Identification of Keratinocyte Mitogens: Implications for Hyperproliferation in Psoriasis and Atopic Dermatitis

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Psoriasis and atopic dermatitis are chronic inflammatory skin diseases characterized by keratinocyte (KC) hyperproliferation and epidermal acanthosis (hyperplasia). The milieu of disease-associated cytokines and soluble factors is considered a mitogenic factor; however, pinpointing the exact mitogens in this complex microenvironment is challenging. We employed organotypic human epidermal equivalents, faithfully mimicking native epidermal proliferation and stratification, to evaluate the proliferative effects of a broad panel of (literature-based) potential mitogens. The KC GF molecule, the T-helper 2 cytokines IL-4 and IL-13, and the psoriasis-associated cytokine cytokine IL-17A caused acanthosis by hyperplasia through a doubling in the number of proliferating KCs. In contrast, IFN-γ lowered proliferation, whereas IL-6, IL-20, IL-22, and oncostatin M induced acanthosis not by hyperproliferation but by hypertrophy. The T-helper 2–cytokine–mediated hyperproliferation was Jak/signal transducer and activator of transcription 3 dependent, whereas IL-17A and KC GF induced MAPK/extracellular signal–regulated kinase/extracellular signal–regulated kinase–dependent proliferation. This discovery that key regulators in atopic dermatitis and psoriasis are direct KC mitogens not only adds evidence to their crucial role in the pathophysiological processes but also highlights an additional therapeutic pillar for the mode of action of targeting biologicals (e.g., dupilumab) or small-molecule drugs (e.g., tofacitinib) by the normalization of KC turnover within the epidermal compartment.

INTRODUCTION

Keratinocytes (KCs) are the major cell type of the epidermis and maintain its structural integrity, contribute to the barrier function of the skin, and regulate immunological cues as part of the innate immune system. KC proliferation is vital for physiological epidermal renewal and for epidermal regeneration after wounding. In this process, interfollicular epidermal stem cells give rise to transit-amplifying cells in the basal layer of the epidermis. After proliferation, daughter cells exit the cell cycle, start to differentiate, and move upward through the stratum spinosum and stratum granulosum to finally end up as terminally differentiated corneocytes before shedding from the skin surface (Lechler and Fuchs, 2005; Weinstein et al., 1984).

In healthy skin, complete epidermal renewal takes approximately 30 days. Accelerated KC turnover or hyperproliferation is required during skin injury. However, under certain circumstances, this tightly regulated process is disturbed, and basal KCs remain hyperproliferative. Besides being related to malignancies (Frost et al., 1966; Wright, 1985), KC hyperproliferation is a classic feature of several benign skin disorders, including the chronic inflammatory skin diseases psoriasis (Leigh et al., 1985; Vanscott and Ekel, 1963; Weinstein et al., 1985) and atopic dermatitis (AD) (Sapuntsova et al., 2002). Although the underlying pathogenesis and pathophysiological processes are distinct, both diseases are typically characterized by epidermal thickening (acanthosis) owing to hyperplasia (increased cell numbers). The resulting excessive skin scaling is due to both disturbed regulation of epidermal proliferation and the terminal differentiation and desquamation process. These disturbances are generally considered to be caused by the cutaneous milieu of potentially mitogenic factors secreted by the inflammatory tissue microenvironment (Guttman-Yassky and Krueger, 2017).

From the early 80s onward, in vitro studies have addressed KC proliferation aiming to identify mitogenic stimuli such as GFs and cytokines that could be relevant for inflammatory diseases or epidermal repair processes. The results of these studies are conflicting and difficult to interpret because of the various culture systems (monolayers vs.
organtoypic models, foreskin vs. adult KCs and different readout parameters (3H-thymidine incorporation, surrogate markers such as keratin 16). This lack of a systematic approach hampered the identification of bona fide KC mitogens. Many of these studies were furthermore not specifically designed to investigate KC (hyper)proliferation but focused on the involvement of the investigated molecules in AD or psoriasis. Clearly, identification of relevant, disease-specific mitogens is ever needed to understand the pathophysiology and to facilitate target drug discovery and development.

We therefore conducted a literature search and screened the identified candidate molecules for their potential as acting KC mitogens using a standardized organotypic epidermal culture system, a concentration series of candidate molecules, and primary epidermal KCs from multiple donors. This comprehensive study uncovered mitogens and calls on the validity of previously reported mitogens. In addition, we provide evidence for the relevance of the signaling pathways associated with the identified mitogens for the induction of KC hyperproliferation.

RESULTS
Selection of candidate molecules involved in KC proliferation
We performed a comprehensive literature search, summarized in Table 1, for selecting candidate mitogens associated with KC hyperproliferation particularly related to psoriasis and AD or wound healing. Studies were screened for information on the effects of soluble factors on KC (hyper)proliferation and epidermal alterations, resulting in a total of 34 potential candidate mitogens that we selected for further functional analysis. This list includes the classical disease-associated cytokines (derived from T helper [Th] 1, Th2, Th17 Th22 cells) but also GFs and serum-derived molecules.

Analysis of KC proliferation in organotypic human epidermis
For evaluating the direct mitogenic potential of relevant factors as listed in Table 1, we employed the widely used and highly standardized organotypic human epidermal equivalent (HEE) model (Niehues et al., 2018), generated from primary adult human KCs in the absence of a dermal substrate to omit any confounding interactions with other cell types. First, we validated that the formation of HEEs using this methodology is based on the proliferation of KCs in the basal layer that generate a complete epidermis over time and not by the stacking of an excess of KCs as known for other models (Mildner et al., 2006; Sen et al., 2010; Truong et al., 2006). Directly after a 24-hour exposure of the HEEs to the thymidine analog 5-ethyl-2’-deoxyuridine (EdU) on day 5 of the air–liquid interface culture, solely basal KCs appeared EdU positive. After a wash-out period of 2 days, EdU-positive cells were found in basal cells, indicative of label-retaining transit-amplifying cells, but also in differentiated KCs of the spinous layer, which correspond to the daughter cells that entered the terminal differentiation program (Figure 1a). This process of KC proliferation and subsequent differentiation in our HEE model system resembles epidermal renewal and KC maturation in vivo.

KC proliferation in organotypic models versus in native human skin
Before detection of hyperproliferative changes on stimulation with the molecules of interest, we first sought to show the proliferative capacity of the HEE model and compare this with that of in vivo native skin. In general, the proliferation rate of KCs in HEEs was, on the basis of the surrogate proliferation marker Ki-67, higher than that in the native skin (Figure 1b). This elevated proliferation rate is commonly seen in organotypic skin and is in this study related to the 8-day period of the air–liquid interface before harvesting. On prolonged air–liquid interface culture periods (up to 20 days), proliferation rates go down, resulting in a thinning of the living epidermis until ultimately all transit-amplifying cells exit the cell cycle marking the end of the organotypic life span (Figure 1c).

To put the mitogenic potential of the tested molecules into perspective regarding hyperproliferation in AD or psoriasis pathophysiology, we compared the epidermal features of normal skin with those of both hyperproliferative skin conditions. The histology of skin biopsies showed the classical morphological hallmarks of AD (e.g., acanthosis, spongiosis) and psoriasis (e.g., acanthosis, rete ridges, parakeratosis) (Figure 2). In normal skin, only a small portion of basal KCs is Ki-67+. In contrast, the acanthotic epidermis of AD and psoriasis skin shows most of all basal KCs as Ki-67+, whereas also in suprabasal cell layers, Ki-67+ cells are found. Semi-automated software-guided quantification analysis estimates a 5.5-fold higher number of Ki-67+ cells in AD and over a nine-fold higher number of Ki-67+ cells in psoriasis in vivo.

Soluble factors affecting proliferation and epidermal morphology in organotypic epidermis
After the validation procedures, mitogenic molecules were individually tested in the HEE cultures. For this first screening, every molecule was tested in a dose series, depicted in Table 2, using HEEs from two different KC donors. General epidermal morphology was assessed, and HEEs were screened for AD and psoriasis phenotypic features. KC proliferation was assessed by Ki-67 protein expression analysis. Results, as summarized in Table 3, indicate that most of the investigated candidates did not alter epidermal morphology nor changed KC proliferation rates. Two candidates (fetal calf serum, TNF-α) detrimentally affected epidermal morphology at higher concentrations. IFN-γ induced hypoplasia and lowered proliferation rates, whereas IL-6, IL-20, IL-22, and oncostatin M induced acanthosis but without any signs of hyperproliferative KCs, illustrating their hypertrophic (cell enlargement) effects on KCs. Epidermal spongiosis (intercellular edema), a typical AD hallmark, was detected for IL-1α and IL-1β. Of the 34 putative mitogens, we found the following four effector molecules to induce hyperproliferation accompanied by acanthosis in our HEE model: KC GF (KGF), IL-4, IL-13, and IL-17A (Table 3 and Figure 3a).
### Table 1. Candidate Mitogens Associated with Keratinocyte Proliferation in Psoriasis and Atopic Dermatitis

| Candidate Mitogen | Species | Model | Link Toward Mitogenicity                                                                 | Ref.                                                                 |
|-------------------|---------|-------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------|
| CCL17             | Human   | In vivo| Atopic dermatitis                                                                      | (Hijnen et al., 2004)                                                |
|                   | Human   | 2D, In vivo | Association with keratinocyte proliferation                                              | (Nakahigashi et al., 2011)                                           |
|                   | Human   | In vivo | Atopic dermatitis                                                                      | (Fujisawa et al., 2002)                                              |
| CGRP              | Human   | In vivo| Psoriasis                                                                               | (Jiang et al., 1998)                                                |
|                   | Human   | 2D    | Association with keratinocyte proliferation                                              | (Yu et al., 2009)                                                   |
|                   | Mouse   | In vivo| Association with keratinocyte proliferation                                              | (Seike et al., 2002)                                                |
| CRNN              | Human   | 2D    | Psoriasis                                                                               | (Li et al., 2019)                                                   |
|                   | Mouse   | In vivo| Association with keratinocyte proliferation                                              |                                                                     |
|                   | 2D, In vivo | Association with keratinocyte proliferation                                              |                                                                     |
| EGF               | Human   | Ex vivo| Association with keratinocyte proliferation and migration                                | (Bhoro et al., 1995)                                                |
|                   | Human   | 3D (HSE) | Association with keratinocyte proliferation and migration                               | (Gibbs et al., 2000)                                                |
|                   | Human   | 2D    | Association with keratinocyte proliferation                                              | (Venuti et al., 1997)                                               |
|                   | Human   | 2D    | Serum contains components enhancing and inhibiting keratinocyte proliferation           | (Bertolero et al., 1986)                                            |
| GM-CSF            | Human   | In vivo| Association with keratinocyte proliferation                                              | (Braunstein et al., 1994)                                           |
|                   | Human   | 2D, In vivo | Psoriasis                                                                               | (Pastore et al., 1997)                                              |
|                   | Mouse   | In vivo| Association with keratinocyte proliferation                                              | (Mann et al., 2001)                                                |
| IFN-γ             | Human   | In vivo| Psoriasis                                                                               | (Uyemura et al., 1993)                                              |
|                   | Human   | 2D, Ex vivo | Decreases keratinocyte proliferation                                                   | (Hattori et al., 2002)                                             |
| IGF-1             | Human   | 2D, In vivo | Elevated IGF-1R expression associated with enhanced keratinocyte proliferation        | (Krane et al., 1991)                                               |
|                   | Human   | 2D, In vivo | IGF-1R expression in proliferative keratinocytes                                        | (Tavakkol et al., 1992)                                            |
|                   | Human   | 2D, In vivo | IGF-1R inhibition reverses keratinocyte hyperproliferation                             | (Wraight et al., 2000)                                             |
| IL-1α             | Mouse   | 2D, In vivo | Association with keratinocyte proliferation                                              | (Hobbs et al., 2004)                                               |
|                   | Human   | 3D (HSE) | Association with keratinocyte proliferation in co-culture with fibroblasts             | (Maas-Szabowski et al., 2000)                                       |
| IL-1β             | Human   | 3D (HSE) | Association with keratinocyte proliferation                                              | (Tanguchi et al., 2014)                                            |
| IL-4              | Human   | In vivo| Psoriasis                                                                               | (Cai et al., 2019)                                                 |
|                   | Human   | 2D    | Association with keratinocyte proliferation                                              | (Yang et al., 2001)                                                |
| IL-5              | Human   | In vivo| Atopic dermatitis                                                                      | (Kondo et al., 2001)                                               |
| IL-6              | Human   | 2D    | Psoriasis                                                                               | (Grossman et al., 1989)                                            |
|                   | Rat     | In vivo| Association with keratinocyte proliferation                                              | (Sawamura et al., 1998)                                            |
| IL-8              | Human   | In vivo| Psoriasis                                                                               | (Steude et al., 2002)                                              |
|                   | Human   | 3D (HSE) | Association with keratinocyte proliferation                                              | (Steude et al., 2002)                                              |
| IL-12             | Human   | In vivo| Atopic dermatitis                                                                      | (Hamid et al., 1996)                                               |
|                   | Human   | In vivo| Psoriasis                                                                               | (Yawalkar et al., 1998)                                            |
| IL-13             | Mouse   | In vivo| Atopic dermatitis                                                                      | (Herrick et al., 2003)                                             |
|                   | Human   | 2D    | Association with keratinocyte proliferation                                              | (Matsumura et al., 2015)                                           |
| IL-15             | Human   | In vivo| Psoriasis                                                                               | (Ruckert et al., 2000)                                             |
|                   | Human   | 2D    | Enhances keratinocyte proliferation                                                     | (Yano et al., 2003)                                                |
|                   | Human   | 2D, In vivo | Association with keratinocyte proliferation and migration                               | (Jones et al., 2016)                                               |
| IL-17A            | Human   | 2D, In vivo | Psoriasis                                                                               | (Charruyer et al., 2017)                                           |
|                   | Mouse   | 2D, In vivo | Association with keratinocyte proliferation                                              | (Wu et al., 2015)                                                  |
|                   | Human   | 2D, In vivo | Association with keratinocyte proliferation, Psoriasis                                 | (Ma et al., 2016)                                                  |

(continued)
| Candidate Mitogen | Species          | Model            | Link Toward Mitogenicity                                | Ref.                      |
|------------------|------------------|------------------|--------------------------------------------------------|---------------------------|
| IL-19            | Human, Mouse     | 2D, In vivo      | Psoriasis                                              | (Witte et al., 2014)      |
|                  | Human Mouse      | 2D, In vivo      | Wound healing                                          | (Sun et al., 2013)        |
|                  | Human            | In vivo          | Atopic dermatitis, Cutaneous T-cell lymphoma           | (Oka et al., 2017)        |
|                  | Human Mouse      | 2D, In vivo      | Atopic dermatitis                                      | (Bao et al., 2014)        |
| IL-20            | Human            | In vivo          | Psoriasis                                              | (Li et al., 2005)         |
|                  | Human            | In vivo          | Psoriasis                                              | (Wei et al., 2005)        |
| IL-21            | Human, Mouse     | In vivo          | Psoriasis                                              | (Caruso et al., 2009)     |
|                  | Human            | In vivo          | Atopic dermatitis                                      | (Mizutani et al., 2017)   |
| IL-22            | Human            | 2D, 3D (HEE)     | Enhances epidermal hypertrophy and keratinocyte migration | (Boniface et al., 2005)   |
|                  | Human Mouse      | 2D, In vivo      | Association with keratinocyte proliferation            | (Ekman et al., 2019)      |
|                  | Human Mouse      | 2D, In vivo      | Psoriasis                                              | (Zheng et al., 2007)      |
| IL-23            | Human Mouse      | 2D, In vivo      | Induces epidermal hyperplasia                          | (Chan et al., 2006)       |
|                  | Human Mouse      | 2D, In vivo      | Psoriasis                                              | (Works et al., 2014)      |
|                  | Human            | In vivo          | Psoriasis                                              | (Nair et al., 2009)       |
| IL-25            | Human Mouse      | 2D, In vivo      | Psoriasis                                              | (Xu et al., 2018)         |
| IL-27            | Human            | 2D, In vivo      | Psoriasis                                              | (Shibata et al., 2010)    |
|                  | Human Mouse      | 2D, In vivo      | Wound healing                                          | (Yang et al., 2017)       |
| IL-31            | Mouse            | In vivo          | Association with keratinocyte proliferation and epidermal acanthosis | (Singh et al., 2016)      |
|                  | Mouse            | In vivo          | Atopic dermatitis                                      | (Dillon et al., 2004)     |
|                  | Human            | 2D, In vivo      | Psoriasis                                              | (Finch et al., 1997)      |
|                  | Human            | 3D (HSE)         | Association with keratinocyte proliferation in coculture with fibroblasts | (Maas-Szabowski et al., 2000) |
| KGF              | Human            | 3D (HSE)         | Association with keratinocyte proliferation            | (Andreidis et al., 2001)  |
|                  | Human            | 2D, In vivo      | Psoriasis                                              | (Finch et al., 1997)      |
|                  | Human            | 2D, In vivo      | Association with keratinocyte proliferation            | (Marchese et al., 1990)   |
| Leptin           | Human Mouse      | 2D, In vivo      | Association with keratinocyte proliferation and involvement in wound healing | (Stallmeyer et al., 2001) |
| OSM              | Human            | 2D, 3D (HEE), In vivo | Atopic dermatitis, Psoriasis                           | (Boniface et al., 2007)   |
|                  | Human            | 2D, In vivo      | Association with keratinocyte proliferation            | (Wang et al., 2020)       |
| PGE2             | Mouse            | In vivo          | Receptor expression associated with enhanced keratinocyte proliferation and tumor formation | (Chun et al., 2010)       |
| TNF-α            | Human            | 2D, In vivo      | Decreases keratinocyte proliferation                   | (Detmar and Orfanos, 1990) |
|                  | Human            | 2D, In vivo      | Psoriasis                                              | (Johansen et al., 2006)   |
| TSLP             | Human            | 2D, In vivo      | Atopic dermatitis                                      | (Sournelis et al., 2002)  |
| VIP              | Human            | In vivo          | Atopic dermatitis, psoriasis                           | (Anand et al., 1991)      |
|                  | Human            | 2D, In vivo      | Receptor expression associated with enhanced keratinocyte proliferation | (Kakurai et al., 2001)    |
|                  | Human            | 2D, In vivo      | Association with keratinocyte proliferation           | (Sung et al., 1999)       |

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; CRNN, cornulin; FCS, fetal calf serum; HEE, human epidermal equivalent; HSE, human skin equivalent; KGF, keratinocyte GF; OSM, oncostatin; Ref, reference; TSLP, thymic stromal lymphopoietin.
KGF induces proliferation through MAPK/extracellular signal–regulated kinase/extracellular signal–regulated kinase in HEEs

Stimulation of HEEs with KGF induced acanthosis through hyperproliferation as witnessed by increased epidermal thickness and a higher number of Ki-67– and EdU-positive KCs from a dosage of 10 ng/ml and higher (Figure 4a–c). No other apparent morphological changes were observed. The results obtained with the organotypic HEE model used in this study correspond to previous studies showing that KGF is a direct regulator of KC proliferation through MAPK/extracellular signal–regulated kinase (ERK) kinase (MEK)/ERK signaling. Indeed, pretreatment of HEEs with a selective blocker of MEK signaling before KGF exposure significantly reduced KGF-induced KC proliferation rates (Figure 4d and e). Other blocking agents targeting non-MEK/ERK signaling cascades did not interfere with KGF-dependent hyperproliferation (Figure 3b). Even at higher concentrations (up to...
| Effector/Inhibitor | Manufacturer (Catalog Number) | Concentrations Tested |
|-------------------|-----------------------------|-----------------------|
| CCL17/TARC        | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human TARC (CCL17), 300-30 |                       |
| CGRP              | Sigma-Aldrich                | 1, 10, and 100 ng/ml  |
|                   | Calcitonin gene-related peptide, C0167 |                       |
| CRNN              | ProSpec                      | 10, 30, and 100 ng/ml |
|                   | Recombinant human cornulin, Pro-797 |                       |
| CGRP              | Sigma-Aldrich                | 0.5, 1, and 5 ng/ml   |
|                   | EGF from murine submaxillary gland E4127 |                       |
| Et-1              | Sigma-Aldrich                | 10, 30, and 100 ng/ml |
|                   | Porcine human endothelin 1, E7764 |                       |
| FBS               | Hyclone Cellbio              | 5, 10, and 20%        |
|                   | FBS, SH30071.03              |                       |
| GM-CSF            | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human granulocyte-macrophage colony-stimulating Factor, 300-03A | |
| IFN-γ             | Hycult Biotech               | 20, 100, and 250 Units/ml |
|                   | Recombinant Human IFN-gamma, HC20310-01 |                       |
| IGF-1             | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human insulin-like growth factor-l,100-11 |                       |
| IL-1α              | Stemcell Technologies        | 10, 30, and 100 ng/ml |
| IL-1β              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-1β, 78076 |                       |
| IL-4               | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-4, 200-04 |                       |
| IL-5               | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-5, 200-05 |                       |
| IL-6               | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-6, 200-06 |                       |
| IL-8               | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-8 (CXCL8), 200-08 |                       |
| IL-12              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-12 p70, 200-12 |                       |
| IL-13              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-13, 200-13 |                       |
| IL-15              | PeproTech                    | 1, 10, and 100 ng/ml  |
|                   | Recombinant human IL-15, 200-15A |                       |
| IL-17A             | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-17A, 200-17 |                       |
| IL-19              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-19, 200-19 |                       |
| IL-20              | PeproTech                    | 10, 30, 100 ng/ml    |
|                   | Recombinant human IL-20, 200-20 |                       |
| IL-21              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-21, 200-21 |                       |
| IL-22              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-22, 200-22 |                       |
| IL-23              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-23, 200-23 |                       |
| IL-25              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-25, 200-25 |                       |
| IL-27              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-27, 200-38 |                       |
| IL-31              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human IL-31, 200-31 |                       |
| KGF                | Sigma Aldrich                | 10, 30, and 100 ng/ml |
|                   | Recombinant human KGF, K1757 |                       |
| Leptin             | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human Leptin, 300-27A |                       |
| OSM                | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human Oncostatin M, 300-10T |                       |
| PGE₂               | Sigma Aldrich                | 10, 30, and 100 μM   |
|                   | Synthetic PGE₂, F0409         |                       |
| TNF-α              | PeproTech                    | 10, 30, and 100 ng/ml |
|                   | Recombinant human TNF-α, 300-01A |                       |
### Table 2. Continued

| Effector/Inhibitor | Manufacturer (Catalog Number) | Concentrations Tested |
|--------------------|-------------------------------|-----------------------|
| TSLP               | PeproTech Recombinant human TSLP, 300-62 | 3, 10, and 30 ng/ml |
| VIP                | Sigma Aldrich Porcine human vasoactive intestinal peptide, V3628 | 0.01, 0.1, and 1 μM |
| Dupilumab          | Dupixent, Sanofi Genzyme      | 10 nM                 |
| Tofacitinib        | Sigma-Aldrich PZ0017          | 450 nM                |
| JakI inhibitor     | Calbiochem JAK inhibitor I, 420099 | 100 nM                |
| MEK/ERK inhibitor  | Sigma Aldrich U0126 monoethanolate, U120 | 100 nM                |
| p38 MAP Kinase inhibitor IV | Sigma-Aldrich SML0543       | 100 nM                |

Abbreviations: CRNN, cornulin; FBS, fetal bovine serum; HEE, human epidermal equivalent; KGF, keratinocyte GF; MEK/ERK, MAPK/extracellular signal–
regulated kinase/extracellular signal–regulated kinase kinase; OSM, oncostatin; TSLP, thymic stromal lymphopoietin.

### Table 3. Screening of all Potential Mitogenic Factors in Dosage Range in HEE Model

| Effector | Morphology (H&E) | Proliferation (Ki-67) | Concentrations Tested | Morphological Changes |
|----------|------------------|-----------------------|-----------------------|-----------------------|
| CCL17/TARC | ![Image](CCL17/TARC) | ![Image](CCL17/TARC) | 10, 30, and 100 ng/ml | ND |
| CGRP     | ![Image](CGRP) | ![Image](CGRP) | 1, 10, and 100 ng/ml | ND |
| CRNN     | ![Image](CRNN) | ![Image](CRNN) | 10, 30, and 100 ng/ml | ND |
| EGF      | ![Image](EGF) | ![Image](EGF) | 0.5, 1, and 5 ng/ml | ND |
| Et-1     | ![Image](Et-1) | ![Image](Et-1) | 10, 30, and 100 ng/ml | ND |
| FCS      | ![Image](FCS) | ![Image](FCS) | 5, 10, and 20% | Toxicity |

(continued)
Table 3. Continued

| Effector | Morphology (H&E) | Proliferation (Ki-67) | Concentrations Tested | Morphological Changes |
|----------|------------------|-----------------------|-----------------------|-----------------------|
| GM-CSF   | ![Image](image1)  | ![Image](image2)      | 10, 30, and **100** ng/ml | ND                    |
| IFN-γ    | ![Image](image3)  | ![Image](image4)      | 20, **100**, and 250 Units/ml | Hypoplasia            |
| IGF-1    | ![Image](image5)  | ![Image](image6)      | 10, 30, and **100** ng/ml | ND                    |
| IL-1α    | ![Image](image7)  | ![Image](image8)      | 10, 30, and **100** ng/ml | Spongiosis            |
| IL-1β    | ![Image](image9)  | ![Image](image10)     | 10, 30, and **100** ng/ml | Spongiosis            |
| IL-4     | ![Image](image11) | ![Image](image12)     | **10**, 30, and 100 ng/ml | Hyperplasia           |
| IL-5     | ![Image](image13) | ![Image](image14)     | 10, 30, and 100 ng/ml    | ND                    |
| IL-6     | ![Image](image15) | ![Image](image16)     | 10, 30, and **100** ng/ml | Hypertrophy           |
| IL-8     | ![Image](image17) | ![Image](image18)     | 10, 30, and **100** ng/ml | ND                    |
| IL-12    | ![Image](image19) | ![Image](image20)     | 10, 30, and **100** ng/ml | ND                    |

*(continued)*
| Effector | Morphology (H&E) | Proliferation (Ki-67) | Concentrations Tested | Morphological Changes |
|---------|-----------------|----------------------|----------------------|----------------------|
| IL-13   | ![Image](image1) | ![Image](image2)     | 10, 30, and 100 ng/ml | Hyperplasia          |
| IL-15   | ![Image](image3) | ![Image](image4)     | 1, 10, and 100 ng/ml  | ND                   |
| IL-17A  | ![Image](image5) | ![Image](image6)     | 10, 30, and 100 ng/ml | Hyperplasia          |
| IL-19   | ![Image](image7) | ![Image](image8)     | 10, 30, and 100 ng/ml | ND                   |
| IL-20   | ![Image](image9) | ![Image](image10)    | 10, 30, and 100 ng/ml | Hypertrophy          |
| IL-21   | ![Image](image11) | ![Image](image12)    | 10, 30, and 100 ng/ml | ND                   |
| IL-22   | ![Image](image13) | ![Image](image14)    | 10, 30, and 100 ng/ml | Hypertrophy          |
| IL-23   | ![Image](image15) | ![Image](image16)    | 10, 30, and 100 ng/ml | ND                   |
| IL-25   | ![Image](image17) | ![Image](image18)    | 10, 30, and 100 ng/ml | ND                   |

(continued)
### Table 3. Continued

| Effector | Morphology (H&E) | Proliferation (Ki-67) | Concentrations Tested | Morphological Changes |
|----------|-----------------|-----------------------|-----------------------|-----------------------|
| IL-27    | ![Image](image1) | ![Image](image2) | 10, 30, and **100** ng/ml | ND                    |
| IL-31    | ![Image](image3) | ![Image](image4) | 10, 30, and **100** ng/ml | ND                    |
| KGF      | ![Image](image5) | ![Image](image6) | 10, 30, and **100** ng/ml | Hyperplasia           |
| Leptin   | ![Image](image7) | ![Image](image8) | 10, 30, and **100** ng/ml | ND                    |
| OSM      | ![Image](image9) | ![Image](image10) | **10**, 30, and 100 ng/ml | Hypertrophy           |
| PGE2     | ![Image](image11) | ![Image](image12) | 10, 30, and **100** µM  | ND                    |
| TNFα     | ![Image](image13) | ![Image](image14) | **10**, 30, and 100 ng/ml | Toxicity (> 10 ng/mL) |
| TSLP     | ![Image](image15) | ![Image](image16) | 3, 10, and **30** ng/ml | ND                    |
| VIP      | ![Image](image17) | ![Image](image18) | 0.01, 0.1, and **1** µM | ND                    |

*Abbreviations: CRNN, cornulin; FCS, fetal calf serum; HEE, human epidermal equivalent; KGF, keratinocyte GF; ND, not detected; OSM, oncostatin; TSLP, thymic stromal lymphopoietin.*

The photographs show the representative images of two keratinocyte donors tested. Testing of all compounds was performed in several experiments, including one or more test compounds in a dosage range indicated in the table, and all experiments included negative controls (a total of nine keratinocyte donors used). The bold and underlined concentration corresponds to the depicted photograph, and either shows the highest concentration tested without any morphological/mitogenic effect or the lowest effective concentration. Photographs show ×40 magnification.
1 μM the p38 MAPK inhibitor did not inhibit KGF-induced hyperproliferation (data not shown).

**Mitogenic effects and signaling pathways of psoriasis- and AD-associated cytokines**

In addition to KGF, we found specific ILs classically known to be elevated in psoriasis and/or AD skin to induce KC proliferation in the organotypic HEEs. IL-17A resulted in a higher number of Ki-67+ cells in the basal layer and the presence of Ki-67+ cells in suprabasal layers of the stratum spinosum (Table 3). Results of the initial screening were verified in a dosage range study with two KC donors, and quantification indicated that IL-17A leads to a doubling of total proliferative KC at a concentration of 1 ng/ml and higher (Figure 5).

To reverse the IL-17A–induced proliferation and investigate the downstream signaling pathways, HEEs were pretreated with secukinumab (anti–IL-17A antibody) and diverse inhibiting agents before IL-17A stimulation. Secukinumab and blocking of the MEK/ERK signaling route reduced the number of proliferating KCs, whereas the inhibitors of Jak (tofacitinib) and MAPK signaling (p38) were unable to dampen the IL-17–induced hyperproliferation (Figure 6a and b). To further dissect the involved signaling events, the phosphorylation (and thus activation) of signal transducer and activator of transcription (STAT) 3 by IL-17A was analyzed. Only, secukinumab and tofacitinib inhibited the phosphorylation of STAT3 (Figure 6c). Because tofacitinib pretreatment did not reduce the numbers of proliferative KCs, IL-17A–mediated hyperproliferation is not likely a result of the activated Jak/STAT3 signaling pathway.

For IL-4 and IL-13 treatment, KC hyperproliferation was also observed (Table 3), and again, an extended dose range study was performed to address the potency of both cytokines. For IL-4 and IL-13, the lowest effective concentration leading to increased Ki-67+ cells was 3 ng/ml (Figure 7a and b). Similar to that of IL-17, Ki-67+ cells were also frequently detected in suprabasal layers. These Th2 cytokines increased the number of proliferating KCs by two-fold in the range of 3 to 30 ng/ml (Figure 7c and d). Next, we evaluated whether hyperproliferation also occurred in a more matured HEE culture after 12, 16, or even 20 days of air–liquid interface culture. In this experiment, the basal proliferation rate is decreasing in time, whereas terminal differentiation and cornification, as witnessed by a thickening of the stratum corneum, are steadily increasing (Figure 8). The Th2 cytokine–mediated hyperproliferation was observed in all conditions and thus occurred irrespective of the basal proliferation rate or differentiation status of the organotypic model.

To verify that the Th2 cytokine–mediated hyperproliferation in KCs was directly related to the IL-4 receptor–activated Jak/STAT signaling cascade (Rawlings et al., 2004), cytokine stimulation was combined with pretreatment of dupilumab (monoclonal anti–IL-4R) and tofacitinib (Jak inhibitor). Both dupilumab and tofacitinib prevented IL-4– and IL-13–mediated hyperproliferation through the inhibition of STAT3/6 phosphorylation in HEEs. Tofacitinib reduced STAT3 phosphorylation even below endogenous levels, but this did not correspond to lowered numbers of proliferating KCs (Figure 9 and Figure 10a and b). Furthermore, inhibition of MEK/ERK or MAPK signaling did not reverse IL-4–induced hyperproliferation (Figure 10c and d). From these results, we can conclude that the Th2 family cytokines IL-4 and IL-13 are direct KC mitogens and act through the activation of Jak/STAT6 signaling but not STAT3 considering the ineffectiveness of phosphorylated STAT3 inhibition on KC proliferation after IL-17 exposure.

**DISCUSSION**

In this study, we aimed to find the direct drivers of KC hyperproliferation in psoriasis and AD. Of the 34 tested candidates, KGF, IL-4, IL-13, and IL-17A met the criteria for...
acting as KC mitogens and could thus serve as potential biomarkers for epidermal hyperproliferation.

In this study, we have chosen a three-dimensional (3D) organotypic HEE model purely composed of KCs to selectively identify direct KC mitogens. The benefits of such models over conventional monolayer cultures are that the epidermal tissue structure allows for the monitoring of differential KC turnover induced by the selected factors and that it allows for the detection of morphological features related to proliferation. Furthermore, in conventional monolayer culture models, KCs already reside in an almost maximum proliferative state, which minimizes the window of opportunity for detection of increased KC proliferation (Rasmussen et al., 2013). One limitation of the HEE model is that the molecules tested negative in our screen could still be indirect mitogens through the activation of other cell types (e.g., immune cells) in the skin that in turn secrete mitogenic factors for KCs. In addition, mitogens that strongly act in concert with other factors are not identified in this study. Furthermore, possible autocrine regulation by molecules produced on cytokine exposure may contribute to the observed effects. Therefore, our study highlights the potency of KGF, IL-4, IL-13, and IL-17A in the pathophysiological processes of AD and psoriasis but does not rule out the involvement of other molecules tested in processes that ultimately lead to KC hyperproliferation.

We found KGF to elevate proliferation in an epidermal (KC-only) model. Previously, KGF was also found to directly

Figure 4. KGF causes keratinocyte hyperproliferation in HEE. HEEs (n = 3) were stimulated with a dosage range of KGF for 72 hours. EdU was incorporated 24 hours before harvesting on day 8 of the air–liquid interface. (a) H&E, Ki-67, and EdU staining on KGF stimulation and (b) quantification of Ki-67- and EdU-positive nuclei/mm length of the HEE. (c) Epidermal thickness (P = 0.008). (d) H&E, Ki-67, and EdU staining and (e) quantification of HEEs pretreated with 100 nM MEK/ERK inhibitor (U0126) for 24 hours and then costimulated with 100 ng/ml KGF for 72 hours (Ki67, P = 0.004; EdU, P = 0.011) (n = 3). *P < 0.05. Bar = 100 μm. EdU, 5-ethyl-2′-deoxyuridine; HEE, human epidermal equivalent; KGF, keratinocyte GF; MEK/ERK, MAPK/extracellular signal–regulated kinase kinase/extracellular signal–regulated kinase kinase.
stimulate KC proliferation in monolayer cultures and in 3D full-thickness models (Andreadis et al., 2001; Erdag et al., 2004; Finch et al., 1997; Marchese et al., 1990). In vivo, after paracrine secretion of KGF by fibroblasts, it binds with high affinity to the KGF receptor, which is solely expressed on epithelial cells (Miki et al., 1992). Studies revealed the involvement of KGF during wound healing (Marchese et al., 1995; Staiano-Coico et al., 1993; Werner et al., 1994, 1992) and in psoriasis (Finch et al., 1997; Kovacs et al., 2005). Furthermore, both IL-19 and IL-20, whose expression is increased in psoriatic epidermis, induce KGF transcripts in CD8⁺ T cells (Li et al., 2005; Wei et al., 2005). KGF may thus be an important contributor to KC hyperproliferation in psoriatic epidermis.

Psoriasis is considered a T-cell–mediated disease, and many of these Th cells and cytokines derived thereof can nowadays be targeted by mAbs, the so-called biologicals. The most recent developments are within the Th17 immune cell axis, and the involvement of IL-17 in psoriasis pathophysiology is widely accepted. The immune cell infiltrates (e.g., Th17 cells, neutrophils) in psoriasis release IL-17, mainly IL-17A, as key effector cytokine and further promote skin inflammatory processes and epidermal alterations typical for psoriasis (Brembilla et al., 2018). Specific evidence toward the role of IL-17A in epidermal hyperproliferation is yet scarce (Lai et al., 2012; Langowski et al., 2006; Wu et al., 2015). In this study, we found IL-17A to double the rate of KC proliferation. In psoriasis, KC hyperproliferation may thus be a consequence of local IL-17A presence near the epidermis. Interestingly, recent studies also call for a pathogenic role of Th17 cells in certain AD endotypes (Suárez-Fariñas et al., 2013), with IL-17 expression elevated in lesional skin (Gittler et al., 2012) and subsets of CD4⁺ Th2 cells may produce IL-17 (Wang et al., 2010). These and our data could suggest a role for IL-17 in AD-related KC hyperproliferation, although selective therapeutic targeting using secukinumab in patients with AD was ineffective (Ungar et al., 2021). In contrast, targeting of the Th2 immune axis by the IL-4R–targeting antibody, dupilumab, is considered effective in AD treatment and widely accepted in daily clinical practice (Beck et al., 2014; Hamilton et al., 2014).

Two members of the Th2 cytokine family, IL-4 and IL-13, have been described not only to drive the inflammatory process that contributes to AD but also to cause reduction of expression of epidermal proteins that are important for epidermal differentiation and barrier formation (Kim et al., 2008; Omori-Miyake et al., 2014). Recently, it was shown that mainly IL-13 signaling pathways dominate in AD and not IL-4 (Tsao et al., 2019). In this study, we now show that both cytokines are direct KC mitogens in organotypic epidermal models. To our knowledge, it was previously unreported for IL-13, whereas for IL-4, increased KC proliferation in vitro has been described but only in monolayer cultures (Yang et al., 2006; Longo et al., 2008). The clinical relevance of these cytokines in AD has been shown in studies using mAbs targeting IL-4Ra (dupilumab) or IL-13Ra (nintedanib)

Figure 5. IL-17A accelerates keratinocyte proliferation in vitro. (a) H&E, Ki-67, and EdU (24-hour incorporation) staining of HEEs stimulated with a dosage range of IL-17A for 72 hours and (b) quantification presented as the number of Ki-67– and EdU-positive nuclei/mm length of the epidermal equivalent (n = 2). Data are presented as mean ± SEM. Bar = 100 μm. EdU, 5-ethynyl-2'-deoxyuridine; HEE, human epidermal equivalent; SEC, secukinumab; TOF, tofacitinib.
et al., 1996). Our results indicate a prime role for the IL-4R–Jak/STAT6 signaling cascade in KC hyperproliferation for both IL-4 and IL-13 because the pretreatment with dupilumab or tofacitinib reduced STAT6 phosphorylation and inverted the Th2 cytokine–induced hyperproliferation. Normalization of epidermal turnover and thereby reduction of acanthosis and scaling may thus be considered a direct therapeutic effect of both.

For IL-17A, previous studies investigating signaling events showed a key role for Act1 and MEK/ERK5 involvement in the proliferation of KCs after imiquimod-induced psoriasis-like inflammation in mice (Ha et al., 2014). In our studies, we also aimed to put the observed in vitro hyperproliferation into perspective concerning the actual hyperproliferation that is seen in lesional skin. Similar to recently described (Damen et al., 2021), proliferative KCs were found both in the basal and suprabasal layers of the HEE. The maximum increase in the number of proliferating cells in the organotypic model is two-fold, which is lower than we found in vivo (psoriasis: nine-fold, AD: seven-fold). One explanation could be of biological origin, namely that the complexity and costimulation of other factors in vivo further drive proliferation rates compared with only by a single exposure to IL-17A, IL-4, or IL-13. In preliminary follow-up studies, we combined cytokine stimulations, yet proliferation rates never exceeded two folds. The in vitro model system may thus be a rate-limiting factor given the time point of cytokine stimulation (between days 5 and 8 of air–liquid interface culture): in this condition, KC turnover is higher than in native healthy skin. This may reduce the detection window, although even after exhaustive culture of 20 days...
Figure 7. Th2 cytokines IL-4 and IL-13 induce hyperproliferation in HEE. A dosage range of IL-4 and IL-13 added during the last 72 hours of air-exposed culture shows their effect on keratinocyte proliferation. (a, b) H&E, Ki-67, and EdU (24-hour incorporation) staining and (c, d) quantification presented as Ki-67 (IL-4, $P = 0.038$; IL-13, $P = 0.05$) and EdU (IL-4, $P = 0.02$; IL-13, $P = 0.011$) positive nuclei/mm length of the epidermal equivalent ($n = 3$). Data are presented as mean ± SEM. Bar = 100 μm. EdU, 5-ethyl-2′-deoxyuridine; HEE, human epidermal equivalent; Th2, T helper 2.
when proliferation rates in the HEE are lower, we again observed a doubled number in proliferation KCs. We postulate that the flat and rigid polycarbonate membrane does not allow for the formation of rete ridges and extension of the basal layer surface such as in native skin to accommodate a larger number of proliferating cells, which hampers the fold increase in proliferating cells and limits acanthosis by proliferation in vitro.

Notwithstanding the potential importance of molecules for which we were unable to detect any effects in our studies, in the following lines, we highlight the few molecules that did increase epidermal thickness, although proliferation rates remained normal. In several KC models, IL-22 (related to both psoriasis and AD) has been shown to increase epidermal thickness (Boniface et al., 2005; Zheng et al., 2007). We also found IL-22 to induce acanthosis; however, this was not due to increased proliferation resulting in hyperplasia but rather by hypertrophy or cell swelling. Remarkably, we even find acanthosis to be more strongly correlated to cell hypertrophy than to hyperplasia because maximum epidermal thickness is found after exposure to nonmitogenic cytokines, such as IL-22 and oncostatin M. Mitogens, such as KGF or Th2 cytokines, increase epidermal thickness to a much lesser extent. Apparently, epidermal acanthosis in hyperproliferative skin diseases may not solely be caused by a higher KC turnover but also by cell enlargement. Strikingly, all hypertrophy-inducing cytokines, IL-6, IL-20, IL-22, and oncostatin M, are known activators of STAT3 signaling (Nguyen et al., 2015). Studies in psoriatic mice have already shown that STAT3 inhibition led to the normalization of epidermal thickness (André á et al., 2013; Miyoshi et al., 2011). Indeed, proliferation rates of murine KC monolayer cultures are higher after IL-17A exposure (Wu et al., 2015). Together with our data on human reconstituted epidermis, the mitogenic effects of IL-17A in the skin are now firmly established.

In summary, our study points at a small set of cytokines that act as direct KC mitogens within the plethora and complexity of the skin inflammatory milieu and elaborates on the epidermis-specific mechanism of action of targeting therapies benefitting their positioning for specific disease endotypes.

MATERIALS AND METHODS

3D HEE culture

Primary human KC isolation and generation of 3D HEEs were performed as described previously (Rikken et al., 2020) and according to the principles of the Declaration of Helsinki. Briefly, primary human KCs were seeded with a density of 150,000 cells into a 24-well transwell system (Nunc; Thermo Fischer Scientific, Waltham, MA) and cultured submerged for 2 days in a proliferation medium (CnT-PR; CELLnTEC, Bern, Switzerland). After 1 more day of submerged culture in differentiation promoting medium (CnT-PR-3D; CELLnTEC), the cultures were lifted to the air–liquid interface and cultured thereafter for a total of 8 days, with refreshing of the medium every other day (Niehues et al., 2017). Cytokines and other tested factors supplemented to the culture medium during days 5–8 of the culture, with refreshing at day 7, in different concentrations are depicted in Table 2. Concentrations of all tested factors were chosen on the basis of experience as well as literature. For signaling studies, inhibitors were added 24 hours before cytokine stimulation and left for costimulation until day 8. To label KC proliferation in time, 10 μM EdU was added into the medium for 24 hours before harvesting at the indicated time points.

Immunohistochemistry

HEEs were fixed in formalin and processed for routine histology. Paraffin sections (6 μm) were stained with antibodies using an indirect immunoperoxidase or immunofluorescence technique (Vectastain, Vector Laboratories, Burlingame, CA) to visualize KC proliferation. Details on antibodies are presented in Table 4.
Quantification of protein expression in immunohistochemical biopsy sections

Image acquisition of immunohistochemical-stained HEE tissue sections was performed by a ZEISS Axio Imager equipped with a ZEISS Axiocam 105 color Digital Camera (Zeiss, Oberkochen, Germany) and a ×10 or ×20 objective. The ZEISS Axiocam 105 color is a compact 5-megapixel camera (2,560 × 1,920 pixels) for high-resolution images with a 1/2.5” sensor. The images were chosen as representative of the whole culture and stored in CZI format. The number of fields ranges from one to three images per slide (depending on the variation of proliferation in the HEE). The images were analyzed with the cell image analysis software CellProfiler (Broad Institute, Cambridge, MA) (McQuin et al., 2018). In CellProfiler, different algorithms are available that can be modified and placed in sequential order to form a pipeline for image analysis. Pipelines for Ki-67 and EdU analysis were created (available on request). Data visualization and statistical analysis were performed using GraphPad Prism (GraphPad software, San Diego, CA). To determine statistical significance between multiple groups (n ≥ 3 groups), the Kruskal–Wallis test (a ranked-based nonparametric test) was used for all data obtained with CellProfiler. If significant, Dunn’s multiple comparison posthoc test was performed (*P < 0.05). In each HEE culture experiment, the human KC donors used are biological replicates.

Western blotting

HEEs were lysed, and after a single cycle of freeze thawing, the lysates were centrifuged at maximum speed for 10 minutes at 4 °C. Actin antibody (Sigma-Aldrich, St. Louis, MO) was used to control equal protein loading. Before immunoblotting, proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes using the NuPAGE system (Life Technologies, Carlsbad, CA). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) was used for detection by the Bio-Rad Universal Hood Gel Imager (Bio-Rad Laboratories, Hercules, CA). All antibodies used are listed in Table 5. Image analysis and quantification of the protein expressions were performed with the Bio-Rad Image Laboratory Software. To determine statistical significance, the Kruskal–Wallis test was used followed by Dunn’s multiple comparison posthoc test.

Data availability statement

No datasets were generated or analyzed during this study.

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CONFLICTS OF INTEREST

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Figure 10. Inhibition of IL-4-/IL-13–induced proliferation. Western blot analysis and quantification of HEEs shown in Figure 9 for (a) IL-4 and (b) IL-13, stained for pSTAT3 (IL-4, \( P = 0.015 \); IL-13, \( P = 0.002 \)) and pSTAT6 (IL-4, \( P = 0.004 \); IL-13, \( P = 0.008 \)) (corrected for \( \beta \)-actin expression, IL-4–IL-13–stimulated HEEs set at 1) (\( n = 3 \)). *\( P < 0.05 \). Data are presented as mean ± SEM. (c) Ki-67 staining and (d) quantification (\( P = 0.037 \)) of HEEs treated with inhibiting agents (450 nM TOF, 100 nM JakI inhibitor, 100 nM p38, 100 nM U0126) 24 hours before costimulation with 10 ng/ml IL-4 for 72 hours (\( n = 3 \)). Data are presented as mean ± SEM. Bar = 100 μm. DUP, dupilumab; EdU, 5-ethynyl-2′-deoxyuridine; HEE, human epidermal equivalent; inh, inhibitor; pSTAT3/6, phosphorylated signal transducer and activator of transcription 3/6; TOF, tofacitinib.

Table 4. Antibodies/Chemicals Used for Immunohistochemistry/Immunofluorescence

| Target     | Antibody Clone                  | Dilution |
|------------|---------------------------------|----------|
| Ki-67      | SP6, Abcam ab16667              | 1:200    |
| Chromatin  | 4′,6-diamidino-2-phenylindol, Boehringer Mannheim | 1:3,000  |
| EdU        | Click-iT EdU Alexa Fluor 488 azide, Thermo Fischer Scientific | 1:1,000  |

Abbreviation: EdU: 5-ethynyl-2′-deoxyuridine.
Table 5. Antibodies/Chemicals Used for Western Blot Analysis

| Target       | Antibody Clone          | Dilution |
|--------------|-------------------------|----------|
| β-Actin      | AC-15, 121M 4846, Sigma Aldrich | 1:100,000 |
| pSTAT3       | Tyr705, Cell Signaling   | 1:2,000  |
| pSTAT6       | pY641, BD Biosciences    | 1:1,000  |

Abbreviation: pSTAT, phosphorylated signal transducer and activator of transcription.

AUTHOR CONTRIBUTIONS
Conceptualization: HN, GR, PLJMZ, JS, EHvdB; Data Curation: HN, GR, PEJvE; Formal Analysis: HN, GR, PEJvE; Funding Acquisition: JS, EHvdB; Investigation: HN, GR, IMJJvVW, EHvdB; Project Administration: HN, GR, EHvdB; Software: PEJvE; Validation: HN, GR, PEJvE, PLJMZ, EHvdB; Writing - Original Draft Preparation: HN, GR, PEJvE, PLJMZ, JS, EHvdB; Writing - Review and Editing: PLJMZ, JS, EHvdB

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