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Étude de la régulation immunitaire dans le psoriasis et le mélanome

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To all my family and friends

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A Lestrigones y a Ciclopes,
o al aira Poseidon nunca temas…….

Ten siempre a Itaca en la memoria.
Llegar allí es tu meta.Mas no apresures el viaje.
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Étude de la régulation immunitaire dans le psoriasis et le mélanome

La peau est la principale barrière de l'organisme contre les agressions physiques et les agents pathogènes microbiens. Il s'agit d'un environnement unique où diverses cellules immunitaires interagissent avec les cellules cutanées pour maintenir l'homéostasie tissulaire et réguler les réponses immunitaires. Une réponse immunitaire dérégulée au niveau de la peau entraîne différentes pathologies. Ma thèse de doctorat comprend deux parties visant à mieux comprendre les réponses inflammatoires de la peau dans des contextes pathogènes, notamment le psoriasis, une des maladies inflammatoires chroniques de la peau les plus courantes et les plus complexes, qui touche 1 à 3 % de la population mondiale, et le mélanome, le cancer de la peau humaine le plus agressif et le plus résistant aux traitements.

PARTIE 1: ***La perturbation de la boucle IL-36 et IL-23/IL17 sous-tend l'efficacité de la thérapie au calcipotriol et aux corticostéroïdes pour le psoriasis***

Dans sa forme la plus commune, le psoriasis en plaques (PP) ou psoriasis vulgaris, présent chez 90% des patients psoriasiques, est caractérisé par une infiltration de cellules immunitaires comprenant des neutrophiles, des DCs et des lymphocytes T dans la peau, ainsi que par un axe cytokinique IL-23/IL-17. Les traitements topiques par des analogues de la vitamine D3, comme le calcipitriène ou le calcipotriol (Cal), ont été utilisés depuis le début des années 1990 pour le traitement de patients souffrant de PP léger à modéré. Ils sont efficaces pour environ 45% des patients et ne provoquent pas d'effets secondaires débilitants sur le long terme. Récemment, un traitement combiné Cal/Betamethasone a été utilisé pour la PP et s'est avéré plus efficace que chacun de ces deux agents. Il a été supposé que le Cal inhibe la prolifération et améliore la différenciation des kératinocytes (KC), et exerce également certains effets immunomodulateurs, mais les mécanismes anti-psoriasis sous-jacents restent à élucider.

Sur la base de ces données cliniques, l'objectif de la première partie de mon étude était d'investiguer le mécanisme par lequel le Cal, seul ou en combinaison avec des corticostéroïdes, régule l'inflammation du psoriasis. En utilisant un modèle expérimental de souris dans lequel une pathologie de type psoriasis est induite par un traitement topique

de la peau avec la crème Aldara (Ald), notre laboratoire a précédemment montré que le traitement topique de Cal inhibait l'axe inflammatoire pivot IL-23/IL-17 et l'infiltration des neutrophiles dans la peau psoriasique de souris induite par Ald. En utilisant des souris Vdrkc$^{-/-}$ avec un knockout conditionnel (KO) chez lesquelles le récepteur de la vitamine D (VDR) est délété sélectivement dans les KC épidermiques, il a été démontré que cet effet suppresseur du Cal est médié par le VDR kératinocytaire. Cela nous a conduit à la découverte d'un mécanisme par lequel le Cal agit sur les KC au lieu de supprimer l'axe inflammatoire IL-23/IL-17 dans le psoriasis. Dans mon étude, j'ai découvert que l'IL-36α et l'IL-36γ (mais pas l'IL-36ß) induites dans les KC épidermiques, étaient efficacement réprimées par le Cal via la signalisation directe du VDR dans les KC de souris. En collaboration avec des cliniciens, nous avons montré que le traitement avec du Cal supprimait à la fois l'expression de l'IL-36α et de l'IL-36γ dans la peau lésée de patients atteints de PP, accompagnée d'une réduction de l'expression de l'IL-23/IL-17. En utilisant une culture *ex vivo* d'épiderme de souris et une culture *in vitro* de kératinocytes primaires humains, j'ai montré que le Cal réprime directement l'expression de l'IL-36α et de l'IL-36γ. Par ailleurs, j'ai disséqué les mécanismes de régulation de l'IL-36α et de l'IL-36γ par le Cal et la dexaméthasone (Dex), et contrairement à l'effet direct du Cal dans les KC, j'ai mis en évidence que la Dex réduisait indirectement l'expression de l'IL-36α et de l'IL-36γ dans la peau psoriasique des souris par le biais des cellules immunitaires.

L'ensemble de nos résultats indiquent que l'association de Cal et de corticostéroïdes perturbe efficacement la boucle de rétroaction positive des cytokines IL-36 α , IL-36 γ et IL-23/IL-17 produites par les cellules épithéliales, révélant ainsi un mécanisme sous-jacent à l'efficacité supérieure de la thérapie combinée Cal et corticostéroïdes pour les patients psoriasiques. Notre étude illustre l'importance centrale de l'interruption de la boucle inflammatoire dans le traitement du psoriasis et suggère que l'IL-36 (kératinocyte) et l'IL-17 (cellules immunitaires) sont les deux nœuds clés pour le ciblage concomitant.

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PARTIE 2: ***Étude du rôle de l'IL-36α/γ dans l'inflammation du psoriasis***

Des études menées par d'autres équipes et par la nôtre ont montré que l'IL-36α et l'IL-36γ (mais pas l'IL-36ß) sont étroitement associées et sur-exprimées dans les KC de la peau psoriasique de la souris et de l'homme, l'objectif suivant de mon étude était de comprendre le rôle de l'IL-36α et de l'IL-36γ dans la pathogenèse du psoriasis. Pour atteindre ce but, nous avons généré un nouvel outil murin avec une délétion simultanée des gènes IL-36α/γ (DKO) en utilisant l'approche CRISPR/Cas9. Lorsque des souris ont été soumises à un protocole expérimental de psoriasis induit par Ald, la peau des souris DKO a montré une réduction de l'hyperplasie épidermique, de l'infiltration des cellules immunitaires dermiques et une réduction de l'expression des gènes IL-17A, IL-22, IL-23p19 et p40, IL-1, S100A8, S100A9 et S100A15 dans la peau. De plus, des analyses par cytométrie de flux avec des marqueurs de surface et intracellulaires dans la peau ont montré que les IL-36α/γ sont essentielles dans le développement du psoriasis ; elles participent à la régulation de l'infiltration des neutrophiles/monocytes/macrophages et à la production d'IL-17A par les cellules Th17 et les cellules T dermiques.

Nous avons ensuite cherché à savoir s'IL-36α et IL-36γ jouent un rôle redondant ou spécifique en utilisant des souris DKO. Il est intéressant de noter que l'injection intradermique d'IL-36α ou d'IL-36γ chez des souris DKO a montré qu'elles favorisent indistinctement l'induction de cellules T dermiques, de cellules T CD4+ et le recrutement de neutrophiles chez les souris WT et DKO. De plus, en utilisant des cultures *ex vivo* d'épiderme de souris DKO en présence d'IL-36α ou IL-36γ, j'ai montré que les deux cytokines induisaient de manière similaire l'expression d'IL-17C, IL-1, IL-1α, S100A7A, S100A8 et S100A9, qui sont potentiellement impliquées dans le recrutement de neutrophiles/monocytes/macrophages.

PARTIE 3*: La lymphopoïétine thymique stromale (TSLP) produite par les cytokines kératinocytes (KC) favorise la croissance et la métastase des mélanomes*

Le mélanome, issu des mélanocytes, est le plus agressif des cancers de la peau chez l'homme. Il a été reconnu que les cellules immunitaires du microenvironnement associé au cancer ont un rôle à la fois promoteur et inhibiteur du cancer. Un phénotype

Résumé

inflammatoire prédominé par des lymphocytes T auxiliaire 2 (Th2) et Treg a été mis en évidence dans certains types de cancer, ce qui semble créer un microenvironnement propice à la tumeur. Dans le contexte du mélanome, la polarisation Th2 a été mise en évidence, mais l'interaction réciproque entre les cellules cancéreuses du mélanome et leur microenvironnement, y compris les KC et les cellules immunitaires, reste largement inconnue. Des études antérieures de mon équipe ont établi le rôle central de la lymphopoïétine thymique stromale (TSLP), une cytokine produite par les KC, dans l'instruction de la réponse inflammatoire Th2 de la peau. Dans le contexte du mélanome cutané, l'expression et le rôle de la TSLP étaient inconnus.

En collaboration avec Yao, W., en utilisant un modèle de souris mélanome dans lequel le tamoxifène induit une mutation BrafV600E spécifique aux mélanocytes avec inhibition de Pten (Braf/Pten), nous avons montré que la production de TSLP était induite dans les mélanomes de souris Braf/Pten. En accord avec les données de la souris, Yao, W. a constaté que l'augmentation de TSLP était également observée dans le mélanome cutané primaire et métastatique chez l'homme. Nous avons montré que l'ablation de la TSLP chez les souris Braf/Pten retardait la croissance et la progression du mélanome, alors que la surexpression de la TSLP a l'effet inverse. Il est intéressant de noter que la TSLP favorise non seulement les cellules Th2 mais aussi les cellules Treg dans les tumeurs et les ganglions lymphatiques drainant les tumeurs par qPCR, IHC et cytométrie de flux. En outre, nous avons également montré que la greffe de cellules B16F10 de mélanome de souris ou de cellules Lu1205 humaines induisait l'expression de TSLP par des KC chez les souris NSG.

Prises ensemble, nos études suggèrent un rôle important de la TSLP dans la promotion de la croissance, de la progression et de la métastase des mélanomes en façonnant le microenvironnement immunitaire associé au mélanome.

Study of the Immune regulation in psoriatic skin and the inflammatory microenvironment in melanoma

Skin is the body's primary barrier against physical insults and microbial pathogens, representing a unique environment in which various immune cells interact with skin cells to maintain tissue homeostasis and to regulate immune responses. Dysregulated immune responses at skin leads to different pathologies. My PhD thesis includes two parts aiming at achieving a better understanding of skin inflammatory responses under pathogenic contexts including psoriasis, one of the most common and complex chronic inflammatory skin diseases, affecting 1-3 % of the worldwide population; and melanoma, the most aggressive and treatment-resistant form of human skin cancer.

PART 1: Disrupting the IL-36 and IL-23/IL17 loop underlies the efficacy of calcipotriol and corticosteroid therapy for psoriasis

In its most common form, plaque psoriasis (PP) or psoriasis vulgaris, presented in 90% of psoriatic patients, is characterized by a skin infiltration of immune cells including neutrophils, dendritic cells and T cells together with a pivotal IL-23/IL-17 cytokine axis. Topical therapy with vitamin D3 analogues, such as calcipotriene or calcipotriol (Cal), have been widely used since early 1990s for treating patients with mild to moderate PP. They are effective in about 45% patients and do not exhibit debilitating effects upon long-term treatment. Recently, a combined Cal/Betamethasone treatment has been used for PP and proved to be more efficacious than either one of these two agents. It has been assumed that Cal inhibits the proliferation and enhances the differentiation of keratinocytes (KCs), and also exerts some immunomodulator effects, but the underlying anti-psoriasis mechanisms remain to be elucidated.

Based on these clinical data, the objective of the first part of my study was to investigate the mechanism of how Cal on its own or in combination with corticosteroids, regulates psoriasis inflammation. Using an experimental mouse model in which psoriasis-like pathology is induced by a skin topical treatment with Aldara cream, our lab previously showed that Cal topical treatment inhibited the pivotal IL-23/IL-17 inflammatory axis and neutrophil infiltration in Aldara-induced mouse psoriatic skin. Using $Vdr^{kc-/-}$ conditional knockout (KO) mice in which vitamin D receptor (VDR) is

ablated selectively in epidermal KCs, it was demonstrated that this suppressive effect of Cal is mediated through keratinocytic VDR. This led us to discover a mechanism that Cal acts on KCs instead of immune cells suppressing the IL-23/IL-17 inflammatory axis in psoriasis. In my study, I found that IL-36α and IL-36γ (but not IL-36β) which were induced in epidermal KCs, were effectively repressed by Cal via direct VDR signaling in mouse KCs. In collaboration with clinical researchers, we showed that Cal treatment suppressed both IL-36α and IL-36γ expression in lesioned skin from patients with PP, accompanied by a reduced IL-23/IL-17 expression. Using *ex vivo* culture of mouse epidermis and *in vitro* culture of human primary keratinocytes (HPKCs), I showed that Cal directly represses IL-36α and IL-36γ expression. Furthermore, I dissected the regulation of IL-36α and IL-36γ by Cal and Dexamethasone (Dex). In contrast to the direct effect of Cal in KCs, I showed that Dex indirectly reduce the expression of IL-36α and IL-36γ in mouse psoriatic skin through immune cells.

Together, our findings indicate that the combination of Cal and corticosteroid disrupts efficiently the epithelial cell-derived cytokines IL-36α, IL-36γ and IL-23/IL-17 positive feedback loop, thus revealing a mechanism underlying the superior efficacy of Cal and corticosteroid combination therapy for psoriatic patients. Our study illustrates a central importance of interrupting the inflammatory loop in psoriasis therapy and suggest that IL-36 (keratinocyte) and IL-17 (immune cells) are the two key nodes for concomitant targeting.

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PART 2: Study of the role of IL-36α and IL-36γ in psoriasis inflammation

Studies from others and ours have shown that IL-36α and IL-36γ (but not IL-36β) are closely associated and increased in KCs in mouse and human psoriatic skin. The next aim of my study was to understand the role of IL-36α and IL-36γ in psoriasis pathogenesis. To achieve this goal, we generated a new mouse tool with a simultaneous deletion of IL-36α/γ genes (DKO) using CRISPR/Cas9 approach. When these mice were

subjected to Aldara model, the skin of DKO mice exhibited reduction of epidermal hyperplasia, dermal immune cell infiltration and reduced expression of IL-17A, IL-22, IL-23p19 and p40, IL-1β, S100A8, S100A9 and S100A7A genes. Furthermore, flow cytometry analyses with surface markers and intracellular staining in the skin showed that IL-36α/γ play and important role in psoriasis development, including neutrophils/monocytes/macrophages infiltration and IL-17A production by Th17 cells and dermal γδT cells.

We next investigated whether IL-36α and IL-36γ play a redundant or specific role using DKO mice. Interestingly, intradermal injection of IL-36α or IL-36γ in DKO mice showed that they promote the induction of dermal γδT cells, $CD4^+$ T cells and neutrophil recruitment indistinctly in WT and DKO mice. Moreover, using *ex vivo* cultures of DKO epidermis in the presence of either IL-36α or IL-36γ, I showed that both cytokines similarly induced expression of IL-17C, IL-1β, IL-1α, S100A7A, S100A8 and S100A9, which are potentially implicated in the recruitment of neutrophils/ monocytes/macrophages.

PART 3: Study of Keratinocyte (KC)-produced cytokine thymic stromal lymphopoietin (TSLP) in cutaneous melanoma

Melanoma, arisen from melanocytes, is the most aggressive of human skin cancer. It has been recognized that immune cells in the cancer-associated microenvironment are related with both cancer-promoting and inhibiting role. A predominant T helper type 2 (Th2) and Treg inflammatory phenotype has been shown in certain types of cancers, which is suggested to create a tumor-permissive microenvironment. In the context of melanoma, Th2 polarization has been evidenced but the reciprocal interaction between melanoma cancer cells and their microenvironment including KCs and immune cells remains largely unknown. Previous studies of my team have established a central role of thymic stromal lymphopoietin (TSLP), a cytokine produced by KCs, in instructing Th2 inflammatory response in skin. In the context of cutaneous melanoma, the expression and the role of TSLP were unknown.

Summary

In collaboration with Yao, W. by using a melanoma mouse model in which tamoxifen induces melanocyte-specific $Braf^{V600E}$ mutation with Pten silencing (*Braf/Pten*), we showed that TSLP production was induced in melanoma from *Braf/Pten* mice. In agreement with mouse data, Yao, W. found that the increase of TSLP was also observed in human primary and metastatic cutaneous melanoma. We showed that the ablation of TSLP in Braf/Pten mice delayed the growth and progression of melanoma, whereas the overexpression of TSLP has the opposite effect. Interestingly, we found that TSLP promoted not only Th2 but also Treg cells in tumor and tumor-draining lymph nodes by qPCR, IHC and flow cytometry analysis. Moreover, we also showed that the grafting of mouse melanoma B16F10 cells or human Lu1205 cells induced TSLP expression by KCs in NSG mice.

Taken together, our studies suggest an important role of TSLP in promoting melanoma growth, progression and metastasis through shaping the melanoma-associated immune microenvironment.

Main Abbreviations

A
αMSH: α-melanocyte stimulating hormone
ADAMTSL5: metalloprotease domain containing thrombospodin type 1 motif-like 5.
AD: atopic dermatitis
ADCC: antibody-dependent cell cytotoxicity
AKI: acute kidney injury
ALD: Aldara
AMPs: antimicrobial peptides
APC: antigen-presenting cell
ARID2: AT-rich interaction domain 2
ASC: apoptosis-associated speck-like protein
ATP: adenosine triphosphate
B
BALF: bronchoalveolar lavage fluid
b-FGF: basic fibroblast growth factor
BSA: body surface area
C
CAF: cancer-associated fibroblast
CAL: Calcipotriol
CDKN2A: cyclin-dependent kinase inhibitor 2A
CDSN: corneodesmosin
CD: crohn's disease
CLA: cutaneous leukocyte-associated antigen
CLRs: C-type lectin receptors
CSD: chronically sun damaged
CT: control
CTLs: $CD8_+$ cytotoxic T cells (also Tc)
CYP27B1: 1α-hydroxylase
CYP27A1: 25-hydroxylase
CYP24A1: 24-hydroxylase
D
d: diameter
DAMPs: damage-associated molecular patterns
DapB: Bacterial Bacillus subtilis dihydrodipicolinate reductase
DC: dendritic cell
DEG: differential expressed genes
DETCs: dendritic epidermal T cells
DEX: dexamethasone.
DNA: deoxyribonucleic acid
DRIP: vitamin D receptor-interacting protein
E
ECDs: extracellular portion
ECM: extracellular matrix
EDC: epidermal differentiation complex
EGFR: epithelial growth factor receptor
ELNs: ears-draining LNs
EP: erythrodermic psoriasis
EtOH: ethanol
F
FCS: foetal calf serum
FDA: Food and Drug Administration
FIMO: Find individual motif occurrences
FLG: filaggrin
G
GPP: generalized pustular psoriasis
GC: glucocorticoids
GR: glucocorticoid receptor
GREs: glucocorticoid-responsive elements GWAS: genome-wide association studies
H
H&E: Hematoxylin and eosin
HDM: house dust mite
HGF: hepatocyte growth factor
HMGB1: high-mobility group box1 protein
HPA: hypothalamic-pituitary-adrenal
HPKCs: human primary KCs
HSP: heat shock protein
4-HT: 4-hydroxytamoxifen
I
IBD: inflammatory bowel disease
ICAM1: intercellular adhesion molecule1
i.d: intradermal
IF: intermedia filaments
IFN: interferon
IHC: immunohistochemistry
IL: interleukin
IL-1RAcP: IL-1 receptor accessory protein
ILNs: inguinal-draining LNs
ILC: innate lymphoid cell
i.p: intraperitoneal
ISH: in situ hybridization

J
JAK: Janus kinase
K
KCs: keratinocytes
Kd: dissociation constant
KRT: keratins
KLK: kallikrein
L
LB: lamellar bodies
LCs: Langerhans cells
LE: left ear
LEKTI: lymphoepithelia Kazal-type-related inhibitor
LN: lymph nodes
LPS: lipopolysaccharide
M
MΦ: macrophages
MAPK: mitogen-activated protein kinase
MAL: myeloid adaptator-like complex
MAMPs: microbe-associated molecular patterns
MAPK: mitogen-activated protein kinase
MBP: mannose-binding receptor
MC: Mast cell
MC1R: melanocortin 1 receptor
MDSC: myeloid-derived suppressor cells
MHC: major histocompatibility complex
miDCs: migratory DCs
MITF: microphthalmia-associated transcription factor
mm: milimiter
MMP: metallo-proteinases
MTX: methotrexate
MyD88: myeloid differentiation factor-88
N
NAFLD: non-alcoholic fatty disease
NER: nucleotide excision repair
NF1: neurofibromin 1
NHEKs: neonatal human epidermal keratinocytes.
NK: Natural killer
NLRP3: Nod-like receptor protein 3
nm: nanometers
NLRs: nucleotide-binding oligomerization domain (NOD)-like receptors
NS: Netherton Syndrome
NT: non-treated
NF-κB: nuclear factor kappa B.
NMOSD: neuromyelitis optica spectrum disorder
O
OA: osteoarthritis
P
PAMPs: pathogen-associated molecular pattern
PASI: Psoriasis area and severity index
pCDs: plasmocytoid dendritic cells
PDGF: platelet-derived growth factor
PGN: peptidoglycans
PI3K: phosphatidylinositol 3-kinase
Ppib: peptidylprolyl isomerase B.
PP: plaque psoriasis
PsA: psoriatic arthritis
PSS-B: peeling skin syndrome type B
PRRs: pattern recognition receptors
PTEN: phosphatase and tensin homolog.
PUVA: Photochemotherapy
PV: psoriasis vulgaris
R
RA: retinoic acids
RE: right ear
RLRs: retinoic acid-inducible gene (RIG)-I-like receptors
ROI: reactive oxygen intermediate
ROS: reqctive oxygen species.
RXR: retinoid X receptor
S
SCF: stem cell factor
SC: stratum corneum
SG: stratum germinativum
SIS: skin immune system
SL: stratum lucidum
SLE: systemic lupus erythematosus
SPINK5: serine protease inhibitor Kazal type 5
SNPs: single nucleotide polymorphisms
SRC: steroid receptor coactivator
SS: stratum spinosum
T
TAM: tumor-associated macrophage
TAN: tumor-associated neutrophils
Tc: cytotoxic CD8+ T lymphocyte
TEWL: trans-epidermal water loss

TERT: telomerase reverse transcriptase
Tfh: follicular helper T cell
TGF: transforming growth factor
Th: T helper cells
TIL: tumor-infiltrating lymphocyte
TIR: Toll/interleukin-1 receptor
TLR: Toll-like receptor
TNF: Tumor necrosis factor
TRAM: TRIF/TRIF-related adaptor molecule
TRIF: adaptor-inducing IFN-β
TRM: tissue-resident memory
TNF: tumor necrosis factor
Treg: regulatory T cells
TSLP: Thymic Stromal Lymphopoietin
TYR: tyrosinase

U

μm: micrometers
UV: ultraviolet
UC: ulcerative colitis

V

VAMPs: viral-associated molecular patterns
VEGF: endothelial growth factor
VDR: vitamin D receptor
VDREs: vitamin D response elements
VEGF: endothelial growth factor

W

WT: wild type

Z

ZO: zona occludens

Abbreviations

INTRODUCTION

1.1- SKIN INTRODUCTION

Skin is the largest organ of the human body with a surface area between 1.5-2m^2 in adults, together with gut and other mucosal surfaces provides to our organism integrity, identity and the interface of the host with the environment (1), (2). Historically, it has been considered as the primary physical barrier that allows exchange with the environment and mediates protection against microbial pathogens, physical and chemical insults including ultraviolet (UV) radiation, allergens and chemical irritants. Besides, the main functions of the skin are thermoregulation, prevention of evaporation (trans-epidermal water loss (TEWL) and electrolytes loss), endocrine and exocrine protein production including vitamin D_3, substances absorption, lipids depot and contains the receptors of sensory stimuli (1), (3), (4). Furthermore, over the years, it has been demonstrated that the skin has a crucial immunological role. The concept of skin immunity was introduced by Streilein (1978) and it was described as a sophisticated "skin immune system" (SIS) by Bos and Kapsenberg in 1986 (5), (6). Nowadays, skin is considered as a complex and dynamic ecosystem comprised by four functional levels:

1) The microbiota constitutes the normal cutaneous commensal flora that covers and inhabited all surface skin area formed by many microorganisms such as bacteria, fungi, and viruses (**Figure 1**). Independent genomic approaches have shown that the predominant skin microbiota are Actinobacteria and Gram-positive bacteria, such as Staphylococcus, Propionibacterium, Corynebacterium and Streptococcus species (7). Their role is the preservation of the stable healthy balance promoting the basal level of immune activation. For instance, Staphylococcus epidermidis (S.epi) protects against other infections. Indeed, S.epi inhibits the colonization by Staphylococcus aureus (S.aureus) blocking the formation of S. aureus biofilms by the secretion of the serine protease Esp (8). However, the colonization of S.epi into the dermis results in inflammation, underlying that for mammalian skin immune system, the location of microbes is crucial to determine their role (1), (6), (9) (**Figure 1**).

2) The chemical barrier function lies in the contribution to preserve hydration and the acidic surface pH between 5.4-5.9 to makes the skin an unfavorable environment for potential pathogens (**Figure 1**). Therefore, the difference between pH level in skin and blood (pH=7.4) act as a secondary defense avoiding the microbe entrance to the

circulation. The skin mechanism that contribute to promote the acidification of the environment are the proteolysis of epidermal filaggrin (FLG) in trans-uronic acid, the production of fatty acids by the stratum corneum and the release of acid electrolytes and lactic acid produced by sweat glands. This environment is convenient for commensal bacteria (10).

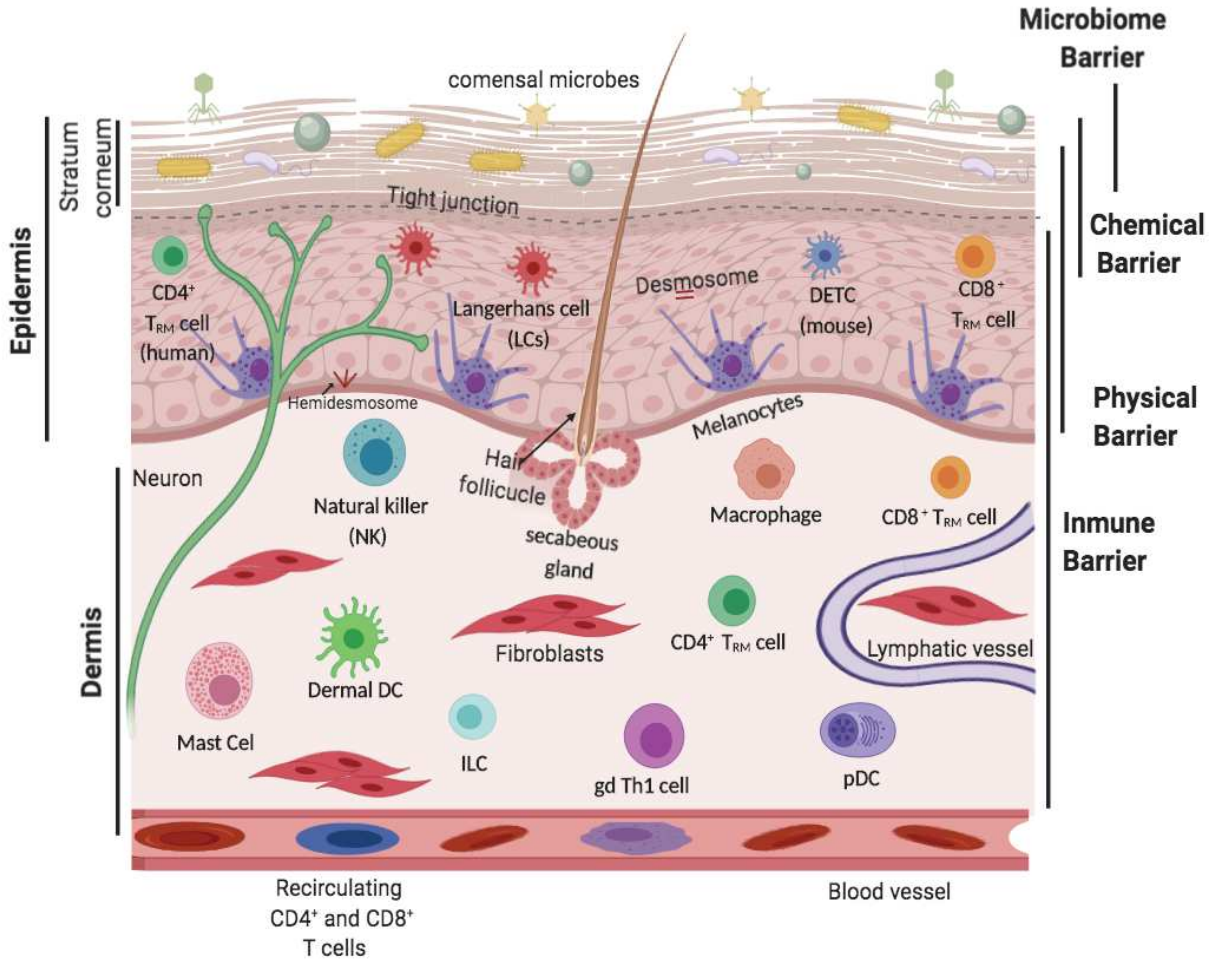


Figure 1: Structure, microbiota and cellular components of the cutaneous barrier in mice and humans. In comparation with human skin, mouse skin has smaller interfollicular skin areas. Human skin has a thicker epidermis (with more cell layers) and dermis than mouse skin. Human epidermis is characterized by epidermal rete ridges (more prominent in psoriasis, denominated papillomatosis) absent in normal mouse epidermis. The most frequent immune cell types in human epidermis are Langerhans cells (LCs), $CD4^+$ and $CD8^+$ tissue-resident memory T ($_{TRM}$) cells. In mouse epidermis, the predominant immune population is $V\gamma5^+$ dendritic epidermal T cells (DETCs). Both human and mouse dermis in steady state are formed by Dendritic cells (DCs), macrophages (MΦ), mast cells, conventional resident-memory αβ T cells, natural killers (NKs) and a small population of innate lymphoid cells (ILCs). In mouse skin, there is an important contribution from recruited γδ T cells to skin immune surveillance and IL-17 production, whereas in human skin the responsible of the surveillance are αβ T cells. Figure adapted from (1), (11), (12) done by Biorender.

3) The physical barrier of epidermis is mainly mediated by corneocytes in the stratum corneum (the structure of the skin is going to be explained further in next sessions). Corneocytes are connected by corneodesmosomes embedded in lamellar layers of lipids such as ceramides, cholesterol and free fatty acids, building the "bricks and mortar". This structure act as the first barrier against pathogens (10). (**Figure 1**)

4) A variety of skin-resident immune sentinels are presented in the different compartments of the skin with the role to promote homeostasis examining environmental antigens and regulating innate and adaptive immune responses. This pull includes initially resident cell populations (Langerhans cells (LCs), dermal dendritic cells (DCs), mast cells, macrophages (MΦ), natural killer (NK) cells, NKT cells and leucocytes: γδ T cells and memory αβ T cells) that mount an immune response through the recruitment of additional cell populations (neutrophils, eosinophils innate lymphoid cells (ILCs) naïve or central memory T and B cells) and an extensive variety of soluble inflammatory mediators (antimicrobial peptides (AMPs), cytokines, and chemokines) interacting with stromal cells (keratinocytes (KCs), fibroblast and endothelial cells, neurons and adipocytes). The crosstalk of these cells induces peripheral tolerance to tissue self-antigens or in the contrary initiates robust immune responses against external agents (5), (10), (13) (**Figure 1**).

Alterations and dysregulations in each component of the skin barrier leads to pathologies such as infections, allergy, cutaneous tumor development or inflammatory diseases as psoriasis, one of the most common and complex chronic inflammatory skin diseases, affecting 1-3 % of the worldwide population (14). In this section of my thesis, I will describe the components and the different functions of the skin. I will be specially focus in the immune function and the role of the skin components in inflammation.

1.1.1- SKIN INFLAMMATION

Inflammation is a coordinated multi-stage complex defense mechanism vital to health, triggered by a harmful stimulus, such as pathogens, damaged cells, toxic compounds, or irradiation, that breach the initial barriers of the skin or mucosal surface of other organs. This

reaction leads soluble and cellular innate signals to initiate inflammatory responses when "innate immune rheostats" fails in the control of the stimuli (15), (16), (17). Initial acute inflammatory responses promote cellular and molecular pathways to minimize injury or infection with the general aim to repair the tissue. However, uncontrolled acute inflammation initiates a chemical signaling cascade and metabolically cells become deprived of oxygen. Therefore, the sodium pump fails, the membrane is disrupted and intracellular mediators (e.g, histamine and bradykinin) are released into extracellular space and promote damage in adjacent cells. At the same time, the total blood flow increases upon arterial dilatation and leukocytes beginning the adhesion to the vessel wall to continue with the transmigration to the injured site. Neutrophils, with a short-live around 7h and MΦ with the ability to survive several months in the affected tissue are considered the first temporary line of defense. They initiate the cell inflammatory process through phagocytosis of debris or microbes (18) (**Figure 2**). Nevertheless, the persistence of the inflammatory response guide to the loss of organ function becoming in a chronic situation, contributing to a variety of inflammatory diseases such as atopic dermatitis (AD), psoriasis and cancer (**Figure 2**).

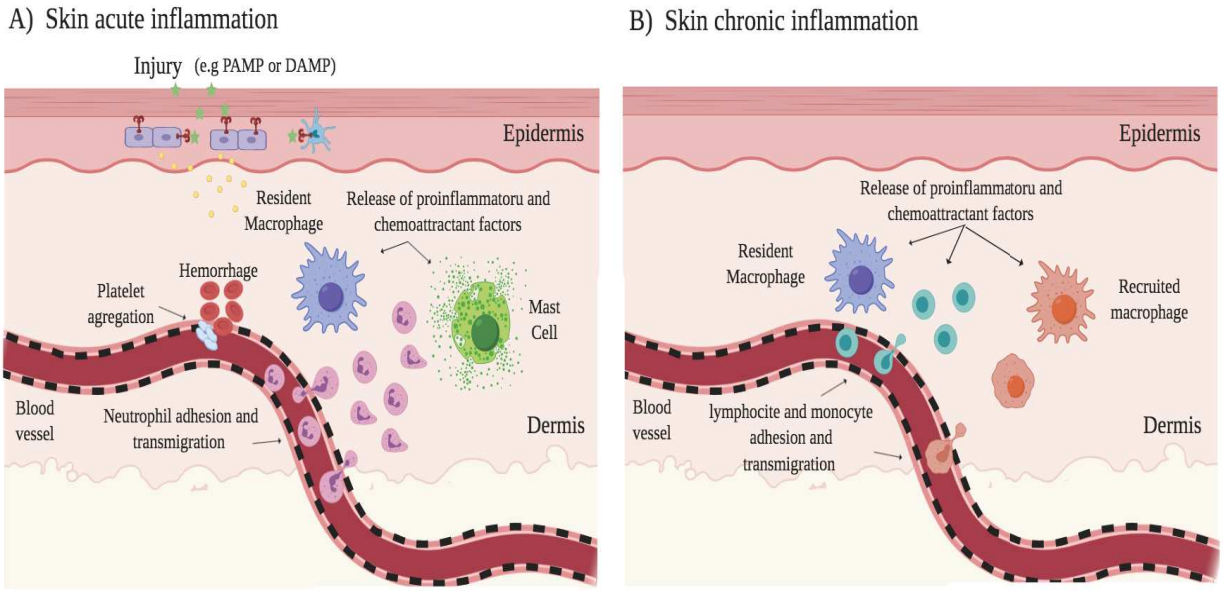


Figure 2. **Acute and chronic skin inflammation cascade. A.** In acute skin inflammation the first damage signal is recognize by immune resident cells (LCs, MΦ and mast cells) and epithelial cells (KCs), which became active and start the release of pro-inflammatory and chemoattractant factors. Additionally, the initial skin injury triggers intravascular processes resulting in neutrophils adhesion and transmigration**. B.** In chronic skin inflammation lymphocytes and monocytes begin the adhesion process and further transmigrate into extravascular space. Transmigrated cells and resident MΦ secrete pro-inflammatory and chemoattractant factors, perpetuating the inflammatory response. Figure adapted from (18), done by Biorender.

Due to the hemodynamic changes and the inflammatory cell responses the results from the local immune response at the tissue level are characterized by 5 cardinal signs described first by Aulus Celsus (30-38 B.C.) and Virchow (1821-1902) including redness (rubor), swelling (tumor), heat (calor), pain (dolor), and loss of tissue function (function laesa) (19). Redness and heat are the consequence of blood vessels vasodilatation, whereas swelling appears caused by edema and the pain is produced by the local release of prostaglandins (20).

1.1.2- OVERVIEW OF SKIN COMPONENTS AND THEIR FUNCTIONS

To understand how the immune system triggers an effective immune response in skin, it is important to consider the interaction between immune and epithelial cells. Therefore, an overview of cellular and structural composition of the skin is crucial to understand the cutaneous immune response. Skin is a highly specialized organ formed by two major components: the epithelium, the connective tissue and additional derivate structures called appendages such as hair follicles, sebaceous and sweat glands (21). Anatomically, the structure of the epithelium compartment is divided in three layers (**Figure 1**): the outermost non-vascularized epidermis, the dermis (formed by connective tissue) and the subcutaneous adipose tissue (hypodermis) (3). Whereas the epidermis creates a well-organized layer principally composed by KCs giving a mechanical protection, the dermis is formed by collagen fibers, fibroblasts, and nerve endings. This anatomical differential organization and composition entails to each of them to preform different functions. Therefore, the thickness of each layer of the skin varies depending on the body region. Indeed, the thinnest layer of the epidermis, is in the eyelid (less than 0.1 mm), whereas the thickest epidermal layer that contains an extra layer, the stratum lucidum, is located in palms and soles of the feed (22).

1) DIFFERENCES BETWEEN HUMAN AND MOUSE SKIN

Although human and murine skin are similar in terms of structural composition, a recent comparative gene array study revealed only 30.2% of identity between the two species, suggesting strong evolutionary modifications (23). The first clear difference is the amount of interfollicular epidermis relative to hair follicle epithelium presented in mice. Moreover, even thought, the most conserved genes in mice are related to barrier structure or function, such as

keratins, cell-to-cell junctions, structural proteins, and cell proliferation-related molecules, the thickness and number of epidermal layers differs between the two species. Human skin, composed by 5 to 10 layers, is relatively thick, firm, and adhered to the adjacent tissues. The epidermal thickness ranged from 76.9 ± 26.2 to 267.4 ± 120.6 μm, women dorsal foot is the thickest epidermis, while the thinnest was found in the women breast. The dermal thickness ranged from 2115 ± 946.4 to 5888 ± 2422.3 μm, men breast is the thickest dermis, while the thinnest dermis was found in the women dorsal hand (24). Nevertheless, murine skin, composed by 2 to 3 layers, presents less than 250 μm and is lax (25), (10). Therefore, these differences reduce the barrier function and enhances percutaneous absorption in mice (26). Histologically is important to mention that, in human skin we can distinguish the epidermal ridges intersected in dermal papillae commonly absent in mice (**Figure 3**). In the immunological context, murine epidermis, in addition to the normal pull of immunological cells mentioned before, contains a specific population of TCRγδ dendritic epidermal T cells (DETCs) fundamental for skin homeostasis and tissue repair (**Figure 1**). Therefore, skin human alterations and genetic specificities between mice and human, should be considered to interpret the inflammatory skin phenotypes observed in mouse models in relation to the mechanisms that drive the pathogenesis of human inflammatory skin diseases (21), (2).

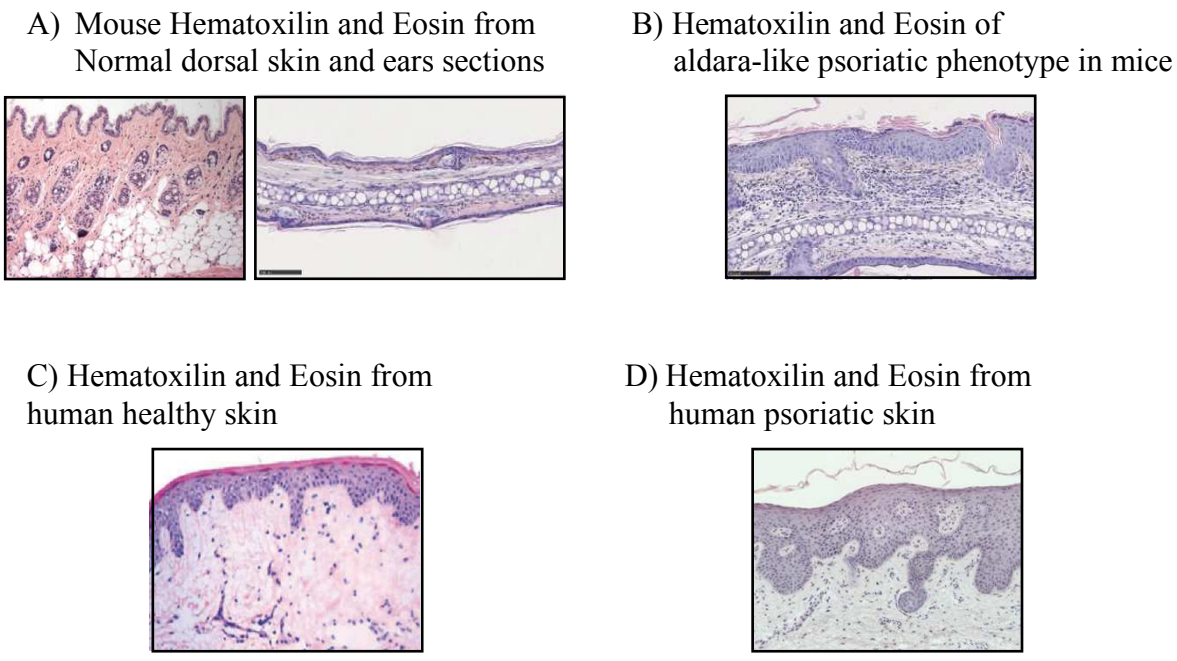


Figure 3: Differences in the histo-structure of the skin between human and mouse skin. A healthy dorsal (left) or ear (right) mouse skin and **B** Aldara-like psoriatic phenotype in the ears of mouse skin. **C** healthy human skin and **D** psoriatic human skin.

2) EPIDERMIS

Human epidermis is a stratified, squamous epithelial layer densely pack between 75 and 150µm (up to 600µm on palms/soles) responsible of the first line of protection, therefore comprises two types of barrier: the physical and chemical/biochemical barrier. It is composed primarily by KCs and DETCs (in mice) and other cell populations such as melanocytes, LCs, Merkel cells, $CD4^+$ T resident memory ($_{RM}$) cells and CD8+T_{RM} cells. It is derived from a single layer of ectodermal progenitor cells in embryos to a multilayered stratified epithelium during development (27). It is a dynamic tissue in which undifferentiated cells are constantly following proliferation cycles in unsynchronized turnover to provide the renewal of the outer layers. Consequently, KCs follow a maturation process climbing the epidermal layer to became corneocytes, cells highly differentiated (21), (22), (28).

- **Layers of epidermis: key features of the epidermal barrier**

Epidermis is commonly divided into four layers according to KCs morphology and location, including the deepest portion denominated basal cell layer (stratum germinativum), the squamous cell layer (stratum spinosum), the granular cell layer (stratum granulosum), the stratum lucidum, and the cornified cell layer (stratum corneum) (29) (**Figure 4**). The epidermal structure changes from anuclear cells in the stratum corneum to distinct hexagonal shaped cells in the stratum basal connected forming a brick wall by junction complexes. The cornification process involves many different components and procedures. Thus, to ensure the proper development of the barrier, a correct regulation and communicative network between all the participants is essential. Here, I will summarize the current state of research concerning the components of the epidermal layer.

-STRATUM GERMINATIVUM (SG)

The basal layer or SG is the deepest layer formed by a single column of epidermal stem cells liable to proliferate and ensure the constant renewal of the epidermis (30) attached to the basement membrane (basal lamina) by hemidesmosomes (desmosomal junctions) and linked by keratins (KRT14-KRT5, keratins role will be discussed in next session). However, not all basal cells have the potential to proliferate (31), after the division one

part of the cells will keep stem cells features to maintain the self-renewal capability and produce further differentiated cells and the other part will continue with the corneocytes differentiation. This phenomenon is preserved by two different division patterns denominated symmetrical and asymmetrical division. In the first case, the two daughter cells obtained are adjacent to the horizontal plane, one of the cells would be continue in the basal layer as a proliferative cell and the other cell could be detached from the lamina and continue with the differentiation. Nevertheless, asymmetric division generates two cells with non-identical cell fates, one daughter remains as a progenitor while the other cell is found in the spinous layer (31). In humans the migration process of a basal cell from the basal layer to the stratum corneous takes at least 14 days, and the transit through the stratum corneous to the outermost epidermis requires 14 extra days (22).

-STRATUM SPINOSUM (SS)

Once KCs are detached from the basal layer, they migrate to the SS composed by 8-10 cell layers. KCs produce KRT10-KRT1, they have lost the proliferative capacity, are irregular, polyhedral and present cytoplasmic "thorns" supporting the connections with other cells in addition to desmosomes and other type of junctions (32). In the cytoplasm KCs present lamellar granules, organelles containing glycoproteins, glycolipids, phospholipids, free sterols, and acid hydrolases, including lipases, proteases, acid phosphatases, and glycosidases, which deliver lipids into the intercellular space (33). Indeed, the enzyme transglutaminase is responsible for the ε–(γ-glutamyl) lysine cross-linking of involucrin into the insoluble cornified envelope (33).

-STRATUM GRANULOSUM

This section of the epidermis possesses between 3-5 layers of KCs with flattened and diamond shape, parallel to the surface of epidermis, connected by TJ junctions (transmembrane proteins including claudins, occludin, and zona occludens (ZO)). The principal role of KCs in this layer, is the synthesis and modification of proteins which participate in keratinization by the "epidermal differentiation complex" (EDC) (34). KCs are characterized by cytoplasmic keratohyalin granules producing proteins, such as filagrin (encoded by *FLG* gene), loricrin, involucrin and keratin filaments. Filagrin and keratin, the principal components of these granules, play an important role in the inter-fibrillary matrix formation, the inner lining of the horny cells. In addition, they are

released to the intercellular space and create the mortar between bricks. The granular cytoplasm also contains vesicular structures that fuse with the plasma membrane, denominated lamellar bodies (LB). These LB contain lipids, corneodesmosins (forming corneodesmosomes, encoded by *CDSN* gene) and kallikreins. When the contents are released into the extracellular space create the 'cornified envelope'.

Gene mutations in *FLG, SPINK5* (serine protease inhibitor Kazal type 5) or *CDSN* for example, produce epidermal barrier dysfunctions. For instance, psoriatic patients present alterations in the expression of *CDSN* and genes of EDC. Allen M. et al, observed that in psoriasis the expression of Cdsn was increase and widespread in multiple layers of stratum spinosum in comparation with the selectively expression of the protein in the stratum granulosum observed in healthy skin. Suggesting that, in psoriasis, Cdsn is expressed by KCs earlier than in healthy skin and leads alterations in the differentiation of KCs in the granular and cornified layers. These alterations maybe promote parakeratosis and the nuclei of KCs persist when the cells migrate to the SC. Another interesting example is the mutation in *SPINK5* encoding for lymphoepithelia Kazal-type-related inhibitor (LEKTI) (35). Mutations in *SPINK5* results in the development of a rare autosomal recessive skin disease denominated Netherton Syndrome (NS) (36). These mutations promote LEKTI functional deficiency and the consequent uncontrolled activation of kallikrein peptidases, including KLK5 and KLK7. The induction of these peptidases produces a dysregulation in the desquamation process and corneocyte elimination (37), (36). Immunologically, NS patients present an increase in Th17 and Th2 inflammatory pathways, including the induction of eosinophilis, TSLP, CCL17 and IgE. Consequently, NS pathology has been linked with AD and psoriasis. Moreover, recently, Malik et al., showed a predominant IL-23/IL-17 and IL-36 signature in NS patients (38).

-STRATUM LUCIDUM (SL)

The SL contains between 2-3 cell layers and is presented only in thicker skin areas such as, palms and soles. The principal role of the SL is the reduction of the friction between SC and stratum granulosum. The principal component is eleidin, a transformed product of keratohyalin.

-STRATUM CORNEUM (SC)

The SC is the uppermost layer of the epidermis formed by 20-30 piles of corneocytes and intercellular lipids. Corneocytes are rich in proteins, such as FLG and low in lipidic matrix, are flattened and denucleated KCs in which the membrane is replaced by the 'cornified envelope'. This structure is essential to blocks the entry or exit of water and water-soluble substances. In homeostasis conditions calcium forms a steep gradient within the epidermis, with the highest concentration in the stratum granulosum (29). The disruption of the SC produces alterations in the barrier permeability breaking the calcium gradient (39) increasing the LB secretion and reducing the levels of loricrin, profilaggrin, and involucrin (40). The turnover process in this layer is controlled by the proteolytic degradation of desmosomes ending with the desquamation and release of corneocytes (41). Nevertheless, the integrity of the barrier is imperfect due to the presence of skin appendages. However, they have useful function to protect the body from mechanical damage, UV light, temperature changes and dryness.

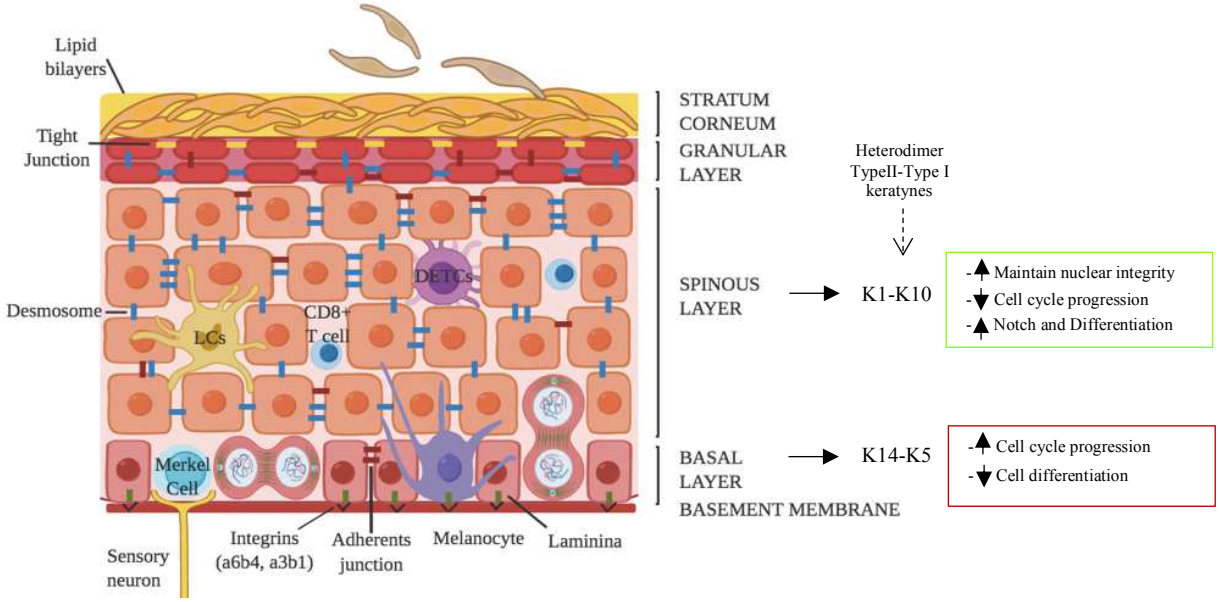


Figure 4: Different epidermal layers. Epidermis is divided in four layers compounded mainly by KCs. In normal epidermis, basal KCs express KRT14-KRT5 to maintain KCs proliferation, promote cell cycle progression and inhibit spontaneous differentiation. In contrast, cells located at the SS express KRT10-KRT1 which promotes terminal differentiation of KCs, inhibits cell cycle progression and maintains cell nuclear integrity. In psoriasis, KCs presents a down-regulation of KRT10-KRT1, up-regulation of KRT14-KRT5 and strong induction of KRT16-KRT6 and KRT17-KRT5/6. KRT16-KRT6 pair maintains cell integrity by increasing cell-cell and cell-matrix contact, decreases cell migration and directionality while maintains mechanical integrity. KRT17-KRT6/5 pair drives KCs hyperproliferation by increasing cell size and increasing protein synthesis. Figure adapted from (42), (43) done by Biorender.

- **Cellular components of the epidermis:**

The resident cell populations presented in epidermis can be generally divided into immune and non-immune cells. Non-immune cell populations are important to maintain the structure and function of the skin, but also contribute to skin immunity, promoting the release of substances that interact with immune cells.

Non-immune cells:

A) KERATINOCYTES (KCs)

KCs, derived from the ectodermal tissue, are the predominant cell type, representing at least 80% of the epidermis. It has been shown that these cells play different important roles that are going to be discuss in detail below. Nowadays, KCs are no longer recognized as a passive protection barrier. For instance, KCs are essential cells participating in the crosstalk between innate and adaptive immune system.

A.1) ESTRUCTURAL FUNCTION

KCs are constantly expose to physical stress consequently their main role is the maintenance of skin structure (21), (2). Therefore, to assure the stability of the tissue, KCs synthesize keratins (KRT), a group of 54 proteins mentioned before that form intermedia filaments (IFs). KRTs are the mayor components of the epithelial cytoskeleton, acting as a flexible scaffold that provide resilience and resistance against mechanical stress. Furthermore, KRTs are also responsible of cell proliferation regulation, migration, adhesion and inflammatory features of KCs (43). KRTs are subdivided in two classes based on their pH: The acidic type I keratins (KRT9-KRT40 encoded by chromosome 17q12-q21) and the neutral–basic type II keratins (KRT1-KRT8 including KRT18 type I encoded by chromosome 12q11–q13) (44). The IFs are formed by heterodimers with longitudinal and lateral interactions between specific pairs of type I and type II keratins. Mutations or abnormal expression of KRTs are associated with diverse skin diseases, such as skin blistering diseases (Epidermolysis bullosa simplex and Epidermolytic hyperkeratosis), psoriasis and skin tumors (42), (43). In normal skin, the expression of KRT14-KRT5 is recognized to be the hallmark of basal KCs forming a cluster of progenitor cells, whereas the expression of KRT10-KRT1 reflects the early differentiation stage of KCs in upper basal layers (42), (5) (**Figure 4**).

A.2) VITAMIN D PRODUCTION

Additionally, KCs in collaboration with ultraviolet (UV) radiation B (UVB light between 290-315 nm wavelength) are implicated in the synthesis of vitamin D participating in the transformation of 7-dehydrocholesterol (7-DHC) precursor. To be active, vitamin D undergoes two hydroxylation reactions: first in the liver by vitamin D 25-hydroxylase (CYP2R1) enzyme to form 25-hydroxyvitamin D, 25(OH)D, also known as calcidiol. Then 25(OH)D is transformed in 1,25-dihydroxyvitamin D (1,25(OH)2D) in the kidneys by 1α-hydroxylase (CYP27B1) to form the active metabolite, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), also known as calcitriol. In addition to the main mechanism, KCs are equipped with the enzymatic machinery (CYP27A1 and CYP27B1) to metabolize vitamin D into calcitriol (**Figure 5**).

Vitamin D is a fat-soluble vitamin with two forms: ergocalciferol (vitamin D_2) in plants and cholecalciferol (vitamin D_3, Vit D_3). The levels of Vit D_3 in human serum are tightly regulated by a feedback mechanism of calcium, phosphorus, parathyroid hormone, fibroblast growth factor and Vit D_3 itself. According to the US Endocrine Society guidelines, Vit D_3 deficiency is defined as a serum level of 25(OH)D below 20 ng/mL (50 nmol/L) (45), (46), (47).

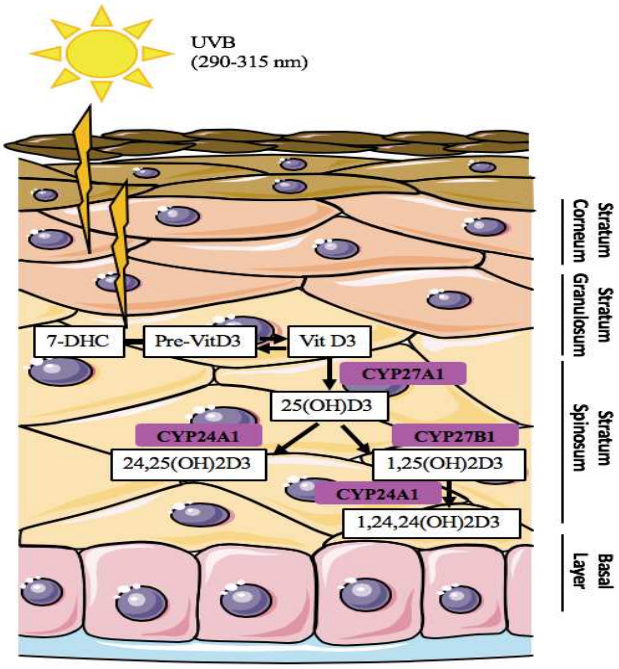


Figure 5: Summary of Vit D_3 pathway in human skin. precursor 7-DHC is transformed in active Vit D_3 under the influence of UVB through 2 subsequent hydroxylation reactions by CYP27A1 and CYP27B1 enzymes. It is rendered inactive through the catabolic enzyme CYP24A1 (47).

The principal function of Vit D_3 is the maintenance of the skeletal architecture through calcium and phosphorus metabolism regulation, but recently, the extra-skeletal effects of vitamin D_3 were discovered. Nowadays, it is known that Vit D_3 affects multiple

functions in skin including KCs proliferation, differentiation, apoptosis, barrier maintenance and immune modulation (**Table 1**). Vit D_3 genomic activity is mediated by the vitamin D receptor (VDR), which forms a heterodimer complex with retinoid receptor (RXR). The VDR-RXR complex is recruited and interacts with the vitamin D response elements (VDREs) present in the promoter of target genes and regulate their expression.

Table 1: Vitamin D effects in KCs.

Effect of Vitamin D in KCs	Function	Reference
Regulation of epidermal differentiation and proliferation	**Direct effect** regulated through VDR interaction in complex with coactivators (DRIP and SRC): -at low concentration (10^{-9} M) enhances KCs proliferation. -at high concentration (10^{-8} M) inhibits KCs proliferation and promotes their differentiation by the reduction of mRNA levels of c-myc and cyclin D1 and the increase in the cell cycle inhibitors p21cip and p27kip. **Indirect effect:** -Vit D_3 in the presence of high concentration of calcium promotes KCs differentiation increasing phospholiphase C-y1 expression.	(48), (49), (50)
Barrier Function	Vit D_3 Promotes the synthesis of structural components (Involucrin, transglutaminase, loricrin and filaggrin) of the cornified envelope inhibiting KCs proliferation.	(45), (48)
Apoptosis	At physiological concentrations prevents apoptosis mediated by phinosine-1-phosphate, MEK/ERK and PI3K/Akt	(47)

vitamin D receptor-interacting protein (DRIP) and steroid receptor coactivator (SRC).

In the context of immune system, the effect of Vit D_3 is dependent on the regulation of AMPs (antimicrobial peptides). Generally, the level of AMPs is low in normal skin, but increases upon barrier disruption. AMPs induction produces CYP27B1 enhancement and subsequently the synthesis of active Vit D_3 in skin. Schauber et al., have shown that as a result of skin injury, cathelicidin levels (an AMP) and Toll-like receptor2 (TLR-2) are increased depending on Vit D_3 mechanism. Besides, 1,25(OH)2D3 and calcipotriol mediate an immunosuppressive action in the skin over the decrease of antigen presentation directly, affecting LCs or by the modulation of cytokine production by KCs (47), (51). Further, it has been shown that calcipotriol induces $CD4^+CD25^+$ T regulatory (Treg) cells, thus mediates tolerance or immunosuppression in the skin preventing subsequent antigen-specific $CD8^+$ T-cell proliferation and IFN-γ production (52).

A.3) IMMUNOLOGICAL FUNCTION

Besides their structural character, in 2009 Nestle et al., identified the innate and adaptive immunological role of KCs in cutaneous inflammation. The innate immune system, considered as the natural immune protection when we born, is designed to directly and rapidly respond to foreign pathogens or injuries by activating unspecific, pre-existing recognition systems and effector mechanisms. The common mechanism can be divided in 4 phases: 1) Recognition of the detrimental stimuli by cell surface pattern receptors; 2) Activation of the inflammatory pathways; 3) Release of the inflammatory markers from neutrophils, eosinophils, MΦ, DCs and endothelial cells such as KCs; and 4) Recruitment of inflammatory cells (5), (10).

1) Recognition of detrimental stimuli by cell surface pattern receptors

In order the inflammatory events mentioned above to begin, pathogens must be detected in compromised tissues. The subset of sentinel innate cells distributed throughout tissues (MΦ, LCs, DCs, and NK cells) and non-immunological cells (KCs, endothelial cells, fibroblast and melanocytes), mediate the detection of injury or invading pathogens by germ-line encoded pattern recognition receptors (PRRs) which recognize:

- pathogen-associated molecular pattern (PAMPs) including highly conserved structures, such as cytosine-phosphate-guanin motifs, heat shock proteins (HSP), peptidoglycans (PGN) and lipopolysaccharides (LPS); leading to the rapid induction of “alarmis” such as AMPs, small cationic and amphipathic molecules (5), (53);
- microbe-associated molecular patterns (MAMPs);
- viral-associated molecular patterns (VAMPs);
- damage-associated molecular pathogens (DAMP) released by host necrotic cells, which originally are intracellular proteins or nucleic acids, such as chromatin-associated protein high-mobility group box 1 (HMGB1), adenosine triphosphate (ATP), uric acid, deoxyribonucleic acid (DNA), and degraded extracellular matrix-like heparan sulphate and hyaluronan.

PRR families include the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and mannose-binding receptor (MBP) (**Figure 6**). They are expressed in the surface and intracellularly to allow for

both extra and intracellular recognition of pathogens with the objective to eliminate the agents or after a longer presence activate more efficient adaptive immune response (54), (55), (56), (57)

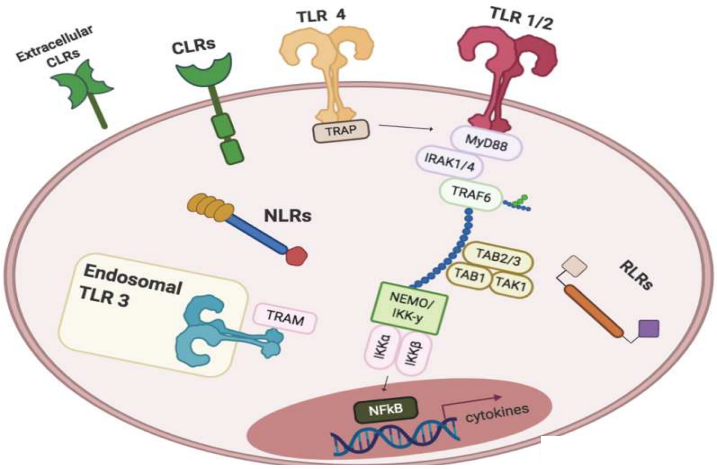


Figure 6. Pattern recognition receptors (PRRs) family. CLRs can be extracellular or anchored to the membrane. TLRs are expressed either at the cell surface or in endosomes. NLRs and RLRs are expressed in the cytosol. Figure adapted from (55), done by Biorender.

TLRs family, composed by 11 and 12 members in humans and mice respectively, is the most well-studied PRRs. TLR1-9 are conserved between mouse and human, whereas TLR10 is non-functional in mouse and TLR11-12 are absent in humans (58). TLRs detect an extended range of PAMPs and MAMPs, including pathogen-derived products such as lipopolysaccharide (LPS) recognized by TLR1, 2 and 4; flaggelin recognized by TLR5; single stranded (ss) by TLRs 7 and 8; double-stranded (ds) RNA by TLR 3; and CpG DNA by TLR9. The receptors presented at the cell surface recognize bacteria whereas endosomal TLRs recognize viral and bacterial nucleic acids (55). (**Table 2**)

Table 2: List of human and mouse TLR's. Adapted from (59) and (60).

Toll-like-receptor	Endogenous ligand	Pathogen	cell distribution
TLR-1:TLR-2 TLR-2:TLR-6	Lipoproteins,Lipomamans, lipoteichoic acid, b-glucans, zymosan	Gram-negative bacteria	Monocytes, DCs, mast cells, eosinophils, basophils, KCs
TLR-3	Double-stranded (ds) RNA; Poly I:C	Viruses with ds RNA genome	Macrophages, DCs, gut epithelium, KCs
TLR-4	Lipopolysacharides; lipoteichoic acid	Gram-negative bacteria	Macrophages, DCs, mast cells, eosinophils KCs
TLR-5	Flagelin	Bacteria	Macrophages, DCs, gut epithelium, KCs
TLR-7	Single-stranded (ss) RNA	Viruses with ssRNA genome	Macrophages, pDCs, eosinophils, B cells
TLR-8	Single-stranded (ss) RNA	Viruses with ss RNA genome	Macrophages, neutrophils

TLR-9	Unmethylated CpG	Viruses/bacteria	pDCs, DCs, eosinophils, B cells, basophils, KCs
TLR-10 (non-functional in mouse)	Unknown	Unknown	pDCs, DCs, eosinophils, B cells, basophils
TLR-11 (absent in humans)	Profilin	Bacteria	Macrophages, DCs
TLR-12 (absent in humans)	Profilin	Bacteria	Macrophages, DCs
TLR-13	Single-stranded (ss) RNA	Bacteria	Macrophages, DCs

pDCs: plasmocytoid dendritic cells

2) Activation of inflammatory pathways

TLR activation by PAMPs and DAMPs triggers quick innate immune responses such as phagocytosis, production of AMPs and the intracellular signaling pathways characterized by the activation of several transcription factors. TLRs binding with the cognate ligand (adaptor proteins) will induce the dimerization of the ectodomain of two TLRs, this allow their interaction with intracellular Toll/interleukin-1 receptor (TIR) domain (**Figure 6**). TLR-1/2 and TLR-2/6 interact with myeloid differentiation factor-88 (MyD88) and adaptator-like (MAL) complex, TLR-3 with the adaptor-inducing IFN-β (TRIF); TLR4 interacts with MyD88/MAL and TRIF/TRIF-related adaptor molecule (TRAM) complexes; the rest of TLRs with MyD88 alone. These complexes activate the "nuclear factor kappa B" (NF-κB), mitogen-activated protein kinase (MAPK) or Janus Kinase (JAK)-signal pathways. This first step entails the antigen processing that induces the adhesion and diapedesis of innate cells by the control of the induction, production and release of cytokines that will be detailed bellow (**Table 3**) (61). Dysregulations of NF-κB, MAPK, or JAK-STAT activity are associated with inflammation, autoimmunity, metabolic diseases and cancer.

- NF-κB pathway

NF-κB transcription factor activation is induced by several stimuli, including PAMPs, intercellular pro-inflammatory cytokines and enzymes. This pathway promotes the production of pro-inflammatory cytokines, and support the cell recruitment, survival, and apoptosis related with immune responses. The family is composed by five related transcription factors: p50, p52, RelA (p65), RelB, and c-Rel. Under physiological conditions, cytoplasmic IκB proteins inhibit NF-κB. Nevertheless, upon PRRs stimuli IκB kinase (IKK), composed by two kinase subunits, IKKα and IKKβ, and a regulatory

subunit, such as IKKγ, is activated through IκB phosphorylation. After activation, IκB ends in its proteasomic degradation and the subsequent release of NF-κB for nuclear translocation and gene transcription activation (16) (**Figure 6**). *Ikbkb*$^{-/-}$ (which encodes IKKβ) selectively in epithelial cells mice and transgenic mice expressing a mutation of the repressor, have been shown to present TNF-dependent psoriasis-like inflammatory skin lesions (11) demonstrating the implication of this pathway in psoriasis.

- MAPK pathway

MAPKs are a family of serine/threonine protein kinases with the role of regulate cell proliferation, differentiation, survival and apoptosis, directly responding to stimuli such as osmotic stress, mitogens, heat shock, and inflammatory cytokines. Mammalian MAPKs include extracellular-signal-regulated kinase ERK1/2, p38 MAP Kinase, and c-Jun N-terminal kinases (JNK). There are at least three components in each MAPK signaling pathway promoting the phosphorylation and activation of the next member. Specifically, ERKs are generally activated by mitogens and differentiation signals, whereas inflammatory stimuli and stress activate JNK and p38 (16).

- JAK-STAT pathway

It is a high conserved pathway through which extracellular factors can control gene expression involving diverse cytokines, growth factors, IFNs, and related molecules, such as leptin and growth hormone. The different ligands activate the receptor-associated JAKs and produce a phosphorylation cascade, creating binding sites for STATs. STATs are cytoplasmic transcription factors that continue with the phosphorylation process and subsequent dimerization before to be translocated to the nucleus and regulate the transcription of inflammatory genes (16). For example, it has been shown that STAT3 is more active in psoriatic skin and this mutation in KCs has been used to create a specific transgenic mouse (K5-Stat3C mouse model) (11).

3) Production of inflammatory markers

The activation of inflammatory cells such as MΦ, KCs or adipocytes induce the production of pro- and anti-inflammatory cytokines, which are small heterogeneous secreted group of proteins (<40 kDa), including IL-1β, IL-6, TNF-α among others, with

the function of promote or inhibit inflammation. Cytokines can have a paracrine function activating neighboring cells by the interaction with specific receptors or as in the case of KCs, autocrine function autoregulating the feedback loop of cytokines release (**Table 3**) (62). The expression of specific cytokines can be a feature of pathological mechanisms and they can be biomarkers for diagnosis, prognosis and therapeutic decisions in different diseases (16). In particular, KCs mediate the production of several chemokines of the CXC family (CXCL8, IP-10), produce a wide group of cytokines including IL-1α, IL-1β, IL-36, IL- 8, IL-13RA1, IL-13, IL-5RA, IL-6 and IL-10, TNF, G-CSF, TGF-β, thymic stromal lymphopoietin (TSLP), type 1 interferons (IFNs), growth factors (GM-CSF, TGF-α) and AMPs (such as β-defensins, cathelicidins or S100 family of proteins) (6). These mediators can exert different functions depending on the context. For instance, IL-1α combined with IL-17 and TNF-α activate innate immunity in skin (62). On the other hand, IL-1α with IL-6, IL-8, TGF-α, MCF and VEGF triggers an allergic reaction to house dust mites (HDM) (62).

The resulting inflammatory environment stimulates the neighboring cells to produce more inflammatory mediators and attracts innate immune cells to the stressed site. Therefore, inflammatory proteins in the blood, including C-reactive protein (CRP), haptoglobin, serum amyloid A, fibrinogen, and α1-acid glycoprotein, help to restore homeostasis and reduce microbial growth independently of antibodies during trauma, stress or infection. Additionally, under inflammatory conditions the antioxidant defense systems are modified. The increase of oxidative stress can induce production of reactive oxygen species (ROS), malondialdehyde (MDA), 8-Hydroxy-2- deoxyguanosine (8-OHdG) and isoprostanes, each of which can activate NF-κB, AP-1, p53, and STAT. Finally, it is important to mention that the alteration of certain enzymes including high-mobility group box 1 (HMGB1), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 promote cell lysis, phagocytosis and apoptosis (63),(15), (62).

Table 3: Summary of cytokines and their functions. Adapted from (64),(61) and (16).

Cytokine	Family	Main sources	Target cells and organs	Function
IL-1α, IL-1β	IL-1	MΦ, monocytes, neutrophils, B cells, T cells, DCs, epithelial (KCs) and endothelial cells.	MΦ	Activation (proliferation and differentiation)
			T cells	
			Hypothalamus	Induction of fiber
			Blood vessels	Pro-Inflammation
			Liver	Induces acute phase protein

IL-36α, IL36β, IL36γ	IL-1	KCs	CDs and MΦ	Pro-inflammation
			Endothelial cells	Promote T cell migration
			T cells	Activate the differentiation
			KCs	Cytokines production
IL-4	IL-4	Th2-cells	T cells	Anti-inflammation, cell differentiation
			B cells	cell differentiation and changes in IgE isotype
			MΦ	IgG and IgE production
IL-6	IL-6	MΦ, T-cells, adipocyte, B cells, DCs, epithelial (KCs) and endothelial cells	Blood vessels	pro-inflammation
			Hypothalamus	Fiber induction
			Liver	Cytokines induction
			B cells	Activation al proliferation
IL-8	CXC	Neutrophils, NK cells, Monocytes, KCs	Endothelial cells	Pro-inflammation, chemotaxis, angiogenesis
IL-10	IL-10	Monocytes, T-cells, B-cells, NK cells, DCs, KCs.	T cells, monocytes, neutrophils	Anti-inflammation, inhibition of the pro-inflammatory cytokines
IL-12	IL-12	DCs, MΦ, neutrophils, T cells	NK cells, Th1 cells, Tc cells.	Pro-inflammation, cell differentiation, activates NK cell
IL-11	IL-6	Fibroblasts, neurons, epithelial cells (KCs)	B cells	Anti-inflammation, differentiation, induces acute phase protein
TNF-α	TNF	Neutrophils, MΦ, NK cells, Monocytes, endothelial cells (KCs), CD4+ lymphocytes, B cells, DCs, adipocyte	Neutrophils and monocytes	cell proliferation, apoptosis, phagocyte cell action
			blood vessels	Pro-Inflammation
			Liver	Cytokines production
IFN-γ	IFN	T-cells, NK cells, NKT cells ; KCs	Cells with nucleus	Pro-inflammation, innate, adaptive immunity anti-viral
GM-CSF	Colony stimulating factors	T-cells, macrophages, fibroblasts	Stem cels	Pro-inflammation, macrophage activation, increase neutrophil and monocyte function
TGF-β		Macrophages, T cells and B cells	Th1 cells	Inhibition of the proliferation
			B cells	Anti-proliferative, induce changes in IgA isotype
			MΦ	Inhibition of cytokine production

The final goal of the innate response is the destruction of the harmful stimuli. For this purpose, among the proinflammatory secretion, MΦ control the immune response by three extra mechanisms. First, MΦ recognize the crystallized fragment (Fc) via FcγRI ($CD64^+$), antibody-dependent cell cytotoxicity (ADCC) and phagocytosis. Alternatively, they express the complement receptor C3bR to recognize the pathogens coated with C3b to promote their lysis and cytokine release. Finally, the last phase promotes apoptotic body removal involving anti-inflammatory mechanisms.

4) Recruitment of inflammatory cells

As mentioned, the released signals after the local activation of resident MΦ, monocytes and other cells including KCs; outcome in the recruitment of neutrophils, monocytes and NK cells. The first cells attracted are neutrophils, followed by monocytes, lymphocytes (NK cells, T cells, and B cells), and mast cells. Monocytes can differentiate into MΦ or DCs and are recruited via chemotaxis into damaged tissues. Neutrophils are key mediators of inflammation, activate T cells via antigen presenting cells and release localized factors to attract monocytes and DCs. Mast cells, reside in connective tissue and on epithelial surfaces, are effector cells that initiate inflammation. Activated mast cell release a variety of inflammatory mediators, including cytokines, chemokines, histamine, proteases, prostaglandins, leukotrienes, and serglycin proteoglycans.

B) MELANOCYTES

Melanocytes are pigment-synthesizing cells derived from the neural crest stem cells that migrate to distal parts of the embryo. In humans, there are approximately 1,500 melanocytes per mm^2 of epidermis mostly located in the basal layer in a ration 1:5 with KCs (65). They express specific proteins such as tyrosinase (tyr), TYRP1, DCT, Pmel17/gp100, MART-1, and MITF allowing their identification. Melanocytes contact with KCs in the superficial layers by dendrites extensions. However, these cells are also present in hair follicles and in the uveal tract of the eye (61). The major role of melanocytes is the production and transfer of melanin pigment to KCs. Melanin is a complex macromolecule that scatters and absorbs UV radiation. It is produced during the conversion of tyrosine to DOPA by the enzyme tyr in a rounded membrane-bound organelle, knowns as melanosome via a series of receptor-mediated, hormone-stimulated and enzyme-catalyzed reactions (66). Epidermal KCs use melanin to protect themselves against UV radiation-induced DNA damage. Once DNA damage appears, KCs stimulate the secretion of α-melanocyte by the activation of the hormone (αMSH), αMSH binds to the melanocortin 1 receptor (MC1R) expressed on melanocytes and subsequently induces synthesis of melanin and melanocytes proliferation. In homeostasis conditions melanocytes are controlled by epidermal KCs following a slow turnover rate in the human epidermis. The KC-derived growth factors released to control the melanocyte maturation include stem cell factor (SCF), endothelin 1 (ET-1), or fibroblast-derived factors such as basic fibroblast growth factor (β-FGF), hepatocyte

growth factor (HGF), or endothelin 3 (ET-3) (67), (68). Disruption of the homeostasis of the epidermal melanin production may triggered continuous proliferation and promote the melanoma development (disease included in the 3rd part of my thesis).

Despite the best-known function of melanocytes is melanin synthesis, accumulating evidence showed that in collaboration with KCs and Langerhans cells, melanocytes participate in the skin barrier protection and have immunomodulatory properties. First, the dendritic shape of melanocytes allows an extensive contact area with KCs, suggesting a complex signaling network. In addition, normal human melanocytes express functional TLRs, such as TLR 1, 2, 4, 5, 7, 9, and 10. Upon TLRs activation melanocytes can produce several pro-inflammatory cytokines including IL-1β, IL-6, TNF-α and chemokines including CCL2, CCL3, and CCL5 (69), (70). These factors will subsequently alert MΦ, neutrophils, fibroblasts and KCs. These inflammatory responses will control melanocyte's immune and metabolic functions leading to skin manifestations including rash, hyper or depigmentation, epidermolysis and psoriasis-like lesions. Beside, some melanocyte cell lines also express major histocompatibility complex class II molecules, intercellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and CD40 antigen, indicating that melanocytes participate in T-cell activation (69). Similarly to KCs, melanocytes may express IFN type I contributing to the first line of defense against viruses in the skin such as HIV, hepatitis C virus (HCV), alphavirus, varicella-zoster, and parvovirus and bacteria including Mycobacterium leprae and Leptospira (70).

C) MERKEL CELLS

Merkel cells are slow-adapting type I mechanoreceptors with oval shaped, located and packed in sites of high tactile sensitivity such as digits, lips, regions of the oral cavity and outer root sheath of the hair follicle. Usually they are grouped into specialized structures known as tactile discs or touch done, attached to basal KCs by desmosomal junctions to play the role of mechanoreceptors (**Figure 4**). Small deformations or alteration of KCs stimulate Merkel cells which produce a chemical signal transmitted to the afferent neuron present in the adjoining KCs which relay the signal to the brain (71).

Immune cells:

A) LANGERANS CELLS

Langerhans cells (LCs) identified in 1868 by Paul Langerhans, represent about 2 to 4% of epidermal cells with hematopoietic origin derived from bone marrow. LCs are located between KCs in homeostatic conditions and are the unique subset of professional antigen-presenting cells (APCs) in epidermis in addition to dermal MΦ, DCs and $CD8^+$ and $CD4^+$ T_{RM} cells (**Figure 1**) (62). They decipher the microenvironmental context and create a network with potential invaders modulating the intensity of the immune response. LCs express C-type lectins on their plasma membrane and langerin (Birbeck granules, CD207) in mice and CD1a, CD11c, CD32, CD324, HLA-DR, Fcγ and Fcε receptors in human (6). CD207 is co-expressed by a different subtype of dermal resident DCs in mice (72). The surface C-type lectins are PRRs with the capacity to recognize MBP and mediated the endocytosis of the pathogen to the Birbeck granule to perform the antigen processing (73). Despite the fact that LCs were considered initially a subset of DCs present in skin to be the "first line fighter" and migrate to lymph nodes (LNs) to present antigens detected, recent studies have demonstrated the involvement of LCs in tolerogenic responses. Thus, LCs are considered a subset of tissue-resident MΦ that gain a DC-like phenotype (74). Upon inflammatory conditions, LCs capture the antigen by the elongation of their dendrites through the epidermal tight junctions and increase their migratory rate to the skin draining LNs. They maturate and finally arrive to the T cell area by up-regulating MHC class II molecules, co-stimulatory molecules (CD40), and CCR7 chemokine receptor essential for migration (21).

B) T CELLS

In addition to LCs and merkel cells, it is estimated that human skin host one million of T cells per cm^2. In homeostasis conditions human epidermis presents $CD4^+$ and $CD8^+$ T_{RM} cells and innate lymphoid cells (ILC) (**Figure 1**). γδ T cells are a subset of innate immune cells residing permanently in the epidermis of mice but not in humans. $CD8^+$ T_{RM} comprise a population of non-circulating memory cells that appear in epidermis after the resolution of skin inflammation in mice and humans. In mice, it has been showed that skin T_{RM} cells persist for over a year, whereas lung T_{RM} cells are maintained

only few months. This epidermal T cells are capable to produce IFN-γ and TNF-α but they proliferate less. Finally, ILCs tissue resident, coming from common lymphoid progenitors, do not express rearranged antigen-specific receptors (75).

B.1) THE CUTANEOUS γδ T CELLS COMPARMENT

γδ T cells are considered as members of the innate immune response first, because the repertoire of γδ chains have less diversity than the classical αβ repertoire. Secondary, γδ T cells present cytotoxic and cytokine-producing ability without TCR engagement by the interaction with different cytokines (76).

In mice, γδ T are classified by Heilig and Tonegawa in Vγ1–Vγ7 subtypes (11). Each subtype is localized in specific organs and are maintained by self-renewal. Their development and migration to the epithelial tissues starts during fetal life and at day E13, the Vγ5 subtype (DETC) produced in the thymus, migrates to the epidermis to maintain skin homeostasis and participate in wound repair. In mice, CCR10 expression in Vγ5 cells is required for efficient homing to the epidermis, where they mainly express IFN-γ and IL-17 cytokines (73).

In humans, γδ T cells are usually classified based on δ chain expression in Vδ1, Vδ2, and Vδ3 subtypes. Vδ1 cells are located in barrier tissues while Vδ2 and Vδ3 are observed in the blood of healthy patients (76). In contrary to murine, human γδ T cells are dependent on TCR engagement for activation and cytokines production. Vδ1 cells in the skin interact with αβ T cells and recapitulate the DETC role, producing KCs growth factors. However, the full repertoire of cutaneous γδ T cells has yet to be fully investigated (75).

C) INNATE LYMPHOID CELLS (ILCs)

Tissue resident ILCs, derived from bone marrow, are subdivided in: ILC1s express the transcription factor T-bet and release IFN-γ, ILC2s express GATA3 and produce the Th2 cytokines IL-5 and IL-13 and ILC3s express RORγt and secrete IL-17 and IL-22. Despite ILCs are tissue-resident cells, they have been detected in the circulation expressing high levels of cutaneous leukocyte-associated antigen (CLA), a skin homing

marker. In mice and humans, all the three groups have been observed in the skin and in homeostasis conditions ILC2s is the largest population. Furthermore, in mice it has been shown that ILC3s are mainly present in epidermis, ILC2s populate the subcutaneous layer and the dermis contains ILC2s and ILC3s. Whereas the presence of ILC1s in the skin is rare. It is known that ILC2s play a role in skin homeostasis by the release of IL-13, amphiregulin and epidermal growth factor (EGR). However, it has been reported that ILC2s are involved in AD. Lastly, ILC3s are related with psoriasis for their capability to produce IL-17A and IL-22 (**Figure 1**) (75).

3) DERMIS

The dermis is an integrated system with mesodermal origin that collaborates with epidermis to keep the properties of both tissues. Its principal function is the body protection providing pliability, elasticity and tensile strength. Additionally, dermis participates in the development of the dermal-epidermal junctions, interacts in the reparation and remodeling of skin after wounds and contains the end of the epidermal appendages. The principal components of the dermal extracellular matrix (ECM) formed by filaments and amorphous connective tissue, are stromal cells such as fibroblasts, fibrocytes, blood and lymph vessels, lymphocytes, plasma cells, DCs, MΦ, mast cells, γδ T cells and ILCs. Collagen represents 70% of skin dry weight and it is a protein with the biggest stress-resistant in skin (22).

Dermis is formed by two components: the papillary and reticular layers which are fused together without clear separation and connected to the epidermis by the basement membrane. The papillary layer is the upper part and it is composed by lax connective tissue. The reticular layer is the profound layer, thicker, with less cells, and form with dense connective tissue and bundles of collagen fibers. In the steady state, few neutrophils, monocytes and αβT cells are presented in the dermis. However, in response to inflammatory stimuli, immune cells are rapidly recruited in the dermis. In the next pages, I will be focus on the explanation of the adaptive immune system in skin inflammation since the role of the innate response has been previously explained.

- **<u>Adaptive immune response in skin inflammation</u>**

Adaptive immune system promotes a delayed but specific response to enhanced immuno-surveillance, called ‘second line of defense’ when the innate immunity is

insufficient to resolve inflammation. The power of the adaptive immunity resides in its unique ability to generate and retain memory for future contacts with the antigen (54).

In general, T-cell functions in inflamed tissue discussed deeper bellow are 1) the promotion of inflammation to eliminate the harmful antigen, 2) stop the immune response to prevent massive collateral tissue damage or cytokines storm and 3) promotion of homeostasis and tissue recuperation after inflammation.

1) Adaptive immune response promoting inflammation response

1.1- Antigen cross-presentation

After the antigen recognition in skin by APCs, including epidermal LCs, dermal MΦ and DCs, the antigen is captured by endocytosis, processed and presented as peptides in the context of MHC to the adaptive immune system, predominantly in draining LNs due to the migratory capacity of DCs. DCs, discovered in 1973 by Steinman and Cohn, are located in LNs (resident DCs, resDCs) and in various non-lymphoid tissues (migratory DCs, miDCs) in an immature state and have the crucial function to process and present antigens to naïve T cells. Consequently, their mayor role is the promotion of the crosstalk between innate and adaptive immunity. They form a complex heterogeneous subset of cells. In mouse skin DCs are classified in either non-conventional plasmacytoid DCs (pDCs), which complete their differentiation in the bone marrow and conventional myeloid DCs (mDCs) which undergo the development process in periphery including the dermis, subdivided in XCR1+ (cDC1s) and CD11b+ (cDC2s). They share functional homology with human $CD141^{hi}CD14^{+}$ DCs and $Cd1a^{+}CD1c^{+}$ DCs, respectively. In homeostasis pDCs population are located in secondary lymphoid organs, and upon pathological conditions, they migrate to the skin, and produce a large amount of IFN-α via TLR7/9 activation (77), (72).

1.2- Classical antigen presentation pathways and T cell activation

Indeed, the first signal of DCs to T cells inside the LNs is mediated by type I and type II MHC molecules. Classically MHC-II process exogenous antigens (from microbes but also apoptotic or infected cells) whereas endogenous antigens are presented by MHC-I. Once the peptide is presented, MHC molecules interact with the specific TCR expressed by T cells. In order to provide the appropriate signal to T cells, DCs expressed costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86), which interact with

CD28 expressed on naïve T cells (**Table 4** include the different costimulatory signals between DCs and T cells). The absence of this secondary signals will lead to T cell anergy, this strategy gives interesting perspective for the development of new immunotherapy treatments in different pathologies as cancer (anti-CLT4, anti-PDL-1). The last crucial signal is given by the cytokines presented in the microenvironment which have the responsibility to define the polarization of the differentiated T cells. After the activation of the antigen-specific T cell, the cell will proliferate, release to the blood, and explore the body to find the damaged tissue. DCs will cooperate to direct the T cell to the skin through the "imprinting" mechanism that is not fully understood yet. However, vitamins, as Vit D_3 produced in skin are accumulated in skin-derived DCs. When T cells are activated by skin-derived DCs, Vit D_3 induces CCR10, CCR4 and CLA expression in T cells, helping the skin addressing (54), (77), (72).

Table 4: Costimulatory signals of the immunoglobulin superfamily (78)

Family	CDs ligands	T cell receptors	Function
Immunoglobulin superfamily (IgSF) with Ig-like domains	CD80(B7-1) CD86(B7-2)	CD28	Strengthen TCR signaling to provide T survival signals and induce other costimulatory receptors
	CD80/86	CTL4	CTL4 has high homology with CD28 and higher affinity. Immunosuppressive effect
	ICOSL	ICOS	Low homology with CD28
	MHC-II	LAG-3	LAG-3 is expressed in Treg and produce immunosuppression
	PD-L1 PD-L2	PD-1	Inhibition of the PI3K/Akt pathway
TNF superfamily (TNFSF)	FasL	TRAILR1 TRAILR2	Induce cell apoptosis
	CD70	CD27	DR3 and 4-1BB are inducible receptors on T cells upon CD28 signaling that interact with 4-1BBL
	OX40L	OX40	Control the late activation and proliferation of $CD4^+$T cells

Furthermore, coming back to the KCs functions, Nickoloff and Turka demonstrated that under specific conditions KCs can act as APCs expressing MHC II activating and maintaining consequently the T cells tolerance. For instance, it has been shown that A431, a KC-derived cell line can present antigens and induce T cell activation and recruitment in a MHCII-dependent manner. Additionally, HaCaT cells and HPV-immortalized KCs present antigens to $CD4^+$ and $CD8^+$ T cells mediating MHCII with the implication of ICAM. Furthermore, it has been shown that in presence of S. aureus KCs can express CD80 activating T cell response (62).

1.3- T cell response

T cells, produced in bone marrow and matured in the thymus, have specialized functions, including cytotoxicity, suppression or control of the responses through the secretion of cytokines. They are classified in two types, αβ T cells and γδ T cells, which differ in the structure of TCR laid out on their membrane.

-In mice, dermal γδ T cells are constitute by Vγ4 and Vγ6 subtypes. These dermal γδ T cells express several hallmarks similar to Th17 cells including RORγt, IL-7R, CCR6, and IL-23R expression as well as ability to produce IL-17. They can be stimulated by IL-23, which leads to their expansion and IL-17 production to promote immuno-surveillance functions (75).

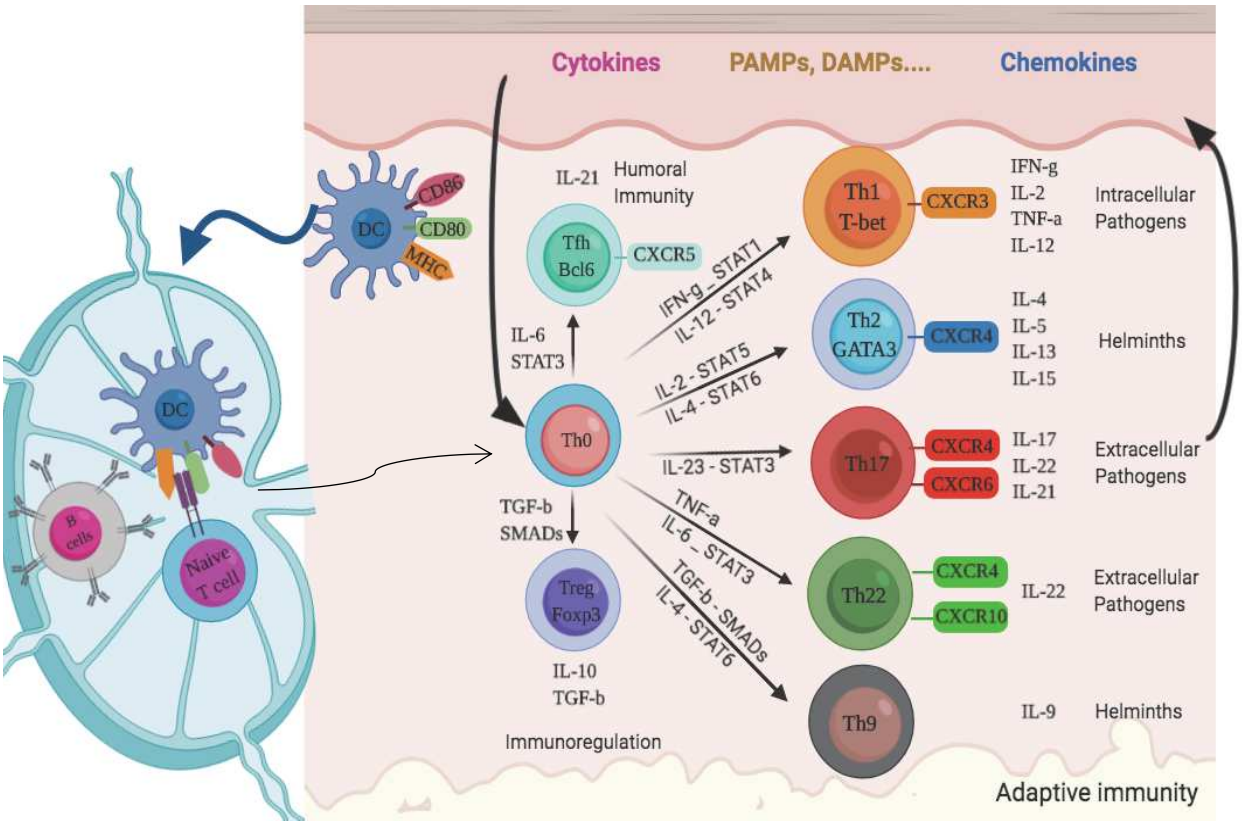
-In general, αβ T cells are classified in two different subpopulations of effector T cells: $CD8^+$ cytotoxic cells (Tc, also denominated CTLs), that recognize antigenic peptides presented by MHC-I molecules and $CD4^+$ helper T (Th) cells, which bind to MHC-II. CTLs promote the cellular lysis of the target cells by the secretion of lysis enzymes such as perforins and granzymes. Additionally, CTLs triggers caspase activation leading to cell apoptosis of cells with intracellular pathogens. Lastly, CTLs produce TNF-α and IFN-γ, which have antitumor and antiviral microbial effects. On the other hand, $CD4^+$ Th cells are essential for T cell-mediated and antibody-mediated response. Th cells are differentiated into two subsets of conventional T cells Th1 and Th2, depending on the cytokine environment and the nature of the antigen. When MΦ and DCs release IFN-α and IL-12, Th1 differentiation is stimulated, these cells produce IFN-γ and promote the recruitment of phagocytic cells. These IFN-γ–producing Th1 cells are $CXCR3^+$ and need the expression of the transcription factor T-bet for their differentiation and activation. The Th2 polarization occurs when naïve $CD4^+$ Th cells secret IL-4. These cells are characterized by $CCR4^+$ chemokine expression and are responsible of the defense against extracellular organisms. They release cytokines such as IL-4, IL-5 and IL-13 and promote the recruitment of eosinophils, basophils and mast cells. Th2 cells express the transcription factor GATA-3 (79).

Recently, several extra subsets of $CD4^+$ Th cells were identified: Th17 (expressing $CCR4^+$ $CCR6^+$), Th22 (expressing $CCR4^+$ $CCR10^+$), Th9 and T follicular helper (Tfh) cells (**Figure 7**) (80). Th17 differentiation is stimulated by the release of IL-23 by APCs,

promoting antibacterial or antifungal immune response through the recruitment of neutrophils and produce IL-17, IL-21 and IL-22. The transcription factors characteristic for the development of IL-17-producing Th17 cells subtype are RORγt and RORα. Th1 and Th17 responses may also promote autoimmunity, whereas Th2 responses may mediate allergy (54). Th22 cells are a subgroup of circulating T cells promoted by TNF-α and IL-6 released from DCs, these cells have skin-homing potential and are responsible of the IL-22 production. Th22 cells are present in inflammatory skin diseases and predominantly found in the epidermal compartment (77). Moreover, the main function of Tfh cells is to provide help to B cells to produce high affinity antibodies and memory B cells to control humoral immunity (81). Lastly, Th9 cells, mainly produce IL-9 providing anti-helminth and anti-tumor activity. The recruitment of T cells in human skin is selective and promoted by adhesion molecules expressed on T lymphocytes (lymphocyte antigen-1) and the corresponding ligands in the skin (E-selectin or intercellular adhesion molecule 1 and vascular cell adhesion protein 1) (12).

Moreover, B cells have membrane-bound immunoglobulins on their surface, so-called B cell receptors (BCRs), that bind and internalize foreign antigens leading to the production and secretion of antigen-specific antibodies. Their principal function is the removal of the pathogens and toxins by direct neutralization or phagocytosis. Furthermore, effector B cells have several functions such as antigen presentation and production of cytokines in skin inflammation. More recently, B cells via IL-10 production are found to inhibit Treg and Th17 responses (76).

Figure 7: Overview of CD4$^+$ T cells heterogeneity in adaptive immune system. After innate immune response, the first phase of adaptive immunity is leaded by the interaction of active APC with naïve T cells. Active T cells migrate from LNs to the damaged place where they induce humoral immunity depending on the stimuli. CD4$^+$ Th cells are subdivided according to the cytokines secretion pattern and by the expression of the different transcription factors in Th1, Th2, Th17, Th22, Th9, Tfh or Treg cells. The resulting microenvironment create a feedback loop re-stimulating epidermal cells mainly KCs to release further mediators. Figure adapted from (77), (76), (77). Done by Biorender (in the following page)



1.4- Immunological Memory

T cell persistence "memory" is the third essential phase of adaptive immunity after T activation and the effector function. After the first stimuli exposition, T cells rich the skin and are maintained as memory populations by growth factors released by KCs and other tissue resident cells.

Recently, Watanabe et. al using a skin xenograft model, in which nude NGS mice were grafted with human neonatal foreskin identified 4 different populations of memory T cells: effector memory (TEM) and resident memory (TRM) both resident cells and migratory memory (TMM) and central memory (TCM), depending on their localization and functions. TEM expresses high levels of CD44 and lack homing addressins (L-selectin and CCR7) in both human and mice (82). They secrete mainly IFN-γ, Th1, Th17, and Th22 proinflammatory cytokines. In contrast TCM express high levels of L-selectin, CCR7, CLA and CCR4, which allow their traffic between LNs and skin. They can also produce IL-2 and Th2 cytokines (IL-4 and IL-13). TRM cells, remain in tissues after infection to re-act in case of antigen challenge. There are two subsets of TRM, the $CD103^{+}$ cells enriched in the epidermis producing IFN-γ, TNF-α, and IL-22. In the

contrary, TRM CD103^{-} cells are in the dermis with a lower effector function. The last subset is TMM expressing CLA^{+}, CCR7^{+} and L-selectin^{-}, which have intermediate functions in cytokine production between TCM and TEM (82).

2) Resolution of the inflammation

The inflammatory mediators released to activate innate and adaptive immunity in epidermal and dermal cells should be controlled after the neutralization of the pathogen to recover tissue homeostasis. These processes include reduction of neutrophils, T cells infiltration by apoptosis and release of anti-inflammatory cytokines (83).

3) Homeostasis and tissue recuperation after inflammation: Regulatory T cells

The last CD4^{+} subtype of T cells is the regulatory T (Treg) cells. This division of cells play the principal role of maintain the immunological homeostasis suppressing self-reactive of inflammatory T cell responses that were not eliminated during the process of negative selection and interact with the innate immune system (84), (85), (86). They are identified by the expression of CD4, CD25 (IL-2 receptor a chain), CD45RA and the transcription factor orkhead box P3 (FoxP3) essential in their development, maintenance and function. FoxP3, which define murine Treg cells, is not exclusively specific for human Treg cells. They represent 5-10% of peripheral CD4^{+} T cells in the circulation and they are a really heterogeneous subgroup (87). The central role of Treg cells in peripheral tolerance was demonstrated in human and mice because the absence of FoxP3 promotes a fatal multiorgan autoimmunity. The development of Treg cells begins in the thymus and is divided in two different pathways. The first group is denominated thymus-derived Treg cells, that differentiate into FoxP3^{+} Treg cells in the thymus following recognition of self-antigen by the TCR. The second group called peripherally derived Treg cells, exit the thymus as naive CD4^{+} T cells and differentiate into FoxP3^{+} Treg cells in the periphery following recognition of their cognate antigen. In the skin thymus-derived Treg cells expressing GATA3 and Nrp1–Helios are the dominant Treg population (88).

For efficiency and efficacy, immune responses often polarize toward a single specific pathway. However, mixed responses may also occur depending of the type of immune response developed and specialized Treg subsets of cells will create a significant level

of complexity (89). Furthermore, it has been identified 4 different populations of Treg cells in human peripheral blood based on the expression of the chemokine receptors:

- $CXCR3^{+}$Tregs, which expressed T-bet are classified as Th1 that produce IFN-γ.
- $CCR6^{+}$ $CCR4^{+}$ Tregs, which produce IL-17 and express RORγt were considering Th17-like Treg cells.
- $CCR6^{+}$ $CCR4^{+}$ $CCR10^{+}$ Tregs, were classified as Th22 cells and secrete IL-22
- CCR4+ Tregs produce IL-4 and express GATA3 were denominated Th2-like Tregs.

Although all Treg populations showed suppressive activity in vitro, they differed in their production of both pro- and anti-inflammatory cytokines, had different expression patterns of Th-associated transcription factors, and proliferate differentially in response to challenge antigens associated with either Th1, Th2 or Th17 cell responses (89).

4) HYPODERMIS

The hypodermis or subcutaneous facia is the deepest layer of skin containing adipose lobules with the end of the skin appendages.

1.2- PSORIASIS

1.2.1 DEFINITION AND EPIDEMIOLOGY

Psoriasis (engl. 'itching condition') is a complex relapsing chronic inflammatory disorder driven by aberrant interactions between the resident epithelial cells and the immune system, which affects mainly the skin and nails, although it has systemic pathological effects. The severity of psoriasis varies markedly between individuals, ranking from minor irritations to intense symptoms (90). It is frequently associated with different comorbidities such as arthritis (denominated psoriatic arthritis, PsA presented in 30% of the patients), as well as cardiovascular diseases and diabetes. Psoriasis is considered inside of the metabolic syndrome diseases including obesity, hypertension and dyslipidemia. Moreover, it has been also associated with crohn's disease (CD), psychological disorders, uveitis, celiac disease, non-alcoholic fatty liver disease (NAFLD), chronic kidney disease and erectile dysfunction (91). Until now, there is no an effective and complete cure. In addition to the pathophysiological characteristics of the disease, studies of quality of life suggest that psoriasis is associated with negative impact in terms of social, emotional and other burdens on patient lives (92).

Psoriasis affects approximately 2-3% of the worldwide population (around 125 million people globally), with a global prevalence between 0.09% and 11.4% (93), (94). It appears most commonly in populations of northern Europe. The prevalence during 1979-2008 in Norway increased from 4.8% to 11.4% and in France was around 5% (calculated by a self-reported psoriasis within a skin cancer screening program (95)). However, psoriasis presents lower prevalence in populations of eastern Asia and in China in the last 25 years the incidence has been increased between 0.17% to 0.59%. Furthermore, it has been shown that the incidence of psoriasis in US population almost doubled between the 1970s to 2009 with a significant increase in pediatric psoriasis group (96). The beginning of the symptoms can appear at any age but the disease is more common from the age of 50 (95). The prevalence of psoriasis in adults ranged from 0.51% to 11.43%, and in children from 0% to 1.37% (94). In 1985, Henseler and Christophers reported two different types of patient cohorts of psoriasis vulgaris (PV) from a database of 2174 patients. In light of this discovery, patients were classified in an early-onset at ages between 16 and 22 years (psoriasis type I) and in a late-onset from 57 to 60 years (psoriasis type II).

1.2.2 CLINICAL AND HISTOLOGICAL CHARACTERIZATION

Psoriasis is defined by epidermal hyperplasia, altered KCs differentiation (high involucrin (IVL)$^+$ cells (97)), hyperproliferation (increased Ki67$^+$ or cytokerantin 16$^+$ cells (98)), infiltration of leukocytes (such as T cells, MΦ and neutrophils), dilation and growth of blood vessels, that appears with different morphology all over the body. However, the principal sites of psoriatic lesions are the elbows, knees, the sacral region, scalp and nails (**Figure 8**) (99). Occasionally, psoriasis plaques may also appear at mechanical trauma sites knowns as Koebner ‘s phenomenon (100). The common symptoms are erythema, scaling of the skin, itching, swelling, burning and bleeding produced by red papules and symmetrical plaques covered with white or silver scales that produce physical changes and deformations (99). Since the symptoms between patients can vary strongly, Fredriksson and Pettersson in 1978 proposed the `Psoriasis Area and Severity Index’ (PASI) method of severity evaluation. Nevertheless, even this tool combines several important features of the disease including erythema, desquamation and the body surface area affected (BSA) calculated based on the ‘one hand method’ assuming that the hand represents 1% of the total BSA, the PASI is not a universal definition for the severity of psoriasis. For instance, it has been shown that the BSA calculation is frequently overestimated and there is a lack of concordance in the scoring system between physicians and it is still unclear if the severity correlates with the presence and intensity of pruritus (101).

Histologically, it is characterized by acanthosis produced by thickened epidermis, in combination with parakeratosis, hyperkeratosis, and elongated rete ridges, called papillomatosis. Acanthosis and papillomatosis are generated by the acceleration of the proliferation of basal KCs followed by a precipitated differentiation process, that outcomes in abnormal nucleated cells in the stratum corneum. Furthermore, the KCs assembling the suprabasal layer present senescent features, contributing to the resistance of the plaques to apoptosis and transformation. Likewise, erythema come out as a consequence of the dilatation of dermal blood vessels within the dermal papillae. During plaque development neutrophils recruited to the damage epidermal site, create a pustule, called Munro‘s microabscesses. Human studies have been reported that the number of infiltrating neutrophils correlates with the severity of psoriasis (102). In addition of neutrophils, other immune recruited cells are found in psoriatic epidermis including CD4$^+$ and CD8$^+$ T lymphocytes (99), (100), (90), (103).

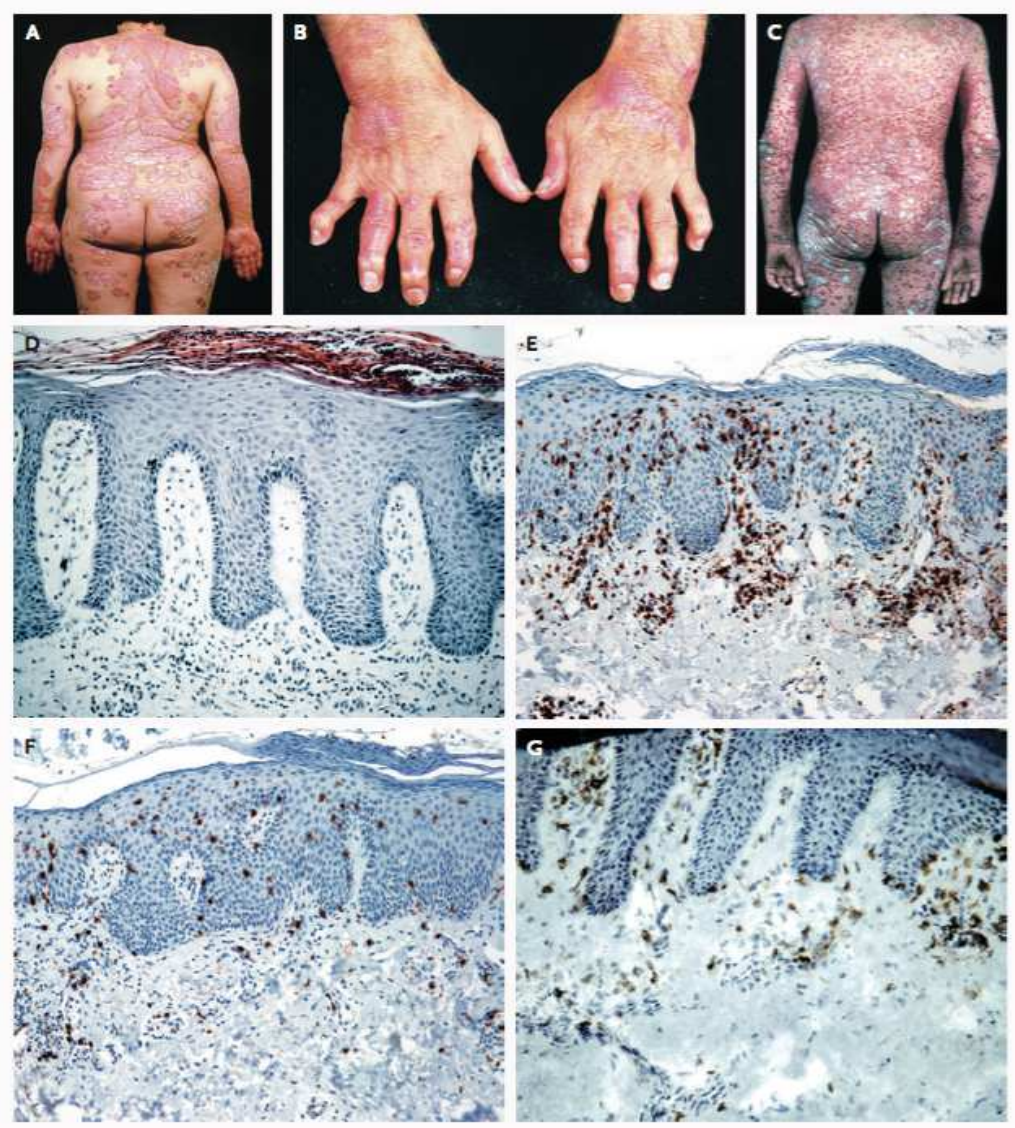


Figure 8. Clinical and Histologic Features of Psoriasis. (**Panels A, B and C**) Erythematous, scaly, sharply plaques in different sizes and shapes. **B)** Psoriatic arthritis (PsA), in hands with nails affected. **D)** Hematoxilin and eosin of psoriatic skin showing the epidermis, parakeratosis, elongated rete ridges, and a mixed cellular infiltrate. **E)** $CD3^+$ T cells. **F)** $CD8^+$ T cells are detected around capillaries of the dermis and in the epidermis. **G)** $CD11c^+$ dendritic cells are detected mainly within the upper part of the dermis. (**E,F,G** 3,3′-diaminobenzidine and hematoxylin contra staining) (photos from Clinical St. John's Institute of Dermatology) (99).

1.2.3 FORMS OF PSORIASIS

Psoriasis vulgaris (PV) also called "plaque psoriasis" is the most common variant form that affect around 80 to 90 % of psoriasis patients. Other typical subtypes are psoriasis guttate, pustulosa palmoplantaris, inverse/flexural psoriasis, psoriatic erythroderma, psoriatic arthritis, and psoriatic nail disease. Patients are classified in the different subgroups depending on the morphology, occurrence or distribution, all described in **Table 5**.

Table 5: Classification of the different psoriatic forms (90)

Type of psoriasis	Occurrence	Location	Morphology	Special features
Psoriasis vulgaris (PV) (plaque-type)	80 to 90%	Elbows, knees, scalp, trunk	Red sharply round or oval plaques covered by silvery scales	High prevalence of pruritus. The plaques can cover large areas of the skin.
Psoriasis guttate	2%; post infectious	Trunk,	Small, scattered papules. typically triggered by Streptococcus A.	Self-limiting, potential co-occurrence with psoriasis vulgaris. Most common in children and people below 30's
Generalized Pustular Psoriasis (GPP)	More common in smoking women over 40 years	Palms and soles	Pustules/blisters of yellow-brown color with scales. Acute and rapidly progressive course and systemic symptoms	Two phenotypes: 1.-pustulosa palmoplantaris (PPP) 2.-Acrodermatitis continua of Hallopeau (ASC): located in distal zones including the tips of the fingers and toes
Psoriasis inversa/flexural	2%	Intertriginous sites	Red erosive erythematous plaques and patches without scales	The symptoms are worse with friction and sweating
Erythrodermic psoriasis (EP) (rare and severe variant)	1% - 2.25%	75% of body surface affected	Inflammation, Erythema, sterile pustules and scaling	Psoriasis is not the only cause; life-threatening. Associated with a Th2 phenotype
Psoriatic arthritis (PsA)	25%	joints	Seronegative inflammatory arthritis	5 different types (distal interphalangeal, mutilans symmetrical, asymmetrical and spondylitis)
Psoriatic nail disease	50%	Nails	Small pits in nail plate with 'oil spots' beneath nail plate	Nail plate can detach and may become thickened and discolored

1.2.4 ETIOLOGY

The etiology of psoriasis remains unclear, nevertheless in the last years several advances have been made in the understanding of this multifactorial disease and there are evidences indicating that is developed through an interplay of genetic, epigenetic, environmental, immune system, infections, and lifestyle factors. Several studies about the family history of psoriatic patients around 1960s revealed that relatives have a genetic predisposition to the development of the disease emphasized in twins. However, the inheritance pattern of psoriasis is complex and could be explained only in few cases (104), (105).

1.2.5 GENETIC FACTORS

Approximately 75% percent of psoriasis patients reported a positive family history. Classical genome-wide association studies (GWAS) performed in different populations during last years have identified at least 12 chromosomal susceptible loci linkage, that can contribute to psoriasis, denominated PSORS1 to 12 (93). Nevertheless, only PSORS1 variants were validated in all experimental cohorts (106). The major susceptibility factor associated with the development of psoriasis (PSORS1), is the variant in the MHC class I molecule HLA-C (107). The HLA-Cw*0602 allelic variant is located on chromosome 6p21, and it is present in up to 60% of psoriasis patients. Individuals who are homozygous for this allele have 5 fold-increased risk compared to heterozygous to develop psoriasis. There are other genes associated with PSORS1 overexpressed in psoriatic epidermis including *ERAP1* (endoplasmic reticulum aminopeptidase 1) that plays a role in processing peptides for loading into MHC class I; *MICA* (MHC class I polypeptide-related sequence A) (93), *CCHCR1* that encodes the coiled-coil α-helical rod protein 1 and *CDSN* (105), (108).

Among all the diverse variants in psoriasis-related genes identified, they can form clusters belongs to the IL-23/Th17 axis, NF-κB signaling pathway, regulation of T-cell function and related with the terminal EDC in KCs. For instance, Nair et al., had identified single nucleotide polymorphisms (SNPs) in *IL-12Bp40, IL-23Ap19* and *IL-23R* genes (the most consistently associated SNP within IL-23R encodes a R381Q amino acid substitution) associated with psoriasis (107). Additionally, other gene variants were identified in genes such as *TNFAIP3*, which encodes for TNF-inducible protein 3; *TNIP1* encoding for ABIN-1; *TRAF3IP2* encoding the signaling adaptor molecule ACT1; *CARD14,* which encodes an adaptor protein highly expressed in KCs, with regulates *TRAF2,* as well as *NFKBIA* encoding for the inhibitory protein IκBα; all of them implicated in the regulation of NF-κB signaling pathway (109). Recently 15 new susceptibility loci responsible to T-cell regulation functions including *RUNX3, TAGAP* and *STAT3* and responsible for the MΦ activation such as *ZC3H12C* were identified in European psoriatic patients (110). Furthermore, PSORS4 include more than 60 genes related with KCs differentiation, maps on chromosome 1q21, including genes encoding for S100 family.

Importantly, patients with GPP, present specific genetic mutations in *IL-36RN*, which leads to the loss of the blocking of the IL-36 downstream signaling pathway (I will discuss about this pathway in next session), and other mutations in *REL, TYK2, RUNX3, STAT3*, and *TRAF3IP2* genes (104), (111).

1.2.6 PATHOGENESIS

The basic pathogenic mechanisms of psoriasis have not yet been fully elucidated. Nevertheless, after a long debate during the last years, it has been demonstrated the important role of immune mechanisms in psoriasis disease. The current hypothesis is based on the crosstalk between autoreactive T cells, DCs and resident KCs as key players in the initiation, progression, and persistence of the disease (112). Therefore, IL-1 family members and the IL-23/IL-17/IL-22 axis appear to be principal drivers, findings supported by the results obtained in the clinical trials of biologic targeting therapies in psoriatic patients (97), (113), (114) (biological therapies are explained in the session 1.2.7).

In general, early upstream events in psoriatic inflammation include the induction of innate immunity pathways by the overproduction of KC-derived AMPs, chemo-attractants and pro-inflammatory cytokines. KCs-secreted molecules are responsible of the activation and recruitment of pDCs, mDCs, neutrophils and MΦ with a consequent presentation of the autoantigen and stimulation of the adaptive immune system. These mediators promote IL-23/IL-17/IL-22 and IL-12/IFN-γ/TNF-α axes. IL-17/IL-22 producing T cells participate in the initial phases, whereas IFN-γ-producing Th1 and CTLs are activated during the chronic phase overproducing inflammatory mediators that self-amplify and sustained the loop (**Figure 9**) (115). In addition to T cells, neutrophils, mast cells, γδ T cells and ILC3 play a role as producers of IL-17 and IL-22 (93). Then, the role of each group of cells is explained more in detail bellow.

1) ROLE OF KERATINOCYTES IN PSORIASIS

   Some years ago, the hyperproliferation and abnormal differentiation of KCs observed in psoriasis was considered as a secondary phenomenon induced by immune activation. However, nowadays it is known that after the skin koebner's phenomenon or the initial injury, KCs have a crucial role in triggering the recruitment of innate immune cells and sustaining the chronic inflammatory phase

of the disease (116). In psoriatic lesions the early-differentiated KCs present an overproduction of involucrin and a downregulation of filaggrin and loricrin (117), (118). Besides, as mentioned above, KCs are the source of different factors such as:

- LL-37, also called human cathelicidin is an "alarmin", released by epithelial cells and human polymorphonuclear cells (PMNs), which can interact with the surrounding MΦ interacting with different surface receptors. In particular, LL-37 affects adenosine triphosphate-receptor P2X7, TLR signaling, epithelial growth factor receptor (EGFR) transactivation and intracellular Ca2+ mobilization (119). Additionally, LL-37 can forms complexes positively charged reacting with negatively charged self-genetic material released by damaged cells (120). LL-37/DNA complex stimulates TLR9 expressed in pDCs promoting PP development by the production of type I IFNs (IFN-α and IFN-β) (121), (122), (123). However, LL-37/RNA complexes stimulate pDCs through TLR7 (124). Consequently, Type I IFNs activate mDCs via TLR8, which can migrate into draining LNs to modulate T cells differentiation and TNF-α, IL-23, and IL-12 secretion in the skin, orchestrating the proliferation of Th17 and Th1 cells, respectively. Furthermore, slan+ monocytes are important pro-inflammatory cells found in psoriasis skin, that respond to LL-37/RNA activation secreting TNF-α, IL-12, and IL-23 (125).

-Keratin-17 is ectopically up-regulated in psoriatic lesional uper-layer of the suprabasal compartment in epidermis participating in psoriasis development (126).

-melanocytic autoantigen ADAMTSL5 (metalloprotease domain containing thrombospondin type 1 motif-like 5) promotes the production of IL-17 or INF-γ activating $CD8^+$ T cells (127). ADAMTSL5 is principally express in melanocytes. However, KCs present a strong staining of this factor that can be associated with its own synthesis or from the deposition of the protein in KCs following the normal melanosome pigmentation transfer of melanin (128).

- β-defensins, including DEFB4A (the functional non-chemokine, ligand for CCR6) which is the most psoriasis-specific AMP, up-regulated by IL-17A and the increase of NETs presented in the inflammatory side (129), (130), (131).

-S100 proteins include, S100A7 (psoriapsin) which participates in wound healing reparation, KC differentiation, nucleocytoplasmic transport, and promoting chemotaxis for $CD4^+$ T cells, neutrophils, and monocytes. The production of S100A7 is increased by IL-19, IL-36γ, and TNF-α. S100A8 and S100A9 forms a heterodimeric complex (132) and S100A15 (koebnerisin), are elevated in psoriatic plaque and decreased by treatment with anti-IL-17A antibody. One of the roles of S100A8/A9 heterodimer is the increase of CXCL1, CXCL2, CXCL3, CXCL8, CCL20, IL-6, and TNF-α, enhancing KCs proliferation (133).

-Lipocalin-2 (LCN2) is expressed in various tissues including liver, lung, kidney, adipocytes, macrophages, and epithelial cells. It is a proinflammatory mediator to enhance the production of several cytokines, such as IL-6, IL-8, CXCL1, CXCL2, and CXCL10, promoting the initiation of the innate immune response (134).

2) ROLE OF NEUTROPHILS

As mentioned before in psoriatic lesions, neutrophils are the first crucial responders of the innate immune system chemoattracted by CXCL1, CXCL2, IL-8, NAP-2, NAP-3, and LPS among others, infiltrating into the dermis and epidermis forming the Munro's microabscesses. In mice it has been showed that neutrophils depletion using anti-Ly-6G monoclonal antibody can ameliorate the psoriasis-like skin inflammation produced by Aldara (Ald) treatment (135) (mouse model that will be explained in detail in next session). Additionally, it has been reported that leukotriene B4 (LTB4), a chemoattractant for neutrophils is up-regulated in human psoriatic lesions (136). Furthermore, several clinical trials suggest that the selective depletion of myeloid linage leukocytes through adsorptive granulocyte and monocyte apheresis improve the symptomatology in psoriatic patients (137), (138). After cell activation neutrophils generate extracellular traps (NETs), which are increased in psoriatic patients. These granules are composed by cell-free DNA, histones, AMPs, danger molecules, and autoantigens. The granules can release their contents into the inflamed area an promote inflammation and DCs activation (139).

3) ROLE OF DCs IN PSORIASIS

Under homeostatic conditions, host nucleic acids are rapidly degraded in the

extracellular environment (140). However, LL-37 as explain above, promote their transport into endosomal compartments of pDCs. Recently, Nestle et al., have shown that pDCs in 'normal pre-psoriatic skin patients' are increased in comparison to healthy control skin, suggesting the implication of these cells in psoriasis (99).

In humans and mouse upon stimulation, monocytes, DCs but also KCs expressed IL-23 (141). IL-23 is a heterodimeric cytokine compounds by IL23p40 chain shared with IL-12 and IL23p19 subunits. The role of IL-23 is the inhibition of IL-10 production and drives the development of IL-17 and IL-22-producing Th17 cells, γδ T, $CD4^+$ T cells and ILC3 leading to neutrophil skin infiltration and the release of the pro-inflammatory cytokines such as IL-1 family (142). In psoriasis mDCs are characterized by the release of IL-23 and the expression of RORγt (RORC) transcription factor in the skin (143). Recent studies showed that intradermal injection of IL-23 into the mouse skin resulted in psoriasis-like inflammation, including immune infiltration and epidermal hyperplasia, which is mediated via Th17 and γδ T cells (144). Furthermore, as mentioned before, GWAS studies revealed the association of psoriasis susceptibility with IL-23 (109). Additionally, It has been reported that IL-23, stimulated by TNF-α in mice and probably in humans, promotes growth and acanthosis in KCs via the JAK2/AKT/STAT3/LMO4 signaling pathway (145).

4) ROLE OF αβ T CELLS IN PSORIASIS

The abnormal regulation of IL-23/IL-17 axis produces changes in the $CD4^+$ and $CD8^+$ T cells infiltration and skin T_{RM} cells. Therefore, Cai Y et al., demonstrated that human dermal $CD3^+$ T cells were induced in psoriatic skin from 1% in healthy skin to 15%. Additionally, they demonstrate that human dermal αβ and γδ T cells have the ability to secrete IL-17 upon IL-23 stimulation, inducing inflammatory cytokines (146).

IL-17 family is comprised by 6 members: from IL-17A to IL-17F and IL-17AF heterodimer produced by different cell types (147). Among them, IL-17A produced from hematopoietic cells, including $CD8^+$ Tc17, γδ T cell, NKs, ILC3 and Th17 cell, has been identified as a key player in infections, inflammation, autoimmune, and cancer (148). In the contrary, IL-17B, IL-17C, and IL-17E, also called IL-25, are preferentially produced by non-hematopoietic cells, including KCs (147). Both

hematopoietic cells and KCs produce IL-17F (149) and KCs-derived IL-17C stimulate Th17 cells to secrete IL-17A. IL-17E, is related to Th2 immune response and allergies. IL-17 receptor (IL-17R) is composed by five members: from IL-17RA to IL-17RE. KCs express IL-17RA/IL-17RC and IL-17RA/IL-17RD (150), (147). Regarding mice studies, it has been shown that the intradermal (i.d.) injection of IL-17C promotes epidermal thickening and neutrophil infiltration (151), data supported by the efficiency of the anti-IL17C antibody in IL-23-induced psoriatic-like skin inflammation model (152). Additionally, mice overexpressing IL-17A in KCs (K14-IL-17A$^{ind/+}$ mice) exhibit severe psoriasiform skin inflammation (153). On the other hand, *Il-17rc*$^{-/-}$ showed reduced and delayed signs of inflammation upon Ald treatment (154). However, in these mice the inflammation was not completely abolished. Similarly, Ald-induced inflammation was strongly reduced in mice with KC-specific deletion in IL-17 receptor *(Il-17r*$^{KC-/-}$*)* (97). All of these studies demonstrate the importance of IL-17 signaling for psoriasiform inflammation, but also suggests an alternative pathway for Ald-induced inflammation.

Additionally, it has been shown that the expression of C/EBPβ protein (a transcription factor highly expressed in KCs located in the upper spinous layer in humans and mouse epidermis) is increased in psoriatic patients and is regulated by IL-17A and TNF-α (169). Moreover, the upregulation of EGFR and cell cycle-related genes, suggests a direct contribution of IL-17 to KC proliferation (97).

5) ROLE OF γδ T CELS IN PSORIASIS

It has been shown that in psoriatic patients the proportion of dermal γδ T cells are increased to 40% in comparison to 15% presented in healthy controls (99), suggesting that γδ T cells may play a role in psoriasis development. In mice, using the Ald model and IL-23 i.d. injection model, it has been shown that γδ T cells KO mice (*TCRδ*$^{-/-}$) presented less inflammation than αβ T cells KO mice (*TCRα*$^{-/-}$). Moreover, *TCRδ*$^{-/-}$ mice reconstituted with Vγ4 and Vγ6 cells restored disease susceptibility (155). Interestingly, it has been demonstrated that Vγ4^{+} cells have memory-like capacity because the cells that persist in the skin after Ald-treatment exhibit features of memory cells upon challenge. Furthermore, Ramirez-Valle et al., demonstrated that the migration and recruitment of dermal IL-17^{+}γδ T cells to the

skin is mediated by CCR2 signaling (156). According with these results, IL-23 injected WT mice presented lower γδ T infiltration into the epidermis upon anti-CCL20 treatment. Additionally, it has been demonstrated that IL-1β can stimulate KCs production of CCL2 and CCL20, which might impact γδ T cell recruitment (nevertheless the source of the chemokine secretion has not been identified yet). Furthermore, it has been showed that activated dermal γδ T cells increase expression of X-linked IL-1 receptor accessory protein-like 1 (IL-1RAPL1) promoting IL-17 production by these cells (75).

In human psoriatic patients γδ T cells are also expanded and produce IL-17A. Laggner et al., showed that Vγ9Vδ2+ cells contribute to psoriasis development expressing IL-17A, IFN-γ, TNF-α and CCR6. In addition, they demonstrated that Vγ9Vδ2+ cells in psoriatic patient blood correlated with the PASI (157). However, it has been recently reported that the majority of IL-17A producing T cells observed in human psoriatic lesions are oligoclonal αβ T cells and not γδ T cells (158). Furthermore, mast cells have been shown to produce IL-17A and IL-22 in human PP (75).

6) ROLE OF ILCs IN PSORIASIS

The susceptibility of Rag-deficient mice to develop psoriasiform inflammation via Ald-treatment indicates that other cells than T cells play a role in psoriasis (159). Following this discovery, Pantelyshin et al., using Rag1/IL-2R deficient mice lacking T cells and ILC, showed that RORγt^{+} γδ T cells and RORγt^{+} ILC contribute to Ald-induced psoriasiform inflammation (160).

Between the three subgroups of ILCs, ILC3s seem to play an important role in the development and maintenance of psoriasis, however, the role of ILC1 and 2 subsets is under debate (75). In general, the frequency of ILCs in total PBMCs in healthy and psoriatic patients is similar. In contrary ILC3s are increased in psoriatic skin and mouse studies using skin xenotransplant models showed that ILC3s alone were able to induce psoriasis similarly to αβ T cells (161). NKp44 has been associated with pro-inflammatory functions in ILC3s, because its activation leads to TNFα production (162), (75). Furthermore, anti-TNFα produce a reduction in the number

of pathogenic NKp44^{+} ILC3s in the circulation, suggesting that a major role of TNF-α in the pathogenesis of psoriasis includes the promotion of pathogenic ILC3s (75).

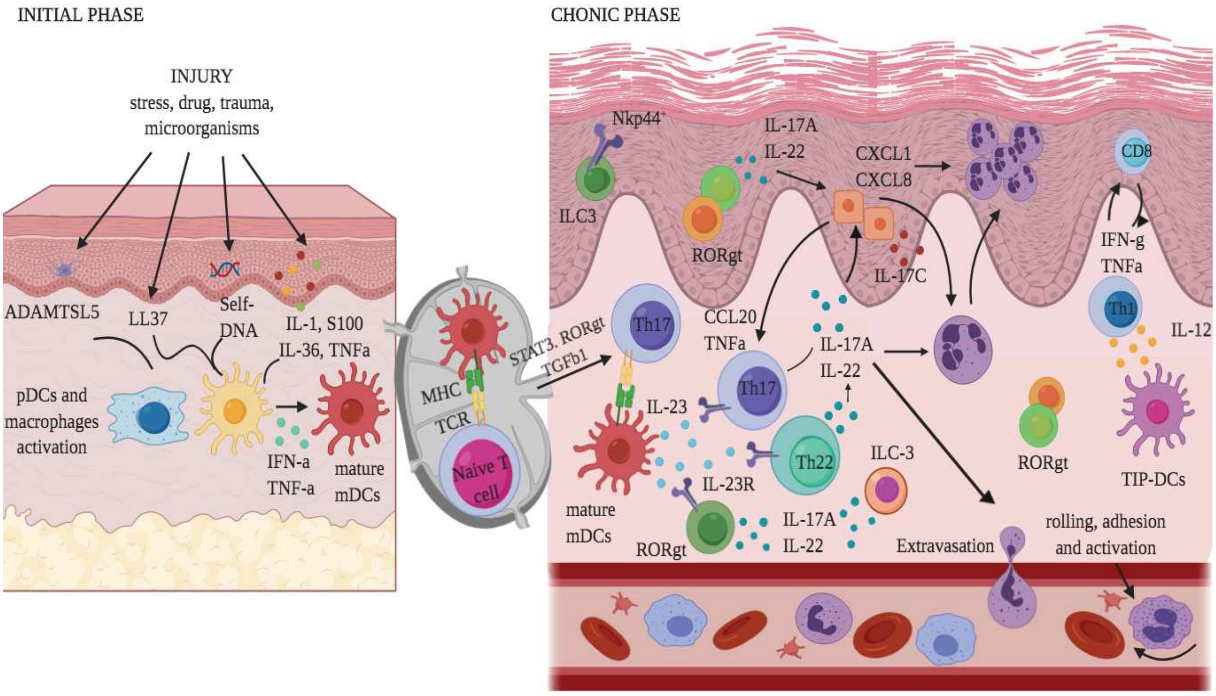


Figure 9: Pathogenic model of psoriasis. The initiation of psoriasis disease is triggered by environmental agents and/or loss of tolerance, leading to pDCs and MΦ activation with the consequent IL-23 production. Active mDCs triggers Th17/Th22 cell polarization in LNs. mDCs producing IL-23 and activated T cells migrate to the skin and produce IL-17, IL-22, and TNF-α. These cytokines act on KCs maintaining the inflammatory loop and epidermal hyperplasia. KCs-derived CCR20 recruits CCR6^{+} cells (IL-23–producing DCs and Th17 cells) to further promote inflammation. CXCL1/2/3/5/8 produced by KCs recruit neutrophils and MΦ into inflamed skin. Besides, IL-23 promotes Th22 cells producing IL-22, which participates in the terminal differentiation and proliferation of KCs. IL-12 produced by activated mDCs promotes the influx of Th1cells. In Both human and psoriatic mouse models, KCs produce CCL2 and CCL20, promoting the recruitment of dermal γδ T cells to the epidermis. These γδ T cells, express RORγt and produce IL-17 and IL-22 driving KCs hyperplasia, neutrophil recruitment and disease progression. Finally, NKp44^{+}ILC3s population contribute to disease progression producing IL-22 and IL-17. Adapted from (163), (75) done by Biorender.

1.2.7 TREATMENT OF PSORIASIS

Nowadays psoriasis is an incurable chronic relapsing disease in which patients often needs long-term treatment to control the symptoms. Numerous topical and systemic therapies

are available for the treatment of the cutaneous manifestations and the therapeutic strategy in each case depends on the disease severity, comorbidities, and individual patient response. In general, patients are classified into two groups: mild or moderate to severe psoriasis, according to the PASI and patient quality of life. Approximately 80% of the patients are included in the mild psoriasis subgroup presenting between 5 to 10% of the BSA affected (164).

Despite the introduction of new biological effective therapies targeting TNF-α, IL-23, and IL-17, the common therapeutic strategy is based on the use of standard conventional topical or systemic treatments because of their convenience, safety, and economic efficiency (92). While mild disease is commonly treated only with topical agents, patients with moderate-to-severe disease with a BSA higher than 5% received topical treatments as adjuvant therapy to reduce the amount of phototherapy or systemic agents (165).

1.- TOPICAL TREATMENTS

The most frequently used topical drugs in patients with mild to moderate psoriasis include corticosteroids and Calcipotriol (Vit D_3 analogue). Tazarotene, tacrolimus, pimecrolimus, emollients and UVB phototherapy can be use as alternative.

1.1 GLUCOCORTICOIDS (GC)

GC are the first line in psoriasis therapy due to their potent anti-inflammatory and antiproliferative activity with wide immunosuppressive effects targeting T cells, APC and inducing apoptosis (166). They are very effective during the first month, but long-term treatments entail side effects, including cutaneous atrophy, groove, persistent erythema, telangiectasia and suppression of the hypothalamic-pituitary-adrenal (HPA), that reduce the efficacy and adherence (165). GC act by two different mechanisms at the cellular level, divided into genomic and nongenomic pathways.

-In the genomic pathway GC binds with the glucocorticoid receptor (GR) activated by cortisol with the subsequent homodimerization and binding with glucocorticoid-responsive elements (GREs) promoting the transcription of anti-inflammatory mediators (167).

-In the nongenomic pathway GC interact with membrane-bound receptors, promoting rapid responses orchestrated in few minutes because the novo protein synthesis is not required acting by modulating the level of activation and

responsiveness of target cells, such as monocytes, T cells, and platelets (167).

1.2 VITAMIN D_3 ANALOGS

The combination of $VitD_3$ analogs, such as Calcipotriol (Cal), and Betamethasone dipropionate is the first-line of topical treatment for mild to moderate PP presenting a higher efficacy than either monotherapy alone (164), (168), (169). The therapeutic use of $vitD_3$ dates back to the 1930s when it was used as an oral agent for osteoporosis on a psoriasis patient who subsequently experienced clearing of psoriatic skin lesions. In light of this discovery, Finamor et al., showed that psoriasis patients treated with 35,000 IU of $VitD_3$ once daily for 6 months, had significant improvement in PASI score with a marked increase in their serum 25(OH)D level. Until 2009, Cal as a cream, solution, ointment or foam was the only topical $VitD_3$ analog available. Two randomized, double-blind clinical trials demonstrated that Cal treatment was efficient in approximately 34% of the patients. Moreover, two long-term studies reported that Cal treatment improved patient rate psoriasis severity (164). Furthermore several clinical trials have demonstrated the efficacy, safety profile and tolerance of other $vitD_3$ analogs like tacalcitol, and maxacalcitol which have little systemic absorption and do not alter systemic calcium homeostasis (170).

In terms of mechanism there are considerable differences between GC and $VitD_3$ analogs. Until now, it has been shown that $VitD_3$ analogs, induce a tolerogenic state in DCs, T cells (166), (171) and enhance the immunosuppressive capacity of Tregs (172). Additionally, $vitD_3$ analogs drive Th2 differentiation inhibiting Th1/Th17 cells (173), (174). In psoriatic patients it has been reported that Cal normalizes the pro-inflammatory cytokines in lesional skin, including IL-17A, IL-17F and IL-8 (169). Moreover Cal-treated KCs presented the inhibition of IL-6, human beta defensin (HBD)-2, -3, S100A7, and S100A15 expression (175), (169). Nevertheless, it remains unclear whether Cal modulates the responsiveness of KCs. Emerging evidences indicate that retinoic acids (RA) and Vit D_3, suppress the gene expression of IL-1 family in “in vitro” studies. Kong et al., reported that Vit D_3 reduce IL-1α activity and suppress the biosynthesis of IL-18 (176). In addition, Ikeda et al., found that Vit D_3 as well as RA suppress IL-1R1 expression in human T cells.

However, the effect of $VitD_3$ is not only related with the modulation of inflammation because in psoriasis $VitD_3$ acts in the regulation of the abnormal epidermal function. It has been showed that $VitD_3$ treatment normalized the expression pattern of integrins, other activation markers like ICAM-1, CD26, HLA-DR and promote the production of LCE and tight junction proteins which were altered on psoriatic skin.

Despite all the evidences demonstrating that $VitD_3$ plays a critical role in psoriasis, the deficiency of $VitD_3$ in psoriatic patient's data are contradictory. Several studies have shown significant lower levels of serum 25(OH)D in psoriatic patients compared to controls. Nevertheless, Wilson et al., showed that $VitD_3$ deficiency is not common in psoriatic patients. However, Richetta et al., showed that among 5 common VDR polymorphisms (A-1012G, FokI, BsmI, ApaI, and TaqI). A-1021G polymorphism was associated with psoriasis in an Italian population and ApaI polymorphism with Chinese psoriatic patients. In contrast, studies in Croatian and Egyptian populations did not find any role of VDR polymorphisms in psoriasis. The presence of these polymorphisms are considered a risk factor for psoriasis development and are associated with the response to different treatments (177).

1.3 PHOTOTHERAPY

Photochemotherapy (PUVA) and narrowband or broadband UVB radiation have been used in psoriasis treatment because they produce the elevation of 25(OH)D level in serum, act via antiproliferative effects (slowing keratinization) and present anti-inflammatory effects (inducing apoptosis of pathogenic T cells). Clinical trials demonstrate that the combination of Cal and UVB treatments reduce more efficiently the PASI score. Nevertheless, this treatment accelerates photodamage and increase the risk of cutaneous malignancies.

1.4 OTHER TOPICAL TREATMENTS

-Tazarotene in combination with topical GC minimize skin irritation.

-Calcineurin inhibitors like topical tacrolimus 0.1% and pimecrolimus 1% are used as a second line of treatment. Topical application is generally well tolerated when used to treat facial and intertriginous psoriasis.

-Anthralin (also known as dithranol) is an effective treatment. However, the mechanism of action is not well understood but may involve anti-inflammatory effects and normalization of KCs differentiation. Skin irritation is an expected side effect that can limit the use of this therapy.

2.- SYSTEMIC TREATMENTS

Moderate to severe psoriasis often requires systemic treatments in combination with phototherapy. Methotrexate (MTX), cyclosporin A and retinoids are the traditional systemic treatment options summarized in **Table 6**.

Table 6: Systemic drugs available for psoriasis treatment

Drug	Mechanism	Application	Side effects
MTX, folic acid analog	Dihydrofolate reductase inhibition blocks thymidine and purine biosynthesis. Induction of lymphocyte apoptosis	subcutaneous or oral administration from 7.5–25mg/weekly	nausea, leucopenia, and increase of liver transaminases
Cyclosporine	Calcineurin inhibition leading to reduced IL-2 (T cell-inhibition)	Short-term oral therapy from 2.5-5.0 mg/kg of body weight	Hypertension, renal toxicity, and non-melanoma skin cancer.
Retinoids Acitretin	It affects transcriptional processes acting through nuclear receptors and normalized KCs proliferation and differentiation	Oral treatment from 0.3–1 mg/kg of body weigh/day.	Cheilitis, effluvium, conjunctivitis,hepatitis, and teratogenicity.
Fumaric acid esters (FAEs)	Not totally clear, glutathione inhibits the NF-kB. DMF: decrease the migratory capacity of slan+ monocytes and inhibits the induction of Th1/Th17 responses.	Oral, approved in 2017 for psoriasis treatment in the EU, Iceland, and Norway	gastrointestinal symptoms
Apremilast	Phosphodiesterase-4 (PDE4) inhibits the hydrolyzation of second messenger cAMP. Reducing the expression of pro-inflammatory cytokines (TNF-α, IFN0γ, and IL-12 increasing IL-10 levels)	Oral	Nausea, diarrhea respiratory infections and nasopharyngitis

3.- BIOLOGICAL TREATMENTS

During the last years, monoclonal antibodies and receptor fusion proteins were under development for the treatment of psoriasis. The advantage of these drugs is that they target specifically the two inflammatory pathways implicated in the development of PP, the IL-23/Th17 axis and TNF-α-signaling. Therefore, they are administered subcutaneously (or intravenously, infliximab) on different weekly schedules (summarized in **Figure 10**)

3.1 TNF-α inhibitors

They are the first-generation of biologics agents and are effective for PP and PsA. Currently, there are four drugs in this category: etanercept, infliximab, adalimumab, and certolizumab.

Etanercept was the first TNF-α inhibitor approved in the treatment of psoriasis by the United States Food and Drug Administration (FDA). It is a recombinant human fusion protein of the receptor portion for the TNF-α ligand fused to the Fc portion of an IgG1 antibody. Infliximab is a chimeric monoclonal IgG1 antibody, and adalimumab is a fully human monoclonal IgG1 antibody. Certolizumab pegol is a pegylated Fab' fragment of a humanized monoclonal antibody against TNF-α. They neutralize TNF-α activity by binding to its soluble and membrane-bound form. In the treatment of psoriasis, they show different response rates: 52% for etanercept, 59% for adalimumab, 80% for infliximab and 83% for Certolizumab.

3.2. IL23/Th17 axis

As previously mentioned, IL-23 drives the expansion of Th17 cells producing inflammatory cytokines as IL-17A, IL-17F, and IL-22. The drugs developed against this axis are divided in:

-IL-23 inhibitors

Ustekinumab was the first monoclonal antibody against the subunit p40 from the dimer IL-23 approved by the FDA for psoriasis, PsA and CD. By targeting p40, ustekinumab blocks Th1 and Th17 response. In clinical trials, this drug demonstrated to be safe, with few side effects, and efficient. Frequent side effects include nasopharyngitis, upper respiratory tract infections, fatigue, and headache. Nowadays there are three fully human monoclonal antibodies targeting specifically p19 available: guselkumab, tildrakizumab, and risankizumab.

-IL-17 inhibitors

Three effective human monoclonal antibodies targeting IL-17 are available. Secukinumab and ixekizumab block IL-17A, whereas brodalumab is directed against the IL-17 RA, inhibiting the biological activity of IL-17A, IL-17F, IL-17A/F, and IL-17E. Secukinumab was the first IL-17A inhibitor approved for PP treatment. Secukinumab and ixekizumab are effective for scalp and nail psoriasis. The frequent adverse effects include nasopharyngitis, headache, upper respiratory tract infection, arthralgia and candidiasis.

-IL22 inhibitors

Fezakinumab, an IL-22 antibody candidate in the treatment of psoriasis was tested in clinical trials. Nevertheless, the studies were discontinued for the absence of clinical improvements in the symptoms of psoriatic patients (178).

3.3. Other candidates under development

Other treatments that has been tested for psoriasis are Tofacitinib and Upadacitinib (JAK inhibitors); piclidenoson, an adenosine A3 receptor inhibitor; serlopitant, a neurokinin-1 receptor antagonist and RORγt inhibitors. In addition, there are currently 13 registered phase III clinical trials testing biosimilars for adalimumab (eight), infliximab (three), and etanercept (two) (179).

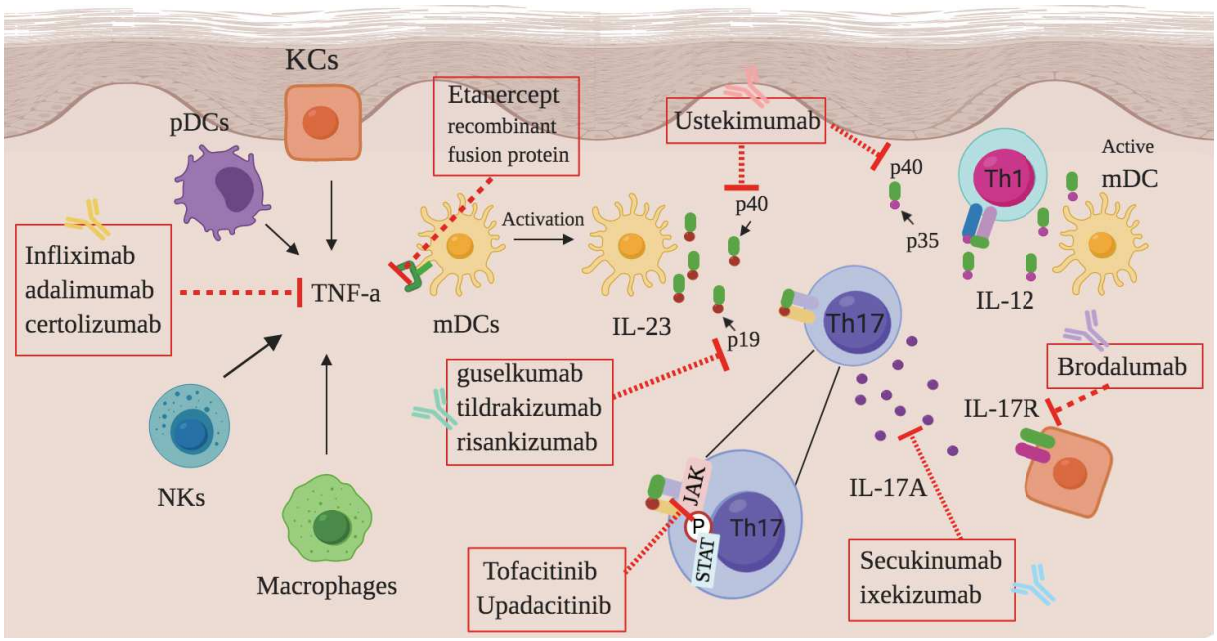


Figure 10: Mechanism of action of the different Biological agents. Infliximab, adalimumab, certolizumab and etanercept belong to the group of TNF-α inhibitors. Ustekinumab, guselkumab, tildrakizumab and risankizumab target IL-23 cytokine. Secuckinumab, ixakizumab and Bridalumab are monoclonal antibodies classified in the IL-17 inhibitors group. Tofacitinib and Upadacitinib are JAK inhibitors. Figure done by Biorender.

1.3- MOUSE MODELS

Despite that psoriasis exclusively affects humans among all animals and the differences between human and mouse skin; animal models of "psoriasiform" inflammation have been developed as a tool to identify the immunological mechanisms underlying psoriasis pathogenesis. Currently, more than 40 mouse models reproducing some features of human psoriasis such as epidermal hyperplasia, vascular proliferation and immune response have been described. Nevertheless, any current system has been yet systematically validated or represents all the molecular features and cellular changes of the human disease.

However, the number of publications using animal models, especially mice, is still increasing and since 1965 more than 20,000 papers were published (180) (**Figure 11**). Because the basic and translational research is often not possible in human psoriatic patients, animal models are an adequate system to discover the pathogenic mechanisms of the disease.

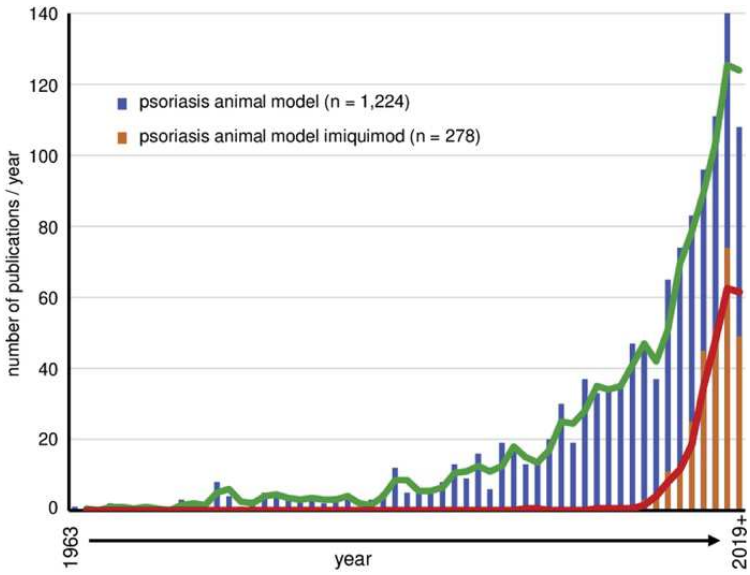


Figure 11: Schematic graph representing the publications on animal models of psoriasis. A recent literature search performed by Schön et al., 2020 highlights the scientific interest in psoriasis research using animal models. Ald mouse model was used in the majority of the publications. The green and red curves show the respective trends (average of 3 years). Graph from (180).

In 2007, Gudjonsson et al. classified several mouse models as acute (inducible), genetically engineered (transgenic) and xenografts (humanized) and spontaneous mouse models (summarized in **Figure 13**) (181).

1. ACUTE MODELS

The mouse model most widely used to understand the pathways involved in psoriasis pathogenesis is the acute Aldara (Ald) model. Upon Ald-treatment mice develop a not chronic inflammatory disease like-psoriasis, but they do not present the comorbidities related with psoriasis, which appear in some of the transgenic models, such as CD18 hypomorphic and in the inducible JunB/c-Jun double KO mice. Ald contains as active ingredient drug Imiquimod (IMQ), also called 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine, is an imadazoquinoline originally developed as a low molecular weight nucleoside analog of TLR7 in mice or TLR7/TLR8 in human capable to promote immune response inducing IFNs expression. Due to its small size and high hydrophobicity, IMQ is formulated for topical administration with isostearic acid as a vehicle. IMQ 5% cream was approved by the FDA in 1997 to treat genital and perianal warts, commonly caused by HSV or human papilloma virus (182).

The inducible inflammatory response developed in murine skin after several doses of Ald mimics some aspects of human psoriasis including phenotypic and histological changes, depends on IL-23/17 axis and the immune cellular recruitment (182). Although the model is simple, with a fast induction of inflammation and low cost there are some practical aspects that should be considered to interpret the results obtained:

1) Inflammation appears in different mouse strains. Nevertheless, there are some differences in local and systemic reactions to Ald treatment between strains. For instance skin inflammation in Balb/c mice is developed faster than in C57BL/6 (182). Additionally, C57BL/6 mice presents severe systemic side effect including dehydration and fever secondary to IL-6 release in serum (163). Moreover, most of the studies have been done in one of the two background strains, even though there are differences in cytokine production and immune cell biased between them (183).

2) Ald can be applied either in the tail, ear or in the shaved back skin. The phenotype of epidermal thickening, parakeratosis, acanthosis and cellular infiltration is better developed in the back skin. However, ears are closer to human skin and the inflammatory response can be followed up measuring the ear-thickness (184), (183).

3) The majority of the studies analyzed Ald inflammatory response after day 3 but first changes including erythema, parakeratosis and inflammatory events such as the up-regulation of type I IFNs, S100A8/A9 and IL-23 genes are observed 24h after the first application. Walter et al, 2013 suggested that these changes rely on the isostearic acid (the vehicle of the cream) and are independent of IMQ (185), (182). Additionally, Ald model in mice dependent on γδ T cells, a minor T-cell population found in healthy human and psoriatic human skin (Matos et al., 2015).

4) Most of the mouse experimental data obtained with Ald model have not been validated in human tissues. Only some genes modified in mice have been identified in GWAS of human psoriasis such as IL-23A, IL36-RN, TRAF3IP2, STAT3. Subsequent studies of the model using microarray expression analyses revealed that the transcriptome of psoriasis-related cytokine response pathways for human PP and the psoriasis-like skin lesions of 5 preclinical mouse models of psoriasis (keratin 14-amphiregulin, keratin 5-Stat3C, keratin 5-Tie2, keratin 5– transforming growth factor-b1, and IMQ) showed strong and statistically similarities in the gene expression profiling particularly in gene expression patterns in epidermis. However, important differences were founded in immune-associated gene expression being the Ald model the less similar to human psoriasis (186).

5) murine TLR7 receptor in MΦ and DCs leading to produce multiple proinflammatory cytokines (187). Nevertheless, murine KCs lack expression of TLR7/8 (188). Independently of TLR7 and MyD88 signaling, IMQ can activate the inflammasome via the NALP3 pathway, promoting the activation of caspase 1 and production of IL-1β (189); Besides it can be a ligand for adenosine receptors with antagonist effect and the vehicle itself has biological activity. The final skin inflammation developed not recapitulates the authentic psoriasis lesions (**Figure 12**)

6) The last limitation of Ald model is that long treatments are not tolerated. For that reason, the study of significant psoriasis comorbidities is not possible. Additionally, it is important to mention that the model is acute and does not recapitulate the chronic nature of the human psoriasis.

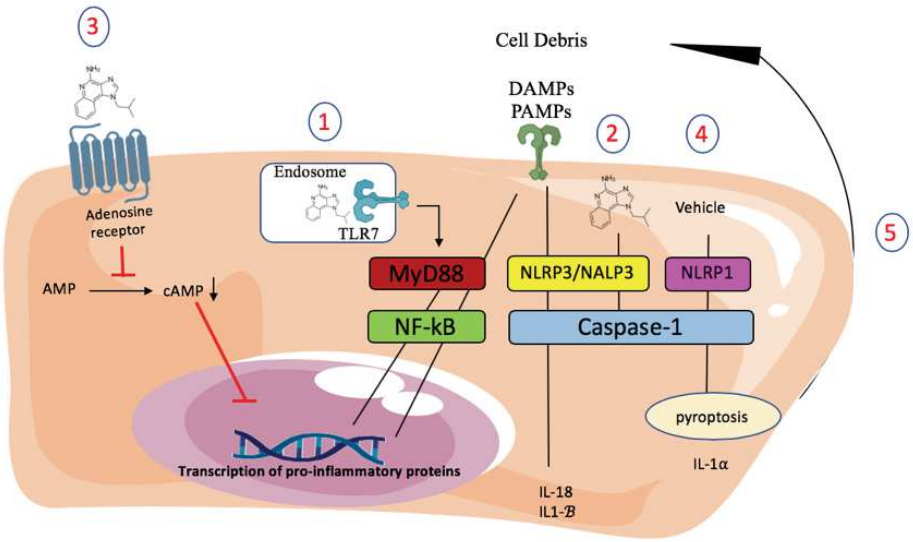


Figure 12: Aldara mechanisms of action. Imiquimod can activate immune response via different pathways including (1) the activation of TLR7/MyD88 pathway. (2) Activation of the inflammasome via NALP3. (3) binding with de adenosine receptor like and antagonist leading to decrease the levels of anti-inflammatory cyclin AMP (cAMP). (4) by the direct activation of the inflammasome by the vehicle. (5) Cell will release IL-1a and cell debris which may in turn activate other nearby cells. Figure adapted from (178)

The most second acute murine model used for psoriasis research is the intradermal injection of IL-23 cytokine which recapitulates activation of critical pathways associated with the disease, including the increase of IL-17 and epidermal and dermal inflammation. Nevertheless, this model is not standardized in the research community and presents similar limitations than Ald model such as the variability in study length, frequency and amount of IL-23 injected and mouse strain used. The third model is the two topical applications per week of 12-O-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, that induce inflammation and epidermal hyperplasia recapitulating the hallmarks of psoriasis (190).

2. GENETICALLY ENGENEERING MODELS

The genetically engineered or transgenic mice models used in psoriasis are classified in whole-body KI or KO and tissue specific and conditional transgenic mice, principle models are summarized in **Figure 13**. The tissue-specific model systems are a very interesting tool that allow further phenotype modifications in a specific cell population or tissue customizing gene expression and controlling the disease manifestations. The K5-CreERT2 JunBfl/fl c-Junfl/fl mouse model is a common example of a tissue-specific inducible KO mouse in which the gene of interest can be repressed by

doxycycline administration. In contrast, the K5-IL-17C and KC-Tie2 mouse models represent tissue-specific overexpression. Nevertheless, these models also have remarkable limitations such as the early prenatal death of the mice for the embryonic modifications, the use of gene modulators like tamoxifen results in potential confounding factor (181), (190).

3. XENOGRAFT (HUMANIZED) MODELS

The xenograft of nonlesional or lesional psoriatic skin on the back of immunocompromised mice allows the development of a closely immunologic and genetic basis of the human disease. Nevertheless, these models present several limitations, including the difficulties of the technical requisites, the variations coming from the quality of the tissue graft, the intrinsic immunologic and genetic differences between the human donors and the influence of murine host factors like the absent of psoriatic-associated human cytokines.

Thus, the complexity of human psoriasis combined with the complexity of mouse biology raise that nowadays there is not a perfect murine model for the study of psoriasis. Nevertheless, the use of the available systems allows us the understanding of this inflammatory disease to elucidate the molecular basis of the inflammatory condition. Additionally, there are "in vitro" (normal human primary KCs) or new ex vivo human systems under development (2D or 3D engineered human psoriatic skin) to study the development of new drugs.

Figure13: Summary of various preclinical mouse models used to study psoriasis disease. Overview of the principal mouse models used in psoriasis research can be divided into directly induced, spontaneous, genetically engineered (both transgenic and knockout) and xenotransplantation. Figure modified from (190), (181), (191).

INDUCED (ACUTE)

- IMQ-induced dermatitis
- TPA application
- Cytokine injection (e.g IL23)

✓
- -Easy to use
- -Not technical difficulties
- -inducible disease
- -Leads to:
- γδT cells, IL-36, IL-23/IL-17 dependent inflammation

✗
- -Non standardized
- -Non suitable for chronic use
- -Influenced by genetic background of mice

SPONTANEOUS

- Homozygous asebia ($Scd1^{ab}/Scd1^{ab}$)
- Flaky skin mice (Ttc^{fsn}/Ttc^{fsn})
- Chronic proliferative dermatitis (*cpdm/cpdm*)

✓
- -Non technical difficulties in generation

✗
- -mice alterations may not reflect human disease

GENETICALLY ENGINEERED

- KO
  - PL/J/CD18 hypomorfic — (mutation in the leukocyte b2 integrin)
  - *Il1rn* $^{-/-}$ in BALB/c — influx of dendritic cells and T lymphocytes to the skin cells
- Tissue-specific overexpression
  - K14-VEGF — PS like-phenotype
  - K14-AREG
  - K14-KGF — keratinocyte growth factor
  - K5-Stat3C — Acanthosis with loss of the granular layer
  - Tg(IL1a)1.1Tsk
- Tissue-specific KO
  - K14-Cre-ikk2$^{fl/fl}$ — Impaired of skin homeostasis
- inudcible KO
  - K5-CreERT2 JunB$^{fl/fl}$ c-Jun$^{fl/fl}$ — The control of cell differentiation and transformation is lost
- Repressible overexpression
  - K5-IL-17C — accelerated angiogenesis
  - KC-Tie2 — acanthosis and proliferation

✓
- -Gene alteration in the full-body
- -Allow the gene-specific effect in vivo

✗
- -single gene alterations do not recapitulate complex diseases
- -limitations in the ability to isolate cellular or tissue-specific gene effects
- -gene alteration in mice may not reflect human disease
- -Often results in prenatal death

XENOTRANSPLANTATIONS

- human skin on AGR129 mice
- human skin on SCID mice
- human skin Athymic nude mice

✓
- -Best model to mimics the celular, phenotypic, and genetic characteristics of human disease in mice

✗
- -Technically difficult
- -Graft phenotype dependent of immune populations already present in skin
- -variability
- -ihuman tissues influenced by host factors

1.4- IL-1 FAMILY CYTOKINES

The Interleukin-1 (IL-1) superfamily cytokines, were described in the early 1980s and originally, they were denominated leukocytic pyrogens and lymphocyte activating factors among other names. Above all, IL-1 cytokines are key signaling molecules in both the innate and adaptive immune systems, mediating the inflammatory response for different stimuli. The family members were linked to innate damaging inflammation. This assumption was done because the cytoplasmic domain of IL-1R (receptor) presents a functional TIR domain with high homology to the TIR domains of TLR (192).

Nowadays, 11 cytokines including IL-1α, IL1-β, IL-1Ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL38; and 10 receptors are considered members of this family (**Figure 14**). In view of their different components in the receptor dimers, the precursor protein size and protein activity they are further classified into four subfamilies. IL-1, IL-33, and IL-36 subfamilies share IL-1RAcP as their secondary receptor, while IL-18 subfamily binds with a different secondary receptor (**Figure 14**).

Exanimating the phylogenetic tree, IL-36 subfamily is compounded by IL-36R antagonist (IL-36Ra), and thee agonists including IL-36α, IL-36β, and IL-36γ (193), (194), (195). The sequences of IL-36α and IL-36γ are more similar each other than IL-36β sequence, suggesting that IL-36α and IL-36γ functions are more related than with IL-36β function. In general features, IL-36 cytokines bind to the IL-36 receptor (IL-36R or IL1Rrp2) and use the IL-1 receptor accessory protein (IL-1RAcP) as a co-receptor (196) (**Figure 14**). In contrast, IL-1Ra, IL-36Ra and IL-38, the three antagonists of the different subfamilies, instead of clustering with their respective agonists, have similar sequences between each other; indicating that there is a conserved sequence and structural requirement for cytokine antagonistic activity. Nevertheless, the antagonist of IL-18, named IL-37, is separated from the other antagonists, suggesting that this antagonist probably follow other binding process (197).

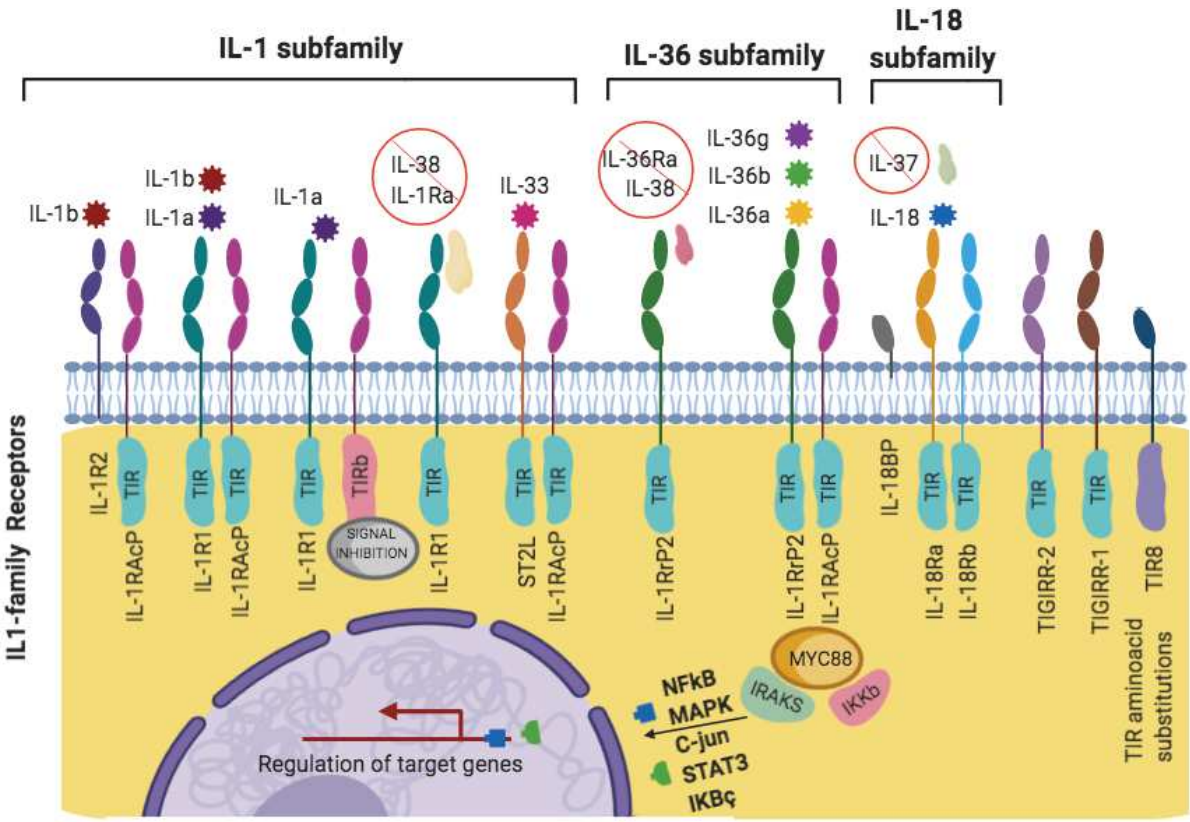


Figure 14: Schematic description of the different receptors and downstream mediators of IL-1 signaling pathways. General representation of the different IL-1 family members, which forms heterodimeric receptor complexes bindings with their specific receptor in combination with the co-receptor IL-1RAcP. For instance, IL-36 agonists bind to IL-36 receptor (IL-36R) while recruiting IL-1RAcp to form the receptor complex. MyD88 binds to the intracellular domains of the IL-36R and IRAKs are recruited to the complex. Activation of the MAPK pathway leads to IκB degradation and NF-κB activation. AP-1 and NF-κB translocate to the nucleus to activate target gene expression. In the contrary, IL-36Ra binds to IL-36R preventing the assembly of functional IL-36 receptor complexes inhibiting activation of the signaling pathway. Figure adapted from (192) done by Biorender.

1.4.1-ORGANIZATION OF THE IL-1 FAMILY MEMBERS

- The IL-1 family consensus sequence

As it was mentioned before, cytokines of IL-1 family and their receptors extensively affect a large spectrum of immunological and inflammatory responses. IL-1 gene with 450kb approximately, is located on chromosome 2q14-q12. This region encodes other cytokines that share gene structure, thus the proteins obtained presents similar secondary and tertiary structures. Specifically, human IL-36 genes are located at the IL-1 locus on chromosome 2q13 sharing 36–46% sequence identity with IL-1 cytokine genes (198), (199). For instance, IL-36Ra and IL-1Ra have 52% homologous amino

acid sequence (51). Indeed, a recent RNA-seq analysis of human primary epidermal KCs treated 8 or 24h with IL-1β or IL-36α/β/γ revealed that KCs transcripts, shared a set of 225 differential expressed genes (DEG) overlapping with the genes altered in PP and GPP skin lesions, as well as genes identified in the GWAS analyses performed in autoinflammatory diseases (200). These findings suggest that IL-1 and IL-36 cytokines have similar intracellular signaling pathways. Nevertheless, disease manifestations produced by aberrant IL-36 signaling are significantly different from those caused by aberrant IL-1 signaling, indicating that these two pathways target different biological processes (196).

In terms of secretion, except for IL-1Ra, all members of the IL-1 family, including IL-36 subfamily, lack a signal peptide and probably they do not follow the canonical endoplasmic reticulum and Golgi dependent secretion mechanism. In the inflammasome, IL-1β and IL-18 are processed by caspase-1 (201) and subsequently released through membrane pores induced by gasdermin D (202). However, despite it has been suggested that IL-36 cytokines follow an alternative pathway than IL-1β and IL-18 such as using microparticles and exosome fractions, the secretion mechanism of IL-36 family remains unknown (203).

Overall, the cytokines precursors are distributed diffusely in the cytoplasm, each precursor contains three-amino acid with a conserved consensus sequence A-X-D, in which A is an aliphatic amino acid, succeed by any amino acid in X and an aspartic acid in D. As illustrated in **Figure 15**, the N-terminal amino acid is located nine amino acids before the consensus sequence, the final sequence after cleavage provides the optimal folding of the cytokine into the barrel shape allowing the receptor binding. In the case of the IL-1β precursor, the N-glycosilation is located at Asn91, followed by a caspase-1 cleavage site (204). On the other hand, IL-36Ra, IL-36α, IL-36β and IL-36γ cleaved forms are between 100 to1000-fold more active than their full-length proteins, but they do not present a characteristic cutting sites for caspase-1 or a signal peptide (205). Additionally, there is low homology among IL-36 cytokine protein sequences in the region of the cleavage site, suggesting that different enzymes may be are the responsible for IL-36 processing (196) (**Figure 15)**. A lot of recently studies tried to elucidate the molecular processing and secretory mechanism of IL36 subfamily. It has been shown, that neutrophil-derived proteases released from active cells including cathepsin G (Cat

G), elastase, and proteinase-3, are key players in the IL36 cytokines post-translational processing (206), (207). In addition, NET-bound proteases, could also be responsible of the process and activation of IL-36 agonists (208). More in detail, the IL-36α activation at Ala4 and Lys3 depends on elastase and Cat G respectively, whereas the IL-36β cleavage at Arg5 is selectively stimulated by Cat G (206). IL-36γ is principally cleaved at Ser18 and Val5 by Cat G and proteinase-3. Additionally, in human epithelial cells, it has been reported that IL- 36γ is processed and activated by Cat S secreted by cells residing in barrier tissues instead of neutrophil-derived proteases (209). Finally, in human primary dermal fibroblast and KCs, IL-36Ra presents a cleavage site in Val2 for neutrophil-derived protease elastase (210).

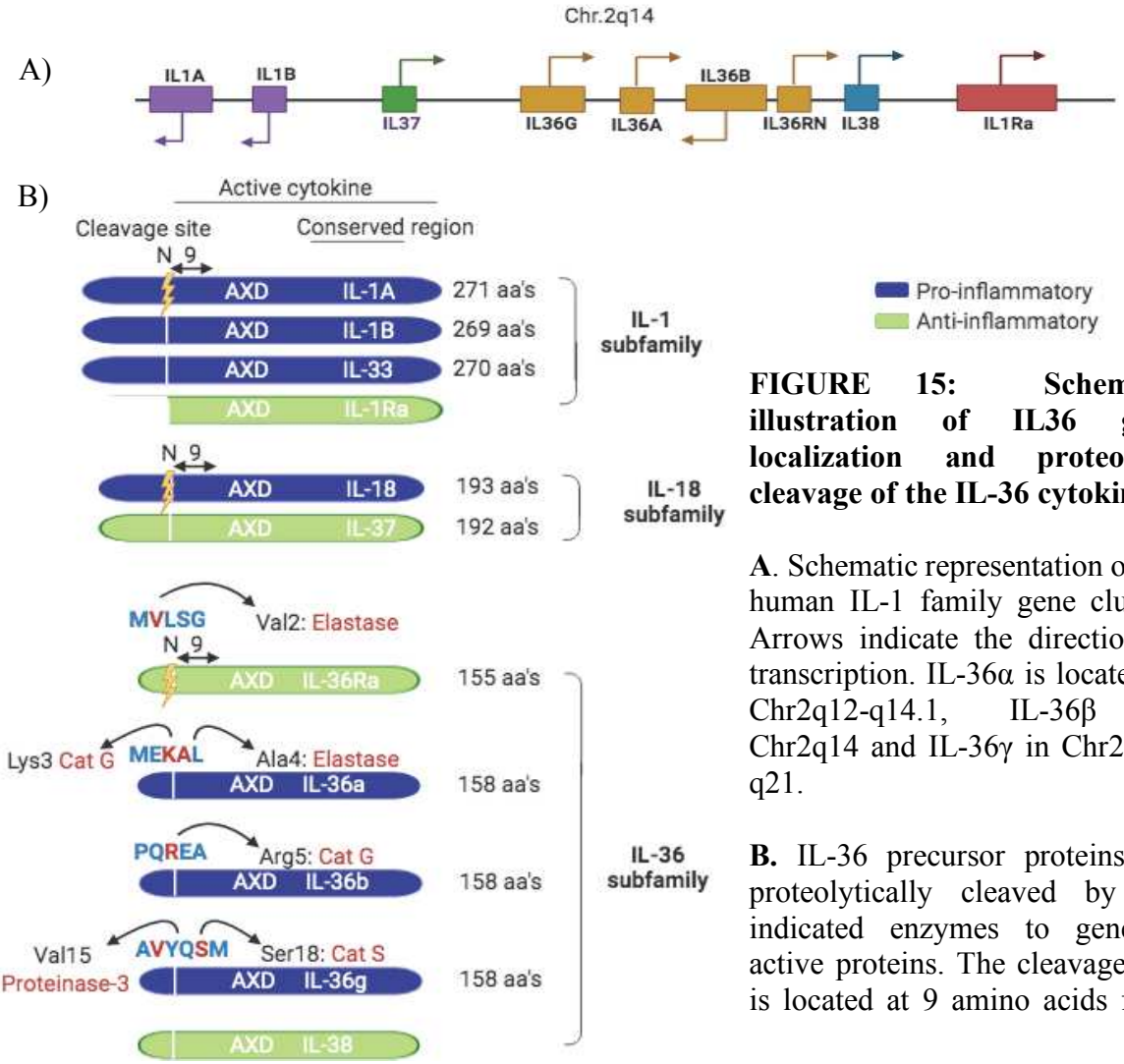


FIGURE 15: Schematic illustration of IL36 gene localization and proteolytic cleavage of the IL-36 cytokines.

A. Schematic representation of the human IL-1 family gene cluster. Arrows indicate the direction of transcription. IL-36α is located in Chr2q12-q14.1, IL-36β in Chr2q14 and IL-36γ in Chr2q12-q21.

B. IL-36 precursor proteins are proteolytically cleaved by the indicated enzymes to generate active proteins. The cleavage site is located at 9 amino acids from

Either Cathepsin G (Cat G) or elastase can process IL-36α at Lys3 and Ala4, respectively. Cat G activates IL-36β by cleaving at residue Arg5, whereas elastase and proteinase-3 cleave IL-36γ at Val15. Cat S activates IL-36γ through cleavage between Glut17 and Ser18. IL-36Ra is activated by neutrophil elastase by cleavage at Val2. IL-36 agonists (IL-36α/β/γ), IL-1 agonists (IL- 1α/β) and antagonists (IL-1Ra/IL-36Ra/IL-38) are grouped into separate bins. Except for

IL-18 and IL-33, all other cytokines in the human IL-1 superfamily are located at Chr2q14.1 region. IL-18 and IL-33 which are at Chr11q23.1–23.2 and 9p23–24 respectively, are clustered at the same locus as IL-36. Figure adapted from (196), done by Biorender.

-The IL-1 family receptors

As illustrated in **Figure 14** and mentioned before, there are 10 members of the IL-1 family receptors. Each family has one cognate receptor and one accessory protein (76). For example, IL-1R1 presented in the membrane binds initially with IL-1α or IL-1β, following by the recruitment of the common co-receptor IL-1RAcP to form the trimeric signaling complex. This conformation allows the binding with the TIR domains of each receptor chain in the intracellular space promoting the binding of MyD88 and triggering the inflammatory signal leading by NFκB activation. IL-1RAcP also exists as a soluble receptor form. However, the binding of IL-1β with IL-1R2, without the cytoplasmic domain, neutralize the heterodimer formation with IL-1RAcP. As mentioned before, all IL-1 family members except IL-18 share the L-1RAcP co-receptor. The ligand binding receptor for IL-33 is IL-33R. IL-36 receptor (IL-36R or IL-1R6) binds IL-36α, IL-36β, IL-36γ and IL-38. Notably, the IL-1 family receptors also contain three receptors (SIGIRR, TIGIRR-1 and TIGIRR-2) with less known family origin and functions (192).

1.4.2- STRUCTURE OF IL-36 SUBFAMILY

The 5 members of the family IL-36α (*Il1f6*), IL-36β (*Il1f8*), IL-36γ (*Il1f9*), IL-36 receptor antagonist (IL-36Ra, *Il1f5*), and IL-38, (*Il1f10*) have been identified in both humans and mice sharing a considerable percentage of sequence homology between themselves; 54% in IL-36α, 62% in IL-36β, 56% in IL-36γ and 91% in IL-36Ra (196).

In this subfamily only, human IL-36γ and mouse IL-36Ra (with 90% of sequence homology with human IL-36Ra) have been determine structurally by either X-ray crystallography or solution state nuclear magnetic resonance (NMR). These techniques showed that as other family members including IL-1α, IL-1β, IL-1Ra, IL-33, IL-18, IL-37 and IL-38; IL-36 cytokines possess a conserved β-trefoil conformation and a central hydrophobic core composed of 12 β-strands, six of which (β1, β4, β5, β8, β9, and β12) are connected by 11 loops forming an anti-parallel β-barrel (**Figure 16**) (14), (192).

Structurally, human IL-36γ and mouse IL-36Ra are similar (rot-mean-square derivation (RMSD) 0.746 Å); nevertheless, their total sequence correspondence is relatively low. The main differences are in β4/5 and β11/12 loop and the structure alignment without these loops results in a higher RMSD at 0.619 Å. It has been reported that by exchanging these two loops, one can tune the agonistic and antagonistic activity of IL-36 (211).

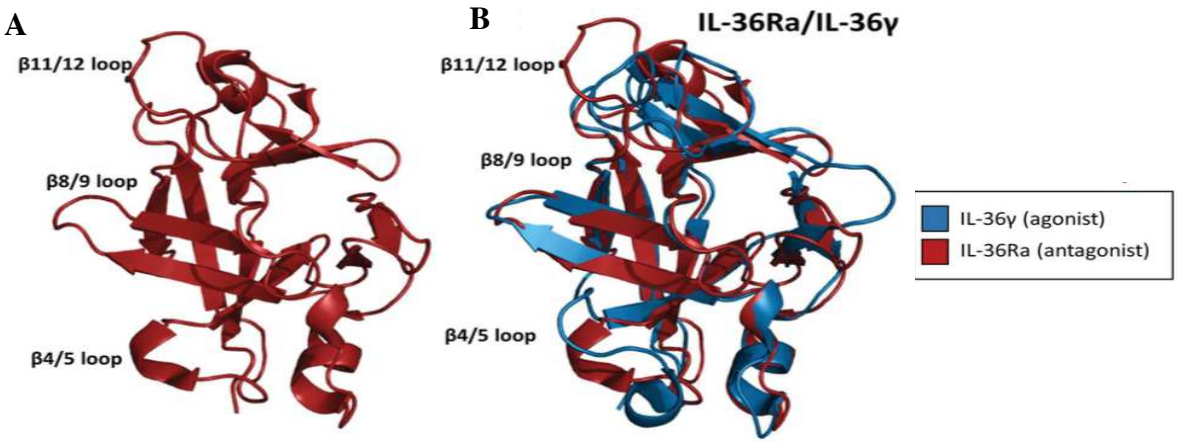


Figure 16: Representation of IL-36γ and IL-36Ra structure. A. Cristal structure of IL-36Ra. **B.** Crystal structure of IL36Ra aligned to crystal structure of Il-36γ. Figure from (75), (192).

1) The IL-36 receptor complex

IL-36R was described by Lovenberg et al., in 1996. It was denominated IL-1R-rp2 and it was considered as an orphan receptor associated to the IL-1 receptor family. Even though mouse and human IL-36R structure has not been elucidated yet, the human extracellular portion (ECDs) has been published by Wang et al., (211). The ECDs hold three immunoglobulin like-domains (D1, D2, D3) and the intracellular portion contains the TIR (**Figure17**).

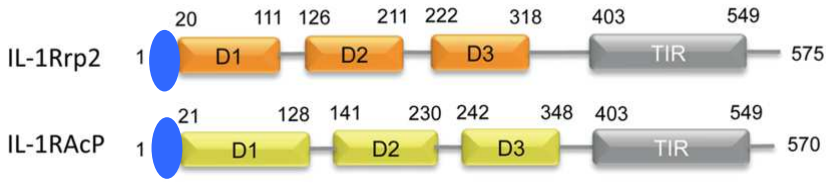


Figure 17: Scheme of the domain organization for IL-1Rrp2 and co-receptor IL-1RAcP. The N-terminal signal peptides are in blue. The three immunoglobulin like-domains are labeled as D1, D2, D3, and the TIR domains are in grey. The numbers above the schematics indicate the approximate residues demarcating the domains. Figure from (212).

Following the same two-step mechanism than IL1 described above, when the truncated fraction of IL36 cytokines binds to the extracellular portion of IL-36R with high affinity (dissociation constant (Kd)=$10^{-5}s^{-1}M$) the co-receptor IL-1RAcP is recruited and promotes the phosphorylation of TIR domains present in each subunit and triggers the activation of the intracellular signaling pathway (213). The first interaction between IL-36R and each cytokine is localized at Asp150 for IL-36α, Asn148 for IL-36β, Ala162 for IL-36γ and Asp148 for IL-36Ra. This first binding promotes hydrogen interactions and the formation of the three-dimensional conformation that favor the binding with IL-1RAcp by the Ser185. The IL-1RAcp final interaction creates the quaternary complex of IL-36R:IL-1RAcP. In contrast, the longer β11/12 loop of IL-36Ra the Asp 148 residue produce a steric obstacle preventing the union of IL-1RacP with IL-36R. The pathway concludes with the expression of inflammatory cytokines by MAPKs, JNKs, and ERK1/2 pathways mediating the activation of AP-1 (activator protein 1) and NF-kB transcription factors (described in previous section) (213).

Despite IL-36α/β/γ binds with the same receptors, depending on the cell type, functional context and interaction with other mediators, each of them may have specific effects and regulatory mechanisms. This hypothesis was supported by Zhou et al., using PR binding assay and orthogonal time-resolved (TR)–FRET assay, they demonstrated that in presence of agonist and antagonist, there is a competitive environment for the occupancy of the receptor binding site. IL-36Ra has a highest affinity for the receptor in comparation with IL36α and IL-36γ affinity. Likewise, the receptor affinity of IL36α (K_D=480±90) is higher than IL-36γ (K_D=1800±200) (213). Nevertheless, specific biological activity of each cytokine remains to be elucidated.

1.4.3- SIGNALING PATHWAYS INDUCED BY IL-36 α/β/γ CYTOKINES

As it was mentioned above IL-36 cytokines activated MAPKs, JNKs, and ERK1/2 pathways and NF-kB transcription factor mechanism. More in detail, it has been reported that IL-36γ activates the phosphorylation of MAPKs, JNK, ERK, p38 MAPK, NF-kB and CREB in bronchial epithelial cells. Whereas IL-36β selectively activates the phosphorylation of p38 MPK in BMDCs. Furthermore, IL-36α and IL36γ stimulation in HT-29 (human colon cancer cell line) and WiDr (colon adenocarcinoma line) activate the phosphorylation cascade through MyD88 adaptor protein complex, phosphorylating

MAPKs, activating NF-kB and AP-1.

1.4.4- IL-36 α/β/γ CYTOKINES REGULATION AND FUNCTIONS

Il-36 cytokines are expressed principally at barrier sited of the organism such as skin, lungs and intestine. Therefore, this subfamily of cytokines plays a principal role in body protection from the environment. The principal function of IL-36 is the driving of the differentiation and maturation of tissue resident immune cells (214), (215) and the recruitment and activation of circulating immune cells to the damaged tissues (214), (194). In homeostasis conditions the expression level of IL-36 family members in KCs, bronchial epithelium, intestinal cells, neurons and glial cells, myeloid DCs (mDCs) monocyte-derived DCs (MDDCs) and MΦ are low in human and mice. Due stimulation, their expression level increase and they promote inflammatory procedures (216), (68), (218) (IL-36 cytokines expressing cells are summarized in **Table 7**). Moreover, IL-36R is mainly express in KCs and fibroblasts in human and mice (217), (218). However, it is also expressed in a lower level in many different cell types with some specie-specific differences between human and mice.

IL-36R expression was detected in mouse bone marrow DCs (BMDCs), bone marrow-derived macrophages (BMM), bone marrow–derived neutrophils (BMN) and splenic $CD4^+$ T cells, but not in $CD8^+$ T and B cells (214). These results were supported by Hashiguchi et al, 2018, they demonstrated that in mice, IL-36R transcript was detected in KCs, mouse embryonic fibroblasts (MEFs), and BMLCs, whereas it was not expressed in γδ T or αβ T and B cells (217). In contrast in humans, Foster et al., detected IL-36R mRNA in monocytes, myeloid DCs (mDCs) and monocyte-derived DCs (MO-DCs). Nevertheless, it was not detected in neutrophils, $CD4^+$ and $CD8^+$ T cells (218), (219). However, mature DCs incubated in the presence of IL-36 and allogeneic $CD4^+$ T cells promote the proliferation of T cells. These results suggest that IL-36 may influence T cell function via APC (218). Furthermore, Dietrich et al., detected high level of IL-36R in human LCs which were activated upon IL-36β stimulation, suggesting a role for LCs in inflammatory skin phenotypes. Additionally, they reported IL-36R expression in human M0 and M2 macrophages, but not in M1 macrophages(220). Moreover, Bridgewood et al., confirmed the expression of IL-36R in human endothelial cells, which contribute to the developed of vascular vessels in psoriasis (221). Most recently,

Catapano et al., demonstrated that pDCs and other skin-resident DCs express IL-36R. In the case of pDC, IL-36 cytokines bind with the receptor, activates TLR-9 and promotes IFN-α production mediated by the up-regulation of *PLSCR1*, a phospholipid scramblase responsible of endosomal TLR-9 translocation (222). All of these findings suggests that T cell activation is driven indirectly by the promotion of other cytokines (218), (222), (223), (224).

Table 7: Cell and tissue expression of IL-36 family cytokines, adapted from (196) and (216)

Cytokine	Cell and tissue expression	Comments
IL-36α	Epithelial cells, B and T lymphocytes, monocytes	Detected During embryonic development (199), (225).
	Respiratory tract: mouse lungs	Expression regulated by the epidermal growth factor (EGF) (219), (226).
	BM, intestine, inflamed synovium, lymph nodes, spleen, tonsils	(227), (228), (229)
IL-36β	Epithelial cells	Expression regulated by the EGF (226).
	Murine neuron/glial cells	(230)
	BM, colon, heart, inflamed synovium, lung, testis, tonsil, B and T lymphocytes, monocytes, DCs, MΦ	In humans. (214), (228), (195), (215)
IL-36γ	KCs and squamous-cell epithelia of the esophagus	(231), (225)
	human THP-1 cells	Induced by LPS highlighting its role in the innate immune response (232)
	DCs	DCs (233) regulated by T-bet
	Bronchial epithelial cells, inflamed synovium, peripheral blood lymphocytes, skin.	In blood lymphocytes extracellular (211 At) α-particles
IL-36Ra	MΦ, monocytes, B cells, DCs principally in skin, brain, tonsils, spleen tissues	(199), (225), (223), (234)

Upon a harmful stimulus, the immune response is orchestrated, and pro-inflammatory mediators are released to promote stimuli clearance. Among these mediators the cytokines implicated in IL-36 induction are TNF-α, IL-6, and IL-8, and several chemokines, such as CXCL1, CXCL2, CXCL8, CCL3, CCL5, and CCL20. Indeed, Carrier et al. demonstrated the induction of the three IL-36 cytokines in human KCs by TNF- α, IL-17, IL-22 and IL-36 itself (223) (in line with the studies of Swindell et al., 2018 (200) and mouse results obtained by Hashiguchi et al., 2018 (217)). Demonstrating that, IL-36 cytokines stimulate KCs, which are a potent source of MΦ, T cell, and neutrophil chemokines enhancing the inflammatory cascade (223), (224), (198).

Additionally, as it was mentioned in **Table 6**, Barksby et al., showed that in monocytic cell line (THP-1) under activation of TLR2/TLR4 by Porphyromonas gingivalis lipopolysaccharide (LPS) and Escherichia coli LPS ligands, IL-36γ expression was induced but IL-36α and IL- 36β were not altered (232).

Furthermore, in mice, it has been shown that intradermal injections of IL-36α promote local inflammation characterized by chemokine expression, leukocyte infiltration and acanthosis (218). Additionally, in murine DCs, IL-36 cytokines induce CD80, CD86 and MHCII leading to IL-12, IL-1β, IL-6, TNF-α and IL-23 production (218), (235). In $CD4^+$ T cells IL-36 induce the production of IFN-γ, IL-4, and IL-17A in a dose dependent manner (214) . These data are in accordance with the results obtained using the IL-36α overproducing mouse model in epidermis (236). Moreover, it has been reported that IL-36 cytokines are essential mediators in the KC-APC crosstalk required to drive Ald-induced psoriasiform dermatitis in mice (237). In this case, stimulation of APC via TLR7 promotes KCs activation and IL-36R-dependent release of chemokines such as CXCL1 and CCL20 and macrophage/neutrophil recruitment to the skin (237) (**Figure 18**). Furthermore, Takaishe et al., demonstrate that the auto-stimulatory loop of IL-36 cytokines in KCs is, regulated by the immunomodulator Regnase 1 (Reg1). Upon Ald-treatment Reg-$1^{-/-}$ mice presented higher IL-36α, this induction was attenuated in Reg-$1^{-/-}$/IL-$36R^{-/-}$ DKO mice (238), (216). Ald-treated *IL-36r*$^{-/-}$ and *Il36rn*$^{-/-}$ mice showed opposite effects, whereas *IL-36r*$^{-/-}$ presented a weaker phenotype *Il36rn*$^{-/-}$ developed stronger psoriasis-like skin inflammation (237).

Thus, the available results suggest that there is a positive feedback loop between IL-36 and IL-17/IL-23 axis and IL-36 cytokines are able to regulate skin inflammation via KCs release and mediate the crosstalk between DCs and T cell to promote immune tissue infiltration, cell activation and KCs abnormal proliferation, contributing to the chronic inflammation presented in psoriasis disease. (I will do a deeper discussion about IL-36 mechanism in psoriasis in next sessions).

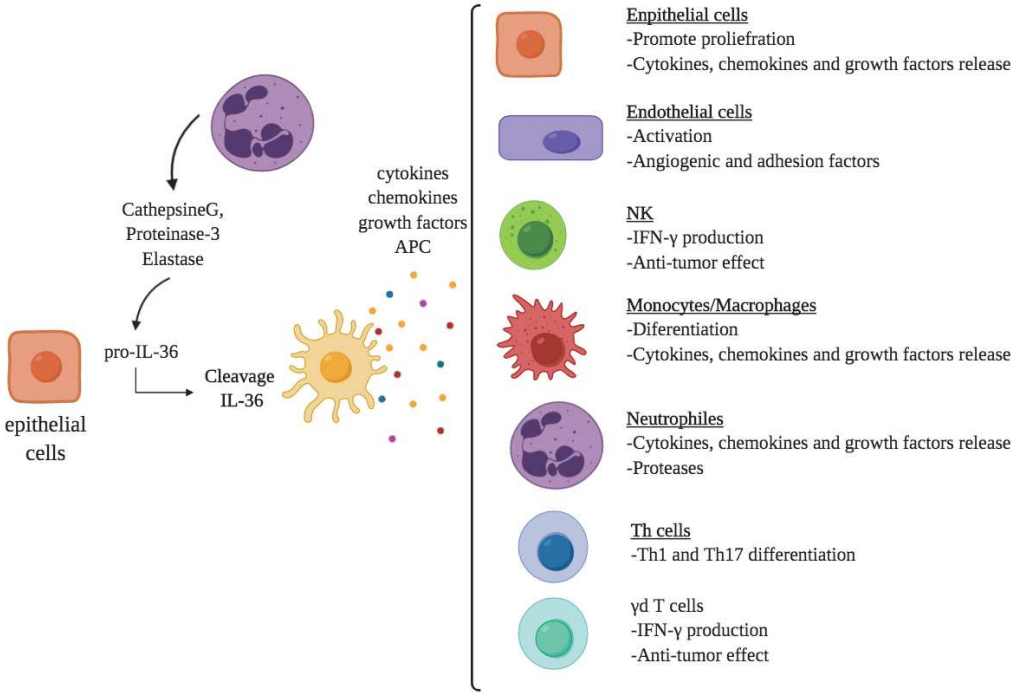


Figure 18: Origin and effects of IL-36 cytokines on different cell types. Pro-IL36 cytokines are released mainly by epithelial cells and processed by several enzymes detailed above to generate the active forms. IL-36 cytokines are able to stimulate KCs and other cells including DCs to enhance pro-inflammatory mediators responsible for several cellular effects in innate and adaptive immune responses. Figure adapted from (192) done by Biorender.

1.4.5- DYSREGULATION OF IL-36 SIGNALING IN INFLAMMATORY DISEASES

Although normal IL-36 signaling participates in the maintenance of tissue homeostasis, as mentioned in previous sessions, induction of IL-36 cytokines has been associated with diverse inflammatory diseases. In psoriasis, IL-36 pathway contributes in both innate and adaptive immune response (196). Besides, recently it has been demonstrated that IL-36 signaling plays a role in other inflammatory pathogenesis such as inflammatory bowel disease (IBD), acute kidney injury (AKI) or pulmonary fibrosis among others, discussed below.

- Skin

IL-36 cytokines, in particular IL-36α, IL-36γ and IL-36Ra, are principally produced by KCs, and in a less extent by dermal fibroblasts and endothelial cells (**Table 6**). Of note, some papers have been reported that IL-36α, β and γ were all induced in human psoriasis (224), (236), (235), (239). Nevertheless, others, demonstrated up-regulation of IL-36α

and IL-36γ, but not IL-36β, were induced in either mouse or human psoriatic samples (228), (240). Additionally, missense mutations in *IL36RN* which affect the function of IL-36Ra were identified in patients with GPP (241), (111). Mouse studies have also pointed out the key role of IL-36 signaling in driving and amplifying psoriatic inflammation together with IL-23/IL-17 axis (237), (219), (242), (240). Additionally, it has been shown that other IL-1 family members as IL-1α and IL-1β are altered in both non-lesional and lesional human psoriasis skin compared with healthy control skin (243). Nevertheless, anakinra an IL-1R antagonist, failed to show efficacy in PP patients (244). Concomitantly, it has been observed that IL-38 expression is down-regulated in the epidermis of human psoriatic lesions during the evolution of the chronic plaque (224). Thus, the available results suggest that there is a positive feedback loop between IL-36 and IL-17/IL-23 axis in psoriasis inflammation.

More in detail, it has been demonstrated that Ald-treated *IL-36r*$^{-/-}$ mice or mice treated with IL-36R antibody are protected from the induction of IL-23/IL-17 axis and psoriasis-like skin inflammation development (237), (242), (219). Additionally supporting these data, *Il36rn*$^{-/-}$ mice and transgenic mice overexpressing IL-36α in KCs exhibit inflammatory skin lesions with some features common to human psoriasis (236), (245), (237). Interestingly, IL-36α was recently suggested to play a primary role in psoriasis pathogenesis, as mice with ablation of IL-36α, but not IL-36β or IL-36γ, failed to develop Ald-induced psoriasis (240). Indeed, as explained before, it has been reported that despite IL-36α and IL-36γ utilize the same receptor (IL-36R/IL-1RacP), IL-36α seems to have a higher affinity than IL-36γ (213). Therefore, IL-36α may drive specified downstream signals. More recently, in vitro experiments in mouse cells demonstrated that IL-36α promotes DC-induced Th17 differentiation (246) and IL-17 production in $CD4^+$ T and γδT cells (247). Together, the above findings indicate that IL-36 cytokines play an important role in innate immune response.

Related with human data, it has been shown using microarrays that IL-36γ is up-regulated in human psoriatic skin compared with healthy skin (248). More recently, D'Erme et al., demonstrate that IL-36γ is the most outstanding marker of psoriasis in comparation with other skin diseases, and its level in blood is closely associated with disease activity (PASI) (249). These data were in accordance with numerous studies,

such as the transcriptome analysis in PP performed by Keermann et al., the GWAS analysis that identified 13 differentially expressed genes (DEGs) in psoriasis patients by Swindel et al., and the transcriptomic analysis of HPKCs treated with IL-36γ versus untreated performed by Mahil et al., (250), (251), (219). Furthermore, Koks et al., and lastly, Traks et al., investigated IL-36γ gene polymorphisms in psoriasis. They found three significantly associated single nucleotide polymorphisms (SNPs) and two haplotypes. Interestingly, none of them affect directly to the peptide sequence of IL-36γ, suggesting that maybe they will affect to the conformation of the protein (252). Additionally, it has been reported that IL-36α it is also induced in human psoriatic lesions compared with healthy skin at RNA and protein level (239), (228), (224).

Based on all of these observations, last year Bachelez et al., reported the results obtained in a phase 1 study, including seven GPP patients treated with a single intravenous dose of a human anti-IL-36R monoclonal antibody. The preliminary results showed that 79.8% of the patients presented improvements at week 4 post-treatment (253). On the other hand, human psoriatic skin transplanted onto immunodeficient mice was normalize when the mice were treated with the anti-IL-36R antibody, demonstrating that the IL-36 axis is required to maintain the lesioned phenotype in human psoriatic skin (242).

Despite the role of IL-36 cytokines in skin inflammation was mainly studied in psoriasis, these cytokines may be implicated in the development of other skin inflammatory disease. For instance, it has been shown that the level of IL-36α is up-regulated in AD patients (239). Additionally, it has been reported an increase of IL-36α expression upon *S. aureus* skin colonization ((247), (254). *S. aureus* is one of the main drivers for AD and transcriptomic analysis comparing pediatric and adult AD skin biopsies reported that pediatric patients presented higher Th17 activation correlated with higher levels of IL-36 expression (255). Moreover, recent studies showed that IL-36 cytokines expression is highly correlated with the severity of skin ichthyoses such as Netherton Syndrome (NS) (256). Furthermore, Mattii et al., reported that IL-36 family members are also highly induced in allergic contact dermatitis and administration of IL-36Ra into patient's skin biopsies significantly reduces the secretion of IL-36 and IL-8. Moreover, multiple lines of evidence from in vitro, in vivo and patient studies suggest

that IL-36 plays an important role in response to herpes simplex virus infection by inhibiting viral replication and by establishing anti-viral defense in the skin (231), (257), (258).

- Joints

It has been shown that in inflammatory joint disorders such as rheumatoid arthritis, psoriatic arthritis (PsA) and osteoarthritis (OA) IL-36 family members are induced (228), (259). Approximately, 20% of rheumatoid arthritis patients had elevation of the IL-36 agonist versus antagonist ratio in synovial fluids. In addition, it has been demonstrated that the induction of IL-36 cytokines promote the expression of pro-inflammatory chemokines in the synovial tissues. However, the expression of IL-36 family members is not correlated with arthritis severity (260).

- Intestine

IL-36α, IL-36γ and IL-36R are up-regulated in patients with Crohn's disease (CD), ulcerative colitis (UC), inflammatory bowel disease (IBD) and Hirschsprung's disease (229), (260), (261), (227). It has been shown that *IL-36rn*$^{-/-}$ mice present reduced innate response upon Citrobacter rodentium colonization (229). Notably, mice treated with anti-IL-36R antibody or *IL-36rn*$^{-/-}$ mice developed less severe fibrosis and colitis (261). Moreover, it has been reported that IL-36 also plays a role in gut healing after injury, likely through the induction of IL-22 (262), (263).

- Lungs

In lungs, it has been reported that IL-36/IL-36R pathway mediates inflammatory response upon infection including house dust mite (HDM), bacteria and viruses, developing an increase in lung injury and mouse mortality (264), (265). For instance, Ramadas et al., demonstrated that IL-36γ was significantly increased in epithelial cells of A/J mice challenged with HDM (266). Similarly, mice infected with Legionella pneumophila, Pseudomonas aeruginosa, Mycobacterium tuberculosis and Mycobacterium bovis BCG (less virulent mycobacteria) presented an increase in IL-36α, IL-36γ but not IL-36β ((264), (267). In children with Mycoplasma pneumoniae, IL-36 was elevated in the bronchoalveolar lavage fluid (BALF) (268). Additionally, in recent reports, IL-36γ was shown to play an important role in clearing Streptococcus pneumoniae in the lungs and to controlling the infection in mice (269).

- Brain

In 1996 Mulero et al., and Loverberg et al., suggested that infections lead to IL-1 mediated fever and appetite suppression. Moreover, IL-36R and IL-36β were detected in mouse glia cells including astrocytes and microglia at high mRNA expression level (179). Nevertheless, IL-36γ and IL-36β failed to induce fever and reduced body weight (179). In the experimental allergic encephalomyelitis (EAE) model of inflammatory demyelinating disease, both IL-36γ and IL-36R were upregulated in the nervous, hematopoietic tissues and "in vitro" microglial cells upon IL-36γ treatment induce mediators implicated in neutrophil recruitment. However, IL-36γ deficient and *IL-36r*$^{-/-}$ mouse developed EAE, suggesting IL-36γ might be a biomarker and not a driver of the disease (270). Recent studies show increased levels of IL-36γ in the serum from multiple sclerosis patients (267) and elevated IL-36α in the serum in patients with neuromyelitis optical spectrum disorder (NMOSD) (196).

4.6 Others

Additionally, elevated levels of IL-36a have been reported in patients with Sjogren's, systemic lupus erythematosus (SLE) and nephritic kidney biopsies (196).

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RESULTS
PART 1

Disrupting the IL-36 and IL-23/IL-17 loop underlies the efficacy of calcipotriol and corticosteroid therapy for psoriasis

Germán B. et al, JCI insight, 2019

INTRODUCTION

As described in detail in the introduction of this thesis, topical therapy with vitamin D_3 analogues, such as calcipotriene or calcipotriol (Cal), have been widely used since early 1990's for treating patients with mild to moderate PP (1). Recently, a combined Cal/Betamethasone (Daivobet ®) treatment has been demonstrated to be more efficacious in psoriasis treatment than either one of these two agents (2), (3), (4), (5). Until now, it has been assumed that Cal inhibits the proliferation and enhances the differentiation of keratinocytes (KCs), and also exerts some immunomodulator effects, but the underlying anti-psoriasis mechanisms remain to be elucidated.

Based on these clinical data, the principal objective of my project was to investigate the mechanism of how Cal on its own or in combination with corticosteroids, regulates psoriasis inflammation. Using an experimental mouse model in which psoriasis-like pathology is induced by a skin topical treatment with Aldara (Ald) cream (6), a previous PhD student in our lab (Wei, R.) showed that topical Cal treatment inhibited the pivotal IL-23/IL-17 inflammatory axis and neutrophil infiltration in Ald psoriasis-like skin inflammation developed in mice. Using $Vdr^{kc-/-}$ conditional knockout (KO) mice in which vitamin D receptor (VDR) is ablated selectively in epidermal KCs, he demonstrated that this suppressive effect of Cal is mediated through keratinocytic VDR. These data suggested that keratinocyte-derived factor (s), which is (are) regulated by Cal via VDR signaling, inhibits IL-23/IL-17 cytokines axis and the psoriatic inflammation.

When I joined the lab, it has emerged that, IL-36 cytokines play a key role in driving and maintaining the IL-23/IL-17 cytokine axis (7), (8), (9), (10). We therefore hypothesized that IL-36 cytokines could be the keratinocyte-derived factors regulated by Cal via VDR signaling. Using the Ald model, I showed that IL-36α and IL-36γ (but not IL-36β) which were induced in epidermal KCs, were effectively repressed by Cal via direct VDR signaling in mouse KCs. In collaboration with clinical researchers, we

showed that Cal treatment suppressed both IL-36α and IL-36γ expression in lesioned skin from patients with PP, accompanied by a reduced IL-23/IL-17 expression. Using ex vivo culture of mouse epidermis and in vitro culture of human primary keratinocytes (HPKCs), I showed that Cal directly represses IL-36α and IL-36γ expression. Furthermore, I dissected the regulation of IL-36α and IL-36γ by Cal and Dexamethasone (Dex). In contrast to the direct effect of Cal in KCs, I showed that Dex indirectly reduce the expression of IL-36α and IL-36γ in mouse psoriatic skin through immune cells.

Together, our findings indicate that the combination of Cal and corticosteroid disrupts efficiently the IL-36α/γ and IL-23/IL-17 positive feedback loop, thus revealing a mechanism underlying the superior efficacy of Cal and corticosteroid combination therapy for psoriatic patients. Our study suggests a central importance of interrupting the inflammatory loop in psoriasis therapy and suggest that IL-36 (keratinocyte) and IL-17 (immune cells) are the two key nodes for concomitant targeting. This part of my work, where I share the co-1st authorship with Wei, R. has been published in JCI insight (2019) (11).

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RESEARCH ARTICLE

Disrupting the IL-36 and IL-23/IL-17 loop underlies the efficacy of calcipotriol and corticosteroid therapy for psoriasis

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Psoriasis is one of the most common skin inflammatory diseases worldwide. The vitamin D3 analog calcipotriol has been used alone or in combination with corticosteroids in treating plaque psoriasis, but how it suppresses psoriatic inflammation has not been fully understood. Using an experimental mouse psoriasis model, we show that topical calcipotriol inhibited the pivotal IL-23/IL-17 axis and neutrophil infiltration in psoriatic skin, and interestingly, such effects were mediated through the vitamin D receptor (VDR) in keratinocytes (KCs). We further reveal that IL-36α and IL-36γ, which have recently emerged as key players in psoriasis pathogenesis, were effectively repressed by calcipotriol via direct VDR signaling in mouse KCs. Accordingly, calcipotriol treatment suppressed IL-36α/γ expression in lesional skin from patients with plaque psoriasis, which was accompanied by a reduced IL-23/IL-17 expression. In contrast, dexamethasone indirectly reduced IL-36α/γ expression in mouse psoriatic skin through immune cells. Furthermore, we demonstrate that calcipotriol and dexamethasone, in combination, synergistically suppressed the expression of IL-36α/γ, IL-23, and IL-17 in the established mouse psoriasis. Our findings indicate that the combination of calcipotriol and corticosteroid efficiently disrupts the IL-36 and IL-23/IL-17 positive feedback loop, thus revealing a mechanism underlying the superior efficacy of calcipotriol and corticosteroid combination therapy for psoriasis.

Introduction

Psoriasis is one of the most common, complex chronic inflammatory skin disorders, affecting 1%–3% of the worldwide population (1, 2). In its most common form, plaque psoriasis (PP; or psoriasis vulgaris) manifests as plaques of red, scaly, and well-demarcated regions of inflamed skin. Histologically, it is characterized by a thickening of the epidermis, with altered proliferation and differentiation of keratinocytes (KCs), and a skin infiltration of immune cells including neutrophils, DCs, and T cells (1). In addition, patients with psoriasis also exhibit signs and symptoms of systemic inflammation, resulting in an increased risk for multiple comorbid conditions, including polyarthritis, cardiovascular pathologies, and metabolic syndrome (3). Even though the mechanisms underlying the complex pathogenesis of psoriasis have not been fully elucidated, there is increasing evidence that the IL-23/IL-17 cytokine axis plays a pivotal role in the inflammatory response in psoriasis (1, 4, 5). It has been recognized that IL-23 secreted by DCs and monocytes/macrophages promotes IL-17–producing T cells, including Th17 cells and γδ$^+$ T cells, which exert pleiotropic effects on the recruitment and activation of other immune cells, such as neutrophils, as well as the hyperplasia of KCs. More recently, an elevated expression of IL-36 cytokines has been found in epidermal KCs of human psoriatic lesional skin (6–9), and IL-36 cytokines have been proposed to be a psoriasis signature (10–12). Mouse studies have also pointed out a key role for IL-36 signaling in driving and amplifying psoriatic inflammation together with IL-23/IL-17 cytokine axis (11, 13–17).

Topical therapies including corticosteroids and vitamin D3 analogues have been used for most patients with mild to moderate PP (1, 18). However, long-term corticosteroid treatments are limited by their side effects.

Authorship note: BG and RW contributed equally to this work.

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Since early 1990s, topical vitamin D3 analogues, such as calcipotriene or calcipotriol (Cal), have been widely used for PP; they are effective in about 45% patients and do not exhibit debilitating effects upon long-term treatment (1, 19). It has been assumed that Cal inhibits the proliferation and enhances the differentiation of KCs, as well as exerts some immunomodulator effects (19), but the underlying antipsoriasis mechanisms remain to be elucidated. Recently, a combined Cal/betamethasone treatment has been used for PP and has proved to be more efficacious than either one of these 2 agents (20, 21). Based on these clinical data, our present study was aimed to elucidate how Cal, of its own or in combination with corticosteroids, regulates the IL-23/IL-17 inflammatory axis in psoriasis, with the ultimate goal of developing more potent, better targeted, and safer treatments.

To this aim, we employed an experimental mouse model in which a psoriasis-like pathology is induced by a topical skin treatment with Aldara (Ald) cream, which contains imiquimod (IMQ) and is currently used in clinic to treat genital and perianal warts, actinic keratosis, and superficial basal cell carcinoma. Since the first report showing that topical Ald treatment induces psoriasis-like skin in mice (22), it has been widely used to create a psoriasis model (see reviews; refs. 23, 24). The relevance of this model to human psoriasis was supported by the frequent clinical incidence of psoriasis upon Ald treatment of cutaneous keratosis (23, 25). Indeed, the inflammation that is induced by topical application of Ald to mouse ears or shaved back skin of mice shows the similarities with human psoriasis and the cytokine expression (23). Particularly, Ald-induced psoriasis-like pathology is dependent on the IL-23/IL-17 axis (23). Using this model, we show in the present study that a topical treatment of Cal suppresses the IL-23/IL-17 inflammatory axis in Ald-induced mouse psoriatic skin. Importantly, by using mutant mice selectively ablated for the vitamin D receptor (VDR) in epidermal KCs, we demonstrate that this suppressive effect of Cal is mediated through keratinocytic VDR. This has led us to uncover a mechanism showing that Cal acts on KCs instead of immune cells (such as T cells, DCs, or Langerhans cells [LCs]; refs. 26, 27) to suppress the IL-23/IL-17 inflammatory axis in psoriasis and, furthermore, to provide evidence about how the combination of Cal and dexamethasone (Dex) effectively disrupt the positive cytokine feedback loop of IL-36 and IL-23/IL-17, which we believe underlies the superior efficacy of the combination therapy for psoriasis.

Results

Topical Cal inhibits the IL-23/IL-17 axis and the neutrophil infiltration in mouse psoriatic skin. Balb/c WT mouse ears were treated daily with Ald from day 0 (D0) to D4 to induce a psoriasis-like skin inflammation and were cotreated with either Cal or ethanol (ETOH, the vehicle for Cal) (Figure 1A). H&E-stained sections from Ald+ETOH ears showed an increased epidermal and dermis thicknesswith a heavy dermal cell infiltration; in contrast, Ald+Cal ears exhibited an attenuated dermal infiltration of inflammatory cells, as well as a decrease in the epidermal and dermal thickness (Figure 1B and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.123390DS1). In contrast, Ald+Cal ears exhibited an attenuated dermal infiltration of inflammatory cells, as well as a decrease in the epidermal and dermal thickness (Figure 1B and Supplemental Figure 1A). Infiltration of neutrophils, one of the manifestations of a psoriasis skin inflammation, was observed in Ald+ETOH dermis (shown by NIMP-R14 staining, Figure 1B), whereas it was much lower in Ald+Cal dermis, indicating that Cal treatment suppresses the skin neutrophil infiltration.

In agreement with previous reports (see review; ref. 23), the Ald topical treatment induced the expression of IL-23p19, IL-23/12p40, IL-17A, and IL-22, as well as the calcium binding proteins S100A7A and S100A8 in skin, which are all characteristic features for psoriatic inflammation (Figure 1C). Strikingly, when mouse ears were cotreated with Cal, the induction of all of these genes was decreased (Figure 1C, compare Ald+Cal with Ald+ETOH), in keeping with a lesser infiltration of neutrophils in Ald+Cal skin (Figure 1B). Note also that the expression level of these genes in WT skin was similar between Cal and ETOH treatments, indicating that a Cal treatment did not impact the basal level of these genes. However, the expression of thymic stromal lymphopoietin (TSLP) was induced upon Cal treatment, as we previously reported (28, 29) (Figure 1C). IHC staining with an antibody against IL-23p19 confirmed that very few IL-23p19$^+$ cells were detected in the dermis from either ETOH- or Cal-treated skin (Figure 1B). In contrast, numerous IL-23p19$^+$ cells were observed in Ald+ETOH skin, most of which were located in the dermis, in agreement with the previous reports showing that dermal DCs and monocytes/macrophages are major cellular sources of IL-23 induced by IMQ/TLR7 signaling in Ald-treated skin (15, 30, 31). The increase in IL-23p19$^+$ cells was abolished in Ald+Cal skin (Figure 1B), which confirmed the data from quantitative PCR (qPCR) analyses (Figure 1C).

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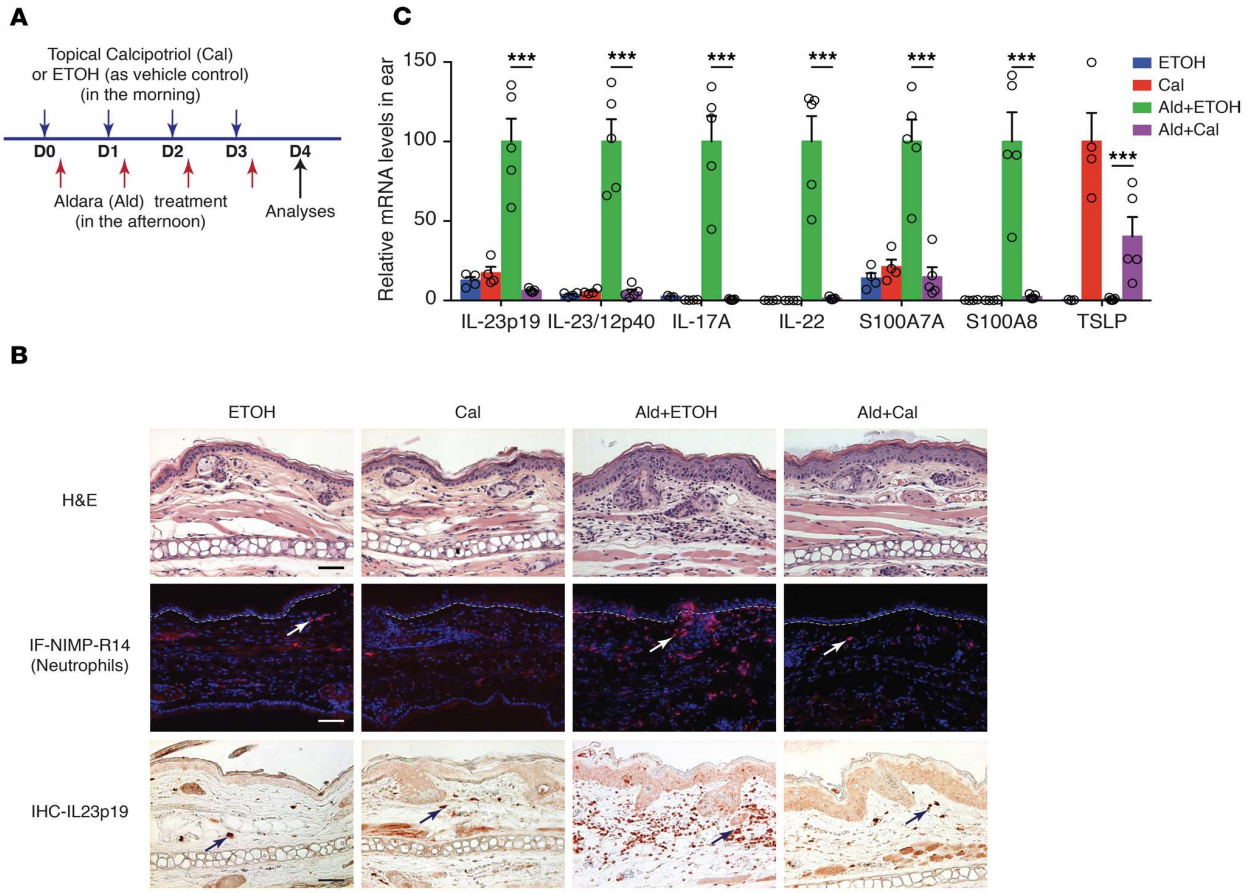

Figure 1. Topical calcipotriol inhibits the IL-23/IL-17 axis and neutrophilia in Aldara-treated skin. (**A**) Experimental protocol. WT Balb/c mouse ears were topically treated with ETOH (vehicle control), calcipotriol (Cal), Aldara (Ald)+ETOH or Ald+Cal every day from day 0 (D0) to D3, with ETOH or Cal treatment in the morning and Ald treatment in the afternoon. Mouse ears were analyzed at D4. (**B**) H&E, immunofluorescent staining with NIMP-R14 antibody (for neutrophils; red corresponds to positive signal, whereas blue corresponds to DAPI staining of nuclei), and IHC staining with IL-23p19 antibody (in dark red) of ear sections. Arrows point to positive signals. White dashed lines indicate the dermal/epidermal junction. Scale bar: 50 μm for all pictures. (**C**) qPCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. ***$P < 0.001$ (2-tailed Student's t test). Data are representative of 3 independent experiments with similar results.

Taken together, these results indicate that a topical Cal treatment inhibits the IL-23/IL-17 axis and the neutrophil infiltration in mouse psoriatic skin.

Keratinocytic VDR mediates the inhibition of the IL-23/IL-17 axis and neutrophilia by Cal. Next, we examined whether the inhibitory effect of Cal on the IL-23/IL-17 axis is mediated through VDR (32). $Vdr^{-/-}$ and WT littermate mice (all in Balb/c genetic background) were subjected to ETOH, Cal, Ald+ETOH, and Ald+Cal treatment, as described in Figure 1A. qPCR analyses of ears showed that, in $Vdr^{-/-}$ mice, Cal failed to inhibit the Ald-induced expression of IL-23p19, IL-23/IL-12p40, IL-17A, IL-22, S100A7A, and S100A8 (Figure 2A), indicating that the inhibition of IL-23/IL-17 by Cal is indeed mediated via VDR. As expected (29), the induction of TSLP by Cal was VDR dependent (Figure 2A).

As VDR is expressed in numerous skin cell types, including KCs, we then asked whether the inhibition of IL-23/IL-17 by Cal could be mediated through keratinocytic VDR. To this end, mice with the ablation of VDR selectively in epidermal KCs (K14-$Cre^{tg/0}$/$Vdr^{L2/L2}$ mice, called here after $Vdr^{KC-/-}$ mice) (29) were subjected to ETOH, Ald+ETOH, or Ald+Cal treatment as described in Figure 1A. Interestingly, Cal failed to inhibit the Ald-induced IL-23/IL-17 axis in $Vdr^{KC-/-}$ mice (Figure 2B), and it was not able to reduce the dermal infiltrate in Ald-treated $Vdr^{KC-/-}$ mice (Figure 2C; H&E staining). IHC staining with IL-23p19 antibody, as well as immunofluorescent (IF) staining with NIMP-R14 antibody, showed that Cal treatment could not reduce the number of IL-23p19$^+$ cells and neutrophils in $Vdr^{KC-/-}$ dermis (Figure 2C).

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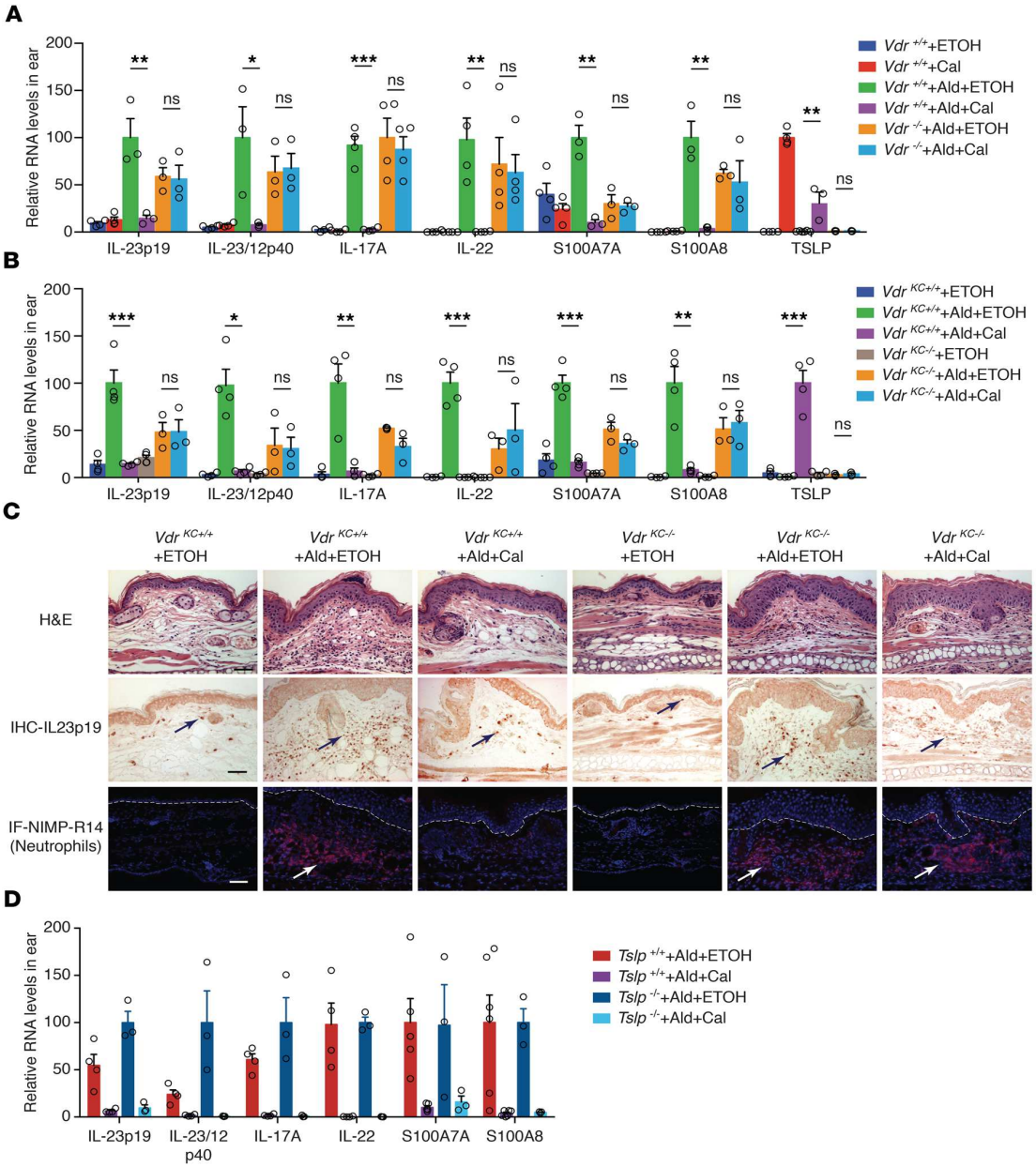

Figure 2. Keratinocytic VDR mediates the inhibition of IL-23/IL-17 and neutrophilia by calcipotriol in mouse psoriatic skin. (**A** and **B**) $Vdr^{-/-}$ mice and their littermate $Vdr^{+/+}$ mice (**A**), $Vdr^{KC-/-}$ mice ($K14\text{-}Cre^{Tg/0}/Vdr^{L2/L2}$) and their littermate $Vdr^{KC+/+}$ controls ($K14\text{-}Cre^{0/0}/Vdr^{L2/L2}$) (**B**) were treated with ETOH, calcipotriol (Cal), Aldara (Ald)+ETOH, or Ald+Cal, as described in Figure 1A. Ears were analyzed by qPCR at D4. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (2-tailed Student's *t* test). Values are mean ± SEM. (**C**) H&E staining, IHC staining with IL-23p19 antibody (in dark red), and immunofluorescent (IF) staining with NIMP-R14 antibody (for neutrophils; red corresponds to positive signal, whereas blue corresponds to DAPI staining of nuclei) among $Vdr^{KC+/+}$ and $Vdr^{KC-/-}$ ear sections. White dashed lines indicate the dermal/epidermal junction. Scale bar: 50 μm. (**D**) qPCR analyses of ears from $Tslp^{-/-}$ mice and their littermate $Tslp^{+/+}$ mice treated with ETOH, Cal, Ald+ETOH, or Ald+Cal, as described in Figure 1A. Ears were analyzed by qPCR at D4. Values are mean ± SEM. Data are representative of 3 independent experiments with similar results.

Together, these data demonstrate that the suppression of the psoriatic inflammation by Cal is dependent on keratinocytic VDR. Note that, as KCs do not express TLR7 either in mice (15) or humans (33, 34) (Supplemental Figure 2), our results do not seem to reflect a crosstalk between VDR signaling and TLR7 signaling in KCs; instead, our data suggest that the Cal/VDR signaling most likely regulates

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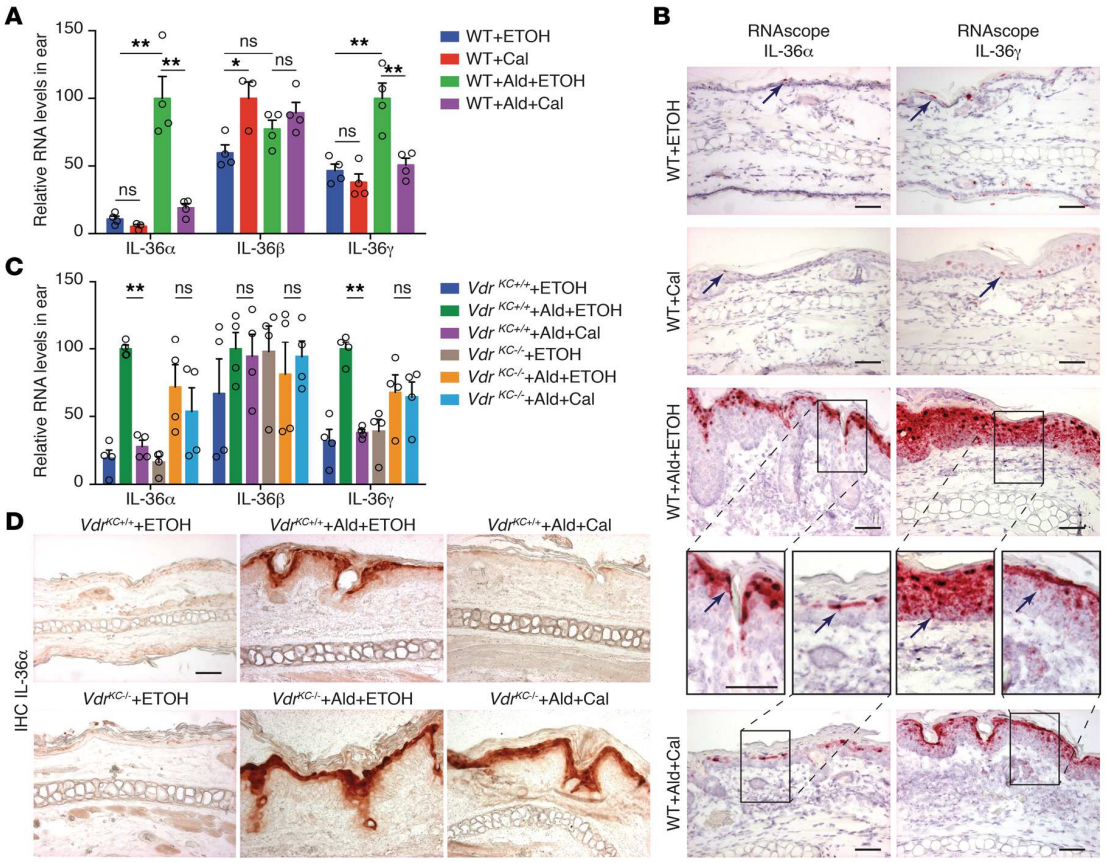

Figure 3. Calcipotriol inhibits IL-36α/γ expression in mouse psoriatic skin epidermis in a keratinocytic VDR-dependent manner. (**A**) qPCR analyses for IL-36 in Balb/c WT ears treated with ETOH, Cal, Ald+ETOH, or Ald+Cal (as described in Figure 1A). *$P < 0.05$; **$P < 0.01$ (2-tailed Student's *t* test). Values are mean ± SEM. (**B**) RNAscope in situ hybridization with IL-36α or IL-36γ probe (signals are in red) on mouse ear sections, counterstained with hematoxylin. Scale bar: 50 μm. (**C**) qPCR analyses for IL-36 in $Vdr^{KC-/-}$ mice ($K14\text{-}Cre^{Tg/0}/Vdr^{L2/L2}$) and their littermate $Vdr^{KC+/+}$ controls ($K14\text{-}Cre^{0/0}/Vdr^{L2/L2}$), treated with ETOH, Ald+ETOH, or Ald+Cal. Values are mean ± SEM. **$P < 0.01$ (2-tailed Student's *t* test). (**D**) IHC staining with IL-36α antibody on ear sections. Positive cells are in dark red. Scale bar: 50 μm. Data are representative of 3 independent experiments with similar results.

certain factors, such as cytokines produced and released by KCs, which consequently regulates a IL-23/IL-17 cascade in immune cells.

We initially hypothesized that the cytokine TSLP produced by mouse KCs mediated the inhibition of the IL-23/IL-17 axis, as Cal is known to induce TSLP overexpression in mouse KCs, thereby driving a Th2 immune response (28, 29, 35, 36), which may counter-regulate the IL-23/IL-17 axis (37). However, when subjected to a Cal treatment, $Tslp^{-/-}$ mice exhibited a similar reduction in Ald-induced expression of IL-23p19, IL-23/IL-12p40, IL-17A, IL-22, S100A7A, and S100A8 as WT $Tslp^{+/+}$ mice (Figure 2D), thus indicating that KC-derived factors other than TSLP are involved in the inhibition of the IL-23/IL-17 axis by Cal.

Cal inhibits IL-36α and IL-36γ expression in mouse psoriatic skin. Evidence has been provided indicating that IL-36 cytokines play a key role in driving and maintaining psoriatic inflammation. Indeed, an elevated expression of IL-36α, IL-36β, or IL-36γ has been reported in human psoriatic lesional skin (6, 7, 9, 11, 12); furthermore, mutations of the IL36RN gene that encodes IL-36 receptor antagonist, leading to an enhanced IL-36 signaling, were associated with generalized pustular psoriasis patients (38, 39). Furthermore, it was shown that IL-36R–deficient mice, as well as mice with blockade of IL-36R, were protected from the Ald-induced IL-23/IL-17/IL-22 axis and psoriasis disease development (11, 13, 15). More recently, it was reported that mice with ablation of IL-36α (but not IL-36β or IL-36γ) failed to develop psoriasis upon Ald treatment (14). We therefore examined whether the Ald-induced IL-36 expression could be modulated by Cal. qPCR analyses showed that IL-36α and IL-36γ, but not IL-36β, were significantly induced in ears from WT mice with Ald+ETOH treatment as compared with those with ETOH treatment (Figure 3A), in agreement with a

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previous report using the Ald model (6). Strikingly, the expression of IL-36α and IL-36γ was greatly diminished in Ald+Cal ears, whereas IL-36β mRNA levels remained unchanged (Figure 3A). Of note, skin treated with WT+Cal and WT+ETOH did not exhibit significant difference in RNA levels of IL-36α and IL-36γ, although a tendency of decrease could be seen in WT+Cal skin (Figure 3A).

We next examined the location of IL-36α– or IL-36γ–expressing cells in Ald-treated skin, using RNAscope in situ hybridization, an assay which exhibits high specificity and sensitivity. In either WT+ETOH or WT+Cal ears, IL-36α was barely detected, while some positive cells for IL-36γ could be seen in the epidermis (Figure 3B). In WT+Ald+ETOH skin, there was a strong increase of IL-36α signal, which was specifically located in the outmost suprabasal layer of epidermis (Figure 3B, left panel). IL-36γ was also induced, but throughout all layers of epidermis, while weaker signals could be seen in the dermis (Figure 3B, right panel). In WT+Ald+Cal ears, the expression of IL-36α and IL-36γ was diminished in the epidermis, whereas IL-36γ signals in the dermis remained unchanged (Figure 3B). These results indicate that Ald induces IL-36α and IL-36γ transcripts located in suprabasal layer and all layers in epidermis, respectively — both of which are downregulated upon Cal treatment.

We then investigated whether the inhibition of Ald-induced IL-36α and IL-36γ by Cal could be mediated through keratinocytic VDR. qPCR results showed that, in $Vdr^{KC-/-}$ mice, Cal failed to downregulate the Ald-induced IL-36α and IL-36γ expression (Figure 3C). To validate these results at the protein level, we performed IHC staining with an IL-36α antibody (a specific antibody against mouse IL-36γ was not available). IL-36α was very weakly detected in ETOH-treated $Vdr^{KC+/+}$ and $Vdr^{KC-/-}$ mice, whereas it was strongly induced in the suprabasal layer of epidermis of both $Vdr^{KC+/+}$ and $Vdr^{KC-/-}$ mice upon Ald+ETOH treatment (Figure 3D). Upon Ald+Cal treatment, the IL-36α signal was greatly decreased in $Vdr^{KC+/+}$ but not in $Vdr^{KC-/-}$ epidermis, demonstrating that Cal inhibits the IL-36α protein production in the suprabasal layer of the epidermis, which is mediated through the keratinocytic VDR.

As it was recently described that, among various mouse strains, Ald response in C57BL/6J (B6) mice were mostly consistent with human psoriasis (40), we further examined whether Cal had a similar effect in Ald-treated B6 mice, as we observed with Balb/c mice. Our kinetic analyses showed that the development of psoriatic inflammation was relatively delayed in B6 mouse ears compared with Balb/c, as a similar skin inflammation was observed at D6 (with 6× Ald) for B6 mice and at D4 (with 4× Ald) for Balb/c mice (Supplemental Figure 3A), in good agreement with what was previously reported (22). Nevertheless, our results confirmed that topical Cal treatment inhibited skin inflammation in Ald-treated B6 mouse ears (Supplemental Figure 3, B and C; compare [(6×Ald)+(6×Cal)] and [(6×Ald)+(6×ETOH)]); moreover, Cal treatment reduced the Ald-induced expression of IL-23p19, IL-23p40, IL-17A, and IL-22, as well as IL-36α/γ in B6 mouse ears (Supplemental Figure 3, D and E), in a similar way as observed in Balb/c mice.

Topical treatment of Cal downregulates IL-36α/γ expression in human psoriasis skin. To explore the human relevance of our findings from mouse models, 4 patients with PP received topical treatment of Cal (Daivonex cream) once per day for 4 days, and biopsies performed before and after treatment on the same lesion were analyzed by qPCR. Results showed that the mRNA expression of human IL-36α (hIL-36α) and hIL-36γ, but not hIL-36β, was highly elevated in all of the 4 PP skin as compared with healthy skin (HS) (Figure 4). This was correlated with the increase of hIL-23p19, hIL-17A, and hIL-22 in PP skin and, to a lesser extent, with hIL-23/hIL-12p40 — but not with hIL-12p35. The level of hTSLP in PP skin showed a mild increase as compared with HS. Most interestingly, upon the 4-day Cal treatment, the levels of hIL-36α, hIL-36γ, hIL-23p19, and hIL-23/12p40, as well as hIL-17A, were all decreased in 3 of the 4 patients (i.e., in patient nos. 1, 2, and 4), while patient no. 3 did not respond to the Cal treatment. Indeed, qPCR analysis of hCyp24A1, a gene known to be positively regulated by vitamin D3, showed that hCyp24A1 was induced upon Cal treatment in patient nos. 1, 2 and 4, but not in the patient no. 3 (Figure 4). We conclude from these results that, in agreement with our mouse data, a Cal treatment reduces the expression of hIL-36α, hIL-36γ, hIL-23p19, hIL-23/12p40, and hIL-17A in human psoriasis skin. The exceptions were for IL-22, which was reduced in only 2 of the 4 patients upon Cal treatment, and for TSLP, whose expression was not upregulated by Cal, in contrast with what was observed in mouse skin (Figure 1C and refs. 28, 29).

Cal, but not Dex, inhibits IL-36α/γ expression in Ald-treated immunodeficient NSG mice. In addition to vitamin D derivatives, topical corticosteroids have been used for therapy of mild to moderate psoriasis (1, 18). Topical treatment with Dex ameliorated the Ald-induced skin inflammation (Figure 5A, upper panel) and reduced the expression of IL-23p19, IL-23/12 p40, IL-17A, and IL-22 (Figure 5B). These observations

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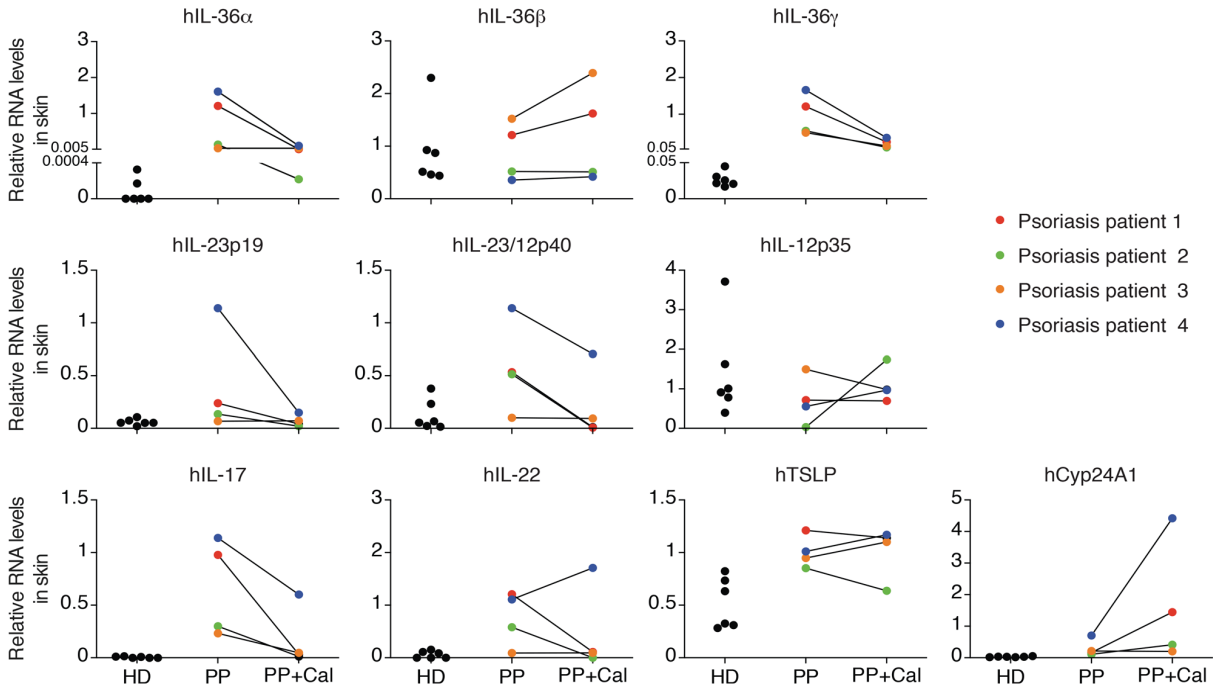

Figure 4. Topical treatment of calcipotriol downregulates hIL-36α and hIL-36γ expression in human psoriasis skin. Skin biopsies were taken from healthy donors (HD), or from lesional skin of plaque psoriatic patients (PP) before and after a 4-day topical treatment with Calcipotriol ointment (DAIVONEX) once a day, and qPCR analyses were performed. Individual values are shown, with the line connecting paired samples from the same patient before and after the calcipotriol treatment.

were in keeping with previous studies showing that Dex inhibited IL-23 expression from DCs (26) or macrophages (41), as well as IL-17/IL-22 expression by T cells (26). Interestingly, we found that the levels of IL-36α and IL-36γ in skin were also reduced (Figure 5B), but such reduction was less prominent as compared with Cal treatment (Figure 3A). IHC staining confirmed that, in WT skin treated with Ald+Dex, IL-36α signals in the suprabasal layer of epidermis were decreased (Figure 5A, lower panel); however, the remaining IL-36α signal in Ald+Dex skin was higher as compared with Ald+Cal skin, in which IL-36α expression was nearly abolished (Figure 3D).

It has been shown that IL-36 cytokines initiate and promote the expression of IL-17/IL-22, but the latter can also induce IL-36 cytokine expression, suggesting a positive feedback regulation loop between IL-36 and IL-17/IL-22 (9, 42). We wondered whether Cal, Dex, or both actually inhibit the expression of IL-36α/γ in a direct manner in KCs. To explore these possibilities, we employed the immunodeficient Nod-Scid *Il2rg*–/– (NSG) mouse line, which lacks T cells, B cells, NK cells, and innate lymphoid cells (ILCs) (43). Ald-treated NSG mice did not exhibit an immune infiltrate in dermis (Figure 5C, upper panel), nor any expression of IL-17 and IL-22, due to the absence of cells producing these cytokines, including Th17 cells, γδ T cells, and ILCs (Figure 5D). However, RNA levels of IL-36α and IL-36γ were significantly increased (compare NSG+Ald+ETOH with NSG+ETOH). IHC staining confirmed an increase of the IL-36α signal in the suprabasal layer of the epidermis of NSG mice treated with Ald+ETOH (Figure 5C, lower panel; compare NSG+Ald+ETOH with NSG+ETOH). Notably, the increase in IL-36α/γ (but not IL-36β) in NGS mice was lower when compared with WT mice, as shown by qPCR analyses (Figure 5D) or IHC for IL-36α (compare Figure 5C with Figure 3D). These results indicate that the expression of IL-36α/γ is directly induced by Ald in the absence of IL-17/IL-22 and that their expression is reinforced by IL-17/IL-22.

We then employed Ald-treated NSG mice to examine whether the inhibition of IL-36α/γ by Cal or Dex was a direct effect on KCs, or an indirect effect subsequent to the decrease in IL-17/IL-22. As shown in Figure 5, C and D, Ald-induced expression of IL-36α and IL-36γ was inhibited by Cal (compare NSG+Ald+Cal with NSG+Ald+ETOH). However, Dex did not have any effect on the expression

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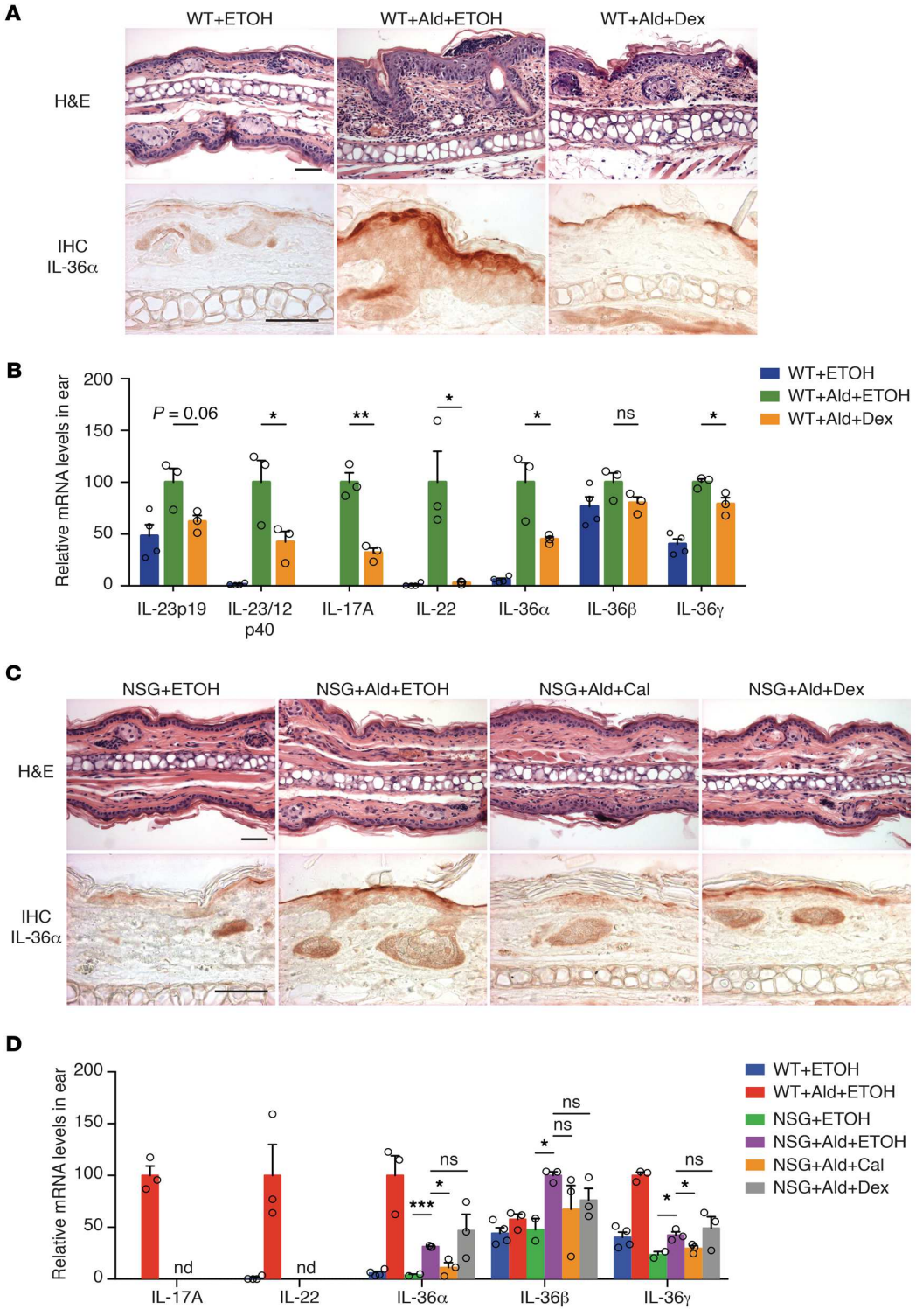

Figure 5. Calcipotriol directly suppresses the expression of IL-36α/γ in mouse skin, but the effect of dexamethasone is indirect. (**A** and **B**) Topical dexamethasone (Dex) reduces IL-23/IL-17/IL-22 as well as IL-36α/γ expression in mouse psoriatic skin. WT Balb/c mouse ears were topically treated with ETOH, Aldara (Ald)+ETOH, or Ald+Dex from day 0 (D0) to D3, with ETOH or Dex in the morning and Ald in the afternoon, as shown in Figure 1A. Ears were analyzed at D4. (**A**) H&E staining (upper panel) and IHC staining with IL-36α antibody (lower panel; positive signals are in dark red). Scale bar: 50 μm. (**B**) qPCR analyses of ears. Values are mean ± SEM. *$P < 0.05$; **$P < 0.01$ (2-tailed Student's t test). Data are representative of 3 independent

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experiments with similar results. (**C** and **D**) Calcipotriol but not dexamethasone inhibits IL-36α/γ in Aldara-treated immunodeficient NSG mouse ears. (**C**) H&E staining (upper panel) and IHC staining with IL-36α antibody (lower panel; positive signals are in dark red). Scale bar: 50 μm. (**D**) qPCR analyses of the treated ears. nd, not detected. Values are mean ±SEM. $^{*}P < 0.05$; $^{***}P < 0.001$ (2-tailed Student's *t* test). Data are representative of 2 independent experiments with similar results.

of IL-36α or IL-36γ in Ald-treated NSG mice (compare NSG+Ald+Dex with NSG+Ald+ETOH). Taken together, these data suggest that the Cal treatment directly inhibits the expression of IL-36α/γ in KCs, whereas Dex treatment indirectly leads to the reduced expression of IL-36α/γ via the suppression of IL-23/IL-17/IL-22 in immune cells.

Cal represses IL-36α/γ expression in mouse epidermal KCs and human primary KCs. To further support that Cal directly regulates the expression of IL-36α and IL-36γ in epidermal KCs, the epidermis of WT Balb/c mouse ears was separated from the dermis, and an ex vivo culture of epidermal sheet was carried out in the presence of Cal or Dex. A significant decrease in IL-36α and IL-36γ expression was observed in the presence of Cal but not of Dex (Figure 6A, left panel). Moreover, this repression was abolished in ex vivo–cultured epidermis from $Vdr^{KC-/-}$ mice (Figure 6A, right panel), indicating that the transcriptional repression of IL-36α/γ by Cal is mediated by keratinocytic VDR.

We next examined whether Cal may repress the expression of both IL-36α and IL-36γ in human KCs. In freshly isolated human primary KCs (HPKCs) from healthy donors, hIL-36γ — but not hIL-36α — RNA was detected by qPCR (Figure 6B; nonstimulated). We stimulated HPKCs in the presence of TNF-α, IFN-γ, and IL-17A, alone or in combination, in order to mimic the psoriatic skin microenvironment (44), and we examined the induction of hIL-36. We found that, when stimulated with either TNF-α, IFN-γ, or IL-17A alone, the expression of hIL-36γ was readily increased in HPKCs; however, hIL-36α remained undetectable (Figure 6B, healthy donor-A) or very low (Figure 6B, healthy donor-B). Among all combinations tested, the triple stimulation with TNF-α+IFN-γ+IL-17A resulted in the strongest stimulation of IL-36α and IL-36γ in both HPKCs derived from healthy donor-A and donor-B (Figure 6B).

We then added Cal or Dex to HPKCs stimulated with the mix of TNF-α+IFN-γ+IL-17A and examined by qPCR their effects on IL-36α/γ expression. Cal did repress both hIL-36α and hIL-36γ, while Dex had no effect on IL-36α expression and weakly decreased IL-36γ expression (Figure 6C). In addition, the Cal+Dex exhibited similar effect as Cal alone (Figure 6C). We conclude that, as observed in mice, Cal — but not Dex — exerts a direct repression on the expression of IL-36α and IL-36γ in human KCs.

Cal and Dex synergistically suppress the expression of IL-36α/γ in the established psoriasis in mice. During the last years, clinical studies have shown that a combined local therapy of psoriasis with Cal and corticosteroids generated the superior efficacy over use of either agent alone (20, 21). We suspected that Cal and Dex could synergistically suppress the expression of IL-36α/γ in the establish psoriatic inflammation. To test that, Balb/c WT mouse ears were first daily treated with Ald from D0 to D3 to induce a psoriatic inflammation and were then posttreated with ETOH, Cal, Dex, or Cal+Dex at D4 and D5 (Figure 7A). Histological analyses of the skin showed a decrease in dermal inflammatory infiltration in both Ald+2×Cal and Ald+2×Dex groups (as compared with Ald+2×ETOH), but most interestingly, the Ald+(2×Cal+Dex) group exhibited a further decrease in skin inflammatory infiltration (Figure 7B, H&E staining) and in thicknesses of the epidermis and the dermis (Supplemental Figure 1B). We then examined the expression of IL-36α and IL-36γ by IHC staining and RNA in situ hybridization. The Cal posttreatment suppressed the IL-36α/γ expression in the established psoriasis (Figure 7B, compare WT+Ald+2×ETOH and WT+Ald+2×Cal). The Dex posttreatment also led to a reduced level of IL-36α and IL-36γ, although this reduction is lesser than that achieved by Cal (Figure 7B). A combined treatment with Cal and Dex resulted in a synergistic suppression of IL-36α expression (as shown by both IHC staining and RNA in situ hybridization), as well as of IL-36γ expression (as shown by RNA in situ hybridization). Similarly, a synergistical reduction by Cal and Dex cotreatment was also observed for IL-23p19, IL-23/IL-12p40, IL-17A, and IL-22 in Ald-treated skin (Figure 7C). Taken together, our data indicate that a cotreatment with Cal and Dex synergistically and efficiently suppresses the expression of IL-36α/γ and IL-23/IL-17/IL-22 in the established psoriasis in mice.

Discussion

In this study, we have explored mechanisms underlying the therapeutic effects of the vitamin D3 analog Cal, on its own or in combination with Dex, in the treatment of PP. Employing an experimental mouse psoriasis model, we demonstrate that topical Cal inhibits the IL-23/IL-17 axis in psoriatic inflammation

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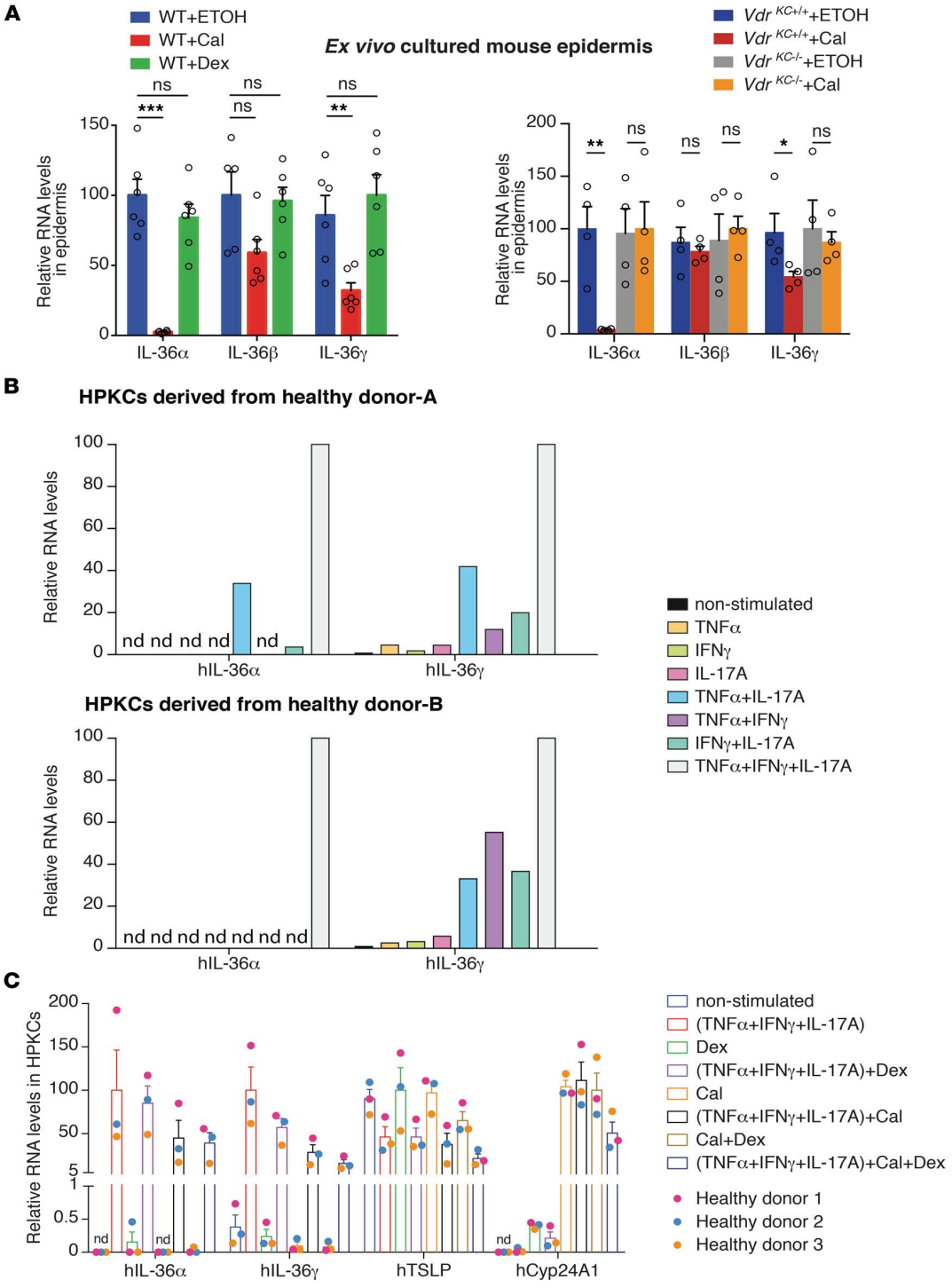

Figure 6. Calcipotriol represses IL-36α/γ expression in mouse epidermal keratinocytes and human primary keratinocytes. (**A**) Calcipotriol but not dexamethasone represses IL-36α/γ expression in ex vivo cultured mouse epidermis in a keratinocytic VDR–dependent manner. Data show the qPCR analyses of cultured epidermis from WT Balb/c mice (left panel) or from $Vdr^{KC+/+}$ and $Vdr^{KC-/-}$ mice (right panel). Values are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (2-tailed Student's *t* test). Data are representative of 2 independent experiments with similar results. (**B** and **C**) Calcipotriol represses IL-36α/γ expression in human primary keratinocytes (HPKCs). (**B**) HPKCs derived from 2 healthy donors were stimulated HPKCs in the presence of TNF-α, IFN-γ and IL-17A, alone or in combination, and they were examined the induction of hIL-36α and hIL-36γ. (**C**) HPKCs derived from 3 healthy donors were stimulated with cytokine cocktails (TNF-α+IFN-γ+IL-17A), in combination with calcipotriol (Cal), dexamethason (Dex), or both for 24 hours, and gene expression was analyzed by qPCR. Dots with the same color (red, blue, or orange) represent HPKCs derived from the same donor no. 1, 2, or 3.

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via keratinocytic VDR signaling. We reveal that Cal directly represses the expression of IL-36α and IL-36γ in mouse epidermal KCs, and we show that this mechanism is conserved in humans. In contrast with Cal, we find that Dex does not directly repress IL-36α/γ expression in mouse or human KCs; however, the topical treatment of Dex indirectly decreases IL-36α/γ in KCs. Furthermore, we obtain evidence indicating that the concomitant suppression of IL-36α/γ by Cal in KCs and of IL-23/IL-17 by Dex in immune cells efficiently interrupt the positive feedback loop of IL-36α/γ and IL-23/IL-17. These results reveal a plausible mechanism for the superior efficacy of the combined Cal/corticosteroid therapy for psoriasis, which is schematically presented in Figure 8.

Our data demonstrate that Cal acts on KC to exert its antiinflammatory effect on psoriasis.. This is different from previous studies proposing that Cal acts on immune cells to modulate IL-23/IL-17 inflammation. For example, it was shown that Cal acted on DCs and T cells in ex vivo–cultured human psoriatic skin explants to inhibit IL-23 and IL-17 expression, respectively (26). More recently, it was reported that Cal suppressed the IL-23 expression by epidermal LCs, which was assumed to be the reason for the inhibition of IL-17 by Cal in Ald-induced mouse psoriasis (27). In our study, we showed that the inhibition of IL-23/IL-17/IL-22 by Cal was abolished in $Vdr^{KC-/-}$ (mice with the ablation of VDR selectively in KCs) (Figure 2, A and B), which demonstrates that this inhibiting effect is actually dependent on keratinocytic VDR. As to IL-23, our IHC staining showed that IL-23$^+$ signals were predominantly detected in dermis but not in epidermis of Ald-treated mouse skin (Figure 2C). This result suggests that Cal inhibits IL-23, which is expressed by dermal DCs or/and monocytes/macrophages, in keeping with other studies suggesting that Langerin-negative dermal conventional DCs (cDCs) produce IL-23 to drive psoriatic inflammation (31) and that LCs have no essential role — or rather an antiinflammatory role — in psoriatic inflammation (45–47).

Recent studies have provided compelling evidence that IL-36 signaling plays a key role in driving and amplifying psoriatic inflammation. Particularly, mice deficient of IL-36R or mice with blockade of IL-36R were protected from the Ald-induced IL-23/IL-17 axis and disease development (11, 13, 15); on the other hand, IL-36R antagonist–deficient ($Il36rn^{-/-}$) mice showed exacerbated pathologies (15). More recently, it has been also shown that IL-36γ induces IL-23 production from psoriasis macrophages (16) and that IL-36α promotes DC-induced Th17 differentiation (17) or directly induces IL-17 on CD4$^+$ T and γδ T cells (48). Of note, we observed that, in contrast with IL-36α and IL-36γ, IL-36β was not significantly induced in either mouse (Figure 3A) or human (Figure 4) psoriatic skin. A similar observation was made by a recent study from Boutet et al. (6), although others reported that IL-36α, -β, and -γ were all induced in human psoriasis (8, 9, 49). Nevertheless, Cal did not repress the expression of IL-36β in either mouse or human psoriatic skin. Therefore, different from IL-36α/γ, IL-36β appears to be neither associated with the IL-23/IL-17 axis in psoriasis nor related to the antiinflammatory effect of Cal.

However, our data do not distinguish whether IL-36α, IL-36γ, or both are primary targets of Cal for its antipsoriasis effect. These 2 cytokines appear to be closely associated in psoriatic inflammation and to be able to interregulate each other (i.e., one induces another) (50, 51). Interestingly, IL-36α was recently suggested to play a primary role in psoriasis pathogenesis, as mice with ablation of IL-36α, but not (or to a much lesser extent) of IL-36β or IL-36γ, failed to develop Ald-induced psoriasis (14). Indeed, despite the fact that IL-36α and IL-36γ utilize the same receptor (IL-36R/IL-1RacP), IL-36α seems to have a higher affinity than IL-36γ to both IL-36R and IL-36R/IL-1RAcP heterodimer (52); therefore, it may drive specified downstream signals. Moreover, IL-36α and IL-36γ may be differently induced in certain physiological or pathological contexts. For example, IL-36α (but not IL-36γ) was shown be promoted by epicutaneous Staphylococcus aureus (48), while IL-36γ was shown to be induced in skin injury (53). It will be interesting to determine the specified versus redundant roles of IL-36α and -γ in cytokine network and inflammatory pathways in psoriasis and other pathologies, which should provide useful information for designing specific targeting strategies.

How the expression of IL-36α/γ is repressed by Cal remains to be further investigated. In our study, we have shown that such repression requires keratinocytic VDR. However, the KO of VDR in KCs ($Vdr^{KC-/-}$) did not lead to a spontaneous upregulation of IL-36α or IL-36γ expression (Figure 3C, comparing $Vdr^{KC-/-}$+ETOH with $Vdr^{KC+/+}$+ETOH); moreover, there was no increase in IL-36α or IL-36γ expression in the Ald-treated $Vdr^{KC-/-}$ skin compared with Ald-treated WT skin (Figure 3C, comparing $Vdr^{KC-/-}$+Ald+ETOH with $Vdr^{KC+/+}$+Ald+ETOH). In other words, in the absence of Cal treatment, VDR on its own does not seem to repress the expression of IL-36α/γ. Besides Cal, we confirmed that the active

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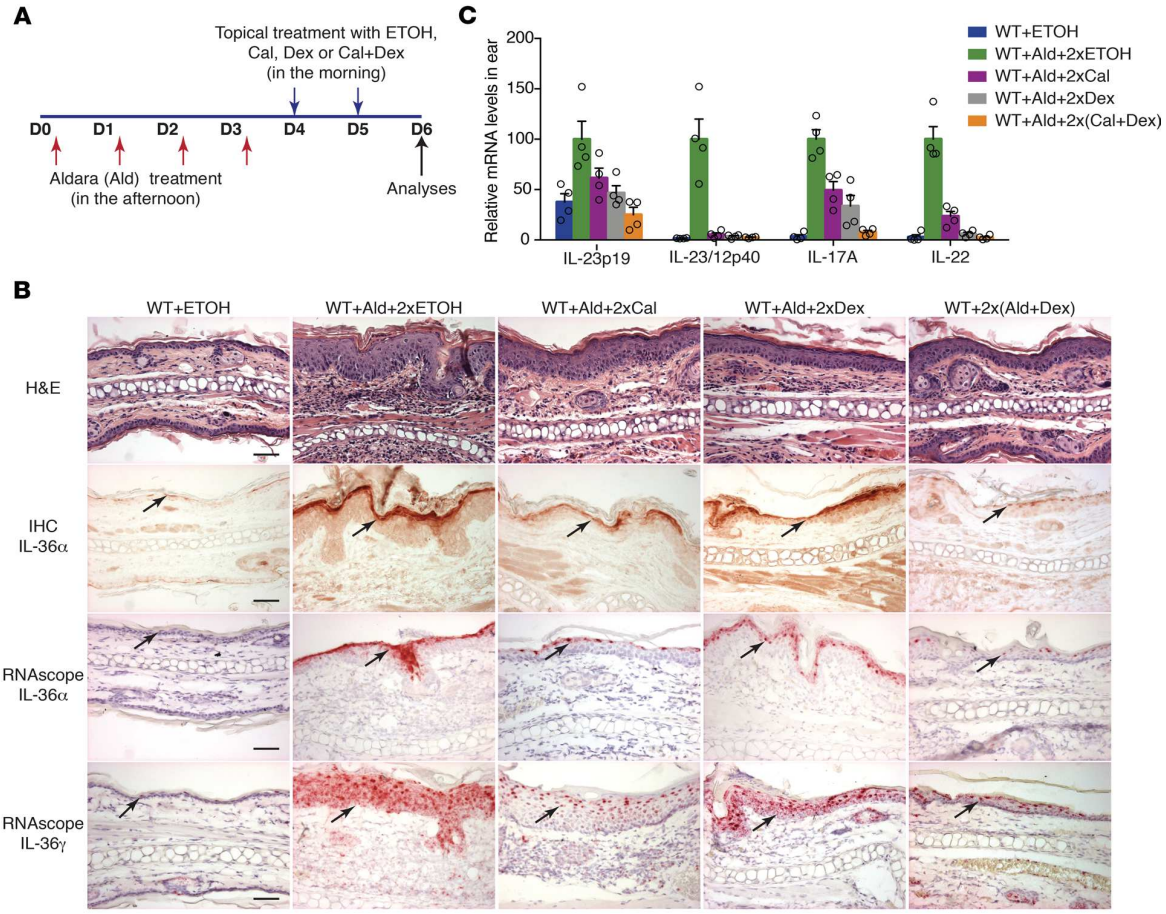

Figure 7. A synergy between calcipotriol and dexamethasone in repressing IL-36α/γ and IL-23/IL-17/IL-22 in the established psoriasis in mice. (**A**) Experimental protocol. WT Balb/c mouse ears were topically treated with Aldara (Ald) from D0 to D3 to induce psoriatic inflammation, followed by 2 times of treatment with ETOH, calcipotriol (Cal), dexamethasone (Dex), or Cal+Dex at D4 and D5. Ears were sampled for analyses at D6. (**B**) Skin sections were used for H&E staining, IHC staining with IL-36α antibody, and RNAscope in situ hybridization with probes for IL-36α or IL-36γ. Arrows point to positive signals. Scale bar: 50 μm. (**C**) qPCR analyses of the treated skin. Values are mean ± SEM. Data are representative of 3 independent experiments with similar results.

vitamin D3, 1α,25-dihydroxyvitamin D3 (calcitriol), also exerted a similar repression of Ald-induced IL-36 expression (Supplemental Figure 4, A–C). These results suggest that the repression of IL-36α/γ by Cal implicates a mechanism of ligand-dependent VDR-mediated transrepression.

Molecular mechanisms underlying the negative regulation of genes by the liganded-VDR are not well understood. Various modes of transrepression have been proposed via different types of negative vitamin D response elements (nVDRE) present in different genes, including putative DR3-type elements (54, 55). Interestingly, Jiang et al. recently identified a DR3 element as a nVDRE in a hIL-36γ promoter (53). To test whether DR3 elements could mediate the transrepression of hIL-36α, we used Find Individual Motif Occurrences (FIMO 4.10) (56) to predict VDREs (MA0693.1, JASPAR 2016) (57) in the promoter region. Within 673 bp upstream of transcription start site (i.e., 1085 bp upstream of translational start codon ATG), we identified 3 potential elements, DR3a, DR3b, and DR3c, after having filtered significant hits with $P < 0.001$ (Supplemental Figure 5A). Among these, DR3c showed the best fit ($P = 5 \times 10^{-5}$). Interestingly, by constructing firefly luciferase reporters for transient transfection to HaCaT cells, we observed that the deletion of DR3c (but not DR3a) in the hIL-36α promoter led to an abolishment of the repression by Cal (Supplemental Figure 5B), suggesting that DR3c functions as one nVDRE to mediate the negative regulation of IL-36α by liganded VDR. However, as it has been recognized that multiple response elements often exist not only in proximal, but also in distal, regions of VDR-regulated genes, our results do not exclude other nVDREs in more upstream regions of the hIL-36α promoter (Supplemental Figure 5C).

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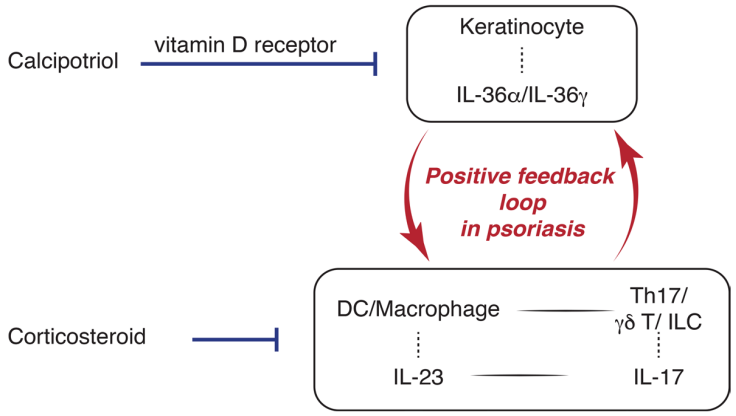

Figure 8. A schematic representation showing that the positive feedback loop of IL-36 and IL-23/IL-17 in psoriasis can be efficiently disrupted by calcipotriol and corticosteroid combination therapy for plaque psoriasis. Calcipotriol acts on keratinocytes to repress the expression of IL-36α/γ, an effect mediated through keratinocytic vitamin D receptor (VDR); on the other hand, corticosteroid acts on immune cells to suppress the IL-23/IL-17 axis (IL-23 produced by DC/macrophages and IL-17 produced by Th17 cells/γδ T cells/innate lymphoid cells [ILC]). By this means, the calcipotriol and corticosteroid combination disrupts efficiently the inflammatory loop, thus achieving a superior efficiency than either agent alone.

Moreover, other atypical nVDREs may exist and function (54, 55). Therefore, ChIP-seq analyses coupled with mutagenesis analyses will be necessary to fully identify the functional nVDREs in the promoters of IL-36α and IL-36γ, as well as to study in depth the regulatory mechanisms.

In contrast with Cal, the decrease of IL-36α/γ expression in mouse psoriasis upon the topical Dex treatment is not mediated by a direct repression. Recently, an interregulation of Th17 cytokines and the IL-36 cytokines has been proposed in psoriasis pathogenesis, which leads to full-blown clinical manifestation (9, 42, 58). Indeed, we showed that, upon Ald treatment, IL-36α/γ expression was induced in the immunodeficient NSG mice (in the absence of IL-17 and IL-22); however, the induction of IL-36α/γ expression was apparently higher in Ald-treated WT mice (Figure 5D). This suggests that IL-36α/γ expression in KCs is positively feedback regulated by cytokines (e.g., IL-17/IL-22) derived from immune cells. It is conceivable that an efficient disruption of the IL-36 and IL-23/IL-17 positive loop can be achieved through the interruption at the 2 critical nodes (i.e., IL-36 in KCs by Cal and IL-23/IL-17 in immune cells by corticosteroid), which thus explain the efficacy of the combined Cal/Dex treatment for psoriasis. It will be interesting to further explore to what extend the disruption of the IL-36/IL-23/IL-17 loop controls various inflammatory mediators identified in psoriasis pathologies, such as TNF-α and IL-6, both of which were observed to be synergistically reduced in Ald-induced psoriatic mice upon Cal/ Dex treatment (Supplemental Figure 6).

In conclusion, our present study has provided potentially novel insights into mechanisms underlying the therapeutic effects of Cal in the treatment of PP, and particularly, it has shown a mechanism underlying the superior therapeutic efficacy achieved by Cal with corticosteroid therapy, which has been recently proved in clinic (20, 21). Based on our findings, it is conceivable that interrupting the inflammatory loop at the IL-36 (KCs) and IL-23/IL-17 (immune cells) dual points should achieve a better effect than monotherapy for psoriasis. In this aspect, it will be highly interesting to test the combination of IL-36–targeting molecules (e.g., Cal) or biologicals (e.g., neutralizing antibodies against IL-36/IL-36R, which are currently under the development) with those therapeutics agents recently developed to target IL-23 or IL-17 pathways (59, 60). It is expected that such a combination will not only improve the efficiency and prevent the recurrences, but also permit the decreased dose of each treatment to reduce side effects, thereby offering potentially new therapeutic strategies for human psoriasis.

Methods

Mice. Mice were bred in IGBMC under specific pathogen-free conditions. Balb/c mice were from Charles River Laboratories, and NSG were from the Jackson Laboratory. *Tslp*$^{-/-}$ (28), *Vdr*$^{-/-}$, and *Vdr*$^{KC-/-}$ (K14-Cre$^{Tg/0}$/ *Vdr*$^{L2/L2}$) (29) mice were all generated by us as described and backcrossed to Balb/c genetic background. Female mice at the age of 8–12 weeks were used in the study.

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Mouse treatment. Ald (3M Pharamaceuticals; MEDA AB Pharma) is a commercially available cream that contains 5% IMQ. Mice received, on a daily basis (in the afternoon) and for 4 consecutive days, a dose of 30 mg Ald cream/ear (corresponding to 0.15 mg IMQ/ear). Cal (2 nmol; MilliporeSigma), 10 nmol of Dex (MilliporeSigma), or the combined solution, in 25 µl ETOH were topically applied (in the morning) as indicated in experimental scheme (Figure 1A and Figure 7A).

Ex vivo culture of mouse ear epidermis. Ears from adult mice were disinfected with 10% povidone-iodine solution and were then washed in distilled water and 70% ETOH. Ears were then separated with forceps and floated in a 4 mg/ml of Dispase (Thermo Fisher Scientific) in PBS in a sterile petri dish, with the dermis side down, and incubated overnight at 4°C. After the dermis was removed, epidermis was washed in PBS and then cultured by floating in a medium without carbonate prepared as described (61): DMEM (Invitrogen) was mixed with DMEM/F-12 1:1 (v/v) mixture (Invitrogen) and then adjusted to pH 7.2. The medium was supplemented with 10% FBS (DUTSCHER), cholera toxin (0.1 nM) (MilliporeSigma), mouse epidermal growth factor (10 ng/ml) (MilliporeSigma), 3,3',5'-triiodo-t-thyronine (2 nM) (MilliporeSigma), human apo-transferrin (5 µg/ml) (MilliporeSigma), human insulin (5 µg/ml) (MilliporeSigma), hydrocortisone (0.4 µg/ml) (MilliporeSigma), penicillin (60 µg/ml) (Invitrogen), and gentamicin (25 µg/ml) (KALYS). Cal (1 µM) or 10 µM Dex was added to the culture medium. After 24 hours of ex vivo culture, the epidermis was harvested for RNA extraction and qPCR analyses.

Human primary KC (HPKC) preparation and culture. HPKC were obtained from surgical samples of healthy breast skin. Skin samples were incubated overnight at 4°C in a trypsin solution (trypsin 0.25%, EDTA 0.1%). Epidermal sheets were then removed from the dermis, and cells were dissociated. HPKC were cultured in KC serum-free medium (SFM) supplemented with bovine pituitary extract (25 µg/ml) and recombinant epidermal growth factor (0.25 ng/ml; all from Invitrogen). HPKC were starved for 24 hours in KC SFM without the addition of growth factors before stimulation for 24 hours in the presence or absence of 20 ng/ml of TNF-α, IFN-γ, and/or IL-17 (all from R&D systems).

Human psoriasis patient study. Skin biopsy samples from patients with moderate to severe PP were obtained from the department of dermatology of Bordeaux Hospital. Patients included in this study did not receive topical or systemic treatments for the final 6 weeks. Patients were never treated with biologics. Patient no. 1 (35 years old; Psoriasis Area and Severity Index [PASI] 32.6; patient no. 2 (44 years old; PASI 6.6); patient no. 3 (21 years old; PASI 20.9); patient no. 4 (55 years old; PASI 37). One skin biopsy was performed on lesional skin before and following 4 days of topical treatment with Cal ointment once a day. Both biopsies were performed on the same psoriatic lesion.

Histopathology and IHC staining. Mouse ears were fixed for 24 hours at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) were stained with H&E or IHC staining.

For IHC staining of IL-36α, paraffin sections were treated with 0.6% H_2O_2 (in PBS) to block the endogenous peroxidase activity before antigen retrieving with citric buffer (10 mmol/l citric acid, pH 6). Slides were then blocked with 5% normal rabbit serum (Vector Laboratories) and incubated with goat polyclonal anti–mouse IL-36α antibody (R&D Systems, AF2297, dilution 1:100). Slides were then washed and incubated with HRP-rabbit anti–goat IgG (DAKO, P0449, dilution 1:100). Staining was then visualized with AEC+ high sensitivity substrate chromogen solution (Dako).

For IHC staining of IL-23p19, paraffin sections were treated with 0.6% H_2O_2 (in PBS) to block the endogenous peroxidase activity before antigen retrieving with citric buffer (10 mmol/l citric acid, pH 6). Slides were then blocked with 5% normal goat serum (Vector Laboratories) and incubated with rabbit polyclonal IL-23p19 (Abcam, ab45420, dilution 1:100). Slides were then washed and incubated with biotinylated goat anti–rabbit IgG and treatment of AB complex (Vector Laboratories). Staining was finally visualized with AEC+ high sensitivity substrate chromogen solution (Dako).

For immunofluorescence staining of NIMP-R14, 10-µm cryosections were fixed in 4% paraformaldehyde, permeabilized with acetone, and blocked with 5% normal goat serum (Vector Laboratories). Slides were then incubated with primary antibody (rat monoclonal anti–NIMP-R14 [anti-neutrophil antibody; Abcam, ab2557, dilution 1:750]). After washing, sections were incubated with CY3-conjugated goat anti–rat IgG antibody (Jackson ImmunoResearch, 112-165-167, dilution 1:400) and mounted with Vectashield medium (Vector Laboratories) containing 4'-6-diamidino-2-phenylindole dinhydrochloride (for nuclear staining; Invitrogen).

RNAscope in situ hybridization. Mouse ears were fixed in 10% of neutral buffered formalin and embedded in paraffin. Freshly prepared 5 µm sections were used for RNA in situ hybridization, following the manufacture protocol for RNAscope Red detection Kit (Advanced Cell Diagnostics). To confirm mRNA

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integrity in the paraffin sections, Mm-Ppib probe (Mus musculus peptidylprolyl isomerase B) was used as a positive control (62). Mm-dapB probe (Bacterial Bacillus subtilis dihydrodipicolinate reductase) was used as negative control. RNAscope probes used for detection of IL-36α and IL-36γ were MmIL-36α (catalog 403651) and MmIL-36γ (catalog 425241).

RNA extraction and qPCR. Total RNA was extracted from mouse ears or human skin biopsies with Trizol reagent (Invitrogen) according to standard protocol. Total RNA from human primary KC cultures was extracted using Nucleospin RNA kit (Macherey-Nagel), according to manufacturer's instructions. Reverse transcription was performed using random oligonucleotide hexamers and amplified by qPCR with a Lightcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) as internal control. The sequences of primers are listed in the Supplemental Table 1. For the analyses of each set of gene expression, an arbitrary unit of 100 was given to the samples with the highest level, and the remaining samples were plotted relative to this value.

Statistics. Data were analyzed using Sigmaplot (Systat Software Inc.) or GraphPad Prism by 2-tailed Student's *t* test or by the Mann-Whitney rank sum test, depending on results from the Kolmogorov-Smirnov test (with Lilliefors correction) for normality and the Levene Median test for equal variance. Data are presented as means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ was considered to be statistically significant.

Study approval. All mouse experiments were performed in accordance with the animal care and ethic committee of IGBMC and Institut Clinique de la Souris (ICS). All studies involving human tissues were approved by the local institutional ethics committee (Hôpital Saint-André Service de Dermatologie). All patients gave their written informed consent.

Author contributions

BG, RW, and ML designed research studies; BG, RW, and PH performed experiments; CM, JS, and KB designed and performed studies on human psoriasis patients and human primary KC cultures; TY performed bioinformatic analyses; CG and JY designed and constructed plasmids for studying promoter activity; BG, RW, and ML analyzed and interpreted data, as well as wrote the manuscript. ML directed the study and supervised the work.

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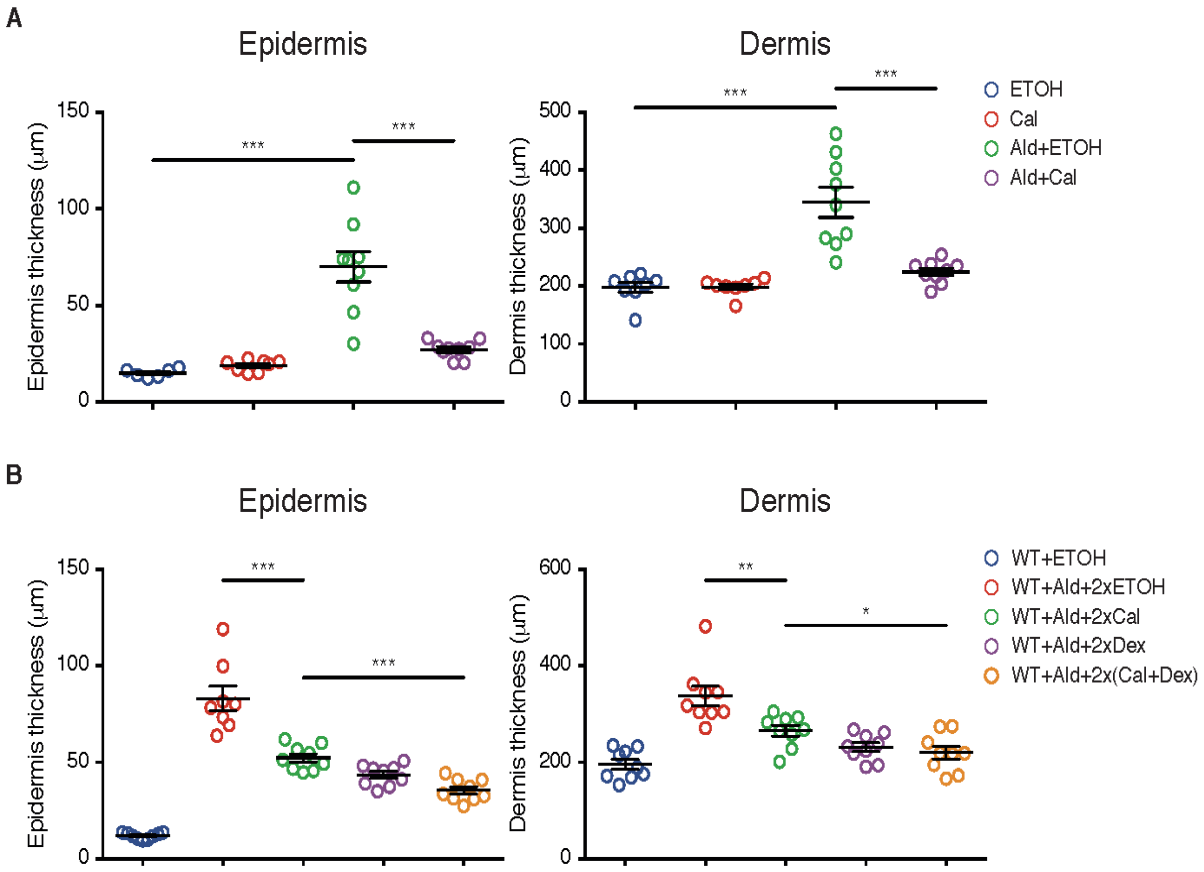
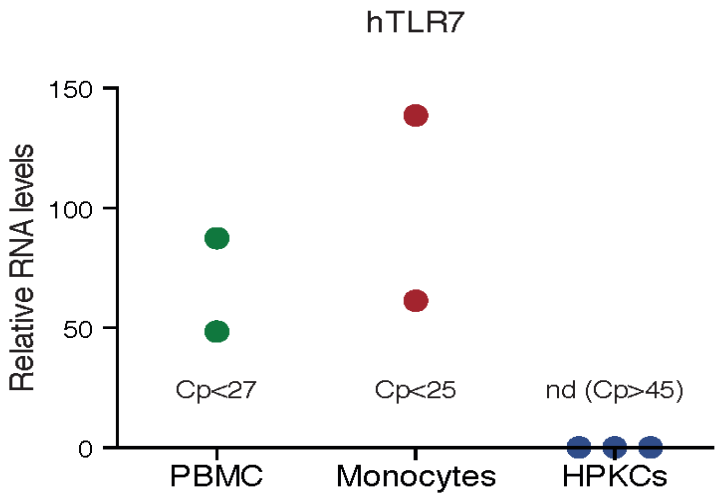
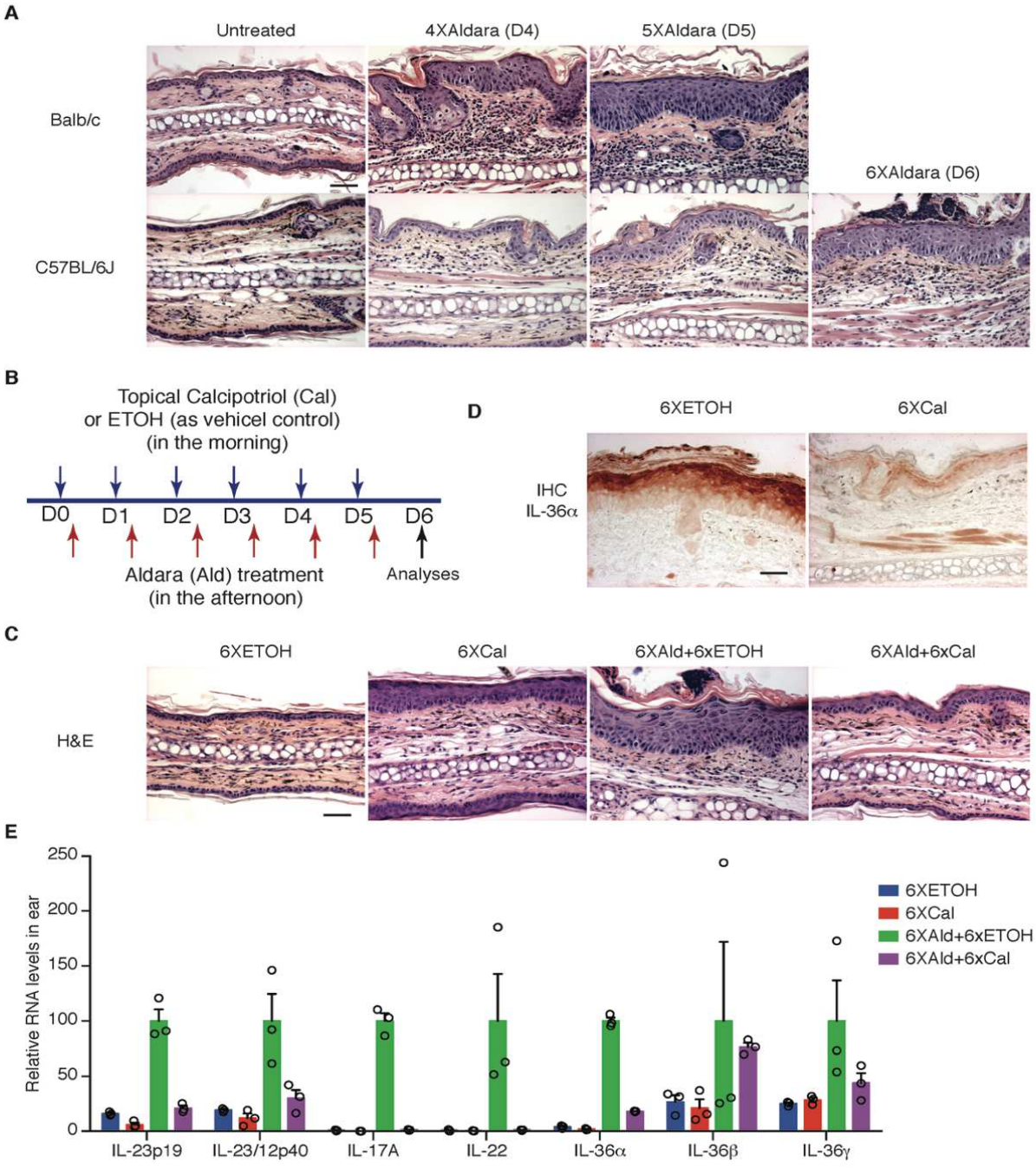


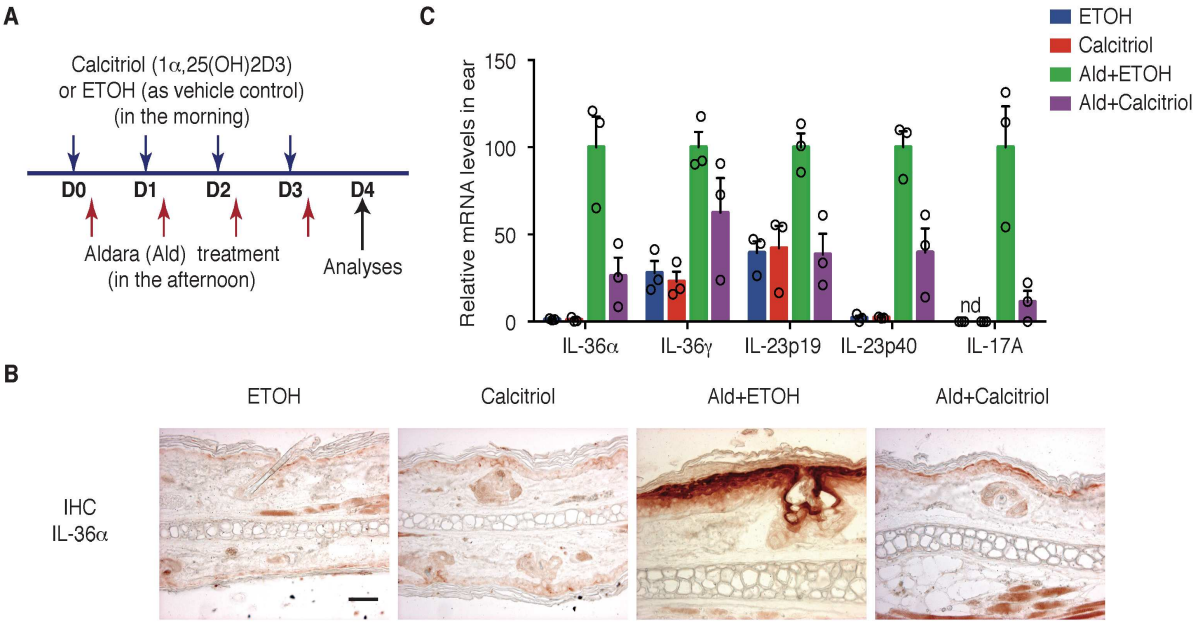
Supplemental Figure 1. Epidermal and dermal thicknesses of the mouse ears with topical treatments. H&E stained slides were digitally scanned using the 20x (0.46 µm/pixe) mode of the Hamamatsu NDP slide scanner (Hamamatsu Photonics, Nanozoomer 2.0HT). Three measurements of the thickness of the epidermis and the dermis were taken from the representative section of each ear using a digital ruler on the virtual slides using the NDP.viewer2 software. (**A**) Wildtype Balb/c mouse ears were topically treated with ETOH (vehicle control), calcipotriol (Cal), Aldara (Ald)+ETOH or Ald+Cal every day from day (D) 0 to D3, as shown in Fig. 1A. (**B**) Wildtype Balb/c mouse ears were topically treated with Aldara (Ald) from D0 to D3 to induce psoriatic inflammation, followed by two times of treatment with ETOH, calcipotriol (Cal), dexamethasone (Dex) or Cal+Dex; as shown in Fig. 7A. Values are mean ± SEM. *P<0.05; **P<0.01; ***P<0.001 (2-tailed student's *t* test).



Supplemental Figure 2. The expression of TLR7 is not detected in human primary keratinocytes (HPKCs). Comparison of RT-qPCR analyses of hTLR7 expression in HPKCs from three independent healty donors and in isolated peripheral blood mononuclear cells (PBMC) and monocytes from two independent healty donors. Primers used to amplify hTLR7 (amplicon length 155bp) were: 5'-TTGGCACCTCTCATGCTCTG-3' (forward) and 5'-ACCATCTAGCCCCAAGGAGT-3' (reverse). Results showed that the expression of TLR7 was not detectable in HPKCs (with amplification cross point Cp>45), while it was well detected in PBMC and monocytes (Cp<27 and 25, respectively).

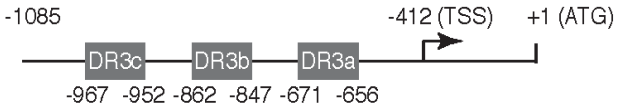


Supplemental Figure 3. Topical calcipotriol inhibits the psoriatic inflammation and the expression of IL-36α/γ expression in Aldara-treated wildtype C57BL/6J (B6) mouse skin. (**A**) Histological analyses of ears sections from wildtype Balb/c and B6 mice upon Aldara treatment (once per day from D0, and analyzed at D4, D5 or D6). Results showed that B6 mice at D6 exhibited comparable inflammation as Balb/c mice at D4. (**B**) Experimental protocol. Wildtype B6 mouse ears were topically treated with ETOH (vehicle control), calcipotriol (Cal), Aldara (Ald)+ETOH or Ald+Cal every day from day (D) 0 to D5, with ETOH or Cal treatment in the morning, and Ald treatment in the afternoon. Mouse ears were analyzed at D6. (**C**) Hematoxylin/eosin (H&E) staining of ear sections. (**D**) IHC staining with IL-36α antibody on ear sections. Bar = 50 μm for all pictures. (**E**) Quantitative RT-PCR analyses (RT-qPCR), showing that calcipotriol treatment inhibited the expression of IL-23, IL-17A, IL-22, as well as IL-36α and IL-36γ, in Aldara-treated B6 mouse ears. Data are representative of two independent experiments with similar results.



Supplemental Figure 4. Topical calcitriol (1α,25(OH)2D3, active vitamin D3) inhibits the psoriatic inflammation and IL-36/IL-23/IL-17 in Aldara-treated skin. (**A**) Experimental protocol. Wildtype Balb/c mouse ears were topically treated with ETOH (vehicle control), Calcitriol (2 nmol), Aldara (Ald)+ETOH or Ald+calcitriol every day from day (D) 0 to D3, with ETOH or calcitriol treatment in the morning, and Ald treatment in the afternoon. Mouse ears were analyzed at D4. (**B**) Immunohistochemical (IHC) staining with IL-36α antibody (in dark red) of ear sections, showing that Calcitriol treatment reduced IL-36α in Ald-treated ear skin. Arrows point to one of positive signals. Bar = 50 μm for all pictures. (**C**) Quantitative RT-PCR analyses (RT-qPCR). Relative RNA levels were calculated using HPRT as internal control. nd, not detected.

A



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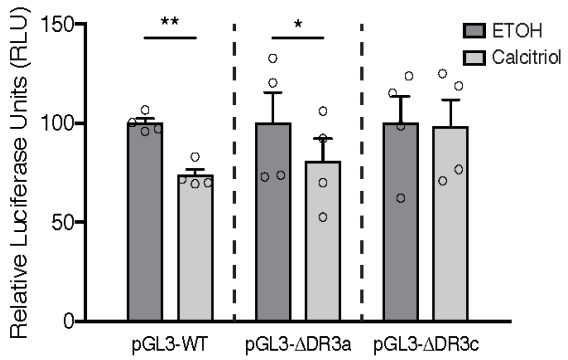


C

DR3 elements	p-value	Sequence	Start	End	Strand
DR3a	0.000225	GACTTCTTCCGGGTCA	-656	-671	-
DR3b	0.000726	AGGGTCACTGGAGTGA	-862	-847	+
DR3c	5.03e-05	GAGTCCATTGAGGTGG	-967	-952	+
DR3d	0.000513	GGGGGCATGGATGGCA	-1169	-1184	-
DR3e	1.28e-05	GGGTTCTGCAGGGTCA	-2152	-2137	+
DR3f	0.000716	CAGTGCTCTGAGCTCG	-2350	-2365	-
DR3g	0.000742	AGGTACTGCGAGCTCA	-2373	-2358	+
DR3h	0.000623	GAGCTCATTCAGGGGA	-2674	-2659	+
DR3i	0.000997	AAGGAGACAGGGTGTA	-3106	-3091	+
DR3j	9.55e-05	CAGTGCACAGGGTTGG	-4209	-4224	-
DR3k	0.000281	TGGTTCTTAGAGGTTG	-4284	-4269	+

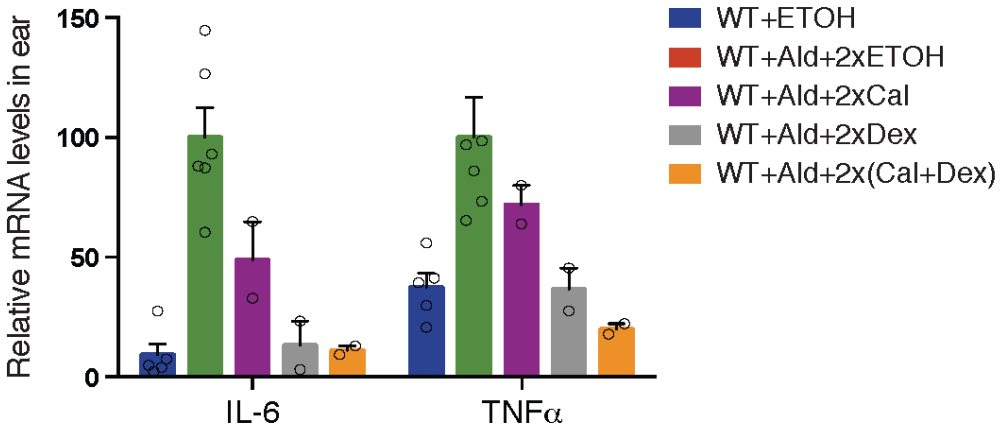
Supplemental Figure 5.

(**A**) Schematic showing of the 1085 bp fragment upstream of ATG (translational starting codon, set as +1) of hIL-36α gene, containing DR3 elements a, b and c. TSS, transcription start site.

(**B**) The 1085 bp fragment upstream of ATG of hIL-36α gene, in its wildtype (WT) form, or the form with the deletion of DR3a or DR3c element, was cloned into the pGL3-Basic vector (Promega) using In-Fusion HD cloning kit (Clontech), to obtain pGL3-WT, pGL3-ΔDR3a, or pGL3-ΔDR3b luciferase reporter constructs (see cloning methods described below). These constructs were transiently transfected into the HaCaT cells using PolyJet (SignaGen Laboratoires). A renilla luciferase plasmid (pRL-CMV) was co-transfected to control for transfection efficiency. Following transfections, the cells were treated with 10-5 M calcipotriol, or ETOH (vehicle control). Cells were lysed 24 h later and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Data are expressed as Relative Units of Firefly Luciferase normalized by the Renilla Luciferase (RLU). Values are mean ± SEM. *P<0.05; **P<0.01 (one-tailed student's t test).

(**C**) DR3 elements within a 5kb promoter region of hIL-36α gene. Red highlights the DR3 elements with the p-value less than 10^{-4}.

Methods for plasmid cloning: the 1085 bp wild type (WT) fragment upstream of ATG of hIL-36α gene was amplified with CloneAmp HiFi PCR premix (In-Fusion HD cloning kit), using HaCaT cell genomic DNA as template with the primer pair, 5'-CGAGATCTGCGATCTCACCCTCATAGACTTACCCCA-3', and 5'- CGGAATGCCAAGCTTTGTGGTGGTGTTGTTTCAGA-3'. The luciferase reporter vector pGL3-basic (Promega) was amplified using the primer pair (5'-AAGCTTGGCATTCCGGTACT-3' and 5'- AGATCGCAGATCTCGAGCC-3'). The two PCR products were used for in-fusion cloning to obtain pGL3-WT. The constructs pGL3-ΔDR3a and pGL3-ΔDR3c were obtained by amplifying the pGL3-WT with the following primer pairs flanking the DR3a or DR3c region and re-ligate each PCR product by In-Fusion cloning (Clontech). Primer pairs for DR3a deletion were: 5'- GTCATCCGTAATGTTTCTCAACTCAGTATCTG-3' and 5'- AACATTACGGATGACTCATCTCTGATGCCACAA-3'. Primer pairs for DR3c deletion: 5'- TAGTAGATCACTATCTCTAGTATAGGTAGCGTGAT-3' and 5'- GATAGTGATCTACTAGAGTGCTCAGGAGCTGGCT-3'.



Supplemental Figure 6. A synergy between calcipotriol and dexamethasone in inhibiting IL-6 and TNFa expression in the established psoriasis in mice. Wild-type Balb/c mouse ears were topically treated with Aldara (Ald) from D0 to D3 to induce psoriatic inflammation, followed by two times of treatment with ETOH, calcipotriol (Cal), dexamethasone (Dex) or Cal+Dex at D4 and D5, as shown in Fig. 7A. RT-qPCR analyses of the treated skin for the RNA levels of IL-6 and TNFα. Data are representative of two independent experiments with similar results.

Supplementary Table 1. Sequences of the primers used for quantitative RT- PCR analysis.

Gene name	Sequence (5' to 3')
HPRT (164 bp)	F TGGATACAGGCCAGACTTTG
	R GATTCAACTTGCGCTCATCTTA
mIL17A (239 bp)	F CCAGGGAGAGCTTCATCTGT
	R ACGTGGAACGGTTGAGGTAG
mIL22 (166 bp)	F CCGAGGAGTCAGTGCT AAGG
	R GCTGATGTGACAGGAGCTGA
mIL23p19 (213 bp)	F AATAATGTGCCCCGTATCCA
	R CTGGAGGAGTTGGCTGAGTC
mIL-23/12p40 (176 bp)	F CCTGAAGTGTGAAGCACCAA
	R AGTCCCTTTGGTCCAGTGTG
mIL36α (187bp)	F CCACGTACATGGGAGTGCAA
	R GGGAAGGCTGCAGACTCAAA
mIL36β (185bp)	F AGATGGTATGGGTCCTGACTGG
	R GCCCTCCATCTCAACACAGC
mIL36γ (200bp)	F GCAGGTGTGGATCTTTCGTAATCA
	R GCAGCAAAGTAGGGTGTCCA
mS100A7A (167 bp)	F CTTGTCCCTGGAGGAGTTGA
	R GCTTGCCCAAGATGTACAGG
mS100A8 (178 bp)	F GGAAATCACCATGCCCTCTA
	R GAGATGCCACACCCACTTTT
mTSLP (194 bp)	F AGCTTGTCTCCTGAAAATCGAG
	R AGGTTTGATTCAGGCAGATGTT
mCyp24 (187bp)	F CCAGCGGCTAGAGATCAAAC
	R CCCCATAAAATCAGCCAAGA
hGAPDH (158bp)	F GTCAAGGCTGAGAACGGGAA
	R AAATGAGCCCCAGCCTTCTC
hIL36α (225bp)	F TGGGGTCGGTCTGCACATAA
	R GGGTCTCCACATGTCGGCAT
hIL36β (185bp)	F CGACAGATGGTGTGGGTCCT
	R TCCCTTGATTCCCAGGTAAACCA
hIL36γ (200bp)	F GAGCAAGGCAGAGGGGATCC
	R GCCACAGACTCAAGGGTGGA
hCyp24 (249bp)	F GGCAACAGTTCTGGGTGAAT
	R TATTTGCGGACAATCCAACA
hTSLP (184bp)	F CCAGGCTATTCGGAAACTCA
	R TGGTGCTGTGAAATATGACCA

- PERSPECTIVES

As discussed in the paper, this study provides novel insights into the mechanisms underlying the therapeutic effect of Cal in the treatment of psoriasis. Furthermore, it gives us the bases to explore other aspects including IL-36 regulation by Cal and IL-36 signaling pathways implicated in skin inflammation presented below:

1) **Cal+Betamethasone (Daivobet ®) treatment represses IL-36α production at protein level in human psoriatic patients.**

In our paper using HPKCs we demonstrated that Cal represses IL-36α and IL-36γ expression (**Figure 6C**). Additionally, we reported that at RNA level, IL-36α and IL-36γ were diminished in psoriatic patients after 4 doses of topical treatment with Calcipotriol oinment (Daivonex ®) once a day (**Figure 4**) and this was associated with the decrease of IL-23/IL-17A/IL-22 expression (**Figure 4**). I further performed IHC using an anti-human IL-36α antibody (R&D, Catalog # AF1078) in paraffin sections from human psoriatic patient skin samples. Results showed that the skin of psoriatic patients presented higher expression of IL-36α in suprabasal layer than healthy donor, similar to our mouse data (human data presented in **Supplemental Figure 7** compared with mouse data presented in **Figure 3D**). After 4 doses of Cal+betamethasone (Daivobet ®) treatment, IL-36α signal in the biopsy from the same patient was not detected (**Supplemental Figure 7)**, validating that Cal in combination with corticoids suppress IL-36α protein production in epidermal KCs in human psoriasis patient.

2) **How is the expression of IL-36α/γ repressed by Cal?**

In our study we have shown that the suppressive effect of Cal in the expression of IL-36α and IL-36γ could be mediated by ligand-dependent transcriptional repression. The molecular mechanisms underlying the negative regulation of genes by the liganded-VDR are not well understood. However, Jiang et al, recently, by using JASPAR database identified five possible VDR binding sites (VDRE) in the hIL-36γ promoter region, from which only one demonstrated to be a functional binding site of VDR with repressive effects in hIL-36γ regulation by chromatin immunoprecipitation and luciferase assays in

neonatal human epidermal keratinocytes (NHEKs) (11). In our paper, we identified 3 potential VDREs in hIL-36α promoter region using the Find Individual Motif Occurrences (FIMO 4.10). By constructing firefly luciferase reporters for transient transfection to human HaCaT cells, we showed that the deletion of one of the three potential elements identified (DR3c) in the hIL-36α promoter lead to the abolishment of Cal repression. It has been known that, not only DR3 elements, but also other atypical elements, could work as VDREs in either proximal or distal promoter regions of VDR-regulated genes (12), (13), (14), (15). Our results do not exclude other VDREs in more upstream or downstream regions of the hIL-36α promoter region. It will be interesting to continue the study of the transcriptional repression of IL-36α and IL-36γ by Vit D_3 using ChiP-Seq in human keratinocyte HaCaT cell line. For that propose, IL-36α and IL-36γ expression would be induced by a cytokine cocktail stimulation including TNF-α, IFN-γ and IL-17A followed by Cal treatment to induce the repression of IL-36α and IL-36γ expression. This system will allow as to identify the functional VDREs in the promoters of hIL-36α and hIL-36γ, following by functional studies by using luciferase report assays or by deleting the VDREs through Crispr/Cas9 strategy.

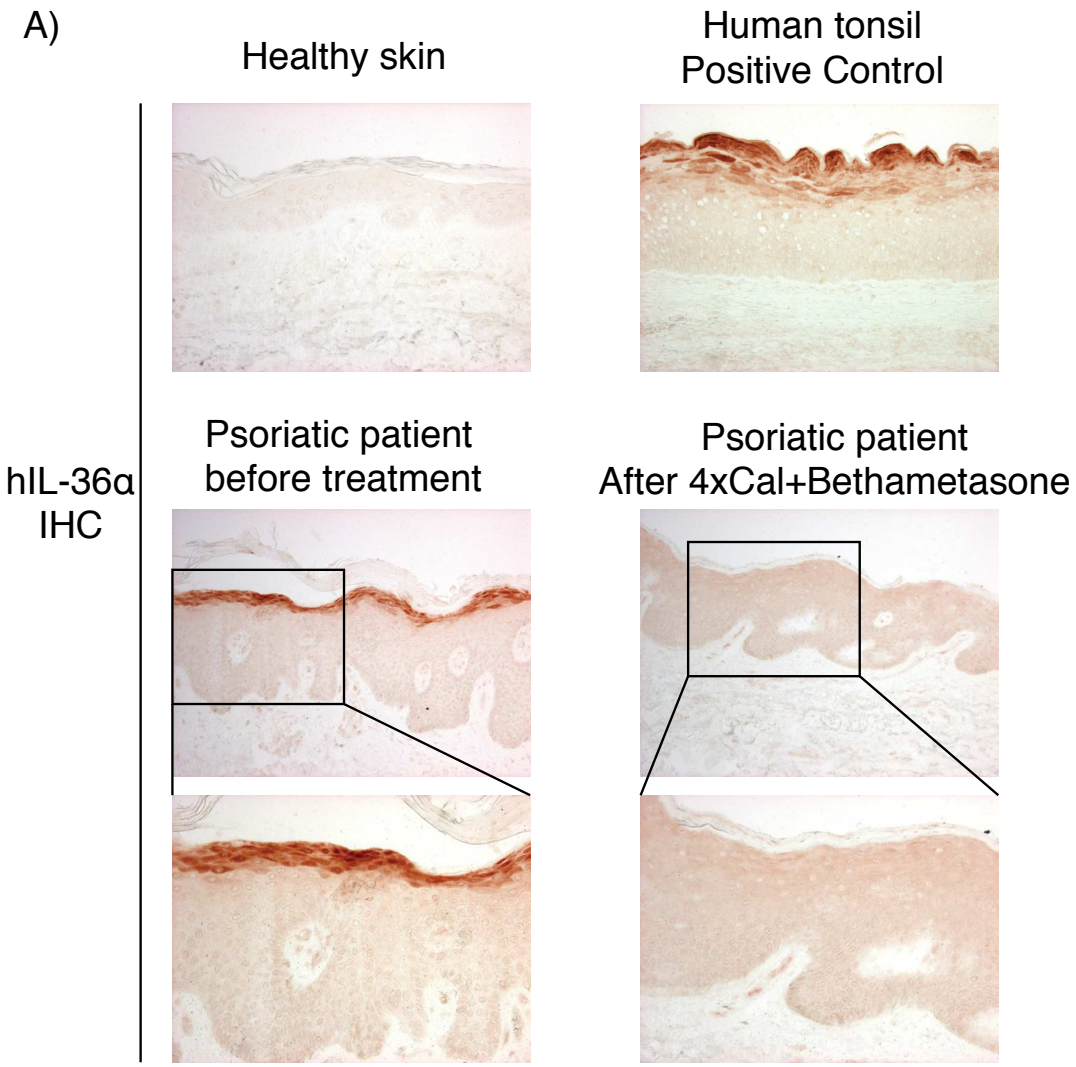
3) Does retinoic acid (RA) regulate IL-36 expression in psoriatic patients?

Another topic to be explored is the effect of RA in IL-36 regulation because RA has been also used as topical treatment in psoriasis. Using ex vivo cultures of mouse epidermis treated with different doses of all-trans RA (atRA), I obtained preliminary data showing that IL-36α expression was reduced in a dose dependent manner. qPCR analyses showed that the expression level of IL-36α was abolished upon atRA 10^{-4}M treatment and atRA effect was weaker at 10^{-5}M or 10^{-6}M. Nevertheless, the effect of atRA in IL-36γ regulation was less clear and the treatment with atRA showed the reduction of IL-36γ expression only at 10^{-4}M but not with the other two concentrations (**Supplemental Figure 8A**). In addition, using in vitro cultures of human keratinocyte HaCaT cell line stimulated with a cytokine cocktail (TNF-α, IFN-γ and IL-17A), I observed that IL-36α induction was diminished in a dose dependent manner by atRA, however this was not the case for IL-36γ **(Supplemental Figure 8B**). Considering these results, it would be interesting to further validate the specific effect of atRA in IL-36α regulation and continue with the exploration of the transcriptional regulation of IL-36 cytokines by atRA

using the Ald mouse model, using in vitro HPKCs and human psoriatic samples before and after atRA treatment.

4) **What is the role of IL-36α and IL-36γ in skin inflammation?**

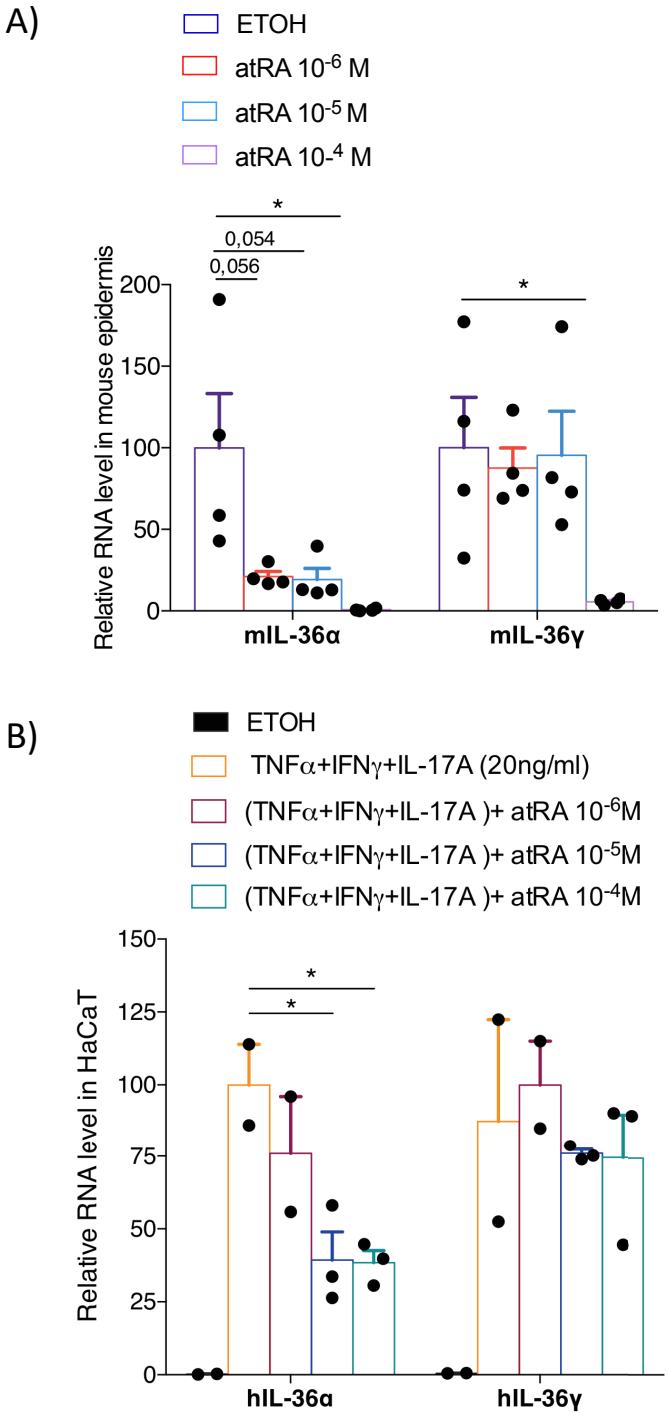
In our paper we showed that IL-36α and IL-36γ (but not IL-36β) are closely associated in mouse psoriatic inflammation and others have reported that IL-36α and IL-36γ regulate each other (10), (16), (17), (18). However, little was known regarding the functional redundancy and specificity of IL-36 cytokines in skin inflammatory responses. For that reason, we decided to explore this aspect and in the next session of my thesis I present and discuss about the results obtained during the last year of my thesis concerning this topic.



Supplemental Figure7. Cal+Dex (DAIBOVET ®) represses hIL-36α/γ production at protein level in human psoriatic patients.

A. Skin biopsies were taken from health donors (HS), or from lesional skin of plaque psoriatic patients (PP) before and after 4-days topical treatment with Calcipotriol (Cal) +Betamethasone (DAIVOBET ®) once a day. Human skin samples were fixed for 24 fours at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5**µ**m) were stained for hIL-36*α*. Human tonsil was used as positive control. Following the antibody data sheep indications, the results showed the hIL-36*α* positive staining is presented in the epidermal region of the human tonsil section.

For hIL-36*α* IHC staining, paraffin sections were treated with 0.6% H2O2 (in PBS) to block the endogenous peroxidase activity before antigen retrieving with citric buffer (10 mmol/l citric acid, pH 6). Slides were then blocked with 5% normal rabbit serum (Vector Laboratories) and incubated with Goat-anti human IL36α-antibody (R&D, AF1078, dilution 1:1000). Slides were then washed and incubated with HRP-rabbit anti-goat IgG (DAKO, P0449, dilution 1:100). Staining was than visualized with AEC+ high sensitivity substrate chromogen solution (DAKO).



Supplemental Figure 8: all-trans retinoic acid (atRA) represses IL-36α/γ expression in mouse epidermis and human keratinocytic HaCaT cells.

A. atRA at different doses (10-6M, 10-5M and 10-4M) represses IL-36α/γ expression in ex vivo cultures mouse epidermis after 24h of culture. quantitative RT-PCR analyses of cultured epidermis from WT C57BL/6N mice. Values are mean ± SEM (n=4). *<p 0.05 (two-tailed Student's t test).

B. atRA represses IL-36α expression induced with TNF-α, IFN-γ and IL-17 (20ng/ml for each cytokine, all from Peprotech) in HaCaT cells after 24h of culture. Data show the quantitative RT-PCR analyses of cultured epidermis from WT C57BL/6N mice. Values are mean ± SEM (n≥2). *p<0.05 (two-tails Student's t test).

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RESULTS
PART 2

PART 2

Study of the role of IL-36α and IL-36γ in psoriasis inflammation

INTRODUCTION

Recently it was reported that IL-36/IL-36R signaling pathway plays an important role in the pathogenesis of psoriasis in human patients and psoriatic mouse models (1), (2), (3), (4). The initial support for the role of IL-36/IL-36R signaling in driving epithelial inflammation arose from the demonstration that transgenic mice overexpressing IL-36α in KCs exhibit lesions with features common to human psoriasis inflammation (5). Moreover, using the Ald mouse model to trigger the development of psoriasis-like skin phenotype, Tortola et al., showed that *Il36r*$^{-/-}$ mice were protected from Ald-induced psoriasiform-like skin inflammation (6). More recently, human and mouse monoclonal antibodies targeting IL-36R signaling were developed. It has been shown that the human IL-36R antibody (MAB92) inhibits the IL-36R-mediated signaling and NFκB and IL-8 cytokines production in HPKCs (7). Additionally, Ganesan et al, developed a mouse IL-36R antibody (MAB04). Using the Ald model and IL-36α/β/γ intradermal (i.d.) injection model, they demonstrated that the intraperitoneal (i.p) administration of MAB04 reduces the skin inflammation and IL-17 cytokine production. In addition, Mahil et al., reported that the intraperitoneal (i.p) administration of IL-36R (M616, Amgen) mouse antibody in Ald-treated mice reduced IL-23/IL-17 and IL-22 cytokine expression accompanied by the reduction of neutrophils and dermal γδT cells infiltration (2).

To our interest, IL-36α and IL-36γ (but not IL-36β) are closely associated and increased in human lesional areas of psoriatic skin patients (8), (9), (10) and both of them are induced in Ald-driven mouse psoriasis in KCs (5), (11), (12), (13), (10). We showed a beneficial role of Cal topical treatment associated with the decrease of IL-36α and IL-36γ (but not IL-36β) in the Ald mouse model, as well as in human psoriatic skin (10). However, whether IL-36α and IL-36γ play a redundant or specific role in psoriatic development remains unclear. Indeed, even though IL-36 cytokines bind with the same heterodimeric receptor complex composed by IL-36R and IL-1R accessory protein (IL-1RAcP) subunits, it has been reported that compared to IL-36γ, IL-36α presented stronger

binding affinity to the receptor (14), suggesting that they could have different biological functions regulating the immune response.

To explore the specific and redundant function of IL-36α and IL-36γ in psoriasis pathogenesis, it is necessary to study with not only IL-36α KO or IL-36γ KO mice (the generation of conditional IL-36α KO mice is under the way in my lab) but also IL-36α/γ double knockout (DKO) mice. However, the IL-36α/γ DKO cannot be obtained by classical crossing of IL-36α KO with IL-36γ KO, because their coding genes *Il1f6* and *Il1f9* are present in the same chromosome separated only by 21kb. Therefore, in collaboration with ICS we used the CRISPR-Cas9 approach to delete both genes (15). We obtained 2 founders in C57BL/6N background (founder number 17 and founder number 18). All the mice used in the study were derived from F2 after crossing the founders with C57BL/6N mice.

First of all, I validated the ablation of IL-36α and IL-36γ in these mouse lines derived from founders' number 17 and 18. The line derived from the founder 17 was used to study in more detail the role of IL-36α/γ in psoriasis-like skin inflammation using Ald model. Further, I explored the specific or redundant function of IL-36α and IL-36γ in the inflammatory response developed in vivo upon the intradermal (i.d.) injection of IL-36α or IL-36γ in DKO mice. Finally, I studied the specific or redundant function of IL-36α and IL-36γ directly in epidermis of DKO mice using ex vivo cultures in presence of IL-36α or IL-36γ.

MATERIAL AND METHODS

Mice

Mice were bred in Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) under specific pathogen-free conditions, where experiments were performed in accordance with the animal care and ethic committee of IGBMC and Institut Clinique de la Souris (ICS). C57BL/6N and Balb/c mice were from Charles River Laboratories. IL-36α/γ DKO (*Il1f6/9* $^{-/-}$*) mice* were generated by the lab using CRISPR-Cas9 strategy in C57BL/6N or Balb/c genetic background. Female mice at the age of 8-12 weeks old were used in the study.

For wild-type (WT) genotyping, the primers for PCR were F1: 3'CAAGGAAAGAATGAGATCCAGGTGG5' and R2:3'AGCATGGAAGACATCCAGTTCCTCC 5'. For IL-36α/γ DKO the PCR primers were F1:3'CAAGGAAAGAATGAGATCCAGGTGG5' and R7:3'ATAGTATAGTTGCTGCTCAAGTATG5' (**Figure 1A**). The thermocycler parameters were: 35 cycles of 30 seconds for denaturation at 94°C, 30 second of hybridization at 65°C and 60 seconds of elongation at 72°C. PCR products were analyzed by 2% agarose gels (m/v) diluted in 400mL of TAE buffer (Tris-Acetate-EDTA) with 10 µg of ethidium bromide.

Mouse treatment

-Aldara treatment

Aldara (Ald, 3M Pharamaceuticals; MEDA AB Pharma, Paris, France) is a commercially available cream that contains 5% imiquimod (IMQ). Mice received on a daily basis (in the afternoon) and for several consecutive days (indicated in each specific experimental protocol) a dose of 30 mg Ald cream/ear (corresponding to 0.15 mg IMQ/ear).

-IL36α/γ intradermal injections

10µl of recombinant IL-36α (Biolegend, #555902) or IL-36γ (Biolegend, #552802) diluted in PBS (0,1 µg/µl) were injected with an insulin syringe with 30G needle (BD #320843) intradermally (i.d.) in mouse ears during 5 consecutives days. Control mice were injected with PBS.

Ex vivo culture of mouse ear epidermis

Ears from adult mice were disinfected with 10% povidone-iodine solution, and then washed in distilled water and 70% ethanol. Ears were then separated with forceps and floated in a 4mg/ml of Dispase (Gibco) in PBS in a sterile petri dish, with the dermis side down, and incubated overnight at 4ºC. After the dermis was removed, epidermis was washed in PBS and then cultured by floating in a medium without carbonate prepared as described previously by Mazzalupo et al., (16): Dulbecco's minimal Eagle's medium (Life Technologies) was mixed with Dulbecco's minimal Eagle's medium/F-12 1:1(v/v) mixture (Life Technologies) and then adjusted to pH 7.2. The medium was supplemented with 10% fetal bovine serum (DUTSCHER), cholera toxin (0.1 nM) (SIGMA), mouse epidermal growth factor (10 ng/ml) (SIGMA), 3,3',5'triiodo-t-thyronine (2 nM) (SIGMA), human apo-transferrin (5 µg/ml) (SIGMA), human insulin (5 µg/ml) (SIGMA), hydrocortisone (0.4 µg/ml) (SIGMA), penicillin (60 µg/ml) (Life technologies), and gentamicin (25 µg/ml) (KALYS). 100 ng/ml of IL-36α (Biolegend, #555902) or IL36γ (Biolegend #552802) were added to the culture medium. After 24 hours of *ex vivo* culture, the epidermis was harvested for RNA extraction and RT-qPCR analyses.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from mouse ears or LNs with Trizol reagent (Invitrogen) according to standard protocol. Reverse-transcription was performed using random oligonucleotide hexamers and amplified by quantitative PCR with a Lightcycler 480 (Roche Diagnostics) and the QuantiTect SYBR Green kit (Qiagen), according to the manufacturer's instructions. Relative RNA levels were calculated using hypoxanthine phosphoribosyltransferase (HPRT) as internal control. The sequences of primers were listed in the below **Table 1**. For the analyses of each set of gene expression, an arbitrary unit of 100 was given to the samples with the highest level, and the remaining samples were plotted relative to this value.

Table 1: Primer sequences

Gene name	(13)Sequence (5' to 3')	
mHPRT (164 bp)	F	TGGATACAGGCCAGACTTTG
	R	GATTCAACTTGCGCTCATCTTA
mIL-17A (239 bp)	F	CCAGGGAGAGCTTCATCTGT

	R	ACGTGGAACGGTTGAGGTAG
mIL-17C (122 bp)	F	TGCTGGAAGCTGACACTCAC
	R	CGTTGATGCATCCACGACAC
mIL-17E	F	ACACCCACCACGCAGAATC
	R	CATGTGGGAGCCTGTCTGTA
mIL-17F (193 bp)	F	TGCCCTGGTTTTGGTTGAAG
	R	GAAGTGCACCCGTGAAACAG
mIL-22 (166 bp)	F	CCGAGGAGTCAGTGCT AAGG
	R	GCTGATGTGACAGGAGCTGA
mIL-23p19 (213 bp)	F	AATAATGTGCCCCGTATCCA
	R	CTGGAGGAGTTGGCTGAGTC
mIL-23/12p40 (176 bp)	F	CCTGAAGTGTGAAGCACCAA
	R	AGTCCCTTTGGTCCAGTGTG
mIL-36α (187bp)	F	CCACGTACATGGGAGTGCAA
	R	GGGAAGGCTGCAGACTCAAA
mIL-36β (185bp)	F	AGATGGTATGGGTCCTGACTGG
	R	GCCCTCCATCTCAACACAGC
mIL-36γ (200bp)	F	GCAGGTGTGGATCTTTCGTAATCA
	R	GCAGCAAAGTAGGGTGTCCA
mS100A7A (167 bp)	F	CTTGTCCCTGGAGGAGTTGA
	R	GCTTGCCCAAGATGTACAGG
mS100A8 (178bp)	F	GGAAATCACCATGCCCTCTA
	R	GAGATGCCACACCCACTTTT
mS100A9 (180bp)	F	AGATGGCCAACAAAGCACCT
	R	TGTGTCCAGGTCCTCCATGA
mTSLP (194bp)	F	AGCTTGTCTCCTGAAAATCGAG
	R	AGGTTTGATTCAGGCAGATGTT
mIFN-γ	F	AACGCTACACACTGCATCTTGG
	R	GACTTCAAAGAGTCTGAGG
mCXCL1 (208 bp)	F	GCTGGGATTCACCTCAAGAA
	R	AGGTGCCATCAGAGCAGTCT
mCXCL2 (153bp)	F	AGTGAACTGCGCTGTCAATG
	R	TTCAGGGTCAAGGCAAACTT
mIL-1α (220bp)	F	TCGGGAGGAGACGACTCTAA
	R	TGGCAATAAACAGCTCTGGA
mIL-1β (230bp)	F	GCCCATCCTCTGTGACTCAT
	R	AGGCCACAGGTATTTTGTCG

Histopathology and immunohistochemical (IHC) staining

Mouse ears were fixed 24h at 4°C in 4% paraformaldehyde and embedded in paraffin. Sections (5µm) were stained with hematoxylin and eosin (H&E) or IHC staining. For IHC staining of mL-36α, paraffin sections were treated with 0.6% H2O2 (in PBS) to block the endogenous peroxidase activity before antigen retrieving with citric buffer (10mmol/L citric acid, pH 6). Slides were then blocked with 5% normal rabbit serum (Vector Laboratories, Burlingame, Calif) and incubated with goat polyclonal anti-mouse IL-36a antibody (AF2297, R&D, dilution: 1:100). Slides were then washed and incubated

with HRP-rabbit-anti-goat IgG (P0449, DAKO, dilution: 1:100). Staining was then visualized with AEC+ high sensitivity substrate chromogen solution (Dako, Glostrup, Denmark).

For immunofluorescence staining of NIMP-R14, 10 µm cryosections were fixed in 4% paraformaldehyde, permeabilized with acetone, and blocked with 5% normal goat serum (Vector laboratories). Slides were then incubated with primary antibody [rat monoclonal anti- NIMP-R14 (anti-neutrophil antibody, Abcam, ab2557, dilution: 1:750). After washing, sections were incubated with CY3-conjugated goat-anti-rat IgG antibody (Jackson ImmunoResearch) and mounted with Vectashield medium (Vector Laboratories) containing 4'- 6-diamidino-2-phenylindole dinhydrochloride (for nuclear staining; Invitrogen).

RNAscope *in situ* hybridization

Mouse ears were fixed in 10% of neutral buffered formalin and embedded in paraffin. Freshly prepared 5 µm sections were used for RNA in-situ hybridization, following the manufacture protocol for RNAscope 2.5 FFPE Red detection Kit (Advanced Cell Diagnostics, Hayward, CA, USA). To confirm mRNA integrity in the paraffin sections, Mm-Ppib probe (Mus musculus peptidylprolyl isomerase B; Cat No.313917) was used as a positive control (56). Mm-dapB probe (Bacterial Bacillus subtilis dihydrodipicolinate reductase; Cat No.310043) was used as negative control. RNAscope probes used for detection of IL-36α, IL-36γ, TSLP and IL1b were MmIL-36α (Cat No. 403651), MmIL-36α (Cat No. 425241), Mm-TSLP (Cat No. 432741), Mm-IL1β (Cat No. 316891).

Cell preparation and Flow cytometry analysis

For skin cells preparation, ears were split into ventral and dorsal halves and incubated 1h 30min at 37ºC with 2,5mg/ml Liberase Thermolysin Low (0,25mg/ml) (Roche, #0540120001) and 0,5mg/ml DNase I (sigma, #DN25-16) in RPMI medium (1% Penicilline/Streptomycine, 1% Sodium Pyruvate, 1% MEM, 2,5% HEPES, 0,001% BME). Digested cells were passed through a 70 µm strainer (Falcon) and resuspended in PBS containing 0,5% BSA and 2mM EDTA. Skin cells were then centrifuged at 350g

and resuspended in PBS containing 1% FCS and 2mM EDTA, counted and used for FACS staining.

For Lymph nodes (LN) cells preparation, LNs were dissociated with piston and passed through a 70 µm strainer (Falcon) resuspended in PBS containing 0,5% BSA and 2mM EDTA. LN cells were then centrifugated at 350g and resuspended in PBS buffer containing 1% of FCS and 2mM EDTA, counted and used for FACS staining.

Surface staining for flow cytometry

To perform flow cytometry analysis, 2 million cells were used for antibody (Ab) staining. Cells were first incubated with anti-CD16/CD32 AB to block unspecific binding, followed by surface markers staining with fluorochrome-conjugated antibodies (**Table 2**) in a 25µl of FACs buffer (1% of FCS+2mM EDTA in PBS) for 10 minutes at 4ºC. Viability staining was performed by adding propidium iodide to a final concentration of 1µg/mL just prior passing the cells to the cytometer.

Intracellular staining

In case of intracellular staining for cytokines, 3 millions of cells were stimulated in vitro at 37ºC for 4 hours in IMDM containing PMA/Ionomycine (eBioscience, 500x stimulation cocktail#00-4970), GolgiSTOP (BD Bioscience, #554714) and 10% FCS. Cells were then proceeded for surface staining as described above before intracellular staining.

For intracellular staining of IL-17A and INF-γ, the BD Cytofix/Cytoperm^{TM} Plus Staining Buffer Set (BD Bioscience #554715) was used according to the manufacturer's procedure. Briefly, after surface staining, cells were fixed and permeabilized by 30 min of incubation of ice in Fixation/permeabilization working solution. After two washes with 1X Permeabilization Buffer using centrifugation for 5 min at 500g, cells were resuspended in PBS containing fluorochromes labeled antibodies against intracellular markers (**Table 2**) and incubated 30 min at room temperature. After two additional washes with 1x Permeabilization Buffer using centrifugation for 5 minutes at 500g, cells were resuspended in PBS containing 1% FCS and 2mL of EDTA. Viability staining was

performed by incubating the cells with Fixable Viability Dye 506 (eBiocience) for 30 min at 4ºC prior to the surface staining.

Table 2: antibodies used for flow cytometry experiments

Marker	Fluorochrome	Clone	Company	Quantity
CD3	FITC	145-2C11	eBioscience	0,5:25
CD45	APC-eFluor780	30-F11	eBioscience	0,06:25
CD4	BV421	GK1.5	BD Biosciences	0,5:25
TCR-β	PE-Cy7	H57-597	BD Biosciences	0,5:25
TCR-γδ	PerCP-Cy5.5	GL3	Biolegend	1:25
IL-17A	PE	TC11-18H10	BD Biosciences	1:25
IFN-γ	Alexa Fluor 700	XMG1.2	BD Biosciences	1:25
Gr-1	PE	RB6-8C5	eBioscience	0,02:25
Siglec-F	Alexa Fluor 647	E50-2440	BD Biosciences	0,5:25
CD8a	Alexa Fluor 700	53-6.7	BD Biosciences	0,5:25

Statistical analysis

Data were analyzed using Sigmaplot (Systat Software Inc. Point Richmond, CA, USA) or GraphPad Prism by t-test, or the Mann-Whitney rank sum test depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance. Data are presented as means and SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ was considered to be statistically significant.

RESULTS

Characterization of IL-36α/γ Double Knockout (DKO) mice with the simultaneous deletion of *Il1f6* and *Il1f9* genes

With the objective to study the IL-36α and IL-36γ downstream pathways, we generated a mouse line with the simultaneous deletion of *Il1f6* and *Il1f9* genes, coding for IL-36α and IL-36γ respectively. The line analyzed here was derived from founder-17 in C57BL/6N background (**Figure 1A**).

I first verified the deletion of IL-36α and IL-36γ in the skin of DEL/DEL mice (named as DKO mice) at RNA and protein level. As in homeostasis conditions, IL-36α and IL-36γ are weakly expressed in the skin, I applied Ald cream from D0 to D5 to induce their expression (**Figure 1B**). qPCR analysis at D6 showed that IL-36α and IL-36γ but not IL-36β RNA expression levels were significantly induced in ears from Ald-treated WT mice as compared with those untreated. As expected, the expression of IL-36α and IL-36γ was not detected in either untreated or Ald-treated DKO mice (**Figure 1C**). IL-36β expression presented a tendency to be higher in Ald-treated DKO mice of Ald-treated WT mice. The total absence of IL-36γ RNA in skin of Ald-treated DKO was also confirmed by RNAscope (**Figure 1D**). IHC staining for IL-36α showed the absence of the IL-36α protein in the epidermis of Ald-treated DKO mice (**Figure 1E**).

Taken together, these results validate that IL-36α and IL-36γ are ablated in the skin of IL-36α/γ DKO mice.

IL-36α/γ DKO mice exhibit an attenuated psoriasis-like skin inflammation induced by Ald at D8

To assess the importance of IL-36α/γ in the development of Ald-induced psoriasis-like dermatitis in skin, DKO and WT littermates mouse ears were treated daily with Ald from D0 to D7 and analyzed at D8 (**Figure 2A**). This experimental protocol was chosen as C57BL/6 mice develop significant inflammation with increase of IL-17A at

this time point. The histological analysis showed that the untreated ears were similar between WT and DKO mice (**Figure 2B**). Ald-treated ears of WT skin showed thickened epidermis and dermis with heavy dermal infiltration of immune cells (**Figure 2B**); in contrast, Ald-treated ears of DKO mice exhibited less thickened epidermis and dermis with an attenuated dermal infiltration of immune cells (**Figure 2B**).

I further performed qPCR analysis of ears. Results showed that Ald topical treatment in WT mice induced the expression of IL-36α, IL-36β, IL-36γ, IL-17A, IL-17F, IL-17C, IL-22, IL-23p19/IL-23p40, as well as the calcium binding proteins S100A7A, S100A8 and S100A9, chemokines CXCL1, CXCL2 and IL-1α and IL-1β in the skin, which are all characteristic features for psoriasis inflammation (**Figure 2C**). Such increase was all diminished (except for CXCL1 gene) in Ald-treated skin of DKO mice (**Figure 2C**). IL-36β expression tended to be higher in Ald-treated than untreated groups but was not significantly different between Ald-treated WT and DKO mice (**Figure 2C**).

These analyses indicate that the absence of IL-36α/γ leads to a decrease in epidermal and dermal thickness, a reduced immune cell infiltration, accompanied by decreased expression of genes related with psoriasis including IL-17A, IL-22, IL-23p19/IL-23p40, S100A7A, S100A8, S100A9, CXCL2, IL-1α and IL-1β.

IL-36α/γ DKO mice exhibit reduced IL-17A expression in TCRγδ^{int} and TCRβ^{+} cells in the skin upon Ald treatment at D8

As IL-17A produced by TCRγδ^{int} T and TCRαβ^{+} T (Th17) cells play an important role in psoriasis development, I next analyzed the protein level of IL-17A using intracellular staining of the skin. The gating strategy from an Ald-treated WT ear sample is shown in **Figure 3A**. From the total skin cells isolated from ears (**Figure 3A(a)**) alive (FVD506-) and singlet cells (**Figure 3A (b-c, c-d)**) were gated. Hematopoietic cells were then gated for CD45^{+} (**Figure 3A(6e)**). CD3 staining was used to identify T cells (**Figure 3A(f)**). They were identified as: CD3^{hi} corresponding to epidermal TCRγδ^{hi} T cells (**Figure 3A(h)**), and CD3^{int} corresponding to TCRγδ^{int} (**Figure 3A(g)**) and TCRβ^{+} T cells (**Figure 3A(g)**). Inside CD3^{int}TCRβ^{+} T cells, CD4^{+} Th cells and CD4^{-} cells corresponding

to $CD8^+$ cytotoxic T cells were gated (**Figure 3A(i)**). The comparison of IL-17A expression in different cells showed that IL-17A is produced by T cells (very little IL-17A could be detected in $CD3^-$ cells) (data not shown). Among T cells, $TCR\gamma\delta^{hi}$ T cells produced very little IL-17A, whereas IL-17A was mainly produced by $TCR\gamma\delta^{int}$ and $TCR\beta^+$ T cells, as previously reported (see reviews: (17), (18)) (**Figure 3A (j, k, l)**).

Flow cytometry analyses showed that Ald-treatment led to a significant increase in the infiltration of $CD45^+$ cells to the skin in WT mice (**Figure 3B**). In contrast, Ald-treated DKO mice presented less $CD45^+$ cells than Ald-treated WT mice (**Figure 3B**). Among $CD45^+$ cells, we observed an increase in $CD3^{hi}$ and $CD3^{int}$ cells in Ald-treated WT mice in comparation with untreated mice. In Ald-treated DKO mice there were comparable $CD3^{hi}$ but less $CD3^{int}$ cells (**Figure 3C**). Among $CD3^{int}$ cells, we further examined $TCR\gamma\delta^{int}$, $TCR\beta^+CD4^+$ and $TCR\beta^+CD4^-$ T cells. In agreement with previous publications (19), (20), we observed that $TCR\gamma\delta^{int}$ and $TCR\beta^+CD4^+$ T cells but not $TCR\beta^+CD4^-$ T cells were increased in Ald-treated WT skin compared with untreated WT mice (**Figure 3D**). Such increase was diminished in Ald-treated DKO mice (**Figure 3D**). The examination of IL-17A showed higher frequencies in $TCR\gamma\delta^{int}$ and $TCR\beta^+CD4^+$ T cells in Ald-treated WT compared to untreated (**Figure 3E**). In contrast, the expression of IL-17A was lower in these subsets of cells in Ald-treated DKO mice (**Figure 3E**).

Together, these results indicate that the absence of IL-36α/γ leads to a reduction in the infiltration of $TCR\gamma\delta^{int}$ and $TCR\beta^+CD4^+$ T cells, as well as to the decrease of IL-17A expression by these cells.

Decrease of $GR1^{hi}$ (neutrophils) and $GR1^{int}$ (monocytes/macrophages) cells in Ald-treated IL-36α/γ DKO mouse skin at D8

Next, I performed surface staining for neutrophils/monocytes/macrophages and eosinophils in the skin. The analysis of flow cytometry showed that $GR1^{hi}$ (neutrophils) and $GR1^{int}$ (monocytes/macrophages) cells were induced in Ald-treated WT mice and this increase was diminished in Ald-treated DKO mice (**Figure 4A**). SiglecF staining did not reveal any increase of eosinophils in Ald-treated WT or DKO mice (**Figure 4A**). Immunofluorescent (IF) staining with GR1 antibody, confirmed that

neutrophils/monocytes/macrophages were barely detected in untreated WT and DKO skin but highly increased in the dermis of Ald-treated WT mice, which was reduced in Ald-treated DKO mice (**Figure 4B**). Interestingly, this reduction of neutrophils/monocytes/macrophages infiltration correlates with the decrease of the gene expression of several chemoattractants including CXCL2, S100A7A, S100A8 and S100A9 analyzed by qPCR in the Ald-treated DKO skin (**Figure 2C**). Together, these data indicate that the absence of IL-36α/γ leads to a reduction in neutrophils/monocytes/macrophages, suggesting that IL-36α/γ play an important role in the recruitment of these cells in Ald model.

IL-17A expression is similar in TCR$\gamma\delta^{int}$ and CD4$^+$TCRβ^+ subpopulations in skin-draining LNs from Ald-treated WT and IL-36α/γ DKO at D8

I further analyzed ear-draining LNs from untreated or Ald-treated WT and DKO mice at D8. I observed that the weight of LNs form Ald-treated DKO was lower than Ald-treated WT mice (**Figure 5A**). qPCR analysis of LNs showed that after Ald topical treatment in WT and DKO mice, IL-17A, IL-17F, IL-22, as well as IL-1β genes were induced as compared with those untreated samples (**Figure 5B**), and the expression of these genes presented a tendency to be lower in Ald-treated DKO mice compared with Ald-treated WT mice (**Figure 5B**). FACs analyses showed an increase of the frequency of TCRγδ$^+$, but not of TCRβ$^+$CD4$^+$ and TCRβ$^+$CD4$^-$ T cells among alive cells in both Ald-treated WT and DKO mice compared with untreated mice (**Figure 5C**). Intracellular staining of IL-17A showed that its frequency was similarly increased among TCRγδ$^+$, TCRβ$^+$CD4$^+$ and TCRβ$^+$CD4$^-$ T cells in Ald-treated WT and DKO (**Figure 5D**). In addition, I examined the IFN-γ expression in each subset of cells. I observed that TCRγδ$^+$, TCRβ$^+$CD4$^+$ and TCRβ$^+$CD4$^-$ T cells in Ald-treated WT mice presented lower expression of IFN-γ compared with untreated WT mice, and no difference was observed between Ald-treated WT and DKO mice (**Figure 5E**).

In summary, these analyses indicate that in the absence of IL-36α/γ skin-draining LNs at D8, the LNs weight is lower but IL-17A frequency in TCRγδ$^+$, TCRβ$^+$CD4$^+$ and TCRβ$^+$CD4$^-$ T cells is not different between WT and DKO mice.

Early neutrophil recruitment to the skin is reduced in IL-36α/γ DKO mice upon Ald treatment at D3

Next, I examined the role of IL-36α/γ at the early-stage of psoriasis development, by performing a shorter protocol of Ald-treatment (3 days from D0 to D2) to compare the phenotype developed in C57BL/6 WT and DKO mice (**Figure 6A**).

H&E staining of paraffin sections of ears revealed that at D3, there was already dermis immune infiltration, which was higher in Ald-treated WT than DKO mice (**Figure 6B**). qPCR analysis of Ald-treated WT mouse ears showed the increase of IL-36α and IL-36γ, indicating that these cytokines are induced at D3. IL-36β expression was similar between WT_NT and DKO_NT mice but was higher in Ald-treated DKO compared with Ald-treated WT mice (**Figure 6C**). RNA levels of IL-17A, IL-17F, IL-22, IL-23p19, IL-23p40, S100A7A, S1000A8, S100A9, CXCL1, CXCL2, IL-1α and IL-1β genes were all increased in Ald-treated WT mice compared with the untreated WT mice (**Figure 6C**). All of them except for IL-17A, IL-23p40, S100A7A and IL-1α presented a tendency to be lower (but not significantly) in Ald-treated DKO mice (**Figure 6C**).

FACS analysis of ears showed that Ald-treated WT mice presented an increase of $CD45^+$ cells which presented a tendency to be lower in Ald-treated DKO mice (**Figure 6D**). Furthermore, analysis of skin T cells by surface staining showed an increased in the frequency of $CD3^{hi}$ in Ald-treated WT and DKO mice in comparison with untreated mice (**Figure 6D**), but no significant increase in the frequency of $CD3^{int}$ cells (**Figure 6D**). Additionally, intracellular staining showed that IL-17A frequencies in these T cell subsets remained unchanged in Ald-treated WT or DKO mice in comparison with the untreated mice (**Figure 6E**).

In addition, Ald-treated WT skin at D3 presented an induction in frequencies of $GR1^{hi}$ (neutrophils) and $GR1^{int}$ (monocytes/macrophages) cells among alive singlet cells (**Figure 6F**). Interestingly, I observed that only $GR1^{hi}$ but not $GR1^{int}$ was decreased in Ald-treated DKO mice (**Figure 6F**). These results suggest that IL-36α/γ plays an important role in mediating the recruitment of neutrophils at early stage in Ald model, before IL-17A induction is evident in $TCR\gamma\delta^{int}$ and $TCR\beta^+$ T cells.

Intradermal (i.d.) injection of IL-36α or IL-36γ promotes TCRγδ^{int}, CD4^{+}TCRβ^{+} cells, neutrophils/monocytes/macrophages and eosinophils recruitment in both WT and IL-36α/γ DKO mice

To assess whether IL-36α and IL-36γ play a redundant or specific role inducing immune response, we intradermally (i.d.) injected IL-36α, IL-36γ or PBS in WT or DKO mouse ears daily for 5 consecutives days (**Figure 7A**). First, qPCR analysis indicated that both IL-36α and IL-36γ cytokines were able to induce each other in WT injected mice. These results were supported by IHC staining for IL-36α, which showed that WT but not DKO mice injected either with IL-36α or IL-36γ presented a high expression of IL-36α (**Figure 7C**), and by RNAscope in situ hybridization, which showed an increase of IL-36γ in WT but not in DKO skin injected with IL-36α or IL-36γ (**Figure 7C** and data not shown). In agreement with the qPCR data, IL-36α-injected WT mice presented more IL-36γ positive signals than IL-36γ-injected WT mice (**Figure 7C**).

Further qPCR analysis showed that i.d injection of IL-36α or IL-36γ induced the expression of several genes related with the development of psoriasis. Specifically, i.d. injection of IL-36α or IL-36γ in WT mice promoted induction of IL-36β, S100A7A, S100A8, S100A9, CXCL1, CXCL2, IL-1β and IL-17C, but not IL-1α. These were similarly observed in IL-36α- or IL-36γ-injected DKO mice (**Figure 7B**). However, the induction of IL-17A, IL-22, IL-23-p19 and IL-23p40 genes (related with T and DCs cells activation) in IL-36α- or IL-36γ-injected WT or DKO mice were all low (cross point > 30 cycles) (data not showed), suggesting that this protocol does not efficiently promote the induction of IL-23/IL-17A as observed in Ald model. Together, these results did not reveal any specific gene induction by IL-36α or IL-36γ, although the level of S100A7A, S100A8, S100A9 and IL-1β seemed to be higher in IL-36α- than IL-36γ-injected WT or DKO mice.

H&E staining of paraffin sections of ears from DKO mice injected either with IL-36α or IL-36γ presented undistinguishable thickened epidermis and dermis. The immune cell infiltration was similarly observed in IL-36α or IL-36γ i.d injected WT or DKO mice compared with PBS injected WT or DKO mice (**Figure 7D**). FACS analyses of the skin

showed that i.d. injection of IL-36α or IL-36γ led to a similar increase of the frequency of $CD45^+$ cells in both WT and DKO (**Figure 8A**). Additionally, frequency of $CD3^{hi}$ among alive cells did not change after the injections (**Figure 8B**), whereas the frequency of $CD3^{int}$ cells among alive cells presented an increase in WT and DKO injected mice (**Figure 8B**) including $TCR\gamma\delta^{int}$, $TCR\beta^+CD4^+$ and $TCR\beta^+$ $CD4^-$ T cells (**Figure 8C**). We did not observe the increase of IL-17A production in these cell populations at this stage, in agreement with the RNA analysis, suggesting that IL-17A induction in these cell populations might be either a later event or related with other factors promoted by Ald.

In addition, the frequency of $GR1^{hi}$ (neutrophils) and $GR1^{int}$ (monocytes/macrophages) was increased in both IL-36α and IL-36γ injected in WT or DKO mice (**Figure 8E**). Moreover, we observed that the frequency of $SiglecF^+$ eosinophils was also increased in WT and DKO mice after the injection of either IL-36α or IL-36γ cytokines in comparison with the PBS i.d injected groups (**Figure 8F**).

These results indicate that i.d. injection of IL-36α and IL-36γ have similar capacities in promoting the recruitment of $GR1^{hi}$, $GR1^{int}$, eosinophils and the increase of $TCR\gamma\delta^{int}$ and $TCR\beta^+CD4^+$ T cells. Their effects on IL-17A expression could not be evaluated because IL-17A production by $TCR\gamma\delta^{int}$, $TCR\beta^+CD4^+$ T cells was not induced in this experimental protocol.

IL-36α or IL-36γ promotes the expression of CXCL1, CXCL2, IL-1*β* and S100 family genes in ex-vivo cultured epidermis from both WT and IL-36*α*/*γ* DKO mice

The above results obtained using the IL-36α or IL-36γ i.d injection protocol suggest that IL-36α and IL-36γ induces the infiltration of neutrophils/monocytes/ macrophages, this is accompanied by an increase of S100A7A, S100A8, S100A9, CXCL1 and CXCL2 which all have chemotactic effects on these cells and these factors can be expressed by immune cells or by epidermal KCs (21). I thus performed an ex vivo culture of epidermis from WT or DKO mice to examine the direct effect of IL-36*α* and IL-36*γ* cytokines in epidermal cells. WT and DKO epidermis was separated from the dermis and ex vivo culture of epidermal sheet was carried out in the presence of IL-36α

or IL-36γ (**Figure 9A**). qPCR analysis showed that WT epidermis treated with IL-36α or IL-36γ presented the induction of IL-36α and IL-36γ genes in WT epidermis indicating that they are able to inter-regulate each other (**Figure 9B**). However, IL-36α or IL-36γ did not induce IL-36β expression in epidermis (**Figure 9B**). Additionally, in both WT and DKO epidermis, treatment with IL36α or IL-36γ similarly induces IL-1β, IL-17C, S100A7A, S100A8, S100A9, CXCL1 and CXCL2 expression (**Figure 9B**).

These results suggest that both IL-36α and IL-36γ are capable to regulate the expression of each other in the epidermis. Additionally, they have similar capacity in promoting the induction of the expression of genes including IL-1β, IL-17C, S100A8, S100A9, CXCL1 and CXCL2 in the epidermis.

-DISCUSSION AND PERSPECTIVES

In this part of my study, I reported that upon Ald-treatment, DKO mice developed an attenuated psoriasis-like skin inflammation characterized by the decrease of neutrophil/monocyte/macrophage skin infiltration, TCRγδ^{int} and TCRβ^{+}CD4^{+} T cells and their IL-17A expression. Furthermore, using a shorter Ald protocol, I showed an early effect of IL-36α/γ in the induction of neutrophil recruitment. In addition, i.d IL-36α- or IL-36γ-injected DKO mice, showed that both cytokines have similar capacity to promote neutrophil recruitment. Moreover, ex vivo cultures of DKO mouse epidermis treated with IL-36α or IL-36γ, presented the induction of the expression of IL-1β, S100A7A, S100A8, S100A9, CXCL1, CXCL2 and IL-17C genes in the epidermis.

In conclusion, our results show that IL-36α/γ play a key role in psoriasis development. According with them my hypothesis is summarized in **Figure 10**. IL-36α/γ are released by KCs and are able to inter-regulate each other (**Figure 10.1**). Moreover, IL-36α/γ promote the release of several epidermal-derived factors, including S100A8/A9, CXCL1, CXCL2, IL-17C, which may participate in the infiltration of neutrophils (**Figure 10.2**), which consequently may induce IL-17A production by the release of several factors that could be IL-1β and NETs. On the other hand, 36α/γ might participate in the initiation of the inflammatory loop interacting with IL-36R expressed in DCs (**Figure 10.3**).

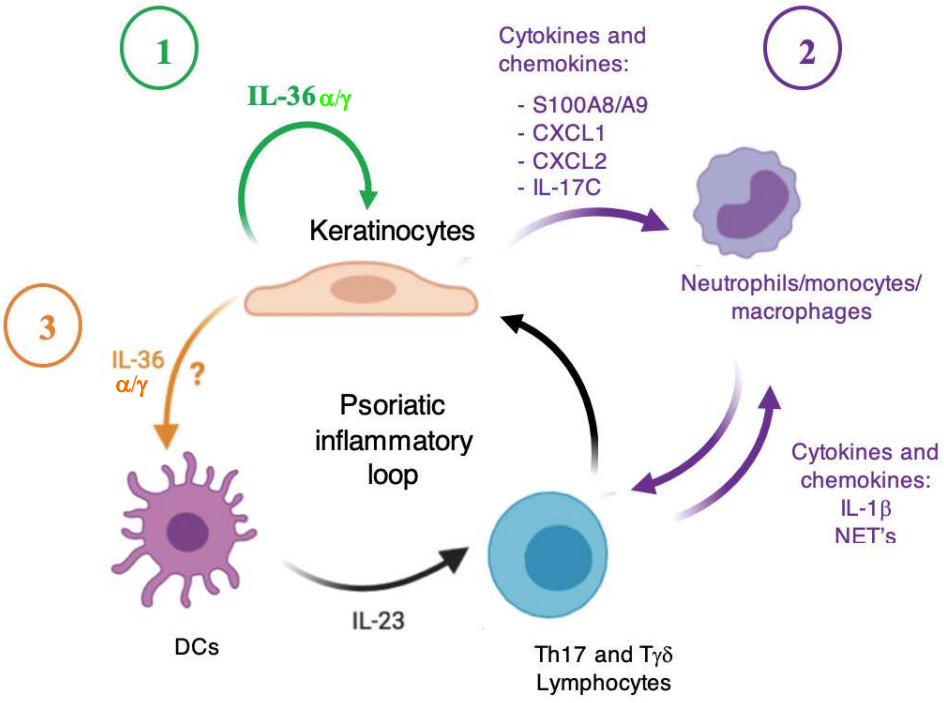


Figure 10: Schematic representation of the IL-36α/γ role in psoriasis development. During psoriasis initiation after the first stimuli (1) activated KCs release IL-36α/γ, which have autocrine effect inter-regulating the expression of themselves and each other in KCs. In addition, (2) IL-36α/γ promote the release of epidermal-derived factors including S100A8/S100A9, CXCL1, CXCL2, IL-17C which promote neutrophil recruitment. (3) The expression of IL-36R by DCs suggest that IL-36α/γ promote the IL-23 production by DCs. IL-23 stimulate the production of IL-17 and IL-22 by lymphocytes (Th17 and γδ T cells) promoting the psoriatic inflammatory loop.

In this session, I will discuss several aspects that we addressed with our last experiments and the next perspectives to continue with the project.

1) Validation of the DKO mouse model

As described in the introduction, our DKO model was generated using CRISPR-Cas9 strategy. To diminish the risk of the off-targets the mice used in the study were derived from F2 after crossing the founders with C57BL/6 mice. Additionally, as mentioned in the introduction of this part, we generated two independent lines of DKO mice (founder-17 and founder-18). When mice from founder-18 were treated with 6xAld DKO mice exhibited a reduction of epidermis and dermis thickness and cell infiltration, accompanied by the decrease of the expression of IL-17A, IL-22, IL-23p19/IL-23p40, S100A7A, S100A8 and IL-1β, thus providing a support for the data obtained from founder-17 (**Supplementary figure 1**).

2) How do IL-36α/γ play a role in the development of psoriasis-like skin inflammation?

In the last decade, several studies pointed out the importance of IL-36 cytokines in psoriasis development (see review: Madonna S. et al., (22)). In our study, upon Ald-treatment, DKO mice presented a weaker phenotype with reduced neutrophil recruitment, TCRγδ^{int} and TCRβ^{+}CD4^{+} T (Th17) cells and decreased in IL-17A production by these cells, confirming that IL-36α/γ play a crucial role inducing psoriasis-like skin inflammation development (**Figure 2 and Figure 3**). Moreover, using a shorter protocol, we observed that IL-36α/γ exert an early role in neutrophil infiltration in Ald model.

It has been shown that the presence of dermal and epidermal microabscesses of neutrophils, is one of the hallmark histologic features of psoriasis (23), which have the function to act as phagocytes and release AMPs including S100A8/A9, LL37 and lipocalin-2 (LCN2, increased in psoriatic patients, (24)) to amplify the inflammatory response. Moreover, several clinical trials suggested that the selective depletion of myeloid linage leukocytes through adsorptive granulocyte and monocyte apheresis in psoriatic patients improve the symptomatology of the disease (25), (26). Similar to human psoriasis, using the Ald model it has been demonstrated that the depletion of neutrophils with an anti-Ly6G antibody ameliorate the disease severity with the reduction in the recruitment of $F4/80^+$ and $CD4^+$ T cells (27), (28), (29).

However, how IL-36α/γ-promoted neutrophil recruitment may promote IL-17A expression in psoriasis development is not clear. One possibility is that IL-1β produced by neutrophils/monocytes/macrophages promotes IL-17A expression by T cells. Supporting this hypothesis, a recent publication from McGinley et al., reported that IL-1β-producing neutrophils and $Ly6C^{hi}$ inflammatory monocytes are an important immune cell population that migrate to the draining LNs, leading to the production of pathogenic $TCR\gamma\delta^+$ T cells, Th17 cells and autoimmunity in a mouse experimental autoimmune encephalomyelitis (EAE) model (30). Our data showed that Ald-treated DKO mice have low IL-1β RNA expression in skin (**Figure 2C**) associated with the reduction of neutrophil/monocyte/macrophage recruitment (**Figure 4**) and accompanied by low induction of IL-17A production in $TCR\gamma\delta^+$ T and Th17 cells (**Figure 3**). Moreover, IL-1β RNAscope showed that the IL-1β expressing cells in Ald-treated WT mice were located along the dermis, correlated with neutrophils/monocytes/macrophages location, which were reduced in Ald-treated DKO mice (**Supplementary figure 2B**). Furthermore, i.d IL-36α- and IL-36γ-injected DKO mice showed similar capacity to promote neutrophil infiltration than injected WT mice (**Figure 8E**) and the induction of IL-1β by qPCR and RNAscope analysis (**Figure 7B** and **Supplementary Figure 2C**), suggesting the implication of IL-36α/γ in the regulation of IL-1β expression level and neutrophil/monocyte/macrophages recruitment which correlates with IL-1β location. To further explore the role of IL-36α/γ in the regulation of IL-1β production by neutrophils we should perform first of all, a surface staining from IL-36α- and IL-36γ-injected or Ald-treated WT and DKO skin samples using Ly6G antibody that is specific for

neutrophils. Moreover, it will be interesting to establish an ex vivo system to co-culture epidermis from DKO mice treated with IL-36α or IL-36γ in the presence of mouse isolated unstimulated neutrophils, to study whether these neutrophils are able to overexpress IL-1β by the factors released by epidermis after IL-36α or IL-36γ treatment.

In response to inflammatory signals, IL-1β is synthesized as a pro-IL1β and needs to be cleavage to gain functional activity. Caspase-1 is the main protease released by neutrophils and macrophages, responsible of this process (31). It will be interesting to confirm whether the IL-1β cytokine detected in our model is the active form that will have the capability to promote Th17 cells performing a western blot from the skin of Ald-treated WT mice or i.d IL-36α- IL-36γ-injected WT and DKO mice. Furthermore, Cai et al., using Ald-treatment in *Il-1r*$^{-/-}$ mice showed that the effect of the increase of IL-1β promotes the proliferation of dermal TCRγδ^{int} T cells and epidermal hyperplasia. Interestingly, they showed that IL-17 induction by IL-23 effect is dependent on IL-1R signaling, suggesting a critical role of this signaling pathway in dermal TCRγδ^{int} T cell activation (32). These data confirm the role of IL-1β in IL-17 production in inflammation.

Another mechanism of crosstalk between neutrophils and IL-36 was proposed by Shao et al., (33). They reported that activated neutrophils in human psoriatic patients and Ald-treated mice form neutrophil extracellular traps (NETs) in skin. These NETs amplify inflammatory responses promoting IL-23/Il-17A axis, IL-36γ and LCN2 release among others by KCs via TLR4/IL-36R signaling. Additionally, this phenotype was decreased upon the blocking of NETs production in Ald-treated mice by Cl-amidine and DNaseI. As neutrophils are the first line of defense, their hypothesis is that NETs contribute to the development of psoriasis-like inflammation promoting IL-17A and IL-36 responses which synergistically promote the induction of neutrophil chemoattractants expression in KCs amplifying skin inflammation (33). It will be interesting to examine the effect of IL-36α/γ in NETs formation. To address this, we could perform ex vivo co-cultures of mouse epidermis treated with IL-36α or IL-36γ in the presence of unstimulated neutrophils to study their capability to form NETs upon IL-36 cytokines treatment. Additionally, its necessary to study the NETs formation in our in vivo i.d IL-36α- or IL-36γ-injection DKO mouse model to explore the implication of NETs in the induction of neutrophil/

monocyte/macrophage and T cells recruitment observed in the DKO mice injected with both cytokines.

3) How does IL-36α or IL-36γ induce neutrophil recruitment and activation?

As it has been illustrated in the summary Figure 10.2, our ex vivo (**Figure 9B**) and i.d injection data in DKO and WT mice (**Figure 7B**) suggest that IL-36α/γ stimuli in epidermis promote the release of cytokines such as IL-17C, chemokines including CXCL1, CXCL2, as well as S100A7A, S100A8, S100A9 factors leading the activation and recruitment of immune cells including neutrophils/monocytes/macrophages to the site of inflammation. Moreover, the release of these factors and the neutrophil infiltration was reduced in Ald-treated DKO mice (**Figure 2C and Figure 4**), suggesting that the ablation of IL-36α/γ regulates the release of these keratinocytic-derived factors and consequently the neutrophil recruitment. In agreement with our results, recently, it has been reported that Ald-treated *Il-36r*$^{-/-}$ selectively in KCs mice presented reduction in the expression of IL-17C, S100A8 and S100A9 in KCs (34), (35).

It has been reported that keratinocyte-derived AMPs, such as LL37, promote the up-regulation of CXCL1, CXCL2, IL-1α, IL-1β, S100A7A, S100A8 and S100A9 by immune and epidermal cells (36), (37). This initial signal promotes the recruitment of neutrophil/monocyte/macrophage to the skin, which consequently will promote the expression of IL-17A by T cells and contribute to the psoriasis inflammatory loop (**Figure 10**). More in detail, it has been showed that S100A8 and S100A9, up-regulated in psoriatic patients (38), are expressed by neutrophils, active monocytes (39) and KCs (40), (41). They form a heterodimeric complex, also called calprotectin and among other functions they have chemotactic activity. In mouse, it has been shown that injection of mS100A8 stimulates early recruitment of neutrophils followed by monocytes over 24h (42). In addition, *S100a9*$^{-/-}$ mice which presented abrogation of S100A8 at protein level (43), upon Ald-model leads to the attenuation of the psoriatic inflammatory phenotype and decreases of IL-1β expression (38). In the other hand, IL-17C is known to be produced by non-immune cells including KCs in mice and humans (44). In humans it has been reported that at RNA level, IL-17C is increased in inflamed skin of psoriatic patients (45). Moreover *Il-17c*$^{-/-}$ and *Il-17re*$^{-/-}$ mice presented lower psoriasis-like inflammatory phenotype upon Ald-treatment (46). Moreover, genome-wide expression

profiling has identified several chemokines including CXCL1, IL-8 (47) and CXCL2 (48) among others secreted in psoriatic skin in response to stress and injury compared to normal skin. Furthermore, it has been shown that IL-36 cytokines induce expression of several pro-inflammatory molecules in KCs including CXCL1, CXCL2, CXCL8 (49), (50), S100A7 and LL37 (51).

It needs to be further confirmed whether the effect of IL-36 cytokines in the induction of S100A8/A9, CXCL1, CXCL2 and IL-17C is in KCs. Our ex vivo system in addition to KCs, contains LCs and TCRγδ^{hi} T cells. Then, it is necessary to perform the sorting of KCs (CD45^{-} cells) to study the genes induced or analyze the expression of these factors in IL36α- or IL-36γ-injected DKO mice at protein level by IHC. Moreover, to study specifically the role of IL-36α/γ in KCs from DKO mice we should perform RNA-seq analysis to examine the genome-wide and identify the genes altered upon IL-36 treatment. To complete this study performed in mice these results should be validated in human systems using monolayer 'in vitro' HPKCs cultures or 3D models of human skin to study the effect of the presence or absence of IL-36α or IL-36γ.

4) Effect of IL-36α and IL-36γ cytokines in DCs

It has been reported that although IL-36R, is expressed mainly in KCs, it is also expressed by DCs (49), (52) and LCs (Dietrich et al., 2016) in human and mice, whereas human T cells or neutrophils do not (49). As it was summarized in the **Figure 10.3**, this finding suggests that IL-36α and IL-36γ could participate in T cell activation through DCs. To have a deeper understanding about IL-36/IL-36R signaling pathway in the development of psoriasis inflammation it is necessary the generation of *IL-36r*$^{DC-/-}$ (mouse line where the IL-36R will be deleted selectively in DCs (CD11c Cre)).

5) Redundant and specific function of IL-36α and IL-36γ

Even though recently, it has been reported that IL36α and γ have different binding affinity for IL-36R, I saw that i.d IL-36α- or IL-36γ-injected WT or DKO mice have similar capacity to promote neutrophil/monocyte/macrophage and T cells recruitment to the skin (**Figure 7 and 8**). However, this protocol was not enough to promote the expression of IL-17A. Considering that we showed the induction of neutrophil/ monocyte/macrophage and T cells infiltration, could be interesting to try a longer

treatment to evaluate if it is possible to promote the expression of IL-17A and the development of the full hallmarks of psoriasis inflammation with this model. Our current results indicate that the effect of IL-36α/γ is principally in the innate immune system and at the same concentration IL-36α and IL-36γ have similar capacity to induce inflammatory processes. Nevertheless, IL-36α showed a tendency (no statistically significant) to have a stronger effect than IL-36γ inducing the expression of some genes including S100A7A, S100A8, S100A9, IL-1β and IL-17C.

Although IL-36α/γ have similar capacity, their functional specificity can be still related with: **A)** Different in vivo expression levels in physiological or pathological situations. IL-36γ is expressed in homeostasis conditions in skin but IL-36α is absent to very low (**Figure 1C, 2C** and Figure 3B first part of the thesis (10). **B)** Different location. IL-36α was specifically located in the outmost suprabasal layer of epidermis, whereas IL-36γ expressing cells were induced throughout all layers of epidermis (**Figure 3B** first part of the thesis). Another interesting observation was that in our ex vivo cultures IL-36α or IL-36γ WT treated epidermis, did not induce IL-36β expression (**Figure 9B**). These results, support that IL-36β is principally express in endothelial cells, whereas IL-36α and IL-36γ are induced by KCs in humans and mice in psoriasis development (22). **C)** Different stimuli. IL-36α was shown to be promoted in skin by epicutaneous S. aureus; whereas IL-36γ was reported to be induced in skin injury. Furthermore, IL-17A and TNF-α induced the expression of IL-36α/β/γ in KCs (12). However, IL-1β and IL-18 amplified IL-36γ production in macrophages (53). Specifically, the release of IL-36γ is induced by poly (I:C) binding with TLR3 in KCs (54). **D)** Different Cleavage. IL-36 cytokines are produced as Pro-IL-36α, IL-36β, and IL-36γ, between 100-1000-fold less active than their active and mature forms (55). However, Milora et al., reported that unprocessed IL-36α is active although its activity after the cleavage is higher (56). Nevertheless, it is not really well known if IL-36β, and IL-36γ need to be cleavage to be active and functional.

Despite that during the last years a lot of publications were highlighting the important role of IL-36γ in psoriasis development (3), Milora et al., suggested that IL-36α but not IL-36γ plays a significant role in driving the psoriatic-like skin inflammation in mice (56). Using a single *Il1f6*$^{-/-}$ (IL-36α KO) and *Il1f9*$^{-/-}$ (IL-36γ KO) mice they

showed that upon Ald-treatment the phenotype developed by *Il1f6*$^{-/-}$ was stronger than *Il1f9*$^{-/-}$, but they only presented histological analyses comparing both models and the analysis was not completed to affirm that IL-36γ does not have a role in psoriasis development. Furthermore, Hashiguchi et al., showed that *Il1f6*$^{-/-}$ mice were refractory to psoriasis-like skin inflammation developed by Ald-treatment (50). In order to explore whether IL-36α has a stronger pathological role in the development of inflammation than IL-36γ, it is necessary to use the new mouse model developed in the lab with the conditional KO of IL-36α in KCs (project underdevelopment) to compare the phenotype that these mice will develop with the results obtained with our DKO mice.

6) Role of IL-36α/γ in Atopic dermatitis (AD)

To finish this discussion, it is interesting to remark that in addition to the fact that IL-36α/γ are recognized as psoriasis biomarkers, during the last years several studies performed with human samples showed that IL-36 family members are upregulated in pediatric and adult lesional AD skin (57), (58). One of the genetic mutations identified as a key player in generalized peeling skin syndrome type B (PSS-B), a severe AD-like syndrome, is corneodesmosin (CDSN). Previously, my lab has reported that *Cdsn*$^{iep-/-}$ mice, in which tamoxifen treatment induce keratinocyte-selective ablation of the *Cdsn* gene, developed strong Th2 and Th17 response with increase of neutrophil recruitment (59). Thus, I decided to use this model to explore whether IL-36α/γ were increased in *Cdsn*$^{iep-/-}$ mice.

Cdsn$^{iep-/-}$ mice were intraperitoneally injected daily from D0 to D2 with tamoxifen to induce disruption of the SC barrier through keratinocyte-selective ablation of the *Cdsn* gene (**Supplementary figure 4A)**. qPCR analyses of cytokines expression in skin at D14 showed that IL-36α, β and γ, as well as IL-17A, IL-22, IL-23p19/IL-23p40 and IL-1β, as previously published by Li et al., tended to be higher in *Cdsn*$^{iep-/-}$ compared with WT control mice **(Supplementary figure 4B)**. RNAscope in situ hybridization showed that in *Cdsn*$^{iep-/-}$ mice there was a strong up-regulation of IL-36α signal specifically located in the outmost suprabasal layer of epidermis (**Supplementary figure 4C**). IL-36γ was also induced throughout all layers of epidermis. Moreover, IL-36α RNA results were confirmed at the protein level by IL-36α IHC (**Supplementary figure 4C**).

The function of IL-36α/γ in the context of PSS-B pathogenesis needs to be further explored. To this aim, we crossed *Cdsn*$^{iep-/-}$ mice (in Balbc background) with IL-36α/γ DKO mice. As our *Cdsn*$^{iep-/-}$ mice have Balb/c background we generated a Balb/c IL-36α/γ DKO mouse (**Supplementary Figure 5)**. This will give us the opportunity to unravel the role of IL-36α/γ in AD, and to discover the key pathways implicated.

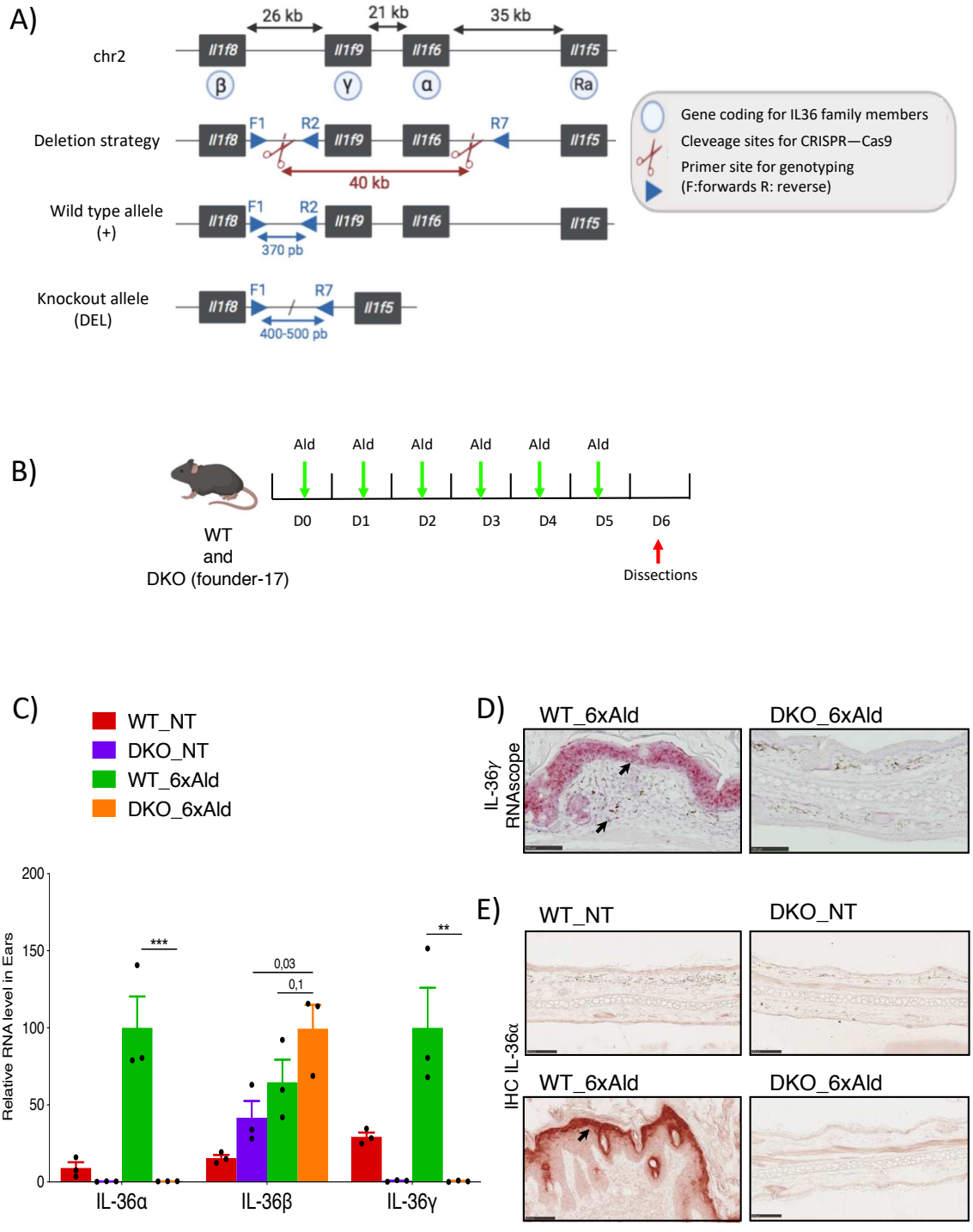


Figure1.Characterization of IL-36α/γ DKO with the simultaneous deletion of *Il1f6* and *Il1f9* genes.

A. Schematic representation of CRISPR-Cas9 cleavage in the locus of *Il1f6* and *Il1f9* genes coding respectively for IL-36α and IL-36γ and genotyping strategy of wild-type (WT) allele (+) amplified by F1 and R2 and DEL/DEL (DKO) allele amplified by F1 and R7. WT band has 377 bp and DKO 430 bp.

B. Experimental protocol. C57BL/6N WT and IL-36α/γ DKO mouse ears were topically treated with Aldara (Ald) or untreated (NT) every day from day 0 (D0) to D5. Mouse ears were analysed at D6.

C. Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n=3). **p<0.01, *** p<0.001 (two-tailed Student's t test).

D. RNAscope in situ hybridization for mIL-36γ probe (signals in red) in WT or IL-36α/γ DKO Ald-treated mouse sections. Black arrows point to one of the positive signal. Counterstained with hematoxylin. Scale bar:100 **µ**m.

E. IHC staining with IL-36α antibody on ear sections. Positive cells are in dark red. Black arrows point to one positive signal. Scale bar:100 **µ**m.

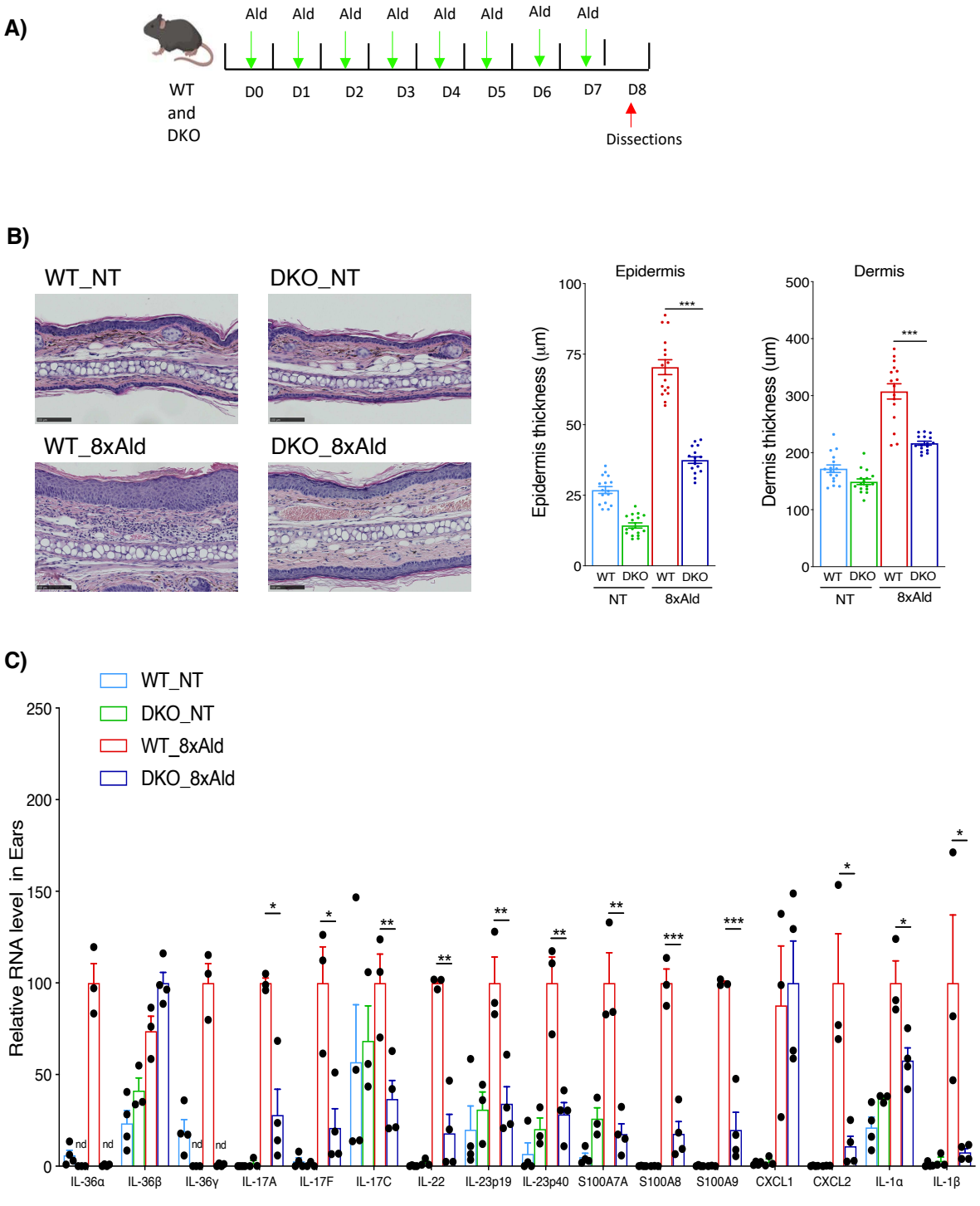


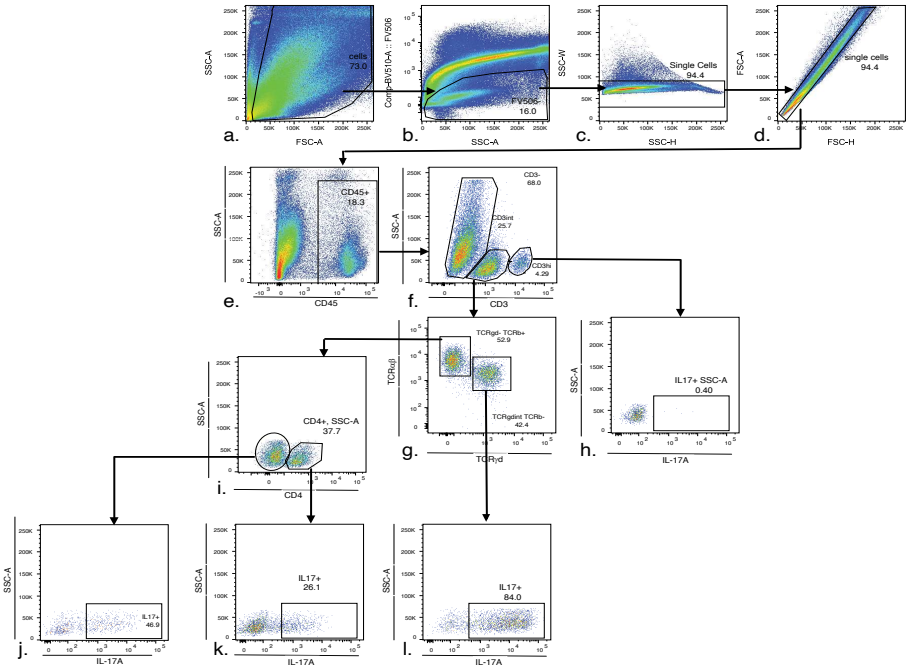
Figure 2: IL-36α/γ DKO mice exhibit an attenuated psoriasis-like skin inflammation induced by Ald at D8.

A. Experimental protocol. C57BL/6N Wild-type (WT) and IL-36α/γ DKO mouse ears were topically treated with Aldara (Ald) or untreated (NT) every day from day 0 (D0) to D7. Mouse ears were analysed at D8.

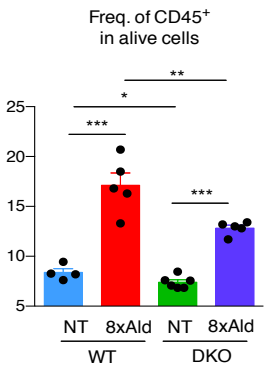
B. H&E staining and measurements of epidermis and dermis thickness from ear sections. Scale bar: 100µm. Values are mean ± SEM. ***P< 0.001 (2-tailed Student's t test).

C. Quantitative RT-PCR analyses of ears. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n≥3). * p<0.05, **p<0.01, ***p< 0.001 (two-tailed Student's t test). Data are representative of 2 independent experiments with similar results.

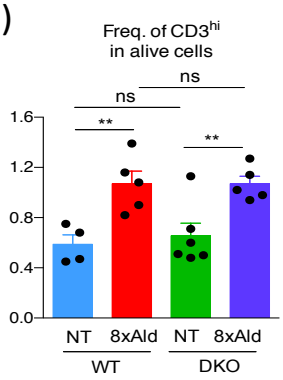
A)

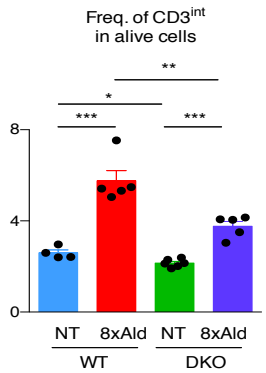

a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.
l.
cells
73.0
Single Cells
94.4
single cells
94.4
CD45+
18.3
CD3int
25.7
CD3hi
4.29
CD4+, SSC-A
37.7
IL17+ SSC-A
0.40
IL17+
46.9
IL17+
26.1
IL17+
84.0
FSC-A
SSC-A
SSC-W
SSC-H
FSC-H
CD45
CD3
CD4
IL-17A

B)

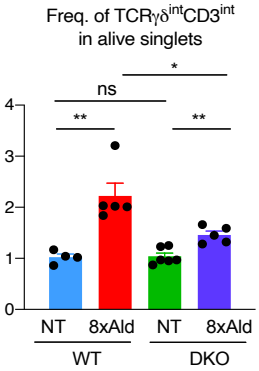

Freq. of CD45+
in alive cells
NT
8xAld
NT
8xAld
WT
DKO

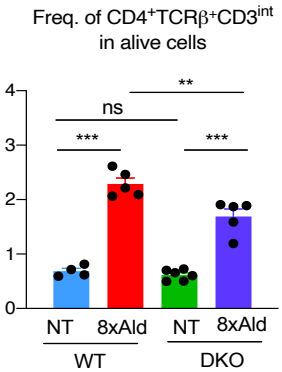
C)

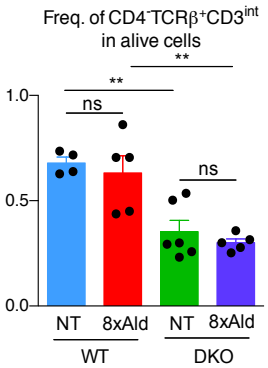

Freq. of CD3hi
in alive cells
ns
NT
8xAld
NT
8xAld
WT
DKO


Freq. of CD3int
in alive cells
NT
8xAld
NT
8xAld
WT
DKO

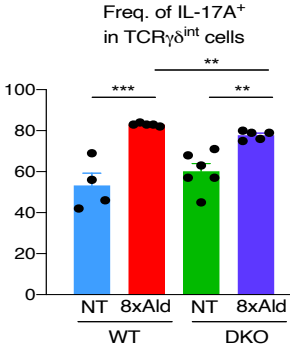
D)

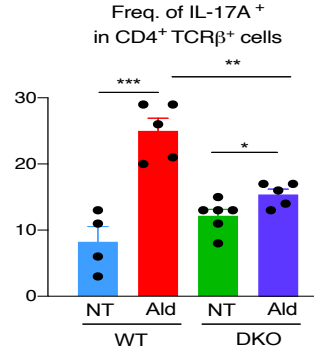

Freq. of TCRγδintCD3int
in alive singlets
ns
NT
8xAld
NT
8xAld
WT
DKO


Freq. of CD4+TCRβ+CD3int
in alive cells
ns
NT
8xAld
NT
8xAld
WT
DKO


Freq. of CD4-TCRβ+CD3int
in alive cells
ns
ns
NT
8xAld
NT
8xAld
WT
DKO

E)


Freq. of IL-17A+
in TCRγδint cells
NT
8xAld
NT
8xAld
WT
DKO


Freq. of IL-17A +
in CD4+ TCRβ+ cells
NT
Ald
NT
Ald
WT
DKO

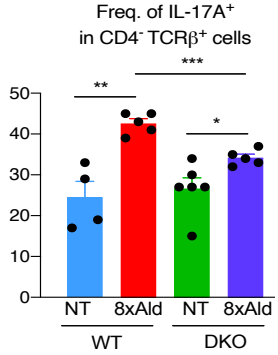

Freq. of IL-17A+
in CD4- TCRβ+ cells
NT
8xAld
NT
8xAld
WT
DKO

Figure 3: IL-36α/γ DKO exhibit reduced IL-17A production in TCRγδ^{int} and TCRβ^{+} T cells in the skin upon Ald treatment at D8.

A. Gating strategy. An Ald-treated WT sample was selected for presenting the scheme. After excluding debris (a), dead cells and doublets (b,c,d), hematopoietic cells were gated for CD45^{+} (e). CD3^{hi} and CD3^{int} cells (f) were further gated to identify T subpopulations (CD3^{hi}TCRδγ^{hi}, CD3^{int}TCRβ^{+} and CD3^{int}TCRδγ^{int}) (g). TCRβ^{+} cells were gated for CD4^{+} and CD4^{-} (i). Finally, IL-17A was gated from each population (h, j, k, l).

B. Frequency of CD45^{+} cells among alive cells.

C. Frequency of CD3^{hi} (left) and CD3^{int} (right) among alive cells.

D. Frequency of TCRδγ^{int} (left), TCRβ^{+}CD4^{+} (middle) and TCRβ^{+}CD4^{-} (right) among alive cells.

E. Frequency of IL-17A^{+} among TCR δγ^{int} (left), TCRβ^{+}CD4^{+} (middle) and TCRβ^{+}CD4^{-} (right) in ears. Values are mean ± SEM. (n≥4) *p< 0.05; **p< 0.01; ***p< 0.001 (one-tail ANOVA test).

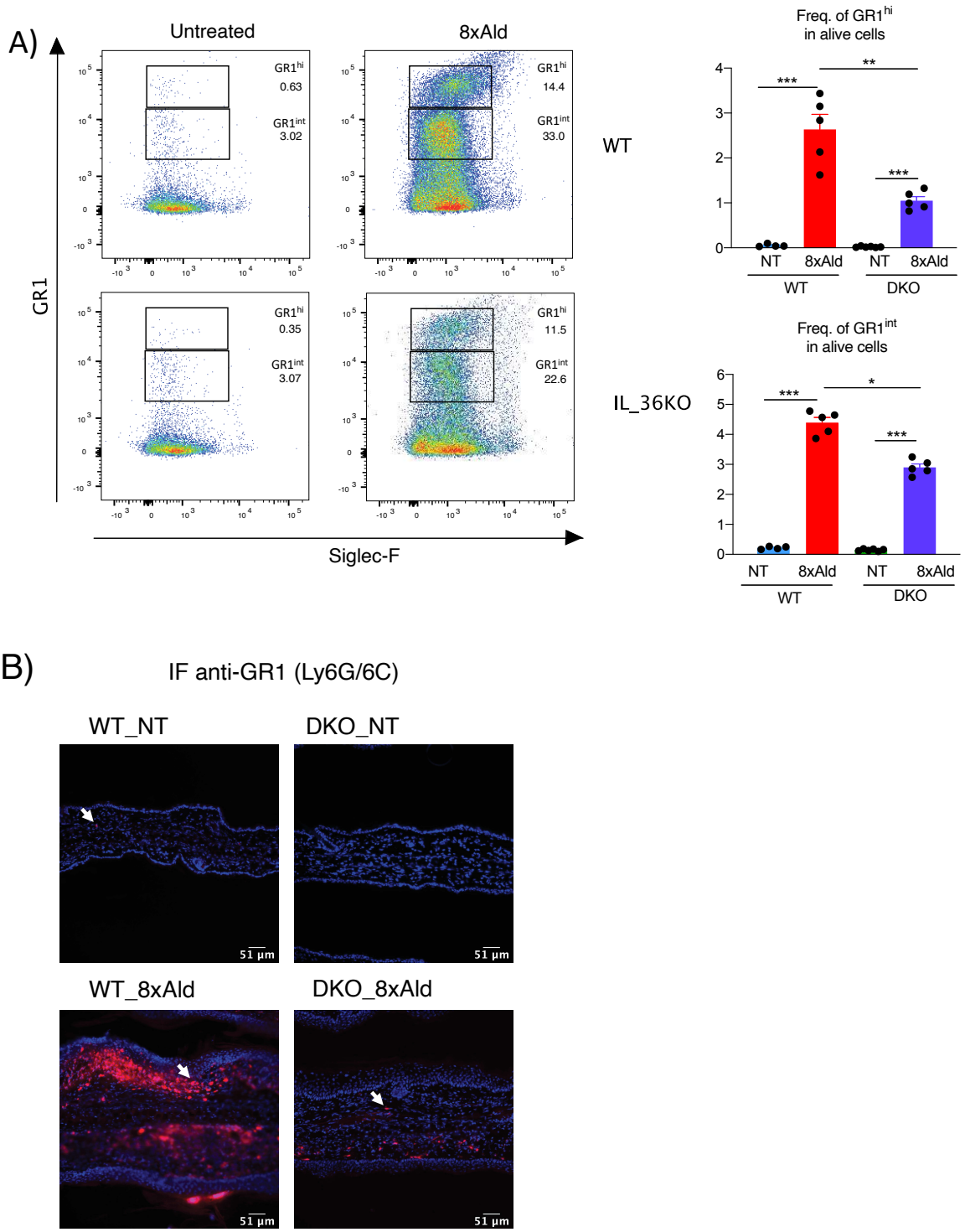


Figure 4: Decrease of GR1^{hi} (neutrophil) and GR1^{int} (monocyte/macrophage) cells in Ald-treated IL-36α/γ DKO mouse skin at D8.

A. Representative FACS plots (left) and comparison of frequency of GR1^{hi} and GR1^{int} in alive cells (right). Values are mean ± SEM. (n≥4) *p< 0.05; **p< 0.01; ***p< 0.001 (one-tail ANOVA test).

B. Immunofluorescent staining with NIMP-R14 (anti-GR1) antibody for neutrophils of mouse ear sections (red corresponds to positive signal, whereas blue corresponds to DAPI staining of nuclei). White arrows point to positive signals. Scale bar: 51 **µ**m.

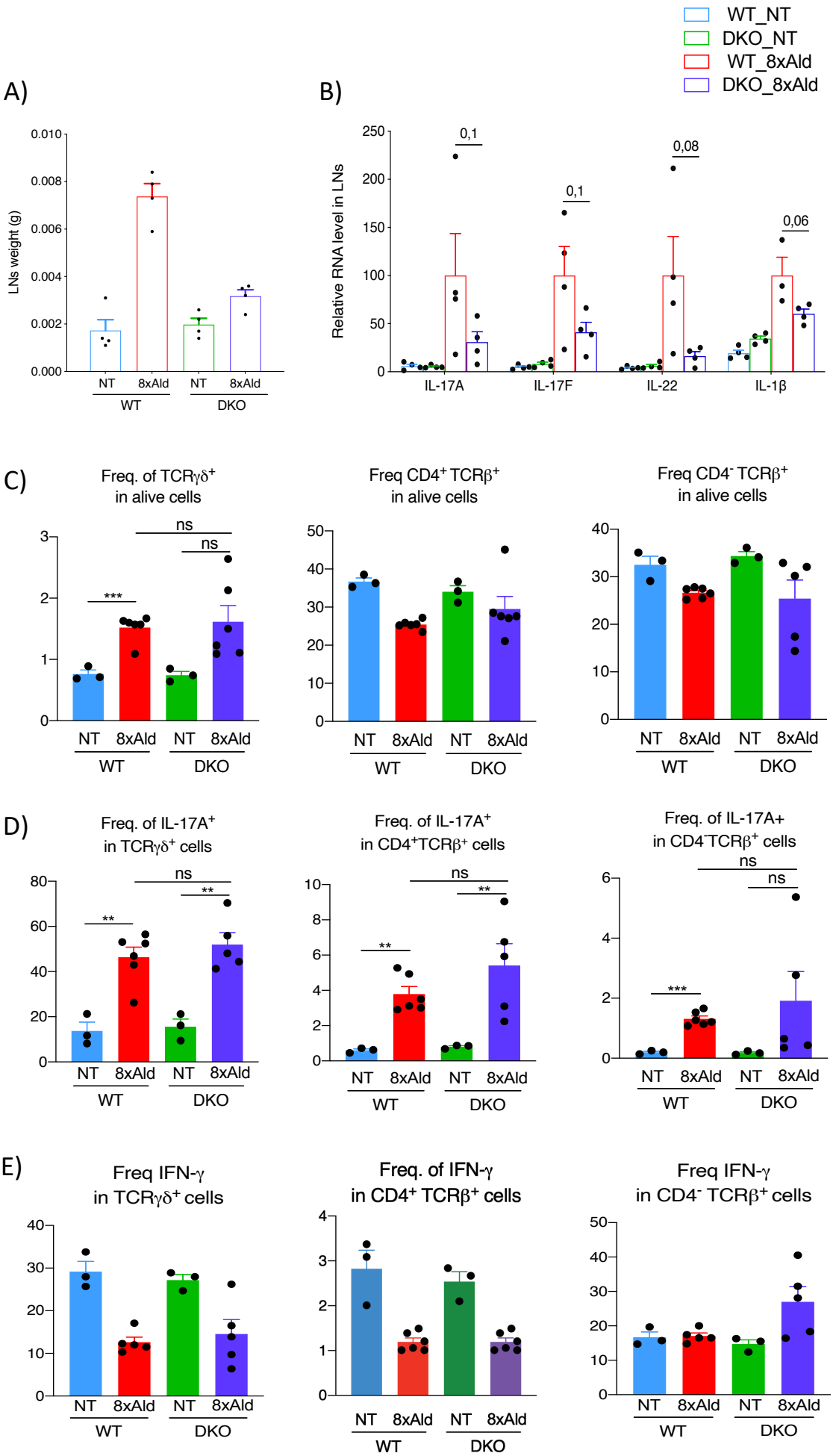

WT_NT
DKO_NT
WT_8xAld
DKO_8xAld
A)
LNs weight (g)
0.010
0.008
0.006
0.004
0.002
0.000
NT
8xAld
NT
8xAld
WT
DKO
B)
Relative RNA level in LNs
250
200
150
100
50
0
0,1
0,1
0,08
0,06
IL-17A
IL-17F
IL-22
IL-1β
C)
Freq. of TCRγδ+ in alive cells
Freq CD4+ TCRβ+ in alive cells
Freq CD4- TCRβ+ in alive cells
ns
ns
***
D)
Freq. of IL-17A+ in TCRγδ+ cells
Freq. of IL-17A+ in CD4+TCRβ+ cells
Freq. of IL-17A+ in CD4-TCRβ+ cells
**
**
**
***
E)
Freq IFN-γ in TCRγδ+ cells
Freq. of IFN-γ in CD4+ TCRβ+ cells
Freq IFN-γ in CD4- TCRβ+ cells

Figure 5: IL-17A expression is similar in TCR$\gamma\delta^{int}$ and CD4$^+$TCRβ^+ subpopulations in skin-draining LNs from Ald-treated WT and IL-36α/γ DKO at D8.

A. C57BL/6N Wild-type (WT) and DKO mouse ears were topically treated with Aldara (Ald) or untreated (NT) every day from day 0 (D0) to D7. Ear-draining lymph nodes (LNs) weights were compared at D8 (n=4).

B. Quantitative RT-PCR analyses of LNs. Relative RNA level were calculated using HPRT as internal control. Values are mean ± SEM. (n=4) (2-tailed Student's t test).

C. Frequency of TCR $\delta\gamma^{int}$ (left), TCRβ^+CD4$^+$ (middle) and TCRβ^+CD4$^-$ (right) among total cells number in LNs

D-E. Frequency of IL-17A$^+$ (C) and IFN-γ (D) among TCR$\delta\gamma^{int}$ (left), TCRβ^+CD4$^+$ (middle) and TCRβ^+CD4$^-$ (right) in LNs. Values are mean ± SEM. (n≥3) **p< 0.01 (one-tail ANOVA test).

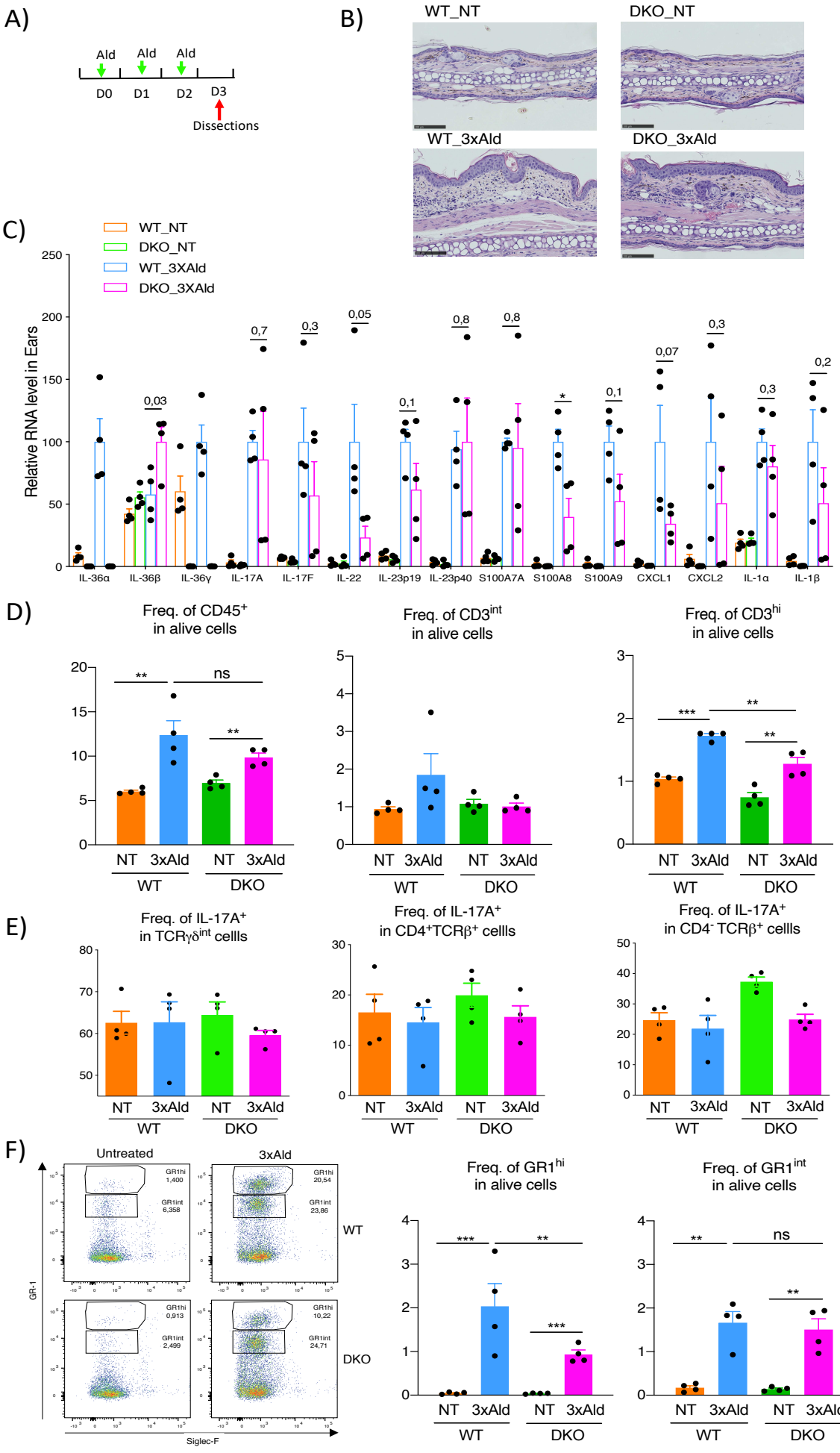

A)
Ald
Ald
Ald
D0
D1
D2
D3
Dissections
B)
WT_NT
DKO_NT
WT_3xAld
DKO_3xAld
C)
WT_NT
DKO_NT
WT_3XAld
DKO_3XAld
Relative RNA level in Ears
250
200
150
100
50
0
0,03
0,7
0,3
0,05
0,1
0,8
0,8
*
0,1
0,07
0,3
0,3
0,2
IL-36α
IL-36β
IL-36γ
IL-17A
IL-17F
IL-22
IL-23p19
IL-23p40
S100A7A
S100A8
S100A9
CXCL1
CXCL2
IL-1α
IL-1β
D)
Freq. of CD45+ in alive cells
Freq. of CD3int in alive cells
Freq. of CD3hi in alive cells
**
ns
**
***
**
**
NT
3xAld
NT
3xAld
WT
DKO
E)
Freq. of IL-17A+ in TCRγδint cellls
Freq. of IL-17A+ in CD4+TCRβ+ cellls
Freq. of IL-17A+ in CD4- TCRβ+ cellls
F)
Untreated
3xAld
GR1hi 1,400
GR1int 6,358
GR1hi 20,54
GR1int 23,86
GR1hi 0,913
GR1int 2,499
GR1hi 10,22
GR1int 24,71
WT
DKO
GR-1
Siglec-F
Freq. of GR1hi in alive cells
Freq. of GR1int in alive cells
***
**
***
**
ns
**

Figure 6: Early neutrophil recruitment to the skin is reduced in IL-36α/γ DKO mice upon Ald treatment at D3.

A. Experimental protocol. Wild-type (WT) and IL-36α/γ DKO mouse ears were topically treated with Aldara (Ald) or untreated (NT) every day from day 0 (D0) to D2. Mouse ears were analysed at D3.

B. H&E staining from WT-NT, DKO_NT, Ald-treated WT and Ald-treated DKO mice. Scale bar: 100µm.

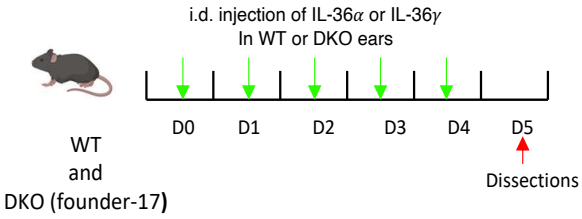
C. Quantitative RT-PCR analyses of ears. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n=4) * $p<0.05$, $p<0.01$, ***$p< 0.001$ (two-tailed Student's t test).

D. Frequency of $CD45^+$ (left), $CD3^{hi}$ (middle) and $CD3^{int}$ (right) among alive cells.

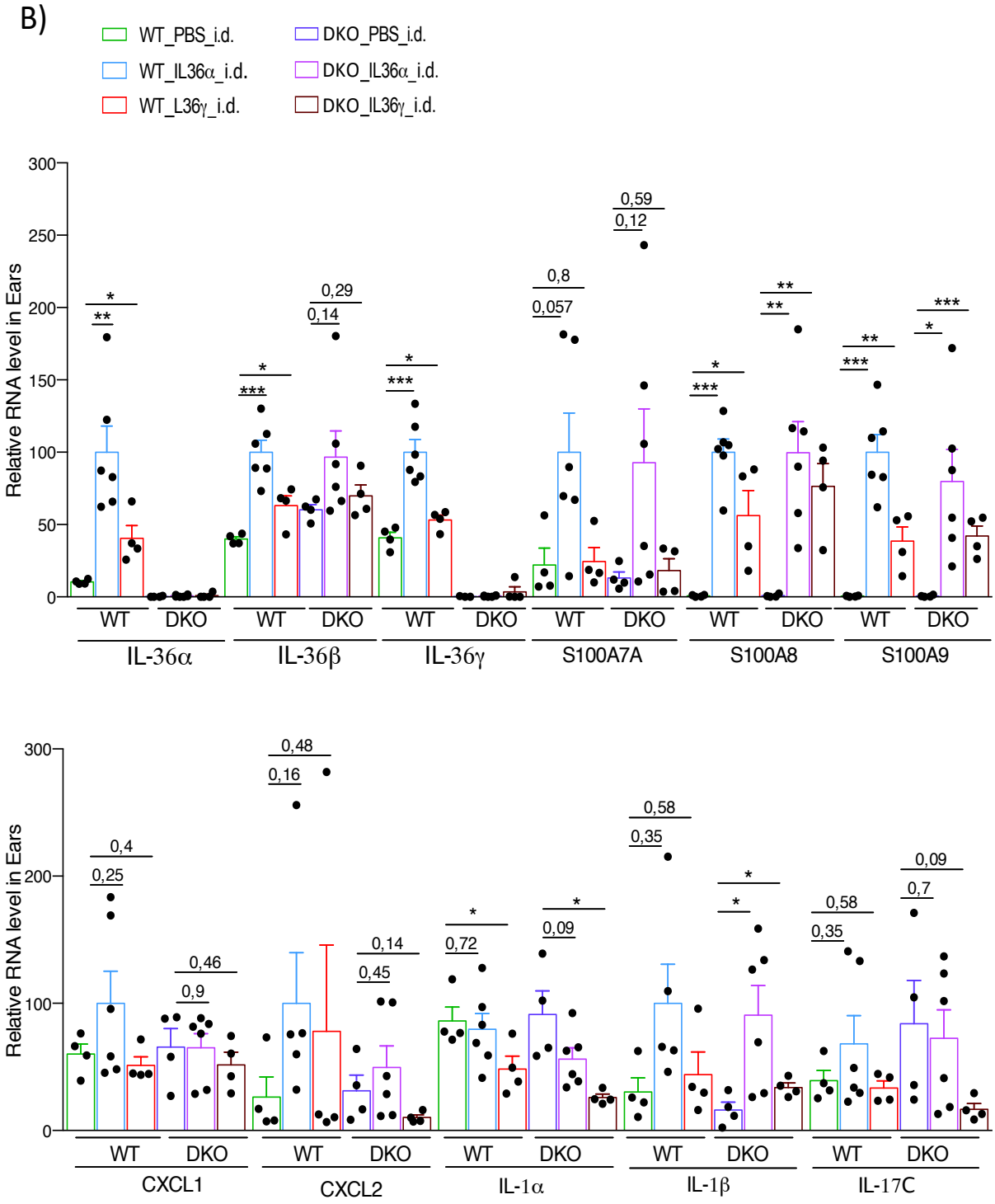
E. Frequency of $IL\text{-}17A^+$ among $TCR\delta\gamma^{int}$ (left) $TCR\beta^+CD4^+$ (middle) and $TCR\beta^+CD4^-$ (right) in ears.

F. Representative FACS plots (left) and Frequency of $GR1^{hi}$ and $GR1^{int}$ in alive cells (right). Values are mean ± SEM. (n=4) *$p< 0.05$; **$p< 0.01$; ***$p< 0.001$ (one-tail ANOVA test)

A)


i.d. injection of IL-36α or IL-36γ
In WT or DKO ears
D0
D1
D2
D3
D4
D5
Dissections
WT
and
DKO (founder-17)

B)


WT_PBS_i.d.
WT_IL36α_i.d.
WT_L36γ_i.d.
DKO_PBS_i.d.
DKO_IL36α_i.d.
DKO_IL36γ_i.d.
Relative RNA level in Ears
IL-36α
IL-36β
IL-36γ
S100A7A
S100A8
S100A9
CXCL1
CXCL2
IL-1α
IL-1β
IL-17C
WT
DKO

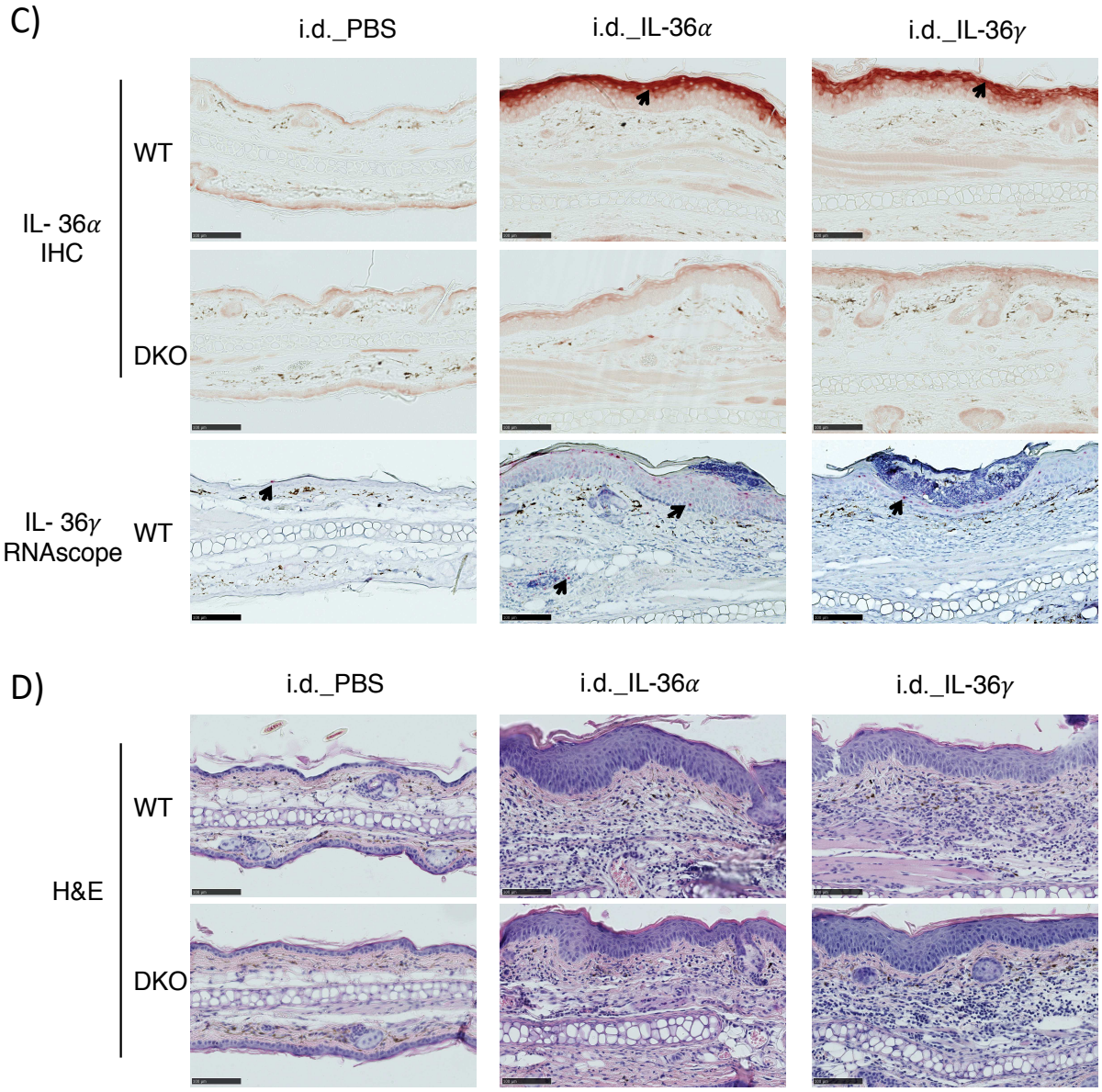
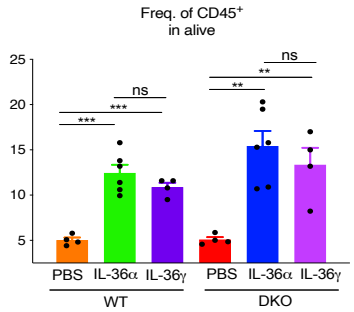


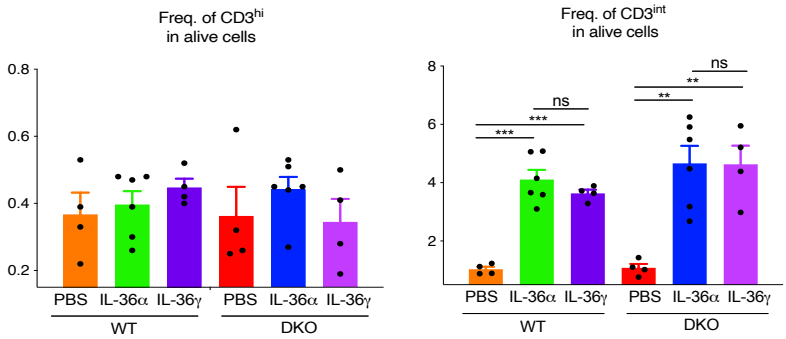
Figure 7: Intradermal (i.d) injection of IL-36α or IL-36γ promotes TCRγδ^{int}, CD4+TCRβ+ cells, neutrophils/monocytes/macrophages and eosinophils recruitment in both WT and IL-36α/γ DKO mice.

A. Experimental protocol. Wild-type (WT) or IL-36α/γ DKO mouse ears were injected intradermally (i.d.) during 5 days with PBS (vehicle) IL-36α or IL-36γ. The mice skin was analysed at D5.
B. Quantitative RT-PCR analyses of ears. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n≥4) * p<0.05, p<0.01, ***P< 0.001 (two-tailed Student's t test). Data are presentative of 2 independent experiments with similar results.
C. IHC staining with IL-36α antibody on ear sections from WT or DKO mouse ears injected with PBS (vehicle), IL-36α or IL-36γ Positive cells are in dark red. Scale bar:100µm. RNAscope in situ hybridization for IL-36γ probe (signals in red) in WT mouse ears injected with PBS (vehicle), IL-36α or IL-36γ, counterstained with hematoxylin. Scale bar:100µm.
D. H&E staining of ear sections from WT and DKO mouse ears injected with PBS (vehicle), IL-36α or IL-36γ Scale bar: 100 µm.

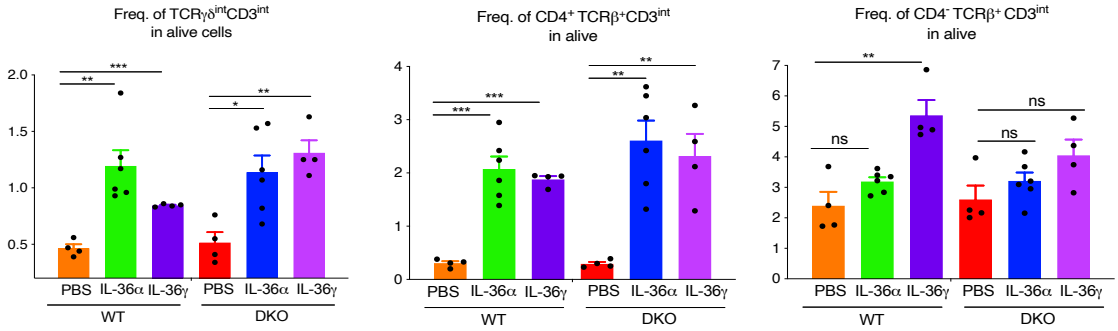
A)


Freq. of CD45+
in alive
PBS IL-36α IL-36γ PBS IL-36α IL-36γ
WT
DKO

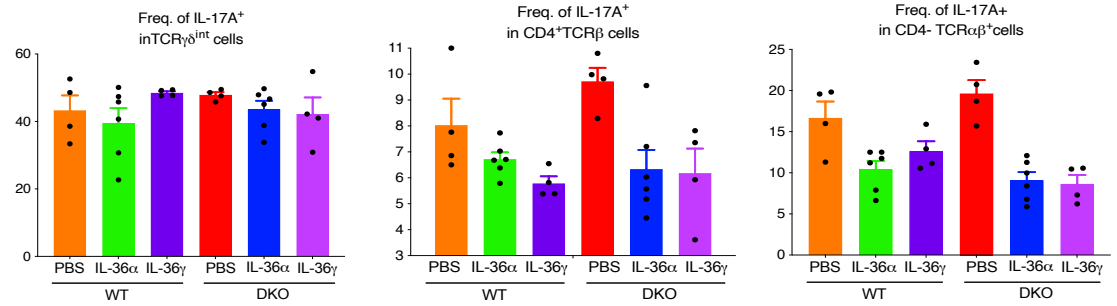
B)


Freq. of CD3hi
in alive cells
Freq. of CD3int
in alive cells
PBS IL-36α IL-36γ PBS IL-36α IL-36γ
WT
DKO

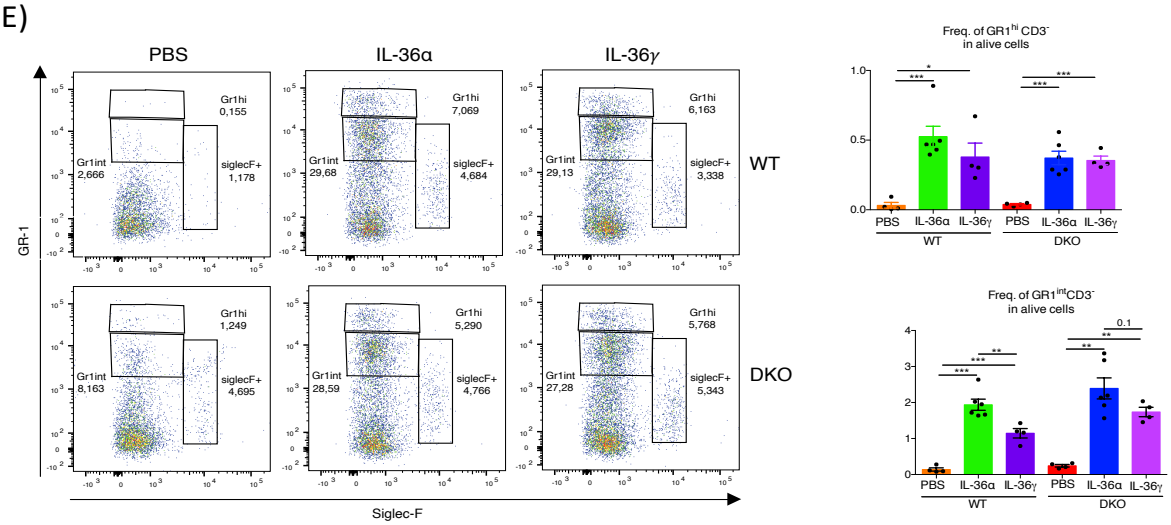
C)


Freq. of TCRγδintCD3int
in alive cells
Freq. of CD4+ TCRβ+CD3int
in alive
Freq. of CD4- TCRβ+ CD3int
in alive
PBS IL-36α IL-36γ PBS IL-36α IL-36γ
WT
DKO

D)


Freq. of IL-17A+
inTCRγδint cells
Freq. of IL-17A+
in CD4+TCRβ cells
Freq. of IL-17A+
in CD4- TCRαβ+cells
PBS IL-36α IL-36γ PBS IL-36α IL-36γ
WT
DKO

E)


PBS
IL-36α
IL-36γ
GR-1
Siglec-F
WT
DKO
Gr1hi 0,155
Gr1int 2,666
siglecF+ 1,178
Gr1hi 7,069
Gr1int 29,68
siglecF+ 4,684
Gr1hi 6,163
Gr1int 29,13
siglecF+ 3,338
Gr1hi 1,249
Gr1int 8,163
siglecF+ 4,695
Gr1hi 5,290
Gr1int 28,59
siglecF+ 4,766
Gr1hi 5,768
Gr1int 27,28
siglecF+ 5,343
Freq. of GR1hi CD3-
in alive cells
Freq. of GR1intCD3-
in alive cells
PBS IL-36α IL-36γ PBS IL-36α IL-36γ
WT
DKO

F)

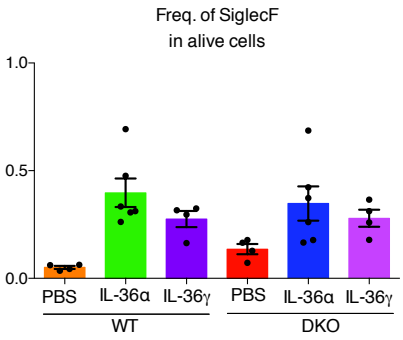


Figure 8: Intradermal (i.d) injection of IL-36α or IL-36γ promotes TCRγδ^{int}, CD4+TCRβ+ cells, neutrophils/monocytes/macrophages and eosinophils recruitment in both WT and IL-36α/γ DKO mice.

A. Frequency of CD45+ cells among alive cells in ears.

B. Frequency of CD3^{hi} (left) CD3^{int} (right) among alive cells in ears.

C. Comparation of the Frequency of TCRδγ^{int} (left), TCRβ^{+}CD4^{+} (middle) and TCRβ^{+}CD4^{-} (right) among alive cells in ears.

D. Frequency of IL-17A^{+} among TCRδγ^{int} (left), TCRβ^{+}CD4^{+} (middle) and TCRβ^{+}CD4^{-} (right) in ears.

E. Representative FACS plot (left) and comparation of the Frequency (right) of GR1^{hi} and GR1^{int}in alive cells.

F. Frequency of SiglecF^{+} among alive cells in ears.
Values are mean ± SEM. (n=4) *p< 0.05; **p< 0.01; ***p< 0.001 (one-tail ANOVA test).

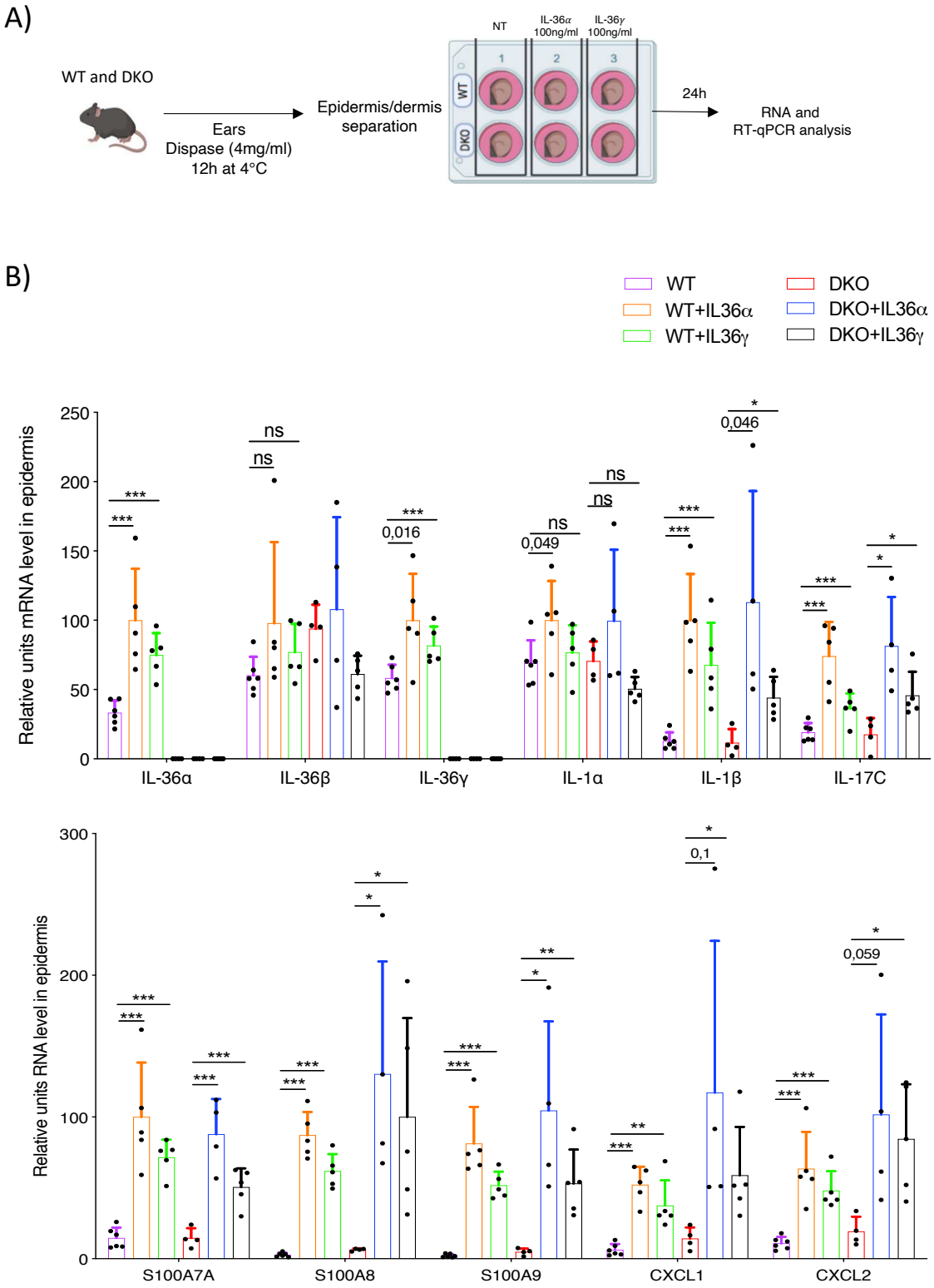
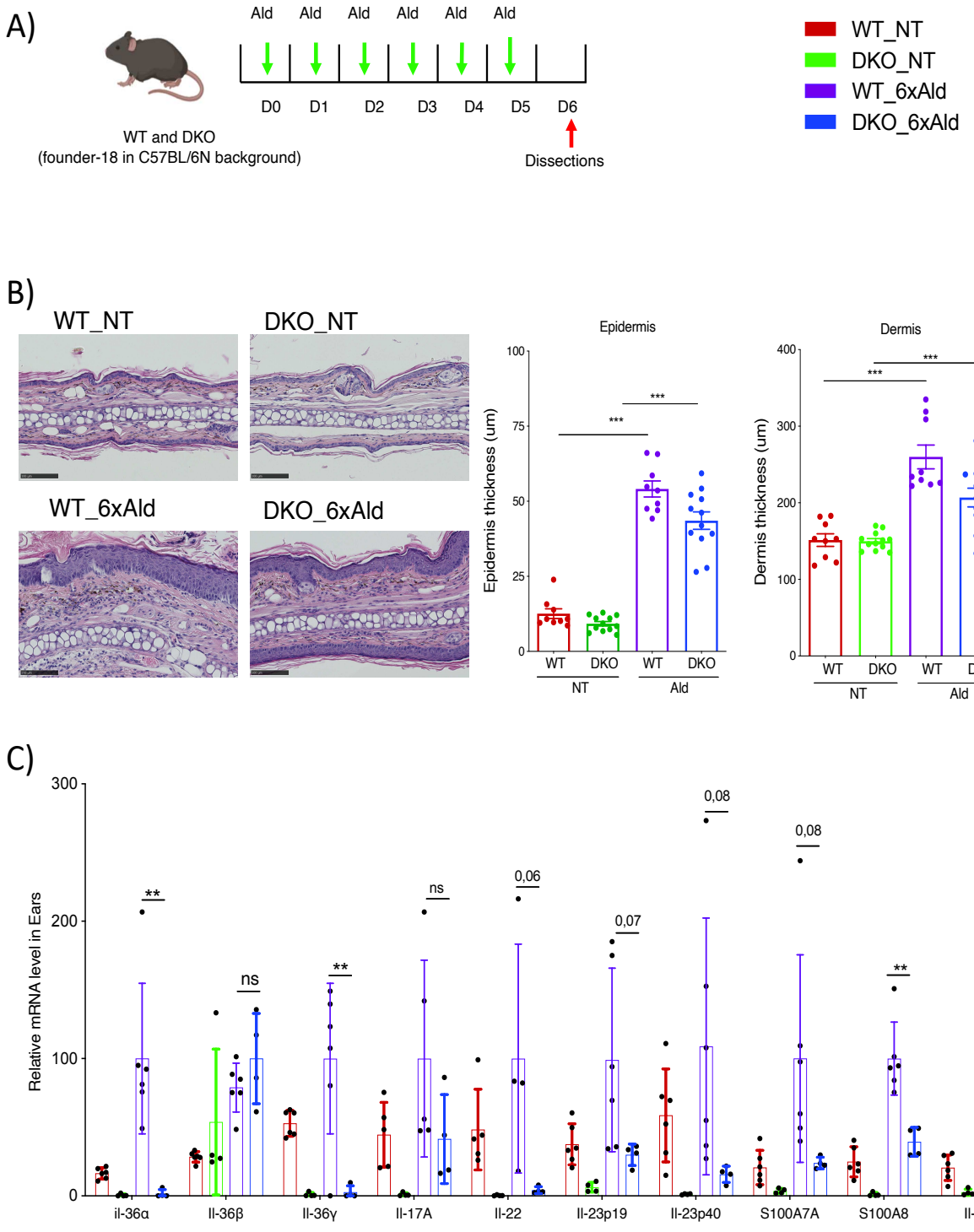


Figure 9: IL-36*α* or IL-36*γ* promotes the expression of CXCL1, CXCL2, IL-1*β* and S100 family genes in ex-vivo cultured epidermis from both WT and IL-36*α*/*γ* DKO mice.

A. Experimental protocol. Epidermis was isolated from WT and IL-36*α*/*γ* DKO mouse ears, cultured ex vivo 24h in the absence or presence of IL- 36*α* or IL-36*γ*.

B. Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. *p<0.5, **p<0.01, ***p< 0.001 (two-tailed Student's t test). Data are representative of 3 independent experiments with similar results

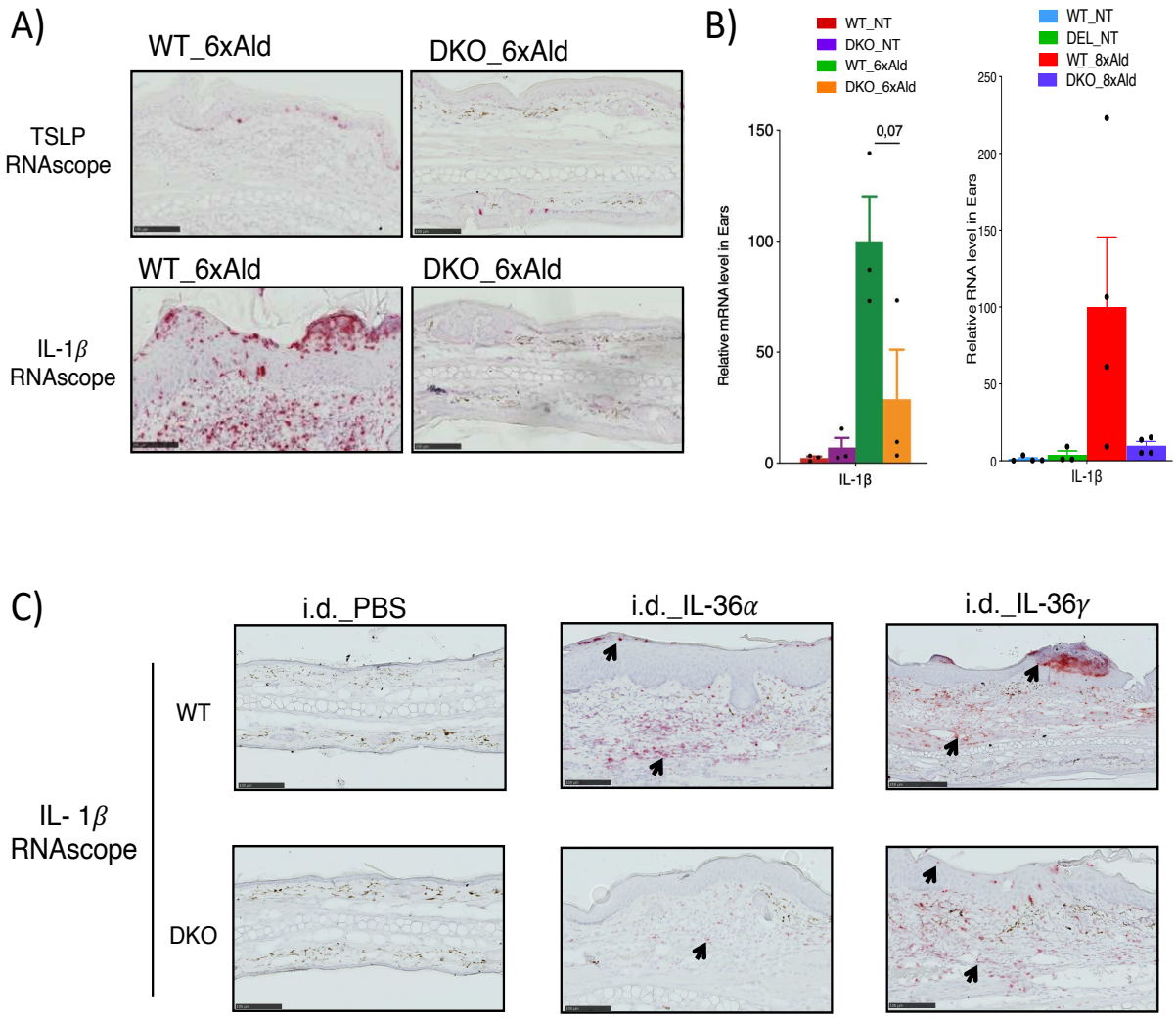


Supplemental Figure 1: IL-36α/γ DKO mice derived from founder-18 exhibit an attenuated psoriasis-like skin inflammation induced by Ald.

A. Experimental protocol. C57BL/6N-18 Wild-type (WT) and IL-36α/γ DKO mouse ears were topically treated with Aldara (Ald) or untreated (NT) every day from day 0 (D0) to D5. Mouse ears were analysed at D6.

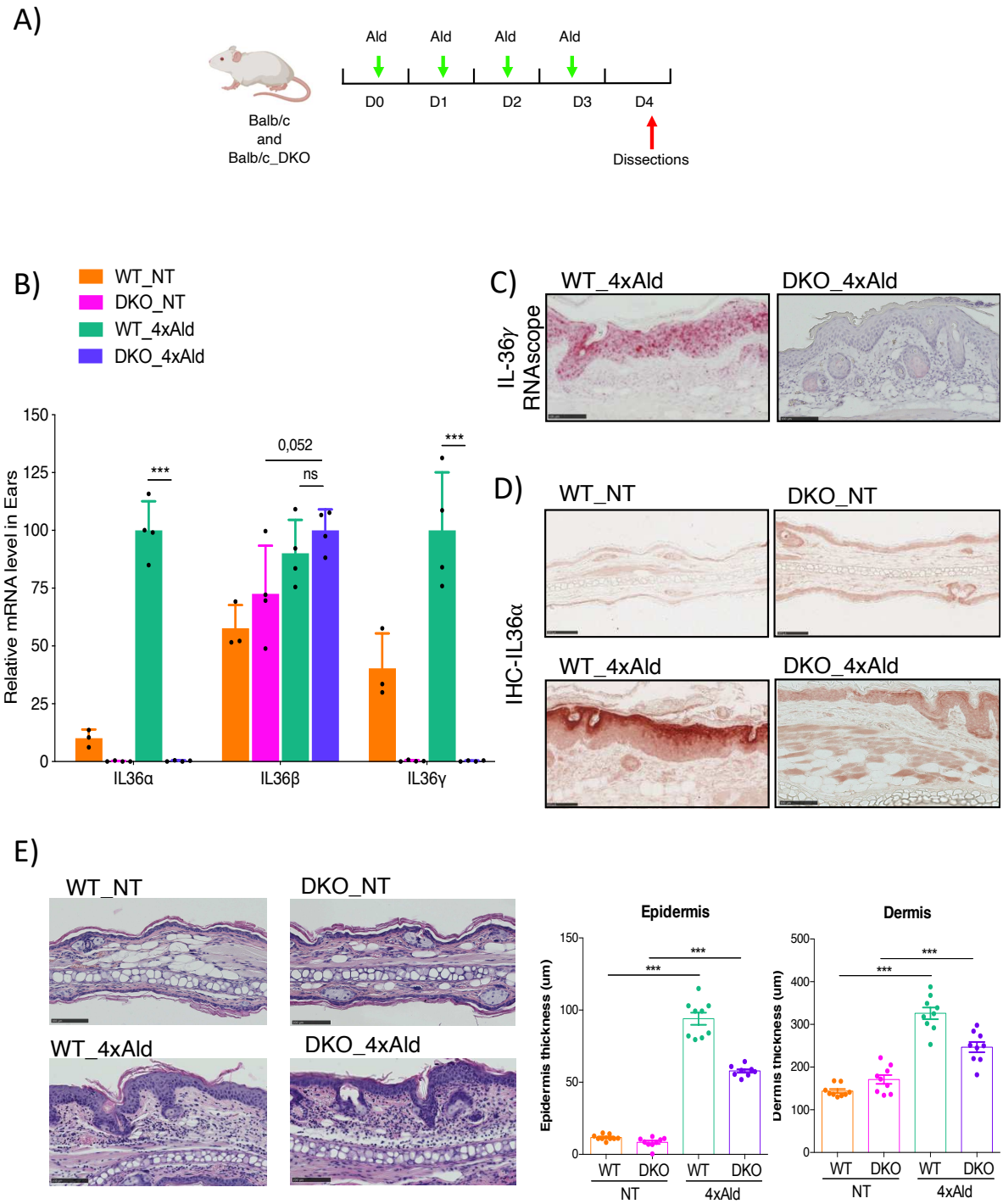
B. H&E staining and epidermal and dermal measurements of ear sections. Scale bar: 100µm. Values are mean ± SEM. ***P< 0.001 (two-tailed Student's t test).

C. Ears qPCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n≥4) **p< 0.01 (two-tailed Student's t test).



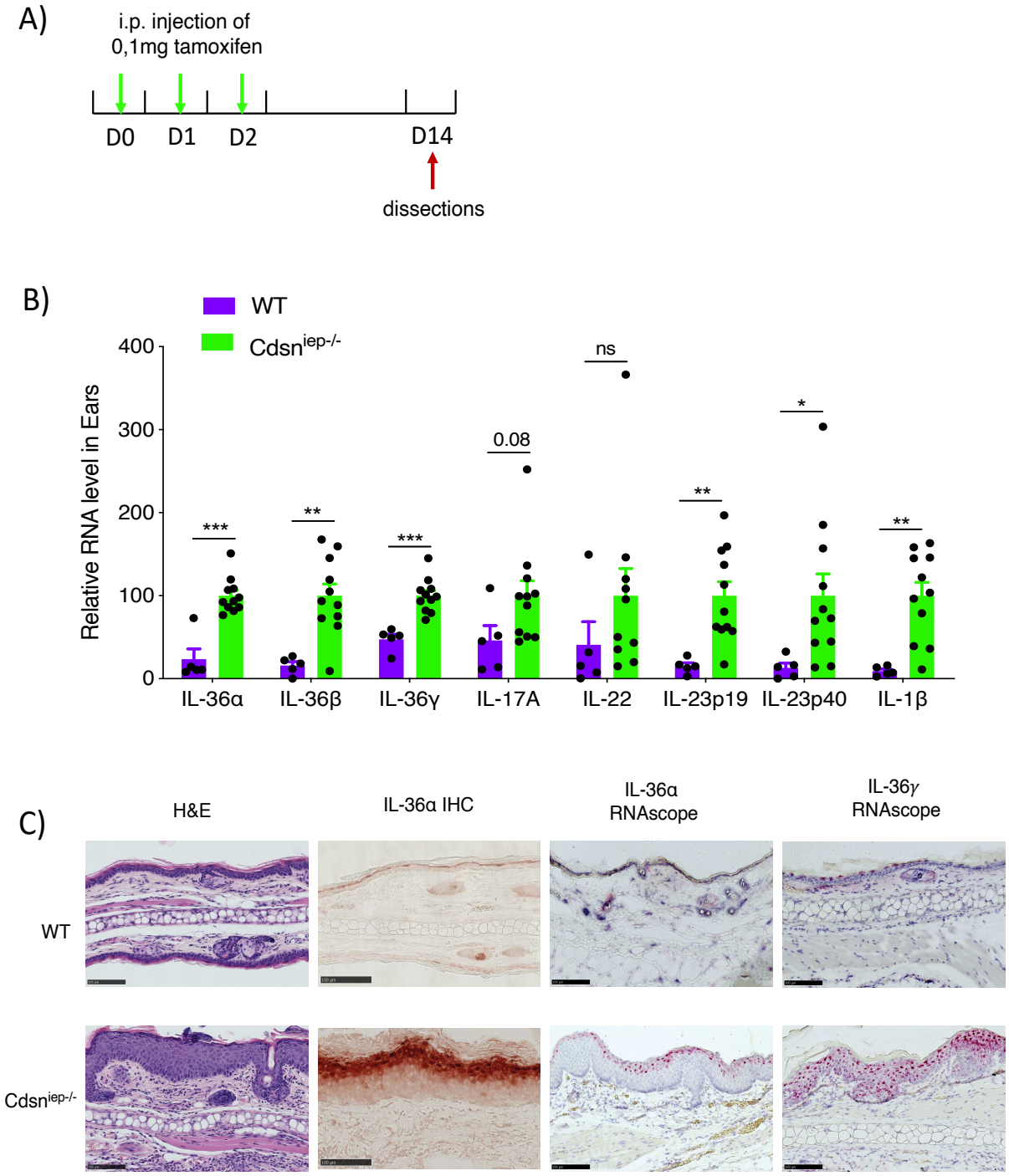
Supplemental Figure 2: IL-1β in DKO mice is reduce in psoriasis-like skin inflammation induced by Ald and is induced after i.d. injection of IL-36α or IL-36γ.

A. Wild-type (WT) and IL-36α/γ DKO mouse ears were topically treated Aldara (Ald) or untreated (NT) every day from D0 to D5. Mouse ears were analysed at D6. RNAscope in situ hybridization for mIL1β and mTSLP probe (signal in red) in Ald-treated WT or IL-36α/γ DKO mouse sections. Black arrows point to one of the positive signal. Counterstained with hematoxylin. Scale bar: 100µm (left). Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are ±SEM. (n=3). (two-tailed Student's t test) (Right).
B. Wild-type (WT) and IL-36α/γ DKO mouse ears were topically treated Aldara (Ald) or untreated (NT) every day from D0 to D7. Mouse ears were analysed at D8. Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n=4)
C. RNAscope in situ hybridization for mIL1β probe (signal in red) in Ald-treated WT or IL-36α/γ DKO mouse sections. Black arrows point to one of the positive signal. Counterstained with hematoxylin. Scale bar: 100µm



Supplemental Figure 3: Balbc IL-36*α*/*γ* DKO mice exhibit the simultaneous deletion of *Il1f6* and *Il1f9* genes.

A. Experimental protocol. Balb/c Wild-type (WT) and DKO mouse ears were topically treated Aldara (Ald) or untreated (NT) every day from D0 to D3. Mouse ears were analysed at D4.
B. Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM (n=4). ***p<0.001 (two-tailed Student's t test).
C. RNAscope in situ hybridization with IL-36*γ* probe (signals in red) on mouse sections, counterstained with hematoxylin. Scale bar: 100**µ**m
D. IHC staining with IL-36*α* antibody on ear sections. Positive cells are in dark red. Scale bar:100**µ**m
E. H&E staining and epidermal and dermal measurements of ear sections. Scale bar: 100**µ**m. Values are mean ± SEM. ***p<0.001 (two-tailed Student's t test).



Supplemental figure 4: Expression of IL-36α and IL-36γ is induce in the skin of mice with the ablation of CDSN in KCs.

A. Experimental protocol. Balbc K14-Cre-ER$^{T2(0/0)}$/Cdsn$^{L2/L2}$ (WT littermates) and K14-Cre-ER$^{T2(tg/0)}$/Cdsn$^{L2/L2}$ mice were injected intraperitoneally with tamoxifen (0.1 mg in 100 µL of sunflower oil) once per day from day 0 (D0) to D2 to generate Cdsn$^{iep-/-}$ mice. Mouse ears were analyzed at D14.

B. Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. Values are mean ± SEM. (n≥4). *p<0.5, **p<0.01, *** p<0.001 (two-tailed Student's t test).

C. H&E staining, IHC staining with IL-36α antibody (signal in brown) and RNAscope in situ hybridization for mIL-mIL-36 α and 36γ probe (signals in red) on ear sections from WT and Cdsn$^{iep-/-}$ mice. Scale bar: 100µm.

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Counterregulation between thymic stromal lymphopoietin– and IL-23–driven immune axes shapes skin inflammation in mice with epidermal barrier defects. J Allergy Clin Immunol. 2016; 138(1):150-161.e13.

RESULTS
PART 3

PART 3

Study of the role of keratinocyte-produced cytokine thymic stromal lymphopoietin in cutaneous melanoma

Collaborating project with Yao. W.

-INTRODUCTION

Malignant skin cancers include melanoma, basal cell carcinoma and squamous cell carcinoma. Melanoma, arisen from melanocytes, is the most aggressive, is treatment-resistant, and is often related with fast progression and a strong tendency for early metastasis (1). Melanoma represents only 4% of skin cancer cases but results in 75% of skin cancer deaths (2). The skin is constantly exposed to multiple complex agents which are capable of triggering the initiation and progression of cancer. These include UV radiation, one of the most important risk factors, as well as chemical products, drugs, stress, and genetic mutations (3). Melanocytes are principally resident in the basal layer of the epidermis and produce melanin which reduces the penetration of damaging UV radiation. The complex interplay of the variety of melanoma-causing factors promotes the transformation of normal melanocytes into melanoma cells. These cells lack the adhesion receptor and have motility, which allows the interaction of melanoma cells with other cells present in the microenvironment (e.g. fibroblasts, endothelial cells, immune cells, soluble molecules, and the extracellular matrix (ECM)), further promoting melanoma cell progression and metastasis (4).

Melanoma progression is divided into five stages. At stage 0 (in-situ melanoma), the melanoma is located as a nevus in the epidermis layer. The transformed melanocytes can progress to stage I with cell invasion and ulcerations. When the tumor progresses to stage II, they invade the vascularized dermis. In stage III, melanoma cells spread to the regional lymph nodes, and at stage IV the distal metastases appear (5). Melanoma presents the highest genomic mutational load amongst all human tumors, with a frequency of somatic mutations ranging widely from 0.1 to 100/Mb (6). The most recurrent somatic mutations in human melanomas include genes that are involved in key signaling pathways of cell proliferation including BRAF (~50% of patients), NRAS (15-20% of patients) and NF1(~ 10% mutually exclusive with BRAF), growth and metabolism (PTEN and c-Kit presented in 30-35% of patients), cell identity (AT-rich interaction domain 2 (ARID2)), resistance to apoptosis (TP53), cell cycle control (cyclin-dependent kinase inhibitor 2A (CDKN2A)), replicative lifespan (telomerase reverse

transcriptase (TERT)) and mutations in nucleotide excision repair (NER) pathways (DNA lesions produced by UV are removed by NER mechanisms. The lack of functional NER pathways outcome with accumulation of UV or chemically induced mutations in the skin and increase the risk of skin malignancies development) (7).

It is well established that solid melanoma is surrounded by different cell types forming the ECM, which include cancer-associated fibroblast (CAFs), endothelial and immune cells such as MΦ, neutrophils, mast cell, DCs, NKs and lymphocytes, creating a heterogeneous tumor microenvironment, or stroma, in which the tumoral cells establish interactions to promote their proliferation and progression (8), (9), (10). It has been recognized that immune cells in the cancer-associated microenvironment are related to both cancer-promoting and inhibiting roles (11), (12). A predominant T helper type 2 (Th2) inflammatory phenotype has been shown in certain types of cancers such as breast and pancreatic cancer (13), (14), where it is suggested to create a tumor-permissive microenvironment. This Th2 phenotype is associated with a worse prognosis in comparison with tumors in which Th1 type responses are predominant. Additionally, Treg one subset of helper T-cells, which regulate immune response and maintain the systemic homeostasis, are used by a large variety of tumors to dampen antitumor immunity (see review Sharma and Rudra (15)). Despite the prognostic significance of Tregs in cancer remains controversial, it has been reported that the increase of Tregs infiltration is a poor prognosis-associated factor in several human solid tumors (16).

In the context of melanoma, it has been reported, Th2 polarization, such as the up-regulation of Th2 type cytokines (IL-4 and IL-13) in the plasma and sentinel lymph nodes of melanoma patients (17), (18), (19). In addition, it has been reported the abundant presence of Treg cells in different types of cancer including melanoma which express surface CTLA-4 at higher levels than tumor-infiltrating $CD8^+$ T cells (20), (21). However, the characterization and the role of local and systemic Th2 and Treg in melanogenesis are unclear. Moreover, the reciprocal interaction between melanoma cancer cells and their microenvironment including KCs and immune cells remains largely unknown.

Previous studies of my team have established a central role of thymic stromal lymphopoietin (TSLP), a cytokine produced by KCs, in instructing Th2 inflammatory response in skin (22), (23), (24), (25). Interestingly, TSLP was recently implicated in carcinogenesis. However, the expression and implication of TSLP in tumor growth and metastasis is controversial. For example, it was shown that TSLP stimulated the growth and progression of

pancreatic cancer (26) and breast cancer (13) by activating DCs and promoting Th2 cell differentiation. On the other hand, two studies related with skin cancer have suggested that the TSLP suppress tumor progression using a mouse model with KC-specific ablation of Notch signalling, in which TSLP is overexpressed (27), (28). In the context of cutaneous melanoma, the expression and the role of TSLP were unknown.

In collaboration with the team of I. Davidson (IGBMC), a former PhD student in our lab, W. Yao, initiated the study of the role of TSLP in melanoma growth and progression using a melanoma mouse model in which tamoxifen induces melanocyte-specific *Braf*^{V600E} mutation with Pten silencing (*Braf/Pten*). The previous study of W. Yao showed that TSLP production was induced in the skin epidermis overlaying melanoma from *Braf/Pten* mice and TSLP levels in serum increased along with melanogenesis. In addition, she observed the increase of TSLP in human primary and metastatic cutaneous melanoma. Furthermore, she showed that the ablation of TSLP in Braf/Pten mice delayed the growth and progression of melanoma associated with a reduced Th2 immune response, whereas the overexpression of TSLP had the opposite effect.

My contribution to this project was to explore questions related with the role of TSLP in Braf/Pten melanoma development. First, I used 4-Hydroxytamoxifen (4-HT), the active form of tamoxifen, to induce a localized tumor allowing better comparison of the tumor growth in Braf/Pten/Tslp$^{+/+}$ and Braf/Pten/Tslp$^{-/-}$ mice. In addition, I collaborated with W. Yao to analyse Th2 and Treg cells associated with mouse melanoma using IHC staining, RT-qPCR and flow cytometry. Moreover, following the previous results obtained by W.Yao showing that the induction of TSLP was predominantly detected in the melanoma-overlaying skin epidermis in Braf/Pten/Tslp$^{+/+}$ mice, we found that grafting of mouse B16F10 or human melanoma cells (Lu1205) induced TSLP expression in the epidermis of NSG mice and further tested whether such induction was mediated by IL-1β.

MATERIALS AND METHODS

Mice and genotyping

Mice bearing the *Tyr::Cre*-ER^{T2} transgene, *Braf*$^{LSL\text{-}V600E/+}$ (13), floxed alleles of *Pten* (A. Suzuki et al., 2001) and *Tslp*$^{-/-}$ (Li et al., 2009) were all kept in C57BL/6J background, and were bred to generate experimental mice with the desired genotypes. Wild type C57BL/6J, NOD.*Cg*-P*rkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) were from Jackson Laboratory. Breeding and maintenance of mice were performed under institutional guidelines, and all of the animal experiments were performed in accordance with European and national guidelines and policies and with approval of the National Ethics Committee.

Tumour induction

8-12-week-old *Tyr::Cre*$^{ERT2(tg/0)}$*::Braf*$^{LSL\text{-}V600E/+}$ and *Tyr*::Cre$^{ERT2(tg/0)}$:: *Braf*$^{LSL\text{-}V600E/+}$: *Pten*$^{lox/lox}$ mice were topically treated with 2 µL of 4-hydroxytamoxifen (4-HT, Sigma, # H6278) (4 mg/mL solution in 100% ethanol) on the dorsal side of ears and in 1cm^2 of dorsal skin for 3 consecutive days to generate Braf and Braf/Pten mice respectively. Tumor growth was monitored twice weekly by digital photography of the skin.

Melanoma cell grafting

Mouse melanoma B16F10 cell line provided by Dr. Lionel Larue (Institut Curie, France) and Lu1205 human cell line derived from human melanoma patients provided by Dr. Irwin Davidson (IGBMC) were used for the grafting experiments. 5x10^4 B16F10 mouse melanoma and 2X10^4 Lu1205 human melanoma cells in 20µl of PBS were injected intradermally (i.d.) into the ears of NSG mice. Tumor growth was monitored twice weekly by digital photography of the skin and analyzed using ImageJ software.

MC903 topical application

MC903 (Calcipotriol, Sigma, Cat No. C4369) was dissolved in 100% ethanol and topically applied on mouse ears (2nmol in 25ul per ear) as described before (Li et al., 2006) (Li et al., 2009).

Quantitative RT-PCR

RNA was extracted using TRI Reagent (Sigma-Aldrich). RNA was reverse transcribed by using random oligonucleotide hexamers and amplified by means of quantitative PCR with a LightCycler 480 (Roche Diagnostics, Indianapolis, Ind) and the QuantiTect SYBR Green kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Relative RNA levels were calculated with hypoxanthine phosphoribosyl- transferase as an internal control. For analyses of each set of gene expression, an arbitrary unit of 1 was given to the samples with the highest level, and the remaining samples were plotted relative to this value. Sequences of PCR primers are shown in **Table 1**.

Table 1: Primer sequences

Gene name	Sequence 5' to 3'	
mHPRT (161 bp)	F	TGGATACAGGCCAGACTTTG
	R	GATTCAACTTGCGCTCATCTTA
mIL-4 (132 bp)	F	GGCATTTTGAACGAGGTCAC
	R	AAATATGCGAAGCACCTTGG
mIL-5 (117 bp)	F	AGCACAGTGGTGAAAGAGACCTT
	R	TCCAATGCATAGCTGGTGATTT
mIL-13 (142 bp)	F	GGAGCTGAGCAACATCACACA
	R	GGTCCTGTAGATGGCATTGCA
mIL-17A (217 bp)	F	CCAGGGAGAGCTTCATCTGT
	R	ACGTGGAACGGTTGAGGTAG
mIFN-γ (238bp)	F	AACGCTACACACTGCATCTTGG
	R	GACTTCAAAGAGTCTGAGG
mMCPT8 (160 bp)	F	GTGGGAAATCCCAGTGAGAA
	R	TCCGAATCCAAGGCATAAAG
mST2 (173bp)	F	TCAATTCACACACGCGGAGA
	R	CCAAAGCAAGCTGAACAGGC
mFoxP3 (bp)	F	TTCATGCATCAGCTCTCCAC
	R	CTGGACACCCATTCCAGACT
mIL-2R (181bp)	F	AACTCCTGGAGCAGCAACTG
	R	TGTTTCCAAGGAGGTGGCTC

F: forward sequence; R: reverse sequence.

Histopathology

Mouse ears were fixed overnight at room temperature in 10% neutral buffered formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin.

Immunohistochemistry and immunofluorescence staining

For immunohistochemistry (IHC) staining of Foxp3, paraffin sections were treated with 0.6% H_2O_2 (Sigma, Cat No. H3410) to block endogenous peroxidase activity before antigen retrieval with citric buffer (10 mmol/L citric acid, pH 6). Slides were then blocked with normal rabbit (Vector Laboratories) incubated with primary antibody (dilution: 1/100). Then the slides were incubated with biotinylated rabbit anti-rat IgG (dilution: 1/300) and treatment of AB complex (Vector Laboratories). Staining was finally visualized with AEC high-sensitivity substrate chromogen solution (Dako, Ref. K3469) and counter- stained with hematoxylin.

RNA *in situ* hybridization (RNAscope)

RNA *in situ* hybridization was performed on freshly prepared 5 µm paraffin sections with the RNAscope 2.5 FFPE Red detection Kit (Advanced Cell Diagnostics, Hayward, CA, USA), according to the manufacturer's protocol. To confirm mRNA integrity in the paraffin sections, Mm-Ppib probe (Mus musculus peptidylprolyl isomerase B; Cat No. 313917) was used as a positive control (Bingham et al., 2017). DapB probe (Bacterial Bacillus subtilis dihydrodipicolinate reductase; Cat No. 310043) was used as negative control. RNAscope Probe-Mm-TSLP (Cat No. 432741) and Mm-IL1β (Cat No. 316891) were used for detection of TSLP or IL1β respectively.

Cell preparation and Flow cytometry analysis

For skin cells preparation, ears were split into ventral and dorsal halves and incubated in small pieces 1h30min at 37ºC with 2,5mg/ml Liberase Thermolysin Low (0,25mg/ml) (Roche, #0540120001) and 0,25mg/ml DNase I (sigma) in RPMI medium. In the other hand for tumor cells preparation, tumors were cut and incubated 1h at 37ºC with the solution of the tumor dissociation kit, mouse (Miltenyi Biotec #130-096-730) diluted in RPMI medium. After the enzymatic digestion, ear, tumoral or LNs cells were passed through a 70um strainer (Falcon) and resuspended in PBS containing 0,5% BSA and 2mM EDTA. Cells were then centrifuged at 350g and resuspended in PBS containing 1% FCS and 2mM EDTA, counted and used for FACS staining.

Surface staining for flow cytometry

To perform flow cytometry analysis, 2 million cells were used for antibody (Ab) staining. Cells were first incubated with anti-CD16/CD32 AB to block unspecific binding, followed by surface markers staining with fluorochrome-conjugated antibodies (**Table 2**) in 25µl of FACs buffer (1% of FCS+2mM EDTA in PBS) for 10 minutes at 4ºC. Viability staining was performed by adding propidium iodide to a final concentration of 1µg/mL just prior passing the cells to the cytometer.

Intracellular staining

For intracellular staining of Foxp3 and GATA3, 3 million of cells were treated with the Foxp3/Transcription factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Briefly, after surface staining, cells were fixed and permeabilized by 30 minutes of incubation of ice in Foxp3 Fixation/permeabilization working solution. After two washes with 1X Permeabilization Buffer using centrifugation for 5 minutes at 500g, cells were resuspended in PBS containing fluorochromes labeled antibodies against intracellular markers (**Table 2**) and incubated 30 minutes at room temperature. After two additional washes with 1x Permeabilization Buffer using centrifugation for 5 minutes at 500g, cells were resuspended in PBS containing 1% FCS and 2mL of EDTA. Viability staining was performed by incubating the cells with Fixable Viability Dye 506 (eBiocience) for 30 min at 4ºC prior to the surface staining.

Table 2: antibodies used for flow cytometry experiments

Marker	Fluorochrome	Clone	Company	Quantity
CD3	FITC	145-2C11	eBioscience	0,5:25
CD45	APC-eFluor780	30-F11	eBioscience	0,06:25
CD4	AF700	GK1.5	BD Biosciences	1:25
CD8a	PerCP-Cy5.5	53-6.7	eBioscience	0,5:25
T1/ST2 (IL33R)	FITC	DJ8	MDBioproducts	0,5:25
FoxP3	APC	FJK-16s	eBioscience	1,5:100
GATA3	PE-Cy7	TWAJ	eBioscience	5:100

Results
Part 3

Ex vivo culture of mouse ear epidermis

Ears from adult mice were disinfected with 10% povidone-iodine solution, and then washed in distilled water and 70% ethanol. Ears were then separated with forceps and floated in a 4mg/ml of Dispase (Gibco) in PBS in a sterile petri dish, with the dermis side down, and incubated overnight at 4ºC. After the dermis was removed, epidermis was washed in PBS and then cultured by floating in a medium without carbonate prepared as described Mazzalupo et al., (29): Dulbecco's minimal Eagle's medium (Life Technologies) was mixed with Dulbecco's minimal Eagle's medium/F-12 1:1(v/v) mixture (Life Technologies) and then adjusted to pH 7.2. The medium was supplemented with 10% fetal bovine serum (DUTSCHER), cholera toxin (0.1 nM) (SIGMA), mouse epidermal growth factor (10 ng/ml) (SIGMA), 3,3',5'triiodo-t-thyronine (2 nM) (SIGMA), human apo-transferrin (5 µg/ml) (SIGMA), human insulin (5 µg/ml) (SIGMA), hydrocortisone (0.4 µg/ml) (SIGMA), penicillin (60 µg/ml) (Life technologies), and gentamicin (25 µg/ml) (KALYS). mIL1β (Biolegend, #575104) at different concentration was added to the culture medium. After 24 hours of *ex vivo* culture, the epidermis was harvested for RNA extraction and RT-qPCR analyses.

Statistical analysis

Data were analyzed using GraphPad Prism and sigma plot by t-test or the Mann-Whitney rank sum test depending on results from the Kolmogorov-Smirnov test (with Lilliefors' correction) for normality and Levene Median test for equal variance. Data are presented as means and SEM. *P < 0.05, **P < 0.01, ***P < 0.001 was considered to be statistically significant.

RESULTS

FoxP3 expression in mouse inguinal-draining LNs and dorsal tumor induced by 4-HT is reduced upon the ablation of TSLP

In the present study, to explore the role of TSLP in mouse melanoma growth, Tyr::Cre$^{ERT2(tg/0)}$::Braf$^{LSL\text{-}V600E/+}$::Pten$^{lox/lox}$ (named as Braf/Pten/Tslp$^{+/+}$) (30) and Braf/Pten/Tslp$^{-/-}$ mice were topically treated with 4-HT. The tumor was locally induced in 1 cm^2 of the dorsal skin by 3 consecutive treatments of 4-HT, one per day from D0 to D2 (**Figure 1A**). This treatment induces the expression of oncogenic Braf^{V600E} in the melanocyte lineage (called Braf mice) and inactivated Pten in melanocytes simultaneously. Pigmented lesion and dorsal melanoma were developed and started to appear around 2 weeks after 4-HT treatment. Pigmented naevi started to generate a small solid tumor (diameter < 2mm) at day 25 (D25), then progressed rapidly to big hypopigmented solid tumors (2mm < d < 5mm) at D34, which continuously grew in size. The volume of the tumor was calculated using the ellipsoid volume formula (1/2 x L x W^2) (31) at D34, D38 and D41. Pigmented naevi appeared first in Braf/Pten/Tslp$^{+/+}$ mice (data not shown) and dorsal tumor grew faster than in Braf/Pten/Tslp$^{-/-}$ mice (**Figure 1B**). In addition, Braf/Pten/Tslp$^{+/+}$ mice presented more pigmented metastases in ingüinal-draining LNs (ILNs) than Braf/Pten/Tslp$^{-/-}$ mice at D44 (**Figure 1C**).

We and others have previously shown that TSLP is key driver in Th2 inflammatory response (22), (24), (25), (32). qPCR analyses of ILNs and dorsal tumors showed that at D44 the expression of Th2-associated markers including IL-13, CCL17, CCL22 was up-regulated in Braf/Pten/Tslp$^{+/+}$ mice compared with CT mice, and abolished in Braf/Pten/Tslp$^{-/-}$ mice. Interestingly, we observed that Treg markers IL2R and FoxP3 were also up-regulated in Braf/Pten/Tslp$^{+/+}$ mice (**Figure 1D and 1E**) and diminished in Braf/Pten/Tslp$^{-/-}$ mice (**Figure 1D and 1E)**. Moreover, IHC analyses showed a reduction of Foxp3+ cells in dorsal melanoma of Braf/Pten/Tslp$^{-/-}$ compared with those of Braf/Pten/Tslp$^{+/+}$ mice (**Figure 1F**).

Taken together, these results show that, Tregs are induced in Braf/Pten/Tslp$^{+/+}$ but reduced upon the ablation of TSLP.

Results
Part 3

Treg immune cells associated with Braf/Pten/Tslp$^{+/+}$ melanoma induced by 4-HT are reduced upon the ablation of TSLP in ILNs and dorsal tumors at D44

To further determine the role of TSLP ablation in Braf/Pten/ Tslp$^{+/+}$ mice, we performed flow cytometry analysis of ILNs and dorsal tumors at D44. In agreement with the Foxp3^{+} qPCR data (**Figure 1B**), intracellular staining of Foxp3 showed that Braf/Pten/Tslp$^{+/+}$ mice presented an increase in the frequency of FoxP3^{+} cells in ILNs compared with CT mice (**Figure 2A**), which was reduced in Braf/Pten/Tslp$^{-/-}$ mice (**Figure 2A**).

Recently, a new subset of Treg expressing ST2, was described in mouse colon (33). Further studies have shown that these cells exhibit higher regulatory function compare to ST2^{-} Tregs in vitro (34), (35). Furthermore, ST2^{+}Treg population has been identified in mouse with KRAS lung (36) and colorectal cancer (37). We then sought to examine whether ST2^{+}Treg cells where affected upon TSLP ablation in our Braf/Pten/Tslp$^{+/+}$ mouse model. We observed that inside FoxP3^{+} cells both Tregs including ST2^{+}Foxp3^{+} (ST2^{+}Treg) and ST2^{-}Foxp3^{+} (Treg) among CD4^{+} cells and their total cell number were reduced in Braf/Pten/Tslp$^{-/-}$ mice (**Figure 2A**). Further characterization of ST2^{+}Foxp3^{+} cells showed that they express high level of GATA3^{+} (**Figure 2B**), as reported previously (33). Frequency of GATA3^{+}Foxp3^{+} and GATA3^{-} Foxp3^{+} among CD4^{+} cells and their total cell number were induced in ILN cells of Braf/Pten/Tslp$^{+/+}$ mice and abrogated in Braf/Pten/Tslp$^{-/-}$ mice (**Figure 2C**).

We also analyzed Tregs in 4-HT induced dorsal tumors at D44. Results showed that Braf/Pten/Tslp$^{+/+}$ mice presented higher frequency of CD4^{+}CD8^{-} cells than Braf/Pten/Tslp$^{-/-}$ mice. On the contrary, Braf/Pten/Tslp$^{-/-}$ mice tended to present higher frequency of CD4^{-}CD8^{+} among CD3^{+} cells (**Figure 3A**). In addition, in dorsal tumoros, Braf/Pten/Tslp$^{+/+}$ mice presented an increase in the frequency of FoxP3^{+} cells among CD4^{+} cells and total cell number compared with CT mice (**Figure 3B**), which tended be lower in Braf/Pten/Tslp$^{-/-}$ mice (**Figure 3B**). Moreover, in Braf/Pten/Tslp$^{+/+}$ mice, ST2^{+}Treg and ST2^{-}Treg cells were induced in comparation with CT mice, and Braf/Pten/Tslp$^{-/-}$ mice showed a reduction of ST2^{+}Treg cells (**Figure 3 B**).

Taken together, our analyses indicate that in the metastatic ILNs and dorsal turmors, Braf/Pten/Tslp$^{+/+}$ mice present an up-regulation of Treg cells including ST2^{+}Treg cells which are diminished upon TSLP ablation.

Topical MC903 treatment promotes ST2^{+}Treg cells in ears draining LNs (ELNs) and ears tumors of Braf/Tslp$^{+/+}$ in mouse melanoma

Previously, W. Yao showed that topical MC903 treatment (a low calcemic vitamin D analog), which has been reported by my lab to induce TSLP expression (38), promotes the development of pigmented lesions on the ears in Braf mice in a TSLP-dependent manner. Here, Braf and Braf/ Tslp$^{-/-}$ mice were topically treatment with 4-HT for 3 consecutive days (from D0 to D2), and from D4 the right ears (RE) were topically treated with MC903 to induce the overexpression of TSLP, whereas left ears (LE) were treated with ethanol (EtOH, vehicle control) (**Figure 4A**).

Intracellular staining of FoxP3 in ELNs at D32 showed that MC903-treated Braf mice did not present an increase of the frequency of FoxP3^{+} cells among CD4^{+} cells compared with CT mice (**Figure 4B**), but interestingly, frequency of ST2^{+}Treg (but not ST2^{-}Treg) among CD4+ cells was increased, which was reduced in MC903-treated Braf/Tslp$^{-/-}$mice (**Figure 4B**). In addition, the frequency of GATA3^{+}Foxp3^{+} among CD4^{+} cells was lower in MC903-treated Braf/Tslp$^{-/-}$ in comparison with MC903-treated Braf mice (**Figure 4C**).

Next, I performed the flow cytometry analysis in ears from MC903-treated Braf and Braf/Tslp$^{-/-}$ mice. In the contrary to ELNs, ear skin from MC903-treated Braf mice presented an increase of total Foxp3^{+} cells among CD4^{+} cells and total cell number compared with CT mice (less prominent than in Braf/Pten mice). In MC903-treated Braf mice, frequencies of ST2^{+}Foxp3^{+} (ST2^{+}Treg) and ST2^{-}Foxp3^{+} (Treg) among CD4+ cells and total cell number were increased; and both populations presented a tendency to be reduced in MC903-treated Braf/Tslp$^{-/-}$ mice. This suggest that the overproduction of TSLP in mouse melanoma induces ST2^{+}Treg cells in Braf mice, and this induction is abrogated upon TSLP ablation.

IL-1β alone does not have the capability to induce TSLP in mouse epidermal cultures

Using NSG mice grafted with B16F10, W. Yao showed that B16F10 cell grafting induced TSLP expression in the skin. I confirmed her results by RNAscope analyses (**Figure 5A**). Moreover, I showed that the grafting of Lu1205 human melanoma cells in NSG mice also induced TSLP production in epidermal KCs at RNA and protein level (**Figure 5C and 5D**).

These results suggest that possibly similar factors from mouse B16F10 and human Lu1205 induce TSLP expression in mouse KCs.

Recently, it has been reported that pancreatic cancer-derived IL-1β and TNF-α activate fibroblasts to produce TSLP (26). To examine whether IL-1β could be the factor promoting TSLP expression, I performed an IL-1β RNAscope analysis of NSG mice grafted with B16F10 which showed an induction of IL-1β expression by dermal cells, most probably neutrophil/monocyte/ macrophage cells. However, ex vivo culture of epidermis from WT mice with IL-1β did not induce TSLP expression in epidermal cells analyzed by qPCR (**Figure 7B**) and by ELISA with culture supernatants (**Figure 7C**). Therefore, other factors than IL-1β may mediate TSLP induction in the epidermis of mouse melanoma.

DISCUSSION AND PERSPECTIVES

Following the discovery by W. Yao showing that TSLP plays a tumor-promoting role for melanoma, we further showed that not only Th2 but also Treg ($FoxP3^+$ cells) are associated with Braf/Pten/$Tslp^{+/+}$ but are reduced in Braf/Pten/$Tslp^{-/-}$ mice. All these results together, suggest that a crosstalk between melanoma cells, skin epidermal KCs, and immune cells plays an important role for melanoma growth and metastasis.

Treg cells are a highly heterogeneous $CD4^+$ T cells, with the principal function of maintain immune homeostasis. However, nowadays it is well-establish that they can play an important role preventing antitumor responses (see review: Sharma and Rudra, (15)) and depending on the context Tregs would express different markers and exert different functions (39). A recent publication from Halim L; et al., presented a transcriptomic analysis of the different circulating Th-like Tregs cells classified based on the expression of chemokines receptors. They identified Th2-like Tregs cells (expressing CXCR4+ and $GATA3^+$) as the main Treg subset presented preferentially in malignant tissues but also in lower percentage in peripheral blood from melanoma and colorectal cancer patients. Moreover, $ST2^+$Treg population has been identified in several cancer mouse models such as KRAS lung cancer (36) and colorectal cancer (37). In agreement with these findings, our results showed that Treg cells including $ST2^+$Tregs are promoted by TSLP in Braf/Pten or Braf tumor and decreased upon ablation of TSLP. To have a better understanding of the role of these $ST2^+$Tregs, further characterization is currently performed in my lab including markers such as OX40, CCR8, the main chemokine receptor found in Treg isolated cells from tumor sites (40), (41) and TIGIT, a co-inhibitory molecule that inhibits Th1 and Th17 cells but not Th2 (42).

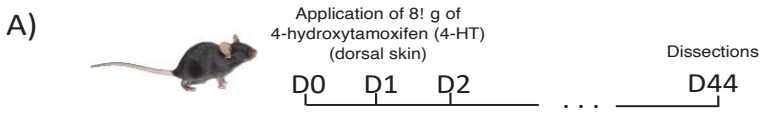
Our results showed that TSLP promotes both Th2 and Treg in melanoma. It was previously reported that the Th2 cytokine, IL-4 was up-regulated in the tumor draining LNs upon B16F10 melanoma cell intravenously injected mice, and the blockage of IL-4 in these mice reduces the size of the tumors developed in lungs (43). It remains to be determined whether Th2, Treg, or both, mediate the tumor-promoting role of TSLP in the context of melanoma.

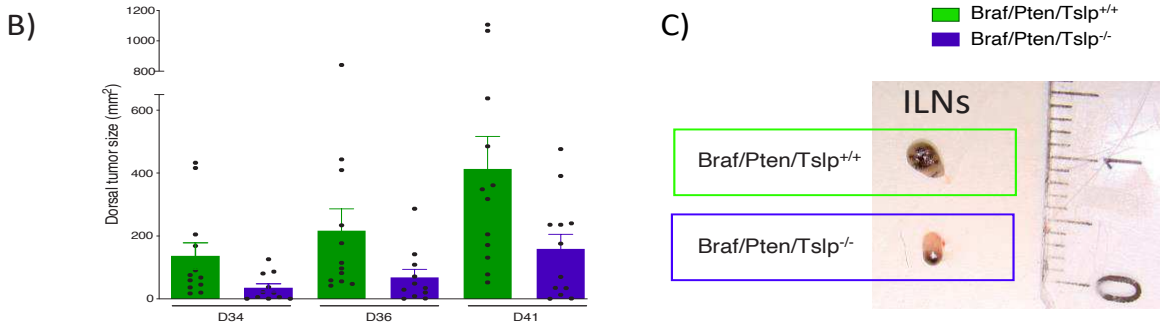
Melanoma is a complex disease with heterogeneous etiological, histopathological and genetic features. Considering the different oncogenes and tumor suppressor genes implicated in melanoma development, numerous genetically engineered mouse models have been

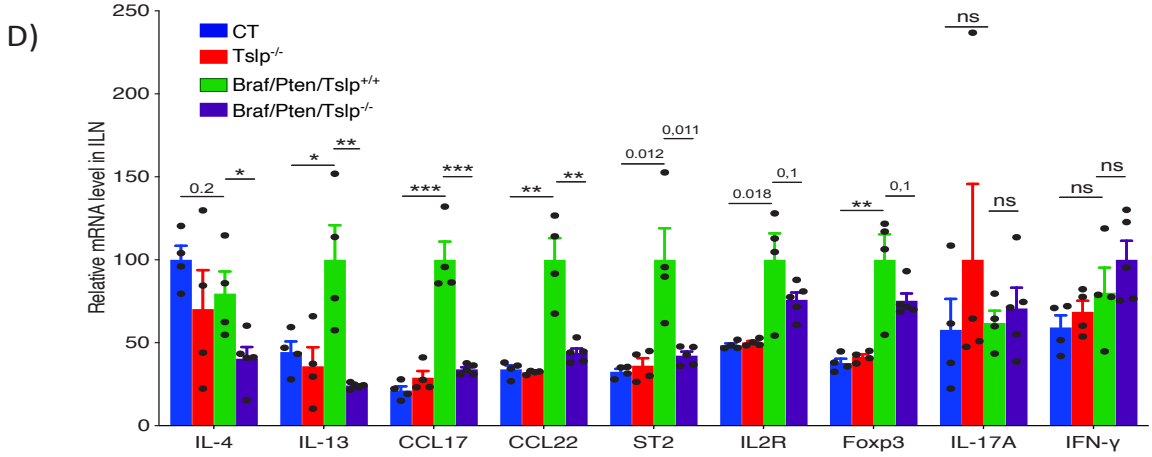
developed providing powerful tools to decipher the molecular mechanisms for melanoma. In our study we used the Braf/Pten model but it would be interesting to explore our results using other melanoma models (44), (45). Moreover, recently, Sun et al., developed a new Braf/Pten model. These mice, instead of used the Tyr-CreER system which target the menalocyte stem cells (MsSCs) located in hair follicle and in the dermis leading to the promotion of tumoral development in the dermis, used the c-kit promoter. C-Kit promoter does not have effect in dermal melanocytes and upon tamoxifen induction targets only the MsSCs located in hair follicle (46). Thus, it can be interesting to examine the role of TSLP using this model mimicking the initial radial growth phase of human melanoma.

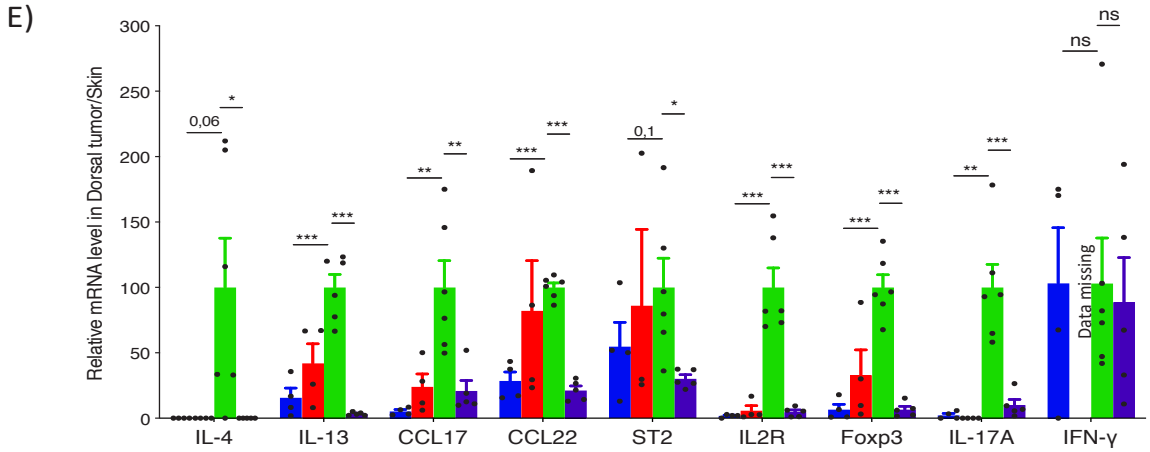
Using our mouse model, W. Yao showed that TSLP is up-regulated in the epidermis of human melanoma biopsies. Additionally, in Braf/Pten mice she observed that TSLP serum levels correlate with the melanoma progression. It needs to be further investigated whether human TSLP expression level could be correlated with the disease progression in human. In collaboration with a medical student who is performing a one-year stage in our lab, we collected more human biopsies from melanoma patients with the objective to study their TSLP expression and the features of the immune cell infiltration in the tumoral microenvironment, in order to explore whether TSLP expression in KCs is correlated with the presence of Th2 and Treg cells. These results will allow us to corroborate our mouse data and establish the role of TSLP in human melanoma.

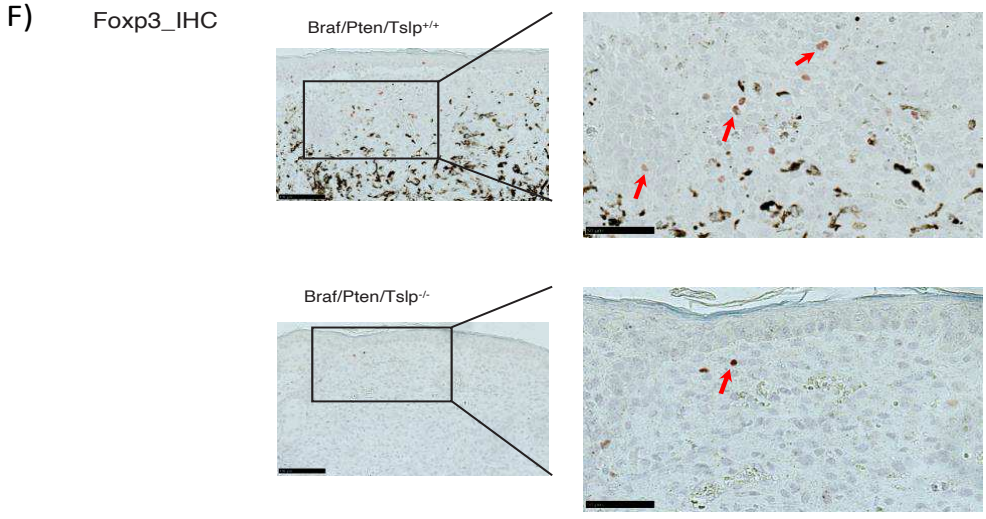
Finally, our NSG grafting experiments with mouse B16F10 and Lu1205 human melanoma cells suggested that there might be from these cells common factors which are capable to induce TSLP in mouse KCs. IL-1β was one candidate because it was shown that the pancreatic cancer-derived factor activate fibroblast to produce TSLP; However, I could not show the evidence that IL-1β induces TSLP production. It remains to be explored what could be the signal(s) derived from melanoma cells inducing TSLP expression. In addition, it is necessary to examine whether these factors are secreted or the contact between tumoral cells and KCs is necessary to promote TSLP expression.


A)
Application of 8! g of
4-hydroxytamoxifen (4-HT)
(dorsal skin)
D0 D1 D2
Dissections
D44


B)
Dorsal tumor size (mm²)
D34
D36
D41
C)
Braf/Pten/Tslp+/+
Braf/Pten/Tslp-/-
ILNs


D)
CT
Tslp-/-
Braf/Pten/Tslp+/+
Braf/Pten/Tslp-/-
Relative mRNA level in ILN
IL-4
IL-13
CCL17
CCL22
ST2
IL2R
Foxp3
IL-17A
IFN-γ


E)
Relative mRNA level in Dorsal tumor/Skin
Data missing
IL-4
IL-13
CCL17
CCL22
ST2
IL2R
Foxp3
IL-17A
IFN-γ


F)
Foxp3_IHC
Braf/Pten/Tslp+/+
Braf/Pten/Tslp-/-

Results
Part 3

Figure1. FoxP3 expression is decreased in Braf/Pten/Tslp$^{-/-}$ mouse inguinal-draining LNs and dorsal tumor.

A. Experimental protocol. Mouse dorsal skin were topically treated with 4OH-tamoxifen (4-HT) ones per day from D0 to D3. Doral tumors and ingüinal-draining LNs (ILNs) were analyzed at D44.

B. Tumor growth was evaluated using the ellipsoid volume formula (1/2 x L x W2) proposed by Perri GC et al., (1963), at D34, D36 and D41. Values are mean ± SEM (n≥12).

C. Representative ILNs photo from Braf/Pten/Tslp$^{+/+}$and Braf/Pten/Tslp$^{-/-}$ mice at D44.

D-E. Quantitative RT-PCR analysis (RT-qPCR) of ILNs and dorsal skin or dorsal tumors of CT, Tslp$^{-/-}$, Braf/Pten/Tslp$^{+/+}$ and Braf/Pten/Tslp$^{-/-}$ mice at D44. Values are mean ± SEM (n≥4). *p<0.05, **p<0.01, ***p<0.001 (two-tailed Student's t test).

F. IHC staining with antibody against Foxp3 on sections of dorsal melanoma from Braf/Pten/Tslp$^{+/+}$ and Braf/Pten/Tslp$^{-/-}$ mice at day 44. Scale bar: 100**µ**m

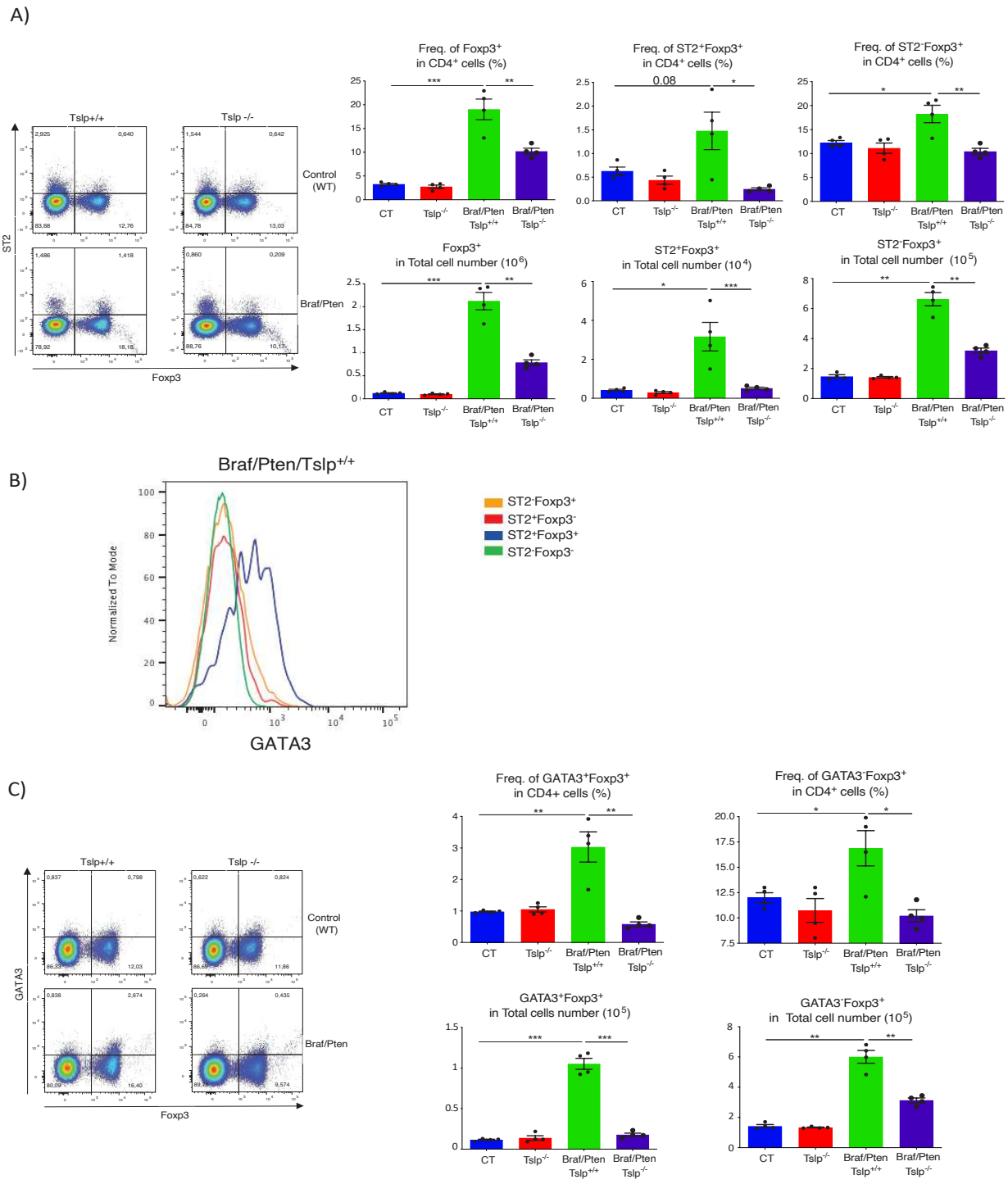


Figure 2: Treg cells associated with Braf/Pten/Tslp$^{+/+}$ melanoma are reduced in Braf/Pten/Tslp$^{-/-}$ ingüinal-draining LNs (ILNs).

A. Representative FACS plots (left) and frequency (right, up) of Foxp3$^+$, ST2$^+$Foxp3$^+$and ST2$^-$Foxp3$^+$ among CD4$^+$ cells or total cell number (right, down) from wild-type (WT), Tslp$^{-/-}$, Braf/Pten/Tslp$^{+/+}$ or Braf/Pten/Tslp$^{-/-}$ in ingüinal-draining LNs (ILNs).

B. Histogram of GATA3 (left) in ST2$^-$Foxp3$^+$, ST2$^+$Foxp3$^-$, ST2$^+$Foxp3$^+$ and ST2$^-$Foxp3$^-$ in ILNs from Braf/Pten/Tslp$^{+/+}$ mice.

C. Representative FACS plots (left) and frequency (right, up) of Foxp3$^+$, GATA3$^+$Foxp3$^+$ and GATA3$^-$Foxp3$^+$ among CD4$^+$ cells or total cell number (right, down) from CT, Tslp$^{-/-}$, Braf/Pten/Tslp$^{+/+}$ or Braf/Pten/Tslp$^{-/-}$ in ILNs. Values are mean ± SEM (n=4). *p<0.05, **p<0.01, ***p<0.001 (two-tailed Student's t test).

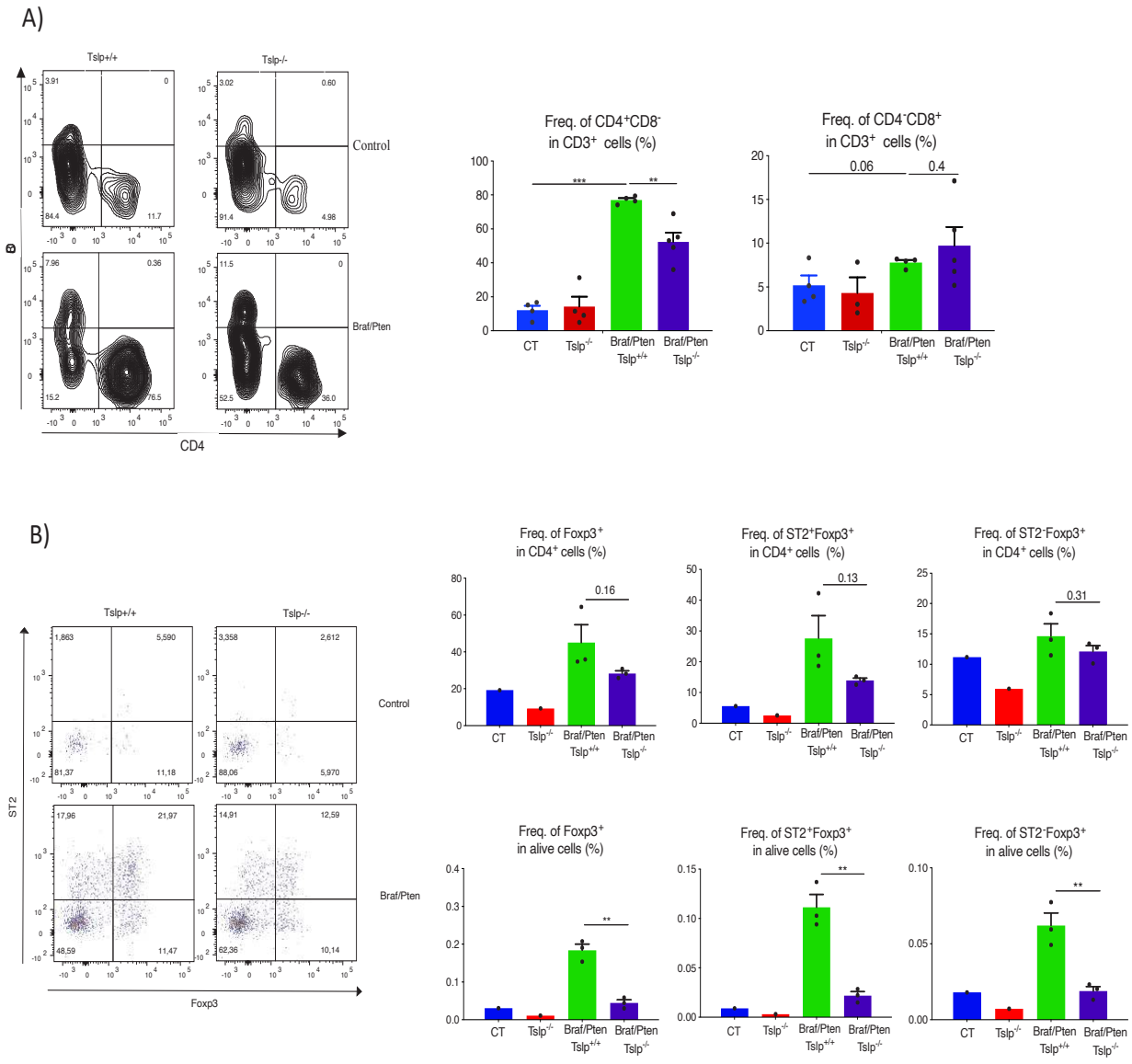


Figure 3: Treg cells associated with Braf/Pten/Tslp$^{+/+}$ melanoma are reduced in Braf/Pten/Tslp$^{-/-}$ mice at D44 in dorsal tumor.

A. Representative FACS plots (left) and frequency (right) of CD4^{+}CD8^{-} and CD4^{-}CD8^{+} among CD3^{+} cells in CT, Tslp$^{-/-}$, Braf/Pten/Tslp$^{+/+}$ or Braf/Pten/Tslp$^{-/-}$ in dorsal tumors.

B. Representative FACS plots (left) and frequency (right, up) of ST2^{+}Foxp3^{+}and ST2^{-}Foxp3^{+} among CD4^{+} cells or total cell number (right, down) from CT, Tslp$^{-/-}$, Braf/Pten/Tslp$^{+/+}$ or Braf/Pten/Tslp$^{-/-}$ in dorsal tumor. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 (two-tailed Student's t test).

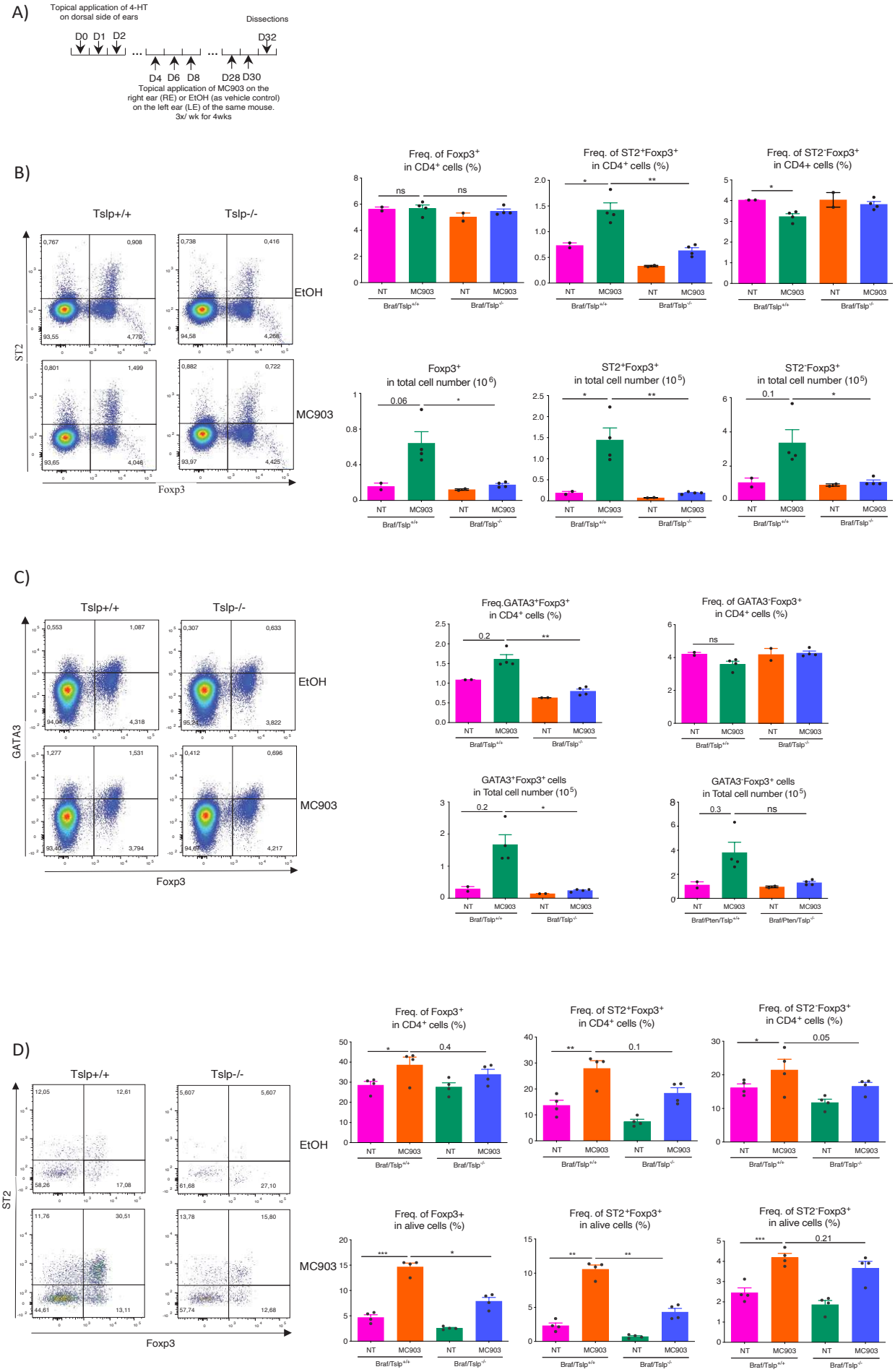

A)
Topical application of 4-HT on dorsal side of ears
Dissections
D0 D1 D2
D32
D4 D6 D8 D28 D30
Topical application of MC903 on the right ear (RE) or EtOH (as vehicle control) on the left ear (LE) of the same mouse. 3x/ wk for 4wks
B)
Tslp+/+
Tslp-/-
EtOH
MC903
ST2
Foxp3
Freq. of Foxp3+ in CD4+ cells (%)
Freq. of ST2+Foxp3+ in CD4+ cells (%)
Freq. of ST2-Foxp3+ in CD4+ cells (%)
Foxp3+ in total cell number (10^6)
ST2+Foxp3+ in total cell number (10^5)
ST2-Foxp3+ in total cell number (10^5)
NT
MC903
Braf/Tslp+/+
Braf/Tslp-/-
C)
GATA3
Freq.GATA3+Foxp3+ in CD4+ cells (%)
Freq. of GATA3-Foxp3+ in CD4+ cells (%)
GATA3+Foxp3+ cells in Total cell number (10^5)
GATA3-Foxp3+ cells in Total cell number (10^5)
Braf/Pten/Tslp+/+
Braf/Pten/Tslp-/-
D)
Freq. of Foxp3+ in alive cells (%)
Freq. of ST2+Foxp3+ in alive cells (%)
Freq. of ST2-Foxp3+ in alive cells (%)

Figure 4: Topical MC903 treatment promotes ST2^{+}Treg cells in ear-draining LNs and ears of Braf/Tslp$^{+/+}$ mouse melanoma.

A. Experimental protocol. Dorsal part of the ears of 8 to 12-week-old CT, Braf/Tslp $^{+/+}$ and Braf /Tslp$^{-/-}$ mice were topically treated with 4-HT for 3 days from D0 to D2. Starting from D4, right ears (RE) were treated with MC903 to induce TSLP expression, while left ears (LE) were treated with ethanol (EtOH, vehicle control) 3 times per week (wk). Ears were analysed at D32.

B. Representative FACS plots (left), frequency (right, up) of Foxp3^{+}, ST2^{+}Foxp3^{+} and ST2^{-}Foxp3^{+} among CD4^{+} cells and total cell number (right, down) of Foxp3^{+}, ST2^{+}Foxp3^{+} and ST2^{-}Foxp3^{+} cells in Braf/Tslp$^{+/+}$ or Braf/Tslp$^{-/-}$ ethanol or MC903 treated in Ear-draining LNs (ELNs).

C. Representative FACS plots (left), Frequency (right, up) of GATA3^{+}Foxp3^{+} and GATA3^{-}Foxp3^{+} among CD4^{+} cells (up, right) total cell number (right, down) of GATA3^{+}Foxp3^{+} and GATA3^{-}Foxp3^{+} cells in Braf/Tslp$^{+/+}$ or Braf/Tslp$^{-/-}$ ethanol or MC903 treated in ELNs.

D. Representative FACS plots (left), Frequency (right, up) of Total Foxp3^{+}, ST2^{+}Foxp3^{+} and ST2^{-}Foxp3^{+} among CD4^{+} cells and total cell number (right, down) of Foxp3^{+}, ST2^{+}Foxp3^{+} and ST2^{-}Foxp3^{+} cells in Braf/Tslp$^{+/+}$ or Braf/Tslp$^{-/-}$ ethanol or MC903 treated in ears skin.

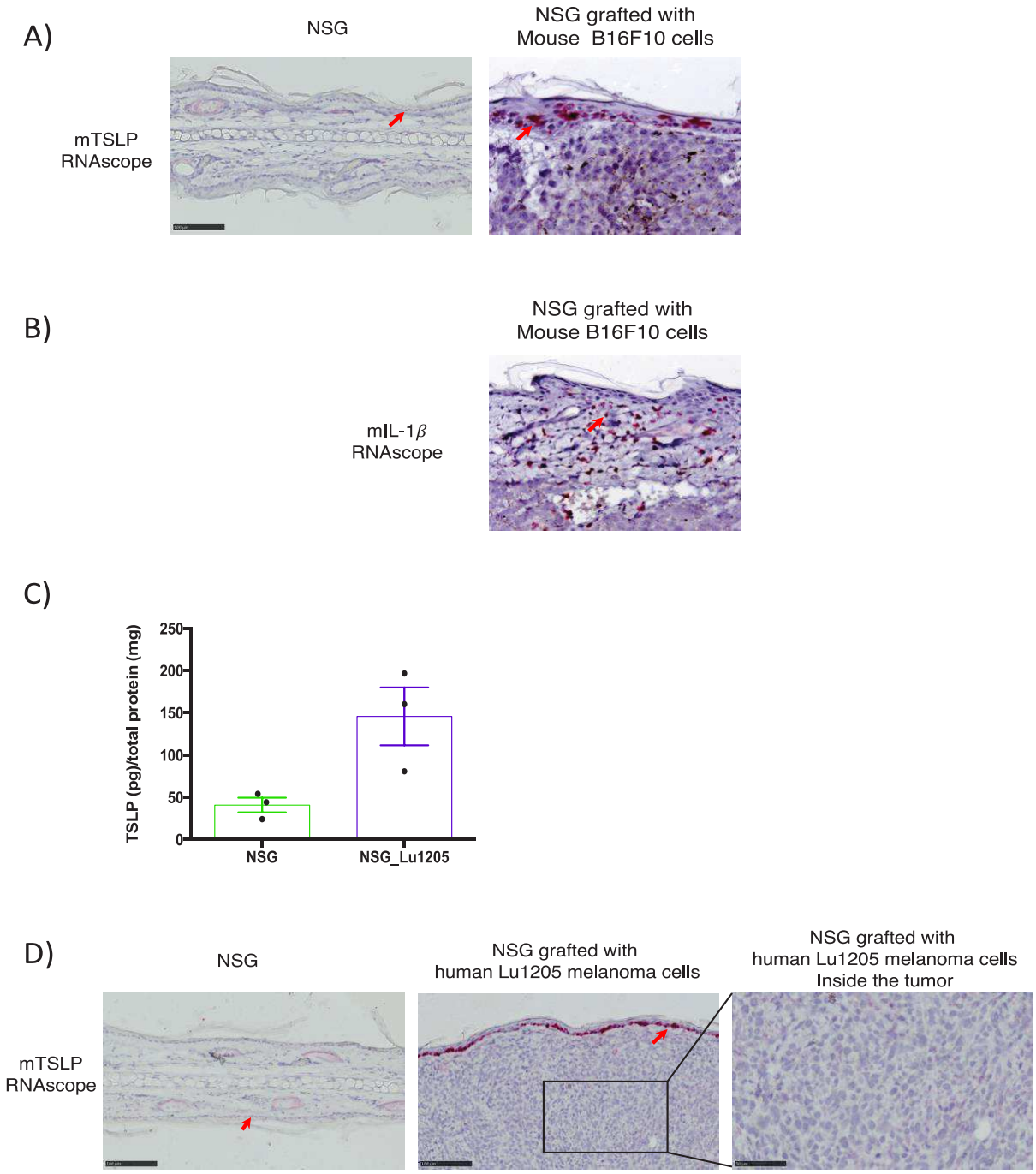


Figure 5: TSLP is induced in NSG mouse skin upon the grafting of mouse B16F10 and human Lu1205 melanoma cells.
$5x10^4$ B16F10 mouse melanoma cells and $2x10^4$ Lu1205 human melanoma cells derived from a metastatic melanoma lesion with $Braf^{V600E}$ mutation were injected intradermally (i.d.) into ears of NSG mice at D0.

A. RNAscope in situ hybridization for TSLP mRNA in B16F10-grafted ears of NSG mice at D35. Red arrows point to one of the positive signals (in red).

B. RNAscope in situ hybridization for IL-1β mRNA in B16F10-grafted ears of NSG mice at D35. Red arrows point to one of the positive signals (in red).

C. TSLP protein level from Lu1205-grafted NSG ears mice at D35 was measured by ELISA. (n=3).

D. RNAscope in situ hybridization for TSLP mRNA in Lu1205-grafted ears of NSG mice at D35. Red arrows point to one of the positive signals (in red). Scale bar= 100 **µ**m

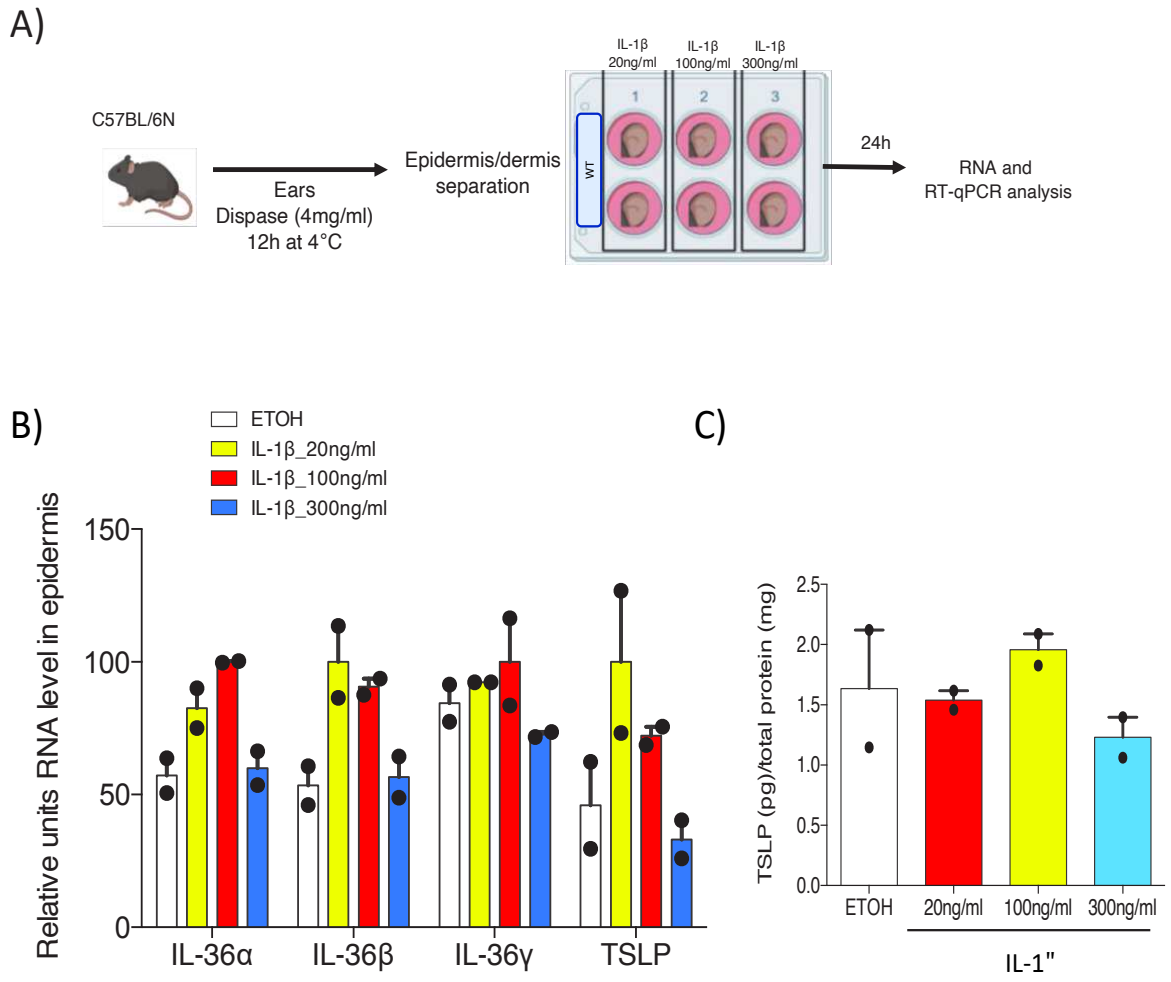


Figure 6: IL-1β alone does not have the capability to induce TSLP in mouse epidermal cultures.

A. Experimental protocol. Epidermis was isolated from WT mouse ears, cultured ex vivo in the absence or presence of different concentrations of IL- 1β.

B. Quantitative RT-PCR analyses. Relative RNA levels were calculated using HPRT as internal control. (IL-36α, IL-36β and IL-36γ were included as a positive control). Values are mean ± SEM (n=2).

C. TSLP protein levels in mouse epidermal supernatants after IL- 1β addition measurements by ELISA. Values are mean ± SEM (n=2).

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Results
Part 3

GENERAL CONCLUSIONS OF THE THESIS

GENERAL CONCLUSIONS OF THE THESIS

Skin is the primary barrier against physical insults and microbial pathogens protecting the body, representing a unique environment in which various immune cells interact with keratinocytes (KCs), fibroblast and melanocytes to maintain tissue homeostasis and to regulate immune responses. The research of my lab during the last years has greatly contributed to the understanding of how KCs, through the release of inflammatory mediators like cytokines, instruct and coordinate the immune responses in skin pathological contexts, such as psoriasis, melanoma and atopic dermatitis.

In this context, my PhD thesis was focus in the understanding of the role of the different factors released by KCs in the development of psoriasis inflammation and in the regulation of melanoma growth through their crosstalk with immune cells presented in the tumoral microenvironment. The immunological function of KCs is based in their capability of the release of different chemokines and cytokines including IL-36α, IL-36γ and TSLP, which promote immune responses.

In the case of psoriasis, we demonstrated that epithelial cell-derived cytokines IL-36α/γ, which are drivers of the immune response developed in human psoriatic skin and psoriatic-skin inflammatory mouse models, are the key targets of Calcipotriol, a VD_3 analog, used topically as 1st line in psoriasis treatment. In addition, we demonstrated that this effect is mediated through the vitamin D_3 receptor (VDR) expressed in KCs. Strikingly, we observed that this effect was synergistically improved by the combination of Calcipotriol with Dexamethasone, which targets IL-23/IL-17 blocking the positive feedback loop of these cytokines in the induction of IL-36α/γ expression in KCs. Based on these results, using IL-36α/γ DKO mice, we observed that the induction of IL-36α/γ cytokines in KCs is important in the development of psoriasis skin inflammation. Specifically, we showed that IL-36α/γ cytokines play an important role in the early-phase of psoriasis promoting neutrophil/monocyte/macrophage recruitment to the skin.

Finally, in the second part of my thesis, we observed that the ablation of the keratinocytic-derived TSLP expression is associated with the delay of the melanoma growth and the reduction of Th2 and Treg cells including $ST2^+$Treg cells in metastatic

inguinal-draining LNs and dorsal tumors induced by OH-tamoxifen. Moreover, by the grafting of mouse B16F10 melanoma cells and human Lu1205 melanoma cells in NSG mice we observed that the tumoral cells were able to promote the expression of TSLP by KCs in the absence of immune cells.

In both cases our data suggest that either in psoriasis skin inflammation or melanoma microenvironment the release of different factors by KCs such as IL-36α/γ or TSLP respectively plays and important role in the pathological development. Therefore, KCs could be considered as important targets in the development of new therapeutical strategies and keratinocytic-derived IL-36α/γ in psoriasis or TSLP in melanoma could be potential biomarkers of the stage and progression of these diseases.

These discoveries give us the opportunity to further elucidate the role of each keratinocytic-derived cytokine in the different pathological conditions. To further understand the IL-36α/γ role in psoriasis I would like to study their implication in NETs formation, IL-1β expressing cells recruitment and whether the induction of these cytokine promote the release of different keratinocytic-derived factors, inducing neutrophil recruitment to the human psoriatic and psoriatic mouse models skin. In addition, it would be interesting to study the role of IL-36α/γ in other inflammatory skin disorders such as in the context of PSS-B pathogenesis, using IL-36α/γ DKO crossed with $Cdsn^{iep-/-}$ mice. Moreover, in the case of melanoma, we would like to characterize more in detail the $ST2^{+}$Treg population presented in melanoma microenvironment of Braf/Pten and Braf mice. Therefore, I would like to study whether $ST2^{+}$Treg cells have a pathological role in melanoma growth performing in vitro co-cultures of melanoma cells with immune cells. In addition, it could be interesting to study other immune cell populations such as macrophages in the melanoma microenvironment upon TSLP ablation. Finally, we would like to further investigate which factors released by tumoral cells are responsible to the induction of TSLP expression in KCs in this pathological situation.

The final objective would be to have a better understanding of the different cytokines and immunological pathways implicated in the development of the two pathological conditions to improve the therapeutic strategies available in clinic.

Beatriz GERMÁN FALCÓN

Étude de la régulation immunitaire dans le psoriasis et le mélanome

Résumé

Ma thèse de doctorat visait à mieux comprendre les réponses inflammatoires de la peau dans le psoriasis et le mélanome. Dans la première partie, j'ai étudié le mécanisme qui sous-tend l'efficacité thérapeutique du Calcipotriol (Cal), un analogue de la vitamine D3 dans le psoriasis. En utilisant un modèle de psoriasis de souris induit par Aldara (Ald), nous avons montré que Cal inhibait l'IL-23/IL-17 et l'infiltration des neutrophiles, par le biais du récepteur de la vitamine D (VDR) dans les kératinocytes (KC). Nous avons en outre révélé que les IL-36/ étaient réprimées par Cal via la signalisation directe du VDR dans les KC de souris et que Cal inhibait également les IL-36α/γ dans la peau lésionnelle humaine. En revanche, la dexaméthasone (Dex) a indirectement réduit l'IL-36α/γ par le biais des cellules immunitaires. De plus, la combinaison de Cal et de Dex a réduit de manière synergique l'IL-36/ et l'IL-23/IL-17 dans le psoriasis établi de la souris. Ces données révèlent le mécanisme qui sous-tend l'efficacité supérieure de la thérapie combinée de Cal/Dex pour le psoriasis.

Dans la deuxième partie, j'ai exploré le rôle de l'IL-36α/γ dans le psoriasis en utilisant une nouvelle génération de souris IL-36α/γDKO. Mes résultats ont montré que l'IL-36α/γ joue un rôle important dans le psoriasis induit par l'Ald, en particulier pour un recrutement précoce du psoriasis, avec l'expression de gènes dans l'épiderme liée au recrutement des neutrophiles.

Dans la 3ème partie, mon travail de collaboration a exploré le rôle de la lymphopoïétine thymique stromale (TSLP) dans le mélanome. En utilisant des modèles de mélanome de souris, nous avons montré que l'expression de la TSLP par les KCs jouait un rôle de promoteur de tumeur pour le mélanome, qui était associé aux réponses des cellules Th2 et Treg.

Mots clés: inflammation, kératinocytes, psoriasis, IL-36, Calcipotriol, vitamine D3, mélanome, TSLP

Abstract

My PhD thesis aimed at achieving a better understanding of skin inflammatory responses in psoriasis and melanoma. In the first part, I studied the mechanism underlying the therapeutic efficiency of Calcipotriol (Cal), a vitamin D3 analog in psoriasis. Using Aldara (Ald)-induced mouse psoriasis model, we showed that Cal inhibited IL-23/IL-17 and neutrophil infiltration, through the vitamin D receptor (VDR) in keratinocytes (KCs). We further revealed that IL-36α/γ were repressed by Cal via direct VDR signaling in mouse KCs and Cal also inhibited IL-36α/γ in human lesional skin. In contrast, dexamethasone (Dex) indirectly reduced IL-36α/γ through immune cells. Further, the combination of Cal and Dex synergistically reduced IL-36α/γ and IL-23/IL-17 in the established mouse psoriasis. These data reveal the mechanism underlying the superior efficacy of the combination therapy of Cal/Dex for psoriasis.

In the 2nd part, I explored the role of IL-36α/γ in psoriasis using a new generated IL-36α/γ DKO mice. My results showed that IL-36α/γ play an important role for Ald-induced psoriasis, particularly for an early recruitment of psoriasis, with the expression of genes in epidermis related with neutrophil recruitment.

In the 3rd part, my collaborative work explored the role of thymic stromal lymphopoietin (TSLP) in melanoma. Using mouse melanoma models, we showed that the expression of TSLP by KCs played a tumor-promoting role for melanoma, which was associated with Th2 and Treg cell responses.

Keywords: inflammation, keratinocytes, psoriasis, IL-36, Calcipotriol, vitamin D3, melanoma, TSLP