Научном већу Института за физику

Београд, 19. 11. 2021.

Предмет:

Молба за покретање поступка за избор у звање истраживач сарадник

Молим Научно веће Института за физику у Београду да покрене поступак за мој избор у звање истраживач сарадник.

У прилогу достављам:

1. Мишљење руководиоца лабораторије са предлогом комисије за избор у звање;
2. Кратку стручну биографију ;
3. Кратак преглед научне активности;
4. Списак објављених радова и других публикација;
5. Потврду о уписаним докторским студијама;
6. Потврду о просеку на основним и мастер студијама
7. Потврду о пријављеној теми докторске дисертације.

С поштовањем,

Анђелија Петровић

истраживач приправник

Анђелија Петровић

Београд, 19. 11. 2021,

Предмет: Мишљење руководиоца лабораторије о избору Анђелије Петровић у звање истраживач сарадник

Анђелија Петровић била је ангажована на пројекту основних итраживања Министарства просвете, науке и технолошког развоја Републике Србије ИИИ41011 под називом "Примене нискотемпературних плазми у биомедицини, заштити човекове околине и нанотехнологијама". Запослена је у Лабораторији за неравнотежне процесе и примену плазме Института за физику у Београду од децембра 2018. године, када је изабрана у звање истраживач приправник. Ради на теми конструисања плазма система и испитивања процеса који се дешавају у систему плазма млаза у контакту са течношћу у циљу третмана течних узорака плазмом под руководством др Николе Шкора. С обзиром да испуњава све предвиђене услове у складу са правилником о поступку, начину вредновања и квантитативном исказивању научноистраживачких резултата Министарства просвете, науке и технолошког развоја, сагласна сам са покретањем поступка и предлажем избор Анђелије Петровић у звање истраживач сарадник.

За састав комисије за избор Анђелије Петровић у звање истраживач сарадник предлажем:

1. Др Невена Пуач, научни саветник, Институт за физику
2. Др Никола Шкоро, виши научни сарадник, Институт за физику
3. Проф. др Срђан Буквић, редовни професор, Физички факултет

Др Гордана Маловић
Научни саветник Института за физику
Руководилац Лабораторије за неравнотежне процесе и
примену плазме

Кратка стручна биографија

Анђелија Петровић рођена је 27.01.1994. године у Крагујевцу. Након завршених основних академских студија на Физичком факултету Универзитета у Београду, уписује мастер академске студије на истом факултету. Мастер академске студије завршава 2018. године са просечном оценом (10.00). Мастер рад под називом „Електрична карактеризација и примене малог преносивог система плазма игле“ урадила је у Лабораторији за гасну електронику на Институту за физику у Београду под руководством др Невене Пуач, научног саветника. Након успешно одбрањеног мастер рада, 2018. године уписује докторксе академске студије на Физичком факултету у Београду смер Физика јонизованог гаса и плазме. Положила је све изабране испите са просечном оценом 10.00.

Анђелија Петровић је у радном односу од децембра 2018. године у Институту за физику у Београду у Лабораторији за неравнотежне процесе и примену плазме и била је ангажована на пројекту „Примене нискотемпературних плазми у биомедицини, заштити човекове околине и нанотехнологијама“ (ИИИ41011). Под руководством др Николе Шкора ради на темама везаним за третман течних узорака плазма млазом.

Списак објављених радова и других публикација:

Радови у врхунским међународним часописима (категорија М21):

Sergej Tomić, Anđelija Petrović, Nevena Puač, Nikola Škoro, Marina Bekić , Zoran Lj. Petrović and Miodrag Čolić, Plasma-Activated Medium Potentiates the Immunogenicity of Tumor Cell Lysates for Dendritic Cell-Based Cancer Vaccines, *Cancers* **2021**, *13*(7), 1626; https://doi.org/10.3390/cancers13071626

Предавање по позиву са међународног скупа штампано у целини (М31)

Anđelija Petrović, Nikola Škoro and Nevena Puač, Treatment of RPMI 1640 cell medium by atmospheric pressure plasma jet, ISPlasma2021/IC-PLANT2021, March 7-11 2021, virtual symposium 08pE15O

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Саопштење са међународног скупа штампано у изводу (М34)

Zoran Lj. Petrović, Nevena Puač, Sergej Tomić, Anđelija Petrović, Nikola Škoro, Marina Bekić, Dragana Vučević and Miodrag Čolić Plasma-activated medium potentiates dendritic cell-mediated anti-tumor response in vitro, Twenty-second International Summer School VEIT, 20 – 24 September 2021, Sozopol, Bulgaria, **PR-7**

Nevena Puač, Sergej Tomić, Anđelija Petrović, Nikola Škoro, Marina Bekić, Dragana Vučević, Zoran Lj. Petrović, and Miodrag Čolić, Immunogenic death of tumor cells induced by plasma activated medium potentiates dendritic cell-mediated anti-tumor response *in vitro* **,** 12th EU-Japan Joint Symposium on Plasma Processing (JSPP-12), 2021, March 29 – April 2021, O - 132

Nikola Škoro, Nevena Puač, Olivera Jovanović, Anđelija Petrović, Zoran Lj. Petrović, Creation and destruction of chemical species in liquids treated by atmospheric pressure plasmas - from gas phase chemistry to bulk liquid, The 1st annual meeting of the COST Action CA18212 "Molecular Dynamics in the GAS phase" (MD-GAS 2020) (February 18. – 21., Caen, France) (2020))

Pavlović O., Škoro N., Lazarević M., Petrović A., Mojsilović S., Puač N., Miletić M, Insight into the anti-tumor mechanism of non-thermal atmospheric pressure plasma in oral squamous cell carcinoma, 7th International Workshop on Plasma for Cancer Treatment (IWPCT-2021), June 29th - 30th 2021, O14

Nikola Skoro, Nevena Puac, Amit Kumar, Olivera Jovanovic, Andjelija Petrovic, Uros Cvelbar, Zoran Lj. Petrovic, Atmospheric pressure plasma treatment and decontamination of water samples, 240th ECS Meeting (October 10-14, 2021), D05-1

Anđelija Petrović**,** Nevena Puač**,** Nikola Škoro, Gordana. Malović and Zoran Lj. Petrović, Electrical characterisation of atmospheric plasma jet during treatments of RPMI 1640 cell medium, XXXIV International Conference on Phenomena in Ionized Gases (XXXIV ICPIG), 2019, July 14th – 19th 2019, TL-22

Предавање по позиву са скупа националног значаја штампано у изводу (М62)

Сергеј Томић, Анђелија Петровић, Невена Пуач, Никола Шкоро, Марина Бекић, Зоран Петровић, Драгана Вучевић, Миодраг Чолић, Атмосферска плазма потенцира имуногеност туморских лизата у туморским вакцинма на бази дендритских ћелија, СРПСКА АКАДЕМИЈА НАУКА И УМЕТНОСТИ, Светски дан имунологије 2021, Одбор за имунологију и алергологију САНУ, Друштво имунолога Србије

Преглед научне активности Анђелије Петровић

Анђелија Петровић бави се дијагностиком система плазма млаза у контакту са течношћу. Плазма млаз на атмосферском притиску је електрично пражњење у околном ваздуху које ствара богато окружење хемијски реактивних честица. Врста и својства ових честица у гасној фази могу се контролисати параметрима плазме. Реактивне врсте створене у гасној фази могу продрети или додатно изазвати реакције са компонентама течности и створити дугоживеће реактивне врсте, између осталих водоник пероксид и азотне врсте (нитрате и нитрате). Овако третирана течност је погодна за даљи третман биолошких узорака, као што су ћелије или ткива. Колегиница Петровић је до сада плазма млазом третирала RPMI 1640 стандардни медијум за узгој ћелија и дестиловану воду. У циљу детаљне дијагностике и оптимизације система пазма млаза за третман ових течности колегиница је извршила електричну карактеризацију система плазма млаза у конфигурацији са две електроде (једна напајана, друга уземљена) и у конфигурацији са једном напајаном електродом. Оптичком емисионом спектроскопијом испитивала је које то ексцитоване врсте постоје у гасној фази при контакту плазма млаза са површином течности. Такође брзом ICCD камером снимала је и проучавала морфологију пражњења у контакту са течношћу. Ефекте третамна испитивала је детекцијом реактивних врста створених у плазмом третиране течности. У ту сврху развила је колориметријске технике за мерење дугоживећих врста као што су водоник пероксид, нитрати и нитрити које се могу користити са обојеним растворима. Физичко-хемијска својства течности третиране плазмом испитривала је помоћу доступне апаратуре и то температуру, растворени O, проводљивост и pH.

У сарадњи са колегама са Института за примену нуклеарне енергије испитивала је ефикасност примене медијума третираног плазмом за третман туморских ћелија како би се произвели туморски лизати за стварање туморске вакцине. Са колегама са Института за биолошка истраживања „Синиша Станковић" испитивала је утицај воде активиране плазмом у лечењу дијабетичких рана на мишевима.

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Article

Plasma-Activated Medium Potentiates the Immunogenicity of Tumor Cell Lysates for Dendritic Cell-Based Cancer Vaccines

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Simple Summary: Dendritic cells (DCs)-based anti-cancer vaccines displayed limited efficacy in clinical trials, mostly due to a lack of protocols for preparing immunogenic tumor antigens used in the vaccine. Here, a unique atmospheric pressure plasma jet was used to prepare a plasma-activated medium (PAM) which induced immunogenic cell death in tumor cells. This procedure increased the efficacy of tumor lysates in enhancing the immunogenicity of DCs according to their increased maturation, production of IL-12, and the capacity to induce cytotoxic CD8 T cells able to kill tumor cells. In contrast to the tumor lysates commonly used in DC vaccines, PAM-tumor lysates lacked the capacity to increase IL-10 production by DCs, and their potential to induce protumorogenic Th2 and regulatory T cells. Cumulatively, these results suggest that the novel method for preparing immunogenic tumor lysates with PAM could be suitable for improved DC-based immunotherapy of cancer patients.

Abstract: Autologous dendritic cells (DCs)-based vaccines are considered quite promising for cancer immunotherapy due to their exquisite potential to induce tumor antigen-specific cytotoxic T cells. However, a lack of efficient protocols for inducing immunogenic tumor antigens limits the efficacy of DC-based cancer vaccines. Here, we found that a plasma-activated medium (PAM) induces immunogenic cell death (ICD) in tumor cells but not in an immortalized L929 cell line or human peripheral blood mononuclear cells. PAM induced an accumulation of reactive oxygen species (ROS), autophagy, apoptosis, and necrosis in a concentration-dependent manner. The tumor lysates prepared after PAM treatment displayed increased immunogenicity in a model of human monocyte-derived DCs, compared to the lysates prepared by a standard freezing/thawing method. Mature DCs loaded with PAM lysates showed an increased maturation potential, as estimated by their increased expression of CD83, CD86, CD40, IL-12/IL-10 production, and attenuated PDL1 and ILT-4 expression, compared to the DCs treated with control tumor lysates. Moreover, in co-culture with allogeneic T cells, DCs loaded with PAM-lysates increased the proportion of cytotoxic IFN-γ+ granzyme A+ CD8+ T cells and IL-17A-producing T cells and preserved the Th1 response. In contrast, control tumor lysates-treated DCs increased the frequency of Th2 (CD4+IL-4+), CD4, and CD8 regulatory T cell subtypes, none of which was observed with DCs loaded with PAM-lysates. Cumulatively, these results suggest that the novel method for preparing immunogenic tumor lysates with PAM could be suitable for improved DC-based immunotherapy of cancer patients.

Keywords: plasma activated medium; dendritic cells; tumor vaccines; Th polarization

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1. Introduction

Cold atmospheric plasma (CAP), also called non-equilibrium atmospheric-pressure plasma (NEAPP), is a partially ionized gas generated under normal atmospheric pressure and ambient temperature [1]. Achieving non-equilibrium conditions at atmospheric pressure proved to be possible in only a limited number of cases. Just recently, CAP sources have become more diversified and are being developed with specific applications in mind. CAP is a source of reactive species, ions, neutral particles and molecules, electrons, and other physical factors such as electromagnetic fields, metastable and excited molecules, weak ultraviolet radiation, etc., while producing only a very weak or negligible heating effect [2]. CAP has been widely used in various fields of modern medicine, such as promoting wound healing, blood coagulation, stem cell differentiation and the treatment of some skin diseases, as an anti-bacterial, anti-viral and sterilization agent [3]. In particular, CAP has recently been tested for cancer treatment [2,4–6]. The therapeutic effect of low-temperature plasma is based on the production of various reactive oxygen and nitrogen species (RONS) [7], such as nitric oxide (•NO) and hydroxyl (•OH) radicals [8]. In contact with the cancer tissue, CAP produced RONS are capable of inducing cell death and this approach has been extensively explored for the treatment of tumor because malignant cells are extremely vulnerable to the effect of RONS and die by necrosis, apoptosis, or necroptosis. In contrast, normal cells are less sensitive to CAP [9]. An indirect approach to using CAP in medicine is the production of a plasma-activated medium (PAM). The gas phase plasma chemistry and the plasma chemistry in the gas/liquid (i.e., cell culture medium) interface induce chemical reactions in the liquid phase. As a result, specific compounds are created in the liquid phase which are responsible for the effects on cells (bacteria, plant cells, human cells, cancer cells, stem cells, etc.). The chemistry, both in a gas phase and liquid phase, depends on the plasma parameters (type of gas mixture, gas flow, concentration of electrons, temperature of electrons, deposited power etc.), and on the type of liquid medium that is being treated by plasma. PAM may be prepared by treating aqueous solutions, including cell culture media, with CAP. In this process, RONS are transported from the gas phase into the liquid surface, dissolved into the medium, and undergo further reactions with dissolved molecules in the aqueous solution. Mixing of the gas-phase RONS with the medium is promoted (in this paper as well) by a strong flow of gas/plasma into the liquid whereby "bubbles" of plasma effluent are formed within the top layer of the liquid.

The interaction of gas-phase RONS with aqueous organics produces other relatively long-lived RONS, such as hydrogen peroxide (H2O2), nitrates, nitrites and organic peroxides (RO2) [8]. Due to the long-lived RONS, PAM has been shown to be as effective in killing cancer cells as direct treatment with CAP, and the effect of both treatments is enhanced by intracellular ROS production [10]. The "treatment dose" depends on the source of the plasma, the time of plasma exposure of the liquid, as well as the period for which the cells or tissue is allowed to remain in contact with PAM [11]. The efficacy of plasma in the treatment of malignant tumors is based on two general principles. The first includes a direct cytotoxic effect caused by RONS- mediated intracellular oxidative stress followed by inactivation by anti-oxidative mechanisms. This phenomenon is of special relevance, knowing that the increase of pro-oxidative mechanisms in the tumor could be beneficial for tumor therapy [12]. The second pathway involves activation of the anti-tumor immune response by molecules released from CAP- and PAM-dependent immunogenic cell death (ICD) [13].

ICD is a type of cell death, also referred to as immunogenic apoptosis, which is characterized by a release or display of damage-associated molecular patterns (DAMPs). The most important DAMPs released from cancer cells during ICD are calreticulin, adenosine triphosphate (ATP), heat-shock proteins, and high mobility group protein B1 (HMGB1). DAMPs are a potent adjuvant for antigen-presenting cells (APCs), especially dendritic cells (DCs), inducing their migration to the regional lymphoid tissue, maturation, and stimulation of a specific anti-tumor immune response [14]. DCs are specialized APCs

playing a key role in the initiation and regulation of innate and adaptive immune responses. Due to their unique properties, DCs have been extensively investigated and used to improve cancer immunotherapy. In this context, many strategies have been developed to target DCs in cancer [15]. One of them is the in vitro generation of DCs from monocytes (MoDCs), loading of these APCs with autologous tumor lysates together with maturation stimuli, and inoculation of thus prepared DCs vaccines in cancer patients [16]. The use of DC vaccines for cancer therapy has been extensively investigated, with more than 200 completed clinical trials to date. The injected MoDCs migrate to the regional lymph nodes where they efficiently induce CD4+ T cell- and CD8+ T cell-mediated anti-tumor responses. MoDCs can be also recruited into the tumor microenvironment (TME) following treatment with ICD inducers, including CAP, with the potency to the prime anti-tumor T-cell responses [15]. The clinical benefit of DC-based vaccines is not as efficient as expected due to the presence of many inhibitory molecules and mechanisms in TME. Therefore, an immunotherapy approach based on improved DC vaccines together with the blockade of inhibitory molecules in TME sounds promising for future tumor therapy [15,17]. Optimization of DC vaccines implies the use of better protocols for preparing tumor antigens, designing new strategies for antigen loading, as well as selection of optimal adjuvants and maturation stimuli. In this context, the use of lysates from tumor cells subjected to ICD could be beneficial, as already well documented [14,18–20]. PAM treatment of tumor cells has not been explored up to the date for the stimulation of MoDCs. This was the reason why we tested the hypothesis that a lysate prepared from PAM-treated tumor cells is superior to the induction of immunogenic DCs than the tumor lysates prepared under the conventional freezing-thawing protocol, commonly employed in clinics.

2. Materials and Methods

2.1. Experimental Setup-Plasma Treatments

Figure 1 illustrates the schematics of the dielectric barrier discharge (DBD) atmospheric pressure plasma jet (APPJ) setup and the plasma treatment of a medium placed in the well of a 24-well plate. The DBD jet consists of a glass tube with an inner diameter of 4 mm and an outer diameter of 6 mm. The two electrodes placed around the tube are 15 mm wide and made of copper tape. The surface of the target grounded through a resistor served as the third electrode. The electrode positioned at 5 mm from the edge of the glass tube was connected to the power supply. Another grounded electrode was placed at a distance of 10 mm from the powered electrode. This electrode was grounded over a second resistor to the same point of the electrical circuit as the ground line of the target. The power supply of the APPJ consists of a function generator (PeakTech DDS Function Generator, PeakTech GmbH, Ahrensburg, Germany), a home-made amplifier, and a high-voltage transformer operating at its resonance frequency of 81 kHz. A sine-wave high-voltage signal was provided to power the APPJ. One high-voltage (HV) probe (P6015A HV probe, Tektronix Inc., Beaverton, OR, USA) and two voltage probes (N2863B voltage probe, Agilent, Santa Clara, CA, USA) were used to record voltage and current waveforms. The current waveforms were obtained at the resistors (R = 1kΩ) in the grounded branches of the electrical circuit. They were used to monitor the stability of plasma and calculate the power delivered from the plasma to the sample. This power was 1.2 W in all experiments. The RMS voltage was 1.9 kV and the RMS current was 2.2 mA. The APJ was operating with 2 slm of He as the working gas. The distance between the ending of the APPJ tube and the surface of the sample was 5 mm in all experiments. After the sample treatment, the production of RONS in PAM was investigated by spectrophotometry and colorimetric methods and the concentrations of nitrite ions, nitrate ions and hydrogen peroxide were determined. The pH value of the PAM-RPMI 1640 was not changed after the plasma treatment.

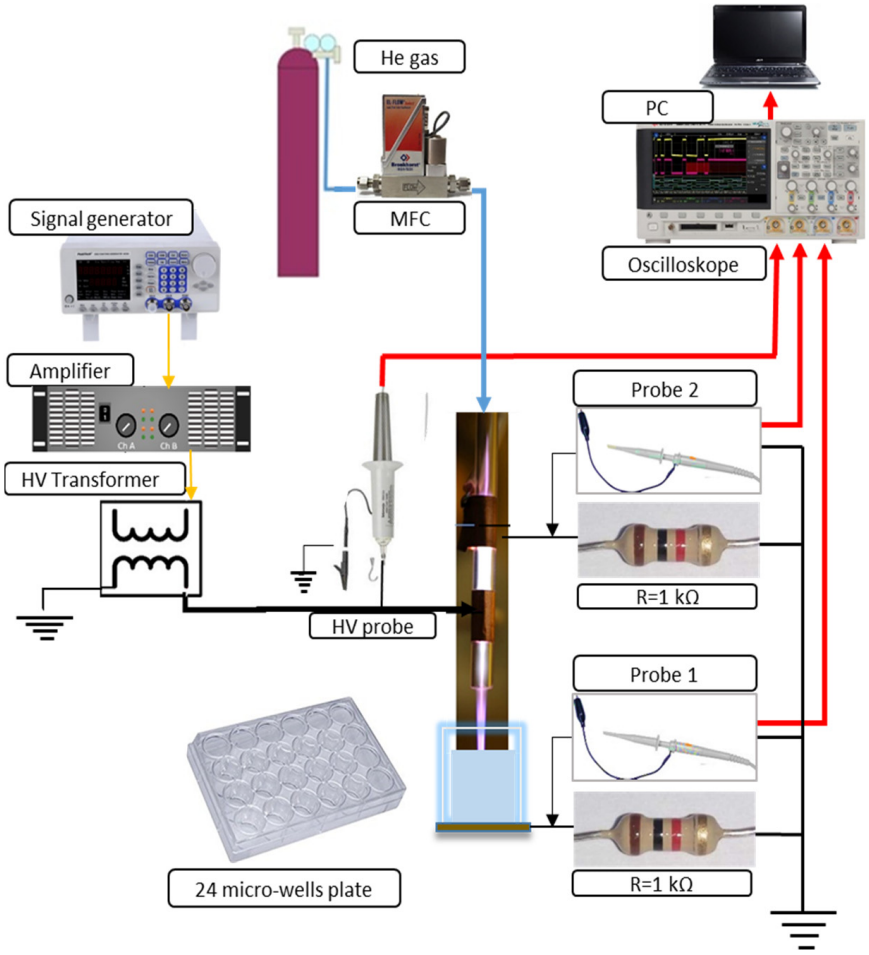

Figure 1. Schematic diagram of the experimental setup.

2.2. Measurement of RONS in the Plasma-Activated Medim

Immediately after the plasma treatment PAM was placed in vials and frozen in liquid N_2. RONS (H_2O_2, NO_2^-, NO_3^-) were measured by using colorimetric measurements. The measurements were performed immediately after the plasma treatment and, as a control, after defrosting the PAM. No differences in RONS concentrations were noticed between these samples (data not shown). This is in accordance with the previously reported results. The good stability of PAM properties in room conditions has been shown to be preserved for 8 to 18 h [21]. Moreover, freeze-thaw procedures for different PAM were studied and it reportedly retains the properties when kept at low temperatures [22].

The H_2O_2 and NO_3^- concentrations of the treated medium were analyzed by using commercial H_2O_2 test stripes (Merckoquant 110011, Merck, Darmstadt, Germany) and commercial NO_3^- test stripes (Merckoquant 110020, Merck, Darmstadt, Germany). The test stripes were scanned by a scanner (Perfection V370 Photo, Epson, Nagano, Japan) in order to analyze their color value. A calibration procedure was performed before the experiments. Six different H_2O_2 concentrations ranging from 0 to 25 mg/L were made by diluting 30% H_2O_2 stock solution in distilled H_2O. The same process of calibration was used for the nitrate concentration. Seven different nitrate concentrations ranging from 0 to 250 mg/L were made by diluting a nitrate stock solution (Nitrate standard solution 200 mg/L NO_3-N in H_2O, 125040, Merck, Darmstadt, Germany) in distilled H_2O. The sum of the color values of the red, green, and blue channel (grey value) for different H_2O_2 and NO_3^- concentrations were plotted in their respective calibration graphs. The exponential fit of the calibration data allows the determination of an unknown H_2O_2 and NO_3^- concentration deposited in the sample during the plasma treatment. Aliquots of 10 µL PAM were taken

immediately after 5 min of plasma treatment time. The plasma treatment was done in triplicate and each time concentration of H_2O_2 and NO_3^- was determined.

To detect nitrite ions (NO_2^-), Griess reagent was added to the PAM (Spectroquant Nitrite test 1.14776.0001, Merck, Darmstadt, Germany). In an acidic solution, nitrite ions react with sulfanilic acid to form a diazonium salt, which in turn reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red-violet azo dye. This dye was determined spectrophotometrically and the absorbance was measured at λ = 540 nm. For the calibration curve, seven different nitrite concentrations ranging from 0 to 1 mg/L NO_2-N were produced by diluting a nitrite stock solution (Nitrate standard solution 40 mg/L NO_2-N in H_2O, 125042, Merck, Darmstadt, Germany) in distilled water. Similarly to NO_3^- and H_2O_2, the concentration of NO_2^- was determined after 5 min plasma treatments. The experiment was done in triplicate.

2.3. Cells

Human melanoma A375, laryngeal carcinoma Hep2, and immortalized mouse fibroblast L929 cell lines were obtained from ATCC (American Type Cell Culture, Manassas, WV, USA) and were stored in 10% DMSO/Fetal calf serum (FCS, Sigma, St. Louis, MO, USA) in liquid nitrogen before the experiments. The cells were thawed in 20x volume of complete RPMI medium, containing basal RPMI 1640, 10% FSC, 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 50 µM 2-mercaptoethanol (2-ME, Sigma, St. Louis, MO, USA), and antibiotics (gentamicin, streptomycin, penicillin) and after washing by centrifugation, they were cultivated at 37 °C, 5% CO_2 up to 80% confluence. After reaching the confluence, the cells were passaged via trypsinization in 0.2% trypsin/0.02% NaEDTA (Sigma, St. Louis, MO, USA) according to standard laboratory procedures.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers who signed consent forms and isolated by using Lymphoprep (Nycomed, Oslo, Norway). All studies involving the usage of human cells were approved by the Ethics committee of the Institute for the Application of Nuclear Energy, University of Belgrade (No. 02/765/2). PBMC were used in direct cytotoxicity assay or as a source for the isolation of monocytes and T cells as negative fractions of Magnetic-activated cell sorting (MACS) using Monocyte isolation kit II and Pan-T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively.

MACS-sorted monocytes were used for the generation of monocyte-derived dendritic cells (DCs) according to a protocol described previously [23]. Briefly, 1×10^6 monocytes were plated in low-adherent 6-well plates (Sarstedt AG & Company, Sarstedt, Germany) and cultivated for 4 days in CellGenix® GMP DC medium (CellGenix, Freiburg im Breisgau, Germany) in the presence of a recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (both at 20 ng/mL, R & D Systems, Minneapolis, MN, USA) to obtain immature (im)DCs. Tumor lysates derived from A375 or Hep2 cells, as described in Section 2.4, were added to the DC cultures on day 4. To obtain mature (m)DC, the cells were treated with LPS from *E. coli* 0.111:B4 (200 ng/mL, Sigma, St. Louis, MO, USA) and human recombinant IFN-γ (20 ng/mL), 4 h after the treatment of DCs with tumor lysates, for the next 16–18 h. Afterward, the DCs were collected and used for phenotype characterization and functional assays with MACS-purified T cells.

2.4. Cytotoxicity Study

PAM was prepared by treating a basic RPMI 1640 medium with CAP. The components for a complete RPMI medium were added immediately afterwards and serial dilutions of PAM were prepared in the complete RPMI medium.

To evaluate the cytotoxic effects of PAM on A375, Hep2, L929 cells (each at 5×10^4 cells per well of a flat 96-well plate) and PBMCs (2×10^5 cells per well of 96-wells plate) were cultivated in complete RPMI medium with serial dilutions of PAM (100%, 50%, 25%, 12.5%, 6.25%, 0%) for 24 h. Supernatants of these cell-cultures were collected and stored at −80 °C for cytokines analysis. The MTT assay was performed on the remaining cells to determine

the metabolic activity of the cells treated with PAM. The corresponding cell-free cultures containing PAM were used as blank controls. After the cultivation, all cultures were washed in phenol red-free RPMI medium twice and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL), Sigma) in phenol red-free RPMI was added for the next 4 h. The formazan crystals were dissolved by using 10% sodium dodecyl sulphate (SDS, Sigma, St. Louis, MO, USA) in 0.01N HCl (Sigma, St. Louis, MO, USA) overnight, and the absorbance was read at 570 nm with a spectrophotometer (ELx800, Biotek, Winooski, VT, USA). The absorbance in cell-free blank controls was subtracted from the absorbance of corresponding experimental cultures. Each of the three experiments performed was carried out in six-plicates. The metabolic activity (MTT%) detected in the treated cultures was expressed as the percentage of the absorbance in non-treated control cultures (100%).

Oxidative stress in the A375 cells treated with different concentrations of PAM (6.25–100%) for 4 h or 24 h was analyzed after trypsinization of cells, by using a Muse® Oxidative Stress Kit (Luminex, Austin, TX, USA), which is based on the reactive oxygen species-sensitive dye dihydroethidium (DHE), according to manufacturer's instruction. Autophagy flux in the A375 cells treated with different concentrations of PAM was evaluated using a Muse ® Autophagy LC3 Antibody-based kit, according to the manufacturer's instructions. The method relies on the detection of membrane-bound LC3 after selective permeabilization of cells which extracts cytoplasmic LC3 but not membrane-bound LC3, and the blockage of lysosomal degradation of LC3 in autophagosomes. Apoptosis of the A375 cells treated with PAM for 24 h was detected with a Muse ® Annexin V & Dead Cell kit, which is based on Annexin V/7AAD staining in Ca^{2+}-containing buffer. The analysis of oxidative stress, autophagy, and apoptosis was performed on a Guava® Muse® Cell Analyzer (Luminex, Austin, TX, USA). The expression of heat-shock protein (HSP) 60 and heat-shock complex (HSC) 70 on the surface of A375 and Hep2 cells was analyzed after 4h exposure of cells to different doses of PAM. After harvesting the cells by trypsinization, the cells were incubated with primary mouse anti-HSP 60 (Clone 24, 1 μg/mL BD Biosciences, San Jose, CA, USA) or mouse anti-HSC 70 (Clone sc-7298, 1 μg/mL Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in PBS/NaN3/5%FCS for 30 min, washed, and then incubated with a secondary anti-mouse IgG-FITC antibody (Sigma St. Louis, MO, USA) for 20 min, followed by fixation and analysis by flow cytometry (BD LSR II).

A375 and Hep2 tumor cells treated with PAM for 24 h, and control non-treated tumor cells, were used for preparing tumor lysates in DC cultures. Totally 5×10^6 live tumor cells were incubated in 500 μL of 100% PAM for 24 h, and a loss of viability >90% in the PAM-treated cultures was confirmed by Trypan blue staining. Both PAM-treated and control tumor cells were then frozen at −80 °C for 10 min and afterward thawed at room temperature for 10 min in an ultrasonic bath, and the process was repeated at least 7 times. After that, 100 μL of complete tumor lysates were added in 2 mL of DC cultures (total 5% vol.) thus providing an equivalent of 1:1 DC: lysed tumor cells, respectively.

Direct cytotoxicity of PAM on DCs was evaluated by treating 4-day DC cultures with 12.5% or 25% of PAM, whereas control DC cultures were treated with equivalent volumes of basal RPMI medium, for the next 24 h. Apoptosis was detected by an Annexin V-FITC/PI staining kit according to manufacturer's recommendations (Thermo Fisher, Waltham, MA, USA) and analyzed on a flow cytometer (LSR II, Becton Dickinson, East Rutherford, NJ, USA). Oxidative status in the DCs treated with PAM for 24 h was assessed by staining the cells with 2.5 μM dihydrorhodamine (DHR) in PBS for 30 min at 37 °C, followed by analysis on the flow cytometer.

2.5. Mixed Cell Reactions

The allostimulatory capacity of DCs (prepared as in Section 2.3) was tested by co-cultivating MACS-purified T cells (1×10^5/well of 96-well U-bottom plate) labeled with CellTrace™ Far Red (1 μM, Thermo Fisher, Waltham, MA, USA), with a different number of DCs (1×10^4, 0.5×10^4, 0.25×10^4 cells/well) for 5 days. To avoid transferring any stimuli

from DC cultures, the cells were washed twice in RPMI medium prior to co-cultivation with T cells. After 5-day co-cultures, the cells were washed in PBS, stained with 7AAD and then analyzed on a BD LSR II flow cytometer. CellTrace Far Red dilution was analyzed after exclusion of doublets and dead (7AAD+) cells, and the percentage of proliferated T cells (CellTrace Far Red ^{low}) was calculated in FCS Express (DeNovo Software, Pasadena, CA, USA). The capacity of DCs to induce allogeneic cytokines production by T cells was analyzed after 5-day co-cultures (1:20 DC:T cell ratio), after treating the co-cultures with PMA (20 ng/mL, Sigma, St. Louis, MO, USA), calcium ionophore (500 µg/mL, Sigma, St. Louis, MO, USA) and monensin (3 µg/mL, Sigma, St. Louis, MO, USA) for the last 4 h to stimulate cytokine accumulation in the primed T cells. Harvested cells were washed in 0.1% NaN3/PBS and stained for surface and intracellular cell markers. To detect cytokines produced in co-culture-supernatants, DC/T cell co-cultures carried out likewise, were treated with PMA and Ca Ionophore for the last 4 h, and the supernatants were collected after centrifugation at 2000 RPM for 5 min.

To evaluate the capacity of DCs to prime cytotoxic T cells, autologous T cells isolated from freshly obtained PBMC were co-cultured with A375 lysate-loaded or non-loaded DCs at a 1:40 DC:T-cell ratio for 6 days. Recombinant IL-2 (10 ng/mL) was added to these co-cultures on day 0 and day 3. After 6 days, the proliferation of autologous T cells was evaluated by staining the cells fixed in ice-cold ethanol (75%) for 2h at -20 °C and washed with PBS, with an anti-human Ki-67 antibody (Wuhan Fine Biotec Co., Wuhan, China) and secondary anti-rabbit IgG Alexa 647 (Abcam, Cambridge, UK), followed by staining with PI (1 ug/mL, Sigma, St. Louis, MO, USA) prior to the analysis of proliferation on a flow cytometer. Cytotoxic activity of the primed autologous T cells towards live A375 tumor cells was carried out by co-cultivating T cells with CellTrace Far Red-labeled live A375 cells (5×10^5 cells) at 1:2, 1:4 and 1:8 A375: T cell ratios for 4 h. After that, the cells were collected and labeled with PI prior to analysis on a flow cytometer. For all mixed cell cultures, T cells cultivated without DCs and A375 cells cultivated without T cells were used as controls.

2.6. Flow Cytometry

The flow cytometry analysis of DCs and T cells was performed on the flow cytometers CyFlow Cube 6 (Sysmex, Kobe, Hyogo, Japan) and LSR II (BD) by staining the cells with the following directly conjugated antibodies: anti-CD83-FITC, anti-CD86-PE, CD86-PerCPCy5.5, anti-CD40-APC, anti-CCR7-FITC, anti-IL17A-Alexa Fluor 488, anti-CD25-PerCPcy5.5, anti-CD127-PE, anti-IL10-APC, anti-HLA-DR-APCCy7 (Biolegend Inc., San Diego, CA, USA), anti-HLA-DR-PerCP (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-ILT4-PE, anti-IFNγ-FITC (R&D Systems Minneapolis, MN, USA), anti-IL-12p40/p70-PE, anti-IL-10-FITC (BioRad, Hercules, CA, USA), anti-CD4-APC, anti-CD8-PerCPCy5.5, anti-Granzyme A-PE (eBioscience, San Diego, CA, USA), anti-Foxp3-FITC, anti-IL-4-PerCP, anti-PDL1-PE (eBioscience, San Diego, CA, USA), anti-CD4-PE (Sysmex, Kobe, Hyogo, Japan), IgG1 negative control-PE, IgG1 negative control-FITC, IgG1 negative control-APC, IgG1 negative control PerCP, (Thermo Fisher, Waltham, MA, USA), IgG2 negative control APCCy7 (Millipore, Burlington, MA, USA). Surface staining with primary Abs was conducted in PBS/0.1% NaN3/0.5% FBS prior to the intracellular staining that was carried out using a flow cytometry fixation and permeabilization kit (Biolegend, San Diego, CA, USA). Signal overlap between the channels was compensated before each analysis using single labeled samples. Non-specific fluorescence was determined according to isotype control antibodies and fluorescence minus one (FMO) controls and at least 5000 cells were analyzed in each sample. Doublets were excluded according to forward scatter (FS) H/FSA, and dead cells were gated-out according to 7-aminoactinomycin D (7AAD) staining, fixable viability dye 620 (BD) staining, or low FSC properties.

2.7. Cytokines

TNF-α, IL-1β, IL-6, TGF-β, IL12-p70, IL-10, IL-23 and IL-27 were measured in DC culture supernatants by a specific duo-set sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) in duplicates according to manufacturer's protocol. The levels of cytokines in DC/T-cell co-cultures were determined by the LegendPlex human Th cytokine panel 13-plex (Biolegend) in duplicates, according to the manufacturer's protocol. Unknown concentrations of cytokines were calculated from standard curves generated with the manufacturer-supplied recombinant cytokines and fitting with a 4-parameter (log) dose/response curve (GraphPad Prism 8, GraphPad Software, San Diego, CA, USA).

2.8. Statistical Analysis

Repeated-measures one-way analysis of variance (RM-ANOVA) was performed, followed by Tukey's multiple comparison test, to analyze differences in means between the different groups of treated cells and control groups (GraphPad Prism 8,). Student's T test was used to evaluate differences in levels of RONS in PAM. Data are presented as means ± SD of the indicated number of independent experiments (different time for experiments with cell lines, different DC donors and/or DC/T cell pairs), and differences were considered significant at p values of ≤ 0.05. For data presented as a heatmap, the values of each cytokine obtained in each experiment were normalized to the range 0–1, according to the following formula:

$$y = \overline{X}\left(\frac{x - \min(x)}{\max(x) - \min(x)}\right) \quad (1)$$

with y- heatmap value; x- level of cytokine in a sample in one dataset, (x)-dataset of one type of cytokine

3. Results and Discussion

3.1. Two-Electrode Plasma Jet Induces Efficient Accumulation of RONS in PAM

A two-electrode dielectric barrier discharge (DBD) atmospheric pressure plasma jet (APPJ) was used as a CAP source (Figure 1) for treating the RPMI 1640 cell culture medium. The power deposited in the effluent discharge in contact with the surface of RPMI 1640 was 1.2 W. The resistors (R = 1 kΩ) in this grounded branch of the electrical circuit used for monitoring current waveforms showed stable plasma throughout the PAM generation. The distance of the APPJ to the treated liquid was kept constant at 5 mm in all experiments and treatment time was 5 min. Plasma treatment did not change the pH of the medium, according to stable phenol-red coloring and the measurements of pH. We have measured the three long-lived RONS and their concentrations in untreated RPMI 1640 and the PAM-RPMI 1640 are shown in Figure 2. In the literature, H_2O_2 is the most commonly detected and characterized, followed by NO_2^-. At the end of the list is the NO_3^- radical, which is scarcely reported albeit it plays an important role in PAM-cell interactions. Their concentration mainly depends on the type of plasma device used, deposited power in the discharge, feeding gas, treatment time, and treated volume. Adding the type of the treated liquid media to this large variety of parameters that can influence the concentration of deposited RONS in the cell medium makes it very difficult to perform a direct comparison of two atmospheric plasma systems [24]. Nevertheless, the described diagnostics provide enough information to ascertain the stability of plasma and identify the most abundant species.

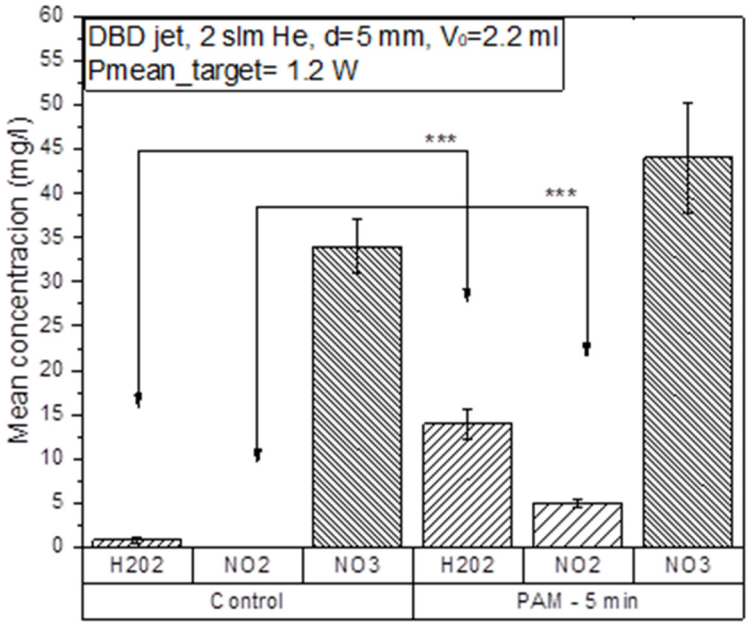

Figure 2. Measured concentrations of RONS in the plasma-activated medium PAM- RPMI 1640 compared to concentrations in the untreated RPMI 1640 medium. Helium flow during the treatments was kept constant at 2 slm and the power deposited in the discharge in contact with the sample was 1.2 W. The distance between the edge of the APPJ and the sample surface was 5 mm in all treatments. *** $p < 0.005$ compared to non-treated control RPMI 1640 (0% PAM) or as indicated by line.

The measured concentration of hydrogen peroxide was 1 mg/L and of nitrate 34 mg/L, while there was no nitrite detected in the untreated RPMI 1640 medium. The largest increase in concentration was observed for hydrogen peroxide, which increased 14 times after the treatment. Hydrogen peroxide is relatively stable and has strong oxidizing properties that can cause lipid peroxidation and, among other roles, serves as a cellular messenger. In the case of PAM-RPMI 1640, the highest yield of H_2O_2 was expected due to the presence of organic molecules in the cell medium (like glucose) [25]. Regardless of some reports in the literature stating that the increase in the concentration of H_2O_2 is generally responsible for the decrease in the viability of cancer cells [22,26], the main reason for this is the synergistic effect with reactive nitrogen species [27]. Therefore, we monitored the concentration of nitrates and nitrites in PAM-RPMI 1640. The concentration of nitrites in PAM was 5 mg/L and the nitrate concentrations increased by 30% compared to the untreated control RPMI medium.

3.2. PAM Induces Immunogenic Cell Death in Tumor Cells

ICD of tumor was reported as beneficial for triggering a DC-mediated anti-tumor response [28]. Increased presence of RONS in PAM makes it a good candidate for inducing ICD in tumor cells. Therefore, we examined the dose-dependent cytotoxicity of PAM towards tumor A375 melanoma cell line and laryngeal carcinoma Hep2 cell line, as well as towards non-tumor cells, such as immortalized L929 cell line and PBMCs (Figure 3a). Results from an MTT assay suggested that even the low doses of PAM (12.5% and 25%) were cytotoxic for A375 and Hep2 cells after 24h culture, but not for L929 cells and PBMCs. Higher doses (50% and 100%) of PAM significantly reduced the metabolic activity of all cell types, but the effect was most prominent in cultures with the tumor A375 and Hep2 cells. According to the ISO standard on the cytotoxicity of medical devices, a 30% reduction in MTT is considered as non-cytotoxic [29], suggesting that the reduction of MTT in the cultures of L929 cells and PBMCs treated with 50% PAM could be considered as non-cytotoxic as well. Our results are in line with previous findings showing that induction of cell death by CAP or PAM treatment is a universal phenomenon in malignant cells [30–33]

in contrast to non-malignant cells, which are more resistant [34]. Towards tumor cells, PAM showed dose- and time-dependent cytotoxic responses in vitro. In addition, the selectivity of PAM against tumor cells is influenced by the medium to be activated and the type of tumor cell lines used [35].

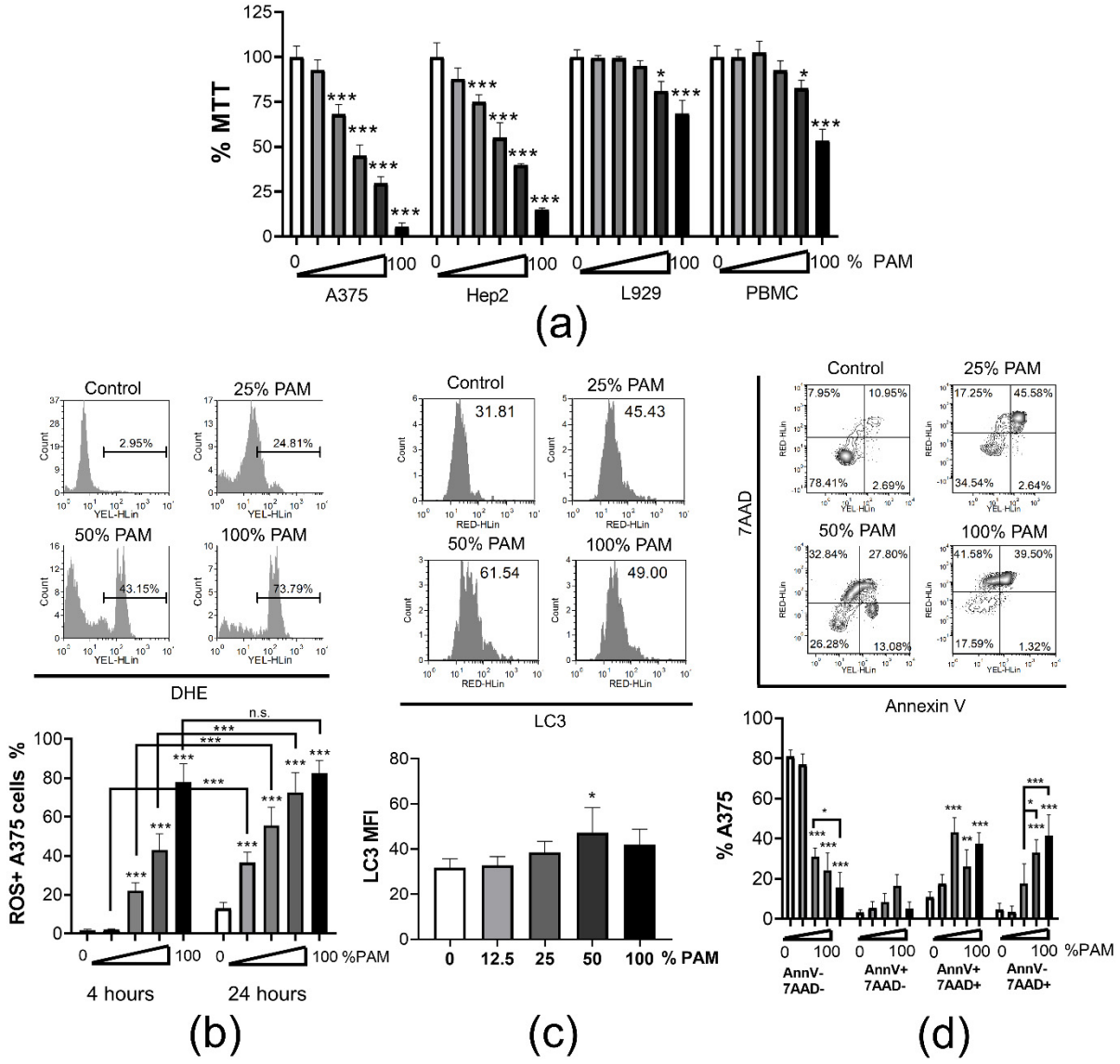

Figure 3. Dose dependent cytotoxicity of PAM. (**a**) Metabolic activity of A375, Hep2, L929, PBMC was assessed by MTT assay after 24 h treatment with serial dilutions of PAM (0% (white bars), 6%, 12.5%, 25%, 50% and 100% (black bars)) in complete RPMI medium. The results are shown as % MTT relative to the non-treated corresponding control cells 100%. Data from two independent experiments, each carried out in triplicates, are shown as the average % of MTT ± SD. (**b**) A representative data on the measurement of oxidative stress by DHE in A375 cells cultivated in the presence different concentrations of PAM for 4 h is shown. The summarized data from two independent experiments, in which the oxidative stress with the different doses of PAM (0% (white bars), 12.5%, 25%, 50% and 100% (black bars)) was measured after 4 h or 24 h as indicated, is shown as mean % of ROS+ cells ± SD. (**c**) A representative histograms on measurements of membranous LC3 expression in permeabilized A375 cells is shown with the indicated total mean fluorescence intensity (MFI), and the summarized data from two experiments is shown as MFI of LC3 ± SD. (**d**) Analysis of cell death by Annexin V/7AAD staining (AnnV−7AAD− viable; AnnV+7AAD− early apoptosis; AnnV+7AAD+ late apoptosis; AnnV−7AAD+ necrosis) in A375 cells is shown, carried out after 24 h cultures in presence of different doses of PAM (0%, 12.5%, 25%, 50%, 100%). Representative dot-plots and summarized data are shown as mean % A375 cells ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ compared to non-treated control cells (0% PAM) or as indicated by line.

High concentrations of RONS, both originating from PAM and produced by PAM-exposed cancer cells were shown responsible for cell death predominantly via induction of oxidative stress [10,36]. Considering that oxidative stress in dying cells is a hallmark of ICD [37], we analyzed oxidative stress in PAM-treated A375 cells by measuring intracellular ROS with dihydroethidium (DHE) staining (Figure 3b). PAM applied at the concentrations of 25% and higher, induced significant accumulation of ROS in A375 cells after 4 h cultures. Thereby, 100% PAM induced oxidative stress in nearly 80% of cells. After 24 h cultures, even the lower doses of PAM (12.5% PAM) induced significant accumulation of ROS in A375. These results are in line with the general mode of PAM and CAP actions in tumor cells [38]. It is known that ROS production by tumor cells treated with PAM depends on PAM-derived RONS (nitrite and H_2O_2) and the generation of initial singlet oxygen ($^1O_2$). This molecule is formed through the complex interaction between NO_2^- and H2O2 and the tumor cells expressing nicotinamide-adenine dinucleotide phosphate oxidase 1 (NOX1), catalase, sodium dismutase (SOD) on their surface [13]. The formation of peroxynitrite ($ONOO^-$) plays a key role in these processes [39]. At the site of inactivated catalase, cell-generated H2O2 enters the cell via aquaporins, depletes glutathione, induces the HOCl signaling pathway and promotes lipid peroxidation and cell death by apoptosis [40,41]. Non-malignant cells lack the expression of NOX1, catalase, and SOD on their surface making them resistant to cell death as long as the concentration of H_2O_2 is below a cytotoxic threshold level [10]. An investigation showed that the cytoprotective effects of mild PAM against oxidative stress in non-malignant cells such as human skin fibroblasts are characterized by the up-regulation of HO-1 mediated by the Nrf2-ARE pathway [42].

Reactive oxygen species and RNS are the key intracellular signal transducers sustaining autophagy [43]. Autophagy is essential for the survival of cancer cells, since it provides the required energy and removes damaged organelles [44]. However, intensive and persistent activation of autophagy leads to programmed cell death [45]. Moreover, autophagy was shown critical for the induction of the ICD of tumor in a mouse model [46]. Therefore, we next measured autophagy and apoptosis in the A375 treated with PAM for 24 h by quantifying membrane-bound LC3, a key marker of autophagosome formation [47], and by annexin-V/7AAD staining, respectively. It was found that autophagy was indeed triggered in the A375 cells treated with 50% PAM, but not with higher or lower doses of PAM (Figure 3c). According to Annexin V/7AAD staining, both increased apoptosis and necrosis were observed in the A375 cells treated with 50% and 100% PAM for 24 h, whereas lower doses of PAM (25%) induced predominantly the apoptosis of these cells (Figure 3d). Apoptosis was described as a dominant mode of cell death induced by PAM. Adachi et al. [48] showed that PAM reduced the mitochondrial membrane potential, down-regulated the expression of the anti-apoptotic protein Bcl2, activated poly (ADP-ribose) polymerase-1 (PARP-1) and released apoptosis-inducing factor (AIF) from mitochondria, suggesting a caspase-independent apoptotic pathway. Aggressive tumors have a different cellular machinery that protects them from the apoptosis caused by anticancer agents, thereby making them drug resistant. Therefore, cancer therapy based on the induction of non-apoptosis has been considered as an alternative approach to treat apoptosis-resistant cancer cells, including necroptosis [48]. However, the effect of CAP and PAM on this form of cancer cell death has not been sufficiently investigated.

In addition to apoptosis and necrosis, the ICD of tumor cells is characterized by the induction of heat-shock proteins, particularly their plasma membrane localization [49], as well as the release of inflammatory mediators, such as HMBG1, IL-1β and others [50]. In line with this, the membrane expression of heat-shock proteins HSP60 and HSC70 on both A375 and Hep2 cells increased after 4 h treatment with high concentrations of PAM (Figure S1a,c). Moreover, significantly higher levels of IL-1β were detected in 24 h culture supernatants of both tumor cell lines in the presence of 50% and 100% PAM, compared to their levels in the control culture supernatants (Figure S1b,d). Considering that heat-shock proteins and IL-1β are strong stimulators of the immune response [50], these results suggested that PAM-treated tumor cells could strongly potentiate immune response as well.

It has been shown that PAM induced apoptotic cell death in a time-dependent manner in endometrial cancer cells. The results correlated with the G2/M-phase arrest at all PAM concentrations and the induction of intracellular ROS accumulation [51]. In addition, PAM induced autophagy as judged by increased intracellular LC3B protein expression simultaneous with a decrease in the phosphorylated mammalian target of rapamycin (mTOR) and phosphorylated AKT protein levels and a decline of autophagy-related (p62 and ATG family) proteins. The autophagy inhibitor MHY1485 rescued PAM-induced cell death by decreasing the expression of LC3B but without the influence on phosphorylation of mTOR and AKT [51]. Adhikari et al. [52] have recently shown that CAP and synimarin nanoemulsion together activate autophagy in melanoma G-361 cells by activating PI3K/mTOR and EGFR pathways, expressing autophagy-related transcription factors and genes. In contrast, a preliminary report by Ando et al. [53] showed that plasma activated infusion (PAI) solution suppressed the autophagy in melanoma and osteosarcoma cell lines by activating the mTOR pathway. The authors suggested that ROS-mediated necroptosis, but not autophagy, plays a dominant role in the cell death induced by PAI. Our results are in accordance with these, showing that the highest doses of PAM induced predominantly necrosis but not autophagy, and the potentiate induction of ICD markers, such as heat-shock proteins and IL-β. However, all three types (autophagy, apoptosis and necrosis) of cell death are seen with 50% PAM, suggesting that further investigation of these mechanisms is necessary.

3.3. Tumor Lysates Prepared with PAM-Treated Cells Potentiate Maturation of Dendritic Cells

Complete tumor lysates are considered attractive and affordable sources of tumor antigens suitable for an autologous anti-tumor DC vaccine [54]. Several clinical trials used whole autologous tumor lysates prepared by multiple freeze–thaw cycles of tumor cells for DC vaccines [55,56]. Necrotic cell death by freeze-thaw enables the release of DAMPs from tumor cells such as HSP70, HSP90, HMGB1, and others [57], driving the maturation of DCs [58]. However, the finding that freeze-thaw necrotic tumor cells could inhibit toll-like receptor (TLR)-induced maturation and functions of DCs [59], opened serious doubts about the immunogenicity of thus prepared tumor lysates [60]. Different protocols for inducing immunogenic tumor lysates have been described including heat-treatment [61], hydrostatic pressure [62], electroporation [63], and others [60]. Nevertheless, it remained unclear how these protocols compare to standard freeze-thaw tumor lysates, and whether the application of PAM for the treatment of tumor cells could improve the immunogenicity of their lysates. Considering that PAM induced the ICD of A375 cells, we next investigated whether the A375 lysates prepared from A375 cells treated with 100% PAM for 24 h (PAM-A375lys) display better effects on DC maturation compared to the lysates prepared from non-treated A375 cells (A375lys). Thereby, LPS/IFN-γ treatment was additionally used as a strong maturation stimulus potentiating the Th1 polarizing capacity of DCs, which is highly desirable in the DC anti-cancer vaccine [64,65].

Both A375 lysates increased the expression of the costimulatory CD86 molecule and reduced CD40 expression by immature (im) DC, whereas PAM-A375lys also increased the expression of HLA-DR by imDC compared to control imDC (Figure 4). Upon LPS/IFN-γ treatment, the expression of all markers tested was up-regulated significantly. Thereby, both lysates additionally up-regulated the CD86 expression by mDC and inhibited LPS/IFN-γ-induced up-regulation of HLA-DR and PDL1 by mDC. However, the PAM-treated A375 lysate displayed an additional stimulatory effect on the up-regulation of CD83 on mDC and reduced the LPS/IFN-γ-induced up-regulation of ILT-4 on mDC significantly. In contrast, the control A375 lysate inhibited LPS-induced up-regulation of CD40 on mDC, unlike the PAM-treated A375 lysate.

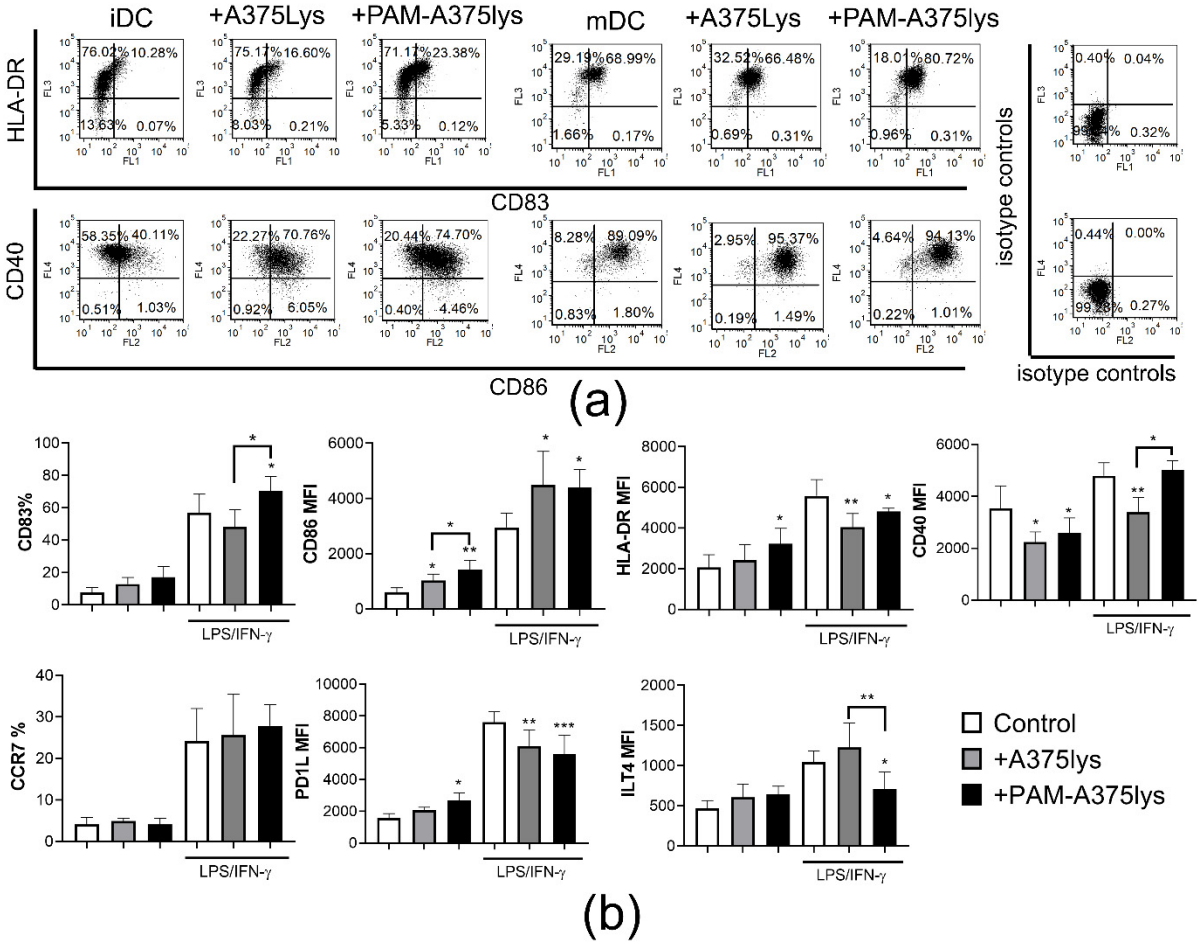

Figure 4. Effects of PAM-treated A375 lysates on phenotypic properties of DCs. Immature DCs differentiated for 4 days in the presence of GM-CSF/IL-4, were treated with the A375 lysate or the PAM-A375 lysate at 1:1 A375: DCs ratio, and after 4 h they were stimulated with LPS/IFN-γ or not, for the next 16 h. (**a**) A representative experiment on CD83/HLA-DR and CD40/CD86 co-expression analysis is shown and, (**b**) the summarized data from 3 independent experiments (different DC donors) are shown for the % of cells expressing the indicated surface marker, or as mean fluorescence intensity (MFI), ±SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs corresponding control DCs (white bars) or as indicated with lines (RM-ANOVA, Tukey's multiple comparison test). Statistical significance between the corresponding iDCs and mDCs was not indicated for clarity.

Miebach et al. [66] showed recently that colorectal cancer cells treated with an argon-based plasma jet increase the expression of CD80 and CD86 by monocyte-derived DCs in co-culture, unlike a neon-based plasma jet, which induced similar weak (<30%) cytotoxicity in tumor cell culture. However, the majority of CAP-treated tumor cells in the DC co-cultures were live in that study, and it remained unclear how they modulate the maturation and functions of DCs triggered by stimuli. Our study is the first report showing that DCs treated with PAM-tumor lysates display a good immunogenic phenotype, especially after the stimulation with LPS/IFN-γ, according to their higher CD83 and lower expression of ILT-4. ILT-4 was demonstrated as an important new checkpoint molecule in tumor immunotherapy involved in the induction of regulatory T cells [67]. On the other hand, CD83 is critically involved in the maturation of DCs and their resistance to pro-tolerogenic effects of IL-10 [68].

3.4. Tumor Lysates Prepared from PAM-Treated Cells do Not Impair High IL-12/IL-10 Production Ratio by DCs

Besides the mature phenotype of DCs, the induction of an efficient anti-tumor response by DCs is marked by their increased IL-12 production or IL-12/IL-10 production ratio [69]. Therefore, intracellular expression of IL-12p40/p70 was analyzed in DCs after the cultures with A375 lysates (Figure 5). It was shown that the A375 lysates did not induce significant IL-12 expression in imDCs. The strongest induction of IL-12p40/p70 expression was detected after LPS/IFN-γ treatment of PAM-A375lys-treated mDCs and control mDCs (Figure 5a), whereas mDCs treated with a control A375 lysate displayed significantly lower IL-12p40/p70 expression.

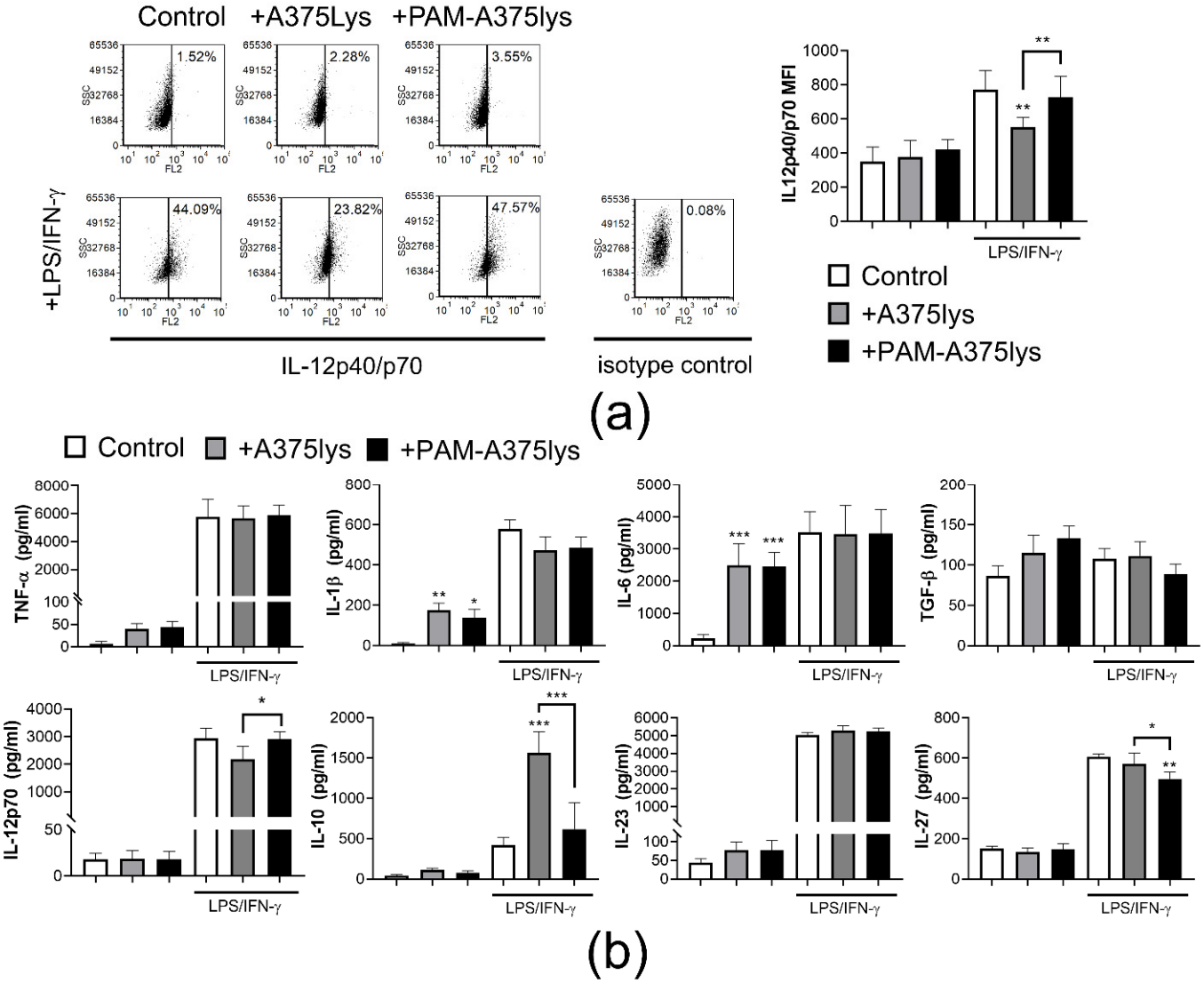

Figure 5. Effects of PAM-treated A375 lysates on cytokines expression by DCs. Immature DCs differentiated for 4 days in the presence of GM-CSF/IL-4, were treated with A375 lysate or PAM-treated A375 lysate at 1:1 A375: DC ratio, and after 4 h they were treated with LPS/IFN-γ or not, for the next 16 h. (**a**) A representative experiment on IL-12p40/p70 expression analysis is shown, and the summarized data from 3 independent experiments (with different DC donors) are shown as mean fluorescence intensity (MFI) ± SD. (**b**) The levels of indicated cytokines detected by ELISA in the cell-culture supernatants are shown as pg/mL ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs. corresponding control DCs (white bars) or as indicated with lines (RM-ANOVA, Tukey's multiple comparison test). Statistical significance between the corresponding iDCs and mDCs was not indicated for clarity.

A similar trend for IL-12 production was observed when protein levels were measured in cell-culture supernatants by ELISA (Figure 5b). However, it was found that control A375lys-treated mDCs produced substantially higher levels of IL-10 compared to PAM-A375lys or control mDCs, suggesting that PAM-A375lys mDCs display a significantly better IL-12/IL-10 production ratio than A375lys-treated mDCs. Previous reports also showed that freeze-thaw tumor lysates stimulate IL-10 production by mouse DCs [59], which could be a consequence of direct inhibitory effects of autocrine IL-10 on the capacity of DCs to produce IL-12 [70]. However, mouse IL-$10^{-/-}$ DCs treated with tumor lysates also displayed a reduced capacity for IL-12 production suggesting that this effect could be independent of IL-10 as well [59]. It remains unclear whether similar mechanisms occur in human DCs treated with tumor lysates, so this requires further investigations.

PAM-A375lys and control A375lys both increased IL-1β and IL-6 production by imDCs but did not modulate their production by mDCs (Figure 5b). The significance of this finding is still not clear, since a NLRP3-regulated increase in IL-1β, and subsequently IL-6 production was reported to promote tumor growth [71]. In contrast, Ghiringhelli et al. [72] showed that the activation of NLRP3-dependent IL-1β production by DCs, triggered via ATP release from dying tumor cells and activation of the P2X7 purinergic receptor, is critical for the efficient priming of IFN-γ-producing CD8 T cells by dying tumor cells. PAM-A375lys-treated mDCs also produced significantly lower levels of IL-27 compared to both control A375lys-treated mDCs and control mDCs. IL-27 has been implicated in promoting cancer progression [73], and high IL-27 levels are associated with advanced cancer [74], most probably due to its capacity to induce regulatory T cells [75]. Therefore, the attenuating effects of PAM-A375lys on IL-27 production by DCs could be interpreted as a desirable effect for the development of a DC cancer vaccine.

To observe whether different kinds of tumor cells induce similar effects on DC maturation and IL-12/IL-10 production ratio, Hep2 tumor cells were used instead of A375 to prepare lysates after the PAM treatment. It was observed that mDCs pre-treated with PAM-Hep2lys express higher levels of CD83 and CD86, lower levels of PDL1, and display a higher IL-12/IL-10 production ratio than the control Hep2lys-treated mDCs, although Hep2lys did not stimulate IL-10 production by DCs (Figure S2a,b). PAM-Hep2lys treated mDCs also displayed higher expression of CD86 and lower expression of PD-L1 compared to control mDCs. These results suggest that the phenomenon of increasing the immunogenicity of tumor lysates by PAM is not limited to a single tumor cell line. However, additional investigations are necessary to evaluate whether this phenomenon applies to primary tumors isolated from cancer patients as well.

We also assessed whether contaminating PAM added to DC cultures along with the lysates (5% PAM) could have directly affected the maturation and the IL-12/IL-10 production ratio by DCs and found no significant effects (data not shown). The cells were also treated with 12.5% PAM, which is more than twice the amount of PAM added with the PAM-A375lys in DC cultures, as well as 25% PAM, as the highest non-toxic dose of PAM for PBMCs (Figure S3). It was found that neither concentration of PAM directly affected oxidative stress and apoptosis in DCs after 24h (Figure S3a,b), nor did it affect the maturation and IL-12 production capacity of DCs (Figure S3c,d). However, 25% of PAM reduced LPS/IFN-γ-induced increase in IL-10 production by these cells. Unlike T and NK cells, which are more susceptible to ROS-inducing treatments [76,77], monocytes and DCs are more resistant due to their stronger anti-oxidative protection systems allowing them to secrete ROS as a part of normal immune functions [12,20,78]. However, an increased presence of exogenous ROS or their prolonged presence could induce depletion of glutathione in DCs leading to their reduced maturation and Th1 polarization capacity [79]. In this sense, 25% PAM induced attenuation of IL-10 production by DCs which could be interpreted as a beneficial effect in tumor therapy. Nevertheless, care should be taken when DCs are exposed to higher doses of PAM or for a longer period, as this could reduce the DC-mediated immune response. Cumulatively, our results suggest

that PAM-treated tumor lysates display good stable immunogenic properties on DCs, and that the effect is not merely a direct consequence of the contaminating PAM.

3.5. DCs Loaded with PAM-A375 Lysate Preserve Th1, Potentiate Cytotoxic CD8 T Cells and Th17 Response

DC-mediated induction of an efficient anti-tumor response involves their increased capacity to induce Th1 and/or Th17 cells [80]. Therefore, the immunogenic properties of DCs loaded with A375 lysates were next investigated in co-cultures with allogeneic T cells to evaluate their T cell polarization capacity (Figure 6). It was found that PAM-A375lys-treated mDCs significantly increased the proportion of Th17 cells (IL-17A$^+$ CD4$^+$ T), IL17$^+$ CD8 T cells and cytotoxic CD8 (IFN-γ^+ Granzyme A$^+$ CD8$^+$) T cells compared to both control mDC and A375lys-treated mDC. According to the unaltered IL-12 production by PAM-A375lys-treated mDCs (Figure 4b), and its role [81], we also did not find altered proportions of Th1 (IFN-γ^+ CD4$^+$T cells) cells in co-culture with these DCs. In contrast, control A375lys-treated DCs reduced the proportion of Th1, Th17 and cytotoxic CD4 T cells (IFN-γ^+ Granzyme A$^+$ CD4$^+$) compared to control mDCs, probably due to their increased capacity to secrete IL-10, which is known to inhibit Th1 and Th17 polarization [82]. Moreover, PAM-A375lys-treated mDCs significantly reduced the proportion of Th2 (IL-4$^+$ CD4$^+$) cells compared to mDCs, unlike A375lys-treated mDCs (Figure 6a,b). In general, mDCs induced lower levels of Th2 cytokines (IL-4, IL-5, IL-13) and higher levels of IL-6, Th9 (IL-9), Th1 (IFN-γ, IL-2, TNF-α) and Th17 (IL17A, IL17F, IL21, IL22) cytokines in DC/T cell co-culture supernatants, as compared to co-cultures with imDCs (Figure 6c). Thereby, PAM-A375lys-treated mDCs were the most potent in relatively lowering the Th2 and increasing the Th1 and Th17 cytokines in co-culture supernatants, confirming that PAM-treated tumor lysates potentiate beneficial anti-tumor properties in DCs. In line with this, Th2 cells demonstrated pro-tumorigenic effects in a mouse tumor model, whereas Th1 and Th17 cells displayed the opposite effects [83,84]. PAM-A375lys-treated mDCs also displayed a significantly higher capacity to stimulate proliferation of allogeneic T cells compared to A375lys-treated mDCs (Figure 6d). These results are in line with the higher levels of IL-2 detected in PAM-A375lys-DC/T co-cultures, better maturation of PAM-A375lys DCs, and their stronger IL-12 production, all of which are critical for the induction of T cell proliferation [85–87].

Analysis of the alloreactive T cell response in DC co-cultures, does not allow direct assessment of the antigen-specific T cells response. Therefore, in a pilot experiment, we additionally tested whether autologous T cells primed with A375 lysates-loaded DCs proliferate and display cytotoxic activity towards live A375 cells after 6-days of priming with DCs. Encouragingly, we observed that the PAM-A375lys-treated mDCs induced the highest proliferation of autologous T cells in co-cultures (Figure S4a). Additionally, T cells primed with PAM-A375lys-treated mDCs displayed significantly higher cytotoxic activity towards live A375 cells after 4h of co-cultures compared to T cells primed with A375lys-mDCs or control mDCs, when the same number of primed T cells was used in the co-cultures (Figure S4b) suggesting that both increased proliferation and increased cytotoxic functions are potentiated by PAM-A375lys-treated DCs.

The increased proliferation and cytotoxic activity of T cells primed with control DCs which were not treated with tumor lysates, could be attributed to the presence of other proteins during co-cultures such as FCS [88]. Therefore, additional investigations on the antigen-specific effects of DCs, especially with cells from cancer patients, are necessary to delineate the antigen-specific and direct modulatory effects of PAM-A375lys on the DCs capacity to induce proliferation and cytotoxic autologous T cells.

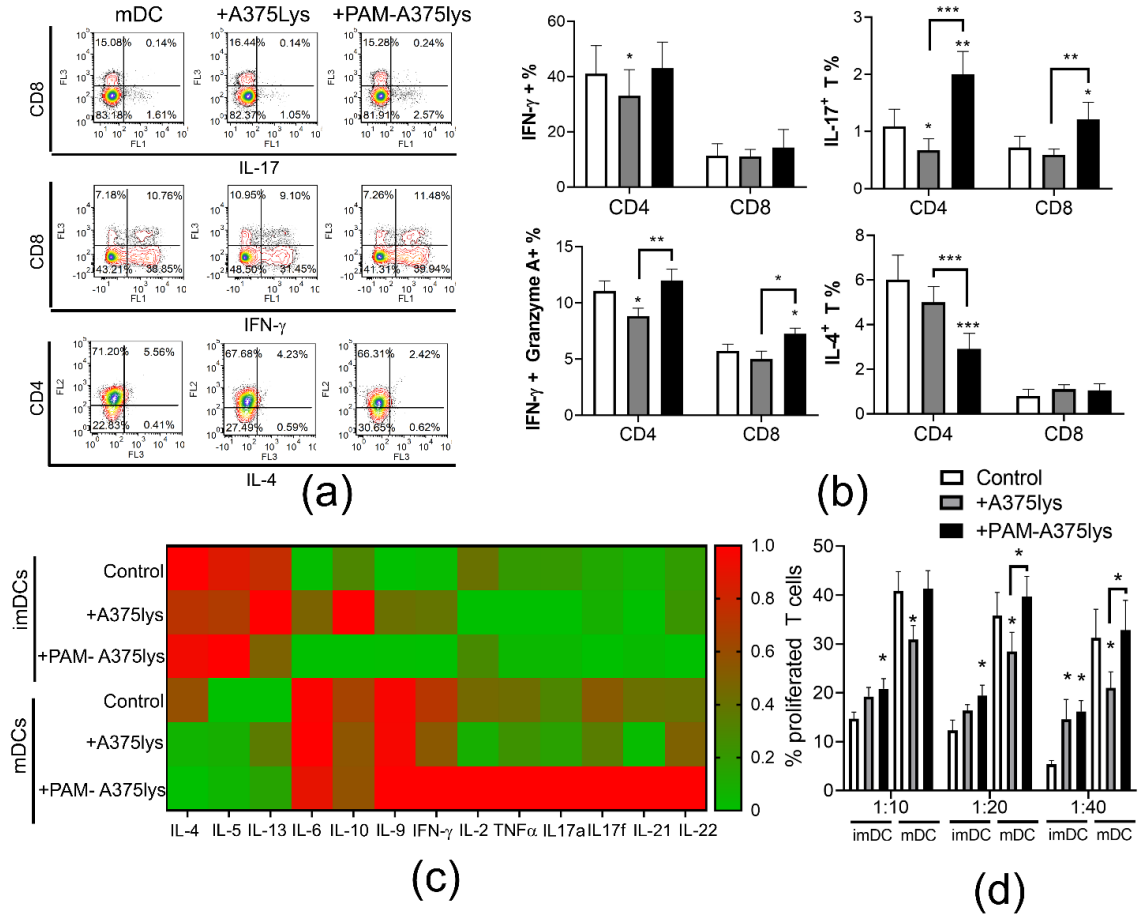

Figure 6. T cell polarization capacity of DCs treated with A375 lysates. (**a**) Immature DCs and mDCs, either treated or not with the A375 lysate (+A375lys) or the PAM-treated A375 lysate (+PAM-A375lys) were co-cultivated with MACS-purified allogeneic T cells (1×10^5/well) at 1:20 (DC: T cell ratio) for 5 days, followed by the stimulation of co-cultures with PMA/Ca Ionophore/monensin for the last 3 h prior to their staining for flow cytometry. Representative dot-plots are shown on the total gated T cells that were co-cultured with the indicated mDCs and afterwards stained to CD4, CD8 and the indicated cytokines. (**b**) The summarized results on the % of cytokine/enzyme expressing cells normalized to 100% of T cells are shown as % ± SD of 3 independent experiments. (**c**) The levels of cytokines from DC/T cell co-cultures as in (**a**), stimulated for the last 3 h with PMA/ Ca ionophore, were measured with the LegendPlex Th13-plex system from the co-culture supernatants. The results are shown as a heatmap wherein each cell represent the level of cytokine normalized in each experiment to the range of 0–1, and averaged from 3 independent experiments, as described in Materials and Methods. (**d**) The allogeneic T cell proliferation was analyzed after 5-day co-cultures of CellTrace Far Red-labeled T cells (1×10^5 cell/well) and different number of DC (1×10^4, 0.5×10^4, 0.25×10^4 cells/well), providing 1:10–1:40 DC: T cell ratios. The percentage of proliferated (CellTrace Far Red ^{low}) T cells is shown as mean ± SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs. corresponding control DCs (white bars) or as indicated with lines (RM-ANOVA, Tukey's multiple comparison test). Statistical significance between the corresponding iDC and mDC was not indicated for clarity.

3.6. DCs Loaded with PAM-Tumor Lysate Do Not Induce Tregs

An efficient immunogenic DC vaccine ought to potentiate the Th1/Th17 response in T cells, but not regulatory T cells, which the suppress immune response and display pro-tumorigenic effects [62]. In line with this, previous reports [89], including our own [23,90], have shown that regulatory IL-10-producing $CD8^+$ T cells display stronger suppressive activity than conventional $FoxP3^+$ $CD4^+$ T cells. Therefore, the presence of conventional CD4 Tregs ($CD4^+CD127^-CD25^+FoxP3^+$) and regulatory $CD8^+$T cells ($CD8^+CD25^+IL\text{-}10^+IFN\text{-}\gamma^-$) was analyzed in DC/T cell co-cultures. It was found that iDCs treated with control A375 lysates induced significant proportions of regulatory CD4 and CD8 T cells, unlike PAM-

A375lys-loaded DCs (Figure 7). Stimulation of A375lys-treated DCs with LPS/IFN-γ did not affect the capacity of these DCs to induce CD4 Tregs, though it did lower their CD8 Treg inducing capacity. The higher capacity of A375lys-treated DCs to induce regulatory T cells is probably a consequence of their higher expression of ILT-4, IL-10, and IL-27 compared to PAM-A375lys-treated DCs. ILT-4 is critical for the induction of $CD8^+CD25^+IL10^+$ regulatory T cells by DCs, as we showed previously by blocking ILT-4 in the DC/T cell co-cultures [23]. IL-10 was shown to induce both regulatory CD8 and CD4 T cells [89], whereas IL-27 potentiates the functions of $CD4^+$ Tregs [91], leading to pro-tumorigenic effects in a multiple gene-deficient mouse model system [92]. Overall, these results suggest that PAM-A375lys induces desirable immunogenic properties in DCs, whereas A375 lysates prepared by the conventional freeze-thaw method could induce adverse effects in DC-vaccines via induction of pro-tumorigenic T cells subsets.

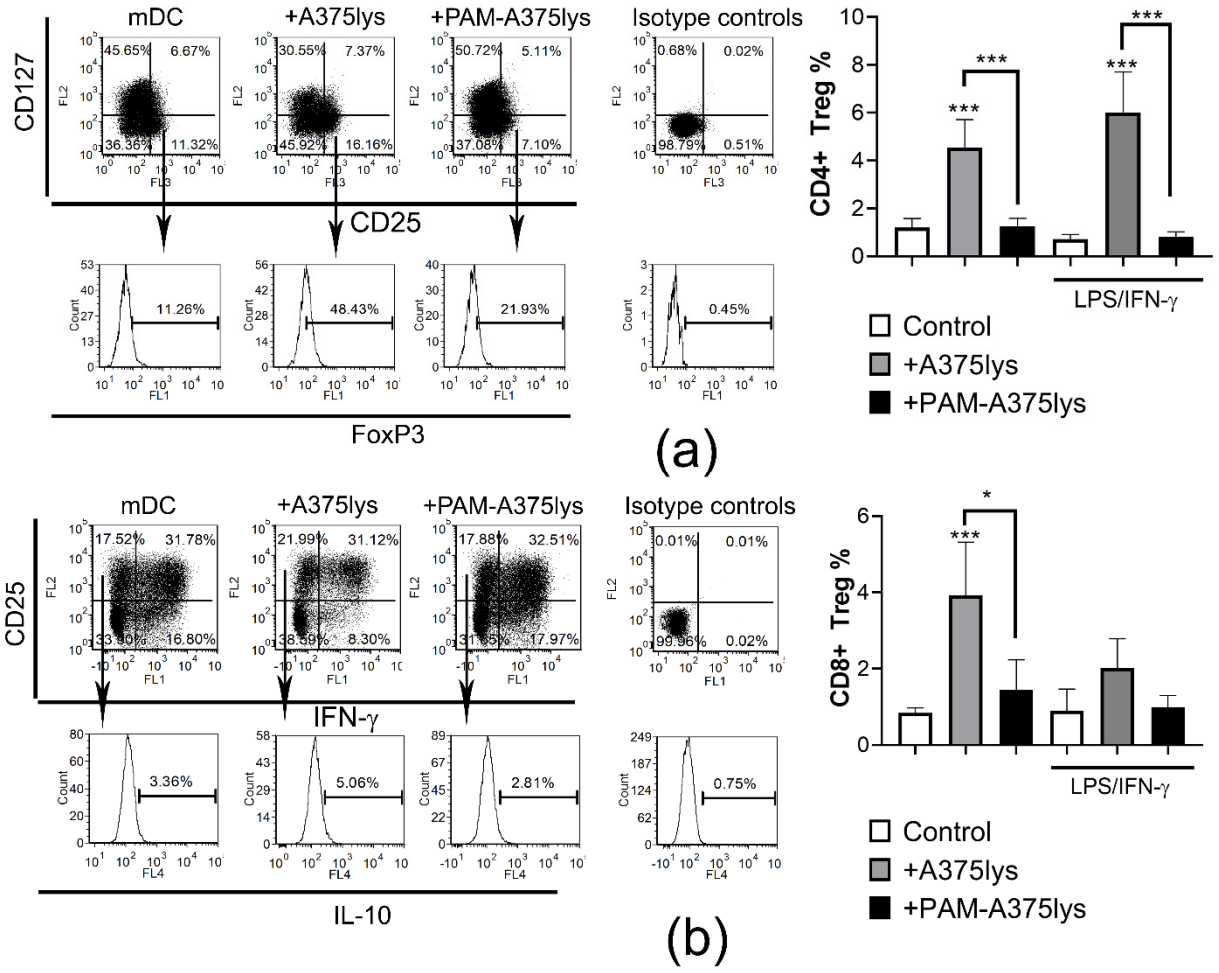

Figure 7. Treg induction capacity of DCs treated with A375 lysates. DCs either treated or not with the A375 lysate (+A375lys) or the PAM-treated A375 lysate (+PAM-A375lys) were co-cultivated with MACS-purified allogeneic T cells (1×10^5/well) at 1:40 (DC: T cell ratio) for 5 days, in the presence of 2 ng/mL of IL-2, followed by the stimulation of the co-cultures with PMA/ Ca Ionophore/ Monensin for the last 3 h prior to their staining for flow cytometry. (**a**) Representative dot-plots are shown on total gated $CD4^+$ T cells after staining to CD25, CD127 and intracellular FoxP3. The summarized results on the % of CD4 Tregs are shown as % ± SD of 3 independent experiments. (**b**) Representative dot-plots are shown on total gated $CD8^+$ T cells after staining to CD25, and intracellular IFN-γ and IL-10. The summarized results on the % of CD8 Tregs are shown as % ± SD of 3 independent experiments. * $p < 0.05$, *** $p < 0.005$ vs. corresponding control DCs (white bars) or as indicated with lines (RM-ANOVA, Tukey's multiple comparison test). Statistical significance between the corresponding iDCs and mDCs was not indicated for clarity.

4. Conclusions

A DBD atmospheric pressure plasma jet was employed as a CAP source in order to obtain PAM by treating RPMI 1640 culture medium. Three main RONS components (H_2O_2, nitrates, and nitrites) were identified in PAM. Tumor cell lines (A375 and Hep2) were more sensitive to the cytotoxic effects of PAM including the stimulation of ROS production, than a non-transformed cell line (L929 cells) and human PBMC. The type of PAM-induced ICD in A375 cells (autophagy, apoptosis, necrosis, membrane expression of heat-shock proteins, and the secretion of IL-1β) depended on applied concentrations of PAM. PAM-A375 lysate potentiated the maturation of DCs by up-regulating CD83 and CD86 expression, simultaneously with a down-regulation of PDL1, and increased the IL-12/IL-10 production ratio by mature DCs, compared to the control A375 lysate without PAM treatment. Mature DCs treated with the PAM-A375 lysate preserved Th1, potentiated Th17 and down-regulated Th2 responses in co-culture with T cells. In addition, such treated DCs increased the frequency and cytotoxic activity of CD8 T cells, along with a reduction in CD8 Tregs frequency. In contrast to the DCs treated with control tumor lysate, which increased the proportion of conventional FoxP3$^+$ CD4 Tregs, the PAM-treated tumor lysate did not potentiate the Treg-inducing capacity of DCs. Cumulatively, our results suggest for the first time that priming of mature DCs with tumor cell lysates prepared by PAM-induced ICD could be explored as a new strategy for the generation of more immunogenic DC-based tumor vaccines.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13071626/s1, Figure S1: Dose dependent effects of PAM on induction of heat shock proteins and IL-1β secretion by tumor cell lines, Figure S2: Effects of PAM-treated Hep2 lysates on phenotype and cytokines production by DCs, Figure S3: Direct effects of PAM on oxidative stress, apoptosis, maturation, and cytokines expression by DCs, Figure S4: Proliferation and cytotoxic activity of T cells primed with DCs.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data is shown in the manuscript and raw data is available from corresponding authors upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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Treatment of RPMI 1640 cell medium by atmospheric pressure plasma jet

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1. Introduction

Atmospheric pressure plasma jets (APPJs) are widely used in the fields of Plasma medicine [1] and Plasma agriculture [2] due to remarkable advantages like simple structure and convenient regulation of their characteristics. The variety of design and construction of atmospheric pressure plasma jets used in the applications in these two fields is enormous. In order to be able to cross reference the effects of one APPJ system with another in a particular application, it is important to investigate the key set of parameters that lead to desired effects.

2. Experimental setup

In this work we aim to investigate the effects of a plasma treatment of the RPMI 1640 cell medium which is then used as Plasma Activated Medium (PAM) with different cell types. We have used two configurations (with one and two electrodes) of dielectric barrier discharge (DBD) type of the APPJ system powered by kHz sine wave high voltage power supply system. He was used as working gas with two different gas flows (1 slm, 2 slm). The distance between the ending of the APPJ tube and surface of the sample was 5 mm. After sample treatment the effects of plasma were investigated by spectrophotometry and colorimetric methods. Concentrations of nitrite ions, nitrate ions and hydrogen peroxide were determined and correlated with the power deposited in the plasma and used gas flow.

3. Results and Discussion

In Fig. 1 we show concentrations of reactive species obtained after 5 min treatment of RPMI 1640 cell medium sample by 2-electrode DBD jet. With the increase of gas flow, nitrites and H_2O_2 concentrations are reduced while nitrates show the same behavior as before any treatment. The reduction for nitrites is threefold while H_2O_2 is reduced by 30%. The change in the flow of working gas produced different reactive species in the medium which will yield different effects on cancer cells.

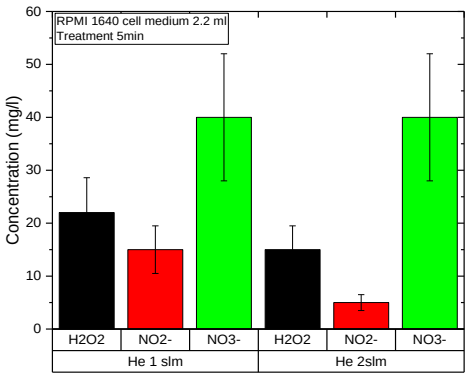

Fig. 1 Reactive species concentration in PAM after 5 min duration and 1.2 W power deposited in the plasma treatment by DBD jet.

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Treatment of DMEM and RPMI 1640 cell medium by DBD type atmospheric pressure plasma jet

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Here we will present the results of the plasma treatment of the DMEM and RPMI 1640 cell culture mediums. Both mediums were treated by Dielectric Barrier Discharge (DBD) type of atmospheric pressure plasma jet. After treatment concentrations of RONS were determined by spectrophotometry and colorimetric methods.

1. Introduction

Plasma medicine is interdisciplinary research field that combines studies in physics, biology, chemistry and medicine [1]. The use of plasma on living cells and tissues has already been thoroughly investigated. Recently, indirect application previously prepared Plasma Activated Medium (PAM) on cells has also attracted great attention, since the cells are normally surrounded or covered with various biological fluids. Therefore, the chemistry induced by the plasma in the aqueous state becomes essential and crucial for the biological outcomes in the treatment of different cell types [2].
In this work we have investigated effects of plasma treatment on cell culture mediums DMEM and RPMI 1640. Atmospheric pressure plasma jets able to generate reactive oxygen and nitrogen species in medium which is then used as PAM.

2. Experimental setup

The physical and chemical properties and potential application of PAM depends on different plasma sources which are employed as well as different medium. We have used two configurations (with one and two electrodes) of dielectric barrier discharge (DBD) type of the APPJ system powered by kHz sine wave high-voltage power supply system (Fig. 1). Helium was used as working gas with the 2 slm gas flow. The distance between the ending of the APPJ tube and surface of the sample, which was contained in a well of a micro-titter plate, was 5 mm. After the treatment the effects of plasma were investigated by spectrophotometry and colorimetric methods. Concentrations of nitrite ions, nitrate ions and hydrogen peroxide were determined and correlated with the different type of medium.

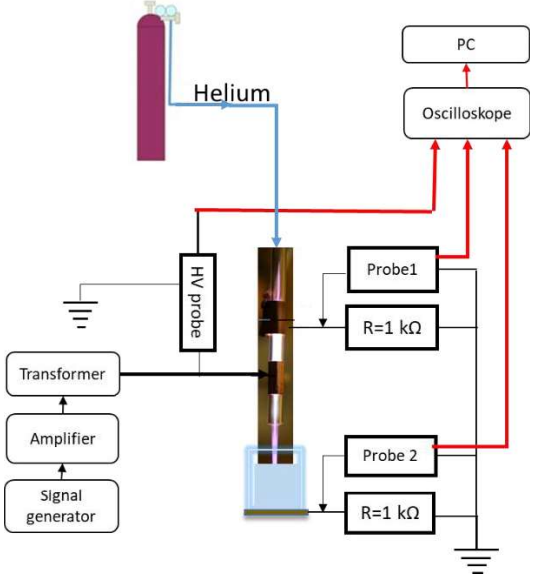

Fig. 1. Schematic diagram of plasm treatment medium by DBD jet

3. Results and discussion

Figure 2 shows the increase in concentrations of reactive species in two different media RPMI 1640 and DMEM after 5 minutes of treatment by 2 electrodes DBD atmospheric pressure plasma jet. Before treatment, the concentration of hydrogen peroxide and nitrate was 1 mg/l and 32 mg/l respectively, while

there was no nitrite in RPMI 1640 medium. There were not reactive species measured by colorimetric and spectrophotometry methods in DMEM medium before the treatment. Although the treatment conditions are the same, the concentration of hydrogen peroxide in PAM-RPMI 1640 is more than 6 times higher than in PAM-DMEM, while the concentration of nitrites is twice as high. Nitrate concentration in PAM-RPMI 1640 also increased after treatment while in PAM-DMEM the colorimetric method did not show any nitrates after treatment as well as before treatment. The differences in concentrations stem from the different chemical compositions of these two mediums that induces different chemical reactions during plasma treatment. Consequently, different concentrations of reactive species in PAM will give different effects on the cells.

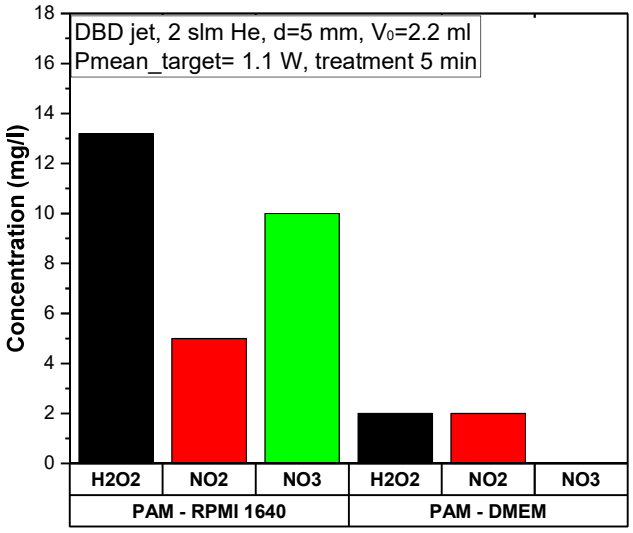

Fig. 2. Increase in reactive species concentrations in PAM-RPMI 1640 and PAM-DMEM after 5 min of treatment and 1.1 W power deposited in the plasma treatment by DBD jet

4. Conclusion

In order to achieve the desired influence of PAM on the cells, the first step is to investigate the effects of plasma treatment on the medium after treatment. It was shown that PAM properties depends on plasma source, treatment time feeding gas and power deposited in the plasma treatment. Here we investigated the influence of the composition of liquid, i.e. type of medium on plasma RONS production. The experimental results show that employment of a Dielectric Barrier Discharge (DBD) type of atmospheric pressure plasma jet produces different RONS concentrations in mediums RPMI 1640 and DMEM while operating with the same working gas and at the same power.

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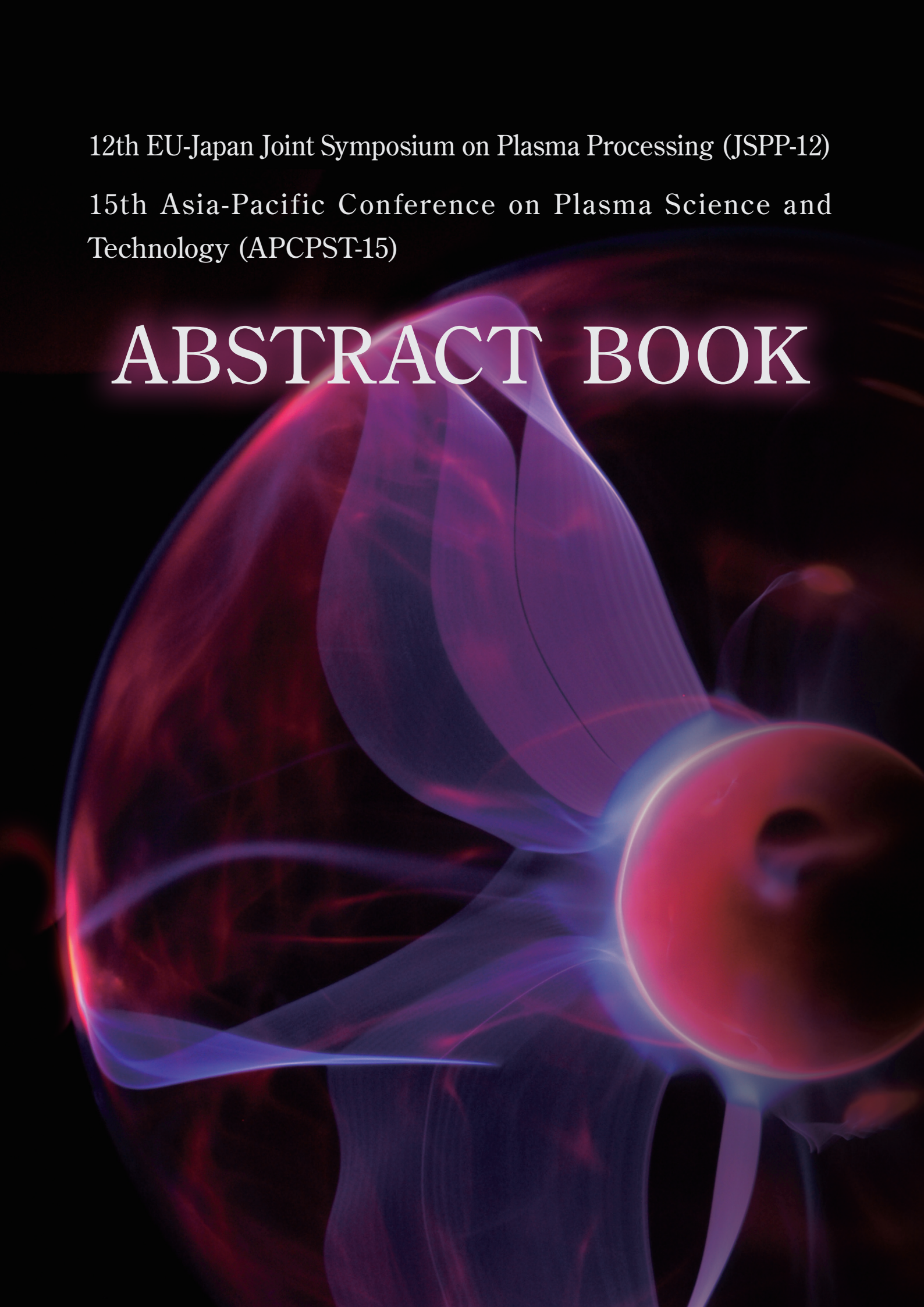
ABSTRACT BOOK



Immunogenic death of tumor cells induced by plasma activated medium potentiates dendritic cell-mediated anti-tumor response *in vitro*

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Non-equilibrium Atmospheric Pressure Plasmas (APPs) are known as a potential tool in various fields in modern medicine. They can promote wound healing and sterilization, blood coagulation, treatment of skin diseases, stem cell differentiation etc. [1-3]. One of the important field of APPs application in medicine is also a treatment of cancer cells [4-5]. The APPs can be used in the direct treatments where plasma is in direct contact with the treated cancer cells or in indirect treatments. In the indirect treatments the APPs are used to treat the liquid media (cell culture media, physiological solution etc.) and this 'activated' liquid media is applied to desired targets.

Either using for direct or indirect treatments, the main reasons for the therapeutic effect of APPs lies in the production of various reactive oxygen and nitrogen species (RONS). In the case of direct treatments, ions and short lived RONS also play a role, while for indirect treatments the main cocktail of active species in liquid media consists of long lived species (like H_2O_2, NO_2^-, NO_3^-, etc.). The cancer cells in contact with plasma produced RONS can go into apoptosis, necrosis or necroptosis while the normal cells are less sensitive to the same treatment parameters. Two main principles are considered responsible for these kinds of effects: oxidative stress due to delivered RONS and the induction of immunogenic cell death (ICD), leading to activation of an efficient anti-tumor immune response. ICD is also referred to as immunogenic apoptosis, which is characterized by an increased expression of damage-associated molecular patterns (DAMPs).

We have used an APP jet (DBD-type) to treat the RPMI 1640 cell medium. The plasma-activated medium (PAM) was used for the treatment of tumor cells and healthy cell lines. We found that produced PAM was highly toxic for tumor cells, but not for immortalized L929 cell line or human peripheral blood mononuclear cells. The PAM treated tumor cells displayed ICD markers and potentiated maturation and anti-tumor functions of human

monocyte-derived Dendritic Cells (DCs), singificantly more than the dead tumor cells prepared by a standard freezing/thawing method. These results support the idea that the PAM could be used efficiently to increase the immunogenicity of tumor antigens and improve clinical protocols for DC-based anti-cancer vaccines.

Acknowledgments: This research was funded by Ministry of Education, Science and Technological Development, grant numbers 451-03-68/2020-14/200019 and 451-03-68/2020-14/200024 and Serbian Academy of Sciences and Arts F31.

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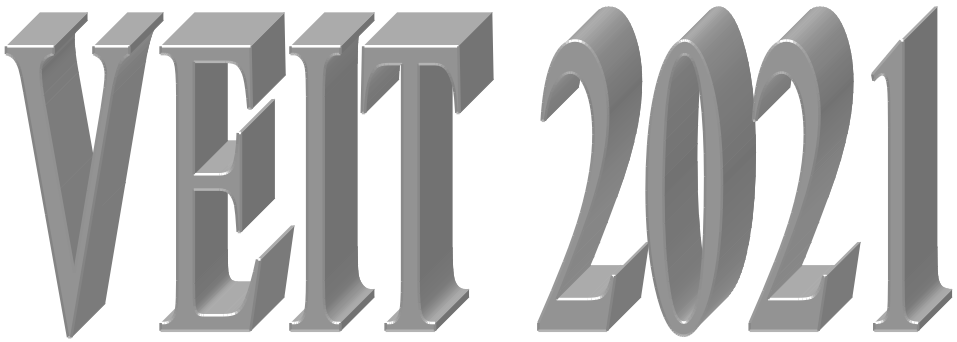
TWENTY-SECOND INTERNATIONAL SUMMER SCHOOL ON VACUUM, ELECTRON AND ION TECHNOLOGIES



20 - 24 September 2021
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PROGRAM
ABSTRACTS

Editors: M. Dimitrova, Ch. Ghelev and E. Vasileva

PR–7

PLASMA-ACTIVATED MEDIUM POTENTIATES DENDRITIC CELL-MEDIATED ANTI-TUMOR RESPONSE *IN VITRO*

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Non-equilibrium atmospheric pressure plasmas (APPs) have been available for many years, albeit with a limited range or sources (streamers, corona, dielectric barrier discharges). Recently, a number of new sources were developed initially in an attempt to move material processing from the realm of vacuum chambers into a possibility for cheaper technologies. It immediately became obvious that such discharges could be applied in medicine or in a wide range of bio technologies where a living tissue or organic materials have to be treated without damage due to increased temperatures. While a range of applications of such plasmas emerged both in medicine (wound healing, blood coagulation, treatment of skin diseases, stem cell differentiation, resistant bacteria sterilization etc.) and in biotechnologies (seed germination, sterilization of containers, food sterilization and extension of shelf life) the primary prize that was in the eyes of most scientists was to employ plasmas in treatment of cancers. Some success was achieved with skin and surface cancers, but application to cancers of inner organs with plasma in gas flow seemed impossible.

Recently, a new paradigm was developed that was to treat water, and later on, liquid media (cell culture media, physiological solution etc.) with plasma in order to pass the active components, i.e. various reactive oxygen and nitrogen species (RONS- H_2O_2, NO_2^-, NO_3^-, etc.), into the liquid and bring it inside the living organism in order to affect the tumor.

The cancer cells in contact with plasma produced RONS can go into apoptosis, necrosis or necroptosis. At the same time, normal cells are less affected by the same treatment. Two main mechanisms are likely to be most responsible for these effects: oxidative stress due to delivered RONS and induction of immunogenic cell death (ICD). ICD represents activation of an efficient anti-tumor immune response (immunogenic apoptosis).

First, we shall discuss whether plasma treatment while inducing apoptosis may contribute to a harmful DNA damage. Resolution of this issue has slowed down application of plasmas in medicine. As the first step in our study, we used an APP jet (DBD-type) with He flow to treat the RPMI 1640 cell medium. The plasma-activated medium (PAM) was used to treat tumor cells and healthy cell lines. We found that PAM was very toxic for tumor cells, but it did not affect strongly the immortalized L929 cell line nor human peripheral blood mononuclear cells. The PAM treated tumor cells displayed ICD markers and triggered efficiently the maturation and anti-tumor functions of human monocyte-derived dendritic cells (DCs). As a suitable

source of tumor antigens, the lysates prepared from PAM-treated tumor cells did not show adverse immunosuppressive effects on DCs, unlike the lysates prepared by a standard freezing/thawing technique. These results may be taken as a proof that PAM may be used efficiently to increase the immunogenicity of tumor antigens and to improve clinical protocols for DC-based anti-cancer vaccines [1].

Acknowledgments: This research was funded by MESTD grants 451-03-68/2020-14/200019 and 451-03-68/2020-14/200024 and Serbian Academy of Sciences and Arts projects F31 and F155.

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PR–8

PLASMA TREATMENT AS A GREEN TECHNOLOGY FOR MODIFICATION OF PLANT TISSUE AND SYNSEEDS

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Plasma Agriculture is a new field of plasma applications where low-temperature plasmas operating at atmospheric pressure gain importance as efficient green technologies [1]. Implemented solely in a gas phase, or in combination with liquids, plasma chemistry is responsible for various effects that result in a higher and faster germination, a better water uptake, anti-bacterial and anti-viral effects, induction of somatic embryogenesis etc. [1-4]. We performed plasma treatments of plant calli that are used in research and industrial production as a tool in biotechnology for genetic manipulation of plants, for micro-propagation, for studies of plant metabolism and cellular development, commercial production of natural products that cannot be chemically synthesized, etc. The treatments of *Irisreichenbachii* Heuffel. calli were performed by using a plasma needle operating at atmospheric pressure. We induced significant morphological and physiological changes in non-embryonic calli toward somatic embryos (SE) formation that were followed also with the enhanced production of arabinogalactan proteins.
Dielectric barrier discharge (DBD) operating in air was used for treatments of synthetic seeds (SynSeeds). Synthetic seeds are artificially encapsulated SE or other non-embryogenic *in vitro*-derived vegetative parts of plants mainly in alginate, which may be used for storage or sowing under *in vitro* or *ex vitro* conditions. The difficulties of sowing artificial seeds directly in soil or

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Atmospheric Pressure Plasma Treatment and Decontamination of Water Samples (invited)

N. Skoro, N. Puac, A. Kumar, O. Jovanovic, A. Petrovic (Institute of Physics Belgrade), U. Cvelbar (Jozef Stefan Institute), and Z. L. Petrovic (Serbian Academy for Sciences and Arts, School of Engineering, Ulster University)

Abstract Text:

As water scarcity becomes widespread concern in many countries, new approaches for water processing are being considered. In order to have the potential to be applied in the industry, these new methods should be primarily environmentally friendly and with low-energy consumption. Employment of non-equilibrium plasma for water treatment entirely satisfies these two points and therefore makes plasma a good candidate. Non-equilibrium (or cold) plasma at atmospheric pressure has so far been successfully used in various applications related to biology and medicine. Plasma rich-chemistry environment produced at room atmosphere with ambient temperature enabled treatment of cells, plants, bacteria, tissues etc. with aims ranging from destruction of pathogens to healing or enhancing the growth in biological systems. Therefore, these applications clearly point out that plasma sources can deliver broad spectrum of treatment conditions.

The latest research direction related to plasma and liquid treatment is plasma agriculture where different approaches for application of plasma in agricultural processes are investigated. Within these novel research area we investigate two possible applications: for the treatment of clean water samples for the production of plasma activated water (PAW); and for decontamination of polluted water containing organic micro pollutants (OMPs). PAW in comparison to clean water is rich in reactive oxygen and nitrogen species (RONS) and this proves to be an important asset when applied to seeds and plants. We showed that germination and plant growth can be enhanced when PAW is applied for seed imbibition and plant watering. On the other hand, the presence of reactive oxygen species in plasma and inside the treated water samples (especially droplets or aerosols) induces the decomposition of different molecules of OMPs. We demonstrated ability of plasma decontamination for several different organic molecules, such as decomposition of Malathion in atmospheric pressure plasma jet with high efficiency, and also for some chemicals used as chemical warfare surrogates. Apart from direct treatment of polluted samples by plasma, we have investigated a possibility of using PAW as a decontamination chemical to decompose organic dye Acid Blue 25 dissolved in water. Along the line of plasma agriculture research we combined the effects of two plasma applications in the plasma processing of agricultural waste water for reuse in irrigation. The experimental results showed that pesticide-containing polluted water can be decontaminated and at the same time enriched with RONS that positively influence germination of maize and radish.

These successful applications unfold many questions related to the plasma properties, plasma gas and liquid phase chemical reactions, an entangled connection between plasma produced reactive species and their chemical interactions with organic molecules in water. In order to make a detailed insight into the treatment process, we performed comprehensive diagnostics of the plasma and of the liquid samples. Our results provide information about electrical and optical plasma properties and make connection with physical parameters of the liquid sample (pH, electrical conductivity). We used colorimetric methods to determine RONS in the treated liquids. To acquire information about plasma-induced decomposition and obtain by-products in the treated samples we performed liquid chromatography coupled with mass spectrometry. All these results provide important knowledge on plasma water treatments and represent another step towards understanding which are the best parameters for treatment monitoring and techniques for up-scaling plasma devices to treat large amounts of water.

Acknowledgement: This study was partially supported by NATO SPS 984555, projects ON141037 and III 41011 of MESTD Republic of Serbia and H2020-MSCA-ITN 812880 Nowelties.

Symposium Selection:
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1st annual meeting of the MD-GAS COST Action

organized in the framework of the
COST Action CA18212 "Molecular Dynamics in the GAS phase"

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Caen, France

About MD-GAS

Emerging highly advanced ion-beam traps and storage rings combined with synchrotrons, X-ray facilities, and high performance computers offer completely new ways to study Molecular Dynamics in the GAS phase (MD-GAS). Cryogenic traps and rings will allow studies of decay and reaction processes involving molecular ions in well-defined conformations and in single or narrow ranges of quantum states.

The MD-GAS COST Action aims to further develop and fully exploit the exceptional potential of the above range of tools to unravel the connection between the initial energy transfer in interactions between isolated molecules or clusters and photons, electrons, or heavy particles (ions, atoms, molecules) and the related molecular dynamics in unexplored time domains ranging from sub-femtoseconds to minutes and hours.

Furthermore, the Action aims to identify reaction mechanisms and routes that lead to the growth of new molecular species, clusters and aerosols. The new knowledge will be important for fundamental atomic and molecular physics, chemical physics, and for applications in radiation therapy and -damage on the nanoscale, astrochemistry, astrobiology, atmospheric science, and climate research.

The MD-GAS COST Action is organized in three Working groups:

- New high-performance instrumentation and experimental methods to study gas phase molecular dynamics at ion-beam storage rings and traps, at synchrotrons and X-ray facilities;
- Survival and destruction of molecules following their processing by heavy particles, electrons, or photons;
- Charge-, energy flow, and molecular growth processes in intermolecular and intra-cluster reactions.

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Thursday 20th February 2020

09:00-09:20	*Inter- and intra-molecular interactions in uracil clusters studied by XPS*	J. Chiarinelli
09:20-09:40	*Fragmentation dynamics of ionized highly excited furan molecules: a combined theoretical and experimental approach*	E. Erdmann
09:40-10:00	*High radiative cooling rates of small clusters*	P. Ferrari
10:00-10:20	*The stability of the smallest carbon cluster dianion:* C_7^{2-}	P. Najeeb
10:20-11:00	Coffee break	
11:00-11:30	Working Group 3 Kick off Meeting - M. Alcamí	
11:30-12:00	*Ion-collision induced reactivity in molecular clusters*	P. Rousseau
12:00-12:30	*Gas-phase molecules through the lens of time-resolved photoelectron spectroscopy*	A. Ponzi
12:30-13:00	*Interaction of low energy electrons with biomolecules and clusters of biomolecules*	J. Kocisek
13:00-14:30	Lunch at the GANIL restaurant	
14:30-15:00	*Highly charged helium nanodroplets*	M. Gatchell
15:00-15:30	*Creation and destruction of chemical species in liquids treated by atmospheric pressure plasmas - from gas phase chemistry to bulk liquid*	N. Skoro
15:30-16:00	*Resonant Inelastic X-ray scattering of chloromethanes*	M. Zitnik
16:00-16:30	*Elastic electron scattering on molecules in the gas phase in the middle energy range*	J. Maljković
16:30-17:30	Coffee break	
16:30-17:30	*Laboratory visit*	
19:00-21:30	Conference diner - Café Mancel, le Château Ducal, Caen	

CREATION AND DESTRUCTION OF CHEMICAL SPECIES IN LIQUIDS TREATED BY ATMOSPHERIC PRESSURE PLASMAS - FROM GAS PHASE CHEMISTRY TO BULK LIQUID

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Large number of recent studies are investigating operation of cold atmospheric pressure (CAP) plasmas in contact with liquids. This is a continuation of the research related to biomedical applications where CAP has proved its excellent potential for sterilization and cleaning of both living tissues and inorganic materials from pathogenic microorganisms [1]. Applications of CAP use chemically reactive gaseous environment that contain reactive oxygen and nitrogen species such as •OH, •NO, H2O2, NO2-, NO3-, HNO3 etc. These species produced in CAP, which is in contact with the liquid, can penetrate and react with molecules in a bulk liquid modifying its physical and chemical properties [2]. This interaction goes through the interfacial region located between the gaseous plasma and a bulk liquid where many important processes involving short-lived species occur. As a result, the treated liquid is activated or, in case of polluted water, decontaminated by plasma [3]. However, in many cases specific plasma-liquid interactions behind the achieved positive results are elusive due to unknown processes in the interfacial region.

Here we will present results of laboratory-scale studies using different plasma source configurations that aim to induce decontamination of polluted water and/or activation of clean water. In all experiments we used plasma jets powered by a continuous kHz signals with the liquid samples placed below the jets. We will show results of detailed plasma diagnostics as well as measurements of basic physico-chemical properties of treated samples in order to reveal the influence of the plasma treatment. Special attention will be devoted to the possibility of assessing reactions in the interfacial region in order to clarify important reactions which exist in the particular treatments.

Acknowledgement: This work is supported by projects ON171037 and III41011 of the MESTD, Republic of Serbia.

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Destruction of cancer cells by plasmas

O14

INSIGHT INTO THE ANTI-TUMOR MECHANISM OF NON-THERMAL ATMOSPHERIC PRESSURE PLASMA IN ORAL SQUAMOUS CELL CARCINOMA

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Oral squamous cell carcinoma (*OSCC*) represents the most malignant neoplasm in oral cavity with a high mortality rate of more than 50%. Despite many advances in different treatment protocols in surgery, radiotherapy and chemotherapy, the long-term survival of patients with diagnosed *OSCC* has remained almost unchanged. Consequently, the need to find a different modality by which to treat cancer, cannot be understated. Cold atmospheric plasma (*CAP*) is a tuneable source of complex chemically reactive components, which allows *CAP* to exert many biological effects on different type of cells, particularly malignant ones. Although increasing number of evidence suggest that *CAP* can induce death of different carcinoma cells, thus offering a promising alternative treatment tool, the mechanisms of the antitumor effects of plasma remain incompletely clear so far. The objective of this study was to evaluate antitumor effect of *CAP in vitro*, along with mechanisms underlying induction of apoptosis. Two different *OSCC* cell lines (*SCC25* and *H400*) were subjected to direct and indirect *CAP* treatment, which implements the use of plasma-activated medium (*PAM*), for different exposure time. The modified plasma needle was used to realize helium-based plasma treatment. Both plasma treatments showed its suppressing effects on cell viability, cell adhesion, cell migration, and apoptotic cell death of *OSCC* cells. In order to understand better the anti-cancer mechanism of *CAP*, the amount of the main reactive oxygen and nitrogen species (*RONS*) produced in plasma-activated medium was measured by spectrophotometric methods. The obtained results demonstrate that both treatment strategies impair *OSCC* cell lines used in this study. The delivery of different levels of plasma-generated reactive oxygen and nitrogen species in cancer environment could be considered in order to explain anti-cancer mechanism of *CAP* in more detail. Interestingly, demonstrated anti-cancer effect of *PAM*, makes plasma an attractive anti-cancer tool that potentially could be used to prevent tumor growth and reach tissues which would be inaccessible for direct plasma treatment. Obtained findings considering anti-cancer features of plasma are promising and encouraging for a potential application of *CAP* in treatment of oral carcinoma. In the meantime, more investigations towards detailed plasma-cancer interactions should be conducted.

Acknowledgments: The study was supported by grant no. III 41011 and OI 175075 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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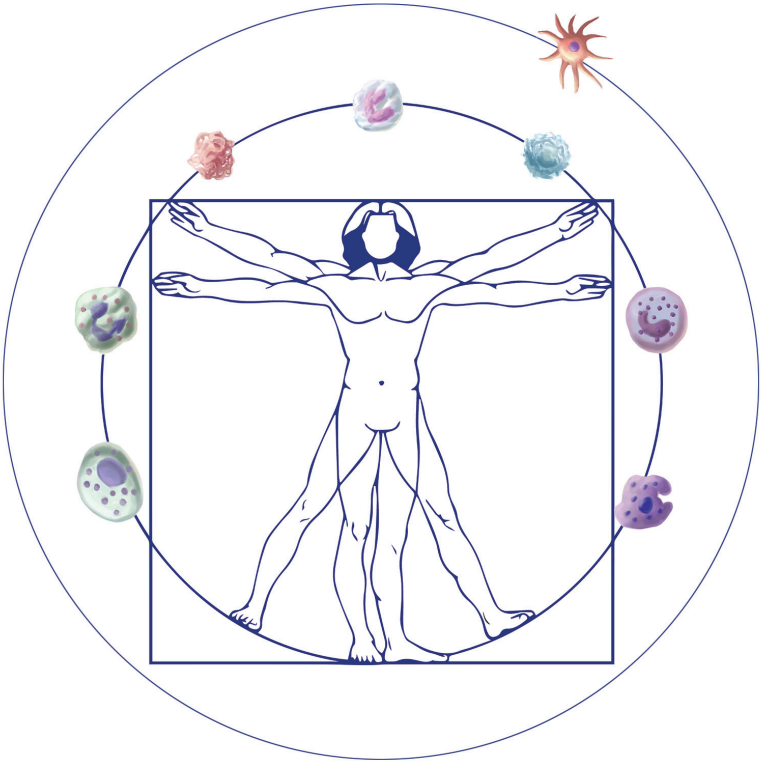

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АТМОСФЕРСКА ПЛАЗМА ПОТЕНЦИРА ИМУНОГЕНОСТ ТУМОРСКИХ ЛИЗАТА У ТУМОРСКИМ ВАКЦИНМА НА БАЗИ ДЕНДРИТСКИХ ЋЕЛИЈА

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Туморске вакцине на бази дендритских ћелија (DC) су веома атрактивне за примену у имунотерапији тумора. Лизати аутологих тумора су најчешће коришћени у клинчкој пракси као извори туморских антигена за DC вакцину. Међутим, имуногеност стандардних туморских лизата који се добијају замрзавањем-одмрзавањем ћелија је често врло слаба, што може смањити ефикасност DC вакцине. Овде је по први пут показано да се применом медијума третираног атмосферском плазмом (енгл. Plasma Activated Medium, PAM) може превазићи проблем слабе имуногености туморских лизата. Наиме, показано је да је PAM веома токсичан за туморске ћелијске линије (Нер2 и A375), али не и за L929 ћелије или за хумане мононуклеарне ћелија периферне крви. Третман туморских ћелија PAM-ом је био праћен повећањем ROS, мембранске експресије *heat-shock* протеина, секреције IL-1β, аутофагије, апоптозе и некрозе туморских ћелија, као маркера имуногене ћелијске смрти. Лизати туморских ћелија третираних PAM-ом су стимулисали већу експресију CD86, CD83, CD40 и продукцију IL-12 од стране зрелих DC, и инхибирали експресију PDL1 и ILT-4, у односу на DC третираних стандардним лизатима. Осим тога, DC третиране PAM-лизатима су имале бољи ало- и ауто-стимулаторни капацитет, индуковале су више IFN-γ+ гранзим A+ CD8+ T ћелија, IL-17A-продукујућих T ћелија, и већу цитотоксичност T ћелија према туморксим ћелијама. DC третиране стандардним туморским лизатима су индуковале више Th2 ћелија и регулаторних CD4 и CD8 T лимфоцита у односу на нетретиране зреле DC, док овај непожељни феномен није запажен након третмана DC PAM-лизатима. Ови резултати показују да туморски лизати припремљени помоћу PAM-а могу знајачно побољшати ефикасност DC вакцина за имунотерапију тумора у клиничкој пракси.

Резултати прихваћени за објављивање у часопису Cancers 2021

Electrical characterisation of atmospheric plasma jet during treatments of RPMI 1640 cell medium

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Atmospheric pressure plasma jets (APPJs) have been extensively used in applications in medicine, biology and, lately, agriculture. Regardless of the application and type of geometry one of the most important parameters for applications is the power deposited in the plasma that is in contact with the sample. Here we will present the results of electrical characterisation of the single and double electrode APPJ. The power deposited to the plasma will be the main parameter that will be later used for correlation with the plasma effects on the sample.

1. Introduction

The plasma medicine and lately plasma agriculture are two main driving fields of the design and construction of plasma systems that operate at atmospheric pressure [1, 2, 3]. The variety of design and construction of atmospheric pressure plasma jets used in the applications in these two fields is huge. In addition, different types of power supplies are employed inevitably leading to very large sets of different plasma parameters that are used in treatments. In order to be able to cross reference the effects of one APPJ system with the another in a particular application, it is important to determine the key set of parameters that lead to desired effects. One of the parameters that can be directly correlated with the effect in the sample generated by plasma is deposited power. Here we will present the results of electrical characterisation obtained with two different electrode geometries of APPJs. As a sample we have used RPMI 1640 cell medium. The effects of plasma treatments were later investigated by spectrophotometry and colorimetry methods and correlated with the power deposited in the plasma.

2. Experimental set up

The APPJ system that we have used consists of glass tube with inner diameter of 6 mm and outer of 8 mm and electrode(s) placed around the tube. We have used two electrode geometries. The first one where only one copper electrode was wrapped around the tube and connected to the power supply. The electrode was 15 mm wide and positioned at 15 mm from the edge of the glass tube. In this case as a second electrode served the surface of the target grounded through a resistor. In the second geometry, an additional electrode of the same width (area) was wrapped around the glass tube at the distance of 15 mm from the powered electrode. This electrode was grounded over a second resistor to the same point of the electrode circuit as the ground line of the target. Resistors served to monitor currents passing through grounded lines. To power the jet in both cases we have used sine wave high voltage power supply system. The system consists of wide range amplifier and additional high voltage transformer operating at its resonance frequency. The wide range amplifier can operate in the range of frequencies from 20 kHz up to 500 kHz. The high voltage transformers that were used had resonance frequencies between 30 kHz and 90 kHz depending on the plasma system impedance load. For electrical characterisation we used high voltage probe and regular voltage probes. As a sample RPMI 1640 cell medium was used. The distance between the edge of the APPJ tube and surface of the sample was 5 mm. The working gas was helium and the flow was kept constant at 2 slm.

3. Results and discussion

In the experiments two sets of electrical characterisation were made. One set consists of high voltage measurements at the powered electrode and measurements of voltage at the resistor (1 kΩ) connected to the grounded electrode that was wrapped around the glass tube. These results for frequencies of 80 kHz and 90 kHz are presented in Figure 1 as P_e.

The other set of results (P_t) presented in Figure 1 represent the power deposited in the branch of the electrical circuit which is grounded through the treated sample. The voltage probe in this case is connected to the resistor (1 kΩ) between the bottom of the plate containing sample and the ground. We can see that the difference for two different

frequencies in the deposited powers can be seen only in the core plasma (P_e). The power deposited in the plasma plume connected to the sample (P_t) does not vary in the magnitude at different frequencies. The only effect is that higher deposited powers are obtained for higher applied voltage values.

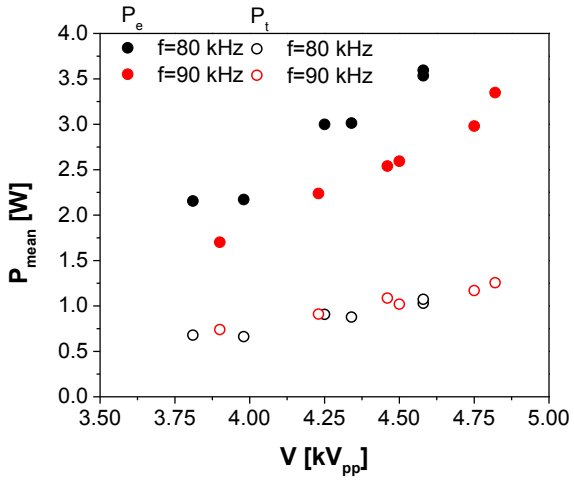

Figure 1. The power deposited in the core plasma (P_e) and the power deposited to the plasma touching the sample (P_t) for two power supply frequencies.

In the case of one electrode APPJ the stability of the discharge depended on the sample properties and on the humidity, both in the jet tube and in the room. The powers (P_t) deposited in the plasma connected to the sample varied significantly from case to case, even for the same applied voltages. Moreover, the maximum value of the deposited power was less than 1 W.

4. Conclusion

In order to determine the key parameter relevant for treatments of cell medium we have used two different configurations of APPJs. One set up consisted of two copper electrodes wrapped around the glass tube while the other had only one electrode on the glass tube of the jet that was connected to the power supply, while the grounded electrode was the treated sample. For two different frequencies of the power supply signal we measured the power deposited in the core plasma and in the branch of plasma connected to the surface of the sample. The difference in measured powers due to the change of the frequencies can be observed only in the core plasma.

Acknowledgments

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	ДОКТОРСКЕ СТУДИЈЕ		

ПРЕДЛОГ ТЕМЕ ДОКТОРСКЕ ДИСЕРТАЦИЈЕ КОЛЕГИЈУМУ ДОКТОРСКИХ СТУДИЈА

Школска година 2020/2021

Подаци о студенту

Име: Анђелија

Презиме: Петровић

Број индекса: 8004/2018

Научна област дисертације: Сизика јонизованог гаса и плазме

Подаци о ментору докторске дисертације

Име: Никола

Презиме: Шкоро

Научна област: Физика јонизованог гаса и плазме

Звање: Виши научни сарадник

Институција: Институт за физику

Предлог теме докторске дисертације

Наслов

PLASMA jet SYSTEM FOR treatment OF Liquid SAMPLES And APPLICATION IN PLASMA MEDICINE
Систем плазма млаза за третман течних узорака и примена у плазма медицини

Уз пријаву теме докторске дисертације Колегијуму докторских студија, потребно је приложити следећа документа:

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3. Фотокопију индекса са докторских студија

Потпис ментора

Датум 15. 09. 2021.

Потпис студента А. Петровић

Мишљење Колегијума докторских студија

Након образложења теме докторске дисертације Колегијум докторских студија је тему

прихватио ☒ није прихватио ☐

Датум

29.09.2021

Продекан за науку Физичког факултета

Универзитет у Београду
Физички факултет
Број индекса: 2013/3009
Број: 2612017
Датум: 09.10.2017.

На основу члана 161 Закона о општем управном поступку ("Службени лист СРЈ", бр. 33/97, 31/2001 и "Службени гласник РС", бр. 30/2010) и службене евиденције, Универзитет у Београду - Физички факултет, издаје

У В Е Р Е Њ Е

Анђелија Петровић

име једног родитеља Војин, ЈМБГ 2701994725071, рођена 27.01.1994. године, Крагујевац, општина Крагујевац-град, Република Србија, уписана школске 2013/14. године, дана 29.09.2017. године завршила је основне академске студије на студијском програму Примењена и компјутерска физика, у трајању од четири године, обима 240 (двеста четрдесет) ЕСПБ бодова, са просечном оценом 9,11 (девет и 11/100).

На основу наведеног издаје јој се ово уверење о стеченом високом образовању и стручном називу **Дипломирани физичар.**

Декан

Проф. др Јаблан Дојчиловић

Универзитет у Београду
Физички факултет
Број индекса: 2017/7025
Број: 2322018
Датум: 26.09.2018.

На основу члана 161 Закона о општем управном поступку ("Службени лист СРЈ", бр. 33/97, 31/2001 и "Службени гласник РС", бр. 30/2010) и службене евиденције, Универзитет у Београду - Физички факултет, издаје

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Анђелија Петровић

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Декан

Проф. др Јаблан Дојчиловић

Република Србија
Универзитет у Београду
Физички факултет
Д.Бр.2018/8004
Датум: 19.11.2021. године

На основу члана 161 Закона о општем управном поступку и службене евиденције издаје се

УВЕРЕЊЕ

Петровић (Војин) Анђелија, бр. индекса 2018/8004, рођена 27.01.1994. године, Крагујевац, Република Србија, уписана школске 2021/2022. године, у статусу: самофинансирање; тип студија: докторске академске студије; студијски програм: Физика.

Према Статуту факултета студије трају (број година): три.
Рок за завршетак студија: у двоструком трајању студија.

Ово се уверење може употребити за регулисање војне обавезе, издавање визе, права на дечији додатак, породичне пензије, инвалидског додатка, добијања здравствене књижице, легитимације за повлашћену вожњу и стипендије.

Овлашћено лице факултета

Република Србија
Универзитет у Београду
Физички факултет
Д.Бр.2018/8004
Датум: 19.11.2021. године

На основу члана 161 Закона о општем управном поступку и службене евиденције издаје се

УВЕРЕЊЕ

Петровић (Војин) Анђелија, бр. индекса 2018/8004, рођена 27.01.1994. године, Крагујевац, Република Србија, уписана школске 2021/2022. године, у статусу: самофинансирање; тип студија: докторске академске студије; студијски програм: Физика.

Према Статуту факултета студије трају (број година): три.
Рок за завршетак студија: у двоструком трајању студија.

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Овлашћено лице факултета