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Letter to the Editor,

Sir,

The expression of IL-21 is promoted by MEKK4 in malignant T cells and associated with increased progression risk in cutaneous T-cell lymphoma

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Short titel: IL-21 is associated with progression risk in CTCL

The expression of IL-21 is promoted by MEKK4 in malignant T cells and associated with increased progression risk in cutaneous T-cell lymphoma

Signaling through Interleukin-2 (IL-2) receptor gamma (IL-2Rg) via the cytokines IL-2, IL-4, IL-7, IL-15, and IL-21 has been implicated in the pathogenesis of cutaneous T-cell lymphomas (CTCL) (Marzec *et al.*, 2008). Extensive research has documented that IL-2, IL-7, and IL-15 stimulate proliferation of malignant T cells, while IL-4 is known to promote a Th2 type inflammatory response within the affected skin. In contrast, the role of IL-21 remains unclear since IL-21 was shown to exert both pro- and anti-tumorigenic responses. On the one hand, IL-21 stimulates anti-tumor cytotoxic T cells and NK cells, which leads to inhibition of tumor growth *in vivo* in a number of cancer models (Sondergaard *et al.*, 2007; Spolski and Leonard, 2014), while on the other hand, it activates the proto-oncogene Signal Transducer and Activator of Transcription (STAT3)(Marzec *et al.*, 2008), which ultimately promotes expression of pro-inflammatory cytokines and survival factors in malignant T cells (Sommer *et al.*, 2004).

Interestingly, IL-21 itself is also a target of STAT3 suggesting the existence of a vicious circle, where IL-21 stimulates STAT3 activation, which in turn up-regulates IL-21 expression (van der Fits *et al.*, 2012). However, IL-21 expression was only partly reduced following inhibition of STAT3 signaling (van der Fits *et al.*, 2012) and IL-21 blockade did not block STAT3 activation (van der Fits *et al.*, 2014) indicating that other pathways are also involved in IL-21 expression in malignant T cells.

The Mitogen-Activated Protein (MAP) KinaseKinaseKinase (MEKK4/ MAP3K4) was recently shown to drive IL-21 expression in $CD4^+$ T cells from patients with

Systemic Lupus Erythematosus (SLE) (Lee *et al.*, 2014), but this has not been documented in CTCL. Yet, a downstream MAP Kinase, p38, is constitutive active in this malignancy and associated with disease progression (Bliss-Moreau *et al.*, 2015).

To elucidate this signaling pathway we addressed whether MEKK4 and p38 were involved in the regulation of IL-21 mRNA expression in patient-derived malignant T cells lines. As shown in Fig. 1A, siRNA-mediated inhibition of MEKK4 resulted in a significant inhibition of MEKK4 (Fig. 1A, left) and IL-21 mRNA expression (Fig. 1A, right) providing the evidence that MEKK4 promotes IL-21 expression in malignant T cells. As expected, siRNA-mediated knock down of STAT3 inhibited IL-21 expression whereas STAT5 knock down had no effect (Fig. 1B). This observation is consistent with previous findings that IL-21 is a STAT3 target gene in malignant T cells. Importantly, STAT3 siRNA had no inhibitory effect on the expression of MEKK4 indicating that MEKK4 is not a down-stream effector of STAT3 (Fig. 1B).

Our findings further revealed that MEKK4 regulates STAT3 phosphorylation. Specifically, siRNA-mediated knock down of MEKK4 resulted in a decrease in serine phosphorylation of STAT3 as judged by Western Blot analysis using a phosphoserine (P-ser727) specific antibody (Fig. 1C, left), whereas the total amount of STAT3 protein was not decreased (Fig. 1C, right). As expected, IL-21 mRNA expression was also inhibited by this treatment (Fig. 1C, right). Importantly, a dual knock down of STAT3 and MEKK4 significantly inhibited IL-21 expression, but to no greater extend than the inhibition of STAT3 or MEKK4 alone (Fig. 1C) implying that both treatments target the same pathway and that ser727 phosphorylation is required for STAT3- mediated IL-21 transcription. Thus, these findings provide evidence for a

link between MEKK4 signaling and STAT3-S727 serine phosphorylation and suggest that MEKK4 augments IL-21 expression via this mechanism. Notably, increased STAT3 serine phosphorylation at residue S727 was previously shown to enhance transcriptional activity of STAT3 (Zhang *et al.*, 1995).

The p38 MAP Kinase induces serine-727 phosphorylation of STAT3 and is a well-established down-stream effector of MEKK4 (Platanias, 2003). Hence, we next addressed the role of p38 in the regulation of IL-21 expression in malignant T cells. To achieve this, we utilized an inhibitor of p38 (SB203580), which blocks IL-2 induced serine phosphorylation of STAT3 (Gollob *et al.*, 1999). As shown in Fig. 1D, SB203580 induced a dose-dependent inhibition of IL-21 expression indicating that p38 indeed promoted the expression of IL-21. Essentially similar results were obtained in two independent experiments using primary malignant T cells (>90% pure as judged from TCR-Vb staining) isolated from peripheral blood from a Sezary Syndrome patient (Fig 1D right). Recently, Aurora kinases A and B were shown to be highly overexpressed and activated in CTCL skin lesions (Humme *et al.*, 2015). As p38 is a down-stream effector of Aurora kinases in other cancers, it is tempting to speculate that Aurora kinases are involved in IL-21 expression by malignant T cells.

Although it is well established that malignant T cells express IL-21 (Marzec *et al.*, 2008; van der Fits *et al.*, 2012), the pathological and clinical relevance of this expression remains unclear. In a recent gene expression analysis and 11 year follow-up on the Boston cohort of CTCL patients, Litvinov et al (Litvinov *et al.*, 2015) documented that IL-21 and IL-21R mRNA expression were highly increased in poor prognosis patient clusters. However, this analysis did not analyze the prognostic value

of specific genes. Therefore, we analyzed for expression of IL-21 mRNA in relation to disease progression in the Boston cohort of CTCL (N=60) patients and compared IL-21 expression between CTCL lesional skin, normal skin samples derived from healthy volunteers (N=6), and benign inflammatory dermatoses that often mimic this malignancy (e.g., psoriasis, pityriasis rubra pilaris and chronic eczema) (N=12) (Litvinov *et al.*, 2010). All patients were enrolled in the IRB-approved study protocol with written, informed consent in accordance with the Declaration of Helsinki (Litvinov *et al.*, 2015).

As presented, in Table S1, IL-21 was expressed in early and advanced stages of CTCL, therefore, suggesting that IL-21 is not simply a surrogate marker for advanced disease. IL-21 was also increased in skin samples from CTCL patients compared to benign inflammatory dermatoses and healthy controls (Fig. 2A). Importantly, IL-21 expression was associated with overall disease progression in CTCL patients, defined as the advancement to the next clinical stage and/or death (Figure 2B). Moreover, IL-21 positivity was associated with increased disease-related mortality (Figures 2C). Furthermore, in our study 19 stage I patient were IL-21^{-} and 18 Stage I patients were IL-21^{+}. Hence, we performed Kaplan-Meier analysis of disease progression from stage I to advanced (≥IIB) stages and/or death. This analysis further confirmed that IL-21 expression is associated with progressive disease even for early stages of CTCL (Figures 2D). These results suggest that there might be an association between IL-21 expression/signaling and disease progression. Notably, p38 and STAT3 activity were also shown to correlate with disease progression (Bliss-Moreau *et al.*, 2015) thereby further highlighting the clinical relevance of our *in-vitro* findings in malignant T cell lines.

In conclusion, we show that IL-21 expression in malignant T-cells is augmented via MEKK4/p38-mediated serine-727 phosphorylation of STAT3 *in-vitro,* and that IL-21 expression is associated with progressive disease.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Map3K4 promotes the expression of IL-21 in malignant T-cells.

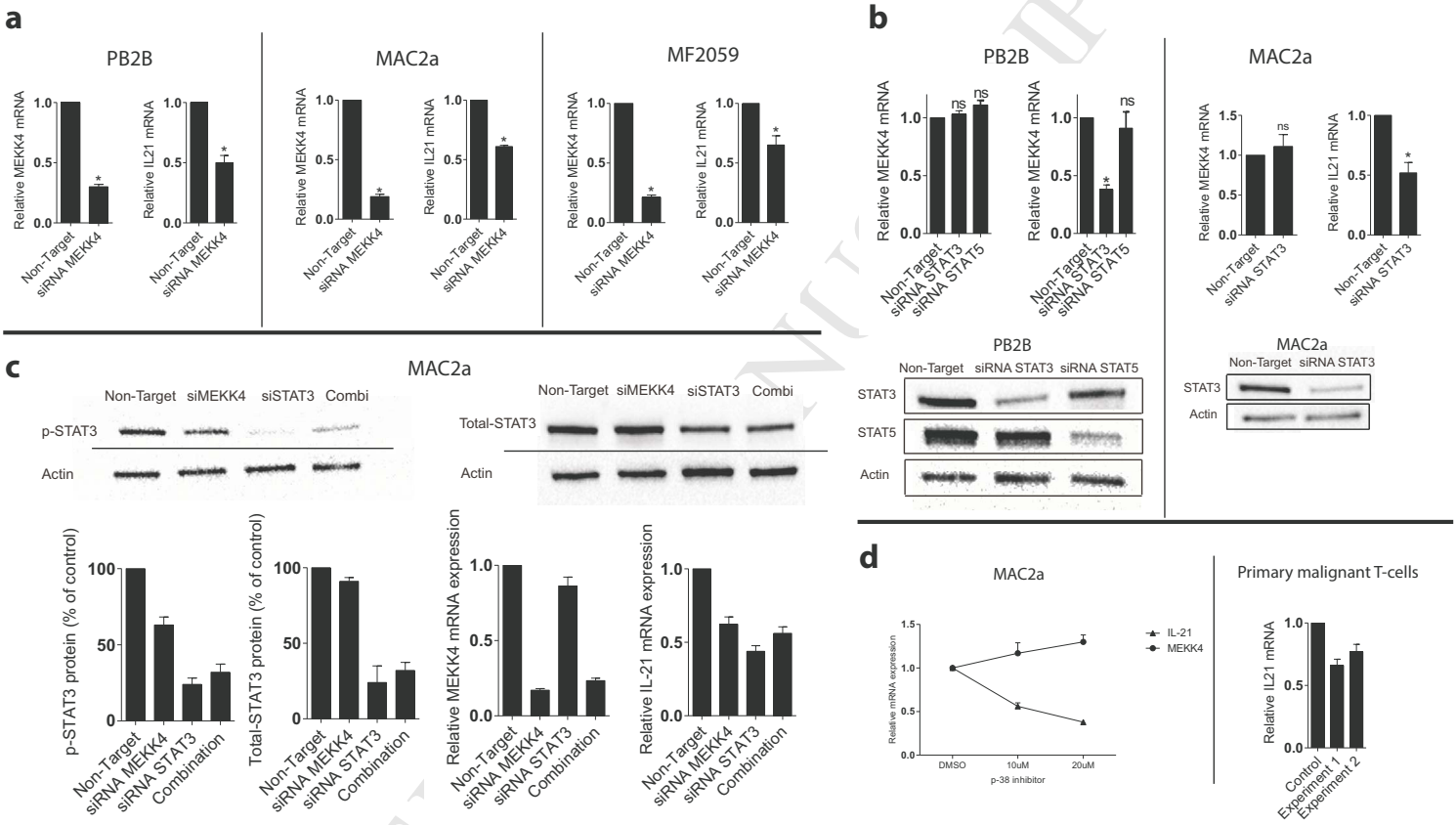
A: MEKK4 and IL21 mRNA expression in malignant T-cell lines obtained from different subtypes of cutaneous T cell lymphoma (MF, SS, and CD30+ anaplastic large T cell lymphoma) after treatment with siRNA against MEKK4 (N=3). **B**: STAT3, STAT5 and Actin (as control) protein expression and MEKK4 and IL21 mRNA expression in two different malignant T-cell lines after treatment with siRNA targeting STAT3 and STAT5 mRNAs (N=3). **C**: Total STAT3, p(ser727)-STAT3, actin (as control) protein expression and MEKK4 and IL-21 mRNA expression in the malignant T-cell line MAC2a after treatment with siRNA targeting MEKK4, STAT3 or combination of MEKK4 and STAT3 (N=2). **D & E**: IL-21 mRNA expression following a treatment with p-38 inhibitor of a CD30+ malignant T cell line, MAC2A, derived from a nonregressing tumor of a patient who had progressed from lymphomatoid papulosis to systemic anaplastic large cell lymphoma (Levi *et al.*, 2000) (N=3)(D) and primary malignant T-cells isolated from peripheral blood from Sezary Syndrome (N=2)(E).

Figure 2. IL-21 is associated with disease progression in CTCL.

A: Relative IL-21 mRNA expression in patients with CTCL, benign inflammatory dermatoses or healthy volunteers. **B-D:** Kaplan-Meier survival curves comparing CTCL patients, with or without IL-21 mRNA expression. **(B)** CTCL patient overall disease progression based on their IL-21 expression status ("progression" defined as progression to a higher clinical stage and/or death, p=0.011). **(C)** CTCL patient disease-specific survival based on their IL-21 expression status,

247 p=0.050. **(D)** Progression of patients with stage I disease to more advance stages

248 (i.e., stage ≥II) based on their IL-21 expression status, p=0.023.


a
PB2B
MAC2a
MF2059
Relative MEKK4 mRNA
Relative IL21 mRNA
Non-Target
siRNA MEKK4
b
PB2B
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siRNA STAT5
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STAT5
Actin
MAC2a
Non-Target siRNA STAT3
d
MAC2a
Relative mRNA expression
IL-21
MEKK4
DMSO
10uM
20uM
p-38 inhibitor
Primary malignant T-cells
Relative IL21 mRNA
Control
Experiment 1
Experiment 2