Rapamycin Sensitive ROS Formation and Na⁺/H⁺ Exchanger Activity in Dendritic Cells

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Key Words

mTOR • Cell volume • Reactive oxygen species • TNF- α • LPS

Abstract

Rapamycin, a widely used immunosuppressive drug, has been shown to interfere with the function of dendritic cells (DCs), antigen-presenting cells contributing to the initiation of primary immune responses and the establishment of immunological memory. DC function is governed by the Na⁺/H⁺ exchanger (NHE), which is activated by bacterial lipopolysaccharides (LPS) and is required for LPS-induced cell swelling, reactive oxygen species (ROS) production and TNF- α release. The present study explored, whether rapamycin influences NHE activity and/or ROS formation in DCs. Mouse DCs were treated with LPS in the absence and presence of rapamycin (100 nM). ROS production was determined from 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence, cytosolic pH (pH_i) from 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence, NHE activity from the Na⁺-dependent realkalinization following an ammonium pulse, cell volume from forward scatter in FACS

analysis, and TNF- α production utilizing ELISA. In the absence of LPS, rapamycin did not significantly modify cytosolic pH, NHE activity or cell volume but significantly decreased ROS formation. LPS stimulated NHE activity, enhanced forward scatter, increased ROS formation, and triggered TNF- α release, effects all blunted in the presence of rapamycin. NADPH oxidase inhibitor Vas-2870 (10 μ M) mimicked the effect of rapamycin on LPS induced stimulation of NHE activity and TNF- α release. The effect of rapamycin on TNF- α release was also mimicked by the antioxidant ROS scavenger Tempol (30 μ M) and partially reversed by additional application of tert-butylhydroperoxide (10 μ M). In conclusion, in DCs rapamycin disrupts LPS induced ROS formation with subsequent inhibition of NHE activity, cell swelling and TNF- α release.

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Introduction

Rapamycin, a widely used immunosuppressive [1-5] and antineoplastic [6-14] drug inhibits the mammalian

target of rapamycin, mTOR [15], a serine/threonine kinase stimulated by nutrients [16, 17], growth factors [18], insulin [19] and leptin [20]. Signaling activating mTOR includes Akt and the extracellular-regulated kinase [21]. mTOR sensitive cell functions include cell proliferation [18] and cell death [22]. mTOR plays a decisive role in the innate immune system and the production of proinflammatory cytokines such as IL-12 and IL-1 β , anti-inflammatory cytokine IL-10, and type I interferon [23]. mTOR participates in the regulation of MHC antigen presentation as well as expression of chemokine receptors and costimulatory molecules [23].

The immunosuppressive effect of rapamycin results in part from inhibition of T-cell proliferation, induction of anergy, modulation of T-cell trafficking, promotion of regulatory T cells and immunostimulation of memory CD8⁺ T-cell differentiation [24-26]. Rapamycin exerts its effects on the immune system further by influencing the function of dendritic cells (DCs) [4, 23, 25-27]. DCs are antigen-presenting cells required for the development of T cell immunity, for the initiation of primary immune responses and for the establishment of immunological memory [28, 29]. The maturation of DCs is governed by Toll-like receptors (TLR), which are activated by bacterial lipopolysaccharides (LPS) and in turn trigger the production of proinflammatory chemokines and cytokines [30-32]. Rapamycin inhibits the maturation of dendritic cells, thus promoting immunologic tolerance [4, 24, 27, 33]. Rapamycin further influences the formation of cytokines such as interferon- α and may, paradoxically improve antigen presentation [24, 34].

According to recent observations, several functions of DCs critically depend on Na⁺/H⁺ exchanger activity [35-37]. The Na⁺/H⁺ exchanger is specifically required for LPS induced dendritic cell swelling and formation of reactive oxygen species (ROS) [36, 37]. ROS in turn triggers TNF- α release [38, 39]. Little is known about an effect of rapamycin on the Na⁺/H⁺ exchanger. In the medullary thick ascending limb, the inhibition of basolateral Na⁺/H⁺ exchanger activity by nerve growth factor (NGF) was blunted rapamycin, pointing to an inhibitory effect of mTOR on the carrier [40].

The present study explored, whether the effect of rapamycin on dendritic cells involves an influence of rapamycin on Na⁺/H⁺ exchanger activity. Bone marrow derived mouse DCs have thus been treated with LPS in the absence and presence of rapamycin and cytosolic pH, Na⁺/H⁺ exchanger activity, cell volume, ROS as well as TNF- α release were determined.

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 C57/BL-6 mice (Charles River, Sulzfeld, Germany) [41, 42]. 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 [43]. 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 bovine serum (FBS), 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, Peprotech 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.

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 4×10^4 cells were analyzed. Staining with FITC-conjugated anti-mouse CD11c (BD Pharmingen, Heidelberg, Germany) was used as a positive marker for dendritic cells [44]. Dendritic cells were further identified by the increase in percentage of CD86 (PE-conjugated anti-mouse CD86, BD Pharmingen, Heidelberg, Germany) and MHCII (FITC-conjugated anti-mouse MHCII, BD Pharmingen, Heidelberg, Germany) after treatment with LPS for 24 hours [44]. After incubating with the respective antibodies 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 (Lipopolysaccharides from *Escherichia Coli* 0111: B4, Sigma, Schnellendorf, Germany) were prepared in culture medium, Vas-2870 (Enzo Life Sciences, Lörrach, Germany) and rapamycin (Tocris Bioscience, Bristol, United Kingdom) were dissolved in DMSO whereas tempol and tert-butylhydroperoxide were diluted 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 % CO₂ atmosphere. Sham experiments were conducted by adding plain DMSO to the respective solutions at a final concentration of 0.1 %. Rapamycin was used at a final concentration of 100 nM [45].

Determination of cell volume

Cell volume was determined by the forward scatter in flow cytometric analysis. Briefly, 4×10^5 cells/ml were taken in a

culture dish and treated with LPS (with or without rapamycin). 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) [46]. Briefly, 4×10^5 /ml cells were taken in a culture dish and treated with LPS (with or without rapamycin). After the treatment, cells were collected and DCFDA (Sigma, Schnelldorf, 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. The percentage of ROS producing cells was calculated by counting only those cells, which produced high levels of ROS.

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)-carboxyfluoresceinacetoxymethylester (BCECF-AM Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Between 10-20 cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH_i values using the high- K^+ /nigericin calibration technique [47]. 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 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^+ [48]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [48]. Assuming that NH_4^+ and NH_3 are in equilibrium between 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 cytosolic pH (pH_i) follow-

ing 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 [49] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 mM $[20/(1+10^{pH_o-pK})]$. The intracellular NH_4^+ concentration ($[NH_4^+]_i$) was calculated from:

$$[NH_4^+]_i = 19.37 \cdot 10^{pH_o-pH_i} \text{ mM}$$

To calculate the $\Delta pH/\text{min}$ 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 solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 , 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4 , 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 5 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.

TNF- α Measurements

TNF- α concentration in DC culture supernatants was 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 ANOVA. $P < 0.05$ was considered statistically significant.

Results

To determine cytosolic pH (pH_i) in bone marrow derived mouse dendritic cells (DCs), BCECF fluorescence was employed. Na^+/H^+ exchanger activity was determined utilizing the ammonium pulse technique. Addition of NH_4Cl to the perfusate resulted in transient cytosolic alkalinization due to NH_3 entry into the cells and subsequent binding of cytosolic H^+ with formation of NH_4^+ (Fig. 1A). Subsequent removal of NH_4Cl resulted in cytosolic acidification due to exit of NH_3 leaving cytosolic H^+ behind (Fig. 1A). The pH_i remained acidic in the absence of Na^+ indicating that the cells did not express Na^+ independent H^+ extrusion mechanisms (Table 1). Addition of extracellular Na^+ was followed by rapid pH recovery revealing Na^+/H^+ exchanger activity (Fig. 1A). Prior to application of lipopolysaccharides (LPS), rapamycin (10 μ M) did not significantly modify DC Na^+/H^+ exchanger activity or pH_i (Fig. 1A).

Treatment of the DCs with 1 μ g/ml LPS was previously shown to stimulate the Na^+/H^+ exchanger activity in a time dependent manner [36, 37]. A maximal effect

Fig. 1. Cytosolic pH and Na⁺/H⁺ exchanger activity in mouse bone marrow derived dendritic cells (DCs) following LPS treatment in the absence and presence of rapamycin. **A.** Representative experiments showing 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 NH₄Cl was added and Na⁺ removed (replaced by NMDG; '0' Na⁺) in a first step (see bars below each tracing), NH₄Cl removed in a second step (NH₄⁺), Na⁺ added in a third step and nigericin (pH_o '7') applied in a fourth step to calibrate each individual experiment. The pH changes were recorded prior to (upper left panel) and 4 hours after exposure to rapamycin alone (100 nM, upper right panel) or to LPS (1 μg/ml) in the absence (lower left panel) and presence (lower right panel) of rapamycin. **B.** Arithmetic means ± SEM (n = 4 independent experiments) of pH_i in DCs (pH_i) prior to (white bar) as well as 4 hours after exposure to LPS (1 μg/ml) in the absence (black bar) and presence (dark grey bar) of rapamycin (100 nM) as well as after exposure to rapamycin (100 nM) alone (light grey bar). **C.** Arithmetic means ± SEM (n = 4 independent experiments) of Na⁺-dependent pH_i recovery in DCs (ΔpH/min) following an ammonium pulse prior to (white bar) as well as 4 hours after exposure to LPS (1 μg/ml) in the absence (black bar) and presence (dark grey bar) of rapamycin (100 nM) and after exposure to rapamycin (100 nM) alone (light grey bar). * (p<0.01) indicates significant difference from respective absence of LPS, # (p<0.01) indicates significant difference from respective absence of rapamycin.

of LPS was seen after 4-hours. Thus this time point was used for the present study. The administration of rapamycin (100 nM) along with LPS virtually abrogated the stimulation of Na⁺/H⁺ exchanger activity by LPS (Fig. 1). Treatment of the DCs with 1 μg/ml LPS had little effect on pH_i in the absence of rapamycin, indicating that cytosolic H⁺ generation was balanced by stimulation of Na⁺/H⁺ exchanger activity. Along those lines, LPS treatment was followed by a significant cytosolic acidification in the presence of 100 nM rapamycin (Fig 1, Table 1).

To explore whether rapamycin influenced the generation of reactive oxygen species (ROS), 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence was determined prior to and following LPS treatment in the absence and presence of rapamycin. As illustrated in Fig. 2, treatment of DCs with LPS resulted within four hours in significant ROS formation, an effect significantly blunted in the presence of rapamycin. Rapamycin, when administered alone, slightly, but significantly reduced intracellular ROS in DCs (Fig. 2). To explore whether LPS induced stimulation of Na⁺/H⁺ exchanger activity depended on ROS formation, further ex-

	pH _i	BC mM/ΔpH _i	0 Na ⁺ Δ pH units/min	+ Na ⁺ Δ pH units/min	Number of cells
control	7.33 ± 0.02	14.4 ± 1.2	-0.08 ± 0.01	0.119 ± 0.012	112
Rapamycin	7.34 ± 0.02	15.4 ± 1.6	-0.06 ± 0.01	0.153 ± 0.022	70
LPS	7.32 ± 0.02	14.3 ± 1.1	-0.06 ± 0.01	0.220 ± 0.015 ^a	96
LPS + Rapamycin	7.23 ± 0.01 ^b	14.5 ± 0.9	-0.05 ± 0.01	0.150 ± 0.014 ^b	141

Table 1. Cytosolic pH (pH_i), buffer capacity (BC, mM/ΔpH_i), Na⁺-independent (0 Na⁺) and Na⁺-dependent (+Na⁺) pH recovery (ΔpH units/min) following an ammonium pulse in bone marrow dendritic cells (DCs) prior to (control) and following a 4 hours treatment with lipopolysaccharide LPS (1 μg/ml) or rapamycin (100 nM) in isolation or combination. ^aindicates significant difference to respective control value, ^bindicates significant difference to respective value in the presence of LPS alone

periments were carried out in the presence of NADPHoxidase inhibitor Vas-2870 (Vas, 10 μM). As shown in Fig. 3, Vas-2870 treatment completely abrogated the effect of LPS, which was mimicked by rapamycin treatment. Vas-2870 (10 μM) alone did not significantly modify Na⁺/H⁺ exchanger activity and there was no additional effect when rapamycin and Vas-2870 were administered together.

Fig. 2. Effect of LPS and rapamycin 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-hr treatment LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of rapamycin (100 nM) or in the presence of rapamycin alone (blue line). B. Arithmetic means $\pm$ SEM (n = 6 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 rapamycin (100 nM) or after exposure to rapamycin (100 nM) alone (light grey bar). *(p<0.05) indicate significant difference from respective control; # (p<0.05) indicates significant difference from respective absence of rapamycin.

Fig. 3. Effect of NADPHoxidase inhibitor Vas-2870 (10 μ M) on the LPS induced stimulation of Na^+/H^+ exchanger activity. Arithmetic means $\pm$ SEM (n = 4 independent experiments) of Na^+ -dependent pH_i recovery in DCs (Δ pH/min) following an ammonium pulse prior to (control) as well as 4 hours after exposure to LPS (1 μ g/ml) alone (LPS) or Vas-2870 (Vas, 10 μ M) alone or with LPS and Vas-2870 (LPS+Vas) or with LPS and rapamycin (LPS+Rapa) or with LPS and rapamycin and Vas-2870 (LPS+Vas+Rapa). *(p<0.01) indicates significant difference from control, # (p<0.01) indicates significant difference from LPS.

Fig. 4. Effect of LPS and rapamycin 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 hour treatment with LPS (1 μ g/ml) in the absence (green line) and presence (pink line) of rapamycin (100 nM) or in the presence of rapamycin alone (blue line). B. Arithmetic means $\pm$ SEM (n = 6 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 rapamycin (100 nM) as well as after exposure to rapamycin (10 μ M) alone (light grey bar). *(p<0.05) indicate significant difference from respective control; # (p<0.05) indicates significant difference from respective absence of rapamycin.

Fig. 5. Effect of rapamycin on LPS-induced $\text{TNF-}\alpha$ release in mouse DCs. Arithmetic means $\pm$ SEM (n = 4 independent experiments) of $\text{TNF-}\alpha$ concentration in the supernatant of DCs cultured for 4 h with LPS (1 μ g/ml) in the absence (LPS) or presence of 100 nM rapamycin (LPS+Rapa). # (p<0.01) indicate significant difference to absence of rapamycin.

To possibly disclose an effect of rapamycin and LPS on cell volume, forward scatter was determined in FACS analysis. As illustrated in Fig. 4, LPS treatment was within 4 hours followed by a significant increase of forward scatter, an effect abrogated in the presence of rapamycin. The application of rapamycin (100 nM) without LPS treatment did not significantly modify the forward scatter of DCs.

Fig. 6. Effect of ROS on LPS-induced TNF- α release in mouse DCs. Arithmetic means $\pm$ SEM ($n = 4-6$ independent experiments) of TNF- α concentration in the supernatant of DCs treated for 4 h with LPS (1 μ g/ml) alone (LPS) or treated with LPS in the presence of ROS scavenger tempol (30 μ M, LPS+Tempol), in the presence of NADPH oxidase inhibitor Vas-2870 (10 μ M, LPS+Vas), in the presence of rapamycin (100 nM, LPS+Rapa) or in the presence of rapamycin and t-butyl hydroperoxide (TBOOH, 10 μ M, LPS+Rapa+TBOOH). #($p < 0.01$) indicate significant difference to LPS alone, *indicates significant difference ($p < 0.05$) from respective absence of TBOOH.

A further series of experiments addressed the effect of rapamycin on LPS induced TNF- α release. To this end TNF- α formation was determined utilizing ELISA. Stimulation of DCs with LPS (1 μ g/ml, 4 h) triggered TNF- α release, an effect blunted in the presence of 100 nM rapamycin (Fig. 5). In order to determine, whether the effect of rapamycin on TNF- α release was secondary to its influence on ROS formation, DCs were stimulated with LPS (1 μ g/ml, 4 h) in the presence and absence of the ROS scavenger tempol (30 μ M), in the presence of NADPH oxidase inhibitor Vas-2870 (10 μ M) alone, in the presence of rapamycin (100 nM) alone, in the presence of rapamycin and Vas-2870 or in presence of rapamycin and the oxidant t-butyl hydroperoxide (TBOOH, 10 μ M). As a result, tempol, Vas-2870 and rapamycin blunted the effect of LPS on TNF- α release and TBOOH partially restored the TNF- α release even in the presence of 100 nM rapamycin (Fig. 6). Thus, inhibition of TNF- α release by rapamycin is at least partially mediated through its effect on intracellular ROS formation.

Discussion

According to the present observations, in murine bone marrow derived dendritic cells (DCs) rapamycin blunts or virtually abrogates the stimulation of Na $^+$ /H $^+$ exchanger (NHE) activity, ROS formation, cell swelling and TNF- α release by lipopolysaccharides (LPS). Thus, rapamycin

interferes with the stimulation of DCs by LPS.

As shown previously [36], the LPS induced stimulation of the Na $^+$ /H $^+$ exchanger activity was critically dependent on the production of reactive oxygen species (ROS). Conversely, ROS production is sensitive to cytosolic pH [50, 51] and thus requires parallel extrusion of H $^+$. Accordingly, the ROS generation in DCs is blunted in the presence of the NHE1 inhibitor cariporide [36]. The Na $^+$ /H $^+$ exchanger activity is stimulated by cytosolic acidification [52] and thus serves to maintain cytosolic pH during H $^+$ generation in parallel to ROS formation. Thus, stimulation of the Na $^+$ /H $^+$ exchanger activity and oxidative burst appear to depend on each other. The present study further demonstrates that TNF- α release is similarly dependent on ROS formation. Accordingly, the inhibitory effect of rapamycin on Na $^+$ /H $^+$ exchanger activity and TNF- α release could be secondary to its inhibitory effect on ROS formation. As a matter of fact, the effect of rapamycin was mimicked by antioxidant tempol and partially reversed by the oxidant t-butylhydroperoxide. As inhibition of the Na $^+$ /H $^+$ exchanger activity with cariporide interferes with ROS formation [36], rapamycin could be similarly be effective by interfering more directly with Na $^+$ /H $^+$ exchanger activity.

ROS production is required for the killing of pathogens [53]. As rapamycin interferes with ROS formation, it is expected to compromise the antibacterial activity of DCs. Activation of the Na $^+$ /H $^+$ exchanger further results in cell swelling. Parallel activation of the Na $^+$ /H $^+$ exchanger and the Cl $^-$ /HCO $_3^-$ exchanger contributes to regulatory cell volume increase in a wide variety of cells [54, 55]. The two carriers accomplish the entry of NaCl together with osmotically obliged water. H $^+$ and HCO $_3^-$ exchanged for NaCl are not relevant for cytosolic osmolarity as they are replenished by cytosolic formation from CO $_2$, which readily crosses the cell membrane [54, 55]. LPS-induced cell swelling is abolished following inhibition of the Na $^+$ /H $^+$ exchanger with cariporide [36], i.e. activation of the carrier accounts for LPS induced cell swelling.

The influence of rapamycin on Na $^+$ /H $^+$ exchanger activity and ROS formation may not only be relevant in the regulation of DC function but presumably contributes to further effects of the drug. Rapamycin and/or inhibition of mTOR has been shown to interfere with cell proliferation and differentiation [56], glucose and amino acid transport [48, 57], protein synthesis [58], muscle regeneration [59] and hypertrophy [60]. Rapamycin further favourably influences polycystic kidney disease [61, 62]. Rapamycin counteracts ageing and extends the

life span [63-65]. Those effects could well involve altered ROS formation and Na⁺/H⁺ exchanger activity. ROS play a critical role in PI3K/Akt/mTOR signaling [66] and stimulation of Na⁺/H⁺ exchanger induced cell swelling is a powerful anabolic signal fostering protein synthesis [55].

In conclusion, rapamycin inhibits LPS- induced stimulation of ROS formation and Na⁺/H⁺ activity, which in turn interferes with the effect of LPS on cell volume and TNF- α release. The observed effects of rapamycin

most likely contribute to the known anti-inflammatory action of the widely used immunosuppressive drug.

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