Original Article

Title:

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

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Running Title: Availability of JAK-inhibitors on Allergic Dermatitis

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Abbreviations: ACD, allergic dermatitis; APC, allophycocyanin; CCR, CC motif receptor; LN, auricular lymph node; BMDC, bone marrow-derived dendritic cell; Con A, concanavalin A; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; IFN, Interferon; IL, interleukin: JAK, Janus kinase; LN, lymph node; LPS, lipopolysaccharide; MHC, major histocompatibility complex class; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin Chlorophyll Protein; STAT, signal transducers and activator of transcription; TDI, toluene-2,4-diisocyanate; TSLP, thymic stromal lymphopoeitin; TNF, tumor necrosis factor; TYK, tyrosine kinase.

A recommended section: Inflammation and Immunopharmacology
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 min before and 4 h after TDI challenge. After scratching bouts and ear thickness were measured, cytokines were determined in challenged skin and cells of the draining lymph node were analysed by means of flow cytometry. In vitro, both JAK-inhibitors significantly inhibited cytokine production, migration and maturation of BMDC. Mice treated orally with JAK-inhibitors showed a significant decrease in scratching behavior; however, ear thickness was not significantly reduced. By contrast, both scratching behavior and ear thickness in the topical treatment group were significantly reduced compared to vehicle treatment group. Cytokine production, however, was differentially regulated by the JAK-inhibitors with some cytokines significantly decreased and some 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, 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 are 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). In order 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 classical 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). In order 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., 2013ab; Fujii & Sengoku. 2013; Gonzales et al., 2014; Ports et al., 2013).

In mammals, four JAK families of enzymes (JAK1, JAK2, JAK3, TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6) are utilized by more than 50 cytokines and growth factors to mediate intracellular signaling (Villarino et al., 2015). In particular, pro-inflammatory cytokines such as interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-6, IL-13, IL-21 and IL-23 have been implicated in inflammatory disease that utilize the JAK pathway (O'Shea et al., 2004). In addition, T_{h}2 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., 2013ab; Fujii & Sengoku, 2013; Gonzales et al., 2014; Ports et al., 2013). However, the exact mechanisms of the anti-inflammatory and anti-itch action of these JAK-inhibitors are still not fully understood. Therefore, the primary objective of the study reported here was to elucidate 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 2 different types of JAK-inhibitors, tofacitinib and oclacitinib, which have been approved by the United States and 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 signalling 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 AD 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).

As dendritic cells are crucial players in allergic diseases, 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 dendritic cell 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 at the anti-itch and anti-inflammatory responses.
Materials and Methods

Reagents. Tofacitinib (CP-690550, C_{16}H_{20}N_{6}O_{6}C_{6}H_{8}O_{7}, IUPAC Name: 3-[(3R,4R)-4-methyl-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]piperidin-1-yl]-3-oxopr opanenitrile) was purchased from Tocris (Minneapolis, MN), and Oclacitinib (PF-03394197, C_{15}H_{23}N_{5}O_{2}S, IUPAC Name: N-methyl-1-[4-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]cyclohexyl]methanesulfonamide) was purchased from Adooq Bioscience (Irvine, CA). Concanavalin A (Con A), lipopolysaccharide (LPS; O127:B8), toluene-2,4,-diisocyanate (TDI), acetone and 2-mercaptoethanol were obtained from Sigma (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 Inc. (Waltham, MA). RPMI1640 medium was from Mediatech Inc. (Manassas, VA). Purified Rat Anti-Mouse CD16/CD32 (Mouse BD FC Block™), anti-mouse CD86 (rat IgG_{2ak}, FITC-conjugated, clone GL1) and major histocompatibility complex class (MHC) II (I-A/I-E, rat IgG_{2bk}, PerCP-Cy™5.5-conjugated, M5/114), and I-A/I-E (rat IgG_{2ak}, Biotin-conjugated, clone 2G9) were ordered from Becton, Dickinson and Company (Franklin Lakes, NJ). Anti-mouse CD3 (rat IgG_{2bk}, FITC-conjugated, clone 17A2), CD11c (hamster IgG, APC-conjugated, N418), CD19 (rat IgG_{2ak}, PE-conjugated, clone 6D5), CD40 (rat IgG_{2ak}, PE-conjugated, clone NLDC-145), and CD207 (Langerin, rat IgG_{2ak}, FITC-conjugated, clone caa828H10) were ordered from Miltenyi Biotec (Auburn, CA). Cy3 streptavidin was from Biolegend (San Diego, CA). DC protein assay kit was from BIO-RAD (Richmond, CA). Recombinant Mouse GM-CSF and CCL19/MIP-3β, ELISAs for IL-1β, -4, -6, -12, TNFα, TSLP, CXCL10/IP-10/CRG-2 and
CCL17/TARC were from R&D systems (Minneapolis, MN). ELISAs for IL-31 was from eBioscience, Inc. (San Diego, CA).

Animals. BALB/cAnN (female, 6-wk-old) were purchased from Charles River Laboratories (Raleigh, NC) and housed in groups of four mice per cage under controlled lighting (a 12-h light-dark cycle), temperature (22 ± 3°C), humidity (55% ± 15%), and ventilation (at least 10 complete fresh-air changes/h). 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. Bone marrow-derived dendritic cells (BMDCs) were generated from bone marrow cells of female BALB/cAnN mice, as described previously (Bäumer et al., 2003; Lutz et al., 1999) with minor modifications. Bone marrow was flushed from femurs of the hind limbs with PBS and taken into BMDCs medium (RPMI 1640 with L-glutamine, 10% heat-inactivated FCS, 50 μmol/l 2-mercaptoethanol, and 20 ng/ml GM-CSF). 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 min at 20°C. The numbers of viable cells were determined with a Cellometer (Nexcelom Bioscience LLC., Laurence, MA) using acridine orange and propidium iodide staining.

JAK-inhibitor Exposure to BMDCs. A short term exposure was performed in mature BMDCs on 8 days old. BMDCs (2.5×10^5 cells/ml) were exposed to 0.1% DMSO (vehicle) and each JAK-inhibitor (at 0.1, 1, or 10 μmol/l) in BMDC medium containing LPS (25 ng/ml) for 24 h. For the long term exposure, immature (3 days 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 μmol/l) 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 μmol/l. After exposure, cytokine production (only in short term exposure, IL-12 and TNFα), migration and phenotypes (MHC II and CD86) in BMDCs were evaluated using the ELISA, chemotaxis assay and FACS, respectively. Two or three independent experiments were performed in DCs from different animals.

**Chemotaxis assay.** Chemotaxis of BMDCs in response to CCL19/MIP-3β was measured in 24-well plates carrying transwell-permeable supports with a 8 μm pore-size polycarbonate membrane. 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-3β. After 90 min of incubation at 37°C, the cells that had migrated to the lower chamber were counted with a Cellometer using the acridine orange and propidium iodide staining.

**Skin dendritic cell 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 h and 30 min before dissection of the mice (6 mice for each group). The ears were rinsed in 75% ethanol and air dried for 10 min. The cartilage-free dorsal halves of split mouse ear skin were cultured in 12-well size plates based on a method of Bäumer et al. (2003). The halves were floated epidermal side up in 1.3 ml BMDC buffer containing 50 ng/ml CCL19/MIP-3β. The halves were changed daily to new wells and new media (including CCL19/MIP-3β). Migrated total cells as well as dendritic cells (CD11c+CD40+ cells) from each ear (day 1~3) were pooled and counted with Cellometer 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), ear halves were weighed.

**Preparation of epidermal sheets for immunohistochemistry.** The preparation and the evaluation of epidermal sheet was performed according to Ratzinger et al. (2002). In short, skin was floated on 0.5 M ammonium thiocyanate for 15 min at 37°C. The Langerhans cells were detected with the biotinylated MHC class II antibody in a 1:200 dilution. Labelling of the antibodies was visualised 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 ten randomly chosen areas per ear were analysed. Six each JAK inhibitor-treated (only 0.1% in acetone/DMSO 7:1) and six vehicle-treated ears were analysed.

**TDI-induced allergic dermatitis model in mice.** TDI-induced and challenged BALB/c mouse was established as an allergic contact dermatitis model, and it has been already 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 a depilatory cream. On the day after depilation, the abdominal skin was stripped 10 times with adhesive tapes. Just after the tape stripping, a 100 μl of 5% TDI in acetone was applied to the stripped epidermis (day 1). On day 2 and 3, 50 μl 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 μl 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 μl of 0.5 % TDI in acetone onto the shaved dorsal region (neck, day 32).
Additionally, the allergic reaction was challenged by application of 20 μl of 0.5 % TDI in acetone onto the mouse ears for measuring the ear swelling. The swelling was calculated by a comparison of the ear thickness (cutimeter, Mitutoyo, Neuss, Germany) before and 24 h after challenge. JAK-inhibitors tofacitinib or oclacitinib were administered orally or topically 30 min before and 4 h after TDI challenge, because the absorption of tofacitinib and oclacitinib was rapid, with plasma concentrations for both tofacitinib and oclacitinib peaking at around 1 h after oral or intravenous administration. Tofacitinib and oclacitinib both have a short half life of 2 and 4 h 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 for topical application to concentrations described below. For each drug, a vehicle-only control group and low- and high-dose groups were set. Oral doses were: Tofacitinib, 10 and 30 mg/kg; 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 the previously published studies (Kudlac 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 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-μm nylon cell strainer in 1 ml RPMI 1640 supplemented with 5% FCS. Cell count was determined using a Cellometer 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 haematoxylin-eosin and evaluated in a blinded manner in respect of cell influx and edema by a semi-quantitative 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 μl RPMI 1640 medium containing 1 mmol/l Pefabloc. The samples were mixed intensively and stored for 30 min on ice. After centrifugation at 3000×g for 10 min at 4 °C, the supernatants were collected and the protein content was determined with DC protein assay kit. IL-1β, -4, -6, -12, -31, TNFα, TSLP, IP-10 and TARC were measured by ELISA.

**Cytokine determination of rostral neck skin.** In order to examine pruritogen evoked cytokine profiles in the affected skin, in a second setting the rostral neck skin were isolated from each mouse 1 h after each JAK inhibitor treatment (only 0.1% in acetone/DMSO 7:1) and 30 min after TDI challenge. Purification of skin tissue and cytokine determination were performed as mentioned in Cytokine determination of ear skin. IL-31, TNFα and TSLP were measured by ELISA.

**Cytokine determination of LNs.** To stimulate T-cell receptor signalling, we cultured single-cell suspensions recovered from LNs (5×10^5 cells/well) for 24 or 96 h with Con A (5 μg/ml) in 48-well plates at 37°C in a 5% CO₂ atmosphere. The levels of cytokines (IL-4, -6, TNFα and TARC) in cell culture medium were measured by using an ELISA.
Flow cytometric analysis of DCs and LNs. To avoid nonspecific binding, $5 \times 10^5$ cells were incubated with 1 µg Mouse BD Fc Block for 5 min at 4°C, followed by incubation with the monoclonal antibodies for 30 min at 4°C in the dark. Cells were washed with 5% FCS in PBS, resuspended at $5 \times 10^5$ cells per tube in 500 µL PBS, and analyzed on a LSR II flow cytometer by using FACSDiva software (BD Pharmingen). 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, Inc., 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 comparison test was applied. When group variances were heterogeneous, data were evaluated by Kruskal–Wallis non-parametric analysis of variance. When differences were significant, Dunnett’s mean rank-sum test was applied. Data are expressed as mean ± 1 S.D. The data was analysed using Prism 4 (GraphPad Software, San Diego, CA, USA).
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 TNFα). Cytokines are critical in the regulation of DC function as well as their capacity to prime T-cell responses. We exposed tofacitinib or oclacitinib to BMDCs for 24 h (short term exposure) and stimulated with LPS to examine effects on mature DCs. TNFα and IL-12 levels of each JAK-inhibitor treated BMDCs 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 DCs migration. However, short term exposure of each JAK-inhibitor had almost no effects on DCs migration (data not shown). Therefore, we next exposed BMDCs to tofacitinib or oclacitinib for 6 days (long term exposure) to examine effects on DC maturation. Both inhibitors significantly reduced the migration of DCs as compared with the values for the vehicle-only control (Fig. 1 C). Migrated DC counts of each JAK-inhibitor treated BMDCs decreased in a dose-dependent manner. Significant differences were found in 0.1 μmol/l concentration of oclacitinib (P < 0.05), 1 and 10 μmol/l concentration 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 co-stimulatory molecules of DCs by FACS. Along with outcomes of DC migration, short term exposure to each JAK-inhibitor had almost no effect on phenotype of MHC II⁺CD86⁺ cells (data not shown). However, a dose-dependent reduction was detectable for the expression of MHC II and CD86 molecules (Figure 1 D and E), which is critical for DCs function. Significant differences were found in 1 and 10 μmol/l concentration of each drug (P < 0.01) compared with the values for the vehicle-only control.

Impact of tofacitinib and oclacitinib on dendritic cell migration ex vivo. To confirm the suppressive effect of tofacitinib and oclacitinib, we next examined the effects of each JAK-inhibitor on skin DCs 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 to vehicle-treated ears (all P < 0.05, Table 1). The cell counts of MHC class II positive cells (that is, Langerhans cells) was significantly lower in vehicle-treated compared to 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 min period 30 min after tofacitinib or oclacitinib were orally administrated (Fig. 2 A). Both low-dose and high-dose of tofacitinib groups displayed significantly less scratching bouts compared with the values for the vehicle-only group (all P < 0.01). Scratching bouts in the high-dose of oclacitinib
group was also significantly less than that 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 h after challenge. (Fig. 2 B). 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 h after TDI challenge. To assess the allergic inflammation in the skin tissue, we measured the levels of related cytokines/chemokines (IL-1β, IL-4, IL-6, TARC, IL-12, IL-31, TNFa, TSLP and IP-10) in the homogenate of ear skin 24 h after TDI challenge. The tofacitinib or oclacitinib treated mice had significantly lower levels of IL-1β, IL-4, IL-6 and TARC than mice treated with the vehicle-only control (Fig. 5). However,
the levels of IL-12, IL-31, TNFα, TSLP and IP-10 were increased by tofacitinib or oclacitinib treatment in 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 min after TDI challenge. In order to examine pruritogen evoked cytokine profiles in the affected skin, we next examined the levels of related cytokines (IL-31, TNFα and TSLP) in the homogenate of rostral neck skin 30 min after TDI challenge. The 0.1% tofacitinib or 0.1% oclacitinib treated mice had significantly lower levels of IL-31, TNFα 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 lymph node 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 measured by flow cytometry 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 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 trend, but the reactions were moderate and significant difference was only observed in CD11c+CD40+ cells in 0.1% treatment group. We also examined the production of related proinflammatory cytokines/chomekines (IL-4, -6, TNFα and TARC) from T cells and DCs after concanavalin A stimulation (Table 3). Corresponding to the numbers of cells, in both tofacitinib
and oclacitinib treatment groups, production of all cytokines decreased in a dose-dependent manner, and statistically significant differences were found in 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) in a 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 to vehicle treatment group, and (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 (Kubo et al., 2014; Meyer et al., 2010), inflammatory bowel disease (Sandborn et al., 2012), and transplant rejection (Vincent et al., 2012). Recently, it has been reported that systemic administration tofacitinib ameliorated the inflammatory responses of oxazolone-induced chronic dermatitis in rats (Fujii & Sengoku, 2013) and clinical symptoms in patients with psoriasis (Bissonnette et al., 2014; Boy et al., 2009). Oclacitinib was the first selective JAK-inhibitor developed for control of pruritus associated with atopic dermatitis by oral administrations in dogs (Collard et al., 2013). However, the exact mechanism of anti-inflammatory and anti-itch responses in allergic skin diseases remains unclear. In this study, we first demonstrated that JAK-inhibitors tofacitinib and oclacitinib acted as effective suppressors to regulate the functions of dendritic cells (DCs) and topically administered JAK-inhibitors display impressive anti-itch and anti-inflammatory responses in a model of allergic contact dermatitis.
DCs are pivotal to both the initiation and maintenance phase of allergic inflammatory diseases. Thus, it can be postulated that an inhibition of DCs functions can at least partly explain an inhibitory action of immunomodulatory substances. This has already been demonstrated for immunomodulators like cyclosporine A, tacrolimus, rapamycin, cilomilast, and glucocorticoids (Bäumer et al., 2003; Chen et al., 2004; Hoetzenecker et al., 2004; Homey et al., 1998; Panhans-Gross et al., 2001). At the beginning, we exposed tofacitinib or oclacitinib to mature BMDCs for 24 h and stimulated with LPS to examine effects on mature DCs. According to the results of our current study, pro-inflammatory cytokine productions (IL-12 and TNFα) were significantly suppressed by means of incubation with each JAK-inhibitor, whereas DCs migration as well as expression of costimulatory molecules was comparable with vehicle control. Although the concentration range tested (0.1 to 10 µmol/l) was higher than published IC50’s for reducing cytokine response, we decided to take these concentrations which have been also used in other published reports which examined the JAK-inhibitor modulated DCs activity in vitro. E.g. Heine et al. (2013) used 10 µmol/l as a highest concentration to study how ruxolitinib (JAK 1 and 2 inhibitor) impairs DCs function. Kubo et al. (2014) also used 10 µmol/l as a highest concentration for tofacitinib. As stated above, 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-3β in human monocytes derived DCs, and Rivas-Caicedo et al. (2009) demonstrated in JAK3 deficient mice that JAK3 is involved in BMDCs 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 effects on DC maturation. In the present study, each
JAK-inhibitor-exposed, LPS-stimulated DCs exhibited a pronounced impairment of their 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 dendritic cell migration assay. Analysis of epidermal sheets demonstrated that, compared to vehicle-treated mouse ears, topical treatment with each JAK-inhibitor leads to an inhibition of Langerhans cell migration. Our BMDCs 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 allergic contact dermatitis (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 & Sengoku. (2013) reported an anti-inflammatory effect in 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.
As we were not able to find an exhibition of dual anti-pruritic and anti-inflammatory effects in allergic dermatitis by systemic application of JAK-inhibitors, we finally attempt 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-inhibitors-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, TNFα and TSLP in the affected skin 30 min 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 results from cytokine determinations of TDI challenged ear skin, topical application of JAK-inhibitors markedly inhibited the production of pro-inflammatory Th2 cytokines including IL-1β, IL-4, IL-6 and TARC. However, Th1 cytokines including IL-12 and IP-10 were up-regulated following JAK-inhibitors exposure. TDI is recognized as a Th2 type allergen and several reports showed 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 in here, Nakagawa et al. (2013) reported that treatment of a pan-JAK inhibitor, Pyridone 6 exhibited 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 Dermatophagoides farinae body extract induced NC/Nga mice chronic atopic dermatitis
Interestingly, our results also demonstrate an elevation in levels of IL-31, TNFα and thymic stromal lymphopoietin (TSLP) 24 h following JAK-inhibitor treatment, whereas topical application of JAK-inhibitors significantly inhibited these cytokine secretions in the affected skin 30 min after TDI challenge. Because it has been reported that epithelial cells and DCs directly communicate to cutaneous sensory neurons via IL-31, TNFα 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. As JAK inhibitors only block the signal transduction but obviously not the secretion of these particular cytokines in a late phase (24 h after challenge), an elevation of these pruritogens might be associated with a possible rebound phenomenon after therapy will be discontinued abruptly. This phenomenon has already been demonstrated for immunomodulators like cyclosporine A and glucocorticoids (Kimata. 1999; Hijnen et al., 2007). Results obtained from the draining auricular lymph node indicate that topical application of both JAK-inhibitors reduced the numbers of T cells and DCs as well as pro-inflammatory cytokine production. Thus, topical application of both JAK-inhibitors might have also an impact on T cells proliferation, DCs migration and cytokine secretion from T cells or DCs. Along with the outcomes in vitro, all effects seen in the vivo setting have been comparable with both JAK inhibitors, tofacitinib and oclacitinib.

To our knowledge, the present study is the first to demonstrate that tofacitinib and oclacitinib exhibit dual anti-pruritic and anti-inflammatory effects in allergic contact dermatitis by topical application. These findings can represent a new treatment option for allergic skin disease like 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 allows 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 and Fukuyama

Conducted experiments: Fukuyama, Ehling, Cook and Bäumer.

Contributed new reagents or analytic tools: Fukuyama, Ehling, Cook and Bäumer.

Performed data analysis: Fukuyama and Bäumer.

Wrote or contributed to the writing of the manuscript: Fukuyama and Bäumer.
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Janus kinase inhibitor oclacitinib (Apoquel(R)) in client-owned dogs with atopic dermatitis.


for the migration of Langerhans cells and dermal dendritic cells from human and murine skin.


**Footnotes**

This work was self-funded.
Legends for Figures

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

**Fig. 2.** Scratching and ear swelling effects of oral administration of JAK-inhibitors on TDI-induced allergic dermatitis. (A) Scratching behavior was induced 30 min after the administration of each JAK-inhibitor and then evaluated for an hour. Results are expressed as mean ± 1 S.D. (n = 7 per group). **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle-only control group. (B) The ear swelling was calculated by a comparison of the ear thickness before and 24 h after TDI challenge. Each JAK-inhibitor was administered orally 30 min before and 4 h after TDI challenge. Results are expressed as the mean ± 1 S.D. (μm, n = 7 per group).
Fig. 3. Scratching and ear swelling effects of topical application of JAK-inhibitors on TDI-induced allergic dermatitis. (A) Scratching behavior was induced 30 min after the application of each JAK-inhibitor and then evaluated for an hour. Results are expressed as mean ± 1 S.D. (n = 12 per group). **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle-only control group. (B) The ear swelling was calculated by a comparison of the ear thickness before and 24 h after TDI challenge. Each JAK-inhibitor was applied topically 30 min before and 4 h after TDI challenge. Results are expressed as the mean ± 1 S.D. (μm, n = 12 per group). **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle-only control group. (C) Representative clinical features of mice of each treatment group.

Fig. 4. Hematoxylin and eosin staining in ear skin of topical application of JAK-inhibitors on TDI-induced allergic dermatitis. (A, B) Histological 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 ± 1 S.D. (n = 6~12 per group). **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle-only control group. (C) Typical microscopic features of skin with hematoxylin and eosin staining. Bar = 50 μm.

Fig. 5. Down-regulated cytokine levels in ear skin 24 h following topical application of JAK-inhibitors. Concentrations of (A) IL-1β, (B) IL-4, (C) IL-6, (D) TARC were determined by ELISA. Results are expressed as mean ± 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 comparison test) vs. vehicle control group.
**Fig. 6.** Up-regulated cytokine levels in ear skin 24 h following topical application of JAK-inhibitors. Concentrations of (A) IL-12, (B) IL-31, (C) TNFα, (D) TSLP, and (E) IP-10 were determined by ELISA. Results are expressed as mean ± 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 comparison test) vs. vehicle control group.

**Fig. 7.** Cytokine levels in dorsal (neck) skin 30 min following topical application of JAK-inhibitors and challenge with TDI. Concentrations of (A) IL-31, (B) TNFα, and (C) TSLP, were determined by ELISA. Results are expressed as mean ± 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 comparison test) vs. vehicle control group.
TABLE 1

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

Results are expressed as mean ± 1 S.D. (n = 6 per group). *: P < 0.05 and **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle-only control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total migrated cells (cells/mg ear tissue)</th>
<th>Migrated CD11c&lt;sup&gt;+&lt;/sup&gt;CD40&lt;sup&gt;+&lt;/sup&gt; cells (cells/mg ear tissue)</th>
<th>Langerhans cell density (cells/mm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-only</td>
<td>3789 ± 875</td>
<td>2184 ± 437</td>
<td>1151 ± 252</td>
</tr>
<tr>
<td>Tofacitinib 0.1%</td>
<td>2534 ± 741 *</td>
<td>1352 ± 435 *</td>
<td>1969 ± 456 **</td>
</tr>
<tr>
<td>Oclacitinib 0.1%</td>
<td>2333 ± 714 *</td>
<td>1494 ± 454 *</td>
<td>2013 ± 178 **</td>
</tr>
</tbody>
</table>
TABLE 2  
Responses in auricular lymph node (LN) of topical application of JAK inhibitors on TDI-induced BALB/c mice  
Results are expressed as mean ± 1 S.D. (n = 6–12 per group). *: P < 0.05 and **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle-only control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>LN weight (mg)</th>
<th>Total cell counts ($\times 10^6$ cells)</th>
<th>CD3$^+$ T cells ($\times 10^6$ cells)</th>
<th>CD11c$^+$CD40$^+$ cells ($\times 10^4$ cells)</th>
</tr>
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<tr>
<td>Intact</td>
<td>2.55 ± 0.30</td>
<td>2.56 ± 0.86</td>
<td>1.23 ± 0.42</td>
<td>0.61 ± 0.29</td>
</tr>
<tr>
<td>Vehicle-only</td>
<td>6.20 ± 0.86</td>
<td>6.83 ± 2.35</td>
<td>2.99 ± 1.07</td>
<td>3.70 ± 1.25</td>
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<tr>
<td>Tofacitinib 0.1%</td>
<td>6.00 ± 0.63</td>
<td>6.22 ± 1.16</td>
<td>2.78 ± 0.53</td>
<td>2.96 ± 0.85</td>
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<tr>
<td>Tofacitinib 0.25%</td>
<td>4.79 ± 0.76**</td>
<td>4.37 ± 0.87 *</td>
<td>2.03 ± 0.45</td>
<td>1.42 ± 0.55 **</td>
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<tr>
<td>Tofacitinib 0.5%</td>
<td>4.10 ± 0.75**</td>
<td>3.91 ± 1.03 **</td>
<td>1.78 ± 0.46 *</td>
<td>0.94 ± 0.51 **</td>
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<tr>
<td>Oclacitinib 0.1%</td>
<td>5.83 ± 1.24</td>
<td>6.01 ± 1.86</td>
<td>2.29 ± 0.59</td>
<td>2.25 ± 0.90 *</td>
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<tr>
<td>Oclacitinib 0.25%</td>
<td>2.89 ± 0.74**</td>
<td>2.68 ± 0.78 **</td>
<td>1.24 ± 0.32 **</td>
<td>0.73 ± 0.27 **</td>
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<tr>
<td>Oclacitinib 0.5%</td>
<td>2.62 ± 0.61**</td>
<td>2.40 ± 0.43 **</td>
<td>1.11 ± 0.17 **</td>
<td>0.49 ± 0.18 **</td>
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</table>
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 ± S.D. (pg/mL; n = 6–12 per group). *: P < 0.05 and **: P < 0.01 (Dunnett’s multiple comparison test) vs. vehicle control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-4</th>
<th>IL-6</th>
<th>TNFα</th>
<th>TARC</th>
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<tr>
<td>Intact</td>
<td>1.9 ± 2.5</td>
<td>1.85 ± 1.42</td>
<td>10.4 ± 14.0</td>
<td>1.5 ± 1.9</td>
</tr>
<tr>
<td>Vehicle-only</td>
<td>217.8 ± 166.1</td>
<td>17.51 ± 5.12</td>
<td>156.7 ± 72.6</td>
<td>205.8 ± 112.0</td>
</tr>
<tr>
<td>Tofacitinib 0.1%</td>
<td>168.2 ± 54.7</td>
<td>13.05 ± 11.26</td>
<td>124.7 ± 53.9</td>
<td>77.5 ± 60.8 *</td>
</tr>
<tr>
<td>Tofacitinib 0.25%</td>
<td>34.8 ± 41.2 **</td>
<td>5.50 ± 2.35 **</td>
<td>22.7 ± 22.8 **</td>
<td>56.8 ± 47.0 **</td>
</tr>
<tr>
<td>Tofacitinib 0.5%</td>
<td>30.3 ± 23.8 **</td>
<td>7.18 ± 3.17 **</td>
<td>40.6 ± 31.5 **</td>
<td>59.5 ± 54.0 **</td>
</tr>
<tr>
<td>Oclacitinib 0.1%</td>
<td>167.7 ± 159.4</td>
<td>9.95 ± 4.36 **</td>
<td>109.4 ± 24.2</td>
<td>75.9 ± 87.0 *</td>
</tr>
<tr>
<td>Oclacitinib 0.25%</td>
<td>26.2 ± 23.7 *</td>
<td>6.35 ± 3.44 **</td>
<td>50.5 ± 56.3 **</td>
<td>37.7 ± 31.4 **</td>
</tr>
<tr>
<td>Oclacitinib 0.5%</td>
<td>8.3 ± 7.86 **</td>
<td>3.70 ± 1.65 **</td>
<td>11.7 ± 7.2 **</td>
<td>11.8 ± 10.9 **</td>
</tr>
</tbody>
</table>
Fig. 1.
**Fig. 2.**

(A) Scratching bouts (60 min)

(B) Ear swelling (μm)
Fig. 3.
Fig. 4.
**Fig. 5.**
Fig. 6.
Fig. 7.