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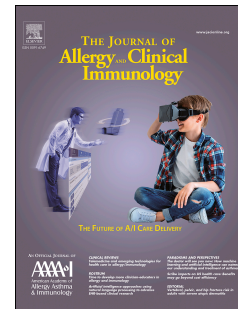
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Original Article**JAK1/2 inhibition impairs the development and function of inflammatory
dendritic epidermal cells in atopic dermatitis.**

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ABSTRACT

Background: Janus kinase (JAK) inhibitors are a new class of therapeutic compounds for dermatological diseases. In atopic dermatitis (AD), data of clinical phase III trials show rapid improvement of pruritus and significant reduction of inflammation within the first weeks with a favourable safety profile. However, their mode of action in AD is not fully understood.

Objective: In our study, we investigate the effect of different JAK inhibitors on cell differentiation, phenotype, and function of inflammatory dendritic epidermal cells (IDEC).

Methods: We analysed the JAK expression in IDEC from *ex vivo* skin and *in vitro* generated IDEC using flow cytometry and PCR. Further, we studied *in vitro* the effect of different JAK inhibitors on IDEC cell differentiation, phenotype, and maturation.

Results: IDEC express JAK1 and JAK2 *ex vivo* and *in vitro*. We found that JAK1 and JAK2 were upregulated during the differentiation from monocytes to IDEC. Conversely, JAK2 inhibition by Ruxolitinib (JAK1/2 inhibitor) or BMS-911543 (JAK2 inhibitor) abrogated the differentiation from monocytes into IDEC. Differentiated IDEC can re-differentiate into a more monocyte-like phenotype in the presence of Ruxolitinib or BMS-911543. Furthermore, we showed that concomitant inhibition of JAK1/2 rather than blocking JAK1 or JAK2 alone, impaired maturation and the release of proinflammatory cytokines upon lipopolysaccharide stimulation.

Conclusion: Our results suggest that inhibition of JAK1/2 impairs IDEC differentiation and function. We provide new insight into the mode of action of JAK inhibitors in AD and highlight the role of JAK1/2 inhibitors for the treatment of AD patients.

KEY MESSAGES

JAK1 and 2 are upregulated during the differentiation from monocytes to IDEC. JAK2 inhibition blocks the differentiation from monocytes to IDEC. Concomitant inhibition of JAK1 and JAK2 is more potent in interfering with IDEC maturation and function of IDEC than blocking JAK1 or JAK2 alone.

CAPSULE SUMMARY

JAK inhibitors show improvement of AD with favourable safety profile. However, the mechanistic role of JAK in inflammatory dendritic epidermal cells (IDEC) remains unclear. We demonstrate that JAK1/2 inhibition impairs generation and function of IDEC.

KEY WORDS

Atopic dermatitis; AD; IDEC; IL-4; IL-13 ; JAK; DC; skin; FcεR

67 **ABBREVIATIONS:**

7-AAD	7-aminoactinomycin D
AD	Atopic dermatitis
cDNA	Complementary DNA
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EC	Epidermal cell suspension
FBS	Fetal bovine serum
FCS	Fetal calf serum
FcεRI	High-affinity receptor for IgE
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IDEC	Inflammatory dendritic epidermal cells
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12p70	Interleukin 12p70
IgE	Immunoglobulin E
JAK	Janus kinase
KC	Keratinocytes
LC	Langerhans cells
LPS	Lipopolysaccharides
MACS®	Magnetic Cell Separation
2-ME	2-Mercaptoethanol
MFI	Mean fluorescence intensity
PBMC	Human peripheral blood mononuclear cells

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pIDEC	Precursor inflammatory dendritic epidermal cells
pJAK	Phosphorylated JAK
RNA	Ribonucleic acid
STAT	Signal transducer and activator of transcription (STAT) protein
TNF α	Tumor necrosis factor alpha

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease affecting up to 20% of the children and 3-8% of the adults in industrialized countries with increasing incidence over the past years¹. The pathophysiology is complex due to the interplay of genetic, immunological, and environmental factors. Recent investigations emphasize mainly 2 aspects (i): The disturbance of the epidermal barrier and (ii) chronic inflammation, both contributing to the clinical phenotype and to the sensitization to allergens^{2, 3}.

In this context, epidermal dendritic cells have been shown to play a major role in bridging innate and adaptive immunity⁴. While resident FcεRI positive Langerhans cells (LC) are responsible for controlling the epidermal-derived tolerance, the FcεRI positive inflammatory dendritic epidermal cells (IDEC) are assumed to be important for the generation and maintenance of the inflammatory reaction⁵⁻⁷.

As in other atopic diseases, the acute inflammatory reaction in AD is dominated by a Th2 immune response and its associated cytokines IL-4 and IL-13⁸. Both cytokines decrease the production of skin barrier proteins such as filaggrin and thereby further alter the skin barrier dysfunction⁹⁻¹¹. However, recent findings suggest that IL-13 may be more important than IL-4 in AD¹².

JAK are a family of cytoplasmatic tyrosine kinases comprising JAK1, JAK2, JAK3, and TYK2 that can associate to type I and type II cytokine receptors. Each cytokine receptor recruits specific combinations of JAK dimers or trimers to the receptor and thereby determinates the signalling cascade¹³. After ligation of a cytokine, JAK get trans-phosphorylated and concomitantly phosphorylate and thereby activate STAT molecules. Once activated, STAT dimers translocate into the nucleus to regulate gene transcription^{14, 15}.

The JAK-STAT pathway plays a central role in haematopoietic cells such as natural killer cells, T cells, and dendritic cells (DC)¹⁶. Whereas loss-of-function mutation of JAK1 and JAK2 is lethal in mice, JAK3 deficiency in mice is associated with impaired lymphocyte development and explains why JAK3 deficiency in humans leads to the clinical presentation of severe combined immunodeficiency syndrome^{17, 18}. In contrast, TYK2-deficient T cells in mice enhance the Th2 response with an increased release of IL-4 and immunoglobulin E (IgE)¹⁹. This elevated Th2 response was observed in a patient with an inactivating TYK2 mutation, severe AD, and high levels of IgE²⁰.

In vitro studies show that pan JAK inhibitors targeting JAK1/2 decrease the number, activation and function of various haematopoietic cells, including T cells, B cells, DC, and mast cells²¹⁻²⁴. In keratinocytes JAK1/2 inhibition reduces hyperplasia and protects keratinocytes from cytotoxic responses²⁵. The JAK2 pathway regulates the expression of MHCs in keratinocytes and initiates the innate immune response in DC^{26, 27}. In a DNCB-induced AD mice model it was shown that a topical JAK1/2 inhibitor reduced the activation of DC, reduced serum IgE levels and decreased skin inflammation²⁸. However, little is known about the distinct effects of selective JAK1, JAK2 and JAK3 inhibitors on immune cells in AD patients since recent clinical trials focused either only on JAK1 selective (Abrocitinib, Upadacitinib) or on pan JAK inhibitors (Ruxolitinib, Baricitinib, Delgocitinib, Tofacitinib).

In this study, we investigate the roles of IL-4 and IL-13 in an *in vitro* model of IDEC and analyse the impact of different pan and selective JAK inhibitors on cell differentiation and function.

METHODS

Patients

Healthy skin was obtained from non-atopic donors undergoing plastic surgery in the Department of Gynaecology, University Hospital Bonn (n=34; age 21-71 years). Caucasian patients with AD (n=22; age 21-82 years; mean total IgE 0.126-18.615kU/ml) according to the criteria of Hanifin and Rajka were included in the study³⁰. They had untreated acute moderate to severe AD, mean SCORAD 55±18. This study was approved by the local ethics committee of the University of Bonn and performed in accordance with the declaration of the Helsinki principle. Informed consent was obtained from all of the participants in this study.

Media and reagents

For IDEC generation and culture, we used RPMI VLE medium supplemented with 500IU/ml human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sanofi-Aventis, NJ, USA), 10uM 2-Mercaptoethanol (2-ME) (Sigma-Aldrich, St. Louis, MO, USA), 10ug/ml IgE (Merck Millipore, Darmstadt, Germany), 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotics/antimycotics (Gibco, Karlsruhe, Germany) as described previously³¹.

JAK inhibitors

For *in-vitro* experiments, JAK inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium in different concentrations ranging from 0.01-1µM according to therapeutic concentrations in plasma-levels of treated patients with Ruxolitinib 10mg tablets³². Beside the pan JAK inhibitor Ruxolitinib (JAK1/2,

InvivoChem, Vernon Hills, IL, USA), the specific JAK inhibitors, Filgotinib (JAK1), BMS-911543 (JAK2), and Decernotinib (VX-509) (JAK3) (InvivoChem, Vernon Hills, IL, USA) were investigated. We added DMSO in equal concentrations as negative control. Prior to our experiments, we investigated apoptosis and cell death using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) or 7-aminoactinomycin D-annexin V (7AAD) (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions (supplement Figure E4).

Immunohistochemistry

For Immunohistochemistry (IHC), we included 5 healthy controls and 5 AD-patients. The staining was performed using the REAL Detection System Alkaline Phosphatase (DAKO, Glostrup, Denmark) with Fast Red as chromogen, pJAK1 (Antibodies online, Lot 8715, ABIN196868, dilution 1:3000) and the pJAK2 (Antibodies online, Lot 8715, ABIN196872, dilution 1:50).

Preparation of ex vivo skin and epidermal cell suspensions

Skin from AD patients was taken by shave biopsy from lesional skin. The skin was cut in 1cm² squares and added to 0.5% trypsin (Sigma-Aldrich, St. Louis, MO, USA). Epidermal cell suspension (EC) was vortexed and filtered through 100µm Nylon filters (Falcon, NY, USA) as described previously³³.

Monocyte isolation and in vitro-generation of human inflammatory epidermal dendritic cells (IDEC)

Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of AD patients by NycoPrep™ (ProGen, Heidelberg, Germany) density-gradient centrifugation according to the manufacturer's protocol. Purity of monocyte

isolation was confirmed by flow cytometry (>90% CD14⁺ cells). 1 million monocytes per ml RPMI VLE medium were seeded in 24 well plates. Cells were cultured with human recombinant IL-4 (75ng/ml, Miltenyi Biotec, Auburn, CA, USA) or IL-13 (100ng/ml, PromoKine, Heidelberg, Germany). IL-13 or IL-4 were added with GM-CSF, IgE and 2-ME to the culture medium on day 0, 2 and 4. On day 6, we exposed IDEC to lipopolysaccharides (LPS) (100ng/ml, InvivoGen, San Diego, CA, USA) for 24 hours to achieve final maturation as described previously³¹.

Flow cytometry

Samples were measured on BD Canto (Becton Dickinson, San Jose, CA, USA) and analysed by Diva software or FlowJo (Becton Dickinson, San Jose, CA, USA). For surface staining, dead cells were excluded by 7-AAD. For intracellular staining, we permeabilized cells with BD Cytofix/Cytoperm kit according to manufacturer's manuscript (Becton Dickinson, San Jose, CA, USA). IDEC isolated from *ex vivo* skin were classified CD1a⁺CD207⁻ and LC CD1a⁺CD207⁺ cells. Monocytes were gated by forward, sideward scatter (FSC/SSC), and CD14⁺. *In vitro* human monocyte derived precursor IDEC (pIDEC) were identified as CD1a⁺CD14⁺, finally differentiated IDEC as CD1a⁺CD14⁻ cells. The MFI was calculated $MFI_{(target)} - MFI_{(isotype)}$. Detailed gating strategy is provided in the Online Repository Figure E1. Antibodies are summarized in the Online Repository Table E1.

RNA isolation and quantitative real-time PCR (pPCR)

Day 7 IDEC were MACS®-sorted for CD1a⁺ (purity >90%) using CD1a beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and embedded in TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). For RNA isolation, we used DNase-free kit (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's

protocol. cDNA was prepared with SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed using iTaq Universal SYBR Green Supermix (Bio-RAD, Hercules, CA, USA) on StepOne Plus Real-Time PCR System. Data analysis was performed on StepOne software v.2.3. (Thermo Fisher Scientific, Waltham, MA, USA) according to $\Delta\Delta CT$ method (relative quantity, RQ). Results were normalized to β -actin encoding mRNA-levels. The oligonucleotides (5'-3') are summarized in Table E3 in the Online Repository.

Determination of cytokine production

Secretion of TNF α , IL-10, and IL-6 by IDEC was analysed in the cell supernatants using a commercially available enzyme-linked immunosorbent assay from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's manuscript. Data readout was performed by BioTek Synergy HT (Winooski, VT, USA).

Statistical analyses

Data are shown as mean $\pm$ SEM. To analyse statistical significance, we used Student *t*-test or Mann-Whitney test. Normal distribution was confirmed by D'Agostino & Pearson omnibus normality and Shapiro-Wilk normality test. Data analysis was performed with Prism ver. 6.01 (1992-2012 GraphPad Software, Inc., San Diego, CA, USA). *P* < .05 was considered significant.

RESULTS

JAK1 and JAK2 are activated in epidermal cells with dendritic morphology.

In a first set of experiments, we investigated the expression of JAK1, JAK2, JAK3, and TYK2 on LC and IDEC from *ex vivo* skin by flow cytometry (Figure 1A). Whereas JAK1 and JAK2 were strongly expressed on LC and IDEC, JAK3 and TYK2 expression was low or not detectable. Although there was no difference in the expression of JAK in LC from healthy donors compared to AD patients, immunohistochemical staining of phosphorylated JAK1 (pJAK1) and pJAK2 on human skin samples revealed a strong activation of epidermal and dermal cells with dendritic morphology in AD patients (Figure 1B).

Inflammatory dendritic cells show a high expression of JAK1 and JAK2 *in vitro*.

Unlike LC, IDEC are absent in healthy skin. In the next step, we investigated whether JAK expression is differently regulated during the differentiation period from monocytes to IDEC. In our *in vitro* model, we studied the JAK expression on monocytes on day 0, precursor IDEC (pIDEC) on day 3, and finally differentiated IDEC on day 7 (Figure 2A). Whereas JAK1 was expressed in >90% of monocytes, pIDEC, and IDEC, JAK3 and TYK2 expression remained low or not detectable accordant to our *ex vivo* results. Interestingly, JAK2 expression increased within the first three days, whereas JAK1 was upregulated in the second differentiation period from day 3 up to day 6 (Figure 2B). The high expression of JAK1, JAK2 and low expression of JAK3 were equally found at the mRNA level of finally differentiated IDEC (Figure 2C).

IDEC can be generated with IL-4 or IL-13.

In established *in vitro* models, monocyte-derived DC are generated with IL-4. However, IL-13 is believed to be the more prominent cytokine in the skin in AD. Since IL-4 type II receptors can equally be activated by IL-4 or IL-13, we were interested whether IDEC can be generated from monocytes if we replace IL-4 with IL-13 in the cell culture medium. During the first 2 days, the number of pIDEC (CD1a⁺CD206⁺) increased independent of stimulation with IL-4 or IL-13 (Figure 3A). On day 4, the number of CD1a⁺ cells reached their maximum. IDEC generated with IL-4 and IL-13 showed the same shift from CD14⁺ to CD1a⁺ cells within 6 days and expressed the same surface and maturation markers in response to LPS (Figure 3B-C).

JAK2 inhibition blocks the differentiation of IDEC generated with IL-4 or IL-13.

Given that JAK1 and JAK2 are highly expressed in IDEC *in vitro* and *ex vivo*, we next investigated the effect of JAK1 and JAK2 inhibition by Ruxolitinib, a JAK1/2 inhibitor, over 4 days in our established IDEC model under IDEC driving conditions using IL-4. Ruxolitinib-exposed monocytes maintained a monocyte-like phenotype. Accordingly, the cells failed to gain CD1a and CD206 expression and remained CD14⁺ (Figure 4A). Next, we checked whether Ruxolitinib treatment could equally interrupt the development from monocytes to IDEC if the cell culture was supplemented with IL-13 instead of IL-4. A single application of 1μM Ruxolitinib (JAK1/2 inhibitor) on day 0 prevented the transformation from CD14⁺ monocytes into CD1a⁺CD206⁺ pIDEC (Figure 4B). To investigate whether this effect can be explained by the potency of Ruxolitinib to block either JAK1 or JAK2, we subsequently chose more selective JAK inhibitors against JAK1, JAK2, and JAK3. Contrary to the Filgotinib (JAK1) and VX-509 (JAK3), only the JAK2 inhibitor BMS-911543 caused a complete suppression of CD1a⁺ cells comparable to the prior effect of Ruxolitinib (Figure 4C).

Precursor IDEC lose phenotypical properties in response to JAK2 inhibition.

In order to investigate if the differentiation into IDEC can be reversed, we analysed the effect of different JAK inhibitors on precursor IDEC (pIDEC). In the presence of Ruxolitinib (JAK1/2) or BMS-911543 (JAK2), cells lost their CD1a and CD206 surface markers and re-expressed CD14, indicating a re-differentiation into a monocyte like phenotype (Figure 5A-B). This effect was equally found in finally differentiated and LPS-activated IDEC, which showed a dose dependent re-expression of CD14 and a reduced CD1a expression in response to increasing concentrations of Ruxolitinib and BMS (Figure 5C). Surprisingly, the FcεRI was significantly downregulated in response to all JAK inhibitors with the strongest effect for JAK2 inhibition (Figure 5A-B).

Inhibition of JAK1/2 reduces LPS induced maturation of IDEC.

Next, we focussed on finally differentiated and LPS-activated IDEC and analysed their maturation capacity in response to different concentrations of Ruxolitinib [0.01μM, 0.1μM, 0.5μM, 1μM] and BMS-911543 [0.01μM, 0.1μM, 1μM]. The dose of 0.5μM Ruxolitinib was enough to significantly decrease CD80 and CD86 compared to LPS activated and DMSO treated IDEC (Figure 6 A). In contrast to JAK1/2 inhibition, the selective inhibition of JAK2 was less effective but also reduced the expression of CD80 significantly (Figure 6B).

JAK1/2 inhibition reduces the release of proinflammatory cytokines by IDEC.

To unravel the impact of JAK inhibitors on IDEC function and their contribution to inflammation in AD, we analysed the effect of JAK inhibition on cytokine release in response to different concentrations of JAK1/2 or JAK2 inhibitors after LPS stimulation. TNFα and IL-6 are important proinflammatory cytokines in AD³⁴⁻³⁷. Both cytokine levels are high in lesional skin of AD patients and correlate with disease

severity³⁸⁻⁴⁰. IL-10 is a Th2-driving cytokine that dampens the production of anti-microbial peptides⁴¹. In line with our previous results, both JAK1/2 and JAK2 inhibition decreased the levels of TNF α and IL-10 (Figure 7). However, the strongest effect was achieved by the combination of JAK1 and JAK2 inhibition. IL-6 cytokine release was actually only impaired when JAK1 and JAK2 were inhibited simultaneously (Figure 7A).

DISCUSSION

Despite clinical improvement of inflammation and pruritus in on-going clinical trials with pan or selective JAK inhibitors, the mode of action of these compounds in the treatment of AD has not been fully understood. In this study, we could show that JAK1 and JAK2 are expressed in epidermal LC and IDEC in healthy skin and AD patients. Immunohistochemical staining reveals a strong activation of JAK1 and JAK2 on cells with dendritic morphology in the upper dermis and epidermis in AD skin, which is absent in healthy controls. Conversely, JAK3 and TYK2 are only weakly expressed or not detectable on LC and IDEC of both healthy and AD patients.

IDEC have been shown to be proinflammatory DC and to play a pivotal role in AD. Whereas LC derive from erythro-myeloid precursors, are self-renewed and more involved in tolerance induction, it is assumed that IDEC derive from peripheral blood monocytes and differentiate into IDEC in peripheral inflamed tissues⁴². It has been shown previously that JAK inhibitors impair DC differentiation²¹. We could demonstrate that JAK2 is upregulated during the first three days of differentiation from monocytes to IDEC *in vitro* whereas JAK1 is constantly expressed in almost all cells. Given that JAK1 is linked to IL-4R α , JAK2 to IL-13R α 1, and JAK3 to IL-2R γ , the high expression of JAK1 and upregulation of JAK2 indicate that IDEC differentiation is preferentially mediated upon IL-4 type II (IL-4 α , IL-13 α 1) receptor and not IL-4 type I signalling (IL-4 α , IL-2R γ)⁴³. Consistently, Lutz et al. showed that DC maturation is maintained in JAK3 knockout mice supporting that IL-4R type II signalling is preferentially responsible for the differentiation from monocytes to DC⁴⁴. This hypothesis is further supported by the observation that IDEC show the same phenotypical surface marker (CD1a, CD206, CD11a, CD11b, CD1b, CD1c, Fc ϵ R1 α) and maturation marker (CD80, CD83, CD86) expression in response to LPS stimulation regardless whether the cells were generated with IL-4 or IL-13.

We showed that JAK1 and JAK2 are highly expressed in IDEC whereas JAK3 and TYK2 expression were only low or not detectable. This observation tempted us to investigate the effect of JAK1/2 inhibition on IDEC cell biology and functionality. We demonstrated that inhibition of JAK1/2 and JAK2 but not of JAK1 or JAK3 alone blocks the differentiation from monocytes into CD1a⁺CD206⁺ pIDEC, indicating that especially JAK2 was responsible for this effect. The likewise delayed response to Filgotinib (JAK1) and VX-509 (JAK3) could be explained by a partial potency against JAK2 at high concentrations but this effect was abrogated on day 4 (Figure 4C). The interruption of monocyte differentiation could derive from the inhibition of IL-4/IL-13 cytokine signalling since these cytokines are crucial for DC differentiation. Already differentiated IDEC re-expressed CD14 and lost CD1a, CD206 positivity in the presence of Ruxolitinib (JAK1/2) and BMS-911543 (JAK2), suggesting a bidirectional development, a re-differentiation into a more monocyte-like phenotype. Furthermore, we showed that inhibition of all JAK subgroups resulted in lower expression of FcεRIα even though the most prominent effect was observed for JAK2 inhibition. This receptor on IDEC is especially important for controlling allergic reactions and initiate inflammation by secretion of proinflammatory cytokines and chemokines⁴⁵. The FcεRIα enables IDEC to mediate IgE-dependent allergen presentation to T cells^{46, 47}. Therefore, low expression of FcεRI especially by JAK2 inhibitors could render IDEC less susceptible to allergic stimuli. Besides phenotypical changes, finally differentiated and LPS-activated IDEC showed a reduced maturation capacity and lower release of proinflammatory cytokines after treatment with JAK1/2 and JAK2 inhibitors. This observation is in line with previous studies on JAK2 knockout mice where deletion of JAK2 resulted in significantly less potent responses to stimulate DC maturation²⁷. Additionally, an impairment of DC differentiation and function by the JAK1/2 inhibitor Ruxolitinib was previously

demonstrated on murine and human PBMC²¹. In contrast to this study, IDEC are characterized by the expression of distinct surface markers, such as CD206 and FcεRI which are important for mediating allergic reactions or bacterial defence⁴⁸. Although DC generated from PBMC are known to produce high amounts of IL-12 in response to LPS stimulation, IDEC failed to release IL-12p70 in our experimental setup, although the secretion of other proinflammatory cytokines such as TNFα, IL-10, and IL-6 were LPS-dependently increased as expected (data not shown). This could be due to a unique distinct function of IDEC to prime T cells into a Th2 rather than a Th1 helper cell type. However, Novak *et al.* described a high release of IL-12 in response to FcεRI stimulation in IDEC, suggesting that IDEC are able to release IL-12 but might fail to release IL-12 in response to LPS stimulus⁴⁹. Another explanation could be the different cytokine milieu. Contrary to both studies, we generated IDEC with IL-13 instead of IL-4. This hypothesis is supported by results of another group showing that secretion of IL-12p70 in response to LPS was only induced by IL-4 but not by IL-13 in human PBMC and in mice. Accordingly, JAK3 knockout mice showed altered concentrations of IL-12p70 after stimulation with IL-4 suggesting that mainly IL-4 type I receptor was responsible for IL-12p70 release and not IL-4 type II⁴⁴. Additionally, we demonstrate that cytokine release of TNFα and IL-10 was dose dependently reduced by Ruxolitinib and BMS-911543. However, JAK1/2 inhibition appeared to be more effective than targeting JAK2 alone. IL-6 was actually not impaired by JAK2 but by JAK1/2 inhibition suggesting an amplified effect on cytokine release of simultaneous inhibition of JAK1 and JAK2. Consistently, the impact of JAK inhibitors on IDEC phenotype was strongest for concomitant JAK1/2 inhibition.

Taken together, our results provide an explanation how JAK inhibitors may decrease inflammation in AD. In particular, JAK2 inhibitors can reduce the number of IDEC,

convert their phenotype, and impair IDEC cell function similar to the effect of JAK1/2 inhibitors on T cells, B cells, and mast cells²¹⁻²⁴. Data from clinical phase 2 and 3 trials for moderate-to-severe AD show that selective JAK1 inhibitors, such as Upadacitinib and Abrocitinib, achieve good clinical responses comparable or even superior to the effect of Dupilumab⁵¹. However, similar to enhanced clinical responses with increasing drug dosages, reduced haemoglobin levels were dose-dependently observed⁵². Since JAK2 interferes with erythropoiesis, the enhanced potency of the higher dosage may be attributed to a partly potency against JAK2 at high dosage^{16, 53}. Therefore, the use of topical formulations is of special interest. Clinical trials of topical JAK1/2 inhibitors, such as Ruxolitinib demonstrate EASI reduction of 71.6% vs. 15.5% (placebo) within 4 weeks without haematopoietic side effects^{54, 55}. Another topical JAK1/2 inhibitor Delgocitinib was approved for AD in Japan confirming the effect of JAK1/2 inhibition in AD. We will need to understand the effect of selective JAK1 and JAK2 inhibitors on other cells that contribute to the inflammatory reaction in AD, such as Langerhans cells, B cells, T cells, and keratinocytes to understand the global impact of JAK inhibition on the pathogenesis of AD.

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FIGURE LEGENDS

Figure 1. JAK1 and JAK2 are activated in epidermal dendritic cells.

A) Epidermal cell suspension was prepared from split skin of healthy controls (HC) or atopic dermatitis (AD) patients and stained for CD1a and CD207 to discriminate CD1a⁺CD207⁺ Langerhans cells (LC), CD1a⁺CD207⁻ inflammatory dendritic epidermal cells (IDEC) and CD1a⁻ keratinocytes (KC) using flow cytometry (left plot). Dot plots (right) show MFI of intracellular JAK1, JAK2, JAK3, TYK2 expression on LC (circlet) and IDEC (triangle) analysed by flow cytometry. Bar graphs represent mean $\pm$ SEM for HC n=8 (white) and AD n=8 (grey) patients. MFI was calculated $MFI_{(target)} - MFI_{(isotype)}$. B) Immunohistochemical staining of phosphorylated JAK1 (pJAK) and JAK2 (pJAK2) on HC and AD human skin. The arrowheads point to pJAK^{pos.} cells with dendritic morphology in dermis and epidermis of AD skin.

Figure 2. IDEC show a high expression of JAK1 and JAK2.

Monocytes were isolated from blood of AD patients and cultivated with GM-CSF, IgE, 2-ME and IL-4 for 7 days. A) Cells were discriminated using flow cytometry on day 0, 3 and 7. Monocytes were determined CD14⁺CD1a⁻, precursor IDEC (pIDEC) CD1a⁺CD14⁺ and finally differentiated IDEC CD14⁻CD1a⁺. B) Intracellular expression of JAK1, JAK2, JAK3 and TYK2 was analysed *in vitro* on monocytes (day 0), pIDEC (day 3) and IDEC (day 7) by flow cytometry. Experiments were performed in triplicates. Bar graphs show mean $\pm$ SEM. C) mRNA expression of JAK1, JAK2, JAK3 and TYK2 relative to actin-levels of monocytes and CD1a sorted IDEC (n=6). Bar graphs show mean $\pm$ SEM. The significance was calculated according to Student *t*-test. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

Figure 3. IL-13 is similarly effective as IL-4 in the generation and maturation of IDEC.

Monocytes were cultivated with IL-4 [75ng/ml] or IL-13 [100ng/ml] under IDEC-driving conditions as described previously. A) Differentiation from monocytes (CD14⁺CD1a⁻) to IDEC (CD14⁻CD1a⁺,CD206⁺) in response to IL-4 or IL-13 supplementation to the culture medium. Flow cytometric analysis was performed on day 0, 1, 2, 4 and 6. B) Expression of phenotypic surface markers and C) maturation markers on finally differentiated and for 24h LPS [100ng/ml] activated IDEC on day 7. Bar graphs show mean $\pm$ SEM of three AD donors.

Figure 4. JAK2 inhibition blocks the differentiation into inflammatory dendritic epidermal cells generated with IL-4 or IL-13.

A) On day 0, monocytes were treated with either DMSO control or Ruxolitinib 1 μ M (JAK1/2 inhibitor), cultivated over 4 days with IL-4 and analysed for the surface expression of CD1a, CD206, and CD14 using flow cytometry on day 0, 1, 2, 3, 4. B) IDEC were generated with IL-13 and treated with either DMSO control or Ruxolitinib 1 μ M over 4 days. A+B) Graphs (left) show mean $\pm$ SEM of 3 AD donors. Dot plots (right) show cell differentiation of one AD donor representative for 3. C) Expression of DC-surface markers on pIDEC generated with IL-13 and treated with specific JAK inhibitors: Filgotinib 1 μ M (JAK1), BMS-911543 1 μ M (JAK2), VX-509 1 μ M (JAK3). Results from one donor representative for 3 are shown.

Figure 5. Precursor inflammatory dendritic epidermal cells lose phenotypical properties in response to JAK2 inhibition.

A) IDEC were generated with IL-13 supplementation under IDEC driving conditions. On day 4, cells were exposed for 48 hours to different JAK inhibitors: Ruxolitinib 1 μ M

(JAK1/2), Filgotinib 1 μ M (JAK1), BMS-911543 1 μ M (JAK2), VX-509 1 μ M (JAK3). Phenotypical surface markers were analysed by flow cytometry on day 6. Box plots represent mean $\pm$ min to max of 6 AD patients. The significance was calculated according to the Student *t*-test. **P* < .05, ***P* < .01, ****P* < .001. B) Histograms depict expression levels of DC-surface markers relative to isotype control (black graph) of one representative patient. C) On day 6, IL-13 generated IDEC were activated with LPS [100ng/ml] and subsequently exposed to either Ruxolitinib [0.1-1 μ M] or BMS-911543 [0.1-1 μ M] for 24 hours. Representative dot plots show percentage of monocytes, pIDEC, and IDEC in response to different concentrations of Ruxolitinib or BMS-911543.

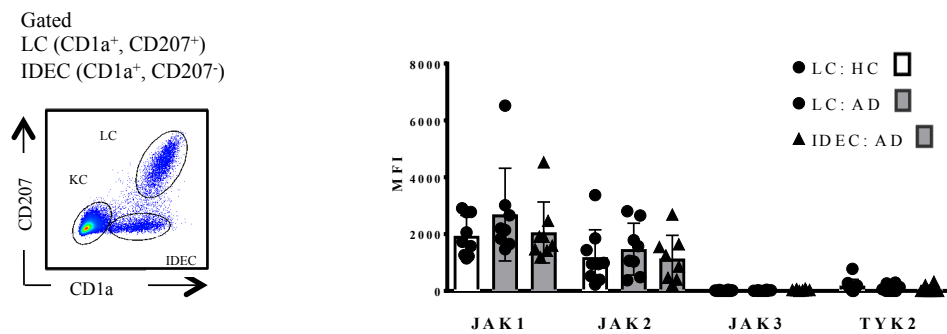
Figure 6. JAK1/2 inhibition reduces LPS induced maturation of inflammatory dendritic epidermal cells.

On day 6, IL-13 generated IDEC were exposed for 24 hours to LPS [100ng/ml] and treated with DMSO, different concentrations of A) Ruxolitinib (JAK1/2) [0.01 μ M, 0.1 μ M, 0.5 μ M, 1 μ M] or B) BMS-911543 (JAK2) [0.01 μ M, 0.1 μ M, 1 μ M] and analysed for surface expression of maturation markers using flow cytometry. Bar graphs show mean $\pm$ SEM for at least 4 experiments. The significance was calculated using the Student *t*-test. **P* < .05, ***P* < .01, ****P* < .001.

Figure 7. JAK2 inhibition reduces the release of proinflammatory cytokines by IDEC.

Monocytes were cultivated with IL-13 [100ng/ml] under IDEC-driving conditions as described before. On day 6, IDEC were exposed to LPS and treated with A) Ruxolitinib (JAK1/2) or B) BMS-911543 (JAK2) for 24 hours. Supernatants were harvested and analysed for the release of TNF α -, IL-10-, and IL-6-levels using an

enzyme-linked immunosorbent assay. Bar graphs show mean $\pm$ SEM of A) $n=5$ and B) $n=4$ AD donors. The significances were calculated according to the Mann-Whitney test. $*P < .05$, $**P < .01$.

A**B**

pJAK1

pJAK2

HC

AD

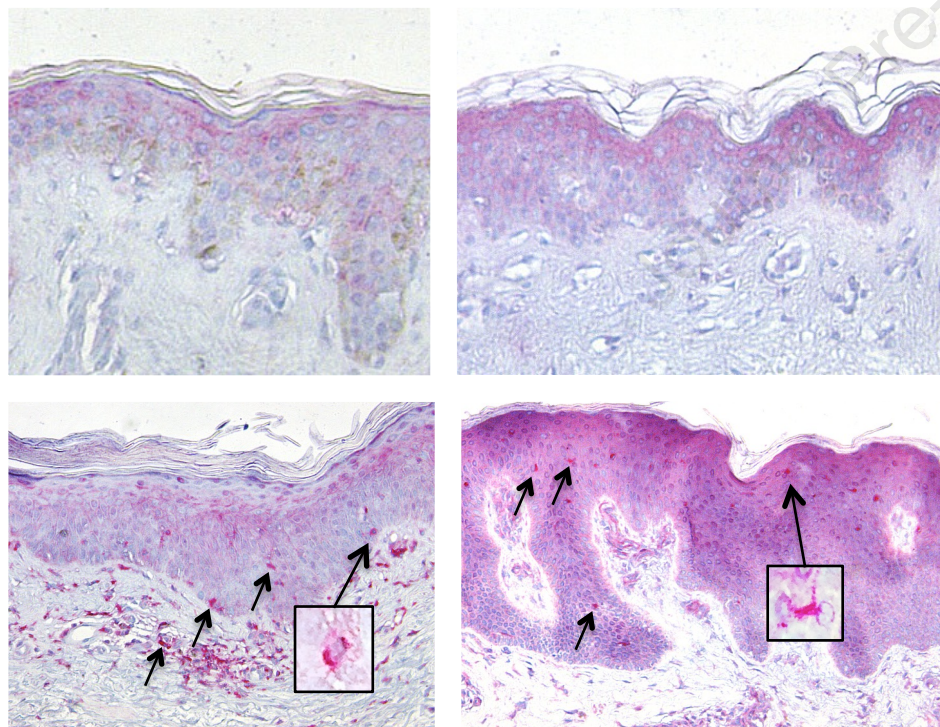


Figure 1

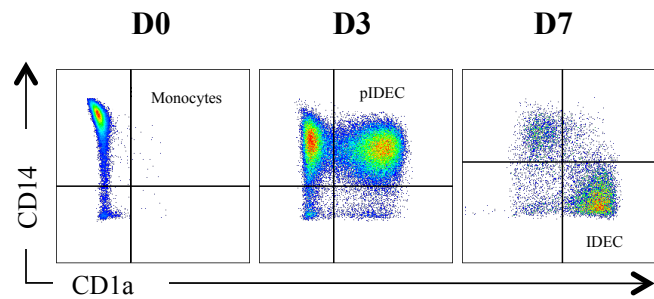
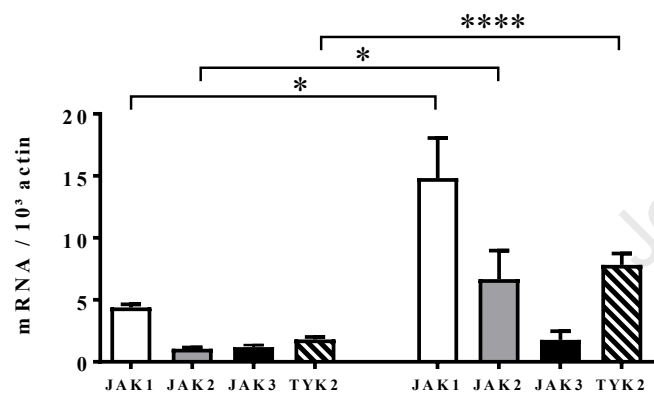
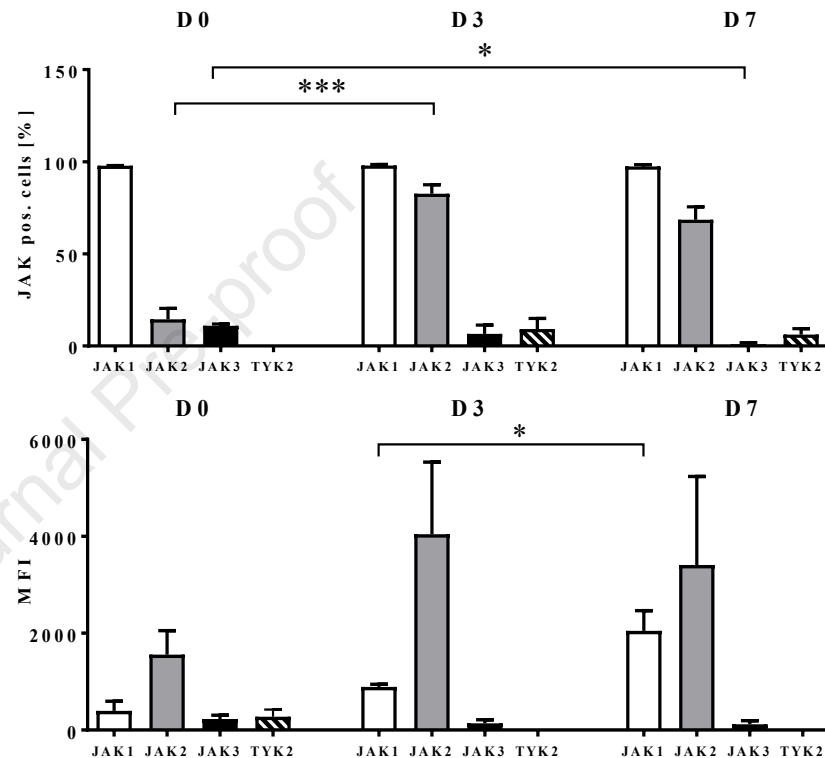
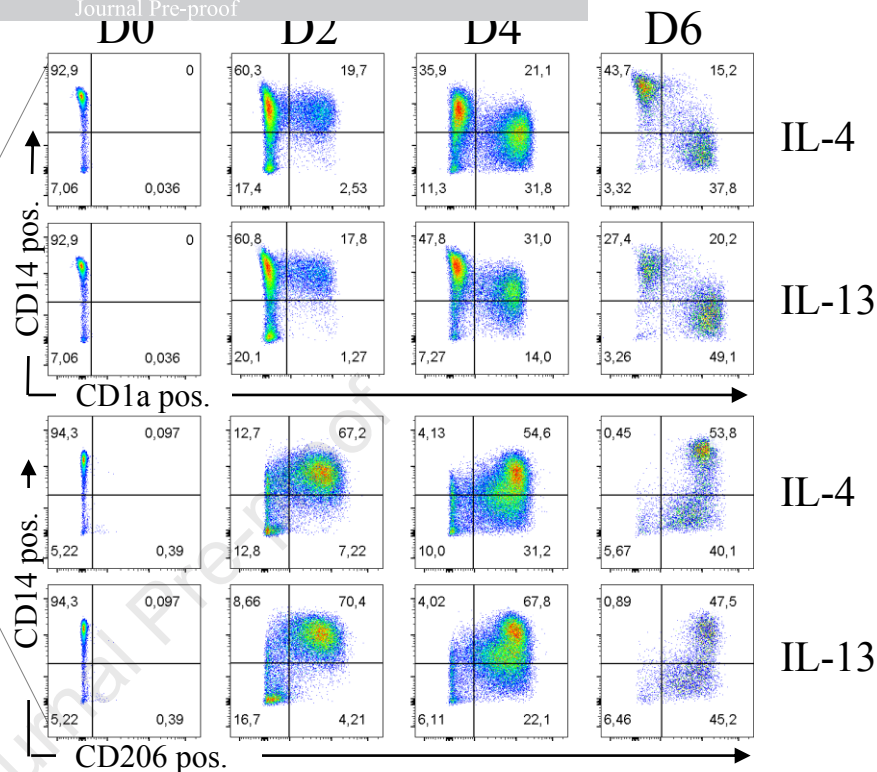
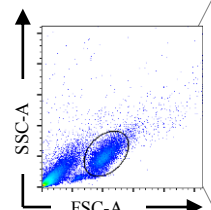
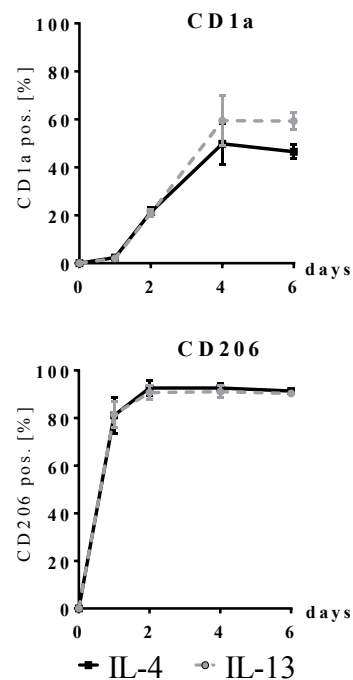
A**C****B**

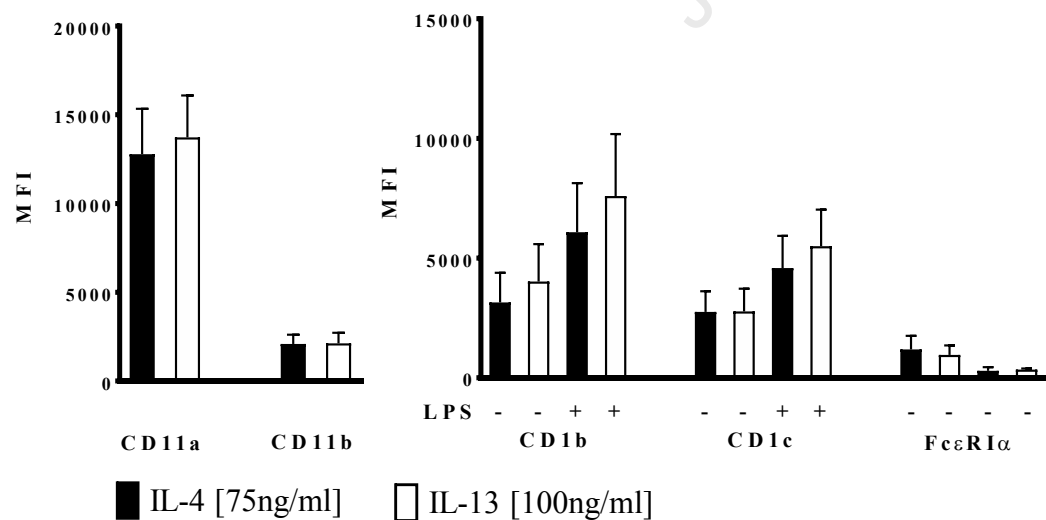
Figure 2

A

Journal Pre-proof



B



C

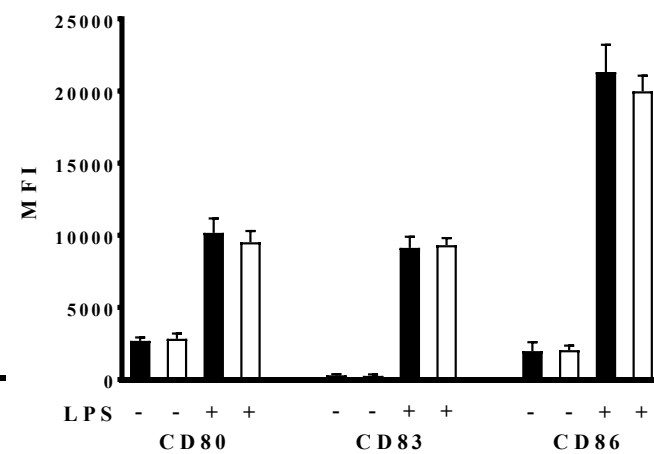


Figure 3

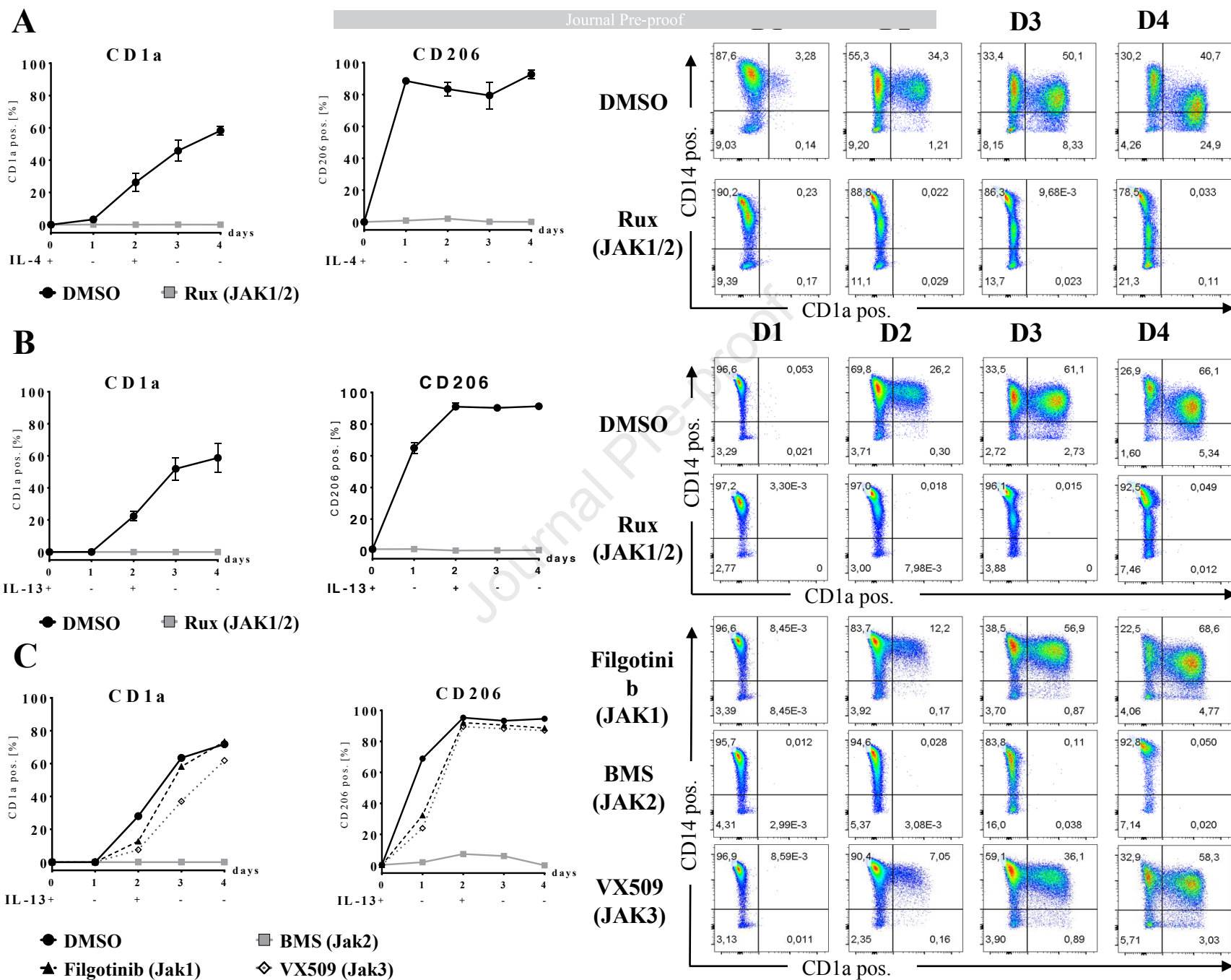


Figure 4

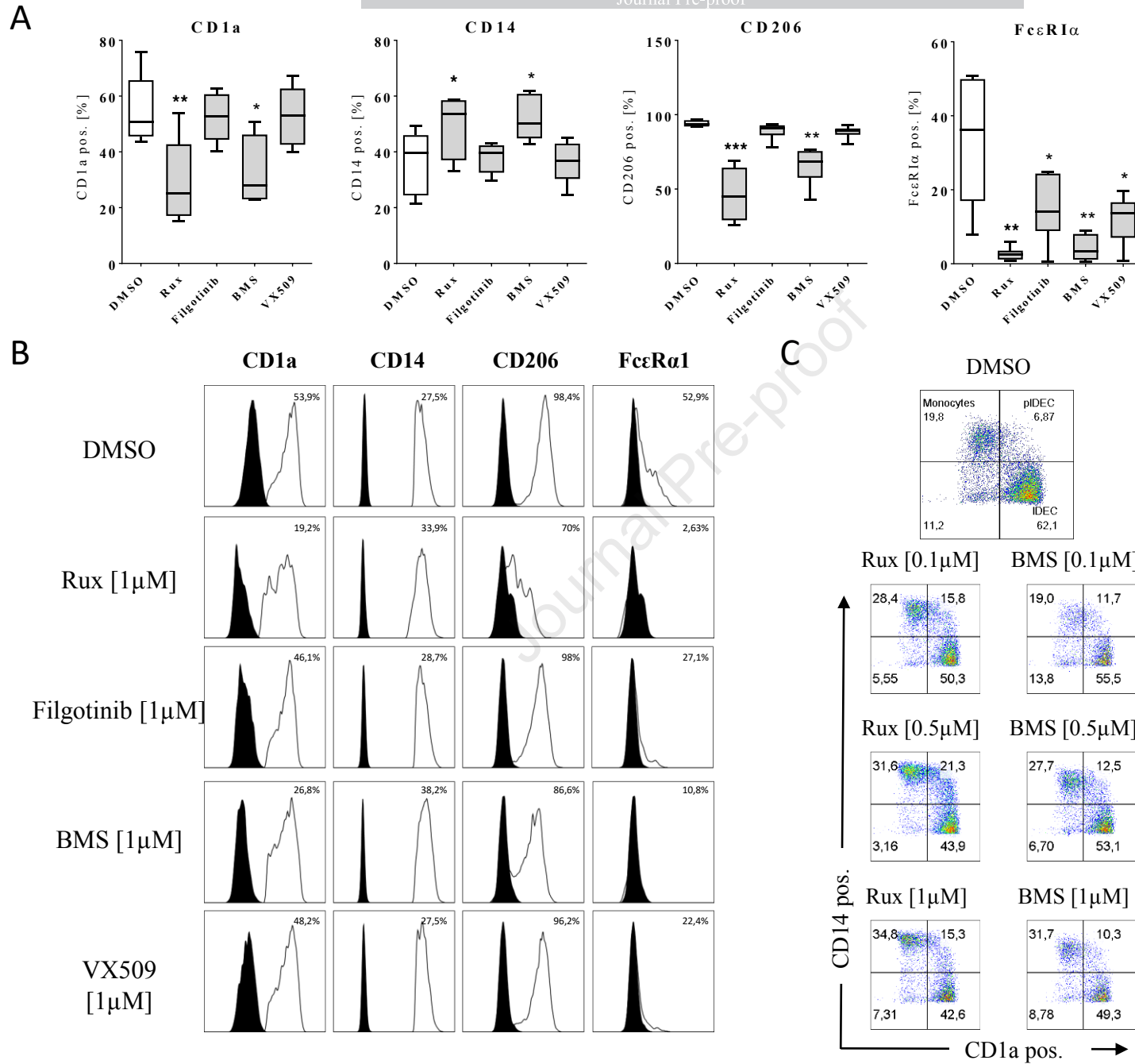


Figure 5

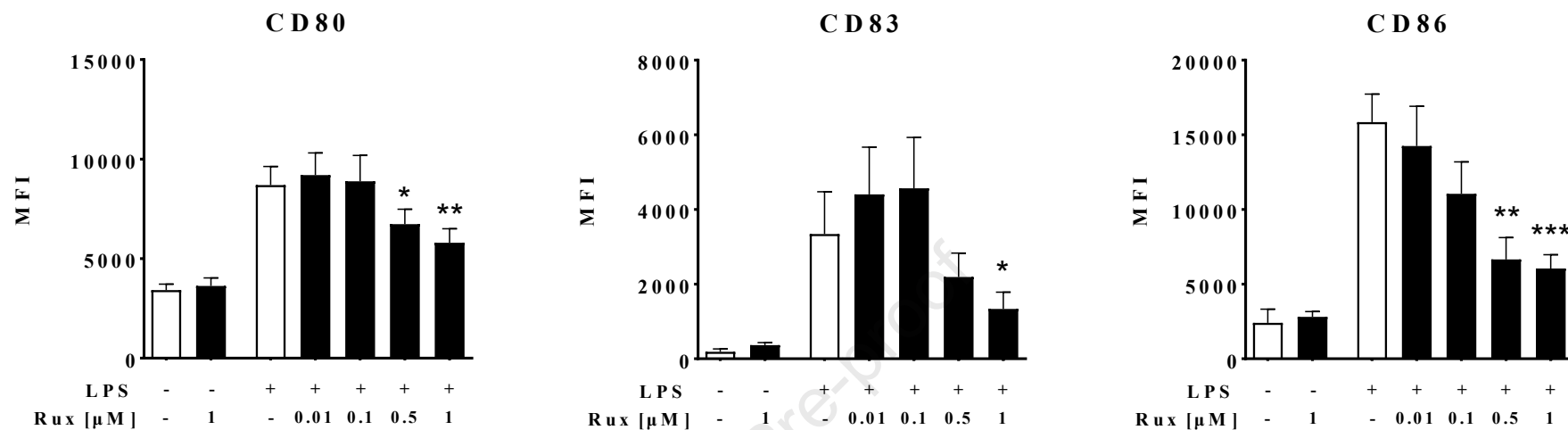
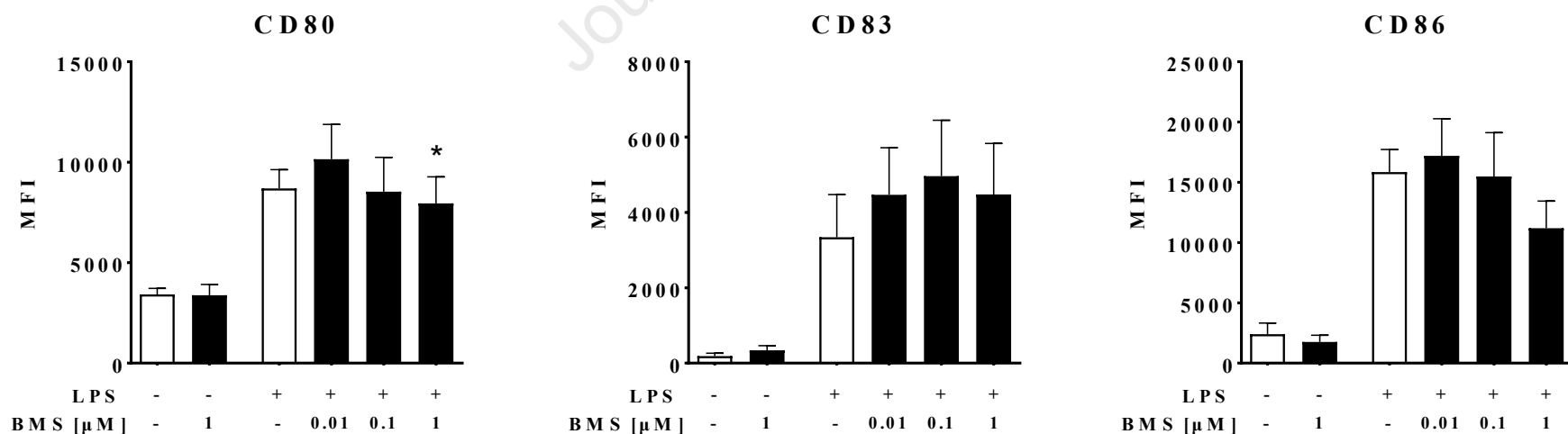
A**B**

Figure 6

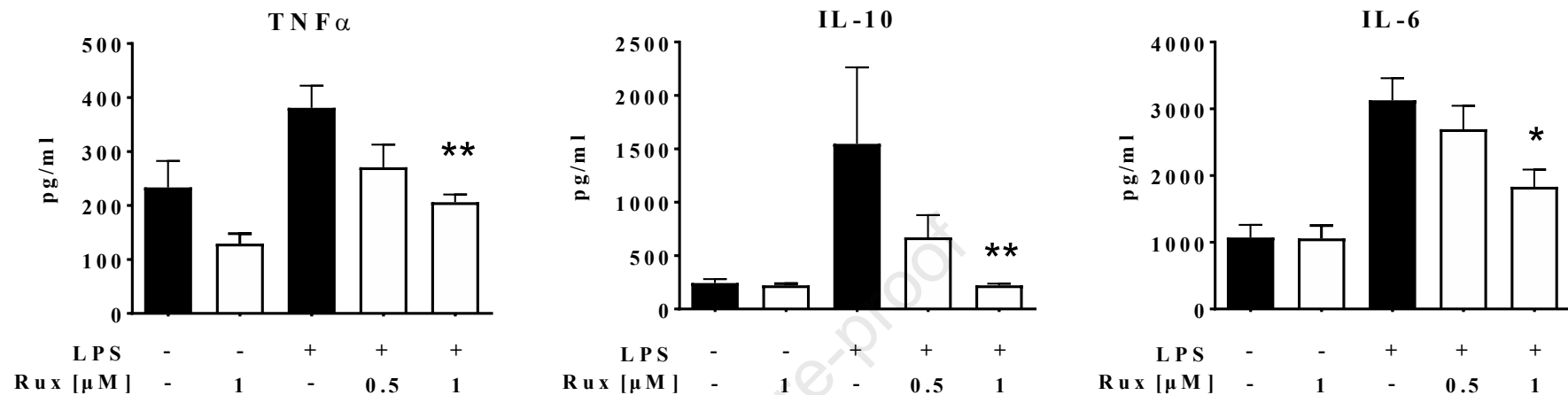
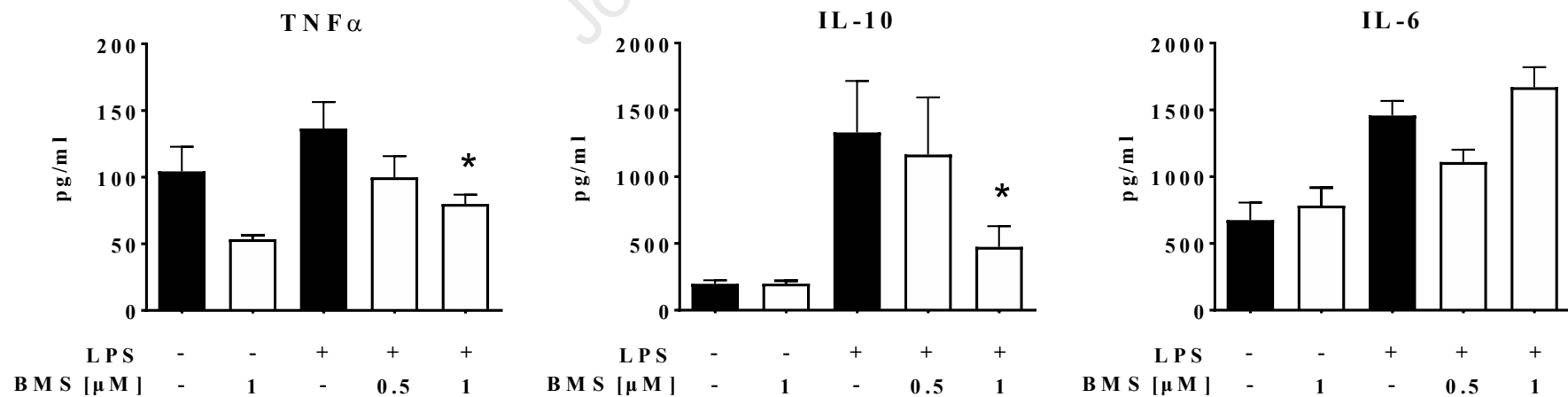
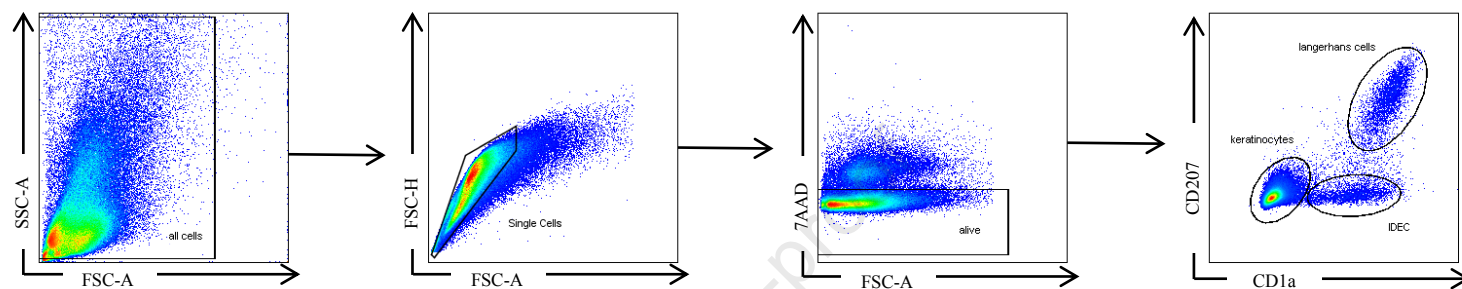
A**B**

Figure 7

A IDEC *ex vivo*



B IDEC *in vitro*

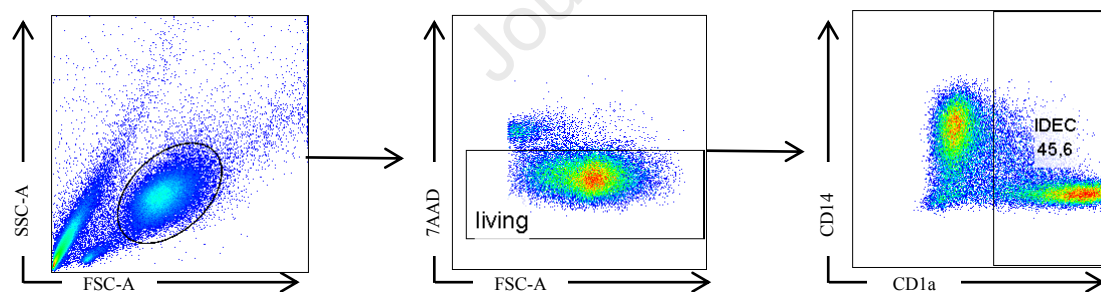


Figure E1

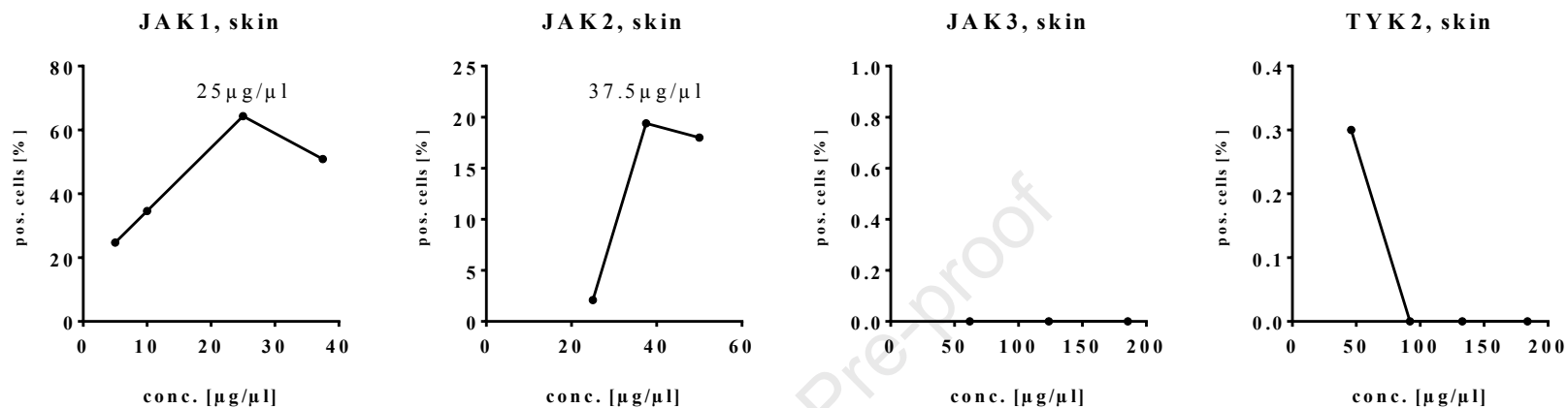
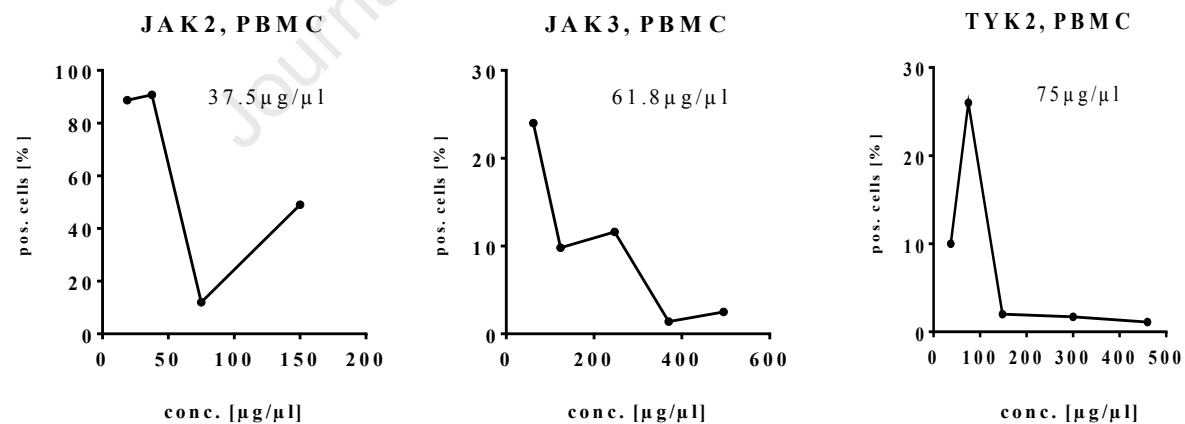
A**B**

Figure E2

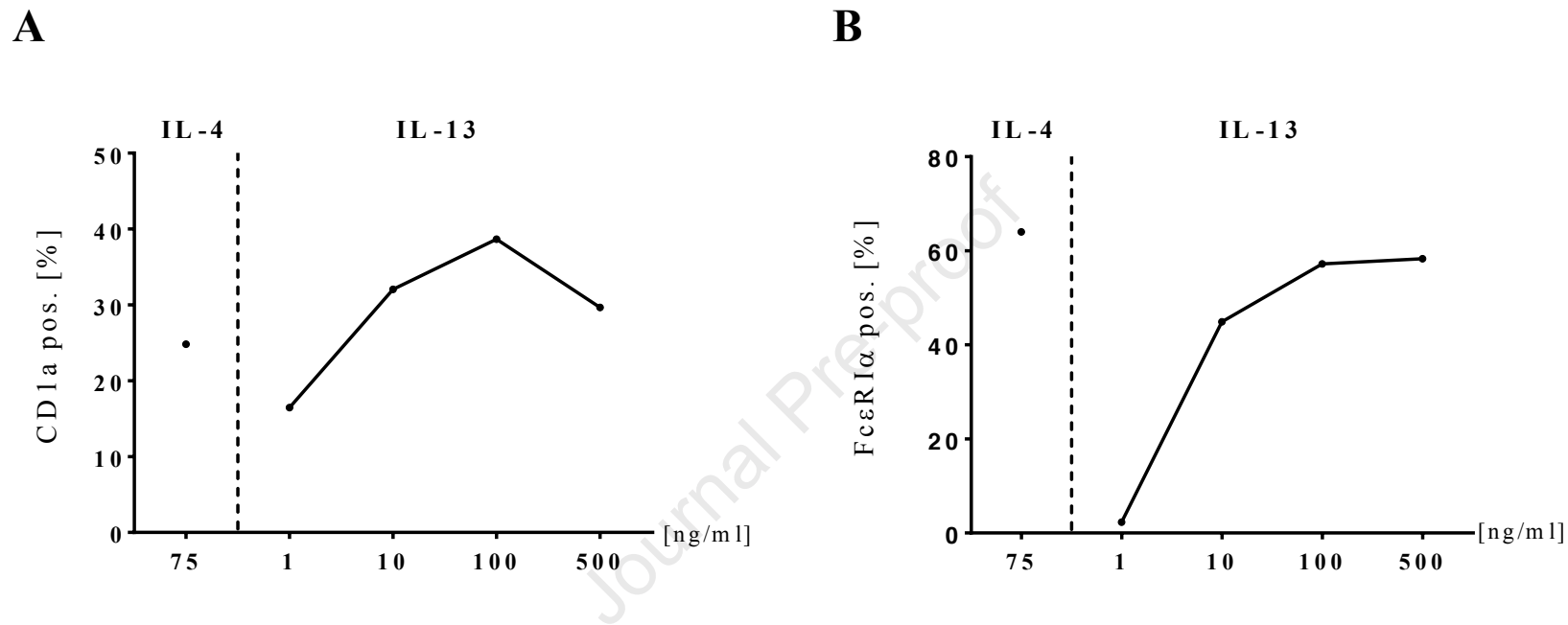


Figure E3

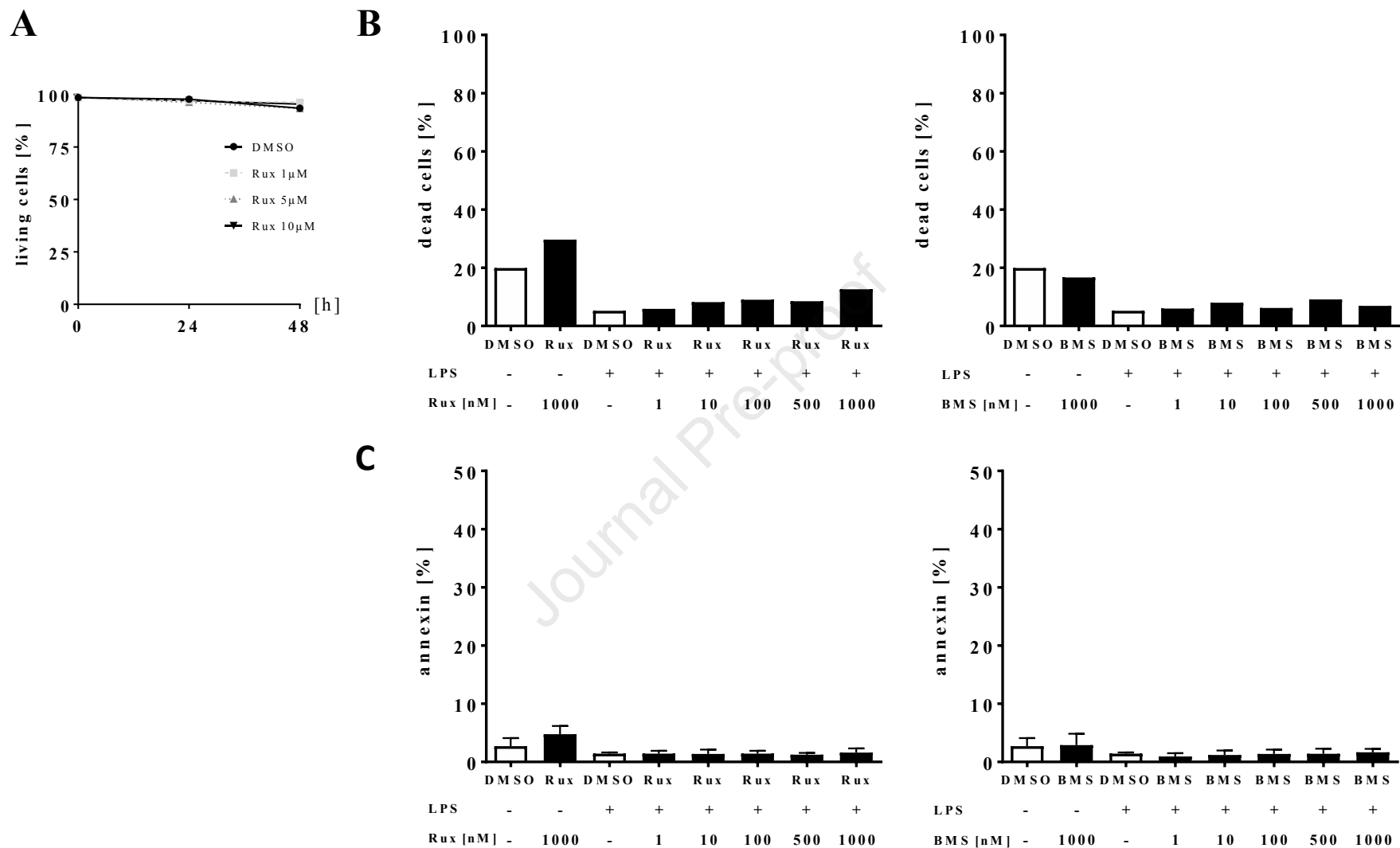
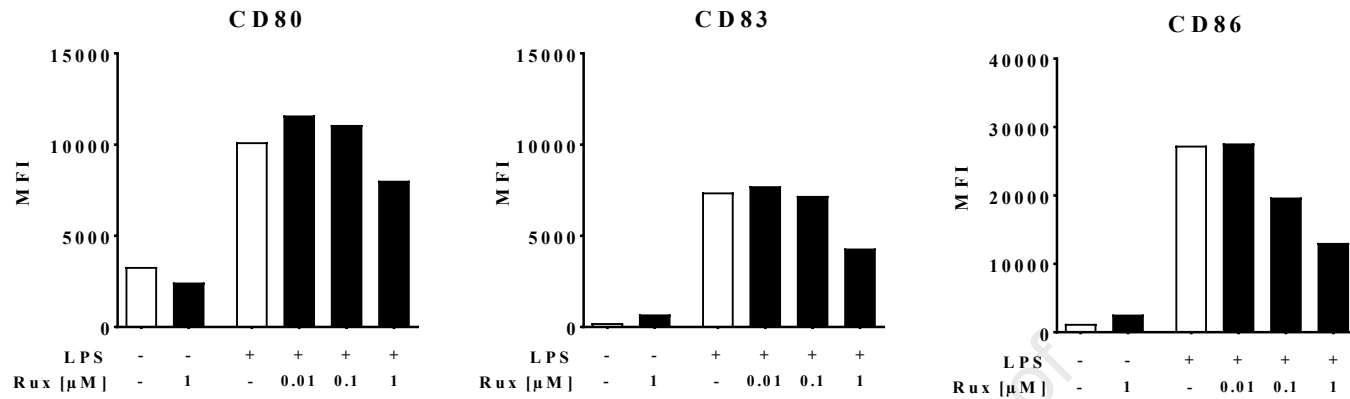
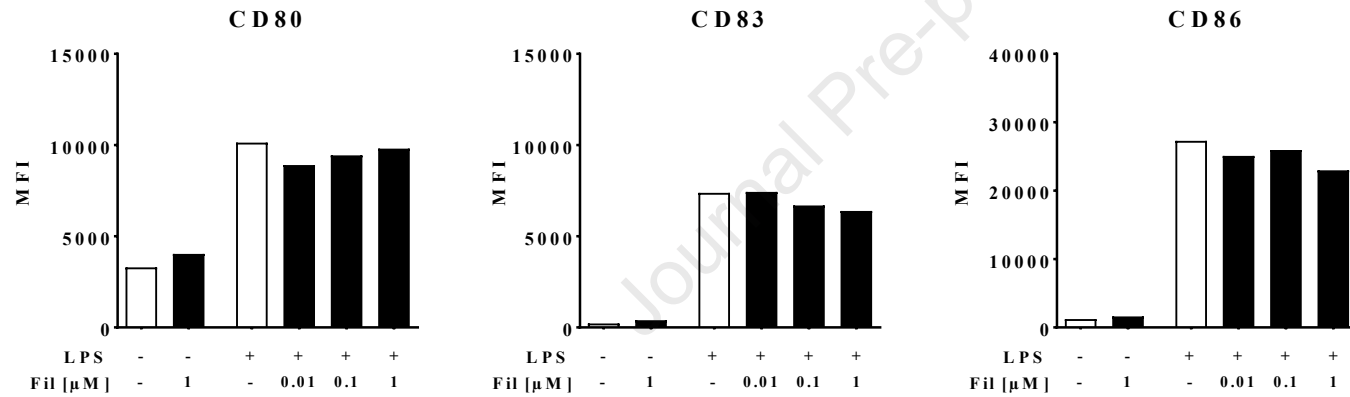


Figure E4

A



B



C

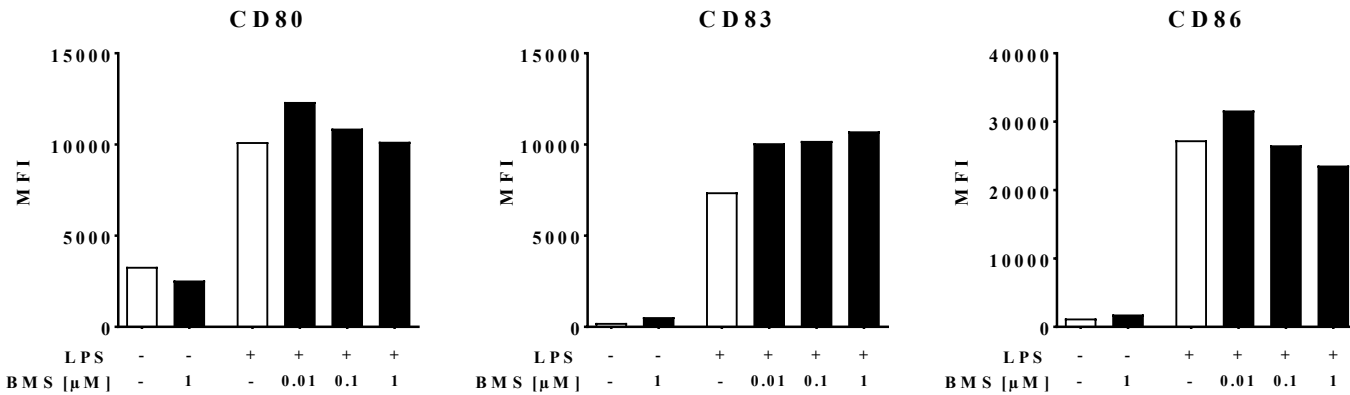


Figure E5