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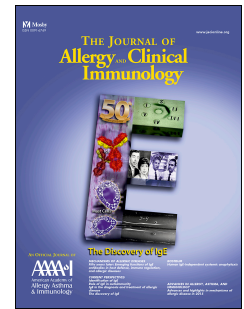
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Letters to the Editor**Title:**

Janus-kinase inhibitors display broad anti-itch properties – a possible link via the TRPV1 receptor

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24 **Capsule summary:**

25 The antipruritic effect of JAK inhibitors is fast and sustained in dogs with atopic dermatitis and
26 in human patients with psoriasis. The inhibitory profiles of these molecules might be more
27 related to their direct inhibition of TRPV1 rather than that of JAKs.

28

29 **Key words:**

30 Itch, Janus-kinase inhibitor, Mice, Oclacitinib, Tofacitinib, TRPV1, Itch, Mice

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To the Editor:

Janus-kinase (JAK) inhibitors are being proposed for the treatment of cancer and inflammatory diseases, such as atopic dermatitis (AD). Their mechanism of action, especially that to reduce itch, remains speculative. The JAK inhibitor oclacitinib is currently approved for the treatment of lesions and pruritus in dogs with AD^{1,2} while tofacitinib is under clinical development for the treatment of the homologous human disease (<https://www.clinicaltrials.gov/ct2/show/NCT02001181>). In rodent and canine models of allergic dermatitis and in human patients with psoriasis and atopic dermatitis, the antipruritic effect of JAK inhibitors is rapidly visible¹⁻³. We suspect that the proposed mechanism of action on pruritus (i.e. a reduction of signal transduction after binding of pruritogenic cytokines, such as IL-31, to their receptors) might not be the only explanation for the fast onset and sustained antipruritic action of JAK inhibitors. To identify additional mechanisms of action of the JAK inhibitors oclacitinib and tofacitinib, we used both *in vitro* (dorsal root ganglia [DRG] neuronal culture) and *in vivo* approaches in itch mouse models that are known to be dependent and independent of JAK signaling pathways. In this study, we observed that the JAK inhibitors oclacitinib and tofacitinib exhibit broad anti-itch properties that appear linked to their inhibition of TRPV1, the transient receptor potential cation channel subfamily V member 1; this receptor is known to play an important role in the transmission of both pain and itch⁴. To illustrate the involvement of TRPV1 in the anti-itch properties of JAK inhibitors, we used three different approaches: first, *in vitro* calcium imaging in DRG neurons; secondly, *in vivo* methods with a mouse model of itch and one of capsaicin-induced pain, and finally, we used TRPV1-transfected AD293 cells.

We used murine primary sensory neurons and compared the responses evoked by several types of pruritogens that are dependent and independent of JAK signaling pathways. We examined the activation of small-diameter DRG neurons (mostly TRPV1-expressing ones) with or without exposure to oclacitinib or tofacitinib using Fura-2 ratiometric Ca²⁺ imaging. *In vitro* treatment with these two JAK inhibitors resulted in a significant reduction in the response of DRG neurons to the following stimuli: IL-31, TNF α , histamine, compound 48/80, chloroquine, protease-activated receptor-2 (PAR-2) activating peptide (SLIGRL-NH₂), capsaicin and a low pH solution (Fig 1, A). However, the response of DRG neurons to serotonin (5-HT) and allyl isothiocyanate (AITC) was not significantly changed as compared to vehicle-only control group after treatment with these JAK inhibitors (Fig 1, A and B).

To examine if the JAK inhibitors also reduced the itch response *in vivo*, we induced scratching responses in mice with all the compounds previously tested on the primary DRG sensory neurons. We confirmed *in vivo* a similar inhibitory pattern after oral administration of the JAK inhibitors compared to the vehicle control group, whereas there was no change in itch behavior following 5-HT (Fig 1, C). It has been shown above that these two JAK inhibitors reduced the itch induced by JAK-dependent cytokines involved in sensory perception, like IL-31³. However, our results indicated a broader action than previously demonstrated with the reduction of itch induced by JAK-independent compounds that included TNF α , histamine, compound 48/80, chloroquine, and SLIGRL-NH₂. Similarly, we also observed a reduction of pain (manifested by wiping behavior) induced by capsaicin, the latter being a specific agonist for TRPV1. In support of this proposed novel mechanism of JAK inhibitors a strikingly similar pattern of reduced scratching behavior to histamine, compound 48/80, and PAR-2 has similarly been shown by a TRPV1 antagonist⁵. Chloroquine interacts not only with TRPV1, but also with the other TRP ion channels⁶. Additionally, IL-31-induced itch is known to be dependent on both TRPV1- and TRPA1-expressing neurons⁷. Interestingly, we did not see any effect of the tested JAK inhibitors on TRPA1-associated sensory perception induced by AITC and 5-HT (Fig 1, A and C)⁸. Following these observations, we hypothesized that the broad antipruritic effect of oclacitinib and tofacitinib could be related to their direct inhibition of TRPV1, rather than a mere JAK inhibition.

To confirm the direct association between TRPV1 and JAK inhibitors, we examined the *in vitro* and *in vivo* responses to IL-31, TNF α , histamine, compound 48/80, chloroquine, SLIGRL-NH₂, and capsaicin in TRPV1-knockout mice. Our inhibitory results are similar to those previously published⁵⁻⁷, and the inhibition pattern mimics that of the JAK inhibitors (Fig 2, A and B).

To test the effects of the JAK-inhibitors in a more clinically-relevant chronic itch setting, we used the toluene-2,4,-diisocyanate (TDI) induced mouse model of chronic allergic itch. First, we observed a significant increase in the expression of TRPV1-receptor positive neurons in the DRG neurons of chronically challenged mice (Fig 2, C and D). Second, TDI-challenged mice topically treated with tofacitinib during the challenge phase showed a significant decrease in itch behavior (Fig 2, E).

To further demonstrate a direct interaction of JAK inhibitors with TRPV1, we performed Ca²⁺ imaging on TRPV1-transfected AD293 cells and studied the effects of oclacitinib and tofacitinib on capsaicin-induced calcium influx. The two JAK inhibitors significantly reduced the

response in TRPV1-transfected cells to capsaicin stimulation (Supplemental Fig 1, A). Finally, we conducted matrix-assisted laser desorption/ionization (MALDI)-ultra-high resolution mass spectrometry (MS) experiments on TRPV1 immunoprecipitates isolated from the TRPV1-transfected AD293 cells (Supplemental Fig 1, B). We could detect higher concentrations of JAK inhibitors in TRPV1-transfected AD293 cells compared to vehicle-treated as well as mock-transfected AD293 cells (Supplemental Fig 1, C and D) although there were still some traces of JAK inhibitors in mock-transfected gel (Supplemental Fig 1, C). As a small Ca^{2+} influx to higher concentration of capsaicin was also found in mock-transfected cells, a low level of TRPV1 expression was likely present in wild type cells. Future studies will include radio-ligand experiments to demonstrate a direct binding to TRPV1.

The inhibition of itch by JAK inhibitors has been reported to be both fast and sustained in dogs and human patients with psoriasis^{1,2,9}. However, this is difficult to explain by just the reduction of itch signaling by a few cytokines, like IL-31. This direct inhibitory action on TRPV1 is likely the missing link, explaining the broad inhibitory action of JAK inhibitors oclacitinib and tofacitinib.

In summary, our results indicate a much more comprehensive relief of itch by JAK inhibitors which is mediated through TRPV1-receptors and not just by inhibiting the signal transduction of itch-inducing cytokines. In addition to itch inhibition, the reduced response to capsaicin by JAK inhibitors indicates an extension of possible benefit in attenuating pain transduction via TRPV1, as already documented in patients with rheumatoid arthritis. Our study provides a novel mechanism for the anti-pruritic role of JAK inhibitors through TRPV1-receptor channels.

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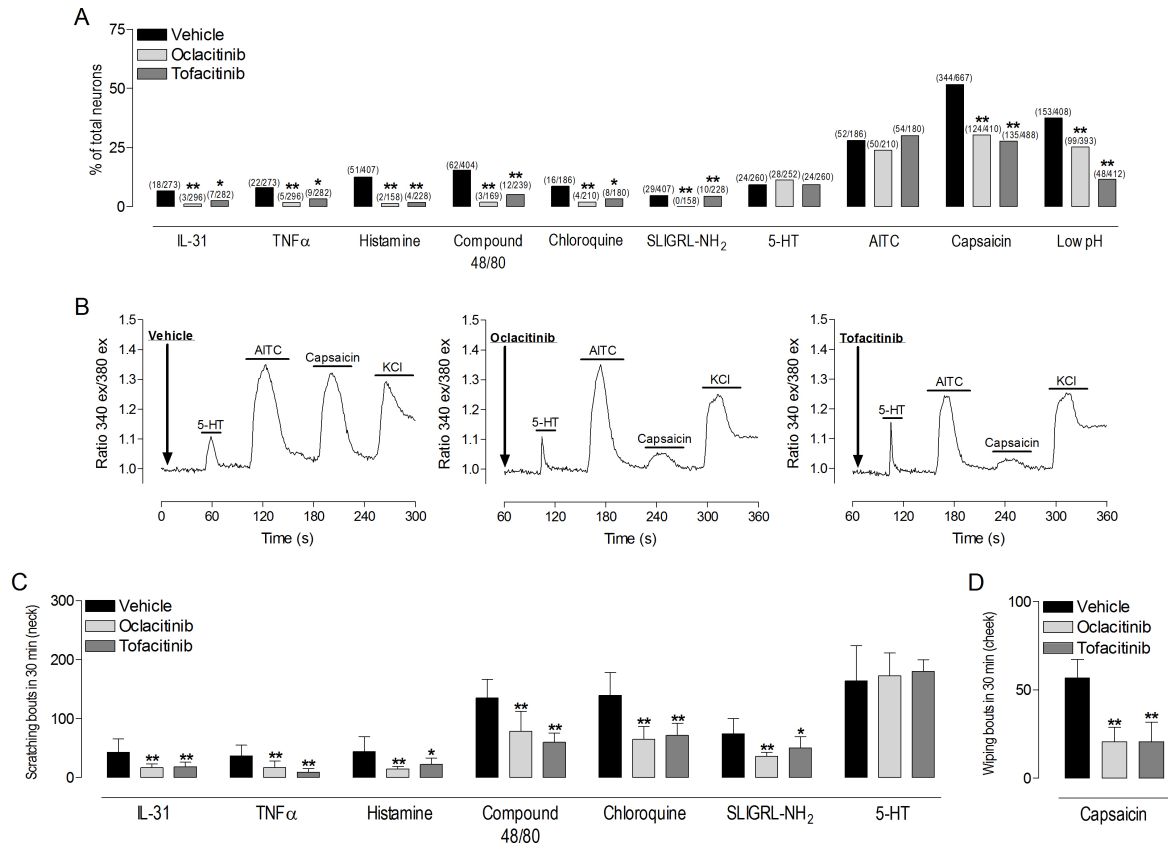
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Legends for Figures

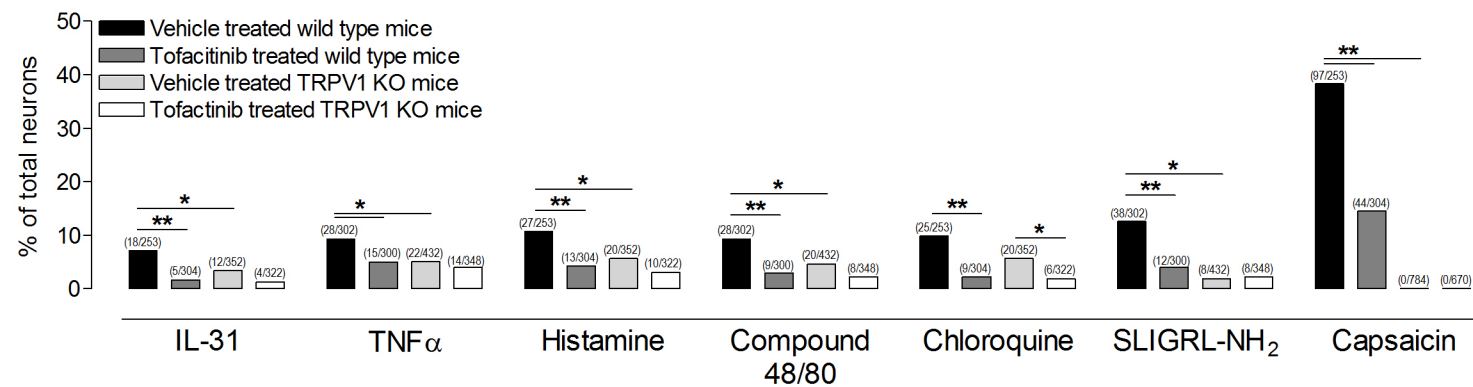
FIG 1. Inhibitory effect of oclacitinib and tofacitinib on pruritogen-induced Ca^{2+} increase in mouse sensory neurons and pruritogen-evoked scratching/wiping behavior in mice. **A** and **B**, Percentage of several pruritogen-induced responses in mouse DRG neurons pre-treated *in vitro* with vehicle-only, oclacitinib or tofacitinib for 1 min. Numbers of neurons tested are given in parentheses. *: $P < 0.05$ and **: $P < 0.01$ (Fisher exact test) vs. vehicle-only group. **B**, Example recordings show cytosolic Ca^{2+} increase (Ratio 340ex/380ex) for a single region of interest (ROI) taken every 100ms. **C**, Scratching or wiping (capsaicin) response after oral administration of JAK-inhibitors to several pruritogens in mice. Results are expressed as mean $\pm$ 1 S.D. ($n = 8$ per group). *: $P < 0.05$ and **: $P < 0.01$ (Dunnett's multiple comparison test) vs. vehicle-only control group.

FIG 2. Inhibitory effect of tofacitinib on pruritogen-induced Ca^{2+} increase in TRPV1 KO mouse sensory neurons, pruritogen-evoked scratching/wiping behavior in TRPV1 KO mouse and TDI-induced chronic itch in BALB/c mouse. **A**, Percentage of several pruritogen-induced responses in TRPV1 KO mouse DRG neurons pre-treated *in vitro* with vehicle-only or tofacitinib for 1 min. Numbers of neurons tested are given in parentheses. *: $P < 0.05$ and **: $P < 0.01$ (Fisher exact test) vs. vehicle-only group. **B**, Scratching or wiping (capsaicin) response after oral

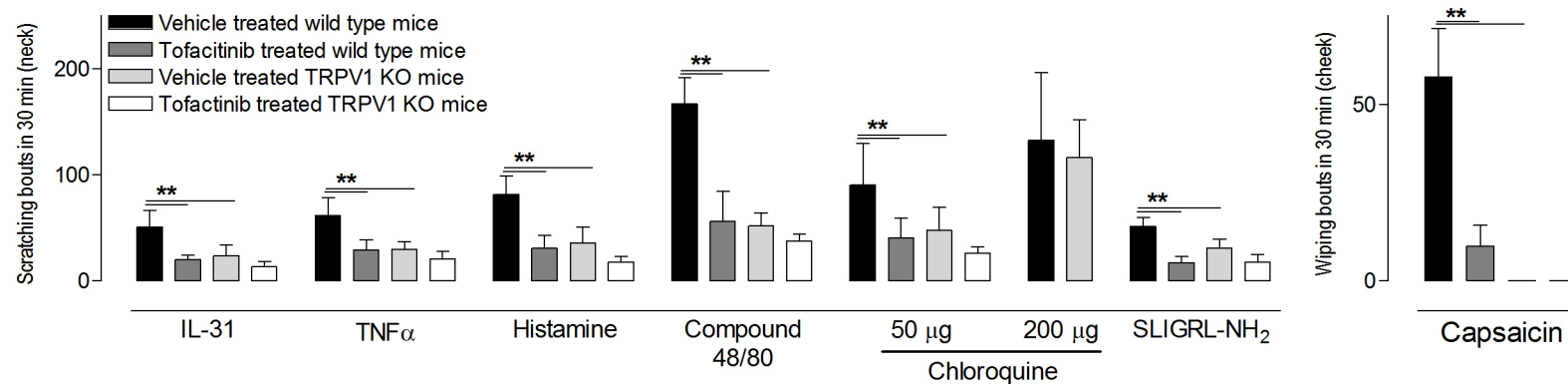
administration of tofacitinib to several pruritogens in TRPV1 KO mice. Results are expressed as mean $\pm$ 1 S.D. (n = 6 per group). *: P < 0.05 and **: P < 0.01 (Student's *t*-test) vs. vehicle-only control group. **C**, Immunohistochemistry of DRG from TDI-induced chronic itch model demonstrates increased number of cells positive for TRPV1-receptors. **D**, Expression of TRPV1-receptor positive neurons in the DRG from TDI-induced chronic itch mice. Results are expressed as mean $\pm$ 1 S.D. (n = 6 per group). *: P < 0.05 (Student's *t*-test) vs. control group. **E**, Scratching response after topical application of tofacitinib during the challenge phase of TDI-induced chronic allergic dermatitis. Results are expressed as mean $\pm$ 1 S.D. (n = 6 per group). **: P < 0.01 (Student *t*-test) vs. vehicle-only control group.



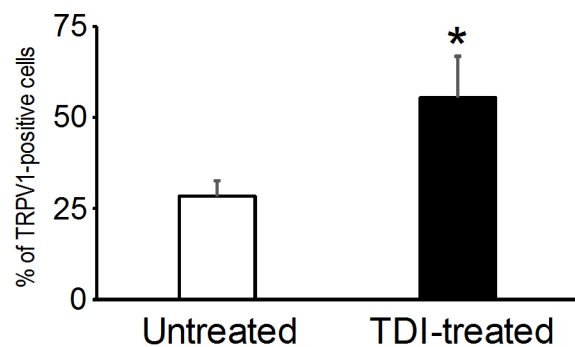
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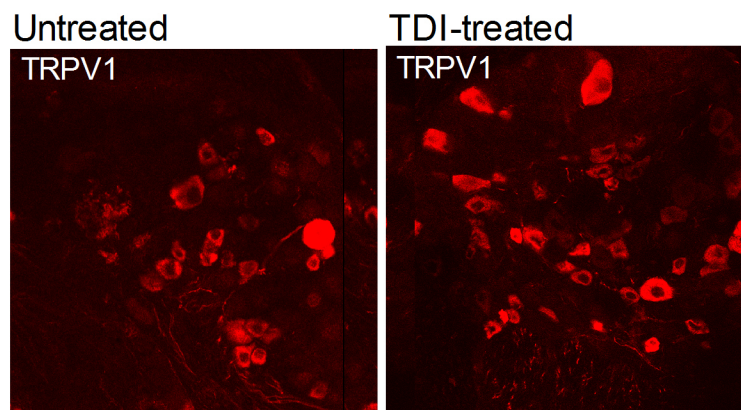
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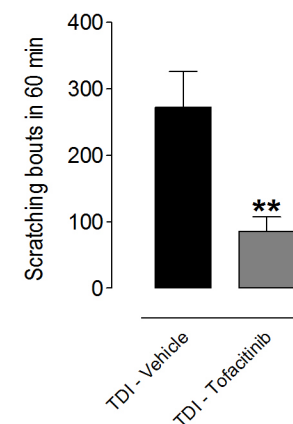
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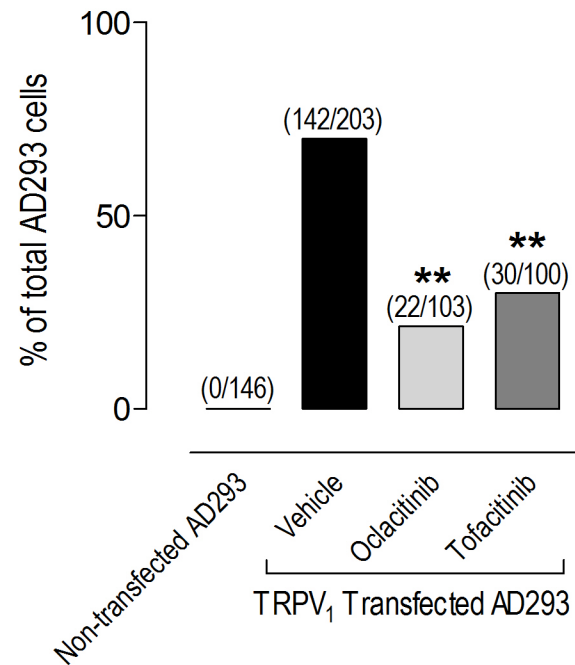
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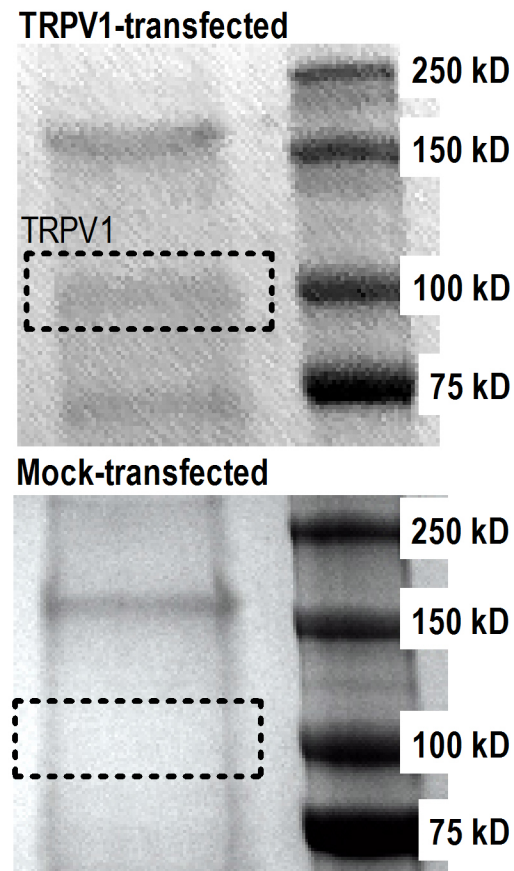
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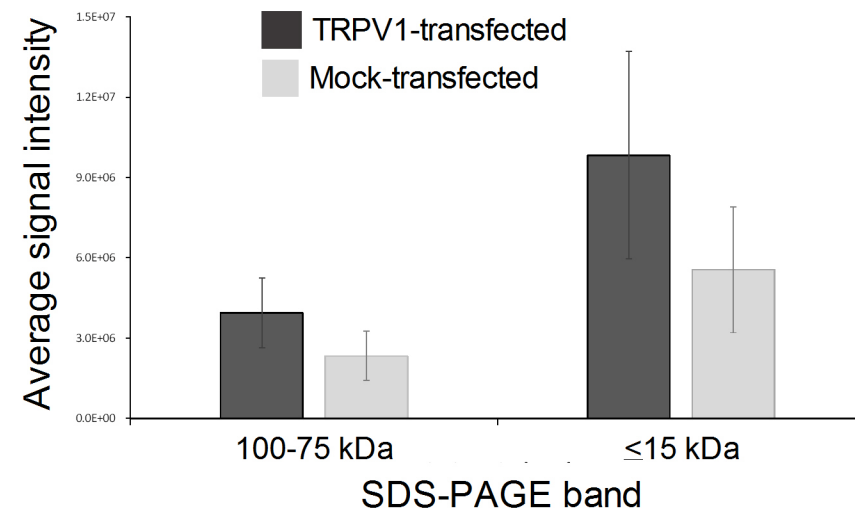
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