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Lauriane Blot

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# THÈSE DE DOCTORAT

## Rôle de CLEC12B dans l'immunité de la peau

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**Spécialité** : Immunologie et Microbiologie

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# Rôle de CLEC12B

## dans l'immunité de la peau

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# Résumé

CLEC12B a été identifié pour la première fois comme étant un récepteur inhibiteur exprimé par les cellules myéloïdes qui neutralise la cytotoxicité médiée par les cellules NK. CLEC12B est un récepteur de lectine de type C (CLR) possédant un domaine ITIM, mais dont la signalisation en aval et le ou les ligands restent en grande partie inconnus. Au cours des 30 dernières années, une fonction de présentation d'antigène des mélanocytes a émergé en raison de leur nature dendritique, de leur position stratégique dans la peau et de leur capacité phagocytaire. Dans un contexte de vitiligo, notre équipe a montré que l'activation de CXCR3B, le récepteur des chimiokines immunitaires CXCL9, CXCL10 et CXCL11, induit l'apoptose des mélanocytes humains en culture. Les mélanocytes restants, activés par la production d'IFN $\gamma$ , expriment des marqueurs de costimulation, déclenchant ainsi la prolifération des lymphocytes T et l'immunité anti-mélanocytaire qui en résulte. De récents résultats de notre équipe ont montré que CLEC12B est principalement exprimé par les mélanocytes humains et joue un rôle important dans la régulation de la pigmentation cutanée, mais également dans la prolifération du mélanome.

Dans ce projet, nous avons cherché à déterminer le rôle de CLEC12B dans l'immunité de la peau en utilisant des mélanocytes humains primaires provenant de donneurs sains. Nous démontrons ici que CLEC12B est essentiel à la production d'IFN $\gamma$  et des chimiokines innées CXCL9, CXCL10 et CXCL11 par les mélanocytes, comme le montre la modulation de l'expression de *CLEC12B* à l'aide de techniques de surexpression ou d'extinction génique. Cette régulation se fait *via* la phosphorylation du domaine ITIM de CLEC12B, comme le montre la forme mutée du gène *CLEC12B*. De plus, CLEC12B peut non seulement moduler la production de chimiokines mélanocytaires, mais il est également capable d'augmenter directement le chimiotactisme des cellules immunitaires de la peau et donc de déclencher une immunité adaptative à long terme. D'un point de vue signalisation, nous montrons que CLEC12B module la voie de signalisation de l'IFN $\gamma$  via l'axe STAT1/IRF1. De plus, CLEC12B potentialise l'effet de l'IFN $\gamma$  dans les mélanocytes activés, induisant ainsi une plus grande production de chimiokines innées et *in fine* une plus grande chimio-attraction des cellules immunitaires. Nous avons également démontré que CLEC12B interagit directement avec *Staphylococcus aureus* et *Escherichia coli* et module une réponse immunitaire innée contre ces bactéries opportunistes présentes sur la

peau via l'axe STAT1/IRF1/CXCL9. Enfin, nous avons montré que CLEC12B détecte des motifs présents sur les mélanocytes, les fibroblastes et les macrophages pro- et anti-inflammatoires, mais son ou ses ligands restent encore à être identifiés. Dans l'ensemble, ces résultats démontrent que CLEC12B est un acteur important dans l'immunité cutanée innée en modulant la production d'IFN $\gamma$  et de chimiokines immunitaires, mais également dans l'immunité adaptative en modulant le chimiotactisme des cellules immunitaires via la phosphorylation de son domaine ITIM. Ce mécanisme présente un grand intérêt car l'IFN $\gamma$  et le recrutement de cellules immunitaires sont des étapes initiales clés impliquées dans l'inflammation de nombreuses pathologies de la peau, faisant de ce récepteur une cible thérapeutique intéressante pour le traitement de maladies infectieuses, des troubles cutanés inflammatoires et pigmentaires, ainsi que du cancer ; tout cela pouvant être directement immuno-régulé par CLEC12B dans les mélanocytes. Cette nouvelle perspective prometteuse reste encore à être testée dans de futures études.

Mots clés : CLEC12B, récepteur lectine de type C, mélanocyte, immunité, IFN $\gamma$ , chimiokines

# Abstract

CLEC12B was first identified as an inhibitory receptor on myeloid cells that counteracts NK cells-mediated cytotoxicity. CLEC12B is a C-type Lectin Receptor (CLR) which possesses an ITIM domain, but ligand and downstream signaling are largely unknown. Over the past 30 years, an antigen-presenting function of melanocytes has emerged due to their dendritic nature, their strategic position in the skin and their phagocytic capacity. In a vitiligo context, our team has shown that CXCR3B activation, the receptor for immune chemokines CXCL9, CXCL10 and CXCL11, induces apoptosis of cultured human melanocytes. The remaining melanocytes, activated by the IFN $\gamma$  production, express co-stimulatory markers which trigger T cell proliferation and subsequent anti-melanocytic immunity. Recent results from our team have shown that CLEC12B is mainly expressed in human melanocytes and plays an important role in the regulation of skin pigmentation, but also in melanoma proliferation.

In this project, we set out to determine the role of CLEC12B in skin immunity using primary human melanocytes from healthy donors. We demonstrate that CLEC12B is critical in production of IFN $\gamma$  and innate chemokines CXCL9, CXCL10 and CXCL11 by melanocytes, as shown by our regulation of *CLEC12B* expression using silencing or overexpression techniques. This regulation was driven by the phosphorylation of CLEC12B's ITIM domain as shown using *CLEC12B* mutated form of the gene. Furthermore, not only can CLEC12B drive melanocyte chemokine production, but it is also capable of directly increase chemoattraction of immune cells in the skin and therefore trigger a long-term adaptative immunity. From a signaling point of view, we show that CLEC12B modulates IFN $\gamma$  signaling pathway through the STAT1/IRF1 axis. Moreover, CLEC12B potentiates the effect of IFN $\gamma$  in primed melanocytes, thus inducing a larger production of innate chemokines and subsequent greater chemoattraction of immune cells. In addition, we have demonstrated that CLEC12B directly interacts with *Staphylococcus aureus* and *Escherichia coli* and modulates an innate immune response against these opportunistic bacteria found on the skin through the STAT1/IRF1/CXCL9 axis. Finally, we have shown that CLEC12B senses motifs present on melanocytes, fibroblasts, and both pro- and anti-inflammatory macrophages, but its exact ligand(s) still remains to be identified. Together, these results demonstrate that CLEC12B is an important player in innate skin immunity by modulating the production of IFN $\gamma$  and immune chemokines, and in adaptative immunity by

modulating the migration ability of immune cells through the phosphorylation of its ITIM domain. This mechanism is of great interest as IFN $\gamma$  and cellular recruitment are key initial steps involved in inflammation of many skin pathologies, making this receptor an interesting therapeutic target for the treatment of infectious diseases, inflammatory and pigmentary skin disorders, as well as cancer; all which may be able to be directly immuneregulated by CLEC12B on melanocytes. This exciting novel prospect remains to be tested in future studies.

Key words : CLEC12B, C-type lectin receptor, melanocyte, immunity, IFN $\gamma$ , chemokines

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# Table of contents

Table of Illustrations .....	14
Abbreviations.....	16
Introduction.....	22
I. The skin .....	22
A. Overview .....	22
B. Structure .....	23
1. Epidermis.....	24
a. Layers.....	24
b. Cellular types .....	26
2. Dermis .....	27
3. Subcutaneous tissue .....	28
C. Functions.....	29
1. Microbiome barrier.....	29
2. Chemical barrier.....	31
3. Physical barrier.....	32
4. Immune barrier.....	33
II. C-type Lectin Receptors (CLRs) .....	35
A. General information about CLRs.....	35
1. Classification, structure, and ligand recognition .....	35

2.	Signaling motifs in CLRs .....	37
3.	Diversity of CLRs ligands affecting the functional outcomes .....	40
4.	Therapeutic potential of CLRs .....	45
B.	CLRs in skin immunity (published review) .....	47
C.	Dectin-1 cluster .....	48
III.	CLEC12B (published review) .....	48
IV.	Melanocytes.....	60
A.	Origin and development .....	60
1.	Embryonic origin and anatomical distribution .....	60
2.	Genes involved in melanocyte development .....	62
B.	Melanocytes as melanin-producing cells .....	63
1.	Skin pigmentation and melanin .....	64
2.	Melanogenesis and related signaling pathways .....	65
3.	Melanosome maturation and transfer .....	68
C.	Melanocytes as antigen-presenting cells .....	71
1.	Antigen-processing and-presentation capacity.....	72
2.	Immune properties of melanization .....	72
3.	Pathogen recognition and production of inflammatory molecules .....	73
4.	Anti-melanocytic immunity .....	74
	Thesis project presentation .....	76
	Results.....	78
	Discussion and perspectives .....	97

Conclusion .....	112
Methods .....	114
References .....	123
Annexes .....	141
I. Publications.....	141
II. Posters and oral communications.....	174
A. Posters.....	174
B. Oral communications .....	177

# Table of Illustrations

## Introduction

*Figure 1: Structure of the skin.*

*Figure 2: Structure of the epidermis.*

*Figure 3: Major cells of the epidermis.*

*Figure 4: Skin barrier functions.*

*Figure 5: Skin commensal interactions with Staphylococcus aureus.*

*Figure 6: Cellular adhesion in the epidermis.*

*Figure 7: Representation of the 17 subgroups of CTLs.*

*Figure 8: Signaling motifs in CLRs.*

*Figure 9: The nature of CLRs ligands.*

*Figure 10: Self and non-self sensing by CLRs.*

*Figure 11: Therapeutic potential of CLRs.*

*Figure 12: Cellular and immunological functions of the Dectin-1 cluster.*

*Figure 13: Embryonic origin and anatomical distribution of melanocytes.*

*Figure 14: Transcriptional regulation of melanocyte development.*

*Figure 15: MC1R signaling pathways.*

*Figure 16: Simplified scheme of the melanogenesis.*

*Figure 17: Developmental stages of melanosomes.*

*Figure 18: Melanosome transfer mechanisms.*

*Figure 29: Melanocyte immune functions.*

*Figure 20: Immune molecules and receptors in melanocytes.*

*Figure 21: Anti-melanocytic immunity.*

## Results

**Figure 1:** *CLEC12B* silencing decreases the production of immune cytokines and chemokines.

**Figure 2:** *CLEC12B* overexpression increases the production of immune cytokines and chemokines through its ITIM domain.

**Figure 3:** *CLEC12B* modulates the chemotaxis of Peripheral Blood Mononuclear Cells (PBMCs) through the production of immune chemokines CXCL9, CXCL10, and CXCL11.

**Figure 4:** *CLEC12B* modulates IFN $\gamma$  pathway through the STAT1/IRF1 axis.

**Figure 5:** *CLEC12B* interacts with *S. aureus* and *E. coli* and modulates an immune response against these opportunistic skin bacteria through the STAT1/IRF1/CXCL9 axis.

**Figure 6:** *CLEC12B* doesn't recognize any mammalian nor microbial glycan structure present on these arrays.

**Figure 7:** *CLEC12B* recognizes protein structures present on melanocytes.

**Figure 8:** IGF2R, CRLF1, HSCB, S100A4, OAF, and LPL are not *CLEC12B* ligands.

**Supplementary Figure S1:** *CLEC12B* isoform 2 doesn't modulate the production of immune cytokines and chemokines.

**Supplementary Figure S2:** *CLEC12B* doesn't modulate IFN $\gamma$  pathway before 24h of IFN $\gamma$  stimulation.

**Supplementary Figure S3:** *CLEC12B* doesn't modulate any pro-inflammatory or innate immune marker at a transcriptional level.

## Methods

**Table 1:** Primer sequences.

**Table 2:** Antibodies.



# Abbreviations

AC	Adenylyl Cyclase
ACTH	Adrenocorticotropic Hormone
AD	Atopic Dermatitis
AMP	Antimicrobial Protein
$\alpha$ MSH	$\alpha$ -Melanocyte-Stimulating Hormone
APC	Antigen-Presenting Cell
CD-MPR	Cation-Dependent Mannose-6-Phosphate Receptor
CHIKV	Chikungunya Virus
CLEC1A	C-Type Lectin Domain Family 1 Member A
CLEC1B	C-Type Lectin Domain Family 1 Member B
CLEC9A	C-Type Lectin Domain Family 9 Member A
CLEC12A	C-Type Lectin Domain Family 12 Member A
CLEC12B	C-Type Lectin Domain Family 12 Member B
CLR	C-type Lectin Receptor
CRD	Carbohydrate Recognition Domain
CREB	cAMP Responsive Element Binding protein
CRLF1	Cytokine Receptor Like Factor 1
CTL	C-type lectin
CTLD	C-Type Lectin-like Domain
CWF	Cell Wall Fraction
CWH	Cell Wall Homogenate
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C Motif Chemokine Receptor

DAMPs	Damage-Associated Molecular Pattern
DAP12	DNAX-Activation Protein 12
DC	Dendritic Cell
DCIR	Dendritic Cell Immunoreceptor
DC-SIGN	Dendritic Cell-Specific Intracellular Adhesion Molecules (ICAM)-3 Grabbing Non-Integrin
DCT	Dopachrome Tautomerase
Dectin-1	Dendritic Cell-Associated C-Type Lectin-1
DHI	5,6-dihydroxyindole
DHICA	DHI-2-carboxylic acid
DOPA	3,4-dihydroxyphenylalanine
EMU	Epidermal Melanin Unit
Esp	Extracellular serine protease
Fc	Fragment Crystallizable
FcR $\gamma$	Fc Receptor $\gamma$
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
hemITAM	hemi-ITAM
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
HSCB	HscB Mitochondrial Iron-Sulfur Cluster Cochaperone
HSP	Heat Shock Protein
HSV-1	Herpes Simplex Virus type 1
GAS	interferon-Gamma Activated Site
IFN	Interferon

IFNGR	Interferon-gamma Receptor
Ig	Immunoglobulin
IGF2R	Insulin Like Growth Factor 2 Receptor
IL	Interleukin
ILC	Innate Lymphoid Cell
INAM	IFN Regulatory Factor 3-Dependent NK-Activating Molecule
iNOS	inducible Nitric Oxide Synthase
IRF	Interferon Regulatory Factor
ISG	Interferon-Stimulated Gene
ITAM	Immunoreceptor Tyrosine-based Activating Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
JAK	Janus Kinase
JNK1	C-Jun N-Terminal Kinase 1
LC	Langerhans Cell
LFA-1	Leukocyte Function-associated Antigen-1
LOX-1	Lectin-like Oxidized Low-Density Lipoprotein(ox-LDL) receptor-1
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
LRO	Lysosomal Related Organelles
M6P	Mannose-6-phosphate
MAPK	Mitogen-Activated Protein Kinase
MBL	Mannose-Binding Lectin
MC1R	Melanocortin 1 Receptor
MDSC	Myeloid-Derived Suppressor Cell
Mincle	Macrophage-Inducible C-Type Lectin

MITF	Microphthalmia-associated Transcription Factor
MR	Mannose Receptor
MSU	Monosodium Urate
NCC	Neural Crest Cell
NFAT	Nuclear Factor of Activated T cell
NF- $\kappa$ B	Nuclear Factor-kappa B
NK	Natural Killer
NKC	Natural Killer Gene Complex
NKG2D	Natural Killer Group 2 member D
NLR	NOD-Like Receptor
NMF	Natural Moisturizing Factor
NT	Neural Tube
OAF	Out At First Homolog
ON	Overnight
Ox-LDL	Oxidized Low-Density Lipoprotein
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Programmed Cell Death 1
PD-L1	Programmed Cell Death 1 Ligand 1
PFA	Paraformaldehyde
PGC1 $\alpha$	Peroxisome Proliferator-Activated Receptor $\gamma$ Coactivator 1- $\alpha$
PMA	Phorbol 12-Myristate 13-Acetate
PMEL17	Premelanosome Protein 17
POMC	Proopiomelanocortin

PRR	Pattern Recognition Receptor
PTEN	Phosphatase and TENsin homolog
PTP	Protein Tyrosine Phosphatase
RIG-I	Retinoic Acid-Inducible Gene I
RLR	RIG-I-Like Receptor
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
RQ	Relative Quantification
RT	Room Temperature
S100A4	S100 Calcium Binding Protein A4
SAMP	Self-Associated Molecular Pattern
SC	Stratum Corneum
SFK	Src Family Kinase
SHP	Src Homology region 2 domain-containing Phosphatase
shRNA	short hairpin RNA
SIGNR3	Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin Receptor 3
SOCS	Suppressor Of Cytokine Signaling
SOX	SRY-Box Transcription Factor
SPPR	Self-PRR
STAT	Signal Transducer and Activator of Transcription
Syk	Spleen tyrosine Kinase
TDB	Trehalose-6,6-Dibehenate
Th	T helper
TLR	Toll-Like Receptor

TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
TYR	Tyrosinase
TYRP1	Tyrosinase Related Protein 1
USF1	Upstream Transcription Factor 1
UV	Ultraviolet
VACV	Vaccinia Virus

# Introduction

## I. The skin

### A. Overview

The human skin is a remarkable organ that serves as the interface between the body and the external environment. The skin is colonized by a plethora of microorganisms, representing the skin microbiota, that lives outside of the body but with the ability to influence systemic behaviors. For decades, it has been thought that the skin has a flat surface of approximately  $2\text{m}^2$ . However, there are approximately 5 million appendages such as hair follicles and sweat glands which greatly increase the epithelial surface area to approximately  $25\text{m}^2$ . This new insight shows how the skin and this microbiota have a greatest potential to influence each other through this special interface (Gallo 2017). The skin performs several important functions that are essential for our overall health and well-being. Beyond its physiological functions that will be presented later, the skin also plays a role in social interactions by conveying emotions through blushing, paleness, or sweating in response to various stimuli. These visual cues can communicate emotional states and contribute to nonverbal communication. Taken together, the multifaceted functions of the skin make it a subject of great interest in various fields, including dermatology, biology, and biomedical research.



## B. Structure

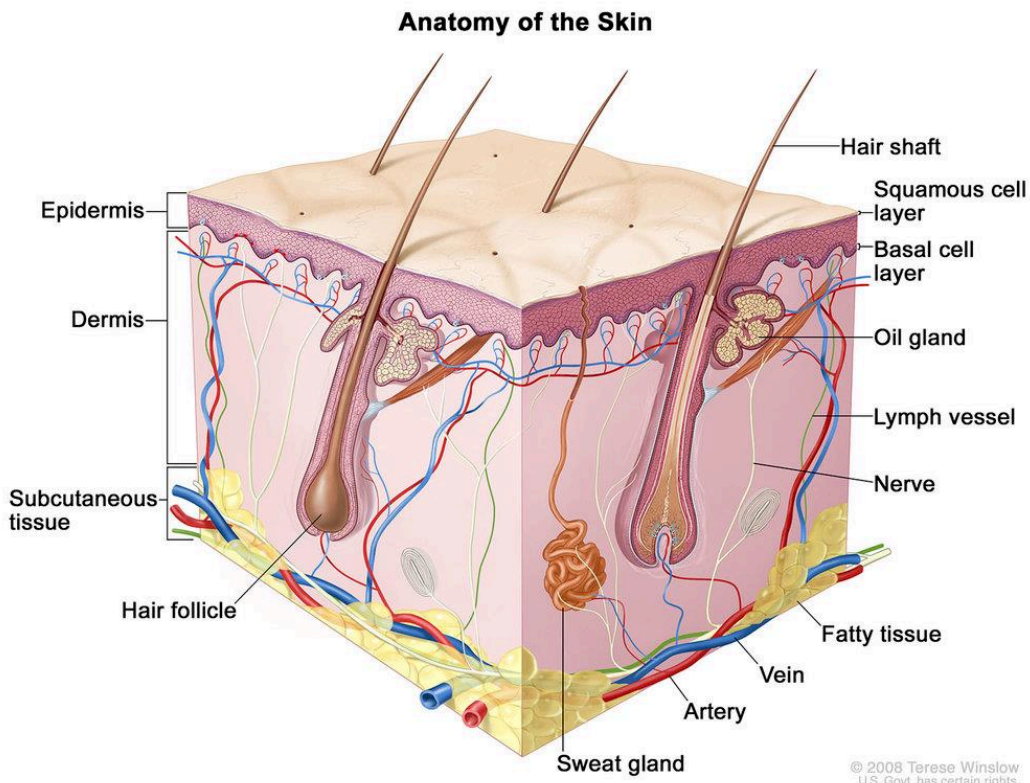


Figure 1: Structure of the skin.

(Extracted from "Skin, Basal Cell And Squamous Cell, Anatomy" on the National Cancer Institute - Visuals online [NCI Visuals Online \(cancer.gov\)](https://www.ncbi.nlm.nih.gov/visuals/)).

The skin is a complex organ with a layered structure. It consists of three primary layers: the epidermis, the dermis, and the hypodermis also known as the subcutaneous tissue (*Figure 1*). Each layer has its own distinct characteristics and functions. The epidermis is the outermost layer of the skin. It provides protection against environmental factors and consists of several distinct sublayers. The dermis is the middle layer of the skin, situated beneath the epidermis. It provides structural support and contains various components including connective tissue, blood and lymph vessels, nerve endings, hair follicles and sweat glands. The hypodermis is the deepest layer of the skin, located below the dermis. It consists mainly of fat cells (adipocytes) that provide thermal insulation, cushioning, and energy storage.

# 1. Epidermis

## a. Layers

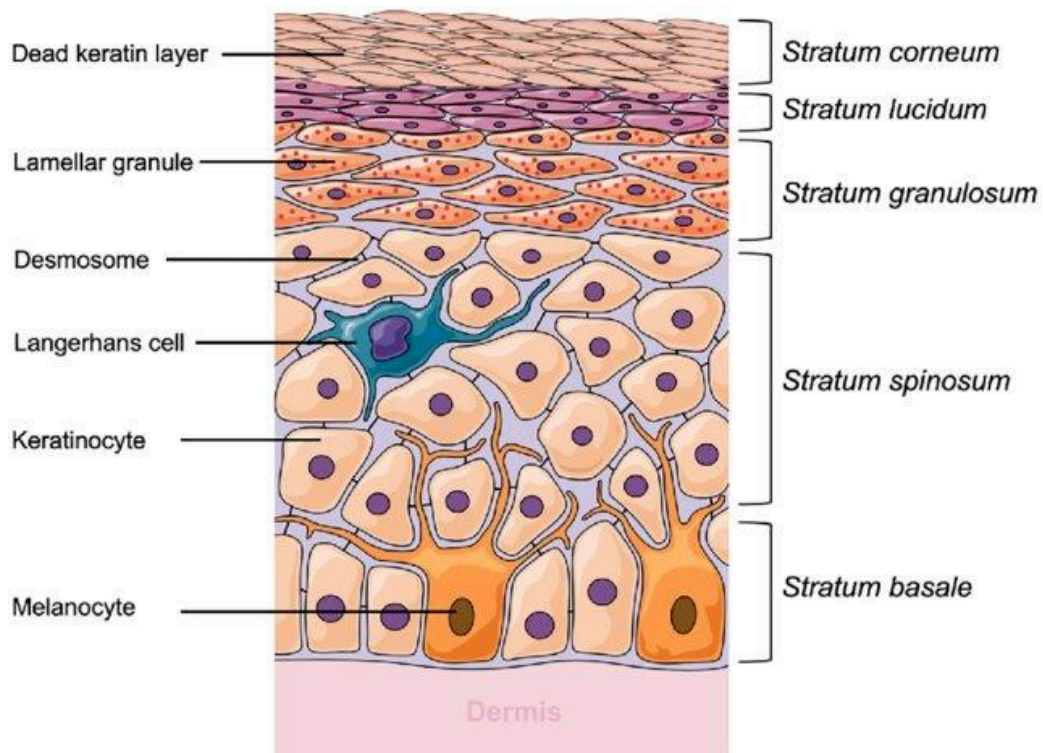


Figure 2: Structure of the epidermis.

(Extracted from (Ramadon et al. 2022)).

The epidermis is a multi-stratified, non-vascularized, keratinized epithelium covering the outer surface of the skin. It functions as a protective barrier against exogenous insult such as pathogens and ultraviolet (UV) light and provides mechanical resistance. Epidermal stratification begins during embryonic development and continues throughout the post-natal life. The different layers of the epidermis are described below (Figure 2).

- **Stratum corneum** (SC) or Cornified Layer is the topmost layer of the epidermis. It is composed of 15-30 layers of flat dead skin cells which are terminally differentiated keratinocytes, also called corneocytes. These corneocytes are rich in keratin, a tough protein that provides strength and resilience to the skin, but they don't contain any nuclei or organelles. This is the layer which varies most in thickness (10-20 $\mu$ m), especially in callused skin (Böhling et al. 2014). The primary function of the cornified layer is to serve as a physical barrier, preventing the entry of pathogens, chemicals, and other harmful substances into the body. It also helps to reduce water loss from the

skin, maintaining its hydration. In skin homeostasis, there is a balance between shedding corneocytes and differentiating keratinocytes derived from a pool of epidermal stem cells (Eckhart et al. 2013). The process of cornified cells shedding to maintain the integrity and renewal of the epidermis is called desquamation which occurs every 2 to 4 weeks.

- ***Stratum lucidum*** is a translucent layer found in thick skin areas such as palms and soles of feet. It consists of 2-3 layers of flattened cells that lack nuclei and other organelles. The dead keratinocytes of the SC are brought up from this layer (Ramadon et al. 2022). The main function of *stratum lucidum* is to provide additional protection to the skin and enhance its barrier function.
- ***Stratum granulosum*** or granular layer is located beneath the *stratum lucidum*. It is composed of granular cells that contain keratohyalin granules and lamellar granules. Keratohyalin granules contribute to the production of keratin, helping to strengthen the skin. Lamellar granules release lipids that act as a waterproof barrier, reducing water loss and maintaining skin hydration.
- ***Stratum spinosum*** or spinous layer is characterized by spiky-shaped cells that are connected to each other by desmosomes, which are protein structures that provide mechanical strength and cohesion to the skin. This layer is involved in cell communication and immune responses. Langerhans cells (LCs), a type of antigen-presenting cells (APCs), are present in the *stratum spinosum*. They play a crucial role in recognizing and presenting foreign antigens to initiate immune responses but also to swallow bacteria or exogenous particles and damaged cells by phagocytosis (Clayton et al. 2017).
- ***Stratum basale*** or basal layer is the innermost layer of the epidermis, attached to the basement membrane by hemidesmosomes that separates the epidermis from the dermis. It is also known as the *stratum germinativum* because of the mitotically active stem cells that are constantly dividing to give rise to keratinocytes that are present in the upper epidermal layers. Merkel cells, functional cells of the sensory system, and melanocytes, melanin-producing cells responsible for skin color, are also found in this layer.

b. Cellular types

## EPIDERMIS

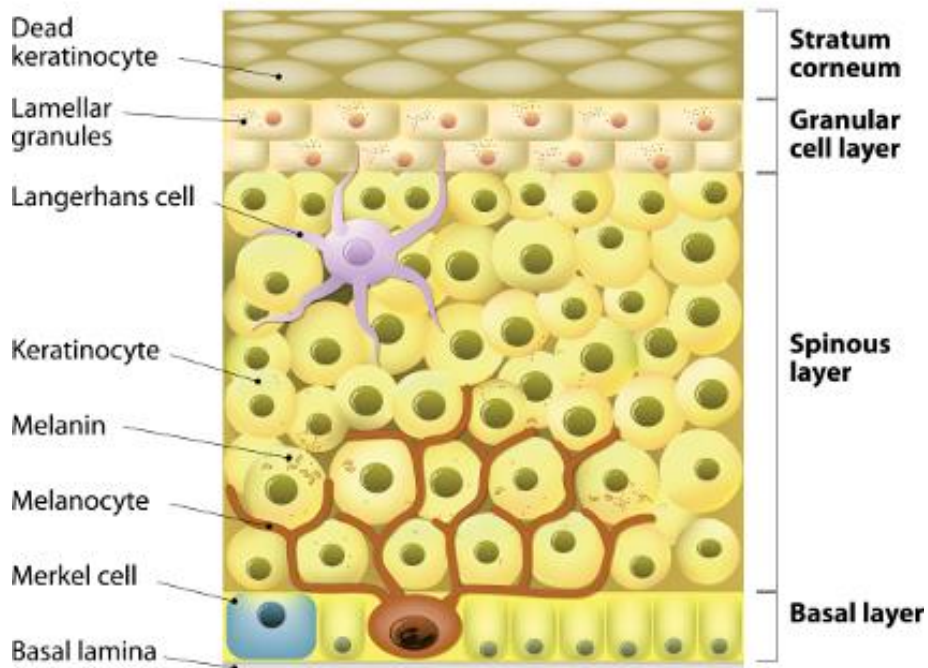


Figure 3: Major cells of the epidermis.

(Extracted from [Skin Cell - Sarah Chapman Skinesis](#))

The epidermis is composed of 4 principal cell types: keratinocytes (80-90%), melanocytes (5-10%), LCs (3-5%), and Merkel cells (2-5%) (Figure 3).

- **Keratinocytes** are the most abundant cells in the epidermis and form most of its layers. They produce keratin, hence their name keratinocytes, which is a tough fibrous protein that provides strength and protection to the skin. As keratinocytes mature and move towards the skin surface, they undergo a process called keratinization or cornification. During this process, they accumulate keratin and eventually become flattened dead cells that form the SC. Keratinocytes contain melanocytes-transferred melanosomes, which are organelles where melanin pigments are synthesized, giving them protection against UV damage. Keratinocytes also play a key role in skin immunoregulation as they are the predominant source of chemokines and cytokines involved in cutaneous immunity (Bitschar et al. 2017).
- **Langerhans Cells** are derived from bone marrow precursors and belong to a group of immune cells called dendritic cells (DCs). They are found in the epidermis, particularly

in the *stratum spinosum* and are named after Paul Langerhans, the German physician who first described them in 1868. They act as sentinels of the immune system in the skin and play a crucial role in recognizing foreign antigens. Once LCs have captured and processed antigens, they migrate from the epidermis to the skin-draining lymph nodes where they present these antigens to other immune cells, such as T cells, initiating adaptive immune responses. One of the defining features of LCs is the presence of Birbeck granules which are rod-shaped or tennis racket-shaped structure. Their exact function is still not fully understood, but they are believed to play a role in antigen uptake and processing (Thornton et al. 2020).

- **Merkel Cells** are oval-shaped cells located in the *stratum basale*, primarily found in touch-sensitive regions of the skin and in areas that require precise sensory perception, such as the fingertips, palms, and soles of the feet. They act as mechanoreceptors and form Merkel cell-neurite complexes with sensory nerve fibers, allowing them to detect and transmit tactile information to the brain.
- **Melanocytes** are specialized pigment-producing cells also located in the *stratum basale*. They produce melanin, the pigment responsible for skin, hair, and eye color. Melanocytes transfer melanin to neighboring keratinocytes, providing protection against UV radiation. These cells will be presented more in detail later as it is the cellular model used in this project.

In addition to these 4 cell types, other cell populations such as lymphocytes, and various inflammatory cells can be found in the epidermis, depending on the specific circumstances and conditions of the skin. These cells collectively contribute to the functions of the epidermis, including protection, immunity, pigmentation, sensory perception, and regeneration.

## 2. Dermis

The dermis is a complex and thick layer of skin, located between the epidermis and the hypodermis. It is mainly composed of fibroblasts which are responsible for producing and maintaining the extracellular matrix of the skin. Fibroblasts synthesize collagen, elastin, and other components of the dermal connective tissue, providing strength, elasticity, and support to the skin. They also play a role in wound healing and tissue repair by producing new collagen fibers. Other cell populations can be found in the dermis such as macrophages, responsible for engulfing and eliminating foreign antigens and cellular debris; mast cells, containing granules

filled with chemical mediators such as histamine and cytokines involved in allergic reactions; lymphocytes involved in skin immunity, and dermal DCs participating in immune surveillance and activation.

The dermis is divided in two main layers: a thin papillary dermis and a thicker reticular dermis. The papillary dermis, the uppermost layer, is composed of thin and loosely organized type I and type III collagen fibers. Its functions are to supply nutrients, oxygen, and immune cells to the epidermis due to the presence of blood vessels and to regulate temperature. The reticular dermis is the deeper and thicker layer of the dermis. It is composed of dense irregular connective tissue, mainly made up of collagen and elastic fibers providing strength, support, and elasticity to the skin. The presence of nerves provides sensory perception and transmit signals between the skin and the central nervous system. The dermis also contains various appendages such as hair follicles, sebaceous glands, and sweat glands. These appendages are embedded in the dermal layer and play essential roles in the regulation of body temperature, lubrication of the skin, and protection.

### 3. Subcutaneous tissue

The subcutaneous tissue, also known as the hypodermis, is the lowermost layer of the skin located beneath the dermis. It is a highly vascularized tissue that anchors the skin to the underlying tissues. It is mainly composed of adipocytes cells grouped into lobules separated by connective tissue septa which contains collagen and elastin fibers that provide support and flexibility to the skin. The adipose tissue serves as fat storage for energy reserve functions and provides cushioning for underlying structures such as muscles and organs. It also acts as insulation to regulate body temperature by reducing heat loss. There also is a connective tissue containing collagen and elastin fibers which gives strength, flexibility, and elasticity. The connective tissue also houses blood vessels, nerves, and lymphatic vessels that supply nutrients, oxygen, and immune cells to the skin. Overall, the hypodermis plays an essential role in maintaining the integrity and functionality of the skin and contributes to the overall well-being of the body.



## C. Functions

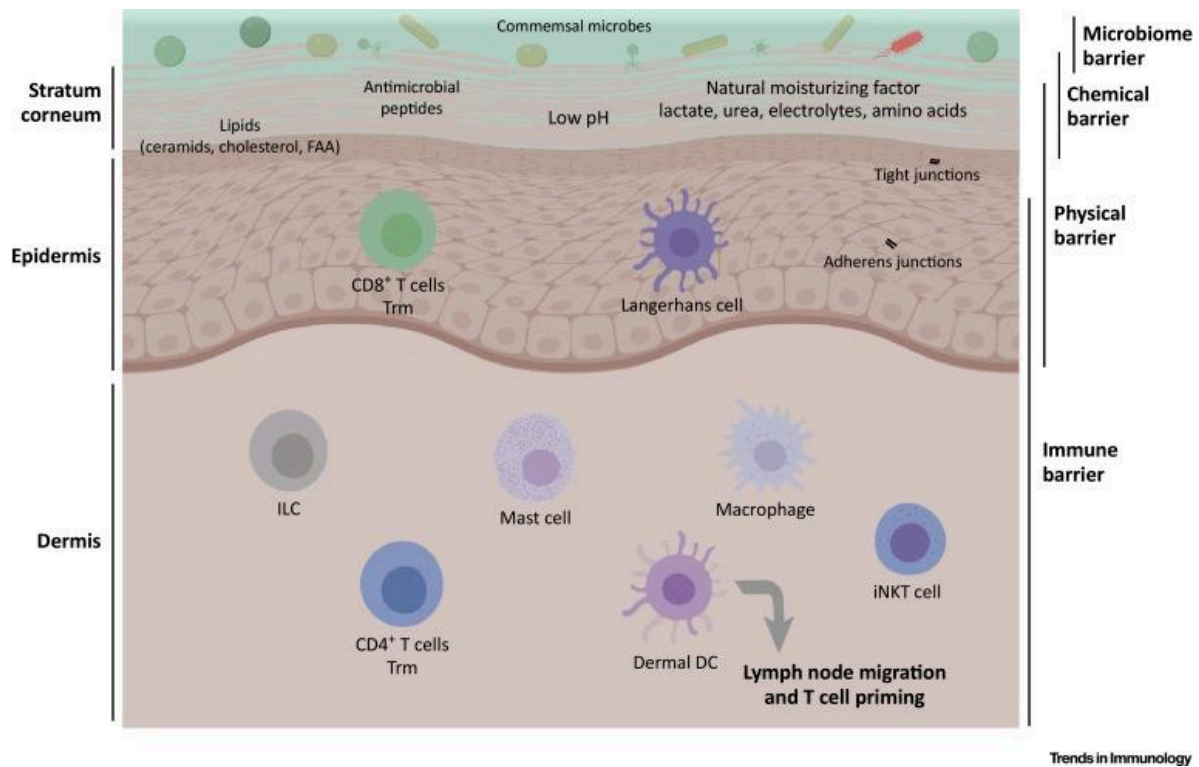


Figure 4: Skin barrier functions.

(Extracted from (Eyerich et al. 2018))

The skin is usually considered as the first defense line of our body, preventing invasion of pathogens and external assaults as well as regulating loss of water and solutes. While the whole skin structure plays a pivotal role in host defense, it is divided into 4 main functional levels: microbiome barrier, chemical barrier, physical barrier, and immune barrier (Eyerich et al. 2018) (Figure 4).

### 1. Microbiome barrier

The microbiome barrier of the skin refers to a plethora of microorganisms residing on the skin surface, known as the skin microbiota, which includes bacteria, fungi, viruses, archaea, and mites (Figure 4). The composition and diversity of this microbiota vary across different body regions and individuals. A typical square centimeter of the skin can shelter approximately one billion bacteria (Weyrich et al. 2015). In contrast to the gut microbiome, the skin microbiota is dominated by *Actinobacteria* with an abundance of Gram-positive bacteria, such as *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* species (Eyerich et al. 2018). These microorganisms play a protective role by competing with potential pathogens for



resources and space, producing antimicrobial substances including bacteriocins and toxic metabolites, and modulating immune responses. For example, commensal *Staphylococcus epidermidis* binds to keratinocytes receptors to inhibit the binding of virulent *Staphylococcus aureus* whereas *S. aureus* releases bacteriocins to inhibit other virulent *Staphylococcus* species (Chiller, Selkin, et Murakawa 2001). Moreover, the skin is an acidic environment (pH 4 to 6) that promotes colonization by commensal bacteria and limits the proliferation of potential pathogenic bacteria such as *S. aureus*, suggesting that this acidity is necessary for skin homeostasis (Proksch 2018).

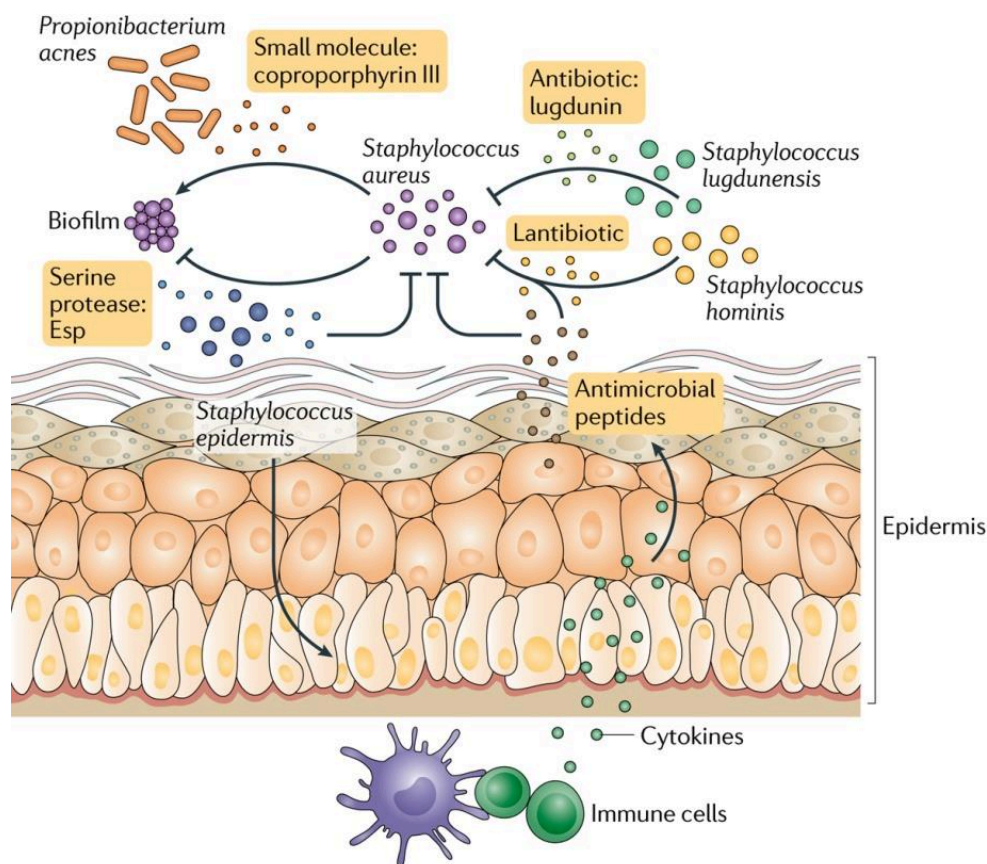


Figure 5: Skin commensal interactions with *Staphylococcus aureus*.

(Extracted from (Byrd, Belkaid, et Segre 2018))

*S. aureus* is a Gram-positive commensal bacterium of humans mainly found on the skin and in the anterior nares which colonizes 10-30% of the population. However, this commensal can turn into a pathogen after penetration of endothelial, epithelial, or dermal barriers, thus causing a plethora of diseases (Horn et al. 2018). Several studies have shown that some *S. epidermidis* strains express the extracellular serine protease (Esp) that inhibits *S. aureus* biofilms formation, by degrading essential proteins for these biofilms, and host epithelial

adhesion (Iwase et al. 2010; Sugimoto et al. 2013) (Figure 5). Moreover, *S. epidermidis* can induce keratinocytes to produce antimicrobial proteins (AMPs) via immune cell signaling to effectively kill *S. aureus* (Figure 5). More recent studies have shown that some *S. epidermidis* and *Staphylococcus hominis* strains can produce lantibiotics, able to synergize with human AMPs, and some *Staphylococcus lugdunensis* strains can produce lugdunin antibiotics to specifically prevent *S. aureus* colonization and survival (Zipperer et al. 2016; Nakatsuji et al. 2017) (Figure 5). On the contrary, it has been shown that the small molecule coproporphyrin III, produced by *Propionibacterium acnes*, can promote *S. aureus* aggregation and biofilm formation (Wollenberg et al. 2014) (Figure 5), highlighting the huge diversity of interspecies interactions in our skin microbiota.

*Escherichia Coli*, discovered in 1885 by Theodore Escherich, is a generally commensal, non-pathogenic faecal bacterium that can live on the skin and mucous membranes without harming the host. Over 95% of *E. coli* strains are harmless, but this bacterium can become pathogenic if the host's defenses are weakened, or if it acquires virulence factors. Although human healthy skin is often exposed to high concentrations of *E. coli*, this bacterium rarely leads to infections. In fact, results have shown that healthy skin keratinocytes secrete an AMP, the 11-kilodalton (kDa) metal ion-binding S100 protein psoriasin (S100A7), which is able to efficiently kill *E. coli*, making the skin particularly resistant to this bacterium (Gläser et al. 2005). A recent study has also reported the isolation of the kallikrein-related peptidase inhibitor SPINK9 as an epidermal antibacterial factor able to kill various *E. coli* strains by entering into the bacterium cytoplasm, but not other bacteria or fungi (Z. Wu et al. 2019).

Together, this microbiome barrier can influence various aspects of our skin physiology, including the other barrier functions, immune responses, and lipid metabolism. The disturbance of the cutaneous microbial balance can result in dysbiosis and promote skin disease and inflammation.

## 2. Chemical barrier

The skin acts as a chemical barrier in preventing loss of water and solutes from the body to the environment and in protecting the body from a variety of environmental insults. The chemical barrier is mainly due to acidic surface pH, natural moisturizing factors (NMFs), epidermal lipids, and AMPs in the SC (Figure 4). As mentioned before, the skin is an acidic environment (pH 4 to 6), known as the acid mantle, which is created by the production of sweat and sebum, as

well as the metabolic activity of skin cells. This acidity inhibits the growth of many pathogens by creating an unfavorable environment for their survival and replication. NMFs, found in corneocytes, comprise lactate, urea, electrolytes, and amino acids whose changes in composition can alter pH, epidermal lipids, and the state of hydration (Eyerich et al. 2018). As for them, epidermal lipids are sebaceous and keratinocyte origin, and they cover the surface of the skin filling the gaps between corneocytes in the SC (Tricarico et al. 2022). They include ceramides (50%), fatty acids (20%), cholesterol ester (15%), and cholesterol (5%) (Asada et al. 2022). AMPs, such as dermcidin, have broad-spectrum antimicrobial activity and can kill or inhibit the growth of certain pathogens in the skin microbiota. They disrupt microbial cell membranes, interfere with microbial metabolism, and/or modulate immune responses. Skin cells also produce AMPs such as lactic acid, produced during glucose degradation, or reactive oxygen species (ROS) that contribute to the acidic pH and have bactericidal properties. These chemical components and mechanisms collectively form the chemical barrier of the skin, working in conjunction with the physical barrier to protect the body.

### 3. Physical barrier

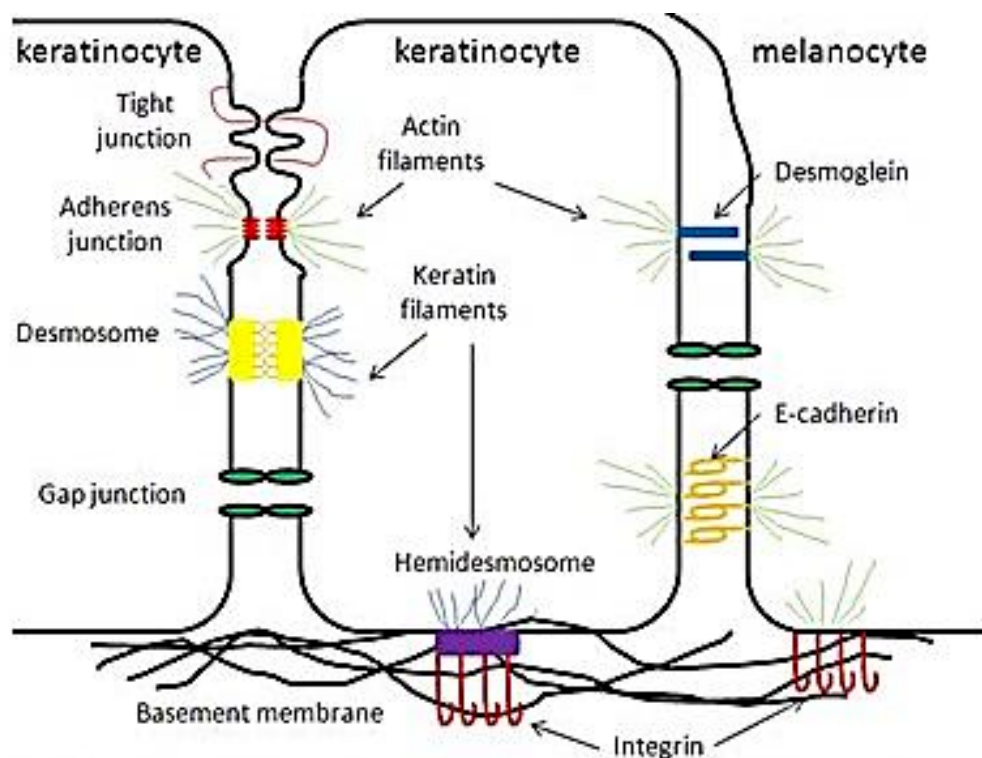


Figure 6: Cellular adhesion in the epidermis.

(Extracted from [Accueil - Archive ouverte HAL](#) – « Morphogenèse du mélanome in situ » from Clément Chatelain)

In addition to its chemical barrier function, the skin also possesses a physical barrier that helps protect the body from harmful substances and water loss. The physical barrier is primarily ensured by the cohesion between the epidermal cells and is mainly localized in the SC of the epidermis (*Figure 4*). It consists of corneocytes connected by corneodesmosomes which are embedded in an intercellular matrix composed of the epidermal lipids discussed earlier, thus forming a hydrophobic matrix. Therefore, the cornified layer is highly impermeable to water, preventing excessive transepidermal water loss and maintaining hydration (Proksch, Brandner, et Jensen 2008). It also prevents the entry of pathogens (such as bacteria, fungi, and viruses) and harmful substances (such as chemicals, allergens, and toxins) into the deeper layers of the skin. This barrier also comprises the nucleated keratinocytes cells underlying the cornified layer which are linked through desmosomes, tight junctions, gap junctions, and adherent junctions to ensure the cellular cohesion (*Figure 6*). Keratinocytes are also linked to adjacent melanocytes through desmoglein and E-cadherin. The adhesion between the epidermis and the dermis involves hemidesmosomes and focal contacts between keratinocytes and the basal lamina (*Figure 6*). Moreover, the different layers of the skin, including the dermis and the hypodermis, provide a mechanical protection with cushioning and support, absorbing and distributing forces that could otherwise harm underlying tissues and organs. In case of physical injuries, such as cuts, scrapes, and wounds, there are mechanisms involved to repair and regenerate the skin and subsequently to restore the integrity of this physical barrier. Altogether, these elements form a solid system that maintains not only cellular cohesion, but also cellular communication, differentiation, migration, proliferation, and to maintain cell permeability.

#### 4. Immune barrier

The immune barrier refers to the complex system of immune cells and molecules present in the skin that help discriminate between self and non-self, identify and eliminate pathogens, initiate inflammatory responses, and provide immune memory for rapid and effective immune defense. This barrier is composed of immediate non-specific innate response elements, as well as adaptive immune responses induced by specific stimuli. It brings together resident cellular actors (such as keratinocytes, fibroblasts, melanocytes, LCs, DCs, mast cells, macrophages, and

resident T cells) and recruited cells (such as monocytes, granulocytes, T cells and natural killer (NK) cells), as well as a large variety of soluble inflammatory mediators like cytokines and chemokines (*Figure 4*). AMPs are also part of the immune barrier and serve as early warning signals to activate and bridge the innate and adaptive immune systems so the term “alarmins” has been proposed to characterize these proteins (Baroni et al. 2012). Various types of T cells, including CD4+ helper T cells and CD8+ cytotoxic T cells, are present in the skin and are crucial for coordinating adaptive immune responses, eliminating infected cells, and modulating immune reactions. Cytokines and chemokines are signaling molecules produced by immune cells which play a crucial role in regulating immune responses, inflammation, and the recruitment of other immune cells to the site of infection or injury. This immune system is well equipped with immune receptors, also known as pattern recognition receptors (PRRs), that sense danger signals *via* pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). These PRRs mainly include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) that will be discussed in detail shortly after as it is the major subject of this project. This immune barrier is highly interconnected with the others as it can sense microbial signals from the microbiome barrier, it is shaped by the condition of the physical barrier, and directly responds to part of the chemical barrier to form an interactive network (Eyerich et al. 2018).

## II. C-type Lectin Receptors (CLRs)

### A. General information about CLRs

#### 1. Classification, structure, and ligand recognition

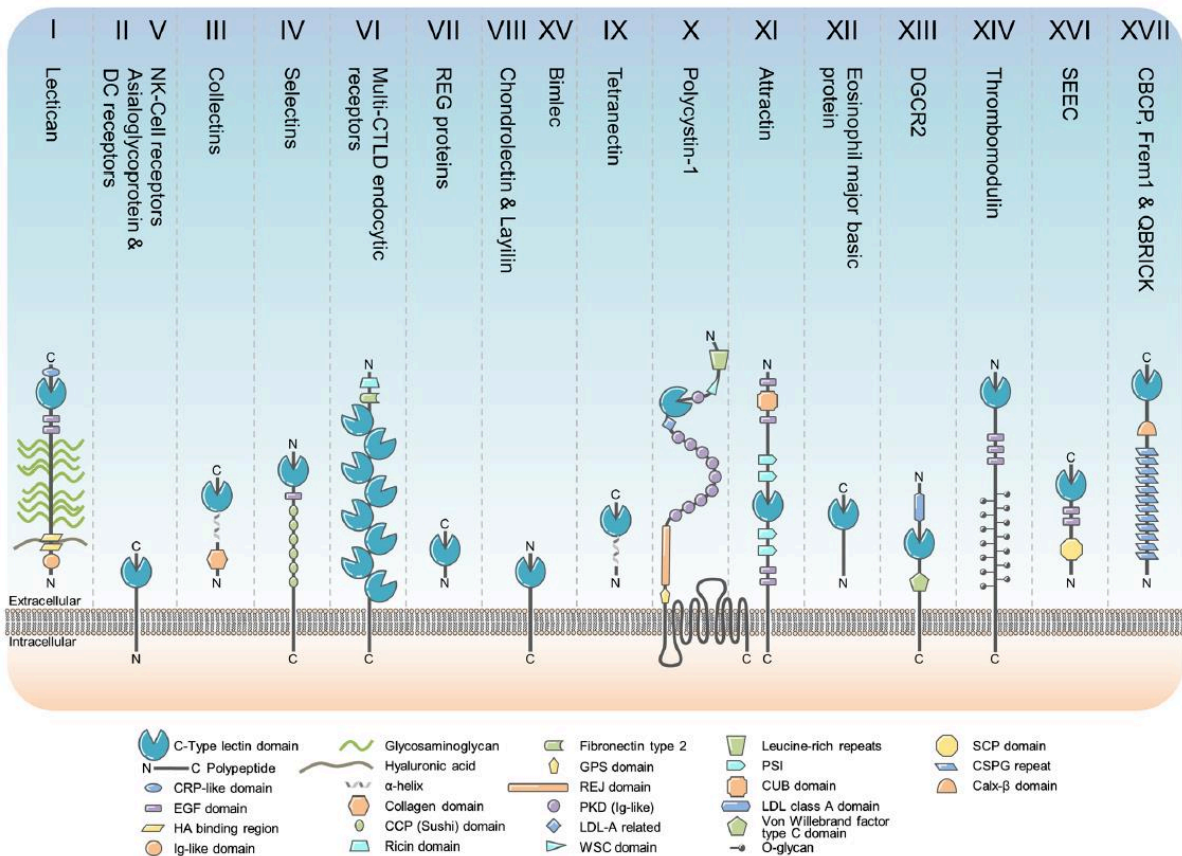


Figure 7: Representation of the 17 subgroups of CTLs.

(Extracted from (Mayer, Raulf, et Lepenies 2017))

Lectins are a structurally diverse class of proteins with the ability to bind carbohydrates specifically and reversibly, but also to agglutinate cells and/or precipitate glycoconjugates (Drickamer 1988). They are found in all kinds of organisms, from plants to bacteria and humans, and may be soluble or membrane bound. Based on their structural characteristics, ligand specificities, and evolutionary relationships, lectins can be categorized into several families, including the C-type lectins (CTLs).

CTLs are a superfamily of more than 1000 members divided into 17 subgroups on the basis of their phylogeny and domain organization (Zelensky et Gready 2005; G. D. Brown, Willment, et Whitehead 2018) (Figure 7), which can be either soluble or transmembrane proteins. There



are 2 types of transmembrane C-type lectin receptors: the type 1 proteins have the C-terminal side of their protein sequence in the cytoplasm whereas the type 2 proteins, which are usually expressed by innate immune cells, have an extracellular C-terminal side. Transmembrane CLR can also be classified into 4 categories according to their intracellular signaling motifs: immunoreceptor tyrosine-based activating motif (ITAM)-coupled CLR, hemi-ITAM-(hemITAM)-bearing CLR, immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing CLR, and a group of CLR lacking typical signaling motifs (Del Fresno et al. 2018) (*Figure 8*). These signaling motifs and subsequent effector responses will be presented shortly after.

As they are multiple structures of CLR depending on their subgroup, we will focus on the group V, also known as the NK-cell receptors (*Figure 7*), to which CLEC12B belongs as it is the focus of this thesis and will be discussed later. They are part of the type 2 transmembrane proteins with an intracellular N-terminal side whose structure includes:

- An intracellular domain, or cytoplasmic tail, with sequences that may enable receptor internalization or motifs that can modulate signaling pathways.
- A short transmembrane domain (about 20 amino acids) mainly composed of hydrophobic residues allowing to anchor the receptor to the cytoplasmic membrane and to expose the receptor on the cell surface. This domain can undergo conformational changes following ligand-induced signal transduction, thus contributing to immune signaling.
- A coiled-coil domain, also named stalk region or neck, composed of alpha helices necessary for oligomerization. The oligomerization ability is influenced by the variability of the length of this domain.
- A single extracellular domain essential for ligand binding.

CTLs were originally characterized by their capacity to bind carbohydrates only in presence of  $Ca^{2+}$  through conserved residues within their carbohydrate recognition domain (CRD), hence their name of "C"-type lectins. These conserved residues include the EPN (Glutamic acid - Proline - Asparagine) and QPD (Glutamine - Proline - Aspartic acid) motifs, which confer specificity for mannose-type and galactose-type carbohydrates, respectively (Weis, Taylor, et Drickamer 1998; Zelensky et Gready 2005). However, many CLR, including the NK-cell receptors, have an extracellular domain similar to the CRD but without the motifs required for  $Ca^{2+}$ -dependent carbohydrate recognition (Weis, Taylor, et Drickamer 1998). Such domains are

called C-type lectin-like domains (CTLDs) and can recognize a broad range of ligands, other than carbohydrates, such as proteins (e.g., F-actin), lipids (e.g., Dipalmitoylphosphatidylcholine) and inorganic compounds (e.g., monosodium urate (MSU) crystals) (Kuroki et Akino 1991; Zelensky et Gready 2005; Ahrens et al. 2012; Neumann et al. 2014). In the end, these CLRs can function as adhesion as well as signaling receptors in many immune functions, such as pathogen and self-antigens recognition, inflammation and immunity to tumor or infected cells.

## 2. Signaling motifs in CLRs

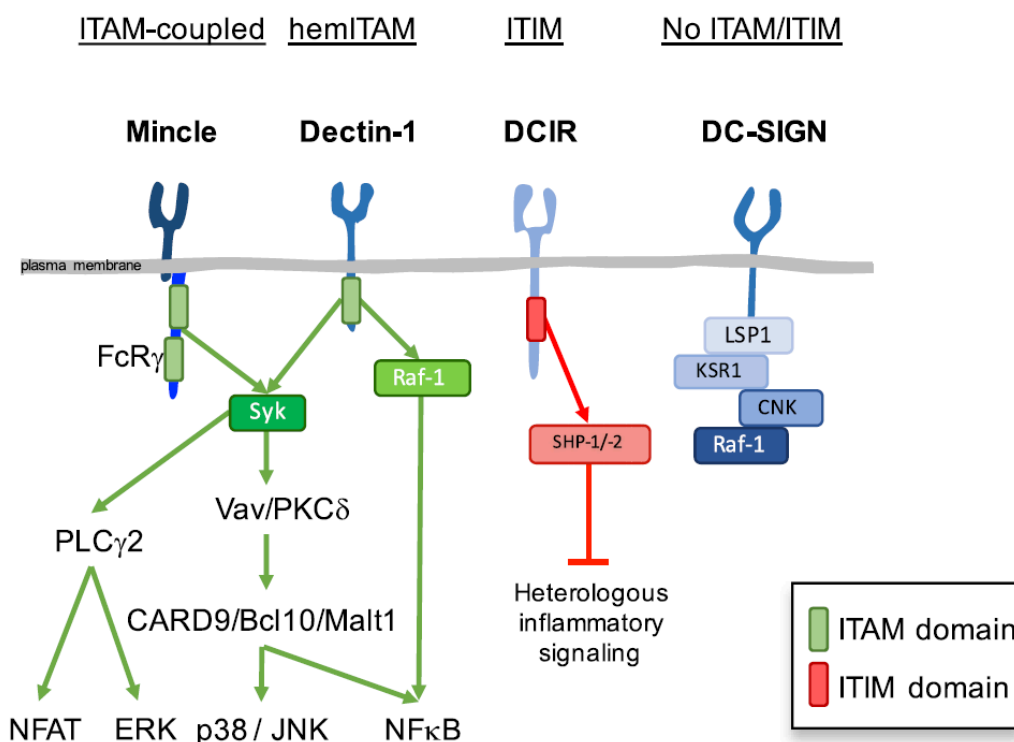


Figure 8: Signaling motifs in CLRs.

(Extracted from (Del Fresno et al. 2018))

As previously mentioned, transmembrane CLRs can be classified into 4 categories depending on their intracellular signaling motif (Figure 8).

- ITAM-coupled CLRs, such as Macrophage-Inducible C-Type Lectin (Mincle or CLEC4E), associate with adaptor proteins, such as Fc receptor  $\gamma$  (FcR $\gamma$ ) chain adaptor or DNAX-activation protein 12 (DAP12), that bear one or more ITAMs characterized by YxxL (Y=Tyrosine, x=any amino acid, L=Leucine) tandem repeats with the following consensus sequence : Yxxl/Lx<sub>6-12</sub>Yxxl/L (Del Fresno et al. 2018).



- hemITAM-bearing CLRs, such as Dendritic Cell-Associated C-Type Lectin-1 (Dectin-1 or CLEC7A), have ITAM-like sequences with only one YxxL signal motif.

These 2 categories of CLRs are considered as activating receptors. Indeed, upon receptor activation, the ITAM motif is phosphorylated, primarily by members of the Src family kinase (SFK) and generates docking sites for the recruitment of spleen tyrosine kinase (Syk) through its SH2 domains. This leads to a conformational change of Syk, followed by an autophosphorylation and subsequent activation. Active Syk binds to proteins in downstream signaling pathways such as PLC $\gamma$ 2 and Vav-PKC $\delta$ , or mediates CARD9-Bcl10-Malt1-dependent activation of Nuclear Factor-kappa B (NF- $\kappa$ B) (Mócsai, Ruland, et Tybulewicz 2010). Dectin-1 can also activate the Raf-1 kinase, thus inducing a Syk-independent non-canonical activation of NF- $\kappa$ B (Gringhuis et al. 2009). In either case, these signaling pathways induce activation of several downstream molecules including mitogen-activated protein kinases (MAPKs) and NF- $\kappa$ B, resulting in the activation of innate immunity and host defense with cellular responses such as phagocytosis, maturation of DCs, and production of inflammatory cytokines (Strasser et al. 2012). In addition, these receptors can also facilitate the development of adaptive immunity, in particular the Th1 response of interferon(IFN) $\gamma$ -producing CD4<sup>+</sup> T cells (Kerrigan et Brown 2011).

- ITIM-containing CLRs, such as Dendritic Cell Immunoreceptor (DCIR or CLEC4A), contain a single S/I/V/LxYxxI/V/L (S=Serine, I=Isoleucine, V=Valine) motif in their cytoplasmic tail. They are usually considered as inhibitory receptors and tend to recognize healthy self to dampen unnecessary immune activation. Ligand engagement by inhibitory receptors results in ITIM phosphorylation by SFKs and recruitment of Src homology region 2 domain-containing phosphatases 1 and 2 (SHP1 and SHP2) to inhibit Signal Transducer and Activator of Transcription (STAT) dimerization, and subsequent activation of interferon-stimulated genes (ISGs) (Barrow et Trowsdale 2006). Upon DCIR activation on DCs, this CLR is internalized into endosomal compartments and its ITIM domain is phosphorylated, allowing the recruitment of SHP1 and SHP2. This results in an inhibition of TLR8-induced TNF and IL-12 production, as well as a decrease in TLR9-induced TNF and IFN $\alpha$  production (T. B. H. Geijtenbeek et Gringhuis 2009).
- CLRs with no ITAM or ITIM can be associated, in the case of Dendritic Cell-Specific Intracellular Adhesion Molecules (ICAM)-3 Grabbing Non-Integrin (DC-SIGN or CLEC4L),

with a signalosome composed of scaffold proteins and the kinase Raf-1 in unstimulated DCs to modulate signaling by heterologous receptors or engage the endocytic machinery contributing to antigen processing and presentation to T cells (Del Fresno et al. 2018). They can also possess internalization motifs in their cytoplasmic tails that are believed to mediate endocytosis of bound ligands.

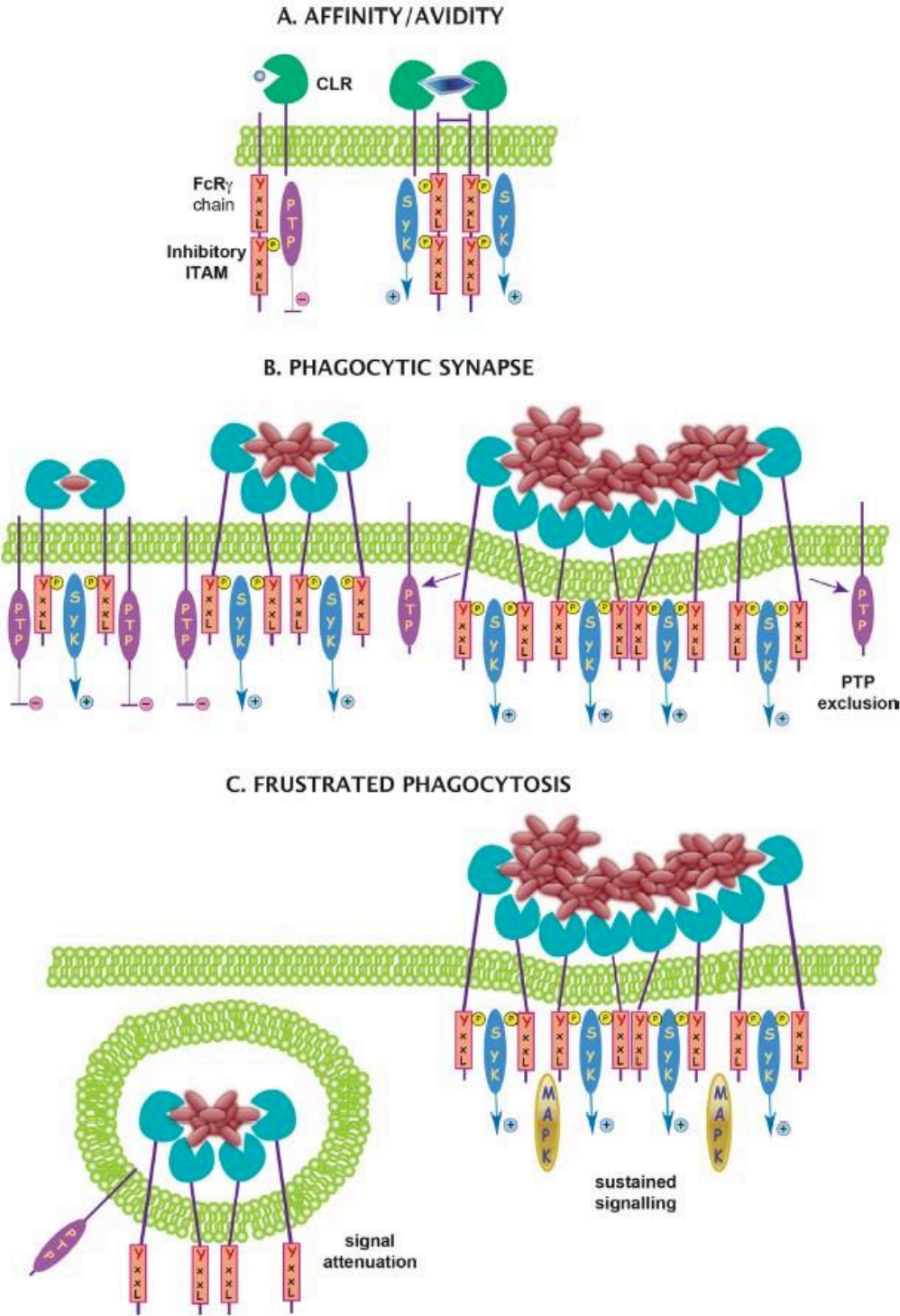
However, the signaling flexibility of CLRs is much more complicated and several studies have shown that hemITAM-bearing and ITAM-coupled CLRs can mediate inhibition whereas ITIM-containing CLRs can mediate activation depending on the environmental and cellular conditions.

For example, *Leishmania major* parasites release a soluble ligand that binds Mincle, triggering an ITAM inhibitory signaling pathway that suppresses DC activation through SHP1 recruitment to evade immune surveillance and dampen adaptive immunity (Iborra et al. 2016). Upon Dectin-1 activation in DCs, FcR $\gamma$  can negatively regulate the Dectin-1 responses in association with SHP1 and Phosphatase and TENsin homolog (PTEN) by decreasing the maturation and cytokine production in DCs, thus inhibiting T cell activation and T helper 17 polarization (Pan et al. 2017). These 2 examples show that the association of phosphatases to the supposed ITAM-activating CLRs can induce the opposite effect, mimicking the ITIM signaling responses.

In contrast to these results, it has been shown that the ITIM-bearing DCIR expression is required for sustained STAT1 phosphorylation and DCIR enables sustained STAT1-mediated type I IFN signaling in DCs (Troegeler et al. 2017). Another study has shown that DCIR exacerbates incidence and pathogenesis of cerebral malaria through an increase in TNF $\alpha$  production in sera, T cell activation in spleen, and T cell migration and sequestration into the brain (Maglinao et al. 2013). DCIR can also act as an attachment receptor for HIV-1 to promote virus uptake into cells (Lambert et al. 2008). Following viral infection, C-Type Lectin Domain Family 12 Member A (CLEC12A or MICAL) activation by MSU released by host dead cells positively activates the TBK1-IRF3 axis of retinoic acid-inducible gene I (RIG-I)-induced IFN-I production through activating SFKs, thereby amplifying an antiviral immune response (K. Li et al. 2019). Although both DCIR and CLEC12A are thought to act as negative regulators of immune cell signaling, these results show that ITIM-containing CLRs can somehow activate some signaling pathways, but whether these receptors deliver a signal on their own through the ITIM domain or require a co-receptor will need further investigation.

Although ITAM and ITIM domains are well-characterized, the exact signaling pathways of most CLRs are still unclear. Altogether, these data illustrate the plasticity of CLRs signaling through their intracellular motifs that can affect the following effector responses, but these responses can also be modulated by the physical nature, affinity, and avidity of the CLRs ligand.

3. Diversity of CLRs ligands affecting the functional outcomes



*Figure 9: The nature of CLR ligands.*

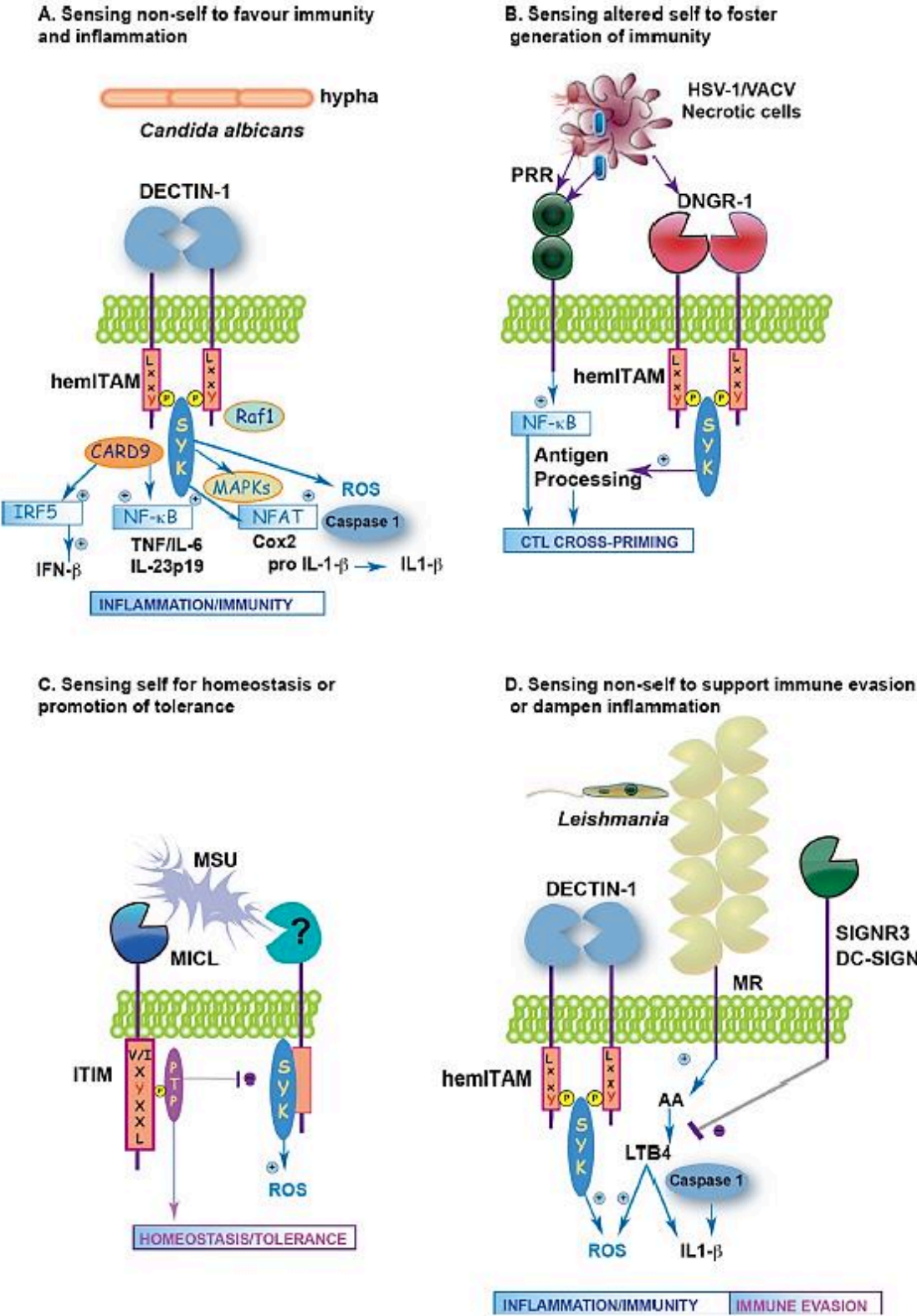
*(Extracted from (Iborra et Sancho 2015))*

Understanding the ligand specificity and functional consequences of CLR-ligand interactions is crucial for unraveling the complex interplay between the immune system and its environment. More precisely, the nature of the ligand is a determining factor as it can affect CLR signaling and subsequent cellular responses. Broadly, CLR ligands can be summarized in four major groups: glycans, (glyco-)proteins, (glyco-)lipids, and crystalline structures (Fischer et al. 2022). Affinity and avidity of the ligand can affect the quantity and duration of signals through the ITAM domain (*Figure 9a*). When an ITAM-coupled CLR binds to a low affinity binding ligand, it induces an hypophosphorylation of the ITAM domain of the associated Fc $\gamma$  chain with only one phosphorylated tyrosine. The resulting inhibitory ITAM conformation preferentially binds to protein tyrosine phosphatases (PTPs) such as SHP1 and SHP2 to inhibit downstream signaling, just like ITIM-bearing CLR (Blank et al. 2009). However, the binding of a high avidity ligand induces the full phosphorylation of the two tyrosines within the ITAM of the Fc $\gamma$  chain, thus allowing the recruitment and activation of Syk and subsequent activating signaling (Mócsai, Ruland, et Tybulewicz 2010).

Whether the ligand is in a soluble monomeric state, or a particulate oligomeric state, influences its affinity/avidity to a given CLR (*Figure 9b*). A soluble ligand doesn't prevent the inhibitory activity of membrane PTPs, thus inducing a poor activating signal. However, if the same ligand is in an oligomeric state, a phagocytic synapse is formed to promote Syk activating signaling associated with an exclusion of the inhibitory PTPs from the receptors. For instance, Dectin-1 signaling is only activated by particulate, but not soluble,  $\beta$ -glucans which cluster this CLR in synapse-like structures from which inhibitory PTPs are excluded, thus allowing Syk association and activation (Goodridge et al. 2011).

Another determining factor of CLR signaling is the size of these ligand particles (*Figure 9c*). Small ligand particles induce an endocytosis of the receptor, leading to an attenuation of inflammatory responses probably through an improved access for inhibitory PTPs. However, large ligand particles induce a delayed or "frustrated" phagocytosis, resulting in a sustained signaling and enhanced inflammation. Regarding Dectin-1, it has been shown that curdlan particles, which are large  $\beta$ -glucan particles, are substantially more potent inducers of pro-

inflammatory cytokine production in comparison to  $\beta$ -glucan microparticles. Due to their large size, curdlan particles can't be engulfed by phagocytes, indicating a "frustrated" phagocytosis correlated with sustained signaling and MAPK activation (Hernanz-Falc3n et al. 2009; Rosas et al. 2008).



*Figure 10: Self and non-self sensing by CLRs.*

*(Extracted from (Iborra et Sancho 2015))*

As described above, the nature of ligands can modulate the CLRs signaling and thus affecting the cellular responses. Another degree of complexity is added to the picture as CLRs can bind more than one ligand and most of them can bind both endogenous and exogenous ligands, resulting in distinct functional responses. Some CLRs can indeed recognize self and non-self ligands, such as DC-SIGN that can bind fucose and high mannose-based ligands present on self receptors, as well as exogenous proteins such as Salp15 in the saliva of *Ixodes* ticks (T. B. Geijtenbeek et al. 2000; Hovius et al. 2008). Other CLRs can only recognize self antigens, such as C-Type Lectin Domain Family 9 Member A (CLEC9A or DNGR-1) recognizing F-actin or CLEC12A sensing MSU upon cellular death (Ahrens et al. 2012; Neumann et al. 2014), while some CLRs can bind a ligand present in various organisms, like Dectin-1 binding to  $\beta$ -glucans present in yeasts, fungi and bacteria (G. D. Brown 2006).

On one hand, sensing exogenous ligands usually favors inflammation and immunity to fight against pathogens (*Figure 10a*). For instance, the Dectin-1 binding to  $\beta$ -glucans from fungi like *Candida albicans* induces NF- $\kappa$ B activation through a Syk-dependent or a Syk-independent non-canonical pathway, resulting in the activation of innate immunity with a production of pro-inflammatory cytokines but also in the induction of an adaptative immunity (Gringhuis et al. 2009; Mócsai, Ruland, et Tybulewicz 2010; Strasser et al. 2012; Cheng et al. 2011; Zhao et al. 2016). However, pathogens can mimic self-inhibitory signals to escape immune surveillance or dampen immunity and inflammation (*Figure 10d*). For example, the murine DC-SIGN homolog Specific Intercellular adhesion molecule-3-Grabbing Nonintegrin Receptor 3 (SIGNR3) favors *Leishmania infantum* resilience through the inhibition of the microbicidal response induced by Dectin-1 and Mannose Receptor (MR or CD206), thus promoting immune evasion (Lefèvre et al. 2013).

On the other hand, sensing endogenous ligands promote communication, cell adhesion, homeostasis, and tolerance (*Figure 10c*). Since our innate immune system can recognize pathogens through PAMPs and damaged self through DAMPs, it has been proposed that it also can recognize healthy self through Self-Associated Molecular Patterns (SAMPs) that could be sensed by inhibitory Self-PRRs (SPPRs) to maintain the baseline non-activated state of innate immune cells and dampen their reactivity following an immune response (Varki 2011). A good

example of SPPR is CLEC12A that binds to MSU and inhibits the MSU-induced Syk-dependent production of ROS probably through the recruitment of the phosphatases SHP1 and SHP2, resulting in a noninfectious anti-inflammatory response (Neumann et al. 2014). Moreover, altered endogenous ligands from transformed or dead cells, so called DAMPs, can foster generation of immunity (*Figure 10b*). For instance, CLEC9A, a receptor for dead cells that recognizes F-actin (Ahrens et al. 2012), is crucial for cross-presentation of necrotic cell cargo associated with herpes simplex virus type 1 (HSV1) or Vaccinia Virus (VACV) to CD8<sup>+</sup> T cells to favor an efficient cytotoxic response (Zelenay et al. 2012; Iborra et al. 2012).

Further investigation is needed to unravel the complexity and plasticity of ligands and signaling motifs contribution into CLRs functional outcomes for the purpose of identifying novel therapeutic targets. For instance, in nature, multivalent ligand presentation at the surface of cells or pathogens overcomes the poor affinity of CLR/carbohydrate interactions. Thus, multivalency is a promising strategy for targeting CLR-expressing cells and such approach could enable the delivery of drugs to specific cells and shape immune responses that are fundamental for vaccination, tolerance induction, or tumor therapy (Lepenies, Lee, et Sonkaria 2013).



#### 4. Therapeutic potential of CLRs

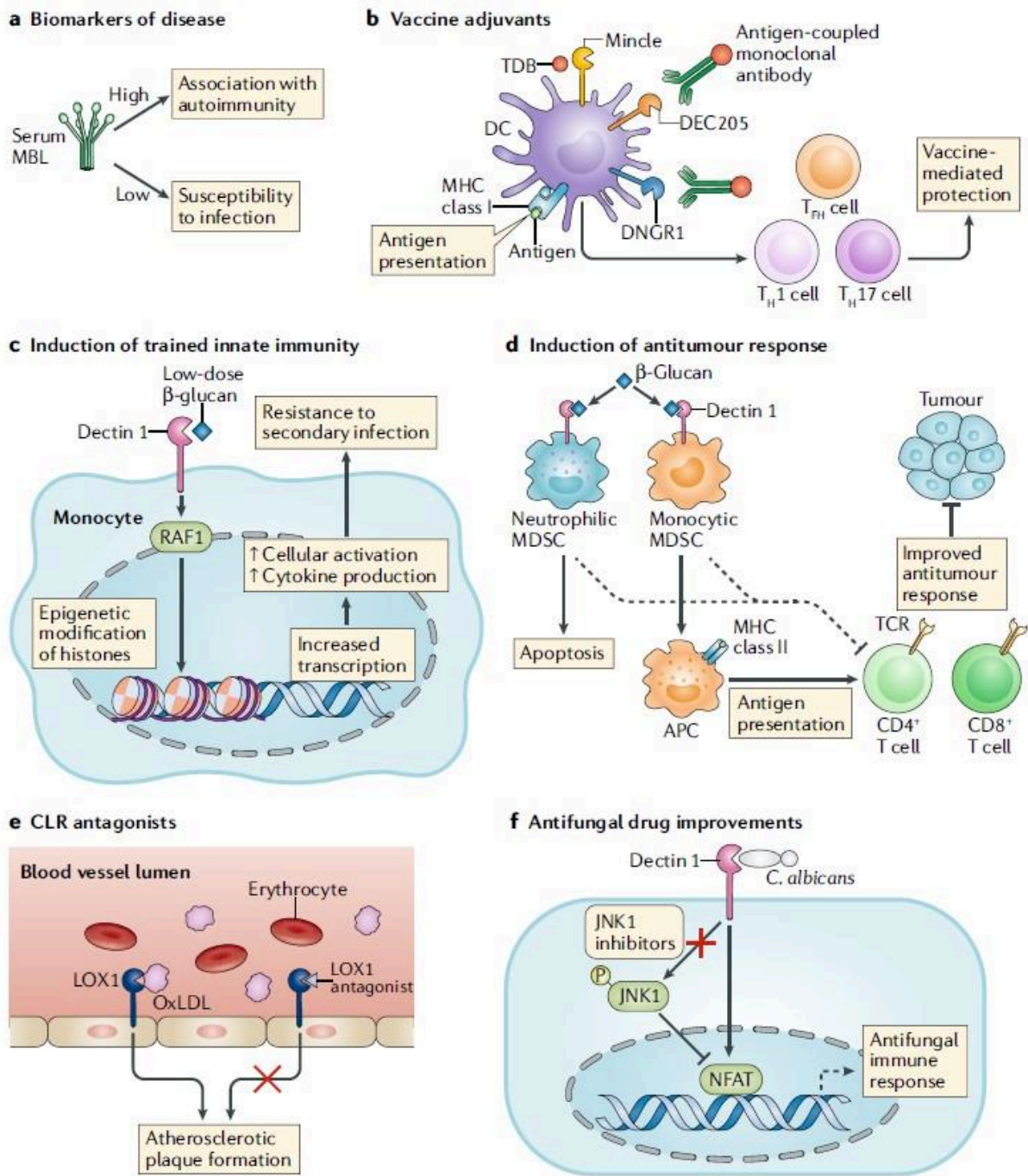


Figure 11: Therapeutic potential of CLRs.

(Extracted from (G. D. Brown, Willment, et Whitehead 2018))

CLRs are mainly expressed by myeloid cells and play a pivotal role in the recognition of pathogens, self-components, and altered glycosylation patterns, triggering and influencing immune cell functions and homeostasis, thus making them attractive targets for future development of novel diagnostics and therapeutic strategies.



CTLs can serve as helpful biomarkers for disease. For example, early detection of serum Mannose-Binding Lectin (MBL) and measurement of its levels can be used to predict a susceptibility to autoimmune and infectious diseases. Plasma-purified or recombinant MBL can therefore be a potential therapeutic target for the treatment of human diseases (Sunil Singh et al. 2016) (*Figure 10a*).

CLRs can improve the efficacy of vaccines through the use of synthetic CLR ligands, such as the Mincle agonist trehalose-6,6-dibehenate (TDB), as adjuvant for protein antigen vaccines (Ostrop et al. 2015), or through the antigen-targeting of CLRs on DCs, such as CLEC9A in the absence of adjuvant (Kato et al. 2015) (*Figure 10b*).

CLRs ligands, such as  $\beta$ -glucan *via* Dectin-1, can trigger “trained innate immunity” through the induction of epigenetic modifications in macrophages and monocytes and subsequent increased cytokine production and cellular activation, providing long-term immune protection and resistance to secondary infection (Van Der Meer et al. 2015) (*Figure 10c*). This trained immunity is important for host defense, vaccine responses and diseases, making it a promising area for new treatments.

Activation of CLRs, such as Dectin-1, can also induce protective antitumor responses. Upon Dectin-1 activation with whole  $\beta$ -glucan particles in myeloid-derived suppressor cells (MDSCs), the neutrophilic MDSCs undergo apoptosis while monocytic MDSCs are converted into potent APCs, thus improving antitumor CD4<sup>+</sup> and CD8<sup>+</sup> T cell-mediated immune responses (Albeituni et al. 2016) (*Figure 10d*).

The use of CLRs antagonists can be another way to use these CLRs as therapeutic targets. For instance, lectin-like oxidized low-density lipoprotein(ox-LDL) receptor-1 (LOX-1 or CLEC8A) acts as a scavenger receptor that binds and internalizes ox-LDL which leads to the formation of atherosclerotic plaques, making the use of LOX-1 antagonists a promising novel target of therapy of atherosclerosis and related disorders (Thakkar et al. 2015) (*Figure 10e*).

Inhibiting CLRs signaling pathways can also induce protective responses and improve antifungal drugs. For example, activation of nuclear factor of activated T cells (NFAT) using inhibitors of C-Jun N-Terminal Kinase 1 (JNK1 or MAPK8) can improve the Dectin 1-induced immune response to *Candida albicans* (G. D. Brown, Willment, et Whitehead 2018) (*Figure 10f*).

Furthermore, the use of soluble fusion proteins of CLR s can provide new tools for therapeutics and diagnostics. Using the immunoglobulin (Ig) crystallizable fragment (Fc) domain as a fusion partner with a given CLR provides soluble, stable, easily purified fusion proteins that have increased avidity properties due to the Fc homodimerization (Willment 2022). For instance, targeting *Pneumocystis carinii* or *Aspergillus fumigatus*  $\beta$ -glucans with the fusion protein Dectin-Fc, composed of the extracellular domain of Dectin-1 linked to the Fc portion of IgG1, enhances host recognition and killing of the pathogens thus promoting survival and resistance against the resulting infections (Rapaka et al. 2007; Mattila et al. 2008; Rodriguez-de la Noval et al. 2020).

Altogether, these examples witness that harnessing the unique properties of CLR s opens new avenues for the development of innovative therapies to enhance immune responses, treat inflammatory diseases including cancers, autoimmune diseases, or allergy, and combat infectious diseases.

#### B. CLR s in skin immunity (published review)

CLR s can sense a broad range of ligands, mainly through DAMPs and PAMPs, to induce immune responses. The recognition of DAMPs promotes sterile inflammatory responses, necessary for tissue repair and regeneration, but can also lead to inflammatory diseases such as cancer and autoimmune diseases. As mentioned above, the skin is colonized by a plethora of microorganisms, called the skin microbiota, and CLR s play a central role in recognizing and discriminating commensals from pathogens through PAMPs to maintain skin homeostasis and fight against infections. However, CLR s can be hijacked by pathogenic microorganisms to escape immune surveillance and initiate diseases.

Hereafter (CLEC12B section) I include a recent review I wrote and published focusing on CLR s in skin immunity, covering their involvement in antitumor, antifungal, antiviral, and antibacterial immunity, and their role in chronic inflammatory skin diseases (Blot, Passeron, et Tulic 2022).

### C. Dectin-1 cluster

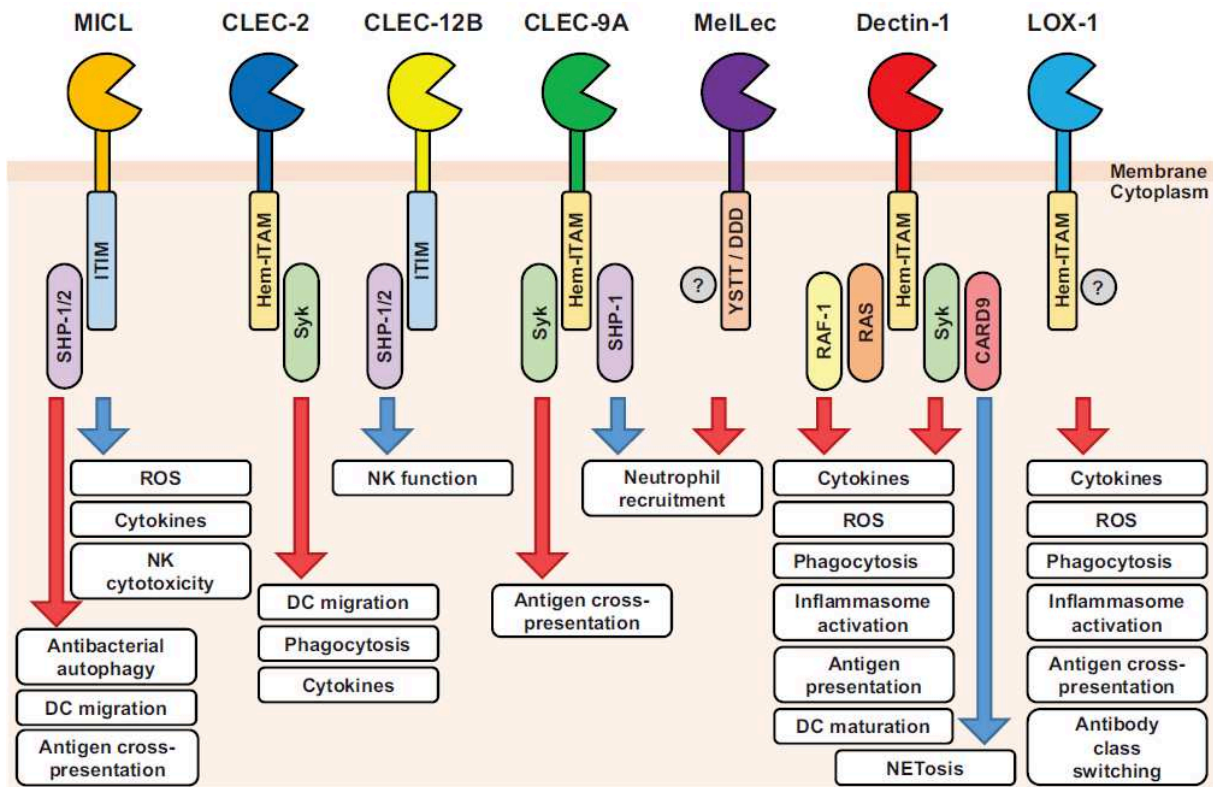


Figure 12: Cellular and immunological functions of the Dectin-1 cluster.

(Extracted from (Tone et al. 2019))

The previously mentioned group V of CLRs, so called NK-cell receptors, contains several families of type II transmembrane CLRs encoded within the natural killer gene complex (NKC), on human chromosome 12. One such family is the Dectin-1 cluster composed of seven related receptors which are mainly, but not entirely, expressed by myeloid cells: CLEC12A, CLEC1B (or CLEC2), CLEC12B (or MAH), CLEC9A, CLEC1A (or MelLec), Dectin-1 and LOX-1 (ordered based on genomic location) (Figure 12). The Dectin-1 cluster plays a key role in immunity and homeostasis, and some of these CLRs functions have already been discussed earlier and in the review below. However, in comparison with the other CLRs in this cluster, CLEC12B is still poorly understood with no ligand identified and its involvement in immunity has not been studied yet. The following chapter summarizes our current knowledge about CLEC12B.

### III. CLEC12B (published review)

In addition to the overview of CLRs in skin immunity, the review below provides all the knowledge on CLEC12B that was available up to 2021, from its identification to its role in

normal skin physiology and in skin pathology (Blot, Passeron, et Tulic 2022). Since then, a few new studies on CLEC12B have been published. Besides its strong expression in skin melanocytes (Sormani et al. 2022), it has been shown that CLEC12B is also expressed on skin mast cells (Iijima et al. 2021). A very recent study has reported that CLEC12B recognizes curdlan and laminarin, both polysaccharides containing  $\beta$ -(1,3) glucans, as well as N-acetylglucosamine (GlcNAc) residues in *Pneumocystis murina* cell wall homogenates (CWH) and in a purified *Pneumocystis carinii* cell wall fraction (CWF) (Kottom et al. 2023). Interestingly, lung mRNA CLEC12B expression is up regulated during infection in the mouse immunosuppressed *Pneumocystis pneumonia* model and CLEC12B silencing induces a significant decrease of TNF $\alpha$  after *P. carinii* CWF stimulation in the mouse RAW macrophage cell line. Another study has shown that CLEC12B has a higher DNA methylation in grade 3 patients with lung adenocarcinoma grading but to date, there is no data linking CLEC12B methylation to lung cancer or chemotherapy sensitivity (Forest et al. 2022). Knowing that CLR orthologs are well conserved across various organisms, multiple sequence alignment showed that CLEC12B found in Nile tilapia fish (*Oreochromis niloticus*), called OnCLEC12B, shares a conservative CTLD with other CLEC12Bs including the human one (Z. Zhang et al. 2022). OnCLEC12B is located at the cell membrane in many organs, including the skin, and its expression is increased after *Streptococcus agalactiae* and *Aeromonas hydrophila* infection. Importantly, OnCLEC12B can bind and agglutinate several Gram-positive and Gram-negative bacteria, including *S. aureus* and *E. coli*, and the addition of Ca<sup>2+</sup> increases its binding and agglutination activities. OnCLEC12B also enhances monocytes and macrophages proliferation, improves *A. hydrophila* and *S. agalactiae* phagocytosis by macrophages, and inhibits NF- $\kappa$ B activation. Taken together, these results show the participation of OnCLEC12B in Nile tilapia immune response to bacterial infection (Z. Zhang et al. 2022).

As previously mentioned, CLEC12B is a CLR predominantly expressed by melanocytes in the skin and, despite its role in pigmentation and melanomagenesis (Sormani et al. 2022; Montaudié et al. 2022), its involvement in skin immunity and the identification of its ligands are waiting to be discovered and have been the focus of my thesis project.

## Review Article

# C-type lectin receptors in skin immunity: Emerging new role for CLEC12B

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### ABSTRACT

C-type lectin receptors (CLRs) are a superfamily of transmembrane proteins, which consist of one or several C-type lectin-like domains and intracellular signaling motifs, such as immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibitory motif (ITIM). CLRs are mostly expressed on antigen-presenting cells and are known to play an important role in both innate and adaptive immunity. As a result, CLRs are involved in numerous physiological functions due to their ability to recognize pathogen-, tumor-, and damaged-associated molecular patterns on pathogens and host cells acting as pattern recognition receptors (PRRs). These immune receptors can respond to signals from the surrounding environment which has a direct and profound effect on the skin, the largest organ in the body and the only one that is in direct contact with the external environmental stimuli. The skin is colonized by a plethora of microorganisms constituting the skin microbiota and plays a central role in host defense against potentially pathogenic microbes including bacteria, fungi, and viruses. Skin dysbiosis has been shown to play a critical role in initiation of skin disease and/or induction of a local inflammatory environment. In this review, we discuss what is known about CLRs in skin immunity and their contribution to skin disease, with a special focus on a newly identified and a promising new CLR, CLEC12B.

**Keywords:** CLEC12B, C-type lectins, melanoma, pigmentation, skin immunity

### BACKGROUND


Lectins were first defined as carbohydrate-binding proteins or glycoproteins agglutinating cells and/or precipitating glycoconjugates. They are found in many organisms and may be soluble or membrane-bound [1]. Lectins can be classified in several categories, but here we are focusing on the C-type lectin receptors (CLRs), originally thought to bind ligands only in the presence of Ca<sup>2+</sup> through conserved residues within their carbohydrate recognition domain (CRD). However, many CLRs have an extracellular domain similar to the CRD lacking the components necessary for Ca<sup>2+</sup>-dependent carbohydrate recognition [2]. Such domains are called C-type lectin-like domains (CTLDs) and they can bind a wide variety of ligands,

other than carbohydrates, such as proteins, lipids, and inorganic compounds [3].

CLRs are a superfamily of more than 1000 proteins divided into 17 groups based on functional and structural characteristics. Usually classified as activating or inhibitory receptors based on their intracellular signaling motifs, some CLRs can induce both positive and negative signals depending on the binding ligand or the environment in order to shape diverse biological responses [4]. They are involved in numerous physiological functions due to their ability to recognize pathogen-, tumor-, and damaged-associated molecular patterns on pathogens and host cells. Most CLRs are expressed on antigen-presenting cells and were shown to play an important role in both innate and

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adaptive immunity [5]. Upon ligation, CLRs can act as innate immune pattern recognition receptors (PRRs) and internalize antigens for presentation to T cells, but also trigger cytokine production and expression of co-stimulatory molecules. Thus, CLRs play also a key role to instruct adaptive immunity through T-cell polarization, attraction of other immune cells, antibody production as well as immunological memory formation [6,7].

CLRs can respond to external signals from the environment and the skin, representing the largest organ in the body, is the most exposed interface with all the environmental stimuli. The skin is colonized by a plethora of microorganisms, representing the skin microbiota, and plays a central role in host defense against potentially pathogenic microbes including bacteria, fungi, and viruses [8]. To sense these pathogens, different types of skin cells express a broad range of PRRs, including CLRs, so that they can recognize and discriminate pathogens from commensal microorganisms to maintain skin homeostasis. Dysregulation of this microbiota can initiate diseases and/or an inflammatory environment.

Rather than providing a complete description of all the CLRs involved in immune homeostasis and skin diseases, already well described by other recent reviews, [7,9] here we focused on what is known about CLRs in skin immunity with a special focus on a newly identified and an emerging role of still poorly understood CLR, CLEC12B.

### **C-TYPE LECTIN RECEPTORS AND ANTITUMOR IMMUNITY**

Antitumor activity is essential for detection and killing of tumor cells. Both innate cells, such as natural killer (NK) cells, and acquired immune cells, especially cytotoxic CD8+ T cells, play important roles in the eradication of tumors. However, some cells can interfere with the antitumor immunity, like regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). Cancer cells can also inhibit the antitumor activity through the interaction between PD-L1 and/or PD-L2, expressed by tumor cells, and PD-1 at the surface of cytotoxic T cells [9]. CLRs are key players of the immune surveillance system and are thought to recognize tumor-specific antigens or neo-antigens to activate anti-tumor immunity, but their precise implication in these immune processes is not fully understood yet.

Melanoma is a very deadly skin cancer arising from melanocytes transformation. Recent reports have highlighted the important role of Dectin-1 (CLEC7A) in melanoma suppression [10-12]. Dectin-1, for Dendritic Cell-Associated C-type Lectin 1, is a type II membrane receptor with a single CTLD and a hem-immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. It is part of the Dectin-1 cluster with

six other CLRs, all encoded in the same locus in both mouse and human genomes [13]. Dectin-1 expressed by dendritic cells (DCs) and macrophages recognize N-glycan structures on melanoma cells and induce the activation of the IRF5-INAM pathway [10,14]. The interferon regulatory factor 5 (IRF5) is a transcription factor involved in the induction of class I interferons and inflammatory cytokines and its activity is critical to induce expression of IFN regulatory factor 3-dependent NK-activating molecule (INAM). This leads to the activation of NK cells through a cell-to-cell contact with DCs which is necessary to enhance the NK cell-mediated killing against melanoma cells [10]. Interestingly, Dectin-1 expressed on NK cells has minor effects on the tumor-killing activity. Another study has shown an antitumor immunity in melanoma through the activation of DCs by curdlan, a selective Dectin-1 agonist. Dectin-1 signaling induces Th9 cell differentiation and production of interleukin-9 (IL-9), leading to an antitumor immunity [11]. More precisely, Dectin-1-activated DCs present an enhanced IL-33 expression, leading to a Th9 priming and antitumor immunity. Blocking IL-33 inhibits the Th9 priming by Dectin-1-activated DCs while adding IL-33 to Dectin-1-activated DCs increases the CD4+ proliferation and inhibits the IL-33-induced Treg priming [12]. Altogether, these results could bring new means for an effective tumoricidal action using Dectin-1 agonistic antibodies or perhaps a combination of Dectin-1 and IL-33 may present a new effective modality of DC-based vaccines in melanoma immunotherapy. Recently, C-type lectin domain family 12 member B (CLEC12B) has been shown to play a role in melanoma, but we'll present these results later in this review.

CLRs can also serve in antitumor vaccination. For example, DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CLEC4L), a type II membrane receptor with one CTLD expressed as a tetramer on DCs, recognizes a wide range of pathogens and can present antigens to MHC-I and MHC-II molecules after internalization in antigen-presenting cells (APCs). This leads to increased CD4+ and CD8+ T cell responses. In a recent study, generation of apoptotic tumor cell-derived extracellular vesicles (ApoEVs) presenting melanoma antigens induced the expression of high-mannose glycans. The DC-SIGN binding to such high-mannose glycans increases the antigen internalization efficiency, resulting in enhanced priming of melanoma-specific CD8+ T cells [14]. These results highlight the importance of using CLRs as promising vaccination tools to shape antitumor T cell responses.

### **C-TYPE LECTIN RECEPTORS AND ANTIFUNGAL IMMUNITY**

Fungi are omnipresent in our environment and the host-associated fungal populations represent the mycobiota.

Although only a few fungal species are considered pathogenic and induce fungal infections, recent studies highlight a pivotal role for the mycobiota in modulating homeostasis and disease susceptibility [15]. Human fungal skin infections are usually superficial, but some fungal species can also lead to extensive skin and subcutaneous tissue infections. Fungal ligands activate a variety of PRRs including CLRs to induce an innate immune response through direct killing of fungi, modulation of mycobiota, and priming of adaptive immune responses. These CLRs can be expressed by skin myeloid cells divided into Langerhans cells (LCs) residing in the epidermis or DCs residing in the dermis. Understanding the role of CLRs in shaping host-fungi interaction and fungal disease susceptibility is necessary to find new therapeutic strategies against fungal diseases [16].

*Candida albicans* is one of the predominant fungi species present in the human mycobiota and usually asymptotically colonizes many niches in healthy individuals, including the skin. However, a fungal dysbiosis can promote the pathogenicity of *C. albicans* leading to infections from superficial dermal infections to systemic ones [17]. During *C. albicans* skin infection, yeast forms are found in the epidermis and invasive filamentous forms in the dermis. On one hand, the yeast form is recognized by Dectin-1 on LCs in the epidermis, leading to an increased production of IL-6 and thus to a Th17 cell differentiation. On the other hand, the filamentous form is recognized by dermal DCs, thus leading to a Th1 cell differentiation [18]. Interestingly, the addition of Dectin-1 ligands, such as curdlan, rescues the ability of filamentous forms to generate Th17 cell response. This suggests that there is no Dectin-1 ligature in the dermis during a *C. albicans* infection but dermal DCs do have the ability to prime Th17 cells. The morphologic change of *C. albicans* from the epidermis to the dermis could be an adaptation to evade immune responses driven, among others, by Dectin-1. In this *in vivo* study, Th17 cells provide protection against skin challenges whereas Th1 cells provide systemic protection [18]. These results reveal a key role of Dectin-1 binding in driving T helper cells responses that provide tissue-specific protection in response to *C. albicans* morphology. However, another study shows that the *C. albicans* filamentous form can induce Th17 responses through the production of IL-1 $\beta$  by macrophages [19]. These *in vitro* methods, using cocultures of macrophages with lymphocytes to measure IL-17 production, describe a Dectin-1/inflammasome pathway as a mechanism that provides a protective Th17 response against *C. albicans* infection. These conflicting data could be explained by the different cellular types used in these two studies (e.g., LCs and DCs versus macrophages) and more importantly, the physiological differences between *in vivo* and *in vitro* studies. Notably, the different Dectin-1 binding between LCs in the

epidermis and dermal DCs leading to different T cell responses cannot be found in cocultures where exogenous Dectin-1 ligands are added to macrophages and lymphocytes.

Dectin-2, for Dendritic Cell-Associated C-type Lectin 2 (CLEC6A), is also a major fungal PRR that can signal *via* Syk pathway to activate DCs and regulate adaptive immune responses to fungal infections. Unlike Dectin-1, Dectin-2 requires an association with FcR $\gamma$  chain for surface expression and coupling to Syk. Although Dectin-2 isn't necessary for innate resistance to *C. albicans* infection, it has an essential role in *Candida*-specific T cell production of IL-17 [20].

Many studies describe how the collaboration of different CLRs can shape specific immune responses to pathogens. *Fonsecaea monophora* is a causative agent of chromoblastomycosis which is a chronic fungal skin infection. This fungal pathogen activates Dectin-1 leading to the activation of the interferon regulatory factor 1 (IRF1) and an enhanced IL-12A transcription for an antifungal Th1 polarization. However, *F. monophora* also binds Mincle (Macrophage-inducible C-type lectin, CLEC4E), a CLR within the Dectin-2 cluster interacting with the FcR $\gamma$  chain which activates protein kinase B (PKB) and induces the proteasomal degradation of IRF1 *via* an E3 ubiquitin ligase Mdm2, leading to an inhibition of IL-12A transcription. Thus, the decreased production of IL-12p35 by human DCs induces a Th2 polarization instead of a Th1 differentiation. This study reveals how the engagement of a CLR, Mincle, can suppress the antifungal immunity mediated by another CLR, Dectin-1, thus making Mincle a potential therapeutic target to redirect Dectin1-induced Th responses [21].

Beyond its protective role against UV damage in human skin, melanin (and more specifically eumelanin) has also antioxidant properties and a protective role against free radicals [22]. However, melanin can be considered as a fungal virulence factor, protecting fungi from the host immune defense by inhibiting host-cell phagocytosis, cytokine production, and apoptosis. MelLec (melanin sensing C-type lectin receptor, CLEC1A), another CLR of the Dectin-1 cluster with one CTLD and an immunoreceptor tyrosine-based inhibitory motif (ITIM) which is expressed by endothelial cells and myeloid cells, can recognize melanized fungal species, more specifically DHN-melanin produced from *A. fumigatus* conidia and other DHN-melanized fungal species [23]. The sensing of fungal DHN-melanin by MelLec is crucial in the control of systemic *A. fumigatus* infection. It has been shown that a single nucleotide polymorphism in the coding region of MelLec, more precisely an amino acid change Gly26Ala in the cytoplasmic tail, increases the risk of disseminated aspergillosis in stem-cell transplant recipients when the



variant is carried by the cell donor. Thus, MeLec might have a myeloid cell-mediated protective role and the identification of donors carrying this single nucleotide polymorphism could help to reduce the incidence of aspergillosis in transplant recipients. These results highlight the importance of melanin in effective fungal pathogenesis as well as in immune defenses against fungal infections, making the fungal melanin system a pertinent target for drug discovery [24].

## C-TYPE LECTIN RECEPTORS AND ANTIVIRAL IMMUNITY

As mentioned, the skin is constantly exposed to potentially pathogenic microorganisms, including viruses. Recognition of viruses by CLRs is complex and crucial as they can recognize and quickly respond to viral PAMPs to shape immune responses and inhibit viral spread within the host. However, some viruses are opportunistic as they can use the CLR machinery to enter host cells, to impair signaling pathways, and to inhibit APC functions [25]. Thus, viruses can avoid immune recognition and antiviral immunity to freely infect host cells.

Human immunodeficiency virus-1 (HIV-1) is mainly sexually transmitted across genital mucosa and establishes a life-long infection when viral DNA succeeds to integrate host genome. It has been shown that DC-SIGN is highly expressed on DCs in mucosal tissues and can bind to HIV-1 glycoprotein gp120 at the surface of immature DCs. Instead of internalizing gp120 into DCs for presentation to T cells and subsequent antiviral immunity, DC-SIGN is exploited by HIV-1 in that it doesn't allow virus entry but captures it to facilitate infection in *trans* of CD4+ T cells in the lymph nodes through binding of gp120 to CD4 [26]. Another study showed that HIV-1 also binds to monocyte-derived macrophages, which lack DC-SIGN on their surface, through the macrophage mannose receptor (MMR), a CLR from the mannose receptor family that can recognize high-mannose oligosaccharides on foreign particles. In a similar way to DC-SIGN, MMR binds to gp120 and captures HIV-1 to mediate transmission of bound virus to T cells. However, HIV-1 longevity is decreased when bound to macrophages in comparison to DCs because of endocytosis-mediated internalization of the virus [27]. These are examples of how a virus can escape antigen processing by hijacking CLRs. Fortunately, some CLRs do protect us against viruses such as HIV-1. In fact, DC-SIGN and MMR can be found on dermal DCs but Langerin (Langerhans cell-specific C-type lectin, CLEC4K) is a type II membrane receptor for mannose expressed on epidermal LCs, [28] meaning that Langerin is the first CLR to encounter HIV-1 in the skin. Langerin binds efficiently to HIV-1 on immature LCs and inhibits LC infection by internalizing and degrading infectious viral particles within Birbeck granules, thus preventing any viral spread to T cells [29].

However, the protective function of Langerin can be sabotaged if the protective layer of LCs in the epidermis is breached, allowing the virus to access to the subepithelial layers of dermal DCs or if there is a maturation of LCs, thus decreasing Langerin expression, or in case of high viral titers. Altogether, their results highlight the crucial involvement of CLRs in HIV-1 pathogenesis.

## C-TYPE LECTIN RECEPTORS AND ANTIBACTERIAL IMMUNITY

The microbial and host factors of the different skin niches drive the microbiota composition. For example, *Staphylococcus* and *Cutibacterium* bacteria are mainly found in sebaceous areas whereas *Corynebacterium*, *Staphylococcus*, and beta-Proteobacteria are rather in moist areas. But this composition can be drastically changed and skin homeostasis disrupted during inflammation processes and diseases [8]. Most of bacterial species found on the skin are commensal or beneficial for our health but there are also pathogenic bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, which can cause local skin or even systemic infections. Bacterial PAMPs can be recognized by different PRRs on skin resident APCs, including CLRs, so that they can discriminate the good guys from the bad guys in this plethora of microorganisms. But just like viruses, some bacteria can exploit CLR interactions to avoid immune recognition and escape antibacterial immunity.

Langerin can interact with both Gram-positive bacteria *S. aureus* and *S. pyogenes* [30,31]. *Staphylococcus aureus* expresses on its surface a wall teichoic acid (WTA) which is a determinant glycopolymer driving host-pathogen interactions. Langerin actually recognizes a particular epitope on this WTA,  $\beta$ -GlcNAc, which is present in almost all *S. aureus* strains, whereas Langerin ligand on *S. pyogenes* is still unknown [30]. This binding increases the expression of co-stimulatory molecules and the production of Th1- and Th17-proinflammatory cytokines, thus inducing skin inflammation. Although Langerin does not interact with  $\alpha$ -GlcNAc, the co-expression of this WTA epitope decreases  $\beta$ -GlcNAc binding and impairs LC responses and pro-inflammatory cytokines production [31]. Moreover, another type II membrane CLR named MGL (Macrophage galactose-type C-type lectin, CLEC10A) interacts with this  $\alpha$ -GlcNAc motif on a particular *S. aureus* lineage, thus increasing pro-inflammatory cytokine production by monocyte-derived DCs [32]. These results suggest that *S. aureus* could evade immune surveillance through the modulation of its own WTA glycoprofiles and subsequent inflammatory responses. In addition, Langerin on epidermal LCs and DC-SIGN on dermal DCs can also interact with another bacterium that can be found on the skin, *Yersinia*



*pestis*, a Gram-negative pathogen causing the plague [33,34]. *Yersinia pestis* is phagocytosed and can invade LCs and DCs through the recognition of the core oligosaccharides of LPS on its surface by Langerin and DC-SIGN, respectively. After being captured by these APCs, *Y. pestis* is delivered to lymph nodes to establish infection and spread across the host body. This mechanism is similar to HIV-1 infection through hijacking of DC-SIGN in order to be captured and transmitted to lymph nodes to spread the infection, as mentioned earlier. However, the protective function of Langerin against HIV-1 does not apply here, as *Y. pestis* exploits these two CLRs to evade antibacterial immunity and to infect the host. Taken together, these studies highlight the importance of CLRs in antibacterial immunity and the need to find therapeutic targets that can block the CLR/pathogen interactions.

### C-TYPE LECTIN RECEPTORS AND CHRONIC INFLAMMATORY SKIN DISEASES

Psoriasis is a chronic inflammatory skin disease characterized by sharply defined, erythematous plaques with silvery scaling. It has been suggested that PAMPs from pathogens and commensal microbiota or alarmins from dead skin cells can activate some CLRs such as Dectin-1 at the surface of LCs [9]. It results in a production of pro-inflammatory cytokines by LCs but also by keratinocytes in the skin and the recruitment of  $\gamma\delta$  T cells and group 3 innate lymphoid cells (ILC3s) to the inflammatory sites. This leads to the recruitment of neutrophils and the activation of keratinocytes to produce inflammatory molecules and antimicrobial peptides, inducing inflammation and keratinocytes proliferation. These innate immune responses have a key role in development of psoriasis [9]. LCs from normal human skin do not express the type I membrane CLR MR (Mannose receptor, CLEC13D) whereas inflammatory epidermal DCs from patients with atopic dermatitis or psoriasis are positive for MR and use it for receptor-mediated endocytosis of mannans [35]. Another study shows that an injection of mannan into mice activates pathogenic inflammatory macrophage responses, leading to the development of psoriasis, psoriatic arthritis, and chronic rheumatoid-like arthritis. MR is one of the receptors binding to mannan and the loss of its expression, in addition to a ROS deficient environment, causes more severe diseases. Importantly, the protective effect of MR is associated with a ROS-dependent regulation of immunosuppressive M2 macrophages [36]. However, MR and DC-SIGN are expressed in CD163+ macrophages and these dermal macrophages are increased in psoriasis in comparison to normal skin and do not express mature DC markers. This subpopulation of activated macrophages produces key inflammatory molecules and contributes to the pathogenic inflammation in psoriasis [37].

In view of these results, the exact role of MR and other CLRs is still unclear in the development of psoriasis and further studies need to be done to elucidate the exact mechanisms.

The spondyloarthropathies, including psoriatic arthritis, cause chronic inflammation of the axial joints, and are thought to be triggered by an abnormal immune response to infection. Some genes associated with this group of diseases are part of the inflammatory Dectin-1/Syk pathway. Administration of curdlan in SKG mice, a model of chronic autoimmune arthritis, shows the development of psoriasiform dermatitis through Dectin-1 ligation and suggests the involvement of innate immunity in this mechanism [38].

Vitiligo is a chronic autoimmune disease characterized by skin and hair depigmentation due to melanocytes loss. Recently, our laboratory has shown that under stress, innate immune cells such as NK and ILC1s in vitiligo skin produce a large amount of IFN $\gamma$  that induces the secretion of chemokines by keratinocytes and melanocytes [39]. These chemokines bind to their receptor CXCR3B at the surface of melanocytes inducing their initial apoptosis and release of self-antigens. The remaining melanocytes express co-stimulatory and adhesion molecules which present the self-antigens to the naive T cells attracted by the chemokines. Thus, the remaining melanocytes are destroyed by CD8+ T cells, leading to skin depigmentation [40]. Interestingly, Natural Killer Group 2D (NKG2D) is expressed in both NK and CD8+ T cells but has different mechanisms of action depending on the cell type. NKG2D is a C-type lectin-like receptor encoded by *KLRK1* gene which is located within the NK complex among other CLRs such as Dectin-1 and MelLec. NKG2D is an activator of NK cell cytotoxicity with a broad range of ligands whereas it is a co-stimulatory molecule requiring TCR activation on CD8 T cells [40]. It has been shown that NKG2D which is expressed by CD8+ effector memory T cells, and its ligands are increased in perilesional skin of vitiligo, leading to the production of pro-inflammatory cytokines [41]. Along with other studies, these data suggest that the recognition of stressed/damaged cells by NKG2D could induce the CD8+ T cell-mediated melanocyte killing in vitiligo skin [42]. Despite the great progress that has been made throughout the past decades in elucidating the mechanisms that cause vitiligo, there is still no cure, but a number of promising therapeutic targets focusing on modulation of innate immunity are emerging [43].

### FOCUS ON CLEC12B

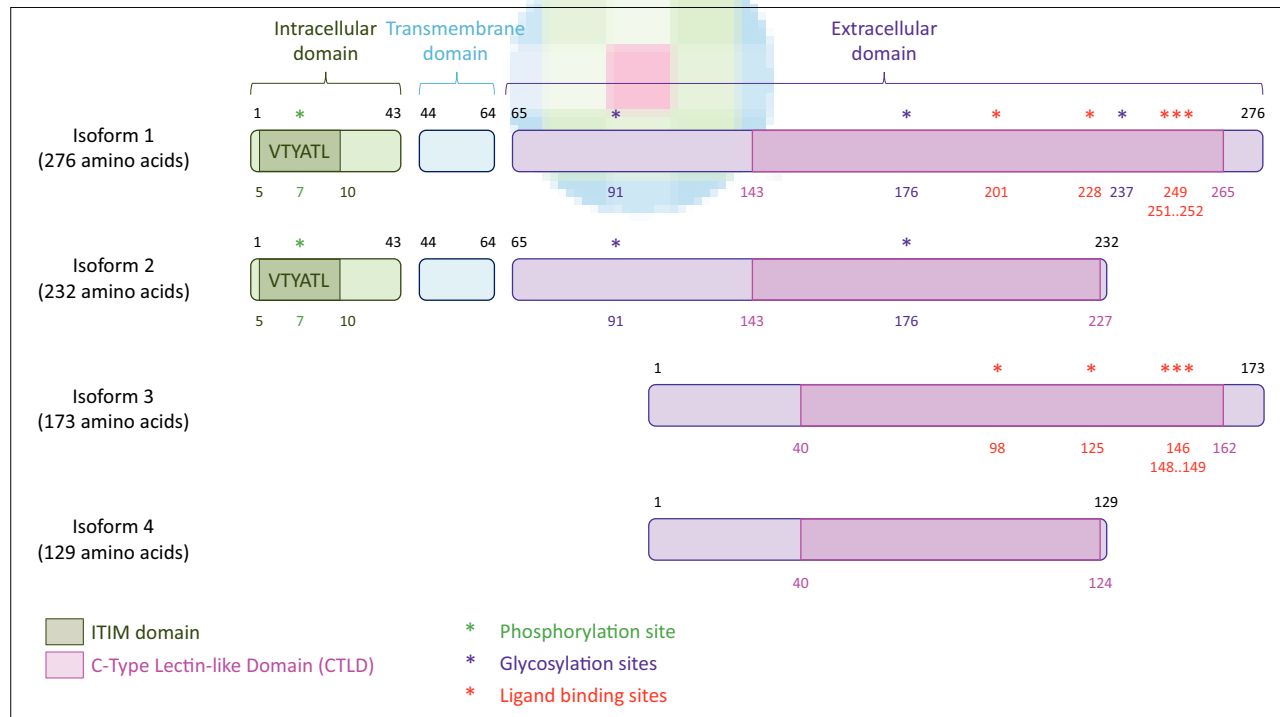
The C-type lectin domain family 12 member B, also named CLEC12B, is part of the Dectin-1 cluster with six other CLRs including CLEC12A, CLEC1B, CLEC9A, CLEC1A, CLEC7A, and

CLEC8A, classified within the group V of CLRs [44]. Although it represents the larger CLR family, it is of importance to highlight that the ligands, partners, and functions of CLEC12B are largely unknown and are only beginning to be understood [13]. This gene is found on mouse chromosome 6qF3 and on human chromosome 12p13.2 and is thought to be expressed in normal human tissues including testis, skin, spleen, and in the bone marrow [45]. Notably, the human and murine homologs of CLEC12B show typical features of C-type lectin-like receptors i.e., amino acid sequences, exon-intron structure, and protein domains. According to a phylogenetic study, CLEC12B is most closely related to Dectin-1 within this gene cluster [46].

### CLEC12B IDENTIFICATION

CLEC12B has been identified by Hoffman *et al.* [47] as an inhibitory receptor on myeloid cells, named after CLEC12A due to their high similarity in structure, function, and chromosomal location. In fact, CLEC12A has been first identified as a myeloid inhibitory CLR, whose gene is also located in the Dectin-1 cluster and is variably spliced, which can recruit both phosphatases SHP1 and SHP2 through its ITIM domain [48]. CLEC12B was

thought to be a potential inhibitory counterpart of NKG2D, because of their high homology in the extracellular domain. However, there was no match in ligand specificity of both receptors and CLEC12B ligands are still to be found. Expression of full-length transcript was found in various tissues except for the brain, in addition to a spliced variant transcript lacking exon 4 and thought to code a non-functional protein. In fact, CLEC12B is found to be differentially spliced because of two independent alternative splicing events. The mRNA coding for a functional type II transmembrane protein shows a cytoplasmic tail containing an ITIM domain connected *via* a transmembrane domain and a stalk domain to a CTLD. However, the three other spliced variants are thought to code truncated and probably non-functional proteins [46] [Figure 1]. It appears that there exists a fifth CLEC12B transcript variant represented as non-coding because of a nonsense-mediated mRNA decay (NM\_001387138.1). At the protein level, CLEC12B is expressed on the human promyelocytic cell line U937 only after phorbol 12-myristate 13-acetate (PMA) stimulation and on *in vitro* differentiated macrophages but not on freshly isolated monocytes or any other blood leukocyte population [47]. Another study identified the porcine homolog of CLEC12B which is expressed on alveolar macrophages, blood DCs and



**Figure 1: CLEC12B isoforms.** The transcript variant 1 shows a cytoplasmic tail containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) connected *via* a transmembrane domain and a stalk domain, to a C-type lectin-like domain (CTLD). The transcript variant 2 differs in the 3' coding region and 3' UTR compared to variant 1. The resulting protein (isoform 2) has a shorter, distinct C-terminus compared to isoform 1. The transcript variant 3 uses an alternate splice site in the central region and initiates translation at a downstream start codon compared to variant 1. The resulting protein (isoform 3) has a shorter N-terminus than isoform 1. The transcript variant 4 uses an alternate splice site in the central region, initiates translation at a downstream start codon, and differs in the 3' coding region and 3' UTR compared to variant 1. The resulting protein (isoform 4) has a shorter N-terminus and a shorter, distinct C-terminus compared to isoform 1. The transcript variant 5 uses an alternate splice site in the 3' region compared to variant 1. This variant is represented as non-coding because the use of the 5'-most expected translational start codon, as used in variant 1, renders the transcript a candidate for nonsense-mediated mRNA decay (NMD)

plasmacytoid DCs but not on monocytes, monocyte-derived macrophages, or monocyte-derived DCs [49]. To assess CLEC12B function, a mutant form has been generated by exchanging the tyrosine residue by a phenylalanine within the ITIM domain so that it can no longer be phosphorylated. Thus, it has been shown that upon phosphorylation of this ITIM, CLEC12B can recruit tyrosine phosphatases SHP1 and SHP2 and act as an inhibitory receptor to reduce the effect of kinases in ITAM-mediated pathways, in particular to inhibit NKG2D-mediated NK cell cytotoxicity [47]. Beyond its ligation to SHP1 and SHP2, CLEC12B is thought to be a putative interaction partner of caveolin-1, a structural protein involved in a variety of cellular functions [50].

### ROLE OF CLEC12B IN NORMAL SKIN PHYSIOLOGY

Recently, our laboratory has identified CLEC12B as a melanocytic gene through a transcriptional analysis of the lesional and non-lesional skin of vitiligo patients in comparison to skin samples of healthy subjects. This analysis showed a strong decrease in key genes involved in melanogenesis in biopsies from lesional skin characterized by a total absence of melanocytes, and CLEC12B was listed among the top melanocytic genes along with dopachrome tautomerase (DCT), melan-A (MLANA), or tyrosinase (TYR) [51]. We also have shown that CLEC12B is significantly more expressed in melanocytes compared to fibroblasts and keratinocytes, which are the main cell types in the skin [52]. These results are in accordance with RNA sequencing dataset from The Human Protein Atlas highlighting that melanocytes are the main cell type expressing CLEC12B, even if it was first identified as a CLR on myeloid cells [47]. We have demonstrated that CLEC12B expression is determined by our skin color whereby the highest expression is seen in light-skin individuals and decreased in highly pigmented skin. Furthermore, we have shown that CLEC12B modulates pigmentation *in vitro* and in reconstructed human epidermis through direct recruitment and activation of SHP1 and SHP2 through its ITIM domain. More precisely, CLEC12B downregulates melanogenesis proteins through the degradation of the c-AMP response element-binding protein (CREB), which is a major transcription factor of melanocyte-inducing transcription factor (MITF) expression [53] [Figure 2]. Taken together, these results demonstrate, for the first time, a regulatory and a physiological function of CLEC12B in the human skin. These insights open exciting new perspectives for potential treatment of pigimentary diseases.

### ROLE OF CLEC12B IN SKIN PATHOLOGY

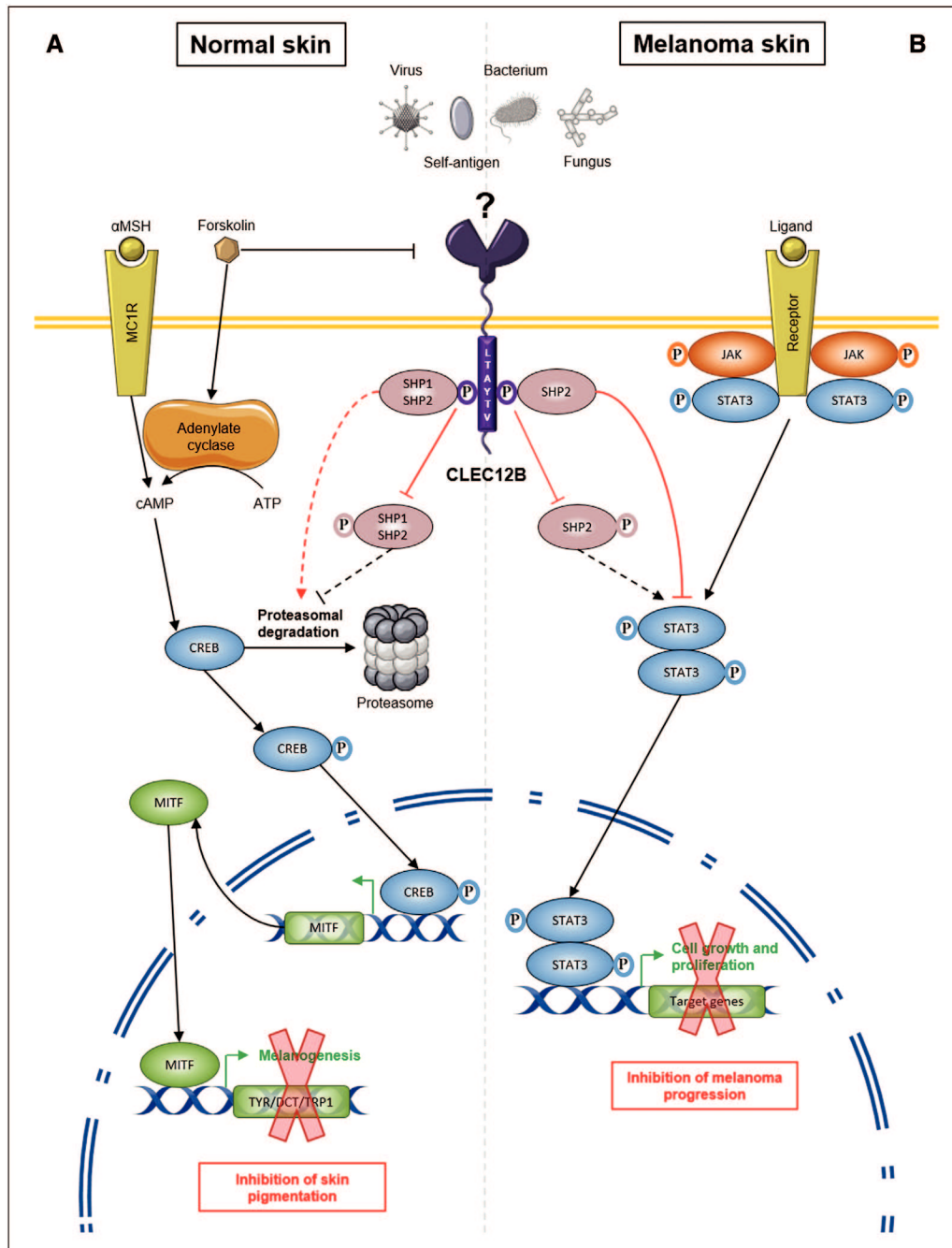
CLEC12B seems to be involved in several diseases but its exact role and function in these pathological processes remain unknown. In the context of skin, CLEC12B expression is significantly increased in chronic otitis with choleastomas in

comparison to healthy skin, and compared to expression of various other CLRs [54]. Furthermore, during the transition from acute to asymptomatic stage in HIV-1 infection, a lot of immunosuppressive genes including CLEC12B are increased in order to modulate the immune system and therefore slow down the disease progression [55]. In patients with a severe form of Behçet's syndrome, CLEC12B is part of a set of inhibitors of inflammation that are downregulated in comparison to patients with milder syndrome and/or controls, suggesting a pro-inflammatory response characteristic of this syndrome, whereas the increased expression of these negative regulators in milder syndromes may protect against severe forms [56]. CLEC12B is differentially expressed in non-ST-segment elevation myocardial infarction (NSTEMI) [57] and is up-regulated in response to *Renibacterium salmoninarum* bacteria in Atlantic salmon during Bacterial Kidney Disease [58].

According to an exome sequencing of a family with two children affected by ganglioneuroma and neuroblastoma, the presence of a potential deleterious mutation in a highly conserved region of the extracellular CTLD has made CLEC12B a candidate cancer predisposition gene [59]. The phosphatases SHP1 and SHP2 (interestingly both recruited by CLEC12B) have been associated with not only skin disorders but many cancers, including melanomas [60,61]. Mutations in the PTPN11 gene, coding for SHP2, is a cause of pigimentary disorders such as Noonan or LEOPARD syndrome [62] and mutations in the *PTPN6* gene, which codes for SHP1, causes inflammatory skin disorders [63]. In the context of melanoma, we have recently shown that CLEC12B plays an important role as a tumor suppressor gene in this skin cancer [64]. Melanoma patients with high CLEC12B expression have a significantly higher median survival than those with low expression. Notably, CLEC12B expression is lower in melanoma and melanoma metastasis than in melanocytic and benign melanocytic lesions. Functionally, we have demonstrated that CLEC12B inhibits melanoma growth through the recruitment of SHP2 on its ITIM domain that further inactivates signal transducer and activator of transcription (STAT) family of proteins (STAT 1, 3, and 5) [Figure 2] and increases p21, p27, and p53, *in vitro* and *in vivo*. Our results have recently been confirmed in a separate study showing a similar suppressor function of CLEC12B in lung cancer cells *in vitro* [65]. Together, these novel results support further studies investigating CLEC12B as a promising new therapeutic target in the treatment of melanoma and lung cancers however its application to other cancers remains to be investigated.

### CONCLUSION

In this review, we have focused on CLRs functions in skin immunity and have shown the extensive roles that this superfamily of proteins is involved in both normal skin physiology



**Figure 2:** CLEC12B signaling pathways and function in the skin (ligands still unknown). (A) In normal skin, CLEC12B recruits both SHP1 and SHP2 through its ITIM domain and decreases their phosphorylation. The with these two phosphatases leads to a downregulation of MITF and downstream melanocytic genes (TYR, DCT, TRP1) through CREB proteasomal degradation. Thus, CLEC12B inhibits melanogenesis and subsequent skin pigmentation [52]. (B) In melanoma skin, CLEC12B recruits SHP2 through its ITIM domain and decreases its phosphorylation. This interaction induces a dephosphorylation of STAT3, leading to the inhibition of cell cycle and proliferation in particularly through an increased expression of p21, p27, and p53. Thus, CLEC12B inhibits tumor growth and subsequent melanoma progression [64]

and pathology. During the past few years, great progress has been made to increase our knowledge about the complexity of their functions in a range of physiological processes including development, skin homeostasis, and skin pigmentation. Discovery of a novel gene, CLEC12B, and its role in skin pigmentation and melanogenesis warrants further exploration. Its newly discovered function involving the recruitment of

phosphatases SHP1 and SHP2 through its ITIM domain confirms the original observations stating that CLEC12B inhibits NK cell cytotoxicity through the interaction with these two proteins. As previously mentioned, these phosphatases are linked to a broad range of biological processes and their interaction with CLEC12B is of interest considering the promising results for CLEC12B described in this review. Knowing that CLRs play a



crucial role in immunity, it would be of interest to decipher the exact signaling pathways in CLEC12B-induced regulation. Future research focusing on discovering CLEC12B ligands is essential to facilitate future studies examining the exact role of CLEC12B in not only skin immunity but in other organs which may open new doors in the treatment of a number of immune disorders.

### Study limitations

The review is based on the analysis of the available literature. The data may lack some evidence from the clinical perspectives.

### Authors' contributions

LB prepared the draft of the review and Figure concepts. TP provided feedback and intellectual input into the review. MKT conceived the research concept and formulated review outline with LB. All authors reviewed and approved final draft of the manuscript. All authors are responsible for the contents and integrity of this manuscript.

### Data availability statement

The authors will be glad to provide raw data of this research, if requested.

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Not applicable.

### Conflicts of interest

There are no conflicts of interest.

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## IV. Melanocytes

### A. Origin and development

Melanocytes represent 5-10% of the cells in the skin and are located in the *stratum basale*. They are specialized cells responsible for skin, hair, and eye pigmentation. Understanding the embryonic origin of melanocytes is essential for comprehending their distribution, function, and association with various skin disorders, including melanoma and vitiligo.

#### 1. Embryonic origin and anatomical distribution

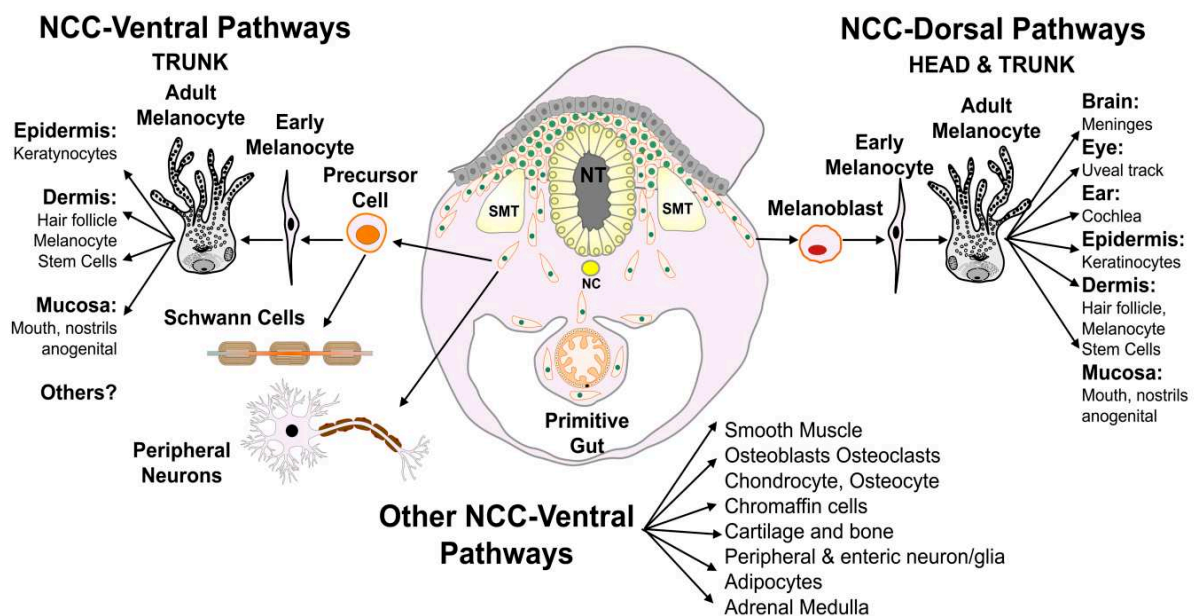


Figure 13: Embryonic origin and anatomical distribution of melanocytes.

(Extracted from (Castro-Pérez et al. 2023))

Neural crest cells (NCCs) are multipotent stem cells arising from dorsal neural ectoderm, at the junction between the neural tube (NT) and the non-neural ectoderm, during early embryonic development (Figure 13). In response to specific stimuli, NCCs undergo an epithelial-mesenchymal transition and gain migratory capacity to delaminate from the NT and populate various regions of the embryo through 2 different waves (Duband 2006; 2010) (Figure 13). The first wave is a dorso ventral migration in the trunk, between the somites and the NT, where the NCCs give rise to a variety of cells such as peripheral neurons, Schwann cell precursors, endocrine glands, or smooth muscles (Figure 13). The second one is dorso lateral in the head and trunk, between the dermomyotome and the superficial ectoderm, leading to the

formation of melanocytes precursors, also known as melanoblasts (Dupin et Le Douarin 2003) (*Figure 13*). However, it has been shown that melanocytes in the skin can also arise from Schwann cell precursors from the neural crest ventral migratory pathway. These precursors can differentiate into both melanocytes and Schwann cells which share development signaling molecules but regulated in an opposing manner (Adameyko et al. 2009).

In both NCC pathways, precursor cells or melanoblasts differentiate into early melanocytes and then in adult melanocytes that primarily populate the skin epidermis for constitutive skin pigmentation and protection against UV radiation. But melanocytes also populate other tissues and organs and have different functions according to their localization in the organism (Plonka et al. 2009) such as:

- Brain: neuroendocrine functions and detoxification.
- Eye: constitutive eye pigmentation and protection against UV in the choroid; vision and metabolism of rod outer segments and retinoids in the retinal pigment epithelium (RPE).
- Ear: balance in the inner ear and hearing in the cochlea.
- Heart: anti-inflammation, reduction and binding of ROS.
- Adipose tissue: cellular survival, reduction and binding of ROS.
- Lung: unknown function but the production and accumulation of melanin can be lethal.
- Skin hair follicles: hair pigmentation, removal of toxic by-products
- Skin hair bulge: melanocyte stem cell reservoir for skin
- Mucosal surface (oral cavity, genital tract, gastrointestinal tract, respiratory tract): immune responses and protection against environmental factors

Interestingly, the neural crest is the source of the melanocytes in the epidermis, dermis, and choroid layers of the skin whereas the neural epithelium of the optic cup is the source of melanocytes in the RPE (Dupin et Le Douarin 2003). All these melanocytes have essential functions in their target organs, and a disturbance in their function or in their number can lead to diseases. Taking the example of the skin, melanoma is a very deadly skin cancer arising from an uncontrolled proliferation of melanocytes whereas vitiligo is an autoimmune disease characterized by a loss of melanocytes in certain skin areas, leading to depigmented patches. Understanding melanocyte distribution in these skin disorders is critical for designing new therapies or dictating the prognosis of the disease.



## 2. Genes involved in melanocyte development

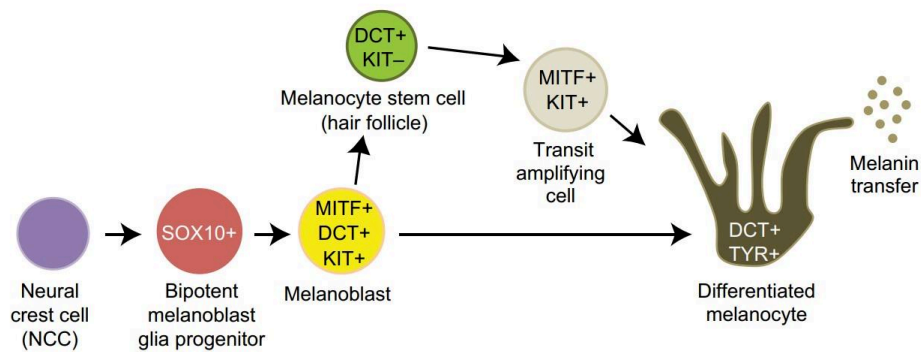


Figure 14: Transcriptional regulation of melanocyte development.

(Extracted from (Mort, Jackson, et Patton 2015))

Melanocyte development is an intricate process orchestrated by a network of genes that regulate various stages from melanoblast specification to differentiated melanocyte function. NCCs are differentiated into bipotent melanoblast glia progenitors via the expression of SRY-Box Transcription Factor 10 (SOX10) (Figure 14), which remains activated in both glial and melanocyte lineages. These progenitors are specified into melanoblasts expressing Microphthalmia-associated Transcription Factor (MITF or Melanocyte Inducing Transcription Factor), Dopachrome Tautomerase (DCT or TRP2) and the receptor tyrosine kinase KIT (or CD117) (Figure 14).

MITF acts as the master regulator of the melanocytic lineage controlling melanocyte development, function and survival (Levy, Khaled, et Fisher 2006). Numerous MITF family member genes exhibiting mutations and/or abnormal expression have been reported in human cancer, including melanoma, and mice with no MITF expression are unable to properly develop melanocytes (Steingrímsson, Copeland, et Jenkins 2004). Many genes encoding the melanin synthesis pathway and components of melanosomes, especially *DCT*, *Tyrosinase* (TYR), and *Tyrosinase Related Protein 1* (TYRP1), turn out to be direct MITF transcriptional targets (Cheli et al. 2010). DCT is involved in the melanin biosynthesis, more specifically in brown/black melanin (eumelanin) synthesis, and is one of the earliest melanoblast markers. KIT expression is induced by MITF and is required for the migration, proliferation, and survival of melanoblasts (H. Yoshida et al. 1996; Opdecamp et al. 1997). WNT signaling also plays a crucial role in melanocyte development through the activation of the WNT/ $\beta$ -catenin pathway

that promotes MITF expression and melanoblast specification (Dunn et al. 2005). Other signaling pathways such as the Notch and Endothelin-1 pathways can regulate MITF expression and activity.

On one hand, some melanoblasts are specified into differentiated melanocytes expressing melanocyte-specific proteins such as DCT, TYR, TYRP1, Premelanosome Protein 17 (PMEL17 or gp100), Melan-A (or MART-1), and/or MITF (Plonka et al. 2009) that are able to produce melanin pigments in the developing embryonic hair follicles (*Figure 14*). On the other hand, another subset of melanoblasts lose the expression of MITF and KIT, but not DCT, and dedifferentiate to form melanocyte stem cells in the hair follicle at the hair bulge (*Figure 14*). These stem cells can give rise to rapidly proliferating transit-amplifying cells expressing again MITF and KIT that differentiate into functional melanin-producing melanocytes in the subsequent hair cycles (Mort, Jackson, et Patton 2015) (*Figure 14*). Intriguingly, melanocyte stem cells become exhausted earlier than other stem cell populations in most animals and humans, resulting in hair greying during ageing (Nishimura, Granter, et Fisher 2005). Thus, differentiated melanocytes can synthesize and transfer melanin to surrounding keratinocytes that will determine skin, hair, and eye color and provide protection against harmful UV radiation.

## B. Melanocytes as melanin-producing cells

The main function of epidermal melanocytes is to produce melanin pigments in specialized lysosome-like organelles, the melanosomes, through a process called melanogenesis. Epidermal melanocytes are oval or fusiform dendritic cells, typically 7 $\mu$ m in length, smaller than keratinocytes, with many extensions, called dendrites, allowing the transfer of melanin granules to neighboring keratinocytes. An epidermal melanin unit (EMU) represents the relationship between one melanocyte and 30-40 associated keratinocytes with a ratio melanocytes to keratinocytes 1:10 in the *stratum basale* (Fitzpatrick et Breathnach 1963). Importantly, other cells can also produce melanin pigments, such as neurons, adipocytes, cells of the RPE, and cells of the iris epithelia and ciliary body of the eye (Hu, Simon, et Sarna 2008; Randhawa et al. 2009).

## 1. Skin pigmentation and melanin

The skin pigmentation can be classified according to how the skin responds to sun exposure, skin injury, or cosmetic procedure. Since the 70s, the most used method of skin phototyping is the Fitzpatrick classification, which is based on a person's genetic predisposition, tendency to sunburn, and ability to tan. This classification was originally developed to help calculating the initial dose of phototherapy in populations with white skin. However, it is based on a subjective vision, highly variable from person to person or from patient to doctor, very dependent on the phrasing of the questions, and doubts have been raised regarding its correlation with constitutive or facultative skin color (Ravnbak 2010).

Contrary to popular belief, our skin color isn't due to the number of melanocytes present in the epidermis as fair skins and dark skins have similar number of melanocytes (Sturm 2009). On one hand, the skin color is determined by the quantity, the type, and the distribution of epidermal melanins, which are genetically controlled and termed natural or "constitutive" pigmentation. To date, more than 600 genes have been identified that affect the skin, hair, and/or eye pigmentation and an updated list of these genes can be found at [Updated list of pigimentary genes \(ifpcs.org\)](#). On the other hand, "facultative" pigmentation is determined by an increased epidermal melanin content, resulting from hormonal factors or environmental factors such as UV light (Sturm 2009). The skin color is also influenced by other pigments such as oxyhemoglobin (red), deoxyhemoglobin (blue), bilirubin (yellow), and carotenoids (yellow, orange, and red), although only melanin can protect the skin from UV light (Gupta et Sharma 2019). There are 2 types of melanins:

- Pheomelanin (red/yellow) that generates superoxide anions and hydroxyl radicals resulting in an endogenous photosensitizer and oxidative DNA damage (Kadekaro et al. 2003).
- Eumelanin (brown/black) that offers a better protection against UV radiations than pheomelanin and is a free radical scavenger able to neutralize ROS with a higher resistance to degradation (Meredith et Sarna 2006).

As a consequence of these statements, the risk of skin cancer is 30 to 40 times higher in lighter skin than in darker one (Hearing 2011).

## 2. Melanogenesis and related signaling pathways

As mentioned before, melanin is synthesized in melanosomes during melanogenesis. This process undergoes different external and internal stimuli that can induce modifications in the quantity and quality of synthesized melanins, the melanocytic dendricity, and the melanosome transfer to surrounding keratinocytes.

UV radiation (UVA and UVB) can penetrate the skin up to the basal layer where they can reach both melanocytes and keratinocytes. UV irradiation can have direct effects on melanogenesis by inducing DNA damage with the formation of pyrimidine dimers. It has been shown that DNA damage and/or DNA damage repair increase the pigmentation in response to UV irradiation (Eller, Ostrom, et Gilchrest 1996). Direct effects of UV also induce the activation of the stress-responsive p38 kinase, which in turn phosphorylates and activates the Upstream Transcription Factor 1 (USF1), resulting in the activation of *TYR* promoter (Galibert, Carreira, et Goding 2001). UV irradiation can also have indirect effects on melanogenesis by inducing the production of different secreted factors, such as  $\alpha$ -Melanocyte-Stimulating Hormone ( $\alpha$ MSH) and Adrenocorticotrophic Hormone (ACTH), by neighboring keratinocytes (Park et al. 2009).

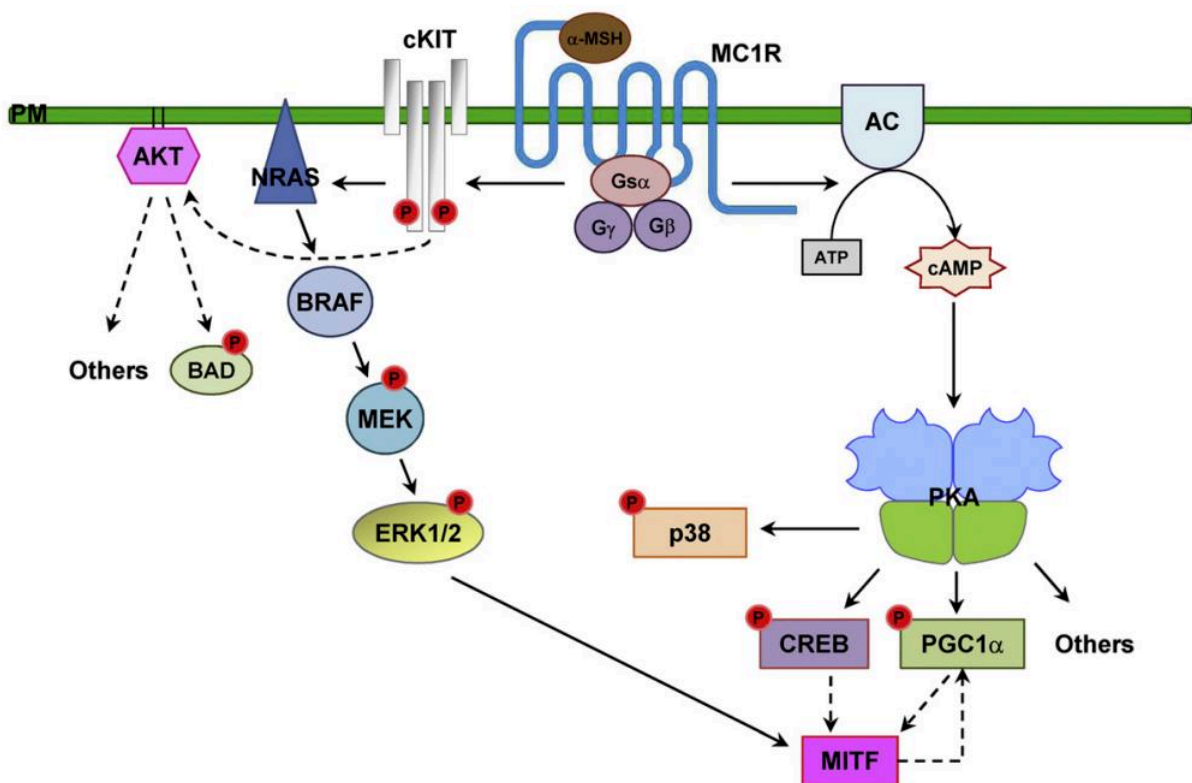


Figure 15: MC1R signaling pathways.

(Extracted from (Herraiz et al. 2017))

The melanotropic hormones  $\alpha$ MSH and ACTH derive from a common precursor, the Proopiomelanocortin (POMC), and are the ligands of Melanocortin 1 Receptor (MC1R) (Tsatmali et al. 2000). UV-induced DNA damage increase the expression of p53, inducing *POMC* transcription (Cui et al. 2007), and UV irradiation stimulate the production of  $\alpha$ MSH, thus inducing an increased expression of MC1R. Upon MC1R binding, the Adenylyl Cyclase (AC) is activated, thus triggering cAMP synthesis and PKA activation (*Figure 15*). PKA activates and phosphorylates cAMP Responsive Element Binding protein (CREB), Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PGC1 $\alpha$ ), and p38, resulting in an increased *MITF* transcription. MC1R also transactivates cKIT to trigger the NRAS-BRAF-MEK-ERK signaling, thus phosphorylating and increasing MITF transcriptional activity (*Figure 15*). MITF increases expression of many melanogenic enzymes necessary for melanogenesis, primarily TYR, TYRP1, and DCT.

The skin immune system also plays its part in skin pigmentation. For instance, mast cells can secrete histamine during inflammation that can bind to H2 receptors on melanocytes. This binding induces the activation of PKA, thus activating the cAMP pathway and *TYR* transcription, resulting in increased melanin synthesis (M. Yoshida, Takahashi, et Inoue 2000). Another study has shown that CD4<sup>+</sup> Th cells produce IL-4, that directly decreases the expression of melanogenesis-associated genes, as well as IFN $\gamma$  and IL-17, that indirectly inhibit the melanogenesis through the production of anti-melanogenic IL-6 (Choi et al. 2013). More precisely, IFN $\gamma$ -induced hypopigmentation is independent of MITF and due to an impediment of melanosome maturation through the Janus Kinase(JAK)1/STAT1/IRF1 pathway (Natarajan et al. 2014).

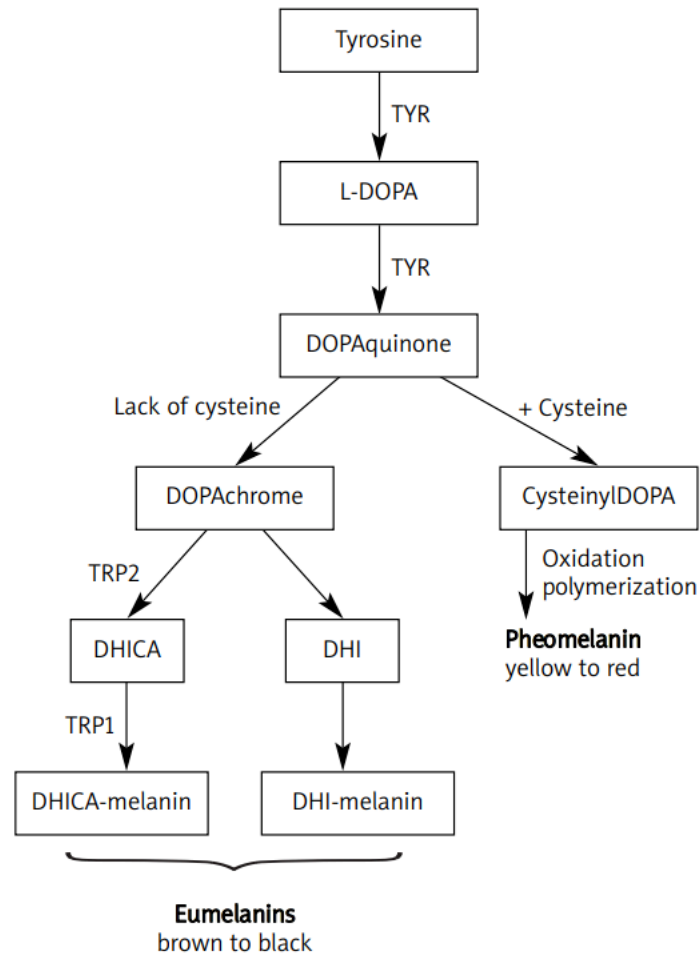


Figure 16: Simplified scheme of the melanogenesis.

(Extracted from (Cichorek et al. 2013))

The enzymes involved in melanogenesis and the availability of their substrates decide about what kind of melanin is produced (Figure 16). The amino acid tyrosine is hydroxylated to L-3,4-dihydroxyphenylalanine (DOPA) which in turn is oxidized to DOPAquinone by the enzyme TYR (Fitzpatrick, Miyamoto, et Ishikawa 1967). When there is cysteine available, it can react with DOPAquinone to give CysteinyLDOPA, which then oxidizes and polymerizes into yellow/red pheomelanin. However, when there is no thiol available such as cysteine, glutathione or thioredoxin, DOPAquinone spontaneously cyclizes to DOPAchrome. In presence of DCT, DOPAchrome can form DHI-2-carboxylic acid (DHICA) which is then converted into a light brown DHICA-melanin (eumelanin) by TYR and TYRP1. But in the absence of DCT, DOPAchrome spontaneously loses carboxylic acid to form 5,6-dihydroxyindole (DHI) which then oxidizes and polymerizes into an insoluble dark brown/black DHI-melanin (eumelanin) (Simon et al. 2009; Hearing 2011; Sugumaran 1991; del Marmol et Beermann 1996). Importantly, melanogenesis

also synthesizes cytotoxic molecules such as quinones and hydrogen peroxide as intermediate products, forcing the melanocyte to partition this process into melanosomes and to increase the level of Bcl-2, an antiapoptotic protein, to protect itself against cytotoxic damage (Sulaimon et Kitchell 2003; Plonka et al. 2009).

The skin color is determined by the ratio of eumelanin to total melanin, while a similar amount of pheomelanin is present in fair and dark skins (Slominski et al. 2004). However, the hair color is determined by the ratio of eumelanin to pheomelanin (Ito et Wakamatsu 2011).

### 3. Melanosome maturation and transfer





Melanosom features	Stage I	Stage II	Stage III	Stage IV
				
Shape	Spherical	Elongated	Elliptical, ellipsoidal	Elliptical, ellipsoidal
Internal structure	–	Matrix fibrils are visible	Matrix fibrils are visible	Matrix fibrils are covered by polymerized melanin
TYR	–	+	+	+
TYRP1	–	+	+	+
TYRP2	–	+	+	+
Melanin synthesis	–	–	Begins, settle on internal fibrils	Filled by melanin
Color			Brown	Dark brown to black

Figure 17: Developmental stages of melanosomes.

(Extracted from (Cichorek et al. 2013))

Melanosomes belong to the lysosomal related organelles (LROs) as they share common features with lysosomes such as lysosome membrane proteins and hydrolases, an acidic pH or the ability to be endocytosed (Raposo et Marks 2007). They are thought to originate from the endoplasmic reticulum of melanocytes (Slominski et al. 2004; Park et al. 2009), but this remains very controversial as others provide arguments in favor of an endosomal origin (Berson et al. 2001). Melanosomes display 4 maturation stages (Figure 17):

- Stage I: corresponds to the organelle synthesis with pre-melanosomes that are small, round, non-pigmented vesicles at the nucleus periphery with an amorphous matrix.

- Stage II: pre-melanosomes become egg-shaped with an organized and structured fibrillar matrix depending on the presence of PMEL17 glycoprotein. At this stage, melanogenesis enzymes TYRP1 and DCT are present, but also an inactive form of TYR so there is still no melanin synthesis.
- Stage III: elliptical/ellipsoidal internal structure where the production of melanin begins, which polymerizes on internal fibrils, resulting in a gradually brown pigmentation of the internal matrix.
- Stage IV: corresponds to a mature melanosome, with uniformly opaque matrix which becomes fully melanized with a loss of TYR activity, that is transported to surrounding keratinocytes.

Interestingly, pheomelanin melanosomes exhibit a different morphology from eumelanin melanosomes with no internal fibril structure.

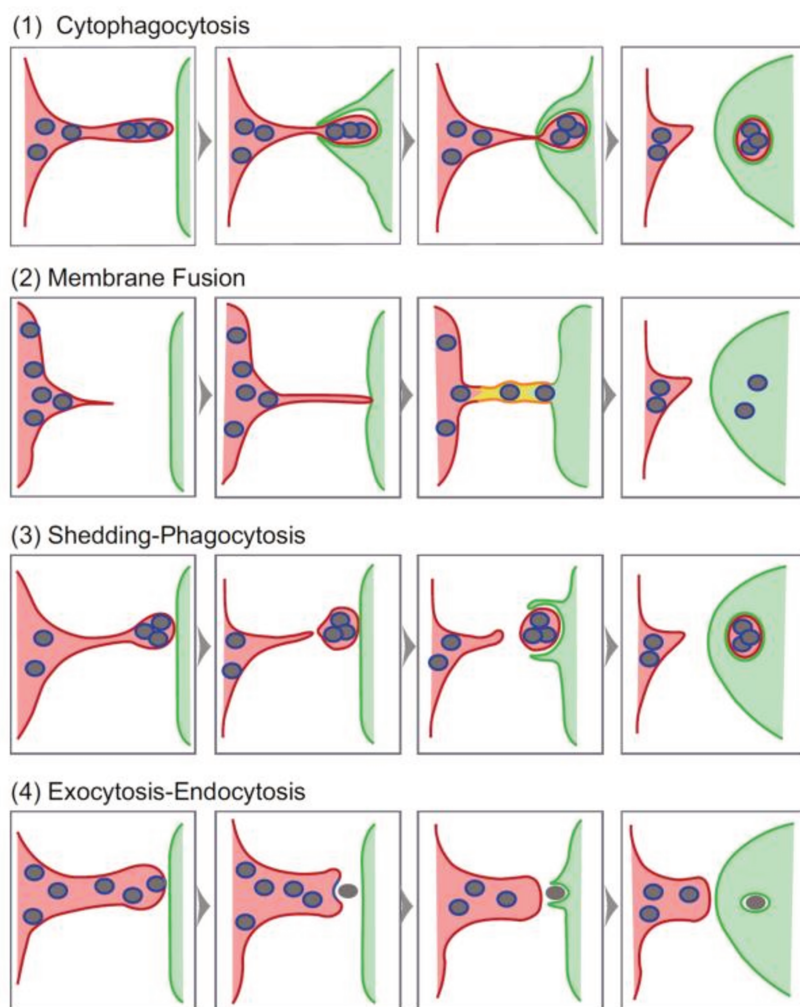


Figure 18: Melanosome transfer mechanisms.



*(Extracted from (Xufeng Wu et Hammer 2014))*

Melanosome transfer from melanocytes to keratinocytes is a dynamic and essential process for skin pigmentation and photoprotection. How melanosomes are transferred is still under investigation, but a few transfer models have been proposed (*Figure 18*):

- Cytophagocytosis: the keratinocyte phagocytizes the melanosome-enriched dendritic tip of the melanocyte.
- Membrane fusion: the melanosome moves through a transient membrane conduit connecting the fused melanocyte and keratinocyte cytoplasms.
- Shedding-phagocytosis: the melanocyte sheds plasma membrane-enclosed melanosome aggregates which are subsequently phagocytosed by the keratinocyte.
- Exocytosis-endocytosis: the melanosome melanin core is exocytosed by the melanocyte into the extracellular space, then this “melanocore” is phagocytosed and/or endocytosed by the keratinocyte.

Melanocytes may choose a certain transfer mechanism depending on local interactions with neighboring keratinocytes or with the underlying dermis. After the melanosome transfer to keratinocytes, they are compartmentalized into Lamp1-positive late endosomes that are transported to the perinuclear region to provide pigmentation and photoprotection. Finally, these melanosomes are thought to be degraded in lysosomes (Fukuda 2021).

### C. Melanocytes as antigen-presenting cells

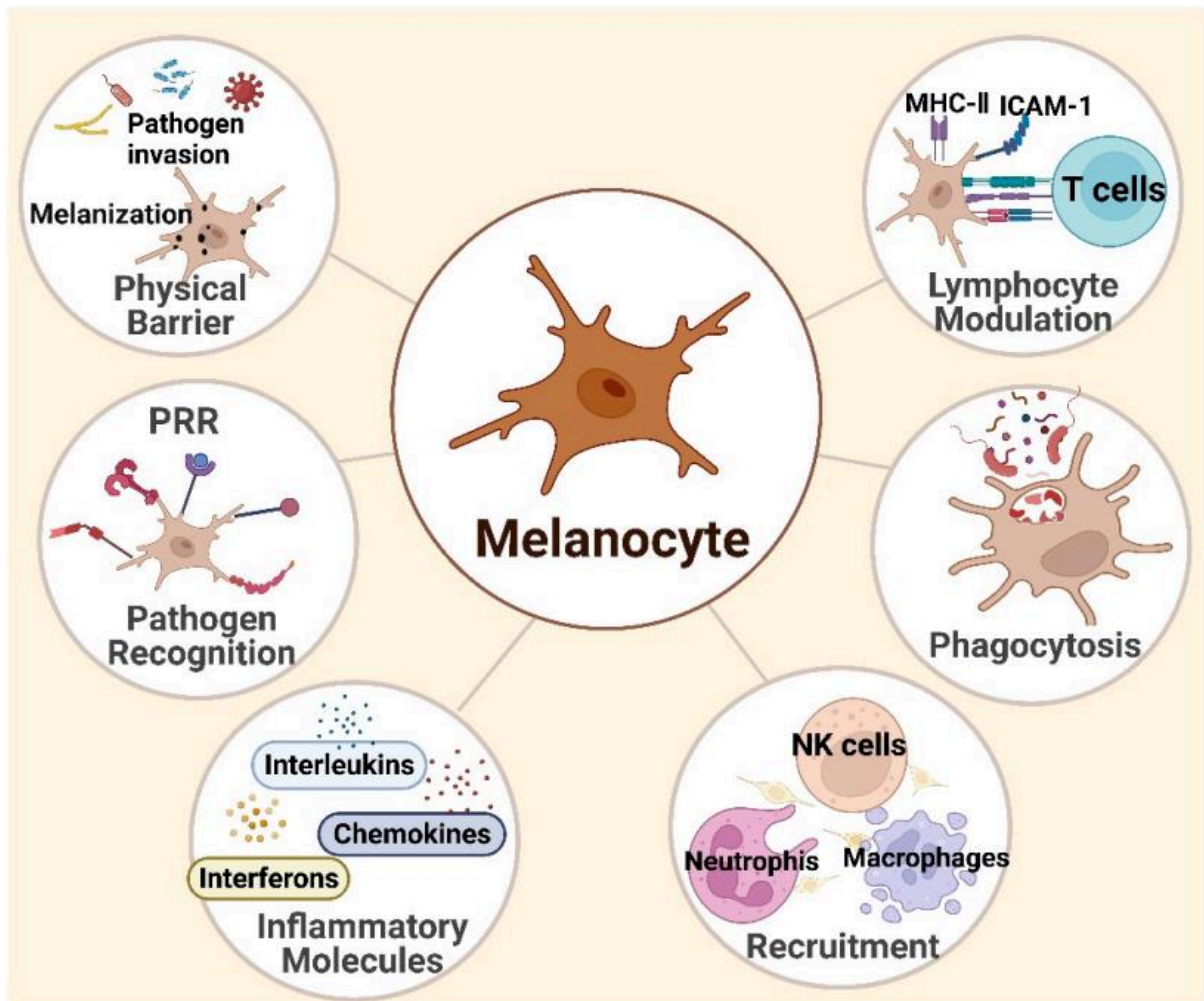


Figure 19: Melanocyte immune functions.

(Extracted from (Y.-Y. Chen et al. 2022))

In addition to their well-known role in producing melanin and determining skin, hair, and eye color, an antigen-presenting function of melanocytes has emerged over the past 30 years. Traditionally, professional APCs in the skin, including dendritic cells, monocytes, and macrophages, can enter the skin from the peripheral circulation and migrate to draining lymph nodes to coordinate immune responses. They have all the machinery necessary to sense, process, and present antigens to subsequently initiate immune responses during homeostasis, inflammation, and disease (Kashem, Haniffa, et Kaplan 2017). However, studies have revealed that melanocytes can also serve as APCs under certain conditions, suggesting a potential role in immune regulation and immunopathology (Figure 19). Importantly, a disturbance in melanocyte homeostasis can lead to several skin disorders. For instance, an inappropriate

melanocyte destruction can lead to vitiligo while an anarchic proliferation can induce melanoma.

### 1. Antigen-processing and-presentation capacity

Melanocytes located in the epidermis basal layer are on the front line to encounter harmful stimuli or pathogens from the outside, in addition to UV radiation. Melanocytes have been shown to express both MHC class I and II molecules, cell adhesion molecule ICAM-1, immunologically cell marker CD40, Fc receptor, to produce immunomodulatory cytokines, and to bind alloreactive T cells (Krasagakis et al. 1991; Smit et al. 1993; Yohn et al. 1990; Lu et al. 2002; Le Poole, Mutis, et al. 1993; Nickoloff et al. 1992) (*Figure 19*). Importantly, ICAM-1 interaction with its ligand Leukocyte Function-associated Antigen-1 (LFA-1) is essential for the interaction between APCs and lymphocytes, cellular cytotoxicity, and T helper (Th) cell function. Upon CD40 ligation, which plays an important role in T cell activation and proliferation and B cell differentiation, co-stimulating and adhesion molecules are increased in melanocytes, indicating an immunocompetent function (Lu et al. 2002). Moreover, melanocytes possess the phagocytic machinery necessary for antigen-processing, in addition to their melanosomes that can act as specialized lysosomal organelles for phagolysosome fusion (Le Poole, van den Wijngaard, et al. 1993) (*Figure 19*). Thus, their strategic position in the skin epidermis, their dendritic nature, and their phagocytosis activity strongly suggest a role in antigen-processing and -presentation. In this context, Le Poole *et al.* have shown that melanocytes can process and present antigenic peptide fragments as well as intact protein preparations to CD4<sup>+</sup> cytotoxic proliferative T cell clones in an Ag-specific and Human Leukocyte Antigen (HLA) class II-restricted manner (Le Poole, Mutis, et al. 1993). Melanocytes can induce a T cell proliferative response, but also undergo T-cell mediated cytotoxicity.

### 2. Immune properties of melanization

As mentioned earlier, the skin immune system modulates the skin pigmentation, but the converse is also true. Interestingly, fair skin undergoes more infections than dark skin (Mackintosh 2001), suggesting that melanocytes and melanization play an immune role. Melanogenesis synthesizes cytotoxic intermediate products, such as quinones, free radicals and hydrogen peroxide that can have antimicrobial activities (Tang 2009), in addition to the enzymes present in melanosomes, such as  $\beta$ -galactosidase, acid phosphatase,  $\alpha$ -mannosidase,

acid lipase, and  $\beta$ -N-acetylglucosaminidase that can neutralize and degrade bacteria (Borovanský et Hach 1999; Dell'Angelica et al. 2000). Melanin also has the ability to limit the nutrient uptake of infectious microorganisms, as well as trapping and killing them (Mackintosh 2001; Burkhart et Burkhart 2005; C. C. Chen et Chen 1995; Fuentes et al. 2014). Moreover, melanization can help the pathogen phagocytosis (Cerenius et Söderhäll 2021) and the transfer of acidic melanosomes from melanocytes to surrounding keratinocytes can help acidifying the cornified layer, thus participating in the skin barrier function, and can have antimicrobial function (Gunathilake et al. 2009) (*Figure 19*).

### 3. Pathogen recognition and production of inflammatory molecules

Factors and receptors	Roles	References
Interleukin-1- $\beta$	MC express IL-1 $\beta$ (and alpha). Tyrosinase activity is inhibited by IL-1 and possibly contributing to hypopigmentation	60,61,11
Tumor necrosis factor- $\alpha$	Inhibit the growth of MC. enhances the expression of VLA-2, ICAM-1 and HLA class I antigens and strongly induced HLA-DR. May decrease pigmentation by reducing melanocortin-1 receptor expression on MC. Together with IL-17 inhibits pigmentation-related signaling and melanin production, and induced keratinocyte production of $\beta$ -defensin 3, an antagonist for melanocortin 1 receptor (MC1R).	61,62,63
Interferon- $\alpha$ , $\beta$	Induced <i>in vitro</i> by viral DNA. pppRNA and Poly I:C can signal to RIG-I and MDA-5 to increase IFN- $\beta$ mRNA expression by MDCL and MC.	11,42,55
Interferon- $\gamma$	Produced by T cells and NK cells. INF- $\gamma$ was recently shown to control directly the melanogenesis process by arresting melanosome maturation. IFN-g can also induce melanocyte senescence and immune-competency (increased released of IL6 and alarmin HSP70)	15
TLR2-4-5	Expressed by MC to detect and respond to bacterial infection; Increased production of IL-6 and IL-8. TLR2/4 can also respond to alarmins (HSP70, HMGB1) to promote local inflammation	64,65
RIG-I, MDA5	Expressed by MC and MDCL. pppRNA and Poly I:C can signal to RIG-I and MDA-5 respectively and induce apoptosis of MDCL but not of primary MC. This may be important for tumor therapy	41
HMGB1	Viral DNA can be detected by RIG-I and AIM2 PRRs to signal apoptosis and cytokine production. MC death may explain the link between DNA virus infection and vitiligo. MC apoptotic or necrotic debris could be perceived as autoantigens by T cells (bystander activation mechanism). Nuclear protein released either by infected-, stressed- or necrotic (e.g. MC) cells. Soluble HMGB1 is a canonical alarmin (danger signal) to alert immune cells by binding to TLR2, TLR4 and/or RAGE. HMGB1 released by UV-irradiated keratinocytes may contribute to angiogenesis and metastasis of melanoma. HMGB1 could be involved in erythema toxicum neonatorum	11,42
		40,66

*Figure 20: Immune molecules and receptors in melanocytes.*

(Extracted from (Gasque et Jaffar-Bandjee 2015))

Several studies have shown that melanocytes express different classes of PRRs, including TLRs 1-5, 7, 9, and 10 (Ahn et al. 2008; Jin et Kang 2010; Yu et al. 2009) (*Figure 20*). We were the first to demonstrate presence of CLR CLEC12B in human melanocytes (Sormani et al. 2022; Blot, Passeron, et Tulic 2022). Presence of PRRs on melanocytes allows them to sense PAMPs present on pathogens and induce immune responses (*Figure 19*). For instance, upon ligation

of TLRs with lipopolysaccharide (LPS), NF- $\kappa$ B and/or MAPK pathways are activated, leading to the production of pro-inflammatory cytokines and chemokines and to an increased melanogenesis (Ahn, Jin, et Kang 2008). These cytokines and chemokines can subsequently induce the recruitment and activation of immune cells in the skin to induce immune responses (Figure 19). Melanocytes secrete a wide range of immunological molecules, some of them are described in Figure 20, such as inducible Nitric Oxide Synthase (iNOS) and  $\alpha$ MSH, which can have anti-inflammatory as well as immunomodulatory activities (Luger et al. 2003; Tam et Stępień 2011). Moreover, under stress conditions, melanocytes can produce Heat Shock Proteins (HSP), such as HSP70, as well as DAMPs, such as High Mobility Group Box 1 (HMGB1) or some Natural Killer Group 2 member D (NKG2D or KLRK1) ligands, leading to lymphocyte recruitment and melanocyte immune-targeted destruction (Mosenson et al. 2014; Plaza-Rojas et Guevara-Patiño 2021).

#### 4. Anti-melanocytic immunity

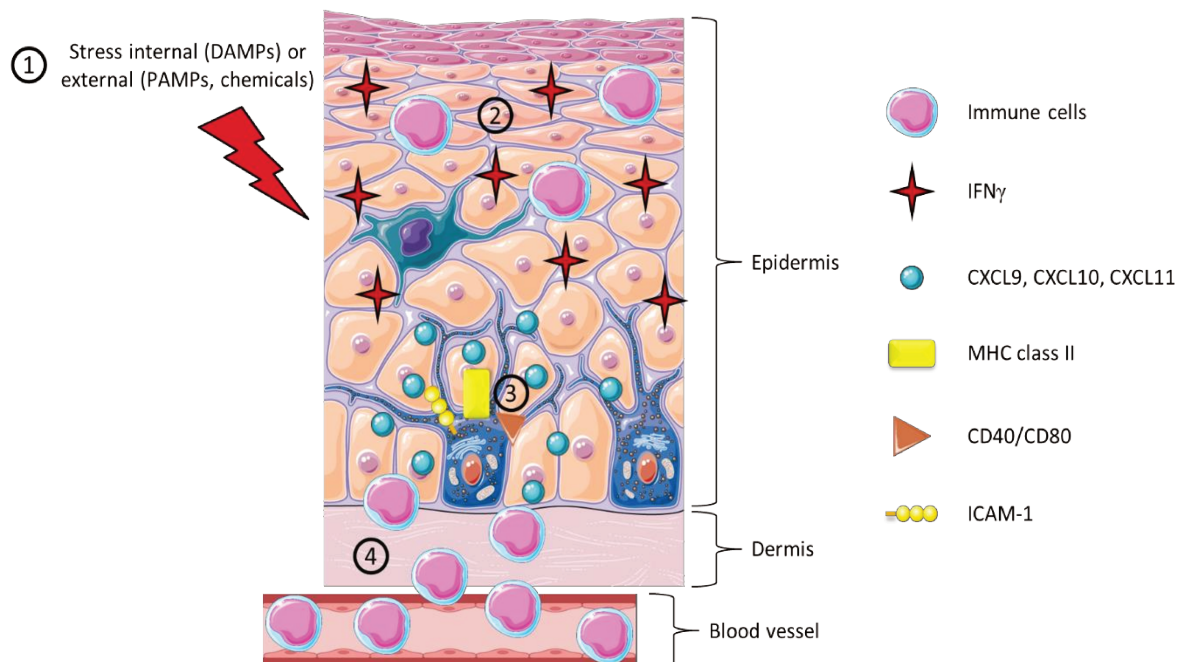


Figure 21: Anti-melanocytic immunity.

(Adapted from [SMART - Servier Medical ART](#) and [Scientific Image and Illustration Software | BioRender](#))

When the skin undergoes an internal or external stress (Figure 21, step 1), the NKs and Innate Lymphoid Cells (ILC) 1 present in the skin start to produce a small amount of IFN $\gamma$ , which is a key immune cytokine (Figure 21, step 2). In healthy conditions, this small IFN $\gamma$  production has

no impact on melanocytes immunogenicity. However, in pathological conditions, such as in vitiligo skin, the basal number of NK and ILC1 innate immune cells is increased and they become more sensitive to stress, leading to enhanced production of IFN $\gamma$ . Subsequently, IFN $\gamma$  induces the production of the C-X-C Motif Chemokine Ligands (CXCL) CXCL9, CXCL10, and CXCL11 predominantly by keratinocytes and also by melanocytes, as well as the expression of co-stimulatory and adhesion molecules, namely MHC class II HLA-DR, CD40, CD80, and ICAM-1, at the surface of melanocytes (*Figure 21, step 3*). These chemokines attract cytotoxic T cells (*Figure 21, step 4*) and bind to their receptor C-X-C Motif Chemokine Receptor (CXCR) 3B found on melanocytes to directly induce their apoptosis, resulting in release of melanocytic antigens. The remaining melanocytes then start presenting these melanocytic antigens to the attracted naïve T cells, that in turn kill the remaining melanocytes, resulting in an anti-melanocytic immunity in the skin (Tulic et al. 2019). TNF $\alpha$  has similar effects as IFN $\gamma$  on melanocytes, inducing apoptosis, inhibition of differentiation, increased production of pro-inflammatory molecules as well as an increased antigen presentation and subsequent immune cell activation (Webb et al. 2015).

Together these data support the concept that melanocytes as antigen-presenting cells can actively participate in skin immunity. Deciphering the huge potential of multifaceted melanocytes is critical in our understanding of their contribution to normal skin immunity and can offer future therapeutic strategies for various skin pigimentary disorders.



# Thesis project presentation

## Background

CLEC12B was first identified as an inhibitory receptor on myeloid cells that counteracts NKG2D-mediated cytotoxicity in NK cells. CLEC12B is a CLR which possesses an ITIM domain but ligand and downstream signaling are largely unknown. CLRs play an important role in immunity by recognizing a broad range of ligands, including PAMPs and DAMPs present on pathogens and host cells. Most of CLRs are expressed on APCs and are involved in production of cytokines and chemokines, but also in antigen processing and presentation, and subsequent T cell activation. Over the past 30 years, an antigen-presenting function of melanocytes has emerged due to their dendritic nature, their strategic position in the skin and their phagocytic capacity. In this context, our team has shown that in a stressed vitiligo skin, NKs and ILC1s produce large amount of IFN $\gamma$ , leading to the production of CXCL9, CXCL10 and CXCL11 by melanocytes and subsequent T cell chemoattraction. These chemokines then bind to their receptor CXCR3B found on melanocytes to induce initial apoptosis of some melanocytes, resulting in the release of self-antigens. The remaining melanocytes, activated by IFN $\gamma$  production, express costimulatory molecules which triggers T cell proliferation. They start to present melanocytic antigens to T cells, initiating an adaptive immune response and subsequent anti-melanocytic immunity. Recent results from our team have shown that CLEC12B is predominantly expressed in human melanocytes and plays an important role in the regulation of skin pigmentation, but also in melanoma proliferation.

## Hypothesis and objectives

To sum up, CLEC12B is a CLR predominantly expressed by melanocytes in the skin. CLRs are mostly expressed by APCs and play a key role in immune responses, in particular through the production of cytokines and chemokines. Melanocytes have an antigen-presenting function, are actively involved in skin immunity, and can be activated by IFN $\gamma$ , thus inducing the expression of APC markers and the production of CXCL9, CXCL10, CXCL11, and subsequent chemoattraction of T cells. Based on the role of CLEC12B in skin pigmentation and melanoma

proliferation, as well as its involvement in several diseases and infections reported by other teams, we hypothesized that CLEC12B has a role in skin immunity through the modulation of the IFN $\gamma$  pathway.

To test this hypothesis, my thesis project has been focusing on:

- Determining whether CLEC12B modulates the production of IFN $\gamma$ , CXCL9, CXCL10, CXCL11 by melanocytes.
- Determining whether CLEC12B modulates the expression of APC markers on melanocytes.
- Determining whether CLEC12B modulates the migration of immune cells.
- Deciphering the signaling pathway of CLEC12B.
- Determining whether CLEC12B modulates immune responses against pathogens.
- Identifying CLEC12B ligands.



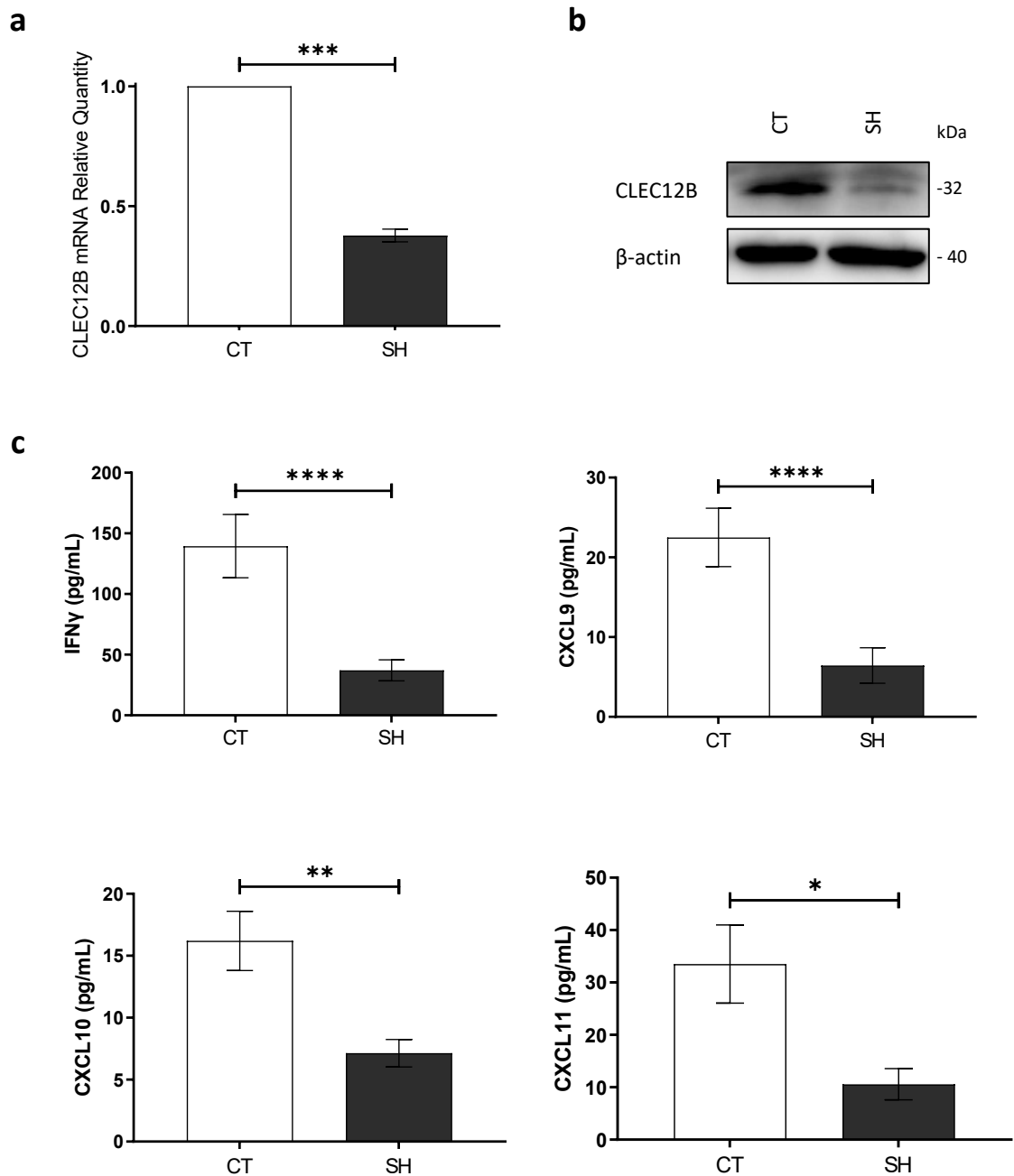
# Results

## ***CLEC12B* silencing decreases the production of immune cytokines and chemokines.**

IFN $\gamma$  is a key immune cytokine which increases melanocyte immuno-competency by enhancing the expression of immune response accelerators (Wang et al. 2014) and induces the production of CXCL9, CXCL10, and CXCL11 by melanocytes (Tulic et al. 2019), which are key regulators of immune cell migration, differentiation, and activation (Tokunaga et al. 2018). To determine whether *CLEC12B* can modulate the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11, we used a lentiviral-mediated silencing of *CLEC12B* with short hairpin RNA (shRNA) (condition SH) in melanocytes. The efficiency of shRNA was analyzed by qPCR (**Figure 1a**) and western blot (**Figure 1b**). The production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 was measured in melanocytes supernatants using ELISA kits. Results showed that IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 were detected in the supernatant of control melanocytes (CT), the most produced being IFN $\gamma$ , and then *CLEC12B* silencing induced a significantly lower production compared to control (**Figure 1c**). These results suggest that *CLEC12B* is involved in the production of immune mediators in melanocytes.

## ***CLEC12B* overexpression increases the production of immune cytokines and chemokines through its ITIM domain.**

Transmembrane CLRs possess intracellular signaling motifs that can modulate signaling pathways and sequence data analysis revealed that *CLEC12B* contains an ITIM sequence (VTYATL: V=Valine, T=Threonine, Y=Tyrosine, A=Alanine, L=Leucine) in its intracellular domain (Hoffmann et al. 2007). To determine whether *CLEC12B* ITIM domain is involved in the regulation of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 production, we generated an ITIM domain mutant by exchanging the tyrosine by a phenylalanine (F) using site-directed mutagenesis, so that the ITIM domain can no longer be phosphorylated. We used a lentiviral-mediated overexpression of *CLEC12B* (condition OV) or a lentiviral-mediated overexpression of ITIM mutant *CLEC12B* (condition OV mutant) in melanocytes. The efficiency of *CLEC12B* overexpression and *CLEC12B* mutant overexpression were analyzed by qPCR (**Figure 2a**) and western blot (**Figure 2b**). As shown in **Figure 1c**, results showed that IFN $\gamma$ , CXCL9, CXCL10, and



**Figure 1: *CLEC12B* silencing decreases the production of immune cytokines and chemokines.**

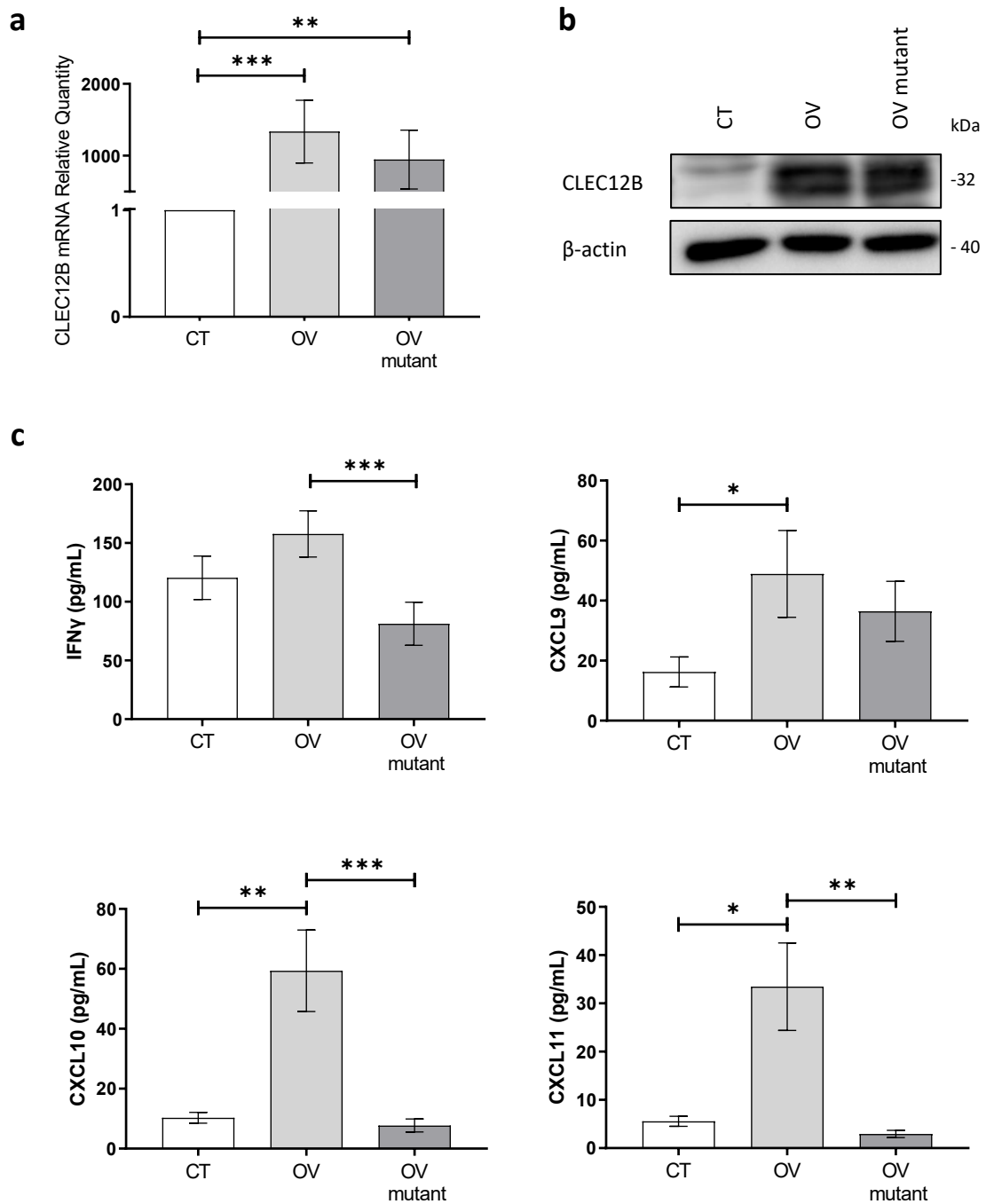
Lentiviral infection with short hairpin RNA (shRNA) was used to silence *CLEC12B* in melanocytes. (a) *CLEC12B* mRNA relative quantity was examined by qPCR. Results were normalized to house-keeping gene *SB34* and expressed as fold change relative to control. (b) *CLEC12B* protein expression level was analyzed by western blot.  $\beta$ -actin was used as the loading control. (c) Production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 by melanocytes was measured by ELISA. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Wilcoxon matched-pairs signed-rank test (a) or Mann-Whitney test (c) and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . CT: control, SH: *CLEC12B* silencing.

CXCL11 were detected in the supernatant of control melanocytes (CT), the most produced being IFN $\gamma$  (**Figure 2c**). *CLEC12B* overexpression induced a significantly higher production of CXCL9, CXCL10, and CXCL11, but not IFN $\gamma$ , compared to CT whereas OV mutant was able to prevent this increase, except for CXCL9 (**Figure 2c**). Overall, these results showed that *CLEC12B* modulates the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 through the phosphorylation of its ITIM domain.

Moreover, *CLEC12B* has three other variants that are thought to code truncated and probably non-functional proteins (Sattler et al. 2010). The transcript variant 2 is the only alternative spliced variant that also has an intracellular ITIM domain (Blot, Passeron, et Tulic 2022), just like the transcript variant 1 coding for the functional type II transmembrane protein used in this project. To determine whether this isoform 2 can also modulate the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11, we used a lentiviral-mediated overexpression of *CLEC12B* isoform 2 (condition OV isoform 2) in melanocytes. The efficiency of OV isoform 2 was analyzed by qPCR (**Supplementary Figure S1a**). Results showed that OV isoform 2 had no effect on the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 compared to control condition (**Supplementary Figure S1b**).

### ***CLEC12B* modulates the chemotaxis of Peripheral Blood Mononuclear Cells (PBMCs) through the production of immune chemokines CXCL9, CXCL10, and CXCL11.**

IFN $\gamma$  binds to the Interferon-gamma Receptor (IFNGR), which is composed of two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains, to induce activation of the downstream signaling pathway (Schroder et al. 2004). Our purpose was to determine whether *CLEC12B* can still modulate the production of CXCL9, CXCL10, and CXCL11 after IFN $\gamma$  stimulation. Results showed an increased production of the three chemokines in control condition after IFN $\gamma$  stimulation (**Figure 3a-b**). Silencing *CLEC12B* significantly decreased the production of CXCL9 and CXCL11 after IFN $\gamma$  stimulation, however the reduction in CXCL10 production did not reach significance (**Figure 3a**). On the contrary, *CLEC12B* overexpression markedly increased the production of all chemokines after IFN $\gamma$  stimulation and potentiated their IFN $\gamma$ -induced production (**Figure 3b**). OV mutant was able to completely prevent the increased production of CXCL9, CXCL10, and CXCL11 similar to CT levels (**Figure 3b**). These results demonstrate that *CLEC12B* modulates the production of CXCL9, CXCL10, and



**Figure 2: *CLEC12B* overexpression increases the production of immune cytokines and chemokines through its ITIM domain.**

Lentiviral infection was used to overexpress *CLEC12B* or to overexpress an ITIM mutated *CLEC12B* in melanocytes. **(a)** *CLEC12B* mRNA relative quantity was examined by qPCR. Results were normalized to house-keeping gene SB34 and expressed as fold change relative to control. **(b)** *CLEC12B* protein expression level was analyzed by western blot.  $\beta$ -actin was used as the loading control. **(c)** Production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 by melanocytes was measured by ELISA. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Wilcoxon matched-pairs signed-rank test **(a)** or Mann-Whitney test **(c)** and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . CT: control, OV: *CLEC12B* overexpression, OV mutant: ITIM mutated *CLEC12B* overexpression.

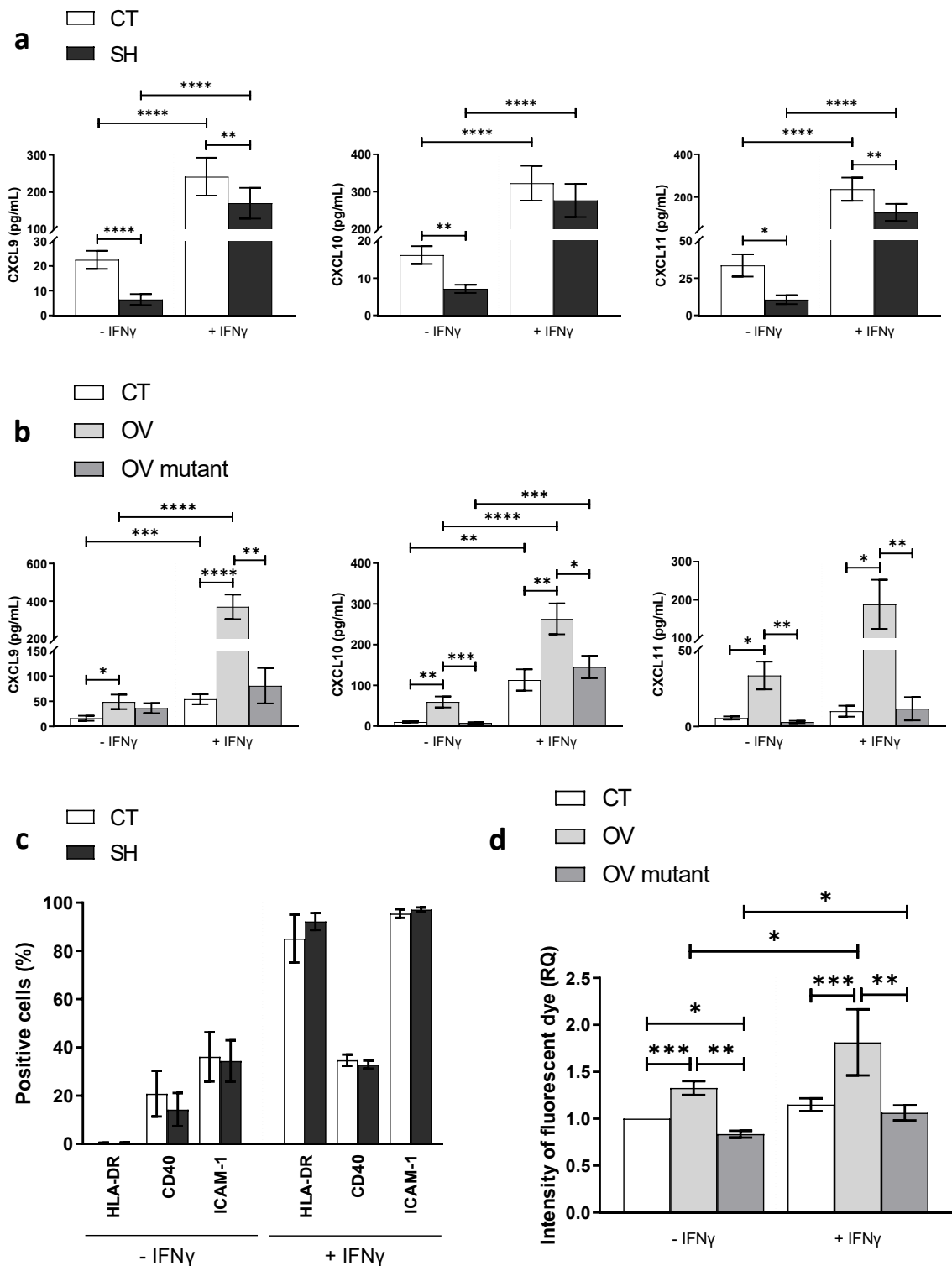
CXCL11, even after IFN $\gamma$  stimulation, through the phosphorylation of its ITIM domain. Furthermore, they show that *CLEC12B* overexpression and IFN $\gamma$  stimulation have a synergistic effect on the production of the three chemokines.

IFN $\gamma$  induces the expression of co-stimulatory and adhesion molecules, namely MHC class II HLA-DR, CD40, and ICAM-1 at the surface of melanocytes (Le Poole, Mutis, et al. 1993; Tulic et al. 2019). To examine whether *CLEC12B* modulates the expression of these APC markers, we used FACS and demonstrated that *CLEC12B* silencing had no effect on the expression of HLA-DR, CD40, and ICAM-1 at the surface of melanocytes, with or without IFN $\gamma$  stimulation (**Figure 3c**). However, IFN $\gamma$  did significantly increase their expression so much so that almost all cells expressed HLA-DR and ICAM-1.

CXCL9, CXCL10, and CXCL11 mainly regulate immune cell migration and induce chemotaxis to recruit immune cells such as cytotoxic lymphocytes, NKs, or macrophages in order to induce adaptative immune responses (Tokunaga et al. 2018). To determine whether *CLEC12B* modulates the chemotaxis of immune cells, we evaluated the migration of Peripheral Blood Mononuclear Cells (PBMCs) from healthy donors in response to the supernatants of unstimulated or IFN $\gamma$  stimulated melanocytes overexpressing *CLEC12B*. We demonstrated *CLEC12B* overexpression to significantly increase melanocyte capacity to attract PBMCs compared to CT, and chemotaxis was completely abolished in *CLEC12B* mutant overexpressing melanocytes (**Figure 3d**). After IFN $\gamma$  stimulation, results showed a tendency to increase chemoattraction in CT (P=0.0637) that was further potentiated in supernatants from OV melanocytes and still abrogated when *CLEC12B* ITIM domain was mutated (**Figure 3d**). Altogether, these results show that *CLEC12B* is involved in cellular recruitment of immune cells through the phosphorylation of its ITIM domain and following modulation of CXCL9, CXCL10, and CXCL11 production.

### **CLEC12B modulates IFN $\gamma$ pathway through the STAT1/IRF1 axis.**

Upon IFN $\gamma$  binding to the IFNGR, the receptor is phosphorylated, allowing recruitment and phosphorylation of STAT1. Activated STAT1 dimers can then translocate to the nucleus and act as transcription factors that bind to the interferon-Gamma Activated Site (GAS) elements of IFN $\gamma$  inducible genes, such as CXCL9, CXCL10, and CXCL11. IRF1 is the most important of these



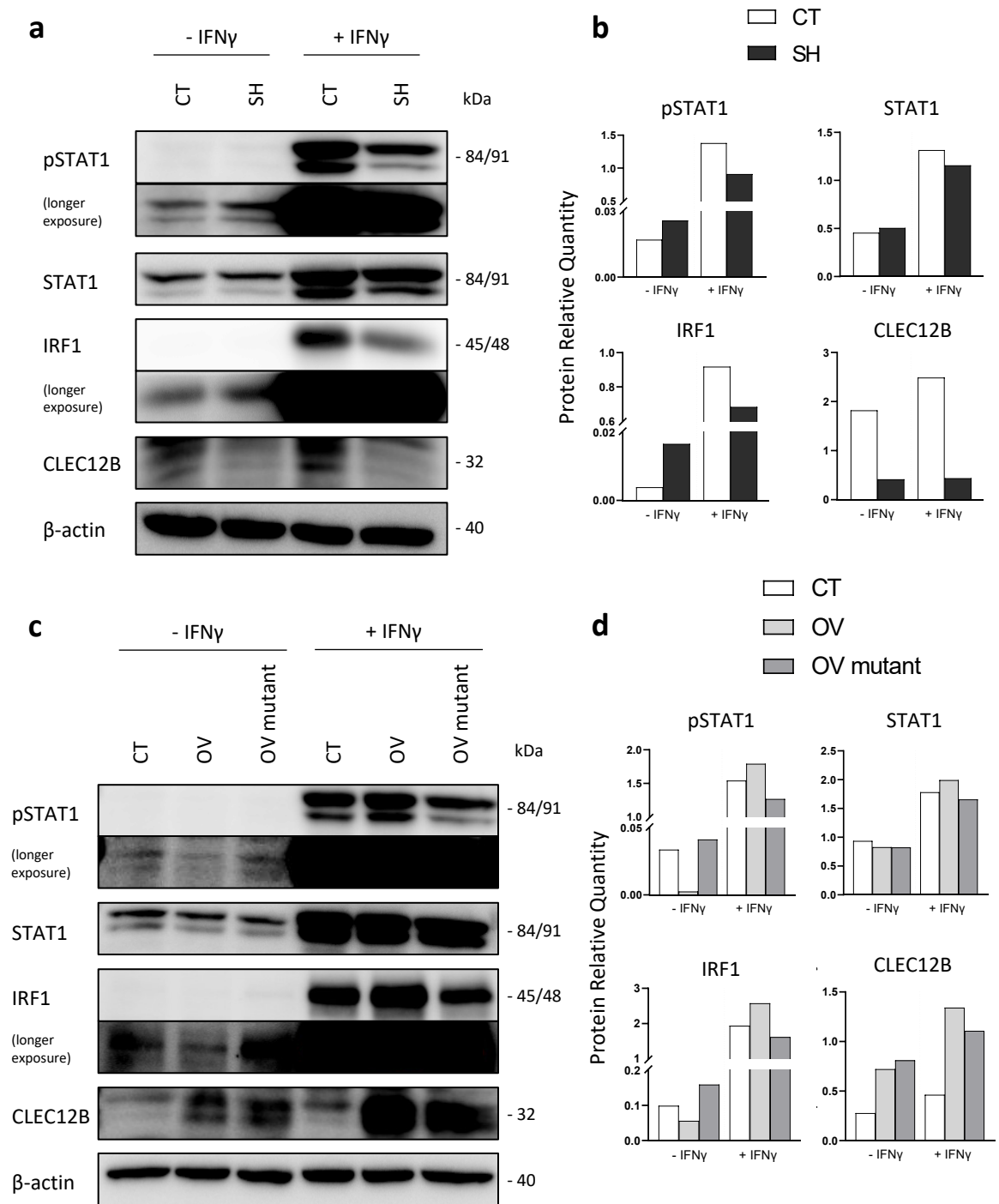
**Figure 3: CLEC12B modulates the chemotaxis of Peripheral Blood Mononuclear Cells (PBMCs) through the production of immune chemokines CXCL9, CXCL10, and CXCL11.**

Lentiviral infection was used to silence *CLEC12B* or to overexpress *CLEC12B* or to overexpress an ITIM mutated *CLEC12B* in melanocytes. (a-b) Production of CXCL9, CXCL10, and CXCL11 in unstimulated or IFN $\gamma$  stimulated (50ng/mL for 24h) melanocytes was measured by ELISA. (c) Proportion of positive cells (%) for immunological and adhesion markers HLA-DR, CD40, and ICAM-1 in unstimulated or IFN $\gamma$  stimulated (50ng/mL for 24h) melanocytes were counted in FACS analysis. (d) Migration of PBMCs in response to the supernatant of melanocytes overexpressing *CLEC12B* or ITIM mutated *CLEC12B* following IFN $\gamma$  stimulation (50ng/mL for 24h) was determined by *in vitro* chemotaxis assay. Results were expressed as fold change relative to IFN $\gamma$ -unstimulated control. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Mann-Whitney test (a,b,c) or Wilcoxon matched-pairs signed-rank test (d) and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . CT: control, OV: *CLEC12B* overexpression, OV mutant: ITIM mutated *CLEC12B* overexpression, SH: *CLEC12B* silencing, RQ: Relative Quantity, HLA-DR: Major Histocompatibility Complex Class II DR, ICAM-1: Intercellular Adhesion Molecule 1.

IFN $\gamma$  inducible genes which in turn acts as a transcription factor binding IRF1-binding site-containing promoters of target genes, including CXCL9, CXCL10, and CXCL11 (Schroder et al. 2004; Kanda et al. 2007; Forero et al. 2019). To understand the mechanisms underlying the regulatory properties of CLEC12B in the production of chemokines, the effects of *CLEC12B* modulation on the expression of STAT1 (phosphorylated and total forms) and IRF1 was analyzed by western blot. In unstimulated melanocytes, silencing *CLEC12B* induced an increase in phosphorylated, but not total, STAT1 and IRF1 expression as shown on western blot analysis (**Figure 4a**) and quantification (**Figure 4b**). In CT melanocytes, IFN $\gamma$  stimulation significantly increased pSTAT1, STAT1, IRF1, and CLEC12B expression which was abolished in SH melanocytes (**Figure 4a-b**). On the contrary, *CLEC12B* overexpression in unstimulated melanocytes decreased the expression of phosphorylated, but not total, STAT1 and IRF1 whereas the ITIM mutant prevented this decrease (**Figures 4c-d**). Upon IFN $\gamma$  stimulation, pSTAT1, STAT1, and IRF1 expression was significantly increased in CT melanocytes and pSTAT1 and IRF1 expression was further potentiated in OV melanocytes, whereas the ITIM mutant abolished the effect (**Figures 4c-d**). Importantly, IFN $\gamma$  stimulation increased CLEC12B expression in CT melanocytes which was further potentiated in OV and OV mutant melanocytes (**Figure 4c-d**). Altogether, these results show that CLEC12B modulates the IFN $\gamma$  pathway through the STAT1/IRF1 axis *via* the phosphorylation of its ITIM domain. Moreover, these data suggest that there is positive feedback between CLEC12B and IFN $\gamma$  where IFN $\gamma$  increases CLEC12B expression and CLEC12B potentiates the effect of IFN $\gamma$  in the downstream signaling pathway and target genes.

Interestingly, the maximum activation of the signaling pathway was reached after 30min of IFN $\gamma$  stimulation and this pathway activation decreased in a time-dependent manner, except for STAT1 expression (**Supplementary Figure S2a**). However, *CLEC12B* silencing (**Supplementary Figure S2b**) or overexpression (data not shown) didn't affect phosphorylated STAT1, total STAT1, and IRF1 expression after 30min or even after 6h of IFN $\gamma$  stimulation. Importantly, there was no increase in CLEC12B expression until 24h of IFN $\gamma$  stimulation.

To assess whether CLEC12B modulates typical pro-inflammatory or innate immune markers, we analyzed the expression of the following genes by qPCR: *TLR2*, *TLR4*, *TLR9*, *MYD88*, *Src*, *Syk*, *IFNGR1*, *IFNGR2*, *CXCR3B*, *SHP1*, *SHP2*, *IL-6*, and *TNF $\alpha$*  (**Supplementary Figure S3**). Results have



**Figure 4: CLEC12B modulates IFN $\gamma$  pathway through the STAT1/IRF1 axis.**

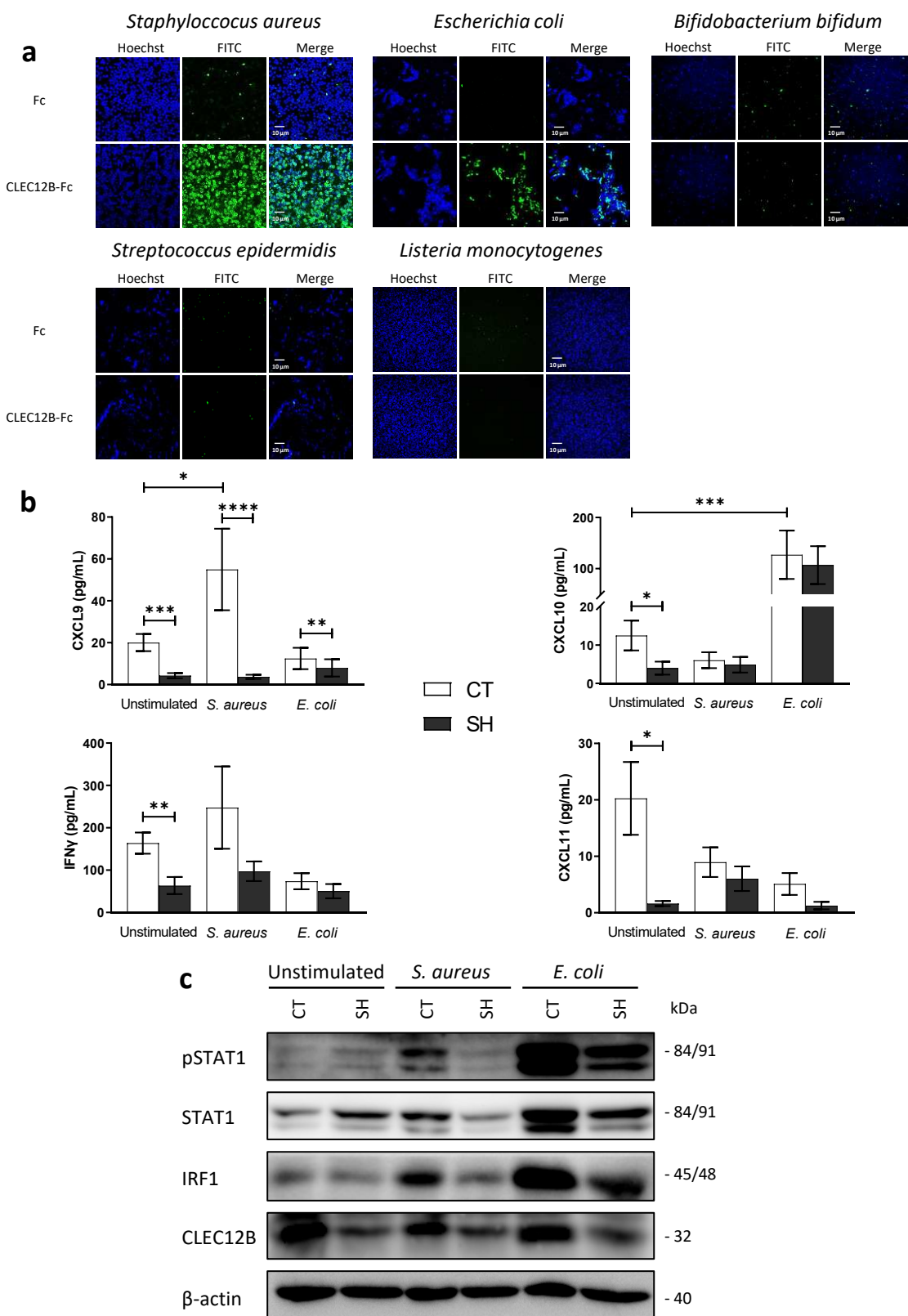
Lentiviral infection was used to silence *CLEC12B* (a) or to overexpress *CLEC12B* or an ITIM mutated *CLEC12B* (c) in melanocytes. pSTAT1, STAT1, IRF1, and CLEC12B protein expression levels were analyzed by western blot in unstimulated or IFN $\gamma$  stimulated (50ng/mL for 24h) melanocytes.  $\beta$ -actin was used as the loading control. (b-d) Western Blot relative quantification of pSTAT1, STAT1, IRF1, and CLEC12B expression reported to  $\beta$ -actin expression was realized using ImageJ. CT: control, OV: *CLEC12B* overexpression, OV mutant: ITIM mutated *CLEC12B* overexpression, SH: *CLEC12B* silencing.



shown that *CLEC12B* silencing (**Supplementary Figure S3**) or *CLEC12B* overexpression (data not shown) didn't modulate any of these genes at a transcriptional level.

**CLEC12B interacts with *S. aureus* and *E. coli* and modulates an immune response against these opportunistic skin bacteria through the STAT1/IRF1/CXCL9 axis.**

CLRs can recognize PAMPs to induce immune responses and fight against pathogens (Mayer, Raulf, et Lepenies 2017). To determine whether CLEC12B can sense exogenous ligands, we incubated different bacteria with the Fc protein alone (as a negative control) or the fusion protein CLEC12B-Fc and visualized a possible interaction using confocal microscopy. We looked at bacterial strains that are typically found on the skin, such as *Staphylococcus aureus* and *Escherichia Coli* which are commensal bacteria that can become pathogenic, or *Bifidobacterium* strains and *Streptococcus epidermidis* which are commensal bacteria that help maintaining skin homeostasis. We also looked at *Listeria monocytogenes* which is a pathogenic bacterium mainly found in the gut that causes the listeriosis and that is one of the most virulent foodborne pathogens. Results showed that CLEC12B-Fc interacted primarily with *S. aureus* but also *E. coli*, however not with *B. bifidum*, *S. epidermidis*, or *L. monocytogenes* (**Figure 5a**), nor with *Bifidobacterium breve*, *Bifidobacterium lactis*, or *Bifidobacterium longum* (data not shown). These data suggest that CLEC12B preferentially interacts with opportunistic bacteria that are mainly found on the skin such as *S. aureus*. To assess whether CLEC12B induces immune responses against *S. aureus* and *E. coli*, we stimulated CT or SH melanocytes with these two bacteria and measured the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 in their supernatants. As previously shown (**Figures 1c-3a**), their production was significantly decreased in unstimulated melanocytes (**Figure 5b**). In CT melanocytes, the production of CXCL9 was significantly increased in response to *S. aureus* and the production of CXCL10 was significantly increased in response to *E. coli*, whereas the production of IFN $\gamma$  and CXCL11 remained insensitive to bacterial stimulation (**Figure 5b**). *CLEC12B* silencing markedly decreased CXCL9 production primarily in response to *S. aureus* but also to *E. coli*, which is in accordance with our IF data, however no significant effect was assessed for CXCL10, IFN $\gamma$ , or CXCL11 (**Figure 5b**). Regarding the downstream signaling pathway, STAT1 (phosphorylated and total forms) and IRF1 expression was significantly increased in response to both *S. aureus* and



**Figure 5: CLEC12B interacts with *S. aureus* and *E. coli* and modulates an immune response against these opportunistic skin bacteria through the STAT1/IRF1/CXCL9 axis.**

(a) Bacteria were incubated with Fc protein alone (negative control, upper panels) or CLEC12B-Fc (lower panels). Bacterial DNA was stained with Hoechst solution (shown in blue). The binding of the CLEC12B-Fc fusion protein to bacteria was detected using a FITC-conjugated anti-human IgG Fc secondary antibody (shown in green). Bacteria were fixed onto cover slips, mounted onto microscopic slides, and visualized using confocal microscopy. The experiment was repeated three times with three randomly selected pictures each, and similar results were revealed. Scale bar indicates 10  $\mu$ m. (b-c) Lentiviral infection was used to silence *CLEC12B* and melanocytes were stimulated with *S. aureus* or *E. coli* (MOI 10) for 24h. (b) Production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 was measured by ELISA. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Mann-Whitney test and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . (c) STAT1, IRF1, and CLEC12B protein expression levels were analyzed by western blot.  $\beta$ -actin was used as the loading control. CT: control, SH: *CLEC12B* silencing.

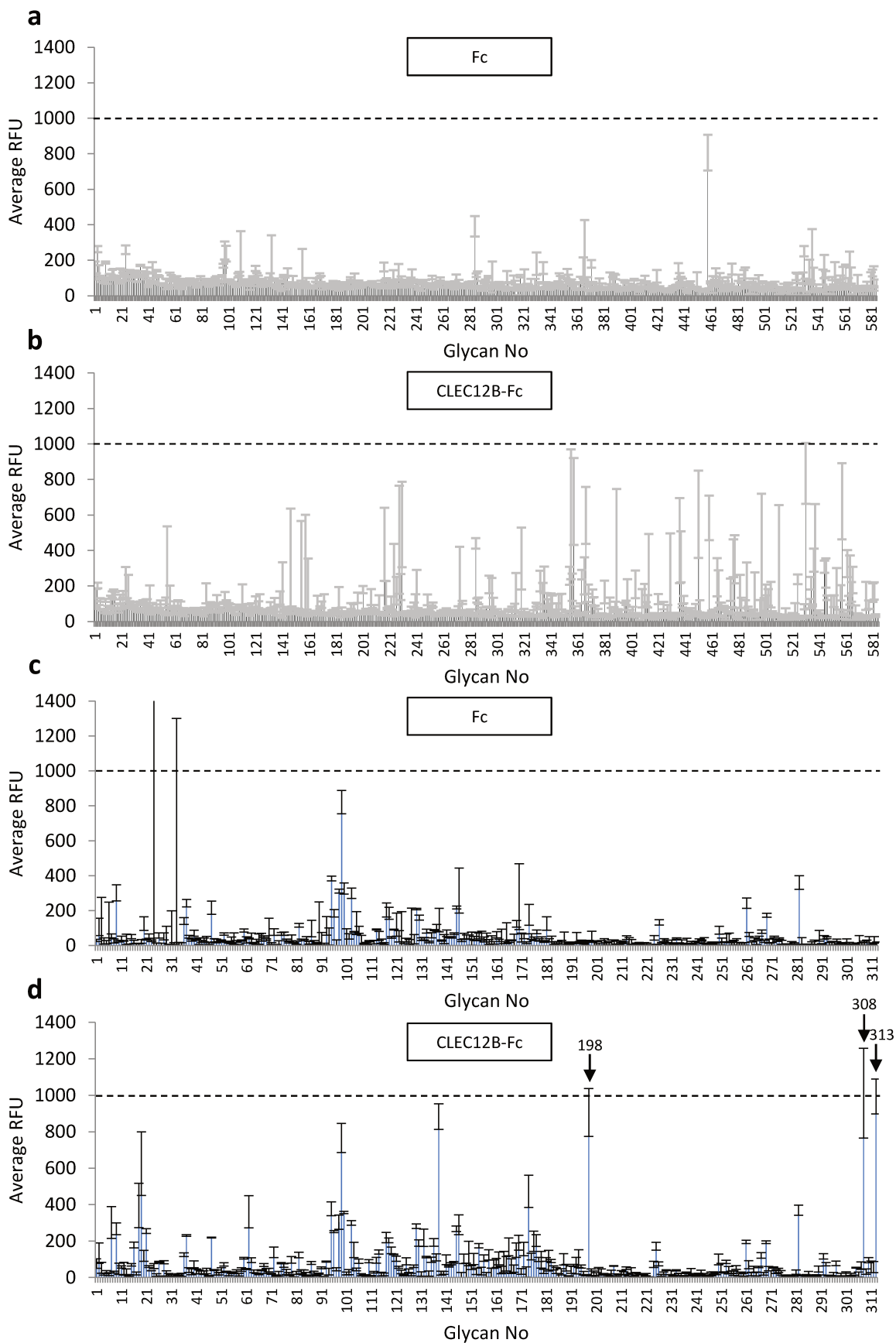
*E. coli* stimulation in CT melanocytes whereas *CLEC12B* silencing prevented the effect (**Figure 5c**). Together, these data show that *CLEC12B* senses exogenous ligands present on *S. aureus* and *E. coli* and modulates an immune response against these opportunistic skin bacteria through the STAT1/IRF1/CXCL9 axis.

**CLEC12B doesn't recognize any mammalian nor microbial glycan structure present on these arrays.**

CLRs can recognize glycan structures expressed by pathogens and host cells to induce immune responses (Vliet, García-Vallejo, et Kooyk 2008). To determine whether *CLEC12B* recognizes a carbohydrate structure, in collaboration with the Consortium for Functional Glycomics (CFG, Harvard University, Boston, USA), we sent the Fc protein alone (as a negative control) or the fusion protein *CLEC12B*-Fc for mammalian and microbial glycan array analyses. These results showed very low specific binding overall (Average RFU < 1000) and high background readings for both mammalian (**Figures 6a-b**) and microbial (**Figures 6c-d**) glycan arrays. A few peaks seemed to appear above the background in the microbial screen: Glycans No 198 (*Shigella boydii* type 9), 308 (Laminarin), and 313 (1-2 Mannan *Acetobacter methanolicus* MB135), but the binding remained very low and below the cut-off (Average RFU < 1000) (**Figure 6d**). Overall, these data suggest that *CLEC12B* doesn't recognize any mammalian nor microbial glycan structure present on these arrays.

**CLEC12B recognizes protein structures present on melanocytes.**

CLRs can sense SAMPs on healthy cells to maintain the baseline non-activated state of innate immune cells and dampen their reactivity following an immune response (Varki 2011). To assess whether *CLEC12B* senses endogenous ligands, we incubated different skin and immune cell types with the Fc protein alone (as a negative control) or the fusion protein *CLEC12B*-Fc and visualized a possible interaction using confocal microscopy. Regarding the three main cell types in the skin, results showed that *CLEC12B* strongly interacted with melanocytes and fibroblasts, but not with keratinocytes (**Figure 7a**). *CLEC12B* also interacted with pro-inflammatory macrophages (M1) as well as anti-inflammatory macrophages (M2) (**Figure 7a**).



**Figure 6: CLEC12B doesn't recognize any mammalian nor microbial glycan structure present on these arrays.**

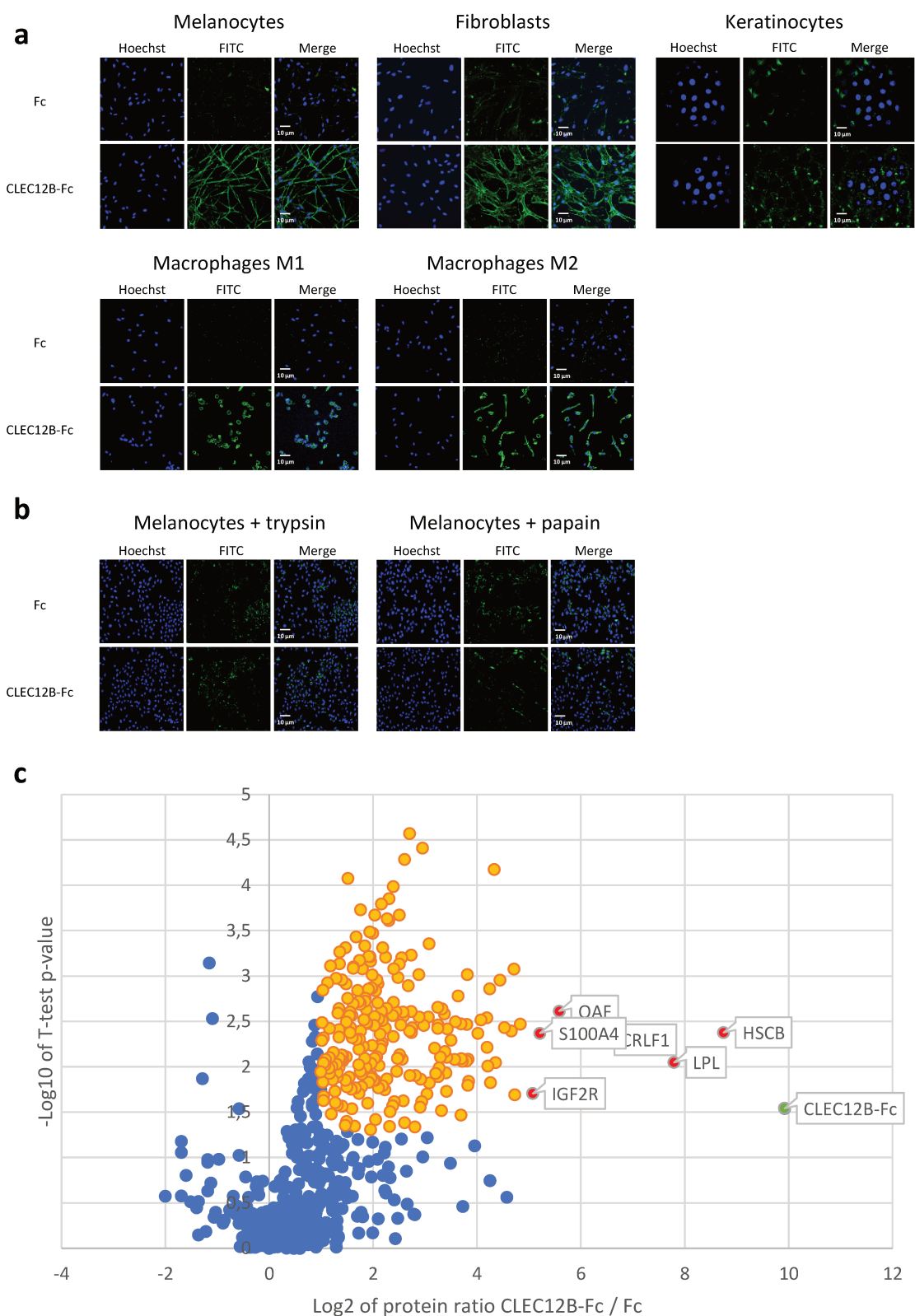
(a-c) Fc protein alone (negative control, 50  $\mu$ g) or (b-d) CLEC12B-Fc (50  $\mu$ g) were incubated with (a-b) the mammalian glycan array of 585 glycans (version 5.1) or (c-d) the pathogen glycan array of 313 glycans (version 1) developed by the Consortium for Functional Glycomics (CFG). Protein binding was revealed by FITC fluorescence using a FITC-conjugated anti-human IgG Fc secondary antibody and expressed as RFU. The average intensity detected from all glycans was calculated and set as baseline. Glycans with average RFU superior to 1000 were considered significant. Error bars represent mean SD replicate determinations. RFU: relative fluorescent units.

CLRs can bind a wide variety of ligands, other than glycans, such as proteins, lipids, or inorganic compounds on host cells (Zelensky et al., 2005). To determine whether CLEC12B recognizes a protein structure on melanocytes, we treated the cells with trypsin (a serine protease) or papain (a cysteine protease) prior to cell incubation with the Fc protein alone (as a negative control) or the fusion protein CLEC12B-Fc and visualized possible interactions using confocal microscopy. Trypsin and papain pre-treatments both completely prevented the interaction between CLEC12B-Fc and melanocytes (**Figure 7b**), indicating that CLEC12B recognizes protein structures present on melanocytes. To identify the protein structures recognized by CLEC12B on melanocytes, we performed a coimmunoprecipitation assay using lysates of melanocytes that were incubated with the Fc protein alone (as a negative control) or the fusion protein CLEC12B-Fc and performed Nanoscale liquid chromatography coupled to tandem mass spectrometry analysis (ProteoToul facility, Toulouse, France). Results identified 6 potential CLEC12B ligands (classified in descending order of their peptide enrichment) (**Figure 7c**):

- Insulin Like Growth Factor 2 Receptor (IGF2R)
- Lipoprotein Lipase (LPL)
- Cytokine Receptor Like Factor 1 (CRLF1)
- S100 Calcium Binding Protein A4 (S100A4)
- Out At First Homolog (OAF)
- HscB Mitochondrial Iron-Sulfur Cluster Cochaperone (HSCB)

**IGF2R, CRLF1, HSCB, S100A4, OAF, and LPL are not CLEC12B ligands.**

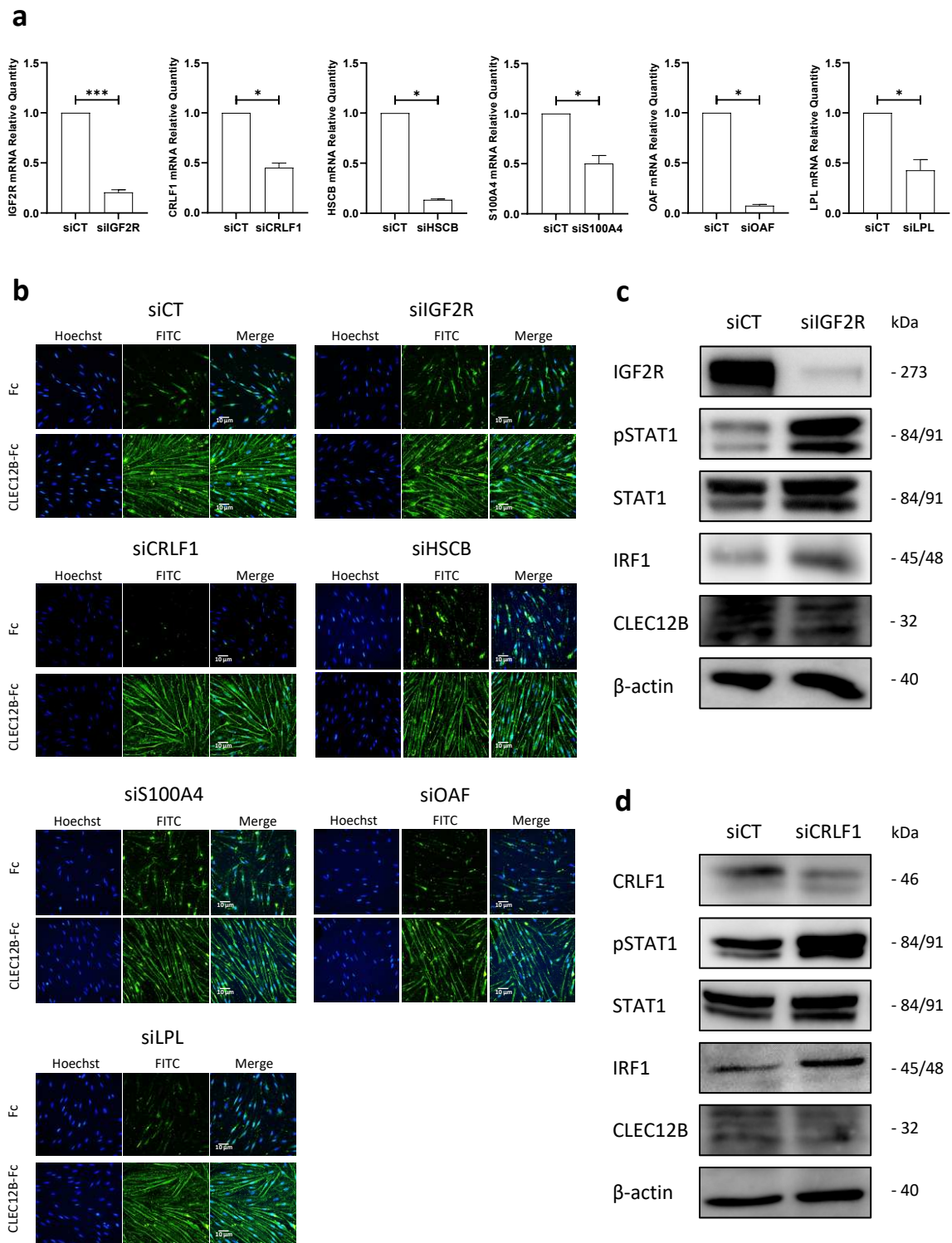
To assess whether IGF2R, CRLF1, HSCB, S100A4, OAF, or LPL are CLEC12B ligands, we used siRNA transfection to silence their expression in melanocytes and we incubated these cells with the Fc protein alone (as a negative control) or the fusion protein CLEC12B-Fc and visualized a possible interaction using confocal microscopy. The efficiency of siRNA was analyzed by qPCR (**Figure 8a**) and western blot for IGF2R and CRLF1 (**Figure 8c-d**). Results showed that *IGF2R*, *CRLF1*, *HSCB*, *S100A4*, *OAF*, or *LPL* silencing didn't prevent the interaction between CLEC12B-Fc and melanocytes (**Figure 8b**), suggesting that none of these proteins are likely to be CLEC12B ligands. IGF2R is a type I transmembrane glycoprotein expressed at the



**Figure 7: CLEC12B recognizes protein structures present on melanocytes.**

(a-b) Cells were incubated with Fc protein alone (negative control, upper panels) or CLEC12B-Fc (lower panels). DNA was stained with Hoechst solution (shown in blue). The binding of the CLEC12B-Fc fusion protein to cells was detected using a FITC-conjugated anti-human IgG Fc secondary antibody (shown in green). Cells were grown onto cover slips, mounted onto microscopic slides, and visualized using confocal microscopy. The experiment was repeated three times with three randomly selected pictures each, and similar results were revealed. Scale bar indicates 10  $\mu$ m. (b) Prior to cell incubation and staining, melanocytes were treated with trypsin (0,05 %) or papain (5  $\mu$ g/mL) for 5 min, then were cytopsined onto microscopic slides. (c) Melanocyte lysates were incubated with Fc protein alone or CLEC12B-Fc and immunoprecipitated using protein G magnetic beads. Samples were analyzed by Nanoscale liquid chromatography coupled to tandem mass spectrometry (NanoLC-MS/MS) and a label free quantification was performed with PROLINE 2.0 software by the ProteoToul facility of Toulouse.

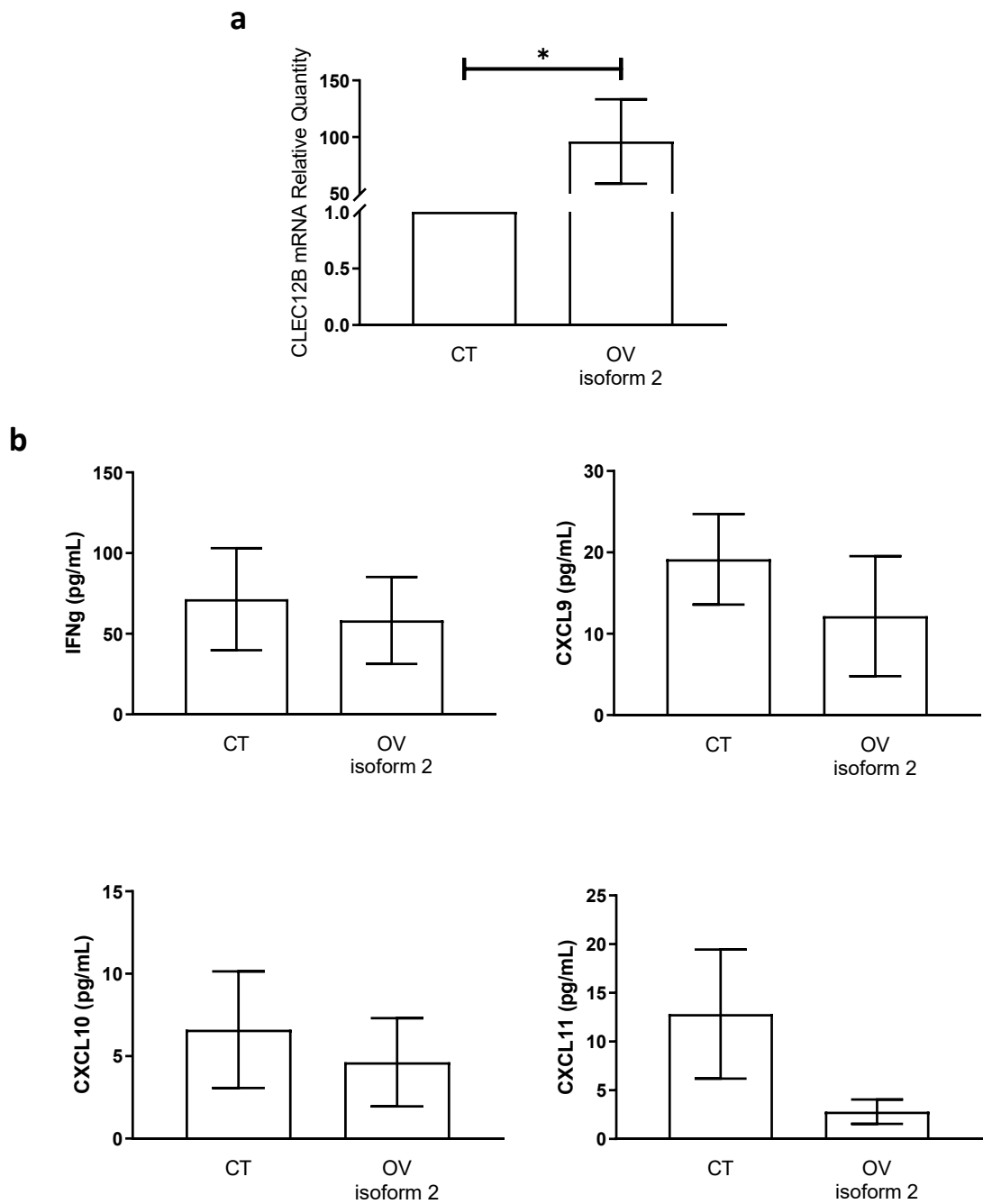
cell surface that binds many different mannose-6-phosphate(M6P)-containing ligands and that is functionally related to the Cation-Dependent M6P Receptor (CD-MPR). Together they form the p-type lectin family and are involved in the trafficking of lysosomal enzymes between the *trans*-Golgi network, endosomes, and lysosomes (J. Brown, Jones, et Forbes 2009). CRLF1 is a soluble type I cytokine receptor that is up-regulated by pro-inflammatory cytokines such as TNF $\alpha$ , IL-6, and IFN $\gamma$ , suggesting that CRLF1 may be involved in regulating the immune system during an inflammatory response. When p28 secretion is chaperoned by CRLF1, the resulting complex can activate STAT1 phosphorylation and possibly SHP2/Ras/MAPK pathway (Crisponi, Buers, et Rutsch 2022). To examine if *IGF2R* and/or *CRLF1* are involved in CLEC12B-induced signaling cascade, we used siIGF2R and siCRLF1 to examine pSTAT1, STAT1, and IRF1 expression. Our results demonstrated that silencing *IGF2R* and *CRLF1* markedly increased pSTAT1, STAT1, and IRF1 expression, without marked changes in CLEC12B expression, as shown on western blot analysis (**Figure 8c-d**). *IGF2R* and *CRLF1* silencing had the same profile as *CLEC12B* silencing on the STAT1/IRF1 axis (**Figure 4a**).



**Figure 8: IGF2R, CRLF1, HSCB, S100A4, OAF, and LPL are not CLEC12B ligands.**

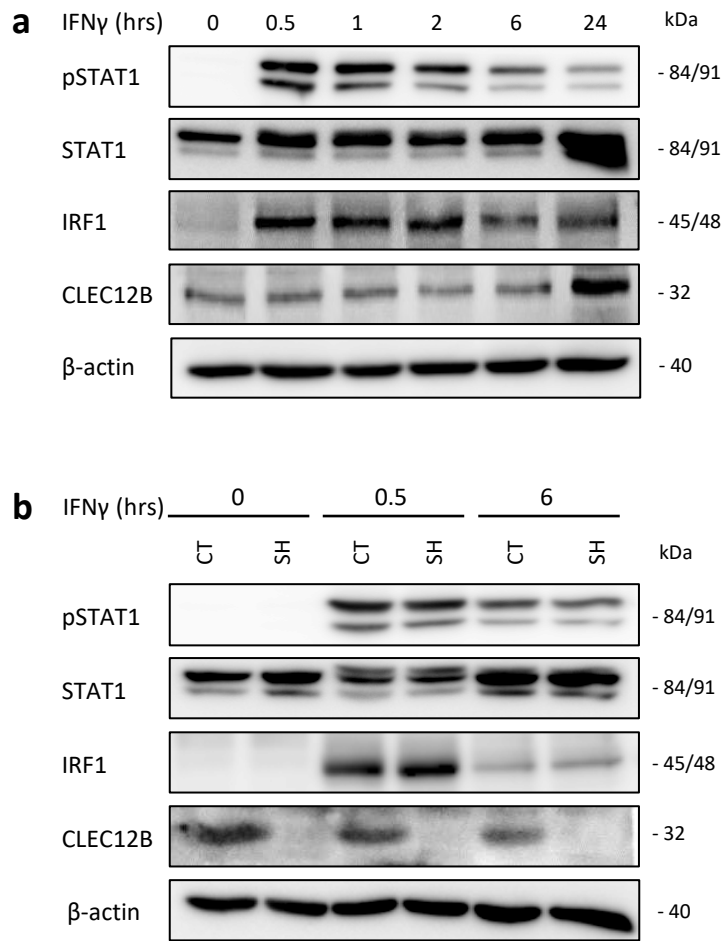
siRNA transfection (50 nM) was used to silence *IGF2R*, *CRLF1*, *HSCB*, *S100A4*, *OAF*, and *LPL* in melanocytes. (a) *IGF2R*, *CRLF1*, *HSCB*, *S100A4*, *OAF*, and *LPL* mRNA relative quantity was examined by qPCR. Results were normalized to house-keeping gene *SB34* and expressed as fold change relative to control. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Wilcoxon matched-pairs signed-rank test and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . (b) Melanocytes were incubated with Fc protein alone (negative control, upper panels) or CLEC12B-Fc (lower panels). DNA was stained with Hoechst solution (shown in blue). The binding of the CLEC12B-Fc fusion protein to cells was detected using a FITC-conjugated anti-human IgG Fc secondary antibody (shown in green). Cells were grown onto cover slips, mounted onto microscopic slides, and visualized using confocal microscopy. The experiment was repeated three times with three randomly selected pictures each, and similar results were revealed. Scale bar indicates 10  $\mu$ m. (c-d) IGF2R, CRLF1, STAT1, IRF1, and CLEC12B protein expression levels were analyzed by western blot.  $\beta$ -actin was used as the loading control. CT: control, si: siRNA silencing.





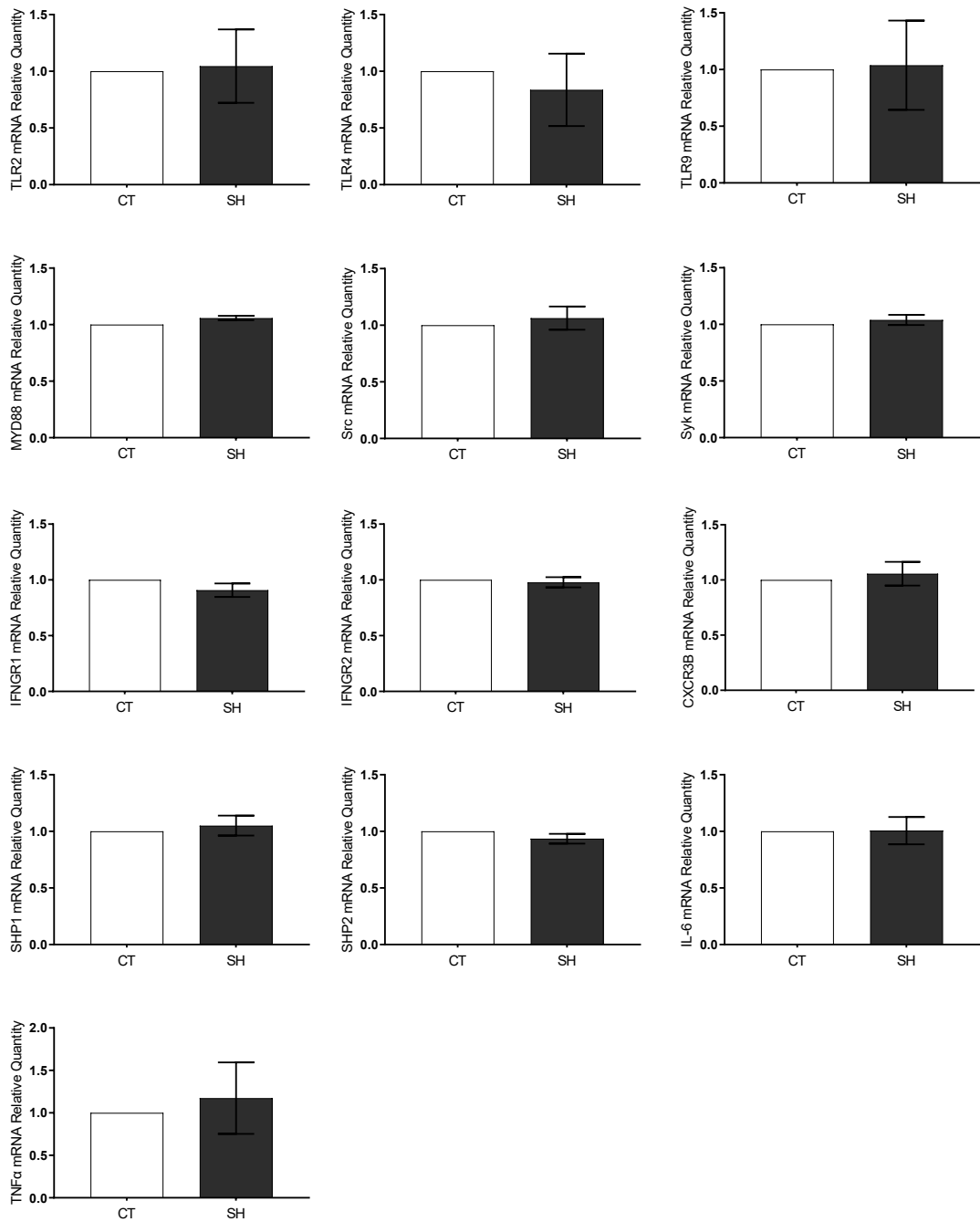
**Supplementary Figure S1: CLEC12B isoform 2 doesn't modulate the production of immune cytokines and chemokines.**

Lentiviral infection was used to overexpress *CLEC12B* isoform 2 (NM\_205852.3) in melanocytes. **(a)** *CLEC12B* mRNA relative quantity was examined by qPCR. Results were normalized to house-keeping gene *SB34* and expressed as fold change relative to control. **(b)** Production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 by melanocytes was measured by ELISA. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Wilcoxon matched-pairs signed-rank test **(a)** or Mann-Whitney test **(b)** and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . CT: control, OV isoform 2: *CLEC12B* isoform 2 overexpression.



**Supplementary Figure S2: CLEC12B doesn't modulate IFN $\gamma$  pathway before 24h of IFN $\gamma$  stimulation.**

(a) pSTAT1, STAT1, IRF1, and CLEC12B protein expression levels were analyzed by western blot in unstimulated or IFN $\gamma$  stimulated (50ng/mL for 0.5, 1, 2, 6 or 24h) melanocytes.  $\beta$ -actin was used as the loading control. (b) Lentiviral infection was used to silence *CLEC12B* in melanocytes. pSTAT1, STAT1, IRF1, and CLEC12B protein expression levels were analyzed by western blot in unstimulated or IFN $\gamma$  stimulated (50ng/mL for 0.5h or 6h) melanocytes.  $\beta$ -actin was used as the loading control. CT: control, SH: *CLEC12B* silencing.



**Supplementary Figure S3: CLEC12B doesn't modulate any pro-inflammatory or innate immune marker at a transcriptional level.**

Lentiviral infection was used to silence *CLEC12B* in melanocytes. *TLR2*, *TLR4*, *TLR9*, *MYD88*, *Src*, *Syk*, *IFNGR1*, *IFNGR2*, *CXCR3B*, *SHP1*, *SHP2*, *IL-6*, and *TNFα* mRNA relative quantity was examined by qPCR. Results were normalized to house-keeping gene *SB34* and expressed as fold change relative to control. Results are shown at the mean  $\pm$  SEM of at least three biological replicates. Differences between groups were tested using nonparametric Wilcoxon matched-pairs signed-rank test and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ . CT: control, SH: *CLEC12B* silencing.

# Discussion and perspectives

Our team described CLEC12B as a melanocytic gene, whose expression is stronger in light-pigmented skin than in dark-pigmented skin, that modulates pigmentation *in vitro* and in human three-dimensional model of reconstructed epidermis (Sormani et al. 2022). CLEC12B was also shown to be significantly less expressed in melanoma cells than in healthy melanocytes and to decrease melanoma proliferation *in vitro* and *in vivo* (Montaudié et al. 2022). Another team showed a similar suppressor function of CLEC12B in lung cancer progression *in vitro* and *in vivo* (Chi et al. 2021). Based on the role of CLEC12B in skin pigmentation and melanoma proliferation, as well as its involvement in several diseases and infections reported by other teams (Q. Li et al. 2009; Oğuz et al. 2016; Kim et al. 2017; B. Zhang et al., s. d.; Derpoorter et al. 2019; Chi et al. 2021; Iijima et al. 2021; Forest et al. 2022; Z. Zhang et al. 2022; Kottom et al. 2023), we speculated that CLEC12B has a role in skin immunity. In this study, we show that CLEC12B is capable of inducing production of immune mediators from human melanocytes. In addition, CLEC12B is capable of modulating cutaneous bacterial responses such as those with *S. aureus* and *E. coli*. The mechanism of CLEC12B-induced immunity in the skin involves increased production of innate CXCL9, CXCL10, and CXCL11 chemokines through the activation of the STAT1/IRF1-mediated pathway, resulting in chemoattraction of PBMCs and therefore trigger of long-term adaptive immunity. Moreover, CLEC12B potentiates the effect of IFN $\gamma$  in primed melanocytes, thus inducing a larger production of innate chemokines and subsequent greater chemoattraction of immune cells. Finally, CLEC12B senses motifs present on melanocytes, fibroblasts, and both pro- and anti-inflammatory macrophages, but its exact ligands still need to be identified. These results demonstrate for the first time CLEC12B to play an important function in the human skin, bridging innate and adaptive immunity.

Due to the slow growing nature of primary human melanocytes, some optimization was performed in Hermes cell lines, however key results were always confirmed in primary human cells. IFN $\gamma$  is a key immune cytokine which increases melanocyte immuno-competency by enhancing the expression of immune response accelerators (Wang et al. 2014) and induces the production of the CXCL9, CXCL10, and CXCL11 by melanocytes (Tulic et al. 2019). Here we demonstrated CLEC12B to modulate the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 in

melanocytes through the phosphorylation of its ITIM domain (**Figures 1-2**), supporting our hypothesis that CLEC12B does have a role in skin immunity. Moreover, our results showed that overexpression of *CLEC12B* isoform 2 in melanocytes had no effect on the cytokine or chemokine production (**Supplementary Figure S1**). This transcript variant 2 is the only alternative spliced variant that also has an intracellular ITIM domain, only one of the two glycosylation sites and none of the five ligand binding sites which are present on the transcript variant 1 (Blot, Passeron, et Tulic 2022), suggesting that these missing sites are necessary for CLEC12B signaling.

After IFN $\gamma$  stimulation, we confirmed results previously published by our team (Tulic et al. 2019) showing that the production of chemokines was significantly increased in control melanocytes (**Figure 3a-b**). However, this increased production of chemokines was markedly higher in control melanocytes from silencing experiments (**Figure 3a**) than in control melanocytes from overexpressing experiments (**Figure 3b**). This can be explained by the difference between the plasmids control used to infect melanocytes (see Methods section), suggesting that the plasmid control in silencing experiments might make melanocytes more sensitive to IFN $\gamma$  stimulation. Silencing *CLEC12B* significantly decreased the production of CXCL9 and CXCL11 after IFN $\gamma$  stimulation, however the reduction in CXCL10 did not reach significance (**Figure 3a**). These results suggest that CLEC12B plays an important role in chemokines production as its inhibition reduces their production and moreover, that CXCL10 is less responsive to its inhibition. On the contrary, *CLEC12B* overexpression markedly increased the production of all chemokines after IFN $\gamma$  stimulation and potentiated their IFN $\gamma$ -induced production (**Figure 3b**). OV mutant was able to completely prevent the increased production of CXCL9, CXCL10, and CXCL11 similar to CT levels (**Figure 3b**). These results demonstrated that *CLEC12B* overexpression and IFN $\gamma$  stimulation had a synergistic effect on the production of CXCL9 and CXCL10. For CXCL11 this didn't reach significance due to a larger variability seen between different melanocytes donors, suggesting a more heterogenous CXCL11 response. Taken together, these data demonstrate that CLEC12B plays an important role in the potentiation of the immune responses following IFN $\gamma$  stimulation. We also showed that *CLEC12B* silencing (**Supplementary Figure S3**) or overexpression (data not shown) in unstimulated melanocytes didn't modulate any of the following typical pro-inflammatory or innate immune markers: *TLR2*, *TLR4*, *TLR9*, *MYD88*, *Src*, *Syk*, *IFNGR1*, *IFNGR2*, *CXCR3B*, *SHP1*,

*SHP2*, *IL-6*, and *TNF $\alpha$*  at a transcriptional level. Previous data from our team similarly showed that *CLEC12B* did not affect CREB transcriptional regulation, even though *CLEC12B* modulates CREB protein expression and induces its proteasomal degradation (Sormani et al. 2022), suggesting that *CLEC12B* is likely to act at translational and post-translational levels. It would be interesting to look at the above pro-inflammatory and innate immune markers, as well as IRF1 and STAT1, after IFN $\gamma$  stimulation to verify whether *CLEC12B* can modulate their transcription in activated melanocytes.

Results from our team and others have previously shown that IFN $\gamma$  induces the expression of co-stimulatory and adhesion molecules, namely MHC class II HLA-DR, CD40, and ICAM-1 at the surface of melanocytes (Le Poole, Mutis, et al. 1993; Tulic et al. 2019). These are important markers notably involved in the formation of an immune synapse between APCs and T cells to induce adaptative immune responses, highlighting the APC function of melanocytes which we can activate through IFN $\gamma$  stimulation to better respond to an antigen and to induce immune responses. Although *CLEC12B* silencing significantly decreased the production of IFN $\gamma$ , we showed that it did not impact these surface markers expression on melanocytes, however IFN $\gamma$  stimulation did significantly increase all these markers (**Figure 3c**). It is known that the expression of these co-stimulatory and adhesion markers is also controlled by other cytokine signaling, such as IL-6, TNF $\alpha$ , IFN $\alpha$ , or IFN $\beta$  (Kessal et al. 2018; Chauhan et al. 2020; Qiu et al. 2022), suggesting that such pathways may compensate for the decreased activation of IFN $\gamma$  signaling when *CLEC12B* is silenced. It would be interesting to look at the effect of *CLEC12B* overexpression on these markers, especially in IFN $\gamma$ -activated melanocytes as there is a synergistic effect between *CLEC12B* overexpression and IFN $\gamma$  stimulation. However, as it may be difficult to see an effect of *CLEC12B* overexpression in IFN $\gamma$  stimulated melanocytes on HLA-DR and ICAM-1 expression because almost all cells express these two markers, immunofluorescence technique could be better suited to examine these changes.

It is known that CXCL9, CXCL10, and CXCL11 mainly regulate immune cell migration and induce chemotaxis to recruit immune cells such as cytotoxic lymphocytes, NKs, or macrophages in order to induce adaptative immune responses (Tokunaga et al. 2018). We demonstrated *CLEC12B* overexpression to significantly increase melanocyte capacity to attract PBMCs compared to CT, and chemotaxis was completely abolished in *CLEC12B* mutant overexpressing melanocytes (**Figure 3d**). After IFN $\gamma$  stimulation, results showed a tendency to increase

chemoattraction in CT melanocytes ( $P=0.0637$ ) that was further potentiated in supernatants from OV melanocytes and still abrogated when CLEC12B ITIM domain was mutated (**Figure 3d**). Altogether, these results demonstrate that CLEC12B is involved in cellular recruitment of immune cells through the phosphorylation of its ITIM domain and subsequent modulation of CXCL9, CXCL10, and CXCL11 production. These findings suggest that CLEC12B could play a role in melanocyte-related pathologies such as vitiligo and melanoma or in pigmentary disorders by inducing innate and adaptive immune responses, and this will be discussed shortly after. Although we did not see changes in co-stimulatory and adhesion molecules expression on melanocytes, future experiments to determine whether CLEC12B can modulate the antigen processing and presentation capacity of unstimulated and IFN $\gamma$ -stimulated melanocytes could be realized using DQ<sup>TM</sup> ovalbumin for instance. DQ<sup>TM</sup> ovalbumin is a self-quenched conjugate of ovalbumin designed especially for the study of antigen processing and presentation that exhibits bright fluorescence upon proteolytic degradation. We could also verify whether CLEC12B can modulate T cells cytotoxic activity using IncuCyte<sup>®</sup> live cell imaging system to monitor real-time detection of melanocyte death before and after IFN $\gamma$  stimulation in presence of cytotoxic T cells, but these experiments were beyond the scope of this thesis.

Upon IFN $\gamma$  binding to the IFNGR, the receptor is phosphorylated, allowing recruitment and phosphorylation of STAT1. Activated STAT1 dimers can then translocate to the nucleus and act as transcription factors that bind to the interferon-Gamma Activated Site (GAS) elements of IFN $\gamma$  inducible genes, such as CXCL9, CXCL10, and CXCL11. IRF1 is the most important of these IFN $\gamma$  inducible genes which in turn acts as a transcription factor binding IRF1-binding site-containing promoters of target genes, including the three previously mentioned chemokines (Schroder et al. 2004; Kanda et al. 2007; Forero et al. 2019). In unstimulated melanocytes, our results showed that *CLEC12B* silencing increased STAT1 phosphorylation and IRF1 expression (**Figure 4a-b**) whereas it significantly decreased the production of IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 (**Figure 1c**). *CLEC12B* overexpression showed the opposite results and *CLEC12B* mutant abrogated the overexpression effect (**Figure 2c, Figure 4c-d**). ITIM-containing CLR are usually considered as inhibitory receptors and tend to recognize healthy self to dampen unnecessary immune activation. Ligand engagement by inhibitory receptors results in ITIM phosphorylation by SFKs and recruitment of SHP1 and SHP2 to inhibit STAT dimerization, and subsequent activation of ISGs (Barrow et Trowsdale 2006). Results from our team and others showed that

CLEC12B can recruit SHP1 and SHP2 through the phosphorylation of its ITIM domain (Hoffmann et al. 2007; Sormani et al. 2022) and that *CLEC12B* overexpression increased the interaction with SHP1 and SHP2 and decreased the phosphorylated forms of the two phosphatases (Sormani et al. 2022; Montaudié et al. 2022). We could hypothesize that there is a period during which SHP1 and SHP2 inhibit the STAT1/IRF1 axis before CLEC12B in turn recruits and inhibits SHP1 and SHP2 to promote the production of CXCL9, CXCL10, and CXCL11. Silencing *SHP1* and/or *SHP2* would be of interest to decipher the exact molecular mechanisms involved in CLEC12B downstream signaling. STAT1 also induces expression of Suppressor Of Cytokine Signaling 1 (SOCS1) which in turn negatively regulates STAT signaling but has a short half-life (1-2 hours) (Baker, Akhtar, et Benveniste 2009). It could be of interest to look at SOCS1 expression when *CLEC12B* is overexpressed or silenced. We could hypothesize that SOCS1 inhibits the STAT1/IRF1 axis in the first few hours but then is degraded so that the signaling can be reactivated to induce the production of CXCL9, CXCL10, and CXCL11.

After IFN $\gamma$  stimulation, we showed that pSTAT1, STAT1, IRF1, and CLEC12B expression was significantly increased in CT melanocytes (**Figure 4**). Silencing *CLEC12B* markedly decreased pSTAT1 and IRF1 expression (**Figure 4a-b**) whereas *CLEC12B* overexpression increased their expression and the ITIM mutant prevented the increase (**Figure 4c-d**). These results are in accordance with the production of CXCL9, CXCL10, and CXCL11 previously described (**Figure 3a-b**). Considering that STAT1 is also involved in IFN $\alpha$  signaling, we looked at the production of IFN $\alpha$  in supernatants from unstimulated and IFN $\gamma$ -stimulated melanocytes when *CLEC12B* is overexpressed or silenced, but we didn't detect any production of IFN $\alpha$  in all the supernatants (data not shown). These results suggest that either melanocytes don't produce IFN $\alpha$  or perhaps that their level of production is really low, and the ELISA kit wasn't sensitive enough to detect these. It could be interesting to look at other inflammatory cytokines as STAT1 is involved in many different signaling pathways, including the IFN $\beta$  pathway, which is the other type I interferon with IFN $\alpha$ , the IL-6 pathway, which is induced by IFN $\gamma$  and has anti-melanogenic effect, or the TNF $\alpha$  pathway, which has similar effects as IFN $\gamma$  on melanocytes and is decreased when *CLEC12B* is silenced after *P. carinii* CWF stimulation in the mouse RAW macrophage cell line. Xingxin *et al.* showed a novel mode of STAT1 regulation where phosphorylated SHP2 sequestered non-phosphorylated STAT1 in the cytosol so that STAT1 homodimerization and nuclear translocation to activate gene transcription were inhibited



(Xingxin Wu et al. 2012). We could hypothesize that upon ITIM phosphorylation, CLEC12B recruits and prevents SHP2 phosphorylation so that STAT1 can be phosphorylated and can induce IRF1 transcription, and subsequent CXCL9, CXCL10, and CXCL11 production. Moreover, different studies stated that SHP2 can exert both positive and negative regulatory actions on cytokine induced JAK-STAT activation (Salmond et Alexander 2006) and SHP1 is positively involved in IFN $\gamma$ -induced STAT activation (You et Zhao 1997). Upon IFN $\gamma$  stimulation, SHP1 and/or SHP2 could work as copartners with CLEC12B to activate the STAT1/IRF1 axis and induce the production of chemokines downstream. Even though ITIM-containing CLRs are usually thought to act as negative regulators of immune cell signaling, they are also able to activate some pathways. For example, the ITIM-bearing DCIR expression is required for sustained STAT1 phosphorylation and DCIR enables sustained STAT1-mediated type I IFN signaling in DCs (Troegeler et al. 2017). Another example is CLEC12A activation by MSU released by host dead cells following viral infection that positively activates the TBK1-IRF3 axis of RIG-I-induced IFN-I production through activating SFKs, thereby amplifying an antiviral immune response (K. Li et al. 2019). CLEC12B was named after CLEC12A due to their high similarity in structure, function, and chromosomal location and just like CLEC12B, CLEC12A has been first identified as a myeloid inhibitory CLR which can recruit both SHP1 and SHP2 through its ITIM domain (Marshall et al. 2004). Considering this information, it is not surprising that CLEC12B can in turn act as a positive regulator of immune signaling. Overall, CLEC12B positively regulates IFN $\gamma$  pathway through the activation of the STAT1/IRF1 axis and potentiates it following IFN $\gamma$  stimulation to subsequently increase the production of CXCL9, CXCL10, and CXCL11 upon phosphorylation of its ITIM domain. However, the exact molecular mechanism remains unclear, especially in unstimulated melanocytes, and whether CLEC12B delivers a signal on its own through the ITIM domain or requires a co-receptor will need further investigation.

Interestingly, CLEC12B expression was only increased after 24 hours of IFN $\gamma$  stimulation (**Supplementary Figure S2a**), and *CLEC12B* silencing (**Supplementary Figure S2b**) or overexpression (data not shown) had no effect on the STAT1/IRF1 axis after 30 minutes or 6 hours of IFN $\gamma$  stimulation. Considering that *CLEC12B* overexpression potentiates the effect of IFN $\gamma$  stimulation on the downstream signaling and target genes, these results suggest positive feedback between CLEC12B and IFN $\gamma$ . A possible mechanism would be that following a stimulus, production of IFN $\gamma$  by other cells in the skin (for instance keratinocytes, NKs, ILCs, or

LCs) activates the STAT1/IRF1 axis to induce the production of CXCL9, CXCL10, CXCL11, and IFN $\gamma$  to maintain an activated signaling, and increases the expression of CLEC12B in melanocytes. Increased CLEC12B potentiates the activation of the STAT1/IRF1 axis and subsequent production of CXCL9, CXCL10, and CXCL11 which in turn can attract immune cells such as cytotoxic T cells towards melanocytes. This proposed mechanism is of great interest as IFN $\gamma$  and recruitment of immune cells are key elements involved in numerous pathologies, suggesting that CLEC12B could be part of immune responses involved in different skin diseases.

Our team has identified expression of CLEC12B to be decreased in lesional skin of vitiligo patients compared to healthy and non-lesional skin through a transcriptomic analysis (Regazzetti et al. 2015). We also showed that when a vitiligo skin undergoes an internal or external stress, the basal number of NK and ILC1 cells is increased and they become more sensitive to stress, leading to enhanced production of IFN $\gamma$  (Tulic et al. 2019). Subsequently, IFN $\gamma$  induces the production of CXCL9, CXCL10, and CXCL11 predominantly by keratinocytes and also by melanocytes. These chemokines attract cytotoxic T cells and bind to their receptor CXCR3B found on melanocytes to directly induce their apoptosis, resulting in release of melanocytic antigens. The remaining melanocytes then start presenting these melanocytic antigens to the attracted naïve T cells, that in turn kill the remaining melanocytes, resulting in an anti-melanocytic immunity in the skin (Tulic et al. 2019). Importantly, chronic IFN $\gamma$  exposure induced apoptosis, cell cycle arrest, and senescence in melanocytes, which is associated with a production of IL-6 and HSP70, thus enhancing melanocyte immune-competency (Wang et al. 2014). Since we showed that CLEC12B modulates the production of CXCL9, CXCL10, and CXCL11 in healthy melanocytes and the following chemoattraction of immune cells, we can envisage that silencing *CLEC12B* in melanocytes from vitiligo patients could decrease the chemokines production, thus decreasing the attraction of cytotoxic T cells and subsequent melanocyte death, making CLEC12B a potential target to prevent and to treat vitiligo by acting at the early stages of melanocyte destruction. Considering that the chemoattraction of cytotoxic T cells and their subsequent infiltration within tumor tissue predicts a better prognosis and is essential for response to checkpoint blocking therapy, CLEC12B could be part of the antitumor immunity in skin cancers such as melanoma.

Recent results from our team showed that CLEC12B expression is lower in melanoma patients compared to benign melanocytic lesions or melanocytes, and that this decreased CLEC12B expression is correlated with poor prognosis and lower survival rate (Montaudié et al. 2022). From a mechanistic point of view, the results showed that *CLEC12B* overexpression recruits the phosphatase SHP2 *via* its ITIM domain, inhibits the activation of STAT3 and increases the activity of the cell cycle inhibitors p53, p21 and p27, thus reducing the proliferation of melanoma cells *in vitro* as well as tumor growth *in vivo* (Montaudié et al. 2022). Moreover, melanoma patients with high expression of the *IFNG* gene have a significantly longer overall survival and IFN $\gamma$  upregulates the expression of Programmed Cell Death 1(PD-1) Ligand 1 (PD-L1) (Karachaliou et al. 2018). p53 plays an important role in IFN $\gamma$ -induced PD-L1 expression in melanoma cells and high PD-L1 expression is associated with an enhanced response to anti-PD-1 immunotherapy (Thiem et al. 2019). IFN $\gamma$  is critical for cancer related immunity by coordinating innate and adaptative antitumor responses whereas STAT3 inhibits the expression and activation of STAT1 and IRFs and further reduces antigen presentation through a decreased expression of HLA molecules (Tripathi et al. 2016). We could hypothesize that overexpressing *CLEC12B* modulates an anti-melanoma immunity through the inhibition of STAT3 and the upregulation of p53 and IFN $\gamma$  pathway, inducing an increased production of innate chemokines CXCL9, CXCL10, and CXCL11 and subsequent recruitment of infiltrating T cells which are critical for antitumor immunity. Thus, CLEC12B could be a potential candidate to treat melanoma by initiating innate and adaptative immunity and by potentiating response to anti-PD-1 immunotherapy, however this hypothesis remains to be tested. Melanoma and vitiligo are both skin diseases with opposite molecular mechanisms as melanoma arises from an uncontrolled proliferation of melanocytes whereas vitiligo is characterized by a loss of melanocytes. Taken together, these results suggest that CLEC12B has a Yin-Yang immune function as it should have a negative impact in vitiligo patients whereas it has a positive impact in melanoma patients (Montaudié et al. 2022), rendering this CLR a very attractive target to treat such pathologies.

Another study from our team, in which I participated as a second co-author, showed that CLEC12B expression depends on constitutive and facultative pigmentation and that CLEC12B modulates melanogenesis (Sormani et al. 2022). These studies were confirmed in three-dimensional reconstructed human skin and human skin biopsies and the mechanism involved

recruitment of SHP1 and SHP2 and the proteasomal degradation of CREB. It has been shown that IFN $\gamma$  indirectly inhibits melanogenesis through the production of anti-melanogenic IL-6 (Choi et al. 2013) and that IFN $\gamma$  stimulation dampens melanogenesis by arresting melanosome maturation through IRF1 in a MITF-independent manner, thus inducing cellular hypopigmentation (Natarajan et al. 2014). These results suggest that, upon IFN $\gamma$  stimulation, the increased activation of the IFN $\gamma$  pathway and its target genes is likely linked to the hypopigmented phenotype observed when *CLEC12B* is overexpressed in melanocytes. Moreover, the IFN $\gamma$  pathway plays a crucial role in pathogenesis of inflammatory skin disorders such as psoriasis, hidradenitis suppurativa, or acne. Elevated IFN $\gamma$  levels are correlated with disease severity in psoriasis and hidradenitis suppurativa and IFN $\gamma$ -induced CXCL9, CXCL10, and CXCL11 are upregulated in psoriatic and acne lesions (Belpaire, van Geel, et Speeckaert 2022). Together, these results suggest that *CLEC12B* could be an interesting target in number of pigmentary or inflammatory skin disorders due to its role in the IFN $\gamma$  signaling pathway modulation.

CLRs can recognize PAMPs to induce immune responses and fight against pathogens (Mayer, Raulf, et Lepenies 2017) and our results showed that *CLEC12B* interacted primarily with *Staphylococcus aureus* but also with *Escherichia Coli* (**Figure 5a**), both commensal bacteria which can be found on the skin and can become pathogenic under certain conditions. Surprisingly, bacteria showed an individual variation in *CLEC12B* binding or even no binding at all. We could hypothesize that *S. aureus* and *E. coli* modulate their surface properties to affect cell wall components, thus interfering with *CLEC12B* recognition, like *Campylobacter jejuni* does (Mayer et al. 2018). A recent study showed that *CLEC12B* from *Oreochromis niloticus* (On*CLEC12B*) bound to *S. aureus* and *E. coli*, among other Gram-positive and Gram-negative bacteria, supporting our own data as On*CLEC12B* contains a conserved CTLD sharing high identities with human *CLEC12B* (Z. Zhang et al. 2022). However, *CLEC12B* did not interact with *Bifidobacterium* strains and *Streptococcus epidermidis*, which are commensal bacteria that help maintaining skin homeostasis, nor with *Listeria monocytogenes*, which is a pathogenic bacterium mainly found in the gut that causes the listeriosis (**Figure 5a**). These data suggest that *CLEC12B* preferentially interacts with potential pathogens that can be found on the skin such as *S. aureus*, although this will need further investigation as we only tested one bacterial strain, *L. monocytogenes*, that is not found at all on the skin. It is important to note that ligand

binding may also be pH sensitive so it might be worth trying different pHs in these CLR-Fc binding experiments. Another limit to this kind of experiment is that we only used whole heat-killed bacteria allowing CLEC12B to bind only external ligands whereas the use of bacterial lysates could have rendered accessible internal components such as glycolipids.

After melanocyte stimulation, we showed a significant increased production of CXCL9 upon *S. aureus* stimulation and a significant increased production of CXCL10 upon *E. coli* stimulation, whereas the production of IFN $\gamma$  and CXCL11 remained insensitive to bacterial stimulation (**Figure 5b**). *E. coli* predominantly stimulates TLR2 and TLR4 and was shown to induce the production of CXCL10 (Gilbert et al. 2013), which could explain the significant induction of CXCL10 production in *E. coli* stimulated melanocytes. Staphylococcal ligands are predominantly recognized by TLR2 and pathogenicity of *S. aureus* strains also induces type I IFN signaling with the binding of IFN $\alpha/\beta$  to their receptor, thus inducing phosphorylation of STAT1 homodimers or STAT1-STAT2-IRF9 heterotrimers, and subsequent activation of ISGs and inflammatory responses (Askarian et al. 2018). Here we showed an increased expression of pSTAT1, STAT1, and IRF1 in response to both *S. aureus* and *E. coli* (**Figure 5c**). Considering that IFN $\gamma$  production remains insensitive to *S. aureus* and *E. coli* stimulation (**Figure 5b**), that CXCL9 production can be induced independently of IFN $\gamma$  by IL-27 or LPS for instance (Ding et al. 2016), and that STAT1 and IRF1 can be part of other signaling pathways than that of IFN $\gamma$ , including TLRs and type I IFN (Feng et al. 2021), we can speculate that anti-*S. aureus* and -*E. coli* innate immune responses are IFN $\gamma$ -independent in melanocytes. Moreover, we showed *CLEC12B* silencing to markedly decrease CXCL9 production primarily in response to *S. aureus* but also to *E. coli*, which was in accordance with our IF data, however no significant effect was assessed for CXCL10, IFN $\gamma$ , or CXCL11 (**Figure 5b**). Regarding the downstream signaling pathway, pSTAT1, STAT1, and IRF1 expression was significantly decreased when *CLEC12B* was silenced in response to both *S. aureus* and *E. coli* (**Figure 5c**). Together, these data show that CLEC12B senses exogenous ligands present on *S. aureus* and *E. coli* and modulates an anti-pathogen innate immune response through the STAT1/IRF1/CXCL9 axis. Immune function and the expression of STAT1/IRF1 proteins in melanocytes overexpressing *CLEC12B* or *CLEC12B* mutant remain to be tested to determine whether CLEC12B can have a synergistic effect on chemokine production upon *S. aureus* and/or *E. coli* stimulation. It would be interesting to determine whether CLEC12B can also modulate an adaptative immune response against these two

bacteria by monitoring real-time detection of melanocyte death in presence of cytotoxic T cells before and after *S. aureus* and *E. coli* stimulation. Before these studies can be contemplated, the exogenous ligands that are sensed by CLEC12B on *S. aureus* and *E. coli* must be identified. Interestingly, *S. aureus* is mainly found in the moist skin of the anterior nares and in parts of the skin where the skin barrier function is disturbed by micro-lesions or in atopic dermatitis (AD) for instance. AD is a chronically relapsing inflammatory disease associated with skin barrier defects and skin dysbiosis. The latter is correlated with a decreased bacterial diversity and an increased *S. aureus* colonization, inducing the production of staphylococcal virulence factors and a decreased expression of AMPs, thus altering the epidermal barrier (Braun et al. 2022). *S. aureus* density on lesional and nonlesional AD skin is correlated with disease severity as well as increased levels of proinflammatory cytokines (Joshi et al. 2023). Staphylococcal virulence factors induce production of IL-1 $\alpha$ , TNF and IL-6 by keratinocytes and decrease TLR2 responsiveness in AD skin (Bitschar et al. 2017). Considering these data, overexpressing *CLEC12B* could help increase the STAT1/IRF1/CXCL9 axis, thus increasing the innate immune response against *S. aureus*, making CLEC12B an interesting target to treat AD by decreasing disease severity and restoring the epidermal barrier.

CLRs have been shown to recognize glycan structures expressed by pathogens and host cells to induce immune responses (Vliet, García-Vallejo, et Kooyk 2008) so we intended to identify CLEC12B ligands using glycan array screening in order to get a deeper understanding of how CLEC12B can influence ongoing immune responses. This research could lead to new strategies for specific targeting of APCs in which CLEC12B ligands are used as adjuvants to elicit downstream signaling and help treat different skin diseases. Results from our collaboration with the Consortium for Functional Glycomics (CFG, Harvard University, Boston, USA) showed that CLEC12B didn't recognize any mammalian nor microbial glycan structure present on the two tested arrays (**Figure 6**). CLRs often display low-affinity binding properties to their ligands. Although a few peaks seemed to appear above the background in the microbial screen: Glycans No 198 (*Shigella boydii* type 9), 308 (Laminarin), and 313 (1-2 Mannan *Acetobacter methanolicus* MB135), the binding remained very low and were below the cut-off value (Average RFU < 1000) (**Figure 6d**). A very recent study has reported CLEC12B to recognize laminarin, a polysaccharide containing  $\beta$ -(1,3) glucans, in *Pneumocystis murina* cell wall homogenates (CWH) and in a purified *Pneumocystis carinii* cell wall fraction (CWF) (Kottom et

al. 2023), which are both fungi that are not found on the microbial screen. Laminarin could be a potential ligand candidate for CLEC12B, but a secondary screening should be performed to confirm these results by measuring the binding affinity through biophysical methods such as surface plasmon resonance, as described in the paper of Bochner *et al.* (Bochner et al. 2005). Even though these two glycan arrays represent many known important epitopes (585 glycan on the mammalian screen and 313 glycans on the microbial screen), they are expected to be only a fraction of different existing glycans as they do not cover glycolipids nor proteoglycans. Moreover, the microbial screen covers a number of very common genus and species, but it represents only Gram-negative bacteria strains so *S. aureus* strains, which are Gram-positive bacteria, or fungi species, such as *P. murina* or *P. carinii*, were not tested on this array. Although these two screens probably only scratch the surface of the whole glycan universe, their coverage was the best available to our knowledge at the time of this study.

CLRs can sense SAMPs on healthy cells to maintain the baseline non-activated state of innate immune cells and dampen their reactivity following an immune response (Varki 2011). Our results showed that CLEC12B strongly interacted with melanocytes and fibroblasts, but not with keratinocytes, and interacted with both pro-inflammatory macrophages (M1) and anti-inflammatory macrophages (M2) (**Figure 7a**). Results from our team showed that *CLEC12B* is significantly more expressed in human melanocytes than in keratinocytes and fibroblasts, and *CLEC12B* expression is almost zero in fibroblasts (Sormani et al. 2022), suggesting that CLEC12B could bind ligands in *cis* on melanocytes where it is predominantly expressed, and in *trans* to communicate with neighboring cells such as fibroblasts. CLEC12B was first identified as an inhibitory CLR expressed on *in vitro* differentiated human macrophages that can recruit SHP1 and SHP2 to counteract NKG2D-mediated signaling (Hoffmann et al. 2007) and macrophages are known professional APCs, so it could be interesting to determine whether CLEC12B can modulate the production of innate chemokines and subsequent immune cells chemotaxis in macrophages.

CLRs can bind a wide variety of ligands, other than glycans, such as proteins, lipids, or inorganic compounds on host cells (Zelensky et al., 2005). Here we showed that trypsin (a serine protease) and papain (a cysteine protease) pre-treatments both completely prevented the interaction between CLEC12B and melanocytes (**Figure 7b**), indicating that CLEC12B recognizes protein structures present on melanocytes. Results from mass spectrometry analysis in

collaboration with ProteoToul facility (Toulouse, France) allowed us to identify 6 potential CLEC12B ligands (classified in descending order of their peptide enrichment) (**Figure 7c**):

- Insulin Like Growth Factor 2 Receptor (IGF2R)
- Lipoprotein Lipase (LPL)
- Cytokine Receptor Like Factor 1 (CRLF1)
- S100 Calcium Binding Protein A4 (S100A4)
- Out At First Homolog (OAF)
- HscB Mitochondrial Iron-Sulfur Cluster Cochaperone (HSCB)

After silencing the expression of the above 6 potential targets, we showed no effect on the interaction between CLEC12B-Fc and melanocytes (**Figure 8b**), suggesting that none of these proteins are likely to be CLEC12B ligands. However, our methods have been focusing on CLR-ligand screening and we only tried fluorescence microscopy to confirm or invalidate the potential candidates from mass spectrometry analysis. It would be worth trying another “qualitative method” such as flow cytometry as this technique allows semi-quantitative comparisons, direct measurement of CLR/ligand interactions and it brings information on a large number of cells for statistical analysis. Indeed, it is hard to have quantitative comparisons between samples or to detect small variations in cellular staining when the target gene is silenced using fluorescence microscopy. Most importantly, we should measure the affinity between CLEC12B and the 6 potential ligand candidates using more “quantitative methods” such as Förster resonance energy transfer (FRET), Quartz crystal microbalance (QCM), or even Surface plasmon resonance (SPR). All of these “qualitative” and “quantitative” methods are well reviewed by Fischer *et al.* (Fischer et al. 2022). We must also consider that affinity and avidity of the ligand might affect the CLR binding, as well as the size of the ligand or whether the ligand is in a soluble monomeric state or a particulate oligomeric state. Although there is a chance that using quantitative methods we might find a putative ligand among these candidates, it is also possible that none of these proteins are CLEC12B ligands. In fact, CLEC12B could bind exogenous ligands, found on *S. aureus* and *E. coli* for instance, but also endogenous ligands found on other cellular types, such as fibroblasts and macrophages that can be in contact with melanocytes. We should try the same experiments described in **Figure 7b-c** in fibroblasts and macrophages to first verify whether CLEC12B recognizes a protein structure on these cells too, then try to identify the potential ligands in these cells, and finally to compare



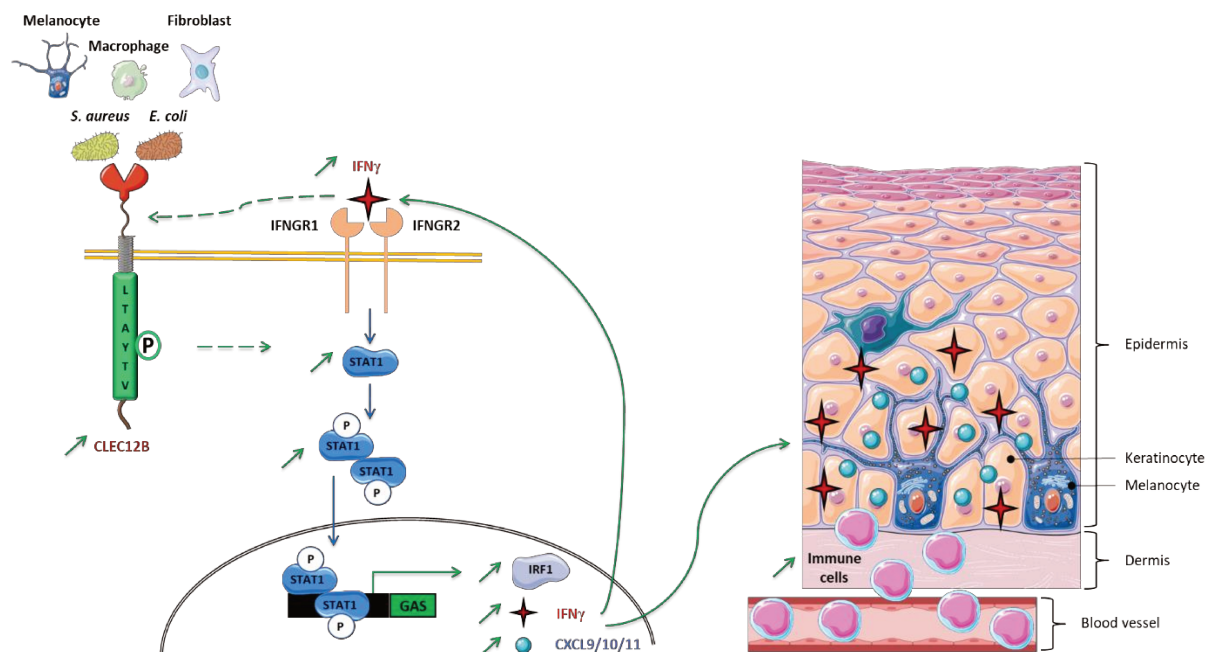
them with the ones obtained in melanocytes. In the same context, we should try to identify the exogenous ligands recognized on *S. aureus* and *E. coli* by mass spectrometry analysis after verifying whether CLEC12B also recognizes protein structures on these bacteria.

The candidates from mass spectrometry analysis could then only be CLEC12B signaling partners that interact with CLEC12B to mediate downstream responses. To test this hypothesis, we showed that silencing *IGF2R* and *CRLF1* markedly increase pSTAT1, STAT1, and IRF1 expression, without marked changes in CLEC12B expression, as shown on western blot analysis (**Figure 8c-d**). *IGF2R* and *CRLF1* silencing displayed the same profile as *CLEC12B* silencing on the STAT1/IRF1 axis (**Figure 4a**), suggesting that IGF2R and CRLF1 may collaborate with CLEC12B as coreceptors or interacting partners to shape different immune responses in response to various stimuli, however this remains to be formally tested in future studies. We decided to focus on IGF2R and CRLF1 as they were the two most relevant targets, biologically speaking, among the 6 candidates. IGF2R is a type I transmembrane glycoprotein expressed at the cell surface that binds many different M6P-containing ligands and that is functionally related to the CD-MPR. Together they form the p-type lectin family and are involved in the trafficking of lysosomal enzymes between the *trans*-Golgi network, endosomes, and lysosomes (J. Brown, Jones, et Forbes 2009). CRLF1 is a soluble type I cytokine receptor that is up-regulated by pro-inflammatory cytokines such as TNF $\alpha$ , IL-6, and IFN $\gamma$ , suggesting that CRLF1 may be involved in regulating the immune system during an inflammatory response. When p28 secretion is chaperoned by CRLF1, the resulting complex can activate STAT1 phosphorylation and possibly SHP2/Ras/MAPK pathway (Crisponi, Buers, et Rutsch 2022). Taken together, these results clearly illustrate the complexity and plasticity of ligands and signaling motifs contribution into CLRs functional outcomes. Despite our best efforts to identify CLEC12B ligands, we were unable to achieve our goal, other than by demonstrating that CLEC12B recognizes motifs on melanocytes, fibroblasts, and macrophages, as well as on *S. aureus* and *E. coli*.

We used the fusion protein mCLEC12B-hFc for the ligand identification studies, whether for the glycan arrays, for the immunofluorescent staining, or for the mass spectrometry analysis. There was no CLEC12B fusion protein commercially available at the time of this study, so we started a collaboration with Dr. Bernd Lepenies (Max Planck Institute of Colloids and Interfaces, Potsdam, Germany) who kindly provided us with the mCLEC12B-hFc he generated following

its own CLR-Fc studies. This fusion protein is composed of the extracellular domain (CTLD) of the murine homolog of CLEC12B (mCLEC12B) fused to the human IgG1-Fc (hFc) (see Methods section). Homo- or heterophilic dimerization is an important determinant of the ligand binding potential of CLEC12B. Many other group V receptors dimerize through cysteines in the stalk region and Hoffmann *et al.* stated that CLEC12B formed disulfide-linked homodimers in transfected cells (Hoffmann *et al.* 2007). However, CLR-Fc fusion proteins allow two CTLDs to be simultaneously presented as a dimeric complex for ligand binding, which is not always sufficient to detect low-affinity binding without further precomplexing (Willment 2022). This could be another explanation why the CLEC12B-Fc fusion protein didn't exhibit any strong interaction with none of the glycans present on the mammalian nor microbial glycan arrays (**Figure 6**). Importantly, the fusion protein was composed of the murine CLEC12B homolog which has 72% identities with the human CLEC12B homolog, and they both share identical features such as ITIM sequence VTYATL, conserved cysteines within the transmembrane and the extracellular regions, and ligand binding sites. However, mCLEC12B doesn't contain the three N-glycosylation sites present in hCLEC12B extracellular domain, which could lead to differences in ligand recognition between the two homologs, as previously demonstrated for Ly-49 and LOX-1 (Mason *et al.* 2003; Kataoka *et al.* 2000). Briefly, Ly-49 is a family of receptors whose binding to class I MHC is regulated by receptor glycosylation as well as LOX-1 whose cell-surface expression and ligand binding depends on N-glycosylation. The glycosylation of CLRs can increase the amount of high molecular weight complexes which can modify the function and accuracy of the CLR-binding properties (Willment 2022). For instance, human, but not mouse, langerin senses wall teichoic acid from *S. aureus*, highlighting the possible differences between mouse and human homologs binding capacity (van Dalen *et al.* 2019). In order to identify CLEC12B ligands, these experiments should be redone with the human homolog of CLEC12B fused to the IgG1-Fc to exclude the potential biological biases induced by the differences between mouse and human homologs and to confirm our own results.

# Conclusion



Taken together, our results shed light on CLEC12B as being an important player in skin immunity. Upon IFN $\gamma$  stimulation, STAT1 undergoes phosphorylation and forms homodimers that translocate into the nucleus to bind GAS, thus activating the transcription of IRF1. IRF1 in turn acts as a transcription factor binding IRF1-binding site-containing promoters of target genes, including CXCL9, CXCL10, CXCL11, but also IFN $\gamma$  to maintain the activation of the pathway. The enhanced production of IFN $\gamma$  increases CLEC12B expression, which in turns potentiates the activation of the IFN $\gamma$  pathway and the production of immune chemokines through the phosphorylation of its ITIM domain. CLEC12B recruits the phosphatases SHP1 and SHP2 on its ITIM domain and decreases their phosphorylation, however the exact mechanism how CLEC12B interaction with SHP1 and SHP2 ends up potentiating the pathway remains unclear. In the end, CLEC12B-mediated increased production of CXCL9, CXCL10, and CXCL11 enhances chemotaxis of immune cells. CLEC12B might sense exogenous ligands on *S. aureus* and *E. coli* as well as endogenous ligands on melanocytes, fibroblasts, and pro- and anti-inflammatory macrophages, even though the exact ligands still need to be identified. Our results also show that CLEC12B modulates an innate immune response against *S. aureus* and *E. coli* through the STAT1/IRF1/CXCL9 axis. These results give light on the key role of CLEC12B

in innate as well as in adaptative immune responses in the skin, making this receptor an interesting therapeutic target for the treatment of pathogenic infections, inflammatory and pigmentary skin disorders, as well as cancer; all which may be able to be directly immuneregulated by CLEC12B on melanocytes.

# Methods

## *Cell culture*

Normal human melanocytes, normal human keratinocytes, and normal human fibroblasts were obtained from the foreskin of healthy young children undergoing circumcision at the Department of Pediatric Surgery, Lenval Hospital, Nice, France. Tissue samples were washed 2 times in ethanol 70% and 2 times in phosphate buffered saline (PBS) containing 1% Antibiotic/Antimycotic (Gibco). After removal of the subcutaneous tissue, samples were cut into 2 mm<sup>2</sup> pieces and incubated overnight (ON) at 4°C with dispase enzyme (Life technologies) containing 1% Antibiotic/Antimycotic. The next day, the epidermis was separated from the dermis using forceps and tissue pieces of dermis were transferred to the bottom of a culture dish and cultured with 10 mL of DMEM/F-12 media (Gibco) supplemented with 10% fetal calf serum (FCS, HyClone) to obtain normal human fibroblasts. The epidermis was incubated within a trypsin/PBS solution (ratio 1:1) containing 1% Antibiotic/Antimycotic for 20 minutes at 37°C. FCS was added to stop the trypsin and the suspension containing dispersed cells was filtered by cell strainer (70µm, Falcon) before being separated in two. After a 5 minute centrifugation at 200 g, the first cell pellet for normal human melanocytes was resuspended in MCDB 153 medium (Sigma Aldrich) supplemented with 1.18 g/L sodium bicarbonate (Invitrogen), 0.1 mM calcium chloride, 1% Antibiotic/Antimycotic, 2% FCS, 5 µg/mL insulin (Sigma Aldrich), 0.5 µg/mL hydrocortisone (Sigma Aldrich), 16 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich), 1 ng/mL fibroblast growth factor (FGF, Promega), 15 µg/mL bovine pituitary extract (Gibco), 10 µM forskolin (Sigma Aldrich). The melanocytes were selected with 20 µg/mL geneticin (Invitrogen) for 2 weeks or until a pure melanocyte population was obtained. Once selected, the NHM were cultured in Cascade Biologics 254 medium (ThermoFisher Scientific) supplemented with Human Melanocyte Growth Supplement (HMGS, Gibco). The second cell pellet for normal human keratinocytes was resuspended in keratinocyte serum-free growth medium supplemented with 30 µg/mL bovine pituitary extract, 10 µg/mL transferrin, 5 µg/mL insulin, 0.3 µg/mL hydrocortisone, 0.39 µg/mL epinephrine, 0.5 mM CaCl<sub>2</sub> and 0.125 ng/mL epidermal growth factor (all PromoCell). Cells were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere.

Three human immortalized melanocytes cell lines were used in this study: Hermes 2A and 2B that are immortalized from Hmel-p16-2 and are p16 null melanocytes immortalized with hTERT, and Hermes 3A that is immortalized from Nohm-1 normal diploid human melanocyte strain and has hTERT and CDK4 expression vectors. The Hermes cell lines were cultured in RPMI 1640 (Gibco) supplemented with 100 units/mL Penicillin (Gibco), 100 µg/mL Streptomycin (Gibco), 10% FCS (HyClone), 200 nM PMA (Sigma), 200 pM cholera toxin (Sigma), 10nM endothelin-1 (Bachem), 10 ng/mL human stem cell factor (Miltenyi) at 37°C in a 5% CO<sub>2</sub> atmosphere.

Polarized M1 and M2b macrophages, as well as bacterial strains *S. aureus*, *E. coli* and *L. monocytogenes* were kindly provided by Dr. Laurent Boyer's team. *Bifidobacterium* strains and *S. epidermidis* were kindly provided by Dr. Hanene Bziouche.

#### *Lentivirus production and transduction*

For *CLEC12B* overexpression, we used CLEC12B Lentiviral Vector pLenti-GIII-CMV-GFP-2A-Puro (Applied Biological Materials) custom (use of CDS of CLEC12B accession number NM\_001129998 for the transcript variant 1 and NM\_205852.3 for the transcript variant 2) and the control plasmid Blank Vector pLenti-CMV-GFP-2A-Puro-Blank (Applied Biological Materials). For *CLEC12B* silencing, we used the MISSION® shRNA plasmid DNA pLKO.1-puro-CMV-TurboGFP™+shRNA (target sequence CAAGAACTCCACCCTAGTGAA) (TRCN0000164269, Sigma Aldrich) and the MISSION® Non-target shRNA Control Vector pLKO.1-puro-CMV-TurboGFP™ (Sigma Aldrich). We used the plasmid psPAX2 (Addgene) as a lentiviral packaging plasmid and the plasmid pCMV-VSV-G (Addgene) as an envelope protein to produce lentiviral particles. We used the jetPRIME™ transfection reagent kit (Ozyme) according to the kit manufacturer's instructions to transfect the 293FT cell line (Invitrogen) for generating lentivirus at high titer using the ViraPower™ lentiviral expression system. Viral supernatant was harvested 24 hours after transfection and filtered through a 0.45 µm cellulose acetate filter. Melanocytes were seeded on six-well plates at a density of 1.5 x 10<sup>5</sup> cells per well and were reverse infected before incubation with the virus for 72 hours. The infected cells were then selected for stable expression using puromycin at 1 µg/mL every two to three days.

### *Site-directed mutagenesis of CLEC12B*

The CLEC12B Y→F mutation was generated by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies), according to the kit manufacturer's instructions. The following primers were used: 5'-GTCTGAAGAAGTGACCTTCGCGACACTCACATTTTC-3' (CLEC12B Y→F forward) and 5'-GAAATGTGAGTGTGCGGAAGGTCACTTCTTCAGAC-3' (CLEC12B Y→F reverse). Mutations were verified by DNA sequencing (Eurofins Genomics).

### *RT-qPCR*

Melanocytes were washed with PBS and RNA were extracted and purified using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The quality and quantity of RNA were determined by spectrophotometric assay at 260nm on the NanoDrop (ThermoFisher Scientific). The reverse transcription reaction was carried out using the Reverse Transcription System kit (Promega) with 1 µg of RNA according to the manufacturer's protocol. The cDNA obtained were amplified by PCR with primers specific to the genes of interest (**Table 1**) using Fast SYBR Green Master Mix (ThermoFisher Scientific) and the samples were run on the StepOne Real-Time PCR System (ThermoFisher Scientific). Analysis by relative quantification (RQ) was carried out using the StepOne Software and the housekeeping gene SB34.

### *Western Blot analysis*

After washing with PBS, melanocytes were lysed on ice using RIPA lysis and extraction buffer (ThermoFisher Scientific) supplemented with Mini cOmplete™ EDTA-Free Protease Inhibitor Cocktail (Roche) and phosphatase inhibitors PhosSTOP (Roche). The supernatant was collected, centrifuged at 13,000 x g for 10 min at 4°C, and assayed for protein concentration using the BCA kit (ThermoFisher Scientific). 20-30 µg of proteins were added to 3% SDS recovery buffer (Euromedex), bromophenol blue and β-mercaptoethanol (Sigma Aldrich) then denatured for 5 min at 95°C. The proteins were then separated by electrophoresis on SDS-page polyacrylamide gels, then transferred to a PVDF membrane (Millipore) before saturation with blocking buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Gelatin, 3% BSA,

0.1% Tween-20) for 1 hour at room temperature (RT). The membranes were then incubated overnight at 4°C with primary antibodies (**Table 2**) diluted in blocking buffer. After three washes in wash buffer (10mM Tris pH 7.6, 150mM NaCl, 1% Triton X-100), the membranes were incubated with secondary antibodies coupled to Horseradish Peroxidase (HRP) (see Appendix 2) for 1 hour at RT. Detection was carried out using enhanced chemiluminescence detection (ECL) (Dutscher) and the chemiluminescent image analyzer LAS-3000 (Fujifilm). Relative expression of target proteins was reported to  $\beta$ -actin expression and quantified using ImageJ software.

### *ELISA*

Melanocytes were seeded on 12-well plates at a density of  $3.5 \times 10^5$  cells per well in 1 mL of medium. 48 hours later, cells were stimulated with IFN $\gamma$  (50 ng/mL) or bacteria *S. aureus* and *E. coli* (MOI 10) in 0.5 mL of medium for 24 hours. IFN $\gamma$ , CXCL9, CXCL10, and CXCL11 production was measured in collected supernatants using commercially available ELISA kits (Peprotech) according to the kit manufacturer's instructions.

### *FACS analysis*

After 24 hours of IFN $\gamma$  stimulation (50 ng/mL), melanocytes were detached using StemPro Accutase (Gibco) and incubated with FcR blocking reagent (Miltenyi) for 10 minutes at 4°C. After washing with PBS, cells were resuspended in MACSQuant Running Buffer (Miltenyi) and seeded in a conical bottom 96-well plate at a density of  $5 \times 10^4$  cells/well. Then cells were incubated with the coupled primary antibodies or control isotypes (**Table 2**) for 15 minutes at 4°C in the dark. After washing, cells were centrifuged at 300 x g for 10 minutes then resuspended in 170  $\mu$ L of MACSQuant Running Buffer before analysis by flow cytometry (MACSQuant10, Miltenyi).



### *Chemotaxis assay*

The chemotaxis of PBMCs was assessed using the CytoSelect™ 96-Well Cell Migration Assay (5 µm, Fluorometric Format) according to the kit manufacturer's instructions (Cell Biolabs). First, melanocytes were seeded on twelve-well plates at a density of  $3.5 \times 10^5$  cells per well in 1 mL of medium and 48 hours later, cells were stimulated with IFN $\gamma$  (50 ng/mL) in 0.5 mL of medium for 24 hours. 150 µL of the cultured supernatants containing the chemoattractants (i.e., CXCL9, CXCL10, CXCL11) were then placed into the feeder wells before the membrane chamber was placed back into the feeder tray.  $1 \times 10^5$  PBMCs from healthy donors resuspended into 100 µL of RPMI 1640 serum-free medium were placed into the wells on top of the membrane chamber. The plate was incubated for 24 hours at 37°C in an atmosphere of 5% CO<sub>2</sub>. Cells that did not migrate were removed from the top of the membrane chamber, and those in the membrane were recovered using 150 µL of the cell detachment solution. 75 µL of the eluate were combined with 75 µL of cells that migrated in the chemoattractant solution (i.e., melanocyte supernatant). The cells were then lysed with the lysis buffer, and the total number of transmigrated cells was quantified using the CyQuant® GR Dye. 150 µL of the mixture was transferred to a 96-well plate suitable for fluorescence measurement and results were read using a fluorescence plate reader at 480 nm/520 nm.

### *Fusion protein*

CLEC12B-Fc fusion protein and the Fc alone as a negative control were kindly provided by Dr. Bernd Lepenies (Max Planck Institute of Colloids and Interfaces, Potsdam, Germany) on a collaborative basis. The generation and production of the CLEC12B-Fc fusion protein was reported by Dr. Lepenies' group (Mayer et al. 2018). Briefly, the cDNA encoding for the mouse CLEC12B CTLD was cloned into an expression vector and fused to the cDNA fragment encoding for human IgG1-Fc. CHO-S cells were transiently transfected with the expression vector construct and soluble CLEC12B-Fc fusion proteins were secreted. The cell supernatant was purified using protein G columns and the presence of CLEC12B-Fc fusion proteins were detected by Western blot.

### *Immunofluorescence*

Bacteria were fixed with 2% paraformaldehyde (PFA) onto cover slides for 10 minutes at RT and cells were grown onto cover slides then fixed with 2% PFA for 10 minutes at RT. After three PBS washes, they were incubated with 500 ng of Fc protein alone (negative control) or CLEC12B-Fc fusion protein diluted in lectin binding buffer (50 mM HEPES, 5 mM MgCl<sub>2</sub> and 5 mM CaCl<sub>2</sub>) ON at 4°C. After three lectin-binding buffer washes, they were incubated with the secondary antibody (**Table 2**) diluted in lectin binding buffer for 2 hours at 4°C in the dark. After PBS washing, DNA was stained with Hoechst solution (Life Technologies) for 5 min at RT. The cover slides were mounted onto Menzel-Glaser Superfrost Plus microscopic slides (ThermoFischer Scientific) with ProLong™ Diamond Antifade Mountant (Invitrogen) and visualized using Nikon confocal A1R microscope or Spinning disk W1 microscope.

### *Glycan Array*

The affinity of the CLEC12B-Fc fusion protein for carbohydrate structures, along with the Fc alone and the secondary antibody (**Table 2**) as negative controls, was tested using the mammalian glycan array of 585 glycans (version 5.1) or the microbial glycan array of 313 glycans (version 1) developed by the Consortium for Functional Glycomics (CFG, Harvard, USA). Data were collected and analyzed by the CFG Core H staff and results can be viewed in full on the Harvard Dataverse website under the following link <https://doi.org/10.7910/DVN/TICFQW>.

### *Immunoprecipitation*

For this experiment, two melanocytes cell lines were used (Hermes 2A and 3A) as large quantity of cellular material was needed which was not attainable with primary cells. After washing with PBS, melanocytes were lysed on ice using RIPA lysis and extraction buffer (ThermoFisher Scientific) supplemented with Mini cOmplete™ EDTA-Free Protease Inhibitor Cocktail (Roche) and phosphatase inhibitors PhosSTOP (Roche). The supernatant was collected, centrifuged at 13,000 x g for 10 min at 4°C, and assayed for protein concentration using the BCA kit (ThermoFisher Scientific). 1 mg of protein lysate was incubated with

Dynabeads™ Protein G (Invitrogen) for 1h30 at 4°C on a rotating shaker for a pre-clearing phase. After removing the beads, protein lysate was incubated with 2 µg of CLEC12B-Fc or Fc alone ON at 4°C on a rotating shaker. Dynabeads™ Protein G were then added to the complex antigen/fusion protein for 1h30 at 4°C on a rotating shaker. After three washes in RIPA buffer, the samples were eluted in 5% SDS, heated for 10 minutes à 70°C, then the beads were removed. The eluted antigen/CLEC12B-Fc or antigen/Fc complexes were sent in quadruplicates for each melanocyte cell line to the ProteoToul facility (Toulouse, France). Samples were analyzed using Nanoscale liquid chromatography coupled to tandem mass spectrometry (NanoLC-MS/MS) and a label free quantification was performed with PROLINE 2.0 software by the ProteoToul facility staff.

#### *siRNA transfection*

Melanocytes were plated in 6-well plates at a density of  $2.5 \times 10^5$  cells per well and reverse transfected with 50 nM siCT (Invitrogen) or siIGF2R, siCRLF1, siHSCB, siS100A4, siOAF, siLPL (all from Horizon Discovery) for 72 hours using jetPRIME™ transfection reagent kit (Ozyme) according to the manufacturer's instructions.

#### *Statistical analyses*

Statistical analyses were performed with Graphpad Prism® 9 software. Differences between groups were tested using nonparametric Wilcoxon matched-pairs signed-rank test for paired differences or Mann-Whitney test for unpaired differences and were considered significant when  $P \leq 0.05$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ .

**Table 1: Primer sequences.**

Target gene	Forward	Reverse
SB34	TGCTCAGTACCCATTCTATCAT	AAGGTAATCCGTCTCCACAGA
CLEC12B Isoform 1	AGGAATTGCCTGTCCATTTTCAT	AGGAATTGCCTGTCCATTTTCAT
CLEC12B Isoform 2	TGGGGATGATGTTTTTGCAG	TCCATGGAAAGCTTGTGTTT
IGF2R	CTGCCGCTATGAAATTGAGTGG	CGCCGCTCAGAGAACAAGTT
CRLF1	CTCTCCCGTGTACTCAACGC	GGGCAGGCCAACATAGAGG
S100A4	GATGAGCAACTTGGACAGCAA	CTGGGCTGCTTATCTGGGAAG
HSCB	AGAGAAGCATTTCGACCCTGGT	AGGAATTGCCTGTCCATTTTCAT
OAF	CAGGAGGATGTCCGGTTCTG	CTCTGGGGCACGTAGAAGTC
LPL	AGGAATTGCCTGTCCATTTTCAT	AGGAATTGCCTGTCCATTTTCAT
TLR2	TTGTGACCGCAATGGTATCTG	TGTTGGACAGGTCAAGGCTTT
TLR4	CCCGACAACCTCCCTTCT	TGCCCATCTTCAATTGTCTG
TLR9	CGTGCAGCCGGAGATGTT	CGTGAATGAGTGCTCGTGGTA
MYD88	GGGCATCACCACACTTGATG	TGCACAACTGGATGTCGCT
Src	AGACGGGAGTCAGAGCGGTTA	GGCGTTGTCGAAGTCAGACA
Syk	CAGCGGGTGAATAATCTCAAG	GCTCATACGGATTGAATGACACA
IFNGR1	TCGATTATGATCCCGAACTACCT	GGAATCGCTAACTGGCACTGA
IFNGR2	TCAGTTCTCCTCTCCCTTGA	TGCTTCTGAAAGGGCCTTGA
CXCR3B	TGCCAGGCCTTTACACAGC	TCGGCGTCATTTAGCACTTG
SHP1	GGAGGCCACGGTCAATGAC	GCCAGTATGGGACGCATTTG
SHP2	GAATATGGCGTCATGCGTGTT	CAGGTCCGAAAGTGGTATTGC
IL-6	ATGTAGCCGCCACACA	CCAGTGCCTCTTTGCTGCTT
TNF- $\alpha$	GCCAGAGGGCTGATTAGAGA	TCAGCCTCTTCTCCTTCTG

**Table 2: Antibodies.**

Experiment	Target gene	Conjugated	Supplier	Reference	Dilution
Primary antibodies					
Western blot	CLEC12B	/	Abcam	ab211452	1/200
	CLEC12B	/	Proteintech	26077-1-AP	1/500
	pSTAT1	/	CST	7649	1/500
	STAT1	/	CST	9172	1/1000
	IRF1	/	CST	8478	1/500
	IGF2R	/	Abcam	ab124767	1/10000
	CRLF1	/	Abcam	ab211438	1/200
	$\beta$ -actin	/	Santa Cruz	sc-47778	1/3000
FACS	CD40	APC	Miltenyi	130-102-547	1/10
	HLA-DR	APC-Vio770	Miltenyi	130-111-792	1/200
	ICAM-1	PE-Vio770	Miltenyi	130-104-031	1/1000
	REA control (S) APC	APC	Miltenyi	130-113-434	1/50
	REA control (S) APC-Vio770	APC-Vio770	Miltenyi	130-113-435	1/50
	REA control (S) PE-Vio770	PE-Vio770	Miltenyi	130-113-440	1/50
Secondary antibodies					
Western blot	Anti-mouse Ig	HRP	Dako	P0447	1/2000
	Anti-rabbit Ig	HRP	Dako	P0448	1/2000
Immunofluorescence	Anti-human IgG Fc	FITC	Invitrogen	A18818	1/500
Glycan array					

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# Annexes

## I. Publications

- **Blot L**, Passeron T, Tulic MK. C-type lectin receptors in skin immunity: Emerging new role for CLEC12B. *Adv Biomed Health Sci* 2022;1:13-22.
- Sormani L, Montaudie H\*, **Blot L\***, Heim M, Cardot Leccia N, Mhaidly R, Verhoeyen E, Regazzetti C, Nottet N, Cheli Y, De Donatis GM, Dabert Gay AS, Debayle D, Taquin Martin H, Gesbert F, Rocchi S, Tulic MK, Passeron T. CLEC12B Is a Melanocytic Gene Regulating the Color of the Skin. *J Invest Dermatol.* 2022 Jul;142(7):1858-1868.e8. doi: 10.1016/j.jid.2021.08.450. Epub 2021 Dec 9. PMID: 34896119. (\* Equal contribution)
- Cluzeau T, Furstoss N, Savy C, El Manaa W, Zerhouni M, **Blot L**, Calleja A, Dufies M, Dubois A, Ginet C, Mounier N, Garnier G, Raynaud S, Rohrlisch PS, Peterlin P, Stamatoullas A, Chermat F, Fenaux P, Jacquelin A, Robert G, Auberger P. Acadesine Circumvents Azacitidine Resistance in Myelodysplastic Syndrome and Acute Myeloid Leukemia. *Int J Mol Sci.* 2019 Dec 25;21(1):164. doi: 10.3390/ijms21010164. PMID: 31881723; PMCID: PMC6981810.



# CLEC12B Is a Melanocytic Gene Regulating the Color of the Skin



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Pigmentation of the human skin is a complex process regulated by many genes. However, only a few have a profound impact on melanogenesis. Transcriptome analysis of pigmented skin compared with analysis of vitiligo skin devoid of melanocytes allowed us to unravel *CLEC12B* as a melanocytic gene. We showed that *CLEC12B*, a C-type lectin receptor, is highly expressed in melanocytes and that its expression is decreased in dark skin compared with that in white skin. *CLEC12B* directly recruits and activates SHP1 and SHP2 through its immunoreceptor tyrosine-based inhibitory motif domain and promotes CRE-binding protein degradation, leading to the downregulation of the downstream MITF pathway. *CLEC12B* ultimately controls melanin production and pigmentation in vitro and in a model of reconstructed human epidermis. The identification of *CLEC12B* in melanocytes shows that C-type lectin receptors exert function beyond immunity and inflammation. It also provides insights into the understanding of melanocyte biology and regulation of melanogenesis.

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## INTRODUCTION

Skin pigmentation is a very complex and tightly regulated process. The melanocytes synthesize melanin, the main skin pigments, within specific organelles called melanosomes that are further transported and transferred to the surrounding keratinocytes (KCs) for protecting them from the harmful effects of UVRs (Miyamura et al., 2007). Among all the pathways involved in the pigmentation process, the MC1R/cAMP pathway is the most critical one. On stimulation of the MC1R receptor at the surface of melanocytes, the cAMP activates MITF through the CRE-binding protein (CREB) (Buscà and Ballotti, 2000; Sato et al., 1997). MITF is the key

transcription factor in pigmentation because it directly regulates the production of the melanocytic enzymes, tyrosinase (TYR), and dopachrome tautomerase (DCT) (Bertolotto et al., 1998). However, besides the MC1R pathway, other pathways can modulate MITF expression and impact melanogenesis. A better knowledge of the factors involved in the regulation of this pathway is critical to understanding the melanogenic process and finding new therapeutic targets for treating pigmentary disorders.

We performed a transcriptome analysis of the lesional and nonlesional skin of patients with vitiligo patients and compared them with the skin of healthy volunteers (Regazzetti et al., 2015). By performing further in-depth analysis of nonlesional vitiligo skin and healthy skin compared with that of lesional vitiligo skin (that are devoid of melanocytes), we segregated the key melanocytic genes. Among the top melanocytic genes, we identified *CLEC12B*. C-type lectin receptors (CLRs) are soluble and transmembrane proteins that are usually involved in the recognition of self-antigens and pathogens (Zelensky and Gready, 2005). Most of the CLRs to date have been described in immune cells. However, some are also expressed by other tissues, including the brain, liver, and kidneys (Lech et al., 2012). In the human skin, KCs, fibroblasts, and endothelial cells express several CLRs (Sheikh et al., 2000; Szolnoky et al., 2001) that are known to be involved in inflammation and immune response (Chiffolleau, 2018; Weis et al., 1998). *CLEC12B* has been reported to be expressed on myeloid cells and to recruit tyrosine phosphatases SHP1 and SHP2 through its immunoreceptor tyrosine-based inhibitory motif (ITIM domain) (Hoffmann et al., 2007). In this study, we showed that *CLEC12B* is highly expressed in melanocytes, with increased expression in light-pigmented skin compared with that in dark skin, suggesting a crucial role of the receptor in pigmentation. Interestingly, we found decreased expression

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Abbreviations: CLR, C-type lectin receptor; CREB, CRE-binding protein; DCT, dopachrome tautomerase; ITIM, immunoreceptor tyrosine-based inhibitory motif; KC, keratinocyte; Ov-CLEC12B, overexpressed *CLEC12B*; sh*CLEC12B*, short hairpin RNA-silenced *CLEC12B*; shRNA, short hairpin RNA; TYR, tyrosinase

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of *CLEC12B* after forskolin stimulation of the cAMP pathway. We showed that *CLEC12B* recruits and activates SHP1 and SHP2, inducing the proteosomal degradation of CREB and the downstream downregulation of MITF and other melanocytic enzymes. Thus, this study unravels *CLEC12B* as a key melanocytic gene that plays a critical role in the regulation of human skin pigmentation, further suggesting that the role of CLRs extends beyond inflammation and immunity.

## RESULTS

### *CLEC12B* is expressed in the human skin by melanocytes

Making use of previously generated transcriptomic data (Regazzetti et al., 2015), we compared nonlesional vitiligo skin and healthy skin with lesional vitiligo skin to segregate the key melanocytic genes. Among all the top melanocytic genes, we found *CLEC12B* to be highly expressed in healthy subjects (Figure 1a) and in the nonlesional skin of patients with vitiligo (Figure 1b), compared with that in lesional vitiligo skin. Interestingly, there was no difference in the expression of *CLEC12B* in the perilesional or nonlesional skin of patients with vitiligo and healthy control skin (data not shown). The decreased expression of *CLEC12B* in lesional vitiligo skin lacking melanocytes is comparable with the degree of decrease observed in known key melanocytic genes such as *DCT*, *MLANA*, *TYRP1*, *TRPM1*, or *TYR* (Figure 1a and b, right panels), suggesting that *CLEC12B* could be an important melanocytic gene. To confirm this hypothesis, an immunofluorescence staining of *CLEC12B* and MITF was performed on normal human skin samples and revealed the coexpression of *CLEC12B* and MITF in melanocytes (Figure 1c). The comparative analysis of the expression of *CLEC12B* mRNA in the three main cell types of the human skin (in vitro cultures of KCs, fibroblasts, and melanocytes from four different donors) using qPCR showed that *CLEC12B* is significantly more expressed in human melanocytes than in KCs and fibroblasts (Figure 1d). In addition, a single-cell RNA sequencing dataset recently added to confirms the enrichment of *CLEC12B* in melanocytes (Supplementary Figure S1). Protein sequence data show that *CLEC12B* possesses a hydrophobic transmembrane domain, suggesting a localization of the receptor at the cell surface. However, immunofluorescence staining of endogenous *CLEC12B* in normal human melanocytes showed mostly a diffuse cytoplasmic subcellular distribution (Supplementary Figure S2a). To verify the specificity of the cytoplasmic immunostaining of *CLEC12B*, we used lentiviral-mediated overexpression of recombinant *CLEC12B* fused with a GFP and observed colocalization between anti-*CLEC12B* and anti-GFP antibodies, hence validating the specificity of our *CLEC12B* antibody (Supplementary Figure S2b). This GFP-fusion *CLEC12B* allowed us to confirm a cytoplasmic distribution of *CLEC12B* but also its expression at the cell surface of melanocytes (Supplementary Figure S2c).

### *CLEC12B* expression depends on constitutive and facultative pigmentation

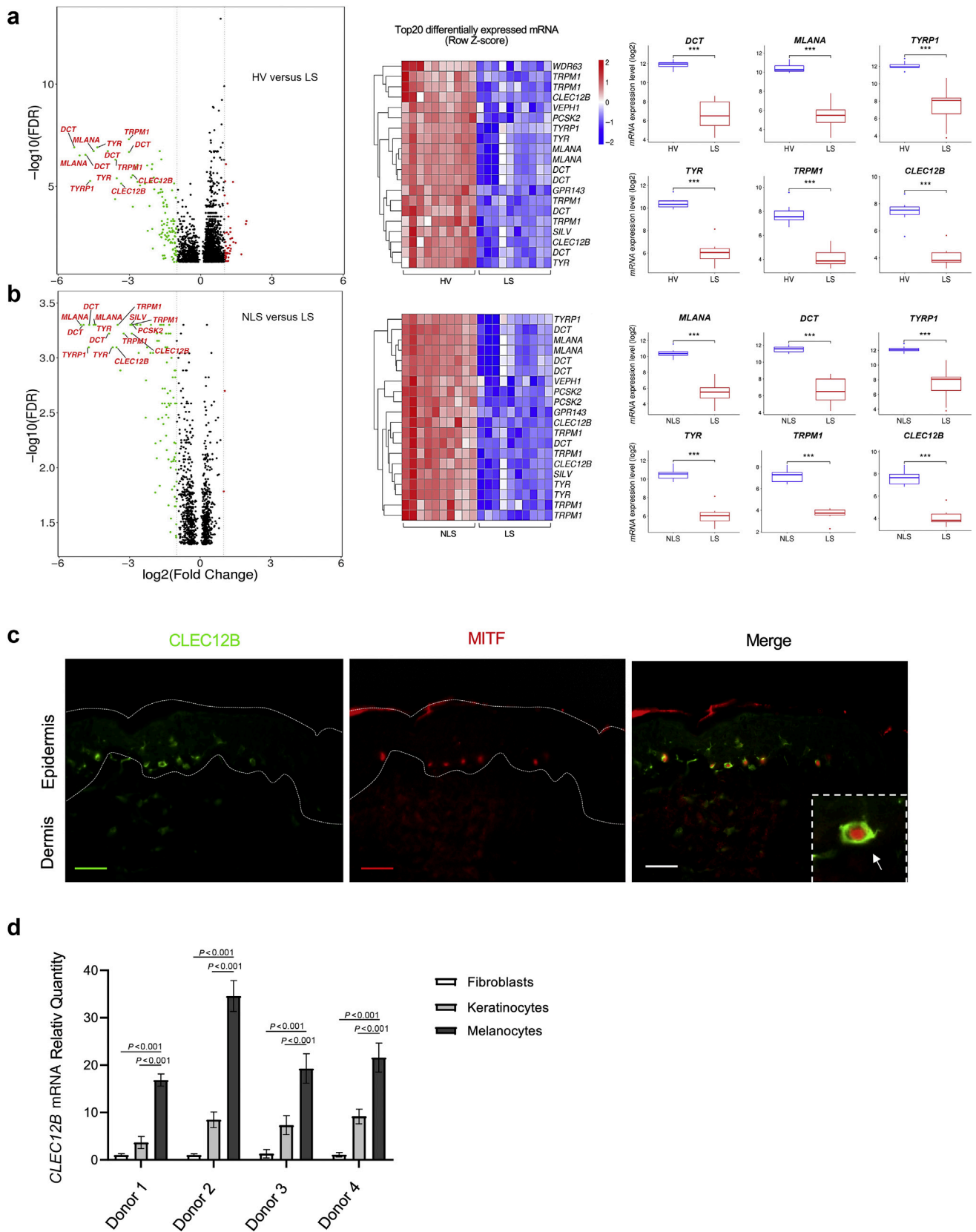
The constitutive color of human skin ranges from extremely fair/light to extremely dark depending on ethnic background and is frequently classified into one of six distinct skin phototypes (Fitzpatrick, 1988). We analyzed the expression of *CLEC12B* according to the skin phototype. *CLEC12B* mRNA

expression levels in cultured melanocytes isolated from light-pigmented skin (phototype I/II, i.e., donors of Caucasian origin) were significantly higher than those in melanocytes isolated from dark-pigmented skin donors (phototypes V/VI, i.e., donors of African origin) (Figure 2a). The immunohistochemistry analysis of *CLEC12B* expression on human skin samples (Figure 2b and Supplementary Figure S2d, e, and f) showed stronger staining of *CLEC12B* (in dark red, localized at the dermal/epidermal border in a characteristic regularly dispersed pattern) on skin sections from light-pigmented donors than that from dark-pigmented donors. The melanin pigments are clearly distinguished by their brown color and their epidermal distribution. In accordance with the single-cell data, we observed some staining in endothelial cells and to a lesser extent also in some basal KCs.

An increase in skin pigmentation over the basal constitutive level is referred to as facultative pigmentation. UVR is the major stimulus of facultative pigmentation in humans by inducing the activation of the cAMP pathway (Buscà and Ballotti, 2000; Sato et al., 1997). We assessed the effect of the cAMP-elevating agent forskolin on *CLEC12B* mRNA expression in normal human melanocytes from moderately pigmented donors. Interestingly, the elevation of cAMP level induced a significant decrease in *CLEC12B* expression (Figure 2c).

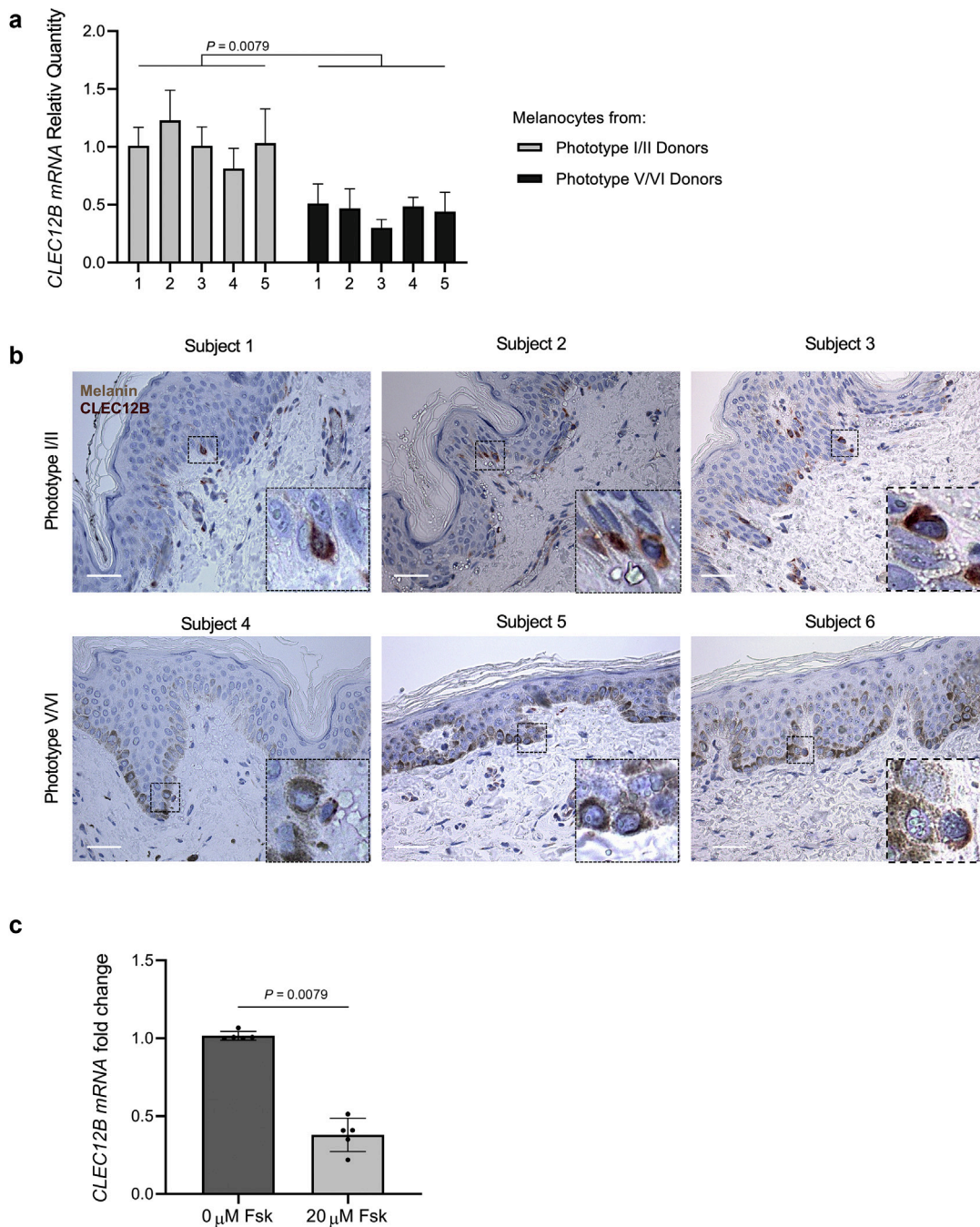
### *CLEC12B* modulates melanogenesis in normal human melanocytes

To investigate the functional role of *CLEC12B* in melanogenesis, we used lentiviral-mediated silencing of *CLEC12B* with short hairpin RNA (shRNA) and a lentiviral-mediated overexpression (i.e., overexpressed *CLEC12B* [Ov-*CLEC12B*]). The efficiency of three different sequences of shRNA was validated by qPCR, and the most efficient constructs were used for the study (90% efficiency in gene knockdown with construct #1 and 85% efficiency in gene knockdown with construct #3) (Figure 3a and Supplementary Figure S3a). In contrast with qPCR results, we were unable to detect any change in the expression of endogenous *CLEC12B* protein by western blot (Supplementary Figure S3b). The construct used to overexpress *CLEC12B* was also validated by qPCR (Figure 3d). In western blot, the predicted molecular weight of *CLEC12B* is 32 kDa. However, we observed that the Ov-*CLEC12B* band appeared at ~40 kDa (Supplementary Figure S3b). We therefore performed proteomic analysis of the band observed between 25 and 35 kDa (which corresponds to the expected molecular weight) and the band at ~40 kDa after *CLEC12B* overexpression (Supplementary Figure S3c and d). The presence of *CLEC12B* peptide was detected only in the 40 kDa band. The *CLEC12B* protein contains three potential sites for Asn-linked glycosylation (Asn-Xaa-Ser/Thr at positions 91, 176, and 237), which might increase the total weight of the protein by 2.5 kDa for each additional site. We therefore hypothesized that *CLEC12B* is glycosylated when overexpressed and reaches 40 kDa. To test this hypothesis, we exposed the cells to tunicamycin, an inhibitor of Asn-linked glycosylation. In the presence of tunicamycin, the molecular weight of the *CLEC12B* protein was reduced to 32–35 kDa, showing that *CLEC12B* is



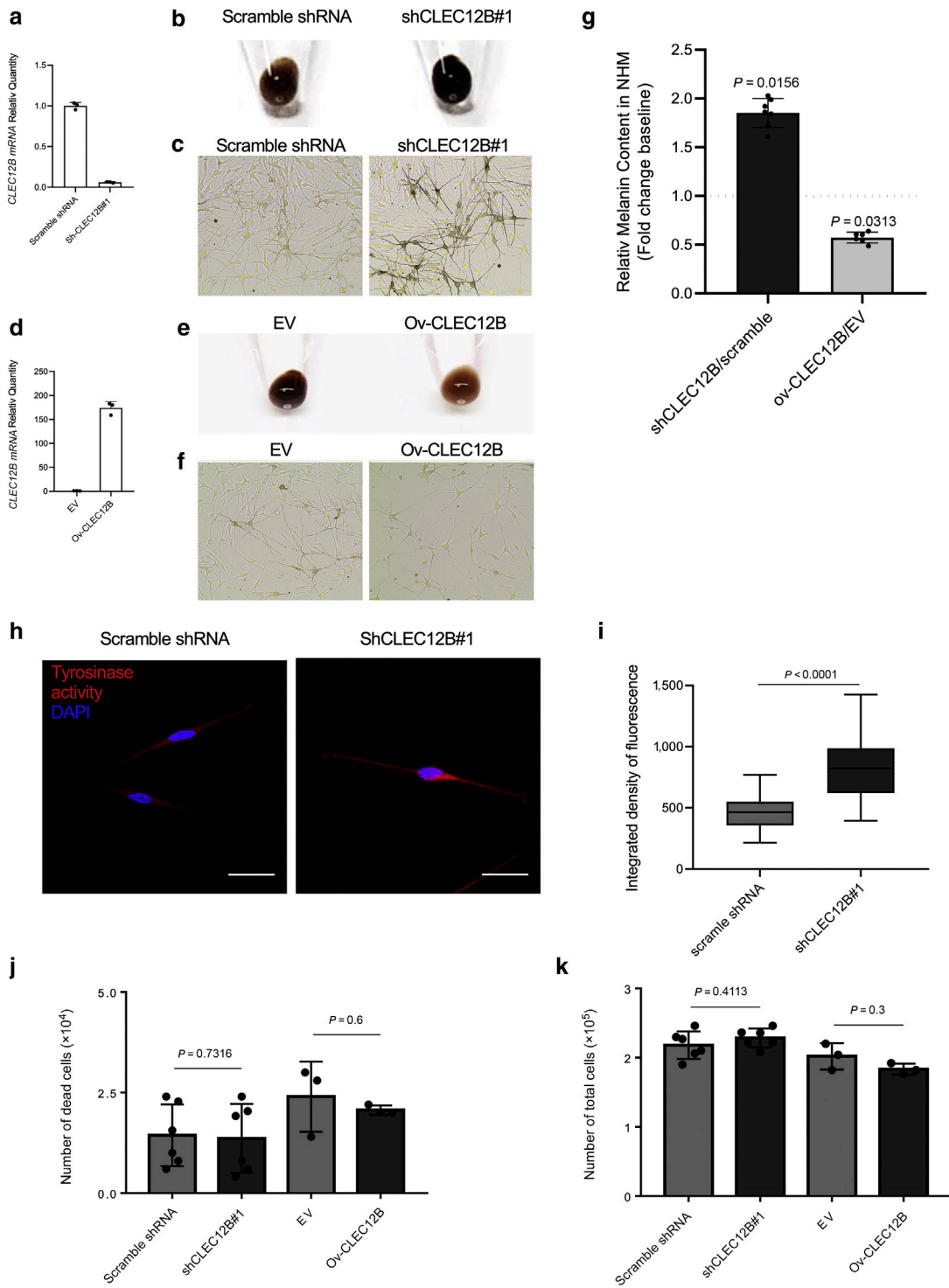
**Figure 1. Identification of *CLEC12B* in human skin.** Microarray data comparing LS from subjects with vitiligo with (a) HV skin or with (b) NLS from subjects with vitiligo, n = 10. Volcano plots (left panels) showing the distribution of differentially expressed mRNA between samples. Heat maps (center panels) depicting the relative mRNA expression changes for the top 20 most differentially expressed genes. Boxplots (right panels) comparing the mRNA expression levels of *CLEC12B*, *TYR*, *TRP1*, *DCT*, and *MLANA* between samples. (c) Immunofluorescence staining performed on healthy human skin showing *CLEC12B* and *MITF* expression in melanocytes. Bars = 25  $\mu$ m. (d) *CLEC12B* mRNA relative quantity was examined by qPCR in normal human fibroblasts, keratinocytes, and





**Figure 2. *CLEC12B* expression depends on constitutive and facultative pigmentation.** (a) *CLEC12B* mRNA relative expression level was examined by qPCR in light- and dark-pigmented donors. qPCR results were normalized to those of house-keeping gene *RPLPO* and expressed as a fold change in mRNA expression relative to that of the light-pigmented donor samples. Data are shown as mean  $\pm$  SD.  $n = 5$  donors. Differences between groups were tested using nonparametric Mann–Whitney test and considered significant at  $P < 0.05$ . (b) IHC staining of *CLEC12B* was performed on human skin samples of light-pigmented skin (phototype I/II = subjects 1, 2, and 3) and dark-pigmented skin (phototype V/VI = subjects 4, 5, and 6). Melanin was visualized as a brown stain, and *CLEC12B* was visualized as a red stain. Inset represents enlarged images of a positive staining zone. Bars = 25  $\mu$ m. (c) Effect of cAMP activation on *CLEC12B* expression. The relative quantity of *CLEC12B* mRNA was determined by qPCR in melanocytes (phototype III/IV) cultured in Fsk-depleted medium (0  $\mu$ M Fsk) or stimulated with Fsk (20  $\mu$ M Fsk) for 48 hours. qPCR results were normalized to those of house-keeping gene *RPLPO* and expressed as fold change relative to control. Error bars represent mean  $\pm$  SD.  $n = 5$  donors. Differences between groups were tested using Mann–Whitney test and were considered significant when  $P < 0.05$ . Fsk, forskolin; IHC, immunohistochemistry.

melanocytes from four donors (phototypes III/IV). qPCR results are normalized to those of house-keeping gene *RPLPO* and expressed as a fold change in mRNA expression relative to fibroblast samples. Data are shown as mean  $\pm$  SD. Two-way ANOVA followed by Tukey’s multiple comparisons test was used; differences were considered significant at  $P < 0.05$ . \*\*\* $P < 0.001$ . FDR, false discovery rate; HV, healthy volunteer; LS, lesional skin; NLS, nonlesional skin.



**Figure 3. *CLEC12B* modulates melanogenesis in normal human melanocytes.** Lentiviral infection was used to silence (shCLEC12B#1) or overexpress *CLEC12B* (Ov-CLEC12B) in phototype III/IV melanocytes. **(a, d)** *CLEC12B* mRNA relative quantity was examined by qPCR. Results were normalized to those of house-keeping gene *RPLPO* and expressed as a fold change relative to the control. Error bars represent mean  $\pm$  SD. The variation of pigmentation observed in **(b, e)** cells pellets and **(c, f)** live cells was quantified in cultured melanocytes using a **(g)** melanin assay. Results were expressed as a fold change of melanin content relative to the controls with the line at 1 to signify normal melanin content. shCLEC12B/scramble:  $n = 7$  donors; Ov-CLEC12B/EV:  $n = 6$  donors. Error bars represent mean  $\pm$  SD. Differences between groups were tested using the Wilcoxon matched-pairs signed-rank test and were considered significant at  $P < 0.05$ . **(h)** Tyrosinase activity was assessed by a tyramide-based tyrosinase assay (Bar = 20  $\mu$ m), and **(i)** fluorescence intensity was quantified using ImageJ analysis tools, with bars representing 5–95% confidence intervals.  $n = 15$  scramble shRNA,  $n = 38$  shCLEC12B#1. **(j)** Viability and **(k)** proliferation assays of melanocytes silenced or overexpressed for *CLEC12B*. Differences between groups were tested using Mann–Whitney test and were considered significant at  $P < 0.05$ . EV, empty vector; NHM, normal human melanocyte; Ov-CLEC12B, overexpressed *CLEC12B*; shCLEC12B, short hairpin RNA–silenced *CLEC12B*; shRNA, short hairpin RNA.

indeed glycosylated when overexpressed (Supplementary Figure S3d).

Transduced cell pellets (Figure 3b and e) and live cells observed by contrast-phase microscopy (Figure 3c and f) showed an increased pigmentation in *CLEC12B*-silenced melanocytes compared with that in scramble shRNA, with decreased pigmentation in cells overexpressing *CLEC12B* compared with that in the cells transduced with the empty vector. The melanin content in transduced melanocytes from six (shRNA-silenced *CLEC12B*/scramble) and seven (Ov-*CLEC12B*/empty vector) different donors was quantified using a spectrophotometric assay. *CLEC12B* silencing induced a significant increase in melanin content compared with the control. On the contrary, the overexpression of *CLEC12B* significantly reduced the melanin content (Figure 3g).

Melanin production requires the TYR enzyme, which catalyzes multiple reactions in the melanin biosynthetic pathway. TYR activity was assessed by a tyramine-based TYR assay, and fluorescence intensity was quantified using ImageJ (National Institutes of Health, Bethesda, MA) analysis tools. Accordingly, we visualized an increase in TYR activity in the *CLEC12B*-silenced melanocytes compared with that in the control (Figure 3h). This augmentation was semiquantified, showing a significant increase in fluorescence intensity in sh*CLEC12B* transduced melanocytes compared with that in the control (Figure 3i). We finally assessed whether *CLEC12B* modulates the viability and the proliferation of melanocytes (Figures 3j and k). We found that modulating *CLEC12B* expression has no significant impact on melanocyte viability or proliferation. Taken together, these results show that *CLEC12B* modulates melanogenesis and has a direct effect on TYR activity in cultured normal human melanocytes.

### ***CLEC12B* modulates melanogenesis in a three-dimensional reconstructed human skin model**

The color of the skin not only reflects the capacity of melanocytes to produce melanin but also their ability to transfer melanin pigment to the surrounding KCs. To further assess the role of *CLEC12B* on skin pigmentation, it is important to study melanocytes in their physiological cellular environment when in direct contact with KCs. Thus, we used a model of human reconstructed epidermis composed of normal human KCs cultured in the presence of normal human melanocytes (phototype III/IV, i.e., moderately pigmented allowing the observation of pigmentation variations) transduced with lentivirus to silence (sh*CLEC12B*#1) or to overexpress *CLEC12B* (Ov-*CLEC12B*). Technically, the seeding ratio between KCs and transduced melanocytes was strictly identical between the different test conditions.

The variation of pigmentation observed in the skin constructs was imaged (Figure 4a) and observed using a transmitted light microscope (Figure 4b). The images were quantified using ImageJ (Supplementary Figure S4a). A Fontana–Masson staining of melanin pigment was performed to visualize the quantity of pigment present in the reconstructed epidermis and to examine the distribution of melanin to the adjacent KCs (Figure 4c). After *CLEC12B* silencing, melanocytes were more dendritic, and the entire epidermis was more pigmented. On the contrary, decreased pigmentation in the epidermis was observed when *CLEC12B* was

overexpressed. These observations were confirmed by semi-quantitation of 30 images per condition using ImageJ software (Figure 4d). Melanin content extracted from the human reconstructed epidermis was quantified using a spectrophotometric assay. In accordance with the observed color of the reconstructed epidermis, the melanin content was significantly increased when *CLEC12B* was silenced and significantly decreased when *CLEC12B* was overexpressed (Figure 4e). The increased melanin content when *CLEC12B* is silenced cannot be explained by an increased number of melanocytes in the reconstructed skin. In contrast, the reduced melanin content in the Ov-*CLEC12B* condition is due to less melanin production but also due to a decrease in melanocyte number (Supplementary Figure S4b and c).

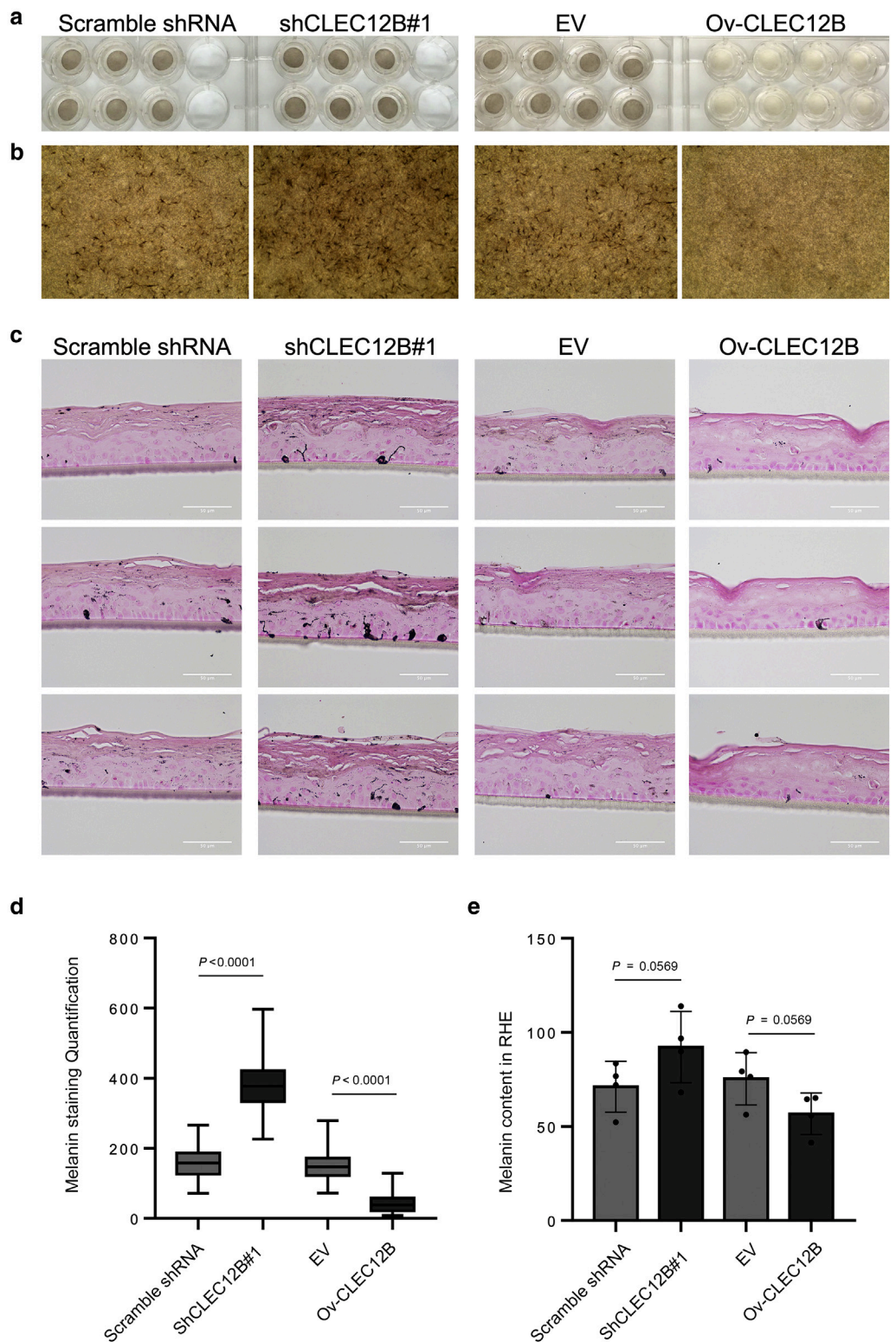
### ***CLEC12B* recruits the phosphatases SHP-1 and SHP-2 by its ITIM domain to regulate MITF and melanogenic proteins through the proteasomal degradation of CREB**

To understand the mechanisms underlying the regulatory properties of *CLEC12B* in melanogenesis, the effects of *CLEC12B* modulation on the expression of melanin synthesis-related proteins (TYR, DCT, and MITF) were analyzed by western blot. Silencing *CLEC12B* markedly increased CREB, phosphorylated CREB, MITF, TYR, DCT, and TRP1 expression levels (Figure 5a, left panel). On the contrary, *CLEC12B* overexpression decreased the expression of these same melanogenesis-related proteins (Figure 5a, right panel). Western blot analysis of the proteins extracted from the reconstructed epidermis showed an increased expression of MITF when *CLEC12B* is silenced and decreased MITF expression when *CLEC12B* is overexpressed (Supplementary Figure S4d). CREB is the main regulator of the MITF expression. To determine whether *CLEC12B* contributed to decreased CREB expression levels through transcription or protein degradation, an RT-qPCR was performed using *CREB*-specific primers. The overexpression of *CLEC12B* showed no effect on *CREB* mRNA levels (Supplementary Figure S5). Thus, we investigated whether the downregulation of CREB expression was caused by proteasomal degradation. Normal human melanocytes overexpressing *CLEC12B* were treated with MG132 (a proteasome inhibitor). Western blot analysis (Figure 5b) and quantification (Figure 5c) showed that MG132 prevented the decreased expression of CREB after *CLEC12B* overexpression. Taken together, these results show that *CLEC12B* induced proteasomal degradation of CREB and the subsequent downregulation of melanogenesis proteins.

Sequence data analysis revealed that *CLEC12B* possesses an ITIM in its intracellular domain. In the literature, it is well-described that ITIM-containing CLRs can recruit and activate the SH2 domain-containing protein tyrosine phosphatases, thus leading to the negative regulation of several downstream signaling pathways (Daëron et al., 2008). Using 293T cells transfected with wild-type or a mutant of the ITIM of *CLEC12B* in combination with SHP-1 or SHP-2, Hoffmann et al. (2007) showed that the wild-type but not the mutant receptor can recruit SHP-1 and SHP-2. To examine whether this mechanism may be involved in *CLEC12B*-induced regulation of melanogenesis by melanocytes, we generated the same mutant by replacing the central tyrosine within the ITIM (which is essential for the phosphatases recruitment)

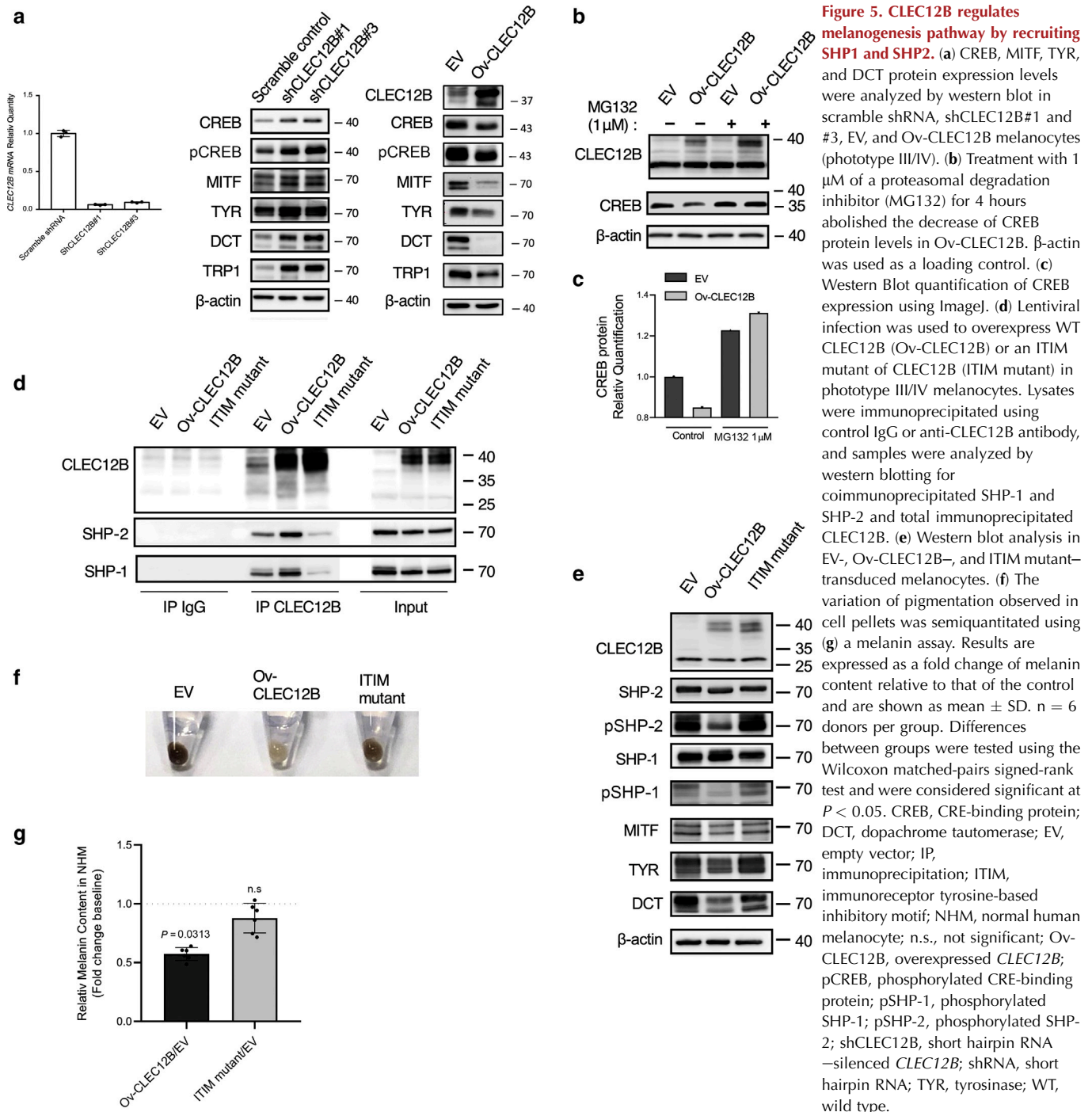


**Figure 4. *CLEC12B* modulates pigmentation in reconstructed human skin.** Reconstructed human epidermis was composed of pooled keratinocytes cultured in the presence of phototype III/IV melanocytes transduced with lentivirus to silence (*shCLEC12B#1*) or to overexpress *CLEC12B* (*Ov-CLEC12B*). The variation of pigmentation was observed (a) in skin constructs and (b) by transmitted light microscopy. Melanin pigments were (c) visualized with Fontana–Masson and Eosin stain and (d) quantified using ImageJ software. Bar = 50 μm; n = 30 quantitated images per condition. Results are shown as box and whisker plots, with bars representing 5–95% confidence interval. Differences between groups were tested using one-way ANOVA test with Sidak’s multiple comparisons and were considered significant at  $P < 0.05$ . (e) Melanin extracted from four different constructs was quantified using a melanin assay. Results are shown as mean ± SD. n = 4 RHE per condition. Differences between groups were tested using the Friedman test with Dunn’s multiple comparisons and were considered significant at  $P < 0.05$ . EV, empty vector; *Ov-CLEC12B*, overexpressed *CLEC12B*; RHE, reconstructed human epidermis; *shCLEC12B*, short hairpin RNA –silenced *CLEC12B*; *shRNA*, short hairpin RNA.



with a phenylalanine amino acid. Coimmunoprecipitation assay using lysates of primary melanocytes stably transduced with empty vector, *Ov-CLEC12B*, or ITIM mutant showed that the endogenous *CLEC12B* recruited SHP-1 and SHP-2; this interaction was increased on *CLEC12B* overexpression and was prevented by the mutant (Figure 5d). We then studied the

relevance of this interaction on phosphatases activation and melanogenesis pathway regulation. Stein-Gerlach et al. (1995) have reported that SHP-2 (also called PTPN11) may modulate its own state of tyrosine phosphorylation. The authors showed that in response to receptor tyrosine kinase stimulation, SHP-2 is immediately phosphorylated; however,



this phosphorylated state is transient (30 seconds) owing to its own phosphatase activity (defined as an intermolecular transdephosphorylation mechanism) (Stein-Gerlach et al., 1995). We observed a similar mechanism in our study with *CLEC12B* overexpression associated with a decrease of the phosphorylated forms of SHP-1 (Y564) and SHP-2 (Y542), therefore demonstrating the catalytic activity of the phosphatases. The effect on phosphorylated SHP-1 and phosphorylated SHP-2 observed after *CLEC12B* overexpression no longer occurred with the mutant receptor. Moreover, the ITIM

mutant (Figure 5e) was able to prevent the decreased protein expression of MITF, TYR, and DCT and reverse the phenotype observed on *CLEC12B* overexpression. (Figure 5e and f). The variation of melanin content observed in transduced empty vector, Ov-*CLEC12B*, and ITIM mutant-transduced melanocytes was confirmed by quantitative spectrophotometry (Figure 5g). Overall, these results showed that *CLEC12B* negatively regulates the melanogenesis pathway and thus melanin production by recruiting the phosphatases SHP-1 and SHP-2 by its ITIM domain.



## DISCUSSION

CLRs were thought to have a restricted role in inflammation and immunity processes. However, attractin, another C-type lectin protein, was shown to play a role in mouse hair pigmentation by interacting with agouti protein and downstream affecting the MC1R/cAMP pathway (He et al., 2001). In this study, we describe *CLEC12B* as a melanocytic gene. The available antibodies we used for assessing *CLEC12B* in western blot did not allow us to detect low protein levels of *CLEC12B*. However, the immunostaining clearly showed the expression of *CLEC12B* in melanocytes in the skin tissues. In accordance with our data, RNA sequencing dataset from The Human Protein Atlas shows that melanocytes are by far the cellular type expressing the most *CLEC12B* (Supplementary Figure S1) even when compared with myeloid cells where *CLEC12B* was first characterized (Hoffmann et al., 2007). We showed that the expression of *CLEC12B* is stronger in light-pigmented skin, whereas it is significantly reduced in dark-skin subjects. The modulation of *CLEC12B* expression in cultured primary melanocytes profoundly affects pigmentation not only in vitro but also in the human three-dimensional model of reconstructed epidermis. Such an impact on the pigmentary phenotypes is quite exceptional and demonstrates, to our knowledge, the previously unreported key role of *CLEC12B* in constitutive pigmentation. We also observed some mild effects of *CLEC12B* in melanocyte proliferation (that was more pronounced in the epidermal reconstructed skin model). We recently showed that *CLEC12B* is significantly less expressed in melanoma cells. The overexpression of *CLEC12B* in melanoma cells reduces their proliferation in vitro and in a mice model (Montaudie et al. J Invest Dermatol In press). However, the impact of *CLEC12B* on melanocyte proliferation is much lower owing to their low proliferation rate than on melanoma cells. The experiments conducted in this study showed that *CLEC12B* promotes the degradation of CREB, which is the major transcription factor of MITF that directly regulates downstream melanogenic enzymes (Buscà and Ballotti, 2000; Sato et al., 1997) and that the recruitment of SHP-1 and SHP-2 by its ITIM domain is involved in this mechanism. Interestingly, germline alteration of *PTPN11* encoding SHP2 have been reported to be involved in the pathogenesis of multiple lentiginos (small hyperpigmented lesions) in LEOPARD syndrome. Motegi et al. (2015) showed that LEOPARD syndrome-associated SHP-2 mutations, which reside in the protein-tyrosine phosphatase domain causing reduced phosphatase activity, might enhance melanin synthesis in cultured human melanoma cells.

To date, nothing was known about *CLEC12B* regulation. In this study, we showed that the stimulation of the cAMP that is the most potent pathway for regulating facultative pigmentation decreases *CLEC12B* expression. Taken together, our results show that CLRs exert function beyond immunity and inflammation. Considering that *CLEC12B* was initially reported to be involved in inflammatory processes, the possible link between inflammation and pigmentation through the modulation of *CLEC12B* warrants further investigation. The identification of *CLEC12B* as a key melanocytic gene fosters a new area of research and opens therapeutic perspectives for treating pigmentary disorders.

## MATERIALS AND METHODS

### Lentivirus production and transduction

Self-inactivating HIV-1–derived vectors were generated by transient transfection of human epidermal KC 293T cells as previously described (Sallets et al., 2018). Viral vectors were produced pseudotyped with vesicular stomatitis virus G. Transfection was performed by using the classical calcium phosphate method. For vesicular stomatitis virus G–lentivirus vector preparation, 3 µg of pHCMV vesicular stomatitis virus G plasmid was cotransfected with a Gag-Pol packaging plasmid (PAX2) and a plasmid encoding a self-inactivating lentivirus vector.

For the overexpression of *CLEC12B*, we used *CLEC12B* Lentiviral Vector pLenti-GIII-CMV-GFP-2A-Puro (catalog number LV796325, Applied Biological Materials, Richmond, British Columbia, Canada) custom (use of CDS of *CLEC12B* accession number NM\_001129998 - transcript variant 1) and the control plasmid Blank Vector pLenti-CMV-GFP-2A-Puro (LV590).

For the silencing of *CLEC12B*, we used the following MISSION shRNA plasmid DNA (Sigma-Aldrich, St. Louis, MO):

1. sh*CLEC12B*#1: NM\_205852/TRCN0000164269/-HPGK-PURO-CMV-TGFP;
2. sh*CLEC12B*#2: NM\_205852/TRCN0000160830/-HPGK-PURO-CMV-TGFP; and
3. sh*CLEC12B*#3: NM\_205852/TRCN0000159325/-HPGK-PURO-CMV-TGFP.

The two constructs with the highest knockdown efficiency (tested using qPCR) were chosen for in vitro experiments. As a negative control, we used the MISSION Non-Target shRNA Control Vector (catalog number SHC002, Sigma-Aldrich).

Viral supernatant was harvested 48 hours after transfection and filtered through a 0.45-µm cellulose acetate filter. A low-speed concentration of the vectors was performed by overnight centrifugation of the viral supernatant at 3,000g at 4 °C. To determine the transduction efficiency and infectious titers of HIV vectors, serial dilutions of vector preparations were added to 293T cells. The infectious titers are expressed as 293T transducing units per milliliter (TU/ml).

Normal human melanocytes were serially passaged and used at passage 3 for viral infection. Cells were seeded on six-well plates at a density of  $2 \times 10^5$  cells per well and infected with a multiplicity of infection of 20. Cells were incubated with the virus for 72 hours. The infected cells were selected for stable expression using puromycin at 1 µg/ml. Infected normal human melanocytes were cultured in medium 254 supplemented with human melanocyte growth supplement (Cascade Biologics, Carlsbad, CA).

### Site-directed mutagenesis of *CLEC12B*

The *CLEC12B* Y→F mutation was generated by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis; Stratagene, Agilent Technologies, Les Ulis, France), according to the kit manufacturer's instructions. The following primers were used (the substituted nucleotides are shown in bold and underlined): 5'-GTCTGAAGAAGTGACCTTCGCGACACTCACATTTTC-3' for *CLEC12B* Y→F-forward and 5'-GAAATGTGAGTGTCGCGAAGGTCACCTTTCAGAC-3' for *CLEC12B* Y→F-reverse. Mutations were verified by DNA sequencing (Eurofins Genomics, Louisville, KY).

### Melanin assay

Melanin content was measured as previously described (Bellei et al., 2010). Briefly, cell suspensions were centrifuged, and pellets were photographed and then solubilized in 120 µl of 0.5 N sodium hydroxide at 80 °C for 1 hour to dissolve melanin. Melanin absorbance was measured spectrophotometrically at 405 nm using a plate reader. Melanin production was calculated by normalizing the total melanin values with protein content.

### Coimmunoprecipitation

After washing with PBS, cells were lysed in NP40 lysis buffer containing protease inhibitors (F. Hoffmann-La Roche, Basel, Switzerland), phosphatase inhibitors (F. Hoffmann-La Roche), and Sodium Orthovanadate (catalog number AK-102V-L, Jena Bioscience, Jena, Germany). The supernatant was collected, centrifuged at 13,000g for 10 minutes, and assayed for protein concentration using the BCA kit (Pierce, ThermoFisher, Waltham, MA). A total of 500 µg of total proteins diluted in 500 µl of lysis buffer was incubated with 2 µg of *CLEC12B* antibody under agitation for 30 minutes at room temperature. After three washes with lysis buffer, the magnetic beads (Ademtech Bio-ademtechs PAG 0463, Ademtech, Pessac, France) were added to the cell lysates and incubated overnight at 4 °C under agitation. The target protein was eluted (PAG elution buffer, catalog number 10701, Ademtech) and analyzed by western blot.

### Histochemistry of reconstructed three-dimensional skin model

The reconstruction of the three-dimensional skin model (SkinEthic RHE) was performed by EPISKIN (Lyon, France) using our in-house sh*CLEC12B* or overexpressing *CLEC12B* melanocytes as described earlier. The reconstructed epidermis was fixed in 4% paraformaldehyde and embedded in paraffin. A total of 5-µm sections were processed for Fontana–Masson staining to visualize melanin pigments and for immunofluorescence staining to visualize melanocytes.

### Immunofluorescence staining (tissue)

Skin samples were supplied by the Departments of Dermatology and Pathology of Centre Hospitalier Universitaire de Nice (Nice, France). After dissection and washes in PBS, skin biopsies were embedded in Tissue-Tek O.C.T medium in cryomolds, snaps frozen in liquid nitrogen-cooled isopentane (2-methyl butane), and stored at –80 °C before cutting. The tissue was cut with microtome into 7-µm sections and fixed on Superfrost Plus slides. After 20 minutes of air drying, sections were fixed in ice-cold acetone (30 minutes at –20 °C) and saturated for 2 hours at room temperature with 5% PBS 3% BSA normal goat serum. Sections were incubated overnight at 4 °C with specific antibodies directed against *CLEC12B* and *MITF* (Supplementary Table S1). The next day, sections were then washed three times with PBS and incubated with fluorescent dye-conjugated secondary antibody (Supplementary Table S1) at room temperature for 1 hour. Slides were mounted with Prolong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific, Waltham, MA). Fluorescence was detected using a confocal microscope (Nikon A1R, Nikon Instruments, Tokyo, Japan) using objective Plan Apo 63× oil 1.4 NA.

### Ethical information

The transcriptomic study was approved by the ethics committee SUD MEDITERRANEE V (N12.034). Patient consent for tissue samples for extracting melanocytes was not required because French laws consider human tissue left over from surgery as discarded material.

Additional methods are provided in the Supplementary Materials and Methods.

### Data availability statement

Datasets related to this article can be found at National Center for Biotechnology Information Gene Expression Omnibus, accession number GSE65127.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### AUTHOR CONTRIBUTIONS

Conceptualization: LS, TP; Data Curation: LS, HM, LB, MH, NCL, RM, EV, CR, NN, YC, GMDD, ASDG, DD, HTM, FG; Formal Analysis: LS, HM, LB, MH, NCL, MKT, TP; Funding Acquisition: TP; Investigation: LS, TP, HM, LB, MH, NCL, RM, EV, CR, NN, YC, GMDD, ASDG, DD, HTM, FG, MKT; Methodology: LS, MKT, TP; Project Administration: TP, SR; Resources: TP, MKT, SR; Supervision: TP, SR, MKT; Validation: TP, MKT, SR; Visualization: LS, TP, HM, LB, MH, NCL, MKT; Writing - Original Draft Preparation: LS, TP, HM, LB, MH, NCL, MKT; Writing - Review and Editing: LS, TP, MKT, SR

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at [www.jidonline.org](http://www.jidonline.org), and at <https://doi.org/10.1016/j.jid.2021.08.450>.

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## SUPPLEMENTARY MATERIALS AND METHODS

### Transcriptomic analysis

A total of 10 patients with active nonsegmental vitiligo, which is defined by the occurrence or the worsening of depigmented lesions in the past 3 months and having hypochromic borders upon Wood's lamp examination, were enrolled in the study after informed written consent was obtained. The study was approved by the ethics committee SUD MEDITERRANEE V (N12.034). A 4-mm skin biopsy was taken from each patient in the center of a vitiligo patch, in the perilesional area (defined as 5 mm outside of the lesion border), and in nonlesional skin located in the same area but at least 3 cm from a depigmented lesion. A 4-mm biopsy was also taken from 10 healthy patients and served as a control that was matched for sex, age, and location.

Biopsies for the microarray analysis were stored in RNA Stabilization Reagent (Qiagen, Venlo, The Netherlands) until use. For RNA extraction, the samples were homogenized with a potter in Qiagen lysis buffer (Qiagen). Total RNA was extracted using RNeasy extraction kits (Qiagen) according to the manufacturer's instructions. RNA quantity was measured using a Nanodrop Spectrophotometer ND8000 (Thermo Fisher Scientific, Waltham, MA). RNA quality was monitored using a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Probes were synthesized and then hybridized on Affymetrix U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA). All chips were normalized using the robust multiarray average method (Bolstad et al., 2003). Only Affymetrix identifiers with expression  $\geq 2 \exp 6$  (64) for at least 7 of 10 samples in at least 1 sample group (lesional, nonlesional, perilesional, or healthy) were selected. Finally, 29,906 of 54,675 identifiers that were initially present were kept for statistical analyses, and thresholds of modulation of 1.5 and  $-1.5$  were selected for further analyses. Data analysis was performed on Array Studio software (OmicSoft, Cary, NC). A two-sided paired Student's *t*-test was performed. The Benjamini-Hochberg procedure was used for correction of multiple testing. The raw data are available at National Center for Biotechnology Information Gene Expression Omnibus, accession number GSE65127.

### Cell culture

Normal human melanocytes, normal human keratinocytes, and normal human fibroblasts were obtained from the foreskin of young children (skin type based on the experimental question) undergoing circumcision. Tissue samples were kindly supplied by the Department of Pediatric Surgery, Lenval Hospital (Nice, France; Dr Kurzenne). The samples were washed with PBS containing 1% antibiotic/antimycotic (Gibco, Life Technologies, Carlsbad, CA) three times for 5 minutes each. After removal of the subcutaneous tissue, tissue was cut into  $2 \times 2 \text{ mm}^2$  pieces. The foreskin samples were incubated with dispase enzyme (4 U/ml, F. Hoffmann-La Roche, Basel, Switzerland) for 12–16 hours at 4 °C. Once the dermis and epidermis of foreskins were separated with forceps, tissue pieces of dermis were transferred to the bottom of a culture dish and cultured with 10 ml of DMEM/F12 (Gibco, Life Technologies) media supplemented with 10% fetal bovine serum to obtain normal human fibroblasts. The epidermis was incubated within a trypsin/EDTA solution

for 20 minutes at 37 °C; the cells were dispersed into cell suspensions and filtered by cell strainer (70  $\mu\text{m}$ , Falcon) before final wash with PBS. Normal human melanocytes were isolated in MCDB 153 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 2% fetal bovine serum (SV30160-03, Hyclone Laboratories, Logan, UT), 5  $\mu\text{g/ml}$  insulin (Sigma-Aldrich), 0.5  $\mu\text{g/ml}$  hydrocortisone (Sigma-Aldrich), 16 nM 12-O-tetradecanoylphorbol 13-acetate (Sigma-Aldrich), 1 ng/ml basic fibroblast GF (Promega, Madison, WI), 15  $\mu\text{g/ml}$  bovine pituitary extract (Gibco, Life Technologies), 10  $\mu\text{M}$  forskolin (Sigma-Aldrich), and 20  $\mu\text{g/ml}$  geneticin (Invitrogen, Waltham, MA) over 2 weeks or until we obtain pure melanocytes population. Melanocytes between passages 2 and 6 were used. Normal human keratinocytes were isolated in a keratinocyte growth medium with a supplement (PromoCell, Heidelberg, Germany). Cells were maintained at 37 °C in a 5% carbon dioxide atmosphere.

To assess the effect of the cAMP pathway activation on *CLEC12B* mRNA expression, normal human melanocytes were cultured in MCDB 153 medium (Sigma-Aldrich) supplemented with 2% fetal bovine serum, 5  $\mu\text{g/ml}$  insulin, 0.5  $\mu\text{g/ml}$  hydrocortisone, 16 nM 12-O-tetradecanoylphorbol 13-acetate, 1 ng/ml basic fibroblast, 15  $\mu\text{g/ml}$  bovine pituitary extract, and 20  $\mu\text{g/ml}$  geneticin and stimulated for 48 hours with 20  $\mu\text{M}$  forskolin (Sigma-Aldrich).

### Western blot analysis

After washing with PBS, cells were lysed in RIPA buffer (catalog number 89900, Thermo Fisher Scientific) containing protease inhibitors (catalog number 04693159001, F. Hoffmann-La Roche) and phosphatase inhibitors (F. Hoffmann-La Roche). After sonication, the supernatant was collected, centrifuged at 13,000g for 10 minutes, and assayed for protein concentration using the BCA kit (Pierce). Total proteins (20–30  $\mu\text{g}$ ) were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to the polyvinylidene fluoride membrane (MilliporeSigma, Burlington, MA). Blocking was performed in Tris-buffer saline containing 3% BSA. Membranes were incubated with the appropriate primary antibodies at a dilution of 1:1,000 overnight at 4 °C. After washing three times, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Bound antibodies were detected using enhanced chemiluminescence plus kit (GE Healthcare, Chicago, IL).

### RT-qPCR

RNA was extracted from cell pellets using RNeasy mini kit (Qiagen, Hilden, Germany) and quantified by spectrophotometry at 260 nm. The cDNA was synthesized in a 20- $\mu\text{l}$  reaction containing 1  $\mu\text{g}$  of RNA and Reverse Transcription system kit (catalog number A3500, Promega) according to the manufacturer's instructions. Real-time qPCR was performed using power SYBER Green mix (catalog number 4367659, Applied Biosystem, Waltham, MA), and specific primers were directed against *CLEC12B* (forward: TGAGGAGAAAACCTGGGCTA; reverse: GCCA-GAGGAGTCCCATGATA) and CRE-binding protein gene *CREB* (forward: TTAACCATGACCAATGCAGCA; reverse: TGGTATGTTTGTACGTCTCCAGA), and results were normalized to the house-keeping gene *RPLPO* (forward:



TGCATCAGTACCCATTCTATCA; reverse:  
AAGGTGTAATCCGTCTCCACAGA).

#### Fontana–Masson staining

Tissue sections were deparaffinized and hydrated with the conventional protocol. Each of the successive steps was followed by three washes in distilled water. Tissue sections were incubated in a warmed (60 °C) ammoniacal silver solution (catalog number HT2001, Sigma-Aldrich) for 30 minutes until the tissue section became brown in color.

The sections were toned by dipping in a gold chloride solution (catalog number HT2002, Sigma-Aldrich) for 30 seconds. The fixation of silver was carried out with 2% sodium thiosulfate (catalog number HT2003, Sigma-Aldrich) for 1–2 minutes. The nuclear contrast was performed with a Nuclear fast red solution (catalog number 3020, Sigma-Aldrich) for 5 minutes. The sections were dehydrated, cleared in xylene, and mounted in a hydrophobic medium (catalog number 4112, Richard-Allan Scientific Mounting Medium, ThermoFisher, Waltham, MA). The sections were examined under a Nikon ECLIPSE Ci microscope (Nikon Instruments, Tokyo, Japan) using dry  $\times 10$  numerical aperture 0.25 and  $\times 20$  numerical aperture 0.40 objectives. Acquisitions were done with a DS – Ri 1 camera (Nikon Instruments).

Pigmentation intensity measurements were done on the slides without counterstaining using ImageJ. Color images were converted to 8-bit images, and tissue regions of interest and thresholds were done manually to select only melanin-containing areas. The sum of intensities (Raw Integrated Density) in melanin-containing areas and total tissue areas were measured. Results were expressed as a ratio between these two parameters (Bolstad et al., 2003; Schindelin et al., 2012). For the statistical analyses, 30 images of each condition were quantified.

#### Immunofluorescence staining (reconstructed epidermis)

Tissue sections were deparaffinized and hydrated with the conventional protocol. Each of the successive steps was followed by three washes in PBS. Sections were saturated for 1 hour at room temperature with PBS 3% BSA, 5% normal goat serum, and 0.15% glycine. Sections were incubated overnight at 4 °C with a specific antibody directed against melanA (see Supplementary Table S1). Sections were then washed three times with PBS and incubated with fluorescent dye–conjugated secondary antibody (Supplementary Table S1) at room temperature for 1 hour. Slides were mounted with Prolong Gold Antifade Reagent with DAPI (Thermo Fisher Scientific). Fluorescence was detected using a confocal microscope (Nikon A1R, Nikon Instruments) using dry  $\times 20$  numerical aperture 0.40 objective. Melanocytes were counted by eye in 30 images of each condition in 10 nonoverlapping fields.

#### Immunofluorescence staining (fixed cells)

Cells were seeded on coverslips for at least 24 hours and subsequently washed with PBS, fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized for 5 minutes with 0.1% Triton X-100, blocked for 1 hour with PBS 3% BSA 5% normal goat serum and incubated overnight at 4 °C with primary antibodies

(Supplementary Table S1). The cells were then washed three times with PBS and incubated with fluorescent dye–conjugated secondary antibody (see Supplementary Table S1) at room temperature for 1 hour. For actin cytoskeleton staining, cells were incubated with Texas Red-X Phalloidin (1:100, catalog number T7471, Life Technologies) for 30 minutes at room temperature. Slides were mounted with DAPI Fluoromount-G (catalog number 0100-20, SouthernBiotech, Birmingham, AL). Fluorescence was detected using a confocal microscope (Nikon A1R) using objective Plan Apo  $\times 63$  oil 1.4 numerical aperture.

#### Immunohistochemical analysis

Skin samples from the Department of Dermatology and Pathology of Centre Hospitalier Universitaire de Nice (Nice, France) were analyzed. Skins from patients of phototypes I/II (n = 5), phototypes III/IV (n = 5), and phototypes V/VI (n = 5) were analyzed. Immunohistochemistry was performed from formalin-fixed, paraffin-embedded tissue sections using the Agilent Dako Autostainer Link 48 (Agilent Technologies, Les Ulis, France) with an antibody against CLEC12B (clone LS-B11313, 1/100, pH9, LSBio, Seattle, WA) according to standard procedures. To perform the revelation of the immunostaining and to amplify the signal, the linker EnVision FLEX+ (Dako Autostainer/Autostainer Plus) was used. Interpretation was performed blindly by one independent pathologist (NCL).

#### Proliferation assays

Cells were plated in 12-well tissue culture plates at a density of 50,000 cells per well in Medium 254 supplemented with human melanocyte growth supplement (Cascade Biologics, Carlsbad, CA). Each condition was plated in triplicate. On day 7, cell viability was determined with 0.4% Trypan Blue Solution (15250061, Gibco).

#### Tyramide-based tyrosinase assay

Tyramide-based tyrosinase assay is performed to assess tyrosinase activity in situ. In the tyramide-based tyrosinase assay procedure, tyrosinase reacts with biotinyl tyramide (structural analog to the natural substrate of tyrosinase), generating biotinylated deposits of the substrate near the enzyme, which are visualized with streptavidin conjugated to a fluorescent dye. Cells were seeded on coverslips for at least 24 hours and subsequently washed with PBS, fixed with 4% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.1% Triton X-100, and blocked with PBS 5% BSA. To enhance assay specificity and avoid potential sources of background, peroxidases are quenched by treatment with hydrogen peroxide 3% for 10 minutes. To block all endogenous biotin, biotin receptors, and avidin binding sites, cells were incubated in AvidinD solution for 15 minutes followed by three washes in PBS before being incubated in biotin solution for 15 minutes (catalog number SP-2001, Vector Laboratory, Burlingame, CA). Cells were incubated with biotinyl tyramide (catalog number NEL700A001KT, PerkinElmer, Waltham, MA) for 15 minutes at room temperature, followed by three washes in PBS. Finally, to detect biotinylated substrates, cells were incubated with streptavidin Texas red conjugate for 1 hour at room temperature. After three washes, slides were mounted with Prolong Gold

Antifade Reagent with DAPI (Thermo Fisher Scientific). Fluorescence was detected using a confocal microscope (Nikon A1R) using objective Plan Apo  $\times 63$  oil 1.4 numerical aperture.

#### Statistical analyses

Statistical analyses were performed with Graphpad Prism 6 software (GraphPad Software, San Diego, CA). Mann–Whitney nonparametric analysis was used to test unpaired differences between two groups. Wilcoxon matched-pairs signed-rank test was used to test paired differences between two groups. For multiple comparisons, we used two-way ANOVA followed by Tukey's post-hoc analysis and Kruskal–Wallis nonparametric test followed by Dunn's posthoc analysis. Differences were considered significant at  $P < 0.05$ .

#### Proteomic identification (master protein with high false discovery ratio)

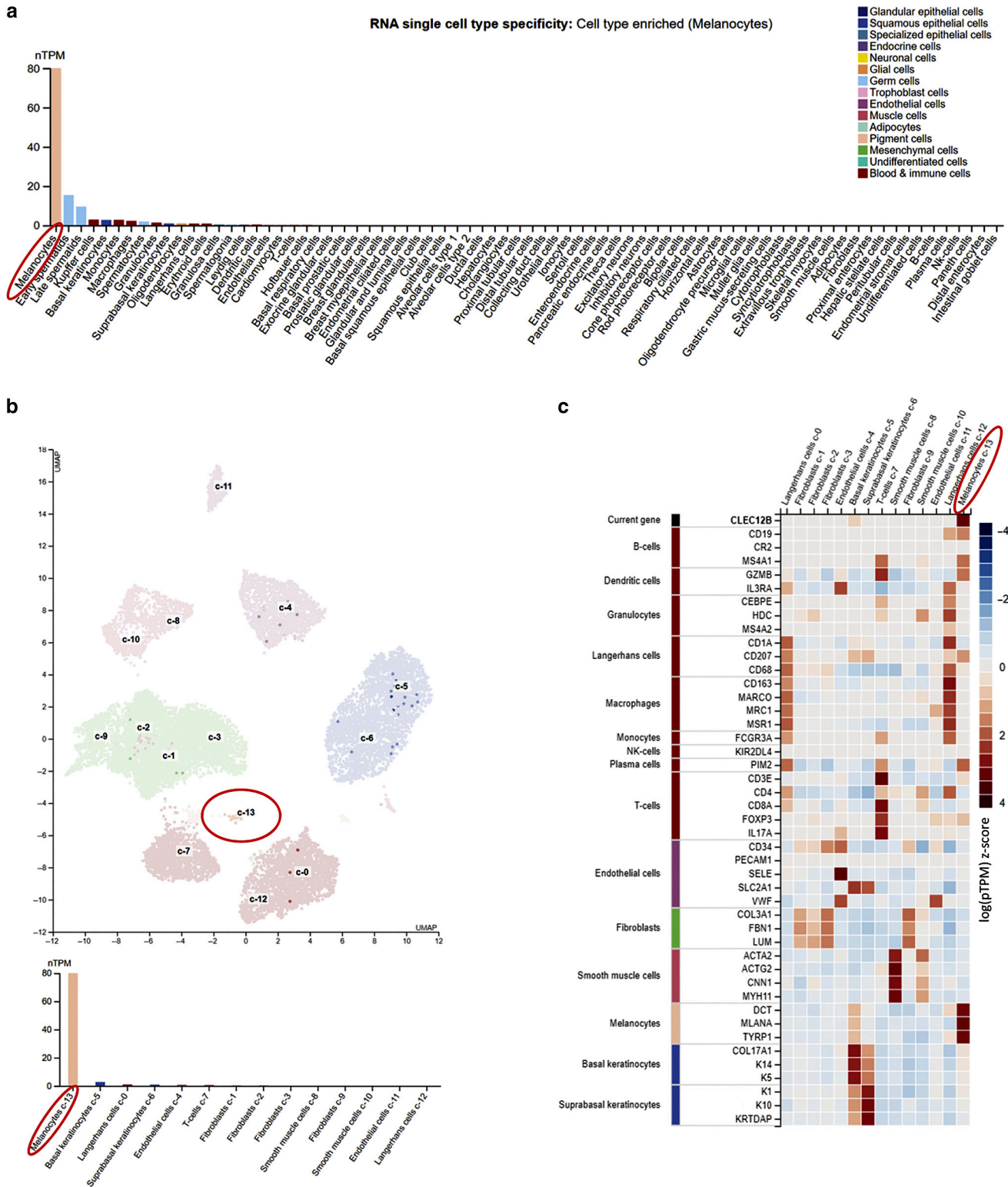
For mass spectrometry analysis, proteins contained in gel slices were reduced/alkylated and digested by treatment with dithiothreitol/iodoacetic acid and trypsin. Peptides extracted were separated using a nano HPLC (Ultimate 3000, Thermo Fisher Scientific). The peptides separation was performed on a 75- $\mu\text{m}$  intradermal  $\times$  250 mm (3  $\mu\text{m}$ , 100  $\text{\AA}$ ) Acclaim PepMap 100 C18 column (Thermo Fisher Scientific) at a flow rate of 300 nL/min. Solvent systems were (i) 100% water and 0.1% formic acid and (ii) 100% acetonitrile and 0.08% formic acid. The following gradient was used:  $t = 0$  minutes 6% B;  $t = 3$  minutes 6% B;  $t = 119$  minutes, 45% B;  $t = 120$  minutes, 90% B;  $t = 130$  minutes 90% B (temperature at 35  $^{\circ}\text{C}$ ). Nano HPLC was coupled to Q-exactive plus mass spectrometer (Thermo Fisher Scientific). mass spectrometry

spectra were acquired at a resolution of 70,000 (200  $m/z$ ) in a mass range of 300–2,000  $m/z$  with an Automatic Gain Control target 3e6 value and a maximum injection time of 100 ms. The 10 most intense precursor ions were selected and isolated with a window of 2  $m/z$  and fragmented by Higher energy C-Trap Dissociation with a normalized collision energy of 27. Tandem mass spectrometry spectra were acquired in the ion trap with an Automatic Gain Control target 2e5 value; the resolution was set at 17,500 at 200  $m/z$  combined with an injection time of 100 ms. Data were reprocessed using Proteome Discoverer 2.1 equipped with Sequest HT. Files were searched against the Swissprot Homo sapiens FASTA database (update of September 2018). Mass accuracy of  $\pm 10$  p.p.m. was used for precursor ions, and 0.02 Da was used for product ions. Enzyme specificity was fixed to trypsin, allowing at most two miscleavages. Because of the previous chemical modifications, carbamidomethylation of cysteines was set as a fixed modification, and only oxidation of methionine was considered as a dynamic modification. Reverse decoy databases were included for all searches to estimate false discovery rates and filtered using the Percolator algorithm at a 1% false discovery ratio.

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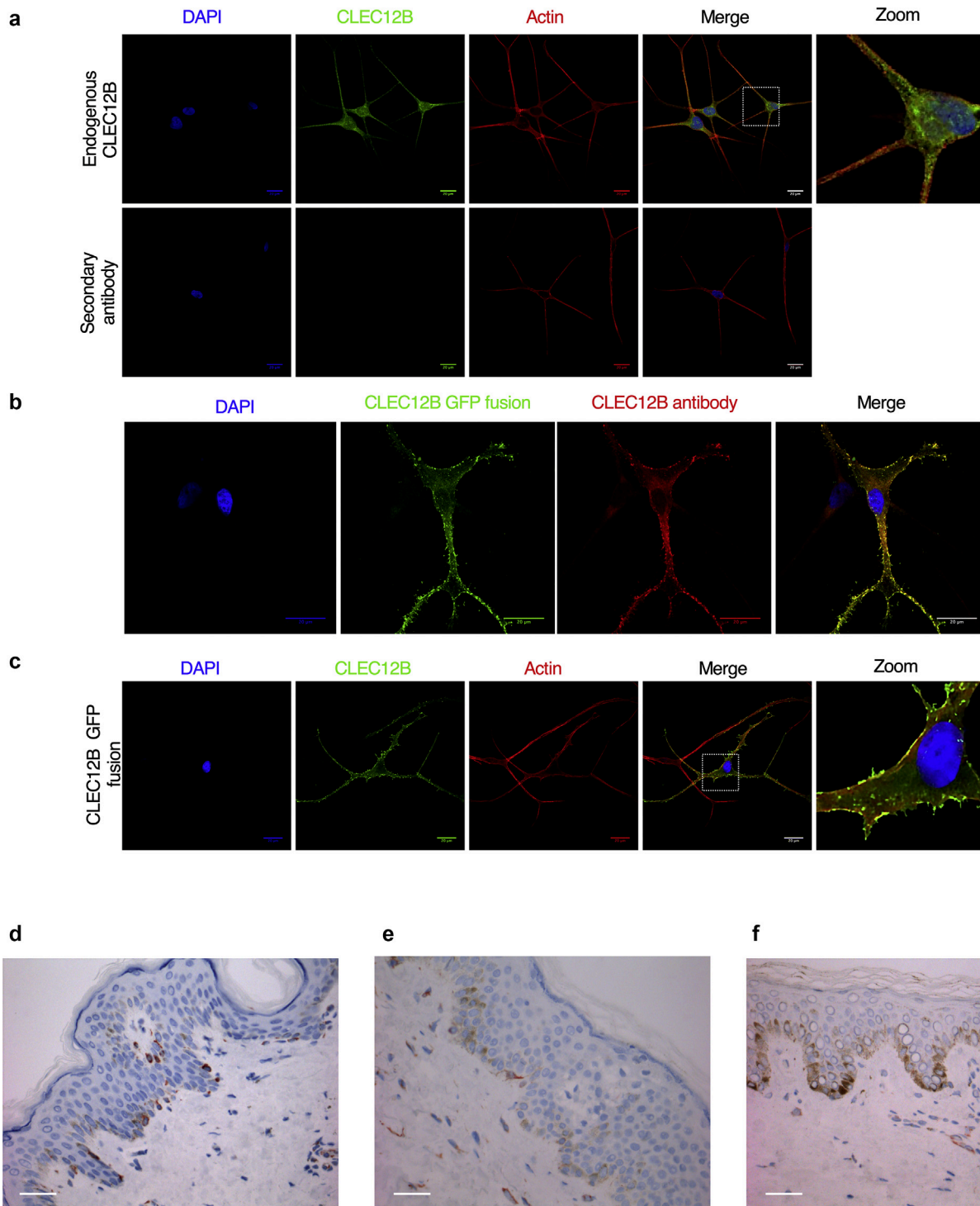
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**Supplementary Figure S1. *CLEC12B* expression is enriched in melanocytes according to the scRNA-seq data analysis from the Human Protein Atlas.** (a) Single-cell transcriptomics data for 13 tissues and PBMCs. These datasets were respectively retrieved from the Single Cell Expression Atlas, the Human Cell Atlas, the Gene Expression Omnibus, and the European Genome-phenome Archive (NX value). (b) RNA expression of *CLEC12B* in the single-cell-type clusters identified in skin visualized by a UMAP plot (top) and a bar chart (bottom) (pTPM indicates transcripts per million protein-coding genes). (c) The heatmap shows the expression of *CLEC12B* and well-known melanocyte markers (dopachrome tautomerase gene *DCT*, *MLANA*, *TYRP1*) in the different single-cell-type clusters of skin. The panel on the left shows which cell type each marker is associated with. NX, normalized expression; scRNA-seq, single-cell RNA sequencing; UMAP, Uniform Manifold Approximation and Projection.





**Supplementary Figure S2. Cytoplasmic and membrane subcellular localization of CLEC12B in melanocytes.** (a) Immunofluorescence staining of CLEC12B performed on fixed melanocytes showing cytoplasm subcellular localization. Alexa fluor 594 phalloidin staining of actin cytoskeleton was used to visualize cell morphology; Alexa fluor 488 secondary antibody was used to reveal CLEC12B staining. (b) Nuclei were stained with DAPI. Immunofluorescence staining of CLEC12B was performed on CLEC12B GFP fusion protein transduced melanocytes. Alexa fluor 594 secondary antibody was used to reveal CLEC12B staining, and DAPI was used for nucleus staining. (c) Lentiviral infection was used to overexpress CLEC12B GFP fusion protein. Alexa fluor 594 phalloidin staining of actin cytoskeleton was used to visualize cell morphology. Bar = 20 µm. IHC staining of CLEC12B was performed on human skin samples of (d) skin phototype I, (e) skin phototype III, and (f) skin phototype VI. Melanin was visualized as brown, and CLEC12B was visualized as red stain. Bar = 25 µm. IHC, immunohistochemistry.



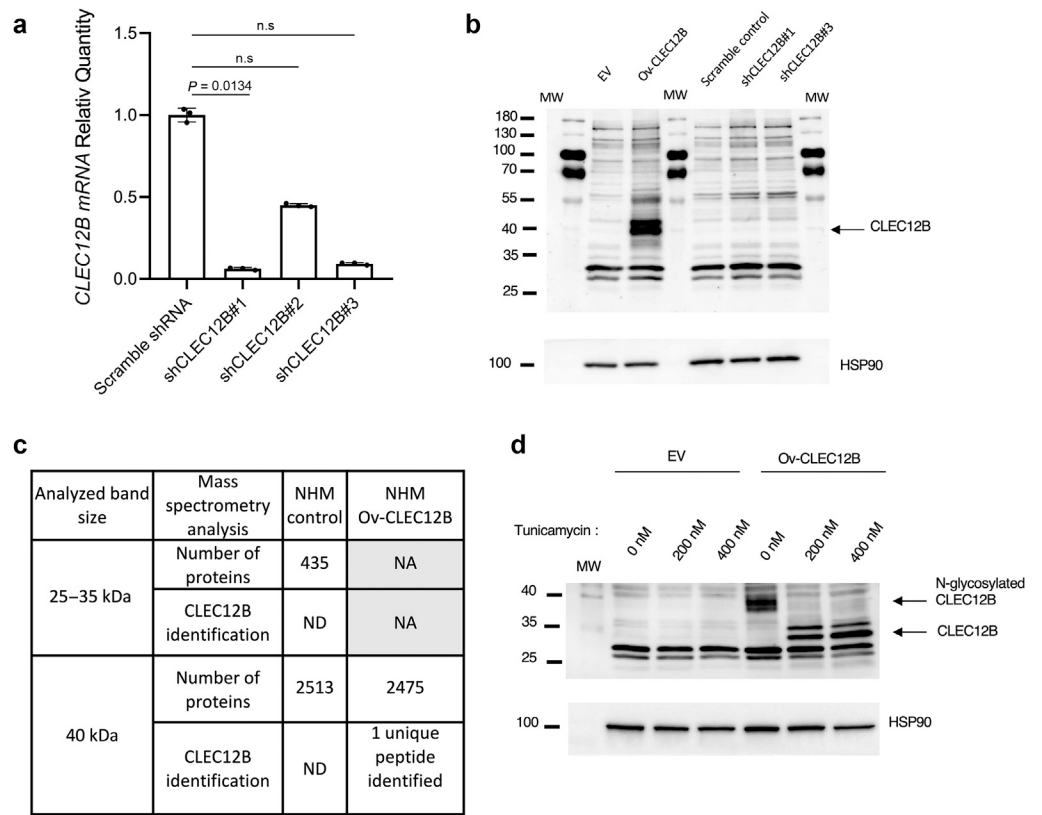
**Supplementary Figure S3.**

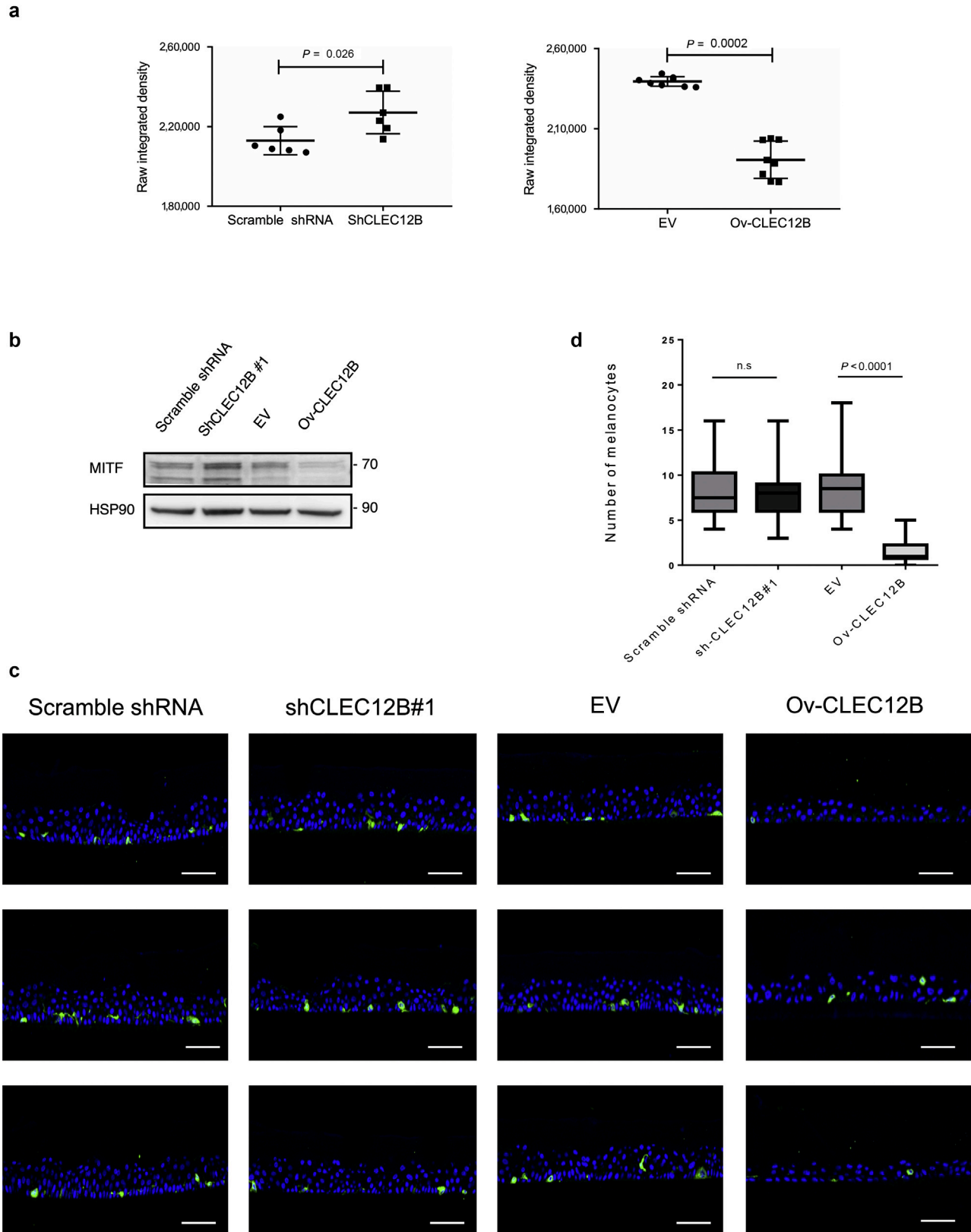
**Glycosylated form of CLEC12B is detected at 40 kDa in CLEC12B-overexpressed melanocytes. (a)**

*CLEC12B* mRNA relative quantity was examined by qPCR in melanocytes transduced with scramble shRNA and sh*CLEC12B* sequence #1, #2, or #3. qPCR results were normalized to those of house-keeping gene *RPLPO* and expressed as fold change relative to scrambled shRNA. Error bars represent mean ± SD. (b) Western blot analysis of *CLEC12B* protein expression level in normal human melanocytes

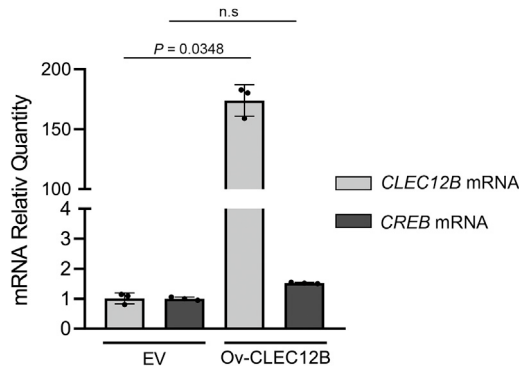
(phototype III/IV) transduced with scramble shRNA and sh*CLEC12B* sequence #1 and #3. HSP90 was used as a loading control. (c) Mass spectrometry analysis of proteins contained into gel slice. Peptides extracted were analyzed by nanoLC-MS/MS. Data reprocessing was done using Protein Discoverer 2.2 against the human-reviewed database (Human\_reviewed\_180910). (d) Glycosylation pattern of *CLEC12B* in normal human melanocytes

transduced with EV or Ov-*CLEC12B* and treated with 200 nM and 400 nM of tunicamycin for 4 hour before protein extraction and western blotting. HSP90 was used as a loading control. EV, empty vector; HSP, heat shock protein; LC-MS/MS, liquid chromatography with tandem mass spectrometry; NA, not applicable; ND, not determined; NHM, normal human melanocyte; n.s., not significant; Ov-*CLEC12B*, overexpressed *CLEC12B*; sh*CLEC12B*, short hairpin RNA-silenced *CLEC12B*; shRNA, short hairpin RNA.





**Supplementary Figure S4. *CLEC12B* modulates pigmentation in reconstructed human skin.** (a) The variation of pigmentation observed in each skin constructs was quantified using ImageJ software and shown as a scatter plot, with bars representing mean  $\pm$  SD (scramble shRNA and shCLEC12B#1:  $n = 6$ ; EV and Ov-CLEC12B:  $n = 8$ ). Differences between groups were tested using Mann–Whitney test and were considered significant at  $P < 0.05$ . (b) Western blot analysis of MITF expression level in protein lysates extracted from the reconstructed epidermis. HSP90 was used as a loading control. The melanocytes were observed by confocal microscopy after immunofluorescence staining of (c) MelanA and (d) counted by eyes. Alexa fluor 647 secondary antibody was used to reveal MelanA staining. Nuclei were stained with DAPI. Bar = 50  $\mu\text{m}$ ;  $n = 30$  quantified images per condition. Results are shown as box and whisker plots, with bars representing 5–95% confidence interval. Differences between groups were tested using one-way ANOVA test with Sidak’s multiple comparisons and were



**Supplementary Figure S5. *CLEC12B* does not affect CRE-binding protein transcriptional regulation.** Relative expression of *CLEC12B* and *CREB* mRNA was determined by qPCR in EV- and *CLEC12B*-overexpressed melanocytes. qPCR results were normalized to those of house-keeping gene *RPLPO* and are expressed as fold change relative to the control. Error bars represent mean ± SD. Differences between groups were tested using Kruskal–Wallis test with Dunn’s multiple comparisons and were considered significant at  $P < 0.05$ . EV, empty vector; n.s., not significant; Ov-CLEC12B, overexpressed *CLEC12B*.

**Supplementary Table S1. List of Antibodies Used in the Study**

Primary Antibody	Source Isotype	Supplier	Catalog Number
<i>CLEC12B</i>	Rabbit	Proteintech	26 077-1-AP
CREB	Rabbit IgG	Cell Signaling Technology	9197
Phospho-CREB (Ser133)	Rabbit IgG	Cell Signaling Technology	9198
SHP-1	Mouse IgG1	BD Transduction Laboratories	610125
Phospho-SHP-1 (Tyr 564)	Rabbit IgG	Cell Signaling Technology	8849
SHP-2	Mouse IgG1	BD Transduction Laboratories	610621
Phospho-SHP-2	Rabbit	Cell Signaling Technology	3751
MITF	Mouse	Abcam	Ab12039
TYR	Rabbit	In house	–
DCT	Rabbit	In house	–
TRP1	Mouse	Abcam	Ab3312
Texas Red-X Phalloidin		Life Technologies	T7471
MelanA	Rabbit	Abcam	Ab51061

Secondary Antibody (WB)	Source Isotype	Supplier	Catalog Number
Goat Anti-Rabbit Immunoglobulins/HRP		Dako	P0448
Goat Anti-Mouse Immunoglobulins/HRP		Dako	P0447
Secondary antibody (IF)			
Alexa Fluor 488		Invitrogen	A11034
Goat anti-Rabbit IgG			
Alexa Fluor 488		Invitrogen	A11032
Goat anti-Mouse IgG			
Alexa Fluor 647		Invitrogen	A21244
Goat anti-Rabbit IgG			



Abbreviations: CREB, CRE-binding protein; DCT, dopachrome tautomerase; IF, immunofluorescence; phospho-CREB, phosphorylated CRE-binding protein; phosphor-SHP-1, phosphorylated SHP-1; phosphor-SHP-2, phosphorylated SHP-2; TYR, tyrosinase; WB, western blot.

← considered significant at  $P < 0.05$ . EV, empty vector; HSP, heat shock protein; n.s., not significant; Ov-CLEC12B, overexpressed *CLEC12B*; sh*CLEC12B*, short hairpin RNA-silenced *CLEC12B*; shRNA, short hairpin RNA.



Article

# Acadesine Circumvents Azacitidine Resistance in Myelodysplastic Syndrome and Acute Myeloid Leukemia

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**Abstract:** Myelodysplastic syndrome (MDS) defines a group of heterogeneous hematologic malignancies that often progresses to acute myeloid leukemia (AML). The leading treatment for high-risk MDS patients is azacitidine (Aza, Vidaza<sup>®</sup>), but a significant proportion of patients are refractory and all patients eventually relapse after an undefined time period. Therefore, new therapies for MDS are urgently needed. We present here evidence that acadesine (Aca, Acadra<sup>®</sup>), a nucleoside analog exerts potent anti-leukemic effects in both Aza-sensitive (OCI-M2S) and resistant (OCI-M2R) MDS/AML cell lines in vitro. Aca also exerts potent anti-leukemic effect on bone marrow cells from MDS/AML patients ex-vivo. The effect of Aca on MDS/AML cell line proliferation does not rely on apoptosis induction. It is also noteworthy that Aca is efficient to kill MDS cells in a co-culture model with human medullary stromal cell lines, that mimics better the interaction occurring in the bone marrow. These initial findings led us to initiate a phase I/II clinical trial using Acadra<sup>®</sup> in 12 Aza refractory MDS/AML patients. Despite a very good response in one out 4 patients, we stopped this trial because the highest Aca dose (210 mg/kg) caused serious renal side effects in several patients. In conclusion, the side effects of high Aca doses preclude its use in patients with strong comorbidities.

**Keywords:** acadesine; azacitidine; apoptosis; MDS; AML; Phase I/II clinical trial

## 1. Introduction

Myelodysplastic syndromes (MDS) represent a group of myeloid neoplasms characterized by blood cytopenia and an increased risk of leukemic transformation. Due to the progressive aging of the population in western countries, the number of MDS patients will significantly increase in the next decades. In this context, MDS will be one of the most challenging issues for hematologists and health care providers in the near future. Azacitidine (Aza, Vidaza<sup>®</sup>), a pyrimidine nucleoside analog is routinely used as the leading treatment for myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) patients ineligible for intensive chemotherapy [1,2]. In MDS patients treated with Aza the overall survival is significantly prolonged by 10 months versus conventional care. Unfortunately, 40% of patients are primary refractory to this drug and 60% of initial responders will relapse in an undefined period. After failure or relapse, overall survival (OS) of these patients is very poor [3].

Aza is a hypomethylating agent that induces apoptosis and cell cycle arrest [4]. We have previously characterized a defect in the mitochondrial pathway of apoptosis in Aza-resistant cells [5]. Concomitantly, we identified a high level of basal autophagy in Aza-resistant cell lines and proposed that induction of autophagic cell death could be a pertinent therapeutic strategy to eradicate Aza-resistant cells [6]. Acadesine (Aca, Acadra<sup>®</sup>) is another nucleoside analog that has proven anti-leukemic effect in hematopoietic malignancies. Indeed, it was shown to induce apoptosis in B-cell chronic lymphocytic leukemia [7–10] and also autophagic cell death in chronic myelogenous leukemia cells [11]. Aca is an AMP mimetic that can induce autophagic cell death via activation of AMPK and inhibition of the mTOR pathway or both [7,12–14].

Autophagy is an essential lysosomal catabolic pathway for the recycling of macromolecules, bulk cytoplasm and damages organelles. It exerts a paradoxical role in the control of cell death and survival [15]. Autophagy is activated under stress conditions such as nutrient deprivation, hypoxia and drugs treatment. The main function of autophagy is to refuel energy to the cell to promote cell survival under adverse conditions, but there is increasing evidence that autophagy can serve *per se* as a cell death mechanism under certain circumstances [16] and can also favor emergence of tumor initiating cells [17–20].

The aim of this study was to evaluate *in vitro*, *in vivo* and *ex vivo* the effect of Aca in MDS and AML cell lines and bone marrow cells from MDS/AML patients. We found that Aca was highly efficient to eliminate Aza-resistant cell lines and primary bone marrow myeloid cells from Aza-sensitive and resistant MDS and AML patients. A phase I/II clinical trial devoted to investigating the efficacy of Aca in Aza-refractory patients was initiated. However, due to serious renal side effects of Aca at the higher dose used in the study, the trial stopped and only 4 patients were fully analyzed. Among them, one exhibited a very good response (80% decrease in blast count). Although high doses of Aca in humans may have a side effect that precludes a chronic use in elderly patients, lower doses of Aca in combination with Aza might could be evaluated in MDS and AML patients.

## 2. Results

### 2.1. Aca Induces Cell Death in an Apoptosis-Independent Manner in MDS Cell Lines

Nucleoside analogs are mandatory molecules for the treatment of MDS and AML patients as exemplified by the beneficial therapeutic effect of Aza in both hematopoietic malignancies. Aca is also a nucleoside analog that has shown promising effect *in vitro* in B cell chronic lymphocytic leukemia [7,8], mantle cell lymphoma [21] and chronic myelogenous leukemia [11,22]. This prompted us to investigate the anti-proliferative and cell death inducing capacity of Aca in myeloid leukemia and more particularly in MDS/AML cell lines. As resistance to Aza is a consistent hallmark of MDS patients [6,23], we also took advantage in the present study of the availability of an Aza-resistant cell line (OCI-M2R) recently generated by our team. As expected, and conversely to Aza-sensitive OCI-M2S cells, OCI-M2R cells were resistant to this drug at 24 and 48 h (Figure 1A,B, right panels). Of note, Aca induced a dose-dependent loss of cell proliferation in OCI-M2S and OCI-M2R cells, as well. In both cell lines,

a maximal inhibition of cell proliferation was obtained for 2 mM Aca (Figure 1A,B, left panels) and the dose of Aca triggering 50% inhibition of cell proliferation (IC<sub>50</sub>) was less than 1 mM at 48 h (Figure 1B, left panel), which is in the range of Aca effect's in other hematopoietic cell lines. We next look for the effect of Aza and Aca on both apoptosis induction and LC3-II accumulation in OCI-M2S and OCI-M2R cells. Aza (1 μM) triggered caspase-3 cleavage at 24 h in OCI-M2S cells but not in their Aza-resistant counterpart, as expected (Figure 1C). At the same time, Aca failed to induce caspase 3 cleavage in OCI-M2S and OCI-M2R cells. Aza was also found to increase LC3-II accumulation in both cell lines, while Aca only moderately increased LC3-II conversion at 6 h in OCI-M2R cells (Figure 1C). Increased caspase-3 enzymatic activity was detected in OCI-M2S cells treated 24 h with Aza, but not in OCI-M2R cells (Figure 1D). Conversely to Aza, Aca failed to increase caspase 3 activity in sensitive and resistant lines. Rather, Aca slightly inhibited caspase 3 activity in OCI-M2S cells (Figure 1D). The increase in caspase 3 induced by Aza matched with the induction of apoptotic cell death already reported in other MDS/AML sensitive cell lines [6,23]. Finally, Aca failed to induce caspase activation in sensitive and resistant cell lines as well, as anticipated from the lack of caspase 3 cleavage illustrated in Figure 1C. As caspase activation was observed following Aza but not Aca treatment, we further investigated the role of apoptosis in the effect of both drugs. Thus, we first assessed the effect of qVD, a pan-caspase inhibitor on Aca-mediated loss of cell proliferation in OCI-M2S and OCI-M2R cells (Figure 2A). The effect of different concentrations of Aca on OCI-M2S and OCI-M2R cell proliferation was unaffected by qVD a pan-caspase inhibitor, indicating that it does not rely on apoptosis induction (Figure 2A), whereas the effect of Aza was impaired by qVD in OCI-M2S cells (Figure 2B) in agreement with the known proapoptotic effect of this drug. As expected, Aza failed to affect cell proliferation in OCI-M2R cells. These findings highlight the implication of another mode of cell death distinct from apoptosis and likely from autophagy in the mechanism of action of Aca.

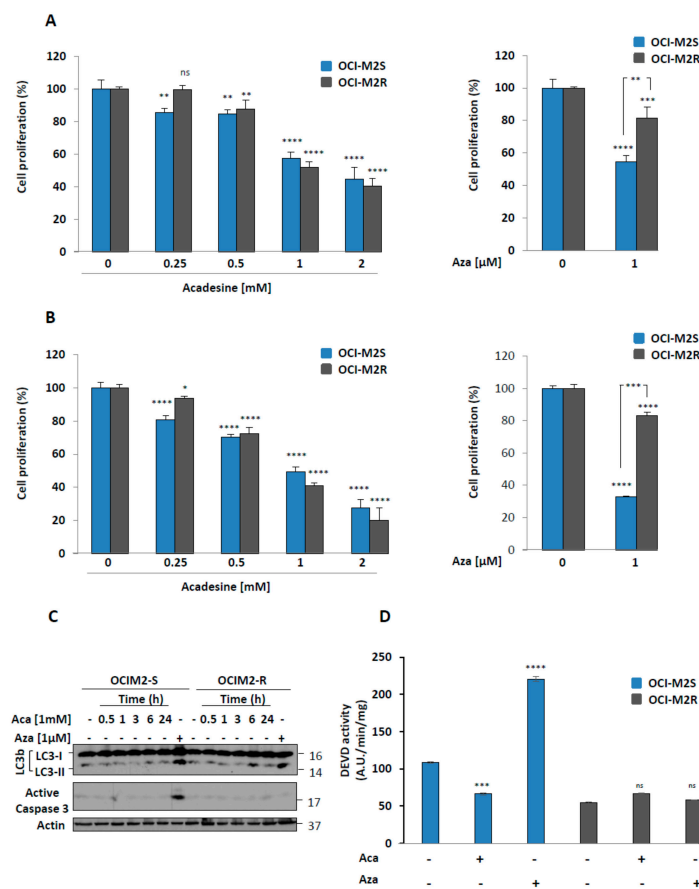
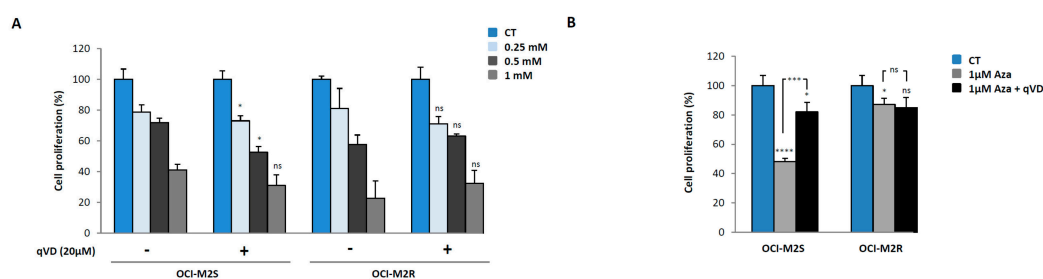


Figure 1. Aca exerts anti-leukemic effects on both OCI-M2S and OCI-M2R cell lines (A) Left panel,



OCI-M2S and OCI-M2R cells ( $0.5 \times 10^6$  cells/mL) were treated or not with increasing concentrations of Aca for 24 h at 37 °C. Right panel, OCI-M2S and OCI-M2R cells ( $0.5 \times 10^6$  cells/mL) were treated or not with 1  $\mu$ M Aza, then in a both experiments cell metabolism was measured using the XTT assay as described in Materials and Methods section. Results are means  $\pm$  SD of 3 different determinations made in triplicate. (B) Left and right panels, OCI-M2S and OCI-M2R cells ( $0.5 \times 10^6$  cells/mL) were treated and analyzed as described in Figure 1A for 48 h at 37 °C. (C) OCI-M2S and OCI-M2R cells ( $1 \times 10^6$  cells/mL) were incubated for various times at 37 °C with 1  $\mu$ M Aza or 1 mM Aca. Whole-cell lysates were prepared, and expression of Poly-ADP-Ribose polymerase (PARP), LC3 and caspase 3 was visualized by western blotting. Actin was used as loading control. (D) OCI-M2S and OCI-M2R cells ( $0.5 \times 10^6$  cells/mL) were treated or not with 1 mM Aca or 1  $\mu$ M Aza for 24 h at 37 °C. Cells were harvested, washed, and lysed in caspase buffer. Caspase-3 activity was evaluated in quadruplicate using Ac-DEVD-AMC as substrate. To allow specific assessment of caspase activity, hydrolysis was followed as a function of time in the presence or the absence of 10 mM Ac-DEVD-CHO. Results were expressed as arbitrary units (a.u.) per min and per mg of proteins and are the means  $\pm$  SD of 4 independent experiments performed in quadruplicate. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , ns: not significant.



**Figure 2.** The anti-leukemic effects of Aca are mediated by caspase independent mechanisms (A) OCI-M2S and OCI-M2R cells ( $0.5 \times 10^6$  cells/mL) were treated with increasing concentrations of Aca for 48 h at 37 °C, in the presence or in the absence of 20  $\mu$ M Z-VAD-fmk, a pan-caspase inhibitor. Cell metabolism was measured using the XTT assay as described in Materials and Methods section. (B) OCI-M2S and OCI-M2R cells ( $0.5 \times 10^6$  cells/mL) were treated with 1  $\mu$ M of Aza for 48 h at 37 °C, in the presence or in the absence of 20  $\mu$ M Z-VAD-fmk, a pan-caspase inhibitor. Cell metabolism was measured using the XTT assay as described in Materials and Methods section. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , ns: not significant.

## 2.2. Aca Efficiently Kills Primary Cell from Aza-Resistant MDS and AML Patient *ex vivo*.

The effect of Aca was next investigated in 6 high-risk (RAEB-2) MDS patients (please see Table 1A-C for patients' characteristics). All patients were clinically refractory to Aza (Table 1A) and accordingly, primary cells from 6 patients were found to be fully resistant to Aza as shown by the lack of Aza effect on cell proliferation, (Figure 3A). Interestingly, Aca induced a dose-dependent decrease of cell proliferation in the blasts of all 6 patients that was highly significant at all the Aca concentrations used (Figure 3A). The same experiments were also conducted in primary cells from 6 AML patients clinically refractory to Aza (Table 1A-C for patients' characteristics). Primary cells from 4 patients were totally insensitive to Aza treatment, whereas 2 patients (red symbols) were partially sensitive to the drug. Once again Aca triggered a dose-dependent and highly significant loss of cell proliferation in the 6 AML samples, independently of the level of resistance to Aza *ex vivo* (Figure 3B).

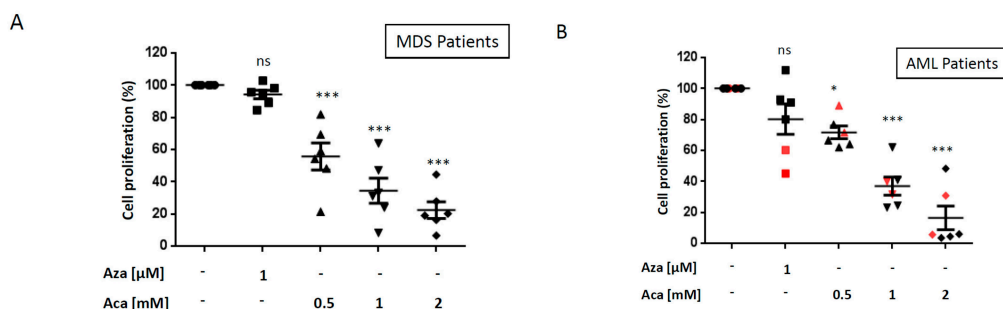
**Table 1.** Patients' characteristics

<b>(A)</b>		<b>n = 12</b>	
<b>WHO Classification</b>			
	RAEB-2	6 (50%)	
	AML 20–30 %	6 (50%)	
<b>IPSS Cytogenetic Risk</b>			
	Good	3 (25%)	
	Intermediate	3 (25%)	
	Poor	6 (50%)	
<b>Cytogenetics Abnormalities</b>			
	Normal Karyotype	3 (25%)	
	+8	1 (8%)	
	7 Abnormalities	3 (25%)	
	5 Abnormalities	3 (25%)	
	Complex > 3 Abnormalities	2 (17%)	
<b>IPSS Classification</b>			
	Low	0	
	Intermediate 1	0	
	Intermediate 2	2 (17%)	
	High	10 (83%)	
<b>Aza Response Status</b>			
	Failure	8 (67%)	
	Relapse	4 (33%)	
	<b>Number of Aza Cycles before Relapse or Failure</b>	6 [1–27]	
<b>(B)</b>			
<b>Patients</b>	<b>Disease</b>	<b>IPSS Cytogenetic Risk</b>	<b>Cytogenetic Abnormalities</b>
#1	RAEB-2	Intermediate	Normal
#2	RAEB-2	Intermediate	Normal
#3	RAEB-2	Intermediate	Normal
#4	RAEB-2	Intermediate	47,XY,+8
#5	RAEB-2	Intermediate	Normal
#6	RAEB-2	Intermediate	46,XY(del(9)(q12q31)/47,idem,+21
#7	AML	Poor	45,XX,-7
#8	AML	Poor	45,XX,t(3;11)(q22,q33),del(9)(q22,q33),-7
#9	AML	Poor	45,XX,-7, inv(3), del11q
#10	AML	Intermediate	46,XY,inv(3)(q21q26)
#11	AML	Poor	46,XX,del(5)(q13q33)/47, idem, del(1)(p3?4),+mar/46,XX
#12	AML	Good	45,X,-Y
<b>(C)</b>			



Table 1. Cont.

Patients	IPSS Classification	AZA Response Status	Number of AZA Cycles before Relapse or Failure
#1	High	Failure	3
#2	Intermediate 2	Relapse	27
#3	High	Failure	6
#4	High	Relapse	9
#5	Intermediate 2	Relapse	19
#6	High	Relapse	26
#7	High	Failure	6
#8	High	Failure	4
#9	High	Failure	6
#10	High	Failure	1
#11	High	Failure	6
#12	High	Failure	6



**Figure 3.** Aca exerts potent anti-leukemic effect in MDS and AML bone marrow cell ex vivo. Bone marrow cells from Aza refractory MDS/AML patients ( $10^6$ /mL) were incubated for 24 h with 1  $\mu$ M Aza or different concentration of Aca. Loss of cell proliferation was assessed using the XTT assay as described in Figure 1. Each assay was performed in quadruplicate. The mean values for each MDS patient and each AML patients are shown on Figure 3 (A) and (B) respectively. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , ns: not significant. The bone marrow cells from two patients (in red) were slightly sensitive to Aza.

### 2.3. Phase I/II Clinical Trial of Aca in MDS/AML Patients

In a previous phase I/II clinical trial [24] in patients with relapsed/refractory chronic lymphocytic leukemia a manageable and predictable safety profile was demonstrated for Aca at single doses between 50 and 210 mg/kg. The main side effects were hyperuricemia and renal impairment that were fully reversible upon allopurinol treatment. There was evidence of anti-leukemic activity on 16/24 patients based on a 20% reduction in B-cell counts. In the same line, we initiated a dose escalation safety and tolerability effect of Aca in Aza-refractory MDS and AML patients. A total of 4 patients were included in the trial and received either 140 mg/kg (3 patients) or 210 mg/kg (1 patient) of Aca (Figure 4A). Patient #1 received 6 cycles, patient #3 received 3 cycles, patients #2 and patient #4 received 2 cycles of Aca. Two patients progressed on Aca (patients #2 and #3) as assessed by the percentage of blast count, one was stable (patient #4). However, the treatment was stopped after 2 to 3 cycles of Aca due to serious renal toxicities (Figure 4B). Patient #1, for whom 6 cycles of Aza were performed, exhibited a very strong reduction (50%) of his blast count after only 2 cycles of Aca and more than 70% after 6 cycles (Figure 4C). Despite encouraging results for one AML patient (patient #1), the trial was stopped essentially due to grade 3 urinary and renal toxicities, that were however reversible after stopping Aca (Figure 4B).

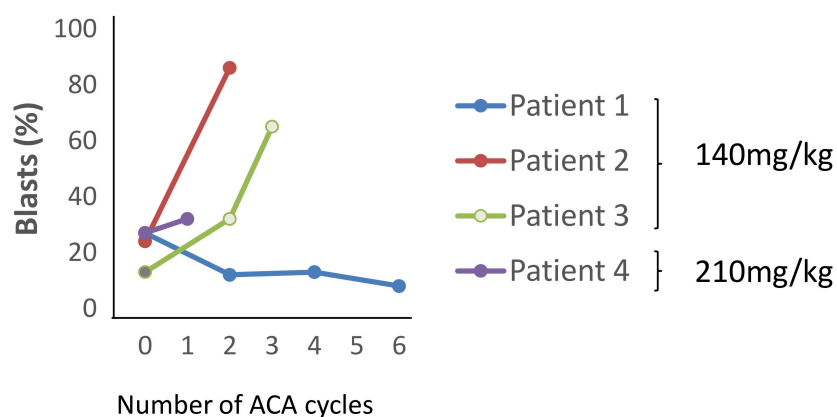
A.

	Patient #1	Patient #2	Patient #3	Patient #4
Age (years)	71	63	74	70
WHO classification	AML	AML	RAEB-2	AML
Blasts (%)	27	24	13	27
Cytogenetic	Good	Int	Poor	Good
IPSS	High	High	High	High

B.

N° ACA cycle	1	2	3	4	5	6
<b>Number of patients / cycle</b>	4	3	2	1	1	1
<b>Number of toxicities /cycle</b>						
Infections	3	1	0	0	0	0
Cardio-Vascularly	0	0	0	0	0	0
Pulmonary	0	0	0	0	0	0
Skin	0	0	0	0	0	0
Intestinal	3	2	0	0	0	0
Neurological	0	0	0	0	0	0
Urinary	2	0	0	0	0	1
Nephrological	3	0	1	0	0	0
Endocrinological	0	0	0	0	0	0
Muscularly	3	1	0	0	0	0
Allergic	0	0	0	0	0	0
Others	3	4	1	0	0	0

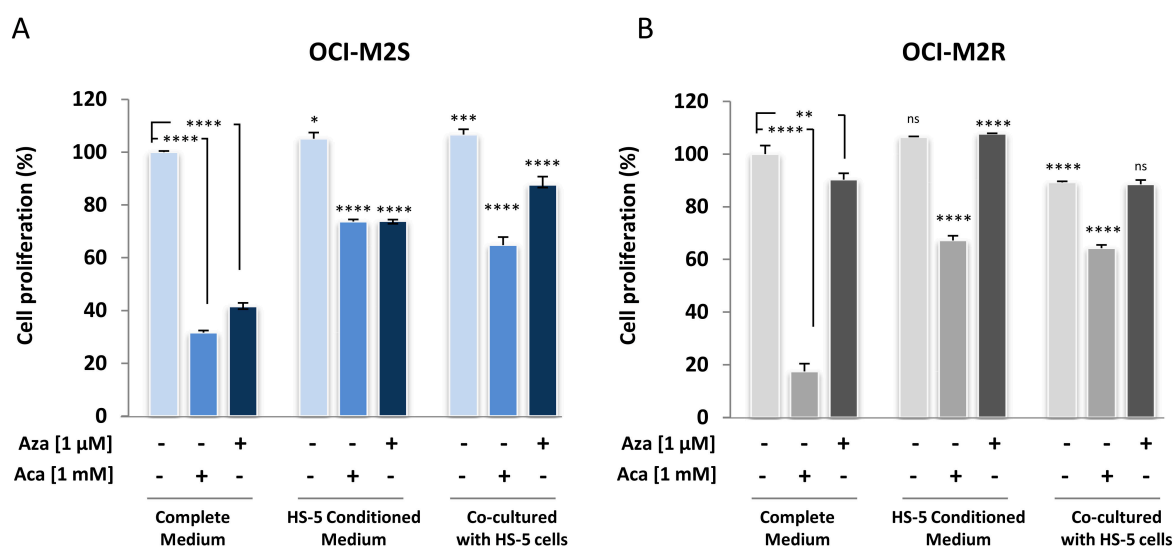
C.



**Figure 4.** Phase I/II clinical trial of Aca in Aza-refractory MDS/AML patients. Four patients (1 MDS, and 3 AML) were enrolled in the assay and treated on Day 1 (D1), D3, D5, D8, D10 and D12 of a 28–56 day-course with Aca at 140 and 210 mg/kg. (A) Characteristics of the four enrolled patients. (B) Main side effects of the 4 enrolled patients. (C) Blast counts of the different patients during the course of treatment.

#### 2.4. Medullary Stromal Cells Inhibit Aca-Induced Cell Death

We next assessed the ability of Aca and Aza to induce cell death in OCI-M2S and OCI-M2R cells co-cultured with human medullary stromal HS-5 cells or HS-5 conditioned medium. As expected, Aza efficiently killed OCI-M2S cells at 1  $\mu$ M (Figure 5A) unlike resistant cells (Figure 5B). Importantly, when OCI-M2S cells were co-cultured for 48 h with stromal cells or in the presence of conditioned medium of HS-5 stromal cells, Aza was strikingly less efficient (Figure 5A). Aca killed OCI-M2S and OCI-M2R cells at 1 mM (Figure 5A,B). Co-culture conditions using either OCI-M2S (Figure 5A) or OCI-M2R (Figure 5B) and a monolayer of HS-5 stromal cells were next performed. Stromal cells diminished Aca-induced cell death in both cell lines (Figure 5A,B). We also wondered whether conditioned medium from HS-5 stromal cells could alone inhibit the effect of Aca. To this aim, OCI-M2S and OCI-M2R cells were grown 1h in complete or HS-5 conditioned medium and next treated with 1 mM Aca. 48 h later, cell proliferation was analyzed (Figure 5A,B). This last experiment clearly shows that the conditioned medium of HS-5 stromal cells inhibited Aca-induced cell death (Figure 5A,B) to roughly the same extent than HS5 stromal cells, suggesting the ability of factors present in the medium partially block antileukemic effect of Aca. In conclusion, even in the presence of HS5 stromal cells or conditioned media collected from HS5 stromal cells, Aca maintained a significant antileukemic activity on OCI-M2R cells.



**Figure 5.** Aca fails to induce its antileukemic effect in MDS/MSC co-culture experiments. (A) OCI-M2S cells were growth in complete, in HS-5 conditioned medium or co-cultured with HS-5 stromal cells. Cells were then treated with 1 mM Aca or 1  $\mu$ M Aza. 48 h later, loss of cell proliferation was assessed using the XTT assay as described in Figure 1. Each assay was performed in quadruplicate. (B) OCI-M2R cells were growth in complete, in HS-5 conditioned medium or co-cultured with HS-5 cells. Cells were then treated with 1 mM Aca or 1  $\mu$ M Aza. 48 h later, loss of cell proliferation was assessed using the XTT assay as described in Figure 1. Each assay was performed in quadruplicate. Statistical analyzes of histograms for “HS-5 Conditioned Medium” and “Co-cultured with HS-5” conditions are compared to the “Complete Medium” used as control. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ , ns: not significant.

### 3. Discussion

MDS defines a group of heterogenous hematologic malignancies that frequently evolved to acute myeloid leukemia. Aza, is an essential treatment for both high-risk MDS and AML patients. However, resistance to Aza is a recurrent clinical problem in treated patients and there is currently no therapeutic option for patients after Aza failure. In the present study, we demonstrate that Aca is able to bypass Aza resistance in MDS and AML cell lines and more interestingly in patient’s samples. We initiated a dose escalation safety and tolerability effect of Aca in Aza-refractory MDS and AML patients. Despite a

very good response in one out 4 patients, we stopped this trial because both doses of Aca (140 and 210 mg/kg) caused serious renal side effects in several patients.

We have previously reported that Aca induces autophagic cell death in Imatinib-sensitive and resistant K562 BCR-ABL expressing CML cell lines. Aca was also very efficient to trigger tumor regression in a xenografted model of imatinib-resistant K562 cells in vivo [11]. From a mechanistic point of view, recent work from the literature also suggested that Aca eradicates CML cells by suppressing activation of the mTOR pathway in BCR-ABL expressing cells [22]. Activation of AMPK or conversely inhibition of mTOR appear therefore as pertinent therapeutic options for the treatment of BCR-ABL expressing malignancies raising the potential use of AMPK activators such as Aca or Metformin in the treatment of refractory CML and Ph(+) acute lymphoblastic leukemia [22].

Previously, we reported that Aca exerts its antileukemic effect by a caspase-independent cell death pathway in CML cells. In the present study, we extended these findings to MDS and AML cell lines and patients. In addition although the mode of cell death induced by Aca was not mechanistically identified in the present study, we established that this drug was unable to induce apoptosis in MDS and AML cell lines and exerted only a minimal effect on autophagy induction, in agreement with an unidentified cell death mechanism.

Aca, has achieved phase I/II clinical trials in B-cell chronic lymphocytic leukemia [24]. A modest reduction of B cell count (20%) was observed for two-thirds of patients. We show here that Aca has also shown some promising anti-neoplastic properties in MDS but also non-negligible side effects at high doses that preclude its use in elderly patients. Nevertheless, the present data underscoring the efficacy of Aca in vitro and in vivo and in one out 4 patients in Aza-resistant MDS/AML cell lines pave the way for reassessing the effect of low and more tolerated doses of Aca (<140 mg/kg/day) in Aza refractory MDS/AML patients. The end pathway major metabolite of Aca is uric acid. In this context, the rapid and effective management of uric acid overload at this dose could help to analyze the anti-leukemic effects of Aca in the longer term.

It is well documented that the bone marrow environment greatly impacts drug availability and efficiency. Of note, it was recently reported that MSCs could participate to the mechanisms of chemoresistance to Aza in MDS. The data presented herein show that factors secreted in the human bone marrow microenvironment by stromal cells can impair the response to Aza, but only moderately affect the response to Aca. We have recently derived new Aca analogs that are one thousand-fold more efficient than Aca [25]. The effect of these inhibitors are currently evaluated in MDS and AML cell lines.

## 4. Materials and Methods

### 4.1. Reagents and Antibodies

IMDM and fetal calf serum (FCS) were purchased from Invitrogen (Villebon sur Yvette, France). Sodium fluoride, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin and Aza were purchased from Sigma (Saint-Louis, MO, USA). Anti-PARP, anti-LC3, anti-caspase 3 and anti-rabbit antibodies were from Cell Signaling Technology (Beverly, MA, USA). Peroxidase-conjugated anti-goat and Peroxidase-conjugated anti-mouse antibodies were from Dakopatts (Glostrup, Denmark). Anti-Actin antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany).

### 4.2. Cell Lines

The human cell lines OCI-M2S and OCI-M2R cells have been generated by iterative additions of increasing concentrations of Aza starting from 0.1  $\mu\text{M}$  until 10  $\mu\text{M}$ . OCI-M2S cells were cultured in the same culture conditions than OCI-M2R cells, all along the selection process that lasted several months. Sensitive and resistant cells were cultured in IMDM medium complemented with 20% SVF, 50 U/mL penicillin, 50 mg/mL streptomycin, and 1 mM pyruvate under 5%  $\text{CO}_2$  in a humidified incubator.

#### 4.3. Bone Marrow Samples

Mononuclear cells were isolated by Ficoll gradient centrifugation from bone marrow cells of MDS/AML patients and were suspended in IMDM medium containing 10% FCS, 50 U/mL penicillin, 50 mg/mL streptomycin, and 1 mM pyruvate under 5% CO<sub>2</sub> in a humidified incubator. Fresh bone marrow samples were treated by Aza or indicated concentration of Aca or other drugs. All patients had diagnosis of MDS with less than 30% of blasts and IPSS scoring intermediate-2 or high or AML. The diagnosis of MDS or AML was based on standard WHO criteria [26]. Patients (Pts) were to receive Aza at the FDA/EMA approved schedule (75 mg/m<sup>2</sup>/d, 7 d/4 weeks). Pts having received ≥1 cycle of Aza and who had bone marrow evaluation after ≥4 cycles, or who died or progressed before completion of 4 cycles were considered evaluable (the last 2 groups were considered as treatment failures). Responses were scored according to IWG 2006 criteria for MDS [27] and for AML [28]. All patients under treatment with Aza were included in protocol NCT01210274 ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). Informed consent was obtained for all patients.

#### 4.4. Assessment of Cell Proliferation:

Cell proliferation was determined with XTT assay purchased from Sigma Roche Applied Science (Penzberg, Germany). Cells (20 × 10<sup>3</sup>) were incubated in a 96-well plate with the indicated concentration of cell death at a final volume of 100 µL. After 24 h, 50 µL of XTT reagent (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) was added to each well. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye in metabolically active cells. The absorbance of the formazan product, reflecting cell proliferation, was measured at 490 nm. Each assay was performed in quadruplicate.

#### 4.5. Western Blot Analysis

After stimulation with various effectors for 24 h, cells were harvested and lysed in buffer containing 1% Triton X-100 and supplemented with protease and phosphatase inhibitors (Roche Diagnostics). Lysates were pelleted, and 50 µg of protein were separated on 12% polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA). After blocking non-specific binding sites, the membranes were incubated with specific antibodies, washed three times and finally incubated with HRP-conjugated antibody for 1 h at room temperature. Immunoblots were revealed using the enhanced chemiluminescence detection kit (Amersham Biosciences, Uppsala, Sweden).

#### 4.6. Caspase 3 Activity Assay

After stimulation, cells were lysed at 4 °C in lysis buffer (0.4 M Na Phosphate PH 6, 150 mM NaCl, 4 mM EDTA, 1 mM PMSF, 10 mg/mL aprotinin and 1% Triton X-100) and lysates were cleared at 10,000 g for 15 min at 4 °C. Each assay, in quadruplicates, was performed with 15 mg of protein prepared from control or stimulated cells. Briefly, cellular extracts were then incubated in a 96-well plate, with 0.2 mM Ac-DEVD-AMC as substrates for various times at 37 °C and Caspase 3 activity is measured after emission at 460 nm (excitation at 390 nm) with or without 1 mM Ac-DEVD-CHO. Enzyme activities were expressed in arbitrary units/mg of protein.

#### 4.7. Phase I-II Clinical Trial of Aca in MDS/AML Patients

GFM Acadesine was a phase I-II trial of Acadesine in IPSS high and int 2 myelodysplastic syndromes, acute myeloid leukemia with 20–30% marrow blasts and chronic myelomonocytic leukemia type 2 patients not responding to azacitidine or decitabine for at least 6 courses or relapsing after a response. The study was sponsored by Groupe Francophone des Myélodysplasies (GFM). GFM-Acadesine received authorization from the French regulatory authority, ANSM, on 29-Mar-2013. The primary objective was to determine the maximal tolerated dose (MTD) and dose limiting toxicities

(DLTs) of increasing doses of IV Acadesine administered on D1, D3, D5, D8, D10 and D12 of a 28 to 56 day-course. The secondary objectives were to evaluate the response rate (according to 2006 modified IWG criteria) and duration, hospitalization duration rates of re-hospitalization for non-hematological toxicities, severe bleeding or febrile neutropenia. EudraCT number: 2012-003120-21.

#### 4.8. Statistical Analysis

All data are presented as mean  $\pm$  S.D. *p*-values were determined using Prism V7.0 software (GraphPad, La Jolla, CA, USA). Unless stated otherwise in the figure legend, comparisons of different groups were made with the one-way ANOVA test with Bonferroni posttest. *p*-values <0.05 (\*), <0.01 (\*\*), <0.001 (\*\*\*) and <0.0001 (\*\*\*\*) were considered statistically significant.

**Author Contributions:** T.C. designed and performed experiments; N.F., A.C., M.Z., W.E.M., L.B., M.D., C.G., A.D., C.S., performed some experiments; T.C., P.A. and G.R. designed experiments and wrote the manuscript; A.J. contribute to the writing of the manuscript and to amendments; A.S., F.C., G.G., N.M., P.S.R., S.R., P.P., and P.F. provides patient's samples; All authors have read and agreed to the published version of the manuscript.

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

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## II. Posters and oral communications

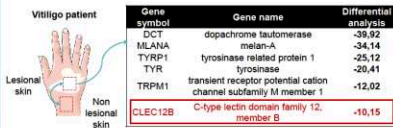
### A. Posters

- JEDNs (Journée des Ecoles Doctorales de Nice)  
25/05/2021, Nice, France
- SFI (Société Française d'Immunologie)  
22-24/11/2022, Nice, France  
Best Poster Presentation

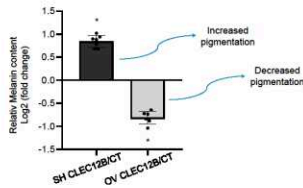
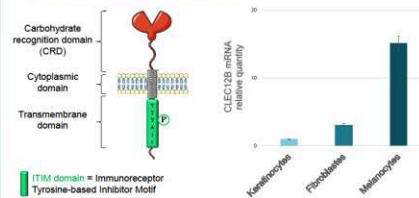
Lauriane Blot<sup>1</sup>, Marjorie Heim<sup>1</sup>, Yann Chelli<sup>1</sup>, Marie Irondelle<sup>1</sup>, Hanene Hadhiri-Bziouche<sup>1</sup>, Orane Visvikis<sup>1</sup>, Els Verhoyen<sup>1</sup>, Bernd Lepenies<sup>3</sup>, Lionel Larue<sup>4</sup>, Laura Sormani<sup>5</sup>, Stéphane Rocchi<sup>1</sup>, Thierry Passeron<sup>1,2</sup>, Meri K Tulic<sup>1</sup>

## Background results

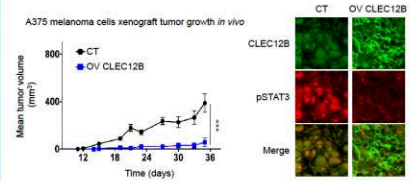
### 1. CLEC12B, a new gene we discovered by a transcriptional analysis of vitiligo skin. *Regazzetti et al, 2015*



### 2. CLEC12B, a C-type lectin receptor predominantly expressed by skin melanocytes which modulates pigmentation. *Sormani et al, Submitted*



### 3. CLEC12B, a tumor suppressor gene in melanoma which regulates STAT activation. *Montaudou et al, In revision*

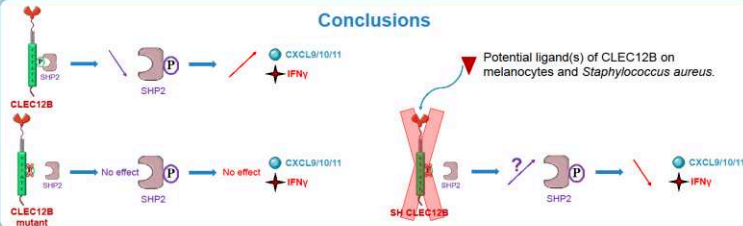


## Context

- CLEC12B (ligand and downstream signaling unknown) is a C-type lectin receptor (CLR) expressed by skin melanocytes.
- Most CLRs are expressed on antigen-presenting cells and are involved in the production of cytokines/chemokines, but also in antigen processing and presentation and subsequent T cell activation.
- Melanocytes are suggested to be antigen-presenting cells.

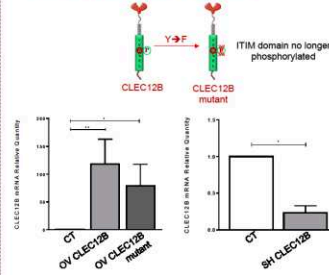
→ Hypothesis: CLEC12B may have a role in skin immunity.

## Conclusions

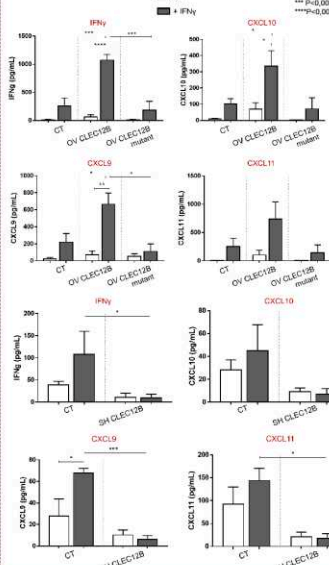


## Results

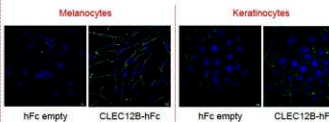
### 1. Overexpression (OV), silencing (SH) and mutating (OV mutant) CLEC12B.



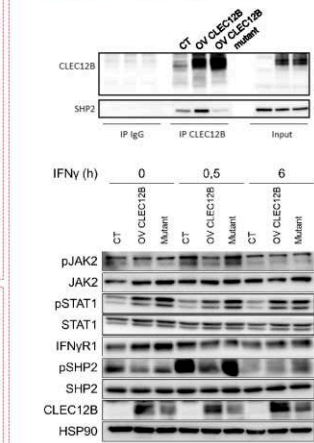
### 3. CLEC12B modulates the production of cytokines/chemokines through its ITIM domain.



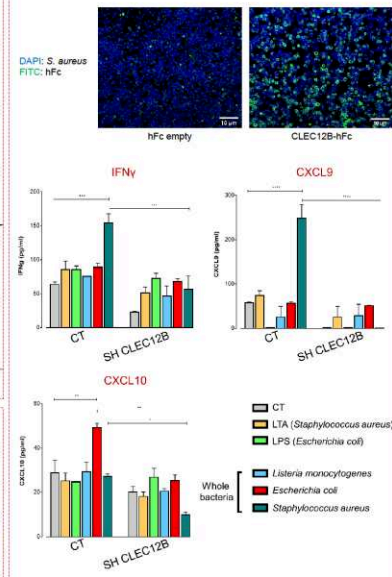
### 5. CLEC12B recognises a ligand on melanocytes but not on keratinocytes.



### 2. CLEC12B binds to SHP2 and regulates IFNγ pathway through its ITIM domain.



### 4. CLEC12B recognises *Staphylococcus aureus* and modulates an immune response against it.

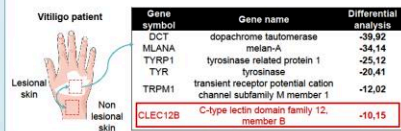


## Perspectives

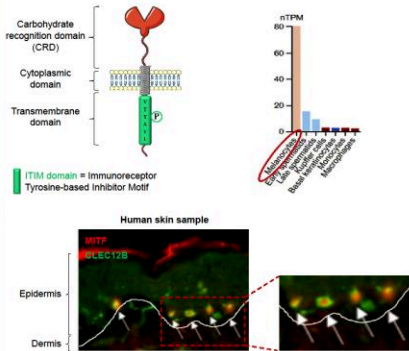
- Downstream signaling of CLEC12B:**
  - Binding of CLEC12B to SHP2
  - Use SHP inhibitors to mimic the effect of OV CLEC12B
  - Effect of SH CLEC12B on IFNγ pathway
  - Transcriptomics/proteomics experiments to find other downstream pathways
- Ligands of CLEC12B:**
  - Test other skin pathogens
  - Glycan array to find specific carbohydrates ligands
- Role of CLEC12B in skin immunity:**
  - Effect of CLEC12B on antigen-presenting cells molecules expression
  - Effect of CLEC12B on antigen uptake and presentation capacity of melanocytes
  - Co-culture of melanocytes and T cells to determine the effect of CLEC12B on T cell activation

## Background results from our team

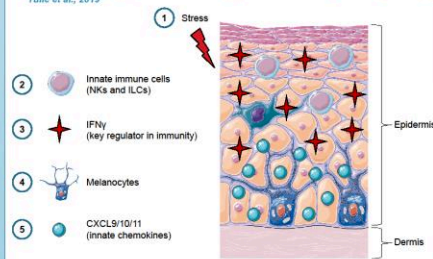
### 1. CLEC12B, a new melanocytic gene we discovered by a transcriptional analysis of vitiligo skin. *Regazzetti et al, 2015*



### 2. CLEC12B, a C-type lectin receptor (CLR) predominantly expressed by skin melanocytes and involved in melanogenesis. *Sormani et al., 2021*



### 3. Skin melanocytes act as Antigen-Presenting Cells (APCs). *Tulic et al., 2019*



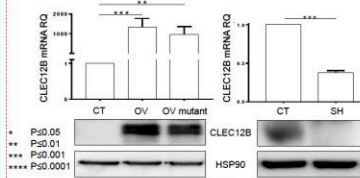
## Hypothesis

- CLEC12B is a CLR predominantly expressed by melanocytes in the skin.
- Most CLRs are expressed on APCs and are involved in the production of cytokines/chemokines, but also in antigen processing and presentation and subsequent T cell activation.
- CLRs can recognize PAMPs & DAMPs present on pathogens and host cells to induce immune responses.
- Melanocytes are suggested to be APCs.

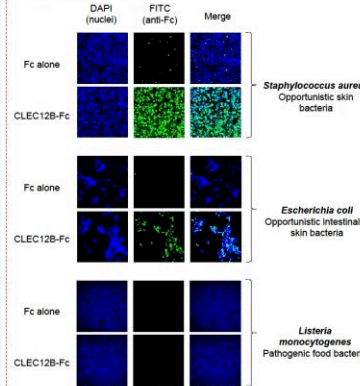
→ CLEC12B may have a role in skin immunity.

## Results

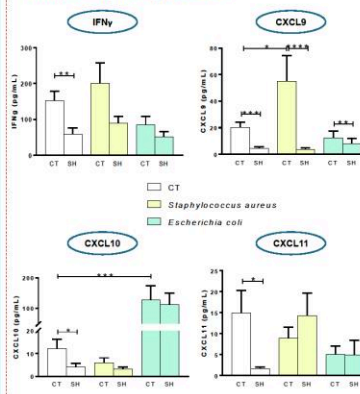
### 1. Tools: overexpression (OV), mutation (OV mutant Y→F) and silencing (SH) of CLEC12B.



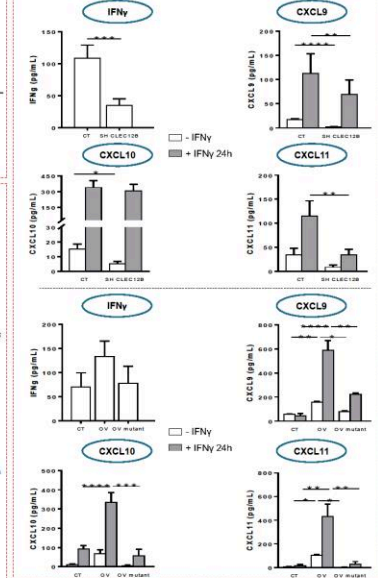
### 2. CLEC12B interacts with skin opportunistic bacteria *Staphylococcus aureus* and *Escherichia coli*.



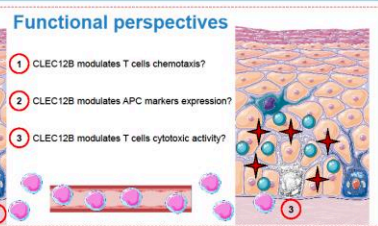
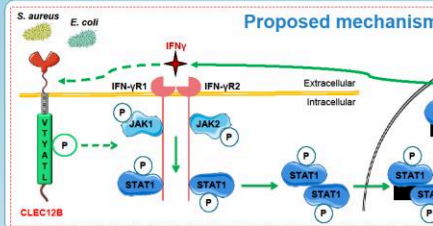
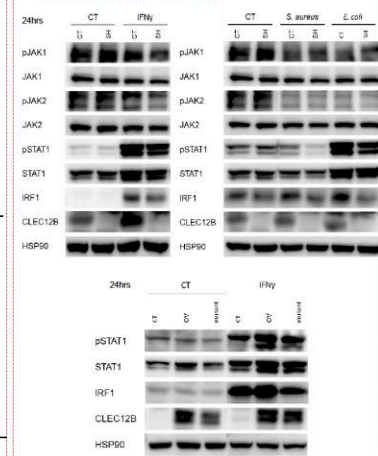
### 3. CLEC12B modulates the production of innate chemokines in response to *S. aureus* and *E. coli*, mainly through CXCL9.



### 4. CLEC12B modulates the production of innate chemokines through the phosphorylation of its ITIM domain.



### 5. CLEC12B modulates the IFNγ pathway in response to IFNγ, *S. aureus* and *E. coli*, mainly through a STAT1/IRF1 axis.



## B. Oral communications

- Internal Seminar at the C3M (Centre Méditerranéen de Médecine Moléculaire) : Role of CLEC12B in skin immunity  
20/03/21, Nice, France
- Animator of a vulgarization science stand at “La Fête de la Science”  
09/10/2021, Nice, France
- IPCC (International Pigment Cell Conference): Role of CLEC12B in skin immunity  
30/05-02/06/2023, Bilbao, Spain  
Travel Award to attend the XXV International Pigment Cell Conference