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

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Pasham V, Rotte A, Yang W, Zelenak C, Bhandaru M, Föller M, Lang F. OSR1-sensitive regulation of Na^+/H^+ exchanger activity in dendritic cells. *Am J Physiol Cell Physiol* 303: C416–C426, 2012. First published May 30, 2012; doi:10.1152/ajpcell.00420.2011.—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. Little is known about extrarenal functions of OSR1. The present study analyzed the impact of decreased OSR1 activity on the function of dendritic cells (DCs), antigen-presenting cells linking innate and adaptive immunity. DCs were cultured from bone marrow of heterozygous WNK-resistant OSR1 knockin mice (osr^{KI}) and wild-type mice (osr^{WT}). Cell volume was estimated from forward scatter in FACS analysis, ROS production from 2′,7′-dichlorodihydrofluorescein-diacetate fluorescence, cytosolic pH (pH_i) from 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein fluorescence, and Na^+/H^+ exchanger activity from Na^+-dependent realkalinization following ammonium pulse and migration utilizing transwell chambers. DCs expressed WNK1, WNK3, NCC, NKCC1, and OSR1. Phosphorylated NKCC1 was reduced in osr^{KI} DCs. Cell volume and pH_i were similar in osr^{KI} and osr^{WT} DCs, but Na^+/H^+ exchanger activity and ROS production were higher in osr^{KI} than in osr^{WT} DCs. Before LPS treatment, migration was similar in osr^{KI} and osr^{WT} DCs. LPS (1 μg/ml), however, increased migration of osr^{WT} DCs but not of osr^{KI} DCs. Na^+/H^+ exchanger 1 inhibitor cariporide (10 μM) decreased cell volume, intracellular reactive oxygen species (ROS) formation, Na^+/H^+ exchanger activity, and pH_i 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 the NKCC1 inhibitor furosemide (100 nM) to values similar to those in osr^{KI} DCs. Oxidative stress (10 μM tert-butylhydroperoxide) increased Na^+/H^+ exchanger activity in osr^{WT} DCs but not in osr^{KI} DCs and reversed the difference between genotypes. Cariporide virtually abrogated Na^+/H^+ exchanger activity in both genotypes and blunted LPS-induced cell swelling and ROS formation in osr^{WT} mice. In conclusion, partial OSR1 deficiency influences Na^+/H^+ exchanger activity, ROS formation, and migration of dendritic cells.

intracellular pH; cariporide; oxidative stress-responsive kinase 1; oxidative stress; cell volume

THE OXIDATIVE STRESS-RESPONSIVE KINASE 1 (OSR1) participates in the signaling of transport regulation during oxidative and osmotic stress (17, 37, 59, 66, 69, 83). The kinase is activated by the WNK (with no K) kinases WNK1 and WNK4, which are in turn activated by hyperosmotic stress (59, 79, 91). OSR1 stimulates the thiazide-sensitive Na-Cl cotransporter (NCC) and the furosemide-sensitive Na-K-2Cl cotransporter (NKCC) and thus influences cell volume, transepithelial transport, renal salt excretion, and GABA neurotransmission (2, 15, 16, 27, 35–37, 59, 80). Mutations of WNK1 and WNK4 may lead to hypertension (20, 24, 37, 74, 84) and autonomic neuropathy (37). OSR1 is similarly considered to participate in the regulation of blood pressure (28, 77–79). Accordingly, OSR1 has been suggested as a potential drug target in the treatment of hypertension (28, 59, 77). The putative role of OSR1 in the regulation of immune cell function, remains, however, elusive.

The present study explored the role of OSR1 in the regulation of cell volume and formation of reactive oxygen species (ROS) in dendritic cells (DCs), antigen-presenting cells involved in the initiation of both innate and adaptive immunity and thus decisive for the regulation of the immune response (1, 3, 23, 58, 76). Exposure of DCs to bacterial wall components such as lipopolysaccharide (LPS) triggers generation of ROS (6, 49, 88), which contributes to pathogen defense (56). Similar to what has been shown in other cell types (25) including macrophages (13), ROS production is paralleled by H^+ generation, which in turn inhibits ROS-generating NADPH oxidase (33). Regulation of cytosolic pH (pH_i) involves the Na^+/H^+ exchanger in a wide variety of cells (13, 53, 55, 90) including macrophages (14, 32, 44, 71, 72) and monocytes (22, 38, 71). The Na^+/H^+ exchanger further participates in the regulation of cell volume (34, 42, 53).

The present study explored the impact of WNK-dependent OSR1 regulation on Na^+/H^+ exchanger activity, cell volume, and ROS formation in bone marrow-derived DCs.

MATERIALS AND METHODS

Animals. All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Dendritic cells were cultured from bone marrow (87) of 7- to 11-wk-old heterozygous OSR1 knockin mice (osr^{KI}) and respective wild-type mice (osr^{WT}). The mice were kindly provided by D. Alessi (Univ. of Dundee, Dundee, UK). As described earlier (57), 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 (Ssniff, Soest, Germany) and tap drinking water.

Cell culture. Bone marrow-derived cells were flushed out of the cavities of the femur and tibia with PBS. Cells were then washed twice with RPMI 1640 medium and seeded out at a density of 2×10^6 cells per 60-mm dish (85). Cells were cultured for 6 days in RPMI 1640 (GIBCO, Darmstadt, Germany) containing 10% fetal calf serum (FCS), 1% penicillin-streptomycin, 1% glutamine, 1% non-essential amino acids, and 0.05% β-mercaptoethanol. Cultures were supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF; 35 ng/ml, Preprotech, Hamburg, Germany) and fed with fresh medium containing GM-CSF on *days 3* and *6*. On *day 7*, >85% of the cells expressed CD11c, which is a marker for mouse DCs (85–87). Experiments were performed on *days 7-8* of DC culture.

Immunostaining and flow cytometry. Cells (4×10^5) were incubated in 100 μl FACS buffer [phosphate-buffered saline (PBS) plus 0.1% FCS] containing fluorochrome-conjugated antibodies at a con-

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centration of 10 μg/ml (86). A total of 2×10^4 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 min at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometric analysis.

Real-time PCR. To evaluate OSR1 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). RNA (2 μg) was reverse-transcribed using oligo$(dT)_{12–18}$ primers and SuperScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Then, quantitative real-time PCR with the Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad, Munich, Germany) was applied using the TaqMan Gene Expression Assay (Hs00362901 m1, Applied Biosystems) to determine OSR1 transcript levels. Reactions were performed using Universal TaqMan Master Mix (Applied Biosystems) in a final volume of 20 μl containing 2 μl of cDNA and the TaqMan Gene Expression Assay as recommended by the manufacturer. Following an initial incubation at 50°C for 2 min and 95°C for 10 min, 40 cycles of thermal cycling at 95°C for 15 s and 60°C for 60 s were performed. Calculated mRNA expression levels were normalized to the expression levels of TBP (Applied Biosystems) in the same cDNA sample. Relative quantification of gene expression was calculated according to the ΔΔCt method.

Western blotting. DCs (2×10^6 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 anti p-NKCC1 (21) (1:5,000) and GAPDH (1:1,000, Cell Signaling) antibodies diluted in 5% milk in PBS and 0.1% Tween 20, washed 5 times, probed with secondary antibodies conjugated with horseradish peroxidase (1:2,000) 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 (Bio-Rad).

Treatments. Cells were exposed either to bacterial LPS (1 μg/ml) or to *t*-butyl hydroperoxide (TBOOH, 10 μM) in the presence or absence of cariporide (10 μM). Stock solutions of LPS were prepared in culture medium whereas the rest of the substances were dissolved in sterile distilled water. The cells were treated by adding the substances to the cell suspension at the indicated final concentration and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Determination of cell volume. Cell volume was estimated from forward scatter in flow cytometric analysis. Briefly, 8×10^5 cells were taken in a culture dish and treated with LPS in the presence and absence of cariporide (10 μM). After the treatment, cells were collected and centrifuged, the pellet was resuspended in FACS buffer, and the forward scatter was analyzed on a FACSCalibur (Becton-Dickinson, Heidelberg, Germany).

Determination of ROS production. ROS production in DCs was determined utilizing 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (68). Briefly, 8×10^5 cells were taken in a culture dish and treated with LPS in the presence or absence of cariporide (10 μM). 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 min 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 analyzed with flow cytometry (FACSCalibur). DCFDA fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

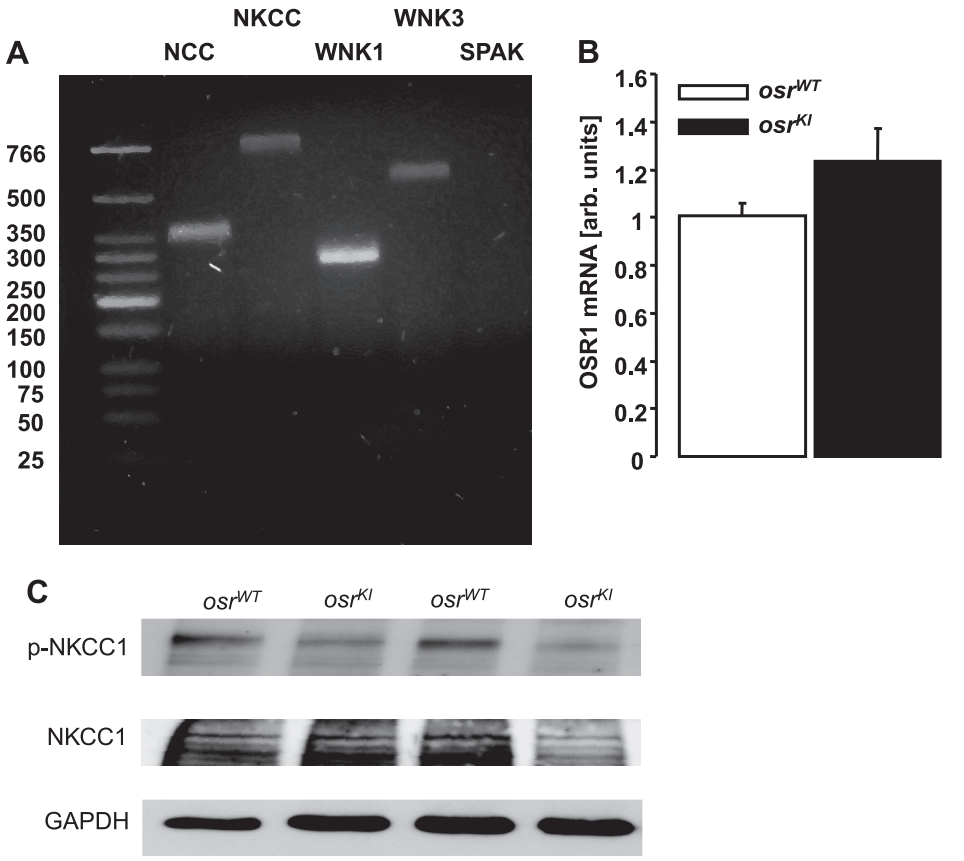

Fig. 1. WNK (with no K kinases) signaling pathway in murine bone marrow-derived dendritic cells (DCs). *A*: agarose gel visualizing the products of RT-PCR reactions amplifying Na-Cl cotransporter (NCC), Na-K-2Cl cotransporter (NKCC), WNK1, WNK3, and SPAK from cDNA of murine DCs. *B*: arithmetic means ± SE ($n = 5$ mice) of relative oxidative stress-responsive kinase 1 (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}).

Measurement of intracellular pH. For digital imaging of pH_i, the cells were incubated in a HEPES-buffered Ringer solution containing 10 μM 2′,7′-*bis*-(carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (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 (63). 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 oil immersion objective (Zeiss Neoplan). 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) and specialized computer software (Metafluor). Between 10 and 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 (81). To this end, the cells were perfused at the end of each experiment for 5 min 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 obtain 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 pH_i due to entry of NH_3 and binding of H^+ to form NH_4^+ (61). The acidification of pH_i upon removal of ammonia allowed calculation of the mean intrinsic buffering power (β) of the cells (61). Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$$\beta = \Delta[NH_4^+]_i / \Delta pH_i$$

where ΔpH_i is the decrease of 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 (9) and at an extracellular pH 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:

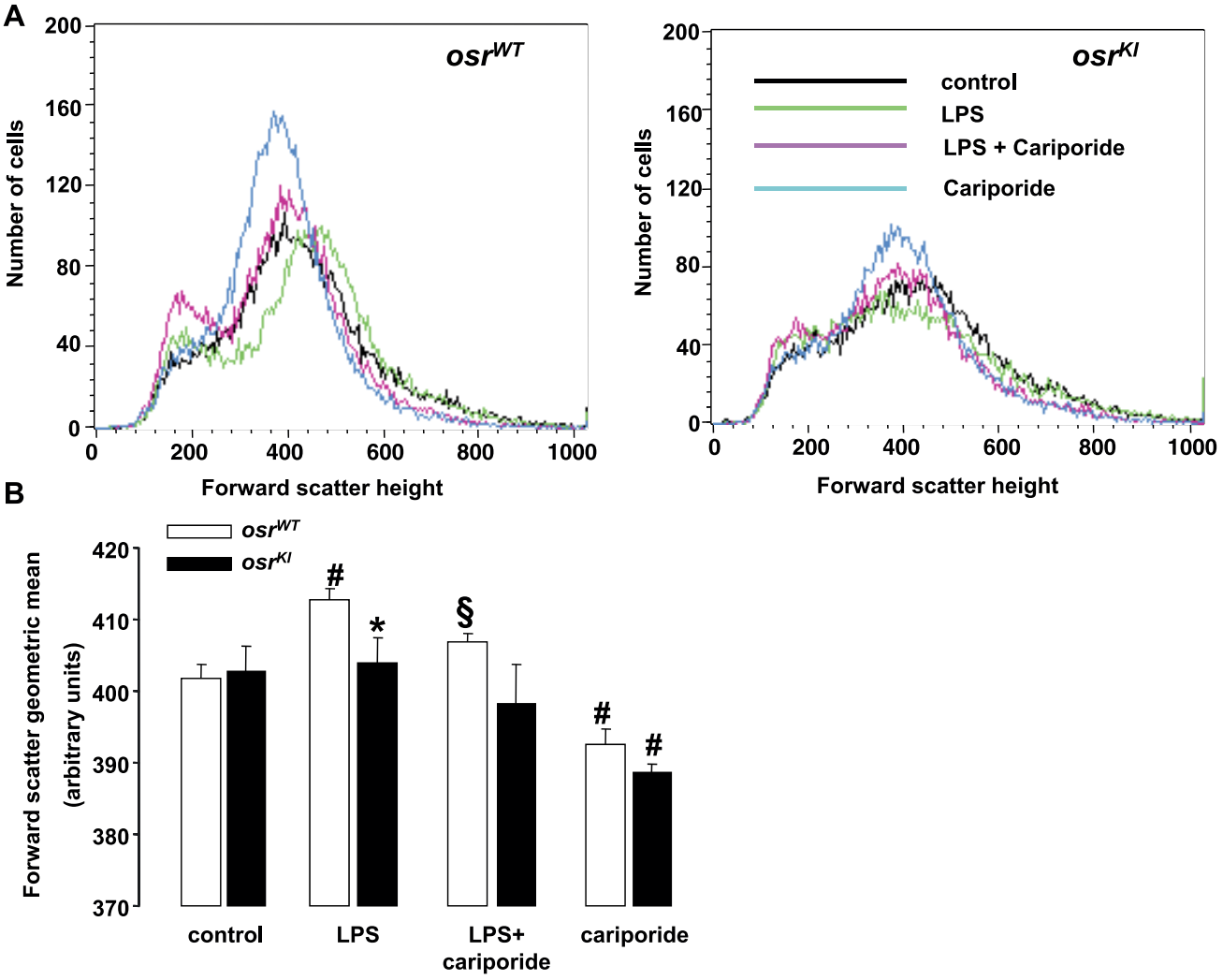

Fig. 2. Forward scatter of DCs from osr^{KI} and osr^{WT} mice following administration of LPS in the presence and absence of cariporide. *A*: representative FACS histograms depicting forward scatter in DCs from heterozygous OSR1 knockin mice (osr^{KI}, *right*) and from wild-type mice (osr^{WT}, *left*) without treatment (control, black line) and after a 4-h 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 ± SE ($n = 6$ 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) before or 4 h following exposure to LPS (1 μg/ml) in the absence or presence of cariporide (10 μM) or in the presence of cariporide alone. *Significant difference ($P < 0.05$) from osr^{WT} DCs; #significant difference ($P < 0.01$) from respective control value; §$P < 0.05$, significant difference from respective absence of cariporide.

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

To calculate the ΔpH/min during realkalinization, 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 the following (in mM): standard HEPES: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 10 glucose, and 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, and 10 glucose (for sodium-free ammonium chloride, 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); and high K^+ for calibration: 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 HEPES, 10 mannitol, and 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.

Determination of migration. For migration assays, transwell inserts (BD Falcon 353097) and BD BioCoat Matrigel Invasion Chambers (354480, BD Biosciences) 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 h. In the following, the nonmigrated 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 for 15 min. The migrated cells were then identified by staining with 4′,6-diamidino-2-phenylindole (DAPI).

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

RESULTS

To determine the presence of a functional WNK signaling pathway in murine bone marrow-derived DCs, RT-PCR was performed. As shown in Fig. 1*A*, DCs express WNK1, WNK3, NCC, and NKCC1 but not SPAK. As illustrated in Fig. 1*B*, OSR1

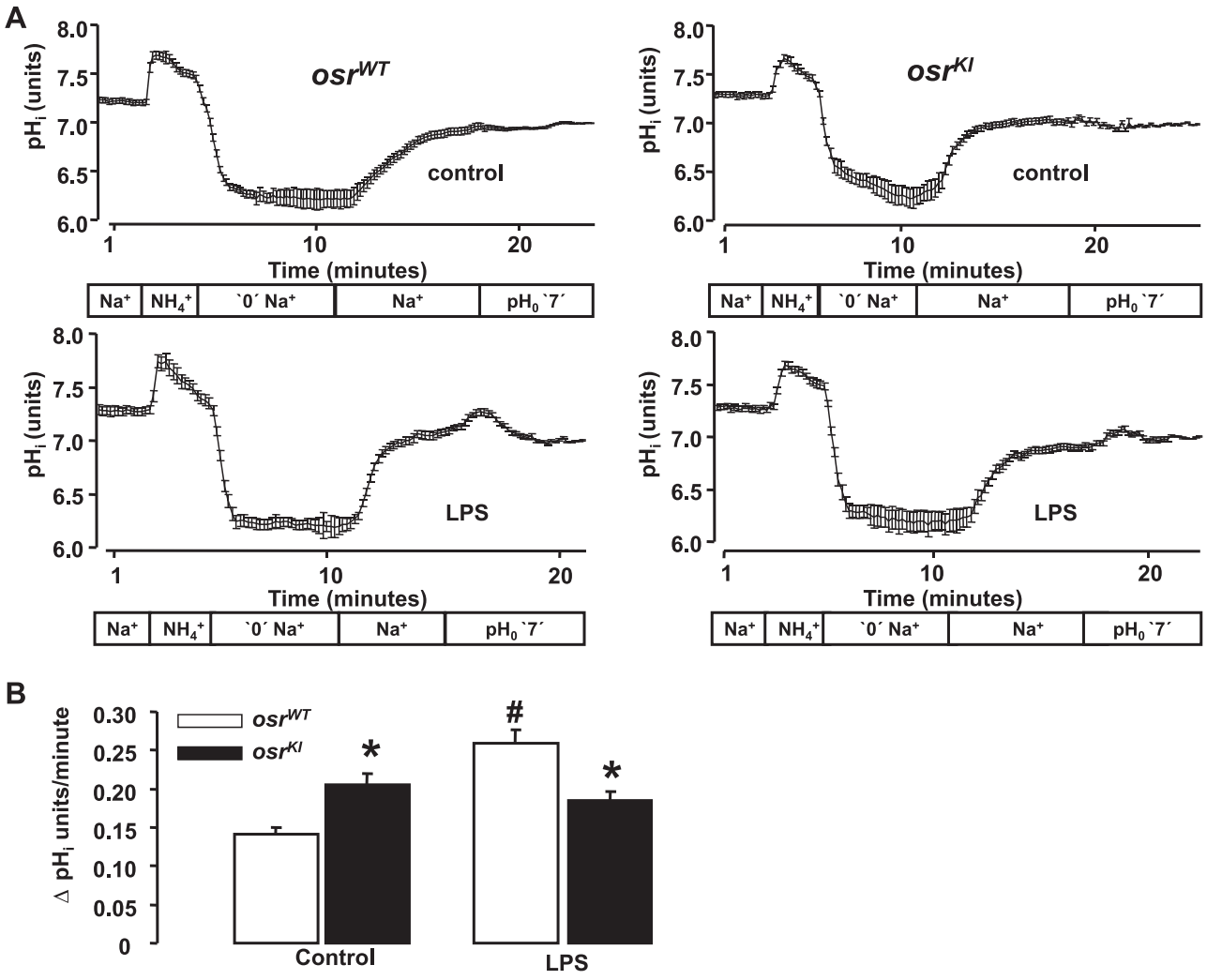

Fig. 3. Cytosolic pH (pH_i) recovery in DCs from osr^{KI} and osr^{WT} mice before and following LPS treatment. *A*: alterations of cytosolic pH in bone marrow-derived DCs following an ammonium pulse. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ was removed (replaced by NMDG) in a first step (see bars below each tracing), NH_4Cl was removed in a second step, Na^+ was added in a third step, and nigericin [extracellular pH (pH_o) 7.0] was applied in a fourth step to calibrate each individual experiment. Representative experiments show the time-dependent alterations of cytosolic pH in DCs from heterozygous OSR1 knockin mice (osr^{KI}, *right*) and from wild-type mice (osr^{WT}, *left*) before (*top*) or following (*bottom*) a 4-h treatment with LPS (1 μg/ml). *B*: arithmetic means ± SE ($n = 6$ 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) before (*left* bars) or following (*right* bars) a 4-h treatment with LPS (1 μg/ml). *Significant difference ($P < 0.01$) from osr^{WT} DCs; #significant difference ($P < 0.01$) from respective value before LPS exposure.

mRNA is readily detectable in DCs and the transcript levels were similar in DCs from OSR1 knockin mice (osr^{KI}) and DCs from wild-type mice (osr^{WT}). Furthermore, the phosphorylation of NKCC1 was lower in osr^{KI} DCs (Fig. 1*C*), indicative of reduced OSR1 activity in osr^{KI} DCs.

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. 2, no significant difference was observed in forward scatter between osr^{KI} and osr^{WT}. Treatment of the DCs with LPS was followed within 4 h by an increase in forward scatter of osr^{WT} DCs, pointing to an increase in cell volume (Fig. 2). 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 cariporide, a specific inhibitor of Na^+/H^+ exchanger 1 (NHE1) (48). As a result, in both genotypes, forward scatter was significantly decreased following treatment of DCs with cariporide (Fig. 2). 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.

The lack of cell shrinkage in untreated osr^{KI} DCs could have been due to lack of OSR1, NCC, and/or NKCC expression in osr^{WT} DCs or due to a compensatory increase in Na^+/H^+ exchanger activity of osr^{KI} DCs. To explore that possibility, cytosolic pH was determined in DCs utilizing BCECF fluorescence. The Na^+/H^+ exchanger activity was determined with the ammonium pulse technique (61). To this end, NH_4Cl was added to the perfusate, leading to NH_3 entry into the cells, binding of H^+ to form NH_4^+ and thus to transient cytosolic alkalinization (Fig. 3). The subsequent removal of NH_4Cl was followed by exit of NH_3 leaving H^+ behind. The H^+ thus retained within the cell led to cytosolic acidification (Fig. 3). In the absence of Na^+, the average cytosolic pH declined further after an ammonium pulse, indicating that the cells did not express significant Na^+-independent H^+ extrusion mechanisms to maintain or recover cytosolic pH (Table 1). Addition of Na^+ was, however, followed by rapid pH recovery, pointing to the operation of the Na^+/H^+ exchanger. The Na^+-dependent pH recovery was blunted in the presence of NHE1 inhibitor cariporide (Table 1). According to the ammonium pulse, the Na^+/H^+ exchanger activity was significantly higher in osr^{KI} DCs than in osr^{WT} DCs.

Treatment of osr^{WT} DCs with LPS (1 μg/ml) was followed within 4 h by a significant increase in Na^+-dependent realkalinization (Table 1 and Fig. 3). 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. 3). The increase in Na^+/H^+ exchanger activity was blunted in the presence of the NHE1 inhibitor cariporide (10 μM) (Table 1). 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. 3). Thus, LPS treatment reversed the difference between the genotypes (Fig. 3).

To determine whether inhibition of NKCC1 with furosemide in osr^{WT} DCs mimics the effects seen in osr^{KI} mice, osr^{WT} DCs were treated with furosemide (100 nM) and Na^+/H^+ exchanger activity was studied. As seen in Fig. 4, treatment of DCs with furosemide led to a significant increase in 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. 4).

Table 1. *Cytosolic pH (ΔpH_i), buffer capacity, and sodium-independent pH recovery in bone marrow-derived dendritic cells isolated from OSR1 knockin mice and from wild-type mice before (control) and following a 4-h treatment with lipopolysaccharide (1 μg/ml) or a 2-h treatment with t-butyl hydroperoxide (10 μM) in the absence or presence of cariporide (10 μM)*

	Intracellular pH, units	Buffer Capacity, mM/pH unit	Sodium-Independent pH Recovery, ΔpH units/min	Sodium-Dependent pH Recovery (NHE Activity), ΔpH units/min	No. 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 h					
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 h + 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

Values are means ± SE. osr^{KI}, oxidative stress-responsive kinase 1 (OSR1) knockin mice; osr^{WT}, wild-type mice; LPS, lipopolysaccharide; TBOOH, *t*-butyl hydroperoxide; NHE, Na^+/H^+ exchanger. *Significant difference ($P < 0.05$) from the respective wild-type value; †significant difference ($P < 0.05$) from the respective control value; ‡significant difference ($P < 0.05$) from the absence of cariporide.

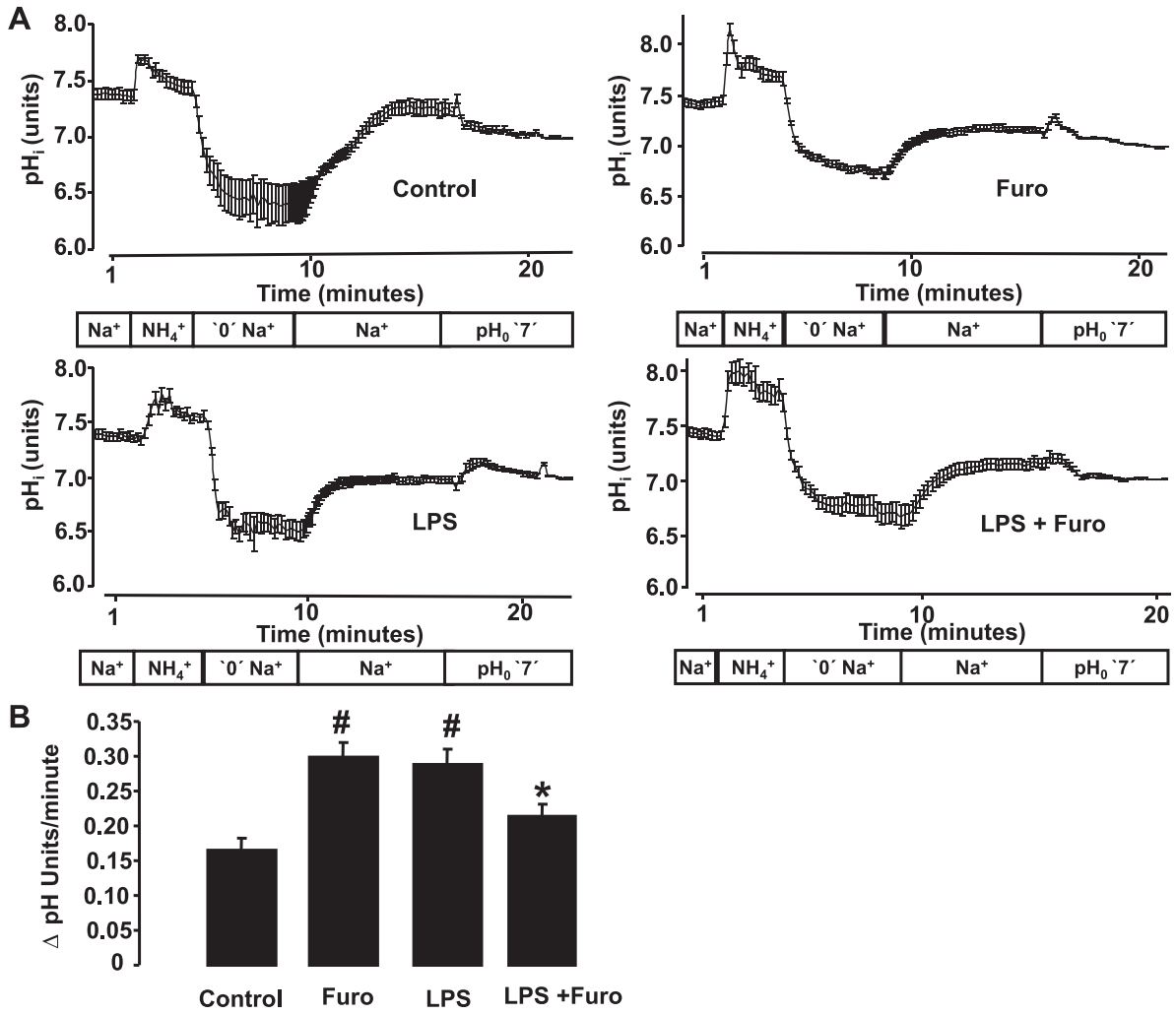

Fig. 4. Cytosolic pH recovery in DCs from osr^{WT} mice before and following LPS treatment in the presence and absence of furosemide (Furo). *A*: representative experiments showing the time-dependent alterations of cytosolic pH in DCs from heterozygous OSR1 wild-type mice before (*top*) or following (*bottom*) a 4-h treatment with LPS (1 μg/ml) in the presence (*right*) and absence of furosemide (100 nM). *B*: arithmetic means ± SE ($n = 6$ independent experiments) of Na^+-dependent recovery of cytosolic pH in DCs (ΔpH/min) following an ammonium pulse in DCs from wild-type mice before (control) or following 4-h treatment with LPS (1 μg/ml) in the presence and absence of furosemide (100 nM). #Significant difference ($P < 0.01$) from control DCs; *significant difference ($P < 0.01$) from respective absence of LPS.

LPS-induced ROS formation has previously been shown to account for the LPS-induced stimulation of Na^+/H^+ exchanger activity (62). Therefore, the effect of oxidative stress on Na^+/H^+ exchanger activity was studied. Treatment of osr^{WT} DCs for 2 h with TBOOH (10 μM) to induce oxidative stress was followed by a significant increase in Na^+-dependent realkalinization (Table 1 and Fig. 5). Again, in the absence of Na^+, no 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. 5). As is apparent from Table 1, the increase in Na^+/H^+ exchanger activity was blunted in the presence of the NHE1 inhibitor cariporide (10 μM). In contrast to osr^{WT} DCs, treatment of osr^{KI} DCs with TBOOH did not significantly modify Na^+/H^+ exchanger activity. Following TBOOH treatment, Na^+/H^+ exchanger activity was significantly lower in osr^{KI} DCs than in osr^{WT} DCs (Fig. 5). Thus, similar to LPS, TBOOH reversed the difference between the genotypes (Fig. 5).

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 1).

Additional studies addressed the role of OSR1 and/or NHE1 in LPS-induced ROS formation. As illustrated in Fig. 6, before 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. 6). 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. 6).

The migratory potential of DCs is dependent on NHE1 activity. Thus, further experiments addressed the CCL21-induced migration of control- and LPS-(1 μg/ml) treated DCs. As presented in Fig. 7, migration of DCs from osr^{KI} mice tended to be less pronounced than migration of DCs from wild-type mice under con-

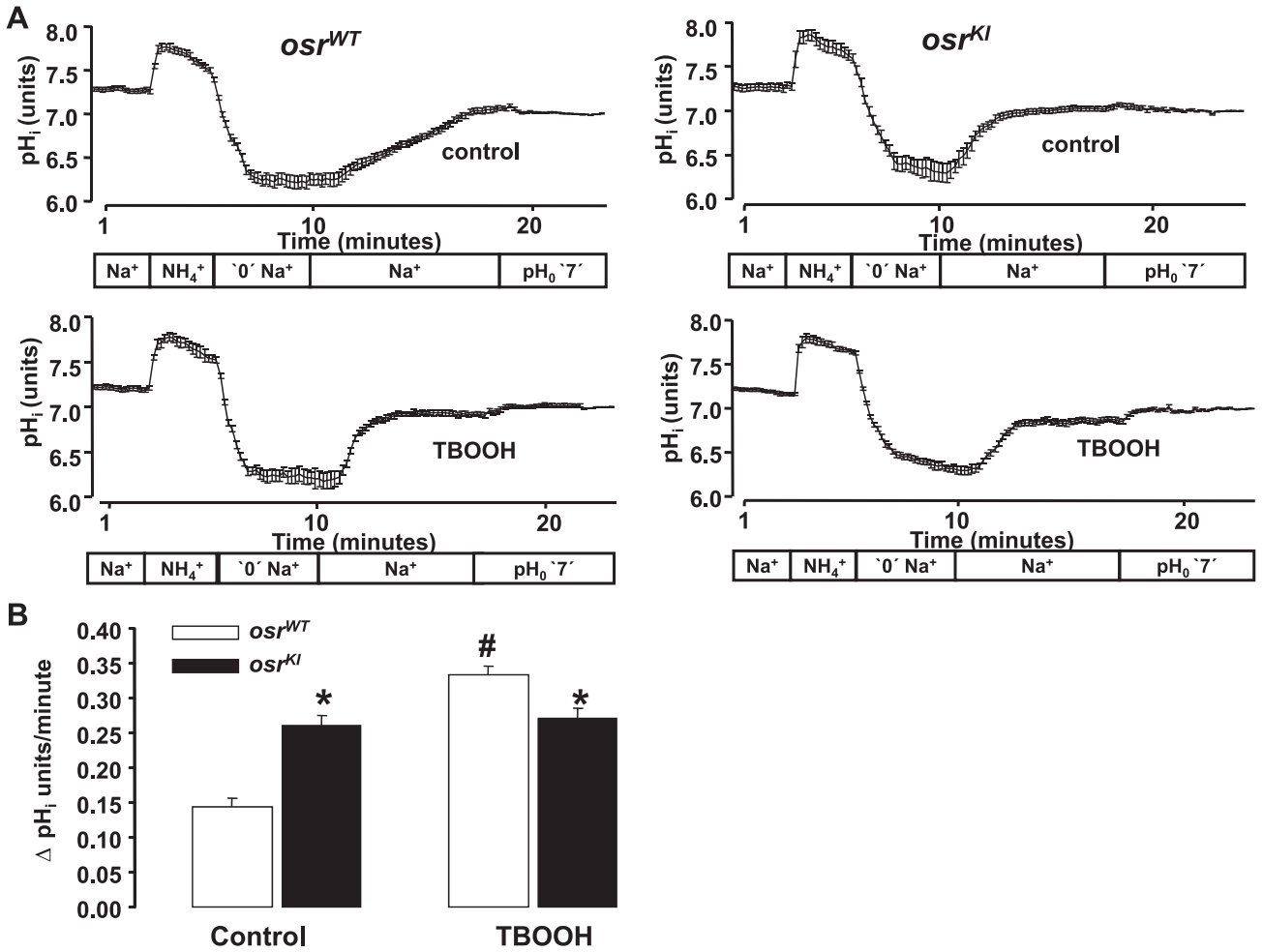

Fig. 5. Cytosolic pH recovery in DCs from osr^{KI} and osr^{WT} mice before and following exposure to *t*-butyl hydroperoxide (TBOOH). *A*: representative experiments showing the time-dependent alterations of cytosolic pH in DCs from heterozygous OSR1 knockin mice (osr^{KI}, *right*) and from wild-type mice (osr^{WT}, *left*) before (*top*) or following (*bottom*) a 2-h treatment with TBOOH (10 μM). *B*: arithmetic means ± SE ($n = 6$ 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) before (control, *left* bars) or following a 2-h treatment with TBOOH (10 μM, *right* bars). *Significant difference ($P < 0.01$) from osr^{WT} DCs; #significant difference ($P < 0.01$) from control.

trol conditions, a difference, 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. 7).

DISCUSSION

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. Similar to what has been reported earlier (62), LPS triggered the formation of ROS, activated the Na^+/H^+ exchanger NHE1, and increased cell volume in osr^{WT} DCs. All of 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 (67). In DCs the NHE1, but not the NHE2, isoform is expressed (62).

As shown earlier (62), Na^+/H^+ exchanger activation by LPS depends on ROS activation. The signaling involves MAP kinases and phosphoinositide 3 kinase (82), well-known stimulators of Na^+/H^+ exchangers (8, 39, 43, 53, 73). Conversely, ROS production is sensitive to cytosolic pH (33). The higher cytosolic pH and Na^+/H^+ exchanger activity in osr^{KI} DCs thus presumably contributes to the higher ROS production in those cells. ROS production is in turn important for antimicrobial activity (56).

Na^+/H^+ exchanger activity is enhanced in osr^{KI} DCs despite the more alkaline pH, which should actually decrease Na^+/H^+ exchanger activity (29). At least in theory, the stimulation of the Na^+/H^+ exchanger in osr^{KI} DCs could result from cell volume regulation. Cell shrinkage leads to stimulation of the Na^+/H^+ exchanger, which operates in parallel to the Cl^-/HCO_3^- exchanger (34, 40). Collectively, the two carriers accomplish the entry of NaCl, followed by osmotically obliged water. The H^+

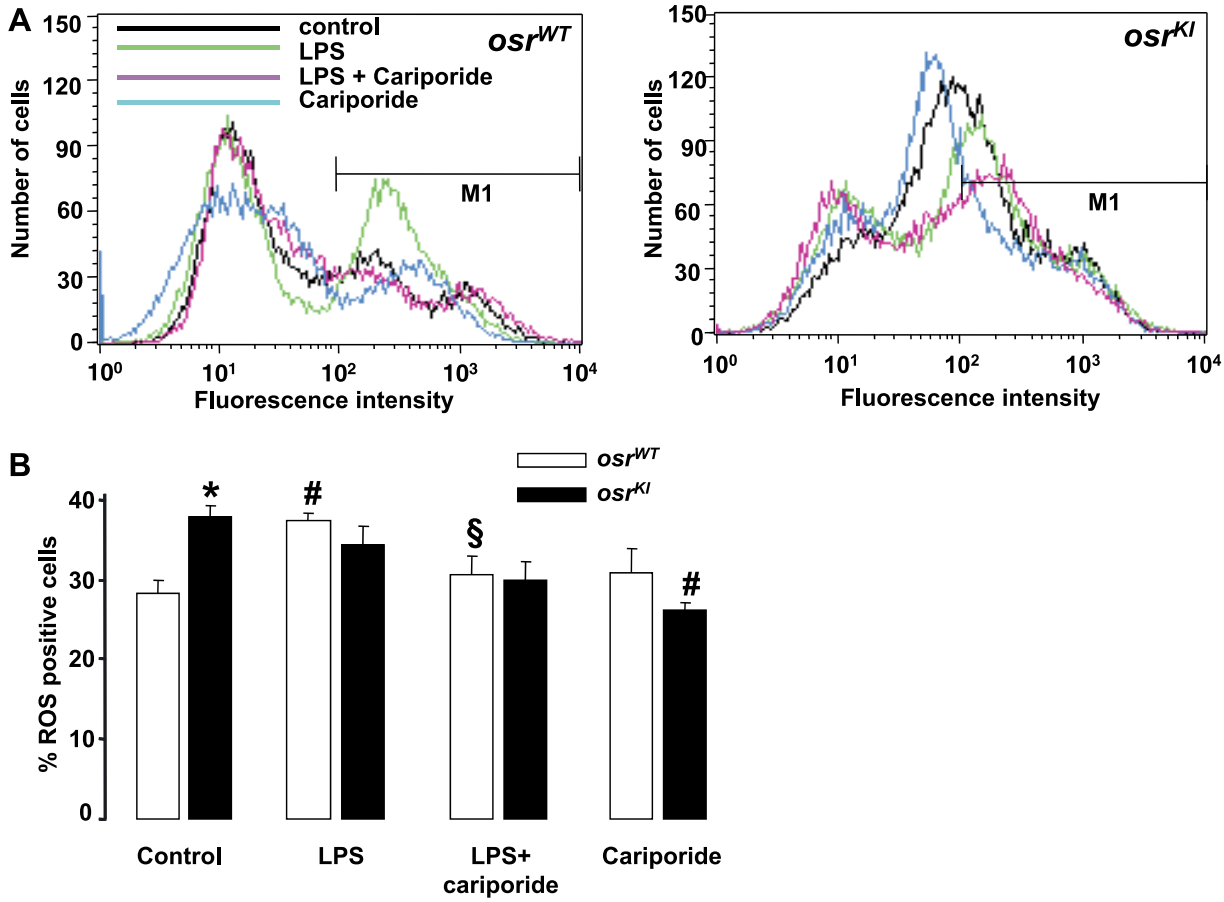

Fig. 6. Effect of LPS on reactive oxygen species (ROS) production of DCs from osr^{KI} and osr^{WT} mice in the presence and absence of cariporide. *A*: representative FACS histograms depicting ROS-dependent 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence in DCs from heterozygous OSR1 knockin mice (osr^{KI}, *right*) and from wild-type mice (osr^{WT}, *left*) without treatment (control, black line) and after a 4-h 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 ± SE ($n = 6$ 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) before or 4 h following exposure to LPS (1 μg/ml) in the absence or presence of cariporide (10 μM) or in the presence of cariporide alone. *Significant difference ($P < 0.01$) from osr^{WT} DCs; #significant difference ($P < 0.01$) from respective control value; §$P < 0.05$, significant difference from respective absence of cariporide.

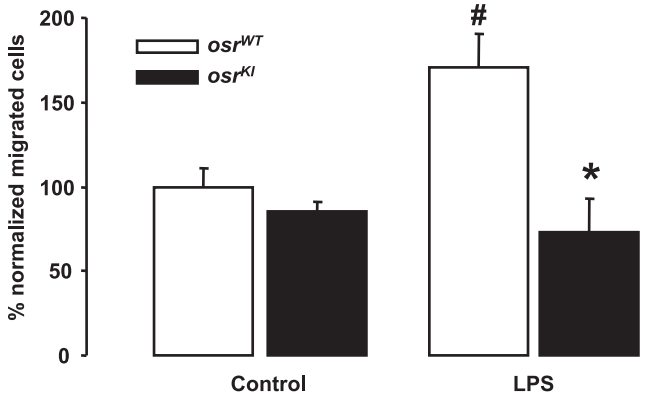

Fig. 7. Decreased migration of DCs from osr^{KI} mice. Arithmetic means ± SE ($n = 6$ independent experiments) of the normalized migration of DCs from heterozygous OSR1 knockin mice (osr^{KI}, black bars) and from wild-type mice (osr^{WT}, white bars) following a 4-h treatment without (control, *left* bars) or with LPS (*right* bars). *Significant difference ($P < 0.05$) from osr^{WT} DCs; #significant difference ($P < 0.05$) from respective control values.

and HCO_3^- extruded in exchange for Na^+ (Na^+/H^+ exchanger) and Cl^- (Cl^-/HCO_3^- exchanger) are osmotically not relevant as they are replenished in the cell by cytosolic formation from CO_2, which easily crosses the cell membrane (34, 40). Along those lines, LPS-induced cell swelling was abolished in the presence of cariporide, an observation highlighting the impact of the Na^+/H^+ exchanger in 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 (2, 15, 16, 27, 35–37, 59, 80). 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 activity, a decreased NKCC activity at least contributes to the upregulation of Na^+/H^+ exchanger activity.

Cell volume may influence the formation of ROS and antioxidative defense (30, 31, 64, 65), and cell shrinkage interferes with

ROS generation (62). Moreover, cytosolic pH modifies a variety of further functions, such as migration, cytokine release, adherence, nitric oxide formation, proliferation, and differentiation of macrophages and/or monocytes (5, 7, 10, 19, 44, 50–52, 54, 60, 71, 75). Na^+/H^+ exchanger activity further fosters (12, 26, 45, 46) or inhibits (4, 41, 47) apoptosis.

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 (3, 18, 70, 89). To fulfill their diverse functions, DCs have to migrate into inflammatory tissues and return to secondary lymphoid sites following microbial challenge (11). 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 (56). 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.

In conclusion, the present study disclosed that, in bone marrow-derived DCs, WNK resistance of OSR1 upregulates Na^+/H^+ exchanger activity, leading to cytosolic alkalinization and fostering formation of ROS. The study thus reveals a completely novel functional consequence of OSR1 inhibition.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

V.P., A.R., W.Y., C.Z., and M.B. performed the experiments; V.P., A.R., W.Y., C.Z., M.B., M.F., and F.L. approved the final version of the manuscript; A.R. and F.L. conception and design of the research; A.R. and M.F. analyzed the data; A.R. and F.L. interpreted the results of the experiments; A.R. prepared the figures; M.F. and F.L. edited and revised the manuscript; F.L. drafted the manuscript.

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