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Autocrine IL-21 Stimulation Is Involved in the Maintenance of Constitutive STAT3 Activation in Sézary Syndrome

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Sézary syndrome (SS) is a cutaneous T-cell lymphoma (CTCL) with malignant CD4+ T cells (SS cells) in skin, lymph nodes, and blood. Signal transducer and activator of transcription 3 (STAT3) is constitutively activated in SS cells, whereas this activation is lost upon *in vitro* culturing, indicating that STAT3 activation observed *in vivo* is the result of activating factors in the micromilieu of the malignant cells. We investigated which factors are involved in STAT3 activation in SS, focusing on cytokines of the common γ-chain family because of their crucial role in T-cell activation. Furthermore, downstream effects of STAT3 signaling in SS cells were assayed. In SS cells, STAT3 was strongly activated by IL-21, and increased expression of IL-21 and its receptor chains was observed in peripheral blood SS cells. IL-21 and IL-21R protein expression was detectable on neoplastic cells in SS skin biopsies. Using short-term culturing experiments, we demonstrate that IL-21 itself and the α-chain of the IL-2 receptor are STAT3 target genes in SS cells, thereby rendering cells more sensitive to stimulation with the T-cell proliferation and activating cytokine IL-2. Combined, our data point toward a pivotal role for an autocrine positive feedback loop involving IL-21 and consequent persistent STAT3 activation in the pathogenesis of SS, thereby indicating IL-21 and IL-21R as new therapeutical targets.

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INTRODUCTION

Cutaneous T-cell lymphomas (CTCLs) are a heterogeneous group of T-cell neoplasms that primarily arise in the skin. Mycosis fungoides, the most common form of CTCL, is a mostly indolent neoplasm that can evolve from skin patches to plaques and eventually tumors containing skin-homing CD4+ memory T cells. Sézary syndrome (SS) is a rare leukemic form of CTCL, often with an aggressive clinical course. It is characterized by generalized erythroderma, lymphadenopathy, and the presence of neoplastic CD4+ T cells (SS cells) in skin, lymph nodes, and peripheral blood.

Persistent activation of transcription factors of the signal transducers and activators of transcription (STAT) protein family has been implicated in the pathogenesis of a wide variety of human cancers, including CTCL. Constitutive STAT3 activation (phosphorylation) was observed in SS cells in the peripheral blood (Zhang *et al.*, 1996; Nielsen *et al.*, 1999; Eriksen *et al.*, 2001; van Kester *et al.*, 2008), as well as in malignant T lymphocytes in skin samples from mycosis fungoides tumors and SS (Qin *et al.*, 2001; Sommer *et al.*, 2004). We previously demonstrated that the STAT3 phosphorylation is lost upon *in vitro* culturing of SS cells (van Kester *et al.*, 2008), indicating that the activation observed *in vivo* is the result of STAT3-activating factors present in the micromilieu of the malignant cells.

Cytokines of the common γ-chain (γc) family are capable of activating STAT3 in various cell types (Yu and Jove, 2004), and have a crucial role in normal T-cell physiology (Rochman *et al.*, 2009). Therefore, a putative role for several of these γc cytokines in the pathogenesis of CTCL has been investigated previously. IL-2, IL-7, and IL-15 can increase the survival of CTCL cells *in vitro* (Dobbeling *et al.*, 1998; Yamanaka *et al.*, 2006), and increased expression of IL-7 and IL-15 was demonstrated in skin and peripheral blood from CTCL patients (Dalloul *et al.*, 1992; Foss *et al.*, 1994; Dobbeling *et al.*, 1998; Leroy *et al.*, 2001; Yamanaka *et al.*, 2006).

IL-21 is the most recently discovered member of the γc family of cytokines. It is produced by activated CD4+ T cells, natural killer (NK) T cells, T follicular helper cells, and T helper 17 (Th17) cells (Monteleone *et al.*, 2009). Its receptor is a dimer comprising the γc chain with its own unique IL-21-specific receptor chain (IL-21R). IL-21R is

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Abbreviations: CTCL, cutaneous T-cell lymphoma; IL-21R, IL-21-specific receptor chain; NK, natural killer; RT-PCR, reverse transcriptase-PCR; SS, Sézary syndrome; STAT, signal transducer and activator of transcription; Th17, T helper 17

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expressed in a variety of immune cells, including B cells, T cells, NK cells, and dendritic cells, as well as in nonimmune cells such as endothelial cells, fibroblasts, and epithelial cells (Monteleone *et al.*, 2008, 2009). IL-21 expression in T cells can be regulated by IL-21 via an autocrine positive feedback loop, involving the activation of STAT3 (Caprioli *et al.*, 2008). This feedback loop is essential for the development of Th17 cells (Nurieva *et al.*, 2007; Wei *et al.*, 2007), and is involved in Th17-mediated autoimmune diseases such as rheumatoid arthritis (Niu *et al.*, 2010).

In this study, we aimed to identify factors that are involved in the maintenance of persistent STAT3 activation in SS, and investigated the existence of an autocrine feedback loop in persistent STAT3 activation. In addition, a role for IL-2RA as STAT3 target gene in the oncogenic process is explored.

RESULTS

STAT3 is activated by IL-21, whereas other common γ-cytokines mainly activate STAT5 in Sézary cells

We previously demonstrated that the persistent STAT3 activation that is observed *in vivo* is lost upon *in vitro* culturing of primary SS cells (van Kester *et al.*, 2008), indicating that autocrine or paracrine STAT3 activation has a role *in vivo*. We assayed STAT3 activation in SS cells, thereby focusing on cytokines of the common γ-chain family. Our data show that STAT3 was consistently activated by IL-21 in all patients studied ($n=6$; Figure 1a), whereas IL-2, IL-7, and IL-15 mainly activated STAT5 (Figure 1b). These data, together with previously published results by Marzec *et al.* (2008), demonstrate that IL-21 is a very potent activator of STAT3, and prompted us to investigate whether autocrine or paracrine stimulation of SS cells by IL-21 might be involved in the constitutive activation of STAT3 *in vivo*.

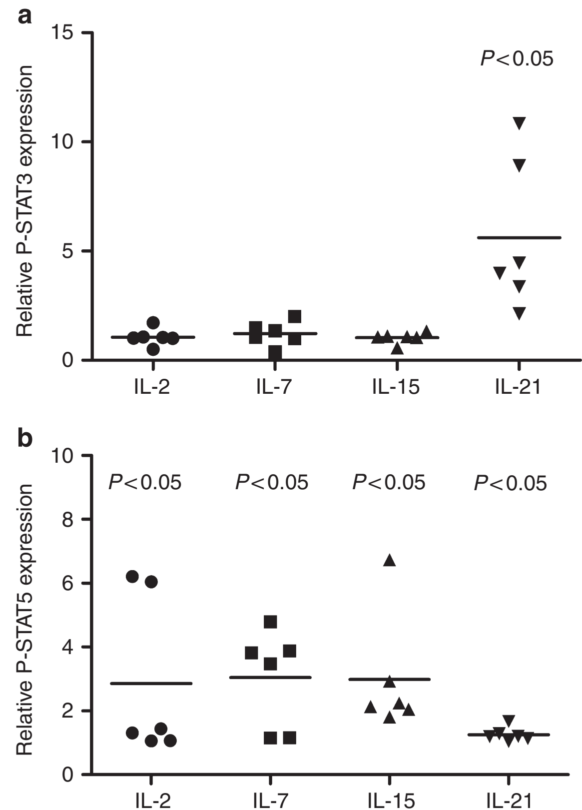

Figure 1. STAT3 is activated by IL-21, whereas other common γ-cytokines mainly activate STAT5 in Sézary cells. CD4+ T cells were isolated from peripheral blood of Sézary patients ($n=6$), and stimulated for 15 minutes with various common γ-cytokines. (**a**) STAT3 and (**b**) STAT5 activation was assayed by flow cytometry using an antibody specific for phosphorylated (P-) STAT3 or STAT5. Each symbol represents the median fluorescent intensity of P-STAT expression in an individual patient relative to the expression in unstimulated cells. The mean is indicated with a horizontal line, and statistically significant differences when compared with unstimulated cells are indicated (Mann–Whitney test).

The expression of IL-21R and IL-21 is increased in peripheral blood Sézary cells

The expression of the IL-21 receptor was analyzed on CD4+ T cells in the peripheral blood of SS patients. mRNA expression of both chains of the IL-21 receptor (common γ-chain (γc) and IL-21R) was significantly increased in CD4+ T cells from peripheral blood of SS patients when compared with CD4+ T cells of healthy donors (Figure 2). In contrast, no significantly increased IL-21R and γc mRNA expression was observed in the activated CD4+ T cells of patients with benign erythroderma when compared with healthy controls (Figure 2). Correlation analysis (Pearson's) revealed a significant correlation between the mRNA expression of γc and IL-21R, in line with their coexpression in a single complex ($P=0.019$).

Flow cytometric analysis of IL-21R protein showed that ~15% of the CD4+ lymphocytes stain positive for IL-21R, both in the peripheral blood of healthy donors and patients with SS (Figure 3a). As in the SS patients, who were analyzed by flow cytometry, over 94% of the CD4+ T cells reacted with a single Vβ-specific antibody, verifying their clonal origin (data not shown), our data demonstrate the existence of IL-21R-negative and -positive subpopulations of SS cells. Analysis of the CD4-negative nontumor lymphocytes (including B cells, NK cells, and CD8+ T cells) demonstrates that a

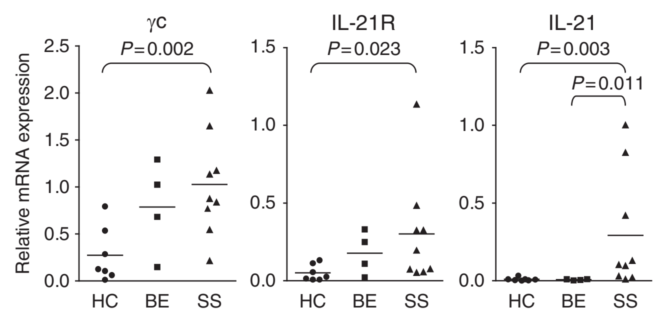

Figure 2. mRNA expression of IL-21 and its receptors are increased in peripheral blood Sézary cells. RNA was extracted from CD4+ T cells isolated from peripheral blood from healthy controls (HC), patients with benign erythroderma (BE), or patients with Sézary syndrome (SS). Expression of both chains of the IL-21 receptor (common γ-chain (γc) and IL-21-specific receptor chain (IL-21R)), as well as IL-21 itself, was assayed by quantitative reverse transcriptase-PCR (RT-PCR) and normalized for expression of a stably expressed reference gene (*ribosomal protein S11* (*RPS11*)). Each symbol represents the expression in an individual patient, and the mean is indicated with a horizontal line. Statistically significant differences ($P<0.05$; Mann–Whitney test) are indicated.

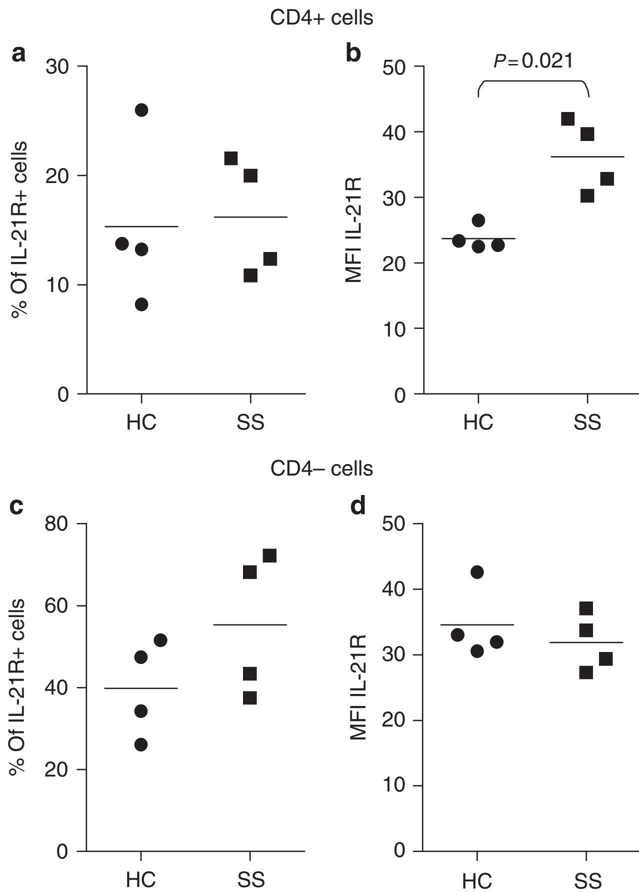

Figure 3. Increased IL-21-specific receptor (IL-21R) protein expression on peripheral blood Sézary cells. IL-21R expression was determined by flow cytometry on peripheral blood mononuclear cells from healthy controls (HC, $n=4$) and patients with Sézary syndrome (SS, $n=4$). The percentage of IL-21R+ cells was assayed in the (**a**) CD4+ and (**c**) CD4− lymphocytes. In addition, the median fluorescent intensity (MFI) of the IL-21R expression on (**b**) IL-21R+ CD4+ and (**d**) IL-21R+ CD4− lymphocytes is depicted. Each symbol represents the percentage or expression in an individual patient, and the mean is indicated with a horizontal line. Statistically significant differences ($P<0.05$; Mann–Whitney test) are indicated.

substantial proportion (30–70%) expresses IL-21R in both healthy donors and SS patients (Figure 3c).

We additionally determined the level of IL-21R protein expression on the IL-21R-positive subpopulations. IL-21R-positive CD4+ SS cells expressed significantly higher levels of IL-21R when compared with IL-21R-postive CD4+ cells from healthy donors (Figure 3b). This is likely not just a consequence of T-cell activation, as IL-21R protein expression was not increased upon stimulation of peripheral blood mononuclear cells with phytohemagglutinin (data not shown). In contrast, IL-21R expression was similar in IL-21R-positive nontumor cells (CD4-negative lymphocytes) from healthy donors and SS patients (Figure 3d), demonstrating that the increased IL-21R expression by CD4+ SS cells is specific for the tumor cells, and not a generalized feature of all lymphocyte subsets in these patients.

Furthermore, the expression of the cytokine IL-21 in peripheral blood Sézary cells was analyzed. Very low IL-21 mRNA expression was detectable in CD4+ T cells from healthy donors and from patients with benign erythroderma, whereas IL-21 expression in Sézary patients was significantly elevated (Figure 2). IL-21 protein levels in the serum were assayed by ELISA. However, in most samples from controls and from SS patients, IL-21 levels were below the detection limit of the ELISA ($<95\,\mathrm{pg\,ml^{-1}}$; data not shown).

Altogether, our data suggest that increased IL-21 expression, together with an increased expression of its receptor, might be involved in autocrine activation of STAT3 by IL-21 in peripheral blood Sézary cells.

SS cells in skin biopsies are positive for both IL-21 and its receptor

To investigate the possibility that IL-21 activates STAT3 in Sézary cells in the skin, immunohistochemical analysis of Sézary skin biopsies ($n=4$) was performed. Sézary cells were identified on the basis of morphology, size, and immunophenotypical analysis of serial sections. Antibodies against phosphorylated STAT3 showed that activated STAT3 protein is present in the nucleus of SS cells, as well as in nonmalignant cells (Figure 4). Although staining of the skin biopsies with an antibody against IL-21 resulted in a brownish background staining, clear cytoplasmic staining was detectable in cells morphologically resembling SS cells (indicated with arrows in Figure 4). In contrast, most cells with a normal nuclear morphology were IL-21 negative (indicated with arrowheads in Figure 4). Immunohistochemical staining with an antibody against IL21R revealed weak positivity of Sézary cells (indicated with arrows in Figure 4), as well as of many other cell types present in the biopsies.

IL-21 expression is regulated by a positive feedback loop via STAT3

Recently, it was demonstrated that the IL-21 gene itself is a direct STAT3 target gene in healthy human T cells, thereby creating a positive feedback loop (Caprioli *et al.*, 2008). We stimulated purified CD4+ SS cells with recombinant IL-21 for various time points and determined IL-21 mRNA expression by quantitative reverse transcriptase-PCR (RT-PCR). IL-21 expression was strongly and transiently increased, with a maximum induction observed after 2 hours (Figure 5). To assess whether this induction was dependent on STAT3 signaling, SS cells were pretreated with the known STAT3 inhibitor Cucurbitacin I (Blaskovich *et al.*, 2003; van Kester *et al.*, 2008), resulting in complete inhibition of IL-21-induced STAT3 activation, whereas IL-2-induced STAT5 activation was only marginally affected (data not shown). IL-21-induced expression of IL-21 mRNA was completely abrogated upon pretreatment of the SS cells with cucurbitacin I (Figure 5). Identical observations were made in CD4+ cells from four SS patients and four healthy donors.

Sézary cells are sensitized for IL-2 stimulation via the IL-21/STAT3 axis

To fully understand the pathological role of STAT3 in SS, identification of its downstream targets is mandatory. It was shown previously that in human T cells, IFN-α enhances the

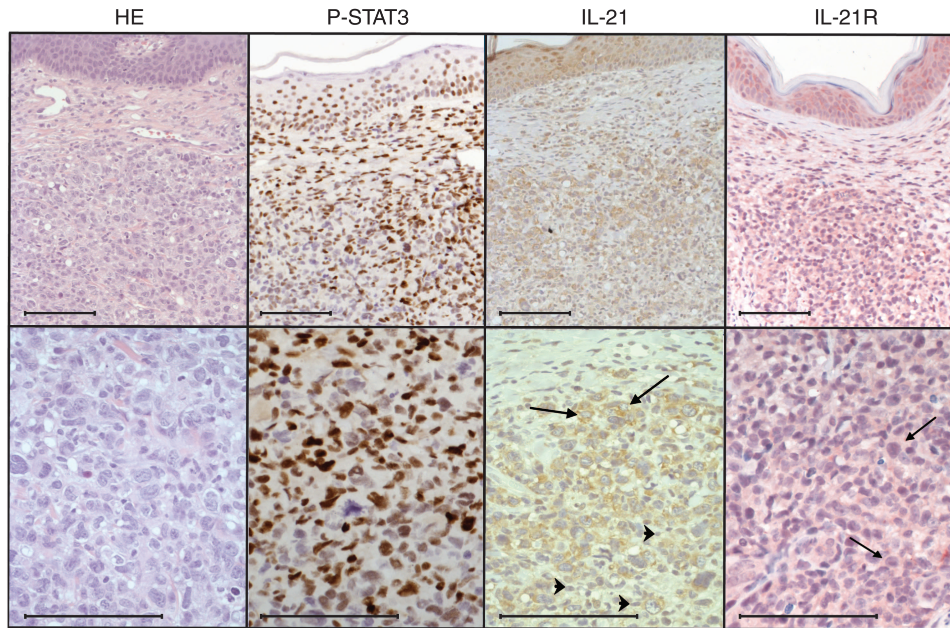

Figure 4. IL-21 and IL-21-specific receptor (IL-21R) expression in Sézary syndrome (SS) cells in skin biopsies. Skin biopsies from SS patients ($n=4$) were stained with hematoxylin and eosin (HE), and with antibodies against P-STAT3, IL-21, and IL-21R. IL-21-expressing SS cells are indicated by arrows, whereas IL-21-negative cells are indicated by arrowheads. Furthermore, SS cells weakly positive for IL-21R are indicated with arrows. Representative examples are shown. Bar = 100 μm.

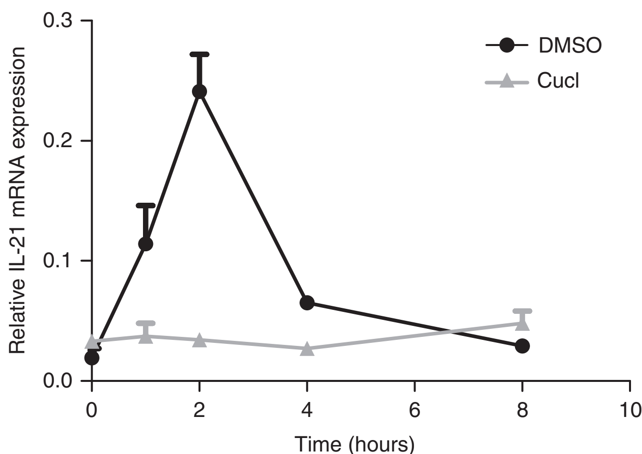

Figure 5. IL-21 mRNA expression is regulated by a positive feedback loop via STAT3. CD4+ cells were isolated from peripheral blood of Sézary patients. Cells were pretreated with cucurbitacin I (CucI) or DMSO for 2 hours, and stimulated with IL-21 for various time points. IL-21 mRNA expression was determined by quantitative reverse transcriptase-PCR (RT-PCR), and expressed relative to mRNA levels of a housekeeping gene (*ribosomal protein S11* (*RPS11*)).

binding of STAT3 to the promoter of the α-chain of the IL-2 receptor (IL-2RA, CD25), resulting in increased IL-2RA expression and IL-2 sensitivity (Matikainen *et al.*, 1999). We therefore assessed IL-2RA mRNA expression in CD4+ cells that were isolated from the peripheral blood from healthy donors, patients with benign erythroderma, and SS patients. CD4+ cells from patients with benign erythroderma display high levels of IL-2RA (Figure 6a), indicative of their activated phenotype ($CD25^{high}$). In addition, in SS cells, IL-2RA mRNA expression is significantly elevated when compared with healthy controls (Figure 6a), in line with the constitutive STAT3 activation that is observed in SS cells *in vivo*. Culturing primary CD4+ SS cells with IL-21 resulted in a transient induction of IL-2RA mRNA expression, with a maximum induction after 2 hours and a return to baseline expression after 8 hours (Figure 6b). Pretreatment of cells with the STAT3 inhibitor cucurbitacin I resulted in a complete abrogation of IL-21-induced IL-2RA mRNA expression, demonstrating that this effect is mediated via STAT3 (Figure 6b). This induction pattern was identical in CD4+ cells from SS patients ($n=3$), as well as from healthy donors ($n=3$).

We furthermore assayed whether the increased IL-2RA expression had functional consequences for the IL-2 sensitivity. SS cells were preincubated with IL-21 to induce IL-2RA expression, and subsequently stimulated with IL-2. As IL-2 stimulation results in phosphorylation of STAT5 in SS cells, we quantified STAT5 activation as readout for the IL-2 responsiveness. Our data show that in three out of five patients, pretreatment of SS cells with IL-21 results in a more pronounced IL-2-induced STAT5 phosphorylation, indicative of an increased IL-2 sensitivity (Figure 6c).

Thus, our data suggest that SS cells are sensitized for IL-2 stimulation via upregulation of the α-chain of the IL-2 receptor via the IL-21/STAT3 pathway.

DISCUSSION

In this paper, we demonstrate that IL-21 is expressed by SS cells in peripheral blood and skin, and is a very potent

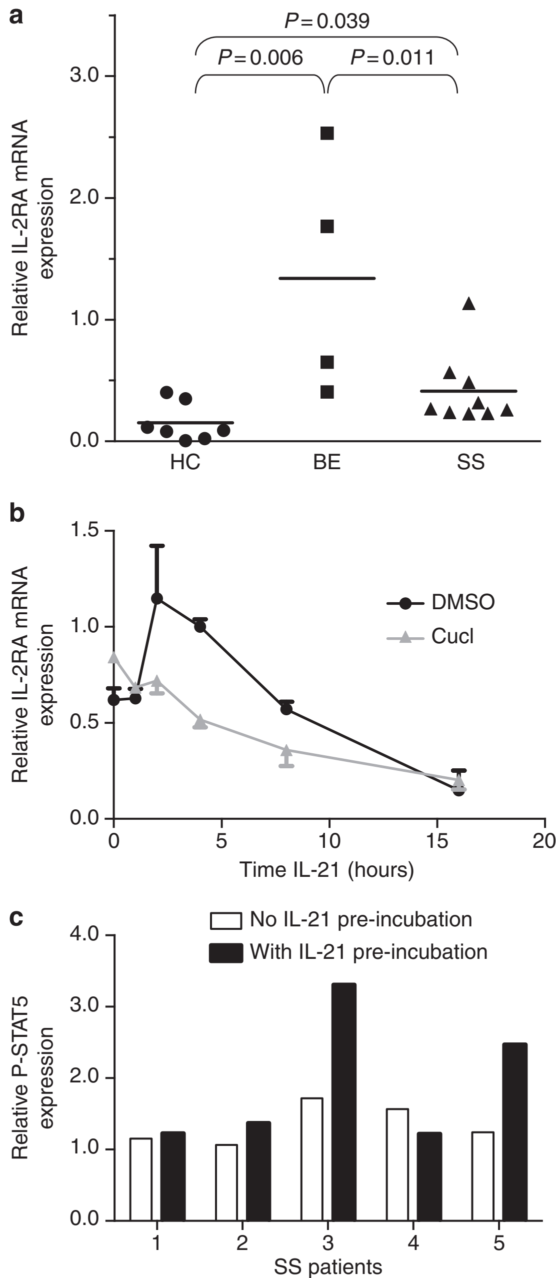

Figure 6. IL-21/STAT3 signaling renders SS cells more sensitive to IL-2. (**a**) IL-2RA mRNA expression was assayed in RNA in CD4+ T cells from healthy controls (HC), patients with benign erythroderma (BE), or patients with Sézary syndrome (SS) using quantitative reverse transcriptase-PCR (RT-PCR), and normalized for ribosomal protein S11 (RPS11) expression. Each symbol represents expression in an individual patient, and the mean is indicated with a horizontal line. Statistically significant differences ($P<0.05$; Mann–Whitney test) are indicated. (**b**) SS cells were pretreated with cucurbitacin I (CucI) or DMSO for 2 hours, and stimulated with IL-21 for the time points indicated. IL-2RA mRNA expression was determined as in **a**. (**c**) SS cells from five patients were incubated overnight with or without IL-21, and subsequently stimulated with IL-2 for 15 minutes. Phosphorylated (P-) STAT5 expression was assayed by flow cytometry, and the median fluorescent intensities (MFIs) of the P-STAT5 staining were calculated relatively to the P-STAT5 MFI in unstimulated cells for each patient.

activator of STAT3 signaling. As IL-21 itself is a STAT3 target gene in SS cells, persistent STAT3 activation in SS cells *in vivo* might be maintained via an autocrine positive feedback loop involving IL-21. Identification of this loop thereby provides new insights into the pathogenic process of SS, potentially indicating IL-21 and IL-21R as new therapeutical targets.

IL-21 can have complex activities on various cell types, resulting in 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). As a consequence, IL-21 has been implicated in the pathogenesis of T-cell-mediated inflammatory (autoimmune) diseases such as psoriasis, rheumatoid arthritis, and systemic lupus erythematosus (Monteleone *et al.*, 2009), thereby making IL-21-inhibiting strategies emerging therapeutic options. On the other hand, boosting IL-21 activity can be beneficial for immunotherapy for cancer by augmentation of CD8+ and NK cell activity. This antitumor activity of IL-21 has been demonstrated in various preclinical mouse tumor models, as well as in phase I clinical trials in patients with advanced metastatic melanoma and renal cell carcinoma (Spolski and Leonard, 2008).

However, it was demonstrated previously that IL-21 also has direct effects on IL-21R+ lymphoid neoplastic cells. IL-21 promotes the growth of multiple myeloma (Brenne *et al.*, 2002), anaplastic lymphoma kinase–expressing anaplastic large-cell lymphoma (Dien Bard *et al.*, 2009), and adult T-cell leukemia (Ueda *et al.*, 2005), and protects Hodgkin's lymphoma cells from apoptosis (Lamprecht *et al.*, 2008). On the contrary, IL-21 can induce apoptosis in follicular lymphoma cells (Akamatsu *et al.*, 2007; de Totero *et al.*, 2010), B-chronic lymphocytic leukemia (de Totero *et al.*, 2006, 2008; Gowda *et al.*, 2008), and diffuse large B-cell lymphoma (Sarosiek *et al.*, 2010). The effects of IL-21 on proliferation and survival of CTCL cells have been studied previously. Culturing CTCL cells with IL-21 protects them from apoptosis, without affecting their proliferation (Marzec *et al.*, 2008; Yoon *et al.*, 2008). We here demonstrate that STAT3 in SS cells is activated by IL-21 in an autocrine manner. Malignant cells from patients with Hodgkin's lymphoma aberrantly produce IL-21 and its receptor, resulting in autocrine STAT3 activation, upregulation of STAT3 target genes, and protection of the Hodgkin/Reed–Stenberg cells from apoptosis (Lamprecht *et al.*, 2008). In analogy, a similar role for the IL-21/STAT3 axis might be involved in the protection of SS cells from apoptosis.

Autocrine activation of T cells by IL-21 was previously demonstrated to be indispensable for the development of murine Th17 cells (Nurieva *et al.*, 2007; Wei *et al.*, 2007). Furthermore, Th17 cytokines IL-17 and IL-21 are direct Stat3 target genes in mice (Durant *et al.*, 2010). Very recently, it was shown that malignant cells in skin lesions of patients with SS and mycosis fungoides express IL-17, and that this IL-17 expression is regulated via the STAT3 pathway (Krejsgaard *et al.*, 2011). Together with our data demonstrating STAT3-dependent expression of IL-21 in SS cells, this implicates that

STAT3 signaling is involved in the regulation of Th17 cytokine expression in CTCL cells.

Our immunohistochemical analysis of SS skin biopsies showed weak expression of IL-21R on not only SS cells, but also on many other cell types including keratinocytes. These data are in line with previous reports demonstrating relatively high expression of IL-21R on keratinocytes in patients with systemic sclerosis and atopic dermatitis, and weak expression in healthy skin (Distler *et al*., 2005; Jin *et al*., 2009). In addition, *in vitro* stimulation of keratinocytes with IL-21 induces cell proliferation (Caruso *et al*., 2009), suggestive of a functional IL-21R on this cell type. It is conceivable that the expression of activated STAT3 in keratinocytes in SS biopsies (Figure 4) results from paracrine activation by IL-21 produced by SS cells. The functional consequence of this paracrine activation remains to be elucidated; in particular, the question of whether this activation of keratinocytes leads to cytokine production that reciprocally affects SS cells needs to be addressed.

A previous microarray study by Marzec *et al*. (2008) demonstrated that only a very limited number of genes are upregulated by IL-21 in a CTCL cell line. This list included IL-2RA, but lacked other genes for which we show upregulation by IL-21, such as the IL-21 gene itself (this study) and miR-21 (van der Fits *et al*., 2011). We think that this discrepancy is due to the early and transient nature of induction of genes by IL-21, whereas the microarray analysis to identify IL-21-responsive genes was conducted at a single time point (4 hours).

In this study, we demonstrate that IL-21 stimulation of primary SS cells results in significantly increased IL-2RA mRNA expression (Figure 6b), resulting in increased IL-2 sensitivity in three out of five patients (Figure 6c). However, we were unable to demonstrate increased IL-2RA protein (CD25) expression on IL-21-stimulated SS cells ($n=5$, data not shown). This discrepancy might have methodological causes, e.g., differences in sensitivity of the different assays (flow cytometry vs. quantitative RT-PCR). Furthermore, the transient nature of the induction of IL-2RA mRNA expression by IL-21 might interfere with the detection of increased IL-2RA protein expression. Alternatively, the lack of IL-21-induced expression of cell-surface IL-2RA might be caused by proteolytic cleavage of IL-2RA from the cell membrane, resulting in secretion of soluble CD25 (sCD25). Stimulation of CTCL cell lines with IL-21 results in increased secretion of sCD25 (Kasprzycka *et al*., 2008), and serum sCD25 levels are elevated in SS patients and correlate with the clinical course and a poor prognosis (Bernengo *et al*., 1993; Wasik *et al*., 1996). However, it is currently unknown how sCD25 is involved in the pathogenesis of cutaneous lymphoma.

In summary, we demonstrate that IL-21 can contribute to the constitutive STAT3 activation that is observed in SS cells via a positive autocrine feedback loop. The continuous activation of this loop renders cells more resistant to apoptosis, and more sensitive to T-cell proliferation and stimulation of cytokine IL-2. This will make this axis potentially interesting for therapeutic intervention by targeting STAT3 or the IL-21/IL-21R couple.

MATERIALS AND METHODS

Patient selection

SS patients (5 men and 10 women, median age 62 years) were diagnosed on the basis of criteria of the World Health Organization (WHO)/European Organization for Research and Treatment of Cancer (EORTC) 2005 classification (Willemze *et al*., 2005; Olsen *et al*., 2007). As control groups, patients with erythroderma secondary to atopic dermatitis (benign erythroderma) and healthy donors were included.

Approval for these studies was obtained from the Leiden University Medical Center review board, and informed consent was provided according to the Declaration of Helsinki Principles.

Cell culturing and stimulation experiments

Heparinized blood was drawn, and peripheral blood mononuclear cells were isolated by Ficoll density centrifugation. From this fraction, CD4+ T cells were isolated by negative selection with magnetic beads (CD4+ T cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the CD4+ cell population was verified by flow cytometric analysis for CD3, CD4, and CD8 expression, and was generally >85%. In addition, earlier experiments demonstrated that >90% of the CD4+ cells comprise malignant cells characterized by expression of a clonally rearranged T-cell receptor.

Cells were cultured in RPMI-1640 supplemented with 10% human AB serum (Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 200 U ml^{-1} IL-2, and 5 ng ml^{-1} IL-7 (PeproTech, Rocky Hill, NJ).

Cells used for stimulation experiments were grown overnight in cytokine-deprived medium, and subsequently stimulated with IL-2 (200 U ml^{-1}), IL-7 (10 ng ml^{-1}), IL-15 (20 ng ml^{-1}; all from PeproTech), or IL-21 (100 ng ml^{-1}; ImmunoTools, Friesoythe, Germany). Cucurbitacin I (Indofin, Hillsborough, NJ) was dissolved in DMSO in a concentration of 50 mM, and diluted in culture medium to a final concentration of 30 μM.

Flow cytometry

The expression of activated STAT3 and STAT5 was determined by flow cytometry using antibodies specifically recognizing phosphorylated STATs, as previously described (van der Fits *et al*., 2011). In brief, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and subsequently permeabilized in 90% ice-cold methanol. After washing, cells were stained with Alexa647-conjugated antibodies against P-STAT3 (Tyr705), and with phycoerythrin-conjugated antibodies against P-STAT5 (Tyr694) (BD Biosciences, Franklin Lakes, NY).

Flow cytometric analysis of IL-21R was performed in non-fixed peripheral blood mononuclear cells, using a rabbit anti-IL-21R antibody (Abcam, Cambridge, UK), followed by FITC-conjugated goat anti-rabbit (Invitrogen, Carlsbad, CA) as a secondary antibody, in combination with anti-CD4-PerCP-Cy5.5 (BD Biosciences).

Samples were acquired on a flow cytometer (FacsCalibur, BD Biosciences), and analyzed using CellQuest software (BD Biosciences). Lymphocytes were gated on the basis of their forward and sideward scatter patterns, and IL-21R expression was assayed on CD4+ and CD4− lymphocytes separately.

Quantitative RT-PCR

The mRNA expression levels of IL-21, IL-21R, γc, and IL-2RA was assayed by quantitative RT-PCR. Total RNA was isolated from 1 to 10×10^6 cells using the RNeasy Mini kit (Qiagen), treated with RQ1 DNase I (Promega, Madison, WI), and converted into complementary DNA using IScript reverse transcriptase (Bio-Rad, Hemel Hempstead, UK), oligo(dT)$_{12-18}$, and random hexamer primers (Bio-Rad). Real-time PCR was performed using the IQ5 instrument and SYBR Green Supermix (Bio-Rad), using the following cycle parameters: 5 minutes at 95 °C, followed by 45 cycles of 15 seconds at 97 °C, and 30 seconds at 60 °C. Sequences of the primers used were as follows: IL-21-forward 5′-CCTGGCAACATGGAGAGG ATTG-3′; IL-21-reversed 5′-TGTGGCGATCTTGACCTTGGG-3′; IL-21R-forward 5′-CCTCACCTGCTGCTTCTCCTC-3′; IL-21R-reversed 5′-TCTTCTTCCATAGCCTCCACAATG-3′; γc-forward 5′-AATTC CCACCCTGAAGAACCTAGAG-3′; γc-reversed 5′-CAGCCAGTCC CTTAGACACACC-3′; IL-2RA-forward 5′-GCGGAGACAGAGGAAG AGTAGAAG-3′; IL-2RA-reversed 5′-GCAGGCAAGCACAACGG ATG-3′; RPS11 (ribosomal protein S11) as described previously (Zuidervaart *et al.*, 2003). Data were analyzed using IQ5 software using the ΔΔCt method (Bio-Rad), and expressed relative to mRNA expression levels of the stably expressed reference gene *RPS11*. Specificity of the PCR products was confirmed by melting curve analysis.

Immunohistochemistry

Immunohistochemical staining was performed on 3 μm sections of formalin-fixed, paraffin-embedded skin samples using standard procedures. After antigen retrieval by boiling for 10 minutes in 10 mM citrate buffer (pH 6.0), tissue sections were incubated with primary antibodies against P-STAT3 (Tyr705; Cell Signaling Technology, Danvers, MA), IL-21R (Abcam), or IL-21 (Lifespan Biosciences, Seattle, WA). Subsequently, sections were incubated with Bright-Vision-HRP (Immunologic, Duiven, The Netherlands), biotinylated swine anti-rabbit-IgG (DAKO, Glostrup, Denmark), followed by the VectaStain ABC kit (Vector Laboratories, Burlingame, CA), or biotinylated goat anti-rabbit-IgG (Vector Laboratories), followed by streptavidin–biotin–peroxidase complex (DAKO), respectively. Immunoreactivity was detected using diamino-benzidine or 3-amino-9-ethylcarbazole, and slides were counterstained with hematoxylin.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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