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

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CCL21 • Ca^{2+} release • I_{CRAC} • SOCE

Abstract
Background/Aims: The protein kinase Akt2/PKBβ 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 Ca^{2+} signaling. We thus explored whether Akt2 regulates DC Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} concentration, thapsigargin-induced release of Ca^{2+} from intracellular stores with subsequent store-operated Ca^{2+} entry (SOCE), ATP-induced inositol 1,4,5-trisphosphate (IP_{3})-dependent Ca^{2+} release as well as Ca^{2+} release-activated Ca^{2+} (CRAC) channel activity were all significantly lower in mature akt2^{-/-} than in mature akt2^{+/+} DCs. Transcript levels of IP_{3} receptor IP_{3}R2 and of IP_{3}R2 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 IP_{3}R2 and ETS1 were similarly higher in akt2^{+/+} than in akt2^{-/-} DCs. The IP_{3}R inhibitor Xestospongin C significantly decreased CCL21-induced migration of akt2^{+/+} DCs and abrogated the differences between genotypes. Finally, knock-down of ETS1 with siRNA decreased IP_{3}R2 mRNA abundance, thapsigargin- and ATP-induced Ca^{2+} 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 IP_{3}R2 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 Cβ (PLC β) [1] (Fig. 8), which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP$_2$) to generate inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ triggers Ca$^{2+}$ release from intracellular stores via IP$_3$, receptors (IP$_3$Rs) in the endoplasmic reticulum (ER). Depletion of Ca$^{2+}$ in ER results in the activation of Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels leading to a massive entry of extracellular Ca$^{2+}$ [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 Ca$^{2+}$-sensing subunit STIM1 and STIM2 [6].

CCR ligation and the resulting release of βγ 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 PKCζ/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 Ca$^{2+}$ ([Ca$^{2+}$]) [3], Akt2 may influence DC migration via regulating components of Ca$^{2+}$ signaling. In lymphocytes engagement of T or B cell receptors leads to the activation of PI3K, which upregulates Ca$^{2+}$ mobilization [12-15]. Accordingly, inhibition of PI3K impairs the rise in cytosolic Ca$^{2+}$ 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 Ca$^{2+}$ signaling in mouse DCs. We show that in mouse dendritic cells, Akt2 enhances migration through up-regulating the expression of IP$_3$R2 via the Akt2-dependent transcription factor ETS1, which results in an increased release of Ca$^{2+}$ from intracellular stores, store-operated Ca$^{2+}$ entry, and current through Ca$^{2+}$ release-activated Ca$^{2+}$ (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 x 10⁶ 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 µg/ml, 24 h, Enzo Life Sciences, Lausen, Switzerland).

**Immunostaining and flow cytometry**

Cells (10⁶) were incubated in 200 µl PBS, containing 0.1% FCS and fluorochrome-conjugated antibodies at a concentration of 10 µg/ml. A total of 5 x 10⁶ 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 IgG2a, 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 x 10⁶ 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 µg/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 µg/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 µl) 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 µl cell culture medium containing immature or LPS (1 µg/ml, 24 h) - matured DCs either untreated or treated with IP₃R inhibitor Xestospongin C (5 µM, Enzo Life Sciences, Lausen, Switzerland) for 3h in a concentration of 1 x 10⁵ cells/ml, allowed to migrate through a polycarbonate mesh (pore size 8 µm) 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 µM, 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 λ = 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×/1.30 oil, Zeiss, Oberkochen, Germany) or a camera (Proxtronic, Bensheim, Germany). Emitted fluorescence intensity was recorded at λ = 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) release was measured following addition of MgATP (100 µ, mol/l, Alfa Aesar GmbH & Co KG, Germany) to the Ca\(^{2+}\)-free solution (see below). To measure SOC entry, changes in cytosolic Ca\(^{2+}\) were monitored upon depletion of the intracellular Ca\(^{2+}\) stores. Experiments were carried out prior to and during exposure of the cells to the Ca\(^{2+}\)-free solution. In the absence of Ca\(^{2+}\), the intracellular Ca\(^{2+}\) stores were depleted by inhibition of the vesicular Ca\(^{2+}\) pump by thapsigargin (1 µM, Molecular Probes). Re-addition of Ca\(^{2+}\) allowed assessing the store-operated Ca\(^{2+}\) entry.

The Ringer solution contained (in mmol/l): 125 NaCl, 5 KCl, 1.2 MgSO\(_4\), 2.2 Hepes, 2 Na\(_2\)HPO\(_4\), 2 CaCl\(_2\), and 5 glucose at pH 7.4. The Ca\(^{2+}\)-free solution contained (in mmol/l): 125 NaCl, 5 KCl, 1.2 MgSO\(_4\), 2 Na\(_2\)HPO\(_4\), 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 \(I_{\text{Ca,AM}}\) 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 (Ins(1,4,5)P\(_3\)), Enzo Life Sciences), pH 7.4.

The liquid junction potential \(\Delta E\) between the CscI-based pipette and the NaCl-based bath solutions estimated according to Barry and Lynch [25] was 1 mV. The data were not corrected for \(\Delta E\).

**Real-time PCR**

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

For amplification the following primers were used (5'->3' orientation): for IP, R1: fw AAG CGG ATG GAC CTG GTG TTA GAA CTG and rev AAT TTT GGT TTC TTG GTG CGT GTC GTA GAA CT; for IP, R2: fw CTG TTC TTC TTC ATC TTC ATC ATC ATC TGG T and rev AGG TTC TTG TTC TTG ATC ATC TGC C TTA G; for IP, R3: fw CTG TTC TAT CGT CAT CAT CGT GTT G and rev AGG TTC TTG TTC TTG ATC ATC TGC C TTA G; for IP, R4: fw CAG CCC ACT TCC TTT TGA TGA and rev TGG TTT TGA AAG GGA ACG AAG AAG AA; for ETS1: fw GAT ATC CTG TGG GAG CAT CTA GAG ATC and rev CAG CTG CAT CCG CCC ACT TCC TTT GTA G; for Tbp: fw CAC TCC TGG CAC ACC AGC TT and rev TGG TCT TTA GGT CAA GAT 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: IP, R2 (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.
Differentiation and maturation of dendritic cells is unaffected by Akt2 deficiency

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

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

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Yang et al.: Akt2- and ETS1-Sensitive IP$_3$R2

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227

Fig. 2. Diminished CCL21-induced increase of intracellular Ca$^{2+}$ 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]. Magitory 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 Ca$^{2+}$ release and store-operated Ca$^{2+}$ 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 Ca$^{2+}$ concentration ([Ca$^{2+}$]). As illustrated in Fig. 2, both slope and peak of CCL21 (75 ng/ml)-induced increase of [Ca$^{2+}$], were significantly less pronounced in LPS-matured DCs from akt2$^{-/-}$ mice than in DCs from akt2$^{+/+}$ mice. Chemokine-induced increase of [Ca$^{2+}$], is known to be due to the release of Ca$^{2+}$ from intracellular stores followed by the activation of store operated Ca$^{2+}$ entry (SOCE) [6, 28]. Therefore, further experiments addressed the impact of Akt2 on Ca$^{2+}$ release from intracellular stores and on SOCE activity in LPS-matured DCs. As indicated in Fig. 3(A, B), the addition of sarcoendoplasmatic reticulum Ca$^{2+}$ ATPase (SERCA) inhibitor thapsigargin (1 µmol/l) in the absence of extracellular Ca$^{2+}$ was followed by an increase of [Ca$^{2+}$], reflecting release of Ca$^{2+}$ from intracellular stores. The slope and the peak of the intracellular Ca$^{2+}$ release were significantly decreased in mature DCs from akt2$^{-/-}$ mice (Fig. 3B). The readddition of extracellular Ca$^{2+}$ in the continued presence of thapsigargin resulted in a rapid increase of [Ca$^{2+}$], reflecting SOCE. Both, peak and slope of SOCE were significantly higher in DCs from akt2$^{+/+}$ mice than in DCs from akt2$^{-/-}$ mice. Release of Ca$^{2+}$ induced by physiological IP$_3$ formation could be assessed through the stimulation of DCs via P2Y receptors with low concentrations of ATP [29]. Accordingly, ATP (100 µM)-induced
release of Ca\(^{2+}\) (measured in the absence of extracellular Ca\(^{2+}\)) was lower in LPS-matured \(\text{akt}^{2/-}\) than in \(\text{akt}^{2/+}\) DCs (Fig. 3C, D).

Reduced SOCE in mature \(\text{akt}^{2/-}\) DCs could be confirmed by whole cell patch clamp recordings. As illustrated in Fig. 3(E-G), the Ca\(^{2+}\) release activated Ca\(^{2+}\) current \(I_{\text{CRAC}}\) triggered
by IP$_3$-induced Ca$^{2+}$ store depletion was significantly higher in LPS-matured DCs from akt2$^{+/+}$ mice than in akt2$^{-/-}$ DCs.

**Fig. 4.** Reduced expression of IP$_3$ receptor 2 in akt2$^{-/-}$ DCs. A. Arithmetic means (± SEM, n=3) of the abundance of mRNA encoding the IP$_3$ receptors: IP$_{3}$R1, IP$_{3}$R2, and IP$_{3}$R3 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 (± SEM, n=6-13) of the IP$_{3}$R2 mRNA abundance in akt2$^{+/+}$ and akt2$^{-/-}$ DCs unstimulated (LPS, 0h) and stimulated with LPS (1 µg/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 (± SEM, n=3-6) of the IP$_{3}$R1, IP$_{3}$R3 and RyR1 mRNA abundance in akt2$^{+/+}$ and akt2$^{-/-}$ DCs unstimulated (LPS, 0h) and stimulated with LPS (1 µg/ml, 1h). * (p<0.05), ANOVA; # (p<0.05), only with unpaired t-test. D. Western blot of whole cell lysate protein of IP$_{3}$R2 and GAPDH in LPS (1 µg/ml, 24 h)-matured akt2$^{+/+}$ and akt2$^{-/-}$ DCs. E. Arithmetic means ± SEM (n=3-16) of IP$_{3}$R2/GAPDH ratio in immature and LPS (1 µg/ml, 4-24 h)-treated akt2$^{+/+}$ and akt2$^{-/-}$ DCs. *(p<0.05), ANOVA, ## (p<0.01), only with unpaired t-test.

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

Impaired Ca$^{2+}$ release, SOCE and I$_{Ca,LO}$ in akt2$^{-/-}$ DCs could have resulted from Akt2 sensitivity of the Ca$^{2+}$ release pathways such as inositoltrisphosphate receptors IP$_3$ Rs or the ryanodine receptor RyR1. As illustrated in Fig. 4A, transcript levels of IP$_3$R1, IP$_3$R3 and RyR1 were similar in immature akt2$^{+/+}$ and akt2$^{-/-}$ DCs, whereas the transcript abundance of IP$_3$R2 was significantly higher in akt2$^{+/+}$ DCs than in akt2$^{-/-}$ DCs. Stimulation of akt2$^{-/-}$ DCs with LPS (1 µg/ml) resulted in a transient upregulation of IP$_3$R2 transcript abundance within 1h in akt2$^{+/+}$ DCs, an effect significantly impaired in akt2$^{-/-}$ DCs (Fig. 4B). Stimulation of DCs with LPS (1 µg/ml, 1 h) further tended to upregulate IP$_3$R1 and IP$_3$R3, an effect reaching statistical significance for IP$_3$R3 (Fig. 4C). The effect of LPS on IP$_3$R1 and IP$_3$R3 was not significantly different between genotypes. In neither genotype, LPS did significantly modify RyR1 transcript levels.
Western blot analysis was employed to elucidate whether the differences in transcript levels were paralleled by similar differences in IP$_3$R2 protein abundance. Prior to maturation and upon LPS (1 µg/ml, 24h)-treatment for 4h, 8h and 12h, the IP$_3$R2 protein abundance was similar in akt2$^{+/+}$ and akt2$^{-/-}$ DCs (Fig. 4D, E). However, 24h after LPS treatment a significant increase of IP$_3$R2 protein abundance was observed in akt2$^{+/+}$ DCs, but not in akt2$^{-/-}$ DCs (Fig. 4D, E).

DC Migration is sensitive to the IP3R inhibitor Xestospongin C

To further address the functional significance of Akt2 sensitive IP$_3$R2 regulation, migratory capacity was tested in LPS-matured DCs from akt2$^{+/+}$ and akt2$^{-/-}$ mice in the absence and presence of the IP$_3$R inhibitor Xestospongin C (Xc, 5 µmol/l, 3h) *(p<0.05), ***(p<0.001), ANOVA; # (p<0.05), only with unpaired t-test.

Fig. 5. Migration of akt2$^{+/+}$ and akt2$^{-/-}$ DCs is sensitive to the IP$_3$R inhibitor Xestospongin C. Arithmetic means ± SEM (n = 5-25) of spontaneous migration and migration in response to either CCL21 (25 ng/ml, 4h, LPS (1 µg/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 IP$_3$R inhibitor Xestospongin C (Xc, 5 µmol/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 µg/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 IP$_3$R2 antibody. B. Arithmetic means (± SEM, n=6) of the Ets1 mRNA abundance in immature akt2$^{+/+}$ and akt2$^{-/-}$ DCs. *(p<0.05), unpaired t-test. C. Arithmetic means (± SEM, n=6-9) of the Ets1 mRNA abundance in akt2$^{+/+}$ and akt2$^{-/-}$ DCs, unstimulated (LPS, 0h) and stimulated with LPS (1 µg/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 µg/ml, 24h)-matured akt2$^{+/+}$ and akt2$^{-/-}$ DCs. E. Arithmetic means ± SEM (n =6) of ETS1/GAPDH ratio in immature and LPS (1 µg/ml, 24h)-matured akt2$^{+/+}$ and akt2$^{-/-}$ DCs. **(p<0.01), ANOVA.
and presence of IP₃R inhibitor Xestospongin C (5 µmol/l). Xestospongin C significantly decreased the migratory activity of CCL21-treated LPS-matured akt2+/+ DCs and abrogated
the differences in migratory activity between DCs from akt2+/+ and akt2-/- mice (Fig. 5A). CXCL12-induced migration of immature DCs was also sensitive to Xestospongin 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, ICRAC 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 ICRAC (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.

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

In the present study we demonstrate that Akt2 up-regulates the expression of IP,R2 in mouse DCs and thus enhances release of Ca\(^{2+}\) from intracellular stores. Accordingly, store-operated Ca\(^{2+}\) entry and current through Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels are decreased in Akt2 deficient DCs. Moreover, we provide evidence that IP,R2 expression in DCs is regulated by the Akt2-dependent transcription factor ETS1. Finally, we show that reduced IP,R2 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 IP,R2 was transiently enhanced in akt2\(^{+/+}\) and to a lesser extent in akt2\(^{-/-}\) DCs. As a consequence, the protein abundance of IP,R2 was increased 24h after LPS addition in akt2\(^{+/+}\) DCs. In akt2\(^{+/+}\) DCs, the protein level of IP,R2 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 IP,R2 expression resulted in an impaired function of IP,Rs in mature akt2\(^{+/+}\) DCs, as demonstrated by reduced Ca\(^{2+}\) release induced by P2Y receptor engagement or by inhibition of sarcoendoplasmatic reticulum Ca\(^{2+}\) ATPase (SERCA) with thapsigargin. Consequently, store-operated Ca\(^{2+}\) entry and IP,R-induced activation of CRAC channels were significantly blunted in Akt2-deficient DCs. Most importantly, increase of cytosolic free Ca\(^{2+}\) concentration upon ligation of the CCR7 was strongly reduced in mature akt2\(^{+/+}\) DCs, which was paralleled by reduced CCR7-dependent migration of those cells. IP,R inhibitor Xestospongin 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 IP,R2 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 Ca\(^{2+}\) through the IP,R2 would be in line with the well established PI3K-dependent upregulation of Ca\(^{2+}\) mobilization in lymphocytes. Phosphorylation of Akt/PKB is compromised and Ca\(^{2+}\) 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, p110\(\delta\) [12]. Diminished Ca\(^{2+}\) mobilization in B cells with either genetic or pharmacologic inhibition of p110\(\delta\) has also been confirmed in other studies [13-15]. The present study suggests that in DCs PI3K-sensitive Ca\(^{2+}\) mobilisation at least partially results from Akt2-dependent IP,R expression. In DT40 B cells and Jurkat cells a seeming inhibiting effect of Akt2 on Ca\(^{2+}\) mobilization has been observed [34]. Whereas the initial amplitude of Ca\(^{2+}\) mobilization was not influenced by Akt2, the subsequent clearance of Ca\(^{2+}\) was accelerated by Akt2, which shortened the increase of cytosolic Ca\(^{2+}\) concentration and blunted the activation of NFAT [34]. The mechanism of Akt2-dependent reduction of Ca\(^{2+}\) mobilization proposed by Martin et al. involves Akt2 interaction with Bcl-2 proteins, which bind to IP,R thus inhibiting Ca\(^{2+}\) release [34]. Accordingly, a cell permeable peptide that blocks the interaction between Bcl-2 and IP,R abrogated the effect of Akt2 [34]. However, enhanced Ca\(^{2+}\) clearance rather than modified Ca\(^{2+}\) 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 IP,Rs have been determined so far. A direct interaction and phosphorylation of IP,R by Akt has been demonstrated [30, 35]. Szado et al. have shown that Akt-dependent phosphorylation of IP,Rs significantly reduces their Ca\(^{2+}\) release activity [30]. Another study of Khan et al. has not revealed any modification of IP,R function by Akt phosphorylation [35]. We could detect phosphorylation of IP,R2 at the Akt specific motif, however no difference in phosphorylated IP,R2 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 IP,R through their anti-apoptotic BH4 domain and to inhibit Ca\(^{2+}\) release [36].
The present study discloses an influence of Akt2 on ETS1-dependent transcription of IP$_3$R2. 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 IP$_3$R2 expression in mouse DCs. The kinetics of ETS1 transcription upon LPS stimulation follows the same pattern as IP$_3$R2 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, IP$_3$R2 mRNA expression was reduced upon ETS1 knockdown. Silencing of ETS1 also resulted in a strong impairment of Ca$^{2+}$ 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 Ca$^{2+}$ mobilization in response to TCR ligation is impaired [31]. In addition, IP$_3$R3 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 IP$_3$R2 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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Yang et al.: Akt2- and ETS1-Sensitive IP3R2


