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Photobiomodulation in dermatology : role of Nrf2 in the anti-inflammatory response of visible light

Sara Salman

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Photobiomodulation en dermatologie :
rôle du facteur Nrf2 dans la réponse
anti-inflammatoire cutanée de la lumière visible

*Photobiomodulation in dermatology: role of Nrf2 in the skin
anti-inflammatory response of visible light*

Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 569, Innovation thérapeutique : du fondamental à l'appliqué (ITFA)
Spécialité de doctorat : Toxicologie
Graduate School : Santé et médicaments. Référent : Faculté de pharmacie

Thèse préparée dans l'unité de recherche
Inflammation Microbiome Immunosurveillance (Université Paris-Saclay, Inserm),
sous la direction du Pr **Saadia Kerdine-Römer**, Professeure.

Thèse soutenue à Paris-Saclay, le 20 novembre 2023, par
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Titre : Photobiomodulation en dermatologie : rôle de Nrf2 dans la réponse anti-inflammatoire de la lumière visible

Mots clés : peau, kératinocytes, Nrf2, photobiomodulation, lumière visible, inflammation

Résumé :

La photobiomodulation (PBM) est une technologie en plein essor qui utilise de faibles niveaux d'énergie de lumière visible ou proche-infrarouge pour stimuler la régénération tissulaire, soulager la douleur et réduire l'inflammation. Elle est désormais un outil précieux en dermatologie cosmétique et médicale pour traiter de nombreuses affections cutanées. À ce jour, les mécanismes cellulaires et moléculaires responsables de l'effet anti-inflammatoire de la PBM n'ont pas été entièrement élucidés. L'objectif de cette thèse est d'étudier les mécanismes mis en jeu dans la réponse anti-inflammatoire cutanée induite par la PBM.

Dans ce travail de Thèse, nous avons adressé le rôle du facteur de transcription Nrf2 dans la réponse anti-inflammatoire à la lumière visible, vu que des études antérieures ont pu mettre en évidence le rôle clé de Nrf2 dans le contrôle de la composante inflammatoire cutanée, induite par des molécules allergisantes. Dans notre étude, nous avons utilisé un modèle de kératinocytes (KCs) humains primaires exposés au 2,4-dinitrochlorobenzène (DNCB), une molécule allergisante mimant un stress pro-inflammatoire. Grâce à des diodes électroluminescentes (LEDs), les KCs ont été exposés séparément à deux longueurs d'ondes : 660 nm (lumière rouge) et 520 nm (lumière verte). Nous avons montré que ces deux longueurs d'ondes permettent l'activation de Nrf2 dans les KCs stimulés. De plus, la réponse inflammatoire mesurée par l'expression des cytokines pro-inflammatoires telles que l'IL-6, l'IL-8 et le TNF- α , est significativement régulée par les deux types de lumières. L'approche par ARN d'interférence nous a permis de montrer que la régulation de la réponse inflammatoire par PBM est Nrf2-

dépendante. En effet, en absence de Nrf2, la lumière est incapable de moduler la composante inflammatoire induite par DNCB.

Pour approfondir notre compréhension des mécanismes anti-inflammatoires, nos recherches se sont concentrées sur le rôle du Récepteur Transitoire Vanilloïde 1 (TRPV1), un canal ionique sensible à la lumière. Dans ce même modèle de KCs, nous avons pu montrer que le blocage de TRPV1 par la capsazépine, inhibe les effets bénéfiques de la lumière verte, suggérant que TRPV1 est une cible cruciale par laquelle la lumière verte exerce son activité anti-inflammatoire dans les KCs. Nos résultats ont également montré que le canal TRPV1 est essentiel pour l'activation de la voie Nrf2 par la lumière verte dans des conditions inflammatoires induites par un stress chimique.

Afin de comprendre comment la lumière peut réguler le microenvironnement cutané, nous nous sommes intéressés aux cellules de Langerhans (LCs), cellules présentatrices d'antigène jouant un rôle crucial dans l'immunosurveillance cutanée. Dans des explants de peau humaine exposés au DNCB, nous avons montré que la lumière rouge régule l'activation des LCs en maintenant leur structure et dendricité, ainsi qu'en réduisant leur migration dans le derme.

Dans l'ensemble, nos résultats soutiennent la fonction régulatrice de la PBM en modulant des cellules clés dans l'épiderme. Elle inhibe la composante inflammatoire primaire des KCs, principalement via la voie Nrf2, et réduit l'activation des LCs. Ces résultats soulignent le potentiel prometteur de la PBM en tant que thérapie non invasive pour les maladies inflammatoires cutanées.

Title: Photobiomodulation in dermatology: role of Nrf2 in the skin anti-inflammatory response of visible light

Keywords: skin, keratinocytes, Nrf2, photobiomodulation, visible light, inflammation

Abstract:

Photobiomodulation (PBM) is a fast-growing technology using low levels of visible or near-infrared light to stimulate tissue healing, relieve pain, and reduce inflammation. It emerges as a valuable tool in cosmetic and medical dermatology for treating numerous skin conditions. To date, the mechanisms responsible for the anti-inflammatory effect of PBM have not been fully elucidated. The aim of this thesis is to investigate the molecular mechanisms involved in the cutaneous anti-inflammatory response induced by PBM.

In this work, we addressed the role of the transcription factor Nrf2 in the PBM anti-inflammatory response, as previous studies have highlighted its key role in regulating skin inflammation in response to chemical molecules. In our study, we used a model of primary human keratinocytes (KCs) challenged with 2,4-dinitrochlorobenzene (DNCB), a contact sensitizer that mimics pro-inflammatory stress. Using light-emitting diodes (LEDs), the KCs were illuminated with two wavelengths separately: 660 nm (red light) and 520 nm (green light). Both wavelengths enhanced Nrf2 activation in stimulated KCs. Additionally, the inflammatory response measured by the expression of pro-inflammatory cytokines such as IL-6, IL-8, and TNF- α was regulated by both lights. RNA interference approach demonstrated that the regulation of the inflammatory response by PBM is Nrf2-dependent, as the absence of Nrf2 prevented light from modulat-

ing the inflammatory response induced by DNCB.

To gain deeper insights into the less-explored mechanisms involved in the green light response, our research focused on investigating the role of Transient Receptor Vanilloid 1 (TRPV1), a light-gated ion channel. In the same primary KC model, we showed that blocking TRPV1 with capsazepine inhibits the beneficial effects of green light, suggesting that TRPV1 is a critical target through which green light mediates its therapeutic activity reducing inflammation in KCs. Interestingly, our findings showed that TRPV1 is essential for the activation of Nrf2 pathway by the green light under inflammatory conditions induced by chemical stress.

To understand how visible light can regulate the skin microenvironment, we investigated Langerhans cells (LCs), antigen-presenting cells that play a crucial role in cutaneous immunosurveillance. In human skin explants exposed to DNCB, we showed that red light regulates the activation of LCs by maintaining their structure and dendricity, and reducing their migration into the dermis.

Overall, our results support the regulatory function of PBM modulating key cells in the epidermis. It inhibits the primary inflammatory response induced in KCs, mainly through the Nrf2 pathway, and reduces LC activation. These findings highlight the potential of PBM as a promising non-invasive therapy for skin-related inflammatory diseases.

Je tiens à exprimer ma gratitude envers le **Pr. Franck Morel** et le **Dr. Nicolas Lebonvallet** pour avoir gentiment accepté d'être rapporteurs de cette thèse. Je les remercie d'autant plus d'avoir porté un intérêt à mon sujet et pour avoir pris sur leur temps pour évaluer la qualité du manuscrit.

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ABBREVIATIONS

A

ACD: Allergic contact dermatitis

AD: Atopic dermatitis

AhR: Aryl Hydrocarbon Receptor

AMP: Antimicrobial peptide

ARE: Antioxidant Response Element

ATP: Adenosine triphosphate

B

DNCB: 2,4-dinitrochlorobenzene

C

CCO: Cytochrome C Oxidase

CHS: Contact hypersensitivity

CpG: Cytosine-phosphate-Guanine

CPZ: Capsazepine

Cul3: Cullin-3

CYPs: Cytochrome P450

D

DAMP: Damage-Associated Molecular Pattern

DC: Dendritic cell

dDC: Dermal dendritic cell

E

ECM: Extracellular matrix

G

GCLC: Glutamate-cysteine ligase catalytic

GSH: Glutathione

H

HO1: Heme oxygenase 1

HSP: Heat-shock protein

I

IFN: Interferon

IL-1 β : Interleukin 1 β

ILC: Innate lymphoid cell

K

KCs: Keratinocytes

Keap1: Kelch-like ECH-associating protein 1

L

LCs: Langerhans cells

M

MAPK: Mitogen-activated protein kinase

MHC: Major histocompatibility complex

MMP: Matrix metalloproteinase

N

Neh: Nrf2-ECH homology

NF- κ B: Nuclear Factor-kappa B

NMF: Natural Moisturizing Factor

NQO1: NAD(P)H quinone oxidoreductase 1

NLRP: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing

Nrf2: Nuclear factor erythroid-2-related factor 2

P

PAMP: Pathogen-associated molecular pattern

PBM: Photobiomodulation

PBMT: Photobiomodulation Therapy

pDC: Plasmacytoid dendritic cell

PDT: Photodynamic Therapy

PRR: Pattern recognition receptor

R

RCTs: Randomized Controlled Trials

ROS: Reactive oxygen species

S

SFN: Sulforaphane

T

Tc: T cytotoxic cell

TGF- β : Transforming growth factor-beta

Th: T helper cell

TLR: Toll-like receptor

TNF- α : Tumor necrosis factor-alpha

Treg: Regulatory T cells

Trm cell: Tissue-Resident Memory T cell

TRPV1: Transient Receptor Potential Vanilloid 1

U

UV: Ultraviolet radiation

Picture a world where a simple beam of light has the power to heal.

That's where my PhD journey begins, exploring the field of Photobiomodulation within a captivating context: the Skin.

PREFACE

PREFACE

Treating skin inflammation is paramount for managing various skin inflammatory diseases and conditions. As traditional pharmacotherapies often carry out several side effects or insufficient responses, there is an increasing need in cosmetic and medical dermatology to explore safer and cost-effective approaches to address skin inflammation. One emerging area of investigation is Photobiomodulation (PBM), using the benefits of “low levels” of visible light to promote therapeutic outcomes, including the control of inflammation.

Unlike traditional medical treatments that start with experimental research and then gradually progress into clinical trials and applications, PBM has taken a somewhat unconventional path. It primarily began as a clinical practice in the early 2000s, using this therapy to address various medical and dermatological concerns. Its clinical success in tissue repair, as well as pain and inflammation management, prompted researchers to delve deeper into understanding its mechanisms. By elucidating how specific wavelengths of light interact with cells and tissues, we aimed to uncover some of the underlying signaling pathways, gene expressions, and cellular responses involved in PBM.

In this thesis, we will investigate how PBM affects our skin and helps with inflammation. We will particularly address the mechanisms of red and green lights in key skin cells, such as keratinocytes and Langerhans cells, focusing on the role of the Nrf2 transcription factor in this response.

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LITERATURE REVIEW

I. PHYSIOLOGY AND PATHOLOGY OF THE SKIN

The skin is a dynamic integumentary system that interacts with the environment and maintains internal homeostasis. It exhibits multiple functions, including sensory perception, energy storage, and acting as a protective barrier. The sensory function lets the skin detect and respond to environmental stimuli by perceiving temperature, pressure, touch, and pain. As the primary interface between our body and the external environment, the skin mainly acts as the first line of defense against chemicals, xenobiotics, physical stresses, and exogenous pathogens through its different barriers: the chemical, physical, immune, and microbial barrier (Proksch *et al.*, 2008; Eyerich *et al.*, 2018). The skin also supports endocrine functions, immune responses, and metabolic processes of endogenous and exogenous compounds. Additionally, the skin helps prevent water loss and regulates body temperature to maintain homeostasis (McLafferty *et al.*, 2012; Kim and Dao, 2022). The skin's unique architecture and the various components of this multilayered system work in conjunction to effectively fulfill these diverse functions. However, the skin is also prone to a range of inflammatory pathologies, which can result from immunological disruptions, genetic and environmental factors.

1. STRUCTURE OF THE SKIN

The skin comprises three main overlying tissues: the epidermis, the dermis, and the subcutaneous tissue, also known as the hypodermis or superficial fascia (Figure 1). The epidermis is the most external layer of the skin and consists of several sublayers of stratified squamous epithelial cells. Its principal function is the protection of the organism against external aggressions. The dermis is the middle layer, composed of connective fibrous tissue containing blood vessels, nerves, hair follicles, and sweat glands. The subcutaneous tissue is the deepest layer of the skin and comprises loose connective and adipose tissues that attach the skin to underlying structures such as muscles and bones (Gilaberte *et al.*, 2016; Kim and Dao, 2022) (Figure 1).

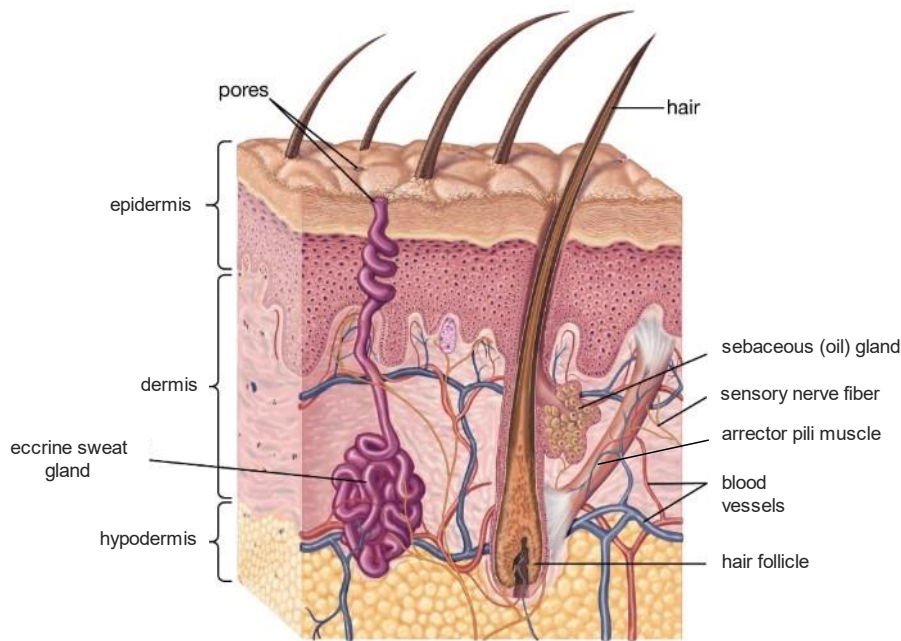


Figure 1. Cross-section of the skin tissue. The skin comprises three main overlapping layers. The epidermis, the most external layer, mainly consists of tightly packed epithelial cells. The dermis is composed of dense connective tissue that houses various structures. Beneath the dermis lies the hypodermis, composed of loose connective and fatty tissues. Adapted from *Encyclopædia Britannica, Inc., 2023*.

1.1. The epidermis

1.1.1. Epidermal cell populations

The epidermis is the skin's outermost layer, whose total thickness can range from 0.05 mm for thin skin (eyelid) to 1.5 mm for thick skin (palms and soles). It is formed by stratified epithelial sublayers composed of keratinocytes (KCs) (Wong *et al.*, 2016). The epidermis hosts some other cell populations such as melanocytes, Langerhans cells (LCs), Merkel cells, and skin-resident T cells, but KCs are by far the most predominant cell type, constituting more than 85% of the total epidermal cell population (Figure 2) (Gilaberte *et al.*, 2016).

Melanocytes, located in the deepest layer of the epidermis, are specialized cells playing a role in pigmentation through the synthesis and transfer of melanin (eumelanin and pheomelanin) to KCs in the form of vesicular structures called melanosomes. Melanin also effectively protects the skin against the harmful effects of ultraviolet (UV) radiation. Merkel cells are also found in the basal layer of the epidermis and are mechano-receptors responsible for detecting tactile stimuli and transmitting them to sensory nerves in the skin (McLafferty *et al.*, 2012; Yousef *et al.*, 2022). KCs and immune cells located in the epidermis, such as LCs and T cells, will be described later in this chapter (see section 2.2).

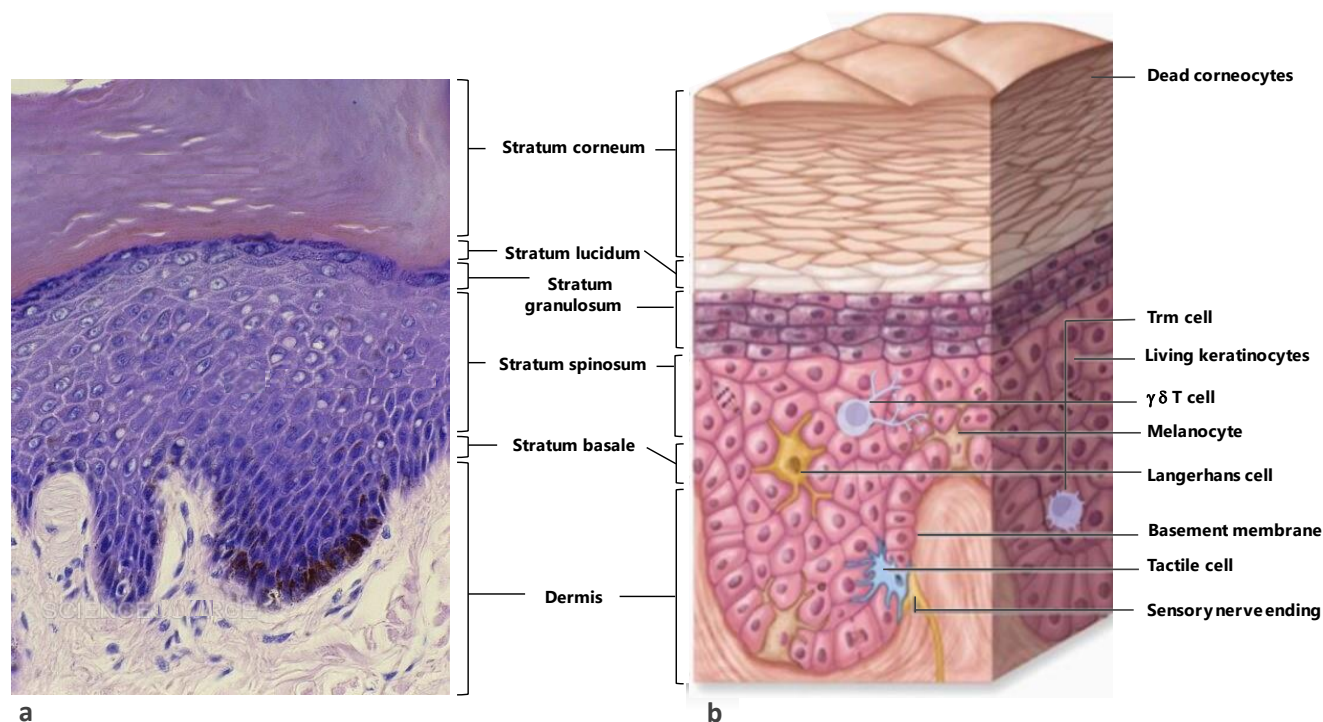


Figure 2. Layers and cell types of the human epidermis. (a) Light micrograph of a vertical cross-section through thick skin showing the sequence of five epidermal layers. x100. Hematoxylin- and eosin-stained human skin samples. (b) Schematic diagram illustrating the sequence of the epidermal layers hosting a range of epidermal cell populations. Trm: Tissue-resident memory T cell. Adapted from Science Source images ©2019 with BioRender.com

1.1.2. Epidermal ultrastructure and KCs differentiation

The epidermis is a stratified keratinized epithelium divided into layers, each with distinct structural and functional characteristics. It consists of four or five layers, depending on its anatomical location. Most of the skin is considered “thin skin” and composed of four layers. While the “thick skin” has a five-layered epidermis and is found on the soles of the feet and palms of the hands. From the depth to the surface, these layers are the *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum* (Figure 2). “Thin skin” lacks the *stratum lucidum* and has a thinner stratum corneum, making it more flexible than thick skin (Fernandez-Flores, 2015; Plotczyk and Higgins, 2019).

Epidermal stratification corresponds to the changes in morphology, gene expression pattern, and metabolism of the KCs when they migrate by differentiating from the depth to the surface of the epidermis. Each layer is characterized by different sets of proteins progressively expressed by KCs while differentiating, typically different types of keratin intermediate filaments (Figure 3). Keratins are fibrous proteins that form heterodimers, assemble into tough insoluble filaments, and

then aggregate into packed macro-fibril bundles to form a dense network at the upper layers, providing strength and protection to the skin (Pillai *et al.*, 2022).

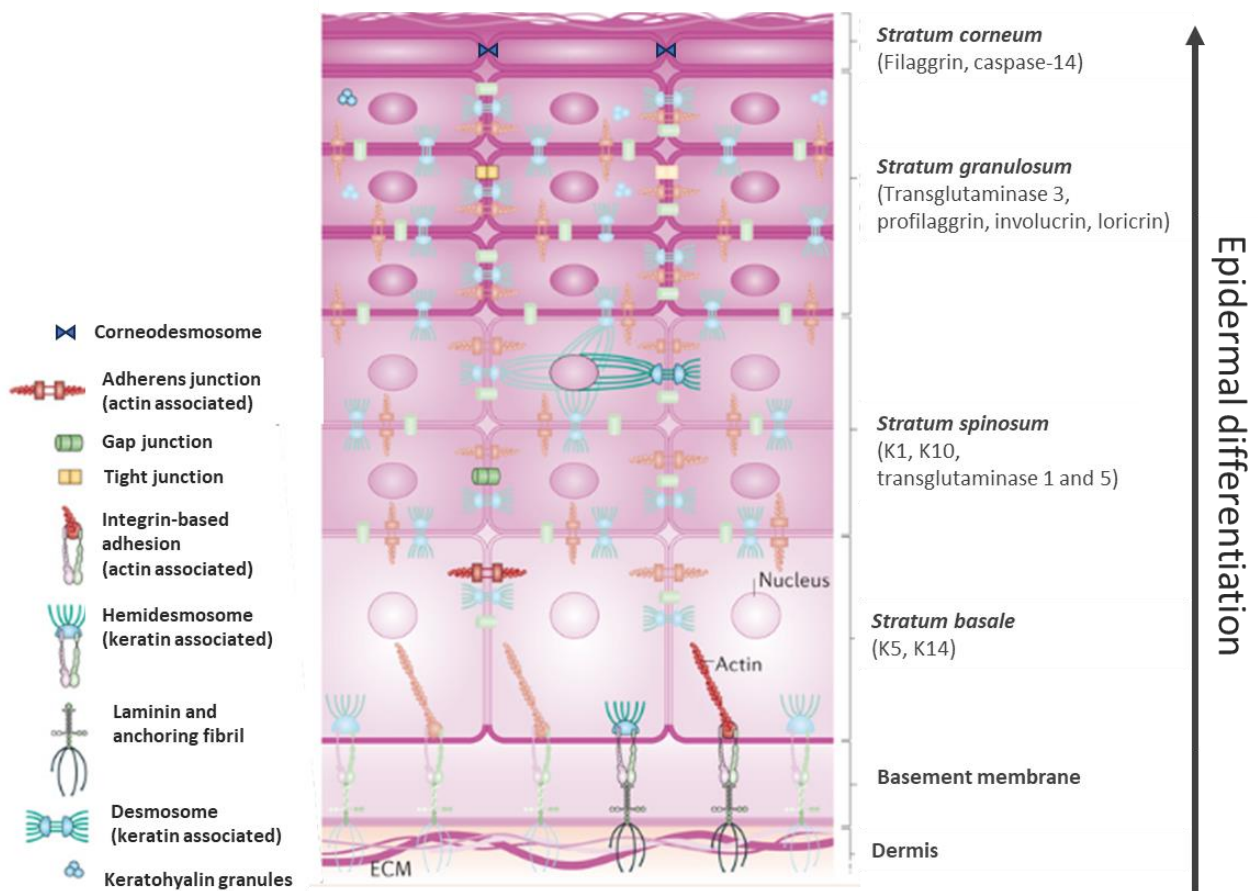


Figure 3. Basic epidermal structure and keratinocyte differentiation. The differentiation process (keratinization) of KCs migrating up through the different epidermal layers is accompanied by the expression of different constitutive proteins characteristic for each differentiation stage. To obtain a fully functional skin barrier, keratinocytes should be connected to each other by intercellular junctions that link intercellular contacts to the cytoskeleton. Adapted from Simpson *et al.*, 2011 and Chieosilapatham *et al.*, 2021.

The epidermis is a dynamic tissue in perpetual regeneration. KCs undergo a continual process of proliferation in the basal layer of the epidermis and differentiation while migrating upwards towards the skin surface before sloughing off as anucleate corneocytes and eventually dying. The constant thickness of the epidermis is maintained by the adequate balance between basal cell proliferation and corneocyte desquamation (Plotczyk and Higgins, 2019). The turnover process of the epidermis is essential for maintaining the integrity and function of the skin barrier and repairing any damage that may occur. The total renewal time of the epidermal turnover process is about 4 to 6 weeks in healthy young adult skin (Maeda, 2017).

- **Stratum basale**

The *stratum basale*, also known as the germinative layer, is the deepest layer of the epidermis. It is mainly composed of a single layer of cuboidal or columnar stem cells that constantly give rise to new KCs. These stem cells are mitotically active and responsible for the constant regeneration of the epidermis. Two models of cell division in basal cells coexist: the symmetrical division, allowing lateral expansion, and the asymmetrical division, leading to vertical expansion toward the upper layers (Lechler and Fuchs, 2005; Clayton *et al.*, 2007). The direction of expansion depends on the orientation of the mitotic spindles (Simpson *et al.*, 2011). Once detached from the basal membrane, KCs change their gene expression profile (Mack *et al.*, 2005; Eckhart *et al.*, 2013). During their maturation, KCs lose their proliferative potential in favor of a differentiation process called keratinization. This maturation is accompanied by a progressive flattening of KCs, which then form the spinous layer (*stratum spinosum*), the granular layer (*stratum granulosum*), up to the ultimate stage of keratinization, the cornified layer (*stratum corneum*).

Basal KCs are characterized by the presence of large nuclei and intermediate filaments of keratin, such as keratin 5 and keratin 14, called tonofilaments, which converge towards desmosomes and hemidesmosomes (Figure 3). Desmosomes are intercellular junctions ensuring cohesion with neighboring KCs, while hemidesmosomes ensure junctions with the basement membrane (basal lamina) at the dermo-epidermal junction. These junctions provide the epidermis with mechanical strength and resistance (Yousef *et al.*, 2022). It was shown that autoantibodies or bacterial toxins that selectively target desmosomal proteins, such as desmogleins, cause pemphigus, a potentially fatal blistering disease, or staphylococcal scalded-skin syndrome (bullous impetigo), respectively (Payne *et al.*, 2004).

- **Stratum spinosum**

The *stratum spinosum*, or squamous cell layer, located above the *stratum basale*, is composed of 8 to 10 layers of actively dividing polyhedral KCs characterized by their spiny appearance caused by the desmosomes between adjacent cells (Figure 3). The transition from the *stratum basale* to the *stratum spinosum* is marked by a change in the expression of keratins: K5 and K14 in the *stratum basale* and K1 and K10 in the *stratum spinosum* (Ter Horst *et al.*, 2018) (Figure 3). This change in keratin expression allows the formation of a more robust network of intermediate filaments. This network, along with desmosomes, strengthens the cell junctions and provides a scaffold and

structural support for the epidermis to withstand mechanical stress and maintain structural integrity (Blanpain and Fuchs, 2009). KCs in the *stratum spinosum* start producing and accumulating involucrin, one of the key tough insoluble proteins that contribute to the formation of the cornified cell envelope by surrounding keratin filaments within corneocytes in the *stratum corneum*. Several types of immune cells, including LCs and Tissue-Resident Memory T cells (Trm), can also be found in this stratum (Konop *et al.*, 2021; Yousef *et al.*, 2022).

- **Stratum granulosum**

The *stratum granulosum* is the most superficial layer of the epidermis that still has living cells. It comprises several layers of flattened KCs held together by tight junctions (Figure 3). KCs of *stratum granulosum* are crucial for the *stratum corneum* homeostasis and for the barrier function through (1) biochemical transformations of keratohyalin granules proteins and (2) the production and release of lamellar bodies content to the extracellular spaces beneath *stratum corneum* forming the lipid-rich lamellae (Figure 3) (Lefèvre-Utile *et al.*, 2021).

KCs in *stratum granulosum* undergo a complex terminal differentiation by which the KCs synthesize and accumulate **keratohyalin granules** rich in newly synthesized proteins, the most relevant being loricrin and profilaggrin, which are key proteins for the formation of the cornified envelope and the structural integrity of the *stratum corneum* (Gilaberte *et al.*, 2016).

As the KCs continue to produce keratohyalin granules, they also begin to lose their nuclei and other organelles, and their cytoplasm becomes filled with dense granules called **lamellar bodies** (Figure 4). These granules consist of lysosomal vesicles containing various lipids precursors organized in laminated layers, including phospholipids, sterols, and glycolipids that are important for the formation and maintenance of the skin barrier homeostasis. Lamellar bodies also contain the enzymes involved in lipid maturation, proteases, and their inhibitors that play a role in desquamation, as well as antimicrobial peptides (AMP) (Ishida-Yamamoto *et al.*, 2018). As the KCs release the lamellar bodies into the extracellular space by exocytosis, the lipid precursors undergo hydrolysis and enzymatic conversion into ceramides, free fatty acids, and cholesterol to form intercellular lipid sheets, constituting a water-repellent barrier preventing excessive water loss (Wertz, 2018).

- **Stratum lucidum**

The *stratum lucidum* is a thin, translucent layer found only in palmoplantar skin. It comprises flattened anucleate KCs presenting particular colorless staining properties and is filled with a protein named eleidin. Eleidin is a transformation product of keratohyalin and is eventually replaced by fully packed keratin as the cells move up toward the skin's surface. The *stratum lucidum* plays a role in the barrier function of the skin by providing an additional layer of protection against mechanical stress and friction (Yousef *et al.*, 2022).

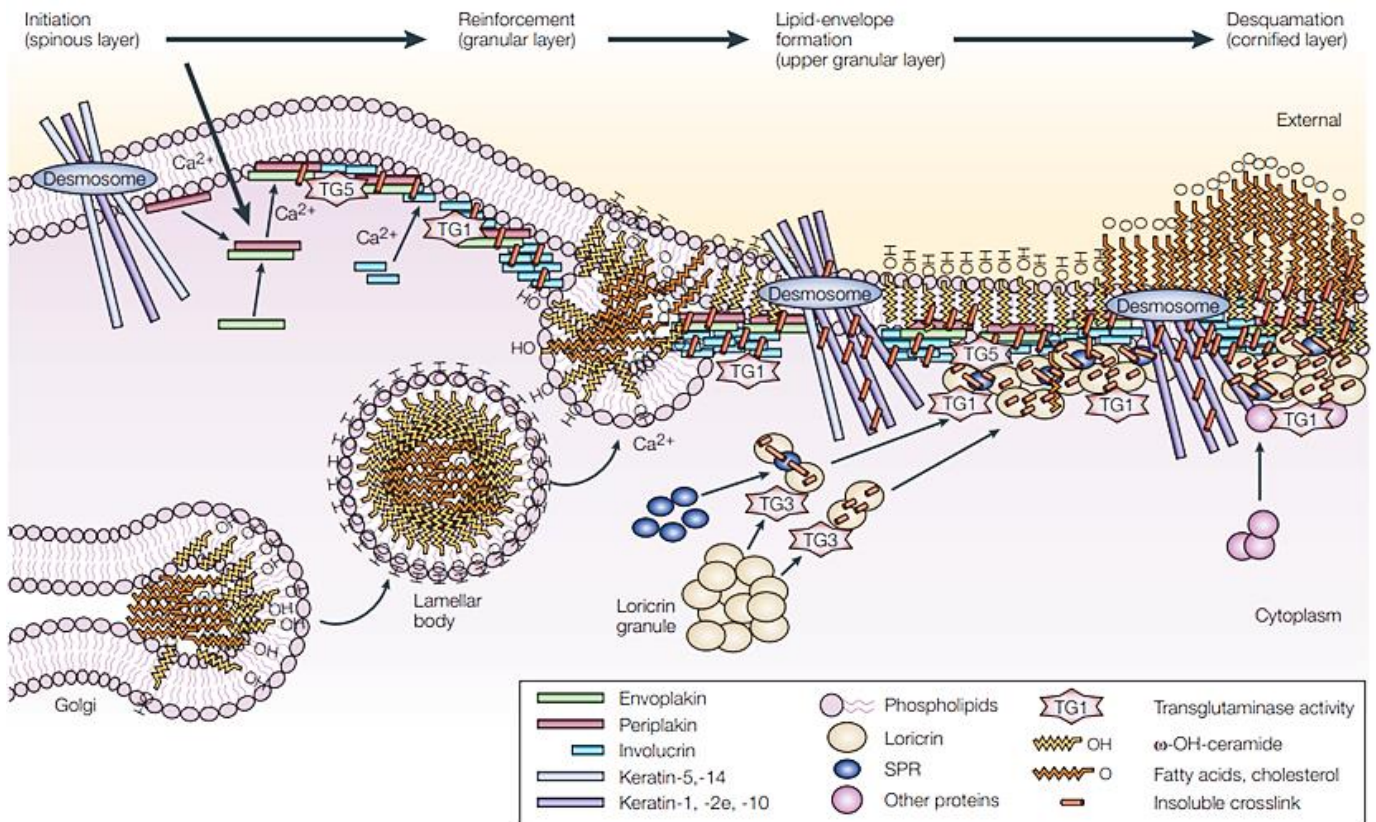


Figure 4. Progressive formation of the cornified envelope. The formation of the cornified envelope in the epidermis involves progressive stages. Initially, in the spinous layer, structural proteins and specific lipids are synthesized, and transglutaminases (TG1 and TG5) play a crucial role in crosslinking proteins under the cell membrane, anchoring them to the desmosome. The reinforcement phase, occurring in the granular layer, involves the covalent attachment of certain lipids to cornified-envelope proteins and crosslinking loricrin to small proline-rich proteins (SPRs) by TG3 and TG1, while lipid envelope formation simultaneously takes place, with lipids from the lamellar body crosslinked to already crosslinked proteins. Finally, during the desquamation phase in the cornified layer, loricrin and other proteins are further crosslinked by TG1 on the protein scaffold, while ω-OH-ceramides, fatty acids, and cholesterol are extruded (Candi *et al.*, 2005).

- **Stratum corneum**

The *stratum corneum* is the outermost layer of the epidermis and is composed of flattened, densely packed corneocytes that are dead KCs linked together by corneodesmosomes (Figure 3). Cornification, the last stage of the keratinization process, is an essential step of *stratum corneum* formation and consists of two major steps: the formation of the intracellular protein network and the formation of the intercellular lipid matrix (Figure 4).

Cornification is a gradual process that starts in the *stratum granulosum* and culminates in the *stratum corneum*. Once dispersed in the cytoplasm, profilaggrin from keratohyalin granules is dephosphorylated and proteolyzed to produce monomeric filaggrin molecules in the *stratum corneum*. Filaggrin is a histidine-rich protein that helps aggregate and strengthen keratin intermediate filaments into dense waterproof macrofibril bundles within the KC cytoskeleton, forming a matrix and causing the cell to collapse (Sandilands *et al.*, 2009). These keratin bundles provide the mechanical resistance of the *stratum corneum*. Other proteins, such as involucrin, loricrin, and small proline-rich proteins (SPRRs), undergo cross-linking facilitated by transglutaminases 1, 3, and 5, resulting in the formation of an insoluble tough protein complex along with keratin bundles just beneath the cell membrane. Transglutaminases play a pivotal role in binding lipids from lamellar bodies to the existing proteins. The exposed lipids form a rigid and insoluble lipid-protein shell known as the cornified envelope (Figure 4) (Candi *et al.*, 2005; Karim *et al.*, 2019).

Furthermore, corneocytes in the *stratum corneum* are surrounded by multiple planar lamellae sheets rich in lipids that are extruded to fill the spaces between corneocytes and effectively seal the skin (Figure 5). The inter-corneocyte lipid cement is a diverse mixture of lipids, with key constituents including ceramides (45-50%), cholesterol (25%), and free fatty acids (15%) (Madison, 2003). The composition and organization of inter-corneocyte lipids are pivotal for establishing the barrier effect. Intercorneocyte lipids are arranged in oriented bilayers. Three types of arrangements confer varying barrier properties: a densely packed orthorhombic arrangement, a less dense hexagonal arrangement, and a disordered liquid arrangement (Figure 5) (Janssens *et al.*, 2012). The latter is predominant in the superficial layers of the *stratum corneum* (Niehues *et al.*, 2018).

The intercellular lipid matrix and the tightly packed keratin filaments of the cornified cell envelope are critical for the functional skin barrier by preventing transepidermal water loss (TEWL)

and the entry of harmful compounds. Within this layer, the corneocytes also secrete defensins, which are part of our first immune defense (Chieosilapatham *et al.*, 2021; Lefèvre-Utile *et al.*, 2021). In the upper layers of the *stratum corneum*, filaggrin is ultimately proteolyzed into amino acids by caspase-14 and other proteases, giving rise to the natural moisturizing factor (NMF), playing a role in the chemical barrier of the skin (see section 2.1). The components of NMF are highly hygroscopic, allowing them to capture and retain water molecules within the corneal layer, thus contributing to hydration maintenance (Eckhart *et al.*, 2013).

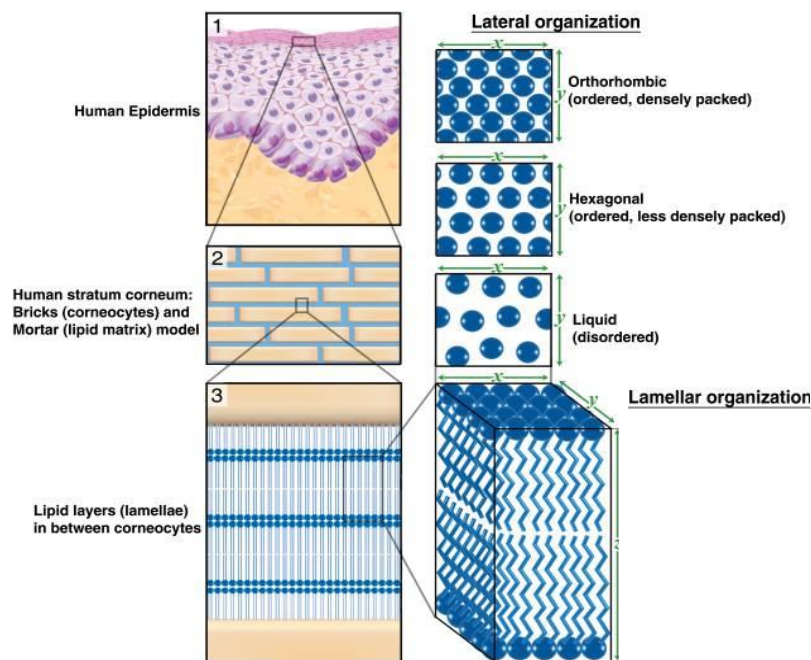


Figure 5. Lamellar and lateral organization of lipids in human *stratum corneum*. (1) In the *stratum corneum*, the outermost layer of the epidermis, the corneocytes are embedded in a lipid matrix, often described as a brick-and-mortar structure (2). The intercellular lipids are arranged in lamellae (3). Laterally, the lipids can adopt three possible arrangements: a highly dense and ordered orthorhombic organization, a less dense and ordered hexagonal organization, or a disordered liquid organization (Janssens *et al.*, 2012).

Cornification is the last terminal differentiation stage, leading to cell death. It results in the collapse of the KCs, which become flattened anucleate squames or thin flakes (Gilaberte *et al.*, 2016; Chieosilapatham *et al.*, 2021). Dead corneocytes are progressively shed off at the surface and continuously replaced by new cells from the underlying layers. Desquamation is not a passive process; it results from complex interactions between kallikreins and cathepsins and their respective inhibitors. It is orchestrated by pH and hydration modifications, ultimately leading to the progressive digestion of corneodesmosomes (Borgoño *et al.*, 2007; Haftek, 2015). Epidermal homeostasis is maintained through the delicate coordination of proliferation, keratinization, cornification, and desquamation processes, ensuring the continuous renewal of the epidermis. These processes and

structures are susceptible to molecular defects underlying numerous dermatoses.

1.2. The dermis

The dermis is a connective tissue beneath the epidermis, providing structural support for the skin. The dermis is the thickest of the three layers of the skin (0.5 to 4 mm). It mainly comprises fibroblasts that secrete the extracellular matrix (ECM). This matrix consists of an interfibrillar mucopolysaccharide gel held together by collagen and elastic fibers synthesized by the fibroblasts. Insoluble collagen fibers make up 80% of the dermis and create the three-dimensional scaffold, giving the skin its tensile strength and toughness, while elastin provides skin flexibility and elasticity, and proteoglycans and fibronectins provide viscosity and hydration. The ECM undergoes a continuous degradation process by proteolytic enzymes, mainly matrix metalloproteinases (MMPs), and is replaced by newly synthesized matrix components, including collagen and fibronectin (Cabral-Pacheco *et al.*, 2020). The maintenance of the dermal matrix structure relies on the balance between MMPs and tissue inhibitors of metalloproteinases. MMPs are crucial for tissue remodeling and are mainly secreted by various cell types, including KCs, fibroblasts, neutrophils, and mast cells (Gilaberte *et al.*, 2016). The production of MMPs is increased in various physiological and pathological processes, such as wound healing, skin aging, and tumor invasion.

The dermis can be divided into the papillary and reticular dermis, which merge without a clear demarcation. The papillary layer is the thin, superficial part composed of loose connective tissue with isolated collagen fibers usually oriented perpendicularly or obliquely to the basal membrane's plane. In contrast, the reticular dermis is formed by dense irregular connective tissue made up primarily of thick collagen fibers in large bundles and elastic fibers that intersect in all directions roughly parallel to the skin surface (Plotczyk and Higgins, 2019; Kim and Dao, 2022). Additionally, there is morphological and functional heterogeneity among fibroblasts in the papillary dermis, reticular dermis, and those associated with hair follicles. Apart from differences in ECM synthesis contributing to the dermal structure, these fibroblast subpopulations exhibit distinct secretion profiles of cytokines and growth factors, impacting keratinocyte growth and differentiation. Moreover, fibroblasts possess different proliferative capacities, including proliferative (mitotically active) fibroblasts and post-mitotic fibroblasts, both essential for dermal homeostasis (Sriram *et al.*, 2015).

The dermis is a vascularized and innervated connective tissue: it contains a deep plexus of blood vessels providing nutritional support to the epidermis and sensory nerves that transmit to the brain pain, itch, pressure sensations, and temperature information. The dermis also houses other specialized structures such as hair follicles, lymphatic vessels, sweat glands, sebaceous glands, and smooth muscles, but also many different populations of myeloid and lymphoid immune cells that either reside in (e.g., mast cells or dendritic cells (DCs)) or traffic through the dermis (Plotczyk and Higgins, 2019).

1.3. The hypodermis

The hypodermis, also known as the subcutaneous tissue, is the deepest layer of the skin. It consists mainly of adipocytes, fat-storage cells organized into lobules with fibrous septa and rich blood and lymphatic supply between them. This adipose tissue serves several essential functions. One of its primary roles is to protect the organs and bones beneath the skin. The adipose tissue also insulates the body by preventing heat loss through the skin, acts as a source of energy since it is a fat reserve, and has an important role in adipose homeostasis, regulating lipolysis and leptin secretion (Wong *et al.*, 2016). Furthermore, findings showed that the production of an antimicrobial peptide by adipocytes is an important element for protection against *S. aureus* skin infection, indicating that adipocytes may constitute a component of skin innate immunity (Zhang *et al.*, 2015). The hypodermis is also an important site for subcutaneous drug administration, as medications injected into this layer can be absorbed more slowly and consistently than injections made into the muscle or other layers of skin.

The thickness of the hypodermis can vary depending on several factors, including body site, weight, age, and sex. Women generally tend to have a thicker hypodermis than men, particularly in the hips and thighs. The hypodermis tends to become thinner as people age, making the skin more susceptible to injury and infection (Plotczyk and Higgins, 2019).

2. FUNCTIONS OF THE SKIN

2.1. Different barrier functions of the skin

Skin functions go far beyond a simple inert mechanical barrier and can be broadly classified as protective (barrier), metabolic, sensing, and maintaining homeostasis (Kim and Dao, 2022; Yousef *et al.*, 2022). All the skin functions are summarized in Table 1.

Table 1. The main functions of the skin layers

Skin layer(s)	Key component(s)/ structure(s) involved	Function(s)
Epidermis	All strata (<i>cell-cell junctions and cytoskeletal proteins, keratin</i>), mainly <i>stratum corneum (protein-enriched corneocytes and lipid-enriched intercellular domains)</i>	Barrier function: Barrier against chemicals, irritants, particles (physical barrier) (Jensen and Proksch, 2009; Köberle et al., 2022)
	Melanocytes and keratinocytes	Barrier against ultraviolet radiation (Photoprotective barrier) (Brenner and Hearing, 2008)
	Langerhans cells, other immune cells, and keratinocytes	Barrier against antigens, haptens, pathogens (immunological barrier) (Chambers and Vukmanovic-Stejic, 2020; Jiang et al., 2020)
	Skin microbiome and acidic pH	Barrier against pathogens (microbiome and chemical barrier) (Elias, 2017; Eyerich et al., 2018)
	<i>Stratum corneum</i> and natural moisturizing factors	Prevents loss of water and electrolytes (Proksch et al., 2008)
	Keratinocytes	Vitamin D ₃ (cholecalciferol) synthesis (Umar et al., 2018)
	Melanocytes	Melanin synthesis and pigmentation (Maranduca et al., 2019)
	<i>Stratum basale</i>	Epidermal turnover (Blanpain and Fuchs, 2009)
Epidermis / Dermis	Keratinocytes, immune cells, and fibroblasts	Wound healing (Reinke and Sorg, 2012)
	Merkel cells (epidermis), Meissner corpuscles (dermis)	Sensory function: Sensing light touch and tactile discrimination (Lopez-Ojeda et al., 2023)
	Sensory nerve fibers (dermis) and mechanoreceptors	Perception of pain, itch, heat, cold, pressure (Lopez-Ojeda et al., 2023)
Dermis	Elastin fibers	Elasticity (Kielty et al., 2002)
	Immune cells	Immunological barrier (Chambers and Vukmanovic-Stejic, 2020)
	Sebaceous gland	Lubrication and waterproofing (Hoover et al., 2023)
Dermis/ hypodermis	Collagen fibers (ECM)	Shock absorption and resilience (mechanical barrier) (Gilaberte et al., 2016)
	Adipose fat	Shock absorption and cushioning (mechanical barrier) (Gilaberte et al., 2016)
	Blood vessels	
	Eccrine sweat glands (dermis)	Thermal regulation (Kim and Dao, 2022)
	Adipose fat (hypodermis)	
Hypodermis	Adipose tissue	Insulation (Kim and Dao, 2022) Calorie/ energy reserve (Kim and Dao, 2022)

As the outermost layer of the body, the skin is required to protect against various environmental threats. Currently, scientists classify the cutaneous barrier between the internal and external environment into four distinct levels, each with specific functions that cross-talk and work together in a coordinated manner: the microbiome barrier, the chemical barrier, the physical barrier, and the immune barrier (Figure 6) (Eyerich *et al.*, 2018; Köberle *et al.*, 2022).

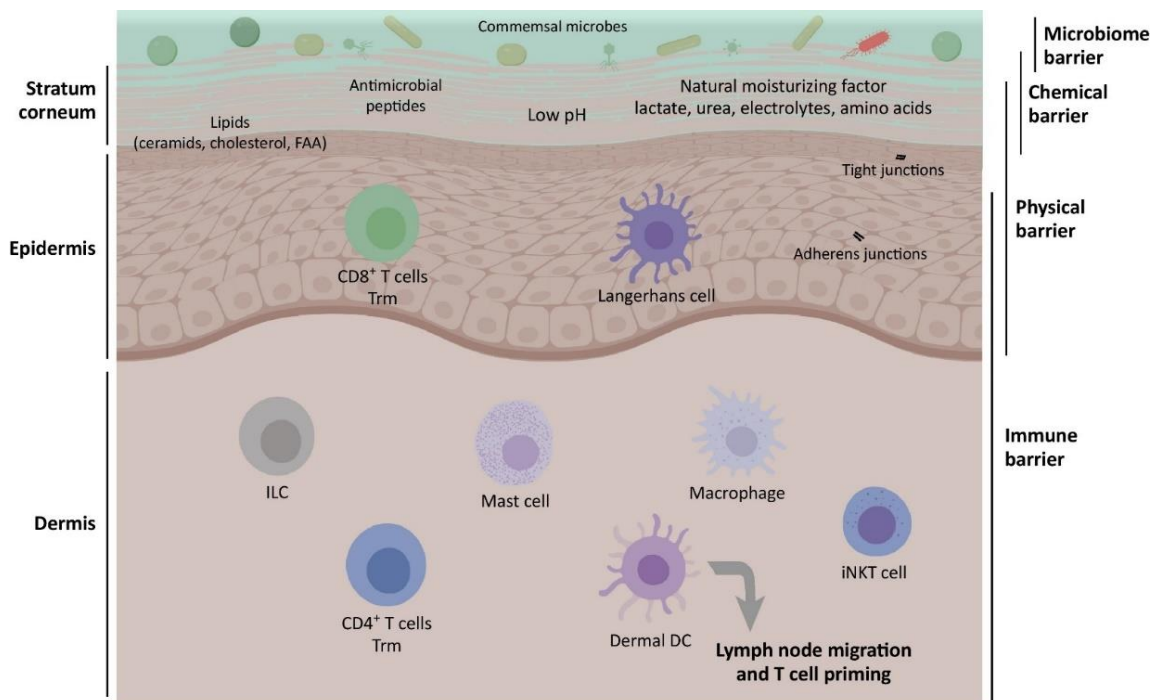


Figure 6. Components of the different levels of the skin barriers

DC: dendritic cell; ILC: innate lymphoid cell; iNKT: invariant natural killer T cell; Trm: Tissue-resident memory T cell (Eyerich *et al.*, 2018)

2.1.1. Microbiome barrier

The skin is colonized by a diverse commensal microbiota, forming the top layer of the cutaneous barriers (Figure 6). The microbiome barrier in healthy skin consists of several microbial communities with relatively stable bacterial, fungal, and viral commensal compositions. Interactions and balances between microorganisms, the skin cells, and the immune system maintain this stability and functionality. The skin microbiome is also regulated by other host environmental factors such as humidity, pH, and lipid content of the epithelial surface (Lefèvre-Utile *et al.*, 2021). Genome sequencing approaches have shown that the skin microbiota is dominated by Gram-positive bacteria, such as *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species, depending on the moisture, oiliness, or dryness of the skin site (Byrd *et al.*, 2018).

Skin commensals are critical in host defense by directly protecting against invading pathogens. Iwase *et al.* showed that the skin commensal bacteria *Staphylococcus epidermidis* or fungi *Malassezia globosa* produces proteases that can inhibit colonization by the pathogenic *Staphylococcus aureus* by blocking the formation of its biofilm (Iwase *et al.*, 2010; Li *et al.*, 2018). Human microbiota can also be a source of bacteriocins (e.g., delta-toxin) or antibiotics (e.g., lugdenin) directly killing major pathogens (Cogen *et al.*, 2010; Zipperer *et al.*, 2016; O'Sullivan *et al.*, 2019). Recently, autoinducing peptides (AIP) isolated from *Staphylococcus simulans* were shown to block methicillin-resistant *S. aureus* quorum sensing, thus disrupting their activity (Brown *et al.*, 2020). Another study revealed that AMPs produced by skin commensal bacteria, such as *S. epidermidis* and *S. hominis*, synergized with human AMP LL-37 and prohibited colonization by *S. aureus* (Nakatsuji *et al.*, 2017). The same team demonstrated that strains of *S. epidermidis* producing 6-N-hydroxyaminopurine, a nucleobase analog that inhibits DNA polymerase activity, reduced the incidence of ultraviolet (UV)-induced tumors in mice, suggesting that the microbiome may also confer protection against skin neoplasia (Nakatsuji *et al.*, 2018).

Furthermore, the skin microbiome can limit pathogen invasion by enhancing, shaping, or educating the host immune system. It was shown that *S. epidermidis* sensitizes primary KCs against *S. aureus* and boosts the innate immune response by increasing KCs production of AMPs, such as Human β -defensin-3 via TLR2/CD36-p38 Mitogen-activated protein kinase (MAPK) signaling (Wanke *et al.*, 2011; Li *et al.*, 2013), or by activating $\gamma\delta$ T cells and inducing perforin-2 expression (Pastar *et al.*, 2020). Commensal bacteria can also regulate and shape adaptive immune surveillance (Nakatsuji *et al.*, 2021). Naik *et al.* showed that microbiota controls the balance of effector and regulatory T cells (Treg) in the skin tissue (Naik *et al.*, 2012), while Linehan *et al.* demonstrated that *S. epidermidis* specifically induces adaptive IL-17A and interferon-gamma (IFN- γ) producing CD8⁺ T cells that were expressing gene signatures related to immunoregulation, including *retinoid X receptor alpha (Rxr α)* and *IL-10*, as well as genes involved in various aspects of tissue repair and wound healing, such as angiogenesis (e.g., *Fgfa*, *Vegfa*), chemotaxis, and ECM production (Linehan *et al.*, 2018).

The fundamental role of skin microflora has been well-established in the wound healing process and skin integrity (Canchy *et al.*, 2023). Adaptation of the tissue to injuries can be mediated by immunity to microbiota, and particularly by the plasticity of the commensal-specific Trm cell, as

it was shown that these commensal-specific type-17 T cells can rapidly release type-2 cytokines such as IL-13 upon wounding thereby, promoting tissue repair (Harrison *et al.*, 2019). It was also shown that *S. epidermidis*-derived lipopeptide 78 inhibits TLR3-mediated skin inflammation induced by endogenous RNA released from necrotic cells after skin wounding. This reduction of inflammation by *S. epidermidis* is critical to ensure normal wound healing (Lai *et al.*, 2009; Li *et al.*, 2019).

So, as described in these examples, skin commensals can restrain inflammation by acting at different levels, directly protecting against pathogens, boosting the innate defense system in the skin, shaping the host's adaptative immunity into an immunoregulatory response, and promoting tissue repair.

2.1.2. Physical barrier

The physical barrier of the skin is a barrier that blocks the percutaneous penetration of chemicals and pathogens due simply to its anatomical resilient structure and composition, referring to the term “physical”. As previously outlined, this barrier is primarily provided by the *stratum corneum* consisting of protein-enriched corneocytes (with the cornified envelope comprising cross-linked tightly packed proteins, cytoskeletal elements, and corneodesmosomes) and lipid-enriched intercellular domains. This robust structure of the *stratum corneum* is often referred to as a “bricks and mortar wall” (Eyerich *et al.*, 2018; Bhattacharya *et al.*, 2019). Components involved in *stratum corneum* formation, such as cornified envelopes, corneocytes, lipids, proteases, protease inhibitors, junctional proteins, and antimicrobial peptides (AMPs), have been implicated in the epidermal barrier. Human ichthyoses and animal knock-out models with dysfunctional epidermal barriers due to mutated proteins involved in *stratum corneum* formation highlight the relevance of these components in the epidermal barrier function (Proksch *et al.*, 2008; Natsuga, 2014). For instance, mutations in keratin-10 were reported in epidermolytic hyperkeratosis (Matsui and Amagai, 2015). Loss-of-function variants in the gene encoding filaggrin are strong predisposing factors for atopic dermatitis (AD) (Palmer *et al.*, 2006) and facilitate enhanced percutaneous allergen priming (Fallon *et al.*, 2009). Similarly, loss of corneodesmosin leads to severe skin barrier disruptions and atopy and eventually to peeling skin syndrome (Oji *et al.*, 2010).

Nonetheless, the entire nucleated epidermis also contributes to the epidermal integrity. It prevents the entrance of harmful substances and pathogenic microorganisms through the intercellular adhesion complexes, in particular the tight junctions between adjacent KCs in the

stratum granulosum, as well as through desmosomes and cytoskeletal elements (keratins) (Figure 6) (Proksch *et al.*, 2008; Ishida-Yamamoto *et al.*, 2018; Chieosilapatham *et al.*, 2021). Tight junction proteins, including claudins, occludin, and zona occludens proteins, are effective in regulating the transport of intermediate-sized and large molecules but also are responsible for tightly sealing the space between adjacent epithelial cells to form a functional barrier of the paracellular pathway (which otherwise allows passage between cells) (Matsui and Amagai, 2015). Defected claudin-1 can lead to neonatal ichthyosis sclerosing cholangitis (NISCH) syndrome (Hadj-Rabia *et al.*, 2004), and knockdown of this protein in mice induces atopic-like skin condition (Tokumasu *et al.*, 2016), demonstrating its crucial role in determining the physical barrier of the skin.

2.1.3. Chemical barrier

The chemical barrier of the skin is the safety belt of acidity that covers the skin, known as the “acid mantle” (Figure 6). The acidic pH of normal human *stratum corneum* ranges from 4.5 to 5.3. The skin employs multiple factors to maintain its acidic pH, including trans-urocanic acid, a metabolite from filaggrin breakdown (Krien and Kermici, 2000), fatty acids within the *stratum corneum* (Fluhr *et al.*, 2001), acidic electrolytes and lactic acid secreted by sweat glands (Thueson *et al.*, 1998), as well as the Na⁺/H⁺ exchanger (Behne *et al.*, 2002).

The acidic pH of the *stratum corneum* plays a critical role in the regulation of the microbiome and serves as a natural defense against harmful pathogens, as it inhibits their growth and colonization. It also conditions the activation of pro-inflammatory cytokines. In addition, the acidic environment is involved in the permeability barrier homeostasis since a relatively low pH is essential for ceramides metabolism, which is integral in forming and maintaining the epidermal permeability barrier (Mauro *et al.*, 1998; Behne *et al.*, 2002; Elias, 2015). Low pH plays one of the most critical roles in the cohesion, integrity, and desquamation of the *stratum corneum*, as well as in the correct organization of the lipids, by regulating their surface structure and stability (Elias, 2017; Bhattacharya *et al.*, 2019).

The skin chemical barrier comprises the “natural moisturizing factors” (NMFs), which refer to compounds that bind and retain water in the *stratum corneum*, maintaining the correct skin hydration level. NMFs consist mostly of free amino acids and their derivatives (pyrrolidone carboxylic, urocanic acid) resulting from filaggrin proteolysis, as well as other small molecules such as lactates, urea and electrolytes secreted through sweat glands (Boer *et al.*, 2016; Lefèvre-Utile *et*

al., 2021). In addition to their hygroscopic function, NMFs contribute to the acidic pH of the skin surface. Changes in the NMFs alter the *stratum corneum* pH and lipids, indicating an interdependence between the chemical and the physical barrier functions (Plasencia *et al.*, 2007; Eyerich *et al.*, 2018). Reciprocally, the correct pH takes part in NMF generation and, thus, in the maintenance of the correct hydration level of the epidermis since proteases involved in filaggrin degradation are pH-dependent. Furthermore, skin hydration in *stratum corneum* is affected by lamellae lipids of the intercellular matrix, such as ceramides and cholesterol and proteins of the cornified cell envelope, which prevent excessive transepidermal water loss (TEWL) and provide a tight, effective barrier (Boer *et al.*, 2016).

2.1.4. Immune barrier

The skin is a site for a wide variety of inflammatory processes, including immunity against infections, tumor immunity, autoimmunity, and allergy. In the early 80s, it was proposed that the skin possesses its own integrated immune system, and the term "skin-associated lymphoid tissues" or "SALT" was primarily introduced to describe this system (Wayne Streilein, 1983; Bos and Kapsenberg, 1986). The immune function is an essential component of the cutaneous barrier and comprises a variety of resident and recruited immune cells present in the epidermis and the dermis (Figure 6), which will be discussed in section 2.2.

Skin immunity consists of an innate and adaptive immune response, sensing and protecting against pathogens and harmful external factors, and mounting memory responses. Unlike adaptative immunity, characterized by an antigen-specific response (Mogensen, 2009), the innate immune system serves as the first line of defense against external aggressions. It is mainly mediated by phagocytic cells like macrophages and antigen-presenting cells (APCs) such as dendritic cells (DCs), providing a nonspecific defense against potential pathogens. The production of AMPs like LL-37, human beta-defensins, and S100A7, mainly by KCs and immune cells, also contribute to rapid responses against nonspecific threats (Herman and Herman, 2019). Additionally, pattern recognition receptors (PRRs) enable skin cells to detect damage or invasion signals and initiate immediate immune responses. These responses involve the release of pro-inflammatory mediators and other signaling molecules that rapidly mobilize additional circulating immune cells to the affected area, leading to inflammation and tissue defense (Eyerich *et al.*, 2009, 2018; McGee *et al.*, 2013; Tsepkolenko *et al.*, 2019).

The immune barrier is widely dispersed throughout the skin (Figure 6), making it closely linked with other levels of the cutaneous barrier. For instance, as described above, there is a well-established synergistic interplay between the microbiome and the innate immunity of the skin as commensal microbes produce their own AMPs, act to enhance the normal production of AMPs by KCs and are beneficial to maintain inflammatory homeostasis by suppressing excess cytokine release after minor epidermal injury (Naik *et al.*, 2015; Nakatsuji *et al.*, 2017; Chinnappan and Harris-Tryon, 2021). Furthermore, innate immunity components can interact with the skin's physical barrier, as it has been demonstrated that the activation of Toll-like receptor 2 (TLR2), a PRR, improves the function of the tight junction by increasing the expression of claudins, zona occludens-1, and occludin (Kuo *et al.*, 2013). In contrast, pro-inflammatory cytokines, such as interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) resulting from innate immune response, have been shown to alter tight junction proteins as an early event in psoriasis (Kirschner *et al.*, 2009). Another interesting example illustrating the cross-talk between cutaneous immune cells and the physical barrier is the effect of E-cadherin ligation that dramatically inhibits IL-5 and IL-13 production by the type 2 innate lymphoid cells (ILC2). E-cadherin is a protein expressed on epithelial cells forming adherens junctions between adjacent cells. Upon barrier disruption, an absence of the E-cadherin ligation activates ILC2 cells via IL-33 signaling, resulting in allergic responses (Salimi *et al.*, 2013). Finally, it was also shown that the chemical barrier impacts immune memory in the skin. The study revealed that the free fatty acids are crucial not only for the functionality of CD8⁺ Trm cells but also for their persistence in the skin tissue, where they mediate protective immunity (Pan *et al.*, 2017).

2.2. Key cellular players in the skin immunity

Various immune and non-immune skin-resident cells collaborate to mount effective immune responses initiated by resident populations in the dermis or the epidermis, such as resident APCs (e.g., LCs and dermal DCs (dDCs)), innate lymphoid cells (ILCs), and Trm cells (Chambers and Vukmanovic-Stejic, 2020). These immune responses progress by recruiting additional cell populations like macrophages and neutrophils to the skin, mainly via chemotaxis. Epithelial and stromal skin cells, namely keratinocytes and fibroblasts, also actively contribute to skin immune responses, participating in immune surveillance, the development of inflammation, and wound healing (Bautista-Hernandez *et al.*, 2017; Jiang *et al.*, 2020).

Over the past few years, researchers have interestingly come to acknowledge that skin-

associated adipocytes situated in the subcutis and lower layers of the dermis modulate skin immune responses by direct interaction with skin-resident cells or by generating several cytokines and adipokines (Guan *et al.*, 2023). Indeed, leptin secreted by adipocytes has been shown to promote pro-inflammatory biomolecules production, such as IL-8 and MMP-1 in KCs (Moonyoung Lee *et al.*, 2018; Palhinha *et al.*, 2019), while adiponectin suppresses TNF- α and INFN- γ and promote anti-inflammatory IL-10 cytokine production in leucocytes (Wolf *et al.*, 2004). Furthermore, differentiating preadipocytes have been recently shown to participate in host defense by producing cathelicidin, a well-known AMP, in a *Staphylococcus aureus*-induced skin infection mouse model (Zhang *et al.* 2015).

All these effector cells contributing to the immune skin barrier communicate through the production of a diverse array of molecules, particularly cytokines, and chemokines, which have different functions depending on the initiating signals and characterizing the activated or infiltrating cell populations. Upon activation of the skin-resident cells, additional immune cells are recruited to the skin by chemotaxis to assist in fighting infections, as well as to eliminate cellular debris and support the healing process. These include various innate immune cells, such as monocytes, neutrophils, and eosinophils, as well as adaptive immune cells, such as naive or central memory T cells and B cells (Richmond and Harris, 2014). Here, we highlight some skin-resident cells actively contributing to skin immunity.

2.2.1. Keratinocytes

2.2.1.1. Keratinocytes as immune sentinels

Long-considered passive components serving the physical cutaneous barrier, KCs are lately described as sentinel cells actively contributing to the immunological barrier of the skin (Nestle *et al.*, 2009; Jiang *et al.*, 2020; Chieosilapatham *et al.*, 2021). Owing to their strategic position between the body and the environment, KCs play a central role in innate local immunity as sensors of danger or stress factors activating *in fine* alert systems in the cell, such as the inflammasome and the Nuclear Factor-kappa B (NF- κ B) signaling (Figure 7).

Danger signals, including pathogen- and damage-associated molecular patterns (PAMPs and DAMPs), can be recognized through a plethora of PRRs expressed by KCs. PAMPs are highly conserved molecules derived from microbial components, such as bacterial lipopolysaccharide (LPS), flagellin, lipoteichoic acid (LTA), or peptidoglycan (PGN) from bacteria walls and its derivatives

(e.g., muramyl dipeptide (MDP)), as well as viral nucleic acids (double-stranded RNA (dsRNA) or unmethylated Cytosine-phosphate-Guanine (CpG) DNA) (Wang and Li, 2020). Whereas DAMPs are endogenous molecules derived from host cells and tissues that are damaged or stressed. DAMPs include molecules released from the ECM as fragments of low-molecular-weight hyaluronan or from the intracellular space like adenosine triphosphate (ATP), high-mobility group box 1 (HMGB1), and

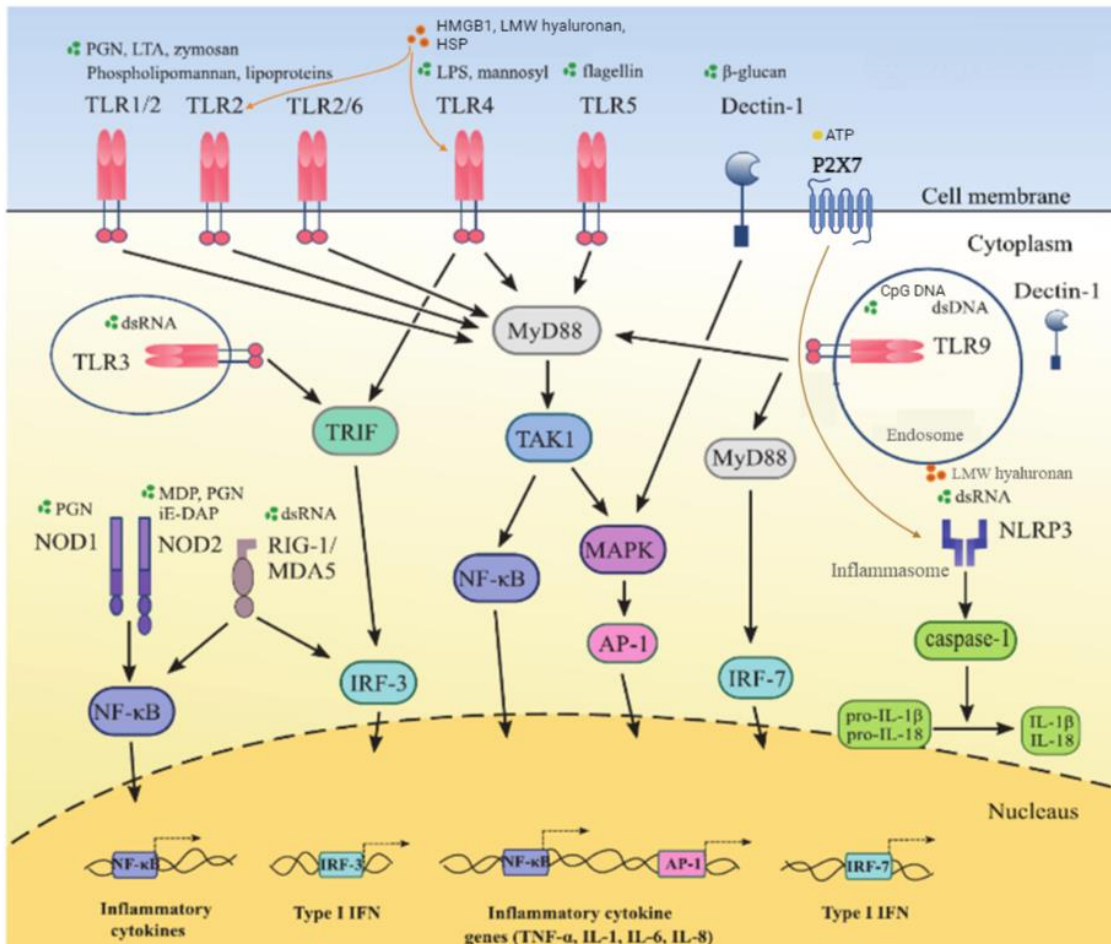


Figure 7. Keratinocytes as sensors of endogenous or exogenous danger. Keratinocytes sense danger signals through pattern recognition receptors (PRRs), culminating in the secretion of different pro-inflammatory mediators via PRRs-mediated signaling pathways. Modified from Wang and Li, 2020

heat-shock proteins (HSPs) (Roh and Sohn, 2018; Wang and Li, 2020).

Human KCs are armed with different types of PRRs, including various **TLRs**, the most important and well-studied PRRs. TLRs are located either on the KCs surface (TLR2, TLR4, TLR5, and TLR1/2 and TLR2/6 heterodimers) or in endosomes (TLR3 and TLR9) (Lebre *et al.*, 2007). Each TLR has different recognition targets (Figure 7). TLRs, except TLR3, recruit as an adaptor molecule, the myeloid differentiation factor-88 (MyD88) inducing subsequently NF-κB translocation into the nucleus and activation of MAPK signaling pathway, both leading to the production of pro-inflammatory cytokines

and chemokines. While TLR3 and TLR4 initiate TIR-domain-containing adaptor-inducing interferon- β (TRIF) dependent pathway leading to type I interferons (IFN- α , β) production. Thus, TLR-induced signaling can be largely divided into MyD88-dependent and MyD88-independent (or TRIF-dependent) pathways (Figure 7) (Wang and Li, 2020).

Other cytosolic members of PRRs include nucleotide-binding oligomerization domain-like receptors (**NLRs**) consisting of NOD1 and NOD2 detecting bacterial PGN fragments or derivatives and activating the production of pro-inflammatory mediators through NF- κ B. NLRs also comprise the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3, forming a complex called NLRP3 inflammasome (Jiang *et al.*, 2020). The activation of this complex leads to pro-caspase-1 cleavage and the subsequent formation of IL-1 β and IL-18, which are crucial to the host defense processes (Dai *et al.*, 2017). The two cytokines promote the secretion of TNF, IL6, and IFN- γ , respectively. Recent evidence by Fenini *et al.* interestingly shows that NLRP1 (rather than NLRP3) is the key inflammasome in human KCs, playing a pivotal role in UV sensing (Burian and Yazdi, 2018; Fenini *et al.*, 2018, 2022). RIG-I-like receptors (**RLRs**), such as retinoic acid-inducible gene – I (RIG-1) located in the cytoplasm, is also a type of PRRs that sense dsRNA and activate NF- κ B as well as interferon regulatory factor 3 (IRF-3), playing a role in antiviral skin defense. C-type lectin receptor (**CLR**), also known as Dectin-1, is expressed in the cell membrane and intracellularly. It recognizes bacterial or fungal cell wall components, β -glucans (Figure 7) (Jiang *et al.*, 2020).

To sum up, the recognition of the cognitive ligands by PRRs on KCs triggers downstream signaling cascades, mainly culminating in the induction of several pro-inflammatory cytokines, such as TNF- α , IL-1, IL-6, IL-18, and IFNs, as well as thymic stromal lymphopoietin (TSLP) and chemokines including IL-8 (that selectively attract neutrophils), CXCL1, CXCL2, CXCL10, CCL2, and CCL20. The various chemokines secreted by KCs drive an influx of different types of inflammatory cell subsets depending on the chemokine signal (Table 2). Another class of molecules exacerbating the inflammatory response consists of cytokine and chemokine receptors upregulated upon KC stimulation. In addition, KCs secrete various growth factors that influence epidermis growth and differentiation, vascular growth, fibroblast proliferation, and nerves (Table 2). Together, these inducible innate mediators orchestrate the early immune response of KCs to pathogenic microbes or damaging insults by activating and recruiting leukocytes to cutaneous sites of inflammation and enhancing phagocytosis and complement- or Natural Killer (NK) T cell-mediated cellular lysis

(Mogensen, 2009).

Table 2. Main immunoregulatory mediators expressed by keratinocytes (Modified from Suter et al., 2009)

Family type of mediators	Components subsets	Selected effect
Interleukins	IL-1, IL-6, (IL-7), IL-8/CXCL8, IL-12, IL-15, IL-17, IL-18, IL-23, IL-3, IL-10	Pro-inflammatory immune response (except for the anti-inflammatory effect of IL-10)
Interferons	IFN- α , - β , - γ	Antiviral action, enhance the activity of macrophages and dendritic cells
Cytokines	TNF- α , TGF- α , β FGF, PDGF, TSLP, TGF- β , α MSH, PGE ₂	Most of the factors are pro-inflammatory; α MSH and PGE ₂ , downregulate the immune response
Leukocyte growth factors	G-CSF, M-CSF, GM-CSF	Stimulate the production and differentiation of granulocytes and macrophages
Chemotactic substances	CXCL8/IL8	Attract PMN
	CCL5/RANTES, CCL2/MCP-1, CXCL10/IP10	Attract monocytes and Th1 cells
	CCL2/MDC, CCL17/TARC	Attract Th2 cells
	CCL20/MIP-3 α	Attract immature LCs and DCs Cutaneous Lymphocyte-associated Antigen ⁺ T cells
	CCL27/CTACK, <i>among others</i>	Attracts skin-homing T cells
Antimicrobial substances	Cathelicidins and defensins, lipocalin	'Natural antibiotics' stimulate the host immune response and have immune regulatory effects
Other receptors	IL-1R, IL-4R, IL-8R, IL-13R, IL-18R, IL-31R, IFN- γ R, TNF- α R, EGFR, TGF- β R, <i>among others</i>	
Immunological molecules	MHC I, MHC II, CD40, CD80/86	Induced by IFN- γ and IL-8, prerequisite for keratinocyte antigen presentation
Growth factors important in inflammation	VEGF, amphiregulin, IL-1	Promotes neovascularization Induces fibroblast proliferation
Adhesion molecules	ICAM, E-cadherin, p120 catenin	ICAM facilitates immune cell recruitment during inflammation and E-cadherin maintains strong adhesion between epithelial cells

CD, cluster of differentiation; CLA, cutaneous lymphocyte antigen; CSF, colony-stimulating factor (G, granulocyte, M, macrophage); FGF, fibroblast growth factor; ICAM, intercellular adhesion molecule; IL, interleukin; INF, interferon; MHC,

major histocompatibility complex; MSH, melanocyte-stimulating hormone; PDGF, platelet-derived growth factor; PGE₂, prostaglandin E₂; PMN, polymorphonuclear cells; TGF, transforming growth factor; TNF- α , tumor necrosis factor-alpha; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.

2.2.1.2. Keratinocytes as initiators of the adaptive immune response

One of the mechanisms by which KCs initiate the adaptive immune response is through cross-talk with LCs (APCs in the epidermis) and dermal dendritic cells (DCs). Upon encountering noxious stimuli (UV, trauma, infection, irritants), KCs produce pro-inflammatory cytokines such as IL-1 β , IL-6, IL-18, and TNF- α , which in turn stimulate the maturation and migration of LCs and dDCs to the lymph nodes where they promote the expansion of Trm CD4⁺ or CD8⁺ T cells. Furthermore, KCs are also involved in the recruitment of leukocytes, including T cells and neutrophils, to the site of inflammation. They produce chemokines such as CXCL9, CXCL10, and CXCL11, which attract T cells to the site of inflammation. KCs also secrete IL-8, a chemokine that plays a key role in recruiting neutrophils. KCs are essential for the retention of these immune cells in the epidermis by expressing adhesion molecules such as E-cadherin (adhesion to LCs) or ICAM-1 (adhesion to leukocytes).

Several studies reinforce the role of KCs in shaping adaptive immunity. KCs activated by chemical sensitizers induced the proliferation of specific T lymphocytes and their polarization to the T helper 1 (Th1) phenotype (Banerjee *et al.*, 2004). Following DNCB exposure, cultured keratinocytes were shown to present cysteine haptened (dinitrophenylated) peptides in multiple HLA molecules believed to potentially stimulate cytotoxic T cells, a key mechanism in skin sensitization (Bailey *et al.*, 2020). Furthermore, KCs can act as non-professional APCs, capable of endosomal processing and presenting antigens like house dust mite protein and parvovirus peptide for CD4⁺ T cells through major histocompatibility complex (MHC) class II and presenting endogenous antigens to CD8⁺ T cells through MHC class I (Black *et al.*, 2007).

2.2.1.3. Keratinocytes as initiators and regulators of inflammation

Besides their role in skin immune defense, KCs are central players in the pathophysiology of many, if not all, inflammatory skin disorders, such as psoriasis and atopic AD, highlighted later in *section 3*. As a matter of fact, upon pathogen or damage sensing, KCs can convert stimuli into the production of cytokines, adhesion molecules, and chemotactic factors responsible for initiating an innate cutaneous inflammation. Among the cytokines expressed by keratinocytes, IL-1 family members and TNF- α are particularly important because they can initiate an independent inflammatory reaction; therefore, they are called “primary cytokines”. The initiation phase may

facilitate or promote an amplification phase as KCs are capable not only of producing a wide repertoire of cytokines, chemokines, and growth factors (Table 2) but also responding to these molecules as they express their receptors, making the KCs both a source and a target of inflammatory mediators (Table 2). By expressing cytokines/chemokines receptors and their corresponding ligands, KCs enter an autocrine amplification loop, further stimulating and recruiting other immunocompetent cells that express the same receptors through a paracrine activity (Suter *et al.*, 2009). Such KCs-mediated amplification loops are responsible for the clinical alterations observed in chronic allergic diseases (Pivarcsi *et al.*, 2004; Purwar *et al.*, 2006). One of the examples of inflammatory skin diseases in which KCs play a critical role is AD. Since the early-onset AD seems IgE-independent, it was suggested that AD is initiated by neuropeptides, irritation, or pruritus-induced scratching that activate KCs, which in turn initiate inflammation by secreting pro-inflammatory cytokines. Thereby, TSLP secretion may drive a Th2 immune response (Liu, 2006). Alternatively, an intrinsic defect in the epidermal barrier function (e.g., due to a mutation in filaggrin) may allow environmental factors (e.g., *S. aureus*, house dust mites) to enter and stimulate KCs, resulting in an inflammatory response through NF- κ B signaling (Esche *et al.*, 2004; Luger *et al.*, 2021).

Recent studies in the past few years have investigated the steroidogenic capabilities of the skin, interestingly showing that KCs are able to synthesize glucocorticoids independently from the central stress response of the hypothalamus-pituitary-adrenal axis, as KCs express enzymes involved in the metabolism of cholesterol to cortisol, including 11 β -hydroxysteroid dehydrogenases. The skin-derived glucocorticoids are important for counteracting inflammation and healthy epidermal development (Hannen *et al.*, 2011; Bigas *et al.*, 2018). It was shown that in psoriatic skin, *de novo* glucocorticoid synthesis, as well as glucocorticoid receptor expression, might be dysfunctional (Hannen *et al.*, 2017). Phan *et al.* demonstrated that a long-term deficiency of CYP11b1 (or 11 β -hydroxylase, an enzyme involved in glucocorticoid synthesis) in the skin resulted in the development of a spontaneous skin inflammation involving type 1 and 17 cytokines, with mononuclear phagocyte infiltration. In the same study, the team showed that experimental models for inflammatory skin disorders lacking skin-derived glucocorticoids resulted in increased contact hypersensitivity (CHS) and psoriasiform skin inflammation. This exacerbated inflammatory response was associated with decreased Treg cells and the involvement of IFN- γ and IL-17A-expressing innate-like unconventional $\gamma\delta$ T cells (Phan *et al.*, 2021).

2.2.2. Skin-resident immune cells

Skin-resident immune cells in the epidermis include **LCs** and two specific subsets of T cells: **$\gamma\delta$ T cells** and **CD8⁺ resident memory T cells (Trm)**. Skin-specific memory T cells gain skin-homing properties after a process known as imprinting, which involves contact with tissue-derived DCs (Edele *et al.*, 2008).

In the dermis, some of the important immunologically relevant cell types include **dDCs**, **macrophages** (CD163⁺ for skin-specific macrophages), **mast cells**, **CD4⁺ and CD8⁺ Trm**, **$\gamma\delta$ T cells**, **CD4⁺ Treg**, **innate lymphoid cells (ILCs)**, and **plasmacytoid DCs (pDCs)** (Figure 6) (Tong *et al.*, 2015; Nguyen and Soulika, 2019; Chambers and Vukmanovic-Stejic, 2020). pDCs are moderately efficient APCs compared to conventional DCs, are very few in normal human skin, and are recruited in inflamed skin during psoriasis, allergy, and viral infection.

2.2.2.1. Langerhans cells

LCs are recognized as the prototypic professional APCs located in the epidermis (*stratum spinosum*). They are star-shaped cells that serve as immune sentries, extending their dendrites through tight junctions towards the *stratum corneum*, enabling them to search for antigens through multiple epidermis layers without compromising the permeability barrier. LCs are characterized by Birbeck granules, a unique intracytoplasmic organelle. They are traditionally distinguished from other cells by the expression of CD1a (MHCII-related molecule) and langerin (CD207, involved in the formation of Birbeck granules), although a subset of dermal DCs was also found to express CD207 (Deckers *et al.*, 2018). LCs originate from the macrophage lineage of fetal liver progenitors, as shown by fate mapping models, instead of DC lineage, as previously thought (Otsuka *et al.*, 2018). However, LCs have similarities with both DCs and macrophages. According to Doebel *et al.*, LCs have a dual identity, as they originate from tissue-resident macrophage lineage but acquire distinct DC-like phenotype and properties upon further differentiation in the epidermis (Doebel *et al.*, 2017).

At a steady state, LCs constantly migrate to lymph nodes to present self-antigen and establish immune tolerance. Upon activation (cutaneous inflammation, injury, infection), LCs showed significant morphological change: they lose their starred shape by retracting their dendrites and adopt rounder cell bodies to facilitate their migration to the skin-draining lymph nodes, where they can initiate an adaptive T cell response. By interpreting the microenvironmental context and depending on the nature of the invading pathogens, LCs determine the appropriate adaptive

immune response, immunogenic or tolerogenic. The cross-talk between LCs and the epidermal cells, including KCs, is essential for programming LCs response (Clayton *et al.*, 2017; West and Bennett, 2017; Yan *et al.*, 2020). As one of the first DCs to encounter microbial antigens, *in vitro* studies have demonstrated that LCs can uptake and process lipid antigens and microbial fragments to present to effector T cells (Hunger *et al.*, 2004). A study provided convincing evidence that LCs can present antigen and prime Th17 cell-mediated immunity to *C. albicans* but fail to generate CD8⁺ T cells (Igyártó *et al.*, 2011). In contrast, human LCs have been found to induce the polarization of naïve CD4⁺ T cells into Th2 cytokine-secreting cells and to efficiently prime naïve CD8⁺ T cells and cross-present soluble viral antigens (Klechevsky *et al.*, 2008). Although initial evidence suggested that LCs play a role in cross-presentation of exogenous protein antigens to CD8⁺ T cells *in vivo* and *in vitro* (Stoitzner *et al.*, 2006; Klechevsky *et al.*, 2008; Holcman *et al.*, 2009), later studies suggest that langerin⁺ DCs from dermal origin, are more likely to cross-present and prime CD8⁺ T cells, while LCs rather induce cross-tolerance (Flacher *et al.*, 2014; Seneschal, Jiang and Kupper, 2014; Kim *et al.*, 2015).

Researchers have long been interested in the potential role of LCs in triggering CHS reactions. However, recent studies have challenged this assumption as LCs' role in CHS and allergic sensitization to antigens remains controversial. Indeed, when LCs were removed using topical steroids, CHS reactions were increased instead of reduced, suggesting that LCs may actually inhibit these reactions (Grabbe *et al.*, 1995). Furthermore, studies using LC-deficient mice have shown that these cells are unnecessary for certain cell-mediated immune responses and may even promote tolerogenic responses by reducing inflammation (Kaplan *et al.*, 2005). A more recent study has demonstrated, using ovalbumin-sensitized LC-depleted mice, the crucial role of these cells in inducing Foxp3⁺ Treg driving the skin-mediated tolerance to ovalbumin in epicutaneous immunotherapy (Dioszeghy *et al.*, 2018). However, these conclusions were refuted by other reports demonstrating that CHS was reduced in mice that only lacked LCs (Zahner *et al.*, 2011). Studies have shown that CD1a⁺ LCs can induce Th17 responses to inflammatory plant lipids in a transgenic mouse model but failed to induce CHS to dinitrofluorobenzene (Kim *et al.*, 2016). This suggests that the type of hapten being presented can influence the polarization of T-cell responses by LCs. The exact role of LCs in tissue homeostasis and disease is still unclear and depends largely on the hapten and specific LCs ablation model used in experiments (Kaplan *et al.*, 2008). In the context of infections, other types of DCs (langerin⁺ CD103⁺ DCs) rather than LCs are likely the primary APCs involved in

mounting an immune response to cytolytic viruses such as herpes (Allan *et al.*, 2003). Thus, despite rapid progress in the field, the exact role of LCs during immune responses has not been fully elucidated.

2.2.2.2. Dermal dendritic cells

Dermal DCs (dDCs) are major sentinels of the skin immune system. In the past, studies have suggested that dDCs are more activated compared to their counterparts in blood circulation, exhibiting higher expression levels of co-stimulatory receptors and displaying a stronger ability to induce T-cell proliferation (Collin and Bigley, 2018). They have several functions: the ability of migration, antigen presentation, and stimulation of T cells. They sample the microenvironment, secrete different cytokines and chemokines, and ultimately either present antigens to the resident T cells or migrate to the lymph node to initiate an adequate immune response (Collin and Bigley, 2018). Unlike LCs, all dDCs are derived from progenitors originating from the bone marrow. They express CD1b, CD1c, CD11c, CD209 (DC-SIGN), and MHC class II on their surface. Two main populations of dermal myeloid DCs have been identified, the CD1c⁺ DCs and the CD141⁺ DCs, both capable of inducing Th1 or Th2 responses (Haniffa *et al.*, 2015; Nguyen and Soulika, 2019). CD141⁺ DCs are potent in inducing Th1 responses and responsible for cross-presenting antigens to CD8⁺ T cells (Jongbloed *et al.*, 2010; Segura *et al.*, 2012).

2.2.2.3. T cells

Naive T cells recognize antigenic peptides presented by APCs through their T-cell receptors (TCR). Activation of T cells leads to the proliferation and differentiation of antigen-specific T cells. T cells can be categorized into two main subpopulations in the skin based on their TCR composition: conventional $\alpha\beta$ T cells, which are the majority, and unconventional $\gamma\delta$ T cells, which represent a smaller population. It should be noted that $\gamma\delta$ T cells or DETCs (for dendritic epidermal $\gamma\delta$ T cells) are cells with dendritic morphology, especially found in rodents and cattle skin. In human skin, the $\alpha\beta$ T cells are predominant, $\gamma\delta$ T cells being found at very low frequencies (less than 10% of T cells in human skin) (McCully *et al.*, 2018). The activation of $\gamma\delta$ T cells can occur through both innate and adaptive pathways, which places them in an intermediate position between innate and adaptive immunity. Although they express a limited T-cell receptor repertoire, they use an array of receptors to monitor epidermal stress. For instance, they recognize microbial antigens or DAMPS from damaged KCs via TLRs, the plexin β 2 upregulated on KCs after wounding via CD100, and ligands such as Rae-1 upregulated during viral infection or tumorigenesis via NKG2D receptor. $\gamma\delta$ T cells produce

a range of effector molecules that contribute to immune defense and tissue repair (Heath and Carbone, 2013; Hu *et al.*, 2022). Accordingly, IL-17-producing $\gamma\delta$ T cells were shown to induce the expression of AMPs in the epidermis, which is essential for antimicrobial defense (MacLeod *et al.*, 2013). They are also responsible for the apoptosis of infected cells through their cytolytic action. Furthermore, mouse $\gamma\delta$ T cells have been primarily shown to sense precancerous cellular dysregulation and negatively regulate carcinogenesis (Girardi *et al.*, 2001; Strid *et al.*, 2009; Shimura *et al.*, 2010) and to participate in wound healing, as for $\gamma\delta$ T cells present in human epidermis (Havran *et al.*, 1991; Toulon *et al.*, 2009; Hu *et al.*, 2022). Whereas in a recent study, it was shown that IL-17 and IL-22 produced by $\gamma\delta$ T cells can act synergistically with other cytokines and are responsible for the delayed healing observed in infected wounds (Lecron *et al.*, 2022). It becomes evident that IL-17A-producing $\gamma\delta$ T cells play a central role in inflammatory skin diseases such as allergic contact dermatitis (ACD), psoriasis, and AD (Jee *et al.*, 2020).

After pathogen or allergen clearance, only a portion of T cells persist and differentiate into memory T cells. Most skin T cells are memory T cells, which provide immunosurveillance and ensure a rapid response to future attacks. Two subpopulations of memory T cells coexist: tissue-resident memory T cells (Trm) and circulating memory T cells (Ho and Kupper, 2019; Nguyen *et al.*, 2019). CD4⁺ and CD8⁺ Trm cells have different tissue localization, surface markers, and cytokine signaling pathways involved in their formation (Nguyen *et al.*, 2019). The CD4⁺ and CD8⁺ Trm are located in the dermal perivascular region and in the epidermis close to LCs, respectively (Ono and Kabashima, 2015). It appears that CD4⁺ Trm can migrate more easily between the skin and circulation compared to CD8⁺ Trm, making them a population of T cells not entirely resident in the skin (Nguyen *et al.*, 2019). Skin Trm cells persist for years and express skin-specific homing antigens, such as cutaneous lymphocyte-associated antigen (CLA) and CCR8. IL-15, TNF- α , Transforming growth factor (TGF β), and IL-33 are involved in the differentiation and residence of Trm in the skin (Tikoo *et al.*, 2018). Trm cells are developed in the context of xenobiotic or toxin exposure, anti-viral responses, allergies, and autoimmune diseases like vitiligo and psoriasis (Ryan *et al.*, 2021). Notably, it was demonstrated that epidermal CD8⁺ Trm rapidly recruit neutrophils that are essential for flare-ups in ACD (Funch *et al.*, 2022).

To maintain immune balance and prevent excessive immune responses, there is a distinct population known as Treg (regulatory T cells). Treg constitute 5-10% of T cells residing in the skin

and are preferentially located near LCs. Tregs are characterized by the expression of CD25, the transcription factor Forkhead box P3 (Foxp3), the cutaneous lymphocyte-associated antigen (CLA), and the CCR4 receptor. They can regulate the response of T cells and the function of APCs, as well as the accumulation of neutrophils during the early stages of inflammation (Matejuk, 2018). CD4⁺ Foxp3⁺ Treg cells with a memory skin resident phenotype have been observed in the dermis, located close to hair follicles. They are immunosuppressive cells that control the extent and duration of the immune response. They exert their suppressive function by mainly secreting the cytokines TGF- β and IL-10 (Kabashima *et al.*, 2019).

3. PATHOLOGICAL SKIN: COMMON INFLAMMATORY SKIN DISEASES

KCs and immune cells residing or recruited to the skin cross-talk permanently through the production of cytokines and chemokines to maintain cutaneous homeostasis, tolerance, and defenses (Bernard *et al.*, 2012). Nonetheless, a coordinated inflammatory response is triggered when needed, controlled by specific cytokines, including IL-1, IL-20, and TNF- α (Albanesi *et al.*, 2005). Inflammation of the skin is found after various external stimuli (e.g., UV radiation, allergen uptake, microbial challenge, or contact with irritants), as well as due to intrinsic, not always well-defined, stimuli (e.g., in autoimmune responses). Often, it is also triggered by a combination of both. The specific responses characterized by the kind of cytokines and immune cells involved and the extent of the reaction depend not only on the trigger but also on the individual genetic predisposition and daily lifestyle, including obesity, alcohol consumption, and smoking (Bäsler and Brandner, 2017; Sawada *et al.*, 2021). One may have a naturally stronger or more reactive immune system, leading to a more vigorous response to potential threats. In contrast, individuals with certain genetic variations may have a reduced ability to mount an effective immune response, making them more susceptible to infections or other skin-related issues.

There are two distinct forms of cutaneous inflammation: acute and chronic. Acute inflammation is a normal self-limiting response to specific infectious agents, internal/external injury, chemicals, and UV exposure. It aims to eliminate the causes of cellular damage, clear or absorb necrotic cells and tissues, and initiate tissue repair. However, prolonged unregulated inflammation is common in many chronic dermatitis, such as AD, psoriasis, rosacea, and ACD. According to a classification of skin inflammatory diseases proposed by Dainichi *et al.*, these inflammatory dermatoses result from a disorder occurring in one of the three layers of the skin

defense: the barrier function (e.g., AD), innate immunity (e.g., acne or rosacea), or adaptive immunity (e.g., ACD or some bullous diseases) (Dainichi *et al.*, 2014). Evidence suggests that healthy skin is also prone to a low-grade, asymptomatic chronic inflammation associated with physiological aging, termed “inflammaging” (Zhuang and Lyga, 2014). The latter is driven by the accumulation of senescent cells in the aging skin, promoting a pro-inflammatory microenvironment that disrupts adaptive immunity and considerably increases the breakdown and remodeling of the extracellular matrix. These impairments alter the function and appearance of the skin with age (Pilkington *et al.*, 2021).

Many pro-inflammatory signaling pathways are activated in the epidermis of patients with inflammatory skin disease, suggesting that KCs have a crucial role in shaping and regulating inflammation in the skin (Pasparakis *et al.*, 2014). Accumulating evidence indicates KCs play an active role in the pathogenesis of many, if not all, inflammatory skin diseases by highly interacting with immune cells, thus initiating or sustaining the inflammatory response (Ni and Lai, 2021).

We herein review the pathophysiology of the most common chronic inflammatory disorders, highlighting the implications and the cross-talk of different skin cells in these dysregulations.

3.1. Psoriasis

Psoriasis is a common chronic inflammatory skin disease with prevalence in adults in 2020, ranging between 0.2% - 2.3% depending on the geographical region or country, and affects around 120 million people worldwide (Parisi *et al.*, 2020). The nature of psoriasis pathogenesis has been recognized as a systemic inflammatory disease as it is significantly associated with several complex extracutaneous comorbidities, such as coronary artery disease, metabolic syndrome, chronic kidney disease, and arthritis (Bu *et al.*, 2022). Psoriasis has several clinical variants, plaque psoriasis being the most frequent phenotype, the most studied, and the less severe form, among others. Chronic plaque psoriasis is characterized by well-demarcated, thickened scaly plaques, typically found on the elbows, knees, and scalp, that are pruritic in about 50% of the cases (Orsmond *et al.*, 2021).

The exact mechanism that triggers psoriasis remains unclear. However, it is accepted that the immune process is activated or exacerbated in genetically susceptible individuals by environmental and internal factors, such as stress, streptococcus infections (Groot *et al.*, 2020), physical injury (Kluger *et al.*, 2017), and drugs as β -blockers or lithium (Balak and Hajdarbegovic, 2017; Awad *et al.*,

2020). The human leukocyte antigen (HLA) class I allele HLA-C*06:02 is by far the main risk allele for developing psoriasis, providing an earlier onset and a more severe course of the disease (Nair *et al.*, 2006). This predisposition in more than 60% of psoriatic patients suggests that the corresponding MHC class I molecule may have a critical role in peptide presentation to pathogenic CD8⁺ T cells. The abundant presence of abnormal activated T cells secreting several cytokines, such as IL-17, IL-22, IL-23, and IFN- γ within psoriatic lesions, and the clinical response of patients to therapeutics that target T-cell derived cytokines or function support the central role of T cells in the pathogenesis of psoriasis. Many investigations have attempted to identify T-cell antigens that might be relevant in psoriasis. In fact, a series of studies has shown that psoriasis is driven by an autoimmune component. In 2014, self-nucleic acids complexed with cathelicidin (LL-37), an AMP essentially produced by damaged KCs, was the first autoantigen to be identified inducing autoreactive CD4⁺ and CD8⁺ T cells and revealing the autoimmune contribution in the pathogenesis of psoriasis (Lande *et al.*, 2014). Other autoantigens have been rapidly identified in the following years, such as keratin 17, disintegrin and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5) expressed by melanocytes, and neolipids associated with phospholipase A2 group IVD (PLA2G4D) originated from mast cells and presented by LCs through CD1a to T cells (Arakawa *et al.*, 2015; Cheung *et al.*, 2016; Yunusbaeva *et al.*, 2018).

The pathogenesis of psoriasis involves two main skin disruptions. Psoriasis is primarily mediated by the **dysregulation of the skin immune barrier** due to immune hyperactivity with dense infiltration of immune cells into the skin. It also involves **physical barrier dysregulations** that result from the hyperproliferation of KCs within the epidermis (hyperkeratosis and acanthosis), the incomplete differentiation of KCs in the *stratum corneum*, and the subsequent disruptions of the intercellular junctions and the lipid-rich matrix (Orsmond *et al.*, 2021) (Figure 8).

On one hand, the immune dysfunction in psoriasis involves **KC activation** and the **alteration of innate immunity** (Figure 8). It is now well known that KCs contribute to psoriasis's early immune pathogenic events as an initiator of cutaneous inflammation (Albanesi *et al.*, 2018; Ni and Lai, 2021). KCs promote psoriatic inflammation by producing autoantigens like LL-37 and keratin 17. A recent study showed that KCs may also potentiate inflammation by inducing differentiation of T cells into Th1 and Th17 cells through direct cell-cell contact in the absence of APCs (Orlik *et al.*, 2020). KCs further promote inflammation in response to stimuli or skin damage through PRR activation and the production of AMPs and pro-inflammatory cytokines and chemokines, such as IL-1 family, TNF- α , IL-

6, IL-8, CXCL1, CXCL10, and CCL20. These mediators initiate innate and adaptive immune activation (Hawerkamp *et al.*, 2022). The innate immune cells initiate psoriatic inflammation by producing cytokines like TNF- α , IL-17, IL-22, and IL-23 that directly trigger KC proliferation, maintain a pro-inflammatory environment, and lead to the subsequent activation of T cells. DCs are considered the most significant innate immune activator in psoriasis. pDCs likely mediate the initial activation of the inflammatory response as they are found in early but not chronic lesions (Albanesi *et al.*, 2009). It was shown that pDCs recognize self-nucleic acids complexed with LL-37 via TLR7 and TLR9 (Lande

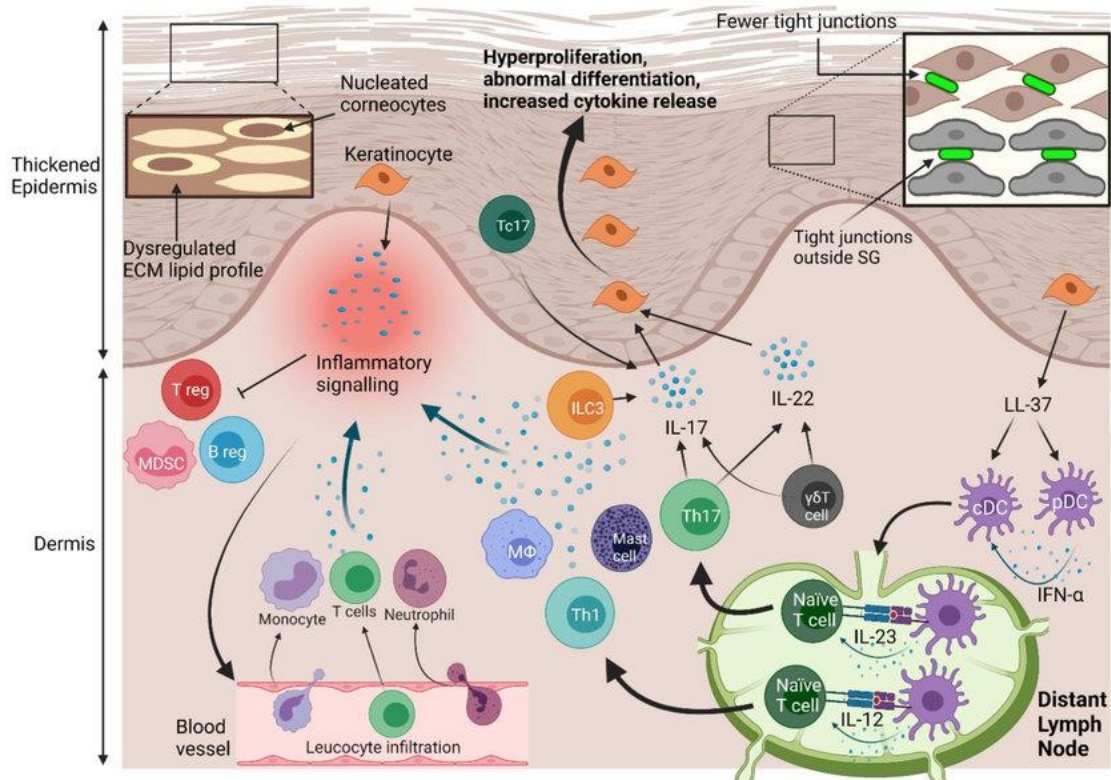


Figure 8. Physical and immune barrier disruption in psoriasis. Psoriasis is caused by a series of events that disrupt the normal functioning of the skin. This includes the hyperproliferation of keratinocytes (KCs), which leads to a thicker epidermis and an increase in proliferation markers. Additionally, decreased expression of tight junction proteins and abnormal expression of tight junctions outside the stratum granulosum further compromise the skin physical barrier function. Dysregulated ceramide production in the stratum corneum lipid-rich ECM contributes to this disruption as well. Aberrant epidermal differentiation, marked by nuclei in some corneocytes, is also observed. KCs proliferate strongly in response to inflammatory cytokines, mainly IL-17 and IL-22. IL-17 is mostly produced by type 3 innate lymphoid cells (ILC3s), T helper 17 (Th17) cells, $\gamma\delta$ T cells, and cytotoxic T cells (Tc17), while IL-22 is produced by Th17 and $\gamma\delta$ T cells. Th17 cells differentiate due to IL-23 signals from dendritic cells (DCs), which can also induce Th1 cell differentiation by releasing IL-12. Plasmacytoid (p)DCs and conventional (c)DCs can be activated by cathelicidin (LL-37) produced by KCs and complexed with self-nucleic acids. An inflammatory environment is created and sustained by cytokines from KCs, DCs, neutrophils, macrophages, and mast cells, leading to T cell, neutrophil, and monocyte infiltration from blood vessels. Inflammatory mediators also inhibit regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells. These dysregulated physical and immune barriers ultimately result in disrupted skin barrier function and in the development of psoriasis. From Orsmond *et al.*, 2021.

et al., 2007), and thus, excessively produce IFN- α which induces the activation of conventional DCs (cDCs) and sensitizes KCs to IL-22 (Tohyama *et al.*, 2012) (Figure 8). Contrastingly, cDCs are associated with autoantigen presentation to T cells in psoriasis as they are found in the lymph nodes and colocalized with T cells in the dermis of the patients. Activated cDCs secrete various cytokines such as TNF- α , IL-6, IL-12, and IL-23 and differentiate naive CD4⁺ T cells into mainly Th1, Th22, and Th17 cells. Furthermore, other innate immune cells present in the lesions, including LCs, neutrophils, macrophages, mast cells, NK cells, ILCs, and $\gamma\delta$ T cells, may play a role in the pathogenesis of psoriasis (Figure 8), but their specific functions are still being explored. Some studies concluded that LCs are anti-inflammatory in psoriasis (Glitzner *et al.*, 2014; Terhorst *et al.*, 2015), while others suggest their function is necessary for inflammation in murine imiquimod-induced psoriasis model (Minseok Lee *et al.*, 2018). These contradictory results can be explained by the presence of different LC subtypes in the skin (Orsmond *et al.*, 2021). Recently, the neutrophil extracellular traps (NETs) have drawn attention as they can induce Th17 differentiation (Lambert *et al.*, 2019) and act as a reservoir for LL-37 and self-nucleic acid complexes. The role of ILCs and $\gamma\delta$ T cells in psoriasis may be especially important in producing IL-17 and IL-22, which are key cytokines in inducing KC proliferation. While T cells, especially Th17 cells, are considered primary drivers of psoriasis, recent research suggests that the ILCs, in particular the ILC3 subset, may be more important in IL-17 production than T cells since deleting T cells from a mouse model of psoriasis does not reduce skin hyperproliferation, while deleting ILCs does (Bielecki *et al.*, 2021).

On the other hand, immunopathogenesis of psoriasis involves **excessive activation of adaptive immunity** (Figure 8). Adaptive immune cells are activated by aberrant signaling of innate immune cells, producing inflammatory mediators that exacerbate psoriatic inflammation. Antigen processing and presentation to T lymphocytes results in CD4⁺ T cell activation and mainly Th1, Th17, and Th22 differentiation, as well as the expansion of CD8⁺ T cells, especially Tc17 (Hu *et al.*, 2021; Orsmond *et al.*, 2021). Both Th17 cells and Tc17 cells are critical elements in psoriasis etiology due to their production of IL-17, which induces KC proliferation and further production of AMPs, chemokines, and IL-1 cytokines (Aghamajidi *et al.*, 2021). Th17 cells are the key adaptive responders in psoriatic lesions as they produce IL-17, IL-22, IFN- γ , and the chemokine CCL20 (Wilson *et al.*, 2007). The upregulation of CCL20 further recruits Th17 cells infiltrating the psoriatic lesions, creating an inflammatory positive feedback loop that drives psoriasis hyperproliferative phenotype (Harper *et al.*, 2009). The strong predisposition for psoriasis of the MHC class I allele *HLA-Cw*0602* suggests

that this molecule is critical in peptide presentation to pathogenic CD8⁺ T cells. The prevalence of Tc17 cells in the peripheral blood of psoriatic patients correlates with the disease severity (Teunissen *et al.*, 2014). These CD8⁺ T cells can release multiple pro-inflammatory cytokines in psoriatic lesions, including IL-17, IL-22, TNF- α , and IFN- γ . IL-23, expressed at increased levels by DCs, macrophages, and KCs in psoriatic lesions, plays a critical role in the activation and survival of IL-17-producing T cells (Teunissen *et al.*, 2014). In addition to the activation of the adaptive immunity, psoriasis may be linked to a **reduction or inactivation of the immunosuppressive cells**, particularly Treg cells, regulatory B cells, and myeloid-derived suppressor cells (MDSCs) (Figure 8). While Treg cells are more prevalent in psoriatic skin, their suppressive function is altered by the pro-inflammatory cytokine milieu, particularly IL-6 and IL-21 (Pietraforte and Frasca, 2023).

While it is evidenced that psoriasis has an autoimmune origin, the exact trigger initiating the pathological immune responses is still unclear. The “egg and hen” theory suggests that it is uncertain whether the autoantigen-specific T cells trigger psoriatic inflammation or whether autoimmunity results from excessive stimulation of innate immunity (Šahmatova *et al.*, 2017). However, it is believed that the abnormal proliferation and maturation of KCs and the activation of the immune system in psoriasis create a self-perpetuating feedback loop that maintains skin inflammation and epidermal hyperplasia (Ten Bergen *et al.*, 2020).

3.2. Atopic dermatitis

AD is a chronic Th2 type inflammatory skin disease associated with cutaneous hyper-reactivity to environmental triggers. AD has a characteristic phenotype, with typically recurrent distributed eczematous skin lesions with itch, often making its diagnosis very simple (Langan *et al.*, 2020). It is a multifactorial disease involving genetic predisposition, alterations in the skin barrier, environmental exposures, and microbiome disruptions in its pathogenesis (Luger *et al.*, 2021).

Defects in filaggrin *FLG* and *FLG2* genes are the most common gene mutations associated with AD, resulting in altered cornified envelope and issues in skin moisturization (Palmer *et al.*, 2006; Liang *et al.*, 2016). Defects in genes encoding other components of the epidermal barrier, such as Small Proline Rich Protein 3 (SPRR3), tight junctions (CLDN1), and protease inhibitors (SPINK5) have also been noted in AD (Luger *et al.*, 2021). A compromised epidermal barrier predisposes the skin to increased permeability of manufactured pollutants and natural environmental allergens, such as house dust mites, cockroaches, pet dander, and multiple pollens, that have all been reported to

induce AD in a subpopulation of patients, through cutaneous exposure (Hostetler *et al.*, 2010). Antigen penetration activates KCs and LCs to produce pro-inflammatory cytokines, such as TSLP and IL-33, and subsequently enhances Th2 immune reactions mediated by IL-4, IL-12, and IL-13 (Nakajima *et al.*, 2019). Furthermore, IL-31 accelerates itch in AD and decreases the quality of life (Otsuka *et al.*, 2011). Therefore, these cytokine-targeted treatments or the recovery of skin barrier function have been developed for the favorable clinical outcome of AD treatment (Ruzicka *et al.*, 2017). In addition to the external trigger that causes atopic skin inflammation, intrinsic factors are involved in the pathogenesis of intrinsic AD. The serum nickel concentration is constitutionally higher in patients with intrinsic AD than in those with extrinsic AD and healthy subjects (Yamaguchi *et al.*, 2015).

There is a global and site-specific dysbiosis in AD involving bacteria, fungi, and viruses (Bjerre *et al.*, 2021). *Staphylococcus aureus* is commonly found in chronic AD lesions, colonizing frequencies of 70% of lesional and 39% of non-lesional sites, with scratching contributing to bacterial binding and proliferation by disrupting the skin barrier and exposing molecules that act as adhesins for *S. aureus*, such as fibronectin, collagens, fibrinogen, elastin, laminin (Maranduca *et al.*, 2020).

II. TREATMENT OF SKIN INFLAMMATION: FOCUS ON THE PHOTOBIMODULATION

Inflammation is a major contributor to many skin diseases and conditions, and the ability to reduce inflammation is of paramount importance in medicine. Over the years, several treatment options have been developed to manage skin inflammation, including topical treatments, such as corticosteroids and retinoids, oral medications, such as antibiotics and immunosuppressants, as well as alternative therapies. One such non-invasive and safe alternative therapy that has recently gained attention is photobiomodulation (PBM). Using low levels of visible light or near-infrared (NIR) radiation, PBM effectively reduces inflammation and improves healing, thus treating various skin conditions, such as acne, herpes lesions, wounds, rosacea, and psoriasis (Jagdeo *et al.*, 2018).

1. DEFINITION OF THE LIGHT

The electromagnetic spectrum represents the range of all electromagnetic waves, including radio waves, microwaves, infrared (IR) radiations, visible light, UV radiations, X-rays, and gamma rays, respectively (Figure 9). Each electromagnetic radiation of the spectrum is defined by its wavelength λ (nanometers (nm)), frequency (hertz), and energy (Joules), with the energy being directly proportional to the frequency of the wave and inversely proportional to its wavelength. Radio waves have the lowest frequency and energy, while gamma rays have the highest frequency and energy. An electromagnetic wave comprises oscillating electric and magnetic fields that can propagate in a vacuum without material support. If we consider the particle aspect of the waves with the advent of quantum theory, an electromagnetic wave can be assimilated to a set of energy packets called photons. Photons are the elementary particles of these waves, have no mass, and mediate electromagnetic interactions. Their properties, including energy, frequency, and wavelength, are determined by the characteristics of the electromagnetic wave they represent (Enwemeka, 2004).

Our skin is exposed daily to broad wavebands composing the solar spectrum received on the earth's surface after the atmosphere filtration. According to the American Society for Testing and Materials, the solar spectrum on the earth's surface comprises UV radiations (UVB and UVA, 290 - 400 nm), visible light, and IR radiations (700 nm - 3000 nm) representing, respectively, 5%, 45% and 50% of the total solar energy reaching the earth's surface (Santhosh and Prasad, 2016; ATSM, 2020;

Geisler *et al.*, 2021). Although photodermatology has primarily focused on the impact of UV radiation on skin health, including DNA damage, photoaging, and carcinogenesis (Narayanan *et al.*, 2010; Bosch *et al.*, 2015; Ganguly *et al.*, 2021), the crucial role of UVB exposure in vitamin D metabolism cannot be ignored (Holick, 2003).

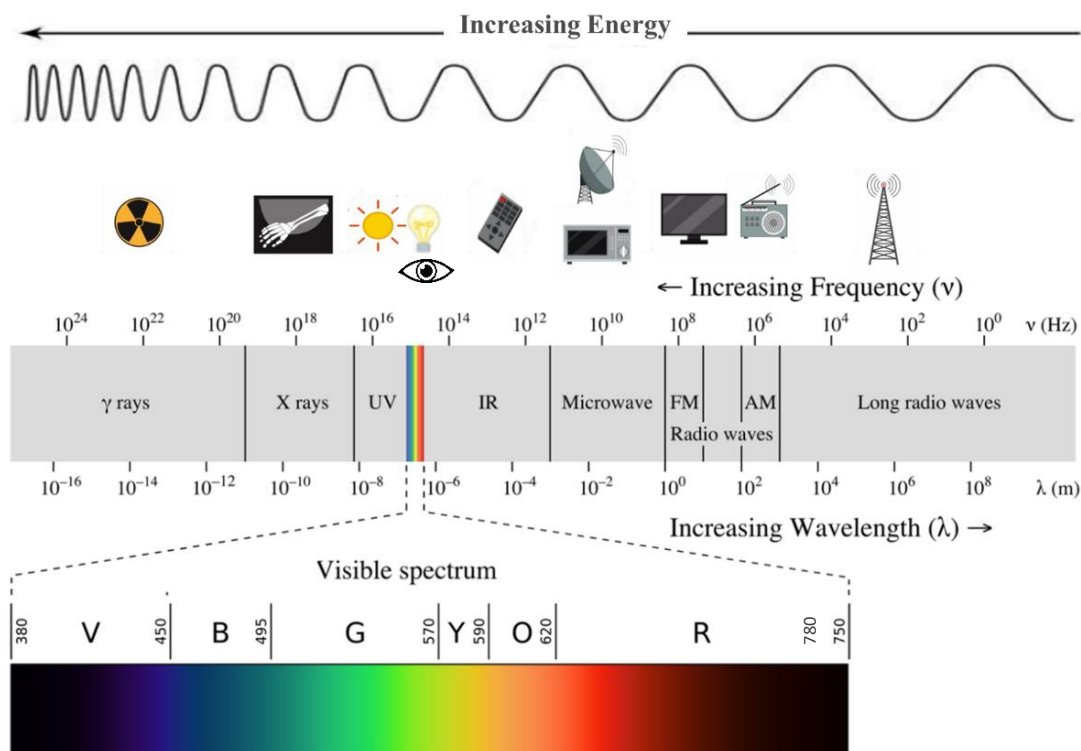


Figure 9. Position of Visible light within the electromagnetic spectrum. The electromagnetic spectrum includes a wide range of electromagnetic waves, including radio waves, microwaves, infrared (IR) radiations, visible light, UV radiations, X-rays, and gamma rays. Each type of radiation is characterized by its wavelength (in nanometers), frequency (in hertz), and energy (in Joules). Radio waves have the lowest frequency and energy, while gamma rays have the highest. Visible light covers a spectrum between 380 nm (dark violet) to 780 nm (barely perceptible red) and varies based on individual eye sensitivity. V: Violet, B: Blue, G: Green, Y: Yellow, O: Orange, R: Red

Visible light comprises a range of wavelengths that can be detected by the human eye and covers a small portion of the electromagnetic spectrum between UV and IR radiations (Figure 9). However, the limits of this visible spectrum are not well-defined, as they can vary among individuals due to differences in eye sensitivity. One can consider that visible light ranges between 400 nm and 700 nm. While the International Commission on Illumination (Commission Internationale de l’Eclairage, CIE) has defined the range of wavelengths that can be perceived by the reference observer as being between 380 nm, corresponding to extremely dark violet, and 780 nm, representing a barely perceptible red (Figure 9) (Slaney, 2016). Within this range, different light colors are distinguished by their respective wavelengths, with violet/blue light having shorter and

more energetic wavelengths than red light. The light that surrounds us every day is called white light. It is composed of a mixture of all the wavelengths constituting the visible spectrum. Light has always been a universal aspect of life, driving several crucial biological responses. Numerous examples of light-induced photochemical reactions can be clearly observed in different biological systems and processes, such as photosynthesis, circadian rhythms, mood regulation, and vision (Gudkov *et al.*, 2017; Blume *et al.*, 2019).

2. HISTORY OF PHOTOTHERAPIES IN DERMATOLOGY

The therapeutic use of light and radiation for human skin diseases has been recognized for centuries. **Heliotherapy**, or the use of sunlight for therapeutic purposes, dates back to ancient times, as early as 1500 BC when Egyptians and Indians used sunlight exposure following the ingestion of plant extract or seeds to treat vitiligo depigmentation (Brodsky *et al.*, 2017). However, the real modern era of phototherapy in dermatology began in the early 20th century when phototherapy was recognized as a medical science thanks to the Danish physician Niels Finsen. In 1903, Finsen was awarded the Nobel Prize in “Medicine and Physiology”, for his breakthrough invention treating skin tuberculosis (lupus vulgaris) by **phototherapy** using sunlight or concentrated electrical light through “Finsen Lamp,” at a time when no anti-inflammatory drugs were available. Finsen has always believed that *M. tuberculosis* bacteria were killed by UV radiation during his therapy. However, subsequent studies on the emitted spectrum using the lens systems and filters he employed revealed that wavelengths ranging between 340 and 550 nm, with transmission peak in the blue light, were responsible for the therapeutic effect (Moller *et al.*, 2005).

Although the seminal work of Finsen received much attention from scientists, the lack of a mechanistic explanation resulted in the limited acceptance of phototherapy within conventional medical practice. It took then several decades for phototherapy to be reintroduced as a powerful tool in dermatology, with the rise of **photochemotherapy** in the 1970s following the development of ultraviolet irradiators emitting high-intensity UVA (Wolff *et al.*, 1976). The previously described treatment of sunlight in conjunction with plant extract used by ancients was the very earliest form of what is now called photochemotherapy, known as Psoralen-UVA (PUVA). PUVA uses UVA and the photosensitizing chemical psoralen, derived from the same plant source, *Ammi majus*, to treat psoriasis and vitiligo (Hönigsmann, 2013; Barros *et al.*, 2021). More recently, in 1992, **UVA1** (340-400 nm) **phototherapy** was introduced first for the treatment of AD and then for other dermatoses

as UVA1 penetrates deeper than UVA2 (320-340 nm) in the skin and, thus, can reach deep dermal components. Phototherapy with UVA1, unlike PUVA, does not use UVA2 and does not require the use of psoralen (Barros *et al.*, 2021).

Another phototherapy that marked an evolution in the field of dermatology in the 20th century was **photodynamic therapy** (PDT) (Allison, 2013). In 1904, the concept of PDT was fortuitously discovered by Oscar Raab and his Professor Herrmann Von Tappeiner in Germany and further advanced by the work of Dougherty in the late 1970s for treating malignant tumors like basal cell carcinoma and lung cancer (Dougherty *et al.*, 1978; Kennedy *et al.* 1990). Although PDT and PUVA approaches both combine phototherapy with photosensitizer agents such as hematoporphyrin derivative or psoralen, respectively, PDT uses visible light, usually in the red or blue spectrum, to activate the photosensitizer, unlike PUVA. PDT also has a different mechanism of action than PUVA, as it is oxygen-dependent and, thus, has different clinical applications. PDT includes nontoxic photosensitizers administered topically, systemically, or induced endogenously by aminolevulinic acid (ALA), accumulating preferentially in pathological tissues. Upon exposure to specific light wavelengths, the excited photosensitizers that absorbed the photons generate reactive oxygen species (ROS), particularly singlet oxygen and superoxide anion, leading to localized cell damage and tissue destruction (Kwiatkowski *et al.*, 2018). Nowadays, PDT is mainly used for acne, actinic keratosis, precancerous lesions, and different types of cell carcinoma, such as basal cell or squamous cell carcinoma, lung cancer, and esophageal cancer, as well as other inflammatory or infectious skin diseases as new sensitizers are being developed (Queirós *et al.*, 2020).

The use of visible light phototherapy has become a cornerstone of modern medicine. It has been widely used to treat neonatal jaundice for the past 60 years to the present day (Cremer *et al.*, 1958). Newborns with hyperbilirubinemia are exposed to sunlight or specific wavelengths, typically blue light, that help break down bilirubin, a neurotoxic product of heme catabolism, by photoisomerization and photo-oxidation. Products are more electrophilic and, therefore, more easily eliminated than bilirubin (Savedra *et al.*, 2021; Choi *et al.*, 2022).

In addition to the historical advancements in phototherapy described above, another major milestone in the therapeutic use of visible light emerged shortly after the invention of the first laser in the early 1960s, as there were immediate concerns about its biological safety. In 1967, Hungarian physician Endre Mester conducted an experiment to investigate the potential carcinogenic effects

of laser radiation on mice (Gáspár, 2009). He divided the mice into two groups, shaving the dorsal hair of both groups and exposing one group to low-power ruby laser treatment (694 nm). Surprisingly, the treated group did not develop tumors, but their hair grew back faster compared to the untreated group (Mester *et al.*, 1968). This observation marked the first evidence of "laser biostimulation". Later, Mester *et al.* demonstrated that low doses of laser can also accelerate wound healing (Mester *et al.*, 1971). Since then, the therapeutic use of low-level visible light from coherent light sources, such as lasers, and noncoherent light sources, like light-emitting diodes (LEDs), has evolved significantly in dermatology and other medical fields, including orthopedics, neurology, and dentistry, among others. Numerous clinical, pre-clinical, and *in vitro* studies have been carried out about low-level visible light since the 2000s, and much has been learned about the mechanistic basis of this rising therapy, now commonly referred to as **photobiomodulation therapy** (PBMT) (Hamblin, 2017).

3. PHOTOBIMODULATION

3.1. Definition

The North American Association for Photobiomodulation Therapy (NAALT) defines **PBM** as the biological changes in the tissue induced by photophysical and photochemical events secondary to the absorption of non-thermal non-ionizing photons in the visible and NIR (750-1000 nm) spectral range by endogenous chromophores (Anders *et al.*, 2019). It follows that **PBMT** is a non-invasive photon therapy based on the principles of PBM aiming to have beneficial therapeutic outcomes including but not limited to the alleviation of pain, reduction of inflammation, immunomodulation, and promotion of wound healing and tissue regeneration (Anders *et al.*, 2019). PBM does not induce thermal damage as the power density used is much smaller than the threshold for photothermal effects ($< 1000 \text{ mW/cm}^2$, depending on the wavelength and tissue type) (Hamblin *et al.*, 2018).

Initially termed "laser biostimulation", more than 60 other names were used in the scientific literature to designate this therapy, the most common being low-level laser therapy (LLLT), causing inconsistencies in terminology (Hamblin *et al.*, 2018). To address this issue, a nomenclature consensus meeting organized by the NAALT and the World Association for Photobiomodulation Therapy (WALT) acknowledged that the term LLLT is broad, although widely used and often-cited Medical Subject Headings (MeSH) term, as the words "low" and "level" are not accurately definable. In addition, the word "laser" is no longer appropriate, as other types of light devices,

such as LEDs and broad-band light, are currently used for this application. The term LLLT may also confusingly include other phototherapies, such as PDT that uses low-dose visible light but requires exogenous chromophores contrary to LLLT. Thus, it has been agreed that PBM is a more suitable and accurate term and should be adopted as the preferred term going forward (Anders *et al.*, 2015).

3.2. Light sources

The most common PBM procedures in dermatology are now performed by the irradiation of the skin with a variety of different light sources, including coherent monochromatic (with narrow bandwidth) light sources, such as lasers, and noncoherent quasimonochromatic (bandwidth of several nanometers) light sources, like LEDs (Van Tran *et al.*, 2021). Broadband (polychromatic) light from halogen or other incandescent-filtered lamps have also been investigated, and promising results were shown *in vivo* in wound healing, burns, and ulcer treatment (Feehan *et al.*, 2018).

The efficiency of LED *versus* laser in PBM is a controversial topic and ongoing debate in the research literature and practice (Heiskanen and Hamblin, 2018). While proponents of lasers argue for their potential superiority based on properties like coherence, polarization, and deeper penetration in the tissue (Moskvin, 2017), other authors concluded that coherence and polarization are lost in the first layers of biological tissues and, thus, are not parameters responsible for the effects of the therapy (de Freitas and Hamblin, 2016; Brochetti *et al.*, 2017). Considerable evidence has accumulated over the last decades suggesting that LEDs are equally effective or even more efficient in certain aspects of PBM, particularly in superficial tissues like the skin (Chaves *et al.*, 2014). At equivalent energy doses, different *in vivo* studies showed that red laser and red LED were both effective in increasing angiogenesis (Corazza *et al.*, 2007; de Sousa *et al.*, 2013), reducing the inflammatory cells (Keshri *et al.*, 2021), and improving skin wounds in rodents (Demidova-Rice *et al.*, 2007; Wu *et al.*, 2015; Keshri *et al.*, 2021), while LEDs were more effective than laser in reducing the wound diameter of diabetic animals or in the earlier reduction of mast cells in wounds (Dall Agnol *et al.*, 2009; De Castro *et al.*, 2014). It was also shown that laser and LED, both in 810 nm NIR wavelength range at pulsed mode, are similarly effective for reducing the inflammatory response and improving burn wound healing in rats (Keshri *et al.*, 2021). Another study observed similar effects using a 660 nm laser (but not 632 LED) and an 850 nm LED (but not 904nm laser), which could be attributed to differences in the treatment regimen and parameters for each exposure (Silveira *et al.*, 2016). This implies that the significant determining parameters for the effectiveness

of PBM are the wavelength and the dose (Salehpour *et al.*, 2018).

LEDs were adopted as a reliable alternative to lasers in PBM. Compared with laser, LED sources possess several advantages, including lower cost, broader coverage of wavelengths (up to ± 10 nm), ease of home use, and enhanced safety. In the past, laser light sources were predominantly used by clinicians, but nowadays, LED-based in-home devices are widely available for patients. Furthermore, in dermatological applications where larger areas of the skin require treatment, LED lights are favored over lasers due to their ability to provide simultaneous irradiation of large tissue regions, which has been a limitation of lasers that typically have small spot sizes (Van Tran *et al.*, 2021). Recently, the use of organic LED (OLED) and quantum dot LED (QLED) has gained attention as next-generation LED light sources in dermatological phototherapy applications since they exhibit unique advantages of homogeneous and narrower width irradiation, less heat generation, flexibility in form, ultra-thinness, and lightweight. These features enable these upgraded LEDs to be an ideal candidate for wearable PBM bandages or belts fitted close to the body and efficiently transmitting light to the skin (Chen *et al.*, 2017; Jeon *et al.*, 2018; Sun *et al.*, 2023).

3.3. Dosimetry

Researchers frequently make critical errors and omissions when describing PBM parameters in their scientific publications, which makes reproducibility impossible. In their reference study, Jenkins and Carroll proposed a list of necessary parameters to be accurately reported by researchers for a comparable and repeatable clinical or experimental study (Jenkins and Carroll, 2011). To fully define a specific PBM procedure, it is necessary to specify many parameters, such as the wavelength, power, beam area, and irradiation time, from which fluence (energy density) and irradiance (power density) can then be calculated (Table 3). It has been shown that the biological effect of PBM depends on the irradiance (power density, number of photons received per second per unit area, mW/cm^2) rather than the fluence (energy density, or the dose, the total number of photons received per unit area during the treatment, J/cm^2) (Lanzafame *et al.*, 2007; Calderhead and Vasily, 2016; Hamblin, 2018). Other parameters regarding the aspect of the wave (continuous or pulsed), pulse structure (Figure 10), number and frequency of treatments, mode of light application (direct contact or at a distance), and combination with other wavelengths should also be reported (Table 3) (Mosca *et al.*, 2019).

Table 3. Definition of the irradiation and the dose parameters in PBM (Chung et al., 2012; de Freitas and Hamblin, 2016; Mosca et al., 2019)

Parameter	Unit	Description
Parameters related to the specific light source, "Irradiation parameters"		
Wavelength (λ)	nm	A measurement of how far a photon will travel in one complete cycle, the distance between two consecutive peaks of a wave
Beam area	cm ²	The cross-sectional area of an emitted light beam, the spatial size of the light beam as it propagates through space or interacts with a target surface
Power output (P)	W	The rate of delivered energy per second, 1 W = 1 J/s
Irradiance (I)	W/cm ²	Power density, the light power (W) over a surface per unit area (cm ²) $I (W/cm^2) = P (W) / \text{Beam area } (cm^2)$
Parameters controlled by the operator, "dose parameters"		
Irradiation time (t)	s (or min)	The total duration of the light treatment
Irradiated area (S)	cm ²	The surface of a specific region of tissue or culture that is exposed to the light during treatment
Energy (E)	J	The amount of energy carried by each photon. A short wavelength = high-energy; a long wavelength = low-energy photons $E (J) = P (W) \times t (s)$
Fluence (F)	J/cm ²	Energy density, the amount of energy (J) over a unit area (cm ²) $F (J/cm^2) = I (W/cm^2) \times t(s) = E (J) / S(cm^2)$
Mode of application	-	The light exposure of the target was made by direct contact with the light source or at a distance from the source (in this case, the distance between the light source and the target should be reported)
Number and frequency of treatments	Hrs, days, or weeks (frequency)	Total number of treatment sessions and the time intervals between each session
Pulse structure (if the wave is not continuous)		
Pulse width	s	The duration the light source is turned "on" during each pulse
Duty cycle	%	Percentage of time light source is actually "on" during the entire treatment. A continuous wave light is always "on" with 100% Duty cycle
Frequency (γ)	Hertz (Hz)	Pulse frequency rate (cycles/s or number of pulses/s), how frequently the pulses are repeated in a pulse train. It is inversely related to wavelength (short wavelength = high frequency, long wavelength = low frequency): $\gamma (Hz) = c (m/s) / \lambda (m)$, where c is the speed of light
Period (T)	s	The time interval between consecutive pulses in a pulse train. It represents the duration from the start of one pulse to the start of the next pulse. $T (s) = 1 / \gamma (Hz) = \text{Time "On"} + \text{Time "Off"}$
Pulse train	s (or min)	A series of pulses that are regularly spaced in time with a consistent period during a single event

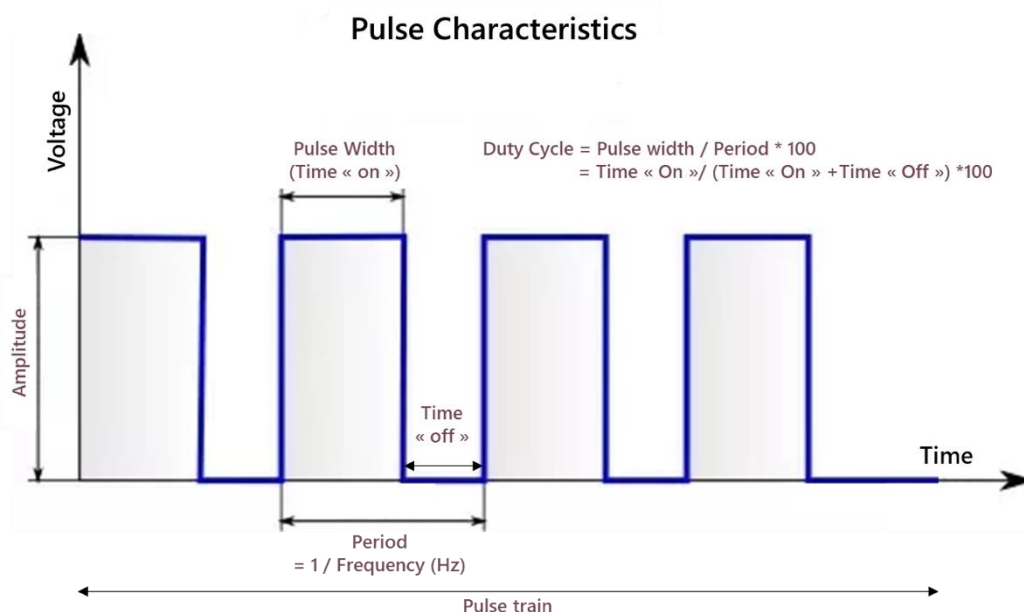


Figure 10. Pulse characteristics of a pulsed wave with 75% Duty Cycle. Duty cycle refers to the ratio of time the pulse is 'on' (pulse width) to the total period of one pulse. For instance, in this representation, the pulse width is 75% of the pulse period.

It has become evident that to achieve positive results with PBM, each dosimetric parameter must be controlled within a limited range of values. Choosing the appropriate values for a large number of illumination parameters is challenging in PBM, resulting in the publication of several negative studies as well as many positive ones (Zein *et al.*, 2018). This complexity is attributed to the biphasic dose response commonly observed in PBM, as described by the Arndt-Schultz curve (Figure 11) (Lee and Oh, 2022; Nie *et al.*, 2023). In line with the concept of “hormesis” when applied to PBM, this relationship demonstrates that low levels of light exhibit superior effects in stimulating and repairing tissues compared to higher light levels. It suggests that there is an optimal range of light dosage for achieving beneficial effects in PBM, while exceeding this range may lead to diminished or even harmful effects (Figure 11) (Huang *et al.*, 2011; Lee and Oh, 2022). Several PBM studies reported this phenomenon in the dermatology field. 660nm, 810nm, and 980nm wavelengths showed a biphasic dose response for the proliferation of adipose-derived stem cells and their ATP production, with different optimum doses (3 J/cm² for 660 and 810 nm vs. 0.3 J/cm² for 980nm) (Wang *et al.*, 2017a, 2017b). Similarly, the viability and the mitochondrial activity of human fibroblasts were higher with lower doses (0.45 and 0.75 J/cm²) of 660 nm laser while decreasing with 3.6 J/cm² (Flores Luna *et al.*, 2020). Another study clearly showed a biphasic dose response of 635 nm lamp on excisional wound healing in mice with small positive effects at 1 J/cm², the most accelerated closure at 2 J/cm², and less important effects at 10 J/cm², while 50 J/cm²

worsened the wound healing time curve (Demidova-Rice *et al.*, 2007).

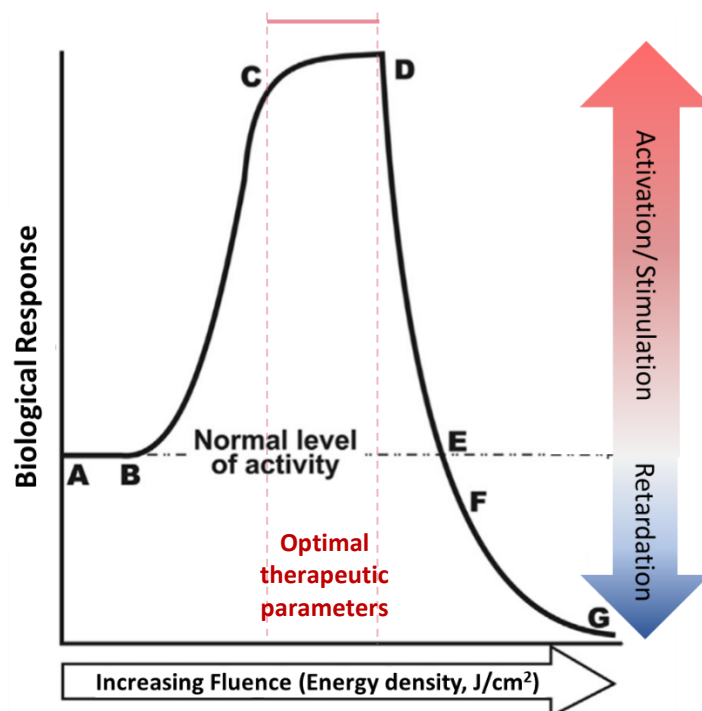


Figure 11. The PBM-adapted version of the Arndt-Schultz curve illustrating how the increased energy density, including the increased power density and/or time of illumination, can have a biphasic dose-response relationship in PBM. For a specific wavelength, from point A to B, the cell or the tissue action spectrum shows no or minimal stimulatory effects, the light doses being insufficient for a significant effect. As the stimulus level increases from point B to C, there is a rapid increase in the stimulatory response. The activity reaches a plateau at points C to D with maximal beneficial effect and then sharply decreases back to normal levels at point E. Beyond point E to point F, with the increasing stimulus strength, activity drops below normal levels, leading to mild retardation, negative or inhibitory effects, ultimately resulting in cell damage or death at point G when the energy density is relatively high. The significant increase in biological activity and the most effective PBM treatment is achieved within the optimal range of parameters that correspond to sections C to D on the curve. Adapted from Calderhead and Vasily, 2016.

The beneficial dosimetric parameters for PBM are usually between 1–100 mW/cm² for power density and 0.1–50 J/cm² for energy density (Markoulli *et al.*, 2021). Studies report successful tissue repair and anti-inflammatory effects from 5–50 mW/cm² at the target tissue depth (Hashmi *et al.*, 2010). De Abreu reported that favorable outcomes on KCs, including viability, proliferation, and migration, were mostly obtained in the 0.1–5.0 J/cm² range for blue, red, and NIR light. Energy densities above 15 J/cm² were ineffective or tended to cause cell death (de Abreu *et al.*, 2019). However, it is believed that the optimal values for illumination would differ according to the wavelength used, the type and depth of the targeted tissue, the intended therapeutic effect, and the patient’s characteristics (skin color, age, amount of hair, and fat). Careful consideration and adaptation of these parameters are necessary to ensure optimal treatment outcomes. Failure to

choose appropriate parameters for individual patients can result in reduced effectiveness or even negative effects of the therapy. These interconnected considerations and patient-specific factors contribute to the complexity of PBM dosimetry, posing challenges for optimization in both research and clinical settings (Hamblin *et al.*, 2018).

3.4. Skin optics and chromophores

When light is applied to the skin, it encounters various optical interactions. These properties include reflection, scattering, and absorption. Reflection occurs when light bounces off the skin's surface. Scattering refers to the random diffuse light redirection due to interactions with skin structures, such as collagen fibers. Absorption refers to the process in which light is absorbed by photoreceptors, also known as chromophores, generally present in the skin, such as melanin and hemoglobin (Hamblin *et al.*, 2018).

To be clinically effective, the light used in PBMT should meet two essential criteria. Firstly, it needs to have good penetration to reach the target cells. Secondly, it should correspond to the absorption spectrum of one or more biologically active photoreceptors (Barolet, 2008).

3.4.1. Photon penetration into the skin

One parameter, above all others, accounts for how deep the light energy will intrinsically penetrate the skin: the wavelength. This is because the optical properties of the skin (absorption and scattering) are wavelength-dependent, meaning that different wavelengths of light interact differently with the skin, influencing the depth to which the light energy can penetrate (Calderhead and Vasily, 2016). Generally, as the wavelength gets longer, absorption and scattering become significantly less (Hamblin, 2017). Thus, longer wavelengths, such as red and NIR (600 – 950 nm), can penetrate deeper into the skin, while shorter wavelengths in the blue-green region are more likely to be absorbed or scattered, limiting their penetration (Figure 12) (Simpson *et al.*, 1998; Van Tran *et al.*, 2021). The penetration depth of NIR is maximal at about 810 nm, and at longer wavelengths greater than 950 nm, water becomes an important absorber, and penetration depth gets shorter again (Figure 12) (Hamblin, 2017).

Wavelengths between 600 and 950 nm (red and NIR) are interesting in PBM because they can penetrate deeply beyond 2 mm into the dermis and can even reach the hypodermis and subcutaneous structures, with potential effects on vessels, muscles, tendons, and nerves. For

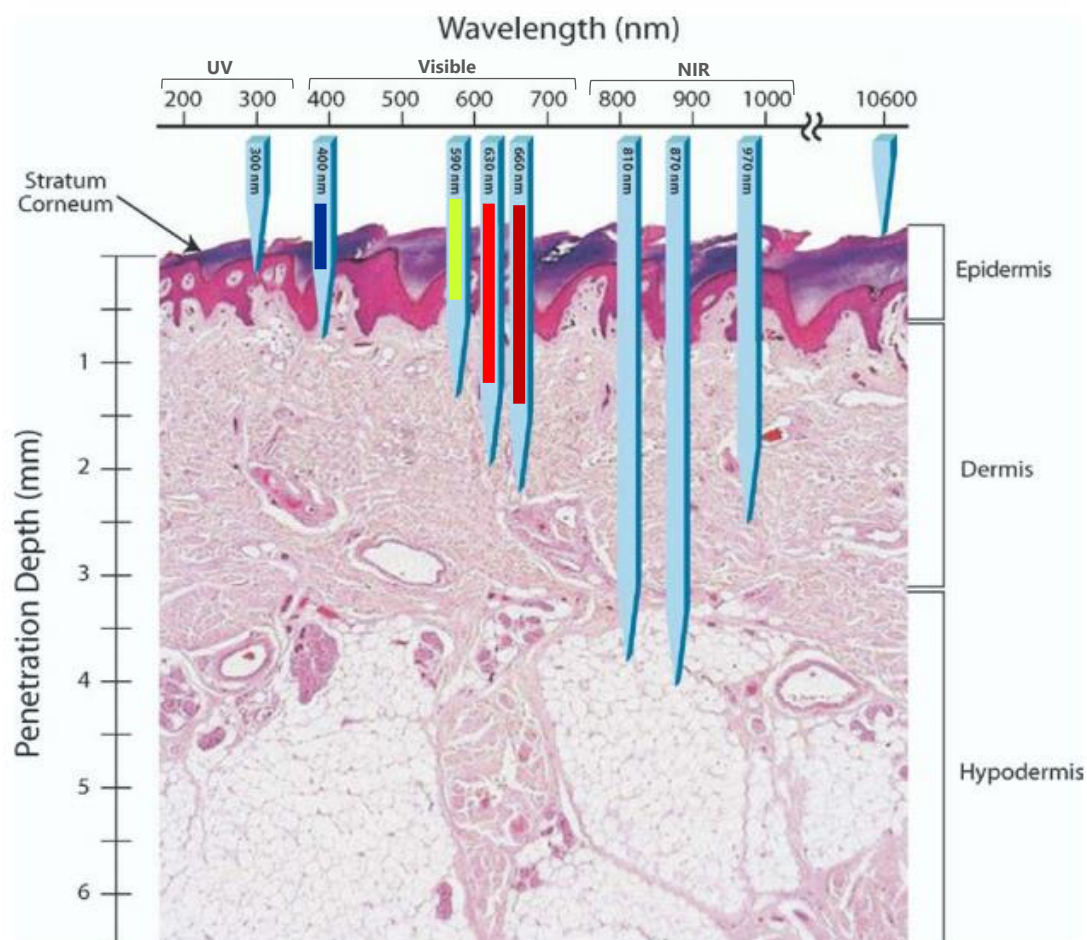


Figure 12. The penetration depth of different wavelengths through the human skin layers. The UV radiations are limited to the epidermis (< 0.1 mm). Blue and green light (400 – 550 nm) penetrates less than 1 mm, primarily affecting the epidermis and the upper dermis. Yellow and orange light (550 – 600 nm) penetrates slightly deeper than blue and green light, reaching the mid-dermis with a penetration depth ranging from 1 to 2 mm. Red light (600 nm – 700 nm) can reach a depth of 2 – 3 mm covering up to the reticular (lower) dermis, while the deep red and NIR (700 – 950 nm) have the deepest penetration into the skin to 3–6 mm, reaching subcutaneous tissues and deeper structures. However, NIR ranging between 950 - 1200 nm are more absorbed and scattered than shorter NIR wavelengths, limiting their penetration depth to less than 2 mm. Adapted from Barolet, 2008.

instance, major cells playing a role in wound healing and rejuvenation are in the dermal extracellular matrix, namely, the fibroblasts, mast cells, neutrophils, and macrophages. However, the green or yellow wavelength does not penetrate deeply enough to stimulate these cells effectively. That is why NIR wavelengths, such as 810 nm, are considered optimal for photoactivating these cells and promoting their therapeutic effects (Calderhead and Vasily, 2016). It is important to note that the percentage of photons that reach the target cells *in vivo* is particularly low, and conclusions from studies conducted on cell cultures cannot be directly extrapolated (Noé, 2014). For instance, the penetration to a depth of 2 cm of low-energy laser radiation is 2.1% for a 675 nm semiconductor laser and only 0.3% for a 632 nm Helium-Neon laser (Kolárová *et al.*, 1999). It is likely to be even

lower for LEDs, whose radiation is not collimated. However, the small number of photons penetrating is sufficient to induce cellular reactions, as evidenced by numerous publications (Musstaf *et al.*, 2019). Many wavelengths in the red (600–700 nm) and NIR (770–1200 nm) spectral regions have shown positive results. However, there is a region between 700–770 nm where the wavelengths have been found to have limited biochemical activity, and results tend to be less satisfactory overall, so they are not used in practice (Hamblin, 2017).

Recently, lower wavelengths, mainly blue and green, have also begun to be explored in PBM but have short penetration depth (Serrage *et al.*, 2019). As a result, they are primarily useful for treating skin conditions located within the epidermis (Sadowska *et al.*, 2021). Their effects remain superficial, so they are often combined with red and NIR wavelengths in clinical applications to optimize therapeutical outcomes (Noé, 2014).

3.4.2. Photon absorption by chromophores

The first law of photobiology states that the photons must be absorbed by specific molecular chromophores for the light to exert an effect on a biological system. Chromophores are the initial photoreceptor molecules that can absorb the incident photon energy at certain wavelengths to initiate a biological effect (Musstaf *et al.*, 2019). Once absorbed, the energy of the photons undergoes a photochemical internal conversion within the chromophore. Photoreceptor molecules are believed to act through either directly absorbing the energy of photons, becoming electrically excited and then directly partaking in chemical reactions, or by the transfer of energy of the absorbed photon to another molecule, which is then able to induce chemical reactions and ultimately a biological effect (Karu, 1999; Musstaf *et al.*, 2019). Here are different chromophores that could be found in the skin: endogenous nucleic acids, aromatic amino acids, urocanic acid, tryptophan, tyrosine, NADPH, NADH cofactors, cytochromes, riboflavins, porphyrins, melanin, protoporphyrin IX, bilirubin, hemoglobin, β -carotene, lipids or water molecules (Sowa *et al.*, 2013).

Some chromophores in the skin are tissue components that can absorb specific wavelengths, thereby limiting their further penetration. These biological components, namely, the pigments (melanin, oxyhemoglobin, and deoxyhemoglobin) and tissue water, have an optimum range of wavelengths at which they highly absorb light (Figure 13) (Chung *et al.*, 2012).

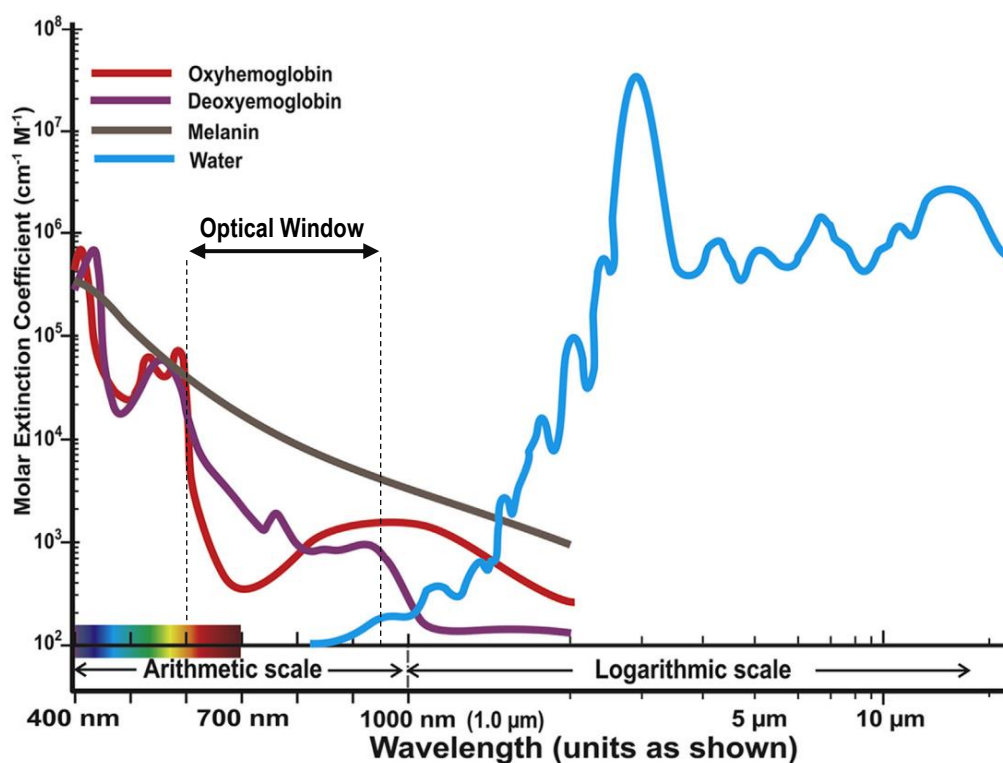


Figure 13. Absorption spectra of main tissue constituents acting as chromophores to visible, NIR and midinfrared wavelengths. The absorption spectra vary among chromophores. Hemoglobin and melanin have high absorption bands at wavelengths shorter than 600 nm. Hemoglobin has two peaks of light absorption within the visible light range. The absorption of light by melanin progressively decreases with increasing wavelengths of light. In contrast, the absorption of light by water molecules generally increases as wavelengths increase and becomes significant for wavelengths over 1000 nm. A “Therapeutic optical window” to light penetration into tissue is defined between 600 and 950 nm in the red and NIR spectral region because of minimized absorption and scattering of light by the most important tissue chromophores. Adapted from Barolet, 2008.

Two major wavelength boundaries, less than 600 nm and more than 1000 nm, impact photon absorption when it comes to PBM application in dermatology. At wavelengths <600 nm, melanin in the epidermis and hemoglobin in dermal blood vessels are major obstacles to photon penetration, as they significantly absorb these photons. Additionally, at wavelengths >1000 nm, water absorbs a significant number of photons, limiting their availability for downstream specific chromophores located, for example, in dermal fibroblasts (Barolet, 2008). Between these two limits, there is a range of wavelengths where laser and LED applications can be explored. This range is the so-called “therapeutic optical window” covering the red and NIR region, from 600 - 950 nm, as mentioned earlier, where the absorption is less likely to happen and, thus, the effective skin penetration of light is maximized (Figure 13). Therefore, although blue, green, and yellow light may significantly affect cells *in vitro*, the use of PBM to target deep cutaneous targets in animals and patients primarily involves red (600-700 nm) and NIR light (780–950 nm) (Chung *et al.*, 2012).

Regarding the chromophores responsible for the PBM effects, three broad classes of primary chromophores have so far been identified. One is the mitochondrial Cytochrome C Oxidase (CCO), mainly absorbing red light and NIR (600-1100nm), another is opsins absorbing UVA – yellow lights (380-560 nm), and flavoproteins that can absorb blue-green lights (400-550 nm), and a third is nanostructured water clusters absorbing NIR (Sharma *et al.*, 2023) (Figure 14).

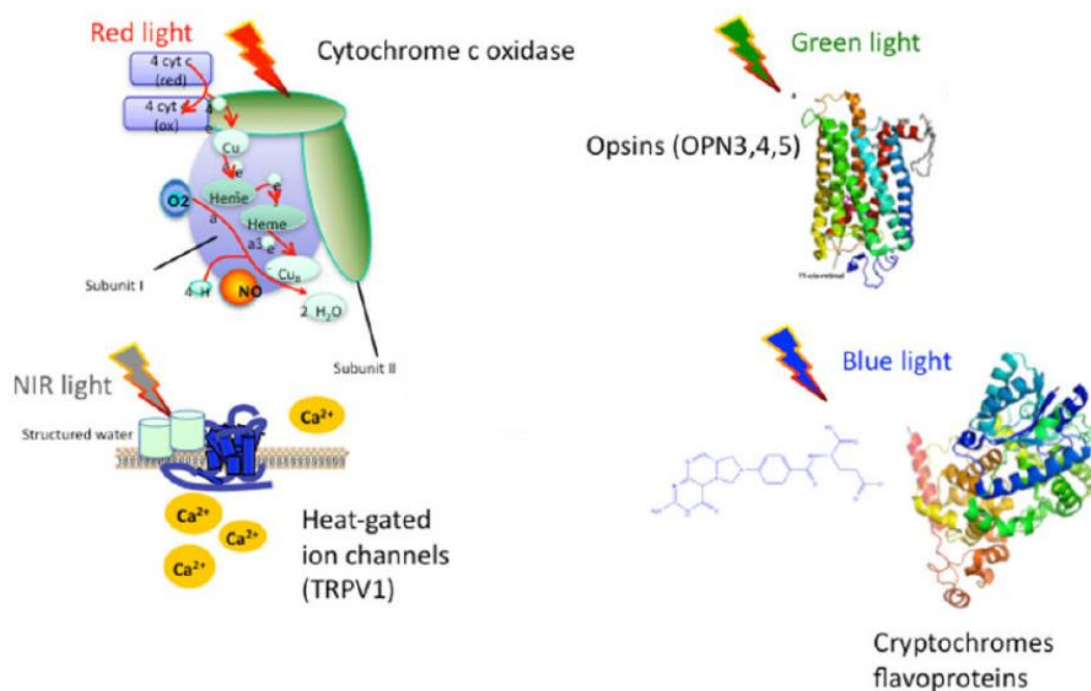


Figure 14. Main chromophores responsible of PBM effects. The primary chromophore involved is cytochrome c oxidase found in the mitochondrial respiratory chain, which predominantly absorbs red and near-infrared (NIR) light through heme and copper. Heat-gated ion channels, including Transient Receptor Potential Vanilloid 1 (TRPV1) mainly responds to NIR (and blue-green light) through the absorption of photons by structured water clusters. Opsins, on the other hand, absorb blue and green light through cis-retinal, while flavoproteins and cryptochromes predominantly absorb blue light via pterin (Hamblin, 2017).

One of the long-standing mechanisms of action of PBM described in the literature is associated with the mitochondria. Mitochondria, cellular organelles present in all cells, are responsible for producing a major proportion of energy in the form of ATP through a series of redox reactions in their membrane-bound respiratory chain. Tiina Karu was a pioneer in suggesting that the beneficial effects of PBM are primarily mediated by chromophores located within the mitochondria (Karu, 1999). In their study, Karu *et al.* aimed to determine the specific photo-acceptor responsible for triggering the biological effects observed at the cellular level (Karu and Afanas'eva, 1995). They measured *in vitro* light absorption peaks during various biological effects, such as DNA synthesis. The measured peaks closely matched the known absorption spectrum of light for

Cytochrome c oxidase (CCO) under its different oxidation states. Based on this observation, they concluded that CCO is the primary capturing entity of red and NIR light (Karu and Afanas'eva, 1995; Karu and Kolyakov, 2005). CCO, the complex IV in the mitochondrial respiratory chain, consists of multiple protein subunits and contains two heme centers, heme-a and heme-a₃, and two copper centers, CuA and CuB centers. All these centers can be in a reduced or an oxidized state, resulting in many possibilities of oxidation states for the enzyme, and each has different absorption spectra. The absorption spectra obtained for CCO in different oxidation states were found to be very similar to the action spectra of PBM, which represents the most efficient wavelengths in mediating the PBM process (Karu, 2010).

Since a relatively broad range of wavelengths has been shown to have beneficial biological effects (at the correct doses), it is unlikely to be only a single chromophore (for instance, CCO) responsible for absorbing the photons. Many researchers suggested that the response of cells to blue or green light is mediated through light-gated ion channels, which enable light to control electrical excitability, intracellular acidity, calcium influx, and other cellular processes. The most likely ion channel is the light-gated channel opsin because the action spectra of the opsin family display peaks in the blue-green spectral region (Mustaff *et al.*, 2019). A study emphasized that blue (420 nm) and green (540 nm) lights are more effective in increasing Ca²⁺ than red or NIR when applied at the same doses, and this calcium influx is generated by the Transient Receptor Potential Vanilloid 1 (TRPV1) cation channels, leading to an inhibition of the proliferation of human adipose-derived stem cells (Wang *et al.*, 2017b). There are also reports of the effectiveness of wavelengths outside the range of absorption of NIR light by CCO, suggesting that wavelengths longer than 900 nm are also absorbed by another chromophore and not just by CCO. The chromophore in these situations is almost certainly water, possibly present in biological membranes in some nanostructured form that is different from bulk water, allowing biological effects without gross tissue heating. Absorption of photons by these water clusters can cause molecular vibration sufficient to perturb ion channels and alter cellular function. However, it is unclear which wavelength CCO absorption ceases and water absorption becomes important (Chung *et al.*, 2012).

PBM is initiated through the interactions between photons and photo-acceptor molecules, resulting in a sequence of primary and secondary events driving the biological effects of PBM. Firstly, photon absorption by its appropriate chromophore generates various triggering molecules and agents capable of inducing a cascade of events involving signaling molecules, effector molecules,

and transcription factors at the molecular level (Figure 15). These molecular events are pivotal in mediating various cellular responses, including cell proliferation, migration, differentiation, and apoptosis, thereby characterizing the cellular aspects of PBM. Ultimately, the combined molecular and cellular effects culminate in broader systemic outcomes, such as the modulation of inflammatory processes, facilitation of tissue repair and wound healing, reduction of edema and pain, and enhancement of muscle performance, thus defining the systemic dimensions of PBM (Barolet *et al.*, 2023).

Various chemical agents, including nitric oxide (NO), ATP, cyclic AMP, Ca²⁺, and ROS, have been associated with light-induced primary effects that act as second messengers for subsequent signaling events (Figure 15) (de Freitas and Hamblin, 2016). The absorption of low-power red light or NIR (700-1100 nm) promotes the photodissociation of NO from CCO. NO originally inhibits CCO activity by non-covalent binding between the heme centers a3 and CuB, leading to competition with oxygen at this site. Consequently, the dissociation of NO by light from CCO increases the enzyme activity and, thus, the respiration rate. This mechanism is believed to drive the observed increase in the production of ATP (Huang *et al.*, 2014; Wang *et al.*, 2017a; Gonçalves de Faria *et al.*, 2021) and ROS (Lavi *et al.*, 2003; Zhang *et al.*, 2008), implicated in the phototransduction pathways associated with PBM (Chung *et al.*, 2012) (Figure 15). Alterations in cellular redox status play a pivotal role in triggering a multitude of intracellular signaling pathways, including those involved in nucleic acid synthesis, protein synthesis, enzyme activation, and progression through the cell cycle. These intracellular responses in the cytosol can subsequently lead to changes in gene expression at the transcriptional level. Several key transcription factors are known to be influenced by shifts in cellular redox state. Notably, crucial transcription factors impacted by these changes include redox factor-1 (Ref-1)-dependent activator protein-1 (AP-1) comprising Fos and Jun, NF-κB, p53, activating transcription factor/cAMP-response element-binding protein (ATF/CREB), and hypoxia-inducible factor (HIF)-1 (Farivar *et al.*, 2014; Barolet *et al.*, 2023).

It is suggested that PBM has more pronounced effects on diseased or damaged cells and tissues compared to healthy cells, primarily attributed to elevated levels of inhibitory NO in unhealthy or hypoxic cells. Notably, the modulation of ROS by PBM was shown to be dependent on the initial physiological condition of the cell at the moment of irradiation. In healthy cells, PBM increases ROS levels, whereas it tends to attenuate them in stressed cells (Barolet, 2008; Hamblin, 2018) (further discussed in *section 4.2* of this chapter).

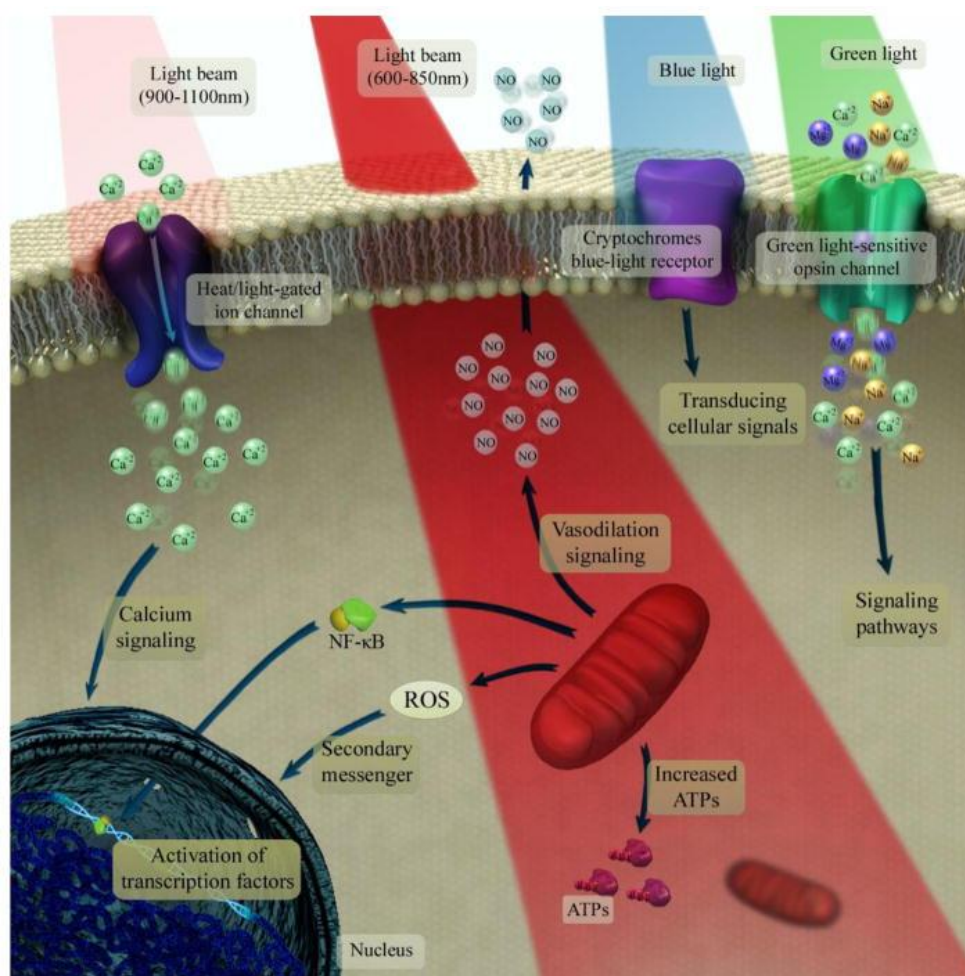


Figure 15. Light chromophores and PBM underlying mechanisms at the cellular and molecular levels. The light within the range of 600-850 nm (red) is mainly absorbed by the cytochrome C oxidase of the mitochondrial electron transfer chain, resulting in secondary mechanisms involving the upregulation of ATP, ROS, and NO. In the range of 900-1100 nm, near-infrared light is absorbed by structured water clusters, which are present in or on heat/light-gated ion channels. This absorption increases the vibrational energy of the water clusters, causing perturbations in the protein structure and opening the ion channels, thereby modulating intracellular Ca^{2+} levels. Green light absorption by opsin photoreceptors activates transient receptor potential channels, leading to non-selective permeabilization to Ca^{2+} , Na^+ , and Mg^{2+} . ROS and cations act as secondary messengers, inducing signaling pathways that are crucial for cellular processes, such as migration and proliferation. Additionally, cryptochromes, a class of flavoprotein blue-light signaling receptors, absorb blue light and appear to activate cellular signals playing a crucial role in regulating the circadian clock (Salehpour et al., 2018).

4. CONTROL OF SKIN INFLAMMATION BY PBM

As an alternative therapy to conventional pharmacological treatments of skin inflammation, PBM offers a non-invasive, non-thermal, and safe approach, effectively reducing inflammation and enhancing the healing process (Avci et al., 2013; Jagdeo et al., 2018). PBM has been shown to exert anti-inflammatory effects at both the cellular and molecular levels, although the exact mechanisms underlying these effects are complex and multifaceted (Hamblin, 2017; Sadowska et al., 2021).

4.1. Cellular anti-inflammatory effects of PBM

One of the primary cellular mechanisms contributing to the anti-inflammatory effect of PBM involves the **modulation of pro-inflammatory mediators**, including cytokines such as TNF- α , IL-1 β , and IL-6. PBM has effectively suppressed the expression and secretion of these cytokines, which are key contributors to the initiation and progression of many chronic inflammatory skin diseases, such as psoriasis or AD (Sharma *et al.*, 2022). *In vitro* studies using TLR-agonists such as bacterial LPS to induce inflammation supported the anti-inflammatory effect of PBM by decreasing pro-inflammatory mediators after red light and NIR exposure. It was shown that the illumination of human gingival fibroblasts with red LED suppressed the secretion of LPS-induced pro-inflammatory mediators, such as IL-6, IL-8, cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂), associated to modulation of MAPK signaling pathway (Choi *et al.*, 2012) and ROS inhibition (Lim *et al.*, 2015). Similarly, LPS-induced IL-6 and IL-8 mRNA expressions were significantly decreased by low-level 805 nm (NIR) laser irradiation of the oral squamous epithelial carcinoma cell line, Ca9-22 (Fujimura *et al.*, 2014). Lim *et al.* compared the anti-inflammatory effect of 635 nm LED with existing COX inhibitors (indomethacin and ibuprofen) and concluded that red light irradiation of human gingival fibroblasts inhibits COX and PGE₂ expression like COX inhibitors, but further able to directly decrease ROS levels and inhibit phospholipase A₂ expression unlike these drugs, and should thus be considered useful as an anti-inflammatory tool (Lim *et al.*, 2007).

Regarding the effects of PBM on the immune cells, it was shown that 810 nm NIR laser has an anti-inflammatory effect on LPS or CpG-activated murine bone marrow-derived DCs, possibly through reduced NF- κ B signaling. PBM decreased IL-12 secretion by DCs and altered the balance of their activation markers: MHCII was upregulated while CD86 was downregulated (Chen *et al.*, 2011b). Fernandes *et al.* showed murine J774 macrophage-like cells activated by LPS and IFN- γ reduced their expression of inflammatory M1 markers, such as TNF- α , inducible nitric oxide synthase (iNOS) and COX-2, following the illumination with 660 nm (red) or 780 nm (NIR) lasers (Fernandes *et al.*, 2015). A recent study of the same group reported that red and NIR lasers could reduce the gene expression of several pro-inflammatory chemokines, such as CXCL2 and CCL3, and cytokines like TNF- α in M1 macrophages. Whereas NIR laser promoted a temporal increase by M2 macrophages of TGF- β 1 expression, a key regulator in tissue repair (de Brito Sousa *et al.*, 2020). These results align with other recent studies demonstrating that NIR increases M2 polarization *in vitro* (Tian *et al.*, 2023) and *in vivo*, where it was associated with accelerated wound healing (Ryu *et*

al., 2022). These results suggest that PBM plays an important role in modulating acute inflammation and influencing the tissue repair process through its differential effects on pro- and anti-inflammatory mediators expression by polarized macrophages.

Growing evidence further supports that PBM regulates skin inflammation using different inflammatory animal models, such as AD, rosacea, or burn wound models. These *in vivo* studies have broadly reported a **decrease in pro-inflammatory mediators** and a **reduction in the infiltration of inflammatory immune cells** into the irradiated site of the lesion. In the DNCB-induced atopy mouse model, illumination with visible red at 650 nm alleviates symptoms of AD, restores cytokine levels of IL-6 and TNF- α back to normal in the spleen, and diminishes epidermal thickness and mast cell counts in skin lesions (Kim *et al.*, 2021).

Rosacea is a chronic inflammatory skin disease involving angiogenesis, immune activation, and inflammatory pathways activation. Rosacea lesions are characterized by the infiltration of several immune cells, including CD4⁺ memory T cells, $\gamma\delta$ T cells, neutrophils, and M1 macrophages, and a decrease in Treg cells. It was recently shown in a rosacea-like mouse model induced by LL-37 peptide intradermal injections that the untraditional yellow-orange (590 nm) light combined with NIR (830 nm) light was able to attenuate the severity of erythema and the expression of inflammatory mediators (S100A9 and p65), together with a decrease in neutrophils, macrophages infiltration in skin lesions and an increase in Treg cells (Wu *et al.*, 2022). In the same mouse rosacea-like model, Jiang *et al.* showed that 590 nm light alleviated inflammation with downregulation of key inflammatory mediators in rosacea, such as IL-6, TNF- α , MMP-9, KLK5, and LL-37 expression. In the same manner, Lee *et al.* demonstrated that LEDs at 630 and 940 nm downregulated TLR-2, KLK5, and LL-37 expressions and protease activity in normal human epidermal KCs *in vitro* as well as in rosacea-like mouse skin, maintaining lower IL-8 protein expression than the control group (Lee *et al.*, 2016). Interestingly, a reduced percentage of degranulating mast cells in the illuminated rosacea lesions was reported, suggesting a newly described mechanism underlying the anti-inflammatory effect of PBM in rosacea (Jiang *et al.*, 2023).

A study on a rat burn wound daily irradiated with 660 nm (red) low-power laser or 850 nm (NIR) LED for 7 consecutive days showed inhibition of IL-6 expression at the site of injury, associated with a decrease in oxidative stress (nitrites, superoxide anion) and an improved cutaneous antioxidant profile, thus, accelerating the inflammatory phase of the wound healing process and the

improvement of skin repair (Silveira *et al.*, 2016). Using the same experimental model, Gupta *et al.* showed no inflammation and faster wound contraction after seven days in the superpulsed NIR 904 nm-treated wounds compared to control or silver sulfadiazine-treated wounds, a commonly used topical burn treatment. Interestingly, this result was associated with less infiltration of inflammatory cells, like neutrophils and lymphocytes, at the irradiated wound site, better neo-vascularization, and re-epithelialization with dense collagen deposition. These observations were explained by a decrease in the TNF- α level at the irradiated-wound site, concomitantly with NF- κ B inhibition; meanwhile growth factors, MMPs that are crucial for tissue remodeling, and cytoprotective HSP-60, and HSP-90 increased post-irradiation (Gupta *et al.*, 2015). Green LED (550 nm, 60 J/cm², 10s), a less documented light than red or NIR, was also able to reduce inflammatory cell infiltration into the burn site, composed predominantly of neutrophils and lymphocytes (Catão *et al.*, 2016).

Nevertheless, third-degree skin burns in rats daily irradiated with 630 nm (red) LED (36 J/cm², 30 sec) showed higher inflammatory infiltrate at days 7 and 14 and failed to modulate it at 21 or 32 days post-irradiation, suggesting that red light might only modulate the number of inflammatory cells in the early stages of the healing process of skin burns and not advanced ones (Simões *et al.*, 2022). However, the increase in immune cells at burn sites may be due to inappropriate irradiation parameters, such as a too-high fluence (36 J/cm² in this study vs. 10 J/cm² in Silveira *et al.* study), or the irradiation time was too short to produce positive effects (30 sec of exposition in this study vs. 10 min of LED application in Silveira *et al.* study).

4.2. Molecular mechanisms of PBM anti-inflammatory effects

Several molecular mechanisms have been proposed to explain the well-established anti-inflammatory effect of PBM. However, conclusive evidence about their exact involvement in the therapeutic action of PBM is still lacking. First, the anti-inflammatory effect of PBM is presumed to be a consequence of ROS modulation in the cells. Since ROS are described as an essential mediator of pro-inflammatory cytokine production, promoting inflammation and tissue damage (Korkina and Pastore, 2009; Biswas, 2016), PBM can indirectly regulate inflammation through its antioxidant activity (Hamblin, 2017). Previous reports described the binary effect of PBM regarding ROS level modulation, as it acts differently depending on the initial state of the cells. For instance, it was shown that the 904-nm laser reduces oxidative stress markers in the wounded skin of diabetic mice, while it increased them in irradiated controls (Tatmatsu-Rocha *et al.*, 2016). Moreover, Huang

et al. found that NIR PBM (810 nm laser, 3 J/cm², 150 sec) reduced intracellular ROS production in primary murine cortical neurons after 1 hour of treatment with chemicals inducing oxidative stress. In contrast, control cells at basal conditions (without oxidative stress) had a slight increase in ROS (Huang *et al.*, 2013). The same wavelength was reported to induce ROS in healthy murine embryonic fibroblasts by activating NF-κB (Chen *et al.*, 2011a). In HaCaTs cells, it was reported that 660 nm laser red light (3 J/cm², 60 sec) favored ROS production 4 min after an oxidative stimulus contrary to 800 nm, 970 nm lasers, or the combination of the three different wavelengths that exerted a reduction of ROS levels. None of these tested laser protocols changes the redox state of KCs at baseline (Rupel *et al.*, 2018). Additional variability could be attributed to the model and the wavelength used, the timing at which ROS levels are assessed following PBM, and the tool used to measure them. It is important to recall that the modulation of ROS by the light follows the biphasic dose-response model, as previously illustrated (George *et al.*, 2018).

Other mechanisms may be involved in controlling inflammatory responses by PBM. Light can modulate various intracellular signaling pathways involved in inflammation, such as NF-κB, MAPKs, and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways. These transduction pathways regulate multiple aspects of immune functions and are key mediators of cytokines production and other inflammatory responses (Liu *et al.*, 2017, 2020). Specifically, PBM has been shown to alter the activity of NF-κB, a transcription factor that plays a crucial role in the regulation of immune and inflammatory responses. PBM has been shown to inhibit the expression and activation of NF-κB, thereby preventing the expression of pro-inflammatory genes (Curra *et al.*, 2015; Gupta *et al.*, 2015). Kim *et al.* reported that the anti-inflammatory effect of violet light (410 nm) in normal human epithelial KCs is NO and proteins S-nitrosylation dependent, resulting in a loss of activity of proteins involved in promoting inflammation such as MyD88 and NF-κB (Kim *et al.*, 2016). Interestingly, it was shown that red light (660 nm) stimulates KCs migration in the early stage of wound healing and KCs differentiation at the final stage by modulating epigenetic events (histone 3 acetylation) and NF-κB expression (de Farias Gabriel *et al.*, 2019). Furthermore, the previously mentioned study of Choi *et al.* demonstrated that PBM modulates p38, JNK, and extracellular signal-regulated kinase (ERK 1/2) phosphorylation in the MAPK signaling pathway, concomitantly to the decrease of pro-inflammatory mediators by gingival fibroblasts (Choi *et al.*, 2012). A similar increase in phosphorylated pERK 1/2 was also reported in the burn wound healing process (Silveira *et al.*, 2016).

ERK 1/2 acts inside the cell to induce mitosis and cell division, thereby initiating signaling cascades related to the healing process (Javierre *et al.*, 2009).

These major observations do not exclude the role of other important mediators and signaling pathways in regulating inflammation by PBM, as proposed by different studies. Becker *et al.* suggested the transcription factor aryl hydrocarbon receptor (AHR) as a possible target for blue light-preventing inflammatory responses in HaCaTs cells (Becker *et al.*, 2016). AHR regulates target genes involved in various biological processes, including xenobiotic metabolism, immune responses, and inflammation. The mRNA expression of the Developmental endothelial locus-1 (Del-1), an inhibitor of lymphocyte function-associated antigen-1 (LFA-1)-dependent neutrophil adhesion, was significantly upregulated by laser irradiation. Del-1, known to have an anti-inflammatory activity, is a regulator inhibiting particularly the IL-17-mediated inflammatory response (Fujimura *et al.*, 2014).

All the observations mentioned above reporting cellular factors modulated by light in correlation with an anti-inflammatory activity lack strong evidence about the direct implication of these factors in the PBM therapeutic effect. Interestingly, using pharmacological inhibitors for CB1 and CB2 cannabinoid receptors, Neves *et al.* showed that these receptors play a crucial role in the inhibition of carrageenan-induced paw edema in mice following plantar irradiation with red light (Neves *et al.*, 2018).

5. CLINICAL APPLICATIONS OF PBM IN INFLAMMATORY SKIN DISEASES

In the past decade, there has been a growing focus on the clinical applications of PBM, primarily due to its stimulatory and anti-inflammatory effects (Hamblin, 2017). This non-invasive therapeutic approach has gained significant attention as it offers an effective strategy with minimal contraindications and negligible side effects. Since the early 2000s, the clinical application of PBM has expanded extensively in various fields, such as rheumatology, orthopedics, dentistry, and neurology, benefiting from numerous innovative methods developed to deliver light to specific target cells or tissues (Dompe *et al.*, 2020). In dermatology, PBM has emerged as a promising versatile therapy for medical or aesthetic applications treating many skin conditions and diseases. As PBM exhibits anti-inflammatory activity, it is particularly beneficial for certain inflammatory skin pathologies, such as acne vulgaris, herpes lesions, psoriasis, eczema, and wound healing. Additionally, PBM has shown positive impacts on various cellular processes, including proliferation, migration, protein synthesis, and cytoprotection, making it advantageous for treating other skin

conditions or diseases like wrinkles, alopecia, vitiligo, UV-photoprotection, and chronic or acute wound healing (Avci *et al.*, 2013; Jagdeo *et al.*, 2018; Sadowska *et al.*, 2021).

The growing literature on *in vitro* and preclinical PBM experiments offers promising prospects for its various indications in dermatology. However, these studies in no way replace controlled clinical trials, although the latter are now sufficiently numerous to arouse interest in the potential of this approach (Noé, 2014; Jagdeo *et al.*, 2018). In their systematic review of LED-based PBM applications, Jagdeo *et al.* provide evidence-based recommendations for the therapeutic use of PBM in dermatology based on published clinical data. Grades of recommendation were assigned based on the Oxford Centre for Evidence-based Medicine, known as Levels of Evidence, used to assess the strength and quality of evidence in medical research and, thus, help researchers and clinicians evaluate the reliability of the findings (Howick *et al.*, 2011). These levels categorize studies based on their design and methodology, ranging from Level 1 (highest level of evidence) to Level 5 (lowest level of evidence). Here is a brief overview of the different grades of recommendation for a specific treatment indication:

- **Grade A** (level 1): High-quality evidence from well-conducted randomized controlled trials (RCTs) or systematic reviews/meta-analyses of RCTs with homogeneity in the results between individual studies. These studies consistently show significant benefits of the treatment and offer robust conclusions about them.
- **Grade B** (level 2-3): Moderate-quality evidence from RCTs with some limitations or well-conducted non-randomized controlled cohort, follow-up, or observational studies. These studies provide good support for the treatment benefits.
- **Grade C** (level 4): Limited-quality evidence from case series, case-control studies, poor-quality RCTs, or observational studies. The evidence may have methodological flaws, inconsistency, or conflicting results. The recommendation is based on a consensus rather than strong empirical evidence.
- **Grade D** (level 5): Very limited or conflicting evidence, including studies with a high risk of bias or insufficient data. The recommendation is primarily based on expert opinion or mechanism-based reasoning.

Below are the main inflammatory skin pathologies that can be treated with PBM.

5.1. Acne vulgaris

Acne vulgaris is a widely recognized skin chronic inflammatory disease that affects over 85% of adolescents and contributes to more than 30% of dermatology cases. The pathogenesis of acne vulgaris often involves *Propionibacterium acnes*, which leads to the development of inflamed lesions. Successful clinical treatment of acne is typically associated with a decrease in the abundance of *P. acnes*. Traditional approaches for acne management include topical and systemic antibiotics, topical retinoids, and chemical peels. However, these methods offer limited effectiveness and may result in side effects like a burning sensation and antibiotic resistance. As a result, phototherapy, particularly using LED lights, has emerged as a promising alternative for acne treatment (Van Tran *et al.*, 2021).

A wide range of LED devices has received clearance from the Food and Drug Administration (FDA) for the treatment of mild and moderate acne. FDA clearance guarantees that these medical devices are both safe and effective when used as directed for this specific clinical application. Jagdeo *et al.* attributed a grade B of recommendation for acne treatment by PBM (Jagdeo *et al.*, 2018). A recent systematic review concluded an overall improvement of acne by blue light across the reviewed studies (Diogo *et al.*, 2021). Several clinical studies showed that blue and red lights decreased significantly inflammatory and non-inflammatory acne lesions, as well as the size of the lesions, compared to no-treatment or the placebo (Gold *et al.*, 2011; Kwon *et al.*, 2013; Ash *et al.*, 2015), or to conventional topical acne treatment such as benzoyl peroxide (Nestor *et al.*, 2016; Cheema *et al.*, 2018). Other studies comparing LED therapy to other phototherapies, such as PDT or intense pulsed laser (IPL), showed that although PBM required more sessions to achieve 90% of lesion clearance, it reported no side effects, unlike the other therapies and, thus, may be safer for home use (Sami *et al.*, 2008; Liu *et al.*, 2014).

The characteristics of the light parameters used are varied across these studies. However, blue and red lights are the most used wavelengths for treating acne in clinical studies, with an average treatment of 4 weeks with a 20-minute exposition twice a week. Both lights exert an antimicrobial effect as light is absorbed by porphyrins generated within sebaceous follicles by *Propionibacterium acnes* (Pei *et al.*, 2015). Porphyrins absorb light wavelengths between 400 and 700 nm, with 415 nm wavelength within the blue light spectrum being most effectively absorbed. Light absorption leads to the photo-excitation of porphyrins and the subsequent release of singlet oxygen and reactive free

radicals that exert bactericidal effects and can damage bacterial cells, including *P. acnes* (Kjeldstad and Johnsson, 1986; Dai *et al.*, 2012). Furthermore, red and blue light have well-established anti-inflammatory properties, modulating cytokine release, and affecting immune cells (Shnitkind *et al.*, 2006; Lim *et al.*, 2007; Fischer *et al.*, 2013). Both lights also influence lipid production in sebocytes and have beneficial effects on acne by suppressing sebum production (Kwon *et al.*, 2013; Jung *et al.*, 2015). Although red light activates porphyrins less effectively than blue light, it is usually applied in order to target sebaceous glands better and actively destroy *P. acnes* bacteria residing in their lower regions due to its deeper tissue penetration in the skin. Red light also exerts anti-inflammatory properties and accelerates tissue healing and regeneration (Greaves, 2016). Combining blue and red light synergizes in attenuating inflamed acne lesions (Papageorgiou *et al.*, 2000; Lee *et al.*, 2007). Recently, a prospective study on moderate to severe acne showed significant improvements in acne lesions using the NIR radiations (785 nm) with a reduction in skin sebum excretion (Szymańska *et al.*, 2021).

5.2. Herpes simplex

Herpes simplex virus type 1 (HSV-1) infection is a widespread condition that primarily affects the oral region, causing various signs and symptoms. The disease follows a natural course consisting of primary infection, latency, and recurrent infections. Recurrent manifestations, known as herpes labialis or cold sores, are characterized by clusters of small, painful, and red papules that develop into vesicles and eventually crust over. Treatment options for herpes labialis included antiviral medications or natural remedies until the rise of PBM (Sousa and Catão, 2022). Today, several LED devices were FDA-cleared for facial herpes simplex treatment using NIR light and were assigned a grade B of recommendation for this indication (Jagdeo *et al.*, 2018). The integrative review of Sousa and Catao concludes that PBM is an effective therapeutic resource, reducing healing time, symptoms, and recurrence of lesions (Sousa and Catão, 2022). In placebo-controlled, double-blinded trials, LED-NIR treatment (3 times/day for 2 days) significantly reduced healing time by at least 2 days in patients with HSV compared to the placebo group (Hargate, 2006; Dougal and Lee, 2013). Similarly, a single-blind RCT comparing the effect of NIR -laser, non-treated control, and acyclovir cream 5% on herpes labialis showed that treatment with diode laser reduced pain severity and the length of recovery time faster than treatment with the antiviral acyclovir or non-treated group (Honarmand *et al.*, 2017).

Other wavelengths were also explored in herpes treatment by PBM, such as red light. In fact, Muñoz Sanchez *et al.* conducted an RCT in which they compared the effect of red light, 670 nm, on herpes labialis with acyclovir, including a 5-year follow-up to observe the period of recurrences. They reported that PBM was an effective therapy for both initial healing and the length of the recurrence periods, and most importantly, with no side effects (Muñoz Sanchez *et al.*, 2012). Recently, antiviral properties were described with blue light that was able to reduce viral replication *in vitro* in the HaCaT cell line and neuronal cell model, along with an increase in cell survival (Zupin *et al.*, 2018; Zupin and Crovella, 2022). In addition to light's antiviral action, blue light's therapeutic properties in treating herpes infections are also based on its anti-inflammatory effects, as recently demonstrated in M1-polarized HSV-1-infected macrophages (Oh *et al.*, 2023). Overall, PBM may be used in the early infection phase to avoid lesion exteriorization and in the latent phase to increase the interval between recurrences. Likewise, PBM may be used in the crust phase of HSV-1 infection to accelerate lesion healing and relieve symptoms (de Paula Eduardo *et al.*, 2014).

5.3. Wound healing

Acute and chronic wounds represent two distinct categories in their healing process. Acute wounds, such as surgical incisions, traumatic injuries, burns, radio-, photo-, or chemo-induced dermatitis, generally follow a predictable and timely healing process. It involves a series of overlapping phases, including hemostasis, inflammation, proliferation, and remodeling (Figure 16). Proper management and interventions can facilitate the healing of acute wounds and minimize complications. In contrast, chronic wounds are characterized by an extended and impaired healing process and remain unhealed after 12 weeks (Fernández-Guarino *et al.*, 2023). These wounds often fail to progress through the normal reparative stages and remain stuck in the inflammatory phase. Common types of chronic wounds include diabetic, venous, and pressure ulcers. Factors contributing to impaired healing in chronic wounds include persistent inflammation, inadequate blood supply, infection, and underlying health conditions (Mosca *et al.*, 2019).

The inflammatory response to wounding is vital to prevent infection and signal downstream events that lead to healing and restoring skin integrity and function. Treatments designed to improve skin regeneration must consider the role of the immune system in skin repair carefully, as many studies show that immune cells and mediators can influence scar formation and disrupt skin regeneration (Schrementi *et al.*, 2018). PBM has shown promise in the treatment of both acute (grade of recommendation, B) and chronic wounds (grade of recommendation, D), as it exhibits a well-established anti-inflammatory effect among others (Jagdeo *et al.*, 2018).

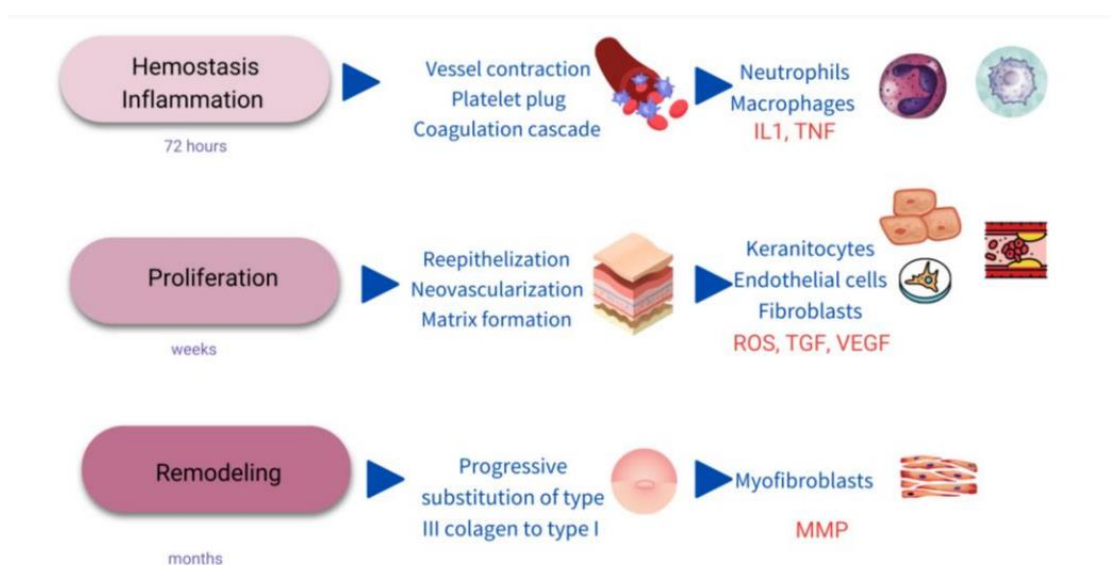


Figure 16. Different stages of wound healing and main actors and mediators contributing to each stage. IL1: interleukin1; TNF: Tumor necrosis factor; ROS: single oxygen radicals; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; MMP: metalloproteinases. From Fernandez-Guarino *et al.*, 2023

5.3.1. Acute wound healing

PBM has been shown to accelerate the resolution of the inflammation phase, enhance angiogenesis, and promote collagen synthesis, leading ultimately to faster wound closure and reduced scar formation (Fernández-Guarino *et al.*, 2023). Additionally, PBM can modulate immune responses, reduce pain, and minimize the risk of infection in acute wounds. Among the various molecular mechanisms of PBM, the activation of TGF- β 1 in the extracellular environment plays a central role in wound healing. TGF- β 1, secreted mainly by platelets and macrophages upon the inflammatory phase, is involved in tissue repair and remodeling. It promotes angiogenesis by stimulating endothelial cell proliferation and migration, which ensures an adequate blood and oxygen supply to the wound and supports tissue regeneration. It also stimulates the production of ECM components, such as collagen and fibronectin, by fibroblasts. Furthermore, TGF- β promotes

the differentiation of fibroblasts into myofibroblasts, which are responsible for wound contraction and regulate the migration and proliferation of keratinocytes, facilitating re-epithelialization (Mosca *et al.*, 2019; Prabhu *et al.*, 2020; Fernández-Guarino *et al.*, 2023). A study highlighted PBM's effectiveness in promoting epithelial cells' functions, particularly expanding their basal colony-forming units representing progenitors and stem cell populations, contributing to re-epithelialization (Khan and Arany, 2016) (Figure 17).

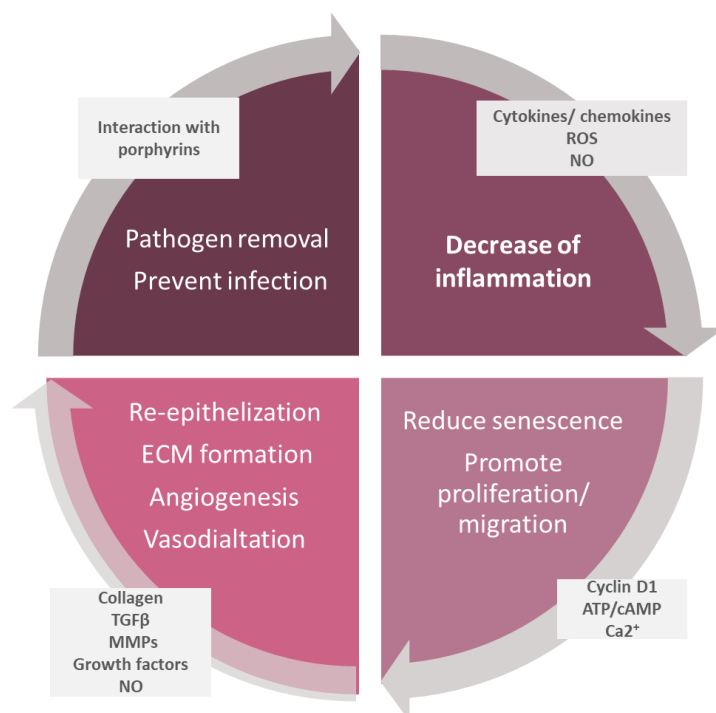


Figure 17. Diagram representing the beneficial effects of PBM in wound healing and the main implicated mediators. PBM contributes to wound healing by acting on different aspects of this reparative process. Firstly, it helps in preventing infections and supports the elimination of pathogens by acting on their porphyrins, which is essential for successful healing. PBM also exerts a remarkable anti-inflammatory effect by modulating immune responses and reducing pro-inflammatory mediators, as well as regulating reactive oxygen species (ROS) and nitric oxide (NO) levels. Moreover, PBM plays a pivotal role in the proliferative phase, mitigating cellular senescence while promoting the vital processes of cellular proliferation and migration. This action involves the regulation of key factors such as cyclin D1, ATP/AMP ratios, and calcium ion (Ca²⁺) dynamics. Furthermore, PBM significantly contributes to the advanced stages of wound healing by promoting re-epithelization and remodeling, facilitating extracellular matrix (ECM) formation, and driving angiogenesis and vasodilation. These effects are mediated through the modulation of collagen production, transforming growth factor-beta (TGF-β), growth factors, matrix metalloproteinases (MMPs), and nitric oxide.

There is a considerable body of literature about wound healing using PBM in experimental models of rodent burns (Gupta *et al.*, 2015; Silveira *et al.*, 2016) or excisions in diabetic rats (Wu *et al.*, 2015; Dungel *et al.*, 2023). The effects of PBM in reducing inflammation and accelerating the healing process have been consistently documented in these studies, as previously discussed. Regarding clinical studies, red and NIR lights have predominantly been used in research related to

wound healing. A human experimental abrasion model treated with a NIR-diode laser (820 nm, 8 J/cm²) exhibited enhanced wound healing compared with sham controls. Interestingly, wounds were created in pairs, and even the adjacent untreated wounds showed enhanced wound healing when compared to both the sham controls, suggesting that the effects of the NIR-laser treatment extended beyond the irradiated area, potentially influencing the overall wound healing process (Hopkins *et al.*, 2004). A pilot study evaluating the healing of nipple trauma by NIR-LEDs (860 nm, 4 J/cm²) in a small group (< 20) of breastfeeding women showed that the treated group needed fewer sessions to heal than the placebo group along with better pain management (Chaves *et al.*, 2012). More recently, Vaghardoost *et al.* evaluated for the first time the effects of red light on the healing process after surgery of a graft-donor site in burn patients. The results showed that local irradiation of red laser accelerates the wound healing process significantly (Vaghardoost *et al.*, 2018). Additional reports provide evidence for the role of LLLT in enhancing the healing of burns (de Oliveira *et al.*, 2018) and in the appearance of surgical scars (Carvalho *et al.*, 2010). Recently, a clinical trial on children with deep burn ulcers showed that PBM could be an effective alternative to skin grafting, significantly decreasing the rate of scar and hypertrophic scar formation (Kazemikhoo *et al.*, 2022). However, high-quality randomized controlled trials with larger sample sizes are still lacking (Glass, 2021). Notably, a prospective randomized trial showed that yellow, red, and NIR lights failed to improve healing compared to placebo after laser ablation on the forearm of 10 volunteers (Le Duff *et al.*, 2022).

Recent development in photobiomodulation has led to the development of a novel biophotonic platform that utilizes fluorescent light energy (FLE) within the visible spectrum of light to heal skin inflammation and wounds. Interestingly, a recent study used blue light on 18 acute second-degree burns case studies associated with a pilot study using an *ex vivo* human skin model. In all cases, complete healing was observed without the need for skin grafting, and there were no adverse events related to the biophotonic platform. In the *ex vivo* human skin model, data suggest that FLE impacts different cellular pathways, including essential immune-modulatory mechanisms, as the treatment with blue light led to an increase in collagen I, fibroblast growth factor, anti-inflammatory cytokines, TGF- β 1, and TGF- β 3 (Mellergaard *et al.*, 2021)

5.3.2. Chronic wound healing

In the context of chronic wounds, PBM offers several potential benefits. It can help stimulate wound bed preparation by promoting granulation tissue formation, collagen deposition, ECM remodeling, and angiogenesis. PBM also exhibits anti-inflammatory effects, reducing chronic inflammation and creating a more favorable environment for healing (Leyane *et al.*, 2021; Bayat *et al.*, 2022) (Figure 17).

Clinical studies have demonstrated the efficacy of PBM in various chronic wound types, such as chronic diabetic ulcers. Chronic diabetic wounds are difficult to treat due to underlying conditions such as angiopathy and neuropathy, resulting in slow healing rates. Conventional treatment options are often insufficient and do not provide satisfactory outcomes. In two double-blinded placebo-controlled RCTs, Frangez *et al.* used a combination of 2 red wavelengths (625 and 660 nm) and an NIR wavelength (850 nm) to irradiate diabetic ulcers. The authors showed a significant increase in blood flow with an improvement in the cicatrization score and a decrease in the wound area compared to the placebo (Frangez *et al.*, 2017; Frangež *et al.*, 2018). Authors from the same group reported in their systematic review about diabetic foot ulcers that all four reviewed RCTs demonstrated positive therapeutic outcomes with light therapy compared to the control, with no adverse events. However, due to some limitations, they recommended additional studies with comparable laser parameters, larger sample sizes, and longer follow-up periods to define the optimal treatment (Tchanque-Fossuo *et al.*, 2016). Other clinical trials using red light to treat diabetic foot ulcers noted a significant improvement in PBM-treated wounds (Feitosa *et al.*, 2015; Mathur *et al.*, 2017).

Regarding other chronic wound types, a double-blinded RCT on 24 patients with a 6-week history of venous leg ulcers showed no difference in wound closure or ulcer size between the red light-irradiated and placebo group after 4 weeks of follow-up. However, mean ulcer pain was reduced by red light (635 nm) compared to placebo (Vitse *et al.*, 2017). Differences in the treatment regimen and study sample size may be responsible for the contradictory results. Therefore, researchers should consider reevaluating successful treatment parameters in larger studies (Jagdeo *et al.*, 2018). An interesting case series study on diabetic patients with pressure ulcers treated with red light demonstrated significant improvements in wound areas following PBM treatments that correlated with increased prohealing factors, including vascular endothelial growth factor

(angiogenesis) and TGF- β (matrix and epithelial closure), whereas TNF- α (inflammation) levels were reduced (Ruh *et al.*, 2018). Consistent with this study, red light PBM positively affected the repair process of rats' chronic wounds, modulating ROS and cytokine production (Pavlov *et al.*, 2021).

Besides red or NIR light, blue light is recently explored in chronic wound treatment (Bayat *et al.*, 2022; Purbhoo-Makan *et al.*, 2022; Prado *et al.*, 2023). Fracalvieri *et al.* conducted a study to investigate the impact of blue light in addition to regular care on the formation of new epidermal cells in lower limb ulcers compared to regular care alone for 8.5 months. Their findings demonstrated that combining blue light and regular care accelerated the wound healing process, particularly in venous leg ulcers, and increased the likelihood of complete wound healing within the 8.5-month timeframe (Fracalvieri *et al.*, 2022). Similarly, Dini *et al.* conducted a study using a portable LED array device emitting blue light to stimulate the healing process in 20 non-healing ulcers. Their results showed that LED blue light positively impacted skin wound repair, improving the wound bed score and reducing patient reports of soreness (Dini *et al.*, 2021).

5.4. Psoriasis

Psoriasis vulgaris is a chronic skin disease characterized by hyperproliferation and reduced differentiation capacity of skin keratinocytes accompanied by an inflammatory response. Phototherapy, whether through natural sunlight or artificial light sources like broadband or narrowband UVB, has been found to improve the symptoms of psoriasis (Lapolla *et al.*, 2011). However, excessive exposure to sunlight or UVB alone can accelerate skin aging and increase the risk of skin cancer. Due to these risks, UV phototherapy is typically administered in hospitals or by specialized practitioners, making it a costly and time-consuming therapy for patients. Fortunately, several studies have demonstrated that UV-free, low-level blue light has beneficial effects in treating psoriasis. It can reduce the proliferation of human KCs, has cytotoxic effects on T cells at low fluencies without harming other skin cells (Liebmann *et al.*, 2010; Yoo *et al.*, 2020), and can inhibit DCs activation (Fischer *et al.*, 2013). Therefore, blue light PBM is a safer alternative to traditional phototherapy methods in treating chronic hyperplastic and inflammatory dermatosis, such as psoriasis vulgaris, receiving a grade C of recommendation (Jagdeo *et al.*, 2018).

Two split-face, double-blinded trials using blue light at 90 J/cm² reported a significant improvement in Local Psoriasis Severity Index (LPSI) scores of the irradiated psoriasis plaques compared to the contralateral untreated plaques after at least 4 weeks of daily treatment (Weinstabl

et al., 2011; Pfaff *et al.*, 2015). Better results were achieved at higher irradiances (200 vs. 100 mW/cm²) and longer treatment duration (12 weeks vs. 4 weeks), where two patients even achieved complete resolution of psoriatic lesions. In another prospective randomized study by Krings *et al.*, the effectiveness of blue light (high irradiance level 600 mW/cm²) was compared to topical calcipotriol, a vitamin D derivative, in the treatment of mild psoriasis vulgaris. Irradiated plaques showed a statistically significant improvement after 12 weeks of daily treatment, with no difference when comparing the efficacy of blue light to topical vitamin D treatments (Krings *et al.*, 2019). The positive effects of blue light were further confirmed in a prospective clinical study involving 30 patients with mild psoriasis vulgaris. The local blue light treatment evaluation showed a statistically significant improvement in the mean LPSI and the Dermatology Life Quality Index (DLQI) scores (Lesiak *et al.*, 2021). Slight hyperpigmentation could be observed as an adverse effect in some of these studies.

However, in one study where 17 patients with plaque psoriasis were treated with blue light (417 nm, 10 J/cm², 8.5 mW/cm²) 3 times per week for 4 weeks, no improvement was observed in the severity scores of the treated plaques (Maari *et al.*, 2003). Treatment failure could be due to a low irradiance or low frequency of treatment sessions if compared to previously described studies.

Although the trials conducted on PBM treating psoriasis are still small and few, the NIR and visible red light with low energy showed promise for treating psoriasis due to their strong penetration and anti-inflammatory action (Zhang and Wu, 2018). In a preliminary study, patients with psoriatic plaques were treated sequentially with LED, delivering continuous 830 and 633 nm in 20 minutes each session for 4-5 weeks. Clearance rates of 60–100% were achieved without any side effects (Ablon, 2010). This study had a small sample size, and the results need further confirmation.

5.5. Atopic dermatitis and eczema

The pathogenesis of AD involves the induction of apoptosis in the skin infiltrated by helper T lymphocytes with the production of singlet oxygen. Therefore, it is logical to propose LED treatment for its anti-inflammatory properties and impact on apoptosis regulation. Only a few studies investigate the use of PBM in treating AD and eczema (grade D of recommendation) (Sadowska *et al.*, 2021). In 1993, Morita *et al.* published a trial on 112 atopic subjects treated with diode laser emitting NIR at 830 nm. He observed a decrease in pruritus in 71% of cases and an improvement in the rash in 62% of cases. Biopsies performed in some cases showed a decrease in ICAM-1 expression

by KCs and a decrease in dermal infiltrates (Morita *et al.*, 1993). In another study, 36 adult patients with severe, chronic AD resisting long-term disease control with local corticosteroids attended full-body blue light irradiation (between 400 and 500 nm, 28.9 J/cm²). In total, two and five treatment cycles were performed over 6 months, and the patients used topical corticosteroids between cycles. At 15 days, 3, and 6 months after starting the study, a decrease in the Eczema Area and Severity Index (EASI) score was observed by 29%, 41%, and 54%, respectively. Reduction in the severity of itching was one of the initial improvement symptoms. In addition, the authors showed improvements in sleep quality as well as in quality of life (Becker *et al.*, 2011). Additionally, the prospective, randomized study showed that blue light (453 nm, 90 J/cm²) used locally was safe and improved the eczema severity index (Keemss *et al.*, 2016). Future studies with larger patient cohorts within a randomized, placebo-controlled clinical trial must confirm these observations.

5.6. Other clinical applications

PBM is finding promising applications for treating other inflammatory skin disorders, including rosacea (Su *et al.*, 2023), oral mucositis (Curra *et al.*, 2015; Bensadoun, 2018; Tam *et al.*, 2020; Finfter *et al.*, 2023), post-radiation dermatitis (Gobbo *et al.*, 2023), and epidermolysis bullosa (Ho *et al.*, 2022). However, additional studies are necessary to optimize protocols and ensure consistent outcomes for these indications.

III. NRF2 AND THE SKIN

1. REACTIVE SPECIES AND OXIDATIVE STRESS

1.1. Redox-active species

A free radical is a partially reduced chemical species with one or more unpaired electrons in a valency shell or outer electron orbit. Thus, it is unstable, short-lived, and highly reactive since it tends to neutralize itself by reacting with other substances, thereby causing oxidation. When this unpaired electron is situated on a nitrogen atom, it is referred to as a reactive nitrogen species (RNS), such as nitric oxide (NO). Alternatively, when this unpaired electron is located on an oxygen atom, it is referred to as a reactive oxygen species (ROS) (Delattre *et al.*, 2007). In biological systems, ROS include oxygen-derived free radicals, such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), and peroxy radical (RO_2^{\cdot}), but also include non-radical oxygen-derived reactive molecules that do not have unpaired electrons, such as singlet oxygen (1O_2) and hydrogen peroxide (H_2O_2) (Villalpando-Rodriguez and Gibson, 2021). Recently, reactive sulfur species (RSS) have been identified as omnipresent molecules in redox biology, playing pivotal roles in cellular functions and redox homeostasis. (Giles *et al.*, 2017). Under physiological conditions, all types of skin cells produce in a controlled manner different reactive species, including ROS, RNS, and RSS (Korkina and Pastore, 2009; Ali *et al.*, 2020). The most important redox-active species produced are those derived from oxygen.

ROS are generated as natural by-products of cellular processes during aerobic metabolism. Mitochondrial respiration is the major source of endogenous enzymatic ROS production. The mitochondrial respiratory chain, responsible for ATP production, can result in the accidental leakage of electrons, producing partially reduced and highly reactive species. Other organelles, including the endoplasmic reticulum, peroxisomes, and lysosomes, contribute to natural ROS generation (Thannickal and Fanburg, 2000; Villalpando-Rodriguez and Gibson, 2021). ROS can also be generated enzymatically by transmembrane-associated oxidases, with the phagocytic NADPH oxidase (NOX) being the most well-characterized. NOX produces $O_2^{\cdot-}$ in phagocytic immune cells, such as neutrophils and macrophages, catalyzing the process of the respiratory burst involved in innate immune response and host defense against pathogens (Phaniendra *et al.*, 2015). Other intracellular enzymes, such as xanthine oxidoreductase and cytochrome P450 family enzymes, also generate ROS

secondary to their main enzymatic function. Non-enzymatic sources of ROS were also described, such as KCs and melanocytes that produce H₂O₂ and superoxide radicals in the photooxidation of pheomelanin by UV radiations (Pelle *et al.*, 2005). Free heme is also a non-enzymatic source of oxidants since heme is an iron chelate with the potential to catalyze iron-dependent reactions leading to ROS generation (Ryter and Tyrrell, 2000).

Apart from endogenous intracellular sources, ROS are also produced as a consequence of cumulative environmental exposures throughout one's life, collectively known as the 'exposome' (Niedzwiecki *et al.*, 2019). The exposome includes the totality of external factors, including but not limited to diets, xenobiotics, chemicals, toxins, pollutants, and physical stressors like UV radiation. Given the diverse and variable nature of these exposures, determining the precise contribution of the exposome to the overall pool of oxidants is challenging (Sies and Jones, 2020).

1.2. ROS in physiological signaling

It is now widely recognized that ROS and other highly reactive molecules play a crucial role as signaling agents in various cellular pathways, referred to as "redox signaling." When produced at relatively low levels, they serve as intracellular and intercellular secondary messengers, essential for activating signaling pathways that regulate crucial biological processes, including cell death/survival, proliferation, differentiation, protein synthesis, angiogenesis, and immune responses (Le Gal *et al.*, 2021) (Figure 18). Among these molecules, H₂O₂ and peroxynitrite (ONOO⁻) have been implicated in numerous cellular signaling cascades. Due to their non-radical nature, these molecules have a longer half-life than most other oxidants, allowing them to migrate away from their production sites and diffuse across membranes (Ali *et al.*, 2020). In KCs, lipid peroxidation has been shown to induce the expression of vascular endothelial growth factor (VEGF) (Nakai *et al.*, 2011). Additionally, the formation of ROS by angiotensin II has been found to increase the levels of epidermal growth factor receptor (EGFR) expression in human KCs (Nakai *et al.*, 2008). ROS activation has been observed to stimulate AKT in human dermal fibroblasts, a kinase that regulates various cellular processes inhibiting apoptosis and favoring cell growth and survival (Nakai *et al.*, 2019). Furthermore, transcription factors such as Activator protein – 1 (AP-1), hypoxia-inducible factor-1 (HIF-1), and NF-κB have been reported to be involved in these redox-modulated pathways (Zhang *et al.*, 2016).

1.3. Redox homeostasis versus oxidative stress

While redox-active species play a vital role in signaling and cellular functions, it is important to recall that they are, by definition, highly reactive molecules with potent oxidizing properties. The reactivity of these species differs substantially among different types of redox molecules. Redox species can cause cellular damaging effects resulting from their interactions with various organic biomolecules within the cell, thus impairing their normal functions. These interactions lead to lipid peroxidation and alterations in membrane lipids, specifically polyunsaturated fatty acids, protein oxidation, DNA strand breakage, RNA oxidation, mitochondrial depolarization, and apoptosis.

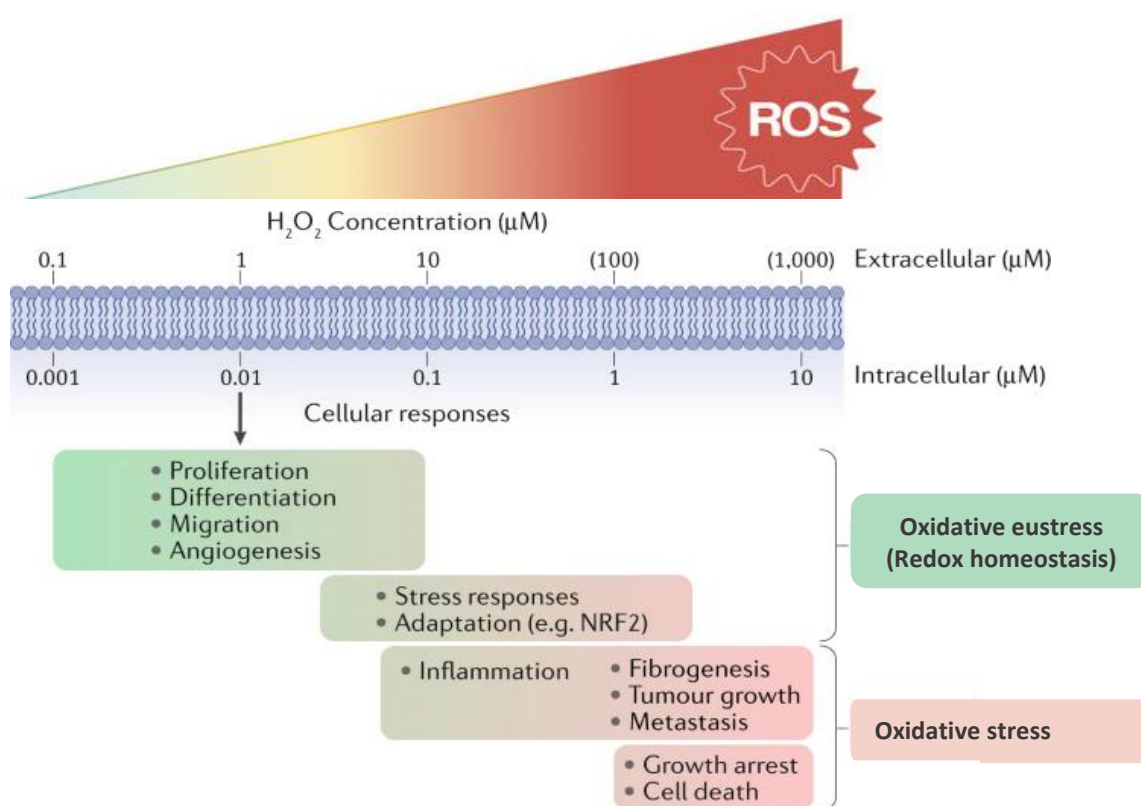


Figure 18. Cellular response depending on ROS (H_2O_2) concentration: from redox homeostasis to oxidative stress. Physiological levels of H_2O_2 , as well as other ROS, play a crucial role in cellular signaling that regulates essential processes, such as cell cycle, growth, differentiation, and various functions. Under normal conditions, cells maintain a balanced steady state known as redox homeostasis to ensure proper functioning. However, excessive concentrations of ROS (typically estimated to be above 100 nM for H_2O_2) have detrimental effects. These supraphysiological levels result in the non-specific oxidation of proteins, leading to disrupted cellular responses and reversible or irreversible damage to biomolecules. This damage can trigger inflammatory responses, growth arrest, and cell death, contributing to the development of pathological conditions collectively known as 'oxidative stress'. Green and red coloring denotes predominantly beneficial (oxidative eustress) or deleterious (oxidative stress) responses, respectively. Experimental high extracellular H_2O_2 exposure is given in parentheses. Adapted from Sies and Jones, 2020.

Therefore, neutralizing redox species by an effective antioxidant defense system is crucial to maintain low concentrations of these oxidants, limiting their effect to the local where they are generated and, thus, preventing cellular damage (Martemucci *et al.*, 2022).

The delicate balance state between the generation and neutralization of redox species within the cell, maintaining normal cellular functions, is called “redox homeostasis”. Redox homeostasis also includes the complex network of redox signaling pathways that activate or inhibit cellular control mechanisms. Through a process known as “oxidative eustress”, minor deviations from the stable redox balance can be regulated to ensure cellular homeostasis is maintained (Schalka *et al.*, 2022) (Figure 18).

In contrast, the imbalance state due to excessive production or inadequate elimination of ROS can lead to “oxidative stress” that arises when redox homeostasis is compromised. Oxidative stress leads to disruption in redox signaling and causes damage to cellular structures and molecules (Schalka *et al.*, 2022) (Figure 18). It affects all classes of macromolecules, mainly lipids, proteins, and DNA, impairing their normal function and compromising cellular processes. Furthermore, the byproducts generated from this damage can act as secondary signals for oxidative stress. Upon exposure of the skin to UV radiations, environmental pollutants, including diesel exhaust fumes, xenobiotics, and chemicals, reactive species are produced excessively. The sustained overproduction of these oxidants overwhelms the skin's antioxidant capacities, decreasing the skin's capacity to respond to the deviations in redox homeostasis, leading to oxidative stress (Chen *et al.*, 2021). A classification of oxidative stress based on its intensity is proposed by Lushchak as a helpful tool to describe experimental data where oxidative stress is induced (Lushchak, 2014).

The newly introduced concept of “**skin redoxome**” represents the cutaneous redox environment, including the generation of reactive species, their neutralization through antioxidants, and the signaling networks responsible for organizing the redox homeostasis (Schalka *et al.*, 2022). It involves the network of redox reactions and redox active species, including radicals, lipids, nucleic acids, proteins, and other molecules that determine the redox environment of cells and tissues, and signaling cascades that regulate the expression of many transcription factors and genes, and mechanisms involved in health and disease. The skin redoxome is intricately connected to the various external factors to which the skin is exposed, collectively known as the exposome, as well as other relevant “omes” that influence the skin's physiological processes and responses. The interactions between the exposome and the skin redoxome also impact the synthesis of proteins

(proteome) and lipids (lipidome), leading to changes in the expression or suppression of various inflammatory elements (Schalka *et al.*, 2022).

1.4. Oxidative stress and skin inflammation

The oxidative stress is implicated in the skin aging process, in promoting and exacerbating inflammation, and plays a role in the pathogenesis of various chronic inflammatory skin diseases, including contact dermatitis, AD, psoriasis, and skin cancer (Nakai and Tsuruta, 2021; Anik *et al.*, 2022; Liu *et al.*, 2023) (Figure 19). For instance, evidence supports increased markers of oxidative stress in AD patients and, especially during AD exacerbation, as well as an altered antioxidant defense (Leveque *et al.*, 2003; Sivaranjani, Rao and Rajeev, 2013; Amin *et al.*, 2015; Ji and Li, 2016). Patients with psoriasis and AD have shown higher levels of urinary nitrate, malondialdehyde, and 8-hydroxydeoxyguanosine metabolites compared to healthy individuals, suggesting that the oxidation of nitric oxide, lipids, and DNA is associated with the development of inflammatory skin diseases (Nakai *et al.*, 2009).

1. Contact dermatitis Activated myeloperoxidase and NADPH oxidase Increased tissue iron levels Reduced activity of GPx Gene polymorphism of MnSOD Increased iNOS expression	6. Skin cancer Reduced activity of SOD Increased iNOS expression
2. Urticaria Reduced activity of MnSOD and GSH	7. Aging Reduced activity of catalase
3. Atopic dermatitis Decreased blood vitamin C levels Increased tissue iron levels Decreased tissue vitamin E levels	8. Vitiligo vulgaris Decreased levels of blood catalase GPx and SOD
4. Psoriasis vulgaris Decreased levels of blood β -carotene, vitamin E, catalase and GPx Increased iNOS expression	9. Alopecia areata Activated XO
5. Acne vulgaris Reduced activity of SOD	10. Wound healing Activated NADPH oxidase. Increased tissue iron levels Increased iNOS expression
	11. Granuloma Activated XO

Figure 19. Reported redox abnormalities in skin inflammatory diseases. Studies reported an increase in the expression of reactive species generators and/or a decrease in the antioxidant defense system, suggesting that oxidative stress plays a role in the pathogenesis of several inflammatory skin diseases. From Nakai *et al.*, 2021.

Two possible mechanisms explain the involvement of oxidative stress in skin senescence and chronic inflammatory skin pathologies (Liu *et al.*, 2023). ROS can directly damage biomolecules within the cell. ROS produced in excess in the skin interact with unsaturated fatty acids, generating lipid peroxides. This enzymatic process creates hydrophilic pores in biological membranes, causing increased permeability of cellular and mitochondrial membranes, oxidative damage, and inflammatory responses (Briganti and Picardo, 2003; J. Chen *et al.*, 2021). In parallel, ROS can mediate cellular signaling pathways, thus affecting gene expressions of pro-inflammatory cytokines (Liu *et al.*, 2023). Oxidative stress-induced skin inflammation is associated with the release of cellular inflammatory factors, such as IL-22, TNF- α , and COX-2, and the expression of these factors is known to be regulated by NF- κ B, MAPK, and JAK/STAT redox-sensitive signaling pathways, as detailed below (Sasaki and Aiba, 2007; Tanaka *et al.*, 2007; Mouchet *et al.*, 2010; Bechara *et al.*, 2018).

Oxidative stress and inflammation are tightly related and interdependent pathophysiological processes often present simultaneously in many pathological skin conditions. Oxidants can promote tissue inflammation by activating multiple pro-inflammatory pathways. While the inflammatory process, in turn, can generate oxidative stress since ROS are produced by cells involved in the host-defense response, which maintains a vicious amplification loop of both events (Biswas, 2016).

1.4.1. Oxidative stress as a promoter of inflammation

Oxidative stress can induce inflammation through the activation of multiple pathways. For instance, ROS induced by organic contact sensitizers contribute to the generation of a pro-inflammatory microenvironment by indirectly activating TLR signaling, thus promoting CHS responses. Indeed, ROS mediate the degradation of high molecular weight hyaluronic acid (high MW HA) of the skin ECM into pro-inflammatory low molecular weight fragments since ROS increase hyaluronidase activity. The HA breakdown products act as DAMPs capable of activating TLR2 and TLR4 (Kaplan *et al.*, 2012). Inhibition of either ROS-mediated or enzymatic HA breakdown using the antioxidant N-acetylcysteine or hyaluronidase inhibitor (aristolochic acid), respectively, prevents sensitization and elicitation of CHS (Esser *et al.*, 2012). Reactive species also shape the immune response to allergens and non-allergen chemicals. For example, KCs-derived ROS can induce type 2 inflammation responsible for AD (Choi *et al.*, 2021). Moreover, it was shown that H₂O₂ can induce inflammation by activating the redox-dependent transcription factor NF- κ B (Flohé *et al.*, 1997). One of the primary mechanisms involves the oxidation and subsequent degradation of I κ B, the cytoplasmic repressor of NF- κ B. H₂O₂ can oxidize cysteine residues within I κ B, leading to their

ubiquitination and subsequent proteasomal degradation. This degradation releases NF- κ B, allowing it to translocate to the nucleus and initiate target gene transcription, including pro-inflammatory cytokine genes. H₂O₂ can also activate upstream signaling pathways that converge on NF- κ B activation. For example, it can activate protein kinases, such as I κ B kinases (IKKs), which phosphorylate I κ Bs and target them for degradation (Lingappan, 2018). Kim *et al.* showed that H₂O₂ modulates IKK-dependent NF- κ B activation by promoting the redox-sensitive activation of the PI3K/PTEN/AKT and NIK/IKK pathways (Kim *et al.*, 2008). Additionally, ROS can modulate the activity of MAPKs, such as p38 and JNK, which are involved in NF- κ B activation. ROS can also modulate the transcription factor's nuclear translocation and DNA binding by modifying the NF- κ B heterodimers (Brigelius-Flohé and Flohé, 2011).

Oxidative stress also plays a crucial role in the activation of the NLRP3 inflammasome that triggers innate immune defenses by promoting caspase-1 activation and the proteolytic processing of pro-IL-1 β and pro-IL-18 into mature IL-1 β and IL-18 inflammatory cytokines (Hua *et al.*, 2013). ROS released from damaged mitochondria can activate NLRP3 inflammasomes, leading to the secretion of IL-1 β and localized inflammation (Zhou *et al.*, 2011). Oxidized mitochondrial DNA has also been found to activate NLRP3 inflammasomes during apoptosis (Shimada *et al.*, 2012). ROS activates inflammasome through MAPK, particularly ERK1/2 downstream activation. ERK1/2 plays a role in determining the cell's pro- or anti-apoptotic state during inflammation. It controls the expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-18 (Harijith *et al.*, 2014). Decreased ROS production reduces ERK1/2 activation and decreased IL-1 β production (Cruz *et al.*, 2007). Moreover, under conditions of oxidative stress, ROS can cause the dissociation of thioredoxin-interacting protein, an inhibitor of the endogenous antioxidant thioredoxin, from thioredoxin and its binding to NLRP3, thereby activating the NLRP3 inflammasome (Zhou *et al.*, 2010; Mohamed *et al.*, 2021).

1.4.2. Inflammation as an inducer of oxidative stress

Under pathological inflammatory conditions, there can be an exaggerated generation of reactive species by the infiltrated phagocytic cells, such as neutrophils and macrophages, producing significant amounts of ROS and RNS, such as O₂^{•-}, [•]OH, NO, and hypochlorous acid (HOCl) to eliminate invading agents. Some of these species can diffuse out of the phagocytic cells, leading to localized oxidative stress and tissue injury (Ali *et al.*, 2020). Apart from the direct production of reactive species by professional phagocytic cells, the non-phagocytic cells can also produce reactive

species in response to pro-inflammatory cytokines. For instance, the pro-inflammatory cytokine IFN- γ and the bacterial pro-inflammatory component LPS have been shown to synergistically enhance ROS production in human pancreatic cancer cell lines and pancreatitis through the TLR-4-NF- κ B pathway (Wu *et al.*, 2013). A study has demonstrated that TLR2, TLR4, and TLR7/8 co-stimulation leads to oxidative stress and an imbalance in the production of pro-inflammatory and anti-inflammatory cytokines (Lavieri *et al.*, 2014).

2. NRF2: A REGULATOR OF THE CELLULAR STRESS RESPONSE

Reactive species must be promptly detoxified to ensure dynamic redox homeostasis within the cell and protect against inflammation. To prevent oxidant damage, the skin possesses an extensive network of antioxidant defense systems, including numerous antioxidant enzymes and non-enzymatic antioxidants (Korkina and Pastore, 2009). Antioxidants are molecules that inhibit, decrease, delay, or completely scavenge the action of free radicals and oxidants and protect the body from oxidative damage. Normally, the presence of antioxidants allows for the clearance of reactive species by directly reacting with oxidants, thereby reducing their oxidative potential (Ali *et al.*, 2020). It is well established that the antioxidant response is mainly orchestrated by the redox-sensitive transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2), an essential factor for maintaining redox homeostasis (Yamamoto *et al.*, 2018).

2.1. The transcription factor Nrf2

Nrf2, encoded by the NFE2L2 gene, belongs to the mammalian cap'n'collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors. This subfamily of transcription factors was first discovered in 1989 with the identification of NF-E2 p45 (Mignotte *et al.*, 1989) and comprises today six transcription factors that were discovered shortly thereafter: Nrf1 (Chan *et al.*, 1993), Nrf2 (Moi *et al.*, 1994), Nrf3 (Kobayashi *et al.*, 1999), and the more distantly related factors Bach1 and Bach2 (Oyake *et al.*, 1996). Nrf2 is the most studied and best-known transcription factor of this subfamily, due to its role as a master regulator of the antioxidant genes expression in response to oxidative stress (He *et al.*, 2020). It is also a cytoprotective transcription factor and could particularly drive detoxification and excretion mechanisms of both organic xenobiotics and toxic metals through regulating the expression of phase II enzymes (Huang *et al.*, 2015; Bellezza *et al.*, 2018). Given its protective functions against endogenous and exogenous stress, Nrf2 is ubiquitous in vertebrates and evolutionary conserved among mammals, with a protein homology ranging from 49% in

zebrafish to 89% in cows when compared to humans (Maher and Yamamoto, 2010).

Over the past three decades, many studies have contributed to better understand the Nrf2 pathway since it was first isolated and identified in 1994 (Moi *et al.*, 1994) (Figure 20). Nrf2 is negatively regulated at the protein level through direct interaction with the dimeric redox sensor, Kelch-like ECH-associated protein 1 (Keap1) (Itoh *et al.*, 1999). The negative regulation of Nrf2 by Keap1 was validated via the generation of Keap1^{-/-} mice, which showed constitutive activation of Nrf2 (Wakabayashi *et al.*, 2003).

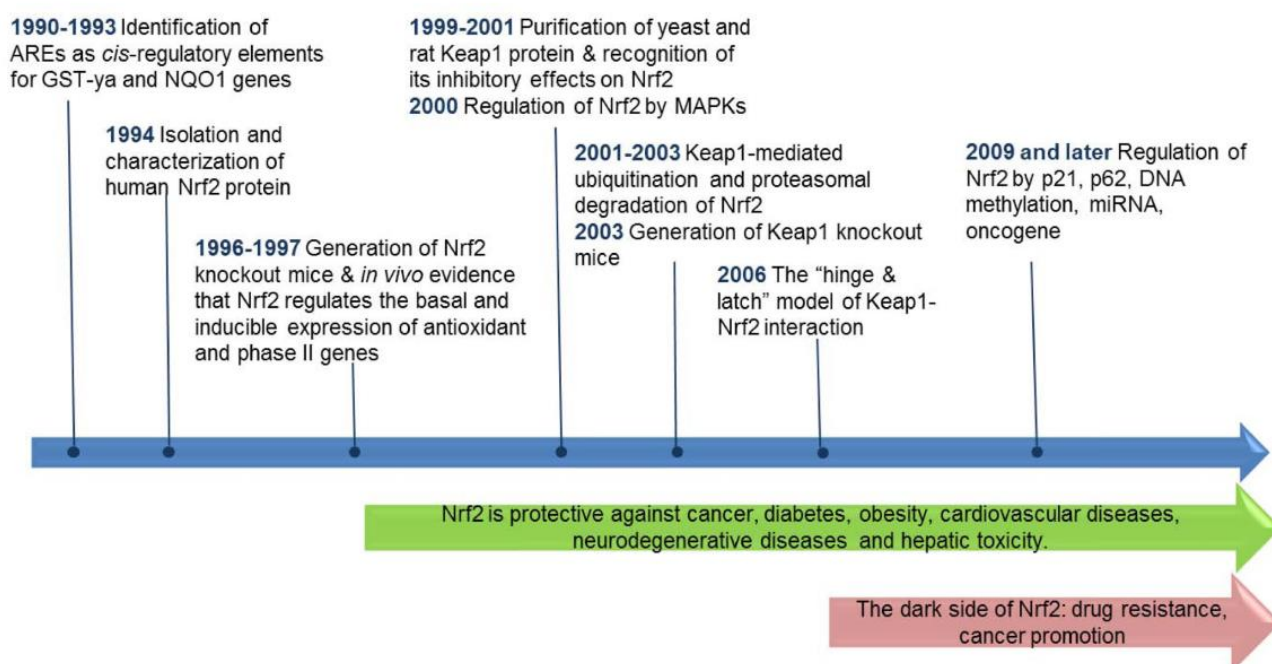


Figure 20. Key research advances in the Nrf2 pathway since 1990. Understanding of Nrf2 pathway has been greatly advanced in the last two decades, with key advances in its regulatory mechanisms including its negative regulation by Keap1, and in its diverse roles in preventing diseases. However, Nrf2’s dual nature is evident, as its hyperactivation in cancer cells can drive aggressive behaviors, emphasizing its central and complex role in cellular processes. From Huang *et al.*, 2015

Nrf2-mediated antioxidant capabilities have been demonstrated to prevent multiple diseases, including neurodegenerative diseases (Song and Long, 2020), cardiovascular disorders (Vashi and Patel, 2021), osteoporosis (Han *et al.*, 2022), and inflammation (Ahmed *et al.*, 2017). However, it should be noted that activating Nrf2 can have a downside as it can promote the survival of cells involved in the development of harmful conditions (Kim and Keum, 2016). For example, in non-small-cell lung cancer, the constitutive activation of Nrf2 in cells with mutated Keap1, a Nrf2 repressor, was found to promote carcinogenesis (Singh *et al.*, 2006). To protect themselves from

oxidative damage, cancer cells increase their antioxidant capacity to maintain a balanced redox state. This is achieved through the hyperactivation of Nrf2, a transcription factor, in various types of cancer. The hyperactivation of Nrf2 in cancer cells has been associated with aggressive proliferation (Okazaki *et al.*, 2020), metastasis (Lignitto *et al.*, 2019), and resistance to chemotherapy (Jeong *et al.*, 2020). This highlights the complex nature of the Nrf2 pathway and reveals its dual role or "dark side" (Wang *et al.*, 2008). The expanding number of targets and functions associated with Nrf2 emphasizes its central importance in cellular organisms.

2.2. Nrf2-Keap1 structures and signaling pathway

2.2.1. Nrf2 structure

Human Nrf2 is a protein composed of seven Nrf2-ECH homology (Neh) domains, each serving specific functions (Figure 21A). **Neh1** contains the basic CNC-bZIP motif, enabling dimerization with the small musculoaponeurotic fibrosarcoma (sMaf) protein and binding to DNA sequences known as the antioxidant response element (ARE). It also contains a nuclear localization signal that facilitates Nrf2 translocation to the nucleus after its release from the KEAP1 molecule (Bellezza *et al.*, 2018). The **Neh2** domain at the N-terminus interacts with Keap1 through amino acid motifs DLG and ETGE. Additionally, seven lysine residues in Neh2 are targeted for ubiquitylation, leading to the proteasomal degradation of Nrf2 (Tong *et al.*, 2006). **Neh3** (C-terminal domain), **Neh4**, and **Neh5** are transactivation domains that bind to co-activators to enhance Nrf2 transcriptional activity and facilitate the transcription of targeted genes (Nioi *et al.*, 2005; J.-H. Kim *et al.*, 2013). **Neh6** is a serine-rich region that negatively regulates Nrf2 stability by promoting its proteasomal degradation independently of Keap1. It achieves this through direct binding with β -transducing repeat-containing protein (β -TrCP), which acts as an adaptor protein for the Skp1-Cul1-F-box protein, SCF E3 ubiquitin ligase complex (Chowdhry *et al.*, 2013). The **Neh7** domain interacts with retinoid X receptor α (RXR α) and contributes to the repression of Nrf2 (Wang *et al.*, 2013).

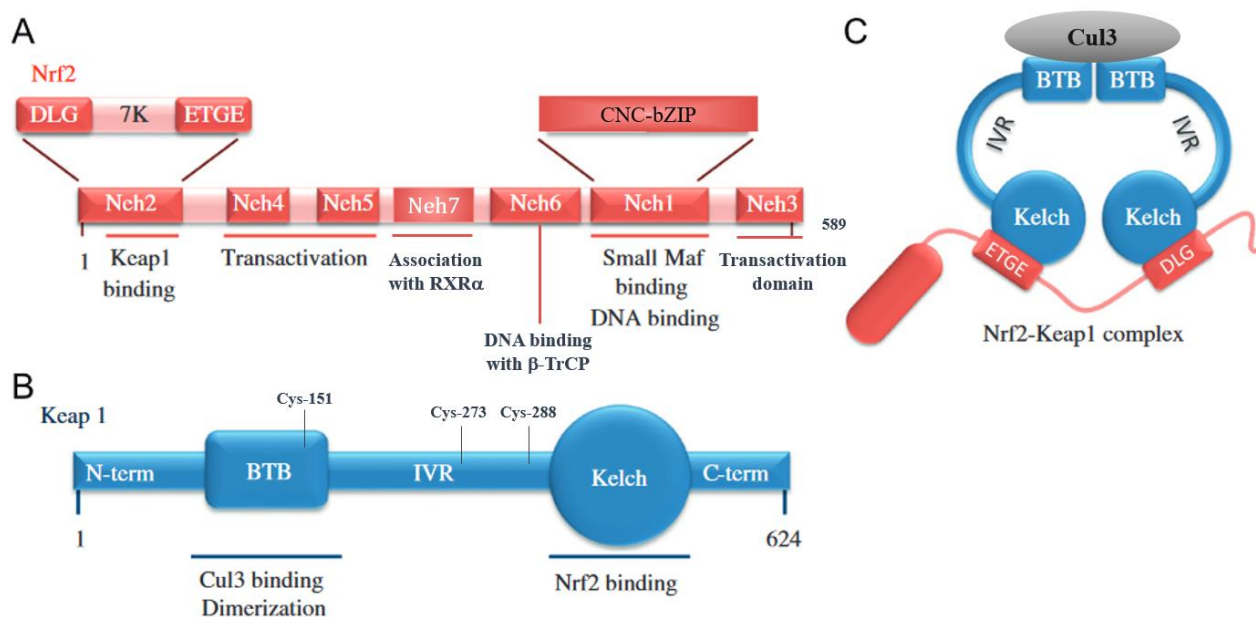


Figure 21. Structure of human Nrf2 and Keap1 proteins, and the Nrf2/Keap1 complex. (A) Structure of Nrf2 with its seven Neh domains. (B) Structure of Keap1 with the main modified cysteine residues. (C) Structure of Nrf2 and Keap1 complex in basal conditions. Adapted from Kansanen *et al.*, 2013.

1.2.1. Keap1 structure

Human Keap1 is a cytosolic cysteine-rich, zinc-finger protein and comprises five domains (Figure 21B). Keap1 possesses approximately 4% of cysteines from all amino acids, while other proteins have about 2% of cysteines as an average content. It has 27 cysteine residues in human homologues, sparsely distributed in the different domains (Kopacz *et al.*, 2020a). These cysteine residues are important stress sensors and are targets for a range of chemicals. The **BTB** (Broad-Complex, Tramtrack and Bric-a-brac) domain senses mainly electrophilic compounds, including the most potent activators of Nrf2, the cyclic cyanoenones, sulforaphane (SFN) found in cruciferous vegetables, and nitric oxide through highly reactive cysteine residue (Cys-151) (Kobayashi *et al.*, 2004; McMahon *et al.*, 2010; Dayalan Naidu *et al.*, 2018). The BTB domains also mediate both the homodimerization of Keap1 and allow the interaction with the cullin-3 (Cul3)-E3 ligase complex (Cullinan *et al.*, 2004) (Figure 21C). The **IVR** region has two critical cysteine residues, Cys-273 and Cys-288, required to repress Nrf2 activity (Yamamoto *et al.*, 2008) (Figure 21B). The IVR region contains the most reactive residues of Keap1, which are direct redox sensors (Dinkova-Kostova *et al.*, 2002). Cysteine- 226, -613, -622, and -624 have been recently identified as specific H₂O₂ sensors (Suzuki *et al.*, 2019). The **Kelch** repeat motifs can bind to the DLG and ETGE motifs of Nrf2 (Figure 21C).

Keap1 acts as a negative regulator of Nrf2 since it targets Nrf2 for proteasomal degradation in the cytoplasm under basal homeostatic conditions and serves as an oxidant and electrophilic stress sensor. Besides these well-known roles of Keap1, recent studies point to Keap1 as an intriguing and potent regulator of cellular function (Kopacz *et al.*, 2020a). Keap1 has been shown to play a role in the regulation of the cell cycle progression (Orthwein *et al.*, 2015; Kopacz *et al.*, 2020b) and the cytoskeleton (Ito *et al.*, 2015; Wu *et al.*, 2018; Kloska *et al.*, 2019). Keap1 also has a role in proteostasis, as it participates in the ubiquitination of proteins other than Nrf2, such as IKK β (Lee *et al.*, 2009; Kim *et al.*, 2010), and was reported to assist in ubiquitin aggregate clearance through autophagy (Fan *et al.*, 2010).

1.2.2. Keap1-Nrf2-ARE pathway

Under normal basal conditions, Nrf2 primarily resides in the cytoplasm, where it forms a complex with Keap1, constantly leading to its degradation by the proteasome (Baird and Yamamoto, 2020) (Figure 22). The Nrf2/Keap1 complex undergoes a cycle of two different conformations in its basal state. Initially, newly synthesized Nrf2 binds to one Keap1 molecule of the Keap1 dimer through a high-affinity ETGE motif, forming an “open conformation”. In this state, Nrf2 is protected from ubiquitination and degradation. Subsequently, the low-affinity DLG motif of Nrf2 binds to the second Keap1 molecule, leading to the formation of a “closed conformation” (Kopacz *et al.*, 2020a). In this closed state, Nrf2 is susceptible to polyubiquitination since Keap1, through its BTB domain, functions as an adaptor facilitating the Cul3-E3 ubiquitin ligase complex to ubiquitylate Nrf2 (Itoh *et al.*, 1999; Suzuki and Yamamoto, 2015) (Figure 22). Ubiquitination of Nrf2 by the Keap1-Cul3-E3 ligase complex leads to its subsequent proteasomal degradation. Nrf2 is an unstable protein with a short half-life of approximately 10 to 30 minutes and a high turnover, allowing for the maintenance of low basal levels of Nrf2 in normal conditions and providing a readily available pool of newly translated protein that can be rapidly stabilized in response to stress (Tonelli *et al.*, 2018).

Upon exposure to electrophilic chemicals or reactive species, modifications occur in the cysteine residues of Keap1, disrupting the Keap1-Nrf2 complex. These modifications initiate the activation of Nrf2 through two proposed mechanisms (Figure 22). First, it is suggested that electrophilic Nrf2 inducers, mainly interacting with Cys-151 in the BTB domain of Keap1, do not **affect binding of Nrf2 to Keap1 but rather induce structural and conformational modifications of Keap1-Cul3 complex leading to Cul3 dissociation**. This results in trapping the Nrf2-Keap1 complex in the “closed conformation”, thus preventing its ubiquitination (Cleasby *et al.*, 2014). Another

proposed mechanism for Nrf2 activation is the “hinge-and-latch” model. According to this theory, inducers were shown to modify cysteine residues in the IVR region of Keap1, disrupting the Keap1-Nrf2 interaction. The Keap1-DLG interaction first dissociates as a latch, while the Keap1-ETGE remains bound as a hinge, making Cul3 inaccessible to generate ubiquitination, thus preventing the degradation of Nrf2 (Suzuki and Yamamoto, 2015; Horie *et al.*, 2021). Consequently, preventing Nrf2 degradation in both cases results in the increased accumulation of newly synthesized Nrf2 molecules. These accumulated Nrf2 molecules are phosphorylated by different kinases and subsequently migrate to the cell nucleus, where Nrf2 forms a complex with one of the sMaf proteins (MafG, MafK, or MafF) (Motohashi *et al.*, 2002) (Figure 22). The presence of sMaf proteins is crucial for activating the transcription of Nrf2 target genes. Indeed, both Nrf2^{-/-} mice and triple knockout mice lacking MafG, MafK, and MafF fail to activate any cytoprotective genes upon stimulation (Motohashi *et al.*, 2004; Katsuoka *et al.*, 2005). The NRF2/sMaf complex activates the expression of approximately 200 genes by binding to a specific DNA binding motif known as ARE (Bellezza *et al.*, 2018). Interestingly, the mapping of Nrf2 DNA binding sites using ChIP-seq analysis has revealed that most of these binding sites are located distally outside the proximal promoter region (Lou *et al.*, 2006; Chorley *et al.*, 2012). Together, these complexes facilitate the transcription of Nrf2 target genes by binding to specific ARE sequences (Baird and Yamamoto, 2020) (Figure 22).

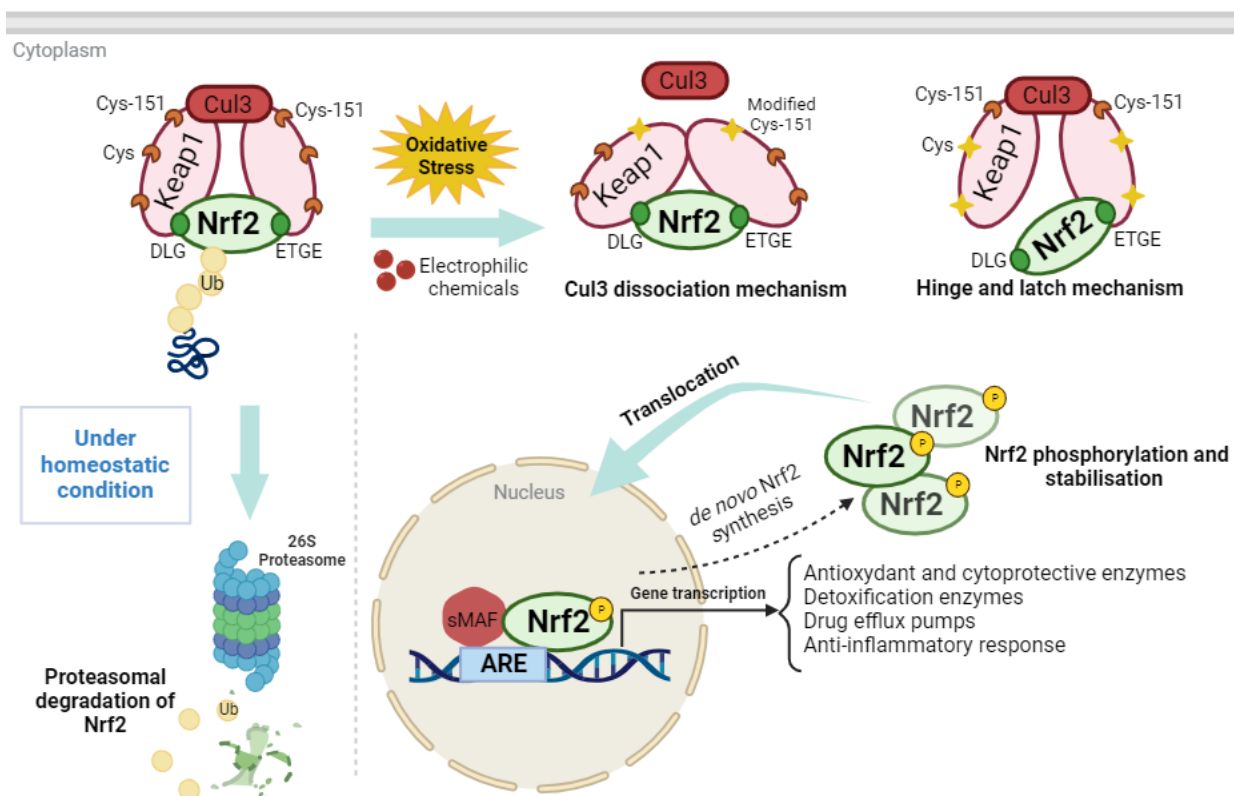


Figure 22. Schematic representation of Keap1-Nrf2 pathway under stressed or basal conditions. In unstressed conditions, Nrf2 forms a complex with Keap1 through its DLG and ETGE motifs and subsequently undergoes ubiquitination by the Cul3- E3 ubiquitin ligase complex, ultimately culminating in its degradation through the 26S proteasome pathway. Under oxidative or electrophilic stress, the cysteine residues within Keap1 are modified, and the activation of Nrf2 can be initiated through two distinct proposed mechanisms. According to hinge-and-latch theory, the modifications of cysteine residues in the IVR region of Keap1 lead to conformational changes that disrupt the binding of the DLG motif of Nrf2 while keeping the ETGE motif intact with Keap1, making Cul3 inaccessible to cause ubiquitination, thus preventing degradation of Nrf2. In the Cul3 dissociation mechanism, Cys151, in particular in the BTB domain of Keap1, undergoes modification by electrophiles or oxidants, leading to the disruption of Keap1-Cul3 complex, which prevents Nrf2 ubiquitination. In both scenarios, preventing the degradation of the Nrf2 pool causes an increased accumulation of the newly synthesized Nrf2 molecules, which translocate to the cell nucleus. There, they engage with small Maf proteins and promote the transcription of Nrf2 target genes through binding to Antioxidant Response Element (ARE) sequences.

2.3. Regulation of Nrf2

The regulation of Nrf2 activity is complex and involves multiple mechanisms and regulation at different levels: regulation of Nrf2 transcription by AhR, NF- κ B, PI3K-AKT signaling pathways or oncogenic proteins, post-transcriptional regulation of Nrf2 by alternative splicing and miRNA, regulation of protein stability, and regulation of its activity on its target genes (Li *et al.*, 2019; Baird and Yamamoto, 2020) (Figure 23).

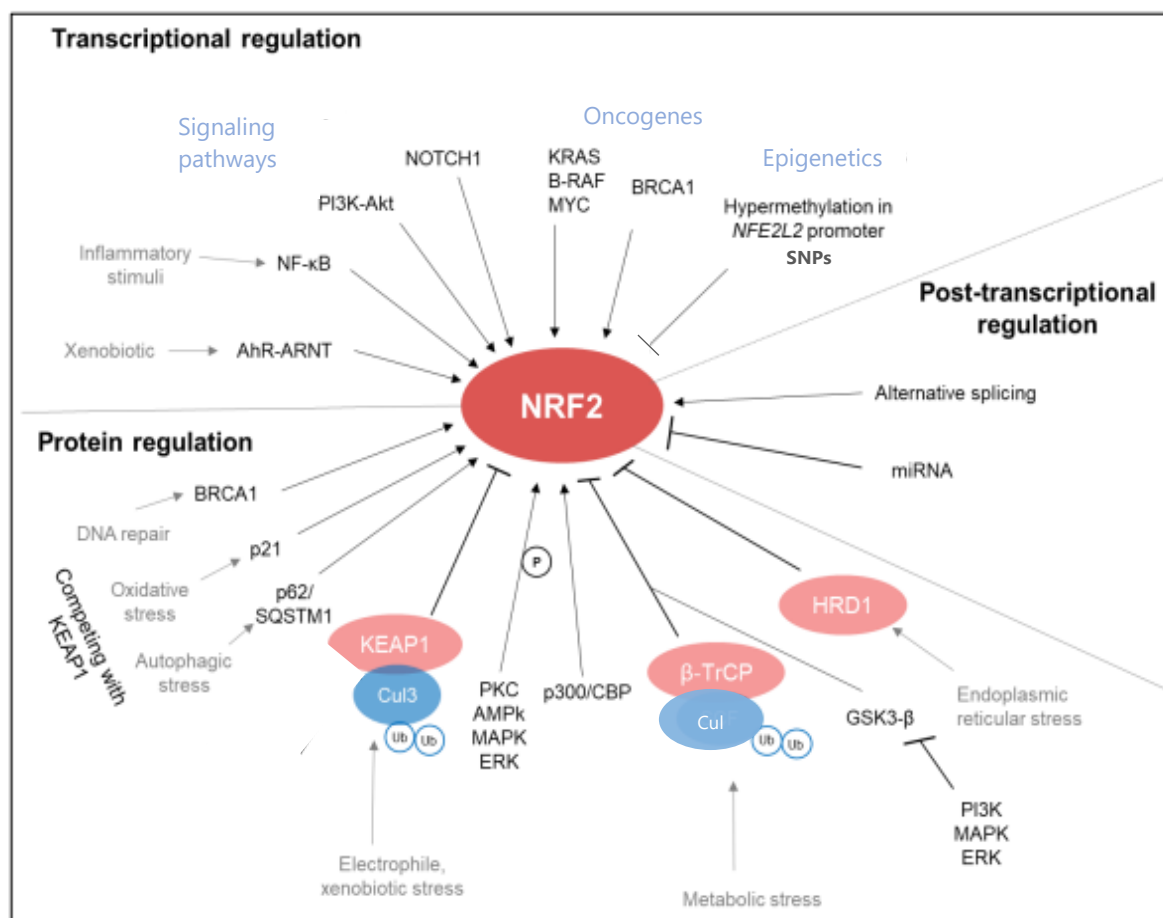


Figure 23. Mechanisms of regulation of Nrf2 activity. Mechanisms include transcriptional, post-transcriptional, and protein regulations. Arrows indicate activating regulation, and bars indicate inhibitory regulation. All text written in light grey are stimuli activating these regulators. β -TrCP, β -transducing repeat-containing protein; AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; B-RAF, v-raf murine sarcoma viral oncogene homolog B1; BRCA1, breast cancer susceptibility 1; CBP, CREB-binding protein; GSK3- β , glycogen synthase kinase-3beta; HRD1, HMG-CoA Reductase Degradation 1; KRAS, Kirsten rat sarcoma viral oncogene homolog; NF- κ B, nuclear factor- κ B; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; SNPs, single nucleotide polymorphisms. Adapted from Tonelli et al., 2018.

The phosphorylation of Nrf2 by kinases, including Protein kinase C delta (PKC δ), MAPK (ERK, JNK, p38), results in Nrf2 stabilization and translocation to the nucleus. Nrf2 can also be activated via acetylation by its transcriptional co-activators, such as p300 and CBP (CREB-binding protein). The p300/CBP complex possesses intrinsic histone acetyltransferase activity, allowing for the acetylation of lysine residues on Nrf2. The DNA-binding domain, Neh1, has the majority of its lysines acetylated. By acetylating Nrf2, CBP promotes the binding of Nrf2 to ARE sequences in the promoters of phase II genes such as NAD(P)H quinone oxidoreductase 1 (NQO1) (Sun et al., 2009). Conversely, the deacetylation of Nrf2 by the deacetylase SIRT1 results from the export of Nrf2 to the cytoplasm,

leading to a decrease in the transcription of Nrf2 target genes (Kawai *et al.*, 2011). Moreover, several cytoplasmic proteins that interfere with Keap1-Nrf2 interaction have been identified. These include ubiquitin-binding protein p62, also known as sequestosome 1 (p62/SQSTM1), a ubiquitin-binding protein that targets protein aggregates for degradation via the autophagic pathway. p62 competes with Nrf2 for the binding with Keap1. An increase of p62 leads to the degradation of Keap1 and a consequent increase in Nrf2 protein stability (Komatsu *et al.*, 2010).

2.4. Downstream targets regulated by Nrf2

While initially associated with oxidative stress, it is now widely recognized that Nrf2 responds to various stressors and participates in various biological processes (He *et al.*, 2020). A classification by Malhotra *et al.* identified three main categories of Nrf2 targets: basal targets expressed under normal conditions, stress-induced targets activated in response to different *stimuli*, and common targets activated both in basal and stress conditions (Malhotra *et al.*, 2010). The expression of Nrf2 target genes is highly dependent on the levels of Nrf2 protein. Depending on the nature and intensity of the *stimuli*, Nrf2 can regulate the transcription of numerous target genes in a dose-dependent manner. Nrf2 basal targets are involved in **mitochondrial physiology and biogenesis**, in **proteostasis** through autophagy regulation and proteasomal assembly, and in **metabolic reprogramming** as it regulates iron and heme metabolism, as well as lipid, glucose, and amino acids metabolism. Whereas stress-induced Nrf2 targets are involved in the **antioxidant defense, xenobiotic detoxification, inflammation and immunity** regulation, **DNA repair**, and prevention of apoptosis (Dodson *et al.*, 2019; He *et al.*, 2020) (Figure 24).

In response to cellular stress, the activation of Nrf2 triggers the expression of cytoprotective genes through AREs. This coordinated gene expression forms a network of enzymes involved in xenobiotic detoxification and the elimination of pro-oxidants, which helps maintain cellular homeostasis.

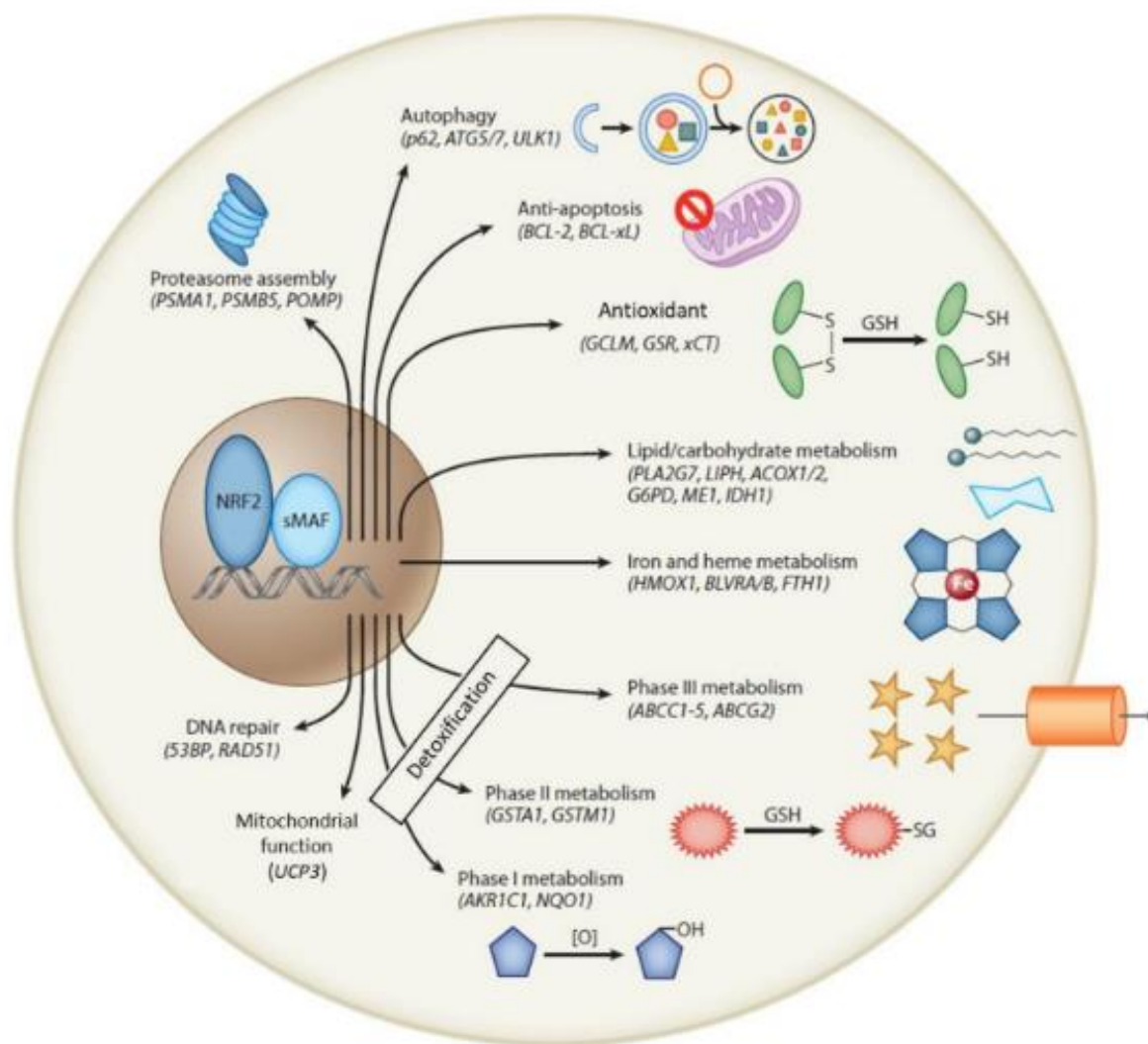


Figure 24. Key cellular functions targeted by Nrf2. Representative target genes of each function are included in parentheses below each transcriptional response. Adapted from Dodsden et al. 2019

To counteract the detrimental effects of reactive species, cells activate their antioxidant defense mechanisms, mainly orchestrated by Nrf2 transcriptional activity. Nrf2 induces general antioxidant pathways by regulating the gene expression of a broad spectrum of antioxidant enzymes mainly involved in ROS neutralization, such as superoxide dismutase (SOD) and catalase, and in glutathione (GSH) synthesis and regeneration (Baird and Yamamoto, 2020; He *et al.*, 2020) (Table 4). GSH is a tripeptide molecule with a thiol group that acts as a potent non-enzymatic antioxidant.

Additionally, the cellular defense mechanism under stressful conditions also involves the removal of potentially harmful xenobiotics through the activation of drug-metabolizing enzymes and drug-efflux transporters. This process helps eliminate molecules that can generate ROS through redox cycling or deplete endogenous antioxidants by removing electrophiles (Bauer, 2014; Dinkova-

Kostova & Talalay, 2008; Tebay et al., 2015). The detoxification enzymes regulated by Nrf2 are categorized into three phases of xenobiotic metabolism:

- **Phase I enzymes**, including NQO1, carbonyl reductases, aldo-keto reductases (AKRs), aldehyde dehydrogenases (ALDHs), and certain cytochrome P450 oxidoreductases, mediate oxidation, reduction, and hydrolytic reactions of toxicants or drugs to activate metabolites (Jung *et al.*, 2013; Dodson *et al.*, 2019; Ross and Siegel, 2021).
- **Phase II enzymes**, such as glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), and UDP-glucuronic acid synthesis enzymes, catalyze conjugation reactions of the metabolites (Chanas *et al.*, 2002; Yueh and Tukey, 2007; Nakamura *et al.*, 2008).
- **Phase III enzymes**, mainly drug efflux transporters like multidrug resistance-associated proteins (MDR), breast cancer resistant protein (BCRP), and ATP-binding cassette family members, transport the conjugated metabolites outside the cell (Maher *et al.*, 2007; Singh *et al.*, 2010).

Table 4. Main antioxidant enzymes encoded by Nrf2 target genes and their principal function (Hayes and Dinkova-Kostova, 2014; Tebay et al., 2015; Dodson et al., 2019; Tu et al., 2019; He et al., 2020)

Primary antioxidant function	Enzyme (Enzyme abbreviation)	Enzyme principal function
ROS neutralization	Superoxide dismutase (SOD)	Metalloenzyme that converts $O_2^{\bullet-}$ into water and H_2O_2
	Catalase (CAT)	Converts H_2O_2 into water and oxygen
	Glutathione peroxidases (GPx)	Reduce H_2O_2 and lipid hydroperoxides using glutathione as a cofactor
	Peroxiredoxins (PRDX)	Reduce peroxides directly
	Thioredoxin 1 (T1)	Reduces oxidized protein thiols and cleaves disulfide bonds
Electrophiles detoxification	NAD(P)H quinone oxidoreductase 1 (NQO1)	Reduces quinones and scavenges $O_2^{\bullet-}$ (Ross and Siegel, 2021)
	Glutathione S-transferases (GST)	Promotes the nucleophilic attack by glutathione on electrophilic molecules
Glutathione (GSH) production and antioxidant recycling	Glutamate-cysteine ligase catalytic (GCLC) and modulator (GCLM) subunits	Catalyze the first and rate-limiting step of GSH biosynthesis
	Glutathione synthetase	Catalyze the final step in GSH biosynthesis
	Glutathione reductase (GSR)	Catalyzes the reduction of oxidized glutathione (GSSG) back to its reduced form (GSH) using NADPH as a cofactor
	Thioredoxin reductase 1 (TXNRD1)	A key enzyme responsible for reducing and regenerating oxidized Trx1 using NADPH
NADPH regeneration	Glucose 6-phosphate dehydrogenase (G6pd), 6-phosphogluconate dehydrogenase (Pgd), Isocitrate dehydrogenase 1 (IDH1), and malic enzyme (Me1)	Promote NADPH production through the pentose phosphate pathway, NADPH being a cofactor for both the GSR and the TXN systems (Wu et al., 2011)
Heme metabolism	Heme oxygenase-1 (HO-1)	Catalyzes heme degradation to carbon monoxide (Zhang et al., 2021)

3. NRF2 FUNCTIONS IN THE SKIN

3.1. Role of Nrf2 in epidermal differentiation

To protect the skin against UV radiation, oxidative stress, and xenobiotic aggressions, all skin cells express Nrf2 at a relatively high level, according to the human protein atlas (Kahremany *et al.*, 2022). Within the epidermis, KCs show an increased expression of Nrf2 (Schäfer and Werner, 2015). Studies have revealed a gradient of Nrf2 expression and activity in normal human epidermal KCs (NHEK) during their differentiation process (Piao *et al.*, 2011). Cells in the upper layers express higher levels of Nrf2 and its target genes than undifferentiated basal cells. Particularly, the expression of NQO-1, an Nrf2 target, is induced at a late stage of differentiation (7 days after cell confluence), while Nrf2 is upregulated a few days earlier (Piao *et al.*, 2011).

Interestingly, Nrf2 plays a role in promoting KC differentiation. Increased Nrf2 accumulation during calcium-induced differentiation has been observed, and experiments with overexpressed Nrf2 in normal human KCs have shown enhanced expression of loricrin and keratin 10, even in the absence of calcium, which is a known promoter of differentiation (Lee *et al.*, 2014). The gradient of Nrf2 coincides with the preferential apoptosis of KCs in the upper layers of the epidermis, particularly in response to UV irradiations and other environmental factors. The higher expression levels of antioxidant enzymes and Nrf2 in the skin's upper layers reinforce the epidermis's antioxidant and detoxification capacity, especially since the superficial layers are more vulnerable to harmful external factors (Schäfer and Werner, 2015).

3.2. Role of Nrf2 in barrier function

Nrf2 is a direct player in the epidermal barrier function, affecting desmosome stability, corneocyte fragility, and keratinocyte proliferation. Transgenic mice with constitutively active Nrf2 exhibit a phenotype characterized by hyperkeratosis, epidermal thickening, and inflammation resembling lamellar ichthyosis (Schäfer *et al.*, 2012). This phenotype is accompanied by an upregulation of differentiation markers, including loricrin, involucrin, and keratin-10, with an impaired corneocyte desquamation, leading to hyperkeratosis. This data suggests that the Nrf2 pathway influences the quality of the skin barrier function (Schäfer *et al.*, 2012). High levels of hypophosphorylated Nrf2 were found in lesional tissue of patients with pachyonychia congenita, a rare skin disease characterized by palmoplantar keratoderma and caused by Keratin 16 gene mutations. This dysfunctional Nrf2 correlated with deficient synthesis of GSH in KCs. Topical

application of SFN, a pharmacological activator of Nrf2 to *Krt16*^{-/-} mice, prevented the clinical lesions and normalized redox balance, suggesting a potential role for Nrf2 in epidermal differentiation and skin integrity (Kerns *et al.*, 2016). In the same manner, previous research has shown that SFN can reprogram the expression of keratins K16 and K17, leading to the restoration of epidermal integrity in genetically modified mice with epidermolysis bullosa simplex (Kerns *et al.*, 2010). Nrf2 also regulates the expression of keratins 6, 16, and 17, which are linked to KC hyperproliferation (Yang *et al.*, 2017). Moreover, Nrf2 increases the expression of mi-RNA 29a and -b that target desmocollin-2, a major desmosomal component (Kurinna *et al.*, 2014).

Further insights were obtained from studying mice with a loricrin gene deletion. Loricrin, a critical component of the cornified cell envelope, plays a vital role in maintaining the skin's barrier function. In loricrin knockout mice, Nrf2 activation occurs in the epidermis, leading to the overexpression of *Lce1* genes. These genes encode glycine/serine-rich proteins similar to loricrin structure that potentially compensate for loricrin deficiency (Ishitsuka *et al.*, 2016). Recent findings have confirmed the coordinated action of Nrf2 and loricrin in ensuring the cornification of the skin barrier. In the absence of loricrin, the lack of sulfur-rich proteins (thiols) in the epidermis disrupts the local redox balance, subsequently mobilizing the Nrf2 pathway to produce lamellar granules (Ishitsuka *et al.*, 2022). Genetic activation of Nrf2 in KCs of a murine model of Netherton syndrome has improved their skin barrier defect by both reducing the expression of pro-inflammatory cytokines and upregulating the secretory leukocyte peptidase inhibitor (SLPI), which in turn inhibits kallikrein 7 and elastase 2 and thus increases the attachment of the stratum corneum by stabilizing desmosomes (Muzumdar *et al.*, 2020). This evidence underscores the role of Nrf2 in strengthening keratinization and promoting epidermal barrier cohesion.

At the molecular level, Nrf2 interacts with AhR, a transcription factor activated by specific chemical ligands such as dioxin and polycyclic aromatic hydrocarbons. AhR regulates the expression of phase I metabolism enzymes (e.g., cytochrome P450 (CYPs)) and proteins involved in epidermal differentiation (e.g., filaggrin, loricrin, involucrin) (Furue, 2020). Intriguingly, it was shown that AhR has an XRE binding site in the promoter of Nrf2 gene (Miao *et al.*, 2005), while Nrf2 reciprocally possesses a binding site in the AhR gene promoter (Shin *et al.*, 2007), suggesting a mutual upregulation of both factors' transcription (Edamitsu *et al.*, 2022). This interaction between Nrf2 and AhR influences the quality of the barrier function, particularly under conditions of stress or in skin diseases characterized by an impaired skin barrier, such as AD or psoriasis. To date, active

ingredients such as tapinarof (topical FDA-approved treatment of psoriasis), coal tar, and glyteer (soybean tar) have this dual benefit (van den Bogaard *et al.*, 2013; Takemura *et al.*, 2018; Hwang *et al.*, 2022; Nogueira *et al.*, 2022).

Recently, a quantitative proteomics study identified a reduced Nrf2 activity and mitochondrial dysfunction in lesional and non-lesional AD patient skin, together with a protein profile reflecting inflammation, defect in KC differentiation, and epidermal stratification (Koch *et al.*, 2023). This data provides further support for the use of Nrf2 inducers, such as bixin (apocarotenoid), in the treatment of AD (Rojo de la Vega *et al.*, 2017; Ogawa and Ishitsuka, 2022). In addition to its potential application in skin diseases, enhancing the barrier function could be useful in protecting the epidermis against harsh environmental factors, such as polluted urban environments and compromised skin conditions, including dry skin and the aging skin (Frantz *et al.*, 2023).

3.3. Role Nrf2 in skin (photo-)aging and photoprotection

The activation of the Nrf2 pathway has been found to protect against the acute effects of UVB and UVA radiations (Kahremany *et al.*, 2022). Genetically modified mice with increased Nrf2 expression demonstrated resistance to UV-induced erythema and the development of precancerous lesions and squamous cell carcinoma. On the other hand, Nrf2 deficiency exacerbates the negative effects of UV radiation on the skin (Schäfer *et al.*, 2010; Saw *et al.*, 2014). Similarly, Nrf2 activators, like sulforaphane or bixin, have also shown protective effects against UV-induced erythema and pigmentation in clinical studies with humans (Talalay *et al.*, 2007; Rojo de la Vega *et al.*, 2017; Kerns *et al.*, 2020).

UV-induced photoaging, characterized by dermal deterioration and the overproduction of MMPs, is associated with oxidative stress (Pilkington *et al.*, 2021). The Nrf2 pathway offers advantages in this context by neutralizing ROS, reducing the “inflammaging” (Pilkington *et al.*, 2021), and alleviating fibrosis and MMP production (Chaiprasongsuk *et al.*, 2017; Wu *et al.*, 2020). Results on photoaging, including wrinkles, induced by UVB in Nrf2-deficient mice have also been published (Hirota *et al.*, 2011). Molecular analysis has provided insights into the mechanisms by which Nrf2 provides photoprotection, including the modulation of antioxidant enzyme production, neutralization of toxic aldehydes, repair of oxidative DNA lesions, and inhibition of pro-inflammatory cytokines (Kahremany *et al.*, 2022). Considering these findings, Nrf2 activators are promising for sun and after-sun products (Rojo de la Vega *et al.*, 2017; Frantz, Rozot, and Marrot,

2023). Evidence from the 3D skin model demonstrates that the protection by skin-derived precursor cells (SKP) against UV-mediated damage is primarily via the PI3K/AKT-mediated activation of the Nrf2/heme oxygenase (HO-1) pathway (Xian *et al.*, 2019). SKP are adult stem cells of dermal origin that have been shown to strongly resist UVB-induced apoptosis and DNA damage via up-regulation of Bcl-2 and Nrf2 (Zhong and Li, 2016). Nrf2 also plays a role in chronological skin aging, as observed in animal models for longevity (Yu and Xiao, 2021; Michalak, 2022).

Furthermore, Nrf2 activation may lower the risk of tumor initiation and promotion by reducing mutagenic DNA lesions and dampening UV-induced inflammation. However, caution is necessary when modulating Nrf2 in cancer treatment, as cancer cells often rely on Nrf2 for survival and resistance to apoptosis. The hyperactivation of Nrf2 in cancer cells has been associated with aggressive proliferation (Okazaki *et al.*, 2020), metastasis (Lignitto *et al.*, 2019), and resistance to chemotherapy (Jeong *et al.*, 2020). Antioxidant intake that may support cancer cell survival should be avoided. Nonetheless, specific Nrf2 activators or agents inducing mild oxidative stress, such as certain polyphenols, may have potential benefits for healthy cells while being detrimental to tumor cells, although further research is needed to confirm this hypothesis (Obrador *et al.*, 2019; Emanuele *et al.*, 2021).

It should be noted that Nrf3, in addition to Nrf2, has been proposed to play a significant role in maintaining a balanced response to UV radiations. Siegenthaler *et al.* investigated the role of Nrf3 in the context of UVR and found that keratinocytes lacking Nrf3 in mice exhibited reduced susceptibility to UVR-induced apoptosis due to increased cellular adhesion. Interestingly, this effect was not dependent on the classical induction of antioxidant defense or the activation of key target genes associated with Nrf2. Furthermore, the study revealed a transient decrease in epidermal NRF2 expression following exposure to physiologically relevant UVB radiation (Siegenthaler *et al.*, 2018).

3.4. Role of Nrf2 in skin inflammation

In addition to the primary function of Nrf2 as a master regulator of xenobiotic detoxification and redox homeostasis within the cells, recent investigations revealed that Nrf2 also plays an essential role in the control of the inflammatory response (Kim *et al.*, 2010; Keleku-Lukwete *et al.*, 2018; Saha *et al.*, 2020). Persistent inflammation is a common characteristic of all pathophenotypes observed in the Nrf2 “diseasome” (Cuadrado *et al.*, 2018). In particular, Nrf2 is a regulator of the skin inflammatory immune response, as several experimental studies have consistently

demonstrated that Nrf2 deficiency is consistently linked to enhanced inflammation, indicating an anti-inflammatory role for Nrf2 (Gęgotek and Skrzydlewska, 2015; Helou, Martin, *et al.*, 2019; Vallion and Kerdine-Römer, 2022). As shown in CHS models induced by chemical sensitizers, such as 2,4-dinitrochlorobenzene (DNCB), ear inflammation and lymphocyte proliferation were exacerbated in mice *nrf2*^{-/-} compared to *nrf2*^{+/+}, demonstrating an essential role for Nrf2 in controlling ACD (El Ali *et al.*, 2013). Another study reveals that Nrf2 controls pro-inflammatory cytokine production in human KCs upon cinnamaldehyde exposure, a well-known skin sensitizer (Vallion *et al.*, 2022). Other data supports the role of Nrf2 in modulating the immune response (Gęgotek and Skrzydlewska, 2015). Nrf2 inhibition in mice alters the phenotype of bone marrow-derived DCs since Nrf2-deficient DCs have impaired GSH levels, reduced phagocytic activity, enhanced expression of MHC class II, and co-stimulatory receptor expression of CD86 and CD80, thereby enhancing T cells stimulatory capacity (Yeang *et al.*, 2012). It was also shown that Nrf2 is a critical factor for chemical-induced skin innate immunity, particularly for the sensitization phase of ACD regulating neutrophil recruitment and accumulation in the skin (Helou, Noël *et al.*, 2019). Furthermore, as mentioned before, Nrf2 disturbances have been implicated in chronic inflammatory skin diseases, such as psoriasis and AD (Vallion and Kerdine-Römer, 2022).

Healing is a complex process involving multiple stages, including transient local inflammation. Once tissue regeneration occurs, restoring homeostasis is crucial, and Nrf2 plays a role in reducing local inflammation and associated ROS levels (Süntar *et al.*, 2021). While Nrf2-KO mice result in a longer duration of inflammation in wounds, it does not significantly affect the duration or quality of healing. However, in diabetic animals, lower Nrf2 expression has more harmful effects, leading to increased oxidative stress and apoptosis in the perilesional area of the skin (Long *et al.*, 2016; Hiebert and Werner, 2019). Nrf2 signaling has been shown as a key event in physical plasma-spurred wound healing, promoting granulation and reepithelialization with a balanced antioxidant and inflammatory response (Schmidt *et al.*, 2019). Using Nrf2 activators or increased Nrf2 expression through Keap1 interfering RNAs improves healing in diabetic conditions. Potent Nrf2 inducers like sulforaphane, cinnamic acid, dimethyl fumarate (DMF), and triterpenoid RTA-408 accelerate healing in diabetes (Rabbani *et al.*, 2017). The precise mechanisms through which Nrf2 influences the healing process are not fully understood. Studies suggest that Nrf2 stimulates the production of chemokine CCL2 by epithelial stem cells, which mobilizes macrophages and, in particular their secretion of EGF, a growth factor that increases KC proliferation for epidermal regeneration

(Villarreal-Ponce *et al.*, 2020). It is also appropriate to consider the role of Nrf2 in reducing the inflammation phase during the healing process since it modulates various key inflammatory mediators such as NF- κ B, NLRP3 inflammasome, and stimulating HO-1, which strong anti-inflammatory properties (Saha *et al.*, 2020). Nrf2 also controls the expression of interleukins IL6 and IL1 β in macrophages to limit pro-inflammatory effects (Kobayashi *et al.*, 2016).

Nrf2 can control the inflammation directly and indirectly (Figure 25). Indirect mechanisms to counteract inflammation involve ROS and RNS modulation by Nrf2 and the crosstalk of the Nrf2 pathway with redox-sensitive key inflammatory pathways, including the inflammasome and NF- κ B (Ahmed *et al.*, 2017) (Figure 25). The NF- κ B pathway and inflammasomes play crucial roles in mediating immune responses during infection and inflammation. During oxidative stress, I κ B, the negative regulator of NF- κ B, is phosphorylated by I κ B kinase (IKK), leading to the release and translocation of NF- κ B into the nucleus. NF- κ B then promotes the transcription of pro-inflammatory mediators such as IL-6, TNF- α , and cyclooxygenase-2 (COX-2). The signaling pathways of Nrf2 and NF- κ B interact through various mechanisms. Studies have shown a competitive binding between Nrf2 and IKK with Keap1, resulting in Keap1-mediated proteasomal degradation of IKK, thus stabilizing I κ B, and inhibiting NF- κ B (Kim *et al.*, 2010; Tian *et al.*, 2012). Furthermore, the activation

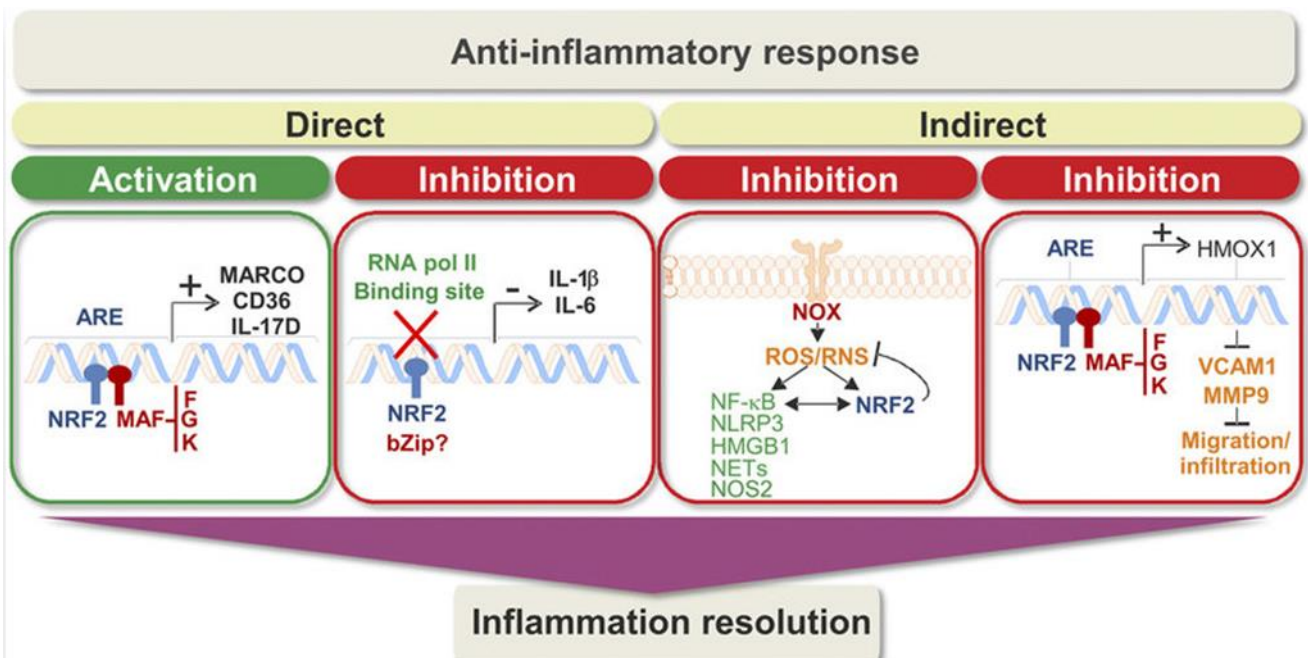


Figure 25. Direct and indirect mechanisms involved in the control of inflammation by Nrf2. Direct mechanisms include both the activation of anti-inflammatory genes like CD36 and MARCO, and the suppression of pro-inflammatory cytokine gene expression, such as IL-6 and IL-1 β , although the specific bZip partner for the latter requires further investigation. Indirect methods involve the modulation of ROS/RNS, the inhibition of immune cell migration/infiltration through HO-1 activity, and the cross-talk of Nrf2 with NF- κ B and NLRP3 pathways aiming to inhibit them and ultimately suppress inflammation. From Cuadrado *et al.*, 2018

of Nrf2 leads to increased activity of HO-1, which also contributes to the inhibition of NF- κ B. Its immunomodulatory function is very well established. It directly inhibits pro-inflammatory cytokines and activates anti-inflammatory cytokines, thus balancing the inflammatory process (Campbell *et al.*, 2021). In parallel, the canonical NF- κ B subunit p65 can affect the Nrf2 pathway. Overexpression of p65 causes the translocation of Keap1 into the nucleus, disrupting the Nrf2-ARE interactions (Yu *et al.*, 2011). Moreover, p65 and Nrf2 compete for the transcriptional co-activator CBP, which acetylates non-histone proteins to enhance the assembly of the transcriptional machinery. Therefore, reducing p65 levels can prevent Nrf2 from being deprived of CBP (Liu *et al.*, 2008).

The activation of NF- κ B is essential for priming the NLRP3 inflammasome, a complex involved in sensing danger signals and promoting inflammation. Conversely, Nrf2 activation can inhibit both pathways, thereby limiting inflammation. The NLRP3 inflammasome responds to various danger signals, including ROS (Rabolli *et al.*, 2016). Nrf2 acts by reducing ROS levels through the activation of antioxidant genes, thus inhibiting the priming of the NLRP3 inflammasome. Recent research has shown that Nrf2 plays a key role in inhibiting the NLRP3 inflammasome by activating TXN1 in cerebral ischemia-reperfusion injury (Hou *et al.*, 2018).

Moreover, the multidomain protein p62, which plays a role in Nrf2 activation by facilitating the autophagosomal degradation of Keap1, is also implicated in inhibiting the NLRP3 inflammasome. This protein, induced by NF- κ B, serves as a crucial connection between Nrf2, NF- κ B, and the NLRP3 inflammasome, contributing to cellular homeostasis and protecting against oxidative stress and inflammation through Nrf2-dependent mechanisms. (Ichimura *et al.*, 2013). Overall, Nrf2 indirectly regulates inflammation through inhibitory interactions with the two major inflammatory pathways, NF- κ B and the NLRP3 inflammasome (Hennig *et al.*, 2018). Additionally, Nrf2 can hinder IL-6 expression indirectly by inducing ATF3 through ARE-dependent mechanisms, which in turn suppresses IL-6 transcription (Hoetzenecker *et al.*, 2012; Bambouskova *et al.*, 2018).

Furthermore, it has been demonstrated that Nrf2 exerts its anti-inflammatory effects by directly suppressing the transcription of pro-inflammatory cytokine genes (Figure 25). ChIP-seq analysis has shown that Nrf2 directly binds close to the promoter regions of IL-6 and IL-1 β genes in an ARE-independent manner, impeding the recruitment of RNA polymerase II and thus inhibiting their induction in macrophages, contrary to the widely accepted view that Nrf2 suppresses inflammation through redox control (Kobayashi *et al.*, 2016). Furthermore, Nrf2 directly upregulates

the expression of MARCO gene, encoding a scavenger receptor required for bacterial phagocytosis, thereby improving bacterial clearance (Harvey *et al.*, 2011). Among direct mechanisms of Nrf2 controlling inflammation, it was recently demonstrated that Nrf2 upregulates the efferocytosis receptor CD36 on skin macrophages and led to an improved capacity to phagocyte damaged and apoptotic neutrophils in response to chemical stress or an inflammatory agent derived from yeast, zymosan, thereby promoting the resolution of inflammation (Kim *et al.*, 2017; Helou, Noël, *et al.*, 2019).

RESEARCH OBJECTIVES

RESEARCH OBJECTIVES

Inflammation is a significant common contributor to numerous acute and chronic skin disorders, making its management paramount in medical and aesthetic dermatology. Traditional pharmacotherapies for inflammatory skin diseases often carry side effects, including immunosuppression, while insufficient responses and relapses remain a challenge. Thus, there is an increasing need within the dermatological field to explore safer and cost-effective approaches as alternative or complementary therapies addressing skin inflammation. One promising area of interest is PBM therapy, a fast-growing therapeutic approach used across various medical domains, including dermatology. PBM uses low-energy visible light or NIR to target skin inflammation and accelerate wound healing and tissue regeneration. Clinical and *in vivo* studies have highlighted its efficacy in treating various dermatoses such as acne, rosacea, AD, and psoriasis. Despite the increasing recognition of PBM's potential in modulating inflammation, the molecular mechanisms driving its anti-inflammatory effects remain elusive. While some studies have reported the modulation by the visible light of different cellular factors, such as NF- κ B and MAPK family members, correlated to anti-inflammatory activity, these observations lack strong evidence about the direct implication of these factors in the PBM therapeutic effect. **We conducted this research to better understand the cellular pathways through which PBM regulates skin inflammation, offering valuable insights into this promising approach.** Our study has two major axes, each serving as the subject of an article. The first axis focuses on the effects of red light, widely used wavelengths in PBM protocols to treat inflammation *in-vivo* and *in-vitro*. The second axis examines the less-explored green light, which has recently demonstrated promising anti-inflammatory properties.

The transcription factor Nrf2 is a key regulator of the cellular defense mechanisms against oxidant and electrophilic stress. It orchestrates the antioxidant and the anti-inflammatory response by regulating the expression of numerous antioxidants, cytoprotective, and detoxification enzymes, as well as modulating the expression of some pro-inflammatory cytokines. Previous reports have particularly emphasized Nrf2's pivotal role of Nrf2 in regulating skin allergic inflammation in response to contact allergens. While only a few studies have reported the effects of PBM on Nrf2 expression and activity, none have delved into Nrf2's role in the observed anti-inflammatory responses. Trotter *et al.* reported a rapid accumulation and nuclear translocation of Nrf2 after

treating the human monocytic cell line, THP-1, with blue light (45 J/cm²), followed by induction of HO-1 expression, an Nrf2-target anti-inflammatory and antioxidative enzyme. These observations were associated with reduced LPS-induced TNF- α and IL-8 secretion (Trotter *et al.*, 2017). In the previous studies, the authors also found that blue light treatment induced upregulation of Nrf2 in A431 epidermoid carcinoma cells and significantly increased levels of HO-1 (Patel *et al.*, 2014). Moreover, Sohn *et al.* showed an upregulation of Nrf2 gene expression and protein accumulation upon treatment of RANKL-stimulated mouse bone marrow-derived macrophages with 635 nm LED (Sohn *et al.*, 2015). Yadav *et al.* recently demonstrated activation of the Nrf2 pathway associated with an acceleration of burn healing in mice treated at 904nm (Yadav *et al.*, 2020). As KCs constitute the major cell population in the epidermis and actively contribute to the inflammatory responses in many skin pathologies, our primary objective of this thesis was to **investigate the implication of Nrf2 in PBM's anti-inflammatory activity using a human primary KC model**. KCs were challenged with the electrophilic compound DNCB, which induces pro-inflammatory stress. To assess Nrf2's role in attenuating the inflammatory response by two wavelengths, 660 and 520 nm, representing the red and green lights, respectively, we used the si-RNA tool to invalidate Nrf2 in primary KCs.

Furthermore, Transient Receptor Vanilloid 1 (TRPV1), a cation channel expressed by KCs, among other cells, has been reported to impact skin inflammation significantly. Although TRPV1 has been described as a potential target activated by the green light, its role in mediating the beneficial effects of the green light in KCs is not fully clarified, presenting an interesting area of investigation. Thus, **our research has also focused on the involvement of TRPV1 in the anti-inflammatory effect of the 520 nm-green LED**. To address this question, we selectively inhibited TRPV1 using its antagonist capsazepine (CPZ) in the same primary KC model. The reduction by the green light of the DNCB-induced pro-inflammatory cytokine expression was assessed when TRPV1 was blocked compared to the control group. As TRPV1 is an effective channel in signal transduction when activated, triggering multiple subsequent signaling pathways, we investigated whether the activation of the Nrf2 pathway by the green light depends on TRPV1 activity, allowing us to gain deeper insights into the molecular mechanisms of green light.

Additionally, KCs interact with other key cells in the epidermis, such as LCs. The latter are antigen-presenting cells crucial in the cutaneous immunosurveillance and initiating an appropriate adaptive immune response (immunogenic or tolerogenic) specific to an antigen. Therefore, **another**

objective of our thesis was to elucidate how PBM regulates the cutaneous microenvironment by acting on LCs. We evaluated the effects of red light (660 nm) on LC activation and migration through Langerin immunostaining in human skin explants exposed to DNCB, providing a more physiologically relevant model than the 2D cultures. This study contributes to a deeper understanding of PBM's immunoregulatory role.

Our research work investigating the anti-inflammatory mechanisms of red and green light unravels the complexities of PBM action in the skin. It highlights the versatility of this therapy as a tool acting on different cellular levels and targets to suppress inflammation. By elucidating how specific wavelengths of light interact with cells and tissues, we aim to uncover the underlying signaling pathways, gene expressions, and cellular responses involved. This knowledge is essential to address skepticism regarding PBM and establish a robust foundation for its use in clinical practice.

EXPERIMENTAL

RESULTS

ARTICLE 1

Article

Photobiomodulation Controls Keratinocytes Inflammatory Response through Nrf2 and Reduces Langerhans Cells Activation

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Abstract: Photobiomodulation (PBM) is rapidly gaining traction as a valuable tool in dermatology for treating many inflammatory skin conditions using low levels of visible light or near-infrared radiation. However, the physiological regulatory pathways responsible for the anti-inflammatory effect of PBM have not been well defined. Since previous studies showed that nuclear factor-erythroid 2 like 2 (Nrf2) is a master regulator of the skin inflammatory response, we have addressed its role in controlling inflammation by PBM. Primary human keratinocytes (KCs) stimulated with 2,4-dinitrochlorobenzene (DNCB) to mimic pro-inflammatory stress were illuminated with two wavelengths: 660 nm or 520 nm. Both lights significantly reduced the mRNA expression of the DNCB-triggered *TNF- α* , *IL-6*, and *IL-8* cytokines in KCs, while they enhanced Nrf2 pathway activation. PBM-induced Nrf2 is a key regulator of the inflammatory response in KCs since its absence abolished the regulatory effect of light on cytokines production. Further investigations of the mechanisms contributing to the immunoregulatory effect of PBM in inflamed human skin explants showed that 660 nm light prevented Langerhans cells migration into the dermis, preserving their dendricity, and decreased pro-inflammatory cytokine production compared to the DNCB-treated group. This study is the first to report that the PBM-mediated anti-inflammatory response in KCs is Nrf2-dependent and further support the role of PBM in skin immunomodulation. Therefore, PBM should be considered a promising alternative or complementary therapeutic approach for treating skin-related inflammatory diseases.

Keywords: Nrf2; keratinocyte; Langerhans cells; skin; inflammation; photobiomodulation; light; immunomodulation



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1. Introduction

The skin is a dynamic organ that fulfills multiple functions to maintain the internal environment's homeostasis. As a primary interface between our body and the outside world, the skin acts as the first line of defense against environmental aggressions (chemicals, microorganisms, and ultraviolet radiation (UV)) [1]. These protections are ensured by the epidermis, the outermost layer of the skin, mainly composed of keratinocytes (KCs), some melanocytes, and immune cells such as resident memory T cells and Langerhans cells (LCs). Long considered passive components of the cutaneous physical barrier, KCs are now described as sentinel cells actively contributing to the immunological barrier of the skin [2–4]. KCs play a central role in innate immunity as sensors of danger signals or stress factors through different types of Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs) [2]. The recognition of the cognitive ligands by PRRs on KCs triggers downstream signaling cascades, such as the nuclear factor kappa B (NF- κ B) pathway leading to the production of pro-inflammatory mediators such as Tumor necrosis factor-alpha (TNF- α), Interleukin (IL)-1, IL-6, and IL-8. These cytokines and chemokines

orchestrate the early immune response of KCs to pathogens and chemical challenges by activating residing immune cells in the skin and recruiting leukocytes to cutaneous sites of inflammation KCs. Therefore, KCs are immunocompetent effector cells that can initiate adequate immune responses to maintain skin homeostasis and defenses and thus have a crucial role in regulating skin inflammation [5].

In physiological conditions, skin cells produce in a controlled manner different reactive species, including reactive species of oxygen (ROS) and nitrogen (RNS) [6,7]. Upon exposure to UV, environmental pollutants, or chemicals, reactive species are produced excessively. The sustained overproduction of these oxidants exceeds the skin's antioxidant capacities, leading to an imbalance state called "oxidative stress" [8]. Oxidative stress and inflammation are interdependent pathophysiological processes, one of which can be easily induced by the other, sustaining a vicious loop of amplification of both events [9]. To ensure redox homeostasis and prevent the damage from redox active species, the skin has an extensive network of antioxidant defense systems, mainly orchestrated by the nuclear factor erythroid-2-related factor 2 (Nrf2) pathway [6]. It is well established that Nrf2 regulates the gene expression of a broad spectrum of antioxidant enzymes, such as superoxide dismutase (SOD) and glutamate-cysteine ligase catalytic subunit (GCLC), cytoprotective enzymes such as heme oxygenase 1 (HO-1), as well as phase II drug detoxification enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) [10,11]. Exposure to oxidative or electrophilic stress leads to the dissociation of Nrf2 from its cytoplasmic inhibitor Kelch-like ECH-associated protein 1 (Keap 1). Nrf2 then translocates into the nucleus and binds to the antioxidant-responsive elements (ARE) of target gene promoters, initiating the transcription of a battery of antioxidants and cytoprotective genes, as cited before [11,12]. In addition to the primary function of Nrf2 as a master regulator of xenobiotic detoxification and redox homeostasis within the cells, recent investigations revealed that Nrf2 also plays an essential role in controlling the inflammatory response [13–15]; in particular, skin inflammation [16,17]. As shown in contact hypersensitivity models induced by chemical sensitizers, such as 2,4-dinitrochlorobenzene (DNCB), ear inflammation and lymphocyte proliferation were exacerbated in mice *nrf2*^{-/-} compared to *nrf2*^{+/+}, demonstrating an essential role for Nrf2 in controlling allergic contact dermatitis [18]. Another study reveals that Nrf2 controls pro-inflammatory cytokine production in human KCs upon cinnamaldehyde exposure, a well-known skin sensitizer [19]. Nrf2 can control inflammation directly and indirectly [20,21]. Indirect mechanisms to counteract inflammation involve ROS and RNS modulation by Nrf2 and crosstalk of the Nrf2 pathway with redox-sensitive key inflammatory pathways, including NF-κB [21]. Recently, it has been described that Nrf2 can also directly inhibit the transcription of pro-inflammatory cytokine genes such as *IL-6* and *IL-1β* through direct DNA binding close to these genes, independently from ARE, contrary to the widely accepted view that Nrf2 inhibits inflammation through redox control [22].

To control skin inflammation, some safe and effective technologies have recently gained attention in medicine, namely photobiomodulation (PBM). PBM, previously known as Low-Level Light (Laser) Therapy (LLLT), consists of exposing tissues to low-energy visible light or near-infrared radiation (NIR) in order to accelerate tissue repair, reduce inflammation, or alleviate pain. Emerging since the early 2000s, PBM is now a valuable non-invasive tool in cosmetic and medical dermatology for treating numerous skin conditions such as rhytides, scars, ulcers, facial herpes simplex with red light and NIR, rosacea and pigmentary disorders with green light, psoriasis and acne with blue light [23,24]. In vitro studies have reported stimulating effects of visible light on various biological processes in the skin, including the proliferation and differentiation of KCs [25], their migration in wounds [26], and the synthesis of proteins such as filaggrin [27]. Several studies have described specific molecular mechanisms inducing the beneficial effects of light on biological systems. T. Karu's work showed that cytochrome C oxidase, located in the mitochondrial respiratory chain, is activated in response to red light and NIR [28]. Its absorption of photons leads to the upregulation of cell repair and survival pathways [28]. In vivo rat burn healing models and in vitro inflammation models induced by lipopolysaccharide (LPS)

show decreased pro-inflammatory cytokines after exposure to red light and NIR [29–31]. Thus far, the endogenous cellular pathways responsible for the well-documented anti-inflammatory effect of PBM have not been fully elucidated. The anti-inflammatory effect of PBM is presumed to be a consequence of ROS modulation. However, it is thought that it is not the only explanation behind this therapeutic property since other signaling pathways are also likely to be involved in reducing inflammation through PBM. Specifically, PBM has been shown to modulate the activity of the NF- κ B and mitogen-activated protein kinase (MAPK) pathways, both involved in the regulation of cytokine production and other inflammatory processes [29,32,33]. To date, only a handful of studies have evaluated the effects of PBM on Nrf2 expression and activity. Yadav et al. recently demonstrated activation of the Nrf2 antioxidant pathway associated with an acceleration of burn healing in mice treated at 904 nm [34]. However, there is no strong evidence for a direct link between the therapeutic action of PBM and the Nrf2 signaling during the inflammatory response.

This study aims to understand the cellular mechanisms by which PBM, mainly red light, regulates skin inflammation. We first investigated the role of Nrf2 in the PBM anti-inflammatory response using a two-dimensional model of primary human KCs challenged with DNCB, a contact sensitizer used to mimic pro-inflammatory stress. Herein, we showed that Nrf2 is a crucial factor controlling DNCB-induced pro-inflammatory cytokines production in KCs upon light exposure. Assessment of the PBM effects on LCs activation in human skin explants challenged with the same sensitizer demonstrated the immunoregulatory role of PBM in the skin as it decreases LCs migration into the dermis.

2. Materials and Methods

2.1. Cell Culture

Human primary epidermal KCs (HPEK) isolated from the juvenile foreskin of multiple donors (>three individuals) were purchased cryopreserved at an early passage from CELLnTEC (Bern, Switzerland). Cells were cultured according to the manufacturer's protocol in CnT-Prime Epithelial Proliferation medium (CnT-PR) fully supplemented, phenol red-free medium with low calcium formulation (0.07 mM) and a combination of Growth factors and Progenitor Cell Targeted factors maximizing retention of proliferative progenitor cells and minimizing cell differentiation (CELLnTec, Bern, Switzerland). HPEK were seeded after thawing or passaging at a density of 4000 cells/cm² and were incubated at 37 °C in a humidified atmosphere under 5% CO₂. The medium was replaced every two days. Cells were passaged at ~80% confluency (day 6) after trypsinization with 0.05% trypsin–EDTA solution (Gibco, Paisley, UK). Cells between the second and fourth passages were used for experiments.

2.2. Chemical Treatment

When reaching 80% confluency, HPEK were detached using 40 μ L/cm² of trypsin–EDTA (0.05%) solution (Gibco, Paisley, UK) and re-plated for at least 18 h in a 24-well plate at a seeding density of 2×10^5 cells per well in 500 μ L CnT-PR medium. The next day, the cells were treated with 25 μ M DNCB (CAS number: 97-00-7; Sigma-Aldrich, St. Louis, MO, USA) for 3 or 6 h. DNCB was diluted in dimethyl sulfoxide (DMSO, CAS number: 67-68-5, Sigma-Aldrich). The final concentration of the solvent in culture media did not exceed 0.1%. Cytotoxicity was evaluated by measuring the fluorescence of propidium iodide uptake using flow cytometry (Attune[®] NxT Acoustic Focusing cytometer; Thermo Fisher, Waltham, MA, USA) and analyzed with FlowJo software (version 8.0.2, BD Biosciences, San Jose, CA, USA).

2.3. Light Source and Photobiomodulation Treatment

HPEK plated at 2×10^5 cells per well in a 24-well transparent plate were illuminated, as described by Guermonprez et al. [35]. Briefly, the light source was pulsed-wave light-emitting diodes (LEDs) placed in the upper lid of a custom-made device allowing

homogeneous and simultaneous irradiation of 12 wells of the plate. LEDs were calibrated with a Qmini 2 spectrometer (RGB Photonics GmbH, Kelheim, Germany) to provide identical light irradiance in each well. Light homogeneity was also controlled for each well (SP90422 Beam Analyser, Ophir Spiricon Europe GmbH, Darmstadt, Germany). Irradiance and homogeneity were measured at the bottom of the wells.

Cells were illuminated at room temperature in air, with two visible pulsed wavelengths separately: 660 nm (red light) or 520 nm (green light), 20 min after DNCB treatment. Light parameters were identical for each wavelength. Cells were illuminated at an irradiance of $12 \pm 4 \text{ mW/cm}^2$ for a total exposure time of 250 s, which conferred a fluence (energy density) of 3 J/cm^2 . The pulse width was fixed at 10 milliseconds, with a duty cycle (DC) of 50%. The distance from the LED to the bottom of the well was 34.5 mm. Cells with no light treatment were used as a negative control (sham group), covered with a black wrap to avoid any light exposition. Following irradiation, cultures were incubated at 37°C , 5% CO_2 until required for further analysis.

2.4. Gene Silencing

Stealth RNAi™ small interfering RNA (siRNA) technology from Invitrogen (Paisley, UK) was used for *Nrf2* knockdown (NFE2L2 HSS107130), targeting the eight different variants of the transcript. The Forward Transfection Protocol using Lipofectamine® RNAiMax Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was performed on KCs according to the manufacturer's instructions. Briefly, HPEK were seeded in 24-well plates at a density of 2.5×10^4 per well in 500 μL of CnT-PR medium one day prior to transfection so that they would be 30–50% confluent at the time of transfection. The next day, the transfection mix (siRNA duplex-Lipofectamine® RNAiMAX complexes) was prepared, and cells were transfected with 50 nM of siRNA *Nrf2* (si-Nrf2 group). Mock transfection (no siRNA) and the scrambled non-silencing siRNA (si-random) with no known human gene homology (Stealth™ RNAi Negative Control-Medium GC duplex; Invitrogen, Carlsbad, CA, USA) were performed in each experiment as negative controls. At 48 h after transfection, the transfected HPEK were used for further experiments. The repression of *Nrf2* and the expression of *Nrf2* target genes were evaluated using Western blot and RT-qPCR, respectively.

2.5. RNA Isolation and RT-qPCR

Total RNA was extracted from HPEK using a silica column-based purification with PureLink™ RNA Mini Kit according to the manufacturer's instructions (Ambion®; Invitrogen, Carlsbad, CA, USA). Total RNA yield was quantified by spectrophotometry (Eppendorf BioPhotometer; Hamburg, Germany), and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA.

A total of 500 ng of RNA was reverse transcribed into cDNA in a total volume of 20 μL with 2.5 μM of oligo (DT) (Promega®, Madison, WI, USA), 0.5 mM of dNTP (MP Biomedicals®, Santa Ana, CA, USA), 1 U/ μL of SuperScript™ IV Reverse Transcriptase (Invitrogen®, Waltham, MA, USA), 1 U/ μL of RNasin (Promega®), 5 mM of Dithiothreitol (DTT; Invitrogen®) and 1X Reverse Transcriptase buffer (Invitrogen®). Reverse transcription products were diluted at 1:20 in RNase-free water and used for quantitative real-time PCR. Quantitative PCR was carried out with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) using the thermal cycler CFX384™ Touch Real-Time System (Bio-Rad, Hercules, CA, USA). All samples were assayed in duplicate. Primer sequences used for cDNA amplification are listed in Supplementary Table S1. Quantification was performed with CFX Maestro Software (version 2.3, Bio-Rad). The results were normalized to the two house-keeping genes expressions: *gapdh* and *hprt*, and were expressed as the fold increase (i.e., ratio of $(1 + E)^{-\Delta\text{Ct}}$ of target genes/ $(1 + E)^{-\Delta\text{Ct}}$ of reference genes, where E is the efficiency and ΔCt is the difference of Ct between treated cells and untreated control cells).

2.6. Protein Extraction and Western Blotting

After pretreatment with DNCB and illumination, HPEK were washed twice in phosphate-buffered saline, trypsinized, and centrifuged into a dry pellet. Cells were resuspended in lysis buffer (20 mM Tris HCl (pH 7.4); 137 mM NaCl; 2 mM EDTA; 1% Triton X-100; 2 mM sodium pyrophosphate; 10% glycerol and H₂O) supplemented with phosphatases and proteases inhibitor cocktail (10 µg/mL aprotinin; 10 µg/mL leupeptin; 1 mM phenylmethylsulfonylfluoride; 1 mM sodium orthovanadate, 100 µg/mL pepstatin and 25 mM β-glycerophosphate). The cells were incubated with the lysis buffer for 20 min in ice and then centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatants containing total cell proteins were stored at −80 °C until use.

After quantification with a BCA protein assay kit (Pierce™, Rockford, IL, USA) for equal protein loading, 40 µg of denatured protein were loaded into 12% SDS-PAGE 1.5 mm gels (TGX-Stain-Free FastCast Acrylamide kit; Bio-Rad, Hercules, CA, USA), electrophoresed and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1 h at room temperature in Tris-buffered saline with 5% nonfat dry milk and incubated overnight at 4 °C with primary rabbit anti-Nrf2 Ab (1:1000 dilution in Tris-buffered saline, 3% BSA; Proteintech®, Ref. 16396-1-AP). The next day, the membrane is washed and incubated for 1 h at room temperature with a secondary antibody conjugated to Horseradish peroxidase (HRP) (Cell Signaling Technology, Ref. 7074). Immunoreactive bands were detected by chemiluminescence when adding the ECL substrate (luminol + peroxide solution) (Clarity Max™; Bio-Rad, Segrate, Italy) and visualized using the ChemiDoc XRS + System (Bio-Rad, Hercules, CA, USA). Band densities were quantified with ImageLab software (version 6.1) and normalized to the total protein loaded.

2.7. Measurement of Nrf2 Transcription Factor Activity (DNA Binding)

KCs were lysed using the Nuclear Extraction kit (Abcam, ab113474, Cambridge, UK). Quantifying proteins in nuclear extracts was performed with a BCA protein assay kit (Pierce™, Rockford, IL, USA). To quantify Nrf2 activation in nuclear extracts, DNA binding was analyzed using a specific colorimetric ELISA-based Nrf2 Transcription Factor Assay Kit from Abcam (Cambridge, UK) (ab207223), as previously described [36]. Briefly, 27 µg of protein nuclear extracts were incubated for 1 h in a 96-well plate where a specific double-stranded DNA sequence containing the Nrf2 consensus binding site (5'-GTCACAGTGA CT CAGCAGAATCTG-3') was immobilized. Active Nrf2 present in the nuclear extract specifically binds to the oligonucleotide. After washing, the plate was incubated for 1 h with the appropriate primary Ab, washed, and then incubated with horseradish peroxidase-conjugated secondary Ab (1:1000) for 1 h at room temperature. The colorimetric readout was quantified by spectrophotometry at 450 nm.

2.8. Cytokines Dosage

KCs were stimulated with 25 µM of DNCB with or without illumination and incubated for 6 h at 37 °C in a humidified atmosphere under 5% CO₂. The level of produced pro-inflammatory mediators (IL-1α, IL-1β, IL-6, IL-8, TNF-α, IP-10, MIP-3α, and MCP-1) was measured in the supernatants, as previously described [37] by Meso Scale Discovery® (Meso Scale Diagnostics, Rockville, MD, USA) multiplex assay, according to manufacturer's instructions. Briefly, the 96-well U-PLEX plate was coated with the different linker-coupled capture antibodies where the linkers self-assemble onto unique spots in each well of the plate. After analytes in the sample bound to the capture reagents, detection antibodies conjugated with electrochemiluminescent labels (MSD GOLD™ SULFO-TAG, Meso Scale Diagnostics, Rockville, MD, USA) bind to the analytes to complete the sandwich immunoassay. The plate was then placed into an MSD instrument (Meso QuickPlex SQ 120MM, Meso Scale Diagnostics, Rockville, MD, USA) where the level of cytokines in the sample was measured and calculated with Discovery Workbench® analysis software (version 4, MSD, Rockville, MD, USA) compared to the standard calibrator.

2.9. Human Skin Explants Preparation and Treatment

Skin biopsies were processed by BIO-EC Laboratory (Longjumeau, France). BIO-EC Laboratory has authorization from the Bioethics group of the general director services of the French research and innovation ministry (registered under n°DC 2022-5373) to use human skin from surgical waste since 5 May 2010. The study was performed following the Declaration of Helsinki after the patients had given consent to use their skin samples by BIO-EC Laboratory.

Full-thickness human skin specimens were obtained from abdominal plastic surgery of a 42 years-old healthy Caucasian woman with a skin phototype II-III. Human skin explants were prepared, as previously described [35]. Briefly, the subcutaneous fat was removed from the skin, and explants of around 12 ± 1 mm in diameter were cut out using a circular scalpel; each condition was tested in triplicate ($n = 3$ explants per batch). Skin explants were then cultured on nitrocellulose filters placed on stainless steel grids at the air-liquid interphase, epidermal side up. The grids were then placed in 6-well culture plates containing proprietary explant medium developed and owned by the BIO-EC. Explants were cultured at 37 °C in 5% CO₂ humidified air. After 24 h of stabilization, 6 µL of DNCB at 0.25% (*w/v*) diluted in 20% DMSO was applied epicutaneously onto the skin explants (6 µL/explant) in pre-evaluated nontoxic concentration. Red light (660 nm) treatment was then dispensed in a pulsed mode (pulse period = 10 milliseconds, DC = 50%) for 250 s using Lightinderm lighting device (Lightinderm, Paris, France). The lighting devices were held as close as possible to the skin explant without any contact with the skin surface. The control explants did not receive any DNCB or lighting treatment. Explants were harvested 24 h after DNCB and light exposure. Every explant was cut in two parts: half were frozen at −80 °C for immunostaining, another half was fixed in buffered formalin for evaluation of the skin morphology according to Masson's trichrome protocol, Goldner variant [38]. The microscopical observations were realized using a Leica DMLB or a BX43 Olympus microscope. Pictures were digitized with a numeric DP72 or DP74 Olympus camera (Olympus, Tokyo, Japan) with cellSens data storing software (Olympus, Tokyo, Japan).

2.10. Immunostaining of Skin Explants

The frozen samples were cut into 7 µm thick sections using a Leica CM 3050 cryostat. Sections were then mounted on Superfrost® (London, UK) plus salinized glass slides. Langerin (CD207) immunostaining was performed on frozen skin sections with a monoclonal anti-langerin antibody (Beckman Coulter, ref. PN IM3449, clone DCGM4, Brea, CA, USA), diluted at 1:200 in PBS-BSA 0.3%-Tween 20 at 0.05% and incubated for 1 h at room temperature. The staining was revealed by goat anti-mouse IgG, cross-adsorbed secondary antibody, Alexa Fluor™ 488 (Invitrogen, ref. A11001, Waltham, MA, USA). The nuclei were counterstained using propidium iodide. The immunostaining was assessed by microscopical observation. The absolute number of langerin-positive cells in the epidermis was counted in 2 different sections from the 3 explants of each batch and was related to the length of the epidermis to obtain the number of langerin-positive cells/cm of epidermis.

TNF-α immunostaining was performed on formalin-fixed paraffin-embedded skin sections with a polyclonal anti-TNFα antibody (Novus biologicals, ref. NBP1-19532, Englewood, CO, USA) diluted at 1:100 in PBS-BSA 0.3%-Tween 20 (0.05%), for 1 h at room temperature, using Vectastain Kit Vector amplifier system avidin/biotin, and revealed by VIP (Vector laboratories, Ref. SK-4600, Burlingame, CA, USA), a substrate of peroxidase giving violet staining once oxidized. Quantifying TNF-α expression in the epidermis was assessed by image analysis and calculation of the surface percentage of the epidermis covered by the staining (stained surface percentage) using cellSens software (version 3.2, Olympus, Tokyo, Japan).

2.11. Statistical Analysis

Statistical Analyses were performed using GraphPad Prism® software (version 8, GraphPad Software-Dotmatics, San Diego, CA, USA). Repeated-measures two-way ANOVA

followed by Tukey’s multiple comparison post hoc test was used to compare different groups, and the homoscedasticity of residuals was checked. Data are expressed as means ± SEM and considered statistically different when the *p*-value < 0.05.

3. Results

3.1. PBM Regulates Pro-Inflammatory Mediators in Stressed KCs

HPEK were stimulated with 25 μM of DNCB, an electrophilic chemical sensitizer used to induce the pro-inflammatory cellular stress. The concentration of DNCB leading to 70% of cell viability was selected. KCs were then illuminated with two different wavelengths separately: 660 nm (red light) or 520 nm (green light), at 3 J/cm² for a total exposure time of 250 s. First, we investigated the ability of these two wavelengths to control the inflammatory response of KCs at the pre-selected dosimetry. The mRNA level of different pro-inflammatory cytokines was assessed by RT-qPCR after 3 or 6 h of DNCB stimulation and light exposure (Figure 1).

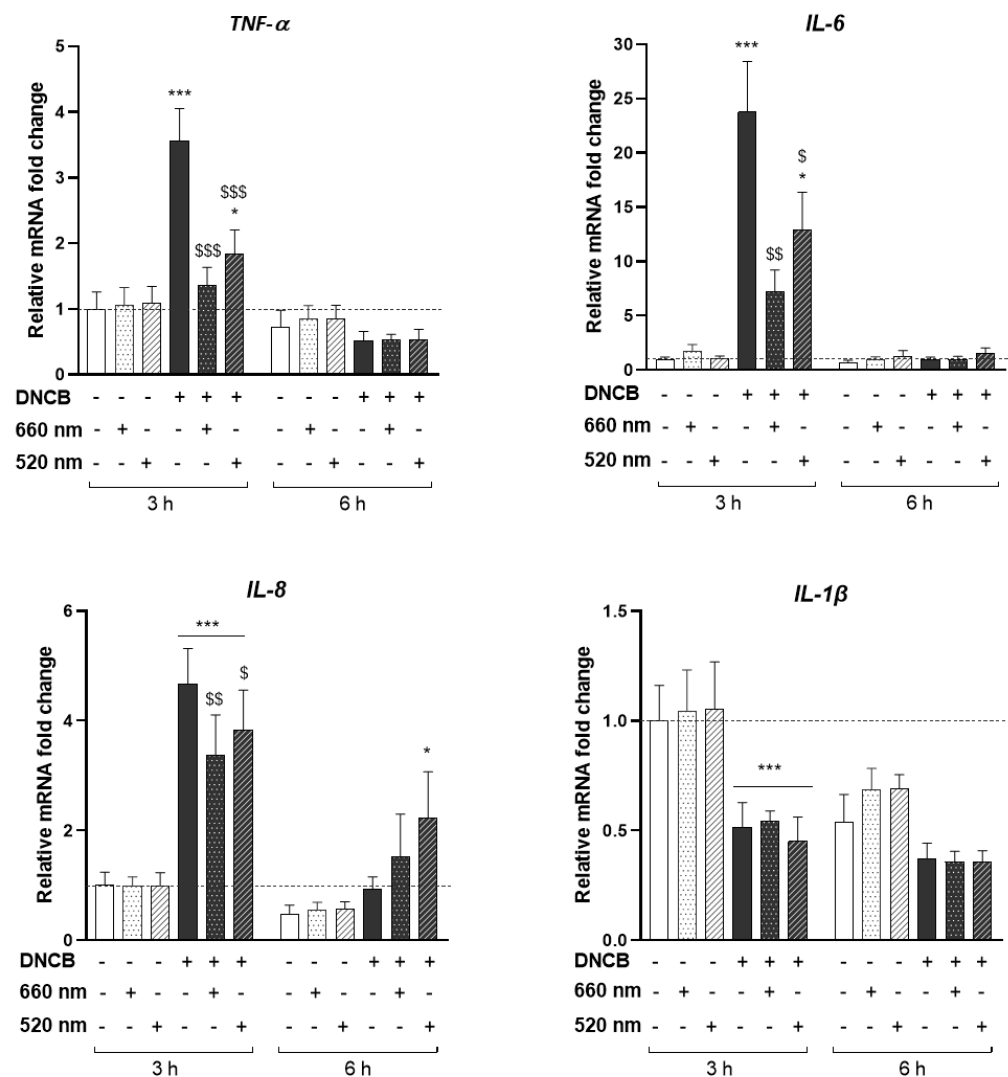


Figure 1. PBM regulates pro-inflammatory DNCB-induced cytokines in KCs. KCs were treated with DNCB or vehicle, with or without red (660 nm) or green (520 nm) light illumination (3 J/cm², 250 s). Pro-inflammatory cytokines gene expression: *TNF-α*, *IL-6*, *IL-8*, and *IL-1β*, were measured by RT-qPCR 3 and 6 h after DNCB and light treatment. Results are expressed as fold change. Data represent mean ± SEM of 5 independent experiments. Two-Way ANOVA followed by Tukey post hoc test, * *p* < 0.05; *** *p* < 0.001 vs. the relative control at 3 h or 6 h; \$ *p* < 0.05; \$\$ *p* < 0.01; \$\$\$ *p* < 0.001 vs. DNCB at 3 h.

LED exposure did not modify the expression of any studied cytokine compared to untreated cells, at basal state (Figure 1), while DNCB induced a significant increase in the expression of *TNF- α* (3.56-fold increase), *IL-6* (23.76-fold increase), and *IL-8* (4.68-fold increase) 3 h post-stimulation. In contrast, *IL-1 β* expression was downregulated by 52% after 3 h of DNCB treatment (Figure 1). Interestingly, red light significantly suppressed the expression of the DNCB-triggered *TNF- α* , *IL-6*, and *IL-8* mRNAs by 62%, 69%, and 28%, respectively, 3 h post-illumination. Green LED exposure likewise significantly decreased the mRNA expression of *TNF- α* by 49%, *IL-6* by 46%, and *IL-8* by 18% compared with DNCB-stimulated conditions (Figure 1). No significant modulation of *IL-1 β* expression due to the illumination was observed. *TNF- α* , *IL-6*, and *IL-8* mRNAs expression were not modified by light upon 6 h of DNCB stimulation. Furthermore, only *IL-8* expression following DNCB plus green light exposure was different from the control evaluated at 6 h post-stimulation (Figure 1).

Altogether, these results showed that PBM did not modulate the transcription of pro-inflammatory cytokines in KCs at the basal state. However, red and green LEDs decreased the expression of cytokines induced by DNCB.

3.2. PBM Enhances Nrf2 Pathway Activation in DNCB-Stimulated KCs

As Nrf2 has been described as a key factor in regulating skin inflammation [17–19,39], we investigated the activation of the Nrf2 pathway in response to light exposure. First, Nrf2 protein accumulation was assessed by Western blot after 3 h of DNCB and light treatment. Our results showed that DNCB significantly increased Nrf2 accumulation compared to untreated control KCs. Red and green LEDs indifferently enhanced Nrf2 accumulation by 30% and 50%, respectively, compared to DNCB-treated KCs (Figure 2A). These observations were associated with a slight increase in Nrf2 DNA-binding to an ARE consensus sequence upon exposure to red (7.3%) and green (12.7%) light compared to DNCB-treated KCs (Figure 2B), showing an increase in the transcriptional activity of Nrf2. Since Nrf2 was shown to be active in the nucleus, the next step was to assess the expression of several Nrf2-target genes by RT-qPCR. DNCB increased mRNA expression of *HO-1* (103-fold increase), *GCLC* (2.09-fold increase), and *NQO1* (1.69-fold increase), while PBM further enhanced the expression of these Nrf2-target genes after illumination with red (113.6%, 53.9%, and 40%, respectively) or green (*HO-1* by 108.3% and *GCLC* by 31.3%) light at 3 h of KCs stimulation (Figure 2C). However, the light-induced upregulation of Nrf2-target genes did not persist 6 h after cell illumination (Figure 2C).

Taken together, these data revealed that PBM reinforced the DNCB-induced accumulation and activation of Nrf2 and slightly increased the expression of the antioxidant target genes.

3.3. Nrf2 Mediates the Anti-Inflammatory Response of the Red Light in KCs

Based on the literature showing the anti-inflammatory role of Nrf2 in KCs [19] and the observed effect of PBM controlling pro-inflammatory cytokines (Figure 1), we suggested that Nrf2 activation might be involved in the control of the PBM-mediated inflammatory response upon DNCB exposure. To address this hypothesis, Nrf2 was invalidated in KCs by siRNA transfection strategy (si-Nrf2) and focused specifically on the red light response after DNCB treatment. Nrf2 accumulation in DNCB-treated Nrf2-invalidated KCs was abrogated by 78.3% compared to its relative si-random control (Figure 3A). In a similar manner, the mRNA expressions of *HO-1*, *NQO1*, and *GCLC* were significantly downregulated by 60.7%, 53.2%, and 49.4%, respectively, in the DNCB-stimulated and light-exposed group (Figure 3A).

To study the response of KCs to red light exposure in the absence of Nrf2, the mRNA expression and secretion of three pro-inflammatory mediators were assessed. As previously described in Figure 1, the red light attenuated DNCB-induced *TNF- α* , *IL-6*, and *IL-8* expression in the si-random control group (Figure 3B). Interestingly, in the absence of Nrf2, illumination of KCs with red light did not reduce the expression of these cytokines

in response to DNCB (Figure 3B). Furthermore, these results correlated with the level of cytokines measured in the supernatant by electroluminescence (Figure 3C). While red light in the control group significantly inhibited DNCB-induced TNF- α , IL-6, IL-8 and also IL-1 α , and IP-10 production, red LED failed to suppress the release of these pro-inflammatory mediators in si-Nrf2 KCs (Figures 3C and S2). These findings implied that the observed anti-inflammatory effect of red light upon DNCB stimulation depends, at least in part, on Nrf2. The production of other mediators such as IL-1 β , MIP-3 α , and MCP-1 was tested, but their secretion level at 6 h post-stimulation was below the assay’s detection limit (data not shown). In our study, Nrf2 downregulation in KCs increased the basal level of IL-1 β transcription at the steady state (Figure S1). IL-1 α was the only cytokine whose secretion was significantly increased in the absence of Nrf2 in the untreated group or in response to DNCB (Figure S2), suggesting that Nrf2 might play a role in the regulation of IL-1 β expression as well as IL-1 α production in KCs.

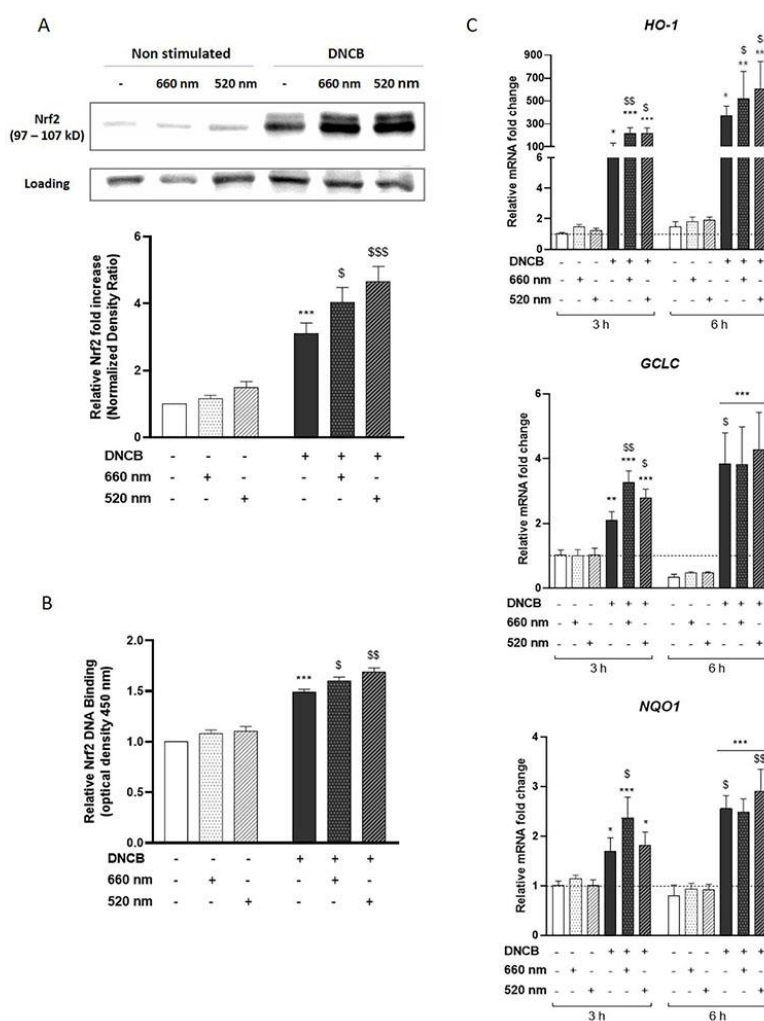


Figure 2. PBM enhances Nrf2-pathway activation in DNCB-stimulated KCs. KCs were treated with vehicle (DMSO 0.1%) or DNCB (25 μ M) and illuminated with either wavelength: 660 nm (red light) or 520 nm (green light). (A) Nrf2 accumulation was assessed by Western blot 3 h after illumination. Histograms correspond to densitometric analysis relative to controls and are adjusted to the stain-free blot. (B) ELISA-based measurement of DNA-binding activity for activated Nrf2 in the nuclear extract, 4 h after KCs treatment. (C) Nrf2 target antioxidant gene expression: *HO-1*, *GCLC*, and *NQO1*, were assessed by RT-qPCR, 3 h or 6 h after light treatment. Data represent mean \pm SEM of at least five independent experiments. Two-Way ANOVA followed by Tukey post hoc test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. the relative untreated control at 3 h or 6 h; \$ $p < 0.05$; \$\$ $p < 0.01$; \$\$\$ $p < 0.001$ vs. DNCB at 3 h.

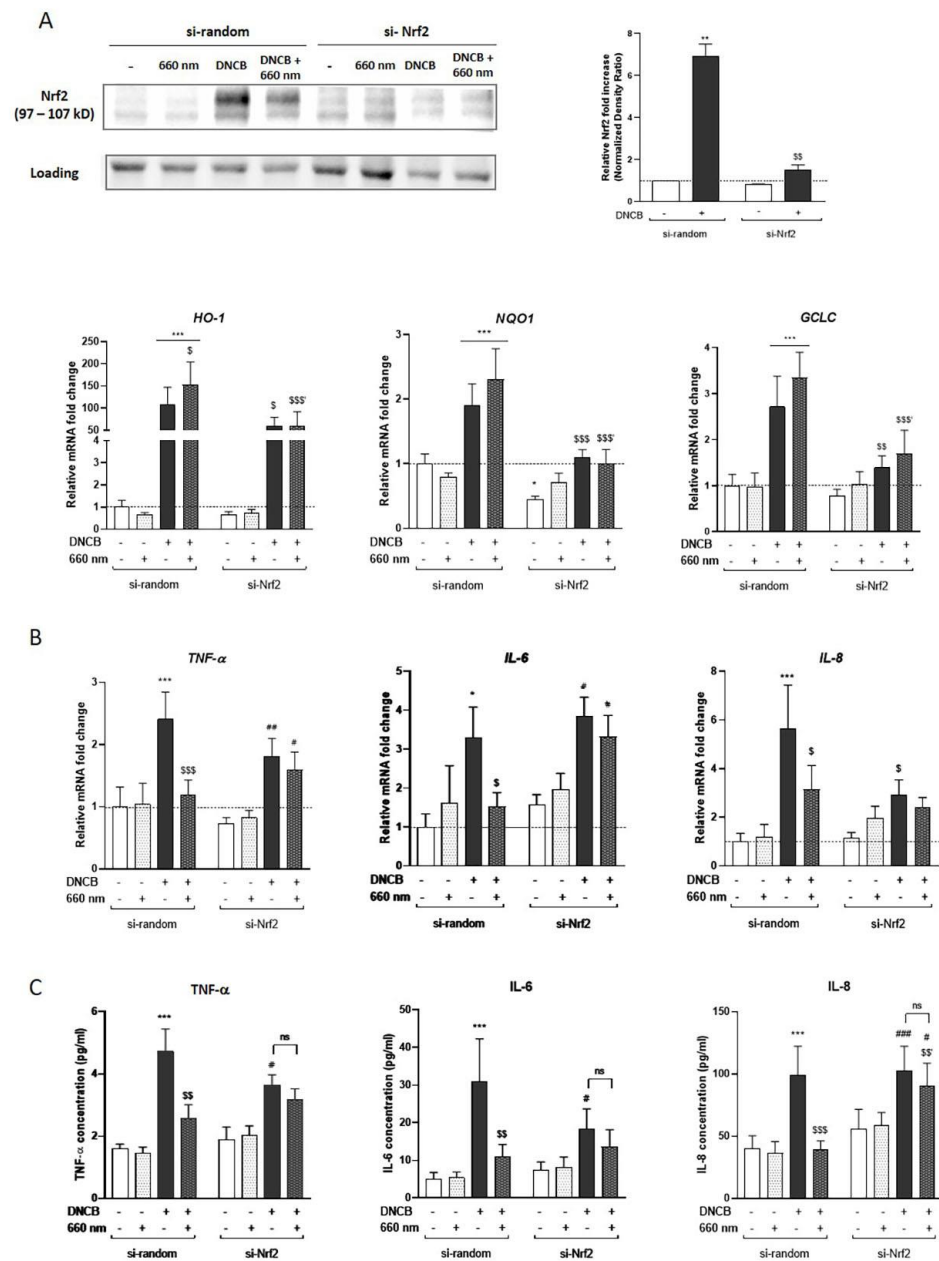


Figure 3. The anti-inflammatory effect of red light in KCs is Nrf2-dependent. KCs were transfected for 48 h by si-Nrf2 or si-random, and then treated with DNCB and exposed to red light. (A) Nrf2 accumulation in the knockdown model was assessed by Western blot 3 h after treatment. The blot is representative of 3 independent experiments. Histograms correspond to densitometric analysis relative to untreated si-random and are adjusted to the stain-free blot. Quantifying the expression of Nrf2 target genes encoding *HO-1*, *NQO1*, and *GCLC* in transfected KCs were assessed using RT-qPCR 3 h after treatment. Induction values were the ratio between gene expressions in treated cells versus gene expression in untreated si-random control. (B) Pro-inflammatory cytokines gene expression: *TNF-α*, *IL-6* and *IL-8*, were assessed by RT-qPCR, 3 h after light treatment. Results are expressed as fold change compared to the untreated si-random control. (C) The inflammatory cytokines level was determined by Meso Scaled Discovery technology in the supernatant of KCs after 6 h of exposure to DNCB with or without red light illumination. Reported data are mean ± SEM of 6 independent experiments. Two-Way ANOVA followed by Tukey post hoc test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. untreated si-random; \$ $p < 0.05$; \$\$ $p < 0.01$; \$\$\$ $p < 0.001$ vs. DNCB-treated si-random, \$\$' $p < 0.01$; \$\$\$' $p < 0.001$ vs. DNCB + 660 nm-treated si-random; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. untreated si-Nrf2. ns means non-significant different.

Overall, our results showed that PBM response controlling DNCB-induced pro-inflammatory cytokines and chemokines is, at least partially, dependent on Nrf2.

3.4. PBM Reduces DNCB-Induced LCs Activation and TNF- α Production in the Epidermis

To investigate the mechanisms of the immunomodulatory response induced by PBM in a more physiologically relevant model, we used an ex vivo model of human skin explants treated with the DNCB for 24 h and exposed to red light. As shown in the microscopical histological evaluation, the solvent (DMSO 20%), the red-light exposure (660 nm, 12 mW/cm² for 250 s), and the DNCB topical application (0.25%) did not induce any significant modification or morphological alterations in the epidermal or dermal structures (Figure S3).

Upon activation, LCs, professional antigen-presenting cells residing in the epidermis, undergo morphological changes and migrate to skin-draining lymph nodes to initiate an appropriate immune response in response to sensitizers such as DNCB [40,41]. To follow LCs migration from the epidermis into the dermis in response to DNCB and light exposure, we immunostained the langerin (CD207), a well-known marker of LCs. The topical application of DNCB (0.25%) induced a significant decrease in the number of langerin-positive cells (CD207⁺) in the epidermis compared to the DMSO excipient batch (40%; $p < 0.001$) (Figure 4A,B). Furthermore, LCs in the DNCB-treated explants had a more constricted and rounded shape, associated with less frequent dendrites than those from the DMSO-treated control group (Figures 4C and S4). DNCB also increased TNF- α expression in the epidermis by 26% (Figure S5) and augmented the level of IL-6, IL-1 α , and IL-1 β ($p < 0.05$) secretion by skin explants compared to the DMSO-treated group (Figure S6). The exposure of explants to red light increased by 23% the number of langerin-positive cells in the epidermis compared to the DNCB alone with a p -value = 0.06 (Figure 4A,B). The LCs surface of red light-exposed tissues following DNCB treatment was significantly increased (DNCB mean = 84 μm^2 ; DNCB + red light mean = 263 μm^2 ; $p < 0.001$) with a higher dendricity (Figures 4C and S4). These observations were supported by a significant decrease of 26% in TNF- α expression level in the epidermis of light-exposed explants (Figure S5). Moreover, our results suggest that exposure to red light slightly decreased pro-inflammatory cytokine secretion in skin explants compared to the DNCB-treated group ($p < 0.1$) (Figure S6), although this effect did not reach statistical significance at the conventional level, likely due to the small number of replicates. Additional studies with larger sample sizes and more statistical power are needed to confirm these findings.

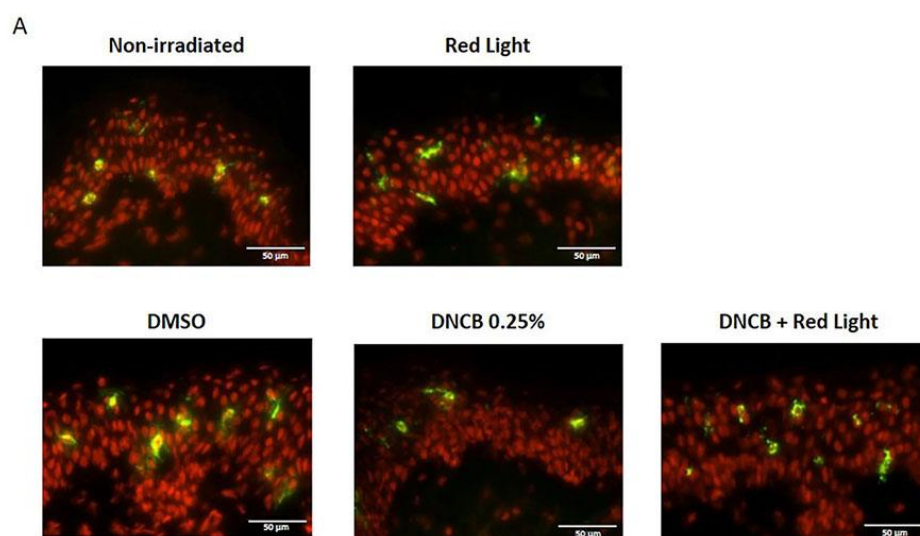


Figure 4. Cont.

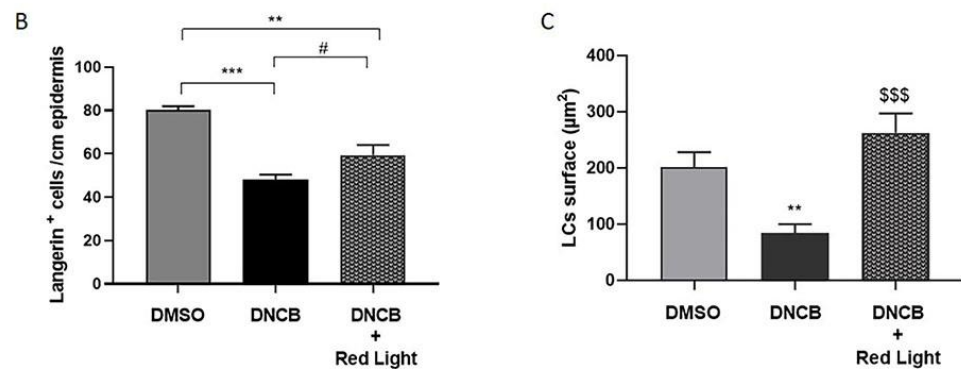


Figure 4. Red light decreases DNCB-induced LCs activation in the epidermis. Human skin explants were topically treated with the excipient (DMSO 20%) or DNCB (0.25% *w/v*) with or without red light exposure (660 nm, 3 J/cm², 250 s). (A) Langerin immunostaining was performed on frozen skin sections 24 h after DNCB treatment and revealed by AlexaFluor 488 (green). The nuclei were counterstained using propidium iodide (red). Scale bars represent 50 µm. (B) The absolute number of langerin-positive cells in the epidermis was counted at 2 different zones for the 3 explants of each batch. Histogram represents the mean ± SEM of 6 measures of langerin-positive cells/cm epidermis. (C) Langerin-positive cells total surface was measured by ImageJ software (version 1.8). Histogram represents the mean ± SEM of cell surface of 10 different measures. ANOVA followed by Tukey post hoc test; ** $p < 0.01$; *** $p < 0.001$ vs. DMSO-treated control group; # $p = 0.06$, \$\$\$ $p < 0.001$ vs. DNCB-treated group.

Taken together, these results implied that red light might modify the skin environment by reducing pro-inflammatory cytokine production induced by DNCB, and prevented LCs activation by inhibiting their migration and maintaining their dendrites.

4. Discussion

Inflammation is a major contributor to many skin diseases, and the ability to reduce inflammation is of paramount importance in medicine. PBM is an increasingly popular therapy for treating skin inflammation and many dermatological diseases using low-energy visible light or NIR [42–44]. Although it has recently gained considerable recognition for its potential to reduce inflammation, the mechanisms underlying the anti-inflammatory effect of PBM remain unclear. We conducted this study to understand the cellular pathways by which PBM can regulate skin inflammation.

Since KCs constitute the major cell population of the epidermis and are a source of inflammation involved in many skin pathologies [2,4,45], we chose to work on a two-dimensional model of primary human KCs to study the benefic effect of PBM. To mimic the pro-inflammatory stress, KCs were exposed to DNCB, and then, the modulation of the inflammatory response by red and green lights was addressed. DNCB is an electrophilic chemical classified as an extreme contact sensitizer [46]. It induces a pro-inflammatory microenvironment and subsequently promotes a complete immune response. Thus, it is widely used for challenging rodents' skin in contact hypersensitivity [18,47] or atopic dermatitis [48,49] models. It has also been used in several *in vitro* studies characterizing the skin sensitization response to allergens in primary KCs or a well-known KCs cell line, HaCaT [50,51]. As previously described, our data showed an increase in the mRNA expression levels of *TNF-α*, *IL-6*, and *IL-8* in response to DNCB. It is consistent with previous reports showing that DNCB treatment specifically increases *IL-6* secretion in HaCaT cells as well as the transcription of *TNF-α*, *IL-6*, and *IL-8* in primary KCs [52,53].

In our study, the illumination with red (660 nm) or green (520 nm) LEDs at 3 J/cm² for 250 s downregulated the expression of the DNCB-induced cytokines, indicating the anti-inflammatory role that plays PBM in KCs. There is growing evidence supporting that PBM serves as a regulator of inflammation. In DNCB-induced atopy mouse model, illumination with visible red (650 nm) alleviates symptoms of atopic dermatitis and restores cytokine

levels such as IL-6 and TNF- α to normal [54]. An *in vivo* study of a rat burn healing model daily irradiated with 660 nm low-power laser (10 J/cm², 20 s) for 7 consecutive days showed inhibition of IL-6 expression at the site of injury, associated with a decrease in oxidative stress and an improved cutaneous antioxidant profile, thus, accelerating the repair of burn wounds [31]. In the same model, green LED (550 nm, 60 J/cm², 10 s), a less documented light than red, was able to reduce inflammatory cells' infiltration to the burn site, composed predominantly of neutrophils and lymphocytes [55]. *In vitro* studies using TLR-agonists such as bacterial LPS to induce inflammation further supported the anti-inflammatory effect of PBM. The illumination of human gingival fibroblasts with 635 nm LED suppressed the LPS-induced IL-6, IL-8, and prostaglandin E₂ release, correlated with modulation of MAPK pathway activation [29]. In the same way, LPS-induced IL-6 and IL-8 mRNA expressions were significantly decreased by low-level 805 nm (NIR) laser irradiation of the oral squamous epithelial carcinoma cell line (Ca9-22) [56]. Fernandes et al. showed a reduction of TNF- α production in murine J774 macrophage-like cells activated by LPS and IFN γ and illuminated with a 660 nm laser (15 mW/cm², 7.5 J/cm²). However, the authors reported an up-regulation of IL-6 by light, in contrast to our result [57]. Although they used the same red wavelength as in our study, at a power density and fluence ranges close to those we used, the difference in IL-6 expression may be explained by the difference in cell type and species (human KCs vs. murine macrophage-like cells), the difference in total light exposure time (250 s vs. 20 s), and the time point of the mRNA expression measurement (3 h vs. 24 h, where the measured expression is no longer a direct effect of light but rather a secondary effect to other cellular processes). IL-6 and IL-8 are "secondary" pro-inflammatory mediators that act in an autocrine manner. Their function is mediated by "primary" cytokines, such as TNF- α , a key regulator of inflammation secreted in response to stimuli [2]. Pro-inflammatory cytokines and chemokines, including IL-6, IL-8, and TNF- α , play a critical role in the initiation and progression of many chronic inflammatory skin diseases, such as psoriasis or atopic dermatitis [58]. Thus, control of their production by PBM can regulate the amplitude and duration of the inflammatory response and modify the course of the disease.

Different mechanisms have been proposed to explain the anti-inflammatory effects of PBM [59–61], but most of these observations lack strong evidence concerning the exact involvement of these mechanisms in the therapeutic action of PBM. The Nrf2 pathway is a key regulator of the response to oxidative stress and inflammation. It plays a pivotal role in the regulation of cellular defense mechanisms, including the promotion of an anti-inflammatory response [14,16,17]. To date, only a few studies have evaluated the effects of PBM on Nrf2 expression and activity. Trotter et al. reported a rapid accumulation and nuclear translocation of Nrf2 after treating the human monocytic cell line, THP-1, with blue light (45 J/cm²), followed by induction of *HO-1* expression, an Nrf2-target cytoprotective enzyme. These observations were associated with reduced LPS-induced TNF- α and IL-8 secretion [62]. The same authors also found that blue light treatment induced upregulation of Nrf2 in A431 epidermoid carcinoma cells and significantly increased levels of *HO-1* [63]. Furthermore, Sohn et al. showed an upregulation of *Nrf2* gene expression and protein accumulation upon treatment of RANKL-stimulated mouse bone marrow-derived macrophages with 635 nm LED [64]. Yadav et al. recently demonstrated activation of the Nrf2 pathway associated with an acceleration of burn healing in mice treated at 904 nm [34]. In line with all these observations, we showed an activation of the Nrf2 signaling by red and green lights in DNCB-stimulated KCs. Indeed, 660 nm and 520 nm LEDs further enhanced Nrf2 accumulation and nuclear translocation compared to the DNCB-treated group. They also increased Nrf2-target genes expression, *HO-1*, *NQO1*, and *GCLC*. To our knowledge, this effect of PBM on Nrf2 pathway activation has not been previously reported in KCs. Since Nrf2 is a key regulator of ROS production, we measured in our study hydrogen peroxide (H₂O₂) in stimulated KCs. No significant modifications of H₂O₂ level were observed after DNCB treatment with or without the illumination of KCs (data not shown), suggesting that PBM-induced Nrf2 does not seem to regulate inflammation

through ROS modulation. Moreover, our study is the first to report that Nrf2 is an essential factor for controlling KCs inflammatory response by the red light, a more commonly used PBM protocol than the green light to treat inflammation *in vivo* and *in vitro*. Indeed, in the absence of Nrf2, the illumination of KCs did not reduce the TNF- α , IL-6, or IL-8 production in response to DNCB.

Since the crosstalk between KCs and LCs is essential for programming LCs response and determining the appropriate adaptive immune response (immunogenic or tolerogenic) [40,41,65], we addressed the immunoregulatory role of PBM in a more physiologically relevant model. We assessed the effect of red LED on LCs in human skin explants treated epicutaneously with DNCB to induce inflammation. In our study, DNCB induced more rounded shrunken LCs with fewer dendrites and promoted their migration from the epidermis into the dermis, indicating activation of LCs. These morphological changes were associated with increased pro-inflammatory cytokine secretion in skin explants and augmented epidermal expression of TNF- α , a marker of skin inflammation and known to play a role in stimulating LCs migration. These findings corroborate previous reports demonstrating an LCs mobilization induced by skin sensitization to DNCB, though TNF- α was not an indispensable requirement for LCs migration in mouse skin [53,66,67]. We showed that the exposure of skin explants to red light (660 nm) prevented the DNCB-induced LCs modifications and migration. It also decreased the expression of TNF- α in the epidermis and tended to regulate the level of some pro-inflammatory cytokine secretion. Thus, this result provides evidence for the immunomodulatory effect of PBM that might contribute to its anti-inflammatory activity preventing allergen sensitization in atopic or allergic dermatitis. Visible red (633 nm) LED has been previously shown to suppress the Th2 immune response induced by topical sensitization of mice skin with a protein antigen, ovalbumin. In the same study, PBM reduced the expression of CD24, a costimulatory surface molecule, among others expressed by LCs [68]. A previous report showed that a single exposure of mouse skin to NIR (30 J/cm²) reversibly suppressed the number of LCs that became round with shortened dendrites, contrary to our observations. However, the reduced density of LCs was correlated to a failure of the skin to induce a contact hypersensitivity reaction, suggesting that NIR induced local immunosuppression, probably by initiating an antigen-specific Treg response [69]. Another study demonstrates that NIR (90 J/cm²) can modulate immune response *in vivo* by activating LCs [70]. Although these studies support the ability of PBM to modulate LCs response, the difference in the LCs activation and mobilization can be attributed to the difference in the wavelengths and the energy densities, which were relatively high compared to the one we used. By impairing cytokine release in the skin microenvironment, PBM may alter the activation of LCs. As Nrf2 activation can control the skin microenvironment [17,20] by regulating the activation of KCs and LCs, it may modulate their ability to elicit an adaptive immunity [18,71]. It is not excluded that PBM may also alter the expression of surface molecules of LCs by a direct mechanism. Further research is necessary to explore the exact mechanisms driving the effects of PBM on LCs activation and to describe the phenotype of LCs in response to PBM to discuss its potential therapeutic applications.

5. Conclusions

In summary, the data presented here support previous studies on the anti-inflammatory effect of PBM. Our findings provide new evidence that the PBM's beneficial activity in human KCs with an inflammatory response is at least dependent on the Nrf2 pathway. Furthermore, the therapeutic effect of PBM in response to DNCB does not seem to involve regulation of the level of H₂O₂ in KCs, but rather a modulation of the skin immune response, probably through the regulation of innate cytokines that are crucial for initiating and perpetuating inflammation. PBM controls TNF- α expression in the epidermis of human explants and prevented the activation of LCs and their migration into the dermis in response to chemical stress.

Altogether, our results support the regulatory function of PBM modulating key immune cells in the epidermis. It acts as an inhibitor of the primary inflammatory response induced by KCs, mainly through the Nrf2 pathway, and reduces LCs activation. Therefore, PBM has a promising future as an effective non-invasive anti-inflammatory therapeutic for skin-related inflammatory diseases, especially those in which Nrf2 has been shown to play a pivotal role as a regulator of inflammation, such as psoriasis, wound healing, or allergic contact dermatitis.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox12030766/s1>, Table S1: Oligonucleotide primer sequences used for RT-qPCR; Figure S1: *IL-1 β* mRNA expression in KCs Knocked down for Nrf2 following DNCB and red LED treatment; Figure S2: IL-1 α and IP-10 levels secreted by KCs knocked down for Nrf2 in response to DNCB and red light exposure; Figure S3: Optical microscopy evaluation of skin explants; Figure S4: Representative images of LCs dendricity; Figure S5: Red light decreases DNCB-induced TNF- α expression in the epidermis; Figure S6: Red light slightly decreases DNCB-induced pro-inflammatory cytokines secretion in the skin explants.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics group of the general director services of the French research and innovation ministry (registered under n^oDC-2008–542, 5 May 2010) authorizing BIO-EC to use human skin from surgical waste.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study is contained within this manuscript or Supplementary Material.

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Abbreviations

ARE: Antioxidant responsive element; DMSO: Dimethyl sulfoxide; DNCB: 2,4-dinitrochlorobenzene; GCLC, Glutamate-cysteine ligase catalytic subunit; HPEK, Human primary epidermal keratinocytes; HO-1, Heme oxygenase 1; IL, Interleukin; KCs, Keratinocytes; LCs, Langerhans cells; LED, Light-emitting diode; MAPK, mitogen-activated protein kinase; MSD, Meso scaled discovery technology; NF- κ B, Nuclear factor kappa; NIR, Near-infrared radiation; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, Nuclear factor erythroid-2-related factor 2; PBM, Photobiomodulation; ROS, Reactive oxygen species; TLR, Toll-like receptor; TNF- α , Tumor necrosis factor-alpha; UV, Ultraviolet radiation.

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Supplementary data

Table S1. Oligonucleotide primer sequences used for qRT-PCR.

Gene Symbol	Gene Name	NCBI Gene Accession Number	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	<i>Glyceraldehyde-3-phosphate dehydrogenase</i>	NM_001357943	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
GCLC	<i>Glutamate-cysteine ligase catalytic subunit</i>	NM_001197115	ACATGGAAGTGGATGTGGACACC	CCTCATCCATCTGGCAACTGTCA
HO-1	<i>Heme oxygenase-1</i>	NM_002133	GGCCTGGCCTTCTTCACCTT	GAGGGGCTCTGGTCCTTGGT
HPRT	<i>Hypoxanthine-guanine phosphoribosyltransferase</i>	NM_000194	GCCAGACTTTGTTGGATTTG	CTCTCATCTTAGGCTTTGTATTTG
IL-1β	<i>Interleukin 1 beta</i>	NM_000576	ACAGACCTTCCAGGAGAATG	GCAGTTCAGTGATCGTACAG
IL-6	<i>Interleukin 6</i>	NM_001371096	TCAATGAGGAGACTTGCCTG	GATGAGTTGTCATGTCCTGC
IL-8	<i>Interleukin 8 / C-X-C motif chemokine ligand 8 (cxcl8)</i>	NM_001354840	TCTCTTGGCAGCCTTCTGA	TGGGGTGGAAAGGTTTGGAG
NQO1	<i>NAD(P)H quinone dehydrogenase 1</i>	NM_001025434	GGGCAAGTCCATCCCAACTG	GCAAGTCAGGGAAGCCTGGA
NRF2	<i>Nuclear factor erythroid-2-like 2 (NFE2L2)</i>	NM_001313903	CTTGGCCTCAGTGATTCTGAAGTG	CCTGAGATGGTGACAAGGGTTGTA
TNF-α	<i>Tumor necrosis factor</i>	NM_000594	CCTCTCTAATCAGCCCTG	GAGGACCTGGGAGTAGATGAG

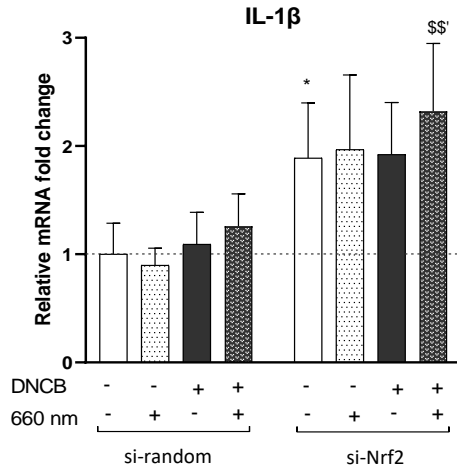


Figure S1.

***IL-1β* mRNA expression in KCs knocked down for Nrf2 following DNCB and red LED treatment.**

KCs were transfected for 48 h by siRNA directed against Nrf2 (si-Nrf2) or random siRNA (si-random). KCs were then treated with DNCB (25μM) and exposed to red light (660 nm, 3 J/cm²). Pro-inflammatory cytokine *IL-1β* gene expression was assessed by RT-qPCR 3 h after light treatment. Results are expressed as fold change compared to the untreated siRNA control random. Reported data are mean ± SEM of 6 independent experiments. Two-Way ANOVA followed by Tukey post-hoc test, * *p* < 0.05 vs. untreated si-random; \$\$' *p* < 0.01 vs. DNCB + 660 nm- treated si-random group.

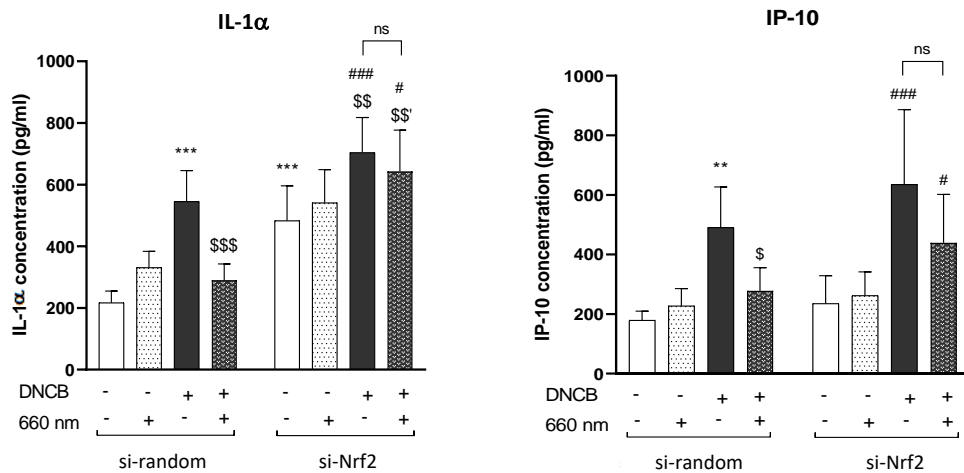


Figure S2.

***IL-1α* and IP-10 levels secreted by KCs knocked down for Nrf2 in response to DNCB and red light exposure.** KCs were transfected for 48 h by siRNA directed against Nrf2 (si-Nrf2) or random siRNA (si-random). KCs were then treated with DNCB (25μM) and exposed to red light (660 nm, 3 J/cm²). *IL-1α* and IP-10 pro-inflammatory cytokines level was determined by Meso Scaled Discovery technology in the supernatant of KCs after 6 h of exposure to DNCB with or without illumination. Reported data are mean ± SEM of 6 independent experiments. Two-Way ANOVA followed by Tukey post-hoc test, ** *p* < 0.01; *** *p* < 0.001 vs. untreated si-random; \$ *p* < 0.05; \$\$ *p* < 0.01; \$\$\$ *p* < 0.001 vs. DNCB-treated si-random group, \$\$' *p* < 0.01 vs. DNCB + 660 nm- treated si-random group; # *p* < 0.05, ### *p* < 0.001 vs. untreated si-Nrf2.

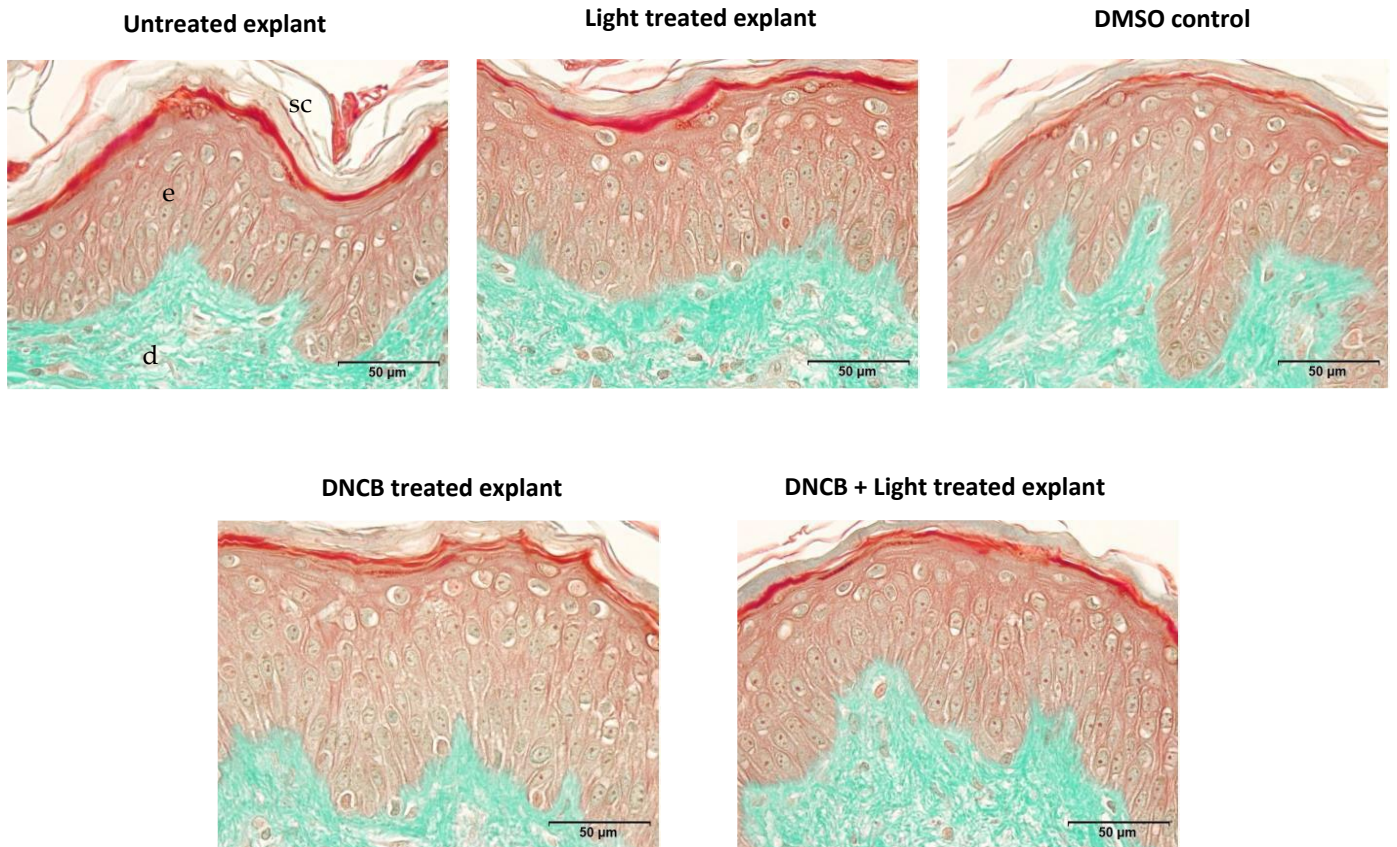


Figure S3.

Optical microscopy evaluation of the skin explants. Skin explants were treated with the excipient (DMSO 20%) or DNCB (0,25% *w/v*), with or without red light exposure (660 nm, 3 J/cm², 250 s). Histological control of the general morphology of the epidermal and dermal structures was assessed after 24 h of treatment by microscopical observation after Masson's trichrome (Goldner variant) staining of formol-fixed paraffin-embedded skin sections. Nuclei (dark brown), cytoplasm (Light greenish red), Keratin (bright red), collagen fibers (green). Images are representative of 3 independent explants from all groups. e, epidermis; d, dermis; sc, *stratum corneum*. Scale bars: 50 μm.

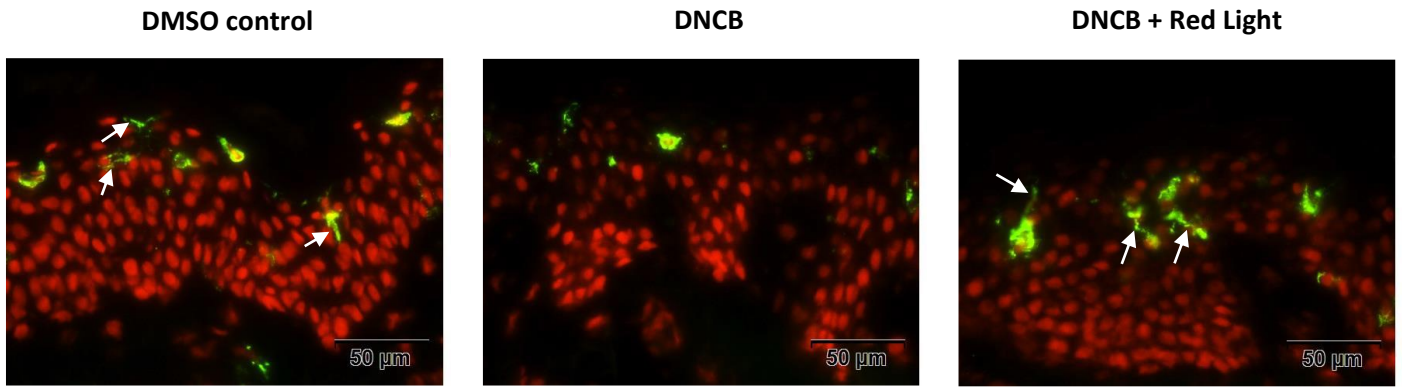


Figure S4.

Representative images of Langerhans cells' dendricity. Skin explants were treated for 24 h with the excipient (DMSO 20%), DNCB (0.25% *w/v*), with or without red light exposure (660 nm, 3 J/cm², 250 s). Langerin immunostaining was performed on frozen skin sections and revealed by AlexaFluor 488 (green). The nuclei were counterstained using propidium iodide (red). The white arrows point to the Langerhans cells' dendritic extensions.

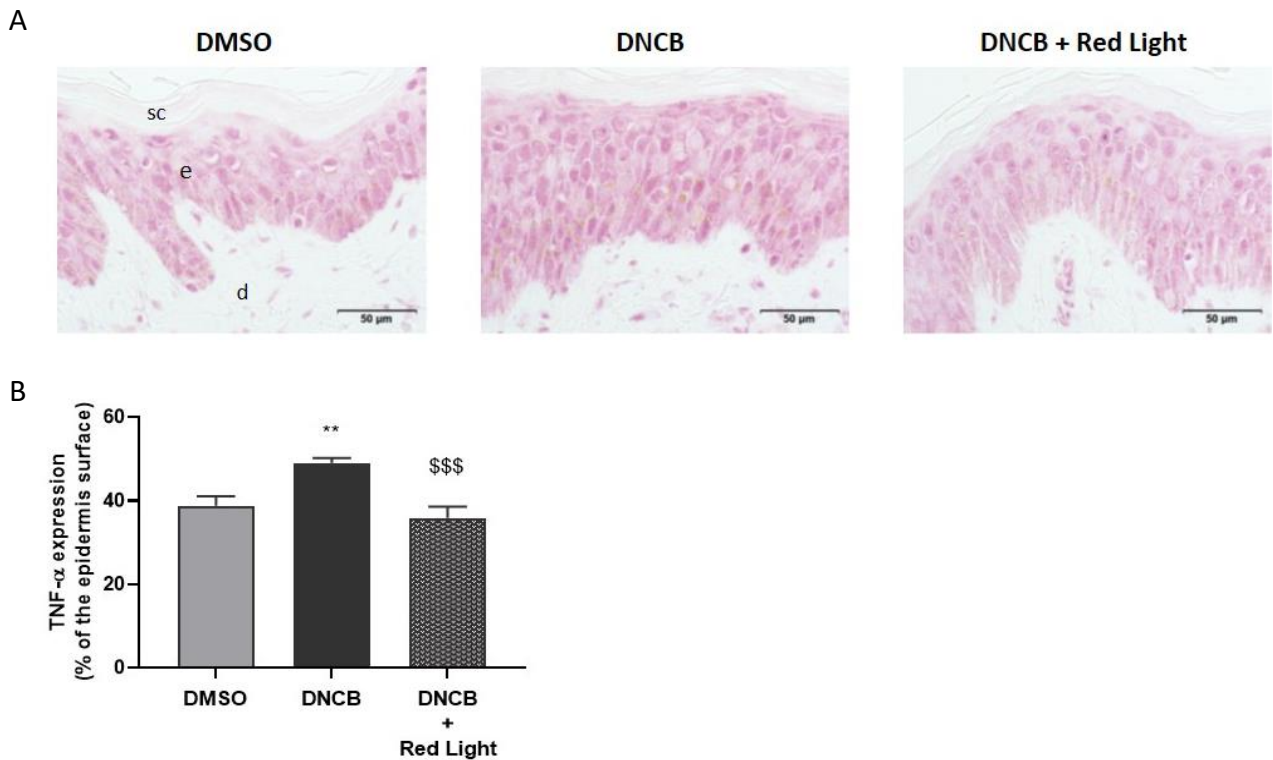


Figure S5.

Red light decreases DNCB-induced TNF- α expression in the epidermis. Human skin explants were treated topically with the excipient (DMSO 20%) or DNCB (0.25% *w/v*) with or without red light exposure (660 nm, 3 J/cm², 250 s). (A) TNF- α immunostaining was performed on formalin-fixed paraffin-embedded skin sections and revealed by VIP, a peroxidase substrate, giving a violet staining once oxidized. The pictures in this figure represent at least 9 images (3 images from 3 different explants for each group). e, epidermis; d, dermis; sc, stratum corneum. Scale bars: 50 μ m. (B) Quantification of TNF- α expression in the epidermis assessed by image analysis and calculation of the surface percentage of the epidermis covered by the staining. Histogram represents the mean \pm SEM of 9 different measures. ANOVA followed by Tukey post-hoc test, ** $p < 0.01$ vs. DMSO-treated control group; \$\$\$ $p < 0.001$ vs. DNCB-treated group.

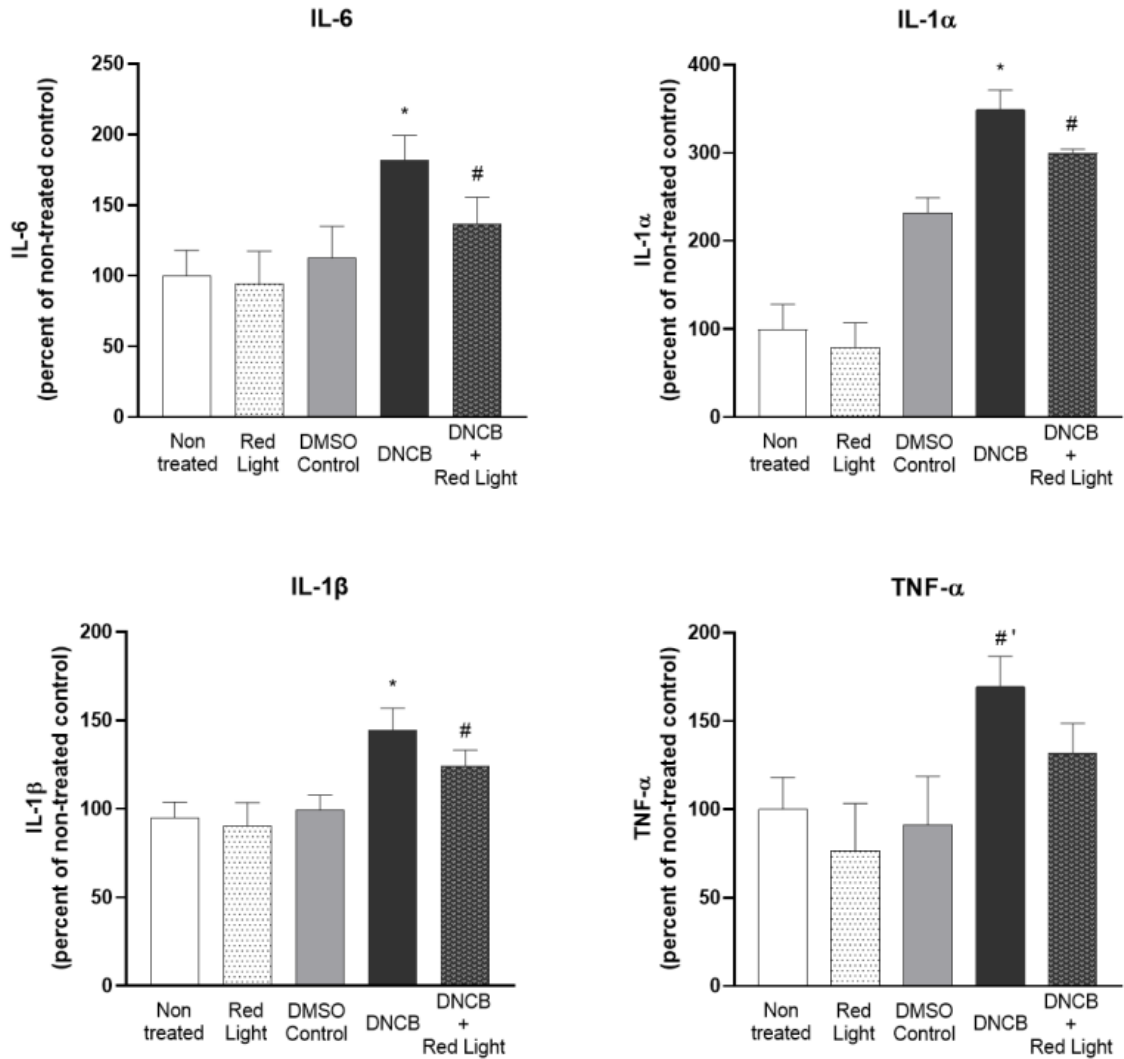


Figure S6.

Red light slightly decreased DNCB-induced pro-inflammatory cytokine secretion in the skin explants. Human skin explants were treated topically with the excipient (DMSO 20%) or DNCB (0.25% *w/v*) with or without red light exposure (660 nm, 3 J/cm², 250 s). Quantification of IL-6, IL-1α, IL-1β and TNF-α secretion level was assessed by Meso Scaled Discovery multiplex assay in the supernatant of the skin explants after 24 h of exposure to DNCB with or without red light illumination. Histogram represents the mean ± SEM of 3 independent measurements obtained from 3 different explants per group. Data were expressed as percentage of non-treated control group. One-way ANOVA followed by Holm-Sidak post-hoc test, * $p < 0.05$ vs. DMSO-treated group; # $p < 0.1$ vs. DNCB-treated group; #' $p < 0.1$ vs. DMSO-treated group.

ARTICLE 2

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Pivotal Role for TRPV1 Channel and Nrf2 Factor in the Green Light-Mediated Control of Keratinocytes Inflammatory Response

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Abstract

Photobiomodulation (PBM) is emerging as a promising approach for managing inflammatory skin disorders. However, the molecular mechanisms governing its effects, especially within the green light spectrum, remain elusive. In this study, we explored the anti-inflammatory mechanisms of green light (520 nm) in primary human keratinocytes (KCs) exposed to the electrophilic sensitizer, 2,4-dinitrochlorobenzene (DNCB), a pro-inflammatory trigger. Our data revealed that green light effectively reduces the mRNA expression of pro-inflammatory cytokines *IL-6*, *IL-8*, and *TNF- α* , induced by DNCB, comparably to the effect of the conventional anti-inflammatory agent, dexamethasone. As we previously demonstrated that the transcription factor Nuclear factor erythroid-2-related factor 2 (Nrf2) is a critical player in the red light response, we investigated its role in the green light anti-inflammatory activity. Green light exposure resulted in the activation of the Nrf2 pathway, as indicated by an increase in Nrf2 accumulation in KCs along with significant induction of Nrf2 target genes, including heme oxygenase 1 (HO-1) and glutamate-cysteine ligase catalytic subunit (GCLC). Invalidation of Nrf2 by si-RNA diminished the green light regulatory effect on pro-inflammatory cytokines, suggesting that Nrf2 is an essential contributor to the anti-inflammatory effect of green light. Additionally, we addressed the role of the Transient Receptor Potential Vanilloid 1 (TRPV1) cation channel, a potential target of green light. We found that blocking TRPV1 with capsazepine (CPZ) abolished the anti-inflammatory effect of green light. This finding highlights TRPV1's integral role in mediating green light beneficial activity in KCs. Importantly, the inhibition of TRPV1 also prevented the green light upregulation of the Nrf2 target genes, suggesting that Nrf2 pathway activation by the green light is TRPV1-dependent. Overall, our study identified two pivotal players in this PBM response: the TRPV1 channel and Nrf2 transcription factor, highlighting the versatility of green light as a modulator of two interconnected systems to control inflammation in KCs effectively. These findings offer potential cost-effective and non-invasive therapeutic approaches for inflammatory skin conditions.

Abbreviations

CPZ: capsazepine

Dex: Dexamethasone

DNCB: 2,4-dinitrochlorobenzene

GCLC: Glutamate-cysteine ligase catalytic subunit

HO-1: Heme oxygenase 1

IL: Interleukin

KC: Keratinocytes

LED: Light-emitting diode

NQO1: NAD(P)H quinone oxidoreductase 1

Nrf2: Nuclear factor erythroid-2-related factor 2

OPN: Opsin

PBM: Photobiomodulation

TNF- α : Tumor Necrosis Factor-alpha

TRPV1: Transient Receptor Potential Vanilloid 1

UV: Ultraviolet

Introduction

Inflammation is a common major contributor to the pathogenesis of many skin diseases and conditions, including chronic and acute inflammatory dermatoses with a significant patient burden¹. Thus, controlling inflammation in dermatology is paramount for treating inflammatory disorders. Currently, pharmacotherapies involving topical medications, such as corticosteroids, immunomodulators, and retinoids, as well as systemic treatments (e.g., immunosuppressants and cytokine-targeted monoclonal antibodies), are commonly used to manage skin inflammation. However, these traditional approaches carry several adverse side effects, compromising patient compliance². Therefore, there is an increasing need to explore safer alternative and complementary therapeutic strategies to effectively address skin inflammation and promote tissue repair with minimal adverse effects. One emerging area of interest is photobiomodulation (PBM), a non-invasive and non-thermal therapy recently gaining attention in dermatology for its potential in treating various skin conditions, such as acne, psoriasis, rosacea, and wound healing with minimal contraindications and no side effects³⁻⁶. PBM therapy, also known as low-level light therapy, uses low levels (< 500 mW/cm²) of visible light or invisible near-infrared (NIR) radiation to modulate cellular responses and elicit beneficial effects⁷. These effects include pain alleviation⁸⁻¹⁰, immunomodulation¹¹⁻¹⁴, tissue regeneration^{3,15-17}, and reduction of inflammation¹⁸⁻²⁰.

While the majority of PBM literature supports its application as a regulator of skin inflammation using red (600 – 700 nm) or NIR (780 -1100 nm) wavelengths²¹⁻²⁵, wavelengths shorter than 600 nm, such as green light (500 – 570 nm), are less commonly researched or reported, as they do not penetrate deeply into the skin layers^{26,27}. Nonetheless, recent studies have shown promising anti-inflammatory effects of green light in various cellular and *in vivo* models, such as reducing inflammatory immune cell infiltration while accelerating wound healing in a rat burn model²⁸ and downregulating chemokines gene expression in human adipose-derived mesenchymal stem cells²⁹. However, the underlying molecular mechanisms driving the anti-inflammatory effect of green light in PBM remain incompletely defined.

The biological effects of PBM are induced by photophysical and photochemical events secondary to the absorption of the non-ionizing photons by the appropriate endogenous photoreceptors^{30,31}. A well-identified photoreceptor for green (and violet-blue) light is the light-sensitive G-protein receptor family, known as opsins (OPNs)⁶, expressed on human KCs, among other cells³². One proposed downstream target of OPN activation is transient receptor potential (TRP) channels, particularly the vanilloid receptor 1 (TRPV1) subtype³³. TRPV1 is expressed on diverse cells in the skin, including KCs, cutaneous sensory neurons, and immune cells such as dermal dendritic cells³⁴⁻³⁷. TRPV1 is a non-selective cation channel activated by several known stimuli, such as capsaicin (the major pungent compound in chili peppers), heat (> 42°C), acidic pH, ultraviolet (UV) radiations, as well as endogenous activators such as endocannabinoids lipids³⁶. Once activated, the opening of the TRPV1 channel permeabilizes the cell membrane to cations in a non-selective manner, with relatively high permeability to Ca²⁺. The calcium influx into the intracellular space acts as a second messenger, initiating several downstream signaling pathways, such as PKC and calmodulin-dependent protein kinase-II (CAMKII), thus contributing to the regulation of inflammatory genes and modulating various biological responses³⁸⁻⁴⁰. It was shown that TRPV1 activation promotes KC differentiation⁴¹⁻⁴³ and migration^{42,44} while it inhibits KC proliferation and enhances its apoptosis^{41,45}. Beyond its role in epidermal homeostasis, TRPV1 participates in cutaneous neuronal functions. It mediates histamine-dependent and independent itch perception⁴⁶⁻⁵¹. In addition, TRPV1, expressed on KCs, plays a key role in sensing and initiating the transduction of thermal, chemical, and inflammatory pain⁵²⁻⁵⁵.

Results

TRPV1 has been evidenced to impact skin inflammation significantly^{38,56}. It is commonly believed that TRPV1 activation has a pro-inflammatory role, as the inhibition or desensitization of the TRPV1 pathway in inflammatory skin diseases such as atopic dermatitis has been shown to reduce the skin inflammation³⁹, leading to an improvement in the epidermal barrier disruptions and the clinical symptoms often associated with these dermatological disorders^{50,57–64}. However, growing evidence highlights TRPV1's anti-inflammatory role as well⁶⁵, particularly in chemical-induced contact dermatitis models⁶⁶. Although TRPV1 was reported as a potential target activated by the green light⁶⁷, its role in mediating the effects of the green light in PBM is not fully clarified, presenting an interesting area of investigation.

PBM using blue, red, or NIR light has also been associated with an upregulation of the transcription factor Nuclear factor erythroid-2-related factor 2 (Nrf2) pathway in different cellular and *in vivo* models^{68–70}. Nrf2 is the master regulator of cellular redox homeostasis⁷¹ and was shown to play a key role in controlling the inflammatory skin response^{72–74}. When cells are exposed to electrophilic stress, Nrf2 is trapped with its cytoplasmic inhibitor, Kelch-like ECH-associated protein 1 (Keap1), in a way preventing its ubiquitination and degradation. Consequently, newly synthesized Nrf2 accumulates in the cytoplasm and translocates into the nucleus, where it binds to the antioxidant-responsive elements (ARE) in target gene promoters. As a result, the transcription of target genes is triggered, encoding a wide range of antioxidant enzymes, such as superoxide dismutase (SOD) and glutamate-cysteine ligase catalytic subunit (GCLC), cytoprotective enzymes such as heme oxygenase 1 (HO-1), as well as phase II drug detoxification enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1)^{75,76}. Our previous study showed that Nrf2 is a crucial factor mediating the anti-inflammatory response of the red light (660 nm) in 2,4-dinitrochlorobenzene (DNCB)-stimulated KCs⁷⁷. However, its involvement in the anti-inflammatory effect of the green light has not been demonstrated yet.

This study aims to elucidate the effect of green light on KC inflammatory response and understand the cellular mechanisms through which PBM modulates inflammation. We first assessed TRPV1 implication in the green light suppressive response within a model of primary human KCs challenged with DNCB, a contact sensitizer used to mimic pro-inflammatory stress. Herein, we provided evidence for the protective role of TRPV1 against chemical-induced inflammation in KCs and highlighted its crucial role in the green light anti-inflammatory activity. We further investigated the role of Nrf2 in the green light response. We demonstrated the critical role of Nrf2 in controlling the expression of DNCB-induced pro-inflammatory cytokines upon green light exposure. Interestingly, the green light-mediated Nrf2 activity on target genes was shown to be TRPV1-dependent, making the green light PBM a versatile tool for skin inflammation management.

Materials and Methods

Cell Culture

Primary human normal epidermal KCs isolated from the skin of multiple juvenile donors (> three individuals) were purchased cryopreserved at an early passage from CELLnTEC (Bern, Switzerland). The Cells were cultured in CnT-Prime Epithelial Proliferation medium (CnT-PR) (CELLnTec, Bern, Switzerland), as previously described⁷⁷. CnT-PR is a phenol red-free, fully supplemented medium with low calcium composition (0.07 mM) and a combination of Growth factors and Progenitor Cell Targeted factors, maximizing retention of proliferative progenitor cells and minimizing cell differentiation. KCs were seeded after thawing or subsequent passaging at 4000 cells/cm² density and incubated at 37 °C within a humidified atmosphere under 5% CO₂. The culture medium was renewed every 48 hours. Passage of cells occurred when they reached approximately 80% confluency (around day 6) using 0.05% trypsin–EDTA solution for trypsinization (Gibco, Paisley, UK). Cells from the second to fourth passages were used for experiments.

Chemical Treatment

Upon reaching 80% confluency, KCs were detached using 40 µL/cm² of trypsin-EDTA (0.05%) solution (Gibco, Paisley, UK). They were re-plated in a 24-well plate at a seeding density of 2×10^5 cells per well in 500 µL CnT-PR medium and incubated for at least 18 hours at 37°C. KCs were treated with 25 µM DNCB (CAS number: 97-00-7; Sigma-Aldrich, St. Louis, MO, USA) for 3 hours at 37°C. DNCB was diluted in dimethyl sulfoxide (DMSO, CAS number 67-68-5, Sigma-Aldrich). The final concentration of the solvent in culture media did not exceed 0.1%. Cytotoxicity was evaluated by measuring the fluorescence of propidium iodide uptake using flow cytometry (Attune® NxT Acoustic Focusing cytometer; Thermo Fisher, Waltham, MA, USA). KCs treated with 10⁻⁶ M of dexamethasone (Dex) (CAS number: 50-02-2) (Merck-Sigma Aldrich, Germany), 1 hour before the DNCB challenge, served as a positive control for the anti-inflammatory activity.

Light Source and Photobiomodulation Treatment

KCs seeded at 2×10^5 cells per well within a 24-well transparent plate were illuminated, as described by Gueronprez *et al.*⁷⁸. The light source employed was pulsed-wave light-emitting diodes (LEDs) positioned in the upper lid of a custom-made device allowing consistent and simultaneous irradiation of 12 wells on the plate. The LEDs underwent calibration with a Qmini 2 spectrometer (RGB Photonics GmbH, Kelheim, Germany) to ensure light irradiance uniformity in each well. Light homogeneity was also controlled for each well (SP90422 Beam Analyser, Ophir Spiricon Europe GmbH, Darmstadt, Germany). The irradiance and homogeneity measurements were taken at the bottom of the well. Cells were illuminated at ambient room temperature, with pulsed 520 nm (green light) wavelength, 20 min after DNCB treatment, at an irradiance of 12 ± 4 mW/cm² for a total exposure time of 250 s, conferring a fluence (energy density) of 3 J/cm². The pulse width was 10 milliseconds, with a 50% duty cycle (DC). The distance from the LED to the bottom of the well measured 34.5 mm. Cells with no light treatment (sham group) served as a negative control, shielded with a black wrap to avoid any light exposition. Following irradiation, cells were maintained at 37 °C, 5% CO₂ until required for subsequent analysis.

Gene Silencing

The stealth RNAi™ small interfering RNA (siRNA) provided by Invitrogen (Paisley, UK) was used for *Nrf2* knockdown (NFE2L2 HSS107130), following previously established protocol⁷⁷. The transfection was performed using Lipofectamine® RNAiMax Transfection Reagent (Invitrogen, Carlsbad, CA, USA). KCs were seeded in 24-well plates at a density of 2.5×10^4 per well in 500 µL of CnT-PR medium one day before transfection, ensuring that the cells reached a confluence of 30–50% at the time of transfection. KCs were then transfected for 48 hours with 50 nM of siRNA *Nrf2* (si-Nrf2 group). In each experimental group, a mock

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transfection (without siRNA) and the scrambled non-silencing siRNA (si-random) lacking human gene homology (Stealth™ RNAi Negative Control-Medium GC duplex; Invitrogen, Carlsbad, CA, USA) were performed as negative controls.

TRPV1 inhibition

One hour before DNCB stimulation, KCs were pre-treated with 20 μM capsazepine (CPZ) (Cas number: 138977-28-3) (Merck-Sigma Aldrich, Germany), a synthetic benzazepine derivative and selective antagonist of the TRPV1 channel. CPZ was dissolved in DMSO, with a final DMSO concentration of 0.05%. CPZ cytotoxicity was assessed with the MTT viability assay.

RNA Isolation and RT-qPCR

Total RNA was extracted from KCs using a silica column-based purification with PureLink™ RNA Mini Kit according to the manufacturer's instructions (Ambion®; Invitrogen, Carlsbad, CA, USA). Total RNA was quantified by spectrophotometry (Eppendorf BioPhotometer; Hamburg, Germany), and the ratio of absorbance at 260 nm and 280 nm was used to assess RNA purity. A total of 500 ng of RNA was subjected to reverse transcription in a total volume of 20 μL with 0.5 mM of dNTP (MP Biomedicals®, Santa Ana, CA, USA), 2.5 μM of oligo (DT) (Promega®, Madison, WI, USA), 1 U/μL of SuperScript™ IV Reverse Transcriptase (Invitrogen®, Waltham, MA, USA), 1 U/μL of RNasin (Promega®), 5 mM of Dithiothreitol (DTT; Invitrogen®) and 1X Reverse Transcriptase buffer (Invitrogen®). cDNA was then used for quantitative real-time PCR after being diluted by 1:20 in RNase-free water. Quantitative PCR was performed with the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) using the thermal cycler CFX384™ Touch Real-Time System (Bio-Rad, Hercules, CA, USA). All samples were analyzed in duplicate. The primer sequences used for cDNA amplification are listed in Supplementary Table S1. CFX Maestro Software (version 2.3, Bio-Rad) was used for the quantification analysis. The results were normalized to the two house-keeping genes expressions: *gapdh* and *hprt*, and were expressed as a fold increase (i.e., the ratio of $(1 + E)^{-\Delta Ct}$ of target genes/ $(1 + E)^{-\Delta Ct}$ of reference genes, where E is the efficiency and ΔCt is the difference of Ct between treated cells and untreated control cells).

Protein Extraction and Western Blotting

As described in ⁷⁷, DNCB-treated and illuminated KCs were washed twice in phosphate-buffered saline before being trypsinized and centrifuged into a dry pellet. Cells were resuspended in lysis buffer (20 mM Tris HCl (pH 7.4); 137 mM NaCl; 1% Triton X-100; 2 mM EDTA; 2 mM sodium pyrophosphate; 10% glycerol and H₂O) supplemented with phosphatases and proteases inhibitor cocktail (10 μg/mL aprotinin; 10 μg/mL leupeptin; 1 mM sodium orthovanadate; 1 mM phenylmethylsulfonylfluoride; 100 μg/mL pepstatin and 25 mM β-glycerophosphate). The cells were incubated with the lysis buffer for 20 min in ice, followed by a 20 min centrifugation at 15,000 rpm, at 4 °C. Following protein quantification with a BCA protein assay kit (Pierce™, Rockford, IL, USA), 40 μg of denatured protein were loaded into 12% SDS-PAGE 1.5 mm gels (TGX-Stain-Free FastCast Acrylamide kit; Bio-Rad, Hercules, CA, USA) for electrophoresis, and then transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1 hour at room temperature in Tris-buffered saline with 5% nonfat dry milk and incubated overnight at 4 °C with primary rabbit anti-Nrf2 Ab (1:1000 dilution in Tris-buffered saline, 3% BSA; Proteintech®, Ref. 16396-1-AP). It is washed and incubated for 1 h at room temperature with a secondary antibody conjugated to Horseradish peroxidase (HRP) (Cell Signaling Technology, Ref. 7074). Immunoreactive bands were detected by chemiluminescence using the ChemiDoc XRS + System (Bio-Rad, Hercules, CA, USA) by adding the ECL substrate (luminol + peroxide solution) (Clarity Max™; Bio-Rad, Segrate, Italy). Band densities were quantified with ImageLab software (version 6.1) and normalized to the total protein loaded.

Statistical Analysis

Statistical Analyses were performed using GraphPad Prism® software (version 8, GraphPad Software-Dot-matics, San Diego, CA, USA). Repeated-measures one-way ANOVA followed by Tukey’s multiple comparison post hoc test was used to compare different groups. Data are expressed as means ± SEM and considered statistically different when the *p*-value < 0.05.

Results

1. Green light PBM Regulates Pro-inflammatory Cytokine Expression in Stimulated KCs

This study triggered the pro-inflammatory cellular stress was triggered in KCs by exposing them to 25 μM of DNCB, an electrophilic chemical sensitizer. The concentration of DNCB resulting in 70% of cell viability was chosen for this study. Following DNCB treatment, KCs were illuminated with 520 nm wavelength at 3 J/cm² for a total exposure time of 250 s. Our primary focus was to evaluate the effectiveness of this wavelength in controlling the inflammatory response of KCs within the defined dosimetry parameters. We aimed to compare the green light activity to a conventional anti-inflammatory agent, Dex (10⁻⁶ M), a well-known glucocorticoid. The mRNA level of different pro-inflammatory cytokines was therefore assessed by RT-qPCR after 3 hours of DNCB stimulation and exposure to light.

Exposure to green LED did not modify the expression of any assessed cytokine compared to untreated cells at the basal state (Figure 1). Conversely, DNCB induced a significant increase in the mRNA expression of *interleukin (IL)-6* (5.06—fold increase), *IL-8* (2.81—fold increase), and *tumor necrosis factor-alpha (TNF-α)* (4.97—fold increase), 3 hours post-stimulation. Interestingly, the green light significantly inhibited the expression of the DNCB-triggered *IL-6*, *IL-8*, and *TNF-α* mRNAs, reducing them by 58%, 45%, and 53%, respectively. Dex likewise decreased the mRNA expression of *IL-6* by 77%, *IL-8* by 59%, and *TNF-α* by 58%, compared with DNCB-stimulated conditions. No notable difference was statistically observed between green light and Dex effects (Figure 1). Collectively, these results showed that PBM using green light did not modulate the transcription of pro-inflammatory cytokines in KCs under basal conditions. However, it effectively decreased the expression of cytokines induced by the DNCB comparably to Dex activity.

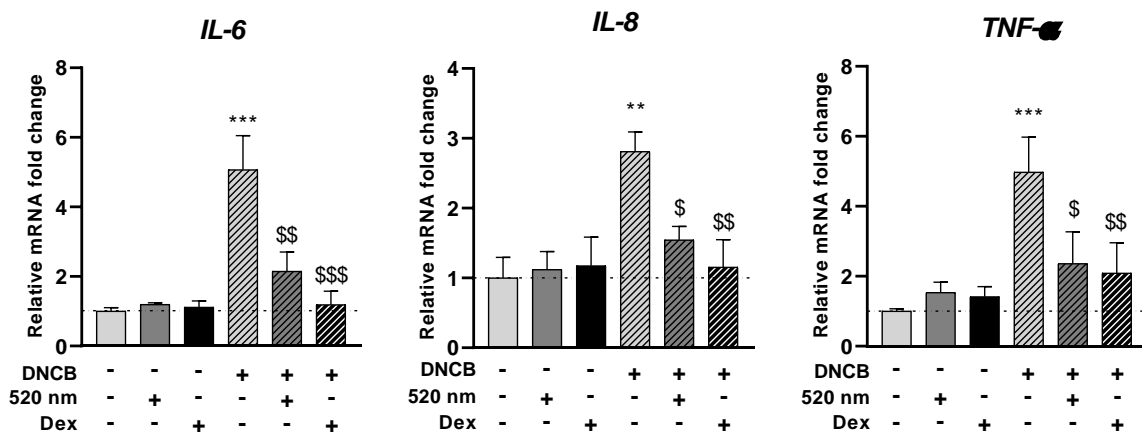


Figure 1. Green light PBM regulates pro-inflammatory DNCB-induced cytokines in KCs. KCs were treated with DNCB or the vehicle, with or without green light illumination (520 nm, 3 J/cm², 250 s). Dexamethasone (Dex) (10⁻⁶ M) served as a positive control for the anti-inflammatory effect. Pro-inflammatory cytokines gene expression: *IL-6*, *IL-8*, and *TNF-α* were measured by RT-qPCR 3 hours after DNCB and light treatment. Results are expressed as fold change. Data represent mean ± SEM of 3 independent experiments. One-Way ANOVA followed by Tukey’s post hoc test, * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001 vs. vehicle control; \$ *p* < 0.05; \$\$ *p* < 0.01; \$\$\$ *p* < 0.001 vs. DNCB-treated group.

2. Green light PBM Enhances Nrf2 Pathway Activation in DNCB-Stimulated KCs

Nrf2 is described as a key factor regulating skin inflammation^{79–82}. While a restricted number of studies have investigated the effects of PBM, such as red light, in Nrf2 expression⁸³, the involvement of Nrf2 in the anti-inflammatory response of green light remains unexplored. Thus, we first investigated the activation of the Nrf2 pathway in KCs in response to the green light exposure, compared to red light (660 nm) in DNCB-treated KCs. Nrf2 protein accumulation was assessed by western blot, 3 hours post-DNCB, and light treatment. Our results showed that DNCB increased Nrf2 accumulation in KCs compared to the untreated control group (Figure 2A). Notably, the green LED was a better enhancer of Nrf2 accumulation (6.52—fold increase) than DNCB (3.7—fold increase) or red LED (5.33—fold increase). However, the difference between the two lights did not reach a statistical significance (Figure 2A). Since the Nrf2 protein was shown to be increased by the green light, the next step was to assess the expression of several Nrf2-target antioxidant genes by RT-qPCR. Our data showed that DNCB increased mRNA expression of *HO-1* (181-fold increase), *GCLC* (2.23-fold increase), and *NQO1* (2.9-fold increase). While green light further enhanced the expression of *HO-1* by 53% ($p < 0.05$), *GCLC* by 19.2% ($p < 0.05$), and *NQO1* by 11.3% ($p = 0.09$) upon illumination of DNCB-stimulated KCs (Figure 2B). Taken together, these data revealed that green light enhanced the DNCB-induced activation of the Nrf2 signaling pathway.

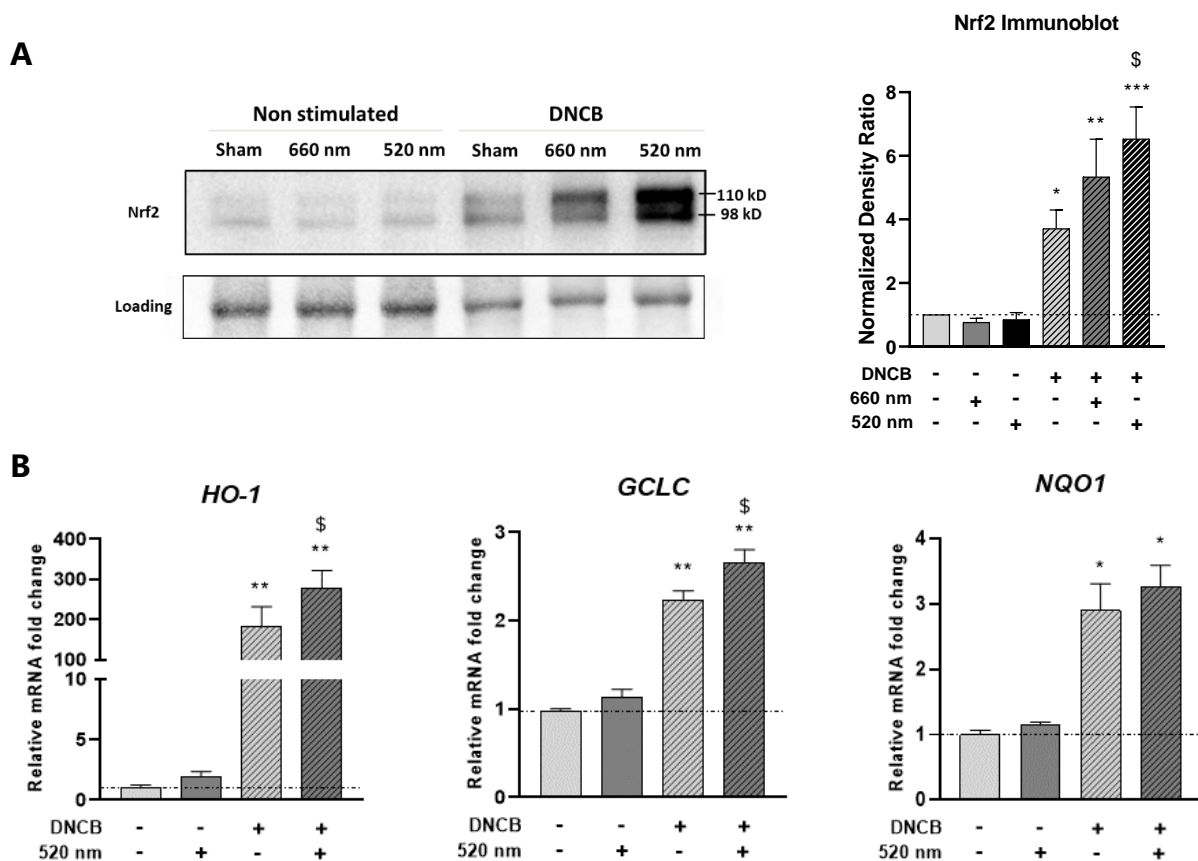


Figure 2. Green light increases Nrf2-pathway activation in DNCB-stimulated KCs. KCs were treated with vehicle (DMSO) or DNCB (25 μ M) and illuminated with either wavelength: 660 nm (red light) or 520 nm (green light). **(A)** Nrf2 accumulation was assessed by western blot 3 hours after illumination. Histograms correspond to densitometric analysis relative to controls and are adjusted to the stain-free blot. **(B)** The expression of Nrf2 antioxidant target genes: *HO-1*, *GCLC*, and *NQO1*, were assessed by RT-qPCR 3 hours after KC treatment. Data represent mean \pm SEM of at least 3 independent experiments. One-Way ANOVA followed by Tukey's post hoc test, * $p < 0.05$; ** $p < 0.01$ vs. vehicle control; \$ $p < 0.05$ vs. DNCB-treated group.

3. The Anti-Inflammatory Response of the Green Light in KCs is Nrf2-dependent

We suggested that Nrf2 activation by the green light might be involved in regulating of the DNCB-mediated inflammatory response. Nrf2 was invalidated in KCs by siRNA transfection strategy (si-Nrf2) to address this hypothesis. The inhibition of Nrf2 accumulation in KCs treated with DNCB reached 80% when using the si-Nrf2 compared to its relative si-random control (Figure S1A). Similarly, the mRNA levels of *HO-1*, *NQO1*, and *GCLC* were significantly downregulated by 70.1%, 81.5%, and 74.8%, respectively, in the group where Nrf2 was invalidated, and KCs were stimulated with DNCB (Figure S1B).

The mRNA expression of three pro-inflammatory mediators was assessed to study the response of KCs to green light exposure in the absence of Nrf2. As described in Figure 1, the green light attenuated DNCB-induced *IL-6*, *IL-8*, and *TNF- α* expression in the si-random control group (Figure 3). Interestingly, in the absence of Nrf2, illumination of KCs with green light did not reduce the expression of these cytokines in response to DNCB (Figure 3). These findings suggest that the anti-inflammatory effect of green light observed upon DNCB stimulation in KCs is dependent on Nrf2.

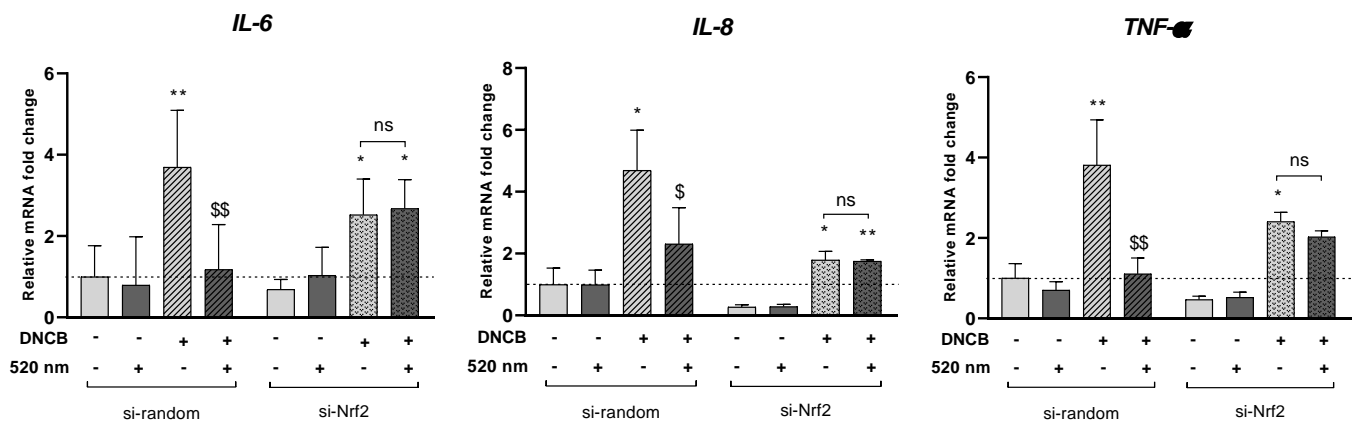


Figure 3. Nrf2 mediates the anti-inflammatory effect of green light in KCs. KCs were transfected for 48 h by si-Nrf2 or si-random, then treated with DNCB and exposed to green light (520 nm, 3 J/cm²). Pro-inflammatory cytokine gene expression: *IL-6* and *IL-8*, and *TNF- α* , were assessed by RT-qPCR, 3 hours following DNCB and light exposure. Results are expressed as fold change compared to the untreated si-random control. Data represent mean \pm SEM of 4 independent experiments. One-Way ANOVA followed by Tukey's post hoc test, * $p < 0.05$; ** $p < 0.01$ vs. relative vehicle control; \$ $p < 0.05$; \$\$ $p < 0.01$ vs. si-random DNCB-treated group. Ns: non-significant.

4. TRPV1 is a crucial green light target alleviating the pro-inflammatory response in KCs

We examined the role of the TRPV1 channel, a green light-activated target^{67,84}, in reducing the pro-inflammatory cytokines upon cell irradiation. TRPV1 is described as a regulator of skin inflammation and immune activation^{47,65}. Therefore, we investigated whether blocking TRPV1 using CPZ (20 μ M) prevents the anti-inflammatory activity mediated by the green light.

Consistent with the above results, green light treatment significantly reduced mRNA expression of *IL-6*, *IL-8*, and *TNF- α* upregulated by DNCB (Figure 4). However, CPZ blocked the beneficial effect of the green light as no significant difference was observed in the CPZ-treated group between the irradiated and non-irradiated KCs stimulated with DNCB (Figure 4). Similarly, DNCB treatment induced a higher level of *TNF- α* expression in the CPZ-treated group compared to the CPZ-untreated conditions. These data indicate that TRPV1 is a key contributor to the anti-inflammatory effect exhibited by the green light and highlight its protective role in

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the KC, particularly under electrophilic stress. Of note, the *IL-8* mRNA level was enhanced by TRPV1 inhibition at the basal state of the control KCs.

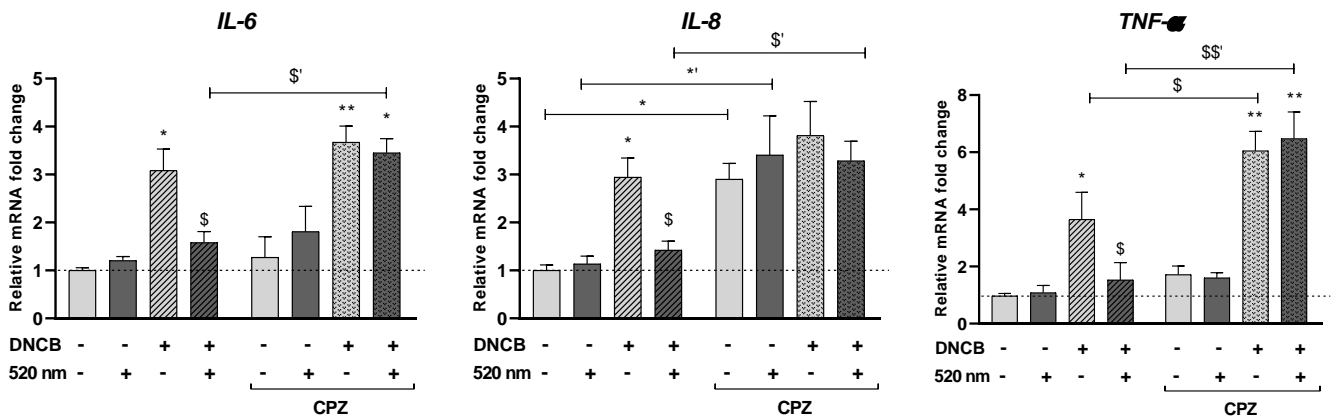


Figure 4. The Green Light attenuates KC inflammatory response through TRPV1. KCs were treated with capsazepine (CPZ) 1 hour prior to DNCB stimulation and green light (520 nm) illumination. Pro-inflammatory cytokine gene expression: *IL-6* and *IL-8*, and *TNF-α* were assessed by RT-qPCR, 3 hours following DNCB and light exposure. Results are expressed as fold change compared to the untreated control. Data ($n=3$) are shown as mean \pm SEM. One-Way ANOVA followed by Tukey's post hoc test, * $p < 0.05$; ** $p < 0.01$ vs. relative vehicle control (with or without CPZ); *' $p < 0.05$ vs. 520 nm- illuminated group without CPZ; \$ $p < 0.05$; vs. DNCB-treated group without CPZ; \$' $p < 0.05$; \$\$' $p < 0.01$ vs. (DNCB + 520 nm) -treated group without CPZ.

5. Green light-induced Nrf2 activity is TRPV1-dependent

The above results suggesting the involvement of both the TRPV1 channel and Nrf2 factor in the green light response drove us to investigate whether TRPV1 mediates the enhanced activity of Nrf2. Therefore, the transcription of Nrf2 target genes was assessed following green light irradiation while blocking TRPV1 channels by CPZ treatment. Our first outcome showed that TRPV1 inhibition partially attenuates the mRNA expression *GCLC* and *NQO1* by approximately 32% in KCs stimulated with DNCB alone. This suggests that TRPV1 plays a role in partially controlling the basal expression of Nrf2 target genes in KCs when exposed to electrophilic chemical stress (Figure 5). Additionally, the upregulation of Nrf2 target genes *HO-1*, *GCLC*, and *NQO1* by the green light in DNCB-stimulated KCs is abrogated when the TRPV1 channel is inhibited (Figure 5). These findings support our hypothesis regarding the effect of TRPV1 on Nrf2 activation and provide strong evidence that the TRPV1 channel is essential for the optimal activation of the Nrf2 pathway by the green light under inflammatory conditions.

Results

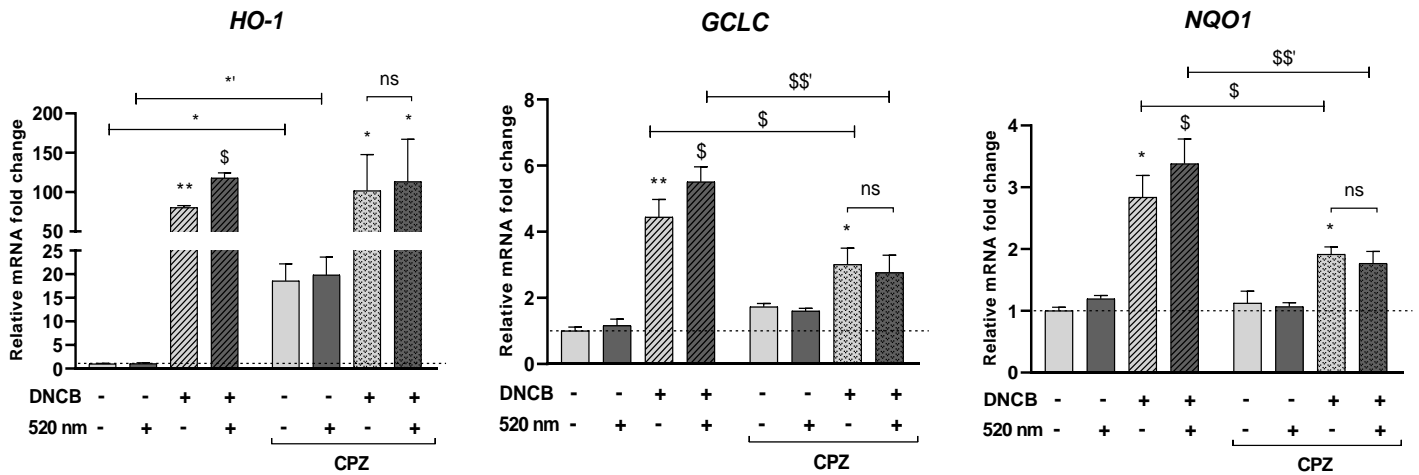


Figure 5. TRPV1 mediates the expression of Nrf2 target genes induced by the green light. KCs were treated with capsazepine (CPZ) 1 hour prior to DNCB stimulation and green light (520 nm, 3 J/cm²) illumination. The expression of Nrf2 antioxidant target genes: HO-1, GCLC, and NQO1 were assessed by RT-qPCR 3 hours after KC treatment. Results are expressed as fold change compared to the untreated control. Data of 3 independent experiments are shown as mean ± SEM. One-Way ANOVA followed by Tukey's post hoc test, * $p < 0.05$; ** $p < 0.01$ vs. relative vehicle control (with or without CPZ); *' $p < 0.05$ vs. 520 nm- illuminated group without CPZ; \$ $p < 0.05$; vs. DNCB-treated group without CPZ; \$\$' $p < 0.01$ vs. (DNCB + 520 nm) -treated group without CPZ. Ns: non-significant.

Discussion

PBM is gaining attention as a safe therapy to alleviate skin inflammation and manage various inflammatory skin disorders^{3,4,5}. A considerable body of research has established the ability of PBM to regulate inflammatory responses both *in vitro* and *in vivo* by modulating the production of pro-inflammatory mediators^{85,86}, reducing immune cell infiltration at the site of inflammation^{19,24}, and inhibiting immune cell activation^{77,87,88}. Despite the well-established efficacy of PBM, the molecular mechanisms underlying these anti-inflammatory effects remain largely undefined. This knowledge gap becomes particularly pronounced when considering the specific impact of the green light (500-570 nm) within the broader spectrum of PBM (400 – 1100 nm). Green light has received less attention in scientific literature than blue, red, or NIR wavelengths. Yet, recent studies have shown promising results regarding its ability to accelerate healing processes and elicit anti-inflammatory effects^{28,33,89}. We conducted this study to investigate the impact of the green light, particularly the 520 nm- LED exposure, on skin inflammation and to elucidate the cellular pathways through which this wavelength may regulate the inflammatory response.

Green light penetrates less than 1 mm into the skin, primarily affecting the epidermis and the upper dermis⁹⁰. Since KCs constitute the major cell population of the epidermis and actively contribute to the inflammatory process in many skin pathologies^{91–93}, we chose to investigate the response of the green light using a two-dimensional model of primary human KCs. To induce the pro-inflammatory conditions, KCs were exposed to DNCB, an electrophilic chemical known as an extreme contact sensitizer⁹⁴. It creates a pro-inflammatory microenvironment, triggering an extensive immune response. Thus, DNCB is commonly used in contact hypersensitivity (CHS)^{80,95} and atopic dermatitis^{96,97} rodent models, as well as *in vitro* studies assessing skin sensitization responses in KCs^{98,99}. Consistent with previous findings^{77,100,101}, our data revealed increased

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mRNA expression levels of pro-inflammatory cytokines, including *IL-6*, *IL-8*, and *TNF- α* , in response to DNCB treatment.

Following KC exposure to 520 nm LED (3 J/cm²), our results demonstrated that green light could effectively suppress the expression of DNCB-induced pro-inflammatory cytokines. This aligns with previous studies that highlighted the role of green light in regulating inflammatory gene expression and protein production in different models, including human adipose-derived mesenchymal stem cells²⁹, collagen-induced arthritis¹⁰², and neuroinflammation¹⁰³ *in vivo* models. However, none of these studies have examined the impact of green light PBM on KC inflammatory response, emphasizing the unique aspect of our research. Remarkably, we observed that the reduction in cytokine expression by green light in our model was comparable to the anti-inflammatory activity of Dex, which is a noteworthy result as glucocorticoids have not typically been used as positive controls for comparing the anti-inflammatory effect of PBM in literature. Instead, other anti-inflammatory agents, such as non-steroidal anti-inflammatory drugs, have been used. For instance, Lim *et al.* compared the anti-inflammatory effect of 635 nm red LED with conventional cyclooxygenase (COX) inhibitors (indomethacin and ibuprofen). They found that red light irradiation of human gingival fibroblasts inhibited COX and PGE2 expression similarly to COX inhibitors, highlighting the potential of PBM in managing inflammation¹⁰⁴. Furthermore, our findings regarding the impact of green light on non-stimulated normal KCs align with previous studies¹⁰⁵, showing no significant modifications in *IL-6*, *IL-8*, or *TNF- α* expression by the light.

To investigate the less-explored molecular mechanisms of green light, we addressed the role of the Nrf2 pathway in its anti-inflammatory response since Nrf2 is recognized as a key factor controlling skin inflammation^{79–82,106}. It is also identified as the master regulator of xenobiotic detoxification and cellular redox homeostasis⁷¹. Only a limited number of studies have explored the effects of PBM on Nrf2 expression, but none has yet demonstrated Nrf2 involvement in the green light response. Trotter *et al.* reported an increase in Nrf2 accumulation and translocation into the nucleus when treating the human monocytic cell line, THP-1, with blue light (45 J/cm²), followed by the induction of *HO-1* expression, an Nrf2-target cytoprotective gene. These observations correlated with a reduction in LPS-induced *TNF- α* and *IL-8* secretion⁶⁹. Additionally, Yadav *et al.* demonstrated that Nrf2 pathway activation is associated with an acceleration in burn healing in mice treated with 904 nm NIR⁷⁰. As reported for the other wavelengths, we showed activation of the Nrf2 signaling by the green light in DNCB-stimulated KCs with an increased stabilization of Nrf2 and enhanced transcription of *HO-1* and *GCLC* target genes. Interestingly, in the absence of Nrf2, the illumination of KCs by the green light did not effectively reduce the *TNF- α* , *IL-6*, or *IL-8* expression in response to DNCB. Consistent with our previous report showing the pivotal role of Nrf2 in the red light activity⁷⁷, this study is the first to report that the anti-inflammatory response elicited by the green light in KCs is, at least in part, Nrf2-dependent. It is known that Nrf2 can exert its anti-inflammatory activity through both direct and indirect mechanisms^{82,107}. The indirect pathways may involve modulating reactive oxygen and nitrogen species and inhibiting redox-sensitive key inflammatory pathways, mainly NF- κ B¹⁰⁷, which was notably reported to be inhibited by PBM in some studies^{12,20,108,109}. It was also shown that Nrf2 can directly inhibit the transcription of pro-inflammatory cytokine genes, such as *IL-6* and *IL-1 β* , by directly binding DNA regions close to these genes, independently of ARE sequences¹¹⁰.

Other potential factors may also contribute to the anti-inflammatory response exerted by the green light. As the TRPV1 cation channel was described as a main target activated by the green light^{67,84,111}, we investigated its involvement in the green light beneficial response. TRPV1, primarily known for its role in pain and itch perception, temperature sensitivity, and epidermal homeostasis, has been recently recognized as a regulator of inflammation^{38,112}. Normal human epidermal KCs were shown to express TRPV1 channel in our

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model, primarily assessed by western blot (data not shown), consistent with previously reported³⁷. Our study demonstrated a compelling link between TRPV1 and the anti-inflammatory effects of green light. The selective inhibition of the TRPV1 channel by its antagonist, CPZ, prevented the green light from effectively controlling pro-inflammatory cytokines in stimulated KCs, suggesting that TRPV1 activation is integral to the anti-inflammatory response elicited by PBM. Intriguingly, Pigatto *et al.* showed that red LED therapy had a significant antinociceptive and anti-inflammatory effect in acute chemical-induced nociception models, with these effects appearing to be mediated in part through interactions with TRPV1 among other receptors¹¹³. In line with this finding, our investigation specifically addressed the effect of green light on KCs, marking the first report of its kind.

The role of TRPV1 in the inflammation process has been a subject of debate⁶⁵, with two opposing viewpoints on whether TRPV1 plays an inflammatory or anti-inflammatory role, as supported by our study. Historically, TRPV1 activation, including via blue light irradiation, was associated with the initiation of inflammatory responses, as studies on KCs have shown that its inhibition attenuated the production of key pro-inflammatory mediators, such as IL-8, IL-1 β , and TNF- α ^{114–116}. In addition, TRPV1 is found to be overexpressed in skin inflammatory diseases such as atopic dermatitis¹¹⁷, psoriasis⁷⁶, sensitive skin syndrome^{119,120}, rosacea¹²¹, and herpes zoster infection¹²². Interestingly, the inflammatory response in these disorders was alleviated by inhibiting or desensitizing the TRPV1 pathway with an improvement in pruritis, pain sensation, and epidermal disruptions^{50,57–64}, further supporting TRPV1's pro-inflammatory role. Conversely, evidence has revealed TRPV1's potential anti-inflammatory and protective properties. It was shown in CHS models that TRPV1 knockout mice exhibited enhanced expression of pro-inflammatory cytokines, along with increased macrophage infiltration and ear swelling^{66,123}, suggesting the protective role of TRPV1 in allergic contact dermatitis. These observations aligned with our results as TRPV1 inhibition aggravated *TNF- α* expression in KCs stimulated with DNCB, a strong contact sensitizer. Moreover, TRPV1 was shown to play a crucial role against exacerbated chemical-induced skin burns and in skin healing as its absence impaired the induction in KCs of growth factors and cytokines essential for the recovery¹²⁴. A significant aspect of the observed anti-inflammatory effect of TRPV1 *in vivo* could be attributed to the pivotal role of TRPV1 signaling in suppressing the activation of key immune cells involved in the inflammatory response, such as dendritic cells and macrophages^{125,126}. Collectively, the TRPV1 channel may exhibit different activities depending on the cell type, context, and trigger. As many *in vivo* studies have analyzed the TRPV1 response in the whole skin tissue, we must consider the overall effect as a result influenced by interactions among various cell types, including neurons, immune cells, and epithelial cells⁶⁵. Further research on specific types of cells is warranted to elucidate the distinct functions of epithelial and non-epithelial TRPV1, providing more precise insights into their roles in inflammation to appropriately target TRPV1 with either agonist, like the green light or antagonist.

TRPV1 are transmembrane receptors with several intracellular ligand-binding sites interacting with negative and positive regulators, thus modulating the channel activity¹²⁷. It remains unclear how green light could activate the TRPV1 channel, whether it occurs through direct photoisomerization of TRPV1 chromophores or indirectly, as a subsequent event following opsins activation^{33,111}. However, the increased calcium influx resulting from green light irradiation can serve as a secondary messenger, triggering multiple subsequent signaling pathways¹²⁸. For instance, a study reported that green light (540 nm) inhibits the proliferation of human adipose-derived stem cells by activating TRPV1 and increasing calcium and reactive oxygen species (ROS)⁸⁴. Furthermore, the Ca²⁺ influx mediated by TRPV1 activation following the treatment with TRPV1 agonists was shown to reduce the inflammatory response. The pre-treatment of endothelial cells with capsaicin before LPS stimulation resulted in reduced production of cytokines and chemokines, decreased expression of adhesion molecules, and lowered activation of NF- κ B, a key regulator factor of the inflammatory responses.

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These suppressive effects were found to be occurring via the activation of the Ca^{2+} /PI3K/Akt pathway, the phosphorylation of endothelial nitric oxide synthase (eNOS), and increased nitric oxide (NO) production ¹²⁹. Thus, TRPV1 might be an effective channel in the phototransduction signals.

As the exact mechanism by which PBM activates the Nrf2 pathway remains unclear, we investigated whether the activation of the Nrf2 pathway by the green LED is a consequence of the light effect on TRPV1 channels. Strikingly, blocking TRPV1 by CPZ abrogated the upregulation by the green light of Nrf2 target genes, *HO-1*, *GCLC*, and *NQO1*, in DNCB-KCs, highlighting the essential role of TRPV1 in Nrf2 activation by the green light PBM. This is consistent with a recent report demonstrating that TRPV1 promotes the nuclear translocation of Nrf2, ultimately inhibiting M1 macrophage polarization and alleviating inflammation in the osteoarthritis model. The authors also highlighted that downstream events of TRPV1 activation, mainly the Ca^{2+} influx and CaMKII activation, facilitated Nrf2 nuclear translocation as ¹³⁰. These findings suggest coordinated crosstalk between TRPV1 and Nrf2 that mediates the anti-inflammatory effects of green light in KCs, highlighting the complexity of the underlying molecular mechanisms.

Interestingly, our results revealed that even without any light irradiation, Nrf2 activation by DNCB is also partially mediated by TRPV1. This finding suggests that DNCB could be a ligand for TRPV1 receptors, thereby indirectly triggering Nrf2 activation. However, a recent study demonstrated that DNCB selectively activates TRP ankyrin 1 (TRPA1) over the other tested members of the TRP channel family ¹³¹. This discrepancy may raise concerns about the non-selective activity of CPZ, which may not only block TRPV1 activity but also affect TRPA1. CPZ was recently found to activate and desensitize the TRPA1 channel, which, along with TRPV1, is involved in nociception and inflammation ¹³². Therefore, results involving CPZ should be interpreted with caution. Further experiments are needed to clarify this hypothesis, as CPZ is widely used in TRPV1 blocking models.

Conclusions

To sum up, our study sheds light on the promising anti-inflammatory potential of green light PBM therapy in the context of skin inflammation. Green light effectively suppressed pro-inflammatory cytokine expression in KCs, comparable to the effects of the anti-inflammatory agent Dex. This study is the first to provide insights into the molecular mechanisms underlying the anti-inflammatory action of green light in KCs. Interestingly, our findings highlighted the critical role of the TRPV1 channel, a well-established green light target, and the Nrf2 pathway in mediating the anti-inflammatory effects of green light in response to a chemical-stress. Importantly, we revealed a link between TRPV1 and Nrf2 in the PBM response, where Nrf2 activation by the green light appears to be dependent on TRPV1 activity. This intricate TRPV1/Nrf2 pathway contributes to the overall anti-inflammatory response of green light in KCs, making the green light a versatile tool that modulates different interconnected pathways and manages inflammation at different cellular levels. Overall, our research contributes to understanding PBM's mechanisms and opens new avenues for harmless and inexpensive therapeutic strategies targeting skin inflammatory conditions. Further investigations are needed to elucidate the specific molecular interactions and other signaling pathways in the green light regulatory network.

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Conflicts of Interest

Sara Salman was funded by Lightinderm. Audrey Rousseaud and Lieve Declercq are employees at Lightinderm and participated in these works as scientific experts. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Article 2

Supplementary data

Table S1. Oligonucleotide primer sequences used for qRT-PCR.

Gene Symbol	Gene Name	NCBI Gene Accession Number	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001357943	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
GCLC	Glutamate-cysteine ligase catalytic subunit	NM_001197115	ACATGGAAGTGGATGTGGACACC	CCTCATCCATCTGGCAACTGTCA
HO-1	Heme oxygenase-1	NM_002133	GGCCTGGCCTTCTTCACCTT	GAGGGGCTCTGGTCCTTGGT
HPRT	Hypoxanthine-guanine phosphoribosyltransferase	NM_000194	GCCAGACTTTGTTGGATTTG	CTCTCATCTTAGGCTTTGTATTTG
IL-6	Interleukin 6	NM_001371096	TCAATGAGGAGACTTGCCTG	GATGAGTTGTCATGCCTGC
IL-8	Interleukin 8 / C-X-C motif chemokine ligand 8 (cxcl8)	NM_001354840	TCTCTTGGCAGCCTTCTGA	TGGGGTGGAAAGGTTTGGAG
NQO1	NAD(P)H quinone dehydrogenase 1	NM_001025434	GGGCAAGTCCATCCCAACTG	GCAAGTCAGGAAGCCTGGA
TNF-α	Tumor necrosis factor	NM_000594	CCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG

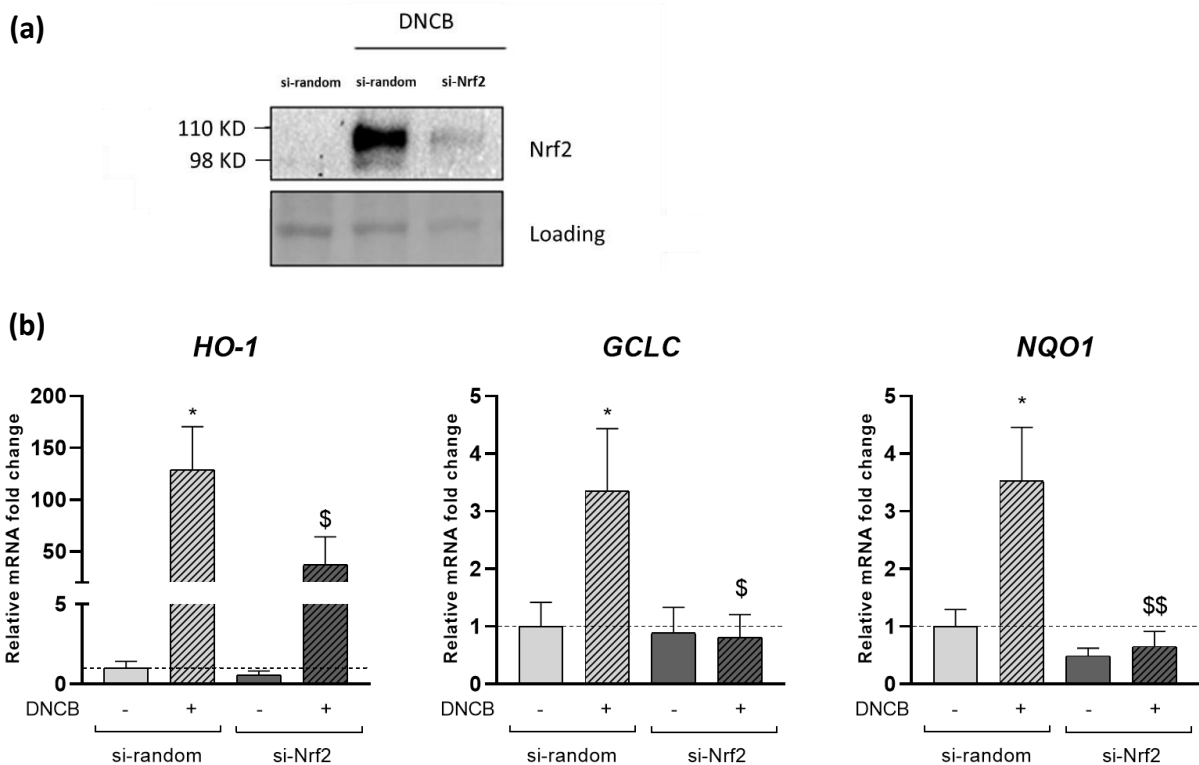


Figure S1. Nrf2 invalidation in KCs. KCs were transfected for 48 h by si-Nrf2 or si-random, then treated with DNCB. (a) Nrf2 accumulation in the knockdown model was assessed by Western blot 3 h after treatment. The blot is representative of 3 independent experiments. (b) mRNA expression of Nrf2 target gene: HO-1, GCLC, and NQO1, were assessed by RT-qPCR, 3 hours following DNCB treatment. Results are expressed as fold change compared to the untreated si-random control. Data represent mean \pm SEM of 4 independent experiments. One-Way ANOVA followed by Tukey's post hoc test, * $p < 0.05$ vs. relative vehicle control; \$ $p < 0.05$; \$\$ $p < 0.01$ vs. si-random DNCB-treated group.

DISCUSSION

GENERAL DISCUSSION

Since its discovery in the late 1960s, PBMT faced challenges in gaining acceptance among patients and the medical community. This was largely due to the lack of understanding of the photochemical and photobiological events leading to the therapeutic effect without generating noticeable adverse effects. In recent years, PBM has witnessed a substantial shift, becoming one of the fastest-growing fields in medicine, supported by an increasing body of literature demonstrating its effectiveness in tissue regeneration, pain relief, and inflammation resorption. This knowledge led to its broader application across medical domains and the development of various specialized devices.

However, there are still two fundamental points of controversy in the field of PBM. First, there is a lack of consensus on numerous dosing parameters associated with PBM, leading to variations in treatment protocols and inconsistent outcomes. When examining the literature, there is a significant disparity in exposure parameters, mainly the fluence and treatment frequency, which complicates the comparison of studies and poses challenges in interpreting the results. The difficulty in establishing a consensus persists due to several factors, mainly the biphasic response of light dependent on wavelengths and tissues and the variation in PBM tools. Furthermore, the predominance of *in vitro* studies about PBM and the challenge of extrapolating findings to clinical settings introduce additional uncertainties and contribute to the ongoing difficulties in establishing a unified approach to PBM. Secondly, despite extensive research on the mechanisms underlying PBM effects, our understanding of this therapeutic modality remains incomplete. While numerous studies have shed light on certain aspects of PBM's anti-inflammatory effects, there is still a need for a comprehensive understanding of the cellular mechanisms responsible for these beneficial effects, particularly in the skin. Therefore, the primary objective of this thesis was to investigate the cellular mechanisms driving the anti-inflammatory effects of green and red lights. By unraveling PBM mechanisms, we aim to fill the existing knowledge gaps, provide insights into the therapeutic potential of PBM in the context of skin inflammation, and potentially optimize its application as a non-invasive and effective treatment option for various inflammatory conditions.

As KCs play a central role in the initiation and regulation of the inflammatory processes associated with many skin diseases, our study primarily focused on investigating the effects of PBM

on these cells. While *in vitro* research on PBM's anti-inflammatory effects exists for various cell types, such as fibroblasts (Choi *et al.*, 2012; Lim *et al.*, 2015; Tripodi *et al.*, 2023), epithelial carcinoma cells (Fujimura *et al.*, 2014), and macrophages (Fernandes *et al.*, 2015; de Brito Sousa *et al.*, 2020; Tian *et al.*, 2023), studies specifically focusing on KCs are relatively limited. In our study, we chose to work on primary human KCs particularly. Despite the challenges associated with culturing primary KCs, they closely mirror the natural physiological responses of KCs within the epidermis, offering a more accurate representation of how PBM may affect skin inflammation in patients. Thus, our research findings can be directly relevant to dermatological conditions, avoiding biases potentially introduced when translating findings from cell lines into clinical applications.

Given the complexity of modifying multiple parameters throughout the experiments, the first part of this work focused on screening various light parameters to identify the optimal ones for our model. In our investigation, we chose to explore the mechanisms of two different wavelengths: 660 nm (red light) and 520 nm (green light). Red light is widely used in PBM protocols to treat inflammation in both clinical and experimental settings, while green light is less explored than the other wavelengths but has recently demonstrated promising anti-inflammatory properties. The initial experiments involved screening different fluences (energy densities), 0.3, 3, and 10 J/cm², by adjusting the combination of different exposure times and irradiances (power densities). Other pulse parameters, including the period (10 ms) and the duty cycle (50%), were maintained consistently with the previous research work developed by our collaborators at Lightinderm®. For our selection process, we assessed ROS production via CM-H2DCFDA and MitoSox probes, along with cell viability (data not shown). Indeed, ROS is an integral aspect of PBM's mechanisms (Hamblin, 2018). In normal cells, PBM at low fluences can generate a relatively low concentration of ROS, acting as secondary messengers and activating signaling pathways involved in crucial biological processes, like cell death/survival, proliferation, differentiation, protein synthesis, and angiogenesis. Conversely, when high fluences are delivered, typically > 10 J/cm², massive concentrations of ROS can be produced able to destroy cellular structures irreversibly and induce cell death (Huang *et al.*, 2011; Ristow, 2014; Hamblin, 2018; Le Gal *et al.*, 2021). Thus, we selected light parameters that induced ROS production without compromising the cell viability in our model, implicating that light-induced ROS concentrations were not too high and did not damage the cells. A power density (irradiance) of 12 mW/cm², an energy density (fluence) of 3 J/cm², and 250 sec of exposure were selected for light treatment with each wavelength. Our results matched the

beneficial PBM dosimetric parameters reported in the literature, usually between 1–100 mW/cm² for power density and 0.1–50 J/cm² for energy density (Markoulli *et al.*, 2021). Studies report successful tissue repair and anti-inflammatory effects from 5–50 mW/cm² at the target tissue depth (Hashmi *et al.*, 2010). De Abreu *et al.* particularly stated that favorable outcomes on KCs, including viability, proliferation, and migration, were mostly obtained in the 0.1–5.0 J/cm² range for blue, red, and NIR light. Energy densities above 15 J/cm² were ineffective or tended to cause cell death (de Abreu *et al.*, 2019).

It becomes evident that PBM has limited effectiveness when applied to normal tissue or cells. To observe its biomodulating effects, it was necessary to generate a stress-induced condition in the KC cultures, thereby simulating the pro-inflammatory microenvironment observed in many skin pathologies. In our study, we established a pro-inflammatory model by treating KCs with 25 µM DNCB, allowing us to assess the reparative potential of PBM. DNCB is an electrophilic chemical classified as an extremely potent contact sensitizer. It is a small hapten molecule (molecular weight = 202.55 g/mol) capable of conjugating proteins or other larger molecules to form sensitizing 2,4-dinitrophenol (DNP) adducts. Notably, in HaCaT cells, DNCB forms preferentially cysteine haptened peptides in the class I MHC molecules capable of priming cytotoxic T cells, a key mechanism in skin sensitization (Bailey *et al.*, 2020). DNCB is commonly used to challenge rodents' skin in CHS (El Ali *et al.*, 2013; Helou *et al.*, 2019) and AD (Hong *et al.*, 2020; Yang *et al.*, 2021) models. It has also been used in several *in vitro* studies characterizing the skin sensitization response to allergens in primary KCs or the KC cell line, HaCaT (Esser *et al.*, 2012; Kim *et al.*, 2012; Parkinson *et al.*, 2020). Our selection of DNCB was further supported by the ability of contact allergens, including DNCB, to promote a pro-inflammatory microenvironment in the skin, referred to as "xenoinflammation," mainly through ROS production by skin cells. This inflammatory response represents an early crucial event for the subsequent adaptative immune response (Martin, 2012). Notably, exposure of KCs to chemical sensitizers induced various pro-inflammatory cytokines, such as IL-1 α and IL-6. As a result, several *in vitro* skin sensitization assays using DNCB and other well-established sensitizers as positive control have proposed assessing KC inflammatory responses to chemical stimuli in order to identify potential contact sensitizers (Son *et al.*, 2013; Chung *et al.*, 2018). Our data revealed an induction in TNF- α , IL-6, IL-8, and IL-1 α production in response to DNCB, consistent with previous reports on human KCs (Pickard *et al.*, 2009; Oh *et al.*, 2019).

General discussion

Intriguingly, some references have characterized DNCB as a photosensitive chemical since it strongly absorbs light above 290 nm (PubChem, 2013; NIST Standard Reference Database, 2005). This suggests that DNCB may undergo photochemical reactions when exposed to UV or visible light, potentially leading to the degradation of the molecule and a decrease in its activity. These photochemical reactions may be influenced by the wavelength and intensity of the light (Ahmad *et al.*, 2016). While the photostability of DNCB raised concern in our study, we conducted a test experiment comparing the DNCB pro-inflammatory effect between two sets of KCs: one stimulated with DNCB that had not been subjected to any light treatment (control), and the other stimulated by DNCB that had been previously exposed to our light irradiation protocol. The results indicated no significant difference in the KC's response to DNCB, regardless of whether it had been exposed to light or not (data not shown), validating the reliability of using DNCB to investigate the effects of PBM.

PBM as a regulator of KC inflammatory response

While numerous *in vivo* studies supported the anti-inflammatory potential of PBM in the skin (Gupta *et al.*, 2015; Kim *et al.*, 2021; Wu *et al.*, 2022), our study is the first to demonstrate the direct control exerted by red and green light over the primary KC inflammatory response to chemical stress. Only a limited number of studies in the literature have evaluated the effects of PBM on KCs, and these studies have reported conflicting results. A study investigating the effect of NIR on HaCaT cells showed that a NIR 1064 nm laser (at 4 and 8 J/cm²) reduced yeast invasiveness in KCs infected with *C. albicans*, downregulated *IL-8* and *TNF- α* expression, and enhanced antimicrobial defense by inducing the expression of the Human β defensin-2 (Baroni *et al.*, 2018). However, mRNA expression in the mentioned study was assessed 24 hours after light treatment, reflecting secondary effects from other cellular processes rather than a direct impact of light, as observed in our 3-hour assessment, adding further complexity in assessing PBM effects. Conversely, other reports investigating red light or NIR effects on HaCat cells reported increased or unmodified pro-inflammatory outcomes (Yu *et al.*, 1996; Kim *et al.*, 2016; De Filippis *et al.*, 2019). These findings may be attributed to the differing conditions under which PBM was tested, as the assessments in these studies were conducted under normal, non-stressed conditions, in contrast to our study, where DNCB was used to challenge the cells. This highlights a fundamental aspect of PBM, which can have opposing effects based on whether cells are healthy or stressed.

Since Nrf2 was previously demonstrated as a key regulator of skin inflammation, we evaluated the implication of this factor in the anti-inflammatory activity induced by red and green light. Our findings indicated that both wavelengths triggered the activation of the Nrf2 pathway. They enhanced Nrf2 stabilization and Nrf2 transcriptional activity by binding to ARE sequences, leading to increased Nrf2 target antioxidant gene expression, such as *HO-1* and *GCLC*, compared to DNCB-treated KCs. Although green light effectively induced Nrf2 accumulation, it failed to significantly upregulate the expression of *NQO1* among other target genes, contrary to red light. This difference in the target genes transcription profiles suggests that these wavelengths may differentially activate the Nrf2 signaling or trigger distinct pathways that could interfere with Nrf2's activity (Edamitsu *et al.*, 2022). Although some studies reported an increase in Nrf2 pathway activation in response to blue or NIR light (Patel *et al.*, 2014; Trotter *et al.*, 2017; Yadav *et al.*, 2020), none have explored the involvement of Nrf2 in the observed PBM anti-inflammatory responses. To address the role played by Nrf2 in PBM, we invalidated Nrf2 in the primary KCs using small interference RNA (siRNA). A significant decrease in Nrf2 accumulation and mRNA expression of the three assessed target genes, *HO-1*, *GCLC*, and *NQO1*, was observed in the invalidated KCs. The inhibition rates of Nrf2 accumulation and target gene expression were correlated with literature that used similar techniques in primary KCs (Marrot *et al.*, 2008; Jeayeng *et al.*, 2017). Interestingly, our study provides novel insights, being the first to report that Nrf2 is an essential factor for controlling KC inflammatory response when exposed to red and green light. Indeed, in the absence of Nrf2, the illumination of KCs failed to reduce the *TNF- α* , *IL-6*, *IL-8*, and *IL-1 α* production in response to DNCB.

The exact mechanism by which PBM activates the Nrf2 pathway remains unclear. To our knowledge, the Nrf2-Keap1 complex does not contain a chromophore group for direct light absorption. We proposed an indirect activation of Nrf2, possibly through the modulation of ROS levels, given that Keap1 is a sensor of the oxidant stress. However, measurements of H₂O₂ levels in our study, at 10 min and 30 min after light exposure, did not show noticeable changes in DNCB-treated KCs (Figure 26). This may indicate that other pathways may be involved in Nrf2 activation, or relying on H₂O₂ as the sole indicator of ROS levels may not fully capture the overall cellular ROS dynamics, especially considering that PBM generates superoxide anion as a major ROS species due to its mitochondrial chromophore, CCO. Apart from disturbing the Nrf2–Keap1 complex through ROS interaction, Nrf2 activation by light may involve crucial phosphorylation steps in the cytoplasm. This stabilization step can be mediated by multiple kinases, including protein kinase C,

phosphatidylinositol-3-kinase (PI3K), and MAPKs. Interestingly, PBM has been shown to modulate the phosphorylation of key kinases in the MAPK signaling pathway, including p38, JNK, and ERK 1/2

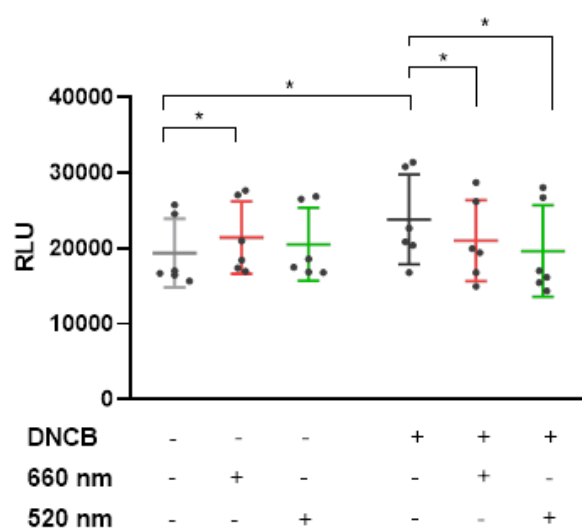


Figure 26. H₂O₂ levels in DNCB-stimulated KCs 30 min after light exposure. Cells were seeded at 100 000 cells/70μl. ROS-Glo™ H2O2 luminescence assay (Promega, USA) was used to analyze oxidative stress in cells 30 min post-exposure to different treatment conditions. A derivatized luciferin substrate is incubated with the sample and reacts directly with H₂O₂ to generate a luciferin precursor. The final luminescence formed by conversion to luciferin was measured using a spectrophotometer (Tristar®, Berthold Technologies). The light signal is proportional to the level of H₂O₂ present in the sample. Results were represented as RLU (Relative Luminescence Unit). Reported data are mean ± SEM of 6 independent experiments. Two-Way ANOVA followed by Tukey post-hoc test, * p < 0.05.

(Choi *et al.*, 2012; Silveira *et al.*, 2016), while also activating the PI3K/AKT pathway (Jere *et al.*, 2022), decreasing pro-inflammatory mediators and enhancing therapeutic activities like wound healing.

Another hypothesis for Nrf2 activation by the light considers the involvement of the AhR pathway since it has been proposed as a promising target candidate for PBM (Figure 27). In a gene expression profiling study, Becker *et al.* particularly observed the induction of AhR battery genes, including CYPs, upon blue light irradiation of HaCaT cells (Becker *et al.*, 2016). This observation suggests that AhR could serve as a potential target for PBM, possibly activated by endogenous ligands generated during the photolytic destruction or photo-oxidation of tryptophan by UV or visible light exposure (Oberg *et al.*, 2005; Diani-Moore *et al.*, 2011; Becker *et al.*, 2016). AhR transcription factor is primarily known for mediating xenobiotic detoxification enzymes, such as CYPs, and playing a role in the expression of the epidermal differentiation complex genes, including filaggrin and the late cornified envelope protein genes (loricrin, involucrin) (Fernández-Gallego *et al.*, 2021). Two different mechanisms probably account for activation of Nrf2 by AhR (Dietrich, 2016): direct upregulation of Nrf2 transcription by AhR, demonstrated for the first time by Miao *et*

al. (Miao *et al.*, 2005), and indirect Nrf2 activation, possibly through ROS generated by CYPs activity (Haarmann-Stemmann *et al.*, 2012; Edamitsu *et al.*, 2022). Some reports even propose that tryptophan-derived metabolites can induce conformational changes in Keap1, leading to Nrf2 dissociation and subsequent nuclear translocation, enhancing cellular defense mechanisms (Xu *et al.*, 2018). Furthermore, AhR pathway can be suggested as another key player contributing to the PBM anti-inflammatory effect (Figure 27). AhR has been implicated in skin inflammatory diseases as a critical regulator of the balance between proinflammatory/ autoimmune and immunosuppressive/ tolerogenic responses depending on the cellular microenvironment and the presence of specific ligands (Fernández-Gallego *et al.*, 2021; Szelest *et al.*, 2021). Interestingly, administration of 6-formylindolo [3,2-b] carbazole (FICZ), a photo-oxidized tryptophan metabolites and ligand of AhR, in the imiquimod model results in attenuated psoriasiform skin inflammation, with reduced expression of the proinflammatory mediators IL-17 and IL-22 (Di Meglio *et al.*, 2014), showing the potential anti-inflammatory role of AhR in the skin.

Another potential factor that may contribute to the anti-inflammatory effect exerted by PBM is the NF- κ B pathway (Figure 27). It is a redox-sensitive inflammatory pathway that regulates multiple aspects of innate and adaptive immune functions and is an essential mediator for pro-inflammatory gene expression and inflammasome regulation (Liu *et al.*, 2017). PBM has been shown to inhibit the expression and activation of NF- κ B in different models, thereby preventing the expression of pro-inflammatory genes (Curra *et al.*, 2015; Gupta *et al.*, 2015; Kim *et al.*, 2016). Furthermore, it is well-established that Nrf2 and NF- κ B signaling pathways interact via multiple mechanisms to regulate inflammation in response to environmental stressors (Vallion and Kerdine-Römer, 2022). Nrf2 can indirectly suppress inflammation by inhibiting NF- κ B, either by preventing I κ B- α proteasomal degradation inhibiting NF- κ B nuclear translocation or by competitive binding between Nrf2 and I κ B kinase (IKK) with Keap1 resulting in IKK degradation and NF- κ B inhibition (Tian *et al.*, 2012).

As the TRPV1 cation channel has been identified as a target activated by the green light (Gu *et al.*, 2012; Wang *et al.*, 2014; Wang *et al.*, 2017b), we thought that it could also be another mediator implicated in PBM anti-inflammatory response. Primarily known for its role in pain and itch perception, temperature sensitivity, and epidermal homeostasis, TRPV1 has been recently recognized as a regulator of inflammation (Ho and Lee, 2015; Caterina and Pang, 2016; Gouin *et al.*,

2017). Our study investigated the role of the TRPV1 channel in the therapeutic response to green light. The selective inhibition of TRPV1 channel by CPZ prevented the green light from effectively controlling pro-inflammatory cytokine expression in stimulated KCs. This result highlights the pivotal role of TRPV1 in mediating the anti-inflammatory response of green light in the DNCB-stimulated model (Figure 27). Further assessment of cytokine production in KC supernatants by ELISA or a multiplex assay would provide additional support for this finding.

The role of TRPV1 in inflammation has always been a topic of debate. Historically, TRPV1 activation was associated with the initiation of inflammatory responses, with studies demonstrating increased production of pro-inflammatory mediators in KCs upon TRPV1 activation by triggers, including through blue light irradiation (Southall *et al.*, 2003; Yoo *et al.*, 2020). In skin diseases like AD and psoriasis, inhibiting or desensitizing the TRPV1 pathway alleviated the inflammatory response and pruritis associated with these disorders (Chen *et al.*, 2021; Park *et al.*, 2022). However, our results align with the other part of the literature revealing potential anti-inflammatory and protective properties of TRPV1, particularly in ACD (Bánvölgyi *et al.*, 2005; Feng *et al.*, 2017) and chemical-induced burn skin reactions (Li *et al.*, 2012). Interestingly, a study supporting our result on TRPV1's role in PBM response showed that red LED (660 nm) elicited an anti-inflammatory effect in acute chemical-induced nociceptive models, and the authors suggested this effect to be mediated by TRPV1 and other TRP channels, such as TRPA1, TRPM8 (Pigatto *et al.*, 2019). The observed anti-inflammatory effect of TRPV1 *in vivo* could be attributed to the role played by TRPV1 signaling in suppressing the activation of key immune cells involved in the inflammatory response, such as dendritic cells and macrophages (Kim *et al.*, 2003; Tóth *et al.*, 2009). The discrepancy in the TRPV1 role in inflammation suggests that this channel may exhibit different actions depending on the cell type, the context, the trigger, and the inhibitor tool used for investigation (e.g., knockout mice, desensitization, or pharmacological antagonism). Further research on specific types of cells is needed to elucidate the distinct functions of epithelial and non-epithelial TRPV1 in different contexts.

The mechanism by which green light activates TRPV1, whether directly or indirectly, remains unclear. Direct activation by light could involve the photoisomerization of TRPV chromophores, while indirect activation may occur as a subsequent event for opsins (OPNs) activation. OPNs are a light-sensitive G-protein receptor family identified as photoreceptors for green and violet-blue photons (Van Tran *et al.*, 2021). Notably, human KCs express different types of light absorbing OPNs,

including cone opsin (OPN1), rhodopsin (OPN2), encephalopsin (OPN3), and peropsin (RRH) (Suh *et al.*, 2020). Most of these OPNs bind to 11-cis-retinal as a chromophore, which undergoes photoisomerization to the all-trans configuration upon photon absorption. The structural change of these chromophores promotes G-proteins activation and initiation of two main signaling pathways involving the cAMP and phosphatidylinositol pathways (Sharma *et al.*, 2023). These two pathways can subsequently influence various biological processes (Kim *et al.*, 2013; Castellano-Pellicena *et al.*, 2019). One proposed downstream target modulated by OPN activation is TRPV1, although the signaling driving this OPN-TRPV1 interaction remains elusive.

TRPV1 is a transmembrane non-selective cation channel with a high permeability to Ca^{2+} once activated. The calcium influx into the intracellular space acts as a second messenger, initiating several downstream signaling pathways, such as PKC, calmodulin-dependent protein kinase-II (CaMKII), and calcineurin/nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) signaling, thus contributing to the regulation of inflammatory genes and modulating various biological responses, including cell proliferation (Ho and Lee, 2015; Gouin *et al.*, 2017b; Xiao *et al.*, 2023). For instance, Ca^{2+} /PI3K/AKT pathway following the TRPV1 activation in endothelial cells was shown to play a role in reducing their inflammatory response (Wang, Cui, *et al.*, 2017). As TRPV1 could be an effective channel in the phototransduction signals, we aimed to investigate the role of TRPV1 in the Nrf2 pathway activation upon green light exposure. Strikingly, blocking TRPV1 by CPZ abrogated the upregulation by the green light of Nrf2 target genes, *HO-1*, *GCLC*, and *NQO1*, in DNCB-treated KCs, highlighting the essential role of TRPV1 as an inducer of Nrf2 signaling in response to green PBM. These findings suggest a coordinated interaction between TRPV1 and Nrf2 that mediates the anti-inflammatory effects of green light in KCs. This is consistent with a recent report demonstrating that TRPV1 promotes the nuclear translocation of Nrf2, ultimately inhibiting M1 macrophage polarization and alleviating inflammation in the osteoarthritis model. The authors also highlighted that downstream event of TRPV1 activation, mainly the Ca^{2+} influx and CaMKII activation, facilitated Nrf2 nuclear translocation (Lv *et al.*, 2021). Future research should focus on the analysis of cytosolic and nuclear Nrf2 protein levels while blocking TRPV1 or chelating Ca^{2+} to confirm whether TRPV1 and calcium influx regulate Nrf2 translocation in the context of green light.

Interestingly, our results revealed that even without light exposure, TRPV1 also plays a role in Nrf2 pathway induction following DNCB treatment. This finding suggests that DNCB could potentially trigger TRPV1 receptors, thereby indirectly inducing the Nrf2 pathway (Figure 27). However, a recent study demonstrated that DNCB selectively activates TRP ankyrin 1 (TRPA1) over the other tested members of the TRP channel family (Wu *et al.*, 2021). This controversy may raise concerns about the non-selective activity of CPZ, which may not only block TRPV1 activity but also affect TRPA1. CPZ was recently found to activate and desensitize the TRPA1 channel, which, along with TRPV1, is involved in nociception and inflammation (Kistner *et al.*, 2016). Therefore, results involving CPZ should be interpreted with caution. Further experiments are needed to clarify this hypothesis, as CPZ is widely used in TRPV1 blocking models.

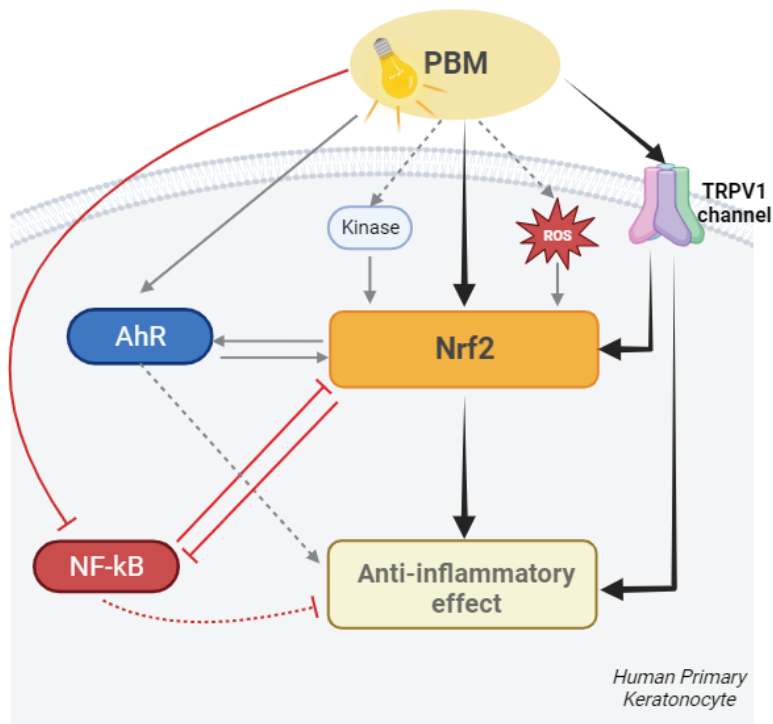


Figure 27. Mechanisms of PBM-induced anti-inflammatory effect in keratinocytes: roles of Nrf2, TRPV1, and beyond. This scheme illustrates the complex interplay of molecular pathways contributing to the anti-inflammatory effect of PBM in keratinocytes. PBM activates the Nrf2 pathway, potentially through indirect pathways that could involve modulation of ROS levels or the phosphorylation by critical kinases in the cytoplasm, such as MAPKs or PI3K. TRPV1 cation channel emerges as a pivotal player, activated by green light and potentially inducing Nrf2 signaling, ultimately enhancing the anti-inflammatory response. Additionally, PBM was demonstrated to activate AhR signaling, possibly through tryptophan photo-oxidation, offering an alternative route that could contribute to the PBM anti-inflammatory. AhR could also directly or indirectly upregulate Nrf2 activation, thereby enhancing the anti-inflammatory activity. Simultaneously, PBM was shown to inhibit the NF- κ B, a key inflammatory factor. Whenever Nrf2 predominates, it further suppresses the NF- κ B pathway, potentializing the PBM beneficial response. The cross-talks Nrf2-NF- κ B and Nrf2-AhR further highlight the complexity of these mechanisms contributing to the therapeutic potential of PBM in skin inflammation. Arrows indicate whether the mechanisms are demonstrated in our study (Black Bold Arrows), described in the literature (Filled Arrows), or mechanisms not yet described (Dash Arrows).

PBM as an immunomodulatory tool to treat skin inflammation

As KCs interact with other key cells in the epidermis, such as LCs, we extended our research to explore how PBM regulates the cutaneous microenvironment by impacting LCs, a key player in skin immunity. LCs act as sentinel cells crucial for cutaneous immunosurveillance, as they are activated by various signals, including the detection of danger signals released by stressed or damaged cells (DAMPs) or from invading microorganisms (PAMPs). Upon activation, LCs undergo maturation, which involves changes in their morphology and surface marker expression. Mature LCs then migrate from the epidermis to regional lymph nodes, where they present antigens to T cells, initiating an appropriate adaptive immune response (immunogenic or tolerogenic) specific to an antigen. LCs also play a role in skin inflammation by releasing pro-inflammatory cytokines and chemokines, which recruit immune cells to the site of inflammation. In our study, we evaluated the effects of red light (660 nm) on LC activation and migration in human skin explants exposed topically to DNCB (0.25%). Skin explants provide a more physiologically relevant model than the 2D cultures, preserving tissue complexity and cell interactions (Lebonvallet *et al.*, 2010).

Using the langerin immunostaining at 24 hours post-treatment, we observed that DNCB induced rounded constricted LCs with fewer dendrites and promoted their migration from the epidermis into the dermis, indicating activation of LCs. Interestingly, the exposure of skin explants to red light preserved LCs star-shaped shape with long branches and prevented the DNCB-induced LC migration. It also decreased the epidermal expression of TNF- α in the epidermis and tended to regulate the level of some pro-inflammatory cytokine secretion. Thus, this result provides evidence for the immunomodulatory effect of PBM that might contribute to its anti-inflammatory activity and prevent allergen sensitization in atopic or allergic dermatitis. Many studies have reported an anti-inflammatory response associated with an immunosuppressing effect of red or NIR PBM by modulating monocytes and macrophages polarization and preventing DC activation (Chen *et al.*, 2009; Chen *et al.*, 2011b; Fernandes *et al.*, 2015; Trotter *et al.*, 2017; de Brito Sousa *et al.*, 2020; Tian *et al.*, 2023). It is important to note that a number of differing protocols were used in these studies regarding the amount, time, and frequency of application, making a comparison of results difficult, especially that higher energy densities (e.g., NIR at 90 J/cm²) were shown to modulate immune response *in vivo* by activating LCs (C.-H. Lee *et al.*, 2016). A previous report showed that a single exposure of mouse skin to NIR (30 J/cm²) reversibly suppressed the number of LCs that became round with shortened dendrites, contrary to our observations. However, the reduced

density of LCs was correlated to a failure of the skin to induce a CHS reaction, suggesting that NIR induced local immunosuppression, probably by initiating an antigen-specific Treg response (Danno and Sugie, 1996).

Although these studies support the ability of PBM to modulate LC response, they were mainly *in vitro* or *in vivo* (rat models), making the results difficult to extrapolate into real human skin. To our knowledge, our study is the first to address the modulatory role of PBM on LCs in human skin explants. However, future research should strengthen our data by deeply characterizing LC cell surface markers and secretion profile in response to PBM and extending the investigation to other key immune cells in the skin, such as dDCs and macrophages, to have more insights into the immunomodulatory mechanisms of PBM, and better address its clinical application in inflammatory skin conditions (e.g., allergic, auto-immune diseases, wound healing). Additionally, we suggest studies following repeated light treatments with a long-term follow up, as some reports pointed on a difference in immune response following PBM between the early and the late time points.

The mechanisms by which red light regulates LC activation are not fully elucidated. However, relying on our earlier mentioned results regarding PBM effects on KCs, we suggest that red light may indirectly suppress LC activation by controlling cytokine release in the skin microenvironment, particularly by KCs, as KCs-LCs crosstalk is essential for programming LCs response (Clayton *et al.*, 2017; West and Bennett, 2017; Yan *et al.*, 2020). It is not excluded that PBM may also alter the expression of surface molecules of LCs by a direct mechanism. Further research is necessary to explore the exact mechanisms driving the effects of PBM on LC activation and to describe the phenotype of LCs in response to PBM to discuss its potential therapeutic applications.

In this study, the immunostaining of Nrf2 and HO-1 in skin explants 24 hours following light treatment revealed a decrease in their expression compared to DNCB-treated conditions. Since this observation was associated with reduced TNF- α levels in the epidermis at the same time point, it may indicate a decreased inflammation and subsequently, reduced mobilization of Nrf2 at 24 h. Thus, given the rapid and brief aspects of PBM responses, this result could potentially be attributed to the late timing of assessment of the Nrf2 pathway, and it is advisable to conduct an early-phase investigation to capture the dynamic changes in Nrf2 activation in the skin.

In the context of our study, it is essential to acknowledge that the effects of PBM differ significantly from natural sunlight. While sunlight may offer some benefits for certain superficial

conditions, known as heliotherapy, several critical distinctions exist. Sunlight's broad spectrum has a peak emission in the green wavelengths (around 500 nm) and gradually decreases in the red and NIR regions, which, while potentially beneficial for superficial issues, does not penetrate deeply into the skin. Additionally, natural sunlight contains a fair amount of harmful UV radiation, so if one exposes himself for a long enough time to get sufficient photons of the correct wavelength to the target tissue, it would likely result in severe sunburn. These distinctions emphasize the need for precise and controlled light therapy like PBM in clinical applications.

The primary advantage of PBM in dermatology is its non-invasive and non-thermal aspect. LED or laser light in the range of 500 to 940 nm are generally considered safe for human exposure, with no or minimal reported adverse effects, such as mild tingling. In contrast, blue light within the 400 to 500 nm wavelength range, in particular violet spectrum (400-440 nm), can potentially have cytotoxic risks to the skin, retina, and various human tissues, a phenomenon commonly referred to as the "blue light hazard" (Van Tran *et al.*, 2021). This cytotoxicity usually occurs at high fluences ($> 50 \text{ J/cm}^2$), which are less commonly used in practice, and mainly results from a massive ROS production in the cell or tissue. Precautions in PBMT should be taken for epileptic patients as certain pulsed lasers or LEDs can trigger seizures. Additionally, it is not recommended to directly expose a developing fetus to light during pregnancy, as the consequences are not documented. It is also advisable to avoid treating areas with primary carcinoma or secondary metastases unless the patient is undergoing chemotherapy. These aspects make PBM a non-invasive therapy that could be safely used as alternative or adjunctive approach to treat skin inflammation.

**CONCLUSION
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PERSPECTIVES**

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Our comprehensive research into the anti-inflammatory mechanisms of red (660 nm) and green (520 nm) light shed light on the promising potential of PBM as a therapy for effectively addressing skin inflammation. Our study highlights the impact of PBM on different key skin cells, with a particular focus on KCs and LCs.

Our investigation reveals that PBM, specifically red and green light, exerts a direct and significant anti-inflammatory effect on KCs. This effect is evidenced by the reduction in the expression of pro-inflammatory mediators. Notably, PBM's response varies depending on the cellular microenvironment, with the most pronounced beneficial effects observed in stressed conditions, such as in our case with DNCB treatment. This result underscores the therapeutic potential of PBM in conditions characterized by challenges like chemical stress. More research should explore the anti-inflammatory potential of PBM in KCs under other pathological scenarios, such as oxidative conditions, decreased pH, hypoxia, or wounding, as literature exploring KC response to PBM remains limited. Furthermore, PBM's ability to regulate cytokine expression, including key players like *IL-6*, *IL-8*, and *TNF- α* , highlights its potential in managing chronic inflammatory skin diseases such as psoriasis and AD (Bernard *et al.*, 2012). By modulating these cytokines, PBM can shape the intensity and duration of the inflammatory response, offering possibilities for altering the course of these diseases.

Our research into the molecular mechanisms of PBM highlights, for the first time, the central role of Nrf2 pathway in the PBM anti-inflammatory response in KCs. Both red and green light stimulate Nrf2 activation, resulting in the upregulating Nrf2 antioxidant and cytoprotective target genes, including HO-1, GCLC, and NQO1. Importantly, the absence of Nrf2 abolishes PBM anti-inflammatory effects, underscoring Nrf2's pivotal role. It will be interesting to address the contribution of Nrf2 in the cutaneous anti-inflammatory effect of PBM using Nrf2 Knockout animal models that consider the interaction of all skin populations. Nonetheless, understanding how PBM could activate Nrf2 pathway remains a research challenge. We suggest an indirect activation of this pathway by light. Thus, future studies should focus on comparing various ROS species and time points to understand their role in the Nrf2 activation by light, given that ROS are common modulators of Keap1. This assessment can be achieved using tools beyond H₂O₂ measurement and

antioxidants such as N-acetylcysteine. Additionally, it will be interesting to investigate the role of the AhR pathway in Nrf2 activation following light exposure by inhibiting AhR activity since AhR was proposed as a potential PBM target. In addition to investigating the Nrf2 pathway, other pathways potentially influencing the anti-inflammatory response induced by PBM need to be explored. The NF- κ B pathway, for example, represents an intriguing area of investigation, as it may interact or compete with Nrf2 activity. Furthermore, studying the activation of key kinases such as p38, JNK, and ERK 1/2 and assessing their role using kinase inhibitors could provide valuable insights into the underlying signaling pathways involved in PBM activity.

Investigating the role of TRPV1 channel via its antagonist, CPZ has shown that the TRPV1 plays a crucial role in the anti-inflammatory response induced by green light in DNCB-stimulated KCs. However, the specificity of this antagonist is still debatable. For this reason, other pharmacological inhibitors or gene inhibition techniques to study the role of TRPV1 in the DNCB-induced inflammatory response should be considered. Our study also highlights a coordinated interaction between TRPV1 and Nrf2, facilitating the anti-inflammatory response of green light. The mechanism by which green light activates TRPV1, whether directly or indirectly, remains unclear. It could involve the photoisomerization of TRPV1 chromophores or downstream signaling through light-sensitive opsins (OPNs) expressed in human KCs. Further research is needed to clarify the signaling pathways underlying the OPN-TRPV1 interaction.

While previous studies have reported the immunosuppressive potential of red or NIR PBM in various models, our study stands out as the first to explore the modulatory role of PBM on LCs in human skin explants. Our research revealed that red light preserves LC morphology and prevents their migration in response to DNCB treatment in human skin explants. This suggests an immunomodulatory role for PBM, which could prevent sensitization to allergens in conditions such as ACD. The mechanisms through which red light regulates LC activation are not fully understood. It is hypothesized that red light suppresses LC maturation and migration directly, or indirectly by modulating the skin microenvironment and cytokine release, particularly by KCs, which interact with LCs to shape the immune response. Further investigations are required to uncover the exact mechanisms of PBM on LC activation and phenotype and understand the kinetics and the order by which skin cells respond to light. Future research should strengthen these findings by characterizing LC surface markers and secretion profiles in response to PBM and extending investigations to other key immune cells in the skin, such as dendritic cells and macrophages. Additionally, as we assessed

LCs at the time point of 24 hours following a single light exposure of the explants, long-term studies following repeated light treatments are needed to understand the differences in immune responses at various time points and provide insights into the application of PBM in inflammatory skin conditions, autoimmune diseases, and wound healing.

Collectively, our comprehensive research into the anti-inflammatory effects of red and green light reveals mechanisms of PBMT in skin inflammation. It underscores the complexities of PBM action in the skin and highlights the multifaceted aspect of this therapy as an approach acting on various cellular targets, including KCs and LCs, and different molecular levels, including Nrf2 pathway and TRPV1 channel, to suppress inflammation (Figure 28). This understanding is pivotal not only for addressing doubts surrounding PBM, but also for establishing a robust groundwork for its use in clinical cosmetic or dermatological practices treating a wide range of inflammatory skin-related concerns. It further opens doors to identifying patient populations that can derive maximum benefit from this therapy, enabling tailored treatment plans and improved patient outcomes.

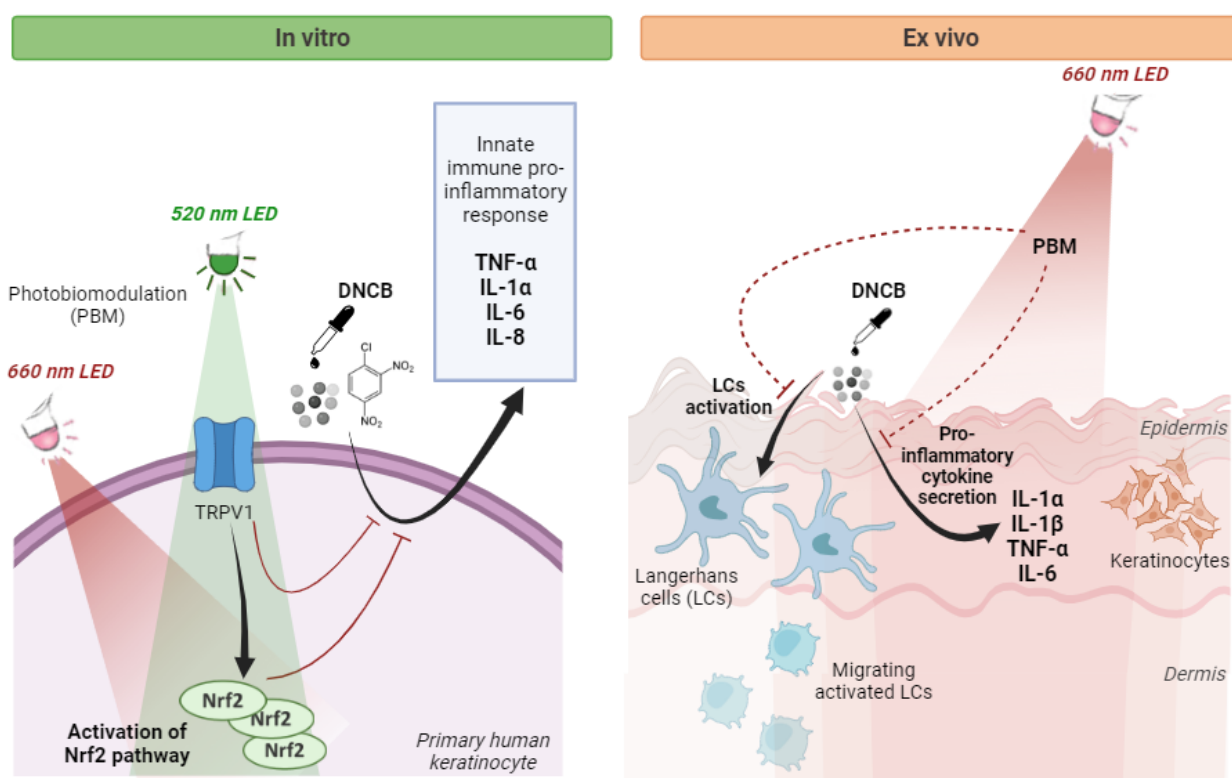


Figure 28. Red and green light anti-inflammatory proposed mechanisms in the skin: from *in vitro* (primary human keratinocytes) to *ex vivo* (human skin explants) studies. In primary human keratinocytes, exposure to green light (520 nm) or red light (660 nm) following stimulation with 2,4-dinitrochlorobenzene (DNCB) resulted in the downregulation of pro-inflammatory cytokines, including IL-6, IL-8, TNF- α . This response was attributed to the activation of Nrf2 pathway. Green light was found to exert its anti-inflammatory effects by modulating TRPV1 channels, which in turn contribute to the Nrf2 activation in keratinocytes. In human skin explants challenged with DNCB, red light was shown to regulate pro-inflammatory cytokine secretion

Conclusion & perspectives

associated with an attenuation of Langerhans cells (LCs) activation and migration, further emphasizing the translational potential of photobiomodulation (PBM). This comprehensive representation underscores the diverse and interconnected mechanisms underlying the anti-inflammatory effects of red and green light, offering insights into the potential therapeutic applications of photobiomodulation in managing skin inflammation.

As we conclude this study, it is worth acknowledging the advancements within the field of PBM. Innovations such as converting UV light into visible light through the application on the skin of spirulina and fluorescent zinc oxide, inspired by the concept of photosynthesis, show the ever-expanding possibilities of using light's therapeutic potential safely and efficiently. Moreover, the development of Light-activated CO releasing molecules (photoCORM), which release carbon monoxide upon exposure to visible light, holds significant promise for both anti-cancer and anti-inflammatory applications (Kottelat and Fabio, 2017).

As wavelengths used in PBM have varying penetration depths into the skin targeting distinct cell populations, and they interact with different chromophores, potentially resulting in different therapeutic mechanisms and effects (de Freitas and Hamblin, 2016; Rupel *et al.*, 2018; Fuchs *et al.*, 2021), combining multiple wavelengths of light in PBM protocols allows for simultaneous targeting of diverse skin layers and cell populations. This approach allows for enhanced therapeutic outcomes to effectively treat skin diseases that usually involve different types of cells in their pathogenesis (Lima *et al.*, 2020; Priyadarshi *et al.*, 2023). Coupling light therapy with serums containing active ingredients is an innovative strategy in dermatological practice to optimize PBM effects. This strategy involves combining antioxidant components or photo-activated molecules, among others, with light therapy, seeking synergistic interactions between the light and serum ingredients to address specific skin concerns like acne, collagen stimulation, and post-procedure care (Guermontprez *et al.*, 2020). It can also be facilitated through at-home skincare devices. Exploring various wavelengths and combinations of light with ingredients further expands the horizons of PBMT as a safe non-invasive approach for addressing skin inflammation in dermatology.

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APPENDIX

APPENDIX

Annex 1: Article published in *Frontiers in Toxicology* (2023).

Ethylhexadecyldimethylammonium bromide, a quaternary ammonium compound, controls inflammatory response through NRF2 pathway in a human immortalized keratinocyte cell line

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Ethylhexadecyldimethylammonium bromide, a quaternary ammonium compound, controls inflammatory response through NRF2 pathway in a human immortalized keratinocyte cell line

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Many everyday products contain quaternary ammonium compounds (QAC) and some of them are known to be skin irritants such as benzalkonium chloride. Others, such as didecyldimethylammonium chloride, have been shown to cause allergic contact dermatitis. Ethylhexadecyldimethylammonium bromide (EHD) is a QAC for which sensitization potential is not clearly known. Therefore, we have studied its mechanism in human keratinocytes (KC), the main cells of the epidermis. We used the well-described human KC cell line KERTr exposed to EHD, cinnamaldehyde (CinA), a well-known skin sensitizer, and a mixture of both. Since chemical sensitizers are known to activate the transcription factor nuclear factor (erythroid-derived 2)-like 2 (NRF2), leading to cellular detoxification and suppressed proinflammatory cytokines, protein or mRNA expression of NRF2 pathway-related enzymes and pro-inflammatory cytokines were investigated by Western blot and RT-qPCR. The activity of the NRF2 pathway on inflammation was studied by RT-qPCR in NRF2-invalidated KERTr cells. We showed that EHD cannot induce the NRF2 pathway, unlike contact sensitizers like CinA. EHD triggers an inflammatory response by inducing the mRNA expression of pro-inflammatory cytokines such as IL-1 β or IL-6. Moreover, mixing EHD and CinA inhibits the effect of CinA on NRF2 expression and mitigates the inflammatory response induced by EHD alone. EHD treatment of KERTr cells in which NRF2 has been invalidated showed an exacerbation of the inflammatory response at the transcriptional level. Hence, EHD may elicit an inflammatory response in KC *via* the NF- κ B pathway, which could lead to irritation when applied to the skin. This inflammation is negatively controlled by the basal activity of the NRF2 pathway.

KEYWORDS

quaternary ammonium, irritation, sensitization, Nrf2, skin inflammation, ethylhexadecyldimethylammonium bromide, keratinocyte, NF- κ B

Abbreviations: ACD, allergic contact dermatitis; CinA, cinnamaldehyde; CV, cell viability; DAMP, damage-associated molecular patterns; DC, dendritic cells; EHD, ethylhexadecyldimethylammonium bromide; KC, Keratinocytes; NMBA, Neuromuscular blocking agents; NRF2, nuclear factor (erythroid-derived 2)-like 2; QAC, quaternary ammonium compounds; ROS, reactive oxygen species.

Introduction

Quaternary ammonium compounds (QAC) are synthetic chemicals commonly found in many products such as cosmetics (*e.g.*, facial cleansers, Sun protection creams or lotions, baby lotions, moisturizers, hair conditioners, hair color, make-up) and biocides due to their antimicrobial, surfactant, and preservative properties. They contain at least one NH_4^+ group linked to a hydrocarbon chain and three other radicals, *e.g.*, methyl or benzyl groups (Buffet-Bataillon et al., 2012). Benzalkonium chloride¹ is the most investigated QAC and is recognized as a strong skin irritant. It may also act as an allergen since it has been implied in some hypersensitivity reactions, including occupational asthma and skin-related symptoms (Bernstein et al., 1994). Whereas sensitization to benzalkonium chloride can occur, it remains quite rare (Dao et al., 2012). Didecylmethylammonium chloride² (DDAC) is a more recent QAC that has been identified as a triggering event of skin inflammation such as allergic contact dermatitis (ACD) (Dejobert et al., 1997; Mowitz and Pontén, 2015).

ACD is a common disease mediated by T-cell. The sensitization phase is induced by contact sensitizers, low molecular weight compounds called haptens. They penetrate the epidermis and form an immunogenic complex with a skin protein (Karlberg et al., 2008). This complex induces an innate immune response in the skin by activating dendritic cells (DC), which have an essential role in initiating the antigen-specific primary immune response (Martin, 2012). They migrate from the skin to the draining lymph nodes, where they present the haptenized protein to T-cell leading to an adaptive response (Karlberg et al., 2008).

Keratinocytes (KC) constitute the major cellular population in the epidermis (Kanitakis, 2001). They respond to inflammatory stimuli through a wide variety of cytokines and pathogen-associated molecular pattern (PAMP) receptors. These cells are also a source of many inflammatory mediators which could initiate a skin inflammatory response and promote the recruitment of inflammatory cells such as neutrophils in the skin (Jiang et al., 2020). After exposure to contact sensitizer, KC release danger signals such as pro-inflammatory cytokines. These signals can be recognized by receptors on the surface of innate immune cells, such as Toll-like receptors (TLR) (Juráňová et al., 2017). Then, some cytokines such as interleukine-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) produced by KC have a role in the maturation and migration of DC during the sensitization phase (Cumberbatch et al., 1997; 2001). Haptens have been shown to induce the NF- κ B (Nuclear factor kappa-light-chain-enhancer of activated B-cell) pathway involved in inflammation (Tak and Firestein, 2001; Christensen and Haase, 2012). Under physiologic conditions, NF- κ B is a complex composed of the proteins p65 and p50. It is sequestered in the cytoplasm by its inhibitor, I κ B. After stimulation of TLR by, *e.g.*, pathogens, I κ B is phosphorylated, ubiquitinated and degraded by the proteasome (Juráňová et al., 2017). Then, p65 is phosphorylated (Zhong et al., 1997). These events lead to the translocation of the complex into the nucleus,

where it notably induces the transcription of chemokines, cytokines such as IL-6, IL-8, TNF- α and proteinases (Juráňová et al., 2017).

Moreover, haptens are electrophilic compounds and generate oxidative stress in the epidermis, activating the NRF2 (Nuclear factor E2-related factor 2) pathway (Christensen and Haase, 2012; Helou et al., 2019). NRF2 is a transcription factor of its target genes like *HMOX1* encoding HO-1 (Heme oxygenase-1) and *NQO1* (NAD(P)H Quinone Dehydrogenase 1). Briefly, under basal conditions, two proteins of KEAP1 (Kelch-like ECH-associated protein 1) hold the protein NRF2 in the cytoplasm and trigger the ubiquitination of NRF2, leading to its degradation by the proteasome. Electrophilic or oxidative stress modifies KEAP1 conformation that liberates NRF2, allowing its nuclear translocation. Then, NRF2 heterodimerizes with a small protein MAF and binds the ARE (Antioxidant response element) sequences to exert its transcriptional function (Tonelli et al., 2018). Moreover, the KeratinoSens is an *in vitro* assay used to assess the sensitization potential of a chemical and is based on NRF2 transcription in a KC model, HaCaT (Emter et al., 2010). NRF2 controls allergic skin inflammation during the sensitization phase induced by a contact sensitizer like cinnamaldehyde (CinA) (El Ali et al., 2013). CinA is used in commercial food, cosmetics, and agriculture (Doyle and Stephens, 2019). Vallion et al. (2022) showed that CinA 100 μM induces the NRF2 pathway and has an anti-inflammatory effect in an *in vitro* KC model, KERTr cells.

NRF2 and NF- κ B pathways interact with each other through a range of complex molecular interactions. The absence of NRF2 is associated with an exacerbation of NF- κ B activity. NF- κ B can have positive and negative effects on the expression of NRF2 target genes. For example, p65 can exert a negative effect on ARE-linked gene expression by different ways and HO-1 has a prominent role in NRF2-mediated NF- κ B inhibition. In fact, the two pathways regulate the fine balance of cellular redox status and responses to stress and inflammation (Wardyn et al., 2015).

The first objective of this work was to study the mechanism of action of a QAC called ethylhexadecylmethylammonium bromide (EHD) in human KC. EHD is classified in category 2 for skin irritation according to CLP (Classification, Labelling, Packaging) criteria³. But it has limited published research describing its sensitization potential. Then, it could be interesting to investigate its effects in human KC. The second objective was to study the potential effects of EHD on the antioxidant and anti-inflammatory effects of CinA 100 μM in KC (Vallion et al., 2022).

Then, KERTr cells were stimulated by EHD, CinA and a mixture of both. After stimulation, expression of NRF2-dependent-ARE-responsive genes and mRNA expression of pro-inflammatory cytokines that can be produced by KC during skin inflammation were measured. Proteins related to NRF2 pathway were also quantified. Our results suggested that, in KC, EHD did not induce detoxification *via* NRF2 pathway but triggered an inflammatory response. Moreover, the anti-inflammatory and antioxidant effects of CinA 100 μM were modulated by EHD when these two compounds were mixed. Our work involving invalidation of *NRF2* in KERTr cells

1 CAS No. 8001-54-5.

2 CAS No. 7173-51-5.

3 Notified classification and labelling according to CLP criteria.

showed the activity of NRF2 pathway on inhibition of EHD-induced inflammatory response in KC.

Materials and methods

Chemicals: EHD (CAS No. 124-03-8), CinA (CAS No. 104-55-2) and Dimethylsulfoxide (DMSO; CAS No. 67-68-5) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). EHD was dissolved in phosphate buffer saline (PBS; GIBCO, Illkirch, France) and CinA in DMSO. All vehicles were used at 0.1% final concentration in culture.

Cell culture: The human KC cell line CCD 1106 KERTr (ATCC CRL-2309™) was purchased from ATCC (Molsheim, France). For passing, the cells were trypsinized once a week (0.05% of trypsin-EDTA 1X; GIBCO) and seeded in a new culture flask at a cell density of 2×10^6 cells/175 cm² in a Keratinocyte-SFM serum-free medium (K-SFM; GIBCO), supplemented with bovine pituitary extract, epithelial growth factor (EGF), penicillin and streptomycin in concentrations of 0.05 mg/mL, 35 ng/mL, 10 U/mL and 10 µg/mL, respectively. The medium was renewed twice a week. The cells were cultured under standard cell culture conditions at a temperature of 37°C and 5% CO₂.

Invalidation of NRF2: NRF2 expression has been invalidated using sh RNA in KERTr cells as described by Vallion et al. (2022). Briefly, plasmid of expression for NRF2 sh RNA (OriGene Technologies GmbH, Herford, Germany) was transferred into KERTr cell using lentiviral transduction. A scrambled sh RNA was used as control. 2×10^6 cells at 50% of confluency were incubated overnight with 10 µg/mL of polybrene and 200 µg/mL of viral particles. Control KERTr cells (KERTr Sh Sc) and invalidated cells for NRF2 (KERTr Sh NRF2) were sorted on a FACS Aria (BD Biosciences, Le Pont-de-Claix, France) based on the GFP (Green fluorescent protein) expression.

Measurement of cytotoxicity: On the day prior to the stimulation, KERTr cells were seeded into 24-well plates (4×10^5 cells/well) in EGF-free culture medium. On the next day, solutions containing different concentrations of EHD (1–30 µM) were added for 24 h. Then, the cells were incubated with propidium iodide (PI; Invitrogen, Illkirch, France). Necrotic cells were stained by PI and were analyzed by flow cytometry using Attune NxT (ThermoFisher Scientific, Illkirch, France) and the FlowJo software (Becton, Dickinson & Company, Franklin Lakes, United States). Results were expressed as the percentage of living cells (PI⁻), and a concentration resulting in 70% cell viability of exposure (CV70) could be determined.

Chemical exposure: The cells were exposed to CinA 100 µM, EHD 10 µM, and a mixture containing both compounds at the same concentration for different exposure times to study the NRF2 pathway and inflammation in KC. Solutions of EHD and CinA were prepared in an EGF-free culture medium to get a concentration equal to their CV70. PBS (0.1%) was used as a negative control. During stimulation, the cells were cultured under standard cell culture conditions at 37°C and 5% CO₂.

Western blot analysis: On the day before the stimulation, KERTr cells were seeded into 6-well plates (2×10^6 cells/well) in an EGF-free culture medium. The solutions containing EHD (10 µM), CinA (100 µM), PBS (0.1%), and a mixture of EHD

(10 µM) and CinA (100 µM) were added for different exposure times. At the end of each time of treatment, cultured KERTr cells were washed twice with cold PBS before lysis in lysis buffer containing 20 mM Tris HCl pH 7.4, 137 mM NaCl, 2 mM disodium EDTA pH 7.4, 1% Triton X-100, 2 mM sodium pyrophosphate, 10% glycerol, 25 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL leupeptin and 50 µg/mL pepstatin (Sigma-Aldrich). The homogenates were incubated for 20 min in ice and then centrifuged at 15,000 × g for 20 min at 4°C. Equal amounts of denatured proteins (30 µg) were loaded onto 10%SDS-PAGE gel (TCX Stain-Free FastCast, Bio-Rad, Marnes-la-Coquette, France) and transferred on polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Membranes were blocked with a tri-buffered saline solution with 3% of bovin serum albumin for 1 h at room temperature. Then, they were incubated with primary antibodies anti-NRF2 (1/1000e, 16396-1-AP, Proteintech, Manchester, United Kingdom), anti-HO-1 (1/1000e, ab13248, Abcam, Paris, France), anti-NQO1 (1/1000e, ab28947, Abcam, Paris, France), at 4°C overnight. After several washings, membranes were incubated with secondary antibodies conjugated to Horseradish peroxidase (HPR) for 45 min at room temperature. To reveal primary antibodies anti-NRF2, anti-rabbit secondary antibodies were used. Primary antibodies anti-HO-1 and anti-NQO1 revelation was carried with anti-mouse secondary antibodies. Immunoreactive bands were detected by chemiluminescence using the ChemiDoc XRS + System (Bio-Rad Laboratories, Marnes la Coquette, France). Bands were quantified with Image Lab software and normalized with the total protein-loaded (Raffalli et al., 2018).

RT-qPCR analysis: The day before the stimulation, KERTr, KERTr Sh Sc, and KERTr Sh NRF2 cells were seeded into 12-well plates (7.5×10^5 cells/well). The solutions containing EHD (10 µM), CinA (100 µM), PBS (0.1%), or a mixture of EHD (10 µM) and CinA (100 µM) were added in wells for 2 h and 6 h. At the end of each time of treatment, cultured cells were trypsinized and centrifuged at 5,000 × g for 3 min at 4°C to get a cell pellet. Cells were then washed twice by centrifugation with cold PBS. Total RNA isolation was performed with PureLink™ RNA Mini (Invitrogen). Total RNA content was measured at 230, 260, and 280 nm by spectrophotometry for quantification and quality assessment. 500 ng total RNA were reverse-transcribed using the thermal cycler T100 (Bio-Rad) in a total-20 µL reaction mixture: 2.5 µM of Oligo (dT) (Promega, Charbonnières-les-Bains, France), 0.5 mM of deoxy-nucleotide triphosphate (dNTP; MP Biomedicals, Illkirch, France), 1 U/µL of Superscript IV Reverse Transcriptase (Invitrogen), 1 U/µL of Recombinant RNasin Ribonuclease Inhibitor (Promega), 5 mM of dithiothreitol (Invitrogen), 5 mM of reverse transcriptase buffer (Invitrogen). 1/20th of each generated cDNA was used for real-time PCR analysis, which was performed using the SYBR Green technology (Sso Advanced universal SYBR Green SuperMix, Bio-Rad) on thermal cycler CFX384 (Bio-Rad). As shown in Table 1, specific forward and reverse primers were used at a final concentration of 0.5 µM for the detection of the investigated genes *HMOX1*, *NQO1*, *IL1A*, *IL1B*, *IL6*, *CXCL8*, *IL23A*, *IL24*, *TNF*, *TSLP*, *ACTB* and *GAPDH* encoding, respectively, for HO-1, NQO1, IL-1α, IL-1β, IL-6, IL-8, IL-23 (subunit α), IL-24, TNF-α, TSLP, β-actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each sample was monitored in duplicate. The results were analyzed using the Bio-

Rad CFX Manager software and were expressed by fold factor calculated by comparing the Ct values obtained from treated and untreated cells (PBS 0.1%) at the same time of exposure and corrected with *ACTB* and *GAPDH* expression.

Statistical analysis: The Mann-Whitney U-test was used. All data are presented as mean values \pm SEM. Measurements were considered significant at a *p*-value \leq 0.05 or \leq 0.1 using GraphPad Prism (San Diego, United States).

Results

A CV70 of EHD equal to 10 μ M in KERTr cells was determined by flow cytometry (data not shown) and was used in this work. For CinA, a value of CV70 equal to 100 μ M had already been determined in previous work. Moreover, the ability of CinA 100 μ M to induce NRF2 pathway had been shown in KERTr cells (Vallion et al., 2022). The effect of the mixture EHD plus CinA on cell viability in KERTr cells was also investigated and did not induce different cell viability after treatment compared to EHD or CinA alone (Supplementary Figure S1).

Study of NRF2 pathway in response to EHD and a mixture containing EHD and CinA

KERTr cells were treated for 2 h or 6 h with EHD, CinA or a mixture of these two compounds. NRF2, HO-1 and NQO1 protein levels were measured by Western blot. Regarding EHD, no accumulation of NRF2 and a low expression of HO-1 ($p \leq 0.1$) were quantified. Level of NRF2 protein was significantly increased following 2-h and 6-h treatment with CinA and the mixture (Figure 1A). However, after 2-h treatment, NRF2 expression was significantly lower with the mixture compared to CinA. Induction in HO-1 protein expression was shown following 6-h treatment with CinA or the mixture. Only a slight induction with EHD was quantified ($p \leq 0.1$). This effect was weaker with the mixture. No change was noticeable concerning NQO1 protein expression.

We measured the mRNA expression of *HMOX1* and *NQO1* by RT-qPCR. Our results indicate that *HMOX1* mRNA expression was significantly increased after 2-h or 6-h treatment with EHD, but the measured values were too low to expect any concrete effect in the cell. Gene expression of both *HMOX1* and *NQO1* was significantly increased following 2-h and 6-h treatment with CinA and the mixture (Figure 1B).

Induction of an inflammatory response by EHD

After 2 and 6 h of incubation of KERTr cells with EHD, CinA, or the mixture of the two, mRNA expression of pro-inflammatory cytokines was measured by RT-qPCR. *IL24* mRNA expression was induced after 6-h treatment with EHD and the mixture ($p \leq 0.05$). A higher expression was measured for *IL1A*, *IL1B*, *IL6* and *CXCL8* after 6-h treatment with EHD ($p \leq 0.05$) and with the mixture ($p \leq 0.1$). An increase in transcript expression of *TNF* was quantified after 2-h and 6-h treatment with EHD ($p \leq 0.1$). Induction of mRNA expression was rapid and important for *IL6*, *CXCL8*, *IL24* ($p \leq 0.05$) and *TNF* ($p \leq 0.1$) in KERTr cells exposed to EHD since a high

mRNA expression was quantified after 2-h treatment. Moreover, statistically significant inhibition of the effect of EHD by CinA on mRNA induction of some of these cytokines is noticeable in KERTr cells for the mixture as shown for *IL6*, *CXCL8*, and *IL24* following 2-h treatment (Figure 2). Ct values measured for *TSLP* transcript expression were not biologically relevant (data not shown).

Inhibition of EHD-induced inflammatory response by NRF2 pathway

Since NRF2 has been shown to have anti-inflammatory properties (Suzuki and Yamamoto, 2017), invalidation of NRF2 expression was carried out to study its potential inhibition on inflammatory response in KC induced by EHD. After stimulation of KERTr Sh NRF2 and KERTr Sh Sc cells by EHD, CinA and the mixture, mRNA expressions of pro-inflammatory cytokines were investigated by RT-qPCR. Each experimental condition was compared to the control (KERTr Sh Sc with vehicle alone) and to its equivalent condition in each KERTr cell type (KERTr Sh NRF2 versus KERTr Sh Sc).

Invalidation of NRF2 in KERTr cells led to an induction of gene expression encoding IL-1 α , IL-1 β , IL-6, IL-8 and IL-24 at the steady state. After stimulation with EHD or the mixture, this induction was increased compared to the control cells. In KERTr Sh NRF2 versus KERTr control, EHD led to a higher expression of *IL24* after 2 h of treatment; *IL1A* and *CXCL8* after 6 h of treatment; and *IL6*, *IL23A* after both times of treatment. The rapid increase in transcript expression of *IL6*, *CXCL8* and *IL24* was again noticeable, but it was mainly higher in KERTr Sh NRF2 cells. Surprisingly, the expression of *IL1B* was decreased after a 6-h treatment of EHD in KERTr Sh NRF2 compared to KERTr control (Figure 3).

Concerning CinA treatment in KERTr Sh NRF2, an increased mRNA expression was quantified for *IL1B* and *IL24* after 6-h treatment; *IL1A*, *IL6*, *CXCL8* and *IL23A* after both times of treatment compared to control. In our study, CinA induced an inflammatory response in absence of NRF2 whereas no inflammatory response could be measured in KERTr Sh Sc as described by Vallion et al., 2022.

In KERTr Sh NRF2, the mixture also caused an increase in *IL1A*, *IL6*, *CXCL8*, *IL23A* and *IL24* expressions after 2 and 6 h of treatment and in *IL1B* expression only after 6 h of treatment (Figure 3). Hence, it implies an inhibitory role of NRF2 on the expression of these genes involved in inflammation and then, on inflammatory response induced by the tested compounds. For *TNF* gene, there were lots of variabilities making any conclusion difficult (data not shown).

After 2-h treatment, a significant decrease in *IL1B* and *IL24* expression with the mixture compared to EHD was measured in both types of KERTr cells (Figure 3). Contrary to normal KERTr cells, no difference between the mixture and the compound alone was measured in KERTr Sh NRF2 cells for *IL6* expression. For *IL1B* expression in KERTr Sh NRF2, after 6 h of treatment, a higher expression can be highlighted with the mixture than EHD or CinA alone.

After 2-h treatment, *CXCL8* expression was lower with the mixture than with EHD in control KERTr cells whereas it was higher in NRF2-invalidated KERTr cells. After 6 h of treatment, the mixture (CinA + EHD) induced a higher expression of *CXCL8* than

TABLE 1 Sequences for primers used in RT-qPCR.

Gene	Forward primer	Reverse primer
<i>HMOX1</i>	5' GGCCTGGCCTTCTTCACCTT 3'	5' GAGGGGCTCTGGTCCTTGGT 3'
<i>NQO1</i>	5' GGGCAAGTCCATCCAACTG 3'	5' GCAAGTCAGGGAAGCCTGGA 3'
<i>IL1A</i>	5' ATCAGTACCTCACGGCTGCT 3'	5' TGGGTATCTCAGGCATCTCC 3'
<i>IL1B</i>	5' ACAGACCTTCCAGGAGAATG 3'	5' GCAGTTCAGTGCATGACAG 3'
<i>IL6</i>	5' TCAATGAGGAGACTTGCCTG 3'	5' GATGAGTTGTATGCTCTGC 3'
<i>CXCL8</i>	5' TCTCTTGGCAGCCTTCCTGA 3'	5' TGGGGTGGAAAGTTTGGAG 3'
<i>IL23A</i>	5' GTTCCCCATATCCAGTGTGG 3'	5' TCCTTTGCAAGCAGAAGTGA 3'
<i>IL24</i>	5' GCTCTCCGGAATAGCAGAAAC 3'	5' TCACAACCTGCAACCCAGTCA 3'
<i>TNF</i>	5' CCTCTCTTAATCAGCCCTCTG 3'	5' GAGGACCTGGGAGTAGATGAG 3'
<i>TSLP</i>	5' CACCGTCTCTGTAGCAATCG 3'	5' TAGCCTGGGCACCAGATAGC 3'
<i>ACTB</i>	5' GGGTCAGAAGGATTCCTATG 3'	5' GGTCTCAAACATGATCTGGG 3'
<i>GAPDH</i>	5' ACCACAGTCCATGCCATCAC 3'	5' TCCACCACCCTGTTGCTGTA 3'

CinA alone. Regardless of the stimulation time, the mixture induced more *IL23A* mRNA expression in KERTr Sh *NRF2* cells compared to EHD. For the comparison with CinA, only the 6 h time point showed a difference with the mixture.

As in normal KERTr cells, *IL24* expression in KERTr Sh *NRF2* cells was decreased with the mixture compared to EHD after 2-h treatment and was increased compared to CinA after 6-h treatment even if invalidation of *NRF2* allowed induction of *IL24* expression by CinA (Figure 3).

Discussion

Contact dermatitis (CD) can be allergic (ACD) or non-allergic, like irritant contact dermatitis (ICD). These inflammatory eczematous skin diseases have different pathophysiological mechanisms. In both cases, danger signals such as DAMP (Damage-associated molecular pattern) and ROS (reactive oxygen species) generated by KC can promote skin inflammation and recruitment of innate immune cells into the epidermis.

ICD is directly caused by the toxic effects of the compound on skin cells leading to unspecific immune responses. Its mechanism is not totally understood, and could imply the disorganization of the lipid layers of cell membranes and the damage of epidermal barrier proteins, e.g., involucrin, filaggrin, or keratin. To promote an ICD, repetitive applications of some irritants may be necessary (Scheinman et al., 2021).

ACD requires the formation of an antigenic complex composed of the hapten and a self-protein (Karlberg et al., 2008). The formation of this neo-antigen depends on different factors, including the hapten's intrinsic properties and the exposure conditions, i.e., the dose, frequency, duration, vehicle, and formulation of the hapten. The sensitization phase can be induced after a first exposure to a contact allergen without symptoms. Then, DC in the skin are activated and can migrate to promote an adaptive immune response in the draining lymph

node. Concerning compounds with weak or very weak sensitizing properties, the sensitization needs to be fully characterized (Scheinman et al., 2021).

To identify contact allergens, most studies focus on KC and DC (Martin, 2012). Indeed, KC are the first cells in contact with the irritant or contact allergens and are considered very active sentinel cells, which are involved in a wide range of inflammatory skin disorders (Jiang et al., 2020).

Some studies have shown that contact sensitizers induce the *NRF2* pathway in KC and DC, whereas skin irritants do not (Ade et al., 2009; Vandebriel et al., 2010). *In vivo* experiments demonstrated that the *NRF2* pathway, activated by contact sensitizer, including CinA, was involved in contact sensitizer-induced inflammation control (El Ali et al., 2013; Vallion et al., 2022).

In our work, we studied the effect of a QAC called EHD on the *NRF2* pathway in a cellular model of human KC. Our results showed that EHD did not efficiently promote *NRF2* expression and its related genes, *HMOX1* and *NQO1*, in KERTr cells. As expected, CinA 100 μ M could induce the *NRF2* pathway (Figure 1) (Vallion et al., 2022). That suggests that EHD may not act as a contact sensitizer in KC. However, it cannot be excluded the possibility that EHD may be a weak allergen like it had been suggested in the controversial case of lanolin (Lee and Warshaw, 2008).

Skin irritation is characterized by inflammation in the skin. It has a known role in ACD, both in the sensitization and elicitation phases (Smith et al., 2002). Moreover, when the skin is readily irritated, it seems more susceptible to contact sensitization (Elsner and Burg, 1993). We, therefore, studied the potential inflammatory effect of EHD in KERTr cells. We showed that EHD triggered an inflammatory response at the transcriptional level since it induced the expression of genes encoding pro-inflammatory cytokines.

IL-6 is a cytokine whose expression can be induced after the application of irritants on the skin. It has a prominent role in the chemoattraction of neutrophils and T-cells (Lee H. Y. et al., 2013). However, *in vivo* studies in mice demonstrated the anti-inflammatory effects of IL-6, which depended on the nature of the irritant (Lee E. G.

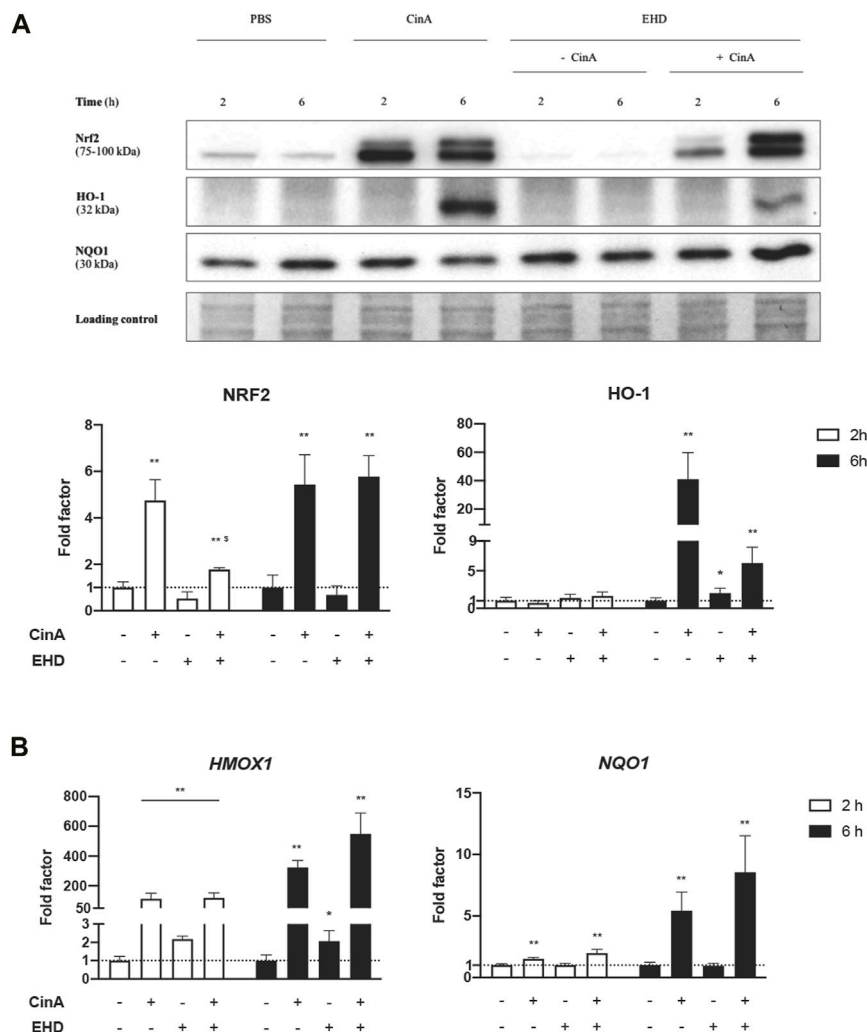


FIGURE 1
EHD does not induce the NRF2 pathway in KERTr cells. KERTr cells were treated for 2 and 6 h with CinA (100 μM), EHD (10 μM), a mixture containing CinA and EHD at the same concentrations, and PBS (0.1%) as vehicle control. **(A)** Effect of EHD on the expression of proteins of the NRF2 pathway. After treatment, protein expression of NRF2, HO-1, and NQO1 were quantified by Western blot with the Imagemag software (Bio-Rad). **(B)** Effect of EHD on the expression at gene level of two NRF2-dependent-ARE-responsive genes, *HMOX1* and *NQO1*. After treatment, the gene expression of *HMOX1* and *NQO1* were quantified by RT-qPCR. Results are expressed as the mean ± SEM of three duplicated independent experiments. * indicates significant differences from untreated control cells at the same time of stimulation (Mann-Whitney test, ** $p \leq 0.05$ and * $p \leq 0.1$), and § indicates significant differences between CinA and the mixture at the same time of stimulation (Mann-Whitney test, $p \leq 0.05$).

et al., 2013). IL-8 is involved in neutrophil recruitment and activation during skin inflammation (Harada et al., 1994). Neutrophils have an essential role in the sensitization and elicitation phases of contact hypersensitivity, an animal model of human ACD. Indeed, neutrophils have a role in DC induction and migration (Weber et al., 2015). Therefore, EHD could promote these events when applied to the skin.

In our study, we measured an increase in *IL1B* expression in KERTr cells, but we could not show a higher production of IL-1β in the supernatants (Supplementary Figure S2). The NALP3 (nucleotide-binding oligomerization domain (NOD)-like receptor family domain-containing protein 3) inflammasome allowing the generation of the active form of IL-1β in KC has been demonstrated as a key regulator of innate immunity in contact hypersensitivity (Watanabe et al., 2007). It suggests that EHD could not activate the NALP3 inflammasome in KERTr cells.

Because NF-κB is a central mediator of pro-inflammatory gene induction (Juráňová et al., 2017), we also investigated its related pathway. After EHD short-term treatments in KERTr cells, it could be noted a decrease in IκBα expression and an increase in phospho-p65 expression by Western blot (Supplementary Figure S3). These signs occurred for NF-κB pathway activation (Juráňová et al., 2017). It can imply that the induction of NF-κB pathway by EHD was the source of the inflammatory response measured at the transcriptional level in KERTr cells. However, the study of inflammatory response at the protein level was more ambiguous because of the high variabilities between experiments. Nevertheless, EHD appeared to trigger an increase in protein expression of IL-6 and IL-8 (Supplementary Figure S2). IL-1β and TNF-α are two stimuli of NF-κB pathway and they could not be clearly found after 24 h of EHD treatment in cell supernatants (Supplementary Figure S2) (Juráňová et al., 2017).

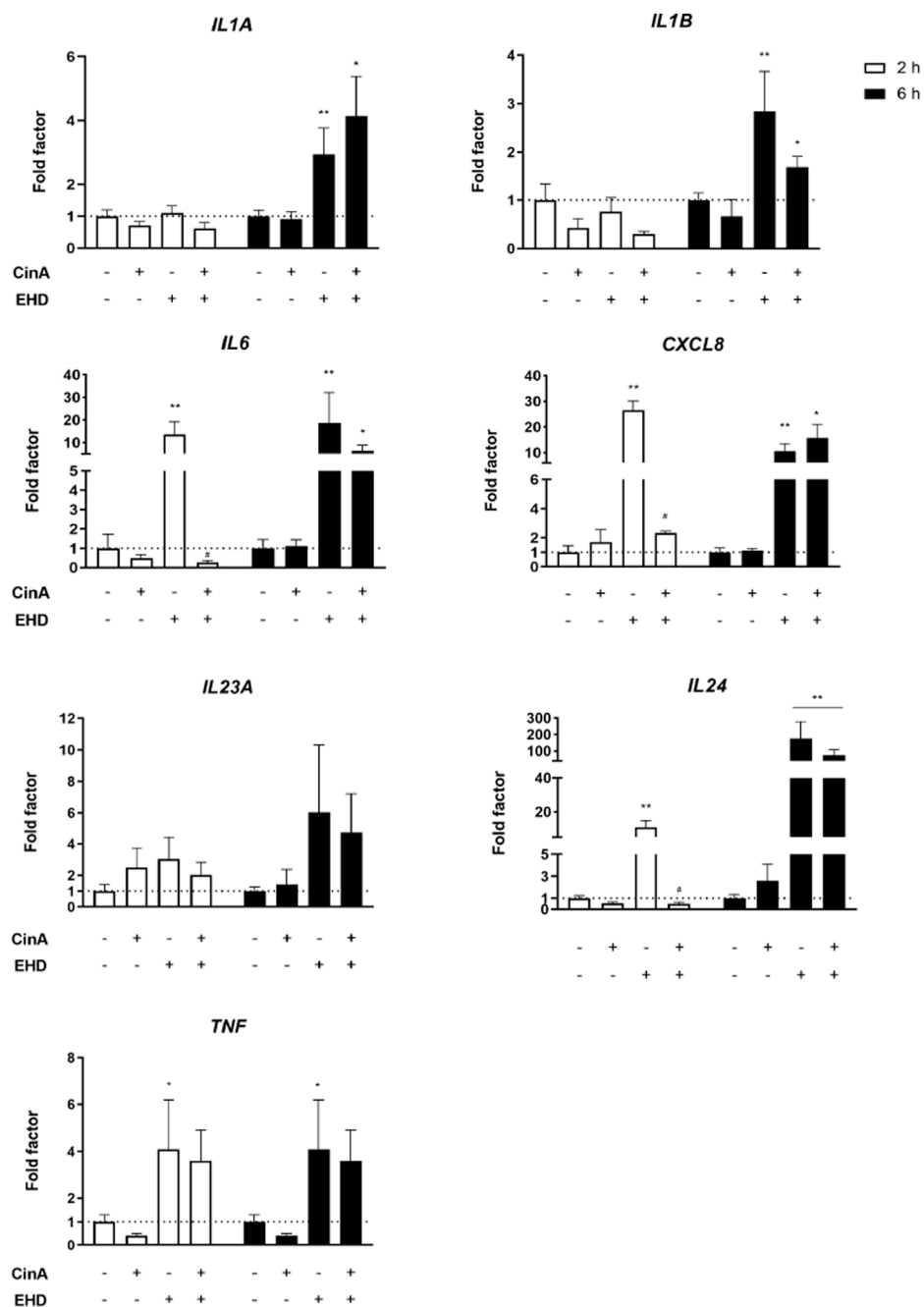


FIGURE 2

EHD induces an inflammatory response in KERTr cells. KERTr cells were treated for 2 and 6 h with CinA (100 μ M), EHD (10 μ M), a mixture containing CinA and EHD at the same concentrations, and PBS (0.1%) as vehicle control. After treatment, expression at gene level of *IL1A*, *IL1B*, *IL6*, *CXCL8*, *IL23A*, *IL24*, and *TNF* encoding, respectively, IL-1 α , IL-1 β , IL-6, IL-8, IL-23, IL-24, and TNF- α , were quantified by RT-qPCR and normalized with *ACTB* and *GAPDH* expression at gene level. Results are expressed as the mean \pm SEM of three duplicated independent experiments. * indicates significant differences from untreated control cells (Mann-Whitney test, ** $p \leq 0.05$ and * $p \leq 0.1$). # indicates significant differences between EHD and the mixture at the same time of stimulation (Mann-Whitney test, $p \leq 0.05$).

Consequently, other *stimuli* such as ROS and DAMP generated by KC exposed to EHD must be sought. If EHD causes oxidative stress in KC, the absence of induction of the NRF2 pathway can result in ROS overproduction which could stimulate the NF- κ B pathway.

Lee et al. (2019) have highlighted the importance of studying the skin sensitization potentials of chemical mixtures that humans could be

occupationally or environmentally exposed to, e.g., DDAC has a mitigated sensitization potential when it is mixed with an increasing amount of ethylene glycol. Nilzen and Wikstrom, (1955) had already demonstrated sensitization to chromium salts and nickel in guinea pigs which could only occur in the presence of a known skin irritant, sodium lauryl sulfate (SLS). We showed that a mixture of EHD (10 μ M) and

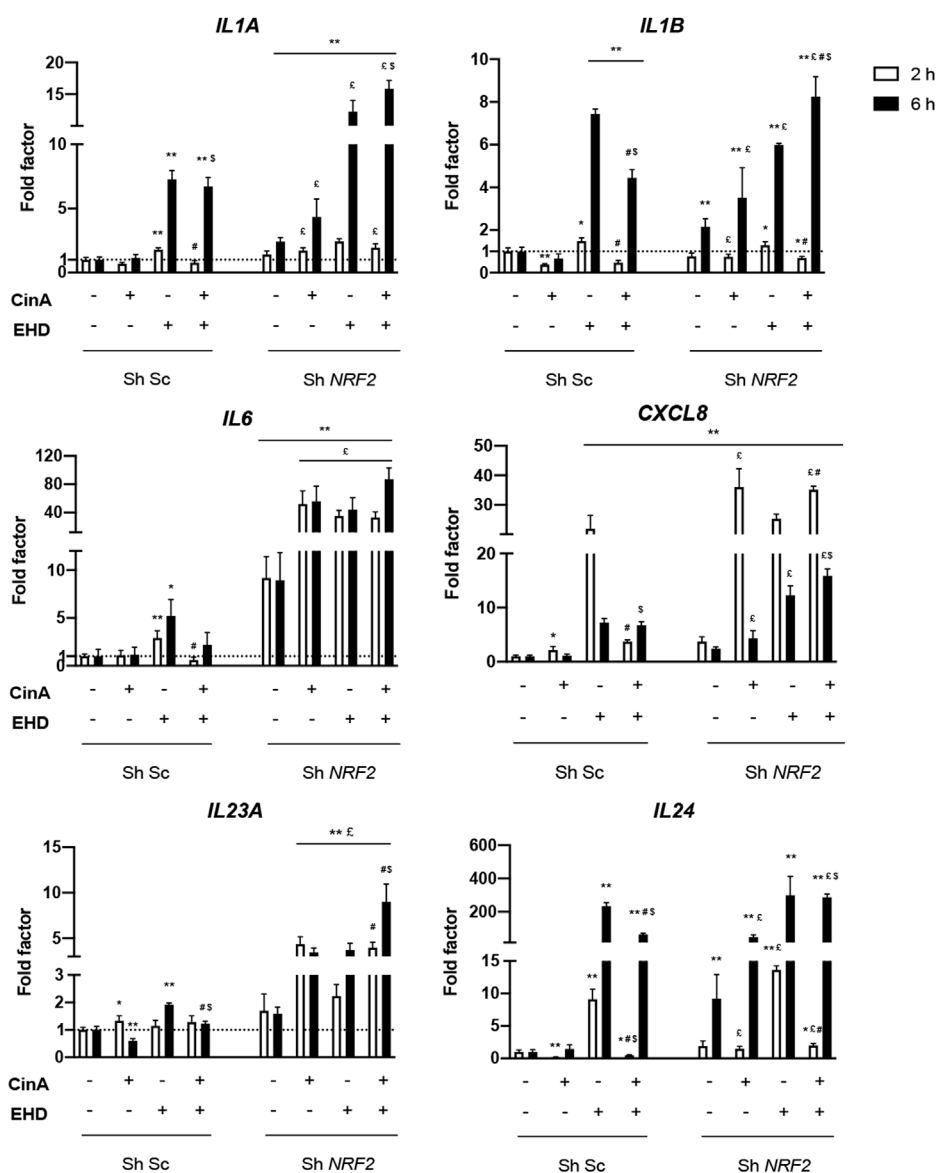


FIGURE 3

NRF2 inhibits inflammation in KERTr cells exposed to EHD and the mixture of EHD and CinA. Control KERTr cells (Sh Sc) and *NRF2*-inactivated KERTr cells (Sh *NRF2*) were treated for 2 and 6 h with CinA (100 μ M), EHD (10 μ M), a mixture containing CinA and EHD at the same concentrations and PBS (0.1%) as vehicle control. After treatment, expression at gene level of *IL1A*, *IL1B*, *IL6*, *CXCL8*, *IL23A*, and *IL24*, encoding, respectively, IL-1 α , IL-1 β , IL-6, IL-8, IL-23, and IL-24, were quantified by RT-qPCR and normalized with *ACTB* and *GAPDH* expression at gene level. Results are expressed as the mean \pm SEM of three duplicated independent experiments. * indicates significant differences from untreated control Sh Sc KERTr cells (Mann-Whitney test, $**p \leq 0.05$ and $*p \leq 0.1$). ϵ indicates significant differences between Sh Sc and Sh *NRF2* for each condition at the same time of stimulation. $\$$ indicates significant differences between CinA and the mixture, at the same time of stimulation, in the same type of cells. # indicates significant differences between EHD and the mixture, at the same time of stimulation, in the same type of cells (Mann-Whitney test, $p \leq 0.05$).

CinA (100 μ M) induced the NRF2 pathway in KERTr cells. More interestingly, NRF2 expression after 2-h treatment with the mixture was diminished compared to CinA treatment (Figure 1). Then, EHD has a rapid and transient inhibitory effect on NRF2 protein level which could result in an inhibition of the antioxidant effect of CinA in KC. However, according to our work, this potential modulation in the cell would occur *via* different ways other than inhibition of HO-1, *HMOX1* or *NQO1* expression. Besides, our studies in KERTr cells revealed that the mixture induced the expression of genes encoding pro-inflammatory cytokines. However, compared with EHD alone, we demonstrated that *IL6*,

CXCL8, and *IL24* expression was lower. This trend was followed by decreased expression of IL-6 and IL-8 in the supernatants (Supplementary Figure S2). This inhibition may be the result of the anti-inflammatory effect of CinA. Indeed, it was shown that CinA exerts its anti-inflammatory effect *via* its ability to induce the NRF2 pathway (Vallion et al., 2022). Then, thanks to *NRF2*-inactivated KERTr cells, we demonstrated an apparent global intensification of the inflammatory response at the transcriptional level when *NRF2* was repressed compared to the control (Figure 3). These findings underlined the efficient anti-inflammatory role of NRF2, even at its basal activity. It is

described in the literature that NRF2 and NF- κ B pathways interact widely with each other (Wardyn et al., 2015). As NRF2 displayed a repressive activity on inflammatory response triggered by EHD or the mixture in KERTr cells, the transcription factor could inhibit the mRNA expression of pro-inflammatory cytokines by interacting negatively with the NF- κ B pathway.

Consequently, the presence of EHD might adversely modulate the skin sensitization potential of CinA in KC. However, we did not demonstrate that EHD was a skin irritant like SLS. Other studies have suggested that irritants may become allergens when exposed to damaged skin (Engebretsen and Thyssen, 2016). Because QAC is found in everyday products with other compounds, it would be interesting to investigate the sensitization potential of QAC in the presence of skin irritants in a cellular model of KC.

Besides, neuromuscular blocking agents (NMBA) are drugs used during anesthesia and are also QAC. They are known to provoke unexpected anaphylactic shock on first injection. The origin of how those patients get sensitized to NMBA remains unclear. A cross-reaction between NMBA and other QAC after a prior skin sensitization of QAC *via* everyday products is suspected to be the cause of these anaphylactic shocks. Then, more investigation about the effects of QAC on KC may also bring new evidence concerning this assumed link (Peyneau et al., 2022).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

RV and SK-R contributed to the conception and methodology; RV, LA, and SK-R performed formal analysis. LA, RV, M-HD, and

SS did the investigation part. SK-R and P-JF brought resources. LA wrote the first draft of the manuscript. RV, SK-R, LA, and P-JF contributed to reviewing and editing. SK-R and RV supervised the project. All authors contributed to the manuscript revision and read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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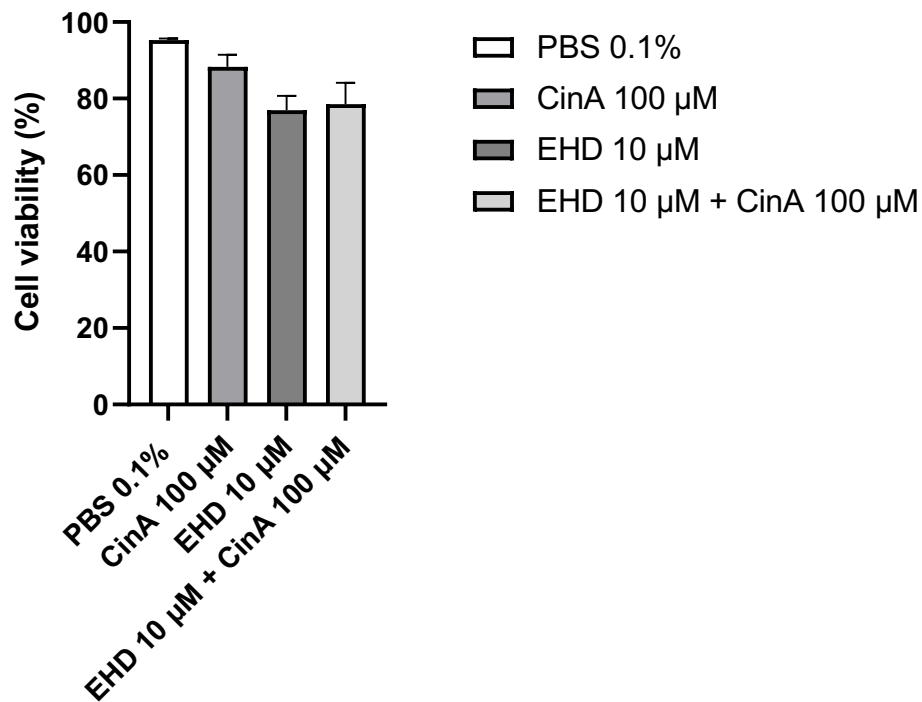
Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ftox.2023.1132020/full#supplementary-material>

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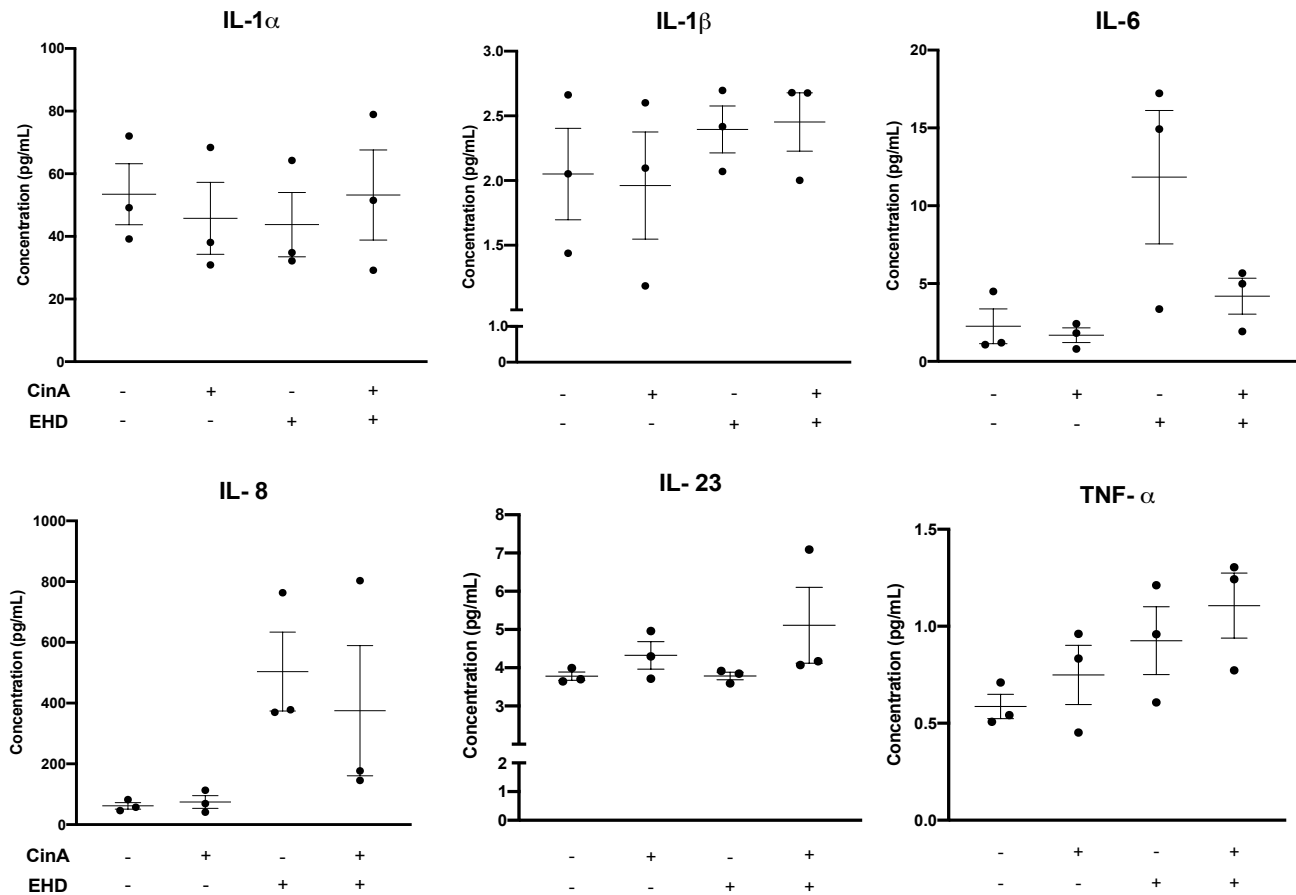
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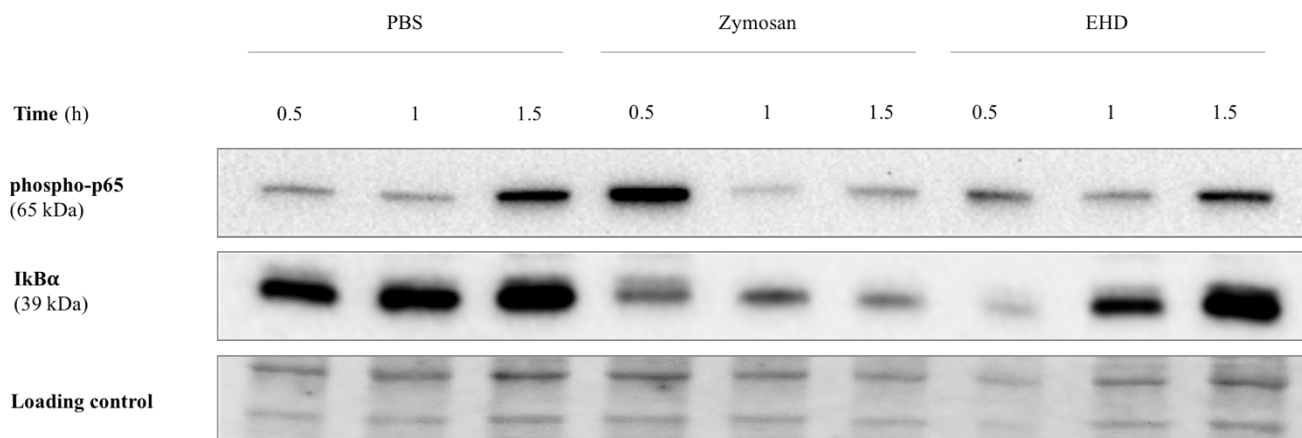
Supplementary Figure 1. There is no difference in cell viability in KERTr cells after treatment with the mixture EHD plus CinA compared to EHD and CinA alone. On the day prior to the stimulation, KERTr cells were seeded into 24-well plates (4×10^5 cells/well) in EGF-free culture medium. On the next day, solutions containing EHD (10 μ M), CinA (100 μ M) or a mixture of CinA (100 μ M) and EHD (10 μ M) were added for 24 h. Then, the cells were incubated with propidium iodide (PI; Invitrogen, Illkirch, France). Necrotic cells were stained by PI and were analyzed by flow cytometry using Attune NxT (ThermoFisher Scientific, Illkirch, France) and the FlowJo software (Becton, Dickinson & Company, Franklin Lakes, USA). Results were expressed as the percentage of living cells (PI).

The results were expressed as the mean \pm SEM of three duplicated independent experiments. PBS is the vehicle control of EHD.



Supplementary Figure 2. EHD may induce secretion of some pro-inflammatory cytokines by KC.

On the day prior to the stimulation, KERTr cells were seeded into 12-well plates (7.5×10^5 cells/well) in supplemented EGF-free K-SFM. The solutions containing EHD (10 μ M), CinA (100 μ M), PBS (0.1 %) and a mixture of EHD (10 μ M) and CinA (100 μ M) were added for different exposure times. After, the culture supernatants were centrifuged at 12,000xg for 10 min at 4°C. The supernatants were collected. Meso Scale Discovery (MSD, Meso Scale Diagnostics, Rockville, USA) U-PLEX® Biomarker Group 1 (Human) assay was performed on supernatants according to the manufacturer's instructions. MSD 96-well plate was read on MSD MESO® QuickPlex SQ 120 Instrument to measure in duplicate IL1- α , IL1- β , IL-6, IL-8, IL-23 and TNF- α protein expression. The results were analyzed using the software MSD Discovery Workbench (Meso Scale Discoveries, Rockville, USA) and were expressed as the mean \pm SEM of three duplicated independent experiments. PBS is the vehicle control of EHD.



Supplementary Figure 3. EHD may activate inflammatory response via NF-κB pathway activation. KERTr cells were seeded into 6-well plates (2×10^6 cells/well) in supplemented EGF-free K-SFM. The solutions containing EHD (10 μ M), PBS (0.1 %) and zymosan (20 μ g/mL; Sigma-Aldrich) were added for 0.5 h, 1 h and 1,5 h. At the end of each time of treatment, cultured KERTr cells were washed twice in cold PBS before lysis in lysis buffer (20 mM Tris HCl pH 7.4, 137 mM NaCl, 2 mM disodium EDTA pH 7.4, 1% Triton X-100, 2 mM sodium pyrophosphate, 10% glycerol, 25 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 mM PMSF, 5 μ g/mL aprotinin, 5 μ g/mL leupeptin and 50 μ g/mL pepstatin). All these products are from Sigma-Aldrich. The homogenates were incubated for 20 min in ice and then centrifuged at 15,000g for 20 min at 4°C. Equal amounts of denatured proteins were loaded onto 10%SDS-PAGE gel (TCX Stain-Free FastCast, Bio-Rad, Marnes-la-Coquette, France) and transferred on PVDF membrane (Bio-Rad). Membranes were successively blocked, incubated with primary antibodies anti-Nrf2 (1/1000e, 16396-1-AP, Proteintech, Manchester, United Kingdom), anti-HO-1 (1/1000e, ab13248, Abcam, Paris, France), anti-NQO1 (1/1000e, ab28947, Abcam, Paris, France), and then with secondary antibodies conjugated to Horseradish peroxidase (HRP). Immunoreactive bands were detected by chemiluminescence using the ChemiDoc XRS+ System (Bio-Rad Laboratories, Marnes la Coquette, France). Bands were quantified with ImageLab software and normalized with the total protein loaded (Raffalli et al., 2018). PBS is the vehicle control of EHD and zymosan is a positive control of the experiment.

References

- Raffalli, C., Clouet, E., Kuresepi, S., Damiens, M.-H., Lepoittevin, J.-P., Pallardy, M., et al. (2018). Editor's Highlight: Fragrance Allergens Linalool and Limonene Allylic Hydroperoxides in Skin Allergy: Mechanisms of Action Focusing on Transcription Factor Nrf2. *Toxicological Sciences* 161, 139–148. doi: 10.1093/toxsci/kfx207.

SCIENTIFIC COMMUNICATIONS

Oral communications

- **Eurotox Congress 2023** – September 2023 -Ljubljana, Slovenia
Awardee of SFT (Société Française de Toxicologie) Travel Grant
- **“Jeunes Toxicologues” seminar** – March 2023
Organized by SFT, SFTG (Société Française de Toxicologie Génétique), STCM (Société de Toxicologie Cellulaire et Moléculaire), and ARET (Association pour la recherche en Toxicologie)
Recipient of the “Best presentation” Award
- **11th ITCASS & 30th ERGECD Meeting** (Immunotoxicologie and Chemical Allergy Speciality section/ European Research Group on Experimental Contact Dermatitis) – November 2022 – Amsterdam, The Netherlands
Awardee of ITCASS Travel Grant Award

Posters

- **STCM Congress** – October 2022 – Dunkerque, France
- **16th ICT International Congress of Toxicology** – Septembre 2022– Maastricht, The Netherlands
- **ESDR 50th Annual Meeting** (European Society for Dermatological Research) – September 2021 – online
- **JED Journée de l’Ecole doctorale ITFA 569** – June 2021 & 2022 –Université Paris Saclay, France