UNIVERSIDAD AUTONOMA DE AGUASCALIENTES

CENTRO DE CIENCIAS BÁSICAS

Título de TESIS:

Estudio del efecto del glicomacropéptido sobre la respuesta *in vitro* del queratinocito al microambiente tisular de la dermatitis atópica.

PRESENTA

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PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS BIOLÓGICAS.

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Aguascalientes, Ags, 14 de noviembre del 2023

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Acrónimos y Abreviaturas

AGCC: ácidos grasos de cadena corta
cGRP: péptido relacionado con el gen de la calcitonina
CO_2: dióxido de carbono
CST: caldo soya tripticasa
DA: dermatitis atópica
DMEM: Dubelco's Modified Eagle Medium
GMP: glicomacropéptido
IFN: interferón
IL: interleucina
ILC: células linfoides innatas
KC: queratinocito
MDC: quimiocina derivada de macrófagos
NGF: factor de crecimiento nervioso
PBS: solución salina de fosfatos
ROS: especies reactivas de oxígeno
RPM: revoluciones por minuto
SBF: suero bovino fetal
TARC: quimiocina regulada por activación y el timo
TGF: factor de crecimiento transformante
Th2: linfocitos T CD4 antígeno-específicos al tipo 2
TNF: factor de necrosis tumoral
TSLP: linfopoyetina del estroma tímico
UFC: unidades formadoras de colonia
V: veces

Resumen

El queratinocito (KC) es el principal componente funcional y estructural de la epidermis, la capa más externa de la piel. La epidermis está altamente especializada en la defensa contra agentes externos y en evitar la pérdida de agua transepidermal. La barrera epidérmica alterada y la diferenciación aberrante del KC están involucradas en la fisiopatología de varias enfermedades de la piel, como la dermatitis atópica (DA). La DA es una condición alérgica, ampliamente distribuida en todo el mundo y para la que todavía no hay cura. Se trata de una enfermedad inflamatoria crónica de la piel generada por la interacción entre factores genéticos y ambientales y caracterizada por una respuesta inmunitaria sesgada hacia el perfil tipo 2. Los pacientes con DA presentan una reducción de la diversidad microbiana en la piel con un aumento de *Staphylococcus* spp. Durante la DA activa hay colonización de la piel lesionada con la bacteria oportunista *Staphylococcus aureus* que generalmente se mantiene en equilibrio gracias a la presencia de bacterias comensales como *Staphylococcus epidermidis*. El glicomacropéptido (GMP) es un péptido bioactivo derivado de la κ-caseína de la leche que se genera por la acción enzimática de la pepsina o la quimosina. Tiene propiedades antialérgicas y restauradoras de la barrera cutánea cuando se administra por vía oral en la DA experimental. El objetivo de este trabajo fue evaluar el efecto del GMP en las respuestas inflamatorias, oxidativas, proliferativas y migratorias de los KCs HaCaT en un modelo de DA *in vitro*. Además, se analizó el efecto del GMP sobre el crecimiento de *S. aureus* y *S. epidermidis* en cultivo, así como sobre la adhesión de ambas bacterias a las células HaCaT en cultivo. Se observó que el GMP protegió a los KCs de la muerte y la apoptosis de forma dependiente de la concentración. El GMP a 6.3 y 25 mg/mL, respectivamente, redujo la producción de óxido nítrico en un 50 % y 83.2 %, así como los lípidos hidroperóxidos en un 27.5 % y 45.18 % en células HaCaT activadas. Mediante el tratamiento con GMP la expresión génica de *TSLP*, *IL33*, *TARC*, *MDC* y *NGF* se regularon significativamente a la baja al compararse contra el control en KCs activados, mientras que se potenció la expresión de *cGRP*. Finalmente, en un microambiente de DA, el GMP a 25 mg/mL estimuló la proliferación de células HaCaT, mientras que concentraciones de 0.01 y 0.1 mg/mL promovieron la migración de las células. En ensayos *in vitro*, el GMP a 6.3 mg/mL estimuló el crecimiento de *S. epidermidis* un 49% y el de *S. aureus* solo en un 25%. Además, el GMP inhibió la adhesión de *S. aureus* a los KCs en cultivo de una manera dependiente de la concentración, llegando a reducir en un 96.9% la adhesión de la bacteria a las células HaCaT a la concentración de 6.3 mg/mL. Contrariamente, el GMP aumentó la adhesión de *S. epidermidis* a los KCs, siendo este efecto inversamente proporcional a la cantidad de GMP añadido al medio. Por lo tanto, con este trabajo demostramos que el GMP tiene propiedades antiinflamatorias y antioxidantes en los KCs, estimula el cierre de heridas en un modelo *in vitro* de DA y disminuye la adhesión de la bacteria oportunista *S. aureus* a las células HaCaT, reforzando la evidencia del GMP como un candidato terapéutico potencial para la DA por su efecto benéfico sobre los KCs.

Abstract

The keratinocyte (KC) is the main functional and structural component of the epidermis, the outermost layer of the skin. The epidermis is highly specialized in defending against external agents and preventing transepidermal water loss. Disrupted epidermal barrier and aberrant KC differentiation are involved in the pathophysiology of several skin diseases, such as atopic dermatitis (AD). AD is an allergic condition, widely distributed throughout the world and for which there is still no cure. It is a chronic inflammatory skin disease generated by the interaction between genetic and environmental factors and characterized by an immune response biased towards the type 2 profile. Patients with AD present a reduction in microbial diversity on the skin with an increase in Staphylococcus spp. During active AD there is colonization of the injured skin with the opportunistic bacteria *Staphylococcus aureus*, which is generally maintained in balance thanks to the presence of commensal bacteria such as *Staphylococcus epidermidis.* Glycomacropeptide (GMP) is a bioactive peptide derived from milk κ-casein that is generated by the enzymatic action of pepsin or chymosin. It has anti-allergic and skin barrier restorative properties when administered orally in experimental AD. This study aimed to evaluate the effect of GMP on the inflammatory, oxidative, proliferative and migratory responses of HaCaT KCs in an in vitro AD model. Furthermore, the effect of GMP on the growth of *S. aureus* and *S. epidermidis* in culture, as well as on the adhesion of both bacteria to HaCaT cells in culture, was analyzed. GMP protected KCs from death and apoptosis in a dose dependent manner. GMP at 6.3 and 25 mg/mL, respectively, reduced nitric oxide by 50% and 83.2% as well as lipid hydroperoxides by 27.5% and 45.18% in activated HaCaT cells. The gene expression of *TSLP*, *IL33*, *TARC*, *MDC*, and *NGF* was significantly downregulated comparably to control by GMP treatment in activated KCs, while that of *cGRP* was enhanced. Finally, in an AD microenvironment, GMP at 25 mg/mL stimulated HaCaT cell proliferation, while concentrations of 0.01 and 0.1 mg/mL promoted the HaCaT cell migration. In *in vitro* assays, GMP at 6.3 mg/mL stimulated the growth of S. epidermidis by 49% and that of *S. aureus* by only 25%. Furthermore, GMP inhibited the adhesion of *S. aureus* to the KCs in culture in a concentration-dependent manner, reducing the adhesion of the bacteria to the HaCaT cells by 96.9% at the concentration of 6.3 mg/mL, this effect being inversely proportional to the amount of GMP added to the medium Therefore, we demonstrate that GMP has anti-inflammatory and antioxidative properties and stimulates wound closure on an AD model of KCs and decreases the adhesion of the opportunistic bacteria S. aureus to HaCaT cells. reinforcing the evidence of GMP as a potential therapeutic candidate for AD due to its beneficial effect on KCs

INTRODUCCIÓN

La piel es el órgano encargado de proteger al organismo de los agentes externos. La epidermis, la capa más externa de la piel, actúa como una barrera para prevenir o dificultar la penetración de antígenos y la invasión de patógenos. Está estructurada por queratinocitos (KCs) en diferentes etapas de diferenciación (Gallegos-Alcala et al., 2021). La alteración de la homeostasis epidérmica debido a defectos funcionales en las proteínas estructurales de los KCs, predisposición genética y desregulación inmunitaria provoca la aparición de la dermatitis atópica (DA), una de las enfermedades de la piel más abundantes en todo el mundo (Gallegos-Alcala et al., 2021; Hadi et al., 2021). Aunque su prevalencia varía según la zona geográfica, la DA se presenta hasta en un 34% de los niños (Hadi et al., 2021). Esta enfermedad suele aparecer en la primera infancia (0-5 años) y puede resolverse antes de la pubertad, aunque persiste en el 2-5 % de los adultos (Barbarot et al., 2018). Se identifica como el comienzo de la marcha atópica, una teoría epidemiológica que propone que el desarrollo temprano de la DA predispone a los pacientes a otras condiciones atópicas en el futuro (Tsuge et al., 2021). Además, la DA representa una carga económica significativa para el sector de la salud y para las familias de los pacientes por lo que se considera un importante problema de salud mundial (Xue et al.,2022).

La DA es una enfermedad inflamatoria de la piel, crónica y recidivante, que se desencadena en pacientes susceptibles tras la constante estimulación de la epidermis con antígenos ambientales. Los KCs están activamente implicados en el inicio, mantenimiento y exacerbación de la inflamación en esta enfermedad. La respuesta inmunitaria disfuncional en la DA se caracteriza por una diferenciación predominante de linfocitos T CD4 antígeno-específicos al tipo 2 (Th2) y la consiguiente sobreproducción del factor de necrosis tumoral (TNF)-α, interleucina (IL)-4, IL-5, IL-9 y IL-22 en la piel lesionada (Gallegos-Alcalá et al., 2021). La linfopoyetina del estroma tímico (TSLP), la IL-33, la IL-25, la quimiocina regulada por activación y el timo (TARC) y la quimiocina derivada de macrófagos (MDC) liberadas por los KCs participan en la diferenciación al perfil Th2, la activación de las células linfoides innatas (ILC)2 y el reclutamiento de linfocitos de tipo 2 en el sitio de la inflamación alérgica (Jahnz-Rozyk et al., 2005; Cayrol et al., 2014; Lee et al., 2010). Aunque la polarización Th2 prevalece en la enfermedad, otras poblaciones celulares como los linfocitos Th1 con producción de interferón (IFN)-γ también participan en la fisiopatología de la fase crónica (Fedenko et al., 2011). Además, el estrés oxidativo tiene un papel importante en la patogénesis de la DA, ya que se ha asociado con respuesta inflamatoria exacerbada y apoptosis de KCs. Los KCs bajo el microambiente Th2 presentan un nivel elevado de estrés oxidativo que puede conducir a la peroxidación de lípidos, la oxidación de proteínas o el daño del DNA, que como consecuencia genera disfunción en las células y por lo tanto deterioro de la función de barrera de la piel (Choi et al., 2021). Asimismo, algunos neuropéptidos, como el factor de crecimiento nervioso (NGF) y el péptido relacionado con el gen de la calcitonina (cGRP), se han asociado con el alargamiento de las terminaciones nerviosas aferentes, la inflamación neurogénica y la sensación de picazón (prurito) característica de la DA (Marek-Jozefowicz et al., 2023). Los antígenos ambientales estimulan a los KCs para que expresen NGF y cGRP de una manera dependiente de especies reactivas de oxígeno (ROS) (Choi et al., 2021). Debido al intenso

prurito, los pacientes con DA se rascan las áreas lesionadas, lo que agrava el daño en la piel, que se exacerba debido a que tienen un proceso alterado de re-epitelización de la herida, ya que las citocinas inflamatorias tipo 2 alteran la capacidad de los KCs para proliferar y migrar adecuadamente (Zhao et al., 2016). El factor de crecimiento transformante (TGF)-β, que participa en múltiples fases de la cicatrización de heridas (Crowe et al., 2000), tiene una expresión más baja en pacientes con DA (Arkwright et al 2001). Por lo tanto, los KCs son sin duda células cruciales en la etapa temprana de la inflamación tipo 2, la perpetuación de la inflamación, el desarrollo de prurito y el daño de la piel en la DA.

Por otro lado, se sabe que los pacientes con DA presentan disbiosis en la microbiota cutánea, con una menor diversidad bacteriana, un sobrecrecimiento del género *Staphylococcus* (Koh et al., 2022) y un incremento en la colonización por *Staphylococcus aureus*. En la DA activa, se ha relacionado la presencia de citocinas inflamatorias producidas por los KCs con el sobrecrecimiento de la cepa patógena *S. aureus* (Domenico et al., 2018).

Desafortunadamente, no existe una cura para la DA. Las terapias están enfocadas a mitigar los principales síntomas de la enfermedad y lograr el control de la enfermedad a largo plazo. Los tratamientos de primera línea son los emolientes, para reparar la barrera epidérmica, y la terapia antiinflamatoria con corticoides tópicos o inhibidores de la calcineurina, para controlar las exacerbaciones agudas y mantener la remisión (Weidinger et al., 2016). El desarrollo de nuevas terapias que controlen los síntomas de la enfermedad y modifiquen las respuestas inflamatoria y oxidativa subyacentes en la DA es un foco de interés actual en investigación. Estos tratamientos modificadores de la enfermedad podrían detener la progresión de la marcha atópica si se utilizan en las etapas tempranas de la DA.

Recientemente, el uso de péptidos bioactivos naturales ha sido ampliamente explorado como tratamiento potencial para diferentes patologías debido a su amplia seguridad y eficacia. En particular, la leche es una fuente de múltiples péptidos con diversas actividades biológicas (Punia et al., 2020). Uno de estos péptidos es el GMP, un péptido de 64 aminoácidos generado en el suero de quesería durante el proceso de elaboración del queso o fisiológicamente durante la digestión enzimática de la leche por la quimosina o la pepsina, respectivamente, el cual corresponde a la región carboxilo-terminal de la κ-caseína bovina escindida (Jollés et al., 1968). Numerosos estudios *in vitro* e *in vivo* le han atribuido importantes funciones biológicas al GMP (Cordova-Davalos et al., 2019). Entre ellos, las actividades antioxidante, antiinflamatoria y antialérgica son importantes para este estudio. Se ha reportado que el GMP disminuye las respuestas de estrés oxidativo en macrófagos y hepatocitos *in vitro* (Li et al., 2017; Cheng et al., 2015). Cuando se dosifica por vía oral en modelos de ratas con DA, reduce la intensidad del edema, la infiltración de células inflamatorias, el prurito y la expresión de citocinas Th2 en las lesiones de DA (Muñoz et al., 2017). Además, la administración de GMP previene o revierte el daño de la barrera cutánea al aumentar la expresión de proteínas estructurales y péptidos antimicrobianos, y al evitar el engrosamiento epidérmico y la colonización *por S. aureus* en el tejido cutáneo afectado (Jiménez et al., 2020). El mecanismo de acción del GMP administrado por vía oral está parcialmente mediado por actividades prebióticas en

la microbiota intestinal y la producción de moléculas inmunomoduladoras, como los ácidos grasos de cadena corta (AGCC) (Jimenez et al., 2020; Jiménez et al., 2016). Sin embargo, no se puede descartar un efecto directo cutáneo del GMP, ya que se ha detectado en sangre tras la ingestión de leche o yogur (Chabance et al., 1998), y se ha documentado previamente una actividad moduladora del GMP administrado oralmente sobre células inflamatorias de la piel, como los mastocitos (Jiménez et al., 2016). El GMP también podría formularse en cremas o ungüentos para aplicación tópica. Por lo tanto, la evaluación de las actividades biológicas del GMP en un modelo de DA en KCs *in vitro* es de gran interés.

HIPOTESIS

Hipótesis

El GMP tiene un efecto protector sobre la respuesta del KC *in vitro* a agentes sensibilizantes o a citocinas inductoras de DA y sobre cambios en la barrera epidérmica asociados a la patología.

OBJETIVOS

Objetivo general

Estudiar el efecto del GMP sobre la respuesta *in vitro* del KC al microambiente tisular característico de la DA.

Objetivos particulares

1. Evaluar el efecto citoprotector del GMP sobre el daño inducido por agentes sensibilizantes en KCs en cultivo.

2. Analizar la actividad antioxidante del GMP en KCs activados por agentes sensibilizantes.

3. Estudiar la expresión de genes relacionados con el desarrollo de la DA en KCs activados por agentes sensibilizantes o por citocinas involucradas en la patología, en presencia o no de GMP.

4. Evaluar el efecto del GMP sobre el crecimiento y la adhesión de *S. aureus* y *S. epidermidis* en KCs en cultivo.

5. Analizar el efecto del GMP sobre la regeneración de heridas y la integridad de la barrera epidérmica en un modelo *in vitro* de lesiones cutáneas tipo DA.

ARTÍCULOS

Artículos

Los metodología, resultados y discusión para cumplir los objetivos 1,2,3 y 5 se describen en el artículo de investigación II titulado: “Glycomacropeptide protects against inflammation and oxidative stress, and promotes wound healing in an atopic dermatitis model of human keratinocytes”. Además, en anexos se detallan la metodología, los resultados y la discusión relacionada con el objetivo 4, los cuales no se incluyeron en los artículos.

I. The keratinocyte as a crucial cell in the predisposition, onset, progression, therapy and study of the atopic dermatitis.

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Review

The Keratinocyte as a Crucial Cell in the Predisposition, Onset, Progression, Therapy and Study of the Atopic Dermatitis

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Abstract: The keratinocyte (KC) is the main functional and structural component of the epidermis, the most external layer of the skin that is highly specialized in defense against external agents, prevention of leakage of body fluids and retention of internal water within the cells. Altered epidermal barrier and aberrant KC differentiation are involved in the pathophysiology of several skin diseases, such as atopic dermatitis (AD). AD is a chronic inflammatory disease characterized by cutaneous and systemic immune dysregulation and skin microbiota dysbiosis. Nevertheless, the pathological mechanisms of this complex disease remain largely unknown. In this review, we summarize current knowledge about the participation of the KC in different aspects of the AD. We provide an overview of the genetic predisposing and environmental factors, inflammatory molecules and signaling pathways of the KC that participate in the physiopathology of the AD. We also analyze the link among the KC, the microbiota and the inflammatory response underlying acute and chronic skin AD lesions.

Keywords: keratinocyte; atopic dermatitis; allergic inflammatory response; keratinocyte differentiation; skin microbiome; in vitro atopic dermatitis models; pharmacological therapy

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1. Introduction

The skin is an organ in constant renewal that covers the body surface. Its total surface area is commonly calculated around 2 m^2 considering variables such as height and weight; however, folds and invaginations, such as hair follicles and sweat ducts, substantially increase the surface area to 25 m^2 [1,2]. The skin fulfills specific functions of protection, restraint, thermal regulation and sensitivity, which are carried out in the three layers that constitute the skin (from bottom to top): hypodermis, dermis and epidermis [3] (Figure 1). The hypodermis is the deepest layer of the skin that lies below the dermis and adjoins the deep fascia that covers the skeletal muscle. It is made up of loose connective tissue and stored fat [4], and cells such as fibroblasts, macrophages, and mainly adipocytes. Main functions of the hypodermis are to work as a caloric reserve, thermoregulation and shock absorber [5]. The dermis is located above the hypodermis and is composed of two main extracellular matrix (ECM) components, i.e., collagen and elastin fibers, which are synthesized by fibroblasts. Afferent nerve endings and arteries also penetrate to the dermal deep region. The dermis has notable functions providing tone, strength, resistance, sensitivity and nutrients to the epidermis [6,7]. In addition, this layer of the skin is of great importance since it contains innate immune cells such as dermal and plasmacytoid dendritic cells (DCs), macrophages, mast cells, γδ T cells and innate lymphoid cells (ILC), crucial in the function of defense in both human and mouse [8–13]. The epidermis is located more externally and contiguous to the ECM and covers the dermis. It is a semi-permeable stratified keratinized epithelium highly specialized in defense against external agents [3,14]. In addition to keratinocytes (KCs), which are the main functional and structural components of epidermal barrier, Langerhans cells, γδ T cells, resident memory

T cells (mainly CD8+), melanocytes and Merkel cells are found in this layer, both in mice and humans [8,10,11,15–18].

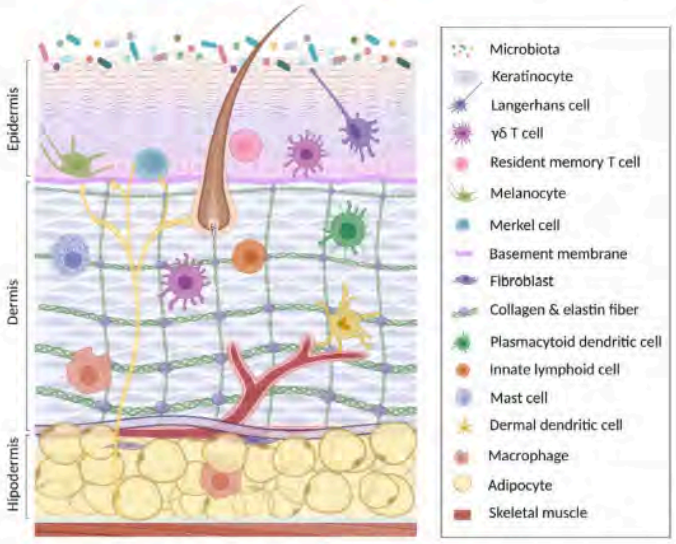

Figure 1. Anatomy of the skin. This image represents the three main layers of the skin, including the most abundant cellular populations in each layer, together with the immune cells present in each anatomical region. Created with BioRender.com (access date: 26 August 2021).

Finally, on the epidermis, the microbiota has an important role in skin health, either modulating the immune response by generating an anti-inflammatory effect or inhibiting the colonization of multiple pathogens [19–21]. Skin microbiota is highly diverse and mainly composed by bacterial, fungal, and viral populations, although microbial biomass is relatively reduced compared to that in the gut and airways [22]. Bacterial skin microbiota is mainly represented by three genera: *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*, which represent over 60% of the bacterial species in the skin [23]. Fungal population on the skin is composed by *Malassezia*, *Aspergillus*, *Penicillium*, *Epicoccum*, and *Candida* [24]. Finally, the skin virome has been poorly studied and although respiratory and enteric virus transmission by hand contact is well documented [25,26], viruses are not normal inhabitants of the skin. Studies in healthy skin have demonstrated the presence of cutaneous human β- and γ-papillomaviruses (HPVs), polyomaviruses and circoviruses [27,28]. All these data highlight the great complexity of the skin microbiota.

In the last decade, the KC has become a target of interest in various pathologies, due to its wide versatility. KCs have the capacity of differentiation, regeneration and interaction with various environmental components, as well as with other epithelial components, both structural and immunological, and they function to maintain a state of balance within healthy skin [29–32]. Alteration of skin homeostasis can generate dermatosis conditions such as psoriasis, seborrheic dermatitis, and atopic dermatitis (AD), among others [33]. All these conditions significantly affect the quality of life of patients. Particularly, AD has aroused particular interest in recent years since records indicate that it affects 15% of the world population. This incidence has been recently altered in the general population, and mainly in health care workers, by the intensive hygiene habits adopted because of the pandemic by SARS-CoV2 [34].

In this review, we summarize the roles of KCs in the context of AD. We first review the biology of the KC, and later review in detail the genetic and immunological profile of the cell that predisposes the beginning and progression of disease, highlighting its interaction with skin microbiota. Current knowledge about the use of the KC as an in vitro model

to study AD is also reviewed. Finally, the pharmacological therapies target to restore KC properties in AD treatment is presented.

2. Keratinocyte in Skin Homeostasis

The KC is the main component of the epidermal barrier. This cell differentiates as it migrates through the different layers of the epidermis, by extension, from stratum basale (SB), through stratum spinosum (SS) and granulosum (SG) to stratum corneum (SC) [3,35]. During cell migration through these strata, the KC undergoes different processes, including proliferation, maturation (or differentiation) and cornification (Figure 2). In general, the differentiation processes of KCs are widely regulated by calcium, which is related with the fact that the highest calcium concentrations are found in the SB and SC [36,37].

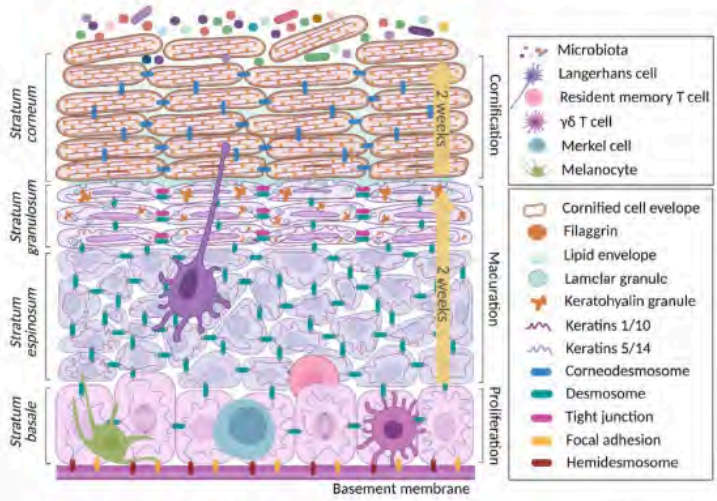

Figure 2. Keratinocyte life cycle. This begins when the keratinocyte (KC) proliferates in the stratum basale. Later, in a course of two weeks, it matures and migrates through the suprabasal layers (spinosum and granulosum stratums) until reaching the top part of the skin, the stratum corneum. Here, the KC acquires the highest degree of maturity and gradually loses viability. Finally, in another two weeks, it moves through the cornified layer to be eliminated by flaking. Created with BioRender.com (access date: 26 August 2021).

2.1. Proliferation Phase

The regeneration of epidermal cells takes place in the epidermal SB, a site where cells are dividing. The basal cells, the main components of this stratum, are cuboidal shaped cells with round to ovoid nuclei and evident tonofilaments within the cytoplasm [38]. In the SB are also located melanocytes that are responsible for skin pigmentation, and Merkel cells that take part in mechanoreception and interact with neurons [15,16,39]. Resident memory T cell and γδ T cells, both with dendritic morphology, are located in this stratum with important implications in local immune responses [40,41].

Basal cells are attached to the ECM of the basement membrane by focal adhesions, mainly β_1 integrins, and hemidesmosomes (HDs). The latter are composed of the $\alpha_6\beta_4$ integrin that extracellularly act as a laminin receptor that preferentially binds to laminin-332 [42,43]. $\alpha_6\beta_4$ integrin is coupled with the transmembrane element bullous pemphigoid antigen (BPAG)-2 (also known as BP180), which act as a functional unity that interacts inside the cell with two plakins, BPAG-1 (also known as BP230) and plectin/HD-1, that form that inner plaque of the HDs and are anchored to cytoskeletal keratin filaments (K)5 and K14 [44–47]. In turn, keratins modulate the location of the HDs [48]. In vitro, it has been demonstrated that K5 and K14 play important roles in promoting phosphatidylinositol

3-kinase-dependent cell proliferation and negatively modulating Notch1-dependent cell differentiation [49]. To a large extent, the cell migration process begins with the activation of the epidermal growth factor (EGF) receptor (EGFR), since it activates tyrosine kinase Fyn, which phosphorylates the cytoplasmic domain of β_4, causing the disassembly of HDs [45].

Basal cells undergo asymmetric perpendicular divisions that cause one daughter cell to maintain adhesion with the ECM and the other cell to continue stratification [29], probably in response to biomechanical signaling modulated by cell-cell contact [50,51]. Adherent junctions, which are related to cell-cell adhesion, organization of the cytoskeleton and cell signaling [52], and desmosomes, which are adhesion joints that provide strength to the skin, are also present in this stratum. The former are composed by P-cadherin and E-cadherin, while the latter are constituted by the cadherins desmoglein (DSM) 2 and 3, and desmocollin (DSC) 3 [51–54].

2.2. Maturation Phase

The maturation phase begins when the daughter cells that leave the SB lose the ability to duplicate and begin their migration through the different strata of the epidermis. This process is accompanied by biochemical and morphological changes before cells die in the SC [30,55].

Far from what was previously thought, HDs in conjunction with focal adhesions facilitate KC migration to the SS [56,57]. The SS is characterized by a lower area consisting of several layers of polygonal KCs with a spiny appearance due to the increased amount of newly formed cell-cell junctions, and an upper area consisting of a layer of elongated and flattened KCs parallel to the epidermal surface. Both keratohyalin granules and lamellar bodies begin to appear in the last layer of the SS, although the latter can be only observed by electron microscopy [38,58,59]. Content of both membrane-bound organelles are described later due to their importance in the cornification process. Langerhans cells are also located in the SS and play important functions in the uptake and processing of antigens [11,60]. On the other hand, cells of the SG are elongated and parallel to the surface of the skin, as are their nuclei, with a high content of basic keratohyalin granules. These cells are connected to the SS and SC cells through desmosomes [38]. Tight junctions (TJs), which are structures composed of membrane proteins from the occludin and claudin families associated with plaque proteins such as zonula occludens (ZO)-1 that facilitate the transport of ions and solutes between neighboring cells [61,62], are formed and overexpressed in this layer [61,63]. Living SG cells gradually begin to reduce their viability, and once they reach the SC are known as corneocytes, which are dead cells devoid of nuclei and organelles.

In addition to morphological changes, the differentiation process is accompanied by structural modifications in cell-cell junctions. When KCs exit the basal layer and migrate upwards into the suprabasal layers, they stop expressing K5/K14 to express K1/K10 to form intermediate filaments, which participate in the dynamics of desmosomes and therefore in the stratification of the epidermis, as has been observed in K1 and K10 knockout mice [64]. It has been observed that desmosomes are larger and more abundant in lower layers of the SS than in the upper layers, reducing even more in the SG [65,66]. These variations, as well as the strength of these interactions, are conditioned by modifications in the homophilic (DSC: DSC) or heterophilic (DSC: DSG) expression of proteins, the latter providing greater strength [66,67]. Furthermore, DSG 2 and 3 of the lowest epidermal layers are exchanged for DSG 1 and 4 in the most differentiated layers. On the other hand, DSC 2 and 3 are ubiquitously expressed in the epidermis, while DSC 1 appears from the SS [52]. In relation to TJs, while claudin 1 has been detected in plaques of the epidermis from the SB to SG, with greater intensity in the suprabasal layers, occludin is present in the SG and partly, in the transition cell layer, while ZO-1 is expressed from the SG to the upper layers of the SS [61]. Concerning adherent junctions, P-cadherin is distributed only in the SB, but E-cadherin is evenly distributed in all layers of the epidermis and is essential for TJ formation, but not for desmosome [68–70]. On the other hand, gap junction connexin (Cx) proteins are transmembrane channels that communicate adjacent cells [71].

The configuration of the union gap is also related to the differentiation of KCs [72]. Thus, Cx43 is particularly expressed in basal cells, while in more differentiated stages there is a change to Cx26, Cx30 and Cx31 [72,73].

2.3. Cornification Phase

Corneocytes are dead cells that present with electrodense, polyhedral, elongated and flattened morphologies, and are joined to each other by corneodesmosomes, serving as the first line of defense against external agents [38,74]. In this layer, the cornified cell envelope (CCE) is formed and reinforced with a lipid envelope. The process of KC migration culminates in desquamation, i.e., the detachment of the corneocytes [30,55].

Cornification is mediated by substances stored in keratohyalin granules and lamellar bodies. The former contain proteins, such loricrin (LOR) and profilaggrin, which together with involucrin (IVL) give rise to the CCE [75–77]. Lamellar bodies begin to be released before the establishment of TJs, in the SG [78]. They store lipids, proteins, enzymes and their inhibitors that provide substrates to the SC during the final phase of cornification, and for timely desquamation [79]. They also contain antimicrobial peptides, such as human beta-defensin (hBD) 2, hBD3, cathelicidin LL-37 and ribonuclease 7, that serve as part of the skin microbial barrier [80–82]. The corneodesmosomes, junction structures that replace to desmosomes, are widely distributed over the entire surface of the corneocytes of the lower layer of the SC, and to a lesser extent in the superficial corneocytes. These arise when corneodesmosin released from lamellar granules in the SG binds to DSG1 and DSC1, giving rise to the corneodesmosome, which represents an important contribution for the CCE in the replacement of plasma cell membrane with the macromolecular deposition of proteins [76,77]. On the other hand, the profilaggrin released from keratohyalin granules is proteolyzed into filaggrin (FLG) monomers that bind to K1 and K10 filaments of the cytoskeleton, providing mechanical strength and flattering of corneocytes in the most external skin layer [83]. In the intercellular spaces of the SG and SC, large amounts of suprabasin (SBSN), a protein that serves as a substrate for the transglutaminase (TGM) 2 and 3, are deposited [84,85]. By the action of TGM, the corneocytes are then heavily cross-linked and make up the CCE. Later, corneodesmosomes are degraded by kalikrein and other enzymes to initiate natural desquamation, which leads to the establishment of an effective epidermal barrier [76,83,86,87]. Desquamation is essential to maintain the thickness and the self-renewal process of the epidermis [88]. Likewise, in the more superficial cornified layer, nonapoptotic caspase 14 is essential in the catabolism of FLG into hygroscopic amino acids, which are, together with their derivatives, important constituents of so-called natural moisturizing factors (NMF). These FLG degradation products provide moisture, maintain acidic pH and protect from UVB-induced damage [83,89,90].

The SC is elemental in the prevention of transepidermal water loss, a marker of the inside-outside barrier; however, this function is favored by cohesion between the corneocytes and because the corneocytes are covered by a lipid sheet made up mainly of cholesterol, phospholipids, and glycoceramides [91,92]. The production of this lipid envelope has been related to the decrease in pH that characterizes this stratum. A healthy SC has an average pH of 4.7 [93] that is maintained by different mechanisms, among them microbiota colonization [94,95]. Differential distribution of moisture and water retention in the skin favors the establishment of a suitable niche for differential colonization of microorganisms [23].

It has been reported that in the last stages of KC differentiation, and the first stages of cornification, there is an increase in the expression of enzymes and antioxidant elements, which together with the structural proteins are fundamental to the homeostasis of the epidermis [96,97]. The main enzymes detected in the epidermis are superoxide dismutase, glutathione peroxidase, and glutathione reductase; however, lipophilic and hydrophilic nonenzymatic antioxidants, such as α-tocopherol and ubiquinol, or ascorbic acid, uric acid and glutathione, respectively, are also found [98].

3. Participation of Keratinocyte in Predisposition, Onset and Progression of the Atopic Dermatitis

AD is a chronic inflammatory disease of the skin characterized by eczematous lesions and a sensation of intense itching. It originates from alteration of the integrity of the epidermal barrier as a consequence of the interaction between genetic and environmental factors [99–102]. Due to skin barrier defects, there is an increase in the permeability to antigens, both of chemical and protein nature. Penetrating antigens interact with KCs, which are described as hyperactive in people with AD, generating exaggerated responses characterized by an excess of cytokines and chemokines that promotes local inflammatory processes. At the onset, lymphocyte T-helper (Th) response is predominantly via Th2/Th22 with slight Th17 participation [103–105], but after chronic allergen exposition, Th1 cells join this enhanced response [104]. The cocktail of cytokines released increases deterioration in the epidermal barrier, inducing disease progression, which is known as the outside-inside-outside hypothesis [106–108]. In the following sections, we review the participation on the KC in different stages of AD, including genetic alterations in the cell that predispose to disease onset, and the KC responses to different stimuli that favor the development, maintenance and progression of AD.

3.1. Genetic Background of the Keratinocyte Predisposing to Atopic Dermatitis

It is widely accepted that AD is a multifactorial disorder. As aforementioned, the inclination to develop the disease results by the interaction of genetical and environmental factors [109]. Although environmental factors play an important role as triggers of AD, genetic factors stand out for predisposition. Family history strongly impacts on the tendency to develop AD. A study in Hungary demonstrated that 65.5% of children with AD had an ascendency of atopic diseases [110], while in Sweden, parental history of asthma and/or allergic rhinoconjunctivitis and allergy to furred animals and/or pollen increased the odds ratio (OR) to 2.0 for AD in children up to 4 years [111]. Genetic predisposition is a highly determinant factor to increase the susceptibility of developing AD. Genetic alterations related to AD, and in which the KC has important participation, include those that represent epidermal barrier dysfunctions and dysregulation of innate immune response.

The human *FLG* gene (*FLG*) is located in a locus named epidermal-differentiation complex on chromosome 1q21 and, as summarized in Table 1, many polymorphisms and loss-of-function (LOF) mutations in *FLG* generate a high risk of developing AD [102]. In homozygotes or compound heterozygotes LOF mutations of the *FLG* gene, the expressed FLG is impaired, which leads to compromised integrity of the epidermal barrier [83]. Although many mutations have been described worldwide, Asian populations have high frequency of the distribution of these genetic variations on AD patients. Commonly, a LOF mutation in *FLG* acquires more importance in AD onset in the first two years of life, while AD development in childhood or adults may be influenced by other factors [112]. It is surprising that some mutations do not affect the levels of NMF, which may indicate that type 2 skin inflammation causes NMF reduction [113,114].

Table 1. Polymorphisms and mutations associated with AD with participation of KCs.

Gene	Polymorphism/ Mutations	Mechanism	Prevalent Populations	OR	Reference
Structural Genes					
FLG	P478S	Prevents the protease cleavage through serine phosphorylation, and then affects the FLG aggregation to keratin filaments	Asian (Taiwan) Asian (Korean)	5.67 1.877	[115,116]
	3321delA	Premature termination codon 41 bases downstream that stops protein translation in filaggrin repeat domain 2	Asian	3.54	[112,117]
	S2554X, S2889X, S3296X, K4022X, R501X	Nonsense mutations	Asian	3.54	[112,117]
	2282del4	Deletion of four base pairs that results in a premature stop codon and complete loss of FLG production	Northern European European American African American	NR 5.6 2.5	[118,119]
Innate immune response					
TLR2	R753Q	Alteration of the function of the intracellular signaling portion homologous to the IL-1 receptor designated as Toll/IL-1 receptor (TIR) domain.	German Italian	NR NR	[120]
	R677W	Associated with reduced NF-κB activation and to increase the risk of bacterial infection			[121]
TLR4	D299G	Impaired dimerization of TLR4 and MD-2 in presence of ligand	Italian	2.46	[122,123]
DEFB1	A692G	Generates an NF-kB transcription factor-binding sequence in the position -20 of the 5′ untranslated region (5′ UTR) with a plausible effect on the expression of hBD2	Mexican	3.21	[124]
	G1654A	In exon 2, meaning a changed Val37Ile next to six conserved cysteine residues that could affect its folding	Mexican	17.37	[124]
TSLP	rs11466749	Means a change 813A/G	European American	0.6	[125,126]
	rs10043985	Means a change 597T/C	African American	0.5	[125,127]
	rs2289276	Means a change 1350C/T	American African	1.8	[125,127]

OR, odds ratio; NR, no reported.

Other genes also expressed in KCs and involved in the innate immune response have been associated with AD development. The Toll-like receptors (TLRs) 2 and 4 have an important role in the activation of the innate immune response on AD [128,129]. For the TLR2 receptor, two clinically relevant single nucleotide polymorphisms (SNPs) with missense changes have been documented, namely R753Q and R677W [130,131], and for TLR4 two SNPs, D299G and T399I, have been described [132]. Another member of the innate immune response secreted by KCs is hBD2 [133]. In the *DEFB1* gene some SNPs have substantial effects on the function of hBD2,with direct impacts on AD onset, namely A692G and G1654A [134,135]. Thymic stromal lymphopoietin (TSLP) is produced by KCs and induces generation of Th2 cells with consequences in the pathogenicity of atopic diseases [136]. Although polymorphisms have been shown to increase the risk for AD (rs2289276), some others have a protective effect (rs11466749 and rs10043985) [125].

Continuous improvement of knowledge concerning polymorphisms that participate in the development of AD, and technological advances in genotyping, will provide better tools to establish strategies for decision making, even before the appearance of the symptoms of AD.

3.2. The Keratinocyte in the Primary Origin of Atopic Dermatitis

Some authors indicate that patients in the first stage of the disease present nonlesional skin that is visibly normal and devoid of clinically apparent disease but presents abnormalities in relation to a normal skin [104,137]. Nonlesional AD skin is characterized by increased epidermal thickness and proliferation index, T-cell infiltration, type-2, -22, -17 cytokine and epidermal S100 protein expression, but a reduction in the expression of proteins of KC terminal differentiation, such as LOR, FLG and IVL, as compared to normal skin [104,138]. Anther protein affected in AD development is SBSN, which is also expressed in the epithelial differentiating layers during cornification [84]. Although its function is not fully understood, it is known that shRNA-mediated SBSN knockdown

promotes poor formation of keratohyalin granules and affects the development of the SG [85]. In intrinsic AD patients, SBSN is substantially decreased, particularly in those with a nickel allergy [85,139]. These cutaneous abnormalities are exacerbated in lesional skin; thus, nonlesional skin shows an intermediary phenotype between normal and lesional skins, though closer to the latter as immune and epidermal alterations in nonlesional skin are associated with disease extent and severity [104,138]. On the other hand, Th2 cytokines are related to some cutaneous characteristics reported in nonlesional skin of AD patients. Nonlesional skin shows a higher expression of intercellular adhesion molecule-1 (ICAM-1) in basal epidermal cells compared to skin biopsies of healthy individuals. However, when the latter are stimulated with interleukin (IL)-4 there is an increase in expression of ICAM-1 and vascular cell adhesion protein (VCAM), two adhesion molecules which have been associated with the attraction of leukocytes in the lesion area [140]. Gene expression profiling of lesional and nonlesional AD skin has identified signature genes with remarkable roles in physiopathology of AD [141]. An analysis of 127 samples from five different studies demonstrated several differentially expressed genes involved in AD grouped in: (i) epidermal development and barrier function (up-regulated in AD: *KRT16*, *COL6A6*; down-regulated in AD: *LOCE2B*, *LOR*, *FLG*, *SCEL*, *AQP9*); (ii) growth factors and inflammation (chemokines *CCL17*, *CCL18*, *CCL22* are commonly upregulated in AD, while growth factors such as *PGF2*, *EREG*, *OGN*, are down-regulated); (iii) epidermal proteases (*KLK5*, *SERPINB3*, *SERPINB4*, *SERPINB7*, *TMPRSS4*, are up-regulated in AD); (iv) antimicrobial function (*MSMB*, *LTF*, *SCGB2A1* are down-expressed; however, *DEFB4* that codifies β-hBD2 is usually up-regulated likely in response to bacterial infection); and finally, (v) epidermal lipids metabolism (*FADS1*, *FAR2*, *FABP7*, *GPD1* are down-regulated in AD) [142]. Furthermore, human KCs stimulated with IL-4 and IL-13 reduce the expression of the barrier proteins LOR and IVN, that are also diminished in nonlesional skin of patients with AD [143]. However, there is still a lot of controversy about the primary origin of AD, since most of the studies related to this first stage in humans were carried out in areas of nonlesional skin from patients with chronic AD. For this reason, an attempt to explain these mechanisms using human KC cultures or murine models has been made.

In addition to the genetic factors mentioned above, environmental factors such as UVB radiation or pollutants have been associated with the deterioration of the skin (Figure 3). UVB radiation induces the production of reactive oxygen and nitrogen species (ROS and RNS) by KCs, which are involved in apoptosis and cell death, and the synthesis of inflammatory mediators and enzymes, such as IL-6, IL-8, prostaglandin (PG)E_2 and cyclooxygenase-2 (COX-2), mainly through MAPK, NF-κB and AP-1 pathways [144,145]. Furthermore, studies in other cell populations have shown that ROS also have an important role in the activation of the NLRP3 inflammasome and the subsequent activation of caspase-1 needed for IL-1β secretion [146]. UVB radiation also stimulates TSLP expression in KCs through HIF-1α-dependent mechanisms via the JNK and ERK pathways [147]. Meanwhile, particulate matter (PM) present in air pollutants and mainly composed of a mixture of metals, organic compounds, materials of biologic origin and ions, has been associated with the incidence of AD in young people [148]. Jin et al. demonstrated that PM can enter into human KCs in vitro and induce the synthesis of IL-8 and matrix metalloproteinase (MMP)-1 in a ROS-dependent manner. However, for PM to penetrate the epidermis, barrier-disrupted skin is necessary, as demonstrated in murine models [149]. KCs can be also activated by air pollutants through the aryl hydrocarbon receptor (AhR), contributing to TSLP and artemin expression [150]. On the other hand, antigens or sensitizing agents of an electrophilic nature activate KC signaling through interaction with cysteine residues (thiol groups) of the cell membrane, producing ROS that induce cell death, ATP release and the degradation of skin hyaluronic acid in compounds of low molecular weight that have been reported as endogenous TLR ligands in inflammatory cells [151,152]. As the activation of KCs by endogenous ligands through TLR4 induces the synthesis of IL-23, a cytokine that stimulates the shift from T CD4 lymphocytes to Th22 [153], it might trigger the development of a specific T-response to antigens passing across the deteriorated

epidermis and induce AD development. Activation of KCs by house dust mite (HDM) allergens may also contribute to AD onset. *Dermatophagoides pteronyssinus* induces NLRP3 inflammasome activation in a ROS and ATP-independent, but cysteine protease-dependent manner, stimulating KCs to release the proinflammatory cytokines IL-1β and IL-18 [154]. In human KCs and murine models, *Dermatophagoides farinae* extract induces TLR1 and TLR6 activation and promotes the synthesis of the innate proallergic cytokines IL-25 and IL-33 through IL-1 receptor-associated kinase 1 (IRAK 1), transforming growth factor (TGF)-β activated kinase-1 (TAK1), IκB kinase and NF-κB pathways, thus conditioning a Th2 response [155].

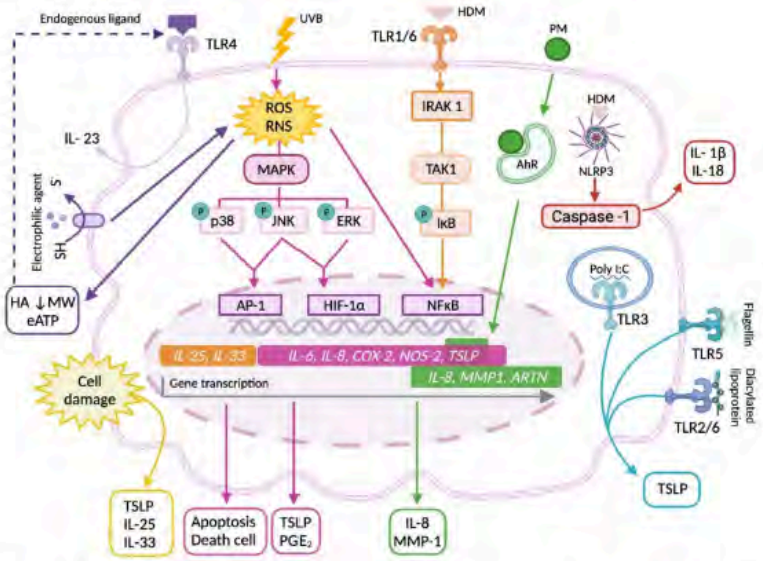

Figure 3. Keratinocyte response to environmental factors. In response to UVB radiation, allergens, endogenous ligands, electrophilic agents, particulate matter present in air pollutants, or ligands of pathogen pattern receptors, KCs activate different signaling pathways that induce apoptosis and cellular death, gene transcription and the release of de novo synthesized type-2 response promoting cytokines and inflammatory mediators, or caspase-1 activation and the subsequent maturation of inflammatory cytokines pro-IL-1β and pro-IL-18. Abbreviations: AhR, aryl hydrocarbon receptor; COX-2, cyclooxygenase-2; eATP, extracellular ATP; HA, hyaluronic acid; HDM, house dust mite; IL, interleukin; MMP-1, matrix metalloproteinase-1; MW, molecular weight; NOS-2, nitric oxide synthase -2; PGE_2, prostaglandin E_2; TSLP, thymic stromal lymphopoietin. Created with BioRender.com (access date: 26 August 2021).

In murine models, it has been observed that deterioration of the skin by itself induces the production of TSLP [156–158], IL-25 [159,160] and IL-33 [161] by KCs. In recent years, TSLP has been proposed as a possible serum marker of AD in humans, together with thymus and activation-regulated chemokine (TARC; chemokine ligand (CCL) 17) [162]. In this context, the production of TSLP can be induced from human KCs by ligands of TLR3 (polyinosinic-polycytidylic acid [poly I: C]), TLR5 (flagellin) or TLR2/6 (diacylated lipoproteins or *Staphylococcus aureus*) [129,163,164], and this effect is enhanced by tumor necrosis factor (TNF)-α, Th2 cytokines and type I interferons (IFNs) [163,165]. The importance of KC-derived cytokines (namely, TSLP, IL-25 and IL-33) is as follows (Figure 4). TSLP actives CD11c DCs, inducing its survival and overexpression of the costimulatory molecules CD40 and CD80 and the chemokines TARC and macrophage derived chemokine (MDC o CCL22) [158,165]. Furthermore, in murine models, TSLP, IL-33 and IL-25 activate ILC2, which is overexpressed in damaged skin [159–161,166]. TSLP-activated DCs together with ILC2 induce allogenic naïve CD4+ T cell proliferation and their Th2 polarization to

produce IL-4, IL-5, IL-13 and TNF-α [158,160,167], which could trigger atopic march, as shown in murine models [157]. Likewise, in response to IL-4 and IL-13, B cells carry out isotype change of the Ig heavy chain to produce allergen-specific IgE antibodies, which collaborate in allergen up-taken by skin DCs to amplify T cell activation [137].

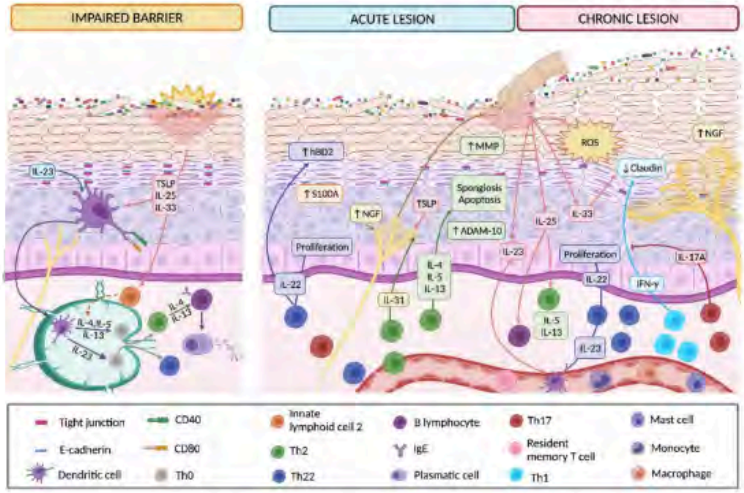

Figure 4. Keratinocyte participation in the onset, development and chronification of atopic dermatitis lesions. Due to an impaired cutaneous barrier, KC-derived cytokines (mainly TSLP, IL-23, IL-25 and IL-33) induce dendritic cell activation and mobilization to nearby lymphatic nodes where they active naïve CD4+ T cell (Th0) and promote Th2 and Th22 polarization. Th2-derived cytokines trigger antibody isotype switching in B lymphocytes to produce IgE. Cytokines produced by Th2 and Th22 cells increase KC proliferation and S100A expression, prompt KC apoptosis and induce cutaneous spongiosis due to a decrease in E-cadherin levels mediated by increased enzymatic activity. These changes are clinically manifested in skin as acute lesions. In this stage, IL-22 and hBD2 mutually enhance their production, perpetuating the inflammatory response associated with AD. Pruritic mediators released by Th2 cells (IL-31) or KCs (NGF and TSLP) increase itching sensation which triggers scratching and worsening of skin lesions. Lesions becomes chronic due to the intensification of the pre-existing Th2 and Th22 inflammatory response enhanced by Th1 and Th17 cytokines. Augmented production of IL-23, IL-25 and IL-33 by KCs maintains Th22 differentiation, up-regulates Th2 cytokine production and diminishes claudin expression, respectively. The decrease in tight junctions, which is enhanced by IFN-γ production, together with the increased levels of NGF, favors epidermal hyperinnervation. Altogether, this inflammatory environment exacerbates the remodeling processes of the epidermis and hyperplasia, while IL-17 down-regulates the IFN-γ effect on claudin expression. Abbreviations: hBD2, human beta-defensin 2; MMP, matrix metalloproteinase; NGF, nerve growth factor. Created with BioRender.com (access date: 26 August 2021).

3.3. Involvement of Keratinocytes in Acute Lesions

Acute skin lesions are usually presented as erythematous, itchy papules with serous exudation [168]. As a consequence of continuous scratching, secondary lesions are generated, which include excoriation and crusted erosion. Subacute lesions may also appear as erythematous scaling papules and plaques [169]. These clinical conditions are similar in humans and dogs, thus dogs are used to understand the inflammatory changes underlying AD skin lesions. A transcriptome study carried out in dogs sensitized with HDM showed that, as a result of epicutaneous challenge with an allergen, acute skin lesions were accompanied by a marked genic expression of Th2 and Th22 cytokines, particularly IL-5, IL-13, IL-31 and IL-22, together with the Th2-promoting chemokines CCL5 and CCL17 [170]. In addition to the pruritogenic cytokine IL-31, other genes encoding pruritogenic pathways were also upregulated in dog biopsies, including several proteases (chymase, tryptase,

cathepsin S), enzymes involved in leukotriene-synthesis, neuromedin-B, and nerve growth factor (NGF) [170]. This Th2 and Th22 dominance coincides with that manifested in acute AD lesions of patients, which are also characterized by an increase in the levels of expression of a subset of epidermal differentiation complex gene products, mainly S100A7 and S100A8 proteins, in the KCs from the upper SS and SG layers [104]. In humans, robust Th2/Th22 activation is accompanied with some IL-17 skewing, which is significantly higher in children than in adults [105]; however, no up-regulation of Th17 cytokines has been detected in acute AD lesions in dogs [170].

This inflammatory environment influences KC structure and, at the same time, the KC-derived mediators create feedback during inflammation (Figure 4). It has been shown that IL-22 increases the proliferation of KCs and inhibits their differentiation through activation of the MAPK signaling pathway and the decrease in the expression of CCAAT enhancer binding protein-α, a transcription factor that regulates the development, proliferation and differentiation of KCs [171]. Th2 cytokines have a negative effect on the integrity of the KC, since they can induce spongiosis by decreasing E-cadherin expression and increasing the intercellular accumulation of hyaluronic acid and inducing cell apoptosis [108,172,173]. The loss of E-cadherin from the KC surface is generated by an increase in the activity of metalloproteases such as ADAM-10 [173]. Elevated levels of MMP-8 and MMP-9 in the SC from the AD acute lesion, might also contribute to the tissue remodeling process [174]. In the acute inflammatory stage associated with AD, increased serum levels of hBD2 correlated with a high level of IL-22 have been described. Particularly, IL-22 enhances hBD2 production by KCs through activation of the signal transducer and activator of transcription (STAT)-3, and hBD2 increases IL-22 production by CD3/CD28-stimulated T cells via JNK and Akt pathways [174]. hBD2 functions as a chemoattractant of immature DC, memory T cells, macrophages, monocytes and mast cells when binding to CCR6 or CCR2 receptors [175–177], with a potential role in the development of the inflammatory and adaptive responses associated with AD. In mice, KC-derived IL-25 induces the release of high levels of IL-13 from ILC2 during AD acute lesions [159]. Finally, in a mice model mimicking human acute AD lesions, the expression of KC-derived IL-33 was augmented and this cytokine was related to an anti-inflammatory effect on the disease [178]; however, in an AD-model in mice overexpressing IL-33, the levels of epidermal claudin 1 were reduced and IL-33 was also able to down-regulate the expression of this TJ protein in KCs assayed in vitro [179], which suggests a possible dual role of IL-33 in AD pathogenesis.

One of the most prominent clinical manifestations of the AD acute phase is pruritus. In this stage, Th2 cytokines, such as IL-4, together with mechanical damage, act synergistically to produce IL-31, a cytokine related to the activation of afferent fibers of neurons in the skin through the IL-31RA receptor [180,181]. In addition, the KC-produced TSLP has the ability to sensitize afferent nerve endings of the skin through the transient receptor potential channels V1 and A1, which trigger a constant itching sensation that induces an increase in the severity of the lesions [182,183]. NGF, produced by KCs and mast cells, is also involved in pruritus, both directly by peripheral nerve sensitization and indirectly by inducing the expression of neuropeptides such as the gene related to calcitonin peptide (cGRP) and substance P [183–186]. Deeper studies are needed to decipher the exact role of cGRP in AD because recently cGRP has been described as a central negative regulator of ILC2-mediated allergic inflammation [187]. Furthermore, it is known that TJs in the SG are involved in the protection of epidermal nerve endings from external stimuli by nerve pruning, a mechanism that is dysregulated when the epidermal barrier is impaired in an AD lesion, promoting the exposure of nerve endings to itch-inducing agents [188].

3.4. Chronic Lesions and KC Participation

If there is a repetitive and persistent exposure to allergens, and the rash and itch of acute lesions progress uncontrolled, patients may develop chronic AD with skin lesions characterized by lichenification and dry fibrotic papules that presents hyperpigmented skin marks [104,168,169]. Even, patients with moderate to severe AD can experience

acute and chronic lesions simultaneously. In chronic stages, the inflammatory process becomes difficult to control. An augmentative and vicious circle is generated in which the damaged epidermis and the activated KCs induce skin dysbiosis and generate a cutaneous inflammatory response, accompanied by the sensation of itching, that triggers the desire to scratch, which subsequently enhances barrier disruption and closes the loop [189,190]. At this stage, there is an intensification of the pre-existing inflammatory response. Th2 and Th22 cytokines are over-expressed, although a notable decrease of IL-4 and IL-4R is detected, accompanied by an upregulation of markers of the Th1 response, mainly IFN-γ and, in a lower degree, of Th17 [104,137,191,192]. In this regard, it has recently been shown that the IL-4α receptor blockade upregulates IFN-γ-producing cells after activation of lymphocytes from AD patients with staphylococcal enterotoxins B [193].

As shown in Figure 4, the large amounts of IL-25 released by KCs in chronic lesions maintain the production of IL-13, mainly by skin accumulated CD4+ T cells, which contributes to the remodeling processes of the epidermis and hyperplasia development [159]. Mediators released from Th22 cells have also been associated with skin remodeling mechanisms by in vitro assays [194]. The constant lesions generated by scratching induce the production of IL-23 by KCs, which have been shown to induce endogenous expression of IL-23 by DCs that, in turn, shift naive CD4+ T cells to Th22 differentiation, which releases high levels of IL-22 causing hyperplasia of the epidermis and chronicity [153,171]. In addition, epidermal hyperinnervation, which is thought to underlie pruritus, has been observed in patients with AD. Analysis of skin biopsies from patients with chronic AD suggests that this may be due to an increase in NGF production and a decrease in the expression of the epidermal innervation regulatory protein semaphorin A3 [195]. Thus, experimental models of dry-skin, both in vivo and in vitro, have demonstrated that a decrease of epidermal nerve densities generates antipruritic effects [196]. In relation to Th1 and Th17 cytokines, little information exists about their effects on KCs in chronic AD. Recently, IFN-γ has been shown to inhibit claudin 1 expression via the JAK/STAT signaling pathway in normal human KCs, which is reflected in the loss of TJ function in a model equivalent to human skin [191]. Strikingly, IL-17A, which is also abundant in chronic lesions of AD, is able to revert TJ dysfunction induced by IFN-γ [191]. On the other hand, it has been observed that after KC damage, large amounts of ROS are expressed in skin biopsies, intensifying inflammatory responses and aggravating skin pathologies such as AD [197]. In summary, all these mechanical and immunological stimuli cause hyperplasia of the epidermis and prominent hyperkeratosis with minimal spongiosis, shaping the characteristic lesions of chronic AD [198]. However, more studies are needed to understand the predominant cellular and molecular mechanisms underlying AD chronic lesions, which will allow a better clinical follow-up of the patient and more efficient treatments.

3.5. Bidirectional Communication between Keratinocyte and Microbiota

Intercommunication of the KC with the complex microbiota that inhabit the skin is a relevant factor in maintaining the balance between health and disease. More than a commensal relation, microbiota and KCs work in a dynamic manner to generate a favorable ecosystem for both parties. However, when this relationship is interrupted, the normal functions of the skin are altered, making it prone to developing skin diseases.

Members of the genus *Staphylococcus* are common inhabitants of the skin; however, staphylococcal infections are frequent in impaired skin in AD. Colonization with *S. aureus* is present in 70% of AD patients with skin lesions and 39% in those with nonlesional skin [199]. *S. aureus* exacerbates the inflammatory process in AD patients by direct interaction of its structural components and released exotoxins with molecular receptors of KCs (Figure 5) [200]. In human primary KCs, TLR2/TLR6 heterodimer activation by diacylated lipopeptides of *S. aureus* induces mRNA expression and release of TSLP [129]. Moreover, Th2 cytokines IL-4 and IL-13 inhibit KC mobilization of hBD3 from the cytoplasm onto the bacterial surface of *S. aureus* [201], which, in turn, favors the continuous activation of TLR2/TLR6 and, consequently, sustained production of TSLP and more Th2 immune

milieu. On the other hand, *S. aureus* produces and secretes toxins (exotoxins) that damage target cells (cytotoxins) or induce exacerbated cytokine production by stimulation of T cell (superantigens) [128]. For example, secreted α2-toxin, also known as phenol-soluble modulin-α2, triggers a potent induction of cell toxicity in KCs isolated from mice [202,203]. In addition, staphylococcal ε-toxin is cytotoxic to KC and causes a proinflammatory reaction by induction of IL-8 by the cell, as well as delaying the proliferative capacity of immortalized human KCs [203]. Superantigens have also been found to activate KCs. In cultured KCs from lesional skin of AD patients, increased expression of IL-1α, IL-1β and TNF-β is induced after staphylococcal enterotoxins A or B, and toxic shock syndrome toxin-1 stimuli, as compared to those from nonlesional skin or from normal skin of nonatopic patients [204]. Altered cytokine secretion in superantigens-challenged KCs may explain the increased inflammation in staphylococcal lesional skin. It is worthy of note that intracellular *S. aureus* invasion is a critical factor that promotes the persistence of chronic infections, since it allows evasion to antibiotic treatments [205]. Bacterial adhesins in *S. aureus* allow bacterial adhesion to KCs and eventually internalization. Exposure of primary KCs to the secreted extracellular adherence protein from *S. aureus* favors bacterial adhesion and internalization, a mechanism that might be mediated by fibronectin as a bridging factor, and integrin α5β1 in KCs [206].

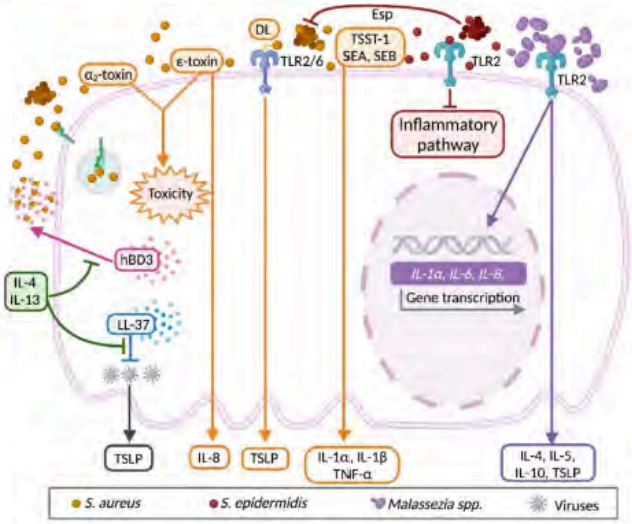

Figure 5. Keratinocyte response to microbiota stimuli under the type 2 immune milieu. KCs recognize bacteria, fungi, and viruses from skin microbiota through specific receptors. Colonization with *Staphylococcus aureus* is present in the skin of AD patients, and the bacteria or their components can activate KCs to secrete proallergic (TSLP) and proinflammatory (IL-1α, IL-1β, IL-8, TNF-α) cytokines, exacerbating the underlying immune response. Exotoxins from *S. aureus* can also damage the cell. Chronic infection of *S. aureus* is partly due to its internalization into KCs through extracellular adherence protein recognition. Th2 cytokines (IL-4, IL-13) avoid *S. aureus* destruction by KC-derived hBD3. As *Staphylococcus epidermidis*/*S. aureus* ratio is diminished in AD skin, the anti-inflammatory and protective effects, and the anti-microbial properties of *S. epidermidis* on KCs and *S. aureus*, respectively, are impaired. In response to *Malassezia* colonization, KCs express proinflammatory IL-1α, IL-6, and IL-8, and release type 2 (IL-4, IL-5, IL-10, TSLP) cytokines. Levels of LL-37 in KCs are down-regulated by type 2 cytokines, increasing viral replication and dissemination of vaccinia, herpes simplex and human papilloma viruses, which at the same time promotes type 2 inflammation through an induction of TSLP expression by the cell. Abbreviations: DL, diacylated lipopeptide; Esp, serine protease; hBD3, human β-defensin 3; LL-37, cathelicidin LL-37; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; TNF, tumoral necrosis factor; TSST-1, toxic shock syndrome toxin. Created with BioRender.com (access date: 30 August 2021).

Staphylococcus epidermidis, a member of the coagulase-negative staphylococci, together with the species *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Staphylococcus capitis*, *Staphylococcus lugdunensis* and *Staphylococcus warneri*, is one of the most prevalent components of the skin microbiota ubiquitously distributed [207–209]. Although *S. epidermidis* is considered a commensal of the skin, eventually it may act as an opportunistic pathogen [210–212], and can even act in a mutualist manner with KCs [94]. Once *S. epidermidis* has colonized external KCs, they stablish a commensal habitat by competing for substrate with other potential pathogens, such as *S. aureus*. Direct production of antimicrobial metabolites has been reported in *S. epidermidis*. The so-called inhibitory-type *S. epidermidis*, isolated from half of volunteer nasal cavities, can reduce biofilm formation of *S. aureus*. This inhibitory capacity is attributed to the *S. epidermidis* serine protease (Esp), which possess serine-protease activity. Remarkably, the microbicidal activity is most observed when combined with hBD2 secreted by KCs [133]. In an experimental model of nasal infection with methicillin-resistant *S. aureus* (MRSA), preinoculated mice with Esp-secreting *S. epidermidis* prevented the nasal colonization by MRSA [213]. In addition to interfering with the colonization of other bacteria on the KC, *S. epidermidis* can modulate the inflammatory response of the KC. It has been demonstrated that the stimulation of primary human KCs by poly I:C through the TLR3 receptor induces the release of inflammatory cytokines such as TNF-α. Noteworthy is that poly(I:C)-treated KCs reduce the production of TNF-α when exocellular lipoteichoic acid from *S. epidermidis* (LTA-Se) is added. Moreover, in vivo experiments have demonstrated that LTA-Se prevents wound-induced IL-6, TNF-α and inflammation in wild-type mice, but this effect is missed in Tlr2−/− mice, suggesting that TLR2 stimulation by LTA-Se prevents inflammatory responses in KCs [21]. Another important relation between KCs and *S. epidermidis* is the capacity to provide an adequate substrate and provide metabolites that improve protection. Autologous application of *S. epidermidis* on the skin of healthy subjects helped to improve skin moisture retention and to keep low acidic skin conditions through the secretion of glycerin, and lactic and propionic acids [94]. These results open the possibility of evaluating *S. epidermidis* application as a new therapy in AD patients.

The main representative of the fungal flora of the skin is lipophilic yeast of the genus *Malassezia* spp. [214]. However, *Malassezia* spp. can behave as opportunistic pathogens and are commonly presented in skin lesions of adult AD patients [215]. Of the nine species found in healthy patients, four of them are frequently presented in patients with mild and severe AD, such as *Malassezia globosa*, *Malassezia restricta*, *Malassezia sympodialis*, and *Malassezia furfur* [216–218]. Skin inflammatory processes in AD can be also mediated by the interaction of KCs with *Malassezia* species due to these fungi modulating the production of proinflammatory mediators [219]. In vitro interaction of the human KC cell line HaCaT, (human, adult, low calcium, high temperature) with *Malassezia* species (*M. furfur*, *M. globosa* and *M. restricta*) induces the expression of IL-8, IL-6 and IL-1α, and this effect is mediated by the activation of TLR2 [220]. Some *Malassezia* species are responsible for the production of type 2 cytokines in KCs. The human primary KCs exposed to *M. globosa* result in an increment of IL-5, IL-10 and IL-13 secretion, whereas *M. restricta* induces IL-4 secretion [221]. As previously mentioned, TSLP has an important role on the induction of the type 2 inflammatory response, and its production has been demonstrated to be induced by *M. globosa* and *M. restricta* in KCs, which might be mediated by the activation of lysophosphatidic acid receptors 1 and 3 of KCs by the lipid layer of *Malassezia* [222].

The impaired skin barrier and the type 2 inflammatory response are important factors that allow higher susceptibility to viral infection in patients with AD [223]. For example, skin from AD patients is characterized by overexpression of the type 2 cytokines IL-4 and IL-13 and, conversely, production of type 1 cytokines IL-12 and IFN-γ is reduced, which in turns reduce the expression of the human cathelicidin LL-37 [224]. This condition makes KCs highly susceptible to experimental infection with the vaccinia virus, which is why AD patients are commonly excluded from smallpox vaccinations since the risk developing eczema vaccinatum is significantly increased [225,226]. Moreover, eczema herpeticum results from the dissemination of herpes simplex virus (HSV)-1 or -2 in AD

patients [227]. In vitro studies have demonstrated that, in the presence of LL-37, the replication of HSV-2 in primary human KCs is reduced in a dose-dependent manner [228]. An altered antiviral innate immune response in KCs has been proven to be due to reduced levels of the transcription factor specificity protein 1, which is related to an enhanced replication of vaccinia virus and HSV in AD patients and leads to the overexpression of TSLP and six members of the family of human kallikreins in the KCs [229,230]. The frequency of AD is also potentially related to the susceptibility and persistence of high-risk HPVs [231]. Certainly, compromised epithelial barrier function in AD makes an easy target for viral infection, and some viruses express proteins able to induce changes toward atopy. In transgenic mice (K14.E7), the expression of the oncoprotein E7 from HPV16 driven by the K14 promoter, which restricts the E7 expression to epithelial cells in the skin, results in the characteristic lesions of AD accompanied by increased production of TSLP and total IgE [232]. Viral dsRNA and a Th2 cytokine milieu might promote type 2 inflammation through an induction of TSLP expression by KCs, suggesting the existence of a vicious cycle between AD and viral infections [163]. In the case of the measles virus, some studies have described an increase in the incidence of AD after vaccination [233], while others have shown a decrease in the risk of AD after measles infection [234], or an improvement in skin lesion or immunological parameters after natural infection or vaccination [235,236]. This controversy was addressed considering the role of KCs in the induction of atopy. When HaCaT cells were exposed to the measles virus, the expression of TSLP and CCL26 (eotaxin-3, a chemotactic factor for eosinophils and basophils) was diminished, while TGF-β was overexpressed. In the same report, a clinical protocol in which patients with moderate AD received a measles vaccination demonstrated that lesional skin reduced the expression of TSLP and CCL26, which was accompanied with reduction of clinical scores of AD [237,238].

Although growing evidence about the interaction of skin with microbiota is more available every day, complete understanding of this complex relationship remains one of the major goals in the field. Undoubtedly, the discover of all mechanism generated by KC-microbiota interactions will provide insight into immunomodulatory activity in AD.

4. The Use of the Keratinocyte as an In Vitro Model of Atopic Dermatitis

The great versatility of KCs has allowed their use as a model to study multiple pathological processes, including AD. These models are highly variable, but can be grouped into two- or three-dimensional groups.

Two-dimensional monolayer cultures are based on the adherence of cells to a glass or polystyrene surface that provides a mechanical support for the cells [238]. In this way, the response of KCs to any damage can be specifically and easily evaluated. The sources of these cells are usually primary human KCs from a healthy patient or with AD, or immortalized human KCs [239]. Monolayer cultures of KCs are achieved due to epidermal differentiation of KCs as a consequence of confluence, cell-cell interactions, or specific factors such as calcium or EGF under conditions of subconfluence [114,240,241].

The advantage of working with KCs from patients with AD as in vitro model of the pathology, is that these cells are already genetically modified and conditioned for the development of pathology [239]. However, the pathological status of AD can be mimicked using normal human epidermal KCs (NHK) or immortalized cell lines. The most widely used cell line for this type of model is HaCaT cell line, that harbors nononcogenic mutations in the *TP53* gene [242], and retains its differentiation properties [243]. It is known that the exposure of KCs to type 2 cytokines, mainly IL-4 and IL-13, induces their differentiation to the pathophysiology conditions of AD. Accordingly, HaCaT cells exposed to both IL-4 or IL-13 attenuate the expression of genes that regulate epidermal cell structure and barrier function in the terminal stage of KC differentiation, such as proteins highly expressed in the SS and SG of healthy skin: K1, K10, DSM 1 and DSC 1. The effect is mediated through mechanisms dependent on the receptor IL-4Rα and STAT-6 at early stages of KC differentiation [244]. Furthermore, human primary KCs treated with both IL-4 and

IL-13 reduce the gene and protein expression of FLG; contrary to up-regulated expression generated by IFN-γ exposure [114]. Likewise, Dang et al. demonstrated that the silencing of *FLG* in NHKs in vitro causes a reduction in the protein expression of K5, K10 and K14, IVL, and TGM 1, along with an increase in LOR, altering the function of the cutaneous barrier mainly at the SC layer level [245]. Furthermore, *FLG* silencing was accompanied by an increase in the generation of IL-4, IL-5, IL-13 and IL-2, as well as a decrease in IL-12 and IFN-γ. In response to cytokines, NHK and HaCaT cells modify in a different manner the gene expression of proteins associated with the CCE. Thus, NHK cells stimulated by IFN-γ, IL-4 or IL-17A, or to a lesser extent with IL-22, decrease the expression of FLG, LOR and K10, and only when stimulated by IFN-γ a significant increase in TGM 1 and TGM 2 is detected [246]. HaCaT cells diminish the gene expression of *K10* and *IVL* only in response to IL-4, and show an increase in *FLG* and *TGM 2* [247], suggesting that HaCaT cells might be a poor model to study the integrity of the epidermal barrier under a cytokine-dependent environment. However, hBD2 transcriptional profiles in response to IFN-γ, IL-17A or IL-4 were similar between HaCaT cells and NHKs, being up-regulated in response to the first two cytokines and down-regulated upon stimulation with IL-4 [246,247].

As previously mentioned, various in vivo experimental studies have shown that AD is a very complex pathology. Although it is characterized by the prevalence of a Th2 environment, IFN-γ has been identified as a crucial cytokine in AD patients with chronic lesions. Therefore, it has been proposed that several in vitro models based on KC stimulation with cytokines can be used for a greater approximation to what really happens at molecular level in AD. Thus, the stimulation of HaCaT cells with IFN-γ together with TNF-α for 6 h induces the expression of IL-1β, IL-6, CCL17, CCL22 and IL-33 at the gene and protein level [248]. Furthermore, after 24 h of exposure with the same cytokines, the up-regulated expression of CCL17, CCL22 and IL-33 is maintained, and a reduction in *FLG* and *LOR* mRNA levels is observed. When cells are stimulated with IL-4, only an increase in IL-13, IL-5 and IL-25 is shown, a phenomenon that is reversed when IL-4 is combined with TNF-α [249]. Therefore, as long as the characteristics of the problem are adequately defined, the stimulation of cultured HaCaT cells with cytokines is a good approach for studying the cellular and molecular aspects of AD. Thus, as the experimental conditions get closer to the real pathophysiology, a better understanding of the disease will be obtained. However, the monolayer models present various limitations, prime among which is their lack of stratification; therefore, the interest in the development and characterization of the three-dimensional models is growing.

Three-dimensional models of human reconstructed epidermis arise from the differentiation of normal human KCs in a chemically defined medium under exposure to an air-liquid interface [250]. There is a variant where the differentiation of KCs starts in a matrix formed by fibroblasts and collagen [251]. Both models emerge as possible strategies in the study of pharmacological treatments for lesions of various origins. Particularly, addition of inflammatory molecules enables the creation of a compromised RE model presenting many AD-like characteristics, such as abnormal differentiation, higher secretion of proinflammatory molecules by KCs and a specific gene expression pattern [252]. In this context, both the IL-4, IL-13, IL-31, TNF-α and the IL-4, IL-13, poli I:C, TNF-α inflammatory cocktails induce edema, better known as spongiosis, within the lower layer of the epidermis of a human reconstructed epidermis model, as well as TSLP secretion by KCs and the down-regulation of *FLG* gene expression [252,253]. In relation to lipid composition, TNF-α alone or combined with Th2 cytokines and IL-31 only affects SC lipid composition, mimicking changes observed in AD patients [253].

5. Pharmacological Therapy to Restore Keratinocytes

The use of topical agents is still the strategy of choice for the treatment of AD. Although severe cases require a combinatory regimen with phototherapy, systemic antibiotics, immunomodulatory drugs or monoclonal antibodies, the modulation of the KC response in AD is key for patient recovery. Mostly, systemic therapies are directed to achieve a

systemic immunomodulation of the cellular and molecular elements involved in the type 2 response [254]. However, the KC is a cell that may actively induce exacerbation of the AD symptoms by the production of proinflammatory and prurigenic/pruritic mediators, so many of the topical treatments are focused on reducing this response.

Moisturizer products are highly recommended to improve moisture content in the SC, which is decreased in AD, as they help to treat xerosis and prevent allergen invasion and relapse of dermatitis, as well as suppression of itching by recovering and maintaining skin barrier functions [255,256]. In a randomized controlled trial of infants with moderate to severe AD, it was revealed that regular emollient use reduced the need for topical corticosteroid use and improved symptoms [257]. The use of topical corticosteroids together with emollients is a valuable therapeutic approach to reduce inflammation and pruritus. Hydrocortisone increases the activity of fatty acid synthase, a key enzyme in fatty acids synthesis in KCs in the SG that promotes the secretion of free fatty acids [258]. Topical betamethasone was shown to normalize epidermal differentiation and reduce epidermal hyperproliferation, although it led to epidermal thinning [259]. Betamethasone also promoted a diminution of the transepidermal water loss in AD patients [259]. However, in a post-treatment phase, an impairment of skin barrier function was measured as the rates of water accumulation returned to initial levels [260]. Although these results indicate the effect of betamethasone on skin hydration, it might also modify the induction of the type 2 cutaneous response since it is able to downregulate TSLP expression in NHK [261]. Although antihistamines are not recommended, they still represent the therapeutic regimen of choice among dermatologist for the treatment of pruritus [262]. Particularly, sedating antihistamines are used in the pediatric population to help patients with a negative impact of AD on sleep. Cetirizine, a histamine H1-receptor antagonist, has shown an anti-inflammatory effect in vitro that is mediated by the reduction of IL-8 production on the macrophage migration inhibitory factor (MIF)-stimulated human KC A431 cell line. Moreover, in a direct form, cetirizine also inhibits MIF production in A431 cells [263]. However, no evidence of improvement of clinical signs of AD was observed in patients treated with oral H1 antihistamines, included cetirizine, as an adjuvant therapy alongside topical agents [264].

Although topical corticosteroids are the standard treatment in AD, inhibitors of calcineurin topically applied, mainly tacrolimus and pimecrolimus, are broadly used since few adverse effects have been reported [265]. Although tacrolimus (FK506) has a relatively high molecular weight (822 Da) and shows good skin penetration activity, it is commonly used topically in conjunction with paraffin-based ointments [266]. The immunomodulatory effect of tacrolimus is mediated after binding immunophilins (mainly FK506-binding protein-12, FKBP-12) and the formed complex (tacrolimus – FKBP-12) then binds to the phosphatase calcineurin and inhibits phosphatase activity, which in turn prevents the nuclear factor of activated T cells (NFAT) dephosphorylation and translocation to the nucleus [267–269]. Hence, it suppresses the activation of T cells by reducing the production of IL-2 and other proinflammatory cytokines. The anti-inflammatory activity of tacrolimus has been observed in KCs. When NHKs are exposed to UVB, the secretion of TNF-α is significantly increased, as in the milieu of AD [270,271]. However, when KCs are treated with tacrolimus, the activation and translocation of NF-kB is reduced in a dose-dependent manner that downregulates the production of TNF-α [271]. Moreover, in TNF-α-stimulated NHKs, the secretion of TGF-β is incremented by tacrolimus and, conversely, the expression of inducible nitric oxide synthase (iNOS) is downregulated, which probably is associated with its therapeutic efficacy in the treatment of AD [272]. The chemokine RANTES (regulated on activation, normal T expressed and secreted) is a potent inductor for eosinophils that is increased in lesional skin of AD patients and is produced by KCs after stimulation with inflammatory cytokines [273]. In Korean AD patients, daily treatment with 0.03% tacrolimus ointment for 8 weeks significantly reduced the number of RANTES-positive cells in lesional skin [274]. In human KCs, the overexpression of RANTES induced by IFN-γ and IL-4 is significantly reduced by 10^{-8} or 10^{-6} M of tacrolimus, indicating its

possible role in the amelioration of AD through KCs targeting [275]. On the other hand, the pimecrolimus (DZ ASM 981) immunomodulatory mechanism of action is similar to that of tacrolimus [267], although less information is available concerning its effects on KCs. In a model of 2,4-dinitrochlorobenzene (DNCB)-induced AD in NC/Nga mice, topical treatment with pimecrolimus reduced the expression of TSLP [276], which, as previously mentioned, is an IL-7-like cytokine highly expressed in KCs after stimuli [277]. In addition, pimecrolimus impaired the activation of NFAT2 in human KCs from the outer root sheath and some of the inner root sheath of the hair follicles [278].

Finally, phosphodiesterase 4 (PDE4) is an enzyme degrading cyclic adenosine monophosphate (cAMP). Among PDE4 inhibitors, apremilast and crisaborole have been broadly used in the treatment of AD, proving to be modest to highly effective in cases of moderate to severe AD [279–281]. In AD patients, PDE4 is highly active in mononuclear leukocytes [282]. Moreover, in IL-1α-stimulated primary human KCs, the mRNA expression of IL-8 and TNF-α is reduced in a dose-dependent fashion in presence of the novel and selective PDE4 inhibitor DRM02 [283]. However, posterior evidence has demonstrated that mRNA expression of TNF-α, IL-1α and CXCL8 in PGE_2-treated NHKs treated with apremilast is reduced, without changes in phosphorylation of the cAMP-PKA-CREB pathway, suggesting an alternative cAMP-independent mechanism that down-regulates these mediators [284]. In human KCs, apremilast reduces the expression of the inflammatory mediators IL-12/IL-23p40, IL-31, CCL5, and alarmins S100A7, S100A8 and S100A12, under stimulation of the type 2 cytokine IL-4 [285]. Then, PDE4 inhibitors demonstrate favorable improvement on the severity of AD by targeting the response of KCs.

6. Conclusions

AD is a heterogeneous skin disease characterized by skin barrier dysfunction, skin inflammation and intense pruritus. Although great advances in the understanding of this cutaneous disease have been achieved over the last years, its pathogenesis is still enigmatic, resulting in a lack of specific treatments. This is further complicated by the lack of data to address whether acute and chronic AD represent progressive stages across a continuum of inflammatory responses, or if each has distinct immunologic mechanisms and diversity among individual patients. However, it is evident that the complex interplay of KCs with environmental agents, skin microbiota, inflammatory cells, and nerves is critical in AD onset, development, progression and chronicity. Current advances in understanding the accurate participation of KCs in AD pathogenic mechanisms may facilitate new drug development, as KC restoration improves local immune dysregulation and avoids cutaneous infection.

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II. Glycomacropeptide protects against inflammation and oxidative stress, and promotes wound healing in an atopic dermatitis model of human keratinocytes

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Glycomacropeptide Protects against Inflammation and Oxidative Stress, and Promotes Wound Healing in an Atopic Dermatitis Model of Human Keratinocytes

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Abstract: Keratinocytes are actively implicated in the physiopathology of atopic dermatitis (AD), a skin allergy condition widely distributed worldwide. Glycomacropeptide (GMP) is a milk-derived bioactive peptide generated during cheese making processes or gastric digestion. It has antiallergic and skin barrier restoring properties when it is orally administered in experimental AD. This study aimed to evaluate the effect of GMP on the inflammatory, oxidative, proliferative, and migratory responses of HaCaT keratinocytes in an in vitro AD model. GMP protected keratinocytes from death and apoptosis in a dose dependent manner. GMP at 6.3 and 25 mg/mL, respectively, reduced nitric oxide by 50% and 83.2% as well as lipid hydroperoxides by 27.5% and 45.18% in activated HaCaT cells. The gene expression of *TSLP*, *IL33*, *TARC*, *MDC*, and *NGF* was significantly downregulated comparably to control by GMP treatment in activated keratinocytes, while that of *cGRP* was enhanced. Finally, in an AD microenvironment, GMP at 25 mg/mL stimulated HaCaT cell proliferation, while concentrations of 0.01 and 0.1 mg/mL promoted the HaCaT cell migration. Therefore, we demonstrate that GMP has anti-inflammatory and antioxidative properties and stimulates wound closure on an AD model of keratinocytes, which could support its reported bioactivity in vivo.

Keywords: bioactive peptides; glycomacropeptide; atopic dermatitis; keratinocytes; cytoprotection; immunomodulation; oxidative stress; wound healing

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1. Introduction

The skin is the organ responsible for protecting the body from external agents. The epidermis, the skin's outermost layer, acts as a barrier to prevent or hinder antigen penetration and pathogen invasion. It is structured by keratinocytes at different stages of differentiation, which are responsible for maintaining skin homeostasis [1]. Disruption of epidermal homeostasis due to functional defects, genetic predisposition, and immune dysregulation causes the onset of the atopic dermatitis (AD), one of the most abundant skin diseases worldwide [1,2]. Although its prevalence varies according to the geographical area, AD occurs in up to 34% of children [2]. This disease commonly appears in early childhood and usually resolves before puberty, although it persists in 2–5% of adults [3]. It is identified as the beginning of the atopic march, an epidemiological theory that proposes that early development of AD predisposes patients to other atopic conditions later in life [4]. In addition, AD represents a significant economic burden for the health sector and patient families and is considered an important global health problem [5].

AD is a chronically relapsing skin inflammatory disease that is triggered in susceptible patients after the constant stimulation of the epidermis with environmental antigens. Keratinocytes are actively implicated in the onset, maintenance, and exacerbation of the inflammation in this disease. The dysfunctional immune response in AD is characterized by a Th2-dominance with an increased production of tumoral necrosis factor (TNF)-α, interleukin (IL)-4, -5, -9, and -22 in lesional skin [1]. Thymic stromal lymphopoietin (TSLP), IL-33, thymus- and activation-regulated chemokine (TARC), and macrophage-derived chemokine (MDC) released by keratinocytes participate in the Th2 differentiation, the activation of innate lymphoid cells (ILC)2, and the recruitment of Th2-type lymphocytes to the site of allergic inflammation [6–8]. Although Th2 polarization is prevalent in the disease, other cell populations such as Th1 lymphocytes with interferon (IFN)-γ production are increased in the chronic phase [9]. Moreover, oxidative stress has an important role in AD pathogenesis, since has been associated with exacerbated inflammation and keratinocyte apoptosis. Keratinocytes under the Th2 microenvironment present an elevated level of oxidative stress that can lead to lipid peroxidation, protein oxidation, or DNA damage, with dysfunctional consequences to the cells and barrier function impairment [10]. Likewise, some neuropeptides, such as nervous growth factor (NGF) and calcitonin gene-related peptide (cGRP), have been associated with nerve ending elongation, neurogenic inflammation, and the itching sensation (pruritus) characteristic of AD [11]. Environmental antigens stimulate keratinocytes to express NGF and cGRP in a reactive oxygen species (ROS)-dependent manner [10]. Because of the intense pruritus, AD patients scratch lesional areas aggravating skin damage, which is exacerbated because they have an altered process of wound regeneration since type 2 inflammatory cytokines impair keratinocyte ability to proliferate and migrate properly [12]. Transforming growth factor (TGF)-β, which participates in multiple phases of wound healing [13], has lower expression in AD patients [14]. Therefore, keratinocytes are undoubtedly crucial cells in the early stage of type 2 inflammation, inflammation perpetuation, pruritus development, and skin damage in AD.

Unfortunately, there is no cure for AD. Therapies are focused on mitigating the main symptoms of the disease and achieving long-term disease control. The first-line treatments are emollients to repair epidermal barrier and anti-inflammatory therapy with topical corticosteroids or calcineurin inhibitors to control acute exacerbations and maintain remission [15]. The development of new therapies that control disease symptoms and modify underlying inflammatory and oxidative responses in AD has attracted research interest. These disease-modifying treatments might stop the progression of the atopic march if used in early stages of AD.

Recently, the use of naturally occurring bioactive peptides has been widely explored as potential treatments to different pathologies due to their broad safety and effectiveness. In particular, milk is a source of multiple peptides with diverse biological activities [16]. One of these peptides is the glycomacropeptide (GMP), a 64-amino-acid peptide generated in the cheese whey during the cheese-making process or physiologically during milk enzymatic digestion. It is cleaved from the carboxyl-terminal region of bovine κ-casein by chymosin or pepsin, respectively [17]. Numerous in vitro and in vivo studies have attributed important biological functions to GMP [18]. Among them, antioxidant, anti-inflammatory, and anti-allergic activities are of importance to this study. It has been reported that GMP decreases oxidative stress responses in macrophages and hepatocytes in vitro [19,20]. When orally dosed in rat models of AD, it reduces the intensity of the edema, the infiltration of inflammatory cell, the pruritus, and Th2 cytokine expression in AD lesions [21]. Additionally, GMP administration prevents or reverses cutaneous barrier damage by increasing the expression of structural proteins and antimicrobial peptides, and by avoiding epidermal thickening and *Staphylococcus aureus* colonization in affected skin tissue [22]. The action mechanism of orally administered GMP is partially mediated by prebiotic activities on gut microbiota and the production of immunomodulatory molecules, such as short chain fatty acids (SCFAs) [22,23]. Nevertheless, a cutaneous direct effect of GMP cannot be ruled out, since it has been detected in blood after milk or yogurt

ingestion [24], and a modulatory activity of oral GMP on skin inflammatory cells, such as mast cells, has been previously documented [23]. GMP could be also formulated in creams or ointments for topical application. Therefore, the evaluation of the biological activities of GMP in an AD model of keratinocyte is of great interest. The aim of this study was to analyze the regulatory capacity of GMP on AD-associated oxidative, inflammatory, and pruritogenic response of human keratinocytes. The effect of GMP on wound closure in an in vitro model of the atopic microenvironment was also studied.

2. Materials and Methods

2.1. Cell Culture and AD Model of Keratinocyte

HaCaT cells (human skin keratinocytes cell line; CLS Cell Lines Service, 300493) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere and 5% CO_2. The cells were harvested from 85–95% confluent monolayer cultures and passaged with the use of 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and 0.038% EDTA (Promega, Madison, WI, USA). Then, cells were washed and resuspended in 1 mL of complete DMEM. Viability and cell count were assessed by trypan blue exclusion assay. Third to seventh passages of cells were used for experiments.

HaCaT cells were plated for 24 h to obtain confluency and later treated with GMP (0.01–25 mg/mL) before and during stimulation with one of the following substances to develop an AD model of keratinocyte: 2,4-dinitrochlorobenzene (DNCB; Sigma, St. Louis, MO, USA) prepared in 0.1% dimethyl sulfoxide as a trigger for cellular death and oxidative stress; hydrogen peroxide (H_2O_2; J.T baker, Phillip Sburg, NJ, USA) as a trigger for cellular death; or IL-4, TNF-α or IFN-γ (all cytokines obtained from PeproTech, Cranbury, NJ, USA) as triggers for inflammatory and pruritogenic gene expression. For this study, LACPRODAN® CGMP-10 (kindly donated by Arla Food Ingredients Group P/S, Viby, Denmark), was used. All the reagents were 0.22 µm filtered before cell culture use.

2.2. MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (Sigma, St. Louis, MO, USA) technique determines the tetrazolium salts reduced by the mitochondrial dehydrogenases of living cells [25]. Cytotoxicity and proliferation assays were performed with 3 or 2 × 10^4 cells incubated with 0.8, 1.6, 3.1, 6.3, 12.5, 25 mg/mL or 0.01, 0.1, 6.3, 25 mg/mL of GMP, respectively, for 12 h in 96-well plates and later stimulated with DNCB 50 µM or H_2O_2 200 µM for 4 h, or with TNF-α/IFN-γ 10 ng/mL mixture for 24 h. When HaCaT cells were incubated with GMP without a later stimulus, concentrations of 0.01, 0.1, 0.8, 1.6, 3.1, 6.3, 12.5, 25 mg/mL were used. Then, the supernatants were removed, and the cell monolayer was incubated with 100 µL of MTT solution (0.5 mg/mL). Four hours later, the formazan crystals were dissolved with 200 µL of isopropanol with 0.04 N HCl. The optical density (OD) of the samples was read at 595 nm and 655 nm (reference) wavelength in a microplate reader (iMarkTM, Bio-Rad, Tokyo, Japan). The cell viability (expressed as percentage) was calculated with the formula: [OD of the test sample/OD control sample] × 100, and the proliferation index as the ratio of the test sample OD compared to the control sample OD.

2.3. Cell Apoptosis Assay

Cell apoptosis was evaluated with the ELISA Cell Death Detection ELISAPLUS kit (Roche Diagnostics GmbH, Mannheim, Germany), to detect histone-associated DNA fragments (nucleosomes) in the cytoplasm as an indicator of late apoptosis. The cells (5 × 10^4) were treated with or without GMP 25 mg/mL for 12 h in 96-well plates and subsequently stimulated with DNCB 50 µM or H_2O_2 200 µM for 4 h. HaCaT cells were lysed using the buffer supplied by the manufacturer, and after centrifugation at 200× *g* for 10 min, supernatants (cytoplasmic fraction) were collected. The ELISA was developed following the

supplier's instructions and the OD was read in a microplate reader at 405 nm wavelength (iMarkTM, Bio-Rad, Tokyo, Japan). Apoptosis levels were represented as nucleosome enrichment factor released into the cytoplasm and calculated as the ratio of OD at 405 nm of the treated cells to that of control cells.

2.4. Nitric Oxide Determination

The nitric oxide (NO) levels were determined by the Griess reaction. In this method, the oxidation of NO in an aqueous solution produces nitrite (NO_2^-), which in the presence of a diazotizing reagent in acidic media and a coupling reagent forms a stable azo compound of an intense purple color [26]. HaCaT cells (3×10^4) were treated with or without GMP at 6.3 or 25 mg/mL for 12 h in 96-well plates and then stimulated with 50 µM DNCB for 4 h. A volume of 100 µL of supernatant was collected and 50 µL of 1% sulfanilic acid in 5% phosphoric acid was added. Five minutes later, 50 µL of 0.5% α-naphthylamine in 5 N acetic acid was added. After 5 min, samples were read at 490 nm and 655 nm (reference) in a microplate reader (iMarkTM, Bio-Rad, Tokyo, Japan). Organic nitrite levels in cell supernatant were calculated by interpolating into a standard curve generated with $NaNO_2$ (0 to 100 µM).

2.5. Measurement of Cellular Hydroperoxide Lipids

Hydroperoxide lipids (LOOH) were evaluated using the FOX 2 method with modifications [27]. This method determines the oxidization of ferrous to ferric ions by LOOH in acidic medium, and the later complexation of ferric ions with xylenol orange to produce a stable purple-blue chromophore. Briefly, HaCaT cells (8×10^5) were incubated with or without GMP at 6.3 or 25 mg/mL for 12 h in 6-well plates and then stimulated with DNCB 50 µM for 4 h. After washing, cells were detached, resuspended in 2 mM Tris HCl, sonicated, and frozen. For LOOH quantification, 20 µL of each sample were added to 180 µL of FOX 2 reagent and incubated for 30 min. FOX2 reagent was freshly prepared with solution A (ammonium ferrous sulfate 250 µM in sulfuric acid 25 mM), and solution B (xylenol orange 100 µM and butylated hydroxytoluene 4 mM); both solutions were prepared in 90% *v*/*v* methanol. The OD was measured at 595 nm in a microplate spectrophotometer (iMarkTM, Bio-Rad, Tokyo, Japan). LOOH levels were calculated by interpolating into a standard curve of tert-butyl hydroperoxide (0 to 50 µM) for the content of cells for each well.

2.6. RNA Extraction, Reverse Transcription, and qPCR

HaCaT cells (8×10^5) were treated with or without GMP at 6.3 or 25 mg/mL for 12 h in 6-well plates and stimulated with 15 µM DNCB or inflammatory cytokines (TNF-α/IFN-γ 10 ng/mL, IL-4 50 ng/mL) at 12 h or 24 h, respectively. For *TGFB1* expression, GMP was used at 0.01 or 0.1 mg/mL. Total RNA was isolated from 2.4×10^6 cells using TRIreagent (Sigma, St. Louis, MO, USA) and quantified with NanoDrop™ 2000 (Thermo Scientific, Waltham, MA, USA). For cDNA synthesis, reverse transcription was performed from 1 µg of RNA with the RevertAid First Strain cDNA Synthesis kit (Thermo Scientific, Waltham, MA, USA) in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. For real-time quantitative PCR, the Maxima SYBR Green/ROX qPCR Master Mix (2×) kit (Thermo Scientific, Waltham, MA, USA) was used in the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Expression levels were determined with $2^{-\Delta\Delta Ct}$ method [28], using GAPDH as housekeeping gene. Primers sequences are listed in Table 1.

Table 1. The primers used in this study.

Target Genes	NCBI Access Number	Primers
TSLP	NM_033035.5	Fw: ATGTTCGCCATGAAAACTAAGGC Rv: GCGACGCCACAATCCTTGTA
IL33	NM_033439.4	Fw: GGAGTGCTTTGCCTTTGGTA Rv: CATTTGAGGGGTGTTGAGAC
CCL22/MDC	NM_002990.5	Fw: GCACTCCTGGTTGTCCTCGT Rv: GACGTAATCACGGCAGCAGA
CCL17/TARC	NM_002987.3	Fw: GTACTTCAAGGGAGCCATTC Rv: CACTCTCTTGTTGTTGGGGT
HMOX1	NM_002133.3	Fw: AAGACTGCGTTCCTGCTCAAC Rv: AAAGCCCTACAGCAACTGTCG
cGRP/CALCA	NM_001033952.3	Fw: TCTAAGCGGTGCGGTAATCTG Rv: CAGTTTGGGGGAACGTGTGA
NGF	NM_002506.3	Fw: TGTGGGTTGGGGATAAGACCA Rv: GCTGTCAACGGGATTTGGGT
TGFB1	NM_000660.7	Fw: CTCCCCACCACACCAGCCCT Rv: GCCACAGCAGCGGTAGCAGC
GADPH	NM_002046.7	Fw: ATCCCATCACCATCTTCCAG Rv: GGCAGAGATGATGACCCTTT

Fw, forward; Rv, reverse.

2.7. Wound Healing Assay

Cell motility was evaluated with the wound healing assay with HaCaT cells [29]. Cells were cultured on 24-well plates covered with fibronectin (10 µg/mL) and DMEM with 10% FBS to confluency. Subsequently, regular medium was replaced with DMEM with 1% FBS for 12 h to maintain cells under serum starvation conditions. Then, the cells were treated with mitomycin C (5 µg/mL) for 2 h to arrest cell proliferation and subsequently washed with PBS. An artificial wound was carefully generated with a sterile 200 µL pipette tip that scratches the confluent cell monolayer to make a cell-free cross along the vertical and horizontal diameter of the well. Cells were washed to remove cell debris and re-coated with fibronectin in DMEM with 1% FBS. After 1 h, wound margins were photographed (initial time) using the camera C-B10 attached to the inverted microscope IM-3 (Optika, Bg, Italy). Then, the cells were treated with TNF-α/IFN-γ mixture (10 ng/mL) to induce type-2 environment and GMP (0.01, 0.1, 6.3, 25 mg/mL) or epidermal growth factor (EGF, 10 ng/mL, as a positive control), for 72 h. Cell migration into de scraped area was photo-documented at 24 h, 48 h, and 72 h. The micrographs were captured with the 4× objective to analyze the wound area using the public software FIYI and with the 10× objective to take representative images. The percentage of wound closure was determined considering the initial wound area in 4 randomly selected fields per condition. Additionally, cells were detached from wells at 48 h after the scratch, and total RNA extraction was performed to analyze *TGFB1* gene expression by quantitative real time-PCR.

2.8. Statistical Analysis

Data were represented as the mean ± SEM. All data were analyzed with GraphPad Prism 8.0 software (Boston, MA, USA). One-way or two-way ANOVA analysis with multi comparison Bonferroni post-hoc test was used to determine statistical significance, stablishing the significance value at $p < 0.05$.

3. Results

3.1. GMP Does Not Present Cytotoxic Activity on HaCaT Cells

First, the viability of human keratinocytes at different concentrations of GMP was analyzed. Results showed that GMP did not have a toxic effect on HaCaT cells at concen-

trations from 0.01 to 25 mg/mL (Figure 1). GMP at the concentration range between 1.6 and 25 mg/mL appeared to stimulate cell proliferation, as cell viability was 40.3% higher when cells were incubated with 25 mg/mL of GMP compared to the control group (GMP 0 mg/mL). Higher GMP concentrations were not tested due to solubility properties and difficulties in filtering the solution.

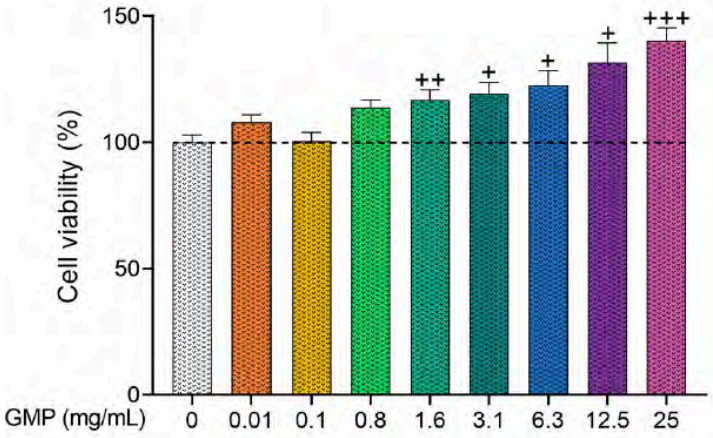

Figure 1. Glycomacropeptide (GMP) has no toxic effect on keratinocytes. HaCaT cells were treated with GMP for 24 h and the percentage of cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) assay. n = 12, 3 independent experiments in quadruplicate. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ vs. control.

3.2. Protective Activity of GMP against Cell Death and Apoptosis

HaCaT cells were incubated with DNCB and H_2O_2, two substances that have been reported to induce cell death and apoptosis in keratinocytes [30,31]. Figure 2A shows that DNCB exposure decreased keratinocyte viability by 58%, while GMP treatment exerted a significant cytoprotective effect, increasing cell viability in a dose-dependent manner at concentrations from 6.3 to 25 mg/mL. Likewise, GMP showed the same dose-dependent protective effect when the HaCaT cell death was stimulated with H_2O_2 (Figure 2B). This effect could be associated with the proliferative response induced by GMP on cells (Figure 1). We choose the GMP concentration of 25 mg/mL to evaluate its effect on keratinocyte apoptosis. DNCB and H_2O_2 increased the level of apoptosis 1.45- and 1.7-fold in HaCaT cells. GMP treatment significantly reduced cell apoptosis to a level similar to that of control conditions, showing a protective role on keratinocyte apoptosis (Figure 2C,D). Based on these results, we decided to use GMP at 6.3 and 25 mg/mL for future experiments.

3.3. GMP Protects Keratinocytes from Oxidative Damage

As oxidative stress has an important role in AD pathogenesis [10], that eventually causes the death of keratinocytes [31], we evaluated the antioxidant effect of GMP on HaCaT cells. Keratinocytes were stimulated with DNCB to induce oxidative stress and the level of NO secreted by the cells was measured. Cell incubation with DNCB led to a 6.1-fold increase in nitrite production compared to control values, but when cells were GMP-treated at concentrations of 6.3 and 25 mg/mL, these levels were reduced by 50% and 83.2%, respectively (Figure 3A). To demonstrate whether GMP was able to avoid cell damage, we analyzed lipid peroxidation as an index of oxidative damage in cell membranes. The value of LOOH in control HaCaT cells was 4.72 µM, but DNCB stimulus significantly increased LOOH levels to 7.99 µM (Figure 3B). GMP treatment reduced the cell accumulation of DNCB-induced LOOH to 5.79 µM and 4.38 µM, restoring the values to those of control

condition with the highest GMP concentration. We also measured the mRNA expression of *HMOX1* to evaluate antioxidant response of the cells. As shown in Figure 3C, DNCB stimulus increased the *HMOX1* gene expression in keratinocytes by 14.6-fold and these levels were reduced by 70.5% and 61.6% when cells were GMP-treated at 6.3 and 25 mg/mL. The three parameters of oxidative stress were similar between control cells and cells treated with GMP in the absence of DNCB, indicating that GMP did not trigger oxidative response in keratinocytes. The antioxidant effect of GMP is related to the decrease in cell death and apoptosis when HaCaT cells are incubated with DNCB in presence of the peptide.

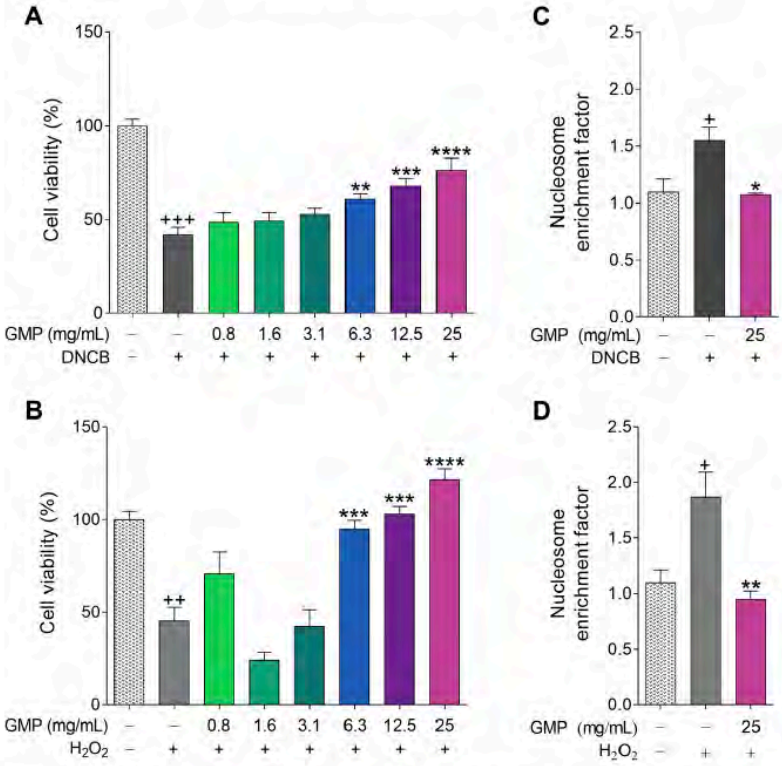

Figure 2. GMP protects keratinocytes against cell death and apoptosis. HaCaT cells were treated with GMP and stimulated with (**A**,**C**) 50 µM 2,4-dinitrochlorobenzene (DNCB) or (**B**,**D**) 200 µM hydrogen peroxide (H_2O_2) to determine: (**A**,**B**) the percentage of cell viability by the MTT assay and (**C**,**D**) apoptosis by ELISA. (**A**) n = 9, 3 independent experiments in triplicate; (**B**,**D**) n = 4 independent experiments; (**C**) n = 3 independent experiments. + $p < 0.05$, ++ $p < 0.001$, +++ $p < 0.0001$ vs. control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. DNCB or H_2O_2.

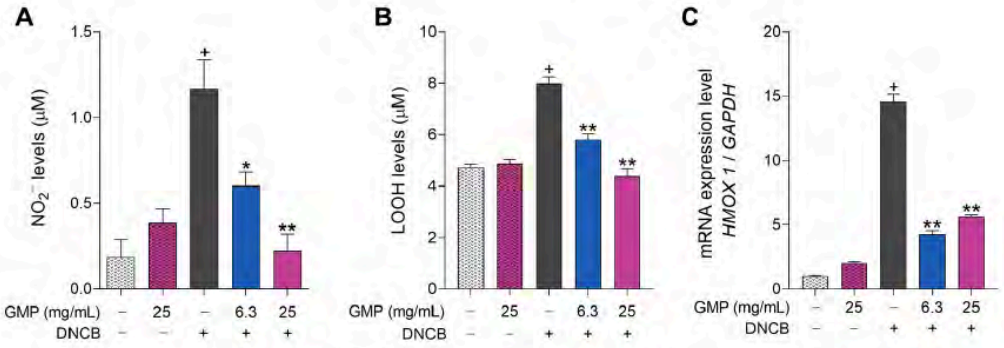

Figure 3. GMP protects keratinocyte against oxidative damage. HaCaT cells were treated with GMP and stimulated with DNCB 50 μM to measure: (**A**) Nitrite (NO_2^-) production and (**B**) Lipid hydroperoxide (LOOH) levels; (**C**) HaCaT cells were treated with GMP and stimulated with DNCB 15 μM to analyze HMOX1 gene expression. (**A**,**B**) n = 9, 3 independent experiments in triplicate; (**C**) n = 4 independent experiments. + $p < 0.0001$ vs. control; * $p < 0.01$, ** $p < 0.0001$ vs. DNCB.

3.4. GMP Down-Regulates Gene Expression Associated with Type-2 Inflammatory Response in Keratinocytes

It is known that in response to barrier disruption and exposure to *S. aureus* and allergens, keratinocytes release alarmins and chemokines, such as TSLP, IL-33, TARC, and MDC, that promote the pro-inflammatory type-2 response characteristic of AD [6,9]. To develop an AD model using keratinocytes, we incubated HaCaT cells with the combination of different cytokines that had been previously reported to induce gene expression related to AD [32]. While levels of *TSLP*, *IL33*, *TARC*, and *MDC* mRNAs were significantly increased in HaCaT cells in presence of TNF-α and IFN-γ (Figure 4), only *TSLP* gene expression was up-regulated when cells were stimulated with TNF-α and IL-4. Thus, we chose TNF-α/IFN-γ mixture to stimulate keratinocyte gene expression in the following experiments.

As shown in Figure 4, the increased gene expression of *TSLP* (3-fold; Figure 4B), *IL33* (3-fold; Figure 4C), *TARC* (2.3-fold; Figure 4D), and *MDC* (1.78-fold; Figure 4E) in HaCaT keratinocytes treated with AD-inducing agents was significantly downregulated by GMP treatment to values similar to the control conditions, showing the efficacy of GMP reducing atopic inflammatory responses. In the absence of stimulus, GMP treatment did not modify the expression of type-2 response stimulating cytokines and chemokines in the cells as compared to control conditions.

3.5. GMP Modifies Gene Expression Related to Itch and Neurogenic Inflammation

It has been reported that the symptom most difficult to control in AD therapy is pruritus, which is associated with aggravation of the lesions [33]. Neuropeptides released by keratinocytes, such as NGF and cGRP, play crucial roles in the itching sensation and type-2 inflammation in AD patients [11,33]. Thus, we examined the effect of GMP on *NGF* and *cGRP* gene expression activated by DNCB and cytokines in keratinocytes. *NGF* expression was increased in DNCB- and TNF-α/IFN-γ-stimulated keratinocytes (Figure 5A,B), with DNCB-stimulation inducing a higher effect compared to when cells were incubated with TNF-α/IFN-γ. GMP treatment significantly downregulated mRNA levels of *NGF* induced by both stimuli in HaCaT cells. The expression level of *NGF* was increased in HaCaT cells incubated with GMP in absence of stimulus (Figure 5B), but this upregulation was avoided by GMP in stimulated cells. *cGRP* gene expression was only significantly upregulated when keratinocytes were stimulated with DNCB (4.6-fold; Figure 5C). Nevertheless, when cells were treated with 6.3 and 25 mg/mL GMP before stimulation, induced levels of *cGRP* mRNA were 1.42- and 3.14-fold higher than without GMP treatment (Figure 5C). Although

cytokines only slightly upregulated *cGRP* gene expression in HaCaT keratinocytes, the expression level was significantly enhanced with GMP treatment (Figure 5D).

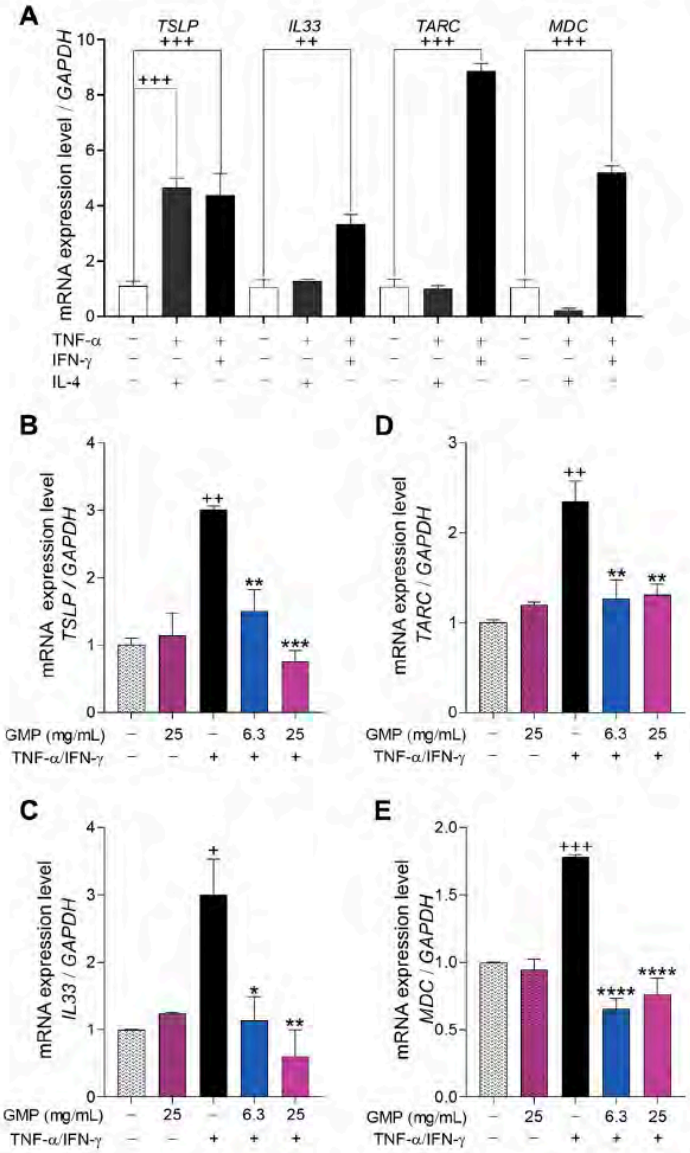

Figure 4. GMP regulates keratinocyte gene expression associated with triggering inflammation in atopic dermatitis (AD). (**A**) In vitro development of an AD model of keratinocytes. HaCaT cells were stimulated with tumor necrosis factor (TNF)-α (10 ng/mL), interferon (IFN)-γ (10 ng/mL) or interleukin (IL)-4 (50 ng/mL) for 24 h. (**B**–**E**) HaCaT cells were incubated with GMP and stimulated with TNF-α/IFN-γ (10 ng/mL). (**B**) *TSLP*, (**C**) *IL33*, (**D**) *TARC* and (**E**) *MDC* gene expression was analyzed by qPCR. n = 3 independent experiments. + $p < 0.05$, ++ $p < 0.001$, +++ $p < 0.0001$ vs. control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. TNF-α/IFN-γ.

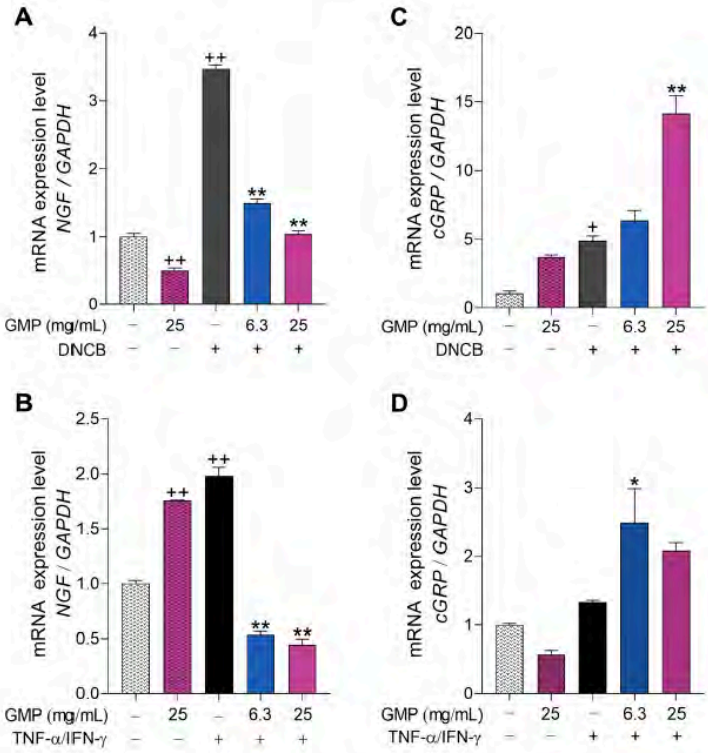

Figure 5. GMP regulates induced NGF and cGRP expression in keratinocytes. HaCaT cells were incubated with GMP and stimulated with: (**A**,**C**) DNCB 15 μM or (**B**,**D**) TNF-α/IFN-γ 10 ng/mL. Gene expression of (**A**,**B**) *NGF* and (**C**,**D**) *cGRP* was analyzed by qPCR. (**A**,**C**) n = 4 independent experiments; (**B**,**D**) n = 3 independent experiments. + $p < 0.01$, ++ $p < 0.0001$ vs. control; * $p < 0.05$, ** $p < 0.0001$ vs. DNCB or TNF-α/IFN-γ.

3.6. GMP Improves Wound Healing in an In Vitro AD Model of Keratinocytes

In AD, recurrent wounds are often generated by constant scratching. The re-epithelialization process of wounds requires the proliferation and migration of keratinocytes to cover the naked dermal surface [34], crucial steps that are altered in AD patients [12]. As previously mentioned (Figure 1), HaCaT cell proliferation was significantly increased in the presence of high concentrations of GMP. Thus, we hypothesized that GMP could be beneficial on tissue repair in an AD model of keratinocytes. We first analyzed the effect of low and high GMP concentrations on keratinocyte proliferation under the influence of AD-inducing cytokines. As shown in Figure 6A, GMP at the concentration range from 0.01 to 6.3 mg/mL did not stimulate the proliferation of keratinocytes under the AD microenvironment. GMP at 25 mg/mL slightly but significantly increased the proliferation index of HaCaT cells ($p < 0.05$). Subsequently, we measured the percentage of wound closure at 24 h, 48 h, and 72 h of GMP incubation. Representative images of the wounds at 72 h are shown in Figure 6B. The results showed that at a short incubation time (24 h), GMP at 0.01 to 25 mg/mL increased would closure to a similar extent to EGF, the positive control, reaching a mean percentage of 30% (Figure 6C). When cells were incubated for longer periods of time with GMP, concentrations of 0.01 mg/mL and 0.1 mg/mL significantly increased wound closure as compared to untreated cells (control), reaching percentages of 38.66% and 33.33% at 48 h and 51.66% and 56.08% at 72 h, respectively, while in control

conditions the percentage of wound closure was 9.08% at both evaluated times (Figure 6C). In our AD model of keratinocytes, EGF did not significantly stimulate the cell migration at any evaluated time.

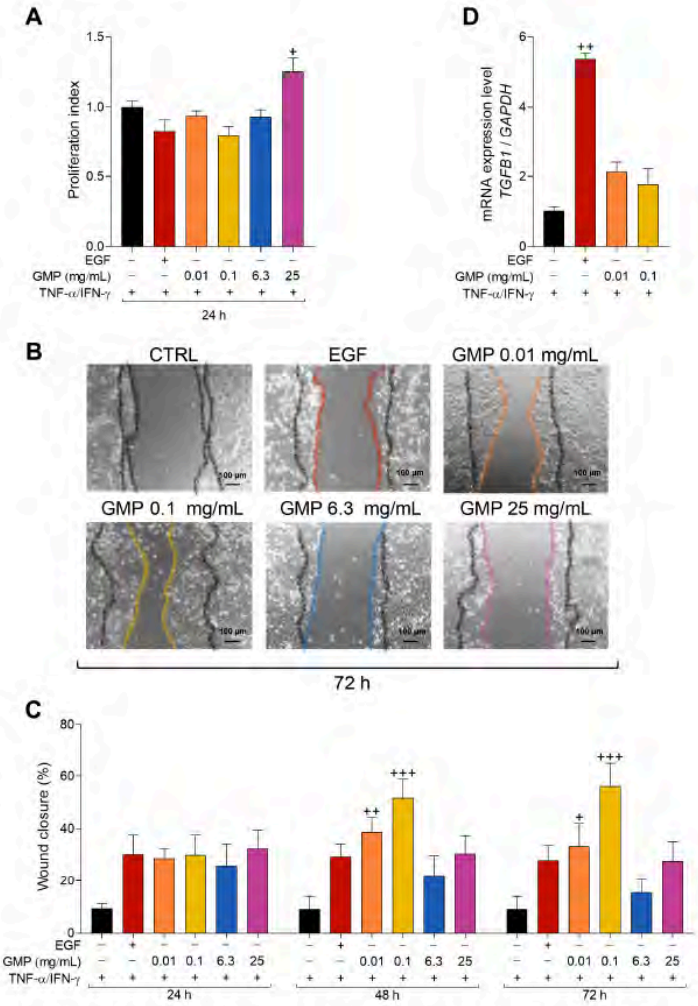

Figure 6. Effect of GMP on proliferation and migration of keratinocytes in an in vitro AD model. (**A**) HaCaT cells were incubated with epidermal growth factor (EGF, positive control) or GMP and stimulated with TNF-α/IFN-γ (10 ng/mL) and the proliferation index was determined. (**B–D**) HaCaT cells were cultured until confluent, incubated with mitomycin C, scratched with a pipette tip, re-coated with fibronectin, and incubated with EGF or GMP plus TNF-α/IFN-γ. (**B**) Representative images at 72 h are shown. (**C**) Distance between wound edges was measured and the wound closure percentage at each indicated time was calculated. (**D**) The gene expression of *TGFB1* was analyzed by qPCR at 48 h. (**A**) n = 6, two independent experiments in duplicate; (**C**) n = 12, 4 randomly selected areas per condition in 3 independent experiments; (**D**) n = 3 independent experiments. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.0001$ vs. control.

During the migration process, the cells undergo an epithelial-mesenchymal transition (EMT), in which TGF-β1 plays an essential role [13]. To evaluate the participation of TGF-β in the stimulated migration of keratinocytes, we analyzed *TGFB1* gene expression in cells incubated with EGF or with GMP concentrations that stimulated cell migration at 48 h after scratching. As shown in Figure 6D, *TGFB1* expression was up-regulated by 4.51-fold in EGF treated cells as compared to control cells. However, *TGFB1* expression was only slightly, but not significantly, increased in cells incubated with GMP.

4. Discussion

Keratinocytes have been positioned as crucial cells in the onset, maintenance, and exacerbation of the AD. This chronic and relapsing skin disease negatively impacts the quality of life of patients and their families, and although it mainly occurs in infancy and childhood, it predisposes patients to other allergic diseases later in life. To date, no cure is available for AD. The most widely used treatments are topical corticosteroids and calcineurin inhibitors, even though resolution is temporary, and it is well documented that they present some adverse effects with long-term application [35]. Thus, it is important to investigate new therapies with the potential to modify the disease, as well as to prevent symptoms. Natural bioactive compounds have captured researchers' attention for this issue. Our group has extensively explored the anti-allergic properties of GMP, showing immunoregulatory, anti-inflammatory, and skin barrier protective activities when orally administered in preclinical models of AD. Nevertheless, there is no information about the effects of GMP on keratinocytes. In the present study, we demonstrate that GMP has no cytotoxic effect on human keratinocytes. Additionally, GMP prevents cell death, apoptosis, and oxidative damage activated by chemical compounds or ROS in human HaCaT cells. In an AD-keratinocyte model, GMP down-regulates the expression of cytokines, chemokines, and neurotrophic factors that trigger the Th2 response, neurogenic inflammation, and pruritus associated to the disease. Under the AD microenvironment, GMP also increases keratinocyte migration, improving wound closures.

GMP represents a good alternative as a protein source in phenylketonuria patients for the elaboration of nutritional supplementation formulas [36]. Different studies suggest the use of GMP as potential therapy in patients that suffer prediabetes or ulcerative colitis [37,38]. Thus, GMP has been extensively explored for general safety issues when orally administered. Using animal models, GMP is reported as non-immunogenic [39]. It is also safe and well-tolerated by humans, with no immunomodulatory effects in healthy adults [40]. Nevertheless, there are no studies about the possible effects of GMP on keratinocytes, either orally or topically administered. In this context, firstly, it should be important to discard GMP toxicity on human keratinocytes. Our results show that GMP does not present cytotoxicity on HaCaT cells when used in a range of concentrations from 0.01 to 25 mg/mL. Previous studies have demonstrated that GMP does not alter the cell viability of human or mouse cell lines at 0.5–2 mg/mL, such as Caco-2/15 human epithelial colonic cells, HepG2 human hepatic cells, and RAW264.7 mouse macrophages [19,41,42]. Our results strengthen the data about the biosafety of GMP.

High level of oxidative stress is involved in the pathophysiology of AD, which eventually induces keratinocyte damage and alters their normal function [43]. The redox imbalance in the cell and the accumulation of ROS have been associated to keratinocyte death [31]. Using activators of oxidative stress in HaCaT cells, we show that GMP has a cytoprotective effect, as it decreases keratinocyte cell death induced by lethal concentrations of both DNCB and H_2O_2 in a concentration-dependent manner. Besides, GMP protects keratinocytes against DNCB-induced oxidative damage, decreasing levels of LOOH and NO. Previous results in RAW-264.7 murine macrophages stimulated with H_2O_2 or LPS demonstrated that pretreatment with intact or hydrolyzed GMP increases cell viability, reduces apoptosis, decreases oxidative stress levels, and increases the activity of antioxidant enzymes [20,42]. Similar antioxidant effects have been reported to GMP hydrolysates in HepG2 mouse hepatocytes [19]. In both macrophages and hepatocytes, this antioxi-

dant activity was mediated through HMOX-1 expression [19,20]. On the contrary, in our results, GMP down-regulated *HMOX1* expression in keratinocytes activated by DNCB, suggesting that GMP is preventing the cell oxidative response and, thus, the HMOX-1 compensatory expression.

HaCaT cell stimulation with the combination of cytokines TNF-α/IFN-γ or TNF-α/IL-4 induces the differential expression of genes that are up-regulated in the skin of AD patients [32]. As previously reported [32], we show that the stimulation of HaCaT cells with TNF-α/IFN-γ better resemble the gene expression that occurs in keratinocytes during AD. Under the influence of these cytokines, we demonstrated that GMP down-regulates the expression level of *TSLP*, *IL33*, *TARC*, and *MDC* in HaCaT cells. TSLP and IL-33 are two of the predominant activators of ILC2s in AD, which are abundant in skin lesions and, once activated, produce the type 2 cytokines IL-5 and IL-13 [44,45]. TSLP is also involved in dendritic cell activation with the subsequent activation of Th2 cell response [46]. In addition, a direct role of TSLP in Th2 differentiation and activation has been recently described [47]. On the other hand, TARC and MDC are chemokines elevated in serum and associated with Th2 lymphocyte attraction and severity of lesional skin in AD patients [6]. We previously reported that when GMP was administered orally in rats, the expression level of Th2 cytokines IL-4, IL-5, and IL-13 was decreased in AD-lesions, which was related to the improvement in clinical signs [21]. Thus, the down-regulatory effect of GMP on the pathological and dominant Th2 immune response might be mediated, at least in part, through decreasing the expression of TSLP, IL-33, TARC, and MDC in keratinocytes. It is also important to consider that inflammatory cytokine expression in keratinocytes, such as IL-33, can be downregulated by metabolites of skin microbiota, and that AD pathogenesis is associated with skin microbial dysbiosis, characterized by a marked reduction in microbial diversity with increment of Staphylococci abundance [1,48]. As orally administered GMP prevents *S. aureus* colonization in a rat model of AD and GMP has been extensively reported as prebiotic [18,22], future works might explore if GMP impairs growth or adhesion of *S. aureus* in keratinocytes in vitro.

Keratinocytes are key source of NGF, a neurotrophic factor that participates in neurogenic inflammation and pruritus [49]. NGF is also involved in the excessive sprouting of cutaneous sensory nerve fibers characteristic of AD [50]. Our results show that GMP reduces the increased expression of NGF induced by cytokines or DNCB in HaCaT cells. These results are in accordance with the anti-pruritic and anti-inflammatory effect of GMP in pre-clinal models of AD [21]. Nevertheless, pruritus can also be triggered by cytokines. An elevated expression of IL-33 is reported in AD lesions of adult patients, which is significantly associated with the itch [51]. Additionally, TSLP released by keratinocytes acts directly on a subset of sensory neurons to trigger robust itch behaviors in animal models [52]. Thus, the decreased expression of *IL33* and *TSLP* in our AD model of keratinocytes might be also causing the abolishment of pruritus in rats prophylactically treated with GMP that has been previously reported [21]. We propose that GMP might be regulating the expression of *NGF*, *TSLP*, and *IL33* through its antioxidant activity. This suggestion is supported by studies showing that ROS production in keratinocytes is linked to the upregulation of mRNA levels of the aforementioned biomarkers of inflammation in AD [10]. On the other hand, we show that HaCaT cells only up-regulate *cGRP* expression when cells were stimulated with DNCB, but not with cytokines. However, in both conditions, GMP significantly enhanced *cGRP* expression. The role of cGRP in AD pathophysiology is controversial. Classically, it is considered to be a neuropeptide involved in skin neurogenic inflammation, participating in pruritus and mainly in vasodilation [11]. Most studies that analyze cGRP in skin are focused on evaluating the density of cGRP-positive fibers in AD patients, observing an increase or no change in cGRP innervation in lesional skin [53,54]. When skin homogenates of AD mice are evaluated, significantly lower cGRP concentration is found as compared to control mice [55]. In accordance, lower cGRP plasma level is found in patients with AD, which is normalized after treatment [56]. These results might suggest a possible immunomodulatory role of cGRP during AD. In this context, the consequences

of increased expression of *cGRP* mRNA in keratinocytes induced by GMP must be exhaustively analyzed in future studies. It is also possible that GMP exerts beneficial effects in experimental AD despite increasing the expression level of cGRP in keratinocytes, because GMP inhibits activation of mast cells [23], cells that, in response to cGRP, release mediators that trigger pruritus and neurogenic inflammation.

In a mouse model that resembles human AD, animals present a delayed wound closure process, and although there is an increased proliferation of keratinocytes, the cells do not migrate efficiently, resulting in a delayed re-epithelization [12]. Based on these observations, we decided to evaluate the effect of GMP on the proliferation and migration of AD keratinocytes in our experimental model. Our results showed that GMP at high concentrations increased keratinocyte proliferation without modifying the wound healing process, and at low concentrations did not affect cell proliferation but increased keratinocyte migration. It is important to highlight that the proliferative effect of GMP on HaCaT cells reported under the control condition (Figure 1) was almost completely lost when cells were under an AD microenvironment (Figure 6A). In the present work, we report for the first time a benefic potential effect of GMP on wound closure. Other bioactive natural substances with anti-inflammatory and antioxidant properties, such as quercetin, also present a potential therapeutic application in wound healing in AD [57]. EMT is a process that occurs in cells as they acquire migratory behavior and is triggered by TGF-β signaling [58]. Particularly, TGF-β promotes migration of HaCaT cells, which is potentiated by EGF [59]. Under our AD microenvironment, EGF, but not GMP, increased TGF-β mRNA expression in HaCaT cells, suggesting that enhancement of migratory activity induced by GMP is not TGF-β-mediated. The anti-allergic effect of oral treatment with GMP is related to systemic TGF-β production, but also to down-regulation of TGF-β expression in the asthmatic lung tissue [23,60]. In addition, the anti-inflammatory and immunoregulatory effect of orally administered GMP on experimental colitis models has been associated with down-regulation of TGF-β expression in colonic tissue, but also with an increase in TGF-β-mediated signaling [61,62]. Thus, although GMP can modify TGF-β expression in other cells, it apparently does not alter its expression in migratory AD-keratinocytes.

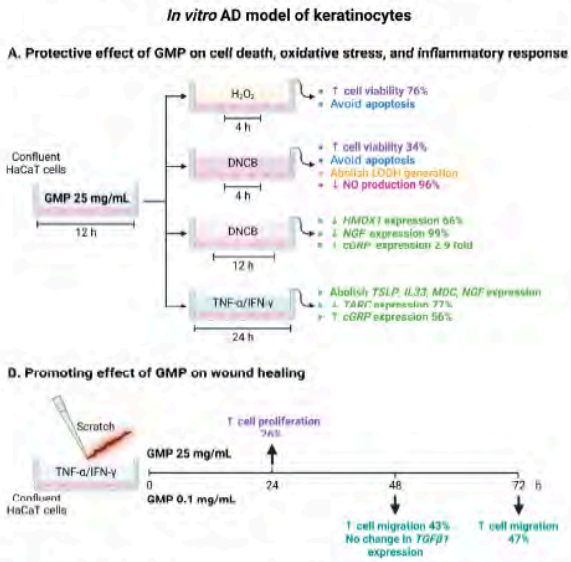

Figure 7. Schematic representation of GMP effects on viability, inflammatory and oxidative response, and the re-epithelization capacity of keratinocytes in an in vitro AD model. Created with BioRender.com (accessed on 3 May 2023).

In summary, we show that GMP at high concentrations presents cytoprotective, anti-inflammatory, and antioxidant activities, and promotes keratinocyte proliferation under an AD microenvironment. Additionally, GMP at low concentrations induces cell migration (Figure 7). Although GMP might be incorporated to topical formulations at different concentrations to enhance a particular bioactivity, GMP at high concentrations could present wound healing effects in vivo in AD lesions, as cell migration is only one step in the complex process of re-epithelialization [34], and excessive ROS levels are proposed as detrimental in the chronic and non-healing wounds in vivo [63]. Thus, in vivo assays are required to define the optimal GMP concentration to be topically applied in AD patients.

5. Conclusions

In conclusion, these results suggest that GMP protects from death, inflammation, and oxidative stress, and stimulates wound healing in an AD model of keratinocytes. This work reinforces the evidence that GMP may be a potential therapeutic candidate for AD, highlighting its beneficial effects on keratinocytes. The limitation of this work is that it comprises an in vitro study in which keratinocytes are alone and under culture conditions, without the contact with other skin cells, molecules, and microbiota that occur during AD and could modify keratinocyte action. Our results support further studies to confirm the bioactivity of GMP in keratinocytes in vivo under the influence of other skin components, as well as to stablish the optimal GMP concentration for topical application, using equivalent skin systems, organoids, biopsies of human skin, or animal models.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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CONCLUSIONES

Conclusión y perspectivas

En este estudio demostramos que:

- El GMP no es tóxico para los KCs.
- Cuando los KCs están en un microambiente atópico en cultivo, el GMP ejerce actividad citoprotectora, antiinflamatoria y antioxidante sobre estas células, además de regular negativamente la expresión de mediadores pruritogénicos.
- El GMP incrementa la proliferación y la migración de los KCs bajo el microambiente atópico, estimulando el cerrado de las heridas *in vitro*.
- El GMP promueve el crecimiento de *S. aureus* y *S. epidemidis in vitro.*
- El GMP disminuye la adhesión de *S. aureus* y aumenta la de *S. epidermidis* a los KCs en cultivo.

Con estos resultados proponemos que el GMP podría incorporarse a formulaciones tópicas que se dirijan a tratar los principales síntomas de la DA. Sin embargo, a pesar de que los estudios *in vitro* nos ayudan a determinar toxicidad, efectos y mecanismos, es necesario realizar estudios *in vivo* para definir la concentración óptima de GMP que se aplicará tópicamente en pacientes con DA. Además, sería interesante realizar estudios de adhesión de *S. aureus* y *S. epidermidis* a KCs bajo un ambiente atópico y co-cultivando ambas bacterias en presencia de GMP.

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ANEXOS

Efecto del GMP sobre el crecimiento y la adhesión de *S. aureus* y *S. epidermidis* en KCs en cultivo

Metodología.

Cultivos celulares

La línea celular de KCs inmortalizados humanos HaCaT (CLS Cell Lines Service, 300493) fue cultivada en medio DMEM (Dubelco's Modified Eagle Medium; Gibco, NY, USA) suplementado con 1 g/L de glucosa, 4 mM de L-alanil-glutamina, 10% de suero bovino fetal (SBF; Gibco, NY, USA) inactivado por calor y 2% de la solución de 5 U/L de penicilina y 5 µg/L de estreptomicina (Sigma-Aldrich, St. Louis, USA). Las células se mantuvieron a 37°C con una atmosfera de dióxido de carbono (CO_2) al 5% y altamente húmeda. Los pasajes se realizaron cuando las células llegaron a una confluencia del 85%. Para cosechar las células, se realizaron dos lavados con solución salina de fosfatos (PBS) pH 7.4 y posteriormente se despegaron incubándolas con una solución de tripsina (Sigma-Aldrich) 0.05% y EDTA 0.25%, 6 min a 37°C, neutralizando la reacción con medio DMEM suplementado. Las células se recolectaron y se centrifugaron a 112 x g durante 7 min a temperatura ambiente. Se decantó el sobrenadante y las células se resuspendieron en medio fresco. Los ensayos fueron realizados cuando las células se encontraron establecidas entre el tercer y séptimo pase. El conteo de células se realizó con el método de azul tripano en hemocitómetro, utilizando el reactivo al 0.4% en PBS.

Cultivos bacterianos

Se trabajó con las cepas de *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 12600) y *Staphylococcus epidermidis* (Winslow and Winslow) Evans (ATCC 14990). Las bacterias se almacenaron en caldo nutritivo (BD Bioxon, Becton Dickinson, México) y glicerol al 15%. Se descongelaron y se colocaron en un matraz de 30 mL con caldo soya tripticasa (CST; BD Bioxon) a 37°C y agitación orbital de 180 revoluciones por minuto (rpm) hasta llegar a la fase exponencial. La biomasa bacteriana se cuantificó por espectrofotometría a una densidad óptica de 600 nm (DO_{600}).

Ensayo de crecimiento bacteriano

Se sembraron 1 x 10^6 bacterias de *S. aureus* o *S. epidermidis* en tubos de ensayo con tapa en 4 mL de CST en ausencia o presencia de GMP (0.01, 0.6, 1, 1.9 y 3.1, 6.3 mg/mL) usando como blanco medio CST adicionado con GMP a cada concentración probada sin microorganismos. Las muestras se incubaron a 37°C con agitación orbital a 180 rpm por 7 h para *S. aureus* y 10 h para *S. epidermidis*. Finalizado el tiempo de incubación se cuantificó la biomasa por DO_{600}.

Ensayo de adhesión bacteriana a KCs

Se sembraron aproximadamente 125 000 KCs de la línea celular HaCaT por pozo en placas de 24 pocillos, se incubaron 48 h en medio DMEM suplementado con SFB al 10% para llevarlas a una confluencia del 85-95% (aproximadamente 400 000 células). Pasado el tiempo, se redujo el SFB al 2% por otras 24 h. Finalmente, se tripsinizaron 2 pocillos para

determinar la densidad celular por pozo por tinción con azul Tripano. Por otro lado, se lavaron las bacterias *S. aureus* o *S. epidermidis* 3 veces colocando 5 mL de PBS y centrifugando a 4577 x g por 7 minutos. Después, éstas se cuantificaron por DO_{600} para que quedaran a una MOI 10 en 500 µL de cada tratamiento respecto a la cantidad de células en cada pozo. Las concentraciones de GMP (0, 0.1, 0.6, 1.9, 6.3 mg/mL) probadas fueron preparadas en medio DMEM con 2% de SFB sin antibióticos.

Previo al cocultivo, las soluciones se adaptaron a las condiciones de cultivo de los KCs por una hora. Posteriormente, 500 µL de cada tratamiento se añadió sobre la monocapa de KCs y se incubaron por 1 h. Finalizado el tiempo, los pozos se lavaron 5 veces con 250 µL de PBS pH 7.4 para eliminar las bacterias no adheridas. Las células se desprendieron de los pozos colocando 250 µL de tripsina por 10 min y se inactivó con 250 µL de DMEM suplementado con SFB al 10% sin antibiótico. Se recolectaron las células en microtubos de 1.5 mL y se centrifugaron a 960 x g durante 10 min a 4°C y se lavaron 2 veces con 200 µL de PBS pH 7.4 centrifugando en las mismas condiciones.

Para cuantificar las unidades formadoras de colonia (UFC) de *S. aureus* o *S. epidermidis* adheridas a los KCs, se eliminó el sobrenadante de la pastilla de células obtenida y se colocaron 500 µL de Triton X-100 (Sigma-Aldrich) al 0.1% en PBS pH 7.4 y se incubó por 30 min a 37°C para lisar las células. Se realizó una dilución 1:10 con PBS pH 7.4 y a partir de ella 6 diluciones seriadas 1:10 en agar soya tripticasa para posteriormente incubarse por 12 h a 37°C. Por último, se cuantificaron las UFC con un contador de colonias Quebec (Reichert Technologies Amelek, New York, USA). y los valores se reportaron en porcentaje de UFC, considerando las UFC del ensayo sin tratamiento como el 100%.

Por otro lado, se evaluó la adhesión bacteriana mediante la cuantificación absoluta de las copias del gen *femA* de *S. aureus* o *S. epidermidis*. A partir de la pastilla de KCs con bacterias adheridas obtenida, se extrajo el DNA genómico con el kit Quick-DNA, fungal, bacterial miniprep kit (Zymo Research, Irvine, USA) siguiendo las instrucciones del fabricante. Se realizó una curva de referencia utilizando diluciones de 10 concentraciones conocidas (de 10^1 a 10^{10} copias/µL) de los plásmidos pTZ57R-femA-SA y pTZ57R-femA-SE generados previamente en el laboratorio (Jiménez et al., 2020). Brevemente, los plásmidos fueron generados mediante clonación de los amplicones de PCR del *femA* de *S. aureus* y *S. epidermidis* (Tabla 1) con el InsTAclone PCR Cloning Kit (Thermo Scientific, Waltham, USA). Los plásmidos resultantes, pTZ57R-femA-SA y pTZ57R-femA-SE, se purificaron con el PureYield Plasmid Maxiprep System (Promega, Madison, USA). Las reacciones de pPCR en tiempo real se desarrollaron con 50 ng de DNA (o 1 µL de plásmidos estándar pTZ57R-femA-SA y pTZ57R-femA-SE de 10^1 a 10^{10} copias/µL), 0.5 µM de oligos para *femA* de *S. aureus* o *S. epidermidis* (según corresponda), 5 µL de buffer Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) y agua libre de nucleasas para completar un volumen final de 10 µL en un StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). Las condiciones de reacción fueron las siguientes: 94°C 3 min; 40 ciclos de 94°C por 30 s, 60°C por 30 s; y una extensión final de 72°C por 3 min. La recta estándar se generó con los datos de Ct para cada dilución *versus* la concentración de número de copias de los plásmidos en cada reacción. Para obtener el número de copias del gen femA de *S. aureus* o *S. epidermidis,* el valor de Ct de cada muestra problema se interpoló con la ecuación de la recta estándar previamente generada.

Tabla 1. Oligonucleótidos para la detección y cuantificación de femA de S. aureus y S. epidermidis.

Oligonucleótido	Secuencia
Fw-femA-SA	TGCCTTTACAGATAGCATGCCA
Rv-femA-SA	AGTAAGTAAGCAAGCTGCAATGACC
Fw-femA-SE	CAACTCGATGCAAATCAGCAA
Rv-femA-SE	GAACCGCATAGCTCCCTGC

Análisis estadístico

Los datos se representaron como la media ± SEM de al menos tres ensayos independientes. Todos los análisis fueron realizados con el software GraphPad Prism 8.0, se usó una prueba ANOVA de una vía con una prueba *post-hoc* Dunnett. La significancia se estableció con un valor de $p<0.05$.

Resultados

Efecto del GMP sobre el crecimiento de S. aureus y S. epidermidis in vitro

Debido a que se sabe que *S. epidermidis* tiene la capacidad de controlar el crecimiento de *S. aureus* (Traisaeng *et al*., 2019), principal patógeno cutáneo en la DA, evaluamos si el GMP modificaba el crecimiento de estas bacterias *in vitro*. Para realizar los ensayos se utilizaron concentraciones de GMP reportadas por otros autores con efecto sobre la disminución de la adhesión de bacterias patógenas (Neeser, 2002) y la concentración de 6.3 mg/mL, que fue la más baja en la que se obtuvierons efectos biológicos en los KCs. En los resultados observamos que el GMP fue capaz de inducir el crecimiento de *S. epidermidis* cuando se incuban por 10 h. Este efecto se observó a partir de la concentración de 0.6 mg/mL de GMP; así, se aumentó la biomasa un 46% incubando con 0.6 mg/mL de GMP, un 47% incubando con 1 mg/mL, un 61% para 1.9 mg/mL y 3.1 mg/mL y finalmente un 49% para 6.3 mg/mL, comparando siempre con la condición control (Figura 1A; $p < 0.001$). Por otro lado, en la Figura 1B, se muestra que cuando el *S. aureus* fue tratado con GMP hubo un incremento en su crecimiento desde la concentración de 0.1 mg/mL, siendo de un 24% ($p < 0.05$). Además, para 0.6 mg/mL de GMP el aumento de la biomasa fue de 40% ($p < 0.001$), para la concentración de 1 mg/mL fue de 38% ($p < 0.001$), con 1.9 mg/mL de 41% ($p < 0.001$), para 3.1 mg/mL de 40% ($p < 0.001$) y finalmente, este incremento fue de 25% ($p < 0.05$) para 6.3 mg/mL. El GMP en el rango de concentraciones entre 0.6 y 6.3 mg/mL promovió el crecimiento *in vitro* de *S. epidermidis y de S. aureus;* sin embargo, este último mostró un menor crecimiento.

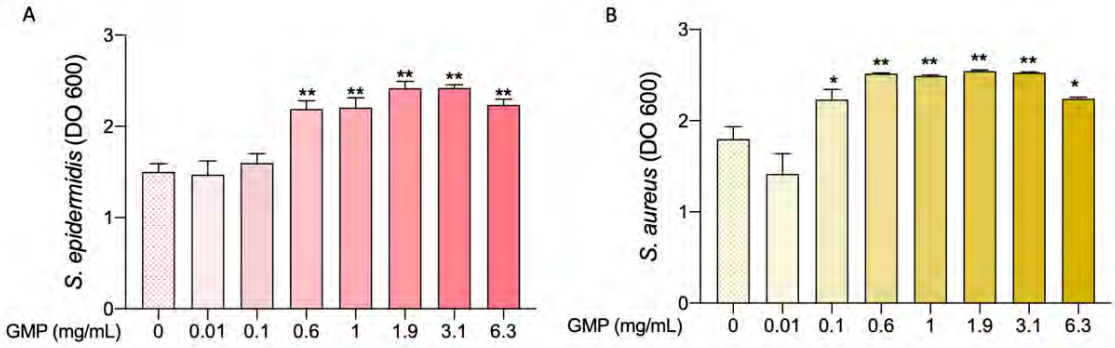

Figura 1. Efectos del GMP sobre el crecimiento bacteriano. A) 1 X 10^6 bacterias de S. epidermidis fueron cultivadas durante 10h con GMP. B) 1 X 10^6 bacterias de S. aureus fueron cultivadas durante 7h con GMP. El crecimiento bacteriano fue determinado midiendo los valores de DO_{600}. Los valores representan la media ± SEM, n=9, tres ensayos independientes por triplicado. Análisis mediante prueba ANOVA con *post-hoc* Dunnett. *p < 0.05, **p < 0.0001 contra las bacterias sin tratamiento.

Efecto del GMP sobre la adhesión de S. aureus y S. epidermidis a los KCs in vitro

Para analizar si el GMP pudiera modificar la colonización de *S. aureus* y *S. epidermidis* en los KCs, se evaluó la adhesión de las bacterias a células HaCaT *in vitro*. La concentración de GMP de 6.3 mg/mL aumentó 4.9 veces (v) la cantidad de UFC de *S. epidermidis* adheridas a los KCs (Figura 2A; $p < 0.05$), y disminuyó en un 67% la de *S. aureus* (Figura 2B; $p < 0.05$). Por otro lado, al medir la adhesión bacteriana cuantificando las copias del gen *femA,* se observó que el tratamiento con GMP aumentó significativamente la adhesión de *S. epidermidis* a los KCs, incrementándola 6.3v con la concentración de 0.1 mg/mL ($p < 0.001$) y un promedio de 4.35v para concentraciones de 0.6 y 1.9 mg/mL de GMP ($p < 0.01$) (Figura 2C). Además, el GMP disminuyó la adhesión de *S. aureus* de una forma dependiente de la concentración, llegando a ser esta disminución hasta del 97% en la concentración de 6.3 mg/mL ($p < 0.0001$; Figura 2D).

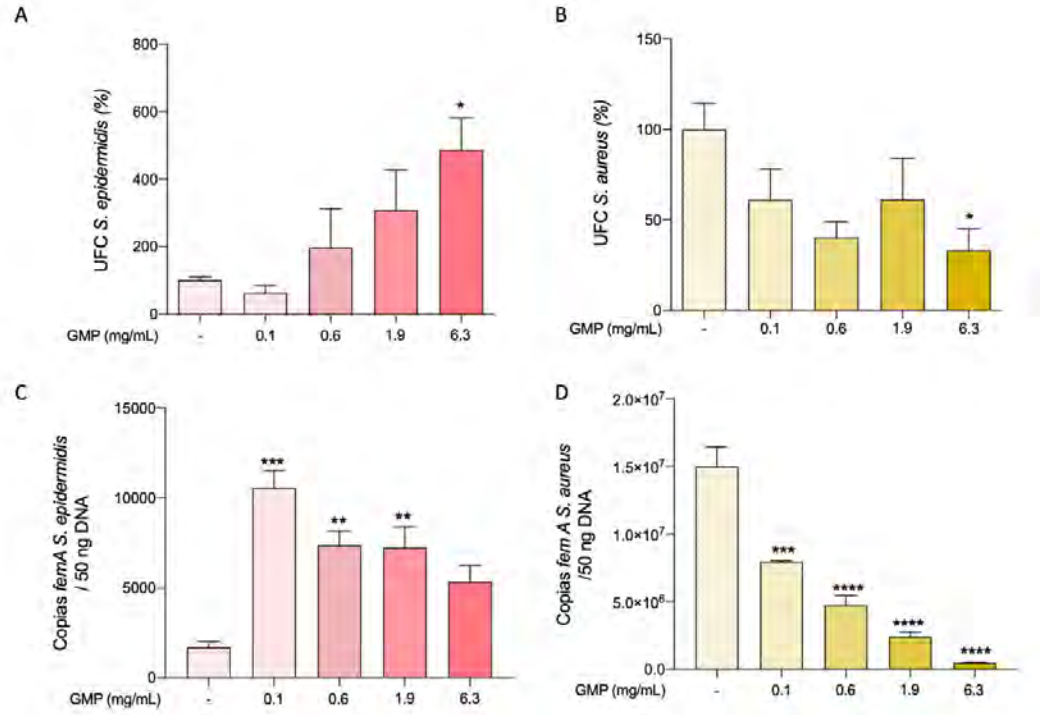

Figura 2. Efecto del GMP sobre la adhesión bacteriana a los KCs. Las cepas de S. epidermidis y S. aureus fueron sembradas en una MOI 10 sobre una monocapa de células HaCaT e incubadas durante 1 hora. A, B) UFCs formadas por las bacterias adheridas a los KCs. B, C) Copias del gen femA de S. epidermidis y S. aureus en la monocapa de células HaCaT. Los valores representan la media ± SEM, A,B n=6 tres ensayos independientes por duplicado; C,D n=3 tres ensayos independientes. Análisis mediante prueba ANOVA con *post-hoc* Dunnett. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 contra el control sin GMP.

Discusión.

La DA se caracteriza por presentar en la piel de los pacientes lesiones en forma de parches eritematosos con exudación y ampollas, con una sensación de prurito intensa difícil de controlar (Weidinger et al, 2016). Así mismo, se conoce que esta piel presenta una susceptibilidad a generar infecciones por *S. aureus*, lo cual se ha relacionado con la presencia de citocinas inflamatorias y disbiosis en la microbiota cutánea (Qiu et al., 2022; Gallegos-Alcalá et al 2021; Domenico et al., 2018). La necesidad de controlar o evitar que un paciente con DA presente una infección se debe a que estas pueden agravar el estado de la lesión afectando la integridad de la barrera epidérmica y volverse sistémicas si no se trata adecuadamente (Ogonowska et al., 2021). En reportes anteriores, nuestro grupo de investigación ha mostrado que el GMP tiene un efecto positivo sobre el control de *S. aureus* en las lesiones de DA experimental, cuando se dosifica oralmente a ratas (Jiménez et al., 2020); así mismo, en un modelo de DA con KCs humanos *in vitro* hemos mostrado que puede proteger contra la inflamación y el estrés oxidativo, así como promover la regeneración de heridas (Gallegos-Alcalá et al., 2023). Nuestros resultados muestran que el GMP promueve el crecimiento *in vitro* tanto de *S. epidermidis* como de *S. aureus,* aunque el efecto es menor para *S. aureus*. El crecimiento de ambas bacterias en presencia de GMP podría deberse al aprovechamiento como fuente de carbono del ácido siálico presente en su estructura, ya que estudios previos han mostrado que este azúcar favorece el crecimiento de *S. aureus* de una forma dependiente de la concentración (Lu et al., 2021). Por otro lado, demostramos que el GMP disminuye la adhesión de *S. aureus*

a los KCs y aumenta la adhesión de *S. epidermidis*. Otros estudios han mostrado que el GMP en concentraciones similares a las utilizadas en este estudio puede disminuir la adhesión a los KCs de algunos de los patógenos más comunes en la piel (*Estreptococcos pyogenes, Candida albicans, Pityrosporum ovale, Trichophyton rubrum, Malassezia furfur*) (Neeser, 2002). Así mismo, se ha reportado que el ácido siálico puede inhibir la adhesión de *S. aureus* a células Caco-2 (Lu et al., 2021), siendo este azúcar abundante en el GMP; pudiendo ser uno de los mecanismos inhibitorios del GMP sobre la adhesión de *S. aureus* a las células HaCaT. El efecto antiinflamatorio mediado por el GMP en los KCs ayudaría de una manera indirecta a evitar la colonización por *S. aureus*, ya que se ha demostrado que las citocinas inflamatorias como IL-1β e IFN-γ potencian el crecimiento de *S. aureus* de manera dependiente de la concentración tanto en su forma planctónica como en forma de biopelículas (Domenico et al., 2018). Por otro lado, el efecto antiinflamatorio del GMP se podría asociar a que favorece una mayor adhesión de *S. epidermidis* al KC, bacteria comensal que favorece la homeostasis cutánea (Zhou et al., 2023).

Se sabe que la superficie de la piel, contrario al intestino, presenta escasez de fibras complejas y carbohidratos necesarios para el metabolismo de los AGCCs, importantes en la regulación del sistema inmune. Sin embargo, la microbiota de la epidermis se enriquece naturalmente de aminoácidos derivados de los KCs. Así, en pacientes con DA, se ha encontrado niveles reducidos de un metabolito derivado del catabolismo de triptófano en la piel, metabolito que ha mostrado un papel importante en el control de la inflamación a través del receptor de aril hidrocarburo de los KCs (Yu et al., 2018). Aunque el GMP no posee triptófano en su estructura (Manguy, & Shields, 2019), otros aminoácidos de su cadena peptídica pudieran ser aprovechados por la microbiota cutánea generando metabolitos con actividad antiinflamatoria. También se ha visto que *S. epidermidis* cultivado *in vitro* con glicerol produce butirato como consecuencia de su fermentación y el butirato puede inhibir el crecimiento de *S. aureus* y la respuesta inflamatoria de los KCs mediante la inhibición de histonas desacetilasas (Traisaeng et al., 2019). Estudios previos reportan la producción de butirato tras la fermentación del GMP por bacterias intestinales en la rata (Jiménez et al., 2016), sugiriendo que una producción potencial de AGCCs por la microbiota cutánea al utilizar al GMP pudiera estar participando en la actividad protectora que realiza este péptido sobre los KCs y sobre la adhesión de *S. aureus*.