Portable air-fed cold atmospheric plasma device for postsurgical cancer treatment

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Surgery represents the major option for treating most solid tumors. Despite continuous improvements in surgical techniques, cancer recurrence after surgical resection remains the most common cause of treatment failure. Here, we report cold atmospheric plasma (CAP)–mediated postsurgical cancer treatment, using a portable air-fed CAP (aCAP) device. The aCAP device we developed uses the local ambient air as the source gas to generate cold plasma discharge with only joule energy level electrical input, thus providing a device that is simple and highly tunable for a wide range of biomedical applications. We demonstrate that local aCAP treatment on residual tumor cells at the surgical cavities effectively induces cancer immunogenic cell death in situ and evokes strong T cell–mediated immune responses to combat the residual tumor cells. In both 4T1 breast tumor and B16F10 melanoma models, aCAP treatment after incomplete tumor resection contributes to inhibiting tumor growth and prolonging survival.

INTRODUCTION

Surgery represents a mainstay in the cure and control of most solid cancers (1, 2). Despite continuous advances in surgical techniques, residual microtumors and/or circulating tumor cells after tumor resection remain a challenge (3–5). It has long been acknowledged that the perioperative inflammation induced by trauma poses a high risk for the development of tumor recurrence and acceleration of local tumor relapse as well as the promotion of tumor invasion and metastasis (6–8). Adjuvant therapies, including chemotherapy and radiation therapy, are commonly used in combination with surgery to prevent disease recurrence and metastasis (9–11). However, these treatments are often associated with severe adverse effects (12–14). Therefore, it is urgently needed to develop effective strategies for postsurgical cancer treatment.

Plasma, the fourth state of matter distinct from the solid, liquid, and gas, is an ionized gas consisting of many reactive species, radicals, and photons and comprises over 99% of the visible universe (15). Lightning, as an example, is one of the most commonly seen plasma in nature. Spurred by the advances in plasma technology, cold atmospheric plasma (CAP), a unique form of plasma with a near room temperature, has attracted increasing attention in biomedical applications (16–18). Leveraging its high bactericidal activity, CAP has been primarily applied for surface sterilization and wound healing (19–21). Recent studies have also validated that the synergistic actions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in CAP could lead to a potent anticancer effect (22, 23).

Given the unique merit of CAP, we hypothesize that CAP therapy could be explored to inhibit disease recurrence and metastasis after tumor resection (2, 24). State-of-the-art CAP devices mostly apply argon or helium as the feeding gas, unavoidably requiring a complex and heavy pressurized gas supply that can make them inconvenient in medical uses (25–27). To overcome this drawback, this study investigates the efficacy of a convenient and portable ambient air-fed CAP (aCAP) device for postsurgical cancer treatment.

Here, we report the application of a simple and portable aCAP device to inhibit local tumor recurrence after surgery (Fig. 1A). The concept of the portable aCAP is inspired by lightning, a giant spark of electricity with gigajoule energy in the ambient air. Lightning is a naturally occurring electrostatic discharge during which two electrically charged regions form between clouds, the air, and/or ground (28, 29). By reducing the energy regime of lightning from gigajoules to joules through adjusting voltages and distances between electrodes, we devise a new portable aCAP. The aCAP device simply uses ambient air as the feeding gas (Fig. 1B) and thus provides an important improvement over conventional CAP devices that require pressurized gas supplies and feed. Theoretically, aCAP can also generate a high level of ROS/RNS because the discharge occurs solely across atmospheric species (i.e., predominantly N2 and O2) and is not facilitated by typical seed gases (e.g., helium and argon). The portability of aCAP makes it especially attractive for postsurgical applications. By the local application of portable aCAP on residual tumor cells at the surgical cavities, ROS/RNS can induce cancer immunogenic cell death (ICD) and release tumor-associated antigens (TAAAs) in situ, evoking effective antitumor immunity. Immature dendritic cells (DCs) will engulf and process TAAAs into peptides during their migration to the tumor-draining lymph nodes (30, 31), where mature DCs can present antigen peptides to T cells, generating cytotoxic T cells. Subsequent T cell–mediated immune response could inhibit tumor recurrence and growth (Fig. 1A).

RESULTS

The design rationale and working mechanism of the aCAP device are illustrated in Fig. 1B. The three-dimensional (3D) printed case
of the aCAP device comprises the fan mount, internal baffle, condenser nozzle, and throat. A fan (~5 V of input voltage) is inserted into the fan mount to draw ambient air into the main body of the device, which is further guided by the baffles through the nozzle to the main throat of the device. At the end of the throat, two pairs of electrodes are placed perpendicular to the airflow to generate aCAP (Fig. 1C). The electrodes are supplied by independent dc-to-ac converters with integrated step-up transformers that can be powered by a battery or its equivalent (fig. S1). The external features of the device can be readily modified to provide wire guides and other structures as necessary. After systemic optimization, the input voltage and the distance between the electrodes of the aCAP device for stable operation and high efficiencies of CAP generation were 6 V and 7 mm, respectively (fig. S2), which was used for the following studies. The root mean square input power for the aCAP device is approximately 30 W. The optical emission spectrum verified the generation of CAP that contains both ROS and RNS (Fig. 1D) (32). The visual observation and temperature monitoring also confirmed the formation of CAP (~24°C at the nozzle area; fig. S3). Figure 1E shows the simulation of airflow for the aCAP device, and its flow rate is around 6.8 liters/min at 5-V input voltage of the fan.

The efficacy of aCAP was first studied in vitro. After aCAP treatment, increased concentrations of both ROS and RNS were detected in the cell culture media (fig. S4) as well as within cells (Fig. 2, A and B), which causes potent tumor-killing effects. A significant amount of cell death in 4T1 breast cancer cells was observed after aCAP treatment, and longer treatment duration led to a significantly higher cancer cell death rate (Fig. 2C). By contrast, aCAP treatment showed a minimal cell killing effect toward DCs at the same test conditions (fig. S5). To verify the effectiveness of aCAP for inducing ICD, we...
measured the level of calreticulin (CRT) (33, 34), an ICD marker on the surface of cancer cells, after aCAP treatment. Increased expression of CRT in 4T1 cells was detected, and the extent of expression was significantly elevated with increased treatment duration (Fig. 2D). These findings were further demonstrated in B16F10 melanoma cells (fig. S6). TAAAs released during CAP-induced ICD can activate DCs and trigger the maturation of DCs. Hence, we evaluated this immunological effect of aCAP by coculture of bone marrow–derived DCs and aCAP-treated cancer cells in a Transwell assay. Encouragingly, an increased percentage of DC maturation was detected as evidenced by the up-regulation of CD80 and CD86 markers (denoting their maturation status) after 4T1 cells were treated with aCAP (Fig. 2E).

To evaluate the performance of aCAP treatment in vivo, we used an incomplete-tumor-resection model to mimic the postsurgical local relapse. After surgical removal of orthotopic 4T1 breast tumors in mice, aCAP was applied on residual tumor cells within the tumor resection cavity with varying treatment duration (Fig. 3A and fig. S7). Increased levels of CRT were detected in the residual tumor tissues in the mice receiving aCAP treatment (Fig. 3B), demonstrating that aCAP treatment could induce ICD in vivo as well. No significant temperature change was observed in the aCAP-treated areas during the treatment, excluding ICD or cell ablation from a photothermal effect and indicating the safe process of aCAP treatment (fig. S7). The percentage of mature DCs in the tumor-draining lymph node was also significantly elevated in the aCAP-treated groups (Fig. 3, C and D). These results indicate that aCAP could effectively evoke a strong antitumor immune response to combat residual microtumors and inhibit tumor recurrence. To validate its antitumor efficacy, we monitored the tumor growth and survival of mice after aCAP treatment (Fig. 3, E to H). Mice treated with aCAP showed significantly improved control of tumor regrowth compared with the untreated group (surgery-only group). Correspondingly, survival was significantly prolonged in mice receiving aCAP treatment, and longer treatment duration led to better outcomes (Fig. 3G). Over 40% of mice survived for at least 60 days when treated with 4 min of aCAP. The body weights of mice were not affected during treatment,
**Fig. 3. aCAP treatment for inhibition of tumor progression in a 4T1-tumor-incomplete-surgery model.** (A) Schematic of the treatment schedule. (B) Quantification of CRT markers on the remaining 4T1 cells after aCAP treatment \((n = 4)\). Data are presented as means ± SD. (C) Representative flow cytometry plots and (D) quantification of DC maturation in vivo in the tumor-draining lymph nodes \((n = 4)\). Cells in the tumor-draining lymph nodes were collected 5 days after the treatments. Data are presented as means ± SD. (E) In vivo bioluminescence imaging of 4T1 tumors after removal of the primary tumor. Three representative mice per treatment group are shown. Tumor resection was done on day 14. (F) Individual and (G) average tumor growth kinetics in experimental groups \((n = 7)\). Growth curves were stopped when the first mouse died. Data are presented as means ± SEM. Statistical significance in (B), (D), and (G) was calculated via one-way ANOVA with a Tukey post hoc test for multiple comparisons. \(*P < 0.05; **P < 0.01; ***P < 0.001.\) (H) Kaplan-Meier survival curves for treated and control mice \((n = 7)\). Statistical significance was compared with the untreated control group and was calculated via the log-rank (Mantel-Cox) test. \(*P < 0.05; **P < 0.01.\) (I) Body weight changes of mice in each group after different treatments. Data are presented as means ± SD \((n = 7)\).
Fig. 4. Post-surgical aCAP treatment triggering T cell–mediated antitumor immune responses. Quantification analyses of intratumoral (A) CD8+ T cells and (B) CD4+ T cells gating on CD3+ cells following various treatments (n = 4). Cells were collected 5 days after the treatments. Data are presented as means ± SD. (C) Quantitative analysis of Ki67 expression in CD3+CD8+ T cells within the tumors 5 days after treatment (n = 4). Data are presented as means ± SD. (D) Representative flow cytometric analyses of CD4+ and CD8+ T cells gating on CD3+ cells in the tumors 5 days after the treatments. (E) Representative flow cytometric analyses of Ki67 in CD3+CD8+ T cells within the tumors 5 days after treatment (n = 4). (F) Representative immunofluorescence staining of CD4+ T cells and CD8+ T cells in the tumors. Scale bar, 50 μm. DAPI, 4',6-diamidino-2-phenylindole. (G) Cytokine levels in the serum from mice isolated 5 days after different treatments. Data are presented as means ± SD (n = 4). Statistical significance was calculated via one-way ANOVA with a Tukey post hoc test for multiple comparisons. *P < 0.05; **P < 0.01; ***P < 0.001.
which is additional evidence indicating minimal systemic toxicity of aCAP treatment (Fig. 3H). In this proof-of-concept study, the longest postsurgical aCAP treatment was set at 4 min. We expect that the extension of aCAP treatment time or repeated treatments could further enhance its therapeutic efficacy.

In an additional experiment to study T cell–mediated immune responses after aCAP treatment, residual tumors were collected 5 days after treatment and were quantitatively analyzed via flow cytometry. The percentages of both CD3$^+$CD8$^+$ and CD3$^+$CD4$^+$ T cells within tumors were significantly increased in the aCAP-treated groups (Fig. 4, A to D). CD3$^+$CD8$^+$ T cells exhibited enhanced proliferative potential as evidenced by the increased coexpression of cell proliferation marker Ki67 (Fig. 4, C and E) (35). Immunofluorescence staining of the residual tumors also indicated the increase in the intratumoral infiltration of CD4$^+$ and CD8$^+$ T cells after CAP treatment (Fig. 4F), which is consistent with the flow cytometry results. Cytokine secretion is also important in the process of antitumor immune responses (36, 37). In a parallel experiment, sera of mice 5 days after treatment were collected and analyzed. Elevated levels of cytokine secretion were observed, including interferon-γ (IFN-γ), tumor necrosis factor–α (TNF-α), interleukin-12p70 (IL-12p70), IL-2, and, IL-10, further substantiating the effective immune response induced by aCAP (Fig. 4G).

To demonstrate that aCAP could be broadly applicable in other postsurgical tumor models, we evaluated its antitumor efficacy in B16F10 melanoma-bearing mice. Mice received aCAP treatment after incomplete tumor resection. Similarly, we found that aCAP significantly promoted anticancer effects as indicated by the enhanced control tumor growth and prolonged survival (fig. S8).

DISCUSSION
Cancer recurrence after surgical resection often occurs in a substantial fraction of patients, causing treatment failure. Here, we have developed a portable aCAP device for postsurgical cancer treatment. This device simply requires ambient air and removes the need for associated complexities related to consumable feed gases such as argon and helium, thus simplifying CAP equipment configurations and more broadly facilitating its applications in medicine. The results of this study show that aCAP-mediated therapy can effectively evoke a T cell–mediated antitumor immune response to inhibit local tumor growth after tumor resection in both 4T1 breast cancer and B16F10 melanoma models, thus prolonging survival in mice. We anticipate that this treatment approach could be also applicable to other types of solid cancer. Moreover, this simple postsurgical treatment strategy holds high promise for potential translation. Further studies associated with the optimization of device parameters and treatment regimen, as well as on large animal models, are expected. In addition, aCAP treatment could be combined with cancer immunotherapies, such as immune checkpoint blockade, to further potentiate the therapeutic outcomes.

MATERIALS AND METHODS
Materials, cell lines, and animals
A fluorometric hydrogen peroxide assay kit was purchased from Sigma-Aldrich. A Griess reagent kit was obtained from Promega. The murine 4T1 breast cancer cell line and B16F10 melanoma cell line were purchased from the UNC Tissue Culture Facility. Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) and penicillin (100 U/ml; Invitrogen) at 37°C in 5% CO₂. Female BALB/c and C57BL/6 mice (4 to 5 weeks) were purchased from the Jackson laboratory. All mouse studies were carried out following the protocols approved by the Institutional Animal Care and Use Committee at the University of California, Los Angeles (UCLA).

Portable aCAP device configuration
The aCAP device was constructed using a 3D printer (LulzBot TAZ 6) at UCLA. The 3D printed unit comprises the fan mount, internal baffle, condenser nozzle, and throat (fig. S1). A low-voltage fan is inserted into the fan mount to draw ambient air into the main body. The air is guided through the nozzle to the main throat of the device. At the end of the throat, two pairs of electrodes are placed perpendicular to the flow to generate the plasma discharge. The electrodes are supplied by a power processing unit (PPU) that includes independent dc-to-ac converters with integrated step-up transformers and is powered by a battery or its equivalent; the fan has its own independent power supply that could be easily integrated into the PPU. The external features of the device can be modified to provide wire guides, housing for transformers, and other structures as necessary for deployment in medical applications. The input voltage, distance between the electrodes, and flow rate of the air plasma device for stable operation and high species delivery efficiency were 6 V, 7 mm, 16.8 liters/min, respectively.

Optical emission spectroscopy of aCAP
A fiber-coupled optical spectrometer from LR1-ASEQ Instruments (Vancouver, Canada) was used to detect aCAP-generated reactive oxygen and nitrogen species (22). The optical probe was placed at a distance of approximately 10 mm from the center of the aCAP device.

In vitro cell viability evaluation after aCAP treatment
4T1 cells or B16F10 cells were seeded in a 24-well plate at a density of 1 × 10⁵ cells per well overnight. Cells were then treated with aCAP for 10, 20, 30, or 40 s and were further incubated for another 24 hours. The cell viability was measured using a Cell Counting Kit-8 following the manufacturer’s protocol. Cells with no treatment were used as control.

In vitro evaluation of CRT levels after aCAP treatment
4T1 cells were seeded in a 24-well plate at a density of 1 × 10⁵ cells per well overnight. Cells were then treated with aCAP for 10, 20, 30, or 40 s and were further incubated for another 24 hours. Thereafter, cells were stained with anti-Calreticulin antibody (Abcam, catalog no. ab92516), followed by flow cytometry analyses [BD LSRII (IMED)].

In vitro DC maturation
DCs were first isolated from the bone marrow following an established method reported previously (36). DC maturation was studied in a Transwell culturing system, where the upper chamber was cultured with 4T1 cancer cells and the lower chamber was cultured with DCs. 4T1 cells were treated with aCAP and were then cocultured with DCs for 24 hours. Thereafter, DCs were collected and stained with the fluorescence-labeled maturation marker–specific antibodies (CD80 [BioLegend, catalog no. 104707, phycoerythrin (PE)–labeled] and CD86
mice. Two weeks later, mice with tumor size about 400 mm$^3$ were divided into $n = 6$). Tumor removal following aCAP treatment was 3 days later, mice with tumor size about 400 mm$^3$ were divided into $n = 3$, and tumors were resected, leaving $\approx 2$ to 5% residual tumor (confirmed by IVIS imaging based on bioluminescence of tumor cells) to mimic residual micrometastatic after surgery. In brief, mice were anesthetized using isoflurane during surgery and treatment. Sterile instruments were used to remove $\approx 95$ to 98% of the tumor [confirmed by In Vivo Imaging System (IVIS) imaging] (1-4). Immediately after surgery, aCAP was applied to the center and $\approx 1$ cm above the surgical sites for the different durations (1, 2, 3, and 4 min). Temperature changes during treatment were monitored by a FLIR thermal camera. The wound was then closed by an Autoclip wound clip system. The tumor volume was measured by a digital caliper and was calculated according to the following formula: width$^2 \times$length $\times 0.5$. Tumor growth kinetics were also recorded using IVIS imaging. Body weights and survival of mice were also recorded. Mice with tumor removal without other additional treatment were used as controls. Animals were euthanized when showing signs of imperfect health or when the size of tumors exceeded 1.5 cm$^3$

B16F10 melanoma tumor model: A total of 1 $\times$ 10$^6$ luciferase-tagged 4T1 cells were transplanted into the mammary fat pad of female BALB/c mice. Eleven days later, mice with tumor size about 400 mm$^3$ were divided into six groups ($n = 6$). Tumor removal following aCAP treatment was similar as described above.

Flow cytometry for in vivo cell analyses
Tumors and draining lymph nodes were collected 5 days after treatment, cut into small pieces, and homogenized to form a single cell suspension. Cells were stained with fluorescence-labeled antibodies. The stained cells were measured on an LSR II (BD Biosciences) and analyzed by the FlowJo software package (version 10.0.7; TreeStar, USA, 2014). For DC maturation tests: CD80 (BioLegend, catalog no. 104707, PE-labeled), CD86 (BioLegend, catalog no. 117343, Brilliant Violet 421–labeled). For T cell analyses: CD3 (BioLegend, catalog no. 100228, Brilliant Violet 421–labeled), CD4 (BioLegend, catalog no. 100408, PE-labeled; catalog no. 100422, PE/Cy7-labeled), CD8 (BioLegend, catalog no. 100712, APC-labeled), and Ki67 (BioLegend, catalog no. 652410, fluorescein isothiocyanate–labeled). Compensation was performed for multicolor analyses. All antibodies were used following the manufacturers’ instructions. Antibodies were diluted 500 times except for CD3 (100 times).}

Cytokine analyses in vivo
Serum levels of IL-2 (BioLegend, catalog no. 431001), IL-12p70 (BioLegend, catalog no. 433607), IL-10 (BioLegend, catalog no. 431414), IFN-γ (BioLegend, catalog no. 430801), and TNF-α (BioLegend, catalog no. 430904) were measured with enzyme-linked immunosorbent assay kits following the manufacturer’s instructions. Serum samples were isolated from mice 5 days after treatment.

Statistical analysis
All results are presented as means $\pm$ SD or as means $\pm$ SEM. Tukey post hoc tests and one-way analysis of variance (ANOVA) were used for multiple comparisons (when more than two groups were compared), and Student’s $t$ test was used for two-group comparisons. The survival benefit was determined using a log-rank test. All statistical analyses were carried out with Prism software package (Prism 5.0; GraphPad Software, 2007). The threshold for statistical significance was $P < 0.05$.

SUPPLEMENTARY MATERIALS
Supplemental material for this article is available at https://science.sciencemag.org/lookup/doi/10.1126/sciadv.abb5686

View/request a protocol for this paper from Bio-protocol.

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Chen et al., Sci. Adv. 2021; 7 : eabg5686 1 September 2021

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Acknowledgments
Funding: This work was supported by grants from the start-up packages of UCLA and Zhejiang University (to Z.G.), National Institutes of Health (R01 CA234343-01A1 to Z.G.), Air Force Office of Scientific Research (FA9550-14-10317, UCLA subaward no. 60796566-114411 to R.E.W.), Air Force Office of Scientific Research (FA9550-21-1-0067 to R.E.W.), Jonsson Comprehensive Cancer Center at UCLA, the start-up packages of McGill University (to G.C.), National Innovation Center for Advanced Medical Devices (to Z.C.), and NSERC Discovery Grant (RGPIN-2021-02669 to G.C.). Author contributions: Z.G., R.E.W., G.C., and Z.C. were responsible for the conception and experimental strategy of the study. G.C., Z.C., Z.W., R.O., D.W., and H.L. performed the experiments and acquired the data. G.C., Z.C., Z.W., R.O., D.W., H.L., R.E.W., and Z.G. interpreted the data. G.C., Z.C., R.E.W., and Z.G. cowrote the manuscript. Competing interests: R.E.W., Z.G., Z.C., and G.C. are inventors on a patent application related to this work filed by the University of California (filed 4 June 2021). Z.G. is a scientific cofounder of ZenCapsule Inc., ZCapsule Inc., and Zenomics Inc. The authors declare that they have no other competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 14 January 2021
Accepted 12 July 2021
Published 1 September 2021
10.1126/sciadv.abg5686

Citation: G. Chen, Z. Chen, Z. Wang, R. Obenchain, D. Wen, H. Li, R. E. Wirz, Z. Gu, Portable air-fed cold atmospheric plasma device for postsurgical cancer treatment. Sci. Adv. 7, eabg5686 (2021).