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Serglycin secretion is part of the inflammatory response in activated primary human endothelial cells in vitro ☆

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ABSTRACT

Background: Endothelial cells have important functions in e.g. regulating blood pressure, coagulation and host defense reactions. Serglycin is highly expressed by endothelial cells, but there is limited data on the roles of this proteoglycan in immune reactions.
Methods: Cultured primary human endothelial cells were exposed to proinflammatory agents lipopolysaccharide (LPS) and interleukin 1β (IL-1β). The response in serglycin synthesis, secretion and intracellular localization and effect on the proteoglycan binding chemokines CXCL-1 and CXCL-8 were determined by qRT-PCR, Western blotting, immunocytochemistry, ELISA and serglycin knockdown experiments.
Results: Both LPS and IL-1β increased the synthesis and secretion of serglycin, while only IL-1β increased serglycin mRNA expression. Stimulation increased the number of serglycin containing vesicles, with a greater portion of large vesicles after LPS treatment. Also, increased intracellular and secreted levels of CXCL-1 and CXCL-8 were observed. The increase in CXCL-8 secretion was unchanged in serglycin knockdown cells. However, the increase in CXCL-1 secretion from IL-1β stimulation was reduced 27% in serglycin knockdown cells; while the LPS-induced secretion was not affected. In serglycin expressing cells CXCL-1 positive vesicles were evenly distributed throughout the cytoplasm, while confided to the Golgi region in serglycin knockdown cells. This was the case only for IL-1β stimulated cells. LPS-induced CXCL-1 distribution was unaffected by serglycin expression.
Conclusions: These results suggest that different signaling pathways are involved in regulating secretion of serglycin and partner molecules in activated endothelial cells.
General significance: This knowledge increases our understanding of the roles of serglycin in immune reactions. This article is part of a Special Issue entitled: Matrix-mediated cell behaviour and properties.

1. Introduction

Endothelial cells have important functions in regulation of blood coagulation, blood pressure, wound healing, filtering of urine in kidneys, as barriers for bacteria and regulation of inflammatory responses in circulation [1]. To participate in such a variety of important functions endothelial cells express cell surface molecules important for cell–cell and cell–matrix interactions and for recognition of pathogens, growth factors and signaling molecules such as cytokines and chemokines. Endothelial cells also express proteoglycans (PGs), including syndecans and glypicans to be exposed on the cell surface, and perlecan and biglycan to be part of the extracellular matrix [2–4]. Endothelial cells also express serglycin, regarded as the major intracellular PG [5]. Serglycin has been shown to be one of the major PGs in cultured human umbilical vein endothelial cells (HUVECs) [6]. In this study it was also shown that serglycin was mainly secreted to apical medium of HUVEC cultured on semipermeable filters to induce polarization. Serglycin was furthermore shown to co-localize to a certain extent with CXCL-1 (GROα) and participate in the regulation of secretion of this chemokine, suggesting that serglycin can participate in inflammatory reactions mediated by endothelial cells. Many of the functions of serglycin studied so far suggest that this particular PG is involved in the intracellular storage, transport, secretion, protection and regulation of several bioactive molecules. These include a wide range of molecules ranging from histamine, chymases, carboxypeptidases, and cytolytic enzymes to cytokines and chemokines [5].

As part of the immune defense endothelial cells respond to lipopolysaccharide (LPS), a structure present in the cell wall of gram-negative bacteria. LPS is a classical pathogen-associated molecular pattern (PAMP) recognized by Toll-like receptor (TLR) 4, activating intracellular signaling proteins and the transcription factors nuclear factor κB (NFκB), activating protein-1 (AP-1) and interferon regulatory factor (IRF) 5 and IRF3 [7,8]. This leads to changes in gene expression, resulting in increased expression of effector-molecules such as cytokines and

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chemokines. TLR4 signaling can be divided into the MyD88-dependent pathway, responsible for the expression of proinflammatory cytokines, and the MyD88 independent/TRIF dependent pathway mediating the activation of type 1 interferon genes [7,8].

Endothelial cells also respond to several cytokines released systemically during inflammatory reactions, including the classical proinflammatory IL-1 cytokines. IL-1β signals via IL-1 receptor type 1 (IL-1R1), also resulting in activation of transcription factors including NFκB, inducing the mRNA expression of a series of genes [9]. Several adaptor proteins, including MyD88 and IRAKs, mediate both TLR4 and IL-1R signaling, and both LPS signaling and IL-1β signaling activate the same intracellular signaling pathways [10]. However, there are also differences in the signaling systems being activated by these two activators relevant for inflammation. Specific molecules for TLR4 signaling include the MyD88 independent/TRIF-dependent pathway [7,10].

To further explore the possible importance and implications of serglycin in endothelial cells in inflammatory reactions we cultured primary human endothelial cells in the presence of LPS or IL-1β and studied the expression, secretion and intracellular localization of serglycin. Results presented suggest that both agents increase serglycin expression and secretion, but that the interactions of serglycin with partner molecules differ depending on type of stimuli. Serglycin secretion is, accordingly, regulated differently by two distinct types of inflammatory stimuli, and is clearly part of the inflammatory response in human endothelial cells.

2. Materials & methods

2.1. Cell culture

Human umbilical cord vein endothelial cells (HUVECs) were isolated from umbilical cords as described [11]. Cells were cultured at 37 °C and 5.0% CO_2 in MCDB-131 medium (Sigma) containing 5 mM glucose and supplemented with 7% heat-inactivated fetal calf serum (FCS, Sigma), basic fibroblast growth factor (bFGF, 1 ng/ml, R&D), hydrocortisone (1 μg/ml, Sigma), epidermal growth factor (EGF, 10 ng/ml, R&D), gentamicine (50 μg/ml, GIBCO Invitrogen) and fungizone (250 ng/ml, GIBCO Invitrogen). The medium was replaced three times a week and cells were used for experiments within three passages. The purity of the endothelial cell cultures was verified by microscopic observations of each culture as well as regular staining for the endothelial cell marker von Willebrand factor (vWF). For experiments, cells were seeded in wells or chamber slides and grown to confluence. Incubations with IL-1β (0.5 ng/ml, R&D) or LPS (1 μg/ml, Sigma) were performed for 24 h in a medium containing 2% FCS.

2.2. Gene expression analysis

HUVEC was cultured as described above. Total RNA was isolated using the E.Z.N.A. Total RNA kit 1 (R6834-02, Omega Bio-Tek) and lysis buffer containing β-mercaptoethanol, according to the manufacturer's instructions. RNA quantity measurements were performed using the ND1000 Spectrophotometer (Saveen Werner) and RNA was stored at −80 °C until further analysis. A quantity of 250 ng RNA was reverse transcribed in a total volume of 20 μl using the "High capacity RNA-to-cDNA kit" (4387406, Applied Biosystems). Quantitative Real-time PCR (qRT-PCR) was performed on an ABI Prism 7900HT (Applied Biosystems) using TaqMan Gene Expression Master Mix (4369016, Applied Biosystems) in a total volume of 20 μl. For gene expression analysis of serglycin (*SRGN*), CXCL-1 (*CXCL1*), CXCL-8 (*CXCL8*) and the endogenous control 60S ribosomal protein L30 (*RPL30*) [12], we used inventoried TaqMan gene expression assay (ID: Hs01004159_m1, Hs00236937_m1, Hs00174103_m1 and Hs00265497_m1). The relative mRNA level for each transcript was calculated by the ΔΔCt method [13,14]. Briefly, the cycle threshold (Ct) values for *SRGN*, *CXCL1* and *CXCL8* were normalized against the Ct values for the housekeeping gene *RPL30* (=ΔCt). For comparison of gene expression in stimulated versus control cells, ΔΔCt was calculated as ΔCt in stimulated cells subtracted the ΔCt for control cells. The fold change in mRNA expression was calculated as $2^{-\Delta\Delta Ct}$. $2^{-\Delta Ct}$ values were calculated for gene expression in response to LPS or IL-1β in siSRGN compared to scramble transfected controls.

2.3. Western blotting

HUVECs were grown in MCDB-131 medium as described, but with FCS reduced to 2%. After 24 h the conditioned medium was collected and cell debris removed by centrifugation. Samples of equal volumes were boiled in Laemmli buffer and subjected to SDS-PAGE on 4–20% gradient gels and electroblotted onto PVDF membranes (Millipore) using the Criterion™ gel system (BioRad). Incubation with primary antibody polyclonal rabbit anti-human serglycin (HPA000759, Atlas Antibodies, 1:100) was followed by secondary antibody HRP-linked goat anti-rabbit (NA934, GE-Healthcare,1:20 000). The membranes were developed using ECL Western Blotting Detection Reagents (GE Healthcare) and finally exposed to films (Amersham Hyperfilm™ ECL). The intensity of the bands was quantified by Carestream Molecular Imaging software.

2.4. Metabolic labeling

HUVEC cultures were metabolically labeled with 0.1 mCi/ml ^{35}S-sulfate (Hartmann Analytic) in RPMI-1640 sulfate free medium (GIBCO Invitrogen) added 5 mM L-glutamine (Sigma) and with FCS reduced from 7 to 2% to increase labeling efficiency. Labeling was performed in the absence (Ctr) or presence of IL-1β or LPS. After labeling for 24 h, the culture medium was collected. Cells were washed in PBS and harvested either in lysis buffer (4.0 M guanidine-HCl, 0.1 M acetate buffer pH 6.5, 2% Triton X-100) or RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Na-deoxycholate, 10 mM EDTA, 10 mM $Na_4P_2O_7$ and phosphatase inhibitor tablet freshly added). In order to remove unincorporated ^{35}S-sulfate, samples were diluted to 1 ml and applied to Sephadex G50 fine (GE Healthcare) gel chromatography columns, with a bed volume of 4 ml. The ^{35}S-macromolecules were eluted in the void volume with 1.5 ml buffer (0.05 M Tris–HCl, 0.05 M NaCl, pH 8), while lower molecular weight molecules remained associated with the column. The amount of ^{35}S-sulfate incorporated in newly synthesized ^{35}S-macromolecules was determined by scintillation counting in triplicates. ^{35}S-macromolecules in HUVEC almost exclusively comprise PGs [15]. Protein content of the cell lysate was determined using Uptima BC Assay protein quantization kit (BioRad), in RIPA-lysates prior to G50 fine gel chromatography or in guanidine-lysates after changing the buffer on the G50 fine column. The results are presented as mean cpm/mg cellular protein.

2.5. Immunoprecipitation

HUVECs were metabolically labeled with ^{35}S-sulfate as described; thereafter the medium was harvested and the cells were washed in PBS and lysed in RIPA buffer. The volumes of both medium and cell lysates were adjusted to the protein content of the cell fractions, and subjected to G50-fine gel chromatography as described above. Samples were incubated over night at agitation and 4 °C with polyclonal rabbit anti-human serglycin (HPA000759, Atlas Antibodies, 1:100) or monoclonal mouse anti-human serglycin (clone 1D8, AbNova, 1:500). In initial experiments medium from the monocytic cell line THP-1 [16] was included as positive control for serglycin, and as negative control unconditioned medium or concentration matched irregular IgG control (Mouse Gamma Globulin, Jackson ImmunoResearch, 015-000-002) was included. Further, 30 μl Protein A/G solution (sc-3003, Santa Cruz) was added and the incubation was continued for 2 h. The samples were then centrifuged and washed three times in 0.05 M Tris–HCl

with 0.15 M NaCl, 0.05% Triton X-100 and 1% BSA, followed by one wash in PBS. Material bound to Protein A/G was finally released by boiling for 5 min in Laemmli sample buffer, centrifuged and loaded on to 4–20% SDS-PAGE. Molecular weight protein markers (BioRad) were run on all gels for size determination. After electrophoresis the gels were dried and subjected to autoradiography.

2.6. Immunocytochemistry

HUVECs grown to confluence on Lab-Tek chamber slides (Nalge Nunc International) coated with 1% (w/v) gelatin from porcine skin were incubated in the absence or presence of IL-1β or LPS for 24 h. The slides were submerged three times in PBS, fixed in 4% paraformaldehyde for 10 min, rinsed in PBS for 10 min and finally dipped in Milli-Q water. The slides were dried and stored at 4 °C until staining. The fixed cells were labeled with affinity purified rabbit anti-human serglycin (1 μg/ml, kindly provided by Professor Niels Borregaard, University of Copenhagen, Denmark), monoclonal mouse anti-CXCL-1 (1 μg/ml, clone 20326, R&D System) and/or mouse anti-CXCL-8 (6 μg/ml, Peprotech) antibodies overnight at 4 °C in a dark humidity chamber. All antibodies were diluted in PBS containing 1.25% BSA and 0.2% saponin. As negative controls, secondary antibodies were included subsequent to incubation with concentration-matched irrelevant IgGs or without primary antibody. The slides were washed for 10 min in PBS and incubated with relevant secondary antibodies; Alexa Fluor 488 conjugated goat anti-rabbit IgG and Alexa Fluor 546 conjugated goat anti-mouse IgG (both from Invitrogen, 1:600) for 90 min at room temperature. This was followed by 10 min wash in PBS, dipped in Milli-Q water, and mounted using SlowFade Gold antifade reagent with DAPI (Invitrogen). Cells were examined using confocal microscopy (Olympus FluoView FV1000, Olympus Corporation) with a plan apochromat 60×/1.35 oil objective. All images were taken as single sections in the z-plane by sequential scanning. Images were then processed using Adobe Photoshop and Adobe Illustrator CS4.

2.7. Serglycin knockdown

Short interfering RNA (siRNA) was used to reduce the expression of human serglycin in HUVECs by using serglycin siRNA (sc-44093, Santa Cruz) and the negative control siRNA (scramble, sc-37007, Santa Cruz) at 0.02 μM. HUVECs were reversely transfected with siPORT (AM4502, Ambion) in Opti-MEM (Invitrogen) transfection medium at a cell density of 200,000 cells/ml (25,000 cells/cm^2) and allowed to adhere. For cells with good viability as judged by visual inspection, this incubation was continued for 24 h. For cells with poorer tolerance for reverse transfection, the transfection was terminated after 5 h and Opti-MEM replaced with MCDB growth medium for 19 h, followed by a new transfection overnight in Opti-MEM. The cells were then seeded in appropriate wells or chamber slides and allowed to adhere. Experiments were continued for 24 h and the silencing efficiency was quantified by qRT-PCR measuring the serglycin transcript as well as Western blotting showing the secreted amount of serglycin.

2.8. ELISA

HUVECs were grown to confluence and stimulated with LPS or IL-1β for 24 h. The conditioned medium was harvested and the cells were washed in PBS and lysed in RIPA buffer. Debris was removed by centrifugation. Both cell associated and secreted levels of CXCL-1 and CXCL-8 were determined by ELISA (human CXCL1 DuoSet DY275, human CXCL8 DuoSet DY208, R&D) according to the manufacturer's instructions. Measurements were performed in duplicate. In initial experiments the protein concentration was determined, and did not differ between scramble transfected and siSRGN transfected cells. Measured amounts of CXCL-1 and CXCL-8 were therefore not normalized against protein and are presented as pg/ml.

2.9. Statistics

Throughout the study the paired *t*-test was used. For the qRT-PCR data, statistics were based on $2^{-\Delta Ct}$ for comparison of response to LPS or IL-1β mRNA expression in siSRGN compared to scramble transfected controls, or $2^{-\Delta\Delta Ct}$ values for a given treatment compared to that of control cells. *p*-Values < 0.05 were considered to be statistically significant. Results are expressed as means with SEM.

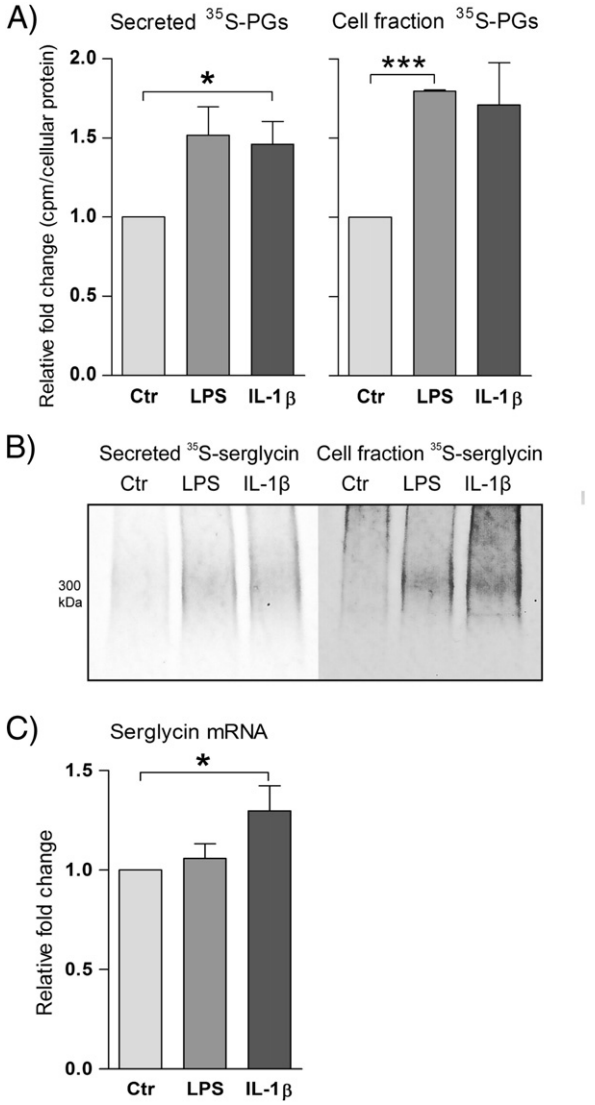

Fig. 1. Effect of LPS and IL-1β stimulation on secreted and cell associated levels of ^{35}S-PGs and the PG serglycin. (A) HUVECs were metabolically labeled for 24 h with ^{35}S-sulfate. The amount of secreted and cell associated ^{35}S-PGs from 9 (secreted; IL-1β) or 3 donors was assessed by scintillation counting and related to cellular protein content. The results are presented as change in cpm/mg protein in LPS or IL-1β stimulated cells relative to unstimulated control cells (Ctr). (B) The effect of LPS and IL-1β stimulation on secreted and cell associated levels of the PG serglycin was determined by immunoprecipitation of ^{35}S-labeled serglycin. Shown here is one representative of three HUVEC donors. (C) Gene expression of serglycin after LPS or IL-1β stimulation was determined by qRT-PCR. Values are expressed as the fold change in stimulated relative to unstimulated control cells. The number of HUVEC donors was 7 for LPS stimulated cells and 9 for IL-1β stimulated cells. The results are presented as mean with SEM. A *p*-value < 0.05 (*) was taken as a significant difference. $p < 0.05$ (*); $p < 0.001$ (***).

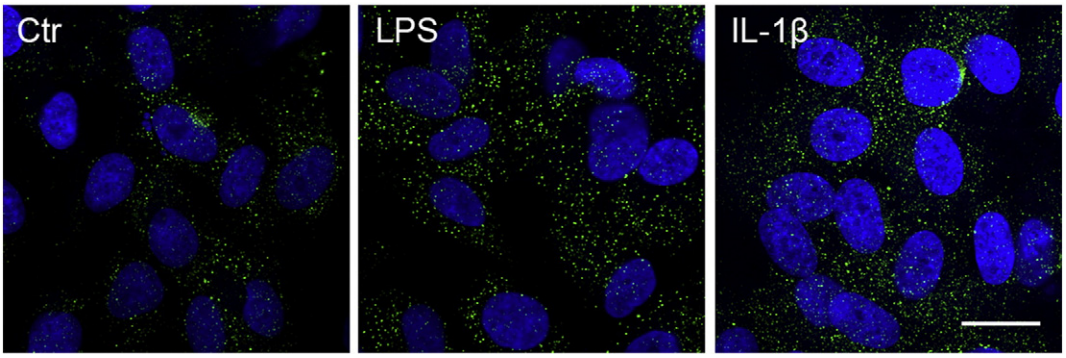

Fig. 2. Intracellular localization and expression of serglycin. Confluent HUVECs cultured on chamber slides were stimulated with LPS or IL-1β, prior to fixation in PFA. Serglycin was detected by immunofluorescence and is visible as green, while cell nuclei are seen as blue after DAPI staining. The intracellular localization and expression of serglycin after LPS and IL-1β stimulation is compared to unstimulated control cells (Ctr). These images are from one of 4 representative HUVEC donors. Scale bar = 20 μm.

3. Results

To investigate the effects of inflammatory conditions on HUVEC, the cells were exposed to LPS and IL-1β and *de novo* biosynthesis of ^{35}S-PGs was monitored after labeling with ^{35}S-sulfate. After 24 h the medium and cell fractions were harvested and subjected to Sephadex G50 fine gel chromatography. The levels of ^{35}S-macromolecules were determined and from Fig. 1A it is evident that both the secreted and cellular contents increased to 1.5 and 1.7–1.8 times respectively, after both LPS and IL-1β treatments. The glycosaminoglycan (GAG) chains decorating all PGs are heavily sulfated, resulting in a total domination of these ^{35}S-macromolecules by ^{35}S-PGs [15]. Thus, these results show that both stimulating agents increase the expression and secretion of ^{35}S-PGs to similar degrees. To analyze the effects of inflammation on ^{35}S-PGs in further detail, conditioned medium and cell fractions from metabolically ^{35}S-sulfate-labeled cell cultures were subjected to immunoprecipitation using an antibody against serglycin, an intracellular PG expressed in HUVEC. The amount of secreted as well as cell associated ^{35}S-serglycin was increased to approximately the same extent after both stimulations, as can be seen from Fig. 1B. This demonstrates that serglycin is one of the PGs contributing to the increased ^{35}S-PG expression and secretion in HUVEC after stimulation with LPS and IL-1β. Further, serglycin mRNA expression was determined using qRT-PCR (Fig. 1C). Serglycin expression was significantly increased after IL-1β stimulation, while no significant difference was observed as a result of LPS stimulation. This suggests that IL-1β and LPS influence serglycin expression through different mechanisms.

Further, the intracellular localization of serglycin in control versus IL-1β or LPS stimulated HUVEC was examined using immunocytochemistry. Fig. 2 shows intracellular serglycin-containing vesicles of different sizes evenly distributed in the cytoplasm in all three cell cultures. The number of these vesicles increased after both LPS and IL-1β treatments. Also, after LPS treatment the portion of large serglycin-containing vesicles increased, clearly evident by visual inspection.

Serglycin has been shown to interact with CCL3 (MIP-1α) [17], and has been implicated in the secretion of both TNF-α and CXCL-1 in macrophages and endothelial cells, respectively [6,18]. In HUVEC, serglycin is previously shown to co-localize with CXCL-1 in type 2 granules [6], while CXCL-8 is found in Weibel–Palade bodies [19,20]. These chemokines are major mediators of the inflammatory response known to be expressed by endothelium upon stimulation [21]. To investigate if the increased secretion of serglycin observed after IL-1β and LPS stimulation could affect chemokine secretion, the levels of chemokines CXCL-1 and CXCL-8 were measured with ELISA. As can be seen from Fig. 3A and B, we found that both the cellular and secreted levels of these two chemokines increased as a result of stimulation. Notably, the cellular content of CXCL-1 was significantly higher after IL-1β stimulation than after LPS stimulation ($p = 0.022$).

This increase in secretion of both CXCL-1 and CXCL-8 could merely be a reflection of the increased activation of HUVEC, possibly unrelated to the increased serglycin expression. To investigate this further, HUVECs were stimulated with either IL-1β or LPS and stained with antibodies against CXCL-1 or CXCL-8. From Fig. 4A, upper panels, it is evident that both LPS and IL-1β increased the number of CXCL-1 positive vesicles. A more pronounced increase in IL-1β stimulated cells supports the ELISA data in Fig. 3A. From Fig. 4A, lower panels, it can be seen that CXCL-8 expression was increased to similar extent after both LPS and IL-1β stimulation, also in line with ELISA data. We have previously shown colocalization of serglycin and CXCL-1 in IL-1β stimulated cells [6].

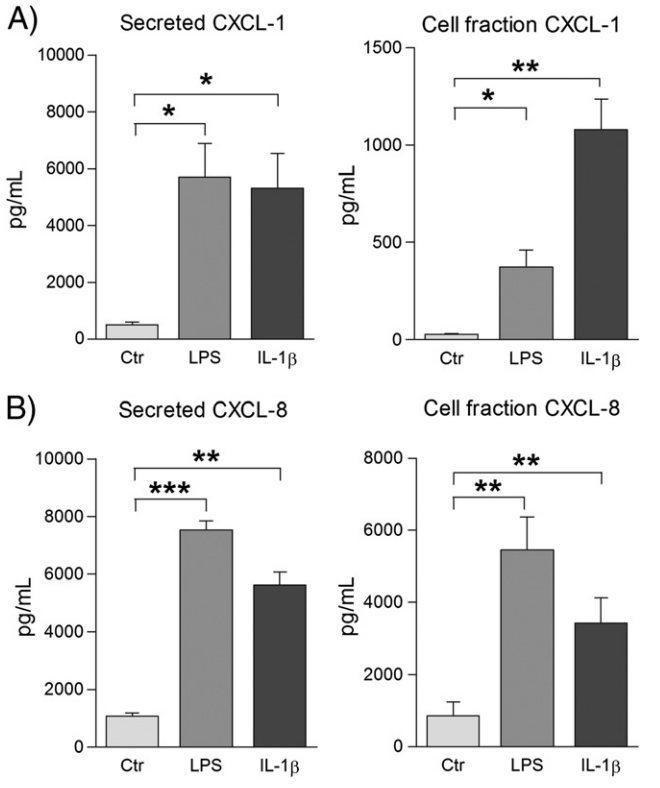

Fig. 3. Effect of LPS and IL-1β stimulation on CXCL-1 and CXCL-8 levels. HUVECs were grown to confluence and stimulated with LPS or IL-1β for 24 h. Both cell associated levels (right) and secretion levels (left) of CXCL-1 (A) and CXCL-8 (B) were determined by ELISA and compared to unstimulated cell cultures (Ctr). For CXCL-1 measurements in cell fraction, the number of HUVEC donors was 7 for IL-1β stimulation and 6 for LPS stimulation. For all or the remainder, the number of HUVEC donors was 4. The results are presented as mean with SEM. A p-value < 0.05 (*) was taken as a significant difference. $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

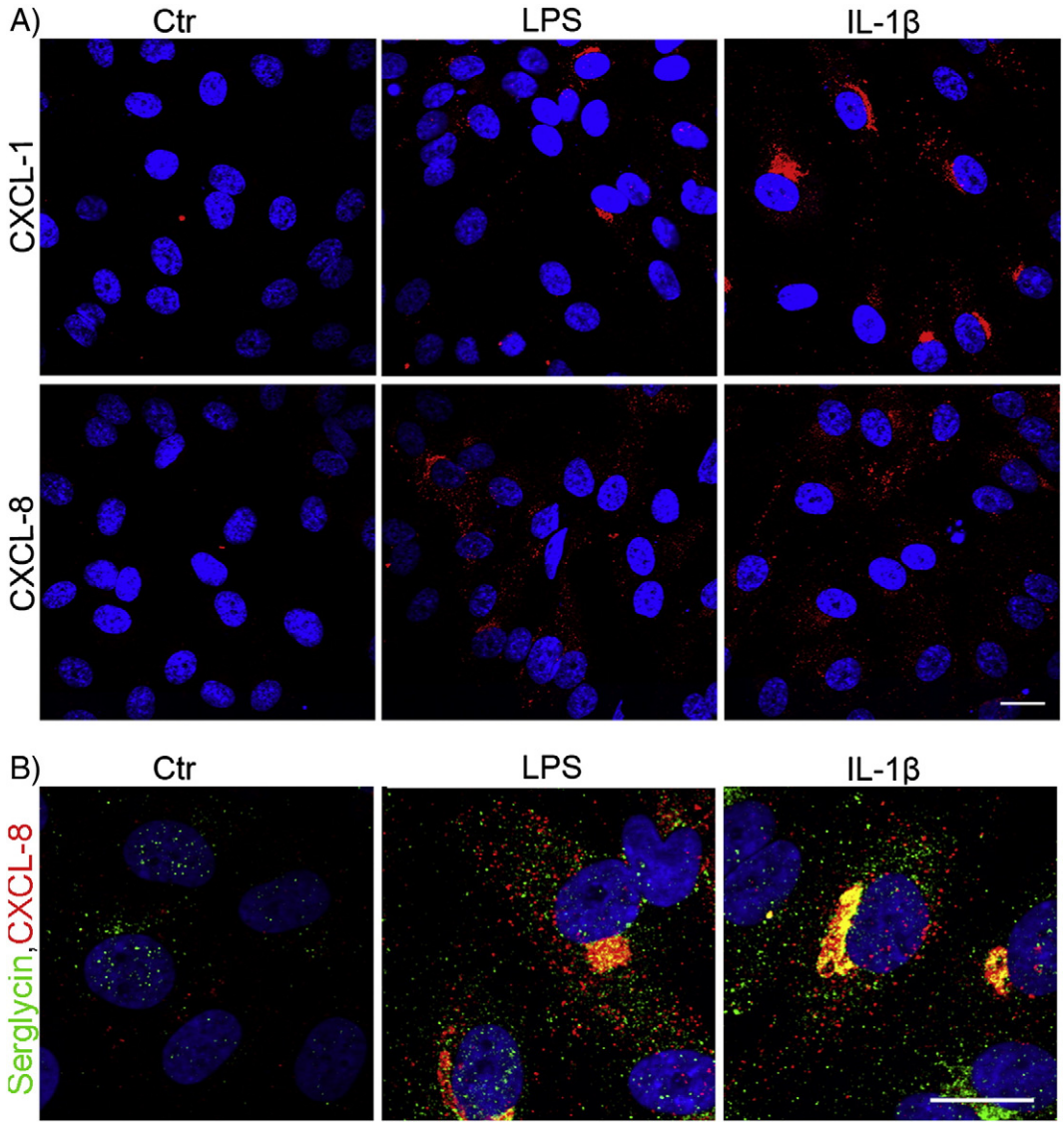

Fig. 4. Effect of LPS and IL-1β stimulation on intracellular localization and expression of CXCL-1 and CXCL-8. Confluent HUVECs cultured on chamber slides were stimulated with LPS or IL-1β. A. CXCL-1 (top panels) and CXCL-8 (lower panels) were detected by immunofluorescence and were visible as red, while cell nuclei were seen as blue after DAPI staining. B. Serglycin visible as green merged with CXCL-8 in red, and co-localization of the two was visible as yellow. Intracellular localization and expression after LPS or IL-1β stimulation is compared to unstimulated control cells (Ctr). Scale bar = 20 μm.

However, no apparent colocalization of serglycin and CXCL-1 was observed upon LPS induction (results not shown). Similarly, co-staining with antibodies against serglycin and CXCL-8 showed very low degree of vesicular colocalization, visible as yellow staining, both in LPS and IL-1β treated cells (Fig. 4B), while colocalization was evident in the Golgi region.

The possible implications of serglycin in regulation of chemokine secretion in stimulated HUVEC were investigated in more detail by making use of siRNA to knock down serglycin expression (siSRGN). Efficient knockdown of the serglycin gene was achieved, as can be seen in Fig. 5A, upper panel. This was further supported by demonstrating a significant decrease of 64.0 ± 14.4% in serglycin secretion by Western blotting of conditioned medium (Fig. 5A, middle and lower panels). Further, secreted levels of CXCL-1 and CXCL-8 were determined in scrambled transfected (siControl) and siSRGN transfected cells using ELISA. In line with the results presented in Fig. 3, in the scramble transfected control cells, the secretion of CXCL-1 and CXCL-8 increased both with LPS and with IL-1β stimulation (Fig. 5B). However, we observed a significantly reduced IL-1β induced CXCL-1 secretion in the serglycin knockdown cells, which is not seen after LPS stimulation. Using qRT-PCR, the levels of CXL-1 and CXCL-8 in knockdown cells were determined in cells from three donors. We found no significant differences in the response to neither LPS nor IL-1β treatment on mRNA expression in siSRGN transfected cells when compared to scramble transfected controls. For CXCL-1 the mean differences were (mean ± SE; *p*-value): Ctr 0.024 ± 0.02; $p = 0.290$, LPS 0.734 ± 0.29; $p = 0.209$, IL-1β 0.725 ± 0.40; $p = 0.123$ and for CXCL-8: Ctr 0.027 ± 0.016; $p = 0.226$, LPS 0.437 ± 0.105; $p = 0.053$ and IL-1β 0.054 ± 0.480; $p = 0.920$. Together, these results suggest that CXCL-1 secretion induced by LPS is independent of serglycin, whereas CXCL-1 secretion stimulated by IL-1β is partly dependent on serglycin. In contrast, secretion of CXCL-8 was not affected by knockdown of serglycin expression, neither in LPS, nor in IL-1β stimulated cells, indicating that CXCL-8 secretion in HUVEC is independent of serglycin.

To investigate the effect of serglycin knockdown on the intracellular distribution of the chemokines, cells were analyzed by immunocytochemistry. An efficient silencing of serglycin is visualized by the absence of green immunofluorescent staining in the serglycin siRNA transfected

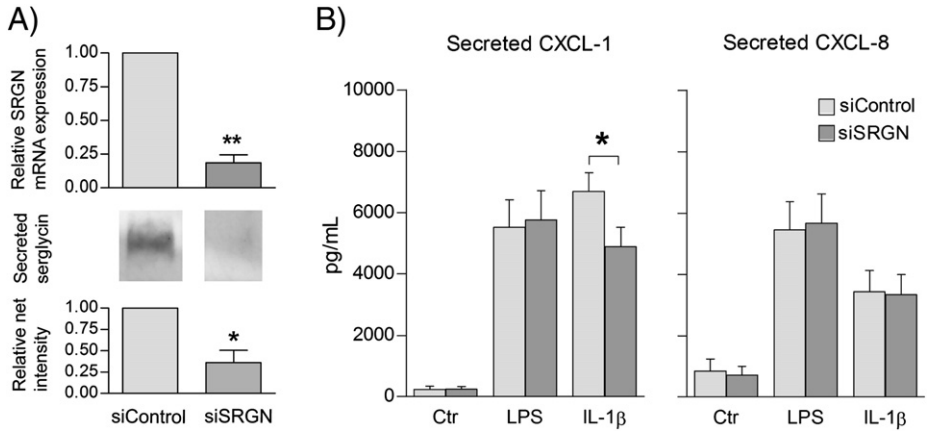

Fig. 5. Effect of serglycin knockdown on cytokine secretion in HUVEC. HUVECs from nine donors were transfected with scrambled siRNA (siControl) or siRNA to SRGN (siSRGN), and stimulated with LPS or IL-1β for 24 h. The efficiency of serglycin knockdown is shown by the relative SRGN mRNA expression determined by qRT-PCR in the siSRGN treated cells compared to the siControl (A, upper panel). Also, the level of secreted serglycin was detected by Western blotting illustrated by one representative blot (A, middle panel) and presented as the relative net intensities of the bands from three donors (A, lower panel). The secretion levels of CXCL-1 (GRO-α) and CXCL-8 (IL-8) were determined by ELISA (B). The release of the cytokines in serglycin knockdown cells (siSRGN) was compared to the scramble control cells (siControl), both in unstimulated and LPS or IL-1β stimulated cells. The number of HUVEC donors was 7, and values are presented as mean with SEM. A p-value < 0.05 (*) was taken as a significant difference.

cells (siSRGN), in comparison to the scramble control cells (siControl, Fig. 6A). As has been shown for the untransfected cells in Fig. 4, also here, CXCL-1 and CXCL-8 expression was increased in scramble transfected control cells after stimulation with LPS and IL-1β (data not shown). From Fig. 6B it is evident that after IL-1β stimulation CXCL-1 positive vesicles were evenly distributed throughout the cytoplasm in serglycin expressing cells, while confined to the Golgi region in serglycin knockdown cells. However, no such effect mediated by serglycin knockdown was observed for the LPS induced CXCL-1 expression. Further, it is evident that CXCL-8 distribution is not affected by the presence of serglycin (Fig. 6C). Taken together these data supports the notion that CXCL-8 storage and secretion in HUVEC is not mediated by serglycin. In contrast, IL-1β stimulated CXCL-1 vesicular presence and secretion was partly dependent on serglycin, while this was not the case for LPS stimulation.

4. Discussion

In this study we demonstrate that serglycin expression and secretion increased in primary HUVEC after stimulation with LPS and IL-1β. These changes were accompanied by increased number of serglycin positive intracellular vesicles and increased size of a large number of such vesicles in LPS stimulated cells. Furthermore we demonstrate that serglycin expression and secretion is differently regulated by LPS and IL-1β. Both increased serglycin mRNA expression and increased PG expression were observed after IL-1β stimulation, whereas no increase in mRNA was observed after LPS treatment. Both types of stimuli did, however, result in increased serglycin secretion. The different effects observed between these two inflammatory stimuli suggest that serglycin expression and secretion is subject to regulation through different types of signaling systems. Furthermore, both stimuli increased both CXCL-1 and CXCL-8 secretion. In addition, the turnover of CXCL-1 was also different, as the cellular content of this chemokine was significantly higher in the cell fractions of IL-1β treated cells than in the corresponding fractions from LPS treated cells. This was not the case for secretion of CXCL-8. Additional evidence for differences in the secretion of CXCL-1 and CXCL-8 was observed after knockdown of serglycin expression. IL-1β induced CXCL-1 secretion was partly inhibited in the serglycin knockdown, which was not the case for CXCL-8 secretion.

In endothelial cells, CXCL-8 is stored in Weibel–Palade bodies and these vesicles have been shown not to contain serglycin [6,22]. Whether CXCL-8 is also present in other types of granules or vesicles in HUVEC has not been determined, but we observed no overt vesicular colocalization of serglycin and CXCL-8. However, serglycin has previously been shown to co-localize with CXCL-1 and through the use of xylosides to inhibit glycosaminoglycan assembly onto core proteins, it was suggested that PGs are involved in CXCL-1 secretion [6]. In this study we now provide further new evidence that part of the secretion of this chemokine from HUVEC depends on serglycin. Hence, the chemokine content of different vesicles in endothelial cells seems to vary between different types of vesicles generated after stimulation. With both LPS and IL-1β stimulation the secretion of serglycin was increased. This finding suggests that serglycin can have functions in formation of some types of vesicles and in interactions with particular vesicular components. However, serglycin is a secretory product and secretion increases after stimulation, which may suggest extracellular functions. Release and delivery of granzyme B to target cells in complex with serglycin has been demonstrated, which demonstrates functions related to both protection and transport of serglycin partner molecules [23]. Our previous demonstration of predominant secretion to the apical medium of serglycin in polarized HUVEC suggests that the primary purpose of serglycin secretion together with partner molecules would be to participate in inflammatory reactions on the endothelial cell surface facing the circulation or in the circulatory system itself. However, the extracellular functions of serglycin secreted to the basal side of endothelial cells, possibly involving modulation of ECM underneath endothelial cells cannot be excluded, but need to be studied further in polarized HUVEC or using in vivo systems, such as mice.

Studies on serglycin in polymorphonuclear leukocytes (PMNs) showed that serglycin was expressed in early stages of promyelocytes and myelocytes but not in mature PMN. Furthermore, by immunostaining presence of serglycin was demonstrated in the trans-Golgi network and immature and secondary granules, but not in mature granules of neutrophil granulocytes [24]. In contrast to our present findings in endothelial cells, serglycin was not detected in the culture medium of granulocytes after stimulation. In contrast to granulocytes, mast cells contain large amounts of serglycin in their storage granules, and the morphology and content of serglycin partner molecules are highly affected when the serglycin gene is deleted [25]. The functions of serglycin in relation to formation of secretory and storage granules and the interactions and regulation of partner molecules may therefore differ to a great extent between cell types. IL-1β stimulates the secretion of CXCL-1. However, in this study, the absence of serglycin by siRNA knockdown resulted in retention of CXCL-1 in the Golgi compartment. This supports a role for serglycin in vesicle formation or in the sorting of CXCL-1 to its proper secretory vesicles, rather than e.g. a role in

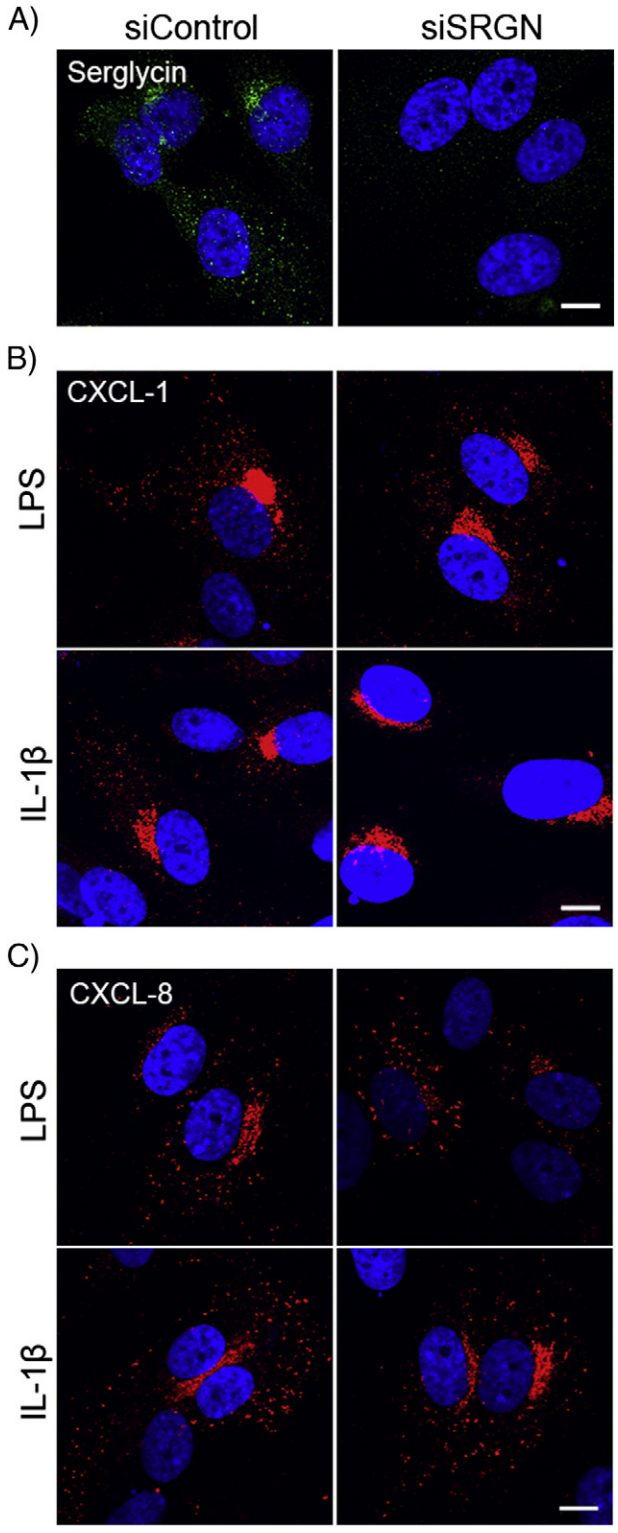

Fig. 6. Effect of serglycin knockdown on intracellular localization of CXCL-1 and CXCL-8. HUVECs were transfected with scrambled siRNA (siControl) or siRNA to SRGN (siSRGN) and grown on chamber slides. (A) The presence of serglycin in the control and the absence of serglycin in the knockdown cells are visualized by the presence and the absence of green immunofluorescent staining of serglycin. Confluent HUVECs were stimulated with LPS or IL-1β. The intracellular localization of CXCL-1 (B) and CXCL-8 (C) in control cells (siControl) and serglycin knockdown cells (siSRGN) is visualized with red immunofluorescent staining. These are representative pictures from 2 out of 3 HUVEC donors for CXCL-1 and 1 out of 2 donors for CXCL-8. Cell nuclei are shown in blue after DAPI staining, and scale bar 10 μm.

promoting storage or stability of this vesicle component. Serglycin is suggested to play a role in early events of granule formation [24], and it has been proposed that the GAG chains play a role in targeting of proteoglycans to their proper cellular or extracellular location [26]. It has recently been demonstrated that serglycin is related to an aggressive cancer cell phenotype [27], and serglycin was detected both in cytoplasm and on cell surfaces in the breast cancer cells, suggesting extracellular functions of this PG.

In this study, we show that both IL-1β and LPS increased the number of serglycin containing vesicles. In addition, cells treated with LPS also contained many larger vesicles with serglycin. A large number of different types of extracellular vesicles have been characterized, such as ectosomes, exosomes and apoptotic bodies, and shown to be important in immune reactions where endothelial cells also play important roles [28]. Several types of microparticles can be secreted from endothelial cells [29] and Weibel–Palade bodies in these cells have been categorized as lysosome-related organelles [30]. The generation of different sizes of serglycin containing vesicles in HUVEC after LPS compared to IL-1β stimulation suggests that the secretory vesicles formed in endothelial cells are dynamic and subject to regulation at different levels and that serglycin may be present in different types of granules and vesicles in HUVEC. Further studies need to focus on which type of intracellular vesicles that contain serglycin and what type of serglycin partners they contain. Whether serglycin is part of several different types of secreted vesicles is a new and fascinating aspect of serglycin biology that needs to be further investigated.

The differences in effects of LPS and IL-1β on serglycin synthesis and secretion suggest that serglycin can be modified through different intracellular signaling pathways in endothelial cells. Defining such differences will be of interest to improve our understanding of the role of serglycin in inflammatory reactions. LPS activation is likely to involve TLR 4, affecting gene expression of inflammatory mediators like cytokines, chemokines and serglycin [7,8]. However, recent studies have shown that LPS can activate cells through TLR4 independent mechanisms involving intracellular LPS sensors, which leads to activation of caspase-11 leading to stimulation of IL-1β secretion [31,32].

Although both LPS signaling and IL-1β signaling activate the same intracellular signaling pathways [10] the differences in effects observed here show that the inflammatory response of HUVEC also involves separate signaling mechanisms. Further analyses on the responsive elements of the serglycin gene [33] could potentially provide further insight into the regulation of serglycin expression after inflammatory stimuli as those used here.

HUVECs are important for different types of inflammatory reactions and endothelial dysfunction is a hallmark of several diseases, such as type I and type II diabetes and atherosclerosis [34,35]. This study shows that serglycin is part of the inflammatory response in endothelial cells and further studies on serglycin in activated HUVEC could provide new insight into the importance of PGs in inflammation.

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