Plasma-generated short-lived reactive oxygen and nitrogen species are the main inducers of immunogenic cancer cell death

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Abstract: The progression of cold atmospheric plasma research in cancer immunotherapy requires understanding of its effectors. In this work, we studied the interaction of melanoma cancer cells with plasma. We showed, using a combination of chemical and biological analyses, that the short-lived reactive oxygen and nitrogen species are responsible for immunogenic cell death. The developed vaccination methodology also validated our *in vitro* findings, demonstrating a way for plasma to be efficiently used for cancer immunotherapy.

Keywords: cold atmospheric plasma, dielectric barrier discharge, short-lived reactive species, EPR analysis, immunogenic cell death, vaccination assay.

1. Introduction

Cancer immunotherapy research has led to significant positive impacts on patient survival. Still, there is a need to develop new treatments that will help improve clinical outcomes, enabling an effective self-sustaining anti-cancer response in patients. One way to achieve this is via induction of immunogenic cell death (ICD) in the tumour. ICD is a type of cell death, governed by "danger signals", of which the most characteristic and studied is surface-exposed calreticulin (CRT) [1].

In recent years, the potential of cold atmospheric plasma (CAP) to induce ICD has been extensively investigated. The high rate of CRT emission due to CAP treatment was reported *in vitro* and *in vivo* [2,3].

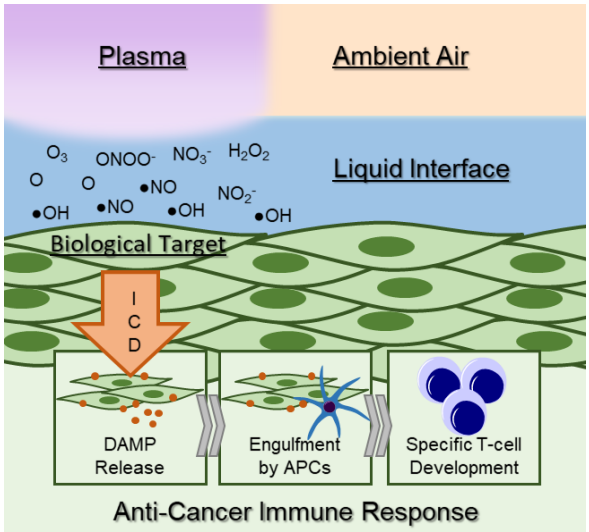

Fig. 1. Elucidating the interaction of cold plasma with cancerous targets enables development of biomedical plasma devices for a robust ant-cancer immune response.

Cold plasma consists of various reactive oxygen and nitrogen species (RONS), ultraviolet radiation, and electric fields, which can interact with the biological target [4] (Fig. 1). To date, studying the interaction of RONS with the biological substrates remains a task at hand, and specifically, the exact RONS which are responsible for the CAP-induced effects. Although the long-lived RONS (such as in plasma-treated media [5]) are often considered the main contributors to plasma treatment effects, it has recently been shown that the short-lived RONS are crucial for efficient CAP anti-cancer treatment of 3D tumours [6].

In this work, we investigated the complex 'cocktail' of RONS produced by a µs-pulsed DBD plasma (17 kV, 50-500 Hz) in contact with a biological target, and elucidated the main RONS which elicit ICD in melanoma cells.

2. Chemical analyses of RONS

Since liquid is an essential part of biological and biomedical milieu *in vitro* and *in vivo*, the interaction of RONS with liquids requires evaluation. Most RONS are generated by CAP in the gas phase, and are further delivered to liquid [7,8]. We monitored various long- and short-lived RONS in plasma-treated PBS (used for *in vitro* experiments). Long-lived RONS (H_2O_2, NO_2^-, NO_3^-) were quantitatively assessed using UV-Vis spectrophotometry [9]. Short-lived RONS (•OH, $O_2^{\bullet-}$/•OOH, •NO, O, $^1O_2$) were monitored by electron paramagnetic resonance (EPR) spectroscopy via various spin trapping reactions [10].

This combination of analyses has revealed that some of the RONS often present in CAP-liquid systems [11] were not present in liquid media by our DBD plasma, such as $O_2^{\bullet-}$ and $^1O_2$. We also acknowledge that other reactive species (e.g. ClO^- formed from Cl^- in PBS [12]) may also be present but were not measured in this study.

Moreover, we quantitatively detected a semi-short-lived RONS: peroxynitrite anion $ONOO^-$. We evaluated its lifetime (s to min) in PBS using an in-house developed colourimetric method [13].

We also monitored the species concentration development over different periods of time, with different pulse frequency. The amount of RONS created by CAP was dependent on the total CAP power rather than on pulse frequency or treatment time alone.

3. *In vitro* studies

We measured cell survival and surface CRT [14] induced by CAP in mouse (B16F10) and human (A375) melanoma cell lines. We used the high frequency discharge (500 Hz) to minimise treatment time (10 s).

We also investigated which RONS caused ICD. For this, we prepared solutions of commercially available RONS (H_2O_2, NO_2^-, NO_3^-, $ONOO^-$) and treated cells with them, as is or together with electromagnetic fields.

Flow cytometry analysis showed reduced cell survival with the RONS mock solution in combination with electromagnetic field. However, the CRT expression of the cells with intact membranes was only elevated when directly treated by plasma. Since the difference between the mock solution and the plasma treatment is short-lived RONS generated by plasma (which are absent in 'mock solutions'), we conclude that the short-lived RONS are largely responsible for ICD induction.

4. *In vivo* validation

We developed a highly efficient vaccination procedure, allowing direct validation of the ICD efficiency. Of the 8 mice vaccinated with our CAP-created vaccine, 6 have survived and 5 were completely tumour-free by the end of the study, compared to those of our negative controls (1 out of 8 surviving). This proves the efficient ICD induction by CAP, and its potential in clinical practices.

5. Conclusions

Using the DBD CAP system, we were able to induce ICD in melanoma cells. The observed effect is attributed to the long-lived and short-lived plasma RONS, rather than physical components of plasma such as electromagnetic fields. Moreover, we show that the short-lived RONS are required to efficiently induce ICD, as demonstrated by CRT expression by cancer cells *in vitro*, and by the vaccination assay *in vivo*. This emphasises the importance of CAP treatment over a combination of the plasma-generated long-lived RONS. In this context, CAP is a unique system for generation and delivery of short-lived RONS, unequalled by any other method.

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