Low FAS/CD95 Expression by CTCL Correlates with Reduced Sensitivity to Apoptosis that Can Be Restored by FAS Upregulation

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FAS expression was generally low in 30 of 31 cutaneous T-cell lymphoma (CTCL) cases (mycosis fungoides/Sezary syndrome, SS) as well as in 5 of 6 large plaque parapsoriasis cases (a CTCL precursor). To investigate this phenomenon, we explored FAS transcript levels, cell-surface FAS protein expression and susceptibility to FAS-mediated apoptosis in four CTCL lines (MyLa, HH, SZ4, and SeAx), freshly isolated leukemic cells from a patient with SS, an acute lymphoblastic leukemia T-cell line (Jurkat), and JFL (a FAS-low variant of Jurkat). Results confirmed low FAS expression by the leukemic SS cells, HH, SZ4, SeAx, and JFL relative to normal peripheral blood mononuclear leukocytes and the other cell lines. There was a direct correlation among FAS transcript level, FAS protein level, and FAS-mediated apoptotic sensitivity in the CTCL samples. When the FAS-deficient cell lines were transfected with a wild-type FAS construct, FAS expression and sensitivity to FAS-mediated apoptosis were restored. In aggregate, these findings provide evidence that like normal T cells, CTCL cells exhibit a mechanistic connection between transcriptional regulation of FAS and sensitivity to FAS-mediated apoptosis, point to the development of FAS deficiency as one molecular mechanism responsible for acquired resistance to apoptosis in CTCL, and indicate that upregulation of FAS expression can restore sensitivity to apoptosis.

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INTRODUCTION

The term CTCL refers to a biologically and clinically heterogeneous group of T-cell neoplasms of the ''skin-associated lymphoid tissue'' that is composed of a subset of peripheral T cells that circulate between the skin and draining lymph nodes through the blood and lymphatics (Wood, 2001). The mycosis fungoides (MF) variant of CTCL typically presents with early skin lesions (flat patches and thin plaques) that have an indolent clinical course. Later there is progression to more clinically aggressive thick plaques and cutaneous tumors. A leukemic variant of CTCL, known as the Sezary syndrome (SS), also exists and is characterized by total body erythroderma. In this study, we use the term CTCL to refer specifically to MF/SS.

There are several lines of evidence suggesting that the pathogenesis of CTCL depends initially on resistance to apoptosis rather than enhanced proliferation to account for the accumulation of tumor cells. Early CTCL begins as flat patches or thin plaques rather than bulky tumors (Wood, 2001). The clinical pace of the disease often remains indolent until patients evolve from the patch/plaque to the tumor phase. Survival correlates highly with TNMB stage—both T stage of skin lesions and overall clinical stage. Early CTCL is a well-differentiated T-cell lymphoma composed of mature CD4+CD45RO+ memory skin-associated lymphoid tissue T cells (Girardi *et al.*, 2004). Similar to normal skin-associated lymphoid tissue T cells, early CTCL tumor cells retain the functional capacity to home to sites of cutaneous allergic reactions (Veelken *et al.*, 1996). Early CTCL tumor cells typically exhibit a low proliferative rate as assessed by mitotic index and Mib-1/Ki-67 staining. As patients undergo tumor progression, the mitotic index and proportion of Mib-1/Ki-67+ cells increase (Dummer *et al.*, 1995; Marrogi *et al.*, 1999). In addition to their lack of proliferation, CTCL tumor cells also appear resistant to apoptosis. Loss of death receptors such as FAS and tumor necrosis factor-R1 associated with blockade of death receptor-mediated pathways was reported among CTCL cell lines (Braun *et al.*, 2007). In CTCL skin lesions, only very rare apoptotic tumor cells were detected by TUNEL assay (Nevala *et al.*, 2001). Consistent with its low proliferative rate and resistance to apoptosis, early CTCL is resistant to chemotherapy that targets rapidly dividing tumor cells (Kuzel *et al.*, 2003).

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Abbreviations: CTCL, cutaneous T-cell lymphoma; FAS-L, FAS-ligand; MF, mycosis fungoides; SS, Sezary syndrome

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In at least some cases of CTCL, there is evidence that defective apoptotic function is due to abnormalities of FAS. This molecule has a central role in T-cell apoptosis by functioning as a major cell-surface death receptor. FAS is expressed relatively strongly by cultured T cells and activated CD45RO+ memory T cells (Li *et al.*, 1999), which is the subset from which CTCL is derived. Nevertheless, FAS protein expression by CTCL has been reported to be variable and frequently weak or negative by several investigators using a variety of immunohistological and flow cytometric techniques (Debusscher and Simonart, 2001; Osella-Abate *et al.*, 2001; Dereure *et al.*, 2002; Contassot *et al.*, 2008). In another report, 8 of 12 tumor-stage MF cases and intraepidermal tumor cells in 4 of 15 patch/plaque MF cases were FAS−. When serial specimens were available for analysis, progression from early to advanced MF was associated with loss of detectable FAS protein in 3 of 4 cases (Zoi-Toli *et al.*, 2000). Reduced or absent FAS protein expression was also noted in the majority of infant acute lymphoblastic leukemias, tumor-stage MF, and cutaneous CD30− non-MF large T-cell lymphoma cases (Debusscher and Simonart, 2001; van Doorn *et al.*, 2002; Wood *et al.*, 2003). In contrast, the majority of benign T cells in cutaneous T-cell pseudolymphomas were FAS+ in 8 of 8 cases (Debusscher and Simonart, 2001). Among peripheral blood tumor cells from MF or SS cases, cell-surface FAS protein expression was highly variable. One study showed 4 of 9 FAS ligand (FAS-L)-resistant SS cases to have deficient FAS expression relative to normal blood lymphocytes (Contassot *et al.*, 2008). Another study also documented FAS− leukemic cells and showed that they were resistant to extracorporeal photopheresis, a therapy known to induce apoptosis in T cells (Osella-Abate *et al.*, 2001). In contrast, FAS+ cases responded to this treatment.

In aggregate, these findings suggest a role for defective apoptosis involving the FAS pathway in the pathogenesis of at least a subset of CTCL cases. Consequently, we assessed FAS expression by tumor cells in samples of MF, SS, and CTCL cell lines and found it frequently to be low relative to other types of T cells. To further explore FAS expression in CTCL, we used various T-cell lines to study the relationships among FAS transcription, FAS protein expression, and sensitivity to FAS-mediated apoptosis. Our findings are consistent with transcriptional regulation of FAS expression in CTCL and with a direct relationship between expression level and sensitivity to apoptosis. Furthermore, we show that upregulation of FAS expression restores sensitivity to apoptosis in FAS-deficient T-cell lines including CTCL. These findings provide an enhanced understanding of FAS expression in CTCL and a rational basis for therapies aimed at increasing tumor cell expression of cell-surface FAS.

RESULTS

CTCL infiltrates generally exhibit only weak FAS expression as detected *in situ* by immunohistology

As shown in Table 1 and Figure 1, T cells in skin biopsy specimens from 31 patients with CTCL and large plaque parapsoriasis (a CTCL precursor) were generally no more than weakly FAS+ compared with autologous epidermal keratinocytes and reactive T cells in lesional skin biopsies of 15 inflammatory skin diseases (chronic spongiotic dermatitis, lichenoid drug reactions, and Jessner's lymphocytic infiltrate). Scattered more strongly FAS+ T cells were observed focally in CTCL and appeared to be tumor-infiltrating lymphocytes based on analysis of semi-serial sections stained with anti-CD8 mAb specific for the cytolytic T-cell subset. In all cases, the CTCL tumor cells were CD4+.

Table 1. FAS expression *in situ* by CTCL is generally weak

Diagnosis	Negative/Weak FAS	Moderate/Strong FAS
CTCL	24 (15 patch/plaque MF, 5 tumor MF, 2 erythrodermic MF, 2 SS)	1 (tumor MF, heterogeneous staining)
Large plaque parapsoriasis (essentially early patch-stage CTCL)	5	1
Chronic dermatoses	0	15 (8 spongiotic dermatitis, 4 benign alopecia mucinosa, 1 Jessner's infiltrate, 1 lichenoid drug dermatitis, 1 lymphomatoid papulosis)

CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; SS, sezary syndrome.
Acetone-fixed frozen sections were stained with a three-stage immunoperoxidase method (monoclonal antibody/biotinylated goat anti-mouse IgG/avidin-HRP) with 3,3-diaminobenzidine chromagen enhanced by copper sulfate and counterstained with methylene blue. Two anti-FAS monoclonals were used separately (APO-1-1 from Alexis and DX2 from Dako). The strongest staining was recorded. Keratinocytes served as an internal positive control. Isotype-matched IgG1 monoclonals of irrelevant specificity served as negative controls.

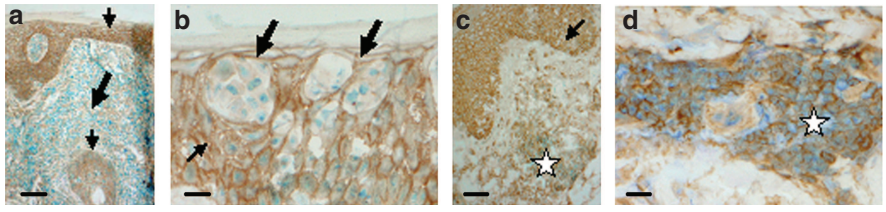

Figure 1. FAS expression *in situ* by CTCL is generally weak. Immunoperoxidase-stained frozen sections showing negative/weak FAS expression by CTCL tumor cells (large arrows) in dermis (**a**) and epidermis (**b**). CTCL tumor cell clusters within epidermis (Pautrier's microabscesses) are shown in **b** (large arrows). This contrasts with much stronger FAS expression by benign T cells (stars) in dermatitis (**c**, **d**) and keratinocytes in epidermis and hair follicles (small arrows) (**a**–**c**). Methylene blue counterstain. (**a**, **c**) Original magnification ×200. (**b**, **d**) Original magnification ×400. Bar = 100 μm in (**a**) and (**c**), and 50 μm in (**b**) and (**d**).

Interestingly, one CTCL case had concurrent lymphomatoid papulosis lesions. This is a clonal T-cell lymphoproliferative disorder rich in CD30+ T cells. Skin lesions undergo spontaneous regression accompanied by lymphoid apoptosis. The cells in the lymphomatoid papulosis lesions were distinctly FAS+, in contrast to the nonregressing CTCL lesions containing cells that were FAS−/weak. Thus, we have a correlation between a clinical/functional property (spontaneous regression by apoptosis) and FAS phenotype. The occurrence of these distinct diseases in the same patient controls for several confounding variables, and further suggests the potential importance of apoptotic pathways in CTCL pathogenesis.

CTCL cell lines and leukemic blood generally exhibit low levels of cell-surface FAS expression

Flow cytometric analysis of four CTCL cell lines (HH, SZ4, SeAx, and MyLa) and seven leukemic CTCL (SS) blood samples demonstrated generally low mean fluorescence intensity of FAS expression except for MyLa cells that were about 10-fold higher (Figure 2). Also analyzed for FAS were pooled normal peripheral blood mononuclear cell, Jurkat (acute lymphoblastic T-cell leukemia line), and JFL (a Jurkat variant with very low FAS expression). As a control to rule out structurally abnormal FAS protein, nucleotide sequence analysis of the CTCL lines revealed no coding region mutations that would create defective FAS proteins that might be expressed but undetectable by flow cytometry. One of the seven SS patients (SS25) had both skin and blood samples studied by immunohistology and flow cytometry, respectively. CTCL cells in both tissue compartments exhibited only low-level FAS expression, suggesting that this was a general feature of this patient's tumor cells rather than a microenvironmentally specific phenomenon. An eighth SS patient also showed low FAS expression by immunohistological analysis of tumor cells in lesional skin. Overall, T cells in 22 of 23 MF, 8 of 8 SS (7 studied by flow cytometry and 1 studied by immunohistology), 5 of 6 large plaque parapsoriasis, HH, SZ4, SeAx, and JFL were low in FAS compared to moderate/high levels of FAS expressed by T cells in 15 of 15 inflammatory dermatoses, normal blood, Jurkat, and MyLa.

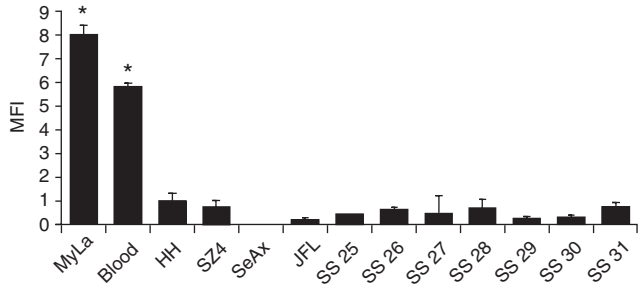

Figure 2. Flow cytometry: FAS expression varies widely among CTCL cell lines and SS blood samples. MyLa, SeAx, SZ4, and HH are CTCL lines. SS25–31 are leukemic CTCL cells from patients with Sezary syndrome. JFL is a Jurkat variant with almost no FAS. Blood is normal PBMC pooled from three donors. Mean fluorescence intensity (MFI) is on *y* axis. Results are the mean of triplicate analyses ± SD. **P*<0.05 relative to other samples.

CTCL cell lines have levels of FAS transcript that parallel levels of FAS protein

We used Northern blotting and real-time RT-PCR (Figure 3) to determine FAS mRNA levels in four CTCL lines: HH, SZ4, SeAx, and MyLa. The results show that FAS transcript level parallels FAS protein expression with HH, SZ4, and SeAx expressing much less mRNA than MyLa. This suggests that reduced FAS protein expression in CTCL reflects reduced FAS mRNA. This relationship is consistent with repeated evidence in the literature indicating that FAS protein is transcriptionally regulated.

Fresh mononuclear leukocytes were isolated from one SS case (SS25) with approximately 60,000 leukemic cells per mm^3. Both FAS transcript and protein levels were low as assessed by real-time RT-PCR and flow cytometry, respectively (Figures 2 and 3). These results resemble our findings among various CTCL lines and suggest that parallels exist between FAS mRNA and protein levels as a general feature of CTCL.

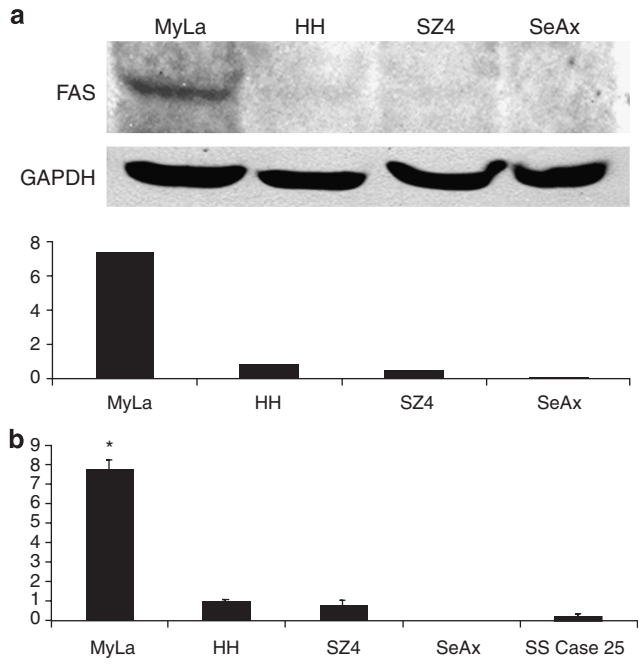

Figure 3. Northern blot and real-time RT-PCR analysis of FAS mRNA level in CTCL cell lines. (**a**) For Northern blotting, total RNA of each cell line was electrophoresed and transferred to nylon membrane, hybridized with biotin-labeled FAS DNA probe or control GAPDH probe, and chemiluminescent signals were detected. The histogram is representative of three blots and shows densities of the FAS blot normalized to GADPH loading control. FAS probe is designed at FAS death domain region of FAS gene (accession number: NM_000043) from nt 1102 to nt 1584. Biotin labeling and chemiluminescent detection kits were from Pierce. (**b**) For real-time RT-PCR, cDNA was synthesized from total RNA of each CTCL cell line (MyLa, SeAx, SZ4, and HH) and SS25 leukemic blood with SuperScript Reverse Transcriptase and Random Primers (Invitrogen). In total, 40 cycles of real-time PCR were performed with SYBR Ex Taq Kit (Takara Biotechnology) and primer set: forward, 5′-TGCCAAGAAGGGAAGGAGTA′; reverse, 5′-CGGGTGCAGTTTATTTCCAC′ on ABI PRISM 7000 Sequence Detection System. Data analysis: the relative expression level of FAS mRNA of each cell line was determined by ABI PRISM SDS software and comparative C_t method analysis. The histogram represents three independent real-time PCR results.

FAS function parallels cell-surface FAS protein level

To directly determine the relationship between FAS protein expression and functional sensitivity of CTCL lines to FAS-mediated apoptosis, we incubated CTCL lines and leukemic CTCL cells (each with different levels of cell-surface FAS) for 16 hours with recombinant FAS-L/FLAG aggregated with anti-FLAG mAb. Aggregation enhances trimerization of cell-surface FAS and the resultant apoptotic signal (Huang *et al*., 1999). Flow cytometric analysis of apoptosis using Annexin V and propidium iodide showed that sensitivity to apoptosis was roughly proportional to FAS expression. The FAS-low samples (JFL; CTCL lines HH, SZ4, and SeAx; as well as leukemic CTCL cells, SS25) were much more resistant to apoptosis than the FAS-high CTCL line, MyLa (see Figure 4; *denotes $P<0.01$ for MyLa compared to others). Thus, there is a consistent relationship within these CTCL specimens: FAS transcript levels correlate with FAS cell-surface protein levels that correlate with FAS function (sensitivity to apoptosis).

Reduced sensitivity to FAS-mediated apoptosis can be restored by upregulation of FAS

Our immunophenotypic studies showed that low FAS expression is common in CTCL. Our cell line studies showed that low FAS expression results in resistance to FAS-L-induced apoptosis in CTCL and other types of T cells. Therefore, we were interested to determine whether upregulation of FAS expression would restore sensitivity to apoptotic signals triggered through the FAS pathway. We transfected FAS-low cell lines JFL, HH, SZ4, and SeAx with plasmids containing green fluorescent protein (GFP) and FAS. The GFP served as a control for effective transfection. Figure 5 shows that the transfections resulted in the expected upregulation of GFP and FAS. As also demonstrated in Figure 5, transfection of cell lines JFL (a FAS-low variant of Jurkat) as well as HH, SZ4, and SeAx (FAS-low variants of CTCL) with the FAS-GFP construct did in fact restore sensitivity to apoptosis mediated by FAS-L. For JFL, there was a 22-fold increase in apoptosis among transfected cells treated with aggregated FAS-L relative to untransfected cells treated similarly. For SeAx, SZ4, and HH the differences were 13-, 6-, and 6-fold, respectively (Figure 5, right upper quadrant values in row 3 versus row 5). Of interest, even in transfected cells not treated with aggregated FAS-L, there was a variable increase in spontaneous apoptosis relative to untransfected cells that was less than twofold for SZ4 and SeAx, threefold for HH, and sixfold for JFL (Figure 5, right upper quadrant values in row 2 versus row 4). This effect was not due to high baseline levels of FAS-L expression because all cell lines tested showed only low-level FAS-L on the cell surface (Figure 6). However, FAS upregulation following transfection did result in a modest parallel increase in FAS-L expression (Figure 7) that together might account for the augmented spontaneous apoptosis observed in transfectants.

DISCUSSION

This study demonstrates wide variability of FAS protein expression in CTCL. There was more than 10-fold difference in cell-surface FAS expression among CTCL specimens as assessed by flow cytometry. Compared to normal blood mononuclear leukocytes, many CTCL samples demonstrated very low FAS levels. These results are consistent with our own immunohistological findings reported here (Table 1) as well as the literature showing variable expression of FAS by CTCL cells in the skin and blood, as detected by immunohistology or flow cytometry, respectively (see 'Introduction' for references).

The literature indicates that FAS expression is transcriptionally regulated in normal T cells. Our current findings are consistent with a similar mechanism of FAS regulation in CTCL and other T-cell neoplasms. Levels of FAS mRNA detected by either northern blotting or real-time RT-PCR paralleled the levels of FAS protein. Similarly, there was also

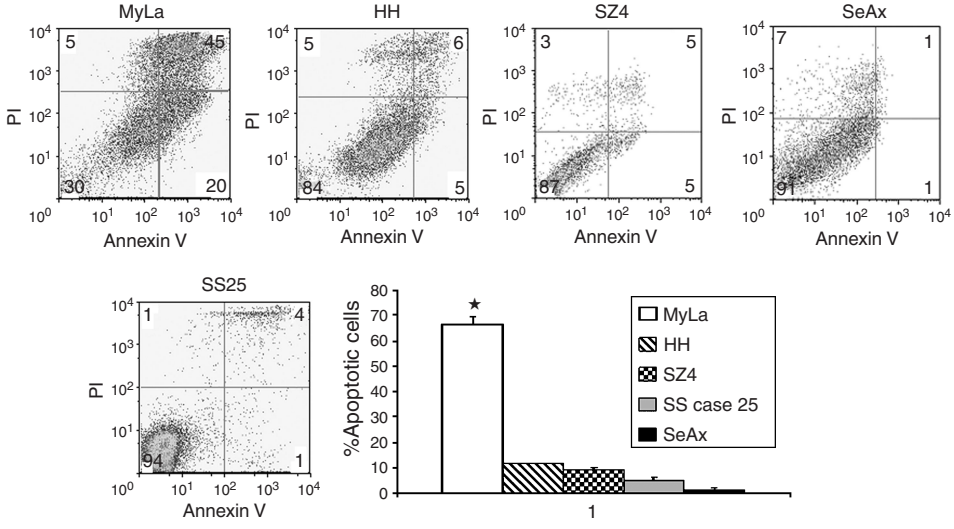

Figure 4. Induction of apoptosis by FAS ligand in CTCL. CTCL lines (MyLa, HH, SZ4, and SeAx) and leukemic CTCL cells (SS25) were studied. Cells were plated at 0.5×10^5 ml^{-1} in 2 ml growth medium in triplicate wells, treated with 50 ng ml^{-1} of FAS-L/FLAG aggregated with anti-FLAG mAb for 16 hours. Apoptosis was determined by flow cytometry using Annexin V–FITC and propidium iodide. *$P<0.05$ relative to other samples.

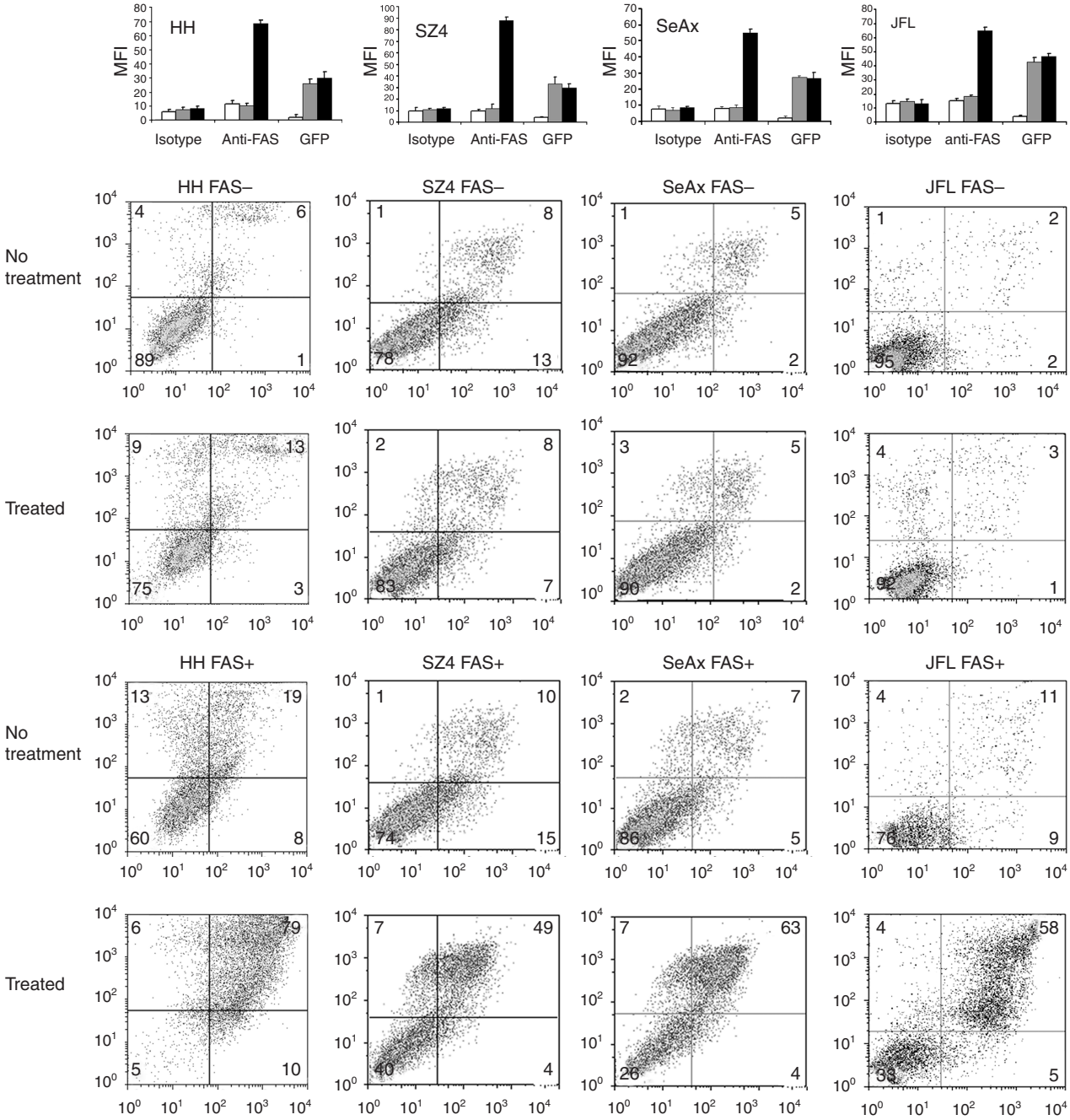

Figure 5. FAS transfection restores sensitivity to FAS-L-induced apoptosis in FAS-deficient CTCL lines (HH, SZ4, and SeAx) and JFL (a low-FAS variant of Jurkat). Row 1 (top): HH, SZ4, SeAx, and JFL cells transfected with pEF-GFP-FAS and control vector pEF-GFP show the expected upregulation of GFP and FAS. Cell-surface FAS was detected by flow cytometry using anti-FAS-PE mAb. Murine isotype-matched antibody conjugated to PE was the negative control. GFP signal was also detected before and after transfection. White bars show untransfected cells, gray bars show control vector-transfected cells, and black bar show FAS vector-transfected cells. Histograms are the average of three experiments. Four lower rows: Apoptosis was induced with FAS-L/FLAG aggregated with anti-FLAG. Apoptosis was measured by flow cytometry using PE–Annexin V and PI staining. Row 2: non-transfected cells; no treatment. Row 3: non-transfected cells; aggregated FAS-L/FLAG treatment. Row 4: FAS-transfected cells; no treatment. Row 5: FAS-transfected cells; aggregated FAS-L/FLAG treatment. FAS-low cells are insensitive to FAS-L (rows 2 versus 3). FAS-transfected cells are highly sensitive to FAS-L (rows 4 versus 5).

a parallel relationship between FAS protein levels and sensitivity to apoptosis mediated by aggregated FAS-ligand (FAS-L). Those CTCL specimens with the greatest expression of cell-surface FAS protein were the most susceptible to apoptosis. Although there are minimal published data correlating relative FAS levels in CTCL with sensitivity to FAS-mediated apoptosis, at least one other study showed an overall relationship between low FAS and resistance to apoptosis in SS (Meech *et al.*, 2001). However, another study found that sensitivity to FAS-mediated apoptosis among neoplastic T-cell lines did not consistently parallel levels of FAS expression (Ni *et al.*, 2005). Differences from our results

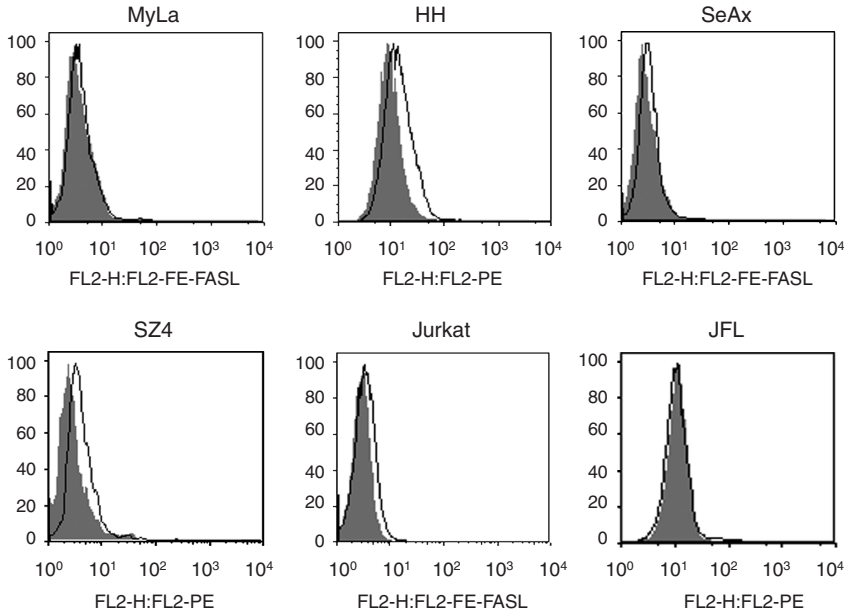

Figure 6. Low baseline expression of cell-surface FAS-L by neoplastic T-cell lines. Flow cytometry using anti-FAS-L mAb (white filled curves) shows minimal FAS-L expression compared to isotype-matched negative controls (gray filled curves).

may be due to technical variations in the apoptotic assays used and/or the nature of the cell lines tested, some of which were HTLV-1 + in this study.

In aggregate, the current findings support a mechanistic linkage in CTCL between transcriptional regulation of FAS protein expression and sensitivity to apoptosis. Our findings indicate that CTCL cases low in FAS are resistant to apoptosis induced by FAS-L. Because this is a major pathway for apoptosis in T cells generally, it is likely that CTCL cells low in FAS have a survival advantage and will exhibit resistance to treatments that work by inducing apoptosis through FAS signaling. In fact, this has been observed in leukemic CTCL patients undergoing extracorporeal photopheresis (Osella-Abate *et al.*, 2001). In the future, assessment of FAS protein levels could be used to define subsets of CTCL that might prove useful for prognostication and predicting response to specific therapies. The relatively strong expression of FAS and CD25 by MyLa indicates that it is probably not representative of MF/SS in general. Nevertheless, it remains useful as a positive control for studies correlating FAS expression with sensitivity to apoptosis.

On the basis of our findings, the low FAS protein expression seen in many CTCL cases appears to be due to low levels of FAS transcripts. Intracellular trapping of FAS protein before it can be expressed on the cell surface is unlikely because FAS mRNA levels parallel FAS cell-surface protein levels. Furthermore, our immunohistological studies would have identified cytoplasmic as well as cell-surface FAS protein. Nevertheless, it is unknown why FAS is reduced in CTCL relative to normal blood T cells and lesional T cells in dermatitis. Possibilities involving reduced transcription include promoter abnormalities such as decreased enhancer function and/or increased transcriptional repressor activity. Other possibilities include decreased transcript stability or increased transcript catabolism. These potential mechanisms are the subject of ongoing studies.

Earlier experiments at the protein level have suggested that CTCL cells are defective in apoptosis mediated through the FAS pathway because of defective signaling through the T-cell receptor/CD3 complex that results in inadequate levels of IL-2, FAS, and FAS-L (Meech *et al.*, 2001; Ni *et al.*, 2005).This proposal awaits further confirmation; however, treatment of circulating SS tumor cells with IL-2 plus the partial protein kinase C agonist, bryostatin-1, did increase the expression of both FAS and FAS-L, as well as apoptosis in response to FAS ligation (Meech *et al.*, 2001). Interestingly, our current data show that FAS upregulation through transfection also results in some FAS-L upregulation. These parallel changes in FAS and FAS-L in response to both bryostatin-1 and FAS transfection suggest the possibility that their regulation may be coordinated. In preliminary studies, we have shown that the green tea polyphenol, (-)-epigallocatechin-3-gallate, and IFN-α can cause reproducible increases in FAS expression by at least some CTCL cell lines (Nihal and Wood, 2007) A similar induction of FAS expression in CTCL by both IFN-α and -γ has been noted by others as well (Contassot *et al.*, 2008). These findings suggest the possibility of upregulating FAS expression in tumor cells in CTCL patients—a goal made more intriguing by our *in vitro* studies showing that FAS upregulation resulted not only in increased apoptosis in response to FAS-L treatment but also in increased FAS-L expression and spontaneous apoptosis.

MATERIALS AND METHODS

Cell lines and plasmids

Human CTCL lines MyLa, SeAx, and SZ4 were generous gifts from Dr Reinhard Dummer, Dr Emmanuel Contassot, and Dr Lars French (University of Zurich); Dr Frank Braun, Dr Jurgen Eberle, and

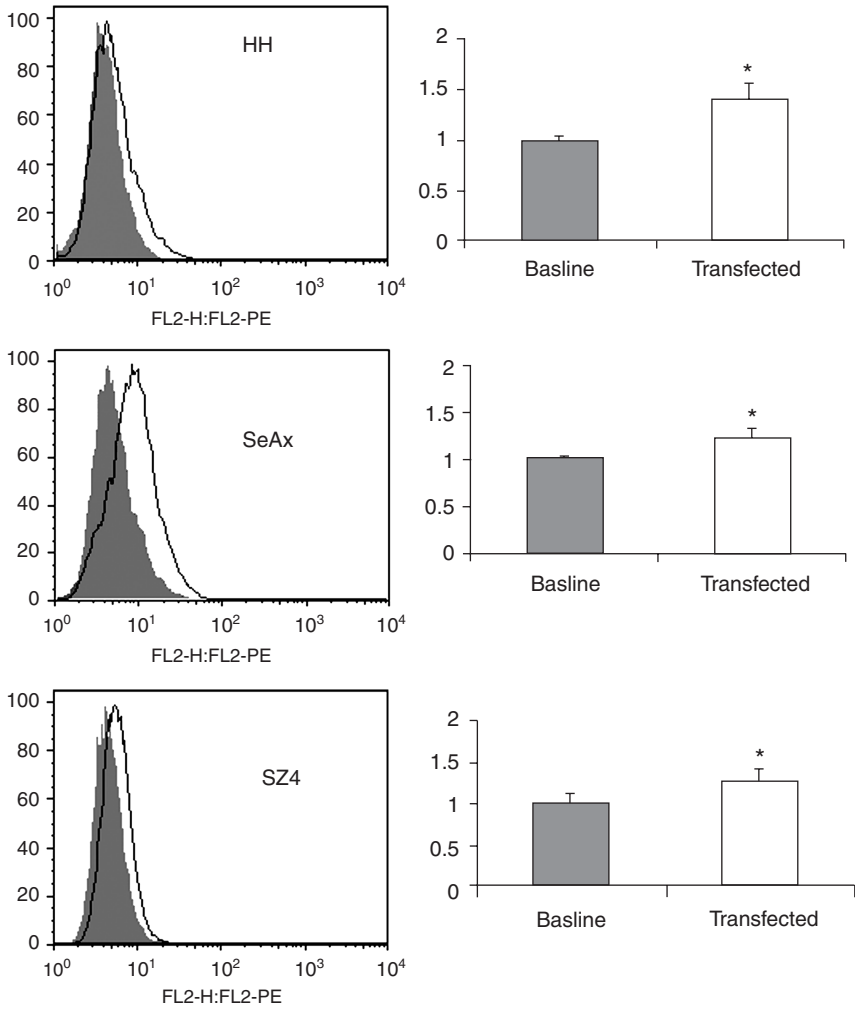

Figure 7. FAS transfection results in modest upregulation of FAS-L in FAS-deficient CTCL lines. HH, SeAx, and SZ4 cells were transfected with pEF-GFP-FAS plasmid and cell-surface FAS-L was detected by murine anti-human FAS-L primary antibody and PE-conjugated secondary antibody. Typical flow cytometry results are shown. Gray filled curves show cells transfected with empty vector and white filled curves show cells transfected with FAS expression vector. Histograms show relative fold increase of the mean florescence intensity (MFI) of PE signal between the two curves. Histograms are the result of three independent experiments. *$P<0.05$ relative to empty vector.

Dr Wolfram Sterry (Charite Medical University of Berlin); and Dr Hetty Carraway (Johns Hopkins University) and were grown in RPMI 1640 containing 2 mM L-glutamine, 1 mM pyruvate, and 10% fetal bovine serum as described earlier (Nielsen *et al.*, 1999). CTCL line HH and acute T-cell leukemia cell line Jurkat are from the ATCC (CRL-2105 and TIB-152), and were grown in the same medium containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. JFL is a Jurkat variant with very low FAS expression and was a gift from Dr Janssen (Lettau *et al.*, 2006). The pEF-GFP and pEF-GFP-FAS plasmids were a generous gift from Dr Vladimir Ivanov (Columbia University, NY). The JFL (Jurkat FAS Low) cell line was a generous gift from Dr Ottmar Janssen (University Hospital, Schleswig-Holstein, Germany).

Lesional skin and blood specimens

All clinical samples were obtained with institutional review board approval. All patients gave informed consent and all protocols adhered to the Declaration of Helsinki Principles. Skin specimens included 23 MF, 2 SS, 6 large plaque parapsoriasis, and 15 benign inflammatory skin diseases as shown in Table 1. Blood specimens were obtained from seven stages III–IV erythrodermic CTCL patients with SS. All had dominant T-cell clonality in their blood detected by PCR or Southern blot analysis of T-cell receptor gene rearrangements. Circulating tumor cells consisted 83–97% of lymphocytes as assessed by cytology (Sezary cell preparation) or T-cell receptor V-beta subfamily immunostaining.

Statistics

Statistical analysis was performed by Student's *t*-test. A *P*-value <0.05 was considered statistically significant and is represented as (*) in the figures.

Immunohistology

We used a three-stage murine mAb/biotinylated goat anti-mouse IgG/avidin–horseradish peroxidase method applied to acetone-fixed frozen sections to assess FAS expression by CTCL and inflammatory skin disease controls. Two different anti-FAS mAbs were used: clone APO-1-1 (Alexis, San Diego, CA) and clone DX2 (Dako, Carpinteria, CA). Positive controls included epidermis, adnexal epithelium, and

endothelial cells within tissue sections. Negative controls included isotype-matched IgG1 murine mAbs of irrelevant specificity. To help identify cell groups, we also stained semi-serial sections for a panel of other antibodies directed against: CD1a, 2, 3, 4, 5, 7, 8, 20, 25, 30; Mib-1; and HLA-DR.

Flow cytometry

Surface FAS expression was determined by staining cells with FITC or phycoerythrin (PE)-conjugated anti-FAS mAb DX2 (Becton Dickinson, San Jose, CA). Surface FAS-L was detected with anti-FAS-L mAb NOK-1 followed by PE-conjugated goat anti-mouse IgG1 secondary antibody (both from BD Pharmingen, San Jose, CA). Isotype-matched mAbs of irrelevant specificity were used as negative controls. Briefly, 2×10^5 lymphoid cells from cultured cell lines and fresh or banked SS blood samples were collected, washed two times with phosphate-buffered saline, and blocked with 1:10 normal goat serum in phosphate-buffered saline for 20 minutes. Cells were immunostained for 30 minutes at room temperature, then washed twice with phosphate-buffered saline, resuspended in FACS buffer (2% BSA in phosphate-buffered saline), and analyzed with a LSR II bench top flow cytometer. Data analysis was performed by FlowJo software.

Northern blotting

Total RNA (10 μg) of each cell line was electrophoresed in a 1% agarose formaldehyde gel and transferred to Biodyne B nylon membranes (Pierce, Rockford, IL). The membrane was prehybridized and then hybridized with biotin-labeled FAS or control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe using Chemiluminescent Hybridization and Detection Kit (Pierce) following the manufacturer's instructions. The chemiluminescent signals were detected by exposure to Blue X-Ray film. The FAS probe is targeted to the death domain region of the FAS gene (accession number: NM_000043) from nt1102 to nt1584 and was labeled with biotin using a biotin-labeling kit (Pierce).

Real time RT-PCR

cDNA was synthesized from total RNA of each cell line with SuperScript Reverse Transcriptase and Random Primers (Invitrogen, Carlsbad, CA). In total, 40 cycles of real-time PCR (30 seconds at 94 °C, 58 °C, and 72 °C) were performed with SYBR Ex Taq Kit (Takara Biotechnology, Japan) using primer sets designed to amplify 110 bp of FAS cDNA and 236 bp of GAPDH cDNA on the ABI Prism 7000 Sequence Detection System. Primer sequences for FAS are: forward, 5′-TGCCAAGAAGGGAAGGAGTA-3′; reverse, 5′-CGGGTGCAGTTTATTTCCAC-3′; and for GAPDH are: forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′.

Apoptosis assay

CTCL cells were plated at $5 \times 10^4\,ml^{-1}$ in 2 ml growth medium and incubated for 16 hours with $50\,ng\,ml^{-1}$ recombinant fusion protein of FAS-L and Flag-tag (Alexis), aggregated with $1\,\mu g\,ml^{-1}$ anti-Flag antibody (Sigma-Aldrich, St Louis, MO). Cells were collected and washed. The percentage of the cells undergoing apoptosis was determined by staining cells with propidium iodide and Annexin V–FITC (1:20; BD Pharmingen) for 15 minutes in the dark, followed by flow cytometric analysis.

Nucleotide sequencing

To screen for mutations in Fas/CD95 exon regions, we used PCR and automated nucleotide sequencing techniques. FAS regions were amplified using genomic DNA and/or cDNA templates. Genomic DNA from CTCL cell lines was extracted using the standard method of proteinase K treatment and phenol/chloroform extraction. The following primers were used to amplify the various coding regions of the FAS exons and for sequencing (F denotes forward primer and R denotes reverse primer in each pair): Exon 2: W196F, 5′-GTTGCTTACTTCAGAAATCAATAA-3′; W197R, 5′-ACTGTAATCTGGATGTTTTGT-3′; Exon 3A: W198F, 5′-ACTTCCCACCCTGTTACCTG-3′; W199R, 5′-CATCACACAATCTACATCTTCTGC-3′; Exon 3B: W200F, 5′-GTACACCAGACAAAGCCCTTTTTC-3′; W201R, 5′-GTGTCAACATAGCACCACAGTAGG-3′; Exon 4: W202F, 5′-CGCGATAACTAATAGTTTCCAA-3′; W203R, 5′-CTCTCAGTCAGTGTTACTTACTTCCCTA-3′; Exon 7: W208F, 5′-CTACAAGGCTGAGACCTGAGTT-3′; W209R, TTTCAAGGAAAGCTGATACCTATT-3′; Exon 8: W210F, 5-TTGTCTTTCTCTGCTTCCATT-3′; W211R, 5′-ATTGGCCTATTACTCTAAAGGATG-3′; Exon 9A: W212F, 5′-TGCTGGAGTCATGACACTAAGT-3′; W213R, 5′-CAATGTGTCATACGCTTCTTTC-3′; Exon9B: W214F, 5′-TAATTGGCATCAACTTCAT-3′; W215R, 5′-GAATTTGTTGTTTTTCACTCTA-3′; Exon 9C: W216F, 5′-GGTTTTCACTAATGGGAATTTCAT-3′; W217R, 5′-CTTCATTGACACCATTCTTTCG-3′.

Total RNA was isolated using Trizol Reagent and cDNA was synthesized using the First-Strand Synthesis System (Invitrogen). FAS gene fragments were amplified by PCR using high-fidelity Taq polymerase (Invitrogen). The following primers were used for PCR amplification and sequencing of a 335 bp FAS cDNA fragment that includes exons 1–3 and part of exon 4: W234F, 5′-ATGCTGGGCATCTGGACCCT-3′; W235R, 5′-GCCATGTCCTTCATCACACAA-3′. The following primers were used for PCR amplification and sequencing of a 338 bp FAS cDNA fragment that includes exons 4–6: W236F, 5′-CATGGCTTAGAAGTGGAAAT-3′; W237R, 5′-ATTTATTGCCACTGTTTCAGG-3′. All automated sequencing was performed at the DNA Biotechnology Center, UW, Madison, WI.

Cell transfection

JFL cells (1×10^6 per sample) were transfected with pEF-GFP-FAS plasmid by using lipofectamine LTX following the manufacturer's instructions. Briefly, cells were plated in six-well plates and 2.5 μg of plasmid and 2.5 μl of PLUS reagent were diluted in 500 μl of serum-free media, 8 μl of lipofectamine LTX was added 5 minutes later. The DNA/lipofectamine mixture was added into cells after 25 minutes of incubation. For the HH SeAx, SZ4 cell lines, 5×10^6 cells were collected and resuspended in 100 μl of Nucleofector Solution V, and 2.5 μg of plasmid was added. The mixture was transferred into an Amaxa-certified cuvette. Program X-001 was used for electroporative transfection using the Amaxa AAD-1001 Nucleofector Device (Amaxa Inc., Gaithersburg, MD).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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