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

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Key Words

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

Abstract

Azathioprine is a powerful immunosuppressive drug, which is partially effective by interfering with the maturation and function of dendritic cells (DCs), antigen-presenting cells linking innate and adaptive immunity. DCs are stimulated by bacterial lipopolysaccharides (LPS), which trigger the formation of reactive oxygen species (ROS), paralleled by activation of the Na⁺/H⁺ exchanger. The carrier is involved in the regulation of cytosolic pH, cell volume and migration. The present study explored whether azathioprine influences Na⁺/H⁺ exchanger activity in DCs. DCs were isolated from murine bone marrow, cytosolic pH (pH_i) was estimated utilizing 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) fluorescence, Na⁺/H⁺ exchanger activity from the Na⁺-dependent realkalinization following an ammonium pulse, cell volume from forward scatter in FACS analysis, ROS production from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, TNFα release utilizing ELISA, and

migration utilizing transwell migration assays. Exposure of DCs to lipopolysaccharide (LPS, 1 μg/ml) led to a transient increase of Na⁺/H⁺ exchanger activity, an effect paralleled by ROS formation, increased cell volume, TNFα production and stimulated migration. Azathioprine (10 μM) did not significantly alter the Na⁺/H⁺ exchanger activity, cell volume and ROS formation prior to LPS exposure but significantly blunted the LPS-induced stimulation of Na⁺/H⁺ exchanger activity, ROS formation, cell swelling, TNFα production and cell migration. In conclusion, azathioprine interferes with the activation of dendritic cell Na⁺/H⁺ exchanger by bacterial lipopolysaccharides, an effect likely participating in the anti-inflammatory action of the drug.

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Introduction

Azathioprine is a powerful immunosuppressive drug [1-4], which is particularly useful in the treatment of inflammatory bowel disease [5-18]. Azathioprine is partially effective by triggering lymphocyte apoptosis [19-22]. Beyond that azathioprine has been shown to inter-

fere with the maturation and function of dendritic cells (DCs) [23, 24], antigen-presenting cells involved in the initiation of both innate and adaptive immunity and thus critically important for the regulation of the immune response [25-29]. Azathioprine has been shown to impair maturation of DCs and to induce a functionally less immunogenic and more tolerogenic phenotype [23, 24].

Following exposure to bacterial wall components such as lipopolysaccharides (LPS), DCs generate reactive oxygen species (ROS) [30-32], which contributes to the killing of pathogens [33]. As previously shown for other cell types [34] including macrophages [35], ROS production is paralleled by generation of H^+ , which acidifies the cytosol and thus influences the activity of the ROS-generating NADPH oxidase [36, 37]. In a wide variety of cells [35, 38, 39] including macrophages [40-47] and monocytes [46, 48, 49] cytosolic pH is regulated by the Na^+/H^+ exchanger, a carrier extruding H^+ in exchange of Na^+ . Stimulation of DCs with LPS has recently been shown to activate the Na^+/H^+ exchanger [50, 51].

Similar to most cells [52, 53] activation of the Na^+/H^+ exchanger increases macrophage [35, 43] and dendritic cell [50, 51] volume. Moreover, the Na^+/H^+ exchanger is known to participate in the machinery of cell migration [51, 54-56].

The present study explored the influence of azathioprine on Na^+/H^+ exchanger activity, ROS production, cell volume, TNF α production and cell migration in murine bone marrow derived DCs.

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) [57, 58]. 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 phosphate buffered saline (PBS) [59]. Cells were then washed twice with Roswell Park Memorial Institute medium (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% fetal calf serum (FCS), 1% penicillin (final concentration 100 Units/ml)/streptomycin (final concentration 100 Units/ml) solution,

1% glutamine solution (final concentration 2 mM), 1% non-essential amino acids (NEAA) solution (final concentration 100 μ M) 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, $\approx 80\%$ of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed at days 7-9 of DCs culture [60].

Immunostaining and flow cytometry

Cells (4×10^5) were incubated in 100 μ l FACS (fluorescence activated cell sorter) buffer (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 fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11c (BD Pharmingen, Heidelberg, Germany) was used as a positive marker for dendritic cells [61]. 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 (FACS-calibur from Becton Dickinson; Heidelberg, Germany).

Treatments

Stock solutions of LPS were prepared in culture medium whereas azathioprine was dissolved in DMSO and cariporide was 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. Sham experiments were conducted by adding plain dimethyl sulfoxide (DMSO) to the respective solutions at a final concentration of 0.1%. Azathioprine was used at a final concentration of 10 μ M based on the previously effective saturating concentrations in other cell types [62, 63].

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 azathioprine). After the treatment, cells were collected, centrifuged, the pellet was resuspended in FACS buffer and analysed with flow cytometry.

Determination of ROS production

ROS production in DCs was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) [64]. Briefly, 2×10^5 cells were taken in a culture dish and treated with LPS (with or without azathioprine). 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. 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 the measurement of cytosolic pH (pH_i), the cells were

incubated in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffered Ringer solution containing 10 μM 2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluoresceinacetoxymethylester (BCECF-AM, Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40x oil immersion objective (Zeiss Neoplan, Germany). BCECF-AM 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. Fluorescence intensity ratio (490/440) data were converted into pH_i values using the high- K^+ /nigericin calibration technique [65]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high- K^+ /nigericin (10 $\mu\text{g}/\text{ml}$) solution (pH 7.0). The fluorescence intensity ratio data thus obtained were converted into pH values using the r_{max} , r_{min} , and pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [66]. The acidification of cytosolic pH upon removal of NH_4Cl allowed calculation of the mean intrinsic buffering power (β) of the cells [66]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3 :

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

where ΔpH_i is the decrease of cytosolic pH (pH_i) following NH_4Cl removal and $\Delta[\text{NH}_4^+]$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[\text{NH}_4^+]_i$ immediately before the removal of NH_4Cl . The pK for $\text{NH}_4^+/\text{NH}_3$ is 8.9 [67] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[\text{NH}_4^+]_o$) is 19.37 mM $[20/(1+10^{\text{pH}_o-\text{pK}})]$. The intracellular NH_4^+ concentration ($[\text{NH}_4^+]_i$) was calculated from:

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

The intracellular pH measured prior to the ammonium pulse manoeuvre was taken as the basal intracellular pH and was used for further analysis. To calculate the $\Delta\text{pH}/\text{min}$ during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9), which could be applied to all measured cells.

The standard Hepes buffered solution was composed of: NaCl (115 mM), KCl (5 mM), CaCl_2 (1 mM), MgSO_4 (1.2 mM), NaH_2PO_4 (2 mM), glucose (10 mM), Hepes (32.2 mM). The sodium free Hepes buffered solution was composed of: N-methyl-D-glucamine (NMDG, 132.8 mM), KCl (3 mM), CaCl_2 (1 mM), MgSO_4 (1.2 mM), KH_2PO_4 (2 mM), Hepes (32.2 mM), mannitol (10 mM), glucose (10 mM). For sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl . The high K^+ solution for calibration was com-

posed of: KCl (105 mM), CaCl_2 (1 mM), MgSO_4 (1.2 mM), Hepes (32.2 mM), mannitol (10 mM) and nigericin (5 μM). 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 (BD Biosciences 354480) were used with a pore diameter size of 8 μm . The transwells were placed in a 24-well cell culture plate containing cell culture medium (750 μl) with or without chemokine ligand 21 (CCL21, 250 ng/ml, Peprotech) in the lower chamber. The upper chambers were filled with 500 μl cell culture medium containing DCs at a concentration of 50,000 cells/ml. The chamber was placed in a 5% CO_2 37°C incubator for 4 hours. In the following, the non-migrated cells were removed by scrubbing with a cotton-tipped swab for two times and washing with PBS. The membrane was removed with a scalpel and fixed in 4% paraformaldehyde (PFA) for 15 mins. The migrated cells were then identified by staining with 4',6-diamidino-2-phenylindole (DAPI).

TNF α measurement

TNF α concentrations in DC culture supernatants were determined by using OptEIA ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. Differences were tested for significance using either Student's t-test with Welch's correction or ANOVA (Dunnett's test) where applicable. $P < 0.05$ was considered statistically significant.

Results

BCECF-AM fluorescence was employed for the determination of cytosolic pH in murine bone marrow-derived dendritic cells (DCs). To estimate Na^+/H^+ exchanger activity, the cells were acidified by an ammonium pulse. To this end NH_4Cl was added to the perfusate leading to NH_3 entry into the cells. The binding of H^+ with formation of NH_4^+ was followed by a transient cytosolic alkalinization (Fig. 1, step II). The subsequent removal of NH_4Cl resulted in exit of NH_3 paralleled by an increase of intracellular H^+ concentration, and thus cytosolic acidification (Fig. 1, step III). In the absence of Na^+ , the cytosolic pH declined further after an ammonium pulse. Thus, the cells did not express sufficient Na^+ -independent H^+ extrusion mechanisms to maintain or recover cytosolic pH (Table 1). The addition of Na^+ , however, was followed by a rapid pH recovery pointing to the operation of a Na^+/H^+ exchanger. Exposure to azathioprine (10 μM) did not significantly modify the Na^+/H^+

Fig. 1. Cytosolic pH and Na^+/H^+ exchanger activity in mouse bone marrow-derived dendritic cells (DCs) under control conditions and following LPS treatment in the absence and presence of azathioprine. A to D. Representative experiments demonstrating time-dependent 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^+ replaced by NMDG (II), NH_4Cl removed in a next step (III), Na^+ added in a fourth step (IV) and nigericin applied in a fifth step (V) to calibrate each individual experiment. The pH changes were recorded in control DCs (A) or DCs treated for 4 hours with azathioprine alone (10 μM , B), with LPS alone (1 $\mu\text{g}/\text{ml}$, C) or with LPS and azathioprine (D). E. Arithmetic means $\pm$ SEM ($n = 3$ independent experiments) of pH_i in DCs (pH_i) prior to (0 h, Control, white bar) as well as 4 hours after exposure to LPS (1 $\mu\text{g}/\text{ml}$) in the absence (black bar) and presence (dark grey bar) of azathioprine (10 μM) or after exposure to azathioprine alone (light grey bar). F. Arithmetic means $\pm$ SEM ($n = 3$ independent experiments) of Na^+ -dependent pH_i recovery in DCs ($\Delta\text{pH}/\text{min}$) following an ammonium pulse prior to (0 h, Control, white bar) as well as 4 hours after exposure to LPS (1 $\mu\text{g}/\text{ml}$) in the absence (black bar) and presence (dark grey bar) of azathioprine (10 μM) or after exposure to azathioprine alone (light grey bar). # indicates significant difference from respective absence of azathioprine, * indicates significant difference from respective absence of LPS.

exchanger activity (Fig. 1B, step IV, and Fig. 1F) and did not significantly modify the intracellular pH (Fig. 1E) in untreated DCs. Treatment with either LPS or azathioprine had no significant influence on cellular buffer capacity (Table 1).

Treatment of the DCs with 1 $\mu\text{g}/\text{ml}$ lipopolysaccharides (LPS) had, within 4 hours, little influence on cytosolic pH in the absence of azathioprine (Fig. 1E). In contrast, LPS treatment was followed by a significant

	BC mM/ ΔpH_i	0 Na^+ ΔpH units/min	Number of cells
control	15.1 $\pm$ 1.4	-0.08 $\pm$ 0.01	88
AZA (4 hr)	16.5 $\pm$ 1.8	-0.06 $\pm$ 0.01	68
LPS (4 hrs)	14.3 $\pm$ 1.1	-0.06 $\pm$ 0.01	104
LPS + AZA (4 hrs)	13.3 $\pm$ 1.1	-0.06 $\pm$ 0.01	193

Table 1. Buffer capacity (BC, mM/ ΔpH_i) and Na^+ -independent (0 Na^+) pH recovery (ΔpH units/min) following an ammonium pulse in bone marrow-derived dendritic cells (DCs) prior to (control) and following a 4 hours (4 h) treatment with lipopolysaccharide LPS (1 $\mu\text{g}/\text{ml}$) or azathioprine AZA (10 μM) alone (LPS, AZA) or combined (LPS+AZA). pH_i and ΔpH units/min in presence of Na^+ are displayed in Fig. 1.

Fig. 2. Effect of LPS and azathioprine 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 hours treatment with LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of azathioprine (10 μ M) or after a 4 hours treatment with azathioprine (10 μ M) alone (blue line). B. Arithmetic means $\pm$ SEM (n = 5 independent experiments) of the percentage of ROS positive DCs incubated for 4 hours without (white bar) or with LPS (1 μ g/ml) in the absence (black bar) or presence (dark grey bar) of azathioprine (10 μ M) or after exposure to azathioprine alone (light grey bar). * (p<0.05) indicate significant difference from untreated (control) group; # (p<0.05) indicate significant difference from LPS in the absence of azathioprine.

Fig. 3. Effect of LPS and azathioprine 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) as well as after a 4 hours treatment with LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of azathioprine (10 μ M) or after a 4 hours treatment with azathioprine (10 μ M) alone (blue line). B. Arithmetic means $\pm$ SEM (n = 5 independent experiments) of forward scatter in DCs incubated for 4 hours without (white bar) or with LPS (1 μ g/ml) in the absence (black bar) or presence (dark grey bar) of azathioprine (10 μ M) or after exposure to azathioprine alone (light grey bar). * (p<0.05) indicate significant difference from untreated (control) group; # (p<0.05) indicate significant difference from LPS in the absence of azathioprine.

decrease of the cytosolic pH of DCs in the presence of 10 μ M azathioprine (Fig. 1E). Similar to what has been shown earlier [50], treatment of DCs with LPS (1 μ g/ml, 4 hours) resulted in a significant increase of Na^+/H^+ exchanger activity (Fig 1C, step IV, and Fig. 1F). In the presence of azathioprine the stimulation of Na^+/H^+ exchanger activity by LPS was significantly blunted (Fig. 1D, step IV, and Fig. 1F).

An additional series of experiments was performed to explore whether azathioprine influences the ROS formation following LPS treatment. To this end the effect of LPS on ROS production was tested in the absence

and presence of azathioprine. As shown in Fig. 2, exposure of DCs to LPS resulted in a significant increase of ROS positive cells within four hours, an effect significantly blunted in the presence of azathioprine. Without stimulation with LPS, the administration of azathioprine had no effect on ROS production (Fig. 2).

An additional series of experiments was performed to elucidate the effect of azathioprine on LPS-induced cell swelling. Cell volume was estimated from forward scatter in FACS analysis. The exposure to LPS was followed by significant cell swelling within 4 hours, an effect partially reversed by the addition of azathioprine (Fig.

Fig. 4. Effect of azathioprine on formation of TNF α in mouse DCs. Arithmetic means $\pm$ SEM (n = 4-6 independent experiments) of TNF α concentration in the supernatant of DCs cultured for 4 hours without (control, white bar) or with LPS (1 μ g/ml) in the absence (LPS, black bar) or presence of 10 μ M azathioprine (LPS+AZA, dark grey bar) or 10 μ M cariporide (LPS+cariporide, striped bars), or cultured for 4 hours with azathioprine alone (AZA, light grey bar). * (p<0.05) indicate significant difference from untreated (control) group, # (p<0.05) significant difference to LPS alone.

Fig. 5. Migration of DCs in the absence and presence of azathioprine. Arithmetic means $\pm$ SEM (n = 4-7 independent experiments) of the normalized migration of DCs following a 4 hours treatment without (control, white bar) or with LPS in the absence (LPS, black bar) or the presence of 10 μ M azathioprine (LPS+AZA, dark grey bar) or following a 4 hours treatment with azathioprine alone (AZA, light grey bar). * (p<0.05) indicate significant difference from untreated (control) group, # (p<0.05) indicates significant difference to LPS alone.

3). Similar to what was observed for ROS production, azathioprine did not significantly modify cell volume in the absence of LPS (Fig. 3).

To explore, whether azathioprine modified cytokine release from DCs, TNF α in the supernatant was determined with ELISA. To this end DCs were stimulated with LPS (1 μ g/ml, 4 h) in either the absence or presence of azathioprine (10 μ M). As illustrated in Fig. 4, in the absence of LPS, secretion of TNF α from DCs was negligible. LPS exposure stimulated the release of TNF α , an effect significantly blunted in the presence of azathioprine. Similar to azathioprine, the Na⁺/H⁺ exchanger inhibitor cariporide (10 μ M) [68] significantly decreased the TNF α production. Thus, LPS induced TNF α production was critically dependent on the activity of the Na⁺/H⁺ exchanger.

An important phenotypic change that occurs in DCs upon maturation is upregulated expression of chemokine receptor CCR7, which allows the interaction of DCs with the chemokines CCL19/MIP3 β and CCL21/6CKine [69, 70]. Under the influence of chemokines, DCs migrate towards lymph nodes and activate the respective lymphocytes. CCL21-induced migration is thus a characteristic feature of mature DCs. As migration is similarly dependent on Na⁺/H⁺ exchanger activity, [51, 54-56], further studies were performed to elucidate the effect of azathioprine on LPS-induced DCs migration. To

this end, a transwell migration assay was performed in DCs treated with LPS in the presence and absence of azathioprine. As shown in Fig. 5, treatment of DCs with LPS led to a significant increase in the percentage of migrated cells, an effect abrogated in the presence of azathioprine. Thus, azathioprine inhibited migration of LPS-treated DCs. In the absence of LPS, azathioprine tended to increase the percentage of migrated cells, an effect, however, not reaching statistical significance (Fig. 5).

Discussion

The present study demonstrates that azathioprine counteracts the stimulation of the Na⁺/H⁺ exchanger, increase of cell volume and triggering of Reactive Oxygen Species (ROS) formation following exposure of dendritic cells to lipopolysaccharides (LPS). The increase in the Na⁺/H⁺ exchanger activity was estimated from Na⁺ dependent realkalinization, which has previously been shown to be virtually abolished in the presence of the NHE1 inhibitor cariporide [50].

As shown earlier [50], stimulation of Na⁺/H⁺ exchanger activity is a prerequisite for LPS induced ROS formation, which is in turn required for the killing of pathogens and thus of pivotal importance for the innate

immune response [33]. ROS production is also sensitive to pH. The activity of the oxidase decreases pH_i that, in turn, inhibits the activity of the oxidase itself [36, 37]. Thus, ROS formation requires extrusion of H⁺, which is accomplished by activation of the Na⁺/H⁺ exchanger [50]. The Na⁺/H⁺ exchanger activity is in turn activated by cytosolic acidification [71] and according to the present observations stimulation of Na⁺/H⁺ exchanger activity by LPS is paralleled by ROS formation. The inhibitory effect of azathioprine on the LPS-induced activation of the Na⁺/H⁺ exchanger presumably contributes to or even accounts for its inhibitory effect on ROS formation.

Activation of the Na⁺/H⁺ exchanger further results in cell swelling. During regulatory cell volume increase the Na⁺/H⁺ exchanger is activated together with the Cl⁻/HCO₃⁻ exchanger [52, 53]. Collectively, the two carriers accomplish entry of NaCl together with osmotically obliged water. The H⁺ and HCO₃⁻ exiting in exchange for NaCl are replenished in the cell by cytosolic formation from CO₂, which can easily pass the cell membrane [52, 53]. The inhibitory effect of azathioprine on LPS-induced cell swelling presumably results from the inhibition of Na⁺/H⁺ exchanger activity. Cell volume influences both formation of ROS and antioxidative defence [72-75]. Cell shrinkage interferes with generation of ROS and the cell swelling following exposure to LPS is thus expected to support ROS formation.

Further functions sensitive to cytosolic pH include migration, cytokine release, adherence, NO formation, proliferation and differentiation of macrophages and/or monocytes [45, 46, 76-85]. We show here that TNFα release is inhibited by the Na⁺/H⁺ exchanger inhibitor cariporide. Accordingly, the inhibition of Na⁺/H⁺ exchanger activity by azathioprine presumably contributes to or even accounts for its inhibitory effect on TNFα release and migration.

Sustained exposure to LPS leads to downregulation of the Na⁺/H⁺ exchanger, which leads to cytosolic acidification. As Na⁺/H⁺ exchanger activity is a function of cell volume, the delayed inhibition of the Na⁺/H⁺ exchanger could result from cell swelling [52, 53]. The cells

remain swollen, however [50], a finding possibly reflecting upregulation of the cell volume regulatory set point, as has been observed following cytosolic acidification [71] or stimulation of cell proliferation [86].

LPS treatment has previously been shown to decrease apoptosis of DCs, an effect abrogated by inhibition of the Na⁺/H⁺ exchanger [50]. As shown in other cell types, activation of Na⁺/H⁺ exchanger activity may enhance [87-90] or inhibit [91-93] apoptosis. The inhibitory effect of Na⁺/H⁺ exchanger activity on apoptosis may result from cytosolic alkalization [91, 92] or from prevention of cell shrinkage [93]. It is tempting to speculate that inhibition of Na⁺/H⁺ exchanger activity could contribute to the stimulation of lymphocyte apoptosis by azathioprine [19-22].

In vivo, azathioprine is converted into several effective metabolites [69, 94-96]. This conversion is unlikely to occur during *in vitro* experiments. The present observations thus reveal effects of azathioprine itself rather than effects of its metabolites.

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, generation of ROS, TNFα release and migration. Azathioprine blunts the effect of LPS on the Na⁺/H⁺ exchanger, cell volume, generation of ROS, TNFα release and migration. Thus, negative regulation of the Na⁺/H⁺ exchanger may play a part in the influence of azathioprine on dendritic cell function.

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