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Short communication

Macrophage-polarizing stimuli differentially modulate the inflammatory profile induced by the secreted phospholipase A_2 group IA in human lung macrophages

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ABSTRACT

In this study we investigated the effects of snake venom Group IA secreted phospholipase A_2 (*sv*GIA) on the release of inflammatory and angiogenic mediators from human lung macrophages (HLMs). HLMs were incubated with lipopolysaccharide (LPS) or *sv*GIA with or without macrophage-polarizing stimuli (IL-4, IL-10, IFN-γ or the adenosine analogue NECA). M2-polarizing cytokines (IL-4 and IL-10) inhibited TNF-α, IL-6, IL-12, IL-1β, CXCL8 and CCL1 release induced by both LPS and *sv*GIA. IL-4 inhibited also the release of IL-10. IFN-γ reduced IL-10 and IL-12 and increased CCL1 release by both the LPS and *sv*GIA-stimulated HLMs, conversely IFN-γ reduced IL-1β only by *sv*GIA-stimulated HLMs. In addition, IFNγ promoted TNF-α and IL-6 release from *sv*GIA-stimulated HLMs to a greater extent than LPS. NECA inhibited TNF-α and IL-12 but promoted IL-10 release from LPS-stimulated HLMs according to the well-known effect of adenosine in down-regulating M1 activation. By contrast NECA reduced TNF-α, IL-10, CCL1 and IL-1β release from *sv*GIA-activated HLM. IL-10 and NECA increased both LPS- and *sv*GIA-induced vascular endothelial growth factor A (VEGF-A) release. By contrast, IL-10 reduced angiopoietin-1 (ANGPT1) production from activated HLMs. IFN-γ and IL-4 reduced VEGF-A and ANGPT1 release from both LPS- and *sv*GIA-activated HLMs. Moreover, IL-10 inhibited LPS-induced ANGPT2 production. In conclusion, we demonstrated a fine-tuning modulation of *sv*GIA-activated HLMs differentially exerted by the classical macrophage-polarizing cytokines.

Abbreviations: ANGPT, Angiopoietin; bFGF, Fibroblast Growth Factor; HIF, Hypoxia Inducible Factor; HLMs, Human Lung Macrophages; INF, Interferon; LBP, LPS-Binding Protein; LPS, Lipopolysaccharide; MMP-9, Metalloprotease-9; NECA, 5-(N-Ethylcarboxamido) adenosine; PDGF, Platelet Derived Growth Factor; *sv*GIA, snake venom Group IA Secreted phospholipase A_2; TAM, Tumor-Associated Macrophages; TNF, Tumor Necrosis Factor; TP, Thymidine Phosporylase; VEGF, Vascular Endothelial Growth Factor.

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1. Introduction

Macrophages are central players in several pathological conditions related to chronic inflammation, such as cancer [1]. Macrophages are characterized by a well-known plasticity, which consists on their ability to modify their functions according to the signals coming from the microenvironment [2]. Looking from a simplified perspective, macrophages can undergo polarization towards two opposite phenotypes. Classical Th1-related cytokines (e.g. IFN-γ) alone or together with microbial molecules [e.g., lipopolysaccharide (LPS)] induce the switch to classical M1 phenotype. Classically activated M1 macrophages are characterized by a high production of effector molecules (e.g., reactive oxygen and nitrogen intermediates) and inflammatory cytokines (e.g., IL-1β, TNF-α, IL-6, IL-12) as well as by a low expression of IL-10. From the functional point of view, they take part into the Th1 response and in resistance against microbes and tumors [2]. By contrast, Th2-related cytokines such as IL-4 and IL-13 induce the alternative M2 phenotype which produces high levels of IL-10 and low levels of IL-12 [3]. M2 polarized macrophages are scarce antigen presenting cells, inhibit Th1 adaptive immune response, play central roles in resistance against parasites, wound healing, tissue remodeling and angiogenesis [1]. However, increasing evidence now definitely indicates that macrophage activation exists on a wide spectrum and cannot easily be binned into defined groups [4–6].

Macrophages infiltrating the tumor (Tumor-Associated Macrophages – TAM) can exert dual roles but a number of evidences suggest they display an M2-like phenotype. Indeed, they share with M2 macrophages a number of functions such as the suppression of the anti-tumor immune response, the remodeling of the extracellular matrix, the promotion of tumor cell invasive behavior and metastatic potential, as well as the support of tumor-associated angiogenesis and lymphangiogenesis [1].

Tumor-derived metabolic derivatives (e.g., hypoxia, lactate, pyruvate, or hydrogen ions) or molecules locally produced or released for tissue damage (e.g., IL-10, M−CSF, LPS, adenosine) [1] can activate proangiogenic programs in TAM [7]. Under the influence of these mediators, TAM express TGF-β, vascular endothelial growth factor A (VEGF-A) and C (VEGF-C), platelet derived growth factor (PDGF), metalloprotease-9 (MMP-9) and some pro-angiogenic chemokines (e.g. CXCL8) which can differentially modulate the various steps of the angiogenic program [8,9]. In the tumor microenvironment, local hypoxia increases the expression of HIF-1 and HIF-2, which in turn activate a pro-angiogenic program in TAM, which lead to the release of VEGF, basic fibroblast growth factor (bFGF) and CXCL8 [10].

Phospholipases A_2 (PLA_2) are able to hydrolyze fatty acids from membrane phospholipids [11]. Among PLA_2 superfamily, secreted PLA_2s ($sPLA_2$s) represent the largest family, being Ca^{2+}-dependent low molecular-weight enzymes. Individual $sPLA_2$s exhibit unique localization (mammalian tissues as well as arachnid, insect, and snake venom), cellular distributions and enzymatic properties, suggesting their distinct biological roles [11,12]. During the activation of the inflammatory cascade, $sPLA_2$s are released in biological fluids and are able to stimulate immune cells through their enzymatic or non-enzymatic (receptor-mediated) activity [11,12].

Both human and venom $sPLA_2$ can efficiently stimulate cytokine production in human lung macrophages (HLMs) [7,13]. Our group demonstrated that $sPLA_2$ group IIA and X promoted the release of VEGF-A and VEGF-C from HLMs by a receptor-mediated mechanism. Adenosine synergistically enhanced $sPLA_2$-induced activation of macrophages by further increasing VEGF-A release [7]. Venom PLA_2 were able to non-enzymatically stimulate neutrophils and to activate cytosolic PLA_2 leading to the production of arachidonate metabolites which in turn promote neutrophil activation and chemotaxis [14]. Group IA (GIA) and group IB (GIB) $sPLA_2$ promoted the production of cytokines (TNF-α, IL-6 and IL-10) and chemokines (CCL2, CCL3, CCL4 and CXCL8) by HLMs. TNF-α and CXCL8 release was dependent on the activation of PI3K/Akt system, the MAPK p38 and ERK1/2 and NF-κB pathways and was independent by their enzymatic activity [15].

LPS is a cell wall component of gram-negative bacteria which can be recognized by TLR4 [16]. The co-receptor CD14 mediates the binding of LPS from the LPS-binding protein (LBP) to the TLR4–MD2 complex expressed on the cell surface and induces the TNF-α and IL-6 production in response to TLR4 [16]. LPS is one of the main activators of the classical M1 polarization, which enable macrophages to kill microorganisms and produce proinflammatory mediators, such as reactive oxygen and nitrogen derivatives, TNF-α, IL-6, IL-23 and IL-12 [2].

The involvement of the major macrophages-polarizing cytokines in the $sPLA_2$-induced production of inflammatory and angiogenic mediators by HLMs has not been defined yet. In this study we explored several modulating effects of different cytokines on the production of several mediators in primary HLMs activated by $sPLA_2$.

2. Methods

2.1. Reagents

The following were purchased: bovine serum albumin, L-glutamine, antibiotic–antimycotic solution (10,000 IU/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B), RPMI 1640, fetal calf serum (FCS) (endotoxin level < 0.1 EU/mL), Percoll®, *sv*GIA $sPLA_2$ (from Naja mossambica mossambica venom) and 5-(N-Ethylcarboxamido) adenosine (NECA) (Sigma Aldrich, St. Louis, MO), IL-4, IL-10 and INF-γ (PeproTech, Rocky Hills, NJ).

2.2. Isolation and purification of human lung macrophages (HLMs)

Macrophages were purified from macroscopically normal lung tissue of patients (hepatitis C virus$^-$, hepatitis B surface Ag$^-$, HIV1$^-$) undergoing lung resection [17]. The study protocol was approved from the Ethics Committee of University of Naples "Federico II" (Prot. 7/19), and informed consent was obtained from 15 donors. The cells were suspended (10^6 cells/mL) in RPMI 1640 with 5% FCS, 2 mM L-glutamine, and 1% antibiotic–antimycotic solution and incubated in 24-well plates. After 12 h, the medium was removed and the plates were gently washed with fresh medium. More than 98% of adherent cells were macrophages, as evaluated by flow-cytometric analysis with the following antibodies: anti-CD45 (Clone REA747, dilution 1:50), anti-HLA-DR (Clone REA805, dilution 1:50) and anti-CD206 (dilution 1:11) Miltenyi Biotec, Germany) (Fig. 1). Samples were analyzed on the MACSQuant Analyzer 10 (Miltenyi Biotec, Germany) and in the FlowJo software, v.10. Doublets, debris (identified based on forward and side scatter properties), and dead cells (identified with Zombie Violet Fixable Viability Kit; BioLegend) were excluded from the analysis. Data were expressed as a percentage of positive cells.

2.3. Isolation of monocytes and differentiation in macrophages

The study protocol involving the use of human blood cells was approved by the Ethics Committee of the University of Naples Federico II, and written informed consent was obtained from blood donors according to the principles expressed in the Declaration of Helsinki (Protocol number 301/12). Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors (HBsAg$^-$, HCV$^-$, and HIV$^-$) obtained from a leukapheresis unit. Leukocytes were separated from erythrocytes by dextran sedimentation. PBMC were purified by Histopaque-1077 (Sigma Aldrich, Milan, Italy) density gradient centrifugation (400 × g for 20 min at 22C). Monocytes were further purified with CD14 microbeads (Miltenyi Biotec, Italy) according to the manufacturer's protocol. To obtain monocyte-derived macrophages (MDMs), monocytes (1.5×10^5 cells/cm^2) were differentiated with M−CSF 50 ng/ml (Miltenyi Biotec, Italy) for 7 days in RPMI 1640 supplemented with 10% FBS (Sigma-Aldrich, Italy) [17].

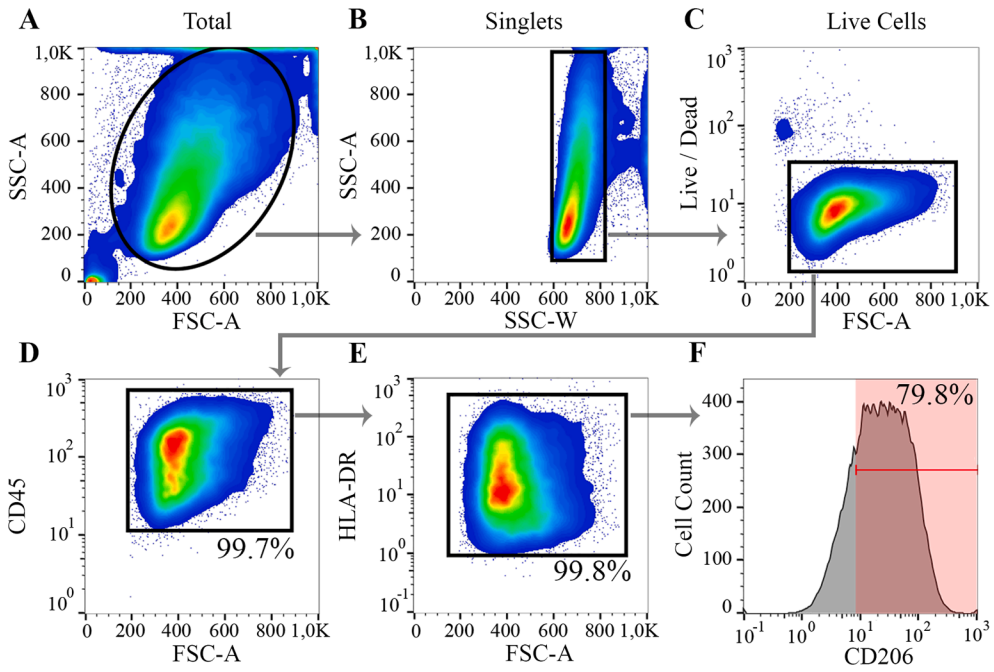

Fig. 1. Representative flow cytometric panels with respect to the gating strategy used to identify human lung macrophages (HLMs). Among total cells (A), after excluding doublets (B) and dead cells (C), macrophages were identified as $CD45^+$ (D) and $HLA\text{-}DR^+$ (E). Expression of CD206 was assessed within $SSC^{high}FSC^{high}CD45^+HLADR^+$ cells (F).

2.4. Cell incubations

Adherent macrophages and MDMs were incubated (37°C, 18 h) in RPMI 1640 containing LPS (100 ng/ml) or *sv*GIA (500 ng/ml) alone or in combination with IL-4 (10 ng/mL), IL-10 (10 ng/mL), INF-γ (1000 U/mL) or NECA (10 μM). Limulus amebocyte Test (MP Biomedicals) was used to check LPS contamination in *sv*GIA preparations used for macrophage stimulation. The test revealed that *sv*GIA preparations were LPS-free (LPS concentration above the detection limit of the assay – 0.125 EU/mL). To further avoid any effect due to unwanted bacterial contaminations, before being used to stimulate macrophages, *sv*GIA (500 ng/mL) and LPS (100 ng/mL) preparations were preincubated (37°C, 30 min) with polymyxin B (50 μg/mL). Cell viability was assessed by trypan blue exclusion and was greater than 95%. At the end of incubation, supernatants were harvested, centrifuged (1000 × g, 4 °C, 5 min) and stored at –80 °C for the subsequent determination of cytokines (TNF-α, IL-6, IL-10, IL-12, IL-1β), chemokines (CXCL8 and CCL1) and angiogenic factors (VEGF-A, ANGPT1 and ANGPT2) concentrations. Lysis of the cells remaining in the plates was carried out by using 0.1% Triton X-100 for total protein quantification by a Bradford assay (Biorad).

2.5. ELISA for cytokines, chemokines and angiogenic factors

The release of soluble mediators in the supernatants of HLMs was measured in duplicate using commercially available ELISA kits for TNF-α, IL-6, IL-10, IL-12, IL-1β, CXCL8, CCL1, VEGF-A, ANGPT1 and ANGPT2 (R&D Systems, Minneapolis, MN). The linearity range of the assay was between 20 and 800 pg/mL for TNF-α, 6 and 200 pg/mL for IL-6, 12 and 400 pg/mL for IL-10, 10 and 2000 pg/mL for IL-12 p40, 3 and 300 pg/mL for IL-1β, 30 and 2000 pg/mL for CXCL8, 15.2 – 1,000 for CCL1, 31.1 – 2,000 pg/mlL for VEGF-A, 156.25 – 10,000 pg/mL for ANGPT1 and 31.1 – 4,000 pg/mL for ANGPT2. Since the number of adherent macrophages and MDMs can vary among the wells and 6 different experiments, the results obtained were normalized for the total protein content in each well, determined in the cell lysates (0.1% Triton X-100) by the Bradford assay.

2.6. Statistical analysis

Data were analyzed with the GraphPad Prism 7 software package. Statistical analysis was performed using an unpaired Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Dunnett's test (when comparison was made against a control) or Bonferroni's test (when comparison was made between each pair of groups). Statistically significant differences were accepted when the *p*-value was ≤ 0.05.

3. RESULTS AND discussion

In this paper, we investigated the effect of some pivotal macrophage-polarizing cytokines on the release of pro-inflammatory and angiogenic factors induced in HLMs by the venom $sPLA_2$ group IA (*sv*GIA) compared to LPS. HLMs were isolated from human lung tissue from a total of 15 patients who underwent surgery for lung cancer. As already reported [17], HLMs (identified as $FSc^{high}SSc^{high}CD45^+HLA^-DR^+$ cells) homogeneously expressed CD206, suggesting an M2-like phenotype (Fig. 1). However, M2 markers can be also expressed by tissue-resident macrophages under the influence of IL-4 [2,17].

In a first series of *in vitro* experiments, we evaluated the release of inflammatory and angiogenic factors produced by HLMs stimulated with *sv*GIA or LPS. Table 1 shows that both LPS and *sv*GIA induced the release of inflammatory cytokines (TNF-α, IL-6, IL-10, IL-12 and IL-1β) and chemokines (CXCL8 and CCL1) from HLMs compared to control. Moreover, both LPS and *sv*GIA caused the release of angiogenic factors such as VEGF-A and ANGPT1. ANGPT2 was released only upon LPS stimulation (Table 1). It is important to note that LPS induced higher levels of TNF-α, IL-6, IL-10, IL-12, IL-1 β, CXCL8 and VEGF-A from HLMs compared to *sv*GIA (Table 1). Thus, the exposure of HLMs to *sv*GIA resulted in a pro-inflammatory profile similar to the one induced by LPS, even though to a lesser extent. It is important to note that all preparations of *sv*GIA were LPS-free. Our results confirm that *sv*GIA plays a major role in the initiation/regulation of lung inflammation and immune responses. In particular, the release of the pro-inflammatory cytokines TNF-α, IL-6, IL-12 and IL-1β indicates that *sv*GIA may enhance the local inflammatory response, whereas the induction of CXCL8 and

Table 1
Human lung macrophages (HLMs) were stimulated with medium alone (Unstimulated) with or without LPS (100 ng/mL) or *sv*GIA (500 ng/mL) for 18 h. Supernatants were harvested, centrifuged twice (1000 × g, 4°C, 5 min) and stored at –80 °C for the subsequent determination of pro-inflammatory (TNF-α, IL-6, CXCL8, CCL1, IL-12, IL-1β, IL-10) and angiogenic (VEGF-A, ANGPT1 and ANGPT2) profiles by ELISA. The results were normalized for the total protein content in each well (by using Bradford assay).

	Unstimulated	LPS	*sv*GIA
TNF-α (ng/mg of proteins)	0.6 ± 0.10	14.0 ± 2.69$^{(*)}$	4.5 ± 1.19$^{(*)(§)}$
IL-6 (ng/mg of proteins)	2.9 ± 0.34	48.3 ± 6.6$^{(*)}$	10.4 ± 0.97$^{(*)(§)}$
CXCL8 (ng/mg of proteins)	89.1 ± 28.08	637.1 ± 59.54$^{(*)}$	405.2 ± 42.54$^{(*)(§)}$
CCL1 (pg/mg of proteins)	31.2 ± 11.84	220.1 ± 47.48$^{(*)}$	110.8 ± 23.18$^{(*)}$
IL-12 (ng/mg of proteins)	0.8 ± 0.006	40.8 ± 1.3$^{(*)}$	2.3 ± 0.1$^{(*)(§)}$
IL-1β (pg/mg of proteins)	1.8 ± 0.6	311 ± 67.1$^{(*)}$	33.8 ± 4.0$^{(*)(§)}$
IL-10 (pg/mg of proteins)	32.0 ± 6.36	525.5 ± 94.12$^{(*)}$	208.0 ± 34.49$^{(*)(§)}$
VEGF-A (pg/mg of proteins)	53.4 ± 12.2	222.8 ± 12.2$^{(*)}$	168.9 ± 8.9$^{(*)(§)}$
ANGPT1 (pg/mg of proteins)	102.2 ± 16.91	326.0 ± 89.15$^{(*)}$	464.2 ± 93.71$^{(*)}$
ANGPT2 (pg/mg of proteins)	24.0 ± 1	42.5 ± 2.5$^{(*)}$	28.5 ± 2.5

Data are reported as mean ± SEM of 6 experiments from different donors and analyzed by using One-Way ANOVA.
*$p < 0.01$ *vs* control; § $p < 0.05$ *vs* LPS.

CCL1 suggests that *sv*GIA promotes the recruitment of neutrophils and T cells at sites of inflammation [2]. Interestingly, *sv*GIA also induced the immunomodulatory molecule IL-10. This effect could be due, at least in part, to the release of TNF-α, the initial cytokine released in the cytokine cascade following LPS exposure [18]. Additional experiments are required to clarify the latter aspect. In addition, we demonstrated that *sv*GIA induced VEGF-A and ANGPT1 release, similarly to LPS, suggesting a role in supporting inflammation-related angiogenic switch.

Interestingly, LPS, but not *sv*GIA, induced ANGPT2 release from HLMs. ANGPTs contribute to multiple pathophysiological aspects such as inflammation, cell survival and cancer. In healthy individuals, expression of ANGPT2 is limited, but in cancer patients the serum levels of ANGPT2 increase and the ratio between ANGPT1 and ANGPT2 decreases accordingly [19]. Since the expression and the release of ANGPT2 can be favored by cytokines such as VEGF-A [20], one could hypothesize that, since LPS is a stronger inducer of the inflammatory response compared to *sv*GIA, the LPS-induced release of VEGF-A was responsible for the induction of ANGPT2 in LPS-stimulated HLMs. However, additional experiments are necessary to address this aspect.

In a second series of *in vitro* experiments, we investigated the effect of some macrophage-polarizing stimuli in modulating LPS and *sv*GIA-induced HLMs activation (Fig. 2). Concerning the pro-inflammatory profile, the M2-polarizing cytokines IL-4 and IL-10 both inhibited the release of TNF-α (panel A), IL-6 (panel B), CXCL8 (panel C), CCL1 (panel D), IL-12 (panel E), IL-1β (panel F), induced by both LPS and *sv*GIA. Our results are in line with published reports describing the multiple anti-inflammatory properties of the prototypic type 2 immunoregulatory cytokine IL-4 [21,22]. In addition, IL-4 inhibited the release of IL-10 too (panel G). The last result was such unexpected, but could be related to the ability of IL-4 of differentially modulate macrophage functions according to the different macrophage populations or the related phenotype [22,23]. Indeed, IL-4 differentially modulated LPS-induced IL-12 and IL-10 production by human peripheral blood monocytes and synovial mononuclear cells [23]. Moreover, in our experimental system, the effect of IL-4 on IL-10 production could be due to the reduction of TNF-α release in both LPS- and *sv*GIA-treated HLMs. Additional experiments are required to clarify this mechanism, but our results add new data on the understanding of the complexity of the different macrophage populations at the inflammatory sites.

Similarly to IL-4, IFN-γ reduced both the LPS- and *sv*GIA-induced IL-12 and IL-10 release (panel E and G) [24]. By contrast, IFN-γ inhibited IL-1β release only in *sv*GIA-activated HLMs (panel F). INF-γ increased the release of CCL1 by LPS- and *sv*GIA-induced HLMs (panel D), suggesting a kind of M2b polarization [25], even in absence of any FcγR engagement. In addition, IFN-γ promoted the release of TNF-α and IL-6 in *sv*GIA-stimulated HLMs to a greater extent than LPS (panel A and B), suggesting an even stronger pro-inflammatory effect. By contrast, IFN-γ did not modulate both LPS and *sv*GIA-induced CXCL8 release from HLMs (panel C).

The potent adenosine A2a receptor agonist NECA inhibited the release of TNF-α (panel A) and IL-12 (panel E) but promoted the release of IL-10 from LPS-stimulated HLMs (panel G), in line with the well-known effect of adenosine in down-regulating classical M1 activation [26]. By contrast, in *sv*GIA-activated HLMs, NECA reduced TNF-α (panel A), CCL1 (panel D), IL-12 (panel E) and IL-1β (panel F) and IL-10 (panel G) release, displaying a less selective anti-inflammatory effect. NECA did not modulate both LPS- and *sv*GIA-induced IL-6 (panel B) and CXCL8 release (panel C).

About the angiogenic profile, the immunomodulatory IL-10 and NECA significantly increased both LPS- and *sv*GIA-induced VEGF-A release (panel H), according to their M2-polarizing properties. On the other hand, IL-10 reduced ANGPT1 production, whereas NECA did not modulate ANGPT1 release in both LPS- and *sv*GIA-stimulated HLMs (panel I). IFN-γ significantly reduced the release of VEGF-A and ANGPT1 from both LPS- and *sv*GIA- activated HLMs (panels H and I), in line with its M1 polarizing effect. Surprisingly, IL-4 inhibited VEGF-A and ANGPT1 release induced by LPS and *sv*GIA-activated HLMs (panels H and I) as well as the release of ANGPT2 induced by LPS (panel J). This inhibitory effect of IL-4 on the release of pro-angiogenic mediators could be due to the significant IL-4-mediated reduction of TNF-α.

In the last group of experiments, we assessed the effect of LPS and *sv*GIA on naive, non-polarized monocyte-derived macrophages (MDMs). Both LPS and *sv*GIA induced the release of the pro-inflammatory cytokines (TNF-α, CXCL8, IL-12 and IL-1β) and VEGF-A from MDMs compared to control (Supplementary Table 1). Moreover, IL-6, CCL1 and IL-10 were released only upon LPS stimulation (Supplementary Table 1). Conversely, ANGTPs were not released by LPS nor *sv*GIA stimulation (Supplementary Table 1). Similarly to HLMs, LPS induced TNF-α, CXCL8, IL-12, IL-1β and VEGF-A to a higher extent compared to *sv*GIA (Supplementary Table 1). Of note, the amount of each single mediator, with the exception of VEGF-A, was higher in HLM compared with MDM supernatants. The latter observation is consistent with the complexity of the TME, which is rich of a plethora of cells and stimuli. These factors could be responsible for the modulation of the macrophage phenotype, leading to a different amount of soluble mediators released by HLMs in response to LPS and *sv*GIA compared to MDMs.

Concerning the effect of the macrophage-polarizing stimuli in modulating LPS and *sv*GIA-induced MDMs activation we found that, similarly to HLMs, IL-4 and IL-10 inhibited the release of the pro-inflammatory mediators, with the exception of IL-10 (Supplementary Fig. 1, panels A-G). In addition, the LPS and *sv*GIA-induced VEGF-A release was inhibited by IL-4 but increased by IL-10 (Supplementary Fig. 1H). IFN-γ reduced the LPS-induced IL-12 release from MDMs (Supplementary Fig. 1E). By contrast, IFN-γ inhibited the release of VEGF-A by both LPS- and *sv*GIA-activated MDMs (Supplementary Fig. 1H). Similarly to HLMs, INF-γ increased the release of CCL1 by LPS-induced MDMs (Supplementary Fig. 1D). Similarly to HLMs, NECA, inhibited the release of TNF-α and IL-12 (Supplementary Fig. 1, panels A and E) from LPS- and *sv*GIA-stimulated MDMs but increased the release of VEGF-A (Supplementary Fig. 1H). Moreover, NECA reduced CCL1 production only in LPS-stimulated MDMs (Supplementary Figure D). IL-1β release was inhibited by NECA from both *sv*GIA-activated HLMs and MDMs (Fig. 2F and Supplementary Fig. 1F).

Collectively, our results demonstrate for the first time that *sv*GIA can

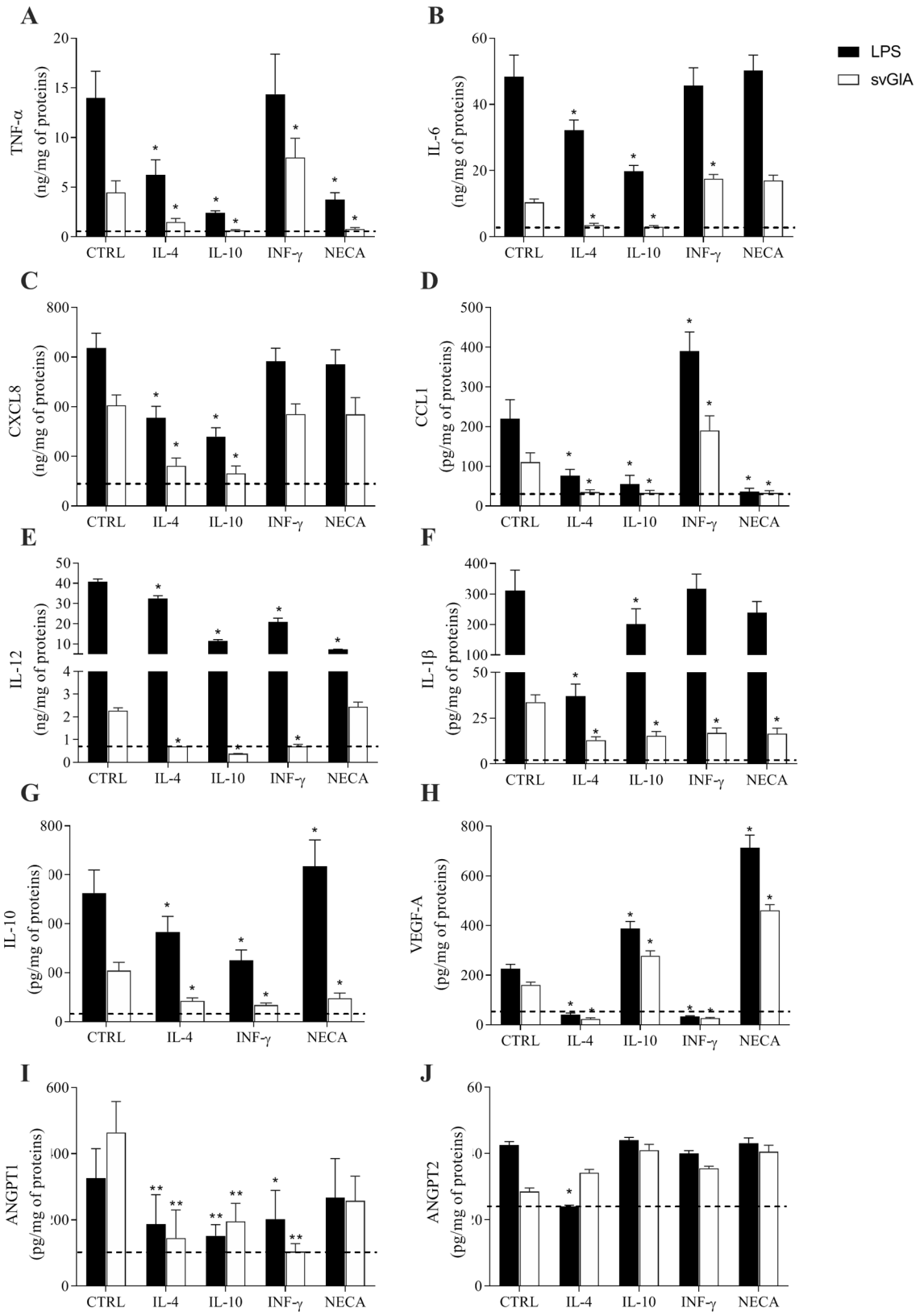

Fig. 2. Pro-inflammatory and angiogenic profiles in LPS- and *sv*GIA-induced HLMs are differentially modulated by IL-4, IL-10, INF-γ and NECA. HLMs were incubated (37°C, 18 h) in RPMI 1640 containing LPS (100 ng/mL – black bars) or *sv*GIA (500 ng/mL – white bars) alone or in combination with IL-4 (10 ng/mL), IL-10 (10 ng/ml), INF-γ (1000 U/mL) or NECA (10 μM). The red line represents the release of mediators in unstimulated cells. At the end of incubation, the supernatants were harvested, centrifuged (1,000 × g, 4 °C, 5 min) and stored at −80 °C for subsequent analyses. TNF-α (A), IL-6 (B), CXCL8 (C), CCL1 (D), IL-12 (E), IL-1β (F), IL-10 (G), VEGF-A (H), ANGPT1 (I) and ANGPT2 (J) concentrations were determined by ELISA and values were normalized for the total protein (measured by Bradford assay) content in each well. The data are reported as mean ± SEM of six different preparations of HLMs from six different donors. *$p<0.05$, **$p < 0.001$ *vs* control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induce the release of several immunomodulatory cytokines, chemokines and angiogenic factors from HLMs and MDMs. In addition, our results extend the role of $sPLA_2$s as relevant players in the scenario of macrophage related inflammation.

CRediT authorship contribution statement

Anne Lise Ferrara: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft. **Maria Rosaria Galdiero:** Conceptualization, Funding acquisition, Validation, Writing - original draft, Writing - review & editing. **Alfonso Fiorelli:** Methodology, Resources, Writing - review & editing. **Leonardo Cristinziano:** Formal analysis, Investigation, Software. **Francescopaolo Granata:** Formal analysis, Methodology, Software. **Giancarlo Marone:** Conceptualization, Visualization. **Rosa Maria Di Crescenzo:** Data curation, Resources. **Mariantonia Braile:** Data curation, Investigation. **Simone Marcella:** Investigation, Visualization. **Luca Modestino:** Formal analysis, Investigation. **Gilda Varricchi:** Methodology, Validation, Visualization. **Giuseppe Spadaro:** Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing. **Mario Santini:** Methodology, Resources, Writing - review & editing. **Stefania Loffredo:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Validation, Visualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References

[1] E. Bonavita, M.R. Galdiero, S. Jaillon, A. Mantovani, Phagocytes as Corrupted Policemen in Cancer-Related Inflammation, Adv. Cancer Res. 128 (2015) 141–171.
[2] A. Sica, A. Mantovani, Macrophage plasticity and polarization: in vivo veritas, J. Clin. Invest. 122 (3) (2012) 787–795.
[3] W. Noel, G. Raes, G. Hassanzadeh Ghassabeh, P. De Baetselier, A. Beschin, Alternatively activated macrophages during parasite infections, Trends Parasitol 20 (3) (2004) 126–133.
[4] S. Chevrier, J.H. Levine, V.R.T. Zanotelli, K. Silina, D. Schulz, M. Bacac, C.H. Ries, L. Ailles, M.A.S. Jewett, H. Moch, M. van den Broek, C. Beisel, M.B. Stadler, C. Gedye, B. Reis, D. Pe'er, B. Bodenmiller, An Immune Atlas of Clear Cell Renal Cell Carcinoma, Cell 169 (4) (2017) 736–749 e18.
[5] M. Cohen, A. Giladi, A.D. Gorki, D.G. Solodkin, M. Zada, A. Hladik, A. Miklosi, T. M. Salame, K.B. Halpern, E. David, S. Itzkovitz, T. Harkany, S. Knapp, I. Amit, Lung Single-Cell Signaling Interaction Map Reveals Basophil Role in Macrophage Imprinting, Cell 175 (4) (2018) 1031–1044 e18.
[6] D. Aran, A.P. Looney, L. Liu, E. Wu, V. Fong, A. Hsu, S. Chak, R.P. Naikawadi, P. J. Wolters, A.R. Abate, A.J. Butte, M. Bhattacharya, Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage, Nat. Immunol. 20 (2) (2019) 163–172.
[7] F. Granata, A. Frattini, S. Loffredo, R.I. Staiano, A. Petraroli, D. Ribatti, R. Oslund, M.H. Gelb, G. Lambeau, G. Marone, M. Triggiani, Production of vascular endothelial growth factors from human lung macrophages induced by group IIA and group X secreted phospholipases A2, J. Immunol. 184 (9) (2010) 5232–5241.
[8] M.R. Galdiero, G. Varricchi, S. Loffredo, A. Mantovani, G. Marone, Roles of neutrophils in cancer growth and progression, J. Leukoc. Biol. 103 (3) (2018) 457–464.
[9] G. Varricchi, S. Loffredo, M.R. Galdiero, G. Marone, L. Cristinziano, F. Granata, G. Marone, Innate effector cells in angiogenesis and lymphangiogenesis, Curr. Opin. Immunol. 53 (2018) 152–160.
[10] C. Murdoch, A. Giannoudis, C.E. Lewis, Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues, Blood 104 (8) (2004) 2224–2234.
[11] G. Lambeau, M.H. Gelb, Biochemistry and physiology of mammalian secreted phospholipases A2, Annu. Rev. Biochem. 77 (2008) 495–520.
[12] J.B. Harris, T. Scott-Davey, Secreted phospholipases A2 of snake venoms: effects on the peripheral neuromuscular system with comments on the role of phospholipases A2 in disorders of the CNS and their uses in industry, Toxins (Basel) 5 (12) (2013) 2533–2571.
[13] M. Triggiani, F. Granata, A. Frattini, G. Marone, Activation of human inflammatory cells by secreted phospholipases A2, Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids 1761 (11) (2006) 1289–1300.
[14] I.B. Sundell, K.A. Aziz, M. Zuzel, R.D. Theakston, The role of phospholipases A2 in the stimulation of neutrophil motility by cobra venoms, Toxicon 41 (4) (2003) 459–468.
[15] F. Granata, A. Frattini, S. Loffredo, A. Del Prete, S. Sozzani, G. Marone, M. Triggiani, Signaling events involved in cytokine and chemokine production induced by secretory phospholipase A2 in human lung macrophages, Eur. J. Immunol. 36 (7) (2006) 1938–1950.
[16] J. Cui, Y. Chen, H.Y. Wang, R.F. Wang, Mechanisms and pathways of innate immune activation and regulation in health and cancer, Hum Vaccin. Immunother. 10 (11) (2014) 3270–3285.
[17] R.I. Staiano, S. Loffredo, F. Borriello, F.A. Iannotti, F. Piscitelli, P. Orlando, A. Secondo, F. Granata, M.T. Lepore, A. Fiorelli, G. Varricchi, M. Santini, M. Triggiani, V. Di Marzo, G. Marone, Human lung-resident macrophages express CB1 and CB2 receptors whose activation inhibits the release of angiogenic and lymphangiogenic factors, J. Leukoc. Biol. 99 (4) (2016) 531–540.
[18] T.L. Hackett, R. Holloway, S.T. Holgate, J.A. Warner, Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an ex vivo study, Respir. Res. 9 (2008) 47.
[19] X. Yu, F. Ye, Role of Angiopoietins in Development of Cancer and Neoplasia Associated with Viral Infection, Cells 9 (2) (2020).
[20] A. Hegen, S. Koidl, K. Weindel, D. Marme, H.G. Augustin, U. Fiedler, Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements, Arterioscler. Thromb. Vasc. Biol. 24 (10) (2004) 1803–1809.
[21] X.Y. Yang, L.H. Wang, K. Mihalic, W. Xiao, T. Chen, P. Li, L.M. Wahl, W.L. Farrar, Interleukin (IL)-4 indirectly suppresses IL-2 production by human T lymphocytes via peroxisome proliferator-activated receptor gamma activated by macrophage-derived 12/15-lipoxygenase ligands, J. Biol. Chem. 277 (6) (2002) 3973–3978.
[22] T. Kucharzik, N. Lugering, M. Adolf, W. Domschke, R. Stoll, Synergistic effect of immunoregulatory cytokines on peripheral blood monocytes from patients with inflammatory bowel disease, Dig. Dis. Sci. 42 (4) (1997) 805–812.
[23] C.S. Bonder, J.J. Finlay-Jones, P.H. Hart, Interleukin-4 regulation of human monocyte and macrophage interleukin-10 and interleukin-12 production. Role of a functional interleukin-2 receptor gamma-chain, Immunology 96 (4) (1999) 529–536.
[24] X. Hu, P.K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T.T. Lu, J.R. Woodgett, L. B. Ivashkiv, IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins, Immunity 24 (5) (2006) 563–574.
[25] M. Sironi, F.O. Martinez, D. D'Ambrosio, M. Gattorno, N. Polentarutti, M. Locati, A. Gregorio, A. Iellem, M.A. Cassatella, J. Van Damme, S. Sozzani, A. Martini, F. Sinigaglia, A. Vecchi, A. Mantovani, Differential regulation of chemokine production by Fcgamma receptor engagement in human monocytes: association of CCL1 with a distinct form of M2 monocyte activation (M2b, Type 2), J. Leukoc. Biol. 80 (2) (2006) 342–349.
[26] G. Hasko, P. Pacher, Regulation of macrophage function by adenosine, Arterioscler. Thromb. Vasc. Biol. 32 (4) (2012) 865–869.