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Resistance of Sézary Cells to TNF- α -Induced Apoptosis Is Mediated in Part by a Loss of TNFR1 and a High Level of the IER3 Expression

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Abstract

Failure to execute an apoptotic program is one of the critical steps and a common mechanism promoting tumorigenesis. Immediate early responsive gene 3 (IER3) has been shown to be upregulated in several cancers. IER3 is a stress induced gene, which upregulation leads to reduction in production of reactive oxygen species (ROS) protecting malignant cells from apoptosis. We observed that malignant lymphocytes from patients with Sézary syndrome (SzS) were resistant to pro-apoptotic dose of tumor necrosis factor- α (TNF- α). The aim of this study was to investigate the role of IER3 in the mechanism of such resistance. CD4⁺ CD26[–] lymphocytes from the peripheral blood of patients with SzS and healthy controls were negatively selected using CD4 and CD26 magnetic beads and analyzed for expression of TNFR1, TNFR2, IER3 expression, and ROS production in response to TNF- α at an apoptotic dose. Sézary cells with a higher level of IER3 expression retained their viability to TNF- α . IER3 upregulation correlated with a decrease level of intracellular ROS and low TNFR1 expression on malignant cells. Targeting IER3 could be of interest for the development of future therapeutic strategies for patients with SzS.

Keywords

Sézary syndrome; Cutaneous T-cell lymphoma; Immediate early response gene 3; Apoptosis; TNF- α

INTRODUCTION

Cutaneous T cell lymphomas (CTCL) are the second most common form of extranodal non-Hodgkin's lymphomas and one of the most frequent T-cell malignancies. Sézary syndrome (SzS) and mycosis fungoides (MF), comprise 55% of CTCL (1), are neoplasms of mature memory-type T lymphocytes expressing cutaneous lymphocyte antigen, integrins $\alpha 4\beta 4$ (2), chemokine receptors CXCR3 (3) and CXCR4 (4) defining their propensity for the skin. The presence of epidermotropic malignant lymphocytes in the blood is characteristic of SzS and

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

O.E. Akilov and I.V. Ustyugova performed experiments. O.E. Akilov, and M.X. Wu analyzed the data. O.E. Akilov, M.X. Wu, and L.J. Geskin conceived and designed the study and wrote the paper. L.D. Falo, Jr. and L.J. Geskin were responsible for the overall project. M.X. Wu, L.D. Falo Jr, and L. J. Geskin provided financial support. All authors discussed the results and commented on the final version of manuscript.

evidence of disease progression in MF (5). Despite recent progress in clinicopathological classification and management of these cutaneous malignancies, specific underlying molecular mechanisms are unknown and are an active field of investigation (6).

Resistance to apoptosis was shown to play a crucial role in the pathogenesis of early CTCL as opposed to uncontrolled proliferation accounting for the tumor expansion (7). Several mechanisms have been shown to contribute to resistance of CTCL cells to apoptosis: activation of the NF- κ B pathway (8), diminished FAS/CD95 expression (7, 9, 10), down-regulation of TRAIL pathway (11), and deletions of the TNF- α -induced protein 3 gene (12). Among multiple inducers of apoptosis, TNF- α plays a distinctive role. The effects of TNF- α on lymphocyte are dose dependent. A high dose of TNF- α is a potent inducer of apoptosis in lymphocytes (13). In contrast, absence of TNF- α may promote lymphocyte survival, as supported by the induction of primary CTCL after treatment with anti-TNF- α antibodies (14–16).

Immediate early response gene 3 (IER3), also known as IEX-1, p22/PRG1, Dif-2, or the mouse homologue *gly96*, is a stress-inducible gene (17–20). IER3 can be rapidly and transiently activated by TNF- α and various other factors (13, 17–23). The IER3 degrades the mitochondrial ATPase inhibitor leading to acceleration of ATP hydrolysis and reduction in reactive oxygen species (ROS) production (24). As a high level of ROS production may cause oxidative stress and mitochondrial membrane disruption leading to apoptosis (25), the upregulation of IER3 protects cells from apoptosis.

The purpose of this study was to further investigate the mechanism of observed resistance of Sézary cells to pro-apoptotic doses of TNF- α . We evaluated TNF-receptor density on the surface of malignant lymphocytes and a downstream of IER3 pathway in response to a pro-apoptotic dose of TNF- α . We found that in addition to a decrease in the level of TNFR1 expression, the level of IER3 induction correlated with down regulation of ROS formation in Sézary cells.

METHODS

Patients

Patients with SzS were enrolled in this IRB-approved study after informed consents were obtained (Table 1). Monoclonal T cell receptor gene rearrangement was detected in all patients by Southern blot and PCR. Peripheral blood flow cytometry revealed loss of CD26 expression on malignant lymphocytes in all patients.

Isolation of CD26+ or CD26– T Lymphocytes from Peripheral Blood

Fifteen ml of peripheral blood was obtained from healthy volunteers and subjects with SzS. Blood samples were directly incubated with whole blood MicroBeads (Miltenyi Biotec, Auburn, CA) for subsequent purification of the CD4 lymphocyte. For CD26 selection, cells were resuspended in CliniMacs PBS/EDTA buffer (Miltenyi Biotec, Auburn, CA) supplemented with 0.5% human serum albumin at 10^7 cells per 100 μ l. To avoid non-specific binding, 20 μ l of FcR Blocking Reagent (Miltenyi Biotec, Auburn, CA) was added. Cells were labeled with CD26 biotin-conjugated antibodies (Miltenyi Biotec, Auburn, CA) for 10 min at 4°C. Thereafter, cells were washed twice and incubated with anti-biotin antibody conjugated to ferrobeads (Miltenyi Biotec, Auburn, CA). Selection of CD26+ and CD26– cells was done by one-step immunomagnetic separation according to the manufacturer's instructions (Miltenyi Biotec, Auburn CA). CD26– cells were collected as a non-bound fraction, while CD26+ cells were eluted with 500 μ l PBS/EDTA/HSA buffer. The purified CD4+ CD26+ and CD4+ CD26– cells were used directly for flow-cytometric assessment of purity. Median purity of each lymphocytes subset was >90.5%.

RT-PCR

Total RNA was isolated from CD26+ or CD26- T lymphocytes from five patients with SzS and five healthy volunteers using RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Contaminated DNA was removed by digestion with DNase I. The resultant RNA was reverse transcribed using ThermoScript reverse transcriptase and random hexamer primers (Invitrogen, Carlsbad, CA). IER3 genes were amplified by TaqDNA polymerase with the following primers: IER3 forward, 5'-CGCAGCCG CAGGGTTCTCTA-3' and reverse 5'-CGGGTGTTGCTGGAGGAAAG-3'; and β -actin forward, 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' and reverse, 5'-CGTCATACTCCTGCTTGCTTGCTGATCCACATCTGC-3'. RNA samples not reverse transcribed were run in parallel as negative controls. The PCR products were separated in 1% agarose and visualized by the Kodak Gel Logic 200 imaging system (Kodak Inc.).

Immunohistochemical Analysis of IER3 Expression

Skin biopsies from seven SzS patients were stained with 1:1000 polyclonal rabbit anti-IER3 antibody (Novus Biological, Littleton, CO). Antigen retrieval was performed in 10 mmol/L of citrate buffer (pH 6) using a pressure cooker. Endogenous peroxidase was quenched with 3% hydrogen peroxide. Blocking was performed with non-immune normal serum. IER3 staining was performed using the EnVision method (Dako Corp., Carpinteria, CA). Immunoreactive cells were visualized with diaminobenzidine chromogenic substrate (Vector ABC, Vector Labs, Burlingame, CA) and counterstained with hematoxylin. Tissue from human adrenal glands served as positive control.

Flow Cytometry

Lymphocytes from peripheral blood of 11 SzS patients and five healthy volunteers were isolated with ficoll-paque gradient, washed twice with PBS, and incubated with 10% normal serum to block non-specific binding before staining. Cell staining was performed with anti-CD4-PE-Cy7 (SK3) from BD Pharmingen (San Diego, CA), anti-CD26-FITC (M-A261) from Serotec (Raleigh, NC), anti-CD120a-AlexaFluor 647 (H398), and anti-CD120b-PE (80M2) from Beckman Coulter (Brea, CA). The cells were evaluated using FACS Aria and CellQuest Software (Becton Dickinson, San Jose, CA). FlowJo software (Tree Star Inc, Ashland, OR) was used for analysis of flow cytometric data.

Viability and apoptosis assays

Cell viability was evaluated by Trypan Blue exclusion assay per the manufacturer's instruction (Cellgro, Mediatech, Inc., Manassas, VA). Apoptosis was measured by flow cytometry with a Annexin-V-FITC Kit (Beckman Coulter, Fullerton, CA), which stained phosphatidylserin on the external surface of apoptotic cells.

Assessment of Intracellular ROS

Freshly isolated cells were washed out of culture medium and left for 10 min in PBS, resuspended at 1.5×10^6 100 μ l in pre-warmed Hank's buffer containing 2% FBS. 1 μ M 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (carboxyl-DCFH-DA) (Invitrogen, Carlsbad, CA) was added to the cell suspension with subsequent incubation at 37°C for 30 min. Washed cells were resuspended in flow buffer and fluorescence emitted in 535 nm was read after excitation at 485 nm. Cells stimulated with 3% H₂O₂ were used as positive control.

Luminex Assay Specifications and Procedure

Peripheral blood was collected from eight patients with SzS and 25 healthy volunteers and was processed within 30 minutes after the draw. 1 ml serum aliquots were prepared and stored at -80°C prior to the Luminex analysis. Cytokines and growth factors were analyzed using xMap™ technology (26): TNF- α was measured using a high-sensitivity kit from Linco/Millipore Research (St. Charles, MO) and TNFRs were measured using kits from Biosource International (Camarillo, CA). The xMap™ serum assays were done in a 96-well microplate format. A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 min with PBS/bovine serum albumin. To generate a standard curve, 5-fold dilutions of appropriate standards were prepared in serum diluent. Standards and patient sera were pipetted at 50 μL per well and mixed with 50 μL of the bead mixture. The microplate was incubated for 1 h at room temperature on a microtiter shaker. Wells were then washed twice with washing buffer using a vacuum manifold. PE-conjugated secondary antibody was added to the appropriate wells following by incubation for 45 min in the dark with constant shaking. After washing twice, assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA). All pipetting procedures were performed by a robotic liquid handler (LabStar, Hamilton, Birmingham, UK). Analysis of data was done using four-parametric-curve fitting (27).

Statistical analyses

The statistical analysis was based on the calculation of arithmetic mean, standard deviation, and/or standard error of mean. The difference between two means was compared by a two-tailed unpaired Student's *t* test without assumption of equal variances. Two-way ANOVA was used to compare two sets of data in TNF- α dose-dependent IER3 expression assays. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Demographics and clinical characteristics of the patients with SzS participating in the study

The clinical characteristics of the patients are summarized in Table S1. Patient age at the time of the study ranged from 49 to 86 with a median age of 70.5 ± 11.6 years (stage IVA1, mean 71.6 ± 9.9 years; stage IVA2, mean 69.5 ± 13.6 years). The male-to-female ratio was 1.2:1. African Americans were 2 out of 11 patients; the rest of the patients were of mixed European descent. The average time from diagnosis with SzS to participation in the study was from 1 to 34 months, on average of 11.4 ± 10.4 months. Peripheral lymphocyte immunophenotyping and PCR for T-cell receptor gene rearrangement (TCR GR) were performed at time of diagnosis in all patients as a part of diagnostic workup. All of our patients had elevated numbers of CD4+ and CD4+CD26- cells.

Loss of TNFR1, but not TNFR2 on Sézary cells is associated with resistance to TNF- α -mediated apoptosis

Malignant lymphocytes were isolated from the peripheral blood of SzS patients and separated from non-malignant cells based on the loss of CD26 expression. The loss of CD26 expression was proposed to be a constant feature of circulating Sézary cells by flow cytometric immunophenotyping (28–30). Morphology of these CD4+CD26- cells derived from SzS patients (= Sézary cells) revealed convoluted cerebriform nuclei characteristic for malignant cells. To assess effects of a pro-apoptotic dose of TNF- α , CD26+ and CD26- T lymphocytes derived from both SzS patients and healthy volunteers were incubated with 100 ng/ml of TNF- α for 3h. We observed that the viability of Sézary cells was more than 90% ($91.5 \pm 5.9\%$), which remained unchanged after TNF- α treatment, in contrast the number of

CD26+ T lymphocytes decreased by 40.8% after co-incubation with TNF- α ($p < 0.001$) (Fig. 1a). The unchanged number of Sézary cells after TNF- α treatment was most likely due to the increasing resistance to TNF- α -induced apoptosis in CD4+CD26- cells as demonstrated by Annexin V staining (reduction of Annexin V staining in $43.3 \pm 5.7\%$ in CD4+CD26+ vs. $0.7 \pm 0.5\%$ in CD4+CD26-, $p < 0.001$) (Fig. 1b).

The effect of TNF- α on the cells is mediated by the binding of two TNF- α receptors: TNFR1 and/or TNFR2. While nearly all cells of the body express TNFR1, limited selective expression of TNFR2 on T lymphocytes secures the pro-survival function of lymphocytes in response to infection, injury, and cancer via proliferation of T cells, differentiation, and recruitment of naïve immune cells (31). We found that a significantly lower percentage of SzS cells expressed TNFR1 and TNFR2, when compared to lymphocytes from healthy controls (Fig. 1c and 1d). Moreover, the level of surface TNFR1 expression on Sézary cells was 2.7 times lower than that on non-malignant cells within the same patient (Fig. S1 and 1c). While the TNFR2 expression was also low on Sézary cells, there were no statistically significant difference between healthy controls and SzS patients (Fig. 1d). We also observed a corresponding elevation of soluble TNFR1, but not TNFR2 in sera from SzS patients in comparison with healthy volunteers (Fig. 1e). The concentration of TNF- α in sera was comparable between SzS patients and control groups (Fig. 1f).

IER3 expression is associated with decreased production of ROS in Sézary cells

Cellular localization of IER3 was studied by immunohistochemistry. In 4/5 of SS patients we observed intranuclear localization of IER3 (round, well-demarcated, or speckled pattern), while one patient had a mixed nuclear and perinuclear pattern (Fig. 2a).

Even small changes in level of expression of IER3 may significantly alter mitochondrial ROS homeostasis (24). Accordingly, we measured ROS level in the CD26+ and CD26- T-lymphocytes in patients with SzS. Carboxyl-DCFH-DA ROS staining demonstrated a significant decrease in the amount of fluorescent oxidized product, carboxyl-DCF indicating a 3.7 times lower level of ROS in malignant CD26- T cells over CD26+ T cells (Fig. 2b). The decrease of ROS in Sézary cells significantly lower in comparison with CD4+CD26- from healthy controls (Fig. 2c).

The level of IER3 expression is not TNF- α -dependent in Sézary cells

We measured the RNA level of IER3 in the SzS patients' malignant and non-malignant cells and in the lymphocytes from the normal volunteers at baseline and after exposure to TNF- α . At the baseline, in non-stimulated cells, the level of IER3 mRNA expression was increased in malignant CD4+CD26- lymphocytes in comparison with non-malignant CD4+CD26+ cells or with CD4+CD26+ or CD4+CD26- lymphocytes derived from healthy volunteers (Figure S2).

Since IER3 expression varies greatly depending on stress stimuli and cytokine microenvironment (13, 18, 22), we evaluated changes in IER3 expression after exposure to TNF- α . We chose the highest dose of 100 ng/ml of TNF- α because it causes the apoptosis of 25% of non-malignant lymphocytes and "synchronizes" the IER3 expression (13). The difference in IER3 expression became even more pronounced revealing the highest expression in malignant lymphocytes (Fig. 3a and b). The dose-dependent expression of IER3 was further evaluated in CD4+CD26- T lymphocytes in response to 5, 10 and 20 ng/ml of TNF- α (Fig. 3c). In agreement with previous findings, in healthy non-malignant lymphocytes the level of IER3 expression was directly correlated with the dose of TNF- α (13). However, in Sézary cells, the IER3 expression was consistently and habitually elevated independent of incremental doses of TNF- α .

DISCUSSION

Failure to execute an apoptotic program is one of the critical steps and a common mechanism promoting tumorigenesis (32–34). Apoptosis can be induced by various stimuli, including TNF- α (35, 36). TNF achieves these effects via distinct signaling pathways through two membrane receptors, TNFR1 (CD120a; p55/60) and TNFR2 (CD120b; p75/80). TNFR1 is ubiquitously expressed on broad cell populations and activates caspase-2 via TRADD signaling leading to apoptosis, while TNFR2 rapidly activates NF- κ B via TRAF2, a transcription factor that leads to cell survival. Differential and adaptable expression of TNFRs on the cell types contributes to control of survival/apoptosis.

TNFRs are membrane proteins with an extracellular domain that can be released from the surface. The high level of soluble TNFR1 in the serum of the patients with SzS together with the profound loss of TNFR1 on the malignant lymphocytes are suggestive of an excessive shedding of this receptor from Sézary cells. The release of the extracellular domain of TNFR1 and the resulting decrease of the number of receptor molecules on the surface was shown to desensitize the cell to the TNF- α effects (37). We found an absence of elevation of TNF- α in SzS patients, resistance of Sézary cells to TNF- α -induced apoptosis, and the loss of TNFR1 on malignant lymphocytes. Our data confirm previous findings in CTCL cell lines (11) and microarray studies of deregulation of TNFR signaling in CTCL (38).

The protective effect of IER3 upregulation in T cells was established by Zhang Y. et al. when apoptosis was triggered by Fas-ligand. The authors observed an inverse correlation of IER3 expression and susceptibility of T cells to Fas-induced apoptosis (39). Interestingly, the T cells at the early stages (≤ 3 days) of activation are largely resistant to Fas-induced apoptosis. Such resistance was not ascribed to the level of Fas or Fas-ligand expression (early activated T cells co-expressed high levels of Fas and Fas-ligand (40)), rather it was attributed to the high level of IER3 expression (39). In a significant proportion of CTCL patients, Fas expression has been reported to be decreased or absent (41) and found to correlate with reduced sensitivity of malignant lymphocytes to Fas-mediated apoptotic signals (7). Possibly, IER3 over-expression in malignant lymphocytes may play some role in this protection as well.

Global gene expression microarray experiments in diverse primary cancer cells (42, 43) and transformed cell lines (e.g., Colo320 colorectal (44), MCF-7 breast (45) carcinoma cells) have identified IER3 as an expression outlier that may have prognostic importance (43, 46). Recently, the role of IER3 in patients with myelodysplastic syndrome were described (46–47). Mice over-expressing IER3 spontaneously develop T-cell lymphoma, which is attributed to alteration in apoptosis sensitivity (including susceptibility to death receptor ligation), cell cycle progression and/or proliferation (48). Zhang et al. generated E μ -IER3 mice by expression of human IER3 gene under the control of an immunoglobulin heavy chain enhancer that drives the expression of the transgene specifically in T- and B-cell lineage. Old E μ -IER3 mice developed T-cell lymphomas with variable CD4 and CD8 phenotype in the spleen. Lymphomas were found to arise from aberrant clonal expansions of T cells expressing a specific TCR (48).

Our findings of over-expression of IER3 in human Sézary cells may shed new light on CTCL pathogenesis. IER3 transcription can be rapidly and transiently activated by irradiation, growth factors, viral infection, and inflammatory cytokines, including TNF- α . Because IER3 is a well-known downstream target for the TNF- α -inducible pathway, we sought to determine the relationships between observed TNF- α -related abnormalities and IER3 expression in patients' malignant and non-malignant lymphocytes. While the level of IER3 expression in non-malignant lymphocytes is directly correlated with the dose of TNF-

α , the IER3 expression in Sézary cells was constantly elevated and was independent of TNF- α dose escalation. This elevated level of IER3 expression correlated with a preserved viability of malignant cells under treatment with apoptotic dose of TNF- α .

We found an over-expression of IER3 and decreased level of ROS in Sézary cells, which were resistant to apoptosis. One of the well-described functions of IER3 is an ability to regulate apoptosis, which is in good agreement with a role of IER3 in the control of mitochondrial oxidative phosphorylation, given that mitochondria are central to the initiation of apoptosis (49, 50). IER3 upregulation leads to ROS reduction due to degradation of the mitochondrial F₁F₀-ATPase inhibitor and acceleration of ATP hydrolysis (24). IER3 over-expression has been reported to reduce cellular ROS not only at the basal level but also after stimulation with a stress inducer (49). At such a low intracellular concentration, ROS probably act as signaling molecules activating oxidant-sensitive signaling to promote cell proliferation and suppression of apoptosis.

Most of our patients exhibited intranuclear localization of IER3 on immunohistochemistry, while only one patient had a mixed nuclear and perinuclear pattern of IER3 distribution. IER3 product is a highly agile protein, which was shown to localize in nuclear regions (51), perinuclearly and within endoplasmic reticulum (13). Localization of the protein to the nucleus or cytoplasm may be important for IER3 to exert its biological activities (52). Nuclear translocation of IER3 was demonstrated to interact directly with Mcl-1 (53). Mcl-1 is an anti-apoptotic member of the Bcl-2 family, which translocates to nucleus to induce a pro-survival effect in response to DNA damage or genomic instability. In the nucleus Mcl-1 triggers Chk1 activation, causes G2 checkpoint arrest, and repairs DNA lesions promoting cell survival (53). These studies strongly support an anti-apoptotic action of IER3 over-expression.

In summary, we demonstrated significant abnormalities in TNF- α signaling pathway, including, among others, significant reduction of the TNFR1 density on Sézary cells and resistance of these cells to pro-apoptotic dose of TNF- α . Moreover, this resistance to apoptosis was not proportionally related to a reduction of TNFR1 density, but could be also explained by high and persistent expression of IER3 regardless of the dose of TNF- α . In fact, levels of ROS were significantly lower in Sézary cells in spite of TNF- α challenge, possibly related to inappropriate over-expression of IER3 and its insensitivity to TNF- α control. Targeting IER3 could be of interest for the development of future therapeutic strategies for patients with SzS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IER3	immediate early response gene 3
SzS	Sézary syndrome

ROS	reactive oxygen species
CTCL	cutaneous T cell lymphoma

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Figure 1.

(a–b) Sézary cells are resistant to TNF- α -induced apoptosis. Lymphocytes from peripheral blood were sorted with microbeads and 0.5×10^6 cells/ml were incubated with 100 ng/ml of TNF- α for 3h. (a) Viability of lymphocytes 3h after incubation with TNF- α . Trypan Blue. 5 SzS patients. (b) FACS analysis of Annexin V-positive cells. Gray, control for autofluorescence of cells; blue, cells incubated with PBS for 3h, stained with Annexin V-FITC antibody; red, cells incubated with 100 ng/ml of TNF- α for 3h, stained with Annexin V-FITC antibody. Representative flow of 5 SzS patients. **(c–d) The loss of TNFR1 and TNFR2 on Sézary cells.** (c) Percentage of TNFR1 positive cells among lymphocytes from 11 patients with SzS and 5 healthy controls. $M \pm SEM$ (*, $p < 0.05$; ***, $p < 0.001$). (d) Percentage of TNFR2 positive cells among lymphocytes from 11 patients with SzS and 5 healthy controls. $M \pm SEM$ (***, $p < 0.001$). **(e) Soluble TNFR1 and TNFR2 in peripheral blood of SzS patients in comparison with healthy volunteers (controls).** Luminex analysis of sera from 8 patients with SzS and 25 age-matched healthy volunteers. **(f) TNF- α in peripheral blood of CTCL patients in comparison with healthy volunteers.** Luminex analysis of sera from 8 patients with SzS and 25 age-matched healthy volunteers.

Figure 2. High IER3 positivity in malignant CD4+CD26⁻ T-lymphocytes is associated with an increased level of ROS

(a) **Representative IER3 staining of Sézary cells in skin biopsy of a representative patient with Sézary syndrome.** IER3 staining was performed as outlined in *Methods*. X1000. Predominant intranuclear localization of IER3 with round, well-demarcated, or speckled pattern. (b) ROS production in malignant lymphocytes (blue tinted) vs. autologous non-malignant cells (red line). Green line represents positive control (3% H₂O₂). (c) Geometric mean of DCF expression in CD4+CD26⁺ vs. CD4+CD26⁻ in 5 SzS patients vs. 5 healthy controls. Bars represent minimum-maximum range of data; a horizontal line represents mean. *, $p < 0.05$

Figure 3. The IER3 is over-expressed in Sézary cells and is not affected by 100 ng/ml of TNF- α (a) Representative RT-PCR of lymphocytes from SzS patient and healthy volunteer after incubation with 100 ng/ml of TNF- α for 1h. (b) mRNA/ β -actin ration. A dot represent a sample from one study subject, and vertical line is the average of the band density (*, $p < 0.05$; **, $p < 0.01$). 5 patients with SzS. 5 healthy volunteers (controls). (c) **The increased baseline level of IER3 expression in Sézary cells is not affected after incubation with TNF- α , while CD26- lymphocytes from healthy volunteers demonstrate variable dose-dependent responses.** Lymphocytes from peripheral blood of patients with SzS and healthy volunteers were sorted with microbeads and 0.5×10^6 cells/ml of CD4+CD26- were incubated with different doses of TNF- α for 1h.