Ca²⁺-dependent Functions in Peptidoglycan-stimulated Mouse Dendritic Cells

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Key Words

K⁺ channels • Kv1 • Store-operated Ca²⁺ channels • Margatoxin • Perhexiline maleate • SKF-96365

Abstract

Peptidoglycans (PGN) from bacterial cell walls may modify the course of an infection with bacterial pathogens. The present study explored the effect of PGN on cytosolic Ca²⁺ activity, cytokine production and phagocytosis of mouse dendritic cells (DCs), essential cells in the initiation and direction of antigen-specific T cell responses. Exposure of DCs to PGN was followed by a rapid increase in cytosolic Ca²⁺ activity ([Ca²⁺]_i), which was due to Ca²⁺ release from intracellular stores and influx of extracellular Ca²⁺ across the cell membrane. In DCs isolated from Toll-like receptor 2 (TLR2) deficient mice the effect of PGN on [Ca²⁺]_i was dramatically impaired. The PGN-induced increase of [Ca²⁺]_i was dependent on voltage-gated K⁺ (Kv) channel activity. PGN-induced increase of [Ca²⁺]_i was significantly blunted by margatoxin (MgTx) and perhexiline maleate (PM), inhibitors of Kv1.3 and Kv1.5, respectively. PGN further stimulated the release of tumour necrosis factor α (TNF α), interleukin-12 (IL-12) and interleukin-10 (IL-10), an effect sig-

nificantly blunted by PM and the specific blocker of store-operated Ca²⁺ channels SKF-96365. Moreover, phagocytic capacity was dramatically increased in PGN-stimulated DCs in the presence of either Kv channel inhibitors or SKF-96365. The observations disclose Ca²⁺ and Kv channel-dependent cytokine production and phagocytosis in PGN-stimulated DCs.

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Introduction

Bacterial peptidoglycans (PGN) are essential components of the bacterial cell wall [1]. They are recognized by host cells [2, 3] and trigger the “Pathogen-Associated Molecular Patterns” (PAMP) [4]. They bind to “Pattern Recognition Receptors” (PRRs) of the host cell and trigger a signaling cascade eventually leading to altered release of pro- and anti-inflammatory mediators [5–11]. Several PGN binding PRRs have been identified: Toll-like receptor 2 (TLR2) as the major PGN receptor, PGN-recognition proteins (GNBP1 und PGRP-SA), and the cytoplasmic Nod1, Nod2 [12, 13]. Involvement of TLR2 in recognition of PGN has become recently a matter of debate. Bacterial PGN was postulated to activate

cells through TLR2, an observation first made in 1999 [14] and then confirmed using TLR2-knockout mice [15]. However, a recent study challenged this established view, indicating that PGN sensing through TLR2 is lost after removal of lipoproteins or lipoteichoic acid (LTA) from cell walls [16], a finding, however, which could not be confirmed by another group [7].

Cells stimulated by PGN include dendritic cells (DCs) [17-19], which are key players in the initiation and direction of antigen-specific T cell responses [20]. DC function is regulated by cytosolic Ca^{2+} -concentration ($[\text{Ca}^{2+}]_i$) [21, 22]. Specifically, alterations of $[\text{Ca}^{2+}]_i$ regulate the production of critically important cytokines, such as interleukin-12 (IL-12), interferon γ (IFN- γ), and interleukin-2 (IL-2) [23]. Moreover, alterations of $[\text{Ca}^{2+}]_i$ determine DC survival [24].

The mechanism of Ca^{2+} entry into DCs includes Ca^{2+} release activated Ca^{2+} (CRAC) channels [25], an ubiquitous signaling mechanism in non-excitabile and excitable cells that is triggered by a reduction in the level of Ca^{2+} in intracellular stores [26]. In DCs activation of CRAC channels was demonstrated upon store depletion induced by dialyzing the cytosol with inositol 1,4,5-trisphosphate (InsP_3) and with high concentrations of the Ca^{2+} chelator BAPTA [25]. Ca^{2+} entry into DCs is enhanced by membrane hyperpolarization [25]. It thus depends on the activity of voltage-gated K^+ channels of the Kv1 channel family [27, 28], which maintain the electrochemical driving force for Ca^{2+} influx [29].

The present study explored the influence of PGN on Ca^{2+} activity and Ca^{2+} -dependent functions in DCs. We show that PGN exposure leads to a transient increase in $[\text{Ca}^{2+}]_i$, an effect impaired by pharmacological inhibition of store-operated channels or by removal of extracellular Ca^{2+} . Moreover, pharmacological inhibition of Kv channels significantly blunts PGN-induced Ca^{2+} entry. PGN-stimulated release of TNF α , IL-12 and IL-10 was significantly decreased by pharmacological inhibition of store-operated Ca^{2+} channels or Kv channels. Finally, we disclosed that inhibition of these channels dramatically up-regulated the phagocytic capacity of DCs.

Materials and Methods

Cell Culture

Dendritic cells (DCs) were isolated from bone marrow of 7-10 weeks old female NMRI mice (Charles River, Sulzfeld, Germany). NMRI mice are widely used in the analysis of dendritic cell function [30-32]. Alternatively, DCs were isolated from

TLR2-deficient mice of C57BL/16 background.

Dendritic cells (DCs) were cultured from bone marrow as previously described [33], with slight modifications. Briefly, bone marrow cells were flushed out of the cavities from the femur and tibia of mice 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% 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. Nonadherent and loosely adherent cells were harvested after 6 days of culture. At day 7, >80% of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed on mature DCs at days 7-9.

Measurement of intracellular Ca^{2+}

For intracellular Ca^{2+} measurements, DCs were either left untreated or were pretreated with the store-operated channel inhibitor SKF-96365 (10 μM , 30 min; Sigma-Aldrich, Munich, Germany), the Kv1.3 inhibitor margatoxin (MgTx, 1 nM, 30 min; Alomone Labs, Jerusalem, Israel), the Kv1.5 inhibitor perhexiline maleate (PM, Sigma-Aldrich, 10 μM , 30 min) or a combination of MgTx and PM. Subsequently, the cells were loaded with Fura-2/AM (2 μM , Molecular Probes, Goettingen, Germany) for another 30 min.

The experiments were performed at 37°C. Intracellular Ca^{2+} was measured prior to and following addition of PGN (from *Staphylococcus aureus*, Sigma-Aldrich) in the absence or presence of extracellular Ca^{2+} . When the cells were pretreated with channel inhibitors, the same inhibitor(s) were added to the bath solutions. Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 CaCl_2 , 2 Na_2HPO_4 , 32 Hepes, 5 glucose, pH 7.4. Nominally Ca^{2+} -free solutions contained: 125 NaCl, 5 KCl, 1.2 MgSO_4 , 2 Na_2HPO_4 , 32 Hepes, 0.5 EGTA, 5 glucose, pH 7.4. For calibration ionomycin (10 μM , Sigma, Germany) was applied at the end of each experiment.

Fluorescence measurements were carried out with an inverted phase-contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany) [34]. Cells were excited alternatively at 340 or 380 nm and the light was deflected by a dichroic mirror into the objective (Fluar 40 $\times$ 1.30 oil, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm and data acquisition was accomplished by using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). The corresponding ratios (F_{340}/F_{380}) were used to obtain intracellular Ca^{2+} concentrations. The following equation was used: $[\text{Ca}^{2+}]_{\text{free}} = K_d \times ((R - R_{\text{min}})/(R_{\text{max}} - R)) \times S_f$ (K_d = dissociation constant of Fura-2; R = ratio of emission intensity at 340 nm excitation, to emission intensity at 380 nm excitation; R_{min} = ratio at zero free Ca^{2+} ; R_{max} = ratio at saturating Ca^{2+} ; S_f = instrumental constant). As a measure for the increase of cytosolic Ca^{2+} activity, the slope and peak of the changes in the intracellular Ca^{2+} concentration were calculated for each experiment.

Fig. 1. PGN exposure increases $[Ca^{2+}]_i$ in DCs. **A.** Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded control and SKF-96365 (10 μ M)-pretreated DCs prior to and following acute addition of peptidoglycan (PGN, 25 μ g/ml; white arrow). At the end of each experiment ionomycin (10 μ M) was added for calibration purposes. For quantification of Ca^{2+} entry peak (ΔnM) and slope ($\Delta nM/s$) of $[Ca^{2+}]_i$ increase following addition of PGN were calculated. **B.** Representative original tracing showing intracellular Ca^{2+} concentrations prior to and following acute addition of PGN (25 μ g/ml) in the absence of extracellular Ca^{2+} . To yield a Ca^{2+} -free environment, EGTA (0.5 mM) was added to the Ca^{2+} -free bath solution. **C.** Mean ($\pm$ SEM) of the peak value (above) and slope (below) of the change in intracellular Ca^{2+} concentrations following addition of PGN (25 μ g/ml) to the bath solution in the absence ($n = 8$, open bars) and presence ($n = 8$, closed bars) of SKF-96365. * ($p < 0.05$) indicate significant difference between treated and untreated cells (two-tailed unpaired t -test). **D.** Concentration dependence of the effect of PGN on intracellular Ca^{2+} as calculated from the peak value (left) and slope (right) of the change in intracellular Ca^{2+} concentrations following addition of PGN to the bath solution ($n = 6-11$). **E.** Pseudocolor images of Fura-2 loaded DCs. Left panels: pseudocolor images at 340nm excitation. Central panels: pseudocolor images of the same cells at 380 nm excitation. Right panels: pseudocolor images resulting from the ratio of both excitation wavelengths of the respective cells. Upper panels were recorded before addition of 25 μ g/ml PGN. Lower panels were recorded after addition of PGN and subsequent influx of extracellular Ca^{2+} . White arrows indicate example cells that show a shift from dark blue (low intracellular Ca^{2+}) to red (high intracellular Ca^{2+}) through addition of PGN.

Patch clamp

Patch clamp experiments were performed at room temperature in voltage-clamp, fast-whole-cell mode according to Hamill et al. [35]. The cells were continuously superfused through a flow system inserted into the dish. The bath was grounded via a bridge filled with NaCl Ringer solution. Borosilicate glass pipettes (1-3 MOhm tip resistance; GC150 TF-7.5, Harvard Apparatus, March-Hugstetten, Germany) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, N.Y., USA). The whole-cell currents were elicited by 200 ms square wave voltage pulses from -90 to +90 mV in 20 mV steps delivered at 20 sec intervals from a holding potential of -70 mV. As shown earlier [28], the currents recovered within 20 sec from their use-dependent inactivation. The currents were recorded with an acquisition frequency of 10 kHz and 3 kHz low-pass filtered.

The cells were superfused with a bath solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 20 glucose and 10 HEPES/NaOH, pH 7.4. The patch clamp pipettes were filled with an internal solution containing (in mM): 80 KCl, 60 K^+ -gluconate, 1 $MgCl_2$, 1 Mg -ATP, 1 EGTA, 10 HEPES/KOH, pH 7.2.

Where indicated a combination of margatoxin (MgTx, 1 nM, Alomone Labs, Jerusalem, Israel) and perhexiline maleate (PM, 5 μ M, Sigma), was added to the bath solution.

Cytokine Measurement

TNF α , IL-12 and IL-10 concentrations in DC culture supernatants were determined by using OptEIA ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

DC phagocytosis assay

Immature or PGN-stimulated DCs (10^6 cells/ml) were suspended in prewarmed serum-free RPMI 1640 medium, pulsed with either, FITC-conjugated dextran (1 mg/ml, Sigma Aldrich) or PE-labelled Latex beads (10 μ l/ml, Sigma Aldrich) and incu-

Fig. 2. Effect of PGN depends on TLR2. A. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded DCs from wild type and $\text{TLR2}^{-/-}$ mice prior to and following acute addition of peptidoglycan (PGN, 25 $\mu\text{g}/\text{ml}$; white arrow). B. Mean ($\pm$ SEM) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} concentrations following addition of PGN (25 $\mu\text{g}/\text{ml}$) to the bath solution to wild type DCs ($n = 8$, open bars) and $\text{TLR2}^{-/-}$ DCs ($n = 13$, closed bars). *** ($p < 0.001$) indicate significant difference between two genotypes (two-tailed unpaired t -test).

bated for 4h at 37°C. Uptake of FITC-conjugated dextran and PE-labelled Latex beads were stopped by adding ice-cold PBS. The cells were then washed three times with ice cold PBS supplemented with 5% FCS and 0.01% sodium azide before FACS analysis.

Immunoblotting

DCs (2×10^6 cells) either unstimulated or pulsed with PGN (60 min) untreated or treated (60 min) with either SKF-96365 (10 μM) or MgTx (1 nM) + PM (5 μM) were snap-frozen in dry-ice ethanol bath. Cell pellets were thawed on ice and washed twice in PBS, then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Samples were stored at -80°C until use for western blotting. Cell lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat-milk in triethanolamine-buffered saline (TBS) and 0.1% Tween-20. Then the blots were probed overnight with antiphospho-I κ B α (Santa Cruz Biotechnology, Heidelberg, Germany) and anti- α/β tubulin (Cell Signaling/New England Biolabs, Frankfurt am Main, Germany) antibodies diluted in 5% milk in PBS and 0.1% Tween-20, washed 5 times, probed with secondary antibodies (sc-2060) conjugated with horseradish peroxidase for 1 h at room temperature, washed final 5 times. Antibody binding was detected with the enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany).

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. Differences were tested for significance using Student's unpaired two-tailed t -test or ANOVA. $P < 0.05$ was considered statistically significant.

Results

As illustrated in Fig. 1, stimulation of DCs with peptidoglycan in a wide range of concentrations (PGN)

resulted in a rapid increase of $[\text{Ca}^{2+}]_i$ (Fig. 1A,D,E). When PGN was applied in the absence of extracellular Ca^{2+} (Fig. 1B) or in the presence of SKF-96365 (10 μM , Fig. 1A,C), a blocker of store-operated Ca^{2+} channels, the increase of $[\text{Ca}^{2+}]_i$ was strongly reduced, but not fully abrogated, indicating that PGN activated both Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} .

The effect of PGN strongly depended on the presence of TLR2. In DCs isolated from TLR2 -deficient mice increase in $[\text{Ca}^{2+}]_i$ was dramatically attenuated (Fig. 2).

DCs have previously been shown to express voltage-gated K^+ channels belonging to the Shaker (Kv1) family, presumably Kv1.3 and Kv1.5 [27, 28]. Accordingly, in patch-clamp experiments, DC currents were determined in the presence and absence of the Kv1.3 channel inhibitor margatoxin (MgTx, 1 nM) and the Kv1.5 channel inhibitor perhexiline maleate (PM, 5 μM). As shown in Fig. 3A, the Kv-like currents were indeed inhibited in the presence of the two blockers. Neither acute application of PGN nor incubation with PGN modified Kv currents in DCs. The current density at +90 mV was 22.1 ± 2.9 pA/pF ($n = 13$) in control cells, and was not significantly changed after acute application of PGN: 22.4 ± 1.5 pA/pF ($n = 5$) or after a 24 h incubation with 25 $\mu\text{g}/\text{ml}$ PGN: 22.8 ± 3.0 pA/pF ($n = 16$). Additional experiments have been performed to test, whether MgTx and PM can modulate the Ca^{2+} entry through store-operated channels. As evident from Fura-2 imaging, MgTx and PM indeed significantly blunted the PGN-induced increase in $[\text{Ca}^{2+}]_i$ (Fig. 3B-G). The effect was particularly pronounced following administration of the two blockers in combination.

Fig. 3. Blocking of Kv channels attenuates the increase in $[Ca^{2+}]_i$ upon PGN stimulation of DCs. A. Original Kv current traces recorded at +90 mV with KCl/K-gluconate pipette solution before and after application of PM (5 μ M) and then MgTx (1 nM). B., D., F. Representative original tracings showing intracellular Ca^{2+} concentrations in Fura-2/AM loaded control DCs and DCs pretreated with MgTx (1 nM) + PM (10 μ M) (B) or MgTx (D) or PM (F) prior to and following acute addition of PGN (25 μ g/ml). C., E., G. Mean ($\pm$ SEM, $n = 6-8$) of the peak value (left) and slope (right) of the change in intracellular Ca^{2+} concentrations for control cells (open bars) and DCs pretreated with MgTx+PM (C), MgTx (E) and PM (G) following addition of PGN to the bath solution. * ($p < 0.05$) and ** ($p < 0.01$) indicate significant difference between two groups (two-tailed unpaired t -test).

To test whether Kv channels and store-operated Ca^{2+} entry, controlled by Kv channel activity, could influence DC functions, production of cytokines was assessed in the presence or in the absence of channel blockers. Bone marrow-derived mouse DCs were shown to produce IL-10, TNF α , and low levels of IL-12 upon stimulation with PGN [36]. TNF α , IL-12 and IL-10 release was analyzed in DCs, which were stimulated for either 4 h (for TNF α production) or 48 h (for IL-12 and IL-10 production) with PGN (25 μ g/ml) in the presence or absence of SKF-96365 (10 μ M) or MgTx (1 nM) or PM (10 μ M) or both MgTx + PM. As shown in Fig. 4, treatment with SKF-96365, PM or MgTx+PM blunted the TNF α , IL-12 and IL-10 release from PGN-stimulated DCs. MgTx alone was without effect suggesting that Kv1.5 rather than Kv1.3 channels are critical regulators of cytokine production in PGN-stimulated DCs.

Innate immune function of DCs involves antigen

uptake. We tested whether channel blockers could affect the capacity of DCs to phagocytose antigen. The uptake of FITC-dextran and latex beads into PGN-stimulated DCs was dramatically enhanced by SKF-96365 or MgTx or PM or MgTx+PM in PGN-stimulated DCs (Fig. 5). Immature cells have higher phagocytic activity. Thus, in unstimulated DCs FITC-dextran uptake was $61.7 \pm 0.7\%$ and latex bead uptake was $42.7 \pm 1.0\%$. None of the channel blockers enhanced the antigen uptake in immature DCs.

We finally addressed the question whether Ca^{2+} or K^+ channel blockers could affect regulation of NF κ B, since this transcriptional factor is crucial for cytokine production in DCs [37]. Phosphorylation of inhibitory molecule I κ B was measured prior to and following activation of DCs with PGN (25 μ g/ml, 60 min) in the presence and in the absence of SKF-96365 or MgTx+PM (Fig. 6). The level of phosphorylated I κ B α was increased by PGN

Fig. 4. Ca^{2+} channel blocker SKF-96365 and Kv channel blocker PM impair TNF- α , IL-12 and IL-10 production by PGN-stimulated DCs. DCs were incubated with or without SKF-96365 (10 μM), MgTx (1 nM), PM (5 μM) or MgTx+PM for 30 min before the stimulation with PGN (25 $\mu\text{g/ml}$). After 4 or 48 h supernatants were collected to measure TNF- α (A) or IL-12 (B) and IL-10 (C), respectively, by ELISA. The results are representative for 4-7 independent experiments. Each experiment was performed in duplicate; values represent the mean $\pm$ SEM of duplicates, ** ($p < 0.01$) and *** ($p < 0.001$) show differences from untreated (control) group, ANOVA.

stimulation, but was not significantly modified by any of the channel blockers tested.

Discussion

The present study reveals that exposure of bone marrow derived dendritic cells (DCs) to peptidoglycan

Fig. 5. SKF-96365, margatoxin and PM enhance phagocytic capacity of PGN-stimulated DCs. Bar diagram representing mean percent ($\pm$ SEM; $n=3-6$) of FITC-dextran (A) or latex bead (B) uptake by PGN (25 mg/ml , 48 h)-stimulated DCs incubated in the absence (control, open bar) or in the presence of either SKF-96365 (10 μM), MgTx (1 nM), PM (5 μM) or MgTx+PM. ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant difference from control (ANOVA).

(PGN) leads to a rapid transient increase in cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$). The peak and slope of PGN induced $[\text{Ca}^{2+}]_i$ increase is significantly blunted in the absence of extracellular Ca^{2+} or in the presence of SKF-96365, a blocker of store-operated calcium channels (I_{CRAC}) [26]. Those observations disclose that PGN-induced increase in $[\text{Ca}^{2+}]_i$ is in part due to activation of Ca^{2+} influx through Ca^{2+} channels in the plasma membrane and in part due to Ca^{2+} release from intracellular stores. These results are in a good agreement with the data obtained recently showing phospholipase C γ -dependent mobilization of Ca^{2+} in bone marrow-derived macrophages and DCs in response to PGN [38]. The effect is only transient, i.e. cytosolic Ca^{2+} is subsequently extruded by uptake into the intracellular stores and extrusion across the cell

Fig. 6. SKF-96365, margatoxin and PM do not affect I κ B α phosphorylation in DCs. Original Western blot of unstimulated (control) and PGN (25 μ g/ml, 60 min)-stimulated DCs untreated or treated (60 min) with either SKF-96365 (10 μ M) or MgTx (1 nM) + PM (5 μ M). Protein extracts were analyzed by direct Western blotting using antibodies directed to phosphorylated I κ B α . Protein loading was controlled by anti- α / β tubulin antibody. One representative experiment out of three is shown.

membrane. We cannot rule out subsequent localized oscillatory increases of Ca²⁺, which may escape detection by determination of bulk cytosolic Ca²⁺ utilizing fluorescence optics but may account for long-lasting effects of Ca²⁺ signaling, such as cell proliferation [39] and maturation [40, 41]. Those Ca²⁺ oscillations have been observed in a multitude of cell types and regulate a wide variety of functions [42–72].

Similar to other immune cells, DCs use store-operated Ca²⁺ entry as the main mode of Ca²⁺ influx [25]. The canonical transient receptor potential (TRPC) channels have also been reported to increase intracellular Ca²⁺ concentrations through coupled plasma membrane receptor stimulation in lymphocytes [69]. Nothing is known, however, about functions of TRPC channels in DCs. Recently Ca²⁺-activated nonselective cation channel TRPM4 was shown to be expressed in DCs where it controls chemokine-dependent DC migration [73]. This channel allows entry of Na⁺, thereby inducing membrane depolarization and decreasing the driving force for Ca²⁺ entry [73]. The TRPM4 channel is also expressed and controls Ca²⁺ homeostasis in T cells [74] and mast cells [75].

The effect of PGN on [Ca²⁺]_i increase is dramatically reduced in TLR2^{-/-} DCs, demonstrating that TLR2 is one of the main receptors for PGN in DCs.

The present observations further reveal that PGN-induced increase in [Ca²⁺]_i depends on Kv channel activity. The channels maintain the potential difference across the cell membrane and thus the electrical driving force for the entry of positively charged Ca²⁺. The effect of the inhibitors reveals that Kv channels in DCs indeed drive the increase of [Ca²⁺]_i upon PGN stimulation. Expression of Kv1.3 and Kv1.5 channels has recently been shown in DCs derived from human blood [27] and mouse bone marrow [28].

We further show that PGN-induced [Ca²⁺]_i increase

in DCs is important for subsequent TNF α , IL-12 and IL-10 production. PGN-mediated Ca²⁺ flux has already been suggested to upregulate cytokine production in DCs and macrophages [38]. The present study provides unequivocal evidence that inhibition of store-operated Ca²⁺ entry impairs TNF α , IL-12 and IL-10 secretion. Moreover, TNF α , IL-12 and IL-10 production is impaired by Kv channel blockers. Furthermore, we demonstrate that phagocytic uptake in DCs depends on channel activity. Interestingly, Ca²⁺ and Kv channel blockers enhanced antigen uptake only in PGN-stimulated cells, which upon PGN-induced maturation lost part of their phagocytic activity. In immature cells that are efficient in antigen uptake the channel blockers did not influence phagocytosis. Ca²⁺ entry is required for the stimulation of DC maturation [21, 22, 76] and maturation leads in turn to decrease of phagocytosis [77, 78].

At the sites of pathogen entry DCs “sample” their environment by phagocytosis, initiating specific immune responses, when they recognize microbes or tissue damage [77]. The efficient uptake of pathogens is thus essential for generation of immunity against an infectious agent. Ca²⁺ and Kv channels thus tune the effective clearance of pathogens during infection.

We further demonstrate that blocking of Ca²⁺ or Kv channels in DCs has no effect on the I κ B α phosphorylation, which is in turn known to regulate the transcription factor NF- κ B. Calcium may up- or downregulate NF- κ B, depending on the cell type [30–32, 79–84].

There are several important questions still to be solved. In contrast to lymphocytes, where the role of store-operated Ca²⁺ entry is well established, the role of those channels in DCs remains ill-defined. In lymphocytes store-operated Ca²⁺ entry upregulates nuclear factor of activated T-cells (NFAT) [85]. The importance of this upregulation is illustrated by patients with a rare form of

hereditary severe combined immunodeficiency [85]. T cells from these patients show impaired formation of multiple cytokines and a selective inability to activate NFAT resulting from a pronounced reduction in I_{CRAC} [85]. In calcium ionophore-treated HL-60 cells, which can under certain conditions differentiate to monocyte/macrophage-like cells, antagonists of the NFAT-activating protein phosphatase calcineurin have been shown to attenuate expression of costimulatory molecules [22]. NFAT activation in DCs was demonstrated upon stimulation of Dectin-1, a C-type lectin, by zymosan [86]. Dectin-1, similar to the T cell receptor (TCR) and the B cell receptor (BCR), has an immunoreceptor tyrosine-based activation motif (ITAM) in its intracellular tail and thus, it seems likely, that NFAT activation in DCs occurs by similar mechanisms to those used in lymphocytes. NFAT activation was shown to regulate IL-2, IL-10 and IL-12 p70, but not TNF α production in zymosan-stimulated DCs [86]. Whether other pattern recognition receptors can activate NFAT in DCs and whether NFAT is important for modulating PGN-stimulated responses remains to be determined.

In conclusion, exposure of DCs to PGN is followed by a rapid increase in $[Ca^{2+}]$, accomplished by both, Ca^{2+} release from intracellular stores and entry of extracellular Ca^{2+} through the store-operated channels. The Ca^{2+} entry requires the cooperation with Kv channels. Accordingly, inhibition of store-operated Ca^{2+} or Kv channels blunts the PGN induced TNF α , IL-12 and IL-10 production and enhances phagocytosis in DCs. The present findings add to an increasing body of evidence that Ca^{2+} -mediated signal transduction pathways serve a central regulatory role in DC responses to diverse antigens, including TLR ligands, intact bacteria, and microbial toxins.

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