Regulation of Ca^{2+} Signaling and Ca^{2+}-Sensitive Functions of Mouse Dendritic Cells by AMP-Activated Protein Kinase and Klotho Protein

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Materials and Methods

Results

Discussions

LIST OF TABLES

Introduction

Materials and Methods

ABBREVIATIONS

ADAM	**A D**isintegrin **a**nd **M**etalloproteinases
AMPK	**AM**P-activated **p**rotein **k**inase
ANOVA	**An**alysis **o**f **va**riance
APC	**A**ntigen-**p**resenting **c**ell
CaMKKII β	**C**almodulin-**d**ependent **p**rotein **k**inase **k**inase II β
$[Ca^{2+}]_i$	**I**ntracellular **Ca^{2+}** concentration **$[Ca^{2+}]_i$**
Ca_v	**V**oltage-gated **Ca^{2+}**
CRAC channel	**C**a^{2+} **r**elease-**a**ctivated **C**a^{2+} channel
CREB	**c**AMP **r**esponse **e**lement-**b**inding protein
CTLs	**C**ytotoxic **T l**ymphocyte
DAG	**D**i**a**cyl**g**lycerol
DC	**D**endritic **c**ell
DBZ	3',4'-**d**ichloro**b**en**z**amyl
EGTA	**E**thylene **g**lycol **t**etraacetic **a**cid
ER	**E**ndoplasmic **r**eticulum
FBS	**F**etal **b**ovine **s**erum
FCS	**F**etal **c**alf **s**erum
FGF23	**F**ibroblast **g**rowth **f**actor 23
FGFR	**FGF r**eceptor
GM-CSF	**G**ranulocyte/**m**acrophage **c**olony-**s**timulating **f**actor

IFN	**I**nter**f**ero**n**
IL	**I**nter**l**eukin
IP_3	**I**nositol-(1,4,5)-tris**p**hosphate
IP_3R	**I**nositol 1,4,5-tris**p**hosphate **r**eceptor
LKB1	**L**iver **k**inase **B1**
LPS	**L**ipo**p**oly**s**accharide
MHC	**M**ajor **h**istocompatibility **c**omplex
NCKX	**K**$^+$-dependent **N**a$^+$/**C**a^{2+} e**x**changer
NCX	**N**a$^+$/**C**a^{2+} e**x**changer
NEAA	**N**on-**e**ssential **a**mino **a**cids
NFAT	**N**uclear **f**actor of **a**ctivated **T** cells
NF-κB	**N**uclear **f**actor **k**appa-light-chain-enhancer of activated **B** cells
NK	**N**atural **k**iller
NMDG	**N**-**M**ethyl-**D**-**g**lucamin
NODs	**Nod**-like receptor**s**
OXPHOS	**Ox**idative **phos**phorylation
PAMP	**P**athogen-**a**ssociated **m**olecular **p**attern
PBS	**P**hosphate-**b**uffered **s**aline
PIP2	**P**hosphatidyl**i**nositol-(4,5)-bis-**p**hosphate
PLCγ2	**p**hospho**l**ipase **C** gamma-2
PMCA	**P**lasma **m**embrane **C**a^{2+}-**A**TPases
PRR	**P**attern **r**ecognition **r**eceptor

ROMK channel	**R**enal **o**uter **m**edullary **K**$^{+}$ channel
RyR	**Ry**anodine **r**eceptor
SDS	**S**odium **d**odecyl **s**ulfate
SEM	**S**tandard **e**rror of the **m**ean
SERCA	**S**arco/**e**ndoplasmic **r**eticulum **C**a^{2+} **A**TPase
SOCC	**S**tore-**o**perated **C**a^{2+} **c**hannel
STIM	**St**romal cell **i**nteraction **m**olecule
TAK1	**T**ransforming growth factor β-**a**ctivated **k**inase
TBS	**T**riethanolamine-**b**uffered **s**aline
T$_{CM}$ cells	**C**entral **m**emory **T** cells
TEA	**T**etra**e**thyl**a**mmonium
T$_{Eff}$ cells	**Eff**ector **T** cells
T$_{EM}$ cells	**M**emory **T** cells
Thr-172	**Thr**eonine 172
T$_{H}$ cells	**T h**elper cells
TNF	**T**umour-**n**ecrosis **f**actor
TLR	**T**oll-**l**ike **r**eceptor
TRP	**T**ransient **r**eceptor **p**otential
TRPC	**T**ransient **r**eceptor **p**otential **c**ation
TRPV	**T**ransient **r**eceptor **p**otential **v**anilloid
T reg cells	**Reg**ulatory **T** cells

SUMMARY

Dendritic **c**ell**s** (DCs) are antigen-presenting cells participating in the regulation of innate and adaptive immunity. The functions of DCs are governed by Ca^{2+} signaling. Ca^{2+}-sensitive functions include DC activation, maturation and migration, as well as formation of the immunological synapse. Upon ligation of their chemokine receptors, such as CXCR4, DCs respond with a fast **i**ncrease of **i**ntracellular **Ca^{2+}** concentrations ($[Ca^{2+}]_i$), which involves release of Ca^{2+} from the stores followed by Ca^{2+} entry through the **s**tore-**o**perated **C**a^{2+} (SOC) channels. While the activity of SOC channels is crucial for DC migration, the SOC-induced increase of $[Ca^{2+}]_i$ is tightly controlled and rapidly terminated, since Ca^{2+} overload impairs DC migration. In DCs, increase of $[Ca^{2+}]_i$ is efficiently blunted and terminated by Ca^{2+} extrusion, which is accomplished by K^+-independent (NCX) and **K^+**-dependent (NCKX) **N**a^+/**C**a^{2+} e**x**changers. Very little is known about how Ca^{2+} signaling and Ca^{2+}-dependent functions are regulated. The present study pursued two aims. The first aim was to investigate the potential role of AMPK in the regulation of cytosolic Ca^{2+} concentration and Ca^{2+}-dependent functions of mouse DCs. The second aim was to study the effects of Klotho in the regulation of the components of Ca^{2+} signaling and Ca^{2+}-dependent functions in DCs.

One of the signaling molecules activated by Ca^{2+} is the energy-sensing **AM**P-activated **p**rotein **k**inase (AMPK), which is known to suppress proinflammatory responses of DCs and macrophages. To explore whether AMPK participates in the regulation of Ca^{2+} entry, Ca^{2+} extrusion and, thereby Ca^{2+}-dependent functions in DCs, bone marrow-derived DCs were obtained from AMPKα1-deficient mice (*ampk*$^{-/-}$) and from respective wild type littermates (*ampk*$^{+/+}$). As measured in transwell chambers, the efficiency of *ampk*$^{-/-}$ DCs to migrate in response to the CXCL12 (75 ng/ml) chemokine was strongly enhanced. Similarly, CXCL12 (300 ng/ml)-induced increase of $[Ca^{2+}]_i$ was much higher in *ampk*$^{-/-}$ DCs than in *ampk*$^{+/+}$ DCs, as measured by Fura-2 fluorescence. Accordingly, when SOC channels were activated by inhibition of the endosomal Ca^{2+} ATPase with thapsigargin, SOC entry was significantly increased in *ampk*$^{-/-}$ DCs. Orai1 protein abundance was enhanced in *ampk*$^{-/-}$ DCs. Moreover, upon removal of external Na^+, activities of both NCX and NCKX were significantly enhanced in *ampk*$^{-/-}$ DCs as compared to *ampk*$^{+/+}$ DCs. Similarly, in patch clamp experiments, the NCX and NCKX currents were both significantly increased in *ampk*$^{-/-}$ DCs. In conclusion, AMPK strongly suppresses DC migration, presumably through inhibition of both, SOC entry and Na^+/Ca^{2+} exchangers in DCs.

SUMMARY

NCKX exchangers are stimulated by immunosuppressive $1,25(OH)_2D_3$. Formation of $1,25(OH)_2D_3$ is inhibited by the anti-aging protein Klotho. Thus, $1,25(OH)_2D_3$ plasma levels are excessive in Klotho-deficient mice (*klotho*^{hm}). The present study explored whether Klotho-deficiency modifies $[Ca^{2+}]_i$ regulation in DCs. DCs were isolated from bone marrow of *klotho*^{hm} mice and corresponding wild type mice (*klotho*$^{+/+}$) and cultured for 7 to 9 days with GM-CSF. According to MHC II and CD86 expression, differentiation and **l**ipo**p**oly**s**accharide (LPS)-induced maturation was similar in *klotho*^{hm} and *klotho*$^{+/+}$ DCs. However, NCKX activity was significantly enhanced in *klotho*^{hm} DCs. The $[Ca^{2+}]_i$ increase upon acute application of LPS (1 µg/ml) was significantly lower in *klotho*^{hm} DCs than in *klotho*$^{+/+}$ DCs, a difference reversed by NCKX-blocker 3',4'-**d**ichloro**b**en**z**amyl (DBZ, 10 µM). CCL21-dependent migration was significantly reduced in *klotho*^{hm} DCs compared to *klotho*$^{+/+}$ DCs. NCKX activity could be enhanced by pretreating *klotho*$^{+/+}$ DC precursors with $1,25(OH)_2D_3$ for the first 2 days after isolation from bone marrow. Feeding *klotho*^{hm} mice a vitamin D deficient diet decreased NCKX activity, augmented LPS-induced increase of $[Ca^{2+}]_i$, and enhanced migration of *klotho*^{hm} DCs, thus dissipating the differences between *klotho*^{hm} DCs and *klotho*$^{+/+}$ DCs. Impaired migration of *klotho*^{hm} DCs could be also rescued by DBZ (10 µM). In conclusion, Klotho deficiency up-regulates Na^+/Ca^{2+} exchange activity and thus blunts the increase of $[Ca^{2+}]_i$ following LPS exposure and CCL21-mediated migration. These effects are in large part due to excessive $1,25(OH)_2D_3$ formation.

ZUSAMMENFASSUNG

Dendritische **Z**ellen (DCs) sind Antigen-präsentierende Zellen, die an der Regulation der angeborenen und adaptiven Immunantwort teilnehmen. Die Funktionalität von DCs wird vom "Ca^{2+}-Signalling" bestimmt. Ca^{2+}- sensitive Funktionen umfassen die Aktivierung, Reifung und Migration von DCs sowie die Bildung der immunologischen Synapse. Auf die Bindung eines Liganden an die membranständigen Chemokin-Rezeptoren, beispielsweise CXCR4, antworten DCs mit einem schnellen Anstieg der zytosolischen Ca^{2+}-Konzentration ($[Ca^{2+}]_i$), der durch den Ausstrom von Ca^{2+} aus den Speichern und den Einstrom durch die "**s**tore-**o**perated **C**a^{2+} (SOC)"-Kanäle bewirkt wird. Die Aktivität der SOC-Kanäle ist wichtig für die Migration der DCs. Gleichzeitig jedoch muss der SOC-induzierte Anstieg von $[Ca^{2+}]_i$ eng kontrolliert und zügig beendet werden, da ein Ca^{2+}-Überfluss die Migration beeinträchtigt. In DCs wird die Erhöhung von $[Ca^{2+}]_i$ durch die Ausschleusung von Ca^{2+} abgeschwächt und schließlich gestoppt. Diese erfolgt über K^+-unabhängige (NCX) und **K**$^+$-abhängige (NCKX) **N**a^+/**C**a^{2+}-Austauscher. Sehr wenig ist darüber bekannt, wie das Ca^{2+}-Signal und Ca^{2+}-abhängige Funktionen reguliert werden. Die vorliegende Studie verfolgt zwei Ziele. Das erste Ziel war, die mögliche Rolle von AMPK bei der Regulation cytosolischer Ca^{2+}-Konzentrationen und Ca^{2+}-abhängiger Funktionen in murinen DCs aufzuzeigen. Das zweite Ziel war, mögliche Effekte von Klotho auf die Regulation von Ca^{2+}-Signalkomponenten und Ca^{2+}-abhängigen Funktionen in DCs zu untersuchen.

Eines der durch Ca^{2+} aktivierten Signalmoleküle ist die Energie-sensitive **AMP**-aktivierte Protein**k**inase (AMPK), die für ihre Unterdrückung proinflammatorischer Antworten von DCs und Makrophagen bekannt ist. Um herauszufinden, ob AMPK an der Regulation des Ca^{2+}-Eintritts oder Ca^{2+}-Ausstoßes und somit Ca^{2+}-abhängiger Funktionen in DCs teilnimmt, wurden DCs aus dem Knochenmark von AMPKα1-defizienten Mäusen (*ampk*$^{-/-}$) und ihren Wildtyp-Wurfgeschwistern (*ampk*$^{+/+}$) angezogen. In *ampk*$^{-/-}$ DCs war die durch das Chemokin CXCL12 (75 ng/ml) vermittelte Migrationseffizienz (gemessen in „Transwell"-Kammern) deutlich gesteigert. Gleichermaßen war der durch CXCL12 (300 ng/ml) ausgelöste $[Ca^{2+}]_i$ –Anstieg (gemessen mittels Fura-2 Fluoreszenz) viel stärker ausgeprägt in *ampk*$^{-/-}$ DCs als in *ampk*$^{+/+}$ DCs. Darüber hinaus war der SOC-Einstrom in *ampk*$^{-/-}$ DCs signifikant erhöht, wenn die SOC-Kanäle durch die Inhibierung der endosomalen Ca^{2+}-ATPase mit Thapsigargin aktiviert

wurden. Die Orai1-Proteinmenge war größer in *ampk*$^{-/-}$ DCs. Darüberhinaus war die Aktivität von NCX und NCKX nach Entfernung von externem Na^+ signifikant erhöht in *ampk*$^{-/-}$ DCs verglichen mit *ampk*$^{+/+}$ DCs. Gleichermaßen waren die Ströme in "patch-clamp" Experimenten von NCX und NCKX signifikant erhöht in *ampk*$^{-/-}$ DCs. Folglich unterdrückt AMPK die Migration von DCs, vermutlich durch eine Hemmung des SOC-Einstroms und der Na^+/Ca^{2+}-Austauscher in DCs.

NCKX Austauscher werden von dem immunsuppressiven $1,25(OH)_2D_3$ stimuliert. Die Bildung von $1,25(OH)_2D_3$ wird gehemmt durch das Anti-Alterungsprotein Klotho. Daher sind $1,25(OH)_2D_3$ Plasmaspiegel in Klotho-defizienten Mäusen (*klotho*^{hm}) stark überhöht. Diese Studie hat untersucht, ob Klotho-Mangel die $[Ca^{2+}]_i$-Regulation in DCs beeinflußt. DCs wurden aus dem Knochenmark von *klotho*^{hm}- und entsprechenden Wildtyp-Mäusen (*klotho*$^{+/+}$) isoliert und für 7-9 Tage mit GM-CSF kultiviert. In Bezug auf MHC II- und CD86-Expression war die Differenzierung und die **L**ipo**p**oly**s**accharid (LPS)-induzierte Reifung ähnlich in *klotho*^{hm} und *klotho*$^{+/+}$ DCs. Allerdings war die NCKX-Aktivität in *klotho*^{hm} DCs signifikant erhöht. Der $[Ca^{2+}]_i$ -Anstieg nach einer akuten Applikation von LPS (1 µg/ml) war signifikant schwächer ausgeprägt in *klotho*^{hm} DCs als in *klotho*$^{+/+}$ DCs, ein Unterschied, der durch den NCKX-Blocker 3',4'-**d**ichloro**b**en**z**amyl (DBZ, 10 µM) aufgehoben wurde. Die CCL21-abhängige Migration war signifikant geringer in *klotho*^{hm} DCs als in *klotho*$^{+/+}$ DCs. Die NCKX-Aktivität konnte verstärkt werden durch Vorbehandlung der *klotho*$^{+/+}$ DC-Vorläufer mit $1,25(OH)_2D_3$ für die ersten zwei Tage nach der Isolation aus dem Knochenmark. Das Füttern der *klotho*^{hm}-Mäuse mit einer Vitamin D-armen Diät erniedrigte die NCKX-Aktivität, vergrößerte den LPS-induzierten Anstieg von $[Ca^{2+}]_i$ und förderte die Migration von *klotho*^{hm} DCs und hob somit die Unterschiede zwischen *klotho*^{hm} und *klotho*$^{+/+}$ DCs auf. Die beeinträchtigte Migration von *klotho*^{hm} DCs konnte auch durch DBZ (10 µM) wiederhergestellt. Schlussfolgernd ist festzustellen, dass der Klotho-Mangel die Na^+/Ca^{2+}-Austauschaktivität heraufreguliert und so den $[Ca^{2+}]_i$ -Anstieg nach LPS-Stimulation und die CCL21-abhängige Migration abschwächt. Diese Effekte liegen zum größten Teil begründet in der übermäßigen Bildung von $1,25(OH)_2D_3$.

1. INTRODUCTION

1.1. Dendritic Cells

The immune system defends the body against infectious organisms (bacterial, parasitic, fungal, viral infections) and against the growth of tumor cells. The cells of the immune system are derived from pluripotent hematopoietic stem cells in the bone marrow (**Fig. 1**).

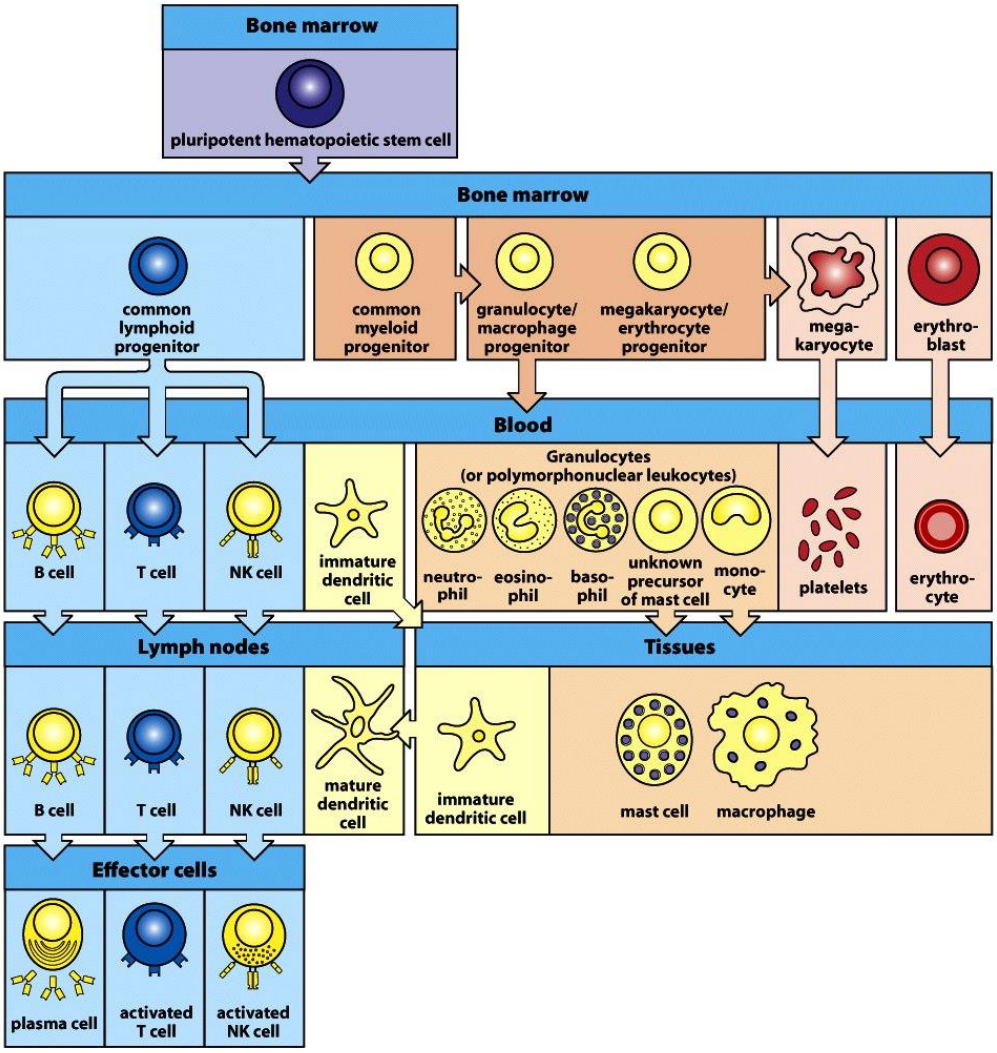

Figure 1. Immune cells arise from pluripotent hematopoietic stem cells in the bone marrow. Taken from (Janeay 2008).

The immune system is divided into antigen-nonspecific innate and antigen-specific adaptive immunity (Fearon and Locksley 1996;Hoffmann et al. 1999;Medzhitov and Janeway 1997). Phagocytic cells (**d**endritic **c**ells (DCs), macrophages and neutrophils), **n**atural **k**iller (NK) cells,

as well as the complement system, and **i**nter**f**ero**n**s (IFNs) belong to the non-specific immune response. Cells of the innate system recognize structures that pathogens express as **p**athogen-**a**ssociated **m**olecular **p**atterns (PAMPs), such as bacterial carbohydrates (**l**ipo**p**oly**s**accharide (LPS), mannose), nucleic acids (bacterial or viral DNA or RNA), bacterial peptides (flagellin, ax21), peptidoglycans and lipoteichoic acids (from Gram-positive bacteria), *N*-formylmethionine, lipoproteins and fungal glucans. PAMPs are detected by **p**attern **r**ecognition **r**eceptors (PRRs). Immune cells are equipped with a variety of PRRs such as **Nod**-like receptors receptor**s** (NODs) (Diebold 2009;Fukata et al. 2009), C-type lectin receptors (Diebold 2009), and **T**oll-**l**ike **r**eceptors (TLRs) (Takeda et al. 2003).

Via the interaction between PAMPs and PRRs, cells of the innate immune system identify pathogens and/or tissue injury and inform the presence of danger to the cells of the adaptive immune system (Matzinger 1994). The antigen- specific adaptive immune system consists of B and T lymphocytes. B and T lymphocytes endow the organism with the ability to recognize and to remember shady pathogens and to mount more rapid and stronger immune responses upon re-exposure to these pathogens in the future.

The linkage between innate and adaptive immunity is accomplished by **a**ntigen-**p**resenting **c**ells (APCs). The major APCs appear to be DCs, which interpret the innate response to the adaptive system (Banchereau et al. 2000;den Haan and Bevan 2000;Steinman and Nussenzweig 2002). The role of DCs as APCs was first discovered by Steinman and Z. Cohn in 1973 (Steinman and Cohn 1973). Ralph M. Steinman was awarded a Nobel Prize in 2011 for his discovery and for establishing the role of DCs in adaptive immunity. DCs emanate from both myeloid and plasmacytoid progenitor cells in the bone marrow (Banchereau et al. 2000). During their lifespan, DCs can undergo a transformation from an immature state to a mature phenotype (**Fig. 2)**. Complete maturation of DCs is thought to require at least 10 to 20 hours (Dauer et al. 2003).

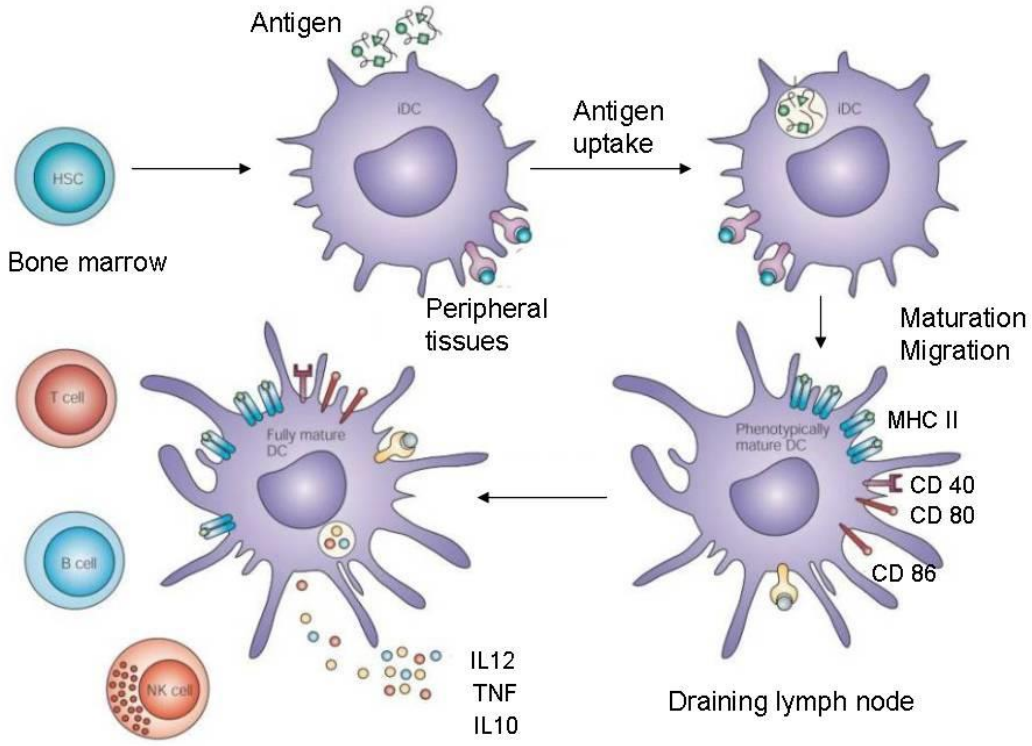

Figure 2. Life span of dendritic cells (DCs). HSCs, hematopoietic stem cells; iDCs, immature dendritic cells; MHC II, major histocompatibility complex II; NK, natural killer; IL12, interleukin-12; TNF, tumour necrosis factor. Taken from (Hackstein and Thomson 2004).

Immature DCs with high phagocytic capacity are found in different tissues such as epidermis, intestine or blood vessel walls. Immature DCs migrate to the site of pathogen entry, recognize pathogenic antigens via PRRs (e.g., TLRs) and phagocytose them (**Fig. 3)**

The expression of TLRs is different between myeloid and plasmacytoid DCs. Myeloid DCs express high levels of TLR1, 2, 4, 5 and 8, and low levels of TLR6, while TLR3, 7, 9 and 10 are not detectable in these cells. In contrast, plasmacytoid DCs express high levels of TLR7 and 9, as well as low levels of TLR1, 6 and 10. TLR1, 2, 4, 5 and 8 are absent in this DC subset (Takeuchi et al. 1999). After antigen capture, immature DCs turn into a mature phenotype, a process influenced by different factors like cytokines and physical environment. The maturation process is associated with morphologic and functional changes. DCs lose phagocytic receptors, adhesive structures, up-regulate co-stimulatory molecules (CD40, CD58, CD86) and the antigen-presenting **m**ajor **h**istocompatibility **c**omplex II (MHC class II) molecule. They futher reorganize their cytoskeleton, acquire high motility and produce pro-inflammatory cytokines, such as **i**nter**l**eukin-12 (IL-12) **t**umour **n**ecrosis **f**actor (TNF) and INFγ (Winzler et al. 1997).

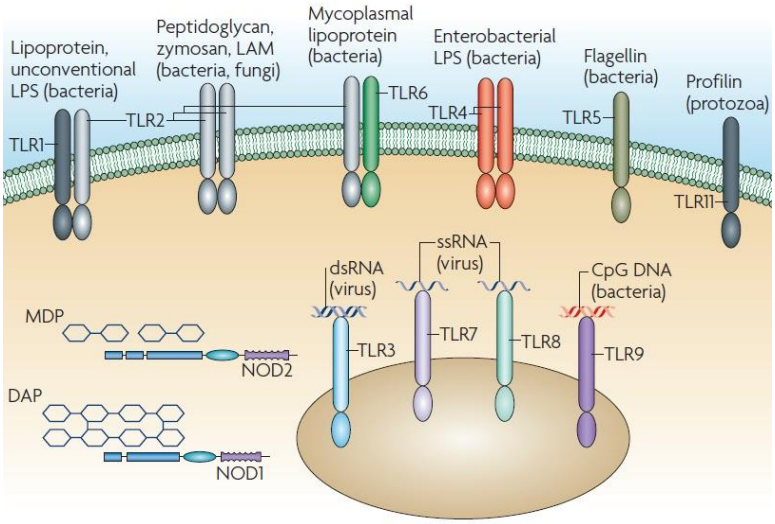

Figure 3. Pattern-recognition receptors (PRRs). TLRs, Toll-like receptors; NLR, Nod-like receptor; DAP, diaminopimelic acid; MDP, muramyl dipeptide; LPS, lipopolysaccharide; LAM, lipoarabinomannan.Taken from (Kaufmann 2007).

Mature DCs migrate to the lymphocyte area of lymphoid organs where DCs translate the pathogenicity of the invader on antigen presenting molecules, MHC class I and class II, on their surface. MHC class II molecules present antigen to $CD4^+$ **T h**elper lymphocytes (T_H) and MHC class I molecules to the antigen-specific $CD8^+$ cells**.**

Upon activation antigen-specific naïve $CD4^+$ T cells proliferate and acquire effector functions. **Eff**ector $CD4^+$ **T** (T_{Eff}) cells differentiate into four main populations: **reg**ulatory **T** cells (T reg cells), T_H1, T_H2 and T_H17 cells. Additionally, two main T cell subsets develop from T_{Eff} cells: **c**entral **m**emory **T** cells (T_{CM}) in lymph nodes and **e**ffector **m**emory **T** cells (T_{EM}) in tissues. T_{CM} cells replicate efficiently and, on the second antigen encounter, develop into terminally differentiated T_{Eff} cells. T_{EM} cells replicate less efficiently than T_{CM} cells (**Fig. 4)** (Kaufmann 2007). Effector $CD8^+$ T cells are **c**ytotoxic cells (**c**ytotoxic **T l**ymphocytes, CTLs) that can recognize and kill virus-infected cells. CTLs induce cell death by release of specialized cytotoxic granules which trigger the inherent apoptotic response.

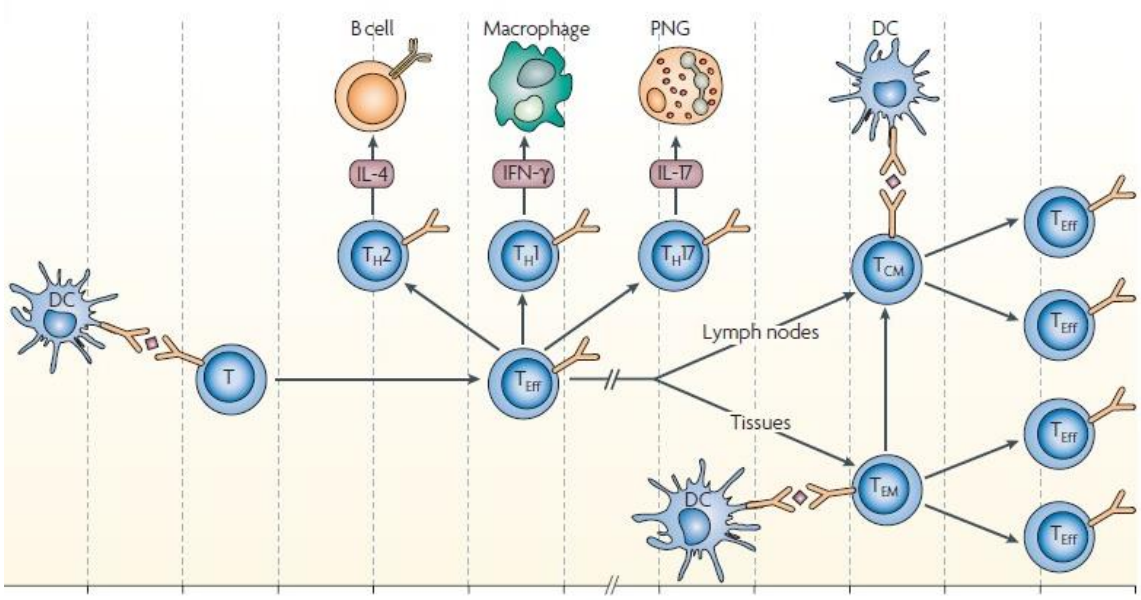

Figure 4. Proliferation of CD4$^+$ T cells. DC, dendritic cells; T, T cells; T_{Eff} , effector T cells; T_H , T helper; T_{CM} ,central memory T cells; T_{EM}, effector memory T cells; PNG, polymorphonuclear granulocyte. Taken from (Kaufmann 2007).

T lymphocytes migrate to the site of infection and induce an immune response. T reg cells produce IL-10 and TGF-β and control and counteract excessive immune responses. T_H1 cells acquire the capacity to produce powerful cytokines such as IFN-γ, IL-2 and TNF to activate macrophages, NK cells, and eosinophils to resist infection by facultative and obligate intracellular microbes (Maldonado-Lopez et al. 1999;Napolitani et al. 2005;Pulendran et al. 1999). T_H2 cells are of central importance for humoral immunity (antibody production) and defense against helminths, with IL-4, IL-5 and IL-13 as main cytokines (Mosmann and Coffman 1989). T_H17 cells producing IL-17 are involved in defense against extracellular bacteria by activating neutrophils and intracellular bacteria by directing T_H1 cells to the site of bacterial replication (Happel et al. 2005).

B lymphocytes become activated by interaction with T_H2 cells and DCs. Activated B lymphocytes migrate into various areas, where they mature into plasma cells, which produce antibodies that neutralize the initial pathogen (Banchereau et al. 2000;Dubsky et al. 2005;Szatmari and Nagy 2008).

After fulfiling their task, DCs undergo apoptosis. Apoptosis in DCs can be regulated by different pathways (Kushwah and Hu 2010). LPS-induced DC maturation initiates DC apoptosis through CD14-mediated NFAT (**n**uclear **f**actor of **a**ctivated **T** cells) activation (Zanoni et al. 2009).

In DCs, many critical processes and functions appear to involve Ca^{2+} signaling. Engulfment of pathogens and apoptotic bodies and antigen processing are accompanied by a rise in **i**ntracellular **Ca^{2+} c**oncentration $[Ca^{2+}]_i$ (Rubartelli and Poggi 1997). DC maturation, including the enhanced expression of MHC class II and costimulatory molecules, is inhibited by chelation of external Ca^{2+} (Koski et al. 1999). Conversely, agents that mobilize intracellular Ca^{2+} can promote DC maturation in the absence of normal cytokine stimulation (Czerniecki et al. 1997). Chemotactic molecules and T cell-derived signals uniformly produce Ca^{2+} increases in DCs, suggesting that Ca^{2+} transients regulate DC migration and their capacity to initiate adaptive immune responses (Chan et al. 1999;Delgado et al. 1998;Dieu et al. 1998;Montes et al. 1999;Yanagihara et al. 1998).

1.2. Mechanisms of Ca^{2+} Signaling in DCs

The calcium ion (Ca^{2+}) plays an important role in various critical functions in the human body. It is the main costituent of the bone: 99% of all Ca^{2+} in the body is stored in bones, and the unbound fraction of the remaining 1% functions as a second messenger regulating different processes, such as fertilization, vision, muscle contraction, nerve conduction, blood clotting, exocytosis, cell division and the activity of many enzymes and hormones (Berridge 1993;Carafoli 2002). $[Ca^{2+}]_i$ in mammalian cells is maintained at a level of 50-100 nM, which is lower than the Ca^{2+} concentration in the serum (Bronner 2001;Schwaller 2009).

In DCs, Ca^{2+} has been shown to regulate maturation, migration, cytokine production and other functions (Czerniecki et al. 1997;Koski et al. 1999;Rubartelli and Poggi 1997). Increase of $[Ca^{2+}]_i$ occurs as an immediate responce to ligation of various receptors, such as TLRs and chemokine receptors (Aki et al. 2008;Barbet et al. 2008;Koski et al. 1999;Matzner et al. 2008;Xuan et al. 2009). Several Ca^{2+} entry pathways have been identified in DCs, such as **C**a^{2+} **r**elease-**a**ctivated **C**a^{2+} (CRAC) channels (Hsu et al. 2001;Matzner et al. 2008), **t**ransient **r**eceptor **p**otential (TRP) channels (Barbet et al. 2008;Basu and Srivastava 2005), **v**oltage-gated **C**a^{2+} (Ca_v channels or **di**h**y**dro**p**yridine (DHP)-sensitive Ca^{2+} channels) (O'Connell et al. 2002;Vukcevic et al. 2008) and purinergic receptors (P2X) (Di Virgilio F. 2005;Ferrari et al. 2000;Mutini et al. 1999;Schnurr et al. 2003) **(Fig. 5).**

Transient increases in $[Ca^{2+}]_i$ can also be mediated by the release of Ca^{2+} from **e**ndoplasmic **r**eticulum (ER) stores via **i**nositol 1,4,5-tris**p**hosphate receptors (IP_3Rs) or/and **ry**anodine **r**eceptors (RyRs). DCs are equipped with all three isoforms of IP3Rs (IP3R1, IP3R2 and IP3R3) (Stolk et al. 2006), and with at least one out of three isoforms of RyR (RyR1) (Heise et al. 2011;O'Connell et al. 2002). Moreover, the TRPM2 channel of the melastatin family has been found to be expressed in endolysosomal compartments in DCs, where it functions as a Ca^{2+} release pathway (Sumoza-Toledo et al. 2011). Deficiency of TRPM2 in DCs reduces chemokine-induced release of Ca^{2+} and secondarily reduces Ca^{2+} entry through the store-operated pathways (Massullo et al. 2006;Sumoza-Toledo et al. 2011).

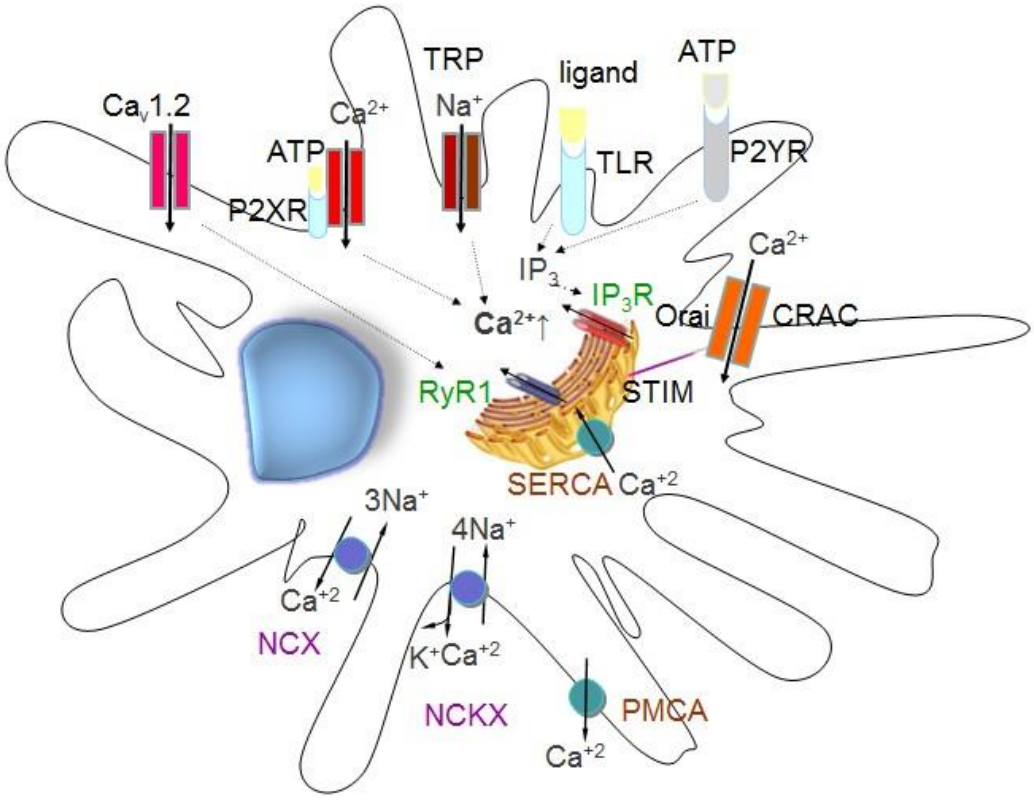

Figure 5. Ca^{2+} signaling in DCs. CRAC, Ca^{2+} release-activated Ca^{2+} channels; TRP, transient receptor potential; Ca_v, voltage-gated Ca^{2+}; P2X, purinergic receptors; SERCAs, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases; NCKX, K^+-dependent Na^+/Ca^{2+} exchanger; NCX, K^+-independent Na^+/Ca^{2+} exchanger; PMCAs, plasma membrane Ca^{2+} ATPases; IP_3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor.

1.3. Ca^{2+} Release-Activated Ca^{2+} (CRAC) Channel

PGN, a TLR2 ligand, and LPS, a TLR4 ligand, have been shown to induce tyrosine phosphorylation of **p**hospho**l**ipase **C** gamma-2 (PLCγ2) **(Fig. 7)** (Aki et al. 2008). PLCγ2

hydrolyzes the phospholipid **p**hosphatidyl**i**nositol 4,5 bis**p**hosphate (PtdIns(4,5)P2 or PIP2), a component of cell membranes, to release soluble **i**nositol 1,4,5-tris**p**hosphate (IP_3) and **d**i**a**cyl**g**lycerol (DAG). Within seconds, IP_3 binds to its receptor IP_3R located on the surface of internal Ca^{2+} stores, ER, to increase $[Ca^{2+}]_i$. Depletion of Ca^{2+} stores activates plasma membrane-localized Ca^{2+} channels known as **s**tore-**o**perated **C**a^{2+} **c**hannels (SOCCs) (Parekh 2003). The best-characterized SOCCs are CRAC channels (Parekh and Putney, Jr. 2005), which were first described in human T cells (Lewis and Cahalan 1989) and mast cells (Hoth and Penner 1992). CRAC channels are highly Ca^{2+}-selective (Prakriya and Lewis 2003). These channels are composed of two functional subunits, the first one forming the pore of the channel called Orai or CRACM (Prakriya et al. 2006;Putney 2007;Vig et al. 2006), which opens upon binding to the second subunit, a Ca^{2+} sensor located in the ER called **st**romal cell **i**nteraction **m**olecule (STIM) (Fahmer et al. 2009;Feske et al. 2006;Gwack et al. 2007;Huang et al. 2006;Peinelt et al. 2006;Penna et al. 2008;Smyth et al. 2010;Vig et al. 2006;Yeromin et al. 2006;Zhang et al. 2005) **(Fig. 6).**

STIM was identified in two repeated RNAi screens, performed in *Drosophila* and HeLa cells, respectively (Feske et al. 2006;Lewis 2007;Oritani and Kincade 1996). Mammals express two STIM proteins, STIM1 and STIM2, with 47% amino acid identity (Williams et al. 2001). STIM is a trans-membrane protein with a paired N-terminal EF-hand inside the ER and cytoplasmic C-terminal protein-interaction domains **(Fig. 6)**. The first EF-hand of each pair binds Ca^{2+} with low affinity, appropriate to the high concentration of Ca^{2+} in the ER (100-700 μM) (Hogan and Rao 2007;Liou et al. 2005;Roos et al. 2005). At resting state, STIM1 exhibits a tubular distribution within the ER membrane (Grigoriev et al. 2008;Honnappa et al. 2009).

In the *Drosophila* RNA interference screen, Orai1 (32.7 kDa) has been identified as a CRAC channel-forming protein (Feske et al. 2006;Honnappa et al. 2009). It has been shown that Orai1 is required for lymphocyte activation, as evidenced by severe immunodeficient phenotypes in patients lacking Orai1 (Feske 2011). Three mammalian homologous CRAC channel pore-building subunits have been identified: Orai1, Orai2 and Orai 3 (Gwack et al. 2007). Each of them contains a cytosolic N terminus, four **t**rans**m**embrane (TM) segments connected by two extracellular and one intracellular loop, and a cytosolic C terminus. All three Orai proteins form highly Ca^{2+}-selective channels within the plasma membrane (Feske et al. 2006;Lewis 2007;Prakriya et al. 2006;Schindl et al. 2008;Vig et al. 2006;Yeromin et al. 2006;Zhang et al. 2005).

Upon store depletion, distributed STIM proteins oligomerize within the ER membrane and then translocate to the cell periphery close to the plasma membrane where they form punctate clusters and activate Orai/CRAC channels (Liou et al. 2007;Luik et al. 2008;Roos et al. 2005;Wu et al. 2006).

The CRAC channel has been shown to be the major Ca^{2+} entry pathway in DCs (Hoth and Penner 1992;Putney and Bird 1993) with Orai2 and STIM2 as the main isoforms (Bandyopadhyay et al. 2011;Hsu et al. 2001;Matzner et al. 2008).

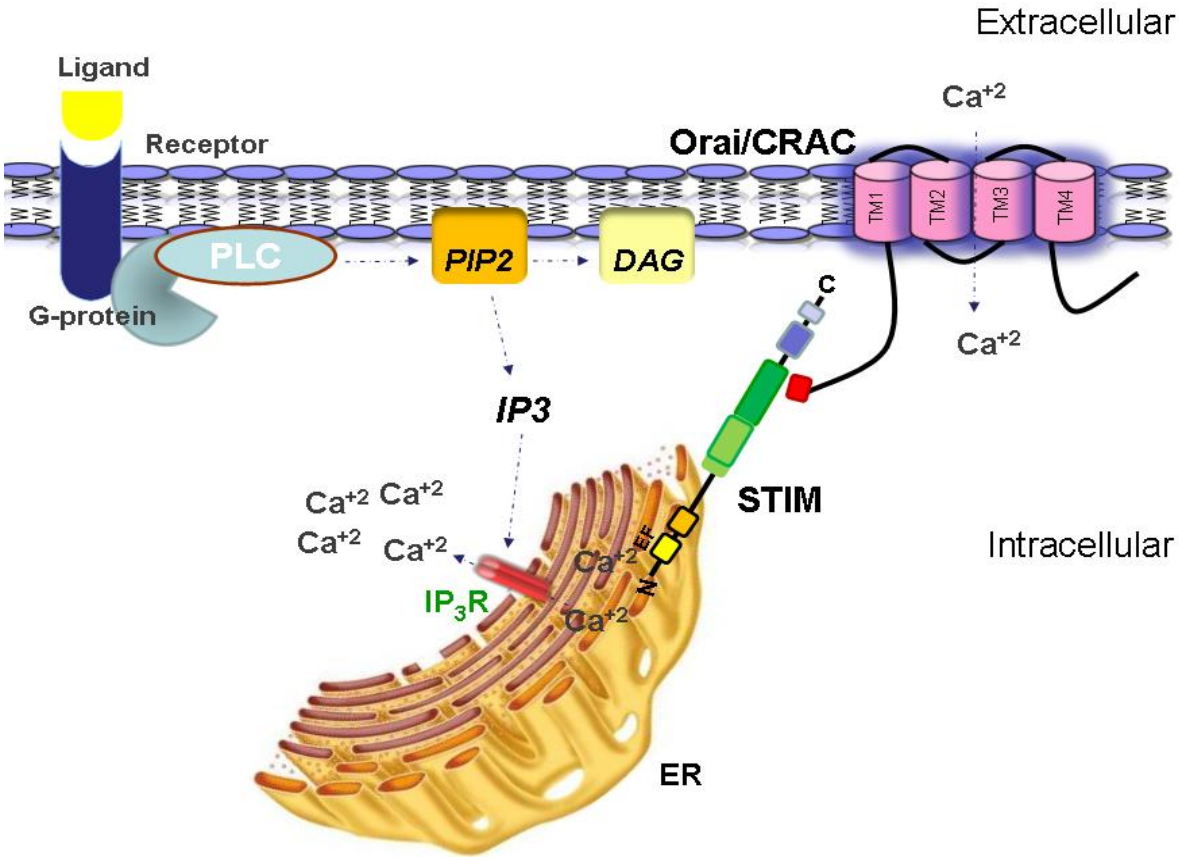

Figure 6. Activation of store-operated Ca^{2+} channels (SOCCs). PLC, phospholipase C; PIP2, phosphatidylinositol 4,5 bisphosphate; IP_3, inositol 1,4,5 trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; STIM, stromal cell interaction molecule; CRAC, Ca^{2+} release-activated Ca^{2+} channels; TM, transmembrane.

1.4. Mechanisms of Ca^{2+} Clearance

The regulation of $[Ca^{2+}]_i$ is important for all cells in order to avoid Ca^{2+} toxicity. Hundreds of cellular proteins have been adapted to bind Ca^{2+}, in some cases simply to buffer or to

lower Ca^{2+} levels, and in other cases to trigger cellular processes. ATPase pumps transport Ca^{2+} into the ER (**s**arcoplasmic/**e**ndoplasmic **r**eticulum **C**a^{2+} **A**TPase**s,** SERCAs) or out of the cell (**p**lasma **m**embrane **C**a^{2+} **A**TPases, PMCA) (**Fig. 5**). SERCA maintains low $[Ca^{2+}]_i$ by exchanging protons for two Ca^{2+} per ATP hydrolyzed, and PMCA transports one Ca^{2+} out of the cell per ATP hydrolyzed. SERCA and PMCA are P-type ATPases, defined by an obligatory aspartyl phosphate intermediate in the pump cycle (Strehler and Treiman 2004). In mouse DCs SERCA1-3 isoforms are known to be expressed, with SERCA2 and SERCA3 being highly expressed (Heise et al. 2011).

Besides SERCA and PMCA, there are other Ca^{2+} clearance mechanisms in cells, such as **K**$^+$-dependent and K^+-independent **N**a^+/**C**a^{2+} e**x**changers (NCKX and NCX). Three NCX and five NCKX isoforms have been identified by molecular cloning in mammalian cells (Herchuelz et al. 2007;Visser et al. 2007;Visser and Lytton 2007). NCX isoforms remove a single Ca^{2+} in exchange for three Na^+ (Yu and Choi 1997), while NCKX isoforms co-transport one K^+ and one Ca^{2+} in exchange for four Na^+ (Lytton 2007). Depending on the prevailing electrochemical driving forces, i.e. the Na^+ and Ca^{2+} concentrations and the membrane potential, Na^+/Ca^{2+} exchangers accomplish Ca^{2+} entry or Ca^{2+} exit (Blaustein and Lederer 1999). The turnover rate of Na^+/Ca^{2+} exchangers is orders of magnitude higher than that of ATP-driven Ca^{2+} pumps (Herchuelz et al. 2007). NCX and NCKX isoforms are expressed in human lung macrophages and blood monocytes (Staiano et al. 2009) as well as murine DCs (**Fig. 5**) (Heise et al. 2011;Shumilina et al. 2010). NCX1-3 and NCKX3-5 isoforms have been demonstrated to be expressed on mouse DCs (Shumilina et al. 2010). Moreover, they have turned out to be the targets of immunosuppressive hormones. A powerful immunosuppressive hormone, 1,25-vitamin D_3 ($1,25(OH)_2D_3$, also known as a calcitriol), up-regulates NCKX1 membrane abundance in mouse DCs (Shumilina et al. 2010). Another powerful immunosuppressor, dexamethasone (which is a synthetic glucocorticoid), up-regulates NCX3 by increasing its transcription and subsequently the membrane abundance of this carrier. The enhanced NCX/NCKX activity in dexamethasone- or $1,25(OH)_2D_3$-treated cells, therefore, accelerates the extrusion of Ca^{2+} and blunts the increase of $[Ca^{2+}]_i$ following stimulation of DCs with LPS. Moreover, the up-regulation of NCX or NCKX by dexamethasone or $1,25(OH)_2D_3$, respectively, contributes to the immunosuppressive effects of those hormones. Thus, CD86 expression which is diminished by dexamethasone or $1,25(OH)_2D_3$ can be restored by pharmacological inhibition of NCX or NCKX, respectively (Heise et al. 2011;Shumilina et al. 2010)**.**

Ca^{2+} transporters are of peculiar importance for DC functions. But very little is known about how this Ca^{2+} transport is regulated. One kinase well known for its role in the regulation of channels and transporters is AMPK.

1.5. AMP-Activated Protein Kinase (AMPK) is Important for Body Energy Homeostasis

The **AMP**-activated **p**rotein **k**inase (AMPK), a ubiquitously expressed serine/threonine kinase, is an intracellular energy regulator. AMPK is expressed in a number of tissues, including liver, brain, skeletal muscle, adipose tissue and pancreas (**Fig. 7**).

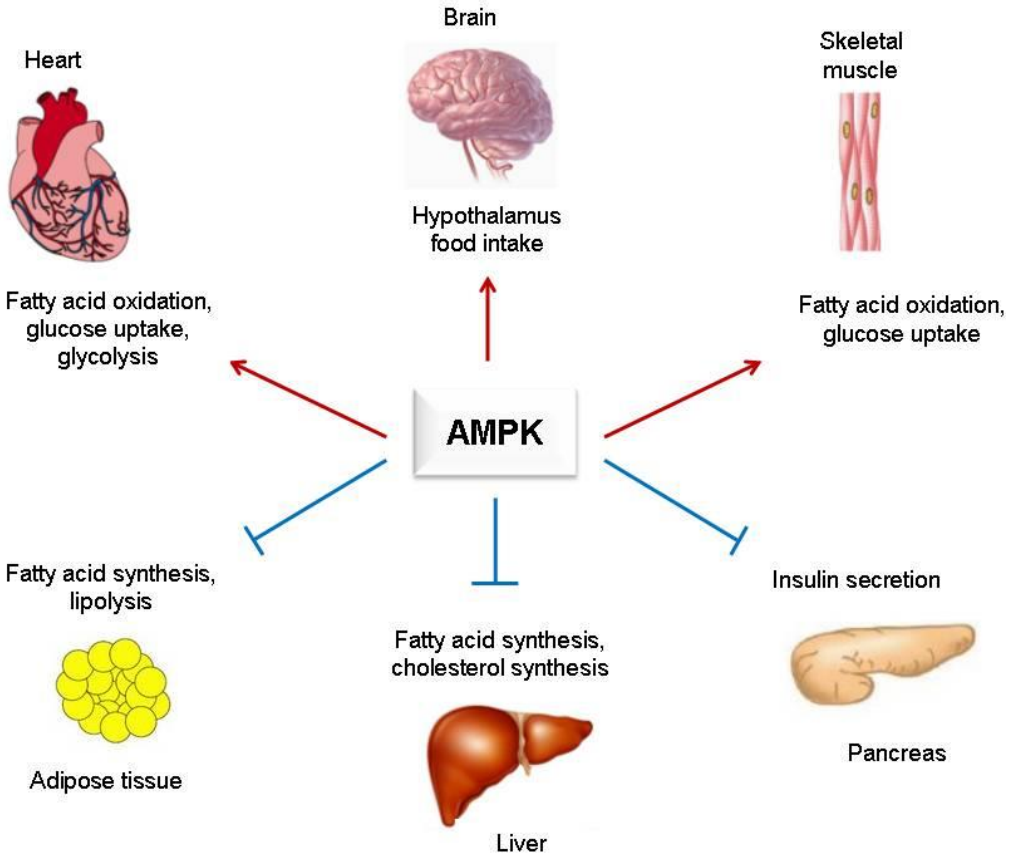

Figure 7. Expression and function of AMP-activated protein kinase (AMPK) in different tissue.

AMPK consists of three protein subunits that together make a functional enzyme (**Fig. 8**) (Hardie et al. 2003).

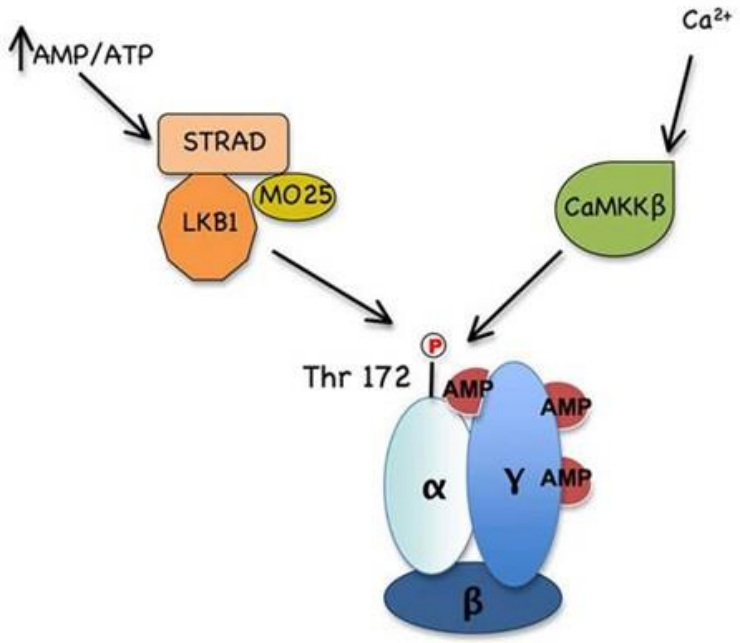

Figure 8. AMP-activated protein kinase (AMPK) phosphorilation. Thr-172, threonine 172; LKB1, liver kinase B1; CaMKKII β, calmodulin-dependent protein kinase kinase II β. Modified after (Viollet et al. 2009).

Those are the catalytic α subunit and two regulatory subunits β and γ, with tissue-specific isoforms existing for each of these three subunits (Hardie et al. 2006). AMPK activation is regulated by at least three mechanisms (Oakhill et al. 2010;Sanders et al. 2007;Shackelford and Shaw 2009;Steinberg and Kemp 2009).

Phosphorylation of the **thr**eonine 172 (Thr-172) residue of the α subunit is crucial for AMPK activation. Thr-172 phosphorylation is accomplished by the upstream protein kinase LKB1 (**l**iver **k**inase **B1**), CaMKKII β (**ca**l**m**odulin-dependent protein **k**inase **k**inase **II β**) and possibly also TAK1 (mammalian **t**ransforming growth factor β-**a**ctivated **k**inase 1) (Hurley et al. 2005;Woods et al. 2003). LKB1, originally identified as a tumour suppressor, exists as a complex with two accessory subunits, STRAD and MO25. The LKB1/STRAD/MO25 complex phosphorylates AMPK in response to an increase in the AMP/ATP ratio (Hardie 2008;Woods et al. 2003). Binding of AMP to the regulatory γ subunit of AMPK promotes allosteric activation, phosphorylation of Thr172 by upstream kinases and inhibition of dephosphorylation of Thr172 by protein phosphatases (Cheung et al. 2000). Moreover, β subunits also play a crucial role in AMPK activation. β subunit myristoylation provides a switch that is a prerequisite for Thr172 phosphorylation (Oakhill et al. 2010).

In addition to an increase in the AMP/ATP ratio, AMPK can be activated by an increase in $[Ca^{2+}]_i$, indicating that AMPK may be activated even in the absence of increased levels of AMP (Bair et al. 2009;Mungai et al. 2011;Towler and Hardie 2007;Zhang et al. 2005). Changes in $[Ca^{2+}]_i$ are sensed by CaMKKII β, which then promotes phosphorylation of the Thr-172 residue of the catalytic α subunit of AMPK (Bair et al. 2009;Mungai et al. 2011;Steinberg and Kemp 2009;Towler and Hardie 2007;Winder and Thomson 2007;Zhang et al. 2009). In contrast, the role of TAK1 in AMPK activation is not fully understood. LKB1 is particularly important for the activation of AMPK in skeletal muscle, whereas CaMKKII β is crucial in the brain (Ronnett et al. 2009).

Active AMPK supports cell survival during energy deprivation (Foller et al. 2009;Hardie 2004;Mcgee and Hargreaves 2008) and promotes energy conservation (Tamas et al. 2006). Thus, AMPK has been found to shut off ATP-utilizing anabolic pathways and turn on ATP-generating catabolic pathways (Horie et al. 2008;Hue et al. 2002;Jensen et al. 2007;Marsin et al. 2002;Mcgee and Hargreaves 2008;Viollet et al. 2009;Winder and Thomson 2007). Active AMPK regulates multiple systems participating in energy homeostasis: it stimulates fatty acid oxidation and glycolysis in heart and skeletal muscle (Hardie et al. 2006;Merrill et al. 1997;Minokoshi et al. 2002;Winder et al. 2000;Yamauchi et al. 2002), inhibits fatty acid and cholesterol synthesis in liver (Henin et al. 1995) and adipose tissue, inhibits lipolysis and lipogenesis in adipose tissue, inhibits insulin secretion by pancreatic beta-cells (Mcgee and Hargreaves 2008;Winder and Thomson 2007), and stimulates food intake in braine. In addition to its effects on cellular metabolism, AMPK influences a great number of other cellular processes, including cell growth and division, apoptosis, gene transcription, protein synthesis and cell polarization (Hardie 2011).

1.6. Role of Active AMPK in the Immune System

The energy regulator AMPK has also been found to play a role in immune cells and their functions. AMPK has been shown to be activated in T cells upon T cell receptor ligation via mechanisms independent of energy stress (Tamas et al. 2006). In 2011, it was shown that the activation of AMPK increased the phagocytic ability of macrophages and neutrophils through a mechanism dependent on Rac1 and formation of actin and microtubule networks (Bae et al. 2011). AMPK acts as a potent counterregulator of macrophage inflammatory function and

promoter of macrophage polarization toward an anti-inflammatory phenotype (Walker et al. 2005).

It is well known that AMPK acts as a negative regulator of the NF-κB (**n**uclear **f**actor **k**appa-light-chain-enhancer of activated **B** cells) pathway in endothelial cells (Hattori et al. 2008;Okayasu et al. 2008) and also in macrophages (Sag et al. 2008). NF-κB is a key element in pro-inflammatory gene expression (Karin and Delhase 2000). Moreover, AMPK has recently been shown to directly phosphorylate CREB (**c**AMP **r**esponse **e**lement-**b**inding protein) at Ser133 causing its activation (Thomson et al. 2008). CREB activation is essential for IL-10 (anti-inflammatory cytokine) production by monocytes (Platzer et al. 1999). Regulation of NF-κB and CREB transcription factors could potentially be the mechanisms explaining the role of AMPK in the regulation of inflammatory responses in macrophages (Sag et al. 2008) (**Fig. 9**).

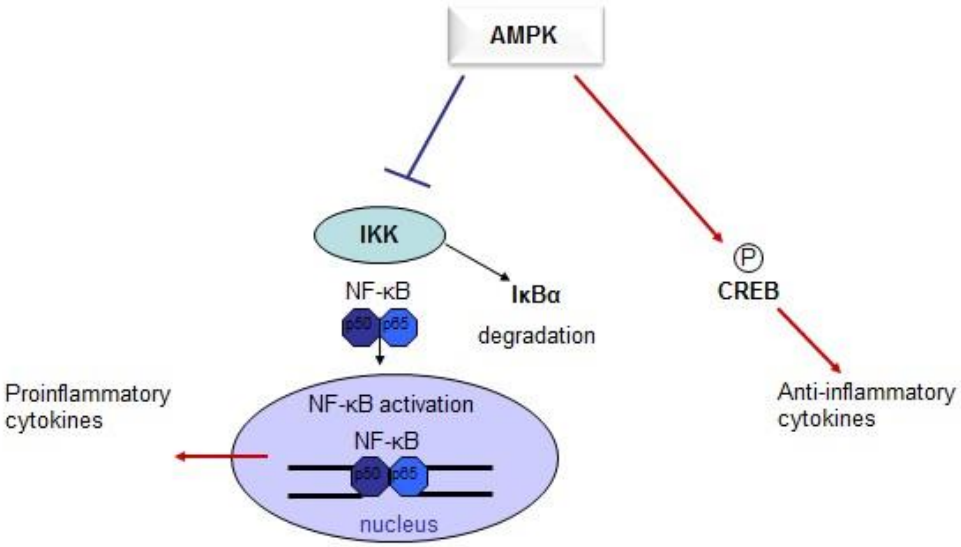

Figure 9. Role of the AMP-activated protein kinase (AMPK) in the regulation of anti-inflammatory and proinflammatory responses in macrophages. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; CREB, cAMP response element-binding protein.

Moreover, TLR-stimulated change in DC metabolism (from mitochondrial **ox**idative **phos**phorylation (OXPHOS) to aerobic glycolysis), which is driven by the PI3K/Akt pathway, is antagonized by AMPK (Murray 2006). Metabolic reprogramming is essential for full DC maturation. TLR-stimulated DC activation is inhibited by the immunosuppressive cytokine IL-10, which was found to partially antagonize the LPS-induced hypophosphorylation of AMPK and, thus, to inhibit TLR-stimulated glycolysis in DCs. In the absence of TLR signals, activated

AMPK, by favoring OXPHOS over glycolysis, functions to enforce a metabolic profile incompatible with DC activation (Krawczyk et al. 2010) (**Fig. 10**).

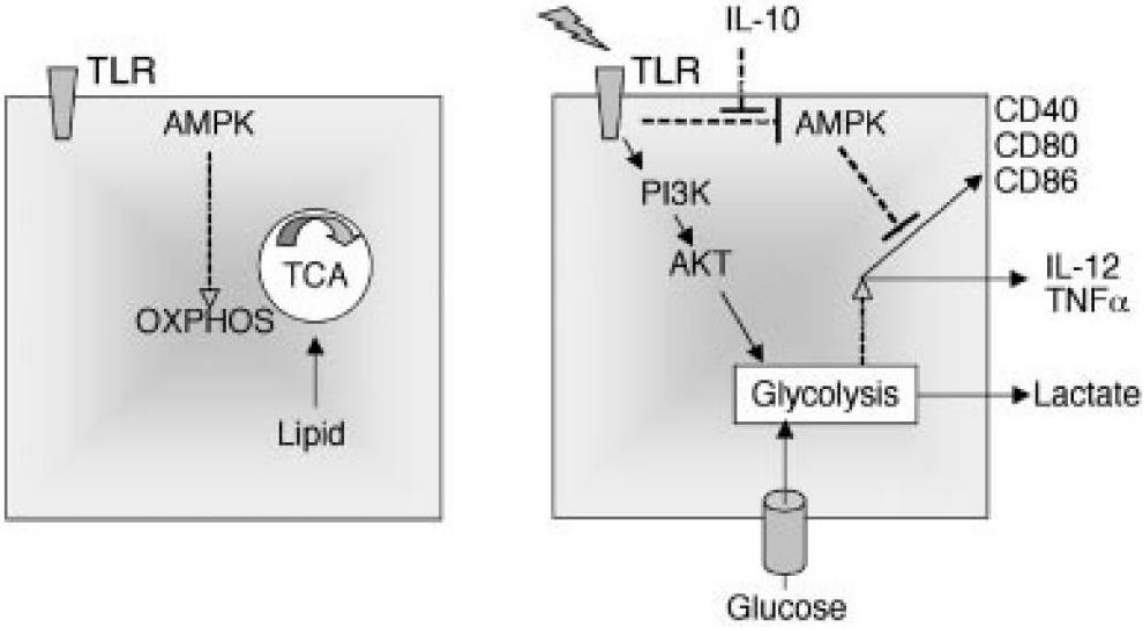

Figure 10. Toll-like receptor (TLR)-induced changes in glycolytic metabolism regulate dendritic cell activation. AMPK, AMP-activated protein kinase; OXPHOS, oxidative phosphorylation; IL-12, interleukin-12; TNF, tumour-necrosis factor; TCA, tricarboxylic acid cycle. Taken from (Krawczyk et al. 2010).

1.7. Ion Channels and Carriers Regulated by AMPK

AMPK has emerged as an important regulator of diverse ion channels and carriers (**Table 1**). General aspects of AMPK functions are to down-regulate ion channel and carrier activity to preserve energy and prevent dissipation of ionic gradients when transporter and exchanger functions might be compromised during conditions of metabolic stress (Andersen and Rasmussen 2012). One of the common mechanisms how AMPK regulates ion channels is via Nedd4-2 (**n**eural precursor cells-**e**xpressed, **d**evelopmentally **d**ownregulated protein 4-2). AMPK induces an increase in Nedd4-2 ubiquitin ligases binding to channels and carriers, such as ENaC, Kir2.1, Kv7.1, KCNQ1, EAAT3, and EAAT4, to initiate their removal from the plasma membrane (Andersen and Rasmussen 2012).

Table 1. Active AMPK regulates ion channels and carriers.

Channels and transporters	*AMPK effect on channel/transporter activity*	*Cell model*	*Mode of action*	*Reference*
CFTR	down-regulation	*Xenopus* oocytes	direct channel phosphorylation	(Hallows et al. 2000)
Nav1.5	up-regulation	ventricular myocytes		(Light et al. 2003)
ENaC	down-regulation	*Xenopus* oocyte, polarized renal epithelial cells	Nedd4-2	(Bhalla et al. 2006)
BK_{Ca}	down-regulation	HEK 293, carotid body type I cell	Direct channel phosphorylation	(Wyatt et al. 2007)
KCa3.1	down-regulation	airway epithelial cells		(Klein et al. 2009)
TREK-1 and TREK-2 channels	down-regulation	HEK293		(Kreneisz et al. 2009)
TASK-3 channels	down-regulation	HEK293		(Dallas et al. 2009)
NHE	up-regulation	HEK		(Rotte et al. 2010)
SGLT1	up-regulation	*Xenopus* oocytes		(Sopjani et al. 2010)
EAAT3 and EAAT4	down-regulation	*Xenopus* oocytes	Nedd4-2	(Sopjani et al. 2010)
KCNQ1	down-regulation	renal epithelial cells	Nedd4-2	(Alesutan et al. 2011a;Alzamora et al. 2010)
Na,K-ATPase	down-regulation	alveolar type II (ATII) cell, human A549 cells		(Gusarova et al. 2011)
Glut 2,	up-regulation	epithelial cells		(Walker et al. 2005)
Glut 4	up-regulation	skeletal muscle		(Park et al. 2009)

Channels and transporters	*AMPK effect on channel/transporter activity*	*Cell model*	*Mode of action*	*Reference*
Kv2.1	up-regulation	HEK293	direct phosphorylation	(Ikematsu et al. 2011)
K_{ATP}	up-regulation down-regulation	ventricular myocytes		(Yoshida et al. 2012)
Kir 2.1	down-regulation	*Xenopus* oocytes	Nedd4-2	(Alesutan et al. 2011b)
TRPC3	up-regulation	HEK 293T cells		(Hirschler-Laszkiewicz et al. 2011)
BK	up-regulation	*Xenopus* oocytes		(Foller et al. 2012)
Kv7.1	down-regulation	epithelial MDCK cell, *Xenopus* oocytes	Nedd4-2	(Andersen et al. 2012)
SMIT (SLC5A3) and BGT1	down-regulation	*Xenopus* oocytes		(Munoz et al. 2012)

However, little is known about AMPK-dependent effects on Ca^{2+}-permeable channels/transporters, which are known to play a crucial role in immune cell functions (Shumilina et al. 2011). The present study thus explored whether AMPK influences Ca^{2+} signaling and Ca^{2+}-dependent functions in murine DCs.

1.8. Klotho Protein

In 1997 Makoto Kuroo and his colleagues discovered Klotho, a protein which is involved in aging (Kuroo et al. 1997). The name Klotho comes from Greek mythology: a Moirae named Klotho or Clotho spins a thread of life. Both the human and the mouse *klotho* gene contains 5 exons which can be alternatively spliced (Shiraki-Iida et al. 1998). The *klotho* gene encodes a single-pass transmembrane protein that is predominantly expressed in the distal tubules of the

kidney, parathyroid glands and choroid plexus of the brain (Kuroo et al. 1997). Deficiency of *klotho* in mice leads to a syndrome resembling aging (Kuroo et al. 1997), whereas overexpression of Klotho in mice extends lifespan (Kurosu et al. 2005). The aging-like phenotype of Klotho-deficient mice includes a shortened lifespan (Klothe-deficient mice suffer premature death around two months of age), hypogonadism, premature thymus involution, ectopic calcification, decreased bone mineral density, skin atrophy, pulmonary emphysema, cognitive impairment, amyotrophic lateral sclerosis-like changes, and hearing loss (Kuroo et al. 1997).

The Klotho protein consists of three domains: a large extracellular domain, a transmembrane domain, and a very short intracellular domain. The Klotho protein exists in two forms: as the membrane Klotho and is secreted form (F**ig. 11**).

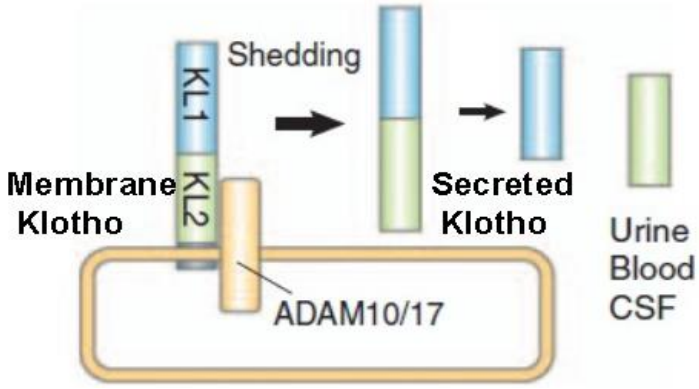

Figure 11. Membrane-bound and secreted Klotho. ADAM10/17, A Disintegrin and Metalloproteinases 10 and 17; KL1/2, Klothe 1/2; CSF, cerebrospinal fluid. Modified after (Huang 2010).

1.9. Membrane Klotho

Three forms of membrane Klotho (αKlotho, βKlotho and γKlotho) have been distinguished (Kuro 2012). These three Klothos function as a co-receptor for the **f**ibroblast **g**rowth **f**actor (FGF) family of hormones (Kuro-o 2008). The FGF family members FGF19 and FGF21 require βKlotho to bind to their respective receptors, FGFR4 and FGFR1c. Complexes of βKlotho/FGFR4 and βKlotho/FGFR1c are expressed in liver and fat tissue respectively, making these the target organs of FGF19 and FGF21 (Kuro 2012). γKlotho is expressed in the kidney in fat tissue and, most abundantly, in the eye. γKlotho forms complexes with FGFR1b, FGFR1c, FGFR2c, and FGFR4 and supports FGFR activation by FGF19 (Kuro-o 2008).

αKlotho makes a complex with FGF receptors FGFR1c, FGFR3c, and FGFR4 to form high-affinity receptors for FGF23 (Kuro 2012;Kuro-o 2006;Kuro-o 2008;Kurosu et al. 2006;Tomiyama et al. 2010;Urakawa et al. 2006). αKlotho is expressed in the kidney and parathyroid glands, thereby rendering these the target organs of FGF23. Activation of FGFR-Klotho receptor complexes by FGF23 reduces the expression of **Na**-dependent **p**hosphate transporters (NaPi2) in the luminal membrane of the proximal tubules, which results in a decreased renal phosphate reabsorption (Razzaque 2009). Moreover, FGF23 binding to FGFR-Klotho receptor suppresses the synthesis of $1,25(OH)_2D_3$, one of the most powerful hormone that regulates Ca^{2+} homeostasis, which is also involved in phosphate metabolism (Yoshida et al. 2002) (**Fig. 12**). $1,25(OH)_2D_3$ increases absorption of Ca^{2+} and phosphate in the intestine (Tsujikawa et al. 2003). Another well-established function of membrane Klotho is regulation of Na^+/K^+ ATPase. Klotho, as a transmembrane protein, increases the membrane abundance and activity of the Na^+/K^+ ATPase (Imura et al. 2007). Klotho is required for the rapid recruitment of Na^+/K^+ ATPase to the cell surface in response to a change in extracellular Ca^{2+} concentration (Imura et al. 2007).

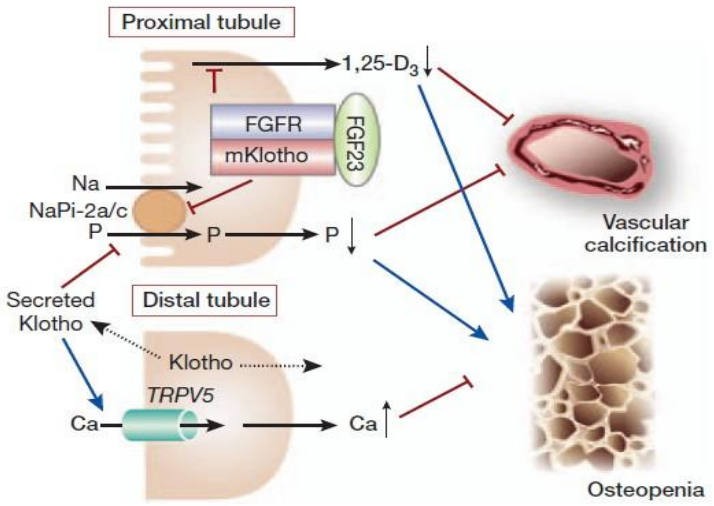

Figure 12. Role of membrane-bound and secreted Klotho in the regulation of phosphate and Ca^{2+} homeostasis. FGF23, fibroblast growth factor 23; FGFR, FGF receptor; mKlotho, membrane Clothe; $1,25\text{-}D_3$, $1,25(OH)_2D_3$; TRPV5, transient receptor potential vanilloid 5. Taken from (Huang 2010).

1.10.Secreted Klotho

The extracellular domain of Klotho consists of two internal repeat sequences of 440 amino acids, named KL1 and KL2 (Ito et al. 2002;Kuroo et al. 1997) (**Fig.12**). The entire

extracellular domain of Klotho can be cleaved by the metalloproteases ADAM10 and ADAM17 (**A D**isintegrin **a**nd **M**etalloproteinase 10 and 17) and released into the extracellular space, and is detectable in blood, urine, and cerebrospinal fluid (Bloch et al. 2009;Imura et al. 2004;Kurosu et al. 2005). Thus, secreted Klotho functions as a humoral factor with pleiotropic activities, including suppression of growth factor signaling, oxidative stress, and regulation of ion channels and transporters (**Fig. 13**) (Hsieh et al. 2010;Kuro-o 2009;Kuro-o 2010;Yamamoto et al. 2005).

1.11. Secreted Klotho as a Regulator of Ion Channels and Transporters

Klotho regulates several ion channels and transporters, some of them being permeable for Ca^{2+}. One of the mechanisms of ion channel regulation by sectered Klotho has appeared to be modification of N-glycans on the surface of the channels, since secreted Klotho exhibits a gylcosidases activity (Kuroo et al. 1997).

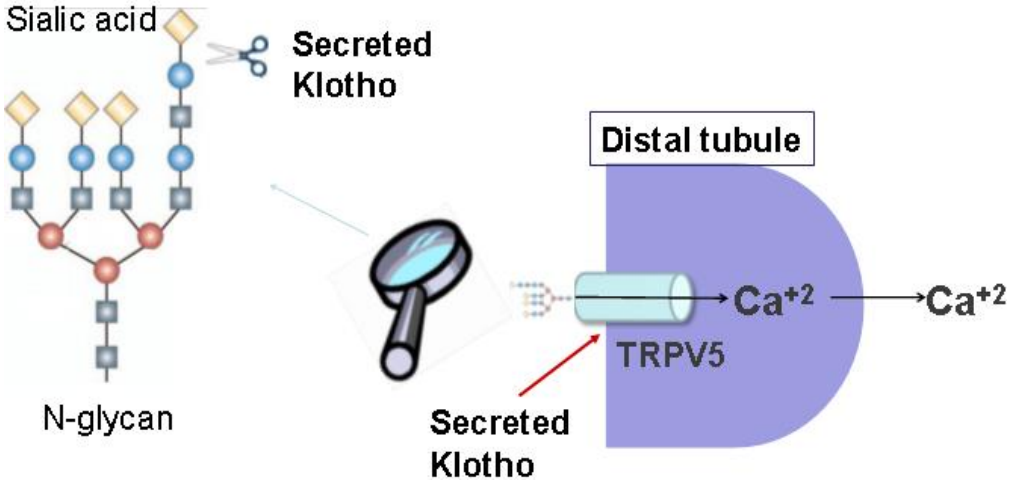

Figure 13. Secreted Klotho removes sialic acid from transient receptor potential vanilloid channel (TRPV5). Modified after (Huang 2010).

Thus, secreted Klotho increases the cell-surface abundance of the epithelial Ca^{2+}-permeable channels TRPV5 (**t**ransient **r**eceptor **p**otential **v**anilloid) (Cha et al. 2008;Chang et al. 2005), TRPV6 (Lu et al. 2008), and renal K^+ channel ROMK (**r**enal **o**uter **m**edullary $\mathbf{K}^+$ channel) (Cha et al. 2009) by removing terminal sialic acids from N-glycans of the channel molecule (Cha et al. 2008) (**Fig. 13**). However, the effect of Klotho on another Ca^{2+}-permeable channel, TRPC6 (**t**ransient **r**eceptor **p**otential **c**ation channels), is opposite to that on TRPV5, TRPV6 and ROMK

channels: the cell-surface abundance of TRPC6 is decreased by Klotho (Huang 2010). The effect of secreted Klotho on TRPC6 is not mediated by the sialidase activity of Klotho, but is related to downregulation of exocytosis of the channel molecules stimulated by serum growth factors (Huang 2010). How secreted Klotho antagonizes the stimulation of exocytosis of TRPC6 by serum growth factors remains unknown. Moreover, secreted Klotho functions as a phosphaturic substance independently of FGF23 via its glucuronidase activity on renal NaPi2a, which results in the inhibition of transporter activity, proteolytic degradation of NaPi2a, and eventually reduced surface expression of the transporter, possibly via its internalization (Hu et al. 2010).

The list of ion channels and transporters regulated by both secreted and membrane Klotho is growing.

As we can see, Klotho, both as a secreted hormone and as a transmembrane protein, can influence Ca^{2+} metabolism directly (via effects on Ca^{2+}-permeable channels) and indirectly (via Na^{+}/K^{+} ATPase or as a coreceptor for FGF23 via regulation of $1,25(OH)_2D_3$). However, nothing is known with respect to the role of Klotho in Ca^{2+} signaling in immune cells.

2. AIMS OF THE STUDY

The first aim of the present study was to investigate the potential role of AMPK (an intracellular energy sensor) in the regulation of cytosolic Ca^{2+} concentrations and Ca^{2+}-dependent functions of mouse DCs.

The second aim was to study the effects of Klotho in the regulation of Ca^{2+} signaling and Ca^{2+}-dependent functions in DCs.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Tissue Culture

Equipment

Names	**Manufacturer and country of origin**
Centrifuge RotiFix 32	Hettich Zentrifugen, Tuttlingen, Germany
Eppendorf pipettes 1000 µl, 100 µl, 10 µl	Eppendorf AG, Hamburg, Germany
Eppendorf cups 1.5 ml	Eppendorf AG, Hamburg, Germany
Heraeus Incubator	Thermo Electron Corporation, Dreieich, Germany
Multiwell™ 6 well	Becton Dickinson Labware, Franklin Lakes, USA
Multi-well plates with 8.0 µm	Becton Dickinson Labware, Franklin Lakes, USA
Neubauer counting chamber	Brand, Wertheim, Germany
Needles BD Microlance™3, 0.55X25 mm	Becton Dickinson Labware, Franklin Lakes, USA
PP-Test Tubes 15, 50 ml	Greiner bio-one, Frickenhausen, Germany
Pipetus® pippetting aid	Hirschmann Laborgeräte, Eberstadt, Germany
Syringde BD 10ml, Luer-Lok™ Tip	Becton Dickinson Labware, Franklin Lakes, USA
Stripette® 5, 10, 25 ml	Coring Incorporated, Corning NY, USA
Tissue Culture Dishes 60x15 mm	Becton Dickinson Labware, Franklin Lakes, USA

Vortex Genie	Scientific Indusreies, Bohemia NY, USA

Chemicals

Names	**Manufacturer and country of origin**
GM-CSF mouse recombinant	Peprotech/Tebu, Cülbe, Germany
L-Glutamine	GIBCO, Carlsbad, Germany
LPS	Sigma, Taufkirchen, Germany
Penicillin-streptomycin	Invitrogen, Karlsruhe, Germany
Phosphate **b**uffered **s**aline (PBS)	GIBCO, Carlsbad, Germany
RPMI-1640	GIBCO, Carsbad, Germany
Trypan blue solution 0,4%	Sigma, Taufkirchen, Germany
1,25-$(OH)_2$ Vitamin D_3	Sigma, Taufkirchen, Germany
ß-mercaptoethanol	Invitrogen, Karlsruhe, Germany

Culture medium composition

Table 2. Complete medium

Substances	**%**
RPMI-1640	
Fetal **b**ovine **s**erum (FBS)	10
L-Glutamine	1

Non-**e**ssential **a**mino **a**cids (NEAA)	1
Penicillin/**s**treptomycin (P/S)	1
ß-mercaptoethanol	0.05

3.1.2. Intracellular Calcium Measurement

Equipment for calcium imaging

Names	**Manufacturer and country of origin**
Discofix® Stopcock for Infusion Therapy	B.Braun, Melsungen, Germany
Centrifuge RotiFix 32	Hettich Zentrifugen, Tuttlingen, Germany
Camera Proxitronic	Proxitronic, Bensheim, Germany
Eppendorf pipettes 1000 µl, 100 µl, 10 µl	Eppendorf AG, Hamburg, Germany
Eppendorf cups 1.5, 2 ml	Eppendorf AG, Hamburg, Germany
Filter Set for Fura-2	AHF Analysentechnik AG, Tübingen, Germany
Filter tips 10, 100, 1000µl	Biozym Scientific, Hess. Oldendorf, Germany
Filter wheel	Sutter Instrument Company, Novato, USA
Heraeus Incubator	Thermo Electron Corporation, Dreieich, Germany
Lamp XBO 75	Leistungselektronik Jena GmbH, Jena, Germany
Metafluor software	Universal Imaging, Downingtown, USA
Microscope Axiovert 100	Zeiss, Oberkochen, Germany

Microscope cover glasses round, 30mm diameter, 0.13-0.16 mm	Karl Hecht KG, Sondheim, Germany
Multiwell™ 6 well	Becton Dickinson Labware, Franklin Lakes, USA
Needles BD Microlance ™3, 1.2X40mm	Becton Dickinson Labware, Franklin Lakes, USA
Objective neo fluar 40x/1.3 oil	Carl Zeiss, Oberkochen, Germany
PP-Test Tubes 15, 50 ml	Greiner bio-one, Frickenhausen, Germany
Syringde BD 10ml, Leur-Lok™ Tip	Becton Dickinson Labware, Franklin Lakes, USA
Syringde BD, Perfusion™ 50ml	Becton Dickinson Labware, Franklin Lakes, USA
Tissue Culture Dishes 35x10 mm	Becton Dickinson Labware, Franklin Lakes, USA

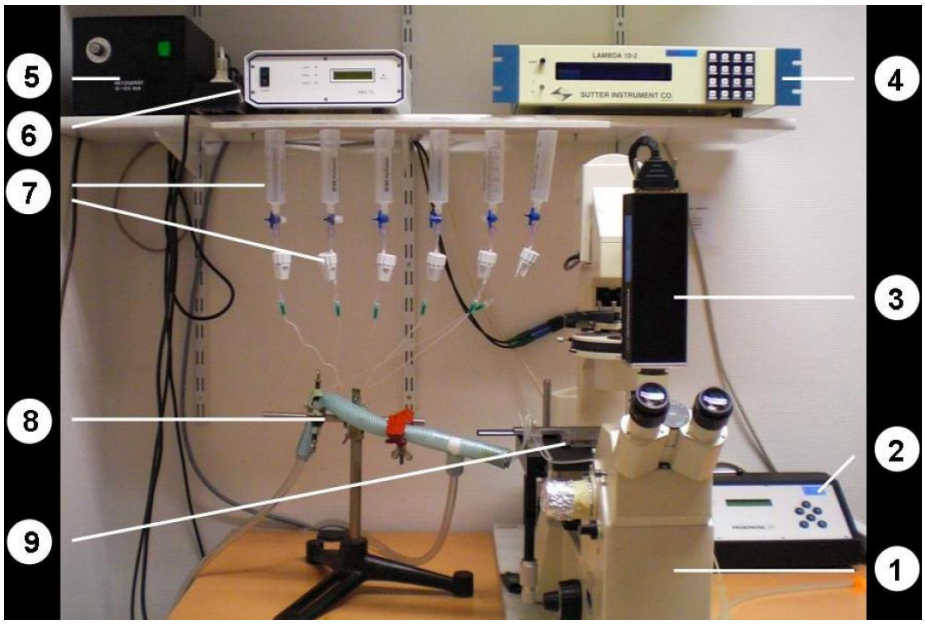

Figure 14. Intracellular calcium imaging setup. 1-Micrsocope, 2-Camera control panel, 3-Camera, 4-Shutter, 5-Light source, 6-Xenon lamp control panel, 7-Perfusion system, 8-Flow heating system, 9-Measuring chamber (Matzner 2010).

Calcium measurement chemicals

Substanses	**Manufacturer and country of origin**
Ampuwa	Fresenius KABI, Bad Homburg, Germany
$CaCl_2$ x 2 H_2O	Carl Roth, Karlsruhe, Germany
CXCL12	Peprotech, Germany
CCL21	Peprotech, Germany
Ethylene **g**lycol **t**etraacetic **a**cid (EGTA)	Sigma, Taufkirchen, Germany
Fura-2 AM	Invitrogen, Karlsruhe, Germany
Glucose	Carl Roth, Karlsruhe, Germany
HEPES	Sigma, Taufkirchen, Germany
Immersol 518F	Carl Zeiss, Göttingen, Germany
Ionomycin	Sigma, Taufkirchen, Germany
KCl	Carl Roth, Karlsruhe, Germany
LPS	Sigma, Taufkirchen, Germany
$MgSO_4$ x 7 H_2O	Sigma, Taufkirchen, Germany
Na_2HPO_4 x 2 H_2O	Sigma, Taufkirchen, Germany
N-**M**ethyl-**D**-**g**lucamin (NMDG)	Sigma, Taufkirchen, Germany
NaCl	Sigma, Taufkirchen, Germany
Poly-L-Lysine	Sigma, Taufkirchen, Germany
Phosphate **b**uffered **s**aline (PBS)	GIBCO, Carlsbad, Germany

Silicone	Grease Carl Roth, Karlsruhe, Germany
Trypan blue solution 0,4%	Sigma, Taufkirchen, Germany
Thapsigargin	Invitrogen, Karlsruhe Germany
3',4'-**d**ichloro**b**en**z**amyl (DBZ)	Sigma-Aldrich, Germany

Calcium measurement buffer composition

Table3. Standard HEPES solution

Substance	**[mM/L]**
NaCl	125
KCl	5
$MgSO_4*7H_2O$	1.2
HEPES	32.2
$Na_2HPO_4*2H_2O$	2
$CaCl_2*2H_2O$	2
Glucose	*5*
pH 7.4 (NaOH); H_2O (at the 37 ^{0}C)	

Table 4. Ca^{2+} free solution

Substance	**[mM/L]**
NaCl	125
KCl	5
$MgSO_4*7H_2O$	1.2

HEPES	32.2
$Na_2HPO_4*2H_2O$	2
EGTA	0.5
Glucose	*5*

pH 7.4 (NaOH); H_2O (at the 37 ^{0}C)

Table 5. Standard Ringer for Na^+/Ca^{2+} exchanger with 40mM KCl (NCKX)

Substance	**[mM/L]**
NaCl	90
KCl	40
$CaCl_2*2H_2O$	2
$MgCl_2*6H_2O$	2
HEPES	10
Glucose	10

pH 7.4 (NaOH); H_2O (at the 37 ^{0}C)

Table 6. 0Na^+-Ringer Solution for Na^+/Ca^{2+} exchanger with 40mM KCl (NCKX)

Substance	**[mM/L]**
NMDG	90
KCl	40
$CaCl_2*2H_2O$	2
$MgCl_2*6H_2O$	2
HEPES	10

Glucose	10
pH 7.4 (HCl); H_2O (at the 37 ^{0}C)	

Table 7. Standard Ringer for Na^+/Ca^{2+} exchanger with 0mM KCl (NCX)

Substance	**[mM/L]**
NaCl	130
KCl	0
$CaCl_2*2H_2O$	2
$MgCl_2*6H_2O$	2
HEPES	10
Glucose	10
pH 7.4 (NaOH); H_2O (at the 37 ^{0}C)	

Table 8. $0Na^+$-Ringer Solution for Na^+/Ca^{2+} exchanger with 0mM KCl (NCX)

Substance	**[mM/L]**
NMDG	160
KCl	0
$CaCl_2*2H_2O$	2
$MgCl_2*6H_2O$	2
HEPES	10
Glucose	10
pH 7.4 (HCl); H_2O (at the 37 ^{0}C)	

3.1.3. Patch Clamp

Technical Equipment

Name	**Manufacturer and country of origin**
Borosilicate glass filaments	Harvard Apparatur, March-Hugstetten, Germany
DMZ puller	Zeitz, Augsburg, Germany
EPC-9 amlifier	Heka, Lamberecht, Germany
ITC-16 interface	Instrutech, Port Washigton, N.Y., USA
Microscope Axiovert 100	Zeiss, Oberkochen, Germany
MS314 electrical micromanipulator	MW, Märzhüuser, Wetzlar, Germany
Pulse software	Heka, Lanbrecht, Germany

Chemicals

Some of the chemicals mentioned above are also for Patch Clamp, therefore the addional chemicals are listed here only.

Chemicals	**Manufacturer and country of origin**
MgATP	Sigma, Taufkirchen, Germany
Tetra**e**thyl**a**mmonium (TEA)	Sigma, Taufkirchen, Germany
CsCl	Sigma, Taufkirchen, Germany
Cs(OH)	Sigma, Taufkirchen, Germany

Patch clamp solution compositions

Table 9. Na^+-based pipet solution

Substance	**[mM/L]**
NaCl	120
KCl	40
TEA-Cl	20
$MgCl_2$	2
Mg-ATP	2
HEPES	10
1 µM free Ca^{2+}	
pH 7.2 (CsOH)	

Table 10. External solution: 130 Na^+ and 0Ca^{2+}

Substance	**[mM/L]**
NaCl	130
KCl	0or 40
TEA-Cl	20
$MgCl_2$	2
HEPES	10
EGTA	**0.5**
Glucose	10
pH 7.2 (CsOH)	

Table 11. External solution:0 Na^+ and 2Ca^{2+}

Substance	[mM/L]
NMDG	130
TEA-Cl	20
$MgCl_2$	2
HEPES	10
$CaCl_2$	2
Glucose	10
pH 7.2 (CsOH)	

3.1.4. Immunostaining and Flow Cytometry

Technical Equipment

Name	Manufacturer and country of origin
FACS Calibur	Becton Dickinson, Heidelberg, Germany
FACS tubes, 1.3 ml, PP, round bottom	Greiner bio-one, Frickenhausen Germany

Antibodies and chemicals

Name	Manufacturer and country of origin
FITC-conjuganted anti-mouse CD11c Clone: HL3 (Armenian Hamster IgG_1 λ2)	BD Pharmingen, Heidelberg, Germany

Fetal **c**alf **s**erum (FCS)	VWR International GmbH, Darmstadt, Germany
PE-conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ)	BD Pharmingen, Heidelberg, Germany
PE-conjugated rat anti-mouse I-A/I-E, clone M5/114.15.2 (IgG2b, κ)	BD Pharmingen, Heidelberg, Germany
Sodium azide	Sigma, Taufkirchen, Germany

Buffers

FACS buffer

PBS

0.1% heat-inactivated FBS

3.1.5. Migration

Technical Equipment

Name	**Manufacturer and country of origin**
BD Falcon™ Cell culture inserts for 24-well plates. 8.0 µm pores, Translucent PET Membrane	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

Chemicals and Kits

Name	**Manufacturer and country of origin**
CCL21	Peprotech/Tebu, Cölbe, Germany
CXCL12	Peprotech/Tebu, Cölbe, Germany
RPMI 1640	GIBCO, Carlsbad, Germany
4',6-**d**i**a**midino-2-**p**heny**l**indole (DAPI)	Invitrogen, Darmstadt, Germany
ProLong® Gold Antifade Reagent	Invitrogen, Darmstadt, Germany
4% Paraformaldehyde	Sigma, Taufkirchen, Germany

3.1.6. Western Blotting

Technical Equipment

Name	**Manufacturer and country of origin**
Agorose Gel electorphoresis chamber	BioRad, München, Germany
Centrifuge 5415R	Eppendorf, Hamburg, Germany
Densitrometer Quentity One	BioRad, München, Germany
Gel tips	Alpha Laboratories, Hampshire, UK
Kodak film	Sigma, Hannover, Germany

Figure **15**. Western blotting equipment. **1-Buffer tank lid with electrode asseply, 2-Membrane, 3-Comb, 4-Glasses 5-Casting Frame, 6-Casting stand 7-Electrode assepmly, 8-Gel holder cassette and foam pads 9- Ice cooling unit 10-power supply.**

Chemicals

Name	**Manufacturer and country of origin**
Acrilamide/bisacrylamide	Carl Roth, Karlsruhe, Germany
BenchMark prestained protein öadder	Invitrogen, California, USA
Cell lysis buffer	Pierce, Bonn, Germany
Detection reagent	GE Healthcare, München, Germany
Glycine	Sigma, Taufkirchen, Germany
Loading buffer (4x)	Carl Roth, Karlsruhe, Germany
Milk power	Carl Roth, Karlsruhe, Germany
Nitrocellulose membrane	VWR, Darmstadt, Germany

Ponceau S	Sigma, Taufkirchen, Germany
Protease inhibitor	Sigma. Taufkirchen, Germany
Sodium **d**odecyl **s**ulphate (SDS)	Sigma, Hannover, Germany
TEMED	Carl Roth, Karsruhe, Germany
Triethanolamine-**b**uffered **s**aline (TBS)	Sigma, Taufkirchen, Germany
Tween-20	Böhriner Ingelheim, Mannheim, Germany

Antibodies

Name	**Manufacturer and country of origin**
phopho-AMPKα (Thr172) antibody or polyclonal rabbit AMPKα	Cell Signaling, Germany
monoclonal rabbit α-tubulin	Cell Signaling, Germany
monoclonal rabbit GAPDH	Cell Signaling, Germany
polyclonal rabbit STIM1	Cell Signaling, Germany
polyclonal rabbit STIM2	Cell Signaling, Germany
polyclonal rabbit Orai1	Proteintech, Manchester, UK
polyclonal goat Orai2	Santa Cruz Biotechnology, Santa Cruz, CA

Buffers

Table 12. Standard migration buffer (Running buffer)

Tris base	25mM

Glycine	190mM
SDS	0.1%
Check the pH; it should be around (do not adjust the pH!!!!!!!!!!)	8.3

Table 13. Transfer buffer

Tris	25mM
Glycine	192mM
Methanol	20%
Check the pH; it should be around (do not adjust the pH!!!!!!!!!!)	8.3

Table 14. Wash buffer

1x PBS	
TWEEN-20	0.05%

Table 15. Solution for preparing 5% stacking gels for Tris-glycine SDS polyacrylamide gel electrophoresis

H_2O	
acrylamide mix	30%
Tris-Cl	(1.0 M, pH 6.8)
SDS	(10%)
ammonium persulfate	10%
TEMED	

Table 16. Solution for preparing resolving gels for 10% resolving gels for Tris-glycine SDS-Polyacrylamide gel electrophoresis

H2O	
acrylamide mix	30%
Tris-Cl	(1.5 M, pH 8.8)
SDS	10%
ammonium persulfate	(10%)
TEMED	

3.1.7. Real Time PCR

Technical Equipment

Name	**Manufacturer and country of origin**
Densitometer	BioRad, München, Germany
Low Profile 96 Well PCR Plate	PEQLAB Biotechnologie GMBH, Erlangen, Germany
CFX96 Real Time System	BioRad, München, Germany
PCR Plate Sealing Films & Foils	Biozym biotech trading GmbH

Chemicals

Name	**Manufacturer and country of origin**
Agarose	Sigma, Taufkirchen, Germany
Chloroform	Carl Roth, Karlsruhe, Germany

DEPC water	Promega, Mannheim, Germany
dNTP mix	Promega, Mannheim, Germany
Ethanol 99.7%	VWR, Darmstadt, Germany
GoTaq® qPCR Master Mix	Promega, Mannheim, Germany
peqGOLD TriFast	PEQLAB Biotechnologie GMBH, Erlangen, Germany
Primers	Invitrogen, Darmstadt Germany
Transcriptor High Fidelity *cDNA Synthesis Kit*	Roche, Mannheim, Germany
2-Propanol (Isopropanol)	Sigma, Taufkirchen, Germany
10 x reaction buffer	Biolabs, Frankfurt, Germany

Primers

Name	**Manufacturer and country of origin**
AMPKα1	Invitrogen, Darmstadt Germany
AMPKα2	Invitrogen, Darmstadt Germany
Klotho (exon 1)	Invitrogen, Darmstadt Germany
Klotho (exon 3/4)	Invitrogen, Darmstadt Germany
Tbp	Invitrogen, Darmstadt Germany

3.1.8. Animals

Experiments were performed in DCs isolated from 6-12 week old AMPKα1-deficient male and female mice (*ampk*$^{-/-}$) and their wild type littermates (*ampk*$^{+/+}$) and from 6-8 week old klotho-deficient mice (*klotho*^{hyp}) and their wild type littermates (*klotho*$^{+/+}$). Description of the *ampk*$^{-/-}$ mice (Viollet et al. 2003) and origin of the *klotho* mice (Kuroo et al. 1997). Congenic strains of Klotho-deficient mice were produced by repeated backcrosses (> 9 generations) to the 129 inbred strains. All animal experiments were performed according to the German animal protection law and approved by the local authorities. The *klotho*^{hyp} and *klotho*$^{+/+}$mice had access to water ad libitum and to control food (Altromin 1310) or vitamin D deficient diet (Altromin, C1017).

3.2. Methods

3.2.1. Culture of Bone Marrow Dendritic Cells

Dendritic **c**ells (DCs) were isolated from bone marrow of *ampk*$^{-/-}$, *ampk*$^{+/+}$ and *klotho*^{hyp}, *klotho*$^{+/+}$ mice following an established protocol (Inaba et al. 1992) with slight modifications (**Fig. 16**). After removing skin and muscle mass from the bone, the bone marrow-derived cells were flushed out of the bone marrow cavity from the femur and tibia with sterile, ice cold PBS using a small needle fixed on a syringe.

The extracted cells were centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was discharged and the cells were re-suspended in complete cell culture medium and centrifuged. Subsequently, the DCs were re-suspended again and counted using a Neubauer counting chamber. Cells were seeded out into 60x15 mm petri dishes at a density of 2 x 10^6 cells per dish. Finally, GM-CSF (35 ng/mL, Preprotech Tebu) was added to the culture media. The cells were cultured for 1 week with changes of the medium on days 3 and 6. For the first medium change, fresh medium as well as GM-CSF was added to the culture. On day 6, nonadherent and loosely attached cells were harvested and the removed volume of the culture medium was replaced by fresh medium and GM-CSF. At day 7, the cells were seeded out into several petri dishes in an amount of 5 x 10^5 cells per dish. For cell treatment, the substances of interest were added to the respective dishes for a certain time period indicated in the respective experiments. Experiments were performed on days 7-9.

DC maturation was induced by treating the cells with **l**ipo**p**oly**s**accharides (LPS from *E. coli*, 1 µg/ml, 24 h, Enzo Life Sciences, Lausen, Switzerland). In some experiments DC precorsors of *kloth*$^{+/+}$ mice were pretreated with 1,25$(OH)_2D_3$ (50nM, Sigma, Taufkirchen, Germany) immediately after isolation from the bone marrow for the first 2 days and then cultured the next 5 days in the absence of 1,25$(OH)_2D_3$.

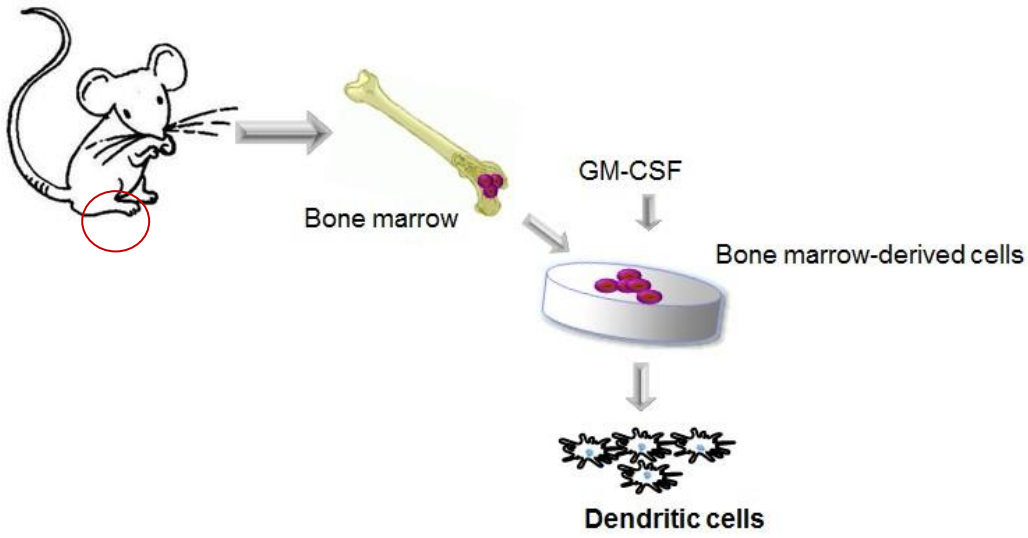

Figure 16. Isolation of Bone marrow derived DCs from the mice. GM, CSF-granulocyte macrophage colony-stimulating factor.

3.2.2. Measurement of Intracellular Calcium

Fluorescence measurements of intracellular calcium concentration $[Ca^{2+}]_i$ were carried out by using an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany) which is connected to: a camera, a light source, a wheel with different excitation filters, a shutter element, a perfusion system inserted into a measuring chamber, a water bath, and a pump to allow a continuous exchange and removal of the added bath solutions (**Fig. 14**). The cells were continuously superfused during each experiment. Experiments were performed at 37 ^{0} C in Ringer solution. 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 CCD 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, and Downingtown, USA). As a measure for the increase of

cytosolic Ca^{2+} concentration, the slope and peak of the changes in the 340/380 nm ratio were calculated for each experiment.

The cells were loaded with Fura-2/AM (2 µM, Molecular Probes, Goettingen, Germany) for 15 min at 37°C. Intracellular Ca^{2+} was measured prior to and following acute addition of CXCL12 (300 ng/ml; Peprotech/Tebu, Cölbe, Germany) or LPS from *Escherichia coli* (1 µg/ml; Sigma-Aldrich, Munich, Germany) to the Ringer solution (**Table 3**).

In order to measure **s**tore **o**perated **C**a^{2+} (SOC) entry, changes in cytosolic Ca^{2+} were monitored upon depletion of the intracellular Ca^{2+} stores.

In order to measure SOC entry, changes in cytosolic Ca^{2+} were monitored upon depletion of the intracellular Ca^{2+} stores. Experiments were carried out prior to and during exposure of the cells to Ca^{2+}-free solution (**Table 4**). In the absence of Ca^{2+}, the intracellular Ca^{2+} stores were depleted by inhibition of the vesicular Ca^{2+} pump by thapsigargin (1 µM, Molecular Probes). Re-addition of Ca^{2+} allowed assessing the store-operated Ca^{2+} entry.

To monitor the activity of K^+ -dependent and K^+ -independen Na^+/Ca^{2+} exchangers, changes in cytosolic Ca^{2+} were determined upon replacement of extracellular Na^+ by **N**-**m**ethyl-**D**-**g**lucamine (NMDG) in Ringer solution for NCX or NCKX. For calibration purposes ionomycin (10 µM, Sigma, Germany) was applied at the end of each experiment.

3.2.3. Patch Clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode according to Hamill et al. (Hamill 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 (**Table 9**). Borosilicate glass pipettes (1-3 MΩ tip resistance; Harvard Apparatus, Kent, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). Voltage clamp step (2 min) was applied to the potential of -80 mV from the holding potential of 0 mV with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered. The liquid junction potential ΔE between the pipette and the bath solutions and between

the salt bridge and the bath solutions were estimated as described earlier (Barry and Lynch 1991;Heise et al. 2011). Data were corrected for the estimated ΔE values.

To measure Na^+/Ca^{2+} exchanger-mediated currents, a Na^+-based pipette solution was used (**Table 9)**. The external solution, (T**able 10)** was either with 0 or 40 mM KCl (for NCX and NCKX, respectively). Na^+/Ca^{2+} exchange currents were elicited by switching to a bath solution in which Na^+ was substituted by $NMDG^+$ (**Table 11**).

3.2.4. Immunostaining and Flow Cytometry

Cells (2×10^6) were incubated in PBS, containing 0.1% FCS and fluorochrome-conjugated antibodies at a concentration of 10 µg/ml. A total of 5×10^4 cells were analyzed in each individual experiment. The following antibodies (all from BD Pharmingen, Heidelberg, Germany) were used for staining: APC Hamster Anti-Mouse CD11c (Clone: HL3), PE-conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ) and PE-conjugated rat anti-mouse I-A/I-E, clone M5/114.15.2 (IgG2b, κ). Following incubation with the respective antibodies for 60 min at 4^0C, cells were washed twice and resuspended in the same buffer and subjected to flow cytometry analysis.

3.2.5. DCs Migration a Assay

For migration assays transwell inserts (BD Falcon 353504) and BD Falcon™ Cell culture inserts for 24-well plates. 8.0 µm pores were used with an 8 µm pore diameter. The transwells were placed in a 24-well cell culture plate containing cell culture medium (750 µl) with or without either CXCL12 (50 ng/ml, Peprotech, for immature DCs) or CCL21 (25 ng/ml, Peprotech, for mature DCs) in the lower chamber. The upper chambers were filled with 500 µl cell culture medium (**Table 2**) containing 5×10^4 cells. The chamber was placed in a 5% CO_2 37°C incubator for 4 hours. In the following, the non-migrated cells were gently removed by cotton tipped swab and PBS wash. The transwells were moved to 4 % PFA (Sigma, Taufkirchen, Germany) and incubated overnight. Membranes were removed by scalpel, placed on slides and stained with DAPI (Invitrogen, Darmstadt Germany). The migrated cells bound to the membrane were then counted.

3.2.6. Western Blotting

To examine the expression of phospho-AMPKα and AMPKα in DCs, cells were lysed with lysis buffer (Cell signaling, Germany) containing protease inhibitor cocktail (Roche) and centrifuged (15,000 g for 20 min at 4°C). Roti Load buffer (Carl Roth) was added to the protein lysate, which then were incubated at 95ºC for 5 min. Proteins were separated by electroporation on 10% SDS-PAGE (**Table 16**). For immunoblotting proteins were electro-transferred onto nitrocellulose membranes and blocked with 10% nonfat milk in **t**riethanolamine-**b**uffered **s**aline (TBS) with 0.1% Tween 20 (TBS-T) at room temperature for 1 h. Then, the membrane was incubated with polyclonal rabbit phopho-AMPKα (Thr172) antibody or polyclonal rabbit AMPKα (1:1000; 62 kDa, Cell Signaling, Germany) or monoclonal rabbit α-tubulin (1:1000, 52 kDa, Cell Signaling) or monoclonal rabbit GAPDH (1:1000, 37 kDa, Cell Signaling) or polyclonal rabbit STIM1 (1:1000, 80 kDa, Cell Signaling) or polyclonal rabbit STIM2 (1:1000, 100 kDa, Cell Signaling) or polyclonal rabbit Orai1 (1:200, 45 kDa, Proteintech) or polyclonal goat Orai2 (1:200, 28 kDa, Santa-Cruz) at 4°C overnight. All antibodies were diluted in TBS-T with 5% BSA. After washing with TBST the blots were incubated with secondary anti rabbit or anti-goat antibody (1:3000, Cell Signaling) for 1 h at room temperature. After washing antibody binding was detected with the ECL solution (Amersham ECL Western Blotting Detection Reagents, GE Healthcare, UK). Densitometer scans of the blots were performed using Quantity One (BioRad, Munich, Germany) (**Fig. 15**).

3.2.7. Real-Time PCR

Total RNA was extracted from mouse dendritic cells or kidney tissue in TriFast (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 (PCR) amplification of the respective genes were set up in a total volume of 20 µl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® Mast Mix (Promega) 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 sec, 55°C for 15 sec and 68°C for 20 sec. For the amplification the following primers were used (5`->3`orientation):

Table 17. Primers

AMPKα1	fw	CAAGCTGTGGCTCACCCA
	rev	GCATAGAGAATGACCCCGCT
AMPKα2	fw	AAGCGAGCGACTATCAAAGACA
	rev	ATGACATTCGCATCGTAGGAG
Klotho (exon 1)	fw	AGATGTGGCCAGCGATAGTTA
	rev	ACTTGACCTGACCACCGAAGT
Klotho (exon 3/4)	fw	CCCTGTGACTTTGCTTGGG
	rev	CCCACAGATAGACATTCGGGT
Tbp	fw	CAAGCTGGAGGTGATCATCG
	rev	TCCACAGTGCTCTTGAATTCG

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad) and all experiments were done in duplicate. Amplification of the house-keeping gene Tbp (TATA binding protein) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the ΔΔct method as described earlier (Pfaffl 2001).

3.3. STATISTICAL ANALYSIS

Data are provided as means ± SEM (**s**tandard **e**rror of the **m**ean), *n* represents the number of independent experiments. Differences were tested for significance using Student's unpaired two-tailed *t*-test or ANOVA (**an**alysis **o**f **va**riance between groups). $P<0.05$ was considered statistically significant.

4. RESULTS

4.1. Expression of AMPKα1 as the Predominant AMPKα Isoform in Mouse Bone Marrow-Derived Dendritic Cells (DCs)

To assess the possible expression of AMPKα in mouse bone marrow-derived DCs we examined abundance of AMPKα1 and AMPKα2 mRNA by real-time PCR. Similar to murine macrophages (Sag et al. 2008), mouse bone marrow-derived DCs express AMPKα1 as the predominant AMPKα isoform and no detectable levels of AMPKα2 mRNA in murine DCs (**Fig. 17A**). Accordingly, DCs were isolated from the bone marrow of AMPKα1-deficient mice (*ampk*$^{-/-}$) and their wild type littermates (*ampk*$^{+/+}$).

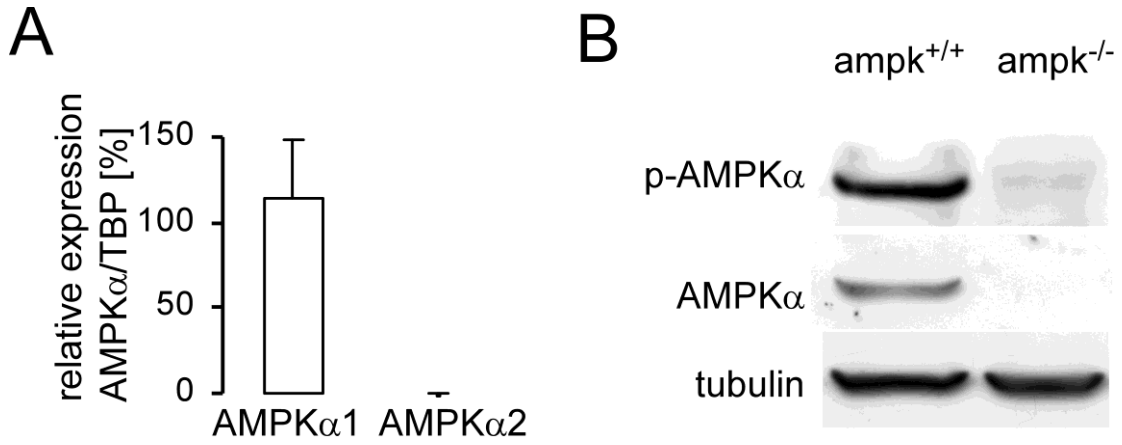

Figure 17. Abundance of AMPKα1 and AMPKα2 transcripts and AMPKα protein in DCs from *ampk*$^{+/+}$ and *ampk*$^{-/-}$ mice.

A. AMPKα1 and AMPKα2 mRNA levels (± SEM, n = 3) determined after isolation from mouse DCs and assessed by real-time PCR using TBP mRNA as a reference gene.

B. Original Western blot of phosphorylated AMPK (upper panel), total AMPK (middle panel) and tubulin (lower panel) protein in *ampk*$^{+/+}$ DCs (left) and *ampk*$^{-/-}$ DCs (right).

No detectable expression of AMPKα protein was observed in *ampk*$^{-/-}$ DCs with antibodies detecting both AMPKα1 and AMPKα2 isoforms (**Fig. 17B**), confirming predominant expression of AMPKα1 in mouse DCs. AMPKα1 was active under resting conditions in *ampk*$^{+/+}$ DCs, as detected by phosphorylation of the Thr-172 residue in its activation loop (**Fig. 17B**).

4.2. Increased Maturation and Differentiation of *ampk*$^{-/-}$ DCs

To check the role of AMPK in the functions of DCs, we measured expression of co-stimulatory molecule CD86 (which is one of the markers of maturation) and MHC II (antigen-presenting molecule) by FACS analysis. According to CD86 and MHC II surface protein abundance on CD11c-positive cells, differentiation of *ampk*$^{-/-}$ DCs was similar to that of *ampk*$^{+/+}$ cells (**Fig. 18** control). In order to induce DC maturation, DCs were treated with a ligand of **T**oll-**l**ike **r**eceptor **4** (TLR4) , **l**ipo**p**oly**s**accharide (LPS, 1 µg/ml, 24 h), as in (Zanoni et al. 2009). Following LPS, the upregulation of CD86 and MHC II was higher in mature *ampk*$^{-/-}$ DCs than in *ampk*$^{+/+}$ DCs (**Fig. 18** LPS), confirming the previous study in which knock-down of AMPK by shRNA fostered maturation of DCs (Krawczyk et al. 2010).

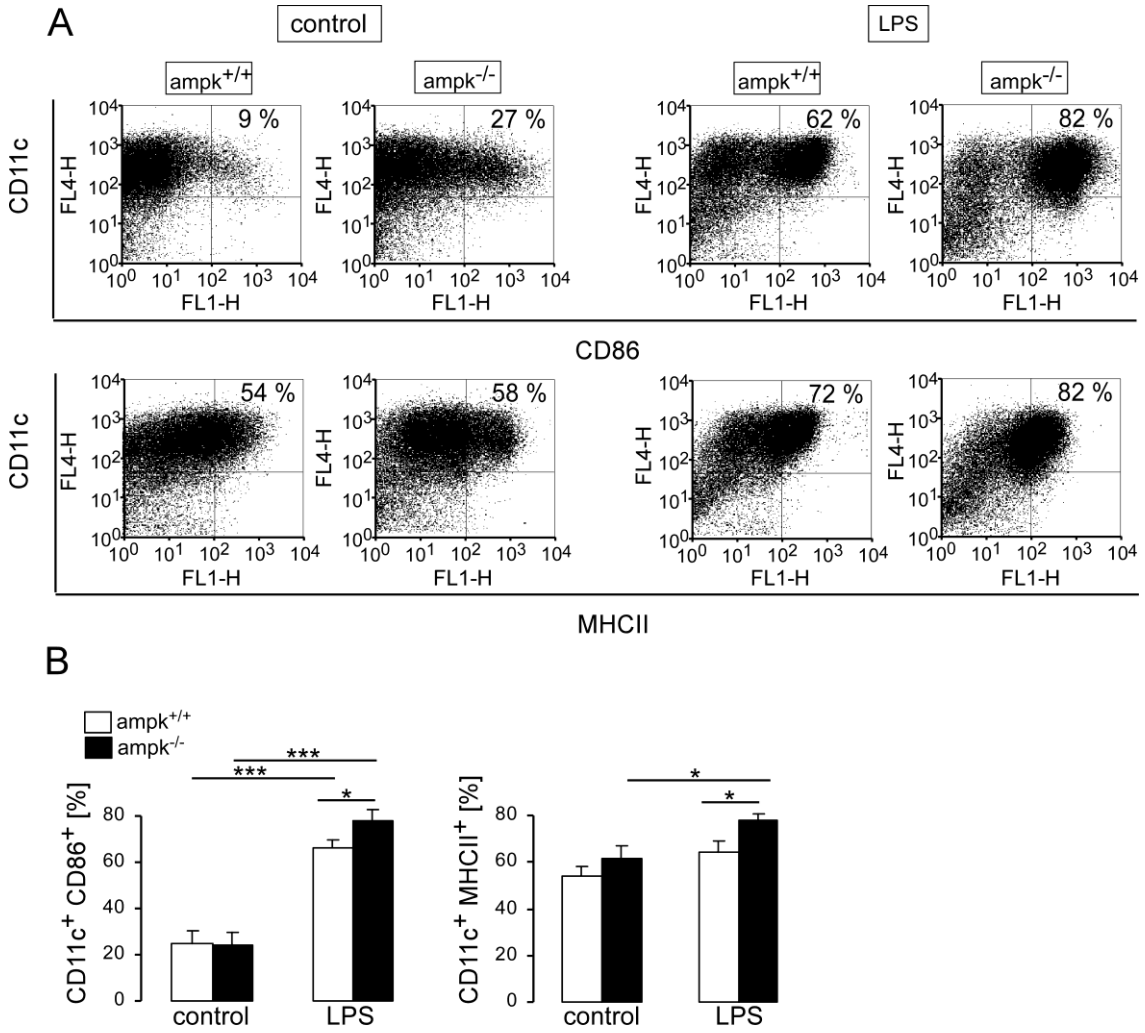

Figure 18. Differentiation and maturation of bone marrow derived DCs from *ampk*$^{+/+}$ and *ampk*$^{-/-}$ mice.

A. Original dot plots of CD11c^{+}CD86^{+} (above) and CD11c^{+}MHC II^{+} (below) DCs at the basal level (control, 1st and 2nd panels) and stimulated with LPS (LPS, 1 µg/ml, 24 h, 3rd and 4th panels) from *ampk*$^{+/+}$ (1st and 3rd panels) and *ampk*$^{-/-}$ (2nd and 4th panels) mice. Numbers depict the percent of cells in the respective quadrants.

B. Arithmetic means ± SEM (n = 7) of the percentage of CD11c^{+}CD86^{+} (left) and CD11c^{+}MHC II^{+} (right) DCs under control and 24 h after LPS stimulation in primary cultures from *ampk*$^{+/+}$ (open bars) and *ampk*$^{-/-}$ (closed bars) mice. * ($p<0.05$) and *** ($p<0.001$), one-way ANOVA with post-test.

4.3. Increased Migration of Immature *ampk*$^{-/-}$ DCs

One of the crucial DC functions include migration (Connolly and Kusner 2007;Shumilina et al. 2011). To check whether AMPK has an effect on DC migration, a transwell chamber assay was employed. Immature DCs have been shown to migrate efficiently to the chemokine CXCL12,

whereas mature DCs migrate vigorously to CCR7 ligands, such as CCL21 (Partida-Sanchez et al. 2004b). Under stimulation with the chemokine CXCL12 (50 ng/ml) the migration was significantly more pronounced in immature *ampk*$^{-/-}$ DCs than in immature *ampk*$^{+/+}$ cells (**Fig. 19A**). The stimulation of migration of LPS-matured DCs by CCL21 (25 ng/ml) was unaffected by AMPKα1 deficiency (**Fig. 19B**).

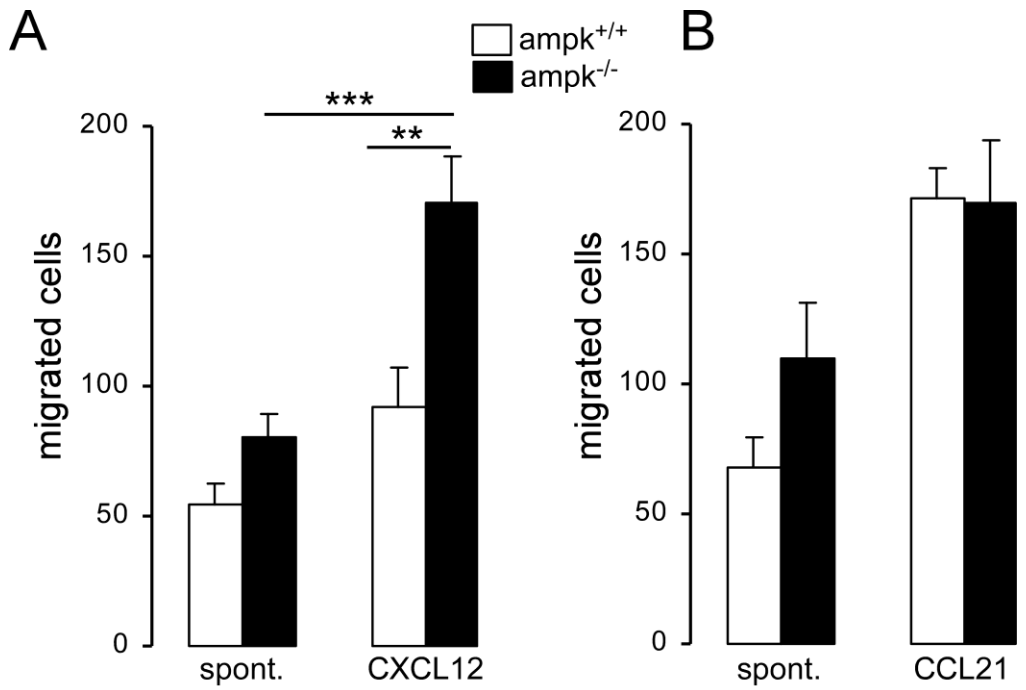

Figure 19. Migration of *ampk*$^{+/+}$ and *ampk*$^{-/-}$ DCs.

A. Arithmetic means ± SEM (n = 7) of migrating *ampk*$^{+/+}$ (open bars) and *ampk*$^{-/-}$ (closed bars) DCs in the absence and presence of CXCL12 (50 ng/ml, 4h). ** ($p<0.01$) and *** ($p<0.001$), ANOVA.

B. Arithmetic means ± SEM (n = 6) of migrating LPS (1 µg/ml, 24 h)-matured *ampk*$^{+/+}$ (open bars) and *ampk*$^{-/-}$ (closed bars) DCs in the absence and presence of CCL21 (25 ng/ml, 4h).

4.4. Active AMPK Downregulates Store-Operated Ca^{2+} Entry.

Both maturation and migration, two processes that were downregulated by AMPK in DCs, are at the same time Ca^{2+} dependent. This suggests that AMPK may influence **i**ntracellular **Ca**$^{2+}$ concentrations ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was determined utilizing Fura-2 fluorescence upon stimulation of the chemokine receptor CXCR4 with its ligand CXCL12. DCs have been shown to respond to

stimulation with 300 ng/ml CXCL12 with fast Ca^{2+} transients (Sumoza-Toledo et al. 2011). Accordingly, exposure of both *ampk*$^{-/-}$ and *ampk*$^{+/+}$ DCs to CXCL12 was followed by an increase of $[Ca^{2+}]_i$ (**Fig. 20 A,B**). Both, the amplitude of this increase (peak, Δ ratio) and the velocity (slope, Δ ratio/s), were significantly increased in *ampk*$^{-/-}$ DCs as compared to *ampk*$^{+/+}$ cells.

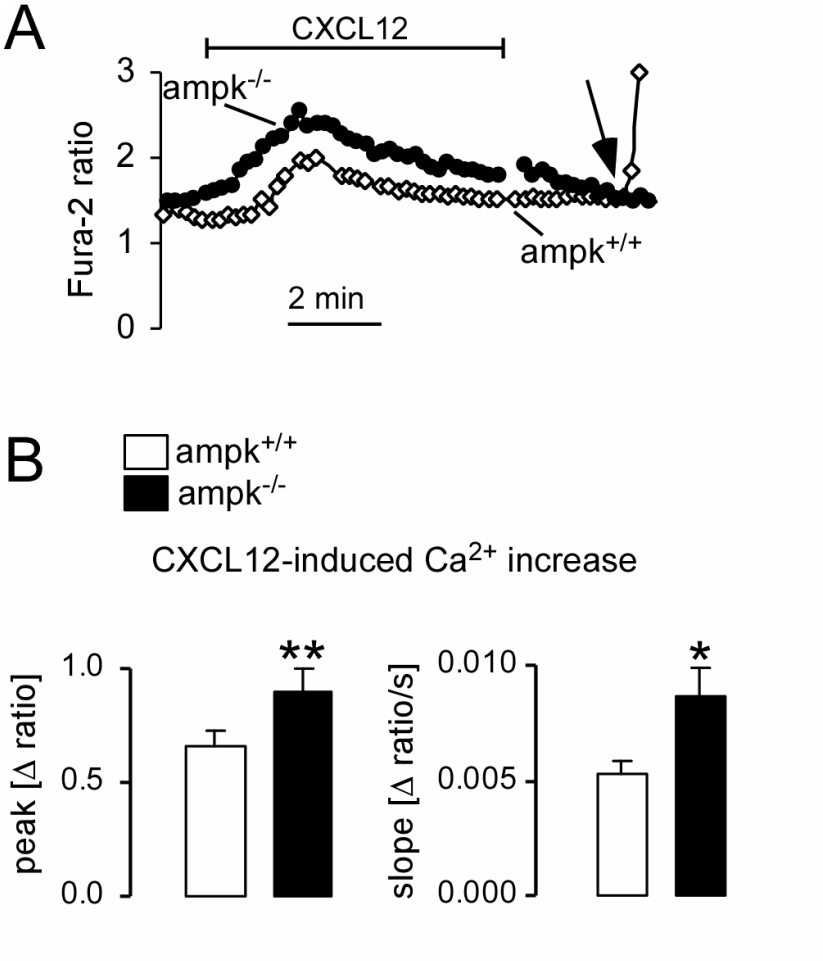

Figure 20. Enhanced CXCL12-dependent increase of $[Ca^{2+}]_i$ in *ampk*$^{-/-}$ DCs.

A. Representative tracing showing the Fura-2 fluorescence ratio in Fura-2/AM loaded *ampk*$^{+/+}$ and *ampk*$^{-/-}$ DCs prior to and following acute addition of CXCL12 (300 ng/ml, white arrow). At the end of each experiment ionomycin (10 µM; black arrows) was added for calibration purposes. For quantification of $[Ca^{2+}]_i$ increase, the slope (Δ ratio/s) and the peak (Δ ratio) were calculated. At the end of each experiment ionomycin (10 µM; black arrows) was added for calibration purposes.

B. Mean (± SEM) of the peak (left) and slope (right) of the change in Fura-2 fluorescence following addition of CXCL12 (300 ng/ml) to *ampk*$^{+/+}$ (n= 16, open bars) and *ampk*$^{-/-}$ (n= 22, closed bars) DCs. * ($p<0.05$), ** ($p<0.01$), two-tailed unpaired *t*-test.

$[Ca^{2+}]_i$ increase upon ligation of DC chemokine receptors involves the release of Ca^{2+} from the

intracellular stores followed by the Ca^{2+} entry through the **s**tore-**o**perated **C**a^{2+} (SOC) channels (Shumilina et al. 2011). To explore, whether AMPK influences Ca^{2+} release and/or SOC entry, *ampk*$^{-/-}$ and *ampk*$^{+/+}$ DCs were treated with the endosomal Ca^{2+} ATPase inhibitor thapsigargin (1 µM). Thapsigargin was first applied in the absence of extracellular Ca^{2+} in order to empty the intracellular stores (**Fig. 21A**). The thapsigargin-induced release of Ca^{2+} from the cytoplasmic stores was similar in *ampk*$^{-/-}$ and *ampk*$^{+/+}$ DCs (**Fig. 21A,B**). The readdition of extracellular Ca^{2+} was followed by a sharp increase of cytosolic Ca^{2+} in *ampk*$^{+/+}$ DCs, an effect significantly enhanced in *ampk*$^{-/-}$ cells (**Fig. 21A,B**).

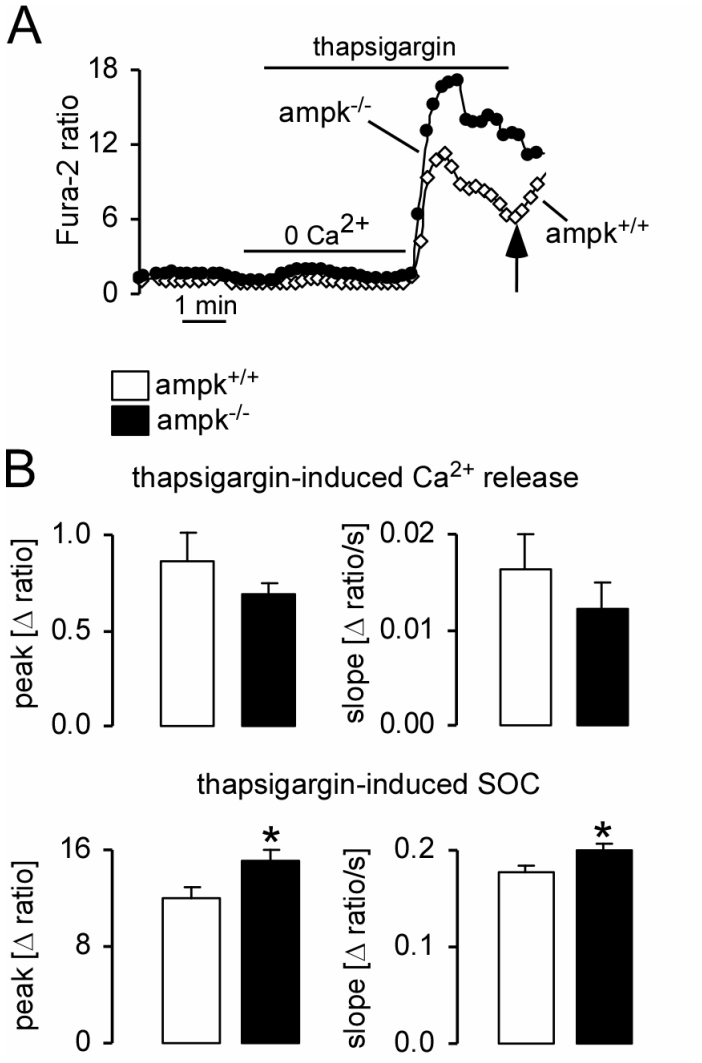

Figure 21. Increased store-operated Ca^{2+} entry in *ampk*$^{-/-}$ DCs.

A. Representative original tracings showing the Fura-2 fluorescence ratios (340/380 nm) in Fura-2/AM loaded *ampk*$^{+/+}$ and *ampk*$^{-/-}$ DCs. Experiments were carried out prior to and during exposure to nominally Ca^{2+}-free bath solution. Where indicated, thapsigargin (1 µM) was added to the nominally Ca^{2+}-free bath solution and the release of Ca^{2+} from the stores was assessed. Readdition of extracellular Ca^{2+} in the presence of thapsigargin reflects the

entry of Ca^{2+} through the SOCs. At the end of each experiment ionomycin (10 µM; black arrows) was added for calibration purposes.

B. Mean (± SEM) of the peak (left) and slope (right) of the change in Fura-2 fluorescence following addition of thapsigargin (1 µM) in the absence (Ca^{2+}-release, upper bars) and in the presence (Ca^{2+} entry, lower bars) of extracellular Ca^{2+} in *ampk*$^{+/+}$ (n= 16, open bars) and *ampk*$^{-/-}$ (n= 34, closed bars) DCs. * ($p<0.05$), two-tailed unpaired *t*-test.

Moreover, the protein expression of the SOC channel pore-forming subunit, Orai 1, was increased, whereas protein abundance of Orai2, and Ca^{2+}-sensing subunits in the ER, STIM1 and STIM2, was unchanged in *ampk*$^{-/-}$ DCs (**Fig. 22 A,B**).

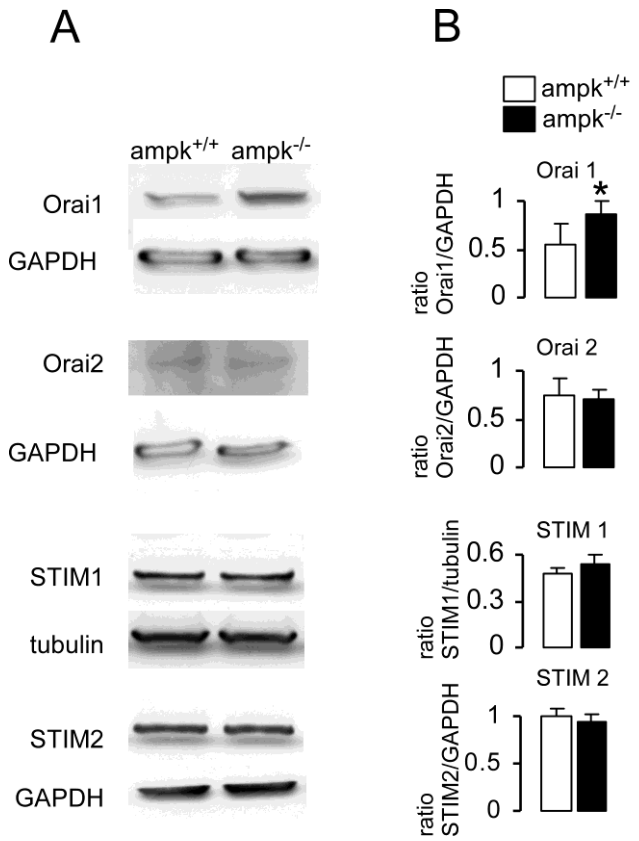

Figure 22. Expression of Orai1, Orai2, STIM1 and STIM2 in *ampk*$^{-/-}$ DCs. Western blot analysis of whole cell lysate protein of Orai1, Orai2, STIM1 and STIM2.

A. Representative experiments showing Orai1-2 and STIM1-2 bands. Blots were stripped and reprobed with a GAPDH or tubulin antibody to determine equal protein loading.

B. Arithmetic means ±SEM of Orai1/GAPDH ratio (n = 4), Orai2/GAPDH ratio (n = 4), STIM1/tubulin ratio (n = 6) and STIM2/GAPDH ratio (n = 6) in *ampk*$^{+/+}$ and *ampk*$^{-/-}$ DCs. *($p<0.05$) at paired comparison.

4.5. AMPK Downregulates K^+-Dependent (NCKX) and K^+-Independent (NCX) Na^+/Ca^{2+} Exchangers

Ca^{2+} signal development is well coordinated and timely terminated through the concerted action of several pumps and transporters situated in the plasma and ER membranes. Extremely important terminators of Ca^{2+} signals in DCs are **K**$^+$-dependent (NCKX) and K^+-independent (NCX) **N**a^+/**C**a^{2+} **e**xchangers (Heise et al. 2011;Shumilina et al. 2010). Therefore, the present study explored the possibility that AMPK could modify in addition Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers. To estimate Na^+/Ca^{2+} exchanger activity, $[Ca^{2+}]_i$ was measured upon removal of external Na^+ in the presence of external Ca^{2+}, a maneuver reversing the driving force and triggering Ca^{2+} influx via Na^+/Ca^{2+} exchangers (Blaustein and Lederer 1999). As illustrated in **Fig. 23**, the increase of $[Ca^{2+}]_i$ following Na^+ removal measured either in K^+-free extracellular solution (to assess NCX) or in K^+-containing bath (40 mM, to analyze NCKX) was significantly higher in *ampk*$^{-/-}$ DCs than in *ampk*$^{+/+}$ DCs.

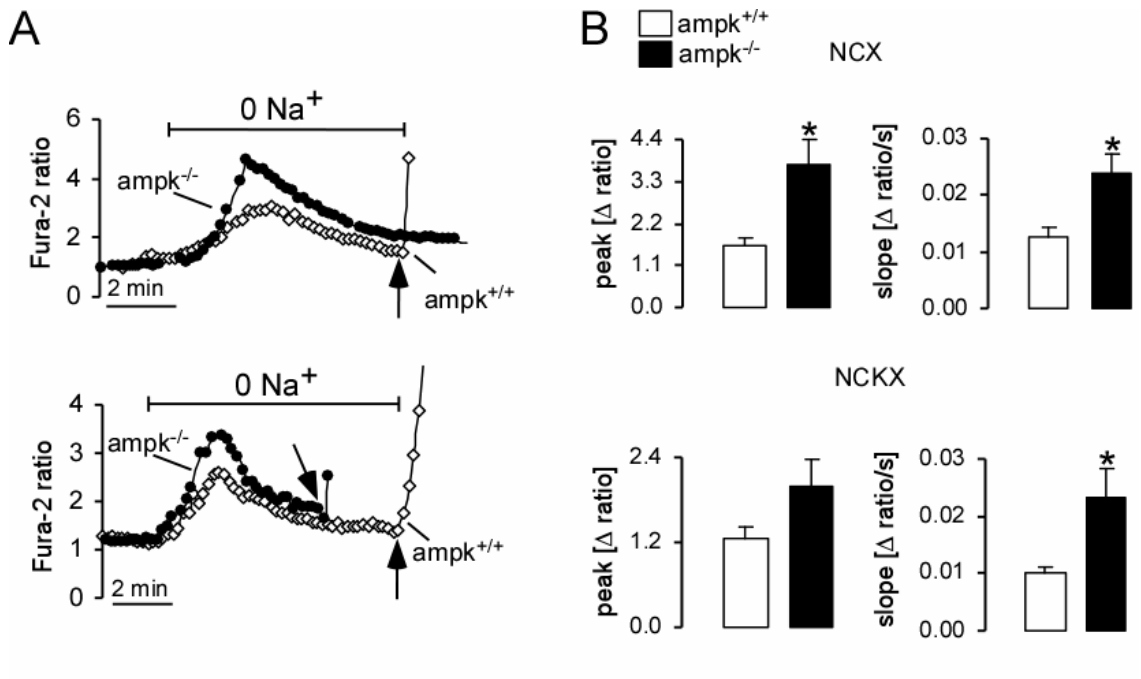

Figure 23. Activity of K^+ dependent and K^+ independent Na^+/Ca^{2+} exchangers in DCs from *ampk*$^{+/+}$ and *ampk*$^{-/-}$mice.

A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded *ampk*$^{+/+}$ DCs (white symbols) and *ampk*$^{-/-}$ DCs (black symbols) prior to and following removal of external Na^+ (0 Na^+) in the presence of 0 mM K^+ (upper panel) or 40 mM K^+ (lower panel). At the end of each experiment ionomycin (10 µM; black arrows) was added for calibration purposes.

B. Arithmetic means (± SEM) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} concentrations in *ampk*$^{+/+}$ DCs (white bars, n = 8) and *ampk*$^{-/-}$ DCs (black bars, n = 11) following removal of external Na^+ in the presence of 0 mM K^+ (upper panels) or 40 mM K^+ (lower panels). * ($p<0.05$), two-tailed unpaired *t*-test.

In an additional approach the patch clamp technique was applied to measure Na^+/Ca^{2+} exchanger currents. The Na^+/Ca^{2+} exchanger currents were determined following reversal of transport mode by creating the respective ionic gradients (Aneiros et al. 2005). Removing extracellular Ca^{2+} in the presence of external Na^+ triggers Ca^{2+} efflux and removing extracellular Na^+ in the presence of extracellular Ca^{2+} triggers Ca^{2+} influx. The holding potential was kept at 0 mV and whole cell currents were recorded upon voltage step to the potential of -80 mV applied for 2 min during the exchange of solutions. Na^+/Ca^{2+} exchanger currents were analyzed as the outward current components calculated as difference in currents (ΔI) elicited by the change from Na^+-containing, Ca^{2+}-free solutions to Na^+-free, Ca^{2+}-containing solutions (**Fig. 24**). Similar to fura-2 fluorescence imaging, the Na^+/Ca^{2+} exchange currents, measured in both, K^+-free and K^+-containing solutions, were significantly higher in *ampk*$^{-/-}$ DCs than in *ampk*$^{+/+}$ DCs (**Fig. 24).**

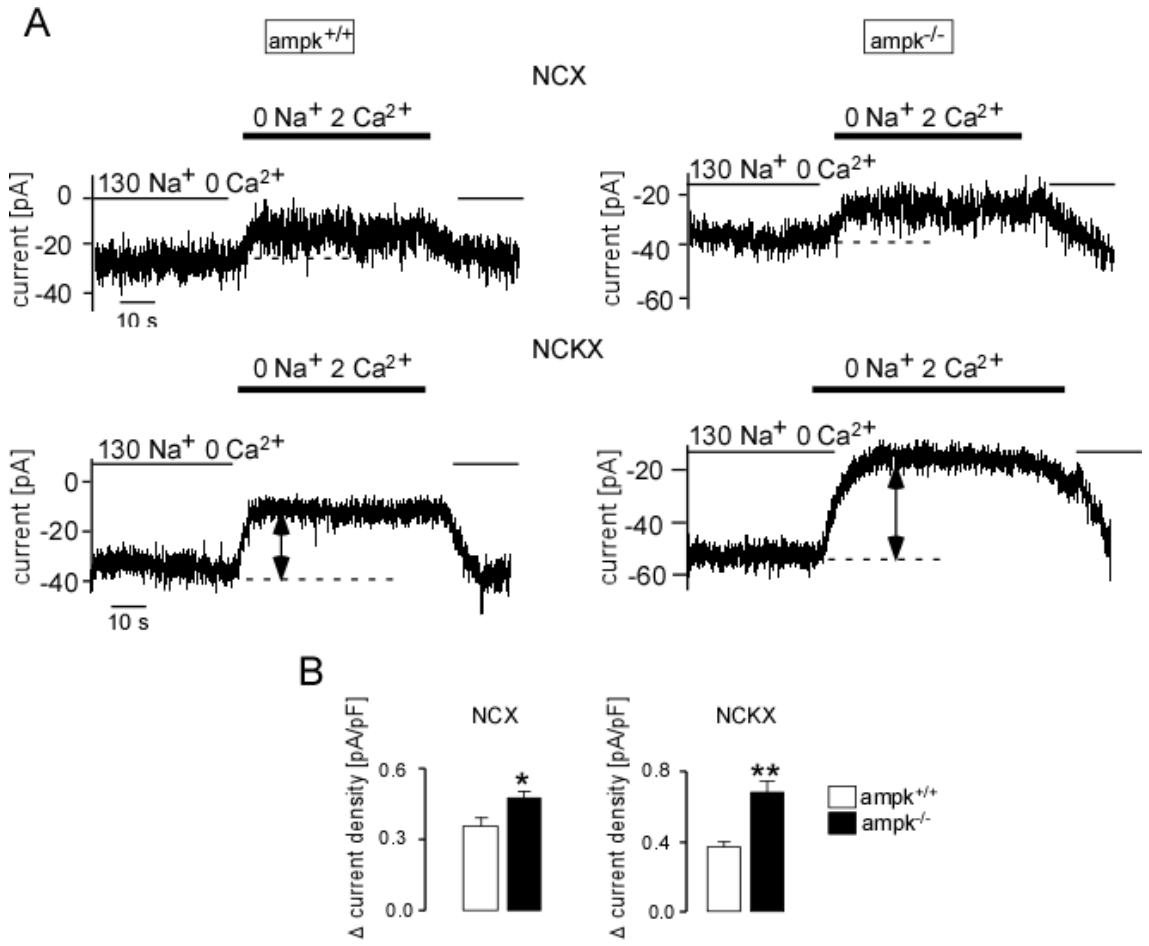

Figure 24. K^+ dependent and K^+ independent Na^+/Ca^{2+} exchanger currents in DCs from *ampk*$^{+/+}$ and *ampk*$^{-/-}$mice.

A. Whole cell currents recorded at -80 mV in *ampk*$^{+/+}$ DCs (left) and *ampk*$^{-/-}$ DCs (right) during the switch between external solutions that contained 0 mM K^+ (upper panels) or 40 mM K^+ (lower panels) and either 130 mM Na^+ and no Ca^{2+} (130 Na^+ 0 Ca^{2+}) or 2 mM Ca^{2+} and no Na^+ (0 Na^+ 2 Ca^{2+}). The internal solution stimulated Na^+ - overload and Ca^{2+} plateau levels (1 µM free Ca^{2+}, 120 mM Na^+, 40 mM K^+). Cesium and TEA^+ were present in the solutions to block K^+ channel currents.

B. Arithmetic means (± SEM) of current density changes (ΔI, pA/pF) at -80 mV in *ampk*$^{+/+}$ DCs (white bars, n = 20) and *ampk*$^{-/-}$ DCs (black bars, n = 20) induced by the switch between external solutions containing 130 Na^+, 0 Ca^{2+} and 0 Na^+, 2 Ca^{2+}. The internal solution was as in A. * ($p<0.05$) and ** ($p<0.01$), two-tailed unpaired *t*-test.

In conclusion, AMPK, an intracellular energy sensor, is an important new regulator of Ca^{2+} signaling in DCs. AMPK downregulating store-operated Ca^{2+} entry and Na^+/Ca^{2+} exchanger activity, is a powerful negative regulator of Ca^{2+}-dependent functions in dendritic cells.

The second project of the present study aimed to elucidate the possible regulation of Ca^{2+} signaling and Ca^{2+}-dependent functions in DCs by the anti-aging protein Klotho, a well-known regulator of several ion channels.

4.6. Transmembrane and Secreted Klotho Transcript Levels are Negligible in Mouse DCs from *klotho*$^{+/+}$ and *klotho*^{hm} Mouse

Klotho protein can be found as a membrane and as a secreted form. Secreted Klotho and transmembrane Klotho transcriptional termination sites are located in exon 1 and in exon 3/4, respectively (Shiraki-Iida et al. 1998). DCs were isolated from the bone marrow of *klotho*^{hm} and *klotho*$^{+/+}$ mice to determine transcript levels of both forms in mouse DCs by RT-PCR analysis. Using the primers for both, the transmembrane (exon 3/4) and the secreted (exon 1) form of Klotho we demonstrated that the Klotho transcript abundance was negligible in mouse DCs. Kidney tissue of Klotho deficient mice (*klotho*^{hm}) and wild type littermates (*klotho*$^{+/+}$) was used as a negative and positive control, respectively (**Fig. 24).**

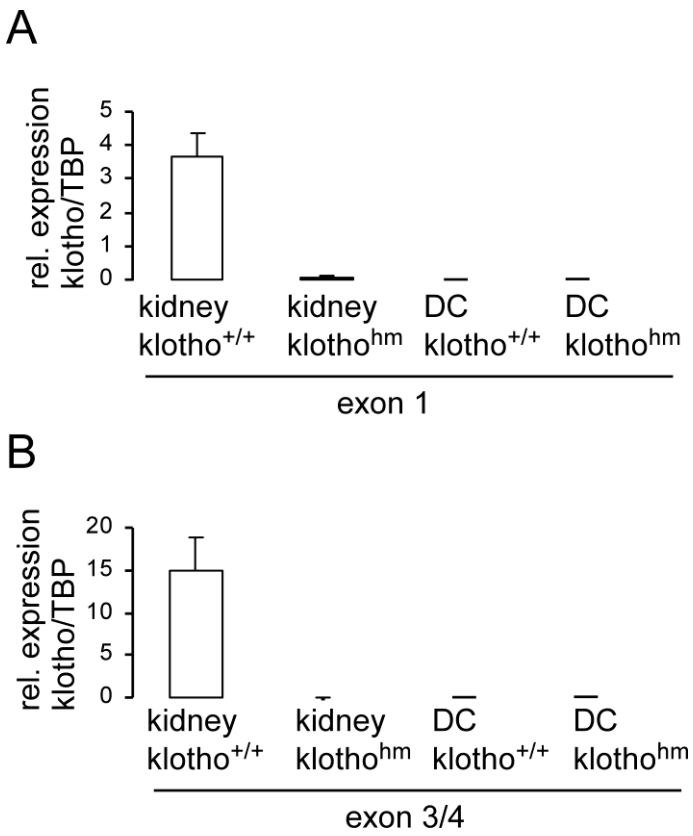

Figure 25. Expression of secreted and transmembrane klotho in DCs from *klotho*$^{+/+}$ and *klotho*^{hm} mice as compared to kidney tissue.

mRNA abundance (± SEM, n = 3) of secreted (exon 1, **A**) and transmembrane (exon 3/4, **B**) form of klotho detected by real-time PCR analysis in DCs and kidneys from *klotho*$^{+/+}$ and *klotho*^{hm} mice using TBP mRNA as a reference gene.

4.7. Klotho Deficiency has no Effect on Maturation and Differentiation of Mouse DCs

To investigate whether Klotho deficiency has an effect on DC maturation and differentiation, DC maturation was induced by LPS (LPS, 1 µg/ml, 24 h). As shown in **Fig. 26**, costimulatory molecule CD86 and antigen-presenting molecule MHC II were similarly expressed on CD11c-positive immature and mature DCs from *klotho*^{hm} and *klotho*$^{+/+}$ mice as analyzed by FACS.

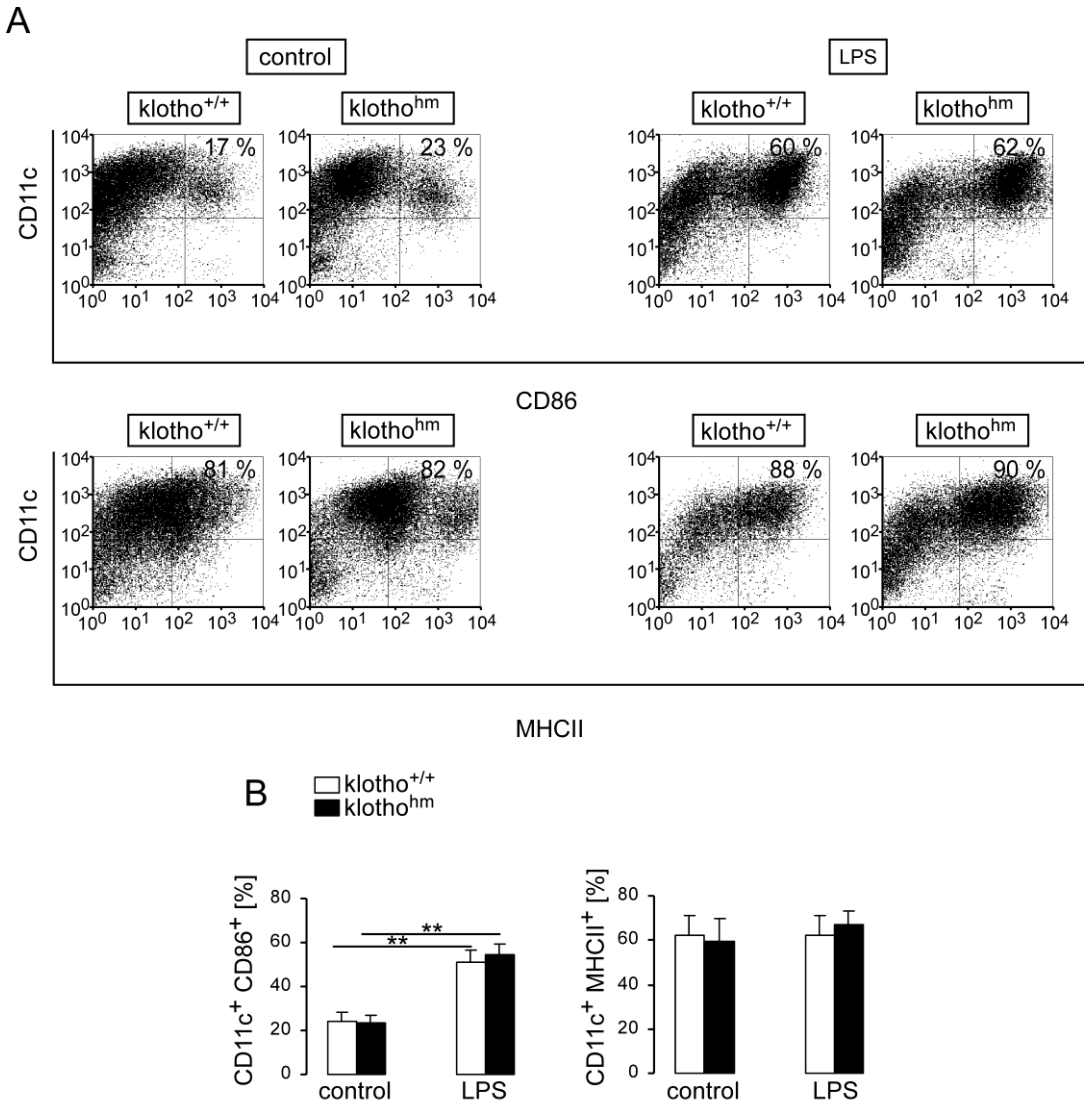

Figure 26. Differentiation and maturation of bone marrow derived DCs from *klotho*$^{+/+}$ and *klotho*^{hm} mice.

A. Original dot plots of CD11c^{+}CD86^{+} (above) and CD11c^{+}MHC II^{+} (below) DCs at the basal level (control, 1st and 2nd panels) and stimulated with LPS (LPS, 1 µg/ml, 24 h, 3rd and 4th panels) from *klotho*$^{+/+}$(1st and 3rd panels) and *klotho*^{hm} (2nd and 4th panels) mice. Numbers depict the percent of cells in the respective quadrants.

B. Arithmetic means ± SEM (n = 4) of the percentage of CD11c^{+}CD86^{+} (left) and CD11c^{+}MHC II^{+} (right) DCs under control and 24 h after LPS stimulation in primary cultures from *klotho*$^{+/+}$ (open bars) and *klotho*^{hm} (closed bars) mice. ** ($p<0.01$), ANOVA.

4.8. LPS-Induced Increase of $[Ca^{2+}]_i$ is Impaired in *klotho*^{hm} DCs, but not in *klotho*^{hm} LVD DCs.

Klotho regulates several ion channels and transporters, some of them being permeable for Ca^{2+} (Cha et al. 2008). To check whether *klotho* has an effect on the SOC entry, the major Ca^{2+} entry pathway in DCs, Ca^{2+} imaging experiments were performed. DCs were stimulated with LPS (100 ng/ml), since LPS is known to stimulate the entry of Ca^{2+} through SOC channels (Matzner et al. 2008). Analysis of $[Ca^{2+}]_i$ revealed that the LPS-induced increase of $[Ca^{2+}]_i$ was significantly lower in DCs isolated from *klotho*^{hm} mice than in DCs isolated from *klotho*$^{+/+}$ mice (**Fig. 27**).

The impaired LPS- induced increase of $[Ca^{2+}]_i$ in *klotho*^{hm} DCs could be completely restored by feeding *klotho*^{hm} mice a low vitamin D diet (*klotho*^{hm} LVD) (**Fig. 27**).

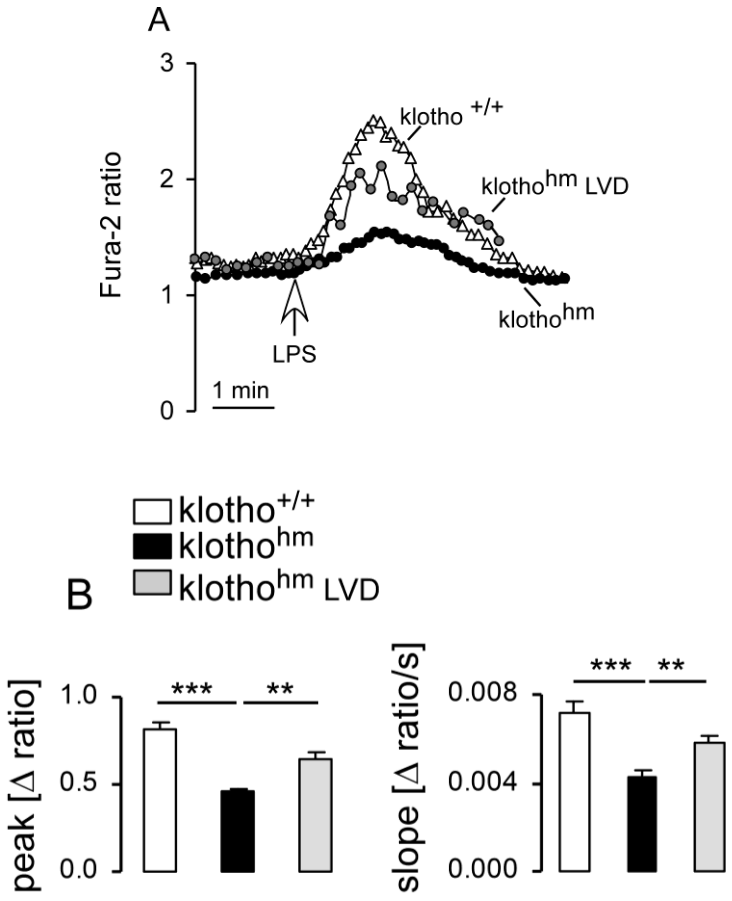

Figure 27. LPS-induced increase of $[Ca^{2+}]_i$ in *klotho*$^{+/+}$, *klotho*^{hm} and *klotho*^{hm} LVD DCs.

A. Representative original tracings showing intracellular Ca^{2+} ratio in Fura-2/AM loaded *klotho*$^{+/+}$ DCs (white symbols), *klotho*^{hm} DCs (black symbols) and *klotho*^{hm} LVD DCs (grey symbols) prior to and following acute addition

of LPS (1 µg/ml; white arrow). For quantification, peak (Δ ratio) and slope (Δ ratio/s) of $[Ca^{2+}]_i$ increase following addition of LPS were calculated.

B. Mean (± SEM) of the peak value (left) and slope (right) of the change in $[Ca^{2+}]_i$ following addition of LPS to *klotho*$^{+/+}$ (n= 66, open bars), *klotho*^{hm} (n= 96, closed bars) and *klotho*^{hm} LVD (n= 79 grey bars) DCs. ** ($p<0.01$) and *** ($p<0.001$), ANOVA.

4.9. NCKX Activity is Increased in *klotho*^{hm} DCs but not in *klotho*^{hm} LVD DCs.

Since vitamin D is a powerful regulator of the K^+-dependent Na^+/Ca^{2+} exchanger NCKX1 in DCs (Shumilina et al. 2010), we analyzed whether Klotho deficiency impacted on NCKX activity. Na^+/Ca^{2+} exchanger activity was estimated by measuring $[Ca^{2+}]_i$ upon removal of external Na^+ in the presence of external Ca^{2+} and K^+ (40 mM). As a result, NCKX-induced Ca^{2+} entry was significantly higher in DCs isolated from *klotho*^{hm} mice receiving control diet than in DCs isolated from *klotho*$^{+/+}$ DCs. Treatment of *klotho*^{hm} mice with a low vitamin D diet significantly decreased the NCKX-induced Ca^{2+} entry in *klotho*^{hm} LVD DCs and thus dissipated the difference of NCKX-induced Ca^{2+} entry between *klotho*^{hm} DCs and *klotho*$^{+/+}$ DCs (**Fig. 28).**

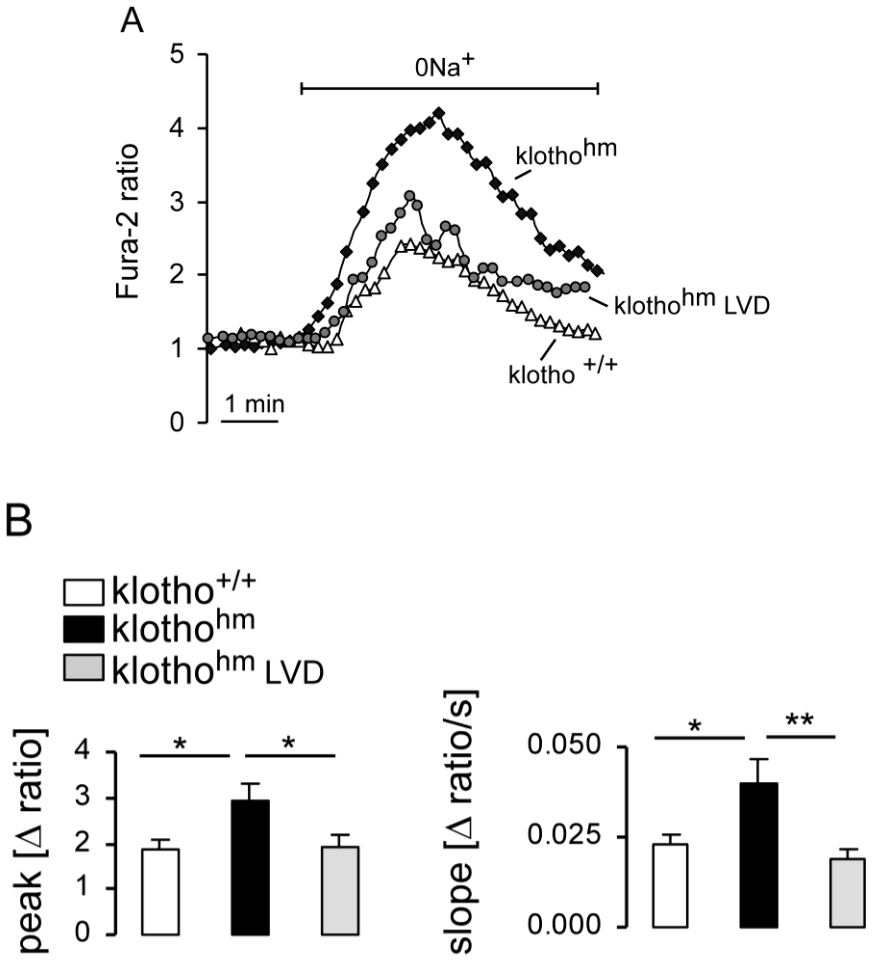

Figure 28. Activity of K^+ dependent Na^+/Ca^{2+} exchangers in DCs from *klotho*$^{+/+}$, *klotho*^{hm} and *klotho*^{hm} LVD mice.

A. Representative original tracings showing intracellular Ca^{2+} ratio in Fura-2/AM loaded *klotho*$^{+/+}$ DCs (white symbols), *klotho*^{hm} DCs (black symbols) and *klotho*^{hm} LVD DCs (grey symbols) prior to and following removal of external Na^+ (0 Na^+) in the presence of 40 mM K^+.

B. Arithmetic means (± SEM) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} ratio in *klotho*$^{+/+}$ DCs (white bars, n = 35), *klotho*^{hm} DCs (black bars, n = 42) and *klotho*^{hm} LVD (n= 37 grey bars) DCs. * ($p<0.05$), ** ($p<0.01$), (ANOVA).

Thus, additional experiments were performed exploring whether *in vitro* pretreatment of *klotho*$^{+/+}$ DC precursors with $1,25(OH)_2D_3$ (50 nM) first two days after their isolation from the bone marrow followed by the next 5 days of culturing in the absence of $1,25(OH)_2D_3$ modified NCKX activity. As shown in **Fig. 29**, the pretreatment with $1,25(OH)_2D_3$ indeed significantly upregulated NCKX-induced Ca^{2+} entry in *klotho*$^{+/+}$ DCs.

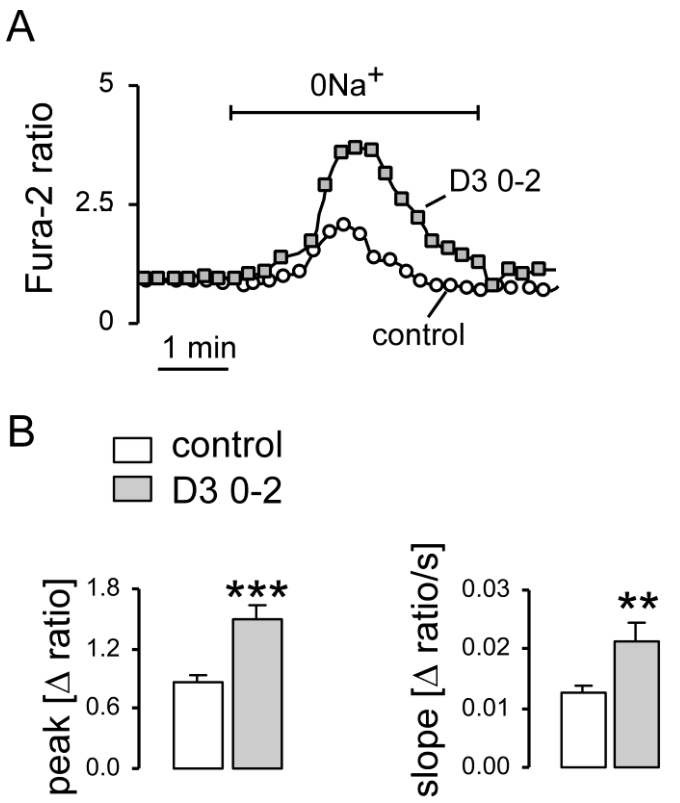

Figure 29. Activity of K^+ dependent Na^+/Ca^{2+} exchangers in *klotho*$^{+/+}$ DCs is enhanced by pre-treatment of DC precursors with $1,25(OH)_2D_3$ during first 2 days after isolation from the bone marrow.

A. Representative original tracings showing intracellular Ca^{2+} concentrations prior to and following removal of external Na^+ (0 Na^+) in *klotho*$^{+/+}$ DCs untreated (control) and treated (D3 0-2) with $1,25(OH)_2D_3$ (50 nM) first 2 days after DC precursor isolation from the bone marrow followed by 5 days of culturing in the absence of $1,25(OH)_2D_3$.

B. Arithmetic means ± SEM (n = 54-85) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} ratio in control and $1,25(OH)_2D_3$ –treated (as in **A**) DCs from *klotho*$^{+/+}$ mice following removal of external Na^+ in the presence of 40 mM K^+. * ($p<0.05$), unpaired t-test.

4.10. The Inhibitory Effect of Klotho on LPS-Induced $[Ca^{2+}]_i$ Increase is Abolished by the Blocker of K^+ Dependent Na^+/Ca^{2+} Exchangers.

As illustrated in **Fig. 30**, the difference in LPS-induced $[Ca^{2+}]_i$ increase between *klotho*$^{+/+}$ DCs and *klotho*^{hm} DCs was dissipated in the presence of NCKX-blocker 3',4'-dichlorobenzamyl

(DBZ, 10 µM). Thus, upregulation of NCKX in *klotho*^{hm} DCs accounted in large part for the blunted LPS-induced increase of $[Ca^{2+}]_i$ in *klotho*^{hm} DCs.

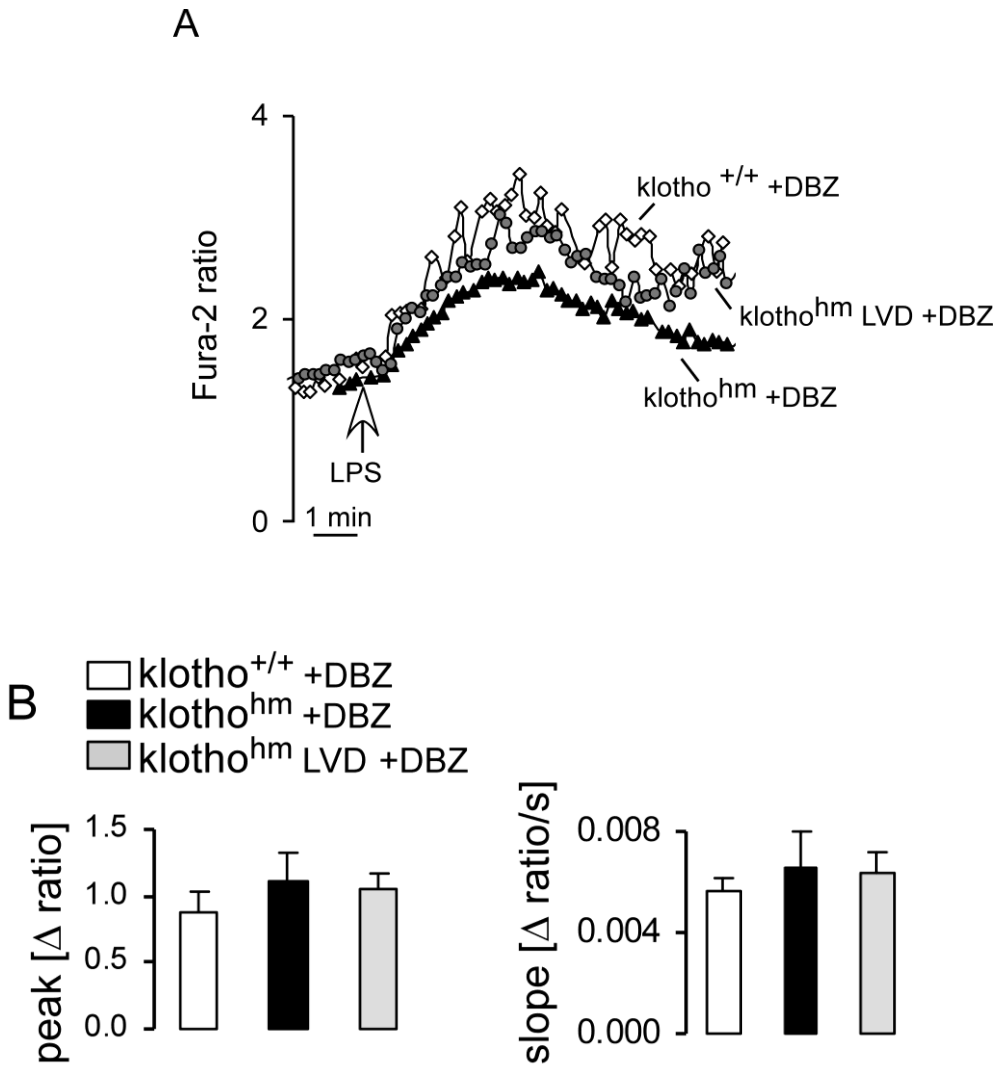

Figure 30. LPS-induced increase of $[Ca^{2+}]_i$ in *klotho*$^{+/+}$, *klotho*^{hm} and *klotho*^{hm} *LVD* DCs in the presence of the K^+-dependent Na^+/Ca^{2+} exchanger blocker 3',4'-dichlorobenzamyl (DBZ).

A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded *klotho*$^{+/+}$ DCs (white symbols), *klotho*^{hm} DCs (black symbols) and *klotho*^{hm} LVD DCs (grey symbols) treated with the K^+-dependent Na^+/Ca^{2+} exchanger blocker 3',4'-dichlorobenzamyl (DBZ,10 µM) prior to and following acute addition of LPS (1 µg/ml).

B. Mean (± SEM) of the slope value (left) and peak (right) of the change in $[Ca^{2+}]_i$ following addition of LPS to *klotho*$^{+/+}$ (n= 29, open bars), *klotho*^{hm} (n= 21, closed bars) and *klotho*^{hm} LVD (n= 21 grey bars) DCs.

4.11.CCL21-Induced Migration of LPS-Matured DCs is Impaired in DCs Isolated from *klotho^{hm}* Mice Fed Control, but not LVD Diet.

The most Ca^{2+}-sensitive DC functions include migration (Connolly and Kusner 2007;Shumilina et al. 2011). Accordingly, migration of immature DCs towards the chemokine CXCL12 and migration of LPS-matured DCs towards CCL21 was analyzed in the transwell chamber assay. Spontaneous and CCL21-induced migration of LPS-matured DCs was significantly less pronounced in *klotho^{hm}* DCs isolated from mice fed control diet than in *klotho$^{+/+}$* DCs (**Fig. 31**). Migration of immature *klotho^{hm}* DCs towards CXCL12 tended to be lower in *klotho^{hm}* DCs than in *klotho$^{+/+}$* DCs, a difference, however, not reaching statistical significance (**Fig. 31**). Migration of DCs isolated from *klotho^{hm}* mice fed LVD diet was not impaired (**Fig. 31**).

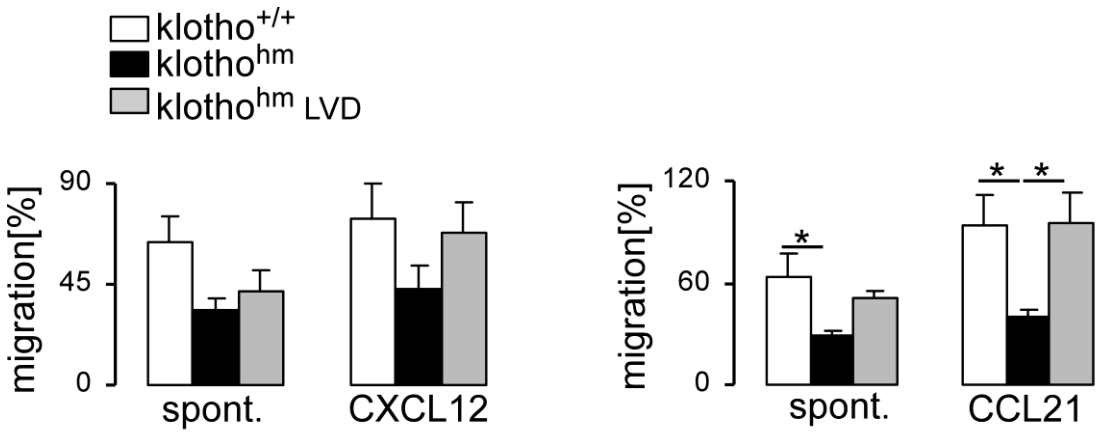

Figure 31. Migration of *klotho$^{+/+}$*, *klotho^{hm}* and *klotho^{hm}* LVD DCs.

A. Arithmetic means ± SEM (n = 8) of migrating *klotho$^{+/+}$* (open bars), *klotho^{hm}* (closed bars) and *klotho^{hm}* LVD (grey bars) DCs in the absence and presence of CXCL12 (50 ng/ml, 4h).

B. Arithmetic means ± SEM (n = 6) of migrating LPS (1 µg/ml, 24 h)-matured *klotho$^{+/+}$* (open bars), *klotho^{hm}* (closed bars) and *klotho^{hm}* LVD (grey bars) DCs in the absence and presence of CCL21 (25 ng/ml, 4h). * ($p<0.05$), ANOVA.

The NCKX blocker DBZ (10 µM, 4h) had no effect on CCL21-induced migration of *klotho$^{+/+}$* DCs or *klotho^{hm}* DCs from the mice fed a LVD diet (Fig. 30). However, DBZ restored the impaired migration of *klotho^{hm}* DCs to the level observed in *klotho$^{+/+}$* DCs (**Fig. 32**).

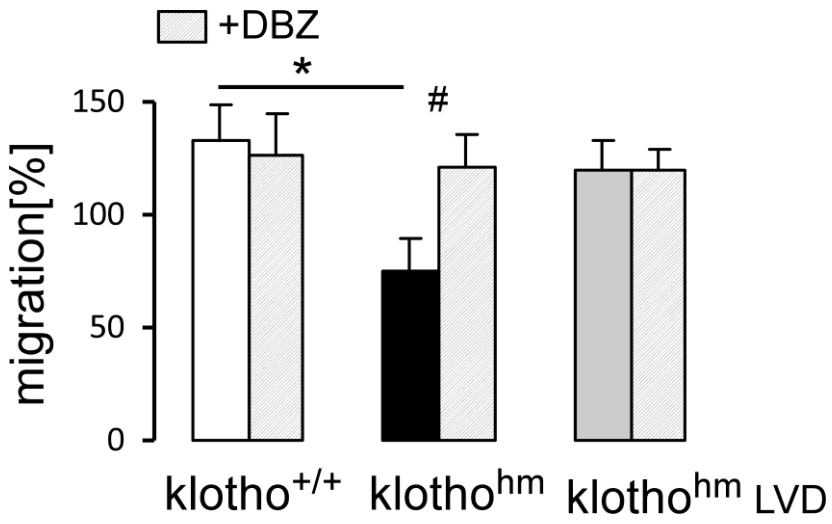

Figure 32. Migration of *klotho*$^{+/+}$, *klotho*^{hm} and *klotho*^{hm} LVD DC DCs in the presence of the K^+-dependent Na^+/Ca^{2+} exchanger blocker 3',4'-dichlorobenzamyl (DBZ).

Arithmetic means ± SEM (n = 8-16) of CCL21 (25 ng/ml, 4h)-induced migration of LPS (1 µg/ml, 24 h)-matured *klotho*$^{+/+}$, *klotho*^{hm} and *klotho*^{hm} LVD DCs measured in the absence or in the presence of DBZ (10 µM). * ($p<0.05$), ANOVA; $^{\#}$ ($p<0.05$), significant difference only with unpaired t-test.

In conclusion, Klotho deficiency leads to sustained up-regulation of Na^+/Ca^{2+} exchange activity and thus blunts the increase of $[Ca^{2+}]_i$ following LPS exposure and CCL21-mediated migration. The effects are in large part due to excessive $1,25(OH)_2D_3$ formation in Klotho deficient mice.

5. DISCUSSION

The present study discloses a novel function of **AM**P-activated **p**rotein **k**inase (AMPK), i.e. inhibition of both, **s**tore **o**perated **C**a^{2+} **e**ntry (SOCE) and Na^+/Ca^{2+} exchanger activity in **d**endritic **c**ells (DCs). One of the most important Ca^{2+} influx pathways in DCs is SOC or capacitative Ca^{2+} entry via **C**a^{2+} **r**elease-**a**ctivated **C**a^{2+} (CRAC) channels, which are activated upon Ca^{2+} release from intracellular stores (Hsu et al. 2001;Matzner et al. 2008). As shown recently, mainly the pore-forming subunit Orai2 and the Ca^{2+}-sensing subunit STIM2 generate SOC entry in DCs (Bandyopadhyay et al. 2011), though Orai1, Orai3 and STIM1 are also expressed in mouse DCs (Matzner et al. 2008). In the present study we show that Orai1 protein expression is enhanced in *ampk*$^{-/-}$ DCs. The isoform Orai1 has most recently been shown to be down-regulated by the ubiquitin ligase Nedd4-2 (**n**euronal precursor cells **e**xpressed **d**evelopmentally **d**ownregulated) (Eylenstein et al. 2011), which has previously been shown to be involved in the AMPK sensitive down-regulation of ENaC (**e**pithelial **Na**$^+$ **c**hannel) (Almaca et al. 2009;Bhalla et al. 2006;Carattino et al. 2005) and KCNQ1 (voltage gated K^+ channels) (Alzamora et al. 2010). Along those lines, the increased SOCE in *ampk*$^{-/-}$ DCs may at least in part result from decreased Nedd4-2 activity and thus decreased ubiquitination and degradation of Orai proteins. The present study does, however, not rule out other mechanisms, such as regulation of the channel by phosphorylation of the channel protein. Hypoxia has been shown to increase cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), which leads to activation of **Ca**$^{2+}$/cal**m**odulin-dependent **k**inase **k**inase-**beta II β** (CaMKK II β) and CaMKK II β-dependent phosphorylation of AMPK (Mungai et al. 2011). Knockdown of STIM1 by siRNA attenuates Ca^{2+} responses to hypoxia and subsequent AMPK phosphorylation (Mungai et al. 2011). The present study shows that AMPK triggers a negative feedback loop with SOC channels.

The inhibition of Na^+/Ca^{2+} exchangers (**K**$^+$-dependent and K^+-independent **N**a^+/**C**a^{2+} e**x**changers (NCKX and NCX) by AMPK in energy depleted cells is a double edged sword. On the one hand, the inhibition of the carrier prevents Na^+ entry through the exchanger. On the other hand, the impairment of Ca^{2+} extrusion augments the increase of $[Ca^{2+}]_i$ following Ca^{2+} entry. As AMPK further regulates Ca^{2+} entry, the impact of AMPK dependent inhibition of Na^+/Ca^{2+} exchangers on $[Ca^{2+}]_i$ is blunted. AMPK is activated by an increase of $[Ca^{2+}]_i$ (Bair et al. 2009;Mungai et al. 2011;Towler and Hardie 2007;Zhang et al. 2009), which can be accomplished by SOC channels (Mungai et al. 2011). Without inhibition of Ca^{2+} entry, the

inhibition of Ca^{2+} extrusion by AMPK would lead to further increase of $[Ca^{2+}]_i$ and thus to cellular Ca^{2+} overload.

AMPK-dependent suppression of Na^+/Ca^{2+} exchangers may be also relevant for other cell types expressing these transporters, such as a wide variety of excitable cells including cardiomyocytes (Reppel et al. 2007;Sipido et al. 2007;Venetucci et al. 2007) and neurons (Canitano et al. 2002;Lytton 2007), retinal rod photoreceptors and smooth muscle cells (Blaustein and Lederer 1999). Moreover, Na^+/Ca^{2+} exchangers are expressed in several nonexcitable cells, such as renal epithelial cells (Schmitt et al. 1999), platelets (Kimura et al. 1999), lymphocytes (Balasubramanyam et al. 1994), neutrophils (Tintinger and Anderson 2004), mast cells (Aneiros et al. 2005;Rumpel et al. 2000) macrophages and monocytes (Aneiros et al. 2005;Staiano et al. 2009).

Inhibition of the cell membrane Ca^{2+} transport molecules presumably leads to a strong AMPK-dependent downregulation of DC migration in response to the chemokine CXCL12. The chemotactic response of DCs to several chemokines is dependent on Ca^{2+} influx (Barbet et al. 2008;Koski et al. 1999;Partida-Sanchez et al. 2004a;Rubartelli and Poggi 1997). Blocking of SOC channels (or depolarizing cells by inhibition of Kv channels) impairs chemokine-dependent migration (Matzner et al. 2008;Xuan et al. 2009). Even though, Ca^{2+} influx is required for DC chemotaxis, Ca^{2+} overload considerably impairs chemokine-dependent DC migration (Barbet et al. 2008). Blockade of NCX leads to an almost complete inhibition of migration which is accompanied by a increase of $[Ca^{2+}]_i$ and an intracellular alkalinisation in MDCK-F cells (Dreval et al. 2005). Therefore, Ca^{2+} signals should be tightly regulated to avoid DC unresponsiveness to chemokines. AMPK may be an elegant regulator of DC migration which acts on both Ca^{2+} entry (via SOC channels) and Ca^{2+} extrusion (via Na^+/Ca^{2+} exchangers).

Suppression of DC migration by AMPK corresponds well to the known role of AMPK in antagonizing DC activation (Krawczyk et al. 2010). Particularly, AMPK has been shown to inhibit Toll like receptor-mediated metabolic transition to aerobic glycolysis, which is essential for DC maturation (Krawczyk et al. 2010). Accordingly, suppression of AMPK expression in DCs by shRNA targeting AMPK subunits results in enhanced LPS (**l**ipo**p**oly**s**accharides)-induced IL-12p40 production and CD86 expression (Krawczyk et al. 2010). AMPK is also a powerful counter-regulator of inflammatory signaling pathways in macrophages (Sag et al. 2008). Inhibition of AMPK expression by RNA interference or

expression of a dominant negative AMPKα1 in macrophages enhanced LPS-induced TNF-α and IL-6 production and diminished the production of anti-inflammatory IL-10 (Sag et al. 2008).

AMPK is phosphorylated in its activation loop in resting DCs (Krawczyk et al. 2010) (and the present study) and resting macrophages (Sag et al. 2008). Moreover, AMPK phosphorylation is diminished rapidly after exposure of DCs and macrophages to proinflammatory agents, such as LPS (Krawczyk et al. 2010;Sag et al. 2008). In contrast, anti-inflammatory cytokines (IL-10, TGFβ) lead to a rapid phosphorylation of AMPK in macrophages (Sag et al. 2008). It is tempting to speculate that downregulation of AMPK activation in LPS-matured DCs explains the present observation that CCL21-dependent migration of mature *ampk*$^{+/+}$ and *ampk*$^{-/-}$ DCs was not different.

In conclusion, according to the present study, AMPK plays a key role in control of Ca^{2+} homeostasis through the regulation of SOCE and Na^{+}/Ca^{2+} exchangers in bone marrow derived DCs. The influence of AMPK on $[Ca^{2+}]_i$, impacts on migration and presumably further Ca^{2+} sensitive functions of DCs as well as other cell types expressing AMPK, SOCE and Na^{+}/Ca^{2+} exchangers (**Fig. 33**).

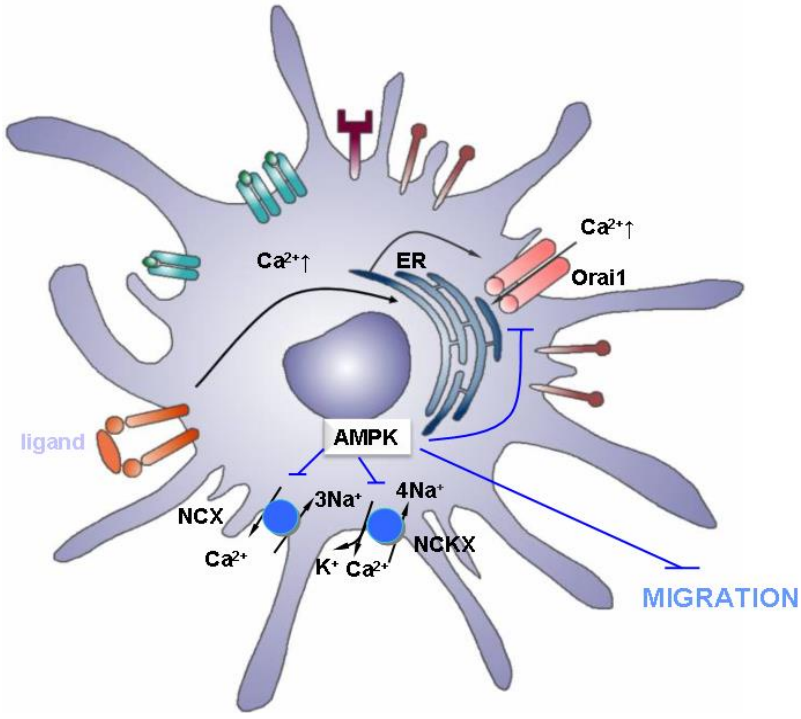

Figure 33. Role of the AMPK in the regulation of Ca^{2+} signaling and Ca^{2+} dependent functions of DCs. AMPK, AMP-activated protein kinase; ER, endoplasmic reticulum; NCKX and NCX, K^{+}-dependent and K^{+}-independent Na^{+}/Ca^{2+} exchangers.

The present study demonstrates that regulation of $[Ca^{2+}]_i$ in DCs is affected by Klotho, an anti-aging protein (Kuroo et al. 1997;Kurosu et al. 2005). Although the expression level of Klotho in mouse DCs was negligible, LPS-induced increase in $[Ca^{2+}]_i$ was significantly blunted in DCs isolated from Klotho-deficient mice (*klotho*^{hm}) as compared to DCs from wild type littermates (*klotho*$^{+/+}$). This effect was largely due to excessive $1,25(OH)_2D_3$ formation in *klotho*^{hm} mice; as it was reversed by feeding *klotho*^{hm} mice a vitamin D deficient diet. As shown earlier (Fischer et al. 2010), the plasma $1,25(OH)_2D_3$ concentrations are similar in *klotho*^{hm} mice receiving a vitamin D deficient diet as in *klotho*$^{+/+}$ mice.

We have previously shown that $1,25(OH)_2D_3$ strongly impaired the LPS-induced $[Ca^{2+}]_i$ increase by stimulation of Ca^{2+} extrusion through NCKX1 (Shumilina et al. 2010). Since the effect of Klotho deficiency on the LPS-induced Ca^{2+} signal was abolished by a vitamin D deficient diet, we explored whether NCKX activity is similarly different in *klotho*$^{+/+}$ DCs and *klotho*^{hm} DCs. As a result, NCKX activity was indeed higher in *klotho*^{hm} DCs than in *klotho*$^{+/+}$ DCs and was again similar in *klotho*^{hm} DCs following treatment of the mice with vitamin D deficient diet and in *klotho*$^{+/+}$ DCs.

The Klotho-dependent regulation of NCKX contributes to or even accounts for the observed sensitivity of LPS-induced $[Ca^{2+}]_i$ increase to Klotho deficiency, since the reduced Ca^{2+} signal upon LPS stimulation in *klotho*^{hm} DCs could be restored by inhibiting NCKX exchangers with the blocker DBZ (3',4'-**d**ichloro**b**en**z**amyl).

By regulating $[Ca^{2+}]_i$ in DCs, Klotho is expected to modify DC functions. The present study discloses a critical role of Klotho in the regulation of DC migration, which is known to be the most sensitive Ca^{2+}-dependent function in DCs (Connolly and Kusner 2007;Shumilina et al. 2010;Shumilina et al. 2011). The effective migration of DCs to the lymph nodes is a prerequisite for their role as regulators of lymphocyte function (Banchereau et al. 2000). Similar to LPS-induced $[Ca^{2+}]_i$ increase, CCL21-dependent migration of DCs was blunted in *klotho*^{hm} DCs, but not in *klotho*^{hm} DCs isolated from mice receiving a vitamin D deficient diet.

How the excessive $1,25(OH)_2D_3$ plasma levels in *klotho*^{hm} mice can affect NCKX activity in DCs isolated as precursors from their bone marrow and cultured one week *in vitro* remains to be investigated. Upon ligand binding, **v**itamin **D** **r**eceptor (VDR) binds to specific genomic sequences in the promoter regions of target genes (vitamin D response elements), and thus recruits transcription factors and co-regulatory molecules on promoters to activate or suppress

gene transcription (Sundar and Rahman 2011). Epigenetic chromatin remodeling has been proposed to be important in the regulation of vitamin D-mediated gene expression involved in various cellular functions (Sundar and Rahman 2011). In DCs, vitamin D has been shown to regulate the NF-kappaB component RelB transcription by chromatin remodeling through recruitment of histone deacetylase HDAC3 (Dong et al. 2005). Accordingly, depletion of HDAC3 attenuated relB suppression by D(3) analogs (Dong et al. 2005). Moreover, epigenetic regulation by $1,25(OH)_2D_3$ has been demonstrated in repression of IL-12B through decreased acetylation of histone H4 and increased trimethylation of histone H3 on the IL-12B promoter and its transcription startsite (Gynther et al. 2011). In the present study we demonstrate that treatment of DC precursors with $1,25(OH)_2D_3$ the first 2 days immediately after isolation from the bone marrow was followed by a sustained upregulation of NCKX activity, which was still apparent 5 days later.

Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers participates in the regulation of $[Ca^{2+}]_i$ in a wide variety of excitable cells including cardiomyocytes (Reppel et al. 2007;Sipido et al. 2007;Venetucci et al. 2007), neurons (Canitano et al. 2002;Lytton 2007), photoreceptors and smooth muscle cells (Blaustein and Lederer 1999). Scince we know that Na^+/Ca^{2+} exchangers are expressed in nonexcitable cells (Aneiros et al. 2005;Balasubramanyam et al. 1994;Kimura et al. 1999;Rumpel et al. 2000;Schmitt et al. 1999;Staiano et al. 2009;Tintinger and Anderson 2004). It remains to be shown whether Na^+/Ca^{2+} exchangers and consequently Ca^{2+}-dependent functions in those tissues are similarly sensitive to Klotho/$1,25(OH)_2D_3$.

In conclusion, the present study demonstrates for the first time a potent influence of Klotho deficiency on Na^+/Ca^{2+} exchangers NCKX and NCKX-regulated LPS-dependent Ca^{2+} signal in bone marrow-derived DCs, unravels the sensitivity of this effect to vitamin D levels and provides evidence for a role of Klotho-sensitive $1,25(OH)_2D_3$ formation in regulating DC migration (**Fig. 34**).

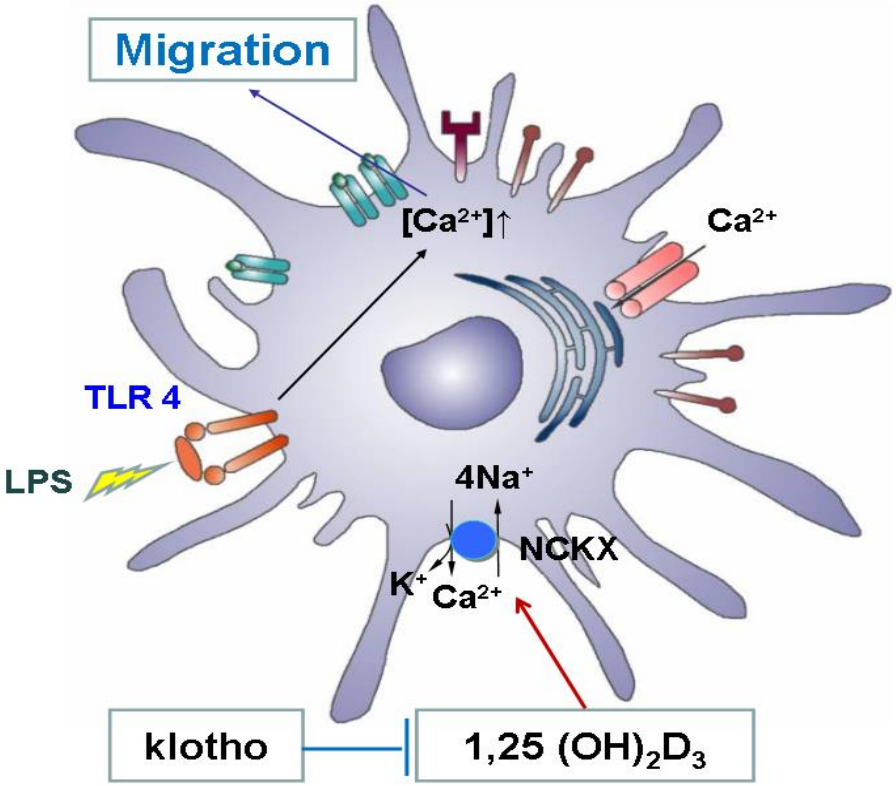

Figure 34. Role of klotho in the regulation of the components of Ca^{2+} signaling and Ca^{2+}-dependent functions in the DCs. NCKX, K^+ dependent Na^+/Ca^{2+} exchanger; $1,25(OH)_2D_3$, vitamin D.

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7. PUBLICATIONS

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3. Effect of dexamethasone on Na^{+}/Ca^{2+} exchanger in dendritic cells. Heise N, Shumilina E, **Nurbaeva MK**, Schmid E, Szteyn K, Yang W, Xuan NT, Wang K, Zemtsova IM, Duszenko M, Lang F. Am J Physiol Cell Physiol. 2011 Jun;300(6):C1306-13. Epub 2011 Feb 9.

4. Expression and functional significance of the Ca^{2+}-activated Cl^{-} channel ANO6 in dendritic cells. Kalina Szteyn, Evi Schmid, **Meerim K. Nurbaeva**, Wenting Yang, Patrick Münzer, Karl Kunzelmann, Florian Lang, Ekaterina Shumilina. Cell Physiol Biochem. 2012; 30(5):1319-32. doi: 10.1159/000343321. Epub 2012 Oct 22.

5. SGK3 Regulates Ca^{2+} Entry and Migration of Dendritic Cells. Schmid E, Bhandaru M, **Nurbaeva MK**, Yang W, Szteyn K, Russo A, Leibrock C, Tyan L, Pearce D, Shumilina E, Lang F. Cell Physiol Biochem. 2012;30(6):1423-35. doi: 10.1159/000343330. Epub 2012 Nov 22.

8. DECLARATION

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

9. CONTRIBUTIONS

The present study describes the role of the energy-sensing AMP-dependent kinase and anti-aging Klotho protein in the regulation of Ca^{2+} signaling and Ca^{2+} sensitive functions of mouse dendritic cells. The majority of the data presented in this thesis was collected from the experiments that I performed personally. I received help in cell culture, Western blotting, immunostaining and flow cytometry maintenance from Evi Schmid, patch clamp from Kalina Szteyn and real time PCR fom Wenting Yang.