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Alfaidi, M., Wilson, H., Daigneault, M. et al. (4 more authors) (2015) Neutrophil Elastase promotes Interleukin-1β secretion from Human Coronary Endothelium. Journal of Biological Chemistry. ISSN 1083-351X

https://doi.org/10.1074/jbc.M115.659029

Neutrophil Elastase promotes Interleukin-1β secretion from Human Coronary Endothelium

Mabruka Alfaidi, Heather Wilson, Marc Daigneault, Amanda Burnett, Victoria Ridger, Janet Chamberlain, Sheila Francis[1].

Department of Cardiovascular Science, Medical School, University of Sheffield, UK

Running title: *Neutrophil elastase promotes endothelial IL-1 secretion.*

To whom correspondence should be addressed: Professor Sheila E. Francis, Department of Cardiovascular Science, Medical School, University of Sheffield, Beech Hill Road, Sheffield, UK S10 2RX. Phone: +44 114 226 1432; Fax: +44 114 271 1862; Email: s.francis@sheffield.ac.uk

Keywords: Interleukin-1β, neutrophil elastase, endothelium, extracellular vesicles, inflammation, atherosclerosis.

Background: The mechanism of IL-1 release from endothelial cells is not fully known.
Results: Neutrophil elastase causes secretion of bioactive IL-1 from endothelial cells via microvesicles.
Conclusion: A mechanistic link between IL-1 secretion from endothelial cells and neutrophil elastase in atherosclerotic plaques is revealed.
Significance: Neutrophil elastase could be a potential target for preventing atherosclerosis.

ABSTRACT

The endothelium is critically involved in the pathogenesis of atherosclerosis by producing proinflammatory mediators, including interleukin-1 beta (IL-1β). Coronary arteries from patients with ischaemic heart disease express large amounts of IL-1β in endothelium. However, the mechanism by which endothelial cells (ECs) release IL-1β remains to be elucidated. We investigated neutrophil elastase (NE), a potent serine protease detected in vulnerable areas of human carotid plaques, as a potential 'trigger' for IL-1β processing and release. This study tested the hypothesis that NE potentiates the processing and release of IL-1β from human coronary endothelium. We found that NE cleaves the pro-isoform of IL-1β in ECs and causes significant secretion of bioactive IL-1β via extracellular vesicles. This release was significantly attenuated by inhibition of neutrophil elastase, but not caspase-1. Transient increases in intracellular Ca^{2+} levels were observed prior to secretion. Inside ECs, and after NE treatment only, IL-1β was detected within LAMP-1 positive multivesicular bodies (MVBs). The released vesicles contained bioactive IL-1β. *In vivo*, in experimental atherosclerosis, NE was detected in mature atherosclerotic plaques, predominantly in the endothelium, alongside IL-1β. This study reveals a novel mechanistic link between NE expression in atherosclerotic plaques and concomitant pro-inflammatory bioactive IL-1β secretion from ECs; this could reveal additional potential anti-IL-1β therapeutic targets and provide further insight into the inflammatory process by which vascular disease develops.

Atherosclerosis is a complex chronic inflammatory disease that involves inflammatory cell recruitment and release of pro-inflammatory cytokines (1). Interleukin (IL)-1β has been implicated in several aspects of vascular inflammation and neointima formation (2). Endothelial cell dysfunction, promoted by IL-1, also plays a central role in atherogenesis, by expression of adhesion molecules and cytokine secretion (3), facilitating leukocyte recruitment

and plaque development. The culmination of two decades of pre clinical experimental studies in the IL-1 field has led to the ongoing phase 3 clinical trial Cankinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) which will test whether blocking IL-1β only will reduce incidence of thrombotic events in patients after myocardial infarction that remain at high risk due to ongoing inflammation (4).

It has generally been assumed that IL-1β is produced predominantly by immune derived cells (5). However, we showed in ischemic heart disease (IHD) patients that atherosclerotic coronary arteries synthesise and express significant IL-1β within the endothelium (6) compared to controls. Experimental studies have also indicated that cultured endothelial cells (ECs) synthesize IL-1β in response to different cytokine stimulations, but that the released IL-1β is low and relatively inefficient (7). It is crucial therefore to understand the mechanism(s) of release of IL-1β from ECs especially since IL-1β acts at a distance rather than just locally in the vessel wall.

IL-1β production is a two-step controlled process, requiring an 'initial' stimulus for transcription/translation of proIL-1β (31kDa) which, in turn, is cleaved by an inflammasome-activated caspase-1 (8) ('a second hit') into a biologically active isoform (17KDa), before secretion. The cleavage of proIL-1β is a crucial step and studies in monocytes show that caspase-1 (a cysteine protease) is a cardinal enzyme in this process(9). There is a spectrum of proposed cellular mechanisms responsible for IL-1 secretion in monocytic cells – including rescue from autophagy and subsequent release, release via microvesicles or multivesicular bodies and terminal release (via pores), dependent on cell type and stimulus intensity (10).

In vivo studies have postulated that IL-1β can also be released in the absence of caspase-1(11) suggesting an alternative, and yet unknown, mechanism by which 'leaderless' IL-1β is secreted. There are other potential enzymes that cleave proIL-1β into its mature form, including the serine proteases (neutrophil elastase, cathepsin G and proteinase 3, (12,13)). It is known that in cell-free systems, these serine proteases cleave purified proIL-1β into a biologically active IL-1β *in vitro* at distinct sites to caspase-1 with production of 18kDa and 20kDa isoforms of IL-1β. However, whether, and to what extent, these proteases could contribute to IL-1β release in cells such as ECs is relatively unknown.

Neutrophil elastase (NE) is a potent serine protease that has wide substrate specificity (14) (15). Experimental studies have potentially focused on the destructive nature of NE, but interesting recent data show that NE can provoke a variety of pro-inflammatory responses such as IL-8 release from bronchial epithelium and TGF-β production in bronchial smooth muscle cells (14). Moreover, deletion of NE in mice leads to reduction of serum inflammatory biomarkers such as TNF-α, MCP-1, and IL-1 (16). One study has also demonstrated NE in macrophage rich human atherosclerotic plaque shoulders (17) and it also appears critical in caspase-1 independent IL-1β generation in NE-induced lung (18) and renal injury (19). In this study, we sought to determine whether NE promotes biologically active IL-1β secretion from vascular endothelium. We show that NE stimulation leads to pro-IL-1β cleavage and increases IL-1β release from coronary artery ECs via a caspase-1 independent, vesicular-release mediated process. Furthermore, we demonstrate that IL-1β is colocalized with NE predominantly in the endothelium in experimental atherosclerosis. This very first demonstration and explanation of active IL-1β release from endothelium potentially provides novel additional strategies for inhibition of IL-1β activity in inflammatory cardiovascular disease.

EXPERIMENTAL PROCEDURES

Human coronary artery endothelial cells (HCAECs) were purchased from PromoCell (Heidelberg, Germany) and cultured in supplemented media according to the

manufacturer's recommendations. The cells, at passage 2-5, were seeded into 6 well plates ($2x10^4$ cells/well) and grown at 37°C/ 5% CO_2 (v/v) until 70% confluent. The first step of stimulation was to up-regulate proIL-1β production by adding cytokines (TNF-α/IL-1α; 10ng/ml each) for 48h as previously described (7). Cells were then washed to remove all traces of stimulating cytokines before the media was replaced with serum free media containing NE (1μg/ml; equating to 60IU). To ensure the stimulating cytokines were completely removed, the final cell wash was tested for presence of IL-1 via ELISA. No cytokines were detected in these washes (data not shown). In some experiments, cells were pre-incubated with NEIII (500μM) (20), caspase-1 inhibitor I (YVAD; 50μM) (21) (8); or BAF1 (50nM) (22) for at least 30 minutes before the addition of NE. At the end of incubations, supernatants were collected and the cells were lysed in ice-cold 1% (v/v) Triton-X100 lysis buffer. Both supernatant and cell lysates were stored at -80°C until analysis was conducted.

Determination of cell viability: Cell viability was evaluated by Trypan blue dye exclusion and by measurement of lactate dehydrogenase (LDH) levels in conditioned media. LDH detected in cell lysates was used as a positive control for total LDH. Levels of LDH were analysed using CytoTox 96 Non-Radioactive cytotoxicity kit (Promega, USA) according to the manufacturer's instructions.

NE activity: NE activity was measured spectrophotometrically using a highly specific synthetic substrate (Elastase Substrate I, MeOSuc-Ala-Pro-Val-pNA; 100μM) as described in detail (14), (23). Briefly, samples (supernatant and lysate) were added to assay buffer (0.45 Tris-Base and 2M NaCl; pH. 8.0) containing Elastase Substrate I for 6h. The rate of the substrate cleavage was measured using a plate reader (Thermo Scientific) at 410nm.

*ELISA for IL-1β***:** The concentrations of IL-1β (pg/ml) in the supernatants and lysates were quantified by ELISA Quantikine kits (R&D systems) according to manufacturer's recommendations.

Apoptosis: Apoptosis, detected via caspase-3/7 activity, was analysed by Caspase-Glo® 3/7 assay (Promega) according to manufacturer's recommendations.

Western blot analysis for IL-1β processing and release: Samples (lysates and concentrated supernatants using 10k Amicon filter devices (ThermoScientific)) were subjected to Western blotting.

Microvesicle isolation: MV isolation was conducted as described (24). Freshly prepared MVs were used for analysis, to avoid false-positive effects caused by leakage of contents from MVs damaged by freeze-thawing.

Flow cytometry measurement of extracellular vesicles (ECV): A pellet of ECV was resuspended in annexinV-binding buffer and labelled with annexin V PE-Cy7 fluorescence according to the manufacturer's instructions (eBioscience, UK). Events were acquired using LSR II flow cytometer (BD Biosciences) and annexin-V positive ECV were enumerated using Accu Count Beads (SPHERO, 0.2-0.9μm) and analysed using FlowJo software (TreeStar).

Measurements of Intracellular Calcium concentration: HCAECs in 96 well plates at a seeding density of $5x10^3$ were treated with cytokines or left untreated for 48h. Fura-4 was then added to the cells according to manufacturer's instructions (Invitrogen). After washing off the dye, the cells were incubated with or without NE and changes in cytosolic Ca^{2+} were recorded using a plate reader (Thermo Scientific) according to manufacturer's recommendations. EGTA (6mM) and Ionophore A3784 (10μM) were used as controls as previously described (25).

Direct effects of NE on rIL-1β /ProIL-1β: NE is known to undergo spontaneous autolysis (26) and has a proteolytic activity against many cytokines such as TNF-α (15). For this reason, we tested the effect of NE with the studied concentration on IL-1β/proIL-1β itself. IL-1β standard (R&D) at a concentration of 125pg/ml and proIL-1β standard (R&D) at a concentration of 10000pg/ml were separately mixed with NE (1μg/ml) and kept in the incubator for 30 min, 2h

and 6h. The samples were stored at -80^{0}C and tested by ELISA and western blot.

Detection of extracellular vesicular shedding: HCAECs ($2x10^4$) were plated in LabTek (Fisher) 8-well chamber slides and subjected to the above-mentioned stimulation conditions. AnnexinV-Alexa Fluor® 488 (Invitrogen) was then added to the cells at 5µl/well. MV shedding was visualised using image acquisition software (Inverted widefield fluorescence microscope Leica AF6000 Time Lapse) after the addition of NE in a 5% CO_2/ 37^{o}C (v/v) heated chamber. The images were captured after 15, 30min, 2h and 6h and analysed using Image J software (NIH). MVs (0.1-1µm) were quantified in blinded samples in a random field of cells.

Immunofluorescence: Cells were fixed with 4% w/v paraformaldehyde and permeabilized in 0.3% v/v Triton-X 100. Non-specific binding was blocked for 30min with 5% v/v goat or rabbit serum in 1% w/v BSA prior to sequential incubation of the cells with primary antibodies: anti-goat IL-1β and anti-rabbit LAMP-1 (1:100 dilutions). Alexa fluor 647 and 488 conjugated secondary antibodies were used in 1% w/v BSA (1:200 dilution). The coverslips were washed with PBS and mounted onto glass slides using media containing DAPI.

The labelling of NE was conducted as previously described (27) using a Microscale Protein Labelling kit (Molecular Probes) according to manufacturer's instructions. 50µg NE was used for the reaction. Final concentration of Alexa647-NE was 0.1 mg/ml in a volume of 100µl.

Determination of IL-1β biological activity: The bioactivity of the secreted IL-1β by HCAECs following NE stimulation was assessed using an IL-8 luciferase reporter assay, sensitive to picomolar concentrations of IL-1β, as previously described (9); (28); (29). Briefly, HeLa cells ($5x10^3$) were grown to 70% confluence in 96 well plates and transfected with a total of 100ng DNA/well; including 60ng pIL-8-luc (reporter) and 40ng pRL-TK (internal control). After 24h, transfection efficiency was assessed and cells were then stimulated for 6h with 0.1nM of IL-1β as a control or with harvested supernatants from HCAEC stimulated with NE. IL-1 beta neutralising antibody (1ug/ml, MAB201, R&D Systems) was used in some wells, to prove specificity. Cells were then lysed with passive lysis buffer (Promega) and transferred to white well plates to assay for luminescence intensity using LARII and Stop and Glo reagents (Promega). Luciferase activity was calculated by normalising to the Renilla luminescence measured in each well according to manufacturer's recommendations (Promega).

Conventional and Immunogold Labelling Electron Microscopy: EC pellets (NE or untreated controls) were processed as previously described (30). Thin sections were immunogold labelled with primary antibodies: anti-goat IL-1β and anti-rabbit LAMP-1 (1:50 dilutions each) for 2h at room temperature. After washing the grids, the sections were incubated with immunogold conjugated secondary antibodies (20nm& 10nm gold particles, Agar Scientific, UK) for 2h. Control experiments were performed by using PBS instead of the primary antibodies and all sections were then post-stained with uranyl acetate and lead citrate.

Mice and diets: ApoE$^{-/-}$ male mice were bred in-house at the University of Sheffield. Food and water were given *ad libitum* under a controlled environment (Temp. 22-25^{o}C, humidity 55±5 and 12h light cycle). At 8-10 weeks of age, the mice were housed individually and fed a high fat, Western-type diet (HFD) containing 21% fat, 0.15% cholesterol and 0.296% sodium over a 12-week duration. Special Diet Services, Witham, UK, supplied the diets. This HFD was used specifically to study the diet effects on atherosclerosis as described (31). All animal care and procedures were closely conducted under ASPA 1986, UK and ethically approved by The University of Sheffield Ethics Committee. At the end of the study, the mice were euthanized and proximal aortae were harvested.

Immunohistochemistry: Sections were used for immunohistochemistry as previously described (32).

Statistical analyses: Results are shown as mean ± standard error of the mean (SEM). Analyses were performed using Graphpad Prism version 6.04 (Graphpad). For multiple comparison tests, one way analysis of variance (ANOVA) followed by Tukey's test was performed. Statistical significance was achieved when the *p* value was less than 0.05.

RESULTS

NE promotes IL-1 release in HCAEC: To assess the contribution of NE to IL-1β secretion, cytokine-primed ECs were treated with varying concentrations of NE in serum free media for different time points. As shown in Figure 1A, after 2h of stimulation, NE at 1μg/ml caused significant (10x) release of IL-1β from cytokine-primed cells (198±24.85pg/ml, $p<0.0001$) compared to primed cells without NE (12.1±4.81pg/ml). This release significantly decreased with higher concentrations of NE (2μg/ml) due to a decrease in cell viability (50±10%). Subsequent experiments, therefore, used NE at 1μg/ml to give the highest amount of IL-1β release without a significant increase in cell death (Figure 1B).

After 6h of stimulation, NE caused a significant increase in IL-1β secretion (Figure 1C) compared to cytokine stimulation alone (272.8±50pg/ml vs. 55.5±17.3pg/ml, $p<0.001$). No IL-1β was detected in the media of unstimulated cells (Figure 1C).

To specifically confirm that the release was due to NE, the cells were pre-treated with the specific neutrophil elastase inhibitor (NEIII), resulting in a significant attenuation of IL-1β secretion. To determine the involvement of caspase-1, cytokine-primed ECs were pre-treated with YVAD-CHO and then with NE. No significant changes in the secreted levels of IL-1β were seen compared to NE alone (Figure 1C). Thus, NE-mediated IL-1β release in endothelial cells was independent of caspase-1.

In unstimulated EC lysates, we did not detect any IL-1β. However, there was a significant IL-1β production inside cells following treatment with proinflammatory cytokines (Figure 1D). The IL-1β levels in the lysates were not significantly different amongst the groups (Figure. 1D). In addition, NE treatment alone did not provoke IL-1β production in the cells (Figure 1D), suggesting no direct effects of NE on IL-1β generation inside the cells. There was also no effect seen on IL-1β production in cell lysates by NE III or caspase-1 inhibitors (Figure 1D).

To confirm that NE was active over the duration of the study, we measured NE activity using quantitative cleavage of a chromogenic specific substrate and interestingly in the lysates harvested from NE-treated cells this was significantly increased (Figure 1E).

Cell lysis as a mechanism of IL-1β secretion was ruled out by the absence of cytosolic enzyme lactate dehydrogenase (LDH) levels in cell supernatants. There were no significant changes in LDH levels following NE stimulation at 2 and 6h compared to control (Figure 1F). Since IL-1 release could be a feature of cell apoptosis, we investigated caspase-3/7 activation in ECs under our stimulation conditions and showed no significant increase in NE treated cells compared to unstimulated ECs, cytokine stimulated cells, or cells in which NE effects are attenuated using NEIII (Figure 1G). In addition, we used propidium iodide (PI) in conjunction with annexin V (PI/AV) to determine if EC were viable, apoptotic, or necrotic at any timepoint used in this study. We did not detect any significant increase in PI/AV staining in NE treated cells compared to untreated controls (data not shown).

In vitro, some proteases, including NE, have been shown to process proIL-1β into mature IL-1β (13). Thus, as the ELISA does not distinguish between pro- and mature- IL-β, we determined which IL-1β isoforms were present, and their relative respective levels, in cell lysates by immunoblotting. Unsurprisingly, unstimulated HCAEC lysates did not contain any detectable IL-1β but full-length proIL-1β (31kDa) was seen in

cytokine-primed cell lysates (Figure 2Ai). However, after the addition of NE, there was cleavage of the 31kDa pro-form associated with a decrease in the 31kDa proIL-1β in cell lysates (Figure 2Ai) that was NE specific as evidenced by inhibition by NEIII but not YVAD. On addition of NE, 20kD and 17kD IL-1β forms were detected (Figure 2Ai). Again, this was NE specific (Figure 2B). Interestingly, NE induced maturation of IL-1β that was not associated with procaspase-1 cleavage or alterations in NLRP-3 levels in lysates as detected by Western blot (Figure 2Aii, iii). Nor did we detect p20 (the product of active caspase-1) in cell media, indicating that cleavage and secretion had not occurred. NE is known for its proteolytic properties, therefore, to confirm that NE cleaved the pro-form, but not the mature form of IL-1, recombinant proIL-1β and recombinant mature IL-1β were incubated with NE in a cell-free system. NE cleaved the pro-form of IL-1 *in vitro* (Figure 2C) but not the mature form (Figure 2D, 2E), supporting the hypothesis that NE specifically cleaves the pro-form of IL-1β only. Taken together, these data clearly suggest that NE increases cytokine-induced IL-1β secretion in ECs via an inflammasome/caspase-1 independent pathway.

NE induced secretion of extracellular vesicles containing bioactive IL-1 from HCAECs: In immune derived cells, protected release of IL-1β (microvesicles, multivesicular bodies (MVB), exosomes) has been proposed as a mechanism for IL-1β trafficking to the extracellular environment (9,33,34). Therefore, we sought to determine whether there was extra-vesicular shedding occurring in response to NE and whether this is associated with IL-1β processing in HCAECs.

Since phosphatidylserine (PS) exposure has been associated with MV shedding in monocytes (9) and EC (24), we used annexin V binding (annexin V-Alexa Fluor 488) as a tool to visualise events in live HCAECs. Small particles (0.1-1μm in diameter) were observed separating from the cells in real time using time lapse imaging over duration of 6h (see video, supplemental material). The first MV generation was at 10 min, with membrane alterations at 30 minutes after NE stimulation with large numbers of MVs observed at later time points (Figure 3). The number of MVs was quantified using flow cytometry and gating for annexin V (Figure 4A). Interestingly, there was a significant increase in the number of MVs isolated from ECs following NE treatment (two to threefold) compared to controls. Importantly, NE inhibition effectively attenuated MV formation and shedding induced by NE and caspase-1 inhibition had no significant effect (Figure 4B).

We next investigated which IL-1β isoforms were inside MVs using immunoblotting. MVs from unstimulated cells contained no IL-1β, and in MVs from cytokine-primed cells (6h), prominent proIL-1β (31kDa) forms were detected (Figure 4C). In MVs isolated from the supernatants of NE treated cells, cleavage of the 31kDa IL-1β isoform to approximately 20kDa-19kDa was observed from as early as 30 minutes (Figure 4D) with further cleavage to the 18 and 15kDa isoforms after 6h (Figure 4C). Treatment of cells with NEIII abolished any cleavage of proIL-1β in these MVs, confirming that these bands are the result of direct NE activity. Cleavage of proIL-1β continued even in the presence of YVAD with more prominent isoforms detected (Figure 4C). MVs were assessed for caspase-1 and NRLP3 content and, interestingly, active caspase-1 p20 and NLRP3 were not detected in MVs isolated from cells treated with NE or NE and YVAD together (data not shown), indicating that intra-vesicle cleavage of proIL-1β is independent of caspase-1 activation. These findings suggest that either NE cleaves the released proIL-1β inside MVs or that NE treated cells continually generate more MVs containing IL-1β as a route of secretion.

We next asked whether the processed IL-1β released into cell supernatants in vesicles as a result of NE activation, was bioactive. We collected total supernatants (containing MVs) from NE-treated or untreated cells for 6h and applied them to HeLa cells expressing an IL-1RI responsive reporter, and measured IL-8 activity. We compared reporter assay output (IL-8) to

media obtained from unstimulated or cytokine-primed EC +/- NE with a positive control (0.1nM recombinant IL-1β) or with an IL-1β neutralising antibody. As shown in figure 4E, supernatants isolated from NE activated ECs significantly increased IL-8 activity compared to unstimulated and cytokine-primed cells and this was completely abrogated by the neutralising antibody. In order to confirm that the bioactivity was due to released IL-1β and not a result of direct NE effects on HeLa cells, NE (1μg/ml) was added to HeLa cells (as a spike) and this showed no significant IL-8 activation. These data indicate that the IL-1β in the MVs is bioactive.

MVs containing IL-1β were confirmed using immunogold TEM and we detected 0.2μm diameter MVs containing immunogold labelled IL-1β only in NE treated cells (Figure 4F).

Mechanisms of IL-1β release in endothelial HCAEC: To study early MV shedding by NE, intracellular calcium changes were assessed. MV release has been linked with transient changes in intracellular calcium $(Ca^{2+})_i$ in specialised secretory cells (35) and IL-1β secretion in macrophages (33). Using a Ca^{2+} sensitive fluorometric dye, we assessed the role of $(Ca^{2+})_i$ in MV formation and release in response to NE and performed experiments in the presence or absence of exogenous Ca^{2+}. In this experiment, $(Ca^{2+})_i$ is released from intracellular stores during an initial stimulation/treatment in Ca^{2+} free media and application of $CaCl_2$ during the second phase of the protocol, allows Ca^{2+} influx inside ECs. In Ca^{2+}-free media, there was a small non-significant increase in cytosolic Ca^{2+} levels in NE treated cells compared to untreated cells after NE stimulation (Figure 5A). However, $(Ca^{2+})_i$ was significantly increased in NE stimulated cells after the addition of $CaCl_2$ compared with unstimulated and cytokine-primed cells (Figure 5B). This finding suggests that NE treatment of EC increases free $(Ca^{2+})_i$ by promoting Ca^{2+} influx into EC and that this calcium influx is associated with MV release.

We detected small MV (approx. 200nm) in response to NE (assessed by flow and electron microscopy), characteristic of exosomes secreted from multivesicular bodies (MVBs) and this led us to study the compartmentalisation of IL-1β in EC (30). HCAECs +/- NE were stained using an immunofluorescence technique for LAMP-1 (a late endolysosomal marker) and IL-1β. Confocal images of cytokine-primed ECs stained for IL-1β and co-stained for LAMP-1 suggested a wide distribution of IL-1β throughout the cytoplasm and no colocalization with LAMP-1 (Figure 5C). Unsurprisingly, no signals were detected for IL-1β in unstimulated ECs (data not shown). However, in ECs incubated with NE for increasing times (30min, 1, 2h), IL-1β was co-localised with LAMP-1 (Figure 5D). Indeed, following a 2h stimulation of EC with NE, the majority of IL-1β was detected in MVBs (Figure 5D).

To confirm that the site of IL-1β processing and secretion was indeed mediated by an endolysosomal mechanism, we evaluated the effect of bafilomycin A1 (BAF1), a lysosomal V/ATPase inhibitor that has been shown to prevent endolysysomal formation (36). Treatment of ECs with BAF1 (50nM) before the addition of NE largely decreased IL-1 levels in the supernatants after 6h, and that was associated with a reduction in pro-IL-1 cleavage in the lysates (Figure 6A, B). We subsequently performed TEM on cytokine-primed HCAECs +/- NE and observed >200nm structures in the cytosol with classical morphological features of multivesicular bodies (MVB) (37). These were detected in close proximity to the plasma membrane in NE-activated ECs but not in unstimulated ECs (Figure 6C, panel i). Interestingly, inside the cells, the majority of IL-1 was detected within the MVBs (Figure 6C, panel ii).

NE is detected in ECs and is colocalized with IL-1β in the endothelium of mature atherosclerotic plaques: To follow the fate of NE in activated ECs, we used Alexa-Fluor 647-labelled NE and performed immunofluorescence staining. After permeabilization, we also labelled the internal endolysosomes with LAMP-1. Surprisingly, NE was detected inside cells. The enzyme co-localised with LAMP-1 (Figure 7A).

Finally, we asked whether NE could be detected in atherosclerotic plaques in mice to ascertain if NE could contribute to local IL-1β generation. Only in well-developed atheromatous lesions of ApoE$^{-/-}$ mice fed a high fat for 12 weeks was IL-1β was detected, predominantly in endothelial cells. Interestingly, in these lesions, NE also appeared to be expressed in the luminal endothelium (Figure 7B) and colocalized with IL-1β-positive stained ECs (Figure 7C).

DISCUSSION

Here we describe for the first time, how coronary artery ECs release IL-1β, which has been a 'holy grail' of endothelial biology for many years. We report that a considerable amount of IL-1β is released from ECs in response to NE via a caspase-1 independent, vesicular pathway. This is supported by the following lines of evidence: 1) Bioactive IL-1β is secreted only in MVs following NE treatment, 2) IL-1β secretion is unaffected by YVAD and occurs without caspase-1/NLRP3 activation and 3) MVB are prevalent in primed NE-treated ECs and contain IL-1β protein. We propose that 'protected release'(10) (i.e. contained within membrane-bound vesicles) of IL-1β is a prevalent mechanism in HCAECs.

The endothelium is fundamental in atherosclerotic plaque development, not only during early lesion development but also later by controlling plaque instability (38). In atherosclerosis, crosstalk between circulating cells such as monocytes and neutrophils and the endothelium can cause ECs to liberate soluble agents perpetuating the cycle of inflammation. Several lines of evidence suggest that IL-1 is an apical cytokine in this process (39) yet its mechanism of release from ECs is largely unknown. Furthermore, the biological pattern of the crosstalk is not completely defined. We hypothesised that NE induces IL-1β secretion from vascular endothelium.

Since caspase-1 has been identified as the main proteolytic enzyme to play a role in proIL-1β cleavage and secretion in monocytes and macrophages (40), we used a specific caspase-1 inhibitor (YVAD-CHO) as a potential means of attenuating IL-1β release. Our data show that caspase-1 appears to be non-essential in EC in this setting for IL-1β cleavage and release by NE. This is at odds with other *in vitro* studies in monocytes (21), but in agreement with more recent data from other cell types (41) and *in vivo* models (42,43). Our findings are also supported by the findings of Guma *et al.* (11), who describe the presence of IL-1β in the synovial fluid of caspase-1$^{-/-}$ mice. Moreover, our data may explain why caspase-1 suppression did not show promise in vascular healing or atherosclerosis progression (32).

It has been previously shown that IL-1 lacks the signal peptide for directing it to the classical ER-Golgi secretory pathway (30). Therefore, IL-1β release has been proposed to occur by distinct mechanisms, including MV shedding and endolysosomal regulation (10). Strikingly, in HCAEC, NE induced MV shedding occurred independent of caspase-1 activity contrasting with previous investigations on immune cells in terms of their caspase-1 dependency of MVs shedding. We suggest that, at least in HCAEC, NE is able to directly cleave the IL-1β precursor, which is associated with protected release in membrane-bound vesicles. In MVs, the prominent forms of IL-1β released (also present inside cells) were 20kDa, 18kDa and 15kDa. These isoforms have been detected previously *in vitro* (13) and although proposed as 5-10 fold less bioactive than the 17kDa isoform, they are active enough for IL-1 to bind to its receptors and initiate signalling. Our study is the first to show in intact cells that NE is capable of cleaving proIL-1β at multiple sites and that these products are bioactive.

A common biogenesis has linked Ca^{2+} regulated MV shedding and IL-1 secretion with an increase in intracellular calcium levels (33). We are in agreement with this and demonstrated that NE transiently increased $(Ca^{2+})_i$ to a maximum after addition of exogenous Ca^{2+}, suggesting that NE mobilized $(Ca^{2+})_i$, mainly by influx of extracellular Ca^{2+}. The secretory pathway identified here has been used to describe the

secretion of other non-classical proteins such as EGF (44). Although a few studies have begun to investigate NE-mediated IL-1β secretion in renal disease and lung ours is the first in cardiovascular inflammation to propose a direct effect of NE on IL-1β release and to link this to a MV release mechanism.

Recent immunofluorescence staining data in murine macrophages has shown that IL-1β does not colocalise with LAMP-1 in macrophages (45). This finding is consistent with our data in EC prior to NE treatment (cytokine-primed cells) where IL-1β appears to be diffusely distributed within the cytoplasm. Significantly, however, 2h after NE treatment, colocalisation increased, strongly suggesting that IL-1β compartmentalisation may be acutely induced by NE and pre-destined for maturation by regulated transport. In ECs primed with cytokines and treated with NE, we observed that MVB with cytosolic IL-1β accumulated within cell invaginations similar to (46). This is consistent with work from Rubartelli *et al* in monocytes (47). Since MVBs were not detected in cells that did not produce IL-1β (33), this strongly suggests that the MVBs are a part of the secretory pathway of IL-1β.

The colocalisation of IL-1β and LAMP-1 suggests that NE may trigger a signalling pathway that allows the IL-1β processing to occur in secretory endolysosomes (MVB). However, the possibility of NE internalisation within ECs, via endocytosis, cannot be ruled out, particularly as we detect enhanced NE activity in NE-treated ECs lysates compared with untreated and cytokine-primed cells. In agreement with others who have shown that NE is internalised by macrophages (48) and tumour cells (27) by active transport, we also detected NE inside EC using confocal imaging. Therefore, it is possible that after internalisation of NE by ECs, NE enters MVBs where it cleaves proIL-1β.

It is possible that NE is also causing release of IL-1β due to a toxicity effect, with NE causing cell damage and leakage of cellular contents. However, at the NE concentration used, this is unlikely to be the case as cell viability was not significantly affected, nor was apoptosis proven. Indeed, IL-1β has been shown to increase caspase 3/7 in cells without inducing apoptosis (49), which is in agreement with our data; NE increases IL-1β levels, which, in turn, affects caspase 3/7, but does not affect apoptosis. We use recombinant NE throughout this study, raising the question of what the natural source of NE would be and whether levels of NE would be enough to activate EC. Previous work by our group has shown IL-β is released from activated co-cultures of ECs and monocytes, at greater levels than from monocytes or ECs alone, and that an unidentified monocyte-derived mediator significantly contributed to this response (50). Taken together with the current findings reported here, we postulate that NE is the mediator released from monocytes and would be the natural source of NE *in vivo*.

Given the continued prominence and topicality of IL-1 in the generation and progression of atherosclerosis (51) we studied the expression of NE *in vivo* in a recognised atherosclerosis preparation: aortic root plaques taken from $ApoE^{-/-}$ mice fed a high fat diet for 12 weeks. Although previous work has detected NE in coronary arteries (6) aortic aneurysms (52) and in carotid plaques (17), our study is the very first to investigate NE distribution in experimental atherosclerosis. Significantly, NE was mainly detected in the endothelium of plaques, and was detected alongside IL-1β. The antibody used for these studies recognises both proIL-1β and mature forms, and we show a clear cellular colocalization with NE in support of our data that NE activates and promotes secretion of IL-1β. This colocalization of NE and IL-1β suggests that the IL-1β observed is likely to be active. We propose that circulating NE is assimilated into developing plaques from degranulating immune cells during their passage through the main vasculature or via vasa vasorum where these exist. This suggestion remains to be clarified, and a causal connection between IL-1β and NE confirmed, in a future study utilising $NE^{-/-}$ mice.

In conclusion, we significantly add to and cement the emerging role of NE in IL-1 induced inflammation. Further, we suggest a novel mechanism for NE-mediated IL-1 secretion by ECs, namely pro-IL-1 processing in the secretory endolysosomes and packaging of mature bioactive IL-1 within MVs for release into the extracellular environment, as part of a single continuum mechanism (figure 8), which is similar to that previously proposed for other cell types (10). We detect NE and IL-1β *in vivo* in the setting of atherosclerosis within endothelium in atheromatous plaques. The pathophysiological relevance of the detection of vesicular IL-1, particularly derived from endothelium, gives ECs the potential to exert a regulatory influence upon atherogenesis and henceforth to become a possible therapeutic target by modulating IL-1 secretion in the local environment.

Our findings have wider application for a better understanding of the role of other important proteases with prominent non-proteolytic and possibly signalling roles such as azurocidin, proteinase 3 and Cathepsin G and provide other avenues for therapeutic targets to limit the influence of interleukin-1.

ACKNOWLEDGMENTS

This work was supported by a PhD studentship to Dr. M Alfaidi from The Medical School, Omar Al-Mukhtar University, Al-Bayda, Libya.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

MA performed all experiments, analysed the results and contributed to writing the paper. HW, MD, AB, VR all helped design, conduct or analyse experiments involving microvesicles. JC and SF conceived and co-ordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

* This work was supported by a PhD studentship to Dr. M Alfaidi from The Medical School, Omar Al-Mukhtar University, Al-Bayda, Libya.

[1]To whom correspondence should be addressed: Professor Sheila E. Francis, Department of Cardiovascular Science, Medical School, University of Sheffield, Beech Hill Road, Sheffield, UK S10 2RX. Phone: +44 114 226 1432; Fax: +44 114 271 1862; Email: s.francis@sheffield.ac.uk

[2]The abbreviations used are: IL-1ra, interleukin-1 receptor antagonist; EC, endothelial cells ; NE, neutrophil elastase; MVs, microvesicles; MVB, multivesicular bodies.

FIGURE LEGENDS

FIGURE 1: NE enhances IL-1β secretion from HCAECs by a caspase-1 independent mechanism. A) IL-1β is released by HCAECs after 48h stimulation with cytokines (TNF-α/IL-1α; 10ng/ml) followed by NE activation (0.5μg/ml - 2μg/ml) for 2h, measured by ELISA (n=3). **B)** Cell viability (measured by trypan blue) is not significantly reduced following exposure to 1μg/ml NE (n=3). **C)** HCAECs, incubated for 48h +/- cytokines then further incubated for 6h in serum free media +/- NE (1μg/ml), in the presence or absence of inhibitors (NEIII; 500μM & YVAD; 50μM), n=5, shows IL-1β release is increased by NE independent of caspase-1. **D)** Levels of IL-1β in cell lysates is not increased following NE incubation, n=5. **E)** Graph showing increased NE activity in EC lysates treated with NE for 6h compared to unstimulated cells, n=3. **F)** LDH levels are unchanged following NE treatment, measured in conditioned media or in cell lysates as a total of LDH, n=3. **G)** Caspase-3/7 activity is unchanged in HCAECs following NE treatment. HCAECs in 96 well plates ($2x10^4$ cells/well) incubated +/- cytokines (TNF-α/IL-1α; 10ng/ml each) for 48h, were subjected to NE (1μg/ml) in serum free media for 6h (n=3). Campthothecin (10μg/ml) was used to induce apoptosis as a positive control. All data are mean ± SEM, analysed by one-way ANOVA with Tukey's multiple comparison multiple test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

FIGURE 2: NE selectively cleaves proIL-1β in primed EC lysates without caspase-1/NLRP3 activation. A) Western blot analysis of cell lysates of primed EC +/- NE, assessed for IL-1β **(i)**, Caspase-1 **(ii)** and NLRP-3 **(iii)**. The blots are representative of n=3, with α-tubulin levels as loading control. For IL-1β, recombinant IL-1β (rIL-1β; 20μg, 17kDa) was loaded as a positive control and represents the commonly detected mature form, whereas proIL-1β (31kDa) indicates the inactive pro-form. For Caspase-1, activated THP-1 cell lysates were used as a positive control for the p20 isoform. **B)** Densitometric analysis of 20kDa IL-1β levels (n=3). **C)** Western blot illustrating the cleavage of recombinant proIL-1β (rProIL-1β; 20μg) by NE. rProIL-1β was incubated at 37°C (5% CO_2; v/v) alone or in the presence of NE (1μg/ml) for 30min, 2h, and 6h. **D)** Western blot for recombinant mature IL-1β (rIL-1β; 20μg) in the presence or absence of NE, shows no cleavage (n=3) and **E)** ELISA for recombinant mature IL-1β shows no difference in levels following NE treatment. Data are mean ± SEM, analysed by one-way ANOVA and Tukey's post-test, *p<0.05, **p<0.01.

FIGURE 3: Neutrophil elastase activates microvesicle shedding from endothelial cells in a time dependent manner. HCAECs were left untreated or treated with cytokines (IL-1α/TNF-α) for 48h and then labelled with annexin-V AlexaFluor®488. After the addition of 1μg/ml of NE, cells were visualized in a heated chamber (5% CO_2 v/v) using a confocal microscope to scan MV release. Images captured after 10 minutes, 30 minutes, 2 hours and 6 hours show an early generation of MVs after 10min of NE stimulation, but more prominent at later time points. Arrowheads indicate fluorescent MVs and arrows represent earliest blebbing in EC treated with NE. Scale bars=50μm; the representative images are from three independent experiments (n=3), and have been digitally altered to remove background fluorescence.

FIGURE 4: NE induces secretion of extracellular vesicles containing bioactive IL-1 from HCAECs A) Flow cytometric characterisation of MV released in response to NE. MVs were isolated and stained with annexin V PE-CY7 as described in the materials and methods**.** Analysis of MVs (red) using Megamex beads (blue) shows they are within the 0.2-0.9μm size limits. **B)** A significant increase in MV stained with annexin V is seen in NE treated cells compared to untreated controls. Analysis was

performed by Flow Jo software, n=3, mean ± SEM, 1-way ANOVA followed by Tukey's post- test, *p<0.05, **p<0.01. **C & D)** Detection of IL-1β in isolated MVs by immunoblotting. Equal amounts of protein (20μg) were loaded in each lane, with rhIL-1β (20μg) used as a positive control (17kDa). Data are representative of n=4. **E)** Luciferase assay for measurement of IL-1β bioactivity in HeLa cells exposed to freshly harvested conditioned media (total supernatants from cytokines primed cells (TNF-α/IL-1α; 10ng/ml each +/- NE; 1μg/ml) or rIL-1β (0.1nM) for 6h +/- anti-IL-1β β (1μg/ml). Specificity for IL-1β is shown by reduction of IL-8 luciferase detection following incubation with IL-1β neutralising antibody. Data are expressed as mean ± SEM, n=3, analyzed by one way ANOVA followed by Tukey's test, ****p<0.0001. **F)** Immunoelectron microscopic analysis of IL-1β in ECs +/- NE treatment. Anti-IL-1β conjugated immunogold (20-nm gold particles, arrows) was used to confirm the present of IL-1β in the MVs (0.2μm) released from the plasma membrane of ECs. Scale bars=0.2μm.

FIGURE 5: Mechanisms contributing to IL-1β release in endothelial HCAEC. A & B) HCAECs were assayed for changes in the cytosolic-free Ca^{2+} in response to the indicated conditions. **A)** No significant change in cytosolic Ca^{2+} levels are seen in Ca^{2+} -free media. **B)** The fluorescent intensity of intracellular calcium changes after 5min of NE stimulation, in the presence of $CaCl_2$. Data are n=6, mean ± SEM, analysed by one-way ANOVA and Tukey's post-test, *p<0.05, ***p<0.001. **C)** IL-1β co-localises with LAMP-1 after NE stimulation. Cells were primed with cytokines (TNF- α/IL-1α; 10ng/ml each) followed by incubation +/- NE (1μg/ml) in serum free media over 2h, before immunostaing for IL-1β (red) and LAMP-1 (green), scale bars; 10μm. **D)** Histogram showing a high correlation between IL-1β and LAMP-1 in ECs after NE activation.

FIGURE 6: NE induces IL-1β release by an endolysosomal dependent mechanism. A) ELISA measuring IL-1β release in conditioned media of HCAECs primed with cytokines (TNF-α/IL-1α; 10ng/ml) ± NE (1μg/ml) ± BAF1 (50nM) after 6h. Experiments are n=3, and data are mean ± SEM, analysed by 1-way ANOVA followed by Tukey's post-test, *p<0.05. **B)** Western blot analysis of lysates harvested from primed HCAECs activated with NE/±BAF1 (50nM) for 6h, 20μg protein loaded per lane with α-tubulin used as a loading control. The blot is representative of three independent experiments. **C) MVB characterization**: *i*) EM analysis showing a full appearance of MVB; multivesicular bodies in NE treated cells in the close proximity of plasma membrane. *ii*) Immunolabelling with anti-IL-1β (20-nm gold particles; arrow) shows IL-1β within MVB (arrowhead). Scale bars=50nm

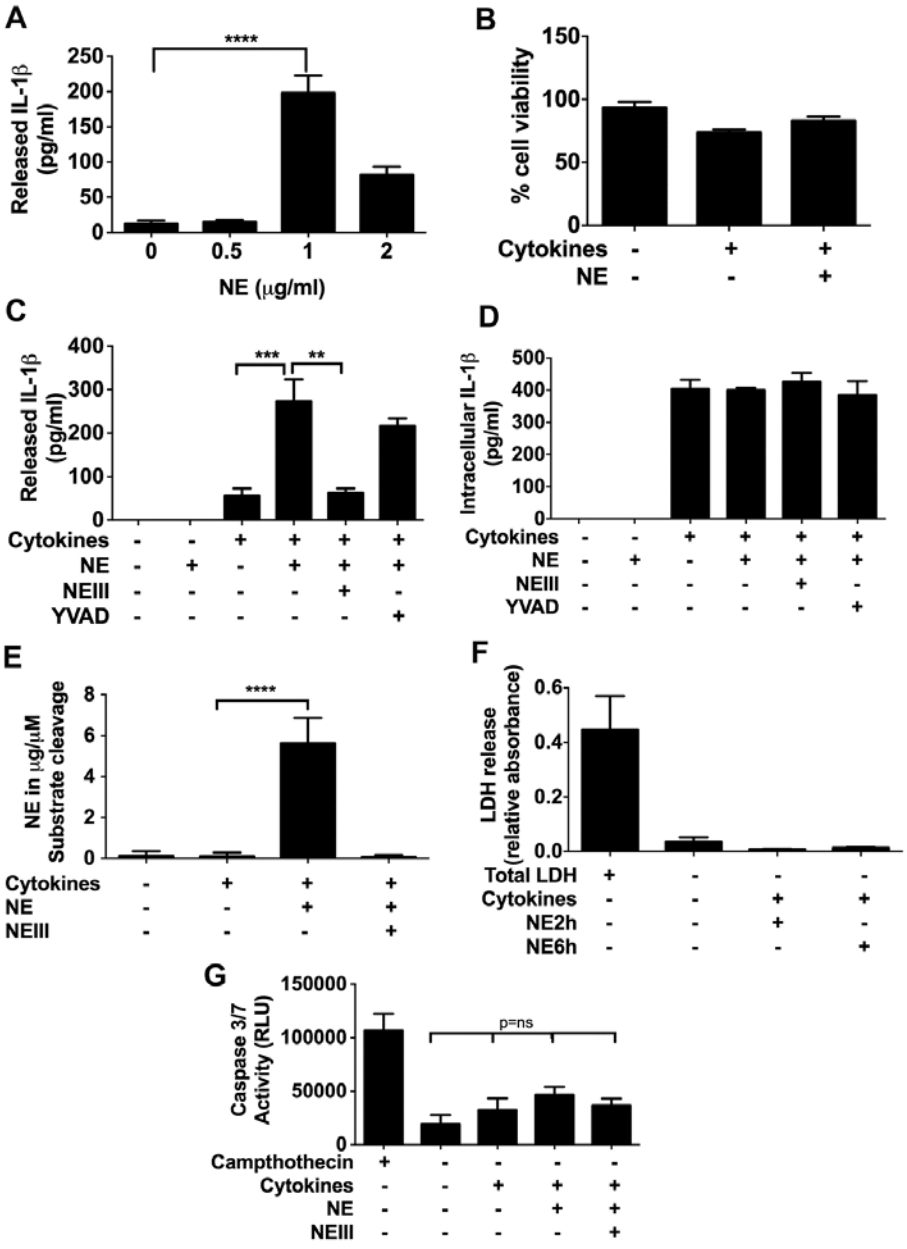
FIGURE 7: NE is detected in ECs and is colocalized with IL-1β in the endothelium of mature atherosclerotic plaques. A) Confocal images showing LAMP-1 and NE in primed ECs after NE treatment. HCAECs were incubated +/- Alexa Fluor 647-labelled (1μg/ml) NE for 2h in serum free media before washing in PBS and colocalisation performed using an antibody against LAMP-1. Confocal images were analysed using Zeiss image and image j software, scale bars=10μm. **B)** Immunohistochemical detection of NE and IL-1β in the luminal endothelium of mouse atherosclerotic plaques. Paraffin embedded aortic sinuses from $ApoE^{-/-}$ mice fed high fat diet for 12 weeks were stained with primary antibodies as indicated. Specificity of staining is confirmed by no primary negative control. Scale bars=200μm. **C)** Colocalisation of IL-1-β, NE and vWF in aortic atherosclerosis. NE positivity was detected predominantly in the endothelium (top right panel; arrows). IL-1β positive endothelium (top left panel) was also detected. The bottom panels show vWF stained endothelium and DAPI for the nuclei. Specificity of staining is confirmed by no primary negative control. Images are representative of histology data obtained from a total of 6 animals. Scale bar = 100μm.

FIGURE 8. Schematic of the proposed mechanism of IL-1β secretion from ECs by NE. NE is released by circulating cells at the site of atheroma and transported by endocytosis inside the diseased endothelium (primed by inflammation) **(i)**. An increase in calcium, due to NE effects, leads to remodelling of the cell membrane and vesiculation **(ii)**, which in turn facilitates the shedding of MVs containing mature IL-1β **(iii)**. ProIL-1β is upregulated in the inflamed endothelium **(iv)**. NE enters MVBs and cleaves the proIL-1β contained within **(v)**. MVBs also fuse to the plasma membrane and release exosomes containing IL-1 **(vi)**.

Video 1. Typical MV shedding after application of NE to ECs was observed.

Annexin V-AlexaFluor®488 labelling to cell membranes was performed first then NE was applied to the cells and monitored in heated chamber (5% CO_2 v/v) using an Inverted widefield fluorescence microscope Leica AF6000 Time Lapse, as indicated in the materials and methods, for 2h. MVs clearly bud off from ECs starting at 10-15 minutes of NE stimulation and continued for 2h. 2min intervals are shown, 200x magnification was used.

Figure 1:


A
Released IL-1β (pg/ml)
****
NE (μg/ml)
B
% cell viability
Cytokines
NE
C
Released IL-1β (pg/ml)
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Cytokines
NE
NEIII
YVAD
D
Intracellular IL-1β (pg/ml)
Cytokines
NE
NEIII
YVAD
E
NE in μg/μM Substrate cleavage
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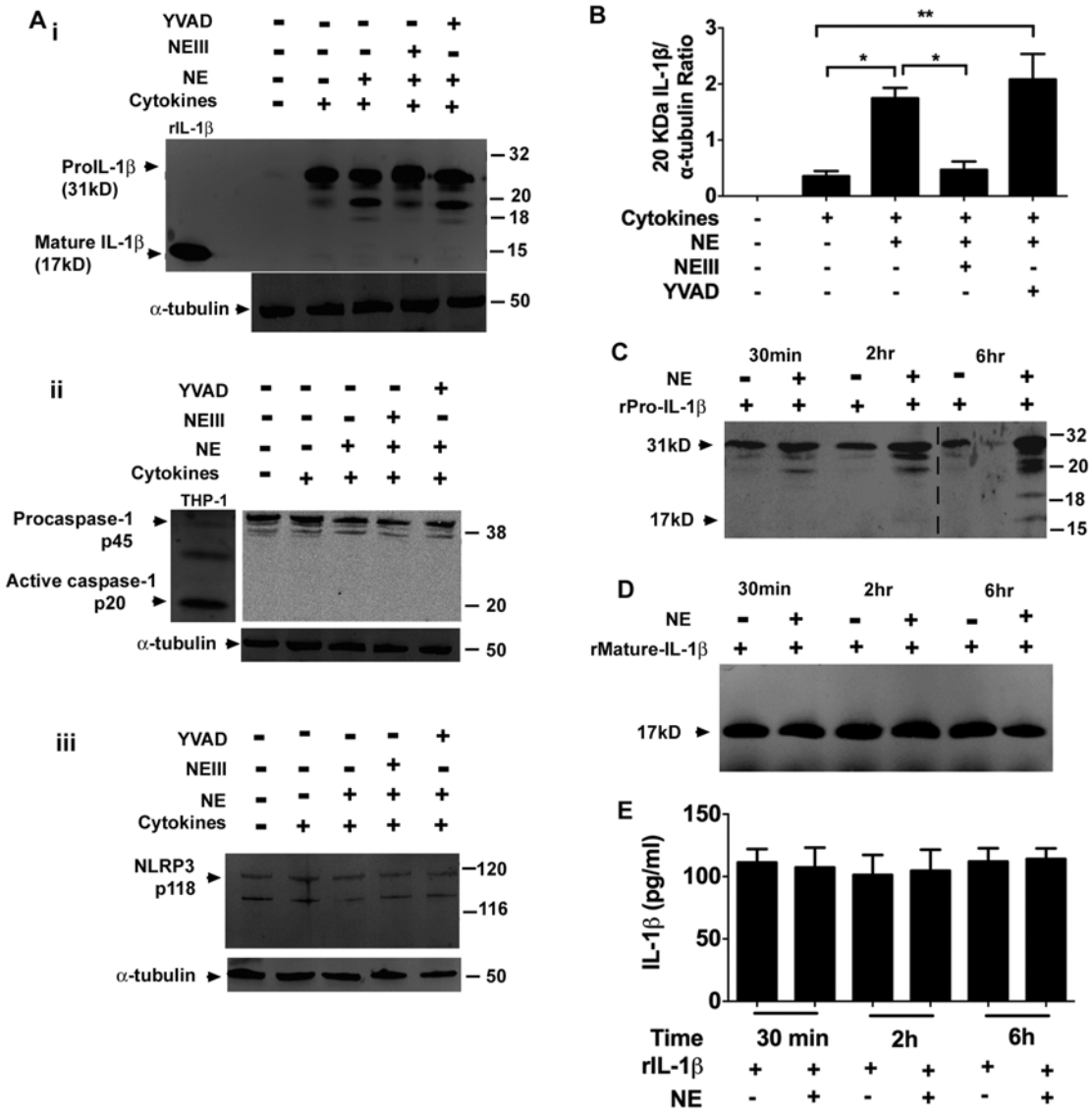
Cytokines
NE
NEIII
F
LDH release (relative absorbance)
Total LDH
Cytokines
NE2h
NE6h
G
Caspase 3/7 Activity (RLU)
p=ns
Campthothecin
Cytokines
NE
NEIII

Figure 2:


A i
YVAD
NEIII
NE
Cytokines
rIL-1β
ProIL-1β (31kD)
Mature IL-1β (17kD)
α-tubulin
32
20
18
15
50
ii
THP-1
Procaspase-1 p45
Active caspase-1 p20
38
iii
NLRP3 p118
120
116
B
20 KDa IL-1β/ α-tubulin Ratio
C
30min
2hr
6hr
rPro-IL-1β
31kD
17kD
D
rMature-IL-1β
E
IL-1β (pg/ml)
Time
30 min
2h
6h
rIL-1β

Neutrophil elastase promotes endothelial IL-1 secretion.

Figure 3:

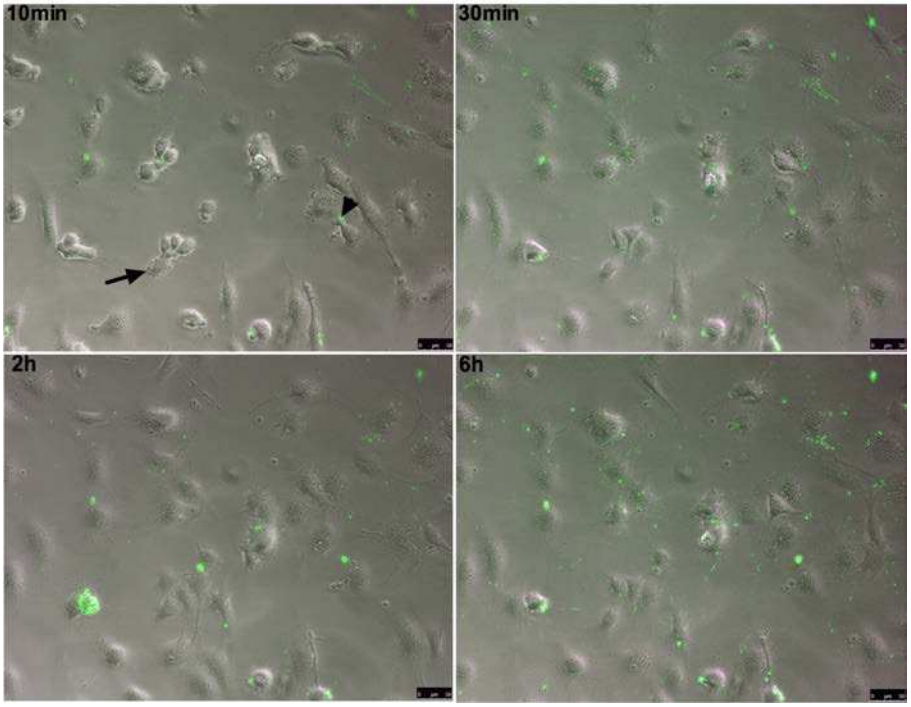
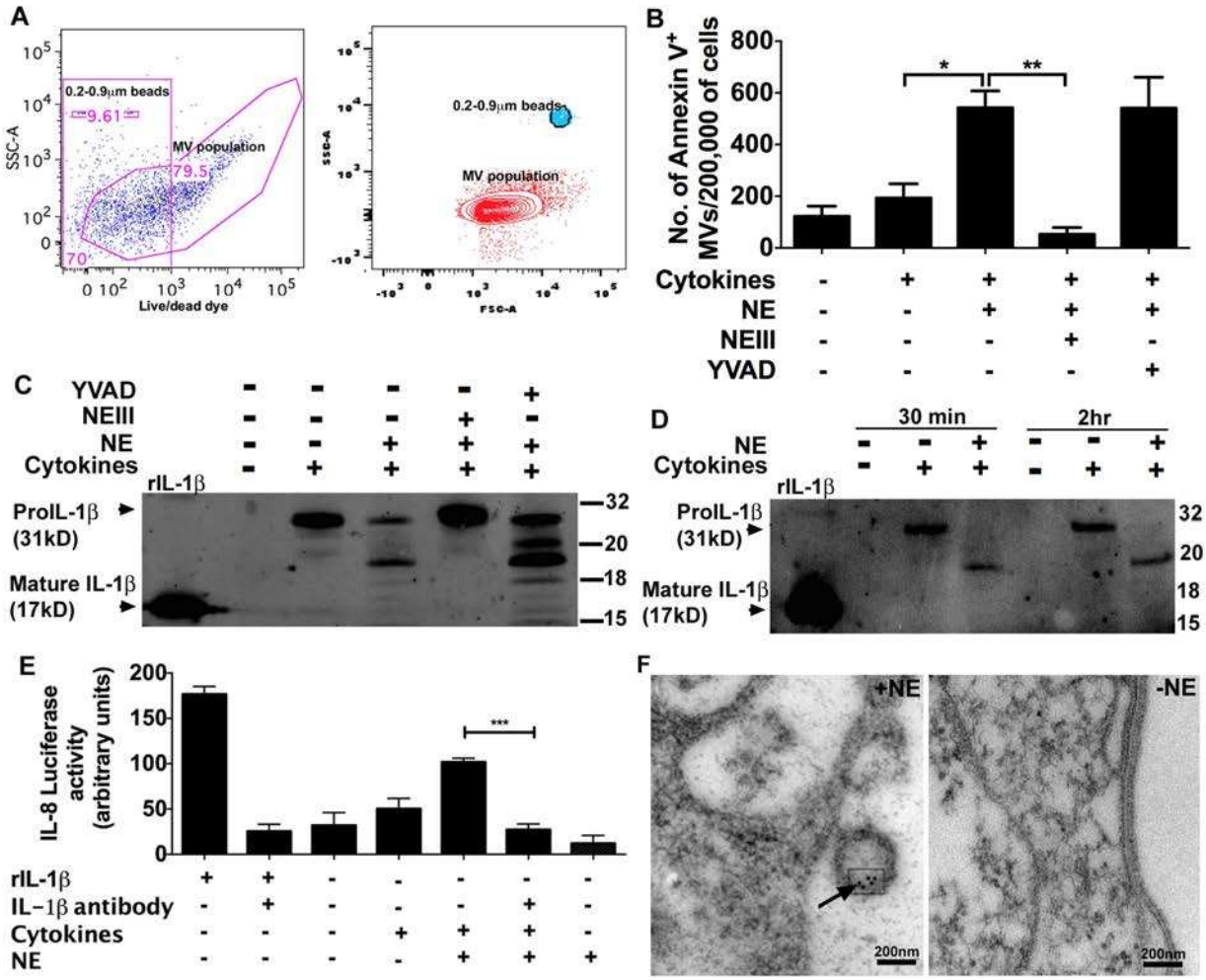
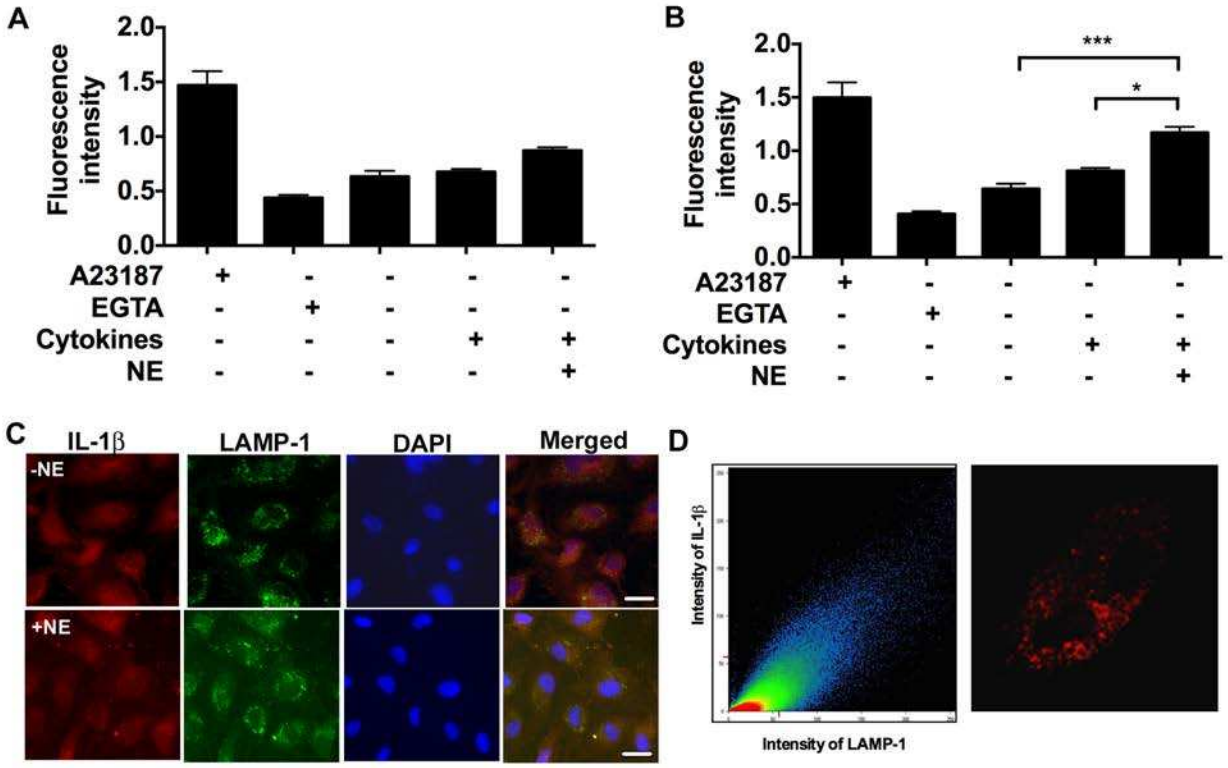


Figure 4:


A
0.2-0.9μm beads
9.61
MV population
79.5
70
SSC-A
Live/dead dye
0.2-0.9μm beads
MV population
FSC-A
B
No. of Annexin V+ MVs/200,000 of cells
800
600
400
200
0
*
**
Cytokines
NE
NEIII
YVAD
C
YVAD
NEIII
NE
Cytokines
rIL-1β
ProIL-1β (31kD)
Mature IL-1β (17kD)
32
20
18
15
D
30 min
2hr
NE
Cytokines
rIL-1β
ProIL-1β (31kD)
Mature IL-1β (17kD)
32
20
18
15
E
IL-8 Luciferase activity (arbitrary units)
200
150
100
50
0
***
rIL-1β
IL-1β antibody
Cytokines
NE
F
+NE
-NE
200nm
200nm

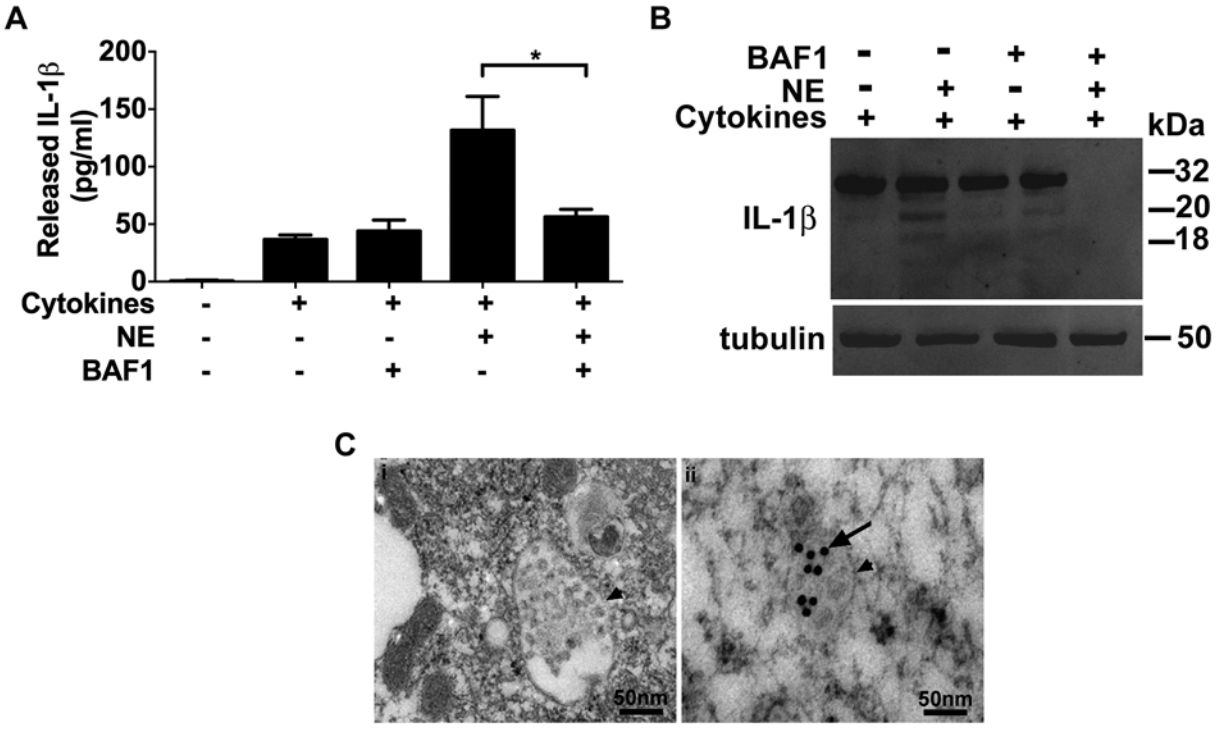
Neutrophil elastase promotes endothelial IL-1 secretion.

Figure 5.



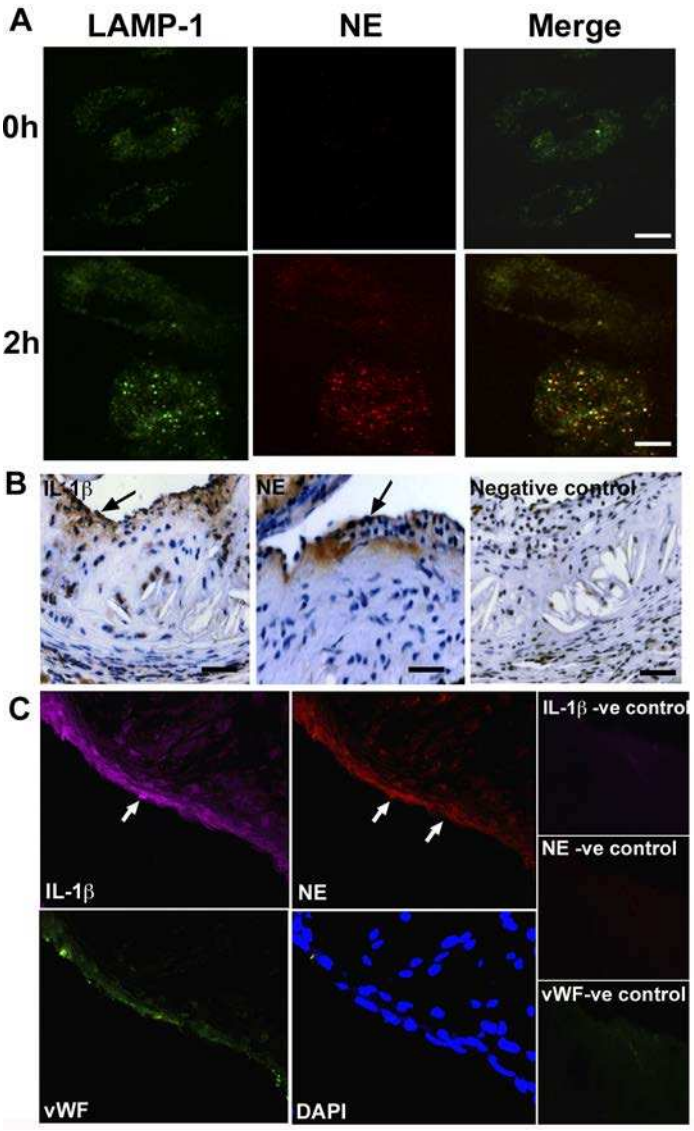
Neutrophil elastase promotes endothelial IL-1 secretion.

Figure 6.



Neutrophil elastase promotes endothelial IL-1 secretion.

Figure 7.



Neutrophil elastase promotes endothelial IL-1 secretion.

Figure 8