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ORIGINAL ARTICLE

Vascular smooth muscle cell phenotype is modulated by ligands of the lymphotoxin β receptor and the tumor necrosis factor receptor

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KEYWORDS
Vascular smooth muscle cells;
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Abstract
Objective: Vascular smooth muscle cells (VSMCs) undergo a phenotypic-switching process during the generation of unstable atheroma plaques. In this investigation, the potential implication of the tumor necrosis factor superfamily (TNFSF) ligands, in the gene expression signature associated with VSMC plasticity was studied.
Material and methods: Human aortic (ha)VSMCs were obtained commercially and treated with the cytokine TNFSF14, also called LIGHT, the lymphotoxin alpha (LTα), the heterotrimer $LT\alpha_1\beta_2$ or with vehicle for 72 h. The effect of the different treatments on gene expression was analyzed by quantitative PCR and included the study of genes associated with myofibroblast-like cell function, osteochondrogenesis, pluripotency, lymphorganogenesis and macrophage-like cell function.
Results: HaVSMCs displayed a change in myofibroblast-like cell genes which consisted in reduced *COL1A1* and *TGFB1* mRNA levels when treated with LTα or LIGHT and with augmented *MMP9* expression levels when treated with LTα. LTα and LIGHT treatments also diminished the expression of genes associated with osteochondrogenesis and pluripotency *SOX9*, *CKIT*, and *KLF4*. By

Abbreviations: DcR, Decoy receptor 3; HVEM, herpes virus entry mediator; JNK, c-Jun N-terminal Kinase; LT, lymphotoxin; LTβR, lymphotoxin beta receptor; NFkB, nuclear factor kappa B; Th, T helper; TNFR, tumor necrosis factor receptor; TNFSF, tumor necrosis factor superfamily; VSMC, vascular smooth muscle cell.

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contrary, all the above genes were no affected by the treatment with the trimer $LT\alpha_1\beta_2$. In addition, haVSMC treatment with LTα, $LT\alpha_1\beta_2$ and LIGHT altered lymphorganogenic cytokine gene expression which consisted of augmented *CCL20* and *CCL21* mRNA levels by LTα and a reduction in the gene expression of *CCL21* and *CXCL13* by LIGHT and $LT\alpha_1\beta_2$ respectively. Neither, LTα or LIGHT or $LT\alpha_1\beta_2$ treatments affected the expression of macrophage-like cell markers in haVSMC.
Conclusions: Altogether, indicates that the TNFSF ligands through their interconnected network of signaling, are important in the preservation of VSMC identity against the acquisition of a genetic expression signature compatible with functional cellular plasticity.

PALABRAS CLAVE
Células musculares lisas vasculares;
Inflamación;
Linfotoxina;
Plasticidad celular

El fenotipo de las células de músculo liso vascular es modulado por los ligandos del receptor de la linfotoxina β y del receptor del factor de necrosis tumoral

Resumen
Objetivo: La transición de placa de ateroma estable a placa inestable implica, entre otros procesos, un cambio fenotípico de las células del músculo liso vascular (CMLVs). En esta investigación, se estudió el posible papel de los ligandos de la superfamilia del factor de necrosis tumoral (TNFSF), en los cambios de expresión génica asociada a la plasticidad de las CMLVs.
Materiales y métodos: Las CMLVs de aorta humana (CMLVah) se obtuvieron comercialmente y se trataron con la citoquina TNFSF14, también llamada LIGHT, la linfotoxina alfa (LTα), el heterotrímero LTα1β2 o con vehículo durante 72 horas. El efecto de los diferentes tratamientos se analizó mediante el estudio de la expresión génica por PCR cuantitativa e incluyó genes asociados con fenotipo miofibroblástico, osteocondrogénico, genes de pluripotencia, genes de linforganogénesis y genes característicos de macrófagos.
Resultados: El estudio de genes asociados a fenotipo miofibroblástico en las CMLVah reveló una reducción de la expresión génica de *COL1A1* y *TGFB1* tras el tratamiento con LTα o LIGHT mientras que el tratamiento con LTα aumentó los niveles de mRNA de *MMP9*. LTα y LIGHT también disminuyeron la expresión de genes de osteocondrogénesis y pluripotencia como *SOX9*, *CKIT* y *KLF4*. Por el contrario, la expresión de los genes anteriores no se vio afectada por el tratamiento con el trímero LTα1β2. El tratamiento de las CMLVah con LTα, LTα1β2 y LIGHT alteró la expresión génica de citoquinas linforganogénicas con una expresión aumentada de los genes *CCL20* y *CCL21* por LTα y una reducción de los niveles de mRNA de *CCL21* y *CXCL13* por LIGHT y $LT\alpha_1\beta_2$, respectivamente. Ninguno de los tres tratamientos alteró la expresión de genes típicos de macrófagos en las CMLVah.
Conclusiones: La presente investigación indica que los ligandos de la familia de los TNFSF a través de su red de señalización, son importantes en la preservación de la identidad de las CMLVs frente a la adquisición de una expresión génica compatible con una mayor plasticidad celular funcional.

Introduction

Cardiovascular disease (CVD) is a clinical manifestation of the atherosclerotic process, currently considered both a metabolic and a chronic inflammatory disease with an active participation of the innate and adaptive immune system.[1] Atherosclerosis lesion represents a profound vascular vessel wall remodeling. The lesion initiates with an endothelial dysfunction which progressively leads to the formation of fibroatheromas with an inflammatory core[2] containing lipid-loaded macrophages and different T helper (Th) and regulatory T (Treg) cells.[3] During the progression of the disease, vascular smooth muscle cells (VSMCs) become migratory and proliferative and undergo a phenotypic-switching process by acquiring characteristics of different cell-types such as myofibroblasts, mesenchymatic cells, macrophages, lymphoid organ organizer cells or osteochondrogenic cells.[4] Studies have shown an important role of the cross-talk between the plaque-stress factors and the functional plasticity of VSMC in atheroma instability and acute coronary syndromes.[5] Therefore, it is of relevance to understand potential

inflammatory mediators that might affect VSMC lesional heterogeneity.

The lymphotoxins (LT) and the tumor necrosis factor superfamily 14 (TNFSF14), also called LIGHT, belong to the TNFSF and constitute with their receptors an important interconnected network signaling in immune homeostasis.[6,7] LIGHT is mainly produced by immune cells and mediates its effects through two receptors, the LTβR and the herpes virus entry mediator (HVEM) while its activity is inhibited by the Decoy receptor 3 (DcR3). LIGHT-signaling through LTβR, a receptor mostly expressed in stromal and epithelial cells, activates both canonical and non-canonical nuclear factor kappa B (NFkB) pathways, exerting important functions in immune response and lymphorganogenesis.[6,7] Through HVEM signaling, a receptor characteristic of T and B cells, LIGHT promotes non-canonical NFKB and c-Jun N-terminal Kinase (JNK) pathways increasing cytokine production, cell survival, and proliferation.[8] The LIGHT/LTβR-HVEM axis becomes more complex due to interactions with the other LT. Thus, LTα exerts cytotoxic effects and forms the homotrimer $LT\alpha_3$ that binds to TNF receptor 1 (TNFR1) and TNFR2. The homotrimer $LT\alpha_3$ can also bind, with low affinity, to HVEM.[6] In addition, LTα and LTβ form the $LT\alpha_1\beta_2$ heterotrimer which is produced by lymphocytic cells, and is essential, through the LTβR/NFKB non-canonical pathway, in lymphoid tissue organogenesis during development.[7] In adulthood, the $LT\alpha_1\beta_2$/LTβR/NFKB interaction is important in immune responses against pathogenic insults. Hence LIGHT, $LT\alpha_3$ and $LT\alpha_1\beta_2$ compete for the same receptors and therefore their actions will depend on their relative abundance in the circulation and within tissues.

It is not therefore surprising that the study of LIGHT and LT signaling through the LTβR/HVEM-dependent pathways in metabolic diseases have yielded discrepant results. Thus, hepatic T cell production of LIGHT in mouse models induces hypercholesterolemia by modulating hepatic enzymes[9] and *Light* gene inactivation alleviates insulin resistance, steatosis and hepatic inflammation.[10] On the other hand, T60N variant of *LYMPHOTOXIN ALPHA* gene has been associated with type 2 diabetes and other features of the metabolic syndrome.[11] However, its deficiency does not affect obesity or insulin resistance in mouse models.[12]

In CVD, discrepant results have been reported as well. LIGHT levels are elevated in coronary disease,[13] clinical heart failure[14] and unstable angina,[15] while a soluble form of LTβR has been observed in human atherosclerosis.[16] In the atherosclerotic *Apolipoprotein e*-deficient (*Apoe*-/-) mice, macrophage specific deletion of *Ltβr* reduced atherosclerosis by augmenting the proresolving $Ly6C^{low}$ monocytes.[17] Consistently, soluble LIGHT acute treatment enhanced proliferant $Ly6C^{hi}$-monocytes and aggravated atherosclerosis.[18] Notwithstanding, in another study, *Ltβr* specific deletion in VSMCs in *Apoe*-/- mice accelerated atherosclerosis indicating atheroprotection in a LTβR-dependent manner which was attributed to T-cell homeostasis induced by a proper functionality of the artery lymphoid organs.[19] Therefore, these studies unveiled a complex role of the TNFSF ligands in atherosclerosis. Notably, genetic inactivation of *Light* aggravated abdominal aneurysm vascular lesions.[20] Specifically, *in vivo* and *in vitro* data indicated that LIGHT/LTβR-signaling disruption provoked dysregulated *SOX9*, *OPN* and *BMP2* gene expression compatible with an osteochondrogenic phenotype which has been previously associated with vascular dysfunction.[21] These results suggest a protective function of LIGHT/LTβR-signaling in vascular injury through the prevention of osteochondrogenic phenotype acquisition.

Given the multiple interactions and connections between the TNFSF ligands and their receptors, in the present investigation we sought to investigate the potential role of the LTα, LIGHT and the $LT\alpha_1\beta_2$ heterotrimer in the VSMC phenotype.

Materials and methods

Human aortic VSMC cell culture experiments

Human aortic (ha)VSMCs were commercially obtained (Invitrogen, C-007-5C, Thermofisher Scientific, Madrid, Spain) and cultured in 231 medium, 20% FBS, 5% of smooth muscle growth factor (Invitrogen, S00725 Thermofisher Scientific) and 2% P/S/A (Lonza, Basel, Switzerland) as described.[20,22] HaVSMCs were kept on a humidified 5% CO_2 atmosphere until treatments and were used until passage 6–7. For expression experiments haVSMC were grown in 20% FBS/DMEM-P/S/A medium on 6-well plates up to 70–80% of confluency and then treated for 72 h in 0.5% FBS/DMEM-P/S/A medium with vehicle, human soluble LIGHT (20 ng/ml, Preprotech, Germany, Hamburg), LTα (at 5 and 10 ng/ml Preprotech, Germany, Hamburg) or with $LT\alpha_1\beta_2$ (at 20 and 100 ng/ml, R&D systems Biotechne, Minneapolis, Minnesota, USA). For protein analysis of the receptors cells were grown as before until confluency and then treated for 24 h in 0.5% FBS/DMEM-P/S/A medium with vehicle or human soluble LIGHT (50 ng/ml). After treatments, cells were rinsed with PBS 1×, collected by centrifugation (500 × *g*, 10 min) snap-frozen with liquid N_2 and stored for gene expression analysis by qPCR.

Gene expression analysis by quantitative real-time PCR (qPCR)

Total RNA was obtained from cultured haVSMCs treated as indicated above using TRIzol reagent following manufacturer recomendations (Invitrogen, Carlsbad, CA, USA). A total of 500 ng of RNA were reverse transcribed with the Maxima First-Strand kit (Fermentas, Waltham, MA, USA). The genes of interest were amplified with Luminaris Color HiGreen High ROX qPCR Master Mix (Fermentas, Waltham, MA, USA) on a 7900 FastSystem thermal cycler and results were analyzed with the formula $2^{-\Delta\Delta Ct}$. mRNA levels were normalized to the GAPDH mRNA levels and relativized to vehicle-treated cells. The primer sequences were obtained from the PrimerBank data base (Massachusetts General Hospital, Harvard University) and are listed in Table 1.

Western Blot analysis

Protein extracts were obtained by homogenization of haVSMCs in the presence of ice-cold lysis TNG buffer (Tris–HCl 50 mM, pH 7.5, NaCl 200 mM, Tween-20 1% vol/vol, NP-40 0.2% vol/vol) supplemented with Complete Mini cocktail, PhosSTOP (Roche, Mannheim, Germany),

Table 1 Sequences of primers used for qPCR expression studies.

Gen	Forward primer	Reverse primer
ACTA2	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC
CCL20	TGCTGTACCAAGAGTTTGCTC	CGCACACAGACAACTTTTTCTTT
CCL21	GTTGCCTCAAGTACAGCCAAA	AGAACAGGATAGCTGGGATGG
CD14	CGCTCCGACATGCATGTG	AGCCCAGCGAACGACAGA
CD68	GGAAATGCCACGGTTCATCCA	TGGGGTTCAGTACAGAGATGC
CD80	AGGGAACATCACCATCCAAG	TGCCAGTAGATGCGACTTTG
CD163	GCTGCATGAATTGCACAGATAT	CGGGATGAGCGACCTGTT
CD206	CGCTACTAGGCAATGCCAATG	TCGTGCAATCTGCGTACCA
COL1A1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
CXCL13	GCTTGAGGTGTAGATGTGTCC	CCCACGGGGCAAGATTTGAA
CXCL16	CCCGCCATCGGTTCAGTTC	CCCCGAGTAAGCATGTCCAC
GAPDH	TGTGGGCATCAATGGATTTGG	ACACCATGTATTCCGGGTCAAT
IFNG	CCAACGCAAGCAATACATGA	TTTTCGCTTCCCTGTTTTAGCT
IL6	CCAGGAGCCCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
KIT	CGTTCTGCTCCTACTGCTTCG	CCCACGCGGACTATTAAGTCT
KLF4	CCCACATGAAGCGACTTCCC	CAGGTCCAGGAGATCGTTGAA
KLF10	GCAACAAGTGTGATTCGTCATAC	CAGCCTCAACATTTAGGTGGG
MCP1	CTCGCCTCCAGCATGAAAG	GGGAATGAGGGTGGCTGCTA
MMP9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
MMP2	TACAGGATCATTGGCTACACACC	GGTCACATCGCTCCAGACT
OCT4	CTGGGTTGATCCTCGGACCT	CCATCGGAGTTGCTCTCCA
SOX9	AGCGAACGCACATCAAGAC	CTGTAGGCGATCTGTTGGGG
TGFB1	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC

beta-glycerophosphate 50 mM (Sigma), 2 mM phenylmethylsulfonyl fluoride (PMSF, Roche) and 200 μM Na_3VO_4 (Sigma). For protein analysis, protein extracts (25–50 μg) were prepared with Laemmli buffer (5 min 95 °C) and subjected to 12% w/v polyacrylamide gel electrophoresis and western blot as described.[10] The following primary (1/200) and secondary (1/2000) antibodies were used to detect the proteins: HVEM (PA5-20237, ThermoFisher), LTβR (ab70063, Abcam) and β-actin (Sigma), anti-mouse IgG-HRP (P0447, Dako) and goat anti-rabbit IgG-HRP (P0448, Dako). The immunocomplexes were detected with an ECL Plus detection kit (ThermoFisher). All antibodies for western blot were acquired and used after checking that validation was performed by the manufacturer company.

Statistical analysis

Quantitative data are presented as the mean ± the standard error of the mean (SEM) and with the single data points. All samples were randomly treated and analyzed by observers blinded to treatments. Treatments and collection of data were obtained at the same time and order to avoid confounding effects. The exclusion criteria were applied when data was out of range of the standard curve in each experiment, when samples were lost during the experimentation and when the (non-iterative) Grubbs test identified outliers. Statistical tests were applied after the determination of normal distribution (Shapiro–Wilk and D'Agostino–Pearson normality tests) and equality of variances (*F* test). Differences were evaluated with unpaired Student's t test, Mann–Whitney *U* test (for nonparametric distribution) and one-way ANOVA followed by Bonferroni multiple comparison test (more than two groups). All statistical tests were run in GraphPad Prism 9.0.0 (GraphPad Prism Software, La Jolla, CA, USA). Differences were considered statistically significant when *p*-values were below 0.05: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Results

Aortic human vascular smooth muscle cells express LTβR and HVEM

LTβR is expressed mainly in stromal and epithelial cells while HVEM is mainly found in immune cells. However, the expression of LTβR and HVEM in liver and adipose tissue[10] and the expression of *LTBR* gene in haVSMC[20] has been previously described. To assess whether the TNFSF ligands can signal in haVSMCs protein analysis was performed. Analysis of both LTβR and HVEM receptors in vehicle- and LIGHT-treated haVSMC indicated the presence of both receptors in these cells with similar levels regardless of the treatment (Fig. 1A, B).

LTα and LIGHT but not LTα1β2 decrease myofibroblast markers in aortic human vascular smooth muscle cells

The potential effect of the LTs and LIGHT in haVSMC phenotype switching was explored by using the expression of different markers for myofibroblast-like cell. Analysis of myofibroblast-like cell genes showed reduced expression of *COL1A1* and *TGFB1* mRNA levels in haVSMCs treated with LTα

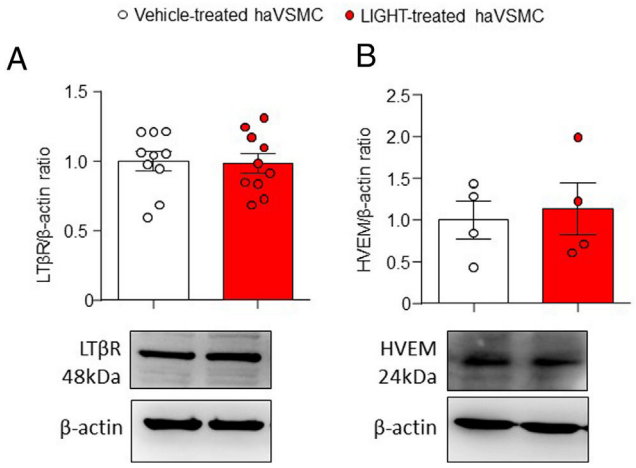

Figure 1 Analysis of the LTβR and HVEM protein content in haVSMC. Protein quantification in the western blot analysis displayed as (A) LTβR/β-actin and (B) HVEM/β-actin ratios in haVSMC treated with vehicle or LIGHT 20 ng/μl overnight. Representative blots are shown for the western blot analysis. Statistical analysis were performed by the Student's *t*-test.

at 10 ng/ml compared with vehicle-treated cells (Fig. 2B, C). HaVSMCs treated with LTα at a lower dose of 5 ng/ml also displayed reduced mRNA levels of *TGFB1* and enhanced expression of *MMP9* compared with vehicle-treated haVSMCs (Fig. 2C, E). No changes were observed in the expression of *ACTA2* or in *MMP2* genes (Fig. 1A, D). Likewise, haVSMCs treated with LIGHT displayed diminished *COL1A1* and *TGFB1* mRNA gene expression (Fig. 2B, C) with no changes in *ACTA2*, *MMP2* or *MMP9* (Fig. 2A, D, E). Treatment of haVSMCs with the trimer $LT\alpha_1\beta_2$ did not affect *ACTA2*, *COL1A1*, *TGFB1* and *MMP9* mRNA levels at any of the two doses tested (20 or 100 ng/ml) (Fig. 3A–D).

Aortic human vascular smooth muscle cells treated with LTα and LIGHT but not display decreased expression of CKIT and KLF4 genes

Next, we evaluated a possible effect of LIGHT and LT signaling in genes related with pluripotency and osteochondrogenic phenotypes. Treatment of haVSMCs with LTα at 10 ng/ml, but not with LIGHT, significantly decreased the expression of *SOX9* (Fig. 4A). Reduced mRNA levels of *CKIT* and *KLF4* (Fig. 4B, C) were also observed in haVSMC treated with LIGHT and with LTα at the both 5 and 10 ng/ml doses. Neither LIGHT or LTα affected the gene expression of *OCT4* or *KLF10* (Fig. 4D, E). Similarly, compared with vehicle-treated controls, haVSMCs treated with $LT\alpha_1\beta_2$ did not display altered mRNA levels of *SOX9*, *CKIT*, *OCT4*, *KLF4* or *KLF10* (Fig. 4F–J).

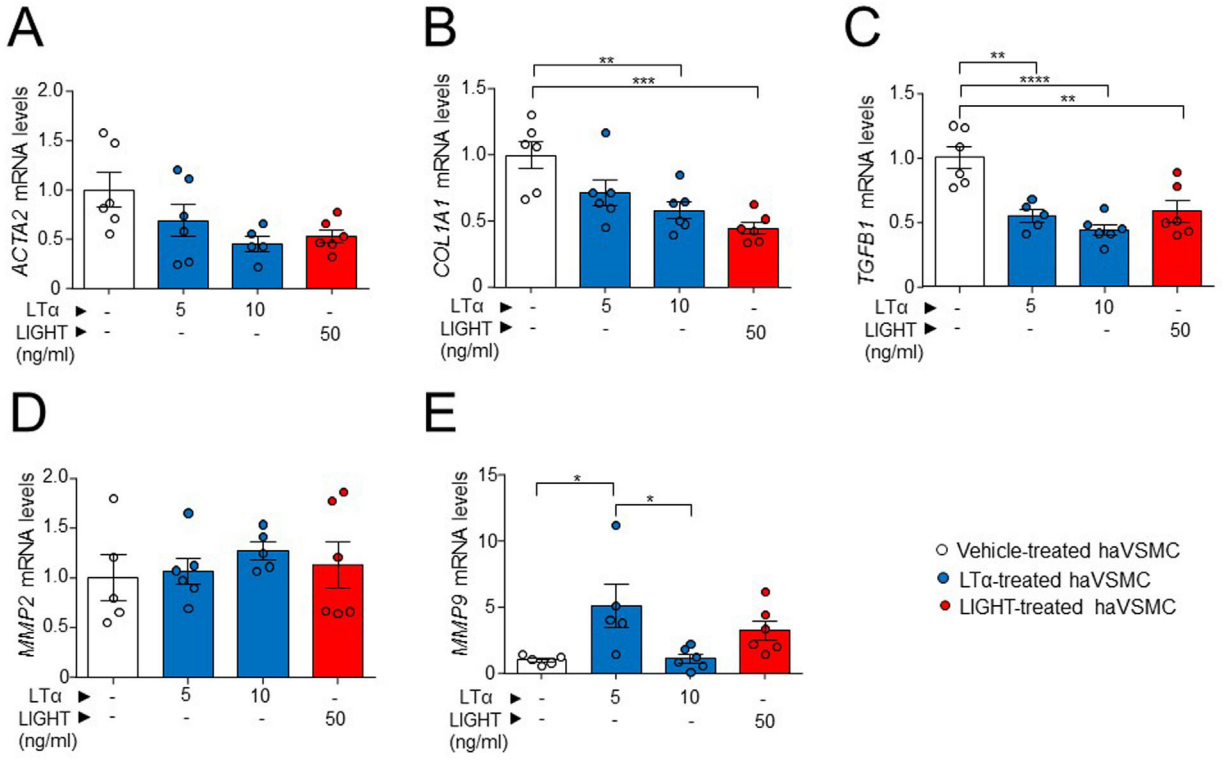

Figure 2 Expression analysis of genes related to the acquisition of haVSMC contractile and secretory phenotype. Relative expression levels of (A) *ACTA2*, (B) *COL1A1*, (C) *TGFB1*, (D) *MMP2* and (E) *MMP9* in ahVSMC treated with vehicle, LTα (5 ng/ml), LTα (10 ng/ml) and LIGHT (50 ng/ml). mRNA levels were normalized with the endogenous gene levels and relativized to the vehicle-treated VSMC mRNA levels. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

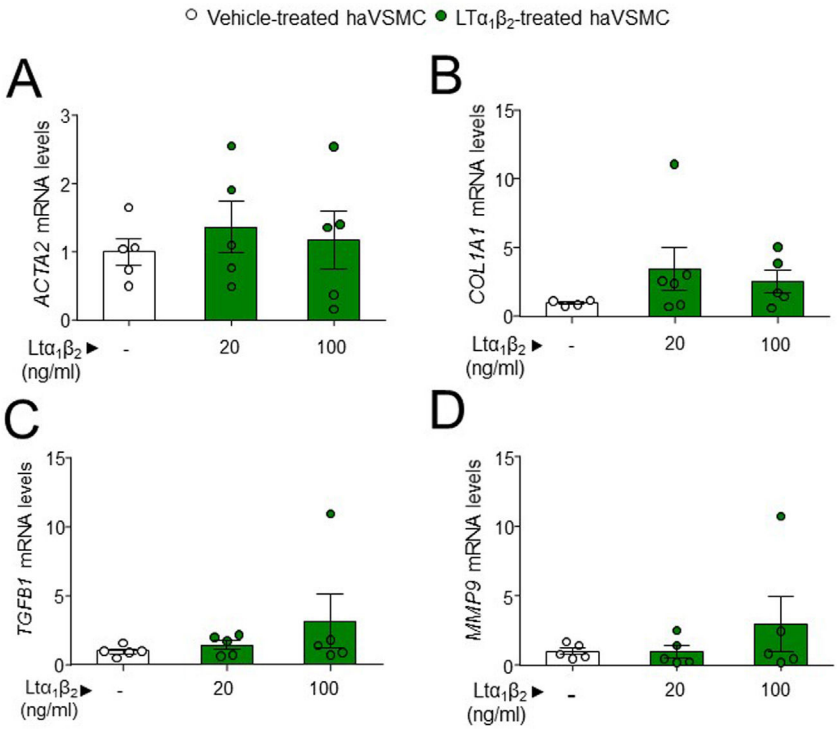

Figure 3 Expression analysis in haVSMC of genes associated to the acquisition of contractile and secretory phenotype. Relative expression levels of (A) *ACTA2*, (B) *COL1A1*, (C) *TGFB1* and (D) *MMP2* in haVSMC treated with vehicle, $LT\alpha_1\beta_2$ (20 ng/ml) and $LT\alpha_1\beta_2$ (100 ng/ml). mRNA levels were normalized with the levels of the endogenous gene and relativized to the vehicle-treated VSMC mRNA levels. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test.

Aortic human vascular smooth muscle cells treated with LTα, LIGHT and $LT\alpha_1\beta_2$ modulate the expression of lymphorganogenic cytokines

One of the described effects of LTβR-dependent signaling in VSMCs is the induction of a cellular phenotype secretor of lymphorganogenic cytokines, hence the production of these were studied. The analysis of haVSMC treated with LTα revealed an increase in the gene expression of *CCL20* and *CXCL16* at the 10 ng/ml dose (Fig. 5A, D) and of *CCL20* at 5 ng/ml dose (Fig. 5A) compared with vehicle-treated VSMCs while the mRNA levels of *CCL21* were unaffected by LTα (Fig. 5C). Unlike LTα, LIGHT did not modify the gene expression of *CCL20* and *CXCL16* (Fig. 5A, D) and surprisingly diminished *CCL21* mRNA levels (Fig. 5B). The mRNA levels of *CXCL13* were unaffected by either LTα or LIGHT treatment (Fig. 5C). Interestingly, the treatment of haVSMCs with the trimer $LT\alpha_1\beta_2$, at both 20 and 100 ng/ml doses, decreased the expression of the *CXCL13* gene (Fig. 5G) while no changes were observed in the other cytokines (Fig. 5E, F, H).

LTα and LIGHT dependent signaling does not affect the expression of macrophage markers in aortic human vascular smooth muscle cells

Previous studies have shown a modulation of macrophage-like cell phenotype through upregulation of *KLF4*.[23] Given the lower *KLF4* mRNA levels observed in haVSMCs treated with LIGHT and with LTα, the expression of macrophage gene markers were also investigated. Although the treatments with both LIGHT and LTα slightly increased the gene expression of the surface membrane markers of macrophage subtypes including *CD14*, *CD80*, *CD206* and *CD183* (Fig. 6A–D) and of phagocytic activity, *CD68* (Fig. 6E) these were no significantly altered. Likewise, no differences were observed in the expression of proinflammatory cytokine genes typically expressed by macrophages, such as *MCP1*, *IL6* and *IFNG*, between vehicle- and LTα- or LIGHT-treated haVSMCs (Fig. 6F–H).

Discussion

Atheroma plaque instability is strongly affected by VSMC functional transdifferentiation[5,22] in which different risk factors and secreted plaque mediators have been importantly implicated.[24] In the present investigation, the potential effect of TNFSF ligands in the modulation of VSMC gene expression associated with functional heterogeneity was explored. Consistent with a preservation of VSMC identity, the treatment of VSMCs with LTα or LIGHT diminished *COL1A1* and *TGFB1* mRNA levels, LTα incubation augmented the expression of *MMP9*, without changing *ACTA2* mRNA levels. Likewise, genes associated with osteochondrogenesis, pluripotency and transdifferentiation such as *SOX9*, *CKIT*, and *KLF4* were also repressed by LTα and LIGHT treatments. Notably, all the above genes were not affected by the treatment with the $LT\alpha_1\beta_2$ trimer. VSMC treatment with all three ligands, LTα, $LT\alpha_1\beta_2$ and LIGHT, altered the expression of well-reported lymphorganogenic cytokines[6,25]

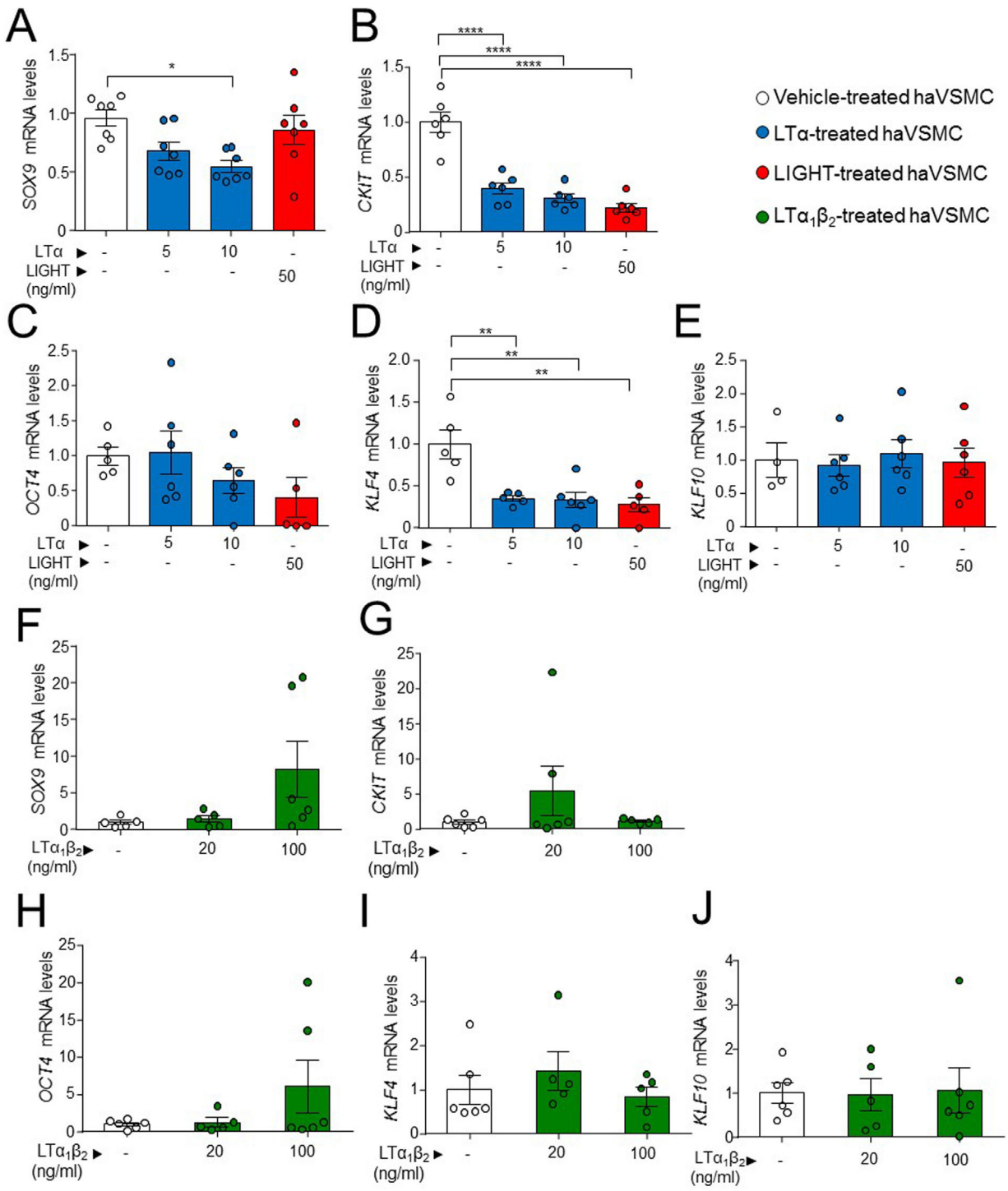

Figure 4 Expression analysis of genes of pluripotency and osteochondrogenesis in haVSMC. Relative expression mRNA levels of (A, F) *SOX9*, (B, G) *CKIT*, (C, H *OCT4*, (D, I) *KLF4* and (E, J) *KLF10* in haVSMC treated with vehicle, LTα (5 ng/ml), LTα (10 ng/ml) and LIGHT (50 ng/ml) (A–) and with vehicle, $LT\alpha_1\beta_2$ (20 ng/ml) and $LT\alpha_1\beta_2$ (100 ng/ml) (F–J). mRNA levels were normalized with the levels of the endogenous gene and relativized to the vehicle-treated VSMC mRNA levels. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$;****$p < 0.0001$.

which included augmented *CCL20* and *CCL21* gene expression by LTα as well as diminished *CCL21* and *CXCL13* by LIGHT and $LT\alpha_1\beta_2$, respectively. Altogether, indicates a role of the TNFSF ligands through their interconnected network of signaling, in the preservation of VSMC identity against the acquisition of a genetic expression signature compatible with a functional transdifferentiation process.

Studies have shown an important role of the cross-talk between the plaque-stress factors and the functional plasticity of VSMC. Thus, VSMC functionality loss consisting

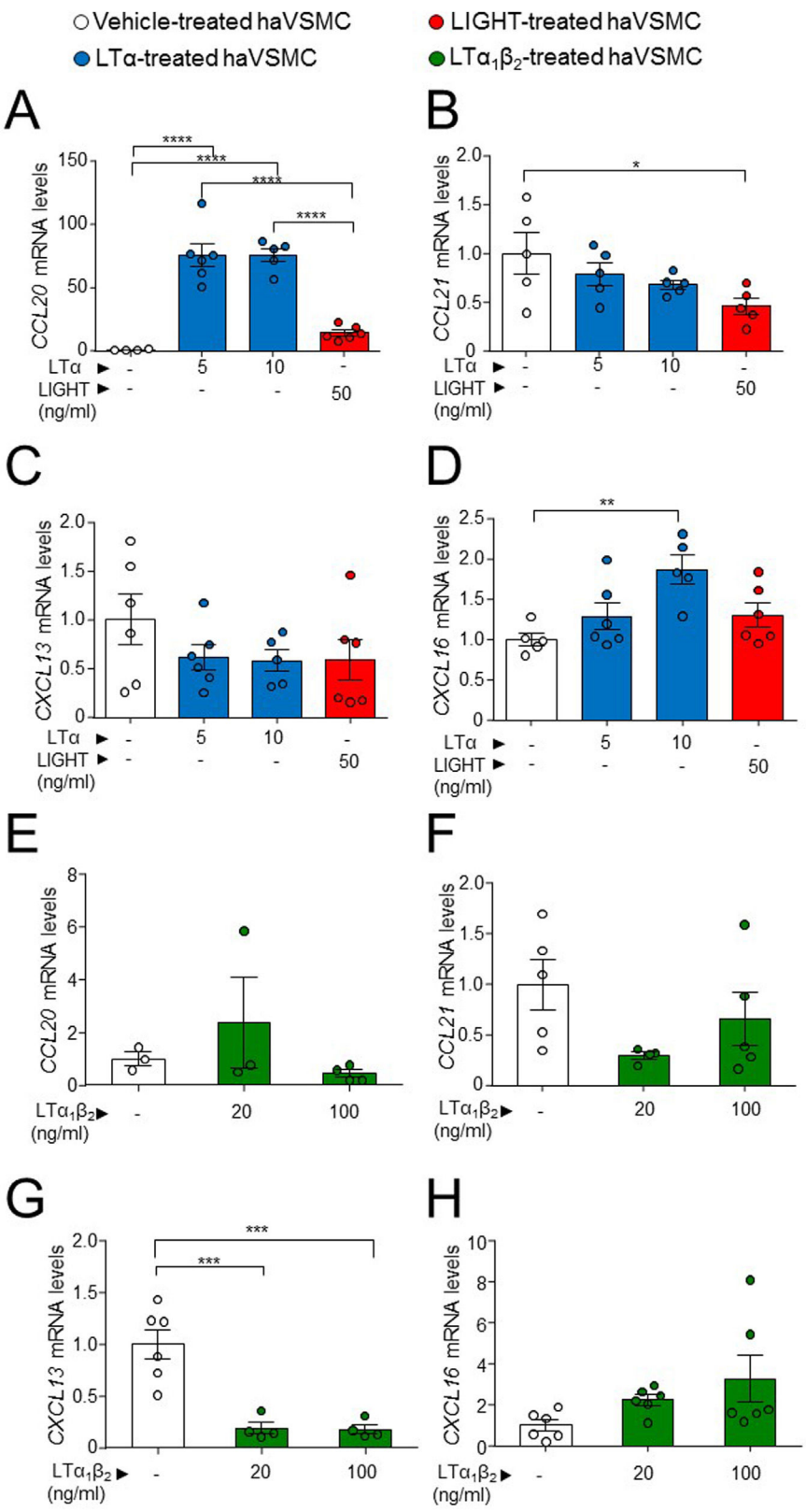

Figure 5 Gene expression analysis of lymphorganogenic cytokines in haVSMC. Relative expression levels of (A, E) *CCL20*, (B, F) *CCL21*, (C, G) *CXCL13* and (D, H) *CXCL16* in ahVSMC treated with vehicle, LTα (5 ng/ml), LTα (10 ng/ml) and LIGHT (50 ng/ml) (A–D) and with vehicle, $LT\alpha_1\beta_2$ (20 ng/ml) and $LT\alpha_1\beta_2$ (100 ng/ml) (E–H). mRNA levels were normalized with the endogenous gene levels and relativized to the vehicle-treated VSMC mRNA levels. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$;****$p < 0.0001$.

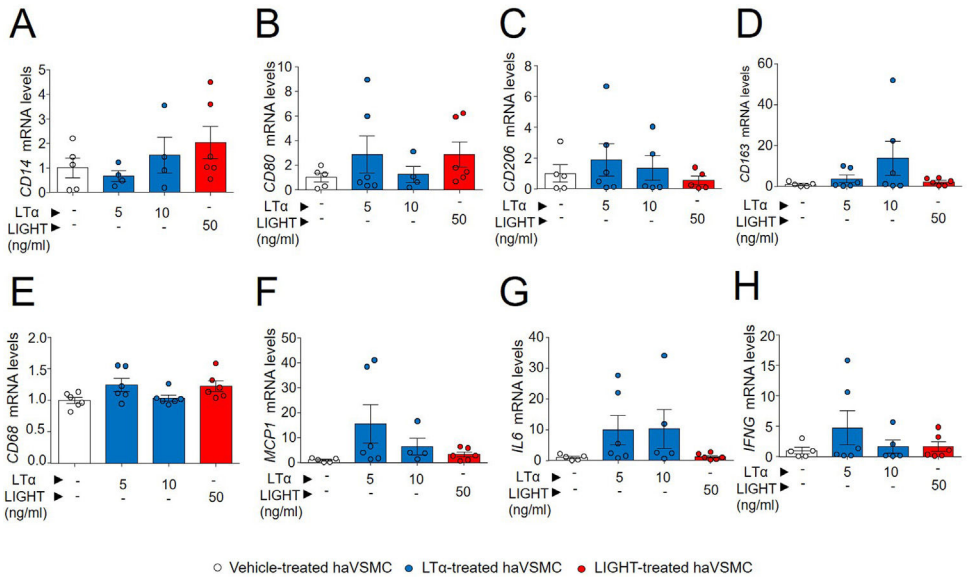

Figure 6 Expression analysis of genes related to macrophage-like cell haVSMC. Relative expression levels of (A) *CD14*, (B) *CD80*, (C) *CD206*, (D) *CD163*, (E) *CD68*, (F) *MCP1*, (G) *IL6* and (H) *IFNG* in haVSMC treated with vehicle, LTα (5 ng/ml), LTα (10 ng/ml) and LIGHT (50 ng/ml). mRNA levels were normalized with the endogenous gene levels and relativized to the vehicle-treated VSMC mRNA levels. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test.

in an acquisition of an inflammatory and apoptotic phenotype promotes plaque instability in insulin resistance states through a CX3CL1-dependet manner.[22] More recent investigations using cellular lineage tracing and single-cell RNAseq in plaque have further extended our knowledge about VSMC plasticity during plaque progression.[23,26] Specifically, *in vivo* VSMC specific deficiency in *Oct4* or *Klf4* in mouse models resulted in two divergent genomic signatures associated with different VSMC fates and function such as osteochondrocyte-like and macrophage-like gene signatures.[23,27] Notably, protective ACTA2+ myofibroblast-like cells forming the fibrous caps were discovered to originate from endothelial-to-mesenchymal transition and macrophage-to-mesenchymal transition, a process dependent of *Pdgfrb* gene.[27]

Our present investigation indicates a gene repression by LTα and LIGHT of *COL1A1* and *TGFB1*, *SOX9*, *CKIT*, and *KLF4* genes, results that are consistent with the above studies indicating important roles of some of these genes in plaque instability through VSMC phenotype switching. On the other hand, LIGHT/LTβR-dependent signaling has as well been linked to atherosclerosis with discrepant conclusions[12,13,15–19] which we believe might be attributed to the intrincated network signaling of the TFNSF ligands. The present investigation is also in agreement with a study describing a protective function of LIGHT in aneurysm lesion[20] and a prevention by LIGHT of *SOX9* gene expression which is associated with an osteochondrogenic and proatherogenic phenotype. Consequently, *in vivo* analysis showed diminished ACTA2+ lesion area and vascular *Col1a1* gene expression,[20] suggesting a protective role for LIGHT by preventing VSMC de-differentiation and plasticity *in vivo* during vascular injury. Of note, as in the present investigation, LIGHT did not affect the *in vitro* gene expression of the *ACTA2* contractile marker in haVSMCs which could be due to a high prevalence of ACTA2+ myofibroblast-like cells[26] which could difficult the detection of other non-contractile cells originated during the transdifferentiation process. Hence, changes in gene expression were performed in the cell culture probably containing a mixed pool of cells with different cell-type phenotypes which could mask gene expression changes belonging to a less prevalent cell-type.

Therefore, a limitation of our results is that our study is based on gene expression techniques and hence future studies would require the detection of active proteins as well as the performance of functional assays to better characterize each cell-type. To avoid the above limitations, future studies should include experimental approaches such as single-cell RNAseq or flow-cytometry analysis using several markers to identify, fractionate and quantify the different cell-types induced by LTα, $LT\alpha_1\beta_2$ and LIGHT.

In addition, a modulation by the TNFSF ligands of lymphorganogenic cytokines, including enhanced *CCL20* and *CCL21* gene expression and diminished *CCL21* and *CXCL13*, has been also observed in the present investigation which suggest a coordinated role of these ligands in cytokine-mediated lymphorganogenesis. In the line of these results, VSMC *Ltbr*-specific deficiency in mice prevented this cell type to acquire a protective lymphorganogenic PDGFRB+ phenotype,[19] and aggravated lesion size, suggesting a role in lymphorganogenesis associated with plaque stability.

Altogether, the present investigation indicates a role of the TNFSF ligands in VSMC gene expression of genes relevant for cellular plasticity and reprogramming events during plaque lesion development and stability. Therefore, these data suggest a possible role of these ligands in cellular plasticity during atheroma development.

Conclusions

The TNFSF ligands through its interconnected network signaling modulate haVSMC gene expression signature associated with cellular plasticity. These results suggest a possible role of the TNFSF ligands in plaque stability.

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Conflict of interests

The authors have no conflict of interests to declare.

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