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## Targeting TREX2: an innovative keratinocyte-based therapy for psoriasis and atopic dermatitis

Ingrid Filgaira Enri

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UNIVERSITAT DE  
BARCELONA

Facultat de Medicina i Ciències de la Salut  
Programa de Doctorat en Biomedicina

# Targeting TREX2: an innovative keratinocyte-based therapy for psoriasis and atopic dermatitis

Memòria presentada per **Ingrid Filgaira Enri**  
per optar al grau de doctora per la Universitat de Barcelona

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A la meva família



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## Abbreviations

|                   |                                                           |
|-------------------|-----------------------------------------------------------|
| <b>ACTB</b>       | Actin- $\beta$                                            |
| <b>AD</b>         | Atopic dermatitis                                         |
| <b>ADMET</b>      | Absorption, Distribution, Metabolism, Excretion, Toxicity |
| <b>AhR</b>        | Aryl hydrocarbon receptor                                 |
| <b>AMPs</b>       | Antimicrobial Peptides                                    |
| <b>ATP</b>        | Adenosine triphosphate                                    |
| <b>bp</b>         | Base pair                                                 |
| <b>BRET</b>       | Bioluminescence Resonance Energy Transfer                 |
| <b>BSA</b>        | Bovine Serum Albumin                                      |
| <b>cAMP</b>       | Cyclic adenosine monophosphate                            |
| <b>cDCs</b>       | Conventional dendritic cells                              |
| <b>cDNA</b>       | Complementary DNA                                         |
| <b>CETSA</b>      | Cellular Thermal Shift Assay                              |
| <b>DAMPs</b>      | Damage-associated molecular patterns                      |
| <b>DC</b>         | Dendritic cell                                            |
| <b>DDR</b>        | DNA damage response                                       |
| <b>DETC</b>       | Dendritic epidermal T cells                               |
| <b>DMEM</b>       | Dulbecco's Modified Eagle Medium                          |
| <b>dsDNA</b>      | Double-stranded DNA                                       |
| <b>dsRNA</b>      | Double-stranded RNA                                       |
| <b>EDTA</b>       | Ethylenediaminetetraacetic acid                           |
| <b>EGF</b>        | Epidermal growth factor                                   |
| <b>ELISA</b>      | Enzyme-Linked Immuno-Sorbent Assay                        |
| <b>ERK</b>        | Extracellular signal-regulated kinase                     |
| <b>FBS</b>        | Fetal Bovine Serum                                        |
| <b>FLG</b>        | Filaggrin                                                 |
| <b>GAPDH</b>      | Glyceraldehyde-3-phosphate dehydrogenase                  |
| <b>GM-CSF</b>     | Granulocyte macrophage colony-stimulating factor          |
| <b>GSK-3 beta</b> | Glycogen synthase kinase 3 beta                           |

|                                 |                                       |
|---------------------------------|---------------------------------------|
| <b>H&amp;E</b>                  | Haematoxylin & Eosin                  |
| <b>hBD</b>                      | Human $\beta$ -defensin               |
| <b>HCA2</b>                     | Hydroxy-carboxylic acid receptor 2    |
| <b>HIF-1<math>\alpha</math></b> | Hypoxia-inducible factor 1-alpha      |
| <b>i.d.</b>                     | Intradermal                           |
| <b>i.g.</b>                     | Intragastric                          |
| <b>i.p.</b>                     | Intraperitoneal                       |
| <b>IC<sub>50</sub></b>          | Half-maximal inhibitory concentration |
| <b>iDCs</b>                     | Inflammatory dendritic cells          |
| <b>IFN</b>                      | Interferons                           |
| <b>Ig</b>                       | Immunoglobulin                        |
| <b>IGF-1</b>                    | Insulin-like growth factor 1          |
| <b>IKK</b>                      | I-kappa B kinase                      |
| <b>IL</b>                       | Interleukin                           |
| <b>IL-4R</b>                    | IL-4 receptor                         |
| <b>ILC1</b>                     | Group 1 Innate lymphoid cell          |
| <b>ILC2</b>                     | Group 2 Innate lymphoid cell          |
| <b>ILC3</b>                     | Group 3 Innate lymphoid cell          |
| <b>ILC</b>                      | Innate lymphoid cell                  |
| <b>IL</b>                       | Interleukin                           |
| <b>IMQ</b>                      | Imiquimod                             |
| <b>IPTG</b>                     | Isopropyl- $\beta$ -D-thiogalactoside |
| <b>IVL</b>                      | Involucrin                            |
| <b>JAK</b>                      | Janus kinase                          |
| <b>JNK</b>                      | Jun N-terminal Kinase                 |
| <b>Krt10/K10</b>                | Keratin 10 (gene/protein)             |
| <b>KC</b>                       | Keratinocyte                          |
| <b>Kd</b>                       | Dissociation constant                 |
| <b>KGF</b>                      | Keratinocyte growth factor            |
| <b>KRT10</b>                    | Keratin 10                            |

|                                |                                                                                   |
|--------------------------------|-----------------------------------------------------------------------------------|
| <b>LC</b>                      | Langerhans cell                                                                   |
| <b>LCN2</b>                    | Lipocalin 2                                                                       |
| <b>LTC4</b>                    | Leukotriene C4                                                                    |
| <b>MAPK</b>                    | Mitogen-activated protein kinases                                                 |
| <b>MBP</b>                     | Maltose Binding Protein                                                           |
| <b>MHC</b>                     | Major histocompatibility complex                                                  |
| <b>MST</b>                     | MicroScale Thermophoresis                                                         |
| <b>MTD</b>                     | Maximum tolerated dose                                                            |
| <b>NETs</b>                    | Neutrophil extracellular traps                                                    |
| <b>NF-<math>\kappa</math>B</b> | Nuclear factor kappa-light-chain-enhancer of activated B cells                    |
| <b>NFAT</b>                    | Nuclear factor of activated T cells                                               |
| <b>NHEK</b>                    | Normal human epidermal keratinocytes                                              |
| <b>NK</b>                      | Natural Killer                                                                    |
| <b>NKT</b>                     | Natural Killer T cell                                                             |
| <b>NLR</b>                     | NOD-like receptor                                                                 |
| <b>NLRP3</b>                   | Nucleotide-binding domain leucin-rich-containing family pyrin domain-containing-3 |
| <b>NOD</b>                     | Nucleotide-binding oligomerization domain                                         |
| <b>NRF2</b>                    | Nuclear factor erythroid 2-related factor 2                                       |
| <b>OD</b>                      | Optical density                                                                   |
| <b>OX</b>                      | Oxazolone                                                                         |
| <b>PAMPs</b>                   | Pathogen-associated molecular patterns                                            |
| <b>PBS</b>                     | Phosphate Buffer Saline                                                           |
| <b>PCR</b>                     | Polymerase Chain Reaction                                                         |
| <b>pDC</b>                     | Plasmacytoid dendritic cell                                                       |
| <b>PDE4</b>                    | Phosphodiesterase-4                                                               |
| <b>PK</b>                      | Pharmacokinetic                                                                   |
| <b>PMSF</b>                    | Phenylmethylsulfonyl fluoride                                                     |
| <b>PRR</b>                     | Pattern-recognition receptors                                                     |
| <b>PsO</b>                     | Psoriasis                                                                         |

|                       |                                                              |
|-----------------------|--------------------------------------------------------------|
| <b>qPCR</b>           | quantitative Polymerase Chain Reaction                       |
| <b>RAR</b>            | Retinoic acid receptors                                      |
| <b>ROS</b>            | Reactive oxygen species                                      |
| <b>RT-PCR</b>         | Reverse Transcription Polymerase Chain Reaction              |
| <b>RT-qPCR</b>        | Reverse Transcription quantitative Polymerase Chain Reaction |
| <b>RXR</b>            | Retinoic X receptors                                         |
| <b>SALT</b>           | Skin-associated lymphoid tissue                              |
| <b>SAR</b>            | Structure-Activity Relationship                              |
| <b>SEC</b>            | Size-Exclusion Chromatography                                |
| <b>SLE</b>            | Systemic lupus erythematosus                                 |
| <b>ssDNA</b>          | Single-stranded DNA                                          |
| <b>STAT</b>           | Signal transducer and activator of transcription             |
| <b>TAK1</b>           | Transforming growth factor-beta-activated Kinase 1           |
| <b>TCR</b>            | T cell receptor                                              |
| <b>TEV</b>            | Tobacco Etch Virus                                           |
| <b>TEWL</b>           | Transepidermal water loss                                    |
| <b>TGF</b>            | Transforming growth factors                                  |
| <b>TLR</b>            | Toll-like receptors                                          |
| <b>TNF</b>            | Tumour Necrosis Factor                                       |
| <b>Treg</b>           | Regulatory T cells                                           |
| <b>TREX1</b>          | Three prime Repair Exonuclease 1                             |
| <b>TREX2</b>          | Three prime Repair Exonuclease 2                             |
| <b>T<sub>RM</sub></b> | Tissue-resident memory T cells                               |
| <b>TSLP</b>           | Thymic stromal lymphopoietin                                 |
| <b>TYK2</b>           | Tyrosine kinase 2                                            |
| <b>UV</b>             | Ultraviolet                                                  |
| <b>VEGF</b>           | Vascular endothelial growth factor                           |
| <b>wt</b>             | Wild type                                                    |

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# Introduction



# 1. Introduction

## 1.1. The skin

The skin is the largest organ of the human body, accounting for a total surface area of 1.85 m<sup>2</sup> and 15% of the total weight (Deniz et al., 2020). It is a complex organ, with diverse physical, chemical and microbiological strategies to protect the organism from external hazardous insults. It also has a crucial role in the host homeostasis preventing it from dehydration, enabling thermoregulation and transmitting sensory inputs (Hsu et al., 2014; Hsu & Fuchs, 2022; Nguyen & Soulika, 2019).

Anatomically, the skin is organized into three layers: the epidermis, the dermis and the hypodermis or subcutaneous fat tissue. The main function of the first is to act as a protective barrier, whereas the main function of the second is to maintain skin's firmness and elasticity (Deniz et al., 2020). In an embryological level, the epidermis and its appendages develop from the surface ectoderm, while the dermis and hypodermis do from the mesoderm (Khavkin & Ellis, 2011; McGrath et al., 2004).

The epidermis is a self-renewing stratified squamous epithelium and the outermost layer of the skin. It can be subdivided into four layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Figure 1A). Cells undergo a terminal differentiation process known as keratinization or cornification that ends up in the formation of a resistant surface formed by cornified cells filled with keratin filaments that lack organelles and nuclei. (Baroni et al., 2012; Ho & Kupper, 2019; Shetty & Gokul, 2012).

The most internal layer of the epidermis is the stratum basale and is composed of a single layer of stem cells known as basal cells that are responsible for producing new keratinocytes (KCs), that will migrate upwards progressively differentiating and creating new layers. In the second layer, the stratum spinosum, KCs attach to each other by desmosomes, a type of protein channels, and contain lamellar granules filled with lipids. In the stratum granulosum, keratin filaments, a component of the cytoskeleton only expressed in epithelial cells, start to aggregate into complex structures by binding to filaggrin (FLG), a filament-associated protein important for the integrity of the epidermis. Cells in this layer start losing organelles and compact themselves forming the outermost layer: the stratum corneum. In this layer, keratinization is finished, which means KCs are

terminally differentiated and form a “bricks and mortar” structure, with KCs bound to one another via desmosomes, and surrounded by lipids that arise from lamellar granules. This structure is both an outside-in and an inside-out barrier, protective and moisture-controlling at the same time. Although KCs are the primary cell type present in the epidermis, other cells such as melanocytes, Langerhans cells (LCs), T cells and Merkel cells can be found there, especially in the stratum basale (Losquadro, 2017; Nguyen & Soulika, 2019; Shetty & Gokul, 2012).

The main cell types in the dermis are fibroblasts, myofibroblasts and immune cells such as macrophages, lymphocytes and mast cells. It is composed mainly of collagen, but also contains elastin, blood vessels, nerve endings, hair follicles, lymphatic vessels, sebaceous glands and sweat glands. The dermis is subdivided into the papillary dermis and the reticular dermis. The first is located under the dermal-epidermal junction, a basement membrane under the stratum basale of the epidermis. The papillary dermis comprises a loosely arranged network of fibrocytes, collagen and blood vessels, which extend into the epidermis to facilitate nutrient transport via capillaries (Losquadro, 2017; Shirshin et al., 2017). The reticular dermis is significantly thicker than the papillary due to its denser concentration of collagen and contains skin appendages such as hair follicles, sebaceous glands and sweat glands (Nguyen & Soulika, 2019; Woodley, 2017).

The deepest layer of the skin is the subcutaneous fat tissue, which functions to provide insulation to the body. It is composed of fibrocytes and adipocytes with high quantities of proteoglycans and glycosaminoglycans, which attract fluid into the tissue and confers it with mucus-like properties (Driskell et al., 2014; Wong et al., 2016). This layer also produces growth factors, adipokines and cytokines and contains immune cells (Cildir et al., 2013).

#### **1.1.1. Immunology of the skin. Immune cells and KC crosstalk**

Beyond serving as a physical barrier, the skin also exerts protective activities through the immune system and can defend against physical and chemical damage, ultraviolet (UV) light radiation, haptens and protein antigens, dehydration and a wide range of microorganisms such as bacteria, viruses, fungi or parasites (Kabashima, 2016; Kabashima et al., 2019).

The immune system of the skin consists of both skin cells and specialized immune cells, collectively known as skin-associated lymphoid tissue (SALT) (Figure 1A). In the skin, there are components from the innate and adaptive immunity. The first, relies on a number of limited germline-encoded receptors and secreted proteins that can recognize features that are shared between pathogens. In contrast, adaptive immunity presents a wide range of antigen receptors that can distinguish very precisely between molecules and create a more specific response. Innate immunity typically serves as the host's initial line of defense, comprising both chemical and cellular barriers capable of detecting microorganisms (Kabashima, 2016; Medzhitov & Janeway, 1997).

Immune cells resident to the skin induce tissue homeostasis and act as sentinels sampling the environment searching for possible dangers. Some of these cells can migrate to lymph nodes and induce tolerance to self-antigens or initiate immune responses. Upon a challenge like UV radiation or trauma, among others, resident cells and recruited cells from the periphery create a response to defend the organism (Nguyen & Soulika, 2019; Pasparakis et al., 2014; Quaresma, 2019). Basically, LCs and KCs sense and react to the challenge by activating dermal dendritic cells (DCs). Plasmacytoid DC (pDCs) activate upon stress signals from KCs and also contribute to the activation of other DC subtypes, along with fibroblasts and Natural Killer T (NKT) cells. The activation of DC leads to the promotion of the clonal expansion of CD8<sup>+</sup> and CD4<sup>+</sup> T cells and the production of cytokines and chemokines that stimulate KCs and DCs, creating an amplifying inflammatory loop (Figure 1B) (Nestle et al., 2009).

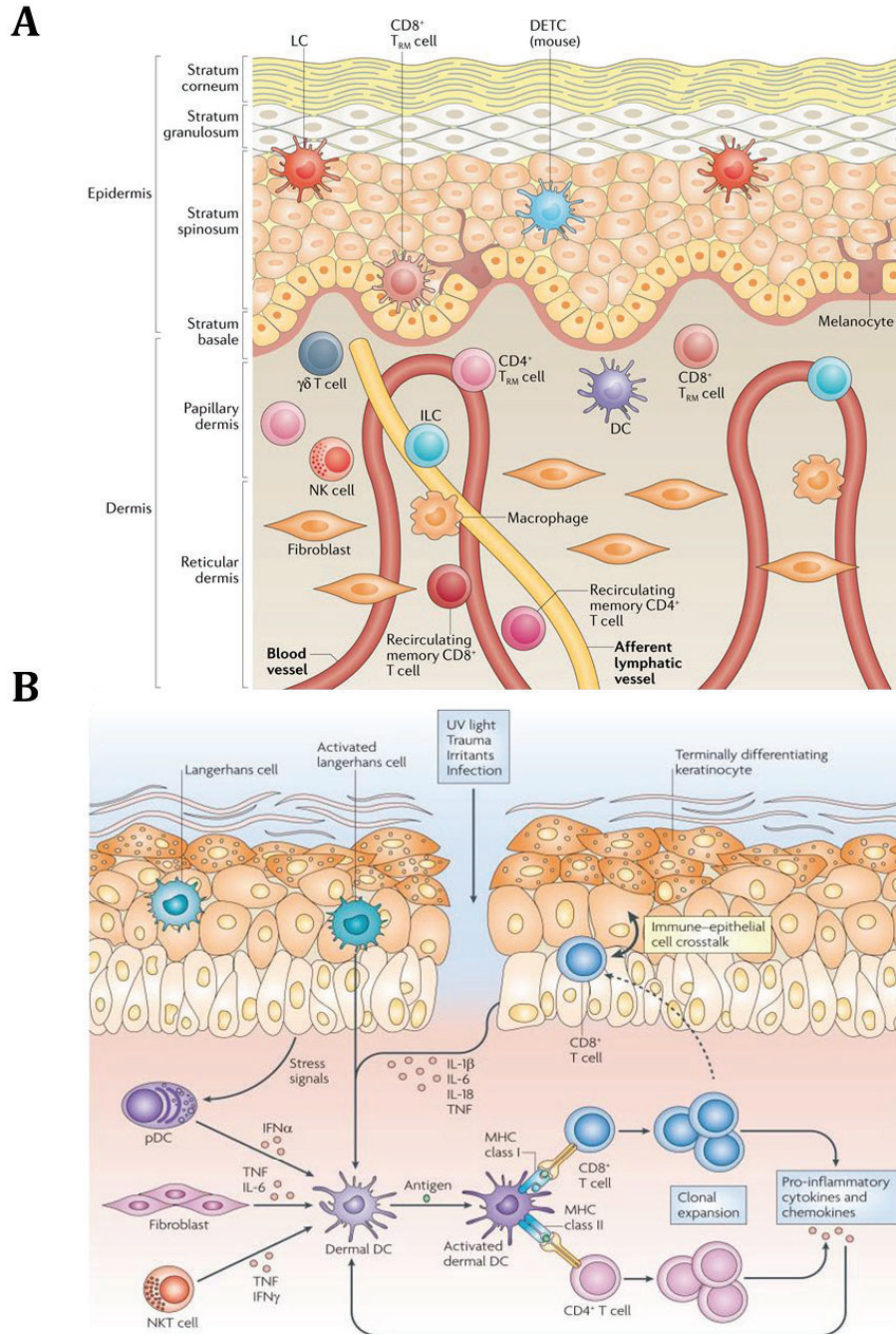


**Figure 1. Skin structure, cells and immunity.** A) The skin: structure and cell components. The epidermis consists of stratified layers of keratinocytes (KCs) that progressively undergo differentiation as they migrate outwards toward the skin's surface. The dermis presents two parts: the papillary and the reticular dermis. In the epidermis, different cell types can be found: Langerhans cells (LCs), CD8<sup>+</sup> tissue-resident memory T cells ( $T_{RM}$ ) and dendritic epidermal T cells (DETC) only in murine skin. The dermis contains: dendritic cells (DCs), macrophages, innate lymphoid cells (ILCs), Natural Killer (NK) and CD8<sup>+</sup>  $T_{RM}$  cells. The structure of the dermis is maintained by fibroblasts and connective tissue. Murine dermis presents  $\gamma\delta$  T cells, while human skin prominently presents  $\alpha\beta$  T cells. The coordination of these cells constitutes an effective system capable of producing immune responses and return to homeostasis (Ho & Kupper, 2019). B) Immune response to stress signals. Triggers such as UV light, trauma, irritants or infection cause barrier disruption and start a coordinated immune response to maintain skin homeostasis. Basically, LC act as sentinels and KCs sense and react to the stimuli by producing pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-18 and TNF) which activate DCs. Plasmacytoid DCs (pDCs) are activated by stress signals derived from KCs and contribute to the activation of dermal DCs by releasing IFN- $\alpha$ . Also, fibroblasts and Natural Killer T cells (NKT) can produce immune mediators that contribute to the activation of dermal DCs, which activate and promote the clonal expansion of skin-resident memory CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Cytokines and chemokines derived from these T cells stimulate KCs and fibroblasts, that amplify inflammation (Nestle et al., 2009).

A detailed description of the features and role of skin cells involved in the immunity of the skin is provided in the subsequent sections.

#### **1.1.1.1. DCs**

DCs are myeloid-derived antigen presenting cells that can be found in several tissues and that present a high capacity to induce T-cell responses (Bourque & Hawiger, 2018; Haniffa et al., 2013; Kashem et al., 2017; Wang & Bai, 2020). They originate from a common macrophage/DC progenitor that ends up differentiating into a common DC progenitor that gives rise to different subsets of DCs (Geissmann et al., 2010; MCGovern et al., 2015; Waisman et al., 2017; Wilcox et al., 2024). Each subset has specific tissue localization, surface markers and function.

DCs sense pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through pattern-recognition receptors (PRR) and can produce different types of cytokines that induce specific T-cell responses (Haniffa et al., 2013; Kashem et al., 2017; O’Keeffe et al., 2015). Depending on the set of cytokines synthesised by activated DC the adaptive response primed will be different (Th1, Th2 or Th17) (Segura et al., 2012). Apart from priming T cell responses, DCs are very important for tissue tolerance (Takenaka & Quintana, 2017; Waisman et al., 2017; Worbs et al., 2017).

Importantly, DCs provide three signals that allow antigen-specific CD4<sup>+</sup> T cells to begin proliferation and differentiation. Firstly, they express on their surface a major histocompatibility complex (MHC) class II/peptide complex for T cell receptor (TCR) recognition expressed by T CD4<sup>+</sup> cells. Secondly, their costimulatory receptors CD40 and B7 induce the proliferation of T cells. Lastly, they secrete several cytokines, including interleukin (IL)-12 and IL-23, among others, that drive CD4<sup>+</sup> lymphocyte maturation (Figure 2) (Baliwag et al., 2015; Lowes et al., 2005; Quaresma, 2019).

#### **1.1.1.1.1. LCs**

LCs are skin-resident myeloid cells with a dendritic morphology found in the epidermis in very close contact with KCs (Deckers et al., 2018; Steinman & Cohn, 1973). These cells anchor to the epidermis in a steady state and have a phagocytic behaviour. Once activated, they extend their processes and can sample the microenvironment without disrupting the

skin barrier, as they can reach both the stratum corneum and the stratum basale (Deckers et al., 2018). These cells are antigen-presenting cells constantly migrating to the skin lymph nodes to promote tolerance in a homeostatic state, but if they sense any danger, they can prime adaptive immune responses, being able to activate both CD8<sup>+</sup> and CD4<sup>+</sup> lymphocytes (Deckers et al., 2018; Griffiths et al., 2005). LCs are known to contribute to various skin diseases such as psoriasis (PsO) or atopic dermatitis (AD) (Elentner et al., 2009; Yoshiki et al., 2014).

#### **1.1.1.1.2. Dermal DCs**

Dermal DCs are antigen presenting cells that can migrate to the lymph nodes and activate and modulate the differentiation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, similarly to LC in the epidermis (Hain et al., 2019). There are several subtypes of DCs in the skin, including pDCs, conventional DCs (cDCs) and inflammatory DCs (iDCs).

pDCs are a unique subtype of DC that migrate to the skin under pathological conditions (Wang & Bai, 2020). They recognise virus or autologous nucleic acids via a type of PRR known as Toll-like receptors (TLR), produce type I interferons (IFN) (IFN $\alpha/\beta$ ) and are implicated in skin disorders such as PsO (Kashem et al., 2017; Nestle et al., 2005).

cDCs are highly effective activating T cells via antigen presentation after activation by inflammatory cytokines, PAMPs and DAMPs (Geissmann et al., 2010; McGovern et al., 2015; Waisman et al., 2017). After activation, they secrete pro-inflammatory cytokines, mainly IL-12 and IL-23, able to activate the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway, involved in PsO and AD pathogenesis (Teng et al., 2015). IL-12 binds to its receptor in naïve CD4<sup>+</sup> T cells and induce the differentiation to Th1 cells, and IL-23 constitutes the cytokine microenvironment necessary for the differentiation to Th17 and Th22 cells (Chiricozzi et al., 2018; Girolomoni et al., 2017; Teng et al., 2015). Moreover, cDCs are crucial inducing immune tolerance through the depletion of autoimmune T cells by expressing anti-inflammatory cytokines such as IL-10 and by prompting the homeostasis of regulatory T cells (Treg) (Liu & Cao, 2015; Manicassamy & Pulendran, 2011; Takenaka & Quintana, 2017).

iDCs are observed in inflamed epidermal tissues of patients with inflammatory skin diseases and also in many parasitic, bacterial and viral infections. These cells express a Fc-like receptor with high affinity for immunoglobulin (Ig) E and is involved in allergic

inflammatory processes (Klechevsky, 2015; Segura & Amigorena, 2014). They deliver antigens to CD4<sup>+</sup> and CD8<sup>+</sup> T cells and are effective in the polarization of Th1, Th2 and Th17 cells by producing a wide variety of cytokines (IL-12, IL-23, Tumour Necrosis Factor (TNF)- $\alpha$ , IL-1 $\beta$ , IL-6 and Transforming growth factors (TGF)- $\beta$ ) (León et al., 2007; Segura et al., 2013; Wang & Bai, 2020).

#### **1.1.1.2. Macrophages**

Macrophages can derive from embryo progenitors that seed in the skin and can self-renew or from circulating monocytes that mature to macrophages once they reach the skin, being the second case the most common (Guilliams et al., 2014; Sheng et al., 2015). There are two main types of macrophages: M1 or pro-inflammatory, that secrete inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and M2 or anti-inflammatory, which can be subdivided into several subtypes, some of which have repairing capacities (Mills, 2015; Mills & Ley, 2014). In reality, macrophages can express markers from both phenotypes and in a steady state basically remove cellular debris (Malissen et al., 2014; Tamoutounour et al., 2013).

#### **1.1.1.3. Mast cells**

Mast cells derive from the bone marrow as progenitors and mature in the skin in response to factors produced by KCs (Hamaguchi et al., 1987; Liu et al., 2010; Thompson-Snipes et al., 1991). This type of cell contains granules that encapsulate histamine, proteoglycans, serotonin, tryptase and/or chymase (Olivera et al., 2018) and are involved in allergic reactions, as they release histamine when they encounter IgE-antigen complexes (Hirano et al., 2018; Shi et al., 2018). Mast cells also produce prostaglandin D2, leukotrienes and a variety of cytokines such as TNF- $\alpha$  and growth factors like vascular endothelial growth factor (VEGF) (Fajt et al., 2013; Nguyen & Soulika, 2019; Stinson et al., 2015; Wernersson & Pejler, 2014).

#### **1.1.1.4. Granulocytes**

##### **1.1.1.4.1. Basophils**

Basophils are primarily found in the blood (<0.5%) as fully mature cells in steady-state conditions but expand in the bone marrow in response to inflammatory signals and have a short lifespan (Kabashima, 2016; Karasuyama et al., 2011; Ohnmacht & Voehringer, 2009). These cells can be activated by a myriad of signals including antibodies (IgE, IgG

and IgD), cytokines (IL-3, IL-18 and IL-33), proteases, TLR ligands and complement factors (Karasuyama et al., 2011; Paul & Zhu, 2010). Once activated, they can produce IL-4, IL-13, IL-6, TNF- $\alpha$ , thymic stromal lymphopoietin (TSLP), histamine and leukotriene C4 (LTC4), antimicrobial peptides (AMPs) and chemotactic factors that can recruit other immune cells (Siracusa et al., 2011, 2012). Several studies have indicated a possible link between basophils and Th2 immune responses, and also an implication in skin diseases such as AD, prurigo, urticaria and bullous pemphigoid, among others (Ito et al., 2011; Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009).

#### **1.1.1.4.2. Eosinophils**

Eosinophils are bone-marrow-derived granulocytes that circulate in the blood and may be recruited upon certain stimulus into the tissues, such as the skin, where some resident eosinophils are present. These cells contain granules loaded with potent and toxic proteins, apart from cytokines and chemokines that are released upon certain stimulus (Long et al., 2015; Melo et al., 2013; Spencer et al., 2006, 2009). Eosinophils, similarly to mast cells, also produce prostaglandin D2, relevant for eosinophilic infiltration of the skin in AD, and leukotrienes (Luna-Gomes et al., 2011). Also, eosinophils are important in the defense against parasites (Jacobsen et al., 2012).

#### **1.1.1.4.3. Neutrophils**

Neutrophils are short-live cells that can be found in the circulation and that migrate into tissues upon recruitment to sites of infection or inflammation (Wang & Jin, 2020). They have several mechanisms of defense including phagocytosis, degranulation, generation of reactive oxygen species (ROS) and the formation of neutrophil extracellular traps (NETs).

The antimicrobial role of neutrophils is closely linked to the release of granules into the surrounding microenvironment after activation. These granules carry a diverse range of antimicrobial molecules including myeloperoxidase, defensins, metalloproteases, lysozyme (Faurischou & Borregaard, 2003)... Activated neutrophils can also generate NETs capable of entrapping pathogens and that are composed of cell-free DNA, histones, AMPs, danger molecules and auto-antigens (van Dam et al., 2019; Wang & Jin, 2020).

Although neutrophils have a crucial role in the first response against pathogens, they may as well contribute to the pathogenesis of different autoimmune and inflammatory

diseases affecting the skin such as systemic lupus erythematosus (SLE), rheumatoid arthritis and PsO (Kaplan, 2011; Nakabo et al., 2022; Wright et al., 2014).

#### **1.1.1.5. Melanocytes**

Melanocytes are cells found in the epidermis that produce melanin, a pigment able to provide colour to the skin. Its function is to protect DNA from UV radiation (Abdayem & Haftek, 2018; Prinz, 2017). They can promote phagocytosis and the production of cytokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$  and chemokines. Melanocytes are implicated in autoimmune diseases such as vitiligo and can be compromised by virus and bacteria (Gasque & Jaffar-Bandjee, 2015; Tsao et al., 2017). In similarity to KCs, they can produce type I IFNs and constitute the first anti-viral line of defense (Natarajan et al., 2014).

#### **1.1.1.6. Innate lymphoid cells**

Innate lymphoid cells (ILC) are a subset of immune cells belonging to innate immunity that in contrast with adaptive lymphocytes lack rearranged antigen receptors and thus lack antigen specificity. They have been divided into different subsets based on the cytokines they express and an assembly of transcription factors: group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), group 3 ILCs (ILC3s) and natural killer (NK) cells. ILC1s produce type 1 cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , while ILC2s secrete type 2 cytokines including IL-4, IL5 and IL-13 (Kiniwa & Moro, 2021; Moro et al., 2009; Vivier et al., 2018; Wagner & Koyasu, 2021). ILC3s produce IL-22 and/or IL-17 (Wagner & Koyasu, 2021) and NK cells release cytotoxic granules and are critical for the defense against infections and tumours (Liu et al., 2021).

ILCs are specially enriched in the skin (epidermis and dermis), where they play an important role in the defense of the skin barrier (Brüggen et al., 2016; Kim et al., 2013). Of note, ILC3s might be relevant for the maintenance of tolerance towards the microbiota of the skin and might be associated to the development of PsO, where the production of IL-17 and IL-22 in response to IL-23 contributes to dermal inflammation (Bielecki et al., 2021; Hepworth et al., 2013; Teunissen et al., 2014; Villanova et al., 2014).

#### **1.1.1.7. T lymphocytes**

There are different types of lymphocytes in the skin:  $\alpha\beta$  and  $\gamma\delta$  T cells.  $\alpha\beta$  T lymphocytes are found both in the epidermis and the dermis and migrate from the periphery upon

inflammation (Davenport et al., 2000; Suwanpradid et al., 2017). The  $\alpha\beta$  subset of T cells comprise multiple subsets, being CD4<sup>+</sup> T cells or T helper cells and CD8<sup>+</sup> T or T cytotoxic cells the most important lineages (Kabashima, 2016).

As previously introduced in Section 1.1.1.1, DC have a very important role inducing the proliferation and differentiation of CD4<sup>+</sup> naïve T cells into different subsets depending on the cytokine milieu (Bourque & Hawiger, 2018). As observed in Figure 2, there are different subsets of CD4<sup>+</sup> T cells: Th1, Th2, Th3, Treg, Th17, Th22, Th25 and Th9. Th1 cells produce IFN- $\gamma$  and protect against intracellular pathogens. Th2 produce IL-4 family cytokines and participate in allergic skin diseases, including AD. Th9 cells produce IL-9 and play an important role in parasite infections, inflammatory diseases and tumours. Th17 produce IL-17 and recruit granulocytes such as neutrophils to defend the skin against bacteria and fungi, apart from playing a key role in PsO and AD. Th22 generate IL-22, which activates epithelial cells and also has a role in PsO and AD. Treg cells are capable of inhibiting immune responses (Quaresma, 2019).

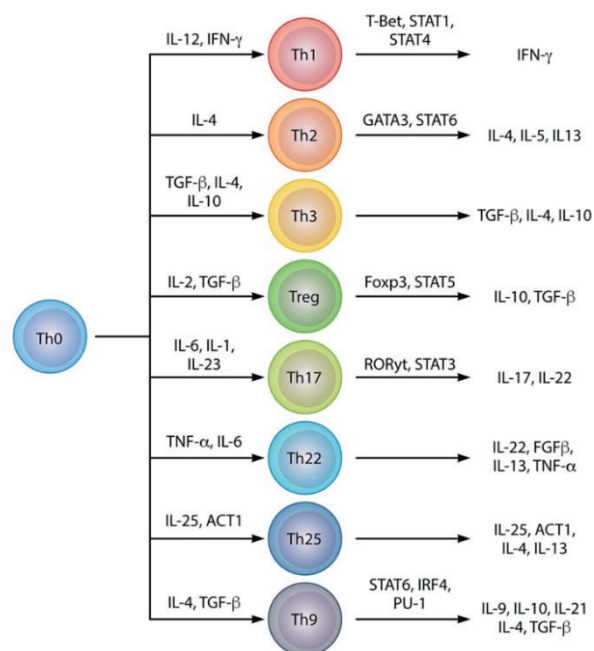


**Figure 2. Subpopulations of T helper cells.** Activation, differentiation factors and cytokines produced (Quaresma, 2019).

In the skin, there's a subtype of  $\alpha\beta$  T cells known as tissue-resident memory T cells ( $T_{RM}$ ) that derive from antigen-specific effector T cells that had infiltrated the tissue as a result of an insult and stayed in distal tissues providing rapid on-site immune protection due to their effector functions (Clark, 2015). Most  $\alpha\beta$  T cells are CD4<sup>+</sup> mainly found in the dermis

(including some Treg cells), whereas in the epidermis there are some CD8<sup>+</sup> T<sub>RM</sub> T cells. Another group of cells that belongs to the  $\alpha\beta$  T cells are NKT cells, which share functions of T and NK cells and behave like innate lymphocytes, secreting cytokines when they come across a pathogen (Chen et al., 2019; Kabashima, 2016).

The second type of T cells in the skin are  $\gamma\delta$  T cells, which have innate-like properties and are enriched in murine tissues (Section 1.1.2). In human skin, there are mainly two subsets of  $\gamma\delta$  T cells: V $\delta$ 1, which are predominant in epithelial tissues, and V $\gamma$ 9V $\delta$ 2, which are present in peripheral blood. Basically, these cells have an anti-tumoral and anti-microbial function (Pang et al., 2012; Thedrez et al., 2007).

#### **1.1.1.8. B lymphocytes**

B lymphocytes are a very minoritarian cell type in the skin in a steady state, but it has been described that their levels are highly increased in cutaneous diseases such as AD (Nguyen & Soulika, 2019; Simon et al., 2008).

#### **1.1.1.9. KCs**

As mentioned previously, KCs are epithelial cells that play an important role maintaining the skin barrier and participating in skin immunity. They can discern between microorganisms that are inherent to the skin and pathological ones (Elbe & Stingl, 1995; Nestle et al., 2009) and have mechanisms to control and influence inflammation (Meisgen et al., 2014).

KCs can produce and release cytokines able to alert the immune system from different danger signals such DAMPs, PAMPs or signals from surrounding cells in the skin. Different types can be distinguished: ILs (IL-1, IL-6, IL-36...), chemokines (CXCL8, CCL20), cytokines (TNF, IFN (IFN- $\beta$ , IFN- $\gamma$ ...)) and TSLP. These cytokines can be stored or transcriptionally regulated, and their main role is to recruit specialized leukocytes (Cordier-Dirikoc et al., 2022; Kamijo et al., 2020; Olaru & Jensen, 2010; Simmons & Gallo, 2024). An updated list of cytokines, growth factors, AMPs and receptors (PRRs, cytokine and growth factor receptors) involved in the signalling mechanisms of KCs in skin immunity can be found in Figure 3. Their implication in PsO and AD will be detailed in Section 1.2.2 and 1.3.2, respectively.

PRR expressed in KCs include TLRs (Lai & Gallo, 2012) that induce a cascade of innate and adaptive immunity once activated. Activation of TLRs expressed on KCs is important to start immune responses that lead to Th1 responses, accompanied by the activation of type I IFNs (Lai & Gallo, 2012; Newton & Dixit, 2012). Also, the activation of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which recognise several intracellular PAMPs and DAMPs, results in the activation of pro-inflammatory signalling pathways through inflammasomes and ends up with the expression of proinflammatory cytokines (IL-1 $\beta$  and IL-18) that activate immune cells (Coll et al., 2015; Feldmeyer et al., 2007; Lai & Gallo, 2012). Also, they produce factors that are crucial for homeostasis and tissue repair (Adachi et al., 2015; Chodaczek et al., 2012; Wang et al., 2012).

Of note, although KCs are the main producers of AMPs, other cell types, including phagocytes and T cells, among others, can also secrete them (Niyonsaba et al., 2017). These molecules defend the skin by destroying infectious microorganisms and act as first line of defense (Albanesi et al., 2005). The most abundant AMPs present in the skin are cathelicidin or LL-37,  $\beta$ -defensins and S100 family proteins (Simmons & Gallo, 2024).

The production of basal levels of AMPs controls the skin microbiota, and upon inflammation and direct activation of PRR by different microorganisms the expression of AMPs is induced (Albanesi et al., 2005; Lai et al., 2009; Liu et al., 2006; Miller & Modlin, 2007). Human  $\alpha$ - and  $\beta$ - defensins serve as chemoattractants for activated neutrophils, T cells and DCs and  $\beta$ - defensins 3 and 4 can recruit monocytes and macrophages (Nguyen & Soulika, 2019; Oppenheim & Yang, 2005; Zhang & Gallo, 2016). Apart from its defensive function, they have shown to induce proliferation, migration, differentiation and to regulate inflammatory responses. Also, they can contribute to different skin diseases such as PsO, AD, rosacea and acne vulgaris, among others (Hattori et al., 2014; Niyonsaba et al., 2017).

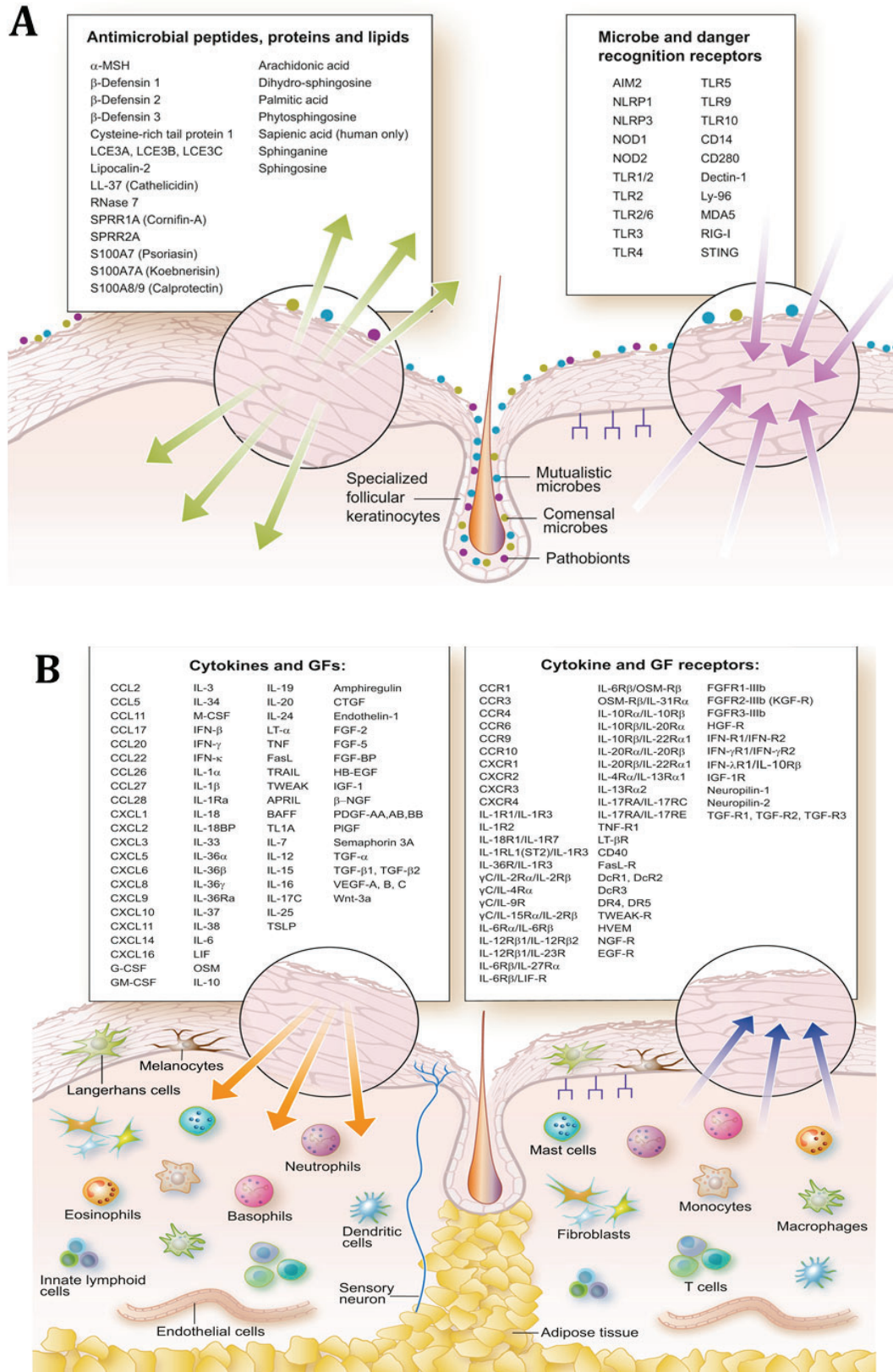


**Figure 3. Role and mechanisms of KCs in skin immunity.** KCs present multiple signalling mechanisms. A) List of antimicrobial molecules generated by KCs and the receptors used to recognize microbial and environmental dangers. B) KCs produce a wide variety of cytokines, chemokines, interleukins (ILs) and growth factors that promote the communication with other cells such as fibroblasts, adipocytes, melanocytes, sensory neurons, macrophages, monocytes, dendritic cells, LC, among others indicated in the image. The activation of some of the presented receptors can alter the function of KCs in response to signals generated by surrounding cells (Simmons & Gallo, 2024).

#### **1.1.1.10. Crosstalk between KCs and immune cells.**

KCs can interact with the skin microbiota and establish functional networks with skin immune cells.

The term skin microbiota refers to the microorganisms present on top of the epidermis (bacteria, virus, fungi and protozoa) (Byrd et al., 2018). The skin microbiota's composition varies between individuals, changes between different locations in the body and is dependent on factors such as thickness of the skin, UV exposure, humidity, pH (Dréno et al., 2016)... Microorganisms resident to the skin present a commensalism relationship with the body, where they can participate in the metabolism, maintain the immune system and protect against pathogens supporting the epidermal barrier (Byrd et al., 2018; Schommer & Gallo, 2013). Such importance does the microbiota have that upon dysregulation, several diseases can appear. An example of this case would be AD, as described on Section 1.3.2.

Microorganisms that reside in the skin produce PAMPs and communicate with KCs, that decide whether an inflammatory response is required. For example, *S. epidermidis*, a microorganism that belongs to the healthy microbiota of the skin, secretes a molecule known as lipoteichoic acid that inhibits the activation of TLR3 in KCs and can also release vesicles that promote the expression of  $\beta$ -defensins, which limits the growth of pathogenic bacteria such as *S. aureus* (Lai et al., 2009; Simmons & Gallo, 2024; Zhou et al., 2023).

T cells and KCs have a tight communication system in which they send each other cytokine signals. Upon stimulation from T cells, KCs can start producing AMPs and pro-inflammatory cytokines that activate and recruit more T cells, establishing this way an amplification system (Nogales et al., 2009; Simmons & Gallo, 2024). As an example of their crosstalk, during type 2 inflammation, scratching promotes barrier disruption and KCs release TSLP and IL-33, which recruits Th2 cells that generate IL-4, IL13 and IL-31 (Komai-Koma et al., 2007; Oyoshi et al., 2010; Tatsuno et al., 2015). All these cytokines affect KC differentiation and promote itchiness, along with an increase of inflammation accompanied by the chemotaxis for more Th2 cells, creating a positive feedback loop (Simmons & Gallo, 2024). Other cytokines like IL-17A, produced by Th17 cells,  $\gamma\delta$  T cells

and NKT cells can regulate the production of AMPs and proinflammatory cytokines from KCs (Sutton et al., 2009; Teunissen et al., 1998).

KCs also interact with macrophages and monocytes, critical players in inflammatory responses. KCs contain a transcription factor known as Nuclear factor erythroid 2-related factor 2 (NRF2) that is translocated to the nucleus upon oxidative stress and activates the expression of CCL2, a chemokine that acts as a recruiter for monocytes (Villarreal-Ponce et al., 2020). Macrophages can also be attracted by CCL2 and produce epidermal growth factor (EGF), that induces the proliferation of KCs, establishing a positive feedback loop, and also the induction of Granulocyte macrophage colony-stimulating factor (GM-CSF), which promotes macrophage infiltration and the maturation of LCs (Gao et al., 2016; Kupper et al., 1988; Mascia et al., 2010).

It is also noteworthy that the communication between granulocytes and KCs plays a crucial role in skin immunology. Neutrophils, similarly to Th17 cells, produce IL-17A that triggers the production of chemokines and AMPs in KCs which at the same time act as chemoattractants for neutrophils, establishing an amplifying system (Liu et al., 2022; Reich et al., 2015; Schumacher et al., 1992). The production of NETs stimulates the production of  $\beta$ -defensins in KCs and the activation of inflammasomes (Brinkmann et al., 2004; Dombrowski et al., 2011; Hu et al., 2016). Other granulocytes such as eosinophils, basophils and mast cells can also respond to cytokines generated by KCs and participate in different signalling processes (Burrows et al., 1991; Iikura et al., 2004; Tomonaga et al., 1986).

ILCs communicate with KCs upon skin injury and get activated by molecules secreted by KCs such as IL-25, IL-33 and TSLP (Leyva-Castillo et al., 2020; Monticelli et al., 2011). Then, they contribute to skin hyperplasia. ILCs can recruit macrophages and also produce IL-17F, which acts like IL-17A (Kim et al., 2013; Simmons & Gallo, 2024).

Fibroblasts, the most abundant cell in the dermis, also interact with KCs. There are different signalling pathways between these two types of cells, but one of the most important is through IL-1 $\alpha$  and IL-1 $\beta$  produced by KCs (Simmons & Gallo, 2024). These cytokines are recognized by their receptor in fibroblasts and activates mitogen-activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) (Barchowsky et al., 2000). Importantly, IL-1 $\alpha$  is present in the cytosol of KCs

and is released when the cell dies, but IL-1 $\beta$  has to be produced and released through inflammasome activation after encountering PAMPs or cytokines (Barchowsky et al., 2000; England et al., 2014; Fenini et al., 2018; Kupper et al., 1986). Fibroblasts respond to IL-1 by producing proinflammatory cytokines such as CXCL1 and IL-8 or CXCL8 (Friedrich et al., 2021; Wei et al., 2019).

Apart from interacting with cells from the immune system, KCs can communicate with sensory neurons and have a role in different types of sensation in the skin (Mikesell et al., 2022; Pang et al., 2015). Moreover, alarmins produced by KCs such as IL-33 and TSLP have been described to activate neurons and cause pruritus (Liu et al., 2016; Tseng & Hoon, 2022; Wilson et al., 2013). This connection between KCs and sensory neurons creates a vicious loop, as scratching damages the skin barrier, KCs release alarmins that increase inflammation and this ends up with a worsened pruritus (Oetjen et al., 2017).

### **1.1.2. Differences between human and murine skin**

Mice (*Mus musculus*) are the most commonly used animal in biomedical research due to several reasons: working with them is cost-effective, they are well-characterized and it is relatively easy to maintain and breed them. Also, they can be genetically manipulated and several models of human diseases have already been established (Justice & Dhillon, 2016; Perlman, 2016). Their use is widely accepted because of the high genetic conservation existing between humans and mice, having only around 300 genes unique to one or another (Waterston et al., 2002). However, there are differences between these species that need to be considered.

In terms of skin, both species present the same layers in the epidermis and dermis, but their thickness is different. While human skin measures around 100  $\mu\text{m}$  and presents 5-10 cell layers, murine skin measures less than 25  $\mu\text{m}$  and only presents 2-3 cell layers (Abdullahi et al., 2014; Gerber et al., 2014; Gurtner et al., 2011; Pasparakis et al., 2014). This fact, makes murine skin percutaneous absorption higher than human skin, and the general skin barrier not as efficient. This should be carefully taken into account when trying to extrapolate data from drug delivery in mice models to humans (Jung & Maibach, 2015; Menon, 2002).

Also, mice present the *panniculus carnosus*, a muscular structure present in the subcutaneous tissue that can contract when suffering from large wounds and that is absent in human skin (Gerber et al., 2014).

Another structure that is different between human and mouse are the rete ridges, invaginations of the epidermis to the dermis only present in the first (Zomer & Trentin, 2018). Importantly, murine skin is covered by hair follicles, whereas these follicles are less abundant in human skin (Zomer & Trentin, 2018).

In terms of immune cells, one of the main differences between human and murine skin are dendritic epidermal T cells (DETCs), only present in the latter. These cells are  $\delta\gamma$  T cells and are especially abundant in mice epidermis, where they are the predominant T cell type (Elbe et al., 1996; Mestas & Hughes, 2004). DETCs sample antigens using their dendrites and produce factors such as insulin-like growth factor 1 (IGF-1) and KC growth factor (KGF) that promote KC proliferation and regulate skin homeostasis and repair in a steady state (Sharp et al., 2005). Upon activation, these cells can secrete cytokines and growth factors such as IL-17A, which is essential for the expression of  $\beta$ -defensins in the skin (Macleod et al., 2013). The AMPs, cytokines and chemokines produced by human and mice are also different: CXCL8 or IL-8, CXCL7 and CXCL11 are not present in mice, while CXCL15 or CXCL14, among others, are not expressed by humans (Di Meglio et al., 2014; Harden et al., 2015; Rademacher et al., 2021)

Main differences are illustrated in Figure 4, where a comparative scheme of mice skin and human skin are displayed.

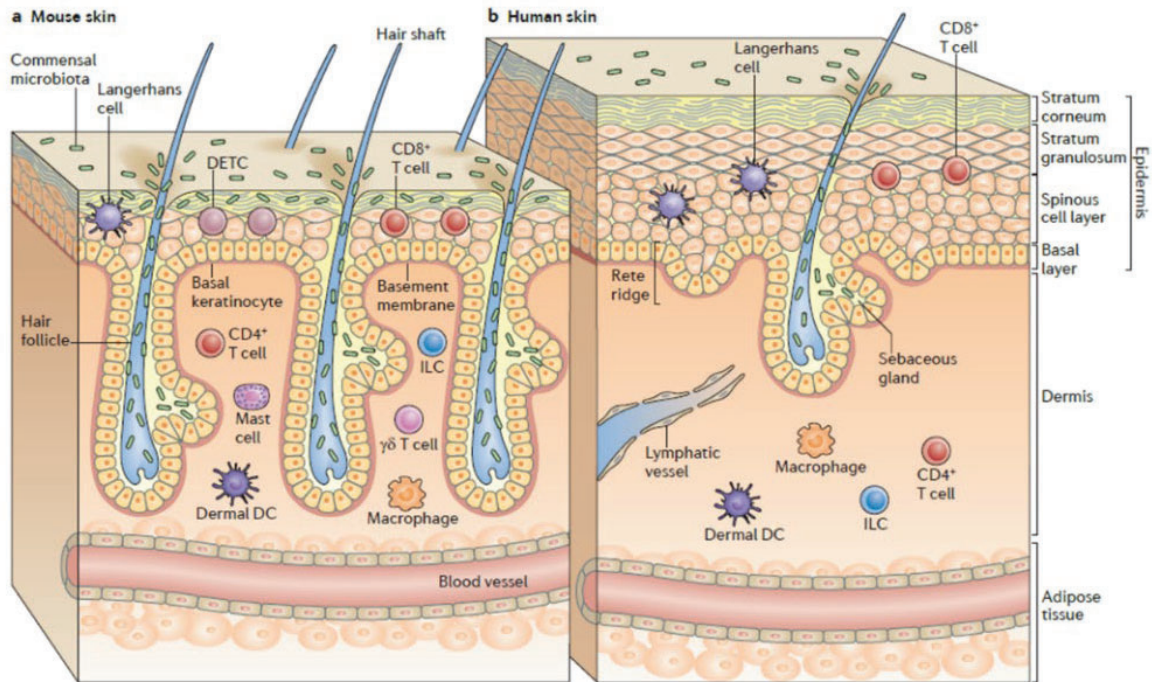


**Figure 4. Structural and cellular differences between murine and human skin.** Mice skin presents more hair follicles, a thinner epidermis, and DETCs cells. Human skin has a thicker epidermis, rete ridges, and LC and CD8+ T cells are the most prominent cells in the epidermis (Pasparakis et al., 2014).

## 1.2. Psoriasis

### 1.2.1. Epidemiology and clinical manifestations

PsO is an inflammatory skin disease affecting around 2-3% of the population worldwide, more than 60 million people (World Health Organization, 2016). It is a highly debilitating condition with significant physical and psychological burdens. The physical hardship basically refers to the itching, pain, burning sensations and bleeding patients suffer, while the psychological load of the disease associates to the visible disfigurement patients present, which can trigger a negative reaction in others. In fact, it has been described psoriatic patients are more likely suffer from depression (up to 20%) and suicidal behaviours (Dubertret et al., 2006; Singh et al., 2017).

The condition prevalence does not distribute equally across the globe. There are specific populations such as Asian and some African where the prevalence is much lower than in Caucasian and Scandinavian populations, where it can reach 11% (Danielsen et al., 2013; Gibbs, 1996; Parisi et al., 2013).

The disease affects men and women equally and the incidence is lower in children than adults. The mean age of onset is 33 years, and there are two different subtypes depending on the onset: early onset (before 40 years, 75% of the cases) and late onset (after 40 years) (Griffiths & Barker, 2007; Tollefson et al., 2010).

Five types of PsO have been reported: plaque or vulgaris, guttate, inverse, pustular and erythrodermic PsO.

- Plaque PsO is the most common form of the disease, accounting for around 90% of the cases and characterized by sharp demarcated erythematous plaques covered by lamellar scales (Figure 5A) (Boehncke & Schön, 2015).
- Guttate PsO presents with an acute onset of small erythematous plaques resembling droplets that mostly affect children and teenagers and is normally triggered by streptococcal infections of the upper respiratory tract likely attributable to the significant antigenic similarities between streptococcal proteins and KC antigens (Besgen et al., 2010; Rendon & Schäkel, 2019). It has been described that a third of the children presenting guttate PsO will develop plaque PsO later in life (Martin et al., 1996).
- Inverse PsO affects folds of skin and is characterized by erosive erythematous plaques and patches (Rendon & Schäkel, 2019).
- Pustular PsO is characterized by multiple sterile pustules (blisters of non-infectious pus) that can be localized or generalized. There are two phenotypes described: PsO pustulosa palmoplantis and acrodermatitis continua of Hallopeau, both affecting hands and feet (Navarini et al., 2017).
- Erythrodermic PsO is an acute condition where 90% of the total body surface is covered in erythematous lesions and inflammation. It can develop from any of the previously described types of PsO and it is a very rare but severe complication of PsO (Boehncke & Schön, 2015).

A series of diseases occur more prevalently in psoriatic patients than in the general population and make life expectancy of psoriatic patients substantially reduced. PsO comorbidities include: psoriatic arthritis, Chron's disease, cancer, depression, non-

alcoholic fatty liver disease, metabolic syndrome and cardiovascular disorders (Griffiths & Barker, 2007; Henseler & Christophers, 1995).

### 1.2.2. Pathogenesis

PsO histopathological hallmarks include epidermal acanthosis (thickening of visible layers), hyperkeratosis (thickened stratum corneum) and parakeratosis (cell nuclei present in the cornified layer). Also, epidermal rete ridges are elongated and blood vessels in the dermis are dilated and twisted. There is an evident inflammatory infiltrate containing T cells, neutrophils, mast cells and macrophages that accumulate in the epidermis forming microabscesses (Figure 5B) (Boehncke & Schön, 2015).

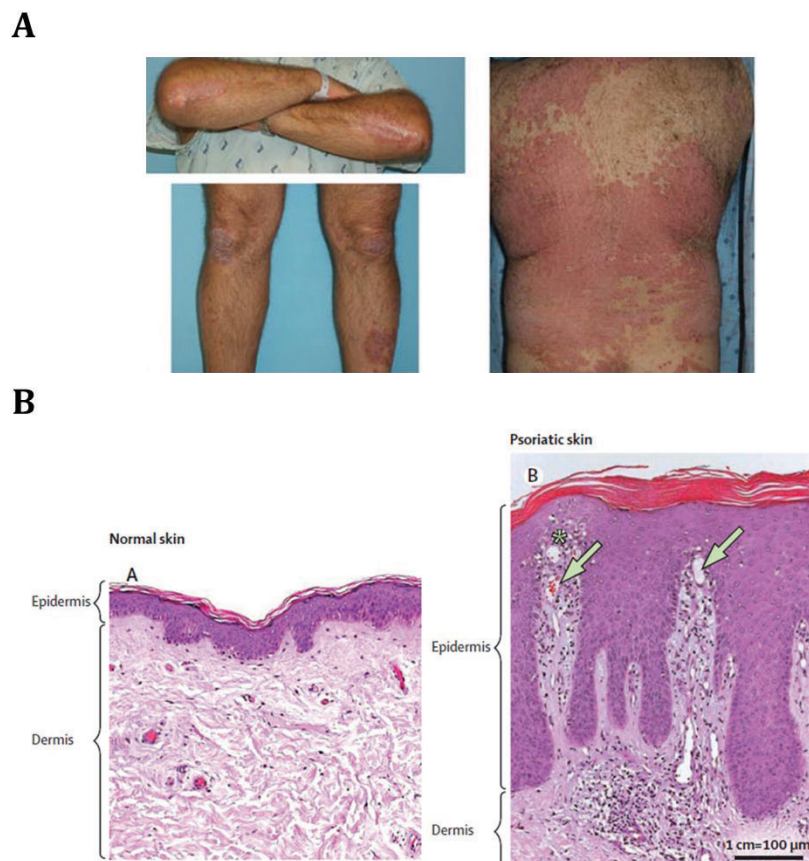


**Figure 5. Clinical and histological characteristics of PsO.** A) Clinical appearance of chronic plaque PsO where erythematous scaly plaques of PsO can be observed in elbows, knees and back (adapted from Lowes et al., 2014). B) Histological features of PsO skin compared to normal skin. Psoriatic skin presents epidermal acanthosis, hyperkeratosis and enlarged rete ridges. Also, blood vessels (arrows) are dilated and an inflammatory infiltrate (asterisk) can be observed). Adapted from Boehncke & Schön, 2015.

Population studies have suggested PsO is an inheritable disease. Many susceptibility loci have been described for PsO, with HLA-Cw6 being the most likely susceptibility allele (Nair et al., 2006; Trembath et al., 1997). Alterations in other genes such as IL-36 receptor or CARD14 have also been linked to the disease, specifically to pustular PsO (Sugiura,

2014). Genetic studies have highlighted crucial pathways involved in the condition: type I IFNs and antiviral signalling, NF- $\kappa$ B, IL-23, IL-17 and antigen processing and presentation (Elder et al., 2010).

The disease develops in susceptible individuals under specific triggers such as stress, infection, alcohol consumption, smoking, exposure to drugs such as lithium, antimalarials and non-steroidal inflammatory agents and sunlight in some circumstances (Griffiths & Barker, 2007).

The central mechanisms of the disease are the following: cross-talk between innate and adaptive immunity and TNF- $\alpha$  central role, the IL-23/Th17 axis and the effect of these immune reactions on other skin cells (Boehncke & Schön, 2015).

Basically, specific triggers can initiate the disease. At the beginning, neutrophils can infiltrate to the skin and release NETs, exosomes, metalloproteinase 9 and IL-17 (Sieminska et al., 2024). KCs get activated and start secreting AMPs such as cathelicidin or LL37,  $\beta$ -defensin and S100 proteins that form complexes with self-genetic material from damaged cells. These complexes stimulate TLR9 in pDC, which produce type I IFN (IFN- $\alpha$  and  $\beta$ ) and signal myeloid DC phenotypic maturation, inducing Th1 and Th17 differentiation and function, including their respective production of IFN- $\gamma$  and IL-17 (Gregorio et al., 2010; Morizane et al., 2012; Morizane & Gallo, 2012; Nestle et al., 2005; Santini et al., 2011). Other T cells such as IL-9 producing Th9 cells or IL-22 producing Th22 cells also get activated (Midde et al., 2021). LL37 also forms complexes with RNA, which stimulate pDC through TLR7 and myeloid cells through TLR8 (Morizane et al., 2012; Morizane & Gallo, 2012). DCs get activated, migrate to lymph nodes and secrete TNF- $\alpha$ , IL-12 and IL-23. The activation of specific populations of T cells facilitates maintaining psoriatic inflammation. Also, Th17 cells produce IL-17, IL-21 and IL-22, which activates the proliferation of KCs in the epidermis. Its proliferation also gets induced by TNF- $\alpha$  and IFN- $\gamma$  and they produce type I IFN, IL-1, IL-6, TNF- $\alpha$ , chemokines and AMPs (Hänsel et al., 2011; Morizane et al., 2012; Nestle et al., 1994). Moreover,  $\gamma\delta$  T cells can produce IL-17A without the IL-23 stimulus (Lee et al., 2015).

The IL-17 family has six members: IL-17A-F. They are produced by different cell types and bind to the IL17 receptor, resulting in the recruitment of the ACT1 adaptor protein. The formation of this complex leads to the activation of intracellular kinases: extracellular

signal-regulated kinase (ERK), p38 MAPK, transforming growth factor-beta-activated Kinase 1 (TAK1), I-kappa B kinase (IKK) and glycogen synthase kinase 3 beta (GSK-3 beta). These kinases result in the transcription of pro-inflammatory cytokines, chemokines and AMPs via NF-kB, AP-1 and C/EBP. Th1 and Th2 cytokines act through JAK-STAT signalling pathways, while Th17 responses are mediated by ACT1 and NF-kB (Gaffen, 2009; Matsuzaki & Umemura, 2018). Figure 6 provides a comprehensive depiction of the intracellular signalling pathways above mentioned, highlighting key players involved (Guo et al., 2023).

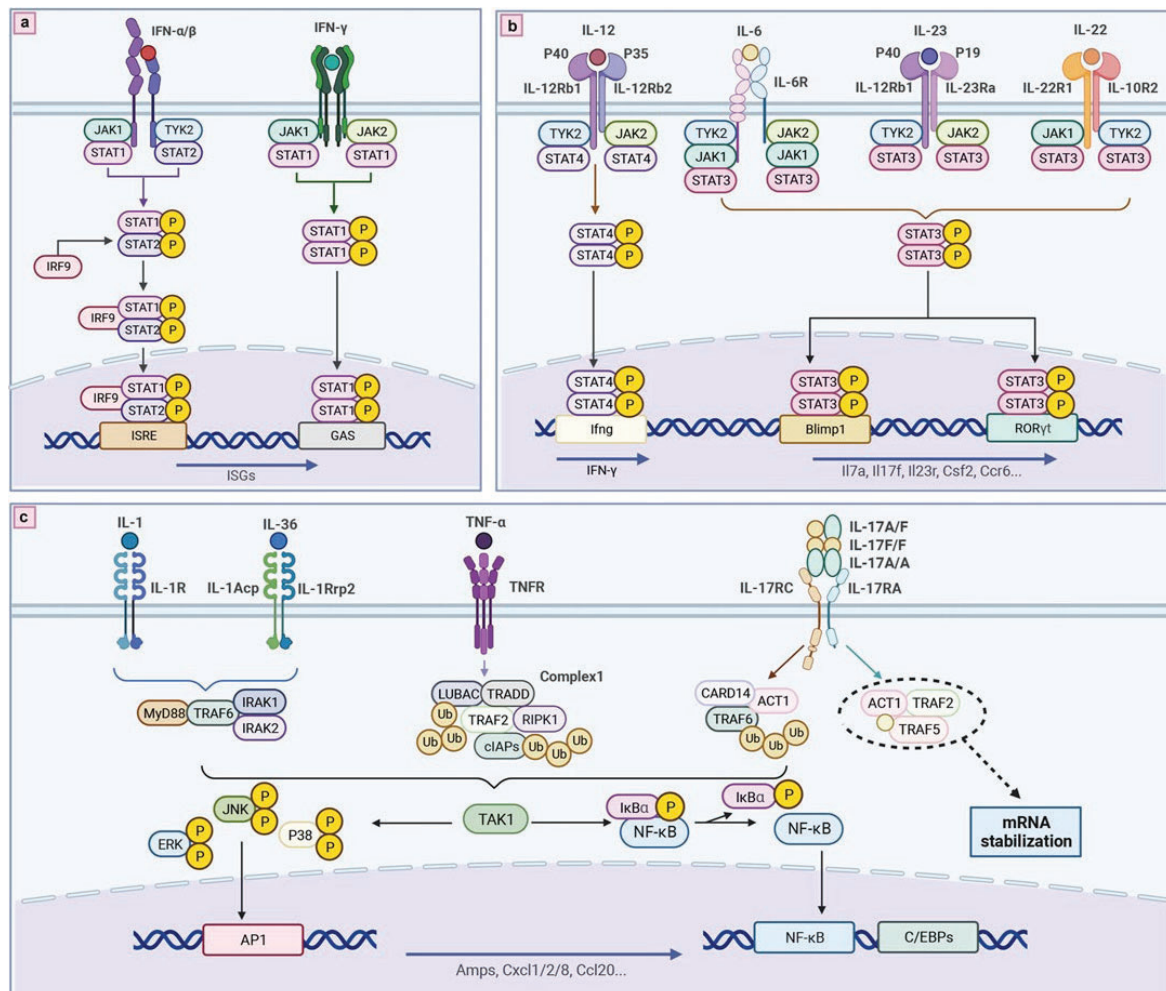


**Figure 6. Signalling pathways involved in the pathogenesis of PsO.** Psoriatic cytokines basically use two signalling pathways: JAK/STAT and TYK2 pathways (a,b) and different cytoplasmic complexes that assemble to activate the NF-kB/MAPK pathway (c). IFN $\alpha/\beta$  and  $\gamma$ , IL-12, IL-6, IL-23 and IL-22 rely on the first, while IL-17, IL-1, IL-36 and TNF- $\alpha$  use the second type of transduction (Guo et al., 2023).

In the late phases of the disease, the skin lesions are highly infiltrated with immune cells, there are huge amounts of cytokines and chemokines and KCs are hyperproliferating. Also, angiogenesis is increased and endothelial cells facilitate the intravasation of immune

cells into the skin (Sieminska et al., 2024). A schematic overview of the early and late phase of the disease can be found in Figure 7.

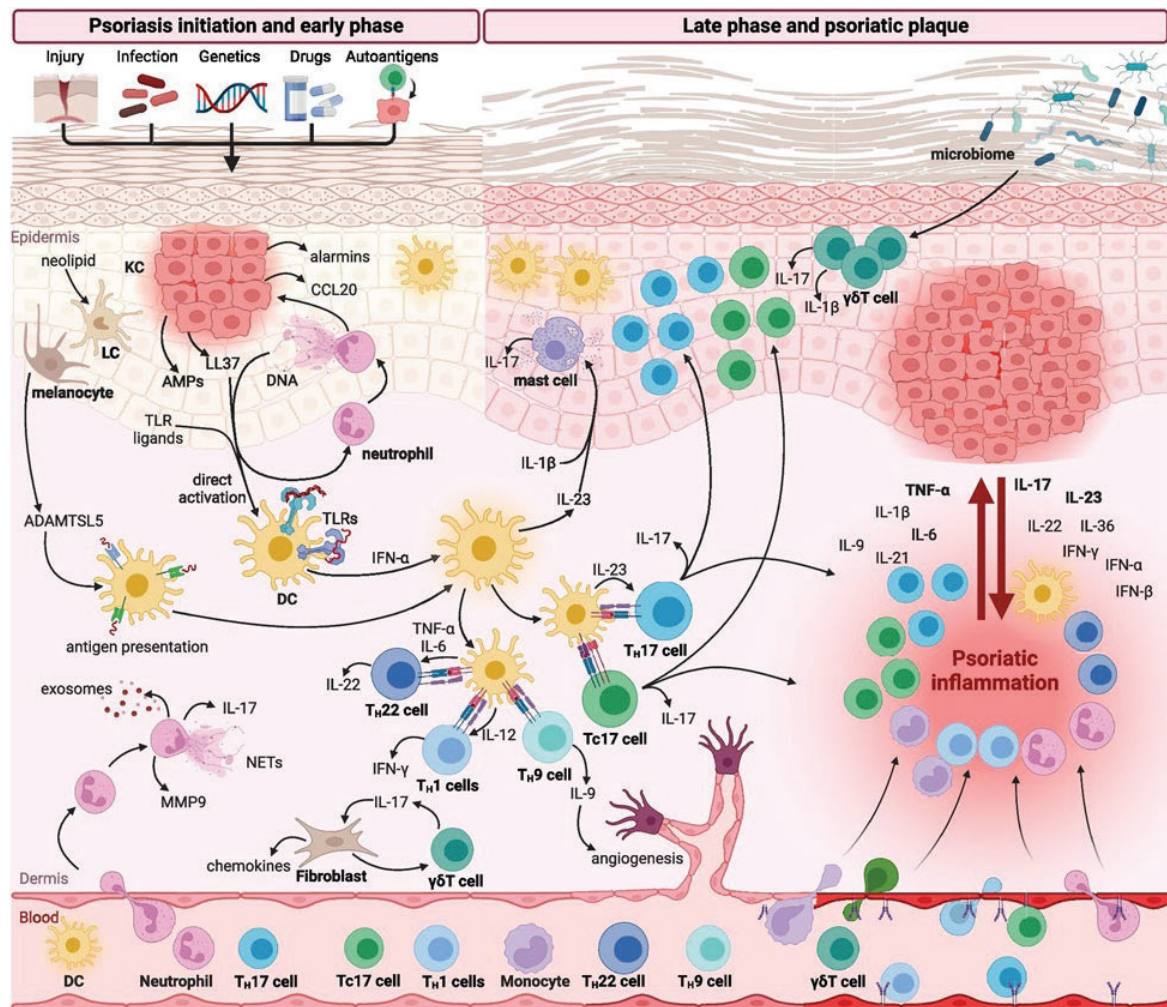


**Figure 7. Pathogenesis of PsO.** The disease is triggered by different factors. In the early phase, neutrophils infiltrate the skin and activate KCs, which produce proinflammatory factors including chemokines, antimicrobial peptides (AMPs) and alarmins. Dendritic cells are activated by these signals and initiate T cell immune response from different subsets (Th17, Th9, Th1, Th22). In the latter phase of the disease, the skin is highly infiltrated with immune cells, there are enormous concentrations of proinflammatory cytokines and chemokines and KCs are hyperproliferating. Angiogenesis and activation of endothelial cells facilitate the infiltration of immune cells from the periphery (Sieminska et al., 2024).

The TNF- $\alpha$ -IL23-Th17 axis has a central role in plaque PsO, but other mechanisms are more prominent in the other variants of the disease. For example, as briefly mentioned before, in guttate PsO streptococcal antigens can induce the expansion of T cells in the skin due to the sequence homology of some of the bacteria and KC proteins. This, might be important in patients with the major histocompatibility HLA-Cw6 allele, as IFN- $\gamma$  responses induced by CD8 $^{+}$  T cells have been reported against self-peptides (Johnston et al., 2004; Leung et al., 1995).

## 1.3. Atopic dermatitis

### 1.3.1. Epidemiology and clinical manifestations

AD, also known as eczema, is a recurrent, chronic, non-infectious inflammatory skin disease that normally develops in childhood and may become a life-long hurdle. Rarely, it can start in adulthood. The prevalence of the disease is around 15-20% among children and 10% in adults (Laughter et al., 2021; Ständer, 2021). Both sexes are affected equally and the prevalence can vary among ethnic groups (McKenzie & Silverberg, 2019; Ständer, 2021). The incidence of the condition has increased in the last decades worldwide, becoming one of the most common skin diseases in childhood (Mallol et al., 2013; Sacotte & Silverberg, 2018).

The clinical features of the disease include eczema-like eruptions, such as erythema, papules, exudative lesions of a specific location, depending on the patient's age, and skin dryness (Avena-Woods, 2017). During childhood, eczema spreads throughout the body and with increasing age, lesions are localized to the arms and legs popliteal flexures, hands, neck, feet and the periocular region (Figure 8A) (Yew et al., 2019). Due to the chronicity of the disease, skin thickening and lichenification, a medical term used for the thickening and hardening of the skin, may occur (Chrostowska-Plak et al., 2013; Silverberg et al., 2018).

Pruritus is a hallmark of AD and its intensity corresponds to the severity of the disease (Yew et al., 2019). It can get worse with stress, sweating, heat, humidity and contact with some types of fabric such as wool (Huet et al., 2019). Itching interferes with patient's lives and causes insomnia and sleep disorder, resulting in a decrease in the quality of life of patients and their families, with effects on productivity and emotional and social life (Chrostowska-Plak et al., 2013; Kahn et al., 2020; Silverberg et al., 2018). Moreover, it has been linked to depression, anxiety and suicidal tendencies (Silverberg, 2019).

Eczema patients are at risk of suffering the "atopic march", a sequence of allergic disorders including food allergies, allergic rhinitis, rhinoconjunctivitis and asthma (Yang et al., 2020). This, is related to skin barrier dysfunction that allows the penetration of allergens and also to a predisposition of the immune system to induce a CD4<sup>+</sup> Th2 cell response with subsequent B-cell antibody production (Ständer, 2021). Also, AD patients have a higher risk of bacterial, viral or fungal skin infections that can result in life-

threatening systemic infections, although very rare, and of having an altered skin microbiome (Droitcourt et al., 2021; Ren & Silverberg, 2020; Wang et al., 2021). Moreover, inflammatory bowel disease and rheumatoid arthritis have been associated with eczema (Silverberg, 2019).

### 1.3.2. Pathogenesis

AD histopathological characteristics are not as pathognomonic or distinctive as PsO ones. However, it has been described that in an acute phase of the disease, epidermal edema breaks the connections established in the epidermis, a process known as spongiosis. Also, there is an infiltration of different cells from the immune system such as T cells and macrophages and an enlargement of the epidermis (acanthosis) (Figure 8B) (Johnson-Huang et al., 2009).

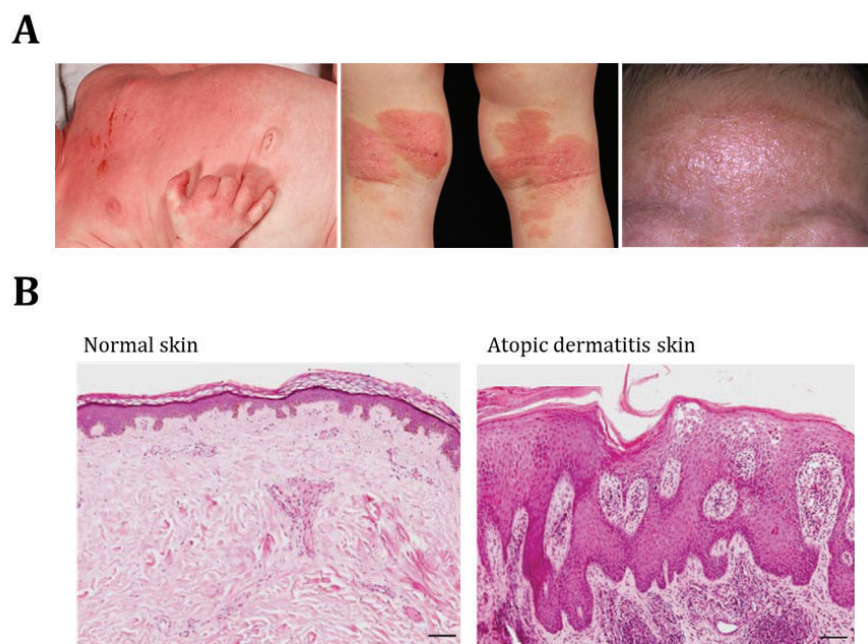


**Figure 8. Clinical and histological characteristics of AD.** A) Clinical manifestations of AD where erythema is observed in the trunk, face and flexor surfaces. Adapted from Weidinger et al., 2018. B) Histological features of AD skin compared to normal skin. AD skin presents epidermal edema, which breaks the connections established in the epidermis (spongiosis). There is acanthosis as a result of scratching and infiltration of T cells, dendritic cells, macrophages, eosinophils and mast cells. Adapted from Johnson-Huang et al., 2009.

Historically, two hypotheses have been proposed to explain the pathogenesis of AD: the “outside-in” hypothesis, which maintains epidermal barrier dysfunction triggers immune dysregulation, and the “inside-out hypothesis”, that suggests immune aberrations are the initial event and stimulation with allergens lead to damages in the epidermal barrier.

Now, the idea is to combine these hypotheses to define the condition (Leung, 2000; Leung & Guttman-Yassky, 2014).

Immune dysregulation, genetic and environmental factors, impaired epidermal barrier integrity and skin microbiome abnormalities are key to AD pathogenesis, both in its initiation and its sustaining in chronic states (Facheris et al., 2023).

Several studies have pointed out a higher incidence of AD in families with history of the condition, noting a possible genetic background (Løset et al., 2019). Thirty-one chromosomal loci containing AD susceptibility genes have been located in the human genome, all related to epidermal barrier function and innate and acquired immune response (Nedoszytko et al., 2020; Paternoster et al., 2015). Mutations in the *FLG* gene are the most relevant, accounting for 30-50% of European patients (Patrick et al., 2021). *FLG* is produced in the upper layer of the epidermis and induces the production of moisturizing factors and lipids that keep KCs together. A loss of function mutation leads to a disruption in skin barrier formation and an increase in transepidermal water loss (TEWL), which results in dry skin, and also to a reduced production of AMPs, which end up in microbial dysbiosis, an alteration of the skin microbiome (Langan et al., 2020; Patrick et al., 2021). A decrease in the expression of tight junction proteins, which regulate the passage of ions, water and solutes and are important for the differentiation and keratinization processes taking place in the epidermis, has also been observed in AD patients (De Benedetto et al., 2011). Not only genetic alterations, but also epigenetic changes that explain the fast increase in allergic diseases in the last years have been described in AD (Bin & Leung, 2016). Genes affected by these changes are mainly involved in KC differentiation, proliferation and innate immune response, such as the *S100A* genes (Mu & Zhang, 2020; Rodríguez et al., 2014).

Skin barrier dysfunction leads to the production of alarmins such as IL-33, IL-25 and TSLP by damaged KCs. These alarmins can activate DCs and type 2 ILCs, that produce IL-5 and IL-13, and will activate eosinophils and Th2 cells (Leung, 2000). T helper cell polarization towards a Th2 lineage induces the production of IL-4, IL-5 and IL13, which stimulate IgE class switching in B cells and eosinophils. IL-33 produced by KCs activates NF-kB and MAPK, which stimulate the production of cytokines related to Th2 response: IL-4, IL-5, IL-13. The JAK-STAT pathway plays a central role in the pathogenesis of AD due to its

involvement in the signal transduction of Th2 cytokines (IL-4, IL-5, IL-13, IL-31 and TSLP). A schematic representation of the signalling pathway along with the key molecules involved, is shown in Figure 9 (Huang et al., 2022).

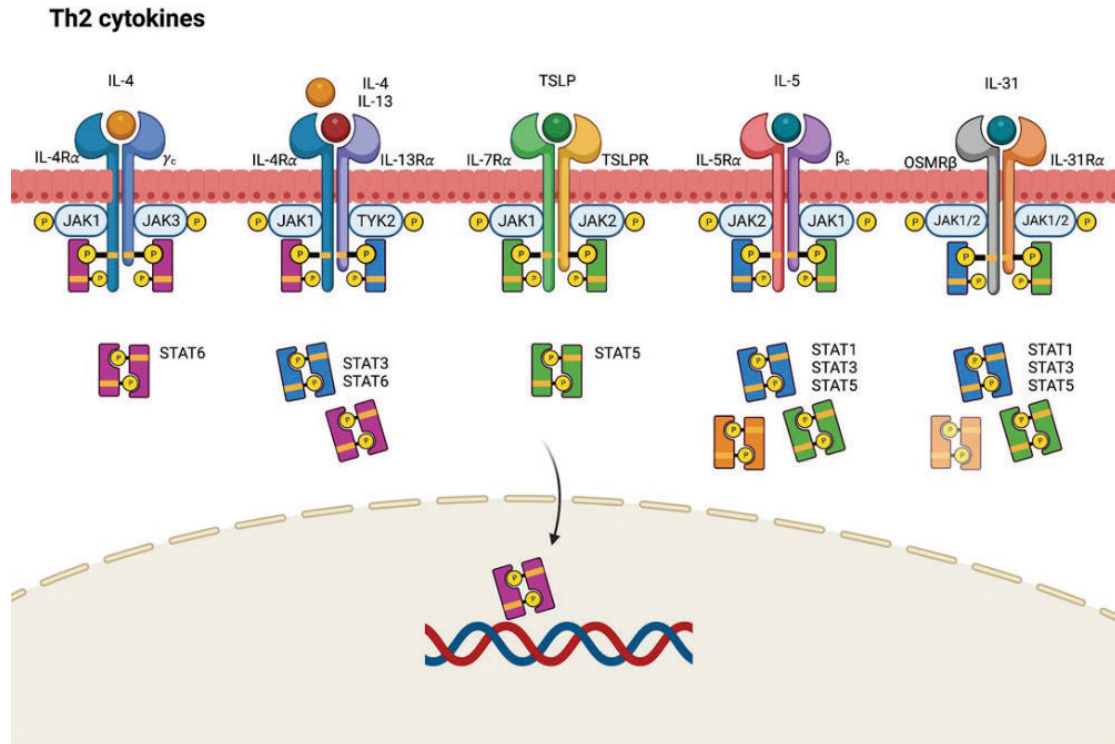


**Figure 9. JAK/STAT signalling pathway involved in the Th2 response of AD.** Schematic view of the main transmission pathways utilised by IL-4, IL-13, TSLP, IL-5 and IL-31 and JAK/STAT molecules involved in the pathogenesis of AD (Huang et al., 2022).

In a more chronic phase of the disease, Th1 cells are recruited and produce IL-2, IL-12, TNF- $\alpha$  and INF- $\gamma$  cytokines (Avena-Woods, 2017; Brunner et al., 2017; Furue et al., 2017; Imai et al., 2013; Klonowska et al., 2018). Due to alterations in the skin barrier, bacteria such as *S. aureus* can appear on the skin and cause infections. This, enhances inflammation and stimulates itching (Meng et al., 2018; Sroka-Tomaszewska & Trzeciak, 2021).

Itching in AD is mediated by unmyelinated C fibres and thinly myelinated A $\delta$  fibres that can be activated by pro-inflammatory cytokines (IL-31, IL-4, IL-13) and peptides released by Th2 cells, eosinophils, neutrophils and mast cells. KCs also release pruritic factors such as TSLP (Kido-Nakahara et al., 2017; Meng et al., 2018; Mollanazar et al., 2015; Yosipovitch et al., 2020). Continuous scratching of the skin damages KCs and causes the release of inflammatory cytokines, activate the Th2 axis and the release of more pruritic factors, that rebind to the sensory nerves and triggers continuous scratching, generating

the known itch-scratch cycle (Yosipovitch et al., 2018, 2020). The role of histamine in AD pruritus is not conclusive.

Even though AD is primarily Th2-driven, contributions of the Th1, Th17 and Th22 axis have also been identified depending on the AD endotype, which are different variants of the disease that have been identified in different age and ethnic groups. For example, Th2 and Th22 lineage responses with lower Th1 and Th17 are very common in European and American populations (Chan et al., 2018), while Japanese AD patients have increased Th17 and a suppression of the Th1 axis (Czarnowicki et al., 2019).

A scheme of AD pathogenesis stages with the main alterations and components is shown on Figure 10.

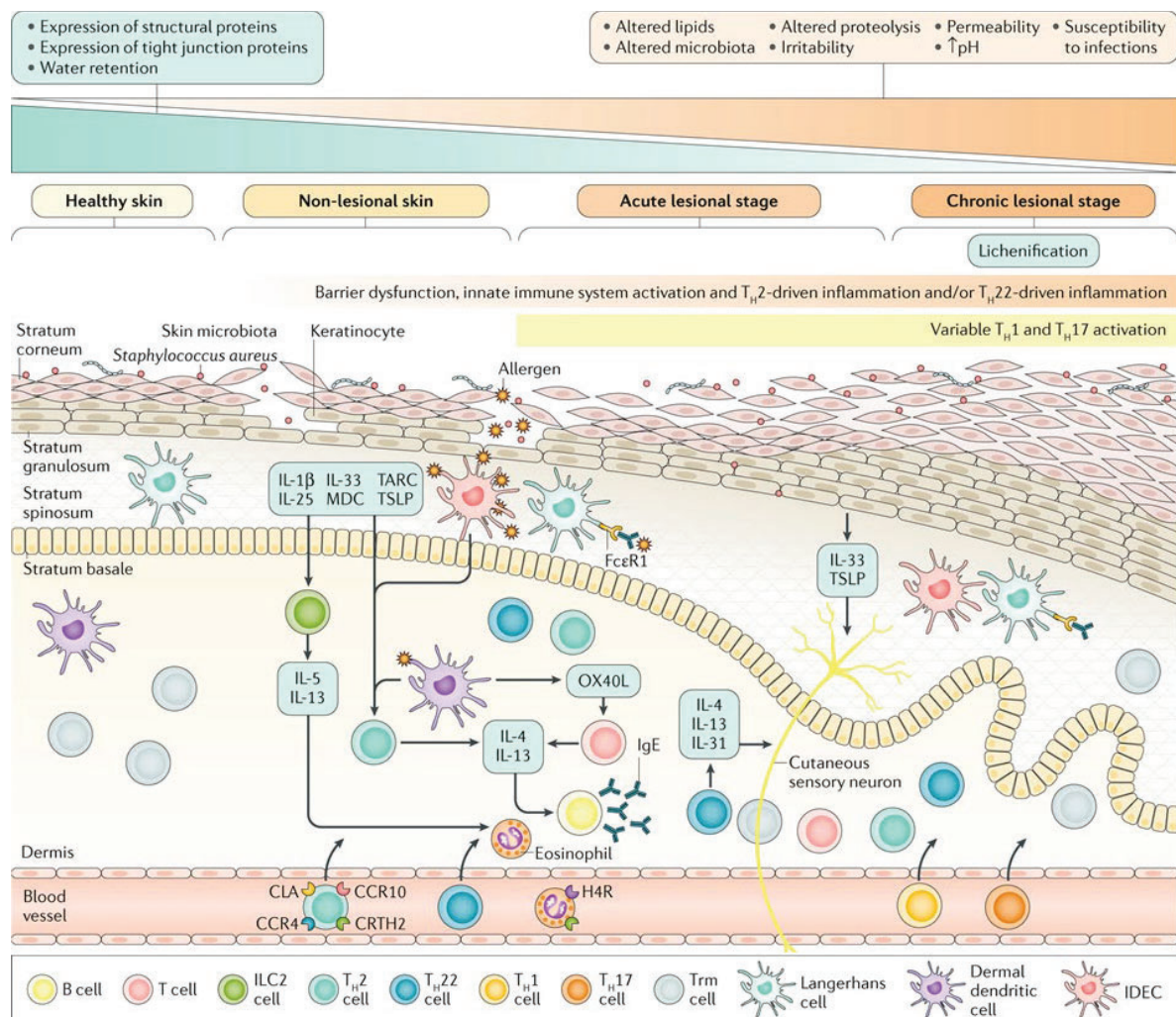


**Figure 10. Pathogenesis of AD.** Skin barrier dysfunction leads to the production of alarmins by KCs that can activate cells from the immune system and induce a CD4 polarization towards a Th2 lineage and activate type 2 ILCs. Dendritic cells uptake antigens and promote type 2 immunity, leading to the production of IgE. Th2 cytokines interact with sensory neurons, intensifying itch. In a more chronic setting, Th1 cells are recruited and produce inflammatory cytokines. Contributions from Th17 and Th22 axis have also been identified (Weidinger et al., 2018).

#### **1.4. PsO and AD therapeutics: an expanding field**

During the last 10 years, skin disease has gained increasing recognition as a significant global health concern. In 2016, the World Health Organization highlighted PsO as a major noncommunicable disease, and AD was identified as a leading cause of non-fatal health burden in skin conditions (World Health Organization, 2016). Recently, a call for comprehensive action to address skin diseases and their global impact has been made in the dermatology field (Bieber, 2023). Advances in the understanding of genetic and immunopathology of PsO and AD, the most common chronic inflammatory skin disorders, has led to the development of new therapies (Boehncke & Schön, 2015; Conrad & Gilliet, 2018; Ständer, 2021; Weidinger et al., 2018). However, this knowledge has been focused on the mechanisms involved in the inflammatory reactions and not the chronicity nor the “tissue memory”, that could have a role explaining the comorbidities each of them present in the long term and the spontaneous regression seen in some children with AD, which could be helpful for the development of new drugs (Bieber, 2023).

A new therapeutical approach taking into account the developmental stages of AD and PsO with candidate targets and drugs has been proposed and aims to prevent or stop the ongoing inflammation and/or facilitate the compensatory mechanisms (Figure 11). This way, both the course of the diseases and their comorbidities could be stopped (Bieber, 2023).

##### **1.4.1. PsO treatment**

Therapeutic options for psoriatic patients are highly influenced by the presence of associated medical conditions. Moderate-to-severe PsO has been defined by several factors: significant effect on the physical, social and psychological wellbeing of the patient, a localized PsO that cannot be controlled with topical treatments and the association with functional impairment or distress (Amatore et al., 2019). Treatment options range from topical therapies or phototherapy for mild PsO to oral systemic treatments and biologics for moderate to severe PsO (Griffiths et al., 2021)

Topical therapies consist on the application of agents like corticosteroids, vitamin D<sub>3</sub> analogues, calcineurin inhibitors, among others in vehicles such as creams, ointments or

gels. These treatments are given to patients with less than 3-5% of affected body surface and their primary downside is poor adherence (Griffiths et al., 2021; Menter et al., 2009).

Phototherapy is based on the application of UV radiation to the skin, which induces immunosuppression by affecting LC, inhibits the hyperproliferation of KCs and angiogenesis and causes the apoptosis of T cells. There are different modalities of phototherapy and the most widely used is narrowband UVB radiation (311-313 nm) two or three times per week. Side effects of this treatment include burning and photocarcinogenesis, apart from being time-consuming (Griffiths et al., 2021; Morita, 2018).

Oral systemic small-molecule-based therapies are used for moderate to severe forms of the disease, mainly as first line of treatment previous to therapies based on biologics. The most commonly used oral therapies for moderate to severe PsO are: methotrexate, ciclosporin, acitretin, fumarates, apremilast, Aryl hydrocarbon receptor (AhR) agonists and more recently, tyrosine kinase 2 (TYK2) inhibitors. These therapeutics interact with ubiquitous targets reducing the immune response in an indirect or direct way, and may present adverse broad side effects, such as immunosuppression. In particular:

- Methotrexate inhibits lymphocyte function by affecting 5-aminoimidazole-4-carboxamide ribonucleotide transformylase activity, increasing adenosine production. This treatment is given once a week and has some important side effects such as bone marrow suppression and cirrhosis, nausea, vomiting, hair loss, teratogenicity and pulmonary toxicity. Methotrexate can also be administered subcutaneously and has proven greater efficacy and less gastrointestinal problems (Allegra et al., 1985; Saurat et al., 2008; Warren et al., 2017).
- Ciclosporin is a calcineurin inhibitor that inhibits the nuclear factor of activated T cells (NFAT) and thereby IL-2 gene expression and T cell activation. It must be used for short periods of time in severe cases or patients who are in a crisis. Their action is very fast, but should not be used for more than one year due to the risk of nephrotoxicity. Moreover, it can present other adverse effects such as hypertension, higher infection risk, nausea, hirsutism, gingival hyperplasia, drug-drug interactions and electrolyte disfunctions (Ellis et al., 1991).

- Acitretin is a synthetic retinoid that decreases proinflammatory cytokines like IL-6 and IFN- $\gamma$  and normalises KC proliferation and differentiation (Dogra et al., 2013; Raharja et al., 2021). Its mechanism of action is not entirely understood; however, it has been described to bind to retinoic acid receptors (RAR) or retinoic X receptors (RXR) and to modulate the transcription of genes codifying proteins implicated in the pathogenesis of the disease. Also, it seems to antagonize other transcription factors and decrease the expression of genes implicated in proliferation, angiogenesis and inflammation (Carretero et al., 2013). Acitretin is not considered a cytotoxic nor immunosuppressive treatment (Menter et al., 2020).
- Fumaric acid esters inhibit the maturation of DCs, induce T cells apoptosis and interfere with leucocyte extravasation (Brück et al., 2018). The mechanism of action of fumaric acid esters is very complex. Active metabolites derived from fumaric acid esters such as monomethyl fumarate, bind to hydroxy-carboxylic acid receptor 2 on the cell surface and react with intracellular glutathione. Also, they modulate the activity of signalling proteins such as NRF2, Nf-kB and intracellular concentrations of cyclic adenosine monophosphate (cAMP). Some studies have also indicated that fumaric acid esters like dimethylfumarate can affect the hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ). The result of these actions causes a downregulation of inflammatory cytokines and a shift from a Th1/Th17 prominent response to a Th2 one (Brück et al., 2018; Ghoreschi et al., 2011; Gold et al., 2012).
- Apremilast is a phosphodiesterase-4 (PDE4) inhibitor that leads to an increase in cAMP and produces a decrease in proinflammatory cytokines (IL-2, IL-12 and TNF- $\alpha$ ) and increase anti-inflammatory cytokines (IL-10). It can cause nausea, diarrhoea and weight loss (Papp et al., 2015).
- Inhibitors of TYK2, a Janus kinase involved in the signalling of IL-23, IL-12 and type I IFNs. Among them, Deucravacitinib is an oral TYK2 inhibitor that binds to the regulatory domain of TYK2 and results in a conformational change in the catalytic domain responsible for binding to adenosine triphosphate (ATP) that results in the inhibition of the protein. It has been approved in the last years and has opened a

new window for the development of therapies (Hawkes et al., 2024; Martin, 2023). Other TYK2 inhibitors are still in phase II clinical trials.

- AhR agonists act on the ligand-activated transcription factor AhR, which is expressed in different types of skin cells (KCs, fibroblasts, mastocytes and melanocytes) and has a role protecting against oxidative stress, epidermal differentiation, melanocyte proliferation and immunomodulation (Lee & Kim, 2023; Salgado-Boquete et al., 2021). An abnormal expression of AhR has been reported in AD and PsO (Mrowietz et al., 2011), and in 2022 a topical agonist of AhR named Tapinarof was approved by the FDA for the treatment of plaque PsO in adults (Elmets et al., 2021).

In the last few years biologics have changed the paradigm in the treatment of PsO. Biologics are recombinant monoclonal antibodies or receptor fusion proteins (human, humanised, or human-mouse chimeric) that target specific mediators of the immune system implicated in PsO. These are mostly administered subcutaneously and can be subdivided in four families: anti-TNF- $\alpha$ , anti-IL-17, anti-IL12 and anti-IL-23 (Bieber, 2023; Griffiths et al., 2021). Biologics are efficacious, used long-term and have a good safety profile. Interestingly, they seem to have a positive impact on reducing, managing or even preventing the emergence of PsO comorbidities such as PsO arthritis and cardiovascular diseases (Bieber, 2023). However, some patients do not respond and the reason is still unknown, although some factors associated include non-adherence, low blood concentrations of biologics probably due to the development of anti-drug antibodies, high body mass and sex differences, being women more prone to non-responsiveness (Iskandar et al., 2018; Menting et al., 2014; Thorneloe et al., 2013; Warren et al., 2019). Another downside of biologics is that they are expensive. In recent years, some low-cost biosimilars have been developed and might allow low-income countries to access them (Griffiths et al., 2021).

The pathophysiology of PsO has been studied in more depth and has been described as more “uniform” than AD’s. The central axis of the disease and also its comorbidities such as psoriatic arthritis or cardiovascular disorders are known and T<sub>RM</sub> T cells have been described to have a role in the chronic stages of the lesions (Bridgewood et al., 2021; Ryan et al., 2021). However, how the natural mechanisms to counteract the inflammation in

both PsO in AD work is uncertain. Current effort is being focused on developing therapies targeting upstream inflammatory cytokines to IL-23 or more downstream at the level of IL-17, and also at the T<sub>RM</sub> level and antigen presentation. Also, the stimulation of Treg cells is being explored as a new therapeutic approach (Figure 11A) (Bieber, 2023).

#### **1.4.2. AD treatment**

In the last ten years, the treatment of AD has evolved significantly due to the discovery of key aspects of its pathogenesis. However, it remains an expanding field with many drugs still under development. AD treatment is selected according to the clinical stage of the disease, the body-surface area involved, age, comorbidities and medications taken by patient and severity of pruritus (Sidbury et al., 2014; Ständer, 2021; Wollenberg et al., 2018).

Patients with AD should always use emollients to improve barrier function, dryness and water loss, even in eczema-free periods, and immunosuppressive topical therapies when eczema happens. For moderate eczema, UV therapy can be applied only in adult patients. Systemic immunosuppressants such as glucocorticoids, cyclosporine or methotrexate are administered to patients with severe forms of the disease. These, however, do not target specific mediators of the disease and can cause liver and kidney dysfunction (Ständer, 2021).

Th2-targeted therapies such as monoclonal IL-4, IL-13, IL-22 and IL-31 receptor antibodies, PDE-4 inhibitors and JAK inhibitors (topical and systemic) have been accepted recently for the treatment of AD. The first human antibody approved for the treatment of AD was Dupilumab, a human antibody that can bind to the alpha subunit of IL-4 and IL-13 receptors and is capable of improving the skin barrier function, reverse the phenotype in the skin and act on immune dysregulation by diminishing Th2-associated chemokines and inflammatory infiltrates (Beck et al., 2014; Cabanillas et al., 2017; Hamilton et al., 2014; Kim et al., 2019). JAK small molecule inhibitors are less selective than monoclonal antibodies against singular cytokines or cytokine receptors but are really effective, as they inhibit the signalling of many proinflammatory cytokines (Figure 6 and Figure 9) (Szalus et al., 2020).

In the case of AD (Figure 11B), interventions proposed before the symptoms start are difficult to consider, as in the absence of reliable biomarkers, all newborn children should

be treated. At this point, treatments aiming to balance the altered skin microbiome are being developed (Bieber, 2022; Harkins et al., 2020; Tham et al., 2020) and the use of emollients is highly accepted as a way of improving the skin barrier (Brown et al., 2020). Once AD lesions start, topical anti-inflammatory therapy using calcineurin, PDE-4 or JAK inhibitors could impact antigen presentation by DC in the skin and also the developing inflammation, mechanisms that lead to a IgE sensitization, which is involved in the generation of AD comorbidities (Bieber, 2022, 2023). Also, targeted therapy against specific mediators such as TSLP and IL-33, or against molecules involved in antigen presentation such as the costimulatory pathway OX40 (expressed on T cells) and OX40L (on antigen presenting cells) are promising (Guttman-Yassky et al., 2023; Han et al., 2017; Leyva-Castillo et al., 2020; Zhu et al., 2011).

Other molecules such as Dupilumab, able to block IL-4 and IL-13, which are crucial for the synthesis of IgE has potential to stop AD and associated pathologies. In a chronic phase of the disease with atopic comorbidities, the optimal approach would be to have biomarkers that would allow a personalised treatment approach in which a combination of biologics or systemic oral agents could be valid (Bieber, 2023; Schwartz et al., 2017; Tanaka et al., 2022). However, the vast majority of agents mentioned in the text and figure are still in clinical developmental phases.

Importantly, the development of new therapies for PsO and AD is still an emerging field with many aspects still unexplored and area for improvement. The search for reliable biomarkers that would make precision medicine a reality in the field could lead to a revolution in the therapeutic pipeline. Also, the discovery of novel targets and approaches would be highly beneficial for the community.

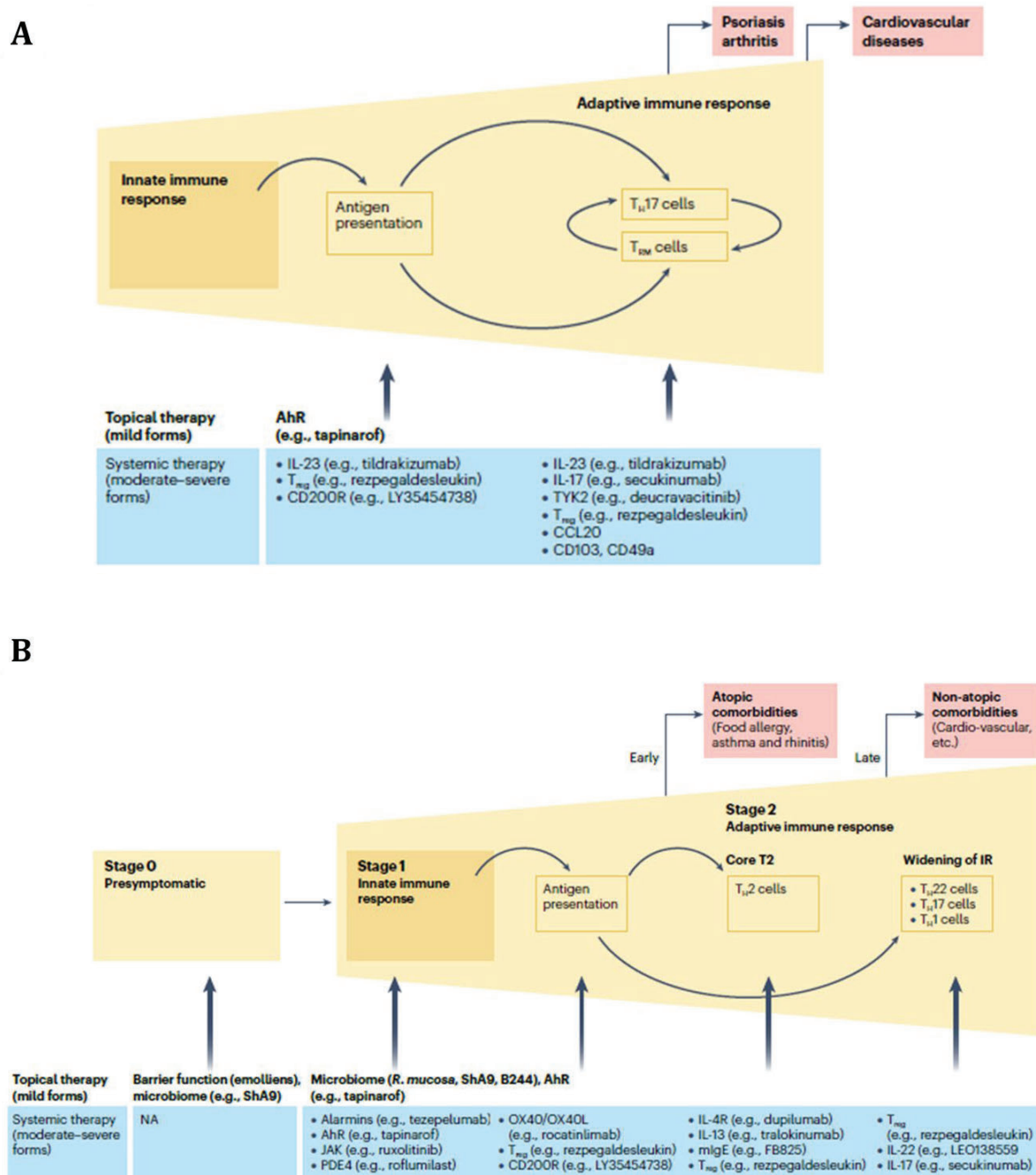


**Figure 11. Scheme of the proposed intervention approach for PsO and AD.** A) In PsO, strategies aiming to affect innate immunity or adaptive immunity, basically antigen presentation and the IL-23-IL-17 axis and T<sub>RM</sub> T cells are proposed to modify the course of the disease (blue boxes). B) AD pathogenesis is divided into three stages: presymptomatic or stage 0, stage 1 in which the innate immune response is activated and stage 2, where the adaptive immune response has initiated (divided into early and late). In the blue boxes, proposed treatment options for every stage are indicated (Bieber, 2023).

## 1.5. Three prime Repair Exonuclease 2

In 1999, the Three prime Repair Exonuclease 2 (TREX2) was identified in a database search due to its homology to the Three prime Repair Exonuclease 1 (TREX1), with whom shares 40% of its general sequence and 80% in their Exo motifs (Höss et al., 1999; Mazur & Perrino, 1999). Even though their DNA binding affinity, expression and functions differ,

their activity and structural conformation is quite similar (Cheng et al., 2018; De Silva et al., 2007).

### 1.5.1. Activity and structure

Nucleases are enzymes that cleave phosphodiester bonds linking nucleotides in nucleic acid sequences. They can be classified in different groups depending on the type of substrate they cleave (RNA for RNases and DNA for DNases) and the position in the nucleic acid chain where they cut (endo- or exonucleases) (Baranovskii et al., 2004; Manils et al., 2022; Zuo & Deutscher, 2001). Endonucleases exert their activity in phosphodiester bonds from inside a polynucleotide chain generating two oligonucleotides and can be sequence or structure specific. In contrast, exonucleases hydrolyse from the outer ends of the nucleic chain (3'-OH or 5'-P) in single or double DNA strands (ssDNA or dsDNA, respectively) in a nonspecific manner (Dehé & Gaillard, 2017; Yang, 2011). Exonucleases take part in different cellular processes such as DNA replication, repair and death and have also been associated to the immune response (Manils et al., 2022). A scheme of the key exonuclease proteins implicated in DNA repair, apoptosis and inflammation is displayed in Figure 12.

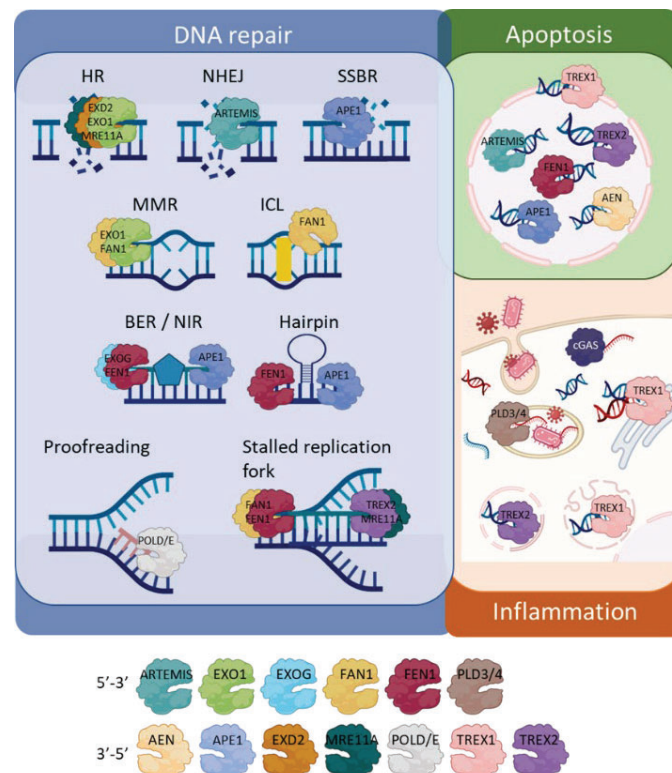


**Figure 12. Exonucleases involved in DNA damage repair, apoptosis and inflammation.** The most important proteins with an exonuclease activity implicated in DNA damage repair, apoptosis and inflammatory processes are depicted. Exonuclease activity (5'-3' and 3'-5') is detailed (Manils et al., 2022).

TREX1 and TREX2 have a robust non-processive 3'-5' exonuclease activity that allows the removal of normal, mismatched, modified or fragmented nucleotides to generate suitable 3' termini for different DNA metabolic pathways involved in the maintenance of genomic stability, ageing and inflammatory and hereditary diseases (Chen et al., 2007; Mason & Cox, 2012; Mazur & Perrino, 1999, 2001a). 3'-5' exonuclease activity has been associated to different types of proteins such as polymerases (pol $\delta$ , pol $\epsilon$ , pol $\gamma$ ), helicases, multifunctional proteins involved in DNA repair, tumour suppressors (NM23-H1, Ape1, WRN, Mre11, Trp53) and autonomous proteins not belonging to multiprotein complexes such as TREX1 and TREX2 (Mason & Cox, 2012; Shevelev & Hübscher, 2002).

In terms of structure, TREX2 and TREX1 are homodimers constituted by two identical subunits that have three domains: the homodimerization domain, the DNA-binding domain and the exonuclease domain (Chen et al., 2007; De Silva et al., 2007; Perrino et al., 2005). Each subunit is composed of five-stranded antiparallel twisted  $\beta$ -sheets surrounded by nine  $\alpha$ -helices that dimerize perpendicularly to the  $\beta$ -sheets creating another  $\beta$ -sheet that extends through the dimer (Figure 13) (Cheng et al., 2018; Perrino et al., 2005). The back part of the dimer is flat and positively charged, and the front contains two negatively charged active sites, each in opposite sides of the molecule. Next to the active sites, there are positive-charged mobile loops that scan DNA looking for 3' free ends, contribute to DNA binding and facilitate its entrance to the active sites (Perrino et al., 2005, 2008).

Both monomers work in a cooperative and synergistic manner. This means that when the active site in one monomer binds to DNA, there is a residue reorganization and a structural change that transmits to the opposing monomer and results in a higher affinity for the substrate in both monomers. This probably means TREX2 prefers to function with both active sites engaged at the same time (De Silva et al., 2009; Perrino et al., 2008).

The structure of TREX2 bound to dsDNA was defined and revealed that when TREX2 is bound to dsDNA with a ssDNA mismatched region, it eliminates the latter until it arrives to the dsDNA segment in a very precise manner. Also, it was seen that TREX2 binds to the 5' termini of DNA to control its function in the 3' end of DNA and that the active sites of the dimer need to release the substrate before releasing the products of the reaction

(monophosphate nucleotides), which seems to point out the non-processive property of the exonuclease (Figure 13) (Cheng et al., 2018).

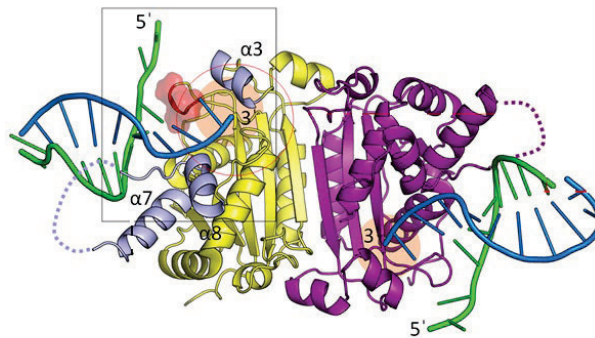


**Figure 13. TREX2 structure bound to dsDNA.** Murine TREX2 is represented in complex with dsDNA. Monomers are coloured yellow and purple and the DNA strands blue and green. Adapted from Cheng et al., 2018.

TREX2 and TREX1 share three conserved exonuclease motifs (Exo I, Exo II and Exo III) and depend on  $Mg^{2+}$  to catalyse DNA. These characteristics make them suitable members of the DnaQ exonuclease family (Mazur & Perrino, 2001a; Perrino et al., 2008). The DnaQ family can be divided in two subfamilies depending on the presence of four carboxylate residues in the active site and a tyrosine (DEDD-y subfamily), or a histidine (DEDD-h subfamily), being TREX2 exonuclease part of the second group. These four conserved carboxylate residues coordinate two  $Mg^{2+}$  ions and histidine deprotonate a molecule of water to produce a nucleophile attack and hydrolyse phosphodiester bonds between nucleotides in DNA (De Silva et al., 2009). A classification of the exonucleases belonging to the DEDD-h subfamily based on their substrate preference and the structural characteristics in binding to DNA can be found on Figure 14. While both TREX2 and TREX1 prefer dsDNA or double-stranded RNA (dsRNA) with a 3' overhang, TREX2 requires a longer interacting double-strand region than TREX1.

TREX enzymes can process ssDNA and dsDNA, but both exonucleases prefer dsDNA with multiple mismatches as substrate, which points out they might proofread mistakes generated by polymerases that do not have an intrinsic exonuclease capacity (Cheng et al., 2018; Höss et al., 1999; Mazur & Perrino, 2001a; Perrino et al., 2008).

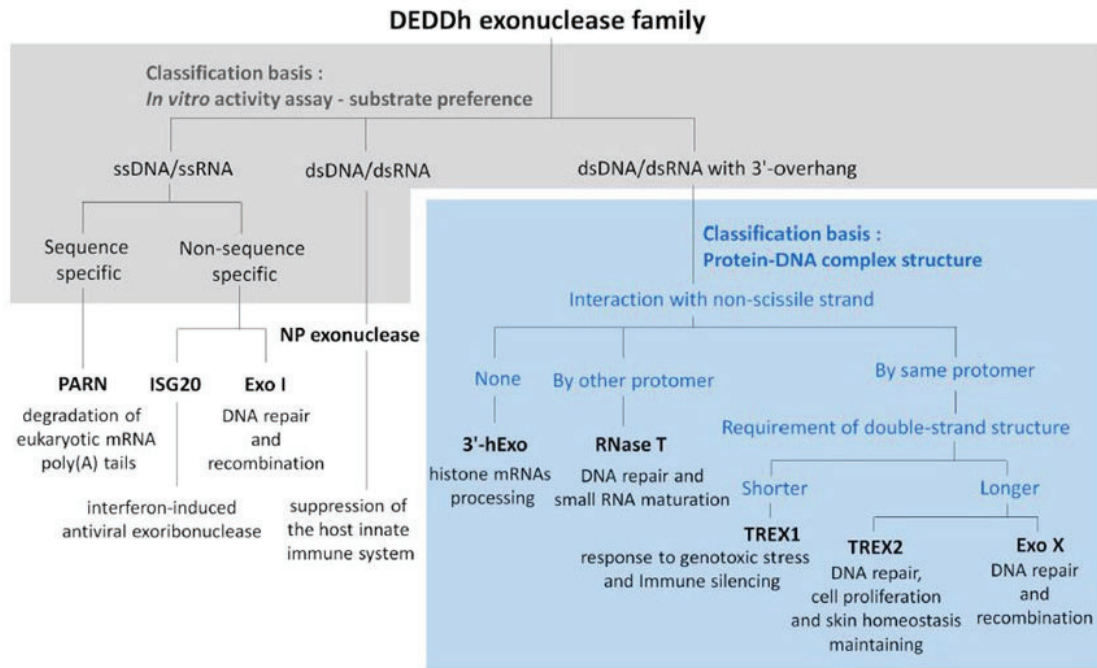


**Figure 14. DEDDh exonuclease family classification.** Exonucleases from the DEDDh family have been classified depending on their substance preference and their DNA-binding structural characteristics. (Cheng et al., 2018).

### 1.5.2.Expression

The human and murine *TREX2* genes (*TREX2* and *Trex2*, respectively) are in the X chromosome and due to alternative splicing three transcripts of the *TREX2* gene have been identified and produce three isoforms of the protein: 236 amino acids (26 kDa), 278 and 279 amino acids (30 kDa) (Chen et al., 2007; Manils et al., 2015, 2016; Mazur & Perrino, 1999; Parra et al., 2009), being the first one the most abundant transcript and the only isoform identified in a protein level. In mice, only the transcript that results in the 236 amino acid isoform and the respective protein have been described (Manils et al., 2015, 2016, 2017; Parra et al., 2009).

*TREX2* expression is restricted to stratified squamous epithelia, and more specifically to the nuclei of KCs, where it is responsible for around 50% of the exonuclease activity produced there. Its expression levels increase upon KC differentiation and has shown to be upregulated in chronic UVB-irradiated skin, actinic keratosis lesions and PsO (Manils et al., 2015, 2016, 2017; Parra et al., 2009).

On the other hand, human *TREX1* gene (*TREX1*), located in chromosome 3, and the murine (*Trex1*), in chromosome 9, codify for a 314 amino acid (32 kDa) protein that presents a

proline-rich domain that allows the interaction with other proteins and an additional C-termini fraction of 76 amino acids that permits its attachment to the endoplasmic reticulum (Brucet et al., 2007; De Silva et al., 2007; Lee-kirsch et al., 2007; Mazur & Perrino, 2001b).

Conversely to KC restricted TREX2 expression, TREX1 is ubiquitously expressed and localized in the endoplasmic reticulum, but can translocate to the nucleus during granzyme A-mediated cell death (Chowdhury et al., 2006).

### **1.5.3.Function**

TREX2 function has been related to skin pathology (Manils et al., 2015, 2016, 2017) and to both genomic stability and instability processes (Chen et al., 2007; Dumitrache et al., 2009; Hasty, 2021; Hu et al., 2013; Ko et al., 2020; Marple et al., 2024). Its physiological importance was determined using *Trex2* knockout mice, where it was established that this exonuclease has a role in skin carcinogenesis and PsO (Manils et al., 2015, 2016; Parra et al., 2009).

This exonuclease has been suggested as a tumour suppressor, though current studies yield conflicting results. In artificial settings, *Trex2*-null and mutated embryonic stem cells showed chromosomal and genomic instability. Still, *Trex2* knockout mice did not present spontaneous tumours, nor decrease on survival, nor chromosomal instability (Chen et al., 2007; Dumitrache et al., 2009; Manils et al., 2015, 2022; Parra et al., 2009). TREX2 function in the DNA damage response (DDR) pathway of embryonic stem cells may vary based on the DNA repair status, being dependent or independent of its exonuclease activity and thereby promoting or preventing replication fork instability and mutations. Still, this effect is not observed in cells from *Trex2* knockout mice or in KCs, where there is a high expression of *Trex2*, contrary to embryonic stem cells where the expression is undetectable (Hu et al., 2013; Ko et al., 2020; Manils et al., 2022; Marple et al., 2024; Parra et al., 2009).

*Trex2* deficiency can increase carcinogen-induced skin carcinogenesis. The reason is that TREX2 has a tumour suppressor role as it promotes the apoptosis of KCs that are under genotoxic stress conditions and favours antitumoral inflammatory responses (Manils et al., 2015; Parra et al., 2009). This exonuclease concentrates in micronuclei and interacts

with  $\gamma$ H2AX, key in genomic stability maintenance and cell death regulation (Manils et al., 2015). When DNA is damaged, TREX2 participates in its repair but mostly contributes to the degradation of fragmented DNA, promoting cell death of damaged KCs and favouring an antitumoral immune response. Therefore, TREX2 has a major role as a proapoptotic tumour suppressor in KC-drive tumours (Manils et al., 2015; Parra et al., 2009).

Furthermore, several studies pointing out the deregulation of TREX2 in cancer support the role of TREX2 as tumour suppressor. An increase in *Trex2* expression was reported well-differentiated tumours of cutaneous squamous cell carcinomas and head and neck squamous cell carcinomas, but not in metastasis (Manils et al., 2015). An epigenetic regulation of *Trex2* was seen in colorectal and laryngeal cancer (Weigel et al., 2019). Also, a reduction on DNA methylation of *Trex2* is associated with a higher expression and survival of patients, while *Trex2* missense mutations and upregulation in colorectal cancer are associated to reduced survival (Weigel et al., 2019).

In the last years, TREX2 has been used in the CRISPR/Cas9 technology to enhance genome-editing efficiency and safety. This methodology is based on modifying a target sequence of DNA by creating double strand breaks. To avoid off target effects or unwanted mutations, the use of modified enzymes that can only induce single-strand breaks or nicks, named nickases, has been explored (Cong et al., 2013; Güell, 2024; Mali, et al., 2013; Ran et al., 2013). When introducing TREX2, the exonuclease can prompt repair mechanisms or make the nicks more suitable for edits, especially when the nicks cause 3' overhanging ends (Güell, 2024; Wang et al., 2023). Also, TREX2 has shown to increase deletion size and prevent perfect DNA repair, avoiding repeated Cas9 cleavage and chromosomal translocations (Bothmer et al., 2017; Chari et al., 2015; Weiss et al., 2020).

Finally, TREX2 is highly expressed in psoriatic lesions (Figure 15A) and its deficiency attenuates the characteristic inflammation in murine models of the disease (Figure 15B). During KC accelerated turnover, TREX2 is overexpressed and recruited to KC's fragmented chromatin, where it is important for the DNA degradation that happens during the process of cornification and apoptosis of KCs. The signals released by cells that are close to death can trigger and amplify inflammation and cause lymphocyte mediated cytotoxicity (Figure 15C) (Manils et al., 2016). Of note, TREX2 is the only nuclease

upregulated in lesional psoriatic tissue compared to non-lesional skin. No other nucleases, including TREX1, are deregulated (Manils et al., 2016).

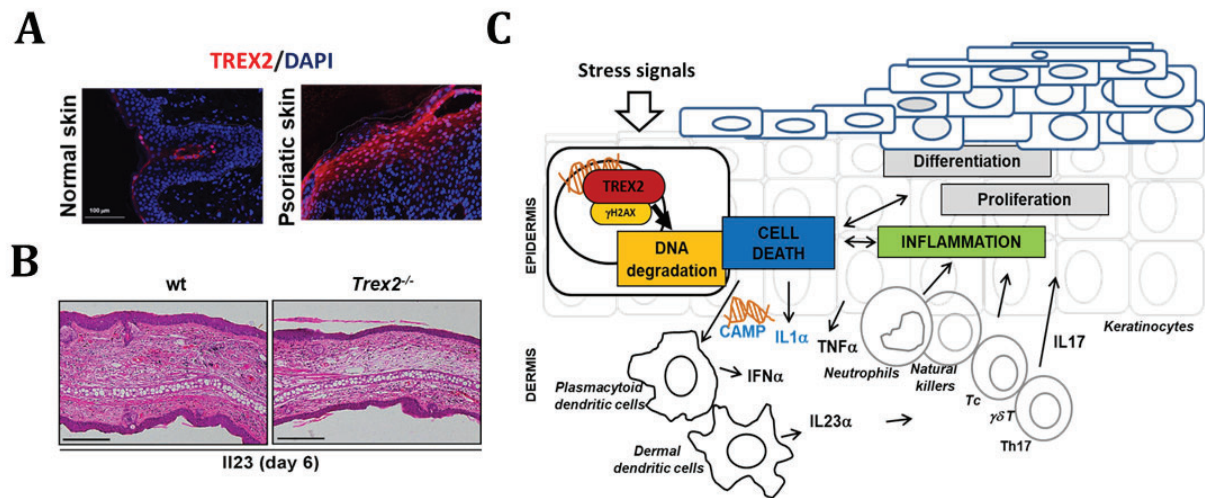


**Figure 15. Role of TREX2 in PsO.** A) Normal and psoriatic skin with TREX2 stained in red and nuclei in blue. TREX2 is overexpressed in psoriatic skin. B) IL-23-induced inflammation is attenuated in *Trex2*<sup>-/-</sup> mice. C) Model scheme of the proposed mechanism by which TREX2 has a role in PsO pathogenesis promoting KC death and inducing inflammation (Manils et al., 2016).

In contrast, TREX1 has an important role in DNA-driven immune responses, being involved in self and foreign DNA degradation limiting the activation of DNA sensing and signalling pathways like the cGAS-STING pathway and preventing aberrant immune responses and autoimmunity (Mohr et al., 2021; Vanpouille-Box et al., 2017; Zhou et al., 2021). The exonuclease is also an interactor of the oligosaccharyltransferase complex in the endoplasmic reticulum and contributes to its function in the N-glycosylation process by reducing glycan-driven immune responses (Hasan et al., 2015). *Trex1* knockout and TREX1<sup>D18N</sup> exonuclease defective mice develop systemic inflammation but do not show spontaneous tumours (Grieves et al., 2015; Morita et al., 2004; Sakai et al., 2017). Therefore, contrasting to the described pro-inflammatory role of TREX2, TREX1 has eminently an anti-inflammatory function (Morita et al., 2004).

In sum, results highlight TREX2 as a novel and unique KC target for KC-based therapies for PsO, directed to the skin and non-immunosuppressive, as it is specifically expressed in KCs playing a proinflammatory role.

# Hypothesis and objectives



## **2. Hypothesis and objectives**

The fact that TREX2 is specifically expressed on KCs, upregulated in psoriatic skin and its loss leads to a decreased inflammation in mice models of the disease, points TREX2 as a candidate target for new KC-based PsO therapies. We hypothesize that the inhibition of TREX2 can be as effective as the most advanced available treatments, which mainly target the immune system, while avoiding their adverse broad immunosuppressive effects. Therefore, blocking TREX2 activity could be a promising non-immune and skin-specific approach to diminish KC-derived inflammation and avert the adverse side-effects associated to systemic treatments targeting the immune system.

In this context, the global aim of the present PhD thesis was to identify, develop and characterize specific and effective TREX2 inhibitory compounds for PsO treatment and other TREX2-driven diseases and further determine their suitability and efficacy for future clinical trials. The specific goals were:

1. To identify and characterize specific, selective, potent and safe TREX2 inhibitory compounds to treat PsO.
2. To evaluate the role of TREX2 in AD and as therapeutic target in this disease.
3. To characterize the molecular mechanisms of TREX2 inhibitors.



# Methodology



### 3. Methodology

#### 3.1. Plasmids and constructions

##### 3.1.1. Subcloning of TREX2 and TREX1 to pMAL-c2X and TREX2 to pMAL-c6T

The codifying sequences for the human (h) and murine (m) isoforms of the exonucleases were subcloned to MBP vectors (pMAL-c2X and pMAL-c6T, New England Biolabs, Figure 16), which permit the introduction of the gene of interest and its fusion to the *malE* gene, codifying for Maltose Binding Protein (MBP) in *E. coli*, that facilitates the purification of the fusion proteins. The promotor of the *malE* gene allows the expression of large amounts of protein when sensitised with isopropyl- $\beta$ -D-thiogalactoside (IPTG). These vectors contain the *lacI<sup>q</sup>* gene, coding for Lac repressor, which prevents the production of the protein under basal conditions until IPTG is present. The vectors also contain the *bla* gene, codifying for  $\beta$ -lactamase, which confers ampicillin resistance. Thus, ampicillin acts as positive selection agent for bacteria growth.

The main difference between the pMAL-c2X and the pMAL-c6T vector lies on the *malE* gene: pMAL-c6T contains a N-terminal hexahistidine tag not present in the pMAL-c2X. Also, the pMAL-c2X presents a cleavage site for Factor Xa, while pMAL-c6T contains one for Tobacco Etch Virus (TEV) protease. These cleavage sites allow the cleavage of the respective tag from the protein of interest.

The subcloning of TREX2 and TREX1 to the pMAL-c2X vector was previously done in the lab (Manils et al., 2015) and was used to purify the protein used in the *in vitro* exonuclease assays as depicted in Section 3.5. The subcloning of TREX2 to the pMAL-c6T vector was part of Maria Fidel's Bachelor's thesis. The production of recombinant HisMBP-mTREX2 was used for the biophysical assays as the cleavage by HisTEV protease was highly efficient and the His tag provided us with additional purification options. Cloning details are depicted in Table 1.

| Vector name            | Vector (bp)     | Insert (bp)         | Insertion site  | Recombinant protein (kDa) |
|------------------------|-----------------|---------------------|-----------------|---------------------------|
| <b>pMAL-c2X-hTREX2</b> | pMAL-c2x (6445) | <i>hTREX2</i> (721) | Sall → Sall     | MBP-hTREX2 (68)           |
| <b>pMAL-c2X-mTrex2</b> | pMAL-c2x (6445) | <i>mTrex2</i> (711) | Sall → Sall     | MBP-mTREX2 (68)           |
| <b>pMAL-c2X-hTREX1</b> | pMAL-c2x (6445) | <i>hTREX1</i> (957) | BamHI → HindIII | MBP-hTREX1 (74)           |
| <b>pMAL-c2X-mTrex1</b> | pMAL-c2x (6445) | <i>mTrex1</i> (957) | BamHI → HindIII | MBP-mTREX1 (74)           |
| <b>pMALc6T-mTrex2</b>  | pMAL-c6T (5247) | <i>mTrex2</i> (711) | Sall → Sall     | HisMBP-mTREX2 (70.6)      |
| <b>pNLF-hTREX2</b>     | pNLF (5887)     | <i>hTREX2</i> (714) | NheI → XhoI     | hTREX2_Nluc (45)          |

**Table 1. Plasmid vector constructions.** Vector and insert details of the constructions used in this thesis.

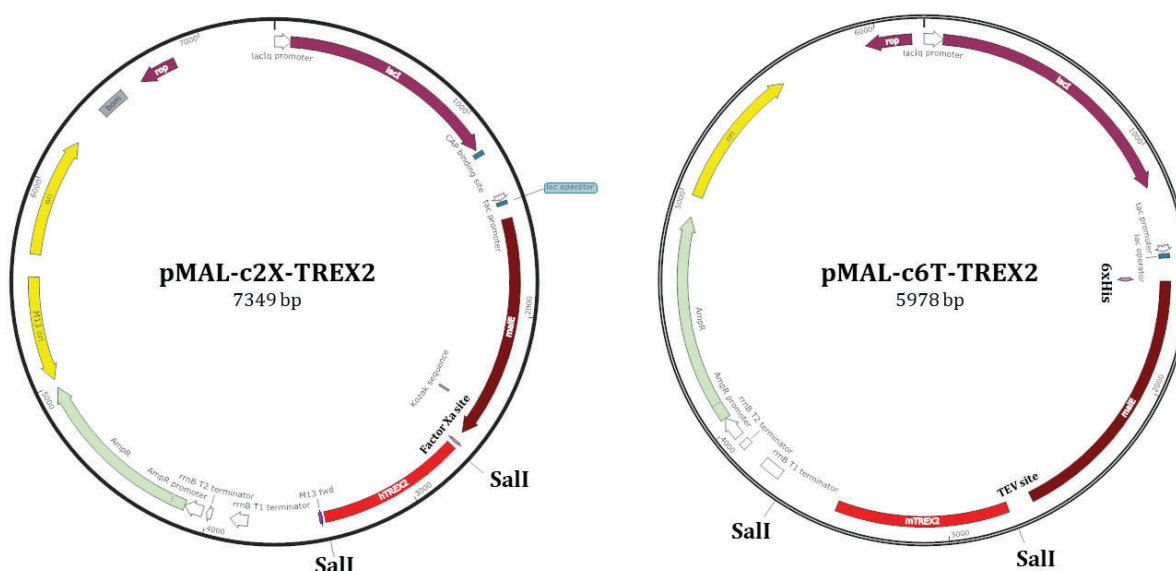


**Figure 16. pMAL-c2X-TREX2 and pMAL-c6T-TREX2 vectors.** In bold, insertion sites, cleavage sites and 6xHis tag.

### 3.1.2. Subcloning of TREX2 to pNLF1 NanoLuc Protein Fusion Vector

The human isoform of TREX2 was cloned into the pNLF1 NanoLuc Protein Fusion Vector (Promega) (Figure 17) for further NanoBRET analysis of target engagement. The NanoBRET target engagement technique is a bioluminescence resonance energy transfer (BRET) based method used to measure interaction between small compounds and proteins within living cells. BRET is used as an indicator of proximity between living cells (Pfleger & Eidne, 2006) and relies on an energy transfer from a luciferase donor to an acceptor fluorophore following the oxidation of a luciferase substrate (Dacres et al., 2012; Wu & Brand, 1994). In NanoBRET, a small luciferase subunit known as Nluc is bound to the protein target of interest and acts as an energy donor to a fluorescent molecule (Hall

et al., 2012; Machleidt et al., 2015; Robers et al., 2015). Several studies have demonstrated it is feasible to use NanoBRET to determine small molecule binding at intracellular targets, also allowing to study the binding kinetics in a real time (Dale et al., 2019; Robers et al., 2015; Vasta et al., 2018).

Basically, TREX2 cDNA was excised from plasmids previously generated in the lab and purified using the QIAGEN QIAquick Gel Extraction Kit (QIAGEN). Vector was dephosphorylated using Shrimp Alkaline Phosphatase (Affymetrix, Inc.) according to its guidelines, and then the TREX2 sequence was ligated to the vector using the Quick Ligation Kit (New England Biolabs) following instructions. After, the ligated vector was transformed to *E. coli* XL1-Blue competent cells following standard protocols. Reaction was incubated on ice for 30 minutes followed by a heatshock at 42°C for 1 minute 45 seconds. Then, samples were centrifuged to concentrate the bacteria that was seeded to LB agar plates (10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl and 15 g/l agar) with ampicillin (20 µg/mL) and incubated at 37°C overnight. Bacterial colonies were grown in 2xYT medium (16 g/l Tryptone, 10 g/l Yeast Extract and 5 g/l NaCl), a nutritionally enriched media, and ampicillin (20 µg/mL) shaking at 37°C overnight. Afterwards, the plasmid was extracted using the QIAprep Spin Miniprep Kit (QIAGEN) and was subsequently digested with NheI (New England Biolabs) and XhoI (New England Biolabs) following manufacturer's recommendations and analysed by agarose gel electrophoresis to identify vector size with correctly inserted cDNA sequence (Figure 17). Some positive constructs were sent sequencing to the Genomic Service of the Scientific and Technological centre from the University of Barcelona (CCiTUB) to further verify correct subcloning and rule out mutations.

The vector constructions as well as the fluorescent tracer needed for NanoBRET technology, indispensable tools to tackle target engagement in a cellular context by means of NanoBRET assays, have been generated to be used in experiments in the near future.



EDTA) and then Column Buffer without EDTA (20 mM Tris-HCl pH 7.4, 0.2 M NaCl) were added to wash the column. Finally, MBP-TREX2 and MBP-TREX1 were eluted by adding a maltose solution (3.6 mg/ml in Column Buffer without EDTA).

To analyse the integrity of the purified proteins, 12% SDS-polyacrilamide gels were used for the SDS-PAGE following the protocol detailed in Section 3.3 and stained using Coomassie Blue (Section 3.3.1.). A minimum of 5 mg of protein per purification was obtained. A representative fraction of each purified fusion protein is shown in Figure 18A. The additional bands seen in the TREX1 purification correspond to partial protein degradation. Even though the bacteria strains used were deficient in many proteases and the inductions of protein were done at room temperature, there was a partial degradation in the murine and human isoforms of TREX1, suggesting the presence of protease targets in TREX1 that are not present in TREX2.

For separating TREX2 from MBP tag, we cleaved the recombinant MBP-mTREX2 with Factor Xa (New England Biolabs) followed by a chromatographic method. As shown in Figure 18B, mTREX2 was efficiently cleaved from MBP by Factor Xa (0.05 µg, New England Biolabs) in column buffer without EDTA upon 6-8 hours at 30°C (Figure 18B). Next, to get rid of MBP and Factor Xa, HiTrap Q FF columns (Cytiva) were used with a NaCl gradient (100 mM-450 mM) (Figure 18C). Unfortunately, this approach proved to be ineffective when samples were analysed by Western Blot. As shown in Figure XC, MBP eluted around 100-200 mM and could be effectively separated from TREX2. However, TREX2 eluted at 450 mM, which coincides with the NaCl concentration (400 mM) at which Factor Xa is known to elute, as indicated in the New England Biolabs protocol: Separating the Protein of Interest from MBP after Protease Cleavage using the pMAL Protein Fusion and Purification System (E8200).

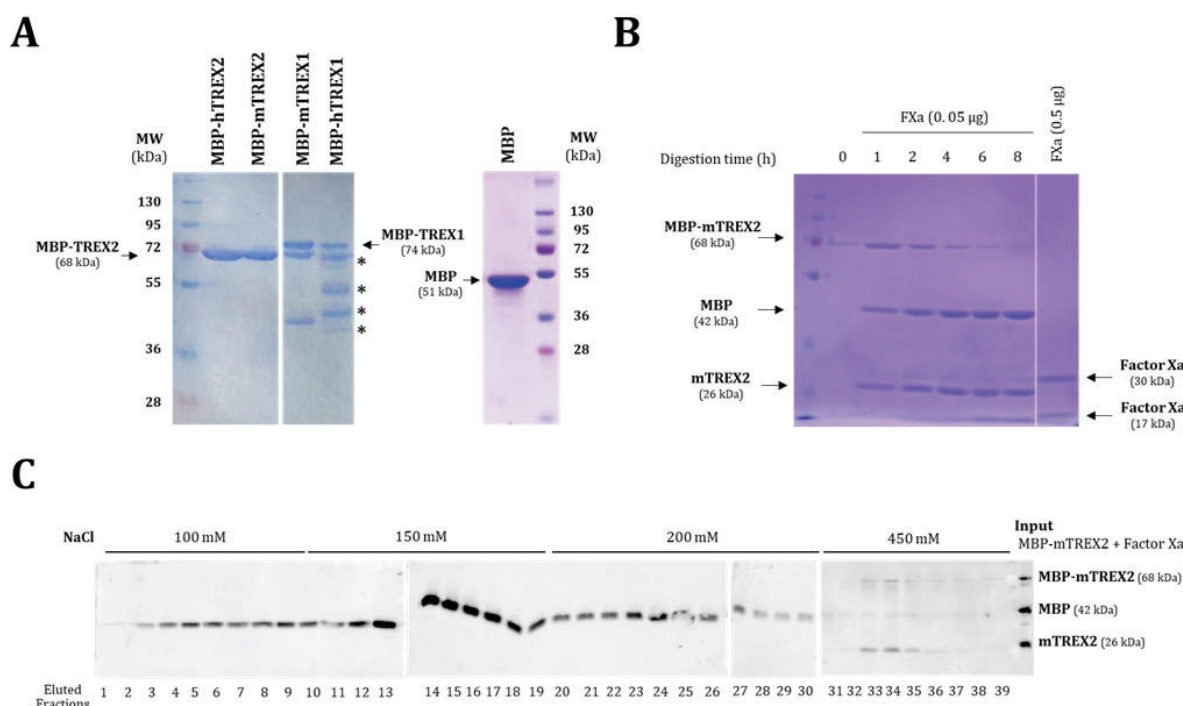


**Figure 18. MBP-TREX2, MBP-TREX1 and TREX2 protein production and purification.** A) Purified MBP (51 kDa) and murine and human TREX2 and TREX1 isoforms bound to the MBP tag (68 and 74 kDa, respectively), approximately 2 µg, analysed by SDS-PAGE. B) Excision of MBP (42 kDa) from mTREX2 (26 kDa) by Factor Xa (0.05 µg) at different time points (1, 2, 4, 6 and 8 hours) is shown. The two subunits of Factor Xa (17 and 30 kDa) are indicated and clearly visualised by loading a higher amount (0.5 µg) of the protease. C) Anion exchange chromatography to separate MBP, mTREX2 and Factor Xa after MBP-mTREX2 cleavage. \* Partially degraded MBP-TREX1.

### 3.2.2 HisMBP-mTREX2 and TREX2 protein production and purification

HisMBP-mTREX2 was induced and purified as indicated in Section 3.2.1, obtaining high production (2.5 mg/ 100 ml culture) and purity levels of recombinant protein as seen in the Comassie staining (Figure 19A). Recombinant protein was cleaved using HisTEV protease (New England Biolabs) at 50 µg/ml for 4 hours at 30°C to separate mTREX2 from HisMBP (Figure 19B).

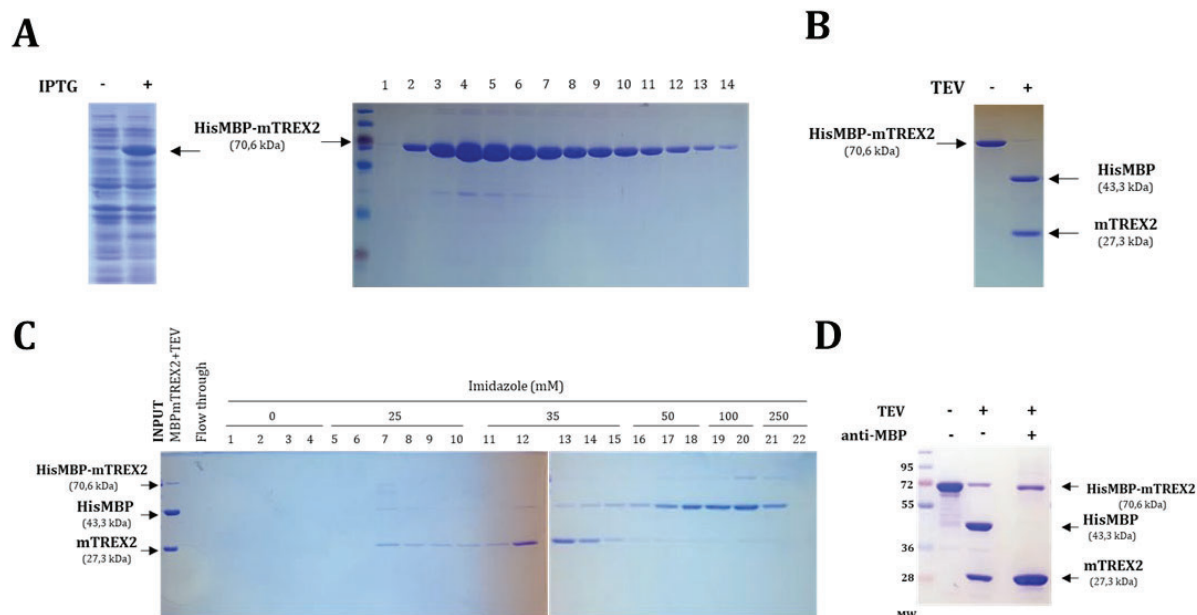
Both MBP and TEV are bound to a histidine tag that has affinity for nickel. Using a HisPure Ni-NTA resin (Thermo Scientific), histidines present in the tag (HisMBP) and in HisTEV protease bind to nickel and theoretically, our protein of interest could be separated because it would bind with less affinity to the column. Then, elution with different concentrations of Imidazole (25, 35, 50, 100 and 250 mM) was performed. However, there was partial coelution of HisMBP and mTREX2 (fractions 12 to 16, Figure 19C), and consequently, this technique was deemed unsuitable and therefore was discarded.

Next, affinity purification using in house anti-MBP antibodies was another method used for separating HisMBP from mTREX2. Briefly, in-house rabbit serum containing anti-MBP

antibodies was diluted in Column Buffer without EDTA (1:10) and incubated with nProtein A Sepharose (Cytiva) for 20 minutes on a rotating platform. Then, the resin was packed and washed with 5 column volumes of Column Buffer without EDTA: first two washes containing 0.1% Tween and last three without. HisMBPmTREX2 digestion with TEV protease was incubated for 1 hour at room temperature in gentle rotation with the antibody-bound resin column. For elution, 3 mL of Column Buffer without EDTA were passed through the column and 0.5 ml fractions were collected and analysed on SDS-PAGE. Fractions containing only mTREX2 were obtained, pooled and showed high purity levels, even though there was still a fraction of uncleaved HisMBP-mTREX2 (Figure 19D). The only drawback of this method was the limited availability of the antibody.

The last, most practical and successful method used for the purification of mTREX2 was Size-Exclusion Chromatography (SEC). For that, a 16/60 S200 column (Cytiva) was used in an AKTA purifier (Cytiva) at 4°C with Column Buffer without EDTA. The flow rate was 0.5 ml/minute and 0.5 ml fractions were collected (Figure 19E). This assay was performed in the EMBL facilities (Hamburg).

More than 60 mg of the HisMBP-mTREX2 protein were purified during this thesis and were used for both crystallisation and MST purposes.



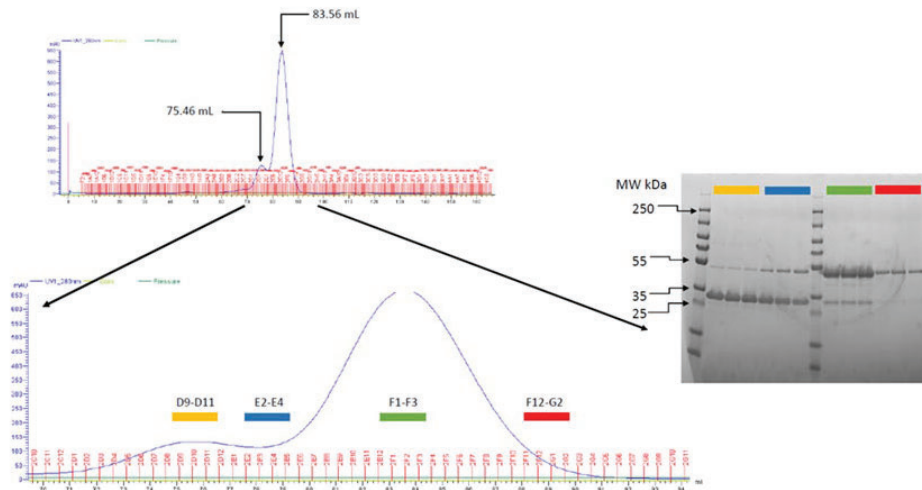
**E**

**Figure 19. HisMBP-mTREX2 and mTREX2 purification.** A) Induction using IPTG (left) and purification of the HisMBP-mTREX2 protein using amylose resin columns analysed by SDS-PAGE and stained with Comassie. B) HisMBP-mTREX2 cleavage using TEV protease (50 µg/ml) for 4h at 30°C. C) Separation of HisMBP and mTREX2 using HisPure Ni-NTA resin co-elutes HisMBP and mTREX2 (fractions 12-16). D) Affinity purification using anti-MBP antibody is able to separate mTREX2 from the HisMBP tag, even though a small fraction of uncleaved HisMBP-mTREX2 is present in the sample. E) Size-exclusion Chromatography (SEC) using an AKTA purifier is able to efficiently isolate HisMBP and mTREX2, though there is still a partial coelution.

### 3.3 Protein extraction, electrophoresis in polyacrylamide gels (SDS-PAGE) and Western Blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5 sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitors: aprotinin (10 µg/µl), leupeptin (10 µg/µl), iodoacetamide (86 µg/µl) and PMSF (1 mM). Lysates were incubated on ice for 30 minutes with occasional vortex followed by centrifugation at 14000 rpm for 15 minutes at 4°C. Supernatants were collected and protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific).

Protein samples were prepared by mixing protein with Laemmli buffer (3x) (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.7% β-mercaptoethanol, 10% glycerol and traces of bromophenol blue) and then were denatured at 95°C for 5 minutes. Protein samples were loaded to 12% polyacrylamide gels (1.5 mm) for protein separation and the electrophoresis was effectuated in SDS-PAGE buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) at 140 V for approximately 1 hour 30 minutes. Then, gels were stained using Comassie Blue or were used for Western Blot.

### 3.3.1 Comassie Blue gel staining

Polyacrylamide gels were fixed in a 7% acetic acid solution for 5 minutes, stained for 5 minutes with Comassie Blue staining solution (50% methanol, 10% acetic acid, 0,25% Comassie Brilliant Blue (SIGMA) and destained using destaining solution (5% methanol and 7,5% acetic acid).

### 3.3.2 Western Blot

After SDS-PAGE electrophoresis, wet transference was performed in Amersham Protran Premium 0.45 NC nitrocellulose membranes (Cytiva) at 4°C for 75 minutes at 100V in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol). After, membranes were blocked in 5% skimmed milk (VWR) in TBS-T (150 mM NaCl, 50 mM Tris pH 7.4, 0.05% Tween 20) for 1 hour at room temperature to prevent non-specific antibody binding. Following a series of washes, a solution of primary antibody diluted in TBS-T and 0.02% Sodium Azide was incubated with the membranes for 1.5 hours at room temperature or overnight at 4°C. Finally, after washes, secondary antibody diluted in TBS-T was applied to the membranes for 1.5 hours at room temperature. Membranes were developed using the Amersham ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences) following manufacturer's indications in a Western Blot imager (Amersham Imager 680). Antibodies used for Western Blot are shown in Table 2.

|                      |                     | Type       | Origin | Dilution | MW (kDa) | Brand   |
|----------------------|---------------------|------------|--------|----------|----------|---------|
| Primary Antibodies   | mTREX2              | Polyclonal | Rabbit | 1:1000   | 26       | Inhouse |
|                      | GFP                 | Monoclonal | Mouse  | 1:2000   | 27       | Roche   |
|                      | HRP- $\beta$ -actin | Monoclonal | Mouse  | 1:20000  | 42       | Merck   |
| Secondary Antibodies | HRP-anti-rabbit     | Polyclonal | Rabbit | 1:10000  |          | Bio-Rad |
|                      | HRP-anti-mouse      | Polyclonal | Rabbit | 1:10000  |          | Bio-Rad |

**Table 2. Antibodies.** List and characteristics of antibodies used for Western Blot in this thesis.

Western Blot results were analysed using ImageJ. Briefly, images were transformed to 8-bit (black and white). Then, a rectangle containing the biggest band of the image was made and the intensity mean of all the bands was measured by putting this square over each band and pressing Control + M to measure. Also, the background was measured putting the square in an empty part of the image. All values were processed in an Excel file: first, inverted values were calculated (255 – mean value), after, net values were created by subtracting the values of the bands minus the background. Lastly, net values of the bands

of interest were divided into net values of  $\beta$ -actin, used as normalizing control, to obtain the relative protein level.

### **3.4 Compounds**

Commercial compounds were purchased from Enamine, Specs, ChemBridge, PHARMEKS and VitaS-M Laboratory. The new synthesised compounds generated for the project were made by Dr Ouldouz Ghashghaei from Prof Rodolfo Lavilla's group (Faculty of Pharmacy, University of Barcelona).

The details of the structure of lead compounds and some related compounds are shown in the present manuscript thesis. The structure of most of the compounds analysed is disclosed in the Patent Cooperation Treaty application (PCT/EP2024/061679) to the European Patent Office protecting newly synthesised TREX2 inhibitor compounds covering composition of matter and their use, especially in PsO and AD.

Commercial compounds were named with the prefix ID and a number, from ID1 to ID84. The names of synthesised compounds were OGP plus a number. The compounds included in the patent were named with the prefix 01- followed by a three-digit number.

### **3.5 Exonuclease activity assay**

To assess the blocking efficacy and selectivity of the compounds, two *in vitro* exonuclease activity assays measuring the degradation of dsDNA have been used and were based on the bibliography (Hasan et al., 2015). Effect of TREX2 inhibitors on TREX2 and TREX1 exonuclease activity was assessed using purified MBP-TREX2 and MBP-TREX1 recombinant proteins and MBP as negative control. The two methodologies employed to ascertain the inhibition of the exonucleases were: (I) A real-time exonuclease activity assay measuring the fluorescence of the complex DNA-SYBR Green I and (II) A time-point exonuclease activity assay evaluating DNA in agarose gels.

#### **3.5.1 Real-time exonuclease activity assay**

This approach relies on the use of SYBR Green I (Invitrogen), a fluorescent agent that binds to dsDNA with high affinity, to determine the quantity of DNA present in the sample. Therefore, when there is degradation of dsDNA, SYBR Green fluorescence is decreased

and therefore there is exonuclease activity. On the contrary, if fluorescence does not decrease, TREX2/TREX1 are not exerting their activity and thus, are being inhibited.

The first step of this assay consists on hybridizing a pair of 31 base pair (bp) long forward (5'CGGTTTGGTTTTGGCGGAGTTTTCGGGTCGG3') and reverse (5'CCCGAAAACCTCCGC CAAAACCAAACCGACCG3') primers in Oligonucleotide Buffer (50 mM Tris-HCl pH 7.4, 5 mM Cl<sub>2</sub>Mg, 10 ng/μl dsDNA) for 1 hour at 37°C, creating two overhanging ends (Figure 20). Next, SYBR Green I (1/5000) is added and incubated at 37°C for 30 minutes so the reagent inserts into dsDNA.

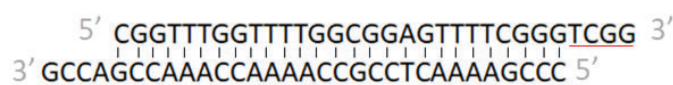


**Figure 20. Representation of the 31 bp dsDNA substrate.** The hybridization of the two 31 bp ssDNA primers gives place to a dsDNA fragment with overhanging ends.

In parallel, TREX2, TREX1 or MBP recombinant proteins were diluted in Protein Buffer (50 mM Tris HCl pH 7.4, 5 mM Cl<sub>2</sub>Mg, 20 mg/ml Bovine Serum Albumin (BSA, Merck)) in previously titrated concentrations in which the proteins had similar activity kinetics (MBP-TREX2, 35 ng; MBP-mTREX1, 100 ng; MBP-hTREX1, 700 ng) (Figure 21A).

The reaction consisted on compound or DMSO (2.5%), protein mix and oligonucleotide mix in a final reaction volume of 20 μl. First, the compound/DMSO and protein were incubated for 15 minutes at room temperature in a 384-well white plate (Greiner). Next, oligonucleotide mix was added to the plate and immediately transferred to a plate reader (CLARIOstar® Plus, BMG LABTECH) at 37°C to read every minute for 40 minutes approximately. This way, a real time measuring of how DNA was being degraded was obtained in the presence and absence of the desired compound.

This throughput approach set in my Bachelor's thesis was finally abandoned as a screening method because of the intrinsic autofluorescence some of the compounds had. This, masked the real effect of the compounds and meant this method was unsuitable for our screening.

### 3.5.2 Time-point exonuclease activity assay

This assay was adapted from the previous protocol and avoids fluorescence measuring. Instead, DNA degradation was measured by DNA electrophoresis in agarose gels. Exonuclease activity was performed using previously titrated concentrations that

depended on the dsDNA fragment size. TREX2 inhibitors at the designated concentrations were preincubated with the indicated proteins for 15 minutes. Reactions were performed in the reaction buffer (20 mM Tris-HCl pH 7.7, 5 mM MgCl<sub>2</sub>, 100 µg/ml BSA, 5% DMSO). Reaction was initiated by adding dsDNA substrates (31 bp or 3000 bp, 200 ng) and terminated using an EDTA solution with loading dye (9 mM EDTA, 60 mM Tris-HCl pH 8, 25% glycerol, 0.125% Orange G). Afterwards, DNA products were subjected to DNA electrophoresis as described in Section 3.6.2.

The recombinant proteins MBP-TREX2 and MBP-TREX1 were titrated so the concentration of each isoform exhibited a similar degradation kinetic of dsDNA. With a 31 bp fragment the concentrations were: MBP-hTREX2 (25 ng), MBP-mTREX2 (15 ng), MBP-mTREX1 (125 ng) and MBP-hTREX1 (700 ng) (Figure 21B). When using the 3000 bp fragment, concentrations were: MBP-hTREX2 (50 ng), MBP-mTREX2 (250 ng), MBP-mTREX1 (150 ng) and MBP-hTREX1 (700 ng). MBP was used as a negative control (35 ng with 31 bp and 100 ng with the 3000 bp substrate). Compounds were tested at concentrations ranging from 1-100 µM to determine the half-maximal inhibitory concentration (IC<sub>50</sub>) range for inhibition of DNA degradation. Those compounds with highest inhibitory capacity, were further tested in a wider concentration range (0.03-100 µM) to calculate a more accurate IC<sub>50</sub>.

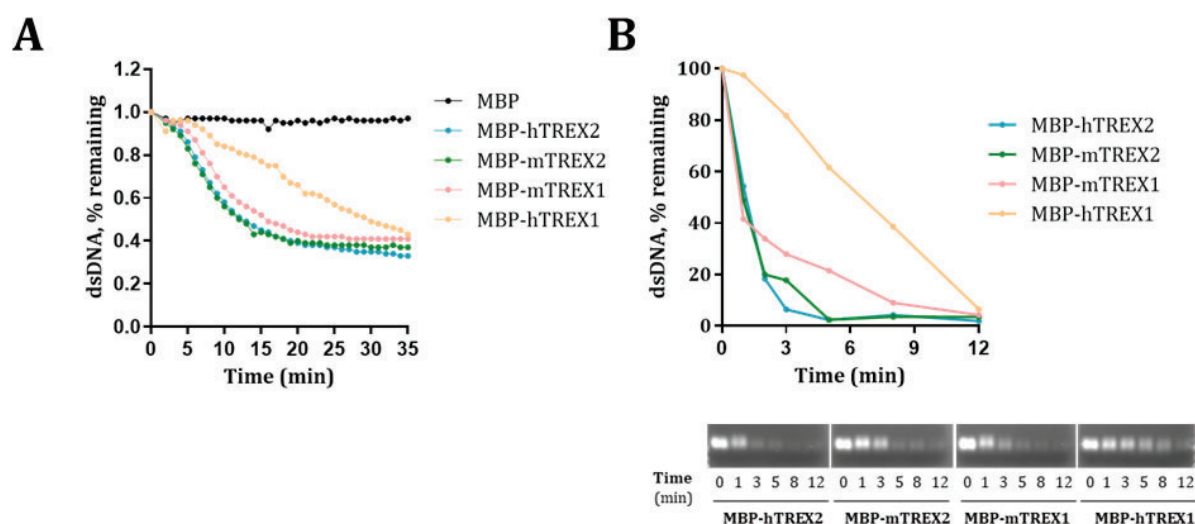


**Figure 21. Analysis of TREX2 and TREX1 exonuclease activity.** A) Real-time exonuclease activity measuring of dsDNA degradation (31 bp) by MBP-hTREX2 (35 ng), MBP-mTREX2 (35 ng), MBP-mTREX1 (100 ng), MBP-hTREX1 (700 ng) and MBP (45 ng). B) Degradation of dsDNA (31 bp) in a time-point exonuclease assay using MBP-hTREX2 (25 ng), MBP-mTREX2 (15 ng), MBP-mTREX1 (125 ng) and MBP-hTREX1 (700 ng). Below, agarose gel from a representative experiment showing the dsDNA degradation of each isoform at different time points.

The 31 bp dsDNA substrate was obtained by hybridizing forward and reverse primers as mentioned in Section 3.5.1. The 3000 bp substrate was obtained from the digestion of a plasmid previously generated in the lab that contained two EcoRI targets. When cut with the restriction enzyme, the fragment of 3000 bp with overhanging ends was obtained. For the digestion of the plasmid, EcoRI (New England Biolabs) in rCutSmart 10x buffer (New England Biolabs) was used following the company recommendations for 1 hour at 37°C. After the digestion, the DNA product was purified using the QIAquick PCR purification kit (QIAGEN) and then was resuspended in a concentration of 0.2 µg/µl in 50 mM Tris-HCl pH 7.4 and 5 mM MgCl<sub>2</sub>.

### **3.6 DNA extraction**

Two different methods were used for DNA extraction from mouse ear punches (30-50 µg) for genotyping. The first was an in-house protocol and the second a commercial kit, the Monarch Genomic DNA Purification Kit (New England Biolabs), which was used according to instructions. For the first, tissue was resuspended in digestion buffer (20 mM Tris pH 8, 100 mM NaCl, 10 mM EDTA and 0.5% SDS, 27.6 µM proteinase K) and was incubated overnight at 55°C. Then, a solution of saturated NaCl was applied to the samples, mixed by inversion and left 10 minutes in ice. Samples were centrifuged at 15000 rpm for 30 minutes at 4°C. Pellet was discarded and the supernatant centrifuged again (process repeated twice). Then isopropanol was added to the samples and left 15 minutes at 4°C in rotation, centrifuged at 12000 rpm for 10 minutes at 4°C and the pellet was washed with 70% ethanol and centrifuged again. Ethanol was removed until the pellet dried and then was resuspended in a Tris-EDTA solution (10 mM Tris-HCl pH 8 and 1 mM EDTA pH 8). DNA quality and concentration were measured in Nanodrop (Thermo Fisher Scientific).

#### **3.6.1 TREX2 genotyping**

The Expand™ High Fidelity PCR System (Roche) was used to perform Polymerase Chain Reaction (PCR) according to the manufacturer's instructions in the MyCycler thermal cycler instrument (Bio-Rad). Primers utilized were: CAGCCTGCTTATCCCAAG and CTTGATGGGCTAGATCTGG. The program used for amplification was the following: 2.5 minutes at 96°C for denaturalization, 39 cycles of 96°C for 20 seconds, 55°C for 45 seconds and 72°C for 2.5 minutes and 1 cycle of 72°C for 10 minutes for the final

elongation. PCR products were run in agarose gels and the expected bands were: 1575 bp (wt) and 585 bp (*Trex2*<sup>-/-</sup>).

### **3.6.2 DNA electrophoresis**

DNA samples were loaded to agarose gels (1% agarose, 89 mM Tris Base, 89 mM boric acid, 2 mM EDTA pH 8) and run in 1x TBE buffer (89 mM Tris Base, 89 mM boric acid, 2 mM EDTA pH 8). Samples were visualized by SYBR-Safe staining (Invitrogen) using an UV transilluminator (GeneFlash, SYNGENE). For the exonuclease assay samples with 31 bp dsDNA substrate, 1.5% agarose gels were used. For the exonuclease activity assay analysis, remaining DNA bands were quantified using the Image Lab software (Bio-Rad).

## **3.7 Mice**

Wild type (wt) and in-house generated *Trex2* knockout (*Trex2*<sup>-/-</sup>) (Parra et al., 2009) mice in a C57Bl/6N background were used for *in vivo* experiments. Animals were housed and bred at the Animal Facilities of the University of Barcelona at the Bellvitge Campus according to the requirements of the Ethics Committee on Animal Use and Care from the Universitat de Barcelona. All animal procedures were carried out in accordance with the ethical standards of the institution with the authorization of the Catalan Government (procedure numbers 9119 and 196/22). All experiments were conducted using male and female aged at least 12 weeks.

### **3.7.1 Mice models of PsO**

#### **3.7.1.1 The IL-23-induced mouse model of PsO**

The IL-23-induced acute mouse model of PsO (Li et al., 2012; Singh et al., 2019) was used to assess the efficacy of the best TREX2 inhibitors selected in terms of potency and selectivity in the exonuclease assay. Daily application of IL-23 in the ear leads to a PsO-like dermatitis with many hallmarks of human PsO such as erythema and scale formation, acanthosis, parakeratosis, infiltration of leukocytes and dilatation of blood vessels (Singh et al., 2019; Wagner et al., 2010; Zheng et al., 2007).

In brief, recombinant mouse IL-23 (Miltenyi Biotec) or vehicle (Phosphate Buffer Saline, PBS) were administered intradermally (i.d.) using a 10 µl Hamilton Syringe (Model 701N, Hamilton) in the ear daily (0.5 µg in 10 µl of PBS in the left ear and 10 µl of PBS in the right) throughout the duration of the experiment. Compounds were administered

intraperitoneally (i.p.), intragastrically (i.g.) or topically as described in Section 3.7.3, once or twice daily the indicated days after starting IL-23 treatment. Samples were taken on the last day of the experiment from euthanized mice and included both ears, half for histological processing (stored in 4% paraformaldehyde at room temperature) and half for RNA (stored in RNAlater, Merck) and protein extraction and stored at -80°C.

### **3.7.1.2 The Imiquimod-induced mouse model of PsO**

The Imiquimod (IMQ)-induced model of PsO (Singh et al., 2019; van der Fits et al., 2009) was used to confirm the effect of the lead compound in another model of the disease. IMQ is a TLR7 agonist and potent immune activator used for the treatment of virus-associated skin abnormalities like actinic keratosis that can exacerbate PsO in patients (Szeimies et al., 2004; Wu et al., 2004). Topical application of IMQ results in many features of human PsO including acanthosis, altered differentiation, vascular phenotype, infiltration of immune cells, abscesses and a T cell response (Gangwar et al., 2022). However, its mechanism of action is still unclear.

For this model, a cream containing 5% IMQ (Immunocare, 50 mg/g) or Vaseline was applied topically in the ventral and dorsal sides of the left and right ear, respectively. Both IMQ and Vaseline in a total dose of 25 mg cream were administered manually every day for 4 days. Compounds were administered i.g. twice-daily starting day 4. Mice were euthanized and samples (same as in Section 3.7.1.1) were taken on day 5. Mice were weighed on the first and final day of the experiment.

### **3.7.1.3 Evaluation of IL-23 and IMQ-induced thickness in mice**

Ear thickness was measured daily using a digital calliper and the increase in the thickness of the ears caused by IL-23 or IMQ was calculated in relation to vehicle, PBS and Vaseline cream, respectively.

## **3.7.2 Mouse models of AD**

### **3.7.2.1 Oxazolone-induced model of AD**

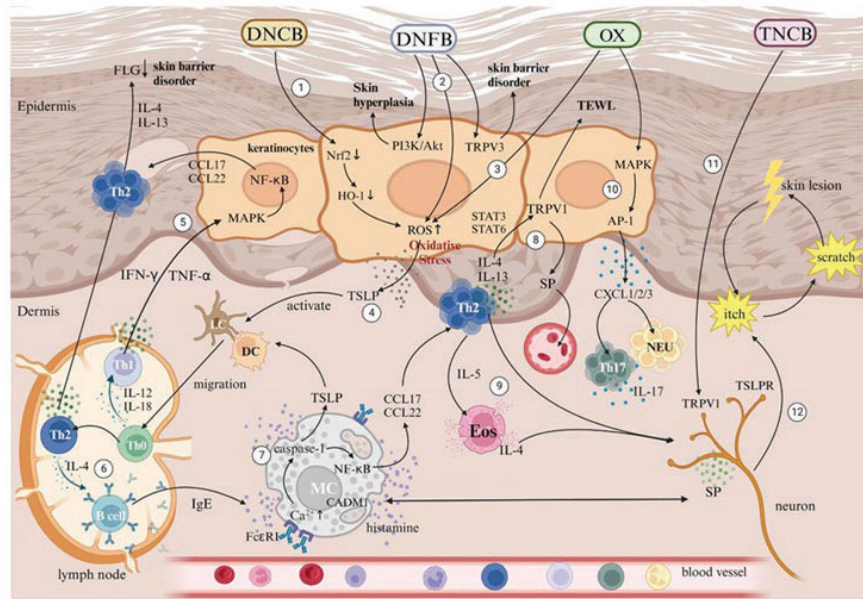
The first model of AD used to test compound efficacy was the Oxazolone (OX) model, short for 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (Merck) (Zachariassen et al., 2017).

OX is a heterocyclic compound that is used as AD inducer. In this model, mice are sensitized with OX and a week after, challenges are applied to the animal for at least two weeks, time needed to shift from a delayed allergy response to a chronic skin disease (Man et al., 2008; Sanjel & Shim, 2022). This model presents erythema, desquamation, edema, TEWL, increased serum IgE and increased cytokines and chemokines (Dong et al., 2022; Lee et al., 2018; Yun et al., 2011). Mechanistically, OX stimulation induces Th2 inflammation in mice, and causes an increase in IL-4 and IL-13 that induce the phosphorylation of STAT3 and STAT6 and leads to the upregulation of TRPV1, which increases dermal blood flow and causes more inflammation (Assas et al., 2014; Chung et al., 2004; Lee et al., 2018; Yun et al., 2011).

TRPV1 upregulation leads to an increased influx of calcium ions in KCs that results in the disruption of epidermal barrier (Yun et al., 2011). OX causes an upregulation of TSLP, which inhibits Treg cells and downregulates the expression of genes important for the maintenance of skin barrier function such as *FLG* (Kim et al., 2023; Kim et al., 2014). OX also activates the MAPK-AP1 pathway and increases the expression of AP1, which leads to an increase in Th17-related chemokines such as CXCL1, CXCL2 and CXCL3, which helps recruiting Th17 cells that secrete IL-17 (Dong et al., 2022).

Finally, OX increases ROS levels by triggering MAPK p38 activation and the phosphorylation of Jun N-terminal Kinase (JNK), that promotes the transcription of NF- $\kappa$ B and increases the production of proinflammatory cytokines like IL-8 and TNF- $\alpha$  (Chang et al., 2018). ROS increase facilitates the accumulation of nucleotide-binding domain leucine-rich-containing family pyrin domain-containing-3 (NLRP3), activates caspase 1, induces the overexpression of IL-1 $\beta$  and stimulates mast cells and the accumulation of leukocytes in the inflammatory site (Lin et al., 2021). An overview of the mechanism of action of OX is shown in Figure 22.

**A**



**B**

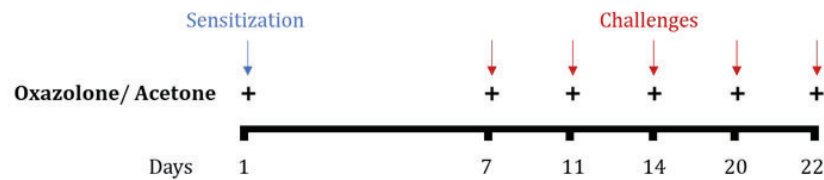


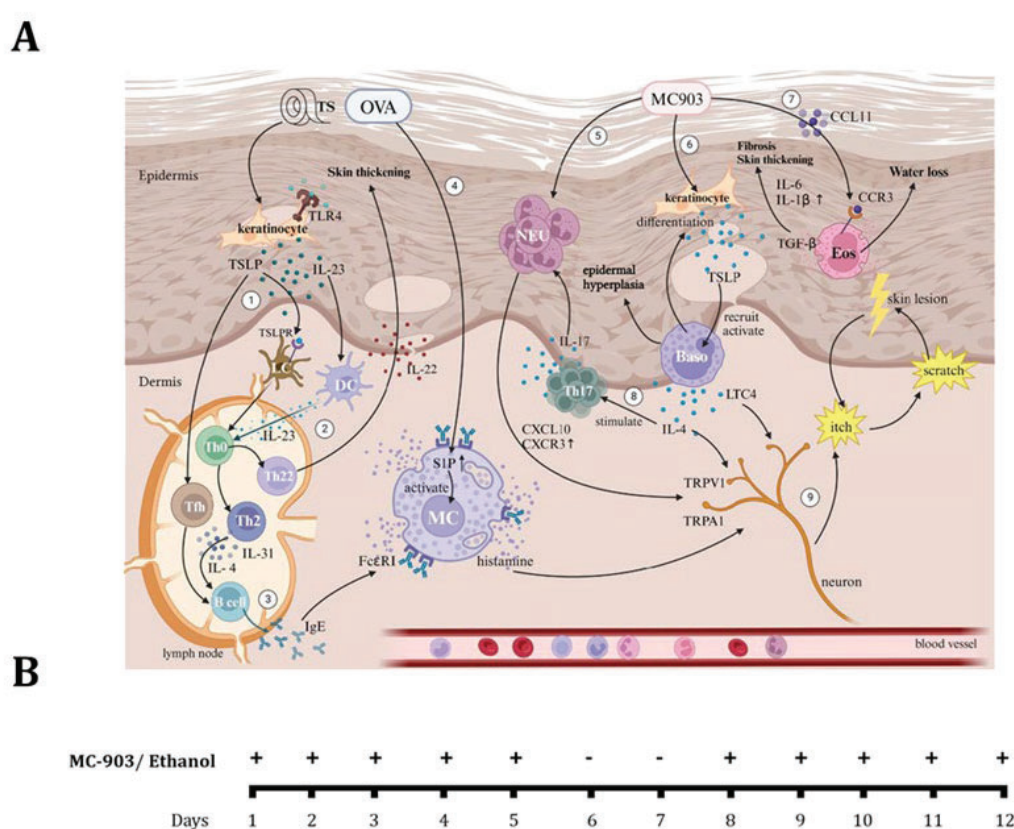
**Figure 22. Oxazolone-induced animal model of AD.** A) Oxazolone (OX) rises ROS and triggers the secretion of TSLP, that activates LC and DC and promotes the differentiation of T cells to Th1, Th2. They induce the transcription of NF- $\kappa$ B and a decrease in FLG leads to skin barrier dysfunction. TRPV1 gets upregulated and causes TEWL. OX also affects MAPK, which increases AP-1 and induce several chemokines that recruit Th17 and neutrophils. The other models displayed in the figure, TNCB, DNCB and DNFB are haptens used to induce AD mouse models as well and present different mechanisms of action (Zheng et al., 2024). B) Experimental approach scheme.

Mice were sensitized with a solution of 1% OX in acetone on both sides of the left ear and only acetone on the right with a micropipette. After 7 days, mice were challenged with a solution of 0.5% OX on the left ear and acetone on the right on days 7, 11, 14, 20 and 22 (Figure 22B). All mice were sacrificed and sampled on day 22. Samples taken are detailed in Section 3.7.1.1. Also, blood was extracted by cardiac puncture using heparinized 5 mL syringes and 25G needles.

### 3.7.2.2 MC903-induced model of AD

Another model of AD used was the well-described mouse model of MC903-induced AD (Moosbrugger-Martinez et al., 2017). MC903-, also known as calcipotriol-, induced model is widely used to study the pathogenesis and assess new therapeutic options for AD. The mechanism of action of MC903 (Figure 23A) basically relies on a series of type 2 immune

responses that induce the production of TSLP by KCs. TSLP stimulates the differentiation to Th2, with the following secretion of IL-4 and IL-13 (Li et al., 2006), and activates basophils, an important source of IL-4 that promotes KC differentiation, the hyperplasia of the epidermis and a dysfunction in skin barrier (Pellefigues et al., 2021; Walsh et al., 2019). Also, acts directly on sensory neurons to induce itch (Wang et al., 2021). Neutrophils and eosinophils are induced by MC903 as well. IL-4 and IL-6 stimulate the JAK/STAT pathway and promotes the expression of ROR $\gamma$ T, a Th17 transcription factor (Han et al., 2023). Significantly, some studies have reported a gender difference in this model, being AD symptoms more severe in females than males (Wang et al., 2022).



were euthanized and sampled on the final day of the experiment. The details of the samples taken are provided in Section 3.7.1.1. Blood was also extracted by cardiac puncture as mentioned in Section 3.7.2.1. Mice were weight on the first and final day of the experiment.

### 3.7.2.3 Evaluation of AD features in MC903 and OX mice models

Ear thickness was measured on MC903 and OX application days using a digital calliper and the increase in the thickness of the ears was calculated in relation to ethanol or acetone, respectively. Arbitrary scores from 0 (not present) to 3 (very severe) were assigned to parameters such as erythema, scaling, erosion and eczema. Scores were evaluated blindly (not knowing the genotype).

The scratching evaluation of mice treated with OX and MC903 consisted on the observation of around 10 mice for 10 minutes by two observers. Importantly, mice scratch with the hind limb as a behavioural response to pruritus, and therefore only that was considered scratching. Facial wiping or grooming, shown in Figure 24, were not considered scratching (Shimada & LaMotte, 2008).



**Figure 24. Differences between scratching, facial wiping and grooming in mice.** Only scratching with hind limbs was considered in the itching evaluation. Facial wiping and grooming were not considered. Adapted from Shimada & LaMotte, 2008.

### 3.7.3 Compound administration

Lyophilized compounds were stored at 4°C until utilized and were prepared immediately prior to the administration on each treatment day. Stock 10 mM solutions were prepared in 100% DMSO and kept away from light as much as possible.

#### 3.7.3.1 Intraperitoneal administration

I.p. administration was utilized in the initial *in vivo* studies before testing i.g. administration. For this, 0,3 mL insulin syringes (BD) were used due to the reduced risk of puncturing vital organs. The formulation vehicle used for i.p. was 10% DMSO, 90%

normal saline. A volume of 100 µl was injected to the lower right or left quadrant of the abdomen with the mice slightly inclined downward to minimize the risk of damaging organs in the peritoneal cavity. Compound concentrations in vehicle were prepared accordingly to administer the indicated doses.

### **3.7.3.2 Intragastric administration**

I.g. administration consists on delivering substances directly into the stomach of the mice through oral gavage using a cannula connected to a syringe. The animal needs to be awake and immobilized during the process. The formulation vehicle for i.g. administration was 10% DMSO, 0.5% carboxymethylcellulose and 89.5% normal saline. Compound was administered at the indicated doses in the vehicle solution.

### **3.7.3.3 Topic administration**

For topic administration, application of 10 µl of vehicle or compound on the dorsal and ventral sides of each ear was performed using a micropipette. In the IL-23-induced model, the formulation vehicle was 10% DMSO in PBS. In the MC-903 model it was 10% DMSO and 90% ethanol. The compound was applied to the ear in a solution prepared with the same proportion as the vehicle.

## **3.8 Enzyme-Linked ImmunoSorbent Assay (ELISA)**

### **3.8.1 IgE quantification**

IgE levels were measured in plasma. Blood samples were collected by cardiac puncture using heparinized syringes. Blood was centrifuged at 300 g for 10 minutes to separate plasma (supernatant) from whole blood. IgE were analysed using the IgE Mouse Uncoated ELISA Kit (Invitrogen) according to the manufacturer's recommendations. Plasma was diluted 1:200 in ELISA's buffer for proper analysis.

## **3.9 Histology based-assays**

In tissue processing, samples are fixed and embedded in paraffin via three steps: dehydration, clearing and infiltration (Aziz & Zeman-Pocrnich, 2022). Briefly, tissue was fixed in 4% paraformaldehyde in PBS for 24h at room temperature and then passed to 70% ethanol to start dehydration until further manipulation. Tissues were inserted into histology cassettes and submerged in 96% ethanol overnight and passed to 100% ethanol

for 3 hours. After that, 1 hour in xylene (clearing) and finally overnight in paraffin and 3 hours in clean paraffin (infiltration). Then, paraffin blocks were made using the Leica Paraffin Embedding Station (Leica). Samples sections of 5 µm were obtained using the microtome HistoCore MULTICUT (Leica) and put on SuperFrost Plus Adhesion Slides (Fisher Scientific) for RNAscope and Immunofluorescence or standard slides for Haematoxylin-Eosin (H&E) and Mastocyte and Eosinophil staining.

Before starting with the staining, samples need to be kept at 60°C for 30 minutes to melt the remaining paraffin. Following, the deparaffination and rehydration process began: 3 cycles of 5 minutes each of xylene, 2 cycles of 5 minutes of absolute ethanol, 2 cycles of 5 minutes of 96% ethanol, 1 cycle of 5 minutes of 70% ethanol and 3 cycles of 5 minutes of distilled water. After this process, samples were hydrated and ready to be stained.

### **3.9.1 Haematoxylin & Eosin staining**

For H&E staining, a series of steps were taken: samples were first submerged for 3 minutes into Mayer's Haematoxylin solution (Merck), washed for 1 minute with tap water, 3 seconds with 1% hydrochloric acid ethanol, washed for 1 minute with tap water, 5 seconds with 1% ammonia water, 1 minute wash with tap water, 1 minute with distilled water and finally 3 minutes with Eosin (Merck) to counterstain. After, a dehydration process started and consisted of 3 cycles of 1 minute each graded series of alcohol solutions (96%, 100%) and finally 3 cycles of 5 minutes of xylene. Lastly, samples were fixed to the slide using DPX mounting medium (Merck).

### **3.9.2 Mastocyte and Eosinophil staining**

Mastocyte and Eosinophil staining was performed using the Eosinophil-Mast Cell Stain Kit (Abcam) following manufacturer's instructions. Briefly, after the deparaffination and hydration of the sections, the Astra Blue Solution was added for 3 minutes and was rinsed twice with distilled water. Then, the Vital New Red Solution was added for 30 minutes in agitation and it was rinsed twice. Afterwards, sections were counterstained with Haematoxylin for 15 seconds and washed using tap water. After, sections were left to dry and were dehydrated through three changes of 100% ethanol of about 20 seconds each. Samples were then dried and mounted using DPX mounting medium (Merck).

### **3.9.3 RNAscope**

The RNAscope assay uses a novel method of in situ hybridization that allows the visualization of RNA molecules in histological samples mounted on slides. The RNAscope RED option was chosen because it offers higher contrast and allows to distinguish more clearly haematoxylin from the red dots in highly pigmented tissues as the skin. The 2.5 HD Reagent Kit RED (ACD, Bio-technique) for mouse samples was used along with the RNAscope Probe Mm-Trex2-C1 according to manufacturer's instructions. In a few words, samples were first deparaffinized and pre-treated with hydrogen peroxide to block endogenous peroxidase activity that could interfere with the dyes. After, samples were applied to a boiling buffer system to undo the cross-linking that occurs during tissue histological processing. Then, a hydrophobic barrier was created and proteases were applied. The next day, the probe recognizing the target of interest was incubated with the samples and a series of amplifying probes were applied. Then, RED dye was added and samples were counterstained with haematoxylin. The HybEZ II Hybridization System oven (ACD, Bio-technique) was used throughout the procedure. Finally, slides were mounted using the VectaMount Mounting Medium (ACD, Bio-technique) and observed under a brightfield microscope as detailed in Section 3.9.5.

### **3.9.4 Immunofluorescence**

After deparaffination and rehydration, heat-induced epitope retrieval was performed to the samples by boiling them for 20 minutes in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9). Then samples were left to cool overnight, and staining was performed the following day. Samples were washed twice with PBS and the tissue was delimited with a hydrophobic marker. All incubations were performed in a humidified chamber. Blocking solution (Fetal Bovine Serum, FBS 20% in PBS) was applied to the samples for 1 hour to avoid unspecific bindings. The primary antibody solution was prepared by diluting the primary antibodies TREX2 (1:50, rabbit origin, inhouse (Parra et al., 2009)) or Keratin 10 (K10) (1:20, mouse origin, Abcam) in blocking solution and applied to the samples for 1 hour. After three washes with PBS, secondary antibody solution was added. Secondary antibody solution was prepared by diluting secondary antibodies, labelled with a fluorophore, anti-rabbit 555 (1:500, Life Technologies) or anti-mouse 488 (1:500, Life Technologies) in blocking solution. After a 30-minute incubation, 3 washes with PBS were

performed. Finally, tissues were incubated for 15 minutes with DAPI (1:20000 in PBS, Sigma) and then were washed with PBS and mounted using ProLong Gold (Invitrogen).

### **3.9.5 Microscopy and image quantifications**

Microscopes utilised for image obtention were Nikon E800, Nikon Eclipse E800 and Carl Zeiss Axio Imager M2 Apotome. The ZEISS ZEN 3.8 software was utilised to process the images. The objective and microscope used are indicated in the corresponding figures.

Quantification of the ear thickness and epidermis in H&E-stained sample sections were performed using the ZEISS ZEN and ImageJ softwares. Five independent measurements of the ear width of at least one section of each sample were performed from images taken at 2.5x or 4x, depending on the microscope used for acquisition. The quantification of the epidermal layers was performed visually in three areas of at least one section of each sample. Mean values of each sample were then used for the representation.

In the case of histological samples stained with Mastocyte and Eosinophil staining, images of the overall surface of the ear were taken at 10x using Carl Zeiss Axio Imager M2 Apotome and were analysed using the ZEN software. There, the width of the ear and the epidermis was calculated taking 6 and 10 independent measurements, respectively. Epidermal layers were quantified as mentioned above. For the Mastocyte and Eosinophil quantification, three different sections of 10x images were used and the numbers of cells were quantified manually. Mean values of each parameter were then used for the representation.

## **3.10 Cell culture**

### **3.10.1 HaCaT and HEK293T cells**

HaCaT cells are a KC cell line derived from spontaneously immortalized human KCs widely used for the study of the skin biology (Boukamp, 1988; Wilson, 2014). On the other hand, the HEK293T cell line derives from human embryonic kidney and is widely used in research. HaCaT and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Merck) supplemented with 10% FBS (Labclinics), penicillin/streptomycin (100 U/ml, 100 µg/ml respectively, Merck) at 37°C and 5% CO<sub>2</sub>. Different dilutions of trypsin were used for splitting these cells: 2.5x for HaCaT cells and 0.1x for HEK293T (10x Trypsin-EDTA stock, Merck).

HaCaT cells with a stable expression of YFP, YFP-hTREX2 and mTRE2-YFP had previously been generated in the lab and cultured in the same conditions described above with geneticin (G418, 100 µg/ml) (Manils et al., 2015).

### **3.10.2 Primary KCs**

#### **3.10.2.1 Normal Human Epidermal KCs**

Normal Human Epidermal KCs (NHEK, PromoCell) are primary human KCs isolated from the epidermis of juvenile human donors. Two proliferation media were used to grow NHEK cells indistinctly: CnT-57 Epithelial Proliferation Medium (CELLnTEC) and KC Growth Medium 2 (PromoCell). Both media were supplemented according to manufacturer's instructions and contain Bovine Pituitary Extract and 1% penicillin-streptomycin-amphotericin B (Merck). Primary human KCs were grown at 37°C and 5% CO<sub>2</sub> until confluent and then changed to CnT-07 Differentiation Medium (CELLnTEC). Medium was changed every second day. All experiments were done with third-passage cells. NHEK cells are primary cells that cannot be split many times, but when needed to passage, we used the DetachKiT (PromoCell), that allows gentle subculturing.

#### **3.10.2.2 Primary murine KCs isolation and culture**

A protocol for the isolation of primary murine KCs was previously established in the lab (Parra et al., 2009) and adapted from existing publications (Blanco et al., 2007; Pirrone et al., 2005). Basically, mouse KCs were obtained from newborn mouse skin (1-3 days old, before hair growth) from C57BL/6N mice in a process that started with the decapitation and limb removal of the mice. Bodies were submerged in 70% ethanol for a minute (sterilization) and maintained in 1% penicillin-streptomycin-amphotericin B (Merck) PBS until further processing. Following that, skin was peeled off the body and applied dermis down to a plate with 1% penicillin-streptomycin-amphotericin B PBS. Next, the skins were passed to a plate with KC Growth Medium 2 (PromoCell) and Dispase (2.5 mg/ml, Merck) for 4 hours at 4°C. Then, the epidermis was removed from the dermis and applied to a plate with KC Growth Medium 2. The epidermis needed to be positioned with the basal layer under the medium and the stratum corneum without being covered with liquid. After, a solution of trypsin (Tryple, Invitrogen) diluted 1:1 with medium was added and incubated at 37°C for 10 minutes. The resulting solution was passed through a 70 µm Cell Strainer (Fisher Scientific) to eliminate epithelial debris and the skins were washed

to get the maximum number of cells. The cellular solution was centrifuged at 210 g, 5 minutes and pellet was resuspended. The number of cells was counted using the Luna II Automated Cell Counter (Logos biosystems) and seeded in the desired concentrations in previously collagenated plates (7 µg/ml collagen I, Millipore). Murine KCs were cultured in KC Growth Medium 2 until day 5, when they were changed to CnT-07 differentiation media. Medium was changed every other day and cells were incubated at 37°C and 5% CO<sub>2</sub>.

### **3.10.3 Cellular model of PsO**

A cellular model of PsO was set treating primary murine and human KCs with IL-17A and TNF-α, key inflammatory cytokines known to upregulate the expression of genes overexpressed in PsO (Chiricozzi et al., 2011; Guilloteau et al., 2010). NHEK cells were treated with human recombinant IL-17A (100 ng/ml, Miltenyi Biotec) and human recombinant TNF-α (Tumour Necrosis Factor α, Miltenyi Biotec) at the indicated doses at day 7 post-confluence for 24 hours. Compounds to be tested were also applied on day 7 for 24 hours at the concentrations described. The final concentration of vehicle (DMSO) was 1%. After that, samples were retrieved for RNA and protein analysis, following standard procedures mentioned in Sections 3.12 and 3.3, respectively. Similar procedure was followed in mouse KCs, but murine recombinant TNF-α (Miltenyi Biotec) and IL-17A (Labclinics) were utilised and applied at day 7, when mouse KCs reach differentiation stage.

### **3.11 Cytotoxicity measurement**

Cytotoxicity studies of TREX2 inhibitors were carried out on HEK293T and HaCaT cells. Proliferation assays were performed using the alamarBlue™ HS Cell Viability Reagent (ThermoFisher).

Cells were seeded in a density of 10000 cells/well in a 96 well-plate in the case of HaCaT cells and 8000 cells/well for HEK293T cells and the compound dissolved in DMSO was added to the cells. The reagent was directly added to cells in culture and incubated for 1-4 hours at 37°C in an incubator, protected from light. Absorbance was measured at 570 nm using 600 nm as a reference wavelength (POLARstar Omega) and normalized to the 600 nm value. Controls with DMSO at the same proportion in which the compound was

dissolved were included in all experiments. The percentage of growth inhibition was calculated following the formula:

$$\% \text{ inhibition} = 100 - ((AO \times 100) / AT)$$

Being AO the absorbance observed in the wells with the compound under study and AT the absorbance in the DMSO controls. The concentration used for the inhibitors was 100  $\mu$ M and compared with those of Cisplatin, an inhibitor of DNA synthesis included as positive control of cytotoxicity.

### **3.12 RNA extraction. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)**

RNA was extracted from NHEK, HaCaT and HEK293T cells using the NucleoSpin RNA/Protein from cells and tissue kit (MACHEREY-NAGEL) according to the kit's guidelines and adjusting the elution volume to the quantity of RNA present in the samples. After purification, RNA was quantified using Nanodrop (Thermo Fisher Scientific).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is used to transcribe RNA into complementary DNA (cDNA) and is necessary to perform quantitative Polymerase Chain Reaction (qPCR). For RNA conversion to cDNA, the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was used according to the guidelines and 1  $\mu$ g of RNA was used for the reaction. To enhance the sensibility and detection on qPCR, TREX2 specific primers were used in the retrotranscription (hTX704R: GCAATACTAGGGCACCAGAC) in addition to random primers. The RT-PCR program followed consisted in: 1 cycle of 10 minutes at 25°C, 1 cycle of 120 minutes at 37°C and 1 cycle of 5 minutes at 85°C.

For the qPCR, reaction mix was set up according to the manufacturer's guidelines in a final volume of 5  $\mu$ l using SYBR Green Master Mix (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) depending on the nature of the amplifying system: primers for the first and TaqMan probes for the second. The probes and primers used are shown in Table 3 and 4. qPCR reaction was performed in the QuantStudio 7 Pro instrument (Applied Biosystems). The protocol used for amplification was: 1 cycle of 2 minutes at 50°C; 1 cycle of 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C; 1 cycle of 15 seconds at 95°C, 60 seconds at 60°C and 1 second at 95°C. RT-qPCR data was extracted using the Design and Analysis software (Applied Biosystems)

and analysed in an Excel file. Basically,  $\Delta C_t$  values were calculated by subtracting the  $C_t$  of the housekeeping gene from the  $C_t$  of the target gene. Relative expression levels were determined using the  $2^{-\Delta C_t}$  method. Data were normalized and statistical analysis was performed using GraphPad prism 10.

| Gene                           | Reference     |
|--------------------------------|---------------|
| <i>Trex2</i>                   | Mm04210320_m1 |
| <i>Defb4</i>                   | Mm00731768_m1 |
| <i>Defb14</i>                  | Mm00806979_m1 |
| <i>Lcn2</i>                    | Mm01324470_m1 |
| <i>Il17c</i>                   | Mm00521397_m1 |
| <i>Tnf-<math>\alpha</math></i> | Mm00443258_m1 |
| <i>Il36<math>\gamma</math></i> | Mm00463327_m1 |
| <i>CXCL8</i>                   | Hs00174103_m1 |
| <i>IVL</i>                     | Hs00902520_m1 |
| <i>TREX2</i>                   | Hs01095167_g1 |
| <i>ACTB</i>                    | Hs99999903_m1 |
| <i>GAPDH</i>                   | Hs02758991_g1 |
| <i>HPRT</i>                    | Hs01003267_m1 |
| <i>IL36<math>\gamma</math></i> | Hs00219742_m1 |

**Table 3. TaqMan probes.** Genes and reference number of Taqman probes (Applied Biosystems) used for qPCR. Murine probes start with M and human with H.

| Gene                           | Primer forward           | Primer reverse           |
|--------------------------------|--------------------------|--------------------------|
| <i>S100a9</i>                  | TAGCCTTGAAGAGCAAGAAGATG  | GTGCTTCCACCATTGTCTGAAT   |
| <i>S100a8</i>                  | TCTTCAAGACATCGTTTGAAAGGA | AGTAGTGACCATTCTTCTGAAGTC |
| <i>Tbp</i>                     | ACTTCGRGCAAGAAATGCTGAA   | TGTCCGTGGCTCTCTTATTCTCA  |
| <i>Actb</i>                    | TGTGCTATGTTGCTCTAGACTTC  | CAGGATTCCATACCAAGAAGG    |
| <i>Tslp</i>                    | TCACTCCCCGACAAAACATT     | TCTGGAGATTGCATGAAGGA     |
| <i>Krt10</i>                   | GTACGAGAAGCATGGCAAC      | GGCATTGTCAATCTGCAGC      |
| <i>Il17a</i>                   | CAGAAGGCCCTCAGACTAC      | CAGCTTCCCTCCGCATTG       |
| <i>Il23a</i>                   | CAAGGACTCAAGGACAACAG     | GGTGTGAAGTTGCTCCATG      |
| <i>TNF-<math>\alpha</math></i> | GGGAAGAGTTCCCAGGGAC      | TATCTCTCAGCTCCACGATT     |
| <i>DEFB4</i>                   | TGATGCCTCTTCCAGGTGTTTT   | TCATGGCTTTTGCAGCATTTTGT  |
| <i>S100A9</i>                  | CCTCGGCTTTGACAGAGTGCA    | ATCTTTTCGCACCAGCTCTTTGA  |
| <i>LCN2</i>                    | CACCACAGCGCCTGCTTCCT     | ACTTCCCCTGGAATTGGTTGTC   |

**Table 4. Primers.** Gene name and sequences of forward and reverse primers used for qPCR.

### **3.13 Biophysical interaction assessment**

#### **3.13.1 Cellular Thermal Shift Assay**

The Cellular Thermal Shift Assay (CETSA) assay is a biophysical technique that allows the study of protein and ligand interaction in intact cells and tissues and is based on the principle of ligand-induced thermal changes in the stabilization of target proteins (Molina et al., 2013; Prabhu et al., 2020). This approach relies on the fact that after a heating step, the soluble protein of a cell correlates to the remaining folded protein in the cell and can be assessed by Western Blot to generate the thermal melting curve or aggregation curve of a protein. Upon ligand binding, there is a shift in the melting curve of the protein that can lead to its stabilization or destabilization and indicates protein-ligand engagement (Dai et al., 2019; Lundgren, 2019; Nagasawa et al., 2020).

The CETSA assay was conducted as previously described (Jafari et al., 2014). In sum, HaCaT cells expressing YFP, YFP-hTREX2 and mTREX2-YFP were expanded in a T-25 flask, grown to 90% confluence, and treated with the inhibitors in a 1% DMSO final concentration for 1 hour at 37°C. Afterwards, cells were trypsinized and centrifuged at 300g for 3 minutes. Cells were resuspended in PBS with protease inhibitors (aprotinin (10 µg/ml), leupeptin (10 µg/ml), iodoacetamide (86 µg/ml) and PMSF (1 mM)) and distributed in PCR tubes for the heat treatment, which was performed using the MyCycler thermal cycler (Bio-Rad). The temperatures used (°C) were: 37, 45, 50, 55, 58, 60, 63, 65 and 70. Next, cells were lysed by freezing and thawing cycles and lysates were centrifuged at 20000g, 4°C for 20 minutes to separate the soluble protein from the precipitated one. Supernatant was then analysed by Western Blot as described in Section 3.3.2.

#### **3.13.2 MicroScale Thermophoresis**

MicroScale Thermophoresis (MST) is a biophysical assay used to quantify interaction between molecules. In our case, we wanted to decipher if there was indeed an interaction between TREX2 and the small compound inhibitors. Basically, this assay measures thermophoretic movement of fluorescent molecules in response to a thermal gradient (Huang & Zhang, 2021). HisMBP-mTREX2 or HisMBP were combined with the Monolith His-Tag Labelling kit RED-Tris-NTA (NanoTemper technologies), which binds to His-tagged proteins and is fluorescent, according to the product guidelines in PBS-0.05% Tween 20 buffer. The protein (100 nM) was mixed with different concentrations of TREX2

inhibitor (15 nM to 0,5 mM) and then loaded into Monolith Premium Capillaries (NanoTemper technologies). A temperature gradient was applied to the samples inside the capillaries and the movement of the fluorescent particles was measured in the Monolith NT.115 instrument (NanoTemper technologies).

MST assays were performed in the European Molecular Biology Laboratory in Hamburg (Germany) during a stay as part of the MOSBRI project that was awarded to the group. MST data was analysed using the eSPC platform, a Data Analysis Platform developed by EMBL, and more specifically the ThermoAffinity software. This was also used to plot the results.

### **3.13.3 Crystallisation**

Co-crystallisation and soaking assays of TREX2 inhibitors and TREX2 have been performed in the European Molecular Biology Laboratory in Hamburg (Germany) following established protocols (Müller, 2017). Briefly, purified mTREX2 in Column Buffer without EDTA was concentrated from 6 to 8 mg/ml. TREX2 inhibitors were dissolved in 100% DMSO to a 10 mM concentration and then diluted in Column Buffer without EDTA to a 1 mM concentration.

Co-crystallisation solution of protein (mTREX2) and ligand (TREX2 inhibitors 01-022 and 01-025) at 1 mM was assessed at 5% DMSO maximal concentration and incubated at room temperature for 10 minutes. The initial crystallization studies of TREX2 showed it crystallises in 1.6 M Trisodium citrate pH 6.5, 0.1 M HCl pH 8.5, 2 M Ammonium Sulphate and 5% isopropanol 2M Ammonium Sulphate. The co-crystallisation solution was mixed in a 1:1 ratio with the crystallisation buffers on 24-well plates using hanging the drop technique. The drops were incubated from one week to one month and protein crystals were fished and flash-frozen in liquid nitrogen for X-ray data collection using the crystallisation buffer supplemented with 10% Glycerol.

For the soaking approach, co-crystallized mTREX2 were used and saturated with ligand at higher concentrations aiming with DMSO concentrations limited at a maximum of 20%. Soaked crystals were collected for X-ray experiments using the same procedure mentioned above.

### 3.14 Screening workflow

This thesis encompasses the process of development of TREX2 inhibitors, from the identification of a druggable hotspot in the TREX2 molecule, to a screening of available and new synthesised compounds, to testing their effect in cells and animal models of PsO and AD and finally demonstrating the target engagement between the protein and the inhibitors. Apart from the activities developed in the group and with our collaborators, several procedures have been outsourced to specialised companies, including the pharmacokinetic (PK) and ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) parameters, among others, and are not included in the present thesis manuscript. Nevertheless, PK and ADMET data was taken into account in the hit to lead selection as described in the screening workflow (Figure 24). Overall, pharmacological and toxicological data obtained from 01-022 TREX2 inhibitor lead supported no broad toxicity and a good pharmacokinetic, pharmacodynamic and bioavailability profile, suitable as potential therapeutics. Moreover, the lead compounds, 01-001 and 01-022, were tested for cytotoxicity in various human cell lines including HaCaT and HEK293T cells, and compared with cisplatin, an inhibitor of DNA synthesis included as positive control of cytotoxicity (Table 5). None of the compounds assessed showed significant cytotoxic effects on cellular proliferation and viability. Also, there was not a cytotoxic effect in hepatic (HEP-G2) nor neuroblastoma cells (SH-SY5Y), as established in assays that were outsourced. Therefore, these compounds would be safe in terms of cell cytotoxicity supporting further development.

|           |                    | Cell line                |         |         |        |
|-----------|--------------------|--------------------------|---------|---------|--------|
|           |                    | HEP-G2                   | SH-SY5Y | HEK293T | HaCaT  |
| Compound  | Concentration (μM) | % cell growth inhibition |         |         |        |
| 01-001    | 100                | 22 ± 1                   | 8 ± 2   | 0,4 ± 2 | -4 ± 4 |
| 01-022    | 100                | 15 ± 3                   | 10 ± 4  | 1,5 ± 4 | -5 ± 2 |
| Cisplatin | 100                | 63 ± 3                   | 95 ± 1  | 34 ± 6  | 82 ± 2 |

**Table 5. Cytotoxicity analysis of TREX2 inhibitors leads.** The effect of the 01-001 and 01-022 compounds (100 μM) on cellular proliferation was assessed on HEP-G2, SH-SY5Y, HEK293T and HaCaT cells and is represented as percentage of cell growth inhibition. Cisplatin acts as positive control of cytotoxicity.

A schematic representation of the overall workflow process of development of TREX2 inhibitors is shown in Figure 24. In brief, the cascade starts with the hit expansion step, where the design and synthesis of the compounds was undertaken and compounds were tested using the *in vitro* exonuclease activity assay. Those compounds with high potency

( $IC_{50} < 5 \mu M$ ) and selectivity ( $>100$  fold) were tested in the IL-23-induced mouse model of PsO and in an *in vitro* KC cellular model of PsO, as part of the Hit-to-Lead step. There, compound's cytotoxicity, hepatic clearance and inhibition of channels hERG and 5-HT2B were assessed to determine their safety profile. Also, the mouse PK was determined in outsourced assays, as well as some of the safety assessment options. Finally, the lead compound efficacy was validated in the IMQ-induced mouse model of PsO. A panel of assays including a safety screen, genotoxic assessment, and CYP P450 assays have been outsourced. In parallel, a number of related activities to determine the mechanism of action, the chemical characterisation, the on-target toxicity, a comparison with reference treatments and an indication expansion of the compounds were or are planned to be addressed, to select the final candidate.

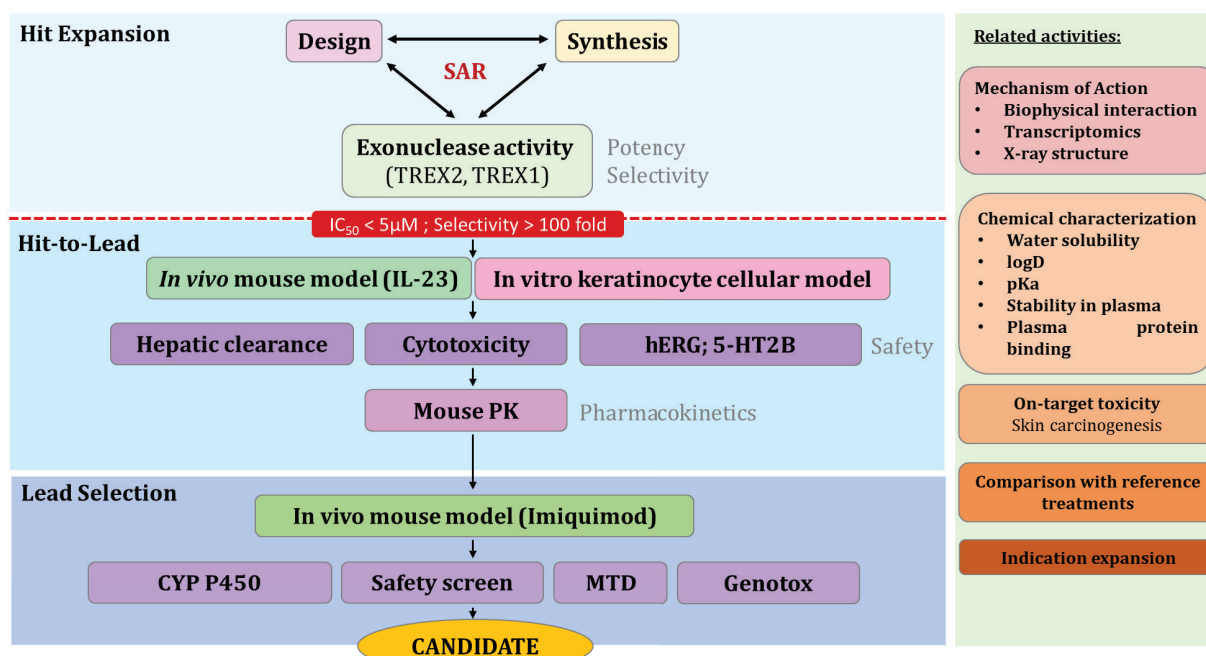


**Figure 25. Cascade/workflow for the Hit to Lead development and selection of the first-in-class TREX2 inhibitors.** Assays undertaken and parameters taken into account are depicted.

### 3.15 Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.3.0 (507). The unpaired t test, Mann-Whitney test, two-way ANOVA with the Šídák's multiple comparison test and one- or two-way ANOVA with the uncorrected Fisher LSD were used, as indicated. P values  $\leq 0.05$  were considered statistically significant.



# Results



## 4. Results

### 4.1. Identification and development of the first-in-class TREX2 inhibitors

The druggability of the TREX2 protein was assessed using computer-aided drug design approaches in collaboration with Prof Xavier Barril's Group (Faculty of Pharmacy and Food Sciences, University of Barcelona). The methodology employed as well as the process involved in this assessment is described in Serena Piticchio's doctoral dissertation (Piticchio, 2020). Briefly, the crystal structure of human TREX2 in the Protein Data Bank (code 1y97) (Perrino et al., 2005) was used to analyse the druggability of the protein using a mixed-solvent molecular dynamic approach. This computational approach is based on simulating the movements of a protein in a mixture of water and organic solvents to determine the parts of the protein with affinity for the latter, which are the potential binding sites (Barril, 2017).

As a result of the analysis, a cryptic allosteric binding site large enough to fit a drug-like molecule that presented hydrophobicity was found in the back part of the TREX2 molecule. The hydrophobicity of the cavity explains why it is cryptic, as it avoids being exposed to aqueous solvents. After, a docking-based virtual screening using an in-house catalogue of commercial compounds with nearly 7 million molecules was conducted against the pocket and identified a group of molecules that were studied to find the best possible interactions. In the end, a selection of 50 compounds were purchased from available chemical compound libraries and tested using an *in vitro* exonuclease assay to determine the effect of the small molecules on TREX2 activity. Afterwards, to improve compound potency, define the chemical space and establish a Markush formula, chemical synthesis of new compounds was undertaken in collaboration with Prof Rodolfo Lavilla's Group (Faculty of Pharmacy and Food Sciences, University of Barcelona). A scheme of the identification of the druggable cavity found in TREX2 and the route from hit expansion to lead optimization and selection is depicted in Figure 26.

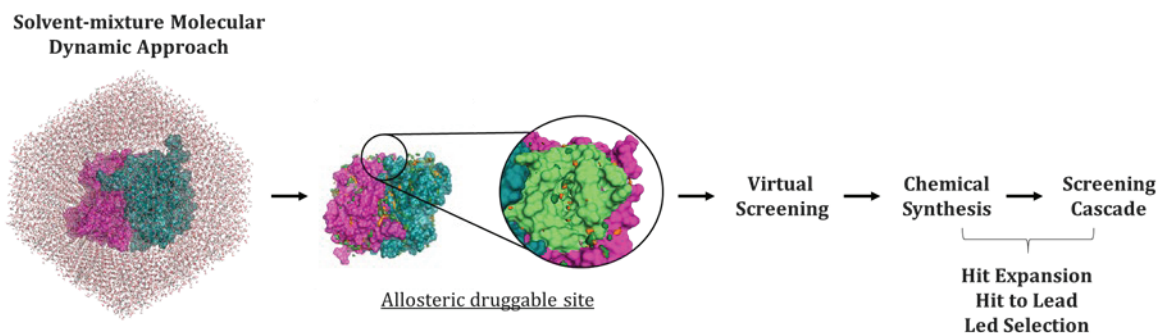


**Figure 26. Discovery of an allosteric druggable hotspot site in the TREX2 dimer and identification of TREX2 inhibitor compounds.** An allosteric binding site was found in the TREX2 molecule by means of a Solvent-mixture Molecular Dynamic approach. Subsequently, a virtual screening was performed with nearly 7 million molecules and 50 small molecules that could potentially bind to the hotspot site were selected. Next, computational analysis and chemical synthesis was undertaken to characterize the first-in-class small molecule TREX2 inhibitors. Adapted from “Molecular recognition and chemical space navigation in drug discovery” by S. Piticchio, 2020, Doctoral dissertation, University of Barcelona.

#### 4.1.1. Hit identification

The 50 compounds selected on the initial virtual screening were tested for inhibitory activity against TREX2 and TREX1 in an *in vitro* exonuclease assay. TREX1 was employed to evaluate the selectivity of the compounds due to the high homology with TREX2. Out of the 50 compounds screened against the murine isoforms of TREX2 and TREX1, only 12 displayed exonuclease activity inhibitory capacity (Figure 27A). Further testing of the active compounds against the human and murine isoforms of TREX2 and TREX1 revealed six of these could block both murine and human TREX2 and TREX1 isoforms (ID4, ID7, ID26, ID30, ID33 and ID48) and five were selective for TREX2 (ID25, ID28, 01-045, ID34 and ID49) (Figure 27B). Among the last, 01-045 and ID49 had a higher capacity to selectively inhibit TREX2 over TREX1 exonuclease.

To further characterize the potency of the 01-045 and ID49 compounds, dose response experiments were performed in order to determine the  $IC_{50}$ . The blocking potency of 01-045 ( $IC_{50}=58.3 \mu M$ ) was slightly higher than ID49 ( $IC_{50}=71.7 \mu M$ ) (Figure 27C). Taking into account the structure of both compounds, modelling of their binding to the TREX2 cavity and their chemical synthesis routes, 01-045 was selected to undertake further hit to lead optimization screening steps.

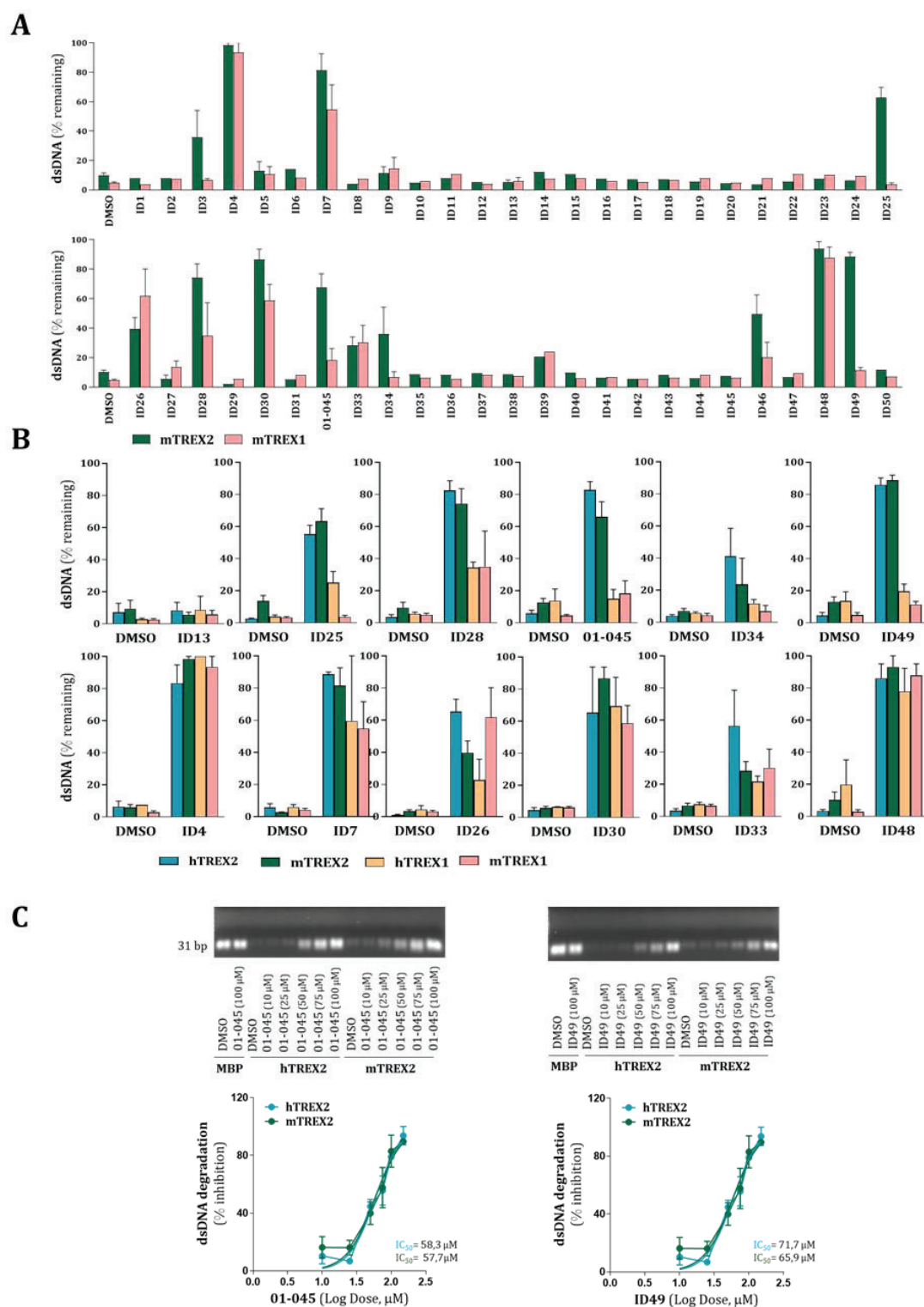


**Figure 27. Analysis of the exonuclease blocking effect of the small molecule compounds targeting TREX2 selected in the virtual screening.** Non-degraded dsDNA is shown in the absence (vehicle, DMSO) and presence of the indicated compounds. A) Effect of the compounds on the exonuclease activity of murine (m) TREX2 and TREX1 isoforms. B) TREX2 inhibitor specificity for human (h) and murine (m) TREX2 and TREX1 isoforms. Representation of the effect of compounds with highest inhibitory capacity on dsDNA degradation. Top, compounds specific for TREX2 (ID25, ID28, 01-045, ID34 and ID49) and a compound without inhibitory action (ID13). Bottom, compounds that can inhibit both TREX1 and TREX2 (ID4, ID7, ID26, ID30, ID33 and ID48). Compound concentration, 100 μM. Mean ± SEM of at least 3 independent experiments for each compound. C) Dose response inhibition of TREX2 exonuclease activity by 01-045 and ID49 compounds. Upper figure, DNA electrophoresis analysis of a representative experiment with different doses of 01-045 and ID49 (10, 25, 50, 75 and 100 μM). Bottom, allosteric sigmoidal curves with mean and SEM of at least three experiments.

The potency of the 01-045 compound (Figure 28A) was further evaluated using a longer dsDNA substrate in the *in vitro* exonuclease assay (3000 bp instead of the 31 bp used in the previous assays). In this case, IC<sub>50</sub> values obtained for TREX2 were 24  $\mu$ M and 6.5  $\mu$ M for the human and murine isoforms respectively, while the values for TREX1 were above 150  $\mu$ M (Figure 28B). Of note, values point out IC<sub>50</sub> decreases when a longer substrate is used, probably closest to an *in vivo* scenario.

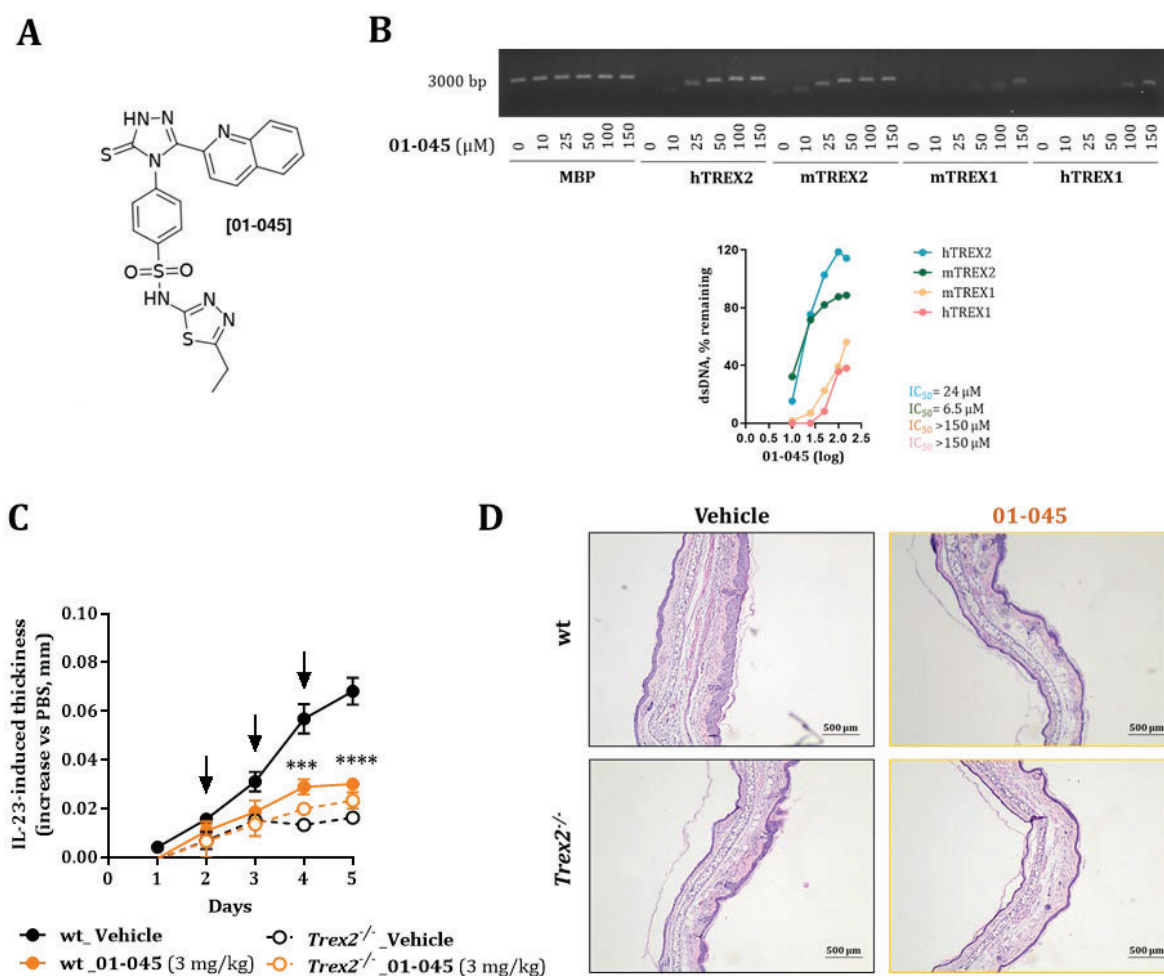


**Figure 28. Potency, selectivity, efficacy and specificity of the 01-045 TREX2 inhibitor.** 01-045 shows selectivity inhibiting TREX2 exonuclease over that of TREX1 *in vitro*, and decreases psoriatic phenotype *in vivo* in the IL-23 mouse model of PsO in a *Trex2* dependent manner. A) 01-045 chemical structure. B) 01-045 dose response inhibition. Top, electrophoresis of non-degraded DNA at the indicated doses of 01-045 (10, 25, 50, 100, 150  $\mu$ M). Beneath, allosteric sigmoidal curve resulting from the quantification of remaining dsDNA. A representative experiment is shown. C) Efficacy of 01-045 in the IL-23 mouse model of PsO. The 01-045 compound triggers a significant reduction of IL-23-induced increase in ear thickness in wt mice, reaching values observed in *Trex2* knockout mice. Left and right ears were treated intradermally (i.d.) with IL-23 or PBS, respectively, once a day. Mice were treated intraperitoneally (i.p.) with 01-045 (3 mg/kg) or vehicle once-daily 1 day after IL-23 first administration. Arrows indicate compound administration. The increase in ear thickness caused by IL-23 in relation to PBS, expressed in millimeters (mm) along time is shown. Values correspond to the mean and SEM of at least 5 wt and 3 *Trex2*<sup>-/-</sup> mice per group. The experiment was conducted independently twice. Analysis of significant differences among untreated and compound treated wt mice: two-way ANOVA with the Šídák's multiple comparison test, \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . D) Representative histological sections stained with H&E of untreated and IL-23-treated ears of wt and *Trex2*<sup>-/-</sup> mice on day 5 of treatment with vehicle or 01-045 compound showing a reduction of ear thickness in wt that is not observed in knockout mice. Microscope Nikon E800 (4x).

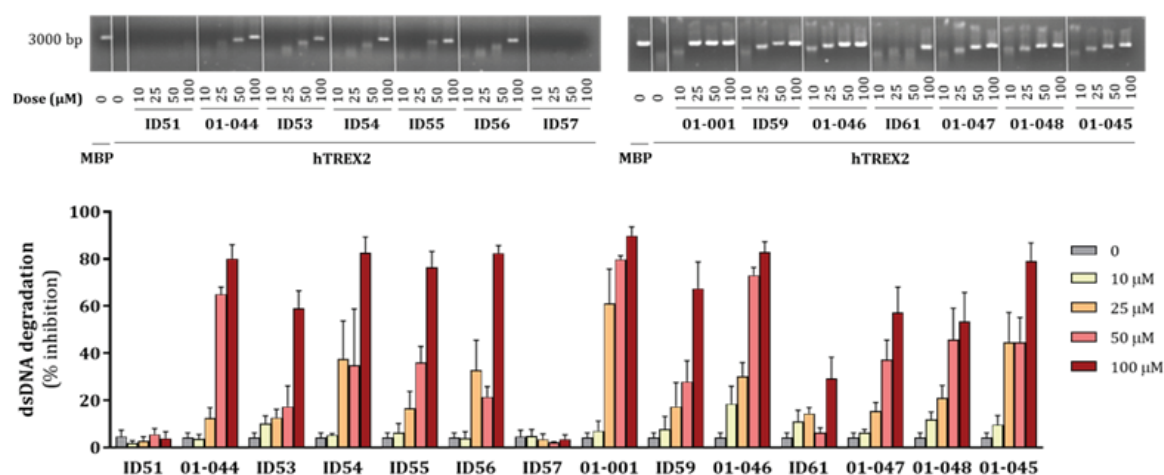
To test the efficacy and specificity of 01-045 counteracting psoriatic inflammation *in vivo*, the IL-23-induced animal model of PsO was used in wt and *Trex2*<sup>-/-</sup> mice. Interestingly, results shown in Figure 28C revealed that the i.p. administration of 01-045 significantly decreased IL-23-induced inflammation, measured as ear thickness in wt but not *Trex2*<sup>-/-</sup> mice, where inflammation was already attenuated, as previously described (Manils et al., 2016). The decrease in ear thickness upon 01-045 treatment was evident at the histological level in the wt ears and not observed in ears from the *Trex2*<sup>-/-</sup> mice (Figure 28D). Overall, data indicate that 01-045 can decrease IL-23 induced inflammation in the IL-23-induced mouse model of PsO in a TREX2-dependent manner, being an initial proof of concept of TREX2 inhibitors as potential therapeutics for PsO.

#### 4.1.2. Hit expansion and Hit to Lead.

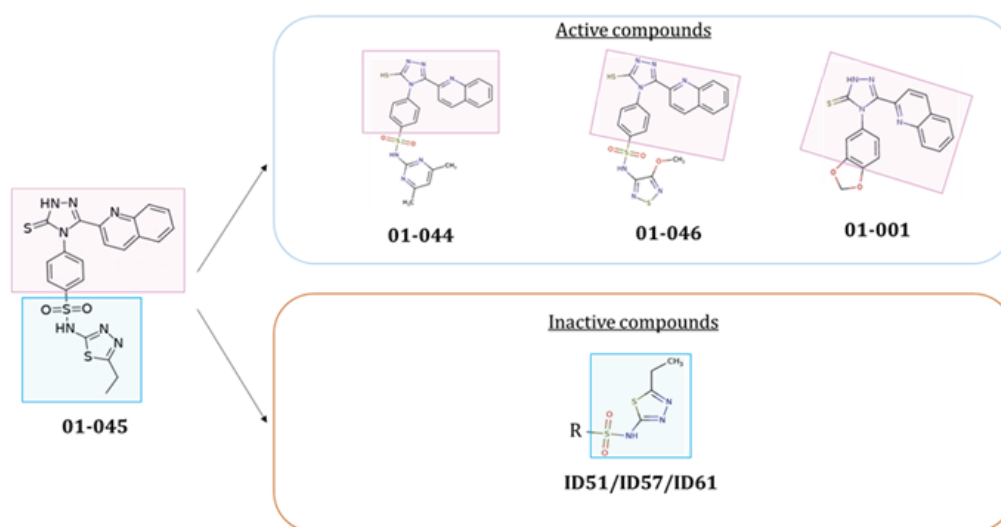
With the initial proof of concept established, hit expansion and Structure-Activity Relationship (SAR) studies were undertaken. We aimed to characterize the chemical space of TREX2 inhibitors and determine the parts of the molecule necessary for TREX2 inhibition to further define the minimal scaffold and the Markush formula of TREX2 inhibitors, the chemical structure representation of a group of related chemical compounds.

For this, a second virtual screening was performed to test available molecules that shared some parts of their chemical structure with the 01-045 compound. The results from this analysis ended up with 13 compounds related to the 01-045 compound that were tested to evaluate their inhibitory capacity against TREX2 in the *in vitro* exonuclease assay. Given that previous assays with the murine and human TREX2 isoforms resulted in similar results, experiments were performed using the human isoform. As seen in Figure 29A, three compounds were inactive or presented very low inhibition (ID51, ID57, ID61), others had medium inhibitory capacity (ID53, ID55, ID56, ID59, 01-047 and 01-048) and the rest highly inhibited TREX2 (01-044, ID54, 01-001 and 01-046). Among the last group, 01-001 stood out as the compound with the highest inhibitory capacity against hTREX2, slightly higher than the 01-045 compound. Moreover, analysis of TREX2 inhibition potency of the compound selected in the second screening allowed to identify motifs of the 01-045 molecule that were necessary for TREX2 inhibition by analysing the active and inactive compounds and their corresponding chemical structure (Figure 29B).

**A**



**B**



**C**

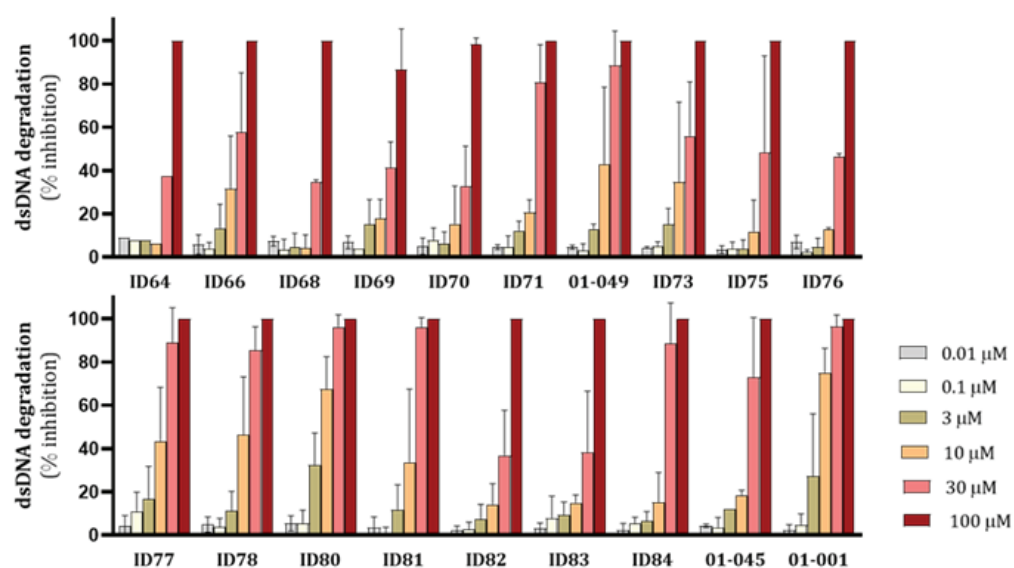


**Figure 29. Analysis of the blocking effect of 01-045 and 01-001-related compounds on TREX2 exonuclease activity.** A) Study of the inhibition of 01-045-related compounds on the exonuclease activity of TREX2. Top, representative agarose gel from an exonuclease assay with the 13 compounds (ID51-01-048) sharing structure with 01-045 and 01-045 at the indicated concentrations (10, 25, 50 and 100  $\mu$ M). Bottom, graph showing the quantification of the inhibitory effect of the indicated compounds on the degradation of dsDNA by human TREX2. Among these, the 01-001 compound stands out as the most potent inhibitor from the set analysed. Mean  $\pm$  SEM of at least 3 independent experiments. B) Overview of the structural motifs of the 01-045 molecule and the related compounds selected by virtual screening. The compounds on top displayed a high inhibitory effect on TREX2 activity and they all share the top motifs (pink frame) of the 01-045 molecule, so that part appears as a scaffold relevant for the inhibition of TREX2. On the other hand, three compounds that contain the bottom motifs (blue frame) of 01-045 (ID51, ID57, ID61), have a very low inhibitory capacity indicating that this part of 01-045 was not necessary for inhibition. C) Dose response inhibition of compounds related to the 01-001 molecule on TREX2 exonuclease activity. Compounds were tested at the indicated concentrations (0.01, 0.1, 3, 10, 30 and 100  $\mu$ M) with the human isoform of TREX2 and a 3000 bp dsDNA. Graphs show the quantification of dsDNA degradation inhibition by the indicated compounds. Mean  $\pm$  SEM in at least 3 independent experiments.

After the discovery of a more potent TREX2 inhibitor, 01-001, this compound was taken as a lead structure for subsequent studies. Then, a third virtual search was performed taking the 01-001 compound as reference molecule to find another group of available compounds with whom it shared some parts of their structure. As a result, 17 01-001-structurally related compounds (ID64-ID84) were purchased and tested for their inhibitory capacity against TREX2 using the *in vitro* exonuclease activity assay. Four compounds (ID65, ID67, ID74 and ID79) were found to be inactive. Dose response analysis of the active compounds showed no improvement of potency compared with 01-001 (Figure 29C). A summary of the IC<sub>50</sub> of the 01-045 and 01-001-related compounds selected in the virtual screenings can be found on Table 6.

| TREX2 inhibitors | TREX2 IC <sub>50</sub> ( $\mu$ M) | TREX2 inhibitors | TREX2 IC <sub>50</sub> ( $\mu$ M) |
|------------------|-----------------------------------|------------------|-----------------------------------|
| ID51             | No inhibition                     | ID70             | 50-100                            |
| 01-044           | 25-50                             | ID71             | 25-50                             |
| ID53             | 50-100                            | 01-049           | 10-25                             |
| ID54             | 50-100                            | ID73             | 50-100                            |
| ID55             | 50-100                            | ID74             | No inhibition                     |
| ID56             | 50-100                            | ID75             | 50-100                            |
| ID57             | No inhibition                     | ID76             | 50-100                            |
| ID59             | 25-50                             | ID77             | 10-25                             |
| 01-046           | 25-50                             | ID78             | 10-25                             |
| ID61             | No inhibition                     | ID79             | No inhibition                     |
| 01-047           | 50-100                            | ID80             | 1-10                              |
| 01-048           | 50-100                            | ID81             | 10-25                             |
| ID64             | 50-100                            | ID82             | 50-100                            |
| ID65             | No inhibition                     | ID83             | 50-100                            |
| ID66             | 25-50                             | ID84             | 25-50                             |
| ID67             | No inhibition                     | 01-001           | 1                                 |
| ID68             | 50-100                            | 01-045           | 10                                |
| ID69             | 50-100                            |                  |                                   |

**Table 6. Potency of 01-045 and 01-001-related compounds inhibiting TREX2.** The IC<sub>50</sub> ( $\mu$ M) values of 01-045 (ID51-01-048) and 01-001 (ID64- ID84) related compounds for the inhibition of TREX2 exonuclease activity are shown.

Dose response assays using recombinant TREX2 and TREX1 protein isoforms demonstrate a selectivity of 01-001 (Figure 30A) inhibiting TREX2 over TREX1. No inhibition of the TREX1 protein is observed. The calculated IC<sub>50</sub> for the human isoform of TREX2 was in the range of 1  $\mu$ M (IC<sub>50</sub> = 1.3  $\mu$ M) (Figure 30B). Therefore, the 01-001 compound displayed a higher potency than 01-045, one order of magnitude, and was confirmed to be highly selective for TREX2.

As the 01-001 compound was the one with the highest potency and selectivity among all the available compounds identified in the virtual screenings, we next analysed its efficacy in the IL-23 animal model of PsO (Figure 30C). Efficacy was tested by i.p. and i.g. administration in wt mice. A significant decrease of IL-23 inflammation measured as ear thickness was observed after both i.p. and i.g. administrations of 01-001, supporting efficacy of 01-001 to treat psoriasis. Therefore, taking into account improved potency, high selectivity and efficacy in vivo reducing IL-23 induced inflammation, the 01-001 compound was selected as a new lead.

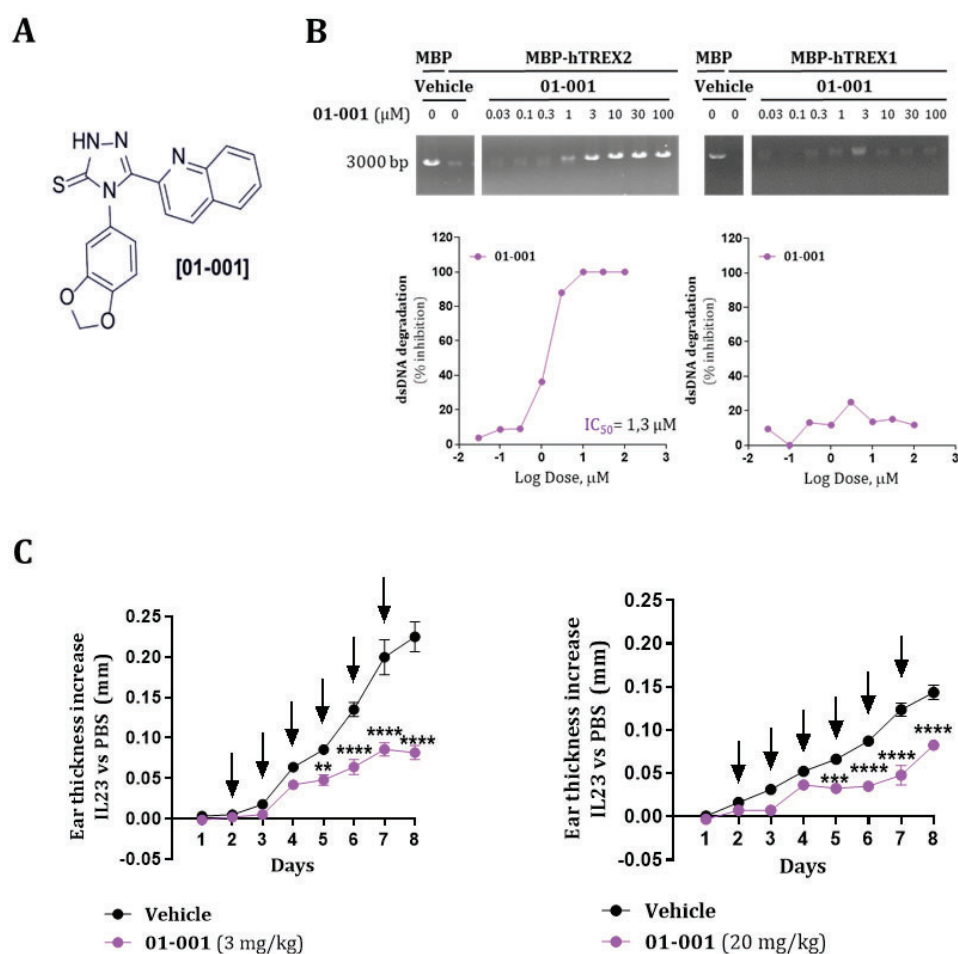


**Figure 30. Potency, selectivity and efficacy of the 01-001 TREX2 inhibitor.** 01-001 potently and selectively inhibits TREX2 exonuclease and exhibits efficacy in the IL-23-induced mouse model of PsO. A) 01-001 chemical structure. B) 01-001 inhibits TREX2 exonuclease activity over that of TREX1. Top, DNA electrophoresis of a representative exonuclease activity experiment with the indicated doses of 01-001 (0.03, 0.1, 0.3, 1, 3, 10, 30 and 100  $\mu\text{M}$ ) and human (h) TREX2 and TREX1 recombinant proteins. Beneath, allosteric sigmoidal curve resulting from the quantification of remaining dsDNA bands.  $\text{IC}_{50}$  value is 1.3  $\mu\text{M}$  for hTREX2 and  $>100 \mu\text{M}$  for hTREX1. C) The 01-001 compound administered intraperitoneally (i.p.) (left, 3 mg/kg) or intragastrically (i.g.) (right, 20 mg/kg) one day after IL-23 first administration triggers a significant reduction of IL-23-induced psoriatic inflammation, measured as ear thickness increase. Left and right ears of the mice were treated intradermally with IL-23 or PBS, respectively, once a day. Arrows indicate compound administration. The increase in ear thickness caused by IL-23 in relation to PBS, expressed in millimetres (mm), along time is shown. Values correspond to the mean and SEM of at least 6 wt mice per group. Analysis of significant differences among untreated and compound treated mice: Two-way ANOVA with the Šídák's multiple comparison test, \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , \*\*\*\*  $p<0.0001$ , compound-treated vs untreated mice.

To deepen into the minimal scaffold necessary for TREX2 inhibition and aiming to optimize inhibitory potency and the PK and ADMET properties, we undertook computational compound design and chemical synthesis. More than 100 new small molecules stemming from 01-001 were synthesised and tested for TREX2 and TREX1 inhibition *in vitro* using the exonuclease activity assay. The potency of the most active compounds ( $\text{IC}_{50} \leq 30 \mu\text{M}$ ) inhibiting TREX2 is depicted on Table 7.

| TREX2 inhibitors | hTREX2 $\text{IC}_{50}$ ( $\mu\text{M}$ ) |
|------------------|-------------------------------------------|
| 01-001           | 1                                         |
| 01-002           | 1-10                                      |
| 01-003           | 10                                        |
| 01-004           | 10                                        |
| 01-005           | 10-30                                     |
| 01-006           | 1-10                                      |
| 01-007           | 1-10                                      |
| 01-008           | 10                                        |
| 01-009           | 30                                        |
| 01-010           | 1-10                                      |
| 01-011           | 1                                         |
| 01-012           | 10-30                                     |
| 01-013           | 10-30                                     |
| 01-014           | 10                                        |
| 01-015           | 10-30                                     |
| 01-016           | 10-30                                     |
| 01-017           | 10                                        |
| 01-018           | 10                                        |
| 01-019           | 30                                        |
| 01-020           | 1-10                                      |
| 01-021           | 10                                        |
| 01-022           | 1                                         |
| 01-023           | 1-10                                      |
| 01-024           | 10                                        |
| 01-025           | 10                                        |
| 01-026           | 30                                        |
| 01-027           | 1-10                                      |
| 01-028           | 10                                        |
| 01-029           | 1-10                                      |
| 01-030           | 1-10                                      |
| 01-031           | 10                                        |

| TREX2 inhibitors | hTREX2 $\text{IC}_{50}$ ( $\mu\text{M}$ ) |
|------------------|-------------------------------------------|
| 01-032           | 1-10                                      |
| 01-033           | 10-30                                     |
| 01-035           | 10-30                                     |
| 01-036           | 10-30                                     |
| 01-037           | 10-30                                     |
| 01-038           | 10-30                                     |
| 01-039           | 10-30                                     |
| 01-050           | 1-10                                      |
| 01-051           | 1-10                                      |
| 01-062           | 10                                        |
| 01-063           | 1-10                                      |
| 01-064           | 10                                        |
| 01-065           | 1-10                                      |
| 01-066           | 10                                        |
| 01-067           | 10                                        |
| 01-068           | 1-10                                      |
| 01-069           | 10                                        |
| 01-070           | 10-30                                     |
| 01-071           | 3-10                                      |
| 01-072           | 1-10                                      |
| 01-073           | 3-10                                      |
| 01-052           | 10-30                                     |
| 01-053           | 3-10                                      |
| 01-054           | 10                                        |
| 01-055           | 3-10                                      |
| 01-056           | 30                                        |
| 01-057           | 10                                        |
| 01-058           | 3                                         |
| 01-059           | 10                                        |
| 01-060           | 3-10                                      |
| 01-061           | 1                                         |

**Table 7. Potency of the first-in-class small molecule TREX2 inhibitors.** The  $\text{IC}_{50}$  ( $\mu\text{M}$ ) values for human (h) TREX2 inhibition of the active ( $\text{IC}_{50} > 30 \mu\text{M}$ ) TREX2 inhibitor compounds are shown.

Selectivity over TREX1 was tested for representative groups of compounds, listed on Table 8. Those compounds with the lowest potency or with no inhibitory capacity are listed in Table 9.

| TREX2 inhibitors | mTREX1 IC <sub>50</sub> (μM) |
|------------------|------------------------------|
| 01-001           | >100                         |
| 01-003           | >100                         |
| 01-022           | >100                         |
| 01-024           | >100                         |
| 01-027           | >100                         |
| 01-028           | >100                         |
| 01-045           | >100                         |

**Table 8. Potency of TREX2 inhibitors on TREX1 exonuclease activity.** The IC<sub>50</sub> (μM) values for mouse (m) TREX1 inhibition of representative TREX2 inhibitor compounds are shown.

| TREX2 inhibitors | hTREX2 IC <sub>50</sub> (μM) |
|------------------|------------------------------|
| 01-034           | 30-100                       |
| 01-040           | 30-100                       |
| 01-041           | No inhibition                |
| 01-042           | No inhibition                |
| 01-043           | No inhibition                |
| OGP-014          | 30-100                       |
| OGP-015          | 30-100                       |
| OGP-019          | 30-100                       |
| OGP-022          | No inhibition                |
| OGP-025          | 30-100                       |
| OGP-040          | 100                          |
| OGP-065          | >100                         |
| OGP-076          | 30-100                       |
| OGP-077          | 100                          |
| OGP-079          | >100                         |
| OGP-081          | 30-100                       |
| OGP-087          | 30-100                       |
| OGP-088          | No inhibition                |
| OGP-098          | 30-100                       |
| OGP-100          | >100                         |
| OGP-101          | 30-100                       |
| OGP-102          | 30-100                       |
| OGP-108          | No inhibition                |
| OGP-128          | 100                          |
| OGP-131          | No inhibition                |
| OGP-133          | 30-100                       |

**Table 9. Non-active TREX2 inhibitor related compounds.** The IC<sub>50</sub> (μM) values for TREX2 inhibition of low or non-active TREX2 inhibitor related compounds are shown.

Of note, to assess sulphur relevance for TREX2 inhibition in the 01-001-related compounds, we synthesised and tested the compounds 01-041, 01-042 and 01-043 (Figure 31). Specifically, the loss of inhibitory capacity in these compounds, where a sulphur is changed to oxygen, methylated or removed, indicate that the central ring of the molecule is essential and that changes in the sulphur atom were not allowed.

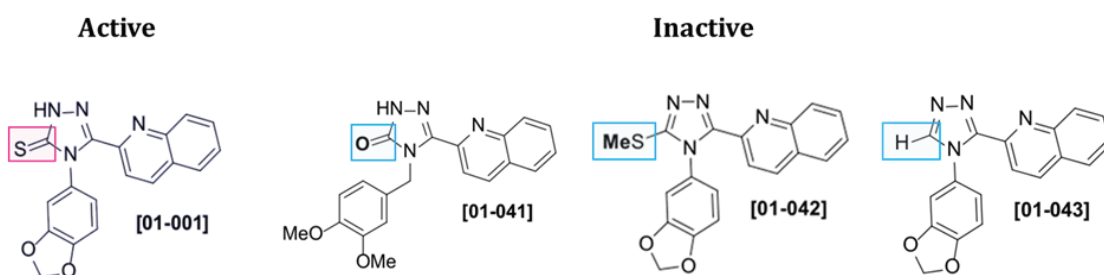


**Figure 31. SAR analysis.** The sulphur (pink frame) is essential for TREX2 inhibitors activity. Schematic overview of active (01-001) and inactive (01-041, 01-042 and 01-043) compounds. Chemical modifications where sulphur is changed to oxygen, methylated or removed render TREX2 inhibitor inactive and are highlighted (blue frame).

Overall, these studies allowed to narrow down the structure necessary for TREX2 inhibition and establish the Markush formula of TREX2 inhibitors (Figure 32). More detailed information on the Markush formula and residues A1 and A2 can be found in the PCT (PCT/EP2024/061679) patent.

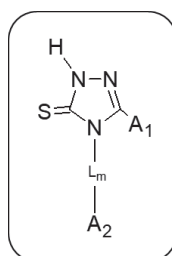


**Figure 32. Markush formula of TREX2 inhibitors.** The structural characteristics of A1 and A2 are disclosed in the PCT (PCT/EP2024/061679) patent.

Computational design and chemical synthesis unveiled the 01-022 (Figure 33A) compound as a 01-001-based compound with a high TREX2 inhibitory activity for TREX2 ( $IC_{50} = 0.8 \mu M$ ), in the sub $\mu M$  range, and high selectivity over the related TREX1 protein (no inhibition was observed at  $100 \mu M$ ) (Figure 33B). Although potency and selectivity properties of 01-001 and 01-022 were very similar, PK and pharmacodynamic parameters together with ADMET properties, pointed 01-022 as an improved new lead.

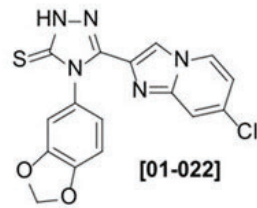
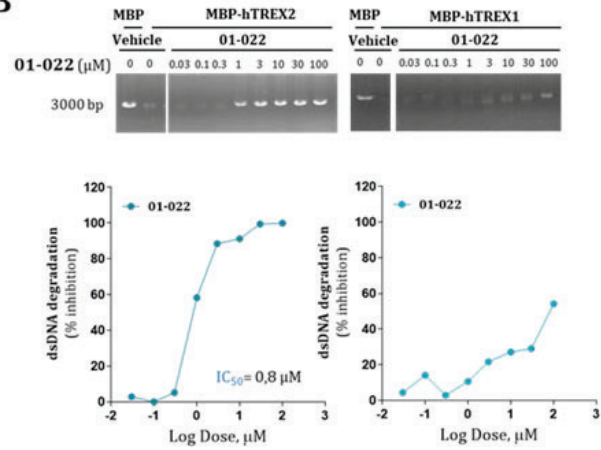
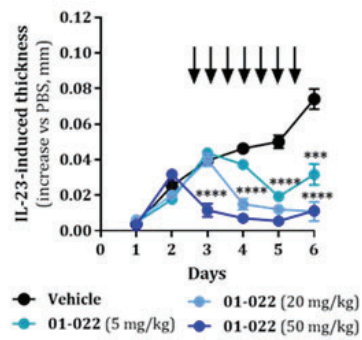
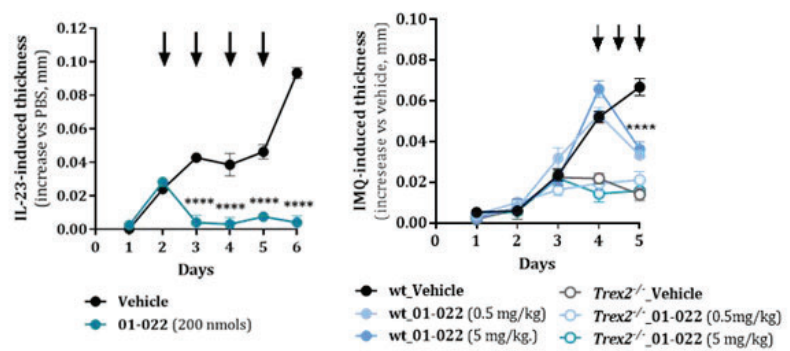
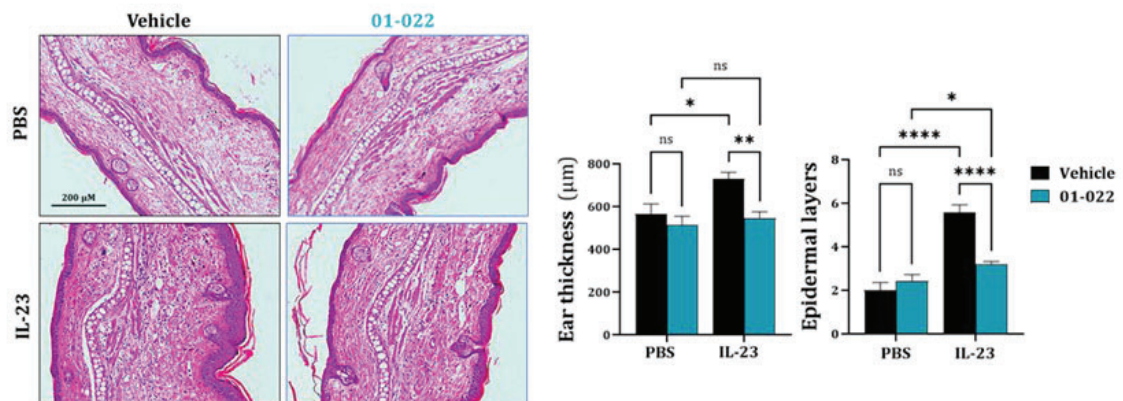
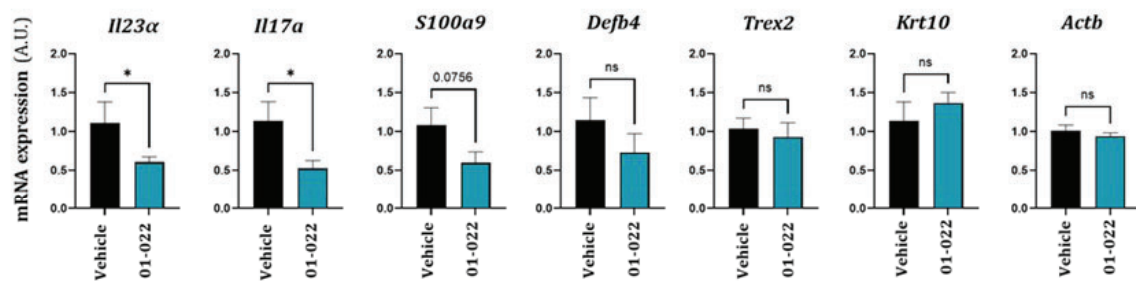
**A****B****C****D****E****F**

**Figure 33. Potency, selectivity, efficacy and specificity of the 01-022 TREX2 inhibitor.** 01-022 potently and selectively inhibits TREX2 exonuclease activity and shows efficacy in the IL-23 and IMQ-induced mouse models of PsO. A) Chemical structure of the 01-022 compound. B) 01-022 inhibits TREX2 exonuclease activity over that of related TREX1. Top, DNA electrophoresis of a representative dose response assay (0.03, 0.1, 0.3, 1, 3, 10, 30, 100  $\mu$ M) on human (h) TREX2 and TREX1 exonuclease activities. Beneath, allosteric sigmoidal curves resulting from the quantification of remaining dsDNA. IC50 value is 0.8  $\mu$ M for hTREX2 and >100  $\mu$ M for hTREX1. C) Efficacy of 01-022 in the IL-23-induced mouse model of PsO. Intragastric (i.g.) (left) and topic (right) administration of the 01-022 compound at the indicated doses two days after the first IL-23 administration causes a significant reduction in IL-23-induced inflammation. Representation of IL-23-induced inflammation as ear thickness increase relative to PBS expressed in millimetres (mm) throughout 6 days. Values correspond to at least 4 animals per group. D) Efficacy of the 01-022 compound in the IMQ-induced mouse model of PsO. 01-022 administered i.g. at the indicated doses on day 4 after daily application of IMQ causes an attenuation of psoriatic inflammation in wt but not *Trex2*<sup>-/-</sup> mice. Representation of IMQ-induced inflammation as ear thickness increase relative to Vehicle (Vaseline cream), expressed in millimetres (mm) throughout 5 days. Values correspond to the mean and SEM of at least 8 wt and 7 *Trex2*<sup>-/-</sup> mice per group. C, D) Arrows indicate compound administration. Analysis of significant differences among untreated and compound treated mice: two-way ANOVA with the Šídák's multiple comparison test, \*\*\* $p$ <0.001, \*\*\*\*  $p$ <0.0001. E) Representative histological sections stained with H&E of untreated and IL-23-treated ears upon Vehicle or 01-022 (20 mg/kg, i.g.) treatment. Quantification of the number of epidermal layers and ear thickness ( $\mu$ m). Microscope Nikon E800 (10x). F) mRNA expression of IL-23-treated ears at day 6 upon vehicle or 01-022 (20 mg/kg, i.g.) treatment. Relative mRNA expression (fold change) of the indicated genes was normalized to the Tbp control gene. A significant reduction in the expression of *Il23 $\alpha$*  and *Il17a*, was observed in the TREX2 inhibitor-treated samples compared to vehicle-treated samples. Analysis of significant differences: unpaired two-tailed Student's t test, \*  $p$ <0.05; ns, not significant. The almost significant p value for S100a9 is indicated.

The efficacy of the 01-022 lead compound was tested in the IL-23- and IMQ-induced mouse models of PsO. As seen in Figure 33C, 01-022 significantly decreased IL-23 induced inflammation measured as ear thickness in a dose dependent manner after i.g. administration. Also, topic administration of 01-022 triggered a drastic decrease of IL-23-induced inflammation. On the other hand, to further validate the efficacy of the compound, 01-022 was tested in another well-established model of PsO: the IMQ-induced mouse model. Noteworthy, 01-022 was administered at day 4, later on than in previous experiment, to test therapeutic efficacy in more challenging conditions. Consistently, a significant decrease of IMQ-derived inflammation was observed upon oral administration of the compound in wt, but not *Trex2*<sup>-/-</sup> mice, confirming the therapeutic efficacy and the specificity of 01-022 lead compound (Figure 33D).

In agreement, the histological analysis of IL-23-treated ears revealed a significant decrease in the ear thickness and epidermal layers from 01-022-treated compared to Vehicle-treated mice (Figure 33E). Moreover, analysis of the mRNA expression of key genes involved in PsO pathogenesis in IL-23-treated ears revealed a significant reduction in the expression of *Il23 $\alpha$*  and *Il17a*, two key cytokines with a crucial role in the pathogenesis of the disease (Figure 33F).

In sum, the 01-022 lead compound has proved to selectively inhibit TREX2 over the closely related exonuclease TREX1 and to decrease PsO-associated inflammation in mouse models of PsO in a TREX2-dependent manner.

## 4.2. Cellular mechanisms of TREX2 inhibitors

To investigate the mechanisms by which TREX2 inhibitors are able to decrease inflammation, a cellular model of PsO using IL-17A and TNF- $\alpha$  was used to treat human (NHEK) and murine KCs (Chiricozzi et al., 2011; Wang et al., 2013). We aimed to determine the effects of the TREX2 inhibitor lead on the expression in KCs of key genes involved in the pathogenesis of PsO to unveil what of the aforementioned effects observed in mice were KC cell autonomous or non-autonomous, as well as TREX2-dependent.

Thus, to unveil the specific effects of the 01-022 TREX2 inhibitor on KCs related to PsO, the expression of key genes related to the pathogenesis of this disease were assessed. In particular, the expression of AMPs like *S100A9*, *S100a8*, *Defb14* or *DEFB4*, known to be upregulated in PsO (Morizane et al., 2010) and that act as chemotactic agents, angiogenic factors and regulators of cell proliferation (Lai & Gallo, 2009; Takahashi & Yamasaki, 2020). Also, the expression of key cytokines involved in the pathogenesis of PsO like *IL-17c*, *IL-36 $\gamma$*  or *TNF- $\alpha$*  (Rendon & Schäkel, 2019; van de Kerkhof, 2022), and other inflammatory mediators like lipocalin 2 (*LCN2*), involved in the inflammation and chemotaxis of neutrophils (Wolk et al., 2017) or *TSLP*, which has a role inducing the proliferation of KCs (van de Kerkhof, 2022). Moreover, the expression of structural, differentiation and skin-barrier genes such as *Involucrin (IVL)*, *Keratin 10 (KRT10)*, *FLG* and *Actin- $\beta$  (ACTB)* were also assessed, along with metabolic gene *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*. Most of these genes are consistently associated to the pathogenesis of PsO, being AMPs or pro-inflammatory genes, all of them reportedly overexpressed in PsO lesions (Büchau & Gallo, 2007; Shao et al., 2016; Towne & Sims, 2012).

In mouse KCs, as seen in Figure 34, *S100a9*, *S100a8*, *Lcn2*, *Il36 $\gamma$*  and *Tslp* were significantly upregulated by IL-17A and TNF- $\alpha$  in wt KCs, as expected (Chiricozzi et al., 2011; Suwarsa et al., 2019), whereas in *Trex2*<sup>-/-</sup> KCs, no upregulation of *Tslp* was observed. Importantly, the expression of these alarmins, diminished significantly after 01-022 treatment in a TREX2 specific manner, as this effect was not observed in KCs lacking *Trex2*. Of note, the expression levels in the presence of 01-022 in IL-17A and TNF- $\alpha$  stimulated wt KCs reached values close to those of stimulated *Trex2*<sup>-/-</sup> KCs. On the other hand, the expression of *Krt10* was diminished upon stimulation with IL-17A and TNF- $\alpha$ , as described (Ma et al., 2008), and no significant variations were caused by 01-022. An increase in the expression

of *Defb4*, *Defb14* and *Il17c* was observed as well, although it did not reach statistical significance. Nevertheless, TREX2 inhibitors had no clear effect on their expression. The expression of *Trex2*, *Tnf-α*, *Gapdh* or *ActB* did not significantly vary after IL-17A and TNF-α nor 01-022 administration. As expected, no expression of *Trex2* is found in *Trex2*<sup>-/-</sup> KCs.

Therefore, a decrease in the expression of alarmins *S100a9*, *S100a8*, *Lcn2* and *Tslp* and the cytokine *Il36γ* was observed upon treatment with 01-022 TREX2 inhibitor in KCs in a TREX2 specific manner. The expression of other genes assessed including *Trex2*, *Defb4* and 14, *Tnf-α*, *Il-17c*, *Krt10* and *ActB* did not vary upon treatment with the small molecule compound, suggesting a specific signature of TREX2 and TREX2 inhibitors in the regulation of these genes.

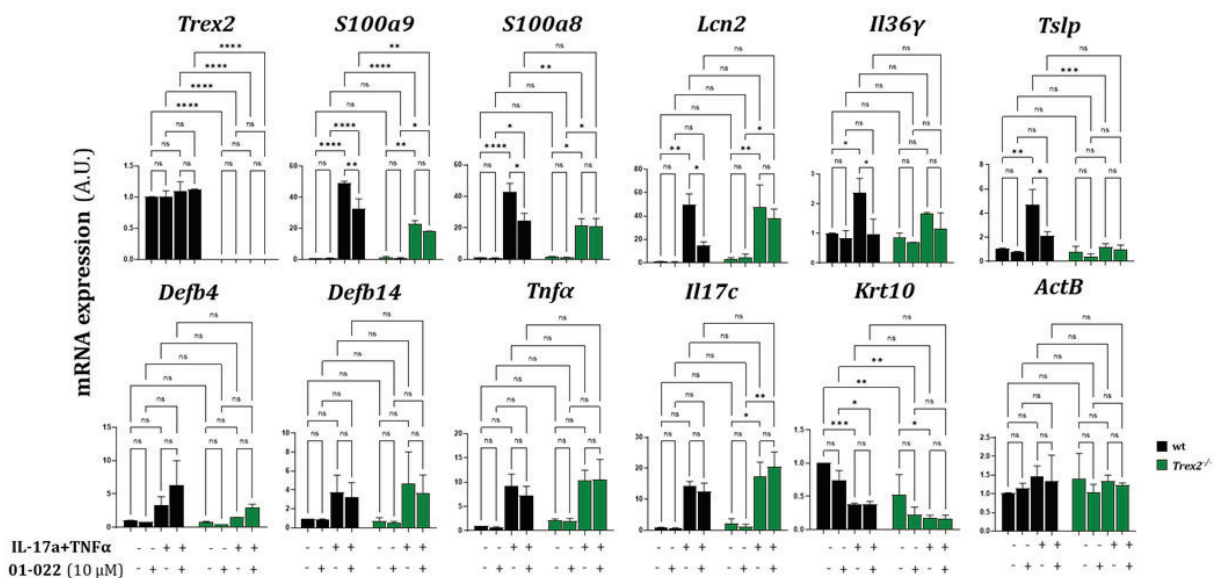


**Figure 34. Effects of the 01-022 TREX2 inhibitor in the KC expression of key genes involved in the pathogenesis of PsO in mouse KCs.** 01-022 reduces the expression of *S100a9*, *S100a8*, *Lcn2*, *Tslp* and *Il36γ* in IL17A and TNFα-treated KCs to values close to those found in *Trex2*<sup>-/-</sup> KCs in a *Trex2*-dependent manner as no effect was seen in knockout KCs. Relative mRNA expression (fold change) of the indicated genes normalized to the control gene *Tbp* in wt and *Trex2*<sup>-/-</sup> mouse KCs after 24-hour treatment with compound (10 μM) and TNFα (10 ng/ml) and IL-17A (100 ng/ml) cytokines. Mean ± SEM of two independent experiments. Significant differences by the two-way ANOVA with the Šidák's multiple comparison test, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, \*\*\*\* p<0.0001; ns, not significant.

In NHEK cells, as seen in Figure 35, IL-17A and TNF-α triggered a significant upregulation of *S100A9*, *DEFB4*, *LCN2*, *CXCL8* and *IVL*, as previously described (Chen et al., 2013; Chiricozzi et al., 2011). Despite not reaching a significant upregulation, other genes also tend to increase with IL-17A and TNF-α such as *IL36γ* (Chiricozzi et al., 2011; Johnston et al., 2011).

Upon 01-022 treatment, the expression of *S100A9*, *DEFB4*, *LCN2* and *IL36γ* clearly decreased in a dose-dependent manner. Interestingly, *LCN2* and *IL36γ*, were also

downregulated in stimulated mouse KCs upon 01-022 treatment. The expression of *CXCL8* was significantly upregulated after IL-17A and TNF- $\alpha$  treatment, as described (Albanesi et al., 1999), but 01-022 did not affect its expression. Also, no effect of 01-022 was seen in the expression of *IVL*, *TREX2*, *TNF- $\alpha$* , *ACTB* and *GAPDH*.

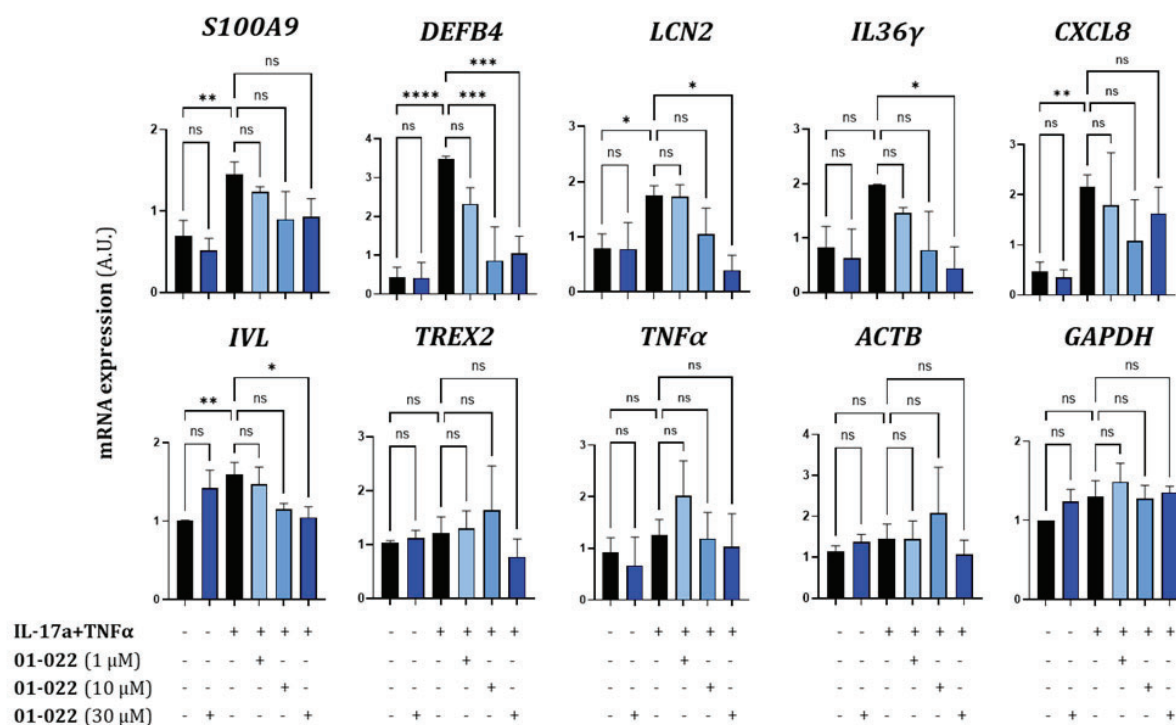


**Figure 35. Effects of the 01-022 TREX2 inhibitor in the expression of key genes involved in the pathogenesis of PsO in primary human KCs.** The 01-022 compound reduced the expression of *DEFB4*, *LCN2* and *IL36 $\gamma$*  in IL-17A and TNF- $\alpha$  treated NHEK cells after 24-hour treatment with Vehicle or the indicated concentrations of compound. Relative mRNA expression (fold change) of the indicated genes normalized to the control gene *HPRT*. Mean  $\pm$  SEM of three independent experiments. Significant differences by one-way ANOVA with uncorrected Fisher's LSD. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ; ns, not significant.

To further prove that the 01-022 TREX2 inhibitor effect in NHEK cells was TREX2 dependent, 01-022 was tested in HaCaT and HEK293T cells, two human cell lines with no detectable expression of *TREX2* (Figure 36A). The first, is a cell line of immortalized KCs and the second has a kidney origin. Also, these cell lines differ in the expression of other genes such as *S100A9*, *DEFB4*, *LCN2*, *IL36 $\gamma$* , *CXCL8*, *IVL*, *TNF- $\alpha$* , *ACTB* and *GAPDH*.

HaCaT cells are a KC immortalized cell line, not primary KCs like NHEK cells, and thus, the expression of several genes is altered, such as those related to KC differentiation. HEK293T cells are not KCs and this might explain why the majority of genes analysed were not expressed. On the other hand, in HaCaT cells (Figure 36B) the induction of *S100A9*, *DEFB4*, *LCN2*, *IL-36 $\gamma$* , *CXCL8* and *TNF- $\alpha$* , and in HEK293T the induction of *LCN2*, *CXCL8* and *TNF- $\alpha$*  upon IL-17A and TNF- $\alpha$  (Figure 36C) did not decrease after 01-022

treatment, supporting the on-target effects of 01-022 TREX2 inhibitors, as *TREX2* is not expressed in these cell lines.

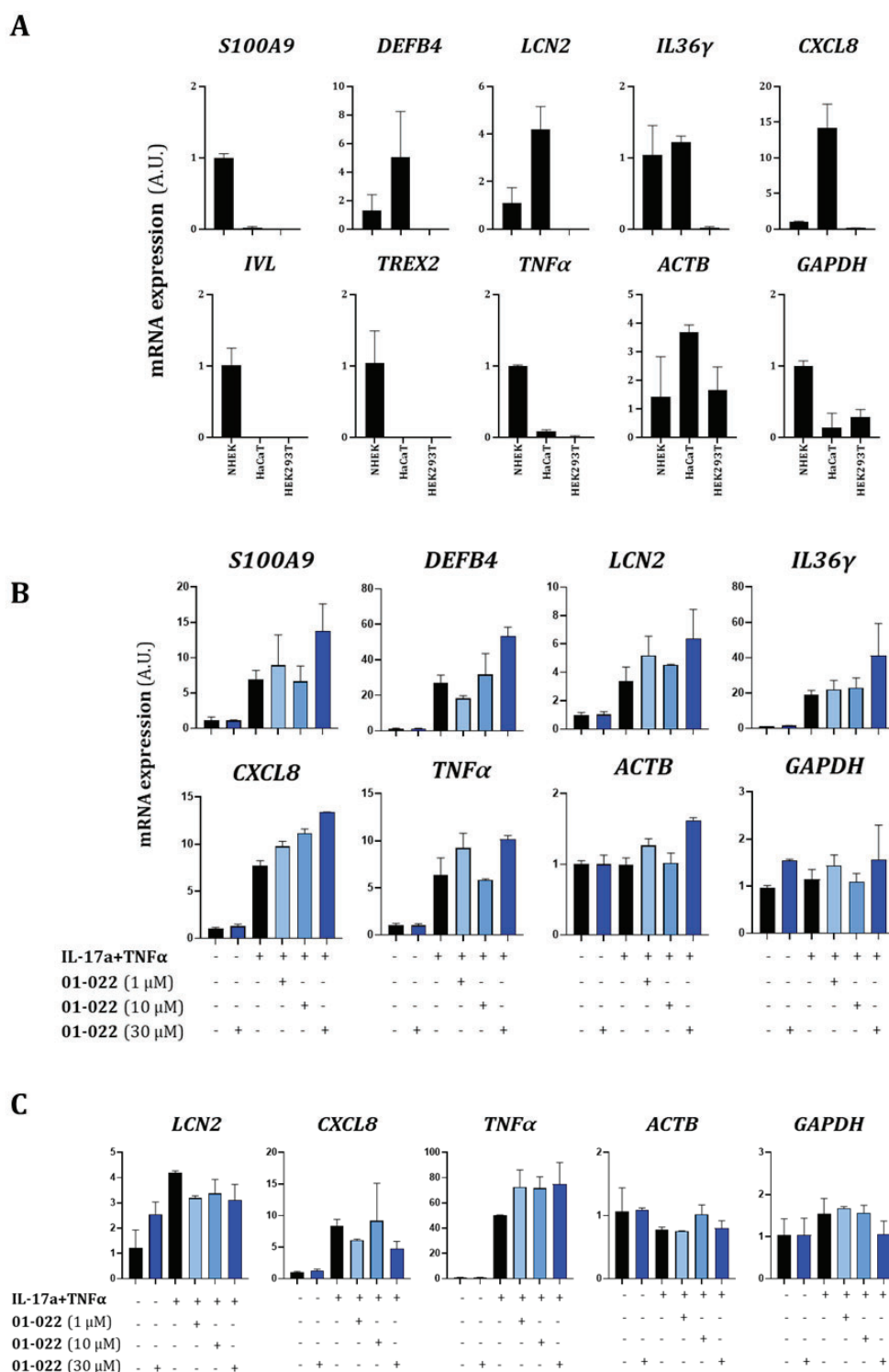


**Figure 36. Effects of the 01-022 TREX2 inhibitor in the expression of key genes involved in the pathogenesis of PsO in HaCaT and HEK293T cells.** The 01-022 compound does not affect the expression of genes involved in PsO pathogenesis in HaCaT and HEK293T cells. A) mRNA expression (fold change) of the indicated genes in HaCaT and HEK293T cells relative to NHEK expression. B) mRNA expression of the genes expressed in HaCaT cells. C) mRNA expression of the genes expressed in HEK293T cells. B, C) Cells were untreated or treated with IL-17A and TNF- $\alpha$  and the indicated concentrations of compound (1, 10, 30  $\mu$ M) for 24 hours. Relative mRNA expression (fold change) of the indicated genes normalized to the control gene *HPRT*. Mean and SD from one experiment.

### 4.3. TREX2 role in AD

#### 4.3.1. TREX2 expression in AD

To determine whether TREX2 has a role in AD, its expression was first analysed on available transcriptomic databases. As shown in Figure 37, *TREX2* expression is higher in AD skin than normal skin, similar to what occurs with *IL-4 receptor (IL-4R)*, a gene known to be involved in AD pathogenesis (Esaki et al., 2015; Tsoi et al., 2019). Interestingly, the expression of the exonuclease in the epidermis of AD lesions is significantly correlated with the AD severity score, suggesting that TREX2 might be involved in AD (Figure 37A). Moreover, the expression of TREX2 was also increased in the DNFB animal model of AD, similarly to IL-4R (Karsak et al., 2007).

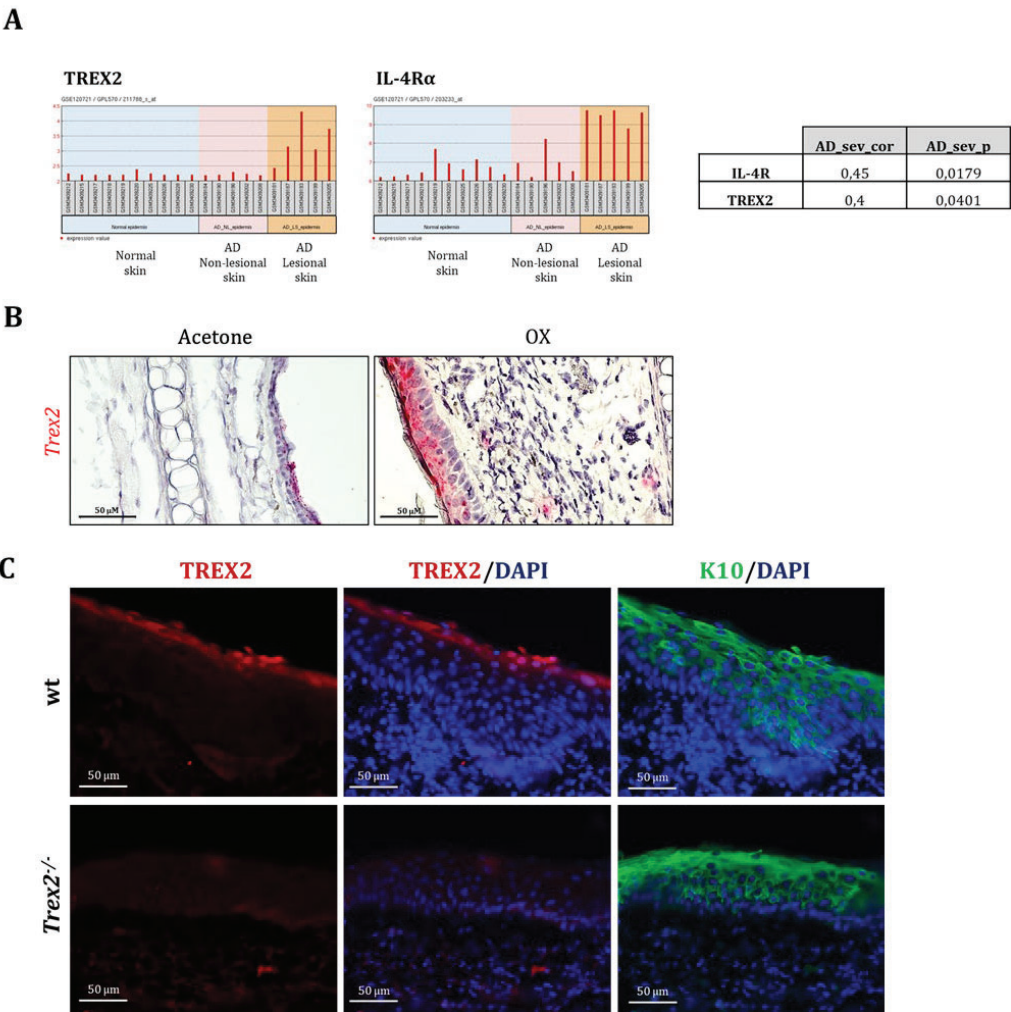


**Figure 37. TREX2 expression in AD.** A) *TREX2* mRNA gene expression data analysis in lesional and non-lesional skin from patients with AD and normal skin from healthy volunteers (GSE120721). Spearman correlation between gene expression and severity of disease indicated (Esaki et al., 2015; Tsoi et al., 2019). Expression of *IL-4R* is also depicted for comparison. B) *TREX2* expression in the OX model of AD. Analysis of *Trex2* RNA expression by RNAscope in the ear skin of mice treated with OX, a mouse model of AD. Red dots representing *Trex2* RNA molecules localize in the epidermis, being more abundant in the upper layers. OX led to an evident increase in *Trex2* RNA expression along the epidermal layers. Microscope used Carl Zeiss Axio Imager M2 Apotome (40x). C) Analysis of *TREX2* protein expression by means of immunofluorescence in the ear skin of wt and *Trex2*<sup>-/-</sup> mice treated with OX. *TREX2* (red) protein localizes in the upper layers of the epidermis. Also, staining of Keratin 10 (K10, green), marker of keratinocyte differentiation, and nuclei (DAPI, blue) are shown. Microscope used Carl Zeiss Axio Imager M2 Apotome (40x).

Next, to confirm and characterize *TREX2* expression in AD, we assessed the mRNA and protein expression levels of *Trex2* by RNAscope and immunofluorescence, respectively, in the OX-induced mouse model of AD. As observed in Figure 37B, OX-treated ears clearly present an induction of *Trex2* mRNA expression that is more abundant in the upper layers of the epidermis. Consistently, the analysis of TREX2 protein expression (Figure 37C) in wt and *Trex2*<sup>-/-</sup> OX-treated skin showed the expression of TREX2 also localised in the upper layers of the epidermis. As expected, no TREX2 staining was present in knockout mice. K10, a marker of KC differentiation, localises also in the upper layers of the epidermis. Hence, the induction of TREX2 in mouse AD lesions was confirmed.

#### 4.3.2. TREX2 role in AD

To assess whether TREX2 has a relevant role in AD two different models, the OX- and the MC903-induced mouse models of AD were used to compare AD phenotype in wt and *Trex2*<sup>-/-</sup> mice.

Analysis of the role of TREX2 in AD using wt and *Trex2*<sup>-/-</sup> animals treated with OX over a period of up to three weeks showed that TREX2 deficiency causes a decrease in the scratching and IgE plasma levels, highlighting the potential role of the exonuclease in the disease (Figure 38A). No changes were observed in the ear thickness between wt and *Trex2*<sup>-/-</sup> mice. The study of several macroscopic parameters including erythema, erosion, scaling and eczema revealed a significant reduction of eczema in the absence of *Trex2* while no changes were observed in the other parameters assessed (Figure 38B).

In Figure 38C, histological analysis of OX-treated ears of wt and *Trex2*<sup>-/-</sup> showed that in the absence of *Trex2*, epidermal thickness and the number of layers in the epidermis were significantly decreased, whereas no differences were observed in ear thickness, consistent with *in vivo* thickness measurements using a digital calliper. Interestingly, eosinophil and mast cell quantification revealed a decrease in the number of eosinophils in the epidermis of *Trex2*<sup>-/-</sup> mice, while mastocyte and eosinophil infiltration in the dermis was very similar in both genotypes (Figure 38C). Consistently, transcriptomic studies in the group have unveiled a significant decrease of inflammatory markers and pathways as well as deregulation of KC differentiation components in the OX-treated ears of *Trex2*<sup>-/-</sup> mice compared to wt (unpublished data). Overall, data from the OX-induced mice model of AD support a relevant role of TREX2 promoting AD-associated itch and inflammation.

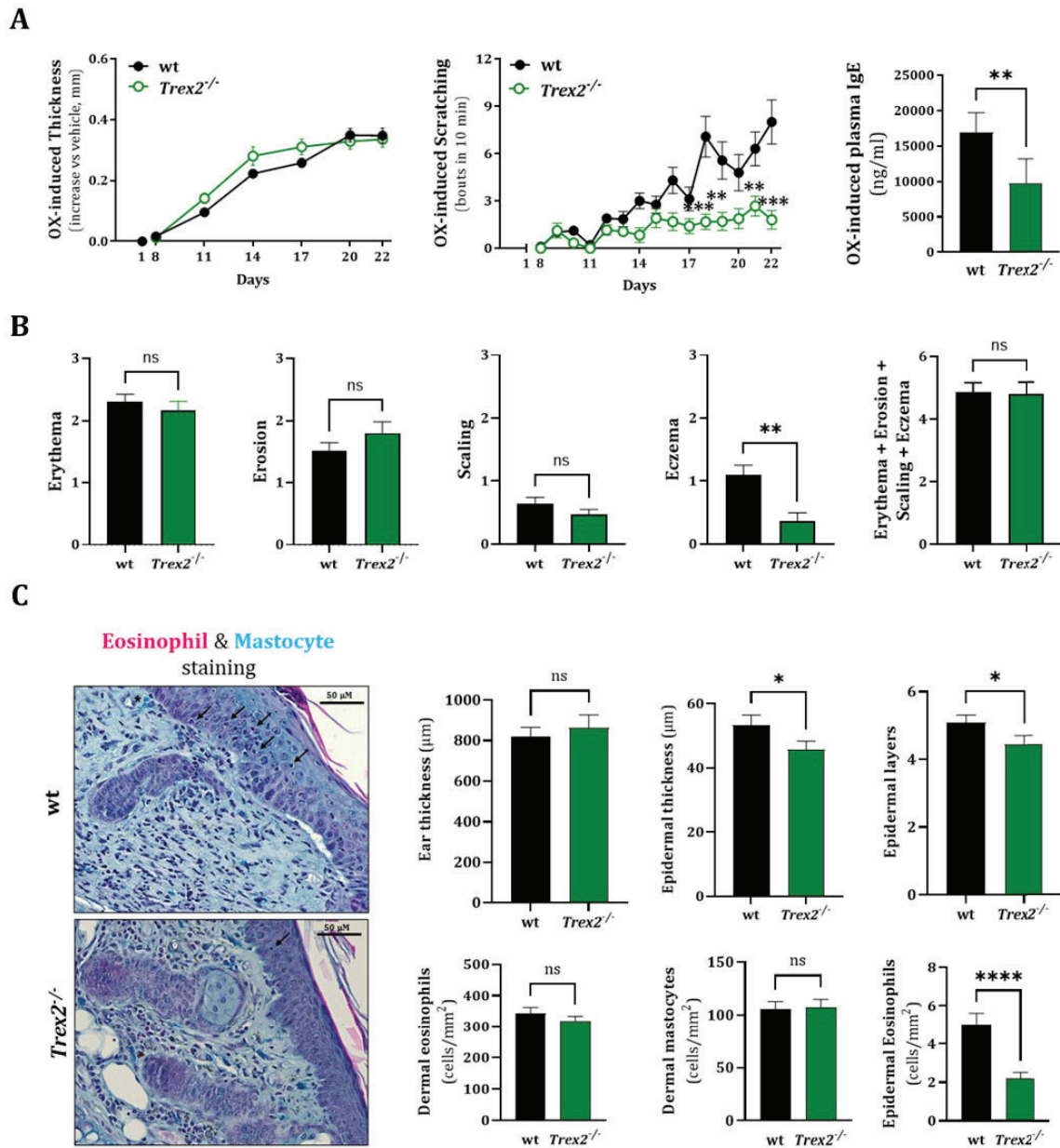


**Figure 38. TREX2 role in the OX-induced mouse model of AD.** TREX2 deficiency ameliorates AD phenotype in the OX-induced AD mouse model. A) TREX2 deficiency reduces itching, eczema and plasma IgE levels, while no changes in ear thickness were observed. Left, ear thickness increase caused by OX relative to Vehicle. Middle, OX-induced scratching as number of bouts in 10 minutes. Right, plasma IgE levels at day 22 are shown. B) Macroscopic parameters at day 22. Erythema, erosion, scaling and eczema were evaluated from 0 (not present) to 3 (very severe). In the right, overall macroscopic score is shown. C) Histological analysis. Trex2 deficiency leads to a reduction in epidermal thickness and layers, along with a decrease in the number of epidermal eosinophils. Left, representative images of eosinophil-mast cells staining of OX-treated ears of wt and Trex2<sup>-/-</sup> mice. Mastocytes are stained bright blue and eosinophils bright red. Epidermal eosinophils are marked with arrows. On the right, a quantification of: ear and epidermal thickness, number of epidermal layers, dermal mastocytes and eosinophils and epidermal eosinophils is shown. Microscope Carl Zeiss Axio Imager M2 Apotome (40x). At least 12 mice per genotype, males and females, were used in two independent experiments. Mean  $\pm$  SEM are shown. Statistical significance between genotypes was determined by two-way ANOVA with the Šídák's multiple comparison test for thickness and scratching evaluation and Mann-Whitney tests for the rest: \*  $p < 0.05$ ; \*\*  $p < 0.01$  \*\*\*\*  $p < 0.0001$ ; ns, not significant.

To further characterize the role of TREX2 in AD we used the MC903-induced model of AD, another model of the disease that recapitulates earliest phases of the disease (Moosbrugger-Martinz et al., 2017). Similar to the OX-induced model, in the MC903-mouse model of AD, scratching and serum IgE were reduced in the *Trex2*<sup>-/-</sup> compared to wt mice (Figure 39A). Moreover, in the MC903-induced model, a significant decrease in ear thickness was observed in the *Trex2*<sup>-/-</sup> compared to wt mice. As seen in Figure 39B, in the absence of *Trex2*, erythema and erosion were significantly reduced and although no changes were observed in scaling nor eczema, the overall score of macroscopic parameters showed significant differences between genotypes. Altogether, data suggest TREX2 deficiency ameliorates MC903-induced AD phenotype, even in a stronger way than OX-induced phenotype. Currently, histological, transcriptomic and immune phenotype characterization are being addressed.

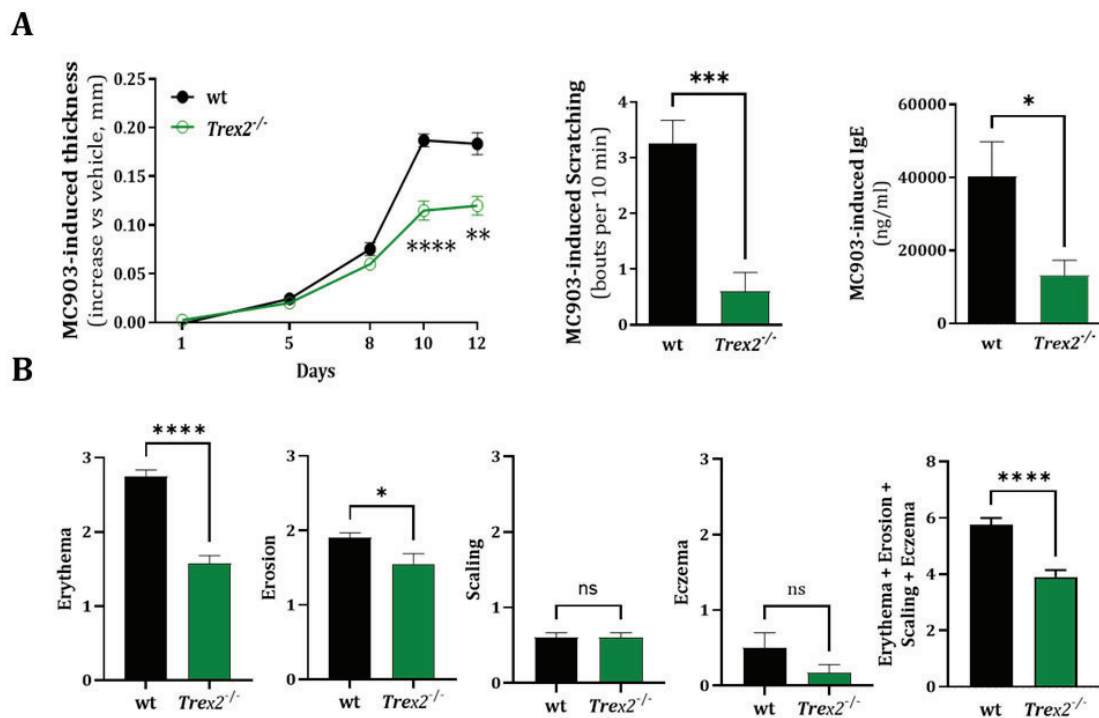


**Figure 39. TREX2 role in the MC903-induced mouse model of AD.** TREX2 deficiency ameliorates AD phenotype in the MC903-induced AD mouse model. A) TREX2 deficiency reduces ear thickness, itching and plasma IgE levels. Left, ear thickness increase caused by MC903 relative to vehicle. Middle, MC903-generated scratching as number of bouts in 10 minutes at day 12. Right, plasma IgE levels at day 12 are shown. B) Macroscopic parameters at day 12. TREX2 deficiency decreases erythema and ear erosion. Erythema, erosion, scaling and eczema were evaluated from 0 (not present) to 3 (very severe). Right, overall macroscopic score is shown. At least 10 mice per genotype, females, were used in two independent experiments. Mean  $\pm$  SEM are shown. Statistical significance between genotypes was determined by unpaired two-tailed Student's t test (A, scratching and IgE; B) and two-way ANOVA with the Šídák's multiple comparison test (A, thickness). \*  $p < 0.05$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ; ns, not significant.

Taken together, data suggest a key role of TREX2 promoting AD-associated itch and inflammation, highlighting TREX2 as a therapeutic target for AD as well. Therefore, next

we tested whether inflammation and itching, main hallmarks of AD pathogenesis, could be reduced upon TREX2 inhibition.

### 4.3.3. TREX2 inhibitors as potential therapeutics for AD

To evaluate the efficacy of TREX2 inhibitors as novel therapeutics to treat AD, the 01-022 TREX2 inhibitor lead was tested in the OX- and MC903-induced mouse models of AD. As seen in Figure 40, the i.g. administration of 01-022 had no effect on OX-induced AD phenotype. In particular, no effect on OX-induced ear thickness, scratching, nor IgE plasma levels were observed (Figure 40A). In a macroscopic level, the compound could decrease erythema and ear erosion, but not eczema or scaling (Figure 40B).

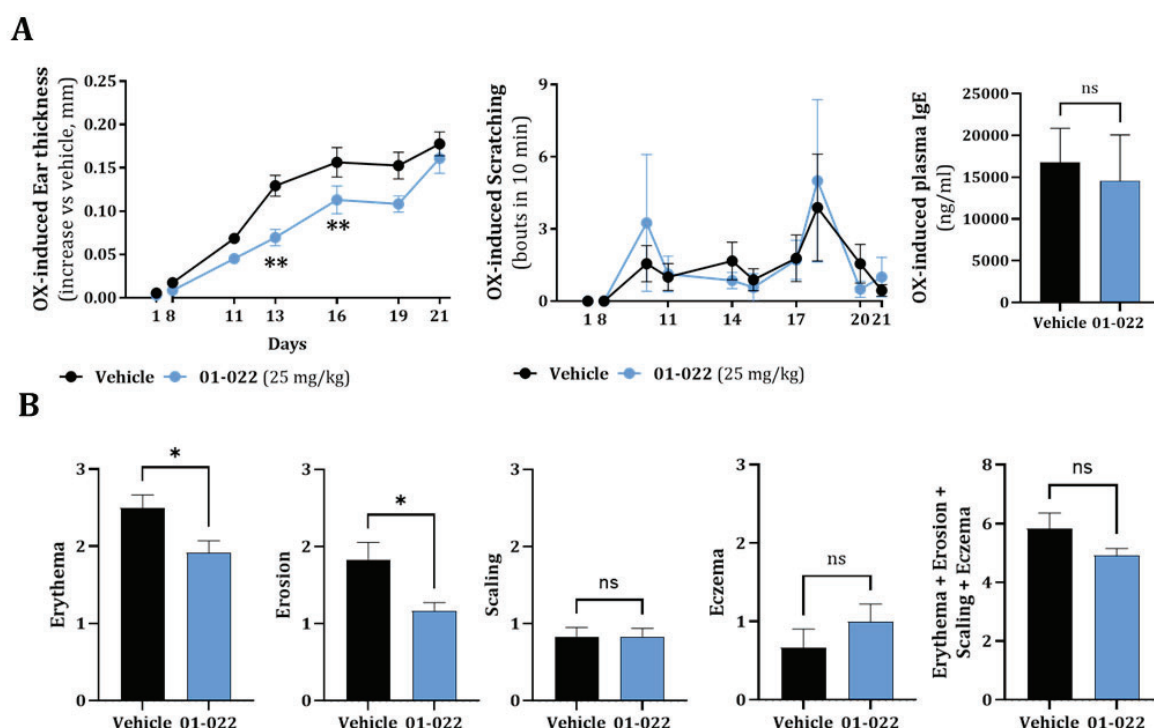


**Figure 40. Assessment of TREX2 inhibitors efficacy as therapeutics for AD in the OX-induced mouse model of AD.** 01-022 TREX2 inhibitor, administered i.g. does not ameliorate major OX-induced AD hallmarks, but erythema and erosion. A) Representation of ear thickness increased induced by OX relative to Vehicle (left) and scratching as number of bouts throughout 10 minutes (middle) along the indicated days. In the right, IgE plasma levels in Vehicle and 01-022-treated mice at day 21 is shown. B) Macroscopic parameters erythema, erosion, scaling and eczema at day 21 are shown. Macroscopic parameters were evaluated from 0 (not present) to 3 (very severe). Mice were treated twice daily from day 1 with the 01-022 compound. At least 8 animals per genotype, males and females were used. Mean  $\pm$  SEM are shown. Statistical significance between treated and untreated was determined by two-way ANOVA with the Šídák's multiple comparison test (A, thickness and scratching) and Fisher LSD test (A, plasma IgE; B) \*  $p < 0.05$ ; ns, not significant.

However, as seen on Figure 41, topic administration of 01-022 after seven days of MC903 treatment led to a reduction of MC903-induced skin thickness, scratching, erythema, erosion and scaling to similar levels of *Trex2*<sup>-/-</sup> mice in a TREX2 dependent manner, as no

effect of 01-022 in these parameters was seen in *Trex2*<sup>-/-</sup> mice, except for erythema. Overall, data support therapeutic efficacy of TREX2 inhibitors to treat AD.

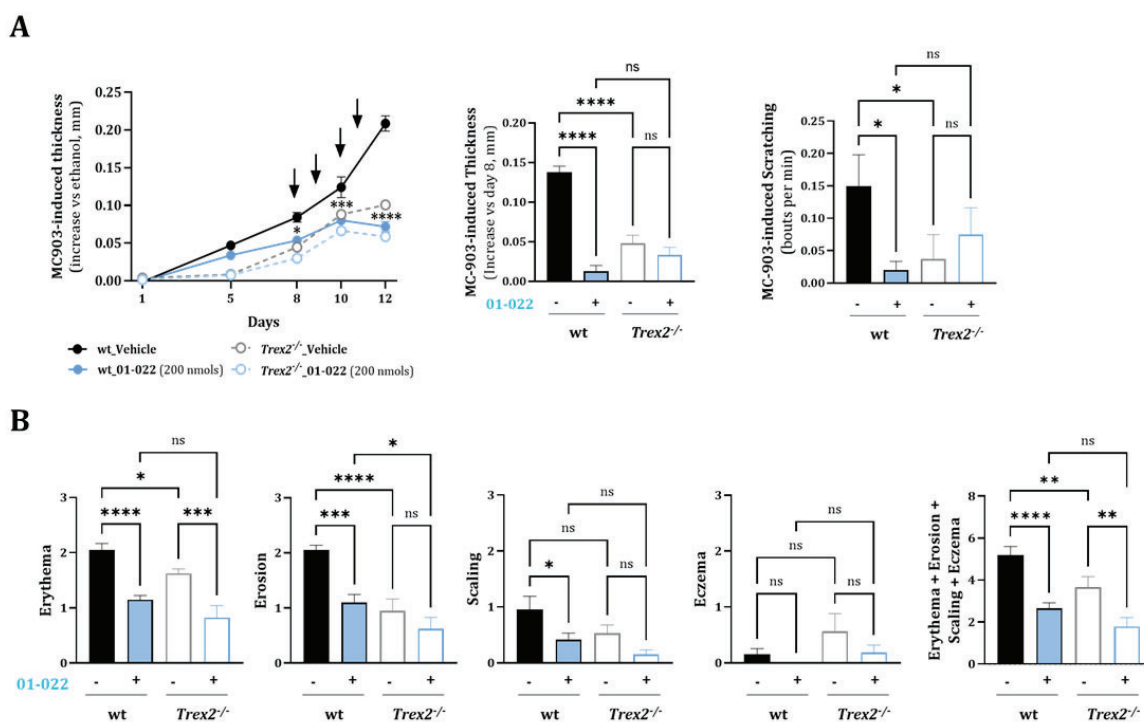


**Figure 41. Assessment of TREX2 inhibitors efficacy as therapeutics for AD in the MC903-induced mouse model of AD.** 01-022 TREX2 inhibitor, administered topically, ameliorates MC903-induced major AD hallmarks in a TREX2-dependent manner. A) Representation of ear thickness increase induced by MC903 relative to Vehicle (ethanol) along the indicated days and from first day (day 8) topic treatment (middle), scratching as number of bouts throughout 10 minutes (right) in vehicle and 01-022-treated wt and *Trex2*<sup>-/-</sup> mice at day 12 are shown. B) Macroscopic parameters were evaluated from 0 (not present) to 3 (very severe). Administration of the 01-022 compound (200 nmols) topically, once a day, since day 8. All values correspond to mean  $\pm$  SEM of at least 9 wt and 8 *Trex2*<sup>-/-</sup> mice in one experiment. Statistical significance between genotypes and treated vs untreated was determined by two-way ANOVA with the Šidák's multiple comparison test (A, thickness) and Fisher LSD test (A, thickness vs day 8, scratching; B) \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ; ns, not significant.

#### 4.4. Biophysical interaction of TREX2 inhibitors with TREX2

Evaluation of target-inhibitor interactions and confirmation of target engagement ensure successful drug development. To that end, several approaches have been undertaken including CETSA, MST and co-crystallisation, among others, to characterise interaction at a cellular, biophysical and molecular level.

##### 4.4.1. Assessment of target engagement by CETSA

Initially, to evaluate the biophysical interaction between TREX2 and the best compounds in a relevant physiological environment, the CETSA approach was used. This assay is based on the fact that proteins unfold as a result of heating even when they are inside cells, and this unfolding leads to precipitation. Thus, the quantification of the remaining

soluble protein as temperature rises in the presence or absence of ligand allows the study of changes in protein stability by comparing the melting curve of a protein in different conditions (Dai et al., 2019; Lundgren, 2019).

The CETSA assay was performed in HaCaT cells stably expressing human and murine YFP-TREX2 recombinant protein isoforms treated with TREX2 inhibitors 01-045 and 01-001 (Figure 42A). Results show a slight change in the melting curve of the protein upon a 24-hour treatment with the compounds, leaning towards the destabilization of the protein (Figure 42B). The melting temperature ( $T_m$ ) of YFP-hTREX2 ( $T_m=62.7^\circ\text{C}$ ) decreased upon 01-001 ( $T_m=58.2^\circ\text{C}$ ) and 01-045 ( $T_m=60.3^\circ\text{C}$ ) treatment. The same effect was observed using the murine isoform of TREX2, where the  $T_m$  from YFP-mTREX2 ( $T_m=64.2^\circ\text{C}$ ) decreased with 01-001 ( $T_m=62.7^\circ\text{C}$ ) and 01-045 ( $T_m=58.5^\circ\text{C}$ ). These changes were not seen in the YFP protein control, in which the  $T_m$  could not be calculated as there was no destabilization of the protein at the working temperatures (Aliye et al., 2015). Yet, additional and more conclusive approaches were needed to support the target engagement of the inhibitors.

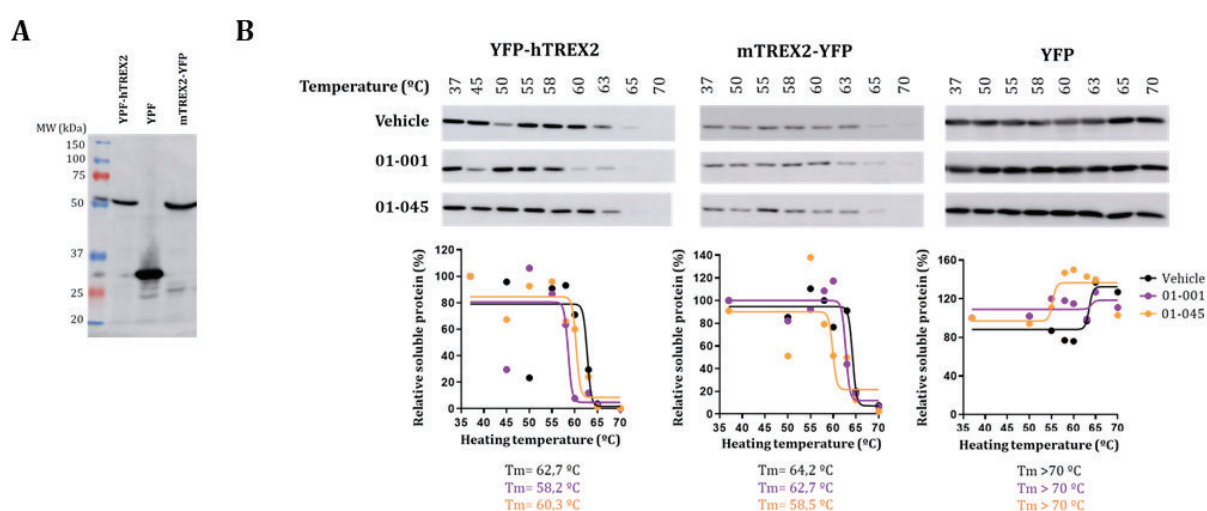


**Figure 42. TREX2 inhibitors target engagement analysis by CETSA. TREX2 inhibitors decrease the  $T_m$  of YFP-TREX2 protein.** A) Expression of YFP (27 kDa), YFP-hTREX2 (53 kDa) and mTREX2-YFP (53 kDa) in HaCaT cells with stable heterologous expression of these proteins. Western blot of total cellular extracts. B) Effect of 01-001 and 01-045 on the thermal stability of TREX2 by CETSA. Top, representative Western Blot analysis of the amount of soluble YFP-hTREX2 (human), mTREX2-YFP (murine) and YFP from HaCaT cells treated with Vehicle (1% DMSO), 01-001 or 01-045 (50  $\mu\text{M}$ ) after heating at the indicated temperatures. Bottom, temperature melting curve graphs with data from the quantification of the bands above. Sigmoidal regression curves fit are represented. The melting temperatures ( $T_m$ ) for YFP-hTREX2 and mTREX2-YFP are depicted. No destabilization of the YFP protein at the temperatures assessed. Representative experiment of two independent experiments.

#### 4.4.2. Assessment of target engagement by MST

The biophysical interaction of TREX2 and the inhibitors was also tested using the MST approach. Basically, this assay measures the thermophoretic movement of fluorescent molecules in response to a thermal gradient. The effect of different concentrations of a non-fluorescent ligand on the movement of the fluorescent molecule, in this case the TREX2 protein, is quantified to detect interaction. If there is interaction between the fluorescent molecule and the ligand, the molecular properties of the molecule change and this has an effect on its movement that can be detected (Huang & Zhang, 2021).

Purified HisMBP-mTREX2 and HisMBP proteins were used for the assay. First, to rule out any non-specific interactions with the HisMBP tag, the 01-071 TREX2 inhibitor was used against the HisMBP-mTREX2 and the HisMBP proteins. As shown in Figure 43A, 01-071 binds to HisMBP-mTREX2, but not to HisMBP. MST analysis showed binding curve plots and dissociation constants ( $K_d$ ) when applying 01-071 to HisMBP-hTREX2 (7.6  $\mu$ M). However, when the compound was tested against HisMBP alone, not a binding curve nor a  $K_d$  could be obtained. Consistently, MST assays indicate that lead compounds 01-045, 01-001 and 01-022 also bind to TREX2 (Figure 43B). Interestingly, the  $K_d$  values obtained for the compounds (7.6  $\mu$ M for 01-045, 5.1  $\mu$ M for 01-001 and 4  $\mu$ M for 01-022) were in the same range as the  $IC_{50}$  for the inhibition of the exonuclease activity (Figure 43C). In agreement, 01-042, a compound that did not show any TREX2 exonuclease inhibitory effect, did not present target engagement to TREX2 as no binding curve nor a  $K_d$  could be obtained. MST experiments were performed in collaboration with Garcia-Alai's group during a short stay in the EMBL facilities in Hamburg (Germany).

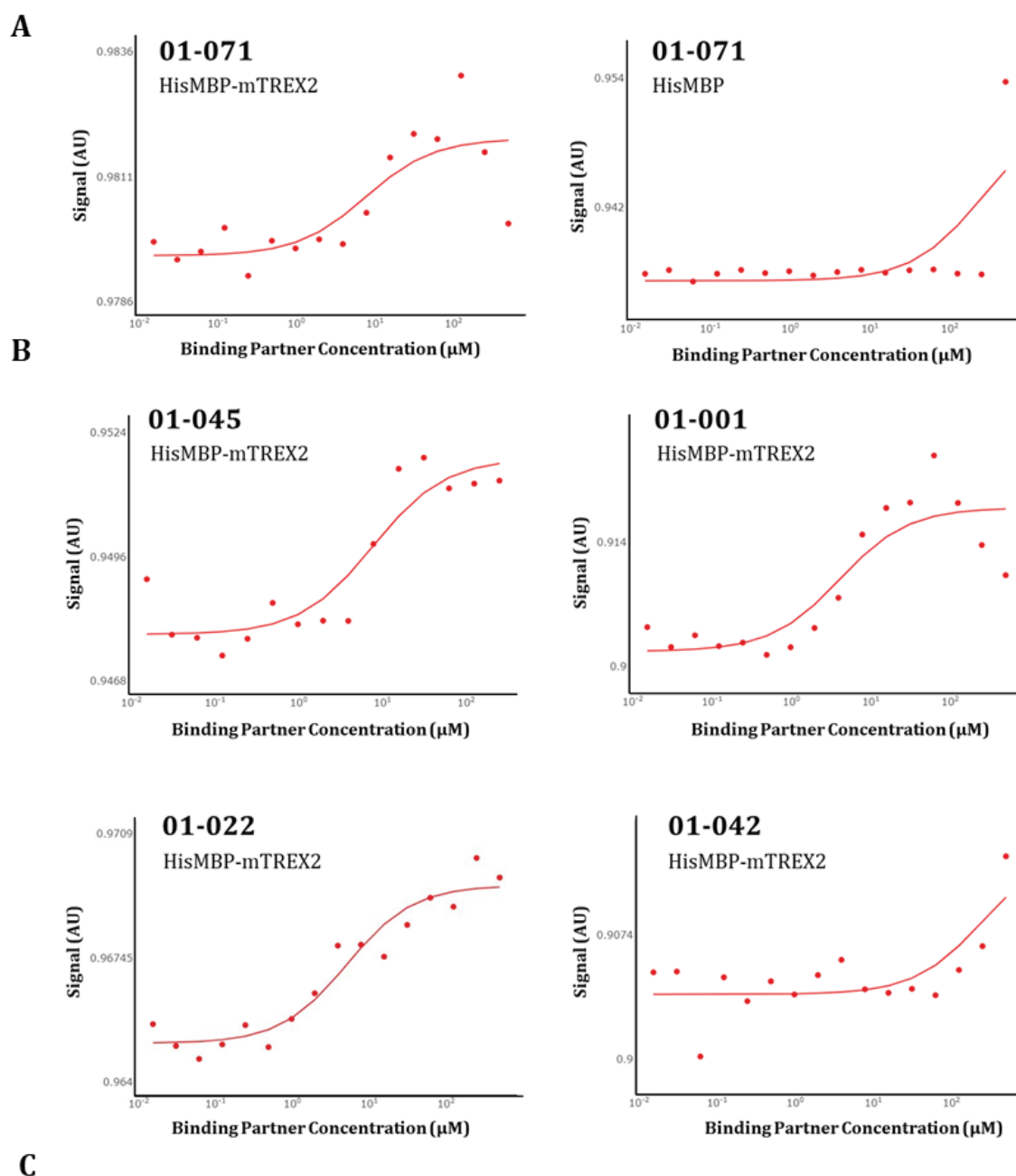


**Figure 43. TREX2 inhibitors target engagement analysis by MST.** A) Binding curve plot of the 01-071 compound with HisMBP-mTRES2 (left) and HisMBP (right). B) Binding curve plot of the indicated compounds with HisMBP-mTRES2. Fitting of the normalized fluorescence traces. The analysis plot was done with the ThermoAffinity software from the eSPC platform created by EMBL. C) Kd ( $\mu\text{M}$ ) and  $\text{IC}_{50}$  of the indicated compounds obtained in the MST and exonuclease activity assays, respectively.

#### **4.4.3. Assessment of target engagement by co-crystallisation**

A definitive proof of the interaction between TREX2 and our small molecule TREX2 inhibitors would be the co-crystallisation of both entities. This advancement would significantly enhance the compound optimization process, as studying their interaction would enable a more efficient and target-driven analysis.

The crystallisation of TREX2 in the presence of TREX2 inhibitors was also performed in the EMBL facilities in Hamburg (Germany) in collaboration with Garcia-Alai's group. The protein used for the crystallisation was mTREX2, which was purified from TEV cleaved HisMBP-mTREX2, as described in Methodology Section 3.3.2, and the TREX2 inhibitor compounds tested were 01-022 and 01-025.

The crystallisation of the mTREX2 protein, previously described (Protein Data Bank code 1y97) (Perrino et al., 2005) was achieved, but no crystal structures with bound compounds have been obtained yet. Upon previous unsuccessful co-crystallisation attempts, we are currently assessing the soaking approach, in which the compound is added to already formed crystals, rather than during crystal formation (Müller, 2017). At the moment, no compound has shown to be bound to TREX2 in any of the crystal structures analysed. However, significant changes in the structure of TREX2 have been observed upon addition of the 01-025 compound.

In particular, recent crystallographic data showed the formation of an intrachain disulphide bond between Cys65 and Cys88 in the presence of the 01-025 compound (Figure 44A), implying unfolding of an alpha helix very closely to the presumed binding site of the TREX2 inhibitors. This conformational change may render the protein inactive, unveiling important insight into the mechanism by which the compounds are able to inhibit the protein.

Interestingly, the induced formation of this disulphide bond by the compound may explain the effect that DTT has on TREX2 inhibitors activity, observed during the first compound screening. As seen in Figure 44B, the presence of DTT in the exonuclease reaction buffer strongly prevented the inhibitory action of the compounds on TREX2 exonuclease activity. Of note, the DTT effect on the compounds that also blocked TREX1 (ID48 and ID7) is not absolute, at least for the human isoform. Taking crystallisation data into account, we hypothesise that in the presence of DTT, a reducing agent, the disulphide

bond cannot form and therefore the activity of the exonuclease after adding TREX2 inhibitors is not blocked. Conversely, in the absence of DTT, the disulphide bond can be formed upon TREX2 inhibitor binding driving conformational alteration in the protein that leads to the inhibition of the exonuclease activity. A deeper characterization of these mechanisms needs to be done.

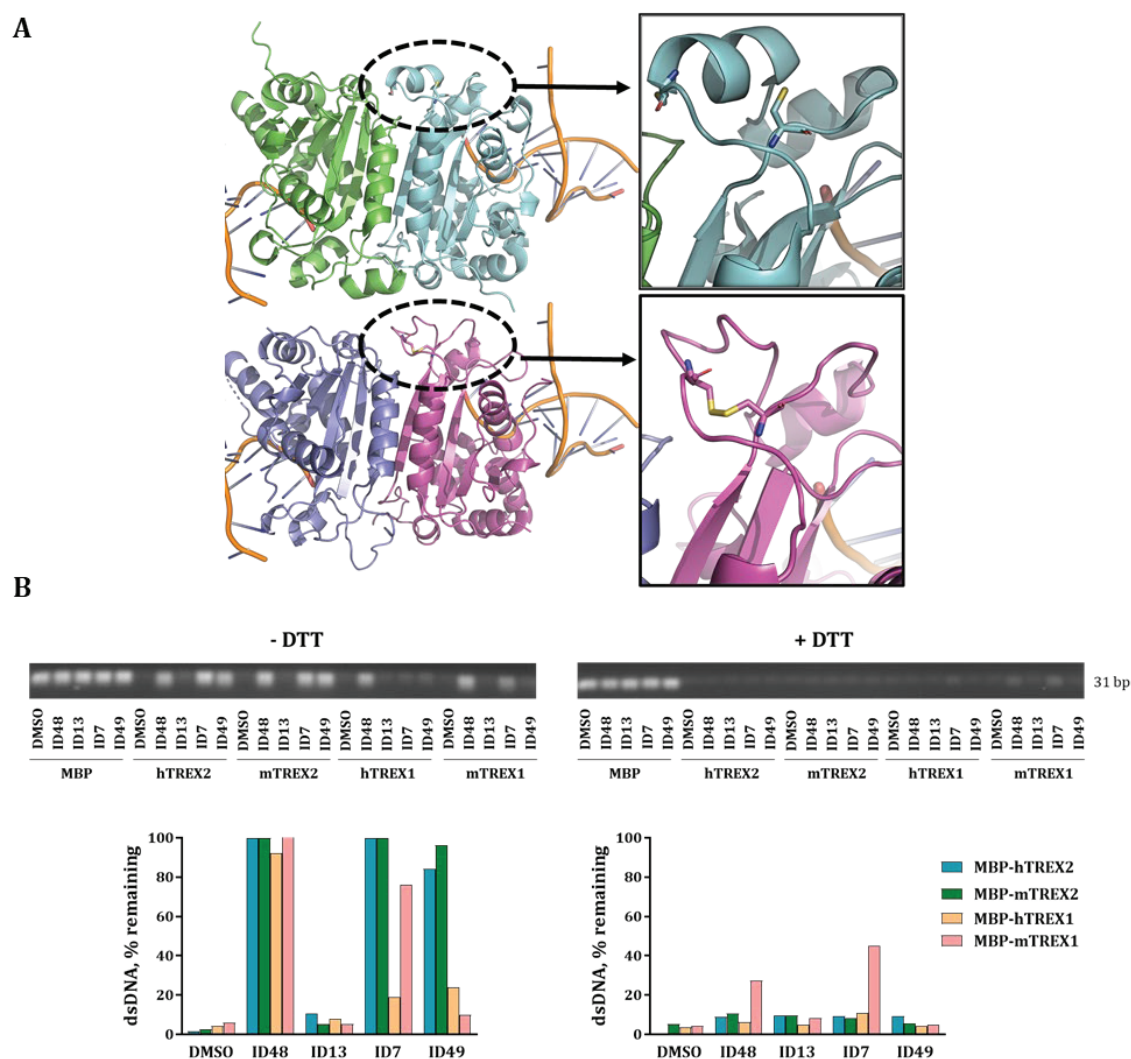


**Figure 44. TREX2 inhibitors target engagement analysis by co-crystallisation.** TREX2 inhibitor-induced formation of the intrachain disulphide bond Cys65-Cys88 could explain the mechanism of action of TREX2 inhibitors. A) TREX2 dimer crystal structures showing folding without (top) and with (bottom) the disulphide bond Cys65-Cys88 were obtained in soaking co-crystallisation experiments with the 01-025 TREX2 inhibitor compound (Cheng et al., 2018; Perrino et al., 2005). B) DTT prevents TREX2 inhibitors inhibition of TREX2 exonuclease activity. The degradation of a dsDNA substrate (31 bp) was analysed in the presence of the indicated compounds (150  $\mu$ M) in the absence and presence of DTT. Graphs show the quantification of remaining DNA. Representative experiment.

# Discussion



## 5. Discussion

The present thesis, points out TREX2 as a KC specific therapeutic target for the treatment of AD, as previously described for PsO (Manils et al., 2016). TREX2 is a 3'-5' non-processive exonuclease that is specifically expressed on KCs (Manils et al., 2015; Parra et al., 2009) and upregulated in both PsO (Manils et al., 2016) and AD skin lesions, as described in this thesis. Moreover, studies of this thesis have decisively contributed to the identification, development and characterization of the first-in-class small molecule TREX2 inhibitors for the treatment of these common inflammatory skin diseases.

PsO and AD have been largely studied, but there are still many aspects unexplored, mainly from the KCs side, and there is yet no cure for them. Currently, there are many therapeutic options for both PsO and AD mostly targeting the immune system, broadly or by blocking specific key actors. However, there are still numerous unmet medical needs. Starting a new era for the development of therapies that do not affect primarily the immune system implies a change in the paradigm of how these diseases are being treated and highlights other players involved, such as KCs, as will be further discussed below.

### 5.1 TREX2 role in AD

In this work, we have confirmed the upregulation of TREX2 in AD mouse models of the disease, in agreement with available transcriptomic databases in other mouse models like the DNFB-induced allergic contact dermatitis model (Karsak et al., 2007), and in human lesional tissue (Esaki et al., 2015; Tsoi et al., 2019) with a significant spearman correlation between TREX2 expression and AD severity score.

Importantly, we have uncovered TREX2 relevance in promoting itch and inflammation, both hallmarks associated to the pathogenesis of AD. We found that TREX2 deficiency triggers a reduction of itching in two different AD mouse models: the OX- and the MC903-induced mouse models of AD, along with a decrease in inflammation.

Reduced inflammation was evident at the macroscopic level in the MC903-induced model, as TREX2 deficiency led to a reduction of MC903-induced ear thickness increase, surrogate marker of inflammation. On the other hand, in the OX-induced model, we did observe a significant decrease in the thickness and layers of the epidermis in the absence of *Trex2* in a histological level, though it did not affect the overall thickness of the ear. In

fact, this lowest epidermal hyperplasia may be related to a reduced inflammation as well. Consistently, parallel RNA-seq transcriptomic studies conducted in the group have revealed that genes associated to AD inflammation and itching pathogenesis, including *Tnf- $\alpha$* , *Il-1 $\alpha$* , *Il-6*, *Il-23 $\alpha$* , *Il-19* and *Il-24* cytokines and *S100a8*, *S100a9* and *Lcn2* alarmins, were downregulated in the OX-treated skin in the absence of *Trex2* (unpublished data). Moreover, transcriptomic studies in the OX-induced model have unveiled a deregulation of genes related to skin differentiation, in agreement with major processes alterations (inflammation and skin differentiation) observed in transcriptomic studies from the PsO IMQ-treated skin in the absence of *Trex2* (Manils et al., 2015), although with a singular AD-related signature. Future assessment of TREX2-dependent effects in the MC903-induced AD model at the histological and transcriptomic level should contribute to elucidate the biological mechanisms and pathways underlying TREX2 role in AD.

Of note, OX- and MC-903 induced models of AD rely on the application of exogenous agents for the induction of an AD-like phenotype and the main difference among them is that the OX-induced model mimics a chronic phase of the disease (Man et al., 2008), being more inflammatory and severe, whilst MC903 resembles a less severe and more acute phase (Moosbrugger-Martinz et al., 2017). Maybe, TREX2 can cause a decrease in thickness in a less severe context. Interestingly, TREX2 deficiency indeed decreases pruritus, one of the most incapacitating aspects of AD (Moniaga et al., 2020) in both models, supporting a key role of TREX2 promoting AD-associated itch.

Moreover, the study of the eosinophil and mastocyte populations in OX-treated ears showed that the number of epidermal eosinophils was greatly reduced in *Trex2*<sup>-/-</sup> mice that may result from a selective diminished specific inflammatory response. Moreover, reduced intraepidermal eosinophil infiltration in *Trex2* deficient mice could be related to the decrease in itch in the absence of TREX2.

A significant decrease in scratching and IgE plasma levels, surrogate markers of itching, in *Trex2* deficient mice compared to wt mice has been observed in both the OX- and MC903-induced mouse models of AD, suggesting a key role of TREX2 promoting itch. IgE mediates the activation of mast cells and eosinophil cells, and ends up in the release of mediators such as histamine (Liu et al., 2011). So, if IgE plasma levels are decreased, probably scratching, that can be stimulated by histamine would be reduced as well (Fukasawa et al., 2022). IgE can also bind to its receptors in DCs, stimulate the uptake of

allergens and its presentation to T cells, which will end up in the activation of T cells (Liu et al., 2011). Factors and cytokines produced by T cells, such as IL-4 and IL-13, and mast cells stimulate the production, maturation and differentiation of eosinophils, that will secrete large numbers of cytokines and chemokines and exacerbate overall inflammation (Bradding et al., 1994; Simon et al., 2004). Therefore, with reduced number of IgE, these events would be ameliorated, shaping intraepidermal eosinophil infiltration. Now, the focus moves to IgE: why are they reduced in the absence of *Trex2* in the OX and MC903 models of AD? Presumably, after the application of OX and MC903, TREX2 might act as a promoter of the death of stressed KCs, as seen in UVB- and IMQ-treated skin (Manils et al., 2015, 2016), ending up in a release of KC alarmins that foster and modulate the inflammatory response axis, by stimulating DC, and cytokine release, which further shape T cell activation and differentiation, that drive B cell differentiation and IgE immunoglobulin class switching. Also, alterations in KCs might end up in problems in the skin barrier, which would translate as an easy entrance for molecules like OX and MC903. Moreover, a deficient skin barrier-function is associated to dryness in the skin, and it is dry skin that induces what is known as “intractable itch”, characteristic of AD lesions (Tominaga & Takamori, 2022). In the absence of TREX2, skin barrier might remain intact making the entrance of these exogenous agents more difficult and the overall response reduced. However, this is just a hypothesis and a deeper analysis into the skin barrier in a gene and protein level should be done in order to confirm what is really happening.

Dry skin itch is a common symptom in both AD and PsO, and it has been described to be not-histamine related, although it is still controversial. Instead, it is cytokines and chemical mediators released from the affected area who induce itch by acting on sensory nerves (Murota & Katayama, 2017). During inflammation, AD patients suffer what is known as skin hyperesthesia, which involves a hypersensitivity to stimuli, and is related to the itch-scratch cycle, in which every time the affected area is scratched, there is an aggravation of the situation (Ikoma et al., 2006). As mentioned above, TREX2 role inducing the death of stressed KCs and inducing the release of cytokines and chemokines could be related both to dry skin, by affecting the skin-barrier, and also to the induction of itch by the release of inflammatory mediators that act on sensory nerves, such as TSLP (Wilson et al., 2013). In fact, we have observed that the cytokine induced expression of *Tslp* is abrogated in *Trex2*<sup>-/-</sup> mice and decreased after TREX2 inhibition in a TREX2 dependent manner in mouse KCs treated with IL-17A and TNF- $\alpha$ , key cytokines in PsO

that are also involved in the late and chronic stages of AD (Figure 10). Therefore, TREX2 role on KCs can be related to the expression of *Tslp* and this can be associated to pruritus. Studies in AD models of KCs treated with IL-4 and IL-13 (Omori-Miyake et al., 2014), will be addressed in the near future to uncover the impact of TREX2 in the expression of *Tslp* and other alarmins, as well as the underlying signalling pathways.

Overall, data support a key role of TREX2 shaping AD-associated immune responses and itching processes and highlight TREX2 as a novel therapeutic target for AD.

## **5.2 Identification, development and characterisation of TREX2 inhibitors**

Here, we have identified, developed and characterized potent, selective, efficacious, and safe small molecule TREX2 inhibitors as novel therapeutics for a singular KC-based, non-immunosuppressive and skin-directed PsO and AD treatments.

The specificity and selectivity of the compounds was established by analysing the effect the compounds had on TREX1, the closest exonuclease to TREX2. TREX1 and TREX2 share a similar dimeric structure and 3'-5' exonuclease activity, but most of their known biological functions do not seem to overlap, as inferred from the unique phenotypes of TREX2 (Manils et al., 2015; Parra et al., 2009) and TREX1 deficient mice (Grieves et al., 2015; Morita et al., 2004). These differential functions may rely in part in their different cellular and subcellular localization as well as affinity and preference for different DNA substrate forms (Cheng et al., 2018; Mazur & Perrino, 2001a). In terms of cell expression, TREX1 is ubiquitously expressed in the cytoplasm of a plethora of cells (Chowdhury et al., 2006; Richards et al., 2007; Simpson et al., 2020), whilst TREX2 is only expressed on KCs and mainly in the nucleus (Manils et al., 2015; Parra et al., 2009). Regarding DNA substrate preference, both exonucleases prefer dsDNA with a 3' overhang, but TREX2 requires a longer double-stranded region than TREX1 (Cheng et al., 2018).

It is essential that the compounds selected are specific for TREX2 and do not influence TREX1 activity because in contrast to TREX2, that has a pro-inflammatory role in stressed KCs, TREX1 has a broad anti-inflammatory role. In particular, TREX1 plays a critical role in the degradation of cytoplasmic self- and non-self-DNA to prevent cGAS/STING activation and further inflammation and autoimmunity (Hemphill et al., 2021; Prabhu et

al., 2020). Exogen or endogen DNA in the cytoplasm can act as a damage signal activating the cGAS/STING pathway, which will induce the production of cytokines and type I IFN (Coquel et al., 2019; Vanpouille-Box et al., 2018). Mutations in the exonuclease domains (N-terminus region) of TREX1 lead to the development of Aicardi-Goutières syndrome and monogenic chilblain lupus, commonly present in patients that suffer SLE (Fye et al., 2011; Günther et al., 2009; Simpson et al., 2020). Mutations in its localization and oligosaccharyltransferase interaction domain leads to retinal vasculopathy with cerebral leukodystrophy (Crow et al., 2006; Lee-kirsch et al., 2007; Simpson et al., 2020; Yan, 2017). So, alterations in TREX1 induce inflammation and autoimmunity and therefore we aim to find compounds that act specifically of TREX2 without affecting TREX1 activity.

The druggable hotspot found in the TREX2 molecule was a cryptic allosteric binding site, which means it can potentially bind a drug-like molecule in a site that is different from the molecule's active site (Huang et al., 2017). In that site, the drug-like molecule will potentially regulate the function of the protein it is bound to (Nussinov & Tsai, 2014). Importantly, one of the main advantages allosteric binding present is that it allows to design drugs that are more specific and selective, as these sites are less conserved than active sites (Casadó-Anguera & Casadó, 2022). In this sense, the druggable hotspot identified in the TREX2 molecule does not share homology with TREX1.

Virtual screening computational chemical design and chemical synthesis resulted in a plethora of compounds that were tested using the *in vitro* exonuclease compound. This process aimed to optimize the molecules by improving their potency to uncover the structural parts necessary for TREX2 inhibition and to define the chemical space and establish a Markush formula for TREX2 inhibitors. As a result of this process, the 01-022 compound was unveiled as a compound with high *in vitro* inhibitory potency (1  $\mu$ M range) and selectivity over TREX1 (>100  $\mu$ M, at least two orders of magnitude).

Relative to the initial lead compound, 01-045, we have achieved an improved potency in the subsequent leads, 01-001 (from 10  $\mu$ M to 1 $\mu$ M, one order of magnitude), maintaining selectivity regarding TREX1. Nevertheless, the two lead compounds 01-001 and 01-022 have an IC<sub>50</sub> in the range of 1 $\mu$ M. During the optimization process, we have not been able to improve the potency of the compounds from low  $\mu$ M (0.8  $\mu$ M) to low nM, probably due to the allosteric nature of the inhibition. The reason why the 01-022 inhibitor was selected

as the current lead compound over the 01-001 compound was due to its improved PK and ADMET properties (not shown), suitable for a future therapeutic use. A panel of assays, including cytotoxicity (Table 5), genotoxicity, CYP P450 and safety screen (not shown), support absence of off-target toxicity in mice.

In terms of on-target safety, although TREX2 deficient mice showed an increased susceptibility to carcinogen-induced skin tumorigenesis, in the absence of DNA damage, TREX2 deficiency does not drive a tumour-prone phenotype (Manils et al., 2015; Parra et al., 2009). In this manner, it is expected that an allosteric inhibition, which does not imply an absolute and permanent inhibition of the function of the protein, will not lead to an increase in skin carcinogenesis. In fact, unfortunately, current available PsO treatments targeting the immune system also have an associated cancer risk as immunity is crucial to face most cancers.

Importantly, several target engagement approaches support TREX2 inhibitors-TREX2 interaction. Thus, TREX2 inhibitors have proved to biophysically interact with the TREX2 molecule as assessed by CETSA, MST and crystallisation. CETSA studies indicate that TREX2 inhibitors induce a destabilization of TREX2. Normally, ligand engagement induces the stabilization of a protein, but there are examples described in the bibliography in which ligands cause the destabilization of its target (Massey, 2018; Nagasawa et al., 2020). Interestingly, we did observe a correlation between the K<sub>d</sub> values obtained by means of MST and the IC<sub>50</sub> values from inhibition of TREX2 in the *in vitro* exonuclease activity assay. Those compounds that were more active in the exonuclease assay display the lowest K<sub>d</sub> in MST, thus a higher binding strength.

So far, the crystal structures with bound TREX2 inhibitor unveiling the binding of TREX2 inhibitors and TREX2 have not been obtained. However, crystal structures from soaking co-crystallization experiments with 01-025 TREX2 inhibitor have revealed the formation of the Cys65-Cys88 disulphide bond, leading to a conformational change in close proximity to the predicted binding site of the compounds. Of note, the two Cys are conserved in human and mouse isoforms. TREX2 inhibitor driven formation of the intrachain disulphide bond and subsequent change in protein conformation may render the protein inactive, uncovering the mechanism by which TREX2 inhibitors act on TREX2 exonuclease activity.

Consistently, DTT, a reductor agent that can preclude the formation of disulphide bonds, was found to prevent TREX2 inhibitors action. By adding DTT the sulphide bond cannot form upon TREX2 inhibitor interaction due to the reduction of two sulphur atoms of cysteine residues, and therefore the protein cannot be inhibited. On the contrary, without adding DTT, sulphur atoms of the two cysteines can form a disulphide bridge upon TREX2 inhibitor binding to TREX2 cavity and render the protein inactive. To further demonstrate the relevance of the formations of disulphide bond Cys65-Cys88 in the mechanism of action of TREX2 inhibitors single and dual mutagenesis of these two cysteine residues is being addressed to determine if the inhibition takes place because of the TREX2 inhibitor-driven formation of this disulphide bond or not.

In an *in vivo* scenario, the 01-022 compound has proved to be effective in models of PsO and AD. Animal models of PsO and AD resemble some aspects of the conditions, they activate relevant pathways of inflammation in the skin of mice that induce a similar phenotype and can be used to study the diseases (Singh et al., 2019; Wagner et al., 2010). Even though some of these models mimic very well the human phenotype, it is important to take into account that they are models, and therefore they don't fully cover the whole pathogenesis of the disease. In addition, it is important to mention that there are significant differences between mice and human, and therefore careful consideration should be taken when translating results from one to another.

The IL-23- and the IMQ-induced models of PsO were used in this work, the first to analyse the efficacy of TREX2 inhibitors in PsO, and the second to validate the effect observed in the first model in a more complex and severe context. These models have been widely used for the study of PsO pathogenesis and the assessment of new therapeutic options (Hedrick et al., 2009; Pan et al., 2021).

The 01-022 compound proved to significantly decrease inflammation in the IL-23-induced mouse model of PsO to levels of untreated ears, as observed by measuring ear thickness *in vivo* and also at a histological level, where the number of layers in the epidermis was reduced after application of the compound. Consistent with a 01-022-mediated decrease in PsO-associated inflammation, a significant downregulation in the *Il17a* and *Il23a* mRNA expression was observed in IL-23 treated ears. This suggests the 01-022 compound can decrease the inflammation by acting on the IL-23/IL-17 axis. The

expression of the other genes assessed, such as AMPs *S100a9* and *Defb4*, differentiation markers as *Krt10* and *Trex2*, *Trex1* and *Actb* was not significantly reduced by 01-022 in IL-23 treated ears. Of note, there was a tendency to a decrease in the expression of *S100a9* and *Defb4*, two genes found to be downregulated by 01-022 in IL-17A and TNF- $\alpha$ -treated mouse and human KCs, respectively, pointing out a KC cell autonomous effect of the TREX2 inhibitor. The relatively lower reduction of the expression of these AMP levels in IL-23 treated skin may be explained by the many cell sources of these proteins. It is worth mentioning that the IL-23 model of PsO is a very elegant method, only affecting one pathway of the many involved that does cause a severe increase in the inflammation. Then, the IL-23-induced PsO model does not mimic the overall complexity of a more severe and chronic phase of the disease pathogenesis.

In contrast, the IMQ model of PsO (van der Fits et al., 2009) provides a more inflammatory model and was used to validate our TREX2 inhibitor lead compound. In the present initial experiment, the administration of the 01-022 compound caused a significant reduction of IMQ-induced inflammation to levels of *Trex2*<sup>-/-</sup> mice, validating the efficacy observed in the IL-23 model of PsO in another and more inflammatory model of the disease.

To further determine the effect of TREX2 inhibition on KCs, where TREX2 is specifically expressed (Manils et al., 2015; Parra et al., 2009), we have used a cellular model of PsO induced by the application of IL-17A and TNF- $\alpha$  (Chiricozzi et al., 2011; Wang et al., 2013). In particular, TREX2 inhibition caused by 01-022 triggered a decrease of upregulated *LCN2*, *IL-36 $\gamma$*  and *DEFB4* in human KCs; and *Lcn2*, *Il-36 $\gamma$* , *S100a9*, *S100a8* and *Tslp* in murine KCs, in a TREX2-dependent manner. The differential signature in AMPs genes observed in human and mouse KCs may rely in differences in gene expression kinetics, KC differentiation stages and human and mouse KC differences. Additional time course experiments need to be done. Also, to circumvent differences between human and murine KCs, future studies will address deletion of *TREX2* in NHEK either using CRISPR/Cas9 approaches or by silencing the gene using a siRNA.

Noteworthy, TREX2 inhibition led to a decrease in the expression of *LCN2* and *IL-36 $\gamma$*  in both mouse and human KCs. *LCN2* is mainly produced by neutrophils and KCs in response to pathogens and inflammation (Wolk et al., 2017) and induces the chemotaxis and activation of neutrophils to perpetuate local inflammation in PsO (Shao et al., 2016). IL-

36γ contributes to KC hyperproliferation and the infiltration of cells from the immune system, like neutrophils. Also, by binding to its receptor it triggers the expression of pro-inflammatory genes (Bassoy et al., 2018; Fischer et al., 2023; Müller et al., 2018; Towne et al., 2004). So, TREX2 inhibition seems to decrease the activation and chemotaxis of neutrophils, among other cells from the immune system and therefore can lead to a decrease in the perpetuation of inflammation.

Also, the induction of AMPs *S100a9*, *S100a8* and *DEFB4* was downregulated by TREX2 inhibition, although *S100a9* and *S100a8* was only observed in mouse KCs, whereas *DEFB4* only in human KCs. Overall, we have observed a 01-022-driven decrease in the expression of several AMPs, *S100a9*, *S100a8* and *DEFB4*, all upregulated in PsO patients (Niyonsaba et al., 2005, 2007, 2009; Smithrithee et al., 2015, Broome et al., 2003; Mirmohammadsadegh et al., 2000). *S100A9* and *S100A8* form a complex known as calprotectin that is increased in active PsO and that seems to be released mainly by lesional KCs, although it can also be expressed by neutrophils and macrophages (Benoit et al., 2006; Mellor et al., 2022; Wang et al., 2018). *S100A9* can act by modulating the cytoskeleton of the cell and recruiting immune cells to inflammation sites and inducing pro-inflammatory cytokines (Vogl et al., 2018; Wang et al., 2018). Human β-defensin (hBD) 2 is encoded by *DEFB4* and its expression is inducible upon inflammation, injury or microorganism encounter (Bayer et al., 2016; Niyonsaba et al., 2006, 2009). It accounts with high antimicrobial capacity and pro-inflammatory functions, as they enhance the production of cytokines and chemokines involved in the pathogenesis of skin diseases (Niyonsaba et al., 2005, 2007, 2009; Smithrithee et al., 2015, Broome et al., 2003; Mirmohammadsadegh et al., 2000). Therefore, TREX2 role in KCs can be associated to the expression and release of AMPs, and thereby contribute to inflammation.

Finally, the expression of *Tslp* is reduced upon TREX2 inhibition in mouse KCs. Of note, consistently the mRNA expression of *Tslp* did not increase upon IL-17A and TNF-α stimulation in *Trex2*<sup>-/-</sup> KCs. TSLP is an allergic mediator that is upregulated in PsO and has a role inducing the proliferation of KCs (van de Kerkhof, 2022). TSLP relevance has been widely described in AD lesions, where it promotes pruritus through the stimulation of sensory neurons (Wilson et al., 2013). Also, it promotes the activation of DCs in AD, which primes a Th2 response (Soumelis et al., 2002). This, as mentioned above, could relate TREX2 to itch promotion in AD.

Basically, the majority of these proteins are related to the proliferation and differentiation of KC and to the innate immune response, mediating the activation of DCs and the recruitment of other immune cells, which ends up in the production of proinflammatory cytokines that keep feeding a self-amplifying inflammatory loop. The 01-022 TREX2 inhibitor can potentially attenuate the inflammatory loop that establishes in PsO lesions by decreasing these proteins, and this might be the explanation to the effects observed *in vivo*. By acting specifically in a KC level, we are observing a decrease in the expression of genes that are involved in the perpetuation of inflammation.

The fact that TREX2 inhibitors led to a reduction of KCs PsO related genes in KC cell cultures indicates a direct action of TREX2 inhibitors in KC, where TREX2 expression is restricted. Further studies are needed to fully characterize the biological mechanisms of TREX2 inhibitors reducing psoriatic and AD inflammatory phenotype in KC and in skin tissue shaping the immune response.

The mechanism by which TREX2 is able to decrease the expression of these genes is still unknown. However, ongoing studies from the group have revealed TREX2 could be involved in signalling pathways of the innate immunity, as TREX2 seems to interact with components of DNA detection signalling pathways (unpublished data). Also, previous studies have proved TREX2 interacts with  $\gamma$ H2AX, involved in DNA repair and cell death (Manils et al., 2015). Therefore, upon the detection of damaged DNA from stressed KCs, TREX2 may drive a change in the expression of genes related to inflammation through its interaction with other proteins.

The efficacy of TREX2 inhibitors was also evaluated in two models of AD: the OX- and the MC903-induced mouse models. The 01-022 compound proved to induce a decrease in AD-phenotype after topic administration, but not i.g., in a dose effective in PsO models, pointing out that probably the effective doses of 01-022 for AD and PsO are quite different and a larger dose of compound is needed to ameliorate AD. In fact, topic administration resulted in the delivery of the highest concentrations of compound to the skin compared with oral administrations, as established in the PK and ADMET studies, explaining therapeutic efficacy of TREX2 inhibitors administered topically to treat AD.

### **5.3 TREX2 inhibitors, a KC-based mechanism vs available immune-based therapies for PsO and AD**

Available PsO and AD therapies mainly target mediators of the immune system, and try to suppress the inflammation once it has already been activated and amplifying. At the moment, multiple biologics have shown a great success in the treatment of the conditions. However, there are challenges that encourage for more research in the field.

The blockage of the TREX2 exonuclease, a target only expressed in KCs, would primarily affect the skin, and this way it would avoid most of the adverse systemic consequences that current treatments targeting key immune system cytokines, pathways and cells have. Also, the importance of targeting KCs, key players in the pathogenesis of AD and PsO, as will be discussed below, would open a new door for another type of treatment not focusing on inflammation itself, but in the initial phases and the amplification systems involved in both diseases.

Research on the pathogenesis of PsO and AD has focused on the cytokines and DC and T cell subsets specifically driving both diseases. This knowledge has contributed to the identification of unique cytokines and mechanisms involved in the pathogenesis of the two chronic skin inflammatory diseases, uncovering targets for available treatment options, extensively described in the introduction of this thesis. However, KCs have been pushed aside diminishing the importance they have in the development of both conditions.

In PsO, it has been well established that KCs act in both the initial phase of the disease and its maintenance. Briefly, initially KC respond to triggers by releasing self-nucleotides and AMPs that promote the activation of pDCs, ending up in the activation of DCs, that start producing pro-inflammatory cytokines (Greb et al., 2016; Perera et al., 2012). Then, KCs act as inflammation amplifiers during the maintenance phase of the disease by proliferating and producing a myriad of chemokines that help recruiting leukocytes (neutrophils, Th17, DCs...), AMPs to generate an innate response and other inflammatory mediators that perpetuate the situation (Griffiths et al., 2021; Hawkes et al., 2018; Lowes et al., 2013; Nestle et al., 2009). Also, as mentioned in the Introduction, KC communicate with several types of cell types, including fibroblasts, endothelial cells and immune cells.

This, makes them a very valuable player in the pathogenesis of the disease, as its constant crosstalk with a variety of cell types helps inflammation maintenance, KC hyperproliferation, infiltration of leukocytes to the lesions (Hawkes et al., 2017; Lowes et al., 2013; Wang et al., 2021; Zhou et al., 2022)... Clearly, KCs seem to have an important and central role in the pathogenesis of PsO. Probably, by affecting KCs using TREX2 inhibitors, the self-amplifying inflammatory loop generated between the cells mentioned would attenuate overall inflammation. Taking into account results from the cellular model of PsO, we have observed a decrease of genes involved in the promotion of inflammation that are expressed by KCs, and therefore by acting directly on TREX2, and having an effect on KCs, the overall amplifying loop KCs take part in could be attenuated.

In AD, KCs have been regarded as effector cells that can trigger abnormal immune responses by producing inflammatory cytokines and chemokines that stimulate the infiltration of immune cells to the skin and end up in the progression of the condition (Albanesi et al., 2005; Das et al., 2022; Girolomoni & Pastore, 2001; Purwar et al., 2006). In contrast to PsO, KCs in AD do not express AMPs due to the inhibition of their expression by Th2 cytokines (Pfalzgraff et al., 2018). They highly express TSLP though, which can promote itching, dysregulates Th2 responses and increases the production of proinflammatory cytokines (Das et al., 2022). Of note, the disruption of the epidermal barrier is also one of the key factors in AD pathogenesis. This disruption ends up in the penetration of microbes and allergens into the epidermis that ends up activating KCs and initiating the inflammatory cascade (Kim et al., 2019). Therefore, KCs have a relevant role in the pathogenesis of AD as well. Differences in the AMPs expression could be the explanation to the events observed in *in vivo* models of AD, where a decrease in scratching is consistent in both models and could be explained by changes in the expression of TSLP, but not inflammation measured as ear thickness increase. This, could be related to the fact that AMPs are not upregulated in AD, and if TREX2 inhibition primarily acts through diminishing these, it could explain why its deficiency is not enough to ameliorate inflammation in a chronic stage of the disease.

Recently, AhR agonist Tapinarof has shown to be effective counteracting PsO and AD (Nogueira et al., 2022; Smith et al., 2017). AhR is a transcriptional factor expressed in KCs, among other cells present in the skin, and that activates the NRF-2 pathway and produce antioxidative enzymes that limit oxidative stress (Bissonnette et al., 2021; Furue et al.,

2019; Smith et al., 2017). It induces the production of loricrin and FLG, which are essential in skin barrier maintenance. Also, it regulates the differentiation of Th17 and Treg cells, downregulating IL-17 and ending up in a reduction of inflammation. Also, it has been suggested it might have some kind of antimicrobial effect on the microbiome of the skin that helps attenuating cytokine production, and therefore inflammation (Haarmann-Stemmann et al., 2021; Smith et al., 2017).

The fact that AhR is expressed in different skin cells and in KCs in particular (Furue et al., 2019), and that its agonist Tapinarof has an effect counteracting inflammation in both PsO and AD, highlights the potential of targeting KCs for treating these conditions.

Other treatment options for moderate to severe cases of PsO and AD range from systemic treatments such as methotrexate or cyclosporine, that induce generalized immunosuppression and have serious adverse reactions, to biologics targeting specific mediators of the immune system that are safer and more effective (Brownstone et al., 2021; Kim et al., 2023; Lee et al., 2020). However, the main problems of biologics are the non-responsiveness of some patients, their high cost and accessibility, though some biosimilars are being created to overcome this issue, and their administration route, as there are patients that prefer non-injectable treatments (Lee & Kim, 2023; Porter et al., 2022).

More recently, TYK2 inhibitors, have proved to be effective for the treatment of both conditions (Chovatiya & Paller, 2021; Nogueira et al., 2020). TYK2 inhibitors comprise a group of oral and topic treatments, and some of them are still in clinical trials (Chovatiya & Paller, 2021). Being small molecules, these treatments have some advantages over biologics, as their costs are significantly reduced (Nogueira et al., 2020). Basically, the inhibition of JAK family members suppress the activity of many cytokines involved in the pathogenesis of PsO and AD, and have also proved to be effective against some associated comorbidities like psoriatic arthritis (Nogueira et al., 2020).

Among all treatment options available for PsO and AD patients, there are still challenges like non-responsiveness, adverse effects and high costs. A deeper understanding on how both diseases work and their comorbidities, the discovery of biomarkers, personalised treatments and the possible combination of therapies depending on the patient's needs would imply an improvement in the quality of life of patients.

TREX2 inhibitors appear as a new potential treatment option, with a new target specifically expressed on KCs, main actors in the pathogenesis of both AD and PsO. By acting on KCs, responsible for the inflammatory loop established in the skin, it would be possible to act at an earlier phase of the disease and not arrive to high levels of inflammation, and this way potentially avoid future comorbidities caused by systemic inflammation, as previously suggested (Bieber, 2023).

In sum, pharmacological blockage of the TREX2 exonuclease, a key KC-specific player involved in the pathogenesis of PsO, using the first-in-class small molecule TREX2 inhibitors show promising results regarding potency, specificity and efficacy as novel therapeutics for PsO and AD (Figure 45). Importantly, significant reduction in key inflammatory mediators involved in the pathogenesis of PsO has been observed upon TREX2 inhibition. In addition, target engagement of TREX2 inhibitors is supported by CETSA, MST and crystallisation approaches.

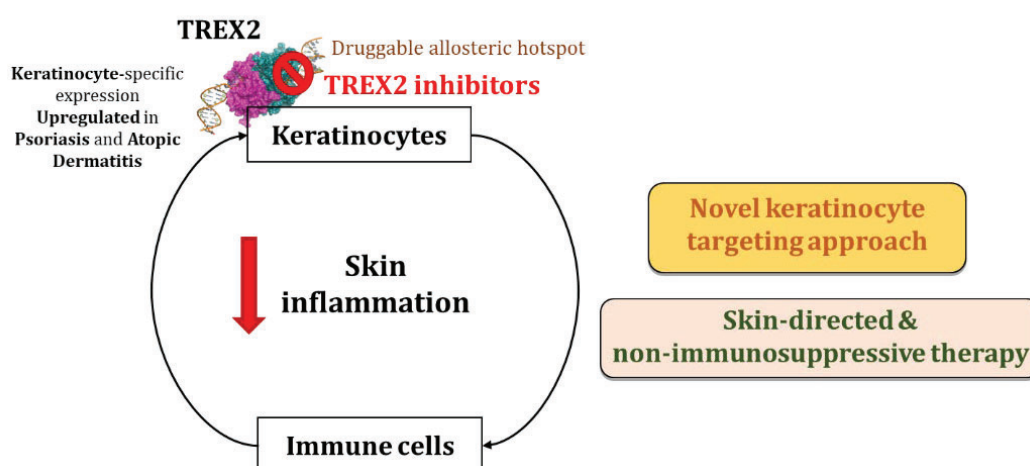


**Figure 45. TREX2 inhibitors as a novel KC-based therapy for PsO and AD.** The skin inflammatory loop involving KCs and immune cells crosstalk can be shut down by inhibition of TREX2 using TREX2 inhibitors, avoiding broad side effects such as immunosuppression, associated to treatments targeting the immune system.

# Conclusions



## 6. Conclusions

- TREX2 is upregulated in AD and its deficiency produces a decrease in itching and inflammation in mouse models of AD, highlighting TREX2 as a key player promoting AD pathogenesis and a new therapeutic target for AD.
- Identification, development and characterization of the first-in-class small molecule TREX2 inhibitors. The essential scaffold, the chemical space and the Markush formula have been defined, selecting 01-022 as the current lead compound.
- TREX2 inhibitors, in particular the lead compound, block potently and selectively TREX2 exonuclease *in vitro* over that of the related TREX1.
- TREX2 inhibitors display efficacy ameliorating the pathogenesis of PsO and AD in mouse models of these diseases in a TREX2-dependent manner, and show effectiveness by oral and topic administration.
- TREX2 inhibitor lead 01-022 reduces the expression of key genes involved in skin inflammation in human and mouse KCs in a TREX2-dependent manner, indicating a KC cell-autonomous and on-target-specific action.
- The interaction of TREX2 inhibitors with TREX2 has been demonstrated by means of the CETSA and MST biophysical approaches. Moreover, crystallisation studies have revealed the TREX2 inhibitor-driven formation of the disulphide Cys65-Cys88 bond leading to structural conformational changes, gaining insight into the possible molecular mechanisms of TREX2 inhibitors.

In sum, we have uncovered the first-in-class small molecule TREX2 inhibitors as novel therapeutics to treat PsO and AD, with a breakthrough KC-based mechanism of action for new, efficacious and more specific therapies for these diseases.



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