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Alteration in Gene Expression in Response to Sequential Red and Near-Violet Light in Human Peripheral Blood Mononuclear Cells

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ABSTRACT

The photobiomodulation paradigm (PBM) takes the advantage of certain photonic wavelengths to regulate the cellular functionality through non-thermal effects and thus in the regulation of gene expression in cellular components. In the current study, the immunomodulatory effects of red low-level laser therapy (LLLT, 632nm) and near-violet light (360nm) on the transcription of the cytokine gene in human peripheral blood mononuclear cells (PBMCs) were investigated. In this regard, PBMCs were put on five discrete experimental cohorts, which included: control, LLLT, UV, and LLLT/UV with a thirty minutes interlude, and LLLT/UV with two hour interlude. The transcriptional measures of interleukin 2 (IL-2), interleukin 10 (IL-10) and tumor necrosis factor alpha (TNF-α) were measured by qualitative real time polymerase chain reactions (RT-qPCR) assays. We have found that LLLT treatment had a significant increasing effect on the IL2 and TNF-α transcription, the characteristic of immune activity; on the other hand, results of the near-violet treatment inhibited IL2 and enhanced the IL10, the measure of the immune activity, respectively. Interestingly, the introduction of LLLT with a UV period of two hours redressed the IL-2 expression and reduced IL-10 levels referring to a restorative impact on mitochondrial-mediated immune stimulatory channels.

These findings reveal that both wavelength and exposure timing critically determine the immunological outcome of PBM in response to photobiomodulation. Optimising temporal sequencing may enable tailored phototherapies for either immune stimulation or suppression, offering potential applications in immunomodulatory and regenerative medicine.

KEYWORDS: Photobiomodulation, Low-level laser therapy, Near-violet irradiation, PBMCs, Cytokine expression, Mitochondrial signaling, Immune modulation.

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INTRODUCTION

Photobiomodulation (PBM) is a developing non-invasive treatment method which employs the non-ionising light at low intensity to regulate the functions of the cells, by photochemical and photophysical mechanisms(ALMusawi.et al.,2016). In contrast to thermal therapies, PBM exerts its action through photon absorption by intracellular chromophores, specifically by the mitochondrial respiratory chain where it leads to biochemical regulation of metabolism, signalling and gene expression. Biological effects are determined by wavelength, fluence and timing parameters and each parameter presents a distinct cellular response spanning activation to the suppression (Hamblin, 2017; Karu & Semyachkina-Glushkovskaya, 2022).

The red spectral range (630-660 nm) of Low-Level Laser Therapy (LLLT) has shown extensive evidence to improve the mitochondrial oxidative phosphorylation by triggering the activity of the cytochrome c oxidase (CCO), resulting in ATP production and the regulated production of reactive oxygen species (ROS). These ROS are secondary messengers involved in intracellular signalling that regulate transcription factor NF-κB and AP-1, and hence induce cellular proliferation, repair, and immune activation (Chung et al., 2012; da Silva et al., 2023). On the other hand, higher-energy photons of near-violet or ultraviolet-A light (approximately 360 nm) are likely to cause oxidative and DNA stress and activate redox-sensitive transcription factors, including STAT3 and p53, to suppress immunosuppressive or anti-inflammatory responses (Pfeifer et al., 2019; Ryu et al., 2020). It is this duality of PBM that is also able to stimulate and suppress immune activity that makes it a potent yet intricate bioregulatory factor. The best ex vivo model to examine the effects of such photobiomodulatory effects is peripheral blood mononuclear cells (PBMCs), which constitutes of lymphocytes (T cells, B cells, NK cells) and monocytes, which are the key players in both adaptive immune response and innate immune response. The direction of the immune system is indicated by the balance between the level of pro-inflammatory cytokines, including interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) and the presence of anti-inflammatory cytokines, including interleukin-10 (IL-10). Cytokines such as IL-2

play a role in the proliferation of T-cells and the activation of the immune response; TNF- α is a central mediator of inflammatory signalling, and IL-10 is a master regulator which restrains overaction of immune response and ensures homeostasis (Couper et al., 2008; Miller et al., 2020).

Recent research has shown that the biologic effect of light radiation is not influenced by wavelength and fluence alone, but the temporal pattern of multiwavelength radiation as well. This phenomenon is referred to as the chronophotobiomodulation one(ALMusawi et al.,2017), which implies that the cell response to the sequencing and timing of exposures to different wavelengths is determined because each wavelength triggers a specific mitochondrial and transcriptional program, which can be synergised or countermeasured (Abdel-Motal et al., 2022; Gao et al., 2023). As an example, red light can cause a short time pro-activating state (increased ATP and ROS signalling) that can be either stabilised or reversed with a second wavelength. Thus, a detailed phenomenon of the interaction of various wavelengths over time is necessary to create effective PBM regimens with immunomodulatory purposes (Yahia et al .,2020). The aim of this research was to examine a successive and also single impact of red light (632 nm, LLLT) and near-violet light (360 nm) on the IL-2, IL-10 and TNF-alpha gene expression in human PBMCs. In particular, the study tried to find out how the differentiation of the period between red and violet exposures (30 minutes vs. 120 minutes) affects the profile of cytokines expression and the general polarity of the immune system. Connecting mitochondrial bioenergetics to cytokine transcriptional changes, the study offers mechanistic knowledge of wavelength- and time-dependent regulation of immune response which has potential application in immune therapy, regulation of inflammation and photomedicine.

MATERIALS AND METHODS

This study complied with the principles of the Declaration of Helsinki (2013 revision). Ethical approval was granted by the Institutional Review Board of Mustansiriyah University – College of Medicine.
Five healthy adult volunteers (age 20–35 years) were enrolled after providing written informed consent. All participants were non-smokers, medication-free for at least two weeks, and had no history of autoimmune, infectious, or chronic metabolic disorders.

BLOOD SAMPLING AND PBMC ISOLATION

Peripheral venous blood (8–10 mL) was drawn aseptically into sterile K_2-EDTA vacutainers (BD Biosciences, USA) and processed within 1 hour to ensure high cell viability.
PBMCs were isolated by density gradient centrifugation as follows:

1. Whole blood was diluted (1:1, v/v) with sterile phosphate-buffered saline (PBS, pH 7.4).
2. The mixture was carefully layered over Ficoll-Hypaque (BioWest, France; density = 1.077 g/mL) and centrifuged at 400 × g for 20 min at room temperature with the brake off.
3. The interphase buffy coat (PBMC layer) was aspirated and transferred to a new tube.
4. Cells were washed twice with PBS (300 × g, 5 min) to remove platelets and residual Ficoll.
5. The final pellet was resuspended in RPMI-1640 medium (BioWest, France) containing:
    - 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA)
    - 1% penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively)
    - 2 mM L-glutamine
    - 1% sodium pyruvate

Cell concentration and viability were assessed by trypan blue exclusion using a Neubauer hemocytometer, and preparations exceeding 95% viability were used for experiments.

CELL CULTURE CONDITIONS

PBMCs were plated in 96-well flat-bottom microplates (Corning, USA) at a density of 1×10^5 cells/cm^2 in 200 µL of culture medium.
Cells were incubated at 37 °C, 5% CO_2, and 95% humidity for 48 h to allow metabolic equilibration prior to light exposure. Cultures were handled in a Class II biosafety cabinet under aseptic conditions.

PHOTOBIOMODULATION SETUP

Laser Source and Dosimetry
Two distinct light sources were used:

Parameter	Red Laser (LLLT)	Near-Violet Source
Wavelength	632 ± 5 nm	360 ± 10 nm
Type	Continuous-wave diode laser (Thorlabs, USA)	Narrow-band UV-A LED array (Ocean Optics, USA)
Beam Diameter	1.0 cm	1.0 cm
Spot Area	0.785 cm²	0.785 cm²
Power Output	30 mW	20 mW
Irradiance	38 mW/cm²	25 mW/cm²
Fluence (Energy Density)	**3.8 J/cm²**	**2.5 J/cm²**
Exposure Time	100 s	100 s
Distance to Cells	2 cm	2 cm

Parameter	Red Laser (LLLT)	Near-Violet Source
Temperature Monitoring	Infrared thermometer (< 37.5 °C)	Same

Energy delivery was verified using a PM100D optical power meter (Thorlabs, USA) before each experiment. To prevent thermal artifacts, temperatures were maintained below 37.5 °C throughout irradiation.

Experimental Design

Cells from each donor were divided into five groups (three replicate wells per condition):

1. Control (G1): No irradiation.
2. LLLT (G2): 632 nm exposure only.
3. UV (G3): 360 nm exposure only.
4. Dual-30 min (G4): 632 nm followed by 360 nm after a 30-min interval.
5. Dual-120 min (G5): 632 nm followed by 360 nm after a 120-min interval.

Plates were irradiated on an anti-reflective surface, ensuring uniform fluence distribution. After exposure, cultures were immediately returned to the incubator for recovery at 37 °C until RNA extraction.

RNA Extraction and Reverse Transcription

Total RNA was extracted using the **ReliaPrep™ RNA Miniprep System (Promega, USA)** under RNase-free conditions. RNA concentration and purity were verified spectrophotometrically (NanoDrop 2000, Thermo Fisher, USA), and only samples with A_{260}/A_{280} ratios between **1.8 and 2.1** were accepted.

Complementary DNA (cDNA) was synthesized from **1 µg of total RNA** using the **GoScript™ Reverse Transcription System (Promega)** according to the manufacturer's protocol. cDNA samples were stored at −80 °C.

Quantitative Real-Time PCR (qRT-PCR)

Gene expression of cytokines **IL-2**, **IL-10**, and **TNF-α** was quantified using **SYBR Green chemistry (GoTaq® qPCR Master Mix, Promega)** on a **3G Tower thermal cycler (Analytika Jena, USA)**.

Cycling protocol:

- Initial denaturation: 95 °C for 10 min
- 40 cycles of:
  - 95 °C for 15 s
  - 60 °C for 30 s
  - 72 °C for 30 s

Primer sequences were validated for efficiency (90–110%) and single-peak melting curves.

GAPDH served as the housekeeping gene. Relative expression was calculated using the $\mathbf{2^{-\Delta\Delta Ct}}$ method, normalizing all groups to the untreated control.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) from three technical replicates per donor.

Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism 10 (GraphPad Software, USA).

A $p < 0.05$ threshold denoted statistical significance. Graphs were $\log_2$-transformed where appropriate to reflect relative gene expression dynamics.

Quality Control and Reproducibility

To underpin reproducibility, all the irradiation procedures were performed in triple on different days. The calibration of the instruments (power meter, pipettes, thermometers) was done on a daily basis. Each PCR run contained negative controls of contamination. No- template controls were negative.

RESULTS

1. General Overview

Qualitative gene expression analysis revealed a distinct wavelength- and timing-dependent regulatory pattern in PBMCs, with clear divergence between immune activation and suppression pathways (as shown in Figure 1–4). The $\log_2$-normalized expression levels of IL-2, IL-10, and TNF-α across the experimental conditions indicated that red and near-violet light exposures modulated cytokine transcription through contrasting mechanisms.

The analysis of qualitative gene expression showed that the unique pattern of wavelength- and time-dependent regulation was observed in PBMCs, and the strong divergence between immune activation and suppression pathways is observed (as it is illustrated in Figure 1–4). The $\log_2$-normalized expression levels of IL 2, IL 10, and TNF alpha under the experimental conditions were found to suggest that the exposure to red and near violet light had opposite effects on the transcription of cytokines

2. Effects of Single-Wavelength Irradiation

Exposure to LLLT (632nm) produced a significant increase in IL 2 expression with a significant increase in TNF a and a relatively small increase in IL 10 (as in Figure 1 and Figure 3). The IL 2 and TNF at $p < 0.01$ and $p < 0.05$ respectively increased about 2.7 and 1.8 fold in comparison to the control, which is the indication of pro-inflammatory and immune activating phenotype and in accordance to T cell activation. On the other hand, the reverse transcriptional effect was observed with near violet (360 0nm) irradiation (Figure 1 and Figure 2). The IL 2 expression of PBMCs in the UV group showed a significant reduction (0.4-fold; $p < 0.01$) and a significant increase in the IL 10 expression (2.5-fold; $p < 0.001$) which indicates a shift to a regulatory or immunosuppressive phenotype that may be prompted by tolerance signalling by oxidative stress.

3. Sequential Dual Exposure and the Role of Recovery Time

(a) 30-Minute Interval (LLLT/UV-30)

The cytokine profile was almost identical to the one of UV alone when PBMCs were exposed to LLLT and subsequently UV after a time of 30 minutes (as shown in Figure 4). In particular, the expression of IL 2 was again repressed (0.5-fold vs. control, $p < 0.01$) whereas the expression of IL 10 was considerably high (2.3-fold, $p < 0.001$). TNF-α had slight variation. This shows that with the reduced interval the UV-dominant signalling was able to prevail in the activation induced by LLLT.

(b) 120-Minute Interval (LLLT/UV-120)

Conversely, a longer time interval between exposures of 120 minutes inverted such a dominance (as shown in Figure 4). The IL-2 expression was recovered to a level much higher than that of the UV and LLLT/UV-30 (2.2-fold; $p < 0.01$), whereas the expression of IL 10 decreased to about the baseline (1.1-fold). TNF-α was also moderately high (~1.5 fold). These data verify that the time interval between exposures is a limiting factor of immune response and allows recovery of mitochondrial activation preceding the next oxidative stress.

4. TNF-α Expression as an Inflammatory Co-Signal

TNF-α in all treatment groups exhibited modulation in line with innate inflammatory signalling, which increased markedly under LLLT and in sequential exposure states showed moderate stabilisation (see Figure 1 -4). Nevertheless, TNF-α was less wavelength time sensitive than IL-2 and IL-10, indicating that its regulation encompasses partly independent inflammatory pathways. Correlation analysis revealed that there is a positive correlation between the TNF-α and IL-2 expression in the LLT and Dual 120 minutes groups (r=0.78) indicating their combined effect in the activity of the immune system. Together, these findings support the view that wavelength and exposure timings are dominant factors of cytokine transcriptional polarity in PBMCs with Figures 1-4 showing the gradual change in phenotypes between immunostimulatory and immunosuppressive.

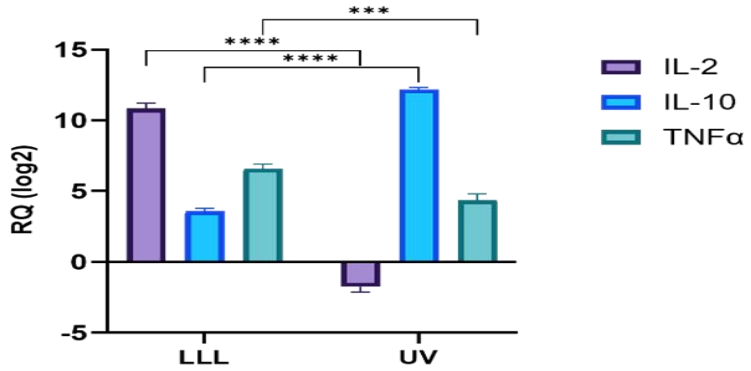

Figure 1: Gene expression of IL-2, IL-10 and TNFα following single wavelength exposure (LLLT vs UV). Gene expression was measured by RT-qPCR and expressed as Log2 of relative quantity to show fold change. *** = $p < 0.001$, **** = $p < 0.0001$

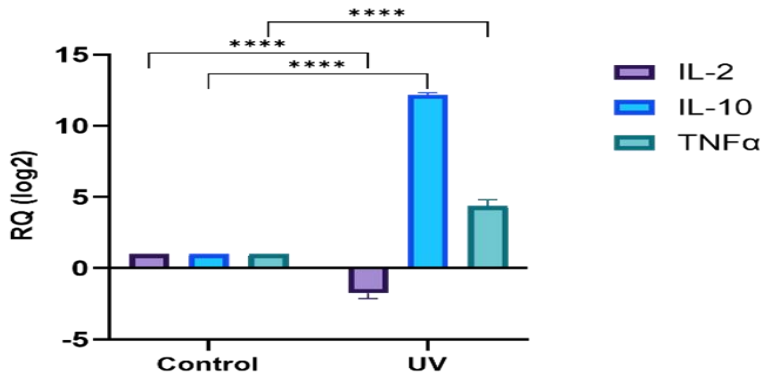

Figure 2: Comparative cytokine expression between Control and UV treated PBMCs, emphasizing IL 10 upregulation. Gene expression was measured by RT-qPCR and expressed as Log2 of relative quantity to show fold change. **** = p <

0.0001

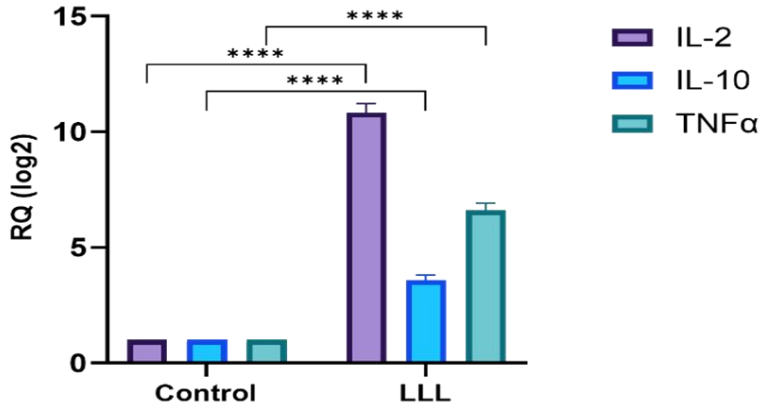

Figure 3: Comparative cytokine expression between Control and LLLT treated PBMCs, highlighting IL 2 and TNF α upregulation. Gene expression was measured by RT-qPCR and expressed as Log2 of relative quantity to show fold change. **** = p < 0.0001

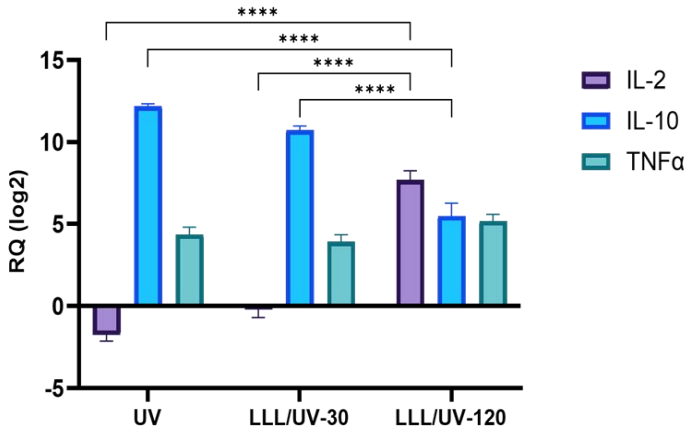

Figure 4: Effects of sequential dual exposure (LLLT/UV - 30 min and LLLT/UV - 120 min) demonstrating interval-dependent immune polarity. Gene expression was measured by RT-qPCR and expressed as Log2 of relative quantity to show fold change. **** = p < 0.0001

DISCUSSION

As the current research paper illustrates, the immunological action of photobiomodulation (PBM) with human PBMCs is conditioned by both the wavelength on which photobiomodulation is practiced and the temporal interaction between consecutive photobiomodulation irradiations. Single and sequential exposures to mitochondrial signalling of IL-2, IL-10 and TNF-2 have indicated that mitochondrial signalling is a dynamic and time sensitive regulator of immune polarity (Figures 1 to 4). The IL-2 and TNF-alpha levels were also greatly increased in response to red light (632 nm), which demonstrates the stimulation of T-cell-related pro-inflammatory reactions. This reaction is consistent with the long-standing position of the cytochrome c oxidase as the major photoacceptor of the red-region photons. Activation of this complex promotes the mitochondrial respiration, accelerates the production of ATP, and generates regulated reactive oxygen species (ROS) that act as second messengers (Hamblin, 2017; Karu and Semyachkina-Glushkovskaya, 2022). This moderate burst of ROS later leads to the activation of NF- 0B and AP-1 transcription factors and leads to the IL-2 and TNF- 0 gene transcription. The available evidence confirms this process because both cytokines exhibited similar patterns of upregulation in LLLT conditions (Figure 3). These results suggest that a cytotoxic stress-inducing stimulation of mitochondria caused by PBM can boost adaptive immune activation without creating cytotoxic stress. On the other hand, near-violet light (360 nm) inhibited IL-2 and increased IL-10, which is in agreement with anti-inflammatory or immunosuppressive repositioning (Figure 2). Photons with short wavelengths have more quantum energy that might cause undesirable oxidative stress and transient DNA damage responses. These factors stimulate redox-sensitive transcriptional factors like STAT3 and p53 that stimulates IL-10 synthesis and inhibits IL-2 synthesis through NF-kB (Ryu et al., 2020; Pfeifer et al., 2019). This mechanistic polarity is the reason why the activation signals of red light pro-activation are antagonized by nearviolet and the dualistic nature of PBM to either activate or inhibit immune activity based on spectral properties. The time-dependent changes in the results between the 30 minutes sequential exposure and the 120 minutes sequential exposure reveals the sensitivity of photobiomodulatory signaling in time (Figure 4). In the case of violet irradiation followed by a red one after 30 minutes only, the pro-activation signature of IL-2 was canceled and substituted with IL-10 upregulation, which can be taken as evidence of the pre-eminence of the oxidative suppressive pathway. Nonetheless, an IL-2 restoration with a 120-minute interval and normalization of IL-10 levels indicate that the mitochondrial activation of red light takes about 2 hours to stabilize, followed by the cell being exposed to additional photic stress. This metabolic

consolidation window is probably the period required to restore mitochondrial membrane potential, detoxify ROS, and stabilize transcriptional NF-kB regulated gene (Gao et al., 2023; Abdel-Motal et al., 2022). Upon consolidation, this state becomes irreversible to later near-violet stimulation thereby maintaining an immunostimulatory phenotype. The intermediate modulation of TNF-a among the groups indicates its role as a secondary system of inflammation in place of an immune-polarizing agent. In this case, the expression of TNF-a was positively correlated with IL-2 ($r = 0.78$) in red-light and Dual-120 min, which confirms the presence of collaboration between TNF-a and IL-2 to enhance T-cell activation via autocrine and paracrine pathway. In part, this autonomous regulation can be explained by the specific promoter sensitivity to oxidative (compared to metabolism signaling) (Biswas, 2020).

Taken together, these findings provide evidence of a mechanistic theory, under which red-light-stimulated mitochondrial stimulation raises ATP and ROS levels to physiological levels, which, in turn, trigger the expression of NF-kB and the expression of pro-inflammatory cytokines. In contrast, near-violet light passes beyond this redox threshold leading to the activation of STAT3 and feedback suppression by IL-10. The interaction between these conflicting routes is time-dependent: in the case of the second exposure that comes before metabolic homeostasis is re-established, suppressive signaling dominates; in case there is sufficient time to rest, the pro-activation state is maintained. The control is a two-color experiment that provides the measurements on a wavelength-timing interaction of PBM, equivalent to so-called chronophotobiomodulation described by Abdel- Motal et al. (2022). It highlights the fact that biological effects are regulated by the photon energy besides dynamic conditioning of bioenergetic machineries within the target cell.

CONCLUSION

This study establishes that time dynamics plays a critical role in the PBM mediated immune modulation. The directionality of immune signalling is dependent on both wavelength and exposure timing, which underlies that photobiomodulation is a sequence-dependent regulatory mechanism and no longer a fixed stimulus. Maximization of these parameters can enable the formulation of custom-made PBM protocols that can induce immune activation or suppression selectively.

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