Ultraviolet light converts propranolol, a nonselective β-blocker and potential lupus-inducing drug, into a proinflammatory AhR ligand

Karim Dorgham¹, Zahir Amoura¹,², Christophe Parizot³,
Laurent Arnaud¹,², Camille Frances⁴, Cédric Pionneau⁵,⁶,
Hervé Devilliers⁶, Sandra Pinto¹, Rima Zoorob¹, Makoto Miyara¹,²,³,
Martin Larsen¹, Hans Yssel¹, Guy Gorochov¹,³ and Alexis Mathian¹,²

¹ Sorbonne Universités, UPMC Univ Paris 06, Inserm UMRS1135, Centre d’Immuno-\nlogie et des Maladies Infectieuses (Cimi-Paris), 83 Bd de l’hôpital, F-75013, Paris, France
² AP-HP, Groupement Hospitalier Pitié-Salpêtrière, institut E3M, Service de médecine interne
², Centre de Référence National pour le Lupus et le Syndrome des Antiphospholipides, Paris, France
³ AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Département d’immunologie, Paris, France
⁴ AP-HP, Hôpital Tenon, Service de dermatologie, Paris, France
⁵ Sorbonne Universités, UPMC, Inserm UMS-29 Omique, Plateforme P3S, F-75013, Paris,
France
⁶ Centre Hospitalier Universitaire de Dijon, Service de médecine interne 2 et centre
d’investigation clinique, Dijon, France

UV light and some medications are known to trigger lupus erythematosus (LE). A common mechanism underlying the immunopathologic effect, resulting from exposure to these two seemingly unrelated factors, remains unknown. The aryl hydrocarbon receptor (AhR) plays a key role in the regulation of IL-22 production in humans and can be activated by both xenobiotics and naturally occurring photoproducts. A significant expansion of Th17 and Th22 cells was observed in the peripheral blood of active systemic LE (SLE) patients, compared to inactive patients and controls. We also show that propranolol, a potential lupus-inducing drug, induced stronger AhR activation in PBMCs of SLE patients than in those of controls. AhR agonist activity of propranolol was enhanced by UV light exposure. MS analysis of irradiated propranolol revealed the generation of a proinflammatory photoproduct. This compound behaves like the prototypic AhR ligand 6-formylindolo[3,2-b]carbazole, a cutaneous UV light-induced tryptophan metabolite, both promoting IL-22, IL-8, and CCL2 secretion by T-cells and macrophages. Finally, LE patients exhibit signs of cutaneous AhR activation that correlate with lesional expression of the same proinflammatory cytokines, suggesting a role for photometabolites in the induction of skin inflammation. The AhR might therefore represent a target for therapeutic intervention in LE.

Keywords: Aryl hydrocarbon receptor · Drug-induced lupus · Interleukin-22 · Propranolol · Ultraviolet light

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Introduction

UV light exposure, pollutants, and numerous pharmaceutical drugs are typical environmental triggering factors for the development of autoimmune diseases, with lupus erythematosus (LE) often being cited as the prototypical disease in this respect [1, 2]. Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the presence of anti-nuclear autoantibodies (ANAs) [3] and inflammation across a large spectrum of organs, while cutaneous lupus erythematosus (CLE) is a form of the disease in which the skin is the first, or only, affected organ [1].

Photosensitivity in LE, defined as an abnormal reaction of the skin to sunlight, is a major feature of most forms of LE that constitutes one of the eleven criteria the American College of Rheumatology uses for the classification of SLE [4]. Lupus patients develop skin rashes and show exacerbations of the cutaneous and systemic manifestations of the disease after exposure to sunlight which account for the seasonal variation of disease activity [5–7]. One of the mechanisms that has been put forward to explain the abnormal photoreactivity in lupus patients is the generation of apoptotic bodies and the translocation of several nuclear antigens, such as SSA/Ro, SSB/La, and Sm, to the cell membrane after exposure to UV light, resulting in presentation of autoantigens and subsequent release of various proinflammatory cytokines [5, 8]. Furthermore, several drugs, such as procainamide, hydralazine, calcium channel blockers, and beta-blockers representing different pharmacological classes have been implicated in the induction of ANAs and, occasionally, in clinically apparent lupus [9, 10]. Drugs and their metabolites may play the role of haptons in drug-altered self-antigen mechanisms. They may also induce the disruption of central immune tolerance and nonspecific activation of T lymphocytes through a process of DNA hypomethylation, which causes the activation of several genes, such as those encoding LFA-1 [9, 11]. A common unifying mechanism underlying the immunopathologic effect resulting from exposure to these two seemingly different triggering factors, i.e. light and drugs, remains unknown.

The aryl hydrocarbon receptor (AhR) is a cytosolic, ligand-dependent, transcription factor that can be activated by structurally diverse synthetic, as well as naturally occurring, chemicals. AhR is normally inactive, bound to several cochaperones, and following ligand binding it translocates into the nucleus where it dimerizes with the AhR nuclear translocator, leading to changes in gene transcription. AhR plays a critical role in xenobiotic detoxification, in particular through the regulation of expression of several cytochrome P450 genes, including CYP1A1, CYP1A2, and CYP1B1 [12, 13]. Recently, this receptor has also been pointed out as playing a key and complex role in the regulation of immune responses [14, 15]. It was initially reported that AhR activation in mouse CD4+ T-cells by 6-formylindolo(3,2-b)carbazole (FICZ), a metabolite derived from tryptophan via UV or visible light exposure [16], increased the proportion of proinflammatory IL-17- and IL-22-secreting cells, referred to as Th17 cells, a lymphocyte population that is implicated in the pathogenesis of numerous autoimmune diseases [17, 18]. Although AhR engagement by FICZ or β-naphthoflavone in human T-cells was found to inhibit IL-17 production [19, 20], it also resulted in an increase in the production of IL-22, giving rise to the generation of so-called Th22 cells that are highly inflammatory in the skin [19–23]. The results from experimental mouse models indicate that AhR-mediated signaling could be linked to autoimmunity, in particular in response to environmental toxics, as well as UV light-induced ligands. However, proof for a role of the AhR in human autoimmune pathologies is lacking. Here, we report that, similar to FICZ, the nonselective β-blocker propranolol, a drug capable of inducing newly positive ANAs in about 10% of patients [24], as well as its photoproducts, augment AhR signaling and induce the secretion of proinflammatory cytokines that contribute to the pathogenesis of this human autoimmune disease.

Results

Th17 and Th22 cell expansion in SLE patients

Th17 and Th22 cells, defined as CD4+ lymphocytes secreting IL-17 and IL-22, respectively, were enumerated in the peripheral blood of patients and controls using flow cytometry (Fig. 1A and B). A significant increase in the median representation of these effectors in active SLE patients was observed, as compared with inactive patients and healthy controls (for IL-17-secreting CD4+ T-cells: 1.27% (0.21–4.39) versus 0.70% (0.17–6.47), p = 0.04; and 0.57% (0.19–1.22), p < 0.0001, respectively; for IL-22-secreting CD4+ T-cells: 0.15% (0.03–1.09) versus 0.10% (0.01–0.74), p = 0.01; and 0.07% (0.02–0.25), p = 0.0004, respectively). This augmentation was not typically found in T-cells from the other systemic autoimmune diseases included in this study, primary Sjögren syndrome and primary antiphospholipid syndrome (Fig. 1B). IL-17- and IL-22-secreting CD4+ T-cell proportions were not significantly different in patients treated with high dose prednisone or immunosuppressive drugs, as compared with the other SLE patients (data not shown).

SLE patients express high levels of CYP1A1 transcripts following stimulation with FICZ

As the AhR pathway is implicated in Th17 and Th22 lymphocyte differentiation, we next assessed AhR responsiveness by PBMC from SLE patients following stimulation with FICZ. It is of note that expression levels of AhR transcripts were similar between SLE patients and healthy controls (Supporting Information Fig. 1). The PBMC response was evaluated by determining the magnitude of CYP1A1 mRNA expression by real-time quantitative PCR [25, 26]. Results from dose-response experiments showed that SLE patients expressed higher levels of CYP1A1 transcripts following activation, as compared with healthy donors (Fig. 2A). This led us to suggest that the AhR pathway might be implicated in
lupus-associated photosensitivity or drug-induced lupus. In order to determine whether these results reflect an intrinsic AHR hypersensitivity, we focused on IL-17 and/or IL-22-producing T-cells that both express AhR and CCR6 receptors [19]. The capacity of FICZ to induce CYP1A1 transcripts in the latter cells was comparable between SLE patients and healthy donors (Fig. 2B), suggesting that higher levels of CYP1A1 expression in SLE patients are likely to be associated with increased numbers of circulating IL-17 and/or IL-22-secreting cells (Fig. 1B), rather than with a hyper responsive state.

AhR agonist activity of some lupus-inducing drugs is potentiated by UV light exposure

Since medication and UV light both represent typical triggering factors of lupus, a panel of lupus-inducing drugs was evaluated for its capacity to induce CYP1A1 mRNA expression in PBMC, prior to or after UV-irradiation. Of note, hydralazine, procainamide, and quinidine, drugs associated with the highest risk of lupus induction [9], are no longer easily available in France and were therefore not tested. Some molecules behave as AhR agonists, especially after their exposure to UV-C light (Fig. 3A). Propranolol, a β-blocker drug, acquired the strongest AhR-activating profile following UV-irradiation. As interindividual variation of AhR responsiveness is described in humans [27], we confirmed in a series of healthy control subjects that UV-C light exposure significantly enhanced AhR agonist activity of propranolol solutions (Fig. 3B). Native propranolol induced stronger AhR activation in SLE patients than in controls (Fig. 3C), although its effects were less pronounced, as compared with the UV-C light-exposed drug. Many molecules belonging to the β-blocker family and structurally related to propranolol have been clearly involved in inducing ANAs and, sometimes, clinically apparent drug-induced lupus [9, 10]. Therefore, in the remainder of the study, we focused on propranolol and the identification of its bio-active photoproduct(s) induced by UV-irradiation.

UV light induces degradation of propranolol

MS full-scan analysis of protonated propranolol detected a major peak corresponding to the expected theoretical m/z of the precursor propranolol ion, i.e. m/z 260 (Supporting Information
T-cells from healthy controls (SLE) were identified by MS/MS analysis as hydroxypro-}

m/z = 259, 276, and 294 (Fig. 4A, B). The relative abundance of the different 4OHP\textsuperscript{a} photoproducts induced by either UV-C or UV-B light exposure is shown in Fig. 4E and F, respectively. In subsequent functional experiments, 4OHP\textsuperscript{a} and its degradation products, generated following its exposure to UV light, were used.

**Propranolol photoproducts are strong AhR agonists**

The 4OHP\textsuperscript{a}, and to a greater extent, UV-C light-exposed 4OHP\textsuperscript{a} (UV\textsuperscript{a} 4OHP\textsuperscript{a}) induced higher expression levels of CYP1A1 mRNA as compared with native propranolol in PBMC of healthy donors (Fig. 5A). These effects were reversed in the presence of CH-223191, a specific AhR antagonist, demonstrating the existence of a cascade of photoproducts with increasing AhR agonist properties, produced following exposure of propranolol to UV light. In order to confirm that 4OHP\textsuperscript{a} photoproducts are bona fide AhR agonists, the effect of UV-light-irradiated 4OHP\textsuperscript{a} was evaluated in an AhR gene reporter assay, involving activation of the xenobiotic responsive element (XRE). As shown in Fig. 5B, both UV-B and UV-C light-irradiated 4OHP\textsuperscript{a} induced GFP-coupled AhR activation in a dose-dependent manner in HepG2 cells, transfected with a XRE-responsive GFP reporter, but not with a noninducible GFP reporter plasmid.

**Propranolol photoproducts induce human T-cells and macrophages to secrete proinflammatory cytokines**

To compare the effect of UV light-induced propranolol derivatives on AhR-mediated cytokine production with that of the canonical AhR ligand FICZ, naive cord blood CD4\textsuperscript{+} T-cells from healthy donors were differentiated under Th17-polarizing conditions, in the absence or presence of FICZ or UV\textsuperscript{a} 4OHP\textsuperscript{a}, and the cytokine production profile was determined. Hierarchical clustering analysis of the cytokine measurements obtained in culture supernatants after 4 days of activation showed that T-cell samples stimulated with FICZ and UV\textsuperscript{a} 4OHP\textsuperscript{a} cosegregate in a single group that is clearly distinct from samples differentiated in the absence of these drugs (Fig. 6A). Both AhR ligands induced a similar cytokine production profile characterized by an increased production of IL-22, IL-8, IFN-γ, GM-CSF, CCL2, and CCL5. Using bivariate analysis, it is shown that the production of each of these cytokines, except CCL5, was significantly increased by the two photoproducts (Fig. 6B). Cytokine fluorometric analysis furthermore confirmed that the proportion of IL-8- and IL-22-secreting CD4\textsuperscript{+} T-cells increased upon FICZ and UV\textsuperscript{a} 4OHP\textsuperscript{a} exposure in vitro (Supporting Information Fig. 3 and Fig. 6C). Of note, both AhR ligands induced a significant decrease in the expression of CCL4 and IL-10 (Fig. 6B).

AhR is also expressed in macrophages, a cell type that plays an important role in organ damage and in the autoimmune response associated with lupus [28], we therefore evaluated the effect of
AhR agonist activity of some lupus-inducing drugs is potentiated by UV light exposure. (A) PBMCs from a healthy subject were stimulated for 6 h with 0.3 μM FICZ or 30 μM β-naphthoflavone or the indicated drugs, with or without prior exposure to UV-C light, and CYP1A1 mRNA expression was determined using RT-qPCR. ACTB was used as the endogenous gene reference. Results are shown as mean ± SD of two replicates. (B) PBMCs from healthy subjects (n = 17) were cultured for 6 h in the presence of 30 μM propranolol or UV-C light-exposed propranolol and CYP1A1 mRNA expression was determined using RT-qPCR with ACTB as the endogenous gene reference. Lines show mean values. Statistical analysis was performed using the paired t-test. (C) PBMCs from healthy donors (n = 9) and SLE patients (n = 11) were stimulated for 6 h with 30 μM propranolol. CYP1A1 mRNA expression was determined using RT-qPCR. ACTB was used as the endogenous gene reference. Statistical analysis was performed using the Wilcoxon matched pairs signed ranks test. (A–C) Data pooled from two independent experiments.

AhR activation in lesional tissue correlates with IL-22, IL-8, and CCL2 expression

We next assessed whether the cytokines whose secretion was induced in vitro by AhR activation were also detectable in situ in skin lesions of spontaneous lupus patients. Of note, drug-induced lupus is rare and skin samples of patients with this disorder were not available. Using real-time PCR, expression of IL-22 and CYP1A1 transcripts was found to be significantly increased in skin biopsies from lupus patients, as compared with healthy controls (p = 0.003 and p < 0.001, respectively), while IL-17 mRNA expression was comparable (Fig. 7A). IL-22 mRNA expression correlated positively with that of CYP1A1 (p = 0.01; r = 0.57) (Fig. 7B). In keeping with the results from in vitro experiments, IL-8 and CCL2 mRNA expression levels were also significantly increased in lupus skin lesions, as compared with those measured in the skin of healthy controls (p = 0.04 and p = 0.006, respectively) (Fig. 7A). Moreover, the expression of both transcripts correlated positively with that of CYP1A1 (p = 0.009; r = 0.62, and p = 0.0002; r = 0.78, respectively) (Fig. 7B). Altogether, we conclude that in situ IL-22, CCL2, and IL-8 expression is increased in concurrence with signs of AhR activation at the site of inflammation.
Figure 4. Mass spectrum analysis of UV light-exposed propranolol. (A) Full-scan analysis of protonated propranolol in an ion trap instrument following positive electrospray after exposure to UV-C light. Enlarged inset shows the mass spectrum between \( m/z \) 270 and 300. (B) Relative abundance of UV-C light-exposed propranolol derivatives after 12 h of exposure. The major peak at \( m/z \) 260 corresponds to the precursor propranolol ion. (C) Relative abundance of UV-B light-exposed propranolol derivatives after 6 h of exposition. The major peak at \( m/z \) 260 corresponds to the precursor propranolol ion. (D) Full-scan analysis of protonated 4-OHpropranolol after exposure to UV-C light. (E) Relative abundance of UV-C light-exposed 4-OHpropranolol derivatives after 48 h of exposition. The major peak at \( m/z \) 276 corresponds to the precursor 4-OHpropranolol ion. (F) Relative abundance of UV-B light-exposed 4-OHpropranolol derivatives after 6 h of exposition. The major peak at \( m/z \) 276 corresponds to the precursor 4-OHpropranolol ion. The precursor ion abundance of propranolol (B, C) and 4-OHpropranolol (E, F) was arbitrary set to 100. (A–F) Data are representative of three independent experiments. (B, C, E, and F) Data are shown as the mean \( \pm \) SD.
Figure 5. AhR agonistic properties of propranolol photoproducts. (A) PBMCs from healthy donors \( (n = 3) \) were stimulated for 6 h in the presence of 3 \( \mu \)M propranolol or 4-OHpropranolol, prior to and after UV-C light exposure, in the presence or absence of 3 \( \mu \)M of the specific AhR antagonist CH-223191. CYP1A1 mRNA expression was determined using RT-qPCR and ACTB was used as the endogenous reference. Data are representative of two independent experiments. Results are shown as the mean \( \pm \) SD and statistical analysis was performed using the Wilcoxon matched pairs signed ranks test. (B) HepG2 cells were transfected with a XRE-responsive GFP reporter and a non-inducible GFP reporter as a negative control. After 24 h of transfection, cells were treated with FICZ (30 nM), UV-B, and UV-C light-exposed 4OHP\(_d\) at 30, 10, and 3 \( \mu \)M and chlorpromazine (CPMZ) at 30 \( \mu \)M for 6 h. Cells were monitored for GFP expression by flow cytometry and the percentage of GFP-positive cells are shown. Data are representative of three independent experiments.

Discussion

As some of the best-known environmental factors that trigger lupus flares are sustained exposition to various xenobiotics, such as drugs [9, 10] and certain environmental pollutants [29–33], we postulated, as suggested by others [34], that the emergence of lupus flares might be associated with the activation of the AhR, a ligand-dependent transcription factor implicated in the xenobiotic detoxification and the regulation of immune responses [14, 35]. In the present study, we show that exposure to UV light of propranolol, a drug that can sometimes induce or aggravate lupus, strongly increased its capacity to activate the AhR and that its downstream photoproducued a proinflammatory AhR signaling pathway, identical to that induced by the natural, UV light-induced tryptophan metabolite FICZ, resulting in a cytokine secretion profile that could also be detected in vivo in patients with spontaneous LE. It can therefore be envisaged that AhR activation plays a role in some drug-induced lupus.

Ligands of the AhR are xenobiotics, not commonly synthesized by living organisms, which typically belong to the family of halogenated aromatics hydrocarbons, including dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and several other families of nonhalogenated synthetic chemicals [36, 37]. Many endogenous molecules and dietary compounds can also directly activate the AhR, like metabolites and UV light photoproduced of tryptophan and other, weaker, ligands, including certain metabolites of arachidonic acid and heme [36, 37]. In the case of drug-induced lupus, the AhR ligand could either be the drug itself or one of its metabolites induced by enzymes or UV exposure. Of note, the 4OHP\(_d\) derivative that we identified after UV light exposure is also a common metabolite produced after enzymatic degradation of propranolol in vivo [38, 39]. Among the different lupus-inducing drugs that we tested, only few had AhR agonistic activity. Yet, only native molecules could be assessed in the experimental protocols because most drug metabolites remain unknown and are not available for testing in vitro.

Whether the photoproducued of propranolol, 4OHP\(_d\), is actually immune-active in vivo needs to be determined, as the concentrations that such liposoluble compounds could reach in the skin or lymphoid tissues are presently unknown. Because the concentrations of 4OHP\(_d\) used in our in vitro experiments exceed by only 3–30 fold the usual propranolol concentrations documented in plasma following oral administration [40], it can be assumed that the results obtained in this in vitro study could be clinically relevant.

In addition to a direct effect of AhR activation on immune activation, other, indirect, pathways leading to autoimmunity can be envisaged. In this respect, it has been reported that lupus-inducing drugs could act as haptens in the context of hapten-neoantigen formation or, alternatively, might induce the exposition of cryptic self-antigens [41, 42]. Both processes are mediated by xenobiotic metabolizing enzymes and could be enhanced by ligand-mediated AhR activation.

It can be envisaged that AhR activation might not only play a role in drug-induced lupus, but also in the much more common, so-called spontaneous, forms of the disease. In spontaneous lupus, the reported increase in anti-dsDNA antibodies levels and disease activity occurring after sunlight exposure [7] could be explained by the production of AhR ligands induced by UV light. Indeed, UV irradiation or visible light exposure of tryptophan generates compounds with high affinity for AhR, among which FICZ is one of the molecules with the strongest AhR agonistic properties [37]. In this
Figure 6. Cytokine and chemokine profiles induced by FICZ and UV light-exposed 4-OHP\textsuperscript{ol}. Naive CD4\textsuperscript{+} T-cells were isolated from healthy cord blood (n = 7) and cultured with anti-CD3/anti-CD28 mAbs, rhTGF-\beta\textsubscript{1}, rhIL-1\beta and rhIL-23 (Ct–1 to –7), in the presence of 0.3 μM FICZ (FICZ–1 to –7) or 3 μM UV-C light-exposed 4OH\textsuperscript{ol} (4OH–1 to –7). The cytokine concentrations were measured in the supernatant after 4 days of culture by multiplex immunoassay and ELISA. (A) Hierarchical clustering of the samples of each of the three conditions according to cytokine concentrations. The scale extends from minimum (green) to maximum (red) values. Individual samples are listed at the top. (B) Statistical analysis of cytokine production using the Wilcoxon matched pairs signed ranks test. (C) Representative analysis of intracellular IL-22 and IL-8 production by CD4\textsuperscript{+} T-cells after 4 days of culture under Th17 lymphocyte differentiation conditions in the presence or absence of FICZ or UV-C light-exposed 4OH\textsuperscript{ol} (UV-C\textsuperscript{+} 4OH\textsuperscript{ol}). Percentages of cytokine-expressing cells are indicated. (D) Cytokine production by in vitro-differentiated macrophages obtained from healthy donors (n = 12), cultured for 6 days in the presence of 400 U/mL GM-CSF and incubated for 48 h with 0.3 μM FICZ or 3 μM UV-C 4OH\textsuperscript{ol}. (A, B) Data are pooled from seven independent experiments. (C, D) Data are pooled from two independent experiments. Statistical significance was determined by Wilcoxon matched pairs signed ranks test. *p < 0.05; **p < 0.01; ***p < 0.001; ns non-significant; Ct = control; Dg = drug.
respect, there is evidence that the synthesis of several related tryptophan photoproducts, along with the expression of the CYP1A1 gene occurs in the skin after UV or sunlight exposure [43, 44]. Sunlight-mediated induction of CYP1A1 activity has already been reported in human lymphocytes [27]. Yet, as all individuals are commonly exposed to AhR agonists of various kinds, it is unlikely that this exposure per se would lead to autoimmunity.

Our results suggest that the higher levels of CYP1A1 expression in PBMC of SLE patients are associated with increased numbers of circulating Th17/Th22 cells that might facilitate the initiation of the inflammatory process by AhR agonists. Numbers of cytokine-producing peripheral blood T-cells were determined following stimulation of the cells with anti-CD3 and anti-CD28 mAbs, rather than the frequently used combination of phorbol esters and calcium ionophore. Although this protocol results in lower numbers of IL-17 and IL-22-secreting cells, it does reflect a more physiological mode of cell activation and the observed difference in the proinflammatory T-cell frequencies, statistically significant albeit small, might have biological relevance. It is of note that the variability in the patients’ response certainly reflects the observed heterogeneity of the lupus disease.

The AhR signaling pathway is involved in the regulation of immune responses via its capacity to favor the differentiation of Th17 cells in mice [17] and IL-22-producing cells in human [19, 20]. We confirm in the present study that AhR stimulation with FICZ drives human CD4+ T lymphocyte differentiation toward a proinflammatory response via the induction of cells producing IL-22, without enhanced IL-17 secretion, and furthermore show that propranolol photoproducts have the same capacity. Our results also corroborate those showing that human in vivo exposure to another environmental AhR ligand, TCDD, induces a selective increase in the frequency of Th22 cells without affecting Treg and T-cells producing IL-17, IL-10, and IFN-γ [45]. Finally, also in macrophages, a cell type involved in the induction of organ damage and autoimmune responses associated with lupus [28], FICZ and propranolol photoproducts were found to increase the production of several cytokines that play an important role in the onset of proinflammatory immune responses, such as IL-8, CCL2, GM-CSF, and G-CSF.

In the skin, activation of the AhR is strongly correlated with cutaneous expression of IL-22, IL-8, and CCL2 transcripts. The production of IL-8 and GM-CSF by T-cells differentiated in the presence of an AhR agonist is reminiscent to that of cutaneous T-cells mediating neutrophilic inflammation in drug-induced and autoimmune diseases, such as exanthematous pustulosis, pustular psoriasis, and Behçet’s disease [46, 47]. More recently, IL-8 was reported to be produced by Th17 clones [48]. The results from the present study confirm the capacity of T-cells to produce IL-8 and extend this notion by showing that its secretion can be increased as a result of AhR stimulation. Although the role of neutrophils in the pathogenesis of SLE is debated [49], the presence of IL-8 in the cutaneous lesions of patients with active disease points to the involvement of neutrophils, at least in certain stages of the disease. It is worth mentioning in this context that the exposure of autoantigens as observed in Lupus might not be limited to the involvement of apoptotic bodies, but also results from NETosis [49].

IL-22 is a key cytokine in the regulation of inflammatory responses, particularly in the skin and the gut, but the precise role of this cytokine in SLE pathogenesis is not known. At present, many authors place IFN-α, rather than IL-22, at the center of the immunologic abnormalities observed in lupus [50–52]. Recently, Bachmann et al. demonstrated that, upon incubation of ephe-"
Figure 7. Expression of IL-22, IL-8, and CCL2 in lupus skin lesions. (A) mRNA expression of IL-17, IL-22, and CYP1A1 (on a first series of biopsies) and of IL-8 and CCL2 (on a second series of biopsies) in the skin of healthy controls and lupus patients was determined using RT-qPCR. ACTB was used as the endogenous gene reference. (B) Statistical correlation between IL-22, IL-8, CCL2, and CYP1A1 mRNA expression was determined using the Spearman’s rank correlation coefficient. (A, B) Data are pooled from two independent experiments. Each dot represents an individual and lines show median values. Statistical analysis was performed using the Mann–Whitney U test.

Clinical features fulfilled the criteria of the European Community Study group in pSS patients [57]. The diagnosis of PAPS was made according to established criteria used [58] in the absence of associated autoimmune disease. Samples of LE-specific skin lesions were obtained from frozen 3 to 4 mm punch biopsies performed for diagnostic purposes on SLE and CLE patients. A first series of 19 biopsies (chronic CLE, n = 10; LE tumidus, n = 6; subacute CLE, n = 2; vasculitis, n = 1) and a second series of 17 biopsies (chronic CLE, n = 4; LE tumidus, n = 4; subacute CLE, n = 6; vasculitis, n = 1; bullous LE, n = 1; Chilblain LE, n = 1) were studied. A systemic form of LE was present in ten and eight patients for the first and the second series, respectively. The control group consisted of 37 healthy volunteers (median age 28 years [20–43], 26 women). The age distributions of the active SLE and PAPS patients were not different than those of the healthy controls (p = 0.68 and p = 0.10, respectively, using the nonparametric Mann–Whitney U test). Patients with inactive SLE and pSS were older than the healthy controls (p = 0.001 and p < 0.0001, respectively). Patients with inactive SLE were older than patients with active SLE (p = 0.01). Control skin biopsies (n = 7 for the first series and n = 9 for the second series) were obtained from healthy donors undergoing plastic surgery. The study was approved by the local ethics committee. Informed consent was obtained from all participants. This research has been conducted in accordance with the Declaration of Helsinki and its subsequent amendments.

Cell isolation and flow cytometry

All blood samples were processed within 4 h of collection. PBMCs were purified by centrifugation over Ficoll–Paque (Eurobio, Les Ulis, France). The cytokine-producing capacity of CD4+ T-cells was assessed as described in reference [59].

Purification of human CD4+CCR6+ T-cells

CD4+ T-cells were isolated from blood of SLE patients and healthy controls, using the RosetteSep Human CD4+ T-cell enrichment kit (StemCell Technologies) and stained with PE mouse anti-human CD196 (CCR6) antibody (BD Biosciences). CD4+CCR6+ T-cells were labeled with anti-PE Microbeads and enriched magnetically using MS MACS columns (both from Miltenyi Biotec). Cells (65–75% purity) were seeded at 5 x 10⁵/mL in a 24-well plate and

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activated with Dynabeads Human T-Activator CD3/CD28 (Invitro- gen Life Technologies) at a 1:1 ratio in the presence of 20 U/mL rIL-2 (Roche Diagnostics, GmbH, Mannheim, Germany). After 10 days of culture, CD4^+CCR6^+ T-cells were enriched once more as mentioned above and 5 × 10^5 cells/mL (90%-99% purity) were stimulated with FICZ at 30 and 300 nM or the same vehicle dilutions (DMSO). After 6 h of incubation, supernatant was removed and cell pellets were processed for RNA extraction.

**Reagents**

Drugs were purchased from pharmaceutical companies. D- penicillamine (Dexolaboratoire), quinine (AGEPS-EPHP), propranolol (AstraZeneca), trimethoprim-sulfamethoxazole (TMP/SMX) (Roche), and isoniazide (Pharmion) were diluted in PBS. Acebutolol (Sanofi-Aventis France), phenytoin (Laboratoires Genopharm), sulfasalazine (Pharmacia S.A.S.), omeprazole (AstraZeneca), propylthiouracile (AGEPS-EPHP), carbamazepine (Novartis Pharma S.A.S.), methylodopa (Merck & Company Inc), chlorpromazine (Sanofi-Aventis France), and minocycline (Sandoz) were diluted in DMSO. 6-formylindolo(3,2-b) carbazole (FICZ, #BML-GR206-0100) was purchased from Biomol (Sandoz) were diluted in DMSO. 6-formylindolo(3,2-b) carbazole (FICZ, #BML-GR206-0100) was purchased from Biomol International (Enzo life sciences, Villeurbanne, France); β-Naphthoflavone (#N3633), CH-223191 (#C8124), β-estradiol (#E4389), and propranolol hydrochloride (#P0884) from Sigma (St. Louis, MO, USA); and 4-hydroxypropranolol (4OHP^2+) (#sc-210061) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

For the drug screening, UV-C irradiation was performed on 30 μM drug solutions for 6 h with a 30-W low-pressure mercury germicidal lamp (predominantly 254 nm; model TUV; Philips Electronic Instruments Inc.), resulting in an irradiation of 100 J/cm^2_. Thereafter, the irradiation of propranolol and 4OHP was performed at 3–6 mM for 12h, except for the mass spectrum analysis of 4OHP^2+ where the molecule was irradiated at 30 mM for 48 h. UV-B irradiation of propranolol and 4OHP was performed at 3 and 6 mM, respectively, with two broad-band UV-B lamps (280–350 nm, model UV21, 15-Watt, Waldmann Eclairage SAS, Reichstett, France), for 6 h resulting in an irradiation of 200 J/cm^2_.

**cDNA synthesis and real-time quantitative PCR**

RNA extraction from cell pellets was performed with a NucleoSpin 96 RNA kit, following the manufacturer's instructions (Macherey-Nagel, Hoerdt, France). cDNAs were synthesized with the Reverse Transcriptase Core Kit and the real-time quantitative PCR (RT-qPCR) assays were performed with the MESA GREEN qPCR MasterMix Plus for SYBR® Assay (both from Eurogentec, Seraing, Belgium). RT-qPCR analysis on skin biopsy cDNAs was performed as described in reference [59] using gene specific primers listed in Supporting Information Table 1. The efficiency of the PCR for each individual target was between 90 and 100% as evaluated in [60]. Relative transcript quantities were calculated as ΔCT values with β-actin (ACTB, GenBank accession No NM_001101.3) and ribosomal protein S9 (RPS9, GenBank accession No NM_001013) as endogenous gene references.

**Mass spectrometry**

The ion trap mass spectrometer HCTultra (Bruker Daltonics, Wissembourg, France) was operated in positive electrospray ionization and the scan modes used were MS, MS/MS, and MS(3). Helium was used for collision-induced dissociation and fragmentation amplitude ranged from 30 to 200% in 40 ms. The scan intervals used in each particular MS(n) experiment are given in the figure captions. Compounds were identified using MassBank Database Service (http://www.massbank.jp) [61] and results reported in reference [62].

**XRE reporter assay**

The Xenobiotic Response Element (XRE) Reporter kit (Qiagen, Coutaouboue, France) was used to monitor the activity of AhR signaling in human cells according to the manufacturer's instructions. HepG2 cells (kindly provided by Dr O. Silvie, CIMI-Paris) were transfected in 24-well plates, using 0.5 μg Cignal XRE-GFP reporter or negative control and 1 μL JetPEI (Polyplus-transfection SA, Illkirch, France). After 24 h of transfection, cells were treated with the indicated concentrations of drugs for 6 h. Chlorpromazine was used as a negative ligand control. GFP expression was monitored by flow cytometry on FACSCan® (BD Biosciences).

**AhR activation**

Fresh PBMCs were seeded in duplicate at 5 × 10^5 cells per well in a 96-well U-bottom plate (BD Falcon^TM) and stimulated as mentioned above with plate-bound anti-CD3 (10 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs. After 16 h of culture, cells were spun, supernatant was removed and 100 μL of drug dilutions in culture medium were added. After 6 h of incubation, cells were collected, spun for 7 min at 1200 rpm, supernatant was removed and cell pellets were processed for RNA extraction.

**Cell culture and cytokine detection assays**

Human primary CD4^+ T-cells were isolated from healthy cord blood (75–80% purity), using the RosetteSep™ Human CD4^+ T-Cell Enrichment kit (StemCell Technologies, Grenoble, France) and were cultured in duplicate with plate-bound anti-CD3 (10 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs in supplemented RPMI 1640 under Th17 cell-polarizing conditions consisting of rhTGF-β1 (Peprotech, Neuilly-Sur-Seine, France; 0.5 ng/mL), rhIL-17 (R&D systems, Lille, France; 10 ng/mL) and rhIL-23 (R&D
systems; 10 ng/mL) alone or in the presence of FICZ (0.3 μM) or UV light-exposed 4-hydroxypropranolol (UV+4OHP; 3 μM). After 4 days of culture, supernatants were collected to be stored at −80°C before analysis for cytokine levels and CD4+ T-cells (>97% purity) were incubated with monensin (GolgiStop™, BD Biosciences) for 16 h before staining for intracellular IL-8 and IL-22 (APC-conjugated anti-IL-8 mAb, clone S8H1 and eFluor660-conjugated anti-IL-22 mAb, clone 22URTI, both from eBioscience).

Cytokine content was determined by LumineX technology, using Invitrogen cytokine human 25-plex panel kit, following the manufacturer's instructions (Life technologies). IL-22 expression was analyzed by ELISA (Human IL-22 DuoSet, R&D Systems).

For macrophage differentiation, PBMCs of healthy donors (n = 12) were incubated on bovine gelatin (Sigma)-coated culture flasks (BD Falcon™) in RPMI-10% FCS at 37°C 5% CO2. After 45 min of incubation, nonadherent cells were discarded, adherent cells were washed twice, detached with Versene (Invitrogen), counted, seeded in 96-well U-bottom plates at a density of 5 × 10⁴ cells/well and cultured in RPMI-10% FCS in the presence of 400 U/mL hGM-CSF (Miltenyi Biotec, Paris, France) in a total volume of 100 μL. After 6 days, culture supernatants were discarded and macrophages were cultured in 200 μL fresh medium alone or in the presence of FICZ (0.3 μM) or UV+4OHP (3 μM). Cytokine content was determined by LumineX technology using Millipore MILLIPLEX MAP human cytokine/chemokine—premixed 26 plex following the manufacturer's instructions (Merck-Millipore, Molsheim, France).

**Statistical analysis**

Values for quantitative variables were expressed as the mean ± SD or as the median and range. Differences between groups were tested using the nonparametric Mann–Whitney U test and the Wilcoxon matched pairs signed ranks test or the parametric paired and unpaired t tests when appropriate. Correlations were calculated using Spearman's rank correlation coefficient. For cytokine analysis, samples with nondetectable values were replaced with zero while those over the detection range were replaced by the detection limits for all samples. In order to compare the relative variation, but not the absolute expression, of cytokine levels between the three experimental conditions, cytokine expression values were normalized for each cytokine and each patient. Hierarchical clustering based on the euclidian distance and Ward's method was used to delineate subgroups of culture conditions based on their cytokine profile. For the cultures of macrophages, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-15, IL-17A, IFN-γ, CXCL10, and TNF-β were below the detection limits for > 66% of the samples and were excluded from further analysis. Statistical analysis was performed using GraphPad Prism, version 5.0 software (GraphPad Software, San Diego, CA, USA) and JMP8 (SAS institute, Cary, NC, USA). All tests were 2-sided and a p value < 0.05 was considered statistically significant.

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**References**

Production of interleukin 22 but not interleukin 17 by a subset of human populations of human IL-17 and/or IL-22 T-cell producers that share a receptor reveals distinct requirements for IL-22 and IL-17 production by TH17 cells. Immunity. 2009. 10: 864–871.


human CD4+ T cells in an aryl hydrocarbon receptor (AhR)-dependent manner. PloS One 2011. 6: e18741.


Abbreviations: AhR: aryl hydrocarbon receptor · CLE: cutaneous lupus erythematosus · FICZ: 6-formylindolo(3,2-b)carbazole · 4OHPr: 4-hydroxypropranolol · HCQ: hydroxychloroquine · LE: lupus erythematosus · SLE: systemic lupus erythematosus · SLEDAl: SLE disease activity index · TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin · UV*4OHPr: UV-light exposed 4-hydroxypropranolol

Full correspondence: Prof. Guy Gorochov, Département d’immunologie, Inserm, UMR-1135, CERVI, Groupement Hospitalier Pitié-Salpêtrière, 83 boulevard de l’hôpital, 75013, Paris
Fax: +33-1-42-17-74-90
e-mail: guy.gorochov@upmc.fr

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