadazol (Geeraert et al. 2010; Köchl et al. 2006; Xie et al. 2010). More recently, Majora et al. demonstrated that Tubastatin-A and the pan-HDAC inhibitor, suberoylanilide hydroxamic acid increased tubulin acetylation and consequently improved autophagic function in human fibroblasts, in the context of Cockayne syndrome (Majora et al. 2018).

Results obtained in this study indicate that HDAC6 inhibition, or knockdown, reduced levels of active NF- κ B as well as the expression of inflammatory markers (ICAM-1, VCAM-1 and MCP-1) in endothelial cells exposed to low SS. These findings are in accordance with previous work which demonstrated an anti-inflammatory effect of Tubastatin-A in epithelial cells, *via* the regulation of the NF- κ B pathway (Wang et al. 2018). Other studies have shown that HDAC6 inhibition downregulates the production of inflammatory cytokines, such as IL-6, IL-1 β , and TNF- α in murine models of arthritis and synovial inflammation, and Freund's complete adjuvant-induced mouse model of inflammation (Ran and Zhou 2019). HDAC6 inhibition has also been reported to reduce TNF- α -induced endothelial dysfunction and prolong survival in murine models of systemic inflammation and injury (Yu et al. 2016). Though the underlying mechanisms seem to be multiple and complex, we found that the autophagic machinery played a central role in endothelial cells. Indeed, reducing levels of the essential autophagy protein ATG5, significantly reduced LC3-lipidation and suppressed the anti-inflammatory effects of HDAC6-inhibition by approximately 70%. This indicates that a functional autophagic process is key to relaying these beneficial effects and is in line with our previous data showing that autophagy is required for atheroprotection under physiological blood flow (Vion et al. 2017). Efficient autophagy may favor an anti-inflammatory endothelial phenotype by limiting the accumulation of damaged organelles, protein aggregates or intracellular pathogens (Mai et al. 2012; Cadwell 2016) and by limiting NF- κ B signaling (Peng et al. 2019).

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323 To further assess the role of HDAC6 in atherosclerotic plaque formation, we used a double
 324 knockout murine model, *HDAC6*^{-/-}/*ApoE*^{-/-}. As previously described *HDAC6*^{-/-} animals were
 325 viable and showed no signs of major defects (Zhang et al. 2008). These mice, and their control
 326 littermates, underwent bone marrow transplantation, which allowed us to focus on the role
 327 played by resident cells in the vasculature in the development of atherosclerotic plaques. We
 328 found that knocking out *HDAC6* reduced atherosclerotic plaque development in the low SS areas
 329 of hypercholesterolemic mice. These effects were not caused by other systemic metabolic
 330 parameters influencing cardiovascular risk, as plasma cholesterol levels, body weight and blood
 331 cell count were not affected by *HDAC6* knockout. In addition to clearing damaged intracellular
 332 material, restoring autophagic flux in endothelial cells exposed to low shear stress, may also aid
 333 in their ability to limit lipid accumulation and the subsequent inflammatory response (Torisu et
 334 al. 2016; Kim et al. 2020). Alternatively, data by Manea et al indicated that administering
 335 suberoylanilide hydroxamic acid to hypercholesterolemic mice reduced the progression of
 336 atherosclerotic lesions through the reduction of oxidative stress in the aorta (Manea et al. 2020).
 337 Furthermore, Chen et al. (2019) found showed that another HDAC6 inhibitor (Tubacin)
 338 mitigates endothelial dysfunction by regulating expression of endothelial nitric oxide synthetase
 339 (J. Chen et al. 2019). Whether or not these alternate pathways are linked to the autophagic
 340 machinery requires further investigation. While our focus in this study has been on endothelial
 341 cells, we cannot exclude that autophagy stimulation may also occur in smooth muscle cells via
 342 *HDAC6*-knockout. This very well might contribute to the anti-atherogenic effects we observed in
 343 our *in vivo* experimental model, since defective autophagy in vascular smooth muscle cells has
 344 been linked to accelerated atherogenesis (Grootaert et al. 2015).

345 Collectively, the main findings of this study indicate that targeting HDAC6 stimulates
 346 autophagic flux, decreases markers of inflammation in endothelial cells, and the formation of
 347 atherosclerotic lesions in mice. These results bolster the idea that autophagy-stimulating
 348 strategies might be beneficial in treating atherosclerosis, and present HDAC6-oriented
 349 interventions as an interesting route.

350

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Conflict of interest: All authors declare nothing to disclose

MATERIAL AND METHODS

Human Umbilical Vein Endothelial cells (HUVECs)

HUVECs were obtained from 20 different donors (11 males, 9 females) (PromoCell; Heidelberg, Germany). Cells were plated at a density of 10,000 cells/cm² and cultured in Endothelial Cell Basal Medium (ECBM, PromoCell; Heidelberg, Germany), supplemented with 2% Fetal Calf Serum (PromoCell; Heidelberg, Germany), growth factors (0.4% ECGS, 0.1 ng/mL EGF, 1 ng/mL β -FGF), 90 μ g/mL heparin, 1 μ g/mL hydrocortisone, 10 μ g/L Amphotericin B (Gibco™ - 15290026), 100 IU/mL Streptomycin (Gibco™-15140148) and 100 IU/mL Penicillin (Gibco™-15140148). Confluent cells were detached with 0.025% Trypsin-EDTA (Gibco™ - 25300-054) in PBS (Gibco™ - 10010023) for 5min at 37°C, followed by washing with complete medium and centrifuging at 600g for 10min.

Lentiviral transduction

Lentiviruses expressing inducible shRNA (Sigma-Aldrich, MISSION™ shRNA inducible vectors) were used to silence HDAC and ATG5. Cells were infected with lentivirus carrying shHDAC6/shATG5 or shControl at MOI 5 for 24 hours in the presence of Hexamethidine Bromide (8 μ g/mL; Sigma-Aldrich - H9268). Transduced cells were selected using puromycin (Sigma Aldrich #P9620) at 1 μ g/mL. HUVECs were treated for about 10 days with Isopropyl β -D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich - I6758) at 1mmol/mL.

In Vitro shear stress system

Confluent HUVECs from passage 2-4 were cultured on glass slides coated with 0.2% gelatin, and placed in a parallel plate chamber perfused with circulating medium for 24 hours, under laminar flow as described earlier (Vion et al. 2017). When specifically stated, cells were treated with Tubastatin-A (3 μ M, Sigma-Aldrich), Rapamycin (1 μ M; Sigma-Aldrich) or Chloroquine (Sigma-Aldrich C6628) and exposed for 24 hours under shear stress conditions. For control

conditions, HUVECs were exposed to vehicle (Dimethyl Sulphoxide; 0.1 μ L/mL, Sigma-Aldrich). For inflammation studies, the cells were treated with TNF- α (1 ng/mL, Peprotech) for the last 12 hours of the experiment.

Western blot analysis

HUVECs were immediately washed twice with PBS and lysed in Radioimmunoprecipitation assay (RIPA) buffer consisting of 150 mmol/L NaCl, 50 mmol/L Tris HCl (pH=7.4), 2 mmol/L EDTA, 2 mmol/L activated orthovanadate, 0.5% deoxycholate, 0.2% Sodium Dodecyl Sulphate and supplemented with CompleteTM Mini protease and phosphatase inhibitors cocktails (Roche, France). Then, the lysates were centrifuged for 15 minutes at 12500g, 4°C to remove cell debris. The supernatant was collected and stored at -80°C until further analysis. Protein concentration was estimated by Lowry's protein assay method (DCTM Protein Assay, BioRad).

Protein samples were denatured using buffer containing Tris-carboxyethyl phosphine hydrochloride (20x XT-Reducing Agent, BioRad-1610792) and separated based on molecular weight by electrophoresis in 4-12% gradient gels (CriterionTM, BioRad-3450123). Proteins were then transferred on either 0.45 μ m nitrocellulose (BioRad) or Polyvinylidene Fluoride (PVDF) membranes; following which the membranes were incubated with Ponceau Red to verify the efficiency of the transfer process. Membranes were blocked with 5% (w/v) milk or Bovine Serum Albumin (BSA) in TBS supplemented with 0.1% Tween-20. To detect the protein of interest, membranes were then incubated with primary antibodies overnight at 4°C, with constant agitation (*Supplementary Table 1*). After three 10-minute washes, the membranes were incubated with secondary antibody coupled with Horseradish Peroxidase (anti-rabbit, anti-rat or anti-mouse, Amersham, GE Healthcare, 1/3000) for 1 hour at room temperature. Immunodetection was performed using ClarityTM Western ECL Substrate, and the chemiluminescent signal was revealed using the Las-4000 imaging system and quantified with MultiGauge software (Fujifilm, Japan)

Immunofluorescence

Cells were fixed with either 100% ice-cold methanol for 20 minutes at -20°C or 4% (v/v) paraformaldehyde (PFA) in PBS, for 5 minutes at room temperature. Cells fixed with PFA were permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. Permeabilized cells were blocked with 5% (w/v) BSA in PBS for 30 minutes and then incubated with primary

antibody overnight at 4°C (*Supplementary Table 2*). The cells were washed with PBS and incubated with secondary antibody conjugated with fluorescent dye, Alexa Fluor®. Lastly, the nuclei were labeled with DAPI. The labeled cells were mounted on coverslips using Fluoromount-G®.

To perform *en face* immunostaining, aortas were harvested from mice and fixed with ice-cold 4% (v/v) PFA for 20 minutes. Residual fat attached to the aorta was removed and dissected along the entire inner side and outer curvature of the arch. Aortas were then blocked with 5% (w/v) BSA in PBS, for 60 minutes at room temperature, and incubated with primary antibody for overnight, 4°C. Following incubation with primary antibody, the aortas were washed multiple times and incubated with secondary antibody conjugated with Alexa Fluor® for 2 hours at room temperature. Subsequently, the aortas were washed with PBS and the nuclei were labeled with DAPI. Labeled aortas were transferred onto glass slides and mounted “*en face*”. The slides prepared were examined using Leica SP8 confocal microscope and analyzed with software ImageJ. For colocalization analysis, ImageJ PlugIn ‘*Colocalization Finder*’ was used and Manders’ coefficients were calculated.

Deacetylase activity assay

To determine the deacetylase enzymatic activity of HDAC6, a fluorescence-based assay was performed (Lemon et al. 2011). Deacetylation of substrate by HDAC6 sensitizes the substrate to the developer solution, which generates a fluorophore. The fluorescence signal is then detected by a fluorimetric plate reader at 460 nm. HUVECs were lysed in PBS buffer supplemented with - 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail (Thermo Fisher). Lysed cells were sonicated and centrifuged at 12500g for 15 minutes, 4°C. The pellet was discarded, and the supernatant was transferred to fresh tubes. Protein concentration was estimated by Micro BCA assay (Thermo Scientific™ - 23235). For the assay, 15 µg of lysates were diluted in 100 µL of PBS and added to a 96-well plate. Where required, 1 µL of the 100x HDAC6 inhibitor of the stock solution were added. Vehicle (DMSO) was added to the controls at the same concentration. The plates were incubated at 37°C for 60 minutes. 5 µL of substrate, specific to HDAC Class II, were added to the wells at a concentration of 1mM and incubated for 2 hours at 37°C. Substrate was bought from Bachem (I-1875; Boc-Lys (Ac)-AMC). 50 µL per well, developer solution was added and incubated at 37°C for 20 minutes. The developer solution consisted of 1.5% Triton X-100, 3 µM TSA (Sigma-Aldrich, T8552), and 0.75 mg/mL trypsin (Gibco™ 15400054), diluted in 1x PBS. AMC fluorescence was measured using a plate

reader, with excitation and emission filters of 360 nm and 460 nm, respectively. Background signals corresponding to the blank solutions was subtracted.

Monitoring autophagic flux using mRFP-GFP-LC3

In order to observe the effect of shear stress on endothelial autophagic flux, we used a LC3 protein, fluorescently tagged with a RFP- GFP tandem, (generous gift from Dr F. Oury, Inserm Paris), according to Vion Kheloufi et al, PNAS 2017 (Vion et al. 2017). The tandem allows us to observe different stages autophagy. At the autophagosomes stage, the LC3 protein is visualized as yellow signal, due to the overlapping of green and red fluorescence signal from GFP and RFP. However, when autolysosomes are formed, the acidic pH from lysosomes causes quenching of GFP. Therefore, the red fluorescent signal corresponds to LC3 present only in the autolysosomes stage. The cells were infected with lentiviruses carrying the plasmid (MOI = 6) in the presence of Hexamethidine Bromide (8 µg/mL; Sigma-H9268) for 24 hours. HUVECs were then exposed to shear stress for 24 hours with or without Tubastatin-A (3 µM; Sigma-SML0044). For experiments where autophagic flux was blocked, HUVECs were treated with Chloroquine (300µM) for the last 6 hours of the shear stress. After exposure to shear stress, the cells were washed with PBS and fixed with 4% PFA for 5 minutes at room temperature, followed by labeling the nuclei with DAPI. The slides were mounted using Fluoromount-G[®] (SouthernBiotech). Images of the prepared slides were taken with the Leica SP8 confocal microscope and analyzed for LC3 positive signal using ImageJ software.

ELISA

Levels of Monocyte Chemoattractant Protein-1 (MCP-1), released by HUVECs, in the media, were assessed by ELISA (R&D, Human MCP-1 Duo-Set DY279). The assay was performed according to manufacturer's protocol.

Animal model

All the mice used in the study were of C57BL/6 genetic background. The double knockout model was generated by crossing *HDAC6*^{-/-} mice, provided by T. McKinsey, University Colorado Denver, USA, and *ApoE*^{-/-} (Charles River Laboratories). All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (no. 07430) and were approved by the institutional ethical committee (no. 02526.02). All experiments were performed on male mice. Since the *HDAC6* gene is found on

the X-chromosome, it was possible to obtain only male littermate control mice to be used for the experiments.

Bone Marrow Transplant

8 weeks old *HDAC6^{-/-}/ApoE^{-/-}* and *HDAC6^{+/+}/ApoE^{-/-}* male mice underwent Bone Marrow Transplant (BMT) procedure, in order to replenish the bone marrow in the mutated models with *HDAC6^{+/+}/ApoE^{-/-}* donor bone marrow. This allowed us to focus on the role of endothelial cells and rule out the potential function of hematopoietic cells in progression of atherosclerosis. 4-6 weeks old *HDAC6^{+/+}/ApoE^{-/-}* donor mice were euthanized by cervical dislocation and the bone marrow was extracted from tibia and femur. The bone marrow was flushed and filtered. The recipient mice received radiation, 9.5 Gray, and were transplanted with donor bone marrow by retro-orbital injections the day after. The mice were allowed to recover for 4 weeks, following which they were put on high fat diet (D12079B, Research Diets; 20% proteins, 50% glucose, 21% lipids). After 10 weeks of high fat diet, the mice were euthanized.

Evaluation of Blood Cholesterol Levels

Mice were anesthetized with 2% isoflurane and blood (75 μ L) was collected by making a sub-mandibular puncture. To prepare platelet-free plasma, the blood collected was centrifuged twice at 2500g for 15 minutes at room temperature. The cholesterol content was determined using Cholesterol FS 10' kit (Diasys).

Isolation of Aortas

Mice were anesthetized using isoflurane (flow was set at 2.5% for induction and 2% for maintenance) and 2L/min of O₂. The abdominal organs were moved aside to expose the inferior vena cava. 500- μ L blood was drawn from the inferior vena cava using a 1 mL syringe and 26G needle primed with sodium citrate. Following blood collection, the diaphragm was cut, and the thoracic cavity exposed. The right atrium was nicked to release the blood. The heart was flushed with PBS via the left ventricle, using a 10 mL syringe and 25G needle. Followed by PBS, the heart was flushed with 1-2 mL ice-cold 4% PFA and PBS to wash out the excess PFA. To allow for better access to the aorta, the liver, lungs, trachea, oesophagus, thymus gland and pulmonary artery, were removed. Using micro-dissection scissors and forceps the excess adipose tissue and adventitia, surrounding the thoracic aorta and the carotids, were removed. The clean aorta was

excised from the heart and placed in 4% PFA for 20 minutes on ice, followed by storage in ice-cold PBS.

Determination of Atherosclerotic Plaque Size On *En Face* Aortas

The aortas were then stained with freshly prepared Oil Red O dye (40% distilled water, 60% Oil Red O solution) for 20 minutes with constant agitation. The solution was prepared by dissolving the dye in isopropanol at a concentration of 5g/L. The aortas were washed with 75% ethanol for 5 minutes to remove the excess stain and mounted “*en face*” on glass slides and observed under microscope. Surface area occupied by the plaque was assessed using software, ImageJ.

Preparation of Spleen

Spleens were then weighed and rinsed twice with ice-cold 0.1 μ m filtered PBS containing 3% FBS. Placing them on 3% PBS-FBS pre-wetted 40 μ m nylon cell strainers (Cat#352340, Thermo Fisher Scientific, USA) suspended over a 50 mL polypropylene tube, the spleens were then mashed using the plunger end of a 1 mL syringe. The cell strainers were then rinsed four times with 1 mL of 3% PBS-FBS. Cell suspension was then centrifuged at 500xg for 10 minutes at 4°C. The supernatant was then carefully aspirated and discarded, and the pellet was then resuspended in 1 mL of 0.1 μ m filtered PBS. 1 mL of red blood cell lysis buffer (Cat# R7757, Merck, USA) was added to the resuspension and incubated at 5 minutes at room temperature. After the incubation, 18 mL of 3% PBS-FBS was added and suspension was centrifuged at 500g for 10 minutes at 4°C. Supernatant was then removed carefully, and pellet was resuspended in 3 mL of complete RPMI media that consisted of RPMI 1640 Medium with GlutaMAX and HEPES (Cat# 72400021, Thermo Fisher Scientific, USA) supplemented with 10% FBS, 1% P/S and 0.1% β -mercaptoethanol (Cat# 1610710, Bio-Rad, USA). Cells were then counted, and a final concentration of 10×10^6 cells/mL was obtained.

Preparation of Bone Marrow

The femur and tibia were isolated from the hind legs of the mouse. Sterilized micronic tubes, with a pore at the bottom, were placed in 1.5 mL Eppendorf tubes. One bone was placed per tube and centrifuged at 10000g for 15 seconds at room temperature. Collected cells were resuspended in 800 μ L PBS, passed through 40micron syringe filter. The samples were then centrifuged at 400g for 10 minutes at 4°C. The pellet was resuspended in PBS to have a concentration of 10×10^6 cells/mL. Bone marrow cells were incubated with the antibody mix for 30 minutes at

4°C. The plate was centrifuged at 500g for 5 minutes at 4°C. The pellet was resuspended in 100 µL of 1% PBS-FBS.

Flow Cytometry

Circulating and splenic immune cell populations were defined as follows: NK cells as NK1.1⁺, neutrophils as CD11b⁺/Ly6C⁺/Ly6G⁺, monocytes as CD11b⁺/Ly6C⁺/Ly6G⁻, dendritic cells as CD11b⁺/Ly6C⁺/Ly6G⁻/CD11c⁺, macrophages as CD11b^{lo/-}/Ly6c⁺/Ly6g⁻/F480^{hi}, activated mature B lymphocytes as CD19⁺/IgM⁺/B220⁺, cytotoxic T lymphocytes as CD3⁺/CD8⁺ and helper T lymphocytes as CD3⁺/CD4⁺. Antibody incubation was performed on ice for 30 minutes (supplemental Table 3) in 96-well plate and 100 µl of PBS-FBS 1% was added post-incubation. The plate was then centrifuged at 500g for 5 minutes at 4°C and supernatant was removed by inverting the plate. A final resuspension of splenocyte pellet with 100 µl of PBS-FBS 1% was performed before transfer to Micronic tubes (Cat# 2517810, Dutscher, France) for flow cytometry analysis (LSRFortessa™, BD Biosciences, USA). Data was analyzed using FlowJo software (BD Biosciences, USA).

Statistical Analysis

Data are expressed as mean ± SEM for all experiments. Comparisons between different SS conditions or between control and treatment conditions were performed by using a Wilcoxon test for *in vitro experiments*. Comparisons between groups of mice were performed by using the Mann–Whitney *U* test or 2-way Anova (with Sidak post-hoc test). Statistical analyses were performed using the GraphPad Prism 7 statistical package. All tests were two-sided and used a significance level of 0.05.

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Figure Legends

Figure 1: Regulation of HDAC6 activity by shear stress. (A) Representative images and analysis of expression of HDAC6 and phosphorylated-HDAC6 by immunofluorescence. HUVECs exposed to high SS (HSS; 20 dyn/cm²) or low SS (LSS; 2 dyn/cm²) for 24 hours, were labeled with HDAC6 (red), or phosphorylated-HDAC6 antibody (red), and the area/cell (μm²) was analyzed (blue=DAPI). Quantification of total HDAC6 (B) and phosphorylated HDAC6 (C) area in endothelial cells exposed to either high SS or low SS. Data represent means ± SEM of 6 independent experiments. (D) Analysis of HDAC6 activity in HUVECs exposed to high SS or low SS for 24 hours. Data represent means ± SEM of 6 independent experiments, normalized to low SS conditions. ns, not statistically different; **P* ≤ 0.05 (Wilcoxon test).

Figure 2: Tubastatin-A treatment increases endothelial autophagy. (A) Western blot analysis of LC3 in HUVECs exposed to either high SS (HSS; 20 dyn/cm²) or low SS (LSS; 2 dyn/cm²) and treated with either vehicle (DMSO: 0.1 μL/mL) or Tubastatin-A (TUBA; 3 μM) for 24 hours. (B) Quantification of the LC3-II/I (left) and LC3-II/GAPDH (right) ratios; data represent means ± SEM of 6 independent experiments normalized to low SS + DMSO. (C) Representative confocal microscopy images of HUVECs exposed to either high SS or low SS and treated with either vehicle (DMSO: 0.1 μL/mL) or Tubastatin-A (TUBA; 3 μM) for 24 hours (blue: DAPI, green: VE-cadherin, red: LC3). (D) Quantification of LC3 area per cell; data represent means ± SEM of 6 independent experiments normalized to low SS + DMSO. **P* ≤ 0.05 (Wilcoxon test).

Figure 3: Autophagic flux is restored in HUVECs exposed to low SS and treated with Tubastatin-A. (A) Representative confocal microscopy images of HUVECs infected with lentiviruses carrying the tandem mRFP-GFP-LC3 plasmid and exposed to high SS (HSS; 20 dyn/cm²) or low SS (LSS; 2 dyn/cm²) for 24 hours, in the presence or absence of Tubastatin-A (TUBA; 3 μM) or Rapamycin (RAPA; 1 μM). Autophagosomes and autolysosomes are denoted by yellow and red signals, respectively. Scale bar: 25 μm. (B) Quantification of the number of autophagy vacuoles per cell. Yellow bars correspond to the number of autophagosomes per cell and red bars correspond to the number of autolysosomes per cell. HUVECs were exposed to either high SS or low SS and treated with either vehicle (DMSO at 0.1 μL/mL), Rapamycin (RAPA; 1μM) or Tubastatin-A (TUBA; 3 μM) for 24 hours. Quantification of the number of autophagosomes (C) and autolysosomes (D) per cell. (E) Quantification of the number of

autophagy vacuoles per cell in HUVECs exposed to low SS and treated with Chloroquine (CHLQ; 300 μ M) and either vehicle (DMSO at 0.1 μ L/mL) or Tubastatin-A (TUBA; 3 μ M) for 24 hours. Quantification of the number of autophagosomes (**F**) and autolysosomes (**G**) per cell in HUVECs exposed to low SS and treated with Chloroquine (CHLQ; 300 μ M) and either vehicle (DMSO at 0.1 μ L/mL) or Tubastatin-A (TUBA; 3 μ M) for 24 hours). Data represent means $\pm$ SEM of 6 independent experiments in which over 100 cells were analyzed per condition. ns, not statistically different; * $P \leq 0.05$ (Wilcoxon test).

Figure 4: Tubastatin-A has anti-inflammatory effect on endothelial cells exposed to low shear stress. (**A**) Western blot analysis of ICAM-1 in HUVECs exposed to high SS (HSS; 20 dyn/cm²) or low SS (LSS; 2 dyn/cm²) and treated with either vehicle (DMSO at 0.1 μ L/mL) or Tubastatin-A (TUBA; 3 μ M) for 24 hours, in the presence or absence of TNF- α (1 ng/mL). (**B**) Western blot analysis of VCAM-1 in HUVECs exposed to high SS (HSS; 20 dyn/cm²) or low SS (LSS; 2 dyn/cm²) and treated with either vehicle (DMSO at 0.1 μ L/mL) or Tubastatin-A (TUBA; 3 μ M) for 24 hours, in the presence or absence of TNF- α (1 ng/mL). (**C**) Quantification of the ICAM-1/GAPDH ratio; data represent means $\pm$ SEM of 6 independent experiments normalized to low SS + DMSO + TNF- α . (**D**) Quantification of the VCAM-1/GAPDH ratio; data represent means $\pm$ SEM of 6 independent experiments normalized to low SS + DMSO + TNF- α . (**E**) ELISA analysis of MCP-1 levels released in the conditioned media of HUVECs exposed to high SS (HSS; 20 dyn/cm²) or low SS (LSS; 2 dyn/cm²) and treated with either vehicle (DMSO at 0.1 μ L/mL) or Tubastatin-A (TUBA; 3 μ M) for 24 hours, in the presence or absence of TNF- α (1 ng/mL). Data represent means $\pm$ SEM of 6 independent experiments. * $P \leq 0.05$ (Wilcoxon test).

Figure 5: Knockdown of HDAC6 increases endothelial autophagy and reduces inflammation. (**A**) Western blots analysis of HDAC6, acetylated α -tubulin, LC3, ICAM-1 and VCAM-1 expression in HUVECs transduced with either shControl (shCtl) or shHDAC6 lentiviruses and exposed to low SS (LSS; 2 dyn/cm²), in the presence or absence of TNF- α (1 ng/mL). Quantification of the HDAC6/GAPDH (**B**), acetylated α -tubulin/GAPDH (**C**), LC3-II/I (**D**), ICAM-1/GAPDH (**E**) and VCAM-1/GAPDH (**F**) ratios; data represent means $\pm$ SEM of 6 independent experiments normalized to shCtl. (**G**) ELISA analysis of MCP-1 levels released in the conditioned media of HUVECs transduced with either shControl (shCtl) or shHDAC6 lentiviruses and exposed to low SS (LSS; 2 dyn/cm²), in the presence or absence of TNF- α (1 ng/mL). Data represent means $\pm$ SEM of 6 independent experiments. * $P \leq 0.05$ (Wilcoxon test).

Figure 6: Tubastatin-A is unable to reduce inflammation in ATG5-deficient endothelial cells. (A) Western blots analysis of ATG5, LC3, ICAM-1 and VCAM-1 expression in endothelial cells transduced with either shControl (shCtl) or shATG5 lentiviruses, exposed to low SS (LSS; 2 dyn/cm²), and treated with vehicle (DMSO at 0.1 μL/mL) or Tubastatin-A (TUBA; 3 μM) in the presence of TNF-α (1 ng/mL). (B) Quantification of the LC3-II/I ratio. Data represent means ± SEM of 6 independent experiments normalized to shCtl. Quantification of Tubastatin-A induced changes in the expression of ICAM-1 (C) and VCAM-1 (D); and concentration of MCP-1 in the conditioned media of HUVECs (E). Data represent means ± SEM of 6 independent experiments. ns, not statistically different; **P* ≤ 0.05 (Wilcoxon test).

Figure 7: HDAC6 knockout increases autophagic flux in atheroprone areas of the aorta. (A) LC3 *en face* staining of the aorta of *HDAC6*^{-/-}/*ApoE*^{-/-} mice and littermate controls *HDAC6*^{+/+}/*ApoE*^{-/-}; n=3 per group; (red, LC3; blue, DAPI). (B) Data represent means ± SEM of LC3 area per cell from 5 different photographic fields per mouse. (C) LC3 and LAMP2A *en face* staining of the aorta of *HDAC6*^{-/-}/*ApoE*^{-/-} mice and littermate controls *HDAC6*^{+/+}/*ApoE*^{-/-}; n=3 per group; (red, LC3; green, LAMP2A; blue, DAPI). (D) Data represent means ± SEM of the percentage of LC3 and LAMP2A colocalization from 5 different photographic fields per mouse. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001 (Two-way Anova and Sidak's post-test)

Figure 8: HDAC6 knockout impairs plaque development in atheroprone areas of the aorta. Chimeric *HDAC6*^{-/-}/*ApoE*^{-/-} mice (n=13) and littermate controls *HDAC6*^{+/+}/*ApoE*^{-/-} (n=14), transplanted with *HDAC6*^{+/+}/*ApoE*^{-/-} bone marrow were fed with a high fat diet for 10 weeks. Representative images (A) and quantification (B) of *en face* Oil Red-O staining of atherosclerotic lesions in the mice aorta. Data represent means ± SEM. ns, not statistically different; ****P* ≤ 0.001 (Mann-Whitney test).

A

B

C

D

A

B

C

D

E

F

G

A

DMSO	+	+	+	+	+	+
TUBA	-	-	-	-	+	+
TNF- α	-	+	-	+	-	+

B

DMSO	+	+	+	+	+	+
TUBA	-	-	-	-	+	+
TNF- α	-	+	-	+	-	+

C

D

E

A

B

C

E

F

A

B