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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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1 **Letter to the Editor,**

2 **Sir,**

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

5

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

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

42

43 Signaling through Interleukin-2 (IL-2) receptor gamma (IL-2Rg) via the cytokines IL-
44 2, IL-4, IL-7, IL-15, and IL-21 has been implicated in the pathogenesis of cutaneous
45 T-cell lymphomas (CTCL) (Marzec *et al.*, 2008). Extensive research has documented
46 that IL-2, IL-7, and IL-15 stimulate proliferation of malignant T cells, while IL-4 is
47 known to promote a Th2 type inflammatory response within the affected skin. In
48 contrast, the role of IL-21 remains unclear since IL-21 was shown to exert both pro-
49 and anti-tumorigenic responses. On the one hand, IL-21 stimulates anti-tumor
50 cytotoxic T cells and NK cells, which leads to inhibition of tumor growth *in vivo* in a
51 number of cancer models (Sondergaard *et al.*, 2007; Spolski and Leonard, 2014),
52 while on the other hand, it activates the proto-oncogene Signal Transducer and
53 Activator of Transcription (STAT3)(Marzec *et al.*, 2008), which ultimately promotes
54 expression of pro-inflammatory cytokines and survival factors in malignant T cells
55 (Sommer *et al.*, 2004).

56

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

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

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

68

69 To elucidate this signaling pathway we addressed whether MEKK4 and p38 were
70 involved in the regulation of IL-21 mRNA expression in patient-derived malignant T
71 cells lines. As shown in Fig. 1A, siRNA-mediated inhibition of MEKK4 resulted in a
72 significant inhibition of MEKK4 (Fig. 1A, left) and IL-21 mRNA expression (Fig.
73 1A, right) providing the evidence that MEKK4 promotes IL-21 expression in
74 malignant T cells. As expected, siRNA-mediated knock down of STAT3 inhibited IL-
75 21 expression whereas STAT5 knock down had no effect (Fig. 1B). This observation
76 is consistent with previous findings that IL-21 is a STAT3 target gene in malignant T
77 cells. Importantly, STAT3 siRNA had no inhibitory effect on the expression of
78 MEKK4 indicating that MEKK4 is not a down-stream effector of STAT3 (Fig. 1B).

79

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

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

94

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

108

109 Although it is well established that malignant T cells express IL-21 (Marzec *et al.*,
110 2008; van der Fits *et al.*, 2012), the pathological and clinical relevance of this
111 expression remains unclear. In a recent gene expression analysis and 11 year follow-
112 up on the Boston cohort of CTCL patients, Litvinov et al (Litvinov *et al.*, 2015)
113 documented that IL-21 and IL-21R mRNA expression were highly increased in poor
114 prognosis patient clusters. However, this analysis did not analyze the prognostic value

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

123

124 As presented, in Table S1, IL-21 was expressed in early and advanced stages of
125 CTCL, therefore, suggesting that IL-21 is not simply a surrogate marker for advanced
126 disease. IL-21 was also increased in skin samples from CTCL patients compared to
127 benign inflammatory dermatoses and healthy controls (Fig. 2A). Importantly, IL-21
128 expression was associated with overall disease progression in CTCL patients, defined
129 as the advancement to the next clinical stage and/or death (Figure 2B). Moreover, IL-
130 21 positivity was associated with increased disease-related mortality (Figures 2C).
131 Furthermore, in our study 19 stage I patient were IL-21⁻ and 18 Stage I patients were
132 IL-21⁺. Hence, we performed Kaplan-Meier analysis of disease progression from
133 stage I to advanced (\geq IIB) stages and/or death. This analysis further confirmed that
134 IL-21 expression is associated with progressive disease even for early stages of CTCL
135 (Figures 2D). These results suggest that there might be an association between IL-21
136 expression/signaling and disease progression. Notably, p38 and STAT3 activity were
137 also shown to correlate with disease progression (Bliss-Moreau *et al.*, 2015) thereby
138 further highlighting the clinical relevance of our *in-vitro* findings in malignant T cell
139 lines.

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

143 *Conflict of interest*

144 The authors declare no conflict of interest.

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223 **Figure legends**224 **Figure 1. Map3K4 promotes the expression of IL-21 in malignant T-cells.**

225 **A:** MEKK4 and IL21 mRNA expression in malignant T-cell lines obtained from
226 different subtypes of cutaneous T cell lymphoma (MF, SS, and CD30+ anaplastic
227 large T cell lymphoma) after treatment with siRNA against MEKK4 (N=3). **B:**
228 STAT3, STAT5 and Actin (as control) protein expression and MEKK4 and IL21
229 mRNA expression in two different malignant T-cell lines after treatment with
230 siRNA targeting STAT3 and STAT5 mRNAs (N=3). **C:** Total STAT3, p(ser727)-
231 STAT3, actin (as control) protein expression and MEKK4 and IL-21 mRNA
232 expression in the malignant T-cell line MAC2a after treatment with siRNA
233 targeting MEKK4, STAT3 or combination of MEKK4 and STAT3 (N=2). **D & E:** IL-
234 21 mRNA expression following a treatment with p-38 inhibitor of a CD30+
235 malignant T cell line, MAC2A, derived from a nonregressing tumor of a patient
236 who had progressed from lymphomatoid papulosis to systemic anaplastic large
237 cell lymphoma (Levi *et al.*, 2000) (N=3)(D) and primary malignant T-cells
238 isolated from peripheral blood from Sezary Syndrome (N=2)(E).

239

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

241 **A:** Relative IL-21 mRNA expression in patients with CTCL, benign inflammatory
242 dermatoses or healthy volunteers. **B-D:** Kaplan-Meier survival curves comparing
243 CTCL patients, with or without IL-21 mRNA expression. **(B)** CTCL patient overall
244 disease progression based on their IL-21 expression status (“progression”
245 defined as progression to a higher clinical stage and/or death, p=0.011). **(C)**
246 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 \geq II) based on their IL-21 expression status, p=0.023.



