Differential sensitivity of lymphocyte subpopulations to non-thermal atmospheric-pressure plasma

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Non-thermal atmospheric-pressure plasmas can possibly be used for several applications in particular in medicine. Plasma treatment can be applied to living tissues and cells, e.g., to induce apoptosis and growth arrest in tumour cells or to improve wound healing. However, detailed investigations of plasma–cell interactions are strongly needed. It is not yet clear whether plasmas will be useful in stimulating immune cells to change their behaviour or function. Therefore, this study focused on the influence of non-thermal atmospheric pressure plasma on cell surface molecules of rat spleen mononuclear cells (MNC) as first important step to gain insight into plasma–immune cells interactions. Rat spleen MNC were treated with plasma by surface dielectric barrier discharge (DBD) at atmospheric pressure in air or argon. Lymphocyte subpopulations and expression of L-selectin, ICAM-1 and LFA-1α expression on T-cells were analysed by flow cytometry 1–48 h after plasma treatment. Plasma changed the ratio of T- and B-cells in favour of B-cells. Of the T-cells the helper T-cells were reduced while cytotoxic T-cells were less affected. L-selectin expressing T-cells were significantly reduced already 1 h after plasma treatment and that of ICAM-1* and LFA-1α*T-cells only after 4 h. These effects were time dependent and less dramatic when using DBD/argon plasma. In conclusion, different lymphocyte subpopulations show different sensitivity to plasma. Adhesion molecules as L-selectin, ICAM-1 and LFA-1α are down regulated by plasma. Whether these results can be used to modify lymphocyte homing or to activate MNC for different applications remains to be clarified.

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Introduction

Plasma is defined as ionized gas and considered as the fourth state of matter. Since it is possible to generate non-thermal plasma at atmospheric pressure, it can be used for treating living tissues and cells, thus becoming increasingly important in medicine. The complex effects of those plasmas are caused by different neutral and charged particles, electric fields, radicals (e.g., reactive oxygen species) and other reactive molecules (e.g., hydrogen peroxide, nitric oxide) as well as different types of radiation, e.g., VUV, UVA, UVB (Dobrynin et al. 2009). Therefore, plasma can be used for several applications, e.g., for disinfection of surgical instruments or catheters (Halfmann et al. 2007; Ehlbeck et al. 2011), management of chronic infected wounds (Kramer et al. 2008; Nosenko et al. 2009; Isbary et al. 2010; Heinlein et al. 2011), dental applications (Lee et al. 2009; Pan et al. 2010), skin regeneration (Foster et al. 2008), treatment of cancer cells (Fridman et al. 2007; Kim et al. 2010a,b).

Plasma has also been shown to affect the human immune system in terms of inducing apoptosis in peripheral blood lymphocytes (Shi et al. 2008). However, it is still in question as to whether plasma can also influence mononuclear cells (MNC) to become more sensitive against tumour cells or to generate regulatory cells important in autoimmune diseases. Detailed investigations of plasma–cell interactions are needed for each of these different applications. It is known that adhesion molecules can be affected by plasma treatment (Haertel et al. 2011) which is important for the wound healing process. However, the extent of down or up regulation of those molecules depends on the plasma source and the treatment time. Plasma is also able to induce apoptosis, which is an important feature when treating cancer cells (Fridman et al. 2007; Kim et al. 2010a,b). These examples show that different objectives demand different requirements from the plasma sources and also from the regimen of plasma treatment.

The purpose of this study was to investigate how non-thermal atmospheric pressure plasma influences mononuclear cells. Such
findings might lead to plasma applications in immunology or cancer treatment. MNC isolated from rat spleens were treated with plasma generated by surface dielectric barrier discharge (surface DBD) at atmospheric pressure in air or argon. Lymphocyte subpopulations as well as L-selectin and LFA-1α expression on T-cells were analysed by flow cytometry 24 h after plasma treatment.

Materials and methods

Materials

Cell culture materials were purchased from TPP (Trasadingen, Switzerland). Roswell Park Memorial Institute Medium 1640 (RPMI 1640) with l-glutamine, fetal calf serum (FCS), Histopaque 1083 and sodium azide (NaNO₃) came from Sigma (Taufkirchen/Deisenhofen, Germany). Penicillin and streptomycin were purchased from Lonza (Verviers, Belgium) and phosphate buffered saline (PBS) came from PAA (Cölbe, Germany). All monoclonal antibodies were obtained from BD Pharmingen (San Diego, USA).

Animals

Male LEW.1W rats (RT1b) were bred at the Division of Laboratory Animal Science of the University of Greifswald and used at the age of 10 weeks. They were housed in a humidity and temperature-controlled facility with light from 6:00 a.m. to 6:00 p.m. They had free access to a standard rat chow (Ssniff, Soest, Germany) containing 0.6% NaCl and fresh tap water ad libitum. All experiments were approved by a governmental committee on animal welfare.

Preparation of mononuclear cells

The spleens were removed from male anaesthetized LEW1.W rats. Mononuclear cells (MNC) were prepared by gradient centrifugation by using a Histopaque 1083 as described earlier (Kuttler et al. 1990). Isolated mononuclear cells were resuspended in 8 ml RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell number was determined with a Neubauer chamber and adjusted to 2 × 10⁶/ml.

Treatment of mononuclear cells with dielectric barrier discharge plasma

For treatment of cells with DBD plasma, 4 × 10⁶ cells were plated in to 60 mm diameter Petri dishes in 4 ml RPMI 1640, 10% FCS. The cells were treated in suspension with surface DBD plasma at atmospheric pressure and air or argon as process gas based on a setup described elsewhere (Hähnel et al. 2007, 2010; Oehmigen et al. 2010). A schematic of the surface DBD is shown in Fig. 1 (Hähnel M., personal communication). The electrode system was made of material for circuit boards and had a thickness of 1.5 mm and a diameter of 50 mm. Both electrodes had an outer diameter of 37 mm and were separated by an epoxy-glass fibre bulk which functioned as dielectric barrier. On the other side of the dielectric the electrode was structured in a concentric ring-shape. The electrode system was mounted into the upper shell of a Petri dish (60 mm diameter). The height of the electrode system in the upper shell of the Petri dish could be adjusted between 2 and 5 mm above the liquid sample in the lower shell of the Petri dish. Between both electrodes a sinusoidal high voltage was applied with a peak voltage of 10 kV and 20 kHz. In order to keep the power input low the high voltage was pulsed with a ratio of 0.4/1.2 ms plasma-on/plasma-off time. Energy of 2.4 mJ was dissipated into the plasma in each cycle of high voltage. The power was 0.25 W/cm². The plasma was formed in a thin layer above the structured side, which faced towards the liquid sample.

Plasma treatment was done for 10, 20 and 60 s. Control cells remained untreated. After a culture period of 1, 4, 24 and 48 h at 37 °C (in a humidified atmosphere of 5% CO₂ and 95% air) the MNC were harvested, centrifuged, and the cell pellet was resuspended in PBS supplemented with 0.1% NaN₃ and 1% FCS (FACS-PBS) or Annexin V staining buffer for flow cytometry. Cells were counted with a Neubauer chamber.

Flow cytometry

Flow cytometry was used to determine lymphocyte phenotypes and expression of L-selectin, ICAM-1 and LFA-1α on T cells by using phycoerythrin (PE)- or fluoresceinisothiocyanate (FITC)- conjugated monoclonal antibodies (Kuttler et al. 1997). Mononuclear cells were stained with the following antibodies: 1A29-PE/R73-FITC (ICAM-1 [CD29] positive T cells), WT.1-PE/R73-FITC (LFA-1α [CD11a] positive T cells), HRL-1-PE/R73-FITC (L-selectin [CD62L] positive T cells), HRL-1-PE/OX38-FITC (L-selectin positive TH cells [CD4]), HRL-1-PE/R73-FITC [L-selectin positive CTL (CD8)], OX33-FITC (B cells, CD45RA).

A minimum of 1 × 10⁵ cells were incubated with the antibodies for 15 min at 4 °C in the dark in a final volume of 70 μl FACS-PBS. Cells stained with the unlabeled antibodies were washed with 4 ml FACS-PBS and incubated with a FITC- or PE conjugated second antibody. After a final washing with 4 ml FACS-PBS stained cells were analysed on a FACSscan (BD, Heidelberg, Germany) by means of an air-cooled 488 nm 15 mW argon laser and detectors for forward scatter, 90° light scatter (side scatter), fluorescence 1 (FL1: FITC = green) and fluorescence 2 (FL2: PE = orange).

Apoptotic cells were determined by using the FITC Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, Germany). Cells were stained with propidium iodide (PI) and FITC Annexin according to the instruction of BD Pharmingen and the staining was analysed on a FACSscan. Early apoptotic cells were defined as PI negative and Annexin V positive. Annexin V positive cells with additional PI uptake were either late apoptotic or necrotic.

The flow cytometric results are expressed as a percentage of cells which stained positive. Moreover, antigen density as mean fluorescence intensity of a staining in log(U) was registered.

Results are given as mean ± SEM of N independent experiments. The number of experiments can be taken from the figures. SigmaStat Software was used to check for statistical significance [Student's
Table 1

Viability (%) of MNC harvested after treatment with DBD/argon plasma and lymphocyte phenotypes (%) as determined by flow cytometry of rat spleen MNC. Control MNC remained untreated. Phenotypes were analysed 24 h after plasma treatment.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Untreated</th>
<th>Treatment time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>10 s</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Viability</td>
<td>86.5 ± 1.1</td>
<td>84.4 ± 2.4</td>
</tr>
<tr>
<td>T-cells</td>
<td>59.4 ± 1.8</td>
<td>60.2 ± 2.6</td>
</tr>
<tr>
<td>B-cells</td>
<td>34.8 ± 1.1</td>
<td>34.5 ± 2.2</td>
</tr>
<tr>
<td>Tn-cells</td>
<td>48.2 ± 1.2</td>
<td>42.1 ± 2.2</td>
</tr>
<tr>
<td>CTL</td>
<td>21.2 ± 0.9</td>
<td>19.8 ± 0.3</td>
</tr>
</tbody>
</table>

*p < 0.05.
**p < 0.01 vs. control.
***p < 0.001 vs. control.

Table 2

Viability (%) of MNC harvested 1, 4, 24 and 48 h after treatment with DBD/air plasma. Control MNC remained untreated (treatment time = 0). Viability was calculated as % of cells in R1 of cells in R1 + R2.

<table>
<thead>
<tr>
<th>Culture time</th>
<th>Treatment time (h)</th>
<th>Treatment time (s)</th>
<th>N</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>95.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>95.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6</td>
<td>6</td>
<td>93.8 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>8</td>
<td>7</td>
<td>94.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>89.9 ± 1.7</td>
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<tr>
<td></td>
<td>20</td>
<td>7</td>
<td>7</td>
<td>73.0 ± 4.8</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>12</td>
<td>7</td>
<td>77.6 ± 1.9</td>
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<td></td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>60.1 ± 5.3</td>
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<td></td>
<td>20</td>
<td>8</td>
<td>8</td>
<td>32.1 ± 2.3</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>7</td>
<td>7</td>
<td>34.4 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>25.0 ± 3.1</td>
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<tr>
<td></td>
<td>20</td>
<td>7</td>
<td>7</td>
<td>15.9 ± 3.6**</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. control.
**p < 0.01 vs. control.
***p < 0.001 vs. control.

Results and discussion

More than 80% of MNC isolated from rat spleens and treated for 10 or 20 s with DBD in argon were viable when analysed after 24 h (Table 1 and Fig. 2E–G). By increasing the duration of treatment to 60 s the amount of viable cells was significantly decreased (Table 1). In contrast, MNC were more sensitive to treatment with DBD in air (Table 2 and Fig. 2B–D). A DBD/air plasma treatment for 60 s and analysis 24 h later caused the death of all MNC while after DBD/argon plasma 67% of MNC were still alive (Table 1 and Fig. 2D and G). DBD/air plasma treated MNC were, therefore, additionally analysed after 1, 4 and 48 h. Plasma was without significant influence or with little effect on viability of MNC analysed after 1 h or 4 h, respectively (Table 1). Viability of control MNC decreased with culture time (Table 2). However, the effect on plasma treated MNC was significantly more pronounced. Analysis of plasma treated MNC after 48 h revealed a high proportion of dead cells (Table 2). Independent of whether MNC were treated with DBD/argon or DBD/air plasma the proportion of cells in R2 (PI positive dead cells) increased with the duration of plasma treatment (Fig. 2B–G). Compared to HaCaT keratinocytes, immune cells showed a very sensitive response to surface DBD plasma. While HaCaT keratinocytes in suspension can be treated for up to 120 s with DBD plasma in air (Haertel B., unpublished data), complete destruction of MNC was already observed after only a 60 s treatment time (Fig. 2D; cells were only found in R2). Human peripheral blood mononuclear cells (PBMC) are similarly sensitive to DBD/air plasma compared to rat spleen MNC (Shi et al. 2008). After being plasma-treated for 30 s only 23% of PBMC were viable. Simultaneously, induction of apoptosis was observed (Shi et al. 2008).

Fig. 2. Representative examples of density plots of mononuclear cells (MNC) isolated from LEW.1W rat spleens. MNC remained either untreated (A) or were treated with DBD/air plasma (B–D) or with DBD/argon plasma (E–G). While viable MNC were identified in region 1 (R1) in R2 dead cells were observed. With increasing time of plasma treatment the proportion of cells in R2 was increasing. This effect was more pronounced using DBD/air plasma (B–D).
Within the gate of living MNC (R1) no increase of early apoptotic cells were found when MNC were treated for 10 s (Fig. 3A). However, increasing the duration of treatment to 20 s a significantly enhanced proportion of early apoptotic cells were detected (Fig. 3A and D). This result was independent on the time of analysis. Surprisingly, already 1 h after plasma treatment a proportion of about 10% MNC was annexin V positive and PI negative. A maximum of early apoptotic cells was observed after 4 h (Fig. 3A). The proportion of late apoptotic/necrotic cells among the gate of living cells remained below 4% (Fig. 3B). Analysing DBD/air plasma treated HaCaT keratinocytes in the gate of living cells 24 h after treatment we excluded Fas expression as late marker for apoptosis.

![Figure 3](image1.png)

**Fig. 3.** Early apoptotic (A) and late/necrotic MNC (B) isolated from LEW.1W rat spleens after plasma treatment. MNC remained either untreated (Con) or were treated with DBD/air plasma for 10 and 20 s. Apoptosis was analysed 1, 4, 24 and 48 h following plasma treatment. Representative dot plots of Annexin V and PI staining are shown from untreated (C) and 20 s DBD/air plasma treated MNC (D) analysed 4 h after plasma treatment. N = 6 (1 h), N = 9 (4 and 24 h), N = 7 (48 h); *p < 0.05, ** and ***p < 0.01.

![Figure 4](image2.png)

**Fig. 4.** Absolute numbers of lymphocyte phenotypes of rat spleen MNC analysed 1, 4, 24 and 48 h following plasma treatment as determined by flow cytometry. MNC remained either untreated (Con) or were treated with DBD/air plasma for 10 and 20 s. (A) αβ T-cells (R73+ cells), (B) CD45RA+ B-cells (OX33+ cells), (C) CD4+ TH (OX38+ cells) and (D) CD8+ CTL's (341+ cells). N = 6 (1 h and 24 h), N = 7 (4 and 48 h); *p < 0.05, ** and ***p < 0.01, ****p < 0.001.
and an increase in the SubG1 phase of cell cycle as sign of apoptosis in treated cells (Haertel B., unpublished data). Additionally, no PI positive HaCaT cells were found after plasma treatment. Up to now, we did not measure annexin V in HaCaT cells.

DBD/argon plasma had no influence on lymphocytes subpopulations up to a treatment time of 20 s investigated 24 h after plasma treatment (Table 1). However, the 60 s treatment period caused a reduced proportion of T and TH1 cells and consequently, an increase of B lymphocytes (Table 1). Using DBD/air plasma this effect was already observed after a treatment period of 20 s when analysed after 24 h (data not shown). Generally, the reduction of T-cells was restricted to the reduction of helper T-cells while CTL were not affected. Similar changes were also observed when the MNC were analysed 4 h after plasma treatment. No changes in relative and absolute composition of lymphocyte subpopulations were observed 1 h after plasma treatment. Calculating absolute numbers of single subpopulations the results underline the reduction of T cells by 20 s plasma treatment when analysed after 4, 24 and 48 h (Fig. 4A). Due to reduction of the number of viable cells an increase of B cell number was not found (Fig. 4B). However, a significant reduction of B cells was only seen after 24 and 48 h. Reduction of T cell number was mainly due to reduction of TH1 cells; reduction of CTL was less pronounced (Fig. 4C and D).

As already mentioned effects of plasma generated at atmospheric pressure are caused by different neutral and charged particles, electric fields, radicals (e.g. reactive oxygen species) and other chemically reactive molecules (e.g., hydrogen peroxide, nitric oxide) as well as different types of radiation (e.g., UVA, UVB). There is no doubt that UV radiation influences the immune system, primarily the skin-associated lymphoid tissue (Roberts et al. 1988). UV radiation can modulate the immune system depending on the dose and kind of application, for example, it can suppress TH1-mediated immune reactions or activate regulatory T-cells (Ullrich 1996). Thereby, UV radiation may play a protective role in autoimmune diseases, for example rheumatoid arthritis, multiple sclerosis or insulin-dependent diabetes mellitus (Ponsonby et al. 2002; Artukovic et al. 2010). It is unclear whether plasma can induce regulatory T-cells and influence thereby T-cell mediated autoimmune diseases, however, it does reduce T-cell number similarly as observed after UVB irradiation (Kohlhardt-Floehr et al. 2010). On the other hand, an increase in intracellular reactive oxygen species, which can also be generated by plasma, induces apoptosis in lymphocytes (Ryazanceva et al. 2010). Hydrogen peroxide has been shown to decrease TH1 cells with no change of TH2 cells (Hagen et al. 2011).

Additionally, L-selectin and LFA-1α were analysed on T-cells surface. L-selectin as cell surface adhesion molecule belongs to a family of adhesion and homing receptors. These receptors are of importance in leukocytes–endothelial cell interactions for entering secondary lymphoid tissues. LFA-1α expressed on nearly all MNC belongs to the integrin family, is involved in cellular adhesion and binds to its ligand ICAM-1 on antigen presenting cells. Thus, this molecule plays a role in T-cell signalling and activation (Bianchi et al. 2000; Perez et al. 2003). Both the proportion and the absolute number of L-selectin expressing T-cells was significantly reduced (Fig. 5A). Of the T cells the TH1 cells were more affected than CTL (Fig. 5B and C). This reduction was already seen 1 h after plasma treatment for 10 s and was most pronounced after 24 h and the 20 s treatment period. The proportion of LFA-1α positive T-cells remained unaffected by DBD/air plasma (data not shown). However, the mean fluorescence intensity of the LFA-1α expression on T-cells was enhanced 4 and 24 h after plasma treatment for 20 s (Fig. 6B). Since the absolute number of LFA-1α positive T-cells decreased it is quite possible that the increase of LFA-1α density is the result of a selection process. The proportion of ICAM-1 positive T-cells was also enhanced by 20 s DBD/air plasma treatment (data not shown). The number of those cells was reduced; however, not as strong as the LFA-1α expressing T-cells (Fig. 6A and B). While L-selectin expressing T-cells were already affected 1 h after plasma treatment (Fig. 5), this was never observed for LFA-1α and ICAM-1 expressing T-cells (Fig. 6). The DBD/argon plasma treatment did not affect these molecules on T-cells.

In conclusion, it has become evident that lymphocyte subpopulations are selectively sensitive to the effects of plasma. The effects are dependent on the duration of treatment as well as on the time after plasma treatment. T-cells react more sensitive to plasma than B-cells, and TH1 cells are more sensitive than CTL. By treating MNC with plasma, adhesion between cells can change, thus affecting cellular functions such as migration and proliferation. Due to the reduction of L-selectin, homing of lymphocytes can also be altered. On the other hand, the threshold for activation of MNC is possibly reduced by plasma treatment due to an increase in LFA-1α. Whether these changes can be used to generate regulatory T-cells, to sensitize immune cells against tumour cells or to modify homing of lymphocytes remains to be clarified. Plasma composition is variable, for example through use of different working gases. On going work are attempting to decipher the composition responsible for the observed effect to design optimal plasma for various requirements.

Fig. 5. Expression of L-selectin on T cells (A), TH1 cells (B) and CTL (C) of rat spleen MNC 1, 4, 24 and 48 h following plasma treatment as determined by flow cytometry. MNC remained either untreated (Con) or were treated with DBD/air plasma for 10 and 20 s. The results are expressed as absolute numbers of cells expressing L-selectin (×10^4). N = 6 (1 h), N = 7 (4 and 48 h), N = 10 (24 h): *p < 0.05, **p < 0.01, ***p < 0.001.
References


