Malignant Cutaneous T-Cell Lymphoma Cells Express IL-17 Utilizing the Jak3/Stat3 Signaling Pathway

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IL-17 is a proinflammatory cytokine that is crucial for the host's protection against a range of extracellular pathogens. However, inappropriately regulated expression of IL-17 is associated with the development of inflammatory diseases and cancer. In cutaneous T-cell lymphoma (CTCL), malignant T cells gradually accumulate in skin lesions characterized by massive chronic inflammation, suggesting that IL-17 could be involved in the pathogenesis. In this study we show that IL-17 protein is present in 10 of 13 examined skin lesions but not in sera from 28 CTCL patients. Importantly, IL-17 expression is primarily observed in atypical lymphocytes with characteristic neoplastic cell morphology. In accordance, malignant T-cell lines from CTCL patients produce IL-17 and the synthesis is selectively increased by IL-2 receptor β chain cytokines. Small-molecule inhibitors or small interfering RNA against Jak3 and signal transducer and activator of transcription 3 (Stat3) reduce the production of IL-17, showing that the Jak3/Stat3 pathway promotes the expression of the cytokine. In summary, our findings indicate that the malignant T cells in CTCL lesions express IL-17 and that this expression is promoted by the Jak3/Stat3 pathway.

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INTRODUCTION

IL-17 is a proinflammatory cytokine that is mainly produced by Th17 cells, but also expressed by other cell types such as γδ T cells, $CD8^+$ T cells, natural killer T cells, natural killer cells, and neutrophils. It is crucial for the host's defense against a range of extracellular pathogens; however, inappropriately regulated expression of IL-17 is associated with the development of a variety of inflammatory and autoimmune diseases (Weaver *et al.*, 2007; Korn *et al.*, 2009; Miossec *et al.*, 2009). A link between chronic inflammation and cancer has long been recognized with several recent studies defining the role of IL-17 in carcinogenesis. It has been shown that IL-17 can act directly on some tumor cells and elicit production of cytokines as well as foster their proliferation and survival. IL-17 can also promote tumor growth indirectly by stimulating the secretion of angiogenic factors such as vascular endothelial growth factor and IL-8 from tumor and/or stromal cells, resulting in increased tumor vascularization. Moreover, IL-17 can induce secretion of chemokines, matrix metalloproteinases, and proinflammatory cytokines from stromal cells, leading to recruitment and activation of inflammatory cells, thereby promoting chronic inflammation. Finally, IL-17 has also been shown to inhibit tumorigenesis, in part by enhancing cytotoxic antitumor responses (Murugaiyan and Saha, 2009; Ji and Zhang, 2010; Zou and Restifo, 2010). Thus, it seems that the role of IL-17 in carcinogenesis is complex, exerting both pro- and anti-tumor effects, depending on the specific context.

Cutaneous T-cell lymphoma (CTCL) defines a heterogeneous group of neoplastic disorders characterized by chronic inflammation and primary accumulation of malignant T cells in the skin. The predominant clinical form of CTCL is mycosis fungoides (MF) (Willemze *et al.*, 2005). In the initial stages of classical MF, patients presents with flat erythematous skin patches that resemble lesions from nonmalignant inflammatory skin disorders like chronic eczema, allergic contact dermatitis, large-plaque parapsoriasis, and psoriasis. These lesions contain dermal infiltrates composed of a variety of inflammatory cells including T cells with normal morphology as well as atypical T cells with a distinctive neoplastic morphology. Typically, the malignant T cells infiltrate the

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Abbreviations: CTCL, cutaneous T-cell lymphoma; IL-2Rβ, IL-2 receptor β chain; MF, mycosis fungoides; siRNA, small interfering RNA; SS, Sézary syndrome; Stat3, signal transducer and activator of transcription 3; γ_c, common cytokine receptor γ chain

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epidermis and form Pautrier microabscesses, which are collections of T cells adherent to dendritic processes of Langerhans cells. During disease progression, the composition of the dermal infiltrates changes and the malignant T cells eventually predominate, leading to the development of overt tumors (Girardi *et al.*, 2004; Kim *et al.*, 2005; Hwang *et al.*, 2008). The progression of MF is sometimes associated with a large cell transformation of the malignant T cells. The prognosis for transformed MF is very poor with a mean survival of 22 months (Vergier *et al.*, 2000). Sézary syndrome (SS) is a particular aggressive variant of CTCL characterized by generalized erythroderma, lymphadenopathy, and the presence of high numbers of T cells with atypical cerebriform nuclei (Sézary cells) in the peripheral blood (Vonderheid *et al.*, 2002).

The etiology and pathogenesis of CTCL remain poorly understood. However, cytokines that signal through receptors containing the common cytokine receptor γ chain (γ_c) have been shown to be vital for the *ex vivo* survival and proliferation of the malignant T cells. Because some of these γ_c cytokines are present in CTCL patients, it has been suggested that they have an important role in the pathogenesis (Dobbeling *et al.*, 1998; Leroy *et al.*, 2001; Yamanaka *et al.*, 2006b; Marzec *et al.*, 2008). Accordingly, the Jak3/signal transducer and activator of transcription 3 (Stat3) pathway is active in the malignant T cells promoting their survival (Zhang *et al.*, 1996; Eriksen *et al.*, 2001; Sommer *et al.*, 2004). In a fraction of CTCL patients, the malignant T cells harbor activating mutations in Jak3 that can confer cytokine-independent activation of the Jak3/Stat3 pathway (Cornejo *et al.*, 2009).

In addition to numerous inflammatory cells, a plethora of cytokines (e.g., tumor necrosis factor-α, IL-1, IL-4, IL-5, IL-15, IL-18, and IL-23) (Hansen *et al.*, 1991; Vowels *et al.*, 1994; Daliani *et al.*, 1998; Dobbeling *et al.*, 1998; Leroy *et al.*, 2001; Doherty *et al.*, 2006; Yamanaka *et al.*, 2006a), chemokines (e.g., CCL17 (chemokine (C-C motif) ligand 17), CCL20, and CCL22) (Ferenczi *et al.*, 2002; Schmuth *et al.*, 2002) matrix metalloproteinases (e.g., MMP2 and MMP9) (Vacca *et al.*, 1997), and angiogenic factors (e.g., vascular endothelial growth factor and IL-8) (Hansen *et al.*, 1991; Krejsgaard *et al.*, 2006) are frequently present in CTCL skin lesions. The malignant progression in a chronic inflammatory environment suggests that IL-17 could be involved in the pathogenesis of CTCL. Supporting this hypothesis, a previous study found IL-17 mRNA in 5 out of 10 biopsies from CTCL patients (Ciree *et al.*, 2004). In this study we show that IL-17 protein is present in 10 of 13 examined skin lesions but not in sera from 28 CTCL patients. Interestingly, IL-17 immunoreactivity is primarily localized to lymphocytes with characteristic malignant cell morphology, strongly suggesting that IL-17 is expressed by the tumor cells *in situ*. Furthermore, malignant CTCL T-cell lines produce IL-17 and the synthesis is selectively increased by IL-2 and IL-15, which are the two IL-2 receptor β chain (IL-2Rβ)-specific members of the γ_c cytokine family. Small-molecule inhibitors or small interfering RNAs (siRNAs) against Jak3 and Stat3 inhibit the IL-17 production, indicating that activation of the Jak3/Stat3 pathway promotes the malignant expression of IL-17.

RESULTS

IL-17 is present in skin lesions but not sera from CTCL patients

To investigate if IL-17 protein is present in CTCL lesions, we performed immunohistochemical stainings of skin biopsy specimens from 13 patients diagnosed with different stages of CTCL and three patients with benign dermatoses using an anti-IL-17 antibody. As shown in Table 1, skin lesions from 10 of the 13 CTCL patients stained positive for IL-17, whereas affected skin from patients with benign dermatoses stained negative ($P<0.05$, Fisher's exact test). A positive IL-17 staining was observed in 1 of 1 MF plaque lesions, 3 of 4 MF tumor lesions, 3 of 4 transformed MF lesions, and 3 of 4 SS lesions, suggesting that IL-17 expression occurs in all stages of CTCL (Table 1).

Figure 1 shows representative pictures of the immunohistochemical stainings of an MF plaque lesion, an MF tumor lesion, SS lesions, and a benign dermatosis. Interestingly, IL-17 positivity was primarily localized to atypical lymphocytes in the dermis and epidermis with characteristic neoplastic morphology, indicating that the malignant T cells express IL-17 *in situ* (Figure 1a–c). Accordingly, most of the lymphocytes in Pautrier microabscesses stained for IL-17, and overall the majority of the epidermotropic lymphocytes were

Table 1. IL-17 expression in CTCL skin lesions

Patient	Diagnosis	IL-17
1	MF plaque	+
2	MF tumor	−
3	MF tumor	+
4	MF tumor	+
5	MF tumor	+
6	MF tumor, transformed	−
7	MF tumor, transformed	+
8	MF tumor, transformed	+
9	MF tumor, transformed	+
10	SS	+
11	SS	+
12	SS	−
13	SS	+
14	Benign dermatosis	−
15	Benign dermatosis	−
16	Benign dermatosis	−

Abbreviations: CTCL, cutaneous T-cell lymphoma; MF, mycosis fungoides; SS, Sézary syndrome.
+ And − indicate that the lesions stained positive or negative for IL-17, respectively.

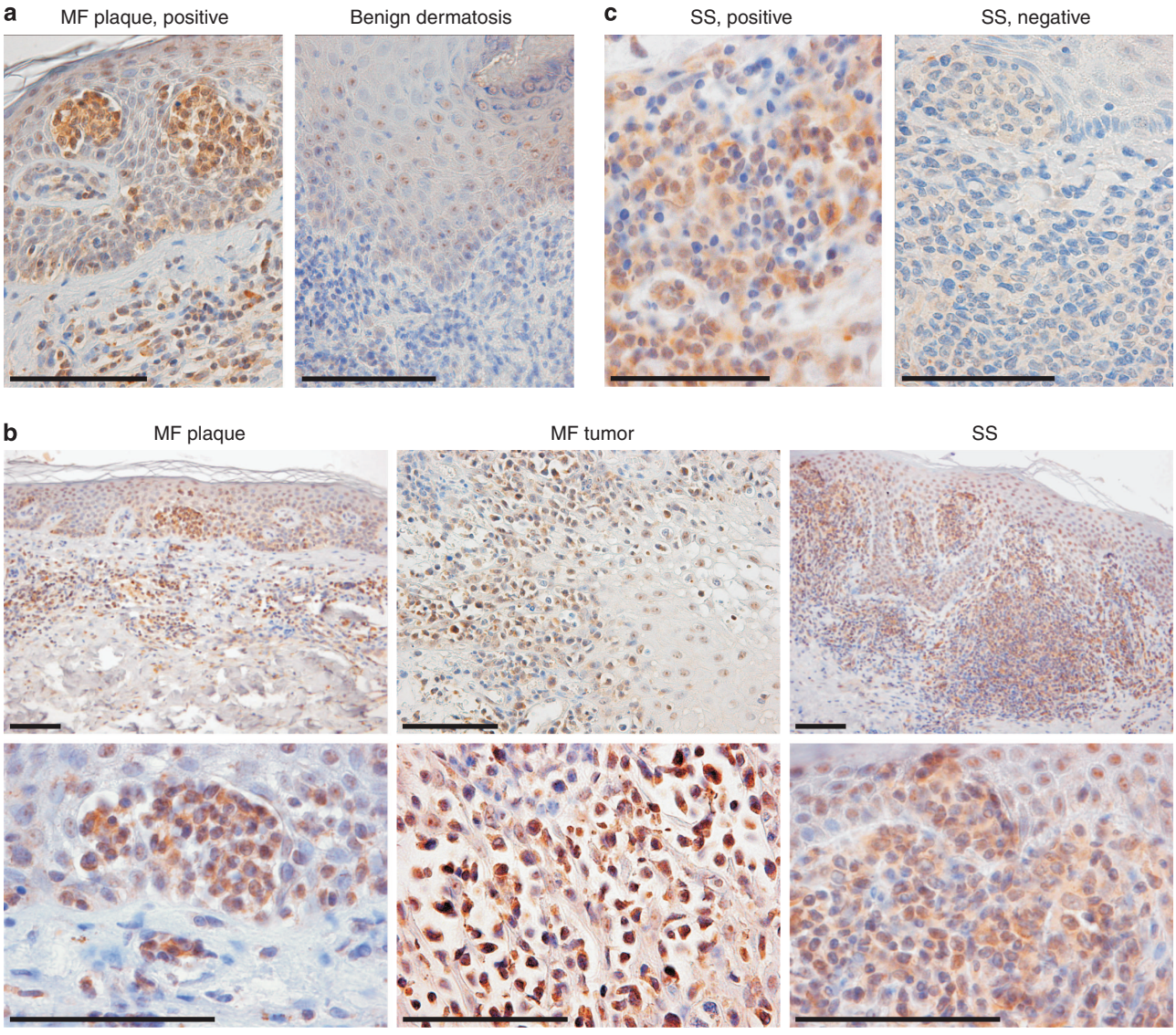

Figure 1. IL-17 is expressed by neoplastic lymphocytes in cutaneous T-cell lymphoma (CTCL) skin lesions. Representative pictures of (**a**) an IL-17-positive plaque lesion and a benign dermatosis, (**b**) a mycosis fungoides (MF) plaque, an MF tumor, and a Sézary syndrome (SS) skin lesion, all positive for IL-17, and (**c**) an IL-17-positive and an IL-17-negative SS skin lesion. Scale bar = 100 μm.

IL-17 positive (Figure 1a and b). It is noteworthy that in all samples (including benign dermatoses) the antibody exhibited some degree of unspecific binding that gave rise to a weak staining that was preferentially localized to the epidermal keratinocytes—a pattern distinctly different from the positive staining in areas of atypical lymphocytes (Figure 1a). One patient with tumor stage MF, one with transformed MF, and one SS patient were IL-17 negative (Table 1 and Figure 1c), indicating that IL-17 expression is a marker of disease heterogeneity as suggested in a previous study on IL-17 mRNA expression in CTCL skin lesions (Ciree *et al.*, 2004).

Having found IL-17 expression in CTCL skin lesions, we asked if IL-17 was also present in the peripheral blood of the patients. Consequently, we examined the concentration of IL-17 in serum samples from 8 healthy donors and 28 patients with different clinical stages of CTCL (Ib, IIa, IIb, III, IVa, and IVb) using an IL-17-specific ELISA with a sensitivity of 10 pg ml^{-1}. As expected, IL-17 was not detected in the sera from the healthy individuals but could be identified in all 8 serum samples spiked with 200 pg ml^{-1} IL-17 with a mean recovery of 100.3% (Figure 2a). However, IL-17 was not detected in the sera of any of the 28 patients, indicating that IL-17 is not present in the peripheral blood of CTCL patients but restricted to the skin lesions (data not shown). Supporting this finding, IL-17 mRNA was not expressed in peripheral blood mononuclear cells isolated from two SS patients with high numbers of malignant T cells in the peripheral blood (Figure 2b).

IL-17 is produced by malignant CTCL T-cell lines

To provide additional evidence that malignant CTCL cells produce IL-17, we analyzed if two malignant (SeAx

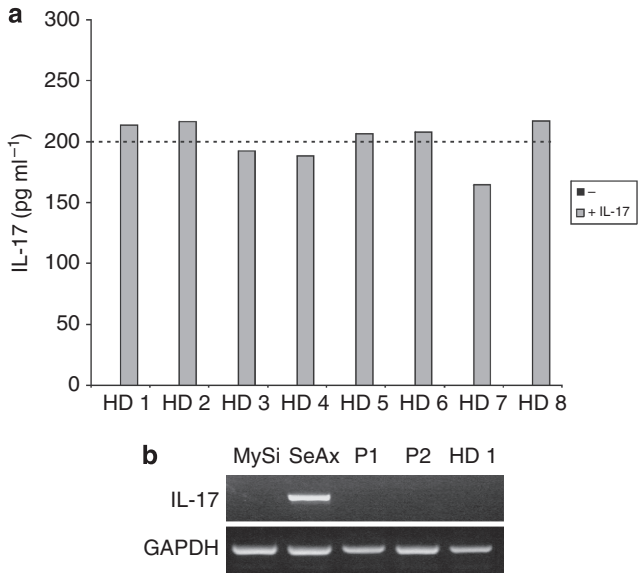

Figure 2. IL-17 is not expressed in the peripheral blood of cutaneous T-cell lymphoma (CTCL) patients. (**a**) Spike-recovery test of the ELISA used for detection of IL-17 in human serum. A total of 200 pg ml^{-1} IL-17 or the corresponding volume of phosphate-buffered saline (PBS; −) was added to sera from eight healthy donors (HD) and the concentration of IL-17 analyzed by ELISA. (**b**) IL-17 reverse transcriptase-PCR (RT-PCR) of a nonmalignant (MySi) and a malignant (SeAx) CTCL T-cell line as well as peripheral blood mononuclear cells (PBMCs) isolated from two CTCL patients (P1 and P2) and a healthy donor (HD 1).

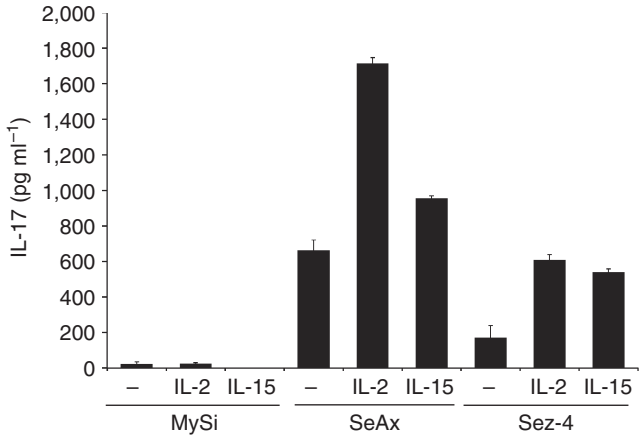

Figure 3. Malignant but not nonmalignant cutaneous T-cell lymphoma (CTCL) T-cell lines express IL-17. Malignant (SeAx, SeZ-4) and nonmalignant (MySi) T cells were cultured for 24 hours with IL-2 (500 U ml^{-1}), IL-15 (20 ng ml^{-1}), or vehicle (−) and the concentration of IL-17 in the supernatant determined by ELISA. Each bar is the mean + SEM of three replicates.

and SeZ-4) T-cell lines and a nonmalignant (MySi) T-cell line established from patients with CTCL expressed IL-17. In accordance with our previous findings, the two malignant T-cell lines but not the non-malignant T-cell line expressed IL-17 mRNA (Figure 2b and data not shown) and secreted IL-17 protein (Figure 3). Interestingly, the secretion of IL-17 was strongly increased when the malignant T cells were stimulated with the IL-2Rβ cytokines IL-2 and IL-15 (Figure 3). The increased levels of IL-17 were not simply a result of increased numbers of the malignant T cells due to the proliferative effect of the IL-2Rβ cytokines, as increased levels of IL-17 were observed before an increase in the total protein levels and as the increase of IL-17 was evident after normalization to the total protein levels (Supplementary Figure S1 online). In contrast to IL-2 and IL-15, stimulation with other γ_c cytokine family members (IL-4, IL-7, IL-9, and IL-21) or IL-23 did not increase the malignant secretion of IL-17 (Supplementary Figure S2 online). It is noteworthy that the malignant CTCL T-cell line HUT-78 did not secrete IL-17 even after cytokine stimulation, which is in accordance with our finding that malignant expression of IL-17 is not observed in all CTCL patients (data not shown).

The expression of IL-17 is promoted by the Jak3/Stat3 pathway

Next, we sought to identify the molecular mechanisms driving the expression of IL-17 by the malignant T cells. The Jak3/Stat3 signaling pathway is normally activated in the malignant T cells in CTCL lesions and is believed to have a central pathogenic role (Zhang *et al.*, 1996; Sommer *et al.*, 2004; Krejsgaard *et al.*, 2009). Because Stat3 has been identified as an important regulator of IL-17 expression in normal T cells (Korn *et al.*, 2009) and IL-2Rβ cytokines increase the Jak3-dependent activation of Stat3 in CTCL cells (Kasprzycka *et al.*, 2008; Marzec *et al.*, 2008), we explored if the Jak3/Stat3 pathway was involved in the IL-17 production by the CTCL cells. As shown in Figure 4, treatment of the SeAx cells with a small-molecule Jak3 kinase inhibitor significantly reduced the expression of IL-17 mRNA and protein, whereas inhibitors against several non-Jak kinases had no significant effect (Figure 4a and b). Similarly, the highly specific Jak3 inhibitor CP-690,550 mediated a dose-dependent inhibition of Stat3 activity that correlated with reduced levels of IL-17 induction (Supplementary Figure S3 online). The downregulation of Jak3 expression by Jak3-specific siRNA also inhibited the IL-17 production, whereas Stat5 siRNA significantly increased the expression of IL-17 from SeAx cells treated with IL-2 (Figure 4c). In contrast, both a small-molecule Stat3 inhibitor and Stat3 siRNA reduced the IL-17 production induced by IL-2 (Figure 5a and b). Immunohistochemical stainings against active Stat3 (pYStat3) on 12 of the CTCL lesions previously analyzed for IL-17 showed that 9 of 11 pYStat3-positive lesions were also IL-17 positive and that one lesion that was pYStat3 negative was also negative for IL-17 (Supplementary Figure S4 online).

DISCUSSION

IL-17 is a proinflammatory cytokine that has been associated with the development of inflammatory diseases and cancer (Weaver *et al.*, 2007; Korn *et al.*, 2009; Miossec *et al.*, 2009). In this study, we show that IL-17 protein is expressed in skin lesions from 10 of 13 CTCL patients. This finding is in keeping with a previously reported identification of IL-17 mRNA in 5 of 10 CTCL skin biopsies. However, the exact cellular source of the IL-17 mRNA was not identified by the reverse transcriptase-PCR-based tissue analysis (Ciree *et al.*, 2004). Here, we found IL-17 protein immunoreactivity primarily

localized to atypical lymphocytes with characteristic neoplastic cell morphology in CTCL skin lesions. Moreover, malignant T-cell lines established from CTCL patients secreted IL-17, indicating that malignant CTCL cells express IL-17 *in situ*. Supporting this notion, Ciree *et al.* (2004) showed that malignant T cells purified from the peripheral blood of CTCL patients produced IL-17 *in vitro* after activation with phorbol 12-myristate 13-acetate and ionomycin. As we did not detect IL-17 in serum or peripheral blood mononuclear cells from CTCL patients, it seems that the malignant T cells in the peripheral blood are not appropriately activated to produce IL-17. This possibility would imply the presence of factors in the skin lesions that locally stimulate the tumor cells to produce IL-17. We found that the IL-2Rβ-specific cytokines IL-2 and IL-15 potently increased IL-17 expression by the malignant T cells. It has previously been shown that IL-15 is produced by keratinocytes in CTCL lesions, identifying IL-15 as one likely factor that could stimulate the expression of IL-17 by the malignant CTCL cells (Dobbeling *et al.*, 1998; Leroy *et al.*, 2001). In line with this hypothesis, other studies demonstrated that IL-15 triggers overproduction of IL-17 in rheumatoid arthritis (Ziolkowska *et al.*, 2000; Yoshihara *et al.*, 2007). Our results show that the IL-2Rβ cytokine-induced expression of IL-17 is mediated through a Jak3/Stat3-dependent mechanism and repressed by Stat5. Because other members of the γ_c cytokine family can increase the activity of Jak3 and Stat3 in the malignant T cells (Marzec *et al.*, 2008), the present findings suggest that the ability of Stat3 to induce expression of IL-17 is subject to regulation by other factors. This conclusion is in agreement with the observation that 2 of the 11 CTCL lesions were pYStat3 positive but not positive for IL-17. However, 9 of 11 CTCL lesions stained positive for both pYStat3 and IL-17 and one lesion that was negative for pYStat3 was also IL-17 negative, which is in accordance with a role of Stat3 in promoting the malignant expression of IL-17.

Ciree *et al.* (2004) found that malignant CTCL cell lines expressed IL-17 receptor A but that exogenous IL-17 did not influence their proliferation or cytokine production, and we obtained similar results in a series of studies of malignant growth *in vitro* (data not shown). Similarly, normal T cells exhibit no or very subtle responses to IL-17, although they express IL-17 receptor A (Gaffen, 2009). Together, these observations suggest that IL-17 does not function as an autocrine factor for the malignant T cells. Receptors for IL-17 are also expressed by fibroblasts, keratinocytes, and epithelial cells. In contrast to T cells, these cell types typically produce a variety of proinflammatory cytokines, chemokines, matrix metalloproteinases, and angiogenic proteins when stimulated

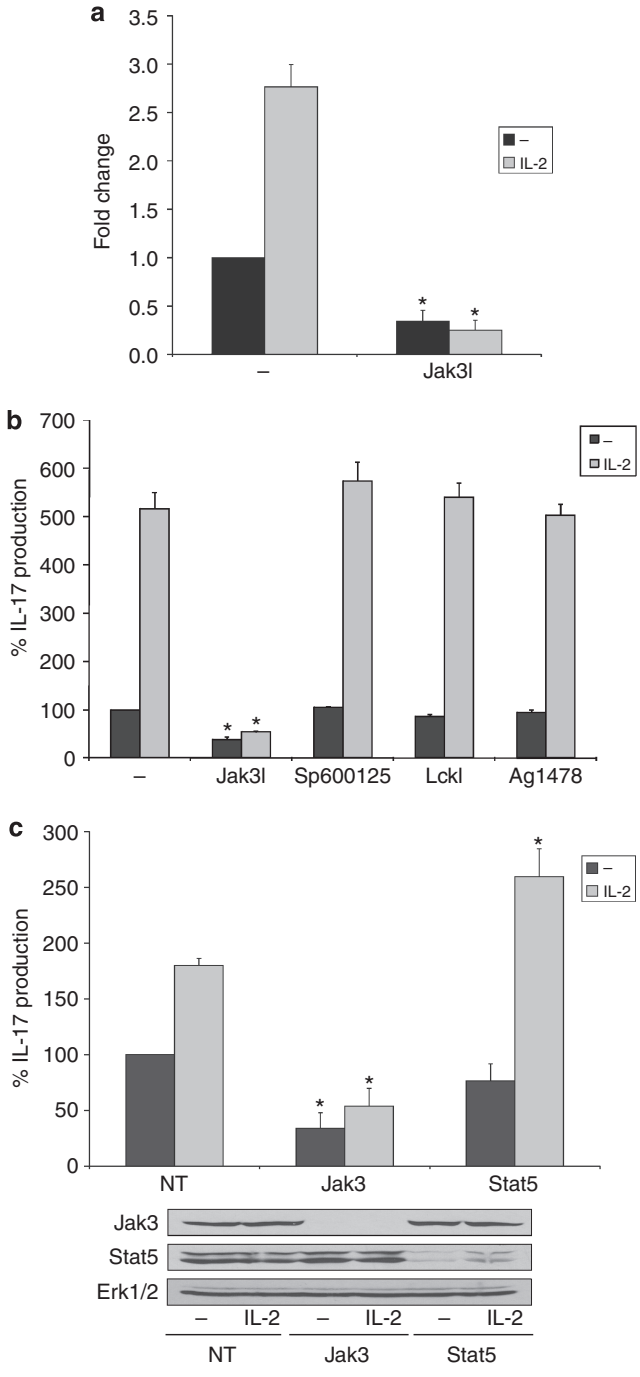

Figure 4. The expression of IL-17 is promoted by Jak3. (**a**) SeAx cells were incubated for 30 minutes with 40 μg ml^{-1} Jak3 inhibitor (Jak3I) or vehicle (–). Then, 500 U ml^{-1} IL-2 or phosphate-buffered saline (PBS; –) was added and the cells incubated for 4 hours further before the relative levels of IL-17 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined by quantitative PCR (qPCR). In each sample, the level of IL-17 mRNA was normalized to that of GAPDH mRNA and depicted as fold change when compared with cells cultured without IL-2 and inhibitor. (**b**) SeAx cells were incubated for 30 minutes with inhibitors against Jak3 (Jak3I, 40 μg ml^{-1}), JNK (Sp600125, 5 μM), Lck (LckI, 5 μM), EGFR (Ag1478, 200 ng ml^{-1}), or vehicle (–) before addition of 500 U ml^{-1} IL-2 or PBS (–). The cells were then incubated for 24 hours and the concentrations of IL-17 in the supernatants determined by ELISA. (**c**) SeAx cells were transiently transfected with nontargeting (NT) small interfering RNA (siRNA) or siRNA against Jak3 or signal transducer and activator of transcription 5 (Stat5). At 24 hours after transfection, the cells were washed and cultured with 500 U ml^{-1} IL-2 or PBS (–) for another 24 hours. Finally, the concentrations of IL-17 in the supernatants were determined by ELISA and the cellular expression of Jak3, Stat5, and extracellular signal-regulated kinases 1 and 2 (Erk1/2) analyzed by western blotting. Bars represent mean + SEM, $n = 3$–4. *$P < 0.05$.

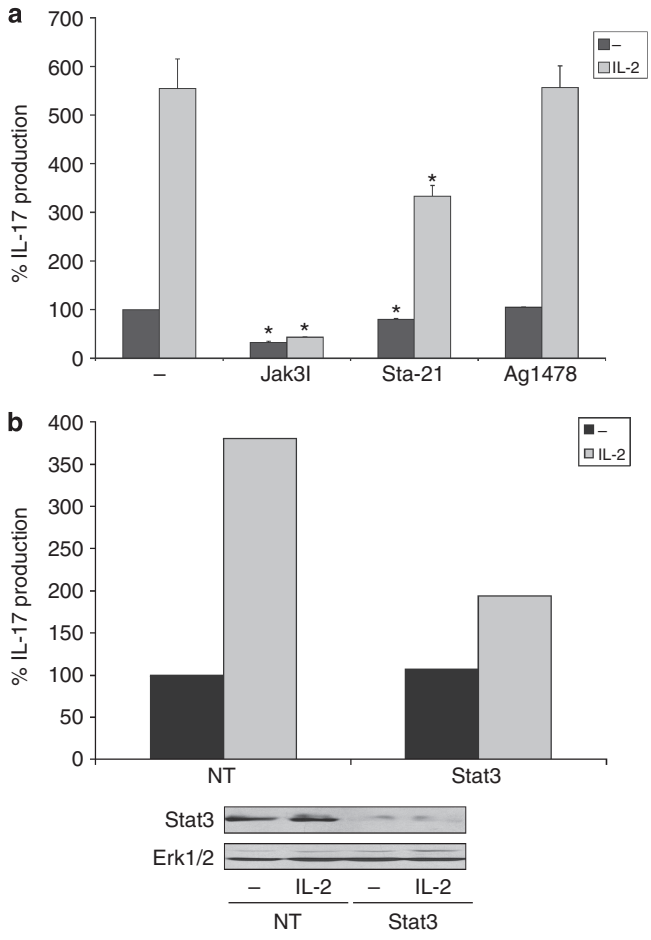

Figure 5. The expression of IL-17 is promoted by signal transducer and activator of transcription 3 (Stat3). (**a**) SeAx cells were initially incubated for 30 minutes with inhibitors against Jak3 (Jak3I, 40 μg ml^{-1}), Stat3 (Sta-21, 40 μM), EGFR (Ag1478, 200 ng ml^{-1}), or vehicle (−). Then, 500 U ml^{-1} IL-2 or phosphate-buffered saline (PBS; −) was added and the cells incubated for 24 hours before the IL-17 concentrations in the supernatants were determined by ELISA. Bars represent mean + SEM, $n = 3$. *$P < 0.05$. (**b**) SeAx cells were transiently transfected with nontargeting (NT) small interfering RNA (siRNA) or siRNA against Stat3. At 24 hours after transfection, the cells were washed and cultured with 500 U ml^{-1} IL-2 or PBS (−) for another 24 hours. Finally, the concentrations of IL-17 in the supernatants were determined by ELISA and the cellular expression of Stat3 and extracellular signal-regulated kinases 1 and 2 (Erk1/2) analyzed by western blotting. Data are representative of two independent experiments.

with IL-17 (Kanda *et al.*, 2005; Koga *et al.*, 2008; Nograles *et al.*, 2008; Gaffen, 2009; Harper *et al.*, 2009; Miossec *et al.*, 2009). Interestingly, CTCL lesions exhibit increased angiogenesis, and several angiogenic and inflammatory proteins that are known to be induced by IL-17 (e.g., tumor necrosis factor-α, CCL20, MMP-9, COX-2, vascular endothelial growth factor, and IL-8) have been reported to be present in CTCL lesions (Hansen *et al.*, 1991; Vacca *et al.*, 1997; Daliani *et al.*, 1998; Schmuth *et al.*, 2002; Mazur *et al.*, 2004; Krejsgaard *et al.*, 2006; Kopp *et al.*, 2010). Therefore, it seems plausible that IL-17 indirectly influences CTCL tumorigenesis by modulating angiogenesis and inflammation; however, whether IL-17 has an overall pro- or anti-tumorigenic role remains to be established. We speculate that IL-2Rβ cytokines, in cooperation with other lesional factors, stimulate the malignant T cells to express IL-17 and that IL-17, in turn, stimulates the stromal cells to secrete angiogenic factors, proinflammatory cytokines, and chemokines that contribute to tumorigenesis by promoting angiogenesis and chronic inflammation. In this context, it is interesting that neutralizing antibodies against IL-17 are now in clinical trails for treatment of inflammatory and autoimmune diseases (Genovese *et al.*, 2010). Furthermore, our findings suggest that drugs targeting the Jak3/Stat3 pathway could inhibit the expression of IL-17. Importantly, several potent and relatively selective inhibitors of Jak3 have been developed. The most clinically advanced compound is the orally active Jak3 inhibitor CP-690,550 that has shown promising results in phase II clinical trials for the therapy of rheumatoid arthritis, psoriasis, and the prevention of renal transplant rejection with relatively few side effects at effective doses (Pesu *et al.*, 2008; West, 2009; Wilson, 2010). The compound is currently in a phase III clinical trial investigating the efficacy and safety in patients with active rheumatoid arthritis on background methotrexate. Therefore, it was of particular interest that CP-690,550 potently abrogated the cytokine-induced IL-17 production from the malignant T-cell lines. As the expression of IL-17 was promoted by Stat3 and repressed by Stat5, selective inhibition of Stat3 activity could also provide a rational strategy for suppression of IL-17 production. However, although several Stat3 inhibitors have been developed, so far no clinical trails of direct Stat3 inhibitors have been published (Yue and Turkson, 2009).

In conclusion, our findings indicate that the malignant T cells in CTCL lesions express IL-17 and that this expression is promoted by the Jak3/Stat3 pathway.

MATERIALS AND METHODS

Reagents

Antibodies against Jak3 and Erk1/2 (extracellular signal-regulated kinases 1 and 2) were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against Stat3 was from Cell Signaling Technology (Beverly, MA) and the Stat5 antibody from Becton Dickinson (Franklin Lakes, NJ). The Lck inhibitor (LckI) and Jak3 inhibitor II (Jak3I; WHI-P154) were from Calbiochem (San Diego, CA), whereas Sp600125, Sta-21, and Tyrphostin Ag1478 were from Enzo Life Sciences (Plymouth Meeting, PA). Finally, DMSO was from Sigma-Aldrich (St Louis, MO), recombinant human IL-2 (Proleukin) from Chiron (Emeryville, CA), and recombinant human IL-15 from Leinco Technologies (St Louis, MO).

Patients and immunohistochemistry

Paraffin-embedded skin biopsies from patients diagnosed with CTCL during the period 1979–2004 were drawn from the archives of the Department of Pathology at Rigshospitalet. For immunohistochemical analysis of IL-17, the biopsies were pre-treated in a microwave oven in TEG buffer (pH 9) and then incubated with an anti-IL-17 antibody (H132; Santa Cruz Biotechnology) diluted 1:100 for 60 minutes. Staining was performed using the DAKO EnVisionHRP/Rabbit kit (DAKO, Hamburg, Germany) as previously described (Gjerdrum *et al.*, 2007). Controls consisted of cell lines

shown to be IL-17 positive and negative by ELISA as well as sections of benign hyperplastic tonsil and benign dermatoses. In accordance with the Declaration of Helsinki Principles (paragraph 25, October 2008) and Danish law, patient consent was not required as the retrospective analyses of the archival tissue biopsies were performed after specific approval and permission by the Danish Ethics Committee (journal no. 01 284225) and the Institutional Review Board. Only tissue sections not needed for diagnosis were used for the analyses and the study was performed in accordance with the Declaration of Helsinki Principles.

Cells

The malignant T-cell lines, SeAx and Sez-4, and the nonmalignant T-cell line, MySi, were established from patients diagnosed with CTCL (Kaltoft *et al.*, 1987; Zhang *et al.*, 2000; Woetmann *et al.*, 2007). The cell lines were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100 $\mu g\,ml^{-1}$ penicillin/streptomycin (all from Sigma-Aldrich), 10% pooled human serum (Blood Bank, State University Hospital, Copenhagen, Denmark), and 10^3 U ml^{-1} IL-2. Before experimental use, the cells were starved for 24 hours in media without IL-2. Peripheral blood mononuclear cells were isolated from two patients diagnosed with SS and a healthy donor as previously described (Kasprzycka *et al.*, 2008). The percentage of malignant T cells in the peripheral blood of the two patients was 62% (P1) and 57% (P2) as judged from the number of CD4 + CD7− T cells. In experiments using small-molecule inhibitors, the inhibitors were not washed out of the culture before addition of cytokines.

ELISA

The concentrations of IL-17 in cell culture supernatants and human serum were measured using the human IL-17 DuoSet ELISA development kit from R&D Systems (Minneapolis, MN) in accordance with the manufacture's instructions. For analysis of IL-17 in human serum, the standard curve samples were resuspended in freshly prepared Reagent Diluent (1% BSA in phosphate-buffered saline) and diluted 1:1 with human serum from healthy donors and the serum samples were diluted 1:1 in Reagent Diluent.

RNA purification, reverse transcriptase-PCR, and quantitative PCR

Total cellular mRNA was purified and reverse transcribed into complementary DNA as described previously (Krejsgaard *et al.*, 2008). For reverse transcriptase-PCR, the complementary DNA was amplified using recombinant Taq DNA polymerase (New England Biolabs, Beverly, MA) with the following primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-forward: 5′-CCATGGAGAAG GCTGGGG-3′, GAPDH-reverse: 5′-CAAAGTTGTCATGGATGACC-3′, IL-17-forward: 5′-TCATCCATCCCCAGTTGATT-3′, IL-17-reverse: 5′-GACCAGGATCTCTTGCTGGA-3′. Quantitative PCR was performed using the Brilliant II SYBR Green quantitative PCR kit from Stratagene (La Jolla, CA) in accordance with the manufacturer's instructions and the samples analyzed on a Mx3000P (Stratagene). For amplification of IL-17, the forward (0.1 μM) and reverse (0.3 μM) primers noted above were used and for amplification of GAPDH the following primers were used: 0.2 μM GAPDH-forward 5′-AAGGTG AAGGTCGGAGTCAA-3′, 0.2 μM GAPDH-reverse 5′-AATGAAGGG GTCATTGATGG-3′.

Transient transfections

Transient transfections were essentially performed as previously described (Sommer *et al.*, 2004) using 0.5 nmol Jak3, Stat3, Stat5, or nontargeting (NT) ON-TARGETplus SMARTpool siRNA (Dharmacon, Chicago, IL) and 2×10^6 cells.

Protein extraction and western blotting

Protein extraction and western blotting were performed as described earlier (Krejsgaard *et al.*, 2006). To ensure equal loading, the total protein concentration of each lysate was determined by Bio-Rad protein Assay (Bio-Rad, Hercules, CA).

Statistics

For statistical analysis, a two-tailed one-sample or two-sample Student's *t*-test with a significance level of 0.05 was used. The asterisk symbol (*) denotes a significant difference ($P<0.05$) between the indicated sample and the sample cultured without inhibitor at the corresponding condition (with or without IL-2).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Ciree A, Michel L, Camilleri-Broet S *et al.* (2004) Expression and activity of IL-17 in cutaneous T-cell lymphomas (mycosis fungoides and Sezary syndrome). *Int J Cancer* 112:113–20

Cornejo MG, Kharas MG, Werneck MB *et al.* (2009) Constitutive JAK3 activation induces lymphoproliferative syndromes in murine bone marrow transplantation models. *Blood* 113:2746–54

Daliani D, Ulmer RA, Jackow C *et al.* (1998) Tumor necrosis factor-alpha and interferon-gamma, but not HTLV-I tax, are likely factors in the epidermotropism of cutaneous T-cell lymphoma via induction of interferon-inducible protein-10. *Leuk Lymphoma* 29:315–28

Dobbeling U, Dummer R, Laine E *et al.* (1998) Interleukin-15 is an autocrine/paracrine viability factor for cutaneous T-cell lymphoma cells. *Blood* 92:252–8

Doherty SD, Ni X, Doherty CB *et al.* (2006) Abnormal expression of interleukin-23 in mycosis fungoides/Sezary syndrome lesions. *Arch Dermatol Res* 298:353–6

Eriksen KW, Kaltoft K, Mikkelsen G *et al.* (2001) Constitutive STAT3-activation in Sezary syndrome: tyrphostin AG490 inhibits STAT3-activation, interleukin-2 receptor expression and growth of leukemic Sezary cells. *Leukemia* 15:787–93

Ferenczi K, Fuhlbrigge RC, Pinkus J *et al.* (2002) Increased CCR4 expression in cutaneous T cell lymphoma. *J Invest Dermatol* 119:1405–10

Gaffen SL (2009) Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* 9:556–67

Genovese MC, Van den Bosch F, Roberson SA *et al.* (2010) LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: a phase I randomized, double-blind, placebo-controlled, proof-of-concept study. *Arthritis Rheum* 62:929–39

Girardi M, Heald PW, Wilson LD (2004) The pathogenesis of mycosis fungoides. *N Engl J Med* 350:1978–88

Gjerdrum LM, Woetmann A, Odum N *et al.* (2007) FOXP3+ regulatory T cells in cutaneous T-cell lymphomas: association with disease stage and survival. *Leukemia* 21:2512–8

Hansen ER, Vejlsgaard GL, Lisby S *et al.* (1991) Epidermal interleukin 1 alpha functional activity and interleukin 8 immunoreactivity are increased in patients with cutaneous T-cell lymphoma. *J Invest Dermatol* 97:818–23

Harper EG, Guo C, Rizzo H *et al.* (2009) Th17 cytokines stimulate CCL20 expression in keratinocytes in vitro and in vivo: implications for psoriasis pathogenesis. *J Invest Dermatol* 129:2175–83

Hwang ST, Janik JE, Jaffe ES *et al.* (2008) Mycosis fungoides and Sezary syndrome. *Lancet* 371:945–57

Ji Y, Zhang W (2010) Th17 cells: positive or negative role in tumor? *Cancer Immunol Immunother* 59:979–87

Kaltoft K, Bisballe S, Rasmussen HF *et al.* (1987) A continuous T-cell line from a patient with Sezary syndrome. *Arch Dermatol Res* 279:293–8

Kanda N, Koike S, Watanabe S (2005) IL-17 suppresses TNF-alpha-induced CCL27 production through induction of COX-2 in human keratinocytes. *J Allergy Clin Immunol* 116:1144–50

Kasprzycka M, Zhang Q, Witkiewicz A *et al.* (2008) Gamma c-signaling cytokines induce a regulatory T cell phenotype in malignant CD4+ T lymphocytes. *J Immunol* 181:2506–12

Kim EJ, Hess S, Richardson SK *et al.* (2005) Immunopathogenesis and therapy of cutaneous T cell lymphoma. *J Clin Invest* 115:798–812

Koga C, Kabashima K, Shiraishi N *et al.* (2008) Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* 128:2625–30

Kopp KL, Kauczok CS, Lauenborg B *et al.* (2010) COX-2-dependent PGE(2) acts as a growth factor in mycosis fungoides (MF). *Leukemia* 24:1179–85

Korn T, Bettelli E, Oukka M *et al.* (2009) IL-17 and Th17 cells. *Annu Rev Immunol* 27:485–517

Krejsgaard T, Gjerdrum LM, Ralfkiaer E *et al.* (2008) Malignant Tregs express low molecular splice forms of FOXP3 in Sezary syndrome. *Leukemia* 22:2230–9

Krejsgaard T, Vetter-Kauczok CS, Woetmann A *et al.* (2009) Ectopic expression of B-lymphoid kinase in cutaneous T-cell lymphoma. *Blood* 113:5896–904

Krejsgaard T, Vetter-Kauczok CS, Woetmann A *et al.* (2006) Jak3- and JNK-dependent vascular endothelial growth factor expression in cutaneous T-cell lymphoma. *Leukemia* 20:1759–66

Leroy S, Dubois S, Tenaud I *et al.* (2001) Interleukin-15 expression in cutaneous T-cell lymphoma (mycosis fungoides and Sezary syndrome). *Br J Dermatol* 144:1016–23

Marzec M, Halasa K, Kasprzycka M *et al.* (2008) Differential effects of interleukin-2 and interleukin-15 versus interleukin-21 on CD4+ cutaneous T-cell lymphoma cells. *Cancer Res* 68:1083–91

Mazur G, Wozniak Z, Wrobel T *et al.* (2004) Increased angiogenesis in cutaneous T-cell lymphomas. *Pathol Oncol Res* 10:34–6

Miossec P, Korn T, Kuchroo VK (2009) Interleukin-17 and type 17 helper T cells. *N Engl J Med* 361:888–98

Murugaiyan G, Saha B (2009) Protumor vs antitumor functions of IL-17. *J Immunol* 183:4169–75

Nograles KE, Zaba LC, Guttman-Yassky E *et al.* (2008) Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *Br J Dermatol* 159:1092–102

Pesu M, Laurence A, Kishore N *et al.* (2008) Therapeutic targeting of Janus kinases. *Immunol Rev* 223:132–42

Schmuth M, Neyer S, Rainer C *et al.* (2002) Expression of the C-C chemokine MIP-3 alpha/CCL20 in human epidermis with impaired permeability barrier function. *Exp Dermatol* 11:135–42

Sommer VH, Clemmensen OJ, Nielsen O *et al.* (2004) In vivo activation of STAT3 in cutaneous T-cell lymphoma. Evidence for an antiapoptotic function of STAT3. *Leukemia* 18:1288–95

Vacca A, Moretti S, Ribatti D *et al.* (1997) Progression of mycosis fungoides is associated with changes in angiogenesis and expression of the matrix metalloproteinases 2 and 9. *Eur J Cancer* 33:1685–92

Vergier B, De MA, Beylot-Barry M *et al.* (2000) Transformation of mycosis fungoides: clinicopathological and prognostic features of 45 cases. French Study Group of Cutaneious Lymphomas. *Blood* 95:2212–8

Vonderheid EC, Bernengo MG, Burg G *et al.* (2002) Update on erythrodermic cutaneous T-cell lymphoma: report of the International Society for Cutaneous Lymphomas. *J Am Acad Dermatol* 46:95–106

Vowels BR, Lessin SR, Cassin M *et al.* (1994) Th2 cytokine mRNA expression in skin in cutaneous T-cell lymphoma. *J Invest Dermatol* 103:669–73

Weaver CT, Hatton RD, Mangan PR *et al.* (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821–52

West K (2009) CP-690550, a JAK3 inhibitor as an immunosuppressant for the treatment of rheumatoid arthritis, transplant rejection, psoriasis and other immune-mediated disorders. *Curr Opin Investig Drugs* 10:491–504

Willemze R, Jaffe ES, Burg G *et al.* (2005) WHO-EORTC classification for cutaneous lymphomas. *Blood* 105:3768–85

Wilson LJ (2010) Recent patents in the discovery of small molecule inhibitors of JAK3. *Expert Opin Ther Pat* 20:609–23

Woetmann A, Lovato P, Eriksen KW *et al.* (2007) Nonmalignant T cells stimulate growth of T-cell lymphoma cells in the presence of bacterial toxins. *Blood* 109:3325–32

Yamanaka K, Clark R, Dowgiert R *et al.* (2006a) Expression of interleukin-18 and caspase-1 in cutaneous T-cell lymphoma. *Clin Cancer Res* 12:376–82

Yamanaka K, Clark R, Rich B *et al.* (2006b) Skin-derived interleukin-7 contributes to the proliferation of lymphocytes in cutaneous T-cell lymphoma. *Blood* 107:2440–5

Yoshihara K, Yamada H, Hori A *et al.* (2007) IL-15 exacerbates collagen-induced arthritis with an enhanced CD4+ T cell response to produce IL-17. *Eur J Immunol* 37:2744–52

Yue P, Turkson J (2009) Targeting STAT3 in cancer: how successful are we? *Expert Opin Investig Drugs* 18:45–56

Zhang Q, Nowak I, Vonderheid EC *et al.* (1996) Activation of Jak/STAT proteins involved in signal transduction pathway mediated by receptor for interleukin 2 in malignant T lymphocytes derived from cutaneous anaplastic large T-cell lymphoma and Sezary syndrome. *Proc Natl Acad Sci USA* 93:9148–53

Zhang Q, Raghunath PN, Vonderheid E *et al.* (2000) Lack of phosphotyrosine phosphatase SHP-1 expression in malignant T-cell lymphoma cells results from methylation of the SHP-1 promoter. *Am J Pathol* 157:1137–46

Ziolkowska M, Koc A, Luszczykiewicz G *et al.* (2000) High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J Immunol* 164: 2832–8

Zou W, Restifo NP (2010) T(H)17 cells in tumour immunity and immunotherapy. *Nat Rev Immunol* 10:248–56