Original Paper

Impact of Janus Kinase 3 on Cellular Ca^{2+} Release, Store Operated Ca^{2+} Entry and $\text{Na}^+/\text{Ca}^{2+}$ Exchanger Activity in Dendritic Cells

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Key Words

ATP • CXCL12 • CRAC channel • SOCE • Intracellular Ca^{2+} release

Abstract

Background/Aims: Janus kinase 3 (JAK3), a tyrosine kinase mainly expressed in hematopoietic cells, participates in the signaling stimulating cell proliferation. The kinase is expressed in dendritic cells (DCs), antigen presenting cells involved in the initiation and regulation of antigen-specific T-cell responses. Dendritic cell function is regulated by cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). Mediators increasing $[\text{Ca}^{2+}]_i$ in DCs include ATP and the chemokine receptor CXCR4 ligand CXCL12. The present study explored, whether JAK3 participates in the regulation of $[\text{Ca}^{2+}]_i$ in DCs. **Methods:** Fura-2 fluorescence was employed to determine $[\text{Ca}^{2+}]_i$, and whole cell patch clamp to decipher electrogenic transport in immature DCs isolated from bone marrow of JAK3-knockout (*jak3*^{-/-}) or wild-type mice (*jak3*^{+/+}). **Results:** Without treatment, $[\text{Ca}^{2+}]_i$ was similar in *jak3*^{-/-} and *jak3*^{+/+} DCs. Addition of ATP (100 μM) was followed by transient increase of $[\text{Ca}^{2+}]_i$ reflecting Ca^{2+} release from intracellular stores, an effect significantly less pronounced in *jak3*^{-/-} DCs than in *jak3*^{+/+} DCs. CXCL12 administration was followed by a sustained increase of $[\text{Ca}^{2+}]_i$ reflecting receptor operated Ca^{2+} entry, an effect significantly less rapid in *jak3*^{-/-} DCs than in *jak3*^{+/+} DCs. In addition, the Ca^{2+} release-activated Ca^{2+} channel (CRAC) current triggered by IP_3 -induced Ca^{2+} store depletion and CXCL12 was significantly higher in DCs from *jak3*^{+/+} mice than in *jak3*^{-/-} mice. Inhibition of sarcoendoplasmatic reticulum Ca^{2+} -ATPase (SERCA) by thapsigargin (1 μM) in the absence of extracellular Ca^{2+} was followed by a transient increase of $[\text{Ca}^{2+}]_i$ reflecting Ca^{2+} release from intracellular stores, and subsequent readdition of extracellular Ca^{2+} in the continued presence of thapsigargin was followed by a sustained increase of $[\text{Ca}^{2+}]_i$ reflecting store operated Ca^{2+} entry (SOCE). Both, Ca^{2+} release from intracellular stores and SOCE were again significantly lower in *jak3*^{-/-} DCs than in *jak3*^{+/+}

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DCs. Pretreatment of *jak3*^{+/+} DCs with JAK inhibitor WHI-P154 (22 μM , 10 minutes or 24 hours) significantly blunted both thapsigargin induced Ca^{2+} release and subsequent SOCE. Abrupt replacement of Na^+ containing (130 mM) and Ca^{2+} free (0 mM) extracellular bath by Na^+ free (0 mM) and Ca^{2+} containing (2 mM) extracellular bath increased $[\text{Ca}^{2+}]_i$ reflecting $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity, an effect again significantly less pronounced in *jak3*^{-/-} DCs than in *jak3*^{+/+} DCs. **Conclusions:** JAK3 deficiency is followed by down-regulation of cytosolic Ca^{2+} release, receptor and store operated Ca^{2+} entry and $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity in DCs.

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Introduction

Janus kinase 3 (JAK3), a tyrosine kinase, is mainly expressed in hematopoietic cells [1, 2]. JAK3 expressing cells include dendritic cells (DCs) [3], antigen-presenting cells contributing to initiation and regulation of antigen-specific T-cell responses [4, 5]. JAK3 is involved in the signaling of cytokine receptors in hematopoietic cells [6-10]. The kinase contributes to the stimulation of cell proliferation as well as the inhibition of apoptosis [11-15]. The gain of function mutation ^{A572V}JAK3 was found in acute megakaryoblastic leukemia [16, 17]. Whether this mutation impacts on Ca^{2+} signaling, remained elusive. Gene targeted mice lacking JAK3 suffer from several disorders affecting immune system and hematopoiesis including impaired B cell development [18]. Jak3 deficiency further interferes with T cell activation leading to decreased IL-2 secretion [18]. In JAK3 deficient mice spleen, peripheral lymph nodes, and thymus are hypoplastic, and intestinal Peyer's patches completely lacking [18]. Jak3-deficiency leads to increased formation of $1,25(\text{OH})_2\text{D}_3$ in the kidney [19] and to enhanced release of IL-12 [20], which is produced by several cell types including dendritic cells [21].

The function of DCs is governed by alterations of cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [22]. $[\text{Ca}^{2+}]_i$ is increased following Ca^{2+} release from intracellular stores accomplished by the IP3 receptor [23]. The emptying of the intracellular stores is followed by activation of Ca^{2+} release activated current I_{crac} [24] leading to store operated Ca^{2+} entry (SOCE) [25-28], which is accomplished by the pore forming Ca^{2+} channel subunits Orai1, Orai2 or Orai3 [29-33] and their regulators STIM1 or STIM2 [34-38]. Cytosolic Ca^{2+} concentration is further a function of Ca^{2+} extrusion by K^+ -independent (NCX) and K^+ -dependent (NCKX) $\text{Na}^+/\text{Ca}^{2+}$ exchangers [39, 40] as well mitochondrial Ca^{2+} uptake or release [41-46]. Agonists stimulating Ca^{2+} release from intracellular stores include purinergic receptor agonist ATP [47], agonists stimulating receptor operated Ca^{2+} entry include CXCL12 [27].

The present study explored, whether JAK3 influences intracellular Ca^{2+} release, SOCE, Ca^{2+} release-activated Ca^{2+} channel activity, and $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity. To this end, DCs were isolated from gene-targeted mice lacking functional JAK3 (*jak3*^{-/-}) and corresponding wild type mice (*jak3*^{+/+}). $[\text{Ca}^{2+}]_i$ in *jak3*^{-/-} and *jak3*^{+/+} DCs was determined utilizing Fura-2 fluorescence, and Ca^{2+} release-activated Ca^{2+} channel current (I_{CRAC}) by whole cell patch clamp.

Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Baden-Württemberg. Experiments were performed with dendritic cells from 7-12 weeks old female and male gene-targeted mice lacking functional JAK3 (*jak3*^{-/-}) and in age- and sex-matched wild type mice (*jak3*^{+/+}) (Charles River, Sulzfeld, Germany) [48]. The mice had free access to water and control food (SSniff, Soest, Germany)

Cell Culture

Dendritic cells (DCs) were cultured from bone marrow of *jak3^{-/-}* and *jak3^{+/-}* mice [27]. Bone marrow derived cells were flushed out of the cavities from the femur and tibia with PBS. Cells were then washed twice with RPMI and seeded out at a density of 2×10^6 cells/10ml per 60-mm dish. Cells were cultured for 8 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10% FCS, 1% penicillin/streptomycin, 1% non-essential amino acids (NEAA) and 0.05% β -mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/ml, Immunotools) and fed with fresh medium containing GM-CSF on days 3 and 6. Experiments were performed on DCs at day 7-9 after isolation.

Measurement of intracellular Ca^{2+}

Fura-2 fluorescence was employed to estimate cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) [49, 50]. To this end, the cells were loaded with Fura-2/AM (2 μM , Molecular Probes, Goettingen, Germany) for 15 min at 37 °C. Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into either the objective (Fluar 40 $\times$ /1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxitronic, Bensheim, Germany). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). The corresponding ratios (F_{340}/F_{380}) were used to obtain intracellular Ca^{2+} concentrations. The following equation was used: $[\text{Ca}^{2+}]_{\text{free}} = K_d \times ((R - R_{\text{min}})/(R_{\text{max}} - R)) \times S_f$ (K_d = dissociation constant of Fura-2; R = ratio of emission intensity, exciting at 340 nm, to emission intensity, exciting at 380 nm; R_{min} = ratio at zero free Ca^{2+} ; R_{max} = ratio at saturating Ca^{2+} ; S_f = instrumental constant). As a measure for the increase of cytosolic Ca^{2+} activity, the slope and peak of the changes in the intracellular Ca^{2+} ratio were calculated for each experiment.

Intracellular Ca^{2+} was measured prior to and following addition of MgATP (100 $\mu\text{mol/l}$, Alfa Aesar GmbH & Co KG, Germany) or CXCL12 (300 ng/ml, Sigma, Germany). Store operated Ca^{2+} entry (SOCE) was determined by extracellular Ca^{2+} removal and subsequent Ca^{2+} readdition in the presence of sarcoendoplasmatic reticulum Ca^{2+} -ATPase (SERCA) inhibitor thapsigargin (1 μM , Invitrogen) [51]. For quantification of Ca^{2+} -entry, the slope (Δ ratio/s) and peak (Δ ratio) of $[\text{Ca}^{2+}]_i$ increase were calculated.

The modified Ringer solution contained (in mmol/l): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 CaCl_2 , 2 Na_2HPO_4 , 32 HEPES (pH 7.4), 5 glucose. the Ca^{2+} -free solution contained (in mmol): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 0.5 EGTA, 2 Na_2HPO_4 , 32 HEPES (pH 7.4), 5 glucose.

The changes in $[\text{Ca}^{2+}]_i$ upon removal of extracellular Na^+ were taken as measure of $\text{Na}^+/\text{Ca}^{2+}$ exchange. N-methyl-D-glucamine (NMDG) was used to replace Na^+ . The Na^+ -containing and Na^+ -free solution contained either 5 mM or 40 mM KCl. Experiments were performed with modified Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 CaCl_2 , 2 Na_2HPO_4 , 32 HEPES (pH 7.4), 5 glucose. To measure $\text{Na}^+/\text{Ca}^{2+}$ exchange the Na^+ -containing solution contained (in mM): 130 or 90 NaCl, 5 or 40 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES (pH 7.4), 5 glucose, and the Na^+ -free solution contained (in mM): 130 or 90 NMDG, 5 or 40 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES (pH 7.4), 5 glucose.

For calibration purposes ionomycin (10 μM , Sigma-Aldrich, Taufkirchen, Germany) was applied at the end of each experiment.

Whole-cell patch clamp

Patch clamp experiments were performed at room temperature in voltage clamp, fast-whole-cell mode according [52-54]. The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). For I_{CRAC} measurements whole-cell currents were elicited by 200 ms square wave voltage pulses from -50 to +50 mV in 10 mV steps delivered from a holding potential of -30 mV. Alternatively, the currents were recorded with 200 ms voltage ramps from -50 to +50 mV. Leak currents determined as the currents at the very beginning of each experiment immediately after reaching the whole-cell mode were subtracted. The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered. DCs were superfused with a bath solution containing (in mmol/l): 140 NaCl, 5 KCl, 10 CaCl_2 , 20 glucose, 10 HEPES/NaOH, pH 7.4. The patch clamp pipettes were filled with an internal solution containing (in mmol/l): 120 CsCl, 35 NaCl, 10 EGTA, 10 HEPES/CsOH, 0.04 inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$, Enzo Life Sciences), pH 7.4. The liquid junction potential ΔE between the CsCl-based pipette and the NaCl-based bath solutions was estimated according to Barry and Lynch [55] and approached 1 mV. The data were not corrected for ΔE .

Statistical analysis

Data are provided as means $\pm$ SEM. n represents the number of the number of independent experiments. Data were tested for significance using ANOVA or Student's unpaired two-tailed t-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

Results

The present study explored the impact of JAK3 on cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) in dendritic cells (DCs). Alterations of $[\text{Ca}^{2+}]_i$ were traced by measurements of Fura-2 fluorescence ratio. Experiments were performed in DCs isolated from bone marrow of JAK3-knockout ($\text{jak3}^{-/-}$) or wild-type mice ($\text{jak3}^{+/+}$). In the absence of agonists and presence of extracellular Ca^{2+} , the Fura-2 fluorescence ratio was similar in $\text{jak3}^{-/-}$ DCs (1.01 ± 0.02 ; $n = 76$) and $\text{jak3}^{+/+}$ DCs (1.08 ± 0.02 ; $n = 76$).

Reduced ATP induced Ca^{2+} release in $\text{jak3}^{-/-}$ DCs

In order to define the impact of JAK3 on agonist induced Ca^{2+} release from intracellular stores, the effect of purinergic receptor agonist ATP in the absence of extracellular Ca^{2+} was tested. As illustrated in Fig. 1, addition of ATP (100 μM) in the absence of extracellular Ca^{2+} was followed by transient increase of Fura-2 fluorescence ratio reflecting Ca^{2+} release from intracellular stores. Amplitude (peak, Δ ratio), and velocity (slope, Δ ratio/s), were both significantly less pronounced in $\text{jak3}^{-/-}$ than in $\text{jak3}^{+/+}$ DCs (Fig. 1). Accordingly, lack of JAK3 compromises ATP induced intracellular Ca^{2+} release.

Reduced Ca^{2+} release and store operated Ca^{2+} entry (SOCE) in $\text{jak3}^{-/-}$ DCs in the presence of SERCA inhibitor

In order to determine SOCE, intracellular stores were emptied by inhibition of sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) with thapsigargin (1 μM) in the absence of extracellular Ca^{2+} . The treatment resulted in a transient increase of Fura-2 fluorescence ratio reflecting Ca^{2+} release from intracellular stores, an effect significantly less pronounced in $\text{jak3}^{-/-}$ than in $\text{jak3}^{+/+}$ DCs (Fig. 2). Subsequent readdition of extracellular Ca^{2+} in the continued

Fig. 1. ATP induced Ca^{2+} release in $\text{jak3}^{+/+}$ and $\text{jak3}^{-/-}$ DCs. A. Representative tracings showing the Fura-2 fluorescence ratio reflecting cytosolic Ca^{2+} activity in $\text{jak3}^{+/+}$ (white symbols) and $\text{jak3}^{-/-}$ (black symbols) DCs. Where indicated, Ca^{2+} was removed from (0 Ca^{2+}) and ATP (100 μM) added to the bath solution leading to release of Ca^{2+} from intracellular stores with subsequent increase of the Fura-2 fluorescence ratio. The amplitude (peak) and the velocity (slope, calculated from the linear fit) of the Fura-2 fluorescence ratio

increase were analysed. B,C. Arithmetic means ($\pm$ SEM, $n = 20$ –37 cells) of the slope (B) and the peak (C) of the change in Fura-2 fluorescence ratio in $\text{jak3}^{+/+}$ (white bars) and $\text{jak3}^{-/-}$ (black bars) DCs following addition of ATP (100 μM) in the absence of Ca^{2+} (Ca^{2+} release). * ($p < 0.05$), ** ($p < 0.01$) indicate significant difference between $\text{jak3}^{-/-}$ and $\text{jak3}^{+/+}$ DCs, unpaired t-test.

Fig. 2. Thapsigargin induced Ca^{2+} release and store-operated Ca^{2+} entry in $\text{jak3}^{+/+}$ and $\text{jak3}^{-/-}$ DCs. A. Representative tracings showing the Fura-2 fluorescence ratio in $\text{jak3}^{+/+}$ (white symbols) and $\text{jak3}^{-/-}$ (black symbols) DCs. Where indicated, Ca^{2+} was removed (0 Ca^{2+}) and sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin ($1 \mu\text{M}$) added to the bath solution leading to release of Ca^{2+} from intracellular stores. Re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin reflects store-operated Ca^{2+} entry (SOCE). The amplitude (peak) and the velocity (slope, calculated from the linear fit) of Ca^{2+} release and Ca^{2+} entry were analysed. B,C. Arithmetic means ($\pm$ SEM, $n = 76$ cells) of the slope (B) and the peak (C) of the change in Fura-2 fluorescence ratio in $\text{jak3}^{+/+}$ (white bars) and $\text{jak3}^{-/-}$ (black bars) DCs following addition of thapsigargin ($1 \mu\text{M}$) in the absence of Ca^{2+} (Ca^{2+} release). * ($p < 0.05$), ** ($p < 0.01$) indicates significant difference from $\text{jak3}^{+/+}$ DCs (two-tailed unpaired t -test). D,E. Arithmetic means ($\pm$ SEM, $n = 76$ cells) of the slope (D) and the peak (E) of the change in Fura-2 fluorescence ratio following re-addition of extracellular Ca^{2+} , reflecting SOCE (Ca^{2+} entry), in $\text{jak3}^{+/+}$ (white bars) and $\text{jak3}^{-/-}$ (black bars) DCs. *** ($p < 0.001$) indicates significant difference between $\text{jak3}^{-/-}$ and $\text{jak3}^{+/+}$ DCs, unpaired t -test.

Fig. 3. CXCL12 induced Ca^{2+} entry in $\text{jak3}^{+/+}$ and $\text{jak3}^{-/-}$ DCs. A. Representative tracings showing the Fura-2 fluorescence ratio reflecting cytosolic Ca^{2+} activity in $\text{jak3}^{+/+}$ (white symbols) and $\text{jak3}^{-/-}$ (black symbols) DCs. Where indicated, CXCL12 (300 ng/ml) was added to the bath solution leading to increase of Fura-2 fluorescence ratio. The amplitude (peak) and the velocity (slope, calculated from the linear fit) of the increase were analysed. B,C. Arithmetic means ($\pm$ SEM, $n = 80$ -94 cells) of the slope (B) and the peak (C) of the change in Fura-2 fluorescence ratio in $\text{jak3}^{+/+}$ (white bars) and $\text{jak3}^{-/-}$ (black bars) DCs following addition of CXCL12 (300 ng/ml). ** ($p < 0.01$), *** ($p < 0.001$) indicate significant difference between $\text{jak3}^{-/-}$ and $\text{jak3}^{+/+}$ DCs, unpaired t -test.

presence of thapsigargin was followed by a sustained increase of Fura-2 fluorescence ratio reflecting SOCE, an effect again significantly less pronounced in $\text{jak3}^{-/-}$ than in $\text{jak3}^{+/+}$ DCs (Fig. 2). Lack of JAK3 thus impairs intracellular Ca^{2+} release and SOCE.

Fig. 4. Ca^{2+} release activated channel current in *jak3*^{+/+} and *jak3*^{-/-} DCs. A. Original tracings of the IP₃ (40 $\mu\text{mol/l}$)-activated current fraction recorded from immature *jak3*^{+/+} and *jak3*^{-/-} DCs following addition of CXCL12 (300 ng/ml). The voltage protocol is indicated (not to scale), whereby cells were held at -30 mV and ramps were applied for 200 ms from -50 mV to +50 mV. B. Mean current-voltage (I/V) relationships of currents normalized to cell capacitance in *jak3*^{+/+} (n=7) and *jak3*^{-/-} (n=6) DCs following addition of 40 μM IP₃ and exposure to the chemokine CXCL12 (300 ng/ml). The voltage protocol is shown (not to scale), whereby cells were held at -30 mV and voltage steps were applied in 10 mV increments for 200 ms from -50 mV to +50 mV. * (p<0.05), ** (p<0.01), unpaired t-test. C. Mean whole-cell conductance of inward currents ($\pm$ SEM, n=6-7) calculated by linear regression of I/V curves shown in (B) between -50 mV and 0 mV. * (p<0.05) indicates significant difference between *jak3*^{-/-} and *jak3*^{+/+} DCs, unpaired t-test.

Reduced CXCL12 induced operated Ca^{2+} entry in *jak3*^{-/-} DCs

In order to determine the impact of JAK3 on Ca^{2+} entry following activation of the chemokine receptor CXCR4, the effect of its ligand CXCL12 was tested. As shown in Fig. 3, the administration of CXCL12 (300 ng/ml) in the presence of extracellular Ca^{2+} was followed by a sustained increase of Fura-2 fluorescence ratio reflecting receptor operated Ca^{2+} entry (Fig. 3). The effect was again significantly less pronounced in *jak3*^{-/-} than in *jak3*^{+/+} DCs (Fig. 3). Accordingly, lack of JAK3 compromises CXCL12 induced Ca^{2+} entry.

Reduced current through store operated Ca^{2+} channels in *jak3*^{-/-} DCs

Whole cell patch clamp recording was employed in order to analyze Ca^{2+} release-activated Ca^{2+} (CRAC) current (I_{CRAC}) in *jak3*^{-/-} and *jak3*^{+/+} DCs. As illustrated in Fig. 4, the conductance of the inward current triggered by Ca^{2+} store depletion with inositoltrisphosphate (IP₃) and stimulation with CXCL12 in the pipette solution was significantly impaired in *jak3*^{-/-} DCs.

Inhibition of Ca^{2+} release and store operated Ca^{2+} entry in *jak3*^{+/+} DCs by JAK3 inhibitor WHI-P154

Additional experiments addressed, whether genetic JAK3 knockout could be mimicked by pharmacological inhibition of JAK3 with WHI-P154. The inhibitor was applied long term

Fig. 5. Effect of Jak3 inhibitor WHI-P154 on thapsigargin induced Ca^{2+} release and store-operated Ca^{2+} entry in *jak3*^{+/+} DCs. A. Representative tracings showing the Fura-2 fluorescence ratio in *jak3*^{+/+} DCs without (white symbols) and with (black symbols) WHI-P154 treatment (22 μM , 24 hours). Where indicated, Ca^{2+} was removed (0 Ca^{2+}) and sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) added to the bath solution leading to release of Ca^{2+} from intracellular stores. Re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin reflects store-operated Ca^{2+} entry (SOCE). The amplitude (peak) and the velocity (slope, calculated from the linear fit) of Ca^{2+} release and Ca^{2+} entry were analyzed. B, C. Arithmetic means ($\pm$ SEM, $n = 66$ -99 cells) of the slope (B) and the peak (C) of the change in Fura-2 fluorescence ratio in *jak3*^{+/+} DCs without (white bars) and with (black bars) WHI-P154 treatment (22 μM , 24 hours) following addition of thapsigargin (1 μM) in the absence of Ca^{2+} (Ca^{2+} release). * ($p < 0.05$), ** ($p < 0.01$) indicates significant difference from *jak3*^{+/+} DCs (two-tailed unpaired *t*-test). D, E. Arithmetic means ($\pm$ SEM, $n = 66$ -99 cells) of the slope (D) and the peak (E) of the change in Fura-2 fluorescence ratio following re-addition of extracellular Ca^{2+} , reflecting SOCE (Ca^{2+} entry), in *jak3*^{+/+} DCs without (white bars) and with (black bars) WHI-P154 treatment (22 μM , 24 hours). * ($p < 0.05$) indicates significant difference from *jak3*^{+/+} DCs (two-tailed unpaired *t*-test). F. Representative tracings showing the Fura-2 fluorescence ratio in *jak3*^{+/+} DCs without (white symbols) and with (black symbols) WHI-P154 treatment (22 μM , 10 min). Where indicated, Ca^{2+} was removed (0 Ca^{2+}) and sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1 μM) added to the bath solution leading to release of Ca^{2+} from intracellular stores. Re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin reflects store-operated Ca^{2+} entry (SOCE). The amplitude (peak) and the velocity (slope, calculated from the linear fit) of Ca^{2+} release and Ca^{2+} entry were analyzed. G, H. Arithmetic means ($\pm$ SEM, $n = 60$ -61 cells) of the slope (G) and the peak (H) of the change in Fura-2 fluorescence ratio in *jak3*^{+/+} DCs without (white bars) and with (black bars) WHI-P154 treatment (22 μM , 10 min) following addition of thapsigargin (1 μM) in the absence of Ca^{2+} (Ca^{2+} release). *** ($p < 0.001$) indicates significant difference from *jak3*^{+/+} DCs (two-tailed unpaired *t*-test). I, J. Arithmetic means ($\pm$ SEM, $n = 60$ -61 cells) of the slope (I) and the peak (J) of the change in Fura-2 fluorescence ratio following re-addition of extracellular Ca^{2+} , reflecting SOCE (Ca^{2+} entry), in *jak3*^{+/+} DCs without (white bars) and with (black bars) WHI-P154 treatment (22 μM , 10 min). * ($p < 0.05$), ** ($p < 0.01$) indicate significant difference from *jak3*^{+/+} DCs (two-tailed unpaired *t*-test).

Fig. 6. $\text{Na}^+/\text{Ca}^{2+}$ exchanger mediated Ca^{2+} entry in *jak3^{+/+}* and *jak3^{-/-}* DCs.

A. Representative original tracings showing the Fura-2 fluorescence ratio in *jak3^{+/+}* (white symbols) and *jak3^{-/-}* (black symbols) DCs prior to and following removal of external Na^+ (0 Na^+) in the presence of 2 mM Ca^{2+} and 40 mM K^+ . The amplitude (peak) and the velocity (slope, calculated from the linear fit) of the Ca^{2+} entry were analysed. **B, C.** Mean ($\pm$ SEM, $n = 33$ -43 cells) of the slope value (**B**) and peak (**C**) of the change in Fura-2 fluorescence ratio following removal of external Na^+ in the presence of 2 mM Ca^{2+} and 40 mM K^+ , reflecting the Ca^{2+} entry, in *jak3^{+/+}* (white bars) and *jak3^{-/-}* (black bars) DCs. * ($p < 0.05$) and *** ($p < 0.001$) indicate significant difference between *jak3^{-/-}* and *jak3^{+/+}* DCs, unpaired *t*-test.

(24h, 22 μM) (Fig. 5A-E) or short term (10 min, 22 μM) (Fig. 5F-J) to *jak3^{+/+}* DCs. As illustrated in Fig. 5F-J, a 24 h and 10 min pretreatment with WHI-P154 (22 μM) blunted significantly both the thapsigargin induced increase of Fura-2 fluorescence ratio reflecting Ca^{2+} release and the increase of Fura-2 fluorescence ratio following readdition of Ca^{2+} in the continued presence of thapsigargin reflecting SOCE. Accordingly, WHI-P154 mimics the effect of genetic JAK3 knockout on both, intracellular Ca^{2+} release and SOCE.

Decreased $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity in *jak3^{-/-}* DCs

Blunted increase of $[\text{Ca}^{2+}]_i$ following stimulation of Ca^{2+} release and/or Ca^{2+} entry could have resulted from accelerated Ca^{2+} extrusion by $\text{Na}^+/\text{Ca}^{2+}$ exchange. Thus, an additional series of experiments explored, whether JAK3 influences $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity was estimated from the alterations of Fura-2 fluorescence ratio following abrupt replacement of Na^+ containing (130 mM) and Ca^{2+} free (0 mM) extracellular bath by Na^+ free (0 mM) and Ca^{2+} containing (2 mM) extracellular bath. As illustrated in Fig. 6, the removal of extracellular Na^+ and addition of extracellular Ca^{2+} was followed by a sharp increase of Fura-2 fluorescence ratio reflecting $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity. The increase of Fura-2 fluorescence ratio was significantly less pronounced in *jak3^{-/-}* than in *jak3^{+/+}* DCs. Accordingly, lack of JAK3 compromises $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity.

Discussion

The present observations reveal a novel function of JAK3, i.e. the regulation of Ca^{2+} release from intracellular stores, of receptor and store operated Ca^{2+} entry (ROCE or SOCE, resp.) as well as $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity in dendritic cells (DCs). All those functions are blunted in DCs isolated from gene targeted mice lacking JAK3 (*jak3^{-/-}*) as compared to DCs isolated from corresponding wild type mice (*jak3^{+/+}*). Thus, those functions are up-regulated by JAK3. The present observations do not allow safe conclusions on the JAK3 sensitive mechanisms influencing Ca^{2+} signaling. In theory, the effects of JAK3 deficiency could have resulted from direct phosphorylation of the effector proteins accomplishing Ca^{2+} release, Ca^{2+} entry and/or Ca^{2+} extrusion, from phosphorylation of other signaling proteins regulating expression or function of the effector proteins, or even more indirectly from altered inflammatory response

and cytokine levels in *jak3*^{-/-} mice. The observation that the effects of genetic knockout on Ca²⁺ release from intracellular stores and SOCE are mimicked by a 10 minutes exposure to the Jak3 inhibitor WHI-P154 may point to phosphorylation of the effector proteins or of signaling molecules directly regulating the effector proteins. The effect of the inhibitor on intracellular Ca²⁺ release is smaller after 24 hours exposure, an observation pointing to partial relaxation of the effect. Along those lines, the differences of cytosolic Ca²⁺ activity ([Ca²⁺]_i) between *jak3*^{-/-} and *jak3*^{+/+} DCs following CXCL12 exposure are only transient. Possibly, JAK3 sensitive CXCL12 induced Ca²⁺ entry is inhibited by increase of [Ca²⁺]_i. In the absence of stimulation, [Ca²⁺]_i is similar in *jak3*^{-/-} and *jak3*^{+/+} DCs suggesting that the JAK3 sensitive mechanisms are only effective following stimulation of the DCs or following emptying of intracellular stores, Ca²⁺ signaling is decisive for migration [56-60], which directs the DCs to cytokines [61], diverse antigens, including Toll-like receptor ligands, intact bacteria, and microbial toxins [5]. Ca²⁺-sensitive signaling plays a critical role in several further functions of DCs, including DC activation, maturation, and formation of immunological synapses with T cells. Moreover, alterations of cytosolic Ca²⁺ trigger immune suppression or switch off of DC activity [22]. Ca²⁺ signaling is further decisive for cell proliferation [62-65]. The impact of JAK3 on cell proliferation is illustrated by the gain of function mutation ^{A572V}JAK3 apparently leading to acute megakaryoblastic leukemia [16, 17]. The impact of JAK3 deficiency on cell proliferation is, however, less obvious, as conflicting results have been reported on development and abundance of DCs in *jak3*^{-/-} mice. The *in vitro* development of *jak3*^{-/-} DCs is seemingly normal [20, 66]. In *jak3*^{-/-} mice normal [66] or decreased [20] abundance of splenic DCs, normal [20] or impaired [66] maturation and normal [66] or enhanced [20] DCs survival have been reported.

In conclusion, lack of JAK3 has a profound influence on Ca²⁺ signaling in DCs, i.e. down-regulation of Ca²⁺ release from intracellular stores, of receptor and store operated Ca²⁺ entry, CRAC channel current, and of Na⁺/Ca²⁺ exchanger activity.

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Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests.

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