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Original Article

AhR mediates an anti-inflammatory feed-back mechanism in human Langerhans cells involving FcεRI and IDO.

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ABSTRACT

Background:

Aryl hydrocarbon receptor (AhR), an important regulator of immune responses, is activated by UVB irradiation in the skin. Langerhans cells (LC) in the epidermis of atopic dermatitis (AD) patients carry the high affinity receptor for IgE, FcεRI, and are crucially involved in the pathogenesis of AD by inducing inflammatory responses and regulating tolerogenic processes.

Objectives:

We investigated AhR and AhR repressor (AhRR) expression and functional consequences of AhR activation in human *ex vivo* skin cells and in *in vitro* generated LC.

Methods:

Epidermal cells from healthy skin were analyzed for their expression of AhR and AhRR. LC generated from CD34⁺ hematopoietic stem cells (CD34LC) were treated with the UV photoproduct and AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ). Cell surface receptors, transcription factors and the tolerogenic tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (*IDO*) were analyzed using flow cytometry and quantitative PCR.

Results:

Epidermal LC and CD34LC express AhR and AhRR. AhR was also found in keratinocytes, which lack AhRR. AhR activation of LC by FICZ caused down-regulation of FcεRI in CD34LC without affecting their maturation. AhR-mediated regulation of FcεRI did not involve any known transcription factors related to this receptor. Furthermore, we could show up-regulation of IDO mediated by AhR engagement.

Conclusions:

Our study shows that AhR activation by FICZ reduces FcεRI and up-regulates IDO expression in LC. This AhR-mediated anti-inflammatory feedback mechanism may dampen the allergen-induced inflammation in AD.

Key words

Ah receptor, atopic dermatitis, Fc epsilon receptor, Langerhans cells, IDO,

Abbreviations:

AhR	aryl hydrocarbon receptor
AhRR	AhR repressor
CD34LC	CD34 ⁺ hematopoietic stem cell-derived LC
CYP	cytochrome P450
FICZ	6-formylindolo[3,2-b]carbazole
IDO	indoleamine 2,3-dioxygenase
KC	keratinocyte
Kyn	kynurenine
LC	Langerhans cell
NHEK	normal human epidermal keratinocytes
rFI	relative fluorescence index
TF	transcription factor
Treg	regulatory T cell
Trp	tryptophan

INTRODUCTION

Immune cells encounter environmental chemicals, also known as xenobiotics, in barrier organs by means of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor (TF). Upon ligand binding in the cytoplasm, the AhR complex is transported into the nucleus where AhR binds to its target structures. This pathway is regulated by the AhR repressor (AhRR), which competes with AhR for the binding site to the AhR nuclear translocator (ARNT), thus forming a transcriptionally inactive complex and initiating a negative feedback loop (1).

Besides xenobiotic molecules, more and more evidence for the existence of endogenous AhR ligands is accumulating, many of them are tryptophan (trp) derivatives. The efficiency of trp metabolites as AhR ligands was reviewed previously (2-4). The high affinity AhR ligand 6-formylindolo[3,2-b]carbazole (FICZ) is physiologically generated from trp by photolysis (5) and is present in human skin upon exposure to UV and visible light (6, 7). It is produced intracellularly from trp upon UVB irradiation and binds to AhR in the cytoplasm inducing nuclear transport of the ligand-bound receptor (8). FICZ is a substrate for the cytochrome P450 enzymes (CYPs), such as CYP1A1, which are strongly induced upon FICZ-mediated activation of AhR. UVB irradiation of human skin induced CYP1A1 and CYP1B1 proving AhR-mediated physiological effects of trp photoproducts (9, 10). Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme that catabolizes trp into kynurenine (kyn) and other metabolites in DC. IDO expression of human DC is also strongly dependent on their maturation status (11). It has been reported that in murine bone marrow-derived DC, AhR was required for the maturation-induced increase of IDO expression and activity (12). Also, in human U937-derived DC, AhR ligands increased IDO expression and function (13). Thereby, autoregulatory feedback loops are initiated restricting AhR signaling.

In murine and human skin, keratinocytes (KC) and Langerhans cells (LC), but also fibroblasts, melanocytes and dermal DC express functional AhR, and can therefore react with AhR activation after UVB irradiation (14). In acute psoriatic lesional skin enhanced expression of AhR and ARNT was observed (15). Moreover, treatment of normal human KC with AhR ligands resulted

in AhR/ARNT colocalization in the nuclei, indicating AhR activation (15). AhR agonists applied to the skin of healthy donors or to non-lesional or lesional skin of psoriasis patients reduced expression of genes characteristic for the “psoriasis transcriptome”(16).

AhR activation via application of coal tar enhanced filaggrin expression in human primary KC as well as in skin biopsies from atopic dermatitis (AD) patients and therefore ameliorated skin barrier defects in AD. Additionally, coal tar leads to Th2 cytokine signaling inhibition through STAT 6 dephosphorylation most likely induced by AhR-regulated activation of the oxidative stress pathway (17). AD is an inflammatory skin disease often occurring concomitantly with other atopic disorders such as asthma. Increased expression of the high-affinity receptor for IgE, FcεRI, on the cell surface of LC is a hallmark of patients suffering from AD (18). UVB phototherapy of AD patients reduced immune cell infiltrates, namely the number of FcεRI-expressing cells in skin lesions (19). Human skin is constantly exposed to sunlight, which can have both, beneficial and adverse effects to cells and organs due to the absorption of photons. Since UVB irradiation activates the AhR, via the trp photoproduct FICZ, and AhR activation strengthens the epidermal barrier and thereby reduces inflammation we here investigated the impact of AhR ligand FICZ on FcεRI-expressing human skin DC.

METHODS

Cord blood and skin preparation

Human cord blood was obtained from the GFO hospitals Bonn, establishment of St. Mary in Bonn from healthy donors. Skin from healthy donors was obtained from 9 female (age 29-50 years) and 2 male (age 25 and 36 years) individuals undergoing plastic surgery at the University of Bonn. This study was approved by the local ethics committee of the University of Bonn and performed in accordance with the declaration of the Helsinki principle. Informed consent was obtained from all participants in this study.

Reagents

The culture medium of CD34LC has been used as described (20). Details of antibodies used for cell surface and intracellular staining are given in Online Repository Table E1. 7-amino-actinomycin D (7-AAD) was from Sigma-Aldrich (St. Louis, MO, USA). SYBR Green was obtained from Biorad (Munich, Germany). Primer used for qPCR are given in the repository Table E2. The AhR ligand FICZ was from Enzo (Lörrach, Germany) and was applied in DMSO from Sigma-Aldrich.

Preparation of epidermal cell (EC) suspensions, epidermal sheets and *ex vivo* skin

After obtaining skin from healthy donors, split-thickness skin (0.4 mm) was immediately prepared by dermatome and incubated with 0.5 % trypsin for 1 h at 37 °C. The epidermis was peeled off and a homogenous EC suspension was prepared and subjected to flow cytometric analysis (21).

Generation and treatment of CD34LC

CD34LC were generated from cord blood-derived CD34⁺ hematopoietic stem cells as described previously (20). At day 7 to 10 of culture, CD34LC were treated with 1 µg/ml FICZ or the solvent DMSO for the indicated time periods. For FcεRI-crosslinking experiments 24-well culture plates were coated with 10 µg/µL anti-FcεRI antibodies or isotype control in PBS for 24 h at 37 °C. After washing CD34LC were applied to the coated plates and incubated at 37 °C for the indicated time periods and then subjected to flow cytometric analysis or qPCR. For qPCR analysis of mRNA expression, CD1a⁺ cells were enriched by CD1a⁺ isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) before cDNA preparation.

cDNA preparation and quantitative real-time PCR (qPCR)

cDNA was prepared using TRIzol[®] and SuperScript[™] II Reverse Transcriptase from Life Technologies GmbH (Darmstadt, Germany) according to the manufacturers' instructions. Gene expression was determined by SYBR Green-based qPCR using the Applied Biosystems StepOnePlus system. Cycling

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conditions were 95 °C for 15 s and 60 °C for 1 min for 40 cycles, followed by a melting point determination. Details of primers are given in Online Repository Table E2. Results were normalized to β -actin encoding mRNA *ACTB* and evaluated using the $\Delta\Delta CT$ method in order to calculate the fold increase. Relative expression levels of mRNA are the ratios of the copy numbers of the respective mRNAs per 1,000 *ACTB* mRNA copies. Templates used for standard curves and primers were obtained from Invitrogen (Carlsbad, USA).

Flow cytometric analysis

For surface expression of Fc ϵ RI and CD83 on CD34LC cells were stained with the respective antibodies or isotype control. For gating, cells were stained with anti-CD1a antibodies and for exclusion of dead cells they were treated with 7-AAD. For intracellular staining CD34LC or EC were first treated with 7-AAD, then permeabilized with BD Cytofix/Cytoperm kit (Becton Dickinson, San Jose, CA, USA) and then stained with antibodies against AhR, AhRR, Langerin and CD1a or the respective isotype controls. LC were gated from the EC suspensions by the expression of both CD1a and Langerin, after dead cell exclusion by 7-AAD. Surface and intracellular markers were measured using FACS Canto and analyzed with FACS Diva software (Becton Dickinson, San Jose, CA, USA) and FlowJo 10.0.5 (TreeStar, Ashland, OR, USA). The relative fluorescence index (rFI) was calculated as follows: $(\text{Mean fluorescence intensity (MFI)}_{\text{surface marker}} - \text{MFI}_{\text{isotype control}}) / \text{MFI}_{\text{isotype control}}$.

Statistical analysis

Statistical analysis of differences due to treatment with AhR ligands were performed by use of the Wilcoxon signed-rank test for paired samples (two tailed) facilitated by IBM SPSS Statistics 22.

RESULTS

***Ex vivo* LC express both AhR and AhRR whereas KC express only AhR.**

It has been shown that human skin expresses *AHR* and *AHRR* mRNA (16) and normal human epidermal keratinocytes (NHEK) expressed high amounts of AhR but showed only marginal expression of the AhRR (22). To analyze, which cell types in human epidermis express AhR and AhRR protein, we stained epidermal cell suspensions of human skin from healthy donors with antibodies specific for AhR and AhRR, respectively. Flow cytometric analysis of EC suspensions revealed that both *ex vivo* CD1a⁺Langerin⁺LC and CD1a⁺Langerin⁻KC expressed AhR. Besides AhR, we could show here for the first time that AhRR was also expressed by freshly isolated LC, however, KC lacked AhRR expression (Fig. 1A and B).

CD34LC express AhR and AhRR mRNA and protein.

The limited numbers of LC from human epidermis is a drawback for their functional investigation. As a model system, we generated LC from cord blood-derived CD34⁺ hematopoietic stem cells (CD34LC) which express CD1a, Langerin and FcεRI (20). These cells were used to further analyze the role of AhR activation in skin APC. The AhR is expressed on CD34LC as reported (23)(24). Furthermore, we could detect that the AhRR is also expressed in human CD34LC (Fig. 2). Treatment of CD34LC with AhR ligand FICZ reduced AhR mRNA level significantly while surface expression was not significantly altered. AhRR mRNA level was not significantly changed by FICZ but surface expression was significantly enhanced.

AhR engagement on CD34LC reduced FcεRI in a transcriptionally controlled manner.

Epidermal LC from AD skin are characterized by their high expression of FcεRI. We investigated the impact of the AhR ligand FICZ on the expression of this receptor in CD34LC. Upon stimulation of CD34LC with FICZ, we observed a massive increase of mRNA of *CYP1A1* and *CYP1B1* compared to cells treated with the solvent DMSO showing functional responsiveness of CD34LC to FICZ (Fig. 3A). Cell surface expression of FcεRI on CD34LC was significantly

diminished upon FICZ induced AhR activation (Fig 3B). This was due to a decrease of *FCER1A* mRNA expression in CD34LC upon AhR-activation (Fig. 3C), whereas expression of the FcεRI gamma chain encoding *FCER1G*, mRNA was unaltered (Fig. 3D). Expression of the FCER1A was previously shown to be regulated by TF, such as PU.1, YY1, ELF1, HMGB1 and HMGB2 (20, 25). We investigated whether AhR stimulation affects expression of these TF thereby reducing FcεRI expression. Expression of *PU.1*, *YY1*, *ELF1*, *HMGB1* and *HMGB2* was not altered upon AhR triggering (data not shown).

UVB irradiation of CD34LC reduced FcεRI surface expression.

Since the AhR serves as sensor for UVB by induction of FICZ (5, 8) we investigated the impact of UVB irradiation on the expression of FcεRI on human LC. CD34LC were irradiated with 20 mJ/cm² or 40 mJ/cm² of UVB light and surface expression of FcεRI was assessed by flow cytometry (Suppl. Fig. 1A). At these dosages, no significant effects of UVB treatment on necrosis, apoptosis or maturation of the cells were observed, as judged by flow cytometric analysis of annexin V/7-AAD and CD83 staining, respectively (data not shown). However, cell surface expression of FcεRI was significantly down-regulated after irradiation with 20 mJ/cm² and 40 mJ/cm² UVB light. UVB-mediated modulation of FcεRI probably occurs at the transcriptional level, since mRNA expression of *FCER1A* was slightly impaired (Suppl. Fig. 1B). AhR downstream targets CYP1A1 and CYP1B1 were moderately upregulated by UV irradiation (Suppl. Fig. 1).

IDO is up-regulated in CD34LC after FICZ-induced AhR activation.

Besides IFNγ stimulation, FcεRI crosslinking was shown to induce the expression of IDO in monocytes of AD patients (26). We found that cross-linking of FcεRI also resulted in increased *IDO* mRNA levels in CD34LC along with an increase of CD83 expression at the cell surface (Fig 4A), indicating maturation of the cells. Interestingly, AhR activation of CD34LC also increased

the expression of IDO (Fig 4B), however did not induce their maturation. Thus, both, AhR and FcεRI ligation may initiate anti-inflammatory signals in human LC.

DISCUSSION

In the present report, we show that UVB irradiation activates AhR signaling in human CD34LC. AhR activation reduces the expression of the high affinity receptor for IgE, FcεRI, and induces the immune regulatory enzyme IDO without affecting maturation of the cells.

Constitutive expression of the AhR has been reported in human monocytes, immature DC and cord blood-derived LC (23). To study the role of AhR signaling in human epidermal APC we analyzed AhR and AhRR expression in skin-derived, CD1a⁺/Langerin⁺ LC. We showed expression of both, AhR and AhRR in human LC, whereas in CD1a⁺/Langerin⁻KC, high expression of AhR but no expression of AhRR was observed. Also NHEK expressed high amounts of AhR but only low amounts of AhRR (22). This is in line with the murine system, where the AhR is also detectable in LC and KC (27). The AhRR, however, is mainly expressed in murine LC but only marginally in KC (28). High AhRR expression in LC controls AhR activation and may be important to balance LC activation by environmental stimuli in the skin.

AhR activation led to the up-regulation of *CYP1A1* and *CYP1B1* in skin biopsies of psoriatic patients and of healthy controls (16). We could show an increase of *CYP1A* and *CYP1B1* expression in CD34LC upon treatment with the AhR ligand FICZ. This is in accordance with reports showing inducible expression of *CYP1B1* in freshly isolated human LC after 7,12-dimethylbenz[a]anthracene stimulation (29) and induction of *CYP1A1* in cord blood-derived LC as well as in human monocytes and DC (23).

LC of AD patients show high levels of FcεRI. They are activated by allergens via specific IgE and initiate pro-inflammatory immune responses (26). Previously, we could show that TLR stimulation of human LC leads to down-regulation of FcεRI *in vitro*, which was accompanied by enhanced LC maturation and might induce LC emigration from the epidermis in AD (20). Here

we report that AhR activation also down-regulates FcεRI expression of human LC, which, however, did not coincide with LC maturation. Interestingly, narrow-band UVB phototherapy of AD lesions reduced the numbers of FcεRI⁺ DC (19). This might be consistent with the observation that AhR activation down-regulated FcεRI expression on APC and this effect may contribute to reduced inflammation after phototherapy. Furthermore, traditional treatment of AD with coal tar has been connected to AhR-signaling in KC (17, 30), indicating that AhR activation in KC attenuates the immune response in AD. Moreover, the topical application of the AhR agonist Tapinarof has been reported to improve the skin inflammatory reaction (31). Our observation supports the concept that the therapeutic activity of this compound could be explained at least in part by the mechanism described herein.

The Trp catabolizing enzyme IDO is another direct target of AhR activation in APC (13, 32). In human skin, IDO expression can be detected in LC, but not in KC (33). IDO expression can be induced in skin by crosslinking of FcεRI in monocytes and skin APC from AD patients and is able to control skin allergy by restricting inflammation and regulating T cell proliferation (26, 34). Exaggerated IDO expression in LC is a predictive biomarker for patients with AD and high risk for Eczema herpeticum (35). Kyn-mediated AhR activation in APC leads to Treg differentiation *in vitro* (12) and UVB-induced activation of the AhR in murine APC induces the generation of Treg which mediate the induction of UVB-induced immunosuppression (36). Thus, besides FcεRI ligation, AhR-stimulation of APC may also contribute to dampen the inflammatory response via IDO induction and may influence T cell proliferation and differentiation in AD.

In summary, we could show that FICZ-induced AhR signaling reduces FcεRI and up-regulates IDO1 expression in CD34⁺LC indicating an immunosuppressive action of AhR signaling in human LC. Therefore, the development of selective moderate AhR-activating agents could be a promising strategy for AD therapy.

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AUTHOR CONTRIBUTIONS

SK and HW initiated and designed the experiments. SS, TS, HK and NL performed the experiments. SK, NH, JV and HW evaluated the data and wrote the manuscript. TB and IF supervised all experiments and edited the final manuscript.

CONFLICT OF INTEREST

The authors have no financial conflicts of interest.

FIGURE LEGENDS

Figure 1: *Ex vivo* isolated human LC express AhR and AhRR, KC express AhR but not AhRR. Flow cytometric analysis of an epidermal cell suspension from healthy skin. LC were defined as CD1a⁺/Langerin⁺ and KC as CD1a⁻/Langerin⁻ living cells. (A) Representative histograms of AhR (top panel, open lines) and AhRR (bottom panel, open lines) staining. Results with control IgGs are shown as filled histograms. (B): Mean values of the relative fluorescence index (rFI) with standard deviation (SD) are shown, n=7.

Figure 2: CD34LC express AhR and AhRR mRNA and protein. (A) *AHR* (A, left panel) and *AHRR* (A, right panel) mRNA from CD1a⁺ CD34LC cells treated with FICZ or DMSO for 24h was quantified by

qPCR, n=6. (B, C) Flow cytometric analysis of FICZ/DMSO-treated CD1a⁺ CD34LC stained with antibodies against AhR or AhRR, as indicated. (B) Representative histograms. (C) Mean values of rFI with SD, n=7.

Figure 3: AhR-mediated decrease of FcεRI in human LC. CD34LC were left untreated or treated with DMSO or FICZ for 24h. (A) *CYP1A1* (n=6) and *CYP1B1* (n=11) mRNA from CD1a⁺ CD34LC was quantified by qPCR. (B) CD34LC treated with FICZ or DMSO were cell surface stained with FcεRI-specific antibodies and analyzed by flow cytometry. CD1a⁺ CD34LC were enriched and change of *FCER1A* (C) and *FCER1G* (D) mRNA expression was measured by qPCR. Mean values with SD are shown, n= 6.

Figure 4: Increase of *IDO1* mRNA expression caused by ligation of FcεRI or AhR in CD34LC. (A) CD34LC were stimulated with FcεRI-specific antibodies (anti- FcεRI) or with irrelevant antibodies (IgG2b). CD1a⁺ CD34LC were isolated and *IDO1* mRNA was quantified by qPCR, n= 6. CD83 (rFI, n=5) on CD1a⁺ CD34LC was determined by flow cytometry (right panel). (B) CD34LC were treated with FICZ or DMSO. *IDO1* mRNA was quantified by qPCR. CD83 (rFI, n=10) on CD1a⁺ CD34LC was determined by flow cytometry (right panel).

Suppl. Figure 1: UVB irradiation reduces FcεRI expression in human CD34LC. CD34LC were irradiated with the indicated dosages of UVB light and were cultured for 6 h. (A) Cells were stained with FcεRI-specific antibodies and analyzed by flow cytometry, n=6. (B) *FCER1A* mRNA expression was determined using qPCR. Bars represent means of ratios of *FCER1A* mRNA copy numbers relative to actin, n=7. (C-D) mRNA levels of the AhR targets *CYP1A1* and *CYP1B1* were determined using qPCR. Bars represent RQ values (fold change compared to untreated cells), n=6.

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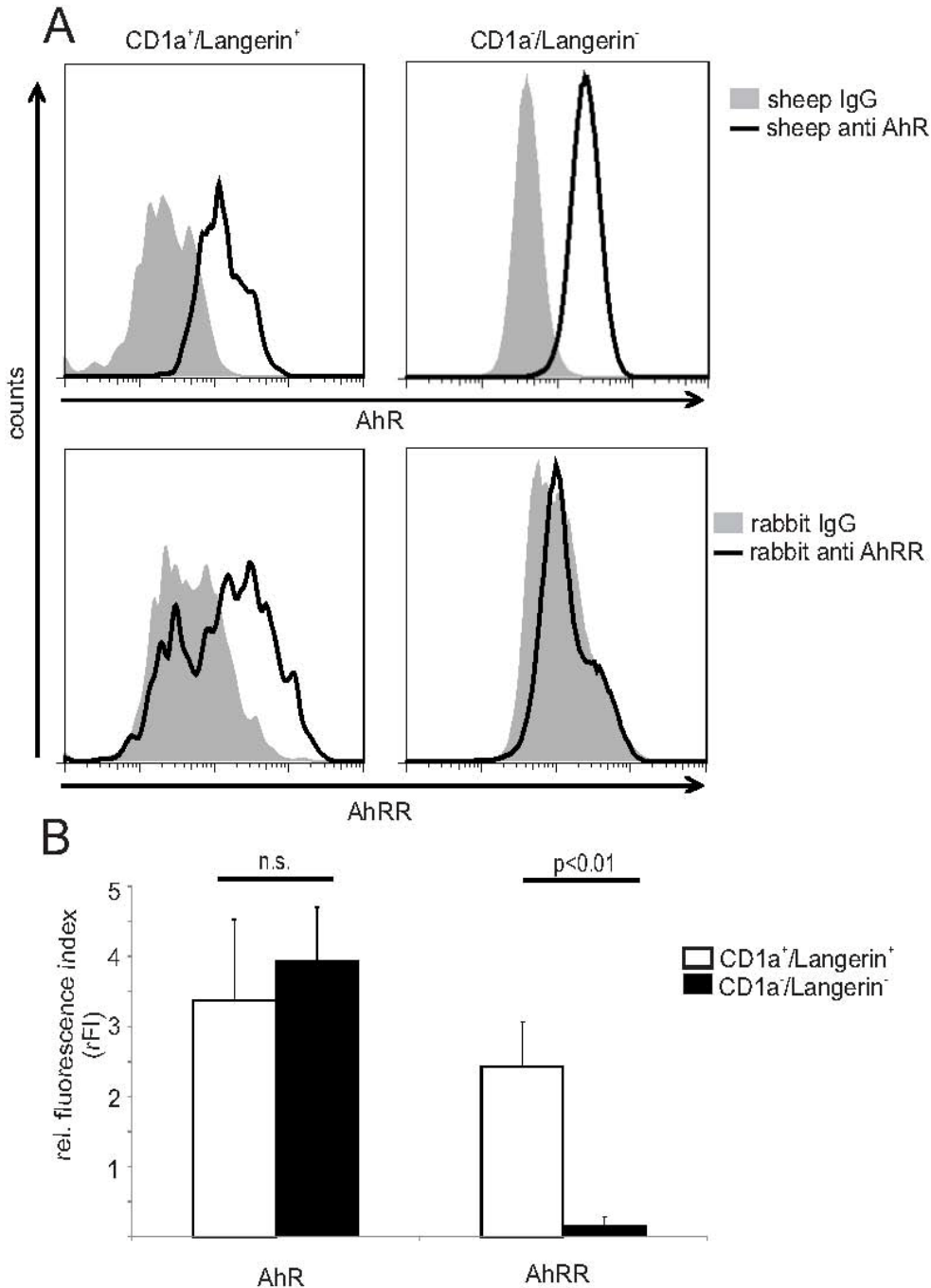


Figure 1

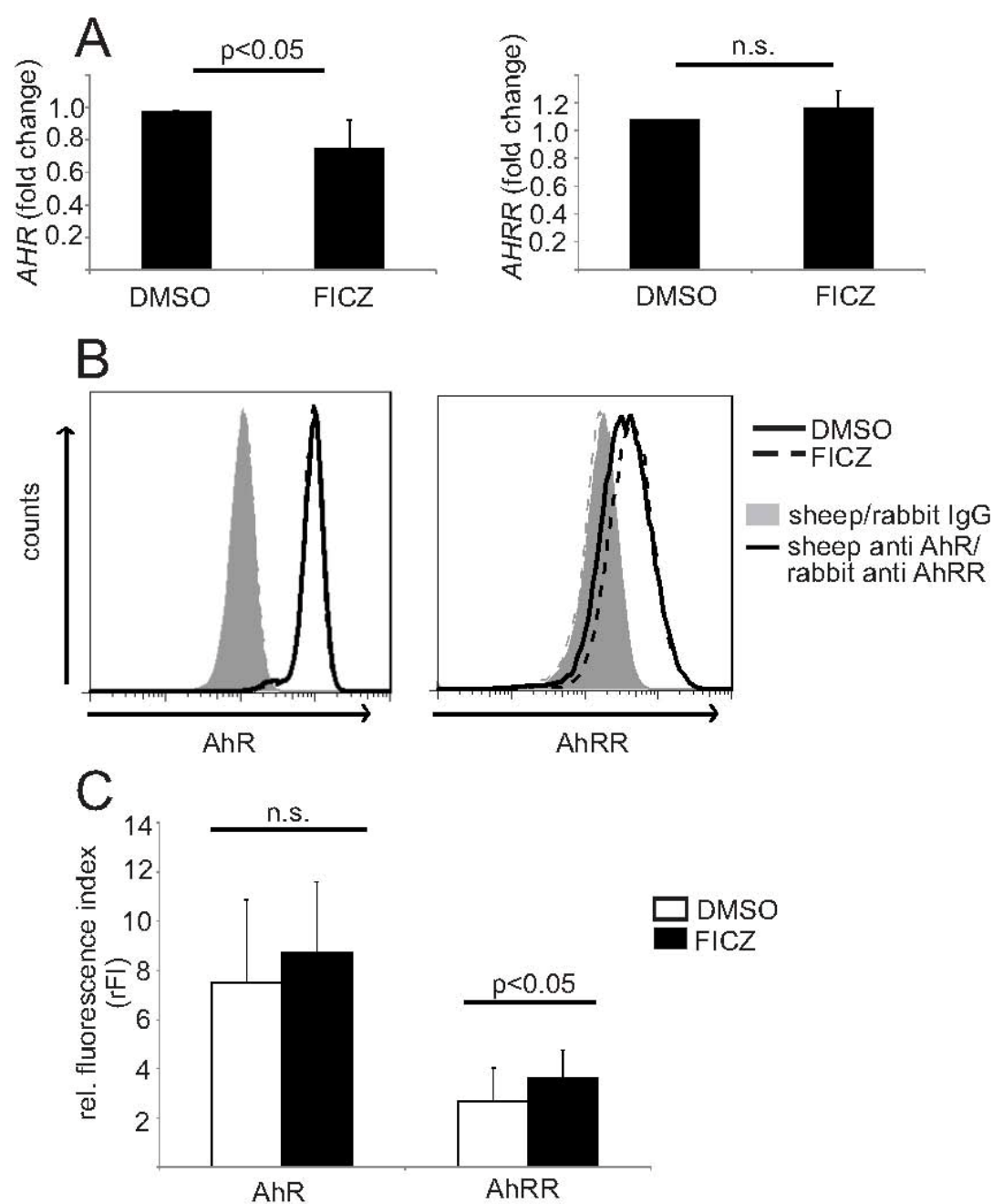


Figure 2

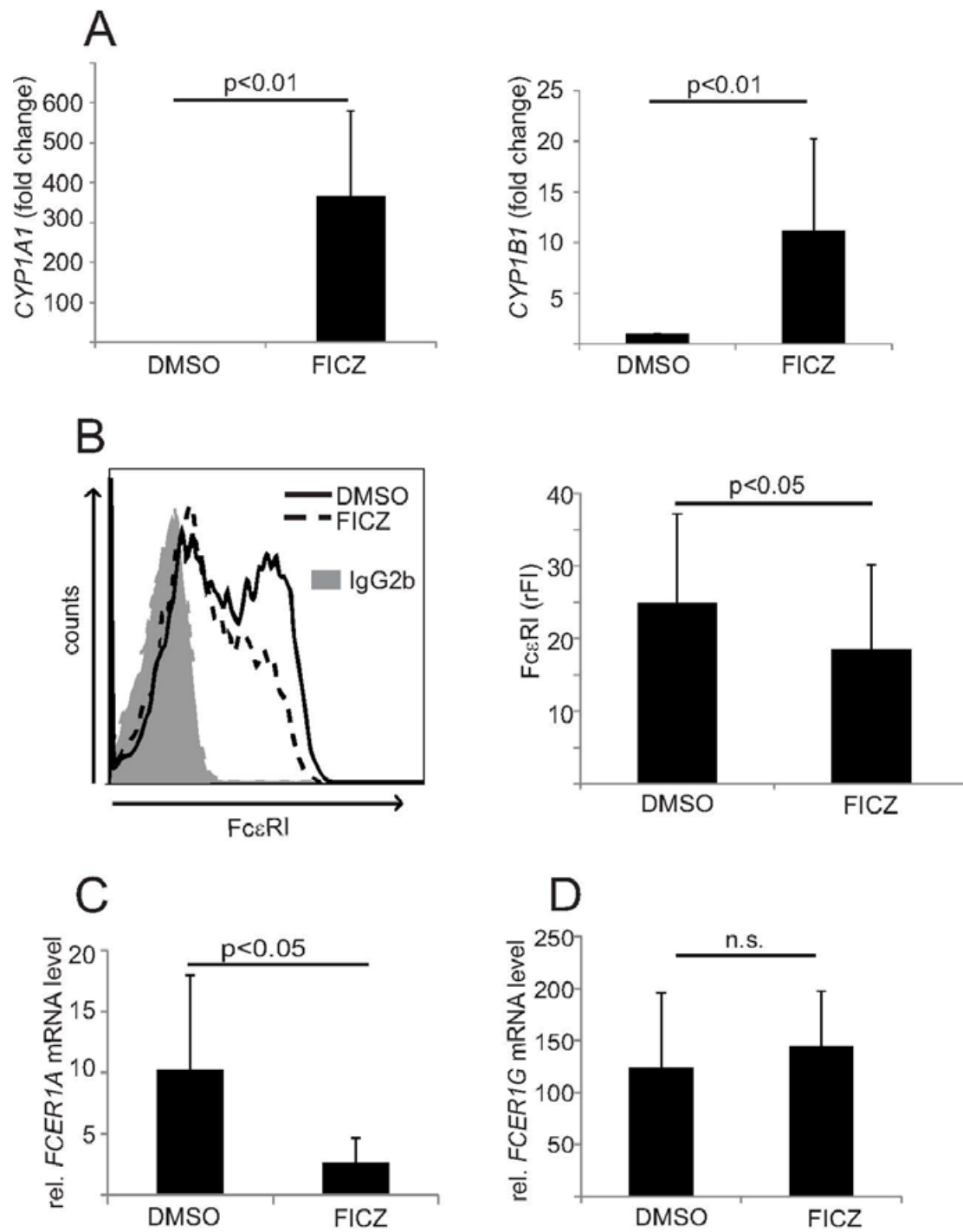


Figure 3

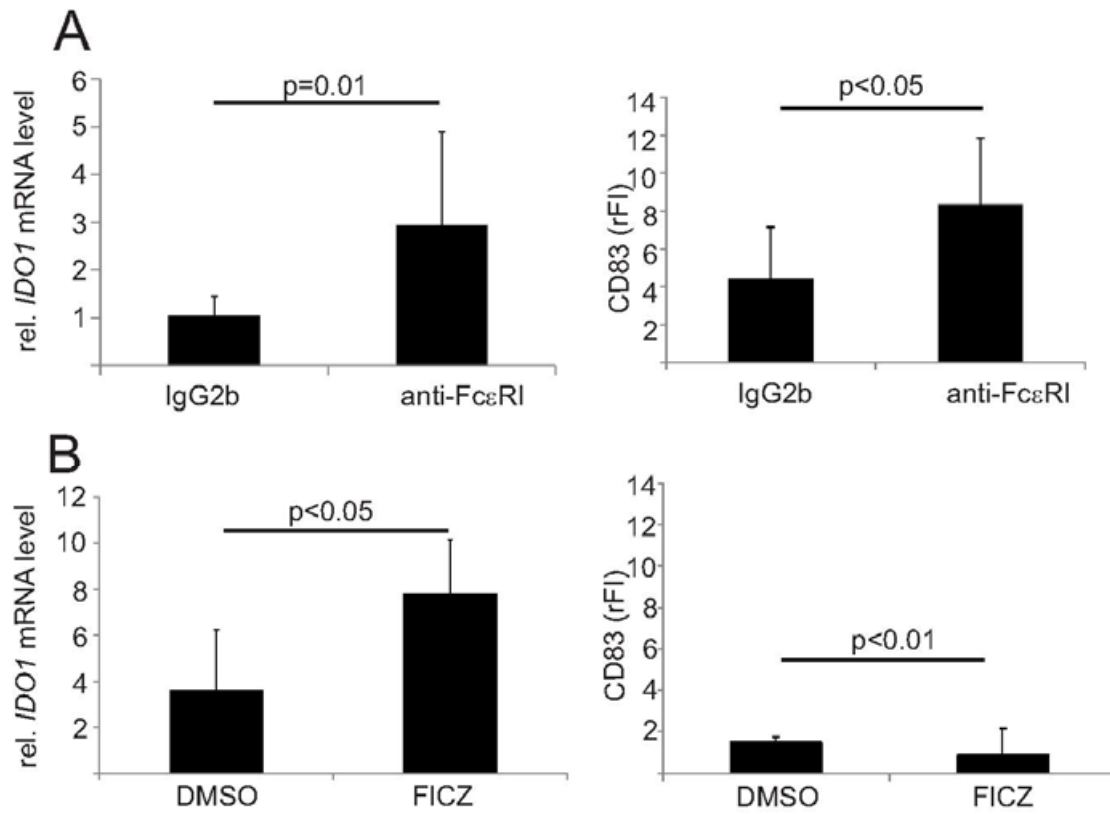


Figure 4