Article

Cold Atmospheric Pressure Plasma Treatment Modulates Human Monocytes/Macrophages Responsiveness

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Abstract: Monocytes are involved in innate immune surveillance, establishment and resolution on inflammation, and can polarize versus M1 (pro-inflammatory) or M2 (anti-inflammatory) macrophages. The possibility to control and drive immune cells activity through plasma stimulation is therefore attractive. We focused on the effects induced by cold-atmospheric plasma on human primary monocytes and monocyte-derived macrophages. Monocytes resulted more susceptible than monocyte-derived macrophages to the plasma treatment as demonstrated by the increase in reactive oxygen (ROS) production and reduction of viability. Macrophages instead were not induced to produce ROS and presented a stable viability. Analysis of macrophage markers demonstrated a time-dependent decrease of the M1 population and a correspondent increase of M2 monocyte-derived macrophages (MDM). These findings suggest that plasma treatment may drive macrophage polarization towards an anti-inflammatory phenotype.

Keywords: cold atmospheric pressure plasma; dielectric barrier discharges; monocytes; monocytes-derived macrophages

1. Introduction

Monocytes are versatile mononuclear phagocytes involved in innate immune surveillance, establishment and resolution of inflammation. When activated, monocytes are recruited from the bloodstream to the site of inflammation where they differentiate into macrophages [1]. The local inflamed microenvironment drives polarization towards activated M1 macrophages, which display a pro-inflammatory phenotype and are involved in severe inflammation leading to tissue damage [2], or towards activated M2 macrophages [3], that display anti-inflammatory properties and are involved in tissue remodeling, wound healing, and efficient phagocytic activity [4]. M1 macrophages express

high levels of major histocompatibility complex class II (MHC II) proteins, including the CD68 marker, and costimulatory molecules CD80 and CD86 [5]. They release reactive oxygen intermediates and several pro-inflammatory cytokines [6]. M2 macrophages are instead characterized by the expression of specific phenotypic markers, such as the mannose receptor-1 (CD206), the scavenger receptors CD163 and CD36 [7,8]. The molecular mechanisms underlying macrophage polarization have not been completely understood, because of the broad spectrum of stimuli affecting the process. However, it has been widely reported that this physiological process is altered in pathological conditions, for example in cancer or autoimmune diseases [9].

In this context, great interest has arisen towards physical stimuli that are able to modulate M1/M2 macrophage polarization [10]. In particular, the possibility to stimulate immune cells through cold atmospheric pressure plasma (CAP) treatment in order to control and drive their activity may pave the way to a vast field of medical applications. CAPs are characterized by a non-equilibrium in temperature between electrons and the heavy species of plasma (i.e., ions, excited species and neutrals) generated by the application of an electric field to a neutral gas. Besides presenting a negligible heat component, CAPs are characterized by several biological active components, such as ions, electrons, reactive oxygen (ROS) and nitrogen (RNS) species, UV radiation, playing a synergic action in the interaction of CAP with biological substrates [11]. Therefore, CAPs may be used for the treatment of cells and biological tissues by properly selecting the most suitable source and operating conditions for plasma generation to avoid any thermal damage [12,13]. Indeed, the Authors have previously shown an effect of CAP in a number of different eukaryotic cell types [14–17]. Immune cells have not been investigated yet thoroughly, although there are indications that their function may be affected. As an example, Kaushik et al. [18], reported the cytotoxic effects of CAP on monocytic lymphoma U937 cells and Bekeschus et al. [19] demonstrated the differential sensitivity of blood mononuclear cell subpopulations to plasma treatment. Focusing on the effects of plasma on monocytes, the plasma treatment of primary human monocytes can activate the pro-proliferative or pro-apoptotic intracellular signaling cascades, depending on plasma treatment time [20]. Several papers have focused on the plasma treatment of macrophagic populations [21–25]. In particular, the direct or indirect plasma treatment of macrophages can increase migration, an important immune cell function against diseases, and anti-tumor function [26,27].

In the present work, we focused our attention on the effects of a Dielectric Barrier Discharge (DBD) CAP source operated in open air on primary human monocytes and in monocyte-derived macrophages (MDM), evaluating their viability, ROS production and membrane markers expression.

2. Materials and Methods

2.1. Monocytes Isolation and Differentiation

The study was conducted in accordance with the Declaration of Helsinki. Ten healthy volunteers were enrolled, after approval of the Research Protocol by the Ethic Committee of Azienda Ospedaliera Maggiore della Carità, Novara, Italy (241CE), and informed written consent. Human monocytes were isolated from heparinised venous blood samples by standard technique of dextran sedimentation and histopaque (density = 1.077 g·cm^{-3}, Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation (400× g, 30 min, room temperature) and recovered by thin suction at the interface, as previously described [28]. Purified monocytes populations were obtained by adhesion (90 min, 37 °C, 5% CO$_2$) in serum free RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM glutamine and antibiotics (Invitrogen, Carlsbad, CA, USA). After 90 min, medium was changed with RPMI added by 10% foetal bovine serum (FBS, Euroclone, Pero, Italy). Cell viability (trypan blue dye exclusion) was usually >98%. For differentiation into monocyte-derived macrophages (MDM), freshly isolated monocytes were cultured in 20% FBS-enriched medium for six days [29,30]. For plasma treatment cells were plated into 12-well plates in 1 mL of fresh medium.

2.2. Plasma Treatment

The CAP adopted in this study and reported in Figure 1A is a DBD source, already tested for biological applications [14,16,31,32] and consisting of a cylindrical brass electrode, 10 mm in diameter, having a hemispherical tip, with 5 mm of curvature radius. The electrode is coated with a 1 mm thick borosilicate glass (relative permittivity $\varepsilon_r = 4.7$), as dielectric layer. The DBD plasma source was operated in open air and powered by a micropulsed generator producing high-voltage quasi-sinusoidal pulses with peak voltage (PV) of 25 kV, pulse repetition frequency (*f*) of 20 kHz, and a duty cycle of 7.5%. In order to enable the plasma generation between the high voltage electrode and the liquid surface, the multiwell plate was positioned onto a grounded counter-electrode (aluminum foil with thickness of 0.13 mm). Plasma treatments were performed by setting the gap between the tip of the plasma source and the surface of the liquid medium at 2 mm. The experimental setup employed for the treatment is schematically reported in Figure 1B. In all the experiments, the temperature of the medium after CAP treatment resulted below the threshold of cytotoxicity. After the treatment cells were incubated 2 h at 37 °C, and 5% CO_2 before further analysis. The setup reported in Figure 1C was used for the time-resolved record of the plasma discharge electrical parameters. A high voltage probe (Tektronix P6015A, Tektronix, Beaverton, OR, USA) was used to measure the voltage waveform. The discharge current was measured by means of a current probe (Pearson 6585, Pearson Electronics, Palo Alto, CA, USA) mounted on the ground cable. Both signals were recorded with an oscilloscope (Tektronix DPO 40034, Tektronix) and subsequently elaborated to estimate the average power.
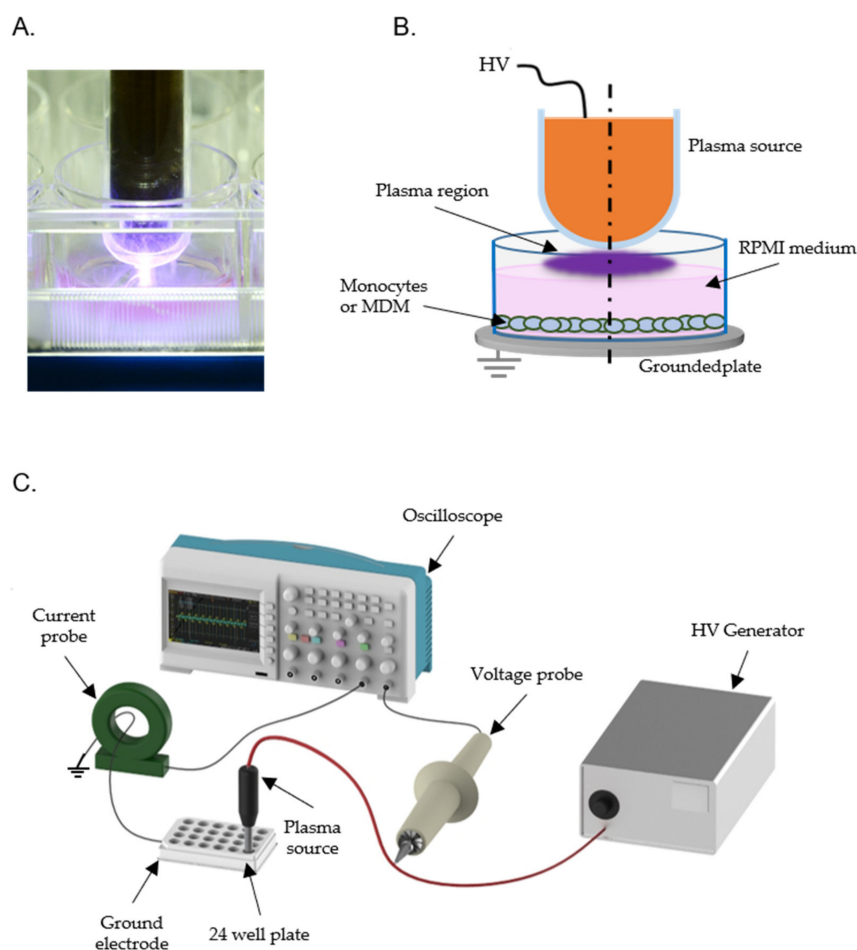
Figure 1. Dielectric Barrier Discharge (DBD) plasma source and setup. (**A**) Picture of plasma generated by a Dielectric Barrier Discharge source on cells; experimental setups employed for (**B**) the plasma treatment of monocytes and monocyte-derived macrophages and (**C**) the electrical characterization.

2.3. Detection of Reactive Oxygen and Nitrogen Species in Plasma-Treated Medium

The Amplex® Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and nitrate/nitrite colorimetric assay (ROCHE, Basel, Switzerland) were used, according to the manufacturer's protocol, to measure the concentrations of hydrogen peroxide and nitrites induced by plasma treatment in 1 mL of cell culture medium. Plasma treated medium was diluted 100 fold in phosphate buffered saline (PBS, a water-based salt solution containing 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl, at pH 7.4) immediately after treatment. The absorbances were measured photometrically with a microplate reader (Rayto, Shenzhen, China).

2.4. Viability Test

To assess potential plasma toxicity in monocytes and MDM, cell viability was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. Cells (1×10^5 cells) were treated with plasma in the above described conditions. Two hours after the treatment, the medium was replaced by the MTT assay solution (1 mg·mL^{-1}; 2 h, 37 °C 5% CO_2; Sigma-Aldrich). Supernatant was removed and DMSO (Sigma-Aldrich) was added in order to dissolve the purple formazan; the absorbance was measured at 580 and 675 nm. Treatment times were 5 s, 10 s and 20 s for monocytes, and 10 s, 20 s and 30 s for macrophages.

2.5. ROS/RNS and Superoxide Anion (O_2^-) Production

2 h after the treatment, the medium was changed and reactive species production was evaluated. O_2^- production was evaluated by the superoxide dismutase-sensitive cytochrome C reduction assay [33]. Briefly, 10^6 monocytes/macrophages treated by CAP were incubated with the cytochrome C (1 mg·mL^{-1}; 2 h, 37 °C 5% CO_2; Sigma-Aldrich) and then the supernatant was read at the spectrophotometer (Perkin Elmer Victor LightPerkin Elmer, MA, USA) at 550 nm. Indeed, cytochrome C reacting with the O_2^- is reduced in ferrocytochrome C whose absorbance is detectable at 550 nm. The results were expressed in nmol cytochrome C reduced/10^6 cells/30 min, using an extinction coefficient of 21.1 mm [34], and correlated with the amount of superoxide anion produced by analyzed cells. Moreover, ROS/RNS and O_2^- productions were also evaluated by flow cytometry analysis (FACS Calibur, BD, San Jose, CA, USA) using the Cellular ROS/Superoxide Detection Assay Kit (AbCam, Cambridge, UK) according to the manufacturer's instructions. The kit provides two fluorescent dye reagents: One (ROS/RNS, green) recognizing reactive species of both oxygen and nitrogen except for the superoxide anion that is detected by the second probe (O_2^-, Orange). Results were analyzed by Flowing Software (version 2.5; PerttuTerho, Turku Centre for Biotechnology, Turku, Finland) and expressed as percentage of cells expressing ROS/RNS or O_2^-. The cut-off for the analysis was based on non-stained sample. Moreover, we analyzed with the same software the mean fluorescence intensity (MFI) as indicator of the mean amount of O_2^- produced by each single cell.

2.6. Flow Cytometry Analysis

Evaluation of surface markers expression was performed by multi-parametric analysis by flow cytometry (FACS Calibur, BD) and analyzed by Flowing Software (version 2.5; PerttuTerho, Turku Centre for Biotechnology). Monocytes and MDM were treated by CAP and after 2 h cells were mechanically detached and stained for FACS analysis. The following antibody panels were used: Anti-CD14 (APC, eBioscience, MA, USA), anti-CD16 (FITC, eBioscience), anti-CD36 (FITC, eBioscience), anti-CD86 (PE, eBioscience), anti-CD163 (PE, eBioscience), and anti-CD206 (PerCp, eBioscience), with 10.000 events acquired. The monocytes and MDM population were defined as CD14$^+$ cells. Data were therefore expressed as the number of CD16$^+$, CD86$^+$, CD36$^+$, CD163$^+$ or CD206$^+$ cells over the number of CD14$^+$ cells (the gating strategy was shown in Figure 2). CD16 and CD86 are representative of M1 phenotype, while CD36, CD163 and CD206 of M2 phenotype.

Comparison between treated and untreated cells was performed and data were expressed as percentage of positive events.
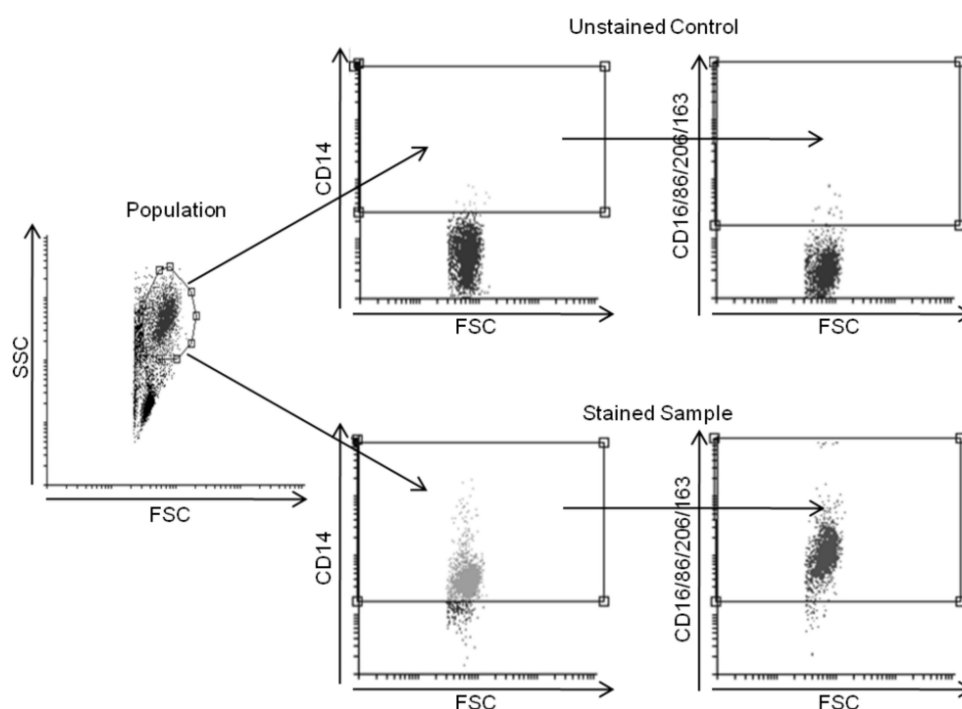
Figure 2. Gating strategy for flow cytometry (FACS) analysis of surface markers in monocytes and monocyte-derived macrophages (MDM). Population was first defined using forward scatter (FSC) and side scatter (SSC) to find viable cells and exclude debris. Then we gated CD14$^+$ cells and on this population we analyzed the expression of the other markers. All the gates were firstly set using unstained control.

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5. Data are presented as mean $\pm$ SEM (standard error of the mean) of "n" independent experiments performed in triplicate on monocytes/macrophages. Data were analyzed by one-way ANOVA non-parametric (Kruskal-Wallis and Dunn's test). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Plasma Treatment and Viability Assay

In Figure 3 is reported the temporal evolution of voltage and current waveforms. Concerning the current waveform is possible to observe multiple peaks both in the positive and negative half-periods of the voltage pulse that may be associated with multiple discharge events. The subsequently processing of waveforms of three independent experiments allowed to estimate an average power density of 1.7 ± 0.1 W.

In Figure 4, the concentrations of hydrogen peroxides and nitrites in 1 mL of culture media after plasma treatments are reported. CAP treatment induced the production of similar concentrations of nitrites (up to about 340 µM after 60 s) in both monocytes and MDM culture medium. On the other hand, hydrogen peroxide concentrations in monocyte culture medium were significantly higher compared to those produced in MDM culture medium.
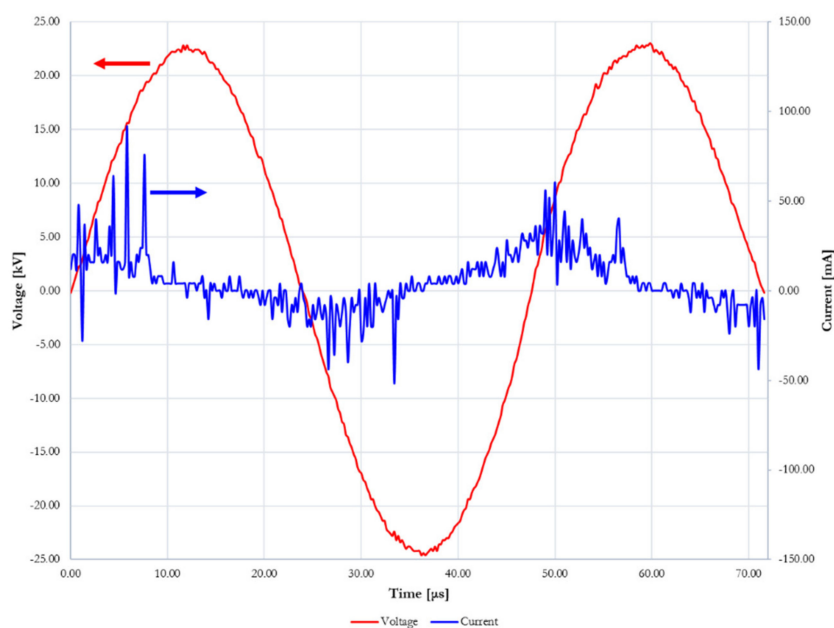
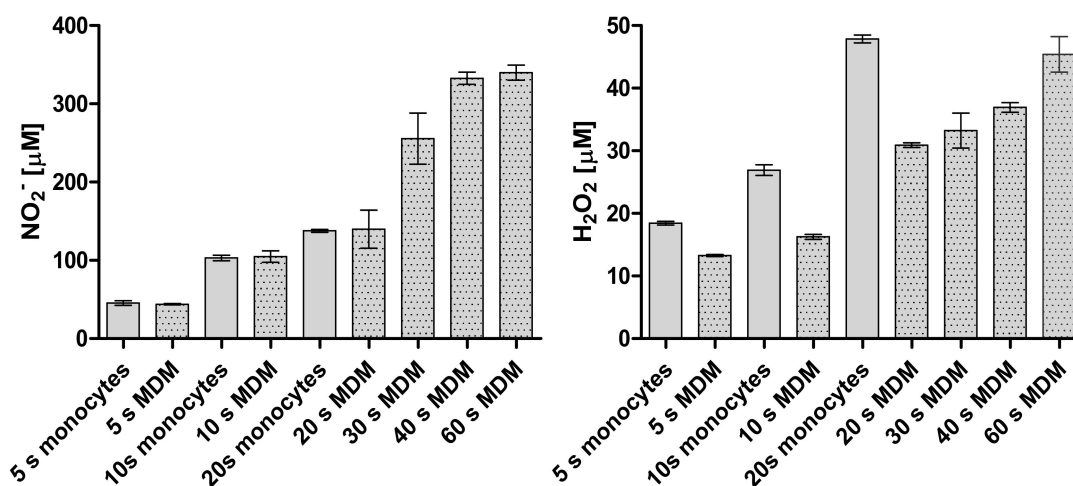**Figure 3.** Typical voltage (blue) and current (red) waveforms recorded during the plasma treatment.

Figure 4. Nitrite and hydrogen peroxide concentrations in plasma treated media. Data are expressed as mean $\pm$ SEM of three independent experiments (n = 9).

Plasma exposure times were selected by evaluating the cytotoxicity induced by CAP in both cell models. Monocytes resulted to be very sensitive to CAP exposure for periods longer than 20 s (data not shown). Therefore, as reported in Figure 5A, the range of exposure times were limited to 5–20 s, despite a significant reduction in cell viability was observed after only 10 s. MDM were at first treated for 20 s, 40 s and 60 s, highlighting a strong reduction of viability after 40 s and 60 s of plasma exposure (data not shown). Accordingly, treatment times of 10 s, 20 s, and 30 s were then performed. In these operating conditions, CAP did not affect MDM viability (Figure 5B), with only a non-significant reduction (20%) of cell viability being registered after 30 s of plasma treatment.
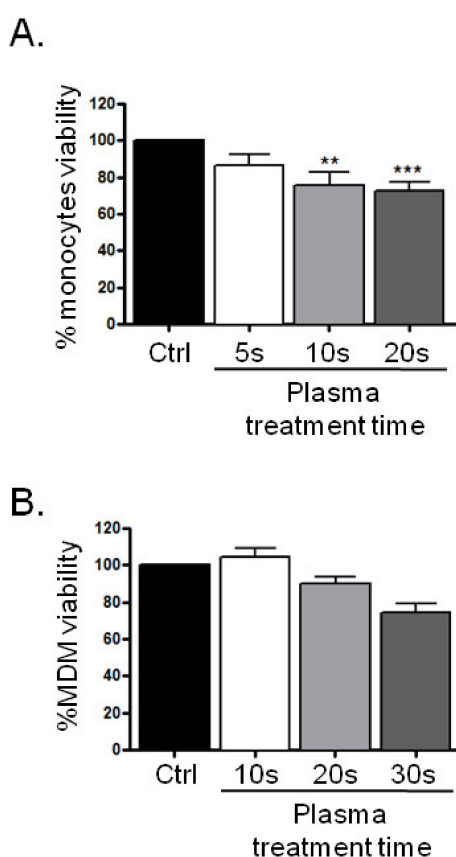
Figure 5. Cell viability after cold atmospheric pressure plasma (CAP) treatment. (**A**) Monocytes treated with CAP for 5 s, 10 s, and 20 s showed a significant reduction of viability already after 10 s of treatment. Data are expressed as mean ± SEM analyzed by Kruskal-Wallis test of 10 independent experiments (*n* = 30). ** *p* < 0.005, *** *p* < 0.001 vs. untreated control (Ctrl). (**B**) MDM treated by CAP for 10 s, 20 s, and 30 s.
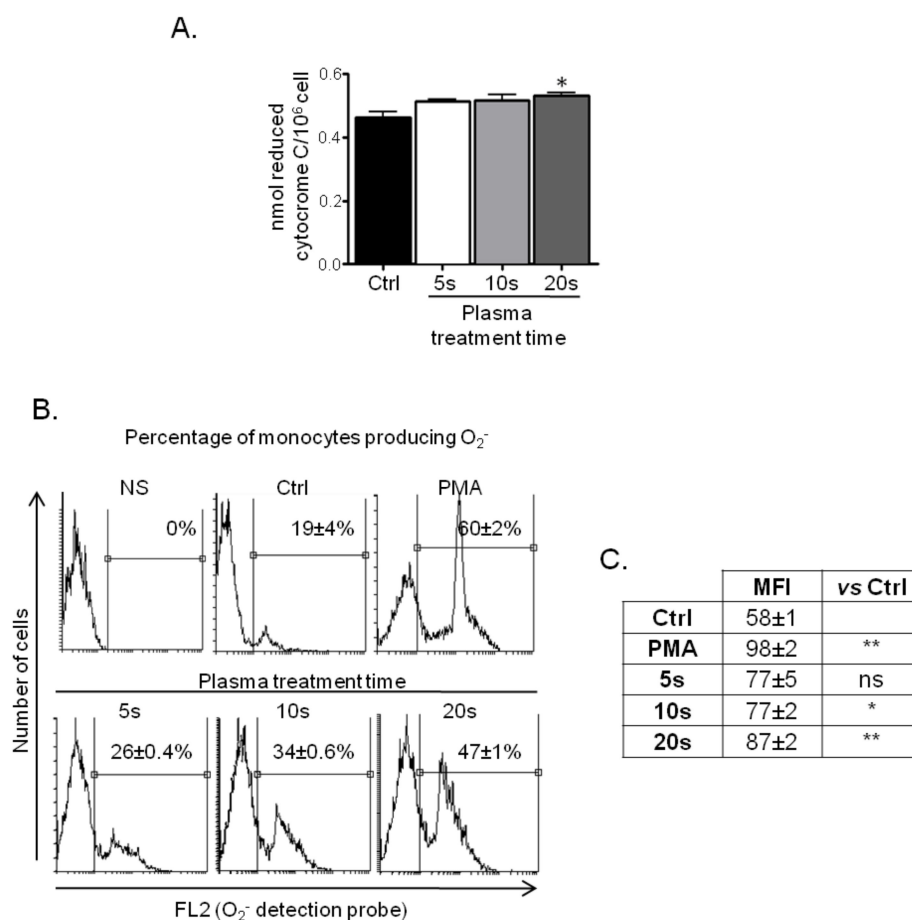
3.2. Plasma Treatment Effects on Monocytes

3.2.1. ROS Production by Treated Monocytes

Since human monocytes are phagocytes and release oxy-radicals upon challenge with appropriate stimuli, we first investigated CAP ability to affect superoxide anion (O_2^-) production, using both an indirect and a direct method (superoxide dismutase-sensitive cytochrome C reduction assay and flow cytometry analysis, respectively), as reported in Materials and Methods. Basal O_2^- production of monocytes amounted to 0.46 ± 0.018 nmol cytochrome C reduced/10^6 cells, while after 20 s of CAP treatment, this was significantly increased by about 0.15 nmol (Figure 6A). This small, but significant, increase might be a direct consequence of the reduced viability observed. The cytofluorimetric assay confirmed this result demonstrating that the percentage of cells producing radical oxygen species moved from about 19 ± 4% of untreated cells to 47 ± 1% after 20 s of CAP treatment (Figure 6B). Moreover, the analysis of MFI, that in this instance represents the amount of superoxide anion produced by cells, revealed a progressive increase (Figure 6C) that become significant at 10 s, confirming that CAP treatment induced not only the oxidative burst in a greater number of cells, but also each cell to produce a higher amount of O_2^-. PMA (positive control of oxidative burst induction) 10^{-6} M was used as positive control of oxidative burst induction [35].

ROS/RNS evaluation in monocytes after CAP treatment could not be performed, since the percentage of expressing cells in the untreated group in our test conditions was high and therefore

poorly responsive to treatments (untreated monocytes 79%±0.7 vs. monocytes treated with PMA 88%±2).

A.

B.

C.

|	MFI	*vs* Ctrl
Ctrl	58±1	
PMA	98±2	**
5s	77±5	ns
10s	77±2	*
20s	87±2	**

Figure 6. Effect of plasma treatment on monocytes superoxide anion production. (**A**) Monocytes were exposed to CAP for 5 s, 10 s and 20 s and results are expressed as levels of nmol reduced cytocromeC/10^6 cells. Data are expressed as mean ± SEM of five independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparisons. * $p < 0.05$ vs. untreated cells (Ctrl); (**B**) Cytofluorimetric assay to assess positive monocytes to the superoxide anion staining. NS, unstained control used to set acquisition parameters; Ctrl, untreated control; PMA, positive control of oxidative burst induction. Results are expressed as percentage mean ± SEM of three independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparisons. (**C**) Mean fluorescence intensity (MFI) analysis expressed as mean ± SEM of three independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparison. * $p < 0.05$; ** $p < 0.005$ vs. corresponding control.

3.2.2. Surface Marker Expression

The expression of specific monocyte surface markers was then analyzed (Figure 6). Freshly isolated monocytes treated with CAP showed a significant reduction (about 40%) of CD86, CD36, CD163 and CD206 already after 5 s of treatment, (Figure 7) while the reduction of CD16 expression became significant after 10 s of plasma exposure.
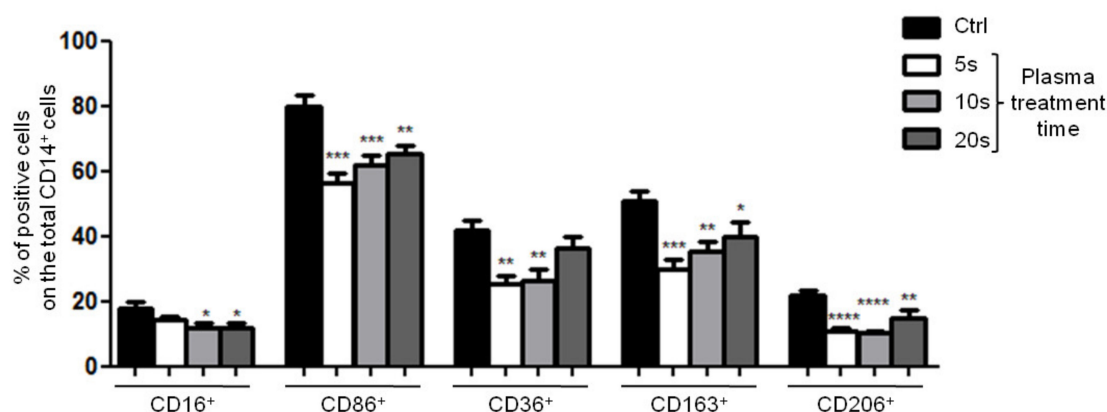
Figure 7. Effect of plasma treatment on membrane markers expression of monocytes. $CD14^+$ cells, treated and untreated (Ctrl), were stained with the indicated antibodies and analyzed by flow cytometry. Results are expressed as the percentage of positive events for each marker on the total of $CD14^+$. Data are expressed as mean $\pm$ SEM of 10 independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparison. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ vs. corresponding control. We have previously shown that untreated monocytes do not change marker expression in a two-hour incubation and therefore the untreated control also depicts the baseline expression [36].

3.3. Plasma Treatment Effects on Monocytes-Derived Macrophages (MDM)

#### 3.3.1. ROS Production by MDM

The production of both O_2^- and other reactive oxygen and nitrogen species (ROS/RNS) in MDM exposed to CAP is shown in Figure 6. MDM had a basal production of O_2^- of about a 0.39 ± 0.04 nmol of cytochrome C reduced/10^6 cells (Figure 8A), and CAP treatments of 10, 20 and 30 s were ineffective in inducing an oxidative burst, as demonstrated by the unchanged percentage of positive cells to the ROS/RNS staining (Figure 8B). In contrast, the number of positive MDM to the O_2^- staining was significantly increased after the longest time treatment (Figure 8C). It is interesting to note that after plasma treatment we can observe the onset of a peak of low-expressing O_2^- cells (represented by the circled peak in the histogram), that gradually increases in parallel with CAP-treatment time. This can be correlated with the emergence of a new population of MDM (in treated cells vs. Ctrl) able to produce basal levels of O_2^-. Moreover, it is important to note that the MFI did not increase (Figure 8D).

3.3.2. Surface Marker Expression

As shown in Figure 9A, in MDM exposed to CAP the expression of the single surface markers was not significantly influenced by CAP treatment, except for CD36, whose expression significantly increased, and CD16, that decreased already after 10 s. This result is in line with the previously presented data: In fact, CAP treatment did not induce the MDM respiratory burst nor a decrease in cell survival even after the longest treatment time. The possibility that CAP exposure could drive polarization of MDM versus M1 or M2 phenotypes was investigated. In fact, freshly isolated monocytes, cultured in 20% FBS-enriched medium, spontaneously differentiate to MDM, defined as M0, an intermediate phenotype (not M1 nor M2), that under different stimuli can shift to one phenotype or to the other. We performed a co-expression analysis for the specific markers of M1 (CD16/CD86) and M2 (CD163/206) on the total of $CD14^+$ population. As demonstrated in Figure 9B, there was a time dependent reduction of M1 population, defined as $CD14^+CD16^+CD86^+$, becoming significant at 30 s, with a correspondent increase to the M2 population, defined as $CD14^+CD163^+CD206^+$.
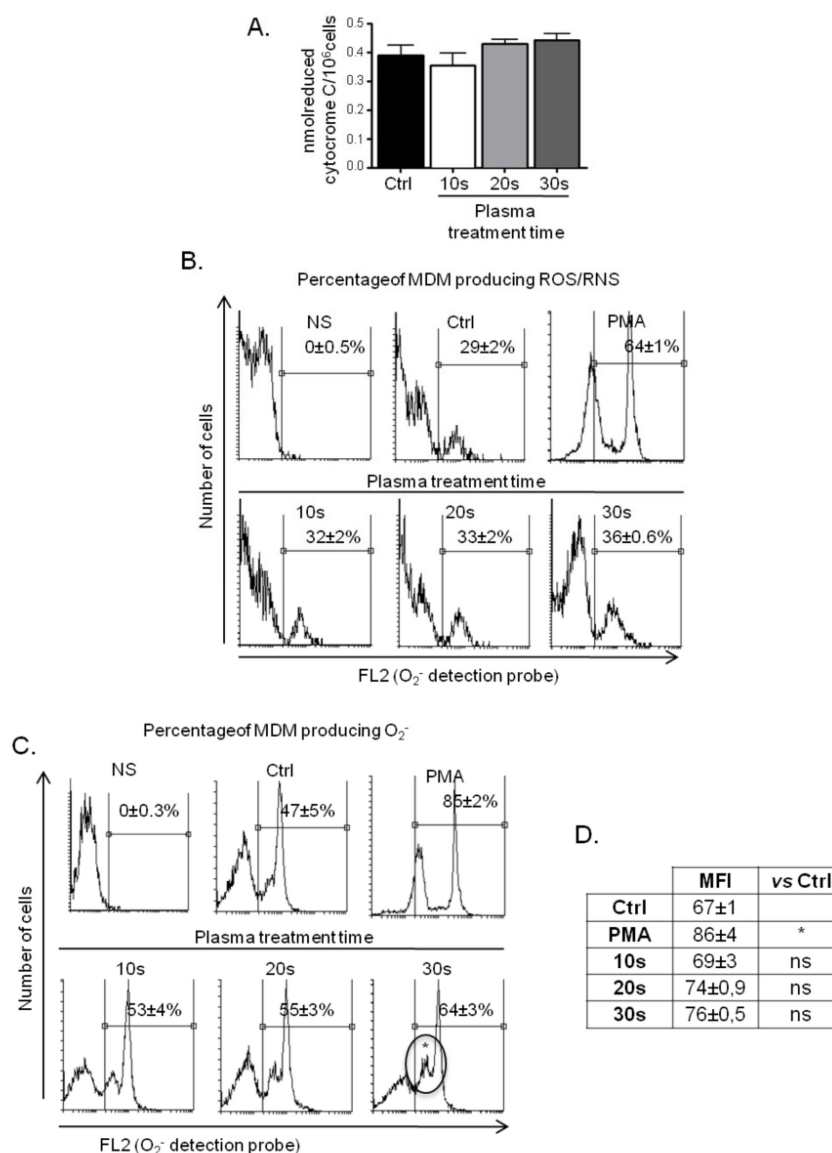
Figure 8. Effect of plasma treatment on reactive species production in MDM. (**A**) Superoxide anion production. Cells were irradiated by CAP for 10, 20 and 30 s, and results are expressed as levels of nmol reduced cytocromeC/10^6 MDM. Data are expressed as mean of five independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparison; (**B**) Cytofluorimetric assay to assess MDM positive to the reactive oxygen (ROS)/RNS detection probe staining after 10, 20 and 30 s of treatment with CAP. Results are expressed as percentage mean of MDM producing ROS/RNS $\pm$ SEM of three independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparison; (**C**) Cytofluorimetric assay to assess MDM positive to the superoxide anion detection probe staining after 10, 20 and 30 s of treatment with CAP. Results are expressed as percentage mean of MDM producing O_2^- $\pm$ SEM of three independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparison. * $p < 0.05$ vs. untreated cell (Ctrl); (**D**) MFI analysis expressed as mean $\pm$ SEM of three independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparisons. * $p < 0.05$ vs. corresponding control. NS, unstained control used to set acquisition parameters, Ctrl, untreated control; PMA, positive control of oxidative burst induction.
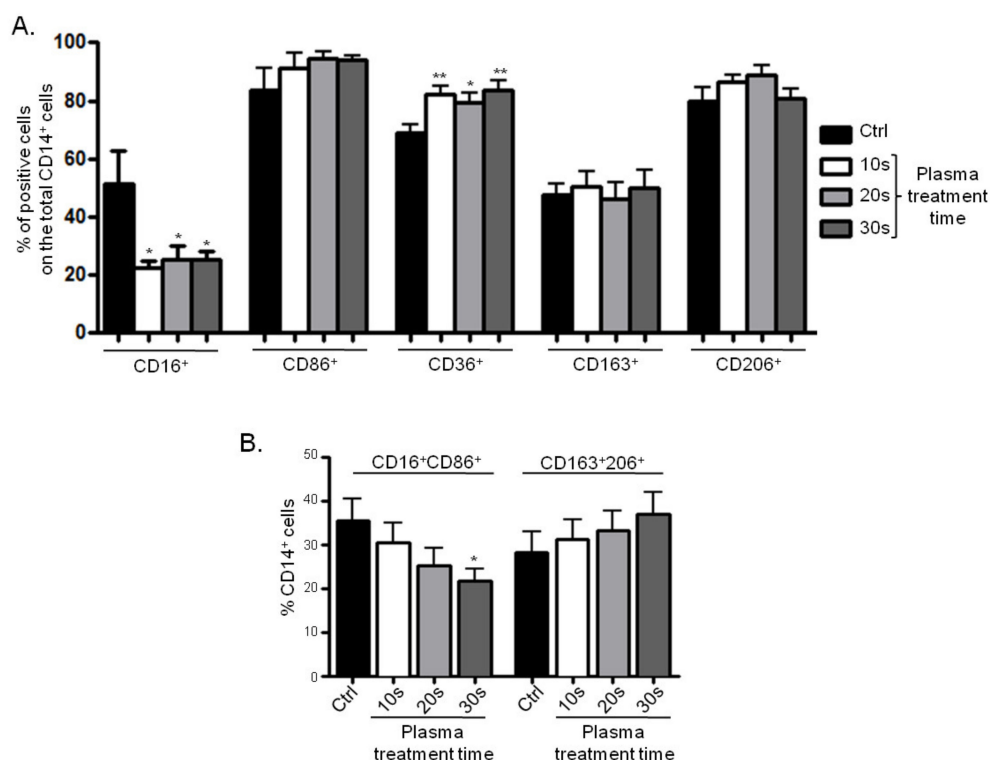
Figure 9. Effect of plasma treatment on MDM phenotype. (**A**) Cyofluorimetric analysis of MDM stained with anti-CD14, anti-CD86, anti-CD36, anti-CD163 and anti-CD206. Results are expressed as the percentage mean of positive events for each marker on the total of CD14$^+$ ± SEM of 10 independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparison. * $p < 0.05$, ** $p < 0.005$ vs. untreated control (Ctrl); (**B**) Cytofluorimetric analysis of M1 and M2 populations represented by the co-expression of CD14/CD16/CD86 and CD14/CD163/CD206 respectively. * $p < 0.05$ vs. untreated cell (Ctrl). Data are expressed as mean ± SEM of 10 independent experiments analyzed by Kruskal-Wallis test and Dunn's test for multiple comparisons. We have previously shown that untreated MDM do not change marker expression in a two-hour incubation and therefore the untreated control also depicts the baseline expression.

4. Discussion

In this study, the effect of a microsecond pulsed DBD on human monocytes and monocyte-derived macrophages was investigated.

The CAP treatment of monocytes and MDM culture medium induced the production of nitrites and hydrogen peroxides. The lower the concentration of FBS, the higher the concentration of hydrogen peroxide, while the concentration of nitrites resulted similar in both treated media. This difference can be ascribed to the differences between MDM and monocytes culture medium in terms of FBS concentrations, scavenger of hydrogen peroxide, but not of nitrites [37]. Moreover, the longer the treatment time, the higher was the concentration of both species in the treated media and the reduction of cell viability for both monocytes and MDM. Similar findings have been reported for several cell lines [16,20,38].

Focusing on monocytes, CAP treatment up to 20 s induced cells to produce of O_2^- and a decrease of surface markers. This effect is unlikely to be due to a change in cell polarization, but is most likely a physiochemical modification, since compensatory increases in cell surface markers were not observed to counteract these decreases. While we did not investigate this further, the decrease of marker expression might be explained by a direct membrane damage (for example peroxidation and subsequent loss of membrane fluidity and elasticity [39]), to a direct protein oxidation [40] or

by other mechanisms, which we have nonetheless not evaluated further. Anyway, it is noteworthy to highlight a slight recovery of the surface marker expression after the impressive 5 s reduction. This could be explained with a rapid anti-oxidative response of cell to the insult [41], but this defense mechanism was not sufficient to restore membrane integrity and keep cell survival. The fact that monocytes are significantly more sensitive to CAP compared to MDM would be in accordance with Bundscherer et al. [42] who demonstrated significant differences between different immune cell types regarding survival after plasma treatment.

On the other hand, CAP treatment for up to 30 s of MDM did not cause an increase in O_2^- production, but only induced a greater number of cells to produce the superoxide anion. As a possible consequence, MDM, terminally differentiated cells, resulted more resistant to CAP treatment. Indeed, CAP treatment did not affect significantly MDM survival. We suggest that these results are strictly connected to the absence of an increase of ROS/RNS and the augmented percentage of O_2^--producing MDM. In fact, as demonstrated by Zhang et al. [43] the polarization versus M2 phenotype is sustained by a proper amount of O_2^-. Indeed, when monocytes are induced to differentiate there is an increased superoxide anion production that triggers the biphasic ERK activation that has a pivotal role for M2 differentiation. In our experiments, CAP stimulated more M0 to secrete basal amounts of superoxide necessary to polarize versus M2, that were represented by the new population previously described in Figure 8C. Moreover, marker expression modifications in MDM were reputed as a true sign of polarization change, since CD16 decrease was counter-balanced by an increase in CD36, while CD163 and CD206 were not affected. In the present study, M2 subsets most represented in CAP treated MDM have not been evaluated. In the future, it will be interesting to explore this aspect as the different M2 sub-populations are involved in different physiological and pathological processes [4,44,45]. This information will therefore provide clues on the clinical applications of CAP.

5. Conclusions

In conclusion, our results suggest that CAP treatment may be able to selectively modulate the effect of some cells over others. Furthermore, CAP could drive macrophage polarization supporting the idea that at the proper operating conditions of plasma treatment it could be possible to direct macrophages versus an anti-inflammatory phenotype. At the same time, our results also suggest the differences in terms of viability of macrophages compared to monocytes after CAP exposure may be due to the different concentration of FBS between the cell culture media. In fact, as reported by Yan et al. [46], it is possible to harness the medium to kill glioblastoma cells by altering the concentration of the serum demonstrating therefore the significant role of media in CAP treatment. In prospect, our results may suggest that CAP technology may find clinical applications in those pathological settings in which macrophages play a prominent role, and in which it can be delivered locally, for example in wound healing, treatment of non-metastatic melanoma difficult to surgically remove or in topical applications for autoimmune disorders, such as psoriasis.

We acknowledge that our results should be read in light of the following limits: (a) We have evaluated changes in monocytes and MDM 2 h after CAP treatment, but events occurring immediately after treatment might be relevant; (b) we have not fully characterized the M2 sub-populations represented in CAP treated cells, neither by FACS nor by real time PCR. This last point, in the future, will yield more precise indications on the possible therapeutic applications of CAP.

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