LTB_4 and 5-oxo-ETE from extracellular vesicles stimulate neutrophils in granulomatosis with polyangiitis

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Abstract

Activation of neutrophils is an important mechanism in pathology of granulomatosis with polyangiitis (GPA). In this study, we evaluated if extracellular vesicles (EV) circulating in plasma of GPA patients could contribute to this process.

EV from plasma of GPA patients in active stage of the disease (n=10) and healthy controls (n=10) were isolated by ultracentrifugation, characterized by flow cytometry (CD63, CD8) and nanoparticle tracking analysis. Targeted oxylipins lipidomics of EV was performed by HPLC-MS2. EV/oxylipins induced neutrophil extracellular traps (NETs) were analyzed by confocal microscopy and released dsDNA was quantified by PicoGreen fluorescent dye. Reactive oxygen species (ROS) production and neutrophils' EV binding/uptake were evaluated by flow cytometry. Brief priming with granulocyte macrophages-colony stimulating factor (GM-CSF) was required for EV-mediated ROS production and dsDNA release. Priming also increased EV binding/uptake by neutrophils observed, however, only for EV from GPA patients. EV from GPA patients had higher concentrations of leukotriene B_4 (LTB_4) and 5-oxo-eicosatetraenoic acid (5-oxo-ETE) as compared to EV from healthy controls. Moreover, neutrophils stimulated with LTB_4 or 5-oxo-ETE produced ROS and released dsDNA in a concentration-dependent manner. These results reveal the potential role of EVs containing oxylipins cargo on ROS production and NET formation by activated neutrophils**.**

Keywords: neutrophils, granulomatosis with polyangiitis, extracellular vesicles, neutrophil extracellular traps, reactive oxygen species, immunology, inflammation, eicosanoids, leukotrienes

Abbreviations:

5-HETE - 5-hydroxyeicosatetraenoic acid

5-oxo-ETE - 5-oxo-eicosatetraenoic acid

BVAS - Birmingham Vasculitis Activity Score

C5a - complement component 5a

cANCA – anti-neutrophil cytoplasmic antibody, cytoplasmic staining pattern

CBC - complete blood count

CRP - C-reactive protein

dsDNA - double-stranded DNA

EV – extracellular vesicles

EV-GPA – extracellular vesicles isolated from the plasma of patients with granulomatosis with polyangiitis

EV-HC – extracellular vesicles isolated from the plasma of healthy controls

FSC/SSC – forward/side scatter

GC - glucocorticosteroids

GM-CSF - granulocyte-macrophage colony-stimulating factor

GPA – granulomatosis with polyangiitis

HMGB1 - high mobility group box 1

LTB_4 - leukotriene B_4

LTC_4 - leukotriene C_4

MnFI - mean fluorescence intensity

NET – neutrophil extracellular traps

NTA - nanoparticle tracking analysis

PBMC - peripheral blood mononuclear cell

PBS - phosphate-buffered saline

PLT – blood platelets

PMN - polymorphonuclear leukocytes

PPP – platelet poor plasma

PR3 – proteinase-3

PR3-ANCA - anti-PR3 antibody

RFU - relative fluorescence units

ROS - reactive oxygen species

TEM - Transmission electron microscopy

TNF-α – tumor necrosis factor alpha

Introduction

Granulomatosis with polyangiitis (GPA) is an infrequent, autoimmune disease with still unknown etiology. Several hypotheses suggest that genetic factors like polymorphisms of proteinase inhibitor (alpha- 1 antitrypsin), cytokine genes variants (IL-10) or some HLA genotypes might be associated with GPA (1, 2). According to *Chapel Hill Consensus* Conference (CHC) among primary systemic vasculitides, GPA is an anti-neutrophil cytoplasmic antibody (with specificity for proteinase 3 - PR3-ANCA or cANCA) associated vasculitis affecting small vessels. This definition implies presence of circulating cANCA and absence or paucity of immune complex deposits in areas of vasculitis by immunohistochemical studies of tissue specimen (3). Among spectrum of cells participating in pathophysiology of GPA, neutrophils are playing the most important role. The current paradigm of GPA assumes neutrophil activation by IgG anti-PR3 (cANCA), which requires direct recognition of PR3 via Fab region of the antibody, and interaction of the Fc part of the antibody with FcγRs on the neutrophil surface (4, 5). This causes neutrophil activation, degranulation, generation of reactive oxygen species and eventually trans-migration through the endothelial cell layer (6–8). However, a novel mechanisms of immune cells activation in pathophysiology of autoimmune disease like GPA can be linked to extracellular vesicles (EV).

Extracellular vesicles are a heterogeneous population of extracellular particles bounded by a phospholipid bilayer. They are released by practically all types of cells and can be detected in various biological fluids. According to the current nomenclature extracellular vesicles are divided into main groups of exosomes and microparticles (9). Exosomes are small (50-150 nm) and are derived from endocytic compartment. Microparticles are larger (100-1000 nm) and produced by budding of the plasma membrane following a loss of asymmetric distribution of phospholipids and reorganization of the cytoskeleton (10). Because distinction between these two population of EV is difficult, classification of EV into exosomes or microparticles is rather dogmatic. Some studies are showing that proteins like CD63, CD81 or CD9 are mostly present in exosomes likely reflecting their origin (11), whereas these markers can also be detected on larger EV (12). It is now recognized that EV are an integral part of the intercellular microenvironment and may act as regulators of cell-to-cell communication (10).

Extracellular vesicles can deliver proteins to the target cells or may participate in transferring receptors between cells. Recently, we observed that tumor-derived EV can induce monocytes/macrophage differentiation (13). It is interesting that EV content may differ from the parent cells, moreover different stimuli may cause release of EV with different cargo (14, 15). Extracellular vesicles can also contain DNA (including mitochondrial DNA), messenger RNA and microRNA but still it is little known about EV lipidomics. Several studies on extracellular vesicles lipids showed that EV can contain active enzymes linked to lipid metabolism like 12-lipoxygenase, phospholipase A2, C and D or lipid mediators like leukotrienes (16–20). The role of extracellular vesicles in GPA and neutrophils activation, especially in the context of lipidomics is still unclear. In this study we analyzed potential of plasma derived extracellular vesicles of GPA patients to activate neutrophils – focusing on oxylipins cargo of EV. We observed that vesicles isolated from GPA patients and not from the healthy controls were able to activate neutrophils and form NETs. These data provide insights into the mechanism by which extracellular vesicles could activate neutrophils and modulate innate immune response under pathological conditions.

Materials and methods

Patients' recruitment and samples collection

We enrolled 10 treatment naïve patients in active stage of GPA and 10 healthy sex and age matched controls. Disease activity was evaluated using Birmingham Vasculitis Activity Score version 3 (BVAS>1). Blood samples were collected in the morning, at least 12h after a meal. Basic laboratory tests (CBC, CRP, anti-PR3 IgG level) were performed in all participants of the study at the time of collection of the peripheral blood. Written informed consent was obtained from all participants of the study and the study protocol was accepted by Jagiellonian University Ethic Committee abiding by the Declaration of Helsinki principles.

Neutrophils isolation

Neutrophils were isolated from healthy donors' peripheral blood collected into tubes Vacutainer (BD Life Sciences, Franklin Lakes, NJ) using Histopaque-1077 gradient centrifugation (Sigma-Aldrich Chemical Co, St Louis, USA) followed by lysis of remaining erythrocytes with ammonium chloride buffer. Purity (>98%) of the neutrophils fraction was ascertained by flow cytometry and cells viability (>95%) was verified by trypan blue exclusion staining. Immediately after isolation, granulocytes were resuspended in X-VIVO 15 medium (Lonza, Basel, Switzerland).

Extracellular vesicles isolation

Extracellular vesicles (EV) were isolated from the platelet poor plasma of 10 treatment naïve patents with active GPA (BVAS 7-22) and 10 healthy sex and age matched controls by centrifugation. Blood samples were taken into Vacutainer tubes with sodium citrate as anticoagulant. After blood collection, samples were centrifuged for 10 min at 1450g (no brake) to obtain plasma, upper three-fourths of plasma was collected and centrifuged again to obtain platelet poor plasma (PPP, 5000g for 5 min no brake). PPP was aliquoted and stored in $^{-}$80°C. Frozen PPP (2.5 mL) was thawed in water bath for 10 min at 25°C and centrifuged for 20 min at 3500g to remove any cryoprecipitate. For EV isolation, collected supernatant was diluted with PBS (7 mL total sample volume) and passed through 0.8 μm syringe filter.

Purification of EV was done by ultracentrifugation in SORVALL WX 80+ ultracentrifuge and T-1270 fixed angle rotor (Thermo Fisher Scientific, Waltham, MA, USA). EV were pelleted by ultracentrifugation (1.5 h, 100000*g*) were washed with PBS and centrifuged again (1.5 h., 100000g). EV pellet was next suspended in 250 µL of filtered PBS, aliquoted and stored in -80 ° C for experiments.

Extracellular vesicles size distribution and surface markers characterization

EV number and size distribution was measured using nanoparticle tracking analysis (NTA, NanoSight LM10, Malvern Instruments, Amesbury, UK). EV samples were diluted 1:1000 with filterd PBS to reduce their number to the linear range of the apparatus, i.e. below $2–10\times10^8$/mL. Measurements video images were analyzed using NTA 3.1 Build 3.1.46 software (Malvern) captured in script control mode (3 videos of 60 s per measurement) using the syringe pump speed 80. A total of 1500 frames were examined per sample.

To rule out any presence of stimulatory endotoxins or anti-PR3 IgG (cANCA) antibodies derived from plasma of GPA patients, EV specimens were tested by a colorimetric method detecting bacterial endotoxins (Pierce Chromogenic Endotoxin Quant Kit, ThermoFisher Scientific, Waltham, MA, USA), and by an indirect immunofluorescence method detecting cANCA (ANCA IFA Granulocyte BIOCHIP Mosaic TEST - EUROIMMUN, Germany).

Evaluation of EV surface markers was performed by flow cytometry as previously described (21, 22). Briefly, EV aliquots (10 µL) were resuspended in PBS, mixed with 5 µL of aldehyde/sulfate-latex beads (4 µm of diameter, Invitrogen, Carlsbad, CA, USA) and incubated at room temperature for 15 min in 1.5 mL Eppendorf tube. After incubation, the content was added with 1 mL of 2% BSA in PBS and incubated overnight with rotation at 4°C. Bead-coupled EV were pelleted by centrifugation at 2700*g* for 5 min, washed with 1 mL of 2% BSA in PBS and centrifuged again. The pellet was resuspended with 50 µL of PBS and stained with the antibodies anti-CD63 (FITC, BD Biosciences, USA) and anti-CD81 (PerCP-eFluor 710, ThermoFisher Scientific, Waltham, MA, USA) for 30 min at room temperature.

Gating of EV-decorated latex beads was on FCS/SSC parameters, thus unbound EV or antibody aggregates were excluded from the analysis (Supplemental Figure S1).

Targeted oxylipins lipidomics of EV was performed by a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Briefly, samples were enriched with chemically identical deuterated standards (Cayman Chemical Co, Ann Arbor, MI, USA), extracted to an organic phase and analyzed using multiple reaction monitoring mode (MRM) tandem mass spectrometry (Qtrap 4000, Applied Biosytems, CA, USA). Details on the analytical procedure are provided in Supplemental Information, Supplemental Tables S1 and S2, and Supplemental Figures S2-S4.

ROS production

Generation of reactive oxygen species was measured in granulocytes by flow cytometry using dihydrorhodamine 123 (DHR 123). Isolated granulocytes (2×10^5/225 µl in X-VIVO 15 medium) were stained with DHR123 (5 µg/mL, Sigma-Aldrich Chemical Co, St Louis, USA) during 5 min incubation in 37°C with gentle shaking (150 rpm). Next, neutrophils were primed with recombinant GM-CSF (25 ng/ml, R&D Systems, Minneapolis, MN, USA) or left without priming for 15 minutes at 37°C and then stimulated with: complement C5a fragment (positive control, 10^{-8} M), EV from GPA patients or healthy controls (25 µL), 5-oxo-ETE or LTB_4 (4-400 pg/mL, Cayman Chemical Co, Ann Arbor, MI, USA). After 1 h, stimulation was stopped by addition of ice-cold PBS. ROS activity of the samples was measured immediately by flow cytometry (BD FACSCanto II) as rhodamine mean fluorescence index (MnFI).

EV binding/uptake by neutrophils

EV specimens were stained with PKH-76 fluorescent tracker dye (PKH67 Fluorescent Cell Linker Kit, Sigma-Aldrich Chemical Co, St Louis, USA) for 5 min in 37°C. Staining reaction was stopped by addition of PBS with 1% BSA and removal of unbound dye by 3 cycles of washing/centrifugation using Amicon Ultra 0.5 mL centrifugal filters (100K, Merck-Millipore, Billerica, MA, USA). After final centrifugation, EV concentrates were restituted to their original volume.

Presence of PKH 67 labeled EV in granulocytes was measured by flow cytometry (BD FACSCanto II). Neutrophils isolated from healthy donors ($0.2\text{x}10^6$/225 µL) were primed with GM-CSF (25 ng/ml) or

left without priming for 15 minutes at 37°C and then incubated with EV (25 µL) for 1 h. After stimulation cells, were washed twice by centrifugation and resuspension in PBS and analyzed.

Quantification of dsDNA release by stimulated human neutrophils

Isolated human neutrophils (5×10^5/225 µL in X-VIVO 15 medium) were primed with recombinant GM-CSF (25 ng/mL, R&D Systems, Minneapolis, MN, USA) or left not primed for 15 min at 37°C and then stimulated with the complement C5a fragment (positive control, 10^{-8} M). EV from GPA patients or healthy controls (25 µL), 5-oxo-ETE or LTB_4 (4-400 pg/mL, Cayman Chemical Co., Ann Arbor, MI, USA) to activate the cells for 1 h. After the incubation, DNase I (EURx, Gdansk, Poland) was added at 2.5 U/mL concentration for the next 10 min. Reaction was stopped by addition of 2.5 mM EDTA, pH 8.0 (Sigma-Aldrich Chemical Co, St Louis, USA). Cells were centrifuged at 190g for 5 min at 4°C. Content of dsDNA in supernatant was measured using Quant-iT Pico Green dsDNA Assay kit and Qubit 3 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA).

Visualization of neutrophil extracellular traps formation

Isolated granulocytes were resuspended in X-VIVO 15 medium ($2.5x10^6$/mL). Cells were primed with 25 ng/mL GM-CSF for 20 min on cleaned glass coverslips. Glass pretreatment included washes with acetone, ethanol, deionized H_2O, baking at 200°C for 1 h. Cells were incubated with 10^{-8} M C5a or EV (25 µL) from GPA patients or healthy controls for 1h at 37ºC in 5% CO_2. After stimulation, staining of live cells with a cell-permeable fluorescent dye MitoSOX Red (5 µM), was performed. Cells were next fixed with 4% paraformaldehyde for 5 min, washed three times in PBS and counterstained with 1 µg/mL Hoechst 33342, next mounted in ProLong Gold medium. Slides were examined and images acquired by confocal laser scanning microscopy LSM 700 (Carl Zeiss Micro Imaging, Jena, Germany) or DMi8 S Platform (Leica Microsytems, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). All comparisons were done using one-way analysis of variance (ANOVA) with Tukey's

post hoc test or t-Student test and Mann–Whitney U test. Descriptive statistics were presented as a mean ± standard deviation or median ± interquartile range. Type I statistical error $P<0.05$ was considered significant.

Results

The study subjects and extracellular vesicles characteristics

All GPA patients were newly diagnosed and treatment naïve, and were anti-PR3 IgG positive. BVAS disease activity score was between 7 and 22 (median = 14). Clinical laboratory tests showed that in comparison with healthy controls, GPA patients had elevated number of polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) (Supplemental Table S3).

EV isolated from GPA patients (EV-GPA) or healthy controls (EV-HC) were in the same size range and concentration (Fig. 1 A and B). Moreover, there were no differences in expression of EV markers. Both EV-GPA and EV-HC expressed CD63 and CD81 (Fig. 1C). All EV samples were free of IgG anti-PR3 antibodies and bacterial endotoxins.

Extracellular vesicles mediated neutrophils activation

To evaluate whether plasma derived EV can participate in granulocytes activation observed in GPA, we performed experiments in which neutrophils isolated from healthy donors were stimulated with EV. Using our preparatory technique, EV 10x concentrated because 250 µL of EV in PBS was obtained from 2.5 mL of PPP. To mimic EV abundance in plasma, neutrophils were stimulated with 25 µL of EV diluted 10-fold in the total cell suspension volume. First, neutrophils were stimulated with EV-GPA or EV-HC alone. In result, no differences were noticed between cells stimulated with EV-GPA or EV-HC and negative control of medium. For the next experiments, neutrophils were primed with GM-CSF (25 ng/mL) before addition of EV. Following stimulation with EV, ROS production (Fig. 2A) and release of dsDNA (Fig. 2B) were observed. These results were present only if EV-GPA were used. Stimulation of primed neutrophils by EV-HC resulted in absence of ROS production or dsDNA release. Moreover, direct microscopy showed that DNA released by intact live neutrophils stimulated with EV-GPA was likely of mitochondrial origin (Fig. 2C). To analyze how priming can influence EV mediated activation of neutrophils, we compared primed or not primed cells incubated with EV stained by fluorescent tracker (PKH67). Biding or uptake of EV was evaluated by flow cytometry. We observed that percentage of PKH67 positive cells (nautrophils/EV aggregates) was higher for primed cells, however, this was evident only for EV-GPA (PKH 67 positive cells: primed vs. unprimed: $P<0.05$, Fig. 3 and Supplemental Figure. S5).

Targeted oxylipins lipidomics of extracellular vesicles

To identify putative stimuli causing neutrophils activation we performed targeted oxylipins analysis of EV using HPLC-MS/MS. Out of 14 quantified oxylipins, the lowest concentration in EV was observed for LTC_4 (EV-GPA: 16.2±8.5 vs. EV-HC: 16.0±8.2 pg/mL, $P>0.05$, Supplemental Figure S6) and the highest for 13,14–dihydro-15-keto PGE_2 (EV-GPA: 818±448 vs. EV-HC: 819±448 pg/mL, $P>0.05$, Supplemental Figure S6). Significant differences between EV-GPA and EV-HC were present for LTB_4 (EV-GPA: 470±176 vs. EV-HC: 215±107 pg/mL, $P<0.05$), 5-oxo-ETE (EV-GPA: 524±279 vs. EV-HC: 221±81.2 pg/mL, $P<0.05$) and 5-HETE (EV-GPA: 62.9±31 vs. EV-HC: 28.9±15.7 pg/mL, $P<0.05$) (Fig. 4).

Oxylipins mediated neutrophils activation

To confirm functional importance of the oxylipins more abundant in EV-GPA we stimulated neutrophils for ROS production and dsDNA release with pure chemical LTB_4 and 5-oxo-ETE. 5-HETE, precursor of 5-oxo-ETE, had no activity at the measured concentration (Supplemental Figure S7). Stimulation of neutrophils with escalated concentrations of LTB_4 or 5-oxo-ETE increased ROS production or release of dsDNA at the minimal concentration of 4 pg/mL. The peak levels of ROS were observed in cells stimulated with 40 pg/mL of LTB_4 or 5-oxo-ETE (Fig. 5A). The highest levels of dsDNA release occurred after stimulation of granulocytes with 400 pg/mL of LTB_4 or 5-oxo-ETE (Fig. 5B). However, alike EV-GPA stimulation, neutrophils activation by LTB_4 or 5-oxo-ETE required a prior GM-CSF priming (Fig. 5C).

Discussion

Circulating EV were already suggested to participate in pathophysiology of autoimmune diseases, including GPA. However, most of the previous studies were limited to the descriptive observations. Hong et al. showed that neutrophils stimulated with ANCA antibodies can release microparticles activating endothelial cells (23). Daniel et al. reported higher plasma level of circulating extracellular vesicles in patients with active stage of GPA (24). In contrast to latter observation, we failed to observe any differences between concentration of EV isolated from plasma of GPA patients or healthy controls. This discrepancy could result from a different methodological approach. Daniel et al. (24) used a low speed (15000g) centrifugation as the isolation method, direct analysis with the use of flow cytometry but no NTA or TEM analysis. Possibly, authors focused on population of large extracellular vesicles like microparticles and apoptotic bodies. Since in the current study ultracentrifugation was used for isolation and NTA analysis confirmed enrichment of small EV, this could explain a difference between counting of large extracellular vesicles in the previous study, and a population of small exosomes and microparticles in the current one.

Studies on EV are currently very popular research area with a contribution of EV in pathophysiology of several disorders confirmed (25–28). However, interactions of EV with granulocytes, especially in autoimmune diseases like GPA are poorly investigated. Study of Kuravi et al. showed that platelet derived EV can promote adhesion of neutrophils to endothelial cells (16). Dieker et al. reported that circulating microparticles isolated from patients with systemic lupus erythematosus participate in neutrophil extracellular traps (NETs) formation by LPS stimulated cells (29). Release of dsDNA by activated neutrophils was also observed in cells stimulated with small EV (30-150 nm) isolated from cell culture supernatants of cancer cells (30). Interestingly, formation of neutrophil extracellular traps by activated neutrophils originally was considered one of the neutrophil death pathways, involving the rearrangement of nuclear and granular architecture which proceeded from the dissolution of internal membranes, followed by chromatin decondensation, cytolysis to formation of nuclear DNA fibers traps (31–33). However, we reported that neutrophils can form NETs consisting of mitochondrial DNA in a

ROS dependent mechanism and remain viable (34, 35). This observation may shed light on a persistence of innate immune response activation in autoimmune disorders.

Mechanism of activation neutrophils in GPA involves binding of self-reactive anti-PR-3 antibodies, which stimulate these cells (4, 5, 36). In the present study stimulation with small EV isolated from the plasma of GPA patients led to ROS production and release of dsDNA in GM-CSF primed neutrophils. The requirement for the neutrophil priming was met by preincubation with GM-CSF prior to stimulation. Higher levels of priming cytokines like TNF-α and GM-CSF were previously documented in GPA by us and others, and these observations may explain occurrence of priming effects *in vivo* (37, 38). Moreover, we reported in patients with GPA that level of circulating cell free mitochondrial DNA is higher in comparison with healthy controls (39). Taken together, presented results demonstrate that neutrophil extracellular traps could be an important mechanism of granulocytes activation in pathophysiology of GPA. Since neutrophil is one of the key players in pathophysiology of GPA the current findings expand the knowledge supporting this idea.

Beside increased binding and uptake of EV by primed granulocytes, the cells activation may result from oxylipins cargo of EV. This has been shown before in studies on LTB_4 or 5-oxo-ETE mediated neutrophil aggregation, adherence, calcium mobilization, ROS production or chemotaxis. All these effects required neutrophils priming with GM-CSF or TNF-α (40–44). Our result on LTB_4 and 5-oxo-ETE stimulation are in line with these studies. The novel finding of the current study is that EV stimulation caused release of dsDNA along with ROS production. This was detectable when primed granulocytes were stimulated with LTB_4 or 5-oxo-ETE, moreover, only EV-GPA had these stimulatory properties containing doubled cargo of LTB_4 and 5-oxo-ETE. We also noticed that EV-HC, which concentration of LTB_4 and 5-oxo-ETE was theoretically sufficient for granulocytes activation did not stimulate primed cells to ROS production or dsDNA release. This could be explained by several factors. First, due to a complexity of EV cargo, EV-HC can contain molecules suppressing neutrophil activation. Anti-inflammatory potential of EV has been reported for neutrophil-derived microparticles isolated from healthy donors or rheumatoid arthritis patients (48, 49). Second, EV-HC might be deficient in some target molecules crucial for internalization by granulocytes. Duchez et al. reported that active 12-

lipoxygenase was essential for neutrophils internalization of platelet derived EV (45), while studies of Maugeri et al. showed that this process required presence of HMGB1 which was absent in microparticles isolated from healthy donors (46, 47). In the current study we did not analyzed any potential proteins involved in EV uptake by granulocytes. However, we observed that priming of neutrophils with GM-CSF resulted with increased EV biding/uptake only for EV-GPA, explaining lack of EV-mediated neutrophils activation when EV-HC were used. However, only experiments using antagonists of receptors BLT1 or 2 and OXER1 could provide additional evidence to confirm the specific role of these two oxylipins.

Mechanism of release and intracellular origin of small EV is difficult to study *ex-vivo*, thus a cellular source of plasma circulating EV remains uncertain. In contrast to large microparticles, classical lineage membrane markers like CD11b for granulocytes or CD42a for platelets can't be used (50). However, some previous studies provided information on the origin of EV by their oxylipins content (51, 52). Duchez et al. reported that in platelet derived exosomes the main arachidonic acid metabolite is 12-HETE (45). In our study high levels of LTB_4 and 5-oxo-ETE were detected, produced by a 5-lipoxygenase (ALOX-5) positive granulocytes. 5-lipoxygenase is also expressed in monocytes, macrophages and dendritic cells (53–55). Study by Esser et al. suggested LTC_4 as the main eicosanoid in exosomes from macrophages and dendritic cells (17), whereas more recently Majumdar et al. reported that during chemotaxis neutrophils release LTB_4 enriched extracellular vesicles (56). Interestingly enough, 5-oxo-ETE is the major metabolite of 5-HETE formed during ROS production in neutrophils (57). We published previously on increased biosynthesis of these two mediators in the asthmatics following positive challenge with a house dust mite allergen, which correlated with the late phase of allergic inflammation (58). Taken together, it is highly plausible that granulocytes are the main source of small EV in patients with GPA. We acknowledge this study lacked appropriate methods to evaluate *ex-vivo* the mechanism of EV formation, and this will require future investigations.

In conclusions, we described a novel observation that primed neutrophils can be stimulated by circulating EV-GPA to produce ROS and release dsDNA. Moreover, by a targeted lipidomics we demonstrated that pro-inflammatory oxylipins of 5-lipoxygenase pathway had increased concentration

in EV-GPA, and their activity could be mimicked by chemically pure LTB_4 or 5-oxo-ETE. The current results also confirm that release of dsDNA by NET formation is important mechanism of neutrophils activation in pathophysiology of GPA.

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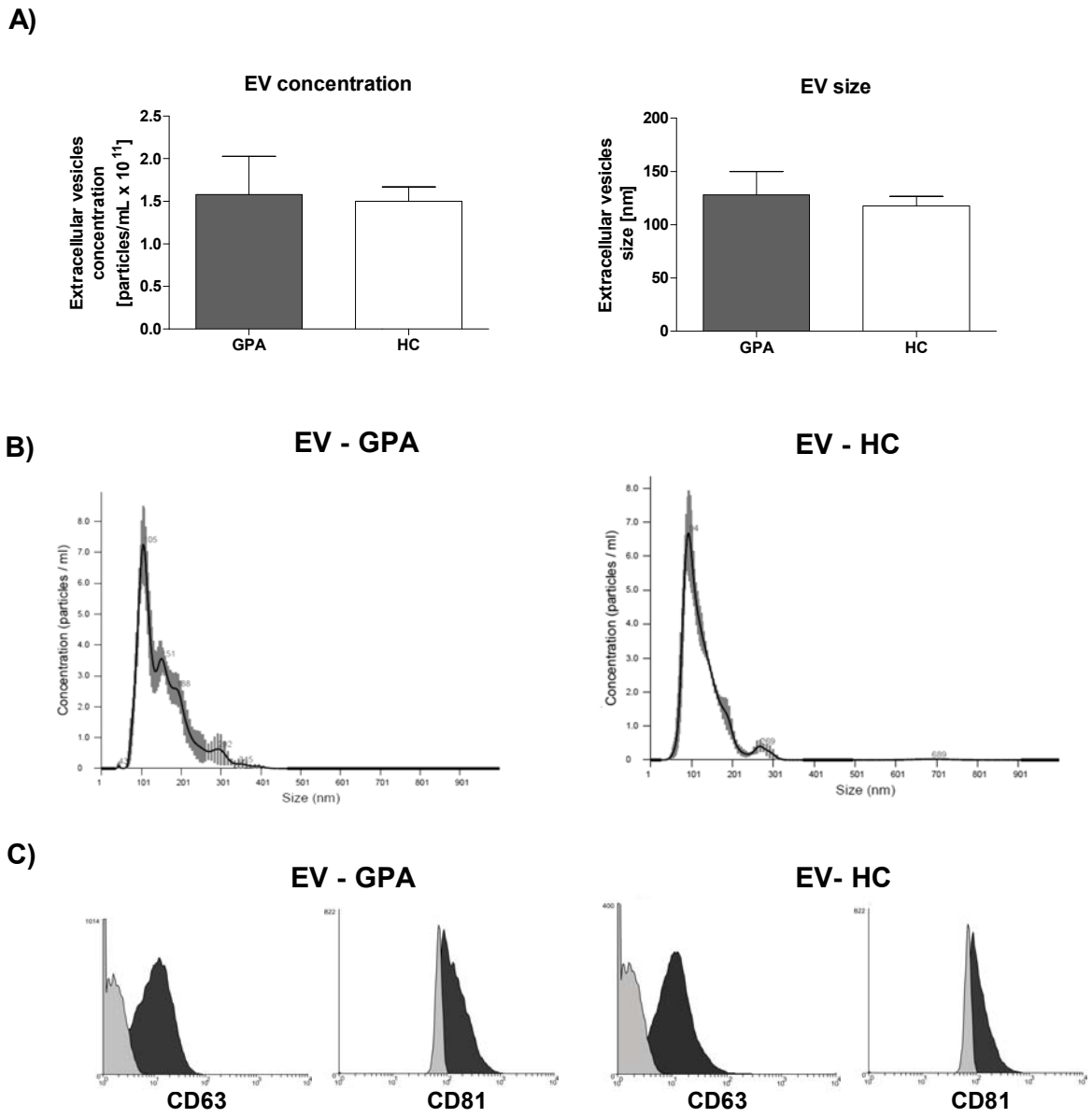

Figure 1. Panel A - Concentration and size distribution of plasma derived extracellular vesicles (EV). Results are presented as mean ± SD. Panel B and C - Representative histograms of size distribution (panel B) and CD63/CD81 expression (panel C) of EV. Left panel – EV isolated from GPA patient, right panel – EV isolated from healthy donor. Panel C –light gray - isotype control, - dark gray CD63 or CD81.

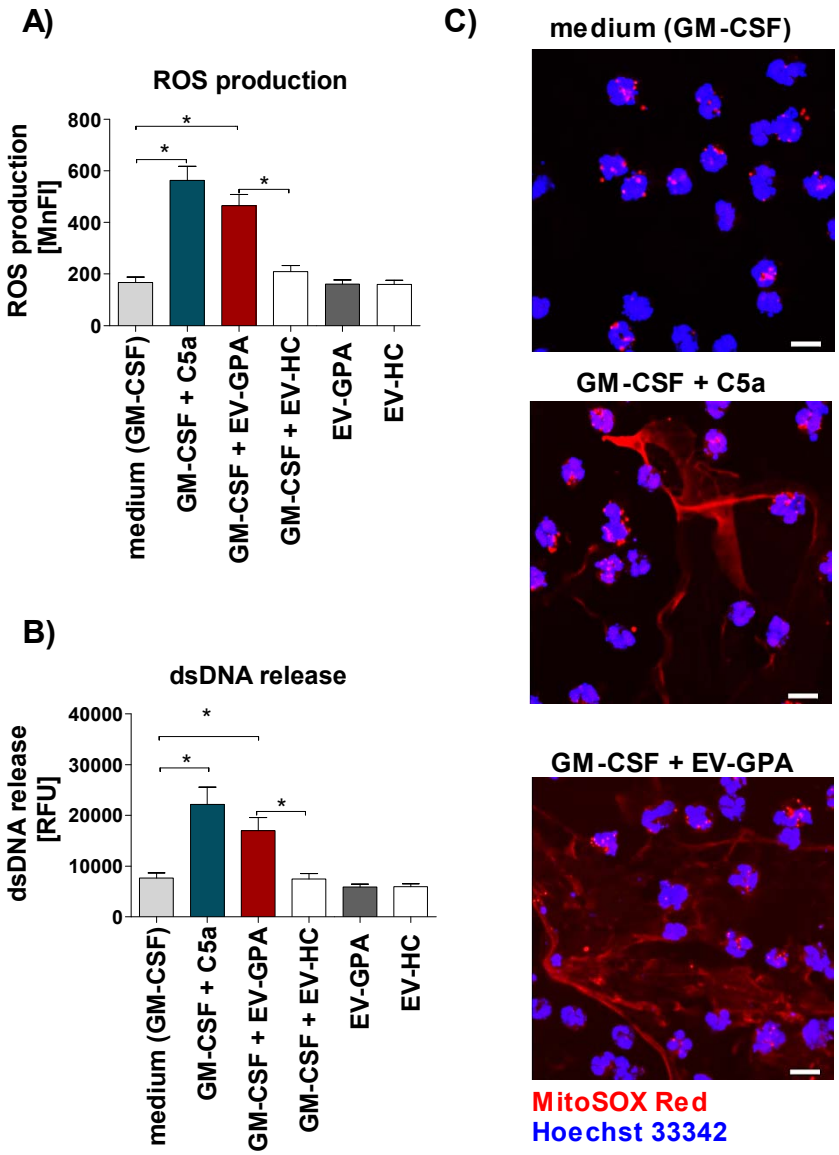

Fig. 2. Extracellular vesicle isolated from GPA patients activates human neutrophils. Neutrophils were isolated from the blood of healthy donors (n=6) and stimulated with C5a (positive control) or EV-GPA (n=10) and healthy controls (EV-HC, n=10). In each experiment cells were primed (GM-CSF, 25 ng/ml) or left not primed for 15 min before stimulation in parallel experiments with EV-GPA or EV-HC. Each EV sample was tested with neutrophils from two different donors. (A) Total ROS production by activated neutrophils was measured by flow cytometry. (B) Quantification of dsDNA in supernatants of activated neutrophils was performed using PicoGreen fluorescent dye. (C) Confocal microscopy. Representative images

of extracellular DNA release following short-term stimulation (total 45 min) of human neutrophils with the simuli. Cells were labeled with 5 μM MitoSOX Red to stain the extracellular DNA and cell nucleus was stained with 1 μg/ml Hoechst 33342. Scale bars are 10 μm. The images were acquired by confocal scanning microscope. GPA-EV - extracellular vesicles isolated from plasma of patients with granulomatosis with polyangiitis, HC-EV - extracellular vesicles isolated from plasma of healthy donors. Results are presented as mean ± SD of MnFI or RFU and were tested with the use of ANOVA with Tukey post-hoc test. *P<0.05 compared with non-stimulated control

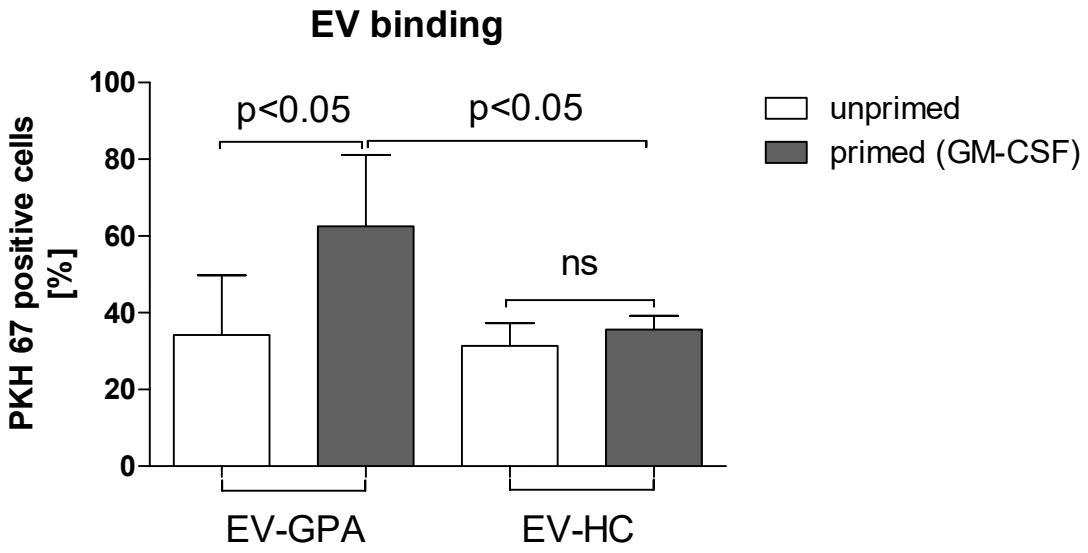

Figure 3. Binding of EV to primed and unprimed neutrophils. Neutrophils isolated from the blood of healthy donors (n=6) were primed with GM-CSF or left not primed for 15 minutes at 37°C, next added with PKH 67-stained EV (isolated from GPA patients or healthy controls) for 1h. Results are presented as median ± interquartile range of PKH67 positive cells and were tested with the use of ANOVA with Tukey post-hoc test. A control of not primed neutrophils added EV not stained with PKH 67 was used (for gating strategy see Supplemental Figure S5).

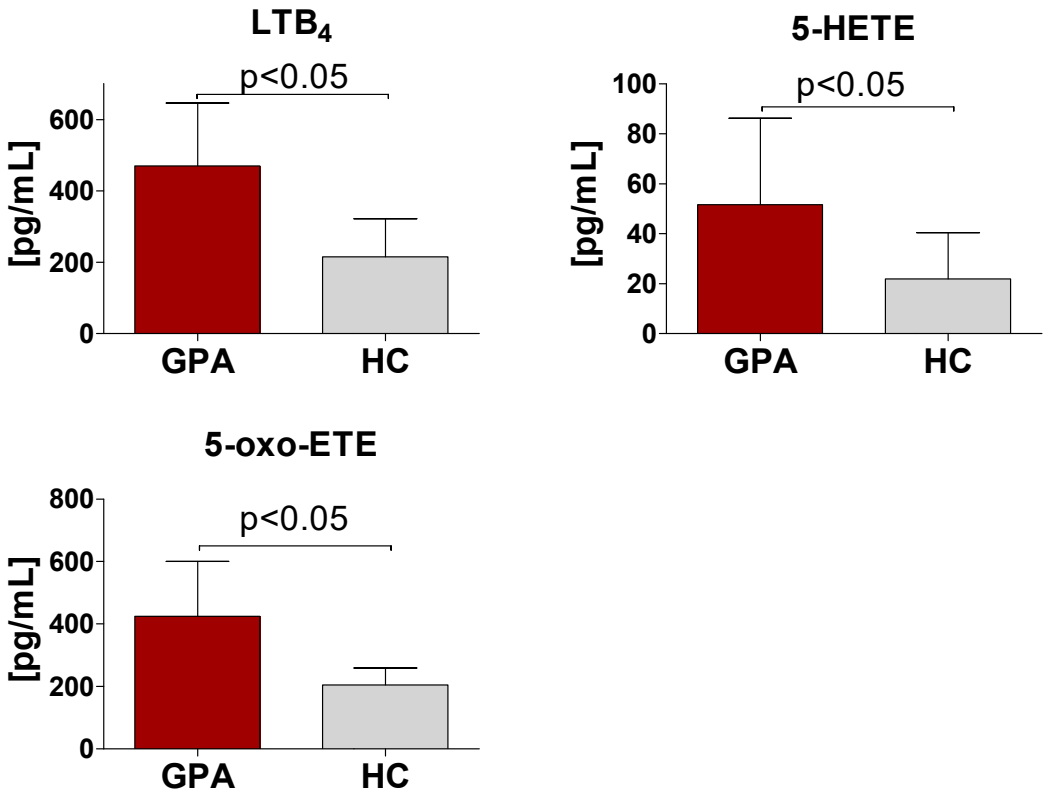

Figure 4. Extracellular vesicles (EV) levels of leukotriene B_4 (LTB_4), 5-hydroxyeicosatetraenoic acid (5-HETE) and 5-oxo-eicosatetraenoic acid (5-oxo-ETE).. EV were isolated from plasma of patients with granulomatosis with polyangiitis (GPA, n=10) and healthy controls (HC, n=10) and targeted oxylipins lipidomics of EV was performed by a high performance liquid chromatography-tandem mass spectrometry. Results are presented as mean ± SD. Differences between studied groups were tested with the use of *t*-Student test.

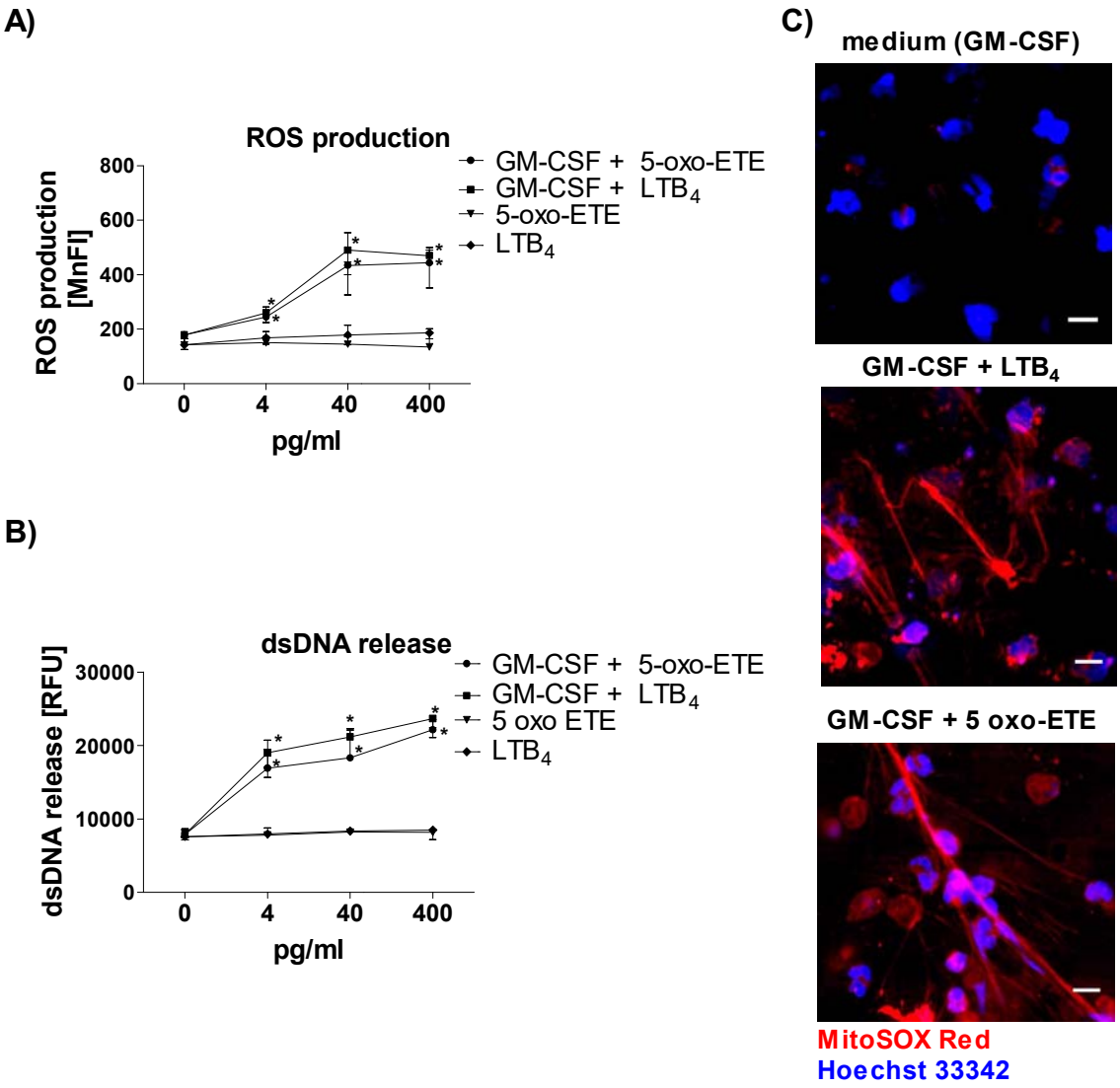

Figure 5. ROS production (panel A) and dsDNA release (panel B and C) by human neutrophils primed with GM-CSF and stimulated with LTB4 or 5-oxo-ETE. (A and B) Neutrophils isolated from the blood of healthy donors (n=6) were primed with GM-CSF (25 ng/ml) or left not primed for 15 minutes at 37°C, and next stimulated with serial dilutions of 5-oxo-ETE or LTB4. Concentration of 40 pg/ml for 5-oxo-ETE or LTB4 is representative for the mean concentration of both eicosanoids in experiments in which cells were stimulated with plasma derived EV isolated from patients with GPA. (C) Fluorescence microscopy. Representative images of extracellular DNA release following short-term stimulation (total 45 min) of human neutrophils

with the indicated stimuli. Cells were labeled with 5 μM MitoSOX Red to stain the extracellular DNA and nucleus stained with 1 μg/ml Hoechst 33342; Scale bars are 10 μm. The images were acquired by fluorescence microscope (DMi8, Leica). Results are presented as mean±SD and were tested with the use of ANOVA and Tukey post-hoc test. *P<0.05 compared with non-stimulated control.