

Expression of nonmuscle cofilin-1 and steroid responsiveness in severe asthma

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Background: Glucocorticoids are the mainstay of asthma therapy; however, a proportion of patients with asthma has a severe form of the disease that fails to respond to therapy. Understanding the molecular mechanisms behind glucocorticoid-insensitive asthma is therefore of clinical importance. Evidence in glucocorticoid-unresponsive Henrietta Lack (HeLa) cells indicated that cofilin-1 could act as an inhibitor of glucocorticoid function.

Objective: To determine whether cofilin-1 expression is abnormally expressed in cells from patients with severe glucocorticoid-insensitive asthma and examine the effect of cofilin-1 overexpression on glucocorticoid function.

Methods: Peripheral blood CD4⁺ T cells were purified from 16 subjects with severe glucocorticoid-insensitive asthma and 16 subjects with mild glucocorticoid-sensitive asthma, and cofilin-1 expression was determined by quantitative real-time RT-PCR and Western blotting. The effect of dexamethasone on cofilin-1 expression was determined in Jurkat T cells, and the effect of cofilin-1 overexpression on anti-CD3/CD28-stimulated IL-2 release was measured.

Results: Peripheral blood CD4⁺ T cells from subjects with severe glucocorticoid-insensitive asthma are less responsive to dexamethasone than cells from subjects with mild glucocorticoid-sensitive asthma. Cells from these patients express significantly ($P < .05$) higher levels of cofilin-1 than cells from subjects with mild asthma. Dexamethasone did not affect cofilin-1 expression in Jurkat T cells. Functionally, dexamethasone suppression of anti-CD3/CD28-stimulated IL-2 was attenuated in Jurkat cells overexpressing cofilin-1.

Conclusion: These results suggest that increased cofilin-1 expression may be important in the regulation of glucocorticoid sensitivity in peripheral blood lymphocytes of patients with severe treatment-insensitive asthma.

Clinical implications: Understanding the mechanisms of enhanced cofilin-1 expression may lead to the development of

new therapies for severe treatment-insensitive asthma.
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Key words: Cofilin-1, peripheral blood T cells, glucocorticosteroids, severe asthma, Jurkat T cells

Asthma is a chronic inflammatory disease of the airways, and as such, glucocorticoids are currently the mainstay of asthma therapy. Glucocorticoids reduce the numbers of inflammatory cells in the airway submucosa, resulting in effective control of symptoms and prevention of exacerbations.¹ However, glucocorticoids are not effective in all patients with asthma, and there is a spectrum of responsiveness.^{2,3} Some patients respond poorly to even high doses of inhaled and oral glucocorticoids and remain unwell despite ongoing therapy, with removal of glucocorticoids exacerbating their disease. For research purposes, these glucocorticoid-insensitive subjects with asthma (SIAs) are usually defined as those patients showing a lack of improvement in lung function after 14 days treatment with high-dose oral glucocorticoids.⁴

T lymphocytes play a key role in the inflammatory response in asthma,⁵ and importantly, T cells of SIA subjects appear to be less responsive to the suppressive effects of glucocorticoids on proliferation than those of patients with mild asthma, thus mimicking the effects of glucocorticoids in the airways.⁶ Furthermore, this differential response is limited to CD4⁺ cells,⁷ particularly when cells are stimulated by T-cell receptor (CD3) cross-coupling with CD28 costimulatory molecules. This suggests that CD4⁺ cells are of greater importance than CD8⁺ cells when trying to understand steroid-dependent asthma.

Recently, cofilin-1 has been implicated as an inhibitor of glucocorticoid receptor action in hormone-resistant HeLa cells.⁸ Here a dexamethasone-resistant clone was analyzed by 2-dimensional gel electrophoresis, and a differentially expressed protein, cofilin-1, was found to inhibit glucocorticoid receptor activity. In addition, overexpression of cofilin-1, in addition to causing cytoskeleton disruption, reduced glucocorticoid function through upregulation of c-Jun.⁸

Nonmuscle type cofilin-1 is dephosphorylated, leading to its activation after anti-CD3/CD28 costimulation, but not after anti-CD3 stimulation alone.⁹ Cofilin-1 binds actin, converting it to its active monomeric form, and transports it into the nucleus. This monomeric (G-) actin can activate RNA polymerase II and therefore promote gene transcription. It also inhibits DNase I, and increased levels

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Abbreviations used

CR: Glucocorticoid-resistant
JNK: c-Jun N-terminal kinase
MA: Mild glucocorticoid-sensitive asthma
SIA: Severe glucocorticoid-insensitive asthma
UK: United Kingdom

of activated cofilin-1 lead to increased inhibition of apoptosis, accounting for the importance of cofilin-1 in the growth and survival of T cells.¹⁰ In untransformed cells, cofilin-1 is activated by dephosphorylation at serine residue 3¹¹ by the phosphatases protein phosphatase 1 and protein phosphatase 2A.¹² In contrast, inactivation occurs via phosphorylation at the same residue by Lin-11 Isl-1 Mec-3 kinase 1.^{13,14}

Here we show that peripheral blood CD4⁺ T cells from SIA subjects are less responsive to dexamethasone than cells from subjects with mild glucocorticoid-sensitive asthma (MAs) and express significantly ($P < .05$) higher levels of cofilin-1 mRNA and protein. Dexamethasone did not directly affect cofilin-1 mRNA or protein expression in Jurkat T cells. However, functionally, dexamethasone suppression of anti-CD3/CD28-stimulated IL-2 production was attenuated in Jurkat cells overexpressing cofilin-1. These results suggest that increased cofilin-1 expression may be important in the regulation of glucocorticoid sensitivity in peripheral blood lymphocytes of patients with SIA.

METHODS

Patient selection

Patients with mild glucocorticoid-sensitive asthma and SIA patients ($n = 16$ per group) attending the Royal Brompton Hospital asthma clinic were selected (Table I). Asthma was defined according to American Thoracic Society criteria as reversible airways obstruction (by $\geq 15\%$).¹⁵ Patients with SIA showed good reversibility to bronchodilator ($22 \pm 8\%$; Table I), and none were current smokers. Two SIA patients were exsmokers with < 5 pack years, and the group had normal transfer coefficient (KCO) values ($104.4 \pm 3.4\%$ predicted). MA was defined as those using inhaled glucocorticoids (400–1000 $\mu\text{g}/\text{d}$) and, as necessary, bronchodilators. SIA was defined according to the European Respiratory Society Difficult Asthma Task Force.⁴ All patients were treated within a tertiary respiratory clinic, and any provoking factors were identified, including allergen exposure,⁴ and managed according to the Royal Brompton Hospital (RBH) severe asthma protocol.¹⁶ All SIAs were using maximal bronchodilator therapy and inhaled glucocorticoids and a stable maintenance oral prednisolone (2–40 mg/d). Lung function and symptoms had previously been documented to worsen on removal of oral glucocorticoid therapy. All subjects gave informed, written consent, and the study was approved by the local Ethics Committee (Royal Brompton Hospital).

CD4⁺ cell purification

CD4⁺ T cells were obtained from 30 mL whole blood by negative selection using T-cell enrichment columns (R&D Systems, Abingdon, United Kingdom [UK]) according to the manufacturer's

TABLE I. Characteristics of patients with MA and SIA*

	MA (n = 16)	SIA (n = 16)
Age (y)	41.9 $\pm$ 4.1	49.7 $\pm$ 3.7
Sex	9 F/7 M	11 F/5 M
FEV ₁ (% predicted)	81% $\pm$ 2%	43% $\pm$ 5%
baseline		
FEV ₁ (% predicted)	98% $\pm$ 2%	64% $\pm$ 5%
post-albuterol		
% Reversibility	18% $\pm$ 3%	22% $\pm$ 8%
Smoking history	0/16	2 Exsmokers/16
Diffusion capacity	ND	104.4% $\pm$ 3.4%
(KCO, % predicted)		
Medication	Bronchodilators, ICS	Bronchodilators, ICS, OCS (2–40 mg/d)

F, Female; ICS, inhaled corticosteroid; M, male; ND, not determined; OCS, oral corticosteroid.

*Means $\pm$ SEMs are shown.

instructions. Purity of the CD4⁺ cell population was tested by flow cytometry as previously described.¹⁷ Cell populations with $>97\%$ purity of CD4 cells were used in the Taqman analysis. In some experiments, cells were stimulated with anti-CD3/anti-CD28 antibodies, and the effect of dexamethasone pretreatment on T-cell proliferation was measured as previously described.¹⁸

RNA extraction and cDNA preparation

RNA was extracted from the purified CD4⁺ cells using an RNeasy (mini) RNA extraction kit (Qiagen, Crawley, UK). Samples were DNase-treated to remove any contaminating genomic DNA, and first-strand cDNA was prepared from 500 ng RNA using Superscript reverse transcription kit (Gibco; Invitrogen Corp, Paisley, UK).

Taqman real-time PCR

Taqman PCR reactions were set up for cofilin-1 (900 nmol/L F: 5'-TCTCGGTGCCCTCTCCTTT and R: 5'-TGACACCATCAGAGA CAGCCA primers and 125 nmol/L probe 5'-carboxyfluorescein-TCCGGAACATGGCCTCCGGT-N, N, N', N'-tetramethyl-6-carboxyrhodamine, denaturation 95°C for 15 seconds and annealing/extension 60°C for 1 minutes for 40 cycles) using a Taqman PCR Mastermix (Applied Biosystems, Warrington, UK). A duplicate set of reactions was run using primers and probe for 18S ribosomal RNA as a cDNA loading control (Applied Biosystems). Samples were quantified against a standard curve containing a 10-fold dilution series of the target DNA (in a plasmid) in the range of 10^8 to 10^1 copies per reaction.

Western blot analysis

CD4⁺ cells were isolated from 50 mL blood as described from 6 of 16 patients per group, and whole cell lysates were prepared. Western blot analysis was performed as previously described¹⁹ using a 1:1000 dilution rabbit antihuman cofilin-1 antibody (Totem Biologicals, Northampton, UK). The expression of β -actin was also detected to confirm equal protein loading.

Dexamethasone treatment of J6 Jurkat cells

J6 Jurkat cells were stimulated with anti-CD3 and anti-CD28 antibodies (500 ng/mL final concentration; BD Pharmingen, Cowley, UK) and incubated at 37°C for 24 hours before being treated with 100 nmol/L dexamethasone (Sigma, Poole, UK). Whole cell lysates were

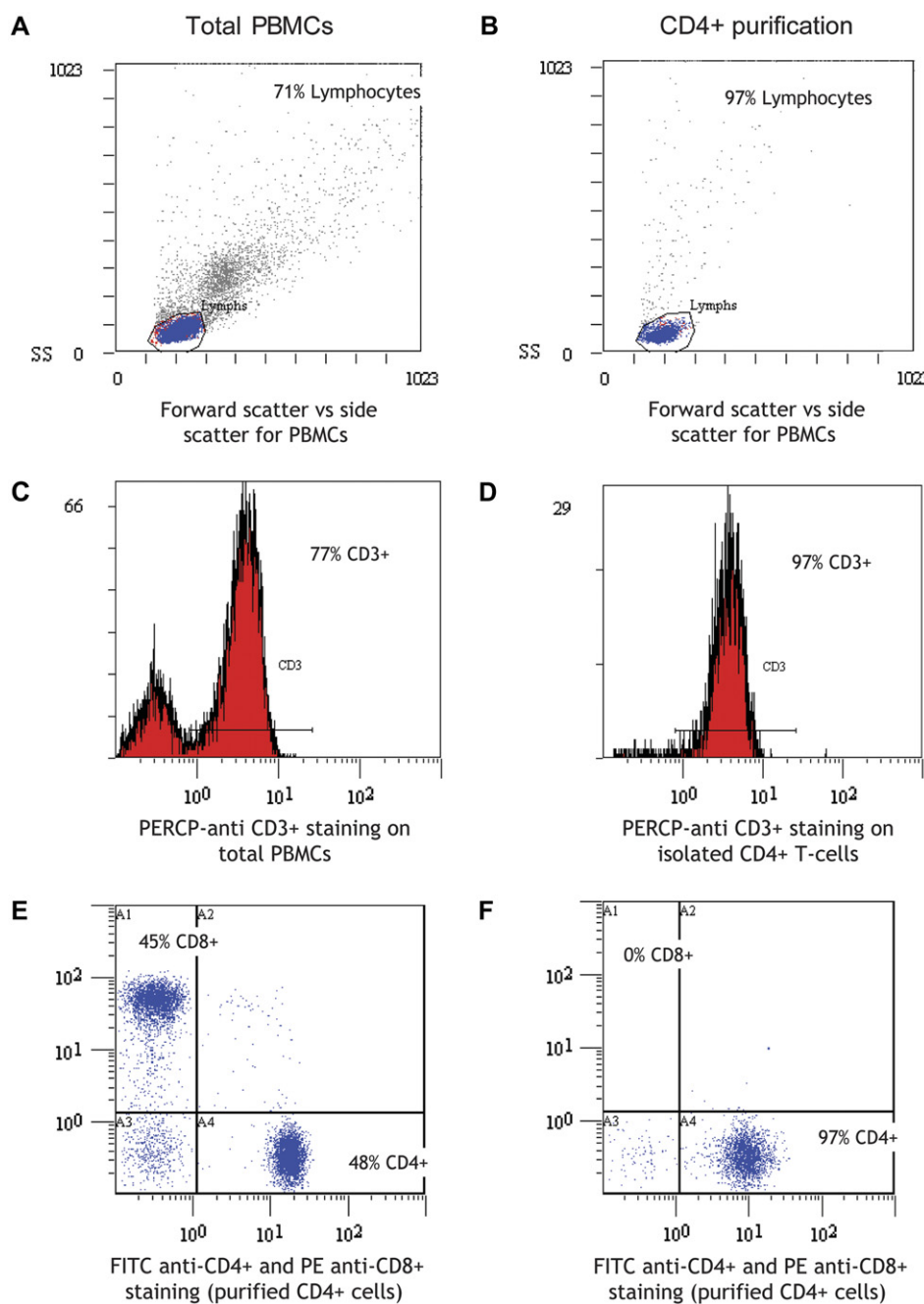


FIG 1. Flow-cytometric analysis showing cell populations present in PBMCs before CD4⁺ cell enrichment (**A**, **C**, **E**) and after enrichment (**B**, **D**, **F**). Lymphocyte enrichment is shown in **B** and **D**. **E** and **F** show CD4⁺ purity after removal of CD8⁺ cells. Results are representative of 16 subjects with MA and 16 subjects with SIA. FITC, Fluorescein isothiocyanate; PE, phycoerythrin.

prepared and Western blots performed as described, and IL-2 and GM-CSF expression in culture supernatants was measured by ELISA (R&D Systems).

Cloning and transfection

Full-length cofilin-1 cDNA was PCR-amplified from a CD4⁺ cDNA library using High-Fidelity Taq/Pwo polymerase (Roche Diagnostics, Lewes, UK). PCR products were purified with a DNA Clean-Up kit (Roche Diagnostics) and inserted into a pCDNA3.1

(directional) V5-His-TOPO vector (Invitrogen Corp), and the correct product was confirmed by sequencing. Plasmids were grown in Top10 competent cells (Invitrogen Corp) and purified by standard procedures.²⁰ Jurkat T cells (10⁷) were electroporated using a Gene-Pulser (Biorad, Hemel Hempstead, UK) at 260 V and 975 μ F with varying amounts of plasmid (5–40 μ g). After optimization of conditions, cells were stimulated 24 hours posttransfection as described, and the effect of cofilin-1 overexpression, as measured by Western blotting, on IL-2 release was determined.

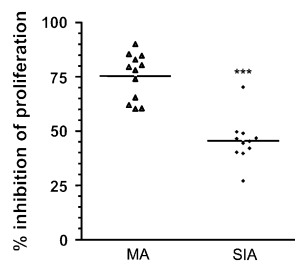


FIG 2. Scatter plot showing that dexamethasone (1 μ mol/L) induced a significantly greater degree of inhibition of anti-CD3/CD28-stimulated CD4⁺ T-cell proliferation in cells from MA compared with those from SIA subjects. $n = 11$; *** $P < .001$.

Statistical analysis

Data are reported as means $\pm$ SEs. Statistical analysis was performed using Prism 3.02 (Graph Pad Software, Inc, San Diego, Calif) with t test and Mann-Whitney test as appropriate. Where indicated, linear regression was performed using Prism 3.02 (Graph Pad Software, Inc). Significance was defined as P value $< .05$.

RESULTS

T-cell proliferation

To perform experiments, it was necessary to obtain pure populations of CD4⁺ T cells. CD4⁺ T cells were enriched to $>97\%$ purity (Fig 1) from MA and SIA subjects and subsequently costimulated with anti-CD3/CD28 antibodies to produce a similar degree of proliferation. Dexamethasone (1 μ mol/L) induced a significantly greater degree of inhibition of proliferation in cells from MA subjects ($75.3 \pm 3.1\%$; $n = 12$) than that seen in cells from SIA subjects ($45.6 \pm 3.1\%$; $n = 11$; $P < .001$), thus confirming that CD4⁺ T cells from SIA subjects were relatively glucocorticoid-insensitive (Fig 2). The degree of repression of proliferation induced by dexamethasone correlated with FEV₁ ($r^2 = 0.56$; $P < .001$).

Expression profiling

Expression profiling of cofilin-1 mRNA was performed in 16 MA and 16 SIA patients. CD4⁺ cells were isolated from peripheral blood samples using negative selection to avoid any cell activation, and purity was checked by flow cytometry. All enriched populations were found to be of $\geq 97\%$ purity. Cofilin-1 mRNA expression was significantly greater in SIA CD4⁺ T cells than in those isolated from glucocorticoid-sensitive MA subjects when expressed as a ratio compared with 18S RNA expression (Fig 3, A; 0.224 ± 0.034 vs 0.421 ± 0.054 ; $P < .01$). The increased expression of cofilin-1 in SIA compared with MA was verified at the protein level by Western blot analysis (Fig 3, B; 0.39 ± 0.05 vs 0.66 ± 0.11 ; $P < .05$). There was a significant inverse correlation between cofilin-1 mRNA expression and FEV₁ ($r^2 = -0.36$; $P < .01$) and cofilin-1 protein expression and FEV₁ ($r^2 = 0.44$, $P < .05$) across all subjects.

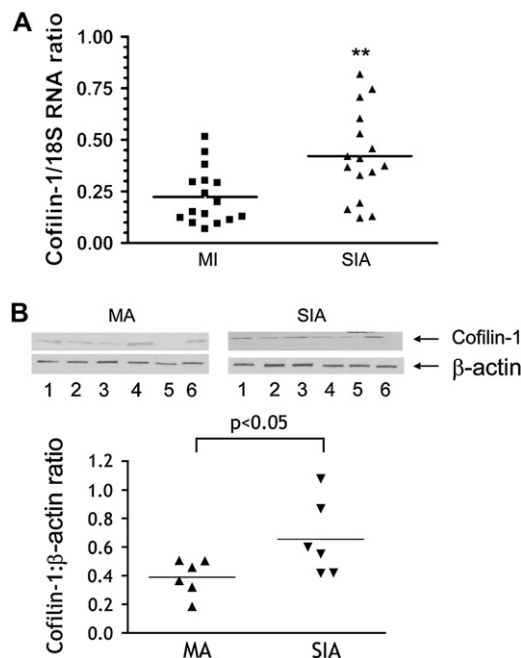


FIG 3. Increased cofilin-1 mRNA (A) and protein (B) in CD4⁺ T cells from SIA and MA subjects. A, Cofilin-1/18S RNA expression in 16 MA and 16 SIA subjects. B, Cofilin-1 protein expression in 6 SIA and 6 MA subjects. β -Actin is a loading control. Lower panel shows scatter plot of cofilin-1/ β -actin expression. ** $P < .01$.

Dexamethasone does not affect cofilin-1 expression

One possible explanation for the enhanced cofilin-1 expression seen in SIA patients was that these cells were exposed to greater levels of glucocorticoids *in vivo*. We therefore examined the time course and concentration-dependent effect of dexamethasone on the expression of cofilin-1 in Jurkat T cells. There was no significant effect of dexamethasone on cofilin-1 expression at any time point or concentration investigated (Fig 4). Similar results were seen in Jurkat T cells transfected with cofilin-1 expression vector. Cofilin-1 expression was ~ 3 -fold higher in cofilin-1-transfected cells than in mock-transfected cells (cofilin-1: β -actin ratio = 5.1 ± 1.6 vs 1.56 ± 0.56 ; $P < .001$), and dexamethasone had no significant effect on this overexpression at 48 hours (Fig 5). The results suggest that the pattern of cofilin-1 expression profiles observed in the patient cohort studied is unlikely to be explained by glucocorticoid therapy in the SIA group and that it is more likely to be a consequence of the disease itself.

Cofilin-1 overexpression reduces dexamethasone suppression of cytokine release

Overexpressing cofilin-1 3-fold, which was within the range of the differences seen between MA and SIA patients, did not affect cofilin-1 localization (data not shown); baseline IL-2 or GM-CSF release, which was below the level of detection; or anti-CD3/CD28-stimulated IL-2 or GM-CSF release (Fig 6). In mock-transfected

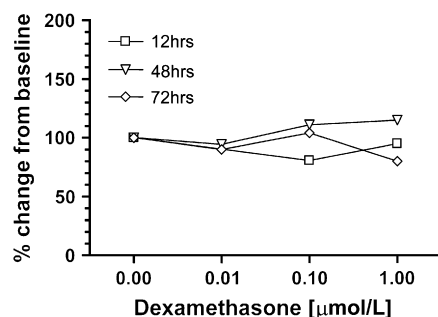


FIG 4. Dexamethasone does not affect cofilin-1 expression in Jurkat T cells. The effect of various concentrations of dexamethasone (0.01–1 μ mol/L) on cofilin-1 protein expression at 12, 48, and 72 hours was examined by Western blotting. Cofilin-1 expression is plotted relative to that of β -actin as a control for protein loading.

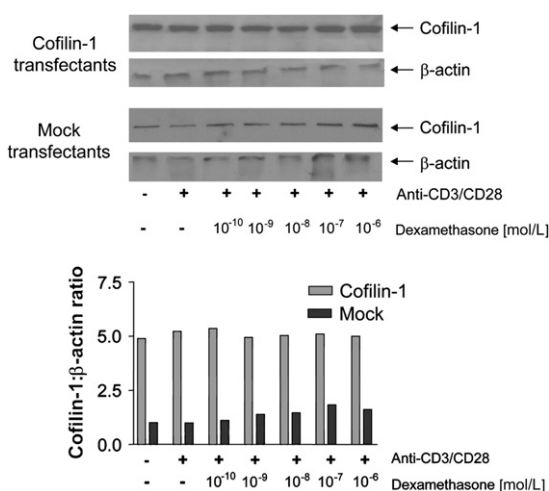


FIG 5. Overexpression of cofilin-1 in Jurkat T cells. Western blot showing cofilin-1 expression in transfected Jurkat T cells (*top*) versus mock-transfected cells (*bottom*) for each treatment. The expression of β -actin is shown to control for protein loading. Graphical analysis of cofilin-1: β -actin ratios as determined by densitometry of Western blots is shown below. Results are representative of 3 independent experiments.

cells, IL-2 and GM-CSF production was reduced at all concentrations of dexamethasone compared with those treated with stimulation alone. However, this suppressive effect was attenuated for those cells overexpressing cofilin-1 and reached significance at 10^{-7} mol/L and 10^{-6} mol/L dexamethasone (Fig 6).

DISCUSSION

The results presented here extend those reported in HeLa cells⁸ to show there is increased expression of cofilin-1 mRNA and protein in peripheral blood CD4⁺ T cells isolated from SIA compared with those isolated from glucocorticoid-sensitive MA subjects. This appears to correlate with reduced dexamethasone sensitivity in these cells

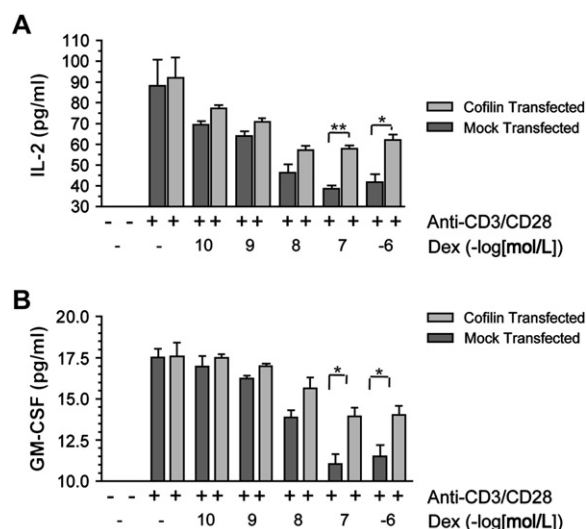


FIG 6. Cofilin-1 overexpression attenuates dexamethasone suppression of (A) IL-2 and (B) GM-CSF release. Histogram showing the effect of dexamethasone on anti-CD3/CD28-stimulated IL-2 and GM-CSF release (pg/mL) from cofilin-1-transfected (*light gray bars*) and mock-transfected (*dark gray bars*) Jurkat cells. Results are presented as means $\pm$ SEMs; $n = 6$. * $P < .05$; ** $P < .01$. Dex, Dexamethasone.

when costimulated with anti-CD3/CD28. Furthermore, overexpression of cofilin-1 in Jurkat T cells produces a similar inhibitory effect on dexamethasone sensitivity to that seen in peripheral blood CD4⁺ T cells from SIA subjects. We have shown that exposure to glucocorticoids per se cannot account for the increased expression of cofilin-1, suggesting that altered cofilin-1 may be associated with reduced glucocorticoid sensitivity in patients with severe asthma.

Resistance to the therapeutic effects of glucocorticoids is also recognized in other inflammatory and immune diseases, including rheumatoid arthritis²¹ and inflammatory bowel disease.²² There is a spectrum of glucocorticoid responsiveness, with the rare glucocorticoid-resistant (CR) patients at one end, but a relative resistance is seen in patients who require high doses of inhaled and oral glucocorticoids (SIA), and both SIA and CR patients present considerable treatment problems.^{23–25}

Importantly for understanding the molecular and biochemical mechanisms underlying insensitivity to glucocorticoids, glucocorticoids are also less effective in inhibiting the expression of inflammatory cytokines from circulating monocytes and T lymphocytes in CR and SIA compared with MA subjects.^{23–25} Previous data indicated that there was a reduction in glucocorticoid responsiveness that was associated with excessive activation of activator protein-1, increased c-Fos expression, and elevated c-Jun N-terminal kinase (JNK) activity in response to inflammatory stimuli, such as TNF- α .^{2,23–25} In another study examining steroid responsiveness in asthma, expression of c-fos mRNA, but not c-jun mRNA, inversely correlated with steroid sensitivity.²⁶ Furthermore, in Crohn disease, steroid resistance is associated with increased epithelial

activation of JNK, p38 mitogen activated kinase, nuclear factor- κ B, and activator protein-1,²⁷ suggesting that drugs targeted at these mediators may be useful therapeutic strategies.

Cofilin-1 is a ubiquitously expressed protein essential for actin filament turnover in mitosis and cell migration.^{13,28,29} In untransformed cells, it is activated by dephosphorylation at serine residue 3¹⁰ and inactivated by phosphorylation at the same residue by Lin-11 Isl-1 Mec-3 kinase 1.^{13,14} Dephosphorylation of cofilin-1 leads to depolymerization of cytoplasmic fibrous actin (F-actin)^{30,31} and nuclear translocation of cofilin-1–actin complexes. Monomeric actin and actin–cofilin-1 complexes have been shown to attenuate apoptosis by inhibition of DNase 1.^{32,33} For this reason, it was not possible to examine the effect of cofilin-1 knockdown on glucocorticoid responsiveness, because previous studies have shown that antisense oligonucleotides result in cell apoptosis.¹⁰ Previous reports on glucocorticoid sensitivity in PBMCs obtained from SIA subjects has suggested that the resistance to glucocorticoid action is lost after 48 hours in culture,¹⁸ indicating that a serum factor may induce insensitivity. Experiments to determine the duration of these differences in cofilin-1 expression may be useful in the future along with an analysis of the day-to-day variations in cofilin-1 expression within the same patient group. This was not possible in our study, because the ethical approval granted prevented patients from being studied a second time. In addition, the overexpression experiments should have been performed in T cells from MA subjects to determine whether steroid sensitivity could be altered; however, this awaits improved and more consistent methods for the transfection of primary T cells.

We were unable to detect any differences in the distribution of lymphocyte populations between the 2 groups of subjects that may account for differences in cofilin-1 expression in this study. A more systematic analysis of T-cell subpopulations such as regulatory T cells (Tregs) or Th17 cells may reveal important differences between patient groups as described by others³⁴; however, this was not possible according to the ethics of the current study.

Specific functional studies of cofilin-1 in T cells have demonstrated its importance in costimulatory pathways of T-cell activation.^{9,33} Costimulatory signals via accessory receptors, such as CD28 and inducible T-cell costimulator, are required to induce T-cell responsiveness and IL-2 cytokine production.^{35,36} Cofilin-1 is only dephosphorylated on activation of the accessory pathways and not after isolated T-cell receptor engagement.^{9,33} Previous studies in glucocorticoid-insensitive HeLa cells show cofilin-1 to be associated with glucocorticoid insensitivity. Furthermore, cytochalasin B, a cytoskeletal disrupting agent, had similar inhibitory effects on glucocorticoid function.³⁷ Interestingly, treatment of cells with cytochalasin B was shown to induce c-Jun,³⁷ providing a possible explanation for the inhibition of glucocorticoid receptor function. Studies in many cells have shown that c-jun and its activating kinase JNK are able to suppress glucocorticoid

function^{38,39} and elevated JNK activity, and enhanced c-jun expression has been associated with SIA.^{2,40} Formal analysis of c-Jun phosphorylation status in transfected Jurkat T cells is required to confirm whether this indeed does occur in these cells.

Studies from our group and elsewhere have reported differences either in glucocorticoid receptor (GR) α nuclear translocation in a subset of SIA subjects or in GR β expression^{24,25} without evidence for changes in GR α expression.^{18,41} In addition, we and others have reported differences in ligand binding affinity and number of binding sites (Bmax) within the nuclei of peripheral blood cells.^{18,24} It is possible, therefore, that changes in cofilin-1 expression in addition to alterations in nuclear GR α and/or GR β expression together have a more profound effect on the ability of a cell, or indeed a patient, to respond to corticosteroids.

In summary, we have shown that peripheral blood CD4⁺ T cells from SIA subjects are less responsive to dexamethasone than cells from MA subjects and that they express significantly higher levels of cofilin-1 mRNA and protein. Functionally, dexamethasone suppression of anti-CD3/CD28–stimulated IL-2 production was attenuated in Jurkat cells that overexpressed cofilin-1. These results suggest that increased cofilin-1 expression may be important in the regulation of glucocorticoid sensitivity in peripheral blood lymphocytes of patients with severe treatment-insensitive asthma.

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