Daniela Ligi, Bruna Lo Sasso, Chiara Della Franca, Rosaria Vincenza Giglio, Luisa Agnello, Marcello Ciaccio and Ferdinando Mannello*

Monocyte distribution width alterations and cytokine storm are modulated by circulating histones

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Abstract

Objectives: Extracellular histone levels are associated with the severity of many human pathologies, including sepsis and COVID-19. This study aimed to investigate the role of extracellular histones on monocyte distribution width (MDW), and their effect on the release of cytokines by blood cells.

Methods: Peripheral venous blood was collected from healthy subjects and treated with different doses of a histone mixture (range 0–200 μg/mL) to analyze MDW modifications up-to 3 h and digital microscopy of blood smears. Plasma obtained after 3 h of histone treatment were assayed to evaluate a panel of 24 inflammatory cytokines.

Results: MDW values significantly increased in a time- and dose-dependent manner. These findings are associated with the histone-induced modifications of cell volume, cytoplasmic granularity, vacuolization, and nuclear structure of monocytes, promoting their heterogeneity without affecting their count. After 3 h of treatment almost all cytokines significantly increased in a dose-dependent manner. The most relevant response was shown by the significantly increased G-CSF levels, and by the increase of IL-1β, IL-6, MIP-1β, and IL-8 at the histone doses of 50, 100, and 200 μg/mL. VEGF, IP-10, GM-CSF, TNF-α, Eotaxin, and IL-2 were also up-regulated, and a lower but significant increase was observed for IL-15, IL-5, IL-17, bFGF, IL-10, IFN-γ, MCP-1, and IL-9.

Conclusions: Circulating histones critically induce functional alterations of monocytes mirrored by MDW, monocyte anisocytosis, and hyperinflammation/cytokine storm in sepsis and COVID-19. MDW and circulating histones may be useful tools to predict higher risks of worst outcomes.

Keywords: COVID-19; cytokine; histones; monocyte; monocyte distribution width; sepsis.

Introduction

Histones are highly conserved, intranuclear, positively charged proteins, which main functions are associated with the maintaining of chromatin stability and the epigenetic regulation of several cellular processes. Increasing evidence shed light on the presence of further extracellular and extranuclear functions for circulating histones. In particular, extranuclear histones can be found in the cytosol and at the cell surface, where they exert anti-microbial effects and promote cell-mediated apoptosis [1]. Moreover, at the extracellular level, histones can be released freely or as a DNA-bound nucleosome passively from dying cells (particularly during necrosis), and also embedded in extracellular traps (ET) during the ETosis process. In this respect, circulating white blood cells extrude ETs in response to a hostile microenvironment, such as during bacterial and viral infections or in sterile conditions due to the activation of immune cells [2]. Extracellular histones induce cellular damages through (I) a direct cytotoxic effect on endothelial cells; (II) the promotion of platelet activation, aggregation, and thrombin generation, resulting in the development of pro-coagulant platelet, endothelial, and monocyte phenotypes; (III) the activation of white blood cells which release cytokines and ROS(2). It is well established that extracellular histones act as damage-

Daniela Ligi and Bruna Lo Sasso have contributed equally as first authors.

Marcello Ciaccio and Ferdinando Mannello have contributed equally as senior authors.

***Corresponding author: Ferdinando Mannello**, Full Professor, Unit of Clinical Biochemistry, Section of Biochemistry and Biotechnology, Department of Biomolecular Sciences-DISB, University of Urbino Carlo Bo, Via A. Saffi, 2, 61029 Urbino, Italy, E-mail: ferdinando.mannello@uniurb.it. https://orcid.org/0000-0001-8112-6139

Daniela Ligi and Chiara Della Franca, Unit of Clinical Biochemistry, Section of Biochemistry and Biotechnology, Department of Biomolecular Sciences-DISB, University of Urbino Carlo Bo, Urbino, Italy. https://orcid.org/0000-0002-5546-8765 (D. Ligi)

Bruna Lo Sasso, Rosaria Vincenza Giglio, Luisa Agnello and Marcello Ciaccio, Institute of Clinical Biochemistry, Clinical Molecular Medicine and Clinical Laboratory Medicine, Department of Biomedicine, Neurosciences and Advanced Diagnostics, BiND, University of Palermo, Palermo, Italy

associated molecular pattern proteins (DAMPs) and promote pro-inflammatory effects by activating TLR2- and TLR4-mediated signalling [3].

Levels of circulating histones in physiological conditions range from 0.79 to 2.30 μg/mL, but they increase in animals or patients with cancer, inflammation, and infections, suggesting an extracellular role of histones in human diseases [4–6]. In this respect, a growing number of recent studies underlined the involvement of circulating histones in classical bacterial and viral sepsis, emphasizing their potential roles both as triggers and therapeutic targets of diseases. Due to their ability to stimulate inflammatory responses, induce endothelium injuries, and activate coagulation in clinical settings such as bacterial sepsis and COVID-19-associated viral sepsis, histones have raised great attention in the scientific community. In fact, it has been demonstrated that histone levels increase with the worsening of the disease, and thus may be useful to stratify patients at higher risk of mortality [7, 8].

Noteworthy, sepsis infections share common laboratory biomarkers, as well [9, 10]. Currently, great attention is paid to the use of monocyte distribution width (MDW), a measure of monocyte heterogeneity, automatically calculated from the dispersion around the mean of the monocyte population volume in whole blood by last-generation hematology analyzers. MDW is an innovative parameter mathematically based on the measure of specific cell volume index and standard deviation of volume distribution within the monocyte population. MDW measures positional parameters with VCS technology (i.e., volume, conductivity, and scatter), using three independent energy sources simultaneously: direct current impedance to measure cell volume of all cell types; radio frequency opacity, to characterize conductivity for the internal composition of each cell; a laser beam to measure light scatter for cytoplasmic granularity and nuclear structure [11].

MDW has been recently FDA-approved and EC-marked as early sepsis indicator, due to its very early availability during the clinical evaluation when sepsis diagnosis could not be suspected [12] (additional references in Supplementary Material), and recent studies underline its possible prognostic role to stratify COVID-19 patients according to the severity and clinical outcomes [11, 13–19].

Monocytes are pivotal players in the innate immune responses against invading pathogens and their activation is characterized by morphological changes (clinically mirrored by MDW alterations) and the release in biological fluids of inflammatory mediators. When the immune system overreacts to an infection or injury, as during critical conditions of sepsis and COVID-19, it activates a hyperinflammatory reaction, named “cytokine storm”. The dysregulated immune response is sustained by a massive release of proinflammatory cytokines, chemokines, and signaling molecules that furtherly attract immune cells, which secrete more cytokines, recruiting even more activated immune cells, thus fueling a dangerous vicious cycle. Despite recruited to block the initial infective stimulus, immune cells and mediators end up attacking the tissues and organs, leading to multiorgan failure and death.

We recently demonstrated that histones trigger MDW changes, mirroring those observed in COVID-19 and sepsis [20], thus emerging as critical contributors of monocyte activation during these conditions. A limited number of data is available instead on the ability of histones to promote hyperinflammatory responses associated with monocyte anisocytosis. The purpose of this study was to investigate the ability of histones to alter MDW index and promote hyperinflammatory responses in peripheral blood cells.

Materials and methods

Sample collection and blood analyses

Healthy subjects (n=8, age range 31–63) were recruited as volunteers among staff at the Department BiND of the University of Palermo and Department DISB of the University of Urbino. Peripheral venous blood was collected in EDTA-K_3 tubes and processed within 4 h from collection. Routine complete blood cell count and MDW were analyzed on an UniCell DxH900 Hematology Analyzer (Beckman Coulter), according to the routine methods and the manufacturer’s instructions. Automated slide preparation (unit SP-100, DI-60 system workflow, Sysmex) was used to obtain May-Grunwald-Giemsa-stained blood smears. This observational non-interventional *in vitro* study was approved by the local Ethical Committee and all investigations have been conducted according to the Declaration of Helsinki principles.

Peripheral blood samples and *in vitro* treatments

Aliquots of 1 mL of EDTA-K_3 whole-blood from each volunteer were treated with a mixture of commercially available histones, including H1, H2A, H2B, H3, and H4 (histone from calf thymus, Sigma, cod. 10223565001) to evaluate the impact of histones at different times and concentrations (0, 50, 100, and 200 μg/mL) on the MDW characteristics and on the release of cytokines from blood cells. Whole blood samples were maintained for 30 min at 37 °C for inducing a “priming” effect on circulating blood cells and then maintained at RT to follow and analyze the MDW modifications at 0, 30, 60, and 180 min after careful inversion avoiding blood cell sedimentation. After 3 h all blood samples were centrifuged (2,000×*g*, 15 min) to obtain plasma for further analyses.

Cytokine release

All plasma samples obtained after 3 h of histone treatments were assayed to evaluate a panel of 24 inflammatory biomarkers through the 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-1β/CCL4, 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 Bio-Plex Manager analysis software (Bio-Plex Manager Software v. 6.1). The protein concentrations (expressed as pg/mL) were calculated through a standard curve. According to the manufacturer's data, the lower detection limit was 0.6 pg/mL, while the mean inter-assay variability was 7.6%.

Statistical analysis

All statistical tests were performed using GraphPad Prism 9.0. Values are expressed as mean ± standard error mean (SEM), unless otherwise specified, and p values <0.05 were considered significant. Differences among groups were determined using one-way or two-way ANOVA followed by a posthoc test (i.e., Tukey's multiple comparison test). Regression analyses were performed through simple linear regression. Correlations among various biomarkers were tested for significance using the Pearson correlation test.

Results

The treatment of whole blood with increasing doses of histones revealed significantly different profiles of monocyte heterogeneity, ranging from round-shaped and reniform nucleus of normal untreated monocytes (Figure 1A) to a progressively enhanced volume, intracellular vacuolization and granularity, membrane alterations and nuclear structure changes, as observed through blood smears (Figure 1B–D).

The treatment of whole blood with histones results in an MDW increase in a time and dose-dependent fashion. In particular, we observed that MDW values in controls ranged from a minimum of 14.31 and a maximum of 20.70 up-to 3 h, and there were not significant differences among all times considered. The treatment with 50 µg/mL of histones induced a significant time-dependent increase of MDW at 30, 60, and 180 min (mean ± SD) vs. respective controls (20.3 ± 1.2, p<0.0027; 21.3 ± 1.4, p<0.0001; 22.3 ± 1.6, p<0.0001, respectively) (Figure 2A and Supplemental Table 1). This result is also confirmed by the significant regression analysis ($Y=0.02039 \times X + 19.06$; $R^2 = 0.4545$; p<0.0001).

The intermediate dose of 100 µg/mL of histones promoted a significant MDW increase of 21.1 ± 1.7 (p<0.0001) after 30 min, 22.1 ± 1.4 (p<0.0001) after 60 min, and 23.7 ± 2.4 (p<0.0001) at 180 min, as also confirmed by the significant regression line ($Y=0.02745 \times X + 19.25$; $R^2=0.5035$, p<0.0001) (Figure 2A and Supplemental Table 1).

The highest dose of 200 µg/mL of histones promoted an MDW increase of 21.9 ± 1.4 (p<0.0001) after 30 min, 22.8 ± 1.4 (p<0.0001) after 60 min, and 24.2 ± 2.6 (p<0.0001) at 180 min, as also confirmed by the significant regression line ($Y=0.02983 \times X + 19.62$; $R^2=0.479$, p<0.0001) (Figure 2A and Supplemental Table 1).

Interestingly, focusing attention on the effects of increasing doses of histones at each time, we observed that besides the time-dependent increase of MDW for each dose, there was also a significant dose-dependent increase of MDW at each time, as demonstrated by the regression lines reported in Table 1.

These histone-induced MDW modifications were not sustained by a change in the monocyte population count, neither in its total count (Figure 1B), nor in the percentage values (Figure 1C), whose minimal variations were distributed within the physiological ranges (2–12 × 1,000/µL and 0.2–1.2% respectively).

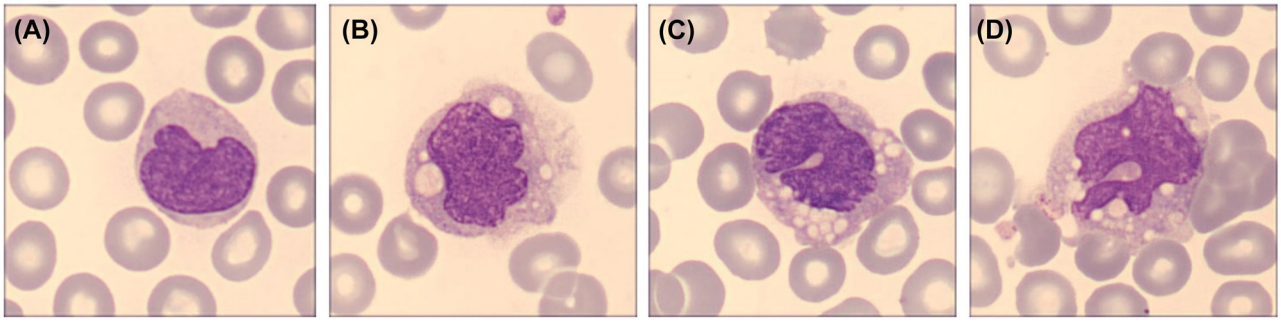

Figure 1: Light microscopy images of peripheral blood monocytes representative of untreated controls (A), HIS 50 µg/mL (B), HIS 100 µg/mL (C), and HIS 200 µg/mL (D) histone-treated whole blood. (May-Grunwald-Giemsa, × 100).

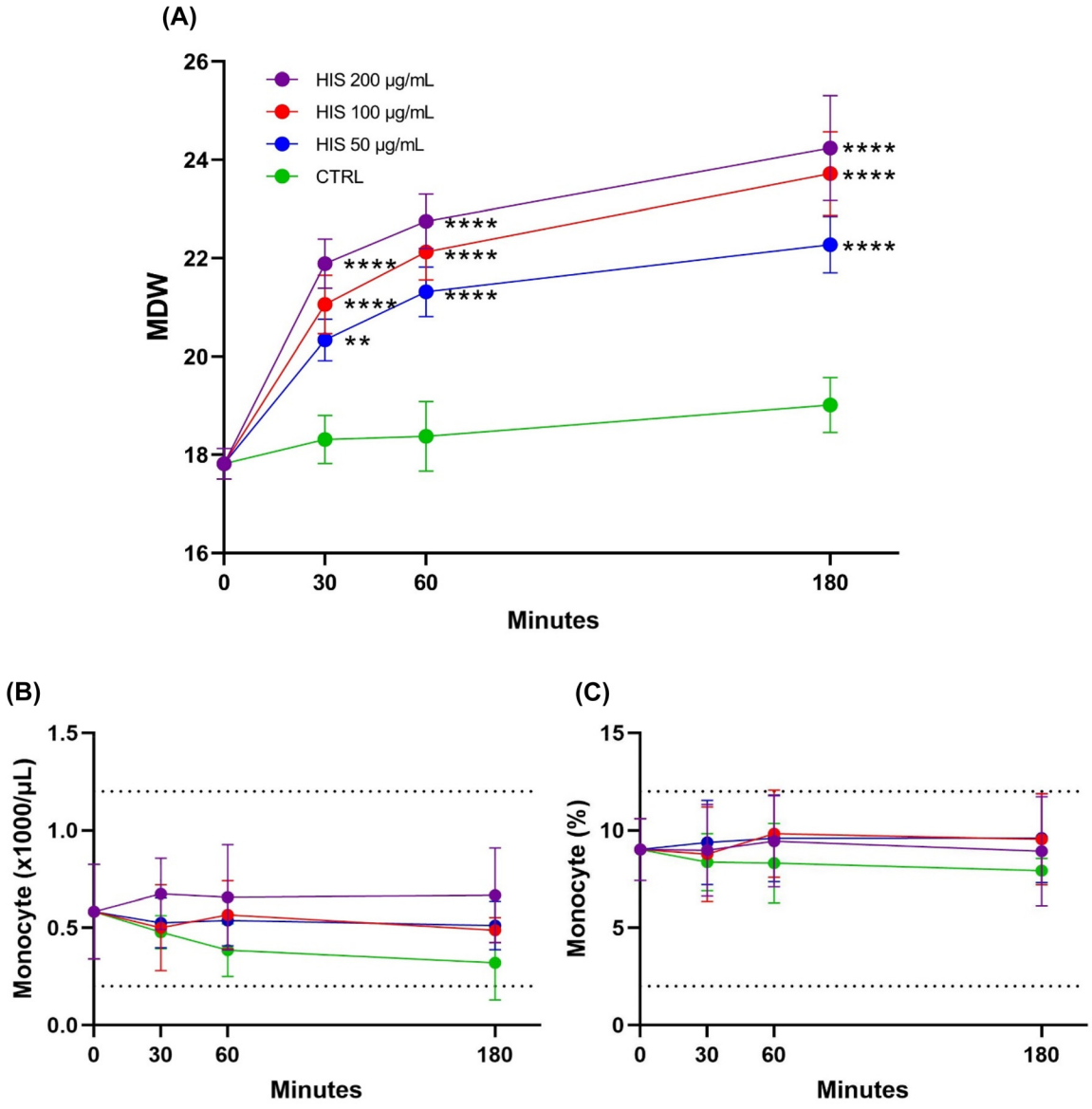

Figure 2: Time- and dose-dependent MDW and monocyte count variations in histone-treated whole blood samples. (A) Time- and dose-dependent MDW index modifications in whole blood samples collected from healthy subjects treated *in vitro* with 0, 50, 100, and 200 µg/mL of histone mixture. Values are expressed as mean ± SEM. (Two-way ANOVA: ** = p: 0.001–0.01; **** = p<0.0001; the comparisons were calculated among treatments and respective controls at each time). (B) Time- and dose-dependent monocyte count (×1.000/µL) and (C) monocyte percentage modifications in whole blood samples collected from healthy subjects treated *in vitro* with 0, 50, 100, and 200 µg/mL of histone mixture. (Two-way ANOVA). The dotted line indicates the cut-off levels.

Table 1: Simple linear regressions exploring dose-dependent MDW changes (time: dependent variable).

	Equation	R^2	p-Value
30 min	Y=0.01649 × X + 18.95	0.4196	<0.0001
60 min	Y=0.01982 × X + 19.44	0.4264	0.0002
180 min	Y=0.02378 × X + 20.37	0.3674	0.0006

After 3 h of treatment with 50, 100 and 200 µg/mL of histones we observed a dose-dependent increase of all cytokines (except for IL-13), as shown in Figure 3 and detailed in Supplemental Table 2.

In particular, we highlighted that G-CSF showed the strongest response to histone treatment; in fact, the levels of G-CSF were significantly increased by 9-fold (p<0.05) after

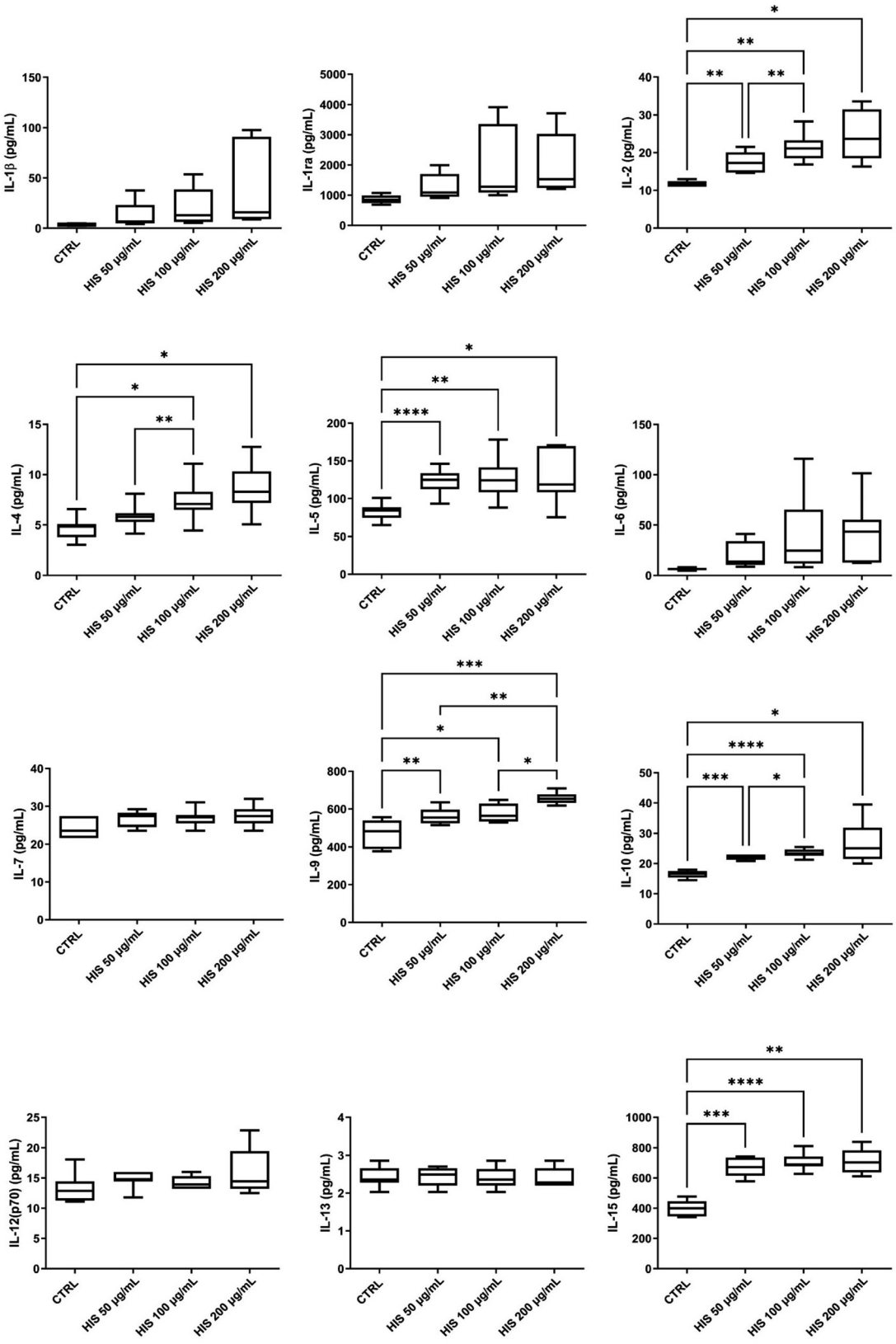

Figure 3: Cytokine values (mean ± SEM) after 3 h of treatment *in vitro* with 0, 50, 100, and 200 µg/mL of histone mixture (one-way ANOVA: *= p: <0.05; **= p: 0.001–0.01; *** =p: 0.001–0.0001; ****= p: <0.0001; comparisons were calculated among treatments and respective controls and among each treatment). Values are expressed as pg/mL.

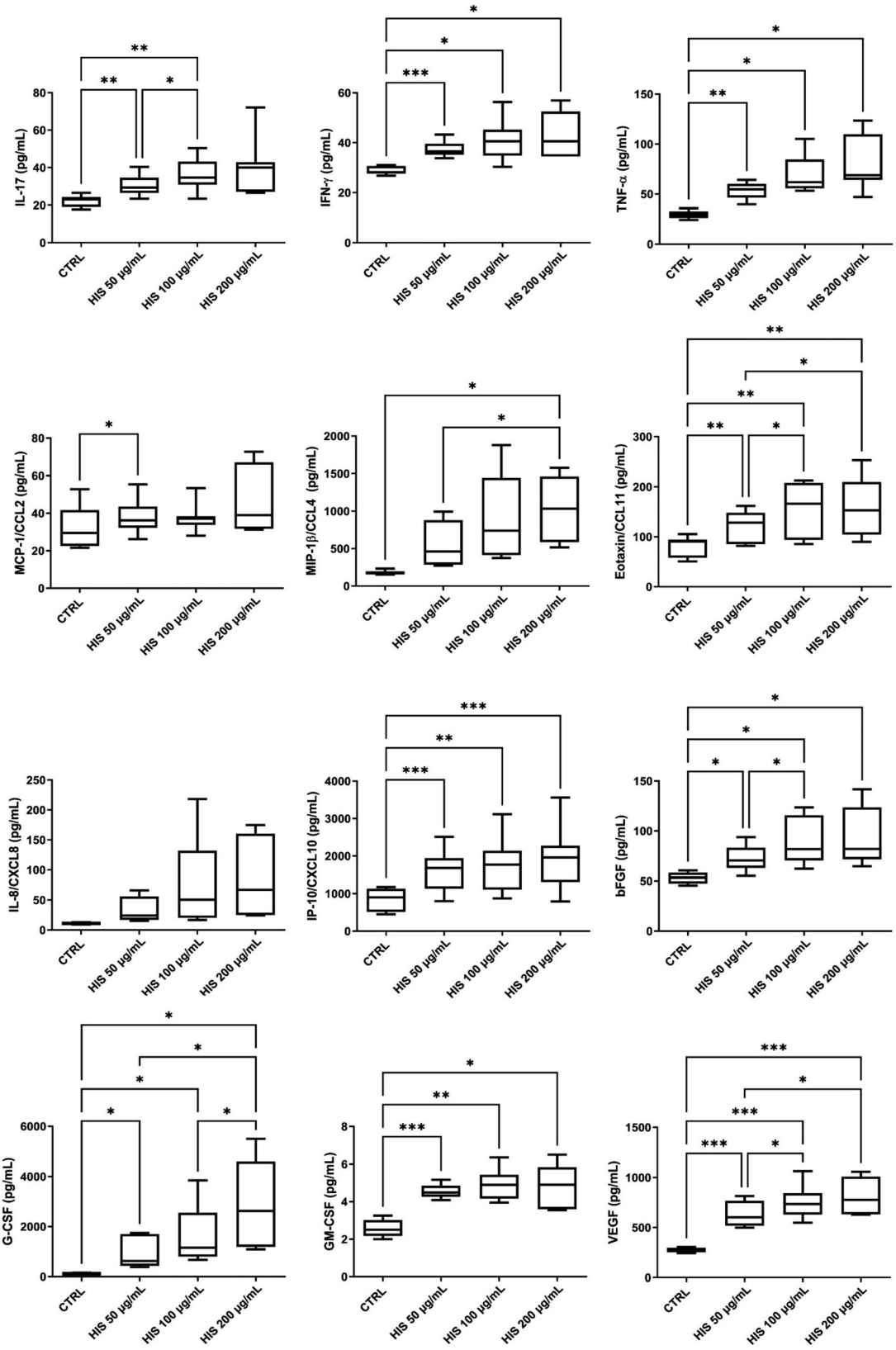

Figure 3: Continued.

treatment with 50 µg/mL of histones, and this trend was almost doubled (about 15-fold, $p<0.05$) with the doubling of the treatment. By using the highest dose of histones, we detected a 27-fold of increase vs. CTR ($p<0.05$). This dose-dependent behaviour is furthermore confirmed by the significant linear regression (Supplemental Table 3).

IL-1β, IL-6, MIP-1β/CCL4, and IL-8/CXCL8 showed a considerable increase of about 3-fold, 6-fold, and 8-fold at 50, 100 and 200 µg/mL, respectively. A significant increase was observed for VEGF, IP-10/CXCL10, GM-CSF, TNF-α, EOTAXIN/CCL11, IL-1Ra, IL-2, and IL-4, after histone treatment with all doses (excepted for IL-1Ra and IL-4 at 50 µg/mL), showing an up-regulation of 1.7-fold, 2.1-fold, 2.4-fold, at 50, 100 and 200 µg/2mL, respectively. A lower but significant increase, ranging from 1.4-fold to 1.6-fold, was observed for IL-15, IL-5, IL-17, bFGF, IL-10, IFN-γ, MCP-1/CCL2, and IL-9. These results are furtherly proven by linear regression analyses, showing significant trends (Figure 3 and Supplemental Table 3).

We furtherly correlated values of each inflammatory cytokine, chemokine, and growth factor measured in plasma samples obtained after 3 h of treatments by Pearson correlation analysis. A plot of all parameters is shown in Figure 4. We found that IL-4, IL-5, IL-6, bFGF, G-CSF, IFN-γ, MIP-1β/CCL4, VEGF, IL-1β, IL-1ra, IL-2, IL-8/CXCL8, and GM-CSF were the biomarkers showing the higher number of significantly positive correlations with other cytokines at the histone dose of 100 µg/mL (Table 2), confirming the presence of an intricate picture of interconnections among proinflammatory and anti-inflammatory cytokines, chemokine and growth factor response induced by histones (further details on the correlations among cytokines in controls, and histone-treated samples are provided in Supplemental Table 4).

Discussion

We demonstrated that extracellular histones can early and increasingly promote morphological changes in the circulating monocyte population similar to those found *in vivo* in classic [21, 22] and viral sepsis [23–25] patients. These alterations are accurately quantified and mirrored by MDW

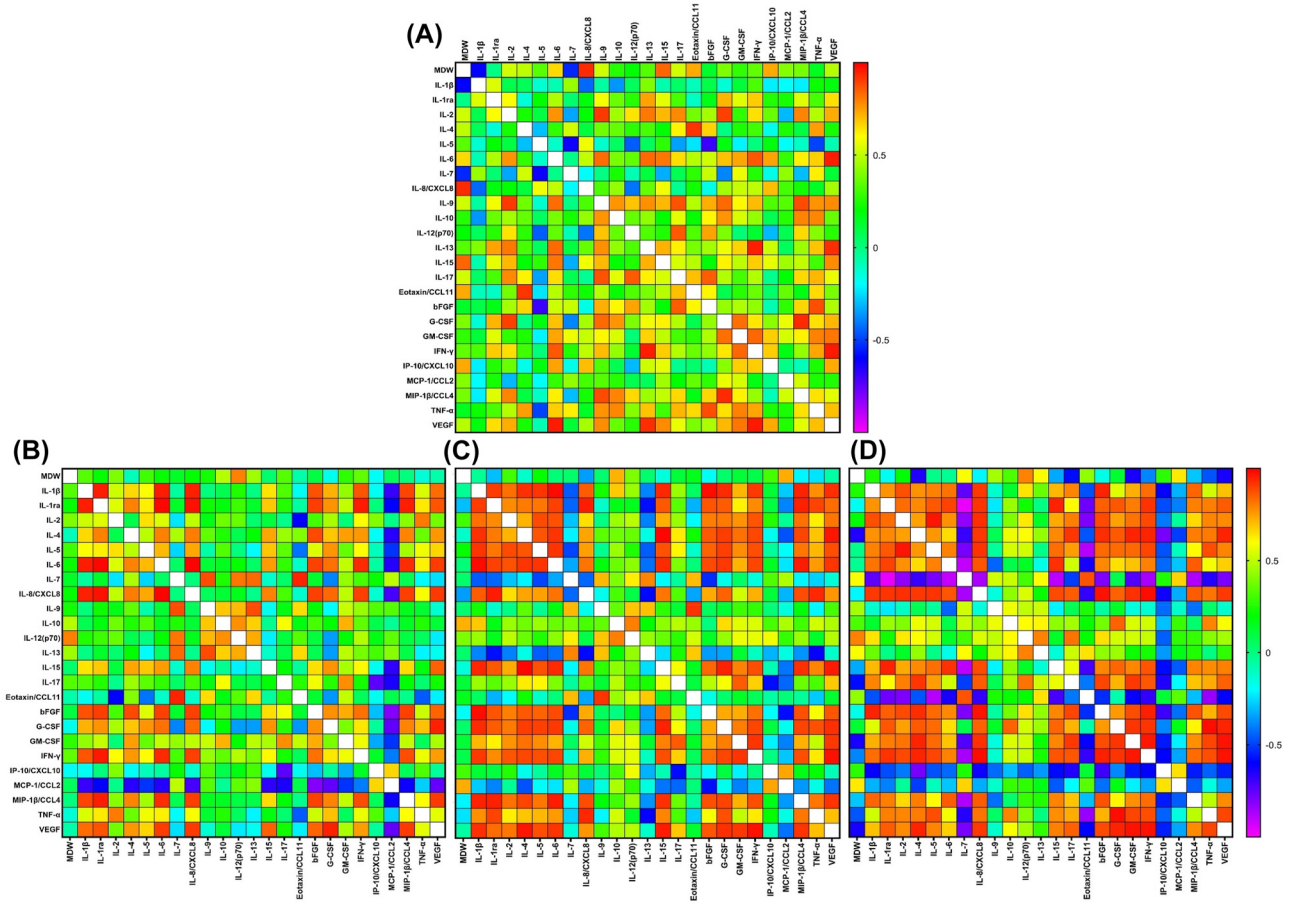

Figure 4: Correlation plot of all parameters determined in this study in controls (A), HIS 50 µg/mL (B), 100 µg/mL (C), and HIS 200 µg/mL (D) of histone-treated samples at 3 h. The plot is based on the Pearson correlation between each pair of biomolecules. On the right, the Pearson correlation coefficient is indicated by the colour gradient.

Table 2: p-Values resulting from Pearson correlation plots of all parameters determined in this study in samples treated 3 h with HIS 100 µg/mL. The dataset is based on the Pearson correlation between each pair of biomolecules.

	MDW	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12(p70)	IL-13	IL-15	IL-17	Eotaxin	bFGF	G-CSF	GM-CSF	IFN-γ	IP-10	MCP-1	MIP-1β	TNF-α	VEGF
MDW		0.9418	0.4722	0.4723	0.7828	0.6244	0.9812	0.9412	0.3931	0.9569	0.0681	0.3774	0.9476	0.7319	0.7964	0.7103	0.6557	0.9425	0.7866	0.8488	0.4619	0.0644	0.9923	**0.0192**	0.6117
IL-1β			**0.0051**	**0.0226**	**0.0030**	**0.0038**	**0.0001**	0.3625	**0.0139**	0.6265	0.1365	0.3884	0.3887	**0.0018**	0.3234	0.9165	**0.0001**	**0.0022**	0.0739	**0.0046**	0.7956	0.5890	**0.0015**	0.7175	**0.0014**
IL-1ra				**0.0225**	**0.0154**	**0.0142**	**0.0025**	0.3226	**0.0002**	0.7776	0.7267	0.7354	0.1330	**0.0060**	0.4083	0.4424	**0.0052**	**0.0065**	0.1025	**0.0220**	0.8188	0.3835	**0.0051**	0.6213	**0.0003**
IL-2					**0.0463**	**0.0038**	**0.0048**	0.5259	0.0817	0.9875	0.3228	0.2603	0.3289	**0.0405**	0.2774	0.6432	**0.0177**	**0.0157**	**0.0256**	**0.0052**	0.5439	0.9451	**0.0208**	0.2443	**0.0127**
IL-4						**0.0053**	**0.0013**	0.6434	**0.0354**	0.4043	0.4079	0.4254	0.7062	**0.0000**	0.0959	0.6017	**0.0062**	**0.0021**	**0.0122**	**0.0032**	0.6820	0.2829	**0.0024**	0.8903	**0.0029**
IL-5							**0.0013**	0.2620	**0.0330**	0.9463	0.3217	0.5899	0.2599	**0.0049**	0.1081	0.7822	**0.0084**	**0.0024**	**0.0240**	**0.0043**	0.9143	0.6827	**0.0020**	0.3657	**0.0143**
IL-6								0.4650	**0.0132**	0.6713	0.4205	0.3520	0.2934	**0.0007**	0.3309	0.9797	**0.0038**	**0.0016**	**0.0462**	**0.0030**	0.7269	0.6066	**0.0017**	0.5581	**0.0002**
IL-7									0.3261	0.0767	0.4410	0.1854	**0.0342**	0.6577	0.9234	0.0780	0.2178	0.7230	0.9236	0.7523	0.4820	0.3451	0.6158	0.1205	0.6468
IL-8										0.6807	0.4967	0.8520	0.1251	**0.0139**	0.4315	0.4104	**0.0396**	**0.0083**	0.1813	**0.0428**	0.7965	0.4908	**0.0046**	0.6235	**0.0041**
IL-9											0.4425	0.0885	0.0553	0.5174	0.7758	**0.0012**	0.8948	0.7713	0.4815	0.5115	0.9748	0.7559	0.8460	0.1837	0.6632
IL-10												0.0560	0.6623	0.3507	0.2681	0.3279	0.9997	0.1122	0.1993	0.2103	0.4988	0.3480	0.1230	0.5608	0.5083
IL-12(p70)													0.3332	0.4313	0.5623	0.2210	0.7549	0.3360	0.1802	0.1923	0.1783	0.5890	0.4113	0.9507	0.3270
IL-13														0.5499	0.5719	0.0631	0.3925	0.4084	0.7736	0.8068	0.4428	0.7409	0.3515	**0.0267**	0.3706
IL-15															0.1297	0.7191	**0.0097**	**0.0004**	**0.0185**	**0.0042**	0.8450	0.3510	**0.0004**	0.9793	**0.0011**
IL-17																0.8081	0.2214	0.1249	**0.0066**	0.0692	0.1271	0.3235	0.1323	0.7202	0.3724
Eotaxin																	0.6980	0.9479	0.7458	0.8887	0.8918	0.9807	0.9806	0.2250	0.9184
bFGF																		**0.0387**	**0.0405**	**0.0121**	0.6458	0.1686	**0.0379**	0.8695	**0.0047**
G-CSF																			**0.0169**	**0.0031**	0.8196	0.7067	**0.0000**	0.6293	**0.0027**
GM-CSF																				**0.0014**	0.6027	0.3767	**0.0251**	0.9276	**0.0449**
IFN-γ																					0.9959	0.5319	**0.0038**	0.8276	**0.0043**
IP-10																						0.0582	0.8550	0.2024	0.6996
MCP-1																							0.6932	0.1310	0.4268
MIP-1β																								0.6108	**0.0031**
TNF-α																									0.8377
VEGF																									

Values in bold, in light grey cells, are referred to p-values <0.05, according to the Pearson correlation analysis.

index changes, whose values are equally increased in COVID-19 (recently overviewed in [26]) and sepsis patients [12, 27].

In particular, we observed a significant time- and dose-dependent enhancement of MDW, that was already significantly increased by the lowest histone dose of 50 μg/mL at the shortest time-point of 30 min. These results suggest that histones exert a potent and early effect on circulating monocyte population, affecting mainly their morphology, including cell volume, cytoplasmic granularity and vacuolization, and nuclear structure, features mirrored by the MDW parameter changes [20], without affecting monocyte count.

According to literature data [28], MDW values in healthy subjects could reach 23.5 when blood was obtained in EDTA tubes. In this respect, our experiments carried out in K_3-EDTA tubes showed that healthy controls had MDW values ranging from 14.3 to 20.7 during time, highlighting the stability of MDW measurements on this model over time.

Up to date, there are no single laboratory tests or specific stand-alone biomarkers with sufficient sensitivity and specificity to timely and accurately diagnose sepsis. In this respect, the measurement of MDW and histone plasma levels may provide useful biomarkers, with possible diagnostic and prognostic value to rule out infection and monocyte activation as critical players in classic and viral sepsis-related inflammation.

Our findings are consistent with the well-known ability of histones to modify cellular and biomolecular pathways of monocytes through direct translocation across cell plasma membrane, endocytosis-independent mechanisms, destabilization of plasma membrane, generation of extracellular vesicles and activation of TLR2/4/9, thus promoting inflammatory reactions and coagulative cascade [2, 29].

Acting as DAMPs, histones activate monocytes promoting not only morphological but also functional changes. In this scenario, we demonstrated that histones promoted a significant release of a wide panel of cytokines from peripheral blood cells. Of note, the cytokine-mediated inflammatory responses resulting from a whole-blood assay model, despite including all circulating blood cell types (WBC, RBC, PLT), has been reported to be representative of the monocyte population, providing better results compared to peripheral blood mononuclear cell and monocyte cultures [30].

In this respect, we observed that histones triggered a significant dose-dependent release of almost all cytokines. Interestingly, G-CSF showed the strongest response to histone treatments. This finding is crucial since G-CSF has been reported to be essential for the onset and amplification of the cytokine storm [31]. CSFs are growth factors implicated in the stimulation of myeloid cell differentiation and proliferation, that once activated synthesize and release further cytokines, thus inducing the cytokine storm [31].

Furthermore, our results highlighted that IL-1β, IL-6, MIP-1β/CCL4, and IL-8/CXCL8 exhibited a relevant dose-dependent increase after histone treatment and it is well-known that these parameters are increased in COVID-19 [32–34], and sepsis [35], suggesting them as crucial soluble mediators during disease progression. A significant increase of VEGF, IP-10/CXCL10, GM-CSF, TNF-α, EOTAXIN/CCL11, IL-1Ra, IL-2, and IL-4, and a lower but significant increase of IL-15, IL-5, IL-17, bFGF, IL-10, INF-γ, MCP-1/CCL2, and IL-9 was observed after histone treatment with almost all doses.

In sepsis conditions, nonsurvivors patients have been demonstrated to present increased levels of IL-2 (7.6-fold), IL-10 (3.1-fold), and MCP-1, IL-15, and GM-CSF (>2-fold) compared to survivors [35]. Moreover, levels of histones in non survivors have been found 28-fold higher compared to survivors' sepsis patients ($p=0.025$) [35].

During sepsis, levels of IL-1β and IL-6 have been reported higher in nonsurvivor vs. survivor patients, suggesting their potential involvement in predicting disease outcome [36–39]. IL-12 has also been reported elevated in sepsis [31].

In COVID-19 viral sepsis, IL-6, IL-2, IL-7, IL-10, G-CSF, IP-10, TNF-α, MCP-1, and MIP-1α have been reported upregulated and suggested to play a crucial role in the pathogenesis of the disease [40]. Similarly, Liu et al. by evaluating a panel of 48 cytokines in the plasma of COVID-19 patients concluded that 38 out of 48 cytokines were remarkably elevated in patients with COVID-19 and there was a strong linear association between severe lung injury and the level of 15 cytokines including: IFN-γ, IFN-α2, IL-1ra, IL-2, IL-4, IL-7, IL-10, IL-12, and IL-17, as well as IP-10, macrophage colony-stimulating factor (M-CSF) and G-CSF [41].

Data from a limited number of COVID-19 patients have shown a cytokine storm in critically ill patients. Here, levels of IL-2, IL-6, IL-10, and IFN- γ were found increased in severe cases of COVID-19 than in mild cases, and a strong inflammatory response during its clinical course was reported to be associated with high morbidity and mortality [42]. In this respect, recent findings indicated that G-CSF levels are also increased in plasma samples from COVID-19 and sepsis patients [40, 43], furtherly corroborating the notion on its fundamental involvement in cytokine storms associated with these conditions.

Interestingly, histones have been demonstrated able to modulate several cytokines including TNF-α, IL-6, IL-10, IL-1β, IL-8, CXCL9, IP-10, MIP-1α, MIP3A, and MCP3 in different experimental models [2, 35, 44–49].

However, no literature data reported such a wide panel of mediators as targets of histone treatment in whole blood model. Most of these mediators are deeply involved in the

regulation of immune cell proliferation, differentiation, and production of new cytokines, leading to a vicious cycle which in turn causes cell and organ damage.

Although the size of the sample is limited, our whole blood assay model and findings demonstrate that histones, acting as DAMPs are potent inducers of a wide array of cytokines and chemokines, simulating *in vitro* the cytokine storm observed *in vivo* in both COVID-19 and sepsis patients, and highlighting that histones are major triggers and contributors during the clinical progression of these pathological conditions.

Being that whole blood assay model is an experimental model that closely and significantly mimes the monocyte responses, and that monocytes are largely implicated in high-inflammatory impact diseases, as classical and viral sepsis, with our findings on MDW changes and associated cytokine release we furthermore provide clear and increasing evidence on the critical roles of monocytes in these conditions.

On these bases, the identification of potential strategies to neutralize histones, and thus histone-mediated damages are warmly encouraged. Several studies suggest that both endogenous molecules (e.g., albumin, C Reactive Protein, Activated Protein C, polysialic acid) and pharmacological treatments (heparins and heparinoids) could directly bind histones, thus limiting their harmful effects on cells and tissues of several organs [6, 46, 50].

Further studies are ongoing to elucidate the involvement of histones in hypercoagulability events in classical and viral COVID-19 sepsis, as well as the possible modulation by heparin compounds.

In conclusion, in the light of the herein emerged new roles and functions of histones as inducers of hyper-inflammatory responses and MDW modifiers, we suggest that monitoring MDW index and histone concentrations in patients with classic and COVID-19 sepsis both upon admission and throughout hospitalization may be a useful parameter to early predict higher risk of worst outcome.

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