been postulated a prerequisite for stable thymic differentiation given the limited self-renewal capacity of thymocyte progenitors. This concept, however, has recently been challenged by several mouse models. When limiting the continuous migration of normal precursors into the thymus experimentally, autonomous thymic function over an extended period of time was still present.\(^5\)

In humans, however, it remains unclear whether long-term thymopoiesis requires continuous seeding or whether an initial engrafment of the thymus with donor progenitors is sufficient. Data from our patients suggest that the period of autonomous thymic function following transplantation is potentially long but not unlimited. An alarming finding in some but not all murine models was an increased risk of intrathymic lymphoid malignancies associated with autonomous thymic function. Although this oncogenic potential has not been detected in patients with SCID after allogeneic HSCT, lymphoproliferative syndrome as observed in 2 of our patients should be watched carefully.

Overall survival was not negatively affected by conditioning in our study, and late complications were limited and commonly related to the underlying SCID variant, particularly DCLRE1C deficiency.\(^6\) Nevertheless, innovative conditioning regimens with reduced toxicity are highly desirable. Likewise, it is important to better understand all factors that determine sustained marrow engrafment and persistent T-cell function, ultimately protecting patients with SCID from infections for their entire lives.

We thank Sandra Steinmann for excellent data management. Naomi Taylor and Richard O’Reilly provided helpful comments.

**REFERENCES**


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**STAT1 gain-of-function compromises skin host defense in the context of IFN-γ signaling**

To the Editor:

Chronic mucocutaneous candidiasis (CMC) is a primary immunodeficiency characterized by defective mucosal and skin host defense mechanisms, due to impaired Th17 responses.\(^1\)\(^2\)\(^3\)\(^4\) It was previously reported that heterozygous mutations in the signal transducer and activator of transcription 1 (STAT1) gene cause autosomal-dominant CMC (AD-CMC).\(^3\)\(^4\) In contrast to loss-of-function mutations of STAT1,\(^5\) STAT1 mutations that cause AD-CMC were found to be gain-of-function (GOF) mutations, leading to cytokine-induced STAT1 hyperphosphorylation. In addition to autoimmune phenomena, cerebral aneurysms, and oropharyngeal and esophageal cancer, the hallmark of STAT1 GOF mutation AD-CMC is severe mucocutaneous fungal infection with predominantly Candida albicans.\(^5\) At the cellular level, STAT1 GOF disturbs the development of Th17 cells, which play an essential role in neutrophil recruitment to the site of infection and induction of antimicrobial proteins in response to Candida, leading to increased susceptibility to fungal infections.\(^3\)\(^5\)\(^2\)\(^3\)\(^4\)

Research has so far been limited to the study of immune cells because of the lack of suitable in vitro tissue models. After keratinocyte isolation we first have cultured the STAT1 GOF keratinocytes next to the healthy wild-type keratinocytes in conventional submerged monolayer cultures. We have compared cell growth and gene expression patterns of keratinocyte expressed genes (filaggrin, keratin 10, late cornified envelope genes, STAT1, interleukin-6/-8/1F9); however, we have not found any differences between the 2 genotype groups (data not shown). For more in vivo–like tissue organization, we then used 3-dimensional human epidermal equivalents (HEEs) generated from patient-derived, genetically defined keratinocytes of 3 patients with CMV and several healthy controls to study the response to Th1 and Th17 cytokines, as important mediators in epithelial host defense (for all experimental details, see this article’s Methods section in the Online Repository at www.jacionline.org). No differences in morphology, epidermal stratification, and expression of proliferation (Ki67) and differentiation proteins (keratin 10, loricrin) were observed between unstimulated wild-type and STAT1 GOF mutation HEEs (Fig 1, A). For more in vivo–like tissue organization, we then used 3-dimensional human epidermal equivalents (HEEs) generated from patient-derived, genetically defined keratinocytes of 3 patients with CMV and several healthy controls to study the response to Th1 and Th17 cytokines, as important mediators in epithelial host defense (for all experimental details, see this article’s Methods section in the Online Repository at www.jacionline.org). No differences in morphology, epidermal stratification, and expression of proliferation (Ki67) and differentiation proteins (keratin 10, loricrin) were observed between unstimulated wild-type and STAT1 GOF mutation HEEs (Fig 1, A). Because keratinocytes are a source of antimicrobial proteins, we also stained for the antimicrobial protease inhibitor we also stained for the antimicrobial protease inhibitor...
SKALP/elafin and for late cornified envelope 3 (LCE3) proteins, a recently discovered group of antibacterial epithelial proteins. SKALP/elafin was present at very low levels in HEEs of both genotypes, whereas LCE3 staining displayed the normal pattern in the upper epidermis. CMC pathogenesis involves impairment of Th17 immunity of T cells and increased IFN-γ-mediated responses. Therefore, we investigated the response of keratinocytes to IL-17, IL-22, or IFN-γ, respectively. Addition of Th17 cytokines IL-17 or IL-22 did not affect Ki67 or keratin 10 expression in keratinocytes (see Fig E1 in this article’s Online Repository at www.jacionline.org). Both cytokines strongly increased the expression of SKALP/elafin, similar to Th17 cytokine-driven psoriasis skin. Addition of IL-17 caused keratinocyte hyperplasia (cell swelling) and decreased loricrin expression (see Fig E1 in this article’s Online Repository at www.jacionline.org). For all changes, no difference was observed between patient and control keratinocytes. IFN-γ stimulation, however, clearly resulted in differences between wild-type and STAT1 GOF keratinocytes (Fig 1, B). Patient-derived HEEs showed disordered epidermal structure, with cornified cells in the spinous layers. IFN-γ showed an antiproliferative effect on both genotypes, as witnessed by the absence of Ki67-positive cells. Moreover, loricrin expression was reduced in IFN-γ-treated cultures of normal cells but was increased in patient cells, suggesting premature terminal differentiation. Keratin 10 staining also indicated disordered differentiation in STAT1 GOF cells. IFN-γ did not have a notable effect on SKALP/elafin expression. In contrast, HEEs from STAT3 loss-of-function keratinocytes showed normal morphology and protein expression, unstimulated as well as on IFN-γ stimulation (see Fig E2 in this article’s Online Repository at www.jacionline.org).

Most strikingly we observed a nearly complete absence of LCE3 proteins in IFN-γ-stimulated STAT1 GOF HEE, which is in sharp contrast with normal HEEs where LCE3 expression increased on IFN-γ stimulation. Future experiments will elucidate whether IFN-γ signaling directly regulates LCE3 or if another mediator, any protein or cytokine, intermediates this protein decrease. Recent findings that LCE3 proteins have broad-spectrum antimicrobial activity prompted us to investigate possible antifungal activity of these proteins. We found that all LCE3 members killed C albicans in a dose-dependent manner (Fig 2, A). Strong anti-Candida activity was observed for LCE3A, LCE3C, and LCE3E (complete killing at 10 μM), with LCE3A being active already at 1 μM. LCE3 proteins had no effect on the noncommensal filamentous fungus Aspergillus fumigatus (data not shown). Together, the absence of antifungal LCE3 protein in STAT1 GOF HEEs on IFN-γ stimulation in vitro and the clinical appearance of fungal infections in patients with CMC at typical body sites in vivo led us to investigate the...
expression patterns of LCE3 protein at various anatomical sites. Earlier analyses already revealed that LCE3 expression is restricted to the skin and oral epithelia. Here, we show a relatively strong LCE3 protein expression in hands and feet, compared with trunk skin (Fig 2, B). Likewise, in oral keratinized epithelium (e.g., gingiva), a substantial proportion of the keratinocyte layers express LCE3 protein.

Our data suggest a pivotal role for keratinocyte-derived LCE3 in specific skin and oral locations to keep Candida overgrowth in check. In patients with STAT1 GOF, IFN-γ-driven epidermal malformation may also lead to impaired barrier function. Speculatively, this and the loss of LCE3 might contribute to the characteristic fungal infections at the hands, feet, and oral mucosa (see Fig E3 in this article’s Online Repository at www.jacionline.org). These observations provide better insight into the predisposition to superficial fungal infection at the typical anatomical sites in patients with CMC with STAT1 GOF mutation. In addition, the extreme severity of AD-CMC compared with syndromic CMC, and patients with defects in IL-17 signaling, might be explained by the fact that in addition to

FIG 2. Antifungal LCE3 proteins expressed at different anatomical locations. A, Mean CFU/mL C. albicans of N = 2 replicates after 2 hours of incubation in the presence of synthetic LCE3 protein. B, H&E (upper panel) and immunohistochemical staining of LCE3 protein (lower panel) at different anatomical sites: trunk (N = 10), foot (N = 2), hand (N = 6), and gingiva (N = 5). Scale bar = 100 μm. H&E, Hematoxylin and eosin.
T\textsubscript{H}17 deficiency there is a local defect of skin development and host defense in the context of immune cell–derived IFN-\(\gamma\). These findings do not at all rule out the impact of defective T\textsubscript{H}17 response in patients with CMC; therefore, we do not propose with our observations that increased IFN-\(\gamma\) signaling in the skin of patients with STAT1 GOF is the sole reason for CMC. However, it could contribute to the severe phenotypes we have observed in patients with STAT1 GOF and could explain why virtually all patients with this mutation will present with mucocutaneous Candida problems.\textsuperscript{E5} Second, this explains that STAT1 GOF mutation skin is normal in the absence of inflammation. However, on inflammation, especially at the feet, hands, and oral cavity, this can result in locally impaired antimicrobial activity. This might also explain that IFN-\(\gamma\) signaling inhibition with the JAK inhibitor ruxolitinib can improve skin lesions without restoring T\textsubscript{H}17 responses, because these defects are theoretically already present before fungal infection. Third, it opens up new treatment strategies for STAT1 GOF skin, namely, blocking IFN-\(\gamma\) signaling, which has been successful in several cases.\textsuperscript{S,9,10,17} With hematological stem cell transplantation it might be possible to fully restore the CMC phenotype because the immune system might overcome any local defects in the skin. However, conceptually this study underscores that it has to be kept in mind that STAT1 GOF might also have effects in nonimmune cells that would necessarily be restored with hematological stem cell transplantation.

In conclusion, we describe an intrinsic defect of STAT1 GOF patients’ skin in the presence of IFN-\(\gamma\). The defective epithelial structure and the loss of the antimicrobial protein LCE3 with activity against \textit{C} \textit{albicans} is likely to contribute to CMC severity in patients with STAT1 GOF mutation. Tissue distribution of LCE3 suggests a possible link of these proteins with the infection predilection sites at the hands, feet, and oral cavity. Moreover, these novel insights put forward another rationale to investigate JAK inhibitors in STAT1 GOF, because it might restore the local skin defects and thus has the potential to lead to clinical benefit.

We thank all patients and volunteers who participated in this study, which is funded by a TOP grant from ZonMw (grant no. 91211052) and also by ZonMW under the name EURO-CMC frame of E-Rare-2, the ERA-Net for Research on Rare Diseases. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Willeke Bloks from the Department of Pathology (Radboudumc) is acknowledged for providing paraffin sections of human tissues.

\textbf{References}


\textbf{Omalizumab effectiveness in asthma-COPD overlap: Post hoc analysis of PROSPERO}

\textbf{To the Editor:}

Asthma and chronic obstructive pulmonary disease (COPD) overlap (ACO), defined as the coexistence of clinical features of asthma and COPD, is common.\textsuperscript{1,2} Although ACO is heterogeneous, common subphenotypes of ACO include those with asthma who are older than 40 years with a history of smoking and/or those who have persistent, progressive, and partially reversible airflow limitation.\textsuperscript{2}

Epidemiologic studies suggest that ACO is associated with higher disease burden, lower quality-of-life scores, and higher health care utilization than COPD or asthma alone.\textsuperscript{1,3} Unfortunately, patients with ACO are often excluded from asthma or COPD clinical trials, including trials for recently approved targeted asthma therapies (eg, dupilumab).\textsuperscript{1}

Treatment of ACO is directed to treat symptoms and airway inflammation and typically comprises inhaled corticosteroids (ICSs) with add-on bronchodilators (long-acting beta-agonists ± long-acting muscarinic antagonists) although ACO phenotypes may be less responsive to ICSs than are patients with asthma.\textsuperscript{3} Omalizumab, a monoclonal anti-IgE antibody approved for patients (≥26 years) with moderate-to-severe allergic asthma inadequately controlled with ICSs, is not approved for patients with COPD or ACO. Efficacy of omalizumab in ACO has been
METHODS

Primary keratinocyte isolation and submerged culture

STAT1 wild-type primary human keratinocytes were obtained from skin derived from abdominal-wall corrections (at least 7 different wild-type donors used). Primary human STAT1 GOF mutation keratinocytes were obtained from biopsies of unaffected skin of the upper buttock of 3 patients with CMC (2 patients with a mutation at c.820C→T; p.Arg274Trp and 1 patient with c.821G→A p.Arg274Gln). These mutations have been reported before to cause CMC. All patients of this study provided written informed consent under a protocol adherent to the Helsinki Guidelines and approved by the Institutional Review Board of the Radboud University Medical Center (UMC). Isolation and cell culture were performed as previously described. Keratinocytes were cultured submerged in 24-well plates using keratinocyte growth medium (KGM BulletKit; Lonza, Basel, Switzerland) as previously described.

HEE culture

HEEs were generated as described previously. Briefly, 100,000 cells were seeded in 24-transwell culture plate (Thincerts, Greiner Bio-One International, Kremsmünster, Austria). After 3 days of submerged culture, the equivalents were lifted to the air-liquid interface and cultured for 8 days. Cytokine stimulation with IFN-γ (250 U/mL), IL-17A (50 ng/mL), or IL-22 (50 ng/mL) was performed during the last 72 hours of air-liquid interface culture, with restimulation after 48 hours.

Immunohistochemistry

Human in vivo tissues for immunohistochemical analysis were obtained from the archives of the Department of Pathology of the Radboud UMC. All tissues and HEEs were fixed in 4% buffered formalin, processed, and embedded in paraffin. Sections (6 μm) were processed for immunohistochemistry and stained for various epidermal proteins (Table E1) with an indirect immunoperoxidase technique.

Fungal strains and growth conditions

Calicibacillus or A. fumigatus (clinical isolates, Department of Medical Microbiology of the Radboud UMC) were inoculated on Columbia agar with 5% sheep blood (Becton, Dickinson and Co, Franklin Lakes, NJ) overnight at 35°C. A single colony of the plate was again inoculated on a new Columbia agar plate with 5% sheep blood plate overnight at 35°C. Colonies were picked and brought into sodium phosphate buffer (pH 7.4, 10 mM) and the transmission of the suspension was determined using a spectrophotometer at 530 nm (PerkinElmer Lambda35; PerkinElmer, Waltham, Mass).

Antimicrobial assay

A 10-fold dilution of the fungal suspension with a transmission value of 90% was used in the antifungal assay (resulting in 10⁵-10⁶ CFU/mL). This suspension was exposed to LCE peptides in an assay volume of 100 μL, for 2 hours at 37°C in a 96-well microplate (Greiner Bio-one). After 2 hours, the suspensions were serially diluted in steps of 10, and 10 μL of each dilution was plated on 5% sheep blood containing Columbia agar plates overnight at 35°C. Antimicrobial effects were determined by counting colony-forming units as previously described.

REFERENCES

FIG E1. Wild-type and STAT1 GOF HEEs stimulated with T_{h}17 cytokines. HEEs were stimulated with (A) 50 ng/mL IL-17 or (B) 50 ng/mL IL-22, respectively, during the last 72 hours of air-liquid interface culture. Photographs show H&E as well as immunohistochemical stainings for Ki67, K10, LOR, SKALP/elafin, and LCE3 protein. Scale bar = 100 μm. N = 2. H&E, Hematoxylin and eosin; K10, keratin 10.
FIG E2. Wild-type and STAT3 LOF HEEs stimulated with IFN-γ. H&E and LCE3 protein stainings of wild-type and STAT3 loss-of-function untreated or IFN-γ-treated HEEs. N = 2 patient keratinocyte donors and N = 2 donors of normal keratinocytes. H&E, Hematoxylin and eosin; LOF, loss-of-function.
FIG E3. Schematic drawing of STAT1 GOF skin defects and *C. albicans* infection. After an inflammatory insult, caused by external stimuli, disturbed epidermal differentiation and loss of LCE3 protein is seen at specific locations of STAT1 GOF skin. This results in aberrant skin barrier function and increased susceptibility to *C. albicans* colonization and infection.
### TABLE E1. List of antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Antibody clone, manufacturer</th>
<th>Host</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>Cytokeratin-10</td>
<td>DE-K10, Euro Diagnostics (Malmo, Sweden)</td>
<td>Mouse</td>
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<tr>
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<td>MIB-1, DAKO (Santa Clara, Calif)</td>
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<tr>
<td>SKALP/Elafin</td>
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<td>Rabbit</td>
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