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The Effect of Photobiomodulation and *Akkermansia muciniphila* on THP-1 Derived Macrophage Polarization Treated with Gliadin Peptide

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Abstract

Introduction: Photobiomodulation (PBM) and *Akkermansia muciniphila* have been shown to be effective in improving inflammatory conditions with positive effects on increasing the population of anti-inflammatory M2 macrophages (MQs). In this study, gliadin-stimulated THP-1 derived MQs were treated with *A. muciniphila* and PBM to evaluate their effects on promoting the polarization of M2 MQs.

Methods: The human monocyte cell line (THP-1) was differentiated to MQs. MQs were stimulated with 200 µg/mL gliadin for 24 hours and then treated with PBM 810 nm alone and in combination with *A. muciniphila* for the following 24 hours to evaluate their effects on MQs polarization. THP-1 derived MQs were also treated with PBM and *A. muciniphila* to evaluate their effects on non-stimulated MQs. CD11b, CD80, and CD206 levels were evaluated by using the flow cytometry technique. Moreover, the expression of some M1 and M2-related cytokines was determined.

Results: PBM therapy of gliadin-stimulated MQs decreased IL-6 and increased TGF-β, IL-10 and TNF-α expression compared with gliadin exposed MQs. PBM along with *A. muciniphila* treatment induced IL-6, TNF-α, and IL-10 expression in MQs in comparison to the untreated group. It also elevated TGF-β, IL-10 and TNF-α levels in gliadin-triggered MQs in comparison to gliadin-stimulated MQ cells.

Conclusion: The result of this study showed the potential of PBMT and *A. muciniphila* for modulating inflammatory responses and MQs polarization. This may open new perspectives to find possible therapeutic targets for celiac diseases.

Keywords: Celiac disease; Photobiomodulation; Macrophage; Gliadin.

Introduction

Celiac disease (CD) is an autoimmune intestinal disorder triggered by an adverse reaction to gluten protein, which is found in wheat, barley and rye, in genetically susceptible individuals.[1,2] Both innate and adaptive immune responses are involved in the pathogenesis of CD.[3] Macrophages (MQs), as critical mediators of the innate immune system, play key roles in CD development. MQs are known as plastic immune cells with both pro-(M1) and anti-inflammatory (M2) phenotypes that can maintain a delicate balance between pro- and anti-inflammatory signals and are involved in several autoimmune diseases.[4,5]

M1 MQs produce pro-inflammatory cytokines like interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) and participate in inflammatory processes. On the other hand, M2 MQs secrete anti-inflammatory mediators such as transforming growth factor-β (TGF- β) and IL-10 and participate in regulating tissue regeneration and resolution of the inflammatory signals.[6-8] M1 and M2 MQs can also convert to each other in response to the local microenvironments, and the unbalanced M1/M2 phenotype is accompanied by the progression of inflammatory disorders.[9-11] Previous studies have shown that gluten peptides can induce activation of MQs to the M1 pro-inflammatory phenotype in CD.[12,13]

Controlling MQs polarization can be an attractive strategy for finding a novel therapeutic target for CD treatment. According to previous reports, Photobiomodulation (PBM) therapy can modulate the inflammation phase and alter the polarization state of MQs.[14-16] PBM therapy

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is a treatment method that uses red or infrared spectra wavelengths to increase photochemical reactions, change cell membrane permeability, increase cell metabolism, reduce inflammation and promote tissue repair.[14-16] Tian et al recommend that near infrared light could stimulate M0-type macrophages and increase the M2/M1 ratio via the PI3K/AKT/mTOR pathway.[17,18]

Moreover, the gut microbiota plays an important physiological role in modulating immunity and may have effects on MQs polarization control.[19] *Akkermansia muciniphila* is an anaerobic gram-negative bacterium found in the gut microbiota with known anti-inflammatory properties.[20] Recent studies have suggested its positive influence on boosting the presence of anti-inflammatory M2 MQs.[7,21,22] Various mechanisms that this bacterium employs to achieve its anti-inflammatory effects are suggested. For instance, the outer-membrane protein Amuc_1100 of *A. muciniphila* triggers TLR2 signaling, resulting in increased IL-10 levels, reduced inflammation, and the maintenance of immune balance. In addition, the metabolites SCFAs and BCFAs from *A. muciniphila* contribute to immune regulation and alleviation of inflammation. Furthermore, *A. muciniphila*-derived extracellular vesicles (EVs) exhibit immune-modulatory properties, impacting gene expression, lipid metabolism, and intestinal barrier integrity, thereby promoting better gut health.[7]

This study aimed to delve deeper into the immunomodulatory effects of *A. muciniphila* and PBM treatments of gliadin stimulated THP-1 derived MQs. By reassessing the cytokine profiles in treated and untreated groups, we aimed to uncover their effects on promoting the polarization of M2 MQs and the underlying mechanisms that drive the therapeutic potential of PBM and *A. muciniphila*.

Materials and Methods

Bacterial Culture

Akkermansia muciniphila MucT strain (ATCC BAA -835) (DSMZ institute, Germany) was cultured anaerobically (N2/CO2 80:20 v/v) in brain heart infusion (BHI) broth supplemented with 2% mucin (Sigma -Aldrich, St Louis, MO, USA) in 10 ml Hungate anaerobic tubes at 37 °C. After the OD600 reached ~1 bacterial suspension was centrifuged at 11,000 g for 20 minutes. The pellet was washed twice with sterile anaerobic PBS and used for further co-culture experiments.

THP-1 Cell Preparation and Treatments

Human monocytes THP-1 cells, as pro-monocytic cell line, were cultured in RPMI 1640 (Roswell Park Memorial Institute medium, Biosera) supplemented with 10 mM Hepes (Gibco, USA), 1 mM sodium pyruvate (Gibco, USA), 1% (v/v) mixture of penicillin and streptomycin (Gibco, USA), and 20% fetal bovine serum (Gibco, USA) at 5% CO_2, 37 °C in a humidified incubator. The culture medium was changed every 48 hours. THP-1 monocytes were differentiated into MQs by 48-hour incubation with 150 nM PMA (phorbol 12-myristate 13-acetate, Santa Cruz Biotechnology, sc-3576) followed by 24-hour incubation in the RPMI medium. Cells were centrifuged, re-suspended in fresh media, and plated in 6-well plates at a cell density of 0.8×10^6/mL 24 hours before the experimental stage.

THP-1 derived MQs were treated with 200 µg/mL gliadin (Sigma-Aldrich, USA) for 24 hours. The gliadin concentration was selected based on previous studies (Jelínková L, 2004). Lipopolysaccharide (LPS)-activated (20 ng/mL) MQs were used as the positive control for the inflammatory condition.

Photobiomodulation Treatment

In the PBM treatment groups, cell cultures were exposed to an aluminum-gallium-arsenide (AlGaAS) diode laser emitting continuous near infrared light ($\lambda = 810$ nm) with a beam diameter of 2 mm (Twin laser; MM Optics, Sao Carlos, SP, Brazil). The laser was positioned on top of the polystyrene transparent cell culture six-well plate (13×10 cm), at a distance of approximately 10 cm. The dose was 1 J/cm^2. In order to eliminate interference from other light sources, the laser exposure occurred in a dark environment.

Moreover, in another group, gliadin stimulated MQs were treated with PBM and *A. muciniphila* (1.5×108 CFU/mL) at the same time for the following 24 hours. THP-1 derived MQs were also treated with PBM and *A. muciniphila* to evaluate their effects on non-stimulated MQs.

RNA Extraction and Real-Time PCR for Gene Expression Analysis

After the incubation, total RNA was extracted from cells by using the Total RNA Purification Mini kit for Blood/Cultured Cell/Tissue (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's instruction. cDNA synthesis was performed by using Transcriptor first strand cDNA synthesis kit (BioFact™, South Korea), and the mRNA expression of IL-6, TGF-β and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the house-keeping gene was assessed by quantitative polymerase real-time qPCR with SYBR Premix Ex Taq (RealQ Plus 2x Master Mix Green-Amplicon, Japan). The Rotor-Gene Q series was employed for the experiments, and the reactions were conducted with the following parameters: an initial denaturation step at 95 °C for 15 minutes, followed by 40 cycles of 20 seconds at 95 °C, and 60 seconds at 60 °C. To determine the changes in gene expression levels, the $2^{-\Delta\Delta Ct}$ Method was employed ($\Delta Ct = Ct^{\text{ gene}} - Ct^{\text{ control}}$). All samples were tested in triplicate for accuracy. The sequences of designed primers are shown in Table 1.

Enzyme-Linked Immunosorbent Assay

After the exposure of cells to various stimuli, culture supernatants were harvested and the levels of IL-10 and TNF-α were assayed by using sandwich enzyme-linked immunosorbent assay (ELISA) kits (KPG, Kerman, Iran) according to the manufacturer's instructions.

Flow Cytometry Analysis

Treated cells were harvested and washed three times with PBS for direct immunofluorescence staining. PE-conjugated anti-CD11b (BioLegend, San Diego, USA) antibody was used as a marker to evaluate THP-1 cell line differentiated MQs. MQs were stained with FITC-CD80 (BioLegend, San Diego, USA) and PerCP- eFlour 710 conjugated anti-CD206 (BD bioscience, USA) antibodies to evaluate the percentages of M1 and M2 MQs, respectively.[7] The data were analyzed by FlowJo software v10 (FlowJo LCC, Ashland, OR, USA).

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism v8 (GraphPad Software, San Diego, CA, USA). Two groups were compared by Student's t-test, and one-way ANOVA was applied for comparisons between multiple experimental groups. *P* values were considered significant at less than 0.05.

Table 1. Sequences for Forward and Reverse Primers Used to Measure Genes Expression by RT-PC

Gene Symbol	Primer Sequence
IL-6	F:5'- CTGGATTCAATGAGGAGACTTGC -3' R:5'- TCAAATCTGTTCTGGAGGTACTCTAGG -3'
TGF-β	F:5'- CAATTCCTGGCGATACCTCAG -3' R:5'- GCACAACTCCGGTGACATCAA-3'
GAPDH	F:5'- AAGAAGGCATGCACAGCTCA -3' R:5'- AAGTGGGTGCAGCTGTTCTC -3'

Results

mRNA Expression of IL-6 and TGF-β

Our results showed that the mRNA expression level of IL-6 increased significantly in MQs treated with gliadin ($P<0.0001$) and in MQs treated with a combination of PBM and *A. muciniphila* ($P=0.004$) relative to untreated MQs. However, the expression of IL-6 was reduced when gliadin stimulated MQs were treated with PBM irradiation for 24 hours compared to the cells that were only exposed to gliadin ($P=0.001$) (Figure 1A). The mRNA level of TGF-β, which decreased in gliadin stimulated cells rather than in untreated cells ($P=0.001$), increased when gliadin-stimulated MQs were treated with both PBM irradiation ($P<0.0001$) and *A. muciniphila,* along with PBM irradiation ($P=0.01$) for the following 24 hrs. In comparison to the cells that were only stimulated by gliadin (Figure 1B).

Protein Expression of IL-10 and TNF-α

According to the results, the treatment of THP-1 derived MQs with gliadin and *A. Muciniphila,* along with PBM induced the secretion of TNF- α and IL-10 ($P<0.0001$). Moreover, PBM irradiation and a combination of PBM irradiation and *A. muciniphila* could significantly increase both IL-10 and TNF-α levels in gliadin stimulated MQs in comparison to gliadin-triggered MQs ($P<0.0001$) (Figure 2).

Flow Cytometry

The flow cytometry technique was used to characterize the effect of gliadin, PBMT and *A. muciniphila* on the MQ cells phenotype. The levels of CD80, as an M1 specific marker, and the levels of CD206, as an M2 specific marker, were evaluated. Our results showed that the incubation of MQs with gliadin induced an increase in the percentage of M1 phenotype compared to the untreated group (Figure 3).

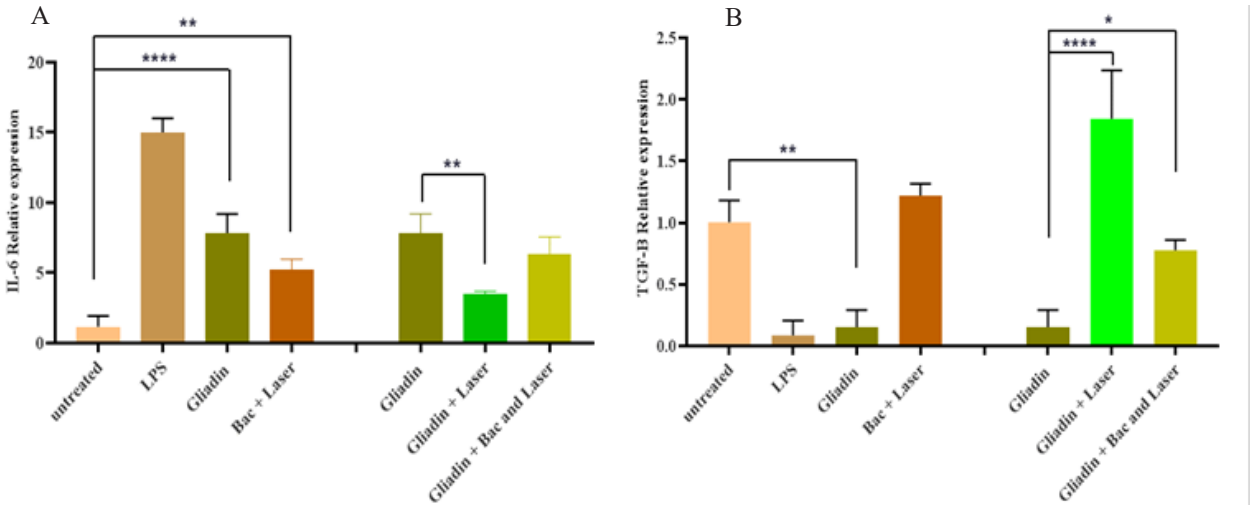

Figure 1. mRNA Expression Level of (A) IL-6 and (B) TGF-β in Different Study Groups. (*$P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$). *Bac, A. Muciniphila*; LPS, lipopolysaccharide

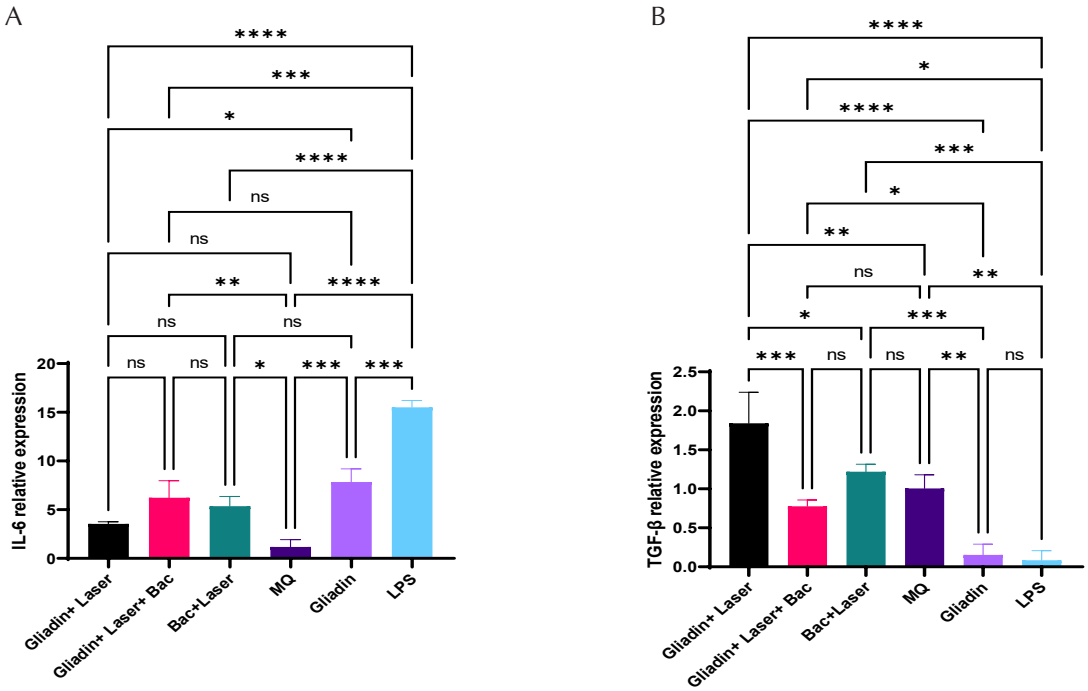

Figure 2. Protein Expression of (A) TNF-α and (B) IL-10 in Different Study Groups. (*$P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$). *Bac, A. Muciniphila*; LPS, lipopolysaccharide

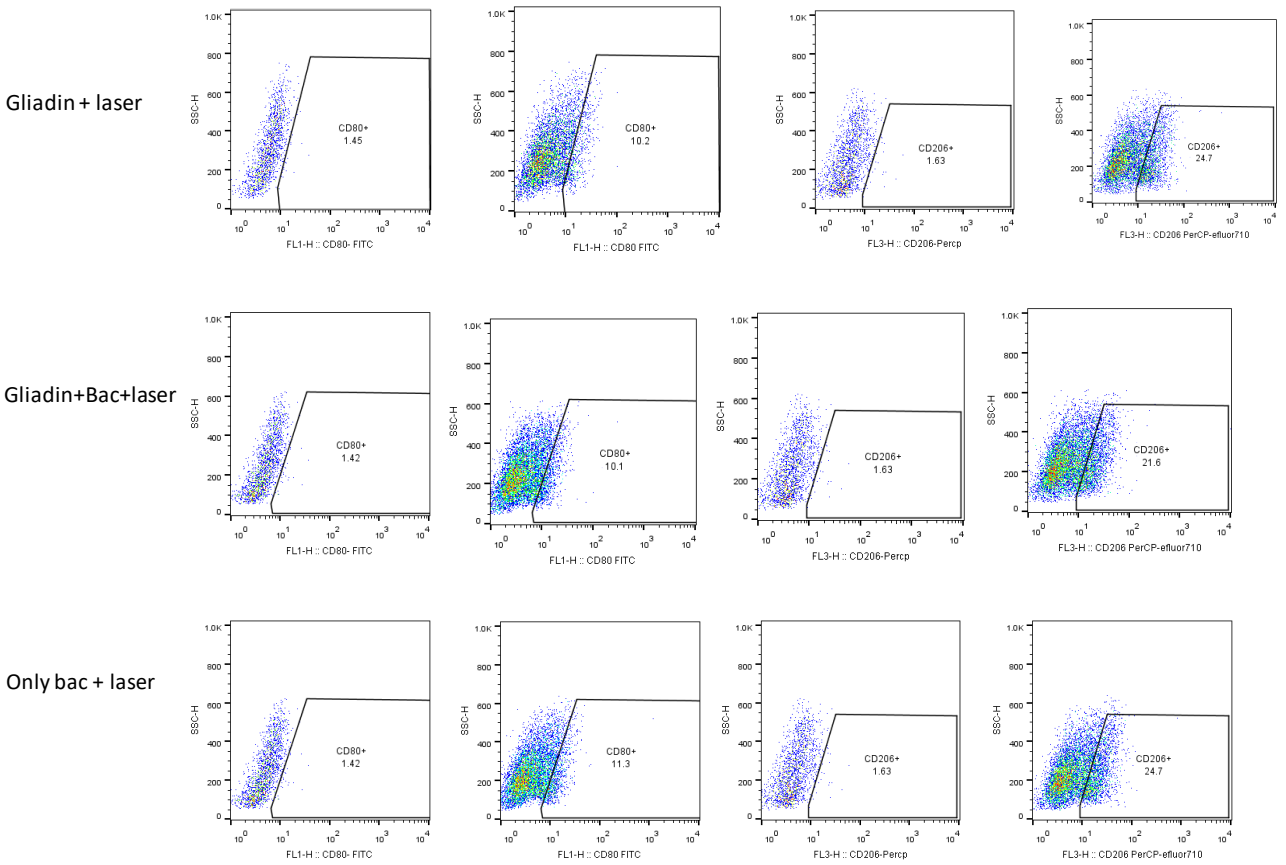

Figure 3. PBM (1 J/cm² of 810 nm) and PBMT+*A. muciniphila* increase the presence of M2 macrophages. Flow cytometry analysis of MQs treated with *A. Muciniphila* and gliadin shows a significant decrease in the number of M1 MQ cells compared with the gliadin group. AK+Gli, *A. Muciniphila* and gliadin; Gli+AK, gliadin and *A. Muciniphila;* AK, *A. Muciniphila;* Gli, gliadin; LPS, lipopolysaccharide. (**$P<0.01$, **** $P<0.0001$). Student's t-test was used to make comparisons between groups.

Moreover, the treatment of gliadin-stimulated MQs with PBMT and PBMT, along with A. *muciniphila,* induced an upregulation in the percentage of cells expressing CD206 (M2 phenotype) relative to the group treated with gliadin alone (Figure 3). Furthermore, the treatment of THP-1 derived MQs with PBMT and PBM, along with *A. Muciniphila,* also resulted in an increased percentage of CD206 + cells compared to untreated cells.

Discussion

The role of the innate immune system in the pathogenesis of CD has been recognized over the years [23]. MQs are the main innate immune response mediators and play a major role in the pathogenesis of CD. These cells have both pro- and anti-inflammatory phenotypes that can be converted into each other in response to microenvironment stimuli. This characteristic has made MQs a good therapeutic target for some immune disorders like CD.[24] The primary aim of this study was to investigate the potential synergistic effects of PBM and *A. muciniphila* on promoting the polarization of M2 macrophages in a gliadin-stimulated inflammatory model. Given the complex interplay between MQ polarization states and inflammatory responses in CD, our study aimed to elucidate how PBM and *A. muciniphila*, individually and in combination, could modulate the phenotypic switch of MQs from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype. By examining the molecular and cellular responses of THP-1 derived MQs to these treatments, we sought to provide insights into novel therapeutic approaches for managing inflammatory conditions associated with CD.

Studies have shown that gliadin can induce an inflammatory immune response in murine MQs or MQs derived from human monocyte cell lines.[25] Moreover, it has been reported that the inflammatory effects of gliadin, along with the irritation of the intestinal microenvironment, can polarize MQs toward the inflammatory M1-like phenotype. This effect has been observed not only in MQs derived from patients with CD but also in MQs of healthy individuals. Additionally, MQs derived from cell lines have shown this response to environmental stimuli like gliadin treatment.[26,27] Consistent with these reports, the present study observed that gliadin treatment of THP-1 derived MQs induced M1 phenotype in MQs, as evidenced by increased levels of CD80, IL-6, TNF-α and reduced levels of TGF-β.[24,28,29]

One of the essential factors that affect the regulation of immune responses is the intestinal microbiota profile,[19] and there is evidence showing the positive effects of *A. muciniphila* on regulating inflammatory responses.[20] Previous clinical research has revealed *A. muciniphila* is one of the beneficial gut microbiomes by increasing mitochondrial oxidation and bile acid metabolism in the gut-liver axis.[30] Furthermore, several studies have shown that PBM, a form of light therapy with therapeutic effects on living tissues at wavelengths of 810 nm, can inhibit inflammation. In fact, it has been confirmed that PBM has a therapeutic effect on inflammation and metabolic disorders.[31,32]

In the present study, it was found that PBM therapy alone and in combination with *A. muciniphila* could increase CD206 expression, which is the M2 MQ phenotype marker. The inhibitory effect of PBM on IL-6 expression in gliadin stimulated MQs and the stimulatory effects of both PBM and *A. muciniphila*, along with PBM, on TGF-β and IL-10 expression by gliadin-triggered MQs were also observed in this study. Moreover, *A. muciniphila*, along with PBM, could induce IL-10 expression in untreated THP-1 derived MQs. Incorporating a detailed analysis of the disparities in the expression levels of pro- and anti-inflammatory cytokines between the PBM + *A. muciniphila*-treated group and the PBM-treated MQs could offer valuable insights into the immunomodulatory effects of the combined therapy approach.

Souza NHC et al. indicated that PBMT at both 660 and 810 nm can modify the MQ phenotypic profile during the muscle repair process.[17] Their study indicated that the PBMT at 810 nm led to a decrease in IL6, TNFα and CD68 + MQs (M1 phenotype), accompanied by an increase in the number of CD206 + MQs (M2 phenotype) and the expression of TGF-β mRNA.[17] In addition, Zhang et al showed that 810 nm irradiation decreased the expression of M1 MQ-specific markers and increased the expression of M2 MQ-related markers.[33] Song et al, in an animal study, indicated that PBMT in rats with spinal cord injury induced the M2 phenotype of MQs.[34] Accordingly, Alves and teammates showed a statistically significant reduction in IL-1β, IL-6 and TNFα levels after low-level laser therapy and inhibition of inflammatory cell proliferation.[35] In a study conducted by Mulhall et al. in a mouse model of experimental periodontitis, it was shown that *A. muciniphila* could change macrophages polarization toward an M2 phenotype and induce IL-10 expression.[21] Keshavarz Azizi Raftar et al. also in their recent *in vivo* study showed that live and pasteurized *A. muciniphila* could successfully maintain homeostasis in colon tissue and increase the RNA level of tight junction proteins.[36] Ashrafian et al in their study showed the positive effect of alive and pasteurized *A. muciniphila* on immune response-related genes in the Caco-2 cell line.[36,37] These findings collectively support the therapeutic potential of PBM therapy and *A. muciniphila* usage in controlling celiac disease.

Moreover, we found that when THP-1 derived MQs were treated with *A. muciniphila* and PBM together, the IL-6 mRNA and TNF-α protein levels increased relative to untreated cells. It may indicate that the anti-inflammatory effect of these agents occurs following any inflammatory stimuli. However, there are reports indicating that TNF-α

can act as an anti-inflammatory agent, which may justify the increased expression level of this cytokine in response to *A. muciniphila* and PBM treatments of untreated THP-1 derived and gliadin stimulated MQs. The increased IL-10 levels in response to gliadin stimulation of MQs might be a sign of immune signals attempting to limit inflammation, which needs to be considered in further evaluations.

Although the data described above strongly suggest that PBM can modulate MQ phenotypes and inflammation after an acute injury followed by gliadin in cell culture with celiac-like condition, further analyses involving *in vivo* methods and, especially, human studies are essential for gaining a better understanding of the role of this therapeutic tool in patients with CD.

Exploring the implications of PBM irradiation and laser therapy within the realm of CD presents a compelling avenue for advancing therapeutic strategies. To address this suggestion, further elucidation of the specific mechanisms by which PBM and laser therapy exert their immunomodulatory effects in the context of CD is warranted. By delving into the intricate interplay between light-based therapies and the pathophysiology of CD, researchers can potentially unveil novel insights that may revolutionize treatment approaches for this autoimmune condition.

There are some limitations that need to be addressed. One major limitation is the absence of *in vivo* studies to confirm the effectiveness and safety of the interventions in a more complex biological system. Cell culture conditions may not fully represent the dynamic interactions and responses that occur within the human body; thus, the extrapolation of these findings to real-world clinical settings should be done cautiously. Additionally, since CD is a multifactorial disorder influenced by genetic, environmental, and immunological factors, the isolated effects of PBM and *A. muciniphila* observed in this study may not fully capture the complexity of the disease pathology. Therefore, further research involving animal models and eventually human clinical trials is necessary to validate the findings and determine the clinical relevance of these interventions in the management of CD.

Conclusion

The result of this study showed the potential of PBMT with an 810nm wavelength and *A. muciniphila* (1.5×10^8 CFU/mL) in modulating inflammatory responses and promoting MQs polarization, offering promising insights for the exploration of novel therapeutic approaches for CD. More investigations are needed to validate and build upon the results obtained in this study. Future studies could delve deeper into the mechanisms underlying the interactions between PBM, *A. muciniphila*, and MQs, and explore their therapeutic potential in *in vivo* models and clinical settings. These avenues of research not only have the potential to broaden our understanding of CD pathogenesis but also hold promise for the development of targeted therapeutic strategies with enhanced efficacy and safety profiles.

Authors' Contribution

Data curation: Somayeh Jahani Sherafat
Formal analysis: Zahra Razzaghi.
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Resources: Somayeh Jahani-Sherafat, Sara Mollaghaei, Nastaran Asri, Mohammad Rostami-Nejad.
Software: Zahra Razzaghi.
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Validation: Zahra Razzaghi.
Writing–original draft: Somayeh Jahani-Sherafat.
Writing–review & editing: Nastaran Asri, Mostafa Rezaei Tavirani, Kaveh Baghaei, Mohammad Rostami-Nejad.

Competing Interests

No potential conflict of interest was reported by the authors.

Ethical Approval

The study was approved by the Ethics Committee of Shahid Behesh University of Medical Sciences (no. IR.SBMU.RETECH.REC.1399.1338).

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