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IL-1β/MMP9 activation in primary human vascular smooth muscle-like cells: Exploring the role of TNFα and P2X7☆

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ABSTRACT

Background: Vascular smooth muscle cells exhibit phenotypic plasticity in response to microenvironmental stimuli and contribute to vascular remodelling through mechanisms only partially understood.
In atherosclerosis, P2X-purinoceptor7 (P2X7) has been related to interleukin-1β (IL-1β) and metalloproteinase 9 (MMP9). The hypoxia-inducible factor-1alpha (HIF1α) was associated to remodelling. Here the activation of IL-1β and MMP9 was studied in relationship to P2X7 and HIF1α in cells exploited from human carotid plaque and internal mammary artery.
Methods and results: Migrating cells expressed HIF1α-regulated canopy FGF-signalling regulator 2 and CD117, and led to primary cells with SMC-like phenotype (VSMC), P2X7^{+}.
We investigated in VSMC the effects of hypoxia, of treatment with tumour necrosis factor-α (TNFα) and/or with P2X7 antagonist, A740003. Quantitative RT-PCR showed that hypoxia unaffected IL-1β and down-regulated MMP9 mRNAs, without activating HIF1α. TNFα increased IL-1β mRNA via NLR Family Pyrin Domain-Containing 3, with production of proIL-1β but no rise of mature IL-1β. Zymography demonstrated that A740003 triggered MMP9 secretion from VSMC. Combination of A740003 with TNFα abrogated this effect. Combination was ineffective on IL-1β activation elicited by TNFα, but down-regulated HIF1α mRNA. A740003 induced the intracellular P2X7 aggregation and differently perturbed lysosome and mitochondria network compared to TNFα.
Conclusions: Cells migration from human arteries leads to partially differentiated VSMC analogous to neointimal cells within atherosclerotic lesions. Down-regulated HIF1α in stimulated VSMC translates in resilience in atherosclerotic lesions. P2X7-independent partial activation of IL-1β elicited by TNFα underlines complexity of the cytokine secretion. Data also supported P2X7 as modulator of MMP9 secretion, important for atherosclerosis progression.

1. Introduction

Smooth muscle cells (SMC) are major stromal contractile components of adult healthy vessels [1], exhibiting low proliferation rate and synthetic activity in vivo, and displaying a variety of phenotypes within contractile and synthetic in the altered vessels of both animal models and human beings [2–7]. These not terminally differentiated but mature cells retain phenotypic plasticity [8], pivotal for the vascular remodelling processes [9]. Exogenous and microenvironmental stimuli may induce SMC phenotypic switching, i.e. changes in the expression of SMC lineage-associated markers [10], and activation of signalling pathways. In vitro differentiation of SMC towards endothelial cells is reported [11], but transdifferentiation in human atherosclerotic lesions is still an object of debate [12].

Phenotypic changes may affect migration and proliferation [1], involved in vessel alteration/thickening and in repair after endothelial injury [13]. It is still not established whether the migratory capability is in vivo acquired upon microenvironmental stimulation by subset of SMC with a specific lineage, or is the consequence of a localization-dependent phenotypic switch.

The involvement in SMC migration/proliferation of the angiogenic growth factor canopy FGF signalling regulator 2 (CNPY2), regulated by hypoxia-inducible transcription factor-1α (HIF1α) has been recently reported [14]. HIF1α may affect the growth of in vitro SMC in hypoxia [15,16], and the expansion of plaque tissue mass leads to local hypoxia, impairing oxygen diffusion capacity in the vessel wall, thus contributing to the lesion progression [17].

☆ The authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. The authors report no relationships that could be construed as a conflict of interest.

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Emerging evidences support the hypothesis that inflammatory mediators of atherosclerosis may converge on the pathway linking interleukin-1 (IL-1), and tumour necrosis factor (TNF-α), which contribute to plaque vulnerability through transdifferentiation, proliferation and activation of SMC [2,6]. Overexpression of IL-1β in atherosclerosis also involve induction of matrix metalloproteinases (MMPs) including gelatinases as MMP9, converting the inactive pro- IL-1β into biologically active forms but also cleaving some mature IL-1β [18]. Subsets of intermediate cells co-expressing markers of SMC and macrophages have been involved in P2X purinoceptor 7 (P2X7)-mediated modulation of IL-1β and of MMP9 in ex-vivo tissue cultures of atherosclerotic carotid artery [5], evidencing the effect of A740003, a specific P2X7 antagonist broadly used in preclinical disease models because of suppressive effect on inflammatory cytokines and vascular remodelling [19].

Here we provide evidence that cells migrating from human atherosclerotic carotid artery plaque (PL) and morphologically normal internal mammary artery (IMA) expressed CNPY2 and led to primary cells with intermediate phenotype macrophage marker-positive SMC-like. We investigated in vitro the response of primary cells either to chronic hypoxia, or treatment with TNFα, focusing on IL-1β/MMP9 activation in relationship to P2X7 and HIF1α.

2. Materials and methods

2.1. Reagents are in Supplementary files

2.1.1. Patient samples

Fragments of carotid artery plaques (PL, atherosclerotic, n = 60) and of internal mammary artery (IMA, not atherosclerotic, n = 20) were harvested as surgery waste from patients submitted either to endoarterectomy (TEA) or to coronary artery bypass grafting (CABG). All patients signed an informed consent and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee at IRCCS San Raffaele Hospital.

2.1.2. Cell culture

PL (after discarding the necrotic core material) and IMA fragments were cut under sterile conditions (from all samples: cubes of about $2mm^3$; from 7 randomly-selected samples: cubes of about $2mm^3$ and rings 2 mm in length). The pieces of tissue (hereinafter referred as "tissue(s)") were cultured in standard CO_2 incubator using 12wells plates and DMEM with 4.5 g/L D-Glucose, L-glutamine and sodium pyruvate, supplemented with 10% heat-inactivated FBS (endotoxin <0.5 EU/mL), 1% penicillin/streptomycin. Low-endotoxin FBS was used to avoid lipopolysaccharide-dependent cytokines release by VSMC [20].

Cells (p0) spread from tissues after a lag time of 15–20 days, reaching 80–90% confluence in 20–30 days. Subsequently p0 cells were split to passage 1 (p1) and tissues moved to a new well to continue the cells release. Percentage of viable p0 cells was evaluated [21]. The procedure was repeated till cells outgrow from tissue, i.e. maximum twice, corresponding to 3 months of tissue culture. Setup experiments showed that VSMC poorly adhered to surfaces using medium with low/no glucose, but well grow in condition mimicking the in vivo normal glucose level (4.5 g/LD-Glucose).

Primary human carotid artery SMC (HCSMC, from single donor 20 years old, Caucasian male, Cat. # 3514K-05a, Cell Application Inc., San Diego, CA) where used as control cells.

VSMC at p1 were treated with: TNFα (100 ng/mL added to culture medium for 24 h), A740003 (selective P2X7 antagonist, 100 μmol/L final concentration into the culture medium for 1 h) either in normoxia or in mild hypoxia (2% O_2 and 5% CO_2 for 5 days). At the end of each treatment, culture supernatants were harvested, cells rinsed in Dulbecco's phosphate buffer (PBS), then either fixed in 2% paraformaldehyde in PBS or in 2.5% glutaraldehyde in cacodylate buffer, or submerged in RNA later solution, or lysed in RIPA buffer. Results derived from at least three replicates/treatment.

After culture, tissue rings were embedded in Killik, frozen in isopentane/liquid nitrogen and stored at −80 °C. Cryosections (10 μm) were obtained by Leica CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany).

2.1.3. Mitotracker, lysotracker and ovalbumin

Living cells plated on chamber slides were incubated (1 h, incubator) in the presence of either Mitotracker (500 nmol/L), or Lysotracker (5 μmol/L) or Ovalbumin (200 μg/mL) into culture medium, then rinsed in PBS.

2.1.4. Immunofluorescence

Paraformaldehyde-fixed cells/cryosections were labelled as previously described [5].

Image panels were mounted with AdobePhotoshopCS, and 3D-renderings obtained with Volocity.

2.1.5. Transmission electron microscopy

Glutaraldehyde-fixed cells were post-fixed in 1% osmium tetroxide, 1% potassium ferrocyanide in cacodylate buffer (1 h, 4 °C). Washing, *en block* stain with uranyl acetate (overnight, 4 °C), dehydration, embedding in Epon 812 and polymerization followed. Ultrathin sections were obtained by Leica Ultracut UCT, seeded on formvar nickel grids and stained with uranyl acetate/lead citrate before imaging using Zeiss Leo 912 Omega transmission electron microscope equipped with Proscan camera and Esivision Pro 3. Software.

2.1.6. Senescence-associated β-galactosidase

Cytochemical protocol was performed on fixed cell following Itahana et al. [22].

2.1.7. Western blot

Western blot was performed as described [5].

2.1.8. Gel zymography

Gelatine zymography was performed on cell supernatants as described [5]. Optical densitometry was performed with FiJi.

2.1.9. ELISA

Human IL-1β was quantified in cell culture supernatants from at least 5 artery/treatment and from HCSMC by ELISA Quantikine kits (R&D System, Minneapolis, MN, USA) following manufacturers' instructions. Luminescence was measured on Infinite F200 microplate reader (TECAN Group Ltd., Männedorf, Switzerland). Samples were run at least in duplicate.

2.1.10. RNA extraction and real-time PCR

Total mRNA was extracted from cells and tissues and quantitative real-time PCR was performed as described [5]. Probes were listed in Supplementary Table 2. Gene expression was presented as $2^{-\Delta Ct}$.

2.1.11. Statistical analysis

Prism7 software was used. Normality of data distribution was assessed by Kolmogorov-Smirnov test. Data from treated samples vs. ctrl were compared with a Wilcoxon signed-ranks test, or *t*-test. Comparison among clinical and experimental data was performed by ANOVA, or non-parametric Kruskal-Wallis or Friedman tests with Multiple Comparison, as opportune. Probability value <0.05 was considered statistically significant.

3. Results

3.1. VSMC migration from artery wall

The artery walls after 2–3 months of tissue culture and cells release showed a diffused paucity of nuclei, except in areas of loose matrix where numerous, spatially disorganized cells were detectable (Fig. 1A, left montage). Elongated cells with similar phenotype escaped from adventitial and sub-endothelial areas with loose matrix of IMA and PL tissues (Fig. S1A). These cells expressed αSMA, sm22 or SMMHC, collagen type I, vimentin, von Willebrand factor, CD68, P2X7, MMP9, FSP1 and weak laminin (Fig. 1B). Among them, several cells were $CD117^+$(c-kit)/$CD34^-$ (Fig. 1B, S1B). Spatially disorganized cells were delimited on the luminal/adventitial side by a cells rim, either multilayered or incomplete. The rims were constituted by cells co-expressing sm22, vWF, FSP1, P2X7 and weak αSMA, and included $Ki67^+$ cells (Fig. 1B, S1B). Outgrowing cells also showed bright signal for CNPY2, (Fig. 1C, left), a marker weakly and focally expressed in the neointima and/or shoulder of freshly harvested PL (Fig. 1C, right).

3.2. Cell culture characterization: morphology and vascular smooth muscle cell phenotype

Proliferating cells were obtained from 90% of the internal mammary artery (IMA) and 75% of the carotid artery plaque (PL) tissues. Cell viability, phenotype changes with passaging, and senescence (Supplementary results, Fig. S1C) were evaluated. To improve experimental reproducibility, primary cells at p1 were used in the successive experiments, if not differently indicated.

Protocols based on outgrowing of cells from human vascular wall get to SMC showing "hills and valleys" pattern and spindle shaped morphology but low degree of differentiation [23]. Preliminarily, we referred to our primary cells as vascular smooth muscle cells (VSMC) and the basic compliance of them to SMC was ascertained.

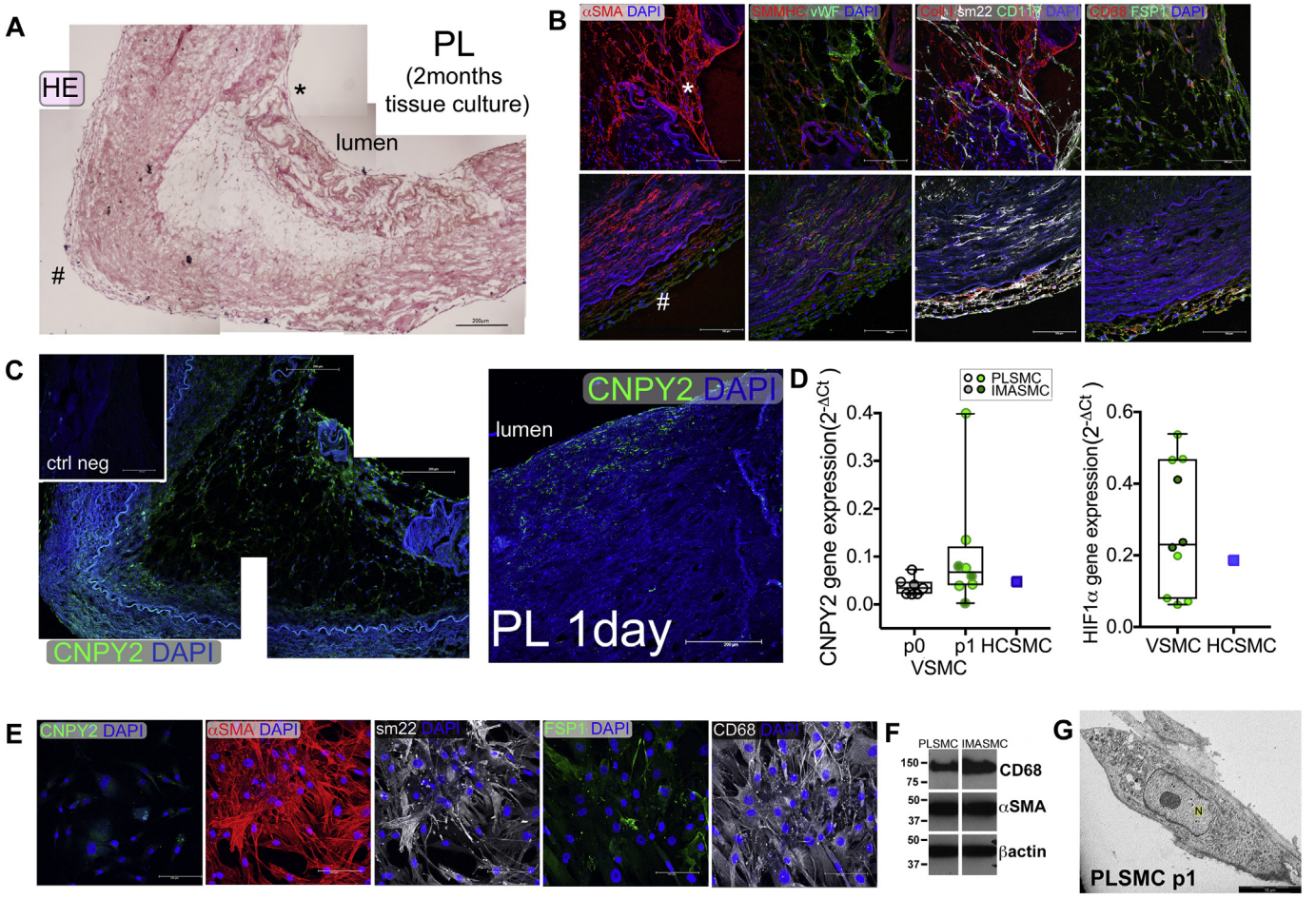

Fig. 1. Characterization of cells outgrowing from PL and IMA tissue rings. Representative montage image from a PL ring cultured for 2 months, stained with Hematoxylin/Eosin is shown (A). Migrating cells characterization is presented in confocal microscopy 2D free projection max images (B). Symbols localize the corresponding luminal (*) and sub-adventitial (#) areas in A and B. Cells in B are stained for αSMA (red), SMMHC (red), vWF (green), Collagen type I (red), sm22 (white), CD117 (green), CD68 (red) and FSP1 (green), nuclei with DAPI (blue). Confocal microscopy from serial section montage of the same PL than in A, labelled for CNPY2 (green) is shown (C, left) displaying outgrowing cells. A negative control for the staining of PL intima is shown (ctrl neg, C left, inset). The presence of CNPY2^{+low} cells (green) in the neointima of another PL cultured for 1 day is displayed (C, right,). CNPY2 (D, left) and HIF1α (D, right) mRNA levels evaluated by real-time PCR in cultured VSMC are plotted ($n \geq 7$). HCSMC are used as control ($n = 1$). Values are presented in boxes with 5–95 percentile and each point represents a single experiment with runs performed in triplicate. Representative confocal microscopy 2D free projection max images from PLSMC labelled for CNPY2 (green), αSMA (red), sm22 (white), FspI (green), and CD68 (white) are presented (E). Nuclei are stained with DAPI (blue). Representative western blot of protein extracts from PLSMC and IMASMC samples showing both αSMA and CD68 is presented (F). Representative transmission electron microscopy image from PLSMC at p1, displaying a spindle cell, is shown (G). Scale bars indicate the magnification.

VSMC demonstrated the in vitro pattern of SMC (Fig. S1A). Spindle shaped cells were more elongated in IMASMC than in PLSMC cultures, where few epithelioid/rhomboid cells were found.

At difference from cells migrating from tissues, VSMC failed to display any cytoplasmic signal for CD117, but displayed several nuclei rich in CD117^{+} dots (Fig. S1D), and expressed both CNPY2 and HIF1α mRNAs (Fig. 1D) in the presence of a barely detectable signal for CNPY2 molecule (Fig. 1E).

Phenotypic analysis of VSMC at confocal microscope demonstrated bright fluorescence for αSMA, sm22, vimentin, collagen type I, laminin and CD68, faint signals for SMMHC and FSP1, and no fluorescence for CD31 (Fig. 1E, S1E). Rare CD45^{+} cells were detected (not shown). No relevant difference between PLSMC and IMASMC was observed. Expression of αSMA and CD68 was confirmed by western blot (Fig. 1F). This pattern indicated the SMC-like nature of VSMC, excluding relevant contamination by endothelial cells, fibroblasts and fibrocytes.

SMC-like structure was confirmed at transmission electron microscope, showing collagen bundles and myofilaments mainly at the cell periphery, no pseudopods nor abundant endoplasmic reticulum (Fig. 1G). Synthetic features as numerous plasmalemmal microvesicles and small irregular vesiculating extrusions were also seen (Fig. S2A–D). Dense vesicles were more abundant in PLSMC than in IMASMC, compatibly with differences in cell metabolism.

The abundance of *exo*-endocytotic vesicles and phagolysosomes was confirmed Ovalbumin and Lysotracker, respectively (Fig. S2E): the non-lipidic composition of vesicles was indicated by OilRedO stain, showing rare cells charged with neutral lipids (Fig. S2F).

No difference either in markers expression or ultrastructural features was appreciated between cells released by the same type of artery from different patients, nor among VSMC from cell populations migrated out of the same tissue at different stages of tissue culture (not shown).

In summary, tissues gave rise to homogeneous populations of VSMC with intermediate phenotype, more committed towards the synthetic than the contractile, i.e. macrophage marker-positive SMC-like. IMASMC and PLSMC slightly differed for organelles composition but not for basic phenotype.

3.3. VSMC model validation: expression of P2X7 IL-1β and MMP9

Our previous study on ex-vivo PL tissue cultures suggested the association of P2X7 to IL-1β/MMP9 in subset of SMC cells expressing sm22

and P2X7 isoform at 54 kD [5]. To ascertain that VSMC represent a suitable model of this subset the expression of P2X7, IL-1β and MMP9 was verified (Fig. 2).

P2X7 and IL-1β mRNAs were lower in VSMC with respect to tissues ($p < 0.001$ for both); MMP9 mRNAs was comparable in VSMC and tissues (Fig. 2A). No differential expression was found between IMASMC and PLSMC (not shown).

At molecular level, P2X7 at 54 kD was broadly detected; the isoform at 100–120 kD found in about 30% of VSMC cultures and that at 75 kD was undetected. Accordingly, cytoplasm localization was always observed, that on the external surface was found in subset of VSMC cultures (Fig. 2B, S3A). Cytoplasmic P2X7 distribution was partially reminiscent of Golgi apparatus pattern, but only contiguity with Golgin-97, residing in the *trans*-Golgi network membrane, was observed (Fig. S3B). Analogously, no co-localization between P2X7 and lysosomes was found (Fig. S3C). Small extra-/intra-cellular vesicles P2X7^{+} were detected (Fig. S3D). IL-1β was barely detectable in VSMC extracts, while present in commercial human carotid SMC (HCSMC) used as control (Fig. 2C).

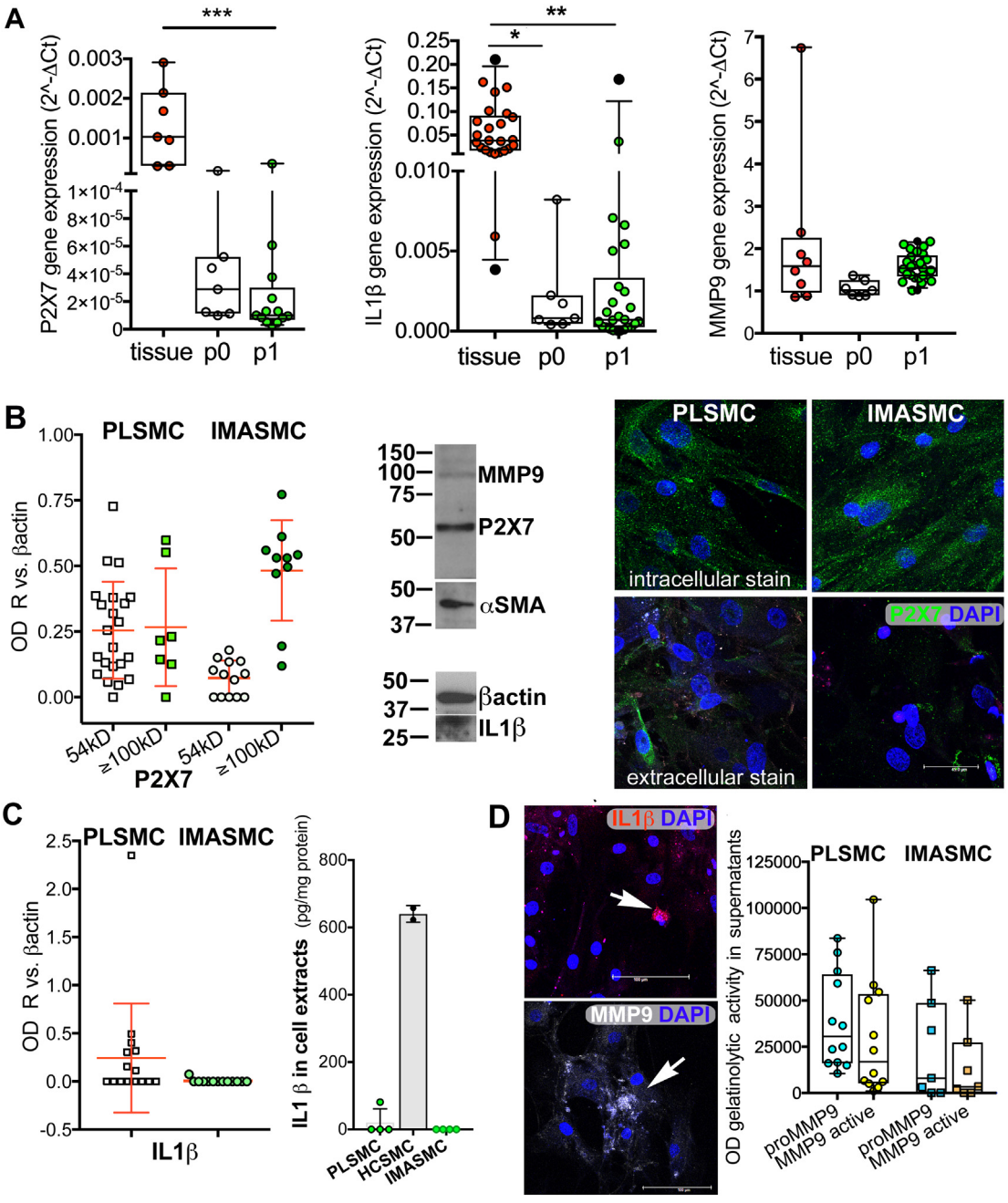

Fig. 2. P2X7 expression in PLSMC and IMASMC. The expression levels of P2X7, IL-1β and MMP9 mRNAs in VSMC at p0 (n = 7 for all) and p1 (n = 13, 26, 25, respectively) vs. tissue extracts (n = 7, 25, 25, respectively) are shown (A). Values are presented in boxes with 5–95 percentile and each point represents a single sample with runs performed in triplicate, (• indicates outlier). Results are analysed by Dunn's multiple comparison test. Densitometry of P2X7 isoforms expression in PLSMC (n = 22) vs. IMASMC (control, n = 13) is presented (B, left). Values are shown in boxes with 5–95 percentile and each point represents a single sample. Representative western blot shows P2X7 at 54 kD, MMP9, αSMA and IL-1β bands (B, middle). Representative confocal microscopy images (B, right) display cellular localization of P2X7 in PLSMC and in IMASMC. Densitometry of IL-1β in PLSMC (n = 17) and IMASMC (n = 13) and cytokine amount in cell supernatants is presented (C). Confocal microscopy images display labelling either for IL-1β (red) or MMP9 (white). Arrows indicate positive cells (D, left). Optical density (OD) analysis of MMP9 gelatinolytic activity in the supernatants of PLSMC (n = 11) and IMASMC (n = 13) is shown (D, right). Values are shown in boxes with 5–95 percentile and each point represents a single sample. Densitometry data are compared by *t*-test. Significant difference are shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. In confocal images nuclei are labelled with DAPI (blue) and scale bars indicate the magnification.

IL-1β fluorescence signal was observed in single cells, and MMP9 granules were focally detected in small clusters of untreated VSMC (Fig. 2D, left). Evaluation of gelatinolytic activity into supernatants indicated a comparable release of MMP9 by PLSMC and IMASMC (Fig. 2D, right).

3.4. VSMC stimulation: effects of hypoxia on IL-1β/MMP9 activation

Following the successful validation, the responsivity of VSMC to hypoxia in terms of IL-1β/MMP9 activation was evaluated.

No up-regulation of IL-1β, NLRP3, MMP9 and P2X7 mRNAs was found in VSMC kept in hypoxia (i.e. cells cultured till 60–70% confluence in normoxia then transferred in 2% O_2, 5% CO_2 hypoxia at for 5 days) with respect to those in normoxia (Fig. 3A, Fig. S4A). No increase in IL-1β or in P2X7 molecules was evident (Fig. 3B Fig. S4B).

Significant down-regulation of HIF1α mRNA ($p = 0.0312$, Fig. 3C) was observed in hypoxia vs. normoxia. CNPY2 mRNA level was unaffected by oxygen supply conditions (Fig. S4C).

3.5. VSMC stimulation: effects of TNFα on IL-1β/MMP9 activation

The TNFα effect on activation of IL-1β/MMP9 was successively investigated.

Treatment with TNFα significantly elicited the expression of both IL-1β and NLRP3 mRNAs ($p = 0.0437$, $p = 0.0340$, respectively) in PLSMC vs. untreated cells (Fig. 3D), and increased pro-IL-1β ($p = 0.0115$) but not affected P2X7 and NLRP3 molecules (Fig. 3E, S6A). Confocal microscope analysis of VSMC treated with TNFα displayed the presence of small cell clusters IL-1β$^+$ (instead of scattered single cells) but no difference in NLRP3 and caspase-1 signals (Fig. 3F).

The occurrence of a rapid processing of pro- IL-1β was tested measuring the IL-1β released by VSMC. The cytokine was undetermined into supernatants from PLSMC and IMASMC treated with TNFα, while secreted IL-1β was measured in supernatants from untreated HCSMC (mean 147.7 ± 8.3 pg/mL), i.e. control cells. No increase in IL-1β amount was assessed in supernatants from HCSMC treated with TNFα (111.2 ± 66.2 pg/mL).

To ascertain whether TNFα led to quantities of mature IL-1β under the detection limit of commercial assays but sufficient for enhancing the release of MMP9, a downstream effect of IL-1β pathway activation, gelatinolytic activity was determined. No differences in pro-MMP9 and active MMP9 gelatinolysis were found in VSMC treated with TNFα vs. untreated (Fig. S4D).

Furthermore, to exclude a bias in the results due to cell death or to effect of the stimuli on phenotype, VSMC were characterized in the presence/absence either of TNFα or hypoxia. The absence of detachment, of relevant phenotype variation and of nuclear fragmentation (Fig. S4E) in the lack of Akt activation (Fig. S4F) demonstrated VSMC survival to TNFα and hypoxia.

To summarize, IMASMC responded to treatments similarly to PLSMC, hypoxia led to down-regulation of HIF1α gene, TNFα elicited

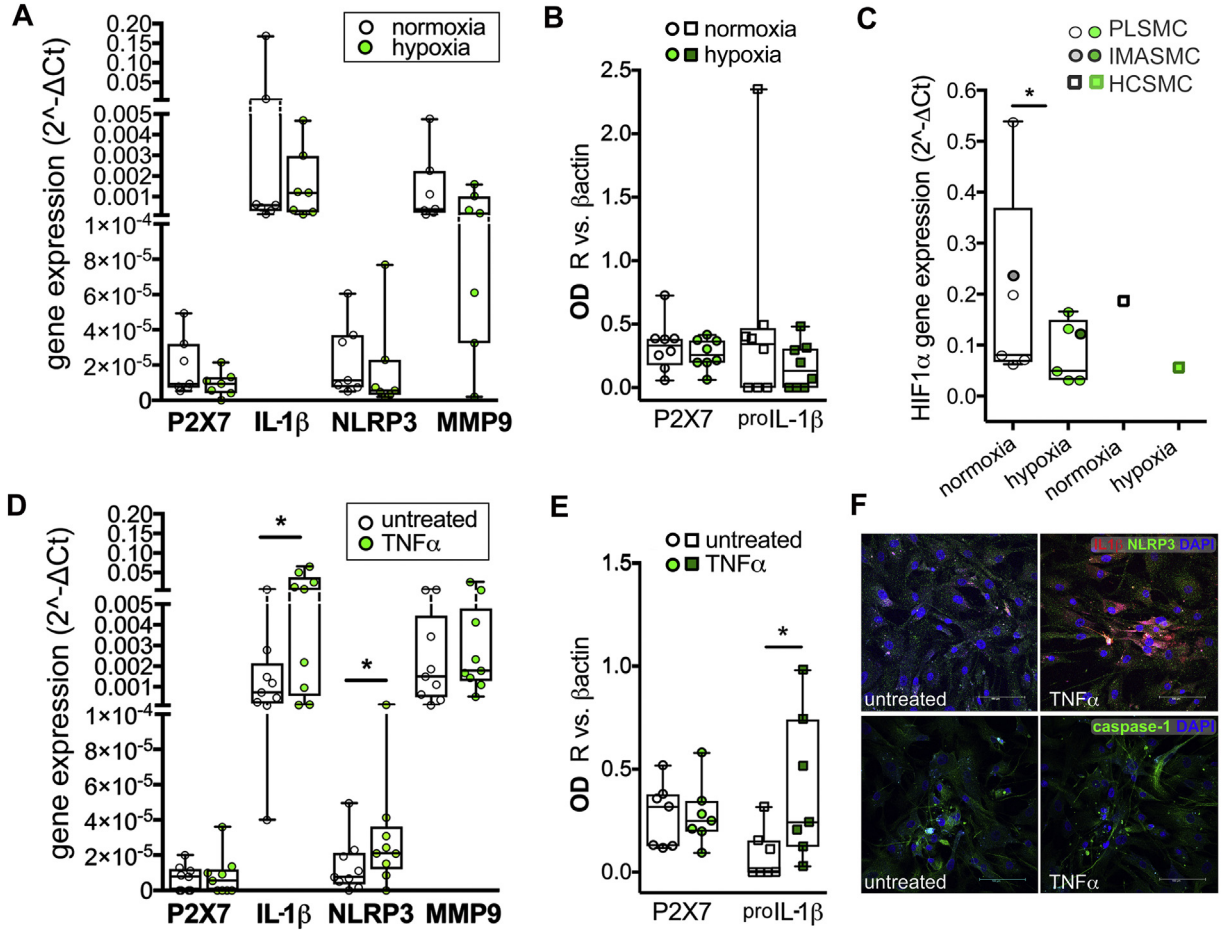

Fig. 3. Effect of atherosclerotic stimuli on VSMC. P2X7, IL-1β, NLRP3 and MMP9 mRNA levels measured by real-time PCR in VSMC challenged with hypoxia (n = 7) vs. those in normoxia (n = 7) are presented (A). Densitometry analysis of P2X7 at 54 kD and of proIL-1β in VSMC in hypoxia (n = 8) and in normoxia (n = 8) is shown (B). HIF1α mRNA level evaluated in VSMC (n = 6) and HCSMC (control cells, n = 1) challenged for 5 days with hypoxia vs. cells in normoxia is plotted (C). P2X7, IL-1β, NLRP3 and MMP9 mRNA levels determination by real-time PCR in VSMC treated with TNFα/untreated (n = 9 for both) is presented (D). Densitometry of P2X7 at 54 kD and of proIL-1β in VSMC treated with TNFα (n = 7) is shown (E). Values are shown in boxes with 5–95 percentile and each point represents a single sample for OD densitometry or single experiment with sample runs performed in triplicate for gene expression. Paired *t*-test was applied. Significant differences are shown as $^*p < 0.05$. Representative confocal microscopy 2D free projection max images from PLSMC treated with TNFα and labelled for IL-1β (red), NLRP3 (green) and caspase-1 (green) are shown (F). Nuclei are stained with DAPI (blue). Scale bars indicate the magnification.

the up-regulation of IL-1β gene translating in immature unsecreted protein.

3.6. VSMC stimulation: effects of P2X7 antagonist on IL-1β/MMP9 activation

IL-1β maturation is vesicle-mediated, and P2X7 regulates multiple types of membrane trafficking responses [24]. Treatment of VSMC with A740003 induced the formation of $P2X7^+$ intracellular aggregates, but not internalization into Golgi or lysosomes, or morphological alterations (Fig. S5A-C). Moreover, A740003 not enhanced IL-1β, P2X7 and MMP9 gene expression in PLSMC (Fig. 4), but slightly increased the release of MMP9 isoforms ($p_{proMMP9} = 0.0244$, $p_{MMP9active} = 0.0020$ vs. untreated cells, Fig. 4B).

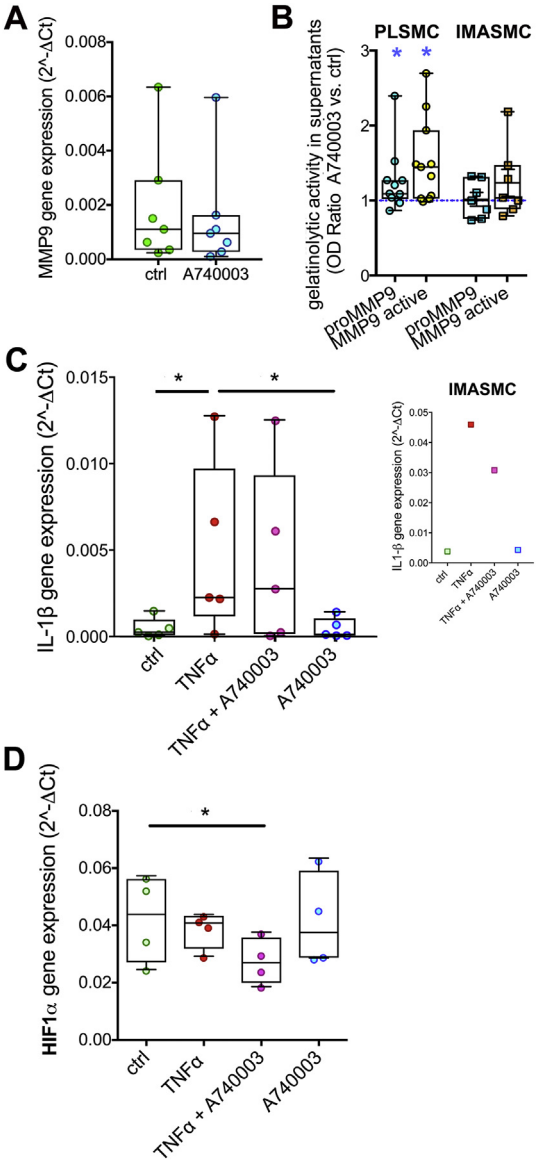

Fig. 4. Effect of TNFα and A740003 on VSMC. Quantifications of MMP9 mRNA level in PLSMC (n = 7) by real-time PCR (A) and of MMP9 gelatinolytic activity in the supernatants of PLSMC (n = 11) and IMASMC (n = 7) treated with A740003 (B) are shown. Wilcoxon signed-ranks test was applied. The levels of IL-1β mRNA by real-time PCR in PLSMC (n = 5) (C) and IMASMC (n = 1) (C, inset) and of HIF1α mRNA in PLSMC (n = 4) (D) are shown. Cells in C and D are treated either with TNFα or A740003 with their combination. Values are shown in boxes with 5–95 percentile and each point represents a single experiment with sample runs performed in triplicate. Significant differences by Friedman test for multiple comparisons are shown as * $p < 0.05$.

3.7. VSMC stimulation: effects of the combination TNFα+A740003

To investigate whether P2X7 antagonism could modulate TNFα effect on IL-1β, subsets of VSMC treated in parallel either with TNFα, or A740003, or A740003 in combination with TNFα (TNFα+A740003), or untreated were compared.

In PLSMC the effect of TNFα+A740003 on IL-1β mRNA was comparable to that exerted by TNFα alone (Fig. 4C). TNFα+A740003 with respect to all the other conditions had no significant effect on the expression of NLRP3, P2X7 and MMP9 mRNAs (Fig. S6B). Preliminary data on IMASMC showed that TNFα+A740003 might rescue IMASMC from TNFα-mediated activation (Fig. 4C inset, Fig. S6B, inset). No variation in IL-1β, NLRP3, P2X7 and caspase-1 molecules was observed in VSMC treated with TNFα alone vs. TNFα+A740003 (Fig. S6A).

Released IL-1β was undetectable into supernatants from PLSMC and IMASMC in the tested conditions. At difference, IL-1β in supernatants from HCSMC treated either with A740003 or TNFα was detected at levels comparable to untreated cells (127.8 ± 1.9 pg/mL, 158.0 ± 5.6 pg/mL, 141.8 ± 3.5 pg/mL, respectively), and TNFα+A740003 increased the cytokine secretion from HCSMC of about 50% (216.2.8 ± 29.0 pg/mL).

These findings underlined the resilience of VSMC levels against stressors and their difference from HCSMC.

At difference from cells treated with A740003, no increase of MMP9 activity in VSMC supernatants followed the treatment with TNFα+A740003 with respect to untreated cells (Fig. S6C).

Moreover, in VSMC treated with TNFα+A740003 but not in those treated either with TNFα or A740003, the expression of HIF1α mRNA decreased with respect to untreated cells (reduction range 24.4%–43.5%, $p = 0.0327$, Fig. 4D).

To explore the effect of A740003 and TNFα on VSMC homeostasis, mitochondrial and lysosomal activities were tested. Mitochondria network displayed more evident filaments in the presence of TNFα alone than in untreated cells, more packaged mitochondria with A740003 (Fig. S6D), and intermediate pattern was found with TNFα+A740003. In VSMC treated either with TNFα or A740003 areas of packaged lysosomes were found, while the lysosome pattern observed with TNFα+A740003 was comparable to that of untreated cells (Fig. S6E).

Experimental findings were unrelated to clinical features of patients.

4. Discussion

We report that $CNPY2^+$ cells may spontaneously escape from areas of loose extracellular matrix of cultured human arteries with/without atherosclerosis, and demonstrate migration/proliferation and dedifferentiation capabilities, important for both physiological wound repair and pathological intima/media invasion [25–27]. Since cell progeny expresses CNPY2 mRNA only, and CNPY2 protein is focally detectable in PL intima, our data not only support the function of HIF1α – regulated CNPY2 as a vascular pro-migratory mediator [14], but hint to a role for CNPY2 in intimal hyperplasia development. Moreover, artery-spreading cells express CD117, retained by several VSMC in the nucleus with a signal reminiscent of speckles, which supports the involvement of c-kit signalling in dedifferentiation of spreading cells [25]. Alongside with the paucity of intravascular proliferation into tissue cultures suggested by poor Ki67, and with expansion of cells at p0, these data suggest a two-step process, i.e. firstly cells migrate outside the native vessel, then start to proliferate. Chemotactic signals might contribute to the process, but their identification overcomes the purpose of our study.

VSMC exhibit an intermediate phenotype, more oriented towards synthetic than contractile SMC, and express the macrophage marker CD68, which is consistent with the phonotypical diversity of SMC [4,28] and the presence of $CD68^{/+}\alpha SMA^+$ cells in atherosclerotic arteries [29]. Macrophages are infrequent in non-grafted IMA [30], likely in relation with physiological athero-resistance [2], but $CD68^+$ cells outgrow

from both arteries. Taken together these findings might support SMC origin for the macrophage marker-positive SMC-like cells identified in human vessels [31], and contribute to shed light on the issue of SMC transdifferentiation in human [12].

In agreement with the theory of a cellular reservoir for the receptor [32] and confirming previous observation [5,33], VSMC mostly express P2X7 at 54 kD, intracellular but targetable by A740003, as suggested by distribution changes. A740003 enhances MMP9 secreted by PLSMC but not by IMASMC supporting the involvement of P2X7 at 54 kD in post-translational modulation of MMP9, and the existence of functional differences between IMASMC and PLSMC. In individuals with atherosclerosis MMP9 is implicated in plaque vulnerability [34] and is associated with risk of myocardial infarction and stroke [35]. However the mechanism of MMP9 regulation is not fully elucidated and the target of individual MMPs still represents a challenge. TNFα, a positive mediator of MMPs regulation and agonist of MMP9 in macrophages [36], in VSMC acts as inhibitor of A740003. These observations might be of potential translational interest in view of MMP-specific therapies.

VSMC resist to TNFα-mediated cell death as macrophages [37], without activating Akt phosphorylation or autophagy as SMC [38]. Brief exposure of SMC to hypoxia has been reported to induce a PI3K-dependent Akt activation leading to induction of HIF1α mRNA [39], and to activate MMP9 [40]. Conversely, chronic hypoxia prolonged the SMC life-span [16,41], but decreases both HIF1α mRNA [42] and secreted MMP9 activity [43]. Similar behaviour is shown by VSMC cultured in 2%O_2 for 5 days to mimic chronic mild hypoxia. Moreover, SMC exposure to hypoxia was reported to increase IL-1β mRNA but not protein [41], and IL-1β transcription was related to HIF1α in lipopolysaccharide-stimulated hypoxic macrophages [44]. Coherently with the down-regulation of HIF1α mRNA, the IL-1β mRNA does not increase in VSMC cultured in hypoxia compared to those in normoxia. In a translational view of these findings, i.e. focusing on atherosclerosis, the down-regulation of HIF1α mRNA could represent a protective mechanism exerted by VSMC against chronic hypoxia.

VSMC are responsive to pro-inflammatory stimulation with TNFα, as both IL-1β and NLRP3 mRNA expression levels increased. The stimulation with IL-1β increased the production of TNFα by bovine aortic SMC [45]. Our findings extend to human VSMC the occurrence of the inverse phenomenon, previously reported in piglet coronary SMC [46]. The power of TNFα activation being partial as not increasing mature IL-1β, thus suggests that further stimuli are required for maturation/secretion. Moreover the ineffectiveness of A740003 in modulating the TNFα-mediated effect indicates that P2X7 is not involved in the first phases of IL-1β pathway modulation in PLSMC. This does not exclude that P2X7 plays a role in the cytokine secretion, and the detected differences in lysosome aggregation are compatible with an antagonism between TNFα and A740003, in the presence of minor changes in mitochondrial network. Due to proatherogenic consequences of lysosomal leakage [47] our findings hint to further studies on the intracellular function of P2X7, in relationship with lysosome activity [48] and atherosclerosis.

4.1. Conclusions and translational perspectives

Our study proposes the spontaneous outgrow of cells from human arteries as a two-step process involving in the migratory phase CNPY2, and leading to homogeneous population of VSMC with intermediate phenotype, analogous to cells within the intima of atherosclerotic lesions [11].

Our data propose a HIF1α/P2X7-independent role for the partial activation of IL-1β/NLRP3 pathway exerted by TNFα in VSMC. Although expanding the pleiotropic effects of TNFα, our results suggest that this factor is unable to exert maturation of IL-1β by VSMC, thus underlining the complexity of the cytokine secretion mechanisms.

In PL both macrophages and SMC bordering the necrotic core express HIF1α and its increase may contribute to atherosclerosis through alteration of SMC proliferation and migration, angiogenesis and lipid metabolism. The maintenance of a low HIF1α mRNA level independently from the applied stimuli could be envisaged as a protective property of VSMC with intermediate phenotype towards vascular alteration–related deleterious effects [49].

Finally, our results provide insights on the modulation of MMP9 exerted by A740003 and TNFα+A740003, which in the perspective of designing selective and clinically useful therapies against vascular negative remodelling warrant further studies.

4.2. Study limitations

VSMC have the advantage of being phenotypically homogenous, but the disadvantage to be contemporary available in limited number, which hindered an extensive experimental lineage campaign by cytofluorimetry, and the cell transfection/gene silencing by siRNAs for further mechanistic studies.

Although in our best knowledge non-P2X7-specific effects of A740003 have never been described, we cannot exclude this possibility.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.12.047.

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