

# Epidermal growth factor receptor/mitogen-activated kinase inhibitor treatment induces a distinct inflammatory hair follicle response that includes collapse of immune privilege

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Linked Article: Fukuda *Br J Dermatol* 2024; 191:658–659.

## Abstract

**Background** Inhibitors of epidermal growth factor receptor (EGFRi) or mitogen-activated kinase (MEKi) induce a folliculitis in 75–90% of patients, the pathobiology of which remains insufficiently understood.

**Objectives** To characterize changes in the skin immune status and global transcriptional profile of patients treated with EGFRi; to investigate whether EGFRi affects the hair follicle's (HF) immune privilege (IP); and to identify early proinflammatory signals induced by EGFRi/MEKi in human scalp HFs *ex vivo*.

**Methods** Scalp biopsies were taken from patients exhibiting folliculitis treated long term with EGFRi ('chronic EGFRi' group,  $n=9$ ) vs. healthy scalp skin ( $n=9$ ) and patients prior to commencing EGFRi treatment and after 2 weeks of EGFRi therapy ('acute EGFRi' group,  $n=5$ ). Healthy organ-cultured scalp HFs were exposed to an EGFRi (erlotinib,  $n=5$ ) or a MEKi (cobimetinib,  $n=5$ ). Samples were assessed by quantitative immunohistomorphometry, RNA sequencing (RNAseq) and *in situ* hybridization.

**Results** The 'chronic EGFRi' group showed CD8<sup>+</sup> T-cell infiltration of the bulge alongside a partial collapse of the HF's IP, evidenced by upregulated major histocompatibility complex (MHC) class I,  $\beta$ 2-microglobulin (B2 M) and MHC class II, and decreased transforming growth factor- $\beta$ 1 protein expression. Healthy HFs treated with EGFRi/MEKi *ex vivo* also showed partial HF IP collapse and increased transcription of human leucocyte antigen (HLA)-A, HLA-DR and B2 M transcripts. RNAseq analysis showed increased transcription of chemokines (CXCL1, CXCL13, CCL18, CCL3, CCL7) and interleukin (IL)-26 in biopsies from the 'chronic EGFRi' cohort, as well as increased IL-33 and decreased IL-37 expression in HF biopsies from the 'acute EGFRi' group and in organ-cultured HFs.

**Conclusions** The data show that EGFRi/MEKi compromise the physiological IP of human scalp HFs and suggest that future clinical management of EGFRi/MEKi-induced folliculitis requires HF IP protection and inhibition of IL-33.

## Lay summary

About 75–90% of people with cancer who are treated with drugs called EGFR inhibitors (EGFRi) and MEK inhibitors (MEKi) will get a skin condition called folliculitis. This is where the hair follicles become inflamed. Despite this, the reasons why some patients develop this are not well understood.

In this study, we had three goals. We wanted to understand how these medications alter the skin's immune response and genetic processes. We also wished to determine the impact of the medications on the immune protection of hair follicles. Finally, we wanted to find early signs of inflammation in hair follicles caused by the medications. We studied scalp samples from people who got folliculitis after long-term EGFRi treatment and compared them to samples of healthy scalp skin. We also examined patients before and after they

Accepted: 3 June 2024

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began EGFRi treatment. In the lab, we exposed healthy hair follicles to an EGFRi called 'erlotinib' or a MEKi called 'cobimetinib'. We then carried out detailed imaging and genetic analyses.

We found that long-term treatment with EGFRi increased certain immune cells (called CD8<sup>+</sup> T cells) in the hair follicle area. This led to a breakdown in the immune protection around hair follicles. A similar breakdown was found in lab-treated healthy follicles. Genetic changes linked to inflammation were also found.

Our findings suggest that EGFRi and MEKi treatments could affect the natural immune defence of hair follicles in the scalp and cause folliculitis. Protecting the immune system and controlling inflammation might be the key to treating people with these drug-related skin conditions.

#### What is already known about this topic?

- Epidermal growth factor receptor inhibitors (EGFRi) and mitogen-activated kinase inhibitors (MEKi) induce folliculitis in 75–90% of patients treated with them.
- While excessive chemokine release may be involved, the pathobiology of this folliculitis is poorly understood.

#### What does this study add?

- Inhibition of the EGFR–MEK–extracellular regulated kinase pathway induces a partial collapse of the physiological immune privilege (IP) of human hair follicles (HFs) that protects them from inflammation, both *in vivo* and *ex vivo*.
- Early on, increased intrafollicular expression of interleukin (IL)-33 may drive EGFRi/MEKi-induced folliculitis and thus deserves therapeutic targeting.

#### What is the translational message?

- A novel strategy for managing EGFRi/MEKi-induced HF toxicity in the future could be to apply topically effective agents that protect/restore the HF's IP and reduce excessive HF secretion of IL-33.

Epidermal growth factor receptor inhibitors (EGFRi) are commonly used in oncology and induce a prominent folliculitis in 75–90% of patients.<sup>1</sup> This suggests that the hair follicle (HF) is highly susceptible to immunological attack following EGFR inhibition.<sup>2–4</sup> EGFR signalling activates mitogen-activated kinases (MEKs), which promotes cell proliferation, survival, differentiation and angiogenesis.<sup>5–8</sup> Folliculitis is also seen in patients following inhibition of MEK, indicating that these adverse effects are a class effect of EGFR–MEK–extracellular regulated kinase (ERK) pathway inhibition.<sup>9–12</sup>

In cancer cells and tumours, overactivation of EGFR inhibits the expression of major histocompatibility (MHC) class Ia molecules via the MEK–ERK pathway, thereby compromising tumour immunosurveillance.<sup>13–15</sup> In contrast, EGFRi or MEKi robustly upregulate MHC class Ia and MHC class II expression not only in cancer patients, mouse models and cancer cell lines,<sup>14–16</sup> but also in cultured human epidermal keratinocytes and in the epidermis of patients treated with EGFRi via NLRP5 (NOD-line receptor family CARD domain-containing 5 and coactivator class II transactivator (CIITA)).<sup>16–23</sup> However, it remains unknown how and why these effects of EGFRi – or any other mechanisms – induce a folliculitis in the majority of treated patients.

Given the fundamental importance of the HF's physiological immune privilege (IP) for HF homeostasis and the central role of MHC class Ia and MHC class II in HF IP collapse and inflammatory hair diseases,<sup>22–27</sup> we hypothesized that EGFRi/MEKi may impair HF IP, thus predisposing the HF to

inflammatory attack. As EGFRi/MEKi are known to promote the secretion of proinflammatory cytokines/chemokines such as CXCL14, CCL2, CCL5, CCL27, interferon (IFN)- $\gamma$ , interleukin (IL)-17A, IL-8 and IL-6 in the epidermis of affected patients,<sup>28,29</sup> we also hypothesized that HF IP collapse may be combined with an abnormal HF expression of selected cytokines/chemokines that attracts an inflammatory cell infiltrate to EGFRi/MEKi-treated HFs, leading to the EGFRi/MEKi treatment-associated folliculitis.

In this study, we critically probed this working hypothesis by (i) characterizing the immunological and transcriptomic effects of EGFRi treatment in densely hair-bearing skin *in vivo*; (ii) interrogating how EGFRi affect the IP of organ-cultured scalp HFs *ex vivo*; and (iii) identifying early events after inhibition of the MEK–ERK pathway so as to identify potential therapeutic targets for mitigating the onset of EGFRi/MEKi-induced HF toxicity.

## Materials and methods

### Biopsy samples

For this study, parietal scalp (rather than truncal skin) biopsies were taken from patients with folliculitis who had been on long-term (i.e. 3–9 months) EGFRi treatment ('chronic EGFRi' group,  $n=9$ ) or from patients prior to commencing and 2 weeks after starting EGFRi treatment ('acute EGFRi'

group,  $n=5$ ), and compared them with healthy scalp skin ( $n=14$ ). Clinical recruitment methods are provided in Appendix S1 and patient demographics in Tables S1–S3 (see Supporting Information).

### Hair follicle organ culture

Tissue samples from hair transplant surgeries were collected. Isolated full-length anagen VI HF s were cultured as previously described.<sup>30</sup> Media were replaced every 48 h with the following additions: erlotinib 5  $\mu\text{mol L}^{-1}$  (Selleckchem, Houston, TX, USA); cobimetinib 1  $\mu\text{mol L}^{-1}$  (Selleckchem); or vehicle control dimethyl sulfoxide (DMSO) 0.5%. Twenty-four-hour HF organ culture was performed for RNA sequencing (RNAseq) experiments and *in situ* hybridization. Seventy-two-hour cultures were used for immunofluorescence (IF) microscopy after snap-freezing HF s and embedding them in optimal cutting temperature [OCT (Tissue Tek O.C.T. Compound; Sakura, Torrance, CA, USA)] medium. As a positive control, HF IP collapse was induced by culturing HF s with 75 IU IFN- $\gamma$  (PeproTech, London, UK).<sup>25</sup>

### Quantitative immunohistomorphometry

Cryosections or paraffin sections were processed for immunohistochemistry (IHC) or IF microscopy as summarized in Tables S4–S6 (see Supporting Information). Representative images for IHC/IF were taken using an Olympus BX53 upright microscope (Olympus, Tokyo, Japan). In scalp skin, epithelial regions of interest were systematically identified based on specific morphological features defined in Figure S1 (a, b; see Supporting Information). Quantitative immunohistomorphometry (qIHM) was performed, as previously described,<sup>31–34</sup> with the number of immune cells analysed using the QuPaths automated cell detection package (version 2.3; <https://qupath.readthedocs.io/en/0.5/>);<sup>35</sup> ImageJ (NIH, Bethesda, MD, USA) was used to measure IF intensity.<sup>26,33,36</sup> For some representative images, the contrast was changed globally within Microsoft PowerPoint, and matching settings were applied to both the test and the control.

### Gene expression profiling (RNA sequencing)

Total RNA was extracted from human scalp skin or from 6–8 full-length anagen VI HF s for each condition using an RNeasy Mini Kit (QIAGEN, Manchester, UK) according to the manufacturer's protocol. Gene expression profiling by RNAseq was performed as detailed in Appendix S2 (see Supporting Information). The adjusted  $P$ -values ( $P_{\text{adj}}$ ) ( $P$ -values corrected with the Benjamini–Hochberg method) were used to determine significant genes ( $P_{\text{adj}} < 0.1$ ).

### *In situ* hybridization

Cryosections (6  $\mu\text{m}$ ) of OCT-embedded HF s were processed for RNA *in situ* detection using an RNAscope 2.5 HD Reagent Kit-RED or Reagent Kit-GREEN (Advanced Cell Diagnostics, Milan, Italy) as described elsewhere.<sup>37–39</sup> Details of probes, detection and analysis are provided in Appendix S3 (see Supporting Information). DapB was used as a negative control and PPIB as a positive control.

### Luminex assay

Supernatants of day-3 organ-cultured HF s were assessed for cytokine content with a ProcartaPlex assay [Thermo Fisher Scientific, Waltham, MA, USA (Appendix S4; see Supporting Information)].

### Statistical analysis

All statistical analysis was carried out in Prism version 9 (GraphPad, La Jolla, CA, USA); specific tests used are discussed in Appendix S5 (see Supporting Information).

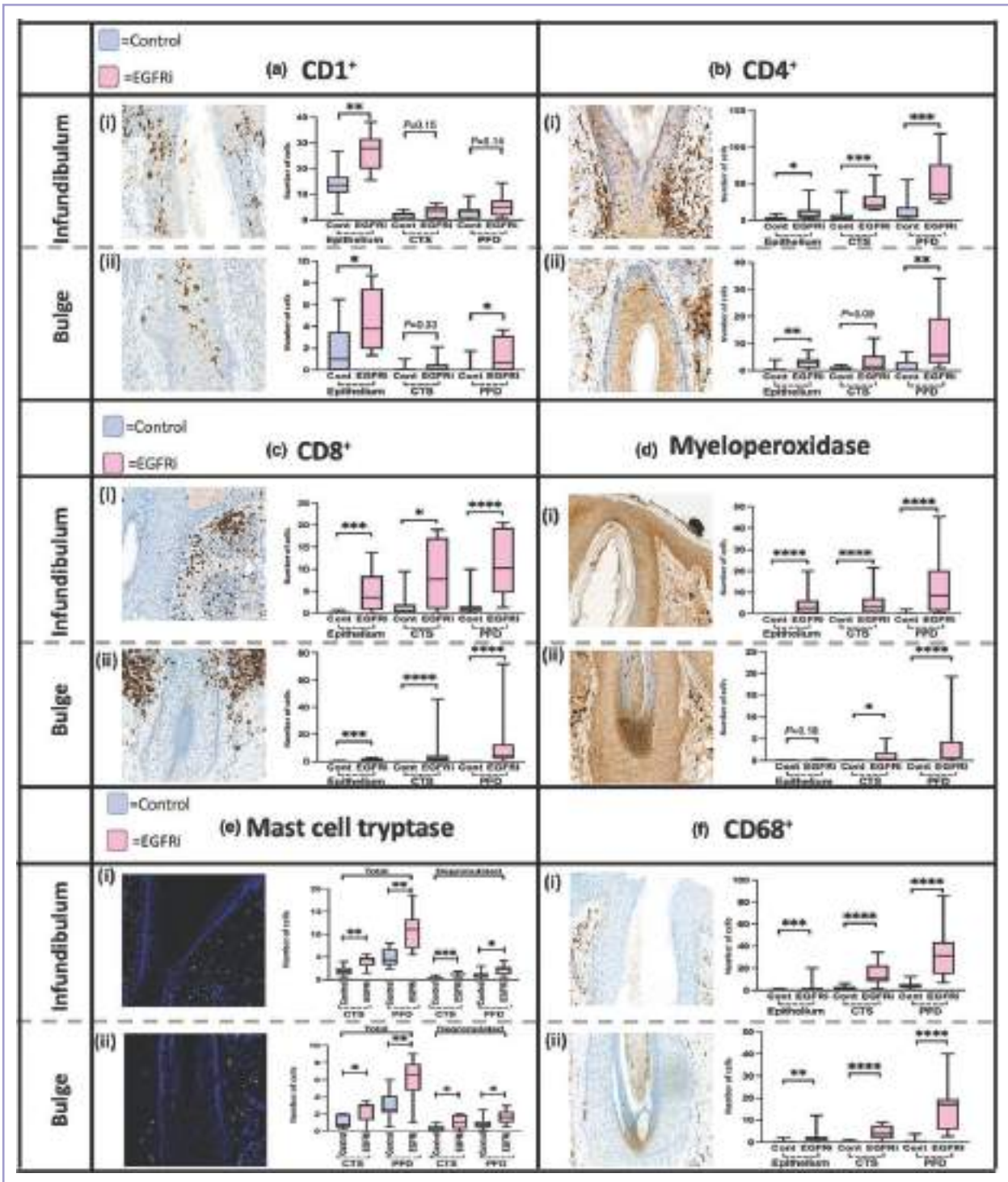
## Results

### Chronic epidermal growth factor receptor inhibitor-induced folliculitis exhibits a distinct inflammatory cell infiltrate

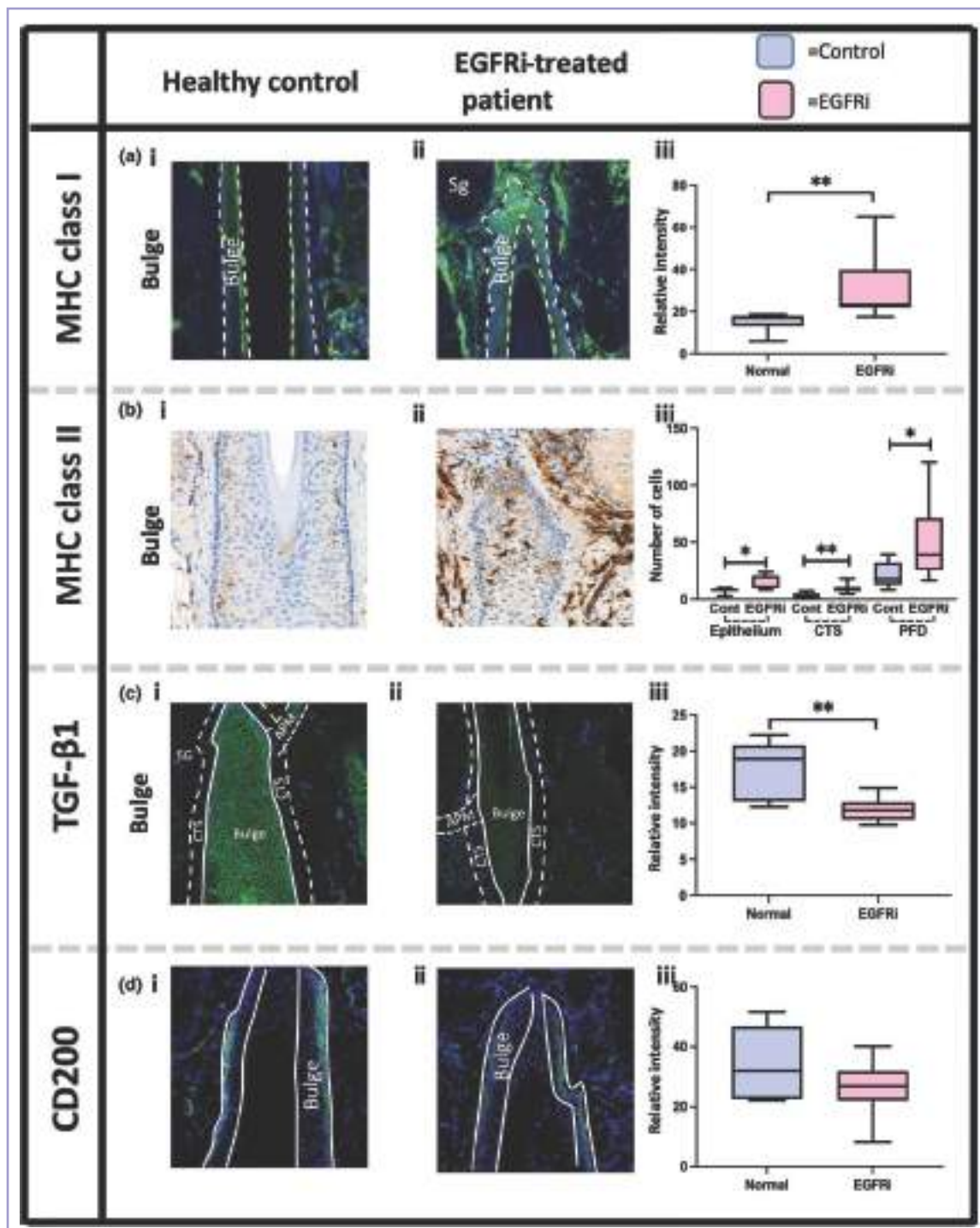
Firstly, we examined scalp biopsies taken from patients treated long term with EGFRi ('chronic EGFRi') who exhibited clinical folliculitis ( $n=9$ ) and compared them with healthy control samples ( $n=14$ ; Tables S1–S3). In line with previous studies,<sup>40–42</sup> histology (haematoxylin and eosin) revealed a dense perifollicular inflammatory infiltrate centred around the HF infundibulum (Figure S1c–h). However, we also noted a distinct – albeit less prominent – infiltrate in and around the HF insertion of the arrector pili muscle (in 73% of follicles; i.e. in the stem cell-rich bulge), a key region of relative HF IP (Figure S1g, h).<sup>26</sup> qIHM demonstrated a significant increase in a wide range of immunocytes with complex co-localization of these cells found not only within the infundibulum, but also around the bulge, including CD1+ Langerhans cells, CD4+, CD8+, neutrophils (myeloperoxidase) and CD68+ macrophages [Figure 1; Figures S2, S3 (see Supporting Information)]. Of particular note, the significantly increased number of CD8+ T cells infiltrating the bulge epithelium in biopsies from the 'chronic EGFRi' cohort compared with healthy controls is a novel and unexpected finding, as bulge infiltration – namely by CD8+ T cells – is characteristic of cicatricial alopecias (Figure 1cii).<sup>26,43,44</sup> Moreover, the number and percentage of degranulated perifollicular mast cells was significantly increased, especially in the peribulge mesenchyme (Figure 1eii). This is interesting, as proinflammatory degranulating mast cells may contribute to HF IP collapse in alopecia areata and lichenplanopilaris.<sup>33,45,46</sup>

### Chronic epidermal growth factor receptor inhibitor-induced folliculitis is associated with impaired hair follicle immune privilege

We next investigated key HF IP markers and found that both MHC class Ia and  $\beta 2$ -microglobulin protein expression were significantly increased in the bulge and proximal outer root sheath (ORS), as well as in the hair bulb, in chronic EGFRi-induced folliculitis compared with healthy controls [Figure 2a; Figure S4a, b (see Supporting Information)], where these autoantigen-presenting molecules are normally absent or expressed at very low level.<sup>23,31</sup> Thus, EGFRi increase the HF's risk of ectopically presenting physiologically sequestered autoantigens via functional  $\beta 2$ -microglobulin-stabilized



**Figure 1** Epidermal growth factor receptor inhibitor (EGFRi) treatment induces a profound inflammatory infiltrate that predominantly affects the infundibulum and isthmus. Immunohistochemical and immunofluorescence staining for (a) CD1a, (b) CD4, (c) CD8, (d) myeloperoxidase (MPO), (e) mast cell tryptase and (f) CD68 in the infundibulum and bulge of EGFRi-treated patients exhibiting a folliculitis. We found that there was a significant increase in the number of Langerhans cells (CD1a), T helper cells (CD4), cytotoxic T cells (CD8), neutrophils and macrophages (CD68) in the epithelium of the infundibulum and isthmus of patients exhibiting folliculitis treated with long-term EGFRi ('chronic EGFRi' patients) compared with the healthy scalp skin of controls (for images of healthy scalp controls, see Figure S2). Data are represented in the adjacent box-and-whisker plots, which show the minimum, maximum, median and interquartile range. Mann-Whitney *U* test comparing the number of immunocytes in healthy control (Cont) scalp skin (*n*=14 patients) vs. scalp skin taken from EGFRi-treated patients exhibiting a folliculitis (*n*=9 patients) in regions of follicular interest, including the epithelium and adjacent connective tissue sheath (CTS) or perifollicular dermis (PFD). \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.0001. Images taken at x20 magnification.



**Figure 2** Epidermal growth factor receptor inhibitors (EGFRi) increase (a) major histocompatibility complex (MHC) class I (MHC-I) and (b) MHC class II (MHC-II) protein expression and decrease the expression of the immunosuppressant protein (c) transforming growth factor (TGF)- $\beta$ 1 in the epithelium of the bulge but do not impact on (d) CD200 expression. Staining of samples of healthy control (Cont) scalp skin ( $n=7$ ) vs. samples from EGFRi-treated patients exhibiting a folliculitis ( $n=7$ ). MHC-II staining for positive cells was analysed in the follicular epithelium and adjacent mesenchyme extending laterally by 30  $\mu$ m (perifollicular mesenchyme) or 100  $\mu$ m (perifollicular dermis; PFD). Mann-Whitney  $U$  test comparing the follicular epithelium of the infundibulum and bulge. \* $P<0.05$ ; \*\* $P<0.01$ . APM, arrector pili muscle; CTS, connective tissue sheath; Sg/SG, sebaceous gland.

MHC class Ia to CD8<sup>+</sup> T cells – a key phenomenological indicator of HF IP collapse.<sup>22,24,47</sup>

From the bulge downward, human HF epithelium does not contain any MHC class II<sup>+</sup> cells under physiological

conditions.<sup>31</sup> Therefore, it is important to note that there was also a major infiltration of MHC class II<sup>+</sup> cells into the ORS in the infundibulum, bulge, proximal outer root sheath and in the bulb in EGFRi-treated patients, which corresponded

to a significant increase of these cells in the adjacent mesenchyme (Figure 2b, Figure S4c). Furthermore, the potent immunoinhibitory cytokine transforming growth factor (TGF)- $\beta$ 1 – one of the key guardians of HF IP<sup>22,24</sup> – was significantly decreased in the bulge and proximal ORS and within the anagen hair bulb of EGFRi-treated patients (Figure 2c, Figure S4d).

However, importantly, protein expression of CD200 – the crucial ‘no danger’ signal that safeguards the bulge IP<sup>15,16,34</sup> – and keratin 15 (K15; a stem cell marker of the bulge) was unaltered in chronic EGFRi-induced folliculitis vs. healthy control (Figure 2d, Figure S4e). Remarkably, protein expression of programmed death ligand 1 (PD-L1), which functions to inhibit T-cell activation,<sup>48</sup> was even upregulated in the infundibulum and bulge (Figure S4f). These phenomena may explain why EGFRi-associated folliculitis does not lead to bulge destruction and scarring alopecia in the majority of patients, despite the partial collapse of bulge IP and the marked bulge infiltration with CD8<sup>+</sup> T cells.

### Gene expression profiling demonstrates that chronic epidermal growth factor receptor inhibitor-induced folliculitis upregulates a distinct set of cytokines and chemokines

Next, we sought to obtain additional pointers to relevant pathomechanisms that may explain the EGFRi-induced folliculitis and skin infiltration. For this, we performed RNAseq analysis on parietal scalp biopsies taken from ‘chronic EGFRi’ cohort of patients with folliculitis ( $n=7$ ) and compared the results with healthy control parietal scalp skin ( $n=9$ ). Using an adjusted  $P$ -value of 0.1, 1129 differentially expressed genes (DEGs) were significantly altered in EGFRi-treated patients compared with control participants [Figure S5a, b; Dataset S1 (see Supporting Information)]. To identify the inflammatory signature associated with EGFRi treatment in densely haired human skin, proinflammatory DEGs were grouped together (Figure S5c). The full public ArrayExpress database is deposited and available at <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13455> (E-MTAB-13455) and <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13454> (E-MTAB-13454).

We found significantly increased steady-state mRNA levels of selected chemokines (CXCL1, CXCL2, CXCL6, CXCL8, CXCL13, CXCL17), cytokines (CCL2, CCL3, CCL4, CCL5, CCL7, CCL14, CCL18, CCL19) and interleukins (IL-21, IL-17F, IL-17A, IL-36 $\gamma$ , IL-23A, IL-32) in patients in the ‘chronic EGFRi’ cohort (Figure S5c). This corroborates the previously reported increased expression of several cytokines and chemokines in EGFRi skin, including CXCL6, CXCL8, IL-36 $\gamma$ , IL-17A, IL-23, IL-21, CXCL2, CCL2 and CCL5.<sup>28,29,49</sup> Importantly, this also identified several novel chronic EGFRi treatment-associated inflammatory, immunocyte-recruiting mediators, namely CXCL13, CXCL1, CCL18, CCL3, CCL7 and IL-26. For example, CXCL13 recruits T follicular helper cells;<sup>50</sup> CXCL1 neutrophils;<sup>51</sup> CCL18 dendritic cells (DCs) and regulatory T cells;<sup>52</sup> CCL3 macrophages and monocytes;<sup>53</sup> CCL7 neutrophils, DCs and natural killer (NK) cells;<sup>54</sup> and IL-26 macrophages, monocytes and NK cells.<sup>55–57</sup> These hypothesis-free transcriptomic data showed that chronic EGFRi treatment induces a profound proinflammatory switch in the signalling milieu of human skin to one that

facilitates and promotes skin infiltration by a wide range of immunocytes.

RNAseq analysis and Ingenuity Pathway Analysis (IPA) also revealed significantly enriched canonical activated T helper (Th)1 and Th2 pathways, presumably with IFN- $\gamma$  the key inducer of human HF IP collapse,<sup>22,25,47</sup> operating as an upstream regulator (Figure S5e). Together with the significant increase in the transcription of MHC class II-related genes (namely *HLA-DRA*, *HLA-DRB5* and *HLA-DPA1*; Figure S5c), these transcriptomic data independently support the protein-level evidence of severe HF IP impairment in EGFRi-treated patients (Figure 2a, b).

### Acute epidermal growth factor inhibitor (EGFRi) treatment effects mirror those of long-term EGFRi-treated patients, except CD8<sup>+</sup> T-cell infiltration of the bulge

We next asked how acute EGFRi treatment affects scalp HF immunology, taking biopsies from parietal nonpustulated scalp areas after 2 weeks of EGFRi treatment. Surprisingly, despite the major differences in treatment duration and associated clinical signs (absence of pustulation/folliculitis), several immunocyte populations were already significantly increased in a manner that closely resembled the pattern found in the infundibulum and bulge of biopsies from the ‘chronic EGFRi’ cohort, including CD1<sup>+</sup>, CD68<sup>+</sup>, CD4<sup>+</sup> and mast cells – with the notable exception of bulge infiltration by CD8<sup>+</sup> T cells (Figures S6, S7; see Supporting Information). This suggests that bulge infiltration is a late-stage phenomenon in the pathogenesis of EGFRi-associated folliculitis.

Just as in ‘chronic EGFRi’ patients with folliculitis, HFs from scalp biopsies from ‘acute EGFRi’ patients showed features of IP dysregulation, including increased MHC class Ia expression throughout the HF epithelium (Figure S8a; see Supporting Information), matched by a decrease in immunoinhibitory TGF- $\beta$ 1 in the infundibulum, bulge and bulb (Figure S8b), and increased PD-L1 expression in the interfollicular epidermis, infundibulum and bulge (Figure S8c). There was also an increase in the number of MHC class II<sup>+</sup> cells throughout the epithelium, connective tissue sheath (CTS) and perifollicular dermis (Figure S9a–e; see Supporting Information). Again, there was no change in CD200 or K15 expression in the HF bulge (Figure S9f).

Thus, these HF IP dysregulation phenomena – predominantly the increase in MHC class Ia and MHC class II<sup>+</sup> cells – may pre-date the pathological influx of immunocytes into the HF epithelium as a potential direct consequence of suppressing EGFR-mediated signalling.

### Epidermal growth factor receptor and mitogen-activated kinase inhibitors upregulate hair follicle expression of major histocompatibility class Ia *ex vivo*

To probe the hypothesis that EGFRi treatment directly induces the HF IP dysregulation phenomena described above, healthy and uninfamed human anagen scalp HFs were microdissected, organ-cultured in serum-free medium,<sup>30</sup> and treated for 3 days with erlotinib 5  $\mu$ g mL<sup>-1</sup> (an EGFRi), 1  $\mu$ g mL<sup>-1</sup> cobimetinib (a MEKi) or vehicle (DMSO 0.5%, as a negative control) in the presence or absence

of the HF IP collapse inducer IFN- $\gamma$  (75 IU, as a positive control).<sup>25</sup> qIHM showed that erlotinib and cobimetinib independently increase MHC class Ia protein expression in the HF epithelium (Figure 3a) but without significantly altering MHC class II (Figure 3bi–iii), TGF- $\beta$ 1 (Figure S10a–c; see Supporting Information) or CD200 protein expression (Figure S10b). This shows that EGFRi/MEKi directly compromise HF IP independently of an influx of extrafollicular inflammatory cells.

Previous studies in HaCaT cells have shown that the synergistic effect of co-culturing EGFRi with IFN- $\gamma$  augments the antigen-presenting effect of IFN- $\gamma$ .<sup>16,20</sup> Therefore, we asked if this is recapitulated in EGFRi-treated HFs. Besides the expected IFN- $\gamma$ -mediated increase of MHC class Ia (Figure 3aiv) and MHC class II (Figure 3biv) expression in the HF epithelium and the increased percentage of MHC class II+ cells in the CTS,<sup>23,26,36</sup> combined treatment with erlotinib or cobimetinib plus IFN- $\gamma$  further upregulated MHC class Ia (Figure 3av, avi) and MHC class II expression vs. IFN- $\gamma$  alone throughout the whole HF epithelium (Figure 3bv, vi).

### Dysregulation of immune privilege-related genes occurs early after intrafollicular epidermal growth factor receptor–mitogen-activated kinase pathway inhibition

Next, we treated HFs from five patients (6–8 HFs per group) with cobimetinib for 24 h *ex vivo*, to characterize specifically early intrafollicular gene expression changes after MEK–ERK pathway inhibition, rather than after EGFR blockade, as the latter also inhibits other downstream signals, including phosphoinositide 3-kinase/Akt and Janus kinase/signal transducers and activators of transcription.<sup>58</sup>

Using an adjusted *P*-value of 0.1, we identified 5947 DEGs between MEKi and control treated HFs (Dataset S2; see Supporting Information). Interestingly, the significantly increased DEGs included MHC class I pathway members (*NLRC5*, *IRF1*, *B2M*, *HLA-B*, *TAP1*) and MHC class II pathway members (*CIITA*, *HLA-DRA*, *HLA-DRB1*, *HLA-DQB1*, *HLA-DPA1*, *HLA-DM*) (Figure 4a–c). Given that *B2M* was one of the most highly expressed transcripts and one of the most significantly upregulated IP-related genes, we independently validated this finding by *in situ* hybridization in HFs treated with cobimetinib (Figure 4d).

This was contrasted by a decrease in steady-state mRNA levels for the immunoinhibitory genes *TGFB1* and *HLA-G*, two well-appreciated HF IP guardians.<sup>23,24,59</sup> However, transcription of the ‘no danger’ signal gene *CD200* was increased (Figure 4a–c).

### Epidermal growth factor receptor/mitogen-activated kinase inhibitors upregulate intrafollicular interleukin (IL)-33 transcription and inhibit soluble IL-33 receptor secretion from scalp hair follicles

We next hypothesized that shared inflammatory DEGs expressed in the organ-cultured HFs and ‘acute EGFRi’ cohort of patients could play a critical role in mediating the initial inflammatory infiltrate following EGFRi (Figure S11a–e). We then compared this ‘acute EGFRi’ dataset

(Dataset S3; see Supporting Information) with our RNAseq dataset derived from organ-cultured HFs (Dataset S2) and identified 127 shared DEGs that were unidirectionally regulated in both ‘acute EGFRi’-treated patients *in vivo* and in MEKi-treated HFs *ex vivo* (Figure S10f). Of these DEGs, two were inflammatory DEGs of particular interest, including *IL33*, encoding the alarmin cytokine IL-33,<sup>60</sup> which was significantly upregulated, while *IL37*, encoding the anti-inflammatory IL-37,<sup>60–63</sup> was downregulated.

As IPA analysis identified IL-33 as an upstream regulator in our ‘chronic EGFRi’ cohort (Figure S5f), we also performed *in situ* hybridization of IL-33. This showed that erlotinib and cobimetinib both increased IL-33 transcription in the bulge and proximal ORS, but not in the hair bulb, after 24 h of HF organ culture [Figure 5a, b; Figure S12 (see Supporting Information)]. This suggests – but does not yet prove – that IL-33 is an important early proinflammatory mediator of EGFRi-induced folliculitis as it has previously been shown to induce macrophage homing to sites of inflammation, as well as promoting interleukin production to cause mast cell, DC, T-cell migration and the production of Th2-associated cytokines.<sup>60,64</sup>

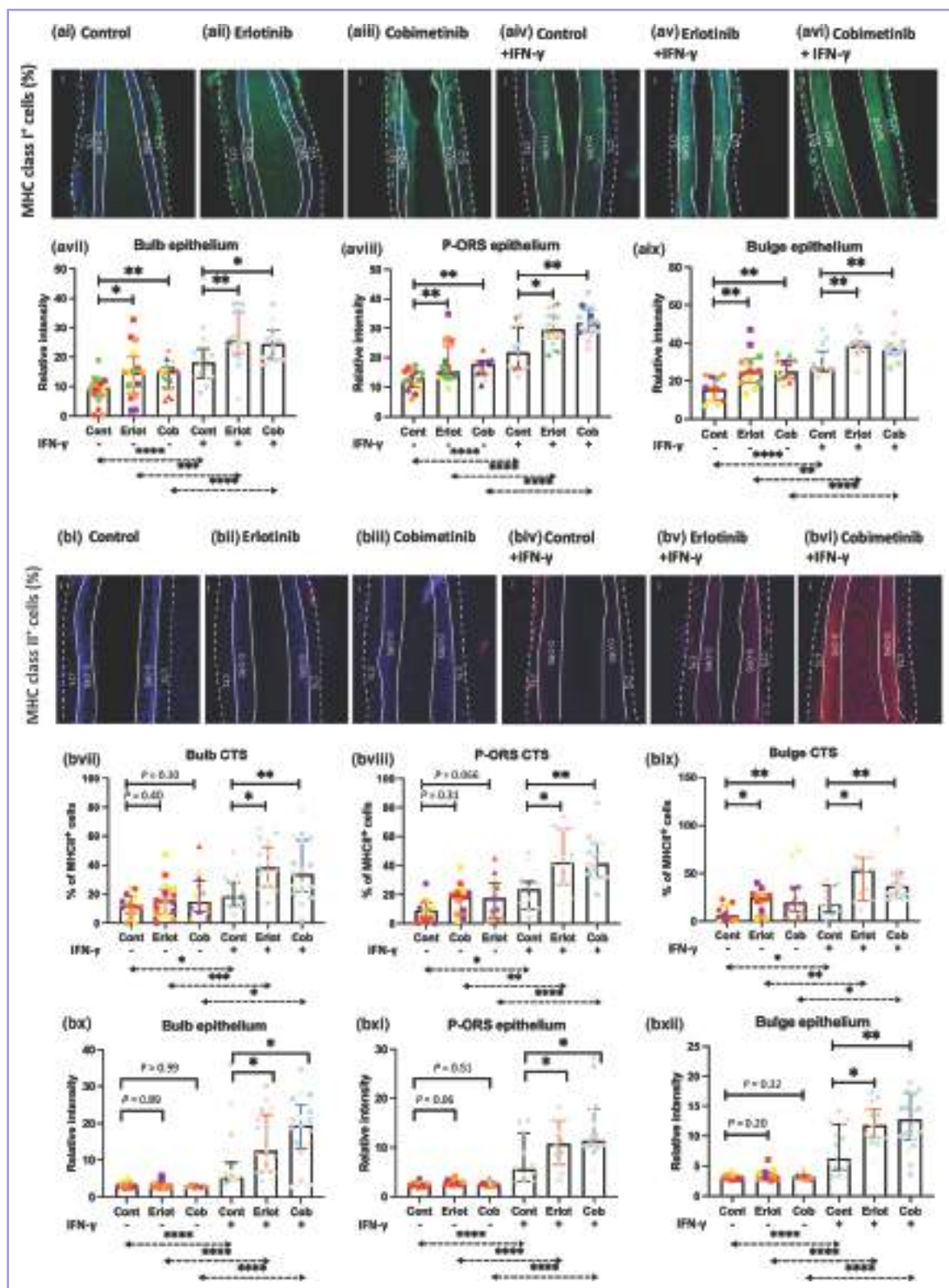
We therefore evaluated the secretion of cytokines, chemokines and interleukins by organ-cultured HFs after 3 days into the media. Interestingly, in the presence and absence of IFN- $\gamma$ , erlotinib and cobimetinib inhibited secretion of the soluble IL-33 receptor (sST2) otherwise known as IL1rL1 (Figure 5e), which acts as a decoy receptor and sequesters free IL-33 to prevent IL-33 signalling.<sup>65</sup> This EGFRi/MEKi-induced decrease in sST2 secretion likely potentiates the proinflammatory effects of enhanced intrafollicular IL-33 signalling.

## Discussion

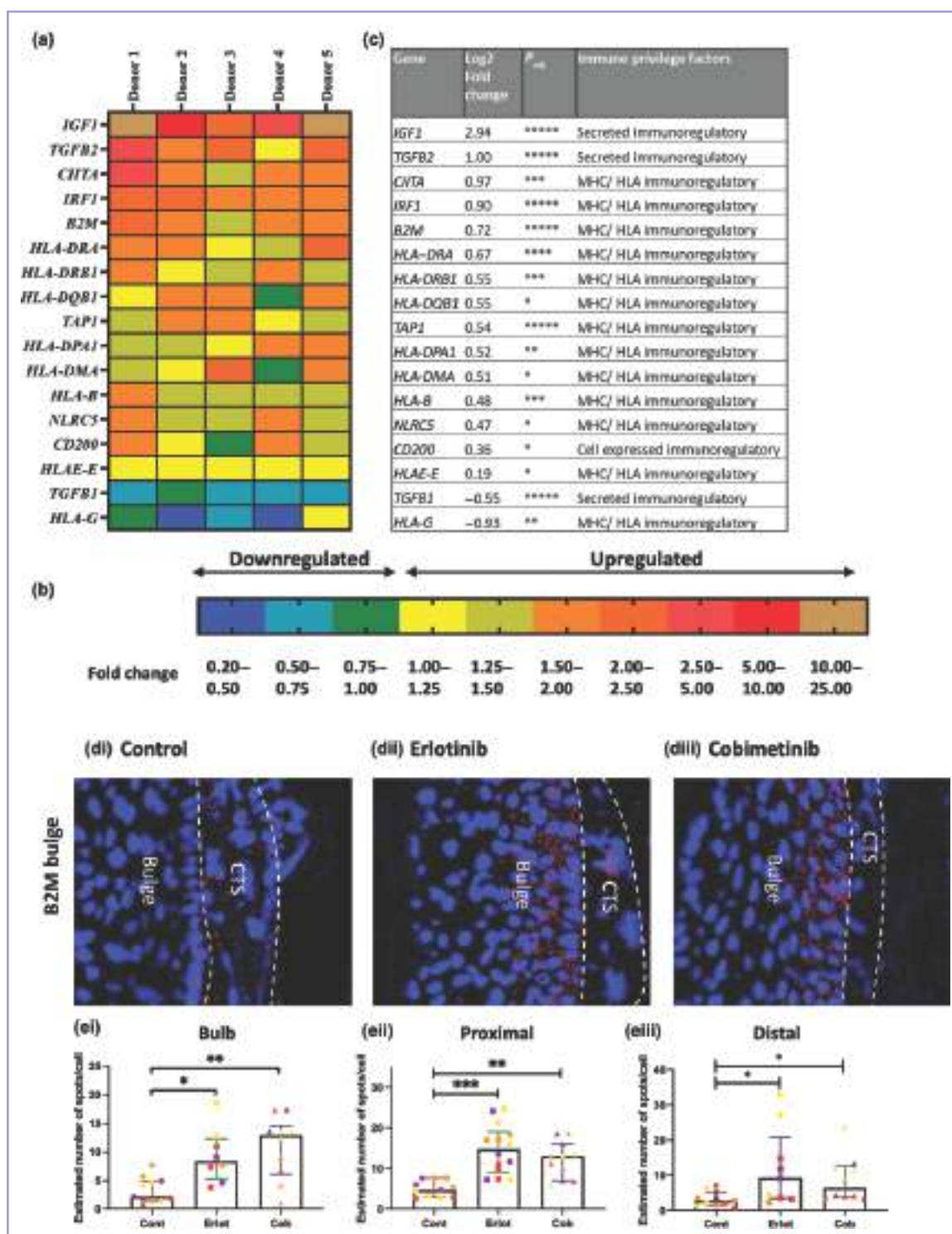
Compared with what has already been elucidated about the pathogenesis of EGFRi/MEKi-associated folliculitis in human skin,<sup>28,49,66</sup> our study provides the clinically important novel insights that – following EGFR inhibition – CD8+ cells invade areas of the HF that exhibit a dysregulated IP characterized by an increase in MHC class Ia,  $\beta$ 2 microglobulin, MHC class II and a decrease in TGF- $\beta$ 1. This research corroborates recent findings by Strobl *et al.*,<sup>67</sup> who demonstrated that EGFR serves a protective role against scarring follicle degeneration in mice, mediated via a reduction in IP. However, our study is the first to show in human HFs that EGFRi treatment directly and severely impairs the physiological IP of human HF by upregulating its autoantigen-presenting capacity in key regions exhibiting IP and by weakening its expression of IP guardians, but it does not result in a complete IP collapse.

That CD200 expression in the bulge is maintained while, unexpectedly, PD-L1 is even upregulated throughout the HF epithelium – in striking contrast with the complete HF IP collapse seen in patients with lichen planopilaris, frontal fibrosing alopecia or alopecia areata<sup>22,26,43,68</sup> – explains why actual hair loss occurs only in a minority of EGFRi-treated patients who develop folliculitis and also why any such hair loss is often reversible.<sup>69,70</sup>

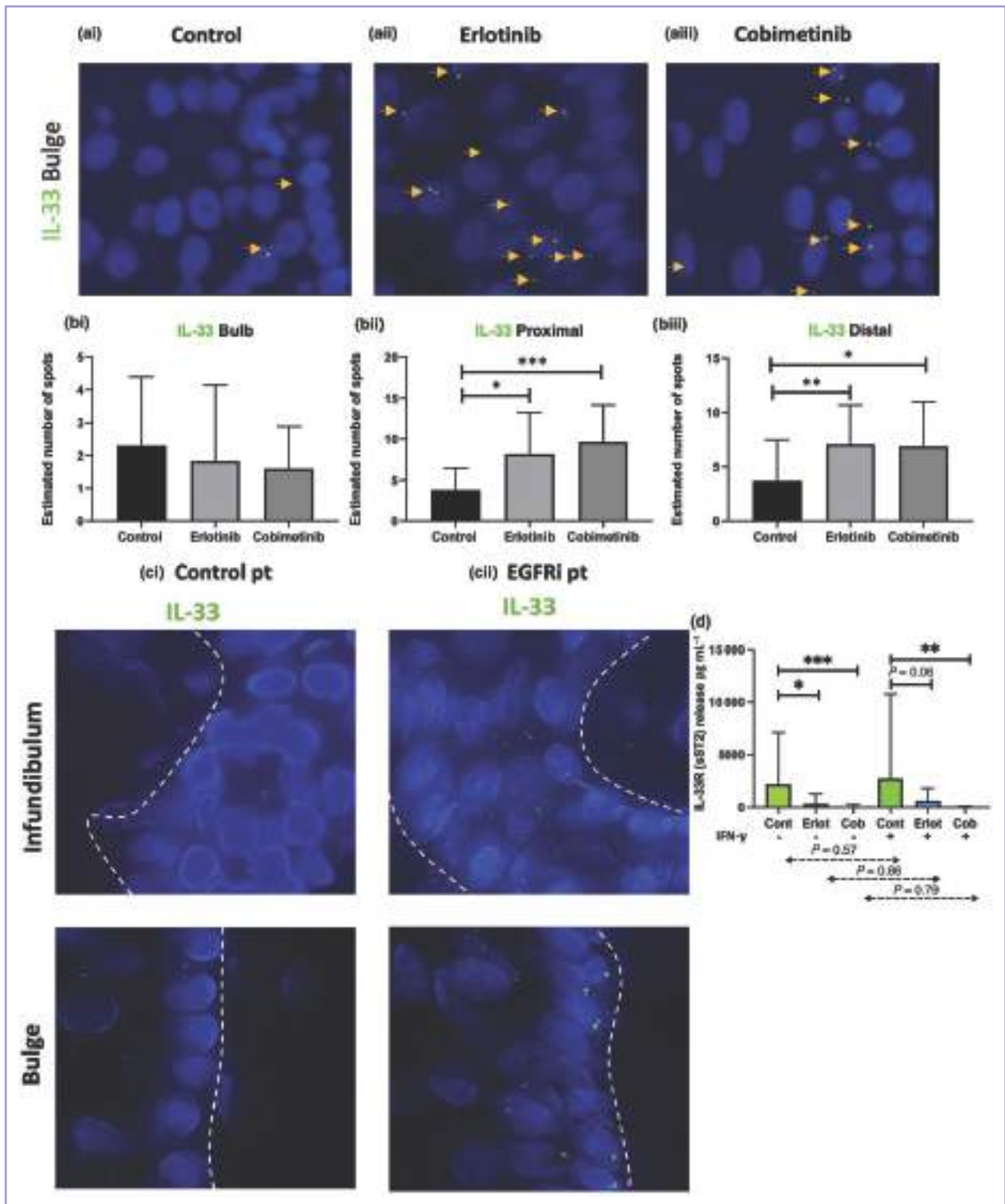
Our IL-33 and sST2 data in organ-cultured HFs and samples from the ‘acute EGFRi’ group are in line with



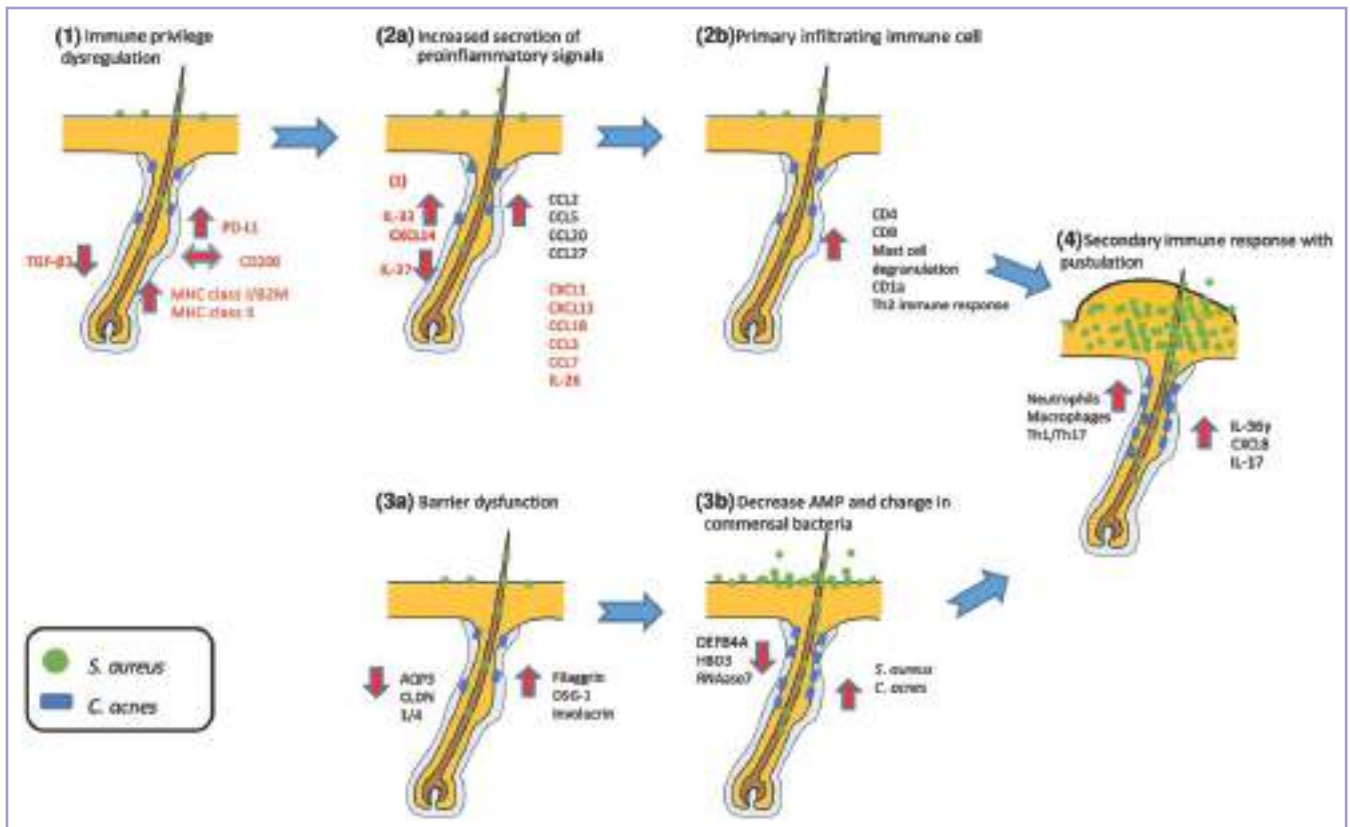
**Figure 3** In the epithelium of the bulge, organ-cultured hair follicles treated with erlotinib (Erlot) or cobimetinib (Cob) both independently upregulated the expression of major histocompatibility complex (MHC) class I molecules. Both erlotinib and cobimetinib potentiate the expression of MHC class I and II molecules in the presence of interferon (IFN)- $\gamma$ . Organ-cultured hair follicles (HF) were treated for 3 days with either dimethyl sulfoxide 0.1% (v/v) ('Control'), erlotinib 5  $\mu\text{mol L}^{-1}$  or cobimetinib 1  $\mu\text{mol L}^{-1}$  in the absence of IFN- $\gamma$  or in the presence of 75 IU IFN- $\gamma$ . Follicles were stained for either (ai–vi) MHC class I or (bi–vi) MHC class II (avii–ix, bvii–ix). The intensity of both MHC class I and class II expression was measured throughout the epithelium of the HF and (bx–xii) the percentage of MHC class II cells was calculated in the connective tissue sheath throughout the HF. Kruskal–Wallis tests were performed and those that reached significance were followed by Dunn's multiple comparison tests. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.0001$  (11–17 HF used per treatment from 4 patient samples). CTS, connective tissue sheath; D-ORS, distal outer root sheath/bulge; DP, dermal papilla; M, hair matrix of the bulb; p-ORS, proximal outer root sheath (images taken at  $\times 20$  magnification).



**Figure 4** RNA sequencing analysis results of immune privilege (IP)-related genes that were significantly expressed in 24-h organ-cultured human hair follicles (HFs) following treatment with the mitogen-activated kinase inhibitor cobimetinib. (a) Heatmap of the expression fold change of IP-related genes ( $P_{adj} < 0.1$ ) from five separate donors following cobimetinib treatment and normalized to control HFs (treated with dimethyl sulfoxide; DMSO) taken from the same donor (8–12 HFs were used for each treatment per donor). (b) Colour assignment for fold change. (c) Summary of the overall transcriptional log<sub>2</sub> fold change of relevant IP-related genes, the adjusted  $P$ -value ( $P_{adj}$ ) and whether individual genes are known to be involved with major histocompatibility complex (MHC)/human leucocyte antigen (HLA) expression, secreted immunoregulatory genes or cell-expressed immunoregulatory genes. \* $P_{adj} < 0.1$ ; \*\* $P_{adj} < 0.05$ ; \*\*\* $P_{adj} < 0.01$ ; \*\*\*\* $P_{adj} < 0.001$ ; \*\*\*\*\* $P_{adj} < 0.0001$ . (d, e) *In situ* hybridization of  $\beta$ 2-microglobulin (B2 M) expression in 24-h organ-cultured HFs treated with either DMSO (Control)/erlotinib 5  $\mu\text{mol L}^{-1}$  or cobimetinib 1  $\mu\text{mol L}^{-1}$ . Images taken at  $\times 40$  magnification. (ei–iii) Mann-Whitney  $U$  test comparing the estimated number of B2 M transcripts in the bulb, proximal or central hair follicular epithelium following incubation with DMSO (cont) vs erlotinib (Erlot) or cobimetinib (Cob); 10–14 HFs used per treatment from 4 patient samples). \* $P < 0.05$ ; \*\* $P < 0.01$ . CTS, connective tissue sheath.



**Figure 5** In organ-cultured hair follicles (HF) the epidermal growth factor receptor inhibitor (EGFRi) erlotinib and the mitogen-activated kinase inhibitor (MEKi) cobimetinib increased the expression of interleukin (IL)-33 in the proximal and distal outer root sheath, which was also seen in EGFRi-treated patients. (ai–iii) *In situ* hybridization of IL-33 expression in 24-h organ-cultured HF treated with dimethyl sulfoxide (DMSO) 0.1% (v/v) (Control), erlotinib 5  $\mu\text{mol L}^{-1}$  or cobimetinib 1  $\mu\text{mol L}^{-1}$ . Images taken at  $\times 60$  magnification. (bi–iii) Kruskal–Wallis tests were performed for *in situ* hybridization results; those that reached statistical significance were then analysed with Dunn’s multiple comparison tests (11–16 HF used per treatment from 4 patient samples). (ci–ii) The increase in IL-33 was also recapitulated in the HF of patients ( $n=2$ ) after 2 weeks of EGFRi treatment. (d) Erlotinib and cobimetinib inhibited the secretion of soluble IL-33R (sST2) from organ-cultured HF in the presence and absence of interferon (IFN)- $\gamma$ . The protein levels of secreted inflammatory proteins in the media of day 3 human organ-cultured HF were detected by a Luminex assay. HF were treated with either DMSO 0.1% (v/v) control (Cont), erlotinib (Erlot) 5  $\mu\text{mol L}^{-1}$  or cobimetinib (Cob) 1  $\mu\text{mol L}^{-1}$  for 4 h followed by the addition or absence of IFN- $\gamma$  75 IU. Protein levels are presented as  $\text{pg mL}^{-1}$ . Kruskal–Wallis tests were performed and those that reached statistical significance were followed by Dunn’s multiple comparison tests. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$  (10–19 HF per condition;  $n=4$  individuals). pt, patient.



**Figure 6** Proposed mechanism of epidermal growth factor receptor inhibitor (EGFRi)-induced folliculitis. We propose that a number of deleterious effects occur in the hair follicles (HFs) of patients following treatment with EGFRi/mitogen-activated kinase inhibitors (MEKi). Novel findings outlined in this paper are highlighted in red. (1) Dysregulation of the immune privilege: increased follicular expression of major histocompatibility complex (MHC) class I (MHC-I),  $\beta$ 2-microglobulin (B2M) and MHC class II (MHC-II) molecules, making the HF highly susceptible to HF-damaging inflammation. (2) Increased secretion of inflammatory cytokines promotes the infiltration of multiple immune cells. (2a) Initially, the increase of proinflammatory peptides, including interleukin (IL)-33, cytokines (CCL2, CCL3, CCL5, CCL7, CCL18, CCL20, CCL27), chemokines (CXCL1, CXCL13, CXCL14) and inhibition of IL-37, an anti-inflammatory interleukin. (2b) The initial inflammatory infiltrate includes CD4<sup>+</sup>/CD8<sup>+</sup> T cells, macrophages, CD1<sup>+</sup> Langerhans cells and degranulating mast cells. (3) A barrier defect occurs in the epithelium and outer root sheath of the HF combined with increased dysbiosis. (3a) Decrease in claudin-1 (CLDN1)/claudin-4 (CLDN4) and an increase in aquaporins (AQP). (3b) Inhibition of antimicrobial peptides [RNase7 and defensin beta 4A (DEFB4A)] increase dysbiosis (*Staphylococcus aureus* and *Cutibacterium acnes*) on the epidermis and within the HF ostia. (4) Secondary immune response: the increase in commensal bacteria, including *C. acnes* increases the Kruppel like factor 4 (KLF4), which induces the secretion of IL-36 $\gamma$  and, consequently, IL-8, which drives neutrophil recruitment resulting in a pustular folliculitis. AMP, antimicrobial peptide; DSG-1, desmoglein 1; HBD, human  $\beta$ -defensin; PD-L1, programmed death ligand 1; Th, T helper.

the observations that this alarmin cytokine is elevated in the sera of patients with EGFRi-induced folliculitis and in EGFR knockout mice,<sup>68,71–74</sup> and enhances IFN- $\gamma$  production by NK cells,<sup>43</sup> as well as neutrophil chemotaxis.<sup>71</sup> If subsequent mechanistic studies confirm that IL-33 signalling does play a key role in driving early pathogenesis events in EGFRi/MEKi-induced folliculitis, it could become a key target of therapeutic intervention, to reduce or prevent this burdensome – and currently almost inevitable – folliculitis. However, EGFRi induces additional deleterious effects, including the expression of Kruppel-like factor 4 (KLF4) from EGFR/MEK-inhibited keratinocytes, which – in turn – promotes the secretion of IL-36 $\gamma$  and, consequently, IL-8, which drives neutrophil recruitment to HFs, manifesting as a pustular folliculitis.<sup>49</sup> Therefore, it is most likely that several different pathways coalesce and culminate in a pustular HF response to EGFRi/MEKi. We propose a possible pathogenic mechanism of EGFRi-induced folliculitis along the hypothetical lines synthesized in Figure 6, which combines our findings with those of prior work.<sup>28,49,66</sup>

Although we have shown a number of interesting findings, these are broadly phenomenological and observational. To translate these findings into the clinic, a more in-depth functional study is required. In particular, future research should prioritize whether agents that strongly downregulate MHC class I or inhibitors of IL-33/agonists of IL-37 prevent the onset of a folliculitis. Fortunately, excellent preclinical research models are available in which these open questions and hypotheses delineated above can be addressed by utilizing human scalp skin xenotransplants on severe combined immunodeficiency mice, which can be reconstituted with a human haematopoietic system,<sup>34</sup> and are thus able to mimic the influx of toxicity-relevant immunocytes such as neutrophils and T cells into human scalp skin transplants.

If our results regarding the ability of EGFRi to perturb the IP of HFs and induce the secretion of IL-33 are corroborated, this could open the door to exciting and novel targeted therapeutics. This may include topical rapamycin,<sup>75</sup> which is known to upregulate the intrafollicular endogenous expression of  $\alpha$ -melanocyte-stimulating hormone (MSH), a known

IP guardian, or treatments that strongly downregulate MHC class I, including topical tacrolimus, super-potent synthetic  $\alpha$ -MSH (afamelanotide), topical aprepitant and vasoactive intestinal peptide receptor agonists.<sup>25,36,76</sup> Alternatively, or in combination, IL-33 inhibitors could prevent the ensuing inflammatory milieu, which is particularly encouraging given the availability of a number of IL-33 inhibitors in current clinical use.<sup>71,77</sup>

### Acknowledgements

The authors wish to thank Professor Bernard Homey, Dr Patrick Twomey and Dr Will Watson for their helpful professional advice and encouragement.

### Funding sources

This work was supported by a Medical Research Council (MR/N025989/1) and supported, in part, by Roche/Genentech. R.B.W. is supported, in part, by the National Institute for Health and Care Research Manchester Biomedical Research Centre. R.P. is supported by a Forst Endowed Scholarship from the Department of Dermatology, University of Miami.

### Conflicts of interest

D.R. is part funded by Roche/Genentech. R.P. is president of a contract research organization that also engages in analyses of drug skin toxicity ([www.monasteriumlab.com](http://www.monasteriumlab.com)), and chief executive officer of a company that develops new actives for skin and hair diseases ([www.cutaneon.com](http://www.cutaneon.com)), but declares no relevant conflicts of interest related to this study.

### Data availability

The data underlying this article are available in the article and [Supporting Information](#).

### Ethics statement

Ethics approval for the use of human tissue was obtained from the North West Research Ethics Committee, UK (Research Ethics Committee 12/NW/0525) and UK North-West Haydock Research Ethics Committee (19/NW/0082). All patients signed informed consent forms before undergoing study-related procedures. This study was conducted in accordance with consensus ethics principles derived from international ethical guidelines, including the Declaration of Helsinki.

### Patient consent

Written patient consent for publication was obtained.

### Supporting Information

Additional [Supporting Information](#) may be found in the online version of this article at the publisher's website.

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