

# Influence of oxygen concentration on T cell proliferation and susceptibility to apoptosis in healthy men and women

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## Abstract

**Introduction.** Much of what we know about the functioning of human T lymphocytes is based on the experiments carried out at atmospheric oxygen (O<sub>2</sub>) concentrations, which are significantly higher than those maintained in blood. Interestingly, the gender differences in the activity of T cells and their susceptibility to apoptosis under different O<sub>2</sub> conditions have not yet been described. The aim of the study was to compare two main markers of lymphocyte function: proliferation capacity and ability to produce cytokines as well as their susceptibility to apoptosis under two different O<sub>2</sub> concentrations, between men and women.

**Material and methods.** 25 healthy volunteers, both males (13) and females (12) were recruited to the study (mean age 25.48 ± 5.51). By using cytometry proliferation parameters of human CD4<sup>+</sup>CD28<sup>+</sup> cells or CD8<sup>+</sup>CD28<sup>+</sup> cells in response to polyclonal stimulation of the TCR/CD3 complex at atmospheric (21%) and physiological (10%) O<sub>2</sub> concentrations using our modified dividing cell tracking technique (DCT) were analyzed as well as the percentages of apoptotic cells. We also determined the levels of IFN-γ, IL-2, IL-10 and IL-17A using Cytometric Bead Array Flex system in cell culture supernatants.

**Results.** CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells from the whole study group were characterized by shorter time required to enter the first (G1) phase of the first cell cycle at 21% compared to 10% O<sub>2</sub>. Both T cell populations performed significantly more divisions at 21% O<sub>2</sub>. The percentages of dividing cells were also significantly higher at atmospheric O<sub>2</sub>. Interestingly, data analysis by gender showed that male lymphocytes had similar proliferative parameters at both O<sub>2</sub> concentrations while female lymphocytes proliferate more efficiently at 21% oxygen. Compared to males, the female CD4<sup>+</sup> cells showed increased susceptibility to apoptosis at both O<sub>2</sub> concentrations. No differences in the levels of cytokines regardless of gender and oxygen conditions were found.

**Conclusions.** We showed that *in vitro* female T cells (both CD4<sup>+</sup> and CD8<sup>+</sup> cells) are more sensitive than male lymphocytes to low O<sub>2</sub> concentration as demonstrated by the decrease in their proliferation dynamics. The effect does not depend on increased apoptosis of female T cells under low O<sub>2</sub> because percentage of apoptotic cells was similar at both O<sub>2</sub> concentrations. (*Folia Histochemica et Cytobiologica* 2017, Vol. 55, No. 1, 26–36)

**Key words:** human T cells; gender; men; women; cell culture; oxygen levels; proliferation; cytokines; apoptosis

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## Introduction

Ever since *in vitro* cell culturing became possible and available, it also became very useful in variety fields of medicine, such as genetics, physiology, oncology or pharmacology. Removing a portion of the medullar plate of an embryonic chicken and maintaining it in a warm saline solution for several days in 1885, Wilhelm Roux could not had been aware that his idea will be turned into laboratory innovative method used so commonly nowadays [1]. We could hypothesize that without cell and tissue culture techniques both physiological and pathological aspects of cells, tissues and even whole body would be rather poorly understood. Still, accumulation of new facts suggests that the interpretation of cell culture data must include some, as yet rarely (if ever), considered parameters. Among those, two of them, the gender of cell donors and the oxygen level in the incubator during cell culture, were studied here.

Even though the differences in males' and females' bodies functioning had been observed and practically applied, for example in anthropomorphic standards, they were ignored by a scientific world for many years. Since scientists have found gender as interesting and significant factor, some papers have been published [2–6]. Gender-related differences were described for the expression in human pluripotent stem cells [2], progenitor cell migration [3], production of many proinflammatory and other cytokines [4–6], as well as maturation of B cells [7] and differentiation of T cells [8].

Lymphocytes seem to be one of the cell types, whose activity may vary depending on sex. Monthly or pregnancy-related changes in estrogens and progesterone concentrations play a key role in immunomodulation. High level of estrogens could release symptoms of autoimmune diseases in women with genetic predispositions by stimulating interferon (IFN) type I responses [6] and favoring the survival of self-reacting B lymphocytes [7]. However, the effect of estrogens on immune cells is dose-dependent — low doses of estrogens promote enhanced  $T_H1$  responses and increase cell-mediated immunity [8], while high doses enhance  $T_H2$  responses [9]. Meanwhile, progesterone suppresses production of tumor necrosis factor alpha (TNF- $\alpha$ ) and interferon gamma (IFN- $\gamma$ ) [10], and stimulates regulatory T cell ( $T_{REG}$ ) differentiation [11]. Different action of sex steroids is associated with the activation of various signaling pathways in a cell [12]. In the pursuance of human biology, it seems that males are not exposed to such hormone fluctuations. However, *in vitro* studies have shown that sex hormones can modulate activity of male pe-

ripheral blood mononuclear cells (PBMC). Athreya *et al.* [13] demonstrated that estradiol significantly decreases in men the percentage of CD4<sup>+</sup> cells following phytohemagglutinin (PHA) stimulation while significantly increases the percentage of CD8<sup>+</sup> cells following interleukin 2 (IL-2) stimulation. Meanwhile, testosterone significantly increased the percentage of CD4<sup>+</sup> cells following IL-2 stimulation. Prolactin enhanced proliferation of T lymphocytes in response to IL-2 or PHA without subset-specific effects. FSH and LH both stimulated IL-2-induced proliferation, especially in physiological concentrations [13]. These results show that sex hormones have subset-specific and dose-dependent effects on T cell activation, which could explain sex-related differences in immune response. Stopińska-Głuszak *et al.* [14] studied the influence of estrogen/progesterone hormone replacement therapy (HRT) on immunoregulatory cytokine release in postmenopausal women. They found that introducing HRT decreases concentrations of IL-2 and IFN- $\gamma$  in PHA-stimulated PBMC cultures. However, there was no correlation between cytokine release in individual patients and changes of serum sex hormone levels. Villacres *et al.* [15] studied the reaction of CD4<sup>+</sup> cells from donors of both sexes after viral infection. According to their results, women were characterized by higher and significant spontaneous release of IFN- $\gamma$  and cytomegalovirus-specific IL-2 secretion.

Based on the methodology descriptions provided in the papers, all of these comparisons were performed under atmospheric oxygen conditions, which gives us information about lymphocytes' behaviour only in laboratory environment, and not in healthy human body. Standard procedure for human cell cultures *in vitro* sets up the composition of ambient gas mixture of 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Other parameters important for maintaining the culture usually are 100% humidity and temperature of 37°C. Even though it is now commonly known that this oxygen concentration is similar to the atmospheric one and therefore is almost two to ten-fold higher than maintained in the blood [16], various authors still consider 21% of oxygen in culture gas mixture as a normal condition and regard all lowered oxygen contents as hypoxic ones [17]. We fully support the view of Atkuri *et al.* [18] that only conditions of *in vitro* experiments closely mimicking the physiological ones, also with respect to the oxygen concentration, would allow to explore the actual *in vivo* processes and that such conditions should be used wherever possible. According to McNamee *et al.* [19], hypoxia is defined as a state of reduced or inadequate oxygen availability. Consequently, physiological tissue oxygen concentration should be considered as normoxia and atmospheric concentration of O<sub>2</sub> during cell culturing

condition as hyperoxia [18, 20], in spite of previous nomenclature.

Various oxygen concentrations, from 1% to atmospheric (21%), were used previously to study the function of lymphocytes, but only a few on the human material [21–24]. It seems that one of the factors influencing the response of lymphocytes at different oxygen concentrations is the type of stimulation. Atkuri *et al.* demonstrated that T lymphocytes stimulated with either concanavalin A (Con A) or combination of anti-CD3 and anti-CD28 antibodies have higher proliferation index at 21% oxygen compared to physiologic (5 or 10%) oxygen concentrations [21]. No difference was seen when lymphocytes were stimulated with PHA [21]. Meanwhile, Krieger *et al.* [22] showed that 5% oxygen enhances PBMC proliferation after stimulation with Con A or pokeweed mitogen (PWM) but not with PHA or staphylococcal enterotoxin B (SEB). According to Naldini *et al.* [23] PHA stimulation at a very low (2%) oxygen concentration stimulates production of IL-2 IL-6 or IFN- $\gamma$ , while inhibits IL-10 secretion. There are also studies showing that 5% oxygen increases PHA or anti-CD3 antibody-mediated proliferation of lymphocytes compared to atmospheric condition, especially in the presence of IL-2 [24]. Extremely low (1%) oxygen concentration, according to some authors [25], increase lymphocyte susceptibility to apoptosis. But there are also results suggesting that T cells under hypoxic conditions in the presence of TCR/CD3 stimuli might be protected from cell death [26].

According to our current knowledge, there is no paper describing activity of T lymphocytes and their susceptibility to apoptosis under different oxygen concentrations taking into consideration gender of participants. Therefore, the aim of the current study was to compare the effects of concentration of O<sub>2</sub> — atmospheric (21%) or physiologic (10%) (comparable to the level of oxygenation of circulating arterial blood [16]) during culture *in vitro*, as well as of donors' gender, on two main markers of lymphocyte function, that is proliferation and cytokine production. To investigate the proliferative capacity, we analyzed time required to enter the first phase of the first cell cycle, the number of cell divisions and the percentage of dividing cell. We also determined the percentages of apoptotic cells. Based on the above mentioned findings, relating cytokine production and oxygen contents during cultures, for analysis of the ability of lymphocytes to produce cytokines under different O<sub>2</sub> concentrations *in vitro* and with respect to gender, we chose IL-2, which is essential for efficient cell proliferation, IFN- $\gamma$ , which is produced by T<sub>h</sub>1 cells, IL-10 — one of the cytokine

produced by T<sub>h</sub>2 cells, and IL-17A, involved in inducing and mediating pro-inflammatory responses [27].

## Material and methods

**Subjects.** After obtaining written informed consent, 20 mL of blood was drawn into EDTA-coated tubes by venipuncture from 25 young and healthy volunteers, both males (13) and females (12). The average age of the examined population was  $25.48 \pm 5.51$  years. All women were recruited in the preovulatory phase of the menstrual cycle, when the progesterone levels are at their lowest. The research was approved by the Bioethical Committee for Scientific Research at the Medical University of Gdansk.

**T cell stimulation and dividing cell tracking.** Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Histopaque™ gradient (Sigma Chemical Co., St. Louis, Missouri, USA) from venous peripheral blood collected in tubes containing EDTA as the anti-coagulant. Twelve millions of PBMCs were stained with 3  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Sigma Chemical Co., St. Louis, Missouri, USA) for 15 min in the dark at 37°C and washed one time with RPMI 1640 medium (Sigma Chemical Co., St. Louis, Missouri, USA) supplemented with 0.5% fetal blood serum (FBS) and then two times with complete culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin). Then cells were stimulated with immobilized monoclonal anti-CD3 antibody (250 ng/2  $\times$  10<sup>6</sup> cells) in 2 mL of complete medium) and incubated up to 5 days in two kinds of culture conditions: one under standard culture conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 100% humidity at 37°C in a typical CO<sub>2</sub> incubator (Barnstead, Merlose Park, Illinois, USA) and one with oxygen concentration lowered to 10%, in CO<sub>2</sub>/O<sub>2</sub> incubator (Binder, Tuttlingen, Germany). Stimulated cells were collected after 72 hours and 120 hours and stained with the following antibodies: R-phycoerythrin-Cy5 (RPE-Cy5)-conjugated anti-CD4 or anti-CD8 (Dako, Glostrup, Denmark) and phycoerythrin (PE)-conjugated anti-CD28-PE (Becton Dickinson, Franklin Lakes, New Jersey, USA, and analyzed with flow cytometry using FACScan instrument (Becton Dickinson). In addition, to determine the levels of different cytokines produced by the stimulated cells, samples of culture supernatants were collected after 72 and 120 hours and quickly frozen at –80°C for subsequent analysis.

**Apoptosis assessment.** Annexin V-PE binding to the cell surface was applied to distinguish between living and apoptotic cells according to protocol of manufacturer (PE Annexin V Apoptosis Detection Kit I, BD Biosciences, Franklin Lakes, New Jersey, USA).

**Cytokine measurement in cell culture supernatants.** BD™ CBA Flex Set system (BD Biosciences) was used according to manufacturer's protocol to determine the concentrations of IFN- $\gamma$ , IL-2, IL-10 and IL-17A produced by stimulated female and male PBMC under different oxygen concentrations. Cytokine concentrations were analyzed with BD CBA software (BD Biosciences).

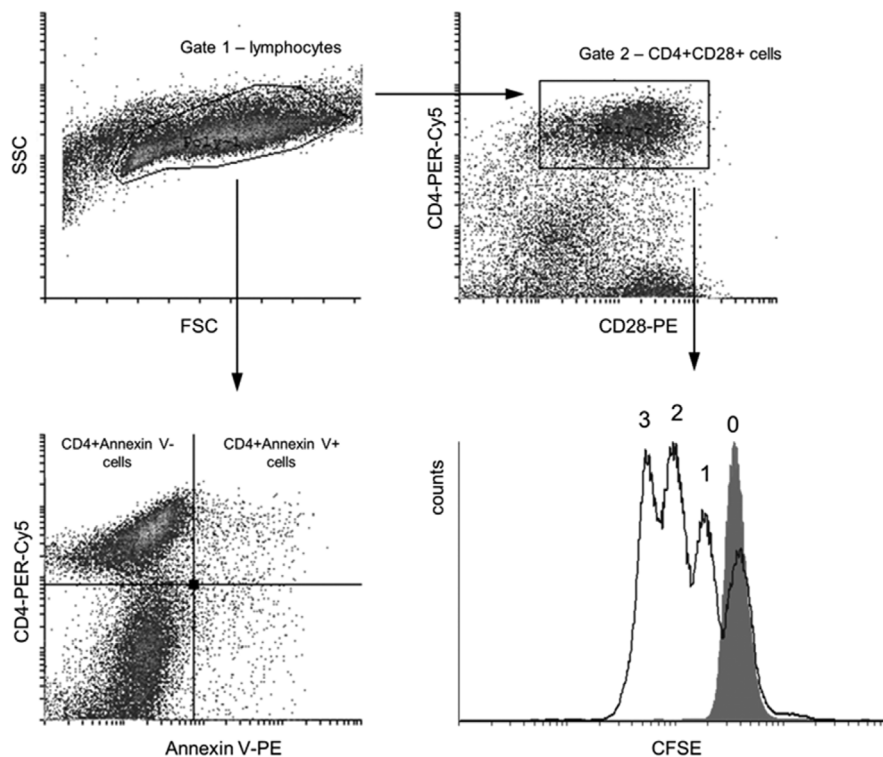
**Cytometric data acquisition and analysis.** The gating strategy (Fig. 1) was as following: proliferating lymphocytes belonging to the populations of interests were selected on the basis of forward and side scatter characteristics and then on their positivity for surface antigens: CD4, CD8 and CD28, which allowed for the discrimination of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> T cells. Thirty thousand events corresponding to the light scatter characteristics of viable lymphocytes were acquired from each sample.

The dividing cell tracking (DCT) method is one of the cell-tracking assays using green fluorescent protein labelling dye, CFDA-SE which, after conversion into CFSE (carboxy-fluorescein succinimidyl ester) inside the cells, allows examining the cell cycle kinetics of T lymphocytes [28]. Apoptotic

CD4<sup>+</sup> and CD8<sup>+</sup> cells were identified as annexin V-positive in the phenotypically distinct populations and additionally in the consecutive generations of dividing lymphocytes visualized by binary diluted CFSE fluorescence [29].

Obtained raw FACS data were analysed with Cyflogic software (©Perttu Terho & ©CyFlo Ltd, Turku, Finland). Calculation of dynamic proliferation parameters including number of divisions per a single dividing cell, percentage of dividing cells in a population and the time required to enter the first (G1) phase of the first cell cycle (G0-G1 transition time) was performed according to our own protocol [29].

**Statistical analysis.** Statistical analysis was done using the Statistica program (StatSoft Inc., Tulsa, Oklahoma, USA). The Kolmogorov-Smirnov and Lilliefors tests were used for testing normality. Since data had non-normal distribution, statistical analysis was done using non-parametric tests: Wilcoxon test for dependent samples (comparison of values obtained for different oxygen concentrations) or Mann-Whitney test for independent samples (comparison of obtained parameter values between men and women), with the level of significance at  $p < 0.05$ .



**Figure 1.** The gating strategy. Proliferating lymphocytes belonging to the populations of interests were selected on the basis of forward (FSC) and side scatter (SSC) characteristic (upper left panel). Next, cells were chosen based on the expression of surface antigens CD4 and CD28 (phenotype CD4<sup>+</sup>CD28<sup>+</sup>, upper right panel) and their proliferation was shown as halving the CFSE fluorescence with each division (lower right panel, fluorescence of control, non-dividing cells is shown as shaded histogram and the numbers of consecutive generations (0-3) are indicated). At the same time, percentage of apoptotic CD4<sup>+</sup> cells was identified as annexin V-positive cells (lower left panel). A similar gating strategy was adopted for CD8<sup>+</sup> cells.

## Results

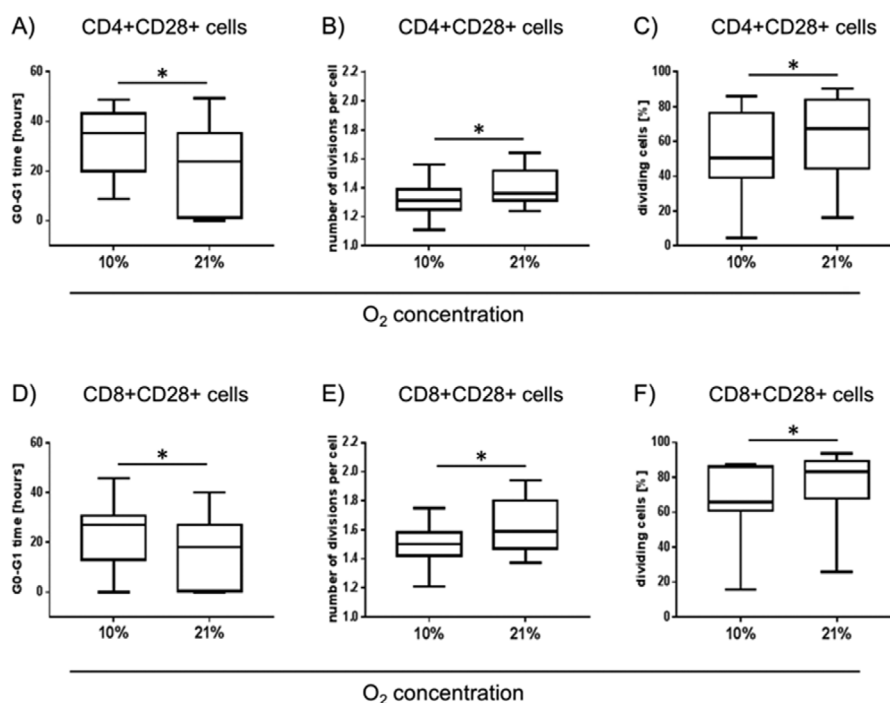
### *Proliferation parameters of T lymphocytes in different oxygen concentrations*

The comparison of the proliferation parameters of anti-CD3-stimulated T lymphocytes depending on the concentration of oxygen was performed in the entire group of volunteers, as well as in each gender separately. Figure 2 presents G0 to G1 transition time, number of divisions per cell and percentage of dividing cells in the populations of CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells in the whole study group. Both lymphocyte populations were characterized by shorter time required to enter the G1 phase of the first cell cycle at 21% oxygen compared to 10% oxygen (Fig. 2A and D). Both CD4<sup>+</sup>CD28<sup>+</sup> (Fig. 2B) and CD8<sup>+</sup>CD28<sup>+</sup> (Fig. 2E) cells performed significantly more divisions per one cell at higher oxygen concentration after 72 hours of stimulation. The percentages of dividing CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells were also significantly higher at 21% oxygen than at 10% oxygen in whole study group (Fig. 2C and F, respectively). After 120 hours of stimulation, the differences observed for cultures incubated for 72 hours were still present, but not statistically significant (data not shown). It is worth mentioning that at both 10% and 21% oxygen

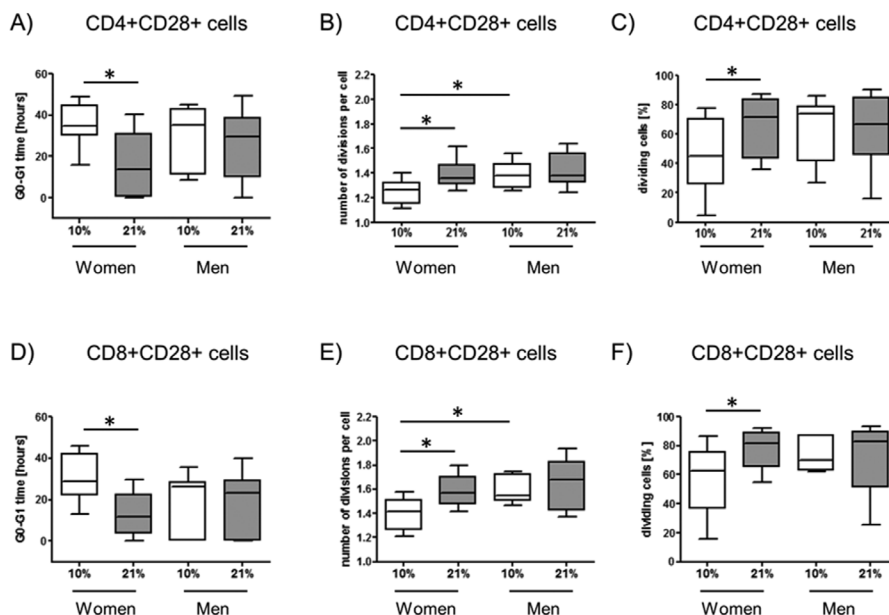
concentrations, CD8<sup>+</sup>CD28<sup>+</sup> cells had shorter G0-G1 time ( $p = 0.001$ ), performed more divisions per cell ( $p = 0.001$ ) after 72 and 120 hours of stimulation compared to CD4<sup>+</sup>CD28<sup>+</sup> cells. The percentages of dividing CD8<sup>+</sup>CD28<sup>+</sup> cells were also significantly higher compared to CD4<sup>+</sup>CD28<sup>+</sup> at both 10% and 21% oxygen ( $p = 0.001$ ).

Then, the results were analyzed in terms of gender (Fig. 3). Female CD4<sup>+</sup>CD28<sup>+</sup> (Fig. 3A) and CD8<sup>+</sup>CD28<sup>+</sup> (Fig. 3D) cells were characterized by significantly shorter G0-G1 time at 21% oxygen concentration compared to 10% oxygen. We found no significant changes in G0-G1 transition time depending on oxygen concentration for the studied T lymphocyte populations isolated from men. Numbers of divisions per one dividing CD4<sup>+</sup>CD28<sup>+</sup> (Fig. 3B) or CD8<sup>+</sup>CD28<sup>+</sup> (Fig. 3E) cell after 72 hours of stimulation were significantly increased at 21% oxygen as compared to cultures incubated in the atmosphere of 10% oxygen, but only in the female group. Moreover, female CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells performed significantly fewer number of divisions per cell compared to male lymphocytes at 10% oxygen.

The percentages of dividing CD4<sup>+</sup>CD28<sup>+</sup> (Fig. 3C) and CD8<sup>+</sup>CD28<sup>+</sup> (Fig. 3F) cells were significantly increased after 72 hours of stimulation at 21%



**Figure 2.** Comparison of the dynamic proliferation parameters of CD4<sup>+</sup>CD28<sup>+</sup> cells (upper panel) and CD8<sup>+</sup>CD28<sup>+</sup> cells (lower panel) after 72 hours of stimulation with immobilized anti-CD3 antibody at 10% or 21% oxygen as indicated, in the whole group. Midpoints of figures present medians ( $n = 25$ ), boxes present 25<sup>th</sup>–75<sup>th</sup> percentiles and whiskers present the minimal and maximal values observed. Differences statistically significant ( $p < 0.05$ ) are marked with \*.



**Figure 3.** Comparison of the dynamic proliferation parameters of CD4<sup>+</sup>CD28<sup>+</sup> cells (upper panel) and CD8<sup>+</sup>CD28<sup>+</sup> cells (lower panel) after 72 hours of stimulation with immobilized anti-CD3 antibody at 10% or 21% oxygen in women (n = 13) and men (n = 12) as indicated on the graphs. Midpoints of figures present medians, boxes present 25<sup>th</sup>–75<sup>th</sup> percentiles and whiskers present the minimal and maximal values observed. Differences statistically significant (p < 0.05) are marked with \*.

oxygen compared to 10% oxygen in the female group. Such difference was not observed in male lymphocyte populations. Percentages of dividing CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells in women group tended to be smaller compared to T cells of men at 10% oxygen, however, these differences were statistically not significant (p = 0.108 for CD4<sup>+</sup>CD28<sup>+</sup> and p = 0.205 for CD8<sup>+</sup>CD28<sup>+</sup> cells).

After 120 hours of stimulation, we still observed high percentages of dividing CD8<sup>+</sup>CD28<sup>+</sup> (p = 0.046) cells at 21% oxygen compared to 10% oxygen in the female group (data not shown). The difference between numbers of divisions after 120 h in culture did not reach statistical significance (p = 0.099 for female CD4<sup>+</sup>CD28<sup>+</sup> and p = 0.386 for female CD8<sup>+</sup>CD28<sup>+</sup> cells). No statistical difference in proliferation parameters of CD4<sup>+</sup>CD28<sup>+</sup> or CD8<sup>+</sup>CD28<sup>+</sup> cells between 21 and 10% oxygen was observed in male lymphocyte populations after 120 hours of stimulation.

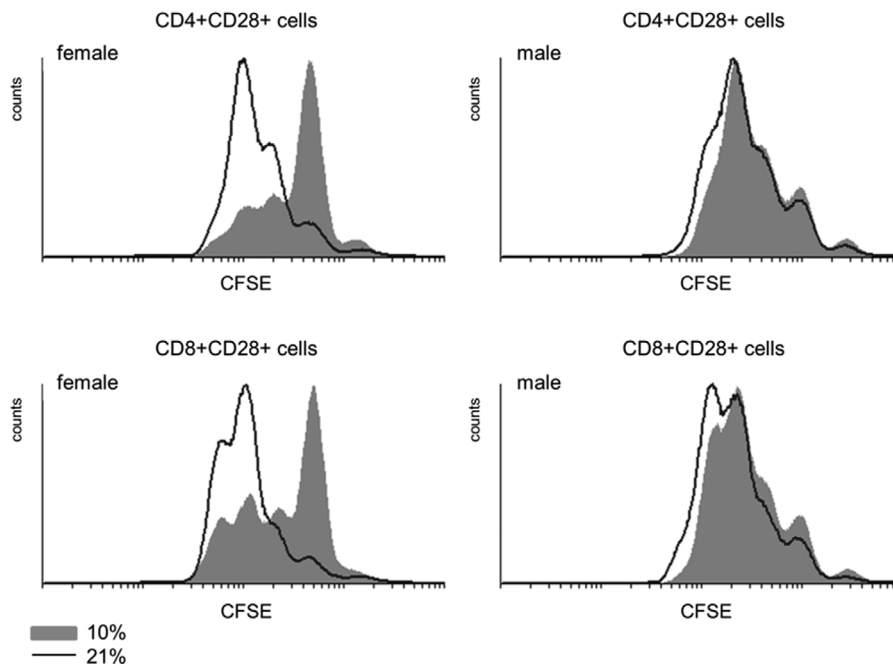
Figure 4 shows representative histograms of CFSE fluorescence of proliferating female and male CD4<sup>+</sup>CD28<sup>+</sup> cells and CD8<sup>+</sup>CD28<sup>+</sup> cells obtained after 72 hours of stimulation at 10% or 21% oxygen. These results clearly demonstrate that female CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells proliferate more efficiently at higher oxygen concentration. Meanwhile, male CD4<sup>+</sup>CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells proliferate at the same rate at both concentrations of oxygen.

#### *Cytokine secretion at different oxygen concentrations*

Analysis of the flow cytometry data for IFN- $\gamma$ , IL-2, IL-10 and IL-17A produced by stimulated female and male PBMCs at 10% or 21% oxygen concentrations revealed no statistically significant differences. However, comparison of the individual changes of cytokine concentrations showed markedly higher concentrations of IFN- $\gamma$  and IL-10 at 21% oxygen in some female samples (Fig. 5).

#### *The influence of different oxygen concentrations on lymphocyte susceptibility to apoptosis*

As the differences in the values of dynamic proliferation parameters described above could possibly be due to different susceptibility of female lymphocytes to programmed cell death under different concentrations of O<sub>2</sub>, we also analyzed the proportions of apoptotic (annexin V-positive) cells among gated CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes. There was no difference in the percentages of apoptotic CD4<sup>+</sup> or CD8<sup>+</sup> cells between 10% and 21% oxygen concentrations in the whole group (Fig. 6A and B, respectively). Analysis of results obtained in both genders revealed that female CD4<sup>+</sup> cells showed increased susceptibility to apoptosis compared to male lymphocytes regardless of oxygen concentration (Fig. 6C). Meanwhile, there was no difference in the percentage of apoptotic CD8<sup>+</sup>



**Figure 4.** Representative histograms showing proliferation pattern of CD4<sup>+</sup>CD28<sup>+</sup> cells and CD8<sup>+</sup>CD28<sup>+</sup> cells illustrated by the CFSE dilution and obtained after 72 hours of stimulation with immobilized anti-CD3 antibody at 10% (grey histograms) or 21% (black line) oxygen in woman and man.

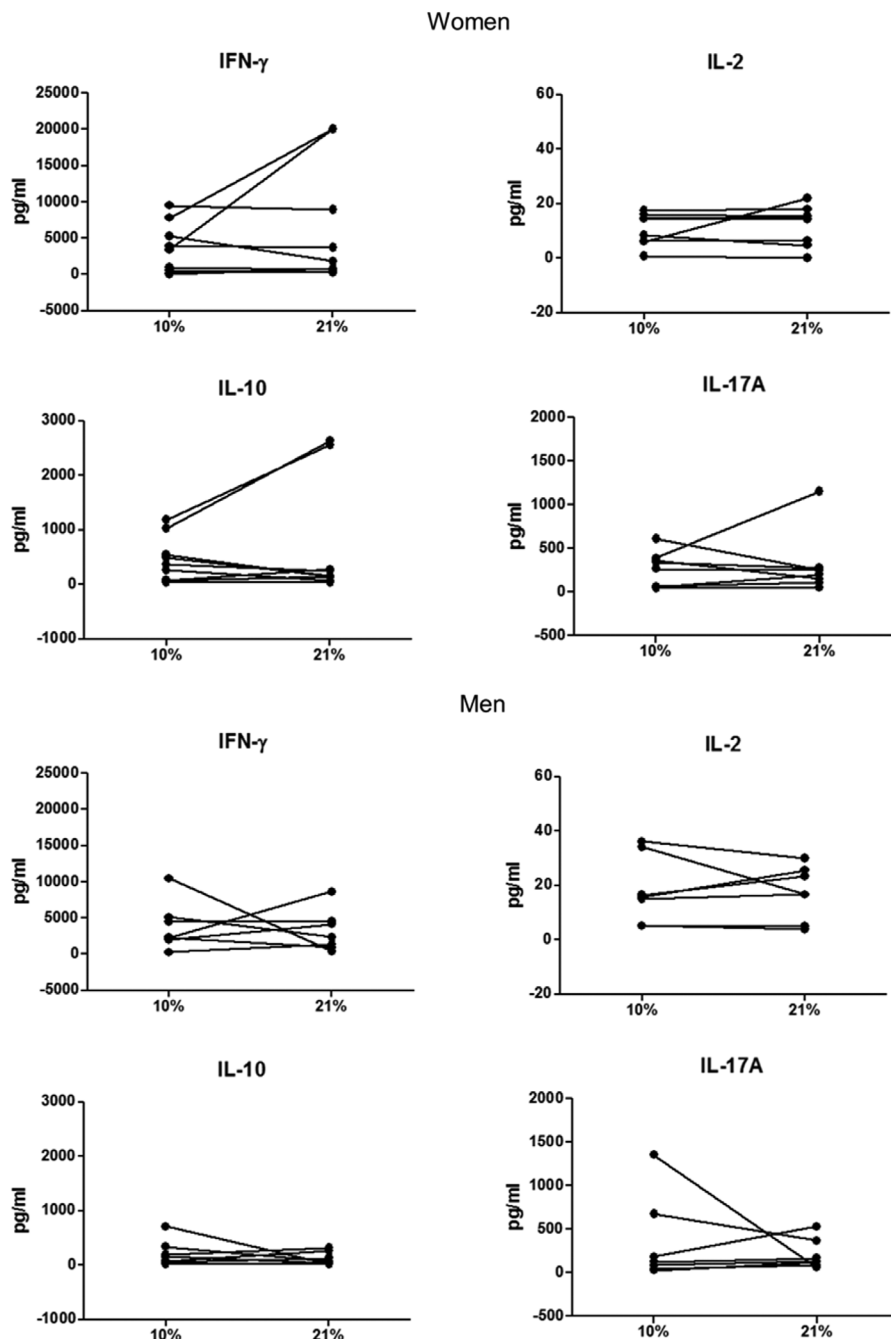
cells between men and women under both oxygen concentrations (Fig. 6D).

## Discussion

Most research on the functioning of immune cells is carried out under the conditions that do not fully reflect physiological conditions, especially with regard to the oxygen concentration in cell culture. This may partly cause conflicting data in the literature, since some authors demonstrate that lymphocytes proliferate more efficiently at atmospheric oxygen levels [21], while others show that proliferation capacity of lymphocytes is higher at low concentrations of oxygen [22, 24]. One of the reasons for the conflicting results could be conducting studies in heterogeneous groups in terms of sex, even if it has long been known that gender can have a significant effect on the activation of lymphocytes [6–11]. For this reason we decided to consider both factors together. To do that we applied our modified DCT technique allowing us to analyze proliferation parameters of T lymphocytes and their ability to produce cytokines in response to specific polyclonal stimulation of the T cell receptor (TCR)/CD3 complex at 21% (atmospheric) and 10% oxygen concentrations.

Our results show that T lymphocytes of healthy people proliferate more efficiently at atmospheric oxygen concentrations after stimulation with anti-CD3 antibody. Both CD4<sup>+</sup> and CD8<sup>+</sup> cells perform more di-

visions per one cell resulting in increased percentage of progeny cells at 21% oxygen. Their improved productivity is the result of shorter time required to enter the first (G1) phase of the first cell cycle. Similar experiments and results in humans were described by Atkuri *et al.* [21] who also reported an increased T lymphocyte proliferation at atmospheric oxygen concentration. They compared influence of different types of stimuli on lymphocyte proliferation. Proliferation index of T lymphocytes was significantly increased in response to both Con A and anti-CD3/CD28 at 20% oxygen compared to physiological (5% or 10%) oxygen concentrations. Our observations coincide with the above-mentioned — the lymphocytes seem to proliferate more effectively at atmospheric oxygen levels. Similarly to us, Naldini *et al.* [23] showed that PBMC were less susceptible to PHA activation at 2% oxygen since their cell cycle was significantly delayed, although they applied 5 times lower oxygen levels than we did. Interestingly, there are also studies presenting contradictory results, *e.g.* Krieger *et al.* [22] reported that PBMC proliferation after stimulation with Con A or PWM but not with PHA or staphylococcal enterotoxin B was enhanced at 5% oxygen. These authors have also suggested that oxygen could differentially influence the growth of PBMC in a mitogen-specific manner [22]. However, it has to be noticed that many authors have investigated proliferative capacity of whole population of PBMC which includes both lymphocytes and

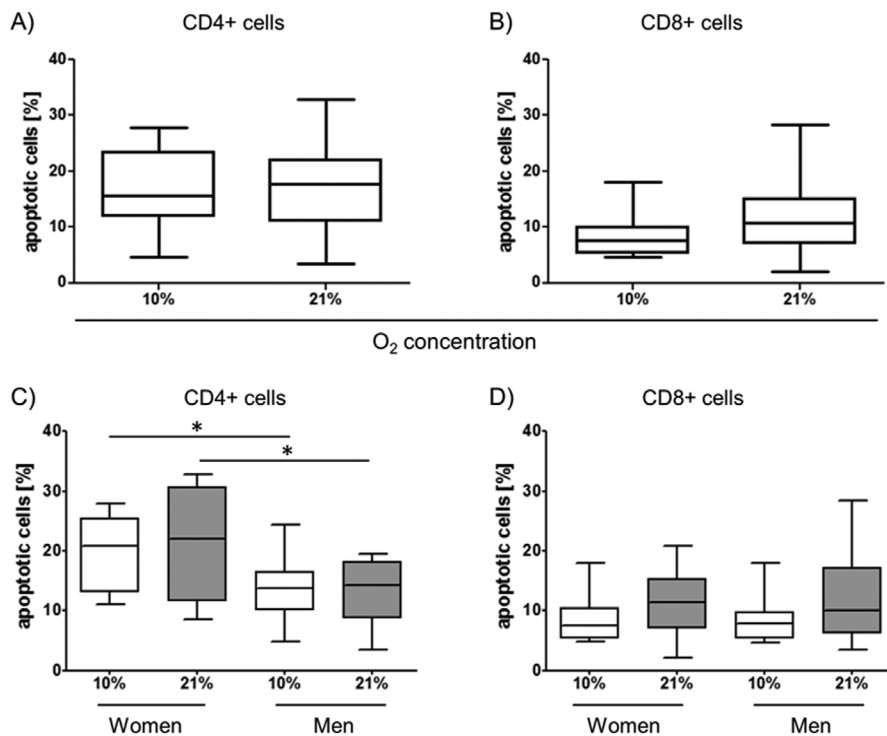


**Figure 5.** Comparison of the individual changes of concentration of IFN- $\gamma$ , IL-2, IL-10 and IL-17A in culture supernatants after stimulation with immobilized anti-CD3 antibody at 10 or 21% oxygen as described in Materials and methods, in the PBMC cultures from women ( $n = 8$ ) and men ( $n = 7$ ). Each set of connected points represents one subject.

monocytes. The recent study by Xu *et al.* [30] suggests that distinct subsets of cells may respond differently to different concentrations of oxygen. These authors showed that in contrast to naive and central memory T cells ( $T_N$  and  $T_{CM}$ ), hypoxia enhanced the proliferation, viability, and cytotoxic activity of effector memory T cells ( $T_{EM}$ ). This may be due to the fact that  $T_N$  and  $T_{CM}$  are located in secondary lymphoid organs, while

$T_{EM}$  cells become concentrated in peripheral tissues in pathologic conditions such as inflammation or tumors where oxygen levels can be extremely low (less than 2%) (reviewed in [17, 31]). However, Xu *et al.* [30] did not give the sex of PBMC donors, while our results showed that male lymphocytes have similar proliferative parameters at both oxygen concentrations. More efficient proliferation observed in our whole study





**Figure 6.** Comparison of the proportions of spontaneously apoptotic (annexin V<sup>+</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> cells after 72 hours of stimulation with immobilized anti-CD3 antibody in 10% or 21% oxygen as indicated, in whole group (upper panel) as well as in women (n = 13) and men (n = 11) (lower panel). Midpoints of figures present medians, boxes present 25<sup>th</sup>–75<sup>th</sup> percentiles and whiskers present the minimal and maximal values observed. Differences statistically significant (p < 0.05) are marked with \*.

group resulted from the fact that female lymphocytes are characterized by increased proliferative capacity at 21% oxygen than at 10% oxygen, however, at high oxygen concentration their proliferation parameters are comparable with that of male lymphocytes. Based on these results, we can assume that investigations of lymphocytes cultured at atmospheric oxygen concentrations yield different results than those one would expect *in vivo*, *i.e.* at low oxygen levels, at least when it comes to T cell proliferation.

We suggest that difference in lymphocyte proliferation at different oxygen concentrations could be caused by effects of various oxygen concentrations on the expression of hypoxia-inducible factor 1 alpha (HIF-1 $\alpha$ ) that regulates cellular response to systemic oxygen levels in mammals. It induces transcription of more than 60 genes, including vascular endothelial growth factor and erythropoietin that are involved in biological processes such as angiogenesis and erythropoiesis, which assist in promoting and increasing oxygen delivery to hypoxic regions [32]. A recent study showed that TCR engagement stimulates HIF-1 $\alpha$  expression in T cells at extremely low oxygen concentration (1%) [33]. Lymphocytes activated in these conditions have reduced proliferation index and are more susceptible

to apoptosis [25]. However, there are also studies demonstrating that HIF-1 $\alpha$  protects T cells stimulated with anti-CD3 antibody from apoptosis in very low (1%) oxygen levels [26]. These differences may be due to various HIF-1 $\alpha$  expression in different lymphocyte populations as has been recently showed by Xu and colleagues [30], which could explain different susceptibility to apoptosis of different T cell subsets [25]. Interestingly, our observations presented in the current work support Larbi *et al.* [25] notion of reduced proliferation, but not of increased apoptosis of T cells under low oxygen concentration. More to the point, our results show that increased susceptibility to apoptosis is not associated with proliferative capacity of T lymphocytes but rather with the gender of cell donors; female Th (CD4<sup>+</sup>) cells, but not the cytotoxic CD8<sup>+</sup> cells, were more susceptible to apoptosis compared to male lymphocytes regardless of oxygen concentration.

Another explanation for the differences in lymphocyte proliferation under different oxygen concentrations proposed by Atkuri *et al.* [18] and by Larbi *et al.* [34] is the intracellular redox state of the cells. They demonstrated that there was a loss of glutathione at both low (5% or 10%) and high (20%) oxygen concentrations, but at the latter O<sub>2</sub> level, the loss was

significantly greater. Thus, the lymphocytes cultured at low oxygen concentrations are subjected to lower oxidative stress than the cells cultured at atmospheric oxygen levels. However, we found that despite of the higher level of oxidative stress at 21% oxygen concentration, cells proliferated more efficiently.

Atkuri *et al.* [21] analysed T lymphocyte population divided into CD4<sup>+</sup> and CD8<sup>+</sup> cells and concluded that the latter cells tend to either proliferate more efficiently or survive better at 5%, 10%, and 20% oxygen. We have obtained similar results; CD8<sup>+</sup>CD28<sup>+</sup> cells had shorter G0-G1 time and performed more divisions per cell compared to CD4<sup>+</sup>CD28<sup>+</sup> cells regardless of oxygen concentration. This could be due to the fact that, according to our observations, female CD4<sup>+</sup> cells are more prone to apoptosis than CD8<sup>+</sup> cells under both oxygen concentrations. Larbi *et al.* [25] suggested that different proliferation capacity of CD4<sup>+</sup> and CD8<sup>+</sup> cells may result from differences in naive and memory subset distributions.

The novel observation of our study is that differences in lymphocyte proliferation and apoptosis concern mainly women. Changes in the proliferation capacity of T cells obtained from women or men could have been related to changes in the levels of cytokines, especially IL-2, which promotes proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells [27]. To our surprise, we found no difference in cytokine concentrations in cell cultures between women and men regardless of oxygen concentration. One possible explanation is that the female volunteers were in the beginning of the preovulatory phase of the menstrual cycle, when the estrogen and progesterone levels are at their lowest. In this case, one could expect the increase in IFN- $\gamma$  production by female cells due to enhanced T<sub>H</sub>1 responses due to low level of estrogen [8]. However, according to Lee *et al.*, percentages of T<sub>H</sub>1/T<sub>H</sub>2 cytokine-producing T cells do not change significantly throughout cycle [36]. Faas *et al.* [37] also have shown that intracellular levels of IFN- $\gamma$ , IL-2 or IL-10 do not vary between the phases of the ovarian cycle.

In our study, gender-related differences in lymphocyte proliferation parameters were observed only at low oxygen concentration. This may be due to the aforementioned differences in the expression of HIF-1 $\alpha$  within various lymphocyte subpopulations in response to low oxygen concentration [29]. This thesis, however, requires confirmation in further studies. Nevertheless, our results show that the use of atmospheric oxygen in a cell culture in a study group heterogeneous in terms of gender can lead to incorrect conclusions regarding the functioning of T lymphocytes. To be credible and solid, all scientists, who investigate human body functions, should

consider use of oxygen concentrations similar to physiological ones.

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