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Original research

IL-20 subfamily cytokines impair the oesophageal epithelial barrier by diminishing filaggrin in eosinophilic oesophagitis

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ABSTRACT

Objective Disruption of the epithelial barrier plays an essential role in developing eosinophilic oesophagitis (EoE), a disease defined by type 2 helper T cell (Th2)-mediated food-associated and aeroallergen-associated chronic inflammation. Although an increased expression of interleukin (IL)-20 subfamily members, IL-19, IL-20 and IL-24, in Th2-mediated diseases has been reported, their function in EoE remains unknown.

Design Combining transcriptomic, proteomic and functional analyses, we studied the importance of the IL-20 subfamily for EoE using patient-derived oesophageal three-dimensional models and an EoE mouse model.

Results Patients with active EoE have increased expression of IL-20 subfamily cytokines in the oesophagus and serum. In patient-derived oesophageal organoids stimulated with IL-20 cytokines, RNA sequencing and mass spectrometry revealed a downregulation of genes and proteins forming the cornified envelope, including filaggrins. On the contrary, abrogation of IL-20 subfamily signalling in *IL20R2*^{-/-} animals resulted in attenuated experimental EoE reflected by reduced eosinophil infiltration, lower Th2 cytokine expression and preserved expression of filaggrins in the oesophagus. Mechanistically, these observations were mediated by the mitogen-activated protein kinase (MAPK); extracellular-signal regulated kinases (ERK)1/2 pathway. Its blockade prevented epithelial barrier impairment in patient-derived air-liquid interface cultures stimulated with IL-20 cytokines and attenuated experimental EoE in mice.

Conclusion Our findings reveal a previously unknown regulatory role of the IL-20 subfamily for oesophageal barrier function in the context of EoE. We propose that aberrant IL-20 subfamily signalling disturbs the oesophageal epithelial barrier integrity and promotes EoE development. Our study suggests that specific targeting of the IL-20 subfamily signalling pathway may present a novel strategy for the treatment of EoE.

INTRODUCTION

Eosinophilic oesophagitis (EoE) is a food allergen-driven chronic inflammatory disease characterised by solid food dysphagia and food impaction in adults and food refusal and failure to thrive in children.^{1,2} Inflammation in EoE is predominated by a type 2 immune response triggered by food antigens.^{3,4} However,

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Eosinophilic oesophagitis (EoE) is a type 2 helper T cell (Th2)-mediated food-associated and aeroallergen-associated chronic inflammatory disease with increasing prevalence and puzzling pathophysiology.
- ⇒ Th2 cytokines interleukin (IL)-4 and IL-13 negatively impact oesophageal barrier integrity, resulting in increased permeability.
- ⇒ Members of the IL-20 cytokine family mediate crosstalk between immune cells and epithelial cells.

WHAT THIS STUDY ADDS

- ⇒ Active EoE is associated with increased expression of IL-20 subfamily cytokines in the oesophagus and serum.
- ⇒ IL-20 cytokines lower the expression of genes involved in oesophageal barrier integrity, including filaggrins.
- ⇒ Abrogation of IL-20 cytokine signalling attenuates experimental EoE in mice.
- ⇒ Inhibition of STAT3 signalling reinforces IL-20 subfamily-mediated reduction of filaggrins and aggravates experimental EoE in mice.
- ⇒ Pharmacological inhibition of ERK rescues IL-20 subfamily-mediated epithelial barrier impairment and attenuates experimental EoE in mice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

- ⇒ Targeting aberrant IL-20 subfamily signalling may represent a novel therapeutic approach for the treatment of EoE.
- ⇒ Ongoing clinical development of ERK inhibitors for several disease entities reflects their therapeutic potential. In EoE, ERK inhibitors could be used to restore epithelial integrity.



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the exact immunological pathways that lead to EoE are still poorly understood. An increasing number of studies illustrate that cytokines such as type 2 helper T cell (Th2)-derived interleukin (IL)-13 elicit transcriptional changes in epithelial cells, altering the integrity of the oesophageal epithelium.^{5–7}

Emerging evidence suggests impaired epithelial barrier integrity is a dominant element in EoE pathophysiology. Genome-wide association studies have identified most associated polymorphisms in the oesophageal epithelium, including variants in the filament aggregating protein filaggrin.^{8–10} Coinciding with this, reduction of proteins, such as filaggrins, desmogleins, occludin and claudins involved in the formation of the apical junction complex is observed in EoE.^{7 11 12} Epithelial barrier impairment by filaggrin reduction results from disturbed protease–protease inhibitor homeostasis and aberrant cytokine signalling in the oesophagus.^{8 13 14}

Growing evidence indicates that the IL-10 cytokine family significantly contributes to the crosstalk between the immune system and epithelial cells. Among these is the IL-20 subfamily, comprising IL-19, IL-20 and IL-24.¹⁵ All IL-20 subfamily members share the type 1 IL-20 receptor formed by IL-20R β and IL-20R α subunits. On the other hand, IL-20 and IL-24 but not IL-19 can also signal through the type 2 IL-20 receptor created by IL-20R β and IL-22R α 1.¹⁶ The activation of both type 1 and type 2 IL-20 receptors results in STAT3 phosphorylation,¹⁶ suggesting overlapping functions of IL-19, IL-20 and IL-24 by receptor sharing and conserved signalling cascades. IL-20 cytokine family members target keratinocytes¹⁷ in the skin, where all three cytokines can induce acanthosis and S100A7 expression.¹⁷ The IL-20 subfamily has been linked to Th2-mediated allergic skin inflammation and allergen-induced airway inflammation.^{18–20} In patients with atopic dermatitis, IL-24 downregulates the expression of filaggrin in keratinocytes.¹⁸ To the same effect, IL-19 and IL-24 mediate epidermal hyperplasia induced by intradermal IL-23 injection in an IL-20R β -dependent manner.²¹ Moreover, transgenic animals overexpressing IL-20 or IL-24 developed epidermal hyperplasia and abnormalities in keratinocyte differentiation,^{15 22} consolidating the relationship between IL-20 subfamily cytokines and epithelial cells. However, it has not yet been studied if the aberrant activity of the IL-20 subfamily affects keratinocyte differentiation in the oesophagus. Thus, we explored whether dysregulation of the IL-20 subfamily could impair the oesophageal epithelial barrier during EoE.

MATERIALS AND METHODS

The resources used are listed in online supplemental tables 4–6. Further details of resources and methods are described in the online supplemental data file.

Oesophageal biopsies from human subjects

Patients referred for diagnostic oesophagogastroduodenoscopy were evaluated for EoE or eligibility as control. Biopsies were taken from the distal and proximal oesophagus and assessed by an independent pathologist. Control subjects were defined as having no endoscopic and histological signs of oesophagitis, nil eosinophils per high-power field (HPF), no history of EoE and no hints of motility disorders.

Patients with EoE were defined as having a confirmed EoE diagnosis or presenting with ≥ 15 eosinophils per HPF in proximal biopsies, symptoms suggestive of oesophageal dysfunction, such as solid food dysphagia or food impaction, and not having any endoscopic and histological signs of GERD.

Detailed patient characteristics are listed in online supplemental table 1.

Patient and public involvement statement

Patients or the public were not involved in our study's design, conduct, reporting or dissemination plans.

Statistical analysis

Data are shown as dot plots and display individual values with medians. GraphPad Prism software was used to generate the graphs and perform statistical analysis. Depending on the experimental setting, p values were calculated using either Mann-Whitney U, Wilcoxon, Kruskal-Wallis or two-way analysis of variance tests. The Grubbs test further analysed the data to identify outliers. P values were shown as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

RESULTS

Elevated IL-20 subfamily cytokines in EoE and type 1 IL-20 receptor expression by the oesophageal epithelium

Recent reports have suggested an association of the IL-20 subfamily with Th2-mediated allergies and allergen-induced airway inflammation,^{18–20} but as of yet, neither their function in the oesophagus nor a potential role in the context of EoE has been studied. Patients with active EoE have significantly increased *IL19* and *IL20* but not *IL24* expression in oesophageal biopsies compared with control individuals (figure 1A), while topical corticosteroid treatment in patients with active EoE reduced *IL19* and *IL20* expression in oesophageal biopsies (figure 1A,B). Serum concentrations of all IL-20 subfamily cytokines, including IL-24, were increased in patients with active EoE compared with control individuals, while treatment with topical corticosteroids lowered IL-20 subfamily cytokines in the serum of patients achieving remission (figure 1C), suggesting for the first time that IL-20 subfamily cytokines may play a role in the active phase of EoE.

To identify cellular targets of IL-20 subfamily cytokines, we performed a RT-qPCR analysis of the IL-20R subunits. The expression analysis indicated *IL20Ra*, *IL20Rb* and *IL22Ra1* expressions in oesophageal biopsies with lower *IL20Ra* and *IL20Rb* expressions in patients with active EoE and topical corticosteroid-treated inactive EoE compared with control individuals (figure 1D). Immunohistochemistry-stained sections from the oesophagus confirmed the predominant expression of IL-20R α and IL-20R β by oesophageal epithelial cells (figure 1E). Collectively, we demonstrate dysregulated IL-20 subfamily cytokine responses in patients with active EoE and expression of the type 1 IL-20 receptor by the oesophageal epithelium.

IL-20 cytokines reduce filaggrin expression in patient-derived oesophageal organoids

To directly address the impact of the IL-20 subfamily on the oesophageal epithelium, we have adopted a protocol to generate oesophageal organoids from patient-derived biopsies.²³ Cells isolated from oesophageal biopsies of control individuals and patients with EoE form self-organising organoids, which proliferate without morphological differences over 11 days (online supplemental figure 1). IL-20R α expression increased during the culture period with the highest expression on day 11, while IL-20R β is constitutively expressed throughout the culture period in controls and EoE (online supplemental figure 1).

We stimulated organoids from control individuals with IL-19, IL-20 and IL-24 to elucidate the effect of the IL-20 subfamily on the transcriptome and proteome of the oesophageal epithelium.^{24 25} Using a principal component analysis (PCA), we observed that interindividual differences dominated the RNA sequencing (RNA-seq) of paired non-stimulated and stimulated organoids. Deeper principal components revealed segregation of non-stimulated from stimulated oesophageal organoids (figure 2A). To gain insights into molecular mechanisms by which

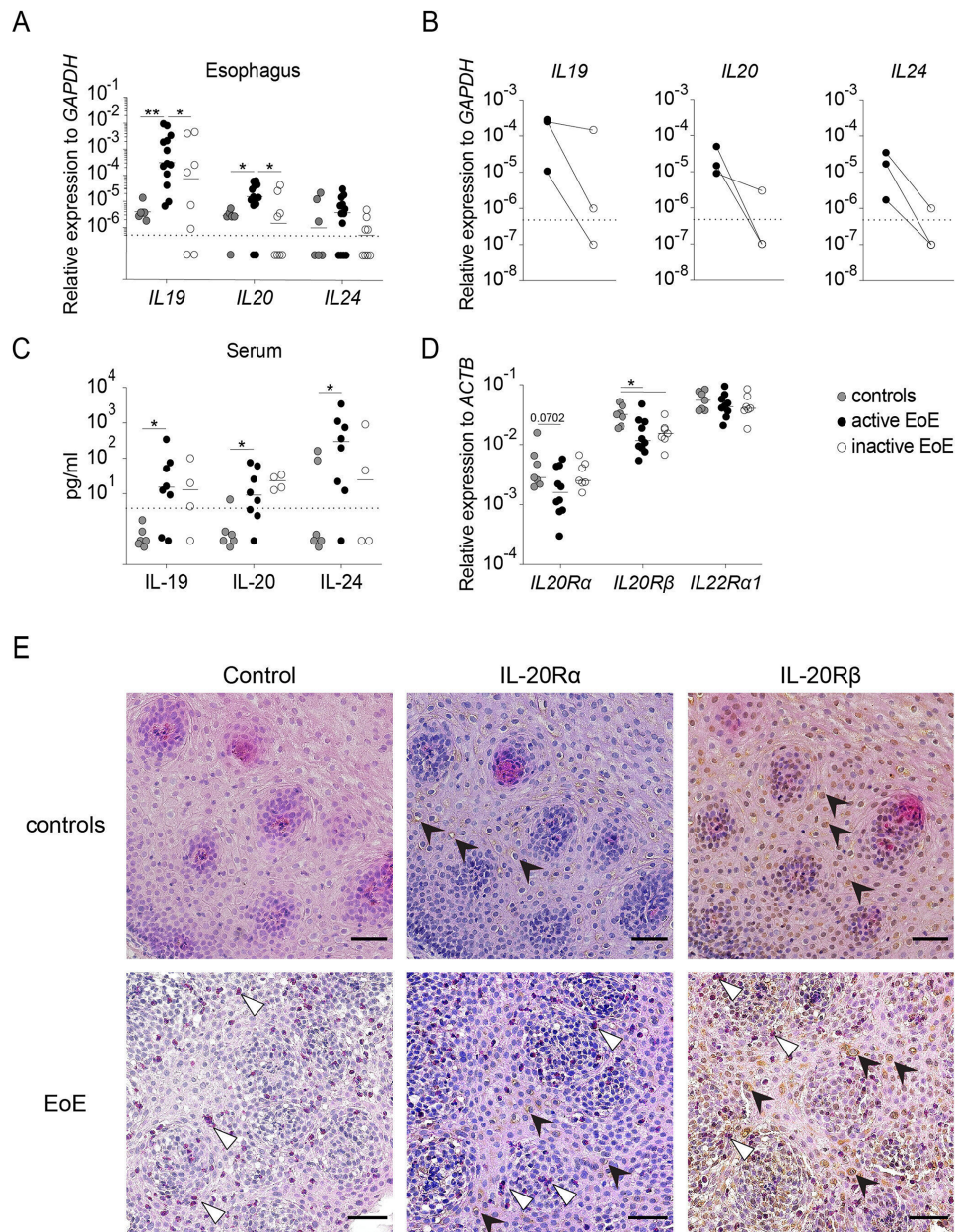


Figure 1 Increased IL-20 cytokines in active EoE. (A) *IL19*, *IL20*, *IL24* mRNA expression levels in the oesophagus of control individuals, active EoE and inactive EoE treated with topical corticosteroids measured by RT-qPCR. (B) Paired *IL19*, *IL20* and *IL24* mRNA expression levels in the oesophagus measured by RT-qPCR before and during treatment with topical corticosteroids. (C) IL-19, IL-20 and IL-24 serum concentrations of indicated groups measured by ELISA. (D) *IL20Rα*, *IL20Rβ* and *IL22Rα1* mRNA expression in control individuals, active EoE and inactive EoE treated with topical corticosteroids by RT-qPCR. (E) Representative control staining, IL-20Rα and IL-20Rβ staining of the proximal oesophagus obtained from control individuals and patients with EoE. White arrowheads indicate eosinophils; black arrowheads indicate IL-20Rα and IL-20Rβ staining. Scale bars, 50 μm. Each dot or line represents one individual with medians. * $P \leq 0.05$, ** $P \leq 0.01$ by Mann-Whitney U test. EoE, eosinophilic oesophagitis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

IL-19, IL-20 and IL-24 affect the oesophageal squamous epithelium, we performed a differential expression analysis between stimulated and non-stimulated oesophageal organoids followed by gene set enrichment analysis (GSEA). Differentially expressed genes included notably keratins (ie, *KRT4*, *KRT13* and *KRT24*), components of desmosomes (ie, *DSG1* and 4, *DSC1* and 2 and *DSP*), members of the tight junction complex (ie, *OCN1* and *CLDN17*), filament-associated proteins, such as *FLG* and *FLG2*, and members of the serine protease inhibitor kazal (SPINK)-type family, such as *SPINK7* (figure 2B,C).

Furthermore, we noted an increased expression of the IL-13 receptor subunits *IL-4Ra*, *IL-13Ra1* and *IL-13Ra2* (online supplemental figure 2). Above all, downregulated genes were associated with keratinisation, cornification, cornified envelope and epidermal differentiation (figure 2D).²⁴

We compared these results to the transcriptome differences previously reported in oesophageal keratinocytes stimulated with IL-13 (GSE65335),²⁶ SPINK7-deficient oesophageal keratinocytes (GSE103356)²⁷ and the EoE transcriptome (GSE58640)²⁸ (online supplemental figure 3). The gene ontology (GO) terms cornification,

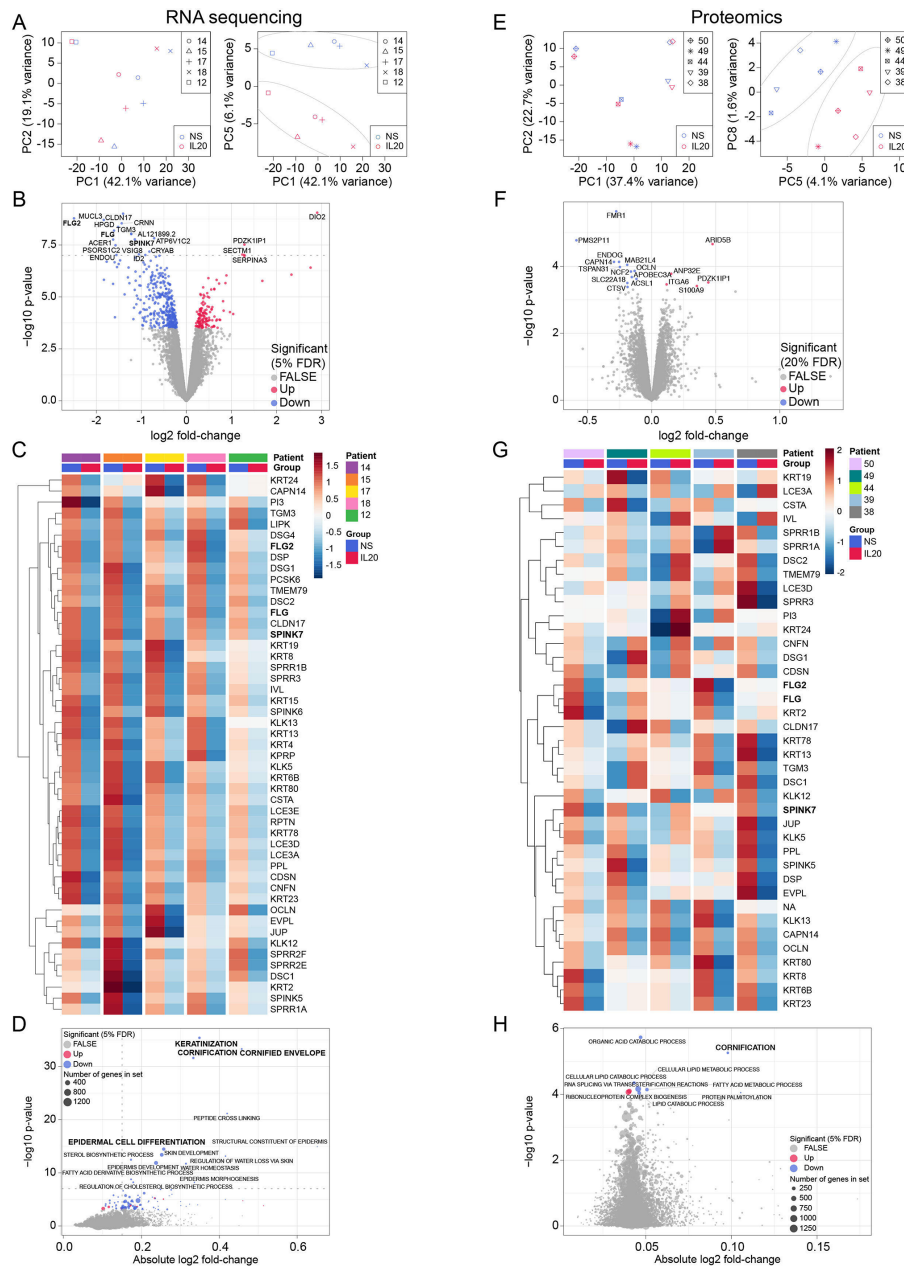


Figure 2 The stimulation of patient-derived oesophageal organoids with IL-19, IL-20 and IL-24 lowers the expression of genes involved in barrier integrity. (A) PCA based on the normalised expression levels in the RNA-seq samples of paired non-stimulated and IL-20 subfamily (IL-19, IL-20 and IL-24) stimulated oesophageal organoids from control individuals (numbered 12, 14, 15, 17 and 18). (B) Volcano plot showing the results of the differential expression analysis between non-stimulated and stimulated human oesophageal organoids. Only genes with a p value lower than 10^{-7} are labelled, and genes of the highest interest for our study are highlighted in bold. (C) Heatmap showing the centred and scaled expression levels of a selected subset of significant differentially expressed genes associated with the epithelium. Genes of the highest interest for our study are highlighted in bold. (D) GSEA results based on differentially expression results from the RNA-seq dataset. The x-axis depicts the average absolute log-fold change across genes within each category. Only categories with a p value lower than 10^{-7} and absolute average log-fold change of >0.15 are labelled. (E) PCA based on normalised protein levels in the proteomics samples of paired non-stimulated and IL-20 subfamily (IL-19, IL-20 and IL-24) stimulated oesophageal organoids from control individuals (numbered 38, 39, 44, 49 and 50). (F) Volcano plot showing the differential protein expression analysis results between non-stimulated and stimulated human oesophageal organoids. All significant proteins are labelled. (G) Heatmap showing the centred and scaled expression levels of a subset of proteins from the genes used (C) and present in the proteomics dataset. Proteins of the highest interest for our study are highlighted in bold. (H) Results of the GSEA based on differentially expression results from the proteomics dataset. GSEA, gene set enrichment analysis; IL, interleukin; PCA, principal component analysis; RNA-seq, RNA sequencing.

cornified envelope, epidermal cell differentiation and keratinisation were downregulated in all four datasets. Interestingly, the GO term keratin filament was only downregulated in our dataset and the EoE transcriptome. Additional IL-20 subfamily-specific effects on the epithelium were reflected by downregulation of the GO terms tight

junction, desmosome, epidermis morphogenesis and apical junctional complex in our but not in the other reported transcriptomic datasets (online supplemental figure 3).

Consistent with the transcriptomic data, proteomics analysis of paired non-stimulated and stimulated oesophageal

organoids from different control individuals displayed high interindividual differences in the PCA. Similarly, segregation of non-stimulated oesophageal organoids from their stimulated counterparts was seen in deeper principal components (figure 2E). Differential expression analysis and GSEA on the protein levels revealed that the extent of changes associated with IL-20 subfamily cytokine stimulation was lower than that observed at the transcriptome level. However, similarly, proteins related to epithelial cornification and differentiation were downregulated (figure 2F–H).²⁵

Despite the altered expression of genes and proteins involved in epithelial cornification and differentiation, we did not observe morphological changes in the organoids on cytokine stimulation (figure 3A). However, RT-qPCR and immunohistochemistry confirmed that stimulating oesophageal organoids from control individuals and patients with EoE with IL-19, IL-20 and IL-24 reduced *FLG*, *FLG2* and *SPINK7* expressions (figure 3B–D), while the receptor expression remained unchanged (figure 3E). Moreover, in patients with active EoE, we observed reduced *FLG*, *FLG2* and *SPINK7* expressions in the oesophagus compared with controls and patients with inactive EoE treated with topical corticosteroids (figure 3F). Immunohistochemistry confirmed reduced *FLG* and *FLG2* levels in the oesophageal epithelium of patients with active EoE, which was partially restored in inactive EoE (figure 3G,H). Together, our data illustrate that the stimulation of patient-derived oesophageal organoids with IL-19, IL-20 and IL-24 reduced the expression of genes and proteins involved in maintaining the epithelial barrier, including the filaggrin family and the serine protease inhibitor *SPINK7*.

Increased IL-20 cytokine expression in murine EoE-like disease model

Because sensitisation to food particles in patients with EoE can occur via the skin,^{29–31} we decided to adopt an experimental EoE mouse model induced by epicutaneous allergen sensitisation (online supplemental figure 4A).³² Ovalbumin (OVA)-skin sensitised and orally challenged (sens+chal) mice developed experimental EoE characterised by oesophageal eosinophil infiltration (online supplemental figure 4B–E). Untreated (ctrl), non-sensitised (non-sens) and sensitised but not challenged (non-chal) animals did not develop experimental EoE (online supplemental figure 4B–E). Consistent with findings in human patients with EoE, mice with experimental EoE demonstrated significantly increased *Il19* and increased *Il20* and *Il24* expression levels in the oesophagus compared with ctrl and non-chal animals (figure 4A). Flow cytometry analysis of *Il19-tdTomato* (*Il19^{tdT}*) reporter animals with experimental EoE indicated that macrophages in the oesophagus of mice with EoE produce *Il19* (online supplemental figure 4F,G).

In the GI tract of mice, the expression of type 1 IL-20 receptor subunits *Il20ra* and *Il20rb* was highest in the oesophagus and forestomach (squamous epithelium) compared with lower expression in the glandular stomach and the small intestine (columnar epithelium) (online supplemental figure 4H). Noteworthy, murine bone marrow-derived macrophages (BMDMs) only express *Il20rb* but not *Il20ra* and *Il22ra1* (online supplemental figure 4I), suggesting that macrophages do not have a functional IL-20 receptor. Consistently, single-cell RNA-seq of CD45⁺ immune cells from mice with experimental EoE indicates that immune cells generally do not express a functional IL-20 receptor (online supplemental figure 4J).³³ In summary and consistent with an observed phenotype in humans, experimental

EoE in mice is characterised by oesophageal eosinophilia and increased *Il19*, *Il20* and *Il24* expressions in the oesophagus.

IL-20R deficiency is protective in the EoE mouse model

Next, we induced the experimental EoE mouse model in wild type (WT), IL-19-deficient (*Il19^{tdT}*) and *Il20R2^{-/-}* mice, in which signalling of all three IL-20 cytokines is impaired. Detection of total IgE and OVA-specific IgE by ELISA confirmed successful sensitisation in WT, *Il19^{tdT}* and *Il20R2^{-/-}* mice (online supplemental figure 5A,B). Chal+sens *Il20R2^{-/-}* mice had significantly reduced eosinophils in the oesophagus compared with WT and *Il19^{tdT}* mice, evidenced by H&E staining (figure 4B,C) and flow cytometry (figure 4D,E) at day 4 of OVA challenge when WT animals have developed severe experimental EoE. While *Il19^{tdT}* mice had reduced eosinophil numbers in the oesophagus compared with WT mice, specific deletion of *Il19* from CX3CR1⁺ macrophages in a novel conditional *Il19*-deficient mouse line (online supplemental figure 5C) suggested that macrophage-derived IL-19 alone does not account for EoE in mice (online supplemental figure 5D–G). Increased oesophageal eosinophilia in chal+sens WT animals was associated with elevated *Il5* and *Il13* expression in the oesophagus (online supplemental figure 5H,I). At the same time, EoE-protected *Il20R2^{-/-}* mice had lower *Il5* and *Il13* expression than WT mice (online supplemental figure 5H,I). Intriguingly, non-chal and sens+chal WT mice had reduced *Flg* and *Flg2* but not *Spink7* gene expression in the oesophagus compared with ctrl and non-sens animals (figure 5A). Both sensitisation with MC903 and OVA challenge in *Il19^{tdT}* and *Il20R2^{-/-}* mice did not reduce *Flg* and *Flg2* gene expression (figure 5B,C). Immunohistochemistry showed reduced suprabasal *Flg2* in the stratum spinosum of the oesophagus with retained expression in the stratum corneum of non-chal and sens+chal WT and *Il19^{tdT}* mice, whereas *Flg2* expression was preserved in all oesophageal epithelial layers of *Il20R2^{-/-}* mice (figure 5D,E). In summary, EoE is attenuated in *Il20R2^{-/-}* mice which may be linked to preserved expression of the filaggrin family in the oesophagus.

IL-20-mediated filaggrin reduction is STAT3-independent

Because signalling through the type 1 IL-20 receptor phosphorylates STAT3,¹⁶ we hypothesised that STAT3 activation is responsible for the IL-20 subfamily-mediated decrease of *FLG* and *FLG2* in squamous epithelial cells. To verify our hypothesis, we stimulated the oesophagus squamous cell carcinoma cell line KYSE-180 with IL-19, IL-20 and IL-24 confirming that the IL-20 subfamily cytokines activate STAT3 (online supplemental figure 6A and online supplemental figure 8A). Interestingly, stimulation with IL-19, IL-20 and IL-24 also resulted in ERK1/2 phosphorylation, while NF-κB (p65) was not activated (online supplemental figure 6B,C, and online supplemental figure 8B,C). Surprisingly, subsequent treatment of IL-20R-expressing KYSE-180 cells (online supplemental figure 6D) with IL-20 subfamily cytokines and the STAT3 inhibitor cucurbitacin 1 resulted in a further decrease of *FLG*, *FLG2* and *SPINK7* expression (online supplemental figure 6E). Accordingly, cucurbitacin 1 also lowered *FLG*, *FLG2* and *SPINK7* in patient-derived oesophageal organoids stimulated with IL-19, IL-20 and IL-24 (online supplemental figure 6F). To corroborate the impact of STAT3 signalling on the oesophageal epithelium, we crossed *Stat3^{fllox/fllox}* with *Krt5-CreERT2* mice to generate animals with a tamoxifen-inducible, squamous epithelium-specific deletion of STAT3. Immunofluorescence confirmed the deletion of epithelial

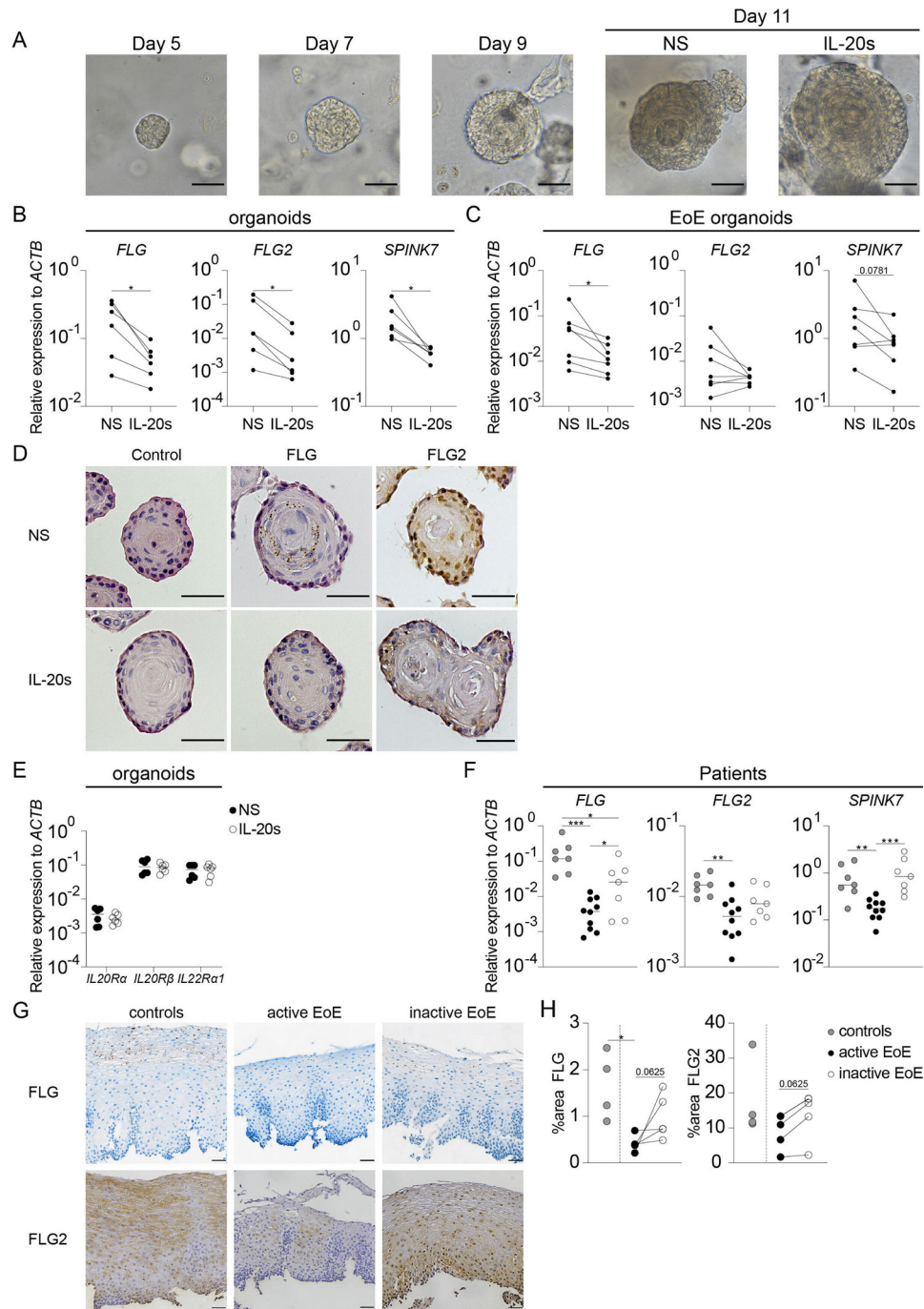


Figure 3 Stimulation with IL-19, IL-20 and IL-24 decreases filaggrin expression. (A) Bright-field images of oesophageal organoids from control individuals at the indicated time. On day 11, organoids were stimulated with IL-19, IL-20 and IL-24 (IL-20s). Scale bars, 50 μ m. (B) *FLG*, *FLG2* and *SPINK7* mRNA expression of paired non-stimulated (NS) and with IL-19, IL-20 and IL-24 (IL-20s) stimulated organoids from control individuals or (C) patients with EoE measured by RT-qPCR. (D) Immunohistochemistry for FLG or FLG2 and control of non-stimulated (NS) and with IL-19, IL-20 and IL-24 (IL-20s) stimulated oesophageal organoids from control individuals. Scale bars, 50 μ m. (E) *IL20R α* , *IL20R β* and *IL22R α 1* mRNA expression in non-stimulated (NS) and with IL-19, IL-20 and IL-24 (IL-20s) stimulated oesophageal organoids. (F) *FLG*, *FLG2* and *SPINK7* mRNA expression in the proximal oesophagus of control individuals, active EoE and inactive EoE. (G) FLG and FLG2 staining with haematoxylin counterstaining in the proximal oesophagus from control individuals, active EoE and inactive EoE. Scale bars, 50 μ m. (H) Quantification of FLG and FLG2 staining using QuPath software. Data are presented as individual values with medians, with each dot or line representing one biological replicate. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ by Mann-Whitney U or Wilcoxon test. EoE, eosinophilic oesophagitis; IL, interleukin; NS, non-stimulated; RT-qPCR, RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

STAT3 on tamoxifen injection (online supplemental figure 6G). Tamoxifen-injected *Stat3 Δ Krt5* mice have increased eosinophils and SiglecF⁺CD45⁺ immune cells in the oesophagus compared with tamoxifen-treated *Stat3^{fllox/fllox}* littermates

demonstrated by H&E staining (figure 6A,B) and flow cytometry analysis (figure 6C–E and online supplemental figure 6H,I), with highest numbers in sens+chal mice. In contrast, Flg2 expression in the oesophagus of *Stat3 Δ Krt5* mice

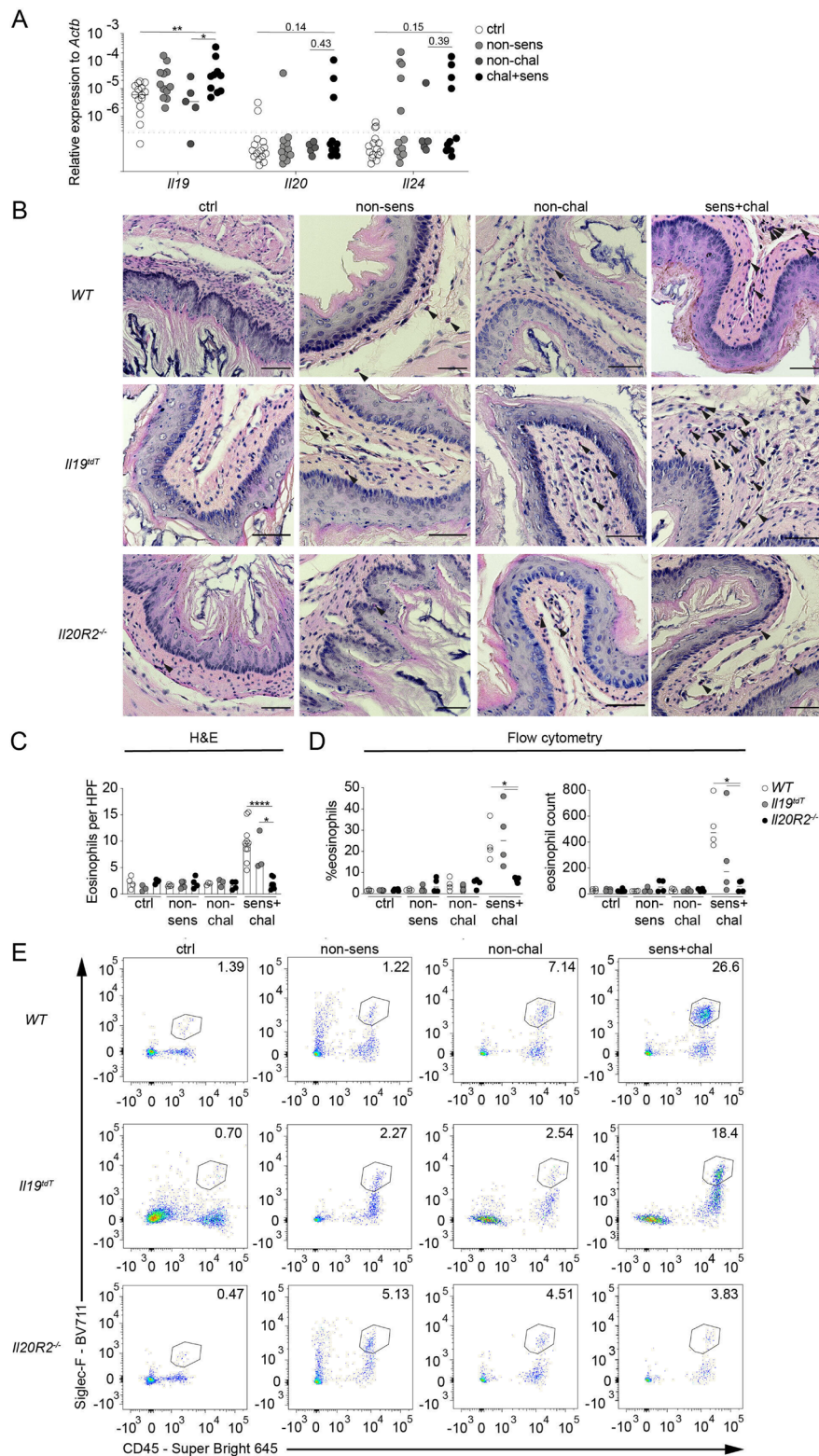


Figure 4 Abrogation of IL-20 cytokine signalling attenuates experimental EoE. (A) *IL19*, *IL20* and *IL24* mRNA expressions in the murine oesophagus by RT-qPCR. (B) H&E staining of oesophageal sections of WT, *IL19^{tdT}* and *IL20R2^{-/-}* animals. Black arrowheads indicate eosinophils. Scale bars, 50 μ m. (C) Quantification of eosinophils (per HPF) from (B). (D) Frequencies and absolute numbers of eosinophil infiltrates in the oesophagus as assessed by flow cytometry. (E) Representative flow cytometry plots of eosinophil frequencies in the oesophagus. Data are presented as individual values with medians, with each dot representing one biological replicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, by Mann-Whitney U test. ctrl, non-sensitised+non-challenged; HPF, high-power field; IL, interleukin; non-chal, sensitised+non-challenged; non-sens: non-sensitised+challenged; sens+chal: sensitised+challenged; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WT, wild type.

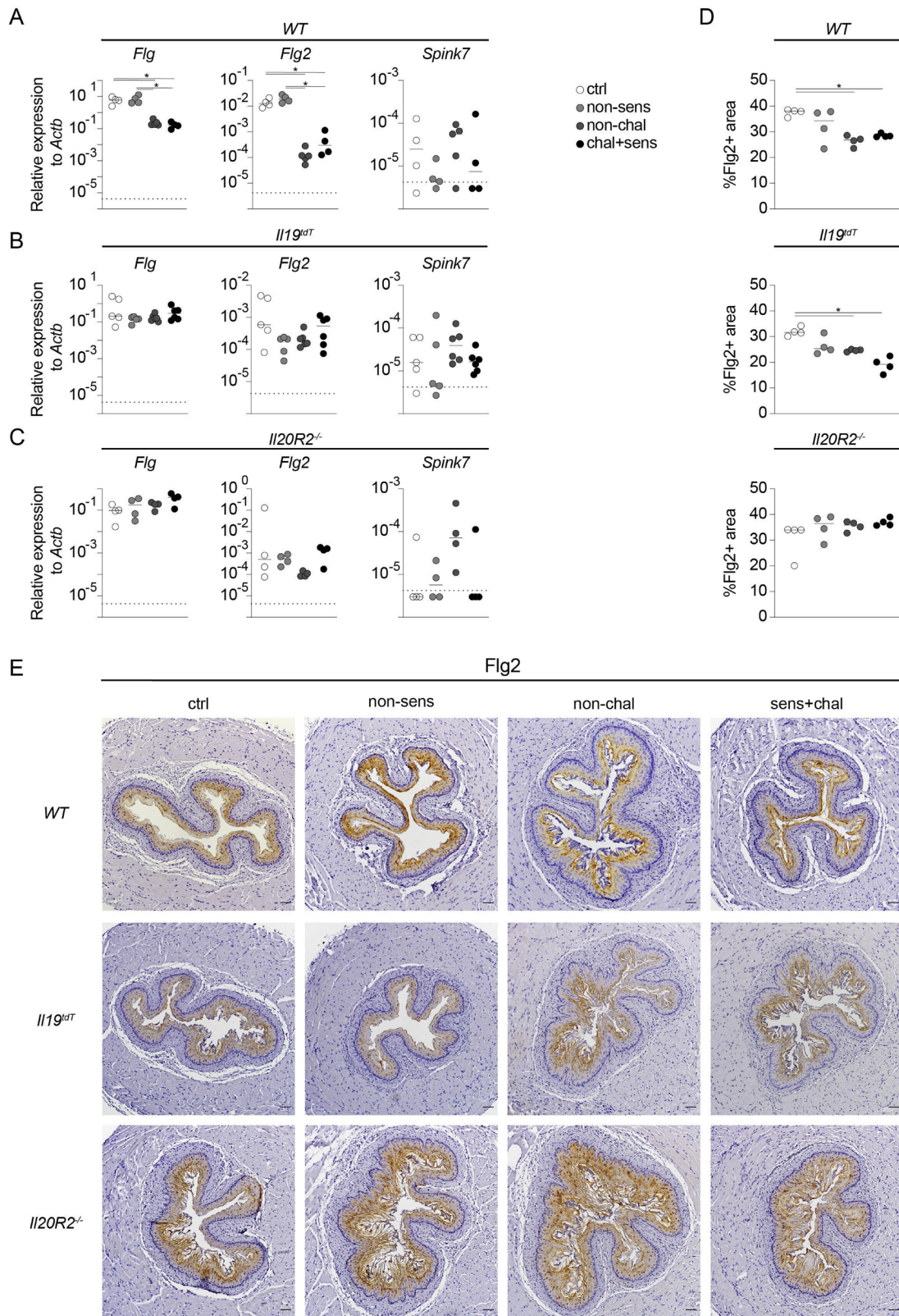


Figure 5 Abrogation of IL-20 signalling preserves filaggrin expression in experimental EoE. (A–C) *Flg*, *Flg2* and *Spink7* mRNA expression in (A) WT, (B) *Il19^{dfl}* and (C) *Il20R2^{-/-}* mice quantified by RT-qPCR. (D+E) Flg2 immunohistochemistry of WT, *Il19^{dfl}* and *Il20R2^{-/-}* animals; (D) percentage of Flg2 stained area (analysed with Fiji (ImageJ V.2.0.0-rc-68/1.52 hour)) and (E) representative images. Scale bars, 50 μ m. Data are presented as individual values with medians, with each dot representing one biological replicate. * $P < 0.05$, by Mann-Whitney U test. ctrl, non-sensitised+non-challenged; EoE, eosinophilic oesophagitis; IL, interleukin; non-chal, sensitised+non-challenged; non-sens, non-sensitised+challenged; sens+chal, sensitised+challenged; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WT, wild type.

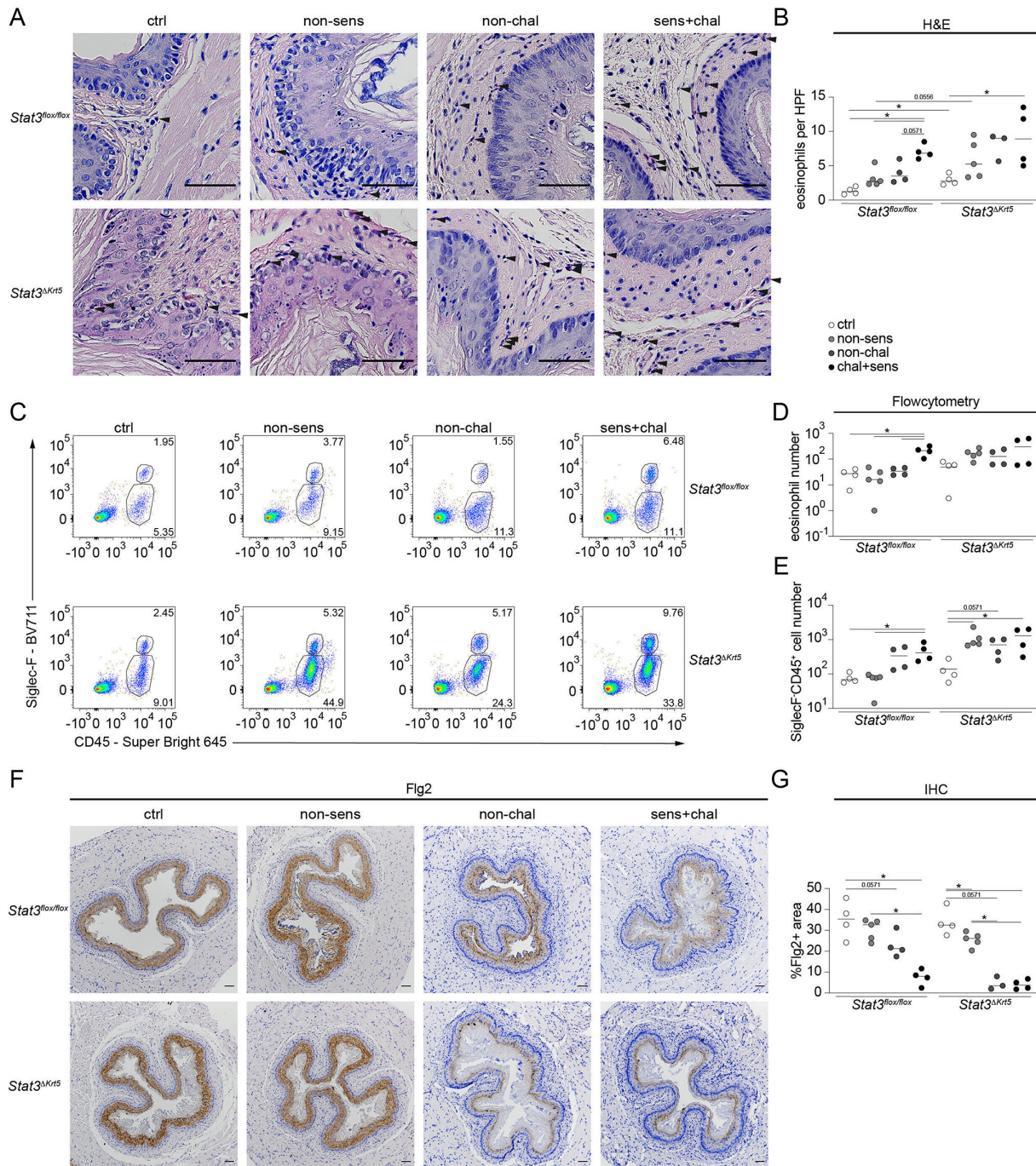


Figure 6 STAT3 deletion in oesophageal epithelial cells aggravates experimental EoE. (A) H&E staining of oesophageal sections obtained from *Stat3^{fllox/fllox}* and *Stat3^{ΔKrt5}* animals. Arrows indicate eosinophils. Scale bars, 50 μ m. (B) Counted eosinophils (per HPF) from (A). (C–E) Flow cytometry of oesophageal cell isolates from *Stat3^{fllox/fllox}* and *Stat3^{ΔKrt5}* mice of indicated treatment groups. (C) Representative flow cytometry blots showing CD45⁺Siglec-F⁺ eosinophils and Siglec-F⁻CD45⁺ immune cells in the oesophagus. Numbers indicate the percentage of eosinophils and Siglec-F⁻CD45⁺ immune cells. Dot blots showing (D) absolute eosinophil and (E) Siglec-F⁻CD45⁺ immune cell numbers in the oesophagus. (F,G) Flg2 staining of oesophagus sections from *Stat3^{fllox/fllox}* and *Stat3^{ΔKrt5}* mice of indicated treatment groups. (F) Representative images; scale bars, 50 μ m; (G) Percentage of Flg2 stained area analysed with Fiji (ImageJ V.2.0.0-rc-68/1.52 hour). Data are presented as individual values with medians, with each dot representing one biological replicate. * $P < 0.05$, by Mann-Whitney U test. ctrl, non-sensitised+non-challenged; EoE, eosinophilic oesophagitis; HPF, high-power field; non-chal, sensitised+non-challenged; non-sens, non-sensitised+challenged; sens+chal, sensitised+challenged.

was drastically reduced in non-chal and sens+chal animals (figure 6F,G). These data reveal that the specific deletion of STAT3 signalling in the squamous epithelium increases epithelial inflammation and interferes with IL-20 subfamily-mediated filaggrin downregulation.

IL-20 subfamily-mediated epithelial barrier impairment is ERK-dependent

Reduced expression of the filaggrin family in human oesophageal organoids stimulated with IL-19, IL-20 and IL-24 prompted us to investigate whether the IL-20 subfamily regulates epithelial

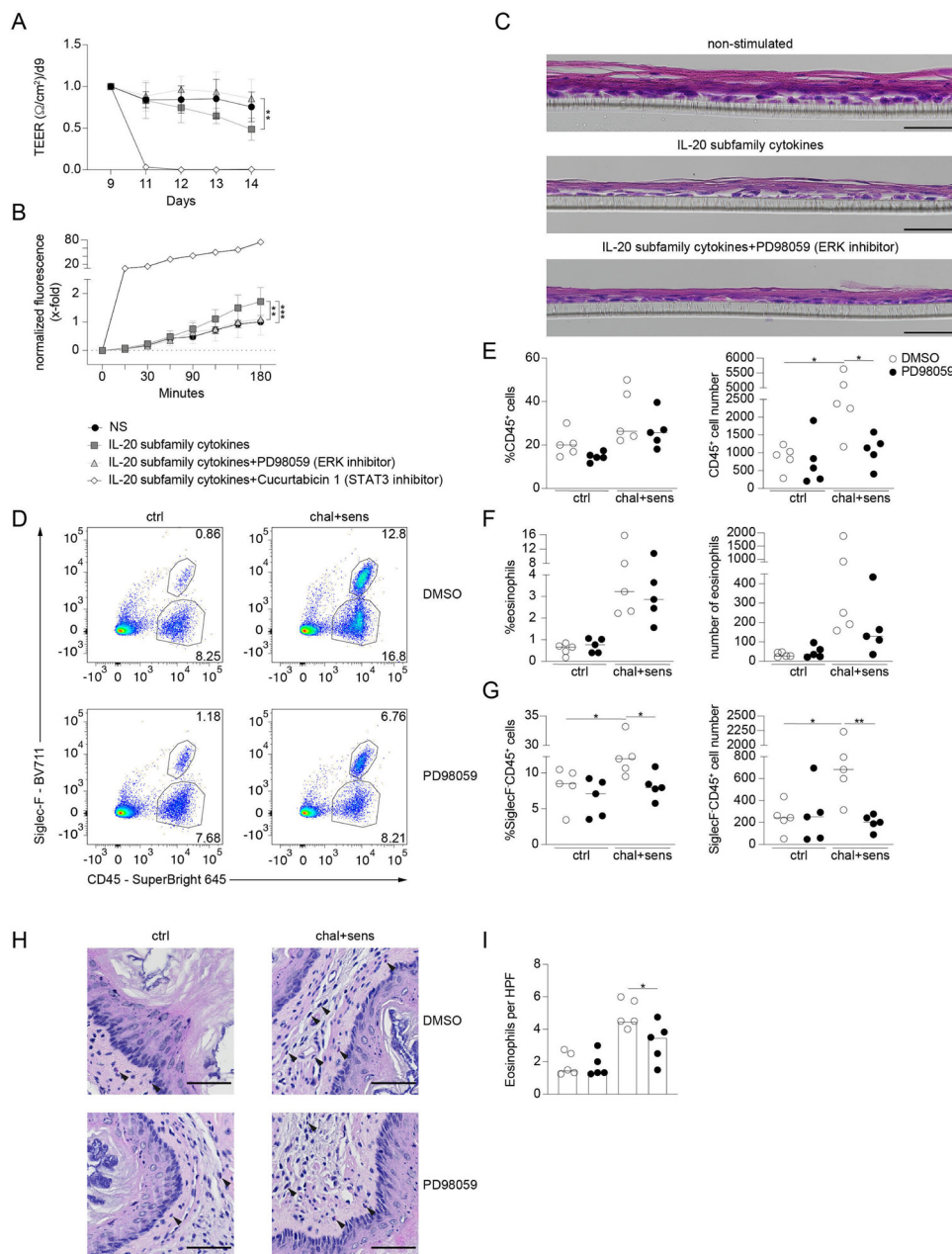


Figure 7 ERK inhibition restores barrier function and attenuates experimental EoE. (A) TEER and (B) macromolecule flux of ALI cultures from primary human oesophageal epithelial cells (n=4) stimulated with IL-20 subfamily cytokines (IL-19, IL-20 and IL-24), IL-20 subfamily cytokines and the ERK inhibitor PD98059, and IL-20 subfamily cytokines and the STAT3 inhibitor cucurtabacin 1. (C) Representative H&E staining of non-stimulated and with IL-20 subfamily cytokines or IL-20 subfamily cytokines and PD98059-stimulated ALI cultures. Scale bars, 50 μ m. (D) Representative dot blots from oesophagus cell isolates measured by flow cytometry of dimethyl sulfoxide (DMSO) vehicle and PD98059-treated ctrl and sens+chal *WT* animals. (E) Percentage and absolute numbers of CD45⁺ cells, (F) Siglec-F⁺CD45⁺ eosinophils and (G) Siglec-F⁺CD45⁺ cells from indicated experimental groups. (H) H&E staining of oesophageal sections of DMSO (vehicle) and PD98059-treated ctrl and sens+chal *WT* animals. Black arrowheads indicate eosinophils. Scale bars, 50 μ m. (I) Quantification of eosinophils (per HPF) from (H). Data are presented as individual values with medians, with each dot representing one biological replicate. Each dot (A,B) represents the mean of four biological replicates; error bars represent SD. **P*<0.05, ***P*<0.01, ****P*<0.001, by Mann-Whitney U test or two-way analysis of variance. ALI, air-liquid interface; ctrl, non-sensitised+non-challenged; DMSO, dimethyl sulfoxide; EoE, eosinophilic oesophagitis; HPF, high-power field; IL, interleukin; NS, non-stimulated; sens+chal, sensitised+challenged; TEER, transepithelial resistance; *WT*, wild type.

barrier integrity in the oesophagus. We, therefore, established air-liquid interface (ALI) cultures from biopsy-derived primary keratinocytes and stimulated them with IL-19, IL-20 and IL-24. Transepithelial electrical resistance (TEER), reflecting the integrity and permeability of the epithelium, was reduced in ALI cultures treated with IL-20 subfamily cytokines, substantiating

the role of this cytokine family in the regulation of epithelial barrier integrity (figure 7A). While adding the STAT3 inhibitor cucurtabacin 1 to the medium aggravated TEER reduction, the addition of the MAPK (ERK1/2) inhibitor PD98059 rescued the IL-20 subfamily-mediated TEER reduction (figure 7A), confirming our previous observations (figure 6 and online

supplemental figure 6). Increased macromolecular flux by fluorescein isothiocyanate (FITC)–dextran (4 kDa) verified increased epithelial permeability in patient-derived ALI cultures stimulated with the IL-20 subfamily cytokines, which was prevented by PD98059 treatment (figure 7B). Consistently, cucurbitacin 1 treatment increased the permeability in the FITC–dextran flux assay (figure 7B). Furthermore, H&E staining visualises the lack of a cornified epithelial layer in patient-derived ALI cultures stimulated with IL-20 subfamily cytokines compared with non-stimulated and PD98059-treated ALI cultures, reflecting reduced epithelial differentiation (figure 7C).

Collectively, these data support a functional impairment of the oesophageal epithelial barrier mediated through IL-20 subfamily cytokine stimulation.

The observation that inhibition of the ERK pathway may forestall epithelial impairment raised the question of whether ERK inhibition could serve as a therapeutic option in EoE. To answer this question, we treated sens+chal^{WT} mice with PD98059, resulting in decreased numbers of infiltrating eosinophils and SiglecF⁺CD45⁺ immune cells in the oesophagus compared with vehicle-treated chal+sens mice (figure 7D–G). H&E staining of oesophageal sections confirmed lower eosinophil infiltration in PD98059-treated sens+chal mice (figure 7H,I). These results suggest that ERK inhibition can prevent IL-20 subfamily-mediated impairment of the oesophageal epithelial barrier attenuating experimental EoE in mice.

DISCUSSION

In this study, by combining transcriptomics and proteomics of patient-derived oesophageal organoids together with functional assays in patient-derived ALI cultures and animal models, we show that dysregulated IL-20 subfamily cytokine expression in EoE downregulates the expression of genes and proteins involved in epithelial cornification and differentiation including the filaggrin family. Our data suggest that the IL-20 subfamily contributes to epithelial barrier impairment in EoE. Targeting the IL-20 subfamily signalling pathway using MAPK inhibitors might be a potential therapeutic strategy to restore epithelial barrier integrity in EoE.

We found increased expression of IL-20 subfamily cytokines in individuals with active EoE, which decreased in inactive EoE after treatment with topical corticosteroids. Importantly, elevated IL-19 and IL-20 concentrations have been noted in the oesophagus and the serum. Nevertheless, further investigations are required to assess whether IL-20 subfamily serum concentrations potentially qualify as biomarkers to predict EoE activity. However, the cellular sources of IL-19, IL-20 and IL-24 in the oesophagus have not been explored. Persisting ambiguities concerning the cellular origins of IL-19, IL-20 and IL-24 lead us to conduct experiments with *Il19^{tdT}* reporter animals, illustrating that macrophages in the oesophagus are one potential source of IL-19. Similar to the skin, fibroblasts and keratinocytes embody different possible sources of IL-20 subfamily cytokines in the oesophagus.³⁴ Because all three cytokines have a typical expression pattern, the genetic development of reporter mouse lines or fate-mapping experiments may help to unravel further sources of IL-20 subfamily members in the oesophagus.

The interplay between immune cells, the cytokines they produce and the epithelium has been proposed to be a decisive factor in the development of EoE.^{8 35–37} Genome-wide association studies and candidate-gene analysis identified EoE risk genes to be expressed mainly by the oesophageal epithelium and a substantial part to be involved in maintaining an intact epithelial barrier.^{9 38 39} Expression

of the type 1 IL20R by the oesophageal epithelium implies epithelial cells as the target of IL-20 subfamily cytokines. Therefore, previous findings showing overexpression of IL-20 and IL-24 results in hyperproliferation of keratinocytes and epidermal hyperplasia,^{15 22} and reports of impaired barrier function in active EoE due to reduced epithelial barrier components^{5–8 11 40} tempted us to elaborate whether stimulation of patient-derived oesophageal organoids with IL-20 subfamily cytokines may disrupt the integrity of the epithelium. Bulk RNA-seq and mass spectrometry-based proteomics revealed a reduction of transcripts and proteins involved in cornification and epithelial differentiation on stimulation of patient-derived oesophageal organoids with IL-20 subfamily cytokines. Apart from the filaggrin family responsible for keratin-filament alignment and prevention of water loss in the stratum corneum,⁴¹ other essential epithelial barrier constituents involved in tight junction, adherens junctions and desmosome formation were downregulated. Although all these factors are likely to play a role in epithelial barrier function, filaggrins were the most extensively regulated candidates on stimulation with the IL-20 subfamily cytokines. The reduction of IL-20 subfamily cytokines and coinciding restoration of filaggrin expression in inactive EoE implies that the IL-20 subfamily-mediated impairment of the epithelial barrier is a reversible consequence of the inflammation and not a constant disease-inherent feature of EoE. Since mutations in the filaggrin family, proposed as EoE risk genes,^{8 9} result in an inherent increase in epithelial permeability,⁴² it is tempting to speculate that the increased expression of IL-20 subfamily cytokines is the outcome of inflammation induced by an inherently increased permeability further amplifying the barrier defect in EoE.

Comparable to human EoE, the experimental EoE mouse model induced expression of IL-20 subfamily cytokines and reduced expression of filaggrins, while the development of experimental EoE and loss of Flg2 was not precluded in IL-19-deficient animals. Disruption of IL-20 subfamily signalling by genetic deletion of IL-20R β , required for both the type 1 and type 2 IL-20 receptors, hampered experimental EoE in *Il20R2^{-/-}* mice and preserved Flg2 expression in the oesophagus. Thus, we concluded that all three IL-20 subfamily members facilitate the development of EoE. Apart from their effects on epithelial cells, members of the IL-20 subfamily may also act on immune cells. Recent work has suggested that IL-19 supports neutrophil development,⁴³ and IL-24 represses the IL-17 cytokine programme in Th17 cells.⁴⁴ Whether the IL-20 subfamily cytokines elicit effects on neutrophils and T cells promoting the development of EoE remains to be explored. The expression pattern of the IL-20 receptor subunits in murine BMDMs and single-cell RNA-seq of CD45⁺ cells from mice with EoE, however, questions a direct effect of the IL-20 subfamily on immune cells in the oesophagus through the known heterodimeric receptors.

Activating the type 1 and type 2 IL-20 receptors by IL-19, IL-20 and IL-24 induces STAT3 phosphorylation.¹⁶ Interestingly, the specific deletion of STAT3 in colonic epithelial cells leads to impaired wound healing, hyperproliferation and high colitis susceptibility.⁴⁵ Conversely, the specific deletion of STAT3 in epithelial cells protected from colitis-associated cancer in mice.^{46 47} In line with this, our experiments with STAT3 inhibition in vitro or its squamous epithelium-specific deletion in vivo suggested that blocking STAT3 signalling further impaired the epithelial barrier. Considering our data, we cannot conclude whether JAK/STAT signalling inhibition, successfully used to treat IBD,^{48 49} also represents an option for the treatment of EoE. However, it must be considered that JAK inhibitors will also constrain STAT activation in immune cells and thereby the production of cytokines facilitating EoE.⁵⁰

However, our investigations suggest that STAT3 activation in conserved signalling cascades through the type 1 and type 2 IL-20 receptors does not explain the impairment of epithelial integrity after stimulation with IL-20 subfamily cytokines.

On the other hand, pharmacological inhibition of ERK1/2 prevented an impaired barrier function in ALI cultures stimulated with IL-19, IL-20 and IL-24, implying IL-20 subfamily-mediated barrier impairment to be ERK1/2-dependent. Comparable observations have been made in the skin, where the treatment of keratinocytes with the specific ERK1/2 inhibitor PD98059 overturned IL-17-mediated filaggrin downregulation.⁵¹ Moreover, the IL-1 family cytokine IL-33 also decreases dermal filaggrin expression in an ERK1/2-dependent manner.⁵² Although we cannot exclude the involvement of other factors in the ERK1/2-dependent regulation of epithelial permeability, IL-20 subfamily signalling seems to display an essential element for epithelial barrier function in the oesophagus. In particular, because alongside IL-20 subfamily-specific downregulation of the GO categories ‘tight junction’, ‘desmosome’, ‘epidermis morphogenesis’ and ‘apical junction complex’, the comparison of our bulk RNA-seq dataset with datasets of IL-13 stimulated oesophageal keratinocytes,⁶ SPINK7-deficient oesophageal keratinocytes¹³ and the EoE transcriptome⁵³ unveiled overlapping downregulation of genes involved in epidermis development. These results further augment the image of coinciding mechanisms regulating epithelial barrier function in EoE. IL-13 has been postulated to be a critical molecular driver of EoE, recapitulating a majority of the EoE transcriptome and similarly inducing epithelial barrier impairment.⁵⁻⁷ Intriguingly, patient-derived organoids stimulated with IL-20 subfamily cytokines upregulated the IL-13 receptor subunits, *IL-4Ra*, *IL-13Ra1* and *IL-13Ra2*. This suggests that the IL-20 subfamily increases epithelial sensitivity for IL-13, reinforcing IL-13-mediated changes in EoE. SPINK7, a member of the SPINK-type family, is highly enriched in the oesophageal epithelium and emerged as an essential regulator of epithelial barrier homeostasis controlling the proteolytic activity of kallikrein 5 and other proteases.^{13 14} Reduced SPINK7 expression in the epithelium of patients with EoE leads to protease–protease inhibitor imbalance resulting in degradation of epithelial barrier proteins and production of proinflammatory cytokines.^{13 14} Although IL-13 broadly regulates oesophagus-specific genes,^{5 6} SPINK7 is not regulated by IL-13.¹³ Remarkably, *SPINK7* was downregulated in patient-derived organoids stimulated with IL-20 subfamily cytokines. Therefore, we suggest specific IL-20 subfamily-mediated effects on the oesophageal epithelium, which position the IL-20 subfamily upstream of SPINK7 and alongside IL-13-mediated changes.

In summary, our study uses in vitro and in vivo models to conceivably establish an ERK1/2-dependent relationship between the IL-20 subfamily and the oesophageal epithelium in the context of EoE. However, before specific targeting of IL-19, IL-20 and IL-24 and their signalling pathway can be considered for restoring epithelial integrity in EoE, the complex interplay between immune cells, the IL-20 cytokines and the oesophageal epithelium needs to be consolidated in further translational and clinical studies. Identifying possible interfaces between the immune system and the oesophageal epithelium will be critical to dissecting the immune response responsible for developing EoE and identifying novel targets for treatment.

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Contributors TK, BK, PW and HM carried out experiments. JR performed bioinformatics analysis of the transcriptomics and proteomics data. FO provided the *IL20R2*^{-/-} mice. SN supported us in establishing a protocol for generating organoids derived from the oesophagus, and MN helped us set up the experimental eosinophilic oesophagitis (EoE) mouse model. TK, AG, PH and JHN recruited patients with EoE. TK and JHN designed the study and wrote the paper. JHN conceived the idea and is the guarantor of the study. All authors discussed the data and read and approved the manuscript.

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