Effect of Bacterial Lipopolysaccharide on Na⁺/H⁺ Exchanger Activity in Dendritic Cells

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Key Words

pH_i • Na⁺/H⁺ exchanger • Cariporide • Oxidative stress
• Cell volume

Abstract

The function of dendritic cells (DCs), antigen-presenting cells linking innate and adaptive immunity, is stimulated by bacterial lipopolysaccharides (LPS), which trigger the formation of reactive oxygen species (ROS). In macrophages, ROS formation is paralleled by activation of the Na⁺/H⁺ exchanger, a carrier involved in the regulation of cytosolic pH and cell volume. The present study explored whether LPS influence Na⁺/H⁺ exchanger activity in DCs. The DCs were isolated from murine bone marrow, cell volume was estimated from forward scatter in FACS analysis, ROS production from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, apoptosis from annexin V binding, cytosolic pH (pH_i) from 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence and Na⁺/H⁺ exchanger activity from the Na⁺ dependent realkalinization following an ammonium pulse. Exposure of DCs to LPS (1 µg/ml) led to a transient increase of Na⁺/H⁺ exchanger activity. Moreover, LPS increased forward scatter and ROS

formation and decreased apoptosis. The NHE1 inhibitor cariporide (10 µM) virtually abrogated Na⁺/H⁺ exchanger activity, inhibited LPS-induced cell swelling, blunted LPS-induced ROS formation and reversed the antiapoptotic effect of LPS. Na⁺/H⁺ exchanger activity was stimulated by oxidative stress and LPS induced stimulation of NHE activity was abolished in presence of ROS chelators (Tempol, Tiron and Vitamin C). In conclusion, LPS treatment transiently upregulates the Na⁺/H⁺ exchanger in DCs, an effect required for the effects of LPS on DC survival, cell volume and ROS formation.

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Introduction

Dendritic cells (DCs) are antigen-presenting cells involved in the initiation of both innate and adaptive immunity and thus critically important for the regulation of the immune response [1-5]. Following exposure to bacterial wall components such as lipopolysaccharides (LPS), DCs generate reactive oxygen species [6-8], which participates in the killing of pathogens [9].

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As shown in other cell types [10] including macrophages [11], ROS production leads to generation of H^+ , which in turn influences the activity of the ROS-generating NADPH oxidase [12, 13]. In a wide variety of cells [11, 14, 15] including macrophages [16-23] and monocytes [22, 24, 25] regulation of cytosolic pH involves the Na^+/H^+ exchanger, a carrier extruding H^+ in exchange of Na^+ . In resident alveolar macrophages, the cytosolic pH is maintained by the H^+ ATPase at a value higher than that required for the activation of the Na^+/H^+ exchanger [16, 17, 19]. Accordingly, the Na^+/H^+ exchanger in this cell type appears not to be critically important for the maintenance of resting cytosolic pH. Following inhibition of the H^+ ATPase, the cytosolic pH is, however, maintained by the Na^+/H^+ exchanger [13]. In peritoneal macrophages, cytosolic pH is more acidic and fully depends on the activity of the H^+ ATPase [13, 26]. As in most cells [27, 28] the Na^+/H^+ exchanger further participates in the regulation of macrophage cell volume [11, 19], which in turn influences a wide variety of cellular functions [28].

The present study explored the influence of LPS on Na^+/H^+ exchanger activity in DCs from murine bone marrow. Experiments were performed in the absence and presence of cariporide, a specific inhibitor of NHE1 [29].

Materials and Methods

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) [30, 31]. Mice had free access to control diet (1314, Altromin Heidenau, Germany) and tap drinking water.

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 2×10^6 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, $\geq 80\%$ 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 (4×10^5) were incubated in 100 μ l FACS buffer (phosphate buffered saline (PBS) plus 0.1% FCS) containing fluoro-

chrome-conjugated antibodies at a concentration of 10 μ g/ml. 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 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 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 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, 2×10^5 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) [32]. Briefly, 2×10^5 cells were taken in a culture dish and treated with LPS (with or without cariporide). After the treatment, cells were collected and DCFDA (Sigma, Schnellendorf, Germany) was added to the cell suspension at a final concentration of 10 μ M. 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 [33]. To this end, the percentage of phosphatidylserine-translocating cells was evaluated by staining with FITC-conjugated Annexin V. In brief 4×10^5 cells were harvested and washed twice with Annexin washing buffer (AWB, 10 mM 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 μ l Annexin-V-Fluos labelling reagent in 1 ml AWB), incubated for 15 minutes at room temperature. After washing with AWB, they were analyzed by flow cytometry.

Measurement of intracellular pH

For digital imaging of cytosolic pH (pH_i) the cells were incubated in a Hepes-buffered Ringer solution containing 10 μ M 2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluoresceinace-

	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
<i>control</i>	7.22 $\pm$ 0.01	17.4 $\pm$ 1.4	-0.090 $\pm$ 0.013	0.169 $\pm$ 0.017	99
<i>LPS 1hr</i>	7.23 $\pm$ 0.02	13.9 $\pm$ 2.1	-0.230 $\pm$ 0.044	0.385 $\pm$ 0.037*	53
<i>LPS + carip 1 hr</i>	7.20 $\pm$ 0.03	16.4 $\pm$ 1.8	-0.229 $\pm$ 0.016	0.023 $\pm$ 0.008 [#]	25
<i>Cariporide 1 hr</i>	7.16 $\pm$ 0.04	14.8 $\pm$ 2.5	-0.116 $\pm$ 0.055	0.056 $\pm$ 0.029*	15
<i>LPS 4 hrs</i>	7.20 $\pm$ 0.02	17.9 $\pm$ 2.2	-0.104 $\pm$ 0.019	0.402 $\pm$ 0.040*	42
<i>LPS + carip 4 hrs</i>	7.18 $\pm$ 0.02	14.4 $\pm$ 0.9	-0.175 $\pm$ 0.041	0.053 $\pm$ 0.011 [#]	31
<i>LPS + Tempol 4 hrs</i>	7.29 $\pm$ 0.01	17.0 $\pm$ 1.1	-0.044 $\pm$ 0.005	0.113 $\pm$ 0.011 [#]	112
<i>LPS + Tiron 4 hrs</i>	7.29 $\pm$ 0.02	20.6 $\pm$ 5.5	-0.055 $\pm$ 0.008	0.133 $\pm$ 0.023 [#]	38
<i>LPS + Vitamin C 4 hrs</i>	7.33 $\pm$ 0.02	19.1 $\pm$ 2.2	-0.054 $\pm$ 0.007	0.114 $\pm$ 0.022 [#]	43
<i>t-butylhydroperoxide 1 hr</i>	7.16 $\pm$ 0.02	18.8 $\pm$ 1.3	-0.063 $\pm$ 0.007	0.241 $\pm$ 0.026*	44
<i>Cariporide 4 hrs</i>	7.14 $\pm$ 0.03*	19.5 $\pm$ 2.2	-0.010 $\pm$ 0.009	0.049 $\pm$ 0.022*	37
<i>LPS 24 hrs</i>	6.97 $\pm$ 0.01*	23.1 $\pm$ 2.3	-0.100 $\pm$ 0.027	0.074 $\pm$ 0.007*	85
<i>LPS + cariporide 24 hrs</i>	6.96 $\pm$ 0.02*	15.6 $\pm$ 3.7	0.002 $\pm$ 0.009*	0.004 $\pm$ 0.003*	25
<i>Cariporide 24 hrs</i>	7.14 $\pm$ 0.03*	18.9 $\pm$ 3.2	-0.074 $\pm$ 0.010	0.006 $\pm$ 0.005*	30

Table 1. Cytosolic pH (pH_i), buffer capacity, sodium dependent and sodium independent pH recovery in bone marrow-derived dendritic cells (DCs) prior to (control) and following treatment with lipopolysaccharide LPS (1 μ g/ml) in absence or presence of cariporide or scavengers. *indicates significant difference from the respective control value, [#]indicates significant difference from the respective LPS treatment.

toxymethylester (BCECF-AM Molecular Probes, Leiden, The Netherlands) for 15 minutes at 37°C. After loading, the chamber was flushed for 5 minutes 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 [34]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K⁺/nigericin (10 μ g/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 NH₄Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH₃ and binding of H⁺ to form NH₄⁺ [35]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [35]. Assuming that NH₄⁺ and NH₃ are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

$$\beta = \Delta[\text{NH}_4^+]/\Delta\text{pH}_i,$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[\text{NH}_4^+]$ is the decrease of cytosolic NH₄⁺ concentration, which is identical to the concentration of [NH₄⁺]_i immediately before the removal of ammonia. The pK for

NH₄⁺/NH₃ is 8.9 [36] and at an extracellular pH (pH_o) of 7.4 the NH₄⁺ concentration in extracellular fluid ([NH₄⁺]_o) is 19.37 [20/(1+10^{pH_o-pK})] mM. The intracellular NH₄⁺ concentration ([NH₄⁺]_i) was calculated from:

$$[\text{NH}_4^+]_i = 19.37 \cdot 10^{\text{pH}_o - \text{pH}_i} \text{ mM}$$

To calculate the $\Delta\text{pH}/\text{min}$ 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 (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 NaH₂PO₄, 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG, 3 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 KH₂PO₄, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH₄Cl); high K⁺ for calibration 105 KCl, 1 CaCl₂, 1.2 MgSO₄, 32.2 Hepes, 10 mannitol, 5 μ M 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.

Statistics

Data are provided as means $\pm$ 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.

Results

BCECF fluorescence was employed to determine cytosolic pH in DCs. To elucidate, whether the Na⁺/H⁺ exchanger is functional in DCs, the cells were acidified by an ammonium pulse. In this maneuver, NH₄Cl is added to the perfusate. NH₃ enters the cells, binds H⁺ to form

Fig. 1. Cytosolic pH recovery in DCs following LPS treatment in the presence and absence of NHE1 inhibitor cariporide. a-d. 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 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. Tracings illustrating alterations of cytosolic pH in typical experiments (a) prior to, or following a (b) 4 hours or (c) 24 hours treatment with LPS (1 $\mu\text{g}/\text{ml}$) and (d) following a 4 hours treatment with LPS (1 $\mu\text{g}/\text{ml}$) in the presence of cariporide (10 μM). e. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) of Na^+ -dependent recovery of cytosolic pH in DCs ($\Delta\text{pH}/\text{min}$) following an ammonium pulse prior to (white bar), following addition of LPS (1 $\mu\text{g}/\text{ml}$) in the absence (black bars) or presence (hatched bars) of cariporide (10 μM) or in presence of cariporide alone (10 μM , grey bars). * indicates significant difference ($p < 0.01$) from control (value prior to LPS). # indicates significant difference ($p < 0.01$) from respective LPS treatment.

NH_4^+ and thus leads to a transient cytosolic alkalinization (Fig. 1). 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. 1). 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. 1, Table 1).

Treatment of the DCs with lipopolysaccharide (LPS, 1 $\mu\text{g}/\text{ml}$) was followed by a marked and statistically significant increase in Na^+ -dependent realkalinization within 1 and 4 hours (Table 1, Fig. 1). 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. 1). The increase in Na^+/H^+ ex-

changer activity was blunted in the presence of the NHE1 inhibitor cariporide (10 μM) (Fig 1). Accordingly, LPS significantly increased the cariporide sensitive realkalinization (from 0.12 to 0.33 ΔpH units/minute). The Na^+/H^+ exchanger activity declined thereafter to values significantly below the Na^+/H^+ exchanger activity prior to LPS treatment (Fig. 1). 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.

The treatment of the DCs with LPS was within 4 hours followed by an increase in forward scatter, pointing to an increase in cell volume (Fig. 2). Forward scatter was not increased following simultaneous treatment of the DCs with LPS and cariporide (Fig. 2). Despite the

Fig. 2. Effect of LPS on forward scatter in mouse bone marrow derived DCs in the presence and absence of cariporide. **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 μ g/ml) in the absence (green line) and presence (pink line) of cariporide. **b.** Arithmetic means $\pm$ SEM ($n = 5$ -6 independent experiments) of forward scatter in DCs incubated for 4 hours without (white bars) or with LPS (1 μ g/ml) in the absence (black bars) or presence (hatched bars) of cariporide (10 μ M). * ($p < 0.05$) indicates significant difference from untreated (control) group; # ($p < 0.05$) indicates significant difference from LPS in the absence of cariporide.

Fig. 3. Effect of LPS on reactive oxygen species (ROS) production in murine bone marrow derived DCs in the presence and absence of cariporide. **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 μ g/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 $\pm$ SEM ($n = 5$ -6 independent experiments) of the percentage of ROS positive DCs incubated for 4 hours without (white bars) or with LPS (1 μ g/ml) in the absence (black bars) or presence (hatched bars) of cariporide (10 μ M) as well as in the presence of cariporide (10 μ M, grey bar) alone. * ($p < 0.05$) indicates significant difference from untreated (control) group; # ($p < 0.05$) indicates significant difference from LPS in the absence of cariporide.

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.

The role of NHE1 in LPS-induced Reactive Oxygen Species (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 LPS-stimulated forma-

tion (Fig. 3). Basal ROS formation tended to be decreased in cariporide treated cells, a difference, however, not reaching statistical significance (Fig 3).

To explore whether ROS formation is required for activation of the Na^+/H^+ exchanger by LPS, ammonium pulse experiments were carried out 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. In the

Fig. 4. Cytosolic pH recovery in DCs following LPS treatment in the presence and absence of ROS scavengers. 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 μ M), (c) in the presence of Tiron (1 mM) or (d) in the presence of Vitamin C (50 μ g/ml). e. Arithmetic means $\pm$ SEM (n = 3-5 independent experiments) of Na⁺-dependent recovery of cytosolic pH in DCs (Δ pH/min) following an ammonium pulse prior to (white bar), following addition of LPS (1 μ g/ml) in the absence of ROS scavengers or oxidants (black bar) or in the presence of Tempol (30 μ M, hatched bars), of Tiron (1 mM, striped bar), or of Vitamin C (50 μ g/ml, crossed bar), or following treatment with t-butylhydroperoxide (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.

presence of ROS chelators LPS induced stimulation of NHE was blunted (Fig. 4, Table 1). Conversely, treatment of the DCs with the oxidant t-butylhydroperoxide (200 mM) was followed by significant increase in the NHE activity (Fig. 4, Table 1). Thus, ROS activation participates in the stimulation of Na⁺/H⁺ exchanger activity by LPS.

Further experiments were conducted to verify whether treatment with LPS and/or cariporide affected cell survival. As shown in Fig. 5, LPS treatment significantly reduced the percentage of Annexin V positive cells pointing to inhibition of DC apoptosis. The effect was virtually abolished when the cells were treated simultaneously with cariporide (Fig. 5). Cariporide alone did not significantly modify DC apoptosis (Fig 5).

Discussion

The present study demonstrates 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 [37]. However, RT-PCR revealed in DCs predominant expression of NHE1 and virtually no NHE2 transcript levels (unpublished observation). 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.

Stimulation of Na⁺/H⁺ exchanger activity is dependent on ROS activation. The present study did not address further signaling involved in LPS induced activation of the Na⁺/H⁺ exchanger. Toll-like receptor signaling involves activation of MAP kinases and phosphoinositide (PI) 3 kinase [38], signaling pathways known to stimulate the activity of Na⁺/H⁺ exchangers [39-43]. Future experiments will be required to define the participation of those and further signaling pathways

Fig. 5. Effect of cariporide and LPS on phosphatidylserine exposure at the surface of mouse DCs. 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 alone (10 µM, blue line). b. Arithmetic means $\pm$ 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.

in the Na^+/H^+ exchanger activation by LPS.

ROS production is instrumental in the killing of pathogens and thus an important element in the innate immune response [9]. Owing to the pH sensitivity of ROS production [12, 13], 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 [44]. The Na^+/H^+ exchanger is expected to be less active without prior acidification. Nevertheless, the ammonium pulse unravels the marked upregulation of dendritic cell 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.

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 $\text{Cl}^-/\text{HCO}_3^-$ exchanger [27, 28]. 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. Besides the Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchanger, several other transport systems participate in regulatory cell volume increase, such as $\text{Na}^+/\text{K}^+2\text{Cl}^-$ cotransport, Na^+ channels and Na^+ -coupled uptake of organic osmolytes [27, 28]. 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 [45–48]. 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 [21, 22, 49–58].

Following sustained exposure to LPS the Na^+/H^+ exchanger activity declines, a phenomenon paralleled by cytosolic acidification. Possibly, the Na^+/H^+ exchanger is inhibited by cell swelling, as Na^+/H^+ exchanger activity is a function of cell volume [27, 28]. Despite downregulation of Na^+/H^+ exchanger activity, the cells remain swollen, however, which may point to an upregulation of the cell volume regulatory set point. Such a setpoint shift has been observed e.g. following cytosolic acidification [44] or stimulation of cell proliferation [59].

According to annexin V binding, LPS treatment decreased apoptosis of DC, 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 [60-63] or inhibit [64-66] apoptosis. The inhibitory effect of Na⁺/H⁺ exchanger activity on apoptosis was explained by the prevention of acidosis [64, 65] or of cell shrinkage [66].

The present 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 Ca²⁺ signaling [30, 31, 67, 68-72]. It must be kept in mind that alterations of cytosolic pH do influence cytosolic Ca²⁺ activity [73-76]. Moreover, some effects of Toll-like receptor stimulation are not mediated by Ca²⁺-dependent signaling [77]. 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 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.

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