Oxygen atoms are critical in rendering THP-1 leukaemia cells susceptible to cold physical plasma-induced apoptosis

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Cold physical plasma has been suggested as a powerful new tool in oncology. However, some cancer cells such as THP-1 leukaemia cells have been shown to be resistant towards plasma-induced cell death, thereby serving as a good model for optimizing plasmas in order to foster pro-apoptotic anticancer effects. A helium/oxygen radio frequency driven atmospheric plasma profoundly induced apoptosis in THP-1 cells whereas helium, humidified helium, and humidified helium/oxygen plasmas were inefficient. Hydrogen peroxide – previously shown as central plasma-derived agent – did not participate in the killing reaction but our results suggest hypochlorous acid to be responsible for the effect observed. Proteomic analysis of THP-1 cells exposed to He/O2 plasma emphasized a prominent growth retardation, cell stress, apoptosis, and a pro-immunogenic profile. Altogether, a plasma setting that inactivates previously unresponsive leukaemia cells is presented. Crucial reactive species in the plasma and liquid environment were identified and discussed, deciphering the complexity of plasma from the gas phase into the liquid down to the cellular response mechanism. These results may help tailoring plasmas for clinical applications such as oxidation-insensitive types of cancer.

Firmly connecting with redox biology, therapeutical effects of cold plasma-generated reactive molecules are investigated in the field of plasma medicine1. The strong advantage of plasma is the parallel deposition of different biologically active reactive species in a localized manner1. In the plasma gas phase, this includes for example hydroxyl radical, nitric oxide, and atomic oxygen1. In plasma-treated liquids, chemistry is further complexed, and typical molecules detected include superoxide anion, hydrogen peroxide, and peroxynitrite4. With adequate concentrations, these species can overwhelm the cells’ antioxidative response, effectively mediating pro-apoptotic redox signalling responses5. Many tumours display an inequity in their redox balance, rendering them more receptive towards oxidation-induced cell death compared to non-malignant cells6. Accordingly, a number of drugs have been tested in clinical trials aiming at further disturbing the redox-balance of cancers7, ultimately inducing apoptosis8. Thus, plasmas applications have been proposed to be a possible asset in oncology as well, as killing has been achieved for various types of cancers in vitro9–11 and in vivo12–14. Although primary monocytes are susceptible to plasma-induced apoptosis15, some malignant cells such as THP-1 leukaemia cells effectively withstand plasma-induced apoptosis16, and rather respond via growth deceleration17. They, therefore, serve as an ideal target to identify which parameters may render a plasma source more effective for oncological applications in oxidation-resistant cancer cells.

Different parameters can be adjusted to alter the plasma generation and thus the reactive species composition. For example, humidity in the feed argon gas was identified to play a highly critical role18. The humidification of a noble gas dramatically increase hydrogen peroxide (H2O2) production in the gas and liquid phase19 whereas addition of oxygen (O2) or nitrogen (N2) enhances the generation of reactive oxygen and nitrogen species (ROS,
A helium (He)-driven atmospheric pressure plasma jet (µAPPJ) in its COST-jet version was used which has been characterized towards its physical and antimicrobial properties. To investigate the biological effects of feed gas alterations, it was humidified and/or spiked with molecular oxygen (O₂) with the idea of altering liquid chemistry, redox reactions, and subsequently eradication of the tumour target cells. In this study, we analysed cell morphology and counts, metabolic activity, apoptosis, and the THP-1 proteome on the one hand, and investigate the plasma-treated liquids on the other hand to determine the mechanism of action as function of the feed gas compositions.

Results

The feed gas composition-dependent THP-1 cell inactivation was mediated via apoptosis. THP-1 cells are known to strongly persist oxidative stress-induced killing. The aim of this work was to identify a feed gas composition that may foster cold plasma-induced eradication of these cells to substantiate its potential use in tumour therapy. THP-1 cells were exposed to a radiofrequency driven plasma jet utilizing He, He/0.6% O₂, He/0.15% H₂O, or He/0.6% O₂/0.15% H₂O as feed gases, and were assayed 24 h later. The conversion of the resazurin to its fluorescent product resorufin via nicotinamide adenine dinucleotide phosphate reduces equivalents identifies the overall metabolic activity of given number of cells. A significantly decreased metabolism could be observed in cells treated with He/O₂ plasma but not with any of the other feed gas conditions used. Flow cytometry was performed to determine the mitochondrial activity on a single cell level using mitotracker orange dye. Highly fluorescent once incorporated into mitochondria, its fluorescence decreases if the mitochondrial membrane potential is lost, which indicates cell death. Only cells exposed to the He/O₂ plasma showed a significant impairment which may have contributed to the overall decreased metabolic activity seen with the resazurin dye. It was asked next whether this finding may also be attributed to a decrease in total cell numbers in the appropriate forward scatter (FSC)/side scatter (SSC) gate. Again, only the He/O₂ plasma condition led to a significant reduction of cells. The data of viable cell counts and metabolic activity are not congruent. This may be a consequence of apoptosis being an active and energy consuming process together with the observation that plasma treatment may increase activity in viable THP-1 cells. Altogether, He/O₂ but no other plasma conditions efficiently reduced THP-1 cell metabolic activity by inducing a down-modulation of mitochondrial activity and via reduction of total cell counts. In none but the He/O₂ condition, cytotoxicity could be observed. We, therefore, assessed morphological features of control and He/O₂ plasma-treated THP-1 cells after 24 h using image cytometry. Treated but not control cells showed features of apoptosis such as membrane blebbing, nuclear degradation with similar side-scatter profiles of dead cells as seen with traditional cytometry.
apoptotic process, THP-1 cells were stained for activated executioner caspases 3/7 (Fig. 3c), and He/O₂ but not He plasma-treated cells were shown to be significantly positive (Fig. 3d). Linking to these apoptotic events, concentrations of interleukin (IL)-8 were significantly decreased in He/O₂ plasma-treated samples as well (Fig. 3e).

Figure 2. He/O₂ but not He, He/H₂O, or He/O₂/H₂O plasma inactivated THP-1 cells. THP-1 cells were exposed to cold physical plasma under different feed gas conditions, and assayed after 24 h. (a) Overall metabolic activity was significantly decreased in the He/O₂ condition, as measured by resazurin to resorufin transformation. (b) Representative histograms of mitotracker fluorescence for each gas condition. (c) Mitotracker fluorescence quantification revealed significant reduction of mitochondrial activity in the He/O₂ condition. (d) Representative FSC/SSC dot plots with the appropriate live and cell count gate. (e) Live cell count quantification demonstrated a significant decrease with the He/O₂ condition. Data are one representative (b,d) or presented as mean ± S.D. (a,c,e) of three experiments. Statistical differences (***p < 0.001) were determined using t-test.
THP-1 cell killing in the He/O₂ condition was mediated via a non-H₂O₂ mechanism. THP-1 cells showed a high susceptibility towards He/O₂ but not He, He/H₂O, and He/H₂O/O₂ plasma. H₂O₂ was previously shown to be an important mediator in plasma cytotoxicity²⁵. Yet, its concentration was decreased in the He/O₂ plasma toxic condition compared to the helium plasma (Fig. 4a). Additionally, the enzymatic antioxidant catalase, a potent scavenger of H₂O₂²⁶, did not inhibit the cytotoxic effect of He/O₂ plasma (Fig. 4b). H₂O₂ contributed, therefore, unlikely to the killing mechanism. To test whether the responsible reactive molecule would be rather short or long lived, THP-1 cells were exposed to plasma-treated medium in presence of absence of the potent thiol-containing non-enzymatic antioxidants N-acetyl-cysteine (NAC) or glutathione (GSH). GSH is among the most abundant proteins in cells, and its oxidized dimer GSSG is readily replenished in cells by glutathione
reductase. Intracellularly, NAC serves as a GSH precursor, and its extracellular antioxidant activity is superior to that of GSH. Similar to direct treatment, helium plasma-treated liquid did not affect cell viability (Fig. 4b). On the contrary, He/O2 plasma-treated liquid exhibited significant cytotoxic activity, although to a significantly lesser extent compared to direct treatment in the He/O2 condition. Data are presented as mean ± S.D. of two experiments. Statistical differences (***/### p < 0.001) were determined using 1-way ANOVA within each gas condition and plasma treatment regimen, and t-test to compare individual values for a single treatment regimen within each gas condition.

Pro-apoptotic and growth deceleration proteins regulated in He/O2 plasma-treated cells. THP-1 cells showed a high susceptibility towards He/O2 plasma. Using proteomic technology, up or down-regulation of proteins in the cellular fraction of THP-1 cells 4 h after plasma treatment with either He or He/O2 with or without the presence of NAC was assessed against gas-treated control cells. Gene ontology analysis identified binding (37%), catalytic activity (33%), structural molecule activity (15%), transporter activity (5%), and receptor activity (4%) as main molecular function categories of proteins that were significantly regulated in He/O2 plasma-treated compared to gas-treated control cells. Six representative significantly up or down-regulated proteins for the He/O2 treated cells are given (Table 1) together with the regulation in the other three samples types. Addition of NAC abrogated the regulation of any protein in plasma-treated samples to the level of untreated gas controls. This corroborates the findings with metabolic activity where NAC fully protected against toxic He/O2 plasma activity on THP-1 cells. Consistent with flow cytometric active caspase 3 detection, an upregulation...
(+11.1-fold) of this protein was also determined in the cellular fraction using mass spectrometry. Other proteins involved in apoptosis, transport or cell growth were also strongly regulated, for example, A-kinase anchor protein 8-like, Exportin-T, Telomeric repeat-binding factor 2-interacting protein, Cell and growth-regulating nucleolar protein (LYAR), and U4/U6.U5 tri-SNRNP-associated protein 1. A pro-immunogenic protein (Squamous cell carcinoma antigen recognized by T-cells 3) was increased as well.

Table 1. THP-1 killing by plasma was accompanied by alterations in protein expression involved in apoptosis and growth retardation. In presence of NAC (2 mM) or vehicle control, THP-1 cells were either left untreated or exposed to either He or He/O₂ plasma. Four hours after treatment, cells were lysed, and proteins were analysed with a proteomic approach using LC/MS. Six representative positively and negatively regulated proteins with at least 2-fold regulation are given in the table for all four treatment regimes. Data sorting is shown in a descending manner for He/O₂ plasma results.

![Figure 5.](image)

Figure 5. He/O₂ plasma generated hypochlorous acid in the liquid via gaseous phase oxygen species. (a) Cell culture medium as loaded with either APF or HPF. Scavengers were added or not, solutions were plasma-treated using either He or He/O₂ as feed gas, and fluorescence of the redox dyes was acquired which was normalized to each respective gas control. APF in general gave a stronger fluorescence signal which was increased in the He/O₂ condition but not using the He plasma. NAC partially scavenged signals. (b) Fifty micromolar H₂O₂ was added to PBS and chloride-free phosphate buffer (PB). Solutions were plasma-treated using either He or He/O₂ plasma. The latter scavenged experimentally added H₂O₂ in PBS but not PB. (c) Singlet oxygen sensor green was added to cell culture medium which was subsequently exposed to either He, He/O₂, He/H₂O, or He/H₂O/O₂ plasma. Fluorescence was acquired and normalized to untreated gas control, and only He/O₂ plasma differed significantly. Data are presented as mean ± S.D. of two experiments. Statistical difference (***(p < 0.001)** was determined using t-test.)
Discussion

Cold physical plasma-derived reactive species are a promising new tool in oncology33. However, some tumours are highly resistant to oxidative stress-induced cell death34. By modulating the reactive species output, it is, therefore, important to identify plasma-settings that also sensitize these cancer cells to apoptosis. Humidification of the carrier gas was previously described to potentiate HO and H2O2 generation of an atmospheric pressure argon plasma jet manifold18. These plasma-derived molecules are strong anticancer agents35. Yet, THP-1 cells resist high H2O2 concentrations and long plasma treatment times16,17,36. By admixing low amounts of oxygen to the feed gas of a helium plasma jet, a redox route was identified that is particularly effective in THP-1 cell killing. In line with classical hallmarks of apoptosis, treated cells showed membrane blebbing and executioner caspase 3 activation37. Interestingly, mitotracker orange fluorescence was also decreased in the viable cell portion. This argues for a destabilized mitochondrial membrane potential23 i.e. at least partial damage in He/O2 plasma-treated cells. The reduction in viability was also reflected by a decrease in constitutively expressed IL-8. This is interesting as oxidant exposure with only moderate apoptotic effects strongly increased IL-8 release in THP-1 cells16,39 and other myeloid cells undergoing plasma-induced cell death40. IL-8 release is subject to redox control41 and a key molecule in regulating inflammation in wound healing42 and cancer43. Its release by e.g. monocytes/macrophages attracts neutrophils to clear infection but also spur inflammation44.

Using proteomics, we identified proteins involved in growth retardation and cell death in He/O2 plasma-treated THP-1 cells. Protein phosphatase 1 regulatory subunit 27 negatively regulates phosphatases, and its significantly decreased recovery (−3.4-fold) reasons for sensation of cell stress resulting in target protein phosphorylation34. Cell and growth-regulating nucleolar protein was significantly increased (+4.1-fold). It is a crucial regulator of ATR-Chk146 which is central in sensing redox stress as well47. This hampers cell cycling that is reflected by significant down-regulation of A kinase anchoring protein 8-like (−8.3-fold) which initiates initial phases of DNA replication48. Vice versa, U4/U6.U5 tri-SNRP-associated protein 1, which is known to induced cell cycle arrest49, was upregulated (+2.6-fold). Paralleling growth deceleration, Exportin-1, important in exporting tRNAs from the nucleus to support protein translation and overexpressed in many tumors due to high proliferation rates50, was significantly down-regulated (−3.4-fold). Similarly, the down-regulation of Telomeric repeat-binding factor 2-interacting protein 1 (−3.4-fold) suggest growth retardation as it phosphorylates the inhibitor of Nrf-B thereby fostering cellular activation51. The protein LYAR is central for cellular growth52 and was significantly reduced (−2.4-fold) in plasma-treated cells. Vice versa, the proliferation marker Ki-67 was found to be decreased (−2.3-fold) whereas levels of cell death executioner caspase 3 drastically increased (+11.4-fold), in line with the flow cytometry experiments. This is underscored by elevated levels of Mitogen-activated protein kinase kinase kinase MLT (+2.1-fold), an upstream regulator Jnk and p38 known to be pro-apoptotic also in other cancer cells53. Interestingly, Histone acetyltransferase p300, a current target for anticancer therapy54, was increased in He/O2 plasma-treated THP-1 cells. Also of potential therapeutic value is the massive elevation of Squamous cell carcinoma antigen recognized by T-cells 3 (SART3) which has been used to potentiate T cell responses in vaccination trials in cancer patients (+11.1-fold)55. It is overexpressed in the majority of adenocarcinomas and squamous cell carcinomas in different tissues as well as in leukemia but not non-malignant cells56.

Several reports conclude that SART3 is among a few targets found in many cancers with value in immunotherapy57-59. Altogether, the toxic plasma condition in THP-1 leukemia not only induced pro-apoptotic proteins but also suggested an enhanced immunogenicity of treated cells.

Significant cell responses were found to be present only in the He/O2, but not in any other feed gas condition. Although not necessarily the single responsible agent60-62, H2O2 has been determined to be central in plasma-mediated cytotoxicity in a number of studies63-66. Here, its direct effects were negligible as results with catalase suggest. Instead, we suspect HOCl to be the active cytotoxic agent for a number of reasons. NAC, an efficient scavenger of HOCl67, abrogated the He/O2 plasma’s cytotoxicity. HOCl is also a known scavenger of H2O268. A full decrease of experimentally added H2O2 was only apparent in the toxic He/O2, but not the non-toxic He plasma conditions. Moreover, scavenging was abolished in the absence of chloride which is apparently essential for the generation of HOCl in liquids69. Both APF and HPF are reported to sense HO and ONOO−70. APF but not HPF is sensitive for HOCl70 and used to detect halogenated acids such as HOCl in phagocytes72 and leukemic cells71. He/O2 but not He plasma increased APF but not HPF fluorescence. These results suggest the presence and activity of HOCl, although a previous report using another plasma jet suggested RNs to be important in THP-1 cell inactivation72. Another possibility is that HOCl acted on cell culture proteins that then have mediated cytotoxic effects. In mammals, HOCl is physiologically generated by myeloperoxidase primarily expressed by neutrophils73. HOCl especially serves to destroy phagocytosed bacteria74. It is also present extracellularly, for example as a by-product of neutrophil extracellular traps generated during infection75. HOCl is highly reactive and capable of inactivating protease inhibitors and lysozyme76. Thus, it has an important role in fighting bacteria and spurring inflammation. Nonetheless, HOCl is readily detoxified by cellular thiols77, albumin78, and cross linking of biological molecules via non-disulfide bonds79, effectively limiting its damage to host cells.

The addition of oxygen to dry helium feed gas was found to be central for plasma to inactivate THP-1 cells in a HOCl-dependent manner. HOCl generation is thought to be mediated via atomic oxygen (1):

\[
O + Cl^- \rightarrow OC\Gamma
\] (1)

Underlining this notion80, O densities in the gas phase of this plasma source have been reported to be markedly elevated in the effective He/O2 plasma (10^15 cm^-3)80 compared to He and He/H2O plasma (10^13 cm^-3)82. Hypochlorous acid can be formed via the reaction of O with Cl−, and our experiments using chloride-free buffer support the significance of this route in our study. In humidified feed gas, the generation of OH− and not O is dominant83 which is reflected in the relatively H2O2 resistant THP-1 cells37 not being strongly affected with these
plasma conditions in the present study. Importantly, O shows a good solubility in liquids as previous work using phenol as target suggest\(^{86}\) which makes its important role in HOCl generation plausible.

A further source of reactivity in the He/O\(_2\) gas mixture could be other excited molecular oxygen species, especially the singlet delta oxygen O\(_2(\Delta g)\) with a gas phase density of around 4 \times 10^{14} \text{ cm}^{-3} with similar power and gas conditions as used in this work\(^{84}\). Yet, its density was previously found to decrease with increasing O\(_2\) concentration (due to fast quenching in collisions with O atoms in the gas phase), limiting its possible impact. Nonetheless, in He/O\(_2\) plasma but no other feed gas condition, singlet delta oxygen was modestly increased in treated medium containing L-histidine, which was technically suboptimal as it can quench singlet oxygen\(^{85}\). According to the following net reaction (2):

\[
\text{HOCl} + \text{H}_2\text{O}_2 \rightarrow \text{Cl}^- + \text{H}^+ + \text{H}_2\text{O} + \text{O}_2(\Delta g).
\]

(2)

Hypochlorous acid oxidizes hydrogen peroxide yielding chloride ions\(^{86}\), water, and singlet delta oxygen (O\(_2(\Delta g)\)). Together with the absence of H\(_2\)O\(_2\), this notion further emphasizes the role of HOCl as an active agent under the discussed circumstances. Consecutively, O\(_2(\Delta g)\) created in this secondary reaction may contribute to the observed effect, but considering its very short lifetime in aqueous, neutral liquids (few µs) compared to HOCl (several minutes)\(^{86}\), to a limited extent only. Notably, ozone production also increases 10-fold in the toxic oxygen setup\(^{81}\). Yet, and in contrast to treating dry surfaces\(^{86}\), modelling studies grant only a minor role to ozone in the liquid\(^{88}\) as it dissolves badly and no reactions with phenols could be detected\(^{81}\).

**Conclusion**

Cold physical plasma has been suggested to be an option for cancer therapy, but some cancers are highly resistant against plasma-induced peroxide stress. By modulating the feed gas of a helium-driven plasma jet by the addition of oxygen, it was demonstrated that the chemistry can be tuned towards other species such as hypochlorous acid. With similar treatment times, this condition severely hampered cell activity and growth by inducing apoptosis whereas plasma treatments dominated by hydrogen peroxide did not. Additionally, immune stimulatory proteins were found to be elevated. Understanding the biological impact of gas and liquid phase plasma chemistry will help improving the plasma’s efficacy in order to tailor them to the therapeutic applications’ needs.

**Materials and Methods**

**Plasma Source.** The COST jet, a proposed reference jet in the European Cost action MP1101 to compare effects of plasma treatments among different laboratories, was used as plasma source (Fig. 1a)\(^{20}\). Helium (99.9999%; Air liquide, France) at a flow of 1.4 slm was used as feed gas. In some experiments, it was admixed with either 0.6% oxygen or humidified helium with approximately 1450 ppm of H\(_2\)O, or both. Humidification was achieved by bubbling 0.3 slm of helium flow through 400 ml of double distilled water and mixing it with 1.1 slm of dry Helium to obtain the total flow of 1.4 slm. (Fig. 1b). Calibrated, USB-controlled flow controllers (MKS instruments, Germany) were used to set and monitor all feed gas conditions.

**Cell culture and plasma treatment.** The leukaemia cell line THP-1 (ATCC TIB-202, Germany) were cultured in fully supplemented RPMI1640 cell culture medium containing 10% fetal bovine serum, 2% glutamine, and 1% penicillin-streptomycin (all Sigma, Germany) in an incubator (Binder, Germany) at 37 °C, 95% relative humidity, and 5% CO\(_2\). For plasma-treatment, 1 \times 10^5 cells in 250 µl of medium were seeded in each well of a 24-well plate (Nunc, Denmark). To some wells, the H\(_2\)O\(_2\) scavenging enzyme catalase (20 µg/ml), the antioxidant GSH (1 mM) or the antioxidant NAC (2 mM) was added (all Sigma). Cells were directly exposed to either each plasma condition or their respective gas controls (plasma off). Alternatively, 1 \times 10^5 cells in 125 µl of cell culture medium received 125 µl of 250 µl medium (indirect approach) plasma-treated for the double amount of time for comparison. Following exposure to plasma or plasma-treated liquid, cells were returned to the incubator for 20 h or 24 h depending on the subsequent analysis.

**Cellular analysis and viability.** THP-1 cells were investigated on a number of properties, such as cell morphology, total metabolic activity, total cell counts, individual mitochondrial activity, and caspase 3/7 activity. For assessment of morphology 24 h after treatment, THP-1 cells were collected and stained with DRAQ5 (BioLegend, UK) prior to image acquisition using an ImageStream Mark X (Merck-Millipore, USA). Cells representative for viable and apoptotic events were analysed using Ideas 6.2 software (Merck-Millipore). For assessment of total metabolic activity 20 h after treatment, resazurin (Alfa Aesar, USA) was added to the wells, and cells were incubated for another 4 h. Supernatants were transferred to 96-well plates and fluorescence was acquired using a microplate reader with λ_em 530 nm and λ_exc 590 nm (Tecan, Switzerland). To assess mitochondrial activity 24 h after treatment, cells were stained with 500 nM mitotracker orange (CMXRos; life technologies, USA) for 20 min at 37 °C, and mitotracker fluorescence of cells in the respective gate was acquired using a CytoFlex flow cytometer (Beckman-Coulter, USA). Mitotracker orange is retained in mitochondria due to their chloromethyl group forming a covalent bond with thiols\(^{86}\). Moreover, the dye fluoresces to a lesser extent upon mitochondrial damage and membrane depolarization\(^{23}\). For total cell counts, cells were aliquoted from the 24-well plates into 96-well plates 24 h after treatment and counted with an attune flow cytometer (Applied Biosystems, USA) capable of measuring absolute particle concentrations. To assess apoptosis 24 h after treatment, THP 1 cells were collected, washed, and stained for 30 min at 37 °C with caspase 3/7 indicator (life technologies). The percentage of cells staining caspase positive was quantified using the CytoFlex flow cytometer.

**Redox-sensitive probes.** Fully supplemented RPMI1640 medium was loaded with 1 µM singlet oxygen sensor, or PBS was loaded with 1 µM of either the fluorescent redox indicators APF or HPF (life technologies).
Both APF and HPF can be oxidized by hydroxyl radicals and peroxynitrite but not hydrogen peroxide whereas only APF is sensitive towards hypochlorous acid29. Two-hundred and fifty microliter was added to each well of a 24-well plate, and wells either received plasma treatment or were left untreated. Subsequently, the liquid was aliquoted into 96-well plates, and fluorescence was acquired using a microplate reader with \(\lambda_{ex} = 485 \text{ nm} \) and \(\lambda_{em} = 535 \text{ nm} \). In order to quantify hydrogen peroxide \((\text{H}_2\text{O}_2)\), plasma-treated cell culture medium or plasma-treated chloride-free phosphate buffer was incubated with amplex ultra red (life technologies) according to the vendor’s instructions, and subsequently quantified against an \(\text{H}_2\text{O}_2\) standard using a Tecan microplate reader with \(\lambda_{em} = 530 \text{ nm} \) and \(\lambda_{em} = 590 \text{ nm} \). Alternatively, cell culture medium was spiked with a known concentration of \(\text{H}_2\text{O}_2\) and the scavenging activity of plasma-introduced reactive components on \(\text{H}_2\text{O}_2\) was assessed in a similar manner.

**Interleukin 8 and global protein expression.** Twenty-four hours after plasma treatment, IL-8 in supernatants of THP-1 cells was quantified using ELISA (BioLegend), and concentrations were normalized to each respective gas control. Four hours after treatment, either eight replicates of untreated or plasma-treated THP-1 cells were pooled into micro-centrifuge tubes, washed, and stored at \(-80 \text{ C}\). Global protein expression was carried out as previously described30. Briefly, peptides were separated by nano-liquid chromatography (Dionex Ultimate 3000; PepMap RSLC column, 75 \text{µm} ID/15 cm), and eluates were ionized by electrospray ionization and analysed by high-resolution mass spectrometry (QExactive, Thermo, USA) mass spectrometer. Data processing was done using Proteome Discoverer 1.4 software (Thermo). Protein candidates were selected upon their involvement in pathways of metabolisms and proliferation as well as on statistical criteria \((\geq \pm 2.0\)-fold expression\). Data were also analysed with Ingenuity Pathway Analysis software (IPA, Qiagen) and free web based applications (PANTHER and Universal Protein Resource).

**Statistics.** Statistical analysis was carried out using prism 7.02 (graph pad software, USA). A significance level of \(\alpha = 0.05\) was set for all statistical analysis, and significantly different data were marked with asterisks \((^* p < 0.05; **p < 0.01; ***p < 0.001)\).

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