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Increased Expression of ITGB 3 in CLL Patient leukemia Cells by Exposure to Cold Physical Plasma and Plasma-treated Medium

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most prevalent hematological cancer, with various medical interventions. In the recent decade, cold physical plasma has become an interesting agent for future cancer therapy. The goal of this study was to see whether cold physical plasma or cold physical plasma-treated liquid (PTL) affected integrin beta 3 (ITGB3) expression, which is hypothesized to mediate an interaction between cancer stem cells and the bone marrow microenvironment, in CLL patients' blood cells. The metabolic activity, cell death pattern, lipid oxidation and ITGB3 gene expression of these treatments was evaluated. Both direct cold physical plasma and PTL exposure enhanced lipid peroxidation in cells of CLL patients, but to a lesser extent in healthy participants. Furthermore, following 48h of cold physical plasma or PTL exposure, the metabolic activity of leukocytes was preferentially reduced in CLL patient leukocytes. In addition, cold physical plasma and PTL treatment elevated ITGB3 mRNA expression in CLL patients' leukocytes compared to untreated and healthy controls. Collectively, our study suggests selective effects of direct cold physical plasma and PTL exposure on blood leukocytes from leukemia patients, but further and more detailed studies are needed to provide additional rationales for such treatment options as future therapy.

Keywords: CAP, cold atmospheric pressure plasma, chronic lymphocytic leukemia, gas plasma technology, NK cells, reactive oxygen species, ROS

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common hematologic malignancy worldwide (1). Increased numbers of mature B cells in the blood reduce the bone marrow's ability to produce non-malignant blood cells and platelets in the fight against infection in CLL patients (2). Evidence suggests a link between the induction of oxidative stress and the use of anti-leukemia chemotherapeutic agents (3-5). Indeed, leukemic cells have increased basal oxidative stress levels, making them susceptible to chemotherapeutic drugs that further increase intracellular reactive oxygen species (ROS) levels (6, 7). It is established that chemotherapy drugs cause irreversible damage not only to malignant but also to normal human cells. Therefore, new avenues for CLL treatments are required.

Cold physical plasma is a low-temperature ionized gas composed of ROS (8). Cold physical plasma can promote cytotoxic effects on tumor cells and tissues. This can be achieved either by direct exposure or through treating the targeted with cold physical plasma-treated liquids (PTL), such as medical products such as saline solutions or cell culture media in the context of basic research (8). PTL is thought to be easier to use, more stable, and less expensive, giving it some advantages in anticancer applications (9). Cold physical plasma PTL treatments are hypothesized to promote cell death in melanoma, glioblastoma, breast, gastric, prostate, and colon cancer (10-13). Only a few studies have focused on the impact of cold physical plasma or PTL treatment on whole blood leukocytes of individuals with leukemia and other hematologic malignancies (14, 15).

Natural killer (NK) cells are essential to the immune system's capability to identify and eliminate cancer cells, including those associated with chronic lymphocytic leukemia (CLL) (16). Boosting NK cell function offers a promising therapeutic approach for individuals with CLL (16). These cells can identify and destroy CLL cells without needing prior exposure, allowing for a quick immune reaction (17, 18). They specifically target cells that present stress signals or unusual surface markers (17). Additionally, NK cells release various cytokines, such as IFN-γ and TNF-α, which can modify the immune response and bolster the activity of other immune cells, such as T cells (17, 18). However, the effectiveness of NK cell function is often hindered by the immunosuppressive environment in CLL (18). Cancerous CLL cells can decrease activating ligands and increase inhibitory ligands, resulting in NK cell fatigue and diminished cytotoxicity (18). Therefore, enhancing NK cell activity could help overcome this immunosuppressive environment, enabling more effective targeting of CLL cells (16).

Integrin beta 3 (ITGB3), also known as CD61/GP3A, is one of the most studied members of the integrin family (19). It has been shown that increased ITGB3 signaling caused by ROS promotes an immune-suppressive environment in solid tumors by recruiting M2 macrophages and neutrophils, thereby contributing to solid tumor metabolism, epithelial-to-mesenchymal transition (EMT), endothelial-to-mesenchymal transition (End-MT), and tumor stemness (20). Unlike in solid tumors, ITGB3 as a mammary progenitor marker may enhance NK cells and T helper 1 (T_H1) cell activation in hematological cancers, thereby increasing antitumor activity (19). ITGB3 appears to play a variable role in immunomodulation depending on several factors (19). Many studies have shown that ITGB3 has therapeutic potential in multiple leukemia (Figure 1) (19). Hence, here we aimed to study the effects of cold physical plasma and PTL on ITGB3 expression in leukocytes of CLL patients.

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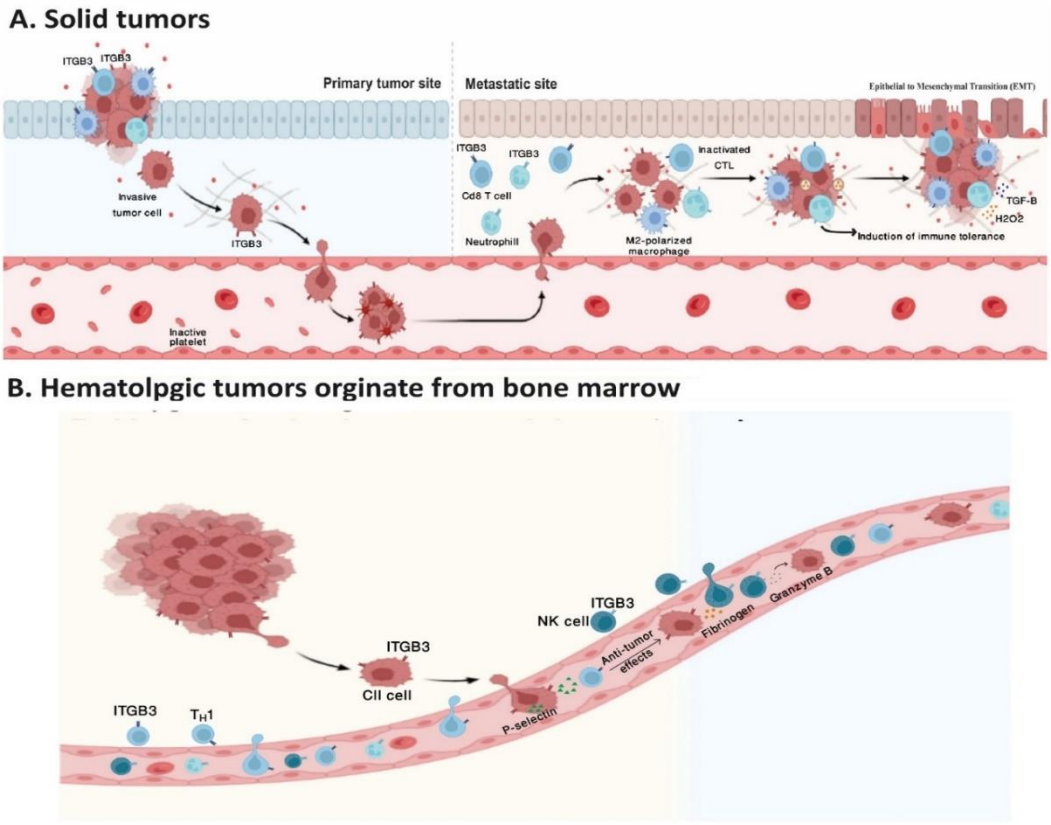

Fig. 1. The numerous roles of ITGB3 in the tumor immune microenvironment. In solid tumors, ITGB3-enhanced signaling promotes immunoregulatory responses by attracting M2 macrophages and neutrophils and inactivating $CD8^+$ T cells. (B) In hematological tumors, ITGB3 amplifies the immunostimulatory responses by activating NK cells and T_H1 cells, boosting antitumor effects.

Materials and methods

Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and the Research Ethics Committee of Mazandaran University of Medical Sciences approved all procedures involving human subjects/patients (ethical approval code: IR.MAZUMS.-REC1398.940) This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and the Research Ethics Committee of Mazandaran University of Medical Sciences approved all procedures involving human subjects/patients (ethical approval code: IR.MAZUMS.-REC1398.940) and was financially supported by the Research and Technology Council of Mazandaran University of Medical Sciences (grant NO. 13861).

Patients and controls

Peripheral blood samples were collected from 12 CLL patients (7 men and 5 women; mean age: 61.7 years) and 12 sex- and age-matched healthy control subjects referred to the Hematology and Oncology Clinic

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of Imam Khomeini Hospital in Sari affiliated to Mazandaran University of Medical Sciences during January 2021 to January 2022. Diagnosis and selection of patients were performed by a hematology-oncology specialist based on white blood cell count, cell morphology, immunophenotyping analysis, clinical symptoms, and microscopic observations of peripheral blood smear according to the standards outlined by the world health organization (WHO). Also, all patients and healthy participants did not receive chemotherapy and immunosuppressive drugs prior to blood collection. They did not have any history of autoimmune diseases, other blood malignancies, or any type of congenital or acquired immunodeficiency. About 10 ml of peripheral blood samples were taken from the antecubital vein and collected into a tube containing heparin sodium anticoagulant. The whole blood samples were transferred to 24-well cell culture plate and maintained at 37 C in 5% humidified CO2 incubator.

Cold physical plasma jet and sample treatment

The experiments were performed with a plasma jet containing two electrodes, which were inserted ~ 7 mm distance apart from each other .The plasma jet length was 15 cm, with the final diameter about 3 cm. The temperature of the plasma was 35.40 °C. The plasma is generated from argon gas with a flow rate of 2.5 standard liters per minute and is fed into the Pyrex tube. The discharge current between the metal wire and the ring electrode is 10 mA and only 5 to 10 percent of this volume is probably evacuated from the tube, which was used for blood sample exposure. Specifically, 1 ml of blood from each volunteer was cultured in 24-well cell culture plates in triplicate per control and treatment group. Direct cold plasma exposure was performed for 120s at a 3-cm distance to the sample surface. This treatment was referred to (direct) plasma treatment. Alternatively, samples were mixed in a 1:4 with plasma-treated PBS (120s). This treatment was referred to as indirect treatment or PTL treatment. Samples were then further incubated at 37^0C in 5% humidified CO2 incubator prior to subsequent analysis.

Malondialdehyde (MDA) measurement

To determine the oxidative stress-induced lipid peroxide assay in cells, MDA levels were measured. Briefly, 48h after treatment, whole blood supernatants of 6 CLL patients and 6 sex- and age-matched healthy control samples were collected. Then, 20% trichloroacetic acid (TCA) was added to the supernatant for precipitation of proteins. Finally, 0.6 % thiobarbituric acid (TBA) was added to the mixture. MDA in the supernatant reacts with TBA to produce an MDA-TBA compound. Then, MDA levels were read at 535 nm by an ELISA reader (BioTek, Winooski, VT, USA) which is directly related to the concentration of MDA. MDA concentration is calculated using the Lambert-Beer law with an extinction coeeficient εM= 155 $mM^{-1}cm^{-1}$ and MDA level (µmols) was determined using following equation:

MDA (µM) = (A sample at 535nm/ Lp × εM) × DF

Lp is the Light path, εM is Molar absorptivity (155 mM-1cm-1) and DF is Dilution factor

Metabolic activity assay

Following 48h incubation after plasma exposure with cold physical plasma or PTL, the metabolic activity of whole blood cells was measured by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma, USA) assay according to the manufacturer's protocol. Briefly, the samples were centrifuged, and the supernatants (blood plasma) were isolated. RBCs were removed using lysis buffer, and leukocyte cells were cultured in isolated plasma. MTT solution (0.5 mg/ml) was added to each well. After 3h under

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standard conditions, the supernatant was replaced with 150 µl of DMSO (Merck, Darmstadt, Germany). The absorbance was measured using a BioTek microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 570 nm.

Annexin V/PI staining for apoptosis

The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect cold plasma effects on cell death and apoptosis in CLL and healthy whole blood leukocytes. Briefly, after 48 h of culture, RBCs were removed by lysis buffer. After that, 3×10^5 leukocytes were resuspended in binding buffer and were stained with 5 µl of Annexin V-FITC solution. Samples were mixed gently and incubated at room temperature in the dark for 15 min. Finally, 2 µL of propidium iodide (PI) solution (1 mg/mL) was added to each sample, and early and late apoptotic cells were analyzed by flow cytometry (Partec, Bergisch-Gladbach, Germany).

Gene expression analysis

Total RNA was extracted from peripheral blood using an RNA extraction kit (YTA, Tehran, Iran), and cDNA was obtained using the Revert Aid first strand kit (Thermo Scientific, Massachusetts, USA). Real-time PCR has been carried out to evaluate mRNA expression of targets in pelleted samples quantitatively using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Massachusetts, USA). The primer sequences are included in Table 1. Gene expression analysis was performed using a Step One real-time PCR device (Applied Biosystems, Foster City, CA, USA). Relative fold change was normalized to the housekeeping gene GAPDH and calculated using the $^{2-\Delta\Delta}$Ct method.

Table 1. Primer sequences were used as follows.

Name	Sequence (5' → 3')
F-integrin β3-Homo	GTAACCTGCGGATTGGCTTCG
R- integrin β3-Homo	TGACACACTCTGCTTCTTCACTTC
F-GAPDH-Homo	GGTGGTCTCCTCTGACTTCAACA
R- GAPDH -Homo	GTTGCTGTAGCAAATTCGTTGT

Statistical Analysis

Results are presented as the mean ± SD of at least four independent experiments. T-test for paired data or 2-way analysis of variances (ANOVA) for repeated measures was used. To perform pairwise comparisons between groups, Tukey's post hoc test was used. Statistical analyses were performed utilizing Prism 6.0 statistical software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Cold physical plasma and PTL exposure induce MDA levels and cytotoxicity in leukocytes of CLL patients

We have previously shown that our atmospheric pressure plasma jet and the cold physical plasma it generates produced ROS and RNS in the treated liquid, such as cell culture medium. In this study, we

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identified oxidative stress damage markers. Blood plasma MDA concentration significantly increased in whole blood of CLL patients and healthy controls treated with cold physical plasma or following PTL exposure (Figure 2A). Cold physical plasma exposure produces RONS that may have a role in conferring cytotoxic action in whole blood leukocytes, including leukemia cells, which could be demonstrated using the MTT assay, as cold physical plasma treatment decreased ($p<0.001$) the viability of the leukocytes in CLL patients (Figure 2B). Here, the metabolic activity of whole blood cells was significantly reduced with 120s of cold physical plasma treatment after 48h ($p<0.001$). At the same time, cold physical plasma-induced cytotoxicity in healthy donor-derived whole blood cells was less pronounced.

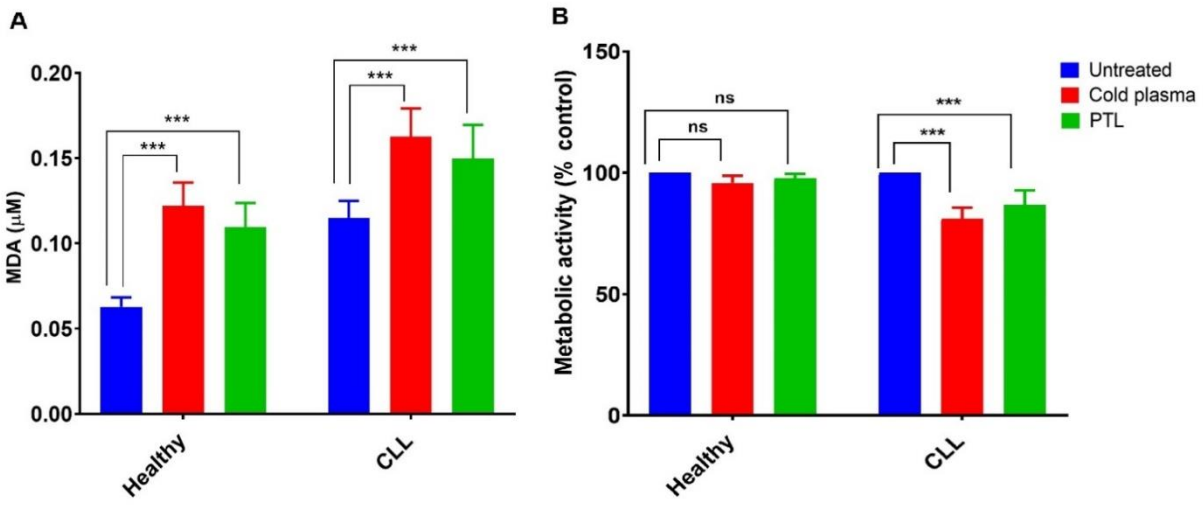

Fig. 2. (A) MDA concentrations in whole blood supernatants of CLL patients and healthy individuals after exposure to cold physical plasma or PTL in comparison to supernatants of untreated cells. The concentration of MDA in CLL patient samples is higher than in healthy individuals. (B) Direct (cold physical plasma) and indirect (PTL) treatment significantly reduced the metabolic activity of whole blood cells of CLL patients as assessed 48h after exposure. Data are expressed as mean + SD of three different experiments. ns = not significant; *** = $p<0.001$.

Cold physical plasma and PTL exposure increased apoptosis in leukocytes of CLL patients

Analysis of Annexin V-FITC/ PI cellular staining utilizing flow cytometry revealed that cold physical plasma exposure at the treatment times applied did not induce apoptosis in healthy leukocytes, which was also found in healthy blood samples exposed to PTL. In leukemia blood samples that also include leukemia cells, the percentage of apoptotic cells (early and late apoptosis) was higher ($p<0.001$) when exposed to cold physical plasma or PTL when compared to untreated samples (Figure 3).

Cold physical plasma and PTL exposure increased ITGB3 expression in leukocytes of CLL patients

Subsequently, the expression of ITGB3 rin was evaluated in cold physical plasma and PTL treatment groups of CLL patients and healthy control leukocytes using real-time PCR. The expression of this gene significantly increased ($p<0.001$) after cold physical plasma and PTL treatment in comparison to untreated controls (Figure 4A-B). By contrast, these treatments had no significant effect on the expression of ITGB3 in healthy control leukocytes. When the expression of ITGB3 was compared to the healthy group, the results indicated that ITGB3 levels in CLL patients were significantly lower ($p<0.01$). However, following treatment

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with cold physical plasma and PTL, the expression of ITGB3 in CLL patients significantly increased ($p<0.001$) compared to the healthy controls (Figure 4C). These results indicated that in response to the cold physical plasma and PTL treatment, the expression of β3 integrin mRNA substantially increased in CLL leukocytes.

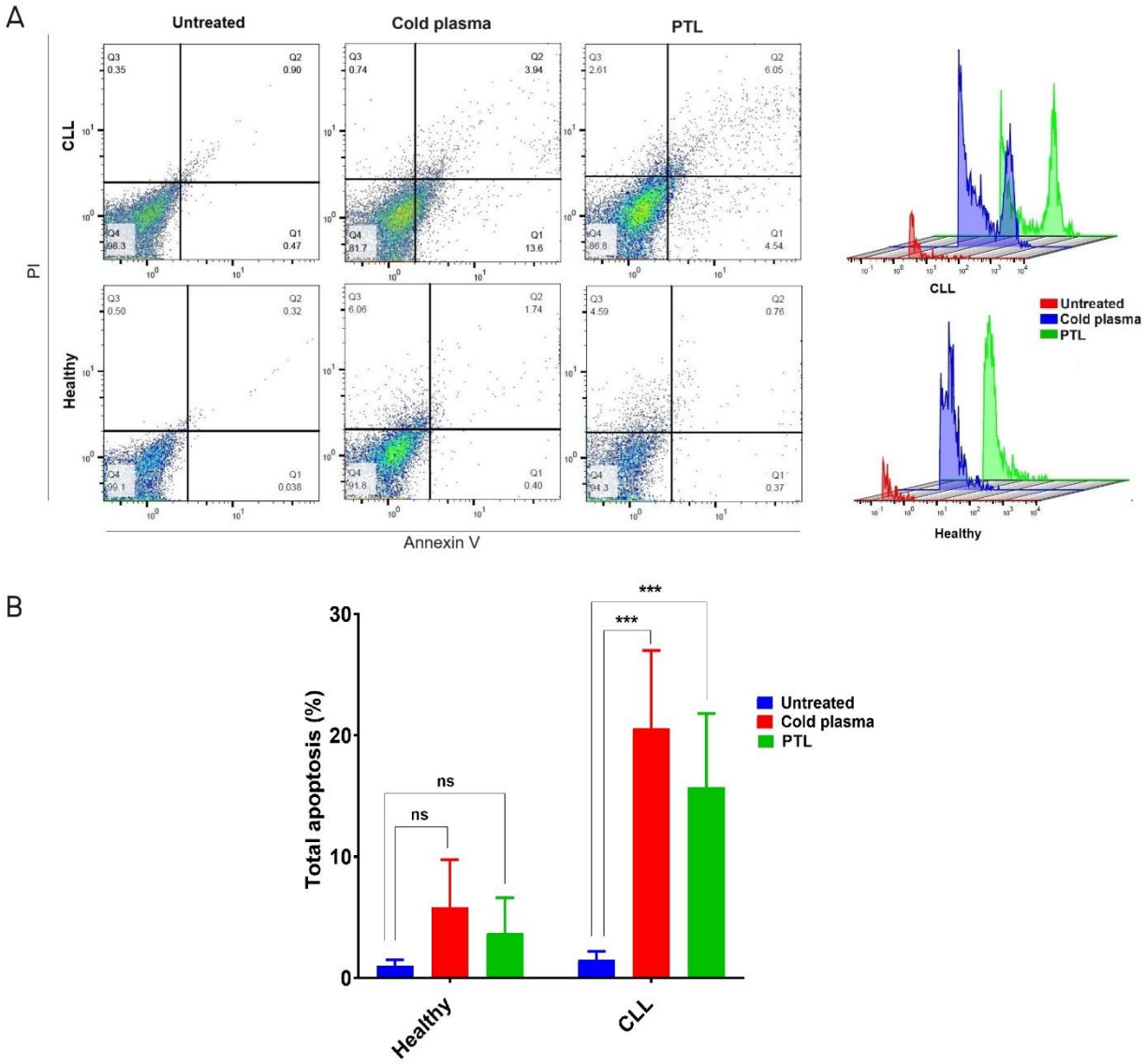

Fig. 3. (A) The effect of cold physical plasma and PTL on apoptosis. Flow cytometry dot plots of whole blood leukocytes stained with Annexin V and PI 48h after cold physical plasma or PTL exposure. The data represent the percentage of viable cells (Q4), early (Q1) or late (Q2) apoptotic cells, and necrotic cells (Q3) and overlay histograms of Annexin V FITC mean fluorescence intensity (MFI) of early and late apoptosis cells demonstrated cold physical plasma and PTL treatment had a greater effect on CLL cells than the healthy controls. (B) Direct (cold physical plasma) and indirect (PTL) treatment significantly increased the total apoptosis percentage of whole blood cells of CLL patients as assessed 48h after exposure. Data are expressed as mean ± SD of three different experiments. ns = not significant; *** = $p<0.001$.

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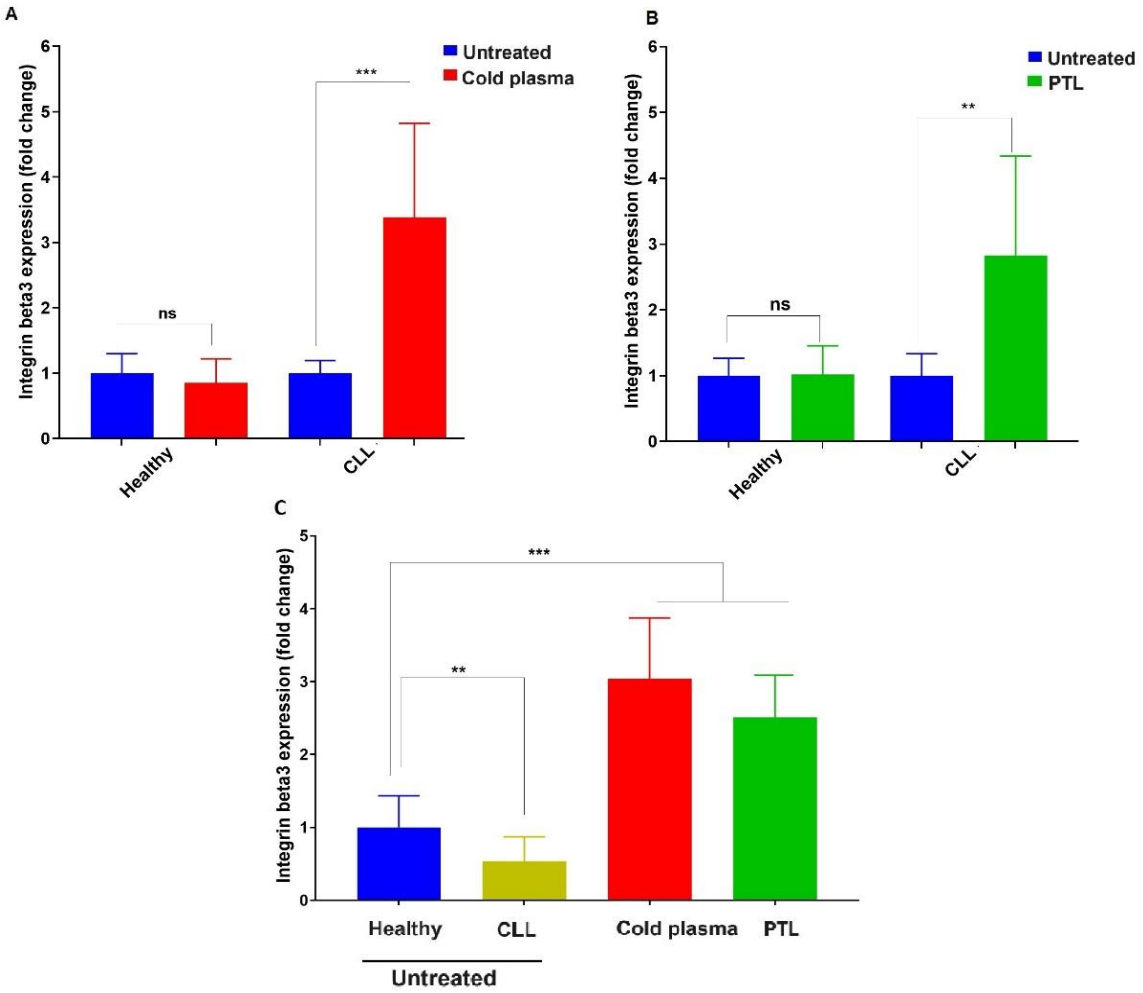

Fig. 4. Cold physical plasma and PTL treatment increased β3 integrin expression in CLL. (A) The effect of cold physical plasma (120s exposure) on β3 integrin expression of total RNA from whole blood of CLL patients and healthy controls (B) The effect of PTL (120s exposure) on β3 integrin expression of total RNA from whole blood of CLL patients and healthy controls. (C) Expression of integrin β3 in CLL patients following treatment with cold physical plasma and PTL compared to the healthy group was measured in the four groups compared with the control. GAPDH was used as housekeeping control. Altered gene expression was calculated as fold changes referring to untreated control group. Data were performed in three independent experiments and expressed as mean + SD. ns = not significant; ∗∗ = P < 0.001 versus the untreated cells.

Discussion

Cold physical plasma and PTL are major sources of ROS, which can trigger apoptosis, necrosis, and/or autophagy in malignant cells (12). Despite the effectiveness of cold physical plasma and PTL on solid tumor cells, only a limited number of studies have explored their effects on hematological malignancies, including CLL (14), which motivated the current study.

Integrin beta 3 (ITGB3) is one of the most extensively investigated members of the integrin family, as it performs several essential roles in the reprogramming of the microenvironment and the growth of cancer

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cells in response to reactive oxygen species (ROS) (19). ITGB3 has been identified as a ROS-regulated protein involved in tumor angiogenesis and metastasis (21). In addition, it appears to play a distinct role in the immune microenvironment of solid and hematologic tumors (19). In our study, we investigated the effects of cold physical plasma and PTL as ROS inducers on cytotoxicity, MDA value, and ITGB3 expression in blood cells of CLL patients and healthy individuals. Our findings showed that cold physical plasma and PTL exposure significantly reduced the metabolic activity of CLL patients' leukocytes, as the amount of lipid peroxidation in CLL patients' leukocytes was much higher than in untreated control and healthy groups. Furthermore, flow cytometry analysis revealed that cold physical plasma and PTL induced apoptosis in the leukocytes of CLL patients but had no negative effect on healthy leukocytes. Because mature B cells account for the vast majority of blood cells in CLL patients (22), it appears that cold physical plasma treatments can affect CLL leukemic cells. Many studies have shown that cold physical plasma or PTL can selectively induce cell death in various tumor cells, such as breast, lung, bladder, melanoma, leukemia, and CLL patients' whole blood, by producing a wide range of RONS (22). Enhanced ROS could play a role in cancer prevention and progression (23, 24). Exposure of H_2O_2 and NO_2^- to cancer cells results in cytotoxic effects based on the duration of exposure to cold physical plasma and PTL, respectively (25).

ITGB3 expression in leukocytes of CLL patients increased in response to cold physical plasma and PTL treatment. ITGB3 has been shown to be a key regulator in boosting TGF-β/H_2O_2/HOCl signal in solid tumors exposed to ROS, as well as having different importance and function in hematological malignancies under the same settings (19, 26, 27). It was found that NK cells exposed to ITGB3 enhanced their cytotoxic activity against K562 cells and AML blasts, implying that ITGB3 promotes NK cell cytotoxicity against leukemic target cells (28). NK cells, unlike cytotoxic T lymphocytes (CTL), do not need antigen-specific identification to lyse their targets (29). When NK cells recognize activated ligands on hematologic malignancy cells, they contribute to the lysis of leukemic blasts by secreting proinflammatory cytokines such as IFN-γ, granzymes, or perforin (29-31). It has recently been demonstrated that NK cells have low cytolytic activity against CLL cells, which can be restored by cytokines such as IL-2/IL-15. However, several mechanisms for NK cell repression in CLL are still unknown (18, 32). Our preliminary findings indicate that cold physical plasma and PTL increased apoptosis and ITGB3 expression in CLL patients' leukocytes. This preliminary data could promote the hypothesis that ITGB3 may act as an NK cell activating ligand in CLL and AML, but regarding some limitations, we could not measure the other genes or proteins that support these findings, therefore more researches are needed to confirm the role of ITGB3 in modulating NK cell activity in CLL.

This study suggests that cold physical plasma or PTL exposure has cytotoxic effects on CLL whole blood cells. In addition, we found an increased ITGB3 mRNA expression due to the treatment, which may point to a potential as an activator of NK cells. More researches will be needed to provide a deeper understanding of cold physical plasma application routes and their potential to modify tumor cells and potentially harness antitumor immunity, including NK cell activation against leukemia cells.

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