Role of Akt2 and SGK1 in Regulation of Dendritic Cells

Bedeutung von Akt2 und SGK1 bei der Regulation von Dendritischen Zellen

Dissertation

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<td>[Ca$^{2+}$]$_i$</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>AM</td>
<td>acetyldimethyl</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca$^{2+}$-induced Ca$^{2+}$ release</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca$^{2+}$ release-activated Ca$^{2+}$ channel</td>
</tr>
<tr>
<td>CRACM 1/Orai1</td>
<td>calcium release-activated calcium modulator 1</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DCFDA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
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<tr>
<td>I_{CRAC}</td>
<td>Ca^{2+} release-activated Ca^{2+} currents</td>
</tr>
<tr>
<td>IFNs</td>
<td>interferons</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP_3</td>
<td>inositol-(1,4,5)-trisphosphate</td>
</tr>
<tr>
<td>IP_3R</td>
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<tr>
<td>RT-PCR</td>
<td>real time polymerase chain reaction</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>NEAA</td>
<td>non-essential amino acids</td>
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<tr>
<td>NCKX</td>
<td>K+-dependent Na+/Ca2+ exchangers</td>
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<tr>
<td>NCX</td>
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<tr>
<td>NK cell</td>
<td>nature killer cell</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDK1</td>
<td>phosphoinositide-dependent kinase 1</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
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<tr>
<td>pDCs</td>
<td>plasmacytoid dendritic cells</td>
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<tr>
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<td>Definition</td>
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<td>-------------------------------------------------</td>
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<tr>
<td>PH</td>
<td>pleckstrin-homology</td>
</tr>
<tr>
<td>PKB /Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane Ca(^{2+})-ATPases</td>
</tr>
<tr>
<td>PtdIns(4,5)P2</td>
<td>phosphatidylinositol-(4,5)-bis-phosphate</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real time polymerase chain reaction</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca(^{2+})-ATPases</td>
</tr>
<tr>
<td>SGK</td>
<td>serum and glucocorticoid inducible kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOCE</td>
<td>store-operated Ca(^{2+}) entry</td>
</tr>
<tr>
<td>SOC()s</td>
<td>store-operated channels</td>
</tr>
<tr>
<td>STIM1</td>
<td>stromal interaction molecule 1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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Abstract

The present study investigates the role of Phosphoinositide 3 kinase (PI3K) pathway in the regulation of dendritic cell (DC) functions. DCs are antigen-presenting cells that provide a link between innate and adaptive immunity and are required for initiation of specific T cell-driven immune responses. The present project concentrates on the possible regulation of DC functions by two PI3K-dependent kinases, Akt2/PKBβ and serum- and glucocorticoid-inducible kinase-1 (SGK1), which share 55\% identity in their kinase domain regions.

Akt2/PKBβ is known to be required for macrophage and DC migration, though the involved mechanisms remained undefined. On the other hand, DC migration is governed by Ca^{2+} signaling. In the present study, we addressed the mechanisms of Akt2-dependent DC migration by studying the possible involvement of Akt2 in Ca^{2+} signaling. DCs were derived from the bone marrow of Akt2-deficient mice (akt2−/−) and their wild type littermates (akt2+/+). Their maturation was induced by lipopolysaccharides (LPS, 1μg/ml). Chemokine CCL21 (25 ng/ml)-induced migration of mature akt2−/− DCs was impaired compared to akt2+/+ DCs. In Ca^{2+} imaging experiments, CCL21 (75 ng/ml)-triggered increase in cytosolic Ca^{2+} concentrations ([Ca^{2+}]_{i}) was also significantly diminished in mature akt2−/− DCs. Moreover, release of Ca^{2+} from intracellular stores induced by thapsigargin (1 μM) and the following store-operated Ca^{2+} entry (SOCE) were reduced in mature akt2−/− DCs. Stimulation of P2Y receptors with ATP (100 μM) resulted in IP_{3} release, which was also significantly decreased in mature akt2−/− DCs. IP_{3}-induced activation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels measured in whole cell patch-clamp experiments was significantly lower in mature akt2−/− DCs. Analysis of the expression of IP_{3} receptor isoforms in akt2+/+ DCs and akt2−/− DCs by RT-PCR revealed a significantly impaired transcript of IP_{3}R2 in akt2−/− DCs. Upon 1h of LPS stimulation mRNA abundance of IP_{3}R2 was increased in akt2+/+ but not in akt2−/− DCs. Accordingly, protein abundance of IP_{3}R2, analyzed by western blot, was reduced in mature akt2−/− DCs. mRNA abundance of transcription factor Ets1, which is known to upregulate the IP_{3}R2 transcription, was significantly lower in immature akt2−/− DCs than in akt2+/+ DCs. Similarly to IP_{3}R2, mRNA level of Ets1 was upregulated by LPS (1h) in akt2+/+ but not in akt2−/− DCs. ETS1 protein had lower expression in mature akt2−/− DCs compared to akt2+/+ DCs. DC migration was sensitive to the IP_{3}R inhibitor Xestospongin C (5 μM), which significantly decreased CCL21-induced migration of akt2+/+ DCs and abrogated the differences between genotypes. Finally, knock-down of ETS1 with siRNA led to impaired RNA abundance of IP_{3}R2, thapsigargin- and ATP-induced Ca^{2+} release from the stores, SOCE and CRAC channel activation, as well as DC migration. In conclusion, Akt2...
upregulates IP3R2 transcription in DCs, presumably by enhancing the expression of ETS1 and this effect may underlie the stimulating effect of Akt2 on DC migration.

On the other hand, PI3K activation suppresses proinflammatory cytokine production in DCs, which limits excessive T helper (Th1) polarization. Little is known about the mechanisms of PI3K-mediated suppression in DCs. The present study explored whether SGK1 can act as a negative regulator of DC functions downstream from PI3K. To this end, DCs were isolated from the bone marrow or from the spleen of gene targeted mice lacking functional SGK1 (sgk1−/−) and their wild type littermates (sgk1+/+). Expression of maturation markers, MHC II, CD86 and CD54, analyzed by flow cytometry, and secretion of interleukin IL-12 upon LPS (100 ng/ml) stimulation, determined by ELISA, were significantly higher and phagocytic capacity, assessed as FITC-dextran uptake, was significantly lower in sgk1−/− than in sgk1+/+ DCs. Moreover, expression of CD86, MHCII and CD54 was also significantly higher in DCs isolated from the spleen of sgk1−/− mice than those from sgk1+/+ mice. Moreover, the nuclear localization of the p65 subunit of the transcription factor NF-κB was significantly enhanced in sgk1−/− DCs. N-myc downstream regulated gene 1 (NDRG1), the physiological substrate of SGK1, which is known to attenuate NF-κB signalling, was similarly expressed in sgk1+/+ and sgk1−/− DCs, however SGK1-dependent phosphorylation of NDRG1 was decreased in sgk1−/− DCs. Silencing of NDRG1 by siRNA in sgk1+/+ DCs led to an enhanced nuclear abundance of p65 compared to the control empty vector-treated DCs. These observations point to an important role of SGK1 in the regulation of DC functions and thus disclose a novel element in the cross-talk between innate and adaptive immunity. Impaired phosphorylation of NDRG-1 in SGK1-deficient DCs may underlie enhanced nuclear localization of NF-κB and, accordingly, increased maturation and IL-12 production.
Abstrakt

In der vorliegenden Studie wurde die Rolle des Phosphoinositol 3 Kinase (PI3K) Signalweges in der Regulation der Funktion Dendritischer Zellen untersucht. Dendritische Zellen sind Antigen-präsentierende Zellen, welche einen Link zwischen angeborener und adaptiver Immunität herstellen und für die Aktivierung von spezifischen T-Zellen erforderlich sind. Das gegenwärtige Projekt konzentriert sich auf die mögliche Regulation der Dendritischen Zelfunktion durch zwei PI3K-abhängige Kinasen, Akt2/PKBβ und die Serum- und Glukokortikoid-induzierbare Kinase-1 (SGK1), welche 55% identische Kinasedomänenregion besitzen.


Darüber hinaus ist die Freisetzung von Ca²⁺ aus den intrazellulären Speichern durch Thapsigargin (1 µM) induziert. Auf dies folgt ein Speicher-abhängiger Ca²⁺ Einstrom (SOCE), welcher in reifen akt2−/− Dendritischen Zellen signifikant reduziert war. Eine Stimulation des P2Y Rezeptors mit ATP (100µM) führte zu einem IP₃-abhängigen Ca²⁺ Ausstrom, welcher in reifen akt2−/− Dendritischen Zellen auch signifikant reduziert war.

Zellen, jedoch nicht in akt2−/− Dendritischen Zellen. Demzufolge war auch die Proteinkonzentration von IP3R2, analysiert mit Hilfe eines Western Blots, in reifen akt2−/− Dendritischen Zellen reduziert.


1. Introduction

1.1 Role of Dendritic Cells

The immune system is composed of two major subdivisions, i.e., the antigen (Ag)-nonspecific innate immunity, which was discovered by Elie Metchnikoff in 1891, and the Ag-specific adaptive immunity, discovered by Paul Ehrlich in 1900 (Kaufmann 2008; Paul 2011).

When the pathogen breaks through the physiological barrier in our body, it will encounter the innate immune system, the first line of defense. The innate immune system has two crucial abilities (Janeway and Medzhitov 2002), i.e., to rapidly recognize pathogens and/or tissue injury and to signal the presence of danger to cells of the adaptive immune system, the second line of defense. Phagocytic cells, nature killer (NK) cells, complement, and interferons (IFNs) are the main players in the innate immune system. These players express a variety of pattern recognition receptors to identify molecular patterns that are associated with microbial pathogens such as bacterial lipopolysaccharide (LPS), carbohydrates, and double-stranded viral RNA.

The adaptive immune system, besides being the second line of defense, also affords protection against re-exposure to the same pathogen (Sun, Beilke et al. 2009). The key features of the adaptive immune system are the abilities to rearrange genes of the immunoglobulin family, permitting the creation of a large diversity of Ag-specific clones and immunological memory.

Each of the major subdivisions of the immune system has both cellular and humoral components to carry out its protective function. Although these two subdivisions have distinct functions, there is interplay between them. Dendritic cells (DCs) act as antigen presenting cells (APCs) and become the link between these two parts (Fig. 1) (Banchereau, Briere et al. 2000; Lipscomb and Masten 2002).

DCs are the most efficient APCs owing to their unique ability of inducing primary immune responses, therefore permitting the establishment of immunological memory. Besides inducing adaptive immune responses to foreign antigens (Ags), DCs are also crucial for maintaining immune tolerance (Lewis and Reizis 2012). DCs were named for their probing, tree-like shapes (from the Greek ‘dendron’, meaning tree), which were first described by R Steinman and Z Cohn in 1973 (Steinman and Cohn 1973).
All DCs are ultimately derived from hematopoietic stem and progenitor cells in the bone marrow (Merad and Manz 2009). Hematopoietic stem and progenitor cells give rise to several distinct progenitors that can be differentiated into one or more DC subsets. DCs can be divided into two major populations: the type I interferon-producing plasmacytoid DCs (pDCs) and conventional DCs (cDCs) expressing the surface marker CD11c. Both populations express class II major histocompatibility complex (MHC-II) (LeibundGut-Landmann, Waldburger et al. 2004; Naik, Sathe et al. 2007; Alvarez, Vollmann et al. 2008). The human pDCs express the surface marker CD123, two additional markers, BDCA-2(CD303) and BDCA-4(CD304), and they are restricted to peripheral blood and bone marrow (Barchet, Blasius et al. 2005; McKenna, Beignon et al. 2005). Unlike human pDCs, mouse pDCs express the cell surface antigens B220 and Ly6C. Moreover, mouse pDCs express CD11c, although in low amounts compared with cDCs, and, except in mice treated with the cytokine Flt3 ligand, they do not express CD123 (IL-3α). Mouse pDC express BST-2 (mPDCA) and Siglec-H and are negative for CD11b. cDCs subset is the predominant DC population in the steady state (Colonna, Trinchieri et al. 2004; de Heer, Hammad et al. 2005). Moreover, upon activation, respiratory cDCs produce a range of inflammatory mediators (de Heer, Hammad et al. 2005; Beaty, Rose Jr et al. 2007). Nonlymphoid tissue cDCs emigrate continuously via lymphatics into draining lymph nodes; similar to the resident populations of lymphoid tissue DCs, most migrant cDCs die in lymphoid tissue and do not reenter the blood circulation. Strong inflammatory stimuli can cause an abrupt decrease in the number of lymphoid cDCs, a consequence of maturation-induced apoptosis and migration (Naik, Sathe et al. 2007).

Figure 1. The life cycle of dendritic cells, obtained from J Banchereau Et Al. Annual Review of Immunology, 2000
1.2 Maturation of Dendritic Cells

DC progenitors are not restricted to bone marrow and can be found in multiple locations. These progenitors can differentiate into immature DCs in peripheral tissues (Ocular Periphery and Disorders, Darlene A. Dartt). Immature DCs express only a small amount of MHC II and no co-stimulatory molecules. A major functional characteristic of immature DCs is the capacity for continuous endocytosis in the steady state (Lutz and Schuler 2002; Wilson, El-Sukkari et al. 2004). Thus, immature DCs are able to capture Ags but are still not able to determine T-cell fate and prime immune responses. Once peripheral tissues are inflamed or infiltrated by microbes, immature DCs become activated (Banchereau, Briere et al. 2000; Lutz and Schuler 2002). After capturing and processing Ags, they migrate to second lymph organs, and give rise to mature DCs that downregulate antigen-sampling functions but become exceedingly powerful at priming T cells (Alvarez, Vollmann et al. 2008).

Mature DCs express high cell-surface levels of antigen-presenting MHC molecules and costimulatory molecules CD40, CD80, CD83, and CD86 (Banchereau, Briere et al. 2000). They produce large amounts of proinflammatory cytokines, such as IL-12, IL-18, and TNFα (Lin, Jacek et al. 2006; Schmidt, Nino-Castro et al. 2012). Thus, DCs are capable of inducing TH1- and TH2-cell differentiation, and cytotoxic T lymphocyte response.

Besides the functional changes, as acquisition of high cellular motility, the morphological changes also accompany DC maturation. This includes a loss of adhesive structures, and cytoskeleton reorganization (Banchereau, Briere et al. 2000; Alvarez, Vollmann et al. 2008; Randolph, Ochando et al. 2008).

1.3 Migration of Dendritic Cells

Due to the complex life cycle, the ability of DCs and their progenitors to migrate is a central feature of DCs and indispensable for their functions within the immune system (Alvarez, Vollmann et al. 2008).

The migration of DCs entails 1) the ability of newly formed DCs or their progenitors to exit their place of birth and enter the blood, 2) the recruitment of the circulating cells into target tissues, 3) the extravascular lodging and interstitial motility needed to sample Ags, 4) the capacity to access lymph vessels to travel either to lymph nodes or back to the blood, and 5) the ability to interact with migrating lymphocytes and other immune cells in a manner that allows the exchange of critical information regarding the nature and context of presented Ags (Alvarez, Vollmann et al. 2008).
As mentioned above, many DCs begin their journey with their release from the bone marrow into the blood and subsequent traffic into peripheral lymphoid and nonlymphoid tissues. In nonlymphoid tissues, DCs eventually proceed into lymph nodes through afferent lymphatics and, in some instances, return back to the blood via the thoracic duct (von Andrian and Mempel 2003; Steinman and Hemmi 2006; Alvarez, Vollmann et al. 2008). Compartmental segregation of lymphoid tissues, which facilitates efficient surveillance and targeted cellular response, relies on the precise trafficking of immune cells between the circulation, tissues, and second lymph organs (von Andrian and Mempel 2003; Ricart, John et al. 2010). However, specific responses require directed cell migration that DCs accomplish through selective expression of chemokine receptors, coupled with generation of specific chemokine in immune organs. Specifically, immature DCs utilize specific chemokine receptor-ligand pathways, such as CCR2-CCL2 and, CCR5-CCL5, and CCR6-CCL20, as well as CXCR3-CXCL10 and CXCR4- CXCL12 (SDF1-α). However, after Ag capture when DCs become mature, they downregulate most of the above mentioned chemokine receptors, with the exception of CXCR4, while upregulating the crucial receptor CCR7. CCR7 has two cognate ligands, CCL19 (ELC, MIP-3 β) and CCL21 (SLC, 6Ckine, Exodus-2), which are highly expressed by peripheral lymphatic endothelial cells as well as LN stroma cells and guide DCs to second lymph organs (Banchereau, Brie et al. 2000; Le, Zhou et al. 2004; Rot and von Andrian 2004). To date 18 human chemokine receptors have been identified, among them four receptors are found on DCs (Table 1). Throughout its migration, DCs also require some other assistance to reach their targeted destination, such as highly regulated coordination of protrusion, retraction, adhesion, detachment, and major dynamic rearrangements of the actin cytoskeleton (Saarikangas, Zhao et al. 2009). Therefore DCs express E-cadherin and elastase and release type IV collagenase that may contribute to their migration (de Noronha, Hardy et al. 2005; Alvarez, Vollmann et al. 2008).

Table 1. Details of human chemokine receptors, obtained from C Murdoch, A Finn. Blood, 2000

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Amino acids</th>
<th>Base pairs</th>
<th>Ligand (high affinity)</th>
<th>Cellular distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR1(GPR5)</td>
<td>333</td>
<td>999</td>
<td>Lymphotactin</td>
<td>T, B, NK</td>
</tr>
<tr>
<td>CXCR1 (IL BRA)</td>
<td>350</td>
<td>1050</td>
<td>IL8, GCP2</td>
<td>N, M, T, NK, Bs, Ms, En</td>
</tr>
<tr>
<td>CXCR2 (IL BRB)</td>
<td>355</td>
<td>1065</td>
<td>IL8, GRO-α, GRO-β, GRO-γ, NAP-2, ENA-</td>
<td>N, M, T, NK, As, Nn, Ms,</td>
</tr>
<tr>
<td>Receptor</td>
<td>Gene Symbol</td>
<td>Accession Numbers</td>
<td>Binding</td>
<td>Function/Expression</td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>---------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>CXCR3</td>
<td></td>
<td>368 1104</td>
<td>IP-10, Mig, I-TAC</td>
<td>Activated T, En,</td>
</tr>
<tr>
<td>CXCR4*</td>
<td>(LESTR, FUSIN)</td>
<td>352 1056</td>
<td>SDF-1α, SDF-1β</td>
<td>Myeloid, T, B, Ep, En, DC</td>
</tr>
<tr>
<td>CXCR5 (BLR1)</td>
<td></td>
<td>372 1116</td>
<td>BCA-1</td>
<td>B</td>
</tr>
<tr>
<td>CX3CR1 (V28)</td>
<td></td>
<td>355 1065</td>
<td>Fractalkline</td>
<td>NK, M, T</td>
</tr>
<tr>
<td>CCR1</td>
<td></td>
<td>355 1065</td>
<td>RANTES, MIP-1α, HCC-1, MCP-2, MCP-3, MIP-5, Ckβ8</td>
<td>N, M, T, NK, B, Ms, As, Nn</td>
</tr>
<tr>
<td>CCR2A</td>
<td></td>
<td>374 1122</td>
<td>MCP-1, MCP3, MCP4</td>
<td>M</td>
</tr>
<tr>
<td>CCR2B↑</td>
<td></td>
<td>360 1080</td>
<td>MCP-1, MCP2, MCP3, MCP4</td>
<td>M, T, B, Bs</td>
</tr>
<tr>
<td>CCR3↑</td>
<td></td>
<td>355 1065</td>
<td>Eotaxin, Eotaxin-2, Eotaxin-3, RANTES, MCP2,3,4, MIP-5</td>
<td>Eo, Bs, T</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td>360 1080</td>
<td>TARC, MDC</td>
<td>T, P</td>
</tr>
<tr>
<td>CCR5↑ (ChemR13)</td>
<td></td>
<td>352 1056</td>
<td>RANTES, MIP-1β, MIP-1α, MCP-2</td>
<td>T, M, MØ, DC</td>
</tr>
<tr>
<td>CCR5</td>
<td>(STRL22, DRY-6, GPR-CY4, CKR-L3)</td>
<td>374 1122</td>
<td>MIP-3α</td>
<td>T, B, DC</td>
</tr>
<tr>
<td>CCR7 (BRL2, EBI1)</td>
<td></td>
<td>378 1134</td>
<td>MIP-3β, 6-C-kine</td>
<td>T, B, DC</td>
</tr>
<tr>
<td>CCR8*↑ (TER1, CKR-L1, ChemR1)</td>
<td></td>
<td>355 1065</td>
<td>I-309</td>
<td>M, Thymus</td>
</tr>
<tr>
<td>CCR9 (GPR-9-6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*CXCR4* and CCR8* are upregulated in certain contexts.
In general, migration of any cell involves three components: the formation and maintenance of cell polarity, activation of motility machinery for linear locomotion, and turning or changing direction of movement in response to gradients of environmental cues (Pollard and Borisy 2003; Philipsborn and Bastmeyer 2007; Petrie, Doyle et al. 2009). Besides sensing of signal gradients, chemotaxis of eukaryotes requires the polar differentiation of the cells. A leading edge called the lamellipodia and a trailing edge called the uropod (or rear end) characterize the polarized migrating cells [general about migration, section 1.3] (Small, Stradal et al. 2002; Philipsborn and Bastmeyer 2007; Alvarez, Vollmann et al. 2008; Saarikangas, Zhao et al. 2010).

Calcium release by the PLC-IP$_3$-IP$_3$R pathway at the rear was thought to be the major source to counteract the biophysics of calcium diffusion and maintain the calcium gradient which is important for maintaining cell polarity [complicated sentence and you call this part Ca$^{2+}$ signaling in DC migration, so now you should really write about DCs] (Clapham 2007; Bononi, Agnoletto et al. 2011). It has been demonstrated that an extracellular Ca$^{2+}$ influx is required for positive feedback at the leading edge of spontaneously polarized macrophages [you may use it for Discussion but not here] (Evans and Falke 2007). Inhibition of extracellular Ca$^{2+}$ influx leads to loss of leading-edge PI3K activity, disassembly of F-actin, cessation of ruffling, and decay of chemoattractant signals (Evans and Falke 2007). Conversely, increasing cytosolic Ca$^{2+}$ enhances membrane ruffling, PI3K activity, and F-actin accumulation.

In DCs, chemotactic chemokines, such as CXCL12 and CCL21, rapidly increase intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) by triggering Ca$^{2+}$ release from intracellular stores followed by the store-operated Ca$^{2+}$ (SOC) entry (Barbet, Demion et al. 2008; Nurbaeva, Schmid et al. 2012). Inhibition of SOC channels leads to profound changes in DC migration. Blocking of SOC channels impairs CCL21-dependent migration (Matzner, Zemtsova et al. 2008; Xuan, Shumilina et al. 2009). On the other hand, ion channels that control Ca$^{2+}$ flux by setting the membrane potential are also important regulators of DC migration. Ca$^{2+}$ influx brings about a strong local depolarization of the cell membrane, and consequently to sustain Ca$^{2+}$ entry, activity of other channels, which can prevent depolarization, is required. In
mouse DCs ion channels that support Ca\(^{2+}\) influx by hyperpolarizing the membrane and thereby providing electrical driving force for Ca\(^{2+}\) entry are voltage-gated K\(^+\) Kv channels (Matzner, 2008) and Ca\(^{2+}\)-activated Cl\(^-\) channel ANO6 (Kalina’s paper). Accordingly pharmacological or genetical inhibition of Kv or ANO6 channels results in a strong impairment of DC migration (Nici’s paper, Kala’s paper). On the other hand, transient receptor potential cation channel of melastatin family TRPM4, which functions as a Ca\(^{2+}\)-activated non-selective cation channel providing under physiological conditions influx of Na\(^+\) and therefore membrane depolarization, breaks the Ca\(^{2+}\) influx and prevents Ca\(^{2+}\) overload (Barbet, Demion et al. 2008). Interestingly, migration of TRPM4-deficient mouse DCs is also impaired, suggesting that [Ca\(^{2+}\)], should be perfectly balanced to enable DC migration.

It has been demonstrated for various cell types that the polar differentiation of migrating cells is established and maintained by generating gradients of Ca\(^{2+}\) concentrations through the concerted action of different Ca\(^{2+}\) channels and transporters.

### 1.4 Mechanism of Ca\(^{2+}\) Release and Ca\(^{2+}\) Entry in Dendritic Cells

Ca\(^{2+}\) can enter the cytoplasm from one of the two general sources: it can be released from intracellular stores, or extracellular Ca\(^{2+}\) can enter the cell across the plasma membrane (Bootman, Collins et al. 2001; Parekh and Putney 2005; Bootman 2006; Putney 2010). These two processes often occur either simultaneously or sequentially.

![Figure 2. Maintaining and using Ca\(^{2+}\) gradients for signaling, obtained from DE Clapham, Cell, 2007](image_url)
The internal stores are held within the membrane systems of the endoplasmic reticulum (ER) or the equivalent organelle, the sarcoplasmic reticulum of muscle cells (Mazzarello, Calligaro et al. 2003; Rizzuto and Pozzan 2006). Ca\(^{2+}\) release from these internal stores is mediated by various channels, of which the inositol-1,4,5-trisphosphate receptor (IP\(_3\)R) and ryanodine receptor (RyR) families have been studied extensively (Mikoshiba 2007; Vanderheyden, Devogelaere et al. 2009; Rossi, Tovey et al. 2011). These two channel classes are regulated by several factors, among which the most important activator is Ca\(^{2+}\) itself.

Calcium entry secondary to calcium release via IP\(_3\)R activation is essential for modulating calcium-dependent processes, particularly in immunity. IP\(_3\) is a second messenger produced through phosphoinositide turnover in response to many extracellular stimuli. The IP\(_3\)R is an IP\(_3\)-gated Ca\(^{2+}\) release channel located on ER. If IP\(_3\) and Ca\(^{2+}\) are bound to an IP\(_3\)R, it can open and Ca\(^{2+}\) will diffuse into the cytosol (Krebs and Michalak 2007; Mikoshiba 2007). IP\(_3\)Rs have three isoforms, IP\(_3\)R1, 2 and 3. Most work has been performed on the ubiquitously expressed IP\(_3\)R1, but it is however clear that various IP\(_3\)R isoforms can subtly differ in their properties (Nadif Kasri, Bultynck et al. 2002; Vermassen, Parys et al. 2004). Their affinity for IP\(_3\) displays a rank-order IP\(_3\)R2>IP\(_3\)R1>IP\(_3\)R3. The open probability of IP3Rs depends on the IP3 concentration and the Ca\(^{2+}\) concentration in the cytosol (Rahman 2012). It increases with increasing IP3 concentration. It is low for low Ca\(^{2+}\) concentration, increases with increasing Ca\(^{2+}\) (100-300nM) and finally decreases again for even higher Ca\(^{2+}\) concentrations (above 300nM) (Bootman, Collins et al. 2001; Foskett, White et al. 2007; Vanderheyden, Devogelaere et al. 2009).

RyRs also contain three different isoforms, RyR1, 2 and 3. RyRs are similar to the IP\(_3\)R, and when stimulated transport Ca\(^{2+}\) into the cytosol (Blaustein, Greger et al. 2001; Wehrens and Marks 2004). It is first demonstrated by O’Connell and his colleagues that immature mouse DCs express functional RyR1 channels, but little or no RyR2 or RyR3, but the expression of RyR1 RNA is reduced dramatically upon maturation (O’Connell, Klyachko et al. 2002). Later, O’Connell has used RyR1 KO mice as a model; their study shows that RyR1 alone is not essential for BMDC functions. Signaling via IP\(_3\)Rs appears to be sufficient to mediate Ca\(^{2+}\) signaling in mouse DCs (Stolk, Leon-Ponte et al. 2006). In contrast, in human DCs, RyR1 is important for the maturation and activation (Bracci, Vukcevic et al. 2007). Recognizing Ca\(^{2+}\) on its cytosolic side establishes a positive feedback mechanism, because a small amount of Ca\(^{2+}\) in the cytosol near the receptor will cause it to release even more Ca\(^{2+}\) (Fill and Copello 2002). The autocatalytic process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) enables the IP\(_3\)Rs and RyRs to communicate with each other to establish coordinated Ca\(^{2+}\) signals (Ashby, Craske et al. 2002; Smith and Parker 2009; Taylor and Tovey 2010). The released Ca\(^{2+}\) is pumped back either into the ER by Sarco-Endoplasmic Reticulum Calcium ATPases (SERCA pumps) or out of the cell via plasma membrane Ca\(^{2+}\) ATPase (PMCA pumps). To maintain low cytoplasmic [Ca\(^{2+}\)]\(_i\), ATPase exchange protons for two (SERCA) or one (PMCA)
Ca\(^{2+}\) per hydrolyzed ATP (Bootman, Collins et al. 2001; Feske 2007; Scharenberg, Humphries et al. 2007). Another extrusion mechanism is the exit of Ca\(^{2+}\) out from the cell via the K\(^{+}\)-independent Na\(^{+}\)/Ca\(^{2+}\) exchangers (NCX, or SLC8A1-3), and K\(^{+}\)-dependent Na\(^{+}\)/Ca\(^{2+}\) - exchangers (NCKX; SLC24A1-5). These transporters exchange one Ca\(^{2+}\) ion for three Na\(^{+}\) ions (NCX) or cotransport one K\(^{+}\) ion with one Ca\(^{2+}\) ion in exchange for four Na\(^{+}\) ions (NCKX). PMCAs and NC(K)X complement each other: PMCAs are effective at maintaining low internal [Ca\(^{2+}\)], over long durations, whereas NCX and NCKX can make the rapid adjustments (Parekh and Putney 2005; Feske 2007).

In spite of its importance, the Ca\(^{2+}\) release phase is transient, sometimes fully deactivating within a few tens of seconds, as the ER Ca\(^{2+}\) stores are necessarily limited. Decreased Ca\(^{2+}\) within the ER triggers a process known as store operated calcium (SOC) entry, in which Ca\(^{2+}\) channels in the plasma membrane are activated to allow Ca\(^{2+}\) to enter from the essentially unlimited extracellular Ca\(^{2+}\) pool to the cytosol (Feske 2007; Scharenberg, Humphries et al. 2007). SOC entry does more than simply providing Ca\(^{2+}\) for refilling the stores, it itself generates sustained Ca\(^{2+}\) signals that control such essential functions as gene expression, cell metabolism and exocytosis. The best characterized SOC channels in DCs are known as ‘calcium release-activated calcium’ (CRAC) channels. DCs have been shown to possess CRAC channels as a main Ca\(^{2+}\) entry pathway (Hsu, O’ Connell et al. 2001). Accordingly, Ca\(^{2+}\) influx is enhanced by membrane hyperpolarization. CRAC channels are highly Ca\(^{2+}\)-selective channels of a low conductance with a characteristic inwardly rectifying current-voltage relationship. The stromal interaction molecule 1 (STIM1) has been identified as the ER-resident Ca\(^{2+}\) sensor and calcium release-activated calcium modulator 1 (CRACM 1); also called Orai1, functions as the pore-forming subunit of CRAC channels (Vig, Peinelt et al. 2006). In mammals, several homologs of these proteins exist: STIM1 and STIM2 in the ER and Orai1, Orai2, and Orai3 in the plasma membrane (Parvez, Beck et al. 2008; Vig and Kinet 2009). A strong functional interaction between STIM1 and Orai1 has been demonstrated by many laboratories. STIM1 and Orai1 are necessary and sufficient to generate CRAC-like currents in vitro (Smyth, Hwang et al.; Park, Hoover et al. 2009; Hogan, Lewis et al. 2010; Srikanth and Gwack 2012). After store depletion, in response to decreased ER Ca\(^{2+}\) concentrations, STIM1 aggregates and translocates to form oligomers, moves to the ER-PM junctions, and close to the regions of Ca\(^{2+}\) influx from the plasma membrane (Hewavitharana, Deng et al. 2007; Carrasco and Meyer 2011; Cheng, Liu et al. 2011).

Whereas much evidence has been accumulated that Ca\(^{2+}\) gradients are indispensable for DC migration, very little is known how ion channels and transporters generating or controlling Ca\(^{2+}\) signals are regulated in DCs. The present study concentrates on a signaling pathway that plays an important role in DC biology, which is the phosphatidylinositol-3-kinase (PI3K) pathway.
1.5 PI3K-PDK1-Akt/SGK1 Pathway

1.5.1 Role of PI3K in DCs

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases essential for diverse physiological reactions. The lipid products produced by PI3Ks include PI(3)P, PI(3,4)P2 and PI(3,4,5)P3 (Rameh and Cantley 1999; Koyasu 2003; Liu, Cheng et al. 2009).

The PI3K family can be divided into three classes—class I, II and III. Each type of PI3K contains a C2 domain and a catalytic domain connected by a helical domain (Walker, Perisic et al. 1999; Bader, Kang et al. 2005). We are interested in the class I PI3Ks, since it is the only class which has been show to activate protein kinase B (PKB; also called Akt) in DCs (Vanhaesebroeck and Alessi 2000; Okkenhaug and Vanhaesebroeck 2003). The class I PI3Ks is subdivided into class IA and the class IB subclasses based on the differences in their activation mechanisms. The class IA PI3Ks are heterodimeric enzymes having three types of the catalytic subunit: p110α, p110β and p110γ (Vanhaesebroeck and Waterfield 1999; Okkenhaug and Vanhaesebroeck 2003; Hirsch, Costa et al. 2007) and five regulatory subunits: p85α, p85β, p55α, p55β and p55γ. The class IA PI3Ks can be activated by tyrosine-kinase-associated receptors, including antigen, co-stimulatory and cytokine receptors (Koyasu 2003; Okkenhaug and Vanhaesebroeck 2003). For example, interleukin 2 (IL-2), IL-3, IL-6, granulocyte colony-stimulating factor and interferons (IFNs) activate class IA PI3Ks.

Figure 3. Structural characteristics of the PI3K family, obtained from S Koyasu, Nature Immunology, 2003

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in many immune cells, including T cells and DCs (Koyasu 2003). The single class IB PI3k enzyme has only one catalytic subunit and one regulatory subunit: p110γ and p101, respectively. P110γ is expressed only in mammals, mainly in leukocytes. The class IB PI3K is activated by G-protein-coupled receptors (GPCRs), including the chemokine receptors (Beer-Hammer, Zebedin et al.; Fruman and Cantley 2002; Saudemont and Colucci 2009).

The preferential substrate of the class I PI3Ks is PI(4,5)P2. The class I PI3Ks can phosphorylate PI(4,5)P2 to generate PI(3,4,5)P3 at the inner leaflet of the plasma membrane. PI(3,4,5)P3 acts as a binding site for pleckstrin-homology (PH) domains (Vanhaesebroeck and Alessi 2000; Tessier and Woodgett 2006). The subset of PH domains bind to PI (3, 4)P2 and PI(3,4,5)P3 with higher affinity than other PIs. Typically, following growth factor activation of PI3K, proteins with PH domains, such as PKB and phosphoinositide-dependent kinase 1 (PDK1), are translocated to the site where PI(3,4,5)P3 is generated at the plasma or endosomal membranes (Scheid, Parsons et al. 2005). There, they interact with other proteins or become activated by PI(3,4)P2 or PI(3,4,5)P3 binding. In fact PKB, which is recruited to the PM by binding PI(3,4)P2 or PI(3,4,5)P3, is considered to be a chief downstream target of PI3K (Vanhaesebroeck and Alessi 2000; Liao and Hung 2010; Yang, Wu et al. 2010).

Many studies indicate that PI3Ks have pleiotropic roles in immune cell activation and function (Koyasu 2003; Okkenhaug and Vanhaesebroeck 2003; Koyasu 2004). In DCs, PI3K is considered to belong to the gate-keeping system, which controls the maturation and survival of DCs, and prevents excessive immune responses (Fukao, Tanabe et al. 2002; Fukao and Koyasu 2003; Shumilina, Zahir et al. 2007; Krawczyk, Holowka et al. 2010). PI3K is activated by LPS, peptidoglycan, CD40 ligand, RANK ligand, and CpG oligonucleotides, all of which induce production of proinflammatory cytokines, such as IL-12, and provide a negative feedback regulation of IL-12 production (Fukao, Tanabe et al. 2002; Fukao and Koyasu 2003).

![Figure 4. PI3K-PDK1 pathway, obtained from Yang KJ, J Biol Chem, 2008](image-url)
1.5.2 PDK1 and PDK1 Functions in DCs

One downstream target of PI3K is PDK1, which is a 63 kDa serine/threonine kinase belonging to the AGC superfamily of protein kinases. It phosphorylates a variety of other protein kinases within the AGC subfamily including PKC isoforms, p70 S6K, Rsk, serum and glucocorticoid-inducible kinases (SGKs) and PKB/Akt (Vanhaesebroeck and Alessi 2000; Woodgett 2005; Tessier and Woodgett 2006; Gurevich, Tesmer et al. 2011). In fact, PDK1 was first discovered by its capacity to phosphorylate PKBα/Akt1 at Thr 308 (Alessi, James et al. 1997; Bayascas, Wullschleger et al. 2008; Calleja, Laguerre et al. 2009).

PDK1 has two domains: the N-terminal catalytic domain and the C-terminal PH domain (Mora, Komander et al. 2004; Yang, Shin et al. 2008). The catalytic domain has three ligand binding sites: the substrate binding site, the ATP binding site, and the docking site. The docking site is also known as PKC related kinase 2-interacting factor-pocket (PIF-pocket), it acts as the binding site for the hydrophobic motif of other AGC kinases (Mora, Komander et al. 2004; Woodgett 2005; Li, Yang et al. 2010). PDK1 activates at least 23 AGC kinases by phosphorylating a specific Thr or Ser residue located within the T-loop of the kinase domain (Vanhaesebroeck and Alessi 2000; Mora, Komander et al. 2004; Komander, Kular et al. 2005). Of note, PDK1 has no hydrophobic motif. The catalytic activity of PDK1 is not acutely regulated, thus some targets are constitutively phosphorylated. The PH domain function is mainly to enable the interaction of PDK1 with PI(3,4)P2 and PI(3,4,5)P3 (Vanhaesebroeck and Alessi 2000; Komander, Fairservice et al. 2004; Tessier and Woodgett 2006). PDK1 only encounters some of its kinase substrates, as Akt/PKB at the plasma membrane. However, PDK1 can also translocate to the nucleus in response to mitogens (Roelants, Torrance et al. 2004; Scheid, Parsons et al. 2005; Woodgett 2005).

In the past few years, the crucial role of PDK1 in embryonal development as well as in normal functions of the liver and heart has been well studied using specific tissue deletion in mice (Lawlor, Mora et al. 2002; Mora, Lipina et al. 2005; Mora, Sakamoto et al. 2005; Zaru, Mollahan et al. 2008; Di, Feng et al. 2010). Several studies have addressed the role of PDK1 in T lymphocytes. PDK1 regulates T cell development and controls the production of IL-4 by Th2 cells (Hinton, Alessi et al. 2004; Nirula, Ho et al. 2006). DCs derived from PDK1 deficient mice have several abnormal aspects in vitro, though a normal in vivo development. PDK1-hypomorphic DCs have a reduced TLR-induced macropinocytosis as well as FcR-mediated phagocytosis but enhanced antigen presentation, increased levels of costimulatory molecules and enhanced production of cytokines (IL-12 and IL-10) (Zaru, Mollahan et al. 2008). Thus, similar to PI3K, PDK1 has also a suppressive effect on DC maturation and cytokine production.
1.5.3 Akt and Akt Functions in DCs

PKB/Akt has been identified as a protein kinase with extensive homology with protein kinases A and C, and was therefore termed PKB (Manning and Cantley 2007). On the other hand, PKB/Akt is the cellular homologue of the viral oncoprotein v-Akt, and is therefore referred to Akt. The Akt family has three isoforms: Akt1, 2 and 3 (also known as PKB α, β and γ), which share a high degree of amino acid identity.

Akt is a 57kDa Ser/Thr kinase with three functional domains: an N-terminal PH domain, a kinase domain and a C-terminal hydrophobic motif (Calleja, Laguerre et al. 2009). PH domain preferentially binds PI(3,4,5)P3 and PI(3,4)P2 over other PIs (Huang and Sauer 2010; Dixon, Gray et al. 2011). The catalytic domain of Akt is structurally similar to other protein kinases of AGC family, including PKA and p70S6K, but is most similar to SGKs (Brunet, Park et al. 2001; Scheid and Woodgett 2003; Woodgett 2005; Loffing, Flores et al. 2006). Akt is cytosolic in unstimulated cells, translocates to the plasma membrane upon activation of PI3K. As indicated earlier, PI3K stimulation leads to the production of PI(3,4,5)P3. PDK1 and Akt are recruited to the membrane via binding of their PH domains to PI(3,4,5)P3 (Koyasu 2003; Okkenhaug and Vanhaesebroeck 2003; Mora, Komander et al. 2004; Yoshizaki, Mochizuki et al. 2007). Colocalization of PDK1 and Akt induces the phosphorylation of Ser 473 at the hydrophobic motif site of Akt, and in further causes the phosphorylation of Thr 308 at the T-loop site of Akt (Mora, Komander et al. 2004; Calleja, Laguerre et al. 2009; Li, Yang et al. 2010). Finally, dually phosphorylated Akt detaches from the plasma membrane and translocates through the cytosol to the nucleus (Manning and Cantley 2007). Activation of Akt and phosphorylation of both these residues are abolished if the cells are incubated with PI3K inhibitors prior to stimulation with agonist. Phosphorylation of both residues, Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain, is essential for maximal activation of Akt1 (Tessier and Woodgett 2006; Manning and Cantley 2007). The Akt2 and Akt3 isoforms are also activated in response to agonists which activate PI3K, by phosphorylation of the residues equivalent to Thr308 and Ser473.

![Figure 5. Classification and domain structure of AKT family members, obtained from S Song, L Salmena, PP Pandolfi, Nature Reviews Molecular Cell, 2012](image)

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18
Ser473 (Crellin, Garcia et al. 2007; Warfel, Niederst et al. 2011). Mutation of either residue of Akt1 to Ala does not prevent the other one from becoming phosphorylated in response to insulin, indicating that the phosphorylation of these residues can occur independently from each other (Vanhaesebroeck and Alessi 2000; Lawlor and Alessi 2001; Richardson, Schalm et al. 2004).

The Akt is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli (Manning and Cantley 2007). Akt plays a key role in multiple cellular processes such as cell survival, growth, proliferation, angiogenesis, metabolism, and migration (Song, Ouyang et al. 2005; Bononi, Agnoletto et al. 2011).

Numerous substrates of Akt have been identified. Thus, Akt may influence plasma membrane and intracellular proteins involved in ionic intracellular homeostasis such as sarco(endo)plasmic reticulum Ca$^{2+}$ ATPase (Rota, Boni et al. 2005) and ryanodine receptors (Barac, Zeevi-Levin et al. 2005). Akt-mediated transcriptional up-regulation of NCX1 and NCX3 has been found in a study on PC12 cells (Formisano et al. 2008). A consensus sequence for Akt phosphorylation (RxRxxS/T) is present in all three types of IP$_3$Rs that indicate the IP$_3$R as a target of Akt (Khan, Wagner II et al. 2006; Pinton, Giorgi et al. 2008; Hwang, Duncan et al. 2009; Martin, Wang et al. 2012). Akt phosphorylates type-I IP$_3$R and therefore inhibits Ca$^{2+}$ release from the ER and prevents Ca$^{2+}$ induced apoptosis in non-cardiac cells (Khan et al. 2006; Szado et al. 2008). Several transcription factors are downstream targets of Akt. Among them are NF-$\kappa$B, CREB, FoxO2 (Tessier and Woodgett 2006), ETS1 (Lavenburg, Ivey et al. 2003).

DCs express Akt1 and Akt2, but not Akt3 (Li, Syrovets et al. 2009). Dongsu et al. has provided evidences that Akt1 regulates DC survival in innate (LPS-driven) and adaptive (CD40-driven) immune responses. In addition, Akt deficiency leads to defective DC activation and survival (Park, Lapteva et al. 2006). Although Akt1 is a predominant family member in bone marrow-derived DCs (BMDCs), Akt2 also plays an important role in the plasmin-triggered chemotactic response of monocyte-derived DCs (Li, Syrovets et al. 2009). Moreover, in macrophages, it has been shown that Akt2 directly mediates the signal transduction pathway, which regulates actin polymerization and chemotaxis (Zhang, Ma et al. 2009). However, very little is known about the mechanisms of Akt2-dependent regulation of migration. In the present project, the role of Akt2 in DC migration and in other Ca$^{2+}$-dependent functions was addressed.

1.5.4 Ets-1

One of the transcriptional factors regulated by Akt is ETS1 (Lavenburg, Ivey et al. 2003). ETS proteins are a family of transcription factors that regulate the expression of a myriad of genes in a variety of tissues and cell types (Remy and Baltzinger 2000;
The name “Ets” stems from a sequence that was detected in an avian erythroblastosis virus, E26 carrying the v-est oncogene (Sharrocks 2001; Pham, Lawson et al. 2007). The newly discovered sequence was called E26 transformation specific sequence or Ets (Blair and Athanasiou 2000; Dittmer 2003). This family was originally defined by the presence of a conserved DNA binding domain, the Ets domain (Span, Manders et al. 2002; Dwyer, Li et al. 2007). ETS proteins are composed of 85 amino acids, with 4 tryptophan repeats DNA-binding domain that recognizes DNA sequences containing a GGAA/T core motif (Sharrocks 2001; Dittmer 2003). In mouse, the ETS family consists of 28 members and it is known to control important biological processes, including cellular proliferation, differentiation, lymphocyte development and activation, apoptosis and migration in different cell types such as B cells, endothelial cells, fibroblasts and fibroblasts (Koskela and Lassila 2003; Wang, John et al. 2005; Gallant and Gilkeson 2006; Dwyer, Li et al. 2007; Geisinger, Astaiza et al. 2012).

Ets1 is the prototype of the ETS family. The ETS1 protein is closely related to ETS2. In humans, the Ets-1 and Ets2 genes are located on two distinct chromosomes, on chromosome #11 and #21, respectively (Watson, McWilliams-Smith et al. 1985; Dittmer 2003). The Ets-1 protein sequence is highly conserved among species. It can be divided into six domains, A-F. The A- and B- domains are regulatory units, C-domain is the activation domain, the D- (or exon VII) domain and F- domain are regulatory domains that control the activity of E- domain, and the E- domain is the DNA binding Ets domain (Sharrocks 2001; Wasylyk, Schlumberger et al. 2002; Dittmer 2003). Ets1 harbors a conserved 85 amino acid DNA binding domain termed the Ets domain. The Ets1 mRNA is subject to alternate splicing to yield three isoforms of the protein: the full length protein p51 or p54 (based on predicted

![Figure 6. The domains of Ets1 protein, obtained from Rui-Xueleng, Autoimmunityreviews, 2011](image)
molecular weight or on its actual migration in SDS-PAGE gels, respectively), p42, a protein lacking the sequences encoded in exon VII and p27, a protein lacking the sequences encoded in exons III-VI (Dittmer 2003; Lulli, Romania et al. 2010). The D- or exon VII domain comprises two regulatory units. The C-terminal unit is a part of an autoinhibitory module; the N- terminal sequence contains a calcium-responsive phosphorylation site. The two units are functionally connected as phosphorylation within the N-terminal sequence increases the inhibitory effect of the C-terminal unit (Dittmer 2003; Russell and Garrett-Sinha 2010; Hollenhorst, McIntosh et al. 2011). Calcium-dependent phosphorylation of the exon VII domain interferes with the activity of the Ets1 autoinhibitory module and decreases the stability of the ETS protein in T-lymphocytes (Dittmer 2003; Mellstrom, Savignac et al. 2008; Lulli, Romania et al. 2010).

ETS1 is expressed in different cell types and is known to play various roles during both physiological and pathological conditions. ETS1 is a transcription factor that is highly expressed within lymphoid cells, in mice, chickens and humans (Remy and Baltzinger 2000; Lelievre, Lionneton et al. 2001). It is known that ETS1 regulates the function of immune cells including B cells, T cells, NK cells and NK T cells (Colucci, Caligiuri et al. 2003; Eyquem, Chemin et al. 2004; Wang, John et al. 2005; Choi, Geng et al. 2011; Grenningloh, Tai et al. 2011; Ramirez, Chandler et al. 2012). Kevin Barton has demonstrated that ETS1-deficient mice produced by gene targeting develop mature erythrocytes, monocytes, neutrophils, and T and B lymphocytes (Barton, Muthusamy et al. 1998). However, loss of ETS1 partially impairs bone marrow-derived B cell development in that there are fewer bone marrow derived B cells and they are inefficient in the pro-B to pre-B transition (Wang, John et al. 2005). Moreover, ETS1 knockout mice have multiple aberrations in T cell development in the thymus and functional responses in the periphery. Many of these early defects in thymocyte differentiation appear to be due to impaired pre-TCR signaling in the absence of ETS1 (Eyquem, Chemin et al. 2004; Higuchi, Bartel et al. 2007). In addition, studies have demonstrated altered cytokine production by ETS1-deficient CD4 and CD8 T cell subsets (Grenningloh, Kang et al. 2005; Zhu, Yamane et al. 2010).

Many Ets-domain transcription factors are known as nuclear targets of many signaling pathways (Sharrocks 2001). The transcription factor Ets2 is one of several transcription factors that have recently been identified as targets of Akt action in mammalian cells (Oikawa 2004).

1.5.5 SGK1

The serum & glucocorticoid inducible kinase (SGK) family exhibits structural similarity to Akt, though it lacks a PH domain. SGK1 is a member of the AGC family of serine/threonine protein kinases, originally cloned by Firestone laboratory as a
glucocorticoid-responsive gene from mammary tumor cells (Loffing, Flores et al. 2006; Tessier and Woodgett 2006). Like Akt, the SGK family is encoded by three genes in mammalian genomes: SGK1, 2 and 3. SGK1 and 3 are ubiquitously expressed, while SGK2 RNA is only present in liver, kidney and pancreas, and at lower levels in the brain (Lang, Bohmer et al. 2006; Loffing, Flores et al. 2006). SGK2 and SGK3 share 80% amino acid identity with SGK1 and with each other in their catalytic domains. However, only SGK1 is responsive to glucocorticoids at the level of transcription (Arteaga, Alvarez de la Rosa et al. 2007; Pao, Bhargava et al. 2010).

Basically, SGKs have two domains: C-terminal catalytic and N-terminal hydrophobic motif domains (Lang, Bohmer et al. 2006; Tessier and Woodgett 2006). Three enzymes differ in the N-terminal domain. SGK2 contains a short N terminus, with no discernable domain, whereas SGK3 has a longer N terminus comprising a phox homology domain, which is another 3’phosphorylated inositol phospholipid-binding domain (Tessier and Woodgett 2006; He, Lee et al. 2011). The catalytic domain of SGKs shares 55% identity with Akt catalytic domain. Moreover, similar to Akt, SGK1 is activated on phosphorylation by PDK1 activated in their turn by PI3K (Vanhaesebroeck and Alessi 2000; Mora, Komander et al. 2004). All three isoforms of SGKs require PI3K activation for function (Woodgett 2005). A distinguishing feature of SGK1 is its stringent stimulus-dependent regulation of its transcription, intrinsic kinase activity, and subcellular localization (Lang, Bohmer et al. 2006; Bononi, Agnollettto et al. 2011). Many extracellular and intracellular factors can upregulate SGK1 mRNA level, such as FSH, serum, and TGFβ (Stockand 2005; Lang, Bohmer et al. 2006). The SGK1 promoter region contains a glucocorticoid response element, a TATA box, SP-1 elements and putative binding sites for p53, AP-1, NFκB, and Ets-2 (Dittmer 2003; Loffing, Flores et al. 2006).

SGK1 has been shown to foster nuclear translocation and activation of NFκB (Lang, Strutz-Seebohm et al. 2010). SGK1 is also involved in the regulation of many other
transcription factors, e.g. forkhead transcription factor Foxo3a and β-catenin (Brunet, Park et al. 2001; Dehner, Hadjihannas et al. 2008).

Moreover, SGK1 regulates a great variety of ion channels, e.g. Na\(^+\) channels: SCN5A, ENaC, ASIC1; cation/Ca\(^{2+}\) channels: TRPV4, TRPV5, TRPV6, Orai1; K\(^+\) channels: ROMK, Kv1.1–5, KCNx/KCNQ1–5; Cl- channels: VSOAC, CIC2, CICKa/bartin, CFTR; glutamate receptor GluR6 and transporters, e.g. NHE3, NKCC2, NCC, NaPiIIb, SMIT, GLUT1,4, SGLT1, NaDC, EAAT1–5, SN1, ASC2, 4F2/LAT, PepT2, and the Na\(^+/\)K\(^+\)-ATPase (Boehmer, Laufer et al. 2008; Boehmer, Palmada et al. 2008; Schuetz, Kumar et al. 2008; Gehring, Zürn et al. 2009; Krueger, Haerteis et al. 2009; Lang, Artunc et al. 2009; Laufer, Boehmer et al. 2009; Lang, Strutz-Seebohm et al. 2010; Sopjani, Kunert et al. 2010).

SGK1 can modulate ion channels/transporters via i) direct phosphorylation as have been shown e.g. for ENaC, TRPV4; ii) increasing protein abundance in the plasma membrane via phosphorylation and thereby inhibition of the ubiquitin ligase Nedd4-2, which prepares its targets for clearance from the membrane (shown for e.g. ENaC, SCN5a, CICKa/bartin, CIC2, Orai1); iii) increasing channel protein abundance in cooperation with NHERF2 (Na\(^+/\)H\(^+\) exchanger regulatory factor 2), a protein mediating trafficking of carriers and channels to the plasma membrane (shown for e.g. ROMK1, TRPV5); iv) upregulation of the transcription factor (NF-κB), which is required for the transcription of channel (Orai1, STIM1) (Lang and Shumilina 2012).

Via the powerful stimulation of Orai1 and STIM1, the components of CRAC (or SOC) channels, which play a pivotal role in immune cell functions, SGK1 has recently been established as an important regulator of immune responses. In mast cells from SGK1-deficient mice, the decreased SOC entry is paralleled by decreased degranulation and a blunted anaphylactic reaction (Sobiesiak, Shumilina et al. 2009; Shumilina, Zemtsova et al. 2010). Along those lines, the decreased Ca\(^{2+}\) influx in STIM1-deficient (Baba, Nishida et al. 2007; Vig, DeHaven et al. 2007) or Orai1-deficient (Vig, DeHaven et al. 2007)(149) mast cells similarly compromises degranulation, cytokine production, and anaphylactic reactions. Moreover, the recent study of our group has demonstrated that upon allergen-induced mast cell activation, SGK1-sensitive upregulation of SOC channels is required for Ca\(^{2+}\)-dependent depolymerization of actin filaments under the plasma membrane (Schmid, Gu et al. 2012). The depolymerization of F-actin ring provides access of mast cell granules to the plasma membrane (Schmid, Gu et al. 2012) and consequently, decreased degranulation of sgk1\(^-/-\) mast cells could be rescued by actin-disrupting drug cytochalasin B (Schmid, Gu et al. 2012). Moreover, SGK1 deficiency decreases Ca\(^{2+}\) entry, degranulation, adhesion, and aggregation of blood platelets (Borst, Schmidt et al. 2012). However, nothing is known about SGK1 functions in DCs. Therefore, the present study explores the possible SGK1-dependent functions in mouse bone marrow-derived DCs.
2. Materials and Methods

2.1 Materials

2.1.1 Cell Culture

I. Equipment

<table>
<thead>
<tr>
<th>Name</th>
<th>Firms and Country of Origin</th>
</tr>
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<tbody>
<tr>
<td>Centrifuge RotiFix 32</td>
<td>Hettich Zentrifugen, Tuttlingen, Germany</td>
</tr>
<tr>
<td>Eppendorf cups 1.5 ml</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>Eppendorf pipettes 1000 µl, 100 µl, 10 µl</td>
<td>Eppendorf AG, Hamburg, Germany</td>
</tr>
<tr>
<td>Heraeus Incubator</td>
<td>Thermo Electron Corporation, Dreieich, Germany</td>
</tr>
<tr>
<td>Needles BD Microlance™ 3, 0.55x25 mm</td>
<td>Becton Dickinson Labware, Franklin Lakes, USA</td>
</tr>
<tr>
<td>Neubauer counting chamber</td>
<td>Brand, Wertheim, Germany</td>
</tr>
<tr>
<td>Pipetus® pipetting aid</td>
<td>Hirschmann Laborgeräte, Eberstadt, Germany</td>
</tr>
<tr>
<td>PP-Test tubes 15, 50 ml</td>
<td>Greiner bio-one, Frickenhausen, Germany</td>
</tr>
<tr>
<td>Stripette® 5, 10, 25 ml</td>
<td>Corning Incorporated, Corning NY, USA</td>
</tr>
</tbody>
</table>
Syringe BD 10 ml Luer-Lok™ Tip | Becton Dickinson Labware, Franklin Lakes, USA
Tissue Culture Dishes 60x15 mm | Becton Dickinson Labware, Franklin Lakes, USA
Vortex | Genie Scientific Industries, Bohemia NY, USA

II. Substance

<table>
<thead>
<tr>
<th>Name</th>
<th>Firms and Country of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetal Bovine Serum (FBS) GIBCO</td>
<td>Peprotech/Tebu, Carlsbad, Germany</td>
</tr>
<tr>
<td>GMCSF mouse recombinant</td>
<td>GIBCO, Cölbe, Germany</td>
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<tr>
<td>L-Glutamine</td>
<td>Carlsbad, Germany</td>
</tr>
<tr>
<td>Lipopolysaccharide E.coli (LPS)</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
<tr>
<td>MEM Non-Essential Amino Acids</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>GIBCO, Carlsbad, Germany</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>GIBCO, Carlsbad, Germany</td>
</tr>
<tr>
<td>Trypan blue solution 0,4%</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
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</table>
# 2.1.2 Calcium Imaging

## I. Equipment

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<tr>
<td>Camera Proxitronic</td>
<td>Proxitronic, Bensheim, Germany</td>
</tr>
<tr>
<td>Centrifuge RotiFix 32 Hettich</td>
<td>Zentrifugen, Tuttlingen, Germany</td>
</tr>
<tr>
<td>Discofix® Stopcock for Infusion Therapy</td>
<td>B. Braun, Melsungen, Germany</td>
</tr>
<tr>
<td>Filter Set for Fura-2</td>
<td>AHF Analysentechnik AG, Tübingen, Germany</td>
</tr>
<tr>
<td>Filter tips 10, 100, 1000µl</td>
<td>Biozym Scientific, Hess. Oldendorf, Germany</td>
</tr>
<tr>
<td>Filter wheel</td>
<td>Sutter Instrument Company, Novato, USA</td>
</tr>
<tr>
<td>Infusion Regulator Dosi-Flow 10</td>
<td>Dahlhausen, Köln/Sürth, Germany</td>
</tr>
<tr>
<td>Lambda 10-2</td>
<td>Sutter Instrument Company, Novato, USA</td>
</tr>
<tr>
<td>Lamp XBO 75</td>
<td>Leistungselektronik Jena GmbH, Jena, Germany</td>
</tr>
<tr>
<td>Metafluor software</td>
<td>Universal Imaging, Downingtown, USA</td>
</tr>
<tr>
<td>Microscope Axiovert 100</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>Microscope cover glasses round, 30mm diameter, 0.13-0.16 mm</td>
<td>Karl Hecht KG,</td>
</tr>
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</table>
Neutral density filters 10, 20, 40, 60%  
AHF Analysentechnik AG, Tübingen, Germany

Objective fluar 40x/1.3 oil  
Carl Zeiss, Oberkochen, Germany

Syringe BD 10 ml, Luer-Lok™ Tip  
Becton Dickinson Labware, Franklin Lakes, USA

Syringe BD Perfusion™ 50 ml  
Becton Dickinson Labware, Franklin Lakes, USA

Tissue Culture Dishes 60x10 mm  
Becton Dickinson Labware, Franklin Lakes, USA

Winged Needle Infusion Set  
Butterfly®-Hospira Venisystems, Donegal Town, Ireland

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### II. Substance

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<tr>
<th>Name</th>
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<tr>
<td>Ampuwa Fresenius</td>
<td>KABI, Bad Homburg, Germany</td>
</tr>
<tr>
<td>CaCl2 x 2 H2O</td>
<td>Carl Roth, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Recombinant Murine Exodus-2 (CCL21)</td>
<td>Peprotech, Hamburg, Germany</td>
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<tr>
<td>Ethylene glycol tetraacetic acid (EGTA)</td>
<td>Sigma, Taufkirchen, Germany</td>
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<tr>
<td>Fura-2 AM</td>
<td>Invitrogen, Karlsruhe, Germany</td>
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<tr>
<td>Glucose</td>
<td>Carl Roth, Karlsruhe, Germany</td>
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<tr>
<td>HEPES</td>
<td>Sigma, Taufkirchen, Germany</td>
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Immersol 518F  
Carl Zeiss, Göttingen, Germany

Ionomycin  
Sigma, Taufkirchen, Germany

KCl  
Carl Roth, Karlsruhe, Germany

LPS  
Enzo Life Sciences GmbH, Lörrach, Germany

MgSO4 x 7 H2O  
Sigma, Taufkirchen, Germany

Na2HPO4 x 2 H2O  
Sigma, Taufkirchen, Germany

NaCl  
Sigma, Taufkirchen, Germany

Thapsigargin  
Invitrogen, Karlsruhe Germany

**2.1.3 Patch Clamp**

I. Equipment

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<th>Name</th>
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<tr>
<td>Borosilicate glass filaments</td>
<td>Harvard Apparatus, March-Hugstetten, Germany</td>
</tr>
<tr>
<td>DMZ puller</td>
<td>Zeitz, Augsburg, Germany</td>
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<tr>
<td>EPC-9 amplifier</td>
<td>Heka, Lambrecht, Germany</td>
</tr>
<tr>
<td>ITC-16 Interface</td>
<td>Instrutech, Port Washington, N.Y., USA</td>
</tr>
<tr>
<td>Microscope Axiovert 100</td>
<td>Zeiss, Oberkochen, Germany</td>
</tr>
<tr>
<td>MS314 electrical micromanipulator</td>
<td>MW, Märzhäuser, Wetzlar, Germany</td>
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</table>
Pulse software  
Heka, Lambrecht, Germany

II. Substance

<table>
<thead>
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<th>Name</th>
<th>Firms and Country of Origin</th>
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<tr>
<td>MgSO4 x 7 H2O</td>
<td>Sigma, Taufkirchen, Germany</td>
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<td>Na2HPO4 x 2 H2O</td>
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<td>Poly-L-Lysine</td>
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<td>D-myo-inositol-1,4,5-triphosphate (IP$_3$)</td>
<td>Enzo, Life Sciences, Germany</td>
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<td>HCl</td>
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<tr>
<td>Immersol 518F</td>
<td>Carl Zeiss, Göttingen, Germany</td>
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2.1.4 FACS

I. Equipment

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<tr>
<td>FACS Calibur</td>
<td>Becton Dickinson, Heidelberg, Germany</td>
</tr>
<tr>
<td>FACS tubes, 1.3 ml, PP, round bottom</td>
<td>Greiner bio-one, Frickenhausen, Germany</td>
</tr>
</tbody>
</table>

II. Reagent
Name | Firms and Country of Origin  
--- | ---  
APC-conjugated anti-mouse CD11c, clone HL3 | BD Pharmingen, Heidelberg, Germany  
2',7'-dichlorodihydrofluorescein diacetate (DCFDA) | Sigma, St.Luis, USA  

### 2.1.5 Cytokine Production

I. Equipment

| Name | Firms and Country of Origin  
--- | ---  
Magellan™ software | Tecan Group Ltd., Männedorf, Switzerland  
Multi well plates; 96 well | Corning Inc., Corning NY, USA  
Sunrise Microplate Reader | Tecan Trading AG, Switzerland  

II. Kit

Name | Firms and Country of Origin  
--- | ---  
Mouse IL-2 ELISA Set | BD Pharmingen, Heidelberg, Germany  
Mouse IL-6 ELISA Set | BD Pharmingen, Heidelberg, Germany  
Mouse IL-12 ELISA Set | BD Pharmingen, Heidelberg, Germany  

### 2.1.6 Migration

I. Equipment
### Name | Firms and Country of Origin
--- | ---
BD Falcon™ Cell culture inserts for 24-well plates. 8.0 µm pores | BD Falcon, Heidelberg, Germany
BD Falcon™ cell culture insert companion plates (24-well, BD cat. no. 353504) | BD Falcon, Heidelberg, Germany
Carl Zeiss Microscopy LSM 700 | Carl Zeiss, München, Germany

### II. Reagent

<table>
<thead>
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<th>Name</th>
<th>Firms and Country of Origin</th>
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<td>CCL21</td>
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<tr>
<td>CXCL12</td>
<td>Peprotech/Tebu, Cölbe, Germany</td>
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<tr>
<td>Xestospongin C, Xestospongia sp</td>
<td>Enzo Life Sciences GmbH, Löffach, Germany</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Sigma, Taufkirchen, Germany</td>
</tr>
<tr>
<td>ProLong® Gold Antifade Reagent with DAPI</td>
<td>Invitrogen, Darmstadt Germany</td>
</tr>
</tbody>
</table>

### 2.1.7 Western Blotting and Nuclear Fractionation

#### I. Equipment

<table>
<thead>
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<th>Firms and Country of Origin</th>
</tr>
</thead>
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<td>Agarose gel electrophoresis chamber</td>
<td>BioRad, München, Germany</td>
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<tr>
<td>Centrifuge 5415R</td>
<td>Eppendorf, Hamburg, Germany</td>
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### Densitometer Quantity One
BioRad, München, Germany

### GE Healthcare film
GE Healthcare, München, Germany

### Nitrocellulose membrane
GE Healthcare, München, Germany

## II. Reagent

<table>
<thead>
<tr>
<th>Name</th>
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</thead>
<tbody>
<tr>
<td>Acrylamide/bisacrylamide</td>
<td>Carl Roth, Karlsruhe, Germany</td>
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<tr>
<td>Cell lysis buffer 10x</td>
<td>Cell Signaling Technology, Frankfurt, Germany</td>
</tr>
<tr>
<td>Complete Protease Inhibitor Cocktail Tablets</td>
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<tr>
<td>Detection reagent</td>
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<td>Glycine</td>
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<td>Loading buffer (4x)</td>
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<td>Milk powder</td>
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<td>peqGOLD Protein-Marker IV</td>
<td>Peqlab, Erlangen, Germany</td>
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<tr>
<td>peqGOLD Protein-Marker V</td>
<td>Peqlab, Erlangen, Germany</td>
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<td>Sodium dodecyl sulfate (SDS)</td>
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<td>TEMED</td>
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<td>Tween® 20</td>
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### 2.1.8 Real Time-PCR

#### I. Equipment

<table>
<thead>
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<tr>
<td>BZO Seal Film</td>
<td>Biozym biotech trading GmbH</td>
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<tr>
<td>CFX96 Real Time System</td>
<td>BioRad, München, Germany</td>
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<tr>
<td>Low Profile 96 Well PCR Plate</td>
<td>PEQLAB Biotechnologie GMBH, Erlangen, Germany</td>
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<td>UV Cuvette</td>
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#### II. Reagent / Kit

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<td>Promega, Mannheim, Germany</td>
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<tr>
<td>dNTP mix</td>
<td>Promega, Mannheim, Germany</td>
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<td>Ethanol 99.7%</td>
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<tr>
<td>GoTaq® qPCR Master Mix</td>
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</tr>
<tr>
<td>peqGOLD TriFast</td>
<td>PEQLAB Biotechnologie GMBH, Erlangen, Germany</td>
</tr>
</tbody>
</table>
2.2 Solutions

2.2.1 Cell Culture Medium

Normal Medium

Substance (RPMI 1640 500ml)

FBS 10%

Penicillin Streptomycin 1%

Glutamine 1%

Nonessential amino acids 1%

β-mercaptoethanol 0.05%

Medium for Silencing

Substance (RPMI 1640 500ml)

Penicillin Streptomycin 1%

Glutamine 1%

Nonessential amino acids 1%

β-mercaptoethanol 0.05%
2.2.2 Intracellular Calcium Imaging

Chemical and Biological Reagents

**Standard HEPES Solution**

<table>
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<th>[mmol/L]</th>
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<tr>
<td>KCl</td>
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</tr>
<tr>
<td>MgSO$_4$7H$_2$O</td>
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<tr>
<td>HEPES</td>
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</tr>
<tr>
<td>Na$_2$HPO$_4$2H$_2$O</td>
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</tr>
<tr>
<td>CaCl$_2$2H$_2$O</td>
<td>2</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
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</tbody>
</table>

pH 7.4 (NaOH); H$_2$O (at the 37 °C)

**Ca$^{2+}$ Free Solution**

<table>
<thead>
<tr>
<th>Substance</th>
<th>[mmol/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>125</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
</tr>
<tr>
<td>MgSO$_4$7H$_2$O</td>
<td>1.2</td>
</tr>
<tr>
<td>HEPES</td>
<td>32.2</td>
</tr>
</tbody>
</table>
Na₂HPO₄·2H₂O  2

EGTA 0.5

Glucose  5

pH 7.4 (NaOH); H₂O (at the 37 °C)

### 2.2.3 Patch Clamp – Pipette Solutions

**Pipette Solution** [mmol/L]

- NaCl 35
- CsCl 120
- EGTA 10
- HEPES 10
- IP₃ 0.04

pH 7.2 (CsOH); H₂O (at the 20 °C)

**Bath Solution** [mmol/L]

- NaCl 140
- KCl 5
- CaCl₂ 10
- Glucose 20
- HEPES 10
pH 7.4 (NaOH); H₂O (at the 20 °C)

2.2.4 FACS

FACS Buffer

PBS

0.1 % heat-inactivated FBS

Cytokine Production

Coating Buffer

0.2 M sodium phosphate

pH 6.5 (HCl); H₂O (at room temperature)

Assay Diluent

1x PBS

heat-inactivated FBS 10 %

Stop Solution

2 M H₂SO₄

2.2.5 Western Blotting and Nuclear Fractionation

Running Buffer

Tris 25 mM

Glycine 250 mM
SDS 0,1 %

**Transfer Buffer**

Tris 25 mM

Glycine 192 mM

Methanol 20 %

pH 8.3 (HCl); H₂O (at room temperature)

**Wash Buffer (TBS-T)**

Tris 50mM

NaCl 150mM

Tween-20 0.05 %

pH 7.4 (HCl); H₂O (at room temperature)

**Nuclear Fractionation Buffer A**

10 mM HEPES

1.5 mM MgCl₂

10 mM KCl

0.5 mM DTT

0.05% NP40

pH 7.9 (HCl); H₂O (at room temperature)
Nuclear Fractionation Buffer B

5 mM HEPES

1.5 mM MgCl₂

0.2 mM EDTA

0.5 mM DTT

26% glycerol (v/v)

pH 7.9 (HCl); H₂O (at room temperature)

2.3 Animals

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⁺/⁺) as well as SGK1- deficient mice (sgk1⁻/⁻) and their wild type littermates (sgk1⁺/⁺) were used in the present study. Male and female mice were studied at the age of 6-12 weeks. Origin of the akt2⁻/⁻ mice, breeding and genotyping were described previously (Cho, Mu et al. 2001). Knockout mice were generated with a targeted disruption in the Akt2 locus by homologous recombination. The targeting vector was designed to insert LoxP sites flanking the sequence containing the coding exons 4 and 5. Mice harboring the targeted allele were identified by Southern blotting and were mated to transgenic mice expressing Cre recombinase driven by a 6-kb 59-flanking sequence from the Brn/Pou3f4 gene to cause germ-line excision of exons 4 and 5. The progeny carrying both the Cre transgene and the targeted allele were mated with wild-type mice to obtain offspring in which the Cre transgene was segregated away and the targeted allele was excised, as determined by the PCR and Southern blotting, respectively. These mice were mated inter se to produce offspring with homozygous deletions of Akt2.

Origin of the sgk1⁻/⁻ mice, breeding and genotyping were described previously (Wulff, Vallon et al., 2002).

A conditional targeting vector was generated from a 7-kb fragment encompassing the entire transcribed region on 12 exons. The neomycin resistance cassette was flanked
by two loxP sites and inserted into intron 11. Exons 4-11, which code for the sgk1 domain, were “floxed” by inserting a third loxP site into intron 3. Targeted R1 ES cells were transiently transfected with Cre recombinase. A clone with a recombination between the first and third loxP site (type I recombination) was injected into C57BL/6 blastocytes. Male chimeras were bred to 129/SvJ females. Heterozygous sgk1-deficient mice were backcrossed to 129/SvJ wild-type mice for two generations and then intercrossed to generate homozygous sgk1+/− and sgk1+/- littermates (Wulff, Vallon et al., 2002). The animals were genotyped by PCR using standard methods.

2.4 Methods

2.4.1 Culture of Bone Marrow Dendritic Cells

Dendritic cells (DCs) were isolated from the mouse bone marrow as described previously (Matzner, Zemtsova et al. 2008; Xuan, Shumilina et al. 2009). Briefly, 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 10^6 cells per 60 mm dish. Cells were cultured for up to 9 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 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 days 7-9. Immature and mature DCs were used in the present study. DC maturation was induced by treating the cells with lipopolysaccharides (LPS from E. coli, 100 ng/ml, 48h or 1 µg/ml, 24 h, Enzo Life Sciences, Lausen, Switzerland).

2.4.2 Isolation of Splenic DCs

Dendritic cells were isolated from the spleen of 8-12 weeks old mice according to the protocol of Stagg et al. (Stagg, Burke et al., 2001). Briefly, single-cell suspensions were prepared by pressing mouse spleen through a gauze cell strainer (BD Pharmingen, Heidelberg, Germany) and washing in RPMI 1640 (GIBCO, Carlsbad, Germany) containing: 10 % FCS, 1 % penicillin/streptomycin, 1 % glutamine, 1 % non-essential amino acids (NEAA) and 0.05 % β-mercaptoethanol. After overnight incubation nonadherent cells were collected and DCs were purified by centrifugation over a 13.7% (w/v) metrizamide discontinuous gradient.
2.4.3 Intracellular Calcium Imaging

To determine cytosolic $\text{Ca}^{2+}$ concentration, 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×/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). As a measure for the increase of cytosolic $\text{Ca}^{2+}$ concentration, the slope and peak of the changes in the 340/380 nm ratio were determined for each experiment.

Intracellular $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ release was measured following addition of MgATP (100 µM, Alfa Aesar GmbH & Co KG, Germany) to the $\text{Ca}^{2+}$-free solution (see below).

In order to measure SOC entry, changes in cytosolic $\text{Ca}^{2+}$ were monitored upon depletion of the intracellular $\text{Ca}^{2+}$ stores. Experiments were carried out prior to and during exposure of the cells to $\text{Ca}^{2+}$-free solution (see below). In the absence of $\text{Ca}^{2+}$, the intracellular $\text{Ca}^{2+}$ stores were depleted by inhibition of the vesicular $\text{Ca}^{2+}$ pump by thapsigargin (1 µM, Molecular Probes). Re-addition of $\text{Ca}^{2+}$ allowed assessing the store-operated $\text{Ca}^{2+}$ entry.

2.4.4 Whole-cell Patch Clamp

Patch clamp experiments were performed at room temperature in voltage clamp, fast-whole-cell mode according to Hamill et al. (Hamill, Marty et al. 1981). The cells were continuously superfused through a flow system inserted into the dish. The bath was grounded via a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (2-4 MΩ tip resistance; GC 150 TF-10, Harvard Apparatus, UK) heat polished with a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS 314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). For $I_{\text{CRAC}}$ 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. The liquid junction potential $\Delta E$ between the CsCl-based pipette and the NaCl-based bath solutions estimated according to Barry and Lynch (Barry and Lynch 1991) was 1 mV. The data were not corrected for $\Delta E$.

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.

### 2.4.5 Migration Assay

For migration assays transwell inserts were used with a pore diameter size of 8 $\mu$m. The transwells were placed in a 24-well cell culture plate containing cell culture medium (500 $\mu$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 $\mu$l cell culture medium containing immature or LPS (1 $\mu$g/ml, 24 h) - matured DCs either untreated or treated with IP$_3$R inhibitor Xestospongin C (5 $\mu$M, Enzo Life Sciences, Lausen, Switzerland) for 3h in a concentration of $1 \times 10^5$ cells/ml. The chamber was placed in a 5% CO$_2$ 37°C incubator for 3h. In the following step, the transwells were placed in 4% PFA for overnight incubation in 4°C, to allow the cells to fix on the membrane. Unattached cells were gently removed with cotton swab, the membrane was then mended on a slide and stained with ProLong® Gold antifade reagent with DAPI (Invitrogen). Cells from five representative areas of each membrane were counted.

### 2.4.6 Cytokine Production

IL-12, IL-6, IL-2 and TNF$\alpha$ concentrations in culture supernatants from DCs treated with LPS (1 $\mu$g/ml, 24 h, for IL-6 measurements), LPS (1 $\mu$g/ml, 18 h, for IL-2 measurements), LPS (100 ng/ml, 48 h, for IL-12 measurements), or LPS (1 $\mu$g/ml, 4 h, for TNF$\alpha$ measurements) were determined by using OptEIA ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

### 2.4.7 DC Phagocytosis Assay

DCs ($10^6$ cells/ml) were suspended in prewarmed serum-free RPMI 1640 medium, pulsed with FITC-conjugated dextran (Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 1 mg/ml and incubated for 3h at 37°C. Uptake was stopped by
adding ice-cold PBS. Then the cells were washed three times with ice cold PBS supplemented with 5% FCS and 0.01% sodium azide before FACS analysis. DCs were analyzed for the uptake of FITC-dextran.

2.4.8 Western Blotting

Protein lysates were separated by SDS-PAGE 10% gel electrophoresis and transferred onto nitrocellulose membrane. Membrane containing the immobilized proteins was incubated for 1 hour at room temperature with 10% non-fat milk in Tris-buffered saline-0.1% Tween 20 (TBS-T), followed by overnight incubation (4°C) with IP$_3$R2 primary antibody (1:750; Santa Cruz,), rabbit polyclonal ETS1 (1:200, 56 kDa, Abcam, ), NDRG1 (1:1000), NfκBp65(1: 1000), rabbit monoclonal GAPDH (1:1000, 37 kDa, Cell Signaling). The following day membrane was washed 3 times in TBS-T and incubated for 1 h at room temperature with anti-rabbit (1:3000, Cell Signalling) or anti-goat (1:3000, 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.

2.4.9 Nuclear Fractionation

Prepare 1 ml of buffer A with added cocktail of usual inhibitors (Roche). Twenty million transfected cells were harvested and washed twice with ice-cold PBS followed by resuspending the cell pellet in 500 μl of buffer A, leave on ice for 10 min. Centrifuge at 4°C at 3000 rpm for 10 min. Remove supernatant and keep it (this will contain everything except large plasma membrane pieces, DNA, nucleoli). On ice resuspend pellet in 374 μl of buffer B and add 26 μl of 4.6 M NaCl. Leave on ice for 30 min. Centrifuge at 24,000 g for 20 min at 4°C. Aliquot supernatant, remove 10 μl for Bradford assay and processed on SDS-PAGE for westernblotting.

2.4.10 Real-Time PCR

Total RNA was extracted from mouse DCs in Tri-Fast (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions. After DNase digestion reverse transcription of total RNA was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany). Real-Time Polymerase chain reaction (RT-PCR) amplification of the respective genes were set up in a total volume of 20 μl using 2 μg of cDNA, 500 nM forward and reverse primer and 2 X GoTaq® Mast Mix (Promega, Mannheim, Germany) according to the manufacturer's protocol. Cycling conditions were as follows: initial denaturation at 95°C for 2 min, followed by 39 cycles of 95°C for 15 s, 58°C for 15 s and 68°C for 20 s. For the amplification the following primers were used (5’->3’orientation):
Table 2. RT-PCR primers sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward sequence (5´-&gt;3´)</th>
<th>Reverse sequence (5´-&gt;3´)</th>
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<tr>
<td>Akt1</td>
<td>CAGCACCCTTCTTTGCC</td>
<td>CAGTCTCAGAGGTGACCTGGG</td>
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<td>Akt2</td>
<td>AGTGAAGACCCACATGA</td>
<td>CCACCTCAGCCCATAGTC</td>
</tr>
<tr>
<td>Akt3</td>
<td>CCGCTCGTTGCCATGC</td>
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<tr>
<td>IP3R1</td>
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<td>IP3R2</td>
<td>CTGTTCTCTCTCATCGCATC</td>
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<td>IP3R3</td>
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<td>RyR2</td>
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<tr>
<td>RyR3</td>
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<td>TGCTTTGGCGCTCTCTACTG</td>
</tr>
<tr>
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<td>CAGCTGGATCGGCCCACCTCCTGTAG</td>
</tr>
<tr>
<td>Ndrg1</td>
<td>ACCCTGAGATGGGTAGAGGTCTCTCTC</td>
<td>CCAATTTAGAATTGCATTCCACC</td>
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<tr>
<td>Tbp</td>
<td>CACTCCTGCCACCCGAGGCTCTCTTTARGTCAAGTTCAGGC</td>
<td>TGCTCTTGCTAGTCCAGCC</td>
</tr>
</tbody>
</table>

Specificity of the PCR product was confirmed by analysis of melting curves.
Real-time PCR amplifications were performed on a CFX96 Real Time System (Bio-Rad). All experiments were done in duplicates. Amplification of housekeeping gene Tbp (TATA binding protein) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved with the Ct method (where Ct is threshold cycle). In addition, PCR products were analysed by agarose gel electrophoresis.

2.4.11 Immunoprecipitation

The cells were washed twice in ice-cold phosphate-buffered saline. The cell pellets were transferred into a cell lysis buffer (Cell Signaling) containing complete protease inhibitor mixture (Roche Applied Science). Lysates were washed with 50µl of protein A/G PLUS agarose beads (Santa Cruz) and then the supernatants were incubated overnight with Phospho-Akt Substrate (RXRXXS*/T) antibody. The supernatant and the antibody mixture was incubated with protein A/G PLUS agarose beads that were washed three times in cell lysis buffer, incubated at 37°C for 1 hour, then heated up to 95 °C for 5 min, and processed on SDS-PAGE for immunoblotting.

2.4.12 Gene Silencing

Specific siRNA sequences for Ets1 (Santa Cruz), negative control (Control siRNA, Santa Cruz) and siRNA sequences for Ndrg1 (Invitrogen) and negative control (Invitrogen) 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 10^6 cells were washed and plated in 6-well plates in 2 ml of serum-free RPMI 1640. The siRNA and the negative control (1000 ng/ml) were incubated with GeneSilencer reagent following the manufacturer’s protocol. Transfection mixture was then added to the wells and incubated overnight. The efficiency of silencing was assessed with RT-PCR and western blotting.

2.4.13 Statistical Analysis

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.
3. Result

Part 1. Akt2- and ETS1-dependent Expression of IP3 Receptor 2 is Required for Dendritic Cell Migration

3.1 Differentiation and Maturation of Bone Marrow Derived DCs

DCs were isolated from gene targeted mice lacking functional Akt2 (akt2<sup>-/-</sup>) and their wild type littermates (akt2<sup>+/+</sup>).

---

Figure 8 Differentiation, maturation and cytokine production of bone marrow derived dendritic cells (DCs) from akt2<sup>+/+</sup> and akt2<sup>-/-</sup> mice

A. Original dot plots of CD11<sup>c</sup>CD86<sup>+</sup> (above) and CD11<sup>c</sup>MHC II<sup>+</sup> (below) DCs at the basal level (control, 1<sup>st</sup> and 2<sup>nd</sup> panels) and stimulated with LPS (LPS, 1 µg/ml, 24 h, 3<sup>rd</sup> and 4<sup>th</sup> panels) from akt2<sup>+/+</sup> (1<sup>st</sup> and 3<sup>rd</sup> panels) and akt2<sup>-/-</sup> (2<sup>nd</sup> and 4<sup>th</sup> panels) mice. Numbers depict the percent of cells in the respective quadrants.

B. Arithmetic means ± SEM (n =7) of the percentage of CD11<sup>c</sup>CD86<sup>+</sup> (left) and CD11<sup>c</sup>MHC II<sup>+</sup> (right) DCs under control and 24 h after LPS stimulation in primary
cultures from akt2+/+ (open bars) and akt2−/− (closed bars) mice. *** (p<0.001), ANOVA.

As illustrated in Fig 8, 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. Following LPS treatment, the abundance of CD86 and MHC II was still similar in DCs from akt2+/+ and akt2−/− mice (Fig. 8A, 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. 8C).

3.2 Impaired Migration of akt2−/− DCs

Migratory capacity of immature akt2−/− DCs in response to the CXCL12 chemokine as well as spontaneous migration was not different from akt2+/+ DCs (Fig. 9A). 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. 9B).

Figure 9. Impaired migration of LPS-matured akt2−/− DCs
A.-B. Arithmetic means ± SEM (n =11-18) of spontaneous migration and migration in response to either CXCL12 (50 ng/ml, 3h, immature DCs, A) or CCL21 (25 ng/ml, 3h, LPS (1 µg/ml, 24h)-matured DCs, B) of akt2+/+ (open bars) and akt2−/− (closed bars) DCs. * (p<0.05), ** (p<0.01), ANOVA.
3.3 Reduced CCL21-dependent Increase of $[\text{Ca}^{2+}]_i$ in \textit{akt2}^{-/-} DCs

Fura2-fluorescence has been employed to determine, whether the difference in migratory activity of LPS-matured \textit{akt2}^{-/-} DCs was paralleled by the differences in the regulation of cytosolic Ca$^{2+}$ concentrations ($[\text{Ca}^{2+}]_i$). As illustrated in Fig. 10, both slope and peak of CCL21 (75 ng/ml)-induced increase of $[\text{Ca}^{2+}]_i$ was significantly less pronounced in LPS-matured DCs from from \textit{akt2}^{-/-} mice than in DCs from \textit{akt2}^{+/+} mice. Chemokine-induced increase of $[\text{Ca}^{2+}]_i$ is known to be due to the release of Ca$^{2+}$ from intracellular stores followed by the store operated Ca$^{2+}$ entry (SOCE) (Barbet, Demion et al. 2008; Nurbaeva, Schmid et al. 2012).

![Figure 10. Diminished CCL21-induced increase in intracellular Ca$^{2+}$ concentration in \textit{akt2}^{-/-} DCs](image)

A. Representative original tracings showing the Fura-2 fluorescence ratios (340/380 nm) in LPS (1 µg/ml)-matured \textit{akt2}^{+/+} DCs (open squares) and \textit{akt2}^{-/-} DCs (closed circles) prior to and following acute addition of CCL21 (75 ng/ml; white arrow). For quantification of Ca$^{2+}$ entry the slope ($\Delta$ ratio/s) and the peak ($\Delta$ ratio) of the fluorescence ratio were calculated by linear regression.

B. Mean ($\pm$ SEM) of peak (left) and slope (right) of the change in Fura-2 fluorescence following addition of CCL21 (75ng/ml) to mature \textit{akt2}^{+/+} (n = 50, open bars) and \textit{akt2}^{-/-} (n = 40, closed bars) DCs. ** (p<0.01), two-tailed unpaired t-test.
3.4 Reduced Ca\(^{2+}\) Release and Store-operated Ca\(^{2+}\) Entry in LPS-matured \(akt2^{-/-}\) DCs

Therefore, further experiments addressed the impact of Akt2 on Ca\(^{2+}\) release from intracellular stores and on SOCE in LPS-matured DCs. As indicated in Fig. 11, the addition of sarcoendoplasmatic reticulum Ca\(^{2+}\) ATPase (SERCA) inhibitor thapsigargin (1 \(\mu\)M) in the absence of extracellular Ca\(^{2+}\) was followed by an increase of \([\text{Ca}\(^{2+}\)]_i\), 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. 11B). The readdition of extracellular Ca\(^{2+}\) in the continued presence of thapsigargin resulted in a rapid increase of \([\text{Ca}\(^{2+}\)]_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 Ca\(^{2+}\) induced by physiological IP\(_3\) formation could be assessed through the stimulation of DCs via P2Y receptors with low concentrations of ATP (Pellegatti, Falzoni et al. 2005). Accordingly, ATP(100 \(\mu\)M)-induced release of Ca\(^{2+}\) (measured in the absence of extracellular Ca\(^{2+}\)) was lower in LPS-matured \(akt2^{-/-}\) than in \(akt2^{+/+}\) DCs (Fig. 11C).

3.5 Impaired CRAC Channel Activity in LPS-matured \(akt2^{-/-}\) DCs

Reduced SOCE in mature \(akt2^{-/-}\) DCs could be confirmed by whole cell patch clamp recordings. As illustrated in Fig. 12, 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.
Figure 11. Reduced Ca\textsuperscript{2+} Release and Store-operated Ca\textsuperscript{2+} Entry in akt2\textsuperscript{+/−} DCs

A. Representative tracing showing the Fura-2 fluorescence ratio in Fura-2/AM loaded LPS (1 µg/ml)-matured akt2\textsuperscript{+/+} and akt2\textsuperscript{−/−} DCs. Experiments were carried out prior to and during exposure to Ca\textsuperscript{2+}-free bath solution. Where indicated, thapsigargin (1 µM) was added to the Ca\textsuperscript{2+}-free bath solution and the release of Ca\textsuperscript{2+} from the stores was assessed. Readdition of extracellular Ca\textsuperscript{2+} in the presence of thapsigargin reflects the entry of Ca\textsuperscript{2+} through the SOC channels.

B. Arithmetic means (± SEM) of the slope (right) and the peak (left) of the change in Fura-2 fluorescence following addition of thapsigargin (1 µM) in the absence (upper bars) and in the presence (lower bars) of extracellular Ca\textsuperscript{2+} to mature akt2\textsuperscript{+/+} (n=117, open bars) and akt2\textsuperscript{−/−} (n=75, closed bars) DCs. *** (p<0.001), two-tailed unpaired t-test.

C. Representative tracing showing the Fura-2 fluorescence ratio in LPS (1 µg/ml)-matured akt2\textsuperscript{+/+} and akt2\textsuperscript{−/−} DCs prior to and following addition of ATP (100 µM) to the Ca\textsuperscript{2+}-free bath solution.

D. Arithmetic means (± SEM) of the slope (right) and the peak (left) of the change in Fura-2 fluorescence following addition of ATP (100 µM) in the absence of extracellular Ca\textsuperscript{2+} to LPS (1 µg/ml)-matured akt2\textsuperscript{+/+} (n=242, open bars) and akt2\textsuperscript{−/−} (n=250, closed bars) DCs. * (p<0.05), two-tailed unpaired t-test.
Figure 12. Impaired currents through store-operated Ca\textsuperscript{2+} channels in akt2\textsuperscript{-/-} DCs

A. Mean current-voltage (I/V) relationships of currents activated by 40 µM IP\textsubscript{3} normalized to cell capacitance in LPS (1 µg/ml)-matured akt2\textsuperscript{+/+} (n=11) and akt2\textsuperscript{-/-} (n=15) DCs. * (p<0.05), two-tailed unpaired t-test.

B. Mean whole-cell conductance of inward currents (± SEM) calculated by linear regression of I/V curves shown in (A) between -50 and -10 mV in akt2\textsuperscript{+/+} (n = 11, open bars) and akt2\textsuperscript{-/-} (n=15, closed bars) DCs. * (p<0.05), two-tailed unpaired t-test.

3.6 Impaired Transcript Level and Protein Abundance of IP\textsubscript{3}R2 in Mature akt2\textsuperscript{-/-} DCs

Impaired Ca\textsuperscript{2+} release, SOCE and I\textsubscript{CRAC} in akt2\textsuperscript{-/-} DCs could have resulted from Akt2 sensitivity of the Ca\textsuperscript{2+} release pathways such as inositoltrisphosphate receptors IP\textsubscript{3}R1-3 or the ryanodine receptor RyR1. As illustrated in Fig. 13A, transcript levels of IP\textsubscript{3}R1, IP\textsubscript{3}R3 and RyR1 were similar in immature akt2\textsuperscript{+/+} and akt2\textsuperscript{-/-} DCs, whereas the transcript abundance of IP\textsubscript{3}R2 was significantly higher in akt2\textsuperscript{+/+} DCs than in akt2\textsuperscript{-/-} DCs. Stimulation of akt2\textsuperscript{+/+} DCs with LPS (1 µg/ml) resulted in a transient upregulation of IP\textsubscript{3}R2 transcript abundance within 1h in akt2\textsuperscript{+/+} DCs, an effect significantly impaired in akt2\textsuperscript{-/-} DCs (Fig. 13B).
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, the IP$_3$R2 protein abundance was similar in *akt2*+/+ and *akt2*−/− DCs. LPS (1 µg/ml, 24 h)-induced maturation resulted in a significant increase of IP$_3$R2 protein abundance in *akt2*+/+ DCs, but not in *akt2*−/− DCs. Accordingly, following treatment with LPS, the IP$_3$R2 protein abundance was significantly higher in DCs from *akt2*+/+ mice than in DCs from *akt2*−/− mice (Fig.13C).

Figure 13. Transcript levels of IP$_3$R1, IP$_3$R3 and RyR1 in immature and treated with LPS-1H *akt2*+/+ and *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 as well as of 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), two-tailed unpaired t-test.

B. Arithmetic means (± SEM, n=7-13) of the abundance of mRNA encoding IP$_3$R2 in *akt2*+/+ and *akt2*−/− DCs unstimulated (LPS, 0h) and stimulated with LPS (1 µg/ml, 1h and 4h). * (p<0.05), ** (p<0.01), *** (p<0.001), ANOVA.

C. Original representative Western blot of whole cell lysate protein of IP$_3$R2 (upper panel) and GAPDH (lower panel) in LPS (1 µg/ml, 24 h)-matured *akt2*+/+ and *akt2*−/− DCs.

D. Arithmetic means ± SEM (n =11-15) of IP$_3$R2/GAPDH ratio in immature and LPS (1 µg/ml, 24 h)-matured *akt2*+/+ and *akt2*−/− DCs. *(p<0.05), ANOVA.
3.7 Xestospongin C Inhibits the Migration of LPS-matured \( akt2^{+/+} \) and \( akt2^{-/-} \) DCs

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 IP\(_3\)R inhibitor xestospongin C (5 µM). 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. CXCL12-induced migration of immature DCs was also sensitive to xestospongin C, however, no significant difference between \( akt2^{-/-} \) and \( akt2^{+/+} \) DCs was observed (Fig.14).

![Figure 14. Migration of \( akt2^{+/+} \) and \( akt2^{-/-} \) DCs in the absence and presence of IP\(_3\)R inhibitor Xestospongin C](image)

Arithmetic means ± SEM (n = 5-25) of spontaneous migration and migration in response to either CXCL12 (50 ng/ml, 4h, immature DCs, A) or CCL21 (25 ng/ml, 4h, LPS (1 µg/ml, 24h)-matured DCs, B) of \( akt2^{+/+} \) (open bars) and \( akt2^{-/-} \) (closed bars) DCs in the absence or in the presence of the IP\(_3\)R inhibitor Xestospongin C (Xc, 5 µM, 3h) *(p<0.05), **(p<0.01), ****(p<0.001), ANOVA; #(* p<0.05), two-tailed unpaired t-test.

3.8 Impaired Expression of the Transcription Factor ETS1 in \( akt2^{-/-} \) DCs

PKB/Akt is known to phosphorylate IP\(_3\)R, reducing its sensitivity to IP\(_3\) and diminishing Ca\(^{2+}\) release (Szado, Vanderheyden et al. 2008). In contrast, reduced IP\(_3\)R2 transcript level and protein abundance in \( akt2^{-/-} \) DCs in the present study suggest that in DCs Akt2 upregulates IP\(_3\)R2 on a transcriptional level. However, we examined the phosphorylation status of IP\(_3\)R2 by immunoprecipitating the lysates with a phosphospecific antibody that detects the consensus Akt phosphorylation sequence (RXRXX(S/T)) and immunoblotting with IP\(_3\)R2-specific antibody (Fig. 15A). IP\(_3\)R2 was phosphorylated in immature as well as
in LPS-matured DCs, however no significant difference was detected between $akt2^{+/+}$ and $akt2^{-/-}$ DCs.

It has been shown that expression of IP$_3$R3 in CD4 T cells requires the ETS1 transcription factor (Nagaleekar, Diehl et al. 2008). In ETS1-deficient CD4 T cells the level of IP$_3$R2 protein was also reduced (Nagaleekar, Diehl et al. 2008). Moreover, activated Akt has been shown to lead to increased Ets1 transcription (Lavenburg, Ivey et al. 2003). Therefore we next examined the transcript level of Ets1 in $akt2^{-/-}$ and $akt2^{+/+}$ DCs. In immature $akt2^{-/-}$ DCs the transcript abundance of Ets1 was significantly impaired compared to $akt2^{+/+}$ DCs (Fig. 15B). Moreover, similar to IP$_3$R2, LPS (1 µg/ml) induced a transient increase in Ets1 transcript level within 1h in $akt2^{+/+}$ DCs, but not in $akt2^{-/-}$ DCs. Accordingly, the protein abundance of ETS1 in LPS-matured $akt2^{-/-}$ DCs was significantly decreased (Fig. 15C).
Figure 15. Transcript level and protein abundance of the transcriptional factor Ets1 in akt2+/+ and 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 IP3R2 antibody.

B. Arithmetic means (± SEM, n=6) of the abundance of mRNA encoding Ets1 in immature akt2+/+ and akt2−/− DCs, as assessed by real-time PCR using Tbp mRNA as a reference gene. *(p<0.05), two-tailed unpaired t-test.

C. Arithmetic means (± SEM, n=6-9) of the abundance of mRNA encoding Ets1 in akt2+/+ and akt2−/− DCs, unstimulated (LPS, 0h) and stimulated with LPS (1 µg/ml, 1h and 4h) as assessed by real-time PCR using TbpP mRNA as a reference gene. **(p<0.01), ANOVA.

D. Original representative Western blot of whole cell lysate protein of ETS1 (upper panel) and GAPDH (lower panel) in LPS (1 µg/ml, 24 h)-matured akt2+/+ and akt2−/− DCs.

E. Arithmetic means ± SEM (n =6) of ETS1/GAPDH ratio in immature and LPS (1 µg/ml, 24 h)-matured akt2+/+ and akt2−/− DCs. **(p<0.01), ANOVA.
3.9 Silencing of Ets1 Impaires the IP$_3$R2 Transcript Abundance upon LPS Stimulation

In order to test whether Akt2-dependent Ets1 expression can underlie decreased IP$_3$R expression and function in akt2$^{-/-}$ DCs, we performed silencing of Ets1 with siRNA (Fig. 16-19). The efficiency of silencing assessed with RT-PCR was 47 ± 11% (n=7) and it was confirmed by western blotting (Fig. 16A). Upon LPS stimulation (1h), Ets1 and IP$_3$R2 transcript abundance were reduced in siEts1 DCs (Fig. 16B).

![Figure 16. Silencing of the Transcriptional Factor Ets1 Reduces IP3R2 Transcritp Abundance upon 1H-LPS Treatment](image)

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 (left).

B. Arithmetic means (± SEM) of the abundance of mRNA encoding Ets1 (n=14, left) and IP3R2 (n=7, right) in LPS (1 mg/ml, 24 1 h)-matured control (control siRNA) and siETS1 DCs, as assessed by real-time PCR using Tbp mRNA as a reference gene. * (p<0.05), two-tailed unpaired t-test.

3.10 Silencing of Ets1 in LPS-matured DCs Reduces Ca$^{2+}$ Release and SOCE

Moreover, thapsigargin-induced (Fig. 17A, B) and ATP-induced (Fig. 17C, D) Ca$^{2+}$ release, thapsigargin-induced SOCE (Fig. 17A, B) were significantly impaired in siEts1 LPS (24 h)-matured DC.
Figure 17. Silencing of the transcriptional factor Ets1 in LPS-matured akt2+/− DCs reduces Ca²⁺ release, store-operated Ca²⁺ Entry.

A. Representative tracing showing the Fura-2 fluorescence ratio in LPS (1 µg/ml, 24 h)-matured control (control siRNA) and siEts1-DCs prior to and during exposure to thapsigargin (1 µM) in the Ca²⁺-free bath solution followed by readdition of extracellular Ca²⁺.

B. Arithmetic means (± SEM) of the slope (right) and the peak (left) of the change in Fura-2 fluorescence following addition of thapsigargin (1 µM) in the absence (upper bars) and in the presence (lower bars) of extracellular Ca²⁺ to LPS (1 mg/ml, 24 h)-matured control (n=55, open bars) and siEts1 (n=103, closed bars) DCs. **(p<0.01), *** (p<0.001), two-tailed unpaired t-test.

C. Representative tracing showing the Fura-2 fluorescence ratio in LPS (1 µg/ml, 24 h)-matured control (control siRNA) and siEtsTS1-DCs prior to and following addition of ATP (100 µM) to the Ca²⁺-free bath solution.

D. Arithmetic means (± SEM) of the slope (right) and the peak (left) of the change in Fura-2 fluorescence following addition of ATP (100 µM) in the absence of extracellular Ca²⁺ to LPS (1 µg/ml, 24h)-matured control (n=38, open bars) and siEtsTS1 (n=61, closed bars) DCs. *** (p<0.001), two-tailed unpaired t-test.
3.11 Silencing of Ets1 in LPS-matured DCs Reduces the I\textsubscript{CRAC} Activity

Similarly, I\textsubscript{CRAC} (Fig. 18A, B) was significantly impaired in siEts1 LPS (24 h)-matured DCs.

![Figure 18. Silencing of the transcriptional factor Ets1 in LPS-matured akt2\textsuperscript{+/+} DCs reduces I\textsubscript{CRAC} activity](image)

A. Mean current-voltage (I/V) relationships of currents activated by 40 µM IP\textsubscript{3} normalized to cell capacitance in LPS (1 µg/ml, 24 h)-matured control (n=20) and siEts1 (n=17) DCs. *** (p<0.001), two-tailed unpaired t-test.

B. Mean whole-cell conductance of inward currents (± SEM) calculated by linear regression of I/V curves shown in (F) between -40 and 0 mV in mature control (n=20) and siEts1 (n=17) DCs. * (p<0.05), two-tailed unpaired t-test.

3.12 Silencing of Ets1 in LPS-matured DCs Impaires Migration

Moreover, CCL21-dependent migration was significantly decreased in siEts1- LPS (24 h)-matured DCs (Fig. 19). Those data strongly suggests that Akt2 upregulates Ets1 transcription leading to enhanced expression of IP\textsubscript{3}R2. Increased Ca\textsuperscript{2+} release, followed by increased store-operated Ca\textsuperscript{2+} entry underlie, at least partially, stimulating effect of Akt2 on DC migration.

![Figure 19. Silencing of the transcriptional factor Ets1 in LPS-matured akt2\textsuperscript{+/+} DCs impaires migration](image)

Arithmetic means ± SEM (n =11) of CCL21 (25 ng/ml, 4h)-induced migration in in LPS (1 µg/ml, 24h)-matured control (control siRNA) and siEts1 DCs. * (p<0.05), two-tailed unpaired t-test.
Part2. Serum- and Glucocorticoid-inducible Kinase 1 SGK1 in the Regulation of Dendritic Cell Functions

3.13 SGK1 Deficiency Fosters DC Differentiation and Maturation

For activation and maturation mouse DCs obtained from bone marrow were cultured with or without LPS (100 ng/ml, 48 h). Cells were collected and stained for MHC class II, costimulatory molecule CD86 (Fig. 20A-D) and the adhesion molecule CD54 (ICAM-1), which mediates DC-T cell interaction (Fig. 20E-I). The basal level of CD86, MHC II and CD54 was higher on CD11c⁺ gated population obtained from SGK1 knockout mice (sgk1⁻/⁻), if compared to CD11c⁺-DCs from the wild-type littermates (sgk1⁺/⁺) (Fig. 20C,D,F). Also stimulation by LPS led to higher receptor abundance in sgk1⁻/⁻ cells (Fig. 20C,D,I), suggesting that SGK1 down-regulates differentiation and maturation of DCs.

Moreover, we isolated DCs from the spleen of sgk1⁻/⁻ and sgk1⁺/⁺ mice. Expression of CD86, MHCII and CD54 was higher in splenic sgk1⁻/⁻ DCs than in splenic sgk1⁺/⁺ DCs (Fig. 21), confirming our observations with bone marrow-derived cells.

3.14 Phagocytic Capacity of sgk1⁻/⁻ DCs is Impaired

The function of DCs as innate immune effectors involves antigen uptake. PI3K is known to play an important role for phagocytic uptake in DCs (Agrawal, Agrawal et al. 2007). Thus we compared the capacity of sgk1⁺/⁺ and sgk1⁻/⁻ DCs to phagocytose antigen by coincubating DCs with FITC-dextran. The phagocytosis of FITC-dextran was found to be significantly reduced in sgk1⁻/⁻ DCs (Fig. 22).
Figure 20. Differentiation and maturation of bone marrow derived DCs from sgk1+/+ and sgk1−/− mice

A., B. Original dot plots of CD11c+CD86$^{\text{high}}$ (A) and CD11c+MHC II$^{\text{high}}$ (B) DCs at the basal level (control, 1$^\text{st}$ and 2$^\text{d}$ panels) and stimulated with LPS (100 ng/ml, 48 h, 3$^\text{d}$ and 4$^\text{th}$ panels) from sgk1+/+ (1$^\text{st}$ and 3$^\text{d}$ panels) and sgk1−/− (2$^\text{d}$ and 4$^\text{th}$ panels) mice. Numbers depict the percent of cells in the respective quadrants.

C., D. Arithmetic means ± SEM (n = 4-5) of the percentage of CD11c+CD86$^+$ (C) and CD11c+MHC II$^+$ (D) DCs under control and 24 h after LPS stimulation in primary cultures from sgk1+/+ (closed bars) and sgk1−/− (open bars) mice. * (p<0.05) and *** (p<0.001) indicate significant difference between sgk1+/+ and sgk1−/− cells (two-tailed unpaired t-test).

E., G. Representative FACS histograms depicting the expression of CD54 in control (E) and LPS-stimulated (100 ng/ml, 48 h, G) sgk1+/+ (grey line) and sgk1−/− (black line) cells.
F., I. Arithmetic means ± SEM (n = 9) of fluorescence intensity (MFI) of the CD54 marker in control (F) and LPS-stimulated (100 ng/ml, 48 h, I) sgk1+/+ (closed bars) and sgk1−/− (open bars) cells. ** (p<0.01) and *** (p<0.001) indicate significant difference between sgk1+/+ and sgk1−/− cells (two-tailed unpaired t-test).

Figure 21. Maturation status of splenic DCs from sgk1+/+ and sgk1−/− mice

A., B. Original dot plots of CD11c+CD86+ (A) and CD11c+MHC II+ (B) splenic DCs from sgk1+/+ and sgk1−/− mice. Numbers depict the percent of cells in the respective quadrants, acquired within the dead cell gate.

C., D. Arithmetic means ± SEM (n = 3–4) of the percentage of CD11c+CD86+ (C) and CD11c+MHC II+ (D) splenic DCs from sgk1+/+ (open bars) and sgk1−/− (closed bars) mice. *** (p<0.001) indicates significant difference between sgk1+/+ and sgk1−/− cells (two-tailed unpaired t-test).

E. Representative FACS histograms depicting the expression of CD54 in sgk1+/+ (grey line) and sgk1−/− (black line) cells.

F. Arithmetic means ± SEM (n = 4) of fluorescence intensity (MFI) of the CD54 marker in sgk1+/+ (open bars) and sgk1−/− (closed bars) cells. ** (p<0.01) indicates significant difference between sgk1+/+ and sgk1−/− cells (two-tailed unpaired t-test).
Phagocytic capacity is impaired in \textit{sgk1}^{-/} DCs

\textbf{A.} Histogram representing the DCs percentage of the uptake of FITC-dextran after 3h by \textit{sgk1}^{+/+} and \textit{sgk1}^{-/-} DCs.

\textbf{B.} Arithmetic means ± SEM (n = 6) of FITC-dextran uptake by \textit{sgk1}^{+/+} (open bars) and \textit{sgk1}^{-/-} (closed bars) DCs. *** (p<0.001) indicates significant difference (two-tailed unpaired t-test).

\subsection*{3.15 \textit{sgk1}^{-/-} DC Secrete Higher Levels of IL-12 in Response to LPS}

We next examined IL-12 production by \textit{sgk1}^{-/-} DCs to determine the involvement of SGK1 signalling in DC cytokine production. DCs were stimulated with LPS (100 ng/ml, 48 h) and IL-12p70 release was analyzed. \textit{sgk1}^{-/-} DCs produced significantly higher amounts of IL-12p70 than \textit{sgk1}^{+/+} DCs (Fig. 23A). No significant difference was observed in the production of IL-10, IL-6 and TNF-\alpha (Fig. 23B-D).
Figure 23. IL-12 secretion is enhanced in sgk1−/− DCs

Arithmetic means ± SEM (n = 18) of IL-12, IL-6, TNFα and IL-10 secretion in cultured sgk1−/− DCs (closed bars) and their wild-type littermates sgk1+/+ (open bars) under control conditions (control) and after stimulation with LPS (100 ng/ml, 48h). ** indicates significant difference from LPS-stimulated sgk1+/+ cells (p<0.01; ANOVA).

3.16 Loss of SGK1 Has No Effect on GSK3

Phosphorylation

Glycogen synthase kinase 3 (GSK3) has been demonstrated as a downstream target of PI3K, which mediates its inhibiting action on IL-12 production in DCs (Ohtani, Nagai, Kondo, Mizuno, Nakamura, Tanabe, Takeuchi, Matsuda, and Koyasu, 2008;Martin, Rehani et al., 2005;Zhang, Katz et al., 2009). We, therefore, studied GSK3 expression and LPS-induced phosphorylation in sgk1+/+ and sgk1−/− DCs. As shown in Fig. 24, GSK3 is similarly expressed in DCs of both genotypes and LPS treatment similarly enhanced GSK3 phosphorylation in sgk1+/+ and in sgk1−/− DCs (Fig. 24).
Figure 24. GSK3 phosphorylation is normal in sgk1−/− DCs

A. Original western blot of the expression of phosphorylated GSK3 protein (upper panel), of total GSK3 protein (middle panel), and of GAPDH protein (lower panel) in sgk1+/+ and sgk1−/− DCs prior to (control) and 1h following addition of LPS (1 µg/ml).

B. Arithmetic means ± SEM (n=3-4 independent experiments) of phosphorylated over total GSK3 protein abundance in sgk1+/+ (open bars) and sgk1−/− (closed bars) DCs prior to (control) and 1h following addition of LPS.

3.17 Loss of SGK1 Leads to Upregulation of NF-κB

The NF-κB family of transcription factors plays a major role in regulating DC development, maturation and function (Li and Verma 2002). To gain further insights into the mechanisms responsible for enhanced APC functions of sgk1−/− DCs, we analyzed p65 nuclear localization in sgk1+/+ and sgk1−/− DCs by western blot of nuclear fraction. Already before stimulation, as well as upon stimulation with LPS (1µg/ml, 10 min) the nuclear localization of p65 was strongly enhanced in sgk1−/− DCs (Fig. 25).

Figure 25. Loss of SGK1 enhances p65 nuclear localization in mouse DCs

Original Western blot of nuclear p65 from sgk1+/+ and sgk1−/− DCs, which were either stimulated with LPS (1 µg/ml, 10 min) or left untreated (control). Protein loading was controlled by anti-Lamin A antibody.
3.18 Loss of SGK1 Results in Reduced Phosphorylation of NDRG-1

N-myc downstream regulated gene 1 (NDRG1) has been shown to attenuate NF-κB signalling (Hosoi, Izumi et al. 2009). Cells overexpressing NDRG1 demonstrate reduced nuclear translocation of p65 (Hosoi, Izumi et al. 2009). Moreover, NDRG1 is a physiological target of SGK1, phosphorylated by SGK1 at Thr328, Ser330 and Thr346 (Murray, Campbell et al. 2004). On the other hand, phosphorylation of NDRG1 at both Ser330 and Thr346 is required for its suppressive action on the NF-kappaB signaling pathway (Murakami, Hosoi et al. 2010). Accordingly we analyzed NDRG1 expression and phosphorylation in unstimulated and LPS-stimulated sgk1+/+ and sgk1−/− DCs. As shown in Fig.26, SGK1-dependent phosphorylation of Thr346 and Ser330 of NDRG-1 was decreased in unstimulated as well as in LPS (1h)-stimulated sgk1−/− DCs.
Figure 26. Phosphorylation of NDRG-1 is reduced in sgk1−/− DCs

A. Original Western blot of sgk1+/+ and sgk1−/− DCs untreated (control) or stimulated with LPS (1 µg/ml, 1h). Protein extracts were analyzed by direct Western blotting using antibodies directed against phospho (p)-NDRG1 at Thr346 (p-(Thr346)-NDRG1) and at Ser330 (p-(Ser330)-NDRG1). Equal protein loading was confirmed with GAPDH antibody.

B. Arithmetic means ± SEM (n =5) of p-(Thr346)-NDRG1/GAPDH and p-(Ser330)-NDRG1/GAPDH ratio in untreated and LPS (1 µg/ml, 1h)-treated sgk1+/+ (open bars) and sgk1−/− (closed bars) DCs. * (p<0.05), ** (p<0.01), ANOVA.
3.19 Knockdown of NDRG1 Results in Enhanced p65 Nuclear Localization

To prove the hypothesis, that diminished phosphorylation of NDRG-1 in sgk1+/− DCs may underline enhanced localization of p65 in the nuclei of those cells, we silenced NDRG1 by siRNA in sgk1+/− DCs. This led to an enhanced nuclear abundance of p65 compared to the control empty vector-treated DCs (Fig. 27).

Figure 27. Enhanced nuclear localization of p65 upon NDRG1 knock-down in sgk1+/− DCs

Western blot analysis of nuclear p65 in untreated or LPS (1 µg/ml, 1h)-stimulated empty vector-transfected and siNDRG1-DCs. Representative experiments showing p65 band and Lamin-A as loading control.
4. Discussion

In the first part of the present study we demonstrate that Akt2 is required for the expression of IP$_3$R2 in mouse DCs and accordingly release of Ca$^{2+}$ from intracellular stores and, as a consequence, store-operated Ca$^{2+}$ entry and activation of Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels are diminished in Akt2 deficient DCs. Moreover, we provide evidence that IP$_3$R2 expression in DCs is regulated by the Akt-2-dependent transcription factor ETS-1. Finally, we show that reduced IP$_3$R2 expression in Akt2-deficient DCs may underlie their defective migration.

IP$_3$R2 protein is similarly expressed in immature nonstimulated akt2$^{+/+}$ and akt2$^{-/-}$ DCs. However, upon LPS-induced stimulation, the transcript abundance of IP$_3$R2 is transiently enhanced in akt2$^{+/+}$ but not in akt2$^{-/-}$ DCs, and accordingly the expression of IP$_3$R2 protein is higher in fully mature akt2$^{+/+}$ compared to akt2$^{-/-}$ DCs. Reduction in IP$_3$R2 expression is paralleled by impaired function of IP$_3$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 as well as IP$_3$-induced activation of CRAC channels is diminished in Akt2-deficient DCs. Most importantly, increase of cytosolic free Ca$^{2+}$ concentration upon ligation of the CCR7 is strongly reduced in mature akt2$^{-/-}$ DCs, which is paralleled by a reduced CCR7-dependent migration of those cells. CCR7-dependent migration was very sensitive to the IP$_3$R inhibitor Xestospongin C, and no difference in migrating ability could be detected between Xestospongin C-treated akt2$^{+/+}$ and akt2$^{-/-}$ DCs. This allows us to conclude that reduced expression of IP$_3$R2 underlies, at least partially, the defective CCR7-dependent migration of Akt2-deficient DCs. Akt2 knockdown has been similarly shown to result in reduced plasmin-triggered migration of human DCs (Li, Syrovets et al. 2010), and CSF-1- and MCP-1-induced chemotaxis of mouse peritoneal macrophages (Zhang, Ma et al. 2009).

Akt2-dependent release of Ca$^{2+}$ through the IP$_3$R2 could 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 anti-IgM crosslinking and to anti-CD3 crosslinking was attenuated in p110δ D910A/D910A mice, in accord with an important role for PI3Ks upstream of Tec family kinase-mediated activation of phospholipase Cγ (PLCγ) and Ca$^{2+}$ signalling by antigen receptorsCa$^{2+}$ flux is attenuated in B and T lymphocytes deficient in the catalytic
subunit of PI3K, p110δ, in response to stimulation of B and T cell antigen receptors, respectively. (Okkenhaug, Bilancio et al. 2002). Diminished Ca^{2+} mobilization in B cells with either genetical or pharmacological inhibition of p110δ has been also confirmed in other studies (Inabe and Kurosaki 2002; Jou, Carpino et al. 2002; Bilancio, Okkenhaug et al. 2006). Jou shows in contrast to the absence of detectable Ca^{2+} mobilization in \textit{PLCγ2}^{-/-} B cells, \textit{p110δ}^{-/-} B cells induce an increase in intracellular Ca^{2+} that is approximately 25% of that observed in wild-type cells. In addition, mobilization of intracellular Ca^{2+} upon anti-CD3 stimulation is unimpaired in \textit{p110δ}^{-/-} T cells. The study of Bilancio in primary splenic B cells, demonstrates the PI3K-dependent fraction of early intracellular Ca^{2+} flux is also completely dependent on p110δ catalytic activity. They suggest that most likely, these functions of p110δ derive from the stimulatory effects of its PIP3 lipid product on the activation of phospholipase C80 (leading to the generation of diacylglycerol and IP3, which induces the release of intracellular Ca^{2+}) and on PDK1. Again, such a key role for p110 is remarkable given the high level of residual class IA PI3K activity in p110δ D910A/D910A cells. Whether Akt-dependent upregulation of IP3R plays a role in those cells needs to be established. However, one study on DT40 B cells and Jurkat cells reports an inhibiting effect of Akt2 on Ca^{2+} mobilization (Martin, Wang et al. 2012). Whereas the initial amplitude of Ca^{2+} mobilization has not been influenced by Akt2, the subsequent clearance of Ca^{2+} is enhanced by Akt2, which results in a reduced duration of Ca^{2+} increase paralleled by a reduced activation of NFAT (Martin, Wang et al. 2012). 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 IP3R to inhibit Ca^{2+} release (Martin, Wang et al. 2012). Accordingly the cell permeable peptide that blocks the interaction between Bcl-2 and IP3R abrogates the effect of Akt2 (Martin, Wang et al. 2012). However, enhanced Ca^{2+} clearance rather than modified Ca^{2+} release measured in the presence of Akt2 may also indicate Akt2-dependent upregulation of SERCA or any other extrusion pathways in this model.

Several mechanisms of Akt-dependent regulation of IP3Rs have been detected so far. A direct interaction and phosphorylation of IP3R by Akt has been demonstrated (Khan, Wagner et al. 2006; Varga-Szabo, Authi et al. 2008). Szado et al. have shown that Akt-dependent phosphorylation of IP3Rs significantly reduces their Ca^{2+} release activity (Varga-Szabo, Authi et al. 2008), however another study of Khan et al. has not revealed any modification of IP3R function by Akt phosphorylation (Khan, Wagner II et al. 2006). We could detect phosphorylation of IP3R2 at the Akt specific motif, however no difference in phosphorylated IP3R2 was observed between \textit{akt2}^{-/-} and \textit{akt2}^{+/+} DCs. Another proposed mechanism involves Akt-dependent regulation of
Bcl-2 anti-apoptotic proteins (Martin, Wang et al. 2012), which are known to interact with IP₃R through their anti-apoptotic BH4 domain and inhibit Ca²⁺ release (Rong, Bultynck et al. 2009). The present study provides another novel mechanism of Akt2- and ETS1-dependent transcription of IP₃R2.

ETS1 is a transcription factor highly conserved throughout evolution, highly expressed in lymphocytes and in tumors (Garrett-Sinha 2013). 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 after 1h treatment with LPS, and although the ETS1 protein level is not increased in fully mature compared to immature akt2⁺/⁻ DCs, mature akt2⁻/⁻ DCs expressed significantly less ETS1 protein than mature akt2²⁺/⁺ DCs. Moreover, IP₃R2 expression is strongly reduced upon Ets1 knock down. Silencing of Ets1 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 to the study on ETS1 deficient CD4 T cells in which Ca²⁺ mobilization in response to TCR ligation is impaired (Nagaleekar, Diehl et al. 2008). Moreover, IP₃R3 has clearly been shown to be under transcriptional regulation by Ets1 (Tamura, Hashimoto et al. 2001; Nagaleekar, Diehl et al. 2008).

Ets1 gene expression is known to be stimulated by activated Akt (Lavenburg, Ivey et al. 2003; Ghosh, Basu et al. 2012; Smith, Findlay et al. 2012). 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 decreased by inhibition of Akt activity (Smith, Findlay et al. 2012). On the other hand, inhibition of PI3k/Akt pathway blocks transcriptional upregulation of Ets1 by ER stress in human melanoma cells (Dong, Jiang et al. 2011) and by PDGF in human aortic vascular smooth muscle cells (Lang, Artunc et al. 2009). Moreover, knockdown of Akt3 reduced the basal levels of ETS1 in melanoma cells, suggesting that the PI3k/Akt pathway may have a role in regulating constitutive expression of Ets-1 (Dong, Jiang et al. 2011). Expression of ETS1 was reduced in akt2⁻/⁻ DCs only upon stimulation with LPS. LPS has been shown to trigger PI3K stimulation with the consequent Akt phosphorylation in mouse DCs (Fukao, Tanabe et al. 2002; Fukao and Koyasu 2003). Thus, LPS-induced activation of Akt2 is probably required for the continuous expression of ETS1 upon DC maturation.
In conclusion, Akt2 upregulates IP$_3$R2 transcription in DCs, presumably by enhancing the expression of ETS1 and this effect may underlie the stimulating effect of Akt2 on DC migration.

Similar to PKB/Akt, SGK1 is activated through PI3K and phosphoinositide-dependent kinase PDK1 (Alessi, Andjelkovic et al. 1996; Kobayashi, Deak et al. 1999; Park, Leong et al. 1999). Recent studies indicate that PI3K and PDK1 may function as an endogenous negative feedback that serves to limit excessive innate immune responses (Fukao and Koyasu 2003; Zaru, Mollahan et al. 2008). SGK1 could act downstream of PI3K and PDK1 to mediate this negative feedback in DCs.

The second part of the present study reveals that in contrast to Akt2-deficiency, lack of SGK1 substantially influences maturation and function of DCs. Accordingly, expression of maturation markers and secretion of IL-12 upon LPS stimulation were enhanced and phagocytic capacity was decreased in bone marrow DCs from gene targeted mice lacking functional SGK1 (sgk1$^{-/-}$) as compared to DCs from wild type littermates (sgk1$^{+/+}$). The differences in genotypes were paralleled by reduced phosphorylation of NDRG-1 and enhanced nuclear translocation of NF-κB in sgk1$^{-/-}$ DCs. Silencing of NDRG-1 in sgk1$^{+/+}$ DCs increased nuclear NF-κB abundance. The present observations thus disclose SGK1 as a new major player controlling DC maturation and provide the mechanism of SGK1- and NDRG-1-dependent NF-κB regulation.

SGK1 seems to be important for development and maturation of DCs since the expression of CD86, CD54 and MHC class II molecules is higher in sgk1$^{-/-}$ cells. Immature DCs are characterized by a high rate of endocytosis, which rapidly decreases during maturation (Banchereau, Briere et al. 2000; Granucci, Zanoni et al. 2003). Thus, in accordance to higher expression of maturation markers, sgk1$^{-/-}$ DCs exhibit reduced phagocytic capacity. This is in agreement with a study on human DCs, showing that pharmacological inhibition of PI3K in DCs results in decreased phagocytosis (Agrawal, Agrawal et al. 2007).

In DCs, monocytes and macrophages, PI3K activation suppresses production of inflammatory cytokines, such as IL-12, triggered by TLR signaling (Fukao, Tanabe et al. 2002; Guha and Mackman 2002; Fukao and Koyasu 2003; Goodridge, Harnett et al. 2003; Fang, Pengal et al. 2004; Aksoy, Vanden Berghe et al. 2005; Kuo, Lin et al. 2006; Jackson, Mulcahy et al. 2010). The present study shows that loss of SGK1 results in a higher level of IL-12 secretion by LPS-stimulated DCs, suggesting that SGK1 may be a key player downstream from PI3K and PDK1 preventing excessive IL-12 production.

The nuclear factor-κB (NF-κB)/REL family of transcription factors plays a central role in coordinating the expression of a wide variety of genes that control immune responses (Ardeshna, Pizzey et al. 2000; Li and Verma 2002). PI3K has been shown to negatively
regulate NF-κB in human monocyte-derived DCs (Aksoy, Vanden Berghe et al. 2005), human monocytes (Guha and Mackman 2002; Martin, Schifferle et al. 2003) and mouse macrophages (Fang, Pengal et al. 2004). Thus, PI3K inhibitors increased IκB kinase (IKK)-α/β phosphorylation and IκB-α degradation with a concomitant increase in NF-κB nuclear translocation upon TLR3 or TLR4 engagement in human DCs (Aksoy, Vanden Berghe et al. 2005). Inhibition of the PI3K pathway enhanced LPS-induced TNF-α and TF gene expression via increased activation of transcriptional factors Egr-1, AP-1 and NF-κB in human monocytes (Guha and Mackman 2002). In Porphyromonas gingivalis LPS-stimulated human monocytes, inhibition of PI3K resulted in the augmentation of NF-κB p65 activation (Martin, Schifferle et al. 2003). Our data showing enhanced NF-κB nuclear localization in sgk1−/− DCs provide evidence that SGK1 is involved in the suppressive effect of PI3K on NF-κB in DCs.

A specific substrate of SGK1, NDRG-1 (N-myc downstream regulated gene 1), which is phosphorylated by SGK1 (and not by Akt) at three different sites (Murray, Campbell et al. 2004), has been shown to attenuate NF-κB signalling (Hosoi, Izumi et al. 2009), when phosphorylated at two SGK1-dependent sites (Ser330 and Thr346) (Murakami, Hosoi et al. 2010). Phosphorylation at Ser330 and Thr346 was strongly reduced in sgk1−/− DCs. Moreover, silencing of NDRG-1 in sgk1+/− DCs led to enhanced nuclear localization of the NF-κB. Therefore, SGK1 may prevent transport of NF-κB to the nucleus via phosphorylation of NDRG-1.

In contrast to studies showing that the PI3K pathway negatively regulates expression of inflammatory genes in macrophages and DCs, the PI3K pathway positively regulates NF-κB activity and NF-κB-dependent gene expression in other cell types. Thus, in airway epithelial cells the PI3K-Akt positively regulate NF-κB transcriptional activity (Thomas, Monick et al. 2002). In 3T3 fibroblasts, overexpression of a constitutively active form of Akt results in NF-κB-dependent gene expression via the activation of IKK and the p38 MAPK (Madrid, Mayo et al. 2001). SGK1 has been shown to phosphorylate IKKβ (Zhang, Cui et al. 2005) or IKKα (Tai, Su et al. 2009) and thus to promote the degradation of IκB. Moreover, SGK1 increases the acetylation of NF-κB through phosphorylation of p300, which also leads to NF-κB activation(Tai, Su et al. 2009). SGK1 is a positive regulator of NF-κB in mast cells (Eylenstein, Gehring et al. 2012) and megakaryocytes (Borst, Schmidt et al. 2012). Moreover, SGK1 is required for the stimulation and nuclear translocation of NF-κB following mineralocorticoid excess (Vallon, Wyatt et al. 2006; Terada, Ueda et al. 2012) and thrombin (BelAiba, Djordjevic et al. 2006). Thus, it is possible that the effect of PI3K pathway in general and SGK1 in particular on NF-κB is dependent on the presence of one or more cell type-specific co-factors.

Interestingly, DCs lacking SGK1, as well as DCs treated with PI3K inhibitors carry the same functional defects as DCs obtained from elderly humans (Agrawal, Agrawal et al. 2007). DCs from aging individuals are similarly less efficient in phagocytic uptake and have increased secretion of cytokines than the younger counterparts (Agrawal,
Agrawal et al. 2007). In elderly subjects decreased phosphorylation of Akt was observed in LPS-stimulated DCs. Possibly, SGK1 participates in the modification of DCs during ageing.

SGK1 is under strong genomic stimulation by glucocorticoids (Firestone, Giampaolo et al. 2003) and 1,25-dihydroxyvitamin D₃ (Akutsu, Lin et al. 2001). Given that both hormones have strong inhibitory effect on DC functions (Yamaguchi, Tsumura et al. 1997; Etten and Mathieu 2005; Lyakh, Sanford et al. 2005; Griffin, Dong et al. 2007; Penna, Amuchastegui et al. 2007; Shumilina, Xuan et al. 2010; Heise, Shumilina et al. 2011), it is intriguing to speculate that their mechanism of action in DCs might include SGK1 activation.

In conclusion, genetic knockout of SGK1 leads to profound changes in DC functions, including changes in maturation, phagocytic capacity and cytokine production. Moreover, at least some of these effects seem to be mediated through the reduced phosphorylation of NDRG-1 and enhanced nuclear localization of the transcription factor NF-κB.
References


Lang, F. and E. Shumilina (2012). "Regulation of ion channels by the serum-and glucocorticoid-inducible kinase SGK1." The FASEB Journal.


Curriculum Vitae

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<tr>
<th>Education</th>
<th>Eberhard-Karls University of Tübingen, Germany</th>
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Publications


Declaration

I hereby declare that this thesis was created with contribution of other laboratory team members.

Contributions

This project describes two cell signaling pathways in dendritic cells, play a crucial role in regulation of physiological functions in those cells. The majority of the data presented in this thesis was collected from the experiments that I performed personally. I received help in calcium imaging from Meerim Nurbaeva and Jing Yan; patch clamp from Kalina szteyn and Ahmad Almilaji; western blotting from Evi Schmid and Antonella Russo, Evi Schmid also performed FACS experiments.