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MIATA COMPLIANT RESEARCH PAPER

Non-thermal plasma induces immunogenic cell death *in vivo* in murine CT26 colorectal tumors

Abraham G. Lin[a], Bo Xiang[b,c], Dante J. Merlino [b], Trevor R. Baybutt[b], Joya Sahu[d], Alexander Fridman[a], Adam E. Snook [b], and Vandana Miller[a]

[a]C. & J. Nyheim Plasma Institute, Drexel University, Camden, NJ, USA; [b]Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, PA, USA; [c]Department of Pediatrics, University of Washington, Seattle, WA, USA; [d]Cutaneous Lymphoma Center, Thomas Jefferson University Hospital, Philadelphia, PA, USA

ABSTRACT

Immunogenic cell death is characterized by the emission of danger signals that facilitate activation of an adaptive immune response against dead-cell antigens. In the case of cancer therapy, tumor cells undergoing immunogenic death promote cancer-specific immunity. Identification, characterization, and optimization of stimuli that induce immunogenic cancer cell death has tremendous potential to improve the outcomes of cancer therapy. In this study, we show that non-thermal, atmospheric pressure plasma can be operated to induce immunogenic cell death in an animal model of colorectal cancer. *In vitro*, plasma treatment of CT26 colorectal cancer cells induced the release of classic danger signals. Treated cells were used to create a whole-cell vaccine which elicited protective immunity in the CT26 tumor mouse model. Moreover, plasma treatment of subcutaneous tumors elicited emission of danger signals and recruitment of antigen presenting cells into tumors. An increase in T cell responses targeting the colorectal cancer-specific antigen guanylyl cyclase C (GUCY2C) were also observed. This study provides the first evidence that non-thermal plasma is a *bone fide* inducer of immunogenic cell death and highlights its potential for clinical translation for cancer immunotherapy.

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Introduction

Cancer treatment strategies in the past have focused on reducing tumor burden through delivery of cytotoxic agents. These methods often do not rely on the patient's adaptive immune responses for the resolution of cancer. Thus, once cells escape treatment, they continue to grow, resulting in tumor recurrence and resistance to therapy.[1–3] Immunogenic cell death (ICD), initially described by Zitvogel, Kroemer, and co-workers, is a modality of death where dying cells stimulate immune responses against dead-cell antigens.[4–6] In cancer therapy, this is advantageous as tumor cells undergoing ICD activate an anti-tumor immune response that is specific for that cancer. Therefore, developing treatments that elicit immunogenic cell death and facilitate the active participation of the patient's adaptive immune system, offer the potential to improve clinical outcomes of cancer therapy.

Plasma, the fourth state of matter, is ionized gas composed of charged particles, active neutral gas species, electric fields, and low amounts of ultraviolet (UV) light.[7–11] Development of plasma systems that can be sustained in atmospheric pressure and at room temperature has opened doors for biomedical applications, including, but not limited to, cancer therapy.[12–14] Mounting evidence demonstrates that these 'non-thermal plasmas' (NTP) can be optimized to destroy tumors with minimal damage to neighboring healthy tissue.[14–17] Decreased tumor burden and prolonged animal survival following direct plasma treatment have been reported, suggesting that plasma should be further explored as a viable candidate for cancer treatment.[18]

The potential of NTP to induce immunogenic cancer cell death is only recently being explored. Bekeschus, et al. has demonstrated that NTP treatment of two murine cell lines *in vitro*, the B16F10 melanoma cells and the CT26 colorectal cancer cells, increased immunogenic cell surface molecules such as major histocompatibility complex I (MHC-I) and surface-exposed calreticulin (ecto-CRT).[19,20] We have reported successful *in vitro* ICD induction in two human cell lines, a radiation-resistant primary nasopharyngeal carcinoma cell line (CNE-1) and the A549 lung carcinoma cell line in response to NTP exposure.[21,22] The mechanism is postulated to be reactive oxygen and nitrogen species (RONS) dependent. NTP-generated RONS rapidly change the oxidative status of cells and induce endoplasmic reticulum (ER) stress pathways in these cells.[19–22] Upregulation of two proteins associated with ER stress and upstream of CRT emission, activating transcription factor 4 (ATF4) and stanniocalcin (STC2), was also demonstrated.[21] Moreover, abrogation of NTP-generated and cell-stimulated RONS tempered the effect of NTP on CRT emission. These reports indicate that NTP-induced ICD is not specific to a single cancer cell type, and merits further investigation into its clinical relevance as an anti-cancer modality. Plasma treatment in animal models of cancers is needed to assess if plasma-induced ICD could benefit patient outcome.

CONTACT Vandana Miller vam54@drexel.edu 200 Federal Street, Suite 500, Camden, NJ 08103.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/koni.

In this study, we used the CT26 murine colorectal tumor model to explore the potential of NTP to induce ICD *in vivo*. NTP generated by a nanosecond-pulsed dielectric barrier discharge (nspDBD) plasma system induced the expression of two key surrogate markers of ICD in these cancer cells: ecto-CRT and secreted adenosine triphosphate (ATP). A vaccination assay, used to determine if a stimulus is a *bone fide* ICD-inducer, showed partial protective immunity against tumor challenge in syngeneic Balb/c mice immunized with NTP-treated CT26 cells. Furthermore, treatment of subcutaneous colorectal tumors expressing the cancer antigen guanylyl cyclase C (GUCY2C) resulted in higher expression of ICD markers in tumors, recruitment of antigen presenting cells (APCs), and generation of more GUCY2C-specific T cells. Together, our findings are the first report that establish the potential of plasma for cancer immunotherapy via ICD.

Results

Plasma induces emission of surrogate markers of ICD

To measure cell death in response to nspDBD plasma, the CT26 colorectal carcinoma cell line was exposed to several plasma energies. Cell viability, quantified with a Muse Cell Analyzer 24 hours after plasma treatment, decreased in an energy dependent manner (Figure 1(a)). As previously described, not all modalities of cell death are immunogenic and capable of initiating anti-tumor effects. The identification of ICD *in vitro* mainly relies on detection of associated damage associated molecular patterns (DAMPs). Therefore, we examined the effect of plasma on cell viability and two DAMP signals in CT26 cells: externalization of CRT and secretion of ATP.[23]

Immunogenicity of dying cancer cells is strongly dictated by surface exposure of CRT.[24] Normally located on the ER membrane, exposed CRT on the cell surface acts as an 'eat me' DAMP signal that facilitates recognition, engulfment, and processing of tumor cells by APCs,[25–27] a critical step for the initiation of an adaptive anti-cancer response.[28–30] Surface-exposure of CRT in response to 10 second plasma exposure was measured 24 hours after treatment. Intact cells were labeled with anti-CRT antibodies, stained with fluorescent secondary antibodies and analyzed using flow cytometry. Our results show that the emission of ecto-CRT on CT26 cells increased in an energy dependent manner, suggesting plasma may increase the immunogenicity of tumor cells (Figure 1(b,c)).

ATP, the most abundant intracellular molecule required for metabolism, is secreted from cells undergoing ICD.[31] It has even been suggested that secretion of ATP follows overlapping pathways with externalization of CRT.[32] Once ATP reaches the extracellular space, it becomes another hallmark of ICD and functions as a 'find me' DAMP signal for recruitment and activation of APCs.[32–34] To detect this secreted DAMP by cells exposed to plasma, the cell culture media was collected 10 minutes after treatment and extracellular ATP was quantified. ATP levels were low at baseline (8.2 nM) and increased 70-fold (582.1 nM) following 300 mJ plasma treatment (Figure 1(d)).

Vaccination with plasma-induced ICD cells provides protection against tumor challenge in mice

To ascertain whether the DAMP signals elicited by plasma could enhance immune responses against cancer, we performed a vaccination assay. Balb/c mice were immunized with CT26

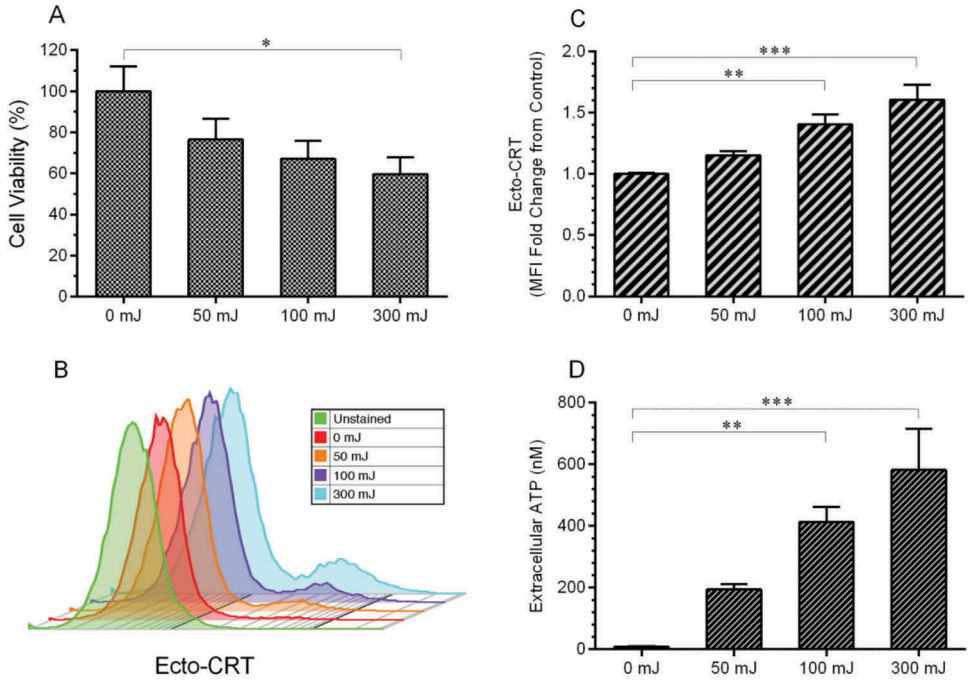


Figure 1. Plasma-induced cell death, surface emission of CRT, and secretion of ATP in CT26 cells. A) Cell viability is indicated by the percentage of live CT26 cells normalized to untreated (0 mJ), 24 hours after plasma treatment. B, C) CRT was detected on the surface of intact CT26 cells 24 hours after plasma exposure. B) Representative histograms and C) mean fluorescence intensity showed increased surface CRT following plasma treatment. D) ATP content was detected in the media 10 minutes after plasma treatment using a chemiluminescent kit. CRT, ATP, and viability data are presented as means ± S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA, Dunnett's multiple comparison test).

cells treated *in vitro* with plasma at the ICD-inducing regime (29 kV, 30 Hz, 1 mm gap distance, 10 seconds). Cells were prepared for inoculation and injected into the left flank as a whole-cell vaccine to allow an immune response to develop. One week after immunization, mice were challenged with live CT26 cancer cells on the opposite flank and tumor growth was monitored twice a week until day 26 when the study was terminated as a subset of the animals reached IACUC-approved endpoints (Figure 2(a)). CT26 cells treated with media only or Cisplatin (50 μM for 24 hours), a non-ICD inducer,[23] were used as controls.

Challenge tumors in the media and Cisplatin groups grew rapidly while tumors in the plasma group developed relatively slowly (Figure 2(b–d)). The mean tumor volume for the plasma immunized group was significantly smaller compared to that of the media group (414.7 ± 104.3 mm^3 vs 847.4 ± 141.5 mm^3; $p < 0.001$) or the Cisplatin group (1041.8 ± 208.3 mm^3) at day 26 (Figure 2(e)). Indeed, 90% of the mice in the plasma immunized group had tumor volumes smaller than the mean tumor volume of the media group (850 mm^3), suggesting that these mice were partially protected by vaccination. Moreover, 3 out of the 10 mice in the plasma group did not develop subcutaneous tumors on the challenge site (Figure 2(f)).

Plasma induces ICD in vivo and stimulates immune cell recruitment

To directly validate whether plasma could induce ICD *in vivo*, CT26 colorectal tumors were established subcutaneously in Balb/c mice and exposed to plasma when they became palpable (Figure 3). A safe operating plasma regimen was first identified by exposing subcutaneous CT26 tumors in Balb/c mice to plasma for various treatment times (10, 25, 50 sec) at set plasma parameters (29 kV and 750 Hz) once daily for five consecutive days. One day and 3 days after the final treatment, tumors were resected with overlaying skin, fixed, and stained with hematoxylin and eosin (H&E) to assess the structural changes in the dermal and epidermal layers of the skin. Ten seconds of plasma treatment resulted in minimal epidermal damage but no changes to the tumor were observed (Figure 4(b,f)). When plasma treatment duration was increased to 25 seconds, thermal and necrotic damage in the epidermis was observed one day after plasma (Figure 4(c)) but not in the underlying tumor. By the third day after treatment, the epidermis appears improved but not fully healed, suggesting that the damage was reversible (Figure 4(g)). The 50-second treatment resulted in considerable damage through all the layers of the skin, and, in fact, tumors just below the skin showed thermal and necrotic damage (Figure 4(d)). By the third day thermal damage was still apparent and changes in collagen had begun to appear. There were also more neutrophils in the skin signifying an inflammatory response (Figure 4(h)).

Based on the results of our safety studies, we chose to investigate if the 10 second plasma treatment over five days induced ICD in the subcutaneous tumors. Tumors were exposed to plasma using three different treatment procedures: i) the same area each day (Plasma 1 Spot), ii) two areas of the tumor each day (Plasma 2 Spots), or iii) different areas of the tumor each day (Plasma Multiple Spots). Three days after the last plasma treatment, tumors were resected, fixed, and sectioned. Immunofluorescence microscopy was performed on tumor sections to identify ICD markers. CRT expression increased in all plasma treatment groups (Figure 5(a)) and was maximum following the "Plasma Multiple Spot" regimen (1.6 ± 0.2 fold, $p < 0.05$). Tumor sections were also stained for High mobility group box 1 (HMGB1), another DAMP signal much like ATP, that recruits inflammatory immune cells and mediates signals between APCs.[35–38] HMGB1 has been observed to translocate from the nucleus to the cytoplasm and extracellular space.[37–39] Here, we observed an increase in immunofluorescence intensity of HMBG1 in all plasma treatment groups (Figure 5(b)), suggesting HMGB1 protein concentration may be elevated following plasma exposure.

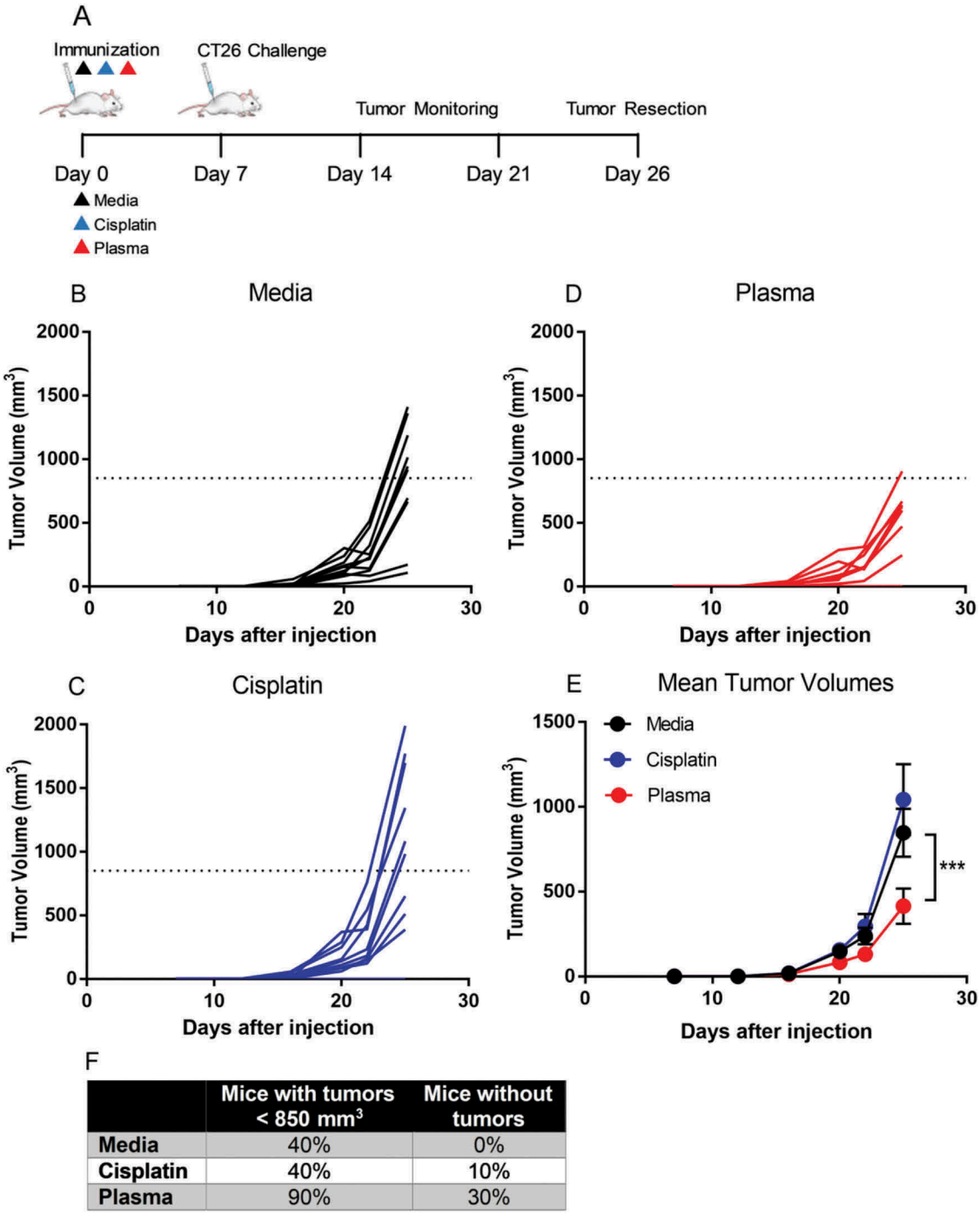
To determine if emitted DAMPs stimulated the recruitment of immune cells into the tumor environment, we also stained for CD45+ (leukocytes) and CD11c+ (APCs) immune cells. Indeed, increased CRT and HMGB1 were associated with more CD45+ (2.0 ± 0.4 fold, $p < 0.05$) and CD11c+ cells (1.8 ± 0.2 fold, $p < 0.05$) following the Plasma Multiple Spots regimen (Figure 5(c,d)). Because CD45 is expressed by all leukocytes, including T cells, B cells, neutrophils, NK cells and others,[40,41] it is possible that other immune cell subsets are also being recruited as a downstream consequence of ICD induction. Representative immunofluorescence images of the multi-spot treatment compared to the untreated are shown in (Figure 5(e)).

Altogether, Plasma Multiple Spots treatments enhanced both emission of DAMPs and recruitment of immune cells in the tumor compared to the other application methods. Therefore, treatment of different spots may be more beneficial compared to repeat treatment of the same area. This treatment condition was used to investigate plasma-induced ICD effects on downstream T-cell response.

Plasma amplifies specific T-cell responses against CT26-GUCY2C tumors

To analyze whether plasma-induced ICD could stimulate an adaptive anti-tumor response, we treated subcutaneous CT26 tumors expressing the colorectal cancer antigen GUCY2C[42,43] (CT26-GUCY2C) in Balb/c mice. Mice were treated with either plasma alone or with plasma in combination with the Ad5-GUCY2C-S1 vaccine. The Ad5-GUCY2C-S1 vaccine was previously shown to safely induce GUCY2C-specific immune responses and antitumor immunity in mice[44–51] and has been translated to human clinical trials.[52] Mice were treated with plasma for five consecutive days and one group was vaccinated with the Ad5-GUCY2C-S1 vaccine, one week after the last plasma treatment (Figure 6(a)). An untreated group and a vaccine only group (Ad5-GUCY2C-S1 vaccination) served as our negative and vaccine controls, respectively.

Splenic GUCY2C-specific T-cell responses were analyzed by IFNγ ELISpot assay 28 days after the initial tumor inoculation. Plasma treatment alone had a marginal effect on GUCY2C-specific responses (22.1 ± 11.9 spots vs 3.0 ± 1.8 spots in Untreated; p = ns). However, plasma treatment prior to vaccination amplified GUCY2C-specific T-cell responses (167.8 ± 41.5 spots vs 109.7 ± 22.3 spots with vaccine alone,



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	Mice with tumors < 850 mm^3	Mice without tumors
Media	40%	0%
Cisplatin	40%	10%
Plasma	90%	30%

Figure 2. Immunization of mice with cancer cells plasma-treated at ICD-inducing regimes reduced the growth rate in challenge tumor. A) Balb/c mice were injected with media-, Cisplatin-, or plasma-treated CT26 cells subcutaneously into the left flank (n = 10 per group). One week later, mice were challenged with live CT26 cells on the opposite flank and monitored for 26 days. Challenge tumors grew more rapidly in mice immunized with cells treated with media (B) or Cisplatin (C) compared to mice immunized with plasma-treated cells (D). Data in B-D indicate tumor growth of each mouse. A dotted line is plotted to indicate the mean tumor volume of the control group (media treatment). E) Mean tumor volumes in the mice vaccinated with plasma-treated cells were significantly smaller compared to those immunized with untreated cells. Data are presented in E as mean value ± S.E.M. ***$p < 0.001$ (Two-way ANOVA, Dunnett's multiple comparisons test). F) The percentage of mice with tumor volumes less than the mean volume of the media group and the percentage of mice that did not develop subcutaneous tumors at the challenge site are displayed.

$p < 0.05$) (Figure 6(b)). This observation supports the potential of plasma to increase the immunogenicity of cancer cells and stimulate canonical pathways required for tumor control.

Typically, immune responses are generated against one or two dominant epitopes of an antigen. When the specificity of immune responses spreads to include subdominant epitopes, new populations of T cells may be generated against the antigen.[53,54] We tested whether plasma and vaccination treatment exposes neoantigens unrelated to the original vaccination target, GUCY2C.[55] We measured T-cell responses against AH1, an endogenous immunodominant MHC class I-associated epitope from gp70 expressed by CT26 tumor

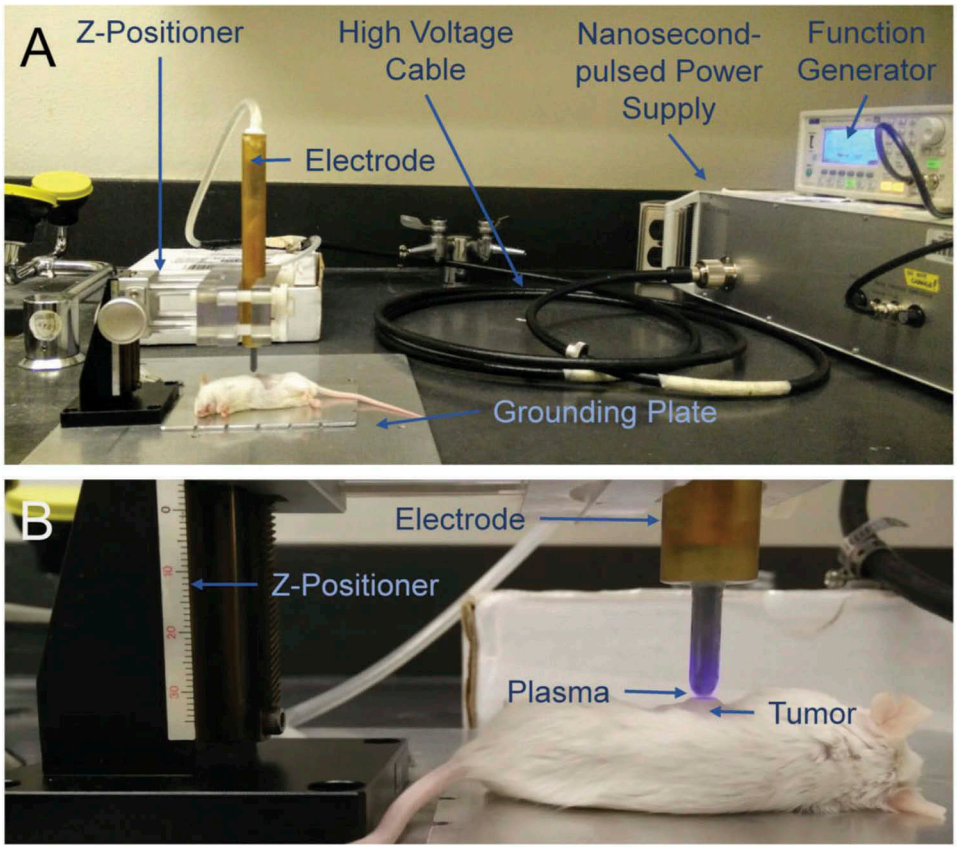

Figure 3. *In vivo* plasma treatment system. A) A nanosecond-pulsed power supply generated 29 kV pulses and a function generator was used to control the frequency of pulses and the plasma exposure time. A z-positioner was used to hold the high voltage electrode in place during treatment, approximately 1 to 2 mm above the target. B) Plasma was generated directly above of the subcutaneous tumor.

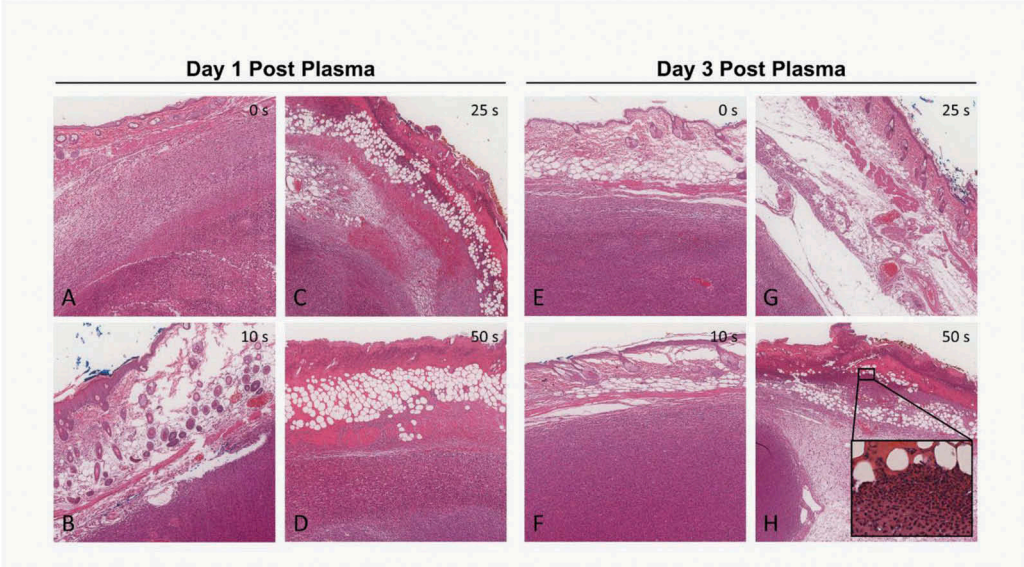

Figure 4. H & E staining of plasma-treated subcutaneous tumors. Tumors with overlaying skin were resected 1 day (A-D) or 3 days (E-H) following the final plasma treatment. Tumors received no treatment (A, E), 10-second treatment (B, F), 25-second treatment (C, G) or 50-second treatment (D, H) daily over the course of 5 days. Images were taken at 20x. Inset in H shows neutrophil infiltration.

cells (Figure 6(c)).[55–57] Mice receiving both plasma and peptide vaccine exhibited a modest increase in the AH1-specific T-cell response (24.2 ± 8.3 vs 11.0 ± 4.3 spots; p = ns). While the study was not powered to detect a statistically-significant change, epitope spreading was observed. Further optimization of plasma treatment to alter tumor microenvironment may

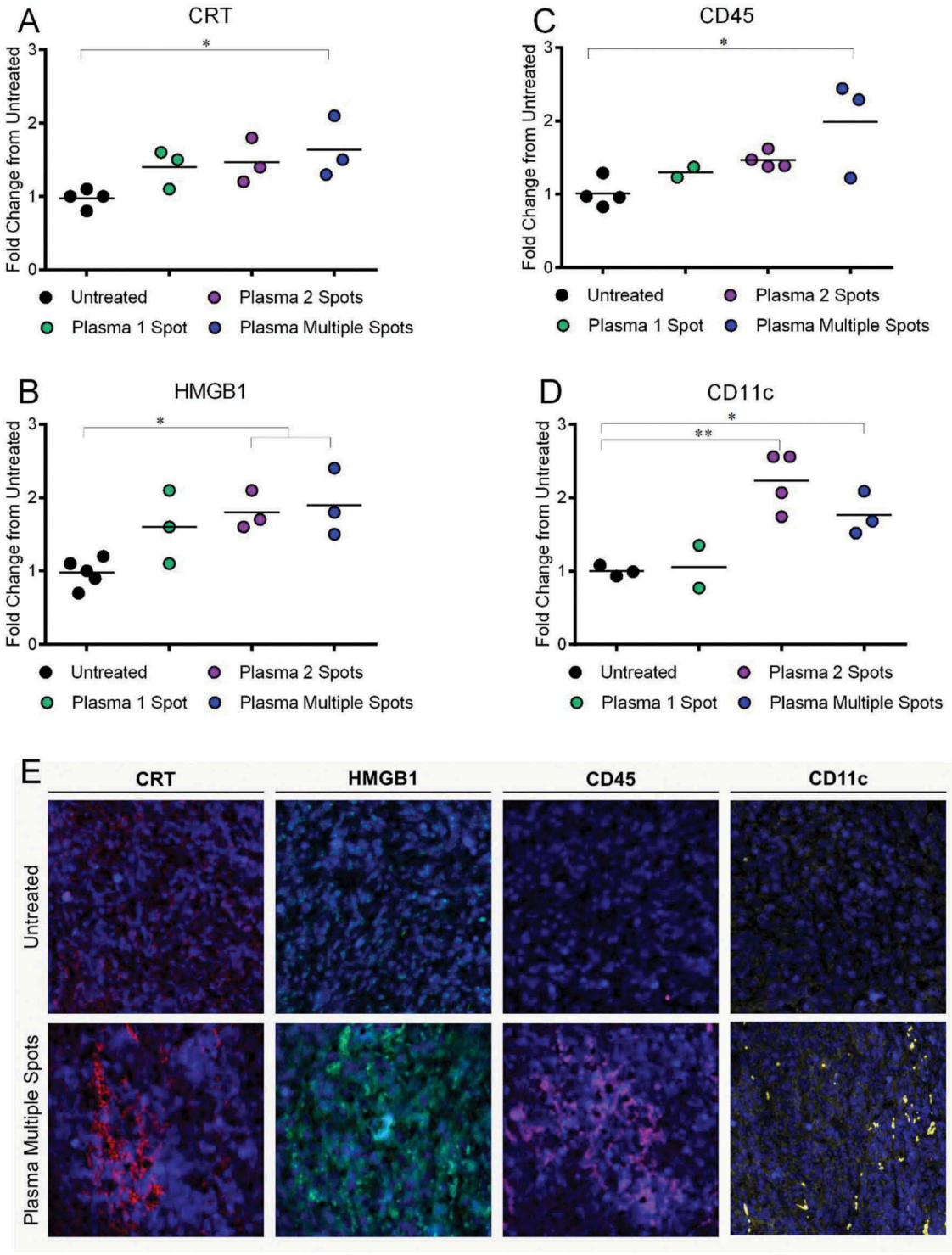

Figure 5. Plasma treatment of subcutaneous tumors in Balb/c mice induced DAMP emission and increased leukocytes and APCs in the tumor 3 days after final treatment. Tumor sections were fixed, stained, and imaged by fluorescence microscopy. Fluorescence intensity of representative sections of the tumor were quantified with ImageJ and normalized to untreated controls. The emission of A) CRT and B) HMGB1 increased in all plasma treatment groups by ~1.5-fold. The presence of leukocytes (C, CD45+ cells) and APCs (D, CD11c+ cells) increased compared to untreated tumors. Data are presented from individual resected tumors. *$p < 0.05$, **$p < 0.05$ (One-way ANOVA, Dunnett's multiple comparisons test). E) Representative images of tumors subject to multi-spot plasma treatment compared to untreated controls are shown (10x, 400μm ˣ 400μm).

produce conditions favorable for epitope spreading. Altogether, we provide compelling evidence that plasma acts as an adjuvant for cancer therapy as shown by the amplification of GUCY2C-specific and AH1-specific T-cell responses in mice. Increased efficacy with the addition of vaccine suggests that plasma may prime the host's immune system and may allow for its use in other combination therapies.

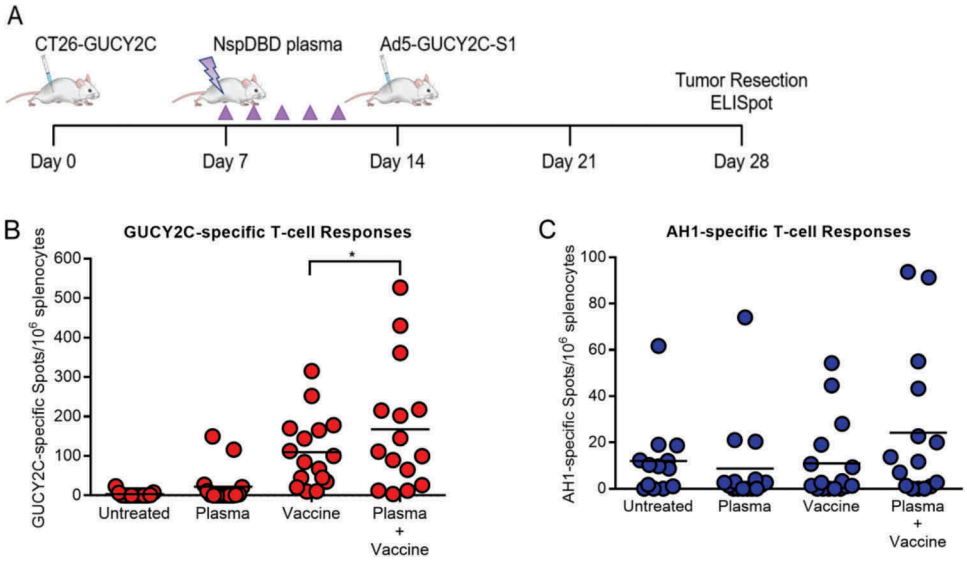

Figure 6. Plasma in combination with vaccination enhanced cancer-specific T-cell responses. A) Mice were challenged with CT26-GUCY2C cells (Day 0) and treated with plasma on day 7. A subgroup of the plasma-treated mice was vaccinated on day 14 with Ad5-GUCY2C-S1. B) GUCY2C-specific and C) AH1-specific T-cell responses were quantified by IFNγ ELISpot in the spleen o.

Discussion

Although significant advancement in conventional tumor-targeted cancer therapies (e.g. surgery, chemotherapy, radiation therapy, etc.) has reduced cancer related morbidity and mortality, this comes at the cost of significant toxicity. Another major challenge is relapse from cells that escape treatment and eventually develop resistance to therapy.[58–60] In contrast, immunotherapy aims to activate the patient's natural defenses to selectively target tumors for resolution of cancer[60–63] with reduced non-specific damage to normal tissue. While current strategies (e.g. adoptive T-cell transfer, checkpoint inhibitors, etc.) are clinically efficacious, several instances of serious adverse effects, including pneumonitis and enterocolitis, have been reported.[64,65] To address these, an ICD-mediated immunotherapy approach is being explored. These treatments stimulate the release of DAMP signals in cancer cells which engage APCs to expose neoantigens and facilitate the initiation of adaptive immune responses.[66,67] The methods to induce ICD include certain chemotherapeutic agents, irradiation, photodynamic therapy with hypericin (PDT-hypericin), and high hydrostatic pressure.[68]

Here, we have demonstrated that non-thermal plasma may be operated for ICD induction and utilized in immunotherapeutic strategies. *In vitro*, nspDBD plasma elicited CRT emission and secretion of ATP from CT26 colon carcinoma cells. Our vaccination study showed that mice immunized with cancer cells treated with ICD-inducing plasma were partially protected against tumor challenge. In agreement with *in vitro* studies, *in vivo* plasma treatment of subcutaneous tumors in mice induced immunogenic cancer cell death. The treatment of multiple spots within the tumor elicited the greatest emission of DAMPs and recruitment of APCs into the tumor area. This treatment condition also led to a tumor-antigen-specific T-cell response. While this is the first report of plasma inducing ICD in an animal model, these results should be confirmed in other cancer models to validate, broaden, and enhance the relevance of plasma as an ICD inducer.

Overall, our results highlight the potential of plasma development for cancer immunotherapy. Further optimization of plasma parameters (applied voltage, pulse frequency, application time, etc.) and treatment schedules must be performed to improve its efficacy. This could be accomplished, in part, by determining the physical and chemical mechanism by which plasma elicits ICD. *In vivo*, it has been reported that anti-tumor effects of plasma are associated with plasma-generated RONS, but the link to immunogenic cell death has not been made.[16] *In vitro*, RONS produced by plasma were demonstrated to be the major effectors for eliciting ecto-CRT and ATP secretion in cancer cells, although potential synergy with the associated pulsed electric fields or UV radiation should not be discounted.[22] Therefore, an in-depth delineation of the specific RONS essential for ICD could help the development of improved plasma systems.

The study of the extracellular microenvironment generated by plasma should be paralleled with investigation of the intracellular mechanisms of induced ICD and the mode of cell death. In 2018, a comprehensive review on the molecular mechanisms of cell death was published by the Nomenclature Committee on Cell Death (NCCD).[69] Twelve major cell death subroutines (intrinsic apoptosis, extrinsic apotosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death, autophagy-dependent cell death, and immunogenic cell death) were defined from morphological, biochemical, molecular, and functional perspectives. To date, six DAMPs have been linked with cell death that is immunogenic [CRT, ATP, HMGB1, type I interferon (IFN), cancer cell-derived nucleic acids, and annexin A1], but not all the underlying mechanisms are clear, and some are dependent on the specific ICD-inducer.[70–76] As these mechanisms become elucidated, understanding the molecular processes following

plasma-induced ICD will be possible and will help the development of this potential therapy.

The scheduling of plasma treatment will also influence clinical outcome, as plasma may also induce bystander effects on other resident or recruited cells in the tumor environment (e.g. macrophages, dendritic cells, effector T cells, etc.). Indeed, in a separate study where mini pigs were exposed to plasma, recruited myeloid cells were detected in the treated areas of the skin one week later,[77] suggesting that local plasma exposure may influence immune cells even in the absence of cancer. Furthermore, several studies have demonstrated that within a defined range of treatment parameters, plasma may stimulate immune cell function (e.g. migration, secretion of cytokines, etc.) *in vitro*.[21,78–81] In depth studies in animal models are required to determine direct plasma effect on cells of the immune system, as the host microenvironment may affect biological outcome. However, this could potentially provide an advantage over radiation or PDT, known ICD inducers, as they are reported to be detrimental to immune cells.[82–84] An indicator that plasma may be immunomodulatory at physiologically safe doses was reported in a study where *Drosophila* exposure to plasma caused differentiation of hematocytes without affecting development or fecundity of the organism.[85]

While the present studies provide preclinical proof-of-concept for plasma immunotherapy in cancer, clinical administration of plasma will be a major challenge for its use in cancer treatment. For superficial cancers such as melanomas, administration of plasma is relatively straight-forward, as cancerous tumors/lesions are easily accessible for direct deposition of plasma-generated RONS. However, treatment of non-superficial cancers is a challenge for the plasma medicine field. One approach to deliver plasma species to deep tumors is through the use of plasma treated liquid (PTL),[86,87] in which media is treated with NTP to enrich dissolved RONS and injected locally in the tumor or perfused through body cavities.[88,89] Utsumi and co-workers demonstrated that injection of PTL locally into subcutaneous tumors can inhibit growth of malignant tumors in mice though the anti-tumor effects are not as prominent as direct plasma treatment.[89] This is likely due to the instability of plasma-dissolved species in the media and the animals' antioxidant capacity.[90] Optimization of PTL generation and storage is required before it finds a role in clinical cancer treatment. A more direct, but invasive approach may involve intraoperative plasma treatment following surgical tumor excision to eliminate cancer cells remaining in the surgical margins. Physicists and engineers are designing different plasma sources and geometries for a less invasive and more focused approach to deliver plasma inside the body.[91–93] Plasma has been shown to propagate along tubes up to several meters in length and with diameters as small as 15 μm.[91,92] The effectiveness of some of these endoscopic plasma devices is being tested in an *in vivo* pancreatic cancer model.[93] For successful clinical application with this approach, a detailed understanding of the RONS delivered to the target from the plasma aperture is critical. Finally, as we show here, immunization with a plasma-created whole-cell vaccine provided protective anti-tumor effects. With optimization of vaccine development and delivery this could be a feasible strategy for plasma-mediated cancer control where plasma acts as an adjuvant.

Ultimately, it is unlikely that a single treatment will be the solution to any type of cancer; a combination of different therapies may be required. Our data suggests that combining plasma with other immunotherapeutic agents may provide additional clinical value (Figure 6). Development of these strategies should be considered based on their effect on the different steps of adaptive immune response progression.[94] For example, plasma-induced ICD could prime the host immune response against tumor antigens, which could be boosted by a targeted vaccine, while checkpoint inhibitor blockade may enhance the therapeutic effect of plasma immunotherapy.

Conclusion

We recently proposed a new paradigm of plasma treatment for cancer: 'plasma onco-immunotherapy'.[95] This approach not only debulks tumors, but also engages the innate immune system via ICD to initiate adaptive immune responses.[95,96] In this study, we demonstrated that plasma is a *bona fide* ICD inducer and can be used alone or in combination with other immunotherapies to generate tumor-specific T-cell responses. With further development of plasma delivery systems and administration protocols, it has potential for clinical translation as a standalone treatment modality or an adjuvant for cancer immunotherapy.

Materials and methods

nspDBD plasma system and treatment parameters

NTP was generated *in vitro* by applying high voltage pulses to a dielectric barrier discharge (DBD) electrode. DBD electrodes used in this study were fabricated in our lab and have a quartz dielectric covering a copper electrode. This prevents current build-up and creates an electrically safe plasma without heating surrounding gas and tissue. A nanosecond pulser (FPG-20-05NM, FID GmbH, Germany) was used to generate high voltage pulses, characterized in our previous work.[8] Briefly, our system produced: 29 kV pulses, 2 ns rise times, 20 ns total pulse duration and 0.9 mJ/pulse.

Cell culture and in vitro plasma treatment

Colorectal cancer cell line CT26.WT obtained from ATCC (CRL-2638). Generation of CT26-GUCY2C cells was described previously.[97] Cells were cultured in complete media: DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Corning Life Sciences, USA). All cells were grown in a humidified environment at 37°C with 5% CO_2 (Panasonic, MCO-19AICUVH-PA, USA). Cells were plated one day prior to plasma treatment in 24-well plates at $3.0x10^5$ cells/mL (0.5 mL/well). Before treatment with plasma, media was removed from each well and cells were washed twice with phosphate buffered saline (PBS). PBS from the second wash was removed from the well right before cells were exposed to plasma in the absence of any liquid. Fresh, complete media (0.5 mL) was immediately added back into the well following exposure to plasma.

For treatment with plasma, a DBD electrode (1.3 cm diameter) was placed 1 mm above cells in the 24-well plate on top of a grounded metal plate with a z-positioner. Since all

liquid was removed, plasma was generated in the gap between the electrode and the plate, directly on the cells by applying high voltage pulses from the nanosecond pulser. Treatment time was fixed to 10 seconds, and pulse frequency was controlled by an external function generator (TTi, TG5011LXT, USA). A range of pulse frequencies were used (50, 15, 30 and 75 Hz), and the combination of plasma treatment parameters produced in the following plasma treatment energies: 50, 100, 300 and 700 mJ, respectively.[8]

Mice and in vivo plasma treatment

Balb/c mice were obtained from Jackson Laboratory (USA), and animal protocols were approved by The Thomas Jefferson University Institutional Animal Care and Use Committee. Subcutaneous tumors were established by injecting 1×10^5 CT26-GUCY2C cells in the flanks of mice, and monitored for growth. Prior to plasma treatment, hair over the tumor area was removed using a chemical depilatory agent to avoid obstruction with plasma generation and treatment. Tumors were treated once daily with plasma beginning on day 7 (for effector T cell development studies) or day 18 (for ICD and recruitment studies) and continued for 5 consecutive days. A smaller DBD electrode (3 mm diameter) was fabricated and used for treatment of mouse tumors. The nanosecond pulser and function generator used for *in vitro* treatment were also used here. Pulse frequency was adjusted to 750 Hz and treatment time was 10, 25 or 50 seconds. Mice were anesthetized with 5% isoflurane and treated on the grounding plate with the electrode positioned approximately 1 mm above the tumor with the z-positioner. Tumor volumes were monitored by measuring 3 orthogonal diameters and calculated using $\frac{4}{3}\pi \times r_1 \times r_2 \times r_3$.

Cell viability assay

Cell viability was determined 24 hours after plasma treatment using a Muse Cell Analyzer (Millipore, USA). Cell suspensions were collected, diluted 1:20 with Muse Count & Viability Reagent (Millipore, USA) and analyzed according to the manufacturer's instructions. The percent viability was determined for each sample.

ATP release assay

Cell supernatant was collected 10 minutes after plasma treatment and extracellular ATP was measured using a luciferin and luciferase-based chemiluminescent kit (Sigma-Aldrich, USA). All procedures were performed and reagents were prepared following manufacturer instructions. Luminescence value was measured by a Photon-Master luminometer (LuminUltra, USA) which was calibrated with the provided UltraClear calibration solution. The measured relative light units were converted into ATP concentration (pgATP/mL). Data were represented at ATP concentration (nM).

Fluorescence detection of surface-exposed calreticulin

CT26 cells were collected 24 hours after plasma treatment and washed twice with blocking buffer (PBS+ 1% heat-inactivated FBS). Cells were then incubated with rabbit anti-mouse calreticulin antibody (ThermoFisher Scientific, USA) in blocking buffer (1:200) for 30 minutes in the dark at room temperature. Following incubation, cells were washed twice with blocking buffer and stained with Alexa Fluor 488 conjugated goat anti-rabbit IgG secondary antibody (ThermoFisher Scientific, USA) at 1:500 in blocking buffer. Cells were incubated at room temperature, in the dark, for 40 minutes. Following staining, cells were washed and fixed with 4% PFA and analyzed by FACS.

Anti-tumor vaccination assay

To prepare the whole-cell vaccine, CT26 cells were treated with either: 1) 300 mJ of plasma, 2) Cisplatin (50 μM) or 3) complete media. Plasma-created vaccine was prepared from cells treated in 24-plates with plasma and cultured in regular media for 24 hours. Cisplatin-vaccine was prepared from cells incubated for 24 hours in Cisplatin media. Media-Vaccine was prepared from cells cultured in regular, complete media for 24 hours. Cisplatin- and media- vaccines were used as controls.

After a 24-hour incubation 3×10^6 cells in 100 μL of PBS were inoculated subcutaneously into the ***left*** flank of Balb/c mice. Seven days later, mice were challenged with 3×10^5 live CT26 cells subcutaneously injected into the ***right*** flank. Tumors were measured twice weekly with calipers and all mice were euthanized on day 28.

H&E staining of tissue sections and damage assessment

Tumors, with overlaying skin, were resected 1 or 3 days after the final plasma treatment and fixed in 10% formalin for at least 48 hours. Tissue was then paraffin-embedded, sectioned with a microtome, deparaffinized and stained with hematoxylin and eosin. Images of stained sections were captured using the EVOS FL Auto Cell imaging system. Sections were evaluated for damaged by a blinded pathologist.

Immunofluorescence staining of tumor tissue

Tumor sections from day 3 after final plasma treatment were used for fluorescence detection of DAMP signals and immune cell recruitment. For antigen retrieval, slides were transferred to a Dako Target Retrieval buffer pH9 (1:10 dilution in H_2O) and boiled for 15 min in a pressure cooker. Slides were then cooled and blocked with blocking solution (10% milk in PBS + 0.3% v/v TritonX + 15 μL Fab donkey anti-mouse IgG (H + L) fragments) for 1 hour at room temperature in a humidified chamber. Following blocking, tumor sections were stained the following antibodies (1:100 in blocking solution) overnight at 4°C in a humidified environment: anti-mouse CRT (PA3-900, ThermoFisher Scientific, USA) and anti-mouse HMGB1 (MA5-16,264, ThermoFisher Scientific, USA) or anti-mouse CD45 (103,101, Biolegend, USA) and anti-mouse CD11c (33,483, Abcam, USA). Tissue samples were then washed four times with PBS + 0.1% v/v Tween (PBST). Secondary antibodies (1:1000 in blocking bluffer) were added: donkey anti-rabbit IgG Alexa Fluor 594 for CRT (A21207, Life Technologies,

USA), goat anti-mouse IgG Alexa Fluor 488 for HMGB1 (115–545-205, Jackson Immuno, USA), donkey anti-rat IgG (H + L) Alexa Fluor 594 for CD45 (A21209, Life Technologies, USA) and goat anti-armenian hamster IgG (H + L) Alexa Fluor 488 for CD11c (127–545-160, Jackson Immuno, USA). Tissue sections were stained for 1.5 hours at room temperature in a humidified environment, protected from light. Following secondary staining, tissue sections were washed four times with PBST and mounted with DAPI (P36935, Molecular Probes, USA). A glass cover slip was placed on top of each tissue section and cured overnight. Sections were viewed under an EVOS FL Auto Imaging System (Life Technologies, USA). Using ImageJ software, mean fluorescence intensity of DAMPs and CD45 +/CD11c+ signals were determined by measuring the intensity of three representative areas on the tissue. Data are presented as normalized mean fluorescence intensity of individual resected tumors.

ELISpot analysis

IFNγ ELISpot assays were previously described.[97] Briefly, on Day 1, multiscreen filtration plates (Millipore, MSIPS4W10) plates were coated with 100 uL per well of anti-IFNγ antibody (BD Biosciences, USA; clone R4-6A2; 10 ug/mL) overnight at 4°C. On day 2, the coating antibody was discarded, the plate washed and blocked. Splenocyes were isolated via mechanical disruption with RBC lysis and added at 1×10^6 cells per well in cRPMI. The following stimulators were added: DMSO (negative control), 10 μg/mL $GUCY2C_{254\text{-}262}$ peptide (JPT, Germany) or 10 μg/mL AH-1 peptide (AnaSpec, USA) for 24 hours in cRPMI. Plates were incubated at 37°C, 5% CO_2 for 24 hours and spots were developed with 2 μg/mL biotinylated anti-IFNγ detection antibody (BD Biosciences, USA; clone XMG1.2) and 2 μg/mL alkaline phosphatase-conjugated streptavidin (ThermoFisher Scientific, USA), followed by NBT/BCIP substrate (ThermoFisher Scientific, USA). Spot forming cells were enumerated using the S6 Universal-V Analyzer automated reader system, software ImmunoSpot v5 (Cellular Technology Limited, USA). Spot parameters were established using automated gating and quantification. Data are presented as antigen-specific spots (normalized by subtracting the DMSO baseline negative control values).

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Disclosure statement

The authors declare no conflict of interest.

ORCID

Dante J. Merlino http://orcid.org/0000-0001-9998-0579
Adam E. Snook http://orcid.org/0000-0001-9216-4560

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