

Topically Administered Janus-Kinase Inhibitors Tofacitinib and Oclacitinib Display Impressive Antipruritic and Anti-Inflammatory Responses in a Model of Allergic Dermatitis

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ABSTRACT

The prevalence of allergic skin disorders has increased rapidly, and development of therapeutic agents to alleviate the symptoms are still needed. In this study, we orally or topically administered the Janus kinase (JAK) inhibitors, tofacitinib and oclacitinib, in a mouse model of dermatitis, and compared the efficacy to reduce the itch and inflammatory response. In vitro effects of JAK inhibitors on bone marrow–derived dendritic cells (BMDCs) were analyzed. For the allergic dermatitis model, female BALB/c mice were sensitized and challenged with toluene-2,4-diisocyanate (TDI). Each JAK inhibitor was orally or topically applied 30 minutes before and 4 hours after TDI challenge. After scratching bouts and ear thickness were measured, cytokines were determined in challenged skin and the cells of the draining lymph node were analyzed by means of flow cytometry. In vitro, both JAK inhibitors

significantly inhibited cytokine production, migration, and maturation of BMDCs. Mice treated orally with JAK inhibitors showed a significant decrease in scratching behavior; however, ear thickness was not significantly reduced. In contrast, both scratching behavior and ear thickness in the topical treatment group were significantly reduced compared with the vehicle treatment group. However, cytokine production was differentially regulated by the JAK inhibitors, with some cytokines being significantly decreased and some being significantly increased. In conclusion, oral treatment with JAK inhibitors reduced itch behavior dramatically but had only little effect on the inflammatory response, whereas topical treatment improved both itch and inflammatory response. Although the JAK-inhibitory profile differs between both JAK inhibitors in vitro as well as in vivo, the effects have been comparable.

Introduction

The prevalence of allergic skin disorders, including atopic dermatitis, allergic contact dermatitis (ACD), and urticaria, has increased rapidly, and development of therapeutic agents to alleviate the symptoms is still needed. According to recent guidelines for the treatment of these allergic skin disorders, therapies focus primarily on reduction of inflammation and symptomatic relief of itch (Ring et al., 2012). To improve and control these symptoms, various pathogenic reactions have to be considered in an individual approach regarding the abnormal reactivity patterns found in the individual patient suffering from allergic skin disorders (Aslam et al., 2014). Nonetheless, there are still limited options and classic topical corticosteroids remain the primary foundation of topical treatment, with topical calcineurin inhibitors preferred for treatment of the face and intertriginous areas (Ring et al., 2012). To open up the option of therapeutic agents for allergic skin disorders, we focused on the Janus kinase (JAK) inhibitors,

which have been recently developed for rheumatoid arthritis and pruritic inflammatory skin diseases (Cosgrove et al., 2013a,b; Fujii and Sengoku, 2013; Ports et al., 2013; Gonzales et al., 2014).

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, and tyrosine kinase 2) and seven signal transducers and activators of transcription (STATs; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) are used by more than 50 cytokines and growth factors to mediate intracellular signaling (Villarino et al., 2015). In particular, proinflammatory cytokines that use the JAK pathway, such as interferon- γ and interleukin (IL)-2, IL-4, IL-6, IL-13, IL-21, and IL-23, have been implicated in inflammatory disease (O'Shea et al., 2004). In addition, T_H2-derived cytokines, including IL-31 and thymic stromal lymphopoietin (TSLP), are ligands for murine and human sensory nerves and have a critical function to evoke itch (Cevikbas et al., 2014). Because these cytokines also interact with JAK, several JAK inhibitors have received a lot of attention recently as a therapeutic agent for major pruritic inflammatory skin diseases (Cosgrove et al., 2013a,b; Fujii and Sengoku, 2013; Ports et al., 2013; Gonzales et al., 2014). However, the exact mechanisms of the anti-inflammatory and

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ABBREVIATIONS: ACD, allergic contact dermatitis; BMDC, bone marrow–derived dendritic cell; Con A, concanavalin A; DC, dendritic cell; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; IL, interleukin; IP-10, interferon- γ inducible protein 10; JAK, Janus kinase; LN, lymph node; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription; TARC, thymus and activation-regulated chemokine; TDI, toluene-2,4-diisocyanate; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

anti-itch action of these JAK inhibitors are still not fully understood. Therefore, the primary objective of the study reported here was to elucidate the anti-inflammatory potential of JAK inhibitors using bone marrow-derived dendritic cells (BMDCs) and a mouse model of ACD.

In this study, we focused on the two different types of JAK inhibitors, tofacitinib and oclacitinib, which have been approved in the United States and the European Union. Both differ in their JAK-inhibitory profile. Tofacitinib is currently in development as an oral formulation for the treatment of several inflammatory diseases, including psoriasis (Meyer et al., 2010). In a cellular setting where JAKs signal in pairs, tofacitinib preferentially inhibits signaling by heterodimers containing JAK3 and/or JAK1 with functional selectivity over receptors that signal via pairs of JAK2 (Meyer et al., 2010). Oclacitinib, currently licensed for the control of pruritus associated with allergic dermatitis in dogs (Gonzales et al., 2014), mainly displays activity against JAK1-dependent cytokines and shows minimal activity against JAK2-dependent cytokines in cellular assays (Gonzales et al., 2014).

Because dendritic cells (DCs) are crucial players in allergic diseases, by controlling the extent and quality of response to allergens (Steinman, 2007), we first elucidated whether the different JAK-inhibitory profiles of oclacitinib and tofacitinib have an impact on DC activation and migration. Although topical treatment is a frequently used method to improve the inflammatory symptoms in major pruritic inflammatory skin diseases, there are only reports with oral formulation products of these two JAK inhibitors thus far. In the current study, we orally or topically administered tofacitinib and oclacitinib during the elicitation phase of the toluene-2,4-diisocyanate (TDI)-induced ACD, and compared systemic and local effects by comparing the anti-itch and anti-inflammatory responses.

Materials and Methods

Reagents. Tofacitinib [3-(3*R*,4*R*)-4-methyl-3-[methyl(7*H*-pyrrolo [2,3-*day*]pyrimidin-4-yl)amino]piperidin-1-yl]-3-oxopropanenitrile) was purchased from Tocris (Minneapolis, MN), and oclacitinib (*N*-methyl-1-[4-[methyl(7*H*-pyrrolo[2,3-*day*]pyrimidin-4-yl)amino]cyclohexyl]methanesulfonamide) was purchased from Adooq Bioscience (Irvine, CA). Concanavalin A (Con A), lipopolysaccharide (LPS) (O127:B8), TDI, acetone, and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO). Pefabloc was purchased from Roche (Basel, Switzerland). Ammonium thiocyanate, phosphate-buffered saline (PBS), methylcellulose, and Tween 20 were ordered from Thermo Fisher Scientific (Waltham, MA). RPMI 1640 medium was purchased from Mediatech (Manassas, VA). Purified rat anti-mouse CD16/CD32 (mouse BD Fc block), anti-mouse CD86 (rat IgG2_{ak}, fluorescein isothiocyanate conjugated, clone GL1), and major histocompatibility complex (MHC) class II (I-A/I-E, rat IgG2_{bk}, PerCP-Cy5.5-conjugated, M5/114) and I-A/I-E (rat IgG2_{ak}, Biotin-conjugated, clone 2G9) were ordered from Becton, Dickinson and Company (Franklin Lakes, NJ). Anti-mouse CD3 (rat IgG2_{bk}, fluorescein isothiocyanate conjugated, clone 17A2), CD11c (hamster IgG, allophycocyanin conjugated, N418), CD19 (rat IgG2_{ak}, phycoerythrin conjugated, clone 6D5), CD40 (rat IgG2_{ak}, phycoerythrin conjugated, clone NLDC-145), and CD207 (Langerin, rat IgG2_{ak}, fluorescein isothiocyanate conjugated, clone caa828H10) were ordered from Miltenyi Biotec (Auburn, CA). Cy3 streptavidin was purchased from Biolegend (San Diego, CA). The DC protein assay kit was purchased from BIO-RAD (Richmond, CA). Recombinant mouse granulocyte macrophage colony-stimulating factor and CCL19/MIP-3 β , enzyme-linked immunosorbent assays (ELISAs) for IL-1 β , -4, -6, -12, tumor necrosis factor (TNF)- α , TSLP, CXCL10/interferon- γ inducible

protein 10 (IP-10)/CRG-2, and CCL17/thymus and activation-regulated chemokine (TARC) were purchased from R&D systems (Minneapolis, MN). ELISAs for IL-31 were purchased from eBioscience, Inc. (San Diego, CA).

Animals. BALB/cAnN (female, 6 weeks old) were purchased from Charles River Laboratories (Raleigh, NC) and housed in groups of four mice per cage under controlled lighting (a 12-hour light/dark cycle), temperature ($22 \pm 3^\circ\text{C}$), humidity ($55\% \pm 15\%$), and ventilation (at least 10 complete fresh-air changes/hour). Standard rodent chow and water were available ad libitum. All aspects of the current study were conducted in accordance with the Animal Care and Use Program of the North Carolina State University (IACUC Protocol No. 13-111-B).

Preparation of BMDCs. BMDCs were generated from bone marrow cells of female BALB/cAnN mice, as described previously (Lutz et al., 1999; Bäumer et al., 2003), with minor modifications. Bone marrow was flushed from femurs of the hind limbs with PBS and taken into BMDC medium (RPMI 1640 with L-glutamine, 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, and 20 ng/ml granulocyte macrophage colony-stimulating factor). On day 3, another 10 ml of BMDC medium was added. At day 6, 10 ml of BMDC medium was replaced. At day 8, nonadherent and loosely adherent cells were collected by means of gentle pipetting and centrifugation at 290g for 10 minutes at 20°C . The numbers of viable cells were determined with a Cellometer (Nexcelom Bioscience, Laurence, MA) using acridine orange and propidium iodide staining.

JAK-Inhibitor Exposure to BMDCs. A short-term exposure was performed in mature 8-day-old BMDCs. The BMDCs (2.5×10^5 cells/ml) were exposed to 0.1% dimethylsulfoxide (DMSO) (vehicle) and each JAK inhibitor (at 0.1, 1, or 10 μM) in BMDC medium containing LPS (25 ng/ml) for 24 hours. For the long-term exposure, immature 3-day-old BMDCs were exposed to the same concentrations as during short-term exposure in the presence of LPS for 6 days. The selected concentrations were adapted from previous reports (Heine et al., 2013; Kubo et al., 2014). The high concentration (10 μM) did not induce any cell toxicity, and particularly by topical administration these concentrations are likely to be achieved in skin. Additionally, we could not find any responses when cells were incubated with less than 0.1 μM concentrations. After exposure, cytokine production (only in the short-term exposure, IL-12 and TNF α), migration, and phenotypes (MHC class II and CD86) in BMDCs were evaluated using the ELISA, chemotaxis assay, and fluorescence-activated cell sorter (FACS), respectively. Two or three independent experiments were performed in DCs from different animals.

Chemotaxis Assay. Chemotaxis of BMDCs in response to CCL19/MIP-3 β was measured in 24-well plates carrying Transwell Permeable Supports with an 8 μm pore-size polycarbonate membrane (Corning, NY). The upper chamber was loaded with 2.5×10^5 cells in 200 μl BMDC buffer. The lower chamber was filled with 600 μl BMDC buffer containing 50 ng/ml CCL19/MIP-3 β . After 90 minutes of incubation at 37°C , the cells that had migrated to the lower chamber were counted with the Cellometer (Nexcelom Bioscience) using acridine orange and propidium iodide staining.

Skin DC Migration Assay. Mice ears were treated with 20 μl of each JAK inhibitor (0.1% in acetone/DMSO 7:1) or with the vehicle 15 hours and 30 minutes before dissection of the mice (six mice for each group). The ears were rinsed in 75% ethanol and air dried for 10 minutes. The cartilage-free dorsal halves of split mouse ear skin were cultured in 12-well size plates based on the method used in Bäumer et al. (2003). The halves were floated epidermal side up in 1.3 ml BMDC buffer containing 50 ng/ml CCL19/MIP-3 β . The halves were changed daily to new wells and new media (including CCL19/MIP-3 β). Migrated total cells as well as DCs (CD11c⁺ CD40⁺ cells) from each ear (days 1–3) were pooled and counted with the Cellometer (Nexcelom Bioscience) and FACS. The viability of the cells was assessed by acridine orange and propidium iodide staining. At the end of the migration assay (day 3) the ear halves were weighed.

Preparation of Epidermal Sheets for Immunohistochemistry. The preparation and evaluation of the epidermal sheets were performed according to Ratzinger et al. (2002). In short, skin was floated on 0.5 M ammonium thiocyanate for 15 minutes at 37°C. The Langerhans cells were detected with the biotinylated MHC class II antibody in a 1:200 dilution. Labeling of the antibodies was visualized by using a Cy3 streptavidin in a 1:4000 dilution using a conventional streptavidine-biotin technique. The specificity for Langerhans cells was checked with a second anti-Langerin staining in some samples. The density of Langerhans cells was counted under the

microscope using 40 magnifications and a calibrated grid. More than 10 randomly chosen areas per ear were analyzed. Six JAK inhibitor-treated ears (only 0.1% in acetone/DMSO 7:1) and six vehicle-treated ears were analyzed.

TDI-Induced Allergic Dermatitis Model in Mice. The TDI-induced and challenged BALB/c mouse was established as an ACD model, which has already been used to evaluate novel therapeutic targets in several previous reports (Bäumer et al., 2003, 2004; Reines et al., 2009; Rossbach et al., 2009). Following a 2-week acclimation period, the abdominal region of each animal was depilated with

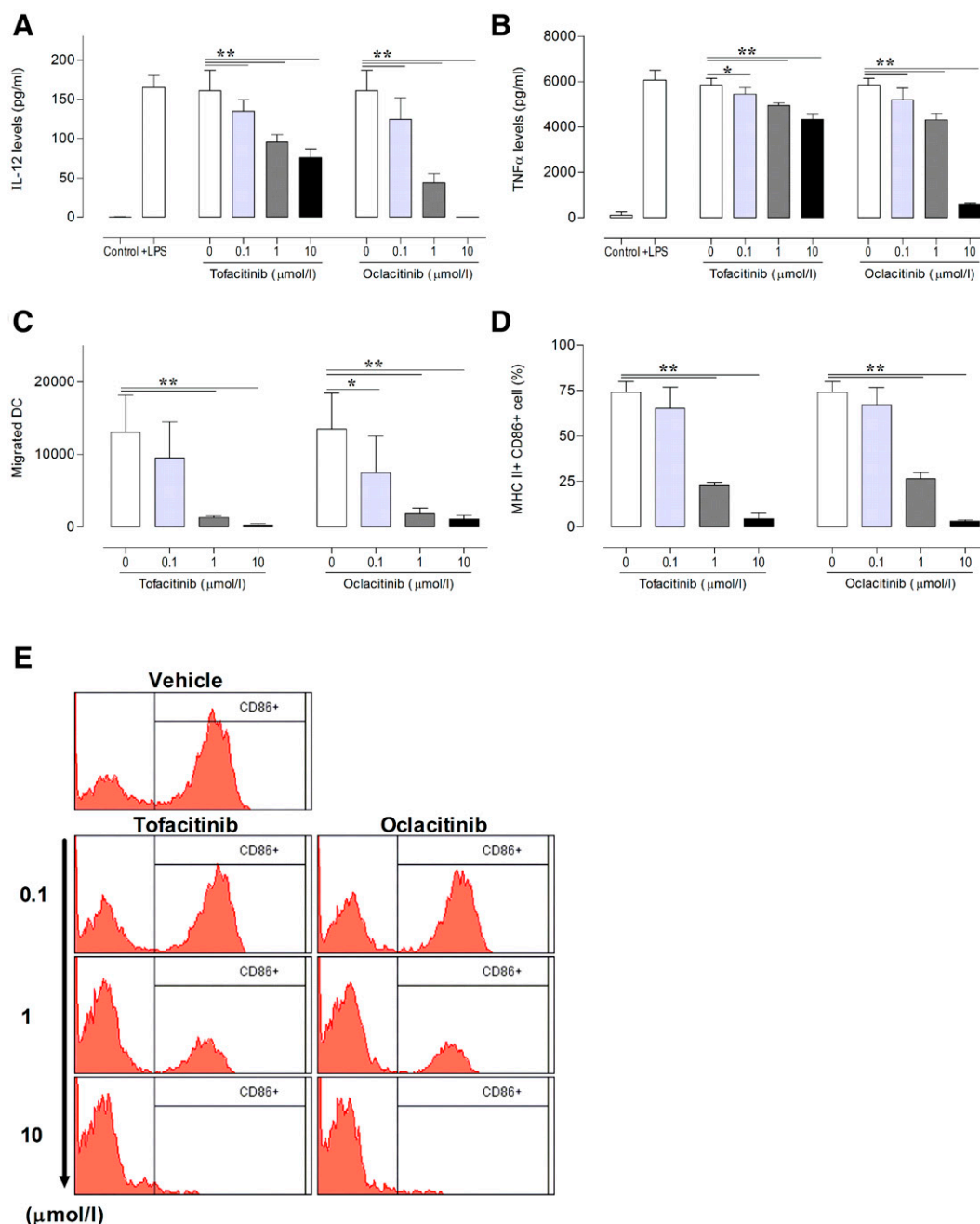


Fig. 1. Effect of BMDC functions by JAK-inhibitor exposure (tofacitinib and oclacitinib). (A and B) Suppression of LPS-induced production of IL-12 (left) and TNFα (right) by short-term JAK-inhibitor exposure. Results are expressed as mean ± 1 S.D. (pg/ml; *n* = 7 per group). **P* < 0.05 and ***P* < 0.01 (Dunnnett's multiple comparisons test) versus vehicle-only control group. (C) Reduced transmigration of BMDCs by long-term JAK-inhibitor exposure. Results are expressed as mean ± 1 S.D. (pg/ml; *n* = 9 per group). **P* < 0.05 and ***P* < 0.01 (Dunnnett's multiple comparisons test) versus vehicle-only control group. (D) Suppression of LPS-induced expression of costimulatory molecules by long-term JAK-inhibitor exposure. BMDCs were stained with anti-CD86 and -MHC class II (I-A/I-E) antibodies. Populations of the MHC class II⁺CD86⁺ cells are expressed as mean ± 1 S.D. (pg/ml; *n* = 8 per group). ***P* < 0.01 (Dunnnett's multiple comparisons test) versus vehicle-only control group. (E) Representative histograms of BMDCs from long-term JAK-inhibitor exposure.

TABLE 1

Effect of topical application of JAK inhibitors on DC migration and Langerhans cell density in BALB/c mice ear skin

Results are expressed as mean $\pm$ 1 S.D. ($n = 6$ per group).

Group	Total Migrated Cells	Migrated CD11c ⁺ CD40 ⁺ Cells	Langerhans Cell Density
	<i>cells/mg ear tissue</i>		<i>cells/mm⁻²</i>
Vehicle only	3789 $\pm$ 875	2184 $\pm$ 437	1151 $\pm$ 252
Tofacitinib 0.1%	2534 $\pm$ 741*	1352 $\pm$ 435*	1969 $\pm$ 456**
Oclacitinib 0.1%	2333 $\pm$ 714*	1494 $\pm$ 454*	2013 $\pm$ 178**

* $P < 0.05$; ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle-only control group.

a depilatory cream. On the day after depilation, the abdominal skin was stripped 10 times with adhesive tapes. Just after the tape stripping, 100 μ l of 5% TDI in acetone was applied to the stripped epidermis (day 1). On days 2 and 3, 50 μ l of 5% TDI in acetone was applied to the same site without tape stripping. The allergic reaction was boosted 21 days later by application of 50 μ l of 0.5% TDI in acetone onto the shaved abdomen (day 25). For measuring the scratching behavior, the allergic reaction was challenged 7 days later by application of 30 μ l of 0.5% TDI in acetone onto the shaved dorsal region (neck, day 32). Additionally, the allergic reaction was challenged by application of 20 μ l of 0.5% TDI in acetone onto the mouse ears for measuring the ear swelling. The swelling was calculated by comparison of the ear thickness (cutimeter; Mitutoyo, Neuss, Germany) before and 24 hours after challenge. The JAK inhibitors (tofacitinib or oclacitinib) were administered orally or topically 30 minutes before and 4 hours after TDI challenge because the absorption of tofacitinib and oclacitinib was rapid, with plasma concentrations for both tofacitinib and oclacitinib peaking at around 1 hour after oral or intravenous administration. Tofacitinib and oclacitinib both have a short half-life of 2 and 4 hours after administration, respectively (Collard et al., 2014; Dowty et al., 2014). Each drug was diluted in a 0.5% methylcellulose/0.25% Tween 20 solution for oral administration, and a 7:1 acetone:DMSO solution for topical application to concentrations described subsequently. For each drug, a vehicle-only control group and low- and high-dose groups were set. Oral doses were as follows: tofacitinib, 10 and 30 mg/kg; and oclacitinib, 30 and 45 mg/kg. Topically administered doses were 0.1, 0.25, and 0.5% for both chemicals. The oral doses of tofacitinib and oclacitinib used in this study were selected based on previously published studies (Kudlacz et al., 2004; Yew-Booth et al., 2012). The topical doses used in this study were selected to avoid systemic toxicity or excessive local irritation (assessed in pilot experiments). Just after measuring ear thickness, mice were anesthetized and sacrificed. The ear auricle was removed from each mouse and stored until they were used for histology and cytokine determination. The auricular lymph node (LN) removed from each mouse was weighed and single-cell suspensions were prepared from the LNs by passage through a sterile 70 μ m nylon cell strainer in 1 ml RPMI 1640 supplemented with 5% fetal calf serum. Cell count was determined using the Cellometer (Nexcelom Bioscience) and acridine orange and propidium iodide staining. Single-cell suspensions were used to analyze cytokine production and for FACS analysis.

Histology. Samples from ear skin were collected and fixed in 4% paraformaldehyde solution. The samples were sectioned and stained with H&E and evaluated in a blinded manner with respect to cell influx and edema by semiquantitative examination (0, no influx, no edema; 1, mild; 2, moderate; and 3, severe influx, severe edema).

Cytokine Determination of Ear Skin. One part of the ear tissue was shock-frozen in liquid nitrogen and stored at -80°C until use. Cytokine determination for ear tissue was performed according to Bäumer et al. (2004). Briefly, mice ears were homogenized under liquid nitrogen, and the homogenates were taken in 200 μ l RPMI 1640 medium containing 1 mM Pefabloc. The samples were mixed intensively and stored for 30 minutes on ice. After centrifugation at 3000g for 10 minutes at 4°C , the supernatants were collected and the protein content was determined with the DC protein assay kit. IL-1 β , -4, -6, -12, -31, TNF α , TSLP, IP-10, and TARC were measured by ELISA.

Cytokine Determination of Rostral Neck Skin. To examine pruritogen-evoked cytokine profiles in the affected skin, in a second setting the rostral neck skin was isolated from each mouse 1 hour after each JAK-inhibitor treatment (only 0.1% in acetone/DMSO 7:1) and 30 minutes after TDI challenge. Skin tissue purification and cytokine determination were performed as mentioned in the section on cytokine determination of ear skin. IL-31, TNF α , and TSLP were measured by ELISA.

Cytokine Determination of LNs. To stimulate T-cell receptor signaling, we cultured single-cell suspensions recovered from LNs (5×10^5 cells/well) for 24 or 96 hours with Con A (5 μ g/ml) in 48-well plates at 37°C in a 5% CO_2 atmosphere. The levels of cytokines (IL-4, -6,

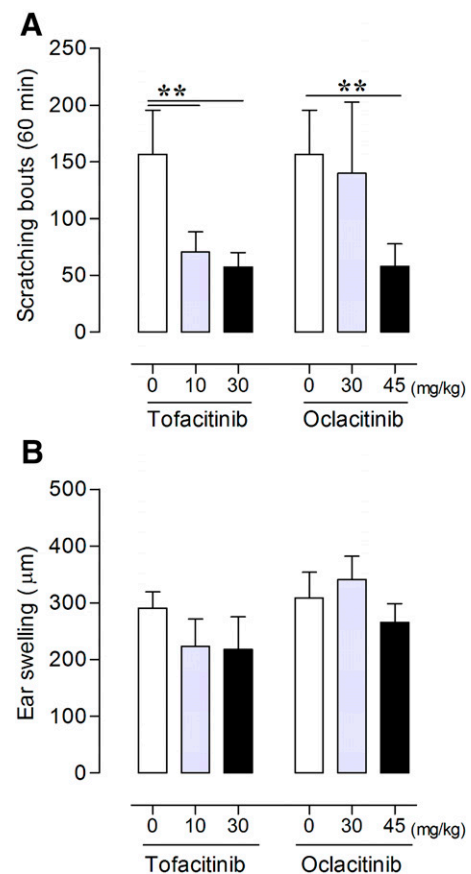


Fig. 2. Scratching and ear swelling effects of oral administration of JAK inhibitors on TDI-induced allergic dermatitis. (A) Scratching behavior was induced 30 minutes after the administration of each JAK inhibitor and then evaluated for 1 hour. Results are expressed as mean $\pm$ 1 S.D. ($n = 7$ per group). ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle-only control group. (B) Ear swelling was calculated by a comparison of the ear thickness before and 24 hours after TDI challenge. Each JAK inhibitor was administered orally 30 minutes before and 4 hours after TDI challenge. Results are expressed as the mean $\pm$ 1 S.D. (μ m, $n = 7$ per group).

TNF α , and TARC) in cell culture medium were measured by using an ELISA.

Flow Cytometric Analysis of DCs and LNs. To avoid non-specific binding, 5×10^5 cells were incubated with 1 μ g mouse BD Fc block (Becton, Dickinson and Company) for 5 minutes at 4°C, followed by incubation with the monoclonal antibodies for 30 minutes at 4°C in the dark. Cells were washed with 5% fetal calf serum in PBS, resuspended at 5×10^5 cells per tube in 500 μ l PBS, and analyzed on a LSR II flow cytometer by using FACSDiva software (Becton, Dickinson and Company). For each sample, 10,000 events were collected and analyzed for expression of antigens.

ELISA for Cytokine. All cytokine levels were measured using an ELISA according to the manufacturer's protocol. The optical density at 450 nm was read using a microplate reader (Sunrise; Tecan US, Morrisville, NC).

Statistical Analysis. Statistical significance of the difference between the vehicle control and treated groups was estimated at the 5% and 1% levels of probability. Data from the vehicle-only control and the tofacitinib- and oclacitinib-treated groups were evaluated by Bartlett's test for equality of variance. When group variances were homogeneous, a parametric one-way analysis of variance was conducted to determine statistical differences among groups. When the analysis of variance was significant, Dunnett's multiple comparisons test was applied. When group variances were heterogeneous, data were evaluated by Kruskal-Wallis nonparametric analysis of variance. When differences were significant, Dunnett's mean rank-sum test was applied. Data are expressed as mean $\pm$ 1 S.D. The data were analyzed using Prism 4 (GraphPad Software, San Diego, CA).

Results

Inhibitory Effect of Tofacitinib and Oclacitinib on Cytokine Production of LPS-Stimulated BMDCs. To assess whether exposure to each JAK inhibitor affects allergic disease in vitro, we focused on murine BMDCs and measured LPS-induced cytokine production (IL-12 and TNF α). Cytokines are critical in the regulation of DC function as well as in their capacity to prime T-cell responses. We exposed tofacitinib or oclacitinib to BMDCs for 24 hours (short-term exposure) and stimulated with LPS to examine the effects on mature DCs. TNF α and IL-12 levels of each JAK inhibitor-treated BMDC decreased in a dose-dependent manner, and statistically significant differences were found compared with the values for the vehicle-only control (* P < 0.05, ** P < 0.01) (Fig. 1, A and B).

Inhibitory Effect of Tofacitinib and Oclacitinib on Migration of LPS-Stimulated Immature BMDCs. We then examined the effects of each JAK inhibitor on DC migration. However, short-term exposure of each JAK inhibitor had almost no effects on DC migration (data not shown). Therefore, we next exposed BMDCs to tofacitinib or oclacitinib for 6 days (long-term exposure) to examine the effects on DC maturation. Both inhibitors significantly reduced the migration of DCs compared with the values for the vehicle-only control (Fig. 1C). Migrated DC counts of each JAK inhibitor-treated BMDC decreased in

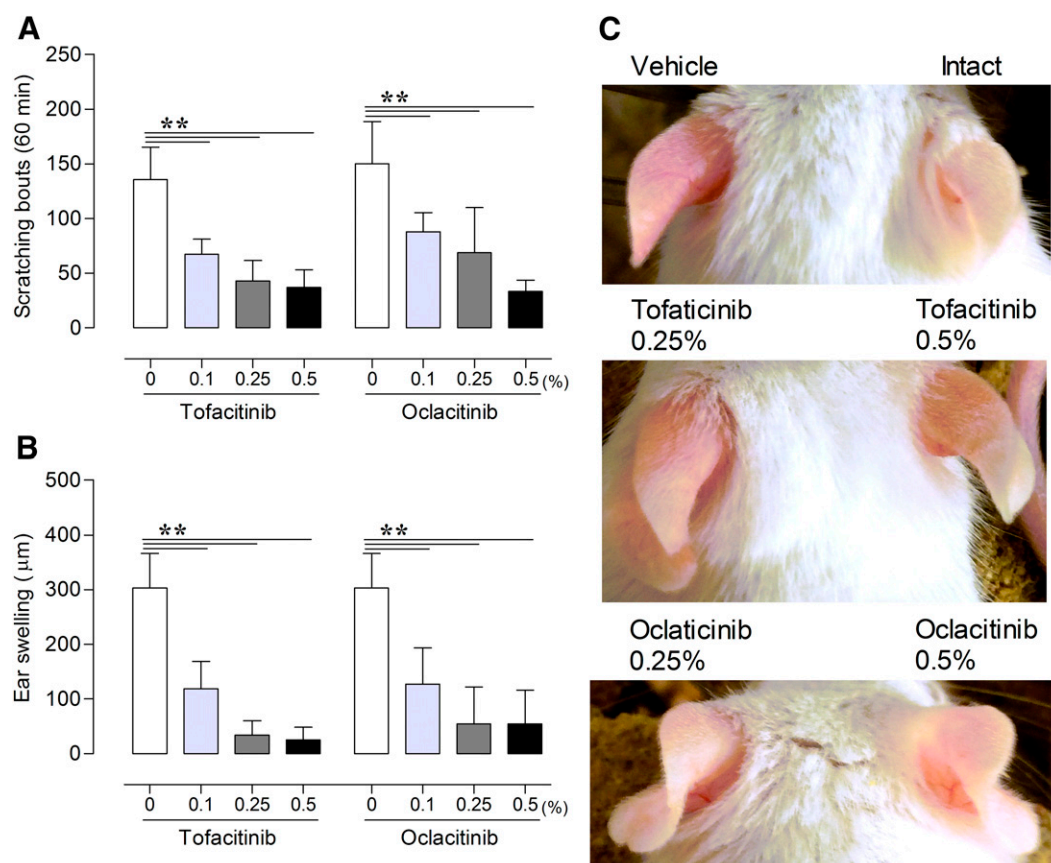


Fig. 3. Scratching and ear swelling effects of topical application of JAK inhibitors on TDI-induced allergic dermatitis. (A) Scratching behavior was induced 30 minutes after the application of each JAK inhibitor and then evaluated for 1 hour. Results are expressed as mean $\pm$ 1 S.D. (n = 12 per group). ** P < 0.01 (Dunnett's multiple comparisons test) versus vehicle-only control group. (B) Ear swelling was calculated by a comparison of the ear thickness before and 24 hours after TDI challenge. Each JAK inhibitor was applied topically 30 minutes before and 4 hours after TDI challenge. Results are expressed as the mean $\pm$ 1 S.D. (μ m, n = 12 per group). ** P < 0.01 (Dunnett's multiple comparisons test) versus vehicle-only control group. (C) Representative clinical features of mice in each treatment group.

a dose-dependent manner. Significant differences were found in 0.1 μM concentration of oclacitinib ($P < 0.05$), 1 and 10 μM concentrations of each drug ($P < 0.01$) compared with the values for the vehicle-only control.

Suppressive Effect of Tofacitinib and Oclacitinib on Costimulatory Molecules of LPS-Stimulated Immature BMDCs. To confirm the suppressive effect of tofacitinib and oclacitinib, we measured the costimulatory molecules of DCs by FACS. Along with the outcomes of DC migration, short-term exposure to each JAK inhibitor had almost no effect on phenotype of MHC class II⁺CD86⁺ cells (data not shown). However, a dose-dependent reduction was detectable for the expression of MHC class II and CD86 molecules (Fig. 1, D and E), which is critical for DC function. Significant differences were found in 1 and 10 μM concentrations of each drug ($P < 0.01$) compared with the values for the vehicle-only control.

Impact of Tofacitinib and Oclacitinib on DC Migration Ex Vivo. To confirm the suppressive effect of tofacitinib and oclacitinib, we next examined the effects of each JAK inhibitor on skin DC migration ex vivo. Topical treatment with tofacitinib (0.1%) and oclacitinib (0.1%) lead to significant reduction of cell migration from mouse ear explants compared with vehicle-treated ears (all $P < 0.05$) (Table 1). The cell counts of MHC class II positive cells (that is, Langerhans cells) were significantly lower in vehicle-treated compared with each JAK inhibitor-treated epidermis (all $P < 0.01$) (Table 1).

Impact of Orally Administered Tofacitinib and Oclacitinib on Scratching Behavior and Ear Swelling in the Challenge Phase of ACD. To examine whether oral exposure to each JAK inhibitor affects itch behavior in mice, we monitored scratching bouts in the 60-minute period 30 minutes after tofacitinib or oclacitinib was orally administered (Fig. 2A).

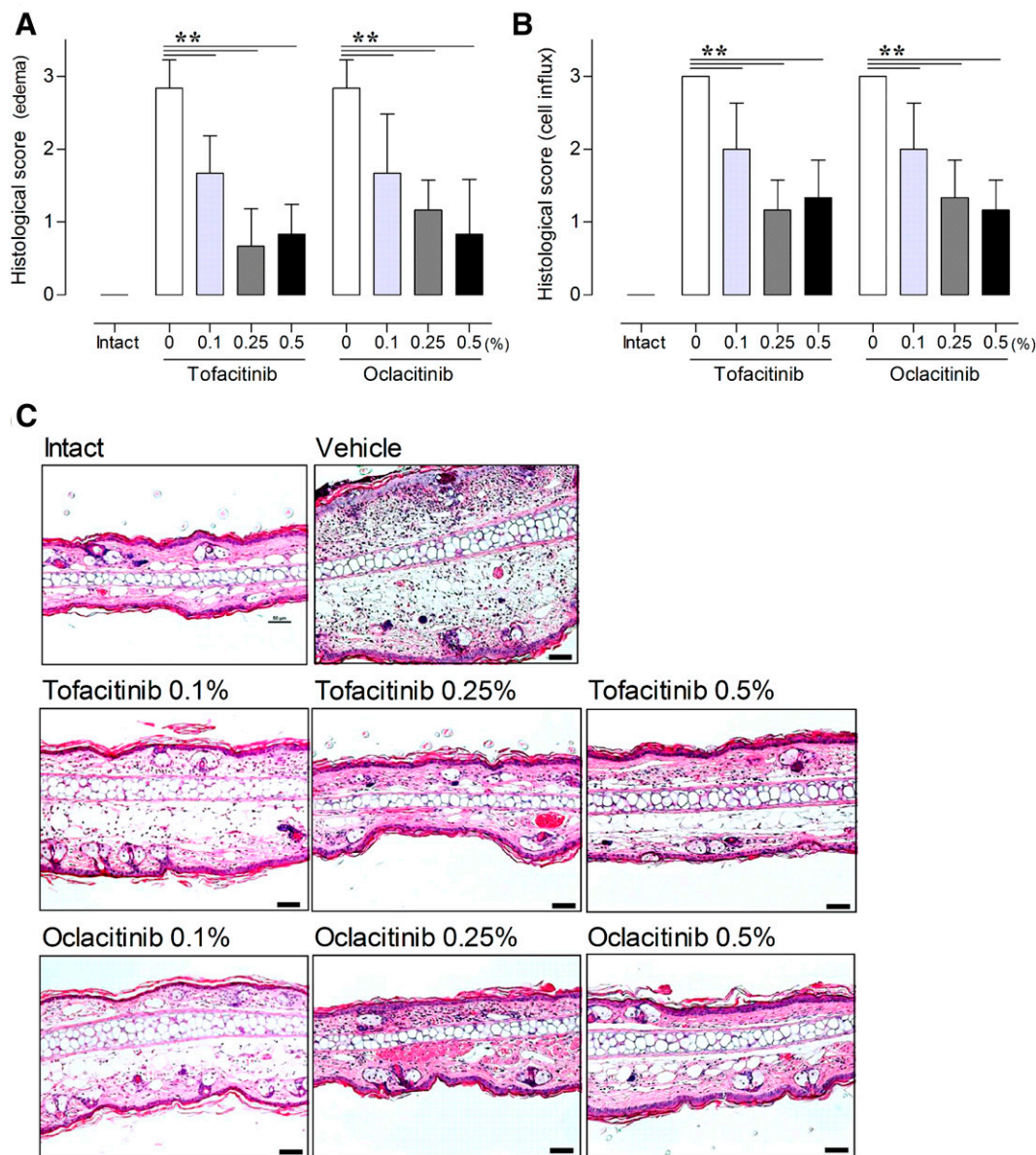


Fig. 4. H&E staining in ear skin of topical application of JAK inhibitors on TDI-induced allergic dermatitis. (A and B) Histologic skin severity scores of edema (top) and cell influx (bottom). Ear skins were treated with nothing (intact), vehicle, tofacitinib (0.1%, 0.25%, and 0.5%), or oclacitinib (0.1%, 0.25%, and 0.5%). Results are expressed as mean $\pm$ 1 S.D. ($n = 6$ –12 per group). $**P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle-only control group. (C) Typical microscopic features of skin with H&E staining. Scale bar, 50 μm .

Both low and high doses of tofacitinib groups displayed significantly less scratching bouts compared with the values for the vehicle-only group (all $P < 0.01$). Scratching bouts at the high dose in the oclacitinib group were also significantly less than in the vehicle-only group ($P < 0.01$). In addition, to examine whether oral exposure to each JAK inhibitor affects skin inflammation, we measured ear thickness and the swelling was calculated by a comparison of the ear thickness before and 24 hours after challenge (Fig. 2B). However, there were almost no changes in ear swelling for both tofacitinib and oclacitinib orally treated mice.

Inhibitory Effects of Topically Applied Tofacitinib and Oclacitinib on Scratching Behavior and Ear Swelling in the Challenge Phase of ACD. Since oral administration only affected scratching behavior, we next examined whether topical treatment to each JAK inhibitor affects itch or skin inflammation. The topical treatment reduced scratching behavior and ear swelling in a dose-dependent manner (all $P < 0.01$) (Fig. 3, A and B). Representative pictures of the ear skin just before sacrifice are shown in Fig. 3C. In addition, each JAK-inhibitor treatment reduced epidermal hyperplasia, parakeratosis, dermal edema, and infiltration of inflammatory cells in the skin. The data revealed that the severity of ACD in the skin lesions was significantly improved by means of topical treatment with both JAK inhibitors (all $P < 0.01$) (Fig. 4, A–C).

Effects of Topically Applied Tofacitinib and Oclacitinib on Cytokine Production in the Ear Skin of the Challenge Phase of ACD 24 Hours after TDI Challenge.

To assess the allergic inflammation in the skin tissue, we measured the levels of related cytokines/chemokines (IL-1 β ,

IL-4, IL-6, TARC, IL-12, IL-31, TNF α , TSLP, and IP-10) in the homogenate of ear skin 24 hours after TDI challenge. The tofacitinib- or oclacitinib-treated mice had significantly lower levels of IL-1 β , IL-4, IL-6, and TARC than mice treated with the vehicle-only control (Fig. 5). However, the levels of IL-12, IL-31, TNF α , TSLP, and IP-10 were increased by tofacitinib or oclacitinib treatment in a dose-dependent manner (Fig. 6).

Effects of Topically Applied Tofacitinib and Oclacitinib on Cytokine Production in the Affected Rostral Neck Skin of the Challenge Phase of ACD 30 Minutes after TDI Challenge. To examine pruritogen-evoked cytokine profiles in the affected skin, we next examined the levels of related cytokines (IL-31, TNF α , and TSLP) in the homogenate of rostral neck skin 30 minutes after TDI challenge. The 0.1% tofacitinib-treated or 0.1% oclacitinib-treated mice had significantly lower levels of IL-31, TNF α , and TSLP than mice treated with the vehicle-only control at this early time point (Fig. 7).

Topical Treatment of Tofacitinib and Oclacitinib Inhibits the Local LN Activation in the Challenge Phase of ACD.

To evaluate the state of activation of T cells and DCs following tofacitinib and oclacitinib topical treatment in the challenge phase of ACD, we used flow cytometry to measure the number of CD3 $^{+}$ T cells and CD11c $^{+}$ CD40 $^{+}$ DCs in auricular LNs (Table 1). We also measured the LN weight and total numbers of LN cells (Table 2). Each parameter of oclacitinib-treated mice decreased in a dose-dependent manner, and statistically significant differences were found in the 0.25% and 0.5% treatment groups (all $P < 0.01$) compared with the values for the vehicle-only control. The 0.1% JAK-inhibitor treatment group showed an increasing

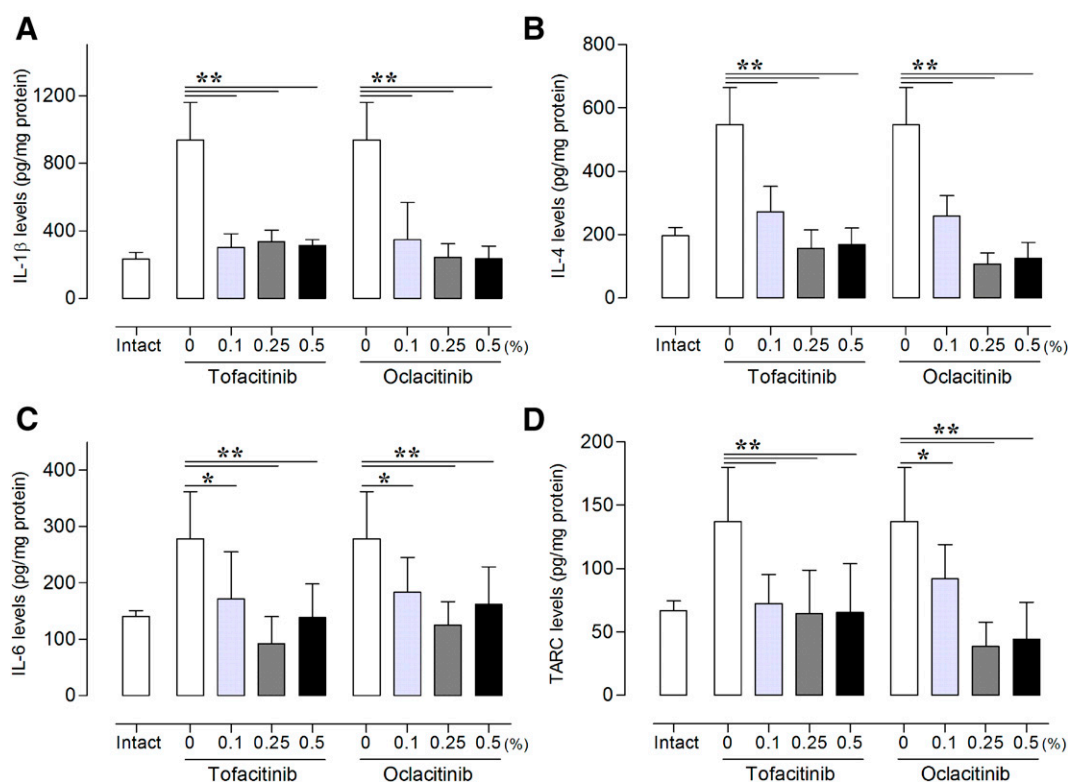


Fig. 5. Downregulated cytokine levels in ear skin 24 hours following topical application of JAK inhibitors. Concentrations of (A) IL-1 β , (B) IL-4, (C) IL-6, and (D) TARC were determined by ELISA. Results are expressed as mean $\pm$ S.D. (pg/ml; $n = 6$ –12 per group). Designations of treatments are as in Fig. 4. * $P < 0.05$ and ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle control group.

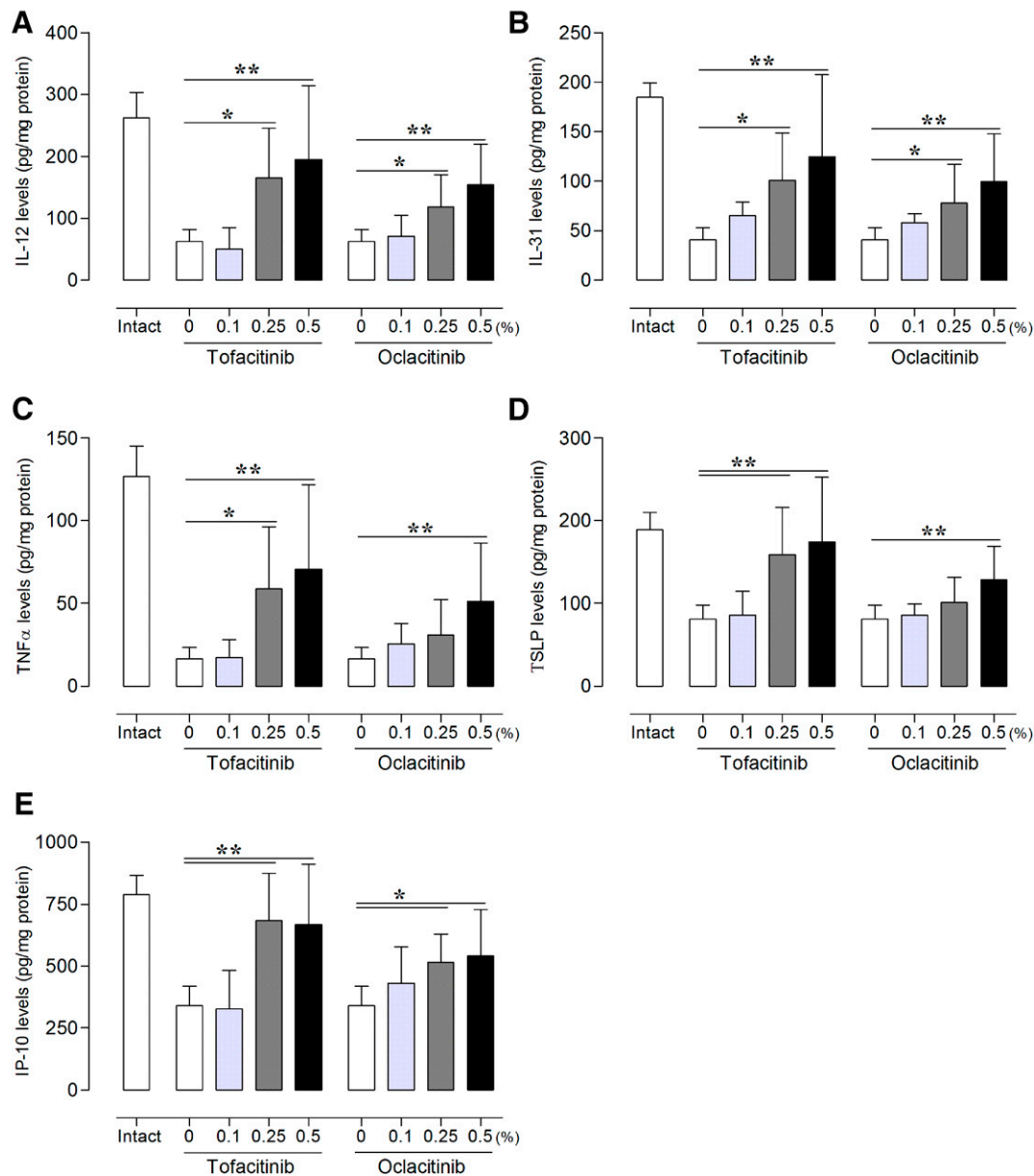


Fig. 6. Upregulated cytokine levels in ear skin 24 hours following topical application of JAK inhibitors. Concentrations of (A) IL-12, (B) IL-31, (C) TNF α , (D) TSLP, and (E) IP-10 were determined by ELISA. Results are expressed as mean $\pm$ S.D. (pg/ml; $n = 6-12$ per group). Designations of treatments are as in Fig. 4. * $P < 0.05$ and ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle control group.

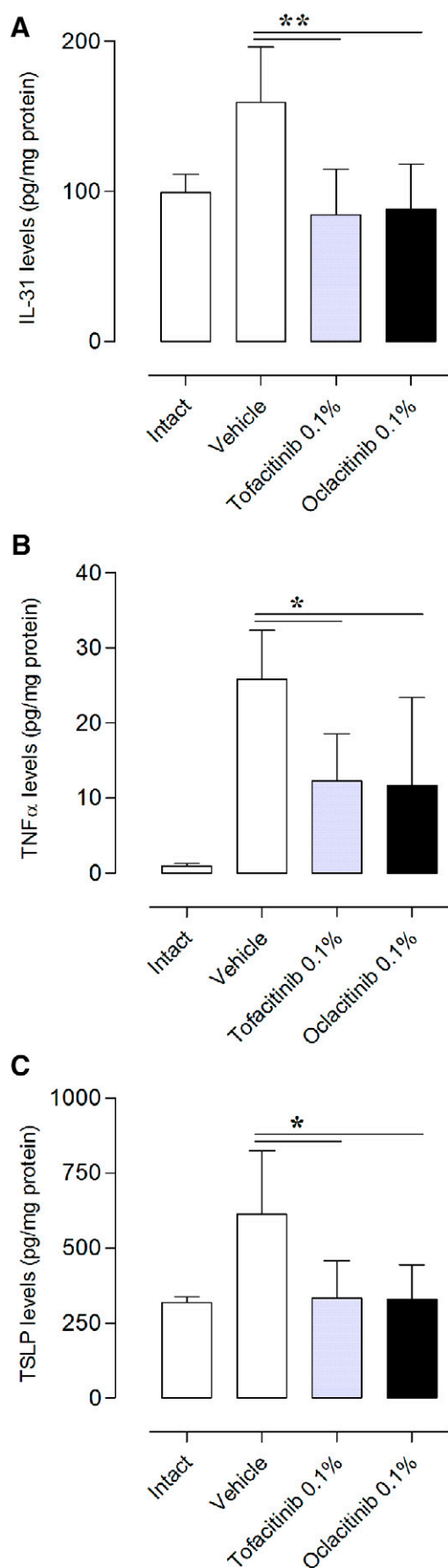
trend; however, the reactions were moderate and significant difference was only observed in CD11c⁺CD40⁺ cells in the 0.1% treatment group. We also examined the production of related proinflammatory cytokines/chemokines (IL-4, -6, TNF α , and TARC) from T cells and DCs after Con A stimulation (Table 3). Corresponding to the numbers of cells, in both the tofacitinib and oclacitinib treatment groups, production of all cytokines decreased in a dose-dependent manner, and statistically significant differences were found in the 0.25% and 0.5% treatment groups.

Discussion

The present study identified several crucial aspects. (1) Both JAK inhibitors significantly inhibited cytokine production, migration, and maturation of BMDCs, corroborated

by a skin DC migration assay. (2) The mouse model of ACD oral treatment with JAK inhibitors resulted in a significant decrease in scratching behavior; however, ear thickness was not significantly reduced. (3) Both scratching behavior and skin inflammation in the topical treatment group were significantly reduced compared with the vehicle treatment group. (4) In vitro as well as in vivo effects of tofacitinib and oclacitinib have been comparable, although they differ in their JAK-inhibitory pattern.

Tofacitinib represents one of the first small molecules developed as a selective inhibitor of JAK3 (Tanimoto et al., 2015), and exhibits anti-inflammatory activities by oral administrations in different models of inflammatory diseases, such as rheumatoid arthritis (Meyer et al., 2010; Kubo et al., 2014), inflammatory bowel disease (Sandborn et al., 2012), and transplant rejection (Vincenti et al., 2012). Recently, it



has been reported that systemic administration of tofacitinib ameliorated the inflammatory responses of oxazolone-induced chronic dermatitis in rats (Fujii and Sengoku, 2013) and clinical symptoms in patients with psoriasis (Boy et al., 2009; Bissonnette et al., 2015). Oclacitinib was the first selective JAK inhibitor developed for control of pruritus associated with atopic dermatitis by oral administrations in dogs (Collard et al., 2014). However, the exact mechanism of anti-inflammatory and anti-itch responses in allergic skin diseases remains unclear. In this study, we first demonstrated that the JAK inhibitors tofacitinib and oclacitinib acted as effective suppressors to regulate the functions of DCs and that topically administered JAK inhibitors display impressive anti-itch and anti-inflammatory responses in an ACD model.

DCs are pivotal to both the initiation and maintenance phase of allergic inflammatory diseases. Thus, it can be postulated that inhibition of DC functions can at least partly explain an inhibitory action of immunomodulatory substances. This has already been demonstrated for immunomodulators such as cyclosporine A, tacrolimus, rapamycin, cilomilast, and glucocorticoids (Homey et al., 1998; Panhans-Gross et al., 2001; Bäumer et al., 2003; Chen et al., 2004; Hoetzenecker et al., 2004). In the beginning, we exposed tofacitinib or oclacitinib to mature BMDCs for 24 hours and stimulated them with LPS to examine the effects on mature DCs. According to the results of our current study, proinflammatory cytokine productions (IL-12 and TNF α) were significantly suppressed by means of incubation with each JAK inhibitor, whereas DC migration as well as expression of costimulatory molecules was comparable with vehicle control. Although the concentration range tested (0.1–10 μ M) was higher than published IC₅₀ values for reducing cytokine response, we decided to take these concentrations, which have been also used in other published reports that examined JAK inhibitor–modulated DC activity in vitro. For example, Heine et al. (2013) used 10 μ M as a highest concentration to study how ruxolitinib (JAK1 and -2 inhibitor) impairs DC function. Kubo et al. (2014) also used 10 μ M as a highest concentration for tofacitinib. As stated previously, JAK/STAT signaling has already been associated with cell migration and modulation of chemokine production. Heine et al. (2013) reported that the JAK inhibitor ruxolitinib can inhibit migratory behavior toward CCL19/MIP-3 β in human monocyte-derived DCs, and Rivas-Caccedo et al. (2009) demonstrated in JAK3-deficient mice that JAK3 is involved in BMDC maturation and CCR7-dependent migration. Due to the importance of proper DC migration to secondary lymphoid organs in order to induce T-cell responses, we further focused on DC migration. We then exposed each JAK inhibitor to BMDCs for 6 days to examine the effects on DC maturation. In the present study, each JAK inhibitor–exposed, LPS-stimulated DC exhibited pronounced impairment of migratory behavior in vitro, and expression of MHC class II and CD86 in DCs was also reduced by each JAK-inhibitor treatment. The in vitro DC migratory responses are confirmed by the skin

Fig. 7. Cytokine levels in dorsal (neck) skin 30 minutes following topical application of JAK inhibitors and challenge with TDI. Concentrations of (A) IL-31, (B) TNF α , and (C) TSLP were determined by ELISA. Results are expressed as mean $\pm$ S.D. (pg/ml; $n = 6$ per group). Dorsal skins were treated with nothing (intact, no TDI challenge), vehicle, tofacitinib (0.1%), or oclacitinib (0.1%). * $P < 0.05$ and ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle control group.

TABLE 2

Responses in auricular LNs of topical application of JAK inhibitors on TDI-induced BALB/c mice
Results are expressed as mean $\pm$ 1 S.D. ($n = 6-12$ per group).

Group	LN Weight	Total Cell Count	CD3 ⁺ T Cells	CD11c ⁺ CD40 ⁺ Cells
	mg	$\times 10^6$	$\times 10^6$	$\times 10^4$
Intact	2.55 $\pm$ 0.30	2.56 $\pm$ 0.86	1.23 $\pm$ 0.42	0.61 $\pm$ 0.29
Vehicle only	6.20 $\pm$ 0.86	6.83 $\pm$ 2.35	2.99 $\pm$ 1.07	3.70 $\pm$ 1.25
Tofacitinib 0.1%	6.00 $\pm$ 0.63	6.22 $\pm$ 1.16	2.78 $\pm$ 0.53	2.96 $\pm$ 0.85
Tofacitinib 0.25%	4.79 $\pm$ 0.76**	4.37 $\pm$ 0.87*	2.03 $\pm$ 0.45	1.42 $\pm$ 0.55**
Tofacitinib 0.5%	4.10 $\pm$ 0.75**	3.91 $\pm$ 1.03**	1.78 $\pm$ 0.46*	0.94 $\pm$ 0.51**
Oclacitinib 0.1%	5.83 $\pm$ 1.24	6.01 $\pm$ 1.86	2.29 $\pm$ 0.59	2.25 $\pm$ 0.90*
Oclacitinib 0.25%	2.89 $\pm$ 0.74**	2.68 $\pm$ 0.78**	1.24 $\pm$ 0.32**	0.73 $\pm$ 0.27**
Oclacitinib 0.5%	2.62 $\pm$ 0.61**	2.40 $\pm$ 0.43**	1.11 $\pm$ 0.17**	0.49 $\pm$ 0.18**

* $P < 0.05$; ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle-only control group.

DC migration assay. Analysis of epidermal sheets demonstrated that, compared with vehicle-treated mouse ears, topical treatment with each JAK inhibitor leads to an inhibition of Langerhans cell migration. Our BMDC results are supported for tofacitinib by the recently published study using human monocyte-derived DCs (Kubo et al., 2014). Although the JAK-inhibitory profile differs between both JAK inhibitors, all effects on murine BMDCs have been comparable.

In the next step, we attempt to examine whether oral exposure to each JAK inhibitor affects both itching and allergic inflammation in a mouse model of Th2-driven ACD. We found that mice treated orally with JAK inhibitors showed a significant decrease in scratching behavior; however, ear thickness was not significantly reduced. Our itching results are supported for oclacitinib by the recently published clinical study in dogs (Gonzales et al., 2014). For tofacitinib, Fujii and Sengoku (2013) reported an anti-inflammatory effect in the oxazolone-induced chronic dermatitis model in rats (effects on itch were not reported). In contrast to our findings, in rats there is a significant decrease of ear swelling with the most pronounced effect at 10 mg/kg. However, the inhibitory action is much more pronounced in the chronic setting after repetitive treatment and challenge. Thus, in our acute setting it can be postulated that oral administration of both JAK inhibitors has a significant impact on pruritus; however, there is only slight impact on inflammatory responses when the JAK inhibitors are administered systemically.

Because we were not able to find an exhibition of dual antipruritic and anti-inflammatory effects in allergic dermatitis by systemic application of JAK inhibitors, we finally attempted to examine whether topical exposure to each JAK inhibitor affects both itch and allergic inflammation. We clearly demonstrated that topical application of JAK inhibitors

prevented the development of ACD in BALB/c mice, reducing scratching behavior and ear swelling in the animals. Edema and infiltration of inflammatory cells were also dramatically reduced in JAK inhibitor-treated mice, indicating the alleviation of dermatitis clinically and histologically. Corresponding to the scratching behavior results, topical application of each JAK inhibitor significantly inhibited the pruritogen-evoked cytokine secretions, including IL-31, TNF α , and TSLP, in the affected skin 30 minutes after TDI challenge. The anti-inflammatory effects in allergic dermatitis by topical application of JAK inhibitors are also supported by the recently published study using topical application of the JAK1/JAK2 inhibitor ruxolitinib in a guinea pig model of delayed-type hypersensitivity (Fridman et al., 2011). According to the results from cytokine determinations of TDI-challenged ear skin, topical application of JAK inhibitors markedly inhibited the production of proinflammatory Th2 cytokines, including IL-1 β , IL-4, IL-6, and TARC. However, Th1 cytokines, including IL-12 and IP-10, were upregulated following JAK-inhibitor exposure. TDI is recognized as a Th2-type allergen, and several reports have shown that topical TDI exposure leads to an increased Th2 cytokine secretion pattern in mice, whereas the Th1 cytokine secretion profile is reduced by TDI (Ban et al., 2006). Corresponding to our results reported here, Nakagawa et al. (2011) reported that treatment of a pan-JAK inhibitor, Pyridone 6, exhibited a therapeutic effect against atopic-like skin inflammation via modulation of helper T-cell differentiation. They demonstrated that secretion of Th2 cytokines IL-13 as well as IL-4 were inhibited by Pyridone 6 in the *Dermatophagoides farinae* body extract-induced NC/Nga mice chronic atopic dermatitis model. Interestingly, our results also demonstrate an elevation in levels of IL-31, TNF α , and TSLP 24 hours following JAK-inhibitor treatment, whereas

TABLE 3

Cytokine production in response to concavalin A stimulation in LNs of topical application of JAK inhibitors on TDI-induced BALB/c mice
Results are expressed as mean $\pm$ S.D. (pg/ml; $n = 6-12$ per group).

Group	IL-4	IL-6	TNF α	TARC
Intact	1.9 $\pm$ 2.5	1.85 $\pm$ 1.42	10.4 $\pm$ 14.0	1.5 $\pm$ 1.9
Vehicle only	217.8 $\pm$ 166.1	17.51 $\pm$ 5.12	156.7 $\pm$ 72.6	205.8 $\pm$ 112.0
Tofacitinib 0.1%	168.2 $\pm$ 54.7	13.05 $\pm$ 11.26	124.7 $\pm$ 53.9	77.5 $\pm$ 60.8*
Tofacitinib 0.25%	34.8 $\pm$ 41.2**	5.50 $\pm$ 2.35**	22.7 $\pm$ 22.8**	56.8 $\pm$ 47.0**
Tofacitinib 0.5%	30.3 $\pm$ 23.8**	7.18 $\pm$ 3.17**	40.6 $\pm$ 31.5**	59.5 $\pm$ 54.0**
Oclacitinib 0.1%	167.7 $\pm$ 159.4	9.95 $\pm$ 4.36**	109.4 $\pm$ 24.2	75.9 $\pm$ 87.0*
Oclacitinib 0.25%	26.2 $\pm$ 23.7*	6.35 $\pm$ 3.44**	50.5 $\pm$ 56.3**	37.7 $\pm$ 31.4**
Oclacitinib 0.5%	8.3 $\pm$ 7.86**	3.70 $\pm$ 1.65**	11.7 $\pm$ 7.2**	11.8 $\pm$ 10.9**

* $P < 0.05$; ** $P < 0.01$ (Dunnett's multiple comparisons test) versus vehicle control group.

topical application of JAK inhibitors significantly inhibited these cytokine secretions in the affected skin 30 minutes after TDI challenge. Because it has been reported that epithelial cells and DCs directly communicate to cutaneous sensory neurons via IL-31, TNF α , and TSLP to promote itch (Trinh et al., 2008; Wilson et al., 2013; Cevikbas et al., 2014), these cytokines play a key role in atopic itch. Because JAK inhibitors only block the signal transduction, but obviously not the secretion of these particular cytokines in a late phase (24 hours after challenge), an elevation of these pruritogens might be associated with a possible rebound phenomenon after therapy is discontinued abruptly. This phenomenon has already been demonstrated for immunomodulators, such as cyclosporine A and glucocorticoids (Kimata, 1999; Hijnen et al., 2007). Results obtained from the draining auricular LN indicate that topical application of both JAK inhibitors reduced the numbers of T cells and DCs as well as proinflammatory cytokine production. Thus, topical application of both JAK inhibitors might also have an impact on T-cell proliferation, DC migration, and cytokine secretion from T cells or DCs. Along with the outcomes in vitro, all of the effects seen in the vivo setting have been comparable with both of the JAK inhibitors, tofacitinib and oclacitinib.

To our knowledge, the present study is the first to demonstrate that tofacitinib and oclacitinib exhibit dual antipruritic and anti-inflammatory effects in ACD by topical application. These findings can represent a new treatment option for allergic skin diseases such as contact hypersensitivity and atopic dermatitis. Topical delivery of tofacitinib and oclacitinib minimizes side effects seen by systemic exposure, such as increases in serum creatinine, increased risk for herpes zoster, and leukopenia (Balagué et al., 2012; Isaacs et al., 2014; Winthrop et al., 2014), as well as allowing for higher levels of cytokine inhibition in local tissue, which may provide prompt and strong relief from both itch and allergic inflammation.

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Authorship Contributions

Participated in research design: Bäumer, Fukuyama.
Conducted experiments: Fukuyama, Ehling, Cook, Bäumer.
Contributed new reagents or analytic tools: Fukuyama, Ehling, Cook, Bäumer.
Performed data analysis: Fukuyama, Bäumer.
Wrote or contributed to the writing of the manuscript: Fukuyama, Bäumer.

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