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Fluorescent light energy in wound healing: when is a photon something more?

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ABSTRACT

Fluorescent Light Energy (FLE) is a unique form of photobiomodulation that stimulates healing, reduces inflammation, and alleviates pain. The system works by exciting a chromophore in a topical substrate, which emits FLE with a broad spectral range (~400-700 nm) that is delivered to the target tissue below. Results from *in vivo* and *in vitro* studies have shown FLE modulates inflammation via down-regulation of pro-inflammatory cytokines such as IL-6 and TNF-α, and stimulates mitochondria biogenesis[1].

A recent study showed FLE-stimulated cells responded more potently compared to cells treated with light from an LED light source ("Mimicking Lamp") designed to generate the same emission spectra and power intensity profile as FLE[2]. FLE-treated human dermal fibroblasts (HDF) experienced up-regulated collagen production, while a minor and non-significant effect was observed for the Mimicking Lamp-treated HDFs. These results suggest that photons generated by FLE either penetrate tissue differently or are absorbed differently compared to photons from a LED light source. Photonic properties of FLE that could impact tissue penetration or absorption may include polarity or coherency, leading to different cellular responses.

To investigate if light polarity may influence cellular responses to FLE stimulation, the present study applied linear and circular-polarizing filters to investigate the influence of FLE's polarity on immune parameters. The data suggest that FLE polarity contributes to its impact on biological systems. Furthermore, the immunemodulatory impact of FLE was investgated in a pilot study on a human *ex vivo* skin model suggesting that central myeloid immune surface markes are modulated by FLE.

Keywords: Fluorescence Biomodulation, Fluorescent Light Energy, Wound Healing, Light Polarity, Cellular Response

1. Introduction

Wound healing is an important physiological process that regenerates skin integrity after trauma resulting from either accident or intent procedure. When trauma occurs, a sequential cascade of molecular and cellular events is triggered to initiate tissue repair processes and regeneration. These events involve the three phases of wound healing: hemostasis/inflammatory reaction, cell proliferation, and tissue remodeling. These stages are not mutually exclusive, but rather overlap through time[1].

Photobiomodulation (PBM), discovered over 50 years ago, has been considered as a possible therapy for wound management. PBM describes the use of visible light to stimulate biological functions in a non-thermal and non-cytotoxic manner. Studies have demonstrated that PBM reduces inflammation, and stimulates healing and tissue repair[2,3]. Advances in understanding how PBM achieves its biological impact have identified endogenous chromophores that are widely expressed in different cell types, including skin cells, as well as in the extracellular matrix. Specifically, PBM has been

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demonstrated to directly activate endogenous chromophores (also known as non-visual photoreceptors) including: cytochrome C oxidase (CCO), a small hemeprotein that is associated with the mitochondria inner membrane and that is sensitive to red and NIR (~610-950 nm) light[4]; flavins, a family of cryptochrome proteins that are involved in the repair of DNA and that are sensitive to blue (~410-500 nm) light[5]; opsins, a family of proteins that are able to modulate calcium channels, thereby impacting intra-cellular calcium levels and that respond to blue and green (~410-550 nm) light[6].

Fluorescence biomodulation (FB), a form of PBM that uniquely employs fluorescence light energy (FLE), has been demonstrated to stimulate healing of both acute and chronic wounds[7,8]. Studies have demonstrated that acute incisional wounds have reduced inflammation, as well as more physiologic re-epithelization and collagen remodeling, resulting in better quality and less visible scars[9,10,11].

The Klox FLE System comprises two components: a light source composed of blue light emitting diodes (LEDs; single peak wavelength between 440 and 460 nm) and a chromophore-containing topical substrate. The topical FLE substrate is an amorphous hydrogel which delivers photonic energy in the form of fluorescence.

A preliminary study suggests that delivery of FLE to the tissue generates more beneficial cellular processes compared with cells and tissues directly illuminated with a direct light source alone, including a direct light source with spectra that matches the FLE spectra, resulting in higher cell viability and less inflammation[12]. Thus, FLE photons appear to induce a different impact on healing compared with traditional PBM. The effects of light on tissue are due to various degrees of absorption of electromagnetic radiation, however, different characteristics of the radiant fluence delivered to the tissue could impact the degree of absorption and therefore the biological impact. Various light related parameters such as power density, monochromatism, coherence, and polarization may impact penetration or absorption of photons.

Polarized light in particular has been utilized for a number of clinical applications including wound healing[13]. Although its exact mechanism of action remains unknown, its advantageous effects over scattered light could be due to its higher capacity to penetrate skin, and thus to reach deeper tissues involved in wound healing. Some *in vitro* studies indicate that polarized light increases fibroblast proliferation and expression of type 1 procollagen, which is essential for the process of wound healing[14]. Furthermore, biological tissues, such as the cornea and the skin, contain near-order light scatterers such as collagen fibrils that could also affect polarization properties of the penetrating light[15].

In the present study, we examined whether altering the polarization state of FLE delivered to *in vitro* cells (fibroblasts) could change production of biological markers involved in healing. These analyses suggest that the biological impact of FLE on the inflammatory phase of wound healing is partly associated with its polarization properties, as measured by IL-6 production from fibroblasts.

2. Material and methods

2.1 Fluorescent Light Energy (FLE) systems:

FLE Systems consist of a multi-LED lamp and a topical photoconverter substrate that has a thickness of 2 mm. The multi-LED lamp delivers non-coherent blue light with a single peak wavelength between 440 and 460 nm. The power density is between 110 and 150 mW/cm^2 at 5 cm. The FLE substrate contains a chromophore embedded in a medium appropriate for the therapeutic application. The FLE substrate is a photoconverter that absorbs some of the light from the blue-emitting multi-LED lamp and, through a Stokes shift in the illuminated chromophore, emits FLE in the range of approximately 510-700 nm.

2.2 Light source Experiments:

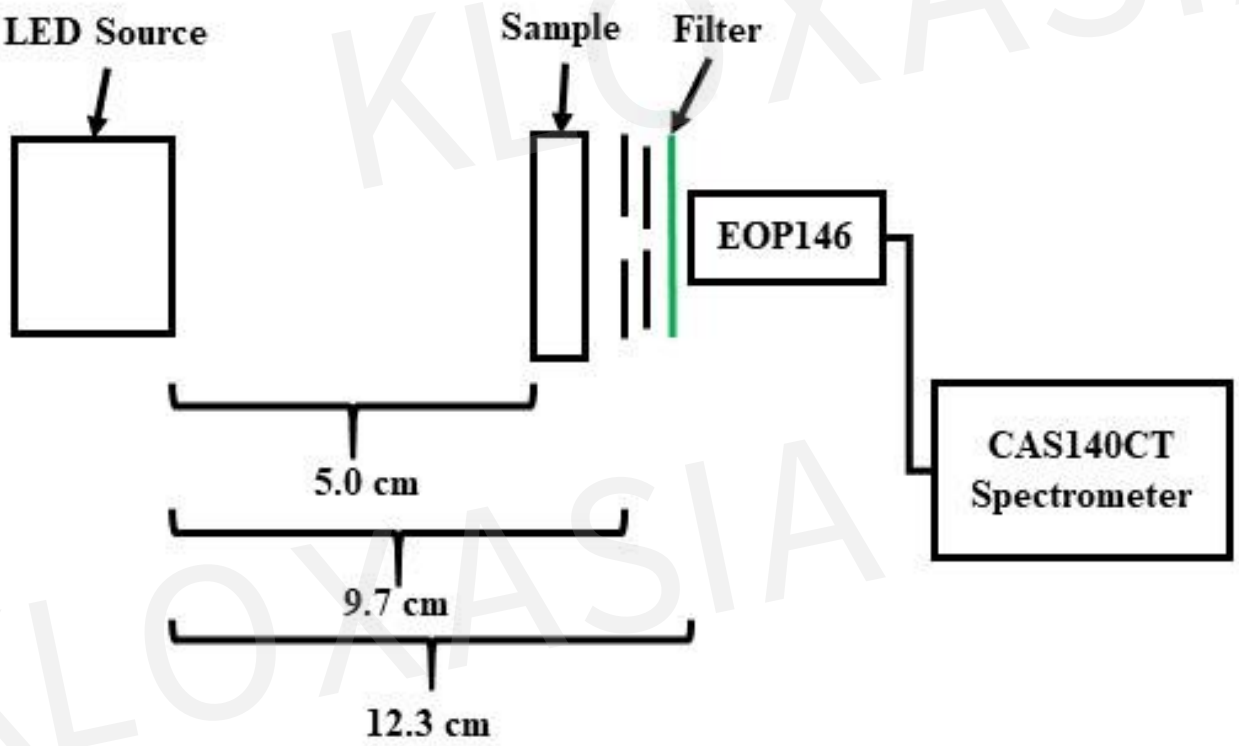
2.2.1 Source of polarized light

We applied a linear polarizing filter or a right handed circular polarizing filter (Edmund Optics, Barrington, NJ, USA) to the FLE substrate. As these filters do not fully transmit the light, we adjusted the LED intensity in the control set (without filter) so that the energy level delivered to the cells in all groups was consistent (7.6 J/cm^2 ± 5%).

2.2.2 Spectra of polarized and non-polorized light

*Equipment used:*The following equipment was used in the experiment, a detector probe EOP146 was fiber coupled to a CAS140CT array spectroradiometer (from instrument systems), with a wavelength range of 357.8 nm to 832 nm. The light source used was a LED light source, using a blue LED with a peak wavelength of 447.6 nm.

Setup description: The detector probe was placed 12.3 cm away from the LED light source. The light from the source was baffled with two baffles ensuring that the only light hitting the probe was coming from the sample. The filter was placed to the right of the sample. The sample was placed 5 cm away from the light source, as shown in the setup below:



2.3 *In Vitro* Experiments:

2.3.1 Cell culture of human dermal fibroblasts

Normal human dermal fibroblast (HDFs) cultures were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured at 37°C and 5% CO_2 in fibroblast basal medium (phenol red-free), supplemented with Fibroblasts Growth Kit-Low serum (ATCC). Culture process was performed seeding HDFs in 12-well plastic plates and incubated for 5-6 hours prior inducing an *in vitro* inflammatory state with 20 ng/ml of pro-inflammatory cytokines IL-1α/β for 18 hours. The next day, the media was replaced with Phosphate Buffer Saline (PBS) during the illumination procedure and then fresh media containing IL-1α/β cocktail was added to continue with the inflammatory stimulus. The culture supernatant (SN) was collected at 6 hours and 24 hours after illumination with FLE system for cytokine analysis.

2.3.2 Analysis of mediators of healing from cell culture

Cytokine secretion: Concentrations of secreted IL-6 was measured in collected supernatants using the Quantikine enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). Absorbance at 450 nm was determined using the Synergy HT microplate reader (Biotek, Winooski, VT USA) and corrected for absorbance at 570 nm.

Cell viability: After collecting cell culture supernatants at 6 hours and 24 hours, a cell viability assay was performed, measuring the mitochondrial metabolic activity (XTT) (Invitrogen, Waltham, MA USA). Absorbance at 450 nm was determined using the Synergy HT microplate reader (Biotek, Winooski, VT USA) and corrected for absorbance at 570 nm.

2.4 *Ex vivo* Experiment:

Affymetrix GeneChip Human Genome Array on ex vivo human skin: Human *ex vivo* full-thickness scalp skin organ cultures were prepared by 4-mm punches and cultured as previously described[16]. Punches (samples) were divided into three groups – 1) untreated control, 2) LED, and 3) FLE – with 2 punches per group. Samples were placed 5 cm from the multi-LED lamp source and illuminated for 9 minutes.

Untreated control samples (group 1) were prepared immediately, while samples from groups 2 and 3 were prepared 24 hours after treatment. Half of each punch was stored in 500 uL RNAlater, overnight at 4°C. RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were transported on ice to Immunology, Frederiksberg, Copenhagen University, and stored at -80°C until analysis. Samples were analyzed by the Center for Genomic Medicine (Copenhagen University Hospital, Denmark) using a Human Gene 2.0 ST Array (includes lincRNA probes) microRNA expression Arrays (Affymetrix, Santa Clara, CA, USA). Raw data was analyzed as previously described[17].

2.5 Data analysis

Results are expressed as a mean ± SD. Normalized IL-6 levels were graphed as the fold change compared to the inflamed cells (stimulated) that were not illuminated (CTRL-ST). The significance of results was analyzed using the Student's *t*-test. Differences between the groups were considered as statistically significant at $*P < 0.05$, $**P < 0.005$, and $***P < 0.0005$. *Ex vivo* date is pilot data with limited replicates therefore no statistics were applied.

3 Results

3.3 Light spectra from multi-LED lamp, FLE and FLE + filters

To examine whether light polarity plays a role in FLE's promotion of biological effects, polarizing filters were applied when treating HDFs. The photonic spectra output from the multi-LED lamp was verified with or without polarizing filters (Fig.1A) as well as from the illuminated topical substrate with or without polarizing filters (Fig.1B). These measurements indicate a reduction in energy by both filters which was consistent across the entire spectrum, thus no alterations in specific wavelengths of the spectrum were observed. To compensate for the loss in energy power outlet from the lamp (adjusted samples) was increased to ensure the same intensity of the fluence spectra was delivered to all treated samples (Fig. 1C and D).

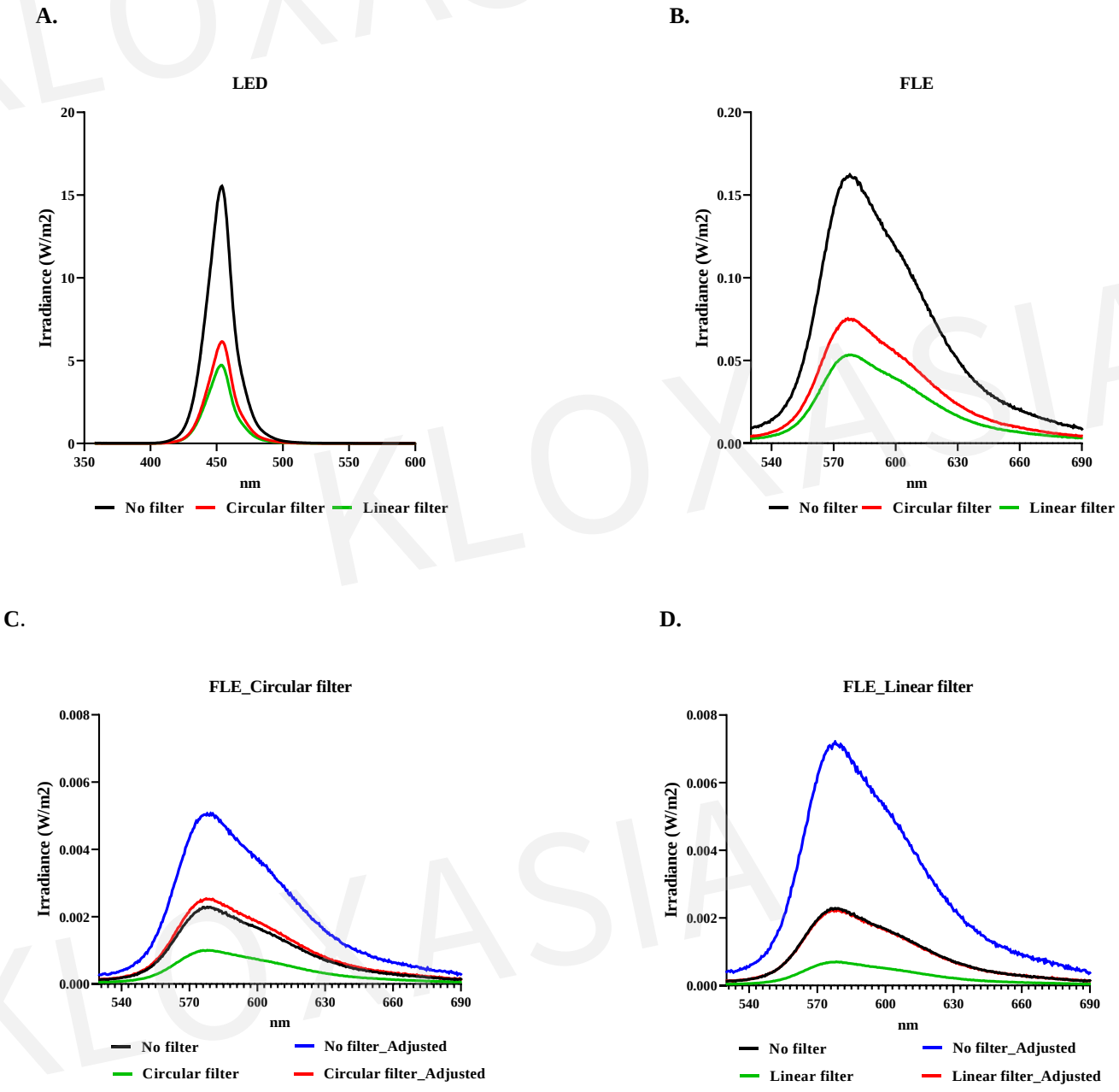


Figure 1. A. The spectra from the LED alone (black) or with polarizing filters (linear or circular: green or red, respectively). **B**. The spectra from FLE alone or filtered by linear or circular polarizers. **C**. The spectra from FLE alone or filtered by circular polarizing filter with and without adjustment of FLE power intensity to deliver the same fluence. Power outlet (adjusted samples) was increased by 43% for the circular filter. **D**. The spectra from FLE alone or filtered by linear polarizing filter with and without adjustment of FLE power intensity to deliver the same fluence. Power outlet (adjusted samples) was increased by 63% for the linear filter.

3.4 Effects of polarity on immune response: IL-6 secretion

We compared the secreted levels of IL-6 in HDFs at 6 and 24 hours after multi-LED +/- substrate (FLE treatment) with or without polarizing filters (Fig.2). FLE decreased IL-6 secretion by more than 50% within 24 hours compared with LED treatment alone, while in fibroblasts exposed to linearly or circularly polarized FLE this inhibitory effect of FLE on IL-6 secretion was significantly abrogated at 24 hours. When compared with FLE irradiated cells without filters, we saw a 63% increase with the circular filter (P=1.4e-4), and 150% increase in IL-6 secretion with the linear filter (P=2.58e-6). Thus the maximum effect of FLE on cellular responses appears to be dependent on the full FLE capacity and availability of non-polarized photons. In addition, polarizing the non-coherent blue light from the multi-LED lamp did not change the fibroblast response when compared to the multi-LED group with no filters.

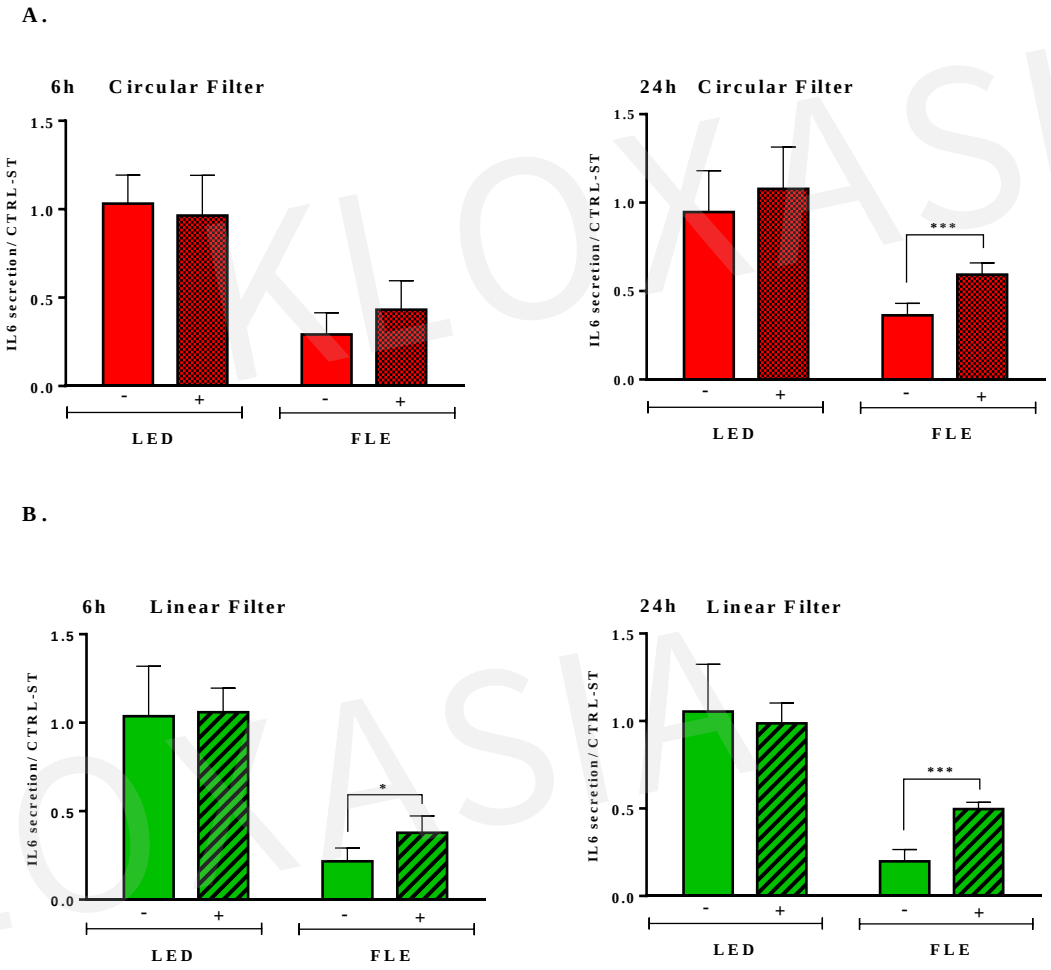


Figure 2. Polarizing filters attenuate the effect of FLE on IL-6 expression from inflamed HDFs. **A**. The application of a right handed circularly polarizing filter significantly decreases the effect of FLE on IL-6 secretion measured 24 hours after illumination. **B**. Similarly, a linearly polarizing filter significantly reduces FLE impact on IL-6 secretion from HDFs as measured 6 and 24 hours post illumination, while linear polarization of LED emitted blue light does not alter its biological effects.$*P < 0.05$, $**P < 0.005$, and $***P < 0.0005$, N=3. HDF cells were inflammatory stimulated using a cocktail of IL1-α, IL1-β. CTRL-ST: control stimulated.

3.3 Myeloid cellular surface markers found to be modulated by FLE

Finally, we investigated (in a pilot study) the effect of FLE on a human *ex vivo* skin model screening for immune modulating factors regulated by FLE. Interestingly, several myeloid immune surface markers were found to be down-regulated on the transcriptional level by FLE treatment (Fig. 3). Langerin (CD207) is expressed on Langerhans cells (LCs) residing in the epidermal layer of the skin, whereas CD1 molecules are markers found on both LCs as well as other dermal Dendritic cells (DDCs)[18,19]. CD1 molecules are major histocompatibility complex I (MHC-I) like molecules that present lipids to responding T-cell[20]. CD209 is expressed on dendritic-like macrophages and has been described to facilitate recognition of *Cutibacterium acnes* (formerly *Propionibacterium acnes*)[21]. These results suggest that FLE treatment target several myeloid cell types and pathways essential for regulating inflammation.

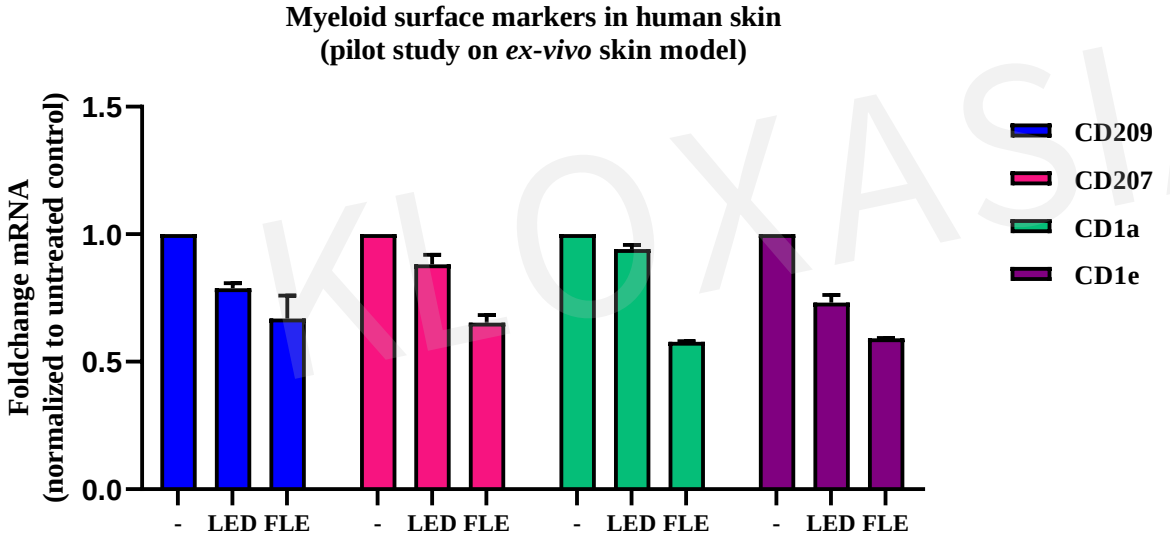


Figure 3. FLE treatment down-regulates myeloid cell surface markers in human skin. *Ex vivo* human skin punches was treated with LED (lamp minus substrate), FLE (lamp plus substrate, or prepared directly after excision without treatment (-). Transcriptional regulation was analysed in total RNA (from full thickness skin samples) 24 hours post illumination.

4 DISCUSSION

Healing of wounds is one of the most complex biological events due to the interplay of different tissue structures and a large number of resident and infiltrating cell types. The process involves soluble mediators, extracellular matrix components, resident cells (keratinocytes, fibroblasts), and resident and infiltrating leukocytes subtypes (e.g. myeloid cells), which participate differentially in the classically defined three phases of wound healing: inflammation, tissue formation, and tissue remodeling[22,23].

Our previous work using *in vitro* and *in vivo* models demonstrated that FLE positively impacts all phases of healing by modulating the inflammatory cytokines, increasing growth factors and stimulating collagen production[24]. The present study investigated the role of light polarization on biological effects of FLE, focusing on IL-6 expression from inflamed HDF cells as an inflammatory marker involved in wound healing. Several reports have suggested that low-level lasers and polarized light may accelerate wound healing. Polarized light has been found to trigger the cellular and humoral defences of the human organism[25]. Our current study, which applies linear and circular polarizing filters, suggests that in addition to their unique wavelength, the photons emitted from the FLE substrate carry other properties that are important to maximize the cellular response. Results also highlight that the unique and complete FLE spectrum is critical for inciting

the biological impacts, as polarizing non-coherent blue light from the multi-LED lamp alone did not change the response by the HDF cells, and only minor effects were observed on central myeloid surface markers regulated in human skin.

Biological tissues are generally composed of oriented structures, which quickly depolarize transmitted light. However certain tissues and cellular structures, such as eye tissues, superficial skin layers, and cell monolayers, are composed of highly organized bipolar lipid layers in their cell membrane allowing a certain degree of light polarization to be transmitted[26]. Several studies report the effect of polarized light on both cells and the expression of physiologically important biomarkers[27,28,29]. The exact mechanism for the unique biological effects of polarized light remain elusive, although the superior penetration capacity of polarized compared to non-polarized light is a plausible hypothesis[30]. Further, it could also be speculated that interaction of polarized light with the bipolar lipid layer of cell and organelle membranes could induce conformation modulation, and subsequently impact activity of the embedded proteins responsible for many cellular activities, including energy production and signal transduction[26].

To further support this study, it will be valuable to test cellular responses in both *in vitro* and *ex vivo* models to the light produced by a mimicking lamp compared to FLE wavelength and intensity but carrying various polarization features. Results of the present study open the door for better understanding the mechanism of action for significant effects of FLE observed in clinical studies[31]. In addition, accurate measurements of polarized light emitted from FLE substrate, as data suggests, will help further develop FLE technology for optimal clinical outcomes.

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