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Chronic venous disease – Part I: Inflammatory biomarkers in wound healing

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ABSTRACT

Venous leg ulcers (VLUs) produce wound fluid (WF), as a result of inflammatory processes within the wound. It is unclear if WF from different healing phases of VLU has a peculiar biochemical profile and how VLU microenvironment affects the wound healing mechanisms. This study was conducted to evaluate the cytokine/chemokine profiles in WF from distinct VLU phases, in WF- and LPS-stimulated monocytes and treated with glycosaminoglycan Sulodexide, a therapeutic option for VLU healing.

WF and plasma were collected from patients with VLU during active inflammatory (Infl) and granulating (Gran) phases. Demographics, clinical characteristics and pain measurements were evaluated. WF, plasma, and THP-1 supernatants were analyzed for 27 inflammatory mediators by multiplex immunoassay. Our results demonstrated that: 1) pain was significantly increased in patients with Infl compared to Gran VLU; 2) cytokine profile of Infl WF was found to be statistically different from that Gran WF, as well significantly increased respect to plasma; 3) LPS- and WF-stimulation of THP-1 cells significantly increased the expression of several cytokines compared to untreated cells; 4) Sulodexide treatment of both LPS- and WF-stimulated THP-1 monocytes was able to significantly down-regulate the release of peculiar inflammatory mediators.

Our study highlighted the importance to understand biomolecular processes underlying CVI when providing treatment for chronic VLU. Identification of inflammatory biomarkers in leg ulcer microenvironment, may provide useful tools for predicting healing outcome and developing targeted therapies.

1. Introduction

Chronic venous leg ulcers (VLUs) represent the most advanced stage of chronic venous insufficiency (CVI), which affects a large part of the worldwide population, causing disability, patient suffering, and an enormous financial burden to healthcare spending with estimates of about 3 billion dollars annually [1,2].

According to the updated Clinical, Etiological, Anatomical, and Pathophysiological (CEAP) classification [3], a venous ulcer has been recently defined as "an open skin lesion of the leg or foot, that shows little or no tendency to spontaneous healing and occurs in the area affected by ambulatory venous hypertension and showing other signs of chronic venous insufficiency" [4], persisting for >30 days [5].

Several hypotheses have been proposed to explain the onset and progression of VLU, and it is widely accepted that superficial and deep vein insufficiency, valvular incompetence, venous reflux and/or obstruction leading to CVI, represent the hemodynamic impairment leading to the development of VLU. In fact, chronic venous hypertension in the lower extremities promotes venous stasis, endothelial cell activation, formation of transcellular gaps between endothelial cells, and finally extravasation of erythrocytes and leukocytes [6].

These latter events involve macrophages, T-lymphocytes and mast cells, which accumulate and activate into the dermis interstitium by

Abbreviations: bFGF, basic fibroblast growth factor; CEAP, Clinical, Etiological, Anatomical, and Pathophysiological; COX-2, cyclooxygenase-2; CVI, chronic venous insufficiency; VLU, venous leg ulcer; ECM, extracellular matrix; ICAM, intercellular adhesion molecule; IL, interleukin; IL-1ra, interleukin 1 receptor antagonist; IFN-γ, interferon gamma; IP-10, interferon gamma-induced protein 10; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; LFA, lymphocyte function-associated antigen; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; PDGF, platelet derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TLR, toll-like receptor; TNF-α, tumor necrosis factors alpha; WF, wound fluid; VCAM, vascular cell adhesion protein; VEGF, vascular endothelial growth factor; VLA, very late antigen.

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increasing exposure of adhesion molecules (e.g., ICAM-1, VCAM-1, LFA-1 and VLA-4, selectins) from endothelial cells and leukocytes, as demonstrated by different studies in animal models and in humans [7–15].

The result of leukocyte-endothelial activation is the release of several cytokines, chemokines, growth factors and proteases. When deregulated they both amplify the leukocyte infiltration and activation, and also impair the functions of resident cells, such as endothelial cells, fibroblast, smooth muscle cells, and myofibroblasts [8,16], finally inducing the inflammatory and proteolytic cascade leading to dermal changes and VLU.

Due to these morpho-functional alterations, the extracellular matrix (ECM) undergoes an intense remodeling. As in physiological conditions ECM acts as a source of hidden and/or latent cytokines and growth factors, and controls chemotactic gradients, any pathological modification of the ECM structure may lead to the aberrant distribution and activity of cytokines, growth factors and proteases [17]. As a consequence, skin damages result further exacerbated, so delaying wound closure.

Whereas normal healing of acute wounds is traditionally divided into four overlapping and timely-limited phases (i.e., hemostasis, inflammation, granulation and remodeling), chronic wounds seem to be blocked mainly in a persistent inflammatory state, which prevents the progression toward the next phases and finally the wound closure [18]. In this regard, "non-healing ulcers are wounds which do not show any reduction in size within 6 months"; in this definition are included both ulcers blocked in the inflammatory phase of wound healing (inflammatory ulcers), and ulcers which, due to conservative or surgical therapeutical procedures, turned granulation phase but did not start to reduce in size (granulating ulcers).

However, it remains unclear why some ulcers heal whereas others are recalcitrant. Therefore, the study of the wound bed microenvironment, and in particular the wound fluid (WF), could represent an important source of biomolecular information, and explain how the disruption of the normal healing process results in ulcer chronicity.

WF consists of fluids, electrolytes, and proteins (enzymes, proteases, and constitutive proteins) derived from the cellular milieu and plasma; its composition is further influenced by pathological processes occurring at the injury site, as such intracellular components are released after cellular damage, cellular and ECM proteins are processed by proteases, and inflammatory mediators and reactive oxygen species are produced by infiltrating leukocytes. In essence, WF reflects the cellular and metabolic events leading to the progression of the wound and/or its healing [19–21].

Several studies [22–32] evaluating the expression and concentration of cytokines and chemokines in chronic ulcer microenvironment suggested the existence of a marked pro-inflammatory condition in non-healing VLU. This pro-inflammatory condition decreases according to the shift from non-healing toward the healing/granulating phase.

Nevertheless, the total amount of each cytokine detected in WF does not necessarily reflects its bioactivity, which can be further influenced by the presence of counteracting anti-inflammatory cytokines, specific cytokine inhibitors, or soluble and membrane-bound receptors which could mask their bioavailability. Moreover, the cytokines could be processed by proteolytic enzymes affecting their activity [25,33].

Several in vitro studies evaluated the effect of WF from chronic and acute wounds added to cell cultures, highlighting that chronic WF showed pro-inflammatory properties [34], decreased cellular mitogenic activities related with growth factor degradation by MMPs [33], and was able to increase MMP expression by cultured dermal fibroblasts [35]. On the contrary, the addition of wound fluids from healing wounds to 3T3 fibroblasts caused a significant increase in proliferation [22], suggesting that healing wounds are characterized by an improved extracellular environment, which is more conductive for the healing response. As a consequence, delayed healing of chronic wounds is not related to a failure in the stimulation of inflammatory and growth factor responses, but to their increased synthesis coupled with a proteolytic imbalance.

On the basis of the underlying chronic inflammatory response, identifying any alteration in growth factor, cytokine and chemokine composition in chronic wounds could potentially help in the development of novel, as well as accurate and objective tests for predicting chronic wound outcome before the appearance of the clinical signs and to provide novel biochemical therapeutic targets.

Since macrophages are one of the major sources of mediators driving the entire healing process, the possibility to better understand and pharmacologically modulate the biochemical and cellular responses induced by different ulcer microenvironments could represent an important tool to promote wound closure [36].

In this respect, we previously investigated the effect of Sulodexide (Alfa Wassermann, Bologna, Italy) in suppressing the inflammatory response in monocyte exposed to lipopolysaccharide (LPS) stimulation [37]. Sulodexide is a mixture of glycosaminoglycans used in the treatment of a number of vascular disorders (e.g. intermittent claudication, peripheral arterial occlusive disease and post-myocardial infarction, post-thrombotic venous disease, and VLU) [38,39], for its established antithrombotic and pro-fibrinolytic activities, as well as for the anti-inflammatory and endothelial-protective effects [40–44]. A body of evidences supported its use as adjuvant therapy in combination with local wound care and bandages for improving healing in patients with persistent venous leg ulcers [4,45–49].

For these reasons, the aims of the present work addressed the following areas: (I) to characterize the inflammatory cytokine profile of WF from inflammatory and granulating VLU, and comparing WF to plasma samples, in order to find early biomarkers of the healing status; (II) to investigate the effects of WF on THP-1 monocyte immune response, with respect to LPS-stimulation; (III) to study the immune-modulatory properties of Sulodexide on LPS- and WF-activated monocytes.

Understanding the difference in cytokine and chemokine profiles in WF during different stages of VLU healing, and how WF affects monocyte activation and cytokine production, will provide insights into the pathophysiology of VLU as well as the identification of potential biomarkers of healing and therapeutic targets. In addition, information from Sulodexide modulation of LPS and WF-treated monocytes, will be imperative to advance our knowledge in the clinical treatment of VLU with therapeutic approach during both the inflammatory and granulating phases of healing [2,38].

2. Materials and methods

2.1. Patient selection and recruitment criteria

Thirty-four patients affected by non healing VLU and admitted to the hospital to undergo surgical debridement and skin grafting, were recruited for studying the cytokine profile of the WF and plasma.

Inclusion criteria: patients of both sexes, older than 18 years, with primary or secondary venous disease, with chronic, non-healing venous leg ulcer both at first episode and relapsing.

Exclusion criteria were: age <18 years, pregnant or breast feeding women, presence of arterial disease, renal insufficiency, insulin-dependent diabetes mellitus, vasculitis, autoimmune disease, cortisone or immunosuppressant or hormonal therapies, previous venous surgery or sclerotherapy.

Data on the medical history of all patients had been recorded, and clinical and duplex ultrasound venous examinations had been performed. Venous pathophysiology was identified according to the clinical aspect and confirmed with Duplex scanning examination. Duplex was performed in the standing position with the weight on the contralateral leg. Venous reflux was elicited by means of calf compression-release maneuver, and diagnosed when venous reflux was >0.5 s in the superficial venous system and >1 s in the deep venous system [50]. The presence of thrombosis was evaluated with compression ultrasounds.

Chronic venous disease was classified according to the CEAP classification [3].

Wound fluids were collected at the initial admission to the hospital when VLU was divided in two groups: I) inflammatory (Infl) and II) granulating (Gran) ulcers. When granulation tissue appeared following sharp debridement during the hospitalization wound fluid examination was repeated. In these cases we performed two wound fluid examinations both at the beginning in the inflammatory stage, and after debridement in granulating phase. Skin grafting or foam sclerotherapy was eventually performed after wound fluid sampling.

All patients underwent biopsy of the ulcer bed to perform quantitative bacterial analysis, and pain was assessed with a visual analog scale (VAS), which rates pain intensity on a scale from 0 to 10, where 0 = no pain; 1–3 = mild pain; 4–6 = moderate pain, 7–10 = severe pain [51].

A written informed consent was obtained from all patients. The study was approved from the local ethics committee (both Barbantini Clinics of Lucca and University "Carlo Bo" of Urbino) and was also in accordance with ethical standards of the Helsinki Declaration of 1975, as revised in 2000.

2.2. Chronic venous ulcer wound fluid protocol

WF was collected by applying cotton gauze to the ulcer bed until saturated, WF-embedded gauze was transferred in a collecting tube without additives or antiproteases, and then centrifuged at 10,000 ×*g*. The supernatant was stored at −80 °C until further analysis. Because WF is the unit of measure in this study, in some cases, patients with an Infl wound that then became Gran, had WF sampled for both wound types.

2.3. Cell culture and treatments

Human monocytic THP-1 (ATCC® TIB-202™) cell line obtained from American Type Culture Collection (Manassas, VA, USA) was grown in standard culture conditions (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% antibiotics) and maintained at 37 °C in humidified air with 5% CO_2. The experiments were performed in serum-free conditions to avoid the recovery of endogenous bovine serum cytokines.

THP-1 cells were seeded at 1,500,000/mL and treated for 18 h with Sulodexide (0.12 LSU/mL) or LPS 10 μg/mL for 8 h, or a combination of LPS plus Sulodexide (LPS 10 μg/mL, 8 h, followed by removal of the culture media, and addition of culture media supplemented with Sulodexide 0.12 LSU/mL for 18 h) to evaluate the effects of Sulodexide on the extracellular release of cytokines both in basal conditions and after the LPS- proinflammatory stimulation.

In addition, THP-1 cell line was stimulated with WF collected from patients with Infl or Gran venous leg ulcers. For this purpose, aliquots (with the same volume) from ten randomly selected samples of WF from each group (n = 10 Infl and n = 10 Gran WF) were pooled. After filtration with 0.45 μm tissue culture filter unit to remove large cell debris and bacteria, both the pooled Infl and Gran WF were immediately diluted in serum-free RPMI 1640 culture media to a final concentration of 5% v/v, where treated cells were grown for 24 h, in the presence or absence of Sulodexide co-treatment to a final dose of 0.12 LSU/mL. Cell viability was assessed by trypan blue exclusion test. Each experiment on serum-free conditioned medium was performed in triplicate in at least two independent experiments.

2.4. Magnetic multiplex immunoassay

Cytokine concentration in WF, plasma and serum free culture media were determined through the 27-plex panel of Pro™ Human Cytokine 27-plex Assay (including: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin/CCL11, bFGF, G-CSF, GM-CSF, IFN-γ, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-bb, RANTES/CCL5, TNF-α, VEGF), a multiplex suspension immunomagnetic assays, based on the use of fluorescently dyed magnetic beads covalently conjugated with monoclonal antibodies specific for the target proteins, according to the manufacturer's instructions (BioPlex, Bio-Rad Labs, Hercules, CA, USA).

Levels of all analytes were determined using a Bio-Plex 200 array reader, based on Luminex X-Map Technology (Bio-Rad Labs, Hercules, CA, USA) that detects and quantifies multiple targets in a 96-well plate with a single small fluid volume. Data were collected and analyzed using a Bio-Plex 200 instrument equipped with BioManager analysis software (Bio-Plex Manager Software v. 6.1). The protein concentrations (expressed as pg/mL) were calculated through a standard curve. Although the commercially available kit of cytokines allow to analyze several biological fluids other than plasma, to exclude in assays the possible WF 'matrix' artifacts caused by possible interference substances, we serially diluted randomly selected WF samples, reanalyzing them for the response linearity. According to the manufacturer's data, the lower detection limit was 0.6 pg/mL, while the mean inter-assay variability was 7.6%.

2.5. Chemicals

Sulodexide was provided by Alfa Wassermann (Bologna, Italy). All chemicals of reagent grade and lipopolysaccharide (LPS from *Escherichia Coli*, code L3129) was obtained from Sigma (Milan, Italy), whereas the sterile compounds for cell culture were from JET BIOFIL Bio-filtration Products Co (Guangzhou, China), and chemicals and reagents for cell culture were from Carlo Erba Reagents S.r.l. (Milan, Italy).

2.6. Statistical analysis

Each variable was expressed as the mean ± standard error of the mean, unless otherwise specified. Statistical analyses were carried out through Fisher Exact test, Mantel-Haenszel χ2, Mann-Whitney, or Student's *t*-test according to variable characteristics. All statistical tests were two-tailed, and significance was set at $p < 0.05$. Data and graphs were analyzed with Prism software for Windows-7, version 3.1 (Graph-Pad, San Diego, CA, USA). We defined this study as pilot preclinical study, therefore we did not determine a power calculation. Accordingly, the results can only be labeled as an exploratory study.

3. Results

3.1. Demographic data

Thirty-four patients with VLU were enrolled in the study. According to the phase of wound healing and to the clinical observation of the ulcer tissues, WF was subdivided into two groups: inflammatory (Infl, n = 32) and granulating (Gran, n = 16).

The biological samples examined were collected from both sexes (19 males and 29 females), with a mean age of 72 years (range: 43–91 years), and presenting ulcers both at first episode (n = 16) or recurrent (n = 32). The mean ulcer duration was 40 ± 47.9 months. The average ulcer size was 12.9 cm^2 (range 0.2–60 cm^2). Complete demographic variables for the study population are presented in Table 1.

3.2. Pain scale

Patient with Infl WF had significantly increased pain scales compared to patients with Gran WF (5.0 ± 0.24 vs. 3.4 ± 0.29, respectively, p = 0.0003)(Table 1).

Table 1
Demographic and clinical characteristics.

Characteristic	Inflammatory	Granulating	p value
Number, n (%)	32 (66.7)	16 (33.3)	
Age range, years	43–91	65–85	
Mean age (±SD), years	69.1 ± 14.8	77.8 ± 6.5	0.03
Median age, years	73	80	0.12
Sex			0.12
Male, n (%)	10 (31.3)	9 (56.3)	
Female, n (%)	22 (68.7)	7 (43.7)	
Comorbidities			
Diabetes, n (%)	10 (31.3)	2 (12.5)	0.29
Hypertension, n (%)	19 (59.4)	15 (93.8)	0.02
Hyperlipidemia, n (%)	18 (56.3)	2 (12.5)	0.01
Smoking, n (%)	6 (18.8)	0 (0)	0.16
Rheumatic disease, n (%)	2 (6.3)	0 (0)	0.55
Ulcer history			0.52
Primary	12 (37.5)	4 (25.0)	
Recurrent	20 (62.5)	12 (75.0)	
Venous insufficiency			0.52
Superficial n (%)	24 (70.6)	14 (73.7)	
Deep, n (%)	5 (14.7)	4 (21)	
Mixed, n (%)	5 (15.7)	1 (5.3)	
Infection	22 (68.8)	0 (0)	<0.001
Duration, months, mean ± SD	48.0 ± 56.6	44.4 ± 85.5	0.79
Surface area, cm^2, mean ± SD	14.6 ± 18.7	9.5 ± 8.0	0.85
VAS score, mean ± SD	5.0 ± 0.24	3.4 ± 0.29	0.0003

VAS: Visual Analog Scale.

3.3. Cytokine profile in venous ulcer wound fluid and plasma samples

A panel of 27 cytokines, including pro-inflammatory and anti-inflammatory cytokines, chemokines, growth factors and colony stimulating factors was used to assess the status of the ulcer microenvironment. These analytes have been measured in all 48 exudates and 10 plasma samples (5 from patients with inflammatory and 5 from patients with granulating venous leg ulcers). We found that all the 27 mediators were detectable in WF, whereas in plasma samples cytokine levels were generally lower, with IL-2 and IL-15 undetectable in all plasma samples.

Comparing the cytokine levels in WF versus plasma obtained from the same patients, it was determined that IL-1β ($p = 0.0040$), IL-1ra ($p = 0.0003$), IL-6 ($p = 0.0002$), IL-8/CXCL8 ($p = 0.0002$), IL-10 ($p = 0.0040$), IL-12 ($p = 0.0295$), IL-17 ($p = 0.0081$), bFGF ($p = 0.0007$), G-CSF ($p = 0.0002$), GM-CSF ($p = 0.0027$), IFN-γ ($p = 0.0047$), MCP-1/CCL2 ($p = 0.0047$), MIP-1α/CCL3 ($p = 0.0093$), MIP-1β/CCL4 ($p = 0.0104$), TNF-α ($p = 0.0003$) and VEGF ($p = 0.0007$) were significantly over-expressed in WF samples, while Eotaxin/CCL11 ($p = 0.0007$), IP-10/CXCL10 ($p = 0.0047$), and RANTES/CCL5 ($p = 0.0207$) were higher in plasma samples (Fig. 1). All numeric data of results are reported in detail in Supplemental file 1.

Comparing the cytokine levels in WF, it was observed that IL-1β ($p = 0.0029$), IL-12 ($p = 0.0002$), IL-10 ($p = 0.0490$), IL-8/CXCL8 ($p = 0.0303$), GM-CSF ($p < 0.0001$), and VEGF ($p = 0.0004$) levels were significantly higher in Infl WF compared with Gran WF (Fig. 2). On the contrary, the chemokines IP-10/CXCL10 ($p = 0.0001$) and RANTES/CCL5 ($p = 0.0009$) and the growth factor PDGFbb ($p = 0.0063$) were found significantly increased in Gran ulcers, compared with Infl ulcers (Fig. 2). All numeric data of results are reported in detail in Supplemental file 2.

3.4. Effects of Sulodexide on LPS-activated THP-1 monocytes

The treatment of THP-1 cell line for 8 h with LPS 10 μg/mL, a well known inflammatory stimulus able to activate monocytes, revealed that almost all of the cytokines and chemokines were increased in cell culture media from LPS-treated THP-1 monocytes, compared to untreated control cells.

Of significance, IL-1β, IL-6, MIP-1α/CCL3 and MIP-1β/CCL4 showed the highest increase in LPS-stimulated THP-1 compared to untreated control cells (>2.5-fold with respect to control cells, with $p = 0.0001$, $p = 0.0002$, $p < 0.0001$, and $p = 0.0007$, respectively) (Fig. 3A, C).

As reported in Fig. 3A–C, IFN-γ, TNF-α, IL-1ra, IL-4, Eotaxin/CCL11, IP-10/CXCL10, MCP-1/CCL2 and PDGFbb were increased in LPS-treated cells compared to controls (range 1.2–2.5-fold of increase compared to controls; $p = 0.0058$, $p = 0.0086$, $p = 0.0013$, $p = 0.0325$, $p = 0.0165$, $p = 0.0115$, $p = 0.0266$ and $p > 0.05$, respectively).

We then assessed the effects of Sulodexide on LPS stimulated or unstimulated THP-1 monocytes. First, the levels of cytokines measured in serum free culture media from THP-1 cells treated with only

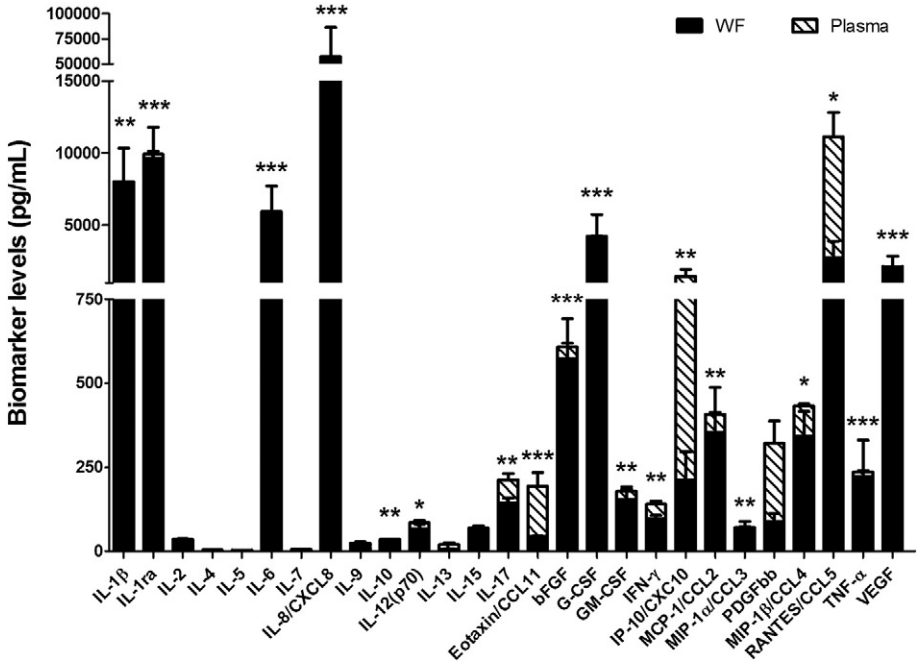


Fig. 1. Levels of cytokines, chemokines, growth factors and colony stimulating factors in WF (n = 10) and plasma (n = 10) samples from the same patient. Values are expressed as pg/mL of the mean ± SEM (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).

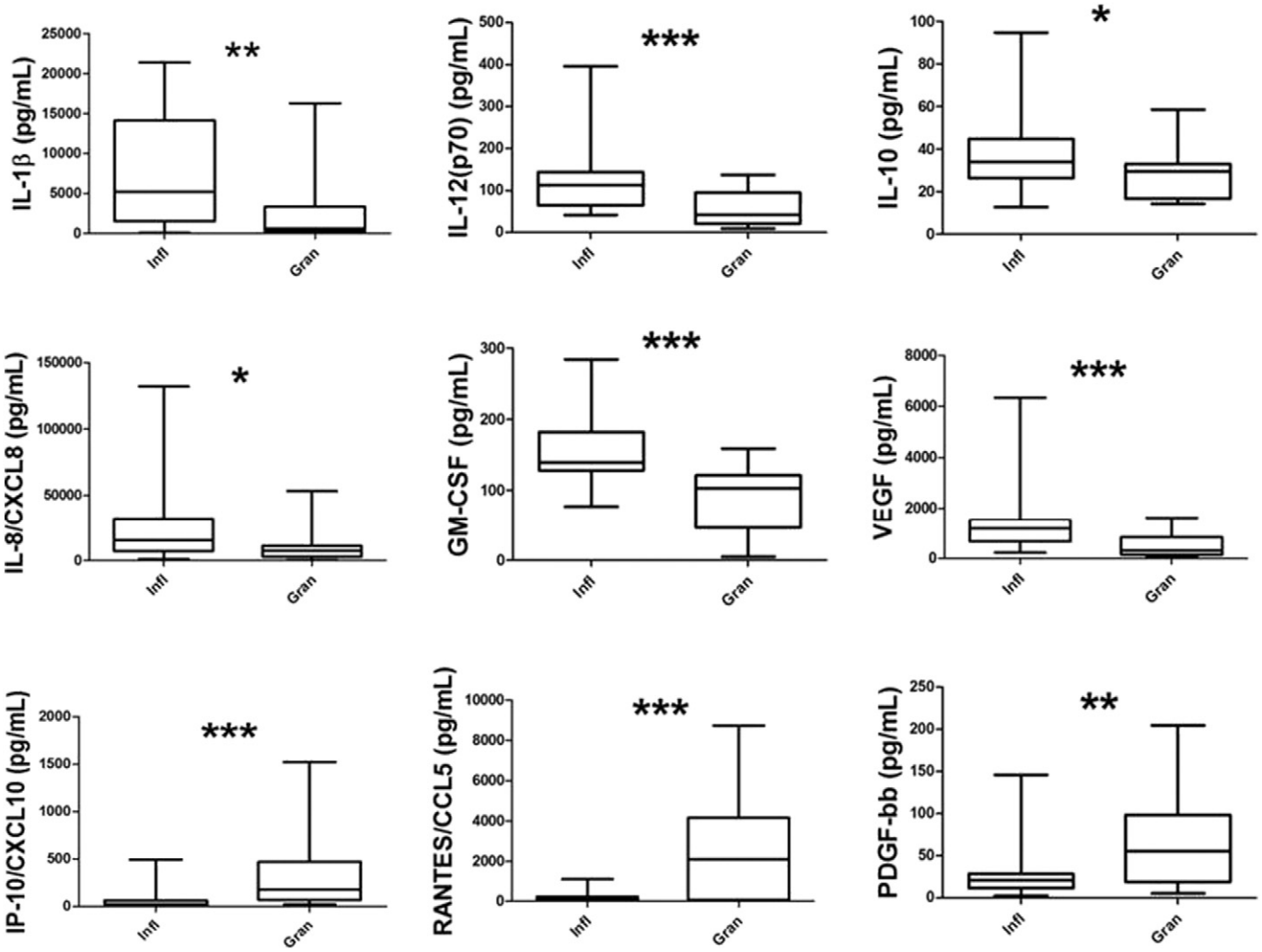


Fig. 2. Levels of cytokines, chemokines, growth factors and colony stimulating factors in inflammatory (Infl, n = 32) and granulating (Gran, n = 16) venous ulcer wound fluids. Values are expressed as pg/mL (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).

Sulodexide 0.12 LSU/mL for 18 h, were not significantly different from the levels observed in control cells (data not shown). For what concerns the treatment of LPS-stimulated THP-1 monocytes with Sulodexide (0.12 LSU/mL) for 18 h, we observed that IL-1β, IL-7, and RANTES/CCL5 were found to be significantly reduced (>40%) after Sulodexide treatment, compared to LPS alone ($p = 0.0007$, $p = 0.0056$, and $p = 0.0038$, respectively). Compared to controls, RANTES decreased by >30% ($p = 0.0014$, Fig. 3A, C).

However, IL-1β levels decreased with Sulodexide treatment ($p = 0.0007$), but remained higher than those found in untreated THP-1 (approximately 2.5-fold, $p = 0.0019$). Similarly, IL-6, TNF-α, IL-1ra, Eotaxin/CCL11, MIP-1α/CCL3 and PDGFbb were found decreased (range 20–40%) in LPS + Sulodexide treated cells with respect to LPS-treated cells ($p = 0.0013$, $p = 0.0084$, $p = 0.0015$, $p = 0.0291$, $p = 0.0008$, and $p = 0.0421$, respectively, Fig. 3).

After the treatment with Sulodexide the levels of IL-6 and MIP-1α/CCL3 remained 2.5-fold higher than controls ($p = 0.0024$, and $p = 0.0014$, respectively, Fig. 3).

In addition, IFN-γ, MIP-1β/CCL4, IL-4 and IP-10/CXCL10 levels were found reduced in LPS + Sulodexide-treated cells compared with LPS-stimulated cells ($p = 0.0101$, $p = 0.0223$, $p > 0.05$, and $p > 0.05$, respectively, Fig. 3), with IP-10/CXCL10 and MIP-1β/CCL4 levels in LPS + Sulodexide-treated cells higher (>25%) than those found in untreated controls ($p = 0.0475$, and $p = 0.0027$, respectively).

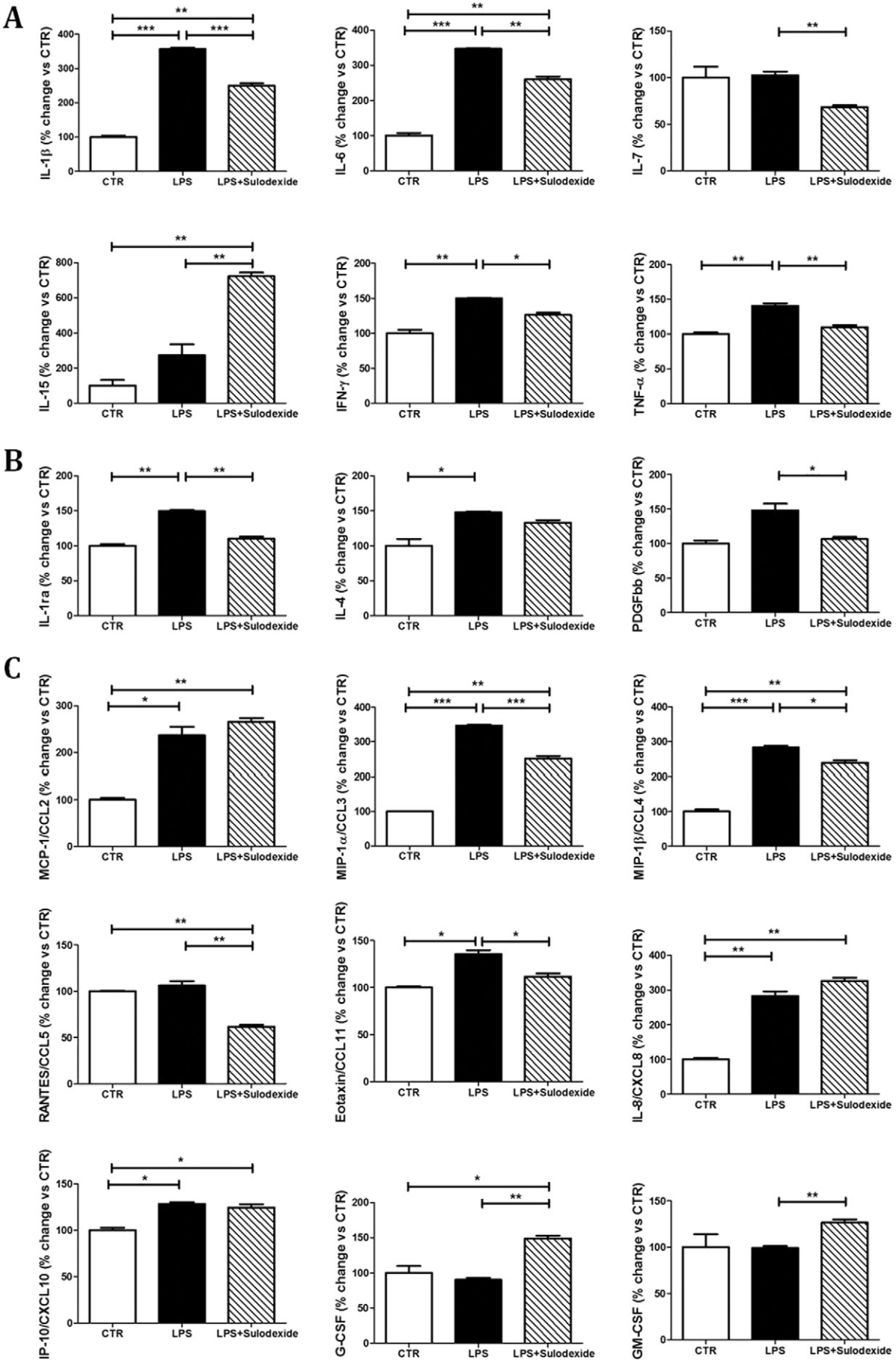
The treatment of LPS-prestimulated cells with Sulodexide, induced a significantly enhanced release of IL-15, G-CSF and GM-CSF compared to both LPS treatment alone ($p = 0.0083$, $p = 0.0012$, and $p = 0.0098$, respectively), and control cells ($p = 0.0015$, $p = 0.0469$, and $p > 0.05$, respectively) (Fig. 3A, C).

3.5. Cytokine profile in WF-stimulated THP-1 cells

The treatment of THP-1 cell line with WF from Infl and Gran venous ulcers led to a significantly increased release of IL-1β (34.4-fold and 20.6-fold, respectively, $p < 0.005$), IL-6 (2.3-fold and 42.6-fold, respectively, $p < 0.05$), IL-8/CXCL8 (7.6-fold and 3.6-fold, respectively, $p < 0.05$), MCP-1/CCL2 (4.7-fold and 3.3-fold, respectively, $p < 0.05$), and IL-15 (3.1-fold and 2.2-fold, respectively, $p < 0.05$) compared to untreated control cells (Table 2).

To a lesser degree, also the pro-inflammatory IL-12, IFN-γ, TNF-α, and the chemokine Eotaxin/CCL11 were found increased in the culture

Fig. 3. Cytokine release profile in LPS- and LPS + Sulodexide-stimulated THP-1 monocytes. A) pro-inflammatory cytokines; B) anti-inflammatory cytokines and growth factors; C) chemokines and colony stimulating factors. Interleukins, chemokines, colony stimulating factors and growth factors are expressed as fold change versus unstimulated THP-1 control cells, referred as 100% (* = $p < 0.05$; ** = $p < 0.005$; *** = $p < 0.0005$).


A
IL-1β (% change vs CTR)
IL-6 (% change vs CTR)
IL-7 (% change vs CTR)
IL-15 (% change vs CTR)
IFN-γ (% change vs CTR)
TNF-α (% change vs CTR)
B
IL-1ra (% change vs CTR)
IL-4 (% change vs CTR)
PDGFbb (% change vs CTR)
C
MCP-1/CCL2 (% change vs CTR)
MIP-1α/CCL3 (% change vs CTR)
MIP-1β/CCL4 (% change vs CTR)
RANTES/CCL5 (% change vs CTR)
Eotaxin/CCL11 (% change vs CTR)
IL-8/CXCL8 (% change vs CTR)
IP-10/CXCL10 (% change vs CTR)
G-CSF (% change vs CTR)
GM-CSF (% change vs CTR)
CTR
LPS
LPS+Sulodexide

media of THP-1 cells treated with both Infl and Gran WF with respect to control cells (range 1.2–1.9 fold, $p < 0.05$, Table 2).

Similarly, the levels of the anti-inflammatory cytokines IL-1ra, IL-4, IL-10 and IL-13 (range 1.2–1.5-fold, $p < 0.05$) and the growth factors PDGFbb and VEGF (range 1.4–2.5-fold, $p < 0.05$) showed a lower increase compared to untreated cells after stimulation with both Infl and Gran WF (Table 2).

On the other hand, IL-2 was found significantly increased by the stimulation with Infl WF (1.2-fold, $p < 0.05$), whereas the release of IL-5, G-CSF and RANTES/CCL5 was significantly induced by Gran WF (1.2-, 6.7- and 1.4-fold, respectively, $p < 0.05$) (Table 2).

All the other mediators were not significantly different in WF-treated compared to untreated control cells (Table 2).

In order to better understand which molecules contained in WF were able to modulate the behavior of human THP-1 monocytes and which of them could be targeted by Sulodexide treatment, we compared the levels of each analyte found in the culture media from Infl versus Gran WF-stimulated cells, after subtracting the level measured in WF to consider only the fraction of biomolecules released by THP-1 cells and avoid interferences with those of WF.

In this respect, we found that levels of the pro-inflammatory cytokines IL-1β, IL-12, IL-15, IL-17, IFN-γ, TNF-α, the chemokines IL-8/CXCL8, Eotaxin/CCL11 and MCP-1/CCL2, as well as GM-CSF and IL-4 were significantly higher in human monocytes treated for 24 h with Infl WF, compared to THP-1 stimulated with Gran WF ($p < 0.05$) (Table 2). However, treatment of THP-1 cells with granulating WF led to a significant increase of IL-6, RANTES/CCL5 and IL-5 with respect to inflammatory WF-treated cells ($p < 0.05$) (Table 2).

Table 2
Cytokine, chemokine, growth factor and colony stimulating factor fold changes in inflammatory (Infl) and granulating (Gran) WF treated THP-1 with respect to untreated control cells.

	Analyte	Infl vs. CTR (p)	Gran vs. CTR (p)	Infl vs. Gran (p)
Pro-inflammatory cytokines	IL-1β	↑↑↑ (**)	↑↑↑ (**)	↑ Infl (*)
	IL-2	↑ (*)	↑	
	IL-6	↑ (*)	↑↑↑ (*)	↑↑↑ Gran (*)
	IL-7	↑	↓	
	IL-12(p70)	↑ (*)	↑ (*)	↑ Infl (*)
	IL-15	↑ (*)	↑ (*)	↑ Infl (*)
	IL-17	↑	↓	↑ Infl (*)
	IFN-γ	↑ (*)	↑ (*)	↑ Infl (*)
	TNF-α	↑ (*)	↑ (*)	↑ Infl (*)
Anti-inflammatory cytokines	IL-1ra	↑ (*)	↑ (*)	
	IL-4	↑ (*)	↑ (*)	↑ Infl (*)
	IL-5	↓	↑(*)	↑ Gran (*)
	IL-9	↑	↑	
	IL-10	↑ (*)	↑ (*)	
	IL-13	↑ (*)	↑(*)	
Chemokines	IL-8/CXCL8	↑↑ (*)	↑ (*)	↑ Infl (*)
	Eotaxin/CCL11	↑ (*)	↑ (*)	↑ Infl (*)
	IP-10/CXCL10	↓	↑	
	MCP-1/CCL2	↑ (*)	↑ (*)	↑ Infl (*)
	MIP-1α/CCL3	↓↓↓	↓↓↓	
	MIP-1β/CCL4	↓	↑	
	RANTES/CCL5	↓↓	↑ (*)	↑ Gran (*)
Colony stimulating factors	G-CSF	↑	↑↑ (*)	
	GM-CSF	↑	↓	↑ Infl (*)
Growth factors	bFGF	↑	↑	
	PDGFbb	↑ (*)	↑ (*)	
	VEGF	↑ (*)	↑ (*)	

↑↑↑ ≥ 10-fold; ↑↑ = 5–10-fold; ↑ ≤ 5-fold.
↓↓↓ ≥ 0.5-fold; ↓↓ = 0.5–0.2-fold; ↓ ≤ 0.2-fold.
** = $p < 0.005$; * = $p < 0.05$.

3.6. Effects of Sulodexide on WF-stimulated monocytes

The co-stimulation of THP-1 cells with 5% WF from Infl venous ulcers and Sulodexide 0.12 LSU/mL revealed that Sulodexide treatment was able to significantly reduce the release of IL-2, IL-12(p70), IL-10 and VEGF ($p < 0.05$, Fig. 4) compared with Infl WF treatment alone. Furthermore, we observed that the co-treatment of monocytes with Sulodexide and Gran WF led to a significant increased secretion of IL-5, IL-17 and GM-CSF ($p < 0.05$) compared to stimulation with only Gran WF (Fig. 4).

4. Discussion

Non-healing wounds result from a variety of cellular activities occurring in the wound microenvironment and interfering with the healing process, delaying the formation of granulation tissue [52]. Therefore, the need for a better comprehension of the biomolecular basis of VLU, together with the need to validate biomarkers able to discriminate ulcer wounds according to their healing phase has led to researches focusing on WF samples mirroring the wound microenvironment.

Several studies hypothesized that chronic wounds are blocked in a persistent inflammatory stage, characterized by excessive white blood cell infiltration, inflammatory mediator release and proteolytic extracellular matrix remodeling. However, the biochemical and molecular switches governing the progression from inflammatory to granulating phase still remains unknown and/or a matter of debate [2,53].

In this regard, we demonstrated that most of the soluble inflammatory molecules (IL-1β, IL-1ra, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-17, bFGF, G-CSF, GM-CSF, IFN-γ, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, TNF-α and VEGF) were over-expressed in WF compared with plasma samples from the same patients. This indicates that WF from VLU is an important source of inflammatory mediators, better reflecting the inflammatory alterations occurring within the ulcer milieu, not necessarily corresponding to a systemic inflammation. As a consequence we could confirm that inflammatory reactions represent locally restricted processes which involve the release of cytokines mainly in the wound area [54].

This was also confirmed by our findings that IL-1β, IL-12, IL-8/CXCL8, IL-10, GM-CSF and VEGF were significantly increased in exudates from Infl compared to Gran ulcers. Several studies [22,32,34,55] described that non-healing ulcers are sustained by increased levels of inflammatory mediators, which decrease during the progression to wound closure, supporting our results. These mediators are, in fact, pivotal players involved in coordinating the function of leukocytes, endothelial cells, fibroblasts, and other cell types to regulate vascular permeability, angiogenesis [26,56], cell proliferation and migration into the wound site, [55,57,58] and extracellular matrix deposition.

Despite an abnormal expression and/or activity of cytokines, chemokines and growth factors, these biomolecules are crucially associated with impaired healing [59], and an appropriate inflammatory phase involving both inflammatory cells and immune mediators is the *sine qua non* condition needed for the early phases of wound repair [32,60,61].

Importantly, levels of IL-10 have been previously compared only in ulcer tissue vs. skin [62–64], demonstrating increased levels of IL-10 in healing WF. The over-expression of IL-10 in Infl WF may represent a response to the high inflammatory state in these wounds, and the VLU environment attempting to compensate by expressing anti-inflammatory cytokines.

The differences in cytokines and chemokines and the degree of inflammations are reflected clinically, in that patients with Infl WF had significantly higher pain scores.

To our knowledge, this is the first description of such a phenomenon in two different VLU wound types, and likely is a result of the differences of inflammatory and anti-inflammatory biomolecules present in the WF.

In fact, pain associated with VLU is induced by the inflammatory reaction in response to wounding, by wound complications (e.g. infection) and by damaged peripheral nerves [65].

In this regard, it has been widely demonstrated that after tissue injury and inflammation, nociceptors are directly sensitized by classical

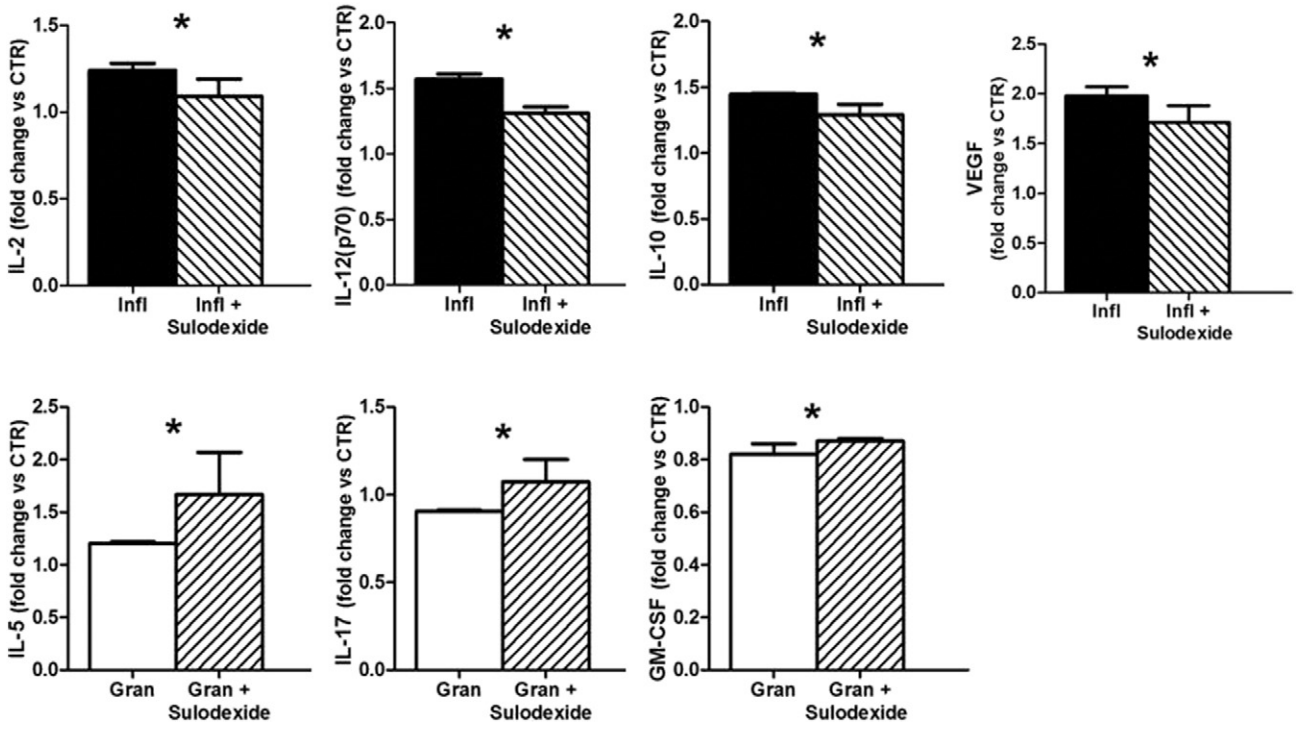


Fig. 4. Cytokine release profile in WF- and WF + Sulodexide-stimulated THP-1 monocytes. Interleukins, chemokines, colony stimulating factors and growth factors are expressed as fold change versus unstimulated THP-1 control cells, referred as 100% (* = $p < 0.05$).

nociceptive mediators (e.g. prostaglandins, and sympathetic amines), and both directly and indirectly by cytokines/chemokines. In fact, some cytokines (IL-1β, TNF-α, IL-6, IL-8/CXCL8, and IL-12) are indirectly involved in hyper-nociception through the activation of COX-2 (and its product prostaglandins) and sympathetic amines [66]. On the other hands, several studies highlighted that pro-inflammatory cytokines and chemokines induce inflammatory and neuropathic pain through direct receptor-mediated actions on afferent nociceptive sensory neurons; these effects are mainly related to the activation of second messengers, modification of ion currents, and change of the excitability [67].

Furthermore, although the inflammatory response seemed to be spatially limited to the wound microenvironment, the biochemical signals necessary for wound closure were also spread to the systemic circulation. In fact, it is noteworthy that RANTES/CCL5, IP-10/CXCL10, and PDGFbb, which were significantly increased in WF from granulating/healing VLU and which might be useful biomarkers of healing [68,69], were also the only molecules found over-expressed in plasma samples compared with WF.

Apart from PDGFbb, which has been already associated with ulcer wound closure, representing a mitogenic signal for fibroblasts [70,71], IP-10/CXCL10 and RANTES/CCL5 has been poorly investigated in VLU. RANTES/CCL5 has been previously associated to an enhanced inflammatory response in VLU, with levels decreasing as the wound proceed to heal [62]. However, more recently it has been reported that RANTES/CCL5, as well as IP-10/CXCL10 and Eotaxin/CCL11, represent crucial chemokines involved in the regulation of white blood cell and endothelial cell precursor mobilization from bone-marrow and dermal tissue in the injury site (necessary "homing process" for tissue repair and to control wound neovascularization) [72–75], and as a potent stimuli for the synthesis of type I collagen and hyaluronan by dermal fibroblasts [72,76].

All these observations pave the hypothesis for a possible double involvement of these mediators, released both locally into the wound site (for granulating phase and tissue repair process) and found systemically in blood circulation (for the homing of recruited precursor cells).

Although from the analyses of WF we could not trace the cell types producing these soluble mediators, it is widely known that monocyte/macrophage cells (one of the most represented cell type infiltrating the ulcer bed) are able to produce a variety of cytokines, chemokines, growth factors, and proteolytic enzymes to guide the evolution of chronic wounds [77,78].

In addition, in response to signals derived from the surrounding microenvironment, monocytes and macrophages undergo reprogramming in distinct functional phenotypes [79,80]. In this respect, we reported that the LPS-induced cytokine production by THP-1 monocytes was characterized by significantly increased levels of IL-1β, IL-6, IFN-γ, TNF-α, IL-1ra, IL-4, IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, and Eotaxin/CCL11 when compared with control cells. Similar findings have been observed through the stimulation of monocytes with WF from inflammatory ulcers.

Our results, probably linked to the observation that many Infl VLU (68.8%) were found bacterial-infected, are in agreement with previous findings [34], suggesting that non-healing WF increased Toll-like receptor (TLR-2 and TLR-4) ligand activity.

In fact, TLR are a group of highly conserved pattern recognition receptors expressed on the cell surface of innate immune cells and non-immune cells of dermis and epidermis. They recognize discrete pathogen molecular patterns, as well as endogenous damage associated molecular patterns released after tissue and cellular damage, and trigger specific pro-inflammatory responses and cytokine release coordinating the wound healing process [81].

Overall, these observations suggested that chronic wounds are characterized by an inflammatory microenvironment, where proteins, enzymes, toxins and TLR-ligands derived from contaminating bacteria alter the biochemical profile of the wound microenvironment, leading to abnormal/non resolving inflammatory responses [34,82]. Of clinical importance, TLR antagonist may be important in VLU treatment and healing, but further research and clinical trials would be required.

According to the recent international guidelines reporting Sulodexide as one of the main therapeutic approaches for VLU [4], and due to the high plasticity of monocyte/macrophage to change phenotype on the basis of

the local stimulation, we decided to administer Sulodexide after LPS-exposure to simulate in vitro the inflammatory activation of monocytes occurring in vivo during CVI.

Our results demonstrated that Sulodexide was able to reduce the release of many important pro-inflammatory cytokines (interleukins, TNF-α, IFN-γ) and chemokines (MIP-1α, MIP-1β, RANTES) and promote the secretion of CSFs. These data suggest that Sulodexide can modulate the expression of cytokines and chemokines in monocytes, and may be important in the mechanisms in healing VLU [37].

Noteworthy was also to determine how Infl and Gran WF affect monocyte activation and subsequent release of biomolecules, and if Sulodexide is able to mitigate the cytokine and chemokine production from WF-stimulated monocytes. We observed that Sulodexide can down-regulate the secretion of IL-2, IL-12, IL-10 and VEGF in Infl WF-treated THP-1, and up-regulate the release of IL-5, IL-17, and GM-CSF in Gran WF-stimulated THP-1, highlighting new potential roles of Sulodexide in VLU healing.

It has been previously demonstrated that heparin showed an anti-inflammatory effect due to the inhibition of the LPS-binding to the CD14 surface receptor in monocytes[83], thus reducing the release of cytokines.

With respect to this study, we added Sulodexide in THP-1 culture media after the removal of the culture media containing LPS, therefore the reduced release of cytokine by Sulodexide was not related to the possible inhibition of the LPS-receptor interaction, since the LPS has already activated its receptor when Sulodexide was added. This would indicate that Sulodexide effects on LPS-stimulated monocytes, could be hypothetically linked to: 1. Downstream inhibition of LPS:CD14 signaling. 2. LPS ligand-receptor uncoupling. 3. Direct inhibition of cytokine transcription and/or translation. 4. Post-translational inhibition. 5. Inhibition of cytokine secretion [37,38,43,44,84].

It is well known that glycosaminoglycans (GAG) exhibit pleiotropic-like effects during inflammatory conditions, depending on the GAG type, sulfated moieties and concentration. In fact, low molecular weight fragments of GAGs, such as those released during the inflammatory-mediated ECM remodeling, can act as signaling molecules to promote white blood cell recruitment in the site of injury [85], as well as high level of GAGs could counteract the inflammatory responses [86]. Both in vivo and in vitro studies have highlighted the ability of Sulodexide to counteract inflammatory responses, by protecting the endothelial layer, by limiting the activity of inflammatory signals, and inhibiting leukocyte activation [38,44].

The modulation of the cytokine release by monocyte may represent the biochemical basis for the anti-inflammatory properties of Sulodexide. As a consequence, reducing the release of inflammatory molecules related to monocyte infiltration and activation in VLU could improve wound repair, and restoring the balance between inflammatory and anti-inflammatory pathways in ulcer microenvironment.

Moreover, the capability of Sulodexide to modulate the cytokine release from activated monocytes may represent a promising approach to coordinate the activity of the phenotypically [79] and functionally [87] different populations of circulating monocytes.

5. Conclusions

We believe that the wounds classified as Infl vs. Gran had a clear clinical distinction and represented two different states of the healing process of VLU, and hence showed differences in the biomolecules present within each sampling of WF. Temporal relationships of various biomolecules found in WF of different VLU wound states need further clarification. Importantly, future works will be required to determine which biochemical and molecular signals in the inflammatory and granulating phases of VLU repair, are predictive of complete ulcer healing and which are predictive of a persistent inflammatory open wound, setting the basis for a better comprehension of the molecular basis of this chronic vascular disease. Moreover, future studies will be required to determine how Sulodexide is able to down-regulate and/or up-regulate a variety of biomolecules in monocytes/macrophages, and improve the ability to target the proper VLU wound type (Infl vs. Gran) for maximum patient's benefit and enhancing the healing process.

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Transparency document

The Transparency document associated with this article can be found in online version.

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