Exploring the IL-21–STAT3 Axis as Therapeutic Target for Sézary Syndrome

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Sézary syndrome is an aggressive cutaneous T-cell lymphoma. The malignant cells (Sézary cells) are present in skin, lymph nodes, and blood, and express constitutively activated signal transducer and activator of transcription (STAT)3. STAT3 can be activated by IL-21 *in vitro* and the IL-21 gene itself is a STAT3 target gene, thereby creating an autocrine positive feedback loop that might serve as a therapeutic target. Sézary cells underwent apoptosis when incubated with Stattic, a selective STAT3 inhibitor. STAT3 activation in Sézary cells did not affect expression of the supposed anti-apoptotic STAT3 target genes *BCL2*, *BCL-xL*, and *SURVIVIN*, whereas expression of (proto)oncogenes *miR-21*, *TWIST1*, *MYC*, and *PIM1* was significantly increased. CD3/CD28-mediated activation of Sézary cells induced IL-21 expression, accompanied by STAT3 activation and increased proliferation. Blocking IL-21 in CD3/CD28-activated cells had no effects, whereas Stattic abrogated IL-21 expression and cell proliferation. Thus, specific inhibition of STAT3 is highly efficient in the induction of apoptosis of Sézary cells, likely mediated via the regulation of (proto)oncogenes. In contrast, blocking IL-21 alone seems insufficient to affect STAT3 activation, cell proliferation, or apoptosis. These data provide further insights into the pathogenic role of STAT3 in Sézary syndrome and strengthen the notion that STAT3 represents a promising therapeutic target in this disease.

INTRODUCTION

Signal transducer and activator of transcription (STAT)3 is a transcription factor that has a critical role in various fundamental cellular processes such as cell development, growth, and homeostasis. Under physiological conditions, STAT3 can be activated by various growth factors or cytokines, mediated by receptor-associated JAK kinases, as well as by non-receptor Tyr kinases (Yu *et al.*, 2007). Upon activation, STAT3 is phosphorylated on the Tyr residue at position 705, followed by dimerization via reciprocal phosphoTyr-SH2 interactions, translocation into the nucleus, and binding to the DNA-binding motifs in the promoter regions of the target genes.

STAT3 was found to be activated inappropriately in a wide variety of human cancers. STAT3 acts as a central mediator of neoplastic cellular transformation (Yu and Jove, 2004; Frank, 2007), and, in certain cellular contexts, its continuous activation is sufficient for oncogenesis (Bromberg *et al.*,

1999). Target genes of STAT3 are involved in various stages of tumor development including proliferation, survival, self-renewal, invasion, and angiogenesis (Yu and Jove, 2004; Frank, 2007). Together, this implies STAT3 as a key player in the oncogenic process, and consequently as a target for the molecular therapy of cancer. Indeed, inhibition of STAT3 by different means can exert anti-cancer effects, mainly by the induction of apoptosis of tumor cells (Frank, 2007).

Sézary syndrome (SS) is a leukemic variant of cutaneous T-cell lymphoma. Malignant cells (SS cells) are CD4+ T cells with a skin-homing memory phenotype, and can be found in patients' skin, blood, and lymph nodes. SS cells show constitutive phosphorylation STAT3 at position Tyr705 (Zhang *et al.*, 1996; Nielsen *et al.*, 1999; Eriksen *et al.*, 2001; van Kester *et al.*, 2008). Inhibition of STAT3 phosphorylation using inhibitors of upstream JAK kinases results in apoptosis of SS cells (Eriksen *et al.*, 2001; van Kester *et al.*, 2008). We recently showed that STAT3 can be activated in SS cells by IL-21 (van der Fits *et al.*, 2012). IL-21 is a cytokine belonging to the common-γ chain family of cytokines. It is produced by different subsets of activated CD4+ T cells and NK-T cells, and has complex activities on various cell types, including enhancement of adaptive T-cell immunity, antibody production, activation and maturation of NK cells, and impairment of regulatory T-cell development and function (Davis *et al.*, 2007). The IL-21 receptor is comprised of two chains, the common-γ chain and an IL-21-specific receptor chain (IL-21R). IL-21R expression can

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Abbreviations: SS, Sézary syndrome; STAT, signal transducer and activator of transcription

be found on a variety of immune cells, as well as on nonimmune cells such as endothelial and epithelial cells (Monteleone *et al.*, 2008, 2009). We previously demonstrated increased expression of IL-21R on Sézary cells when compared with CD4+ cells from healthy donors (van der Fits *et al.*, 2012). As IL-21 is a direct STAT3 target gene in healthy T cells (Caprioli *et al.*, 2008), as well as SS cells (van der Fits *et al.*, 2012), an autocrine positive feedback loop can be involved in the activation of STAT3 and in the subsequent resistance to apoptosis in SS cells.

In this study, we investigated the feasibility of therapeutic targeting of the IL-21—STAT3 autocrine loop in SS. We show that selective blocking of IL-21 does not affect apoptosis or cell proliferation. In contrast, inhibition of STAT3 using Stattic induces apoptosis and simultaneously attenuates cell proliferation.

RESULTS

Stattic causes rapid apoptosis of SS cells

Treatment of SS cells with AG490 or Cucurbitacin I (JSI-124) results in decreased STAT3 phosphorylation, accompanied by induction of apoptosis (Eriksen *et al.*, 2001; van Kester *et al.*, 2008). As both compounds do not specifically target STAT3 but rather function as inhibitors of upstream JAK kinases, we investigated the effects of treatment of SS cells with Stattic, a compound specifically targeting the SH2 domain of STAT3, thereby preventing STAT3 dimerization, activation, and nuclear translocation (Schust *et al.*, 2006).

Stattic causes a dose-dependent inhibition of the viability of SS cells, demonstrated for primary cells as well as the Sézary cell lines SeAx en HuT-78. Both primary cells and cell lines show a comparable sensitivity toward Stattic (Figure 1a). This decreased viability was caused by the induction of apoptosis, as revealed by the detection of active caspase-3 as early as 6 hours after addition of Stattic (Figure 1b). In addition, flow cytometric analysis for propidium iodide and AnnexinV revealed a decrease in the percentage of viable cells, whereas the percentage of apoptotic and dead cells was increased (Figure 1c). Similar effects of Stattic were observed for CD4+ cells from healthy donors. However, the level of induction of apoptosis seems less in healthy CD4+ cells than in SS cells (Figure 1c).

IL-21 induces (proto)oncogenes rather than anti-apoptotic genes

Many STAT3 target genes regulate cellular processes important in oncogenesis (reviewed in the study by Frank, 2007). In this study, we examined a number of previously described STAT3 target genes involved in regulation of proliferation and apoptosis. Primary SS cells were treated for various time points with IL-21 to activate STAT3, and expression of putative STAT3 targets was assayed by quantitative RT-PCR. In line with previous studies (van der Fits *et al.*, 2011), the oncogenic *pri-miR-21* was significantly induced in SS cells, thereby serving as a positive control (Figure 2a). Interestingly, expression of anti-apoptotic genes *BCL2*, *BCL-xL*, and *SURVIVIN* was not induced by IL-21, whereas only marginal induction was observed for *MCL1*. In contrast, (proto)oncogenes *TWIST1*, *PIM1*, and *MYC* were strongly induced by IL-21 (Figure 2a).
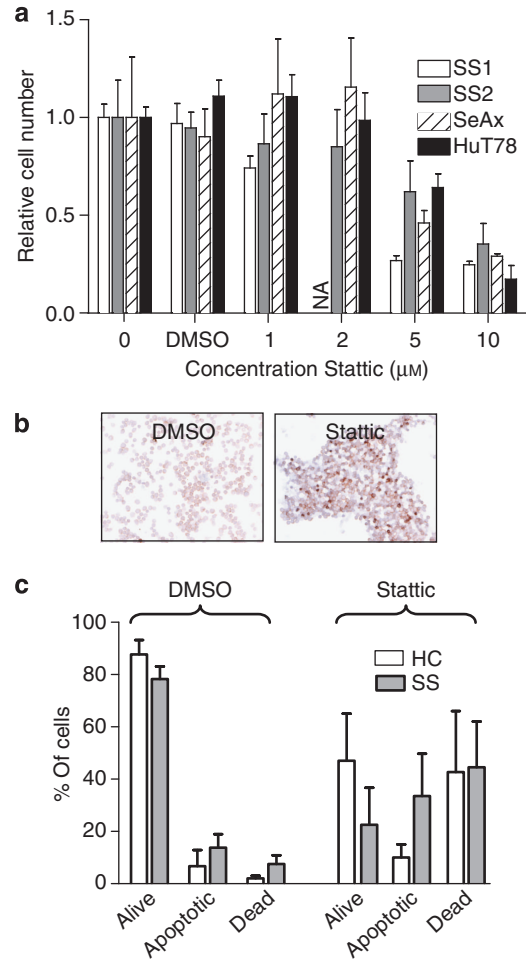
Figure 1. Specific inhibition of signal transducer and activator of transcription (STAT)3 by Stattic results in apoptosis of Sézary cells. (**a**) Primary Sézary syndrome (SS) cells (*n* = 2), SeAx or HuT-78 cells were cultured with Stattic or DMSO. After 72 hours, cell viability was assayed using the WST test, and calculated relative to cells cultured in medium. NA, not assayed. (**b**) Primary SS cells were cultured for 6 hours with 10 μM Stattic or the volume equivalent DMSO, centrifuged onto glass slides, and stained for active caspase-3. A representative example of four patients is shown. (**c**) CD4+ cells from healthy donors or Sézary patients were cultured for 24 hours with 10 μM Stattic, or the volume equivalent of DMSO. Apoptosis was assayed by flow cytometry. Alive cells were defined as being double negative for AnnexinV and propidium iodide (PI). Apoptotic cells were AnnexinV+ and PI−, whereas dead cells were AnnexinV+ and PI+. The mean % of cells ± SEM is shown for *n* = 3 (healthy controls, HC) or *n* = 4 (Sézary patients, SS).

Blocking IL-21 does not influence SS cell survival

We previously demonstrated that IL-21 is a potent inducer of STAT3 in SS cells (van der Fits *et al.*, 2012). To investigate whether IL-21 and the IL-21 receptor (IL-21R) can serve as therapeutic targets, we assayed the effect of blocking IL-21 using IL-21R-Fc chimera protein. The efficacy of this protein in Sézary cells was experimentally validated in dose-response and time course experiments (data not shown). Incubation of primary SS cells with IL-21R-Fc chimera for 72 hours did not consistently affect cell numbers (data not shown, *n* = 4) or cell cycle progression (data not shown, *n* = 4). In addition,
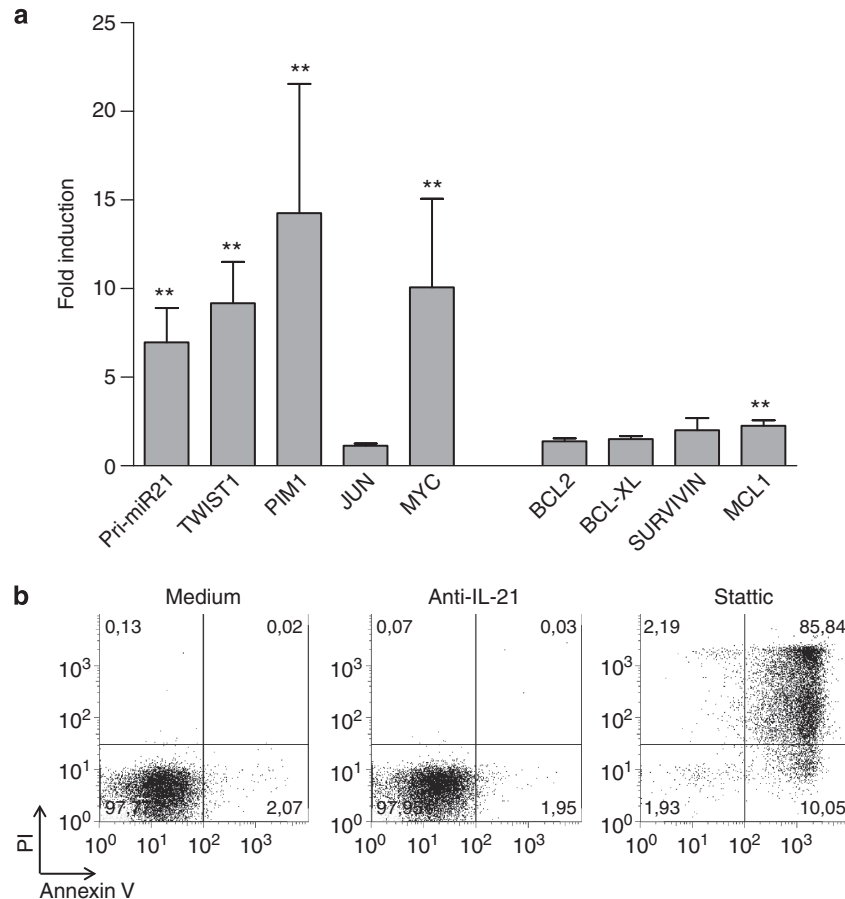
Figure 2. IL-21 induces expression of (proto)oncogenes rather than anti-apoptotic genes, and blocking IL-21 does not affect apoptosis. Primary Sézary syndrome (SS) cells were stimulated with IL-21 for 1, 2, 4, and 8 hours and RNA was extracted. mRNA expression of the genes indicated was determined by quantitative reverse transcriptase in real-time PCR (QPCR), and calculated relative to expression in non-stimulated cells. As induction of the different genes in each patient did not occur with identical kinetics, we determined the maximum fold induction for each gene and patient. For each gene, the mean of this maximum fold is depicted ± SEM for $n = 6$ patients. Asterisks indicate significant differences (Wilcoxon rank test, $P < 0.05$) (**a**). Primary SS cells were incubated with $10\,\mu g\,ml^{-1}$ of IL-21R-Fc chimera (anti-IL-21) or $10\,\mu M$ Stattic for 72 hours, and apoptosis was assayed by flow cytometry for AnnexinV and propidium iodide (PI). The experiment shown is representative of $n = 2$ (**b**).

blocking IL-21 did not result in apoptosis, whereas STAT3 inhibition by Stattic strongly induced apoptosis of SS cells (Figure 2b).

CD3/CD28 stimulation of SS cells induces IL-21 expression and STAT3 activation, which is unaffected by blocking autocrine IL-21 signaling

The lack of effect of blocking IL-21 on survival of SS cells might be due to the relative inert state of SS cells cultured *in vitro*. To bypass this, we assayed the effect of blocking IL-21 in SS cells simultaneously activated by CD3/CD28-coated beads.

Stimulation of CD4+ cells from healthy donors and SS patients with CD3/CD28-coated beads resulted in secretion of IL-21 protein in the culture supernatant (Figure 3a), whereas IL-21 was hardly detectable when cells were left unstimulated (data not shown). Interestingly, IL-21 secretion by SS cells was increased compared with cells from healthy donors when stimulated overnight, whereas this difference was not observed anymore when cells were stimulated for 3 days

(Figure 3a). For SS cells, we additionally showed that this induction of IL-21 protein secretion was accompanied by increased mRNA expression (Figure 3b).

Addition of IL-21R-Fc chimera to the culture medium did not result in attenuation of IL-21 RNA and intracellular IL-21 expression (Figures 3b and d), but significantly reduced the level of IL-21 protein in the culture medium (Figure 3c), demonstrating that the secreted IL-21 is efficiently captured by the IL-21R-Fc chimera. The CD3/CD28-induced IL-21 expression was accompanied by elevated activation of STAT3, which was not affected by addition of the IL-21-blocking protein (Figure 3e). In addition, blocking autocrine IL-21 in CD3/CD28-activated SS cells did not exert any effect on the expression of STAT3 target genes *pri-miR-21*, *TWIST1*, *PIM1*, and *MYC* (data not shown). In contrast, selective targeting of STAT3 in CD3/CD28-activated SS cells using Stattic resulted in decreased IL-21 expression, STAT3 activation (Figure 3), and expression of STAT3 target genes (data not shown).

Summarized, CD3/CD28 activation of Sézary cells results in increased IL-21 production and activation of STAT3. Inhibition
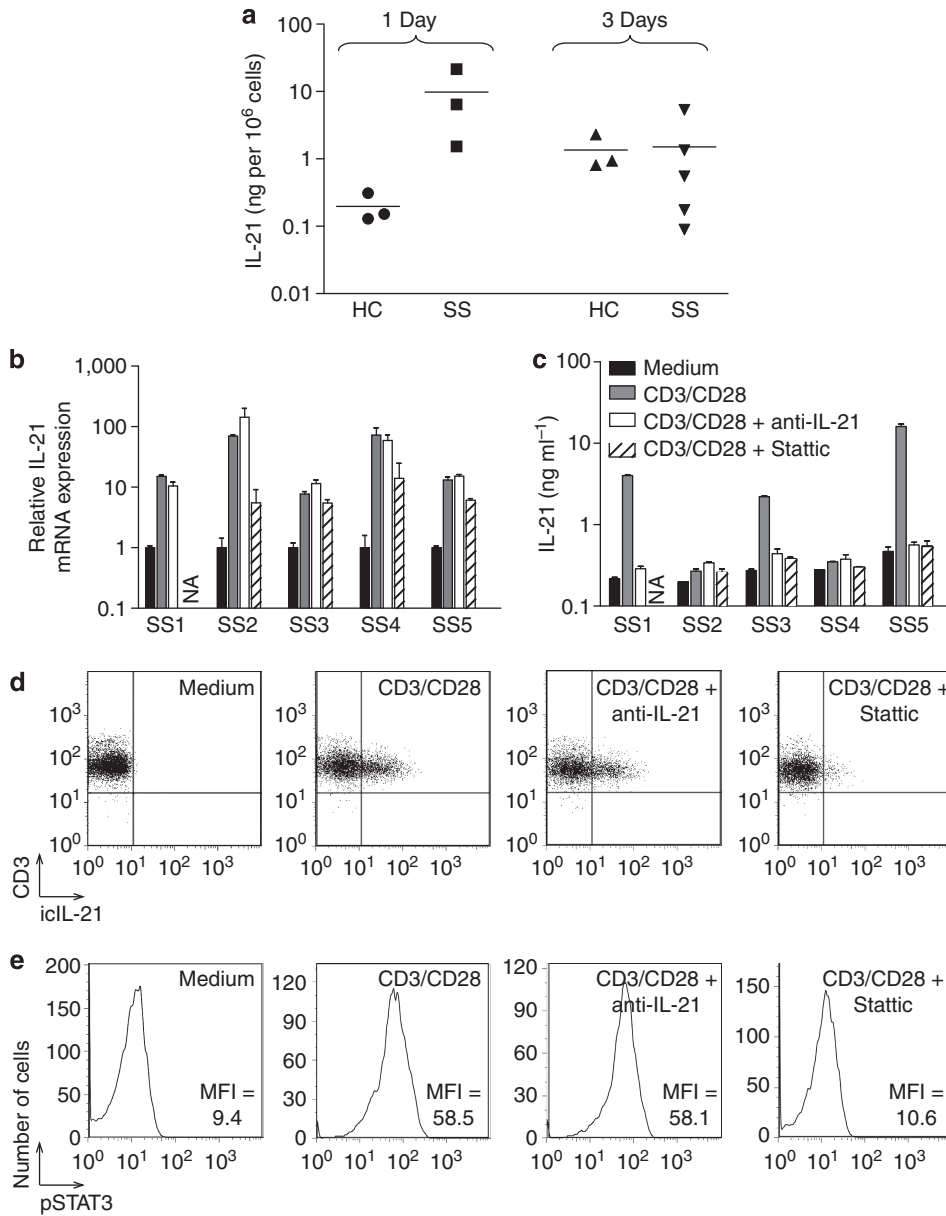
Figure 3. Polyclonal stimulation of Sézary cells results in IL-21 production and signal transducer and activator of transcription (STAT)3 activation, which is not influenced by anti-IL-21. CD4+ cells isolated from healthy controls (n=3, HC) or Sézary patients (n=3 or 5, SS) were stimulated with CD3/CD28 beads for 1 or 3 days. IL-21 protein secretion in the culture supernatant was assayed by ELISA (**a**). Primary Sézary cells (n=5) were cultured for 3 days in medium, medium with CD3/CD28 beads, or with CD3/CD28 beads + anti-IL-21, or with CD3/CD28 beads and Stattic. IL-21 expression was assayed on mRNA level by quantitative reverse transcriptase in real-time PCR (QPCR) (**b**) or on protein level by ELISA (**c**). In addition, intracellular IL-21 was detected by flow cytometry. Cells were stained for expression of CD3 and IL-21. The experiment shown is representative for n=2 (**d**). pSTAT3 expression was assayed by flow cytometry, and the median fluorescent intensity (MFI) is indicated in the figure. The experiment shown is representative of n=3 (**e**). NA, not assayed.

of autocrine IL-21 feedback signaling does not effectively downregulate STAT3 activation and expression of STAT3 target genes. Similar results were obtained using SS cells mitogenically stimulated by PMA/ionomycin or PHA (data not shown).

Blocking IL-21 does not affect CD3/CD28-induced proliferation, whereas blocking STAT3 does

Polyclonal stimulation of SS cells by CD3/CD28 beads resulted in the formation of clusters of cells, indicative for cell proliferation and/or activation. This was not affected by addition of IL-21R-Fc chimera, whereas cluster formation was prevented by Stattic (Figure 4a). This observed cell activation was reflected in the cell numbers obtained after culture (Figure 4a). Cell proliferation analysis revealed that SS cells cultured in medium alone were inactive and not proliferating, i.e., in G1 phase (Figure 4b). Upon culturing with CD3/CD28-coated beads, cells in G2 and S phase could be recognized. Percentages of cells in G2 and/or S phase were not affected by co-culture with IL-21-blocking protein, whereas the addition
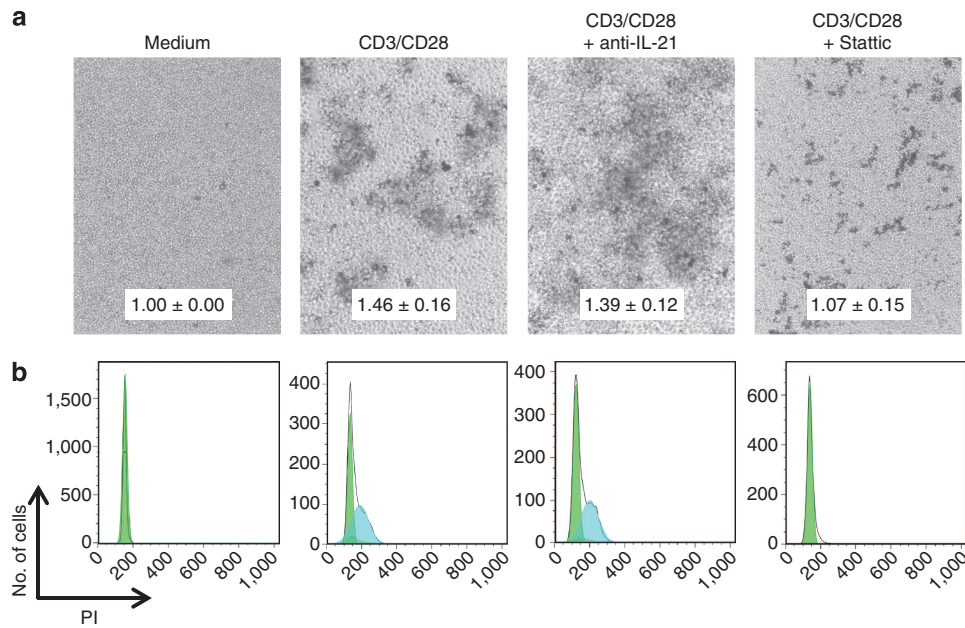
Figure 4. Polyclonal stimulation of Sézary cells causes cell proliferation, which is unaffected by anti-IL-21, but reduced by Stattic. Primary Sézary syndrome (SS) cells were cultured for 3 days with the stimuli indicated. Cells were photographed and counted. Relative cell numbers ± SEM are indicated (**a**, $n = 4$). (**b**) Cellular DNA of the cultured SS cells was stained by propidium iodide (PI), and the cell cycle status was assayed by flow cytometry. The peaks with different DNA content represent cells in G1 and G2 phase. The example shown is representative of $n = 5$.

of Stattic strongly prevented cell proliferation (Figure 4b). Stimulating SS cells with mitogenic stimuli as PMA/ionomycin or PHA instead of CD3/CD28 yielded in similar results (data not shown). To exclude the possibility that the observed CD3/CD28-induced proliferation was caused by admixed reactive CD4+ cells, these cell cycle experiments were repeated using SS cells that were nearly 100% pure, based on an aberrant immunophenotype (i.e., loss of CD7 expression). This yielded identical results, demonstrating that the observed CD3/CD28-induced cell proliferation was within the malignant cell population (data not shown).

In conclusion, polyclonal or mitogenic stimulation of SS cells results in proliferation of cells, which is dependent on STAT3 signaling but is independent of the secretion of IL-21.

DISCUSSION

We demonstrate here that blocking IL-21 does not affect STAT3 activity, apoptosis, or proliferation of primary SS cells, indicating that inhibition of IL-21 signaling is not sufficient to interfere in these processes. In contrast, blocking STAT3 using Stattic results in increased apoptosis and attenuation of cell proliferation, further substantiating the critical role for STAT3 in the oncogenic process in SS.

Our data show that STAT3 inhibition by Stattic can cause apoptosis of SS cells cultured *in vitro* in the absence of co-stimulation by CD3/CD28 (Figure 1), and in the absence of detectable STAT3 phosphorylation (Figure 3e). Similar observations were made very recently in colorectal cancer cell lines in which treatment with Stattic resulted in significant increased sensitivity toward chemoradiotherapy while phosphorylated STAT3 was not detectable. Off-target effects of

Stattic in these experiments were excluded by demonstrating identical effects of shRNA-mediated silencing of STAT3 (Spitzner *et al.*, 2014). These puzzling observations in our and this recently published study might be explained by technical issues, for e.g., by the inability to detect low levels of phosphorylated STAT3 that can still have a physiological role. In addition, the effects of Stattic on unphosphorylated STAT3 can serve as an alternative explanation for the observed results. Stattic selectively inhibits the SH2 domain of STAT3, irrespective of its phosphorylation status (Schust *et al.*, 2006). It has become increasingly clear that unphosphorylated STAT3 can also drive gene expression, among others, of well-known oncogenes, and has been implicated in oncogenesis (Yang *et al.*, 2005; Yang and Stark, 2008).

Stimulation of SS cells by IL-21 results in STAT3 activation, coinciding with increased expression of (proto)oncogenes *miR-21*, *TWIST1*, *PIM1*, and *MYC*. This nicely correlates with the previously reported overexpression of *miR-21* (van der Fits *et al.*, 2011), *TWIST1* (van Doorn *et al.*, 2004), and MYC (Vermeer *et al.*, 2008) in SS, and indicates that these genes are downstream STAT3 target genes mediating the oncogenic effects of STAT3. STAT3 binding to its target genes is cell type– and context-dependent, as illustrated by the limited overlap between data sets of genome-wide studies on different cell types (Chen *et al.*, 2008; Kwon *et al.*, 2009; Hutchins *et al.*, 2012). Studies on selected candidate genes identified several *bona fide* STAT3 target genes mediating the oncogenic function of STAT3 in various tumor types. miR-21 was previously shown to be directly regulated by STAT3 and pathologically involved in multiple myeloma (Loffler *et al.*, 2007). In addition, expression of the transcription factor

TWIST1 is under direct control of STAT3 in breast cancer cells and is involved in mediating (part of the) oncogenic function of STAT3 (Cheng *et al.*, 2008). We observed that treatment of SS cells with IL-21 results in STAT3 activation, accompanied with increased expression of *TWIST1* and *miR-21*, thereby indicating that these genes are candidate STAT3 target genes in SS as well. Previous gene expression analysis of Sézary cells treated with IL-21 for 4 hours identified a very limited set of upregulated genes, including *PIM1* (Marzec *et al.*, 2008), which was confirmed in our study. Other putative STAT3 target genes identified in our study (i.e., *miR-21*, *TWIST1*, and *MYC*) were not identified by Marzec, probably as a consequence of the transient nature of the induction of these genes.

Blocking IL-21 in Hodgkin lymphoma cell lines results in reduction of STAT3 activity and expression of STAT3 target genes (Lamprecht *et al.*, 2008). Also, in ALK-positive anaplastic large-cell lymphoma cell lines, STAT3 activity was efficiently blocked by targeting IL-21 or IL-21R (Dien Bard *et al.*, 2009). In contrast, we demonstrate that blocking the autocrine IL-21 loop in SS cells did not have a significant effect on STAT3 activation or on expression of its target genes, although efficient downregulation of IL-21 secretion in the medium was confirmed. STAT3 can be activated by multiple cytokines and growth factors (Murray, 2007; Yu *et al.*, 2007), including cytokines that are (over)expressed in SS, such as the anti-inflammatory IL-10 (Dummer *et al.*, 1996). In addition, IL-31, whose overexpression in SS was demonstrated recently (Ohmatsu *et al.*, 2012), is capable of inducing, among others, phosphorylation of STAT3 (Cornelissen *et al.*, 2012). It is clear that further research should be conducted to elucidate whether the constitutive STAT3 activity in SS *in vivo* can be attributed to a single cytokine or growth factor, or whether this is a result of a combination of factors.

IL-21 can have tumor-promoting effects on IL-21R-positive malignant lymphocytes. Hodgkin lymphoma cell lines are protected from apoptosis by IL-21 (Dien Bard *et al.*, 2009), and IL-21 induces proliferation of human myeloma cells, while inhibiting apoptosis (Brenne *et al.*, 2002). In ALK-positive anaplastic large-cell lymphoma, cell survival was significantly enhanced by IL-21, whereas this was reduced by inhibiting IL-21R (Dien Bard *et al.*, 2009). IL-21 did not induce proliferation of SS cells, but ameliorated apoptosis (Marzec *et al.*, 2008; Yoon *et al.*, 2008). Our study shows that blocking IL-21 does not affect survival, proliferation, or apoptosis of primary SS cells, both in resting and activated cells, under the experimental conditions used. The polyclonal and mitogenic stimulation of cells might induce a plethora of activator pathways, which cannot be inhibited by blocking a single cytokine.

In conclusion, although our results do not support a critical role for IL-21/IL-21R signaling in survival of Sézary cells, targeting STAT3 in SS still seems an attractive therapeutic option. STAT3 inhibition using the selective inhibitor Stattic results in SS cell apoptosis and inhibition of cell proliferation. As constitutive STAT3 activation is observed in a wide variety of solid and hematological tumors, much research effort is put into developing STAT3-blocking strategies (reviewed in the study by Masciocchi *et al.*, 2011). Several direct inhibitors of STAT3 have entered clinical trials, such as the synthetic compounds OPB-33121 and OPB-51602, as well as the STAT3 decoy and antisense oligonucleotides (www.clinicaltrials.gov). In addition, it is becoming clear that numerous anti-cancer compounds originally developed to target other pathways, or for which the underlying anti-tumor mechanisms are not completely understood, can cause a reduction of STAT3 activity, thereby putatively contributing to the beneficial effect. (for e.g., Zhang *et al.*, 2008; Tiffon *et al.*, 2011; Gupta *et al.*, 2012).

MATERIALS AND METHODS

Patient selection and cell isolation

Eleven patients (8 females, 3 males; median age 65 years, range 47–82 years) were diagnosed with SS on the basis of the criteria of the WHO/EORTC classification (Willemze *et al.*, 2005; Olsen *et al.*, 2007). Patient characteristics are shown in Table 1. In addition, blood was drawn from three healthy donors. CD4 + cells were purified as previously described (van der Fits *et al.*, 2011). Approval for these studies was obtained from the Leiden University Medical Center review board, and written informed consent was provided according to the Declaration of Helsinki Principles.

Cell culturing and stimulation experiments

The Sézary cell lines HuT-78 (ATCC number TIB-161), SeAx (kindly provided by Dr K Kaltoft) (Kaltoft *et al.*, 1987), and primary CD4 + cells were cultured as described previously (van der Fits *et al.*, 2011).

For the identification of STAT3 targets in Sézary cells, cells were cultured overnight in a cytokine-deprived medium, and subsequently stimulated with 100 ng ml^{-1} of IL-21 (ImmunoTools, Friesoythe, Germany) for 1, 2, 4, or 8 hours. Polyclonal stimulation of Sézary cells was achieved using CD3/CD28 beads (Dynabeads Human T-activator CD3/CD28, Invitrogen Dynal AS, Oslo, Norway) at a ratio of 1:5 (beads:cells). Stattic (Calbiochem, San Diego, CA) was dissolved in DMSO (100 mM), and used at a final concentration of 10 μM, or indicated otherwise. Blocking exogenous IL-21 was achieved using recombinant IL-21R-Fc chimera (R&D systems, Minneapolis, MN) at a concentration of 10 μg ml^{-1}.

Flow cytometry

STAT3 activation was assayed using antibodies directed against phosphorylated STAT3 (BD Biosciences, Franklin Lakes, NJ) as described previously (van der Fits *et al.*, 2011).

Flow cytometric detection of apoptosis was performed by staining cells with allophycocyanin-conjugated antibodies against AnnexinV and propidium iodide using the apoptosis detection kit (eBioscience, San Diego, CA).

To assay the cell cycle, cells were fixed in 2% paraformaldehyde for 15 minutes at room temperature, followed by cold 70% ethanol for 15 minutes at 4 °C. RNA was digested by 100 μg ml^{-1} RNase A (Qiagen, Hilden, Germany), and DNA was stained using propidium iodide (eBioscience).

For detection of intracellular IL-21, cells were stimulated for 1 hour with the stimuli indicated, before incubation with 10 μg ml^{-1} Brefeldin A (Sigma, Zwijndrecht, The Netherlands) for another 5 hours. Cells were fixed in 2% paraformaldehyde, followed by staining with allophycocyanin-conjugated CD3 antibodies (BD Pharmingen, Breda,

Table 1. Patient characteristics

Patient number	Sex	Age	Treatment	CD4/CD8 ratio	Total lymphocyte count ($10^9 l^{-1}$)
1a	F	71	Prednisone 10 mg	162	18.5
1b	F	72	Prednisone 10 mg Chlorambucil 2 mg	105	20.0
2	F	82	Prednisone 20 mg Radiotherapy	14	0.3
3	F	65	Prednisone 10 mg	18	2.3
4	F	64	Prednisone 10 mg	17	5.4
5	M	63	None	19	3.3
6	F	63	Prednisone 20 mg Chlorambucil 2 mg	130	4.5
7	M	47	None	23	8.6
8	M	47	None	15	1.1
9	F	47	Prednisone 20 mg	35	6.4
10	F	79	None	34	4.9
11a	F	69	Prednisone 5 mg Chlorambucil 2 mg	81	5.0
11b	F	73	Prednisone 20 mg Chlorambucil 2 mg	98	3.2
11c	F	74	Prednisone 10 mg Chlorambucil 2 mg	152	8.3
12	M	81	Prednisone 10 mg Chlorambucil 2 mg	203	35.0

Table 2. Sequences of the primers used for quantitative reverse transcriptase in real-time PCR

Gene	FW primer	REV primer	Reference
Pri-miR-21	5′-CATTGTGGGTTTTGAAAAGGTTA-3′	5′-CCACGACTAGAGGCTGACTTAGA-3′	(Loffler *et al.*, 2007)
TWIST1	5′-CACTGAAAGGAAAGGCATCA-3′	5′-GGCCAGTTTGATCCCAGTAT-3′	This study
PIM1	5′-GTCCAAAATCAACTCGCTTGC-3′	5′-GAAACCCGAGCTCACCTTCTT-3′	This study
JUN	5′-TGACTGCAAAGATGGAAACG-3′	5′-CAGGGTCATGCTCTGTTTCA-3′	This study
MYC	5′-GGTGCTCCATGAGGAGACAC-3′	5′-CAGCAGAAGGTGATCCAGACTC-3′	This study
BCL2	5′-GCCCTGTGGATGACTGAGTA-3′	5′-GGCCGTACAGTTCCACAAAG-3′	This study
BCL-xL	5′-CTTGGATGGCCACTTACCTG-3′	5′-CTGCTGCATTGTTCCCATAG-3′	This study
SURVIVIN	5′-GTTGCGCTTTCCTTTCTGTC-3′	5′-TGGTTTCCTTTGCAATTTTGT-3′	This study
MCL1	5′-TCCCCTGCCATCCCTGAACTC-3′	5′-CGTGAAAGATGAAAGGTCTGTGGAC-3′	This study
IL-21	5′-CCTGGCAACATGGAGAGGATTG-3′	5′-TGTGGCGATCTTGACCTTGGG-3′	(van der Fits *et al.*, 2012)
ARF5	5′-TGCTGATGAACTCCAGAAGATGC-3′	5′-CGGCTGCGTAAGTGCTGTAG-3′	(van Kester *et al.*, 2012)
ERCC3	5′-ATATCCAAGGTAGGTGACACTTCG-3′	5′-TTGTACTCTTCTGCAACCATCCC-3′	(van Kester *et al.*, 2012)
TMEM87A	5′-CATCTGGACAACCATGAAGTTCAG-3′	5′-AGGATCATGGAGAACAGCAAGC-3′	(van Kester *et al.*, 2012)

The Netherlands). Hereafter, cells were permeabilized in phosphate-buffered saline with 0.2% BSA and 0.5% saponine (Sigma). Intracellular staining was performed using PE-conjugated antibodies against IL-21 (eBioscience), or an isotype control (BD Pharmingen).

Samples were acquired on a flow cytometer (FacsCalibur, BD Biosciences), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Quantitative PCR

mRNA expression levels of various genes were assayed in total RNA isolated from cultured Sézary cells using the RNeasy Mini kit (Qiagen). RNA was treated with DNase I (Promega, Madison, WI) and converted into cDNA by IScript reverse transcriptase (Bio-Rad, Hemel Hempstead, UK), oligo(dT)$_{12\text{-}18}$ and random hexamer primers (Bio-Rad).

Real-time PCR was performed in a 384-well format on a CFX384 PCR detection system (Bio-Rad), using iQ SYBR Green Supermix (Bio-Rad), and the following cycle parameters: 5 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C, 20 seconds at 60 °C, and 20 seconds at 72 °C. Specificity of the PCR products was confirmed by melting curve analysis. Primer sequences are listed in Table 2. Data were analyzed using CFX Manager software (Bio-Rad) applying the ΔΔCt method. Expression was normalized to the stably expressed reference genes ARF5, ERCC3, and TMEM87A (van Kester *et al.*, 2012).

Immunocytochemistry

SS cells were cultured for 6 hours with 10 μM Stattic or a volume equivalent of DMSO. Cytocentrifuge preparations of these cells were prepared using a Shandon CytoSpin III cytocentrifuge (Thermo Scientific, Breda, The Netherlands). Cells were fixed in acetone, blocked with 5% normal goat serum, and subsequently incubated with a polyclonal rabbit antibody against the active form of caspase-3 (BD Biosciences). Biotinylated goat-anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was used as secondary antibody, followed by streptavidin–biotin–peroxidase complex (DAKO, Glostrup, Denmark). Immunoreactivity was detected using 3-amino-9-ethylcarbazole, and slides were counterstained with hematoxylin.

IL-21 ELISA

Culture supernatants were analyzed for IL-21 protein expression using a commercially available ELISA kit (eBioscience) according to the instructions provided by the manufacturer. Detection limit of the ELISA was generally 150 pg ml^{-1}.

Analysis of cell survival

To measure cell survival, primary SS cells were incubated with Stattic in the concentrations indicated in a 96-well plate (six wells per condition). After 72 hours, cell proliferation reagent WST-1 (Roche, Mannheim, Germany) was added, and absorbance at 450 and 650 nm (reference wavelength) was measured after 8 hours. The number of viable cells was calculated relative to the numbers of non-treated cells.

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