Neutralizing IL-22RA1 improves histological and molecular alterations associated with atopic dermatitis pathogenesis

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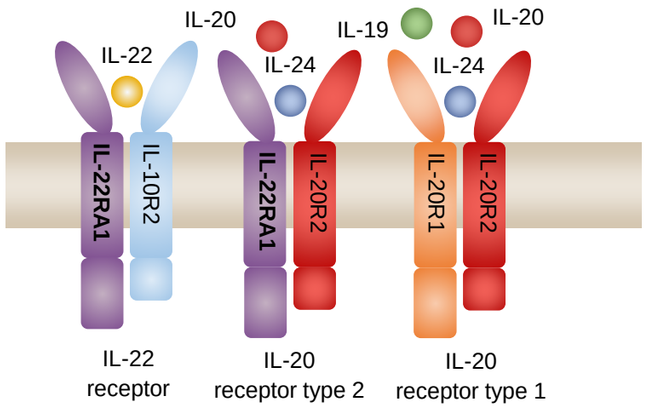
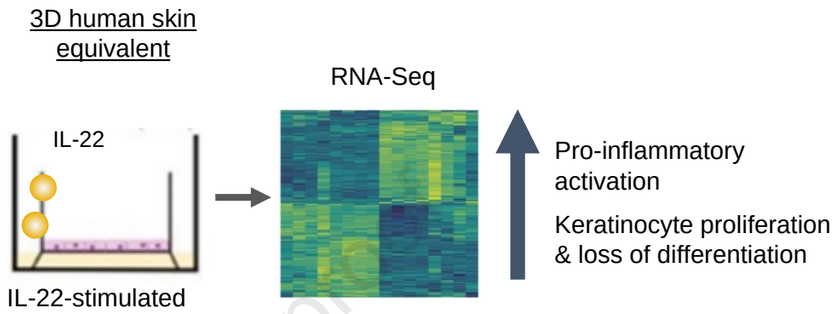
Neut[illegible] and molecular alterations associated with atopic dermatitis pathogenesis

Wasserer et al., J Allergy Clin Immunol (2025)

Background: IL-22RA1



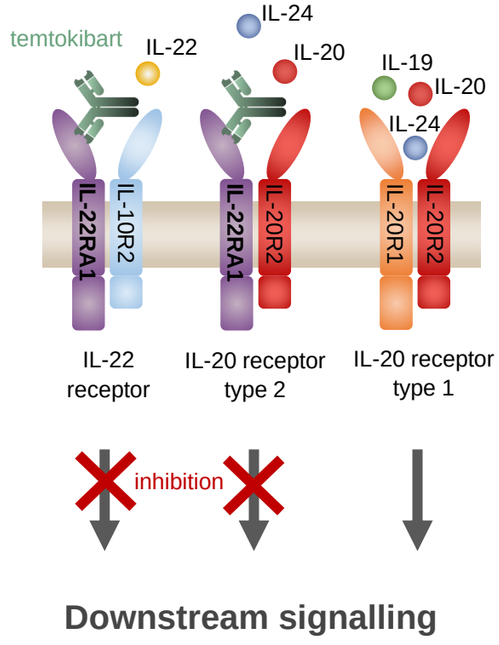
IL-22 stimulation in healthy skin models



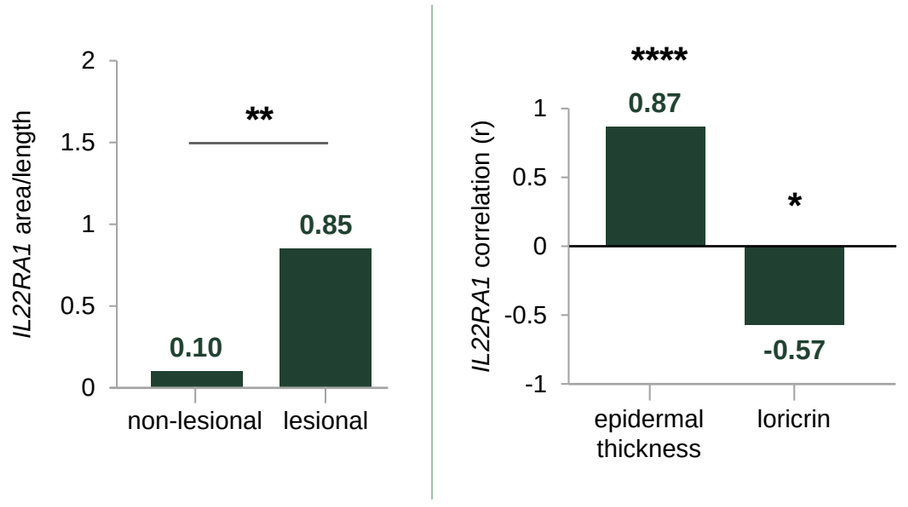
Conclusion

IL-22/IL-22RA1 axis functionally contributes to AD pathogenesis:

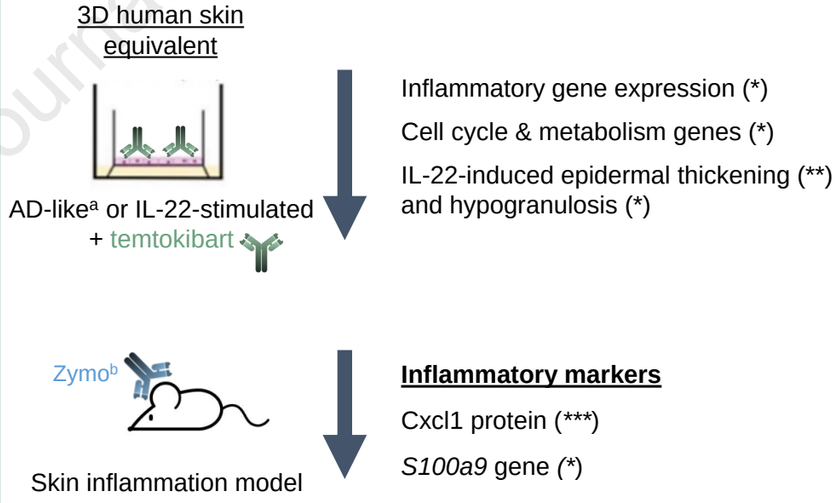
- Increased immune activation
- Keratinocyte proliferation
- Epidermal thickening
- Altered cell cycle & metabolism



IL-22RA1 expression in lesional AD skin



IL-22RA1 inhibition in AD models



(*) *P*<0.05, (**) *P*<0.01, (***) *P*<0.001, (****) *P*<0.0001. [a]AD-like skin models were a 3D skin equivalent stimulated with T-cell supernatant isolated from human AD lesional skin. [b]Zymo is a surrogate murine monoclonal antibody for temtokibart.

AD, atopic dermatitis; IL, interleukin; R1, receptor alpha subunit; R2, receptor beta subunit; RA1; receptor alpha subunit 1; RNA-Seq, RNA sequencing; TSN, T-cell supernatant.

Title: Neutralizing IL-22RA1 improves histological and molecular alterations associated with atopic dermatitis pathogenesis

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Abstract

BACKGROUND: A subgroup of patients with atopic dermatitis (AD) do not show sufficient improvement with current systemic therapies, highlighting the heterogeneity of the chronic inflammatory skin disease and the need for novel treatments.

OBJECTIVES: In this study, we investigated the pathogenic contribution of the IL-22/IL-22 receptor (IL-22RA1) axis to AD skin inflammation in in vitro, ex vivo, and in vivo models to evaluate the therapeutic potential of blocking this axis.

METHODS: IL-22RA1 expression in AD skin was assessed by *in situ* hybridization. Inhibition of the IL-22/IL-22R signaling cascade was evaluated in a human AD *in vitro* model (3D skin equivalents) and a TPA mouse model using temtokibart, a humanized antibody directed against IL-22RA1.

RESULTS: *IL22RA1* was highly expressed in the epidermis of lesional AD skin versus non-lesional control skin; expression correlated positively with epidermal thickness and negatively with the barrier integrity marker loricrin. IL-22 stimulation in 3D skin equivalents induced a specific molecular signature associated with lack of terminal differentiation, altered lipid metabolism, and increased immune response. Inhibition of IL-22RA1 with temtokibart showed significant improvements in skin barrier integrity at the histological and molecular levels. IL-22RA1 inhibition in a skin inflammation mouse model with Zymo, a surrogate murine anti-IL-22RA1 monoclonal antibody for temtokibart, reduced local expression of Cxcl1 and *S100a9*.

CONCLUSIONS: These findings suggest that the IL-22/IL-22RA1 axis functionally contributes to AD pathogenesis. Thus, blocking IL-22RA1 represents a potentially valuable new therapeutic option.

Clinical Implications: IL-22/IL-22RA1 contributes to AD pathogenesis. IL-22 inhibition improves histological and molecular changes in AD and represents a potential new therapeutic option.

Capsule Summary: IL-22RA1 inhibition improves histological and molecular alterations associated with the pathogenesis of atopic dermatitis and represents a potential new therapeutic option.

Keywords: atopic dermatitis; IL-22; IL-22RA1; skin inflammation, temtokibart; treatment

Abbreviations:

AD, atopic dermatitis

DEG, differentially expressed genes

EASI, Eczema Area and Severity Index

ELISA, enzyme-linked immunosorbent assay

HE, hematoxylin and eosin

IFN, interferon

IHC, immunohistochemistry

IL, interleukin

IL-22R, Interleukin-22 receptor

ILC3, type 3 innate lymphoid

IPA, ingenuity pathway

68 ISH, *in situ* hybridization

69 JAK, Janus kinase

70 MT, Masson's trichrome

71 PCR, Polymerase chain reaction

72 SEM, standard error of mean

73 Th, T helper

74 TSLP, thymic stromal lymphopoietin

75 TSN, T-cell supernatant

76

INTRODUCTION

Atopic dermatitis (AD) is a common, highly heterogeneous, type 2 inflammatory skin disease characterized by a large variety of disease endotypes.[1–3] The key driver of AD pathogenesis is an exaggerated T helper (Th) 2 immune response with the related cytokines interleukin (IL)-4 and IL-13. However, numerous other type 2 cytokines and molecules (including IL-22, IL-33, IL-36, IL-5, IL-1a, thymic stromal lymphopoietin [TSLP], and immunoglobulin E) contribute to AD pathogenesis and account for the heterogeneity amongst patients with AD.[1,4]Long-term systemic therapies for AD include cyclosporine, biologics (dupilumab, tralokinumab, lebrikizumab), and Janus kinase (JAK) inhibitors.[3] Cyclosporine was the first drug approved for AD; however, its long-term use is associated with some unfavorable side effects.[5] Dupilumab targets IL-4Ra, tralokinumab and lebrikizumab target IL-13, and JAK inhibitors block downstream signaling of multiple AD-relevant cytokines including IL-4, IL-13, IL-5, and IL-31, among others.[1,6,7] Despite up to three-quarters of patients with AD achieving 75% reduction from baseline in the Eczema Area and Severity Index on these therapies, up to ~25% of patients may not achieve this clinical endpoint, underlining the heterogeneity of the disease and the need for additional treatment options.[8–16]

IL-22 is a member of the IL-10 family of cytokines and is mainly produced by Th22 and type 3 innate lymphoid (ILC3) cells.[17] In the skin, IL-22 enhances the production of pro-inflammatory cytokines, chemokines, antimicrobial peptides, and acute phase proteins.[18–20] It also induces increased proliferation of keratinocytes while inhibiting their terminal differentiation process, causing downregulation of essential barrier proteins such as filaggrin and loricrin, thus leading to a compromised skin barrier integrity.[19,21–25] IL-22 levels are elevated in both serum and lesional skin in patients with AD and serum levels correlate with disease severity.[26–31] IL-22 binds with high affinity to the IL-22 receptor (IL-22R), which consists of a specific IL-22RA1 subunit and the IL-10R2 subunit.[32–34] While IL-10R2 is widely expressed, IL-22RA1 is primarily

expressed by epithelial cells in various tissues, with highest expression in the pancreas, skin, and gastrointestinal tract.[35] IL-22RA1 additionally can form heterodimers with IL-20R2, forming the IL-20 type 2 receptor, allowing the signaling of the other IL-10 family members IL-20 and IL-24 that further supports skin barrier impairment and have been associated with itch.[36] [17] However, IL-20 and IL-24 can also signal through the IL-20 type 1 receptor, independent of the IL-22RA1 subunit.[37,38]

IL-22 as a therapeutic target has been investigated in a phase 2a trial with a monoclonal antibody targeting IL-22 (fezakinumab); while the primary endpoint was not met in the overall study population, a subgroup of patients with high lesional IL-22 levels at baseline showed significant clinical and molecular improvement by IL-22 blockade, further underlining the pathologic contribution of IL-22.[39,40] In this study, we investigated the pathogenic interplay of immune-derived IL-22 and epithelial-based IL-22RA1 in AD skin inflammation in in vitro, ex vivo, and in vivo models to evaluate the therapeutic potential of blocking this axis. We considered that targeting IL-22RA1 may be more efficacious for AD treatment than blocking IL-22 alone, as it would additionally block signaling of IL-20 and IL-24 through the IL-20 type 2 receptor, which may contribute to disease pathology. [36]

MATERIALS AND METHODS

Detailed information on materials and methods is provided in the Supplementary Materials section.

Study Cohorts and Ethics Compliance

All skin materials were sampled in accordance with national legislation in the country of origin and the Declaration of Helsinki protocol after written informed consent. Tissue samples for the generation of 3D skin equivalents and TSN, cell isolation, histological analyses, and other *in vitro* assessments were acquired from healthy donors and patients with AD in accordance with the local ethics committee at the Klinikum Rechts der Isar, Technical University Munich (5590/12, 2773/10). Anonymized human skin samples from healthy donors used for ex vivo assessments were obtained following reduction surgery of abdomen or breast through Biopredic International (cat# PEA089, France).

Summary of the study design

To characterize the spatial expression of IL22RA1 and IL22 in AD epidermis, lesional AD and non-lesional control skin sections were analyzed by ISH, with further immunohistochemistry (IHC) to detect DEFB4, loricrin, and T cells, along with epidermal thickness measurements via Masson's trichrome (MT) staining. To investigate IL-22RA1 function, 3D skin equivalents were created using primary human keratinocytes. These models were stimulated with recombinant IL-22 or T-cell supernatants (TSN) in the presence or absence of the anti-IL-22RA1 inhibitor, and analyzed through RNA sequencing to determine IL-22's molecular role in AD. The effects of IL-22RA1 inhibition were also tested in an in vivo mouse model using an anti-IL-22RA1 antibody (Supplementary Materials).

Statistical analyses

Data derived from ISH, FACS analysis, ELISA results qPCR, and HE stainings were analyzed using GraphPad Prism 9 software and visualized as mean ± standard error of mean (SEM). Comparison of disease or treatment groups was performed as indicated in figure legends. Significance level was defined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****). For the transcriptome analyses, a log2 fold-change of 1 and a *P*-value of $P < 0.05$ was used as cut-off for DEGs. Q (false-discovery rate) was used to adjust for multiple testing. Ingenuity Pathway Analysis (IPA) was performed for the IL-22 molecular signature and DEGs were annotated using the STRING database (https://string-db.org/).[83,84] All networks were visualized in Cytoscape (https://cytoscape.org/).[85]

RESULTS

IL22RA1 expression is elevated in lesional AD skin and correlates with markers for epidermal barrier integrity

In lesional AD skin, IL22RA1 was exclusively expressed in epithelial cells, whereas its binding partners, IL10RB and IL20RB showed an additional expression across various other cell types including immune cells (Fig.1A, Fig. S1A). In keratinocytes *IL22RA1* was preferentially expressed in differentiated keratinocytes, in vivo and in 3D skin equivalents (**Fig. 1B, G**). Within the keratinocyte compartment, *IL22RA1* was co-expressed with the IL10RB chain to bind IL-22 and to a higher degree with the ubiquitously expressed IL20RB chain to bind IL-20 or IL-24 (**Fig. 1A, C).** However, these data might be biased by the outermost cells in the epidermis (expressing the highest levels of IL22RA1, 1B+D S1B) being the most difficult to separate and include in single cel suspensions, and hence they might have been excluded from this dataset. In situ hybridization revealed a significantly higher expression of *IL22RA1*(P=0.0097) in the entire epidermis in lesional compared to non-lesional skin (**Fig.1D, E, Fig. S1B**) and its

expression positively correlated with epidermal thickness (r=0.87; *P*<0.0001), downstream expression of the proinflammatory antimicrobial peptide *DEFB4* (r=0.64; *P*=0.006), and negatively with loricrin coverage (r=-0.57; *P*=0.016) as a marker of barrier integrity (**Fig.1F**).

In line with the elevated *IL22RA1* expression, its ligands *IL22*, *IL20* and *IL24* were significantly increased in AD lesions (**Fig. 1H, Fig. S1C**). Here, IL20 and IL24 were mainly expressed in the epithelial compartment (keratinocytes and fibroblasts) with expression of *IL24* in all keratinocyte subpopulations and a pronounced expression of *IL20* in undifferentiated and proliferating cells (**Fig.1H, I**). In contrast, *IL22* was exclusively expressed in immune cells (T cells and ILCs) (**Fig.1H, J**). Digestion of human AD biopsies further revealed that 1.63% of all skin cells expressed IL-22, with $CD3^+CD4^+$ T cells being the dominant producer (85.45% ±4.88 $CD4^+$ vs 8.94% ±4.93 $CD8^+$, *P*=0.031) (**Fig.1K, L**). This is also in alignment with the IL22 ISH stainings (Fig S1C), showing that IL22 is produced by only a few single cells in lesional AD skin, however, quantitative IL-22 expression in these cells seems very high. Additionally, immune cells that produce IL22 seem to migrate into the epidermis so that IL22 is produced in close proximity to the IL-22 receptor).

Identifying the molecular signature of IL-22/IL-22RA1 in a human *in vitro* model of AD

Interaction of immune cells, especially T cells, with epithelial cells has been shown to be crucial for AD pathogenesis. To further investigate the contribution of the IL-22/IL22RA1 axis to disease pathology, 3D skin equivalents were generated using primary human keratinocytes from healthy donors (n = 8)).

that were stimulated with either recombinant IL-22 alone (n = 8) or lesional AD TSN (n=5; **Fig.2A**) to mimic AD inflammation in vitro. TSN were generated from activated T cells isolated from AD lesional skin biopsies and contained a complex mixture of AD-relevant cytokines including e.g. IL-4, IL-13 and IL-22 (**Fig.S2, TableS1**). Compared with unstimulated 3D skin equivalents (control), stimulation with IL-22 alone differentially regulated 266 genes (142 up-regulated and 124 down-regulated vs control) that are associated with pathways indicating an increased chemotaxis of immune cells, keratinocyte proliferation and differentiation, and antimicrobial defense (Fig.S3). Consistent with this, pathways such as *defense response against bacteria* and *inflammatory response* were activated and pathways as skin development suppressed (**Fig.S3, TableS2, TableS3**). The TSN, with its more complex AD-relevant cytokine composition, regulated more genes (687 differentially expressed genes [DEG], 474 up-/213 down-regulated vs control). In total, 89 genes overlapped with the IL-22 DEG list, signifying IL-22–specific genes (**Fig.2B**). Further annotation of these 89 genes using the STRING database and classification into eight categories revealed that most of these genes fulfill functions in forming the epithelial barrier (33%), play a role in metabolism (20%) or the immune response (16%), or possess enzymatic activity (10%) (**Fig.2C**).

In summary, IL-22 induced a unique molecular signature in epithelial cells leading to deregulation of the epidermal barrier and enhanced inflammation as well as antimicrobial defense.

Blocking IL-22RA1 efficiently inhibits the production of chemo-attractant and antimicrobial peptide molecules in vitro and ex vivo

To investigate the therapeutic potential of neutralizing the IL-22/IL-22RA1 axis, a fully humanized antibody inhibiting signaling via the IL-22RA1 was identified (temtokibart). The inhibitory capacity of temtokibart was determined by incubating primary keratinocytes with

211 temtokibart concentrations of 0.001–300 nM prior to activation with an AD cytokine mixture. The
212 secretion of the chemo-attractant CCL2 was dose-dependently inhibited, with full inhibition of
213 the IL-22 signal at temtokibart concentrations of 1–10 nM (**Fig.3A**). As *IL22RA1* and DEFB4
214 expression correlated significantly in lesional AD skin (Fig.1F), skin biopsies from healthy
215 donors were preincubated with temtokibart in doses ranging from 0.1–1000 nM or motavizumab
216 as isotype control ex vivo prior to IL-22 stimulation leading to an efficient reduction of *DEFB4*
217 expression after 24-hours at a concentration of 10nM (**Fig.3B**). An EC50 value of 3.7 nM was
218 estimated for temtokibart and full Emax was achieved at antibody concentrations approaching
219 300 nM. Furthermore, a temtokibart concentration of 300 nM significantly inhibited the TSN-
220 induced expression of *DEFB4* in a 3D skin equivalent (P=0.0105; **Fig.3C**). Further details
221 regarding the affinity determination of temtokibart can be found in **TableS5**.

222 Blocking IL-22RA1 with temtokibart efficiently inhibited IL-22–induced increases in the
223 chemo-attractant cytokine CCL2 and antimicrobial peptide DEFB4, both AD disease-related
224 proteins, ex vivo and in vitro.

225 **IL-22RA1 inhibition restores cell cycle and metabolism in AD inflammation**

226 To identify the transcriptomic alterations induced by IL-22RA1 inhibition, RNA
227 sequencing of TSN-stimulated 3D skin equivalents (n=5) with or without pre-incubation with
228 temtokibart 300 nM was performed. 468 DEGs were significantly (P<0.05) regulated by
229 temtokibart inhibition, of which 428 genes could be distinctly mapped in a STRING database
230 protein-protein interaction analysis (**TableS6**, **TableS7**). Markov clustering (MCL) of the 428
231 genes identified 74 clusters. 289 DEGs could be displayed in a network as only nodes (**Fig.4A**).
232 Functional enrichment analysis (based on KEGG, Gene Ontology, Reactome,
233 COMPARTMENTS, STRING subgroups) [83,84] for the largest five MCL clusters revealed an
234 impact of temtokibart on cell cycle and metabolic activity (**Fig.4B-F**).

Specifically, cluster 1 included genes and pathways linked to ubiquitination, a process of post-translation protein modification important for a large spectrum of cell metabolism including cell growth, inflammation, and cell differentiation (**Fig.4B**, **TableS8**). Cluster 1 was characterized by TRIM family proteins that mark proteins for degradation by the proteasome and regulate apoptosis. [41] Cluster 2 and cluster 4 were related to RNA synthesis and RNA metabolism, respectively (**Fig.4C, E, TableS8**), highlighting the restructuring of the epidermal barrier at the gene expression level. Cluster 3 included genes and pathways involved in cell cycle mechanisms and chromatin condensation (**Fig.4D**, **TableS8**) reflecting the potential of temtokibart to reduce IL-22 mediated hyperproliferation. Finally, cluster 5 united genes and pathways related to protein translation (**Fig.4F**, **TableS8**). Similar results were obtained with a GO term analysis that highlighted effects on cell cycle progression, RNA metabolism and cell trafficking (**Fig.S4 A, B, TableS9**).

In line with the unsupervised MCL method and subsequent pathway analysis, among the top 100 temtokibart up- and down-regulated DEGs were genes involved in epidermal barrier formation and inflammation (**Fig.4G, H** and **TableS4**). IL-22RA1 inhibition with temtokibart significantly induced expression of *DLX1* and *IL37* (both $P<0.05$) and thereby enhanced the suppression of inflammatory cytokine signaling. It further fostered lipid metabolism by enhanced expression of *ELOVL6* and *ETNK1* (both $P<0.05$) and restored epidermal integrity by increasing Loricrin expression. No effect on regulation of keratins was observed (**Fig.S4C**). In contrast, temtokibart inhibited extracellular matrix remodeling by reduced expression of *PRSS53* and *MMP3* (both $P<0.05$) and epidermal growth by downregulation of *TUBB3* and *EPGN (P*<0.05 and *P* 0.01, respectively). In addition, a well-known IL-22 induced antimicrobial peptides, *S100A7A*, *S100A9* and *DEFB4* were reduced, highlighting the broad role of IL-22RA1 blockade in epidermal barrier integrity.

Taken together, transcriptome analysis of temtokibart-treated 3D skin equivalents demonstrated effects of IL-22RA1 inhibition on cell cycle, metabolism, and epidermal barrier integrity, and thus may improve skin architecture in AD skin lesions.

Blocking IL-22RA1 function in 3D skin equivalents restores epidermal barrier integrity

To understand the impact of blocking IL-22RA1 on histological hallmarks of AD, 3D skin equivalents (n=3) stimulated with recombinant IL-22 or TSN with or without preincubation with temtokibart 300 nM were subjected to HE staining and analyzed for epidermal thickness (acanthosis) and granulosis. IL-22 stimulation significantly increased acanthosis compared with unstimulated controls (P=0.001) (**Fig.5A**). Blocking IL-22RA1 with temtokibart significantly inhibited this increase (P=0.0034) (**Fig.5A**). In addition, IL-22 stimulation significantly reduced the amount of keratohyalin granulae (hypogranulosis) compared with control (P=0.031). This decrease was also significantly inhibited by temtokibart (P=0.0313) (**Fig.5B**).

Similar to IL-22 stimulation, IL-22–containing TSN also induced epidermal thickening (p = 0.009) (**Fig.5C**) and hypogranulosis (P=0.031) (**Fig.5D**). However, the TSN-stimulated equivalents presented with significantly less epidermal thickness compared with IL-22–stimulated equivalents and, histologically, resembled spongiosis (defined as intercellular fluid, **Fig.5C**) rather than hypertrophy or hyperproliferation of keratinocytes (**Fig.5A**). The hypogranulosis was significantly inhibited by temtokibart (P = 0.031) (**Fig.5D**).

In addition, we could show that IL-22 mediates the secretion of IL-20 by fully confluent primary human keratinocytes and that this effect could be significantly blocked by temtokibart highlighting a shutdown of a potential self-amplifying circle. IL-24 production, in contrast, was only marginally induced by IL-22 and not affected by temtokibart treatment, however this effect might be explained by the stucturaly instability of IL-24 [37,42] (**Fig.5E**).

In summary, IL-22RA1 inhibition with temtokibart can efficiently prevent IL-22–mediated acanthosis,hypogranulosis and secretion of IL-20.

IL-22RA1 inhibition impacts skin inflammation in vivo

To verify the in vitro results in an in vivo model, the effects of blocking IL-22RA1 were investigated using Zymo, a surrogate murine anti-IL-22RA1 monoclonal antibody for temtokibart, in a mouse model of local skin inflammation induced by topical application of 20 µL 0.01% phorbol 12-myristate 13-acetate (TPA) (**Fig.6A**). Local protein expression of Il-22 and Cxcl1, and mRNA expression of *S100a9*, were assessed. In mouse ear tissue topically stimulated with TPA, significantly higher protein levels of inflammatory marker Cxcl1 were observed in comparison to the control (P<0.0001) (**Fig.6B**). This effect was reversed by inhibition of IL-22RA1 with Zymo (P<0.0001) (**Fig.6B**). A significant increase of both Cxcl1 and Il-22 protein levels in ear tissue was observed following application of TPA (both P<0.001; **Fig.6C, D**). Cxcl1 was reduced upon IL-22RA1 inhibition (P=0.0005; **Fig.6C**), whereas IL-22 was not affected (P=0.07; **Fig.6D**). Gene expression levels of inflammatory marker *S100a9,* which are elevated in AD during the transition from non-lesional to lesional skin (*40,41,43*), were highly upregulated in TPA-stimulated ear tissue and significantly downregulated by IL-22RA1 blockade (P=0.0021; **Fig. 6E**).

In summary, in vivo data show that IL-22RA1 blockade can reduce key proinflammatory proteins in a murine skin inflammation model.

DISCUSSION

Increased knowledge of AD pathophysiology has enabled the development of specific biologics targeting the IL-4/IL-13 pathways and small molecules inhibiting JAKs. However, some patients respond insufficiently, and additional treatment options are needed. [43–46] In this work, we analyzed the contribution of IL-22RA1 to skin inflammation using in vitro, ex vivo, and in vivo models and highlight the potential of targeting the IL-22/IL-22RA1 axis in AD.

For AD, several studies have demonstrated elevated serum IL-22 levels in patients with AD compared with healthy individuals, and high levels of IL-22 and Th22 cells were observed in lesional AD skin from adults.[26–31] Additionally, IL-24 and IL-20, members of the IL10 familiy, have been shown to be upregulated in lesional AD skin[36,47] and both mediate its signal either via IL22RA1/IL20RB, or IL20RA/IL20RB. In this study, we now add further information on an increased expression of *IL-22RA1* in AD epidermis, detected dominantly in the proliferating and differentiated kerationcytes-compared with non-lesional controls, which has only previously been demonstrated in lesional skin of patients with psoriasis.[48] Our correlation analyses further showed a positive correlation between *IL-22RA1* expression and epidermal thickness and the proinflammatory antimicrobial peptide *DEFB4*, respectively. This is in line with previous findings showing that IL-22 increases epidermal thickness in reconstituted human epidermis[19,20], and induces expression of *DEFB4* in keratinocytes.[35,49] In addition, we observed a negative correlation between *IL-22RA1* expression and loricrin, a barrier integrity molecule. The impairment of the epidermal barrier and differentiation of keratinocytes is a hallmark of AD and others have shown that IL-22 downregulates loricrin as well as other important barrier and differentiation molecules like profilaggrin.[19,50–52] Interestingly, the fact that IL-22RA1 expression is significantly increased in the skin during AD pathology and is highly co-expressed not only with the IL10RB subunit, but also with the IL20 receptor IL-20RB subunit, seems to further amplify the local inflammation already fueled by the increased IL-22 expression and the

redundant IL-20 and IL-24 cytokines. Altogether, these findings underline the negative impact of IL-22, IL-20 and IL-24 on the skin barrier, implying that the increased chronic presence of the IL-22 receptor in AD lesional skin is disadvantageous.

To investigate the pathophysiological role of the IL-22/IL-22RA1 axis in AD, we developed an in vitro model using 3D skin equivalents stimulated with T-cell supernatants (TSN) containing various AD-relevant cytokines, including IL-22. Stimulating these models with IL-22 alone confirmed its known functions, such as inducing chemokines, antimicrobial peptides, and dedifferentiation of the epidermal barrier, characterized by hyperkeratosis, hyperproliferation, and hypogranulosis.[18,20,35,52–54] Comparing the molecular signatures of IL-22 and TSN revealed that 33% of IL-22-specific genes were present in the TSN environment, with 95.5% regulated similarly. This underscores the suitability of our AD model to investigate IL-22 and IL-22RA1 in a context that closely resembles in vivo conditions.

For this purpose, an IL-22RA1-inhibiting antibody temtokibart and a surrogate murine anti-IL-22RA1 monoclonal antibody for temtokibart (Zymo) were developed and used for further in vitro and in vivo studies, respectively. This antibody not only fully blocks the activity of IL-22, but also of the IL-22RA1-dependent part of the IL-20 and IL-24 signaling. Therefore, we showed that temtokibart prevents epidermal abnormalities induced by IL-22, such as acanthosis, hypogranulosis, cell cycle activity, and alterations in metabolism in vitro and ameliorates inflammation in vivo in a murine model of skin inflammation. Specifically, IL-22 as well as IL-22–containing TSN induced epidermal thickening and hypogranulosis of 3D skin equivalents that was prevented by temtokibart, except for TSN-induced acanthosis. However, the TSN-stimulated equivalents presented with significantly less epidermal thickness compared with IL-22–stimulated equivalents which, histologically, resembled spongiosis rather than hypertrophy or

hyperproliferation. As TSN contains both IL-4 and IL-13, which have been previously described to induce spongiosis, as well as IFN-γ, which mediates apoptosis by Fas-FasL interaction[55–59] and TNF, which induces spongiotic dermatitis[60], the observed morphological differences might be due to the heterogenous cytokines in the supernatant that interact with IL-22.

The observed histological effects of IL-22 on barrier integrity were confirmed at the molecular level as well. Genes associated with tissue remodeling such as the matrix metalloprotease MMP3, the epidermal growth factor EPGN, the calcium binding protein, and anti-microbial peptides S100A7A, S100A9, the serine protease PRSS53 and CXCL1 were downregulated upon IL-22RA inhibition.[61–64] In contrast, genes associated with lipid metabolism such as the fatty acid elongase ELOVL6, and the ethanolamine kinase ETNK1, as well as genes associated with anti-inflammatory capacity such as the transcriptional regulator of TGFb family members DLX1 and the anti-inflammatory cytokine IL-37 were induced.[65–68] These results highlight the ability of temtokibart to restore barrier integrity and reduce inflammation in a complex cytokine environment. Additionally, Temtokibart also suppresses the IL-22 induced production of IL20 and IL-24, cytokines with overlapping functions that might be capable of compensating IL-22 activity.[20]

Despite the limitations of our in vitro study, such as the heterogeneity of keratinocyte donors and small sample sizes, our in vitro data were supported by in vivo studies demonstrating inhibition of Cxcl1 and *S100a9* expression by IL-22RA1 blockade with Zymo in a murine inflammatory skin model. Both inflammatory markers are known to be induced in lesional AD skin and possess antimicrobial activity and the ability to recruit immune cells into the tissue[69–74] and were also upregulated molecules in common IL-22/TSN signature in the in vitro study. S100A9 has been shown to be highly elevated in the transition phase from non-lesional to lesional skin, functioning as an alarmin and underlining its potential role in initiation of skin inflammation.[50,75]

Considering that AD is a very heterogeneous disease with a broad variety of subtypes, it is of high interest to identify new treatments with different mode of actions. In this manuscript, we present in vitro and in vivo data supporting IL-22RA1 as a new promising target for treatment of a skin barrier disrupted by high levels of IL-22, differentiating from the currently approved therapies.[1,71,76,86] Indeed, temtokibart has shown promising results in patients with moderate to severe AD in a phase 2a clinical trial[77], and a phase 2b study is ongoing.

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Conflict of interest

Sophia Wasserer received speaker's and/or travel fees from Janssen-Cilag Pharma, Novartis Pharma, Lilly, Almirall, BMS and Sanofi. **Britta C Martel** is employee, and a shareholder of LEO Pharma A/S. Thomas Litman and Tine Skak-Nielsen are former employees of LEO Pharma A/S and shareloders of LEO Pharma A/S. **Josephine Hebsgaard**, **Mette Sidsel Mortensen**, **Malene Bertelsen**, and **Birgitte Ursoe** were employees of LEO Pharma A/S at the time that the study was conducted. **Sidsel Mortensen** and **Malene Bertelsen** are shareholders of LEO Pharma A/S. **Manja Jargosch** and **Natalie Garzorz-Stark** declare no conflict of interest. **Anna Caroline Pilz** received speaker's, consulting and/or travel fees from Abbvie, ALK-Abello, Bristol Myers Squibb, Boehringer Ingelheim, LEO Pharma, Novartis, UCB. **Tilo Biedermann** gave advice to or received honoraria for talks or research grants from the following companies: Abbvie, Alk-Abelló, Boehringer-Ingelheim, Celgene-BMS, LEO Pharma, Lilly Deutschland

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Data and materials availability: All data are available in the main text or the supplementary materials.

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List of Supplementary Materials

Fig S1-S4

Materials and Methods

Data file for Tables S1 to S9

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690 **Figure Legends**

691

692 **Fig. 1. IL-22RA1 expression and its binding partners are elevated in lesional AD skin and correlate with of**
693 **epidermal barrier integrity.** (**A**) Expression analysis of *IL22RA1* and its co-receptors in a single cell dataset of
694 lesional AD skin (n=4). (**B**) Frequency of *IL22RA1* positive undifferentiated, proliferating and differentiated
695 keratinocytes. (**C**) Number of single- and double receptor positive cells per AD patient. (**D**) Representative image of
696 *IL22RA1* in situ hybridization (ISH) stained lesional AD skin samples and non-lesional control skin. (**E**) positive area
697 per length of skin sections stained with *IL22RA1* specific ISH probes in non-lesional control (n=6) and lesional AD
698 (n=17) samples; **P*=0.0097. (**F**) Pearson correlation coefficient of *IL22RA1*-positive ISH area with markers of
699 epidermal integrity and AD severity. Epidermal thickness measurements were performed using Masson's trichrome
700 staining, and the presence of DEFB4, loricrin, and T cells was detected using immunohistochemistry. (**G**)
701 Representative *IL-22RA1* ISH of a formalin-fixed paraffin-embedded 3D skin equivalent (n=6). (**H**) Single cell analysis
702 for expression of *IL22*, *IL20* and *IL24* in lesional AD dataset (n=4). (**I**) Single cell expression of *IL22*, *IL20* and *IL24* in
703 keratinocytes and (**J**) T cells and Innate lymphoid cells (ILC). (**K**) Representative flow cytometry staining of IL-22
704 protein in full skin digestions of AD biopsies (n=6). (**L**) Percentage of IL-22 producing cells amongst $CD4^+$ and $CD8^+$ T
705 cells in AD full skin digestions (n=6). **P*<0.05; ***P*<0.01; ****P*<0.01; *****P*<0.0001)

706

Fig. 2. The IL-22 signature consists of genes associated with epidermal barrier, metabolism, and inflammation in an AD-like in vitro model. (**A**) In vitro study design with 3D skin equivalents. (**B**) Differentially expressed genes (DEG) in 3D skin equivalents stimulated with TSN (687 DEG (474 up-/213 down-regulated)) or IL-22 (266 DEG (142 up/124 down)) versus unstimulated 3D skin equivalents (control), and the overlap of both (89 DEG (52 up/36 down)). DEGs were defined as log2 fold-change (log2FC)>1, $P<0.05$. (**C)** Annotation via STRING of DEGs regulated by TSN or IL-22 and categorized into 8 categories. The log2FC was visualized as heatmap. The list of all DEGs and their annotation is summarized in **Table S4**.

Fig. 3. Blocking IL-22RA1 with temtokibart efficiently inhibits the production of chemo-attractant and antimicrobial peptide molecules in vitro and ex vivo. (**A**) Dose-dependent inhibition of CCL2 secretion by 2D cultured human keratinocytes after incubation with temtokibart upon stimulation with an AD cytokine mix. (**B**) A representative experiment from 7 experiments showing dose dependent inhibition of IL-22 mediated effects on *DEFB4* mRNA expression by temtokibart in ex vivo biopsies from healthy donors (n=7) preincubated with temtokibart (black circles) or isotype control motavizumab (black squares) and subsequently stimulated with IL-22 for 24 hours. (**C**) Relative gene expression of *DEFB4* in 3D skin equivalents (n=3) pre-incubated with temtokibart 300 nM for one hour and subsequently stimulated with TSN for 24 hours. Comparison of groups was performed with Wilcoxon Test. **P*<0.05.

Fig. 4. Transcriptome analysis of an in vitro AD model reveals impact of IL-22RA1 inhibition on cell cycle, metabolism, and barrier integrity. (**A**) Visualization of the top five clusters from Markov Clustering (MCL) in a degree sorted circular layout. The size of the node represents the p-value in a discontinuous scale. Continuous color-coded scale of nodes represents the log2FC. (**B-F**) Functional enrichment analysis of the largest five MCL clusters based on GO, KEGG, Reactome, COMPARTMENTS, and STRING databases. X-axis shows the strength of each enriched pathway and continuous color scale of each bar graph represent the false discovery rate (FDR). Representative terms with an FDR < 0.05 were selected. **B)** cluster 1: ubiquitination, (**C**) cluster 2: RNA synthesis, (**D**) cluster 3: cell cycle and chromosome condensation, (**E**) cluster 4: RNA metabolism, (**F**) cluster 5: translation. Selected up- (**G**) and down-regulated (**H**) DEGs from the top 100 DEGs regulated by temtokibart. DEGs displayed as normalized counts (n = 5). Significance levels of DEGs are displayed and defined as **P*<0.05.

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737

738 **Fig. 5. Blocking IL-22RA1 with temtokibart in 3D skin equivalents prevents IL-22–induced thickening of the**
739 **epidermal layer (acanthosis) and hypogranulosis.** Representative 3D skin equivalent sections (n=3) and their
740 quantification of (**A**) acanthosis and (**B**) hypogranulosis upon IL-22 stimulation versus unstimulated 3D skin
741 equivalent (control), and prevention of acanthosis and hypogranulosis, respectively, by pre-incubation with
742 temtokibart 300 nM. Representative 3D skin equivalent sections and their quantification upon TSN stimulation are
743 shown in panels (**C**) for acanthosis and (**D**) for hypogranulosis. Black bars in the histology sections showing
744 acanthosis indicate measured thickness in µM (panels A and C). For the quantification of acanthosis, the narrowest
745 and the widest epidermal parts were selected for each section and in total, four measurements were performed for
746 each section. Granulae in 3D skin equivalent sections in panels B and D were detected and visualized as yellow dots
747 using QuPath. For the quantification of granulae, the total area (µm^2) covered by granulae was measured at two
748 different sites of each section. (**E**) Detection of secreted IL-20 and IL-24 by ELISA in fully confluent primary human
749 keratinocytes upon stimulation with IL-22 in presence or absence of temtokibart. Comparison of groups was
750 performed with Wilcoxon Test. **P*<0.05, ***P*<0.01, ****P*<0.001.

751

752 **Fig. 6. Blocking IL-22RA1 in mice using Zymo, a surrogate murine anti-IL-22RA1 monoclonal antibody for**
753 **temtokibart, reduced Cxcl1 and *S100a9*.** (**A**) In vivo study design. Protein levels of CXCL1 (**B, C**) and Il-22 (**D**)
754 measured by ELISA in ear tissue. In Panel B, data are presented as % of isotype control calculated as: ((measured
755 value - mean of control)/(mean of isotype control - mean of control))x100%. Data compiled from 5 separate
756 experiments (**B**) and one single study (**C, D**). In Panels C and D, data are shown as measured in pg/mL. **E)** qPCR
757 results on *S100a9* regulation by Zymo-mediated inhibition of IL-22RA1 in homogenized ear tissue; data retrieved
758 from a single study. In Panels B–E, significance levels have been calculated using unpaired t-test with Welch's
759 correction and are displayed as **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001. TPA, phorbol 12-myristate 13-
760 acetate.

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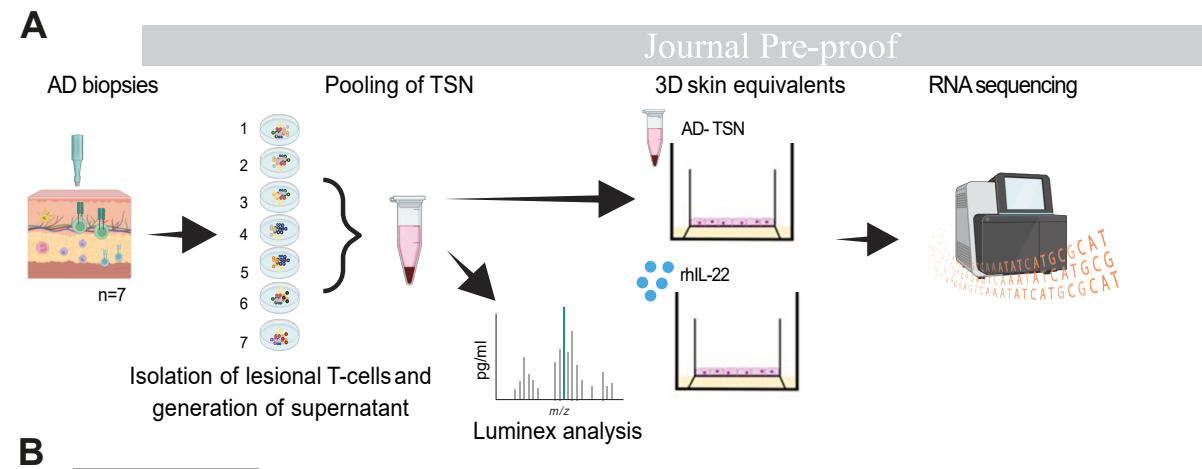
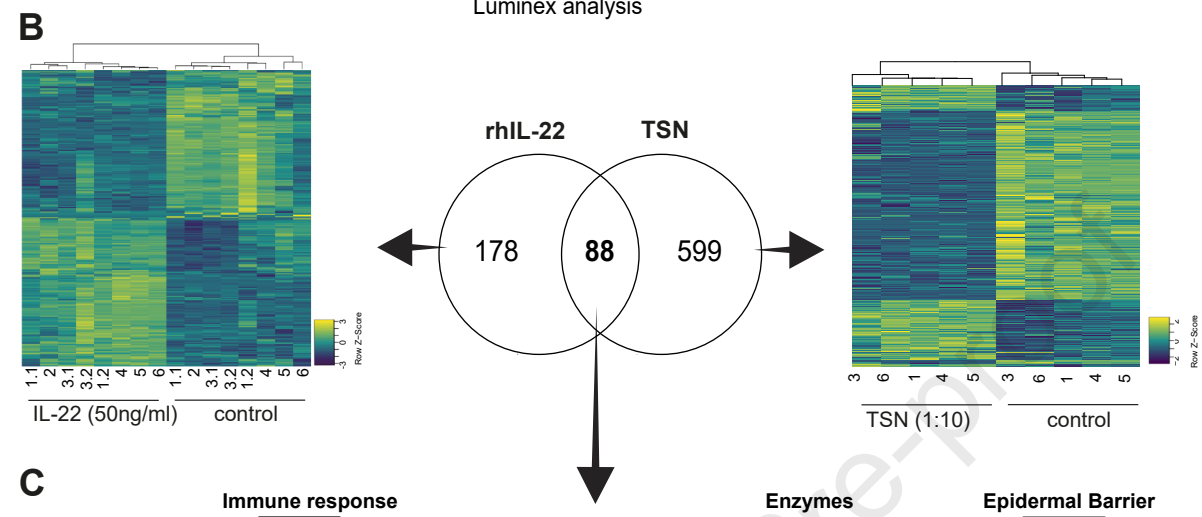
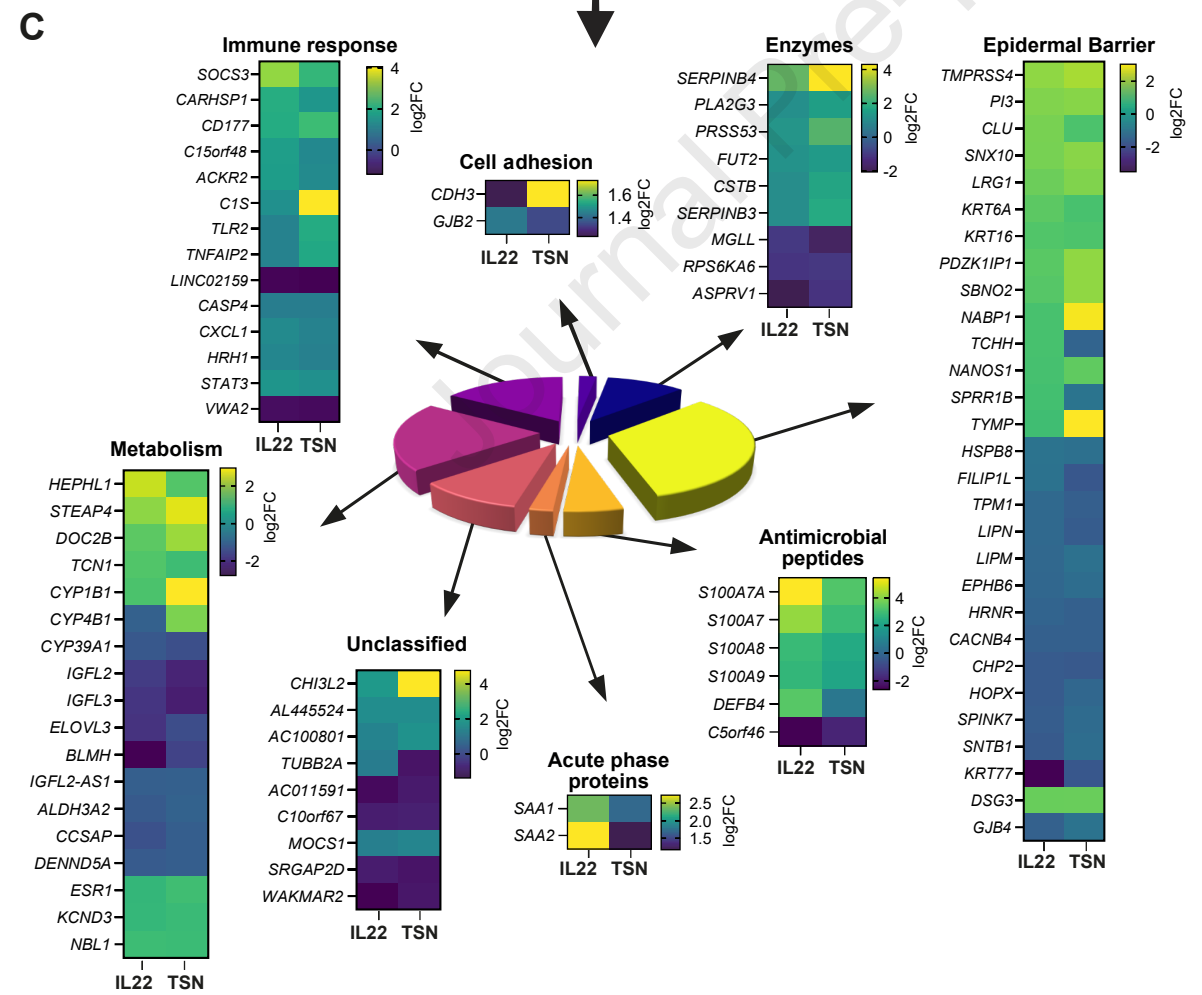
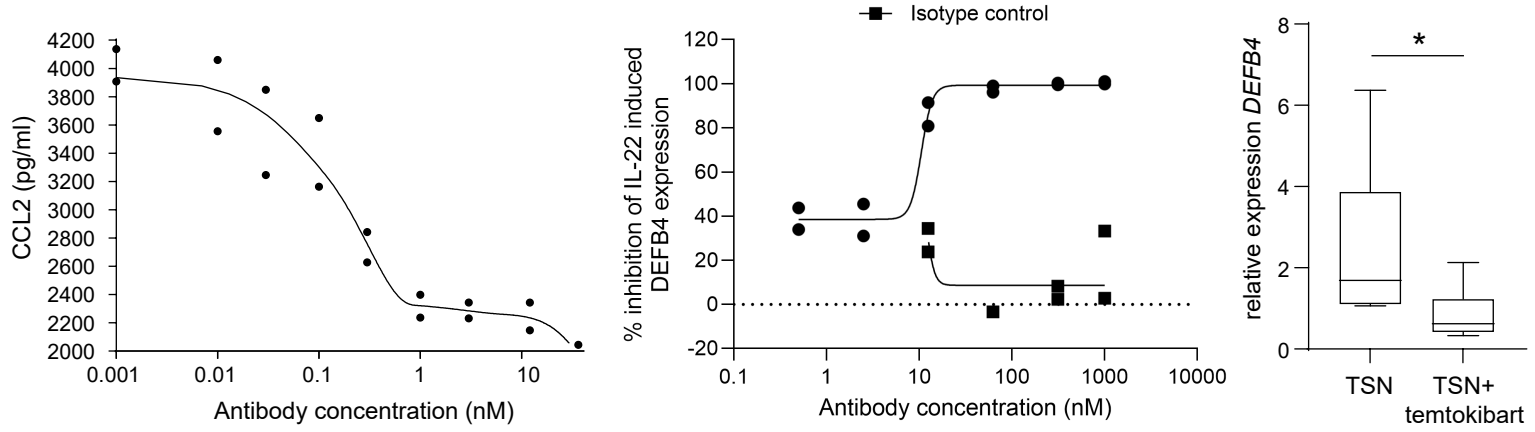
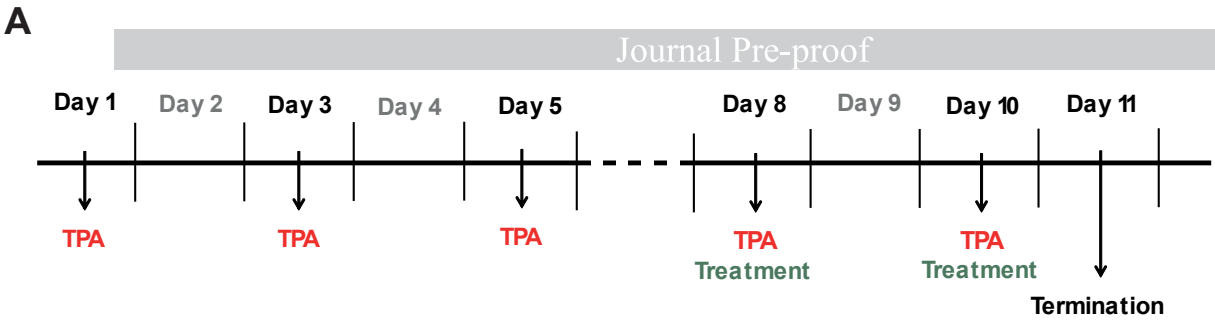
Fig 2

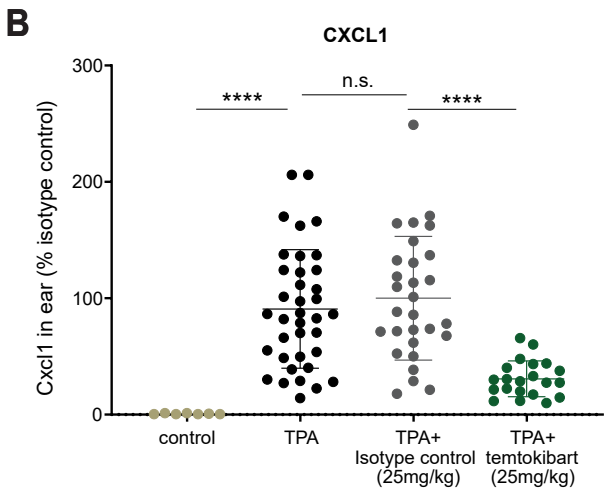


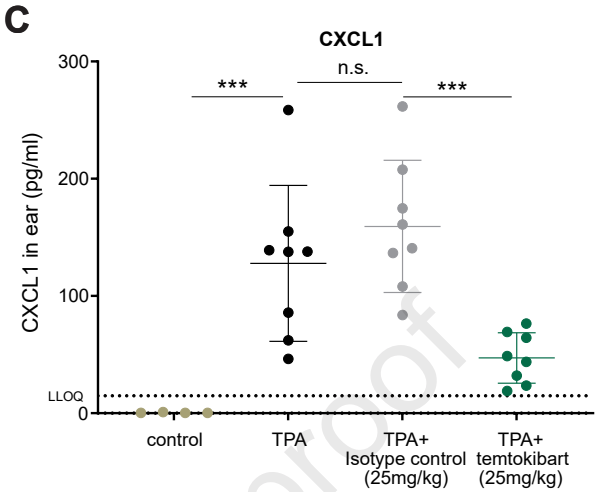


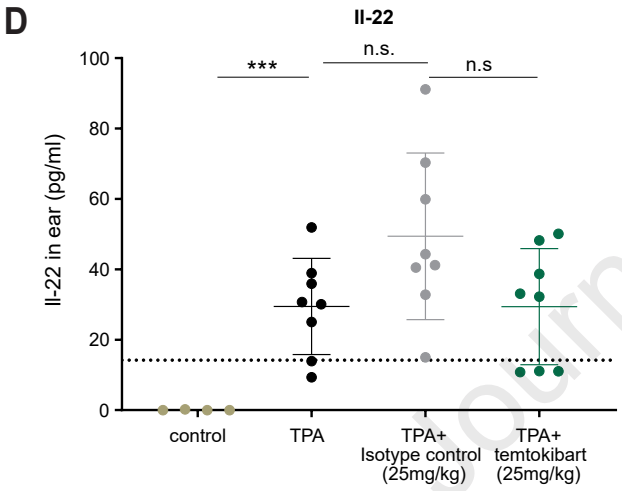












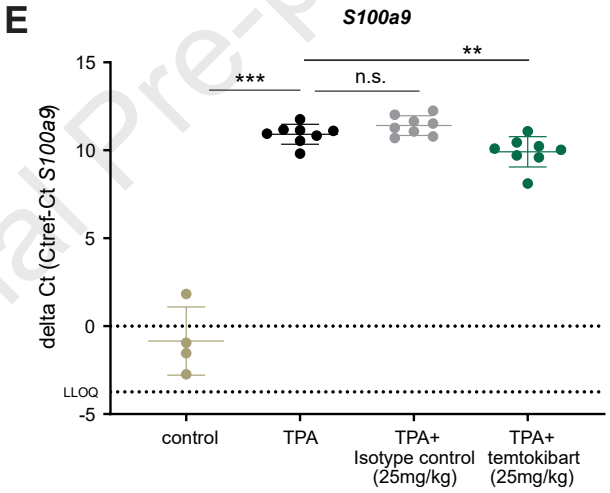


Fig 1

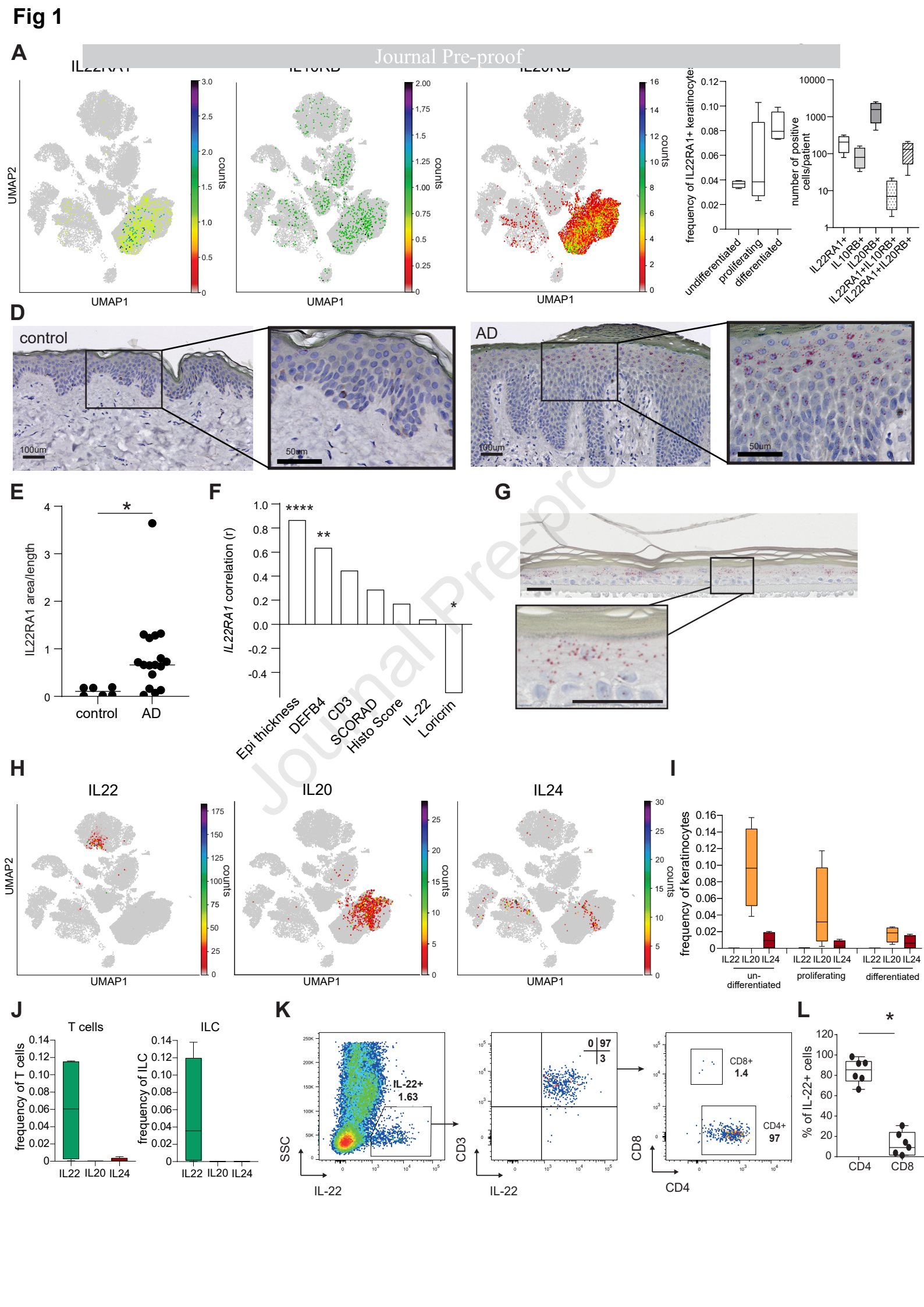
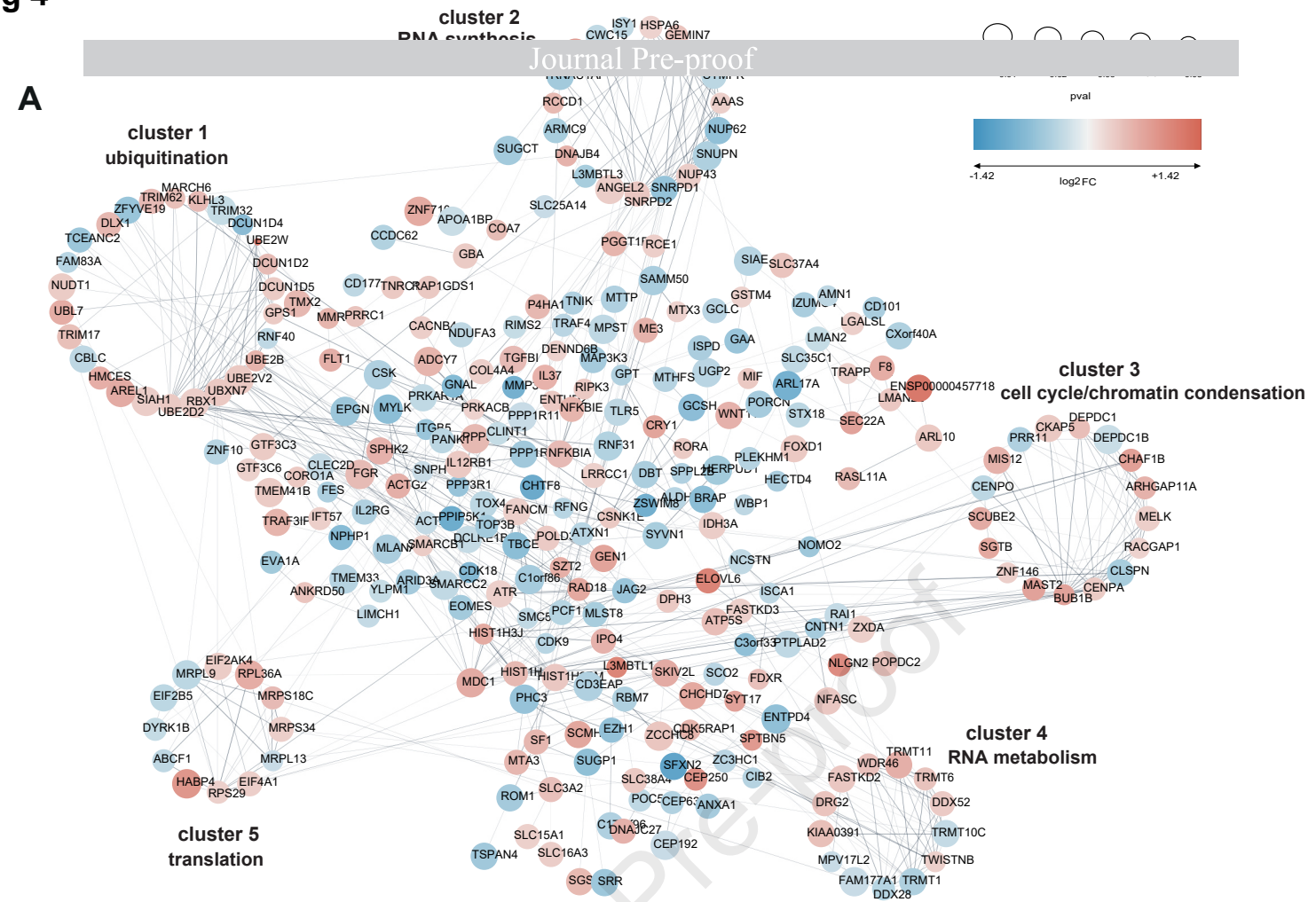
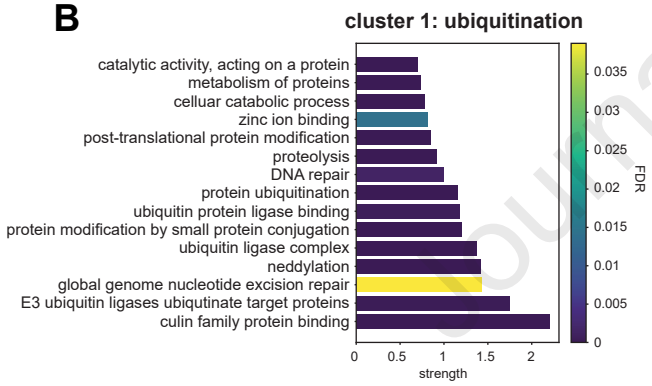
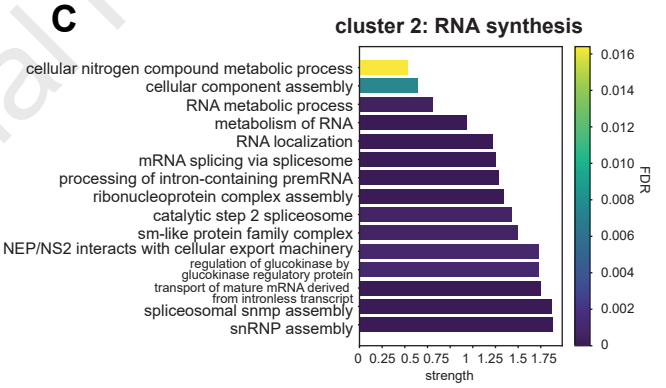
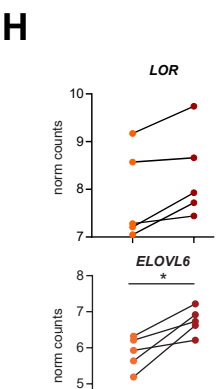


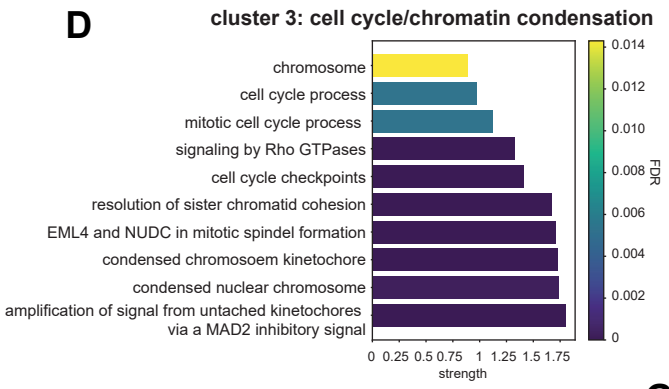
Fig 4

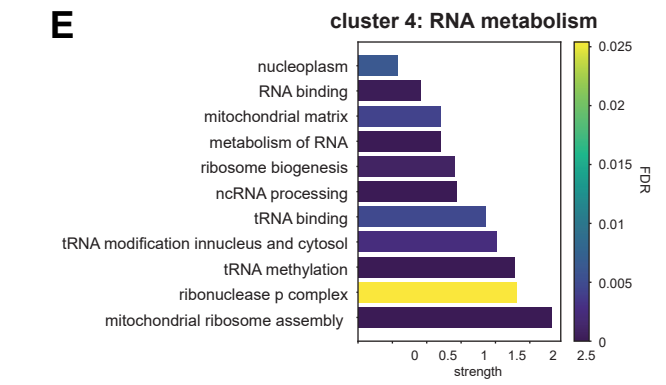


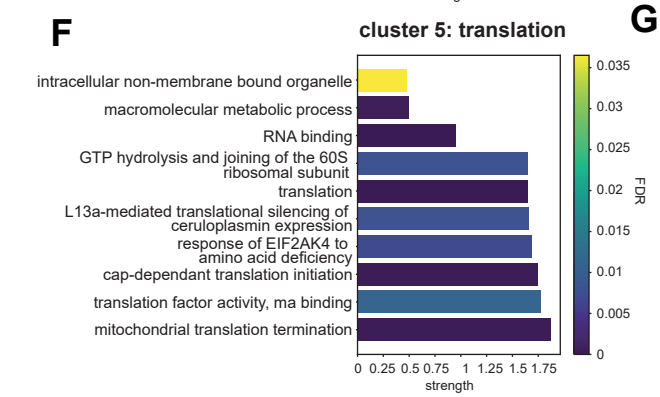












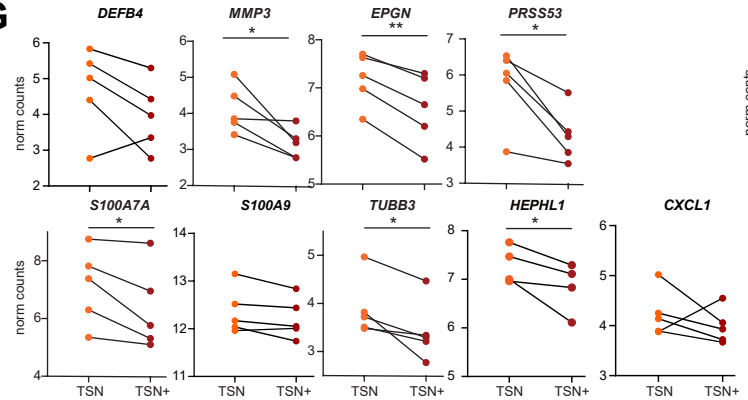


Fig 5