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Culprit drugs induce specific IL-36 overexpression in Acute Generalized Exanthematous Pustulosis

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Short title: IL-36 overexpression in Acute Generalized Exanthematous Pustulosis

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ABSTRACT

Acute generalized exanthematous pustulosis (AGEP) is a severe adverse cutaneous drug reaction. Although an involvement of drug-specific T cells has been reported, the pathophysiology of AGEP and mechanism of neutrophilic skin inflammation remains incompletely understood. Recently, mutations in *IL-36RN*, the gene encoding the IL-36 receptor antagonist, have been reported to be more frequent in AGEP patients and pustular psoriasis. Here, we show that IL-36 cytokines, in particular IL-36 γ , is highly expressed in lesional skin of AGEP patients, keratinocytes and macrophages being a major source of IL-36 γ . Such an IL-36 γ overexpression could not be observed in patients with drug-induced maculo-papular rash (MPR). *In vitro*, the causative drug specifically induced IL-36 γ release either directly by peripheral blood monocytes from AGEP patients, or indirectly by keratinocytes in presence of autologous peripheral blood mononuclear cells. Such culprit drug induction of IL-36 γ secretion *in vitro* was specific for AGEP and involved the sensing of drug/albumin complex as danger signal by Toll-like receptor 4. Our results suggest that IL-36 γ secretion by monocytes/macrophages and keratinocytes in response to culprit drug exposure likely plays a key role in the pathogenesis of AGEP.

INTRODUCTION

Acute generalized exanthematous pustulosis (AGEP) is a severe cutaneous adverse drug reaction (ADR) caused mainly by antibiotics, antimalarials and antifungals (Sidoroff et al., 2001). Clinically, AGEP is characterized by a disseminated eruption of sterile nonfollicular pustules on the background of a widespread erythematous skin eruption, accompanied with fever and peripheral blood neutrophilia. To date, the pathophysiology of AGEP remains not fully elucidated. However, AGEP is currently considered as a T cell-mediated disease (Pichler, 2003) and is currently seen as a delayed type hypersensitivity reaction. Indeed, drug-specific T cells are suspected to play a central role in AGEP as evidenced by the high levels of T cell stimulation induced by causative (culprit) drugs as measured by the lymphocyte transformation test (LTT) (Anliker and Wuthrich, 2003). Furthermore, drug-specific CD4⁺ and CD8⁺ T cells have been derived *in vitro* from AGEP patients' peripheral blood. Most of these drug-specific T cells (Nishio et al., 2007) produce IL-8 (Britschgi and Pichler, 2002, Britschgi et al., 2001), a powerful neutrophil chemoattractant. IL-8-producing T cells are therefore, considered to be the cause of neutrophil survival and recruitment to the skin during the course of the disease (Schaerli et al., 2004). It has also been suggested that Th17 effector cytokines, namely IL-17 and IL-22, stimulate keratinocytes to produce IL-8, and that keratinocyte-derived IL-8 also contributes to neutrophil accumulation in AGEP patients' skin (Kabashima et al., 2011). Hence, T cell- and keratinocyte-derived IL-8 has been proposed to be responsible for the recruitment of neutrophils to the intraepithelial pustules.

AGEP shares certain clinical and histological features with pustular psoriasis. Recently, genetic studies identified mutations in the IL-36 receptor antagonist gene (*IL-36RN*) in patients with general pustular psoriasis (Kanazawa et al., 2013, Marrakchi et al., 2011, Onoufriadis et al., 2011, Sugiura et al., 2012). Interestingly, mutations in *IL-36RN* have also

been reported in AGEP (Navarini et al., 2013), suggesting that IL-36 signaling dysregulation is involved in the physiopathology of both pustular diseases. IL-36 receptor antagonist (IL-36Ra) inhibits the binding of IL-36 α , β and γ to their receptor. A mutation in *IL-36RN* may therefore result in exacerbated IL-36 signaling leading to the production of IL-1, IL-6 and IL-8 and subsequent neutrophilic skin infiltration with pustule formation.

IL-36 cytokines have emerged as important cytokines mediating inflammatory responses in the skin. Indeed, several reports have shown that all IL-36 isoforms are overexpressed in psoriatic skin (Debets et al., 2001, Gresnigt and van de Veerdonk, 2013, He et al., 2013, Johnston et al., 2011). In keratinocytes, production of IL-36 family members can be induced by TNF, IL-17 and IL-22, cytokines known to be involved in psoriasis which further supports an important role for IL-36 in this disease (Carrier et al., 2011). Recently, it has been reported that antigen-presenting cells located in human skin, express high levels of IL-36R and are responsive to IL-36 cytokines (Dietrich et al., 2016), an observation that further supports an important role of IL-36 in skin biology.

Here, we provide evidence that drugs causing AGEP can specifically trigger IL-36 cytokine production by peripheral blood monocytes via Toll-like receptor 4 and by keratinocytes from AGEP patients and subsequently induce IL-8 by peripheral blood mononuclear cells in an IL-36-dependent manner. These observations are supportive of a drug-specific dysregulation of IL-36 signaling as a possible driver of AGEP pathogenesis, and identify myeloid cells and keratinocytes as important players in AGEP.

RESULTS

Enhanced IL-36 expression in lesional skin of AGEP patients

First, we performed gene expression profiling (GEO Accession number GSE121421) using Affymetrix Human Exon 1.0 ST chips (see supplementary material for description). Total RNA were extracted from lesional skin from patients with AGEP (n=8) or maculo-papular rash (MPR, n=6), and from skin from healthy individuals (n=7). The hierarchical clustering of genes differentially expressed in AGEP, MPR and healthy skin revealed a perfect segregation of these three conditions based on the gene expression profile of individual samples and the magnitude of change in gene expression (Fig.1a and Table S1). Interestingly, the IL-36 γ gene (*IL1F9*) was found to be overexpressed in AGEP skin when compared to healthy skin (ratio=3.06, p=0.0006; Table S2) and MPR (ratio=2.54, p=0.005; Table S3). Using quantitative PCR, we then analyzed the expression of *IL-36* in skin biopsies from AGEP (n=16) and maculo-papular rash (MPR), a milder form of cutaneous reaction to drugs (n=16). The expression of *IL-36* α , β and γ was found to be upregulated in AGEP skin when compared to MPR (*IL-36* α 15-fold, p<0.001; *IL-36* β 28-fold, p<0.001; *IL-36* γ 32-fold, p<0.001) and normal skin (*IL-36* α 7-fold, p<0.001; *IL-36* β 1.35-fold, p<0.05; *IL-36* γ 3-fold, p<0.001) (Fig. 1b). The expression of *IL-36RN* was found to be higher (4.5-fold, p<0.001) in AGEP skin as compared to MPR whereas it was not different to that found in normal skin. *IL-36R* was similarly expressed in both diseases.

The relative expression of *IL-36* β was however low as compared to *IL-36* α and γ . Therefore, we further analyzed the expression and tissue distribution of *IL-36* α and γ by labeling AGEP and MPR lesional skin biopsies with antibodies to both cytokines. *IL-36* α and γ were found to be highly expressed in pustular and peri-pustular regions of AGEP skin (Fig.1c). Both keratinocytes and dermal immune cells infiltrating pustular areas were found to produce IL-

36 α and γ . In contrast, IL-36 γ was weakly expressed in MPR skin biopsies and IL-36 α was barely detectable. In AGEP lesional skin, IL-36 γ was predominantly expressed by keratinocytes in the epidermis and by immune cells infiltrating the dermis in pustular areas. Also, IL-36 γ staining was particularly intense in non-pustular epidermis surrounding the pustules in AGEP. As previously reported (Song et al., 2016), IL-36 α was also expressed in pustules from patients suffering from pustular psoriasis (PP) and, like in AGEP biopsies, IL-36 γ was the predominant form in the epidermis (Fig. 1c). Neither IL-36 α , nor IL-36 γ could be detected in skin specimens from healthy donors (Fig. S1).

IL-36 production by keratinocytes and macrophages in lesional skin of AGEP patients

To identify which cell types release IL-36 α and γ in AGEP skin, we co-labeled AGEP biopsy sections with antibodies to IL-36 α and γ and antibodies to CD68 to identify macrophages, and to CD3 to identify T-cells (Fig. 2). IL-36 α was predominantly expressed by CD68⁺ macrophages (41.17% \pm 4.71 of positively labeled cells) and to a lesser extent by keratinocytes and CD3⁺ T cells (27.33% \pm 2.17 and 22.33% \pm 2.39 of positively labeled cells, respectively). IL-36 γ was predominantly expressed by keratinocytes and CD68⁺ macrophages (44.08% \pm 4.47 and 43.17% \pm 3.31 of positively labeled cells, respectively) and to a lesser extent by CD3⁺ T cells (15.17% \pm 1.26 of positively labeled cells). In contrast, IL-36 α and IL-36 γ were not expressed by neutrophils present in pustular areas (Fig. S2).

Rapid culprit drug-specific induction of IL-36 γ in monocytes in AGEP

Immunofluorescence analyses of AGEP biopsies suggested that, in addition to keratinocytes, immune cells may also represent an important source of IL-36 γ in the skin. Therefore, we assessed whether peripheral blood mononuclear cells (PBMC) taken from AGEP patients 8, 9

and 20 more than 6 months after the ADR - when in complete remission - were still able to respond directly to the causative drug. To this end, PBMC from patients having experienced AGEF were exposed to their respective causative drug or an irrelevant control drug – that did not cause AGEF in the tested patients - in ELISPOT plates. The number of IL-36 γ ⁺ spots was counted after 1, 2, 4, 6 and 8 hrs. IL-36 γ release was detected in PBMC from AGEF patients already after 1 hr of exposure to their respective culprit drug (1.7-fold more spots than control drug, $p < 0.001$) and reached a plateau after 4 hrs (2.7-fold more spots than control drug, $p < 0.001$) (Fig. 3a, left panel), whereas no increase in IL-36 γ production was observed with control drugs that did not cause AGEF in the tested patients (Fig. 3a, right panel and Fig. S3). In contrast, none of the culprit drugs involved in AGEF did induce IL-36 γ secretion in PBMC from MPR patients with proven sensitization to the same drug. Similarly, none of the drugs used was able to induce IL-36 γ secretion in PBMC from healthy blood donors. Of note, IL-36 γ production was concentration dependent and was exclusively observed in PBMC from patients exposed to the drug having caused AGEF (Fig. S3) whereas none of the drugs used in this study was able to induce IL-36 γ in drug-sensitized MPR patients' or healthy donors' PBMCs even at the highest concentration.

Since immunofluorescence analyses of AGEF skin lesions revealed that macrophages and, to a lesser extent, T cells are possible sources of IL-36, we sorted CD14⁺ (monocytes) and CD3⁺ cells (T cells) from patients' PBMC and exposed them to culprit or control drug to evaluate IL-36 γ secretion by ELISPOT (Fig. 3b). Whereas the control drug did not induce IL-36 γ secretion in PBMC or in CD14⁺ and CD3⁺ cells, the culprit drug was able to induce IL-36 γ secretion in CD14⁺ cells at levels similar to those observed with total PBMC. In contrast, the culprit drug was not able to induce IL-36 γ secretion in CD3⁺ PBMC. These results suggest that PBMC from patients having experienced AGEF are able to respond directly and

specifically to the causative drug and that monocytes/macrophages are an important source of IL-36 production in response to culprit drug exposure.

IL-36 induction by culprit drugs in keratinocytes and PBMC from patients having experienced AGEP

By immunolabeling, both keratinocytes and macrophages were positively labeled for IL-36 α and γ in pustular areas of AGEP biopsies (Fig. 2). To assess a possible cross-talk between macrophages and keratinocytes, and the relative contribution of each cell type to drug-induced IL-36 secretion, we performed co-culture experiments using autologous hair follicle-derived keratinocytes and PBMC from three patients having experienced AGEP but in remission at the time of blood and hair collection (at least 6 months after disappearance of symptoms, patients 8, 9 and 20). Keratinocytes and PBMC were cultured either alone or together using a transwell culture system. To formally exclude the contamination of one cell type by the other, we confirmed the purity of each cell type by qPCR using primers to CD45 (PBMC marker) and ITGA6 (keratinocyte marker) on both PBMC and keratinocytes after a 6hr-coculture (Fig. S4). When keratinocytes and PBMC were each cultured alone, only PBMC showed elevated IL-36 γ mRNA levels after exposure to the culprit drug. Interestingly, both keratinocytes and PBMC showed culprit drug-specific IL-36 γ gene expression when cultured together (Fig. 4). The addition of soluble IL-36Ra did not reduce IL-36 expression, suggesting that, at the analyzed time point, there was no auto- or paracrine amplification and/or induction of IL-36 gene expression by IL-36 released by PBMC or keratinocytes. Taken together, these results indicate that, in culprit drug-induced responses of patient-derived keratinocytes and monocytes, IL-36 γ is the predominant form of IL-36 induced. Furthermore, in response to culprit drug exposure IL-36 is expressed directly by PBMC and indirectly by keratinocytes.

IL-36 secretion by AGEP patients' PBMC is induced by TLR4 recognition and requires albumin

Toll-like receptors (TLR) are innate immunity receptors signaling through NF- κ B and leading to the transcription of inflammatory cytokines including IL-36 γ (Aschan et al., 1990, Lian et al., 2012). A treatment of AGEP patients' PBMC exposed to culprit drug with an inhibitory peptide to the TLR adapter protein MYD88 (Pepinh-MYD) led to a decreased IL-36 γ secretion as compared to vehicle. Moreover, the inhibition of TLR4 signaling with a neutralizing antibody also resulted in a decreased IL-36 γ secretion by PBMC exposed to culprit drug whereas a neutralizing antibody to TLR2 had no effect on IL-36 γ expression (Fig. 4b). Since MyD88 is also involved in IL-1RI/IL-1 signaling, we also treated drug-exposed PBMC from AGEP patients with a recombinant IL-1 receptor antagonist (IL-1Ra). IL-1Ra was also able to partially reduce IL-36 production in response to the culprit drug, however to a significant lesser extent than the TLR4-neutralizing antibody.

Albumin is the most abundant serum protein and is known to have high ligand-binding capacities (Fasano et al., 2005). It has also been reported to be a major target for drugs such as amoxicillin and to be required for the induction of immediate hypersensitivity reactions to drugs (Ariza et al., 2016). To assess whether albumin was required for IL-36 γ secretion by AGEP patients' cells exposed to culprit drugs, cells were first exposed to culprit and control drugs in the absence of proteins in the culture medium (serum-free). In the latter condition, culprit drug-induced IL-36 γ secretion was dramatically decreased (Fig. 4c). Interestingly, the medium supplementation with albumin restored the ability of PBMCs to secrete IL-36 γ in response to drugs. Together, these results suggest that drug-albumin complex is sensed by TLR4 as danger signal in patients having experienced AGEP.

Selective, IL-36-induced IL-8 production by patients' PBMC

IL-8 has been previously reported to be produced by drug-specific T cells in AGEF patients (Britschgi et al., 2001) and in accordance with this, IL-8 expression was significantly upregulated in AGEF skin when compared to healthy skin (ratio=2.25, $p=0.01$; Table S2) but also when compared to MPR (ratio=2.24, $p=0.008$; Table S3) as revealed by Affymetrix gene expression array (Fig. 1a). We could confirm this by qPCR (Fig. 5a, left panel). Immunohistochemistry analyses further confirmed that IL-8 was overexpressed in AGEF as compared to MPR and healthy skin and that cells of the infiltrate in pustular regions of AGEF skin were the main source of IL-8 (Fig. 5a, right panels). As previously reported, IL-36 is an inducer of IL-8 (Marrakchi et al., 2011, Towne et al., 2011, Zhang et al., 2017). Consistently, the exposure of PBMCs from AGEF patients to their respective culprit drug resulted in IL-8 production that was inhibited by recombinant IL-36Ra in a dose dependent manner (Fig. S5). Also, using the keratinocyte/PBMC co-culture model described above, we assessed whether IL-36 could promote IL-8 gene expression, and if so, which cell type could produce IL-8 in response to IL-36. IL-8 gene expression was induced by culprit drugs in PBMC cultured alone - confirming our ELISPOT data - whereas it was barely detectable in isolated keratinocyte cultures. Levels of IL-8 gene expression in patients' PBMC were further increased (4.6-fold increase over control-drug exposure, $p<0.05$) when co-cultured with autologous keratinocytes and this, selectively in response to culprit drug (Fig. 5b). In contrast, keratinocytes co-cultured with PBMCs produced only marginal amounts of IL-8. Interestingly, in this autologous co-culture model, the upregulation of IL-8 gene expression in AGEF patients PBMC was abrogated by IL-36Ra. Altogether, these results indicate that PBMC are the main source of drug-induced IL-8 production, and that IL-8 production is dependent on PBMC- and/or keratinocyte-derived IL-36.

DISCUSSION

T cells and neutrophils are considered to be major players in AGEP (Britschgi et al., 2001, Feldmeyer et al., 2016). It has been proposed that drugs causing AGEP elicit drug-specific T cell responses, and that these T cells secrete IL-8 (Britschgi et al., 2001). Recently, mutations in the IL-36RN gene have been reported in AGEP (Navarini et al., 2013), suggesting that a deregulation in IL-36 signaling may contribute to the pathophysiology of AGEP. Our data is supportive of this hypothesis, showing that culprit drugs can directly induce IL-36 γ secretion in AGEP patients' cells *in vitro*. Analysis of IL-36 gene and protein expression *ex vivo* is also supportive of the above and, furthermore, reveal that macrophages and keratinocytes are the main producers of IL-36 in the pustular areas of AGEP skin. IL-36 γ was found to be the predominant form of IL-36 expressed in AGEP skin biopsies. Interestingly, causative drugs were able to induce IL-36 γ and subsequent IL-8 production in PBMC taken from patients months to years after AGEP resolution whereas these cytokines could not be induced by drugs in healthy control donors who had never experienced an adverse drug reaction. This is, to our knowledge, the first experimental and translational evidence that the innate immune system is able to mount fast pro-inflammatory response selectively to the culprit drug and exclusively in AGEP patients via the production of IL-36 γ . Indeed, such a production by monocytes and keratinocytes from patients could be observed as early as 1 hr after culprit drug exposure *in vitro*. To further support a role for innate immunity in AGEP, we also provide evidence that TLR4 is involved in the sensing of culprit-drug/albumin complex as a danger signal inducing IL-36 γ exclusively in PBMC from patients having experienced AGEP.

Keratinocytes are a major source of IL-36 cytokines in cutaneous inflammation, particularly in psoriasis (Carrier et al., 2011). Our data suggests that keratinocytes are also an important source of IL-36 in the skin in AGEP. However, co-culture experiments revealed that

keratinocytes exposed to culprit drugs were only able to produce high amounts of IL-36 γ when cultured in presence of autologous monocytes and culprit drug. This observation suggests that, when exposed to the causative drug, patients' monocytes release a soluble factor that further stimulates IL-36 γ production by keratinocytes. This factor remains to be identified but our results using IL-36Ra suggest that it is very likely not IL-36 itself at an early time point. Indeed, unlike what has been reported in psoriasis (Carrier et al., 2011), we could not observe any IL-36 induction by IL-36 itself in AGEP patients' keratinocytes at the early time-point of 6 hrs after drug stimulation as evidenced by the lack of effect of IL-36Ra.

We also observed that the use IL-1Ra led to a reduced IL-36 γ production by AGEP patients' PBMCs exposed to culprit drugs. A great overlap in IL-1 family gene upregulation by IL-1 β and IL-36 γ has been recently reported (Swindell et al., 2018). This overlap includes genes encoding IL-1 β and IL-36 themselves that can be induced by either cytokine suggesting an IL-1 β -IL-36 γ amplifying loop in PBMC stimulated by culprit drugs. The extent of IL-36 γ inhibition by IL-1Ra was however significantly lower than the one seen with a blocking antibody to TLR4. The important role of IL-36 in response to certain drugs in AGEP patients is further supported by the inhibition of IL-8 by IL-36Ra, the latter cytokine being already suspected to cause neutrophil recruitment and survival in lesional skin during the course of AGEP (Schaerli et al., 2004).

Like in other inflammatory diseases such as psoriasis (Nickoloff, 2006), contact hypersensitivity (Watanabe et al., 2007) or sunburn (Feldmeyer et al., 2007), keratinocytes may actively participate in the pathogenesis of AGEP by secreting IL-36, which could contribute to the herein observed and previously reported induction of IL-8 gene expression by macrophages. IL-8 may also originate from drug-specific T cells in AGEP (Britschgi et al., 2001). However, the mechanisms leading to drug-specific T cell priming and recruitment to the skin in AGEP have not been elucidated to date. Unlike murine T cells, human T cells do

not express IL-36R (Foster et al., 2014) but, in AGEP, IL-36 may nevertheless contribute to T cell-mediated immune responses through its strong stimulatory effects on antigen-presenting cells (Vigne et al., 2011). Our findings that IL-36 γ can be induced by a drug very early in innate immune cells do not exclude a delayed drug-specific T cell response but whether the induction of IL-36 γ in monocytes by culprit drugs and the activation of drug-specific T cells are dependent events in AGEP pathogenesis or not remains to be investigated.

Th17 cells may also be involved in AGEP as revealed by their previously reported increased frequencies and elevated IL-22 blood levels in AGEP patients (Kabashima et al., 2011). IL-17 is known to increase IL-8 secretion by keratinocytes (Pennino et al., 2010) in allergic contact dermatitis (Albanesi et al., 1999) and in psoriasis (Chiricozzi et al., 2011). Our *in vitro* data revealed however that in response to IL-36, the major source of IL-8 was patients' monocytes, consistently with the previously reported elevated expression of IL-36R and potential strong responsiveness to IL-36 by dermal myeloid cells (Dietrich et al., 2016). Although keratinocytes also express high levels of IL-36R (Dietrich et al., 2016), IL-8 production by keratinocytes was marginal as confirmed by qPCR in co-culture experiments and immunohistochemistry where IL-8 was only detected in the peri-pustular immune infiltrate (Fig. 5).

Although mutations in the *IL-36RN* gene have been identified in AGEP patients, no other marker(s) of genetic predisposition have been formally identified for AGEP to date. Noteworthy, our data shows that a deregulation of the IL-36-IL-8 axis can occur independently of *IL-36RN* mutations in AGEP, suggesting that other factors influencing the susceptibility to an IL-36-mediated proinflammatory response to culprit drugs are at play in AGEP patients.

Overall, we provide evidence that IL-36 is involved in AGEP pathogenesis, and identify monocytes and keratinocytes as potential key producers of IL-36 in this ADR. We also

identify TLR4 as an essential pattern recognition receptor involved in the sensing of drug/albumin complex as a danger signal in AGEF patients. Further supportive of our findings is the very rapid onset of the disease after the first intake of a drug, suggesting an early response mediated by the innate immune system. However, the mechanisms or factors conferring such a responsiveness to certain drugs to monocytes and keratinocytes in AGEF patients remain to be identified and require extensive investigations. Given that monocytes/PBMC of all AGEF patients tested have an enhanced propensity to produce IL-36 γ upon culprit drug exposure, the identification of predictive markers that would help identify patients prone to pathological IL-36 γ production upon exposure to certain medications may be achievable.

MATERIALS AND METHODS

Patients and biological material collection

The characteristics of all studied AGEF patients from the Dermatology Department of the University Hospital of Zürich are presented in Tables 1 and Table S4. All human samples were collected after informed written patient consent with approval of Local Ethics Committees and according to the Declaration of Helsinki Principles. 4-6mm punch skin biopsies were taken from lesional skin of patients in the acute phase of the ADR. Healthy skin samples were obtained from the Department of Plastic Surgery, University Hospital of Zürich. For paraffinization, skin samples were fixed in formalin 4% overnight. For RNA isolation, samples were snap-frozen in liquid nitrogen and stored at -80°C . Peripheral blood from patients was taken both in the acute phase of the disease and under healthy conditions (at least 6 months after the ADR). Healthy controls were obtained from the Blood Donation Center (Schlieren, Switzerland). Peripheral blood mononuclear cells (PBMC) were isolated using a density gradient (Ficoll-Paque, Pharmacia, Glattbrugg, Switzerland).

Monocyte and T cell purification

Where indicated, CD14^{+} monocytes were sorted from PBMC by positive selection using magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). CD3^{+} cells were sorted from the CD14^{-} fraction by negative selection using a Pan T Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. Purity was assessed by flow cytometry (Facsanto A, Becton-Dickinson) using mouse anti-human CD45-PeCy7 (Biolegend, San Diego, CA), mouse anti-human CD3-APC (Biolegend) and mouse anti-human CD14-FITC (Becton-Dickinson, Franklin Lakes, NJ).

Primary keratinocyte culture

Hair follicles were collected from patients at least 6 months after resolution of the ADR. Hair follicle extremities containing outer root sheath were cut, washed in complete keratinocyte medium (Strittmatter et al., 2016) and incubated for 3 minutes in 1mg/ml Dispase II, followed by three washing steps in medium. The hair was then plated on J2 feeder cells in complete medium and cultured until small colonies of keratinocytes become visible. Cells were splitted at 80% confluence and passaged three times before being used for experiments.

Co-culture experiments

PBMC and keratinocytes used in these experiments were collected at least 6 months after resolution of the ADR. 70.000 AGEF hair follicle keratinocytes were plated on a 12mm transwell plate with 0.4µm pore polycarbonate membrane inserts (Corning, NY, USA) overnight to allow them to adhere. The following day, keratinocyte medium was replaced by Gibco Opti-MEM medium (Thermo Scientific) and 1×10^6 PBMC were added inside the inserts, followed by addition of culprit drug (Amoxicillin 1mg/ml; Letrozole 100nM; Vancomycin 500µg/ml) or control drug (Metamizole 100µg/ml; Carbamazepine 10µg/ml; Amoxicillin, 1mg/ml) or vehicle (RPMI medium containing 10% FBS) in both compartments at the same concentration +/- IL-36 inhibition by an IL-36RA (R&D Systems, 1µg/ml). After 6 hours, cells were harvested and RNA was isolated for quantitative RT-PCR analysis.

Toll-like receptor and IL-1 inhibition

PBMC were isolated from AGEF patients and cultured in ELISPOT plates. Cells were stimulated with the culprit drug in the presence or absence of the MyD88 inhibitory peptide Pepinh-MYD (Invivogen, San Diego, CA, 25mM), anti-TLR2 antibody (R&D Systems, Clone # 383936, 5µg/ml), anti-TLR4 antibody (R&D Systems, Polyclonal Goat IgG, 5µg/ml),

control IgG antibody (5 μ g/ml) and IL-1RA (Anakinra, Biovitrum AB, 1 μ g/ml). Untreated cells and irrelevant control drug served as controls.

Statistical analyses

Differences between groups were assessed using one-way Anova followed by Turkey's post-test. Differences were considered significant when: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ and **** $p \leq 0.0001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Table 1. AGEP patients – Patch and Prick tests, LTT, culprit drugs and presence of *IL36RN* mutations

Nr.	Patch test	Prick	LTT	Drug	<i>IL36RN</i>
1	N.A.	negative	negative	Amoxicillin	n.d.
2	N.A.	N.A.	N.A.	Terbinafin	n.d.
3	negative	negative	negative	Metformin	No mutation
4	negative	negative	N.A.	unknown	No mutation
5	positiv	positive	negative	unknown	n.d.
6	negative	negative	negative	Lonsoprazol / Clarithromycin	n.d.
7	N.A.	N.A.	N.A.	unknown	n.d.
8	N.A.	N.A.	positive	Amoxicillin	No mutation
9	N.A.	N.A.	positive	Letrozole	n.d.
10	positive	positive	positive	Amoxicillin	No mutation
11	N.A.	N.A.	N.A.	Amoxicillin	n.d.
12	N.A.	N.A.	N.A.	Floxapen	n.d.
13	negative	negative	N.A.	Ceftriaxon	n.d.
14	N.A.	N.A.	N.A.	Ciprofloxacin / Metamizol / Paracetamol	n.d.
15	N.A.	N.A.	N.A.	Amoxicillin	n.d.
16	positive	positive	negative	Novalgin	n.d.
17	negative	negative	N.A.	Ibuprofen / Pure Life Cleanse	No mutation
18	N.A.	N.A.	N.A.	Terbinafin	n.d.
19	positive	positive	positive	unknown	n.d.
20	N.A.	N.A.	N.A.	Vancomycin	n.d.
21	N.A.	N.A.	positive	Fluconazole	n.d.
22	N.A.	N.A.	N.A.	Unknown	n.d.
23	N.A.	N.A.	N.A.	unknown	n.d.
24	N.A.	N.A.	N.A.	Amoxicillin	n.d.
25	N.A.	N.A.	N.A.	Amoxicillin	n.d.
26	N.A.	N.A.	positive	Acetazolamide	n.d.
27	N.A.	N.A.	N.A.	Cotrimoxazole Cotrimoxazole /	n.d.
28	Positive	Positive	negative	Codein / Paracetamol	
29	N.A.	N.A.	Positive	Lisinopril	n.d.

n.d.: not determined; in bold: cells from these patients have been used in experiments shown in figures 3, 4 and 5.

FIGURE LEGENDS

Figure 1. IL-36 α and IL-36 γ are overexpressed in pustular regions of AGEP lesional skin.

(a) Gene expression hierarchical clustering in lesional skin biopsies from patients suffering from AGEP (n=8) and MPR (n=6) analyzed using Affymetrix Human Exon 1.0 ST chips and (b) quantitative RT-PCR analysis of IL-36 α , IL-36 β , IL-36 γ , IL-36R, IL-36RN in skin biopsies from AGEP patients (n=16), MPR (n=16) and normal skin (NS; n=5). (c) Immunohistochemical analysis of lesional skin biopsies revealed that IL-36 α and IL-36 γ are overexpressed at the site of pustules in AGEP and PP controls. Representative pictures of 18 AGEP, 10 PP and 18 MPR cases are shown (scale bar in the large panels=500 μ m; scale bar in the small panels=100 μ m). Neither IL-36 α , nor IL-36 γ were detectable in normal skin (NS) from healthy donors (Fig. S1a). Bottom panels show the semi-quantitative evaluation of IL-36 α and IL-36 γ labeling by immunohistochemistry in the pustular epidermis, non-pustular epidermis, and dermis (Fig. S1b) of lesional skin biopsies from patients with AGEP (n=18), PP (n=10), MPR (n=18) and normal skin (NS) from healthy donors (n=5). Expression levels were qualified as very strong (++++), strong (+++), moderate (++) , weak (+) or absent (0). *p<0.05; **p<0.01; *** p<0.001; **** p<0.0001.

Figure 2. IL-36 γ is expressed by keratinocytes and immune cells in AGEP skin.

Pustule-containing sections of AGEP skin samples were co-labeled with antibodies to CD68 (green), CD3 (green) and IL-36 α (red) (a) or IL-36 γ (red) (scale bar in the large panels=500 μ m; scale bar in the small panels=20 μ m) (b). The percentage of epidermal cells (keratinocytes), CD68 and CD3-labeled cells among the IL-36 α or IL-36 γ -labeled cells was

determined (c). Cell nuclei appear in blue (DAPI). The mean \pm SD of 12 examined patients is shown (patients 8-10 and 20-28). * p <0.05; *** p <0.001.

Figure 3. PBMC and monocytes taken from AGEP patients more than 6 months after the ADR selectively secrete IL-36 γ in response to culprit drug exposure.

(a) PBMC from AGEP or MPR patients as well as healthy donors were cultured in IL-36 γ ELISPOT plates for 8 hrs in presence of the culprit drug having caused AGEP or MPR, respectively (left panel) or a control drug (right panel). The number of spots was counted 1, 2, 4, 6 and 8 hrs after drug exposure. The means \pm SD of 3 different AGEP patients (no. 8, 10 and 21), 3 MPR patients and 3 healthy blood donors are shown. (b) CD14⁺ monocytes and CD3⁺ T cells were isolated from AGEP blood with a purity of >96% (right panel) and were cultured in IL-36 γ ELISPOT plates (left panel) in presence of the culprit drug or a control drug and compared to total PBMC. The number of spots was counted 8 hrs after drug exposure (lower panel). The mean \pm SD of 3 different patients (patient no. 8, 9 and 10) is shown. ** p <0.01; *** p <0.001.

Figure 4. IL-36 γ is specifically induced by culprit drugs in PBMC and keratinocytes and is dependent on TLR4 and albumin..

(a) Autologous PBMC and keratinocytes (KC) from patients having experienced AGEP but in remission at the time of blood and hair collection were cultured either alone or together in a transwell system allowing for soluble factor-mediated interactions. Patients' cells were exposed to the culprit drug (Amoxicillin 1mg/ml, patient 8; Letrozole 100nM, patient 9; Vancomycin 500 μ g/ml, patient 20) or a control drug (Metamizole 100 μ g/ml, patient 8; Carbamazepine 10 μ g/ml, patient 9; Amoxicillin 1mg/ml, patient 20) in presence or absence of IL-36RA (1 μ g/ml). After 6 hrs of culture, RNA was extracted from KC (left panels) and PBMC (right panels) to measure IL-36 γ mRNA levels. Relative *IL-36 γ* expression in KC and

PBMC cultured either alone or in co-culture as indicated and exposed to the culprit drug or a control drug in presence or absence of IL-36Ra are shown for each tested patient. Means \pm SD of 3 replicates are shown. **(b)** PBMC from AGEF patients were pretreated with vehicle, MyD88 inhibitory peptide, neutralizing antibodies to TLR2 and TLR4 or recombinant IL-1 receptor antagonist (IL-1RA) and exposed to culprit drug for 6 hours. An ELISPOT assay was performed and the number of IL-36 γ ⁺ spots were counted using an ELISPOT reader. Untreated cells or cells exposed to an irrelevant control drug were used as basal IL-36 γ secretion controls. Mean \pm SD of the 5 tested patients is shown. ***p<0.001. **(c)** PBMC from AGEF patients were exposed to culprit drug for 6 hours in medium containing 10% FBS or in serum free medium or in serum free medium supplemented with 2.5 mg/ml human serum albumin (HSA) as indicated. An ELISPOT assay was performed and the numbers of IL-36 γ ⁺ spots were counted using an ELISPOT reader. Untreated cells were used as basal IL-36 γ secretion controls. Mean \pm SD of the 5 tested patients is shown. *p<0.05; ***p<0.001. Representative IL-36 γ ELISPOT pictures are shown below the graph.

Figure 5. IL-8 is expressed in lesional skin biopsies from patients suffering from AGEF and is induced in patients' PBMC by IL-36.

(a) Quantitative RT-PCR analysis of IL-8 in lesional skin biopsies of patients suffering from AGEF (n=16), MPR (n=16) and normal skin (NS; n=5). Immunohistochemical analysis of lesional skin biopsies revealed that IL-8 is expressed at the site of pustules in AGEF in dermal immune cells but not by keratinocytes (scale bar in the left panels=300 μ m; scale bar in the right panels=50 μ m). Representative picture of 18 AGEF cases is shown (upper panels). IL-8 was not detectable in normal skin from healthy donors (lower panels). **(b)** Quantitative PCR analysis of IL-8 gene expression in PBMC (upper panels) and KC (lower panels) from patient

8 (Amoxicillin 1mg/ml), patient 9 (Letrozole 100nM) and patient 20 (Vancomycin 500µg/ml), cultured either alone or in co-culture as indicated and exposed to the drug having cause AGEP or a control drug in the presence or absence of IL-36Ra. Gene expression reported as $2^{-\Delta CT}$, which represents the target gene expression relative to the reference gene (RPL27). Mean±SD of the 3 tested patients is shown. The figure illustrates a representative experiment that was repeated 3 times for each patient. *p<0.05; **p<0.01; ***p<0.001.









