Function and Regulation of Na^{+}/ H^{+} Exchanger in Dendritic Cells

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1. SUMMARY

Dendritic cells (DCs) are specialised antigen presenting cells, linking innate and adaptive immunity. They are stimulated by bacterial lipopolysaccharides (LPS), which trigger the formation of reactive oxygen species (ROS). In macrophages, ROS formation is paralleled by the activation of the Na^+/H^+ exchanger, a transporter involved in the regulation of cytosolic pH and cell volume. The present study was undertaken to unravel the possible role of Na^+/H^+ exchanger in the activation of DCs.

In the first step, using quantitative real time PCR analysis the expression of Na^+/H^+ exchanger (NHE) isoforms in bone marrow-derived mouse DCs was analysed. The expression of NHE1 was highest among the various isoforms known to be localised in the cell membrane, so the study focussed on the functional significance of NHE1 isoform.

Exposure of DCs to LPS, within 4 hours led to a gradual cytosolic acidification paralleled by a transient time and dose dependent increase of Na^+/H^+ exchanger activity. Moreover, LPS increased forward scatter in FACS, reflecting increase of cell volume, enhanced ROS formation, decreased apoptosis and stimulated release of TNF-α. An NHE1 inhibitor cariporide (10 µM) significantly blunted the effects of LPS on Na^+/H^+ exchanger activity, on cell swelling, on ROS formation, on TNF-α secretion as well as antiapoptotic effect of LPS. Na^+/H^+ exchanger activity was stimulated by oxidative stress as induced by tert-butyl-hydroperoxide (10 µM) and LPS induced stimulation of NHE activity was abolished in the presence of ROS chelators (Tempol, Tiron and Vitamin C). These data indicate that LPS treatment leads to ROS formation, which in turn leads to transient upregulation of the Na^+/H^+ exchanger in DCs. On the other hand, upregulation of Na^+/H^+ exchanger is required for the effects of LPS on DC survival, cell volume and ROS formation.

The function of DCs is regulated by the phosphoinositide 3 (PI3)-kinase pathway. On the other hand, PI3-kinase is an important regulator of diverse transporters including the Na^+/H^+ exchangers (NHE). The next step of this study was to elucidate the role of PI3-kinase in regulation of NHE activity, cell volume, ROS formation and migration. LPS-induced upregulation of Na^+/H^+ exchanger activity, cell swelling, enhancement of

ROS production and stimulation of migration were all significantly blunted by PI3K inhibitors Wortmannin (1 µM) or LY294002 (10 µM) . The present observations disclose a critical role of PI3K signalling in the regulation of DC function following exposure to LPS.

The oxidative stress responsive kinase 1 (OSR1) is activated by WNK (with no K kinases) and in turn stimulates the thiazidesensitive Na-Cl cotransporter (NCC) and the furosemide sensitive Na-K-2Cl cotransporter (NKCC) thus contributing to transport and cell volume regulation. Little is known about extrarenal functions of OSR1. The present study analysed the impact of decreased OSR1 activity on the function of DCs. For this purpose, DCs were isolated from bone marrow of heterozygous WNK resistant OSR1 knock in mice (osr^{KI}) and wild type mice (osr^{WT}). DCs express WNK1, WNK3, NCC, NKCC1 and OSR1. NKCC1 phosphorylation was reduced in osr^{KI} DCs. Cell volume and cytosolic pH were similar in osr^{KI} and osr^{WT} DCs, but Na^+/H^+ exchanger-activity and ROS-production were higher in osr^{KI} compared to osr^{WT}DCs. Prior to LPS treatment, migration was similar in osr^{KI} and osr^{WT} DCs. LPS (1 µg/ml), however, increased the migration of osr^{WT} DCs but not of osr^{KI} DCs. NHE1 inhibitor cariporide (10 µM), which virtually abrogated Na^+/H^+ exchanger activity in both genotypes, decreased cell volume, intracellular ROS formation, and cytosolic pH to a greater extent in osr^{KI} than in osr^{WT} DCs. LPS increased cell volume, Na^+/H^+ exchanger activity, and ROS-formation in osr^{WT}DCs but not in osr^{KI}DCs and blunted the difference between osr^{KI} and osr^{WT} DCs. Na^+/H^+ exchanger activity in osr^{WT} DCs was increased by NKCC1 inhibitor furosemide (100 nM) to values similar to those in osr^{KI} DCs. Oxidative stress (induced by 10 µM tert-butyl-hydroperoxide) increased Na^+/H^+ exchanger activity in osr^{WT} DCs but not in osr^{KI} DCs and reversed the differences between the genotypes. Cariporide blunted LPS induced cell swelling and ROS formation in osr^{WT} DCs. In conclusion, partial OSR1 deficiency influences Na^+/H^+-exchanger-activity, ROS-formation and migration of dendritic cells.

2. Zusammenfassung

Dendritische Zellen (DZ) sind spezialisierte, Antigen-präsentierende Zellen, die angeborene und erworbene Immunität verbinden. Sie werden durch bakterielle Lipopolysaccharide (LPS) stimuliert, was zur Bildung reaktiver Sauerstoffspezies (ROS) führt. In Makrophagen führt die Bildung von ROS zu einer Aktivierung des Na^+/H^+-Austauschers, eines Transportproteins, das an der Regulation des cytosolischen pH-Wertes und des Zellvolumens beteiligt ist. Die vorliegende Arbeit wurde durchgeführt, um die mögliche Rolle des Na^+/H^+ Austauschers bei der Aktivierung von DZ zu untersuchen.

Im ersten Schritt wurde mit Hilfe der Real Time PCR die Expression der Isoformen des Na^+/H^+ Austauschers in DZ analysiert. Von den verschiedenen Isoformen die in der Zellmembran exprimiert werden, war die Expression des NHE1 am höchsten, weshalb sich diese Studie auf die funktionale Signifikanz der NHE1-Isoform konzentriert.

Die Behandlung der DZ mit LPS führte innerhalb von 4 Stunden zu einer schrittweisen cytosolischen Ansäuerung begleitet von einem zeit- und dosisabhängigen Anstieg der Aktivität des Na^+/H^+-Austauschers. Außerdem führte die Behandlung mit LPS zu einem Anstieg des Vorwärtsstreulichtsignals bei der Durchflusszytometrie was die Erhöhung des Zellvolumens zeigt, des weiteren zu vermehrter ROS-Bildung, verminderter Apoptose und einer erhöhten Freisetzung von TNF-α. Der NHE1-Inhibitor Cariporid (10 µM) hob die Effekte von LPS auf die Na^+/H^+-Austauscher-Aktivität, auf das Zellvolumen, auf die Bildung von ROS, auf die TNF-α Sekretion und auch auf die antiapoptotischen Effekte von LPS auf. Die Aktivität des Na^+/H^+-Austauschers wurde durch oxidativen Stress (induziert durch 10 µM Tert-butyl-hydroperoxid) stimuliert und eine durch LPS hervorgerufene Stimulation der NHE-Aktivität wurde in Gegenwart von ROS-Chelatoren (Tempol, Tiron und Vitamin C) aufgehoben. Abschließend lässt sich sagen, dass die Behandlung mit LPS zur Bildung von ROS führt, was wiederum eine vorübergehende Hochregulierung des Na^+/H^+ Austauschers in DZ zur Folge hat. Eine Hochregulierung des Na^+/H^+ Austauschers ist ebenfalls erforderlich für die Effekte von LPS auf das Überleben von DZ, das Zellvolumen und die Bildung von ROS.

Die Funktion von DZ wird durch den Phosphoinositol 3 (PI3)-kinase Signalweg reguliert. Zudem ist die PI3-kinase ein wichtiger Regulator diverser Transporter einschließlich des Na^+/H^+ Austauschers (NHE). Im nächsten Schritt dieser Arbeit soll die Rolle der PI3-kinase bei der Regulation der NHE Aktivität, des Zellvolumens, der Bildung von ROS und der Migration aufgeklärt werden. LPS-induzierte Effekte wie der Anstieg der Na^+/H^+-Austauscher-Aktivität, die Zunahme des Zellvolumens, die Steigerung der Bildung von ROS sowie die Stimulation der Migration wurden durch die PI3K Inhibitoren Wortmannin (1 µM) oder LY294002 (10 µM) signifikant reduziert. Die vorliegenden Beobachtungen weisen auf eine kritische Rolle des PI3K-Signalweges bei der Regulation der Funktion von DZ nach Behandlung mit LPS hin.

Die oxidative-stress-responsive-kinase 1 (OSR1) wird durch den WNK (with no K kinases) Signalweg aktiviert und stimuliert ihrerseits die Thiazid-sensitiven Na-Cl Kotransporter (NCC) und die Furosemid-sensitiven Na-K-2Cl Kotransporter (NKCC) und trägt so zur Regulation des Elektolyttransportes und des Zellvolumens bei. Es ist wenig über extrarenale Funktionen von OSR1 bekannt. Die vorliegende Studie beschäftigt sich mit dem Einfluss von verminderter OSR1-Aktivität auf die Funktion von DZ. Dafür wurden DZ aus dem Knochenmark von heterozygoten, WNK-resistenten OSR1 knockin Mäusen (osr^{KI}) und Wildtyp Mäusen (osr^{WT}) gewonnen. Die DZ zeigten die Expression von WNK1, WNK3, NCC, NKCC1 und OSR1. Die NKCC1 in den osr^{KI}DZ zeigten weniger Phosphorylierung. Das Zellvolumen und der cytosolische pH-Wert waren ähnlich in den osr^{KI} und osr^{WT}DZ, aber sowohl die Aktivität des Na^+/H^+ Austauschers als auch die Bildung von ROS waren in den osr^{KI}DZ stärker erhöht als in den osr^{WT}DZ. Vor der Behandlung mit LPS war das Migrationsverhalten von osr^{KI} und osr^{WT}DZ ähnlich. LPS (1µg/ml) führte jedoch zu einem Anstieg der Migration bei den osr^{WT}DZ, nicht jedoch bei den osr^{KI}DZ. Der NHE1-Inhibitor Cariporide (10µM), der die Aktivität des Na^+/H^+-Austauschers in beiden Genotypen nahezu aufhob, ließ in osr^{KI} DZ das Zellvolumen stärker abnehmen als in osr^{WT}DZ und erniedrigte auch deutlicher die intrazelluläre ROS-Produktion, und den cytosolischen pH-Wert. Die Behandlung mit LPS führte zu einem Anstieg des Zellvolumens, der Na^+/H^+-Austauscher-Aktivität, und der ROS-Produktion in osr^{WT}DZ jedoch nicht in osr^{KI}DZ und hob die Unterschiede zwischen osr^{KI} and osr^{WT}DZ auf. Die Na^+/H^+-Austauscher-Aktivität in den osr^{WT}DZ wurde durch

den NKCC1-Inhibitor Furosemid (100 nM) auf Werte ähnlich denen der *osr*^{KI}DZ erhöht. Oxidativer Stress (10 µM Tert-butyl-hydroperoxid) erhöhte die Na^+/H^+ Austauscher-Aktivität in den *osr*^{WT}DZ aber nicht in den *osr*^{KI}DZ und revidierte den Unterschied zwischen den Genotypen. Die Behandlung mit Cariporide kehrte den LPS-vermittelten Anstieg des Zellvolumens und der ROS Produktion in *osr*^{WT} DZ um. Daraus lässt sich schließen, dass eine partielle OSR1 Defizienz die Na^+/H^+ Austauscher Aktivität, die ROS-Bildung und die Migration von dendritischen Zellen beeinflusst.

3. INTRODUCTION

Immune system

Immune system consists of the network of molecules, cells, tissues and organs that work together to protect the body. The cells involved are white blood cells, or leukocytes, beloging either to innate or to adaptive immunity that combine to seek out and destroy disease-causing organisms or substances.

Innate immune system

The innate immune system is a non specific first line of the defense mechanism for protecting the host from an invading microbial pathogen. The innate immune system includes phagocytic cells, natural killer (NK) cells, complement system and interferons (IFNs). Leukocytes, macrophages and dendritic cells, which engulf and kill microbes, are collectively called phagocytes. The cells of the innate immune system recognize, and respond to, pathogens in a non-specific way. Innate immunity is found in all classes of plants and animals. Recent studies have shown that the innate immune system possesses a greater degree of specificity than previously believed, and is highly developed in its ability to discriminate self from foreign pathogens. Innate immune recognition is mediated by pattern recognition receptors (PRRs), which are germline encoded, and each receptor has broad specificities for conserved and invariant features of microorganisms[1]. PRRs selectively bind to essential components of pathogens, known as pathogen associated molecular patterns (PAMP). Some of the most important PRRs are the toll like receptors (TLRs)[2].

The major functions of the vertebrate innate immune system include[2-4]:

- recruiting immune cells to sites of infection, through the production of cytokines.
- activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes.
- the recognition and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells.

- activation of the adaptive immune system through a process known as antigen presentation.
- acting as a physical and chemical barrier to infectious agents.

The components of innative immunity

Natural Killer Cells (NK cells) are bone marrow derived granular lymphocytes, spread throughout the body, which mediate cellular cytotoxicity, produce chemokines and inflammatory cytokines, such as IFN-γ and TNF-α[5-11]. They are an important constituent of innate resistance to viruses and bacteria and also provide immune surveillance against the development of tumours[12-18]. NK cells also interface with adaptive immunity by stimulating dendritic cells and by promoting T cell responses[19].

Eosinophils are white blood cells (WBCs), they produce and store diverse biologically active molecules, including cytotoxic, cytostimulatory proteins, lipid mediators, chemo-tactic peptides and cytokines[20-22]. They also participate in killing of multicellular parasites and certain infections in vertebrates[23]. Along with mast cells, they also control mechanisms associated with allergy and asthma[24].

Basophils, are the granulocytes, which are rapidly recruited into the bone-marrow, small intestine, blood stream, and other tissues during helminth infections and allergic inflammation[25;26]. They are probably important mediators for inducing and maintaining the Th2 response because they are an important source of the cytokine interleukin-4 and exert functions similar to antigen-presenting cells (APCs)[27;28].

Neutrophils are the WBCs, which play an important role in host defense against microbial pathogens and in the inflammatory reaction.They generates reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and hypochlorous acid (HOCl)[29].

Mast cells are resident in several types of tissues, mainly skin, airways, and intestines. Mast cells can participate in direct killing of organisms by phagocytosis and ROS production [30;31]. Mast cells are also involved in adaptive immunity, since they can present antigens and secrete cytokines and chemokines[30].

Macrophages are specialized phagocytic cells, found in all tissues. The major functions of macrophages are antigen presentation, phagocytosis, and immunomodulation[32;33]. Macrophages also secrete IL-1, IL-6, TNF, and INF-α/β—cytokines[32].

Dendritic cells are specialised antigen presenting cells; they also play a key role in phagocytosis. DCs are discussed in a separate section.

Neutrophils, monocytes, dendritic cells, mast cells and macrophages are collectively called as phagocytic cells. Their role is to phagocytose (engulf and then digest) cellular debris and pathogens, either as stationary or as mobile cells.

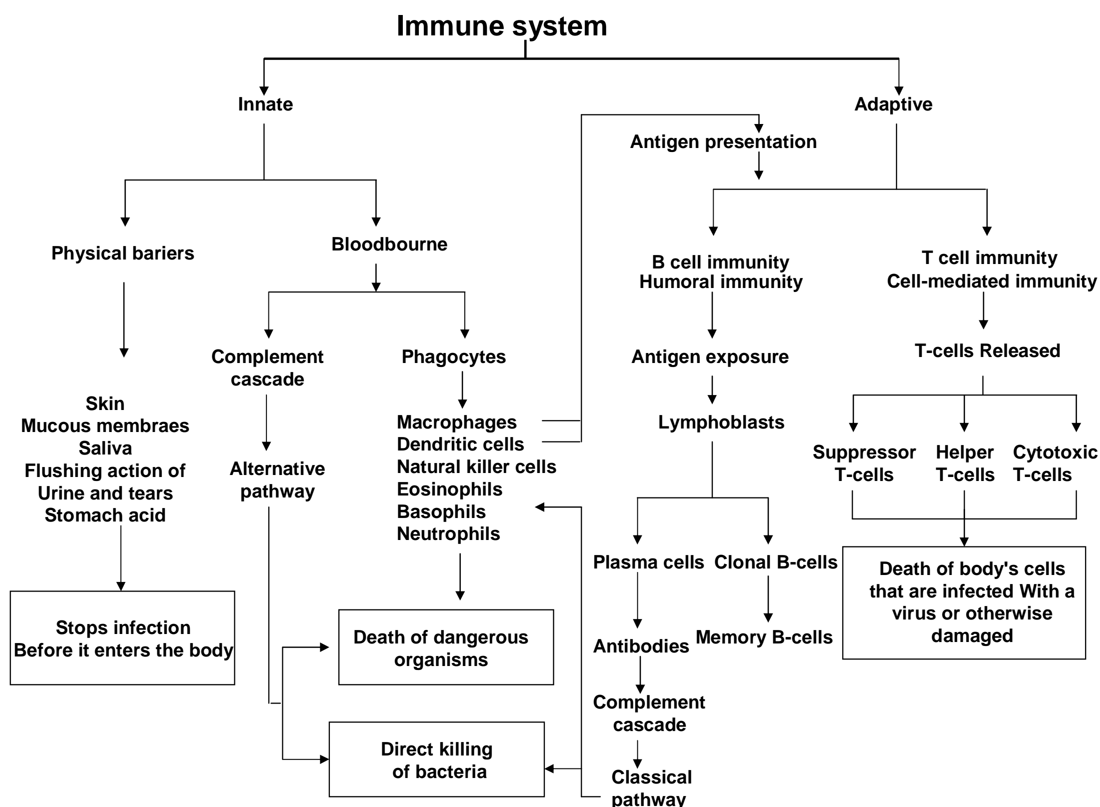


Figure 1:Immune system

Adaptive immune system

Vertebrates depend on innate immune responses as a first line of defense, but they can also mount much more sophisticated defenses, called adaptive immune responses. The innate responses call the adaptive immune responses into play and both work

together to eliminate pathogens. Unlike innate immune responses, the adaptive responses are highly specific to the particular pathogen that induced them. They can also provide long lasting protection. A person who recovers from measles, for example, is protected for life against measles by the adaptive immune system, although not against other common viruses, such as those that cause mumps or chickenpox[34].

Adaptive immunity is further divided into humoral immunity and cellular immunity. Humoral immunity is involved in the eradication of microbes present in the blood or fluid by generating antibodies, which are produced by B-cells. On the other hand, cellular immunity is responsible for the eradication of cancer cells and microbes hidden inside cells, and is mediated by killer T-cells. T-cells and B-cells express unique T-cell receptors (TCRs) and B-cell receptors (BCRs), respectively and recognize a vast number of different antigens. TCRs and BCRs are generated by DNA recombination during the differentiation of T and B-cells. Each TCR and BCR is composed of a variable region, encoded by different gene segments and a constant region. Each member of the gene segment, encoding the variable region, is randomly joined to the other members, resulting in the creation of a huge diversity of receptors. When a huge repertoire of TCRs and BCRs are generated in a ready made manner, the repertoire includes receptors that react with components of the host. Lymphocytes harbouring self reacting receptors are then excluded during differentiation. When a pathogen invades the body, T and B-cells with the corresponding receptors are activated, and killer T-cell development and antibody production are induced. At the same time, memory T and B-cells are generated[35].

Dendritic cells

Dendritic cells (DCs) are APC involved in the initiation of both innate and adaptive immunity and thus critically important for the regulation of the immune response[36-38]. They are unique among APCs and have been referred to as “professional” APCs, since the principal function of DCs is to present antigens, and since only DCs have the ability to induce a primary immune response in resting naive T lymphocytes[39]. To perform this function, DCs are capable of capturing antigens, process and present them on the cell surface along with appropriate costimulation

molecules. They aslo play an important role in the development of T cell immunity and the establishment of immunological memory[40;41].

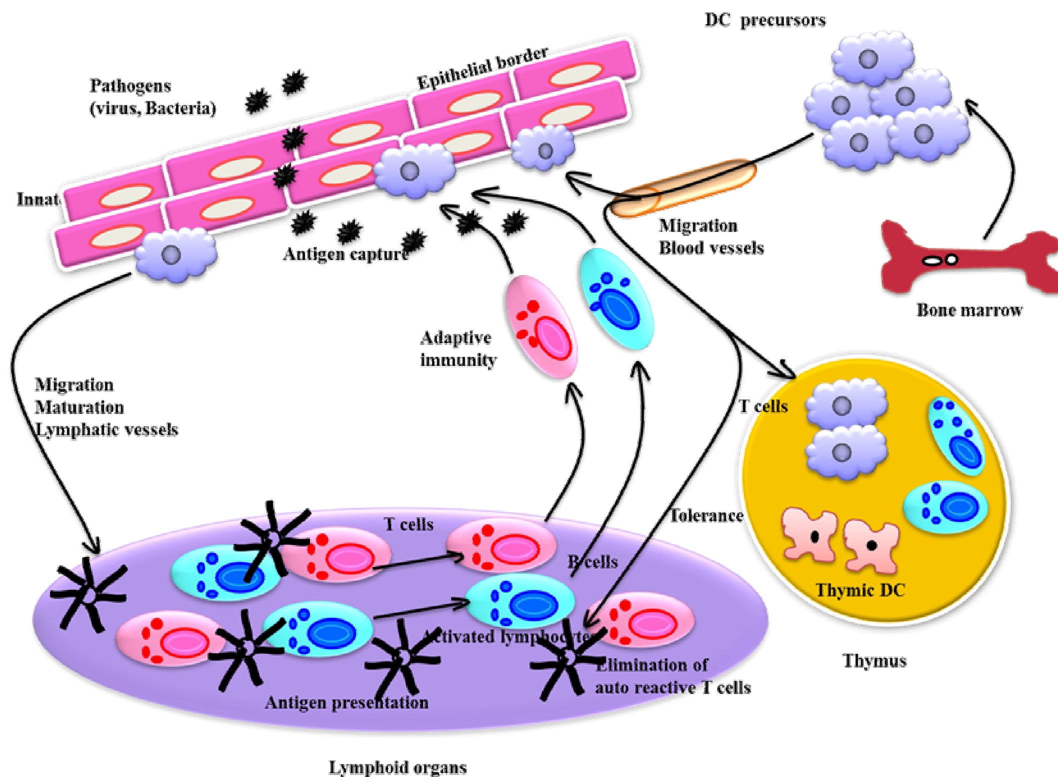


Figure 2: DC biology - differentiation, migration, antigen presentation, and tolerance[42].

DCs are surveillance cells, generated in the bone marrow, which migrate as precursor cells to sites of potential entry of pathogens such as skin, respiratory tract, and lung, where they reside as immature cells in the epithelia of skin and mucosal tissues (Fig. 2). DCs of bone marrow origin express myeloid markers CD13, CD33 and CD11c and have a common progenitor with monocytes, macrophages and granulocytes. They are involved in stimulating naive T-cells. In contrast, lymphoid DCs, which develop simultaneously with T-cells in the thymus from a common population and express lymphoid markers such as CD8a, are involved in the removal of potentially autoreactive

T lymphocytes upon their development. Immature DCs from myeloid origin also have a crucial role in the maintenance of peripheral tolerance to self antigens[43]. They are involved in the differentiation of regulatory T-cells required for the maintenance of self-tolerance[43]. Resident immature DCs have the ability to take up antigens, via both receptor and non-receptor mediated mechanisms and degrade them in endocytic vesicles to produce antigenic peptides capable of binding to major histocompatibility complex (MHC). In response to danger signals, DCs increase production of proinflammatory cytokines (IL-10, IL-6, IL-12, IL-4 and TNFα), expression of co-stimulatory molecules (CD40, CD80, CD86, MHC class II, and ICAM-1) [44] and acquisition of the responsiveness to homeostatic chemokines, including CCL19 and CCL21 via upregulation of the chemokine receptor CCR7. Subsequently, DCs enter the draining lymph nodes in the T-cell rich zone, present the processed antigens to T lymphocytes in an MHC restricted fashion [41;45] to induce their activation and differentiation into effector cells. After antigen presentation, mature DCs are programmed to undergo apoptosis. Some immature DCs can migrate directly into thymus and lymphoid tissues, where they participate actively in the T-cell education or selection and the elimination of autoreactive T-cells. Activated T-cells eliminate microbes and B-cells mature into plasma cells secreting antibody that neutralize pathogens[42].

The role of dendritic cells in innative and adaptive immunity

Phagocytosis

Along with macrophages and neutrophils, DCs are considered as professional phagocytes. However, unlike other phagocytes, DCs are potent antigen-presenting cells and are not directly involved in immediate pathogen clearance. Like macrophages, DCs are present in all peripheral tissues and accumulate at the sites of pathogen entry. Immature DCs express a large array of phagocytic receptors, including lectins, scavenger receptors, and pathogen receptors[37]. DCs also express a variety of TLRs and other PRRs. Different DC subpopulations express different phagocytic receptors, performing therefore a selective uptake of different particles. For example, splenic CD8+ DCs take up apoptotic bodies much more efficiently than other DCs in the spleen [46]. CD8- DCs, in contrast, phagocytose Leishmania more efficiently than the CD8+ subset[47;48].

After taking up pathogens, infected or apoptosing cells, DCs process antigens derived from these particles into peptides and load these peptides on MHC class I or MHC class II molecules. DCs take up pathogens in peripheral tissues, undergo particular maturation programs selectively in response to different pathogens, migrate to lymphoid organs, and present antigen to T lymphocytes to initiate antigen-specific immune responses. Thus, DCs are specialized in linking innate and adaptive immune responses, rather than directly eliminating pathogens[49](fig. 3).

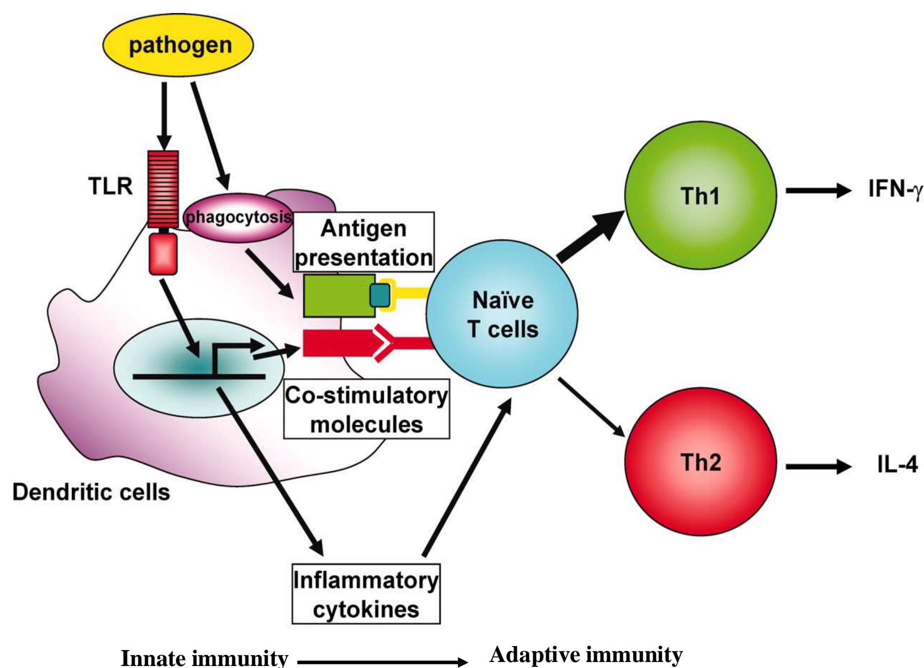


Figure 3:Innate and adaptive immunity[49]

Migration

During their life cycle, DCs migrate from the blood to peripheral tissues and from peripheral tissues to lymphoid organs. Migratory properties of DCs are of fundamentally importance for their function. The migratory capacity of DCs following antigen capture is regulated by chemokines released by the target tissue and by modulation of surface

adhesion molecules. Differentiation and migration of DCs are parallel processes that follow a unidirectional path from progenitors in the bone marrow[50;51], then precursors migrated in bloodstream[52], and finally fully differentiated, immature DCs residing in DC pool at the peripheral tissues. After migrating into peripheral tissues, DCs survey the environment, sample antigenic materials, and travel through afferent lymphatics to reach draining LNs where they present the collected antigens to T cells[53;54]. Stimulation of DCs with microbial products and inflammatory cytokines leads to induction of DC maturation and up-regulation of CCR7, a chemokine receptor that drives their migration to the lympoid organs[55;56]. CCR7 has two ligands, CCL21, which is produced by endothelial cells of lymphatic vessels and stromal cells present in the T cell zone, and CCL19, which is produced by stromal cells and mature DCs in the T cell zone[57-60]. These chemokines attract CCR7 receptors of mature DCs to migrate to lympoid organs.

T cell activation

DCs are the most effective APCs for inducing maturation of naive T cells because they express the highest concentration of MHC class II molecules on their surface. MHC class I peptides are categorized by endogenous antigenic processing via the cytosolic pathway, and MHC class II peptides are categorized by exogenous antigenic processes via the endocytic pathway. MHC class II molecules engage specific T cell receptors[37]. Moreover, DCs are equipped with costimulatory molecules. DC costimulatory molecules include members of the B7 family, TNF family and intracellular adhesion molecules, which are critical for the activation of T cells and for the proper homing of DCs before and after antigen capture. If this costimulatory molecule signal is missing, then the T cell is normally anergized, becoming unreactive to activation.

The ability of DCs to induce $CD4^{+}$T cells to differentiate into the Th1 or Th2 phenotype is dependent on IL-12 production. IL-12 is a cytokine, mainly produced by DCs in response to antigenic stimulation. It is known as a T cell-stimulating factor, which can stimulate the growth and function of T-cells. It also plays a key role in the development of Th1 responses, leading to IFN-γ and IL-2 production by Th1 cells. These cytokines can, in turn, promote T cell responses and macrophage activation[61]. IFN-γ in

synergy with other cytokines, such as TNF-α, activates macrophages to control intracellular infection[61;62].

Immune tolerance

Tolerance is the specific inability of a host to respond to antigens, which is generated both centrally and peripherally. Central tolerance occurs in the thymus for T cells and in the bone marrow for B-cells. T cells that might inadvertently respond to DCs carrying self-peptides are deleted during ontogeny in the thymus. T cells that fail to respond to stimuli in the thymus die from neglect, while T cells that recognize MHC/peptides with high avidity undergo apoptosis and are deleted, this latter process is called negative selection. T cells that recognize self with low avidity in the thymus survive, a process called positive selection, and reach the periphery where they respond only to antigens presented in the context of self MHC. Thymic epithelial cells are responsible for presenting self-peptides in the context of MHC for positive selection. Both thymic DCs and thymic epithelial cells contribute to negative selection[63-66].

Peripheral tolerance mechanisms include T cell death, T cell anergy, and active suppression by T regulatory cells. In the normal host if self-antigens are presented, no T cells should be available to respond, because of central tolerance induction. However, if T cells recognize only low levels of MHC/peptide, have a low affinity for their cognate ligand, or receive no costimulation from DCs, they become anergic or undergo apoptosis. For example, immature DCs treated with IL-10 fail to mature and, as a result, induce anergy in responder T cells[67]. Once generated, anergic T cells can suppress development of an immune response by directly suppressing the expression of MHC class II, CD80, and CD86 on DCs in culture[68]. T regulatory cells play a role in the expression of tolerance and it is likely that these cells are stimulated initially by DCs[69;70]. T regulatory cells that secrete suppressive factors like IL-10, TGF- b, both IL-10 and TGF- b, and those that suppress by cell-cell contact can suppress Th0, Th1, and Th2 T cells. T regulatory cells are typically generated when responder T cells are stimulated repeatedly in the presence of high levels of IL-10[71], a cytokine that can be secreted by DCs. DCs secrete IL-10 and inhibit T cell proliferation, cytokine secretion and IgE production in an antigen-specific manner[72].

Generation of memory

The role of the DC in CD8 T cell memory is not clearly known but it has been reported that memory CD8 T cell persistence was dependent on the balance of IL-15 with IL-2[73]. DCs secrete IL-15 [74;75] therefore playing an indirect role in CD8 T cell memory.Van Essen et al. [76]has demonstrated that CD4 T cell memory depend on DCs to process and present antigen. The source of the antigen was thought to be antigen-antibody complexes on follicular dendritic cells (FDCs). This study also demonstrated a requirement for B cells to maintain memory development, likely to facilitate the development of FDCs and to secrete the complex-forming antibody[64].

B cell stimulation/function

Beside activating naive T cells, DCs also play a role in stimulating B cells in both lymph node T cell areas and germinal centers[37;77]. DCs stimulate B-cells indirectly by activating T-cells to upregulate CD40L and secrete B cell helper factors. Additionally DCs can also interact directly with CD40-activated naïve B cells to induce proliferation within the paracortical areas of the lymph node[77]. Furthermore DCs can play a role in B cell differentiation into IgM-secreting plasma cells [78;79] through IL-12 dependent mechanism. However, plasma cell differentiation can also be facilitated by other DC cytokines[78]. DCs can capture and present unprocessed antigen to B cells and induce an IgG switch both in vitro and in vivo[80] A unique FDC population exists in the germinal centers of secondary lymphoid tissue and is important in B cell recall responses[81]. FDCs along with memory B cells play an important role in maintaining serum antibodies for long periods after exposures to infectious agents[64].

Reactive oxygen species

Reactive oxygen species (ROS) are oxygen-derived small molecules, including oxygen radicals [superoxide (O_2•), hydroxyl (•OH), peroxyl (RO_2•), and alkoxyl (RO•)] and certain nonradicals that are either oxidizing agents and/or are easily converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($1O_2$), and hydrogen peroxide (H_2O_2)[82]. ROS are produced in response to growth factors, cytokines, G protein–coupled receptor agonists, or shear stress[83].

ROS have important signaling properties in many cells, including DCs. DCs are equipped with a membrane localized electron transport system, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, that reduces molecular oxygen to superoxide anions at the expense of NADPH[84-86]. Activation of DCs may thus initiate/include ROS-mediated autocrine/paracrine regulatory functions. NADPH-oxidase is activated by a large number of receptor-binding agonists, including particles that trigger phagocytosis, chemoattractants, and other “danger molecules.” The precise receptor repertoire thus determines the responsiveness of the cells to a particular agonist [87]. A role for NADPH oxidase in phagosomal function was reported because NADPH oxidase activity was required to the efficient killing of intracellular Escherichia coli in human DCs [88]. The phagosomal pH in DCs is controlled through an equilibrium of the activities of two multimolecular complexes present on the membrane of these compartments: the NADPH oxidase NOX2 and V-ATPase [47] and H+ channel[89].

ROS affect the maturation state, the production and secretion of cytokines, [85;88;90;91]and the antigen presenting capacity of DCs. The cells become functionally more efficient when exposed to ROS. Thus, ROS could serve as endocrine regulators of DC function and thereby influence the nature of the ensuing immune response. There is evidence that ROS change the outcome of the DC-Tcell interaction[85]. Even though ROS-deficient DCs have retained the capacity to induce T cell proliferation in vitro, T-cells activated in the absence of ROS exhibit an altered differentiation profile[88].

Toll Like Receptors

Initial recognition of microbes in the body is based on germ line encoded pattern recognition receptors (PRR) that selectively bind to components of pathogens, know as pathogen associated molecular patterns (PAMP). Some of the most important PRRs are the TLRs, 13 of which have been recognized in mammals: TLR1 to TLR13[92],. TLRs together with the Interleukin-1 receptors form a receptor superfamily, known as the "Interleukin-1 Receptor/Toll-Like Receptor Superfamily", all members of which have a so-called TIR (Toll-IL-1 receptor) domain in common. They recognize the PAMPs of bacterial, fungal and viral components (Fig. 4)[35].

Activation of TLRs signaling pathways leads to the induction of various genes that function in host defence, including inflammatory cytokines, chemokines, MHC and co-stimulatory molecules. TLRs are abundant on the surface of macrophages, neutrophils and DCs, as well as on the epithelial cells lining the lung and gut.

Lipopolysaccharides (LPS) were first identified as most potent TLR ligands. LPS are found in the outer cell walls of Gram-negative bacteria and are recognized by TLR4 [93;94].Lipid containing components from the cell walls of a variety of microorganisms are recognized by TLR2 and related TLRs, such as TLR1 and TLR6 [95-97]. TLR5 is involved in recognition of flagellin which elicits mucosal immune responses by acting on epithelial cells or macrophages. TLR5 is also expressed in DCs residing in the mucosa [98]. Bacterial and viral DNAs are recognized by TLR9. Their activity is dependent on unmethylated CpG motifs, which are more abundant in bacterial than in mammalian DNA[99]. Single-stranded viral RNA is recognized by TLR7 and also by its close relative, TLR8 [100;101]. These interactions are critical for sensing RNA viral infection. RNA viral infection also induces the production of double stranded RNA (dsRNA) in infected cells, and these dsRNAs can act as immune adjuvants after recognition by TLR3[102]. TLR11 has been shown to be expressed in bladder epithelial cells and mediate resistance to infection by uropathogenic bacteria in mouse [103].

Individual TLRs are differentially distributed within the cell. TLR1, TLR2 and TLR4 are expressed on the cell surface. In contrast, TLR3, TLR7, TLR8 and TLR9 have been shown to be expressed in intracellular comparments such as endosomes [49]. The latter is understandable because nucleic acids are embedded inside the pathogens. In the endosome of virus infected cells, the nucleic acids are released from the virus and encounter their respective TLRs. Consistent with this, nucleic acid-recognizing TLRs are expressed mainly in phagocytes[104].

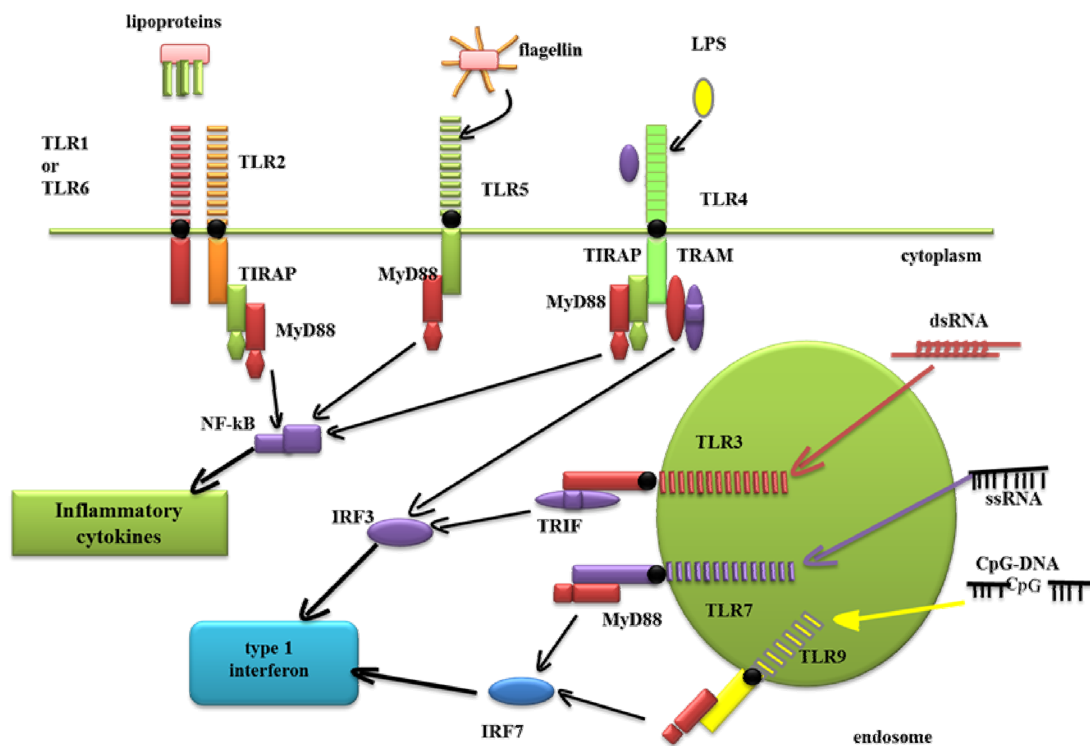


Figure 4:TLR ligands and signalling[35]

TLR signalling pathways

MyD88-dependent pathway

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. The TIR domain is conserved among all TLRs, except for TLR3. In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, has been first characterized to play a crucial role[105]. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of the death domains of both molecules (Fig. 4). Toll-interacting protein (TOLLIP) can also associate with IRAK and the TIR domains of the receptors, and recruits IRAK to the receptor complex [106]. IRAK is activated by phosphorylation and then associates with TNF receptor-associated

factor 6 (TRAF6) [107] leading to the activation of TAK1 and MAP kinase kinase 6 (MKK6) signaling pathways, which inturn leads to the activation of c-Jun N-terminal kinase (JNK), NF-κB and p38 MAP kinase respectively [108].

Macrophages and DCs derived from MyD88 knockout mice do not produce the cytokines IL-1β, TNF-α, IL-6 and IL-12 when stimulated with LPS, polyIC, MALP-2 or CpG, which signal through TLR4, TLR3, TLR2 and TLR9, respectively[102;109-111]. Consequently, MyD88-deficient mice are completely resistant to endotoxic shock [109].

MyD88-Independent pathway

MyD88-deficient bone marrow derived DCs (BMDCs) stimulated with LPS, polyIC or CpG fail to produce IL-12 or IL-6. However, they can still induce activation of NF-κB, JNK and p38, which upregulate expression of MHC and co-stimulatory molecules, such as CD80 and CD86, when treated with LPS or polyIC, but not when stimulated with CpG[102;110;112]. These results indicate that TLR4 and TLR3 are sufficient for DC maturation by MyD88-independent signaling pathway(s). MyD88-dependent signalling pathway is necessory for the induction of IL-6 and IL-12[110;112].

TIR domain–containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) shown to function downstream of TLR4[113;114]. TIRAP possesses a C-terminal TIR domain like MyD88 but lacks an N-terminal death domain. TIRAP associates with the TIR domain of TLR4, and a dominant-negative form of TIRAP inhibits TLR4 mediated activation, but not TLR9 or IL-1R mediated activation of NF-·B, indicating that TIRAP regulate activation of the MyD88- independent pathway[114]. TIRAP also associates with the protein kinase PKR and two PKR-regulatory proteins, PACT (PKR-activating protein) and p58, indicating that PKR functions downstream of TIRAP. Indeed, PKR can be activated by LPS thrugh the MyD88-independent pathway [114] but PKR-deficient cells shows impaired LPS signalling [115]. Taken together, this indicates that TLR4 uses two adaptors with TIR domains MyD88 and TIRAP which control activation of distinct signal-transduction pathways[114].

TLR independent signaling

TLRs recognize pathogens at either the cell surface or lysosome/endosome membranes, but not in the cytosol. The cytosolic pathogens are detected by various cytoplasmic PRRs, which activate a number of signaling pathways. They are the NLR proteins and the CARDhelicase proteins. These protein families are involved in the recognition of bacterial and viral components, respectively [116]. NLRs consist of a C-terminal LLR domain, a central NOD and an N-terminal effector domain that initiates signalling. The minimal components of peptidoglycan are recognized by NOD1 and NOD2, leading to NF-κB activation and inflammatory response induction [117]. Consistently, macrophages lacking either NOD1 or NOD2 fail to produce cytokines in response to the corresponding ligands [118]. In addition to NOD1 and NOD2, several NLRs are present in the cytoplasm. These NLRs are involved in inflammasome formation and the production of mature IL-1 and IL-18[35;119].

Fibroblasts lacking both MyD88 and TRIF can still induce the IFN in response to RNA virus infection, indicating the existence of TLR-independent virus detectors[120;121]. An RNA helicase, retinoic acid-inducible gene I (RIG-I), recognise viral invasion in cytoplasm and induces type 1 IFNs in a TLR-independent manner [122;123]. RIG-I possess two N-terminal caspase-recruitment domains (CARDs) followed by an RNA helicase domain. The CARDs are responsible for signal transduction, which leads to the activation of NF-κB and IRF3/7 via their adaptor molecule, IFN-β promoter stimulator 1 (IPS-1), which is located on the outer membrane of mitochondria [124;125].

Phosphatidylinositol 3-kinases in the TLR signalling

Phosphatidylinositol 3-kinases (PI 3-kinases or PI3Ks) are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking [126]. PI 3-kinase is a lipid kinase that catalyzes the transfer of the γ-phosphate group of ATP to the D-3 position of PtdIns (3,4,5)P3 (PIP3), and targets Akt/PKB, Bruton's tyrosine kinase (Btk), PDK, atypical PKCs, phospholipase Cg and other enzymes. Previous studies have show that PI 3-kinase is involved in the regulation of DC functions[127-130]. PI3-kinase suppresses the IL-12 production triggered by TLR signalling and limits the Th1 polarization [131]. Inhibition of PI3-

kinase enhances IFN-β synthesis [132]. On the other hand, LY294002, a PI3 kinase inhibitor abolished NF-κB activation and IL-23 production[133]. Moreover, the class I_B PI3 kinase p110γ crucially regulates neutrophil chemoattractant-induced migration to the site of infection[134-136] PI3 kinase also plays an important role in the ROS production by the binding to $p40^{phox}$[137;138].

PI3 kinase pathway is also involved in the regulation of Na^+/H^+ exchanger in fibroblasts. Downstream targets of the PI3 kinase pathway include the serum and glucocorticoid inducible kinase SGK1, which in turn upregulates the Na^+/H^+ exchanger[139]. However, nothing is known about the role of PI3K in the regulation of Na^+/H^+ exchanger activity of DCs.

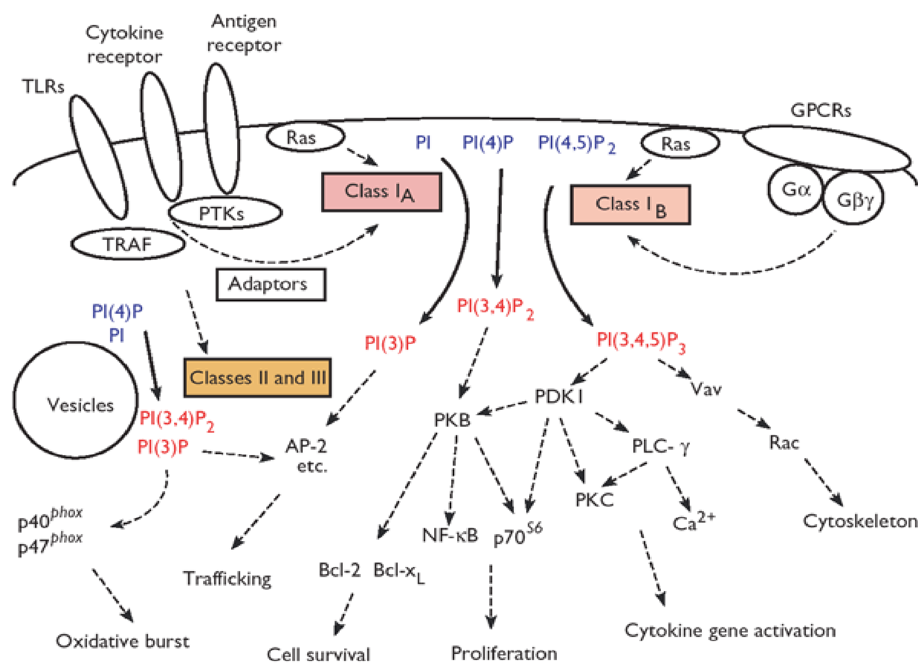


Figure 5: Signal transduction pathway involving PI3Ks in immune cells [140]

The oxidative stress-responsive kinase 1

The oxidative stress-responsive kinase 1 (OSR1) was originally identified and named because of its sequence similarity to SOK1 (Ste20/oxidant stress responsive kinase-1) [141]. OSR1 is commonly expressed in all tissues but most abundant in heart and skeletal muscle [141]. The distribution of OSR1 at the organ or tissue level largely overlaps with SPAK [142;143]. OSR1 and SPAK have been suggested to be coupled to cellular events such as cell differentiation, cytoskeleton rearrangement, cell proliferation, and transformation[144-148]. In addition, extensive biochemical and physiological studies demonstrate that OSR1 and SPAK are also involved in the regulation of ion homeostasis and volume control in mammalian cells. OSR1 is activated by WNK (with no K kinases) and in turn stimulates the thiazide-sensitive Na-Cl cotransporter (NCC) and the furosemide-sensitive Na-K-2Cl cotransporter (NKCC) thus contributing to transport and cell volume regulation. For example, during hyperosmotic stress, OSR1 and SPAK interact with and activate NKCC1 ($Na^+/K^+/2Cl^-$ Cotransporter-1). WNK1 is reported as a substrate of Akt, a kinase strongly regulated by insulin, and Thr-60 of WNK1 is phosphorylated by insulin [149]. It was reported that PI3 Kinase and WNK4 are involved in the insulin induced phosphorylation of SPAK and NCC[150]. However little is known about extra renal functions of OSR1 and the possible role of OSR1 in the activation of DCs.

Intracellular pH and immune response

The maintenance of intracellular pH (pH_i) in a controlled physiological range is critical for normal cellular functions. In fact, the activity of intracellular enzymes, the interaction of cytoskeletal elements and the rate at which cells grow and differentiate depend on pH_i[151]. In immune cells the plasmamebrane transporters V-type H^+ ATPase (V-ATPase), NHE, Na^+-dependent and independent Cl-/HCO3- exchangers and voltage-gated H^+ channels contribute to pH_i homeostasis [152-154]. In this connection, V-ATPase and NHE-mediated H^+ efflux counteract the dangerous effects of excessive intracellular acidification, whereas Na^+ dependent and independent $Cl^-/HCO3^-$ exchangers can in addition protect the cells from cytosolic alkalinization by controlling the efflux of bicarbonate [155-157]. In addition, upon depolarization of the plasma

membrane and/or strong intracellular acidifaction, voltage-gated H^+ channels create a H^+ efflux, and thus contribute to pH_i homeostasis[158].

Several phagocytic functions are strictly connected to the cellular acid-base status. Generally, an intracellular acidification represents an inhibitory condition for chemotaxis, cell migration, ROS generation and the release of cytokines, but at same time it promotes phagocyte spreading and adherence. Moreover, the acid base status also influences monocytic differentiation[159-161]..

Sodium/ Hydrogen Exchanger

Among several H^+ transport systems utilizing by the cells to minimize significant pH_i fluctuations, the best known system is represented by the sodium/ hydrogen exchanger (NHE) family. NHE exchanges Na^+ for H^+ according to their concentration gradients, thus promoting the regulation of pH_i and cell volume [162]. However, the function of NHE is not restricted to pH_i homeostasis but seems to play a key role also in the modulation of proliferation, differentiation, survival, apoptosis, migration as well as cytoskeletal organization [49;158;163-167] (Fig.6).

The nine members of the NHE family described so far (NHE1–9) show a particular tissue distribution pattern. The first five isoforms are expressed largely at the plasma membrane, whilst the other isoforms (NHE6–9) have been shown to reside predominantly intracellularly, although NHE8 has been shown to reside also in the plasma membrane of epithelial cells [168;169]. The isoform NHE-1 is found in the plasma membrane of most mammalian cells and is normally described as the housekeeping isoform[165]. It regulates cell volume and pH, cell morphology and cytoskeletal organization. Other isoforms have a more restricted tissue distribution and appear to regulate more specialized functions. NHE-2, NHE-3 and NHE-4 are expressed predominantly in the kidney and gastrointestinal tract, while NHE-5 is expressed mainly in the brain[165;170]. Two other classes of NHE isoforms NHE-6 and NHE-7 seem to be exclusively localized in intracellular organelles such as mitochondrial and trans-golgi, respectively. These isoforms are also expressed in tissues with high metabolic rates such as heart, brain and skeletal muscle [171;172]. Recently, new isoforms NHE-8 and NHE-9

have been discovered but their intracellular localization is not yet completely elucidated [173;174].

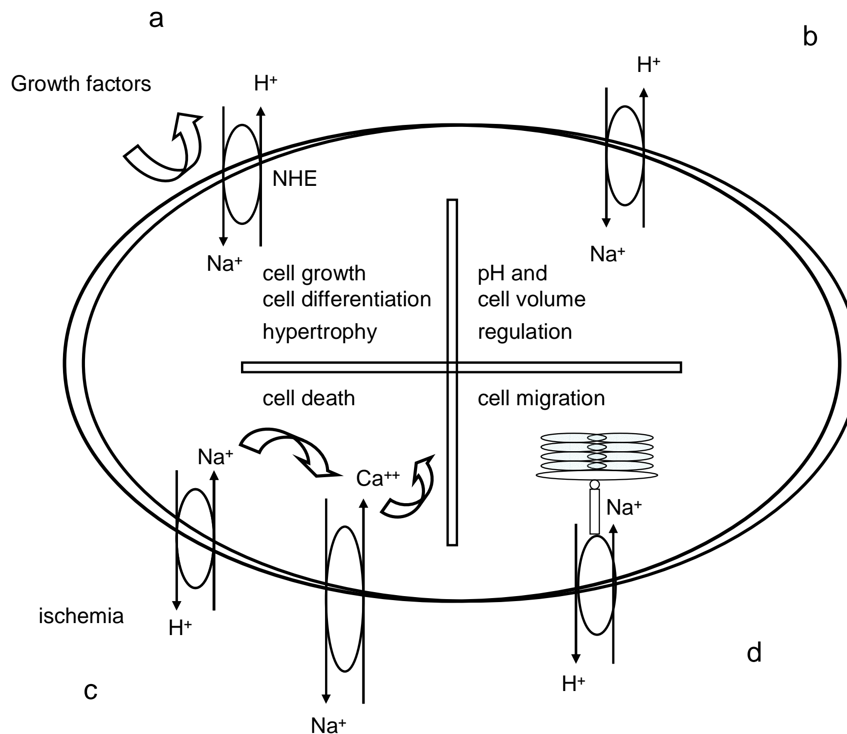


Figure 6:Physiological functions of NHE-1[166].

(a) Growth factors can stimulate NHE-1, this leads to increased cell growth, cell differentiation and hypertrophy. (b) NHE-1 exchanges Na^+ for H^+ according to their concentration gradient and promotes the regulation of cytoplasmic pH (pH_i) and cell volume. (c) During ischemia–reperfusion NHE-1 activation increases intracellular Na^+ thus stimulating the Na^+/Ca^{2+} exchanger; the subsequent increase of intracellular calcium induces cell death. (d) NHE-1 through its interaction with ERM proteins can operate on cytoskeletal proteins and modulate cell migration[166].

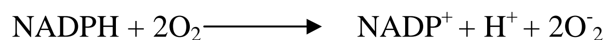
It has been reported in monocytic cells that NHE contributes to pH_i regulation at rest or after intracellular acidification[175;176]. As reported earlier for macrophages, also in monocytic cell line U937 the role of these transporters in pH_i regulation seems to be strictly dependent on the initial setpoint of pH_i [177;178].

Role of NHE in ROS formation

ROS are diffusible and short-lived, localizing the ROS signal at the precise subcellular compartment after receptor activation is essential for specific redox signaling events. Several enzymes, including the mitochondrial electron transport system, xanthine oxidase, cytochrome p450, NADPH oxidase, uncoupled NO synthase (NOS) and myeloperoxidase, have been reported to produce ROS. However, the major source of ROS appears to be the NADPH oxidase. In phagocytic cells, NADPH oxidases consist of membrane-associated cytochrome b558, comprising the catalytic gp91phox and regulatory p22phox subunits, and cytosolic components including p47phox, p67phox, p40phox, and the small GTPase Rac1 [179]. In nonphagocytic cells, several homologues of gp91phox (also termed as Nox2) including Nox1, Nox3, Nox4, and Nox5, as well as the dual oxidases (Duox; Duox1 and Duox2), have been identified [180;181]

The generation of ROS has been connected to stress responses, apoptosis, aging and death [182;183]. In recent years, however, the “bad reputation” of H_2O_2 and other ROS molecules has been changed. These molecules are now being recognized as molecules of life that are essential to the proper development and proliferation of the cells. It has been known for some time that low doses of H_2O_2 have mitogenic effects and can mimic the function of growth factors [184;185].

The enzyme NADPH oxidase plays a crucial role in host defense by producing ROS. Phagocytic cells through the NADPH oxidase dependent ROS generation can counteract and kill invading microbes[186]. NADPH dependent ROS production occurs according to the following reaction:



$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O^-_2$$

In macrophages and other specialized phagocytic cells, NADPH oxidase activity is diminished by an increase in cytosolic H^+ concentration. In this connection, NHE could play a homeostatic role to counteract a potential cytosolic acidification.

It has been reported that in Na^+ depleted medium or in the presence of amiloride, cytoplasmic acidification was observed in liver macrophages upon activation of NADPH oxidase by zymosan or PMA. No such effects were observed at physiological concentrations of Na^+ [187].

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Role of NHE in cell volume regulation

The maintenance of adequate cell volume is one of the most important factor not only in defining intracellular osmolality and cell shape, but also in the regulation of other cellular functions, such as transepithelial transport, migration, growth, survival, and the regulation of intracellular metabolism[188].

Exposure of cells to hypertonic medium or cellular loss of osmolytes leads to exit of water according to the osmotic gradient and thus to cell shrinkage. The following regulatory cell volume increase is accomplished by ion uptake [189]. Cell shrinkage leads to activation of the Na^+-K^+-$2Cl^-$ cotransporter and/or the combined activation of the Na^+/H^+ exchanger in parallel to the Cl^-/HCO^-_3 exchanger [189]. The H^+ and the HCO_3^- extruded by the Na^+/H^+ exchanger and the $Cl^-/HCO3^-$ exchanger, respectively, are replenished in the cell from CO_2 via H_2CO_3. The net effect of those two carriers is thus NaCl entry. Na^+ accumulated by either Na^+-K^+-$2Cl^-$ cotransport or Na^+/H^+ exchange is extruded by the Na^+/K^+ ATPase in exchange for K^+. Thus, the transporters eventually lead to uptake of KCl. Several Na^+-K^+-$2Cl^-$cotransporter [190] and Na^+/H^+exchanger isoforms [189] have been cloned, which do not all serve cell volume regulation. For instance, the Na^+/H^+ exchangers NHE-1, NHE-2 and NHE-4 are activated by, and NHE-3 is inhibited by cell shrinkage [189].

It has been reported that in the alveolar macrophages, NHE exerts a minor role for pH_i setpoint regulation, but it seems to play a key role for cellular volume regulation in hyperosmotic conditions[191]. Since it is known that modification of cell volume represents an upstream signal able to activate intracellular messengers and thus promote different cellular responses [192], it is conceivable that NHE could be a crucial target for any phagocytic activity coupled with cellular volume alterations. Several phagocytic functions, including chemotaxis and the response to chemoattractants depend on cellular volume, and NHE-dependent volume regulation could represent an important step for phagocytic cell migration to different microenvironments such as interstitial fluids, tumor tissues and abscesses [192].

Role of NHE in migration

Cell migration is important for many physiological and pathophysiological processes such as embryogenesis, immune defence, wound healing or metastasis. In fibroblasts along with intracellular pH and cell volume regulation, NHE1 is also involved in cell migration[193]. NHE1 acts downstream of RhoA and contributes to the integrin-induced cytoskeletal reorganization[194]. NHE1 mutations in fibroblasts that disrupt ERM binding, but not ion translocation, leads to impaired organization of focal adhesions and actin stress fibers, and an irregular cell shape and impaired adhesions[195]. Thus, the colocalization of ERM proteins and NHE1 appears to facilitate modulation of cytoskeletal dynamics required for cell migration. NHE1 is required for the cytoskeletal anchoring and polarity and its activity makes a significant contribution to cell adhesion, motility and migration[193].

Rationale for the present studies

The stability of cytoplasmic pH (pH_i) in a controlled physiological range is critical for normal cell functions such as the activity of intracellular enzymes, the interaction of cytoskeletal elements, cell proliferation and differentiation [151]. In case of dendritic cells (DCs), several phagocytic functions are strictly connected to intracellular pH_i such as chemotaxis, cell migration, reactive oxygen species (ROS) generation, cytokine release, phagocyte spreading and adherence[159-161;166]. Moreover, it has been shown that the acid-base status also influences monocytic differentiation[176]. Several H^+ transport systems in cells aid in minimising significant pH_i fluctuations.The sodium/ hydrogen exchanger (NHE) family, is one such system, which exchanges Na^+ for H^+ based on their concentration gradients. Indeed, Na^+/H^+ exchangers have been reported to regulate cell proliferation, differentiation, survival, apoptosis, migration as well as cytoskeletal organization[158;163-167].

Bacterial lipopolysaccharides (LPS) are known stimulators of DCs and are known to trigger the formation of ROS[85;196]. In macrophages, ROS formation is paralleled by activation of the Na^+/H^+ exchanger[191;197-202]. However, the role of Na^+/H^+ exchangers in the DC activation has not been studied in detail and it is interesting to

verify the effect LPS on Na^+/H^+ exchangers and the significance of Na^+/H^+ exchangers in DC functions.

On the other hand it has been shown that regulation of DC function involves the phosphoinositide 3- kinase (PI3 kinase) pathway[127-130]. PI3 kinase is activated by LPS and provides a negative feedback regulation of IL-12 production[129;131]. Therefore, it is also important to elucidate whether the Na^+/H^+ exchanger in DCs is regulated by PI3-kinase dependent signaling and whether PI3-kinase sensitive Na^+/H^+ exchanger activity participates in the regulation of DC functions.

It has been also reported that PI3-Kinase and WNK4 are involved in the insulin induced phosphorylation of SPAK and NCC[150]. The oxidative stress-responsive kinase 1 (OSR1) is activated by WNK (with no K kinases) and in turn stimulates the thiazide-sensitive Na-Cl cotransporter (NCC) and the furosemide-sensitive Na-K-2Cl cotransporter (NKCC) thus contributing to transport and cell volume regulation along with NHE. Thus, it is necessary to know whether the decreased OSR1 activity alters the DC function.

AIMS OF THE STUDY

1. To study the expression of Na^+/H^+ exchangers in DCs and to identify the prominent isoforms expressed in DCs.
2. To determine the effect of LPS on Na^+/H^+ exchanger activity in DCs and to study their possible role in the activation of DCs.
3. To analyze the role of PI3- kinase in the regulation of Na^+/H^+ exchanger activity.
4. To elucidate the significance of cell volume regulatory mechanisms in DC functions using cells obtained from mice with decreased OSR1 activity.

4. MATERIALS AND METHODS

MATERIALS

Equipment

Eppendorf Centrifuge 5415R	Hinz GmbH, Hamburg, Germany.
Eppendorf Pipets 1000µl, 200µl, 20µl, 10µl	Eppendorf, Hamburg, Germany.
Fluorescence microscope	Axiovert, Zeiss, Jena, Germany.
Fluorescence Microscopy low lightCCD camera	Proxitronic, Bensheim, Germany.
LSM 510 confocal microscope	Zeiss, Jena, Germany.
Lambda 10-2 Sutter Instrument	Novato, USA.
Lamp ebx 75 isolated	Leika, Jena, Germany.
Light cycler	Roche Diagnostics, Mannheim, Germany.
FACS-Calibur	Becton Dickinson, Heidelberg,Germany.
FACS tubes, 1,3ml, PP, round bottom	Greiner bio-one, Frickenhausen.
Incubator	Heraeus, Hanau.
MagNa Lyser	Roche Diagnostics, Mannheim, Germany.
Mastercycler gradient	Eppendorf, Hamburg, Germany.
Metaflour Image Analyzer software	MDS Analytical Technologies, Toronto,Canada.
Microflow Biological Safety Cabinet	Nalge Nunc, Wiesbaden-Bierbach, Germany.
Microscope Stemi 2000	Zeiss, Jena, Germany.
Milli-Q	MILLIPORE, S.A. Molsheim France. Germany.
pH Meter 765	Knick, Zweibrücken, Germany.
Vortex	Labnet Abimed, Langenfeld, Germany.

Chemicals

Chemical	Supplier
$BaCl_2x2H_2O$	Carl Roth, Karlsruhe, Germany.
BCECF (2’,7’,-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein)	Molecular Probes, Leiden, Netherlands.
BD FACS Flow solution	Becton Dickinson, Heidelberg
Buffer –formamide	Sigma, Taufkirchen, Germany.
BSA bovine serum albumine	Sigma, Taufkirchen, Germany.
$CaCl_2x2H_2O$	Carl Roth, Karlsruhe, Germany.
Cariporide	
DCFDA	Sigma, Schnelldorf, Germany.
Diethyl ether	Carl Roth, Karlsruhe, Germany.
EDTA (Ethylenediamine tetraacetic acid)	Sigma, Taufkirchen, Germany.
Ethanol absolute (99%)	Sigma, Taufkirchen, Germany.
ECL kit (enhanced chemiluminescence)	Amersham, Freiburg, Germany.
Formaldehyde solution	Sigma-Aldrich, Taufkirchen, Germany
Glucose	Carl Roth, Karlsruhe, Germany.
GM-CSF	Preprotech, Tebu-bio, Rocky Hill, NJ.
HCl	Sigma, Taufkirchen, Germany.
HEPES	Sigma, Taufkirchen, Germany.
$K_2HPO_4x2H_20$	Sigma, Taufkirchen, Germany.
KCl	Carl Roth, Karlsruhe, Germany.
KH_2PO_4	Sigma, Taufkirchen, Germany.
LPS (Lipopolysaccharide) from *Escherichia coli*	Sigma-Aldrich, Germany.
LY294002	Sigma-Aldrich, Germany.
Lysis buffer	Pierce, Rockford, USA.
Mannitol	Sigma, Taufkirchen, Germany.

$MgSO_4$	Sigma, Taufkirchen, Germany.
NaCl	Sigma, Taufkirchen, Germany.
N-Methyl-d-glucamine (NMDG)	Sigma, Taufkirchen, Germany.
Nigericin	Sigma, Taufkirchen, Germany.
PBS (Phosphate buffer saline)	GIBCO, Karlsruhe.
Phorbol ester	Sigma, Taufkirchen, Germany
Protease inhibitor cocktail	Sigma-Aldrich, Taufkirchen, Germany
RPMI 1640	GIBCO, Carlsbad, Germany
Sterilium	Carl Roth, Karlsruhe, Germany.
TBOH	Sigma-Aldrich, Taufkirchen, Germany.
TEA buffer (triethanolamine acetic anhydride)	Sigma, Taufkirchen, Germany.
Tempol	Sigma, Taufkirchen, Germany.
Tiron	Sigma, Taufkirchen, Germany.
Tris buffer	Sigma, Taufkirchen, Germany.
Tween-20	Sigma-Aldrich, Taufkirchen, Germany.
Wortmannin	Sigma-Aldrich, Taufkirchen, Germany.

Kits and Antibodies

FITC-conjugated anti-mouse CD11c	**BD Pharmingen, Heidelberg, Germany**
TNFα ELISA kits	BD PharMingen, Heidelberg, Germany.

Animals

All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities. Dendritic cells (DCs) were cultured from bone marrow of 7-11 weeks old female C57/Bl-6 mice (Charles River, Sulzfeld, Germany)[144;203], heterozygous OSR1 knockin mice (osr^{KI}) and respective wild type mice (osr^{WT}). osr^{KI} and osr^{WT} mice were kindly provided by D. Alessi, University of Dundee, UK. As described earlier[204], the knockin mice carry a mutation of the T-loop Thr residue in OSR1 (Thr185) to Ala preventing activation by WNK isoforms. Mice had free access to control diet (1314, Altromin Heidenau, Germany) and tap drinking water.

METHODS

Cell Culture

Bone marrow-derived cells were flushed out of the cavities from the femur and tibia with PBS. Cells were then washed twice with RPMI and seeded out at a density of 2 x 10^6 cells per 60-mm dish. Cells were cultured for 6 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10 % fetal calf serum (FCS), 1 % penicillin/streptomycin, 1 % glutamine, 1 % non-essential amino acids (NEAA) and 0.05 % β-mercaptoethanol. Cultures were supplemented with granular monocyte colony stimulating factor (GM-CSF, 35 ng/mL, Preprotech Tebu) and fed with fresh medium containing GM-CSF on days 3 and 6. At day 7, ≥80% of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed at days 7-9 of DC culture.

Immunostaining and flow cytometry

Cells (4 x 10^5) were incubated in 100 µl FACS buffer (phosphate buffered saline (PBS) plus 0.1% FCS) containing fluorochrome-conjugated antibodies at a concentration of 10 µg/ml. A total of 2 x 10^4 cells were analyzed. Staining with FITC-conjugated anti-mouse CD11c (BD Pharmingen, Heidelberg, Germany) was used as a positive marker for dendritic cells. After incubating with the antibody for 60 minutes at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometric analysis.

Treatments

Stock solutions of LPS were prepared in culture medium whereas all other substances were dissolved in sterile distilled water. The cells were treated by adding the substances to the cell suspension at the indicated final concentrations and incubating accordingly at 37°C in a humidified 5 % CO_2 atmosphere.

Determination of cell volume

Cell volume was determined by the forward scatter in flow cytometric analysis. Briefly, 2 x 10^5 cells were taken in a culture dish and treated with LPS (with or without

cariporide). After the treatment, cells were collected, centrifuged, the pellet was resuspended in FACS buffer and analysed with flow cytometry (FACS-calibur from Becton Dickinson; Heidelberg, Germany).

Determination of ROS production

ROS production in DCs was determined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA)[205]. Briefly, 2 x 10^5 cells were taken in a culture dish and treated with LPS (with or without cariporide). After the treatment, cells were collected and DCFDA (Sigma, Schnelldorf, Germany) was added to the cell suspension at a final concentration of 10 µM. After 30 minutes of incubation in the dark at 37°C, cells were centrifuged and the pellet was washed twice with ice-cold PBS. The pellet was then resuspended in FACS buffer and the fluorescence was analysed with flow cytometry (FACS-calibur from Becton Dickinson; Heidelberg, Germany). DCFDA fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Phosphatidylserine translocation

Apoptotic cell membrane scrambling was evidenced from annexin V binding to phosphatidylserine at the cell surface[206]. To this end, the percentage of phosphatidylserine-translocating cells was evaluated by staining with FITC-conjugated Annexin V. In brief 4x10^5 cells were harvested and washed twice with Annexin washing buffer (AWB, 10mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). The cell pellet was resuspended in 100 ml of Annexin-V-Fluos labelling solution (Roche) (20 ml Annexin-V-Fluos labelling reagent in 1 ml AWB), incubated for 15 min at room temperature. After washing with AWB, cells were analyzed by flow cytometry.

Measurement of intracellular pH

For digital imaging of cytosolic pH (pH_i), cells were incubated in a Hepes-buffered Ringer solution containing 10 µM 2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluoresceinacetoxymethylester (BCECF-AM Molecular Probes, Leiden, The

Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Between 10-20 cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH_i values using the high-K^+/nigericin calibration technique[207]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K^+/nigericin (10 µg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+[207]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (ß) of the cells[207]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$$ß = \Delta [NH_4^+]_I / \Delta pH_i,$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 [208] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 mM $[20/(1+10^{pHo-pK})]$. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from:

$$[NH_4]_i = 19.37 \cdot 10^{pHo-pHi} \text{ mM}$$

To calculate the ΔpH/min during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 5 µM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C.

Real time PCR

To evaluate OSR1 or NHE transcript levels, mRNA abundance was determined by quantitative real-time PCR. To this end, total RNA was isolated using the Trifast Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA was reverse-transcribed using oligo$(dT)_{12\text{-}18}$ primers (OSR1) and random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScript II Reverse Transcriptase. Then, quantitative real time PCR was performed with the BioRad iCycler iQ^{TM} Real-Time PCR Detection System.

OSR1 mRNA expression was analysed using the TaqMan Gene Expression Assay Reactions were performed using Universal TaqMan Master as per the manufacturer's reccomendations (Step1: 50°C for 2 minutes : Step 2: 95°C for 10 minutes; Step 3: 40 cycles of thermal cycling at 95°C for 15 sec. and 60°C for 60 sec)

NHE mRNA expression was analysed using the iTaq Fast SYBR Green system according to manufacturer's reccomendations (Step 1: 95°C for 2 min; Step 2: 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 68°C for 20 sec).

Calculated mRNA expression levels were normalized to the expression levels of TBP in the same cDNA sample. Relative quantification of gene expression was calculated according to the ΔΔCt method.

For the amplification we used the following primers (5'-3'orientation):

Nhe1, fw TCTGATGGTGCTGGCAGTAG rev; ATGTCCCAGGTCTTACACGC

Nhe2, fw TTGAAATGGCAGAGACAGGGA rev CATTTCGCCTGGCGTGAG;

Nhe3, fw GTCACCCAGGATGTAGCCTCTG rev GGTGGCACCCTGGATAGGAT;

Nhe4, fw ATGCGGAAAGGCCAGAGTCT rev TCTCTCCTTGCTGCCTGAGG;

Nhe6, fw TGCCTGGCTCTTTCGGATG rev AGCAGGCTGGGAGTGTGGTA;

Nhe7, fw GCAGGAAAGTGCATGGATATTCA rev GGAGAGTGGTGGTTAGCGGG;

Nhe8, fw CTCGTGGACATCGAGGATGC rev AGGTGCTCTGACTCGATGGC;

Nhe9, fw ACGAAGACAGAGAGTGCACAGCT rev GTGGTGGTCAGTGGAGGGC;

Tbp, fw CACTCCTGCCACACCAGCTT rev TGGTCTTTAGGTCAAGTTTACAGCC.

OSR1, Fw TTTCTCAGCTGAGGTCTCCCCG

Rev TGCCATCATCAGGAATATCCGAGCC

Western blotting

DCs (2 X 10^6 cells) were washed twice with PBS, then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich, Schnelldorf, Germany). Samples were stored at -80°C until use for Western blotting. Cell lysates were separated by 7.5 % SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% BSA in triethanolamine-buffered saline (TBS) and 0.1% Tween-20. Then the blots were probed overnight with primary antibiodies anti p-Akt (1:1000, Cell Signaling), anti Akt(1:1000, Cell Signaling), GAPDH (1:1000, Cell Signaling) and anti p-NKCC1 [209] (1:5000) antibodies diluted in 5% milk in PBS and 0.1% Tween-20, washed 5 times, probed with secondary antibodies conjugated with horseradish peroxidase (1:2000) for 1 h at room temperature, and washed final 5 times. Antibody binding was detected with the enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany). Densitometer scans of the blots were performed using Quantity One (BioRad).

Determination of migration

For migration assays transwell inserts (BD Falcon 353097) and BD BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences 354480) were used with a pore diameter size of 8 µm. The transwells were placed in a 24-well cell culture plate containing cell culture medium (750 µl) with or without chemokine ligand 21 (CCL21, 250 ng/ml, Peprotech) in the lower chamber. The upper chambers were filled with 500 µl cell culture

medium containing DCs at a concentration of 50,000 cells/ml. The chamber was placed in a 5% CO_2 37°C incubator for 4 hours. In the following, the non-migrated cells were removed by scrubbing with a cotton-tipped swab for two times and washing with PBS. The membrane was removed with a scalpel and fixed in 4% paraformaldehyde (PFA) for 15 mins. The migrated cells were then identified by staining with 4',6-diamidino-2-phenylindole (DAPI).

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test or ANOVA and only results with $p < 0.05$ were considered statistically significant.

5. RESULTS

Effect of bacterial lipopolysaccharide on Na^+/H^+ exchanger activity

Quantitative real time PCR analysis was carried out to identify the prevailing Na^+/H^+ exchanger isoforms expressed in the dendritic cells. As illustrated in Fig. 7, the DCs expressed mainly the NHE1 and NHE6 isoforms. Further studies were aimed at elucidating the functional significance of NHE1 isoform which is known to be expressed in the cell membrane as compared to NHE6 isoform which is known to be expressed in mitochondrial membrane[210].

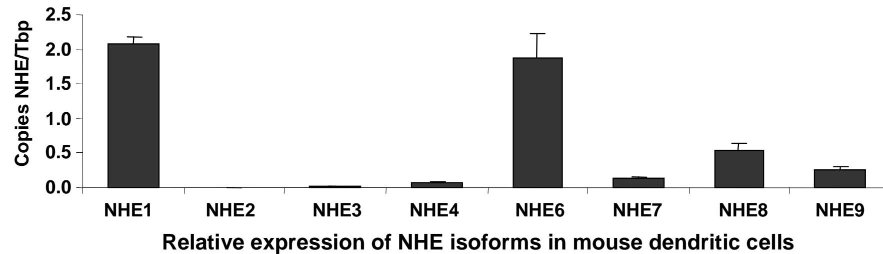

Figure 7:NHE expression in mouse bone marrow derived DCs.

Arithmetic means ± SEM (n = 3 independent experiments) of relative NHE mRNA expression in DCs.

To determine, whether the Na^+/H^+ exchanger is functional in DCs, the pH recovery of the cells after ammonium pulse was measured using the BCECF fluorescence. In this maneuver, NH_4Cl is added to the perfusate. NH_3 enters the cells, binds H^+ to form NH_4^+ and thus leads to a transient cytosolic alkalinization (Fig. 8). The subsequent removal of NH_4Cl leads to exit of NH_3 leaving H^+ behind. The H^+ trapped in the cell leads to cytosolic acidification (Fig. 8). In the absence of Na^+, the average cytosolic pH declined further after an ammonium pulse, revealing that the cells did not express sufficient levels of Na^+-independent H^+ extrusion mechanisms to maintain or recover cytosolic pH (table 1). The addition of Na^+ led, however, to a rapid pH recovery pointing to the operation of a Na^+/H^+ exchanger. The Na^+-dependent pH recovery was blunted in the presence of NHE1 inhibitor cariporide (Fig. 9, table 1).

Treatment of DCs with LPS did not significantly affect the buffer capacity of the cells (table 1). Accordingly, the relation between H^+ transport across the cell membrane and cytosolic pH was comparable in the presence and absence of LPS. As revealed from the ammonium pulse experiments, a 4-hr LPS treatment was followed by a marked and statistically significant increase in Na^+-dependent realkalinization (table 1, Fig. 8). In order to determine the concentration of LPS triggering half maximal effects, NHE activity of DCs was studied at varying LPS concentrations (100 ng/ml - 10 µg/ml). A sigmoidal dose response curve was obtained (Fig. 8b) with a peak response at 1 µg/ml LPS. The following experiments were performed at saturating LPS concentration (1 µg/ml). Further experiments were performed to elucidate the time course of the LPS effect on NHE activity. As shown in Fig 8c, LPS (1 µg/ml) increased NHE activity within one hour.

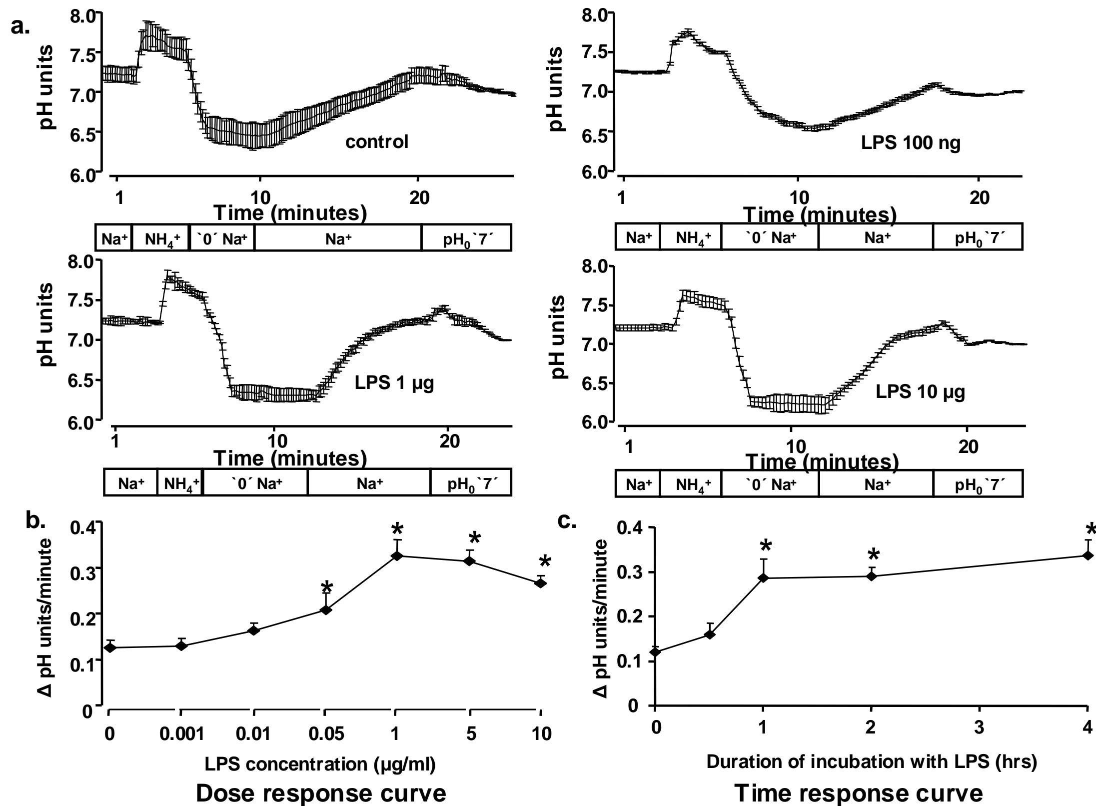

Figure 8: LPS treatment leads to stimulation of NHE activity in DCs

Alterations of cytosolic pH (pH_i) in bone marrow derived DCs following an ammonium pulse. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG) in a first step (see bars below each original tracing), NH_4Cl removed in a second step, Na^+ added in a third step and nigericin (pH_o 7.0) applied in a fourth step to calibrate each individual experiment.

a. Original tracings (± SEMs) illustrating alterations of cytosolic pH in typical experiments prior to (upper left) and 4 hours following treatment with LPS 100 ng/ml (upper right) or LPS 1 µg/ml (lower left) or LPS 10 µg/ml (lower right panels).

b. Dose response curve of LPS: Arithmetic means ± SEM (n = 3 independent experiments) of Na^+-dependent recovery of cytosolic pH in DCs (ΔpH/min) following an ammonium pulse prior to (LPS ‘0’ µg/ml) or 4 hours following treatment with varying concentrations of LPS. * indicate statistically significant difference from control (LPS ‘0’ µg/ml).

c. Time response curve of LPS: Arithmetic means ± SEM (n = 3 independent experiments) of Na^+-dependent recovery of cytosolic pH in DCs (ΔpH/min) following an ammonium pulse prior to (time point ‘0’ hrs) or following treatment LPS at different time points. * indicate statistically significant difference from control (time point ‘0’ hrs).

To determine NHE1 contribution in LPS stimulated NHE activity, further series of experments was performed with specific NHE1 inhibitor cariporide. Treatment of DCs with LPS (1 µg/ml) was followed by a marked and statistically significant increase in Na^+-dependent realkalinization within 1 and 4 hours (table 1, Fig. 9). Again, in the absence of Na^+ there was a further acidification, i.e. accelerated realkalinization following LPS treatment was due to Na^+-dependent H^+ extrusion pointing to stimulation of Na^+/H^+ exchanger activity (Fig. 9). The increase in Na^+/H^+ exchanger activity was blunted in the presence of the NHE1 inhibitor cariporide (10 µM) (Fig 9). Accordingly, LPS significantly increased the cariporide sensitive realkalinization (from 0.12 to 0.33 Δ pH units/minute). The Na^+/H^+ exchanger activity declined thereafter to values significantly below the Na^+/H^+ exchanger activity prior to LPS treatment (Fig. 9). Treatment of DCs with LPS did not significantly modify cytosolic pH within the first 4 hours, indicating that the Na^+/H^+ activity required cytosolic acidification and/or that it was paralleled by enhanced H^+ generation. LPS did not affect the buffer capacity of the cells (table 1). Accordingly, the accelerated realkalinization following LPS treatment was due to enhanced H^+ transport across the cell membrane and not due to decreased buffer capacity.

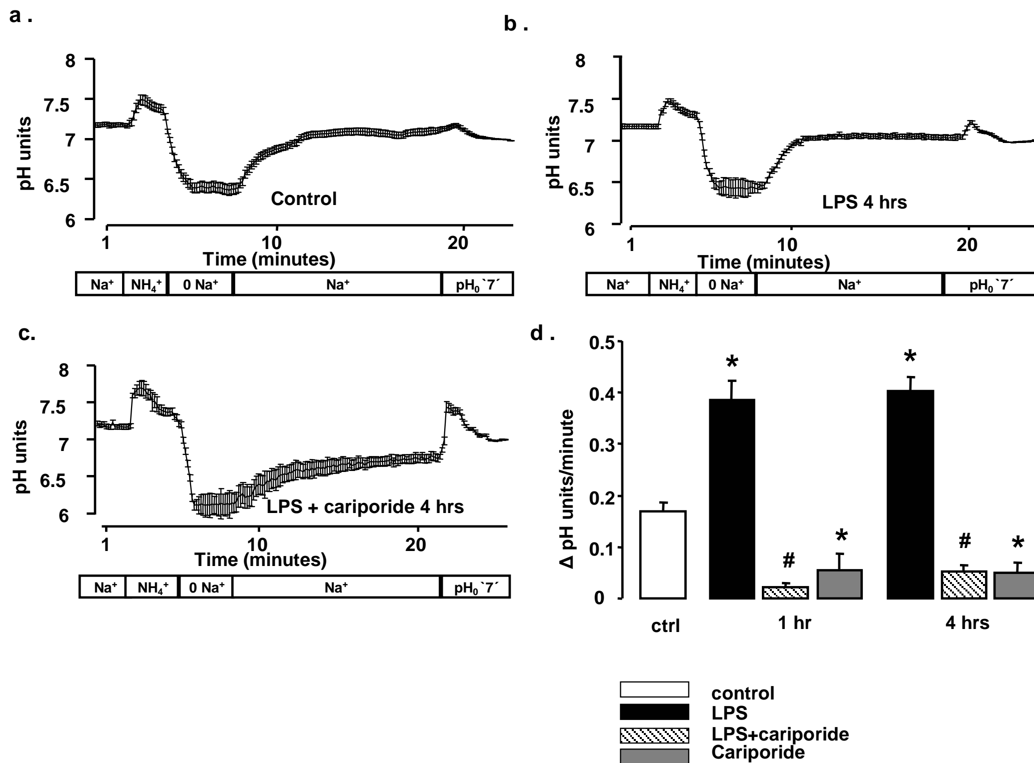

Figure 9: LPS induced NHE stimulation is sensitive to NHE1 inhibition.

a-c. Alterations of cytosolic pH in typical experiments (**a**) prior to, or following a 4 hours treatment with LPS (1 µg/ml) (**b**) in the absence and (**c**) in the presence of cariporide (10 µM).

d. Arithmetic means ± SEM (n = 4 independent experiments) of Na^+-dependent recovery of cytosolic pH in DCs (ΔpH/min) following an ammonium pulse prior to (white bar), following addition of LPS (1 µg/ml) in the absence (black bars) or presence (hatched bars) of cariporide (10 µM). Alternatively, cariporide was added without LPS (grey bars). * indicates significant difference (p<0.01) from control (value prior to LPS). # indicates significant difference (p<0.01) from respective LPS treatment.

Na^+/H^+ exchangers are known to regulate the cell volume and are known to be critically important for the Reactive oxygen species (ROS) formation in the DCs. Therefore, cell volume and ROS formation was studied by FACS analysis in LPS-stimulated DCs. The treatment of DCs with LPS was within 4 hours followed by an increase in forward scatter, pointing to an increase in cell volume (Fig. 10). Forward scatter was not increased following simultaneous treatment of DCs with LPS and cariporide (Fig. 10). Despite the decline of NHE activity, cell volume remained larger at

later stages of LPS treatment (not shown), which may point to a set point shift of cell volume regulation.

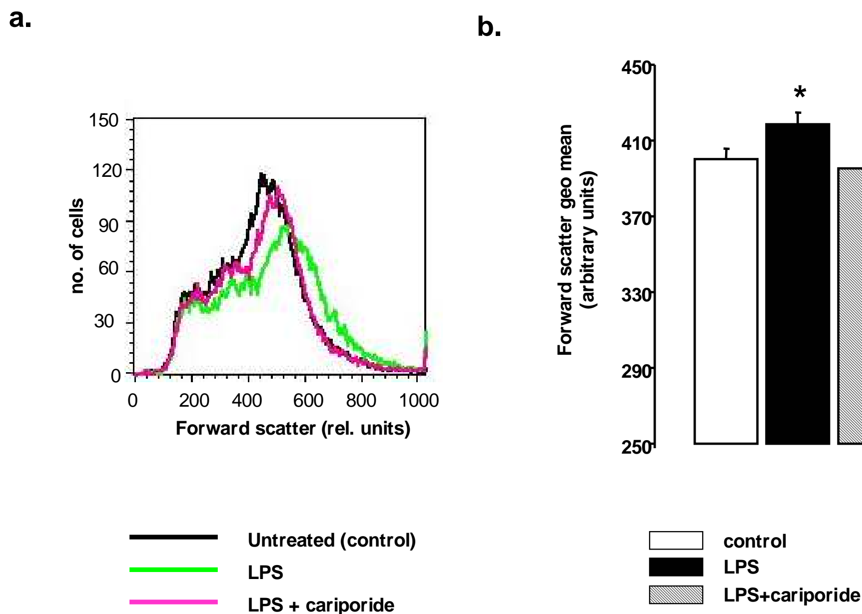

Figure 10: LPS induced cell volume increase is sensitive to NHE1 inhibition.

a. Representative FACS histograms depicting the forward scatter in murine DCs without treatment (control, black line) and after a 4 hours treatment with LPS (1 µg/ml) in the absence (green line) and presence (pink line) of cariporide.

b. Arithmetic means ± SEM (n = 5-6 independent experiments) of forward scatter in DCs incubated for 4 hours without (white bars) or with LPS (1 µg/ml) in the absence (black bars) or presence (hatched bars) of cariporide (10 µM). * ($p<0.05$) indicate significant difference from untreated (control) group; # ($p<0.05$) indicate significant difference from LPS in the absence of cariporide.

The role of NHE1 in LPS-induced ROS formation was studied in DCs in the presence and absence of cariporide. LPS enhanced the formation of ROS, an effect again significantly reduced upon simultaneous treatment with cariporide, pointing to a critical role of NHE1 in ROS formation (Fig. 11). ROS formation tended to be decreased in

cariporide treated cells, a difference, however, not reaching statistical significance (Fig 11).

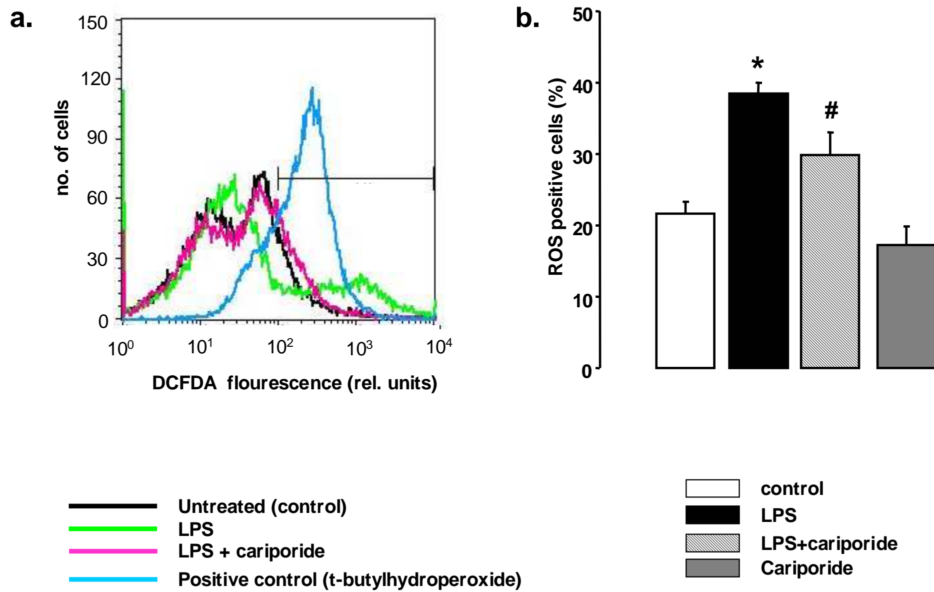

Figure 11: LPS induced ROS production is sensitive to NHE1 inhibition.

a. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in murine DCs without treatment (control, black line) as well as after a 4 hours treatment with LPS (1 µg/ml) in the absence (green line) and presence (pink line) of cariporide. Cells treated for 30 minutes with the oxidant t-butylhydroperoxide (0.3 mM) were used as positive control (blue line).

b. Arithmetic means ± SEM (n = 5-6 independent experiments) of the percentage of ROS positive DCs incubated for 4 hours without (white bars) or with LPS (1 µg/ml) in the absence (black bars) or presence (hatched bars) of cariporide (10 µM) as well as in the presence of cariporide (10 µM, grey bar) alone. * ($p<0.05$) indicate significant difference from untreated (control) group; # ($p<0.05$) indicate significant difference from LPS in the absence of cariporide.

To explore whether ROS formation is required for activation of the Na^+/H^+ exchanger by LPS, ammonium pulse experiments were carried in DCs treated with LPS in presence of ROS scavengers. Three different known ROS scavengers (Tempol, Tiron and Vitamin C) were used at previously reported concentrations[211]. In the presence of ROS chelators LPS induced stimulation of NHE was blunted (Fig. 12, table 1). Conversely, treatement of DCs with the the oxidant t-butyl hydroperoxide (200µM) was followed by a significant increase in the NHE activity (Fig. 12, table 1). Thus, ROS activation participates in the stimulation of Na^+/H^+ exchanger activity by LPS.

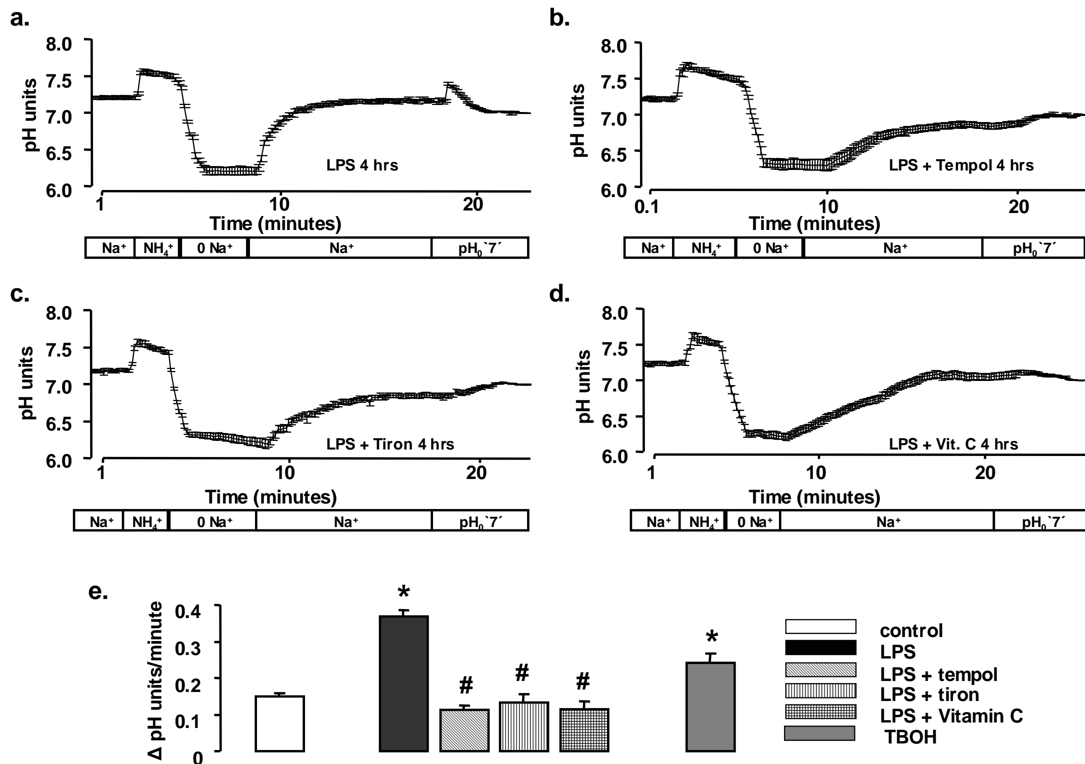

Figure 12: LPS induced NHE stimulation is dependent on intracellular ROS.

a-d. Tracings illustrating alterations of cytosolic pH in typical experiments in 4- hour LPS treated DCs (**a**) in the absence of ROS scavengers, (**b**) in the presence of Tempol (30 µM), (**c**) in the presence of Tiron (1 M) or (**d**) in the presence of Vitamin C (50 µg/ml).

e. Arithmetic means ± SEM (n = 3-5 independent experiments) of Na^+-dependent recovery of cytosolic pH in DCs (ΔpH/min) following an ammonium pulse prior to (white bar), following addition of LPS (1 µg/ml) in the absence of ROS scavengers or oxidants (black bar) or in the presence of Tempol (30 µM, hatched bars), of Tiron (1 M, striped bar), or of Vitamin C (50 µg/ml, crossed bar), or following treatment with t-butyl hydroperoxide (TBOH, 200 mM, light grey bar). * indicates significant difference (p<0.01) from control (value prior to LPS). # indicates significant difference (p<0.01) from respective LPS treatment.

table 1:Cytosolic pH (ΔpHi), buffer capacity and sodium independent pH recovery in bone marrow-derived dendritic cells (DCs) prior to (control) and following treatment with lipopolysaccharide LPS (1 µg/ml).

	Intracellular pH (units)	**Buffer Capacity (mM/pH unit)**	**Sodium independent pH recovery (Δ pH units/minute)**	**Sodium dependent pH recovery (NHE activity, Δ pH units/minute)**	**Number of cells**
control	7.22 ± 0.01	17.4 ± 1.4	-0.090 ± 0.013	0.169 ± 0.017	99
LPS 1hr	7.23 ± 0.02	13.9 ± 2.1	-0.230 ± 0.044	0.385 ± 0.037*	53
LPS + carip 1 hr	7.20 ± 0.03	16.4 ± 1.8	-0.229 ± 0.016	0.023 ± 0.008#	25
Cariporide 1 hr	7.16 ± 0.04	14.8 ± 2.5	-0.116 ± 0.055	0.056 ± 0.029*	15
LPS 4 hrs	7.20 ± 0.02	17.9 ± 2.2	-0.104 ± 0.019	0.402 ± 0.040*	42
LPS + carip 4 hrs	7.18 ± 0.02	14.4 ± 0.9	-0.175 ± 0.041	0.053 ± 0.011#	31
LPS + Tempol 4 hrs	7.29 ± 0.01	17.0 ± 1.1	-0.044 ± 0.005	0.113 ± 0.011#	112
LPS + Tiron 4 hrs	7.29 ± 0.02	20.6 ± 5.5	-0.055 ± 0.008	0.133 ± 0.023#	38
LPS + Vitamin C 4 hrs	7.33 ± 0.02	19.1 ± 2.2	-0.054 ± 0.007	0.114 ± 0.022#	43
t-butylhydroperoxide 1 hr	7.16 ± 0.02	18.8 ± 1.3	-0.063 ± 0.007	0.241 ± 0.026*	44
Cariporide 4 hrs	7.14 ± 0.03*	19.5 ± 2.2	-0.010 ± 0.009	0.049 ± 0.022*	37

* indicates significant difference from the respective control value.

# indicates significant difference from the respective LPS treatment.

Further experiments were conducted to verify whether treatment with LPS and/or cariporide affected cell survival. As shown in Fig. 13, LPS treatment significantly reduced the percentage of Annexin V positive cells pointing to inhibition of dendritic cell apoptosis. The effect was virtually abolished when the cells were treated simultaneously

with cariporide (Fig. 13). Cariporide alone did not significantly modify DC apoptosis (Fig 13).

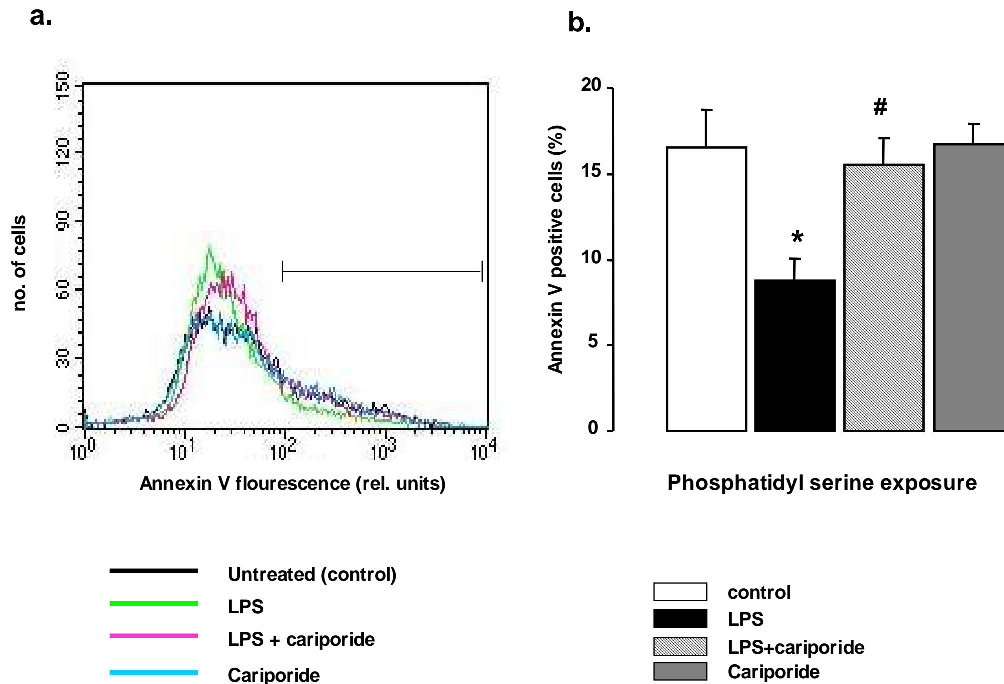

Figure 13: Cariporide abrogates effects of LPS on dendritic cell phosphotidyl exposure.

a. Representative FACS histograms depicting the expression of annexin V in DCs cultured for 24 hours without (control, black line) or with LPS (1 µg/ml) in the absence (green line) and presence (pink line) of cariporide (10 µM) or with cariporide (10 µM, blue line)

b: Arithmetic means ± SEM (n = 3 independent experiments) of the percentage of annexin V-positive DCs following a 24 hours culture without (control, white bar) or with LPS (1 µg/ml) in the absence (black bar) or presence (hatched bar) of cariporide (10 µM) or with cariporide alone (10 µM, grey bar). * ($p<0.05$) indicates significant difference from control, # ($p<0.05$) indicates significant difference from LPS.

To explore, whether NHE modified cytokine release from DCs, TNF-α in the supernatant was determined with ELISA. To this end DCs were stimulated with LPS (1 µg/ml, 4 h) in the absence or presence of the Na^+/H^+ exchanger inhibitor cariporide (10 µM)[212]. As illustrated in Fig. 14, the release of TNF-α following exposure to LPS was significantly blunted in the presence of cariporide. Thus, LPS induced TNF-α production was critically dependent on the activity of the Na^+/H^+ exchanger.

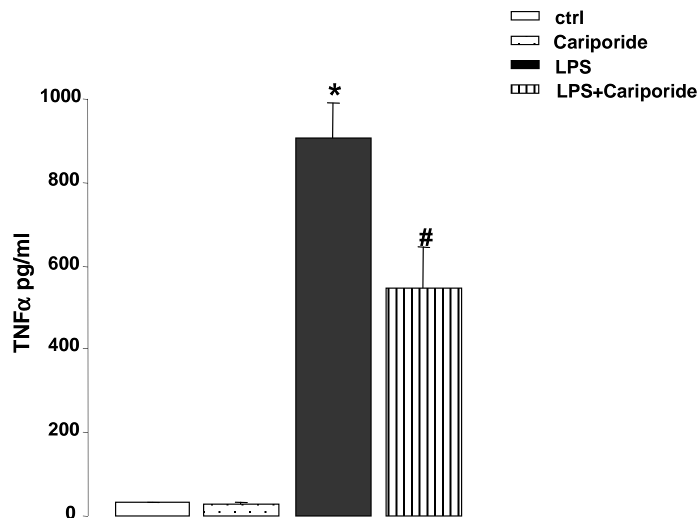

Figure 14: Cariporide abrogates LPS induced TNF-alpha secretion in DCs.

Arithmetic means ± SEM (n = 4 independent experiments) of TNF-α concentration in the supernatant of DCs cultured for 4 h with LPS (1 µg/ml) in the absence (black bar) or presence of 10 µM cariporide (striped bars) as well as in the presence of cariporide (10 µM, pointed bar) alone. * ($p<0.05$) indicates significant difference from control, # ($p<0.05$) significant difference to LPS alone.

Phosphoinositide 3-Kinase Dependent Regulation of Na^+/H^+ exchanger activity

Phosphoinositide 3 kinase (PI3 kinase) pathway has been previously reported to be involved in activation of dendritic cells by LPS[213]. Since NHE1 is important in cell volume regulation, ROS production and release of TNFα, and since PI3 kinases are known to be involved in regulation of NHE1 activity[214], further set of experiments focussed on the possible correlation between PI3 kinase activation and stimulation of NHE1 activity by LPS. Phosphorylation of Akt is a key downstream event of PI3 kinase signaling and therefore western blot analysis was performed to determine Akt phosphorylation as a readout of PI3 kinase activity in LPS treated DCs. The total protein abundance of Akt was not significantly different between untreated and LPS treated DCs, but the phosphorylated Akt protein was significantly higher in DCs treated with LPS indicating the activation of the PI3 kinase pathway (Fig.15).

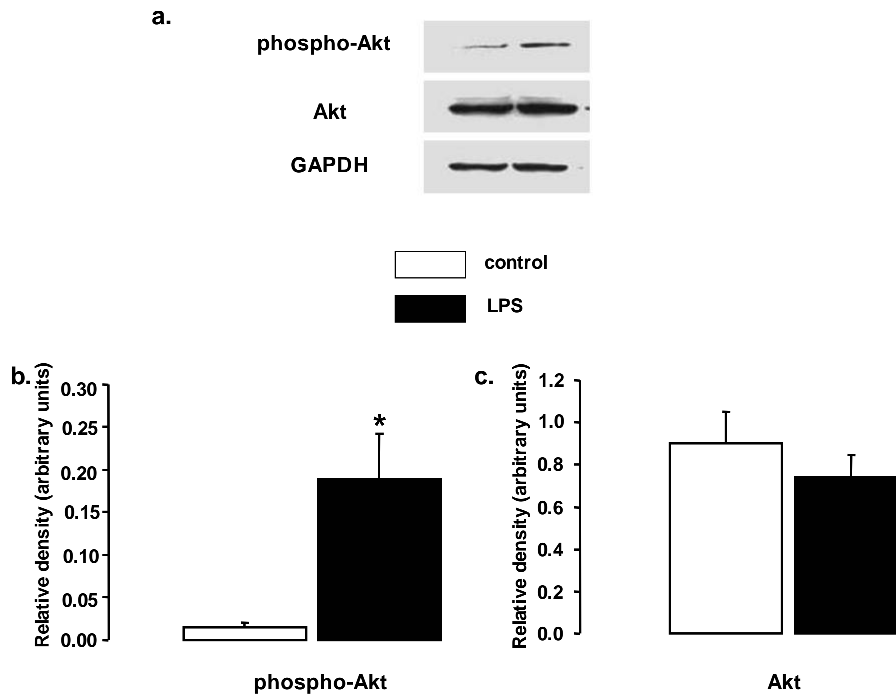

Figure 15: LPS treatment leads to activation of PI-3 kinase pathway.

a. Original Western blot illustrating the phosphorylated Akt (upper panels), total Akt (middle panels) and GAPDH (lower panels) prior to and 4 hours following LPS treatment.

b. Arithmetic means ± SEM (n = 3 independent experiments) of phosphorylated Akt abundance (relative to GAPDH) in LPS treated (closed bars) and control DCs (open bars). * ($p<0.05$) indicates significant difference between LPS treated and untreated DCs.

c. Arithmetic means ± SEM (n = 3 independent experiments) of total Akt abundance (relative to GAPDH) in LPS treated (closed bars) and control DCs (open bars).

In a next step, the role of PI3 kinase dependent signaling for the regulation of the Na^+/H^+ exchanger activity was determined. Previous experiments showed that a 4-hr LPS treatment lead to a marked and statistically significant increase in Na^+-dependent realkalinization (Fig. 13b). To determine the possible role of PI3 kinase signalling, LPS induced upregulation of the NHE activity was further studied in the presence of varying concentrations of phophoinositide (PI) 3 kinase inhibitors wortmannin (0.1 µM – 5 µM)

and LY294002 (1 μM – 50 μM). Inhibition of the PI3 kinase by wortmannin or LY294002 blunted the increase in Na^+/H^+ exchanger activity following LPS treatment in a dose dependent manner (Fig. 16b). The effect of LPS on NHE activity was virtually abolished in the presence of 1 μM wortmannin and 10 μM LY294002 (Fig. 16b). The concentrations of wortmannin (1 μM) and LY294002 (10 μM) were used in further experiments. In the absence of LPS inhibition of PI3 kinase did not have any effect on the Na^+/H^+ exchanger activity and prior to LPS treatment the rate of Na^+-dependent pH recovery was similar in the absence or presence of wortmannin (1 μM) or LY294002 (10μM) (table 2).

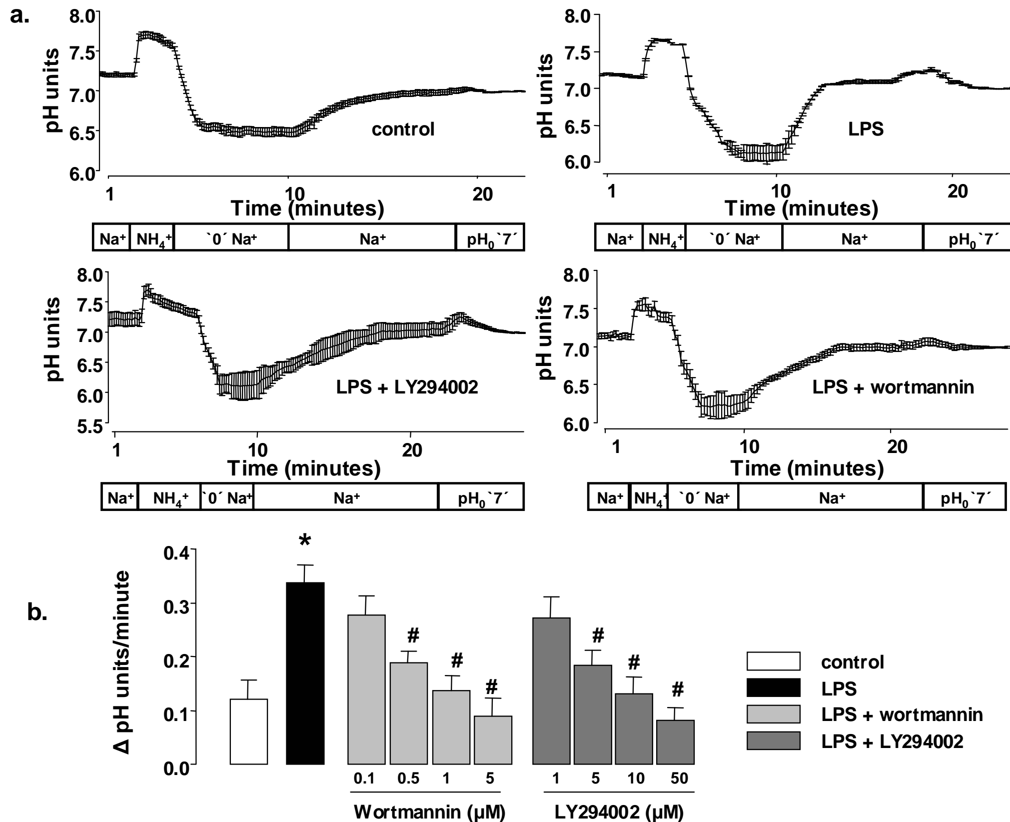

Figure 16: LPS induced NHE stimulation is dependent on PI-3 kinase pathway.

a. Original tracings (± SEMs) illustrating alterations of cytosolic pH in typical experiments prior to (upper left) and 4 hours following treatment with LPS (1 µg/ml) in the absence (upper right) or presence of 1 µM Wortmannin (lower right) or 10 µM LY294002 (lower left panels).

b. Arithmetic means ± SEM (n = 3 independent experiments) of Na^+-dependent recovery of cytosolic pH in DCs (ΔpH/min) following an ammonium pulse prior to (white bar) or 4 hours following treatment with LPS in the absence (black bar) or the presence of 0.1-5 µM Wortmannin (light grey bars) or 1-50 µM LY294002 (dark grey bars). * ($p<0.05$) indicate statistically significant difference from control, # ($p<0.05$) indicates significant difference from absence of PI3 kinase inhibition.

table 2:Cytosolic pH (ΔpHi), buffer capacity and sodium independent pH recovery in bone marrow-derived dendritic cells (DCs) prior to (control) and following treatment with lipopolysaccharide LPS (1 µg/ml). Effect of PI3 kinase inhibitors.

	Intracellular pH (units)	**Buffer Capacity (mM/pH unit)**	**Sodium independent pH recovery (Δ pH units/minute)**	**Sodium dependent pH recovery (NHE activity, Δ pH units/minute)**	**Number of cells**
control (0.1 % DMSO)	7.22 ± 0.02	17.9 ± 1.9	-0.019 ± 0.013	0.121 ± 0.035	44
LPS	7.17 ± 0.02	13.5 ± 2.0	-0.222 ± 0.033*	0.343 ± 0.037*	96
Wortmannin	7. 24 ± 0.05	13.9 ± 3.8	-0.033 ± 0.016	0.144 ± 0.039	20
Wortmannin + LPS	7.15 ± 0.02	19.5 ± 1.5	-0.112 ± 0.020#	0.136 ± 0.028#	48
LY294002	7.22 ± 0.05	12.2 ± 2.2	-0.051 ± 0.012	0.153 ± 0.043	30
LY294002+LPS	7.28± 0.03	16.9 ± 2.5	-0.032 ± 0.010#	0.157 ± 0.033#	37

* ($p<0.05$) indicates significant difference from the respective control value.

($p<0.05$) indicates significant difference from the respective value in the absence of PI3 kinase inhibitor wortmannin or LY294002.

As the effect of LPS on NHE activity was abolished in the presence PI3 kinase inhibitors, experiments were conducted to verify whether the downstream effects of NHE activation, i.e, cell volume increase, ROS production were influenced by PI3 kinase inhibitors.

According to FACS analysis, the treatment of the DCs with LPS for 4 hours was followed by an increase in forward scatter, reflecting cell swelling (Fig. 17a, b). Treatment with wortmannin (1 µM) and LY294002 (10 µM) abrogated the effect of LPS on cell volume of DCs. In the absence of LPS, neither wortmannin (1 µM) nor LY294002

(10 µM) significantly modified the forward scatter (Fig 14b).

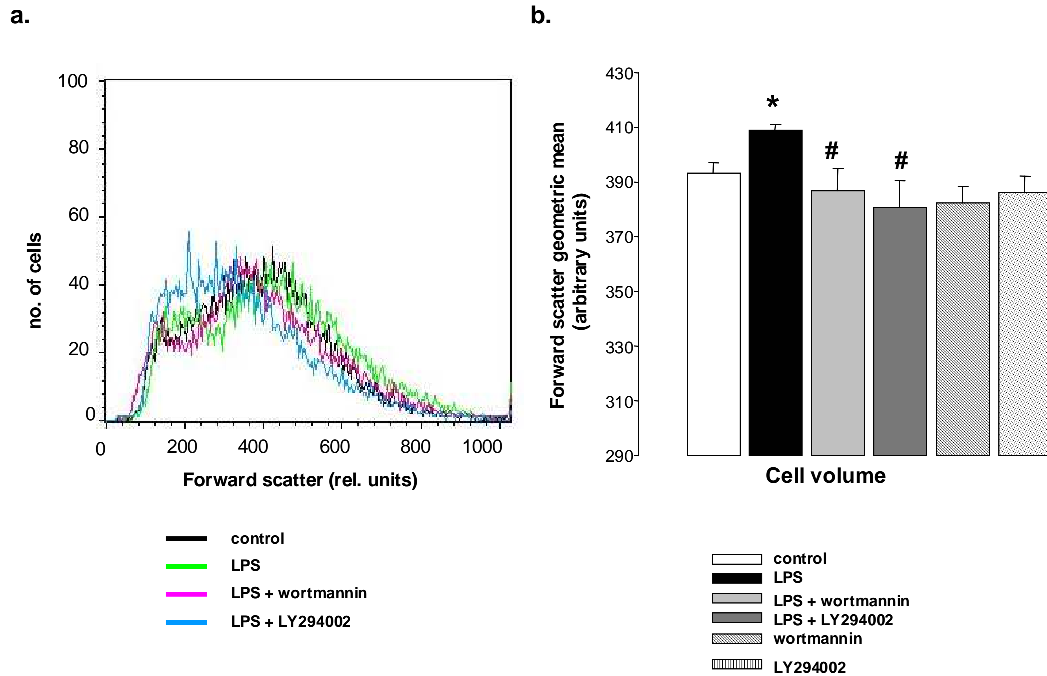

Figure 17: LPS induced cell volume increase is abrogated upon inhibition of PI-3 kinase pathway.

a. Representative FACS histograms depicting the forward scatter of murine DCs without treatment (control, black line) as well as after 4 hours treatment with LPS (1 µg/ml) in the absence (green line) and presence of 1 µM Wortmannin (pink line) or 10 µM LY294002 (blue line).

b. Arithmetic means ± SEM (n = 3 independent experiments) of the forward scatter of DCs prior to (white bar) or following a 4 hours treatment with LPS in the absence (black bar) or the presence of 1 µM Wortmannin (light grey bar) or 10 µM LY294002 (dark grey bar) or in the presence of 1 µM Wortmannin alone (hatched bar) or in the presence of 10 µM LY294002 alone (dotted bar). * ($p<0.05$) indicate statistically significant difference from control, # ($p<0.05$) indicates significant difference from absence of PI3 kinase inhibition.

2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence revealed that LPS-stimulated formation of ROS was abolished by treatment with wortmannin (1 µM) and LY294002 (10 µM) (Fig. 18a, b). Again, in the absence of LPS, neither wortmannin (1 µM) nor LY294002 (10 µM) significantly altered ROS formation (Fig 18b)

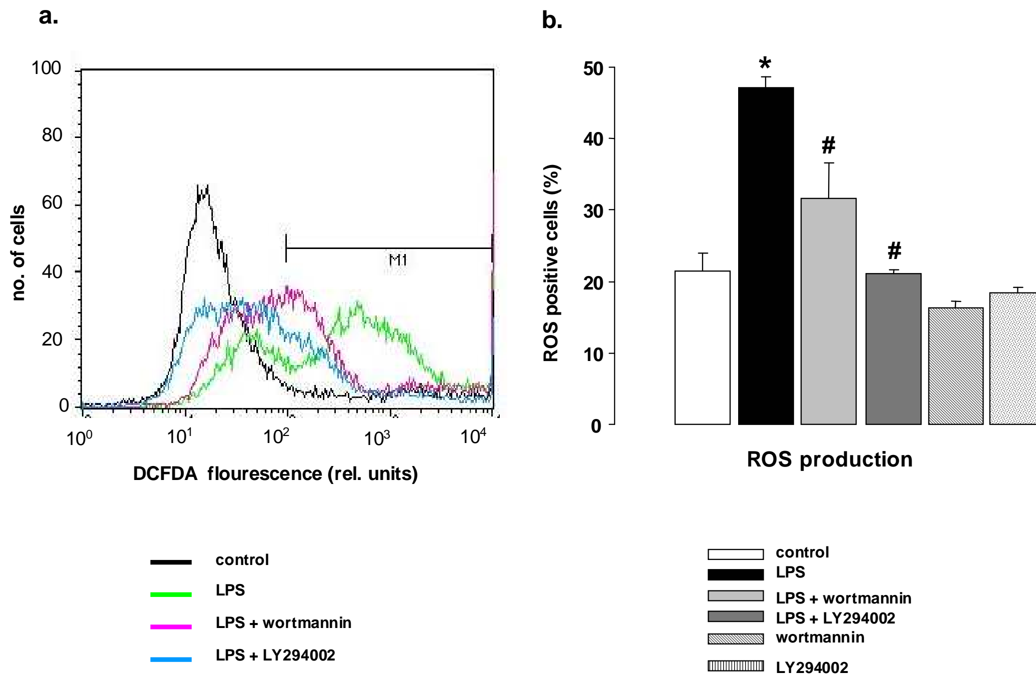

Figure 18:LPS induced ROS generation is blunted upon treatment with PI-3 kinase inhibitors.

a. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in murine DCs without treatment (control, black line) as well as after a 4 hours treatment with LPS (1 µg/ml) in the absence (green line) and presence of 1 µM Wortmannin (pink line) or 10 µM LY294002 (blue line).

b. Arithmetic means ± SEM (n = 3 independent experiments) of the percentage of ROS positive DCs prior to (white bar) or following a 4 hours treatment with LPS in the absence (black bar) or the presence of 1 µM Wortmannin (light grey bar) or 10 µM LY294002 (dark grey bar) or in the presence of 1 µM Wortmannin alone (hatched bar) or in the presence of 10 µM LY294002 alone (dotted bar). * ($p<0.05$) indicates statistically significant difference from control, # ($p<0.05$) indicates significant difference from absence of PI3 kinase inhibition.

Na^+/H^+ exchangers are also known to be critically important for cellular migration[215]. Accordingly, a migration assay was performed in the DCs treated with LPS in the presence and absence of wortmannin (1µM) or LY294002 (10 µM). A transwell migration assay disclosed a stimulatory effect of LPS on migration. The effect

was again significantly blunted in the presence of wortmannin (1 µM) or LY294002 (10 µM) (Fig. 19).

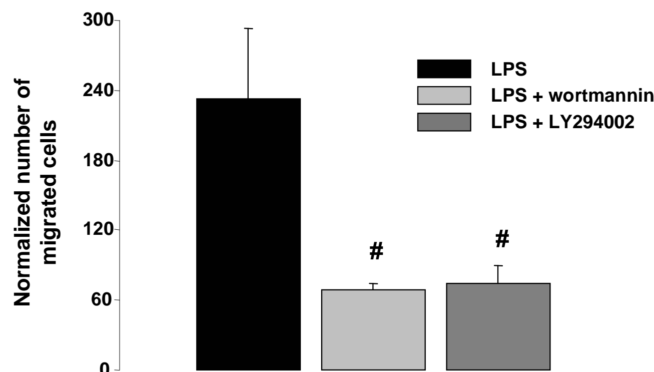

Figure 19: LPS induced DC migration is inhibited upon blocking the PI-3 kinase signalling.

Arithmetic means ± SEM (n = 12 cells out of 4 mice each) of the normalized migration of DCs following a 4 hours treatment with LPS in the absence (black bar) or the presence of 1 µM Wortmannin (light grey bar) or 10 µM LY294002 (dark grey bar). # indicates significant difference from absence of PI3 kinase inhibition. The number of LPS treated cells migrating in response to CCL21 in presence or absence of PI3 kinase inhibitors were expressed in percent of migrating cells receiving no LPS and no inhibitor but the respective solvent.

OSR1-sensitive regulation of Na^+/H^+ exchanger activity

To explore further molecular mechanisms regulating NHE activity in dendritic cells, the role of oxidative stress responsive kinase, OSR1 was studied.

To determine the presence of a functional WNK signalling pathway in murine bone marrow-derived dendritic cells (DCs), RT-PCR was performed. As shown in Fig. 20a. DCs express WNK1, WNK3, NCC and NKCC1 but not SPAK. As illustrated in Fig. 20b, OSR1 mRNA is readily detectable in DCs and the transcript levels were similar in OSR1 knockin mice (*osr*^{KI}) and wild type mice (*osr*^{WT}) DCs. Furthermore, the phosphorylation of NKCC1 was lower in *osr*^{KI} DCs (Fig. 20c), indicative of reduced OSR1 activity in *osr*^{KI} DCs.

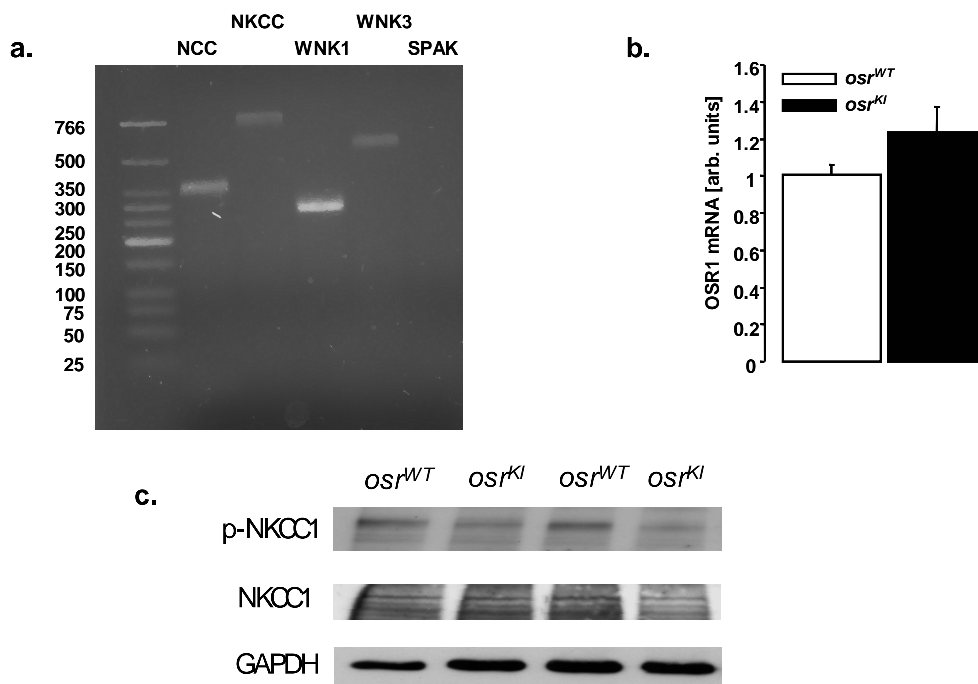

Figure 20 : WNK signalling pathway in murine bone marrow-derived dendritic cells

a. Agarose gel visualizing the products of RT-PCR reactions amplifying NCC, NKCC, WNK1, WNK3 and SPAK from cDNA of murine DCs.

b. Arithmetic means ± SEM (n = 5 mice) of relative OSR1 mRNA abundance in murine DCs from heterozygous OSR1 knockin mice (*osr*^{KI}) and wild type mice (*osr*^{WT}).

c. Original Western blot illustrating the expression of phosphorylated and total NKCC1 in DCs from heterozygous OSR1 knockin mice (*osr*^{KI}) and wild type mice (*osr*^{WT}).

As OSR1 is involved in regulatory cell volume increase, the present study determined the forward scatter of DCs in FACS analysis as a measure of cell volume. As illustrated in Fig. 18, no significant difference was observed in forward scatter between osr^{KI} and osr^{WT}. Confirning previous results, treatment of osr^{WT} DCs with LPS was followed within 4 hours by an increase in forward scatter pointing to an increase in cell volume (Fig. 21). In contrast, the forward scatter of osr^{KI} DCs was not significantly modified by LPS treatment. As a result, following LPS treatment forward scatter was significantly higher in osr^{WT} DCs than in osr^{KI} DCs. To elucidate the contribution of the Na^+/H^+ exchanger, experiments were performed in the absence or presence of the NHE1 inhibitor cariporide. As a result, in both genotypes forward scatter was significantly decreased following treatment of DCs with cariporide (Fig 21). The decrease of forward scatter tended to be higher in osr^{KI} DCs than in osr^{WT} DCs, a difference, however, not reaching statistical significance.

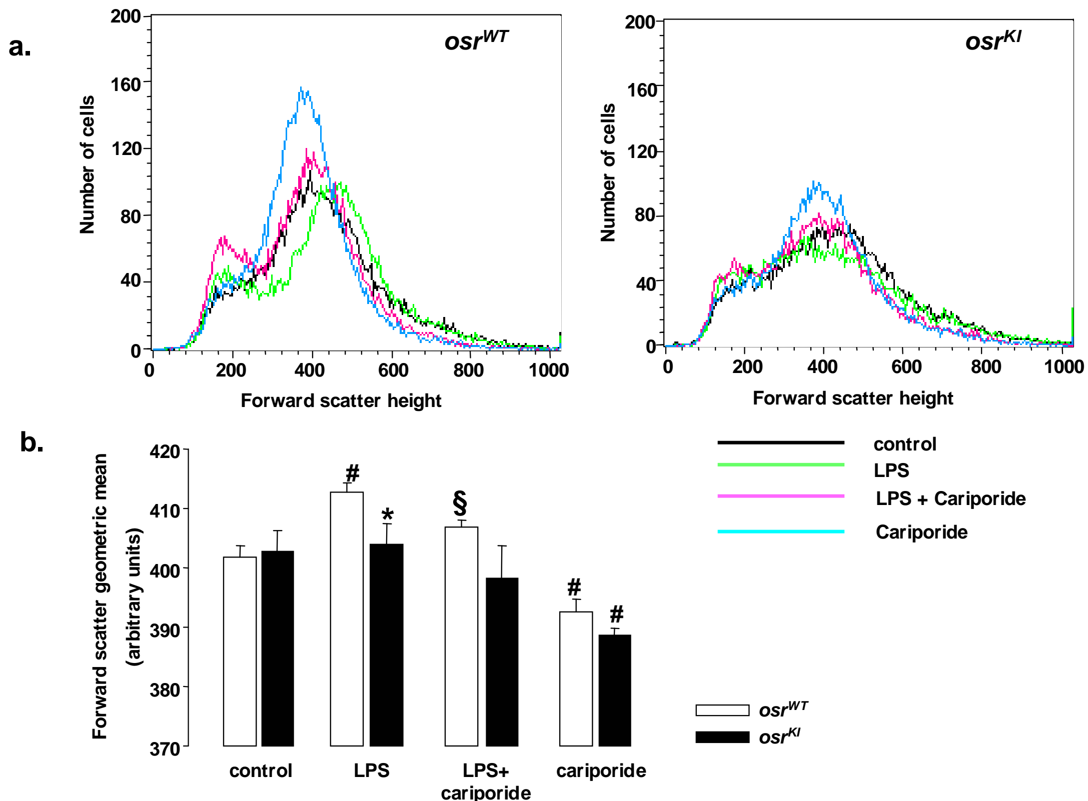

Figure 21:LPS induced cell volume increase is blunted in OSR KI mice.

a. Representative FACS histograms depicting the forward scatter in DCs from heterozygous OSR1 knockin mice (osr^{KI}, right panel) and from wild type mice (osr^{WT}, left panel) without treatment (control, black line) and after a 4 hours treatment with LPS (1 µg/ml) in the absence (green line) and presence (pink line) of cariporide or in the presence of cariporide alone (blue line).

b. Arithmetic means ± SEM (n = 6 independent experiments) of forward scatter in DCs from heterozygous OSR1 knockin mice (osr^{KI}, black bars) and from wild type mice (osr^{WT}, white bars) prior to or 4 hours following exposure to LPS (1 µg/ml) in the absence or presence of cariporide (10 µM) or in the presence of cariporide alone. * indicates significant difference ($p<0.05$) from osr^{WT} DCs, # indicates significant difference ($p<0.01$) from respective control value, § ($p<0.05$) indicate significant difference from respective absence of cariporide.

The lack of cell shrinkage in untreated osr^{KI} DCs could have been due to the lack of OSR1, NCC and/or NKCC expression in osr^{WT} DCs or due to a compensatory increase in Na^+/H^+ exchanger activity in osr^{KI} DCs. To explore that possibility, cytosolic pH was determined in DCs utilizing BCECF fluorescence. According to the ammonium pulse, the

Na^+/H^+ exchanger activity was significantly higher in *osr*^{KI} DCs than in *osr*^{WT} DCs.

As in all wild type DC studied before, treatment of *osr*^{WT} DCs with lipopolysaccharide (LPS, 1 µg/ml) was followed within 4 hours by a significant increase in Na^+-dependent realkalinization (table 3, Fig. 22). Again, in the absence of Na^+ further acidification was observed, i.e. accelerated realkalinization following LPS treatment was due to Na^+-dependent H^+ extrusion pointing to stimulation of Na^+/H^+ exchanger activity (Fig. 22). The increase in Na^+/H^+ exchanger activity was blunted in the presence of the NHE1 inhibitor cariporide (10 µM) (Table 3). In contrast to what was observed in *osr*^{WT} DCs, treatment of *osr*^{KI} DCs with LPS did not significantly modify Na^+/H^+ exchanger activity. Accordingly, following LPS treatment, Na^+/H^+ exchanger activity was significantly lower in *osr*^{KI} DCs than in *osr*^{WT} DCs (Fig. 22). Thus, LPS treatment reversed the difference between the genotypes (Fig. 22).

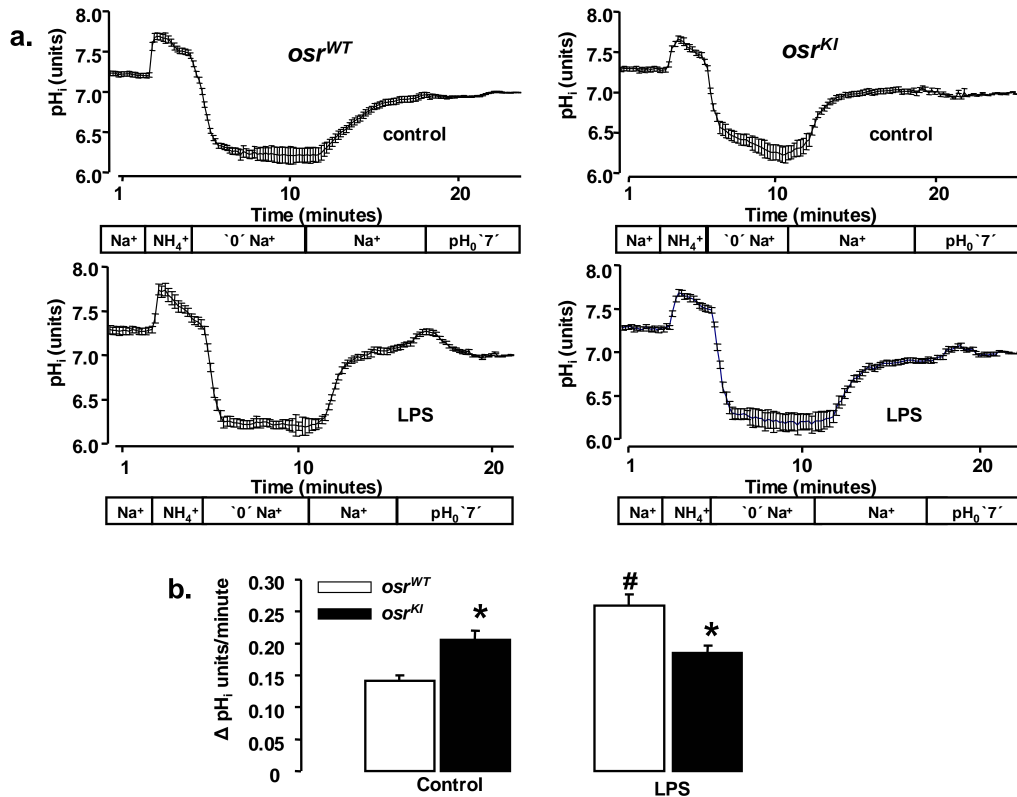

Figure 22:Defective regulation of NHE activity by LPS in OSR KI mice.

a. Alterations of cytosolic pH (pH_i) in bone marrow-derived DCs following an ammonium pulse. Representative experiments showing the time-dependent alterations of cytosolic pH in dendritic cells (DCs) from heterozygous OSR1 knockin mice (*osr*^{KI}, right panels) and from wild type mice (*osr*^{WT}, left panels) prior to (upper panels), or following (lower panels) a 4 hours treatment with LPS (1 µg/ml).

b. Arithmetic means ± SEM (n = 6 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) following an ammonium pulse in DCs from heterozygous OSR1 knockin mice (*osr*^{KI}, black bars) and from wild type mice (*osr*^{WT}, white bars) prior to (left bars) or following (right bars) a 4-hour treatment with LPS (1 µg/ml). * indicates significant difference (p<0.01) from *osr*^{WT} DCs, # indicates significant difference (p<0.01) from respective value prior to LPS exposure.

To determine, whether inhibition of NKCC1 with furosemide in *osr*^{WT} DCs mimic the effects seen in *osr*^{KI} mice, *osr*^{WT} DCs were treated with furosemide (100 nM) and the Na^+/H^+ exchanger activity was studied. As seen in Fig. 23, treatment of DCs with

furosemide led to significant increase in the basal Na^+/H^+ exchanger activity. In analogy to what had been observed in osr^{KI} DCs, the LPS-induced stimulation of Na^+/H^+ exchanger activity was reversed in the presence of furosemide (Fig. 23).

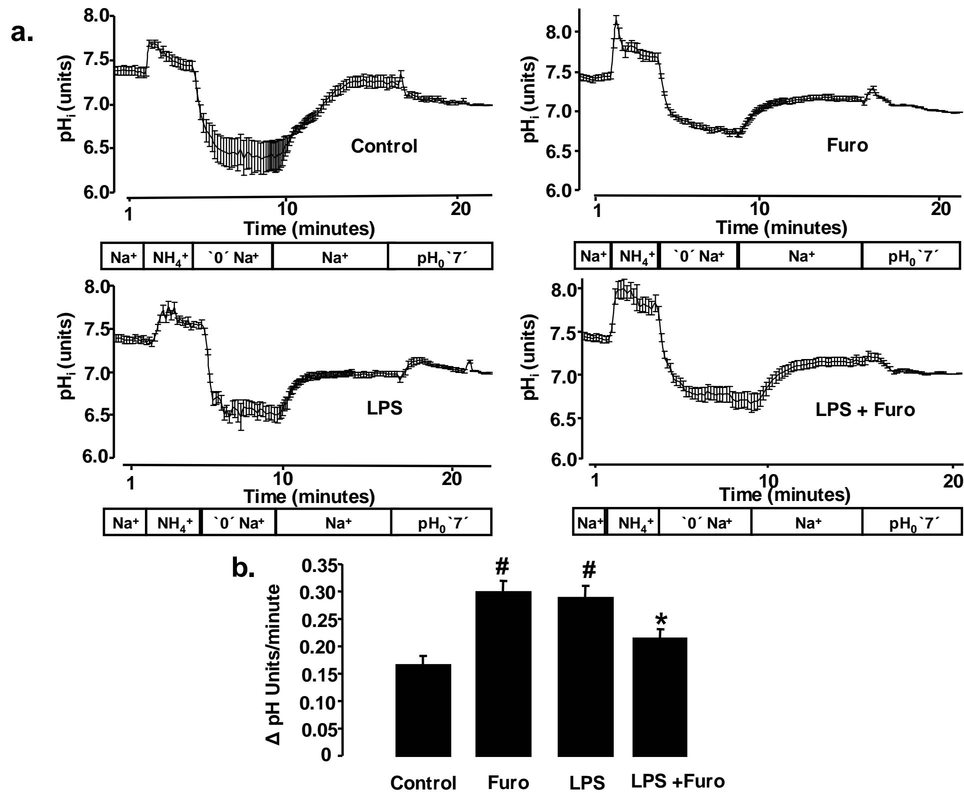

Figure 23: NKCC1 inhibitor furosamide treatment of DCs reveals an OSR KI type phenotype.

a. Representative experiments showing the time-dependent alterations of cytosolic pH in DCs from heterozygous OSR1 wildtype mice prior to (upper panels), or following (lower panels) a 4-hr treatment with LPS (1 µg/ml) in the presence (right panels) and absence of furosemide (Furo, 100 nM).

b. Arithmetic means ± SEM (n = 6 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) following an ammonium pulse in DCs from wild type mice prior to (control) or following 4-hr treatment with LPS (LPS, 1 µg/ml) in the presence and absence of furosemide (Furo, 100 nM). # indicates significant difference ($p<0.01$) from control DCs, * indicates significant difference ($p<0.01$) from respective absence of LPS.

Previous experiments have shown LPS-induced ROS formation to account for the LPS-induced stimulation of Na^+/H^+ exchanger activity (Fig. 12). Therefore, effects of oxidative stress on Na^+/H^+ exchanger activity were studied. Treatment of osr^{WT} DCs for 2 hours with tert-butyl-hydroperoxide TBOOH (10 µM) to induce oxidative stress was followed by a significant increase in Na^+-dependent realkalinization (table 3, Fig. 24). Again, in the absence of Na^+ further acidification was observed, i.e. accelerated realkalinization following LPS treatment was due to Na^+-dependent H^+ extrusion pointing to stimulation of Na^+/H^+ exchanger activity. In contrast to osr^{WT} DCs, treatment of osr^{KI} DCs with TBOOH did not significantly modify Na^+/H^+ exchanger activity. Following TBOOH treatment the Na^+/H^+ exchanger activity was significantly lower in osr^{KI} DCs than in osr^{WT} DCs (Fig. 24). Thus, similar to LPS, TBOOH reversed the difference between the genotypes (Fig. 24).

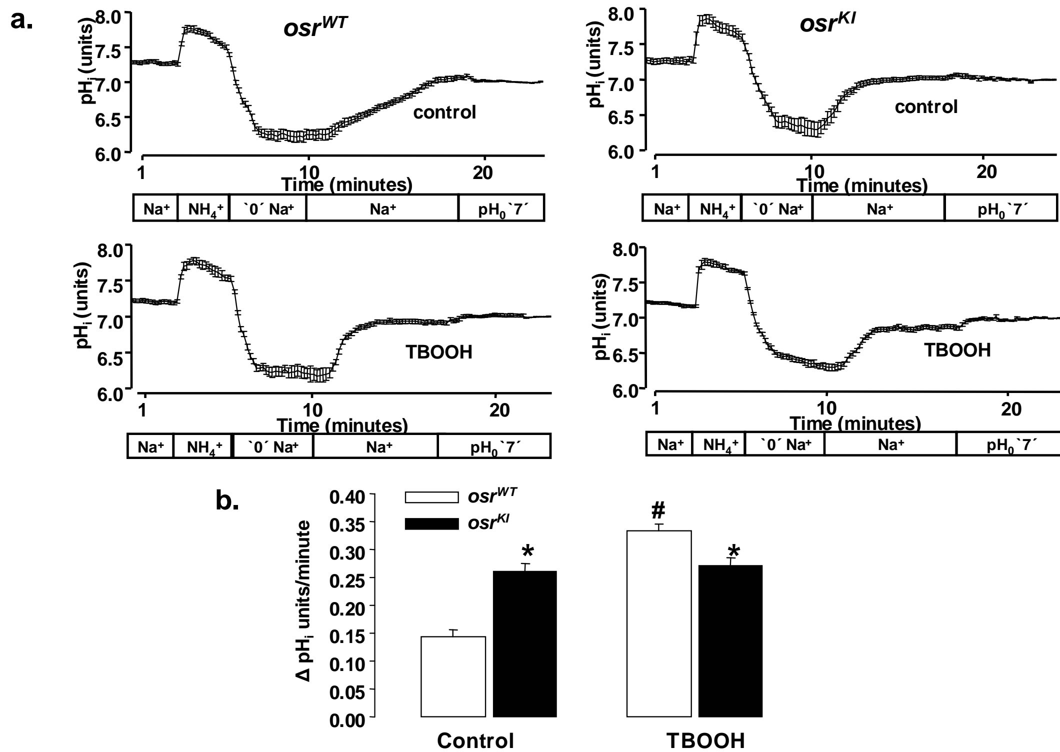

Figure 24: ROS induced stimulation of NHE activity is blunted in OSR KI mice.

a. Representative experiments showing the time-dependent alterations of cytosolic pH in DCs from heterozygous OSR1 knockin mice (osr^{KI}, right panels) and from wild type mice (osr^{WT}, left panels) prior to (upper panels), or following (lower panels) a 2-hr treatment with t-butyl hydroperoxide (TBOOH, 10 µM).
b. Arithmetic means ± SEM (n = 6 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) following an ammonium pulse in DCs from heterozygous OSR1 knockin mice (osr^{KI}, black bars) and from wild type mice (osr^{WT}, white bars) prior to (control, left bars) or following a 2-hr treatment with t-butyl hydroperoxide (TBOOH, 10 µM, right bars). * indicates significant difference (p<0.01) from osr^{WT} DCs, # indicates significant difference (p<0.01) from control.

The buffer capacity of the cells was not significantly different between osr^{KI} DCs and osr^{WT} DCs and was not significantly modified by exposure to LPS or oxidative stress (table 3).

Table 3: Cytosolic pH (ΔpHi), buffer capacity and sodium-independent pH recovery in bone marrow-derived dendritic cells (DCs) isolated from OSR1 knockin mice (osr^{KI}) and from wild type mice (osr^{WT}) prior to (control) and following a 4 hours treatment with lipopolysaccharide LPS (1 µg/ml) or a 2 hr treatment with t-butyl hydroperoxide (TBOOH, 10 µM) in the absence or presence of cariporide (10 µM).

		Intracellular pH (units)	Buffer Capacity (mM/pH unit)	Sodium-independent pH recovery (Δ pH units/minute)	Sodium-dependent pH recovery (NHE activity, Δ pH units/minute)	Number of cells
Control	osr^{WT}	7.33 ± 0.01	15.8 ± 0.9	-0.066 ± 0.007	0.141 ± 0.009	199
	osr^{KI}	7.35 ± 0.01	15.1 ± 1.1	-0.055 ± 0.009	0.206± 0.015*	150
Cariporide alone	osr^{WT}	7.28 ± 0.03	18.3 ± 1.5	-0.070 ± 0.008	0.032 ± 0.012§	41
	osr^{KI}	7.26 ± 0.04	17.8 ± 1.4	-0.044 ± 0.022	0.012 ± 0.004§	40
LPS 4 hrs	osr^{WT}	7.35 ± 0.01	14.5 ± 2.2	-0.053 ± 0.006	0.259 ± 0.018#	200
	osr^{KI}	7.33 ± 0.01	14.4 ± 0.9	-0.065 ± 0.006	0.185 ± 0.012*	172
LPS 4 hrs + cariporide	osr^{WT}	7.31 ± 0.03	13.7 ± 3.4	-0.071_ ± 0.030	0.026 ± 0.004§	46
	osr^{KI}	7.34 ± 0.01	13.6 ± 1.4	-0.042 ± 0.006	0.019 ± 0.004§	40
TBOOH	osr^{WT}	7.24 ± 0.02#	16.0 ± 1.4	-0. 054 ± 0.014	0.229 ± 0.027#	77
	osr^{KI}	7.23 ± 0.02#	14.0 ± 1.2	-0.061 ± 0.011	0.186 ± 0.019#	98
TBOOH + cariporide	osr^{WT}	6.99 ± 0.04§	14.3 ± 1.2	-0.044 ± 0.017	0.026 ± 0.009§	42
	osr^{KI}	6.99 ± 0.02§	15.7 ± 1.4	-0.068 ± 0.017	0.022 ± 0.008§	47

* ($p<0.05$) indicates significant difference from the respective wild type value.

($p<0.05$) indicates significant difference from the respective control value.

§ ($p<0.05$) indicates significant difference from the absence of cariporide.

Additional studies addressed the role of OSR1 and/or NHE1 in LPS-induced ROS formation. As illustrated in Fig. 25, prior to LPS treatment, ROS formation was significantly higher in osr^{KI} DCs than in osr^{WT} DCs. Cariporide treatment did not affect the intracellular ROS in osr^{WT} DCs but significantly decreased ROS formation in osr^{KI} DCs (Fig. 25). LPS enhanced the ROS formation in osr^{WT} DCs, but did not significantly alter ROS formation in osr^{KI} DCs. Accordingly, following LPS treatment, ROS formation was not significantly different between osr^{KI} DCs and osr^{WT} DCs (Fig. 25).

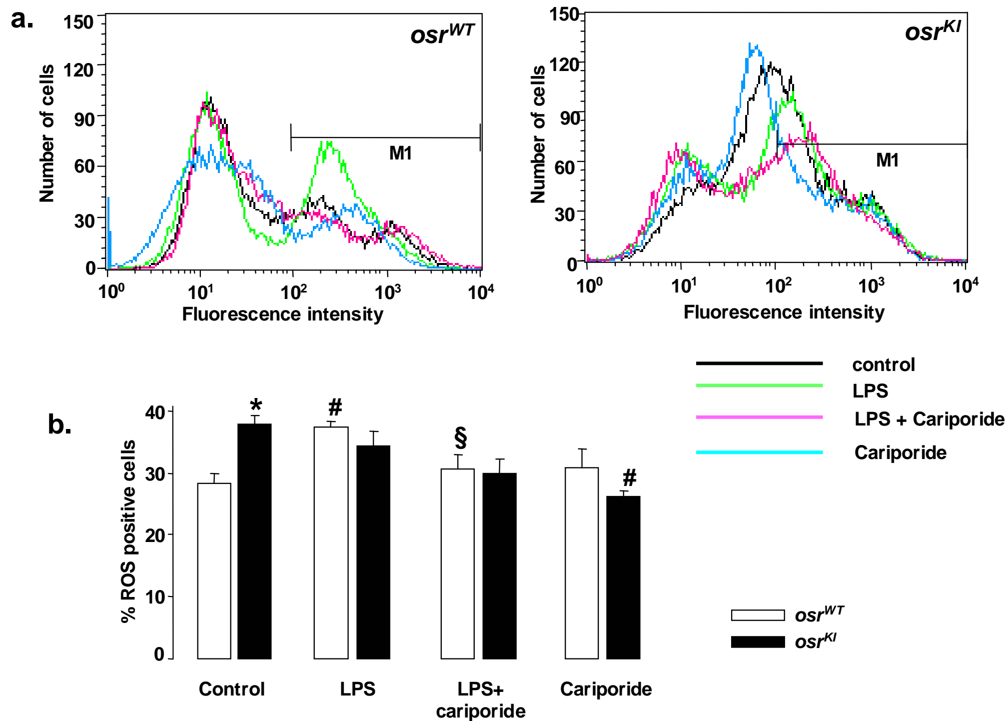

Figure 25: Defective production of intracellular ROS in OSR KI mice.

a. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in DCs from heterozygous OSR1 knockin mice (*osr*^{KI}, right panel) and from wild type mice (*osr*^{WT}, left panel) without treatment (control, black line) and after a 4 hours treatment with LPS (1 µg/ml) in the absence (green line) and presence (pink line) of cariporide or in the presence of cariporide alone (blue line).

b. Arithmetic means ± SEM (n = 6 independent experiments) of the percentage of ROS-positive DCs from heterozygous OSR1 knockin mice (*osr*^{KI}, black bars) and from wild type mice (*osr*^{WT}, white bars) prior to or 4 hours following exposure to LPS (1 µg/ml) in the absence or presence of cariporide (10 µM) or in the presence of cariporide alone. * indicates significant difference (p<0.01) from *osr*$^{+/+}$ DCs, # indicates significant difference (p<0.01) from respective control value, § (p<0.05) indicate significant difference from respective absence of cariporide.

Further experiments addressed the CCL21-induced migration of control- and LPS (1 µg/ml) treated DCs. As presented in Fig. 26, migration of DCs from *osr*^{KI} mice tended to be less pronounced than migration of DCs from wild type mice under control conditions, a dfifference, however, not reaching statistical significance. LPS treatment

markedly stimulated migration of osr^{WT} DCs but did not significantly affect migration of osr^{KI} DCs. Accordingly, upon LPS treatment migration of osr^{KI} DCs was significantly less pronounced than migration of osr^{WT} DCs (Fig. 26).

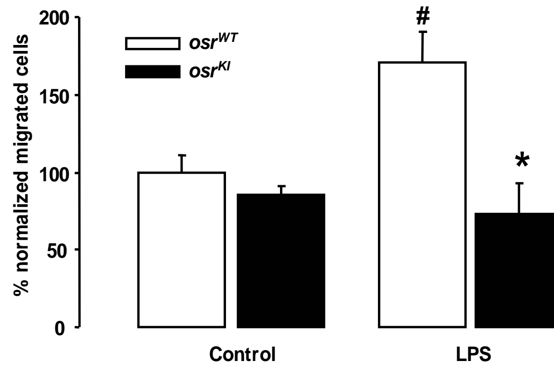

Figure 26: Decreased migration of DCs from osrKI mice

Arithmetic means ± SEM (n = 6 independent experiments) of the normalized migration of DCs from heterozygous OSR1 knockin mice (osr^{KI}, black bars) and from wild type mice (osr^{WT}, white bars) following a 4 hours treatment without (control, left bars) or with LPS (LPS, right bars) * indicates significant difference ($p<0.05$) from $osr^{+/+}$ DCs, # indicates significant difference ($p<0.05$) from respective control value.

6. DISCUSSION

Effect of bacterial lipopolysaccharide on Na^+/H^+ exchanger activity

The first part of the study demonstrates that murine bone marrow derived DCs express functional Na^+/H^+ exchangers. Unlike reported for resident alveolar macrophages [197;198;200;202] and peritoneal macrophages[191;198;202], bone marrow derived DCs recover their cytosolic pH following cytosolic acidification mainly by H^+ extrusion via the Na^+/H^+ exchanger and not by Na^+ independent H^+ pumps or channels.

The study also shows that LPS increases cell volume and triggers the formation of Reactive Oxygen Species (ROS), effects paralleled by and requiring activation of the Na^+/H^+ exchanger NHE1. The increase in the Na^+/H^+ exchanger activity is evidenced from accelerated Na^+ dependent realkalinization, which is virtually abolished in the presence of the NHE1 inhibitor cariporide. The inhibitor may affect in addition the NHE2 isoform[216]. However, RT-PCR revealed in DCs predominant expression of NHE1 and virtually no NHE2 transcript levels. Along those lines, we could not find any published evidence that NHE2 is expressed in DCs. Expectedly the inhibitor does not modify the Na^+-independent pH changes.

ROS production is instrumental in the killing of pathogens and thus an important element in the innate immune response[186]. Owing to the pH sensitivity of ROS production[202;217], it requires parallel extrusion of H^+. Thus, activation of the Na^+/H^+ exchanger may support ROS production. It should be pointed out, however, that the ammonium pulse experiments analysed Na^+/H^+ exchanger activity following marked cytosolic acidification, which is known to stimulate Na^+/H^+ exchanger activity[218]. The Na^+/H^+ exchanger is expected to be less active without prior acidification. Nevertheless, the ammonium pulse unravels the marked upregulation of DC Na^+/H^+ exchanger activity by LPS treatment. The inhibitory effect of cariporide suggests that the Na^+/H^+ exchanger is relevant for ROS formation even without prior acidification. However, since NOX2 activity leads to a strong membrane depolarization and NHE as an electroneutral exchanger can only be responsible for pH but not for charge compensation, NOX2 also relies on the activity of the H^+ channel Hv1 in DCs[219].

The activation of the Na^+/H^+ exchanger further leads to cell swelling. During regulatory cell volume increase the Na^+/H^+ exchanger operates in parallel to the Cl^-/HCO_3^- exchanger[220;221]. The tandem accomplishes the entry of NaCl together with osmotically obliged water. The H^+ and HCO_3^- exiting in exchange for NaCl are osmotically not relevant as

they are replenished in the cell by cytosolic formation from CO_2, which can easily pass the cell membrane[220;221]. Besides the Na^+/H^+ and Cl^-/HCO_3^- exchanger, several other transport systems participate in regulatory cell volume increase, such as $Na^+/K^+,2Cl^-$ cotransport, Na^+ channels and Na^+-coupled uptake of organic osmolytes[220;221]. The observation that LPS-induced cell swelling is abolished in the presence of cariporide highlights the significance of the Na^+/H^+ exchanger in cell volume maintenance of LPS-treated DCs.

Cell volume has previously been shown to influence both, formation of ROS and antioxidative defence [222-225]. Cell shrinkage has been shown to interfere with generation of ROS and the increase in cell volume following exposure to LPS may thus support the formation of ROS. Besides ROS formation and cell volume, cytosolic pH modifies migration, cytokine release, adherence, NO formation, proliferation and differentiation of macrophages and/or monocytes[159-161;226-234].

According to annexin V binding, LPS treatment decreased apoptosis of dendritic cells, an effect again abrogated in the presence of cariporide. Notably, addition of cariporide in the absence of LPS did not significantly modify the survival of DCs. In other cell types the Na^+/H^+ exchanger activity has been shown to foster [235-238] or inhibit [239-241] apoptosis. The inhibitory effect of Na^+/H^+ exchanger activity on apoptosis was explained by the prevention of acidosis[240;241] or of cell shrinkage[239].

The release of TNF-α following exposure to LPS was significantly blunted in the presence of the Na^+/H^+ exchenger inhibitor cariporide, similar to other cells, such as monocytes[230] and alveolar epithelial cells[242;243]. Thus, LPS induced TNF-α production was critically dependent on the activity of the Na^+/H^+ exchanger.

The present part of the study highlights the importance of Na^+/H^+ exchanger-dependent regulation of cell volume and cytosolic H^+ concentration for survival and proper function of DCs. Previous observations have shown that several DC functions, such as maturation, antigen presentation, cytokine production and migration, depend on Ca^{2+} signaling[144;203;244-249]. It must be kept in mind that alterations of cytosolic pH do influence cytosolic Ca^{2+} activity [250-253]. Moreover, some effects of Toll-like receptor stimulation are not mediated by Ca^{2+}-dependent signaling[254]. Those functions may in part be mediated by Na^+/H^+ exchanger activity and the resulting alterations of cytosolic pH and/or cell volume.

In conclusion, the present part of the study discloses that exposure of bone marrow derived DCs to LPS stimulates the activity of the Na^+/H^+ exchanger. The stimulation of the carrier is required for the subsequent increase in cell volume and generation of ROS.

Phosphoinositide 3-Kinase Dependent Regulation of Na^+/H^+ exchanger activity

Toll-like receptor signaling involves activation of MAP kinases and phosphoinositide (PI) 3 kinase[255], signaling pathways known to stimulate the activity of Na^+/H^+ exchangers[139;256-259]. Present experiments were conducted to determine the participation of PI3 kinase signaling pathway in the Na^+/H^+ exchanger activation by LPS.

The present study further reveals that stimulation of the Na^+/H^+ exchanger activity requires functional PI3 kinase. Accordingly, pharmacological inhibition of the PI3 kinase by either wortmannin (1µM) or LY294002 (10 µM) abrogates the stimulating effect of LPS on Na^+/H^+ exchanger activity.

Inhibition of PI3 kinase further disrupts the effect of LPS on the formation of ROS, It indicates that PI3 kinase is involved in ROS production, which is important in DC fuction[88;90;91].

The activation of the Na^+/H^+ exchanger by LPS further leads to cell swelling. Again, the effect of LPS on cell volume requires functional PI3 kinase, and is presumably related to the activation of the Na^+/H^+ exchanger.

LPS further stimulate the migration of DCs. *In vivo*, DCs migrate to peripheral tissues and from there to lymphoid tissues[254]. The function is again dependent on PI3 kinase activity and presumably again involves PI3 kinase dependent activation of the Na^+/H^+ exchanger. The carrier has previously been shown to support the migration of a wide variety of cells[165;166;260;261].

Pharmacological inhibition of PI3 kinase in DCs influences further functions including K^+ channel activity and release of IL-12[127;131;262]. Whether or not those functions depend on activation of the Na^+/H^+ exchanger remains to be shown.

The PI3 kinase is activated by IGF1 and insulin[263-265]. Those hormones are thus expected to influence regulation of cytosolic pH, cell volume and ROS formation in dendritic cells.

In conclusion, the present part of the study discloses that exposure of bone marrow derived DCs to LPS stimulates the activity of the Na^+/H^+ exchanger, an effect requiring functional PI3 kinase.

OSR1-sensitive regulation of Na+/H+ exchanger activity

The present study reveals that WNK resistance of OSR1 affects Na^+/H^+ exchanger activity and formation of ROS in DCs. In the absence of LPS cell volume was similar in DCs from osr^{KI} and osr^{WT}, but Na^+/H^+ exchanger activity and formation of ROS were significantly higher in osr^{KI} DCs than in osr^{WT} DCs. Similarly, LPS triggered the formation of ROS, activated the Na^+/H^+ exchanger NHE1 and increased cell volume in osr^{WT} DCs. All those effects were blunted or even absent in osr^{KI} DCs. Accordingly, LPS reversed the differences of ROS formation, Na^+/H^+ exchanger activity and cell volume between osr^{KI} DCs and osr^{WT} DCs. The increase in Na^+/H^+ exchanger activity in osr^{WT} DCs following LPS treatment was virtually abolished in the presence of cariporide, which inhibits both NHE1 and NHE2[216]. In DCs the NHE1, but not the NHE2 isoform is expressed (fig.7).

As shown in the present study, Na^+/H^+ exchanger activation by LPS depends on ROS formation. Conversely, ROS production is sensitive to cytosolic pH[217]. The higher cytosolic pH and Na^+/H^+ exchanger activity in osr^{KI} DCs thus presumably contributes to the higher ROS production in those cells.

Na^+/H^+ exchanger activity is enhanced in osr^{KI} DCs despite the more alkaline pH, which should actually decrease Na^+/H^+ exchanger activity[218]. At least in theory, the stimulation of the Na^+/H^+ exchanger in osr^{KI} DCs could result from cell volume regulation.

The cell volume regulatory stimulation of the Na^+/H^+ exchanger may be required because of the lacking OSR1-dependent stimulation of NKCC in osr^{KI} DCs. OSR1 is known to stimulate the NKCC and to participate in cell volume regulation[145;266-272]. It is tempting to reason that OSR1 deficiency leads to decreased NKCC activity, requiring enhanced activity of the Na^+/H^+ exchanger for cell volume maintenance. We cannot rule out, however, the possibility that OSR1 regulates Na^+/H^+ exchanger directly, e.g. by phosphorylating the carrier protein. As furosemide treatment of osr^{WT} DCs similarly increases Na^+/H^+ exchanger, a decreased NKCC activity at least contributes to the upregulation of Na^+/H^+ exchanger activity.

ROS also affect the maturation state, the production and secretion of cytokines, and the antigen presenting capacity of DC[85;88;90;91]. T-cells activated in the absence of ROS exhibit an altered differentiation profile[88].

The present study did not address the functional significance of OSR1-sensitive DC function. DCs are antigen-presenting cells critically important in the regulation of innate and adaptive immunity[37;43;273;274]. To fulfill their diverse functions, DCs have to migrate

into inflammatory tissues and return to secondary lymphoid sites following microbial challenge [275]. The blunted stimulation of migration following LPS treatment is expected to compromise the function of osr^{KI} DCs. On the other hand, augmented ROS formation of osr^{KI} DCs is expected to foster the removal of pathogens[186]. However, the difference of ROS formation between osr^{KI} and osr^{WT} DCs is lost following stimulation with LPS and enhanced ROS production in immature DCs may not be relevant for the power of the immune response. Clearly, further studies will be required to elucidate the impact of reduced OSR1 activity on the immune system.

7. ABBREVIATIONS

µM	Micromolar
2-ME	2-mercaptoethanol
AIF	Apoptosis inducing factor
ANOVA	Analysis of Variance between groups
APC	Antigen presenting cell
AWB	Annexin washing buffer
BCR	B cell receptor
CCL21	Chemokine ligand 21
DC	Dendritic cell
ERK	Extracellular signal-regulated protein kinase
ECL	Enhanced chemiluminescence
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharides
MAPK	Mitogen-Activated Protein Kinase
mg	Milligram
MHC	Major histocompatibility complex
Min	Minute
ml	Milliliter
mM	Millimolar (mmol/L)
NHE	Na^+/H^+ exchanger
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential Amino Acid Solution
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK	Natural killer cell
NKCC	Na-K-Cl cotransporter

OSR1	Oxidative stress-responsive kinase 1
P/S	Penicillin-streptomycin
PBS	Phosphate-Buffered Saline
PI3 kinase	Phosphoinositide 3 kinase
PS	Phosphatidylserine
RPMI	Roswell Park Memorial Institute
ROS	Reactive Oxygen Species
SEM	Standard error
TBOOH	Tert-butyl-hydroperoxide
TBS	Triethanolamine-buffered saline
TCR	T cell receptor
Th	T heper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
WBC	White blood cell

8. References

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