

INVESTIGATIVE REPORT

Expression of Allograft Inflammatory Factor-1 in Inflammatory Skin Disorders

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Allograft inflammatory factor-1 (AIF-1) is an evolutionarily conserved, inflammatory protein produced by activated macrophages during chronic transplant rejection and in inflammatory brain lesions. Since T-cell-mediated inflammation is common to various dermatoses and nothing is known about AIF-1 in skin, we studied its protein expression at the tissue level and regulation in monocytic cell lines by various agents. Using immunohistochemistry, we found that AIF-1 is expressed at low levels in normal skin, but is highly upregulated in various inflammatory skin disorders, such as psoriasis, lichen planus, graft-versus-host disease and mycosis fungoides. The main cell types expressing AIF-1 in affected skin are macrophages and Langerhans' cells. We also show by real-time PCR that AIF-1 mRNA levels in monocytic THP-1 and U937 cell lines are significantly upregulated by retinoic acid as well as a number of cytokines. We conclude that AIF-1 may mediate survival and pro-inflammatory properties of macrophages in skin diseases. Key words: CD-163; Langerhans' cell; macrophage; immunohistochemistry; retinoic acid.

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Allograft inflammatory factor-1 (AIF-1) was originally cloned from activated macrophages in rat atherosclerotic allogeneic heart grafts undergoing chronic transplant rejection (1). The full-length AIF-1 is a 147 amino-acid (17 kDa) interferon- γ (IFN- γ) inducible Ca²⁺-binding protein. AIF-1 expression has been associated with inflammatory lesions of the central nervous system, stimulation of smooth muscle cell proliferation and the pathogenesis of insulin-dependent diabetes mellitus (2). However, it is not only associated with allograft inflammation, but could also function in cell-mediated autoimmune responses.

We have previously studied the α -helical coiled-coil rod homolog (HCR) as a psoriasis candidate gene located in the PSORS1 locus in the major histocompatibility complex (MHC) region on chromosome 6p21.3 (3).

In the process of identifying genes co-regulated with HCR (see <http://microarray.genomecenter.columbia.edu/cgi-bin/find-links.cgi>), we found a set of genes to be consistently co-expressed with HCR, among them AIF-1, which mapped to the same MHC region on chromosome 6 known for clusters of genes involved in the inflammatory response. Since T-cell-mediated inflammation is one of the features of psoriasis, and since no data exist on AIF-1 in cutaneous biology, we decided to investigate the relevance of AIF-1 in skin.

We show here that AIF-1 is expressed at low levels in normal skin, but is highly upregulated in various inflammatory skin disorders. The main cell types expressing AIF-1 in affected skin are macrophages and Langerhans' cells. We also show that AIF-1 mRNA levels in monocytic cell lines are strongly upregulated by retinoic acid as well as a number of cytokines.

MATERIALS AND METHODS

Clinical specimens

Formalin-fixed paraffin-embedded specimens of psoriasis, non-lesional and lesional ($n=10$), X-linked ichthyosis ($n=3$), prurigo nodularis ($n=3$), atopic dermatitis ($n=2$), mycosis fungoides ($n=3$), lichen ruber planus ($n=2$), pityriasis rubra pilaris ($n=4$), chronic wounds ($n=5$), normally healing timed wounds ($n=3$), graft-versus-host reaction ($n=4$) and normal skin ($n=4$) were obtained from the Department of Dermatopathology, University of Helsinki, Finland. The diagnoses were confirmed by an experienced dermatopathologist (LJ). The use of the archival human samples was approved by the relevant ethics committee and followed the Declaration of Helsinki Guidelines.

Immunohistochemistry

Immunostaining was performed using the avidin-biotin-peroxidase complex technique (Vectastain ABC kit; Vector Laboratories, Inc. Burlingame, CA, USA). Diaminobenzidine (DAB) was used as chromogenic substrate, Mayer's solution as counter-stain, and goat polyclonal antibodies diluted 1:600 to target the AIF-1 protein (ab5076, Abcam, Cambridge, UK). Sections were pre-treated by 30 min incubation in 1 \times Target Retrieval Solution buffer (DAKOcytation) in a 95°C water bath. As negative controls, sections were treated with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). Serial sections were stained for Langerhans' cells (CD1a; MTB1, 1:20, Novocastra Laboratories, Newcastle Upon Tyne, UK), macrophages (CD163; 10D6, 1:100, Novocastra Laboratories) and T cells (CD3; A0452, 1:75, Dako A/S, Glostrup, Denmark).

Double-immunostaining for AIF-1 and CD163 or CD1a was performed with peroxidase-anti-peroxidase technique combining Vector mouse and goat kits. After pre-treatment in 95°C citrate buffer, pH 6.0, CD163 antibodies (1:100) or CD1a antibodies (1:15) were incubated on sections for 1 h at 37°C and detected using aminoethylcarbazole (AEC) (red) as chromogenic substrate. After the protein blocking solution treatment, sections were incubated with AIF-1 antibodies (1:600) overnight at 4°C and the immuno-signal was detected using DAB with nickel enhancement (brown-black) as a chromogenic substrate. Semi-quantitative immunoreactivity grading for AIF-1 (Table I) was based on the number of positive cells, which was assessed as follows, using 200× magnification of a light-field microscope: + a few AIF-1 positive cells; ++ moderate number of positive cells; +++ a large number of positive cells. The slides were analysed independently by two experienced investigators (LJ and US-K).

Cell cultures and stimulations

Expression of AIF-1 mRNA was studied in two monocytic cell lines, THP-1 and U937. The cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) (GIBCO Invitrogen Life Technologies, Paisley, UK), 1 mM sodium pyruvate, 10 mM HEPES, 100 U penicillin and 100 µg/ml streptomycin (Sigma, St Louis, MO, USA, and Roche Molecular Biochemicals, Indianapolis, IN, USA). The medium for THP-1 cells was further supplemented with 0.05 mM β-mercaptoethanol. Medium was changed three times a week and cell density kept at 0.2–1×10⁶ cells/ml. To study the regulation of AIF-1, 1.4×10⁶ monocytes in 2 ml cell culture were plated on 6-well plates and grown overnight. Cells were depleted of serum overnight prior to 24 h of stimulation with tumour necrosis factor-α (TNF-α, 10 ng/ml, Sigma), basic fibroblast growth factor (bFGF, 10 ng/ml, Sigma), epidermal growth factor (EGF, 10 ng/ml, Sigma), insulin-like growth factor-1 (IGF-1 100 ng/ml, Sigma), vascular endothelial growth factor (VEGF, 10 ng/ml, Sigma), interleukin-1 beta (IL-1β, 5 U/ml, Roche Molecular Biochemicals), IFN-γ, 10 ng/ml, (Sigma), dexamethasone (Dxm, 1×10⁻³M, Sigma), all-trans retinoic acid (RA, 1×10⁻⁶ M, Sigma) or lipopolysaccharide (LPS, 10 ng/ml, Sigma). As controls, cells incubated in fresh serum-free medium for 24 h were used. Total cellular RNA was extracted from the cells using the RNeasy miniprep-Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. 250 ng of total RNA was reverse transcribed into cDNA using the SuperScriptTMIII First-Strand kit (Invitrogen, Carlsbad, CA, USA) with oligo dT, and used as a template for reverse transcript PCR and quantitative real-time PCR (qRT-PCR). All cell stimulations were carried out three times and each sample was run in triplicate in the qRT-PCR.

Table I. Expression of allograft inflammatory factor-1 (AIF-1) by immunohistochemistry in various dermatoses

Sample	Macro-phages	Langerhan's cells	Lympho-cytes	Neutro-philis
Psoriasis	+++	++	+	-
Mycosis fungoides	+++	+++	+	-
Pityriasis rubra pilaris	+	+	+	-
Lichen planus	+++	+	-	-
X-linked ichthyosis	+	+	-	-
Prurigo nodularis	+++	+	+	+
Atopic dermatitis	+	+	-	-
Graft-versus-host	++	+	+	-
Chronic venous ulcer	++	-	+	+
Acute wound	+	+	+	+

Reverse transcript PCR and quantitative real-time PCR (TaqMan RT-PCR)

AIF-1 primers used for reverse transcript PCR amplification were: forward 5'-GACCTTAATGGAAATGGCGATA-3' and reverse 5'-ATCTCTTGCCAGCATCATC-3'. GAPDH primers were used as an endogenous control (4). PCR was carried out in 25 µl reactions containing 2 µl cDNA, 1× reaction buffer for HotStarTaq polymerase, 200 nM of each primer, 200 µM dNTPs and 0.75 U of HotStarTaq polymerase (Qiagen). PCR was carried out as follows: 95°C for 15 min, (95°C for 20 s, 56°C for 30 s and 72°C for 60 s) times 40 cycles for AIF-1 and 25 cycles for GAPDH, followed by 72°C for 3 min. For qRT-PCR, commercially available gene expression assays were used (TaqMan[®] Gene Expression Assays, Applied Biosystems, Foster City, CA, USA). Two assays for AIF-1 (assay identification and order numbers: Hs00741549g1 and Hs00610419g1) labelled with FAM[™] reporter dye, and as an endogenous control GAPDH (4310884E) labelled with VIC[™] reporter dye was used. The reaction was performed in 10 µl volume mix containing 2 µl cDNA, 1× pre-formulated assay mix and 1× TaqMan[™] Universal Master Mix and run as follows: 50°C for 2 min, 95°C for 10 min (95°C for 15 s and 60°C for 1 min) times 40. The reaction was performed with the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems).

Statistics

The statistical significance of the differences in mRNA expression levels in TaqMan analysis was calculated using Student's unpaired, two-tailed *t*-test.

RESULTS

AIF-1 was detected by immunohistochemistry in several different cell types in samples representing various inflammatory dermatoses (Table I). It was expressed by macrophages particularly in psoriasis, lichen planus, mycosis fungoides, and graft-versus-host disease (Fig. 1A–C, G, H). AIF-1 was also frequently detected in Langerhans' cells, particularly in psoriasis, lichen planus and mycosis fungoides (Fig. 2A, B). Subsets of positive lymphocytes were seen in, e.g. mycosis fungoides (Fig 2C, D) and chronic wounds. More rarely, positive neutrophils were detected, e.g. in wounds or prurigo nodularis (Fig. 2E). The degree of inflammation clearly correlated positively with the number of AIF-1 positive cells (Fig. 1A, B versus D). In normal skin, scattered macrophages and dendritic cells expressed AIF-1 (Fig. 1D–F). AIF-1 protein was particularly upregulated in psoriasis, lichen planus, graft-versus-host disease and mycosis fungoides (Table I). However, keratinocytes were never positive in any of the samples examined.

Since AIF-1 was expressed particularly by macrophages on tissue level, we studied AIF-1 mRNA expression and regulation by qRT-PCR using two different monocytic cell-lines, THP-1 and U937, treated with various agents for 24 h. In THP-1 cells, AIF-1 mRNA was significantly upregulated by RA, IFN-γ, IL-1β, and VEGF, but downregulated by TNF-α and bFGF (Fig. 3A). EGF, IGF, LPS and Dxm had no significant

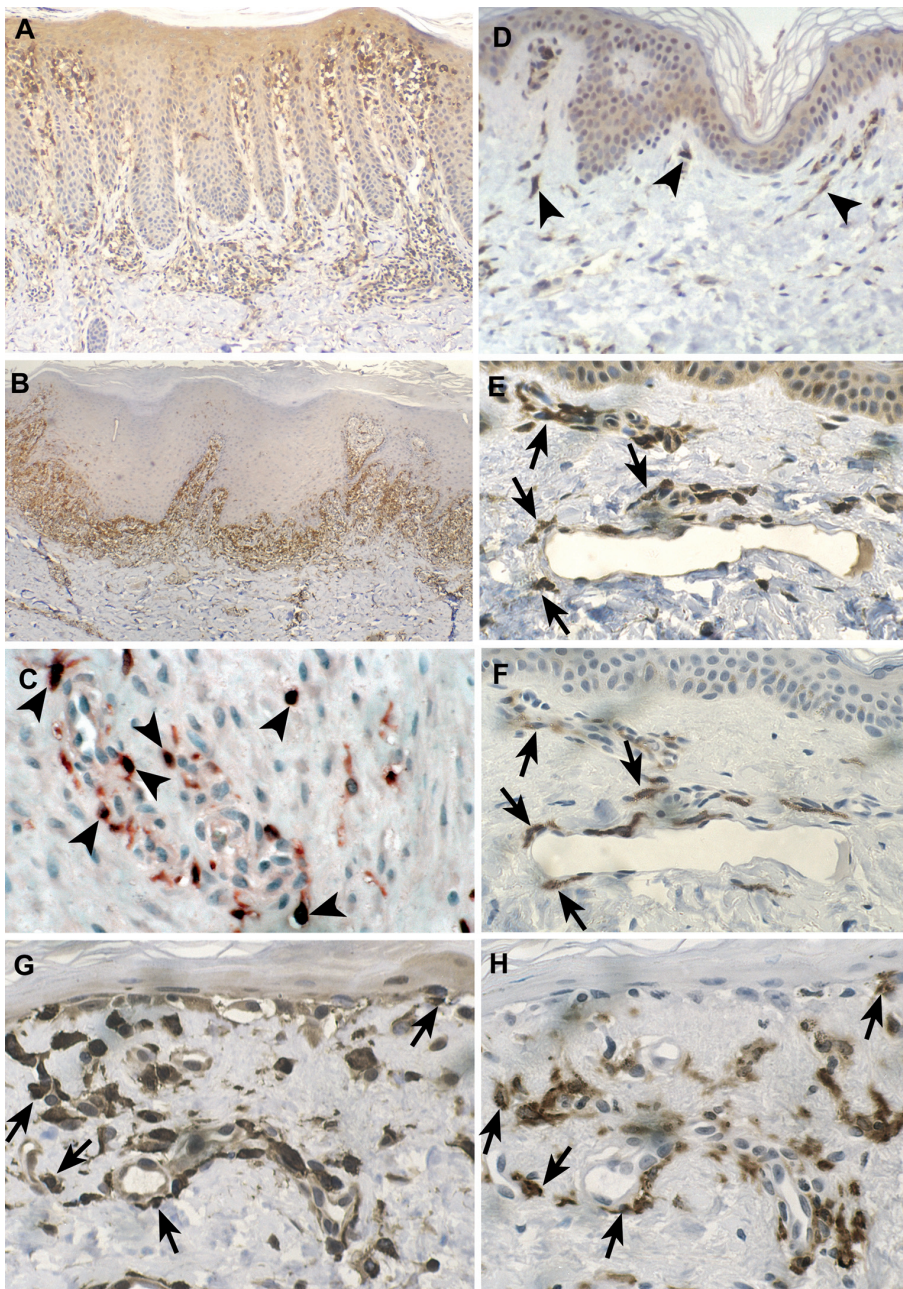


Fig. 1. Allograft inflammatory factor-1 (AIF-1) is upregulated in macrophages in autoimmune and inflammatory dermatoses. Immunostainings for AIF-1 protein in (A) psoriasis and (B) lichen planus. (C) Double immunostaining of a chronic wound reveals that a subset of CD-163 positive macrophages (red) express AIF-1 protein (dark brown-black) (arrowheads). AIF-1 is expressed by occasional stromal cells in normal skin (D; arrowhead, E; arrow) and most of these are macrophages as assessed by CD-163 staining in serial sections (F). In graft-versus-host-disease the predominant cell type expressing (G) AIF-1 are (H) CD-163 positive macrophages. Arrows in E and F, and G and H, respectively, depict corresponding cells. Magnification: (A, B) $\times 100$, (D) $\times 200$ and (C, E–H) $\times 400$.

effect on AIF-1 expression in THP-1 cells. In U937 cells, AIF-1 was significantly upregulated by RA and EGF and downregulated by Dxm (Fig. 3B). Stimulations with IGF, VEGF, IFN- γ , IL-1 β , TNF- α , bFGF or LPS had no significant effect. The only agent that upregulated AIF-1 in both cell lines was RA. AIF-1 mRNA was also detected by qRT-PCR in testis, placenta and skin (Fig. 4).

DISCUSSION

AIF-1 is an evolutionarily conserved protein produced by activated monocytic cells in a variety of traumatic, inflammatory and degenerative lesions. It has been shown to be involved in inflammatory responses and has documented expression in human cells, such as macro-

phages, neutrophils (1) and activated T-lymphocytes, and in various human tissues, such as transplanted hearts (5), brain (6) and endometriotic tissue (7). But so far AIF-1 expression has not been studied in the skin.

We detected AIF-1 by immunohistochemistry in several different cell types in samples representing various inflammatory dermatoses. Our results are in line with previous data on the presence of AIF-1 in human macrophages in transplanted human hearts (1), in CD3-positive lymphocytes in human coronary arteries with vasculopathy (5), its expression by rat intestinal dendritic cells (8) and in neutrophils of rat cardiac allografts (1). The degree of inflammation clearly correlated positively with the number of AIF-1 positive cells, which was substantial in psoriasis, lichen planus,

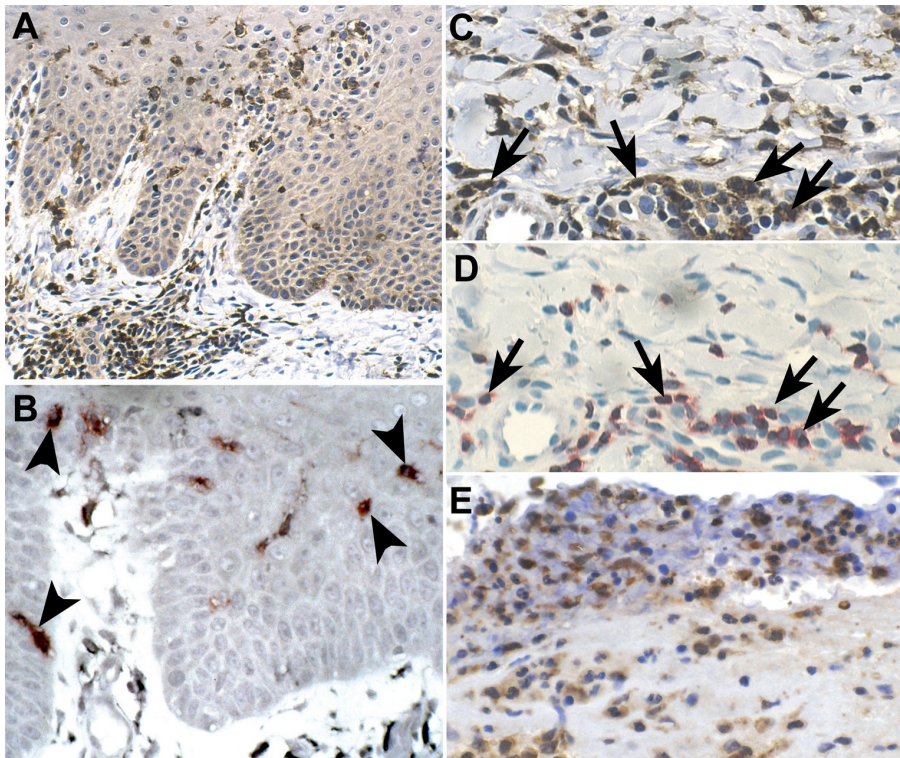


Fig. 2. Allograft inflammatory factor-1 (AIF-1) can also be expressed by dendritic cells, a subset of lymphocytes, and neutrophils in skin disorders. (A) Expression of AIF-1 protein by Langerhans' cells in psoriasis. (B) Double immunostaining for AIF-1 protein (dark brown-black) and CD1a positive Langerhans cells (red) in mycosis fungoides demonstrates that in addition to macrophages, also Langerhans cells produce AIF-1 (arrowheads). (C) Staining for AIF-1 protein in mycosis fungoides and (D) a serial section stained for CD3-positive lymphocytes shows that a subset of them express AIF-1. (E) AIF-1 expression in neutrophils at the surface of a healing wound. Arrows in (C) and (D) depict corresponding cells. Magnification: (A) $\times 200$, (B–F) $\times 400$.

graft-versus-host disease and mycosis fungoides. In these disorders, AIF-1 may play a role in the immune activation and function of macrophages (7) or influence lymphocyte activation after inflammatory stimuli (5).

So far the only agents with a documented inducing effect on AIF-1 have been IFN- γ in rat macrophages (1), IL-1 β and TNF- α in CRL-2192 rat macrophage cell line (9) and T-cell-conditioned media in vascular smooth muscle cells (10). The anti-inflammatory drug sodium salicylate downregulates AIF-1 mRNA (9). Here we have studied two human monocytic cell lines using

nine different agents, all with documented inflammatory properties, and eight of them showed significant up- or down-regulation of AIF-1 mRNA in at least one of the two cell lines. THP-1 and U-937 cell lines are derived from the monocyte/macrophage lineage, but U937 represents a later maturation stage (11). Interestingly, the regulation pattern differed between the two monocytic cell lines, indicating a rather specific function of AIF-1, where the variation might be dependent on the maturation stage of the cells. Furthermore, the regulation of AIF-1 seems cell-type specific, as LPS stimulates its

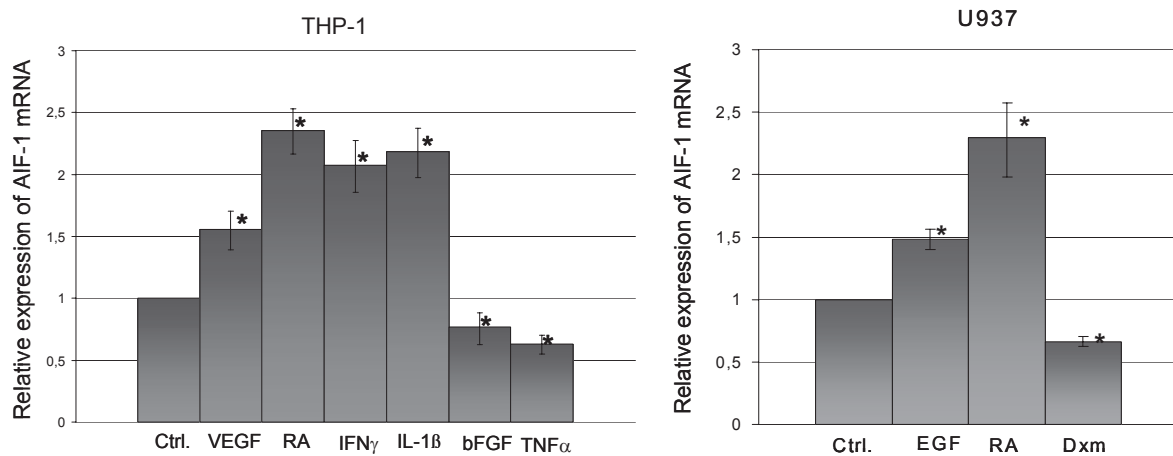


Fig. 3. Relative expression of allograft inflammatory factor-1 (AIF-1) mRNA in non-stimulated and stimulated U937 and THP-1 monocytes assessed by quantitative real-time PCR. Results are shown relative to mRNA levels from non-stimulated U937 and THP-1 monocytes, assigned the value 1. (A) In THP-1 cells AIF-1 was significantly upregulated by vascular endothelial growth factor (VEGF), retinoic acid (RA), interferon- γ (IFN- γ), interleukin-1 beta (IL-1 β) and downregulated by basic fibroblast growth factor (bFGF) and tumour necrosis factor- α (TNF- α). (B) AIF-1 mRNA expression in U937 monocytes was significantly upregulated by endothelial growth factor (EGF) and RA and downregulated by dexamethasone (Dxm). * $p < 0.05$.

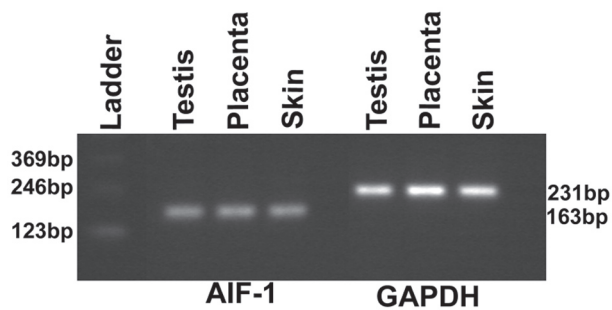


Fig. 4. Reverse transcript PCR showing expression of allograft inflammatory factor-1 (AIF-1) (163 bp) and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (231 bp) in human testis, placenta and skin.

expression in the brain (12) but not in our monocytic cell lines. RA was the only agent inducing AIF-1 mRNA in both cell lines. Retinoids are widely used anti-psoriatic drugs that inhibit keratinocyte proliferation and counteract inflammation (13). At the tissue level, Dxm reduces AIF-1 positive macrophages in the brain (14). We found Dxm to downregulate AIF-1 in U937 cells.

In conclusion, our results suggest that AIF-1 expression may play a role in mediating survival, migration and pro-inflammatory properties of macrophages in skin disorders. Furthermore, the use of corticoids or retinoids in certain skin diseases may alleviate them partly due to their regulatory effects on AIF-1.

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