Influence of Dexamethasone on Na⁺/H⁺ Exchanger Activity in Dendritic Cells

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Key Words

pH_i • Na⁺/H⁺ exchanger • Glucocorticoids • Cell volume • Immunosuppression

Abstract

Glucocorticoids regulate the function of dendritic cells (DCs), antigen-presenting cells linking innate and adaptive immunity. Glucocorticoids influence the function of other cell types by modulating the activity of the Na⁺/H⁺ exchanger (NHE), a carrier involved in the regulation of cytosolic pH and cell volume. The present study explored whether dexamethasone influences Na⁺/H⁺ exchanger activity in DCs. The DCs were isolated from mouse bone marrow, cell volume was estimated from forward scatter in FACS analysis, cytosolic pH (pH_i) utilizing BCECF fluorescence and Na⁺/H⁺ exchanger activity from the Na⁺ dependent realkalinization after an ammonium pulse. Treatment with the glucocorticoid dexamethasone (100 nM; 1, 4, 16 and 24h) significantly decreased pH_i (≥4 h) and gradually increased Na⁺/H⁺ exchanger activity (=16 h). The stimulation of Na⁺/H⁺ exchanger activity by dexamethasone was virtually abrogated by glucocorticoid receptor blocker mifepristone (1 μM) and NHE3 inhibitor dimethyl amiloride (5 μM), but not prevented by NHE1 inhibitor cariporide (10 μM). Dexamethasone

treatment significantly increased SGK1 mRNA levels. Stimulation of Na⁺/H⁺ exchanger activity by dexamethasone was blunted in DCs lacking SGK1. Dexamethasone treatment did not significantly alter ROS formation but significantly decreased the forward scatter. Exposure of DCs to lipopolysaccharide (LPS, 1 μg/ml) led to a transient increase followed by a decline of Na⁺/H⁺ exchanger activity and to enhanced forward scatter as well as ROS formation, all effects significantly blunted in the presence of dexamethasone (100 nM). In conclusion, glucocorticoid treatment decreased pH_i and cell volume, effects paralleled by upregulation of Na⁺/H⁺ exchanger activity in DCs. Moreover, glucocorticoids blunted the stimulation of Na⁺/H⁺ exchanger activity, cell swelling and ROS formation following LPS treatment.

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Introduction

Glucocorticoids are powerful suppressors of the immune response [1-5]. They accomplish their immunosuppressive effect in part by inhibiting the maturation and function of dendritic cells [2, 6-11], key players in the regulation of the immune response [12].

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Glucocorticoids prevent the upregulation of MHC class II and costimulatory molecules and thus decrease the ability of DCs to present antigens and elicit a T cell response [2]. In addition, glucocorticoids impair the production of several cytokines, such as interleukin (IL)-6, IL-12 and tumor necrosis factor (TNF)- α [1, 6]. Glucocorticoids foster the endocytic activity of DCs [13], a property of immature DCs, which are characterized by strong antigen capture, low antigen presentation and low T cell stimulation.

Glucocorticoids exert their effects primarily by binding to the glucocorticoid receptor, a member of a large superfamily including receptors for other steroid hormones, thyroid hormone, vitamin D3, retinoic acid, and a number of orphan receptors [5]. The cellular mechanisms involved in the regulation of DC maturation by glucocorticoids are ill-defined. Glucocorticoids stimulate the expression of the κ B inhibitor (I κ B), which binds the nuclear factor NF- κ B thus preventing the transcription factor from entering the nucleus and triggering gene transcription [14, 15]. Glucocorticoids further stimulate the expression of GILZ (Glucocorticoid-Induced Leucine Zipper gene), which similarly contributes to the inhibition of NF- κ B [16].

In other cells, glucocorticoids modify cell function in part by regulating the activity of the Na⁺/H⁺ exchanger [17-28], an effect mediated in part by up-regulation of the serum and glucocorticoid inducible kinase SGK1 [27, 28]. Na⁺/H⁺ exchangers play a decisive role in the regulation of cytosolic pH [29-31] and cell volume [32, 33]. Along those lines, SGK1 expression is markedly up-regulated by cell shrinkage [34]. Cell volume influences a wide variety of further cellular functions [33]. Specifically, cell swelling influences formation of reactive oxygen species (ROS) and modifies cellular antioxidative defence [35-38]. In DCs, activation of Na⁺/H⁺ exchanger activity is in turn sensitive to ROS formation [39].

However, little is known about pH regulation of DCs and nothing is known about glucocorticoid regulation of DC Na⁺/H⁺ exchanger activity. The present study thus explored the influence of dexamethasone treatment on the regulation of cytosolic pH in DCs from murine bone marrow.

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 (DCs) were cultured from bone

marrow of 7-11 weeks old female C57/Bl-6 mice (Charles River, Sulzfeld, Germany). Mice had free access to control diet (1314, Altromin Heidenau, Germany) and tap drinking water.

Cell Culture

Dendritic cells (DCs) were cultured from bone marrow of 7-11 weeks old female C57/Bl-6 mice [40, 41]. Bone marrow derived cells were flushed out of the cavities from the femur and tibia with PBS. Cells were then washed twice with RPMI 1640 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 % FCS, 1 % penicillin/streptomycin, 1 % glutamine, 1 % non-essential amino acids (NEAA) and 0.05 % β -mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/mL, 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 DCs 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 fluorochrome-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 antibodies for 60 minutes at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometry analysis.

Determination of cell volume

Cell volume was determined by the forward scatter in flow cytometric analysis. Briefly, 2×10^5 cells were collected, centrifuged, the pellet was resuspended in FACS buffer and analysed with flow cytometry (FACS-calibur, Becton Dickinson, Heidelberg, Germany).

Determination of ROS production

ROS production in DCs was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Sigma, Schnelldorf, Germany) [42]. Briefly, 2×10^5 cells were collected and DCFDA was added to the cell suspension at a final concentration of 10 μ M. 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 analysed with flow cytometry. DCFDA fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular pH

For digital imaging of cytosolic pH the cells were incubated in a HEPES-buffered Ringer solution containing 10 μ M 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 sticking to the outside of the cells. 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 8 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 values using the high-K⁺/nigericin calibration technique [43]. 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 performed 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⁺ [44]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [44]. Assuming that NH₄⁺ and NH₃ are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

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

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and Δ[NH₄⁺]_i 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 [45] 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})]. The intracellular NH₄⁺ concentration ([NH₄]_i) was calculated from:

$$[NH_4]_i = 19.37 \times 10^{pH_o - pH_i}.$$

The calculation of the buffer capacity required that NH₄⁺ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na⁺, indicating that there was no relevant further exit of NH₄⁺. 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 (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 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 Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from mouse dendritic cells in Trizol (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After DNase digestion reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse tran-

scriptase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 µl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x iTaq Fast SYBR Green (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Amplification of the house-keeping gene Tbp (TATA box binding protein) was performed to standardize the amount of sample RNA. Cycling conditions were chosen as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 68°C for 20 sec. For the amplification the following primers were used (5'→3' orientation):

Nhe1, fw TCT GAT GGT GCT GGC AGT AG rev; ATG TCC CAG GTC TTA CAC GC;
Nhe2, fw TTG AAA TGG CAG AGA CAG GGA rev CAT TTC GCC TGG CGT GAG;
Nhe3, fw GTC ACC CAG GAT GTA GCC TCT G rev GGT GGC ACC CTG GAT AGG AT;
Nhe4, fw ATG CGG AAA GGC CAG AGT CT rev TCT CTC CTT GCT GCC TGA GG;
Nhe6, fw TGC CTG GCT CTT TCG GAT G rev AGC AGG CTG GGA GTG TGG TA;
Nhe7, fw GCA GGA AAG TGC ATG GAT ATT CA rev GGA GAG TGG TGG TTA GCG GG;
Nhe8, fw CTC GTG GAC ATC GAG GAT GC rev AGG TGC TCT GAC TCG ATG GC;
Nhe9, fw ACG AAG ACA GAG AGT GCA CAG CT rev GTG GTG GTC AGT GGA GGG C;
Tbp, fw CAC TCC TGC CAC ACC AGC TT rev TGG TCT TTA GGT CAA GTT TAC AGC C.

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad) and all experiments were done in duplicate. Relative quantification of gene expression was achieved using the Δct method as described earlier [46].

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.

Results

BCECF fluorescence was employed to determine cytosolic pH (pH_i) in bone marrow derived mouse dendritic cells (DCs). As shown in Table 1, the exposure of DCs to dexamethasone (100 nM) was followed by sustained cytosolic acidification, an effect reaching statistical significance at =4 hours incubation.

The ammonium pulse technique was utilized to determine the Na⁺/H⁺ exchanger (NHE) activity of DCs. In this manoeuvre, NH₄Cl is added to the perfusate. NH₃

Table 1. Cytosolic pH (pH_i), buffer capacity ($mM/\Delta pH_i$) Na^+ -independent ($0\ Na^+$) and Na^+ -dependent ($+Na^+$) pH recovery ($\Delta\ pH\ units/min$) following an ammonium pulse in bone marrow dendritic cells (DCs) prior to (control) and following treatment with lipopolysaccharide LPS ($1\ \mu g/ml$) or dexamethasone Dex ($100\ nM$) in isolation (LPS, Dex) or combination (LPS+Dex). ^aindicates significant difference to control value, ^bindicates significant difference to respective value in the presence of LPS alone.

	pH_i	$mM/\Delta pH_i$	$0\ Na^+$ $\Delta\ pH\ units/min$	$+Na^+$ $\Delta\ pH\ units/min$	Number of cells
Control	7.29 ± 0.01	17.1 ± 1.0	-0.057 ± 0.007	0.132 ± 0.010	317
Dex (1 hr)	7.26 ± 0.02	21.4 ± 1.6	-0.093 ± 0.021	0.103 ± 0.009	74
Dex (4 hrs)	7.21 ± 0.01^a	17.4 ± 1.9	-0.112 ± 0.014^a	0.155 ± 0.012	174
Dex (16 hrs)	7.20 ± 0.03^a	16.4 ± 1.8	-0.073 ± 0.017	0.251 ± 0.032^a	47
Dex (24 hrs)	7.15 ± 0.01^a	20.0 ± 1.1	-0.094 ± 0.006^a	0.252 ± 0.013^a	325
LPS (4 hrs)	7.29 ± 0.01	17.9 ± 2.2	-0.090 ± 0.016	0.344 ± 0.032	104
LPS + Dex (4 hrs)	$7.19 \pm 0.01^{a,b}$	17.3 ± 1.6	-0.144 ± 0.017^a	$0.182 \pm 0.016^{a,b}$	130
LPS (24 hrs)	6.97 ± 0.01^a	20.8 ± 1.9	-0.089 ± 0.027	0.074 ± 0.007^a	132
LPS + Dex (24 hrs)	$7.17 \pm 0.01^{a,b}$	21.6 ± 2.2	-0.047 ± 0.003^b	0.135 ± 0.012^b	272

Fig. 1. Cytosolic pH recovery in mouse bone marrow derived dendritic cells (DCs) following dexamethasone treatment. Alterations of cytosolic pH (pH_i) in bone marrow derived dendritic cells (DCs) following an ammonium pulse. To load the cells with H^+ , $20\ mM\ NH_4Cl$ was added and Na^+ removed (replaced by NMDG; $0\ Na^+$) in a first step (see bars below each tracing), NH_4Cl removed in a second step (NH_4^+), Na^+ added in a third step and nigericin ($pH_0\ 7'$) applied in a fourth step to calibrate each individual experiment. A. Time dependent alterations of cytosolic pH (pH_i) prior to (left upper panel), 1 hour (right upper panel), 4 hours (left lower panel) and 24 hours (right lower panel) following treatment with dexamethasone ($100\ nM$). B. Arithmetic means $\pm\ SEM$ ($n = 4$) of Na^+ -dependent pH_i recovery in DCs ($\Delta pH/min$) following an ammonium pulse prior to (control, white bar) and 1, 4, 16 or 24 hours following addition of $100\ nM$ dexamethasone (light grey bars). * indicates significant difference from control (value prior to dexamethasone).

enters the cells, binds H^+ to form 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 pH did not recover from this acidification after an ammonium pulse, revealing that the cells did not express a Na^+ independent H^+ extrusion mechanism. The addition of Na^+ led, however, to a rapid pH recovery pointing to the operation of a Na^+/H^+ exchanger. Exposure to dexamethasone ($100\ nM$) did not significantly modify the Na^+/H^+ exchanger activity within the first hour, but led to a gradual and sustained significant increase of the Na^+/H^+ exchanger activity reaching statistical significance at $= 16$ hours incubation (Fig. 1).

To explore, whether dexamethasone treatment led to genomic upregulation of NHE isoforms, the transcript levels of the NHE1-9 isoforms were determined by RT-PCR. As shown in Fig. 2, the highest transcript levels were observed for NHE1, NHE6 and NHE9. The transcript levels of none of those three NHE isoforms were significantly modified by treatment of the DCs with dexamethasone. Instead, dexamethasone treatment upregulated the transcript levels of NHE2 and NHE3, which remained, however, by far lower than those of NHE1, NHE6, or NHE9, even after treatment of the DCs with dexamethasone (Fig. 2).

To identify the isoform involved in the increased NHE activity after dexamethasone treatment, experiments were performed in the presence of NHE1 inhibiting

Fig. 2. Effect of dexamethasone on the expression of Na^+/H^+ exchangers in mouse bone marrow derived dendritic cells (DCs). Arithmetic means $\pm$ SEM ($n = 3$) of NHE transcript levels in DCs pretreated for 24 hours without (control, white bars) or with (light grey bars) 100 nM dexamethasone. Total RNA was isolated and NHE mRNA levels assessed by real-time PCR using Tbp mRNA as a reference gene. * indicates significant difference from control (absence of dexamethasone).

Fig. 3. Inhibitors of dexamethasone induced stimulation of NHE activity. Arithmetic means $\pm$ SEM ($n = 4$) of Na^+ -dependent pH_i recovery in DCs ($\Delta\text{pH}/\text{min}$) following an ammonium pulse prior to (- dex) and 24 hours following addition of 100 nM dexamethasone (+ dex) in the absence and presence of A. cariporide (10 μM) and dimethylamiloride (DMA, 5 μM) as well as B. mefiprestone (mefi, 1 μM). * indicates significant difference from respective absence of dexamethasone; # indicates significant difference from control; § indicates significant difference from dexamethasone alone.

Fig. 4. Role of SGK1 in the dexamethasone induced stimulation of NHE activity A. Arithmetic means $\pm$ SEM ($n = 3$) of SGK1 mRNA levels (over Tbp mRNA levels) in DCs incubated for 24 hours without (- dex) or with (+ dex) dexamethasone (100 nM). B. Arithmetic means $\pm$ SEM ($n = 4$) of Na^+ -dependent pH_i recovery in DCs ($\Delta\text{pH}/\text{min}$) following an ammonium pulse prior to (- dex) and 24 hours following addition of 100 nM dexamethasone (+ dex) in DCs from *sgk1*^{+/+} (white bars) and from *sgk1*^{-/-} (black bars) mice. * indicates significant difference from control, # indicates significant difference from respective wild type mice.

cariporide (10 μM) and in the presence of the NHE3 inhibiting dimethyl amiloride (5 μM). As shown in Fig. 3A, the presence of cariporide significantly reduced the NHE activity in both, control and dexamethasone treated cells, but did not abrogate the difference between untreated and dexamethasone treated DCs. In contrast, dimethylamiloride had no effect on the control cells but virtually abolished the dexamethasone induced NHE ac-

tivity, an observation suggesting that dexamethasone up-regulates NHE activity at least in part by up-regulating NHE3.

Further experiments were performed using the competitive antagonist of the glucocorticoid receptor, mefiprestone (1 μM). As a result, the administration of mefiprestone abrogated the effect of dexamethasone on the NHE activity (Fig. 3B.).

Fig. 5. Cytosolic pH recovery in mouse bone marrow derived dendritic cells (DCs) following treatment with both LPS and dexamethasone. A. Time dependent alterations of cytosolic pH (pH_i) following a 4 hour (upper panels) and 24 hour (lower panels) treatment with LPS (1 $\mu\text{g/mL}$) in the absence (left panels) and presence of dexamethasone (100 nM, right panels). B. Arithmetic means $\pm$ SEM ($n = 4$) of Na^+ -dependent recovery of cytosolic pH in DCs ($\Delta\text{pH}/\text{min}$) following an ammonium pulse prior to (white bar) and following treatment for 4 hours (middle panel) and 24 hours (right panel) with LPS (1 $\mu\text{g/mL}$) in presence (grey bars) and absence (black bars) of dexamethasone (100 nM). * indicates significant difference from control, # indicates significant difference from respective absence of dexamethasone.

Fig. 6. Effect of LPS and dexamethasone on ROS formation in mouse bone marrow derived dendritic cells (DCs). A. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in DCs without treatment (control, black line) as well as after a 4 hour treatment (left panel) or after a 24 hour treatment (right panel) with LPS (1 $\mu\text{g/mL}$) in the absence (green line) and presence (pink line) of dexamethasone or with dexamethasone alone (blue line). B. Arithmetic means $\pm$ SEM ($n = 4-8$) of the percentage of ROS positive DCs incubated for 4 hours (left panel) or 24 hours (right panel) without (white bars) or with LPS (1 $\mu\text{g/mL}$) in the absence (black bars) or presence (dark grey bars) of dexamethasone (100 nM) or with dexamethasone alone (light grey bars). * indicate significant difference from untreated (control) group; # indicate significant difference from LPS in the absence of dexamethasone.

Dexamethasone is known to upregulate the expression of the serum and glucocorticoid inducible kinase SGK1, which is in turn known to participate in the regulation of NHE3 activity [34]. SGK1 transcript levels were therefore studied in dexamethasone treated cells and NHE activity was studied in SGK1 knockout DCs. As shown in Fig. 4A, dexamethasone treatment (100 nM, 24 hours) significantly increased SGK1 transcript levels and lack of SGK1 abrogated the stimulation of NHE activity by dexamethasone (Fig. 4B).

NHE activity has previously been shown to be stimulated by exposure of DCs to lipopolysaccharides (LPS) [39]. Thus, an additional series of experiments was performed to study the influence of dexamethasone on the effect of LPS on pH_i and NHE activity. Following treatment with LPS (1 $\mu\text{g/mL}$) alone, the pH changed little within 4 hours but declined markedly following 24 hours (Table 1). Treatment of the DCs with LPS in the presence of dexamethasone (100 nM) led to a significant decrease of the cytosolic pH of DCs within 4 hours. How-

Fig. 7. Effect of LPS and dexamethasone on forward scatter in mouse bone marrow derived dendritic cells (DCs). A. Representative FACS histograms depicting the forward scatter in DCs without treatment (control, black line) and after a 4 hour (left panel) or 24 hour treatment (right panel) with LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of dexamethasone or with dexamethasone alone (blue line). B. Arithmetic means $\pm$ SEM (n = 4-8) of forward scatter in DCs incubated for 4 hours (left panel) or 24 hours (right panel) without (white bars) or with LPS (1 μ g/ml) in the absence (black bars) or presence (dark grey bars) of dexamethasone (100 nM) or with dexamethasone alone (light grey bars). *indicate significant difference from untreated (control) group; #indicate significant difference from LPS in the absence of dexamethasone.

Fig. 8. Effect of mepiprestone on ROS formation and cell volume in mouse bone marrow derived dendritic cells (DCs). A. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in DCs without treatment (control, black line) as well as after a 24 hour treatment with LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of dexamethasone, treatment with LPS in the presence of both dexamethasone and mepiprestone (yellow line) or with dexamethasone alone (blue line) or with dexamethasone and mepiprestone (red line). B. Arithmetic means $\pm$ SEM (n = 4-6) of the percentage of ROS positive DCs incubated for 24 hours in the absence (white bar) or presence of LPS (1 μ g/ml) in the absence (black bar) or presence (dark grey bar) of 100 nM dexamethasone, with LPS in the presence of dexamethasone and 1 μ M mepiprestone (striped bar), in the presence of dexamethasone alone (light grey bars) or in the presence of dexamethasone and mepiprestone (dotted bar). *indicate significant difference from untreated (control) group; #indicate significant difference from LPS in the absence of dexamethasone. C. Representative FACS histograms depicting the forward scatter in DCs without treatment (control, black line) and after a 24 hour treatment with LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of dexamethasone, treatment with LPS in the presence of both dexamethasone and mepiprestone (yellow line) or with dexamethasone alone (blue line) or with dexamethasone and mepiprestone (red line). D. Arithmetic means $\pm$ SEM (n = 4-6) of forward scatter in DCs incubated for 24 hours in the absence (white bars) or presence of LPS (1 μ g/ml) in the absence (black bar) or presence (dark grey bar) of dexamethasone (100 nM), with LPS in the presence of dexamethasone and (1 μ M) mepiprestone (striped bar), in the presence of dexamethasone alone (light grey bars) or in the presence of dexamethasone and mepiprestone (dotted bar). *indicate significant difference from untreated (control) group; # indicate significant difference from LPS in the absence of dexamethasone; § indicate difference from respective absence of mepiprestone.

ever, dexamethasone blunted the further acidification after 24 hours (Table 1). Treatment of DCs with LPS (1 μ g/ml) was followed by a significant increase (4 hours)

and then a significant decrease (24 hours) of Na^+/H^+ exchanger activity (Fig 5). The administration of dexamethasone along with LPS blunted both, the transient stimu-

lation and the delayed inhibition of NHE activity by LPS (Fig. 5).

The stimulation of NHE activity in DCs by LPS was reportedly [39] dependent on the generation of reactive oxygen species (ROS). Accordingly, an additional series of experiments was performed exploring the influence of dexamethasone on ROS formation in the absence and presence of LPS. Dexamethasone administered alone did not modify ROS formation in DCs (Fig. 6). Treatment of DCs with LPS was followed within four hours by a significant increase in the ROS positive cells, an effect significantly blunted in the presence of dexamethasone (Fig. 6).

In a further series of experiments the effect of dexamethasone and/or LPS on cell volume was studied utilizing forward scatter in FACS analysis. Treatment with dexamethasone alone was followed within 4 hours by a slight but significant reduction in cell volume and within 24 hours by a more pronounced cell shrinkage (Fig. 7). The exposure to LPS was followed within 4 and 24 hours by significant cell swelling. The LPS induced cell swelling was abrogated in the presence of dexamethasone.

Additional experiments were performed on the effect of dexamethasone in the presence and absence of the competitive glucocorticoid receptor antagonist mifepristone (1 μ M). As a result, in the presence of mifepristone the effect of dexamethasone on the ROS formation was not abrogated but the effect of dexamethasone on cell volume was blunted (Fig. 8).

Discussion

The present study reveals that glucocorticoids, powerful immunosuppressive hormones, decrease cytosolic pH, decrease cell volume and stimulate the Na^+/H^+ exchanger (NHE) activity in bone marrow derived dendritic cells (DCs). Moreover, dexamethasone blunts both the transient stimulation and the delayed decline of NHE activity by lipopolysaccharides (LPS). Thus, similar to what has been observed in other cell types [17-28], glucocorticoids do regulate Na^+/H^+ exchanger activity in dendritic cells.

The stimulation of NHE activity following dexamethasone treatment may be partially the result of transcriptional upregulation of the carriers. Dexamethasone treatment is followed by a significant increase of the mRNA encoding the NHE isoforms NHE2 and NHE3. Previously, NHE3 has similarly been shown to be genomically upregulated by glucocorticoids in other cell

types [24, 27, 47-49]. However, even after dexamethasone treatment, the transcript levels of NHE2 and NHE3 were still one order of magnitude lower than those of the isoforms NHE1, NHE6 and NHE9, which according to the present study are the predominant isoforms in bone marrow derived murine dendritic cells. Nevertheless, the effect of dexamethasone was not abrogated in the presence of the NHE1 inhibitor cariporide but in the presence of the NHE3 inhibiting dimethylamiloride.

The upregulation of NHE activity by dexamethasone is at least in part due to the observed cytosolic acidification and cell shrinkage, as NHE is sensitive to both cytosolic pH and cell volume [50-52]. The present observations do not allow safe conclusions regarding the cause underlying cytosolic acidification and cell shrinkage. Possibly, dexamethasone stimulates bicarbonate permeable anion channels in DCs, which would lead to cellular acidification due to cellular bicarbonate loss and by the same token to cell shrinkage due to loss of Cl^- and accompanying K^+ . Glucocorticoids may activate several types of anion channels, an effect partially due to upregulation of the serum and glucocorticoid inducible kinase SGK1 [34]. Additional studies will be required to explore whether dexamethasone similarly influences anion channels in DCs.

Dexamethasone did not influence ROS formation in the absence of LPS but virtually abrogated the stimulation of ROS formation by LPS. In human monocytes dexamethasone abrogated the stimulation of NADPH oxidase by LPS, an effect paralleled by and at least partially due to downregulated expression of the NADPH oxidase component p67-phox and gp91-phox [53]. In a previous study, the LPS induced oxidative burst was required for the stimulation of NHE activity by LPS [39]. Conversely, the NHE1 inhibitor cariporide blunted LPS-induced ROS formation [39].

The present study unravels a powerful effect of dexamethasone on cytosolic pH regulation in DCs. Previous observations have shown that several DC functions, such as maturation, antigen presentation, cytokine production and migration, depend on Ca^{2+} -signaling [40, 41, 54-59]. In contrast, little is known about the role of Na^+/H^+ exchanger sensitive pH and cell volume homeostasis in the regulation of DC maturation and function. Whether or not the altered NHE activity under the influence of dexamethasone contributes to the immunosuppressive effect of the hormone, remains to be determined.

The present observations may not only be relevant for the regulation of the immune system by glucocorticoids but similarly important for effects of other

hormones and xenobiotics, such as $1,25(\text{OH})_2\text{D}_3$ [60] and PPARgamma agonists [61].

In conclusion, the present study discloses that the glucocorticoid dexamethasone enhances the activity of the Na^+/H^+ exchanger in bone marrow derived DCs. Further studies are required to elucidate the impact of this effect on DC maturation and its role in the immunosuppressive action of this hormone.

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