

β -amyloid context intensifies vascular smooth muscle cells induced inflammatory response and de-differentiation

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Summary

Several studies have shown that the accumulation of β -amyloid peptides in the brain parenchyma or vessel wall generates an inflammatory environment. Some even suggest that there is a cause-and-effect relationship between inflammation and the development of Alzheimer's disease and/or cerebral amyloid angiopathy (CAA). Here, we studied the ability of wild-type $A\beta_{1-40}$ -peptide (the main amyloid peptide that accumulates in the vessel wall in sporadic forms of CAA) to modulate the phenotypic transition of vascular smooth muscle cells (VSMCs) toward an inflammatory/de-differentiated state. We found that $A\beta_{1-40}$ -peptide alone neither induces an inflammatory response, nor decreases the expression of contractile markers; however, the inflammatory response of VSMCs exposed to $A\beta_{1-40}$ -peptide prior to the addition of the pro-inflammatory cytokine IL-1 β is greatly intensified compared with IL-1 β -treated VSMCs previously unexposed to $A\beta_{1-40}$ -peptide. Similar conclusions could be drawn when tracking the decline of contractile markers. Furthermore, we found that the mechanism of this potentiation highly depends on an $A\beta_{1-40}$ preactivation of the PI3Kinase and possibly NF κ B pathway; indeed, blocking the activation of these pathways during $A\beta_{1-40}$ -peptide treatment completely suppressed the observed potentiation. Finally, strengthening the possible *in vivo* relevance of our findings, we evidenced that endothelial cells exposed to $A\beta_{1-40}$ -peptide generate an inflammatory context and have similar effects than the ones described with IL-1 β . These results reinforce the idea that intraparietal amyloid deposits triggering adhesion molecules in endothelial cells, contribute to the transition of VSMCs to an inflammatory/de-differentiated phenotype. Therefore, we suggest that acute inflammatory episodes may increase vascular alterations and contribute to the ontogenesis of CAA.

Key words: amyloid beta peptide; cerebral amyloid angiopathy; IL-1 β ; inflammation; PI $_3$ K; de-differentiation and vascular smooth muscle cells.

Introduction

From a histo-pathological point of view, Alzheimer's disease (AD) is characterized by an age-dependent formation of amyloid β ($A\beta$)-containing plaques, accumulation of Tau protein-related

neuro-fibrillary tangles (NFT), and neuronal loss in selective brain regions. Post-mortem brains of patients with AD as well as transgenic mouse models also display an increased expression of inflammatory mediators and, although much debated, several studies link the use of anti-inflammatory drugs with a reduction in risks of the disorder. Indeed, it has been shown that (i) there is a significant reduction in the activation of microglial cells in the brain of long-term NSAIDs users (Mackenzie & Munoz, 1998); (ii) disruption of memory by $A\beta$ appears dependent on Cyclooxygenase-2 (COX-2)-mediated PGE $_2$ signaling at the synapses, which is blocked by NSAIDs (Kotilinek *et al.*, 2008); (iii) COX-2 expression is induced in neurons by $A\beta$, glutamate and inflammatory cytokines (Bazan, 2001; Blanco *et al.*, 2010) and PGE $_2$ levels are increased in patients with AD (Montine *et al.*, 1999); and (iv) blocking interleukin-1 β (IL-1 β) signaling rescues cognition, attenuates Tau pathology and restores neuronal β -Catenin pathway function in an AD model (Kitazawa *et al.*, 2011). Regarding this evidence, inflammation has been proposed either as 'a driving force of Alzheimer disease' or at least as 'the third important component of the disease.'

Consistent with the importance of inflammation in AD, it has been shown that $A\beta$ peptide-activated microglial cells trigger the release of inflammatory molecules directly toxic to neurons. Microglia-derived factors shown to be toxic to neurons include nitric oxide combined to superoxide anions, reactive oxygen species, tumor necrosis factor- α (TNF α), IL-1 β , a protease-resistant toxin induced by interaction with microglial heparan sulfate, complement proteins, and cathepsin B. Moreover, activated microglia cells recruiting astrocytes actively enhance the inflammatory response triggered by extracellular $A\beta$ deposits; they also initiate local cytokine-mediated acute-phase response activation of the complement cascade and induction of inflammatory enzyme systems such as the inducible nitric oxide synthase, multiple forms of PLA $_2$, and the prostanoid generated by COX-2; these factors, either alone or in concert, can contribute to neuronal dysfunction, microglial toxicity and cell death. Finally, many cytokines such as IL-1 β , TNF α alone or combined with IFN γ , and chemokine signaling (CXCR2 signaling) promote $A\beta$ production by modulating γ -secretase activity in neurons or increasing levels of endogenous BACE1 [β -Site APP cleaving enzyme-1, (Zhao *et al.*, 2011)]. For review, the study described by Heneka *et al.*, (2010) is referred.

Abnormal accumulation of $A\beta$ not only occurs in brain parenchyma but also occurs in cerebrovasculature, either around the capillaries perfusing the cerebellum, cerebral cortex and leptomeninges [capillary cerebral amyloid angiopathy (CAA)], or within the media of medium and large arteries (arterial CAA) irrigating the central nervous system (Thal *et al.*, 2008). Regarding the one form affecting the medial layer of the vessel wall, it is associated with vascular smooth muscle cells (VSMCs) degeneration, resulting primarily in a loss of tonus, and ultimately, in a disruption of the

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vessel wall integrity possibly leading to intracerebral hemorrhages and hypo-infusion (Attems *et al.*, 2011). In the late 1990s, it had been suggested that degeneration of the cerebrovasculature resulting in hemorrhages may be at least partially mediated by vasculature inflammation. This hypothesis was built upon several observations. Monocyte/macrophage marker-positive foci/cells colocalized with HCHWA-Dutch arterial A β (Maat-Schieman *et al.*, 1997); in sporadic CAA, cerebrovascular amyloid deposition, mainly composed of A β ₁₋₄₀ peptides, was associated with increased recruitment or activation of monocyte/macrophage lineage cells (Yamada *et al.*, 1996); more recently, data in both mouse models and A β -related angitis patients suggested a critical implication of cross-talk between endothelial cells, macrophages and T cells in the modulation of cerebrovascular amyloid deposition and CAA development (Weiss *et al.*, 2011). *In vitro*, several A β -peptides induce both CD40 expression, IL-1 β and interferon- γ secretion from human aortic endothelial cells; this latter finding also suggested that, although pro-inflammatory cytokines may be produced by recruited T cells and macrophages, A β also functions as a direct inflammatory stimulator of parietal cells (Suo *et al.*, 1998). In addition, because cytokines modulate the expression of multiple genes including their own and those of their receptors, Suo *et al.*'s, data (1998) were the first suggesting that A β -induced cytokine production amplifies the initial A β -induced inflammation, being responsible for an amplification loop of the inflammatory molecular cascade. However, if the influence of A β peptides on endothelial cells has been well documented that of A β peptides on VSMCs inflammatory response remains ambiguous.

Here, we show that the inflammatory response of IL-1 β -treated VSMCs was highly enhanced when previously exposed to wild-type A β ₁₋₄₀ peptide (A β ₁₋₄₀); A β ₁₋₄₀ peptide alone did not induce any inflammatory response in VSMCs. Using a pharmacological approach, we also demonstrate that this 'sensitization' process occurs through A β ₁₋₄₀ preactivation of the PI $_3$ K and possibly NF κ B pathway and that it accelerates VSMCs de-differentiation. Finally, we evidenced that the inflammatory context generated by endothelial cells exposed to A β ₁₋₄₀ peptide has similar effects than the ones described with IL-1 β , giving substance to a possible *in vivo* relevance to our findings.

Results

A β ₁₋₄₀ does not induce any VSMCs inflammatory response

To determine whether A β ₁₋₄₀ accumulation could trigger an inflammatory response from VSMCs, we first assayed the expression or the secretion of two inflammatory markers, COX-2 and Prostaglandin E₂. The concentration of A β ₁₋₄₀ peptide used was 50 μ M and the length of treatment was 24 h. Treatment with IL-1 β (10 ng mL⁻¹), a pro-inflammatory cytokine, served as positive control and allowed VSMCs to fully trans-differentiate toward an inflammatory state (Clement *et al.*, 2006). As shown in Fig. 1A,B, whereas IL-1 β greatly induced the expressions of COX-2 and the secretion PGE₂, A β ₁₋₄₀ was inefficient. Similar results were obtained when tracking MMP-9 (Fig. 1C,D), a matrix metalloprotease known

to be involved in inflammatory diseases including peripheral arterial diseases (Busti *et al.*, 2010). Indeed, MMP-9 mRNA levels (estimated by quantitative, RT-PCR Fig. 1C) and MMP-9 activity (measured by gelatin zymography, Fig. 1D) remained as undetectable in A β ₁₋₄₀-treated cells as in vehicle-treated cells (Ctl). As expected (Jung *et al.*, 2003; Blaise *et al.*, 2012), (i), MMP-2 mRNA levels was increased more than 6-fold ($P < 0.001$) in cells treated with A β ₁₋₄₀, when compared to cells with vehicle (Fig. 1E); and (ii) the active-form of the MMP-2 protein (referred to as Act. MMP-2, Fig. 1D) was clearly detected in A β ₁₋₄₀-treated VSMCs. Of note, the bands observed in vehicle correspond to nonmature forms of the enzyme (Fig. 1D). Altogether, these results clearly evidenced that an A β ₁₋₄₀-treatment alone does not trigger VSMCs transition toward an inflammatory phenotype.

A β ₁₋₄₀ highly enhanced IL-1 β -induced inflammatory response in VSMCs through PI $_3$ K and NF κ B activation

Because A β -peptide accumulation generates vessel wall inflammation by inducing endothelial cell cytokine production and monocyte entry (Li *et al.*, 2009; Vukic *et al.*, 2009), we thought of revisiting the previous experiments mimicking this situation *in vitro*. This consisted of evaluating the inflammatory response of VSMCs in an amyloid context. To do so, VSMCs were sequentially treated with 50 μ M of A β ₁₋₄₀ peptide and exposed to 2 ng mL⁻¹ of IL-1 β before measuring the expression and/or secretion of inflammatory markers. (Both treatments were 24 h; the details of the whole protocol are illustrated in Fig. 2A). As shown in Fig. 2B,C, 24-h-pretreatment with A β ₁₋₄₀ strongly enhanced the IL-1 β -initiated-COX-2 expression and PGE₂ secretion. Similar results were obtained monitoring MMP-9. Indeed, A β ₁₋₄₀-pretreatment also increased IL-1 β -induced MMP-9 mRNA expression (Fig. 2D); this translated into potentiated MMP-9 enzymatic activity in VSMCs when compared to cells treated with IL-1 β alone (over 4-fold, considering the MMP-9 bands normalized to pro-MMP2, Fig. 2E). To be mentioned, A β ₁₋₄₀ had a similar potentiating effect on MMP-9 and COX-2 expression when followed by TNF α treatment; nevertheless, the level of expression of COX-2 and MMP-9 in TNF α -treated VSMC is very weak and remains very low after the sensitization by A β ₁₋₄₀-treatment (Fig. S1). These results establish that the A β ₁₋₄₀ peptide sensitizes VSMCs to IL-1 β and suggest that it may regulate one or several molecular entities involved in the IL-1 β -induced inflammatory response of these cells.

Using pharmacological compounds such as the PI $_3$ K inhibitor LY₂₉₄₀₀₂, the selective (BAY₁₁₋₇₀₈₂) or nonselective (MG132) inhibitor of the NF κ B pathway, we showed that the inhibition of either of these two pathways clearly abrogated the effect of IL-1 β on COX-2 expression (Fig. 3A) and MMP-9 secretion (Fig. 3B). In addition, 50 μ M of A β ₁₋₄₀ rapidly (within 15 min) triggered a significant phosphorylation of AKT reflecting a PI $_3$ K pathway activation, which decreased in less than 15 min (Fig. 3C). Finally, treating VSMCs transfected with the Ig κ -CONA-Luc vector with A β ₁₋₄₀ for 24 h resulted in enhancing luciferase activity by ~12-fold when compared to vehicle-treated cells ($P < 0.001$, Fig. 3D). As mentioned in the materials and methods section, the Ig κ -Cona-Luc vector carries a luciferase reporter gene under the control of three synthetic copies

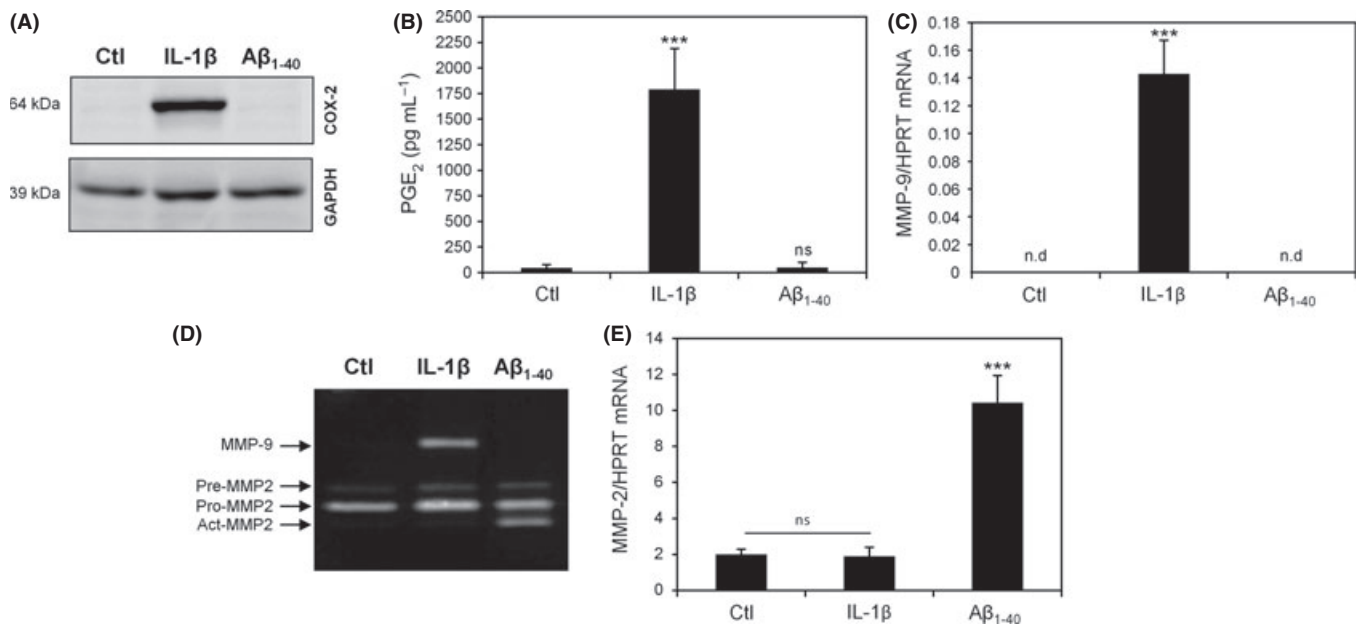


Fig. 1 A β_{1-40} peptide does not induce vascular smooth muscle cells (VSMCs) inflammatory response. Serum-starved cells were treated for 48 h with vehicle (control), interleukin-1 β (IL-1 β) (10 ng mL⁻¹) or A β_{1-40} peptide (50 μ M). (A) Cyclooxygenase-2 (COX-2) and GAPDH Western blot representative of four independent experiments. (B) PGE₂ release (pg mL⁻¹). Values are the mean \pm SD of four independent experiments in duplicate; ***, $P \leq 0.001$ vs. vehicle-treated cells referred as control (Ctl), n.s., not significant vs. control. (C) and (E) MMP-9 and MMP-2 mRNA normalized to HPRT. Data represent the means \pm SD of four independent experiments in triplicate; n.s., not significant, n.d., not detectable and ***, $P < 0.001$ compared with control (Ctl). (D) VSMCs secreted MMP-2 and MMP-9 activities evaluated by zymography. Pro- or active-form of MMP-2 and pro-MMP-9 were visualized after Coomassie Blue staining. The zymography represents 4 independent experiments.

of the NF κ B consensus responsive element cloned upstream of the conalbumin transcription start site; the Cona-Luc control vector is identical, except that it does not contain NF κ B sequences. The basal luciferase activity of the CONA-Luc control vector remained unchanged; as expected, a slight activity of the Ig κ -CONA-Luc vector was detected in vehicle-treated cells, reflecting the basal activity of the NF κ B pathway in VSMCs (Shin *et al.*, 1996). Interesting enough, neither I κ B α phosphorylation nor I κ B α degradation, both usually characteristics of NF κ B activation, was observed when A β_{1-40} treatment was performed for a short period of time (Fig. 3C). Altogether, these results (i) corroborate previous data obtained in SMC demonstrating that COX-2 and MMP-9 expressions can be induced by PI3K (Hsieh *et al.*, 2006; Lee *et al.*, 2007) and NF κ B pathways (Duggan *et al.*, 2007; Liang *et al.*, 2007); and (ii) demonstrate that A β_{1-40} rapidly activates the PI3K pathway and belatedly triggers NF κ B pathway activation. Therefore, we next evaluated whether A β_{1-40} -activation of PI3K and NF κ B pathways was involved in VSMCs sensitization to IL-1 β .

To this end, VSMCs were first treated with 50 μ M of A β_{1-40} for 24 h, with or without NF κ B (MG132) or PI3K (LY294002) inhibitors; then, after removing the cell medium containing A β_{1-40} and inhibitors, VSMCs were exposed to 2 ng mL⁻¹ of IL-1 β for a 24-h-additional treatment before evaluating the expression of inflammatory markers (see Fig. 4A for the protocol). The reason for using only the MG132 proteasome inhibitor and not the specific NF κ B inhibitor BAY₁₁₋₇₀₈₂ is that MG132 is a reversible compound, whereas BAY₁₁₋₇₀₈₂ is not; as PI3K and NF κ B inhibitors abrogated IL-1 β -induced expression of COX-2 and MMP-9 (Fig. 3A,B), the compounds used during A β_{1-40} pretreatment had to be reversible in order to avoid

affecting IL-1 β -activated pathways. As shown in Fig. 4B, inhibition of the PI3K pathway during A β_{1-40} cell exposure significantly reduced IL-1 β -induced MMP-9 mRNA expression ($P < 0.001$) (upper panel), MMP-9 secretion (middle panel), and COX-2 expression (lower panel) to levels observed for cells not subjected to A β_{1-40} before IL-1 β treatment. Importantly, since attesting for the reversibility of LY294002, preincubation of the cells with this compound alone did not prevent the effect of IL-1 β on MMP9-transcript (Fig. 4B upper panel, bar 6 vs. 2). Analogous results were obtained when performing similar experiments with the MG132 molecule (Fig. 4C). Lastly, we also observed that LY294002 and MG132, combined with A β_{1-40} -pretreatment, annealed the A β_{1-40} -dependent potentiation of PGE₂ secretion initiated by IL-1 β (Fig. 4D, bars 8 and 12 vs. 4, $P < 0.001$). As a whole, this last set of experiments demonstrated that the increase in the IL-1 β -dependent inflammatory response induced by A β_{1-40} exposure involves PI3K and NF κ B activation.

A β_{1-40} highly enhanced IL-1 β -induced VSMCs de-differentiation through PI3K and NF κ B activation

Because vessel wall inflammation drives a SMC de-differentiation characterized by a loss of the contractile apparatus, we thought of examining this process in an amyloid context. To do so, cells were exposed to A β_{1-40} peptide for 48 h before being treated with IL-1 β for 72 h; the loss of the contractile phenotype was tracked by measuring the expression of α -actin and SM22, two markers of differentiated contractile VSMCs (Shanahan *et al.*, 1993). As expected, the expressions of α -actin and SM22 transcripts were

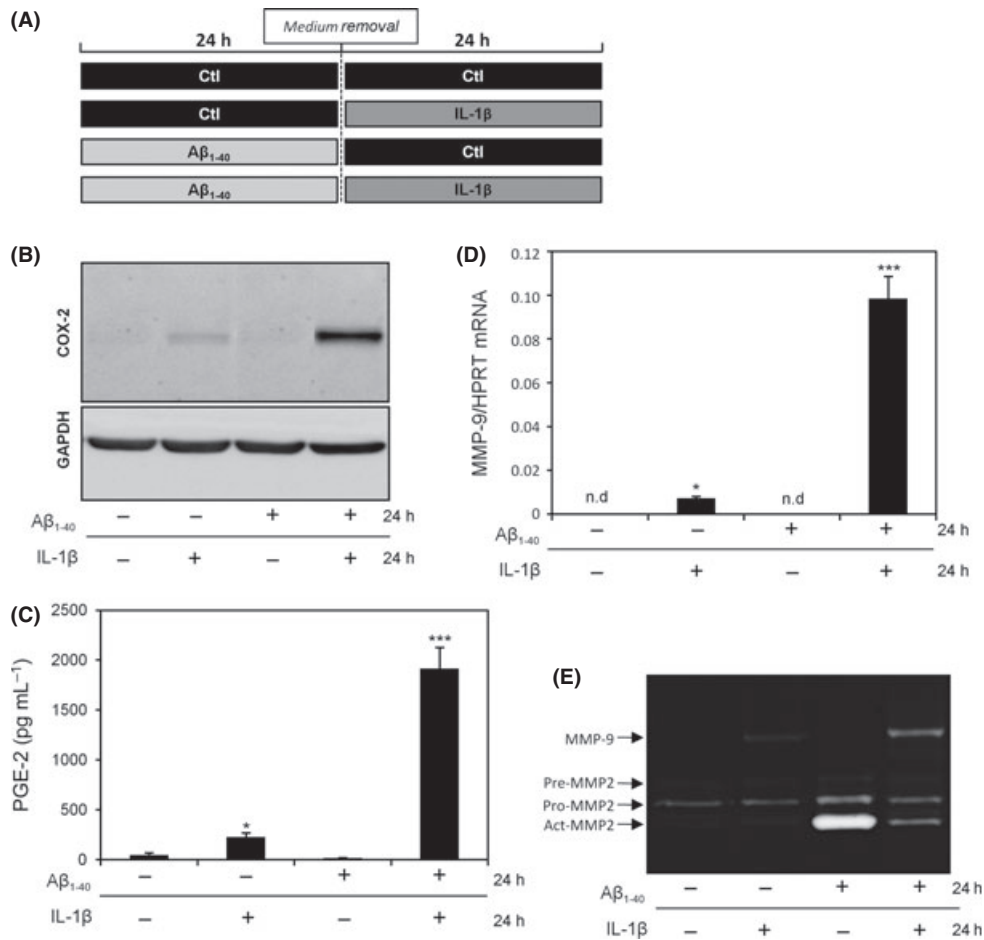


Fig. 2 A β_{1-40} peptide enhances the IL-1 β -induced inflammatory response in vascular smooth muscle cells (VSMCs). (A) Experimental procedure. Cells were treated for 24 h with A β_{1-40} peptide (50 μ M), or vehicle (control). After medium removal and washes in PBS, VSMCs were treated with IL-1 β (2 ng mL⁻¹) or vehicle (ctl) for an additional 24 h. (B) COX-2 and GAPDH Western blot representative of four independent experiments. (C) PGE₂ release (pg mL⁻¹). Values are the mean \pm SD of four independent experiments; n.s., not significant, *, $P \leq 0.05$, and ***, $P < 0.001$ compared with control cells. (D) MMP-9 transcripts normalized to HPRT. Data represent the means \pm SD of four independent experiments in triplicate; n.d., not detectable, *, $P \leq 0.05$ and ***, $P < 0.001$ compared with control cells. (E) VSMCs secreted MMP-2 and MMP-9 activities evaluated by zymography. Pro- or active-form of MMP-2 and pro-MMP-9 were visualized after Coomassie Blue staining. The zymography represents four independent experiments.

decreased in cells treated with IL-1 β alone (Fig. 5A, bar 2 vs. 1, $P < 0.001$); however, this decrease was much more important when pre-exposed to A β_{1-40} peptide prior to IL-1 β treatment (Fig. 5A, bar 4 vs. 2, $P < 0.001$). Similar results were obtained when tracking protein expressions. Indeed, the intensities of α -actin and SM22 bands in Fig. 5B lane 4, (corresponding to proteins extracted from cells exposed to both A β_{1-40} peptide and IL-1 β) were much lower than in Fig. 5B lane 2 (corresponding to proteins extracted from cells exposed to IL-1 β alone); α -actin and SM22 labeling, as well as the number of α -actin and SM22-positive cells, were much lower in cells preincubated with A β_{1-40} prior to IL-1 β (Fig. 5C, column 4) compared with cells exposed to IL-1 β alone (Fig. 5C, column 2). Of note, similar results were obtained for SM-Calponin and SM-MHC (Fig. S2). Here again, the inhibition of PI₃K pathway by LY₂₉₄₀₀₂ blocked the effect of A β_{1-40} on SM22 and α -actin transcript expressions in IL-1 β -treated VSMCs. Indeed, the increased inhibition of α -actin and SM22 expressions returned to a level comparable to cells treated with IL-1 β alone (Fig. 5A, bar 8 vs. 7, $P < 0.001$).

Consistently, LY₂₉₄₀₀₂ treatment translated into a marked recovery of the expression of both of these contractile proteins. Indeed, their corresponding bands after LY₂₉₄₀₀₂ treatment were almost as intense as in control/untreated cells (Fig. 5B, lane 8 vs. 1); analogous conclusions could be made out of immuno-cytochemistry experiments (Fig. 5C, column 8 vs. 1). Altogether these results demonstrated that A β_{1-40} exposure to VSMCs aggravates IL-1 β 's effect on VSMCs de-differentiation through a PI₃K pathway preactivation.

Conditioned medium obtained from A β_{1-40} -exposed endothelial cells culture increases the expression of inflammatory markers in A β_{1-40} -exposed VSMCs and aggravates their de-differentiation

Previous studies have demonstrated that amyloid peptides-exposed endothelial cells produce pro-inflammatory mediators including cytokines (Suo *et al.*, 1998; Vukic *et al.*, 2009). Considering the vessel structure where endothelial cells dialog with VSMCs, we next

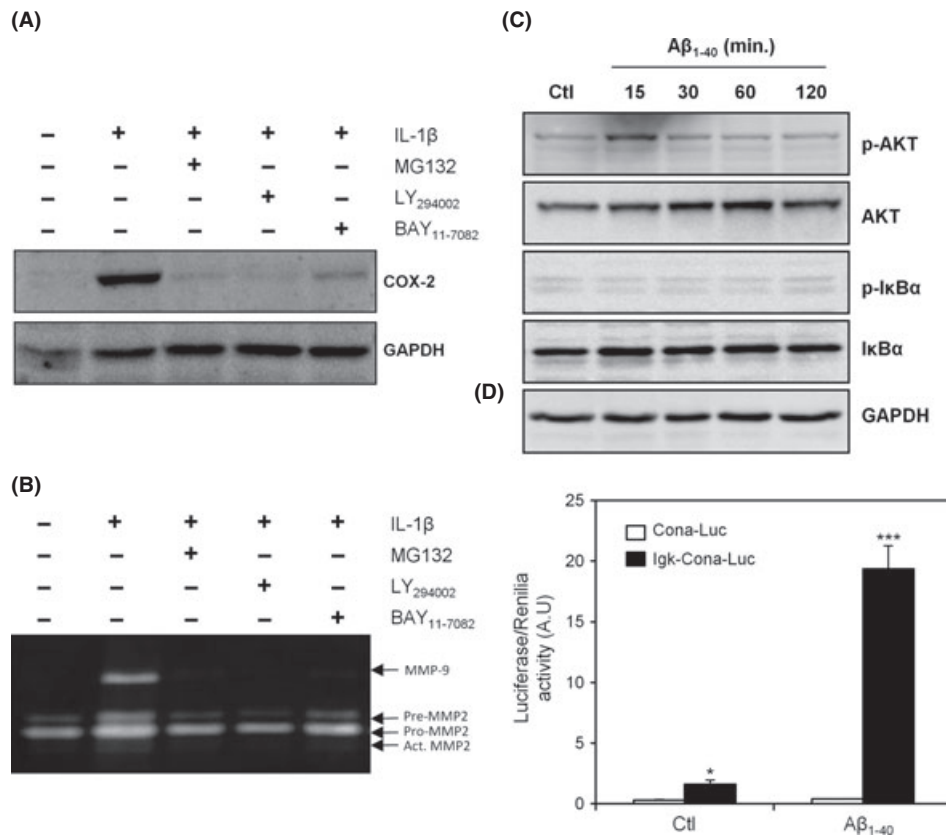


Fig. 3 Activation of PI $_3$ K and NF κ B pathways is involved in IL-1 β -induced inflammatory response and both pathways are activated by A β_{1-40} peptide. (A), (B). Vascular smooth muscle cells (VSMCs) were treated with IL-1 β (10 ng mL $^{-1}$) for 24 h or with the vehicle, and with the PI $_3$ K-specific inhibitor LY₂₉₄₀₀₂ (50 μ M), the NF κ B pathway-specific inhibitor BAY₁₁₋₇₀₈₂ (5 μ M) or the MG132 (200 nM), a proteasome inhibitor often used as a NF κ B pathway inhibitor. (A) COX-2 and GAPDH Western blot. (B) VSMCs secreted MMP-2 and MMP-9 activities evaluated by zymography. Pro- or active-form of MMP-2 and pro-MMP-9 were visualized after Coomassie Blue staining. The zymography is representative of three independent experiments. (C) VSMCs were treated with A β_{1-40} peptide (50 μ M) for the indicated length of time. Phosphor-AKT, AKT, phosphor-I κ B α , I κ B α , and GAPDH Western blots, represent 2–3 independent experiments. (D) VSMCs were transfected with Cona-Luc or Igk-Cona-Luc reporter constructs (and Renilia vector for normalization) for 24 h. Cells were then treated with A β_{1-40} peptide (50 μ M) or vehicle for an additional 24 h. Results are expressed as the ratio of luciferase and renilia activity in arbitrary units (A.U.) and are the means of two independent experiments in triplicate, *, $P \leq 0.05$ and ***, $P < 0.001$ compared with control/vehicle-treated (Ctl) Cona-Luc-transfected cells.

investigated how the inflammatory context generated by endothelial cells (EC) exposed to A β_{1-40} peptide could influence A β_{1-40} -enhanced VSMCs transition toward an inflammatory phenotype. Of note, 'Amyloid' VSMCs or ECs refer to VSMCs or ECs pre-exposed 48 h to A β_{1-40} peptide; nonamyloid cells refer to VSMCs or ECs pre-exposed 48 h to A β_{1-40} peptide vehicle. To do so, we generated *in vitro* conditioned media by incubating mouse brain endothelial cells with or without 50 μ M of A β_{1-40} peptide for 72 h; these condition media were referred to as CM-EC-A β or CM-EC-Ctl, respectively. As shown in Fig. 6B, 'amyloid' VSMCs were much more responsive to CM-EC-A β when compared to 'nonamyloid' VSMCs or 'amyloid' VSMCs receiving CM-EC-Ctl. Indeed, (i) whether at the messenger or at the protein level, the induction of COX-2 expression by CM-EC-A β was always significantly higher when VSMCs were previously exposed to A β_{1-40} (Fig 6B, bar & lane 4 vs. 2, $P < 0.01$); (ii) similar observations could be made for PGE₂ secretion (Fig 6C, bar & lane 4 vs. 2, $P < 0.01$); and (iii) although CM-EC-Ctl triggered an increase in COX-2 expression and PGE₂ secretion from 'amyloid' VSMCs (the reference being the effect of CM-EC-Ctl on vehicle-treated VSMCs, Western blot Fig. 6B lane 3

vs. 1 and Fig. 6C, bar 3 vs. 1, $P < 0.01$), CM-EC-A β systematically have a significantly higher effect on these parameters when compared to CM-EC-Ctl (Fig. 6B and C, bar & lane 4 vs. 3, $P < 0.01$).

When tracking VSMCs de-differentiation (by measuring SM22 and α -actin transcript expressions Fig. 6D left and right panels, respectively), we also evidenced that A β_{1-40} exposition prior to CM-EC-A β aggravates the deleterious effects of inflammation (bars 4 vs. 3, $P \leq 0.05$). Of note, CM-EC-A β added to 'nonamyloid' VSMCs did not have any effect when compared to CM-EC-ctl (Fig. 6D, bar 2 vs. 1); conversely, CM-EC-ctl on 'amyloid' VSMCs triggered a significant decrease in these markers (Fig. 6D, bar 3 vs. 1, $P < 0.001$).

When revisiting these experiments in presence of LY₂₉₄₀₀₂, we further emphasized the importance of the PI $_3$ K pathway in the A β_{1-40} -sensitization process of VSMCs to an inflammatory context. Indeed, here again LY₂₉₄₀₀₂ annealed the CM-EC-A β induced raised of COX-2 expression (Fig 6B bar & lane 8 vs. 4, $P < 0.01$) and PGE₂ secretion (Fig. 6C bar 8 vs. 4, $P < 0.001$). Interestingly enough, the small but significant rise of COX-2 expression and PGE₂ secretion

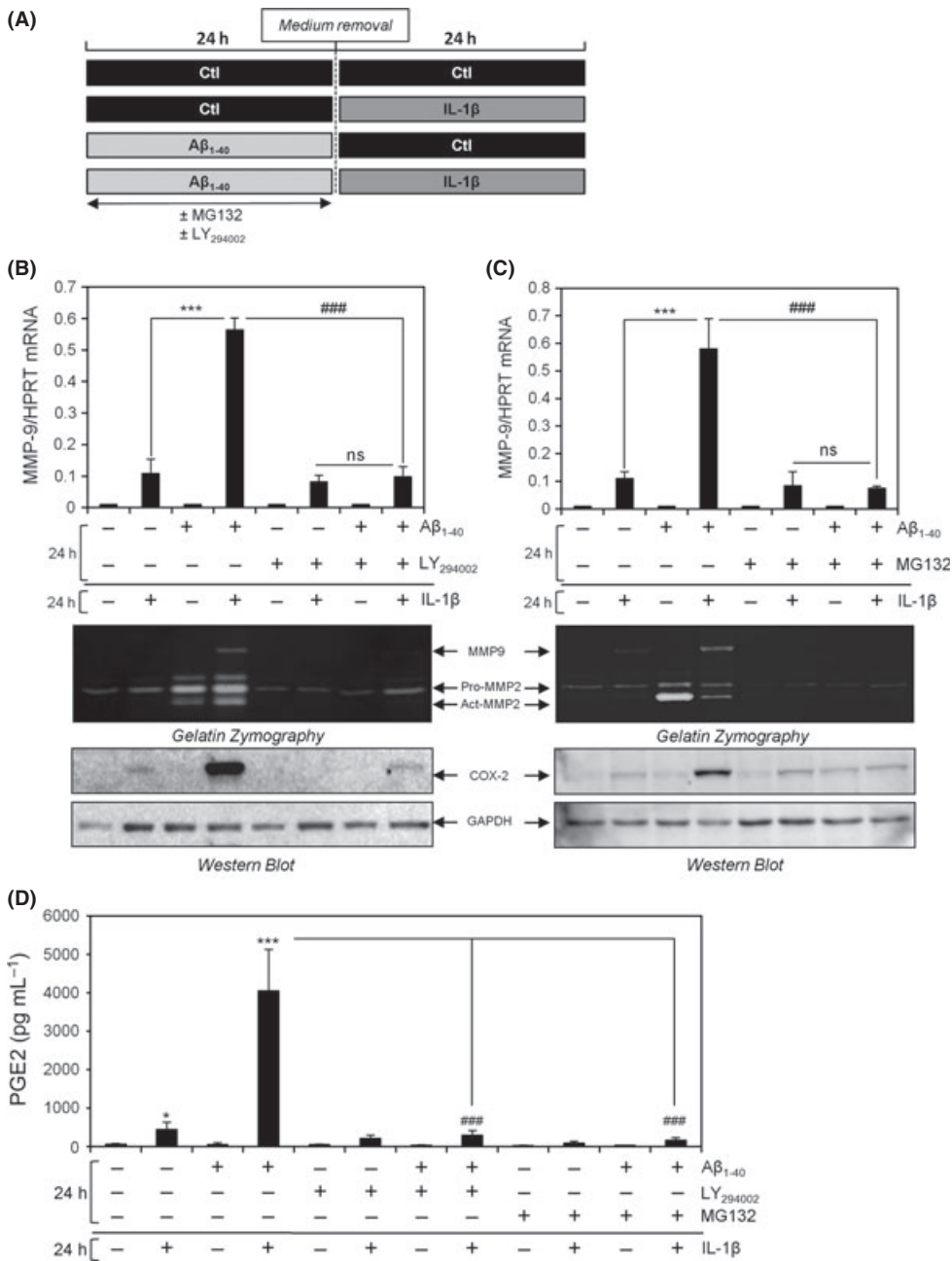


Fig. 4 The enhancement of the interleukin-1 β (IL-1 β)-initiated inflammatory response induced by the A β_{1-40} peptide is abolished by the PI3K-specific inhibitor LY294002 or by the proteasome blocker MG132. (A) Experimental procedure. Cells were incubated for 24 h in the presence of A β_{1-40} peptide (50 μ M), or vehicle with or without LY294002 (50 μ M), or MG132 (200 nM). After medium removal and washes in PBS, vascular smooth muscle cells (VSMCs) were treated with IL-1 β (2 ng mL $^{-1}$) or vehicle for an additional 24 h. (B), (C) upper panel. Effect of LY294002 (B) and MG132 (C) on A β_{1-40} -enhanced MMP-9 mRNA expression initiated by IL-1 β . Data represent means \pm SD of three independent experiments performed in triplicate, ***, $P < 0.001$ compared with IL-1 β -treated cells; ###, $P < 0.001$ compared with A β_{1-40} /IL-1 β -treated cells; n.s not significant compared with LY294002/IL-1 β -treated cells. (B), (C) middle panel. Effect of LY294002 (B) and MG132 (C) on secreted MMP-2 and MMP-9 activities determined by zymography. Pro- or active-form of MMP-2 and pro-MMP-9 were visualized after Coomassie Blue staining. (B), (C) lower panel. Effect of LY294002 (B) and MG132 (C) on COX-2 expression. COX-2 and GAPDH were immuno-detected with appropriate antibodies. Zymography and Western blot represents three independent experiments. (D) Effect of LY294002 and MG132 on A β_{1-40} enhanced PGE $_2$ secretion initiated by IL-1 β . Data represent means \pm SD of four independent experiments * $P < 0.05$ and *** $P < 0.001$ compared with control-vehicles-treated cells; ###, $P < 0.001$ compared with A β_{1-40} /IL-1 β -treated cells.

induced by CM-EC-ctrl on AP_{1-40} -treated VSMCs is also completely blocked by incubation with the LY294002 compound (Fig. 6B Western blot lane 7 vs. 3 and Fig. 6C bar 7 vs. 3, $P < 0.01$). Finally, PI3K inhibition also prevented 'amyloid' VSMCs sensitization to the

inflammatory context generated by EC exposure to A β_{1-40} ; indeed, it restored α -actin and SM22 transcript expressions to levels similar to vehicle-treated VSMCs receiving CM-EC-A β (Fig. 6D bar 8 vs. 4, $P < 0.01$ and $P \leq 0.05$). Altogether, these results (i) corroborate

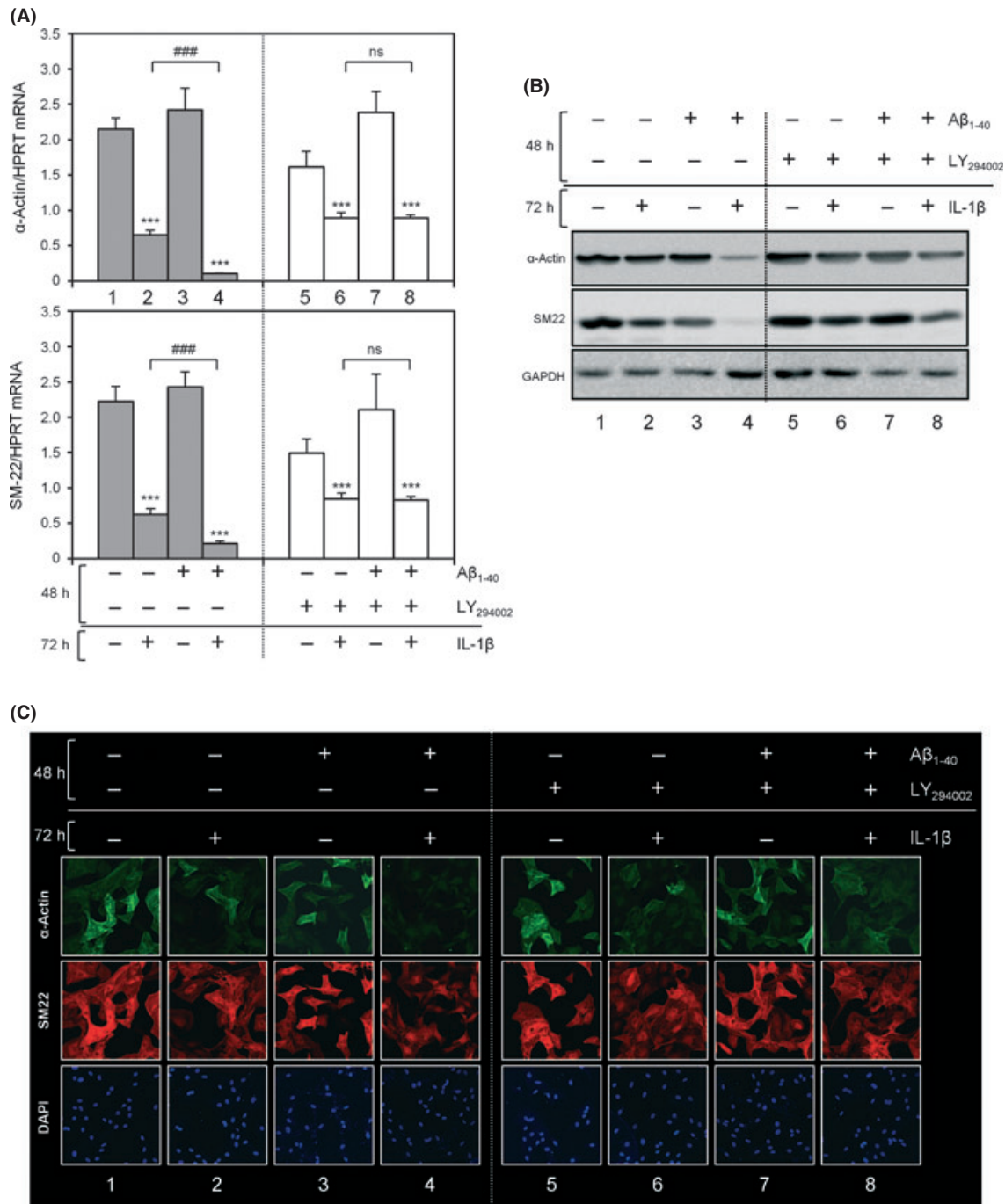


Fig. 5 Aβ₁₋₄₀ peptide enhances the deleterious effects of IL-1β on the vascular smooth muscle cells (VSMCs) contractile apparatus through the activation of the PI₃K pathway. Cells were treated for 48 h with Aβ₁₋₄₀ peptide (50 μM), or vehicle with or without LY294002 (50 μM). After medium removal and washes in PBS, VSMCs were then treated with IL-1β (2 ng mL⁻¹) or vehicle for an additional 72 h. (A). α-actin and SM22 transcripts normalized to HPRT. Data represent the means ± SD of three independent experiments in triplicate, ***, *P* < 0.001 compared with control/vehicle-treated cells; ###, *P* < 0.001, compared with Aβ₁₋₄₀/IL-1β-treated cells, n.s., not significant. (B). α-actin, SM22 and GAPDH were immuno-detected with appropriate antibodies. The Western blot shown is representative of three independent experiments. (C). Immunostaining on PFA-fixed VSMCs using α-actin and SM22 antibodies; the secondary antibody used for α-actin immunodetection is coupled to FITC (green); the one used for SM22 is coupled to Dylight 549 (red). Cell nuclei were DAPI-stained. Slides were analyzed by confocal microscopy (40×).

published data showing that endothelial cells exposed to an amyloid context generate on their own a strong inflammatory response (Suo *et al.*, 1998; Vukic *et al.*, 2009); (ii) reinforce the hypothesis as to whether ‘amyloid’ VSMCs, being at the vicinity of endothelial cells,

amplify the initial Aβ-induced inflammation, therefore possibly responsible for an amplification loop of the vascular inflammatory statute; and (iii) underline the importance of PI₃K pathway in these processes.

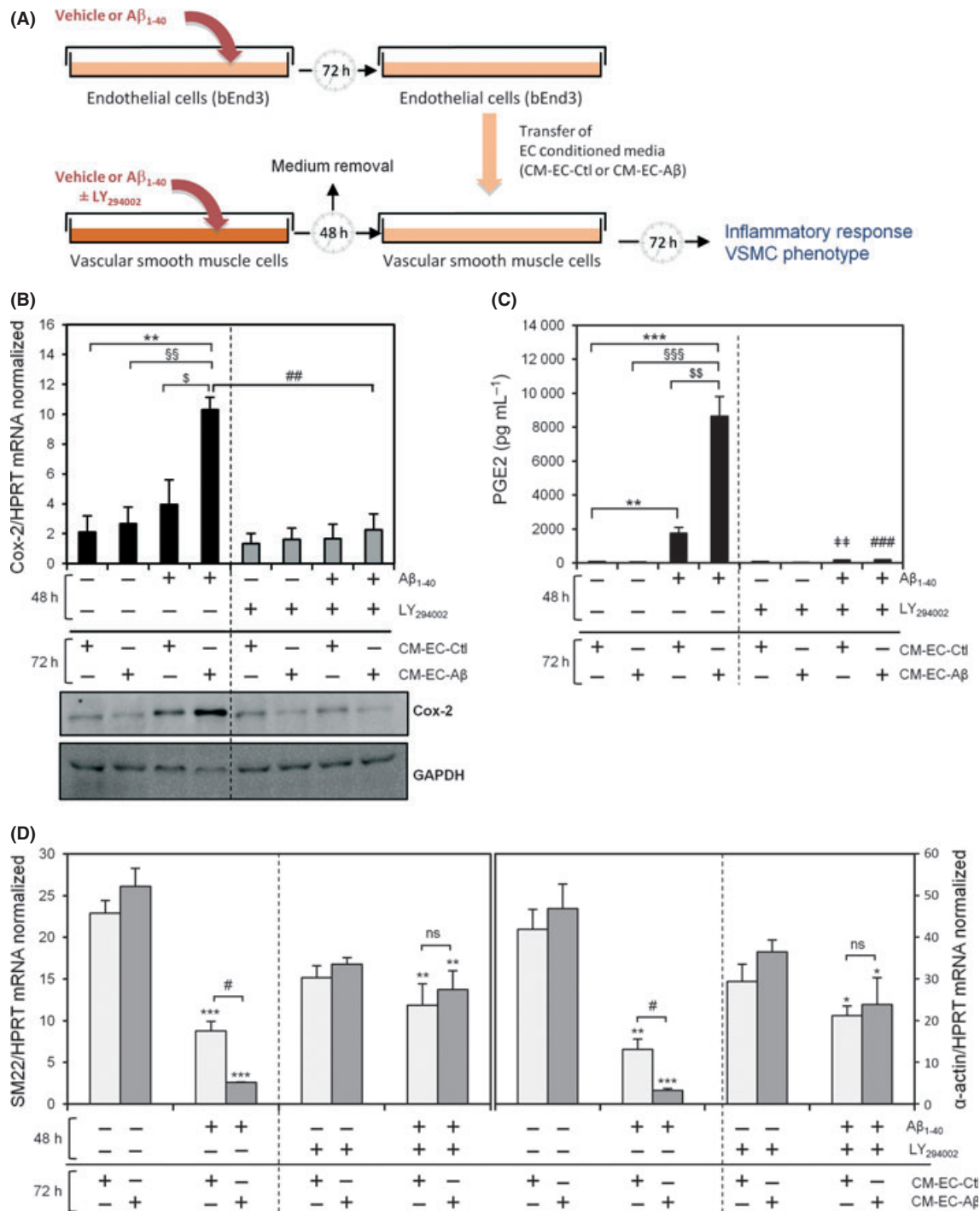


Fig. 6 A β_{1-40} -exposed endothelial cell culture medium increases the expression of inflammatory markers in amyloid vascular smooth muscle cells (VSMCs) and aggravates their de-differentiation. (A) Experimental procedure. VSMCs were treated for 48 h with A β_{1-40} peptide (50 μ M), or vehicle, with or without LY₂₉₄₀₀₂ (50 μ M). After medium removal and washes in PBS, VSMCs were exposed to the medium conditioned by endothelial cells previously treated (or not) for 72 h by vehicle [CM-endothelial cells (EC)-Ctl] or 50 μ M of A β_{1-40} peptide (CM-EC-A β). PGE₂ secretion, COX-2 and contractile markers expression were assayed by RT-PCR and normalized to HPRT expression. Data represent the means \pm SD of 3 independent experiments in triplicate; **, $P < 0.01$ compared with vehicle-treated cells; §§, $P < 0.01$ compared with vehicle-treated VSMCs exposed to CM-EC-A β ; \$, $P \leq 0.05$ compared with A β_{1-40} -treated VSMCs exposed to CM-EC-Ctl; ##, $P < 0.001$, compared with A β_{1-40} -treated VSMCs exposed to CM-EC-A β . Lower panel. COX-2 and GAPDH Western blot representative of 3 independent experiments. (C) VSMCs secretion of PGE₂ induced by conditioned medium. Data represent the means \pm SD of four independent experiments, **, $P < 0.01$ and ***, $P < 0.001$ compared with vehicle-treated VSMCs exposed to CM-EC-Ctl; §§§, $P < 0.001$ compared with vehicle-treated VSMCs exposed to CM-EC-A β ; §§, $P < 0.01$ compared with A β_{1-40} -treated VSMCs exposed to CM-EC-Ctl; ###, $P < 0.001$ compared with A β_{1-40} -treated VSMCs exposed to CM-EC-A β . (D). α -actin and SM22 transcripts normalized to HPRT. Data represent the means \pm SD of three independent experiments in triplicate, ***, $P < 0.001$, **, $P < 0.01$, and *, $P \leq 0.05$ compared with vehicle-treated VSMCs exposed to CM-EC-Ctl; #, $P \leq 0.05$, compared with A β_{1-40} -treated VSMCs exposed to CM-EC-Ctl, n.s., not significant.

Discussion

It has been shown that CAA-related inflammation might contribute to the pathogenesis of the disease. However, the effects of amyloid deposition on VSMCs inflammation and the molecular mechanisms involved remain unknown. Here, we first demonstrated that the A β ₁₋₄₀ peptide does not induce an inflammatory response in VSMCs because A β ₁₋₄₀ peptide treatment did not result in PGE₂ or MMP-9 secretion or in COX-2 expression. However, A β ₁₋₄₀-peptide treatment clearly hypersensitized VSMCs to a pro-inflammatory context. Indeed, when exposed to the pro-inflammatory cytokine IL-1 β or a medium conditioned by 'amyloid' endothelial cells, 'amyloid' VSMCs over-expressed and/or released these above-mentioned parameters; moreover, this phenomenon was accompanied with a decreased expression of both smooth muscle α -actin and SM22, two contractile markers. Therefore, we now claim that inflammation in vessels displaying A β ₁₋₄₀ amyloid deposits, although beginning with endothelial cells is amplified through 'amyloid' VSMCs. This hypothesis is further supported by the fact that, in our model, this amplification only starts when A β ₁₋₄₀ concentration reach 25 μ M, corresponding to the appearance of oligomeric forms (Fig. S3). This phenomenon, associated with VSMCs de-differentiation ultimately leading to VSMCs degeneration could cause, or at least aggravate, the vascular alterations characteristic of CAA, resulting in hemorrhage, cerebral hypo-perfusion, and stroke. It could also play a fundamental role in the development of Alzheimer's disease and be responsible for associated neuro-degeneration and cognitive impairment.

The lack of effect of A β ₁₋₄₀ alone on VSMC-contractile proteins expression objectified here (Fig. 5) is consistent with Chow *et al.*'s results; indeed, although using A β ₁₋₄₂, they showed in cultured VSMC that exogenous A β -peptide did not affect the expression of the serum responsive factor (SRF), an interacting transcription factor that orchestrate a VSMC-contractile/differentiated phenotype (Chow *et al.*, 2007). This further support the hypothesis as to whether the increase in SRF observed in AD VSMC is not a consequence of accumulated A β -peptides.

The absence of inflammatory response from A β ₁₋₄₀-treated VSMCs is consistent with Suo *et al.*'s data showing that, among parietal cells, A β ₁₋₄₀ peptide mainly triggered the production of pro-inflammatory mediators from endothelial cells (Suo *et al.*, 1998). However, Previti *et al.* (2006) reported that VSMCs exposed to the E22Q Dutch-mutant A β ₁₋₄₀ (Dutch A β ₁₋₄₀)-peptide secrete significant amounts of interleukin-6 (IL-6) (Previti *et al.*, 2006). Similar to IL-1 β , members of the IL-6 cytokine family including IL-6, IL-11, and oncostatin M, increase the expression of COX-2 and promote the release of PGE₂ in several cellular models (Tai *et al.*, 1997; Osuka *et al.*, 1998; Bernard *et al.*, 1999); in addition, they are thought to mediate the effect of IL-1 β on COX-2 expression in SMCs (Lahiri *et al.*, 2001). Rather than being controversial, we believe that these data support, once again, a possible difference between the ontogenesis of Dutch familial CAA and sporadic forms of CAA (characterized by Dutch and WT-A β ₁₋₄₀ deposition, respectively) which we recently reported (Blaise *et al.*, 2012).

The molecular mechanism responsible for the hyper-sensitization of 'amyloid' VSMCs involves A β ₁₋₄₀ preactivation of signaling

pathways triggering COX-2 and MMP-9 expression in VSMCs, namely the PI₃Kinase and possibly NF κ B pathways (Hsieh *et al.*, 2006; Duggan *et al.*, 2007; Lee *et al.*, 2007; Liang *et al.*, 2007). Indeed, A β ₁₋₄₀ treatment of VSMCs induces AKT phosphorylation and NF κ B activity (Fig. 3) and their inhibition (conducted with LY₂₉₄₀₀₂ and MG132, respectively) abrogates this phenomenon (Fig. 4). Whether the MG132 effect could be attributed to the inhibition of NF κ B pathway could be a matter of debate as this compound inhibits the 26S complex of the proteasome, thus affecting the degradation of numerous ubiquitinated proteins and, consequently, many cellular processes [see (Demasi & Laurindo, 2012) for review]. Nevertheless, if A β ₁₋₄₀ peptide actually induces NF κ B signaling, this effect would likely depend on PI₃kinase activation. Indeed, (i) direct activation of NF κ B usually occurs within 15 min in VSMCs (Katsuyama *et al.*, 1998) and this is not the case here (see Fig. 3C); (ii) the A β ₁₋₄₀-induced NF κ B activation occurs later than A β ₁₋₄₀-induced PI₃Kinase activation (Fig. 3C); and (iii) several studies have demonstrated a cross-regulation between PI₃K and NF κ B pathways (Reddy *et al.*, 2000; Madrid *et al.*, 2001). Even more convincing, Cheng *et al.* (2010) showed that the inhibition of PI₃K by LY₂₉₄₀₀₂ compound attenuates the IL-1 β -induced recruitment of the activated p65 subunit of NF κ B to the MMP-9 promoter region (Cheng *et al.*, 2010).

Because PI₃K/Akt pathway is involved in the induction of IL-1-Receptor1 (Teshima *et al.*, 2004), we proposed that one of the mechanisms involved in the hypersensitization of 'amyloid' VSMCs to IL-1 β includes the increase in expression of this receptor. This scenario is strengthened by our results showing that A β ₁₋₄₀-treatment significantly enhanced the expression of IL-1-receptor1 (Fig. S4). Of note, the hypothesis as to whether A β ₁₋₄₀ exposition enhances the expression of receptors binding pro-inflammatory mediators was already enounced by Suo *et al.* (1998) and served to explain how A β ₁₋₄₀-treated VSMCs may contribute to the development of an inflammatory process within vessels.

Considering that A β ₁₋₄₀ alone significantly activates the PI₃kinase/Akt and possibly NF κ B pathways, the lack of its effect on MMP-9 and COX-2 expressions could be surprising (Fig. 1 and Fig. 3). In fact, this lack is consistent with the need of being triggered by an inflammatory context; it also reveals that IL-1 β induces distinct signaling pathways. Because p42/p44 MAPK, p38 MAPK, and the JNK pathways have been shown to be involved in IL-1 β -induced COX-2 and MMP-9 (Bartlett *et al.*, 1999; Laporte *et al.*, 1999; Liang *et al.*, 2007) expression, we hypothesize that they are the pathways activated here by IL-1 β .

Adding medium conditioned by 'amyloid' endothelial cells to A β ₁₋₄₀-exposed VSMCs potentiates the expression of inflammatory markers and further down-regulates contractile markers. The fact that 'amyloid' VSMCs were hypersensitized to a medium conditioned by A β ₁₋₄₀-exposed endothelial cells highlights the possible *in vivo* relevance of our *in vitro* study. More accurately, it also substantiates that endothelial cells exposed to A β -peptides release pro-inflammatory mediators (Suo *et al.*, 1998; Vukic *et al.*, 2009). Interestingly, conditioned medium derived from A β ₁₋₄₀-treated endothelial cells did not have any effect on control (*i.e.* A β ₁₋₄₀ unexposed) VSMCs; this emphasizes the possible importance of

the amyloid content within the vessel in increasing the inflammation due to VSMCs. In this regard, it would be much of interest to determine how a decrease in LRP-mediated amyloid- β clearance influences the inflammation-related CAA progression; AD individuals with CAA showed elevated levels of SRF and MYOCD, A β accumulation and significantly lower levels of LRP, compared with age-matched, nondemented controls without CAA (Bell *et al.*, 2009).

As the effect of conditioned medium derived from A β ₁₋₄₀-treated endothelial cells is very similar the effect of IL-1 β on 'amyloid' VSMCs, one may suggest that IL-1 β is a major component of this medium. Nevertheless, the pro-inflammatory cytokines IL-1 β , IL-2, IL-6, TNF α and IFN- γ were poorly detected whether in vehicle or in A β ₁₋₄₀-treated endothelial cell conditioned medium (Fig. S5). On the other hand, we evidenced a reproducible increased secretion of LIX (CXCL5) and MCP-1 from A β ₁₋₄₀-treated endothelial cells compared to vehicle-treated endothelial cells (fold 1.5 ± 0.035 and 1.44 ± 0.66 , respectively). Because CXCL5 is a small cytokine produced in response to IL-1 β (Chang *et al.*, 1994), one may suggest CXCL5 as the relaying cytokine responsible for 'amyloid' VSMCs phenotypic transition toward a de-differentiated/inflammatory state. In partial support of this suggestion, one of its receptors (namely CXCR2) is involved in airway SMC trans-differentiation (Al-Alwan *et al.*, 2012). It is also likely that MCP-1 participates to the phenotypic modification of 'amyloid' VSMCs; indeed, it is directly involved in inducing a phenotypic transition of VSMCs through the PI₃K pathway (Selzman *et al.*, 2002).

In conclusion, these *in vitro* experiments combined with the use of medium conditioned by endothelial cells on VSMCs allow a close approximation of the *in vivo* situation driving inflammation in CAA; however, macrophages and cell systems are also major players in the inflammatory process. Therefore, experiments need to be conducted on transgenic mouse models which develop cerebral and vascular amyloid plaques [APP23, (Winkler *et al.*, 2001)] to accurately study the vascular changes and the inflammatory processes taking place during CAA. In addition, the importance of MCP-1 and LIX secretion by ECs exposed to A β ₁₋₄₀ peptide should be evaluated by performing experiments similar to those described in this study by either inhibiting MCP-1 or LIX expression in endothelial cells, or by blocking their receptors in VSMCs.

Experimental procedures

Reagents

The source of reagents is given in Table S1 (Supporting information). The A β ₁₋₄₀ peptide purity is > 95%. A β ₁₋₄₀ peptide was solubilized at 2 mM in ultrapure water and frozen. The same batch was used for all experiments.

Cell culture

Mouse brain endothelial cells (bEnd.3/LGC Standards, Molsheim, France) were grown in Dulbecco's Modified Eagle's Medium (4.5 g L⁻¹ glucose), 10% FCS and antibiotics. Rat VSMCs were

isolated as described by Blaise *et al.*, (2012). Experiments were performed on cells at passages from 2 to 6 for VSMCs or from 3 to 13 for bEnd.3 cells. Confluent cells were made quiescent growing them in a serum-free medium 12 h before treatment. Experiments were performed in serum-free medium. Media conditioned by endothelial cells were centrifuged 5 min 10 000 \times g before contacting them with VSMCs. Conditions of cell treatment are indicated in the Figure legends. All incubations were performed at 37 °C in a 5% CO₂ atmosphere. Cells were exposed to A β ₁₋₄₀-peptide diluted in culture medium at 50 μ M for 24–48 h.

RT-PCR assays

Total RNA was extracted from VSMCs using the RNeasy kit (Qiagen, Hilden, Germany). RT-PCR assays were performed as described by Blaise *et al.*, (2012). The forward and reverse primer sequences used to amplify the cDNA are given in Table 1.

Protein extraction and Western blot

Protein extraction, Western blot and detection signal procedures were as described by Blaise *et al.*, (2012). Antibodies are given in Table 2.

Zymography, PGE₂, and luciferase assays

Gelatin zymography of conditioned media samples was performed as described by Blaise *et al.*, (2012). PGE₂ secretion was evaluated with an enzyme immunoassay kit from Cayman Chemical SPI-BIO (Massy, France). For luciferase activity, VSMCs were transfected by adding a total DNA/Fugen HD ratio of 2:3 according to the Roche Diagnostics instructions. VSMCs were transfected with 1 μ g of firefly-luciferase reporter plasmid-Igk-cona- or control vector cona-luc- and 50 ng of pRL-TK (Renilla-luciferase reporter gene under the

Table 1 Summary of PCR primers

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number
HPRT	aggacctctcg aagtgt	atccctgaagtg ctcattata	NM_012583.2
MMP-2	atcgcccatca tcaagttc	catgttctcgat gggtgtc	NM_031054.2
MMP-9	ttcgagggag cgtcctat	ttagtggtgcag gcagagta	NM_031055.1
COX-2	ggaagtctttg gtctgggtg	tcttgatcgtctctct atcagta	NM_017232.3
SM22	tatggcagcag tgagag	ctttcttcataaacc agttggga	NM_031549.2
α -actin	accagattatg tttgagacc	cagagtcagca caatacca	NM_031004.2
SM-Calponin	ttaaccgaggtc ctgcctac	gctggtgggtcg tatttctgg	NM_031747.1
SM-MHC	tgagaggaagaag atggctca	tgtagttctgtct ggcagctt	NM_001170600.1

Table 2 Summary of primary antibodies

Antibodies	Species	References
Anti-phospho-Akt	Rabbit monoclonal	4046; Cell Signaling technology, Inc.
Anti-Akt(pan)	Rabbit monoclonal	4691; Cell Signaling technology, Inc.
Anti-α-actin	Mouse monoclonal	clone1A4; Dako
Anti-SM22	Rabbit polyclonal	ab-14106; Abcam (Paris, France)
Anti-SM-Calponin	Mouse monoclonal	C2687; Sigma-Aldrich (Saint Quentin Fallavier, France)
Anti-SM-MHC (MYH11)	Goat polyclonal	sc-79079; Santa-Cruz Biotechnology, Inc. (Tebu-Bio, Le Perray en Yvelines, France)
Anti-COX-2	Goat polyclonal	sc-1745; Santa-Cruz Biotechnology, Inc.
Anti-Aβ1-40	Mouse monoclonal	ab-7501; Abcam
Anti-phospho-IκBα	Mouse monoclonal	sc-8040; Santa-Cruz Biotechnology, Inc.
Anti-IκBα	Rabbit polyclonal	sc-871; Santa-Cruz Biotechnology, Inc.
Anti-GAPDH	Goat polyclonal	sc-20357; Santa-Cruz Biotechnology, Inc.

control of thymidine-kinase promoter, as internal control). Transfected cells were treated for 24 h with Aβ1-40 peptide. Cells were harvested in reporter lysis buffer; lysates were assayed for luciferase activities using a dual luciferase assay kit (Promega, Lyon, France) and were normalized by the ratio of firefly and *Renilla* luciferase activities. The firefly-luciferase reporter plasmid- Igκ-cona-luc made of 3 NFκB sites cloned upstream of the minimum conalbumin promoter comes from Dr R.Weil, Institut Pasteur, Paris.

Immunocytochemistry

Immunocytochemistry was performed as described by Blaise *et al.*, (2012). After treatment, cells were washed, fixed, permeabilized and incubated with primary antibodies (anti-α-actin or -SM22). Incubation with DyLight 488- and DyLight 549-conjugated secondary antibodies was performed for 1 h, Hoescht staining for 5 min at RT. Coverslips were mounted with Dako mounting medium (Dako, Carpinteria, CA, USA). Cells were examined on a Leica SP5 confocal fluorescent microscope. Acquisition parameters were established for vehicle-treated cells and were unchanged for all experimental conditions.

Statistical analysis

Data are reported as the mean ± SD. Values were compared between groups with the Welch’s unpaired, corrected *t*-test.

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Authors contribution

I. Limon and R. Blaise have access to all data and take responsibility for data and accuracy of the analysis. All the authors approved the final manuscript version. R. Blaise, G. Béréziat and I. Limon contributed to the study conception and design. R.Blaise, C. Rouxel, N. Trabelsi and A. Vromman contributed to the acquisition of the data. R. Blaise, I. Limon and A. Vromman contributed to the analysis and interpretation of the data. R. Blaise and I. Limon contributed to the manuscript preparation. R. Blaise and A. Vromman contributed to the statistical analysis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1 Reagents and sources.

Fig. S1 $\text{A}\beta_{1-40}$ effect on tumor necrosis factor- α (TNF α)-treated vascular smooth muscle cells (VSMCs).

Fig. S2 Expression of SM-Calponin and SM-MHC in vascular smooth muscle cells (VSMCs) treated by $\text{A}\beta_{1-40}$ and IL1 β .

Fig. S3 Conformational state of $\text{A}\beta_{1-40}$ and its dose-effect on IL1 β -induced vascular smooth muscle cells (VSMCs) inflammatory response.

Fig. S4 $\text{A}\beta_{1-40}$ effect on the expression of the IL-1-R1 in vascular smooth muscle cells (VSMCs).

Fig. S5 Inflammatory molecules in vehicle- vs. $\text{A}\beta_{1-40}$ -treated endothelial cells culture medium.