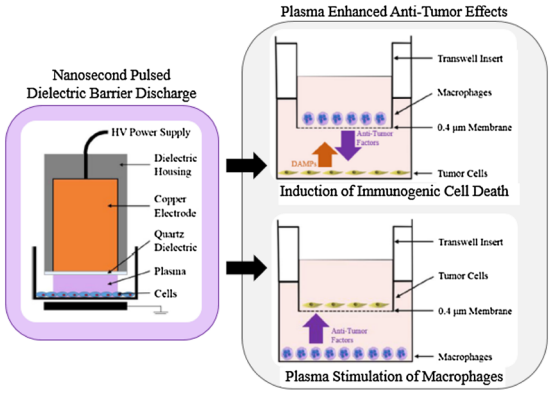
Uniform Nanosecond Pulsed Dielectric Barrier Discharge Plasma Enhances Anti-Tumor Effects by Induction of Immunogenic Cell Death in Tumors and Stimulation of Macrophages

Abraham Lin, Billy Truong, Arthur Pappas, Lawrence Kirifides, Ahmed Oubarri, Shuyang Chen, Shaojun Lin, Danil Dobrynin, Gregory Fridman, Alexander Fridman, Nianli Sang, Vandana Miller*

Several have shown plasma to be efficacious against cancer cells in vitro and in vivo with minimal damage to non-cancerous cells and tissue. Most are focused on direct influence of plasma on tumor cells. However, the body's immune system also plays a crucial role in the control of cancer. A new concept of immunogenic cell death (ICD) has been emerging, where through this modality of cell death, an immune response may be stimulated. The goal of the presented study was to explore a regime of plasma treatment that induces ICD in tumor cells and stimulates anti-tumor effects in macrophages- a key immune cell type involved in the initiation of immunological responses. Here, we show that treatment with uniform nanosecond pulsed dielectric barrier discharge (nspDBD) plasma directly activated anti-tumor effects in macrophages. We further show macrophage activation following the expression of plasma-induced damage-associated molecular patterns (DAMPs) in tumor cells, characteristic of ICD.



A. Lin, B. Truong, A. Pappas, L. Kirifides, A. Oubarri, Dr. D. Dobrynin, Dr. G. Fridman, Prof. A. Fridman, Dr. V. Miller
A. J. Drexel Plasma Institute, Drexel University, 200 Federal Street Suite 500, New Jersey, Camden 08103, USA
E-mail: vmiller@coe.drexel.edu
Dr. S. Chen, Dr. N. Sang
Department of Biology, Drexel University, 3245 Chestnut Street, Pennsylvania, Philadelphia 19104, USA
Dr. S. Chen, Dr. N. Sang
Sidney Kimmel Cancer Center, Philadelphia, USA
Dr. S. Lin
Department of Radiation Oncology, Fujian Provincial Cancer Hospital, Fuzhou, China

1. Introduction

Use of non-thermal plasmas is currently being explored for cancer therapy.[1–6] Local application of plasma on tumors in vivo has led to reduced tumor volume and increased animal survival, but complete remission of cancer through the use of plasma has not yet been attained.[2–4] Tumor cells harbor an arsenal of survival strategies that makes complete elimination of cancer difficult.[7] Included among these is resistance to cell death and the ability to evade immunological surveillance.[7,8] Additionally, cancers are associated with immune suppression.[9] Therefore, an attractive form

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

DOI: 10.1002/ppap.201500139

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

of therapy is one that increases cancer cell susceptibility to death, enhances recognition of poorly immunogenic cancer cells by the immune system, and subverts immunosuppression.[8–10]

In recent years, a new concept of immunogenic cell death (ICD) has emerged.[11] This modality of cell death stimulates specific immune responses against antigens from stressed and dying cells.[12,13] Although mechanisms that promote ICD are still unclear, a number of factors known to elicit immune responses have been identified.[14–17] These factors are known as damage-associated molecular patterns (DAMPs). DAMPs, such as adenosine triphosphate (ATP), are actively secreted by dying cells; alternatively, previously intracellular molecules like calreticulin (CRT) become exposed on the cell surface.[15] Secreted ATP acts as a prominent "find me" signal for activating receptors on immune cells and may lead to proinflammatory cytokine release.[18,19] CRT is a protein on the endoplasmic reticulum (ER), a eukaryotic organelle that is responsible for the synthesis, folding, and modification of proteins used by the cell.[15,20] During ICD, the ER responds by upregulating stress genes, such as activating transcription factors (ATF4, ATF6) and stanniocalcin (STC2), causing CRT to be translocated from the ER to the cell surface.[20–23] This ecto-CRT functions as an "eat me" signal and mediates the engulfment of tumor cells by macrophages and dendritic cells, cells of the innate immune system.[11] These cells migrate to immune organs where tumor antigen presentation to cells of the specific immune system occurs resulting in the generation of a systemic, specific anti-tumor immune response.[13,15] Therefore, in contrast to the current use of plasma for local treatment of tumors, plasma-induced ICD could be a viable approach for systemic immunotherapy of cancer and requires investigation.

We have previously shown that plasma can stimulate macrophage function in an in vitro migration assay.[24] Macrophages are key immune cells originating from the bone marrow progenitor cells. These cells are released into the bloodstream where they develop into monocytes. After circulating in the bloodstream, monocytes take residence in different tissue and differentiate into tissue-specific macrophages, displaying different phenotypes depending on the local environment.[25,26] Classically activated macrophages, M1 macrophages, are considered pro-inflammatory.[27] They secrete factors and cytotoxic substances, such as tumor necrosis factor α (TNFα) and reactive oxygen and nitrogen species capable of killing cancerous cells.[28–30] We hypothesize that besides migration, plasma can also directly activate macrophages to exert anti-tumor effects.

In the present study, we explored the potential for plasma to induce ICD in tumor cells and evaluated whether the DAMP signals associated with ICD enhance anti-tumor effects of macrophages. We also investigated whether plasma alone can stimulate macrophages to enhance killing of tumor cells. We utilized a uniform nanosecond pulsed dielectric barrier discharge (nspDBD) and exposed the CNE-1 nasophylangeal carcinoma cell line and the THP-1 macrophage cell line to a range of plasma doses in 24-well tissue culture plates. To investigate anti-tumor effects of macrophages stimulated by either plasma or DAMP signals from tumor cells, a transwell insert system was used to co-culture macrophages and tumor cells together. Our results indicate that anti-cancer activity of macrophages is enhanced by plasma. Treatment of cancer cells in the same regime results in a dose-dependent increase in ATP secretion and ER stress. Furthermore, these DAMP signals enhanced CNE-1 cell killing following co-culture with non-plasma-treated macrophages. This is the first report of non-thermal plasma-enhanced anti-tumor effects through stimulation of macrophages both directly by plasma and indirectly via DAMP signaling from plasma induced ICD of tumor cells.

2. Materials and Methods

2.1. Cell Culture

The radioresistant nasopharyngeal carcinoma (NPC) cell line, CNE-1, was originally derived from NPC patients in China. We obtained the CNE-1 cell line from Dr. Shaojun Lin's lab, Department of Radiation Oncology, Fujian Provincial Cancer Hospital, Fuzhou, Fujian, China. The CNE-1 cell line was cultured in RPMI-160 (RPMI) (Corning Life Sciences, USA) with 10% fetal bovine serum (FBS) (Corning Life Sciences, USA) and 1% penicillin/streptomycin (PS) (Life Technologies, USA). A human acute monocytic leukemia cell line (THP-1), was obtained as a kind gift from Dr. Kara Spiller (Drexel University, Philadelphia, PA, USA). These cells were cultured in RPMI supplemented with 10% heat-inactivated FBS and 1% PS. All cells were grown at 37 °C with 5% CO_2 in a humidified atmosphere.

2.2. Differentiation of THP-1 Monocytes to M0 Macrophages

THP-1 cells were differentiated into M0 macrophages one day prior to co-culture or plasma treatment. For co-culture with tumor cells, THP-1 cells were seeded in 6.5 mm transwell inserts with 0.4 μm pore size polyethylene terephthalate membrane (Corning Life Sciences, USA) at a concentration of 1.55×10^5 cells/insert. Cells were differentiated into M0 macrophages by the addition of 0.5 μL of 100 μL/mL phorbol 12-myristate 13-acetate (PMA) (Sigma–Aldrich, USA) to each well. The inserts were placed into a 24-well plate (Corning Life Sciences, USA) with 600 μL of THP-1

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Table 1. nspDBD treatment parameters.

Parameter	Value
Excitation	Nanosecond pulsed
Voltage	29 kV
Energy per pulse	0.9 mJ/pulse
Rise time	2 ns
Pulse width	20 ns
Treatment time	10 s
Application distance	1 mm
Set Frequency	5, 15, 30, and 75 Hz
Calculated Total dose	47, 141, 282, and 705 mJ

culture media, to prevent cells in the transwell inserts from drying. Cells were incubated overnight at 37 °C with 5% CO_2 in a humidified atmosphere and phosphate buffered saline (PBS) was used to wash the differentiated cells the next day. PBS was removed immediately before co-culture with CNE-1 cells. For plasma treatment, THP-1 cells were seeded into 24-well plates at a concentration of 7.0×10^5 cells/well. These cells were also differentiated into M0 macrophages with 0.5 μL of PMA (100 μL/mL). PBS was used to wash the differentiated cells following overnight incubation and removed immediately before plasma treatment.

2.3. Nanosecond Pulsed Dielectric Barrier Discharge Plasma Parameters

nspDBD plasma was produced by applying a positive voltage pulse to a high-voltage electrode placed 1 mm above the cells in the wells.[31] Plasma pulse characteristics and methods of measuring energy per pulse of a single uniform nspDBD plasma discharge were defined in our previous work.[32] Briefly, the nanosecond pulser (FPB-20-05NM, FID GmbH, Germany) generated a 29 kV pulse with a 20 ns pulse width and with rise times of 2 ns. Treatment duration was fixed at 10 s and the frequency of the pulses was controlled with an external function generator (TTI, TG5011 LXT). The energy per pulse of a single uniform nspDBD plasma discharge in a 24-well plate was calculated by measuring voltage and current without displacement current and found to be 0.9±0.1 mJ/pulse. Table 1 shows the operating parameters for our nspDBD plasma treatments.

2.4. Non-Thermal Plasma Treatment and Co-Culture with Macrophages

For experimental assessment of CNE-1 cell viability and DAMP signals following plasma, cells were seeded at a concentration of 1.5×10^5 cells/well and grown to confluence in 24-well plates with 500 μL of complete cell culture media one day prior to treatment. Media were removed, cells were washed with PBS, and PBS was removed right before treatment. Immediately after treatment, 500 μL of fresh media were added to the wells and cells were incubated at 37 °C with 5% CO_2 for 1 and 24 h. Subsequent viability, extracellular ATP, and ER stress analysis were performed after incubation. Mock plasma treatment of cells, represented as 0 mJ, was performed in an identical manner but without the generation of plasma to serve as our negative control. A schematic drawing of our electrode and setup is shown in Figure 1.

To determine whether DAMP signals released by tumor cells undergoing ICD can enhance anti-tumor effects in macrophages, CNE-1 cells were seeded into 24-well plates, treated with plasma as stated above, and co-cultured with THP-1 M0 macrophages. Following plasma treatment of the tumor cell line, 750 μL of CNE-1 complete media was added to each well. The transwell inserts with the M0

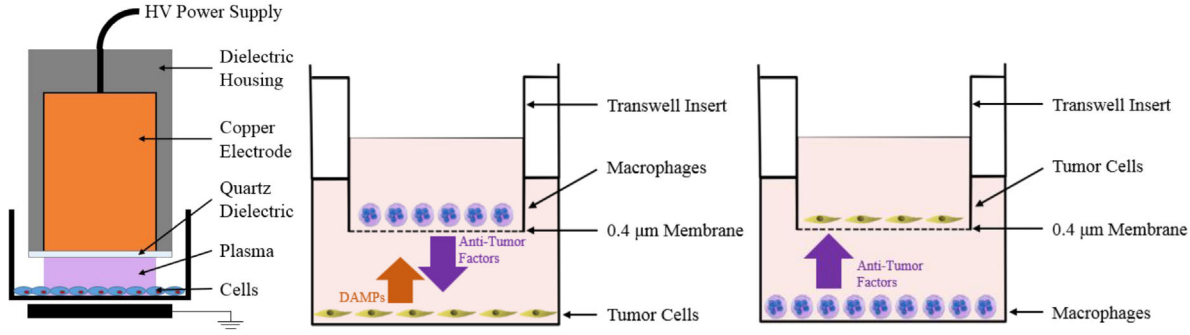


Figure 1. nspDBD treatment and co-culture system. (Left) Media were removed from each well and plasma was generated and applied directly onto the cells by a high-voltage electrode. Immediately following treatment, fresh media were added into the well. (Middle) Tumor cells and macrophages were co-cultured in a transwell insert system. The bottom of the transwell insert was porous (0.4 μm pores) which kept two cell types separate, but allowed for the exchange of various factors in the media. (Right) To determine if plasma can stimulate Mo macrophages cells to increase anti-tumor effects, Mo macrophages were treated with plasma and co-cultured with CNE-1 cells.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
DOI: 10.1002/ppap.201500139

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

macrophages were then placed into the wells, and 750 μL of THP-1 cell culture media was added to the inserts. This system allowed for the exchange of DAMP signals and cytokines secreted into the media through the porous membrane (Figure 1). Media in the wells and inserts were replaced at 24 h and cells were cultured for a total of 48 h before subsequent assessment of CNE-1 cell viability.

To evaluate whether plasma can stimulate THP-1 M0 macrophages cells to functionally increase anti-tumor effects, M0 macrophages were treated with plasma and co-cultured with CNE-1 cells (Figure 1). CNE-1 cells were seeded into the inserts at a concentration of 2.0×10^4 cells/insert one day prior to co-culture. THP-1 cells were seeded and differentiated as described above. Immediately before plasma treatment of M0 macrophages, PBS was removed from the wells. After treatment, 750 μL of THP-1 complete media were immediately added, and the transwell inserts with the CNE-1 cells were placed into the wells. Cells were cultured together for 48 h, and CNE-1 cell viability assessment was performed following 48 h incubation.

2.5. Cell Counts and Viability Measurement and Analysis

Cell viability was determined via fluorescence staining with 100 μg/mL of propidium iodide (PI) (Invitrogen, NY), a DNA-binding fluorescent reagent that permeates damaged cell membranes. Cells were stained at 1 and 24 h post plasma treatment for direct killing studies; cells were stained at 48 h post for co-culture experiments. Cell counts and viability data were collected with an image cytometer (Nexcelom Bioscience, USA) and further analysis, including size gating, was done on the FCS flow cytometry software (DeNovo Software, USA).

2.6. Extracellular ATP Measurement

An adenosine 5′-triphosphate (ATP) bioluminescent somatic cell assay kit (Sigma–Aldrich) was used to determine extracellular release of ATP from plasma-treated CNE-1 cells. ATP reacts with luciferin which is catalyzed by firefly luciferase found in the assay mix to form adenyl-luciferin. The adenyl-luciferin then reacts with oxygen to produce oxyluciferin, adenosine monophosphate, carbon dioxide, and light. The light is measured in the PhotonMaster luminometer (LuminUltra, PM10146). By first calibrating the luminometer with the provided UltraClear calibration solution, the measured relative light units (RLU) is converted into pgATP/mL. This assay was performed on the cell culture media of the plasma-treated CNE-1 cells 10 min after treatment.

All reagents were prepared following the protocol provided in the kit. The ATP assay mix working solution was prepared by diluting the ATP assay mix stock solution 1:25 fold with the dilution buffer. 100 μL of working solution was added into 12 × 55 mm test tubes (Hack, USA) and allowed to stand at room temperature for 3 min.

Following plasma treatment, 50 μL of supernatant was collected from each well and placed into labeled Eppendorf tubes with 100 μL of ultrapure water (Sigma–Aldrich). 50 μL of the cell solution in the Eppendorf tubes were then transferred into the 12 × 55 mm test tubes containing 100 μL of ATP assay mix working solution. RLU were immediately measured with the PhotonMaster to determine extracellular ATP concentration.

2.7. Western Blot Analysis for ER Stress

Proteins from plasma-treated CNE-1 cells were collected 24 h after treatment. Cells were washed three times with cold PBS and lysed with 100 μL urea buffer (8 M urea, 10 mM Tris, 10% glycerol, 1% SDS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mix, and pH 6.8). Cells were then scraped with a mini cell scraper and the suspension was collected in Eppendorf tubes. A 1 mL syringe was used to pipet the solution up and down 5–10 times to homogenize the cells. Tubes were then frozen at −80 °C until Western blot analysis was to be performed. Western blotting was performed as previously described.[33] Protein concentrations were determined by Protein Assay Kit I (Bio-Rad, USA). The same amount of protein was loaded into Bio-Rad Mini-protein TGX precast gels or self-made 8% SDS–PAGE gels. The proteins were then transferred into Bio-Rad PVDF membrane, blocked with 5% non-fat milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, and pH 7.5), and incubated with desired antibodies. Band signals were developed by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA).

2.8. Statistical Analysis

All experimental data points were from triplicate samples and plotted as the mean ± standard error of the mean (SEM). Data were analyzed by Student's *t*-test to determine significance between data points. A value of $p \leq 0.05$ was considered statistically significant and indicated with an asterisk (*) when comparing treated and untreated cells. A plus sign (+) was used to indicate a value of $p \leq 0.01$.

3. Results and Discussion

3.1. Effect of Uniform nspDBD Plasma on CNE-1 Cell Viability

The viability of CNE-1 cells following uniform nspDBD treatment was first established. Pulse frequencies for

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

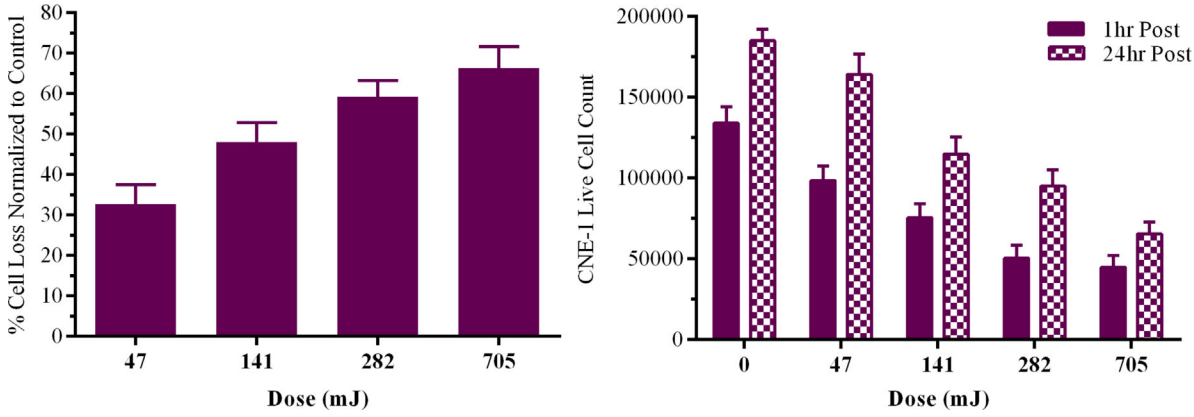


Figure 2. Effect of uniform nspDBD on viability of CNE-1 cells at 1 and 24 h. Viability was determined by trypsinizing the cells in each well, staining with propidium iodide and quantifying in the Cellometer. (Left) Cell viability 1 h post plasma treatment was normalized to mock-treated cells (control) and represented as percent cell loss. Cell loss increased dose-dependently from 32% following 47 mJ treatment to 66% for 705 mJ treatment. (Right) All plasma-treated cells continued to proliferate 24 h post treatment as determined by the increase in live cell counts. Data are represented as mean ± SEM.

plasma treatment were 5, 15, 30, and 75 Hz corresponding to 47, 141, 282, and 705 mJ dose treatments. Cells were collected 1 and 24 h after treatment, stained with PI, counted in the Cellometer, and analyzed for viability. Cell viability 1 h following treatment was normalized to the mock-treated group (control) and represented as cell loss, shown in (Figure 2). A dose-dependent increase in cell loss following treatment was observed ranging from 32% cell loss for the lowest dose treatment to 66% cell loss following the highest dose treatment. Live cell counts increased in all plasma-treated groups at 24 h (Figure 2) suggesting that surviving cells recovered and continued to proliferate.

3.2. nspDBD Plasma Stimulates Extracellular ATP Secretion

ATP is the most abundant intracellular molecule required for metabolism.[13] When cells experience physical or chemical stress, ATP can be released and acts as a messenger to stimulate diverse responses.[13,19] As a DAMP signal, ATP is an important chemoattractant for monocytes and dendritic cells and also promotes secretion of key pro-inflammatory cytokines, such as IL-1β and IL-18 from immune cells.[15,18] ATP has also been associated with increased macrophage maturation and increased cytotoxicity of NK cells, other cell types of the innate immune system.[18,19]

CNE-1 cells were treated with plasma and incubated in RPMI 10% FBS and 1% PS for 10 min to allow time for ATP to be secreted into the media. ATP analysis was performed on the supernatant as described in the methods section. Extracellular ATP concentration in the supernatant of our mock-treated cells, represented as 0 mJ dose treatment, was 9.0 nM. ATP concentration increased after plasma treatment from 45.4 nM for low-dose treatments to 789.7 nM for our highest treatment (Figure 3). These results suggest that at this uniform plasma regime, plasma can stimulate CNE-1 cells to secrete ATP available for immune stimulation.

3.3. nspDBD Plasma Induces ER Stress

In order to examine whether nspDBD plasma can trigger ER stress, we measured the protein levels of two cellular

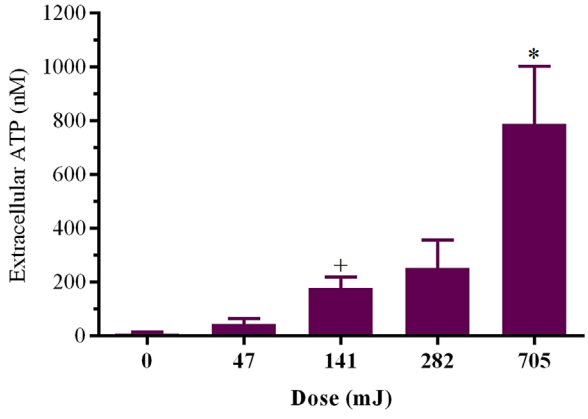


Figure 3. Dose-dependent increase in extracellular ATP concentration in the supernatant following plasma treatment. The media of CNE-1 cells were collected 10 min after plasma treatment, and an ATP bioluminescent kit was used to determine extracellular ATP concentration in the media. RLU measurements were taken in the PhotonMaster luminometer. While mock treated cells (0 mJ) secreted 9.0 nM of ATP into the media, ATP secretion increased following plasma treatment, from 45.4 to 789.7 nM for 47 and 705 mJ dose treatments respectively. Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{+}p < 0.01$.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
DOI: 10.1002/ppap.201500139

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

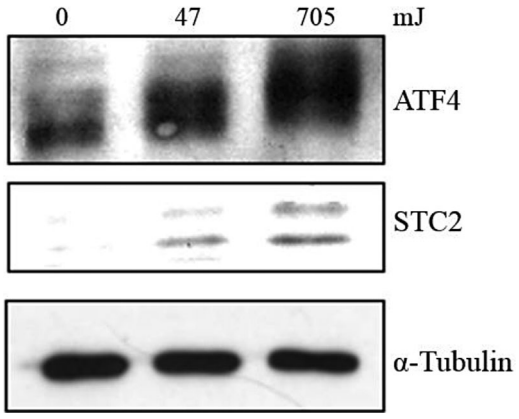


Figure 4. Plasma treatment triggers ER stress response. CNE-1 cells were treated with plasma at 47 and 705 mJ doses. Cells were then lysed at 24 h, protein samples were collected, and Western blot analysis was performed. Protein levels of ATF4 and STC2 were examined as indicators of ER stress, and α-Tubulin was used as a loading control. ATF4 protein levels increased in a dose-dependent manner, indicating that plasma can induce ER stress, which is needed to translocate CRT to the cell surface during ICD.

indicators of ER stress, activating transcription factor 4 (ATF4) and stanniocalcin 2 (STC2), in response to our lowest and highest dose treatments.[21,22] ATF4 is involved with the control of amino acid metabolism and redox chemistry following activation during ER stress.[21] STC2, a secretory glycoprotein, is overexpressed and secreted to the extracellular matrix upon a variety of cellular stresses including hypoxia, low glucose and low glutamine.[34] Irradiation usually activates the ER-stress-related ATF4-STC2 pathway as part of cellular response to damage of cellular components.[35] Western blot analysis of cellular proteins demonstrated that ATF4 and STC2 expression increased in a dose-dependent manner 24 h post treatment with nspDBD plasma (Figure 4). These data indicate that nspDBD plasma treatment triggers ER stress. These stress proteins are upstream of another hallmark of ICD, ecto-CRT.[13,21,22] Following ER stress, intracellular CRT is translocated to the surface of the cell that increases immunogenicity of poorly immunogenic cells. This process is crucial for the engulfment of tumor cells by macrophages and dendritic cells, which is required for subsequent antigen processing and presentation.

3.4. Enhanced CNE-1 Cell Death Following Plasma Treatment by ICD-Mediated Macrophage Stimulation

To examine whether plasma-induced DAMP signals enhance macrophage anti-tumor effects, CNE-1 cells were treated at 141 and 282 mJ and cultured for 48 h with non-plasma-treated THP-1 M0 macrophages in transwell inserts. These treatment doses were selected based on our previous results.[24] Cell viability assay was performed on the CNE-1 cell line 48 h post treatment as previously described. Cell counts were normalized to CNE-1 cells cultured alone and grown without the macrophage cell line (control). Non-plasma-treated CNE-1 cells co-cultured with M0 macrophages exhibited a 10% cell loss (Figure 5). Following plasma treatment and co-culture, cell loss increased to 44 and 57% for 141 mJ and 282 mJ treatments, respectively. This was significantly more than direct killing with plasma (29 and 42% for 141 and 282 mJ treatments, respectively). We propose

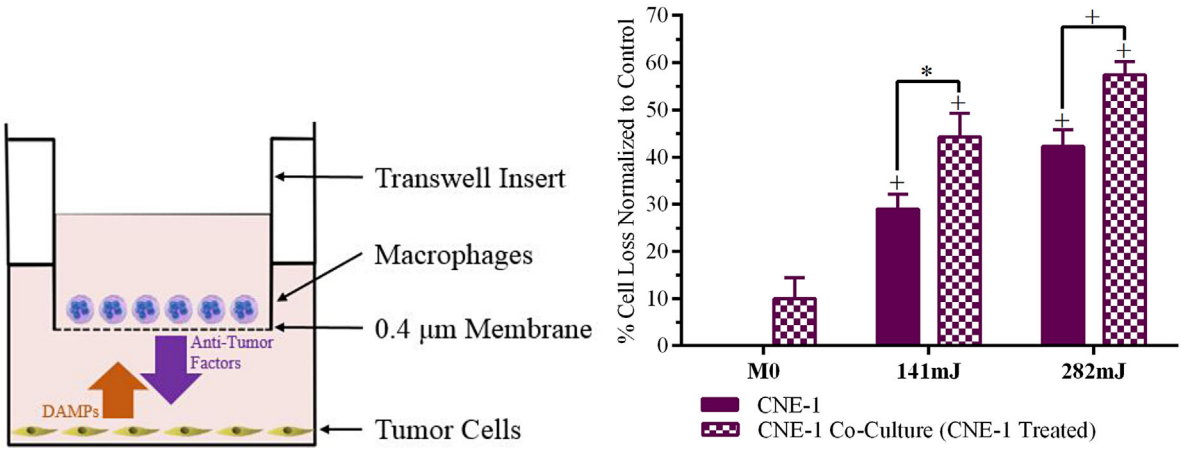


Figure 5. Plasma treatment of CNE-1 cells enhanced anti-tumor effects of macrophages. (Left) Following plasma treatment, CNE-1 cells were cultured for 48 h with Mo macrophages in 0.4 μm pore transwell inserts. (Right) Cell viability following treatment and co-culture was normalized to CNE-1 cells that did not receive plasma and that were cultured alone (control). Non-plasma treated CNE-1 cells cultured with Mo macrophages had 10% cell loss while 141 and 282 mJ dose treatments and co-culture elicited 44 and 57% cell loss, respectively. Effect of plasma alone on CNE-1 cells was 29 and 42% cell loss at 141 and 282 mJ, respectively. Data are represented as mean ± SEM. $^{*}p < 0.05$, $+p < 0.01$.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

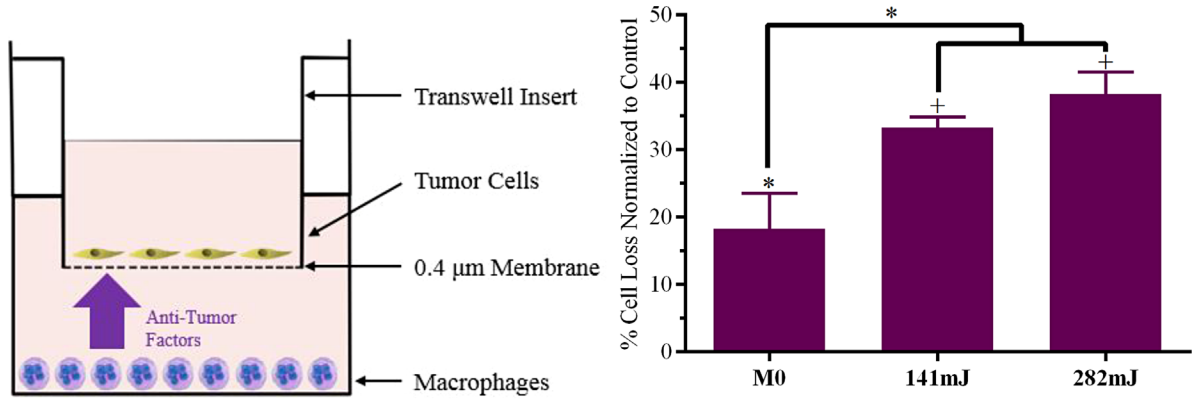


Figure 6. Plasma-treated macrophages enhanced anti-tumor effects. (Left) THP-1 Mo macrophages were treated with plasma and co-cultured with the CNE-1 tumor cell line in the transwell insert system. (Right) CNE-1 cell viability was analyzed 48 h post co-culture and normalized to the control, cells that were cultured alone. Macrophages by themselves without plasma treatment (Mo) induced 18% cell loss while those that were treated with 141 mJ plasma elicited 33% cell loss. 282 mJ plasma treatment caused 38% cell loss compared to the control. Data are represented as mean ± SEM, $^{*}p < 0.05$, $+p < 0.01$.

that the increased extracellular ATP (Figure 3) activates macrophages that cause this enhanced anti-tumor effect.

3.5. nspDBD Treatment of M0 Macrophages Enhances Anti-Tumor Effects

In our past work, we have shown that nspDBD plasma can influence functional capabilities of macrophages in an in vitro wound-healing model.[24] As macrophage migration was stimulated at certain regimes of plasma treatment, we hypothesized that other functions, particularly anti-tumor effects, could also be influenced. To test this, we treated THP-1 M0 macrophages with plasma and cultured them with CNE-1 cells in transwell inserts. While non-plasma treated M0 macrophages elicited 18% CNE-1 cell loss, 141 and 282 mJ plasma stimulated macrophages elicited 33 and 38% cell loss, respectively (Figure 6). It is important to emphasize that in this experimental setup, the CNE-1 tumor cell line never came in direct contact with plasma.

The conditioned media of classically activated monocyte-derived human macrophages and THP-1 macrophages has been shown to inhibit proliferation of colon cancer cell lines (HT-29 and CACO-2) in a dose-dependent manner.[36,37] Classically activated macrophages or M1 macrophages respond to challenges by generating an inflammatory response that includes secretion of pro-inflammatory cytokines.[27,30] On the other hand alternatively activated M2 macrophages are immunosuppressive and can release anti-inflammatory cytokines.[27] Engström et al. cultured M1 macrophages in media for 48 h. This media, which they referred to as conditioned media (CM), caused an inhibitory effect on cell proliferation when it was transferred and used to culture HT-29 and CACO-2 colon cancer cell lines. However, CM from M2 macrophages did not elicit this response. The cytokine and chemokine expression profiles of both classes of macrophages were examined to identify the factors responsible for proliferation inhibition. Compared to M2 macrophages, M1 macrophages released a variety of cytokines/chemokines, including tumor necrotic factor α (TNFα), interleukin 6 (IL6), CXCL1, RANTES, etc., to a much greater extent.[36] It is these factors, secreted from plasma-stimulated M0 macrophages, which may be responsible for the observed CNE-1 cell death.

4. Conclusion

This is the first report of enhanced tumor cell killing by uniform nspDBD plasma-elicited immunogenic cell death of cancerous cells resulting in stimulation of macrophages indirectly. Following treatment, levels of indicators of ICD, ATP secretion, and ecto-CRT, were augmented in the CNE-1 tumor cell line. This was associated with increased expression of ER stress proteins, ATF4, and STC2. Co-culture with unstimulated THP-1 M0 macrophages significantly reduced cell viability compared to tumor cells treated with plasma and cultured without macrophages (Figure 5). This indicates that stress signaling from tumor cells can stimulate macrophage function via factors secreted by tumor cells. Furthermore, the same regime of plasma treatment directly enhanced anti-tumor effects of M0 macrophages as seen by the reduced CNE-1 viability following co-culture in Figure 6. Taken together, these results suggest that the use of plasma could enhance anti-tumor effects through both the induction of ICD in tumor cells and augmentation of macrophage function. Identification of the cytokines and ROS species secreted by macrophages following plasma treatment that

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim
DOI: 10.1002/ppap.201500139

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

16128869, 2015, 12, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/ppap.201500139 by Drexel University, Wiley Online Library on [20/11/2026]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

are responsible for the anti-tumor activity is still required and ongoing. Future studies involve the application of plasma to in vivo tumor models where all the components of the immune system beyond macrophages, such as dendritic cells, T cells, and natural killer (NK) cells will be present. This will allow for establishment of specific systemic immune responses against tumors through the simultaneous treatment of cancerous cells and immune cells in the local tumor environment.

Acknowledgements: We would like to thank Dr. Kara Spiller for the THP-1 cell line and the following Drexel undergraduate cooperating education program students for their assistance with this work: Laureen B. Saba and Saibou Traore.

Received: July 20, 2015; Revised: July 20, 2015; Accepted: September 28, 2015; DOI: 10.1002/ppap.201500139

Keywords: damage-associated molecular patterns; immunogenic cell death; nanosecond pulsed dielectric barrier discharge; plasma medicine

[1] C.-H. Kim, J. H. Bahn, S.-H. Lee, G.-Y. Kim, S.-I. Jun, K. Lee, S. J. Baek, *J. Biotechnol.* **2010**, *150*, 530–538.
[2] M. Vandamme, E. Robert, S. Pesnel, E. Barbosa, S. Dozias, J. Sobilo, S. Lerondel, A. Le Pape, J. M. Pouvesle, *Plasma Process. Polym.* **2010**, *7*, 264–273.
[3] M. Keidar, R. Walk, A. Shashurin, P. Srinivasan, A. Sandler, S. Dasgupta, R. Ravi, R. Guerrero-Preston, B. Trink, *Br. J. Cancer* **2011**, *105*, 1295–1301.
[4] L. Brullé, M. Vandamme, D. Riès, E. Martel, E. Robert, S. Lerondel, V. Trichet, S. Richard, J.-M. Pouvesle, A. Le Pape, *PLoS ONE* **2012**, *7*, e52653.
[5] M. Keidar, A. Shashurin, O. Volotskova, M. A. Stepp, P. Srinivasan, A. Sandler, B. Trink, *Phys. Plasmas* **2013**, *20*, 057101.
[6] F. Utsumi, H. Kajiyama, K. Nakamura, H. Tanaka, M. Mizuno, K. Ishikawa, H. Kondo, H. Kano, M. Hori, F. Kikkawa, *PloS ONE* **2013**, *8*, e81576.
[7] D. Hanahan, R. A. Weinberg, *Cell* **2011**, *144*, 646–674.
[8] K. E. de Visser, A. Eichten, L. M. Coussens, *Nat. Rev. Cancer* **2006**, *6*, 24–37.
[9] L. Zitvogel, L. Apetoh, F. Ghiringhelli, G. Kroemer, *Nat. Rev. Immunol.* **2008**, *8*, 59–73.
[10] I. Mellman, G. Coukos, G. Dranoff, *Nature* **2011**, *480*, 480–489.
[11] A. D. Garg, D. Nowis, J. Golab, P. Vandenabeele, D. V. Krysko, P. Agostinis, *Biochim. Biophys. Acta.* **2010**, *1805*, 53–71.
[12] C. Sun, H. Wang, S. Mao, J. Liu, S. Li, J. Wang, *Immunol. Lett.* **2015**, *164*, 65–71.
[13] G. Kroemer, L. Galluzzi, O. Kepp, L. Zitvogel, *Annu. Rev. Immunol.* **2013**, *31*, 51–72.
[14] H. P. Harding, I. Novoa, Y. Zhang, H. Zeng, R. Wek, M. Schapira, D. Ron, *Mol. Cell* **2000**, *6*, 1099–1108.
[15] D. V. Krysko, A. D. Garg, A. Kaczmarek, O. Krysko, P. Agostinis, P. Vandenabeele, *Nat. Rev. Cancer* **2012**, *12*, 860–875.
[16] A. Tesniere, T. Panaretakis, O. Kepp, L. Apetoh, F. Ghiringhelli, L. Zitvogel, G. Kroemer, *Cell Death Differ.* **2008**, *15*, 3–12.
[17] A. Tesniere, L. Apetoh, F. Ghiringhelli, N. Joza, T. Panaretakis, O. Kepp, F. Schlemmer, L. Zitvogel, G. Kroemer, *Curr. Opin. Immunol.* **2008**, *20*, 504–511.
[18] C. M. Cruz, A. Rinna, H. J. Forman, A. L. Ventura, P. M. Persechini, D. M. Ojcius, *J. Biol. Chem.* **2007**, *282*, 2871–2879.
[19] L. Vitiello, S. Gorini, G. Rosano, A. la Sala, *Blood* **2012**, *120*, 511–518.
[20] C. X. Santos, L. Y. Tanaka, J. Wosniak, Jr., F. R. Laurindo, *Antioxid. Redox Signal.* **2009**, 2409–2427.
[21] D. T. Rutkowski, R. J. Kaufman, *Dev. Cell* **2003**, *4*, 442–444.
[22] D. Ito, J. R. Walker, C. S. Thompson, I. Moroz, W. Lin, M. L. Veselits, A. M. Hakim, A. A. Fienberg, G. Thinakaran, *Mol. Cell Biol.* **2004**, *24*, 9456–9469.
[23] T. Panaretakis, O. Kepp, U. Brockmeier, A. Tesniere, A. C. Bjorklund, D. C. Chapman, M. Durchschlag, N. Joza, G. Pierron, P. van Endert, *EMBO J* **2009**, *28*, 578–590.
[24] V. Miller, A. Lin, G. Fridman, D. Dobrynin, A. Fridman, *Plasma Process. Polym.* **2014**, *11*, 1193–1197.
[25] L. Bingle, N. Brown, C. Lewis, *J. Pathol.* **2002**, *196*, 254–265.
[26] C. E. Lewis, J. W. Pollard, *Cancer Res.* **2006**, *66*, 605–612.
[27] F. O. Martinez, S. Gordon, *F1000prime Rep.* **2014**, 6.
[28] K. L. Spiller, R. R. Anfang, K. J. Spiller, J. Ng, K. R. Nakazawa, J. W. Daulton, G. Vunjak-Novakovic, *Biomaterials* **2014**, *35*, 4477–4488.
[29] K. E. Iles, H. J. Forman, *Immunol Res.* **2002**, *26*, 95–105.
[30] S. Dupré-Crochet, M. Erard, O. Nüβe, *J. Leukocyte Biol.* **2013**, *94*, 657–670.
[31] G. Fridman, A. Shereshevsky, M. M. Jost, A. D. Brooks, A. Fridman, A. Gutsol, V. Vasilets, G. Friedman, *Plasma Chem. Plasma Process.* **2007**, *27*, 163–176.
[32] A. Lin, N. Chernets, J. Han, Y. Alicea, D. Dobrynin, G. Fridman, T. A. Freeman, A. Fridman, V. Miller, *Plasma Proces. Polym.* **2015**.
[33] S. Chen, C. Yin, T. Lao, D. Liang, D. He, C. Wang, N. Sang, *Cell Cycle (just-accepted)* **2015**.
[34] S. Qie, D. Liang, C. Yin, W. Gu, M. Meng, C. Wang, N. Sang, *Cell Cycle* **2012**, *11*, 3679–3690.
[35] S. Lin, Q. Guo, J. Wen, C. Li, J. Lin, X. Cui, N. Sang, *J. Exp. Clin. Cancer Res.* **2014**, *33*, 1–11.
[36] A. Engström, A. Erlandsson, D. Delbro, J. Wijkander, *Int. J. Oncol.* **2014**, *44*, 385–392.
[37] A. Hedbrant, A. Erlandsson, D. Delbro, J. Wijkander, *Int. J. Oncol.* **2015**, *46*, 37–46.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim