

Cigarette smoke-exposed neutrophils die unconventionally but are rapidly phagocytosed by macrophages

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Pulmonary accumulation of neutrophils is typical for active smokers who are also predisposed to multiple inflammatory and infectious lung diseases. We show that human neutrophil exposure to cigarette smoke extract (CSE) leads to an atypical cell death sharing features of apoptosis, autophagy and necrosis. Accumulation of tar-like substances in autophagosomes is also apparent. Before detection of established cell death markers, CSE-treated neutrophils are effectively recognized and non-phlogistically phagocytosed by monocyte-derived macrophages. Blockade of LOX-1 and scavenger receptor A, but not MARCO or CD36, as well as pre-incubation with oxLDL, inhibited phagocytosis, suggesting that oxLDL-like structures are major phagocytosis signals. Specific lipid (β -carotene and quercetin), but not aqueous, antioxidants increased the pro-phagocytic effects of CSE. In contrast to non-phlogistic phagocytosis, degranulation of secondary granules, as monitored by lactoferrin release, was apparent on CSE exposure, which is likely to promote pulmonary inflammation and tissue degradation. Furthermore, CSE-exposed neutrophils exhibited a compromised ability to ingest the respiratory pathogen, *Staphylococcus aureus*, which likely contributes to bacterial persistence in the lungs of smokers and is likely to promote further pulmonary recruitment of neutrophils. These data provide mechanistic insight into the lack of accumulation of apoptotic neutrophil populations in the lungs of smokers and their increased susceptibility to degradative pulmonary diseases and bacterial infections.

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Subject Category: Immunity

Tobacco smoking is strongly associated with multiple respiratory infections, such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* and *Legionella pneumophila*,¹ and with several chronic inflammatory pulmonary diseases, particularly asthma, interstitial lung disease and COPD.² Collectively, these tobacco-associated infectious and inflammatory diseases are a major cause of mortality and morbidity across the globe.

Pulmonary neutrophil influx, often massive, occurs in response to bacterial infection and is also a common feature of the chronic inflammatory lung diseases. Compromised neutrophil anti-bacterial capacity has been proposed as a major cause of increased susceptibility to infectious diseases in smokers,¹ while the inappropriate, tobacco-induced, necrosis-exacerbated release of neutrophil-derived proteolytic enzymes, particularly elastase and matrix metalloproteinase (MMP)-8 and -9, has been proposed as a key event in development of destructive lung diseases.³

The best studied tobacco-induced lung pathology is COPD.³ In this disease, there is a clear relationship between the numbers of neutrophils present and both lung function and emphysema.⁴ The large numbers of neutrophils in COPD is generally explained by increased neutrophil chemotaxis and recruitment; or by delayed efferocytosis because of suppressed apoptosis; or by alterations to the complex system of redundant receptors and polyvalent recognition molecules that enables efficient detection of specific molecular patterns on the dying cell surface.⁵ It has been hypothesized that abnormal regulation of neutrophil apoptosis, promoting longevity and necrotic death, may contribute to the ineffective resolution of inflammation in COPD.⁶ Competing cell signals influence neutrophil longevity and dictate if these cells die by necrosis, apoptosis or autophagic cell death in which degradative dismantling of damaged organelles and proteins is apparent.⁷ Although it is known that necrotic imbalance promotes inflammation and that apoptosis coupled with effective phagocytotic clearance helps resolve pro-inflammatory

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Abbreviations: COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; hMDM, human monocyte-derived macrophage; PS, phosphatidylserine; PE, phycoerythrin; PI, propidium iodide; SR-A, scavenger receptor A

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responses, recent evidence suggests that autophagy is also important in pathogen clearance and in directing the intensity of innate cascades.⁷ However, the precise interrelationships between autophagic, necrotic and apoptotic processes remain to be established.

Although there is general support for the phenomenon of dysregulated neutrophil apoptosis in COPD,⁶ some of the available data are conflicting. The proportion of apoptotic sputum neutrophils in subjects with COPD, healthy smokers and healthy controls has been reported not to differ, suggesting that the inhibition of apoptosis cannot explain the increased numbers of airway neutrophils in this disease.⁸ Makris *et al.*⁹ have recently shown a significantly increased ratio of apoptotic to total induced sputum neutrophils in COPD patients compared with healthy controls. Pletz *et al.*,¹⁰ on the other hand, have shown that neutrophil apoptosis is suppressed in acute exacerbations of COPD.

Certainly, tobacco smoke and components can directly or indirectly alter innate cell death processes. For example, Aoshiba *et al.*¹¹ reported that nicotine alone could suppress neutrophil apoptosis in a dose-dependent manner. Tobacco is a rich source of multiple pro-oxidants with oxidative stress thought to have a major role in inducing imbalance in innate cell homeostasis in COPD.¹²

However, it has become apparent that the mechanisms underlying tobacco-induced and/or COPD-related neutrophil apoptosis may not be entirely conventional and may even overlap. Unusual expression profiles of surface markers of cell activation are apparent in apoptotic neutrophils from subjects with COPD, most notably, upregulated $\alpha_M\beta_2$ integrin (Mac-1; CD11b/CD18; complement receptor-3).¹³ It has also been shown that cigarette smoke extract (CSE) treatment almost totally abrogated the activity of caspase-3, a key proteolytic enzyme in the spontaneous apoptosis cascade, yet this did not influence neutrophil apoptosis.¹²

What has yet to be fully ascertained is if tobacco smoke induces alterations to specific pro-phagocytosis surface markers ('eat me' signals); if tobacco-associated changes to such cell cycling markers influence subsequent phagocytotic clearance; if phagocytosis of smoke-exposed neutrophils results in pro-inflammatory or in non-phlogistic efferocytosis; or if alleviation of oxidative stress through anti-oxidant treatment may affect tobacco-moderated neutrophil cell death and efferocytosis. Furthermore, tobacco smoke contains

>4000 components many of which could simultaneously induce overlapping pro-necrotic, pro-autophagic and pro-apoptotic cellular events. This would help explain the large conflicts between existing data on tobacco-induced cell death in neutrophils where one type of cell death has usually been studied in isolation. Therefore, we set out to examine these concepts, as well as the ability of smoke-exposed neutrophils to combat bacterial challenge (*Staphylococcus aureus*), using CSE-exposed primary human neutrophils.

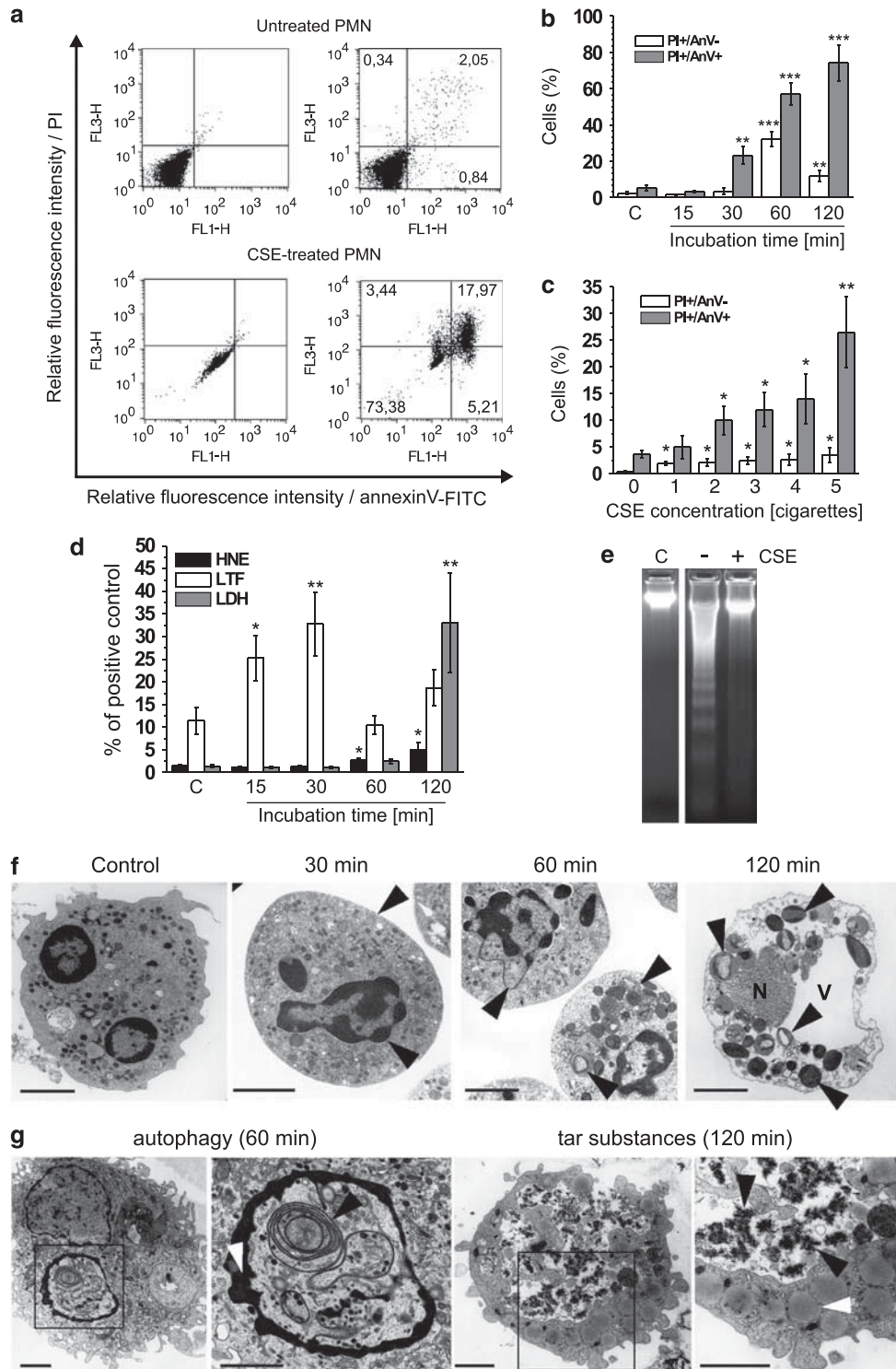
Results

CSE induces an atypical cell death in primary human neutrophils. Typical autofluorescent shift, phosphatidylserine relocalization and DNA accessibility patterns of primary neutrophils exposed to CSE are shown in Figure 1a. CSE-induced phosphatidylserine relocalization (annexin V staining) was clearly time- (Figure 1b) and dose- (Figure 1c) dependent. CSE-exposure induced the rapid (15 min) release of the secondary granule protein, lactoferrin, but not the primary granule marker, elastase, implying CSE-induced secondary granule-specific degranulation (Figure 1d). Indeed, CSE-induced lactoferrin release was inhibited by cytochalasin D (data not shown) and is, therefore, a dynamic process. After longer incubations with CSE (120 min), increased release of elastase, lactoferrin, as well as the cytoplasmic protein, lactate dehydrogenase, was apparent (Figure 1d). Similarly, permeability to Trypan blue occurred only after a 120 min to exposure to CSE (data not shown). Thus, longer CSE exposures induce membrane permeability and/or necrotic characteristics in human neutrophils. Interestingly, spontaneous apoptosis over 24 h, as assessed by DNA laddering, was completely inhibited by CSE (Figure 1e). TUNEL and comet assays of DNA fragmentation also failed to detect committed neutrophil apoptosis following CSE (data not shown). Transmission electron microscopy (TEM) was used to examine CSE-exposed neutrophils for ultrastructural characteristics consistent with apoptosis, autophagy and/or necrosis. As shown in Figure 1f, intact cell membrane and normal nuclear morphology were apparent after short CSE-exposures. In keeping with the lack of molecular and morphological signs of committed apoptosis, there was no detectable decrease in

Figure 1 CSE induces atypical cell death in primary human neutrophils. (a) Typical autofluorescence shift (left panels) or annexin V and propidium iodide (PI) staining (right panels) of control neutrophils (upper panels) or neutrophils treated with CSE from five cigarettes for 30 min (lower panels). (b) Intensity of annexin V and PI staining is dependent on time of neutrophil exposure to CSE. (c) Intensity of annexin V and PI staining is dependent on dose of neutrophil exposure to CSE (0–5 cigarettes). (d) Membrane integrity in CSE-treated neutrophils. Short CSE (prepared from five cigarettes) exposure times (15 min) induce lactoferrin release from primary human neutrophils, while longer exposure (120 min) is required to induce neutrophil elastase and lactate dehydrogenase release. Enzymes present in the culture medium of necrotic (sonicated) neutrophils acted as the positive controls (100%). (e) CSE exposure inhibits DNA fragmentation in neutrophils. DNA was extracted from freshly isolated neutrophils (negative control, C) and neutrophils incubated for 24 h with (+) and without (–; positive spontaneous apoptosis control) pretreatment with CSE (30 min) and subjected to electrophoresis. (f) TEM analysis of neutrophils exposed to CSE from five cigarettes for up to 120 min. An intact cell membrane, regular nuclear morphology (black arrowheads) and cell rounding are apparent (30 min). Numerous autophagosomes (60 min) and autophagosomes with visible remnants of organelles and tar substance (120 min) appear on longer CSE exposure times. Disruption of nucleus (N) and extensive vacuolization (V) is also visible at 120 min. Scale bars represent 2 μ M. (g) TEM micrographs showing extensive autophagy (60 min) and accumulation of tar-like substances (120 min) in CSE-treated neutrophils that have been engulfed by hMDMs. The left (60 min) enlargement shows numerous autophagosomes (containing mitochondria) with the black and white arrowheads indicating autophagosomal membranes and a spacious phagosome, respectively. In the right (120 min) enlargement, electron-dense acicular inclusions within macropinosome-like compartments (black arrowheads) and atypical neighboring lysosomes (white arrowhead) are apparent. hMDMs in both panels demonstrate membrane ruffling. Scale bars represent 2 μ M. Data presented are mean (\pm S.D.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, relative to controls, C

the mitochondrial potential in neutrophils exposed to CSE for 30 min (data not shown). Longer exposure times revealed ultrastructural characteristics more consistent with autophagy (60 min) and necrosis (120 min) (Figure 1f). Analysis of TEM images showing neutrophils engulfed by human monocyte-derived macrophages (hMDMs) strengthen the

concept of autophagy as an important mechanism of cell death under the influence of CSE (Figure 1g). Here, autophagy was particularly intense in phagocytosed neutrophils following 60 min of CSE-exposure, when multiple autophagosomes encompassing mitochondria, or other organelles, were clearly visible (Figure 1g, left panels).



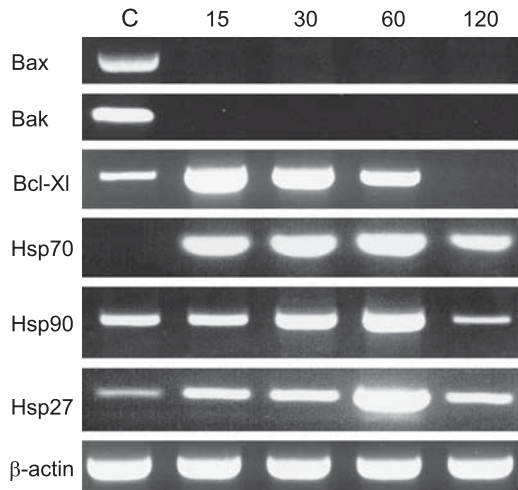


Figure 2 Accumulation of selected pro- and anti-apoptotic transcripts in CSE-treated neutrophils analyzed by semiquantitative RT-PCR. Indicated mRNAs were measured in neutrophils following CSE exposure for 15, 30, 60 and 120 min. Control neutrophils (C) were incubated in culture medium 120 min. Cells were treated as described in the legend for Figure 1b and d. The presented data are derived from a single representative experiment out of three performed. β -actin was used as a reference transcript

Following prolonged CSE exposure (120 min), ultrastructural characteristics more typical of necrosis were prominent (Figure 1g, right panels). Massive neutrophil accumulation of tar-like substances was also obvious at 120 min.

Activity of selected pro- and anti-apoptotic genes in CSE-treated neutrophils. As can be seen in Figure 2, CSE-exposure completely suppressed the activity of *bax- α* and *bak1* in human neutrophils. Enhanced activity of *hsp27* and *hsp90 α* were also apparent, with an increase transcript signal noted over a 1-h period, while induction of *bcl-xl* peaked at 15 min. Although no activity was observed in control cells, *hsp70* was rapidly induced by CSE.

CSE-exposed neutrophils are effectively phagocytosed by hMDM. Despite complex and atypical cell death patterns, CSE-exposed neutrophils were effectively phagocytized by naive hMDM in a time- (Figure 3a) and dose- (Figure 3b) dependent manner, reflected by hMDM-internalized neutrophil-specific elastase activity.

Expression of CD31 and CD47 is preserved on CSE-treated neutrophils. CD31 and CD47 are normally downregulated during apoptosis.^{14,15} As shown in Figure 4, CSE did not significantly influence neutrophilic expression of these major anti-phagocytotic proteins. Thus, preservation of CD31 and CD47 lends further support to the concept of the initiation of atypical cell death pathways in CSE-exposed neutrophils.

LOX1 and scavenger receptor A (SR-A) hMDM scavenger receptors are largely responsible for the recognition and engulfment of CSE-exposed neutrophils. As shown in Figure 5, pre-incubation of

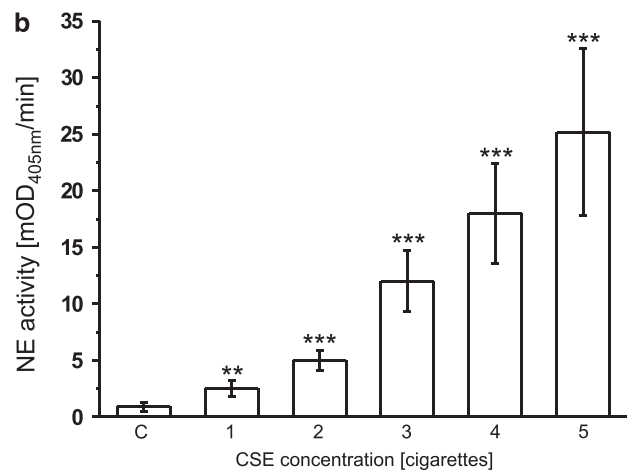
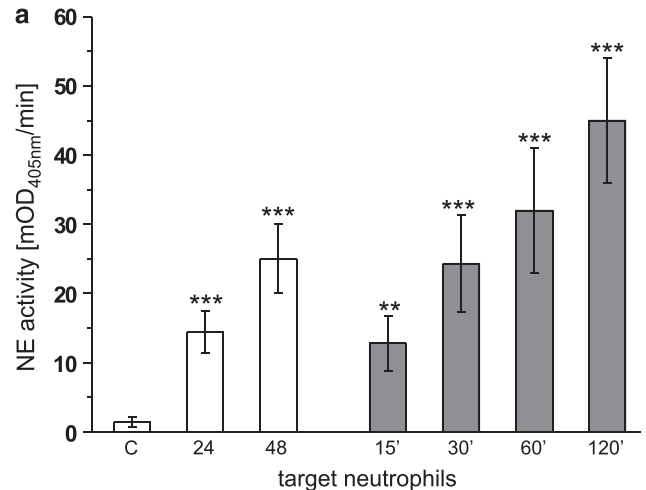


Figure 3 Neutrophil exposure to CSE increases efficiency of phagocytosis by hMDMs in a (a) time- and (b) concentration-dependent manner. (a) Phagocytosis by hMDMs of fresh, healthy neutrophils (C), early (24 h) and late (48 h) spontaneously apoptotic neutrophils, as well as neutrophils treated with CSE prepared from five cigarettes for 15, 30, 60, or 120 min (37 °C). hMDM-neutrophil ratio, 1:20. (b) Phagocytosis by hMDMs of fresh, healthy neutrophils (C), as well as neutrophils treated with CSE (37 °C, 30 min) prepared from one to five cigarettes. hMDM-neutrophil ratio, 1:20. Non-ingested neutrophils were removed by intensive washing of the hMDM monolayer. hMDMs were solubilized with detergent and the neutrophil elastase activity was measured in lysates as described in materials and methods. The intensity of phagocytosis is expressed as mOD/min of the substrate turnover catalyzed by neutrophil-derived elastase. Data presented are mean (\pm S.D.). ** $P < 0.01$, *** $P < 0.001$, relative to controls, C

hMDMs with oxLDL inhibited phagocytosis of CSE-exposed neutrophils by >60%. Furthermore, phagocytosis of CSE-pretreated neutrophils was even more efficiently blocked when oxLDL treatment was supplemented with β -carotene or quercetin. The oxLDL interference with phagocytosis implied involvement of scavenger receptors in the recognition of CSE-exposed neutrophils by macrophages. To verify the implication that hMDM scavenger receptors recognizing oxidized lipids were involved in the recognition and phagocytosis of CSE-treated neutrophils, specific mAbs known to block selected scavenger receptors (SR-A, MARCO, LOX1 and CD36) were employed. Although

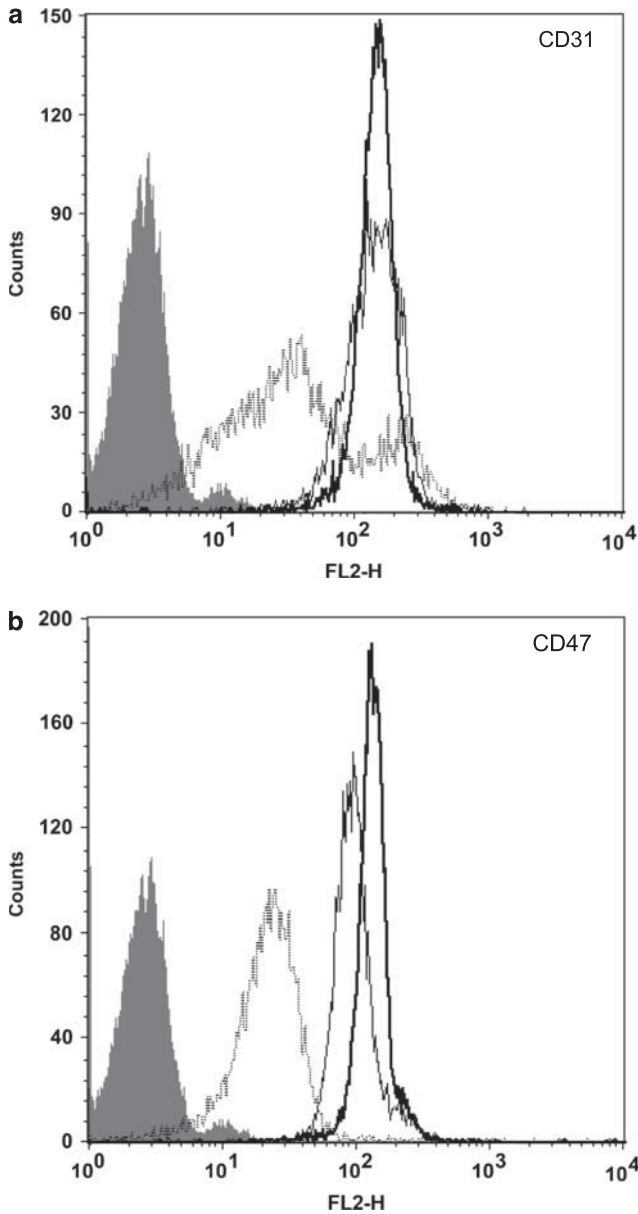


Figure 4 Expression of CD31 and CD47 is preserved on CSE-treated neutrophils. Fresh, healthy (thin line), apoptotic (dotted line), and CSE-exposed (thick line) neutrophils were stained with PE-labeled anti-CD31 (a) or anti-CD47 (b) antibody and analyzed by flow cytometry. Gray areas represent the corresponding isotypic antibody controls for untreated cells. Results of one representative experiment of three are presented

blockade of MARCO and CD36 suppressed the uptake of neutrophils in a limited manner, specific blocking of SR-A and LOX1 receptors inhibited phagocytosis by approximately 60 and 80%, respectively.

hMDM phagocytosis of CSE-exposed neutrophils is non-phlogistic. Phagocytosis of necrotic, and perhaps autophagic, cells has been shown to induce a pro-inflammatory response in macrophages.¹⁶ However, a non-phlogistic response to phagocytosis clearly is preferable for

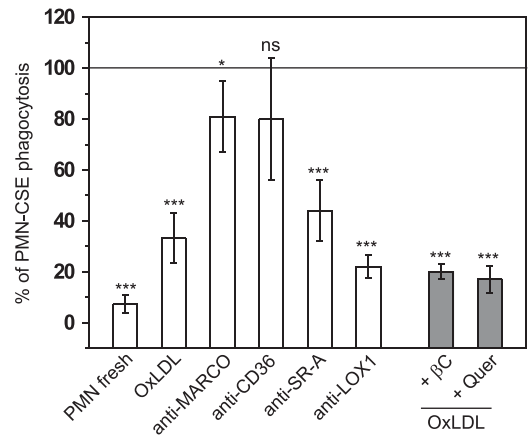


Figure 5 LOX1 and SR-A hMDM scavenger receptors are key mediators of CSE-exposed neutrophil recognition and engulfment by hMDMs. Control hMDMs and hMDMs pre-incubated (20 °C, 20 min) with mAbs specific for individual scavenger receptors (MARCO, CD36, SR-A and LOX1; all 10 μg/ml) and their capacity to phagocytose control (freshly isolated) and CSE-exposed neutrophils over 2 h established by measurement of the activity of internalized neutrophil elastase. Competitive inhibition by oxLDL (50 μg/ml) of phagocytosis of neutrophils pretreated with CSE in the presence and absence of β-carotene or quercetin was also assessed. Results are expressed as percentage of the positive (100%) established by monitoring phagocytosis of CSE-treated neutrophils. Data presented are mean (± S.D.). **P*<0.05; ****P*<0.001, compared with CSE only (100% control)

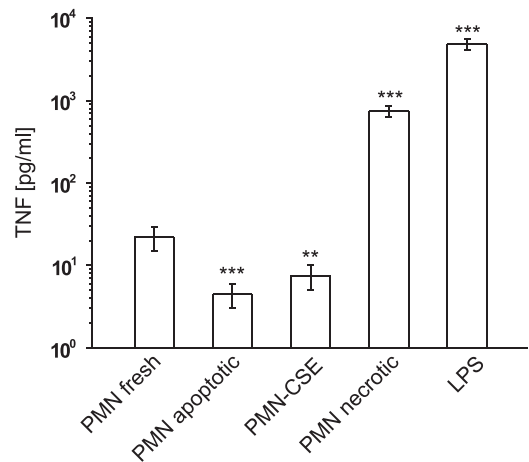


Figure 6 hMDM phagocytosis of CSE-exposed neutrophils is non-phlogistic. hMDMs were co-cultured for 4 h with fresh; apoptotic; necrotic; or CSE-treated (30 min) neutrophils and TNF release into cell-free culture supernatants measured by ELISA. *E. coli* LPS stimulation served as the positive control. Data presented are mean (± S.D.). ***P*<0.01, ****P*<0.001, compared with control (fresh) neutrophils

the resolution of inflammation.¹⁶ Here, we show that hMDM phagocytosis of CSE-exposed neutrophils did not induce pro-inflammatory cytokine (TNF) production, as presented in Figure 6. This is in sharp contrast to the robust pro-inflammatory response, comparable to that elicited by LPS, elicited from hMDMs on phagocytosis of necrotic neutrophils. The dramatic variation in inflammatory potential of CSE-exposed and necrotic neutrophils again hints at atypical, non-necrotic, cell death mechanisms in neutrophils encountering CSE.

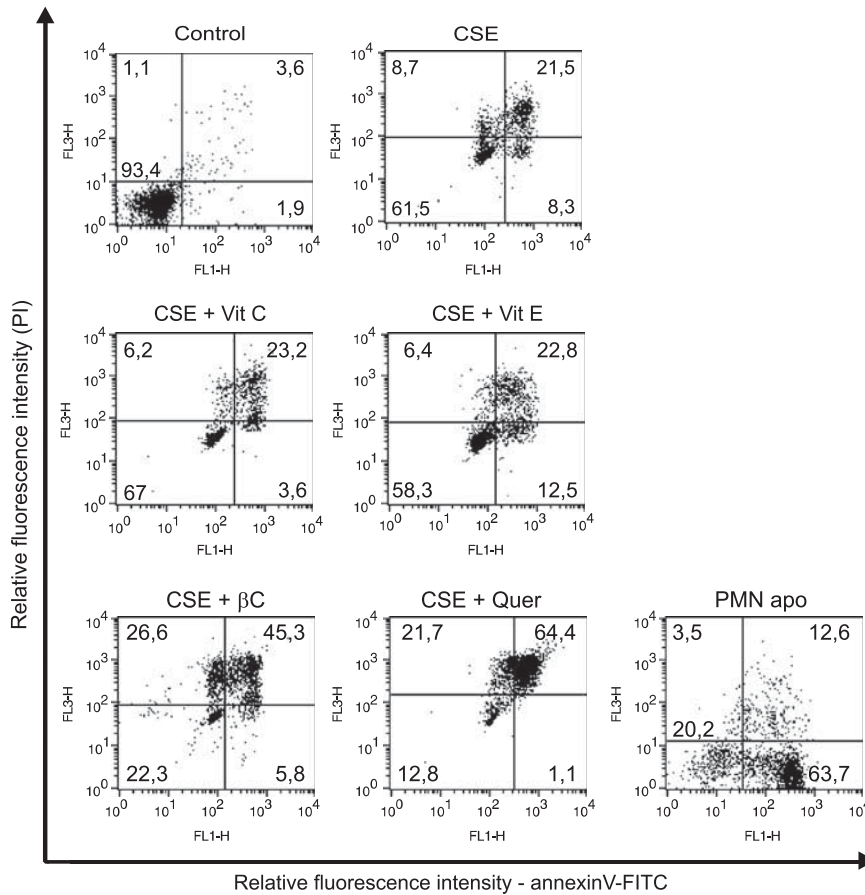


Figure 7 β -Carotene and quercetin potentiate the cytotoxic activity of CSE. Neutrophils were exposed to CSE (37 °C, 30 min) with and without several major antioxidants; Vit C (vitamin C, ascorbic acid); Vit E (vitamin E, α -tocopherol); β -C (β -all trans β -carotene); and Quer (quercetin) and annexin V and PI staining determined by flow cytometry. Freshly isolated (control) and spontaneous apoptotic (PMN apo) neutrophils served as the negative and positive controls, respectively. Dot-plots are from one representative experiment out of three

β -carotene and quercetin enhance annexin V⁺/PI⁺ neutrophils and hMDM phagocytosis of CSE-exposed neutrophils. As shown in Figure 7, the addition of lipid antioxidants (β -carotene or quercetin) to CSE, but not soluble antioxidants (vitamin C and vitamin E), significantly potentiated the proportion of annexin V⁺/propidium iodide (PI)⁺ neutrophils. Similarly, β -carotene or quercetin treatment significantly increased the phagocytosis of CSE-exposed neutrophils by hMDMs (Figure 8).

CSE-exposed neutrophils do not effectively phagocytose *S. aureus*. The ability of respiratory tract neutrophils to effectively phagocytose, and subsequently destroy, bacterial pathogens is critical in the prevention of pulmonary infectious diseases. As is clearly shown in Figure 9, CSE-treatment of neutrophils rendered these granulocytes ineffective in ingesting two different strains of *S. aureus*. Indeed, the suppression of phagocytotic capacity was dramatic, with <15% of CSE-exposed neutrophils containing fluorescein isothiocyanate (FITC)-labeled bacteria, compared with over 60% of control, unexposed neutrophils.

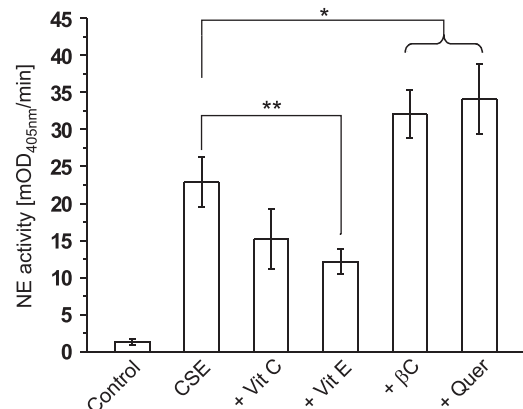


Figure 8 β -Carotene and quercetin enhance the phagocytosis-inducing activity of CSE. The capacity of hMDMs to phagocytose freshly isolated neutrophils as well as neutrophils exposed to CSE (37 °C, 30 min) with and without several major antioxidants; Vit C (vitamin C, ascorbic acid); Vit E (vitamin E, α -tocopherol); β C (β -all trans β -carotene); and Quer (quercetin) was determined, as described elsewhere. Data presented are mean (\pm S.D.). * P <0.05; ** P <0.01 between indicated means

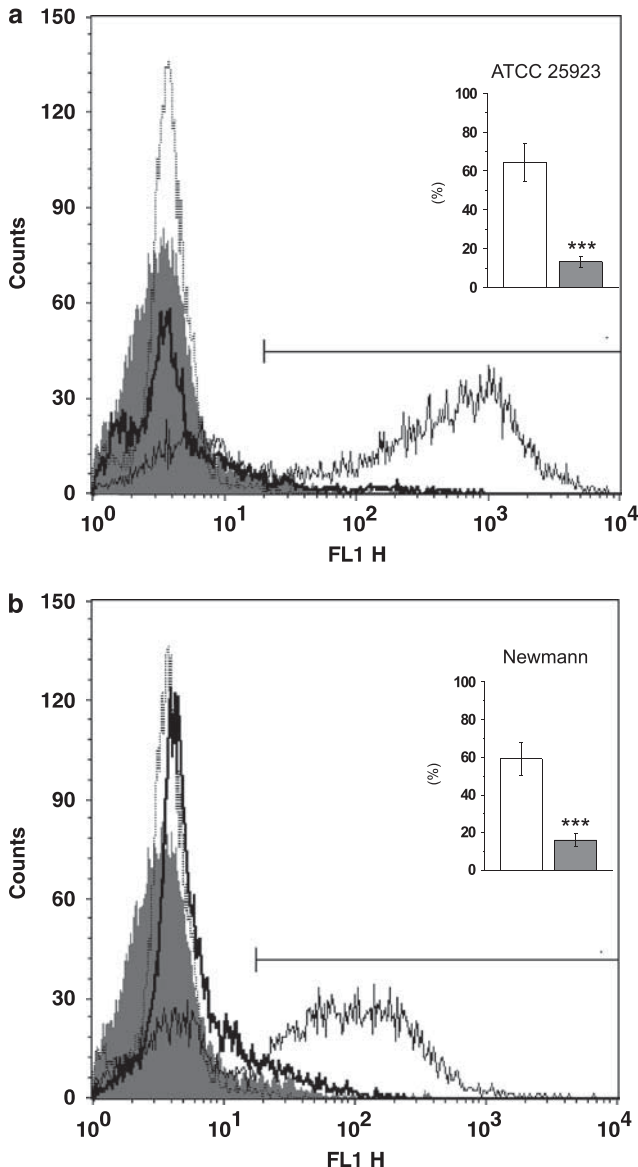


Figure 9 CSE-treated neutrophils lose their ability to effectively phagocytose *S. aureus*. Phagocytosis of opsonized, FITC-labeled ATCC 25923 (a) and Newmann (b) *S. aureus* strains was assessed by flow cytometry. Representative traces of bacteria engulfed by control neutrophils and CSE-treated neutrophils are demarcated by thin and thick lines, respectively. Autofluorescence signals are demarcated by the gray areas (untreated neutrophils) and dotted lines (CSE-treated cells) and correspond to neutrophils without ingested bacteria. Inset graphs represent the mean (\pm S.D.) percentage of bacteria-containing neutrophils with the white and gray bars representing control and CSE-exposed neutrophils, respectively. *** $P < 0.001$, compared with unexposed, control neutrophils

Discussion

Tobacco smoke is known to exert profound effects on neutrophils. For example, cigarette smoke and nicotine have been reported to suppress the respiratory burst^{17,18} and the ability to kill pathogenic bacteria,¹⁷ while reports are conflicted on the influence of smoking on neutrophil chemotaxis.¹⁹ What is clear, however, is that neutrophil accumulation occurs in the lungs of smokers and that neutrophil numbers correlate with

destructive pulmonary disease progression.⁴ The manner of cell death is a critical element in determining inflammatory intensity. However, the available evidence for an effect of tobacco smoke on neutrophil longevity is controversial. Some investigators have reported that nicotine can suppress neutrophil apoptosis; others have concluded that high nicotine doses did not influence apoptosis in freshly isolated peripheral neutrophils; while others have suggested showing nicotine to induce DNA cleavage in myeloid cells and to be a potent inducer of apoptosis in systemic neutrophils.¹⁹ An accurate composite picture of the influence of cigarette smoke and neutrophil longevity is difficult to generate because of a wide variation in experimental models and in methods of generating tobacco smoke preparations. In this study, therefore, we have examined multiple aspects of cell death in, and efferocytosis of, CSE-exposed neutrophils, all in a single experimental model employing primary human neutrophils, primary hMDMs and a readily reproducible CSE.

One recent report shows that total leukocytes isolated from smokers exhibit a significant suppression of multiple proapoptotic genes (*C1D*, *MTCBP-1*, *CTCF*, *IKIP*, *MAF* and *YWHAQ*), while activity of the anti-apoptotic gene, *CTMP*, is induced compared with leukocytes from non-smokers.²⁰ Herein, we show that CSE induces an atypical type of cell death in neutrophils that includes features of apoptosis (PI staining and PS relocalization; non-phlogistic response of hMDMs to neutrophil phagocytosis), autophagy (ultrastructural characteristics) and necrosis (leakage of granule and cytoplasmic components; persistence of CD31 and CD47 expression). Furthermore, such CSE-exposed neutrophils were effectively cleared by professional phagocytes, indicating the induction of cell surface molecular patterns identifying them as damaged and/or dying cells.

In light of these results, it is evident that the treatment of neutrophils with CSE triggered an atypical cell death pathway where some early features of apoptosis appeared but were not followed by executive phase. Even the spontaneous apoptotic program was dramatically inhibited. These data are in keeping with our previous study in which myeloid (HL-60) cells were differentiated into neutrophil-like cells in the presence of nicotine. Molecular signals consistent with early apoptosis were apparent, yet committed apoptosis did not occur.¹⁷ Our data are also in keeping with several clinical studies that have shown that apoptotic neutrophils represent only a small percentage of the total pulmonary population in several tobacco-exacerbated diseases, including acute respiratory distress syndrome (7.4×10^5 neutrophils/ml; 1.6% apoptotic cells)²¹ and pneumonia (1.1×10^6 neutrophils/ml; 0.3% apoptotic cells),²² while others have reported a dramatically reduced percentage of neutrophils undergoing spontaneous apoptosis in healthy smokers and subjects with COPD compared with non-smokers.¹⁰ Furthermore, recent studies have reported increased autophagy in mouse lungs subjected to chronic cigarette smoke exposure, and in pulmonary epithelial cells exposed to CSE,²³ and may be induced by oxidative stress and mediated by sirtuin-1 (SIRT1).²⁴

The gene products of *bax- α* and *bak1*, members of the *bcl2* family are known to interact with mitochondria and promote apoptosis. Although present in significant amounts in control

cells, *bax- α* and *bak1* mRNAs were not detectable following 15-min incubation, indicating a suppression of intrinsic apoptosis in CSE-exposed neutrophils. Conversely, cells responded to CSE treatment with the sharp increase in expression of *bcl-xl*, the protein product of which prevents cytochrome C release within mitochondria and is thus anti-apoptotic. Thus, transcriptional profiling of key pro- and anti-apoptotic genes in CSE-exposed neutrophils reflects the suppression of the executive phase of apoptosis noted in biochemical assays and electron microscopy.

To estimate the response of general cytoprotective mechanisms to CSE, we analyzed the expression of major representatives of three families of heat shock proteins genes (*hsp27*, *hsp70* and *hsp90 α*). Heat shock proteins are highly conserved often multi-functional proteins best characterized as molecular chaperones, which protect the native conformation of other proteins, guarding against structural inactivation and irreversible multimeric aggregation. Thus, induction of heat shock proteins in response to stress serves to help protect the cell against the initial insult and to augment recovery. Elevated heat shock protein expression can also provide tolerance to a wide array of stressors and resistance to apoptosis.²⁵ In the case of *hsp70*, in which mRNA was absent in control cells, we observed immediate and extensive expression throughout the CSE treatment. HSP 70 is anti-apoptotic and inhibits both caspase-dependent and -independent apoptotic pathways.²⁶ Expression of *hsp90 α* and *hsp27* increased their expression moderately with apparent maximum at 60 min. HSP27 is a small heat shock protein that suppresses cell death signaling in neutrophils.²⁷ HSP90 has been shown to localize to mitochondria in tumor cells, with HSP90 inhibition leading to the induction selective apoptosis.²⁸ Thus, the rapid CSE induction of *hsp90 α* , *hsp27* and *hsp70* transcripts is in keeping with suppression of overt apoptosis in smoke exposed neutrophils.

Although relocation of phosphatidylserine (PS) from the inner to the outer cell surface is a universal early marker of apoptotic cells and a prerequisite for their successful engulfment by phagocytes, several other cellular 'eat me' signals are recognized, including altered carbohydrates, oxidized lipids, binding sites for thrombospondin and collectins (e.g., mannose-binding lectin, surfactant protein A and C1q), as well as calreticulin, an endoplasmic reticulum chaperone.²⁹ These markers are recognized by an equally large variety of receptors on phagocytes, including CD14, CD68, CD91 (LRP), SR-A, ATP-binding cassette transporter 1 (ABC-1), complement receptors, integrin $\alpha_v\beta_5$ and $\alpha_v\beta_3$ /CD36/thrombospondin complexes.⁵ These receptors mediate the clearance of endogenous cells directly or indirectly via PS-binding proteins.⁵ Herein, through the use of oxLDL and blocking antibodies to selected scavenger receptors, we show that LOX1 and SR-A scavenger receptors were largely responsible for the recognition and engulfment of CSE-exposed neutrophils by hMDMs.

Interestingly, the addition of β -carotene or quercetin to CSE resulted in increased PI-staining as well as engulfment of the treated cells. In the case of β -carotene, this may be explained by the pro-oxidant properties of this lipid. However, no pro-oxidant activity has been reported for quercetin so far. The oxLDL blocking and pro-oxidant effect of β -carotene, taken

together, strongly suggest that CSE-induced neutrophil surface ox-LDL-like sites thus providing 'eat me signal' for macrophages.

Furthermore, it has been previously established that the type of cell death of ingested host cells can dictate the inflammatory response of professional phagocytes on engulfment. Macrophage ingestion of apoptotic cells has been shown to be non-phlogistic, while necrotic and autophagic cells generally seem to induce a pro-inflammatory reaction.³⁰ As we show that CSE-exposed neutrophils exhibit atypical cell death patterns that include features of necrosis, autophagy and apoptosis, we decided to establish whether or not neutrophil phagocytosis elicited a pro-inflammatory response in hMDMs. CSE-treated neutrophils did not stimulate macrophages to produce significant amounts of TNF when compared with necrotic neutrophils and less, even, than measured for fresh neutrophils. This was perhaps surprising because, following longer treatment with CSE, a majority of neutrophils were PI-positive. The distinctive autophagosomes found in most of CSE-treated neutrophils also suggested a potential for a phlogistic response in hMDMs to phagocytosis.³⁰

However, while phagocytosis of CSE-exposed primary human neutrophils was efficient and did not elicit inflammation, CSE-exposed neutrophils did exhibit degranulation. At lower doses of CSE, cytochalasin D-inhibited degranulation was specific to secondary granules, using lactoferrin release as a surrogate marker. Other secondary granule components presumably released alongside lactoferrin include, neutrophil collagenase (MMP-8), myeloperoxidase, NADPH oxidase, alkaline phosphatase and the anti-microbial molecules, lactoferrin, LL-37 and lysozyme. Increased MMP-8 has been associated with COPD.³¹ Thus, the tobacco-induced release of neutrophil secondary granule components could add to the pro-inflammatory and/or degradative burden and contribute to COPD progression and the exacerbation of other chronic, tobacco-induced inflammatory diseases.

We have previously reported that nicotine exposure inhibits the respiratory burst in myeloid cells, concomitant with a reduced capacity to clear periodontopathogenic bacteria, and that these phenomena are likely to be dependent on the $\alpha 7$ acetylcholine receptor and linked to the cholinergic anti-inflammatory pathway.¹⁷ Here, we show that CSE-inhibits neutrophil uptake of another pathogen, *S. aureus*. Although *S. aureus* is a rare pathogen in COPD exacerbations, some strains are the exclusive cause of necrotizing pneumonia, an infectious disease with high mortality rate.³² Significantly, pneumonia is much more frequent in smokers than non-smokers.¹ The observed dramatic inhibition of phagocytosis fits well into the overall picture of chronic and recurrent bacterial infections in COPD patients.

It should be noted that, compared with other similar studies, for example, Stringer *et al.*¹², high CSE concentrations (20–100% CSE) were employed herein. However, the high CSE concentrations were counterbalanced by short periods of neutrophil treatment, generally 30-min incubations. We hypothesize that such an approach reflects the peak 'window of contact' between cigarette smoke and pulmonary neutrophils, that is, before the instable, reactive intermediates in smoke are diffused and/or metabolized to less active or inert molecules.

In summary, the highly effective LOX1 and SR-A-driven, non-phlogistic phagocytosis by hMDMs of CSE-exposed neutrophils that die by unconventional mechanisms, would be expected to have a major role in limiting pulmonary inflammation in smokers. In contrast, degranulation of secondary granules and – at long exposure times, leakage of primary, secondary and cytoplasmic contents – would be expected to enhance inflammation and tissue destruction. Furthermore, CSE-induced suppression of bacterial uptake by neutrophils is consistent with bacterial persistence in the lungs of smokers and is likely to promote further pulmonary recruitment of neutrophils responding to bacterial-derived chemotactic stimuli. Our finding of a highly complex, atypical cell death pattern in CSE-exposed neutrophils, which includes features of cellular stress, apoptosis, necrosis and autophagy, may help explain the conflicting literature in this area. Certainly, the consequences of neutrophil exposure to CSE are multifarious and a deeper understanding of their role in smoking-induced inflammatory pulmonary diseases, such as COPD, is required in order to develop improved preventative measures and therapeutic strategies.

Materials and Methods

Materials. The following microbes and materials (source) were employed: *S. aureus* Newman and ATCC 25923 (ATCC, Chicago, IL, USA); Ficol-Paque PLUS (Amersham Biosciences, Uppsala, Sweden); 96- and 24-well cell culture plates, 5 ml round-bottom polystyrene tubes and OptEIA TNF, IL-1 β and IL-6 assays (Becton Dickinson Co., Franklin Lakes, NJ, USA); six-well culture plates (Sarstedt, Newton, NC, USA); glutaraldehyde (Polysciences Inc., Warrington, PA, USA); TSB medium, osmium tetroxide, propylene oxide, Epon resin and uranyl acetate (Fluka Chemie, Buchs, Switzerland); oligo (dT)₂₀ primer, SuperScript III reverse transcriptase, trizol reagent, PBS, RPMI 1640 and 10% heat-inactivated FCS (Gibco Invitrogen Corp., Paisley, UK); BSA, copper sulfate, citrate buffer, NaCl, EDTA, L-glutamine, ethanol, DMSO, ascorbic acid, β -carotene, quercetin, *N*-acetyl-L-cysteine, diphenylene iodonium, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, *Escherichia coli* 0127:B8 LPS, FITC, Tris buffer, Pappenheim stain, gentamycin, hexadecyltrimethylammonium bromide and custom-designed primers (see Table 1) (Sigma-Aldrich, Poznań, Poland); polyvinylalcohol (Merck, Hohenbrunn, Germany); Marlboro cigarettes (Philip Morris Polska SA, Kraków, Poland); lactoferrin ELISA Kit, ethanol and α -tocopherol (Merck KGaA, Darmstadt, Germany), annexin V-FITC kit (Bender MedSystems, Vienna, Austria) and Trypan blue (Fisher Scientific, Gliwice, Poland). Lactate dehydrogenase was assayed using a Cytotoxicity Detection Kit (Roche, Warsaw, Poland). The following antibodies were purchased: anti-CD14 and CD16 (clone TÜK4, and DJ130c, respectively, DakoCytomation Denmark A/S, Glostrup, Denmark), isotype controls as well as anti-CD11b, anti-CD209, phycoerythrin (PE)-conjugated anti-CD31 and anti-CD47

(clones ICRF44, DCN46, WM-59 and B6H12, respectively, Becton Dickinson Co.), anti-SR-A (clone SRA-E5, Trans Genic Inc., Kobe, Japan) and anti-LOX-1, anti-MARCO and anti-CD36 (clones 23C11, PLK1 and FA6-152, respectively, Cell Sciences, Canton, MA, USA). Taq Buffer, dNTPs and LC Taq polymerase were obtained from Fermentas (St. Leon-Rot, Germany).

Isolation, maintenance and CSE treatment of primary human myeloid cells. Primary human neutrophils and hMDMs were isolated from de-identified, EDTA-treated whole blood obtained from healthy, non-smoking donors supplied by the Red Cross, Krakow, Poland, in compliance with the requisite confidentiality assurance on human subjects.

Neutrophils were isolated by Ficol-Paque PLUS density gradient and erythrocyte sedimentation in 1% polyvinyl alcohol with any residual erythrocytes removed by hypotonic lysis, as previously described.³³ Neutrophils were maintained in complete neutrophil medium (RPMI 1640 supplemented with L-glutamine (2 mM), gentamycin (50 μ g/ml) at 37 °C, 5% CO₂. Cell purity was typically > 95% pure on Pappenheim staining. Cell viability was typically > 99%, as determined by Trypan blue exclusion. Freshly isolated, non-apoptotic neutrophils (5 \times 10⁶ cells/ml) were exposed to CSE (1 ml) or control conditions (1 ml PBS) in 5 ml round-bottom polystyrene tubes (37 °C, 5% CO₂, for the times indicated in the Figures). Cells were washed once in complete neutrophil medium and harvested by centrifugation (280 \times g, 5 min, 20 °C), before resuspension under the required experimental conditions and density, as described below.

hMDMs were derived from PBMCs isolated using a Ficol-Paque PLUS density gradient and plated at 4 \times 10⁶/ml in 24-well Primaria cell culture plates in complete macrophage medium (RPMI 1640 supplemented with 2 mM L-glutamine, 50 μ g/ml gentamycin and 10% pooled heat-inactivated human serum). Plates were incubated (5% CO₂, 37 °C, 2 h) and non-adherent PBMC removed by washing with complete macrophage medium. Adherent cells were cultured in regularly changed complete macrophage medium for a minimum of 7 days. After non-enzymatic detachment of cells, hMDM phenotype was confirmed by CD14-, CD11b-, CD16- (> 90% positive) and CD209-specific (< 1% positive) immunofluorescence using flow cytometry. The adherent cells acquired typical macrophage morphology; exhibited extensive phagocytic activity against live *S. aureus*; and were resting (they did not produce IL-1 β , TNF or IL-6, as determined by ELISA, according to the kit manufacturer's instructions). Cell viability was typically > 99%, as determined by Trypan blue exclusion.

Preparation of a CSE. Immediately before experimentation, CSE was freshly generated from filtered Marlboro cigarettes, essentially as described by Aoshiba et al.³⁴ Cigarette smoke was bubbled through 10 ml PBS in a gas trap connected to a vacuum pump. CSE preparations were sterile filtered (0.22 μ m), cooled on ice and used within 15 min. CSE was used at a concentration of 100% in all experiments, unless otherwise noted.

Induction of apoptosis in CSE-exposed neutrophils

Flow cytometry. The proportion of apoptotic neutrophils in CSE-exposed populations was determined by flow cytometry using annexin V-FITC kits and a FACScan flow cytometer (Becton Dickinson). CSE-induced apoptosis was compared with spontaneous neutrophil apoptosis, induced by 24-h incubation in

Table 1 Primer sequences for selected pro- and anti-apoptotic neutrophil genes

Gene	Primer sequence	Product size (bp)
<i>Bax-α</i>	Forward: 5'-CTGACATGTTTTCTGACGGC-3' Reverse: 5'-TCAGCCCATCTTCTCCAGA-3'	290
<i>Bak1</i>	Forward: 5'-TGAAAATGGCTTCGGGGCAAGGC-3' Reverse: 5'-TCATGATTTGAAGAATCTTCGTACC-3'	642
<i>Bcl-xl</i>	Forward: 5'-TTGGACAATGGACTGGTTG-3' Reverse: 5'-GTAGAGTGGATGGTCAGTG-3'	765
<i>Hsp70</i>	Forward: 5'-TTTGACAACAGGCTGGTGAACC-3' Reverse: 5'-GTGAAGATCTGCGTCTGCTTGG-3'	590
<i>Hsp90α</i>	Forward: 5'-GCTGTGCCGTTGGTCTGTGC-3' Reverse: 5'-GGTTCTCCTTCATTCTGGTGC-3'	1498
<i>Hsp27</i>	Forward: 5'-CCAGAGCAGAGTCAGCCAGCAT-3' Reverse: 5'-CGAAGGTGACTGGATGGTGA-3'	576
β -Actin	Forward: 5'-AGCGGGAAATCGTGCGTG-3' Reverse: 5'-GGGTACATGGTGGTGCCG-3'	307

complete neutrophil medium, 5% CO₂, 37 °C, as described by Guzik *et al.*³⁵ as well as to neutrophils in which necrosis was induced by brief cell disruption with a hand-held sonicator (10 × 1 s pulse, 50 W, 30 kHz; model UP50H sonicator, Dr. Hielscher, Teltow, Germany) and confirmed by Trypan blue uptake.

DNA-laddering. Late apoptosis was monitored by the presence of ladder-like DNA fragments, as described by Guzik,³⁶ in CSE-exposed (30 min) and control neutrophils (fresh, unexposed neutrophils and neutrophils induced into apoptosis by 24-h incubation in complete neutrophil medium).

Apoptotic ultrastructure. The ultrastructural consequences of neutrophil treatment with CSE were determined by TEM. Rested (37 °C, 5%CO₂, 2 h, complete neutrophil medium) CSE-exposed and unexposed cells were fixed (2.5% glutaraldehyde in PBS buffer, pH 7.4, RT, 60 min), rinsed with PBS, and post-fixed (1% osmium tetroxide, RT, 100 min). After dehydration in an ethanol gradient and propylene oxide, neutrophils were embedded in Epon resin. Ultrathin sections (LKB-NOVA microtome, Vienna, Austria) were contrasted (9% uranyl acetate) and examined (JEM 1200 EX electron microscope (JEOL, Tokyo, Japan), 80 kV).

Induction of necrosis in CSE-exposed neutrophils

Membrane integrity and degradation. The CSE-induced release of primary (elastase); secondary (lactoferrin) granule; and cytoplasmic (lactate dehydrogenase) neutrophil components, by CSE-exposed and control neutrophils was monitored by: *p*-nitroaniline (37 °C, 405 nm, 30 min) release from the elastase substrate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, as previously described,³⁷ using a Molecular Devices microplate reader (Molecular Devices, Sunnyvale, CA, USA); and by lactoferrin and lactate dehydrogenase ELISA Kits (Calbiochem, EMD Biosciences Inc., La Jolla, CA, USA), according to the manufacturer's protocols. Cell viability was assessed by Trypan blue exclusion.

Necrotic ultrastructure. The ultrastructural consequences of neutrophil treatment with CSE were determined by TEM, as described above.

Induction of autophagy in CSE-exposed neutrophils. TEM, as described above, was also used to examine ultrastructural features consistent with autophagy in CSE-exposed and control neutrophils. Furthermore, flow cytometry data were examined for unusual patterns of annexin V/PI labeling.

Anti-phagocytosis markers on the surface of CSE-exposed neutrophils.

In addition to relocation of phosphatidyl serine, surface expression of two key anti-phagocytotic markers (CD31 [PECAM-1] and CD47) was monitored by immunofluorescence using PE-conjugated anti-CD31, anti-CD47 or isotype control monoclonal antibodies (30 min, 4 °C) with 10⁴ cells were acquired per analysis. Flow cytometric data were analyzed using the CellQuest v3 program (Becton Dickinson) to determine the percentage and mean fluorescence intensity of positive cells.

Analysis of selected pro- and anti-apoptotic transcripts.

Total RNA was extracted from neutrophils (2 × 10⁷) using Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. RNA samples (2 μg) were used for cDNA synthesis reactions in a total volume of 20 μl containing 10 μl of each RNA sample, 0.5 μg anchored oligo (dT)₂₀ primer and 200 U of SuperScript III reverse transcriptase, according to the protocol provided with the enzyme. RNA samples were treated with RNase-free DNase to remove all genomic DNA before the RT reaction. PCRs were set up in a total volume of 50 μl containing: Taq Buffer with KCl (Fermentas, provided with the enzyme), 0.2 mM dNTPs, 200 nM of each primer, 1.5 mM MgCl₂, 2 U of LC Taq polymerase (Fermentas), 2 μl of each cDNA and sterile water. Reactions (30 cycles) were carried out in a GeneAmp 2400 thermocycler (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) under the following conditions: 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1.5 min (*bak1* and *hsp70*) or 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min (*bax-α*, *bcl-xl*, *hsp90α*) or 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min (*hsp27* and *β-actin*). Each thermal profile was ended with the final extension at 72 °C for 15 min. The reaction products were resolved in nondenaturing 2% agarose gel and visualized by staining with ethidium bromide. All primers were custom synthesized and their sequences are listed in Table 1. The primers were designed to match sequences within separate exons (except for the *hsp70* encoded by a single exon) to avoid the contribution of genome-templated product in the signal analysis.

Phagocytosis of CSE-treated neutrophils by hMDMs

Neutrophil elastase activity in hMDMs. On purification, hMDMs were routinely negative for neutrophil elastase activity (*data not shown*), as expected.

Therefore, neutrophil elastase was employed as a surrogate marker of neutrophil engulfment by macrophages, essentially as described by Guzik *et al.*³⁵ Briefly, CSE-exposed and control neutrophils (20 : 1) were added to hMDM monolayers in complete macrophage medium in 24-wells plates (2 h, 37 °C, 5% CO₂). hMDM monolayers were washed vigorously with ice-cold PBS to remove bound but non-ingested neutrophils then lysed with 0.1% hexadecyltrimethylammonium bromide (15 min, 37 °C). In all, 100 μl of lysate was transferred to a 96-well plate in four replicates and 100 μl *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (1 mM in Tris buffer, pH 7.5) added. *p*-Nitroaniline release was monitored (30 min, 37 °C, 405 nm). Neutrophil phagocytosis is represented by hMDM-internalized neutrophil elastase activity, reported as mOD (*p*-nitroaniline release)/min.

Visualization of neutrophil phagocytosis. For TEM analysis of phagocytosed neutrophils, hMDMs were cultured on glass coverslips in six-well culture plates hMDMs, trypsinized, then fixed and stained as described for neutrophils, above.

Blockade of hMDM scavenger receptors. In order to determine which scavenger receptors have a mechanistic role in the phagocytosis of CSE-exposed neutrophils, hMDMs were pretreated with scavenger receptor-specific mAbs or oxLDL. Briefly, before the addition of CSE-exposed and control neutrophils in phagocytosis assays, described above, hMDM monolayers were incubated (20 °C, 20 min) with anti-SR-A; anti-LOX-1; anti-MARCO; or anti-CD36 mAbs (all final concentration 10 μg/ml) in 0.5 ml complete medium with serum replaced by BSA (0.5%). After removal of scavenger receptor-specific antibodies, neutrophil phagocytosis assays were performed. Alternatively, hMDM monolayers were incubated in a suspension of 50 μg/ml (final concentration) of oxidized LDL isolated by the method of Havel *et al.*³⁸ and Cu²⁺ (copper sulfate)-catalyzed oxidation.³⁹ Neutrophil phagocytosis assays were performed with oxLDL present in the interaction medium during phagocytosis.

Non-phlogistic versus pro-inflammatory hMDM response to phagocytosis of CSE-exposed neutrophils. Following phagocytosis of CSE-exposed neutrophils, as described above, 4- and 24-h hMDM secretion of TNF was determined by ELISA, according to the instructions provided. Unstimulated hMDMs and hMDMs stimulated with *E. coli* 0127:B8 LPS (2 ng/ml) served as negative and positive controls, respectively.

S. aureus uptake by CSE-treated neutrophils.

S. aureus strains Newman and ATCC 25923 were grown in TSB medium (18 h, 37 °C), washed twice with a large volume of PBS, and viably labeled with 0.1% FITC in PBS (37 °C, 2 h). After washing, FITC-labeled bacteria were opsonized in the presence of 10% pooled human serum (30 min, 37 °C), washed again and suspended in PBS (10⁹ bacteria/ml). Cell numbers were calculated from standard densitometric curves established at 540 nm. CSE-exposed and control neutrophils were incubated with and without opsonized *S. aureus* suspensions at a leukocyte: bacterial ratio of 1 : 20 (30 min, 37 °C) in a 1 ml total volume. One ml ice-cold complete neutrophil culture medium was added and free bacteria removed by centrifugation (110 × *g*, 5 min). To exclude adherent, but non-phagocytosed, bacteria from analysis, FITC fluorescence was quenched with Trypan blue, as previously described.⁴⁰ Bacterial phagocytosis data are presented as the fraction of neutrophils with internalized fluorescent bacteria.

Effect of anti-oxidants on neutrophil apoptosis, bacterial uptake and phagocytosis by hMDMs.

In some experiments, the following antioxidants (final concentration) were added to CSE: ascorbic acid (100 μM, vitamin C), 10–20 μM α-tocopherol (10–20 μM, vitamin E), all-trans-β-carotene (1–2 μM, vitamin A, DMSO solvent), quercetin (1–2 μM, flavonoid, DMSO solvent) as well as solvent controls.

Statistical analyses. All experiments were performed in triplicate, unless otherwise stated. The data are presented as mean (± S.D.). All statistical analyses were performed using GraphPad Prism v2.0 (GraphPad, San Diego, CA, USA). Statistical significance was assessed at 5% and calculated using Student's *t*-test.

Conflict of interest

The authors declare no conflict of interest.

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