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Differential induction of ATF3 and HO-1 in myeloid cells and keratinocytes via Dimethylfumarate or Cyclosporine A

Stefanie Müller, Nikola Smatlik, Marc Burian, Kamran Ghoreschi, Martin Röcken, Amir S. Yazdi*

Department of Dermatology, Eberhard Karls University, Liebermeisterstr. 25, D-72076 Tübingen, Germany

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ABSTRACT

Background: Chronic inflammatory skin diseases are characterized by controlled proliferation of keratinocytes. Here, activating transcription factor 3 (ATF3) might play a fundamental role. In these inflammatory diseases, proliferation is controlled and only rarely leads to cancer development which can be supported by an inflammatory microenvironment. ATF3 is a dual function protein as it suppresses pro-inflammatory IL-6 and IL-8, but also acts as a pro-oncogenic factor by the suppression of p53. We therefore analyzed ATF3 expression comparing myeloid cells with keratinocytes.
Objective: To dissect the bi-modal role of ATF3 we pharmacologically induced ATF3 and analyzed its influence on cytokine expression and secretion in a cell type specific manner.
Methods: Since inflammatory skin diseases can be treated systemically with Cyclosporin A or Dimethylfumarate we stimulated myeloid cells and primary human keratinocytes with these drugs and analyzed gene expression by quantitative real-time PCR. Cytokine secretion was measured by ELISA.
Results: In the present study, we could show that ATF3 is induced in PBMCs by DMF and weakly by Ebselen, while CsA is the most prominent inducer of ATF3 in keratinocytes without enhancing HO-1 transcription. Further we could show that induction of stress by LPS treatment elevates IL-1β and IL-6 and weakly ATF3 transcription in PBMCs. While transcription of both cytokines is elevated, LPS treatment mediates IL-6 secretion with only little IL-1β secretion. Treatment with DMF dampens LPS-induced transcription.
Conclusions: Taken together, our results shed light into the different carcinogenic potential of CsA and DMF, which both target ATF3. Collectively our data demonstrate that CsA strongly induces pro-carcinogenic ATF3 in keratinocytes, whereas ATF3 induction by DMF in myeloid cells acts anti-inflammatory.

1. Introduction

The most important inflammatory skin diseases such as psoriasis, lichen planus or atopic dermatitis are characterized by a hyperplasia of the epidermis. In the vast majority of cases hyperproliferation is controlled and almost never leads to cancer with the exception of lichen planus. One key factor which might control and regulate proliferation is the activating transcription factor 3 (ATF3) which is a member of the cAMP responsive element-binding (CREB) family of transcription factors. It has been shown earlier that ATF3 can dimerize with other ATF/CREB proteins including ATF2, c-Jun and Jun B [1]. Already ATF3 alone is an important transcriptional modulator that limits the inflammatory response by controlling the expression of various cytokines and chemokines. It is known that, ATF3 critically regulates the function of different immune cells, thus ATF3 expression in macrophages is necessary for governing basal INF-β expression [2]. In resting macrophages, NK cells and CD4+ T cells ATF3 expression is maintained at a low level. Upon activation, ATF3 expression is induced. Furthermore, ATF3 can directly interfere with NF-κB and AP-1 driven promoters, resulting in decreased expression of inflammatory cytokines, such as Interleukin (IL)-6 [3]. Due to the decreased expression of inflammatory cytokines ATF3 is able to limit the intensity of the inflammatory responses. One of the most potent inducers of ATF3 is Cyclosporine A (CsA), a potent immunosuppressant used in organ transplantation to prevent rejection. Besides this clinical application CsA is used

* Corresponding author at: Department of Dermatology, University of Tübingen, Liebermeisterstr. 25, 72076 Tübingen, Germany.
E-mail address: Amir.Yazdi@med.uni-tuebingen.de (A.S. Yazdi).

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for treatment of patients with rheumatoid arthritis, atopic dermatitis, psoriasis, Crohn's disease and nephrotic syndrome. CsA dampens lymphocyte activation by forming a complex with cyclophilin to block the phosphatase activity of calcineurin that in turn decreases the production of inflammatory cytokines produced by T lymphocytes [4]. In addition to its desired anti-inflammatory effect, CsA strongly increases the risk of non-melanoma skin cancer in patients, particularly after chronic UV exposure [5], via the suppression of p53-dependent senescence [6].

Carcinogenesis is usually induced by oncogenic mutations and is often supported by an inflammatory microenvironment. Although ATF3-dependent p53-suppression leads to cancer promotion, ATF3 inhibition significantly increases the expression of proinflammatory cytokines, especially of IL-6 [7] or IL-8 [8]. The interplay between putative pro-oncogenic and anti-inflammatory aspects of ATF3 is important for the treatment of psoriasis or multiple sclerosis with Dimethylfumarate (DMF). Besides inducing ATF3 transcription [9], DMF can induce type II dendritic cells via increased hemoxygenase-1 (HO-1) and activating the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway [10]. Despite long-term treatment for many years, patients that receive DMF for psoriasis have no increased risk of non-melanoma skin cancer. Thus, both DMF and CsA induce the pro-tumorigenic ATF3, but only CsA treatment increases the risk of non-melanoma skin cancer. This raises the question, under why the induction of ATF3 results in uncontrolled hyperproliferation of the skin finally progressing to skin cancer only upon CsA, but not after DMF treatment. In patients with cutaneous squamous cell carcinoma (SCC) which represents the most important cutaneous complication following organ transplantation, ATF3 is specifically induced, and its expression is strongly potentiated by combination of Cyclosporine A and UV treatment [11].

Due to this clinical and experimental evidence it is still unknown whether ATF3 functions as a tumor suppressor or as tumor promoter. In the present study, we investigate in a cell type specific manner under which conditions ATF3 can be induced, and how this relates to cytokine expression and secretion.

2. Materials and methods

2.1. Isolation and cultivation of primary cells

Primary keratinocytes were isolated from healthy human foreskin by using 10 mg/ml Dispase (Roche, Basel, Swizerland) to separate the epidermis from the dermis as described[12]. The epidermal layer was separated into single cells and cultivated in CnT-07medium (CELLnTEC, Bern, Swizerland). Peripheral human blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll gradient centrifugation. For adherence of PBMCs, cells were cultured for 1 h in RPMI (Biochrom, Berlin, Germany) without FCS and the adherent cell fraction was further cultivated in RPMI with 10% FCS for 24 h.

2.2. Induction of ATF3 in PBMCs and keratinocytes

Primary human keratinocytes and PBMCs were stimulated with Dimethylfumarate (DMF) (70 μM and 35 μM) (Sigma Aldrich, St. Louis, US), Cyclosporin A (10 μM and 5 μM) (Enzo Life Sciences GmbH, Lörrach, Germany) and Ebselen (40 μM and 20 μM) (Enzo Life Sciences GmbH, Lörrach, Germany) for 2 to 6 h to induce ATF3 on mRNA level. In addition, keratinocytes were treated with 100 nM of 12-*O*-Tetradecanoylphorbol-13-acetate (TPA, phorbol 12-myristate 13-acetate, PMA) (Invivogen, San Diego, US).

2.3. Activation of the NLRP3-inflammasome in myeloid cells

In addition to the initial induction of ATF3 by DMF PBMCs were treated with 100 ng/ml LPS (Invivogen, San Diego, California) for 2 h. To activate the NLRP3-inflammasome cells were additionally treated with 5 mM ATP (Invivogen, San Diego, California) for 30 min.

2.4. RNA isolation and gene expression analysis

Total RNA (Peq Gold total RNA Kit Sline, VWR, Erlangen, Germany) and reverse transcription (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, Thermo Fischer Scientific, Waltham, Massachusetts) were performed according to the manufacturer's instructions. Quantitative real-time-PCR (Roche LightCycler 480 system) was performed to determine gene expression using the LightCycler 480 instrument (Roche, Basel, Swizerland). The expression of the specified genes was calculated relative to the expression of the housekeeping gene β-actin. Primer and probe sequences are listed in Table 1.

2.5. Cytokine analysis

Human IL-6 and IL-1β secretion was measured in cell-free supernatants using R&D ELISA kits (Human IL-1 beta/IL-1F2 Duoset ELISA, Human IL-6 DuoSet ELISA) according to the manufacturer's instructions.

Table 1
Oligonucleotide primers and LightCycler hybridization probes.

Target gene	Accession no.	Primer	Primer sequence	Purpose
ATF3	P18847	ATF3 A	AAATGCTGCTTCTCGTTCTTGA	qPCR
		ATF3 S	GGAGTGCCTGCAGAAAGAGTC	qPCR
		ATF3 P	6FAM-TCCTCAATCTGAGCCTTCAGTTCAGCA–BBQ	Hybridization Probe
ACTB	P60709	ß-Actin F	AGCCTCGCCTTTGCCGA	qPCR
		ß-Actin R	CTGGTGCCTGGGGCG	qPCR
		ß-Actin TM	6FAM-CCGCCGCCCGTCCACACCCGCC–BBQ	Hybridization Probe
IL1A	P01583	IL1 A F	GAAGGCTGCATGGATCAATCTGT	qPCR
		IL1 A R	GTGAGGTACTGATCATTGGCTCG	qPCR
		IL1A TM	6FAM-CGGGAAGGTTCTGAAGAAGAGACGGT–BBQ	Hybridization Probe
IL1B	P01584	IL–1B S	CAGGGACAGGATATGGAGCAA	qPCR
		IL–1B R	ATGTACCAGTTGGGGAACTG	qPCR
		IL–1B TM	6FAM-AGAATCTGTACCTGTCCTGCGTGTTGAA–BBQ	Hybridization Probe
IL6	P05231	IL–6 U	CCAGAGCTGTGCAGATGAGTACA	qPCR
		IL-6 B	CCTGCAGCTTCGTCAGCA	qPCR
		IL-6 TM	6FAM-CATTTGTGGTTGGGTCAGGGGTGGT–BBQ	Hybridization Probe
HMOX1	P09601	HMOX1 F	ACTGCGTTCCTGCTCAACAT	qPCR
		HMOX1 R	GCATAAAGCCCTACAGCAACTG	qPCR
		HMOX1 TM	6FAM-CAGGAGCTGCTGACCCATGACACCAA–BBQ	Hybridization Probe

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