Original Paper

Akt2- and ETS1-Dependent IP₃ Receptor 2 Expression in Dendritic Cell Migration

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Key Words

CCL21 • Ca²⁺ release • I_{CRAC} • SOCE

Abstract

Background/Aims: The protein kinase Akt2/PKB β is a known regulator of macrophage and dendritic cell (DC) migration. The mechanisms linking Akt2 activity to migration remained, however, elusive. DC migration is governed by Ca²⁺ signaling. We thus explored whether Akt2 regulates DC Ca²⁺ signaling. **Methods:** DCs were derived from bone marrow of Akt2-deficient mice (*akt2*^{-/-}) and their wild type littermates (*akt2*^{+/+}). DC maturation was induced by lipopolysaccharides (LPS) and evaluated by flow cytometry. Cytosolic Ca²⁺ concentration was determined by Fura-2 fluorescence, channel activity by whole cell recording, transcript levels by RT-PCR, migration utilizing transwells. **Results:** Upon maturation, chemokine CCL21 stimulated migration of *akt2*^{+/+} but not *akt2*^{-/-} DCs. CCL21-induced increase in cytosolic Ca²⁺ concentration, thapsigargin-induced release of Ca²⁺ from intracellular stores with subsequent store-operated Ca²⁺ entry (SOCE), ATP-induced inositol 1,4,5-trisphosphate (IP₃)-dependent Ca²⁺ release as well as Ca²⁺ release-activated Ca²⁺ (CRAC) channel activity were all significantly lower in mature *akt2*^{-/-} than in mature *akt2*^{+/+} DCs. Transcript levels of IP₃ receptor IP₃R2 and of IP₃R2 regulating transcription factor ETS1 were significantly higher in *akt2*^{+/+} than in *akt2*^{-/-} DCs prior to maturation and were upregulated by LPS stimulation (1h) in *akt2*^{+/+} and to a lower extent in *akt2*^{-/-} DCs. Following maturation, protein abundance of IP₃R2 and ETS1 were similarly higher in *akt2*^{+/+} than in *akt2*^{-/-} DCs. The IP₃R inhibitor Xestospongine C significantly decreased CCL21-induced migration of *akt2*^{+/+} DCs and abrogated the differences between genotypes. Finally, knock-down of ETS1 with siRNA decreased IP₃R2 mRNA abundance, thapsigargin- and ATP-induced Ca²⁺ release, SOCE and CRAC channel activation, as well as DC migration. **Conclusion:** Akt2 upregulates DC migration at least in part by ETS1-dependent stimulation of IP₃R2 transcription.

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Introduction

Migration of dendritic cells (DCs) is of utmost importance for the induction of both protective immunity as well as immunological tolerance. Upon stimulation with pathogen-derived antigens or endogenously generated “danger” molecules, activated DCs acquire a migratory phenotype associated with the upregulation of the chemokine receptor CCR7 [1]. The G protein-coupled receptor CCR7 is the dominant mediator of DC mobilization to the T cell compartment of lymphoid organs [1, 2]. CCR7 engagement by the chemokines CCL19 or CCL21 leads to the activation of phospholipase C β (PLC β) [1] (Fig. 8), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ triggers Ca²⁺ release from intracellular stores via IP₃ receptors (IP₃Rs) in the endoplasmic reticulum (ER). Depletion of Ca²⁺ in ER results in the activation of Ca²⁺ release-activated Ca²⁺ (CRAC) channels leading to a massive entry of extracellular Ca²⁺ [3] (Fig. 8). Blocking of CRAC channels impairs CCR7-dependent migration of DCs [4, 5]. In mouse DCs the predominant components of CRAC channels include the pore-forming subunit Orai1 and Orai2 and the Ca²⁺-sensing subunit STIM1 and STIM2 [6].

CCR ligation and the resulting release of $\beta\gamma$ subunit from the activated G protein also leads to the activation of phosphatidylinositol 3' kinase (PI3K) and downstream Akt/PKB [1] (Fig. 8). Akt is known to play a pivotal role in regulating chemotaxis in leukocytes, including DCs [1]. Of the three Akt isoforms, only Akt1 and Akt2 are expressed in DCs [7]. Whereas the major function of Akt1 seems to be regulating DC survival and maturation [8], Akt2 has been shown to be pivotal for migration of DCs and macrophages [7, 9, 10]. Thus, plasmin-triggered chemotactic response of human monocyte-derived DCs [7], as well as colony-stimulating factor-1 (CSF-1)- or monocyte chemoattractant protein-1 (MCP-1)-induced chemotaxis of mouse peritoneal macrophages [9] is Akt2-dependent. We have previously shown that CCR7-dependent migration is impaired in Akt2-deficient bone marrow-derived mouse DCs [10].

The mechanisms linking Akt2 activity to DC motility remained elusive. In macrophages Akt2 functions upstream of PKC ζ /LIMK/cofilin directly regulating CSF-1-induced actin polymerization [9]. In epithelial cells, Akt2 has been shown to upregulate migration by modulating the transcription of genes required for migration [11]. Since DC migration is extremely sensitive to cytosolic concentrations of free Ca²⁺ ([Ca²⁺]_i) [3], Akt2 may influence DC migration via regulating components of Ca²⁺ signaling. In lymphocytes engagement of T or B cell receptors leads to the activation of PI3K, which upregulates Ca²⁺ mobilization [12-15]. Accordingly, inhibition of PI3K impairs the rise in cytosolic Ca²⁺ concentration upon T or B cell receptor ligation [12-15]. Moreover, another downstream target of PI3K, the serum and glucocorticoid inducible kinase SGK1, which shares 55% identity with Akt in their kinase domain regions [16], is a powerful regulator of CRAC channels [17-21]. The present study explored the mechanisms of Akt2-dependent migration and a possible involvement of Akt2 in the regulation of Ca²⁺ signaling in mouse DCs. We show that in mouse dendritic cells, Akt2 enhances migration through up-regulating the expression of IP₃R2 via the Akt2-dependent transcription factor ETS1, which results in an increased release of Ca²⁺ from intracellular stores, store-operated Ca²⁺ entry, and current through Ca²⁺ release-activated Ca²⁺ (CRAC) channels.

Materials and Methods

Mice and Cells

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Akt2-deficient mice (*akt2*^{-/-}) and their wild type littermates (*akt2*^{+/+}) were used in the present study. Origin of the mice, breeding and genotyping were described previously [22]. Male and female mice were studied at the age of 6-8 weeks.

Dendritic cells (DCs) were isolated from mouse bone marrow as described previously [4, 5]. 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 per 60 mm dish. Cells were cultured for up to 9 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10% FCS, 1% (vol/vol) penicillin/streptomycin (100x, PAA), 2 mM glutamine, 1% (vol/vol) non-essential amino acids (MEM NEAA, 100x, Gibco) and 0.05% β -mercaptoethanol. β -mercaptoethanol was added to the medium since it is a potent reducing agent often used in mouse (but not human) cell culture medium to prevent toxic levels of oxygen radicals [23].

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 days 7-9. DC maturation was induced by treating the cells with lipopolysaccharides (LPS from *E. coli*, 1 μ g/ml, 24 h, Enzo Life Sciences, Lausen, Switzerland).

Immunostaining and flow cytometry

Cells (10^6) were incubated in 200 μ l PBS, containing 0.1% FCS and fluorochrome-conjugated antibodies at a concentration of 10 μ g/ml. A total of 5×10^4 cells were analyzed in each individual experiment. The following antibodies (all from BD Pharmingen, Heidelberg, Germany) were used for staining: Allophycocyanin (APC) Hamster Anti-Mouse CD11c (Clone: HL3), phycoerythrin (PE)-conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ) and PE-conjugated rat anti-mouse I-A/I-E, clone M5/114.15.2 (IgG2b, κ), which reacts with the mouse major histocompatibility complex (MHC) class II I-A/I-E molecules. Following incubation with the respective antibodies for 60 min at 4°C, cells were washed twice and resuspended in the same buffer and subjected to flow cytometry analysis. For MHC II expression only the cells with high expression, which form a clear subpopulation on FACS diagrams, were analyzed (MHC^{high}).

Gene silencing

Specific siRNA sequences for ETS1 (Santa Cruz), negative control (Control siRNA, Santa Cruz) were synthesized and annealed by the manufacturer. siRNA transfection was carried out using the GeneSilencer siRNA transfection reagent (Genlantis, San Diego, CA, USA). 4×10^6 cells were washed and plated in 6-well plates in 2 ml of serum-free RPMI 1640. The ETS1-siRNA and the negative control siRNA (1 μ g/ml) were incubated with GeneSilencer reagent following the manufacturer's protocol. Transfection mixture was then added to the wells and incubated 48 hours. The efficacy of silencing was assessed with RT-PCR and western blotting.

Cytokine Production

IL-6 and IL-12 concentrations in culture supernatants from DCs treated with LPS (1 μ g/ml, 24 h) were determined by using OptEIA ELISA kits (BD Pharmingen) according to the manufacturer's protocol.

Migration

For migration assays the transwells were placed in a 24-well cell culture plate containing cell culture medium (750 μ l) with or without either CXCL12 (50 ng/ml, Peprotech, for immature DCs) or CCL21 (25 ng/ml, Peprotech, for mature DCs) in the lower chamber. The upper chambers were filled with 500 μ l cell culture medium containing immature or LPS (1 μ g/ml, 24 h) - matured DCs either untreated or treated with IP₃R inhibitor Xestospongin C (5 μ M, Enzo Life Sciences, Lausen, Switzerland) for 3h in a concentration of 1×10^5 cells/ml, allowed to migrate through a polycarbonate mesh (pore size 8 μ m) at 37°C for 3h. The cells migrated to the other side of the transwell were stained with DAPI, cells from five representative areas of each membrane were counted.

Measurement of intracellular Ca²⁺

To determine cytosolic Ca²⁺ concentration, the cells were loaded with Fura-2/AM (2 μ M, Molecular Probes, Goettingen, Germany) for 15 min at 37°C. Fluorescence measurements were made with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany) [6]. Cells were excited alternatively at $\lambda = 340$ (filter 340/26) or 380 (filter 387/11) 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 $\lambda = 505$ (filter 495/10) nm and data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). As a measure for the increase of cytosolic Ca²⁺ concentration, the slope and peak of the changes in the 340/380 nm ratio were determined for each experiment.

Intracellular Ca²⁺ was measured prior to and following addition of CXCL12 (300 ng/ml) or CCL21 (75 ng/ml) to the Ringer solution (see below). Alternatively, the Ca²⁺ release was measured following addition of MgATP (100 μ, mol/l, Alfa Aesar GmbH & Co KG, Germany) to the Ca²⁺-free solution (see below). In order to measure SOC entry, changes in cytosolic Ca²⁺ were monitored upon depletion of the intracellular Ca²⁺ stores. Experiments were carried out prior to and during exposure of the cells to the Ca²⁺-free solution. In the absence of Ca²⁺, the intracellular Ca²⁺ stores were depleted by inhibition of the vesicular Ca²⁺ pump by thapsigargin (1 μM, Molecular Probes). Re-addition of Ca²⁺ allowed assessing the store-operated Ca²⁺ entry.

The Ringer solution contained (in mmol/l): 125 NaCl, 5 KCl, 1.2 MgSO₄, 32.2 Hepes, 2 Na₂HPO₄, 2 CaCl₂, and 5 glucose at pH 7.4. The Ca²⁺-free solution contained (in mmol/l): 125 NaCl, 5 KCl, 1.2 MgSO₄, 2 Na₂HPO₄, 32.2 Hepes, 0.5 EGTA, 5 glucose, pH 7.4 (NaOH).

Whole-cell patch clamp

Patch clamp experiments were performed at room temperature in voltage clamp, fast-whole-cell mode according to Hamill et al. [24] as described in [4]. 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 CaCl₂, 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 (Ins(1,4,5)P₃, Enzo Life Sciences), pH 7.4.

The liquid junction potential ΔE between the CsCl-based pipette and the NaCl-based bath solutions estimated according to Barry and Lynch [25] was 1 mV. The data were not corrected for ΔE.

Real-time PCR

Total RNA was extracted from mouse DCs in TriFast (Pqlab, Erlangen, Germany) according to the manufacturer's instructions as described in [6].

For amplification the following primers were used (5'→3' orientation): for IP₃R1: fw AAG CGG ATG GAC CTG GTG TTA GAA CTG and rev AAT TTG TGC TGT GTG CTT CGC GTA GAA CT; for IP₃R2: fw CTG TTC TTC TTC ATC GTC ATC ATC ATC G and rev GAA ACC AGT CCA AAT TCT TCT CCG TGA; for IP₃R3: fw CTT CTT TAT CGT CAT CAT CAT CGT GTT G and rev AGG TTC TTG TTC TTG ATC ATC TGA GCC A; for RyR1: fw TGG GGC ACT ACA ATA ACT TC and rev AGC AGA ATG ACG ATA ACG AA; for ETS1: fw GAT ATC CTG TGG GAG CAT CTA GAG ATC and rev CAG CTG GAT CGG CCC ACT TCC TGT GTA G; for Tbp: fw CAC TCC TGC CAC ACC AGC TT and rev TGG TCT TTA GGT CAA GTT TAC AGC C.

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad) and all experiments were done in duplicate. The house-keeping gene Tbp (TATA binding protein) was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔCT method as described earlier [26].

Western Blot and Immunoprecipitation

Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membrane. After blocking in 10% nonfat dried milk in Tris-buffered saline solution (TBS-T, pH 7.4) with 0.01% Tween-20, blots were incubated with primary antibodies as follows: IP₃R2 (Santa Cruz), ETS1 (Abcam), rabbit monoclonal GAPDH (Cell Signaling, USA). Membranes were washed in TBS-T with 0.01% Tween-20 and incubated with HRP-coupled anti-rabbit (Cell Signalling) or anti-goat (Santa Cruz) secondary antibody and washed again in TBS-T. For detection, membrane was blotted with ECL reagent (GE Healthcare), exposed to X-ray film (GE Healthcare) and developed.

Cell lysates were immunoprecipitated by using Phospho-Akt Substrate (RXRXXS*/T) antibody covalently bound protein A/G PLUS agarose beads (Santa Cruz), size-fractionated by SDS-PAGE, and immunoblotted.

Fig. 1. Differentiation, maturation, cytokine production, and migration of bone marrow derived dendritic cells (DCs) from *akt2*^{+/+} and *akt2*^{-/-} mice. A. Dot plots of CD11c⁺CD86⁺ and CD11c⁺MHC II^{high} DCs at the basal level (control) and stimulated with LPS (1 µg/ml, 24 h) from *akt2*^{+/+} and *akt2*^{-/-} mice. Numbers depict the percent of CD11c⁺CD86⁺ or CD11c⁺MHC II^{high} cells. B. Arithmetic means ± SEM (n = 7) of the percentage of CD11c⁺CD86⁺ and CD11c⁺MHC II^{high} DCs under control and 24 h after LPS stimu-

lation in *akt2*^{+/+} and *akt2*^{-/-} DCs. * (p<0.05), *** (p<0.001), ANOVA; # (p<0.05), only with unpaired *t*-test. C. Arithmetic means ± SEM (n = 8) of IL-6 and IL-12 secretion in LPS (1 µg/ml, 24h)-stimulated *akt2*^{+/+} and *akt2*^{-/-} DCs. D.-E. Arithmetic means ± SEM (n = 11-18) of spontaneous migration and migration in response to either CXCL12 (50 ng/ml, 3h, immature DCs, D) or CCL21 (25 ng/ml, 3h, LPS (1 µg/ml, 24h)-matured DCs, E) of *akt2*^{+/+} and *akt2*^{-/-} DCs. * (p<0.05), ** (p<0.01), ANOVA.

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. Differences were tested for significance using Student's unpaired two-tailed *t*-test or ANOVA. *P*<0.05 was considered statistically significant.

Results

Differentiation and maturation of dendritic cells is unaffected by Akt2 deficiency

DCs were isolated from gene targeted mice lacking functional Akt2 (*akt2*^{-/-}) and their wild type littermates (*akt2*^{+/+}). As illustrated in Fig 1, the surface abundance of both, the costimulatory molecule CD86 and antigen-presenting molecule MHC II, was similar on CD11c⁺ DCs from *akt2*^{+/+} and *akt2*^{-/-} mice. In both genotypes, exposure for 24 hours to bacterial lipopolysaccharides (LPS, 1 µg/ml) resulted in a significant increase of the CD11c⁺CD86⁺ population and CD11c⁺MHC II^{high} cells (with a high expression of MHC II). Following LPS

Fig. 2. Diminished CCL21-induced increase of intracellular Ca²⁺ concentration in *akt2*^{-/-} DCs. A. Representative tracing showing the Fura-2 fluorescence ratios (340/380 nm) in LPS (1 µg/ml)-matured *akt2*^{+/+} DCs and *akt2*^{-/-} DCs upon acute addition of CCL21 (75 ng/ml). B. Mean (± SEM) of peak and slope of the change in Fura-2 fluorescence following addition of CCL21 (75 ng/ml) to mature *akt2*^{+/+} (n = 50) and *akt2*^{-/-} (n = 40) DCs. ** (p < 0.01), unpaired t-test.

treatment, the abundance of CD86 and MHC II was still similar in DCs from *akt2*^{+/+} and *akt2*^{-/-} mice (Fig. 1A,B). The release of interleukin 6 (IL-6) and 12 (IL-12) was again similar in LPS-matured DCs from *akt2*^{+/+} and *akt2*^{-/-} mice (Fig. 1C).

Impaired migration of LPS-matured *akt2*^{-/-} DCs

It has been shown that immature DCs migrate towards the chemokine CXCL12, whereas mature DCs migrate towards CCR7 ligands, such as CCL21 [27]. Migratory capacity of immature *akt2*^{-/-} DCs in response to the CXCL12 chemokine as well as spontaneous migration was not significantly different from *akt2*^{+/+} DCs (Fig. 1D). However, in contrast to LPS-matured *akt2*^{+/+} DCs, LPS-matured *akt2*^{-/-} DCs failed to significantly enhance migration in response to the chemokine CCL21 (25 ng/ml) (Fig. 1E).

Reduced Ca²⁺ release and store-operated Ca²⁺ entry in *akt2*^{-/-} DCs

Fura2-fluorescence has been employed to determine, whether the difference in migratory activity of LPS-matured *akt2*^{-/-} DCs was paralleled by differences in the regulation of cytosolic Ca²⁺ concentration ([Ca²⁺]_i). As illustrated in Fig. 2, both slope and peak of CCL21 (75 ng/ml)-induced increase of [Ca²⁺]_i were significantly less pronounced in LPS-matured DCs from *akt2*^{-/-} mice than in DCs from *akt2*^{+/+} mice. Chemokine-induced increase of [Ca²⁺]_i is known to be due to the release of Ca²⁺ from intracellular stores followed by the activation of store operated Ca²⁺ entry (SOCE) [6, 28]. Therefore, further experiments addressed the impact of Akt2 on Ca²⁺ release from intracellular stores and on SOCE activity in LPS-matured DCs. As indicated in Fig. 3(A, B), the addition of sarcoendoplasmatic reticulum Ca²⁺ ATPase (SERCA) inhibitor thapsigargin (1 µmol/l) in the absence of extracellular Ca²⁺ was followed by an increase of [Ca²⁺]_i, reflecting release of Ca²⁺ from intracellular stores. The slope and the peak of the intracellular Ca²⁺ release were significantly decreased in mature DCs from *akt2*^{-/-} mice (Fig. 3B). The readdition of extracellular Ca²⁺ in the continued presence of thapsigargin resulted in a rapid increase of [Ca²⁺]_i, reflecting SOCE. Both, peak and slope of SOCE were significantly higher in DCs from *akt2*^{+/+} mice than in DCs from *akt2*^{-/-} mice. Release of Ca²⁺ induced by physiological IP₃ formation could be assessed through the stimulation of DCs via P2Y receptors with low concentrations of ATP [29]. Accordingly, ATP (100 µM)-induced

Fig. 3. Reduced Ca²⁺ release and store-operated Ca²⁺ entry in *akt2*^{-/-} DCs. **A.** Representative tracing showing the Fura-2 fluorescence ratio in LPS (1 μg/ml)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs. Where indicated, thapsigargin (1 μmol/l) was added to the Ca²⁺-free bath solution and the release of Ca²⁺ from the stores was assessed. Readdition of extracellular Ca²⁺ in the presence of thapsigargin reflects SOCE. **B.** Arithmetic means (± SEM) of the slope and the peak of the change in Fura-2 fluorescence following addition of thapsigargin (1 μmol/l) in the absence (Ca²⁺ release, upper bars) and in the presence of extracellular Ca²⁺ (SOCE, lower bars) to mature *akt2*^{+/+} (n=117) and *akt2*^{-/-} (n=75) DCs. *** (p<0.001), unpaired *t*-test. **C.** Representative tracing showing the Fura-2 fluorescence ratio in LPS (1 μg/ml)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs upon addition of ATP (100 μmol/l) to the Ca²⁺-free bath solution. **D.** Arithmetic means (± SEM, n=242-250) of the slope and the peak of the change in Fura-2 fluorescence following addition of ATP (100 μmol/l) as in C. * (p<0.05), unpaired *t*-test. **E.** Original tracings of the IP₃ (40 μmol/l)-activated current fraction recorded from LPS (1 μg/ml)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs after stimulation with a ramp voltage. The voltage protocol is shown (not to scale), whereby cells were held at -30 mV and ramps were applied for 200 ms from -50 mV to +50 mV. **F.** Mean current-voltage (I/V) relationships of currents activated by 40 μM IP₃ normalized to cell capacitance in LPS (1 μg/ml)-matured *akt2*^{+/+} (n=15) and *akt2*^{-/-} (n=11) DCs. 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), unpaired *t*-test. **G.** Mean whole-cell conductance of inward currents (± SEM, n=11-15) calculated by linear regression of I/V curves shown in (F) between -40 mV and 0 mV. * (p<0.05), unpaired *t*-test.

release of Ca²⁺ (measured in the absence of extracellular Ca²⁺) was lower in LPS-matured *akt2*^{-/-} than in *akt2*^{+/+} DCs (Fig. 3C, D).

Reduced SOCE in mature *akt2*^{-/-} DCs could be confirmed by whole cell patch clamp recordings. As illustrated in Fig. 3(E-G), the Ca²⁺ release activated Ca²⁺ current I_{CRAC} triggered

Fig. 4. Reduced expression of IP₃ receptor 2 in *akt2*^{-/-} DCs. **A.** Arithmetic means ($\pm$ SEM, $n=3$) of the abundance of mRNA encoding the IP₃ receptors: IP₃R1, IP₃R2, and IP₃R3 and the ryanodine receptor RyR1 in immature *akt2*^{+/+} and *akt2*^{-/-} DCs as assessed by real-time PCR using Tbp mRNA as a reference gene. * ($p<0.05$), unpaired *t*-test. **B.** Arithmetic means ($\pm$ SEM, $n=6-13$) of the IP₃R2 mRNA abundance in *akt2*^{+/+} and *akt2*^{-/-} DCs unstimulated (LPS, 0h) and stimulated with LPS (1 μ g/ml, 1h, 4h, 8h and 24h). * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), ANOVA; # ($p<0.05$), only with unpaired *t*-test. **C.** Arithmetic means ($\pm$ SEM, $n=3-6$) of the IP₃R1, IP₃R3 and RyR1 mRNA abundance in *akt2*^{+/+} and *akt2*^{-/-} DCs unstimulated (LPS, 0h) and stimulated with LPS (1 μ g/ml, 1h). * ($p<0.05$), ANOVA; # ($p<0.05$), only with unpaired *t*-test. **D.** Western blot of whole cell lysate protein of IP₃R2 and GAPDH in LPS (1 μ g/ml, 24 h)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs. **E.** Arithmetic means $\pm$ SEM ($n=3-16$) of IP₃R2/GAPDH ratio in immature and LPS (1 μ g/ml, 4-24h)-treated *akt2*^{+/+} and *akt2*^{-/-} DCs. * ($p<0.05$), ANOVA, ## ($p<0.01$), only with unpaired *t*-test.

by IP₃-induced Ca²⁺ store depletion was significantly higher in LPS-matured DCs from *akt2*^{+/+} mice than in *akt2*^{-/-} DCs.

Reduced expression of IP₃ receptor 2 in *akt2*^{-/-} DCs

Impaired Ca²⁺ release, SOCE and I_{CRAC} in *akt2*^{-/-} DCs could have resulted from Akt2 sensitivity of the Ca²⁺ release pathways such as inositoltrisphosphate receptors IP₃Rs or the ryanodine receptor RyR1. As illustrated in Fig. 4A, transcript levels of IP₃R1, IP₃R3 and RyR1 were similar in immature *akt2*^{+/+} and *akt2*^{-/-} DCs, whereas the transcript abundance of IP₃R2 was significantly higher in *akt2*^{+/+} DCs than in *akt2*^{-/-} DCs. Stimulation of *akt2*^{+/+} DCs with LPS (1 μ g/ml) resulted in a transient upregulation of IP₃R2 transcript abundance within 1h in *akt2*^{+/+} DCs, an effect significantly impaired in *akt2*^{-/-} DCs (Fig. 4B). Stimulation of DCs with LPS (1 μ g/ml, 1 h) further tended to upregulate IP₃R1 and IP₃R3, an effect reaching statistical significance for IP₃R3 (Fig. 4C). The effect of LPS on IP₃R1 and IP₃R3 was not significantly different between genotypes. In neither genotype, LPS did significantly modify RyR1 transcript levels.

Fig. 5. Migration of *akt2*^{+/+} and *akt2*^{-/-} DCs is sensitive to the IP₃R inhibitor Xestospongine C. Arithmetic means $\pm$ SEM (n = 5-25) of spontaneous migration and migration in response to either CCL21 (25 ng/ml, 4h, LPS (1 μ g/ml, 24h)-matured DCs, A) or CXCL12 (50 ng/ml, 4h, immature DCs, B) of *akt2*^{+/+} and *akt2*^{-/-} DCs in the absence or in the presence of the IP₃R inhibitor Xestospongine C (Xc, 5 μ mol/l, 3h) * (p<0.05), ** (p<0.01), *** (p<0.001), ANOVA; # (p<0.05), only with unpaired t-test.

Fig. 6. Reduced expression of the transcription factor Ets1 in *akt2*^{-/-} DCs. A. The lysates of immature and LPS (1 μ g/ml, 24h)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs were immunoprecipitated with a phosphospecific antibody that detects the consensus Akt phosphorylation sequence (RXRXX(S/T)) and then immunoblotted with IP₃R2 antibody. B. Arithmetic means ($\pm$ SEM, n=6) of the Ets1 mRNA abundance in immature *akt2*^{+/+} and *akt2*^{-/-} DCs. * (p<0.05), unpaired t-test. C. Arithmetic means ($\pm$ SEM, n=6-9) of the Ets1 mRNA abundance in *akt2*^{+/+} and *akt2*^{-/-} DCs, unstimulated (LPS, 0h) and stimulated with LPS (1 μ g/ml, 1h and 4h). ** (p<0.01), ANOVA; # (p<0.05), only with unpaired t-test. D. Western blot of whole

cell lysate protein of ETS1 and GAPDH in LPS (1 μ g/ml, 24h)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs. E. Arithmetic means $\pm$ SEM (n = 6) of ETS1/GAPDH ratio in immature and LPS (1 μ g/ml, 24h)-matured *akt2*^{+/+} and *akt2*^{-/-} DCs. ** (p<0.01), ANOVA.

Western blot analysis was employed to elucidate whether the differences in transcript levels were paralleled by similar differences in IP₃R2 protein abundance. Prior to maturation and upon LPS (1 μ g/ml) treatment for 4h, 8h and 12h, the IP₃R2 protein abundance was similar in *akt2*^{+/+} and *akt2*^{-/-} DCs (Fig. 4D, E). However, 24h after LPS treatment a significant increase of IP₃R2 protein abundance was observed in *akt2*^{+/+} DCs, but not in *akt2*^{-/-} DCs (Fig. 4D, E).

DC Migration is sensitive to the IP₃R inhibitor Xestospongine C

To further address the functional significance of Akt2 sensitive IP₃R2 regulation, migratory capacity was tested in LPS-matured DCs from *akt2*^{+/+} and *akt2*^{-/-} mice in the absence

Fig. 7. Silencing of the transcription factor ETS1 in LPS-matured *akt2*^{+/+} DCs reduces Ca²⁺ release, store-operated Ca²⁺ entry, I_{CRAC} and migration. A. Western blot analysis of whole cell lysate protein of ETS1 in immature control (control siRNA) and siETS1-DCs. Representative experiments showing ETS1 band and GAPDH as loading control. B. Arithmetic means (± SEM) of the Ets1 (n=14) and IP₃R2 (n=7) mRNA abundance in LPS (1 mg/ml, 1 h)-treated control (control siRNA) and siETS1-DCs. *(p<0.05), unpaired *t*-test. C. Fura-2 fluorescence ratio in LPS (1 μg/ml, 24 h)-matured control (control siRNA) and siETS1-DCs upon exposure to thapsigargin (1 μmol/l) in the Ca²⁺-free bath solution followed by readdition of extracellular Ca²⁺. D. Arithmetic means (± SEM) of the peak and the slope of the change in Fura-2 fluorescence following addition of thapsigargin (1 μmol/l) in the absence and in the presence of extracellular Ca²⁺ to LPS (1 mg/ml, 24 h)-matured control (n=55) and siETS1 (n=103) DCs. ** (p<0.01), *** (p<0.001), unpaired *t*-test. E. Fura-2 fluorescence ratio in LPS (1 μg/ml, 24 h)-matured control (control siRNA) and siETS1-DCs upon addition of ATP (100 μmol/l) to the Ca²⁺-free bath solution. F. Arithmetic means (± SEM, n=38-61) of the peak and the slope of the change in Fura-2 fluorescence following addition of ATP (100 μmol/l) as in E. *** (p<0.001), unpaired *t*-test. G. Mean I/V relationships of currents activated by 40 μmol/l IP₃ normalized to cell capacitance in LPS (1 μg/ml, 24 h)-matured control (n=20) and siETS1 (n=17) DCs. *** (p<0.001), unpaired *t*-test. H. Mean whole-cell conductance of inward currents (± SEM, n=17-20) calculated by linear regression of I/V curves shown in (G) between -40 and 0 mV. * (p<0.05), unpaired *t*-test. I. Arithmetic means ± SEM (n=11) of CCL21 (25 ng/ml, 4h)-induced migration in LPS (1 μg/ml, 24h)-matured control (control siRNA) and siETS1 DCs. * (p<0.05), unpaired *t*-test.

thapsigargin (1 μmol/l) in the Ca²⁺-free bath solution followed by readdition of extracellular Ca²⁺. D. Arithmetic means (± SEM) of the peak and the slope of the change in Fura-2 fluorescence following addition of thapsigargin (1 μmol/l) in the absence and in the presence of extracellular Ca²⁺ to LPS (1 mg/ml, 24 h)-matured control (n=55) and siETS1 (n=103) DCs. ** (p<0.01), *** (p<0.001), unpaired *t*-test. E. Fura-2 fluorescence ratio in LPS (1 μg/ml, 24 h)-matured control (control siRNA) and siETS1-DCs upon addition of ATP (100 μmol/l) to the Ca²⁺-free bath solution. F. Arithmetic means (± SEM, n=38-61) of the peak and the slope of the change in Fura-2 fluorescence following addition of ATP (100 μmol/l) as in E. *** (p<0.001), unpaired *t*-test. G. Mean I/V relationships of currents activated by 40 μmol/l IP₃ normalized to cell capacitance in LPS (1 μg/ml, 24 h)-matured control (n=20) and siETS1 (n=17) DCs. *** (p<0.001), unpaired *t*-test. H. Mean whole-cell conductance of inward currents (± SEM, n=17-20) calculated by linear regression of I/V curves shown in (G) between -40 and 0 mV. * (p<0.05), unpaired *t*-test. I. Arithmetic means ± SEM (n=11) of CCL21 (25 ng/ml, 4h)-induced migration in LPS (1 μg/ml, 24h)-matured control (control siRNA) and siETS1 DCs. * (p<0.05), unpaired *t*-test.

and presence of IP₃R inhibitor Xestospongine C (5 μmol/l). Xestospongine C significantly decreased the migratory activity of CCL21-treated LPS-matured *akt2*^{+/+} DCs and abrogated

Fig. 8. Tentative model of Akt2-dependent Ca²⁺ signaling and migration in DCs. Red arrows represent the novel contribution of the present study.

the differences in migratory activity between DCs from *akt2*^{+/+} and *akt2*^{-/-} mice (Fig. 5A). CXCL12-induced migration of immature DCs was also sensitive to Xestospongine C. However, CXCL12-induced migration of immature DCs was not significantly different between *akt2*^{-/-} and *akt2*^{+/+} DCs (Fig. 5B).

PKB/Akt has previously been shown to phosphorylate IP₃R, reducing its sensitivity to IP₃ and diminishing Ca²⁺ release [30]. We thus examined the phosphorylation status of IP₃R2 by immunoprecipitating the lysates with a phosphospecific antibody that detects the consensus Akt phosphorylation sequence (RXRXX(S/T)) and immunoblotting with IP₃R2-specific antibody (Fig. 6A). IP₃R2 was phosphorylated in immature as well as in LPS-matured DCs. However no significant difference was detected between *akt2*^{+/+} and *akt2*^{-/-} DCs.

Reduced expression of the transcription factor ETS1 in akt2^{-/-} DCs

According to previous observations, expression of IP₃R3 in CD4⁺ T cells requires the ETS1 transcription factor [31]. In ETS1-deficient CD4 T cells the level of IP₃R2 protein was also reduced [31]. Moreover, activated Akt has been shown to lead to increased ETS1 transcription [32]. Therefore we next examined the transcript level of ETS1 in *akt2*^{-/-} and *akt2*^{+/+} DCs. The transcript abundance of ETS1 was significantly lower in immature *akt2*^{-/-} DCs than in immature *akt2*^{+/+} DCs (Fig. 6B). Moreover, similar to its effect on IP₃R2, LPS (1 μg/ml) induced a transient increase in ETS1 transcript level within 1h in *akt2*^{+/+} DCs, but not in *akt2*^{-/-} DCs (Fig. 6C). Accordingly, the protein abundance of ETS1 in LPS-matured *akt2*^{-/-} DCs was significantly decreased (Fig. 6D, E).

Silencing of the transcription factor ETS1 in LPS-matured akt2^{+/+} DCs reduces Ca²⁺ release, store-operated Ca²⁺ entry, I_{CRAC} and migration

In order to test whether Akt2-dependent ETS1 expression contributes to decreased IP₃R2 expression and function in *akt2*^{-/-} DCs, we suppressed ETS1 expression with siRNA (Fig. 7). The efficiency of silencing assessed with RT-PCR was 47 ± 11% (n=7) and was confirmed by western blotting (Fig. 7A). Upon LPS stimulation (1h), ETS1 and IP₃R2 transcript abundance were reduced in siETS1 DCs (Fig. 7B). The discrepancy in the IP₃R2 transcript abundance shown in Fig. 4B and in this series of experiments is most probably due to the influence of siRNAs on DC maturation [33]. Thapsigargin-induced (Fig. 7C, D) and ATP-induced (Fig. 7E, F) Ca²⁺ release, thapsigargin-induced SOCE (Fig. 7C, D), as well as I_{CRAC} (Fig. 7G, H) were significantly impaired in siETS1 LPS (24 h)-matured DCs. Moreover, CCL21-dependent migration was significantly decreased in siETS1- LPS (24 h)-matured DCs (Fig. 7I). Those data strongly suggest that Akt2 upregulates ETS1 transcription leading to enhanced expression of IP₃R2, increased Ca²⁺ release and increased store-operated Ca²⁺ entry, which at least partially account for the effect of Akt2 on DC migration.

Discussion

In the present study we demonstrate that Akt2 up-regulates the expression of IP₃R2 in mouse DCs and thus enhances release of Ca²⁺ from intracellular stores. Accordingly, store-operated Ca²⁺ entry and current through Ca²⁺ release-activated Ca²⁺ (CRAC) channels are decreased in Akt2 deficient DCs. Moreover, we provide evidence that IP₃R2 expression in DCs is regulated by the Akt2-dependent transcription factor ETS1. Finally, we show that reduced IP₃R2 expression contributes to or even accounts for impaired migration of Akt2-deficient DCs (Fig. 8).

Within 1 hour of LPS-induced maturation, the transcript abundance of IP₃R2 was transiently enhanced in *akt2*^{+/+} and to a lesser extent in *akt2*^{-/-} DCs. As a consequence, the protein abundance of IP₃R2 was increased 24h after LPS addition in *akt2*^{+/+} DCs. In *akt2*^{-/-} DCs, the protein level of IP₃R2 also tended to be higher 24h after LPS than prior to LPS treatment, a difference, however, not reaching statistical significance. The apparent inability of *akt2*^{-/-} DCs to strongly upregulate IP₃R2 expression resulted in an impaired function of IP₃Rs in mature *akt2*^{-/-} DCs, as demonstrated by reduced Ca²⁺ release induced by P2Y receptor engagement or by inhibition of sarcoendoplasmatic reticulum Ca²⁺ ATPase (SERCA) with thapsigargin. Consequently, store-operated Ca²⁺ entry and IP₃-induced activation of CRAC channels were significantly blunted in Akt2-deficient DCs. Most importantly, increase of cytosolic free Ca²⁺ concentration upon ligation of the CCR7 was strongly reduced in mature *akt2*^{-/-} DCs, which was paralleled by reduced CCR7-dependent migration of those cells. IP₃R inhibitor Xestospongine C inhibited CCR7-dependent migration and abrogated the difference in migrating ability between *akt2*^{+/+} and *akt2*^{-/-} DCs. This allows us to conclude that reduced expression of IP₃R2 underlies, at least partially, the defective CCR7-dependent migration of Akt2-deficient DCs. Akt2 knockdown has similarly been shown to result in reduced plasmin-triggered migration of human DCs [7], and CSF-1- and MCP-1-induced chemotaxis of mouse peritoneal macrophages [9].

Akt2-dependent release of Ca²⁺ through the IP₃R2 would be in line with the well established PI3K-dependent upregulation of Ca²⁺ mobilization in lymphocytes. Phosphorylation of Akt/PKB is compromised and Ca²⁺ flux in response to stimulation of B and T cell antigen receptors is attenuated in B and T lymphocytes deficient in the catalytic subunit of PI3K, p110δ [12]. Diminished Ca²⁺ mobilization in B cells with either genetic or pharmacologic inhibition of p110δ has also been confirmed in other studies [13-15]. The present study suggests that in DCs PI3K-sensitive Ca²⁺ mobilisation at least partially results from Akt2-dependent IP₃R expression. In DT40 B cells and Jurkat cells a seeming inhibiting effect of Akt2 on Ca²⁺ mobilization has been observed [34]. Whereas the initial amplitude of Ca²⁺ mobilization was not influenced by Akt2, the subsequent clearance of Ca²⁺ was accelerated by Akt2, which shortened the increase of cytosolic Ca²⁺ concentration and blunted the activation of NFAT [34]. The mechanism of Akt2-dependent reduction of Ca²⁺ mobilization proposed by Martin et al. involves Akt2 interaction with Bcl-2 proteins, which bind to IP₃R thus inhibiting Ca²⁺ release [34]. Accordingly, a cell permeable peptide that blocks the interaction between Bcl-2 and IP₃R abrogated the effect of Akt2 [34]. However, enhanced Ca²⁺ clearance rather than modified Ca²⁺ release measured in the presence of Akt2 may result also from Akt2-dependent upregulation of SERCA or any other extrusion pathway in this model.

Several mechanisms of Akt-dependent regulation of IP₃Rs have been determined so far. A direct interaction and phosphorylation of IP₃R by Akt has been demonstrated [30, 35]. Szado et al. have shown that Akt-dependent phosphorylation of IP₃Rs significantly reduces their Ca²⁺ release activity [30]. Another study of Khan et al. has not revealed any modification of IP₃R function by Akt phosphorylation [35]. We could detect phosphorylation of IP₃R2 at the Akt specific motif, however no difference in phosphorylated IP₃R2 was observed between *akt2*^{-/-} and *akt2*^{+/+} DCs. Another proposed mechanism involves Akt-dependent regulation of Bcl-2 anti-apoptotic proteins [34], which are known to interact with IP₃R through their anti-apoptotic BH4 domain and to inhibit Ca²⁺ release [36].

The present study discloses an influence of Akt2 on ETS1-dependent transcription of IP₃R2. ETS1 is a transcription factor highly conserved throughout evolution and highly expressed in lymphocytes and in tumors [37, 38]. ETS1 seems to mediate the upregulating effect of Akt2 on IP₃R2 expression in mouse DCs. The kinetics of ETS1 transcription upon LPS stimulation follows the same pattern as IP₃R2 with a rapid and transient upregulation of ETS1 transcript abundance 1h after LPS treatment. Mature *akt2*^{-/-} DCs expressed significantly less ETS1 protein than mature *akt2*^{+/+} DCs. Moreover, IP₃R2 mRNA expression was reduced upon ETS1 knock down. Silencing of ETS1 also resulted in a strong impairment of Ca²⁺ release induced either by inhibition of SERCA with thapsigargin or by ligation of P2Y receptors with ATP. These results are in accordance with the study on ETS1-deficient CD4 T cells in which Ca²⁺ mobilization in response to TCR ligation is impaired [31]. In addition, IP₃R3 has clearly been shown to be under transcriptional regulation by ETS1 [31, 39].

ETS1 gene expression is known to be stimulated by activated Akt [32, 40, 41]. In prostate cancer cells elevated Akt (v-akt murine thymoma viral oncogene homolog) activity has been demonstrated to increase ETS1 protein levels and exogenous ETS1 expression is sufficient to rescue invasive potential, which decreases following Akt inhibition [41]. On the other hand, inhibition of the PI3K/Akt pathway blocked transcriptional upregulation of ETS1 by ER stress in human melanoma cells [42] and by PDGF in human aortic vascular smooth muscle cells [43]. Moreover, knockdown of Akt3 reduced the basal levels of ETS1 in melanoma cells, suggesting that the PI3K/Akt pathway may play a role in regulating constitutive expression of ETS1 [42]. Expression of ETS1 was reduced in *akt2*^{-/-} DCs only upon stimulation with LPS. LPS has been shown to stimulate PI3K with the subsequent Akt phosphorylation in mouse DCs [44, 45]. Thus, LPS-induced activation of Akt2 is probably required for the continuous expression of ETS1 upon DC maturation.

Conclusion

Akt2 upregulates IP₃R2 transcription and protein expression in DCs presumably by enhancing the expression of ETS1. The effect contributes to or even accounts for the stimulating effect of Akt2 on DC migration.

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