Ruxolitinib alleviates inflammation and fortifies skin barrier function through dampening IL-13

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Abstract (244w)

Background:

Atopic dermatitis (AD) is a prevalent inflammatory skin disorder characterised by an impaired skin barrier, dysregulated immune system, and pruritis. Emerging pharmaceutical therapies for AD include selective Janus kinase (JAK) inhibitors such as ruxolitinib, the first dual JAK1/JAK2 inhibitor approved by U.S. Food and Drug Administration.

Objective:

This study aimed to evaluate the effects of ruxolitinib on AD-related symptoms using mouse and human skin models.

Methods:

AD-related symptoms were assessed in MC903/ruxolitinib-treated mice, including ear swelling, histological analysis, pruritus, serum biomarker quantification, and immune cell analysis. Additionally, immunohistochemistry and transcriptome analysis were conducted on AD-related cytokine-treated reconstructed human skin (RHS) and ruxolitinib-treated human skin explants with and without tape-stripping.

Results:

Ruxolitinib-treated mice exhibited reduced inflammation, including decreased ear swelling, and diminished pruritus. Furthermore, reductions in immune cell populations, including T cells, and serum biomarker IL-13 were observed in ruxolitinib-treated mice. Transcriptome analysis revealed increased STAT3 expression and decreased skin barrier

gene FLG in AD-related cytokine-treated RHS. Regardless of tape-stripping, ruxolitinib-treated skin explants exhibited decreased *IL13RA1* expression and increased expression of skin barrier genes *FLG*, *FLG2*, and *LOR*.

Conclusion:

Ruxolitinib treatment in mice resulted in decreased inflammation and pruritus, along with increased expression of skin barrier proteins through downregulation of IL-13. Consistently, ruxolitinib-treated human skin explants demonstrated enhanced expression of skin barrier proteins, while IL-13 treatment of RHS led to downregulation of these proteins. These findings support data from human clinical trials indicating reduced SCORAD, pruritus, and inflammatory phenotypes in AD patients treated with ruxolitinib.

Key words

atopic dermatitis; ruxolitinib; JAK/STAT; MC903; reconstructed human skin; skin explants

1. Introduction

Atopic dermatitis (AD) is a prevalent pruritic inflammatory skin disorder with heterogenous clinical presentations in terms of age of onset, nature and severity of the disease which can be explained by the complex and differing drivers of the disease (1). Pathophysiological drivers of AD include an impaired skin barrier, a dysregulated immune system and pruritus (2). Genome-wide association studies have uncovered underlying genetic mutations that underpin AD presentation, with several risk loci such as *FLG*, *IL6R* and Th2 signalling molecules being significantly associated with the disease (3). AD is associated with cutaneous inflammation, which includes an activation of the T-cell response, specifically Th2 immune response and an increased activation of other immune cells including skin resident dendritic cells, innate lymphoid cells, and Langerhans cells (4).

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway is a critical inflammatory pathway in AD pathophysiology mechanisms. The JAK family of proteins include JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). Upon cytokine activation, they interact with various cytokine receptors to mediate signalling to a group of molecules called STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6). During cytokine activation, STATs, which reside in the cytoplasm, are phosphorylated by activated JAKs and translocate into the nucleus to act as transcription factors, driving gene expression of specific target genes (5, 6). Several cytokines signal through the

JAK/STAT pathway, including the IL-2, IL-3, IL-6, IL-10 family of cytokines, several of which are upregulated during AD pathogenesis (5, 7).

The use of biologics, like dupilumab, a blocker of IL-4R, has achieved significant success in the treatment of AD since it was approved in 2017 (8-10). However, there remain patients who are refractory to these treatments, indicating the need for new/complementary treatment options (10-12). The key roles that JAK/STAT signalling play in cytokine signal transduction suggest they are an important class of molecules to be targeted for ameliorating AD (13). Delgocitinib is the first generation pan-JAK inhibitor to alleviate AD-like inflammation in animal studies and was approved for use in 2020 in Japan (14-16). Current development of this drug is focused on chronic hand eczema (9, 17). First-generation of JAK inhibitors like delgocitinib act broadly to inhibit three or all four of the JAK family molecules, and newer JAK inhibitors are more selective by inhibiting 1 or 2 of the JAK molecules (7). There are currently several topical JAK inhibitors that have been approved for use to treat AD (9) [delgocitinib (14-16), ruxolitinib (18, 19)], and systemic [baricitinib (17, 20-22), upadacitinib (23-26) and abrocitinib (27-32)]. Several other JAK inhibitors (gusacitinib, tofacitinib, ifidancitinib, brepocitinib, jaktinib, cerdulatinib, ATI-1777, CEE321 and ivarmacitinib) are in various phases of clinical trials for AD treatment (7, 9, 13, 33, 34).

Ruxolitinib (Opzerula), a dual inhibitor of JAK1 and JAK2, was the first JAK inhibitor approved by the FDA for use in AD, in 2021 (35). Ruxolitinib cream has shown

therapeutic benefits in AD patients, with a major improvement of the Eczema Area and Severity Index (EASI), Investigator Global Assessment (IGA) response and itch over time (18, 19, 36). Despite its promising therapeutic potential, the use of JAK inhibitors should also be explored with care, as ubiquitous JAK blockade may result in biological toxicities, especially in certain groups of at-risk patients (37-39). Therefore, the characterisation of JAK inhibition in mouse and human skin models is important to tease out the effects of both short- and long-term treatments. Furthermore, the central role JAK/STAT signalling in transducing the cytokine stimulation means that JAK inhibitors are increasingly explored to address other dermatological diseases that have a dysregulated cytokine profile, such as alopecia areata, psoriasis, psoriasis arthritis and vitiligo (40, 41). Thus, the present study sought to understand how ruxolitinib affects inflammation, a key characteristic of the disease, in a well-established AD mouse model and human skin models.

2. Materials and Methods

2.1 Animal Experiments

Animal studies were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Biological Resource Centre (BRC), A*STAR. Mice were bred and maintained in specific pathogen-free (SPF) conditions in BRC, A*STAR. 8-week-old female C57BL/6 mice were used for the MC903 mouse model. Inhalatory anaesthesia was administered to immobilize the animals during measurements and treatment procedures. MC903 (or Calcipotriol) (2nmol/mouse) (Cayman Chemical) was topically applied to the dorsal and ventral sides of both ears daily for 14 days. During this period, mice received daily oral gavage of vehicle (10% DMSO, 20% PEG400, 10% Tween80, 60% 0.1MNaHCO3) or ruxolitinib (0.05mg per gram of mouse weight) (MedChemExpress). Daily monitoring of mouse weights was conducted to monitor their health, with no significant weight loss recorded with these drug concentrations. Ear phenotypes were documented by imaging (Canon S110 Zoom lens 5x) before (Day 1) and after the 14-days treatment regime (Day 14).

2.2 Ear swelling and pruritis measurements

Ear swelling was measured daily using an ear calliper (Peacock dial thickness gauge, Ozaki). Video recordings were conducted to quantitate pruritus, with mice individually placed in cages to acclimatize for 30 minutes before each 1-hour recording session. The mice were recorded on day 13 of the 14-days treatment. Subsequently, videos were reviewed manually in a blinded manner, and scratch counts were recorded. A single

scratch count was defined as “lifting of either hind limb to scratch at the face, neck, or side and replacing the paw onto the floor, regardless of the number of scratching strokes that occurred between the first lift and final lowering of the hind limb”(42).

2.3 Histology

Harvested mouse ears were halved and fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 48 hours. Following fixation, tissues were dehydrated, paraffin embedded, and sectioned to 5 μm sections and were mounted on positively charged microscope slides (Leica). For immunohistological analyses, skin tissue sections were deparaffinized and heat induced antigen retrieval (HIER) was conducted on deparaffinized sections under pressure at 121 °C. Subsequently, sections were blocked for 1 hour in 10% goat serum (Dako) before being incubated overnight with primary antibodies (Supplementary table 1) at 4 °C in a humidified chamber. Secondary antibodies (EnVision, Dako) were applied for 30 minutes at room temperature, and peroxidase activity was detected using diaminobenzidine tetrahydrochloride substrate (Dako, Glostrup, Denmark). Images were acquired using an Olympus CX41 and Olympus CX43 bright field microscopes.

In situ hybridization was performed using the RNAscope® assay (Advanced Cell Diagnostics, a Bio-Techne brand), following the manufacturer’s protocols for formalin-fixed paraffin-embedded (FFPE) tissue sections. Briefly, 5 μm FFPE sections were

mounted on slides, baked, deparaffinized, and subjected to hydrogen peroxide treatment and target retrieval, followed by protease digestion to ensure optimal probe penetration. Sections were then incubated with RNAscope® target-specific probes (Mm-Il13 [312291], Mm-Il13ra1 [437541], Mm-Polr2a [312471], DapB [310043]). Signal detection was performed using the RNAscope® 2.5 HD Red chromogenic detection kit, which generates red punctate dots representing individual RNA transcripts. Sections were then counterstained with hematoxylin and imaging was conducted using an Olympus CX43 brightfield microscope.

2.4 Preparation of ear tissues and flow cytometry

Cell suspensions preparations from ear tissues and flow cytometry methods were conducted according to previously described methods (43). Briefly, mouse ears were separated into dorsal and ventral halves and incubated in RPMI-1640 (Sigma-Aldrich) with 1 mg/mL dispase (Sigma-Aldrich) for 1 hour at 37°C. The separated tissues were dissected and incubated in RPMI containing 10% serum, collagenase IV (0.2mg / ml, Sigma-Aldrich) and DNase I (20000U / ml, Roche) for 1 hour at 37°C. Subsequently, cells were then passed through 19 G needle and filtered through a 100 µm cell strainer (BD Falcon) to obtain a homogeneous cell suspension. The single-cell suspensions from the epidermis and dermis were stained with fluorophore-conjugated antibodies at 4 °C for 40 minutes. The antibody panels were similar for both tissue types, with the exception of the PE-conjugated antibody: CD64 was used for the dermis, while CD103 was used for the epidermis. Following staining, cells were washed and counterstained with DAPI.

Multi-parameter analyses and sorting of labelled cell suspensions (Supplementary table 1) were performed on a LSR II and FACSAria II (Becton Dickinson, San Jose, USA), respectively. Data were analysed with FlowJo software (Tree Star Inc. Ashland, USA). Fluorochrome monoclonal antibodies (mAbs) used in the manuscript are listed in Supplementary table 1 and details of the flow analysis gating strategy applied are in Supplementary Fig. 2.

2.5 Serum biomarker measurements

After the 14-days of treatment, serum samples were collected from mice for Luminex analysis using the ProcartaPlex customized panel (Thermo Fisher Scientific) to quantify the following targets: Eotaxin; IFN-gamma; IL-10; IL-13; IL-17 A; IL-1 beta; IL-31; IL-33; IL-4; IL-5; MCP-1; TARC; TSLP.

Harvested supernatants and standards were incubated with fluorescent-coded magnetic beads pre-coated with respective antibodies in a black 96-well clear-bottom plate overnight at 4˚C. Following incubation, plates were washed 5 times with wash buffer (PBS with 1% BSA [Capricorn Scientific] and 0.05% Tween-20 [Promega]). Sample-antibody-bead complexes were incubated with biotinylated detection antibodies for 1 hour and washed 5 times with wash buffer. Subsequently, streptavidin-PE was added and incubated for another 30 mins. After another 5 times washes, sample-antibody-

bead complexes were resuspended in sheath fluid for acquisition on the FLEXMAP® 3D (Luminex) using xPONENT® 4.0 (Luminex) software. Data analysis was done on Bio-Plex Manager™ 6.1.1 (Bio-Rad). Standard curves were generated with a 5-PL (5-parameter logistic) algorithm, providing values for both mean florescence intensity (MFI) and concentration data.

2.6 Reconstructed human skin (RHS) and human skin explants

3D reconstructed human skin (RHS) were generated as previously described (44). Briefly, on day 1, a layer of rat tail type 1 collagen (Falcon) matrix (containing human fibroblasts) was deposited onto polyethylene terephthalate (PET) membranes (Falcon) to polymerize for 24 hours. On day 2, human keratinocytes were seeded on the matrix, and cultured for 24 hours before the culture being transitioned to the air-liquid interface on day 3. Subsequently, the cultures were fed with media to induce epidermal differentiation. Both control and cytokine-treated RHS were maintained in differentiation culture for a total of 14 days, with cytokines IL-4 (R&D Systems #204-IL; 20ng/ml) and IL-13 (R&D Systems #213-ILB; 20ng/ml) added on day 10 and collected on day 14. Triplicate samples were prepared for each experimental condition.

For human skin biopsy explants, 8mm human skin biopsy explants were obtained from healthy skin donors, with approval from the NHG domain specific review board (NHG DSRB 2018/09945) at National University Health System (NUHS). Informed consent was obtained from the skin donors. Skin explants were generated by punching with an 8mm

punch and cultured at 37°C with 5% CO_2 for 24 hours either with DMSO (control) or with 1μM of ruxolitinib. Skin was subjected to 20 rounds of stripping using Tegaderm (3M) prior to biopsy punching for the "stripping" condition. Quadruplicate samples were prepared for each experimental condition. Each biopsy was halved, with one half fixed in 10% formalin for FFPE samples and the other half for bulk RNA transcriptomics.

2.7 Transcriptomics analyses

Total RNA extraction was conducted using TRIzol reagent (Invitrogen) and RNeasy Micro Kit (Qiagen), followed by assessment of RNA quality through Nanodrop, agarose gel electrophoresis, and Agilent 2100 analysis (RIN≥7.0). Subsequently, poly(A) RNA was enriched using oligo(dT) beads and rRNA removed before RNA sequencing was performed at NovogeneAIT Genomics using an Illumina HiSeq4000 platform.

Paired-end reads were aligned using STAR version 2.2.1 against the GENCODE GRCh38 primary assembly. The aligned reads were summarized to the gene level using featureCounts version 1.6.3, with -p option and GENCODE v30 gene annotation. Differential expression analyses were carried out using edgeR, with genes of FDR<0.05 considered significant. Additionally, gene ontology analysis was conducted using DAVID Bioinformatics Resources (45, 46) and visualization of volcano plots and significant gene ontology pathways were generated using GraphPad Prism version 10.

2.8 Statistical analyses and graphs

Graphs were generated using GraphPad Prism version 10 for macOS, GraphPad Software, Boston, Massachusetts USA, and statistical significance were determined using unpaired t-tests and two-way ANOVA tests, where appropriate, and stated in the figure legends.

3. Results

3.1 Ruxolitinib attenuates AD associated inflammation, pruritis, immune cell infiltration and Th2 serum biomarkers in the MC903 mouse model

MC903 model is a highly reproducible dermatitis mouse model that is widely used to simulate AD (47-49). Application of MC903 on mouse ears for 14 days induced visible signs of dermatitis, including erythema, edema and flakiness. Notably, mice that are treated with ruxolitinib exhibited improved ear appearance, characterized by reduced erythema and dermatitis (Fig. 1A). To track dermatitis induction in MC903-treated mouse ears, we measured the ear thickness using a calliper over the 14-days treatment. In vehicle-treated mice, MC903 led to gradual thickening of the ears, which was significantly attenuated in ruxolitinib-treated mice (Fig. 1B).

To assess the efficacy of oral ruxolitinib in suppressing JAK/STAT pathway activation in the MC903 model, we evaluated phosphorylated STAT3 Y705 as a marker of JAK/STAT activation in immunohistochemical analyses. The ears of MC903- treated mice displayed increased JAK/STAT pathway activation evidenced by a significant increase in phosphorylated STAT3 Y705 staining in keratinocyte nuclei within the epidermis and in immune cells in the dermis (Fig 1C). A modest increase in phosphorylated STAT6 Y641 was also observed (Supplementary Fig 1). In contrast, ruxolitinib-treated mice exhibited significantly reduced levels of phosphorylated STAT3 Y705 , indicating JAK/STAT pathway inactivation (Fig. 1C). Video recordings conducted on experimental mice at day 13 of the 14-day treatment revealed an increase in scratch counts in the MC903 vehicle

group compared to controls. Conversely, mice treated with ruxolitinib exhibited a statistically significant reduction in scratch counts (Fig. 1D).

To identify immune cell infiltrates during the acute phase of inflammation, we conducted a comprehensive high-dimensional analysis of the ear cell populations using flow cytometry (Supplementary Fig. 2A). MC903 induced an accumulation of $CD45^+$ immune cells into the ear and lymph nodes (Fig. 1E, Supplementary Fig. 2C) and significantly, ruxolitinib attenuated the increase of $CD45^+$ immune cells, specifically T cells (including DETCs) induced by MC903 application (Fig. 1E).

Next, to delineate the evolution of the driver of the inflammation, serum biomarkers from mice treated with 14 days of ruxolitinib were quantified using the Luminex xMAP technology that can measure several analytes simultaneously. Thymic Stromal Lymphopoietin (TSLP) is a master switch for Th2 inflammation in AD (50) and is overexpressed in AD skin lesions. In our experiment, TSLP was activated in the mice treated with MC903 (Fig. 1F). However, expression of TSLP is not decreased in our ruxolitinib-treated mice when compared to vehicle-treated mice. Significantly, Th2 markers, IL-5 and IL-13, were increased in MC903 treatment and supressed in ruxolitinib-treated mice (Fig. 1F). Levels of IL-17A was increased with MC903 and decreased with ruxolitinib but non-significantly (Fig. 1F) Although, some markers linked to AD, such as TARC, Eotaxin, IL-4, IL-33, were investigated, no significant changes were observed in our mice (Supplementary Fig. 3). The suppression of some Th2 markers like

IL-5 and IL-13 in ruxolitinib mice could explain the reduced inflammation in ruxolitinib-treated mice.

3.2 IL-13-treated RHS have increased STAT3 and decreased skin barrier RNA transcripts and proteins

To investigate the potential mechanism underlying the reduction of IL-13 in ruxolitinib-treated mice and its impact on alleviating AD-related symptoms, we conducted transcriptomic analysis of control and IL-13-treated RHS. Analysis of differentially expressed genes (DEGs) in IL-13-treated RHS revealed enrichment of gene ontology biological processes associated with skin barrier function, including epidermis development, cell adhesion, and keratinization (Fig. 2A). IL-13-treated RHS expressed increased inflammatory skin disease linked genes- *CCL26*, *PRR9*, *CYSLTR2*, *IL13RA2* (Fig. 2B, Supplementary table 2). And notably, key skin barrier genes such as *FLG*, *IVL*, *and HRNR* were significantly downregulated in IL-13-treated RHS (Fig. 2C). Among the STAT molecules examined, *STAT3* exhibited a significant increase in IL-13-treated RHS (Fig. 2D). Next, we investigated the effects of both IL-4 and IL-13 (Th2 cytokines) on STAT3 and skin barrier proteins using immunohistochemistry. Immunohistochemistry experiments revealed that these AD-linked cytokines elicited an increase in STAT3 and a reduction of skin barrier protein, FLG and LOR (Fig. 2E and Supplementary Fig. 4).

3.3 Ruxolitinib enhances skin barrier proteins in human skin explants and MC903-dermatitis mice

To validate the results obtained from MC903-treated mice and IL-13-treated RHS, we investigated the effects of ruxolitinib on IL-13 signaling and skin barrier genes in human skin explants. We employed 2 skin explant models; *de novo* non-stripped skin explants and skin explants subjected to 20 rounds of stripping with tegaderm to simulate weakened skin barrier conditions. Immunohistochemistry revealed a reduction in phosphorylated STAT3 Y705 in samples treated with 1μM ruxolitinib (Fig. 3A, Supplementary Fig. 5). Of interest, control skin explants that were subjected to cell culture experiments for 24 h expressed significant levels of phosphorylated STAT3 Y705 compared to skin explants that were not subjected to tissue culture conditions (Fig. 3A). Transcriptomics analyses of both types of skin explants (non-stripped and stripped) treated with ruxolitinib showed an increase of skin barrier genes -*FLG*, *FLG2* and *LOR* and a decrease of *IL13RA1* (Fig. 3B, Supplementary table 3) indicative of reduced IL-13 signalling. Interestingly, the stripped human skin explant model treated with ruxolitinib exhibited a more pronounced increase in RNA levels of skin barrier genes and a greater reduction in IL13RA1 compared to the non-stripped skin explant model. When we probed for the effect of ruxolitinib on the RNA of IL-4 and IL-13 pathway members, we found that *IL13RA2, IL4* and *IL13* RNA are not statistically significantly regulated in ruxolitinib-treated skin explants and that IL13RA1 is the only member that is significantly regulated (FDR<0.05) (Supplementary Table 3), suggesting that the decrease of IL13 receptor subunit alpha 1 is a possible mechanism for IL-13 signalling blockade post ruxolitinib treatment. Probing for Il13 and Il13ra1 expression revealed increased IL13ra1 protein and mRNA levels in both the epidermis and dermis of MC903-treated mouse skin. Treatment with ruxolitinib significantly reduced Il13ra1 expression

(Supplementary Fig. 6). Similarly, Il13 protein levels were elevated following MC903 treatment and were attenuated by ruxolitinib. These findings suggest that Il13 and Il13ra1 are distributed across both epidermal and dermal compartments, indicating their involvement in impairing skin barrier function, are likely through effects on epidermal protein expression, as well as influencing immune activity in the dermis.

Significant top gene ontology biological processes affected by ruxolitinib treatment in both *de novo* and stripped explants included several skin barrier-related pathways (Fig. 3C). Immunohistochemistry experiments showed that ruxolitinib restores FLG expression in stripped skin explants (Fig. 3D, Supplementary Fig. 5). However, ruxolitinib did not affect FLG expression in non-stripped skin explants (Supplementary Fig. 5). Transcriptomic analysis of non-stripped and stripped ruxolitinib-treated explants uncovered several AD-linked genes (*FLG2*, *IL12A*, *SPINK7*, *CXCL9*) (Supplementary Fig. 7). In the same vein, expression of skin barrier proteins Flg and Ivl were increased in ruxolitinib-treated mice (Fig. 3E, Supplementary Fig. 8).

The skin barrier comprises structural proteins and lipids that work together to maintain its integrity and protective function. Among these lipids, ceramides are critical components essential for barrier function (51). To assess the impact of ruxolitinib on skin lipid metabolism, we examined the expression of ceramide synthase and fatty acid elongase genes in the human skin explant model. At the transcriptomic level, ruxolitinib treatment did not result in consistent or significant changes in the expression of genes

involved in ceramide or fatty acid synthesis (Supplementary Fig. 9). Together, these results suggest that ruxolitinib enhances skin barrier integrity primarily through effects on structural proteins rather than lipids.

These findings underscore the effectiveness of ruxolitinib treatment in enhancing the integrity and functionality of the skin barrier to boost skin health in both human and mouse skin models, highlighting its therapeutic utility in managing AD.

4. Discussion

This study characterises the effects of ruxolitinib-treated mice in the mouse MC903 dermatitis and human skin models (Fig. 4). Our mouse study aligns with previous results of reduction of mouse nerve innervation and itch shown by Oetjen *et al.* during JAK/STAT signalling inhibition in MC903-treated mice (52). They employed 2 models, disruption of JAK1 signalling using a JAK1 conditional knockout, and 7 days of ruxolitinib (i.p.) treatment to block JAK/STAT signalling, and in both models, they were able to show a reduction of itch and the number of dorsal root ganglion (DRG) neurons innervating the tissue. Ruxolitinib has also been shown to reduce pruritis-induced behaviours in the IL-33-transgenic dermatitis model (53). To add to this, our study specifically narrowed down that ruxolitinib fortifies the skin barrier through the dampening of IL-13.

The migration of immune cells from blood vessels into the site of dermatitis contributes to the observed increase in immune cell populations in mice undergoing dermatitis (Fig. 1E). Additionally, differentiation, and proliferation of various immune cells within the skin during AD pathogenesis may further drive disease progression. Notably, the neutrophil population in MC903-treated mouse ears was increased, and in ruxolitinib-treated mice, neutrophil numbers decreased slightly but non-significantly (Supplementary Fig. 2B). Walsh *et al.* have previously hypothesized that neutrophils have an important role in the induction of itch in the MC903 model (48), hence the decrease in neutrophils in this mouse model could potentially result in the reduction of itch (Fig. 1D). JAK1/2 inhibition have also been reported to block the development and function of dendritic epidermal cells in AD (54). Several immune cell subsets that were not investigated in our study, but have been reported to play important roles in AD pathogenesis, include eosinophils (55-57), group 2 innate lymphoid cells (58) and mast cells (58).

Our investigations center on IL-13 as a key driver of AD pathogenesis and skin barrier dysfunction, with ruxolitinib treatment suppressing IL-13 and reversing skin barrier pathology. Luminex results of blood serum from MC903-treated mice detected low levels of Il-4 (Supplementary Fig. 3) , a key Th2 cytokine. Consequently, we focused our experiments with investigating the effects of IL-13-treated reconstructed human skin rather than IL-4 treatment. The role of IL-5 in the pathogenesis of AD has been investigated (59). Mepolizumab, a humanized monoclonal antibody targeting IL-5, was

evaluated in clinical trials for AD treatment but did not demonstrate clinical efficacy. These findings suggest that while IL-5 may contribute to AD-associated inflammation, targeting IL-5 alone is insufficient to produce therapeutic benefit. A limitation of our study is the absence of dose titration for ruxolitinib treatment on the mice to ascertain its dampening effect on Il-5 and Il-13. Given the complex interplay among cytokines, it is possible that other cytokine activities contribute to the observed skin barrier defects. Future studies should explore a broader range of ruxolitinib doses and examine how modulation of individual cytokines influences skin barrier integrity.

In addition to targeting IL-13 cytokine signalling, ruxolitinib may exert broader modulatory effects that contribute to barrier restoration, including effects on other cytokines that are not deeply explored in the current study. Given that ruxolitinib has been shown to inhibit IL-22 in cutaneous squamous cell carcinoma (60) and IL-22 blockade has demonstrated significant improvements in severe atopic dermatitis (9, 61), further investigation into whether ruxolitinib can suppress IL-22 to strengthen the skin barrier is required. Ruxolitinib has been reported to suppress several cytokines including IL-6, IL-10, IL-12, IL-17, IL-1β, IFN-γ, TNF-α, CCL2, CCL5, and CXCL10 in various studies (62-65). However, in our study, IL-6, IL-12, TNF-α, CCL5 and CXCL10 were not examined. Our results showed that systemic IL-10, IL-1β, IFN-γ and CCL2 (Supplementary Fig. 3) had no significant reduction, IL-17 showed a decreasing trend, though not statistically significant (Supplementary Fig. 3). These findings suggest that while ruxolitinib has a documented capacity to modulate a broad set of cytokines, its

effects are context-dependent and not universally observed across all inflammatory markers. And our reported effects of IL-13 may not be the only mechanism that ruxolitinib works through to alleviate AD.

TSLP is a key alarmin which increased in AD and the MC903 mouse model. TSLP production, which is primarily driven by vitamin D receptor (VDR) activation (49), is not significantly inhibited by ruxolitinib. We did, however, detect a decrease in Th2 cytokines Il-5 and Il-13. This reduction aligns with reduced Th2 skewing, leading to attenuation of AD symptoms. Interestingly, using a different TSLP-induced dermatitis mouse model, Scuron *et al.* showed that ruxolitinib downregulated TSLP signalling, by detecting a reduced mRNA levels of Il7r (53). Li *et al.* have shown that in the MC903 model, cytokines like Il-5, Il-13 and Il-31 are fully TSLP dependent, but cytokines like Il-4, Il-6, Il-10 are only partially dependent on TSLP (66). Our data demonstrate that ruxolitinib does not alter circulating levels of TSLP, an upstream alarmin involved in initiating Th2 inflammation. Instead, the reduction in IL-13 levels likely dampens Th2 activation by disrupting the feed-forward loop that perpetuates this immune response (67).

Our findings reveal a significant reduction of specific immune cells, particularly T lymphocytes (Fig. 1E), as well as serum biomarkers Il-5 and Il-13 (Fig. 1F). This suggests that the reduction of Il-13 (Fig. 1F) may be due to the reduction of T cells in the system (Fig.1E, Supplementary Fig. 2). In addition to T cells from the adaptive immune system,

innate lymphoid cells (ILCs) from the innate immune system produce Th2 cytokines, IL-4, IL-5, IL-13, IL-31, that drive AD pathogenesis (68). A limitation to our study is that we did not probe for the effect of ruxolitinib on ILCs. Given the observed decrease in infiltrating T cells (Fig. 1E) and a concomitant decrease in Il-5 and Il-13 (Fig. 1F), we attributed the effect of ruxolitinib on dampening Il-13 to the decrease of T cells. However, the possible role of ILCs in the process should be explored in the future.

IL-13 is a key cytokine in maintaining homeostasis of the skin. Healthy skin expresses an IL-13 transcriptomic signature that supports the differentiation of dermal dendritic cells in response to allergic sensitization (69). IL-13 is essential for priming the skin to support a Th2 response, while reducing Th17 response, both of which are important immune axes in inflammatory skin conditions like AD. Interestingly, spatial transcriptomics of AD skin lesions has revealed that IL-13 expression is localized to specific disease-specific regions in lesional skin, particularly the middle and basal epidermis and the upper layers of the dermis (70). Regions surrounding IL-13 transcript-positive spots are characterized by gene signatures, suggesting IL-13's role in producing an inflammatory microenvironment. This underscores the importance of dampening IL-13 to treat AD's inflammatory profile. RNA sequencing results from ruxolitinib-treated skin explants revealed that IL13RA1 (Supplementary Fig. 6) is reduced, suggesting a possible mechanism by which ruxolitinb ameliorates AD through reducing IL-13 signaling, which warrants further investigation.

In our study, RNA sequencing analysis of keratinocytes exposed to Th2 cytokine IL-13 revealed a decrease in keratinization-related genes and proteins in our experiments. Conversely, treatment with ruxolitinib enhanced the expression of skin barrier genes in both human skin explants and MC903-treated mice (Fig. 4). This observation aligns with previous findings highlighting the detrimental impact of the IL-13/periostin/IL-24 pathway and Th2 cytokines on filaggrin expression and barrier function (71, 72). The efficacy of pan-JAK inhibitor delgocitinib (JTE-052) in rescuing skin barrier disruption further underscores the promising role of JAK/STAT pathway blockade in fortifying the skin barrier (14), indicating that the blockade of the JAK/STAT pathway is highly effective in boosting skin health.

Our findings show that IL-13 levels (Fig. 1F) and IL13RA1 (Supplementary table 3) transcript levels are reduced with ruxolitinib treatment to diminish the inflammatory actions of the cytokine. However, we did not directly investigate the molecular mechanisms by which ruxolitinib regulates IL-13 or IL13RA1 expression. In particular, the impact of ruxolitinib on known transcriptional regulators of IL-13 (73) remains to be elucidated (Supplementary Fig. 7A).

Dupilumab, a human monoclonal antibody against IL-4RA receptor subunit shared by IL-4 and IL-13, is utilized in AD treatment (37). It inhibits downstream signalling following binding of IL-4 and IL-13 to the receptor. Similarly, our study demonstrates that ruxolitinib, like dupilumab, can also block IL-13 signalling to result in attenuated AD-

symptoms and better skin health. Given that ruxolitinib is a more affordable treatment, could serve as a cost-effective alternative to dupilumab, which is a more costly treatment regime.

Our findings are congruent to those published in mouse models and human trials (18, 19, 36, 53). The characterisation of the effects of ruxolitinib treatment on the MC903 model will help the understanding the effects of ruxolitinib and JAK1/2 inhibition in AD.

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Figure Legends

Fig. 1. Ruxolitinib attenuates AD associated phenotypes in the MC903 mouse model.

(A) Representative photographs of MC903-treated mice following daily treatment of vehicle and ruxolitinib. Images were taken on Day 14 of their 14-day treatment regimen. (B) Daily measurement of ear swelling throughout the 14-day treatment period using an ear calliper. Graph represents changes in ear thickness relative to Day 1. Statistical analysis is performed with a two-way ANOVA with Tukey's test. Each data point represents an individual mouse, with 9 mice in each group. (C) Immunohistochemical assessment of pSTAT3 Y705 expression in ears of mice across the 4 treatment groups (ethanol/ vehicle; MC903/vehicle; ethanol/ ruxolitinib: MC903/ruxolitinib). (D) Scratch counts recorded in MC903-treated mice receiving ethanol/vehicle; ethanol/ruxolitinib; MC903/vehicle; MC903/ruxolitinib treatments. Each data point represents scratch counts from an individual mouse and each treatment group has 8-14 mice and an unpaired t-test was performed to determine statistical significance. (E) Flow cytometry analysis of immune cell populations in mouse ears with quantification of $CD45^+$ cells and T cells (including DETCs). Unpaired t-tests were performed to determine statistical significance. (F) Measurements of serum biomarker levels of TSLP, Il-5, Il-13 and Il-17A in mice at the end of the 14-days of treatment regime. Data is presented for different treatment groups: ethanol/vehicle, MC903/vehicle, and MC903/ruxolitinib and each data point represents an individual mouse, and each treatment group has 4-6 mice and unpaired t-tests were performed to determine statistical significance.

Fig. 2. Transcriptomic analysis and immunohistochemistry of cytokine-treated RHS.

(A) Top 10 gene ontology biological processes identified in significant differentially expressed genes (DEGs) of IL-13-treated RHS. (B) Illustration of top 30 upregulated and downregulated DEGs of IL-13-treated RHS. (C) Volcano plot illustrating DEGs in IL-13-treated RHS. (D) RNA levels of STAT genes in IL-13-treated RHS. [Orange bar denote statistically significant increased log fold change of RNA levels (false discovery rate, FDR<0.05); blue bar denote statistically significant decreased log fold change of levels (false discovery rate, FDR<0.05) while grey bars denote non-significant log fold change of RNA levels). (E) Immunohistochemistry depicting STAT3, skin-expressed proteins, filaggrin and loricrin, in control, IL-4 and IL-13, treated RHS. Experiments were performed in triplicate.

Fig. 3. Treatment effects of ruxolitinib on human skin biopsy explants and effects of ruxolitinib on the human and mouse skin barrier.

(A) Immunohistochemical analysis of pSTAT3 Y705 activation in explants treated with DMSO (control) or 1μM ruxolitinib, both in non-stripped and stripped conditions. Staining was performed in 4 samples, with a representative image shown. (B) Volcano plots illustrating DEGs in explants treated with ruxolitinib under both non-stripped and stripped conditions. (C) Identification of the top 5 gene ontology biological processes associated with differentially expressed upregulated genes in both non-stripped and stripped human skin explants treated with ruxolitinib. (D) Immunohistochemistry illustrating filaggrin expression in control and ruxolitinib-treated stripped human skin

explants. Staining was performed in 4 samples, with a representative image shown. (E) Immunohistochemistry illustrating expression of skin-expressed proteins, filaggrin and involucrin in MC903-treated mice that were treated with vehicle control or ruxolitinib. Staining was performed in 3 samples, with a representative image shown.

Fig. 4. Effect of ruxolitinib in a MC903 mouse model for AD. Created with BioRender.com.

Supplemental Information

Supplementary Fig. 1. Immunohistochemical assessment of pSTAT6 Y641 expression in ears of mice across the 4 treatment groups (ethanol/ vehicle; MC903/vehicle; ethanol/ ruxolitinib: MC903/ruxolitinib).

Supplementary Fig. 2. (A) Flow analysis gating strategy: Illustration of the gating strategy employed for flow analyses of immune cell subsets in the epidermis and dermis of MC903-treated mouse ear tissues. (B) Flow cytometry results of immune subsets in the ear tissues. (C) Flow cytometry results of immune subsets in the ear-draining lymph nodes.

Supplementary Fig. 3. Serum biomarker measurements: Quantification of cytokines IL-1β, IFNG, MCP-1, TARC, Eotaxin, IL-4, IL-31, IL-33 and IL-10 in mice at the conclusion of

the 14-day treatment regimen (ethanol/vehicle; MC903/vehicle; MC903/ruxolitinib). Each data point represents an individual mouse, and each treatment group has 4-6 mice.

Supplementary Fig. 4. Summary of immunohistochemistry analysis performed in triplicate RHS samples: (A) STAT3, (B) pSTAT3 Y705, (C) filaggrin, (D) loricrin and (E) involucrin in control RHS and IL-4- and IL-13- treated RHS.

Supplementary Fig. 5. Summary of immunohistochemistry analysis: STAT3, pSTAT3 Y705, filaggrin, involucrin and loricrin in stripped and non-stripped human skin explants treated with DMSO and 1μM ruxolitinib.

Supplementary Fig. 6. Transcriptomic fold change of reported transcription factors that regulate IL-13 in ruxolitinib- treated stripped human skin explants. (B) Immunohistochemistry and RNAscope illustrating expression of Il13 and Il13ra1 in MC903-treated mice that were treated with vehicle control or ruxolitinib.

Supplementary Fig. 7. Top 30 DEGs of ruxolitinib-treated stripped and non-stripped human skin explants.

Supplementary Fig. 8. Summary of immunohistochemistry analysis performed in triplicate mice: (A) filaggrin and (B) involucrin protein expression in MC903/vehicle and MC903/ruxolitinib-treated mice.

Supplementary Fig. 9. Transcriptomic fold change of ceramide synthases and fatty acid elongases derived from RNA sequencing of stripped skin biopsy explants that were treated with 1μM ruxolitinib.

Supplementary Table 1. List of antibodies utilized in the study.

Supplementary Table 2. Differentially expressed genes in IL-13 treated RHS versus control RHS (FDR <0.05).

Supplementary Table 3. Differentially expressed genes in ruxolitinib-treated skin explants (stripped and non-stripped) (FDR <0.05).