Plasma Medicine 3(4): 267-278 (2013)

Cold Physical Plasma Treatment Alters Redox Balance in Human Immune Cells

Sander Bekeschus,[a,b*] Thomas von Woedtke,[a] Axel Kramer,[c] Klaus-Dieter Weltmann,[a] & Kai Masur[a]

[a]ZIK *plasmatis* @ Leibniz-Institute for Plasma Science and Technology (INP Greifswald), Felix-Hausdorff-Str. 2, 17489 Greifswald, Germany; [b]Institute of Immunology and Transfusion Medicine, Department of Immunology, University of Greifswald, Ferdinand-Sauerbruch-Str. 1, 17489 Greifswald, Germany; [c]Institute of Hygiene and Environmental Medicine, University Medicine, Greifswald, Walther-Rathenau-Str. 49 A, 17489 Greifswald, Germany

[*]**Address all correspondence to:** sander.bekeschus@inp-greifswald.de, Tel. 0049 3834 554 3948

ABSTRACT: Cold atmospheric pressure plasma is a promising tool for various biomedical applications. Particularly, treatment of cells and tissues in diseases such as chronic wounds possesses high potential. However, detailed knowledge of how plasma mediates its actions on cells is necessary to explore its potentially beneficial effects in clinical settings. Previous studies have shown that plasma induces oxidative stress. We confirmed this hypothesis by showing that plasma significantly oxidized glutathione (GSH), a major cellular reductant. In plasma-treated cells we found elevated levels of GSH, pointing to a change in cellular redox balance. Oxidative stress can induce apoptosis and plasma-mediated apoptosis has been shown before measuring phosphatidylserine exposure. Using primary human immune cells, we investigated what events precede this reaction. Apoptosis is an active cellular process and accordingly it was dependent on incubation temperature after treatment. Damage of mitochondria was linked to apoptosis previously and plasma treatment resulted in mitochondrial oxidation and reduced mitochondrial membrane potential. Further, we measured a treatment time dependent activation of executioner caspase 3 which is known to be crucially involved in apoptosis. Together, our results suggested that plasma-mediated oxidative stress reactions in eukaryotic cells are in line with the foregoing research in redox biology. Establishing this link will help anticipate results in future research and clinical studies involving cold atmospheric pressure plasmas.

KEY WORDS: apoptosis; cold atmospheric pressure plasma; oxidative stress; plasma medicine; redox balance

I. INTRODUCTION

In plasma medicine, medical applications and their physiological and biochemical principals are investigated.[1] An important indication of cold atmospheric pressure plasma is the treatment of nonhealing chronic wounds.[2–4] To understand how plasma mediates its effects on cells investigations of mechanistic interactions are crucial. It is known that plasma generates reactive oxygen and nitrogen species (ROS/RNS) while their kind and quantity is dependent on the plasma source used and settings applied, e.g., composition of the feed gas.[5–8] Generated species then oxidize biological molecules and cells and prolonged plasma treatment initiates apoptosis.[9,10] The treatment time needed

to initiate programmed cell death depends on the cell type and the cellular condition, e.g., activation.[11–13] The present study investigated events preceding cold plasma-mediated apoptosis. We studied human immune cells, as they are one important element in wound healing.[14] Recently, we have shown that plasma oxidized mononuclear cells and apoptotic events occurred after prolonged treatment.[10,15] Cells, however, have defense systems against oxidation.[16] In particular, thiols protect against oxidative stress by detoxifying ROS/RNS such as free radicals and peroxides[17] which are normal by-products of cellular metabolism.[18] The largest pool of free thiols constitutes glutathione (GSH), an essential antioxidant present in millimolar concentrations inside cells.[19,20] We therefore investigated the degree of oxidation and relative quantity of extracellular and intracellular GSH, respectively. If strong oxidation tips the redox balance, apoptosis may occur and mitochondria are tightly entangled in this process.[21] Hence, we investigated mitochondrial oxidation and membrane potential after plasma treatment. Apoptosis is an active process and accordingly we investigated its dependency on incubation temperature. Finally, caspases play an essential role in cell death[22] and we showed that caspases 3/7 were active in plasma-treated cells.

II. MATERIAL AND METHODS

A. Cell Isolation

Upon donation at the blood bank (University Medicine of Greifswald, Germany) healthy blood donors gave written and informed consent about anonymous usage of blood for research purposes in the case that the donation is not suitable for clinical application. Isolation of human peripheral blood mononuclear cells (PBMCs) was done by density gradient centrifugation with lymphocyte separation medium (PanBioTech, Germany) as described before.[23] Subsequently, cells were suspended at 1×10^6 /ml in medium (RPMI1640 without phenol red) containing 10% fetal bovine serum and 1% glutamine/penicillin/streptomycin (all from Sigma, Germany). One milliliter of suspension was seeded per well in 24-well plates (Sarstedt, Germany) prior to plasma treatment. Before and after experiments, cells were incubated at 37°C with 5% CO_2 in a cell culture incubator (Binder, Germany) if not stated otherwise.

B. Plasma Source and Treatment

Before usage of the atmospheric pressure argon plasma jet “kinpen 09” (neoplas, Germany) the jet was left running with gas for at least 1 h to deplete the tubing of residual humidity as suggested before.[5] Using a mass flow controller (MKS Instruments, Germany) and applying three standard liters per minute of argon gas, plasma was generated applying a voltage of 2–6 kV_{pp} at a frequency of 1.1 MHz. For treatment of cell suspen-

sion, a programmable computer-driven *xyz* table (Nanotec, Germany) hovered the jet above the center of each well as described in detail previously.[10] Controls using argon gas only were carried out and always resembled untreated control (data not shown).

C. Assessment of GSH

GSH (Sigma, Germany) was added to cell culture medium (final concentration 50 µM) and 1 ml was added to wells of a 24-well plate. After treatment, 900 µl were added to cuvettes containing 100 µl PBS spiked with 200 µM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma, Germany). After incubation for 5 min, absorbance was analyzed spectrophotometrically (Specord Plus 210, Analytik Jena, Germany) at 412 nm.

D. Plasma Effects on Intracellular GSH and Mitochondria

For assessment of intracellular GSH, PBMC were plasma-treated and after 2, 6, and 24 h collected into 12×75 mm tubes (Sarstedt, Germany). After washing in PBS with divalent cations, cells were stained with anti-CD4 allophycocyanin (APC), 7-aminoactinomycin (7-AAD, both from BioLegend, USA), and 10 µM of ThiolTracker violet (Life Technologies, USA) for 30 min in the incubator. After washing, cellular fluorescence was acquired using flow cytometry (Gallios, Beckman-Coulter, USA) and 405-nm laser excitation collecting ThiolTracker fluorescence with a 525-nm band-pass (40-nm) filter. For assessment of mitochondrial oxidation PBMCs were incubated with 10 µM MitoTracker orange CM-H_2TMRos dye (Life Technologies, USA), washed, and resuspended in medium. Cells were then plasma-treated, harvested, washed, and fluorescence was acquired by flow cytometry. The viability dye 4′,6-diamidino-2-phenylindole (DAPI, Sigma, Germany) was used to discriminate live from dead cells. Mitochondrial membrane potential was assessed using the probe JC-1 (Enzo, Germany). Briefly, PBMC were plasma-treated and after 2, 6, and 24 h collected into FACS tubes. After washing, cells were stained with anti-CD4 APC, DAPI, and 2 µM JC-1 for 15 min at room temperature in the dark. After washing, cellular properties were acquired by flow cytometry.

E. Assessment of Apoptosis by Flow Cytometry

For kinetics of phosphatidylserine exposure, PBMCs were plasma-treated and stained at different time points with Annexin V and anti-CD4 APC in Annexin V binding buffer (all BioLegend, USA). For incubation temperature experiments, cells were incubated for 24 h at 4°C (fridge), 37°C (incubator), or room temperature, after treatment. Cells were acquired by flow cytometry in binding buffer. Procedure was similar for caspase 3/7 staining where CellEvent probe (Life Technologies, USA) and Annexin V PE-Cy7 (BioLegend, USA) were used instead of Annexin V FITC and DAPI.

F. Software and Statistics

For analysis of flow cytometry standard 3.0 files, Kaluza (Beckman-Coulter, USA) and FlowJo (Treestar Software, USA) were used. Calculations were done using Excel (Microsoft, USA). Statistical analysis was carried out using prism 6.04 (GraphPad software, USA). In GSH kinetic experiments, repeated-measures two-way analysis of variances (ANOVA) was applied comparing means for each time point separately and applying Sidak post-testing. Similar analysis was applied for comparison of percentages of caspase-positive cells except that each plasma treatment time was compared to its according time point control. For comparison of intracellular thiol content, a ratio paired *t*-test was used comparing mean fluorescence intensities of control and plasma-treated samples for each time point separately. This was also done for comparison of MitoTracker orange mean fluorescence intensities. Significance levels were indicated as follows: *α=0.05, **α=0.01, and ***α=0.001.

III. RESULTS AND DISCUSSION

Human PBMCs were treated with cold plasma to investigate its influence on oxidation and programmed cell death. PBMCs contain dozens of different cell types or subsets which also partially display different sensitivity towards oxidative stress.[24,25] Hence, by including only $CD4^+$ T helper cells in flow cytometric PBMC analysis we focused on a single cell type in all experiments. This lymphocyte subset was particularly suitable for the following reasons: (i) its high sensitivity allows for subtle discriminations of plasma effects[11,15]; (ii) unlike monocytes/macrophages, lymphocytes are not potent producers of ROS/RNS themselves[26]; (iii) their frequency in chronic wounds is elevated[27]; and (iv) lymphocyte responses to oxidative stress have been investigated by others in redox biology, possibly allowing a transfer of mechanisms to plasma-mediated redox changes in cells.

A. Oxidation by Cold Plasma Affected Extra- and Intracellular GSH

GSH constitutes and regenerates the majority of free thiols in cells, scavenging reactive species to keep redox homeostasis.[28,29] Plasma treatment oxidized GSH in medium quickly and significantly (Fig. 1). Thirty minutes after treatment about 50% (25 μM) GSH was oxidized. GSH-oxidation was continuous, suggesting a stable oxidant being present in plasma-treated medium. We recently showed that plasma deposits hydrogen peroxide in medium which may account for this effect.[15] Next, intracellular GSH concentrations were investigated. The probe employed measures reduced thiols and thus gives an estimate of intracellular GSH levels.[30] Increased dye fluorescence in living (7-AAD-negative) cells was found (Fig. 2A), differing significantly to controls 2 and 6 h after plasma treatment (Fig. 2B). Twenty-four hours after treatment, dead cells were

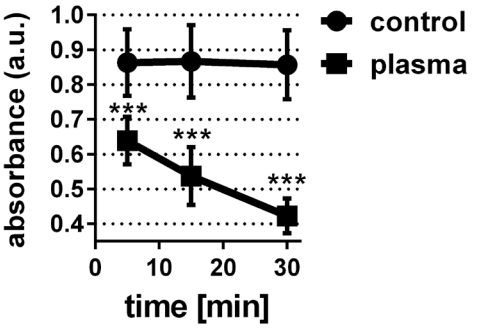

FIG. 1: Plasma treatment oxidized glutathione. Medium was spiked with 50 μM GSH and plasma-treated for 1 min. DTNB was added and absorption read over 30 min. GSH oxidation significantly decreased with incubation time as determined by two-way ANOVA. Data are presented mean±standard deviation of three independent experiments

more abundant in treated (Fig. 2D) compared to control samples (Fig. 2C) and contained less GSH. Although upregulation of GSH in treated cells compared to controls was somewhat unexpected, it was in line with previous findings attributing increased levels of GSH an important role in apoptosis.[31] Likewise, dead cells (7-AAD-positive) were likely to have stopped metabolizing, possibly explaining decreased GSH levels in them. GSH is very important for T cell functions including nonoxidative mediated cell death, cytokine production, and regulation by regulatory T cells.[32–35] In vivo studies revealed no direct correlation of oxidative stress and intracellular GSH levels, underlining our findings.[36] However, the authors found a stronger modulation of GSH in mitochondria.

B. Plasma Induced Mitochondrial Oxidation and Membrane Depolarization

Mitochondria are the power plants of the cell.[37] Using a MitoTracker probe, we found that plasma treatment increased oxidation in mitochondria (Fig. 3A) significantly (Fig. 3B). The probe accumulates rapidly in mitochondria and is sensitive to intracellular redox changes[38,39] confirming our findings. Using the lipophilic and cationic probe JC-1, we also assessed mitochondrial membrane potential and found a decrease after plasma treatment (Fig. 3C). In its monomeric form, it exhibits green fluorescence while upon aggregation in polarized mitochondria its fluorescence shifts to red.[40] The probe reenters the cytosol after mitochondrial membrane depolarization, and green fluorescence therefore indicates loss of membrane potential.[41] Two or six hours after plasma treatment, only a few cells were single positive for green fluorescing JC-1 (Fig. 3C, Q4) while abundant cells stained double positive 6 h after exposure. This suggested the presence of

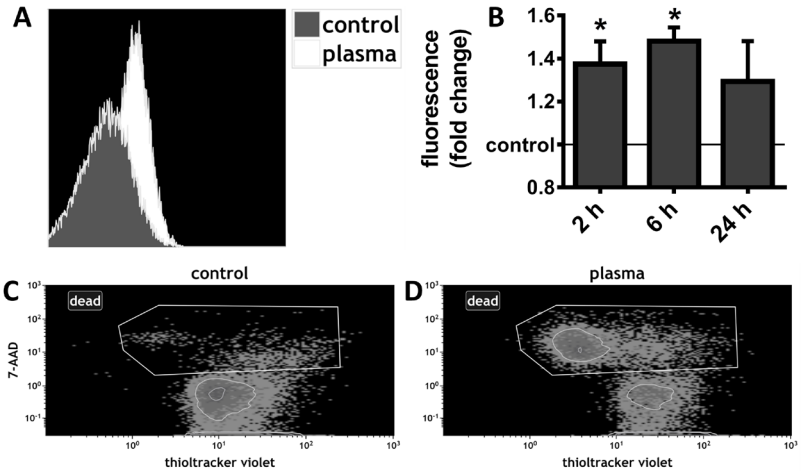

FIG. 2: Intracellular glutathione increased in living cells after plasma treatment. PBMC were treated with plasma (20 s) and collected either immediately, after 4 h, or after 24 h. After staining, fluorescence of ThiolTracker dye was measured via flow cytometry and mean intensity in living T lymphocytes calculated. Shown is a representative fluorescence overlay 4 h after treatment (A). There was significantly more GSH present in plasma-treated lymphocytes compared to controls (B). Twenty-four hours after treatment, dead cells contained less free thiols compared to living cells (C, D). Data are presented mean±standard error (B) or representative (C) of four independent experiments

intact mitochondria in otherwise apoptotic cells as membrane depolarization implied.[41] ATP production of mitochondria is required during the first 120 min in apoptosis initiation,[42] explaining low membrane depolarization after 2 h in our experiments.

C. Plasma-Induced Apoptosis was an Active Process and Involved Caspases

Apoptosis is a process allowing controlled disassembly of cells for removal and is central in, e.g., organ development, immune system specificity, or inflammation control.[43] It is known that plasma initiates apoptosis as we have shown before in lymphocytes.[10] Although considered an early marker of programmed cell death in eukaryotic cells,[44] we here show that phosphatidylserine was not exposed until 810 h after treatment (Fig. 4A). Bearing in mind the loss of mitochondrial membrane potential (Fig. 3C), key apoptotic events took place much earlier, however. Not only in apoptosis many enzymes exhibit temperature-dependent activity.[45] Accordingly, the incubation temperature significantly influenced cell survival after plasma treatment (Fig. 4B), suggesting a decreased enzyme activity necessary for apoptosis initiation. The enzyme caspase is central in cell death and was shown to be redox modulated.[46] The subtypes caspase 3/7 are of special

importance[47] and activated caspases were significantly increased between 6 and 12 h (Fig. 5B). In contrast to previous studies, there was a nonsignificant increase already after 6 h in T lymphocytes.[48] Caspase activation after plasma treatment was detected before[49,50] but not in primary immune cells and not linked to changes in mitochondrial membrane potential, GSH levels, and onset of phosphatidylserine exposure. In this context, it would be of interest to investigate the expression of Bax or cytochrome c release. Both precede mitochondrial depolarization and caspase activation and are linked likewise.[51,52] Further, these cellular processes require fully functional and metabolically active cells.[53] Thus, cold plasma-induced redox changes in cells may be able to mediate damage in different cellular compartments but are far from rendering eukaryotes fully inactive as, e.g., in necrosis.

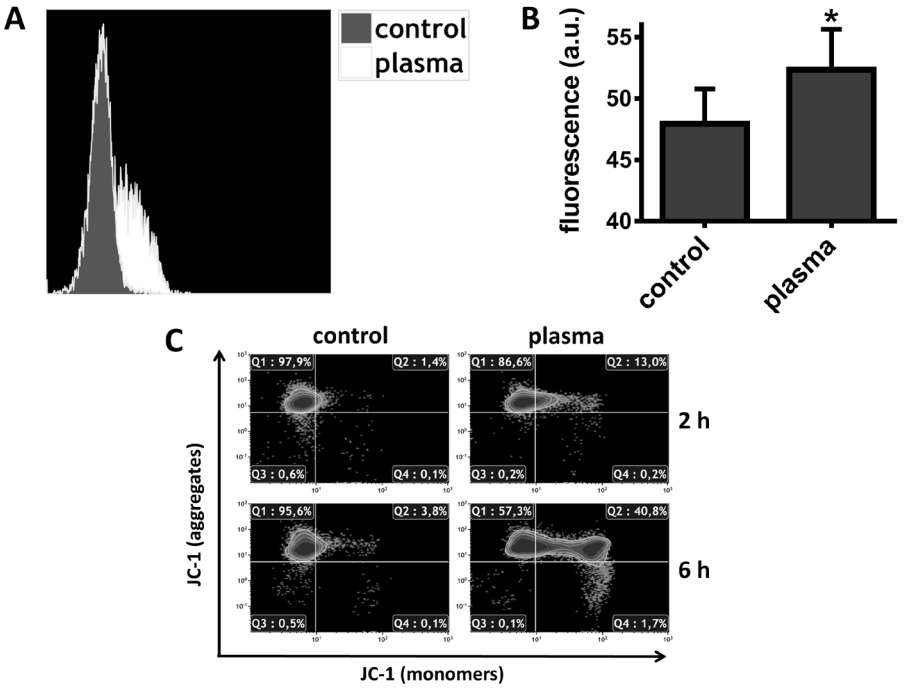

FIG. 3: Plasma-treatment changed mitochondrial oxidation and membrane potential. PBMC were stained with mitotracker probe, plasma-treated (20 s), and changes in fluorescence were measured by flow cytometry. Fluorescence overlay showed increased mitochondrial oxidation (A). Increase in mean fluorescence intensity (B) was significant (mean±standard deviation of three independent experiments, ratio paired *t*-test, $p<0.05$). For measurement of mitochondrial membrane potential, cells were plasma-treated and incubated for 2 or 6 h before staining with JC-1 and cell surface markers. Decrease of relative incidences of cells in dot plot quadrant Q1 indicated loss of mitochondrial potential (C) in plasma-treated T lymphocytes (shown is one representative of five independent experiments)

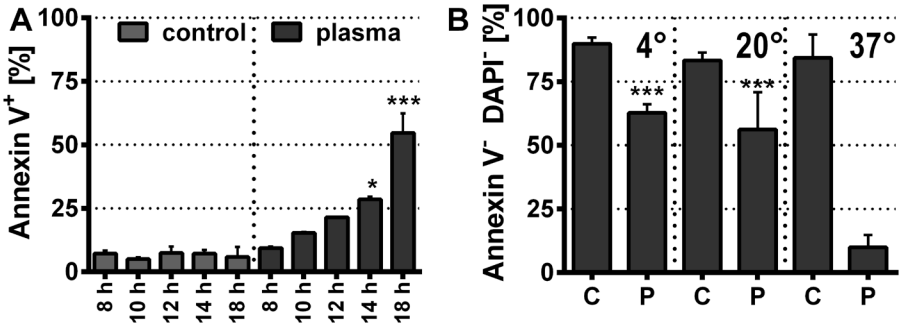

FIG. 4: Plasma-mediated apoptosis was an active process. PBMC were plasma-treated (60 s), incubated for various time points (37°C), and stained with Annexin V and surface markers (A). Percentage of apoptotic cells increased compared to control between 8 and 10 h following exposure to plasma. Second, cells were incubated for 24 h under different thermal conditions. After staining with Annexin V and DAPI, viable cells were determined by flow cytometry. Survival rates of cells incubated at 4°C or room temperature differed significantly from those incubated at 37°C. Survival rates in control cells did not differ between incubation temperatures. Data are presented mean±standard deviation (B) or representative (A) of three independent experiments. Significance was determined by two-way ANOVA

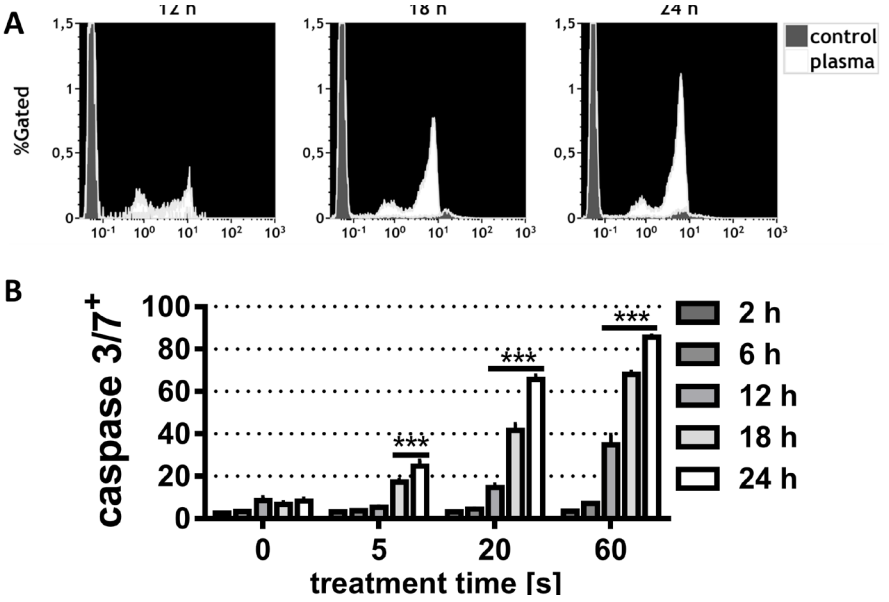

FIG. 5: Plasma cytotoxicity involved caspase activation. PBMC were plasma-treated, stained for caspase 3/7 activity after different time points and measured by flow cytometry. Caspase-positive cells were present 12 h after treatment and increased for longer incubation times (A). Caspase activation was treatment time dependent and increased significantly 12 h after treatment of 20 or 60 s, respectively (B) Data are presented mean±standard deviation of three independent experiments. Significance was determined by two-way ANOVA (p<0.001)

IV. CONCLUSION

It was possible to demonstrate how cold physical plasma changed the redox balance in lymphocytes, finally evoking apoptosis. While extracellular GSH was oxidized by plasma, cytosolic GSH levels increased indicating an active production, possibly to restore redox homeostasis. Mitochondria are key regulators of apoptosis and plasma quickly led to their oxidation and membrane depolarization, indicative of apoptosis initiation. Exposure of phosphatidylserine appeared between 8 and 10 h after oxidative challenge but was temperature dependent. Finally, caspase activation was linked to plasma-induced programmed cell death. All results were in line with findings in redox biology and suggested that plasma effects in cells were not mediated by alternative molecular pathways.

ACKNOWLEDGMENTS

The authors thank Professor Dr. Andreas Greinacher (Head of Department of Transfusion Medicine, University of Greifswald, Germany) for allocating buffy coats, and Anne Müller for technical assistance. This work was realized within the framework of the multidisciplinary research center ZIK *plasmatis* which is funded by the German Federal Ministry of Education and Research (Grant no. 03Z2DN11)

REFERENCES

1. von Woedtke T, Reuter S, Masur K, Weltmann K-D. Plasmas for medicine. Phys. Rep. 2013;530:291–320.
2. Kramer A, Lindequist U, Weltmann KD, Wilke C, von Woedtke T. Plasma medicine—its perspective for wound therapy. GMS Krankenhhyg Interdiszip. 2008;3:Doc16.
3. Kramer A, Lademann J, Bender C, Sckell A, Hartmann B, Münch S, Hinz P, Ekkernkamp A, Matthes R, Koban I, Partecke I, Heidecke CD, Masur K, Reuter S, Weltmann KD, Koch S, Assadian O. Suitability of tissue tolerable plasmas (TTP) for the management of chronic wounds. Clin Plasma Med. 2013;1:11–8.
4. Klebes M, Ulrich C, Kluschke F, Patzelt A, Vandersee S, Richter H, Bob A, von Hutten J, Krediet JT, Kramer A. Combined antibacterial effects of tissue-tolerable plasma and a modern conventional liquid antiseptic on chronic wound treatment. J Biophotonics. 2014. doi: 10.1002/jbio.201400007.
5. Winter J, Wende K, Masur K, Iseni S, Dünnbier M, Hammer MU, Tresp H, Weltmann K-D, Reuter S. Feed gas humidity: a vital parameter affecting a cold atmospheric-pressure plasma jet and plasma-treated human skin cells. J Phys D Appl Phys. 2013;46:295401.
6. Pipa A, Reuter S, Foest R, Weltmann K. Controlling the NO production of an atmospheric pressure plasma jet. J Phys D Appl Phys. 2012;45:085201.
7. Reuter S, Tresp H, Wende K, Hammer MU, Winter J, Masur K, Schmidt-Bleker A, Weltmann K-D. From RONS to ROS: Tailoring plasma jet treatment of skin cells. IEEE Trans Plasma Sci. 2012;40:2986–93.
8. Graves DB. The emerging role of reactive oxygen and nitrogen species in redox biology and some implications for plasma applications to medicine and biology. J Phys D Appl Phys. 2012;45:263001–42.
9. Partecke L, Evert K, Haugk J, Doering F, Normann L, Diedrich S, Weiss F-U, Evert M, Huebner N, Guenther C, Heidecke C, Kramer A, Bussiahn R, Weltmann K-D, Pati O, Bender C, von Bernstorff W. Tissue tolerable plasma (TTP) induces apoptosis in pancreatic cancer cells in vitro and in vivo. BMC

Cancer. 2012;12:473–82.
10. Bekeschus S, Masur K, Kolata J, Wende K, Schmidt A, Bundscherer L, Barton A, Kramer A, Bröker B, Weltmann K-D. Human mononuclear cell survival and proliferation is modulated by cold atmospheric plasma jet. Plasma Process Polym. 2013;10:706–13.
11. Bekeschus S, Kolata J, Müller A, Kramer A, Weltmann K-D, Bröker B, Masur K. Differential viability of eight human blood mononuclear cell subpopulations after plasma treatment. Plasma Med. 2014 (in press).
12. Bundscherer L, Bekeschus S, Tresp H, Hasse S, Reuter S, Weltmann K-D, Lindequist U, Masur K. Viability of human blood leucocytes compared with their respective cell lines after plasma treatment. Plasma Med. 2014 (in press).
13. Bundscherer L, Wende K, Ottmüller K, Barton A, Schmidt A, Bekeschus S, Hasse S, Weltmann K-D, Masur K, Lindequist U. Impact of non-thermal plasma treatment on MAPK signaling pathways of human immune cell lines. Immunobiology. 2013;218:1248–55.
14. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature. 2008;453:314–21.
15. Bekeschus S, Kolata J, Winterbourn C, Kramer A, Turner R, Weltmann K-D, Bröker B, Masur K. Hydrogen peroxide: a central player in physical plasma-induced oxidative stress in human blood cells. Free Radical Res. 2014;48:542–9.
16. Sies H. Strategies of antioxidant defense. Eur J Biochem.1993;215:213–9.
17. Winterbourn CC, Hampton MB. Thiol chemistry and specificity in redox signaling. Free Radical Biol Med. 2008;45:549–61.
18. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol. 2004;55:373–99.
19. Kidd PM. Glutathione: systemic protectant against oxidative and free radical damage. Altern Med Rev. 1997;1:155–76.
20. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Biol Med. 2001;30:1191–212.
21. Fleury C, Mignotte B, Vayssière J-L. Mitochondrial reactive oxygen species in cell death signaling. Biochimie. 2002;84:131–41.
22. Hampton MB, Fadeel B, Orrenius S. Redox regulation of the caspases during apoptosis. Ann N Y Acad Sci. 1998;854:328–35.
23. Boyum A. Separation of leucocytes from blood and bone marrow. Scand J Clin Lab Invest. 1968;21 Suppl 97:77–89.
24. Haertel B, Volkmanna F, Woedtke Tv, Lindequist U. Differential sensitivity of lymphocyte subpopulations to non-thermal atmospheric-pressure plasma. Immunobiology. 2012;217:628–33.
25. Harlin H, Hanson M, Johansson CC, Sakurai D, Poschke I, Norell H, Malmberg K-J, Kiessling R. The CD16–CD56bright NK cell subset is resistant to reactive oxygen species produced by activated granulocytes and has higher antioxidative capacity than the CD16+CD56dim subset. J Immunol. 2007;179:4513–9.
26. Schütt C, Schilling T, Krüger C. sCD14 prevents endotoxin inducible oxidative burst response of human monocytes. Allergie Immunologie. 1991;37:159–64.
27. Loots MAM, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. Soc Invest Dermatol. 1998;111:850–7.
28. Eaton DL, Bammler TK. Concise review of the glutathione S-transferases and their significance to toxicology. Toxicol Sci. 1999;49:156–64.
29. Hampton MB, Orrenius S. Redox regulation of apoptotic cell death in the immune system. Toxicol Lett. 1998;102:355–8.
30. Mandavilli BS, Janes MS. Detection of intracellular glutathione using ThiolTracker violet stain and fluorescence microscopy. Curr Protoc Cytom. 2001; doi: 10.1002/0471142956.cy0935s53.

31. Franco R, Cidlowski J. Apoptosis and glutathione: beyond an antioxidant. Cell Death Differ. 2009;16:1303–14.
32. Chiba T, Takahashi S, Sato N, Ishii S, Kikuchi K. Fas-mediated apoptosis is modulated by intracellular glutathione in human T cells. Euro J Immunol. 1996;26:1164–9.
33. Liang C, Lee N, Cattell D, Liang S-M. Glutathione regulates interleukin-2 activity on cytotoxic T-cells. J Biol Chem. 1989;264:13519–23.
34. Liang S, Lee N, Finbloom D, Liang C. Regulation by glutathione of interleukin-4 activity on cytotoxic T cells. Immunology. 1992;75:435.
35. Yan Z, Garg SK, Banerjee R. Regulatory T cells interfere with glutathione metabolism in dendritic cells and T cells. J Biol Chem. 2010;285:41525–32.
36. Albrecht SC, Barata AG, Großhans J, Teleman AA, Dick TP. In vivo mapping of hydrogen peroxide and oxidized glutathione reveals chemical and regional specificity of redox homeostasis. Cell Metab. 2011;14:819–29.
37. Sheftel AD, Lill R. The power plant of the cell is also a smithy: the emerging role of mitochondria in cellular iron homeostasis. Annals Med. 2009;41:82–99.
38. Poot M, Pierce RH. Detection of changes in mitochondrial function during apoptosis by simultaneous staining with multiple fluorescent dyes and correlated multiparameter flow cytometry. Cytometry. 1999;35:311–7.
39. Karbowski M, Kurono C, Wozniak M, Ostrowski M, Teranishi M, Soji T, Wakabayashi T. Cycloheximide and 4-OH-TEMPO suppress chloramphenicol-induced apoptosis in RL-34 cells via the suppression of the formation of megamitochondria. Biochim Biophys Acta. 1999;1449:25–40.
40. Cossarizza A, Baccaranicontri M, Kalashnikova G, Franceschi C. A new method for the cytofluorometric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimid azolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun. 1993;197:40–5.
41. Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A. JC-1, but not DiOC6 (3) or rhodamine 123, is a reliable fluorescent probe to assess ΔΨ changes in intact cells: implications for studies on mitochondrial functionality during apoptosis. FEBS Lett. 1997;411:77–82.
42. Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med. 1997;185:1481–6.
43. Edinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol. 2004;16:663–9.
44. Rimon G, Bazenet CE, Philpott KL, Rubin LL. Increased surface phosphatidylserine is an early marker of neuronal apoptosis. J Neurosci Res. 1997;48:563–70.
45. Somero GN. Temperature adaptation of enzymes: biological optimization through structure-function compromises. Annu Rev Ecol System. 1978:1–29.
46. Hampton MB, Orrenius S. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett. 1997;414:552–6.
47. Porter AG, Jänicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 1999;6:99–104.
48. Takahashi A, Hanson MGV, Norell HR, Havelka AM, Kono K, Malmberg K-J, Kiessling RVR. Preferential cell death of CD8+ effector memory (CCR7- CD45RA-) T cells by hydrogen peroxide-induced oxidative stress. J Immunol. 2005;174:6080–7.
49. Ahn HJ, Kim KI, Hoan NN, Kim CH, Moon E, Choi KS, Yang SS, Lee J-S. Targeting cancer cells with reactive oxygen and nitrogen species generated by atmospheric-pressure air plasma. PLoS One. 2014;9:e86173.
50. Ishaq M, Kumar S, Varinli H, Han Z, Rider AE, Evans MD, Murphy AB, Ostrikov KK. Atmospheric gas plasma-induced ROS production activates TNF-ASK1 pathway for the induction of melanoma cancer cell apoptosis. Mol Biol Cell. 2014:mbc. E13-0-0590.
51. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. Bax-induced caspase activation and apoptosis via cytochromec release from mitochondria is inhibitable by Bcl-xL. J Biol Chem.

1999;274:2225–33.
52. Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998;281:1309–11.
53. Chiarugi A. “Simple but not simpler”: toward a unified picture of energy requirements in cell death. FASEB J. 2005;19:1783–8.