

Reciprocal regulation of interleukin-17A and interleukin-22 secretion through aryl hydrocarbon receptor activation in CD4⁺ T cells of patients with vitiligo

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Abstract. Previous studies have shown the participation of the cytokines interleukin (IL) 17A and IL22 in the development of vitiligo. The aryl hydrocarbon receptor (AhR) functions in the pathogenesis of vitiligo and can modulate cytokine production. The aim of the present study was to determine the relationship between AhR activation and the secretion of IL17A and IL22 in CD4⁺ T cells in vitiligo. A total of 20 newly diagnosed patients with progressive, unstable vitiligo and 20 healthy controls were recruited. CD4⁺ T cells and skin samples were collected. Immunohistochemistry, ELISA, reverse transcription-quantitative PCR, western blotting and RNA interference experiments were performed. The expression of AhR was significantly lower in the CD4⁺ T cells and skin, both lesional and nonlesional, of patients with vitiligo compared with healthy subjects. AhR expression was markedly lower in nonlesional compared with lesional skin of patients with vitiligo. The expression levels of IL17A and IL22 were significantly higher in patients with vitiligo compared with healthy subjects. Knockdown of AhR significantly increased the production of IL17A and markedly decreased IL22 levels in the CD4⁺ T cells of patients with vitiligo. *Ginkgo biloba* extract EGb 761 activated AhR, inhibited IL17A secretion and enhanced IL22 release in the CD4⁺ T cells of patients with vitiligo. In conclusion, reduced AhR expression is associated with progressive, unstable vitiligo. Activation of AhR with *G. biloba* extract EGb 761 may have therapeutic potential for decreasing IL17A levels and increasing IL22 levels in patients with vitiligo.

Introduction

Vitiligo is an acquired depigmentation disorder caused by the progressive destruction of melanocytes (1). Vitiligo pathogenesis involves defects that are intrinsic to melanocytes and the autoimmune targeting of these cells (2). Although cytotoxic CD8⁺ T cells serve as the effector arm in autoimmunity, CD4⁺ T helper cells and relevant cytokines appear to play important roles in the development of vitiligo (3).

Interleukin (IL) 17A is primarily expressed by the T helper (Th) 17 subset of CD4⁺ T cells, which is characterized by the expression of retinoic acid receptor-related orphan receptor (ROR) A and RORC genes (4). IL22 is an IL10-family cytokine that is produced by Th17, $\gamma\delta$ T, natural killer T (NKT), innate lymphoid and Th22 cells (5). Recent studies have shown the participation of IL17A and IL22 in the development of vitiligo (6-9). Studies have also demonstrated that systemic, tissue and cellular levels of IL17A and IL22 are elevated in vitiligo (10,11). Although IL17A is widely accepted to be involved in the pathogenesis of vitiligo, its role in the disease remains controversial. IL22 has opposing effects, from pro-inflammatory to protective, but it has been proposed that in acute inflammation IL22 is protective, while in more chronic settings it is pathogenic (12-14). Together, these data imply that modulation of IL17A and IL22 is beneficial in the treatment of progressive vitiligo and that these cytokines may represent an effective therapeutic target.

The aryl hydrocarbon receptor (AhR) is a cytosolic transcription factor expressed in many different cell types and is a member of the basic helix-loop-helix/Per-Arnt-Sim family (15). AhR activation, nuclear translocation and formation of a complex with the AhR nuclear translocator complex results in the regulation of a number of target genes (15). AhR activation is involved in multiple biological processes, including immune response, endocrine secretion and metabolism of low molecular-weight chemicals (16). Previous studies by the authors of the current study have shown that AhR activation is involved in immune dysregulation in CD4⁺ T cells (17) and inflammation in keratinocytes (18).

In the past decade, the role of AhR in the pathogenesis of vitiligo has generated significant interest. Studies have

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demonstrated that AhR expression is lower in vitiligo lesions compared with normal skin (19,20). Therefore, AhR activation has been suggested as a potential therapy for vitiligo (21). The traditional herbal supplement *Ginkgo biloba* (*G. biloba*) is used for its purported health benefits. It has been suggested to have beneficial effects on senile dementia, peripheral arterial occlusive disease and on various neurosensory disturbances (22). There are multiple reports of promising results for *G. biloba* in the treatment of vitiligo (23-26). EGb 761 is a standardized extract of *G. biloba* leaves, with 22-27% flavonoid glycosides (primarily quercetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B and C and 2.6-3.2% bilobalide) (27). However, whether *G. biloba* extract can modulate the AhR pathway and, therefore, the production of IL17A and IL22 in CD4⁺ T cells in vitiligo remains to be elucidated.

The aim of the present study was to investigate the expression of AhR and its role in the regulation of IL17A and IL22 in CD4⁺ T cells of patients with vitiligo. The effects of *G. biloba* extract on AhR activation and the expression of IL17A and IL22 in CD4⁺ T cells from patients with vitiligo were also studied.

Materials and methods

Subjects. Based on the revised vitiligo classification (1), 20 patients with progressive, unstable vitiligo (age, 16-58 years; mean age, 23 years; 14 females and 6 males) and 20 age- and sex-matched healthy controls (age, 18-60 years; mean age, 27 years; 14 females and 6 males) were recruited from patients at the Department of Dermatology, Shanghai General Hospital. Progressive, unstable disease was defined arbitrarily as the appearance of new lesions in the preceding 3 months. Clinical and demographic data were collected; two dermatologists reviewed all cases. Relevant clinical information regarding the study subjects is presented in Tables I and SI. The study was approved by the Human Ethics Committee of Shanghai Jiaotong University and written informed consent was obtained from each subject.

Isolation, culture, and treatment of CD4⁺ T cells. Blood samples (~15 ml) were collected from all participants. CD4⁺ T cells were purified by negative selection using RosetteSep Human CD4⁺ T Cell Enrichment Cocktail (StemCell Technologies, Inc.) followed by density gradient centrifugation using Histopaque (Sigma-Aldrich; Merck KGaA). The purity of CD4⁺ T cells (>95%) was evaluated by flow cytometry. Briefly, Peripheral blood mononuclear cells (PBMCs) were washed twice with 200 μ l ice-cold fluorescence-activated cell sorting (FACS) buffer, centrifuged at 4°C and 200 x g for 5 min and fixed with 100 μ l BD Cytotfix/Cytoperm (BD Biosciences) for 20 min at 4°C. PBMCs were then washed twice and resuspended in 100 μ l ice cold FACS buffer containing 1% FITC-conjugated anti-human CD4 Antibody (cat. no. 60016FI, Clone OKT4; StemCell Technologies, Inc.) and incubated at 4°C for 30 min. PBMCs were again washed twice, and CD4⁺ T cells were resuspended in 200 μ l FACS buffer. CD4⁺ T cell counts were performed by flow cytometry (Fortessa; BD Biosciences). Data were analyzed with FlowJo v10 (FlowJo LLC). Cells were cultured in X-VIVO 15 medium (Lonza Group,

Ltd.) supplemented with 10% human AB serum (Valley Biomedical Products and Services, Inc.) at 37°C under 5% CO₂. CD4⁺ T cells (1x10⁶ cells/well) were cultured in 24-well plates with plate-bound anti-CD3 (eBioscience; cat. no. 16-0037) and anti-CD28 (eBioscience; cat. no. 16-0289; both 2 μ g/ml). Where indicated, cells were treated with 7-aminoactinomycin D (7-AAD; BD Biosciences), *G. biloba* extract EGb 761 (Dr. Willmar Schwabe GmbH), AhR antagonist CH223191 (3 μ M; Sigma-Aldrich; Merck KGaA) or AhR agonist [4-(3-chloro-phenyl)-pyrimidin-2-yl]-(4-trifluoromethyl-phenyl)-amine VAF347 (50 nM; Sigma-Aldrich; Merck KGaA).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using the RNeasy Mini kit (Qiagen, Inc.). RT was performed using the PrimeScript RT-PCR kit according to the manufacturer's protocol (Takara Bio, Inc.). qPCR was performed on an Mx3000P qPCR system (Agilent Technologies, Inc.) using SYBR Premix Ex Taq (Takara Bio, Inc.). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 20 sec. Target gene expression was normalized to the housekeeping gene GAPDH and analyzed using the 2^{- $\Delta\Delta$ C_q} method (28). The primer pairs used for the qPCR are shown in Table SII.

Western blot analysis. CD4⁺ T cells were lysed and protein was extracted using the Complete Lysis-M reagent (Roche Applied Science). Protein concentration was measured using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (20 μ g) were dissolved in NuPage LDS Sample Buffer (Invitrogen; Thermo Fisher Scientific, Inc.) and 10% NuPage Sample Reducing Agent (Invitrogen; Thermo Fisher Scientific, Inc.). Protein lysates were boiled at 70°C for 10 min prior to loading. Electrophoresis was conducted on 4-12% NuPage Bis-Tris gels (Invitrogen; Thermo Fisher Scientific, Inc.) at 200 V for 40 min. The separated proteins were transferred onto PVDF membranes (Invitrogen; Thermo Fisher Scientific, Inc.) and blocked in TBS containing 2% BSA (Sigma-Aldrich; Merck KGaA) and 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA). Membranes were probed with the following primary antibodies: Anti-AhR mouse monoclonal IgG antibody (1:1,000; cat. no. ab2769; Abcam), anti-cytochrome P450 1A1 (CYP1A1) rabbit polyclonal IgG antibody (1:500; cat. no. ab3568; Abcam) or anti-GAPDH rabbit IgG antibody (1:50; cat. no. FL-335; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following primary incubation, membranes were incubated with anti-rabbit (1:5,000; cat. no. ab205718; Abcam) or anti-mouse (1:5,000; cat. no. ab205719, Abcam) horseradish peroxidase-conjugated IgG secondary antibodies for 2 h at room temperature. Protein bands were visualized using the WesternBreeze Chemiluminescent kit (Invitrogen; Thermo Fisher Scientific, Inc.).

Immunohistochemistry. Skin tissue specimens were obtained from the biopsies of lesion and nonlesion areas from vitiligo patients. Normal skin tissue specimens were obtained from healthy volunteers. All skin tissue samples were fixed with 10% buffered formalin for 1 day at room temperature. The paraffin-embedded tissue blocks were cut into 4- μ m tissue

Table I. Clinical characteristics of the subjects.

Characteristics	Vitiligo patients	Healthy controls
Number	20	20
Male/female ratio	6/14	6/14
Age, years	23.40±0.99	27.44±1.36
Skin type, n (%)		
III	15 (75)	17 (85)
IV	5 (25)	3 (15)
BSA involved, n (%)		
≤10%	4 (20)	N/A
>10%	16 (80)	N/A
Mucosa involved, n (%)	10 (50)	N/A
Hair involved, n (%)	6 (30)	N/A

BSA, body surface area; N/A, not applicable.

sections, deparaffinized using xylene for 10 min, rehydrated through a graded series of ethanol, followed by blocking of endogenous peroxidase activity in 0.3% H₂O₂ in methanol for 30 min at room temperature. Antibody-binding epitopes were retrieved by pressure-cooking the tissue sections in 10 mmol/l sodium citrate buffer (pH 7.0; LSI Medience Corporation) for 10 min. Non-specific binding was blocked using 10% goat serum (cat. no. 50062Z; Thermo Fisher Scientific, Inc.) for 10 min at room temperature. The sections were then incubated with anti-AhR antibody (1:100; cat. no. ab2769; Abcam), anti-CD4 antibody (1:100; cat. no. ab67001; Abcam), anti-IL17A antibody (1:100; cat. no. ab189377; Abcam) or anti-IL22 antibody (1:100; cat. no. ab134035; Abcam). Biotinylated anti-mouse antibodies (1:200; cat. no. SP KIT-C3, Fuzhou Maixin Biotech Co., Ltd.) were applied for 15 min in a humidified chamber at room temperature. A DAB kit (DAB-0031; Fuzhou Maixin Biotechnology Development Co., Ltd.) was used to generate chromogenic signals. PBS was used as the negative control.

Quantification. Quantification of immunohistochemical staining and western blotting bands was performed using Image J software (version 1.48 v; National Institutes of Health). For evaluation of immunohistochemical staining, the epidermis was selected as the region of interest (ROI) and the staining intensity of the ROI was quantified. The number of cells in the ROI was then counted. The relative staining intensity was defined as the intensity of 100 cells and calculated using the formula: Relative staining intensity (arbitrary units) = intensity of ROI/cell number x 100.

ELISA. IL17A and IL22 in serum and culture supernatants were measured using commercial immunoassay kits (cat. nos. ab83688 and ab119543; Abcam) according to the manufacturer's instructions.

Small interfering RNA (siRNA) transfection. Transfection with AhR-targeted specific siRNA was performed as previously described (13). Briefly, siRNA targeted against AhR

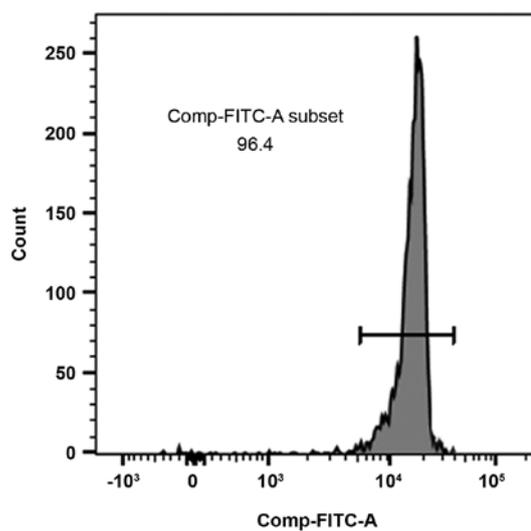


Figure 1. Representative flow cytometry plot showing the purity of the CD4⁺ T cells. CD4⁺ T-cell counts were performed by flow cytometry and the results showed that cell purity was >95%.

(si-AhR; cat. no. s1200) and siRNA consisting of a scrambled sequence (si-control; cat. no. AM4611) were purchased from Ambion (Thermo Fisher Scientific, Inc.). CD4⁺ T cells cultured in 24-well plates were incubated with 3 ml HiPerFect Transfection Reagent (Qiagen SAS) containing 10 nM siRNA and 0.5 ml culture medium. Following incubation for 48 h, siRNA-transfected CD4⁺ T cells were treated as indicated. Transfection showed no effect on cell viability, as demonstrated by microscopic examination (data not shown).

Cell viability. The 7-AAD cell viability assay was performed as previously described (29). Since 7-AAD is a fluorescent DNA intercalator that binds to double stranded DNA, live cells with intact membranes are identified by the exclusion of 7-AAD, which penetrates dead and damaged cells to label DNA.

Statistical analysis. Data are presented as the mean ± SEM. Data were analyzed using a Student's t-test or one-way ANOVA followed by Tukey's or Dunnett's multiple comparison tests. Relationships were determined by correlation analysis. All analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Identification and purity of CD4⁺ T cells. Flow cytometric analysis showed that the purity of the CD4⁺ T cells was >95% (Fig. 1).

AhR expression is low in the CD4⁺ T cells and the skin of patients with vitiligo. Since different reports have presented contradictory results (30,31), the expression of AhR in patients with vitiligo was assayed. AhR mRNA expression levels were significantly lower in the CD4⁺ T cells of patients with vitiligo compared with healthy subjects (Fig. 2A). To determine if the level of AhR expression was indicative of functional AhR protein, the expression of CYP1A1, a known AhR-regulated gene, was examined. Low expression of AhR mRNA was

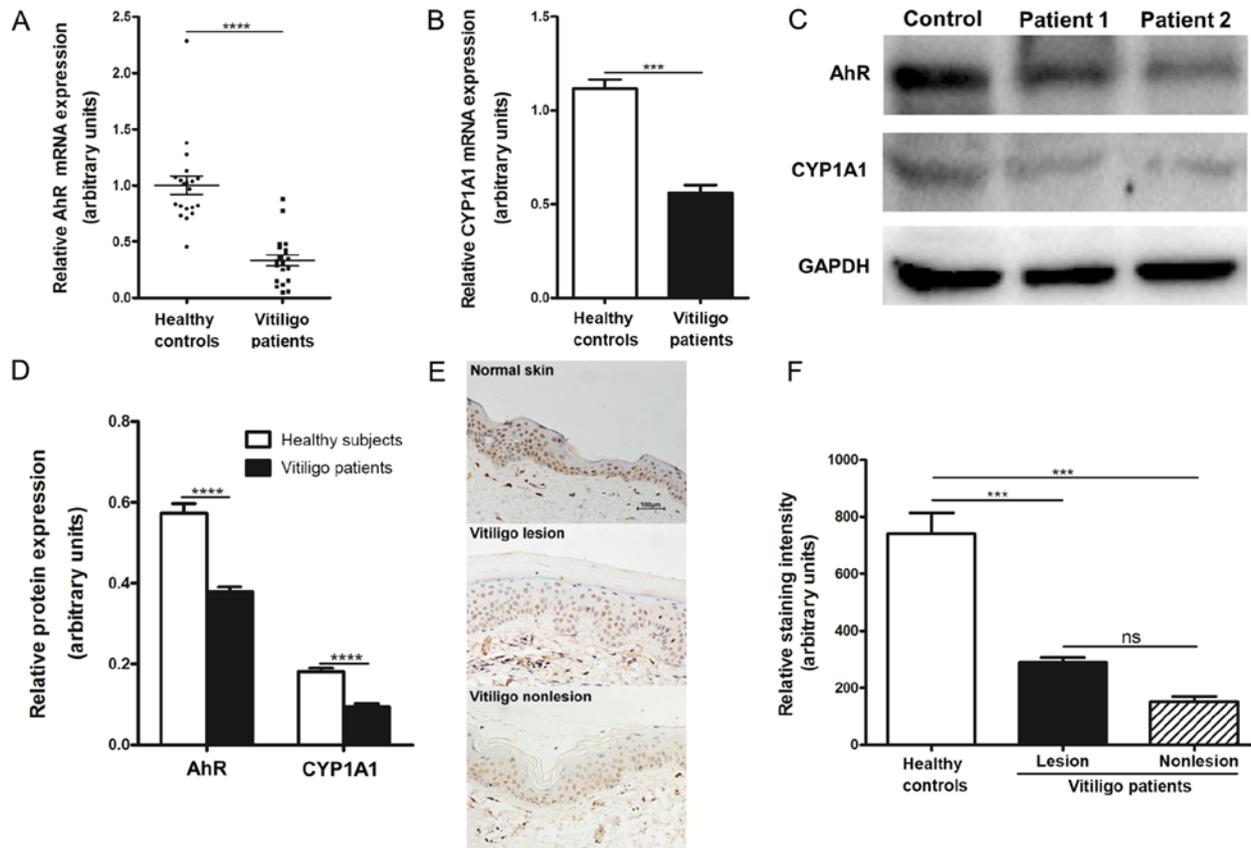


Figure 2. AhR expression is decreased in the CD4⁺ T cells and skin of patients with vitiligo. (A) AhR mRNA and (B) CYP1A1 mRNA levels in CD4⁺ T cells isolated from patients with vitiligo (n=20) and healthy controls (n=20). (C) Representative western blot and (D) quantitative analysis of AhR and CYP1A1 protein levels in CD4⁺ T cells isolated from patients with vitiligo (n=20) and healthy controls (n=20). (E) Representative immunohistochemical and (F) quantitative analyses of skin samples from patients with vitiligo (n=20) and healthy controls (n=10). Scale bar = 100 μ m. ***P<0.001 and ****P<0.0001. AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 1A1; ns, not significant.

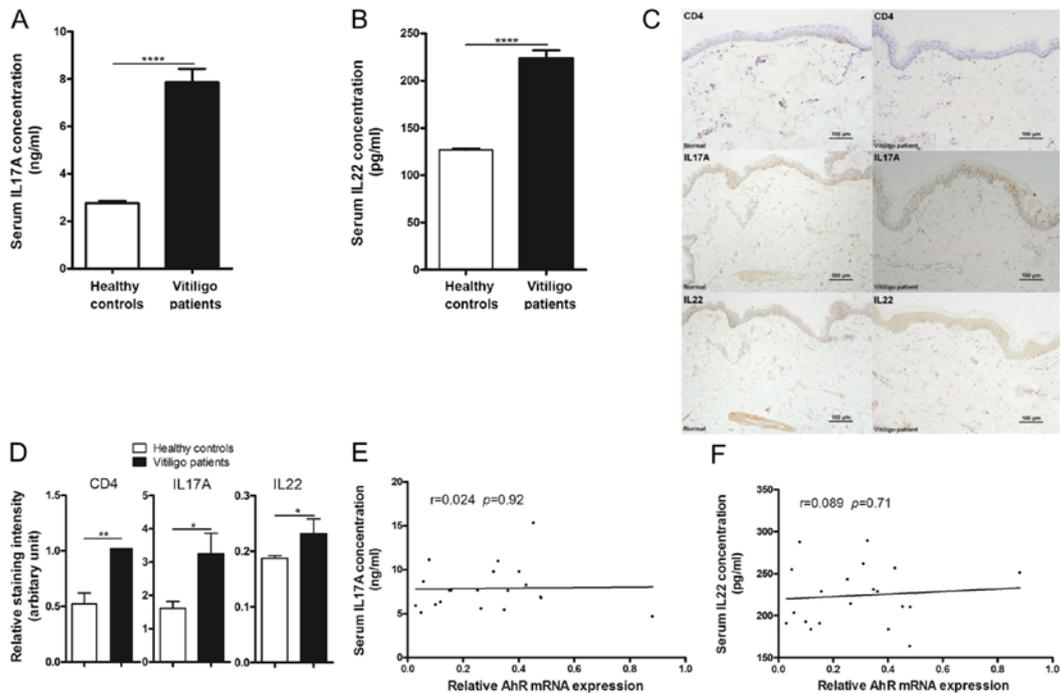


Figure 3. IL17A and IL22 production are upregulated in the serum of patients with vitiligo. Serum (A) IL17A and (B) IL22 measured by ELISA in peripheral venous blood samples collected from patients with vitiligo (n=20) and healthy controls (n=20). (C) Representative immunohistochemical and (D) quantitative analyses of skin samples from patients with vitiligo (n=20) and healthy controls (n=10). Scale bar = 100 μ m. Correlation between serum (E) IL17A levels and AhR mRNA levels in CD4⁺ T cells and (F) serum IL22 levels and AhR mRNA levels in CD4⁺ T cells of patients with vitiligo (n=20). *P<0.05, **P<0.01 and ****P<0.0001. IL, interleukin.

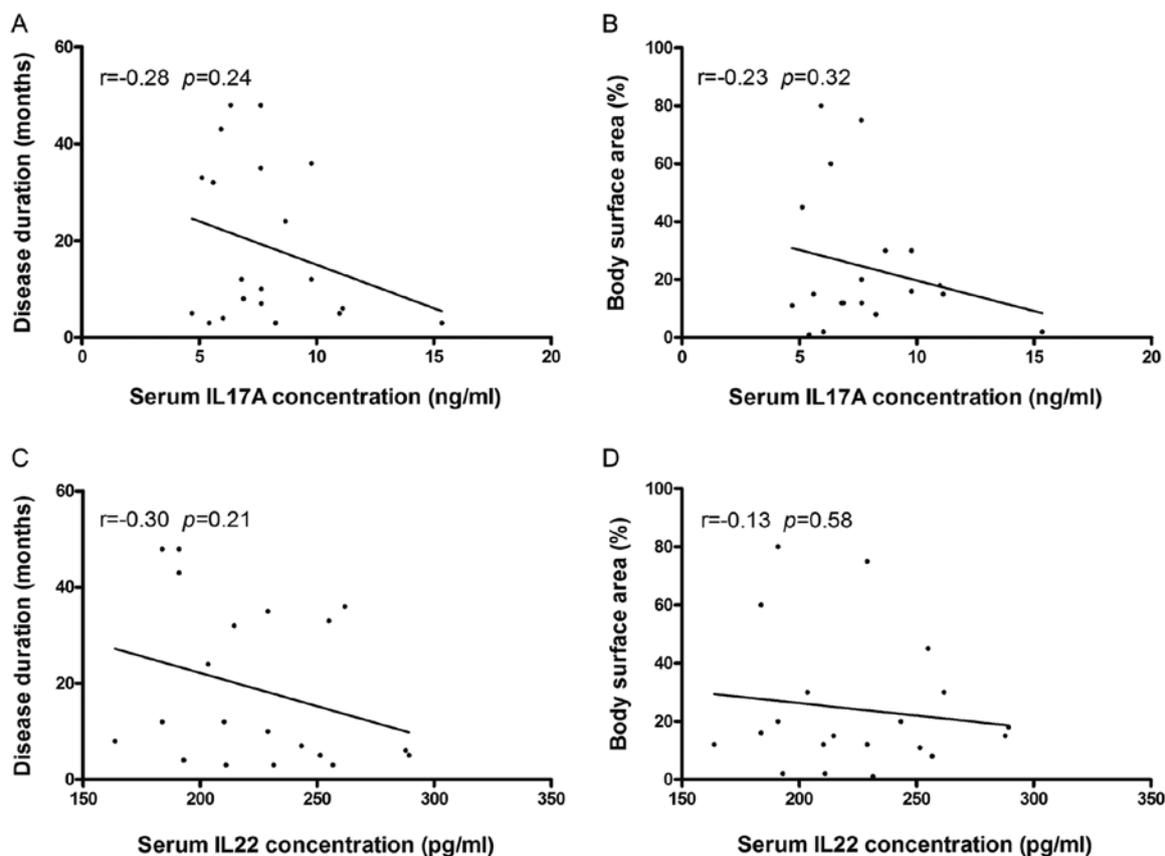


Figure 4. Correlation between serum IL17A and IL22 levels and clinical manifestations of patients with vitiligo. Correlation analyses between (A) serum IL17A levels and disease duration and (B) serum IL17A levels and body surface area in patients with vitiligo (n=20). Correlation analyses between (C) serum IL22 levels and disease duration and (D) serum IL22 levels and body surface area in patients with vitiligo (n=20). IL, interleukin.

associated with reduced CYP1A1 mRNA expression in the CD4⁺ T cells of patients with vitiligo compared with healthy controls (Fig. 2B). Consistent with this observation, western blotting revealed that the protein levels of AhR and CYP1A1 were significantly decreased in the CD4⁺ T cells of patients with vitiligo compared with healthy subjects (Fig. 2C and D).

The expression of AhR in skin samples from patients with vitiligo was subsequently investigated by immunohistochemical analysis. Significantly lower levels of AhR protein in the nonlesional and lesional skin of patients with vitiligo were found compared with the normal skin of healthy subjects (Fig. 2E and F). Levels of AhR expression were markedly lower in nonlesional skin compared with lesional skin in patients with vitiligo. Collectively, these data suggested decreased functionality of AhR in patients with vitiligo.

IL17A and IL22 levels are higher in patients with vitiligo. Since T cell-mediated autoimmunity and altered cytokines are involved in vitiligo pathogenesis, cytokine IL17A and IL22 production in patients with vitiligo and healthy subjects were investigated. Significantly higher concentrations of IL17A and IL22 were detected in the serum of patients with vitiligo compared with healthy subjects (Fig. 3A and B). Similarly, immunohistochemical data showed that CD4⁺, IL17A and IL22 expression were significantly increased in the skin tissues of patients with vitiligo compared with healthy subjects (Fig. 3C and D).

No correlation was observed between AhR mRNA expression in CD4⁺ T cells and serum levels of IL17A or IL22 (Fig. 3E and F). Furthermore, no significant correlation was observed between clinical manifestations (disease duration and body surface area involvement) and the serum concentration of either cytokine (Fig. 4).

AhR knockdown increases secretion of IL17A, but not IL22, from CD4⁺ T cells of patients with vitiligo. A previous study suggested that AhR expression plays an essential role in Th17 subset development, and AhR deficiency increased the expression of IL17A in AhR^{-/-} mice (32). Additionally, AhR has been reported to be associated with Th22 subset development (33). Transfection of si-AhR in the CD4⁺ T cells of patients with vitiligo reduced AhR expression at the protein level compared with the non-transfected and si-control transfected cells (Fig. 5A and B). Furthermore, IL17A secretion by the si-AhR-transfected CD4⁺ T cells of patients with vitiligo significantly increased after 72 h compared with si-control-transfected cells (Fig. 5C). However, IL22 production was reduced, although not significantly, after 72 h compared with si-control-transfected cells (Fig. 5D).

The effects of AhR knockdown on the expression of RORC and forkhead box protein P3 (FoxP3), which are transcription factors for the Th17 and Treg subsets, respectively, were examined. The mRNA expression of RORC was significantly upregulated in the CD4⁺ T cells of patients with vitiligo 24 h following transfection with si-AhR compared with

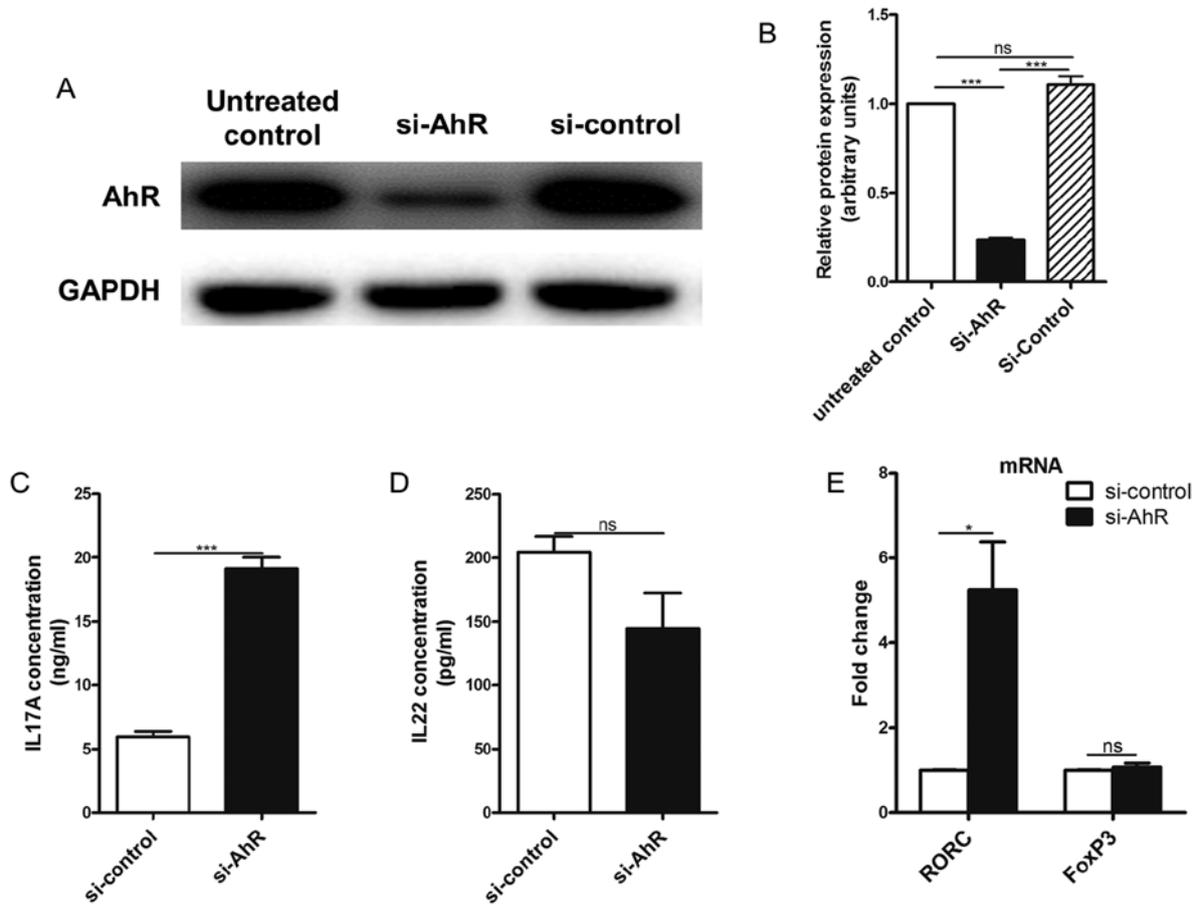


Figure 5. AhR knockdown regulates the production of IL17A, but not IL22 in cells of patients with vitiligo. (A) Representative western blot and (B) quantitative analysis of AhR expression in si-control or si-AhR-transfected CD4⁺ T cells from patients with vitiligo stimulated with anti-CD3 and anti-CD28. (C) IL17A and (D) IL22 levels measured by ELISA after 72 h of transfection. (E) RORC and FoxP3 mRNA levels in si-control or si-AhR-transfected CD4⁺ T cells of patients with vitiligo after 24 h of transfection. n=3. *P<0.05 and ***P<0.001. AhR, aryl hydrocarbon receptor; si-AhR, small interfering RNA targeting AhR; IL, interleukin; RORC, retinoic acid receptor-related orphan receptor C; ns, not significant.

si-control-transfected cells, while the levels of FoxP3 mRNA expression were similar in the CD4⁺ T cells of patients with vitiligo transfected with si-AhR and si-control (Fig. 5E).

AhR activation by G. biloba extract EGb 761 reciprocally regulates IL17A and IL22 production in CD4⁺ T cells of patients with vitiligo. CD4⁺ T cells were treated with EGb 761 at concentrations of 0-200 µg/ml for 24 h. The 7-AAD cell viability assay indicated that EGb 761 was non-toxic at concentrations ≤100 µg/ml. By contrast, the toxicity of EGb 761 increased in a concentration-dependent manner at concentrations >120 µg/ml (data not shown).

Next, the ability of 100 µg/ml EGb 761 to activate AhR was assessed. VAF347, a well-established specific AhR agonist, was used as a positive control to monitor the effects of AhR activation. Following 6 h of EGb 761 or VAF347 treatment, an increase in transcript abundance for CYP1A1 was observed in the CD4⁺ T cells from both patients with vitiligo and healthy subjects compared with controls (Fig. 6A). However, the expression of CYP2E1 mRNA, which is not an AhR target gene, was unaffected (data not shown). The addition of EGb 761 did not influence AhR mRNA expression in cells either from patients with vitiligo or healthy subjects compared with controls (Fig. 6B).

The effects of AhR activation on the production of IL17A and IL22 were then evaluated. CD4⁺ T cells from patients with vitiligo and healthy subjects were treated with EGb 761 or VAF347 for 72 h, followed by measurement of IL17A and IL22 secretion by ELISA. As shown in Fig. 6C, treatment with EGb 761 decreased IL17A production by >60% in the CD4⁺ T cells of healthy subjects, but only by ~45% in those patients with vitiligo, compared with the untreated CD4⁺ T cells.

Treatment with the AhR antagonist CH223191 suppressed the inhibitory effects of EGb 761 on IL17A production. Basal IL22 production was not affected by CH223191 treatment (Fig. 6D). EGb 761-induced IL22 production increased by ~25% in the CD4⁺ T cells of healthy subjects and by ~18% in those of patients with vitiligo, compared with the untreated CD4⁺ T cells. CH223191 inhibited the upregulation of IL22 induced by EGb 761 in the CD4⁺ T cells of both patients with vitiligo and healthy subjects. These findings suggested that AhR activation by *G. biloba* extract EGb 761 regulated IL17A and IL22 production in CD4⁺ T cells from patients with vitiligo.

Discussion

Recent reports have implicated AhR as an important factor in the pathogenesis of vitiligo. Consistent with Wang *et al.* (31),

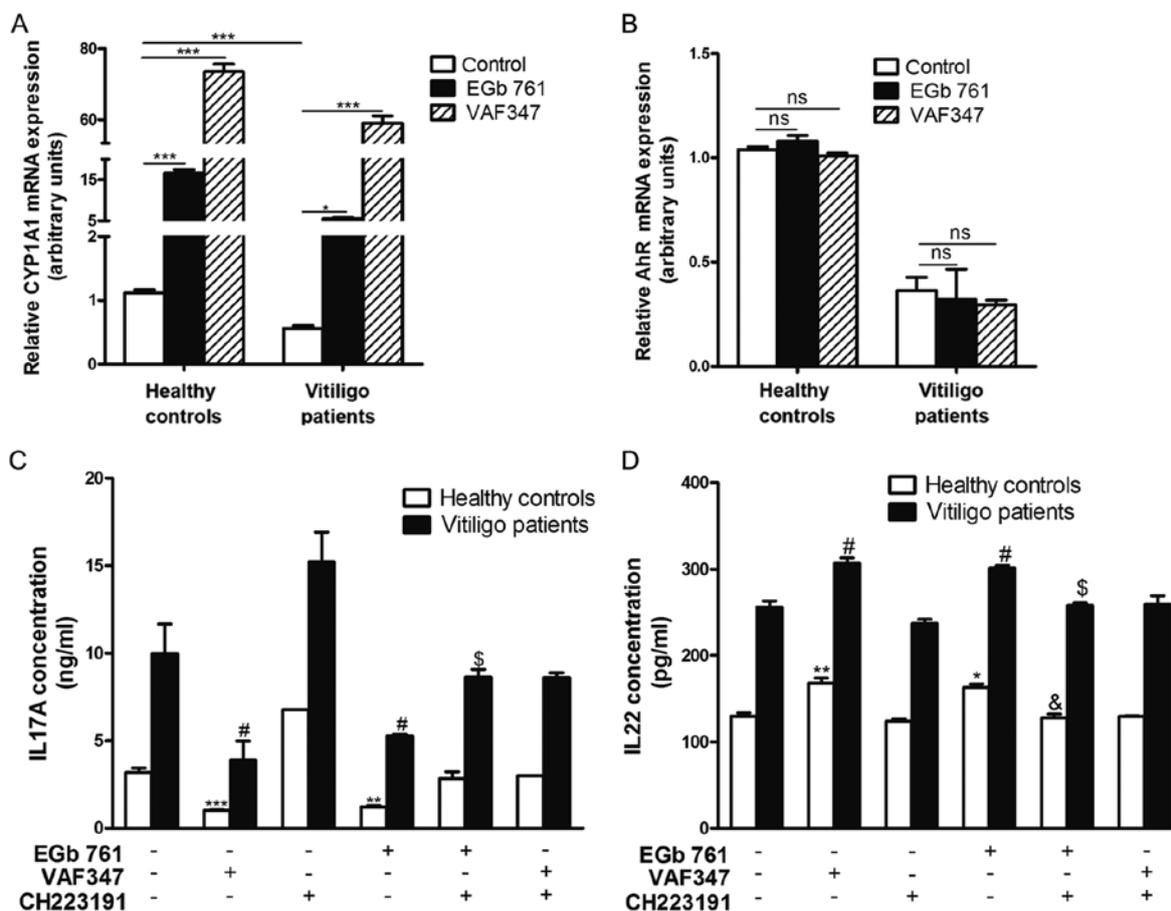


Figure 6. AhR activation by *Ginkgo biloba* extract EGb 761 regulates IL17A and IL22 production in CD4⁺ T cells of patients with vitiligo. Cells from patients with vitiligo and healthy subjects were treated with EGb 761 or VAF347, with or without CH223191. (A) CYP1A1 and (B) AhR mRNA levels after 6 h of treatment. * $P < 0.05$ and *** $P < 0.001$. (C) IL17A protein levels measured in the culture medium by ELISA after 72 h of treatment. ** $P < 0.01$ and *** $P < 0.001$ vs. untreated healthy control; # $P < 0.05$ vs. untreated vitiligo; \$ $P < 0.01$ vs. EGb 761-treated vitiligo. (D) IL22 protein levels measured in culture medium by ELISA after 72 h of treatment. * $P < 0.05$ and ** $P < 0.01$ vs. untreated healthy control; # $P < 0.001$ vs. untreated vitiligo; \$ $P < 0.01$ vs. EGb 761-treated vitiligo; & $P < 0.05$ vs. EGb 761-treated healthy control. n=3; ns, not significant. AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 1A1; EGb 761, *Ginkgo biloba* extract EGb 761; VAF347, AhR agonist VAF347; CH223191, AhR antagonist CH223191.

the present data showed that AhR transcript levels were significantly lower in patients with vitiligo compared with controls. By contrast, Behfarjam and Jadali (30) reported that AhR mRNA levels were significantly elevated in PBMCs of patients with vitiligo; this difference might be explained by the fact that patients with inactive vitiligo were examined in the latter study. It was previously reported that epidermal AhR expression is significantly decreased in patients with vitiligo (19,20), consistent with the present results. The present study demonstrated that AhR protein expression was significantly lower in the lesional and nonlesional skin of patients with vitiligo compared with the normal skin of healthy controls. Furthermore, AhR expression was markedly higher in lesional compared with nonlesional skin of patients with vitiligo, which might represent a feedback response to counteract inflammation (19). Collectively, the results of the aforementioned *in vivo* studies suggest that an intrinsic defect in AhR expression could constitute a major susceptibility factor for the development of vitiligo.

IL17A and IL22 play roles in various autoimmune diseases (11,34). IL17A is widely accepted to be involved in the pathogenesis of several autoimmune diseases, such as psoriasis and vitiligo, whereas IL22 has been reported to

be protective in acute inflammation, such as in protecting the intestine from viral infection in an experimental animal disease model, and pathogenic in chronic settings, such as in atopic dermatitis and rheumatoid arthritis (11-14). There are conflicting reports regarding their expression levels in vitiligo. In line with Elela *et al* (10), the present study showed that the serum concentrations of IL17A and IL22 were significantly higher in patients with vitiligo compared with healthy controls. By contrast, other studies reported no difference in the serum concentrations of IL17A and IL22 in vitiligo (19,31). These discrepancies may be explained by the dependence of inflammatory cytokine secretion on disease status, as both progressive and stable patients were included in previous studies (19,31), while only progressive patients were assessed in the present study. No obvious expression of either cytokine was detected in skin by immunohistochemistry (data not shown), which is inconsistent with results from a previous study (10). This might be explained by very mild skin inflammation in the patients with vitiligo in the present study.

AhR mRNA expression did not exhibit a correlation with the serum levels of either cytokine. These findings suggest that CD4⁺ T cells might not be the only source of serum IL17A and IL22 in patients with vitiligo, since both cytokines

can be produced by a variety of cells, including NK and $\gamma\delta$ T cells (5,35). Although several studies have reported that serum IL17A levels are positively correlated with vitiligo duration and the extent of body area involvement (36,37), no such correlation was noted in the present study. There are two potential explanations for these differences. First, the sample size of the present study was small, with only 20 patients. Second, all patients in the present study had active disease, which is significant as it has been reported that IL17A may be better correlated with active disease than with other clinical manifestations (38).

In the present study, the suppression of AhR expression in cultured CD4⁺ T cells from patients with vitiligo by AhR-targeted siRNA treatment resulted in a significant increase in IL17A transcript levels compared with those in si-control-transfected cells. AhR knockdown also increased the expression levels of mRNA encoding RORC, the master transcription factor for Th17 differentiation, but not FoxP3, consistent with a previous report (32). The inhibitory effect of AhR knockdown on IL22 transcript abundance was incomplete in CD4⁺ T cells from patients with vitiligo, suggesting the involvement of additional mechanisms. IL22 production has previously been reported to be regulated by mechanisms involving both AhR-dependent and -independent pathways (39).

A previous study demonstrated that AhR activation might be beneficial for the treatment of vitiligo (21). In the present study, treatment of CD4⁺ T cells with *G. biloba* extract EGb 761 activated AhR. This is consistent with findings by Rajaraman *et al* (40), which showed that *G. biloba* extract activated AhR in MCF-10A human mammary epithelial cells. EGb 761 primarily contains flavonoid glycosides (27). Different flavonoids have different affinities for AhR and the strength of AhR modulation is dependent on their structures (41). Quercetin, the primary flavonoid glycoside in EGb 761, is a well-established activator of AhR (41). Both the expression levels and activation of AhR have been reported to have important roles in modulating biological processes. For example, deficiency of AhR increases the expression of IL17A in CD4⁺ T cells from AhR^{-/-} mice (32), and TCDD, which does not alter AhR expression, has been reported to affect CD4⁺ T cell differentiation through activating AhR (42). In the present study, AhR mRNA expression was not influenced by EGb 761 treatment in the present study. Thus, EGb 761 may be hypothesized to exert its biological function through activating AhR, not by modulating its expression.

In the present study, EGb 761 inhibited IL17A and increased IL22 secretion in the CD4⁺ T cells from patients with vitiligo and healthy controls. The extent of regulation of either cytokine in patients with vitiligo was less compared with that in healthy controls, which might be due to a decrease in AhR functionality in the CD4⁺ T cells of patients with vitiligo. The AhR antagonist CH223191 *per se* did not significantly influence the expression of IL22. Pié *et al* (39), showed that IL22 expression was reduced in the presence of CH223191, while Rohlman *et al* (32), reported that AhR deficiency had no significant effect on the expression of IL22. These contradictory results might be associated with the different cells used in the studies, since Pié *et al* (39) used PBMCs and IL22 is produced in a variety of cells including Th17, $\gamma\delta$ T, NKT, innate lymphoid and Th22 cells (5). IL22 expression is regulated by AhR-independent pathways (39). The changes in IL17A and

IL22 secretion induced by EGb 761 in the present study were nearly suppressed by CH223191, suggesting specific regulation of these two cytokines by AhR, at least in CD4⁺ T cells responding to EGb 761.

In conclusion, the present study demonstrated that AhR expression is significantly reduced in CD4⁺ T cells of patients with vitiligo, and that activation of AhR by *G. biloba* extract EGb 761 reciprocally regulates IL17A and IL22 production in CD4⁺ T cells from patients with progressive unstable vitiligo, although the exact mechanisms merit further investigation. The present data also support the hypothesis that AhR may be a potential therapeutic target for the treatment of vitiligo.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

BL, YX, XM and YS performed the experiments and analysed the data. BL, YX and XM wrote the manuscript. YS, WS and ZW designed and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Human Ethics Committee of Shanghai Jiaotong University. Written informed consent was obtained from each subject.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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