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Letter to the Editor

Jak3 contributes to the activation of ALK and Stat3 in ALK+ anaplastic large cell lymphoma

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To the editor: We read with great interest the manuscript entitled 'Inhibition of ALK enzymatic activity in T-cell lymphoma cells induces apoptosis and suppresses proliferation and STAT3 phosphorylation independently of Jak3' by Marzec *et al*.[1] In this study, the authors showed in ALK+ anaplastic large cell lymphoma (ALCL) cells that the effects of two selective inhibitors of Jak3 on Stat3 phosphorylation, cell proliferation, and the cell cycle were mediated via direct inhibition of ALK, but not Jak3, the natural target of these inhibitors. The authors concluded that exclusively ALK, and not Jak3, activates Stat3 in this type of malignant lymphoma.

These conclusions contradict our previously published results that showed Jak3 to be physically associated with ALK and that it contributes to ALK and Stat3 activation in ALK+ ALCL cells.[2] Thereafter, Crockett *et al*[3] further identified Jak3 and Stat3 in the NPM-ALK protein complex, which indirectly implicates a functional interaction between these proteins. Previous studies by the group of Marzec *et al*[1] as well as by us also showed that Shp1, a physiologic inhibitor of Jak3,[4] is defective in ALK+ ALCL cell lines and tumors.[5,6] Furthermore, we recently showed that Jak3 is constitutively activated and that this activation significantly correlates with ALK expression in ALCL primary tumors.[7] These findings provide strong evidence that Jak3 plays a significant pathogenetic role in ALK+ ALCL via interaction with ALK and Stat3.

We have several concerns regarding the design of the studies and the conclusions drawn by Marzec *et al*.[1] The WHI-P131 and WHI-P154 compounds were originally structured as Jak3 inhibitors.[8] The authors concluded that these compounds induce a previously unidentified inhibitory effect on ALK. This hypothesis is intriguing, alas remained unexplained by the authors, considering the lack of significant homology between Jak3 and ALK.[9]

Marzec *et al*[1] based a major part of their conclusions on an experiment in which they studied the effect of the Jak3-selective inhibitors on phosphorylated ALK and Stat3 (pALK and pStat3, respectively) levels after transfection of ALK into the murine pro-B acute lymphoblastic leukemia cells BaF3. This experiment was primarily designed on the assumption that BaF3 cells lack the expression of Jak3. To our and others' knowledge[10,11] (in addition to a personal communication with Dr Robert Kirken, The University of Texas, El Paso, TX, USA), BaF3 cells express Jak3. In an experimental study, we used standard co-immunoprecipitation techniques and confirmed that Jak3 is expressed and constitutively activated in BaF3 cells (Figure 1). Furthermore, we demonstrated that at 12 h, WHI-P154 induces concentration-dependent decrease in pJak3 levels in these cells (Figure 1). Similar results were obtained with WHI-P131 (not shown). Therefore, the conclusions drawn by the authors from the transfection studies performed in BaF3 cells do not exclude that the decrease in pALK and pStat3 levels after treatment with Jak3 inhibitors in fact resulted from the direct inhibition of Jak3, which further confirm our previous findings regarding the functional interaction between Jak3, ALK, and Stat3.

Marzec *et al*[1] also based a major part of their conclusions on the effects of a pan-Jak inhibitor, which showed less pronounced effects in human

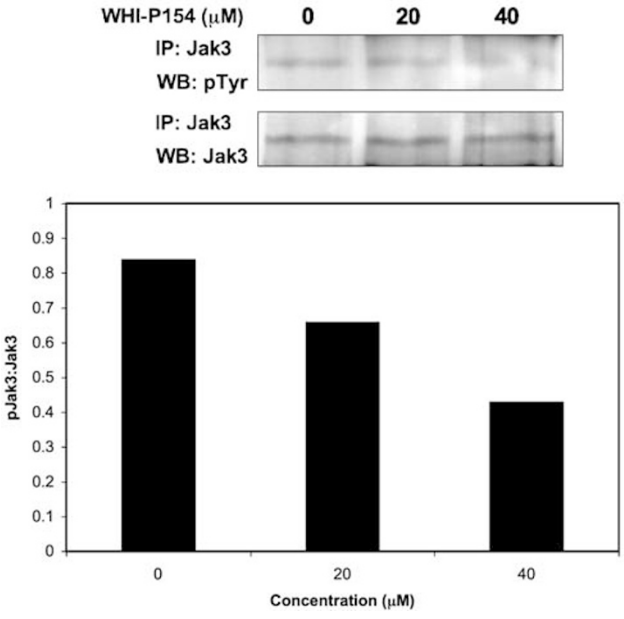

Figure 1 Immunoprecipitation and Western blot studies showing that Jak3 is expressed and constitutively activated in BaF3 cells (upper panel). Selective inhibition of Jak3 by WHI-P154 induced concentration-dependent decrease in pJak3 levels at 12 h. Similar changes were not detected in Jak3 levels. The lower panel shows a densitometry scanning of the bands shown in the upper panel. There was a gradual decrease in pJak3:Jak3 ratio with increasing the concentration of WHI-P154. The antibodies used in this experiment were purchased from Sigma (Jak3) and Santa Cruz Biotechnology (pTyr). Similar results were obtained using WHI-P131 (data not shown). IP: immunoprecipitation; WB: Western blotting.

ALK+ ALCL cells compared to ALK− T-cell lymphoma cells. According to the manufacturer (JAK inhibitor I; Calbiochem), the pan-Jak inhibitor induces significantly variable inhibitory effects on Jak1, Jak2, Jak3, and Tyk2. It is of note that the inhibitory effects of this compound on Jak2 and Tyk2 are five times more potent than its effect on Jak3. Importantly, the characteristics of the pan-Jak inhibitor, including IC_{50}, were established based on murine, not human, Jaks. Furthermore, the pan-Jak inhibitor blocks IL-2- and IL-4-dependent proliferation of T-lymphocytes as well as the phosphorylation of Stat5 in unidentified mechanisms. Owing to the ill-defined characteristics, specificity, and effects of the pan-Jak inhibitor, it is rather difficult to draw convincing conclusions from these experiments. For example, it is possible that the lack of significant effects of the pan-Jak inhibitor on the proliferation of ALK+ ALCL cells was due to other unknown, yet significant effects that resulted from the interaction of the pan-Jak inhibitor with other kinases/signaling pathways. In contrast, the more selective inhibitors of Jak3 induced significant inhibitory effects in ALK+ ALCL cells. As BaF3 cells express Jak3, the limited effect of the pan-Jak inhibitor compared with the more pronounced effect of the Jak3-selective inhibitors on pALK and pStat3 levels is not surprising and actually these findings further support that Jak3 plays a significant role in ALK/Stat3 activation. Similarly, it is not unexpected that the inhibitory effects of the pan-Jak inhibitor on ALK kinase activity were lacking compared to the pronounced inhibitory effects of the selective inhibitors of Jak3 on ALK enzymatic activity in ALK+ ALCL cell lines, which further confirm our previous findings and provide another evidence that Jak3 contributes to the constitutive activation of ALK in these tumors.

To evaluate direct inhibition of Jak3 on pStat3 levels in ALK+ ALCL cells, Marzec *et al*[1] used a compound synthesized in their laboratory based on a previously published structure of CP-690,550 (Pfizer).[12] The authors incubated ALK+ ALCL cells with this compound for 1 h and used Western blotting techniques to demonstrate that there was no significant change in pStat3 levels. Notably, the effect of this compound on Jak3/pJak3 levels was not shown in the manuscript. Assuming that this compound induces a significant decrease in pJak3 levels, its effect on pStat3 levels in ALK+ ALCL cells should have been studied over a gradually increasing range of time points and by using co-immunoprecipitation. To further support their hypothesis, Marzec *et al*[1] used a commercially available Jak3 siRNA in one ALK+ ALCL cell line and showed that there was no effect on pStat3 level. It is of note that the transfection efficiency of Jak3 siRNA and the baseline level of Jak3 were not shown. Additionally, changes in pJak3 were not explored, considering that a fraction of the active form of Jak3 might still have been present. The authors showed that Jak3 siRNA completely abolished Jak3 in ALK+ ALCL cells. In our experience and others with the siRNA technique, including Jaks siRNA, is that it induces approximately 40–60% downregulation of the target protein.[13] Similar to the previous experiment, the changes in pStat3 levels were studied using Western blotting and not co-immunoprecipitation. In addition, pStat3 levels were evaluated at only two time points: 24 and 48 h.

It has been previously shown in different systems that Jak3 interacts and activates Stat3.[14–16] Considering this fact as well as previous studies in ALK+ ALCL cell lines and primary tumors that demonstrated that Jak3 is highly expressed, constitutively activated, and physically associated with ALK, we believe that excluding a significant role of Jak3 in ALK/Stat3 interaction is not logical. We also believe that ALK plays a pivotal role in the pathogenesis of ALK+ ALCL; however, oncogenesis, including lymphomagenesis, is complex and involves numerous interacting molecules and pathways. In 2002, Zhang *et al*[17] proposed that Stat3 is constitutively activated in ALK+ ALCL via multilevel deregulatory mechanisms. We find it surprising that the same group today denies a significant role of Jak3, the physiologic activator of Stat3, in these tumors. We agree with the authors that targeting ALK might represent a potential therapeutic modality in ALK+ tumors; however, this hypothesis should not preclude the fact that other pathways, for example, Jak3 signaling, should be explored and evaluated for their therapeutic potential in these tumors.

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