

ORIGINAL ARTICLE

Atopic Dermatitis, Urticaria and Skin Disease

Vemurafenib acts as an aryl hydrocarbon receptor antagonist: Implications for inflammatory cutaneous adverse events

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Abstract

Background: In recent years, the BRAF inhibitor vemurafenib has been successfully established in the therapy of advanced melanoma. Despite its superior efficacy, the use of vemurafenib is limited by frequent inflammatory cutaneous adverse events that affect patients' quality of life and may lead to dose reduction or even cessation of anti-tumor therapy. To date, the molecular and cellular mechanisms of vemurafenib-induced rashes have remained largely elusive.

Methods: In this study, we deployed immunohistochemistry, RT-qPCR, flow cytometry, lymphocyte activation tests, and different cell-free protein-interaction assays.

Results: We here demonstrate that vemurafenib inhibits the downstream signaling of the canonical pathway of aryl hydrocarbon receptor (AhR) in vitro, thereby inducing the expression of proinflammatory cytokines (eg, *TNF*) and chemokines (eg, *CCL5*). In line with these results, we observed an impaired expression of AhR-regulated genes (eg, *CYP1A1*) and an upregulation of the corresponding proinflammatory genes in vivo. Moreover, results of lymphocyte activation tests showed the absence of drug-specific T cells in respective patients.

Conclusion: Taken together, we obtained no hint of an underlying sensitization against vemurafenib but found evidence suggesting that vemurafenib enhances

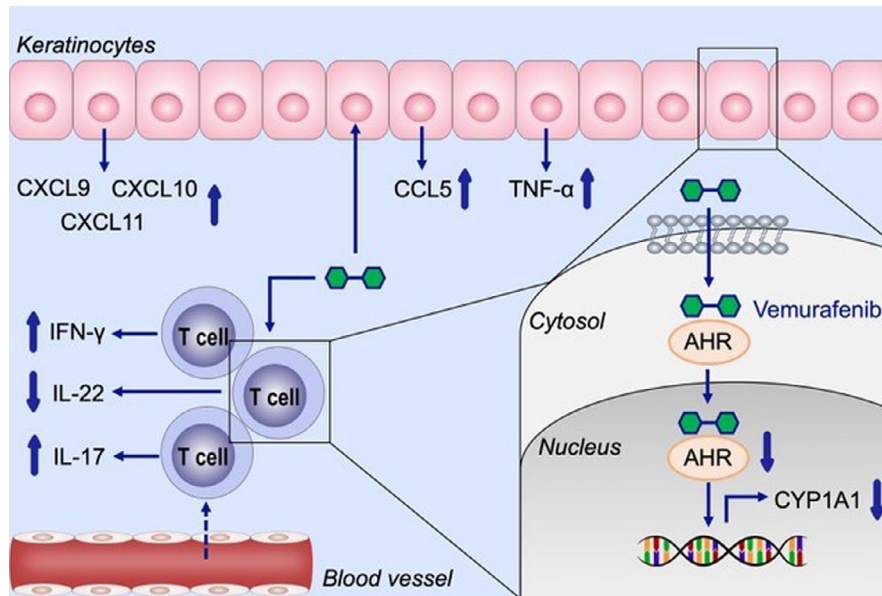
Abbreviations: ADR, adverse drug reaction; AhR, aryl hydrocarbon receptor; BRAFi, BRAF protein kinase inhibitor; EGFRi, EGFR inhibitor; h, hours; LAT, lymphocyte activation test; SI, stimulation index; VIR, vemurafenib-induced rashes; XRE, xenobiotic response element.

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proinflammatory responses by inhibition of canonical AhR signaling. Our findings contribute to our understanding of the central role of the AhR in skin inflammation and may point toward a potential role for topical AhR agonists in supportive cancer care.

KEYWORDS

aryl hydrocarbon receptor, drug eruption, lymphocyte activation test, melanoma, vemurafenib



GRAPHICAL ABSTRACT

Vemurafenib-induced rashes are characterized by an increased expression of IFNG, TNFA, and chemokines including CCL5, CXCL9, CXCL10 and CXCL11. Vemurafenib acts as aryl hydrocarbon receptor (AHR) antagonist by inhibiting AhR translocation to the nucleus and leading to downregulation of cytochrome P450 family 1 subfamily A member 1 (CYP1A1). Vemurafenib induces IL-17 and IFN- γ expression in T cells, while inhibiting IL-22 expression.

1 | INTRODUCTION

The introduction of immune-checkpoint inhibitors (eg, ipilimumab, pembrolizumab, nivolumab), BRAF (vemurafenib, dabrafenib) and MEK inhibitors (cobimetinib, trametinib), has revolutionized the treatment of advanced melanoma. The first of its class BRAF protein kinase inhibitor (BRAFi) vemurafenib received FDA approval in August 2011.¹ Although vemurafenib is generally well tolerated, cutaneous adverse events occur in up to 70% of treated patients² and include inflammatory maculopapular rashes (up to 37%), photosensitivity (12%), or secondary skin tumors (20%).¹ Cutaneous adverse effects represent a serious threat to patients' therapy adherence and may lead to dose reduction or therapy cessation. Despite clinical relevance, molecular and cellular mechanisms of vemurafenib-associated rashes (VIR) have remained largely elusive. Interestingly, Chapman and coworkers reported that pretreatment with the proinflammatory, anti-cytotoxic T lymphocyte-associated antigen blocking antibody ipilimumab was associated with a higher frequency (up to 70%) of VIRs.³ The authors proposed that increased numbers of severe grade 3 rashes are

the result of hypersensitivity drug reactions to vemurafenib driven by immune-checkpoint inhibition. In patients who developed grade 3 rashes vemurafenib therapy was paused until the rash resolved and were successfully retreated with lower doses of vemurafenib.^{3,4} These clinical observations point toward either drug hypersensitivity through allergic sensitization or a pharmacological mechanism enhancing cutaneous inflammations. Here, we set out to investigate the underlying molecular mechanisms of VIRs. To this end, we characterized the phenotype of the rashes, performed lymphocyte activation tests (LAT), and investigated potential interactions with the signaling of the aryl hydrocarbon receptor (AhR) representing a well-known xenobiotic receptor and central regulator of inflammatory processes.⁵⁻⁷

2 | MATERIAL AND METHODS

2.1 | Human samples

Punch biopsies were obtained from patients with VIRs after obtaining informed consent. For skin explants, punch biopsies were

taken from healthy skin. Buffy coats from healthy donors were obtained from the Institute of Hemostasis and Transfusion Medicine, University Hospital Duesseldorf. The guidelines of the Declaration of Helsinki were deployed, and the study was authorized by the local ethics committee (No: 1928).

2.2 | Animal samples and preparation of cytosol

Hartley guinea pigs were obtained from Charles River (Wilmington, DE, USA). Guinea pig hepatic cytosol was prepared as described⁸

and stored properly until use. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

2.3 | Culture of human cells and explants

The culture of ex vivo explants, keratinocytes, and T cells is described in the supplemental methods.

The tested concentrations of vemurafenib (up to 100 $\mu\text{mol/L}$)^{9,10} and dabrafenib^{10,11} correspond to published studies. Cell viability

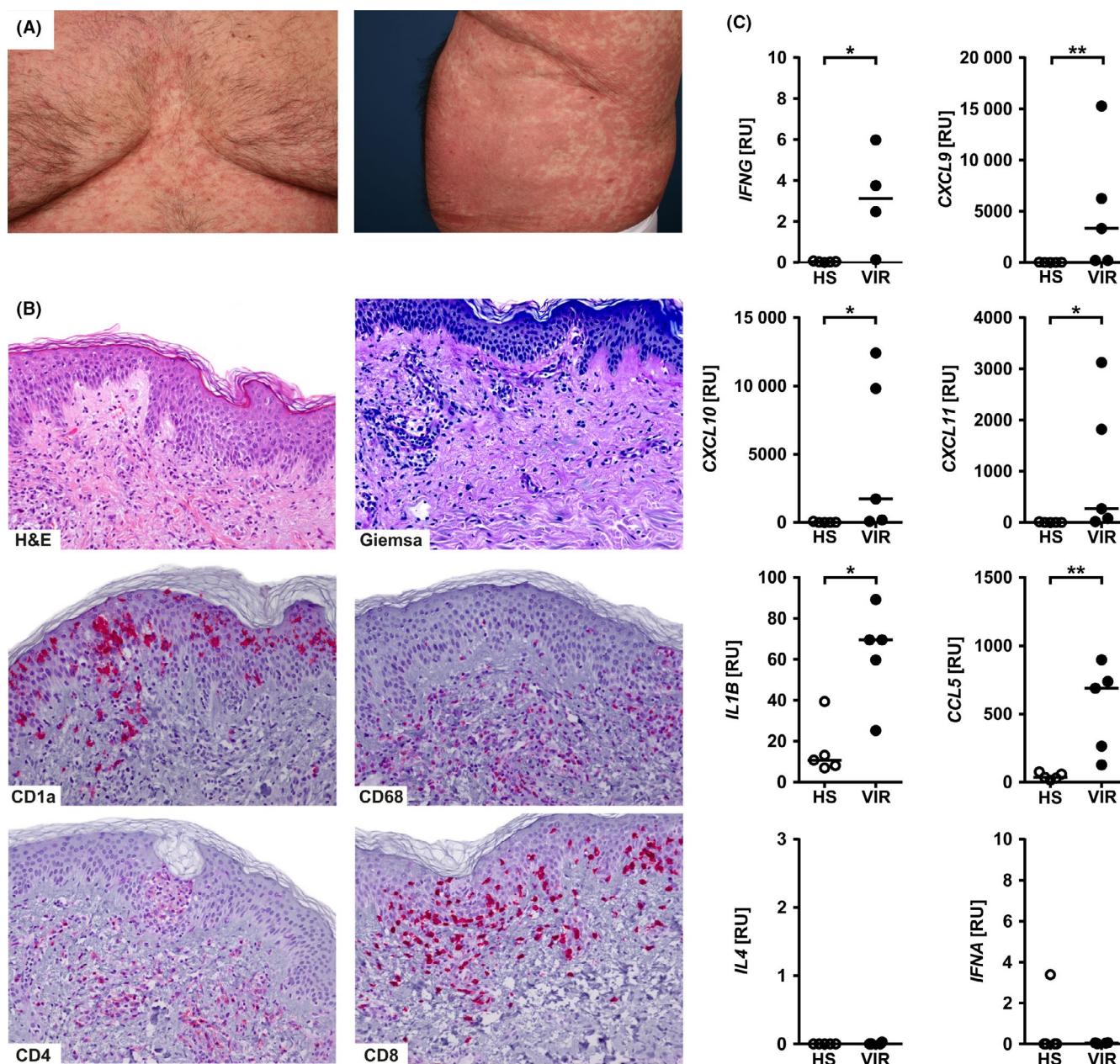


FIGURE 1 Clinical, histologic, and molecular characterization of vemurafenib-induced skin rashes. (A), Representative patient with generalized maculopapular rash. (B), Hematoxylin and eosin (HE) stain, Giemsa stain, and immunohistochemical analysis of CD1a, CD68, CD4, and CD8 in lesional skin of one representative patient. (C), semi-quantitative PCR analysis of cytokine and chemokine expression in healthy skin (HS, $n = 5$) compared to lesional skin of vemurafenib-induced rashes (VIR, $n = 4-5$). qPCR values are shown as relative units compared to 18S rRNA expression. Data are presented as single values and median. Mann-Whitney U test was used to evaluate significant differences (* $P < 0.05$, ** $P < 0.01$)

was confirmed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (not shown).

2.4 | RNA extraction and qPCR

Biopsies were homogenized in TRIzol® using a POLYTRON PT2500E (KINEMATICA AG). RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions, reverse transcribed into cDNA and analyzed by quantitative real-time PCR (ABI PRISM® 7000 Sequence Detection System/ QuantStudio 6 Flex; Thermo Fisher Scientific).¹²

2.5 | Statistics

Statistical significances were assessed with Mann-Whitney *U* tests or Kruskal-Wallis test with Dunn's postcorrection and calculated using GraphPad Prism 5.03 (GraphPad software, Inc). Statistical significances were depicted as follows: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Additional methods are described in the supplemental methods.

3 | RESULTS

3.1 | Vemurafenib-induced inflammatory rashes are characterized by a dense lymphohistiocytic infiltrate

Patients (*n* = 5; 67-76 years) with VIRs and healthy controls (*n* = 5; 52-74 years) were included in our analysis. Patients presented with a generalized maculopapular rash with small papules and macules without scaling (Figure 1A). Histopathologic evaluation of lesional skin biopsies demonstrated a superficial dermatitis without epidermal changes, with mild spongiosis or subtle vacuolar interface changes. Immunohistochemistry revealed a lymphohistiocytic infiltrate with equally distributed CD4⁺ and CD8⁺ T cells (Figure 1B). We did not observe any prominent infiltrates of eosinophils, neutrophils, or mast cells.

3.2 | Vemurafenib-induced inflammatory rashes are characterized by a predominant T_H1 signature

We next analyzed the expression of signature cytokines in lesional skin (VIR, *n* = 4-5) compared to healthy controls (HS, *n* = 5). Our analyses revealed a significant induction of T_H1-associated cytokine *IFNG*, IFN-γ-induced chemokines *CXCL9-11*, *CCL5* (Figure 1C), and a significant upregulation of homeostatic chemokines *CCL27* and *CXCL14* (Figure S1B). Moreover, proinflammatory cytokines and chemokines

such as *CCL2* and *IL1B* were found to be upregulated (Figure 1C and Figure S1B). Although we observed increased expression levels of the T_H2-associated chemokines *CCL1*, *CCL17*, *CCL18*, and *CCL22*, no relevant regulation was detected for *IL4*. Furthermore, *IL17A* or *IFNA* were not induced in lesional skin (Figure 1C and Figure S1B). Taken together, we observed a predominant upregulation of T_H1-associated chemokines.

3.3 | Vemurafenib induces inflammatory cytokines and chemokines in vitro

Next, we analyzed the recruitment pathways directing leukocyte subsets to sites of vemurafenib-induced cutaneous inflammation. We performed comprehensive analyses of cytokine and chemokine expression in whole skin explants, primary human keratinocytes, and T cells treated with vemurafenib in vitro. In skin explants, vemurafenib alone altered the expression of *CCL5* and *CCL18*, which tended to be upregulated, mimicking the expression profile of VIRs (Figure 2A). In keratinocytes, vemurafenib induced a significant expression of *TNF* and *CCL5* (Figure 2A). In T cells, an early upregulation of *IFNG* after 6 h was observed, and after 24 h, transcription and protein levels were increased (Figure 2B). Further, *IL17A* was upregulated after 6 h and 24 h of vemurafenib treatment, yet at overall low expression levels (Figure S2A). *IL4* expression was induced by vemurafenib at negligible levels (Figure S2B).

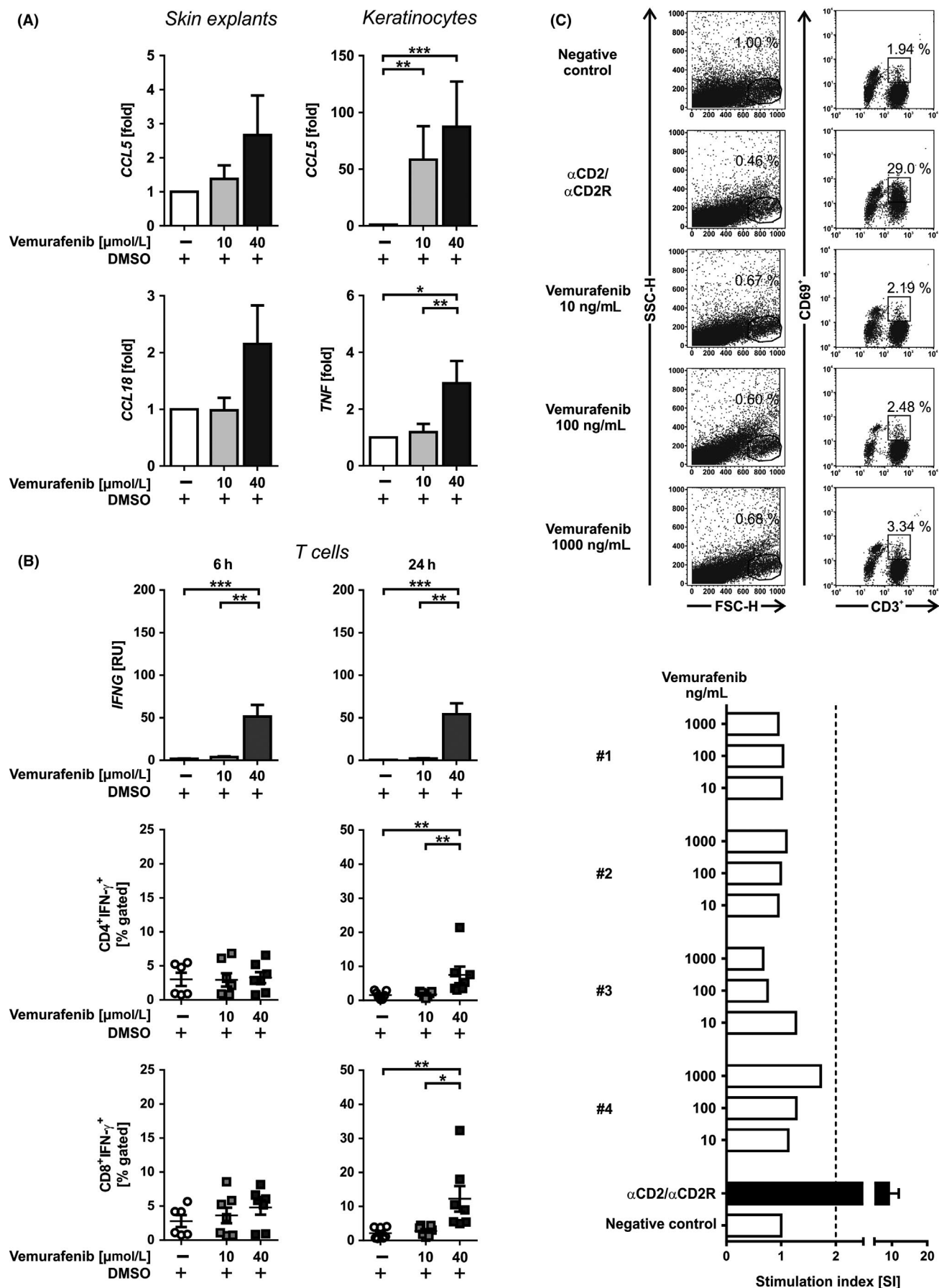
3.4 | Absence of circulating drug-specific T cells in patients with vemurafenib-induced rashes

To discriminate between allergic or nonallergic pharmacologic effects, we performed LATs with leukocytes obtained from patients suffering from VIRs (*n* = 4). To distinguish between allergic and nonallergic patients, Beeler *et al* suggested a stimulation index (SI) cutoff value of 2.¹³ Using this threshold, we did not detect vemurafenib-specific T cells in any patient. Hence, VIRs are rather a result of a nonallergic, pharmacologic mechanism than a result of a specific sensitization against the drug (Figure 2C,D).

3.5 | The structure of vemurafenib is compatible with binding to AhR in silico

To identify nonallergic pharmacologic mechanisms as potential cause of VIRs, we focused on the well-known xenobiotic receptor AhR that binds and regulates the metabolism of drugs and other xenobiotics. Recent findings indicate that the loss of the AhR results in cutaneous inflammation.¹⁴ Several protein kinase inhibitors

FIGURE 2 Vemurafenib induces cytokines and chemokines in skin explants, keratinocytes and T cells, whereas it does not sensitize T cells. (A, B), Skin explants (*n* = 6), keratinocytes (*n* = 14-15) and total T cells (*n* = 9-14) were treated with vemurafenib [10; 40 μmol/L]. qPCR-values are shown as mean + SEM of fold change normalized to 18S rRNA expression compared to DMSO. (B), IFN-γ expression of CD4⁺/CD8⁺ T cells (*n* = 7) after treatment, represented as single values and mean. (C, D), Analysis of CD69⁺CD3⁺ lymphocyte activation after incubation with vemurafenib (one representative patient). Stimulation indexes (SI) were calculated as fold increase of the CD69 upregulation after vemurafenib stimulation of all four patients compared to control. Kruskal-Wallis test with Dunn's postcorrection was used to evaluate significances (**P* < 0.05, ***P* < 0.01, ****P* < 0.001)



modulate AhR activity in an agonistic or antagonistic manner.¹⁵ The AhR ligand indirubin also binds kinase domains in the same ATP-competitive way as does vemurafenib,^{16–19} suggesting some level of cross-reactivity between ligands for AhR and kinases. AhR binds to [³H]TCDD and other aromatic hydrocarbon ligands (PAS-B, residues 277–393) through its second PAS domain.²⁰ The dimensions and molecular characteristics of vemurafenib are within the range of characteristics displayed by *bona fide* AhR ligands (ie, $\sim 14 \times 12 \times 5$ Å planar, hydrophobic ring structures with some hydrophilic moieties²⁰). To computationally probe vemurafenib binding to the AhR PAS-B domain, we inferred the atomic structure of PAS-B based on $\sim 26\%$ identical known PAS-B domain structures. The structural models of PAS-B showed a hydrophobic cavity, in shape and stereochemical character similar to the ligand-binding pocket of the HIF-2 α PAS domain (PDB 4xt2), suggesting that PAS-B binds ligands in the same way as does HIF-2 α PAS. Using a conservative in silico docking approach that allowed ligand flexibility but kept the protein structure rigid, we observed that indirubin and vemurafenib can be accommodated in the PAS-B pocket without steric clashes (Figure 3A and 3B). The calculated binding energies of indirubin and vemurafenib were very similar (-8.29 and -7.91 kcal/mol, respectively, corresponding to dissociation constants K_d of 1–3 $\mu\text{mol/L}$) supporting that vemurafenib may be a relevant AhR binding partner.

3.6 | Vemurafenib is an AhR antagonist

To test the hypothesis that vemurafenib is an AhR ligand, we used human HepG2 hepatoma cells, stably transfected with a luciferase reporter gene driven by two AhR binding sites.²¹ HepG2 cells were exposed to vemurafenib alone or in combination with the AhR agonist benzo[a]pyrene (BaP). Basal luciferase reporter gene activity was slightly reduced following incubation with 40 $\mu\text{mol/L}$ vemurafenib (data not shown). Similarly, incubation with 40 $\mu\text{mol/L}$ vemurafenib markedly reduced the level of AhR-dependent luciferase activity by BaP, suggesting that vemurafenib is acting as an AhR antagonist (Figure 3C). By contrast, luciferase assays did not show any inhibitory effect of dabrafenib or encorafenib on AhR activity in relevant concentrations (up to 1 $\mu\text{mol/L}$)^{10,22} (Figure S3A/B). Dabrafenib and encorafenib even significantly increased AhR-dependent luciferase activity at low concentrations of 0.1 $\mu\text{mol/L}$ (in the absence of BaP). The multikinase inhibitor sorafenib showed a slight trend toward AhR inhibition at pharmacological concentrations of 10 $\mu\text{mol/L}$ ²³ in the presence of BaP (Figure S3C). A similar inhibitory trend was seen for the novel BRAF inhibitor PLX8394 (Figure S3D).

To determine the mechanism by which vemurafenib reduces AhR-dependent gene expression, we examined its ability to directly bind to AhR. Hydroxyapatite ligand-binding analysis using guinea pig hepatic cytosolic AhR revealed that vemurafenib competitively inhibits [³H]TCDD, a high-affinity AhR ligand, from binding to AhR. This inhibition occurs in a vemurafenib concentration-dependent manner; with maximum competitive inhibition observed at 40 $\mu\text{mol/L}$ and hence in line with our micromolar affinities as calculated in silico (Figure 3D). While this analysis confirmed that vemurafenib is

a direct ligand for AhR, it does not provide any information as to whether vemurafenib is binding as an AhR agonist or antagonist. To address this question, we utilized a gel retardation analysis to assess the ability of a chemical to stimulate AhR transformation and DNA binding, a characteristic of AhR agonists. Although incubation of guinea pig hepatic cytosol with vemurafenib did not stimulate AhR transformation or DNA binding, it did produce a concentration-dependent decrease in TCDD-stimulated AhR transformation and DNA binding (Figure 3E). Ligand and DNA binding analyses confirmed the ability of vemurafenib to act as an AhR antagonist (ie, it exhibits no AhR agonist activity). Against the background of previous studies that have demonstrated species-specific differences in AhR ligand-binding specificity, respective DNA binding analysis studies were repeated using in vitro synthesized human AhR and ARNT (the AhR dimerization partner required for AhR DNA binding).^{5,24} Similar to the results obtained with guinea pig hepatic cytosolic AhR, vemurafenib antagonized the ability of TCDD to stimulate transformation and DNA binding of the human AhR (Figure 3F). As expected, vemurafenib treatment inhibited basal as well as BaP-induced nuclear translocation of AHR in human keratinocytes (Figure 3G). Taken together, these results confirm the ability of vemurafenib to act as an AhR antagonist.

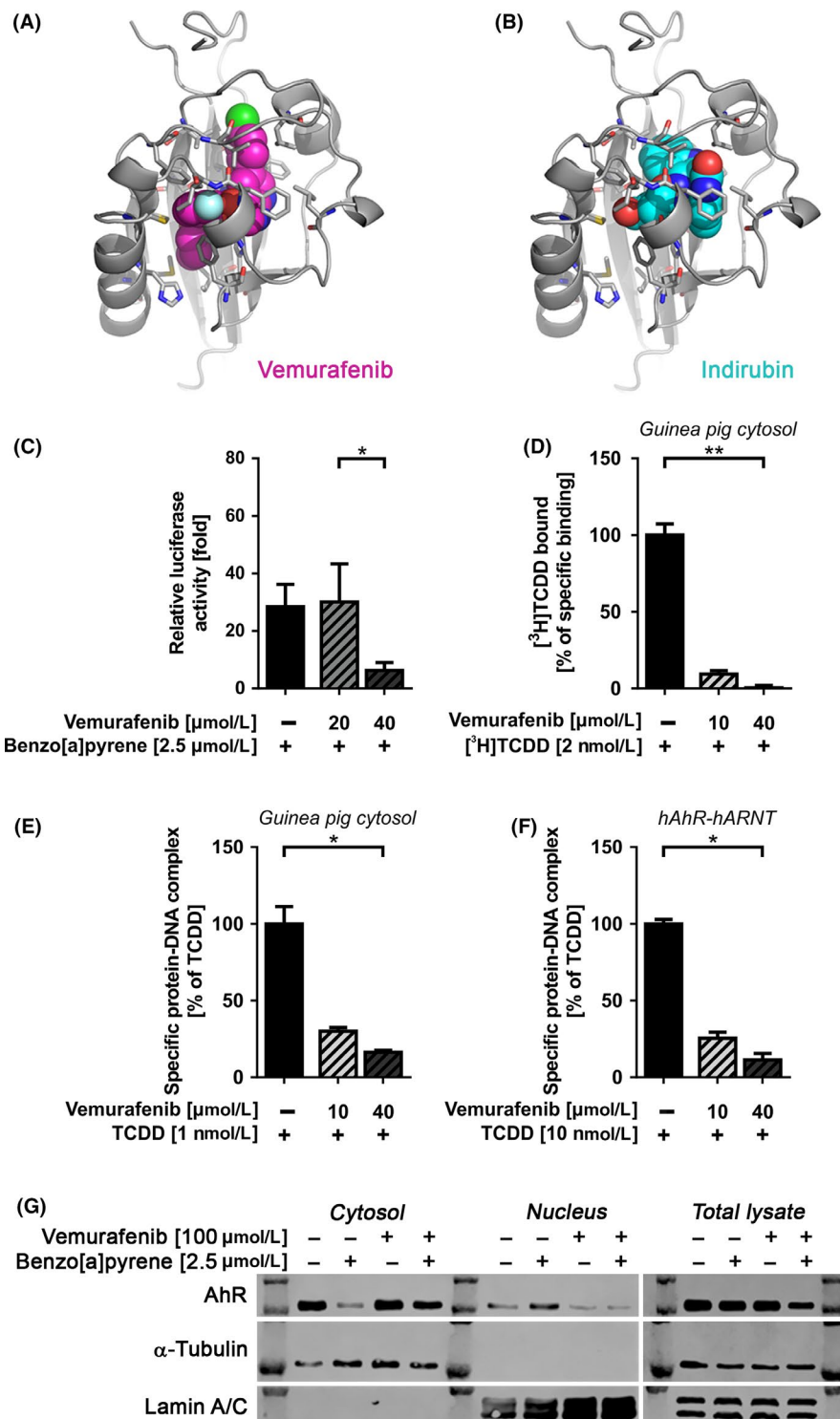
3.7 | Vemurafenib impairs CYP1A1 expression in vitro, ex vivo, and in vivo

In order to prove the AhR antagonism of vemurafenib in vivo and in vitro, we assessed the expression of AhR and AhR-related genes in lesional skin of VIRs compared to healthy controls as well as in skin explants, keratinocytes, and T cells. As an additional control, we analyzed papulopustular rashes of cancer patients treated with epidermal growth factor receptor (EGFR) inhibitors, which we have demonstrated to be caused by nonallergic, pharmacologic mechanisms.²⁵

AHR was significantly upregulated in VIRs (Figure S4A). In contrast, no regulation of AHR was observed in EGFR inhibitor (EGFRi)-induced rashes or healthy controls (Figure S4C). Next, we assessed a dysregulation of AhR signaling and analyzed the endpoints of the canonical and noncanonical pathway of AhR signaling with two representatives: Prostaglandin-endoperoxide synthase 2 (COX2) and cytochrome P450 (CYP) 1A1. COX2 was significantly upregulated in VIRs as compared to healthy controls (Figure S4B). CYP1A1 gene expression was significantly downregulated in VIRs compared to healthy controls (Figure 4A). In line with these results, CYP1A1 protein expression was found to be reduced in lesional skin (Figure 4B). Furthermore, we observed a downregulation of CYP1A1 after stimulation with vemurafenib in skin explants, keratinocytes, and T cells in vitro (Figure 4C). Vemurafenib did not only reduce the basal CYP1A1 expression but also it decreases it when skin explants have been stimulated with the AhR agonist FICZ (Figure 4C).

Comparing vemurafenib to dabrafenib in terms of T cell stimulation, we found that dabrafenib did not induce IFN- γ and IL-17 at gene or protein level in the pharmacological relevant levels up to 1 μM

FIGURE 3 Vemurafenib is an AhR antagonist. (A, B), In silico docking of vemurafenib and indirubin to PAS-B homology models (gray). Best docking poses for are shown for vemurafenib (A, van der Waals sphere representation with carbons colored in magenta) and indirubin (B, carbons in cyan). Residues of PAS-B forming the cavity are highlighted as stick models. (C), Reporter gene analyses using a XRE-HepG2 reporter cell line treated with vemurafenib [20; 40 $\mu\text{mol/L}$] combined with benzo[a]pyrene [2.5 $\mu\text{mol/L}$]. Luciferase activities were offset to protein concentration and normalized to DMSO. (D), Ligand binding was analyzed in guinea pig cytosol, using 2 nmol/L [^3H]TCDD in combination with vemurafenib [10; 40 $\mu\text{mol/L}$]. DNA binding was assayed in guinea pig cytosol (E) or using in vitro synthesized AhR/ARNT complexes (F) with TCDD combined with vemurafenib [10; 40 $\mu\text{mol/L}$]. Values are normalized to [^3H]TCDD (D) or TCDD (E/F). Kruskal-Wallis test with Dunn's postcorrection was used to evaluate differences (* $P < 0.05$, ** $P < 0.01$). (G), AhR translocation into the HaCaT cell nucleus after 2h treatment with vemurafenib [100 $\mu\text{mol/L}$] or in combination with benzo[a]pyrene [2.5 $\mu\text{mol/L}$]. One representative plot of two experiments is displayed showing cytosolic and nucleus fractions together with the total lysate and the controls α -tubulin and lamin A/C



(Figure 4F/G). Furthermore, dabrafenib did not affect CYP1A1 gene expression and IL-22 protein expression in CD4⁺ T cells (Figure 4F/G).

4 | DISCUSSION

Despite its clinical efficacy, vemurafenib therapy is associated with a wide range of adverse events including arthralgia, fatigue,

and skin toxicities.²⁶ Cutaneous side effects are predominant and occur in up to 70% of the patients.² Four different cutaneous reaction patterns have been described: (a) inflammatory reactions; (b) hair/ nail changes; (c) melanocytic disorders/proliferations; and (d) keratinocytic proliferations.²⁷ Proliferative alterations have been systematically analyzed and are contributed to a pharmacologic, paradoxical activation of the RAS-RAF-MEK-ERK signaling pathway.²⁸ This concept is supported by clinical observations showing

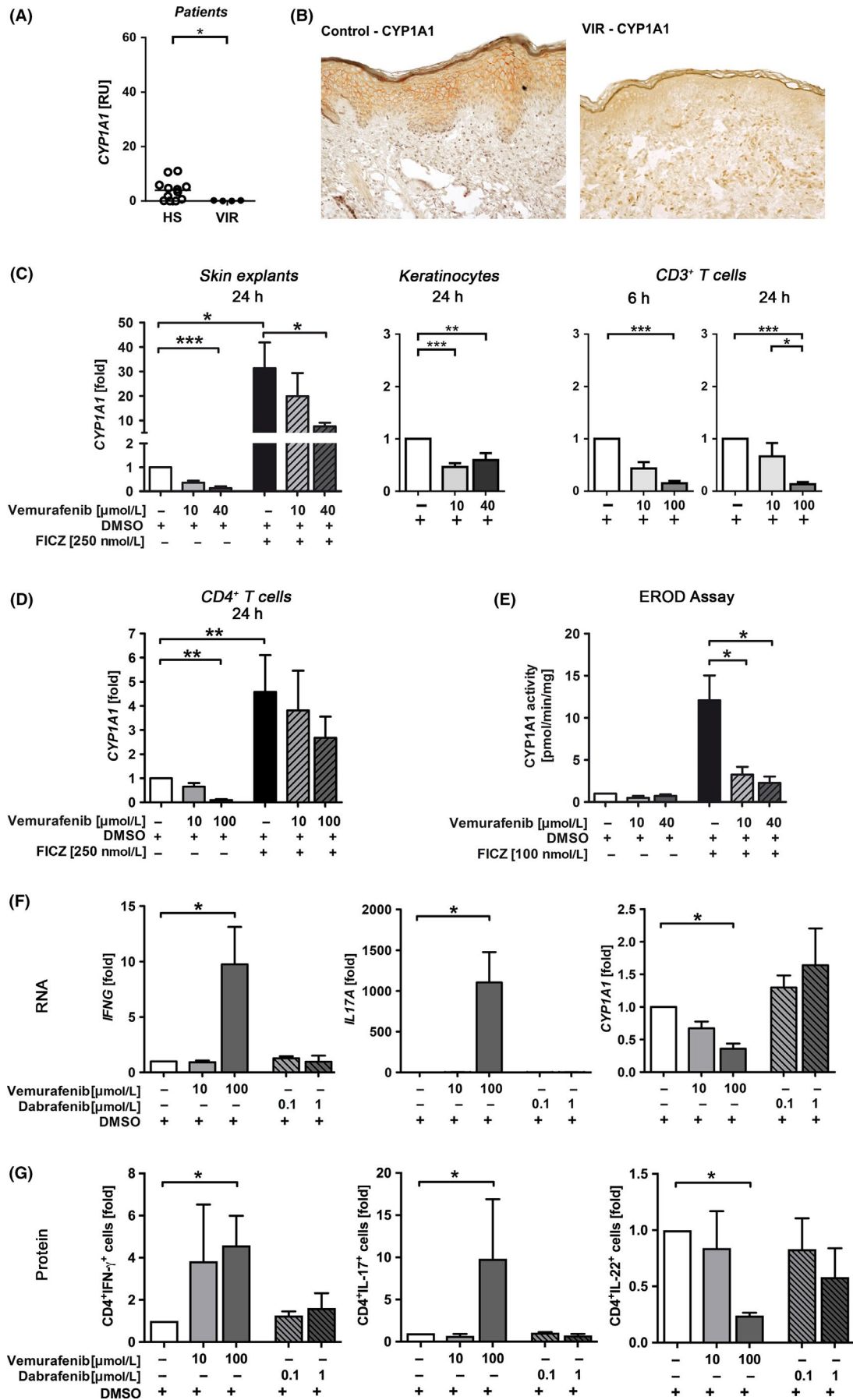


FIGURE 4 CYP1A1 expression is impaired by vemurafenib in vitro and in vivo. Expression of CYP1A1 mRNA in lesional skin (VIR, $n = 4-5$) compared to healthy controls (HS, $n = 11$) (A), representative immunohistochemistry analysis of CYP1A1 protein in lesional skin versus control (B) and CYP1A1 mRNA expression (C) in primary keratinocytes ($n = 14-15$), skin explants ($n = 6$), and CD3⁺ T cells ($n = 9-15$). (D) CYP1A1 expression in purified CD4⁺ T cells treated with vemurafenib alone or in combination with FICZ for 24h ($n = 5$). qPCR values are shown as fold change relative to 18S rRNA expression and compared to normalized DMSO control. (E) Ethoxyresorufin-O-deethylase (EROD) Assay on primary keratinocytes to detect CYP1A1 activity in presence of vemurafenib ($n = 4$). CYP1A1 activity has been adjusted to protein concentration and normalized to DMSO. (F), CD4⁺ T cells ($n = 4$) were treated with vemurafenib [10; 100 $\mu\text{mol/L}$] or dabrafenib [0.1; 1 $\mu\text{mol/L}$]. *IFNG*, *IL17A* and *CYP1A1* gene expression values are shown as fold change normalized to 18S rRNA expression compared to DMSO. (G), IFN- γ , IL-17 and IL-22 protein expression of CD4⁺ T cells ($n = 4$) as fold change to DMSO after treatment with vemurafenib or dabrafenib. Data are presented as mean + SEM. Kruskal-Wallis test with Dunn's postcorrection and Mann-Whitney U test was used to evaluate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

that BRAFi-induced proliferative lesions regress under concurrent administration of MEK inhibitors (MEKi).^{29,30} By contrast, co-administration of MEKi results in a slight increase in incidence and severity of inflammatory rashes (35% for vemurafenib plus placebo versus 39% for vemurafenib plus cobimetinib).²⁹ These observations support the hypothesis that inflammatory rashes and proliferative alterations are caused by different mechanisms.

From a clinical perspective, VIRs present as maculopapular drug exanthemas, pointing toward a T cell-mediated hypersensitivity reaction.³¹ Indeed, immunohistochemical analyses of lesional skin revealed a lymphohistiocytic infiltrate. However, we did not observe prominent infiltrates of eosinophils. Our analyses of cytokines revealed a T_H1-signature with an induction of dendritic cell (DC)-associated as well as T cell-derived inflammatory mediators, such as *TNF*, *IL1B*, and *IFNG*, and the T_H1-associated chemokines *CXCL9-11*. In addition, the increased expression of *CCL27* and *CXCL14* facilitates the recruitment of T cells as well as macrophages.³² The upregulation of *IL1B* indicates an activation of the inflammasome in macrophages and/or other cells of the innate immune system. Conversely, we did not detect a relevant induction of T_H2-cytokines, *IFNA*, or T_H17-associated cytokines. To test our hypothesis that VIRs are caused rather by pharmacologic than allergic mechanisms, we performed LATs of blood samples obtained from patients with VIRs. Although stimulated T cells expressed IFN- γ RNA and protein, LATs failed to detect vemurafenib-specific T cells in our collective. To conclude, the negligible numbers of eosinophils, the absence of an allergy-associated T_H2 signature, and the absence of drug-specific T cells, indicate that VIRs are caused by nonallergic pharmacological reactions rather than a drug-specific sensitization. This hypothesis is further supported by an observed induction of chemokines, including *CCL2*, *CCL5*, *CCL27*, and *CXCL14*, which also play a central role for the pathogenesis of nonallergic, pharmacologic, papulopustular rashes induced by EGFRi.²⁵ In contrast, nonimmediate allergic drug reactions are associated with a T_H1 chemokine signature.^{33,34} To assess whether the induction of inflammation is caused by a direct pharmacological effect of vemurafenib on skin or by indirect, systemic effects, skin explants were stimulated with vemurafenib ex vivo. In line with the cytokine-chemokine signature observed in patient samples in vivo, we found a marked induction of *TNFA* and *CCL5*. Respective in vitro analyses in human keratinocytes and T cells showed similar results, indicating that structural cells and immune cells are targets of vemurafenib and central regulators of vemurafenib-induced inflammation in the skin.

Although vemurafenib and dabrafenib are both potent inhibitors of the BRAF kinase, reported incidences for inflammatory rashes are significantly higher for vemurafenib (41% vs. 30%), suggesting that these rashes are rather not caused by a class-specific effect of BRAFi but by another, so far unknown vemurafenib-specific mechanism.³⁵ A putative target for vemurafenib is the xenobiotic receptor AhR, which has been shown to be modulated by several other protein kinase inhibitors in an either agonistic or antagonistic manner.^{7,15,36-38} Interestingly, AhR is highly expressed in skin and other barrier organs.⁵ Upon binding of an agonistic ligand, AhR stimulates expression of a battery of genes in a tissue- and cell-specific fashion.^{5,24} Activation of the canonical signaling pathway involves ligand-dependent translocation of AhR into the nucleus, where it forms a dimer with the AhR nuclear translocator (ARNT) resulting in the binding to an upstream regulatory region of respective target genes.⁵ In addition, AhR activation leads to stimulation of noncanonical signaling pathways, which may trigger proinflammatory responses.^{5,39,40} As recently postulated, canonical and noncanonical AhR signaling pathways are tightly balanced in healthy skin thereby contributing to skin homeostasis.⁴¹ In fact, our analysis revealed a decreased number of CYP1A1 transcripts (as marker for canonical AhR signaling) in vemurafenib-induced rashes, which was paralleled by elevated mRNA levels of COX2 (as marker for noncanonical AhR signaling). Of note, a recent study using an imiquimod mouse model of psoriasiform dermatitis indicates that loss of AhR results in exaggerated cutaneous inflammation.⁴² Accordingly, treatment of wild-type mice with AhR agonists attenuated psoriasiform skin inflammation.⁴² AhR activation by coal tar was shown to reduce proinflammatory responses and restore epidermal barrier functions in a 3D in vitro model of atopic dermatitis.⁴³ Furthermore, inflammatory responses in lipopolysaccharide-challenged AhR-deficient mice are characterized by induction of TNF- α , IL-1 β , IL-6, IL-18, and IFN- γ ,⁴⁴ a cytokine pattern closely resembling the one observed in VIRs.

Di Meglio *et al* identified that deficiency or antagonism of the nonhematopoietic skin compartment is necessary and sufficient for the development of skin inflammation.⁴² Their results ruled out a central role for DCs or macrophages but did not exclude a possible involvement of T or B cells in AhR-mediated skin inflammation. In line with these results, we did not observe significant effects of vemurafenib on DCs (not shown) but demonstrate that vemurafenib downregulates the AhR prototype target gene CYP1A1 in keratinocytes and T cells in vitro as well as skin explants ex vivo.

These findings are supported by the observation that CYP1A1 gene expression was down-modulated in VIRs in vivo. By contrast, CYP1A1 was not altered in EGFRi-associated rashes as compared to healthy donors. This indicates that VIRs are likely triggered by disturbance of canonical but not noncanonical AhR signaling that involves the c-Src/EGFR/ERK pathway.⁴⁰ Our hypothesis that vemurafenib acts as a potent AhR antagonist is further supported by reporter gene analyses, showing that vemurafenib treatment inhibited both basal as well as BaP-induced activity of the AhR-dependent luciferase construct. In fact, competitive ligand-binding assays and determination of DNA-bound AhR/ARNT protein provided evidence that vemurafenib is an antagonistic ligand of AhR. In contrast to a recent study on MCF-7 breast cancer cells, reporting that vemurafenib treatment induces nuclear translocation of AhR (Corre et al.⁴⁵), our data clearly show that the BRAF inhibitor retained AhR in the cytosol of solvent—as well as BaP-treated keratinocytes—and thus behaves as a pure antagonist. The molecular mechanism responsible for this apparent cell-specific differences remains to be elucidated. As opposed to vemurafenib, dabrafenib did not interfere with AhR and dabrafenib-treated patients develop cutaneous rashes at lower incidences.^{46,47} Taking in account our hitherto results, it is also not surprising that immunostimulatory immune-checkpoint inhibitors, such as ipilimumab, nivolumab, or pembrolizumab, can aggravate the severity of vemurafenib-induced rashes.^{3,4,48} Up to 70% of patients treated with vemurafenib develop a drug eruption. The initial trigger remains unclear. Unknown factors such as infectious triggers, environment-/food-related xenobiotics or an individual genetic predisposition may favor the spontaneous onset of the observed rashes.

Taken together, our results indicate that vemurafenib-induced inflammatory rashes are caused by a pharmacologic mechanism rather than a specific sensitization against the drug. Moreover, our results imply that this mechanism is not a class-specific, direct inhibition of BRAF, but rather a vemurafenib-specific inhibition of the AhR. Interestingly, Smith *et al.* identified a naturally derived topical AhR agonist called tapinarof (GSK2894512) capable of inducing CYP1A1 expression together with an improvement of murine AhR-mediated imiquimod-induced psoriasiform skin lesions.⁴⁹ Against this background, we hypothesize that activation of AhR for instance via an AhR agonist-rich diet, (eg, brassica vegetables containing indole-3-carbinol), or via topical application of nontoxic AhR ligands may represent a novel strategy to prevent or treat VIRs.⁵⁰

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

HCH, AK, SM, and PAG designed, performed, and analyzed most of the experiments. MP helped in performing the luciferase assays and implemented the AhR translocation assay. KMR conducted the EROD assays. AAS and MSD designed and performed the AhR binding experiments. AAM and STA conducted the in silico computational modeling. AD performed flow cytometry experiments. SAB did the immunohistochemistry experiments and helped with microscopy. PO, MEL, and JK provided suggestions and discussions throughout the study. SM supervised the study and wrote the manuscript together with HCH, AK, PAG, TH, and BH.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplemental figures

Supplemental figure 1

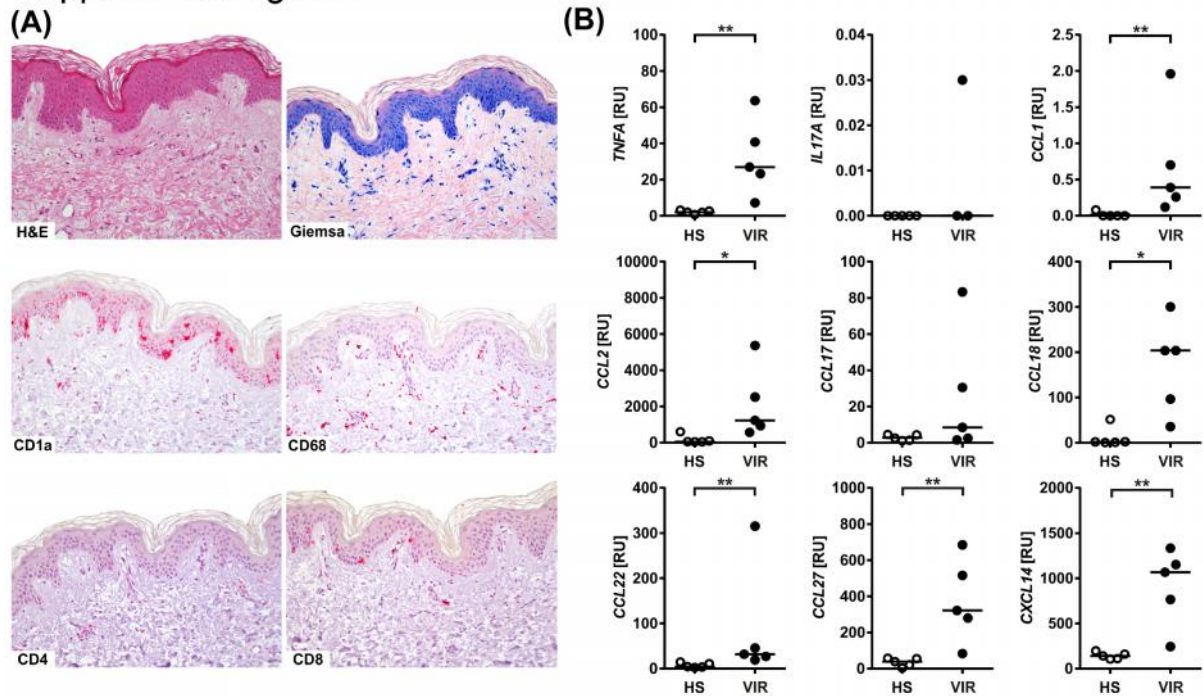


Figure S1: **Vemurafenib-induced rashes are characterized by a broad upregulation of cytokines and corresponding chemokines.** (A), Hematoxylin and eosin (HE) stain, Giemsa stain and immunohistochemical staining of CD1a, CD68, CD4 and CD8 in control skin of one representative patient. (B), Semi-quantitative PCR analysis of cytokine and chemokine expression in healthy skin (HS, n=5) compared to lesional skin of vemurafenib-induced rashes (VIR, n=4-5). qPCR-values are shown as relative units compared to 18S rRNA expression. Data are presented as single values and median. Mann-Whitney U test was used to evaluate significant differences (* $p < 0.05$, ** $p < 0.01$).

Supplemental figure 2

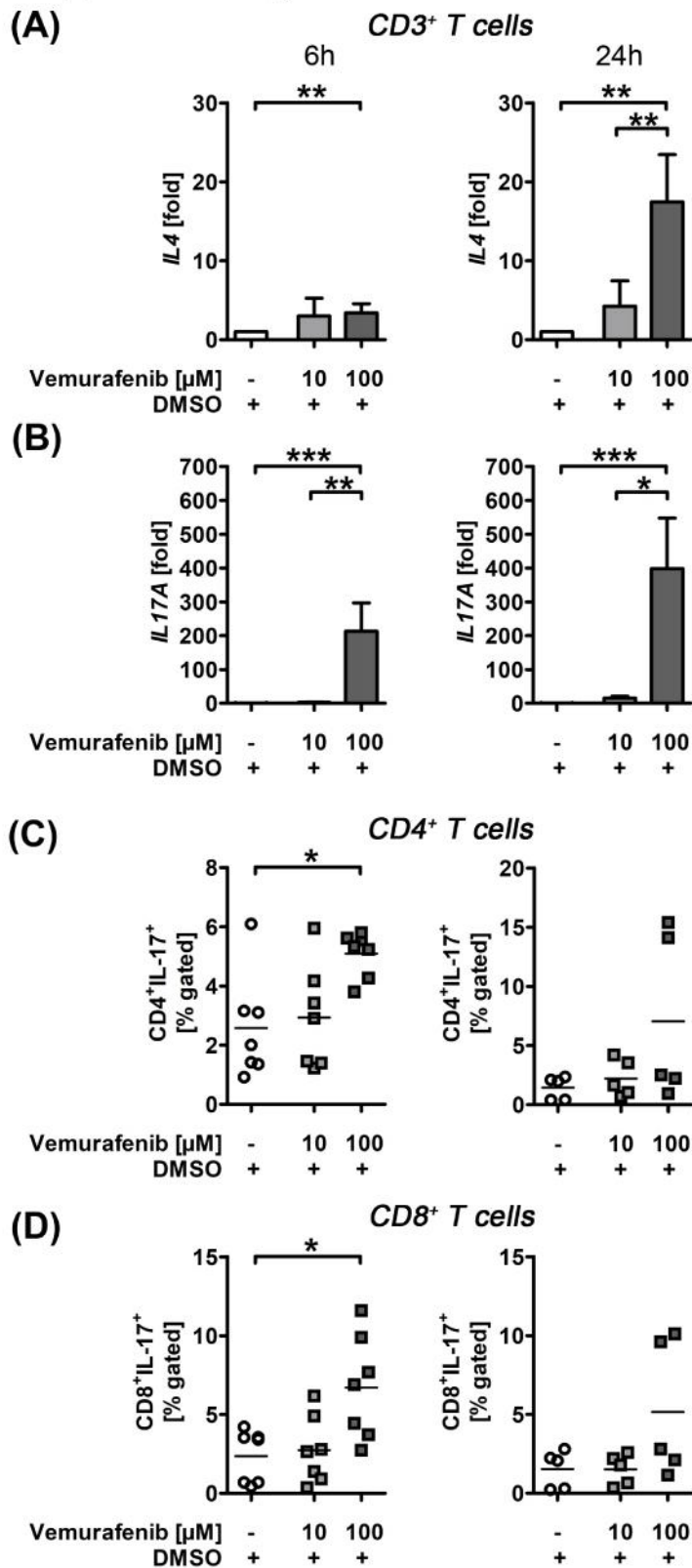


Figure S2: *IL4* and *IL17* expression in T cells treated with vemurafenib. T cells were treated with vemurafenib [10 or 100 μ M] and an equal dose of DMSO for 6 h or 24 h. Semi-quantitative PCR analysis of *IL4* ((A), n=8-14) and *IL17A* expression ((B),

n=9-15) in vemurafenib-treated T cells from healthy donors. qPCR-values are shown as fold change normalized to 18S rRNA expression. Data is presented as mean + SEM. IL-17 protein expression of CD4⁺ T cells ((C), n=5-7) and CD8⁺ T cells ((D), n=5-7) after treatment, represented as single values and mean. Kruskal-Wallis test with Dunn's post correction was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

Supplemental figure 3

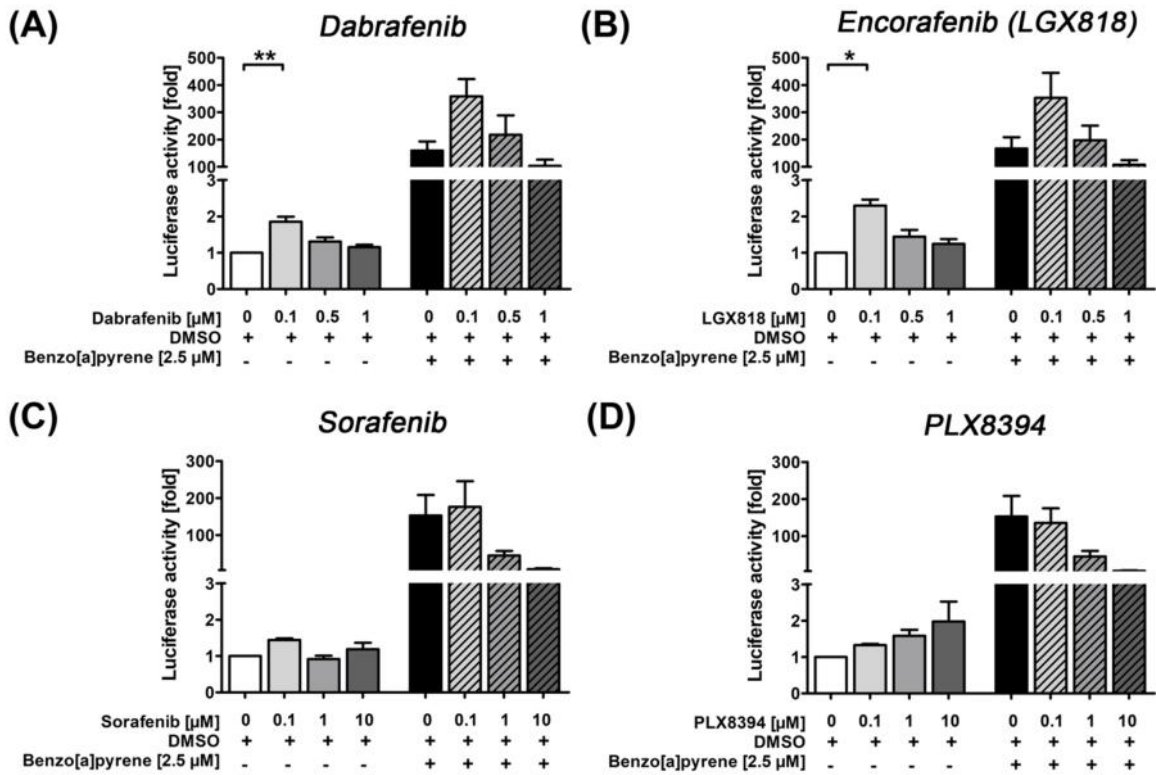


Figure S3: AhR activity under stimulation with different BRAF inhibitors. Reporter gene analyses using a XRE-HepG2 reporter cell line treated with dabrafenib [0.1; 0.5; 1 μ M] (A), encorafenib (B, LGX818) [0.1; 0.5; 1 μ M], sorafenib (C) [0.1; 1; 10 μ M], or PLX8394 (D) [0.1; 1; 10 μ M], or DMSO or a combination of the respective inhibitor with benzo[a]pyrene [2.5 μ M] (n=3-4). Luciferase activities were offset to protein concentration, hereafter DMSO control was used to normalize all values. Kruskal-Wallis test with Dunn's post correction was used to evaluate significant differences (*p<0.05).

Supplemental figure 4

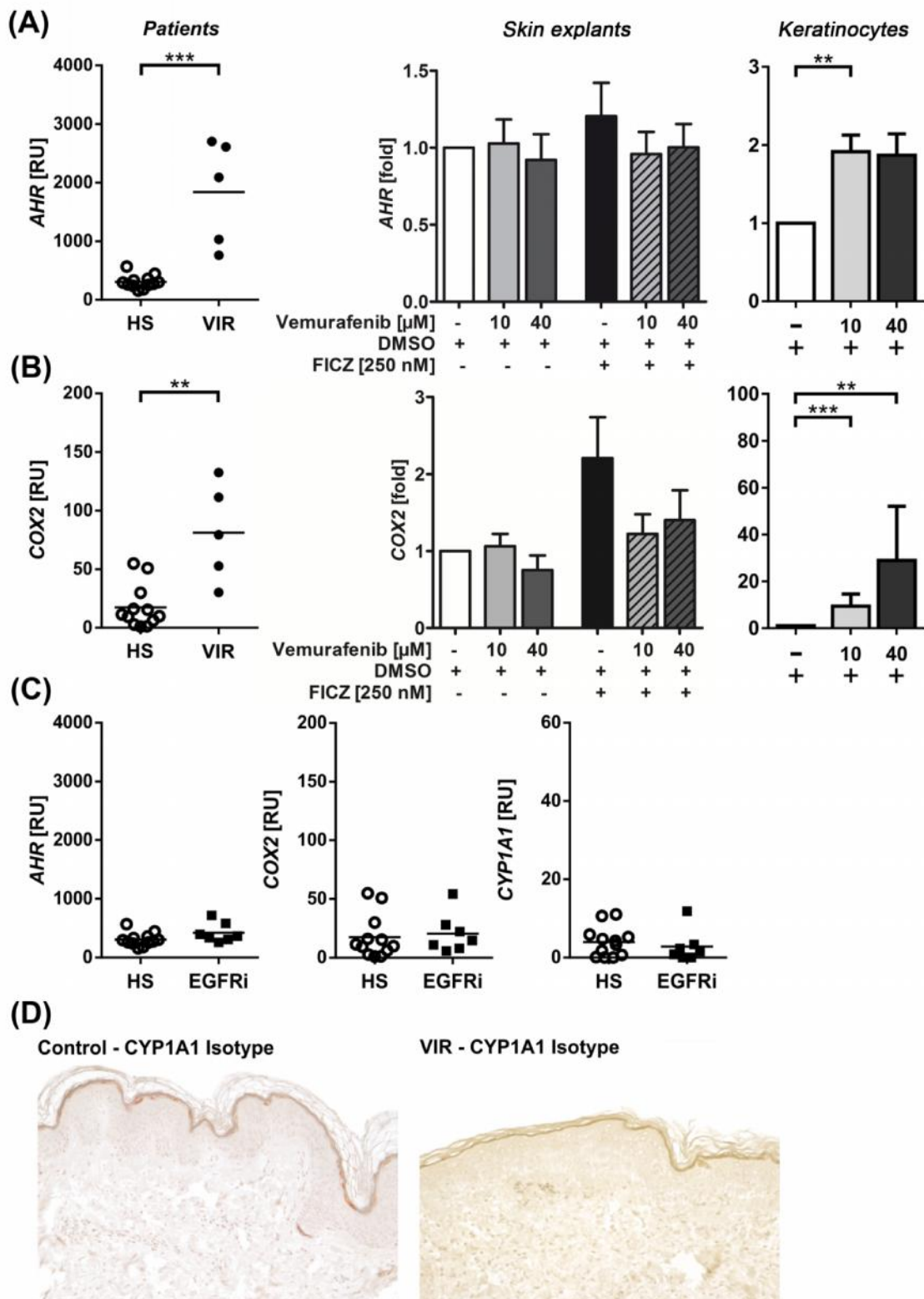


Figure S4: **AhR signaling is impaired by vemurafenib *in vitro* and *in vivo*.** Expression of *AHR* (A) and *COX2* (B) and in patients with vemurafenib-induced rashes (VIR, n=4-5) compared to healthy controls (HS, n=11), as well as vemurafenib [10 μ M; 40 μ M] treated skin explants (n=6) and primary keratinocytes (n=14-15).

qPCR-values are shown as fold change relative to 18S rRNA expression and compared to normalized DMSO control. C, Gene expression of *AHR*, *COX2* and *CYP1A1* in patients receiving erlotinib (EGFRi, n=7) compared to healthy controls (HS, n=11). Immunohistochemical staining of CYP1A1 isotype (D) is shown in VIR compared to control skin. Kruskal-Wallis test with Dunn's post correction was used to evaluate significant differences (*p<0.05, **p<0.01, ***p<0.001).

Supplemental methods

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded skin biopsies of VIRs and controls were stained with antibodies against CD1a, CD68, CD4, and CD8 (Dako Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocols.

Histochemistry detecting CYP1A1 was performed as follows: Skin sections were incubated at 60°C overnight before deparaffinization. After rehydration using a graded alcohol series, the antigens were demasked using Tris/EDTA buffer (pH9) together with boiling for 20 min. Slides were then washed with PBS and subsequently incubated with H₂O₂ for 10 min to block peroxidase. After another washing step in PBS, the samples were incubated with human serum, streptavidin and 0.3% TritonX-100 in PBS for 30 min at room temperature. Then the CYP1A1 antibody (A-9; 1:50; Santa Cruz) was applied to the skin sections and incubated overnight at 4°C. On the next day the sections were washed with PBS and then incubated with the biotinylated secondary antibody (anti-mouse IgG1) for 30 min at room temperature. After washing, streptavidin HRP was applied to the sections and incubated for 45 min. The staining was then developed using 3-amino-9-ethylcarbazole (AEC) substrate and the sections were counterstained using hematoxylin. All histologic images were taken with an Eclipse Ni-U microscope and a DS-Ri1 camera (Nikon).

Lymphocyte activation tests (LAT)

T cell activation was determined quantitatively via flow cytometry using BD FastImmune (BD Biosciences, San Jose, CA, USA). Whole blood obtained from patients with VIRs was incubated with or without different drug concentrations for 4 h at 37°C, stained against CD3 and CD69 (BD Biosciences, San Jose, CA, USA). Flow

cytometric analyses were performed using FACSCalibur (BD Biosciences) and analyzed with CELLQuest-Pro (CellQuest Inc., Tampa, FL, USA).

Culture of human cells and explants

For *ex vivo* explants, 4 mm skin punches were incubated with DMEM +10% FCS +1% Penicillin/Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), +/- vemurafenib (10; 40 μ M; Selleckchem, Munich, Germany) or DMSO for 5 days at 37°C. On day 5, one volume of medium containing additives and/or 250 nM 6-formylindolo(3,2-b) carbazole (StressMarq Biosciences, Cadboro Bay, Canada) was added for 24 h.

Human keratinocytes from foreskin were treated with vemurafenib for 24 h. Keratinocytes were lysed in TRIzol[®] Reagent (Thermo Fisher Scientific) for RNA extraction.

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using Ficoll centrifugation. T cells were enriched using human CD3⁺ T cell enrichment columns (bio-techne, Wiesbaden, Germany) or by using the human CD4⁺ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD3⁺ T cells were cultured in X-vivo-15 (Lonza, Verviers, Belgium) and exposed to vemurafenib (10; 100 μ M) or controls for 6 h or 24 h. CD4⁺ T cells were activated using the human T Cell Activation/Expansion Kit (Miltenyi Biotec) overnight in RPMI medium (Thermo Fisher Scientific) supplemented with 10% FCS and 1% Penicillin/Streptomycin, before treatment with vemurafenib (10; 100 μ M), dabrafenib (0.1; 1 μ M; Selleckchem) or vemurafenib combined with 250 nM FICZ for 24h. All T cells were used for FACS staining or for RNA extraction.

Flow cytometry

Flow cytometric analysis was performed with T cells treated additionally with GolgiStop and GolgiPlug (BD Biosciences) 4 h before the incubation ended. Cells were blocked with human serum, stained against CD4, CD8 (BD Biosciences) and permeabilized for intracellular IFN- γ (bio-technique), IL-17 (bio-technique) and IL-22 (Thermo Fisher Scientific) staining using Cytofix/Cytoperm (BD Biosciences). Samples were analyzed analogously to LAT or using the CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA) together with the CytExpert software.

Computational modeling

SwissModel ¹, RaptorX ² and iTASSER ³ produced the homology models that were used for flexible ligand docking with AutoDock 4.2 ⁴. Docking was performed using a Lamarckian genetic algorithm (population size = 100, 1,500,000 energy evaluations, $\leq$ 25000 generations, number of top individuals to automatically survive to next generation = 1, mutation rate = 0.02, crossover rate = 0.8, 10 docking runs, random initial positions and conformations, probability of performing local search on an individual in the population = 0.06).

AhR ligand and DNA binding assays

Competitive ligand binding to AhR was performed using guinea pig hepatic cytosol. Cytosol was incubated with 2 nM [³H]2,3,7,8-tetrachlorodibenzo-p-dioxin ([³H]TCDD) +/- 200 nM 2,3,7,8-tetrachlorodibenzofuran (TCDF) or vemurafenib for 1 h at room temperature and [³H]TCDD specific binding determined using the hydroxyapatite binding assay as described ^{5,6}. Measuring the ability of vemurafenib to stimulate AhR transformation and DNA binding (AhR agonist activity) or to inhibit the ability of

TCDD to stimulate AhR transformation and DNA binding (AhR antagonist activity) was carried out using a gel retardation analysis with guinea pig hepatic cytosol or *in vitro* synthesized human AhR/ ARNT ^{6,7}.

AhR/XRE-dependent reporter gene assay

Reporter gene analyses were done with XRE-HepG2 reporter cell line, grown in RPMI1640 containing 3.7% NaHCO₃, 10% FCS, 1% Penicillin/Streptomycin and 0.8 mg/ml G418. Cells were treated with vemurafenib (20; 40 µM), dabrafenib (Selleckchem; 0.1; 0.5; 1 µM), encorafenib (LGX818; MedChemExpress, Monmouth Junction, NJ, USA; 0.1; 0.5; 1 µM), sorafenib (MedChemExpress; 0.1; 1; 10 µM), PLX8394 (MedChemExpress; 0.1; 1; 10 µM), or DMSO +/- benzo[a]pyrene (2.5 µM). After 24 h, cells were harvested and luciferase activities were determined using the luciferase assay system (Promega, Mannheim, Germany) in a Multi-Biolumat LB9505C (Berthold, Wildbad, Germany). Concentrations were determined by BC Assay protein quantification kit (Bio-Rad, Munich, Germany). Luciferase activities were normalized to protein concentration; each sample was analyzed in triplicate.

AhR translocation assay

HaCaT keratinocytes were incubated with vemurafenib (100 µM) or combined with benzo[a]pyrene (2.5 µM) for 2h. The cells were then harvested and the isolation of nuclear and cytoplasmic extracts was followed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Waltham, MA, USA) ⁸. The cytosolic and nuclear fractions were then subjected to western blot analyses as described by Frauenstein *et al.* ⁹ using antibodies against AhR (D5S6H; Cell Signaling Technology, Cambridge, UK), α-tubulin (11H10; Cell Signaling Technology) and lamin A/C (4C11; Cell Signaling Technology).

Ethoxyresorufin-O-deethylase (EROD) assay

The EROD assay is a kinetic method that measures the fluorescent product resorufin^{10,11}. The substrate 7-ethoxyresorufin is converted to resorufin by CYP1A1. Therefore the fluorescent intensity is directly correlated to CYP1A1 concentration.

Primary human keratinocytes were seeded in 48 well plates and incubated in presence of FICZ, vemurafenib or both at the indicated concentrations for 24h. Cells were then washed and incubated in presence of 2.5 μ M 7-ethoxyresorufin and 10 μ M of dicumarol, a reductase inhibitor prior to fluorescence measurement. Using a resorufin dilution series and by determination of protein concentration by CBQCAC kit (Life technology, USA), the enzyme activity is calculated.

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