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# Decrease of Tumor-infiltrating Regulatory T Cells Using Pentoxifylline: An *Ex Vivo* Analysis in Triple-negative Breast Cancer Mouse Model

**Mohammad Hossein Kazemi[1,2], Mahdieh Shokrollahi Barough[1,2], Alireza Ghanavatinejad[3], Zahra Momeni-Varposhti[4], Samaneh Khorrami[1,5], Behnam Sadeghi[2,6], and Reza Falak[1,5]**

*[1] Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*
*[2] Department of ATMP, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran*
*[3] Department of Immunology, Pasteur Institute of Iran, Tehran, Iran*
*[4] Hematopoietic Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
*[5] Immunology Research Center, Institute of Immunology and Infectious Disease, Iran University of Medical Sciences, Tehran, Iran*
*[6] Division of Pediatrics, Department of Clinical Science, Translational Cell Therapy Research (TCR), Intervention and Technology (CLINTEC), Karolinska Institutet, Stockholm, Sweden*

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## ABSTRACT

Triple-negative breast cancer (TNBC) is the most aggressive type of BC with the highest percentage of tumor-infiltrating lymphocytes (TILs). Hence, TIL therapy is considered a promising approach to target TNBC. Depletion of regulatory T cells (Tregs) in TILs can improve the antitumor function of TIL therapy. Pentoxifylline (PTXF) is a xanthine derivative that can modulate the nuclear factor kappa B (NF-κB) signaling and probably affect the Treg proportion in TILs. We aimed to evaluate the *ex vivo* effect of PTXF on the proportion of Treg cells in the TILs derived from a mouse model of TNBC.

The 4T1 cells were inoculated subcutaneously to BALB/c mice to induce TNBC. TILs were isolated from tumor tissue by enzymatic digestion and cultured alone or with 4T1 cells for 24, 48, and 72 h in the presence of interleukin (IL)-2 and different concentrations of PTXF. The toxicity of PTXF and its effects on Tregs proportion as well as cytokine production was evaluated using MTT assay, flow cytometry, and ELISA, respectively.

PTXF had no significant impact on the viability of TILs. Both 500 and 1000 μg/mL of PTXF decreased the proportion of Tregs in a dose-dependent manner. The level of interferon-γ and tumor growth factor-β in TILs supernatant were increased and decreased, respectively.

**Corresponding Authors:** Reza Falak, PhD;
Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 8670 3248, (+98 912) 275 3942, Fax: (+98 21) 8862 2652, E-mail: Falak.r@iums.ac.ir

Behnam Sadeghi, MD, PhD;
Department of ATMP, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran. Tel: (+98 21) 8879 1120, (+46 73) 958 3970, Fax: (+98 21) 8879 6208, E-mail: Behnam.sadeghi@ki.se

Our data suggest that *ex vivo* treatment of TILs with pentoxifylline could decrease the proportion of Tregs in the conventional IL-2-mediated TIL expansion and change the cytokine balance of TILs in favor of antitumor immune response.

**Keywords:** Breast neoplasms; Pentoxifylline; Regulatory T-lymphocytes; Tumor-infiltrating lymphocytes

## INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed malignancy in women accounting for their highest cancer mortality.[1] BCs are divided into four general types, based on the overexpression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her-2).[2] Triple-negative BC (TNBC) lacks the overexpression of ER, PR, and Her-2. It accounts for about one-fifth of all BCs and is the most prevalent BC in young women.[3] Lack of hormone receptors, resistance to chemotherapy and targeted therapies, heterogeneity, and high rate of metastasis and post-surgery recurrence make TNBC the most challenging BC type with the lowest survival rate.[1,4] TNBC is the most immunogenic BC on which many immunotherapy studies have been performed with promising results.[1,5]

There is ample evidence to suggest that lymphocytes infiltrated in the tumor environment are actively capable of inhibiting tumor progression. The presence of immune cells has been used as a prognostic factor for BC.[6–8] Tumor-infiltrating lymphocytes (TILs) are a heterogeneous population of immune cells that can penetrate the tumor site. The infiltration rate of TILs in various solid tumors is different and depends on the immunogenicity of the tumor.[9,10] The TIL percentage in tumor biopsies will be considered a favorable prognostic factor if the dominant population of these cells does not have immunosuppressive functions.[6,11] Literature has shown that isolated TILs from malignant breast tissues are capable of performing antitumor activities and cytokine secretion in the face of autologous tumor tissues from which they have been isolated.[12,13] TNBC has been reported to have the highest levels of TIL (≥20%) among the various types of BCs, and therefore, cell therapy with expanded TILs might be one of the promising personalized medicine for this type of cancer.[14,15] A serious challenge of TIL therapy is the presence of tumor-promoting immune cells such as regulatory T cells (Tregs), which can reduce the antitumor function of TILs. Hence, targeting and reducing Tregs in the TIL population can improve the efficacy of TIL therapy.[16,17] Recent studies have shown that targeting c-Rel, one of the multiple components of the nuclear factor (NF)-κB complex, reduces Treg cells at the tumor site.[18,19] Using *c-rel* knocked-out mice or c-Rel inhibitors, recent studies showed that targeting c-Rel can improve the tumor treatment by reducing the population and proliferation of Treg cells at the tumor site.[18,20] One of the chemical inhibitors of c-Rel, wprovedoven specific effects on c-Rel, is Pentoxifylline (PTXF).[21] PTXF is a competitive nonselective phosphodiesterase inhibitor with specific effects on c-Rel.[18] PTXF is a xanthine-derivative FDA-approved drug for patients with intermittent claudication, stomach cramps, muscle aches/cramps, and muscle weakness caused by peripheral artery disease.[22,23] In a mouse model of melanoma, treatment with PTXF, similar to *c-rel* genetic deletion, reduces Treg and improves the antitumor response.[18] Very few studies have been performed on the effects of this drug on tumor-infiltrating cells, and there are still inconsistencies in the effects of this drug on the immune cells involved in the tumor. In this study, we investigated the *ex vivo* effects of PTXF on the proportion of Tregs in TILs derived from the BALB/c mouse model of TNBC.

## MATERIALS AND METHODS

### Tumor Inoculation and Mice Model

The 4T1 cell line (TNBC cell line appropriate for BALB/c mice) was obtained from Pasteur Institute, Tehran, Iran (ATCC code: CRL-2539). Cells were propagated in RPMI-1640 containing 10% fetal bovine serum (FBS) (Gibco, US) and harvested at the confluency of <80% and the viability of >95%, washed, and suspended in sterile phosphate buffer saline (PBS). Five female BALB/c mice (6–8 weeks old) were subcutaneously injected by $10^5$ cells/mouse in the flank. Mice were housed at the animal research facility of the Breast Cancer Research Center,

Motamed Cancer Institute, ACECR, Tehran, Iran. Mice were observed daily and humanely euthanized by $CO_2$ inhalation if the tumor size exceeded 15 mm in diameter (final tumor volume did not exceed 1800 $mm^3$) or any signs of metastasis or suffering were seen in mice. The project was approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran (IR.IUMS.FMD.REC.1397.220). The animal maintenance and experiments were carried out according to the standard national and international regulations for laboratory animals.

**Tumor Digestion and TIL Isolation**

The euthanized mice were floated into ethanol 70% and transferred under a sterile cabinet for tumor dissection. The tumors were dissected by surgery and washed several times using sterile PBS containing 5X PenStrep solution (500 IU/mL Penicillin and 500 µg/mL Streptomycin) (Gibco, US). The tumor masses were mechanically cut into 1-2$mm^2$ pieces in a petri-dish containing RPMI-1640 with 1X PenStrep solution (100 IU/mL Penicillin and 100 µg/mL Streptomycin) using scalpel blades. Enzymatic digestion was carried out with a cocktail of 0.2% collagenase IV (Gibco, US) and 10 U/mL DNase I (Sigma-Aldrich, US) in RPMI-1640 for 40 min in a shaker incubator at 37°C and 90 rpm. The digestion process was stopped by adding FBS. The suspension was passed through a 70 µm cell-strainer (SPL, South Korea) to obtain single cells and centrifuged at 1000xg for 5 min to remove the enzymes and proteolyzing components. The cell pellet was resuspended in RPMI-1640 and added gently to the same volume of Ficoll 1.077 (GE Healthcare, Sweden) and centrifuged at 800xg for 20 min. The compacted cells between Ficoll and culture medium were harvested, washed, and their viability was checked by trypan blue dye on a hemocytometer chamber.

**MTT Assay**

TILs were seeded triplicate in 96-well plates (SPL, South Korea) at a concentration of $2\times10^5$ cells/well in the complete medium (RPMI-1640 with 1X PenStrep solution and 10% FBS) containing 150 U/mL inteinterleukins (IL)-2 (MiltenyiBiotec, Germany). PTXF was added to the culture medium at the concentration of 125, 250, 500, 1000, and 2000 µg/mL. The plates were incubated for 24, 48, and 72h in a humidified 37°C cell-culture incubator with 5% $CO_2$. The control group only contained TILs with a complete culture medium (without PTXF), and in the positive control group, TILs were treated with 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, US) as a toxic agent. After 24, 48, and 72h incubation, 20 µL of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide) solution (Sigma, St. Louis, US) was added into the wells. After 4h incubation, the supernatant was slowly removed, and the precipitated formazan crystals were solubilized in 100 µL of DMSO for 30 min. The optical density of each well was measured at 570 nm in parallel to standard wells.

**Treatment of TILs with Pentoxifylline**

TILs were cultured in a triplicate manner in a 48-well plate at the concentration of $10^6$ cells/mL in the complete medium; containing 150 U/mL IL-2. The treated groups were incubated with 500 or 1000µg/mL of PTXF in a humidified cell-culture incubator with 5% $CO_2$ for 24, 48, and 72 hours. The non-treated control group received only IL-2 without PTXF. After incubation, cells were harvested in separate tubes for flow cytometric analysis.

**Flow Cytometry**

Immunophenotyping of TILs was carried out using surface staining for FITC-conjugated CD3 (clone: 145-2C11, BD Bioscience, US), APC-conjugated CD4 (clone: GK1.5, BioLegend, San Diego, US), PE-conjugated CD8 (clone: 53-6.7, BD Bioscience, US), and FITC-conjugated CD25 (clone: 7D4, BD Bioscience, US). One microgram of antibody per $10^5$ cells was added in PBS with 2% FBS, and then cell suspension was incubated for 30 min at 4°C in the dark. The intracellular staining was performed for FOXP3 evaluation using FOXP3 Fix/Perm Buffer Set (BioLegend, San Diego, US) and PE-conjugated anti-FOXP3 antibody (clone: MF23, BD Biosciences, US), according to the manufacturer protocol. All fresh experiments were checked for dead cell percentage using 7-AAD viability dye (BioLegend, San Diego, US). The flow cytometry analysis was carried out using an Attune NXT flow cytometer (Invitrogen, US), and data were analyzed by FlowJo software (Tree Star).

**Co-culture of TILs with Tumor Cells and Cytokine Assay**

TILs were co-cultured triplicate in 96-well culture plates at a concentration of $5\times10^5$ cells/mL with 4T1 cells at the ratio of 5:1 in the complete medium containing 150 U/mL IL-2 with or without 500 and 1000 μg/mL of PTXF and incubated in a cell-culture incubator for 24, 48, and 72h. After the incubation period, supernatant samples were gently collected for cytokine assay, and the levels of interferon (IFN)-γ and tumor growth factor (TGF)-β were measured using the enzyme-linked immunosorbent assay (ELISA) Kits (eBioscience, San Diego, US), according to the manufacturer recommended protocol.

**Statistical Analyses**

All tests were performed in triplicate. Based on the normal distribution of data, one-way ANOVA and Student T-test were employed to compare means. The results were analyzed and illustrated using GraphPad PRISM 8 with a $p<0.05$ significant index.

## RESULTS

**Viability of Pentoxifylline-treated TILs**

The effects of five doses of PTXF on the viability of TILs were measured at three-time points using an MTT assay (Figure 1). We used PTXF untreated TILs as control and adjustment of cell viability percentage. A toxic concentration (10%) of DMSO was used as the positive control.

As it is represented in Figure 1, the results of the Student T-test showed that all doses of PTXF in all three-time points had no significant effects on the cell viability in comparison with the untreated control, except for treatment with 2000 μg/mL of PTXF treatment for 48h and 72h, which significantly decrease the cell viability ($p<0.01$).

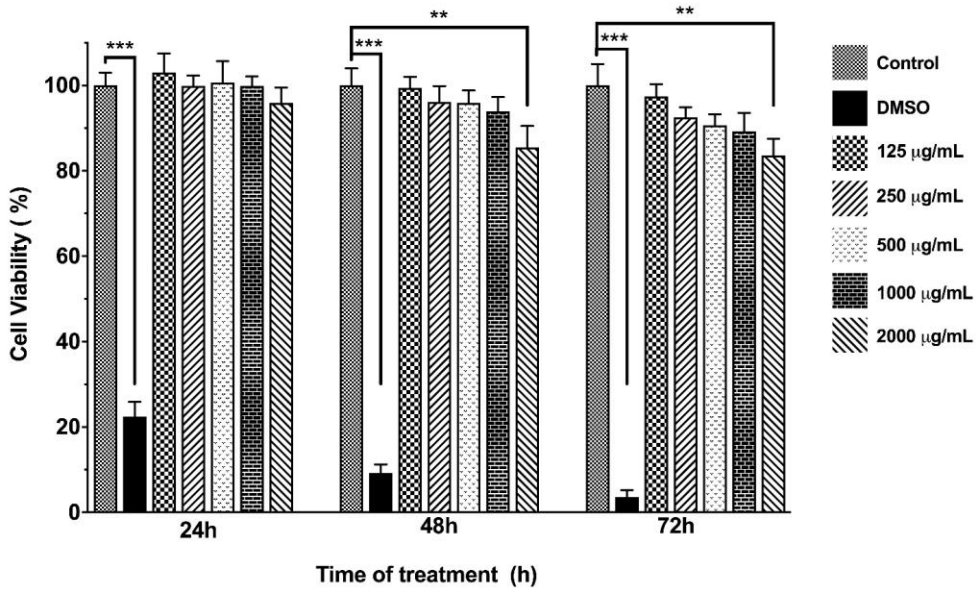


**Figure 1. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium bromide (MTT) assay for evaluation of the effects of different doses of Pentoxifylline (PTXF) on the viability of tumor-infiltrating lymphocytes (TILs) in three time-points: All columns in each time-point are presented as mean ± standard deviation (SD) and were compared to their control using the Student T-test. The stars above the bar show a significant difference between columns. DMSO. Dimethyl sulfoxide; **$p$<0.01; ***$p$<0.001**

***Ex vivo* Effect of Pentoxifylline on the Tumor-infiltrating Tregs**

TILs were treated with 500 or 1000 μg/mL of PTXF for 24, 48, and 72h, then harvested for flow cytometric analysis to determine the effects of PTXF on the percentage of Tregs. All analyses were performed on the single cells gated as illustrated in Figure 2A. Over 95% of the cells were alive based on a


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7AAD live/dead cell stain. A representative gating on $CD3^+$ $CD8^+$ cells and $CD3^+$ $CD4^+$ cells are shown in Figure 2B and 2C, respectively. The $CD25^+FOXP3^+$ population gated from $CD4^+$ cells were counted as Tregs. Figure 3 shows a representative gating strategy on the Treg population in TILs treated with either 500 or 1000 μg/mL of PTXF as well as untreated control group, in three time-points.

Moreover, the bar chart illustrated a comparison between the Tregs percent in each group (Figure 4) using a one-way ANOVA test. It is shown that PTXF treatment decreased Tregs in all three-time points of treatment in a dose-dependent manner. The 24 h and 48 h treatment of TILs with either 500 or 1000 μg/mL of PTXF significantly decreased the proportion of Tregs compared to the untreated control group. The most noticeable decline in Treg frequency is seen in the 1000 μg/mL-48 h treated group, which is approximately one-third of the control group ($p$<0.001), and half of the 500 μg/mL-48 h treated group ($p$<0.01). The 72 h treatment of TILs with PTXF also decreased the Tregs in both PTXF treatment groups; however, the changes were not significant (Figure 4).

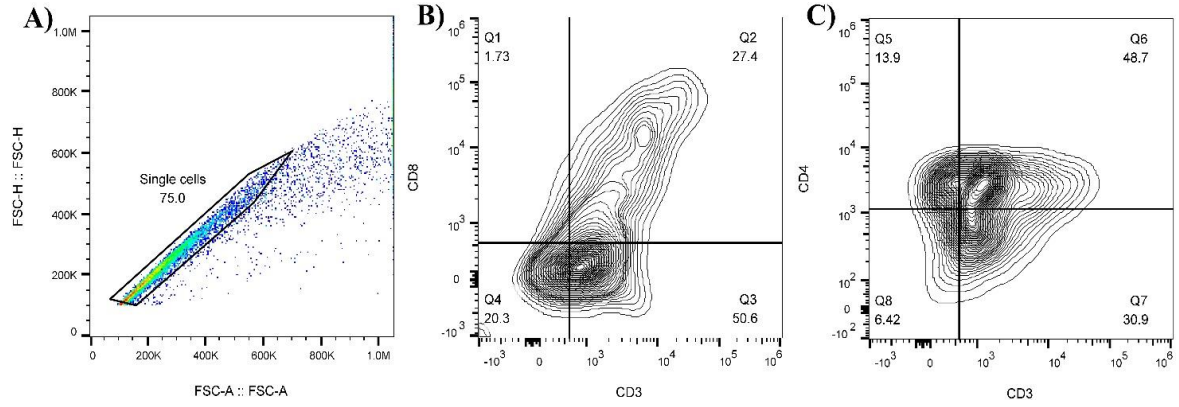


**Figure 2. A representative flow cytometry gating strategy on tumor-infiltrating lymphocytes (TILs): A) Single cells were gated on diagonal population, based on the height of forwarding scatter (FSC-H) to the area of forwarding scatter (FSC-A). B) $CD3^+$ $CD8^+$ cells ($CD8^+$ T cells) were gated from single cells and shown in a quadrant chart. C) $CD3^+$ $CD4^+$ cells ($CD4^+$ T cells) were gated from single cells and shown in a quadrant chart.**

**Cytokine Assay**

TILs were co-cultured with a 4T1 cell line (5:1 ratio) in the presence of IL-2 and PTXF (500 or 1000 μg/mL) at three-time points. The levels of IFN-γ (Figure 5A) and TGF-β (Figure 5B) were measured in the supernatant. According to the one-way ANOVA test, twenty-four hours of treatment of TILs with PTXF showed no significant changes in IFN-γ levelevelspared to the control group. However, 48h treatment with 1000μg/mL and 72h treatment with either 500 or 1000 μg/mL of PTXF significantly increased IFN-γ compared to the control group ($p$=0.021,0.044,0.022, respectively). The effect of PTXF in 48h treatment was somewhat dose-dependent. The maximum significant increment of IFN-γ production in supernatant was detected in 48 h treatment with 1000 μg/mL of PTXF in which the IFN-γ level was approximately 1.55-fold higher than the control group (P=0.021). In 48h treatment with 1000 μg/mL, the IFN-γ level was slightly higher than those treated with 500 μg/mL. The levels of IFN-γ in 24 h and 72 h treatment with 500 and 1000 μg/mL of PTXF were comparable (Figure 5A).

The TGF-β level in supernatant was lower in PTXF treated groups compared to the control group, but this difference is only significant in groups treated with 1000 μg/mL of PTXF for 48h and 72 h, and also in the group treated with 500 μg/mL of PTXF for 72h ($p$<0.001). The group treated for 72 h with either 500 or 1000 μg/mL of PTXF showed a comparable decrease in the amount of TGF-β whereas the 48h treatment with 500 μg/mL of PTXF did not significantly reduce the TGF-β level compared to the control group (Figure 5B).

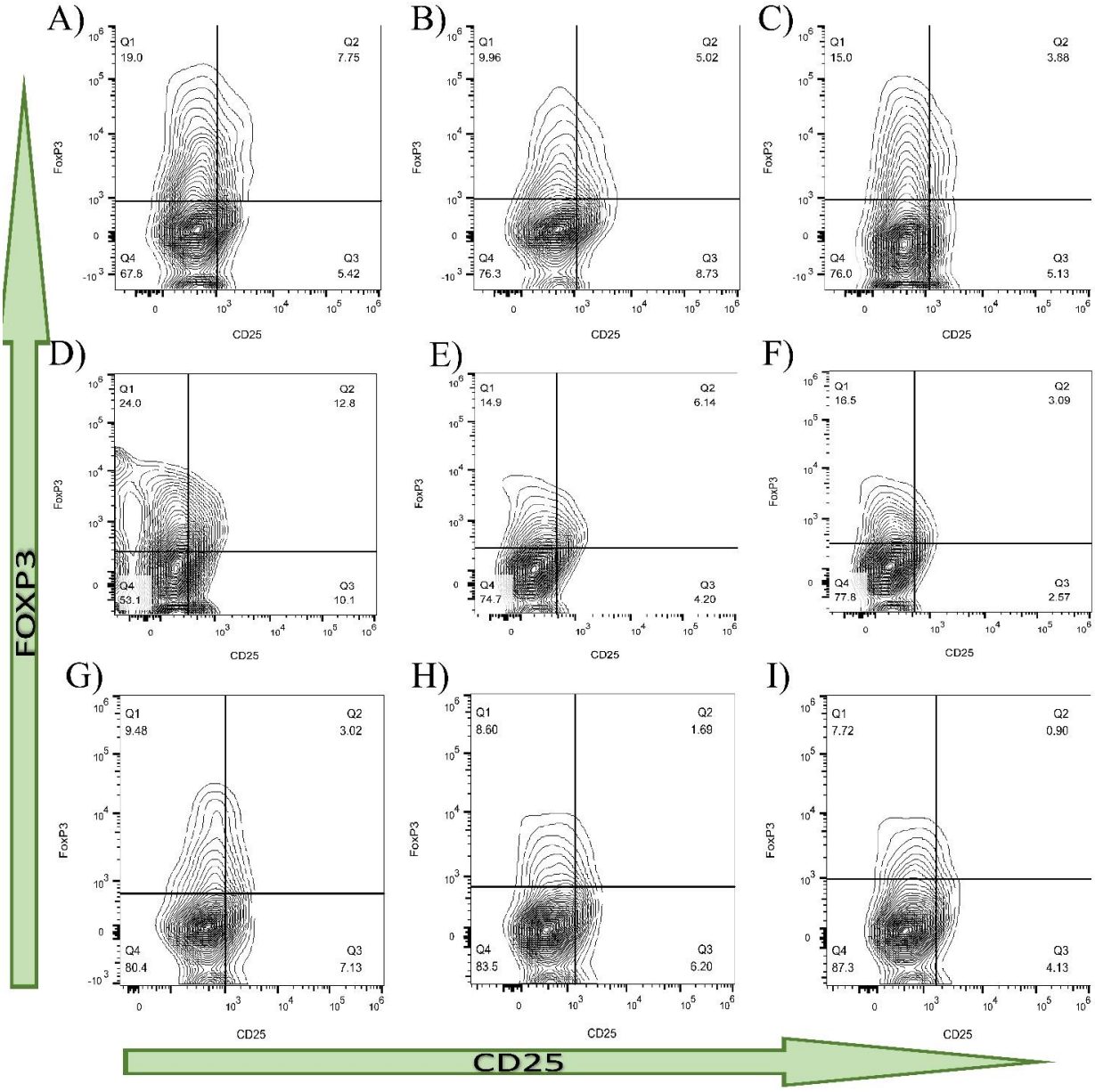


**Figure 3. Flow cytometry quadrant charts of regulatory T cells (Tregs) in Pentoxifylline (PTXF) treated and untreated tumor-infiltrating lymphocytes (TILs) to evaluate the percent of Treg cells in TILs. Quadrant charts are based on CD25 and FOXP3. All populations are gated from $CD3^+$ $CD4^+$ populations, and gating was performed based on the unstained tube and gating controls. A,B,C) TILs received no PTXF as control (A) or 500 (B) or 1000 (C) μg/mL of PTXF for 24h. The 48h (D, E, F) and 72h (G, H, I) treatments were performed as same as for 24h treatment for untreated control groups (D and G), groups treated with 500 (E and H) or 1000 μg/mL (F and I) of PTXF.**

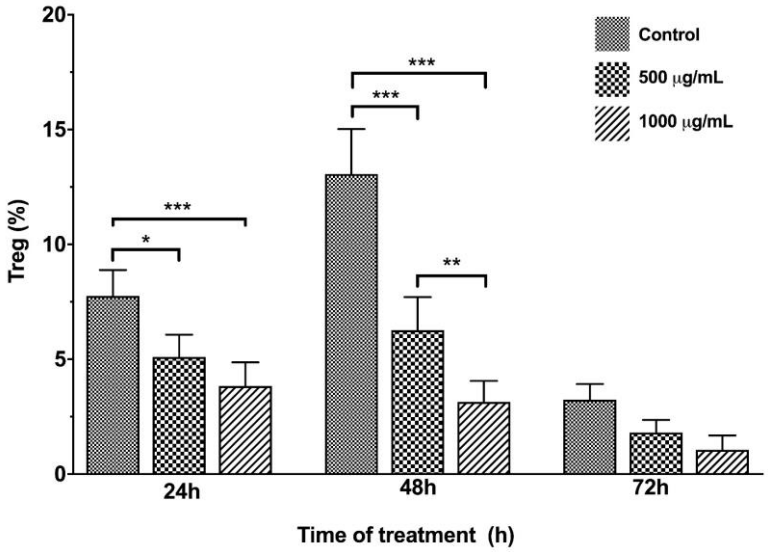


**Figure 4. Bar chart showing the changes in the proportion of regulatory T cells (Tregs) in tumor-infiltrating lymphocytes (TILs) following different periods of treatment with different doses of Pentoxifylline (PTXF). Data are presented as mean ± standard deviation (SD) and comparisons were carried out, using a one-way ANOVA test. Stars above the bars show significant differences between columns. *$p$<0.05; **$p$<0.01; ***$p$<0.001**

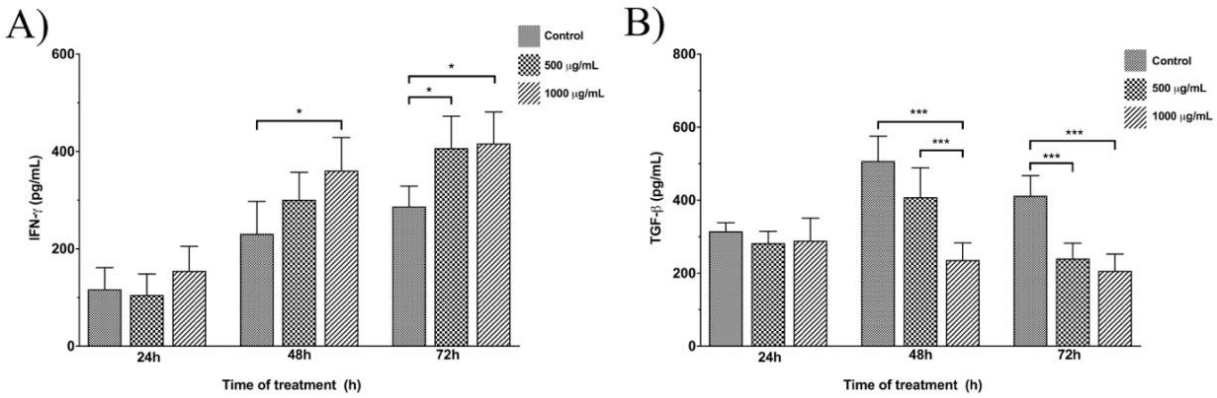


**Figure 5. Bar charts showing the secreted interferon (IFN)-γ and tumor growth factor (TGF)-β in the supernatant of tumor-infiltrating lymphocytes (TILs) following treatment with Pentoxifylline (PTXF) and co-cultured with tumor cells. TILs were treated with two doses of PTXF and co-cultured with 4T1 cells (5:1 ratio) in three time periods. IFN-γ (A) and TGF-β (B) in the supernatant are measured and shown by mean standard deviation (SD) and compared to each other using a one-way ANOVA test. Stars above the bars show significant differences between columns. *$p$<0.05; ***$p$<0.001**

## DISCUSSION

Immunotherapy using TILs is a personalized treatment with promising results in recent years.[24–26] Recent studies showed favorable responses to the transfusing TILs expanded by IL-2 in chemo-resistant melanoma patients.[27,28] The prognostic role of TILs in BC has been entered into the clinical guidelines so that the quantity and quality of TILs could be valuable factors for BC prognosis and treatment response.[6–8]

However, utilizing TILs as a therapeutic modality in BC is not well investigated and has a long way to go. The high proportion of immunosuppressive cells in the breast TILs might be one of the critical impediments in the TIL therapy of BC.[17,29] Therefore, investigating the methods that reduce and re-educate the immunosuppressive cells can be very promising for improving the efficiency of this treatment.

An efficient TIL therapy requires the tumor-reactive effector TIL pool with a minimum of suppressor cells. The researchers aim to remove regulatory cells such as Treg, which actively exist within the tumor-infiltrating cells, and suppress antitumor immune cells, resulting in an adverse response to TIL therapy.[30,31] The conventional method to expand TILs includes IL-2, which is also an essential factor for Treg expansion.[32,33]

Despite advances in cell targeting, specific methods for targeting Tregs are still challenging. Recent studies showed that targeting c-Rel, one of the multiple components of the NF-κB complex, reduces Treg cells at the tumor site.[18,34] FOXP3 is the essential transcription factor for the development and function of Tregs so the downregulation of FOXP3 could profoundly inhibit the regulatory functions of Tregs.[35,36] Studies have shown that the FOXP3 production and function are dependent on c-Rel.[37,38] So, using c-Rel inhibitors might decrease the Tregs proportion in TILs. Here, we attempted to investigate the effect of *ex vivo* treatment of TNBC-isolated TILs with PTXF on the proportion of Tregs.

The TILs were extracted from the tumor mass of the BALB/c mouse model of TNBC using optimized enzymatic digestion. The first thing we explored was the effect of PTXF on the viability of TILs. The previous studies have used 300-3000 μg/mL final concentrations of PTXF in the in vitro treatment,[39–41] and one study showed relatively significant results for overnight treatment of TILs with 500 μg/mL of PTXF.[18] Therefore, we decided to evaluate the effects of 500 μg/mL, as well as two higher concentrations and two lower concentrations of PTXF on the viability of TILs. As the results of the MTT assay illustrated, the different doses of PTXF (up to 1000 μg/mL) have no significant effects on the viability of TILs during three days of treatment, and thus, can be used in the *ex vivo* treatment. The negligible effect of *ex vivo* treatment with PTXF was previously reported even in higher concentrations.[39–41] Given the proven safety of PTXF in previous studies, we decided to use the highest possible concentrations of PTXF that are safe in the *ex vivo* treatment to achieve the best results. Hence, we choose both 500 and 1000 μg/mL for *ex vivo* treatment at three time-points of 24, 48, and 72h to optimize the dose and time of PTXF treatment.

We found that *ex vivo* treatment of TILs with both 500 and 1000 μg/mL of PTXF could significantly decrease the Tregs in a dose-dependent manner. The highest proportion of Treg was seen in 48h, probably induced by the exogenous IL-2. Although stimulated TILs produce IL-2 in an autocrine manner, the PTXF is proven to inhibit the production of IL-2.[21] Therefore, the exogenous IL-2 in the culture with PTXF is necessary. It has been shown that the PTXF could decrease the proliferation of T cells, but in the case of adding exogenous IL-2, the anti-proliferative effect of PTXF was abrogated.[42] Hence, PTXF can eliminate the concern of Treg dominancy in the IL-2-mediated conventional method of TILs proliferation.

Grinberg-Bleyer and colleagues conducted a comprehensive study on the target of c-Rel and p65 in the TILs in a mouse model of melanoma, concluding that targeting c-Rel and not p65 would result in the reduction of activated Treg cells at the tumor site.[18] The use of PTXF, similar to the genetic deletion of *c-rel*, reduces Treg in *ex vivo* and *in vivo*, and its use improves the antitumor response. Using *c-rel*-knocked out mice, they proved that the PTXF effect in reduction of Treg is *c-rel*-dependent, and PTXF treatment in *c-rel* knocked out mice could not further decrease the Tregs. Interestingly, they claimed that *c-rel* deletion or PTXF does not completely inhibit all Tregs and only decreases the activated Tregs in the tumor site. The resting Tregs, which are mainly involved in preventing autoimmunity, are substantially dependent on P65 than c-Rel, thereby not significantly decreased or inhibited by PTXF.[18] It could be interpreted in a way that c-Rel targeting by PTXF could diminish pro-tumor activated Tregs without much concern about autoimmunity and severe inflammation. Several studies have thoroughly evaluated the inhibitory effect of PTXF on c-Rel in both mRNA and protein levels.[18,21] Therefore, we did not evaluate the expression of c-Rel in our study.

In the next step, we measured the amount of IFN-γ and TGF-β in the stimulated TILs culture supernatant to evaluate the changes in one of the antitumor and one of the pro-tumor cytokines following PTXF treatment. Expectedly, the level of TGF-β was decreased while the IFN-γ was increased in PTXF treated TILs

supernatant. Although a piece of literature reported an inhibitory effect of phosphodiesterase inhibitors on IFN-γ production,[43,44] PTXF treatment has been reported to have no inhibitory effect on IFN-γ production from T cells and even lead to higher IFN-γ in melanoma TILs.[18,45,46] Interestingly enough, PTXF has been reported to promote IFN-γ-producing Tregs.[18] The lowering effect of PTXF on TGF-β has also been reported, suggesting the improvement of antitumor capabilities following PTXF treatment.[18]

The limitations of this study that are suggested to be investigated in future projects are 1. The effect of PTXF in tumor-infiltrating Tregs should be confirmed in various types of human TILs. 2. Although the effect of PTXF on c-Rel inhibition is confirmed in several studies,[18,21] investigating the other effects of PTXF on the cellular and molecular signaling of cells present in the tumor microenvironment could be helpful to optimize its use in immunotherapy; 3. Combining PTXF with other immunotherapeutic modalities might have synergistic effects in reducing and inhibiting immunosuppressive cells; 4. More functional *ex vivo* and *in vivo* analyses are required to confirm the antitumor effects of PTXF in TIL therapy. To sum up, our findings confirm the previous study[18] on melanoma-infiltrating lymphocytes about decreasing Tregs proportion in the breast tumor-infiltrating lymphocytes as well as improvement of pro-inflammatory cytokine balance in TILs supernatants. Using IL-2 is a conventional way to expand TILs which expand Tregs as well and even much more. Using PTXF during this process could dramatically reduce the concern of Treg increment, providing a cost-effective way to TILs expansion. Regarding the safety of PTXF in several human studies and its FDA approval, its clinical application in tumor treatment seems much more feasible than those interventions that still have concerns of safety and a long way to be commercialized.[47–49] Given the synergistic antitumor effect of PTXF along with checkpoint inhibitors and also the ability of PTXF to inhibit A2 adenosine receptor,[50–53] combinations of such drugs in future investigations could lead us to maximize the effect of TIL expansion and TIL therapy.

## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## ACKNOWLEDGEMENTS

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