Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

CpG-ODN-mediated TLR9 innate immune signalling and calcium dyshomeostasis converge on the NFκB inhibitory protein IκBβ to drive IL1α and IL1β expression

Robyn De Dios,[1] Leanna Nguyen,[1] Sankar Ghosh,[2] Sarah McKenna[1] and Clyde J. Wright[1]

[1]*Section of Neonatology, Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO, and* [2]*Department of Microbiology & Immunology, College of Physicians and Surgeons, Columbia University, New York, NY, USA*

doi:10.1111/imm.13182
Received 23 December 2019; revised 31 January 2020; accepted 11 February 2020.
Correspondence: Dr Clyde J. Wright, Associate Professor, Section of Neonatology, Department of Pediatrics, Children's Hospital Colorado and University of Colorado School of Medicine, Perinatal Research Center, 13243 East 23rd Avenue, Mail Stop F441 Aurora, CO 80045, USA.
Email: clyde.wright@cuanschutz.edu
Senior author: Dr. Clyde J. Wright

Summary

Sterile inflammation contributes to many pathological states associated with mitochondrial injury. Mitochondrial injury disrupts calcium homeostasis and results in the release of CpG-rich mitochondrial DNA. The role of CpG-stimulated TLR9 innate immune signalling and sterile inflammation is well studied; however, how calcium dyshomeostasis affects this signalling is unknown. Therefore, we interrogated the relationship between intracellular calcium and CpG-induced TLR9 signalling in murine macrophages. We found that CpG-ODN-induced NFκB-dependent IL1α and IL1β expression was significantly attenuated by both calcium chelation and calcineurin inhibition, a finding mediated by inhibition of degradation of the NFκB inhibitory protein IκBβ. In contrast, calcium ionophore exposure increased CpG-induced IκBβ degradation and IL1α and IL1β expression. These results demonstrate that through its effect on IκBβ degradation, increased intracellular Ca^{2+} drives a pro-inflammatory TLR9-mediated innate immune response. These results have implications for the study of innate immune signalling downstream of mitochondrial stress and injury.

Keywords: cytokines; inflammation; monocytes/macrophages; rodent; transcription factors; transgenic/knockout mice.

Introduction

It is increasingly recognized that sterile inflammation underlies many disease states.[1,2] Central to the pathogenesis of sterile inflammation is the innate immune response to non-microbial activators. Cellular injury downstream of trauma, ischaemia/reperfusion or toxin exposure releases organelle components into the cytosol and intracellular contents into the surrounding extracellular space. Some of these released molecules contain damage-associated molecular patterns (DAMPS), which serve as ligands for both intracellular and cell surface pattern recognition receptors (PRRs). Unchecked activation can lead to a pro-inflammatory state and subsequent immune pathology, many of which are macrophage-derived interleukin (IL)1 dependent.[1,2]

When sequestered in their native intracellular location, DAMP-containing components represent no danger to the host. Several mitochondrial components are well-established DAMPs and can trigger the innate immune response and sterile inflammation.[3,4] Mitochondrial DNA is rich in unmethylated CpG motifs and is a recognized ligand for endosomal toll-like receptor (TLR)9.[5] Mitochondrial DNA-TLR9 activation signals exclusively through the adaptor protein myeloid differentiation primary response 88 (MyD88) leading to pro-inflammatory

Abbreviations: BMDM, bone marrow-derived macrophages; CpG, 5′—C—phosphate—G—3′; CpG-ODN, CpG-oligodeoxynucleotides; DAMPs, damage-associated molecular patterns; IL1, interleukin-1; IL1α, interleukin 1 alpha; IL1β, interleukin 1 beta; IκBα, NF-kappa-B inhibitor alpha; IκBβ, NF-kappa-B inhibitor beta; IκBε, NF-kappa-B inhibitor epsilon; mRNA, messenger ribonucleic acid; MyD88, myeloid differentiation primary response 88; NE-PER, nuclear and cytoplasmic extraction reagent; NFκB, nuclear factor kappa-light-chain-enhancer of activated b-cells; PRRs, pattern recognition receptors; TLR4, toll-like receptor 4; TLR9, toll-like receptor 9; T-PER, tissue protein extraction reagent

nucelar factor kappa-light-chain-enhancer of activated b-cells (NFκB) activation.[3,4,6–9] Thus, any cellular stress causing mitochondrial injury can result in innate immune signalling and sterile inflammation.

Exogenous CpG-oligodeoxynucleotides (ODN) stimulate TLR9 mediate innate immune signalling, and have been used to study the pathogenesis of sterile inflammation. CpG-ODN has been used to study atherosclerosis,[10–14] ischaemia-reperfusion,[11,15–19] infection/sepsis,[20–37] and various autoimmune diseases.[38–50] Importantly, mitochondrial stress and injury has also been implicated in the pathogenesis of atherosclerosis,[51] ischaemia-reperfusion,[52,53] infection,[54,55] and various autoimmune diseases.[56] However, mitochondrial stress and injury leads to other pathological changes that impact innate immune signalling beyond CpG-rich DNA release.[57–60] Previous reports have shown that cytosolic calcium levels dictate the degree and duration of TLR4-mediated innate immune signalling,[61–67] in part due to its effect on NFκB signalling.[62,68–71] The NFκB inhibitory protein IκBβ is particularly susceptible to alternations in intracellular calcium, as calcium-mediated activation of calcineurin is a key step in IκBβ inactivation and subsequent NFκB activation.[72,73] The degradation kinetics of IκBα and IκBβ dictates the magnitude and duration of NFκB target gene expression,[74–77] which directly affects IL1α and IL1β expression.[78–81] Previous reports have demonstrated that exposing astrocytes[33] and pulmonary endothelial cells[82] to CpG-ODN results in IκBα degradation, while IκBβ was not evaluated. Exposing WEHI-2312 B lymphoma cells to CpG-ODN results in degradation of both IκBα and IκBβ,[83] but whether this occurs in non-cancerous cells is unknown. While it is well known that mitochondrial stress can disrupt intracellular calcium homeostasis,[84–86] how this affects CpG-ODN TLR9-NFκB signalling is unknown.

Therefore, we sought to interrogate how altering intracellular calcium affected CpG-induced TLR9-mediated innate immune signalling in macrophages. We found that in RAW 264.7 macrophages, CpG-ODN induced IκBβ degradation and subsequent NFκB activation. This resulted in increased expression of the MyD88-dependent primary response genes IL1α and IL1β, which was inhibited by pharmacological NFκB blockade. Both calcium chelation (EGTA-AM) and calcineurin inhibition (FK-506) attenuated IκBβ degradation, NFκB activation, and IL1α and IL1β expression. In contrast, exposure to a calcium ionophore (A23187) increased CpG-induced IκBβ degradation, and IL1α and IL1β expression. Finally, using bone marrow-derived macrophages (BMDMs) lacking or overexpressing IκBβ, we demonstrated that this key inhibitory protein is both necessary and sufficient for CpG-ODN-induced IL1 expression. These results demonstrate that through its effect on IκBβ degradation, increased intracellular Ca^{2+} drives a pro-inflammatory TLR9-mediated innate immune response. These results have implications for the study of innate immune signalling downstream of mitochondrial stress and injury, and reveal potential therapeutic targets to attenuate inflammation associated with TLR9 activation.

Materials and methods

Cell culture

RAW 264.7 macrophages (ATCC) were cultured in Dulbecco's modified Eagle's media (DMEM 1 ×) containing 4·5 g/l D-glucose, L-glutamine and sodium pyruvate. Media was supplemented with 10% fetal bovine serum, 2% HEPES, 1% GlutaMAX and 1% penicillin/streptomycin. Cells were seeded 2 days prior to exposures. Seeding densities were as follows: 2×10^6 cells/10-cm dish for cytosolic and nuclear extracts, and 450 000 cells/well on a six-well plate for whole-cell lysates and mRNA isolations. BMDMs were collected from 10–12-week-old male B6 and IκBβ$^{-/-}$ mice (kind gift of Dr Sankar Ghosh), and were cultured for 7 days as previously described before usage in experiments.[87]

CpG exposures and pharmacological NFκB inhibition

RAW 264.7 cells were exposed to CpG-ODN (0·3–3 μM, 0–24 hr; InvivoGen tlrl-1668, San Diego, CA, USA). To pharmacologically inhibit NFκB activity, cells were exposed to Bay 11-7085 (1–10 μM for 1 hr; Sigma-Aldrich B5681, St. Louis, MO) prior to CPG exposure. BMDMs derived from B6 and IκBβ$^{-/-}$ mice were exposed to CPG-ODN (0·3 μM; InvivoGen tlrl-1668).

Calcium imaging, calcium chelation, calcineurin inhibition and calcium ionophore exposures

To interrogate the role of intracellular calcium on CPG-induced NFκB signalling, RAW 264.7 cells were pre-treated with Fluo-4, AM (5 μM for 30 min; ThermoFisher F14201, Waltham, MA), EGTA/AM (250 μM for 30 min; Santa Cruz Biotechnology sc-203937, Dallas, TX), or FK-506 (10 μM for 24 hr; Selleckchem S5003, Houston, TX) before being exposed to CpG DNA. RAW 264.7 cells were co-treated with calcium ionophore A23187 (10 μM; Sigma-Aldrich C7522). BMDMs were co-treated with calcium ionophore A23187 (0·5 μM; Sigma-Aldrich C7522).

Fluorescent imaging of the calcium probe was captured using a standard FITC filter, and the Olympus IX83 microscope and Olympus DP80 camera using Olympus CellSens software.

Protein isolation and Western blot analysis

Whole-cell lysates were collected by scraping cells off culture vessels using tissue protein extraction reagent (T-

PER; ThermoFisher Scientific), while cytosolic and nuclear extracts were collected using the nuclear and cytoplasmic extraction reagent (NE-PER) kit (ThermoFisher Scientific). Whole-cell, cytosolic and nuclear extracts were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen, Waltham, MA), and proteins were transferred to an Immoblin-FL membrane (Millipore, Burlington, MA). The membrane was then probed with antibodies against IκBα (Cell Signaling 4814, Beverly, MA), IκBβ (Invitrogen PA1-32136), Calnexin (Enzo Life Sciences 6956, Farmingdale, NY), cREL (Cell Signaling 12707), p65 (Cell Signaling 6956 & 8242), p50 (Abcam 32360, Cambridge, MA) and Lamin B (Santa Cruz Biotechnology sc-6217). Blots were viewed using the LiCor Odyssey imaging system, and densitometric analysis was performed using ImageStudio (LiCor, Lincoln, NE).

mRNA isolation, cDNA synthesis and analysis of relative mRNA levels by real-time quantitative polymerase chain reaction (RT-qPCR)

Cells were scraped off culture vessels using a mixture of RLT, and mRNA extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Germantown, MD). mRNA purity and concentration were measured using the NanoDrop (ThermoFisher Scientific) before proceeding to synthesize cDNA using the Verso cDNA synthesis kit (ThermoFisher Scientific). Relative mRNA levels were evaluated by RT-qPCR using exon spanning primers IL1α (Mm00439620_m1), IL1β (Mm01336189_m1), IRG1 (Mm01224532_m1) and CxCL10 (Mm00445235_m1), TaqMan gene expression and StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA). Relative quantification was performed by normalization to the endogenous control 18s using the cycle threshold (ΔΔCt) method.

Results

CpG-ODN-induced TLR9 innate immune signalling induces degradation of the NFκB inhibitory proteins IκBα and IκBβ

First, we sought to determine if CpG-ODN-mediated TLR9 innate immune signalling in macrophages proceeded through IκBα and IκBβ. Therefore, we exposed RAW 264.7 cells to CpG-ODN (0·3, 1 or 3 μM) for 1 hr and evaluated cytosolic IκB levels. We observed IκBβ degradation following exposure to all doses (Fig. 1a). In these dose−response experiments, there was minimal effect on cytosolic IκBα levels, but only one time point was evaluated. Having noted the greatest degradation at a CpG-ODN exposure of 3 μM, this dose was used in the subsequent experiments.

We next sought to determine the time course of IκBα and IκBβ degradation in CpG-ODN-exposed macrophages. We noted significant degradation of IκBα at 0·5 and 1 hr of exposure (Fig. 1b,c), and significant degradation of IκBβ at 0·5 hr of exposure that continued through 8 hr of exposure.

To confirm that these findings were not limited to the RAW 264.7 murine macrophage cell line, we exposed BMDMs to CpG-ODN (0·3 μM, 1–5 hr). At the time points evaluated, we found significant degradation of IκBα at 1 hr of exposure (Fig. 1d,e), and IκBβ at 5 hr of exposure (Fig. 1d,e). These results demonstrate that CpG-mediated innate immune signalling proceeds through both IκBα and IκBβ in macrophages.

CpG-ODN-induced TLR9 innate immune signalling induces nuclear translocation of cRel, p65 and p50

Having noted CpG-ODN-induced IκB degradation, we next evaluated for NFκB subunit nuclear translocation. Within 30 min of exposure, nuclear levels of p65 and cRel had increased significantly (Fig. 2a,b). Nuclear levels of p50 took longer to significantly increase (Fig. 2a,b). We evaluated later time points (16–24 hr), and found that nuclear levels of both cRel and p50 remained significantly elevated (Fig. 2c,d).

We next evaluated CpG-ODN-exposed BMDMs for NFκB subunit nuclear translocation. At 1 hr of exposure, nuclear levels of p65 and p50 had increased significantly (Fig. 2e,f). These results demonstrate that CpG-mediated innate immune signalling results in nuclear translocation key activating NFκB subunits in macrophages.

CpG-ODN-induced IL1 gene expression is NFκB dependent

Given the association between sterile inflammation and IL1, we next evaluated CpG-ODN-exposed macrophages for IL1α and IL1β expression. In RAW 264.7 cells, expression of both IL1α and IL1β significantly increased within 1 hr of exposure, and remained significantly elevated through 5 hr of exposure (Fig. 3a). Having noted that a single exposure to CpG-ODN induced NFκB nuclear translocation that lasted through 24 hr of exposure (Fig. 2c,d), we evaluated IL1α and IL1β at 24 hr of exposure. At this time point, expression of both IL1α and IL1β remained significantly elevated (Fig. 3b). Similarly, expression of both IL1α and IL1β increased significantly in CpG-ODN-exposed BMDMs (Fig. 3c).

To implicate NFκB signalling IL1α and IL1β expression, RAW 264.7 cells were pre-treated with the NFκB inhibitor BAY 11-7085 prior to exposure to CpG-ODN. Pre-treatment with BAY 11-7085 (1–10 μM, 1 hr) resulted in a consistent inhibition of CpG-ODN-induced IκBα and IκBβ degradation at doses of 5 and 10 μM (Fig. 3d,

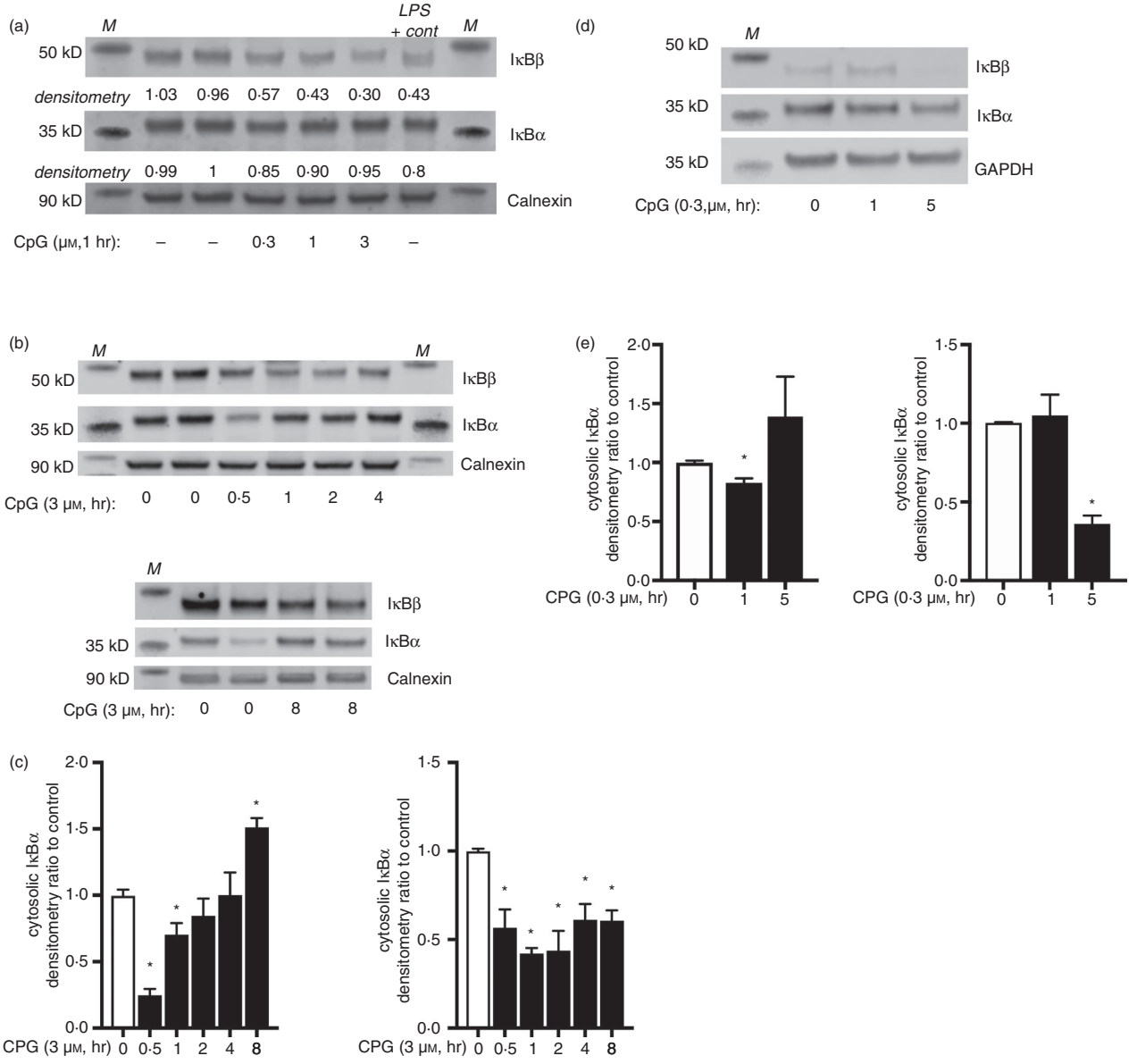

Figure 1. Exposure to CpG-ODN causes dose- and time-dependent IκBβ and IκBα degradation. (a) Representative Western blot of whole-cell lysates from RAW 264.7 exposed to CpG-ODN (0·3–3 μM, 1 hr). Whole-cell lysate from RAW 264.7 exposed to lipopolysaccharide (LPS; 1 μg/ml, 1 hr) as the positive control and calnexin shown as the loading control. Densitometric values normalized to calnexin for individual lanes are provided below each lane. (b) Representative Western blot of cytosolic extracts from RAW 264.7 exposed to CpG-ODN (3 μM, 0–8 hr) with calnexin shown as the loading control. (c) Densitometry ratio to control of IκBα and IκBβ in cytosolic extracts from RAW 264.7 exposed to CpG-ODN. Values shown as means ± SEM; $n = 6$/time point. *$P < 0{\cdot}05$ versus unexposed control. (d) Representative Western blot of cytosolic extracts from bone marrow-derived macrophages (BMDMs) exposed to CpG-ODN (0·3 μM, 0–5 hr) with GAPDH shown as the loading control. (e) Densitometry ratio to control of IκBα and IκBβ in cytosolic extracts from BMDMs exposed to CpG-ODN. Values shown as means ± SEM; $n = 3$/time point. *$P < 0{\cdot}05$ versus unexposed control.

e). Preventing inhibitory protein degradation resulted in an attenuated CpG-ODN-induced nuclear translocation of both p50 and p65 (Fig. 3f). Furthermore, BAY 11-7085 pre-treatment resulted in a dose-dependent decrease in CpG-ODN-induced IL1α and IL1β expression (Fig. 3g). These results demonstrate a key role of NFκB signalling in CpG-ODN-induced IL1α and IL1β expression.

TLR4-mediated innate immune IκBβ/NFκB signalling is Ca^{2+} dependent

Previous reports have demonstrated that Ca^{2+}-mediated activation of calcineurin is a key step in IκBβ inactivation and subsequent NFκB activation,[72,73] but whether this is relevant to TLR-mediated innate immune

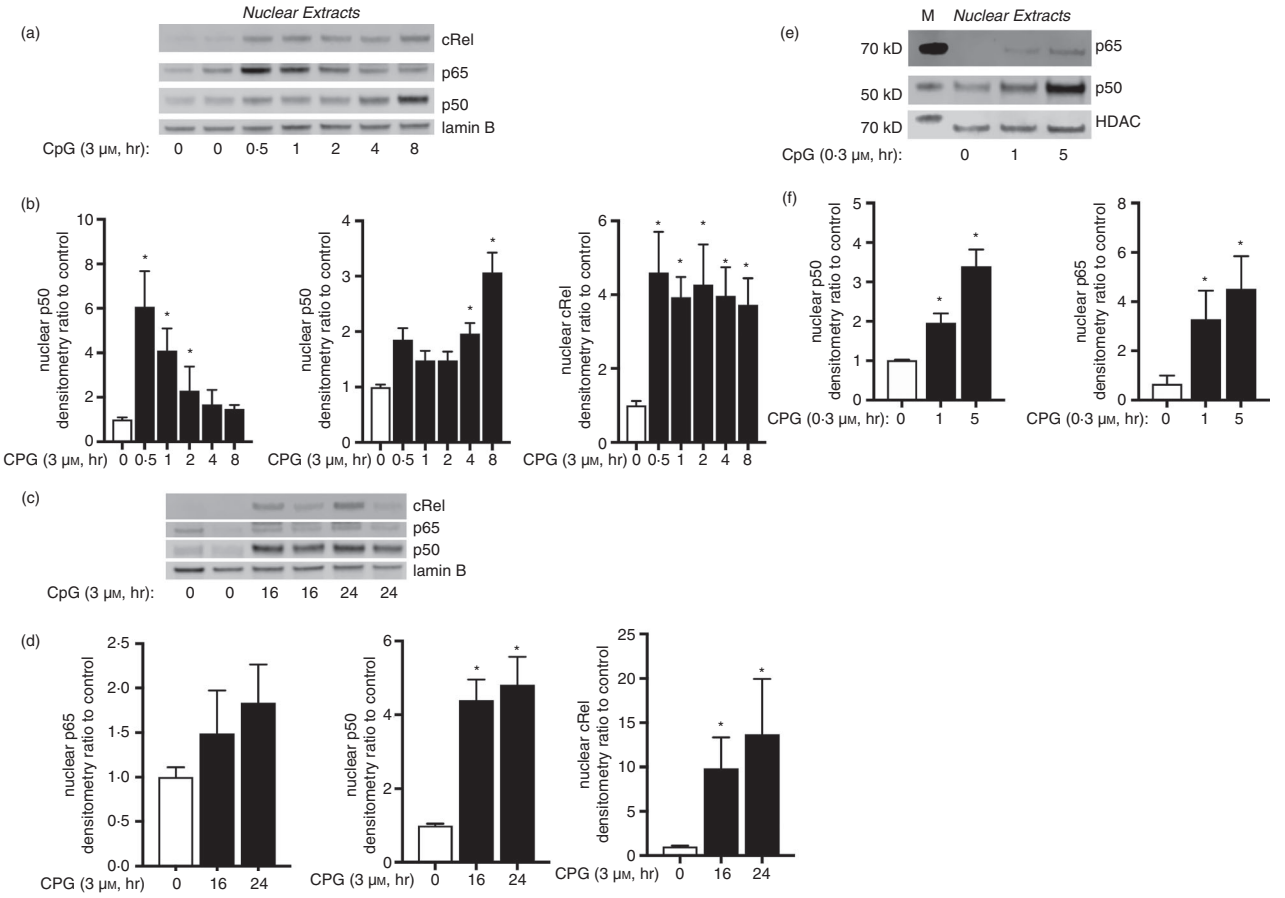

Figure 2. Exposure to CpG-ODN leads to nuclear translocation of the NFκB subunits cREL, p65 and p50. (a) Representative Western blot of nuclear extracts from RAW 264.7 exposed to CpG-ODN (3 μM, 0–8 hr) with lamin B shown as the loading control. (b) Densitometry ratio to control of p65, p50, cREL in nuclear extracts from RAW 264.7 exposed to CpG-ODN. Values shown as means ± SEM; $n = 6$/time point. *$P < 0{\cdot}05$ versus unexposed control. (c) Representative Western blot of nuclear extracts from RAW 264.7 exposed to CpG-ODN (3 μM, 0–24 hr) with lamin B shown as the loading control. (d) Densitometry ratio to control of p65, p50, cREL in nuclear extracts from RAW 264.7 exposed to CpG-ODN. Values shown as means ± SEM; $n = 6$/time point. *$P < 0{\cdot}05$ versus unexposed control. (e) Representative Western blot of nuclear extracts from bone marrow-derived macrophages (BMDMs) exposed to CpG-ODN (0·3 μM, 0–5 hr) with HDAC shown as the loading control. (f) Densitometry ratio to control of p65 and p50 in nuclear extracts from BMDMs exposed to CpG-ODN. Values shown as means ± SEM; $n = 3$/time point. *$P < 0{\cdot}05$ versus unexposed control.

signalling is unknown. The role of IκBβ in mediating TLR4-mediated NFκB signalling is well characterized.[78,79] Thus, RAW 264.7 cells were exposed to either a cell-permeant calcium chelator (EGTA-AM), a calcineurin inhibitor (FK-506) or a calcium ionophore (A23187) and lipopolysaccharide (LPS), and NFκB signalling was assessed. Previous reports have shown that RAW 264.7 cells are sensitive to the calcium ionophore A23187.[67] Both EGTA-AM (Fig. 4a,b) and FK-506 (Fig. 4c,d) pre-treatment resulted in a dose-dependent inhibition of LPS-induced IκBβ degradation. Additionally, exposure to A23187 in the absence of LPS resulted in significant IκBβ degradation (Fig. 4e,f). These results demonstrate that intracellular calcium has a direct effect upon innate immune IκBβ/NFκB signalling.

TLR9-mediated innate immune IκBβ/NFκB signalling is Ca^{2+} dependent

We next evaluated the role of Ca^{2+} in TLR9-mediated IκBβ/NFκB signalling. We found increased signal from RAW 264.7 cells pre-treated with the cell-permeable fluorescent calcium indicator Fluo-4 within 1 hr of exposure to CpG-ODN (Fig. 5a,b). Objectively, we found that fluorescent signalling increased by 50% when the entire surface area of the 35-mm culture dish was evaluated. We found that both EGTA-AM (Fig. 5c,d) and FK-506 (Fig. 5c,d) inhibited CpG-ODN-induced IκBβ degradation in a dose-dependent manner. Furthermore, CpG-ODN-induced IL1α and IL1β expression was significantly attenuated by EGTA-AM (Fig. 5e) and FK-506 (Fig. 5f).

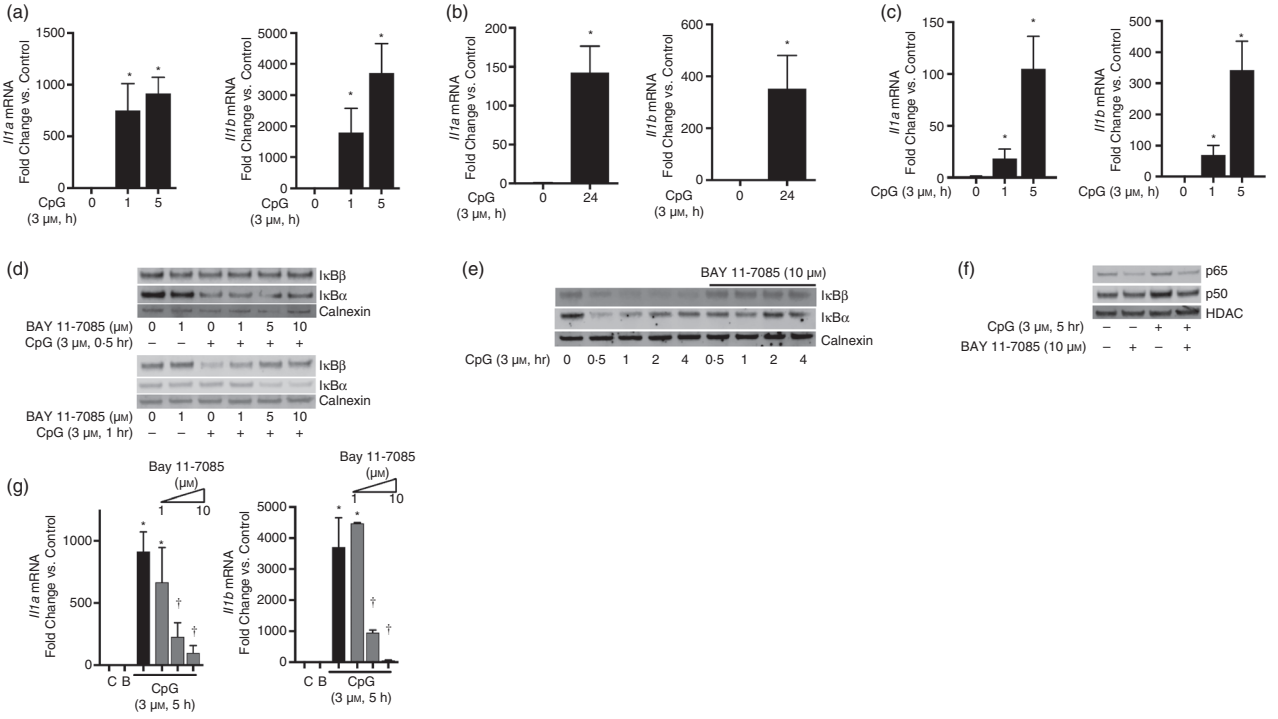

Figure 3. CpG-ODN-induced expression of IL1α and IL1β is attenuated by the NFκB inhibitor Bay 11-7085. (a) Fold-increase in IL1α and IL1β mRNA expression in RAW 264.7 following CpG-ODN exposure (3 μM, 0–5 hr). n = 6/time point. *P < 0·05 versus unexposed control. (b) Fold-increase in IL1α and IL1β mRNA expression in RAW 264.7 following CpG-ODN exposure (3 μM, 24 hr). n = 6/time point. *P < 0·05 versus unexposed control. (c) Fold-increase in IL1α and IL1β mRNA expression in bone marrow-derived macrophages (BMDMs) following CpG-ODN exposure (0·3 μM, 0–5 hr). n = 3/time point. *P < 0·05 versus unexposed control. (d) Representative Western blot of whole-cell lysates from RAW 264.7 pre-treated with the NFκB pharmacological inhibitor BAY 11-7085 (0–10 μM, 1 hr) followed by CpG-ODN exposure (3 μM, 0–1 hr) with calnexin shown as the loading control. (e) Representative Western blot of cytosolic extracts from RAW 264.7 pre-treated with the NFκB pharmacological inhibitor Bay 11-7085 (10 μM, 1 hr) followed by CpG-ODN exposure (3 μM, 0–4 hr). Calnexin shown as the loading control. (f) Representative Western blot of nuclear extracts from RAW 264.7 pre-treated with the NFκB pharmacological inhibitor Bay 11-7085 (10 μM; 1 hr) followed by CpG-ODN exposure (3 μM, 0–5 hr). HDAC shown as the loading control. (g) Fold-change in IL1α and IL1β mRNA expression in RAW 264.7 pre-treated with the NFκB pharmacological inhibitor BAY 11-7085 (1–10 μM, 1 hr) followed by CpG-ODN exposure (3 μM, 5 hr). n = 5/time point. *P < 0·05 versus unexposed control. $^{\dagger}P$ < 0·05 versus CpG-ODN exposed.

These results confirm that Ca^{2+}-mediated activation of IκBβ/NFκB signalling links CpG-ODN and IL1α and IL1β expression.

Calcium ionophores act synergistically to increase CpG-ODN-mediated IL1 expression

The effect of the calcium ionophore A23187 on CpG-mediated IκBβ/NFκB signalling was tested next. Exposure to A23187 alone resulted in significant degradation of IκBβ [Fig. 6a (lanes 3 and 4) and b]. Furthermore, simultaneous exposure of RAW 264.7 cells to CpG-ODN and A23187 resulted in significant degradation of IκBβ [Fig. 6a (lanes 7 and 8) and b] compared with CpG exposure alone [Fig. 6a (lanes 5 and 6) and b]. Consistent with this finding, CpG-ODN plus A23187 induced significantly higher IL1α and IL1β expression compared with CpG-ODN alone in RAW 264.7 cells (Fig. 6c). Similarly, CpG-ODN plus A23187 induced significantly higher IL1α and IL1β expression compared with CpG-ODN alone in BMDMs (Fig. 6d).

Targeting IκBβ inhibits CpG-ODN IL1 expression

Having demonstrated the effects of intracellular Ca^{2+} on TLR9-mediated IL1α and IL1β expression, we sought to further interrogate the role of IκBβ. To do this, we used BMDMs isolated from IκBβ$^{-/-}$ mice, as well as IκBβ-overexpressing (AKBI) mice. Previous work has demonstrated that NFκB signalling that proceeds through individual inhibitory proteins confers specificity to the resulting transcriptome, including the expression of IL1.[74,75,78,79,88,89] Thus, cellular systems and organisms lacking a specific IκB isoform reveal isoform-specific target genes. In IκBβ$^{-/-}$ BMDMs, CPG-ODN-mediated signalling cannot proceed through IκBβ but does so exclusively through IκBα (Fig. 7a,b). Of note, CpG-ODN-induced IL1α and IL1β expression is significantly

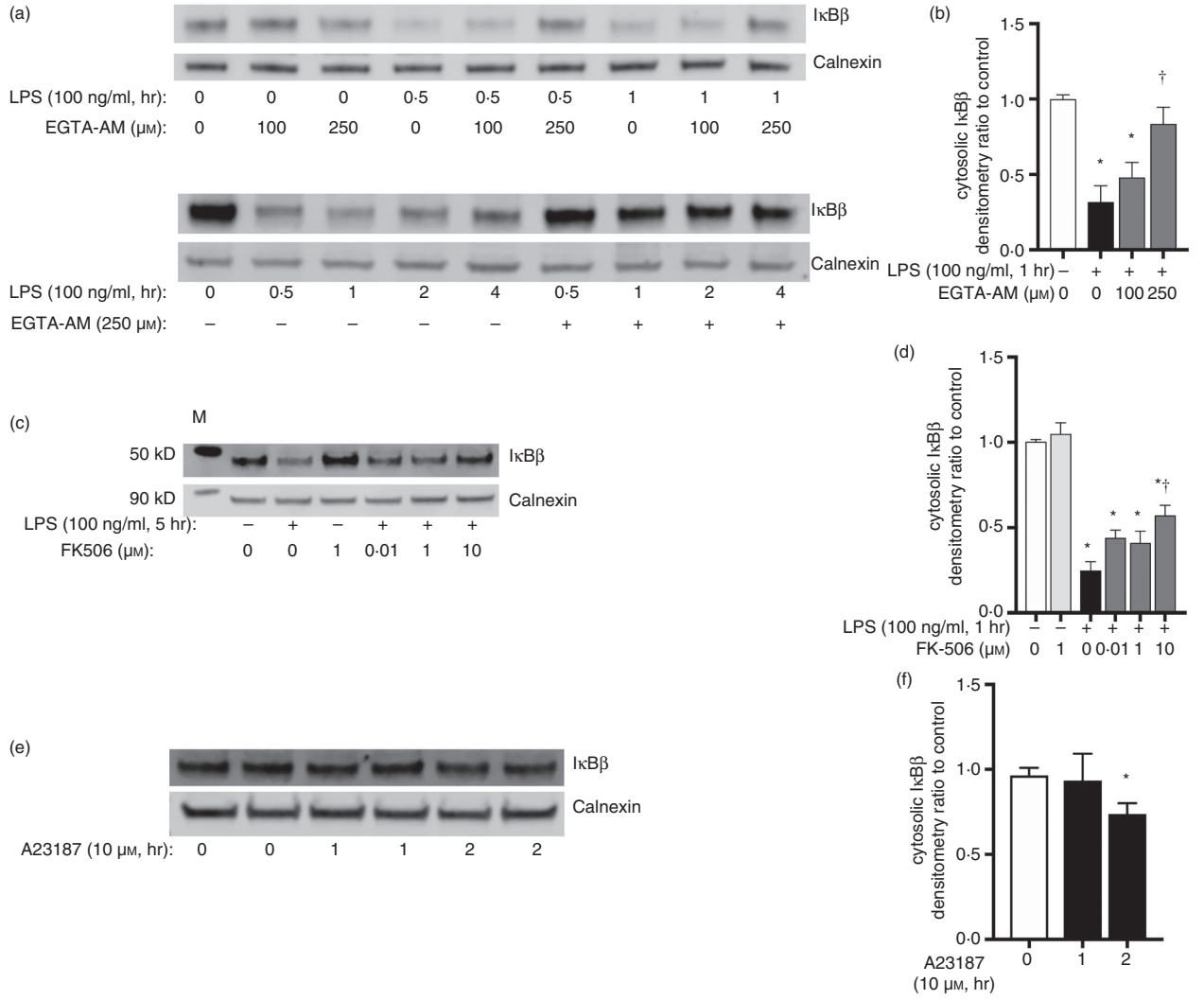

Figure 4. Lipopolysaccharide (LPS)-induced TLR4 mediated IκBβ degradation is responsive to alterations in intracellular calcium. (a) Representative Western blot of whole-cell lysates from RAW 264.7 pre-treated with calcium chelator EGTA-AM (250 μM, 0·5 hr) followed by LPS exposure (100 ng/ml, 0–4 hr) with calnexin shown as the loading control. (b) Densitometry ratio to control of IκBβ in whole-cell lysates from RAW 264.7 following pre-treatment with the cell-permeable calcium chelator EGTA-AM followed by LPS exposure. Values shown as means ± SEM; $n = 5$/time point. *$P < 0{\cdot}05$ versus unexposed control. (c) Representative Western blot of whole-cell lysates from RAW 264.7 pre-treated with calcineurin inhibitor FK-506 (0·1–10 μM, 24 hr) followed by LPS exposure (100 ng/ml, 5 hr) with calnexin shown as the loading control. (d) Densitometry ratio to control of IκBβ in whole-cell lysates from RAW 264.7 following pre-treatment with calcineurin inhibitor FK-506 followed by LPS exposure. Values shown as means ± SEM; $n = 5$/time point. *$P < 0{\cdot}05$ versus unexposed control. †$P < 0{\cdot}05$ versus LPS exposed. (e) Representative Western blot of whole-cell lysates from RAW 264.7 exposed to calcium ionophore A23187 (10 μM, 0–2 hr) with calnexin shown as the loading control. (f) Densitometry ratio to control of cytosolic IκBβ in RAW 264.7 lysates following exposure to calcium ionophore A23187. Values shown as means ± SEM; $n = 3$/time point. *$P < 0{\cdot}05$ versus unexposed control.

suppressed in IκBβ$^{-/-}$ BMDMs (Fig. 7c). Due to overexpression of the inhibitory protein IκBβ (Fig. 7d), CpG-ODN-induced NFκB signalling (Fig. 7d,e) and the expression of IL1α and IL1β mRNA are blunted in AKBI BMDMs (Fig. 7f). To overcome the attenuated signalling associated with IκBβ overexpression, AKBI BMDMs were simultaneously exposed to CpG-ODN and A23187. Exposure to CpG-ODN and A23187 significantly increased IL1α and IL1β expression compared with CpG-ODN or A23187 alone (Fig. 7g).

Discussion

We found that in macrophages, CpG-ODN-mediated TLR9 signalling proceeds through IκBβ/NFκB signalling. Furthermore, CpG-ODN-induced IL1α and IL1β are NFκB dependent. Importantly, we found that intracellular calcium flux plays an important role in CpG-ODN-mediated TLR9 activation and IL1 expression. Specifically, CpG-mediated degradation of the NFκB inhibitory protein IκBβ and subsequent IL1 expression is attenuated

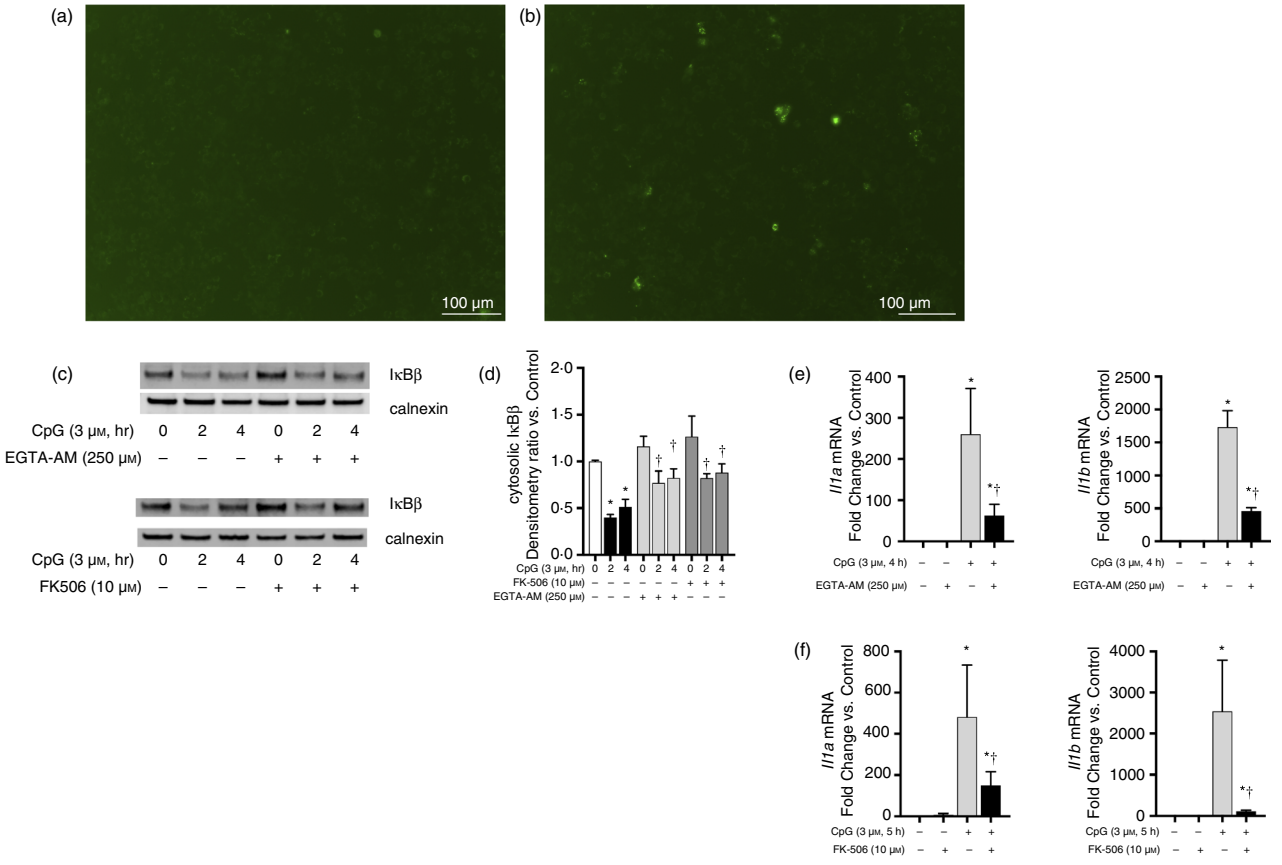

Figure 5. Treatment with EGTA-AM or FK-506 prior to CpG exposure reduced IκBβ degradation as well as IL1α and IL1β expression. Representative immunofluorescence staining of (a) control and (b) CpG-ODN exposed (3 μM, 1 hr) RAW 264.7 macrophages pre-treated with the cell-permeable calcium-sensitive fluorescent indicator Fluo-4, AM. Images were captured with the same settings. Internal scale bar: 50 μm. (c) Representative Western blot of whole-cell lysates from RAW 264.7 pre-treated with calcium chelator EGTA-AM (250 μM, 0·5 hr) or calcineurin inhibitor FK-506 (10 μM, 24 hr) followed by CpG-ODN exposure (3 μM, 0–4 hr) with calnexin shown as the loading control. (d) Densitometry ratio to control of IκBβ in whole-cell lysates from RAW 264.7 lysates following pre-treatment with the cell-permeable calcium chelator EGTA-AM or calcineurin inhibitor FK-506 followed by CpG-ODN exposure. Values shown as means ± SEM; n = 5/time point. *$P < 0{\cdot}05$ versus unexposed control. $^{\dagger}P < 0{\cdot}05$ versus CpG-ODN exposed. (e) Fold-change in IL1α and IL1β mRNA expression in RAW 264.7 pre-treated with calcium chelator EGTA-AM (250 μM, 0·5 hr) followed by CpG-ODN exposure (3 μM, 0–4 hr). n = 5/time point. *$P < 0{\cdot}05$ versus unexposed control. $^{\dagger}P < 0{\cdot}05$ versus CpG-ODN exposed. (f) Fold-change in IL1α and IL1β expression in RAW 264.7 pre-treated with calcineurin inhibitor FK-506 (10 μM, 24 hr) followed by CpG-ODN exposure (3 μM, 0–5 hr). n = 5/time point. *$P < 0{\cdot}05$ versus unexposed control. $^{\dagger}P < 0{\cdot}05$ versus CpG-ODN exposed.

with calcium chelation or calcineurin inhibition. Conversely, exposure to calcium ionophore alone results in IκBβ degradation and IL1 expression, and exposure to ionophore and CpG-ODN amplifies these effects. Finally, using BMDMs lacking or overexpressing IκBβ, we demonstrate that this key inhibitory protein plays a central role in CpG-ODN-induced IL1 expression. These results reveal potential therapeutic targets to attenuate the pro-inflammatory effects of IL1 associated with sterile inflammation.

These results are interesting because they implicate a specific NFκB inhibitory protein isoform in the regulation of CpG-ODN-mediated IL1 expression. Canonical NFκB signalling proceeds through phosphorylation and proteolysis of the IκB family of inhibitory proteins. Three cytoplasmic inhibitory proteins have been identified: IκBα, IκBβ and IκBε.[76] Much has been learned about the IκB isoforms by evaluating the inflammatory stress-induced NFκB transcriptome in cells and mice lacking the expression of individual IκBs.[74,75,78,79,88,89] Each isoform uniquely contributes to the degree and duration of NFκB activation, as well as the NFκB transcriptome. Multiple studies have implicated IκBβ in TLR4-mediated IL1β expression.[78–80,90,91] However, this is the first report linking IκBβ to IL1 expression following CpG-ODN TLR9-mediated innate immune signalling.

Our data demonstrate that intracellular calcium affects CpG-ODN-mediated TLR9 signalling. The effect of intracellular calcium on TLR9-mediated innate immune signalling is not unexpected. These findings are consistent

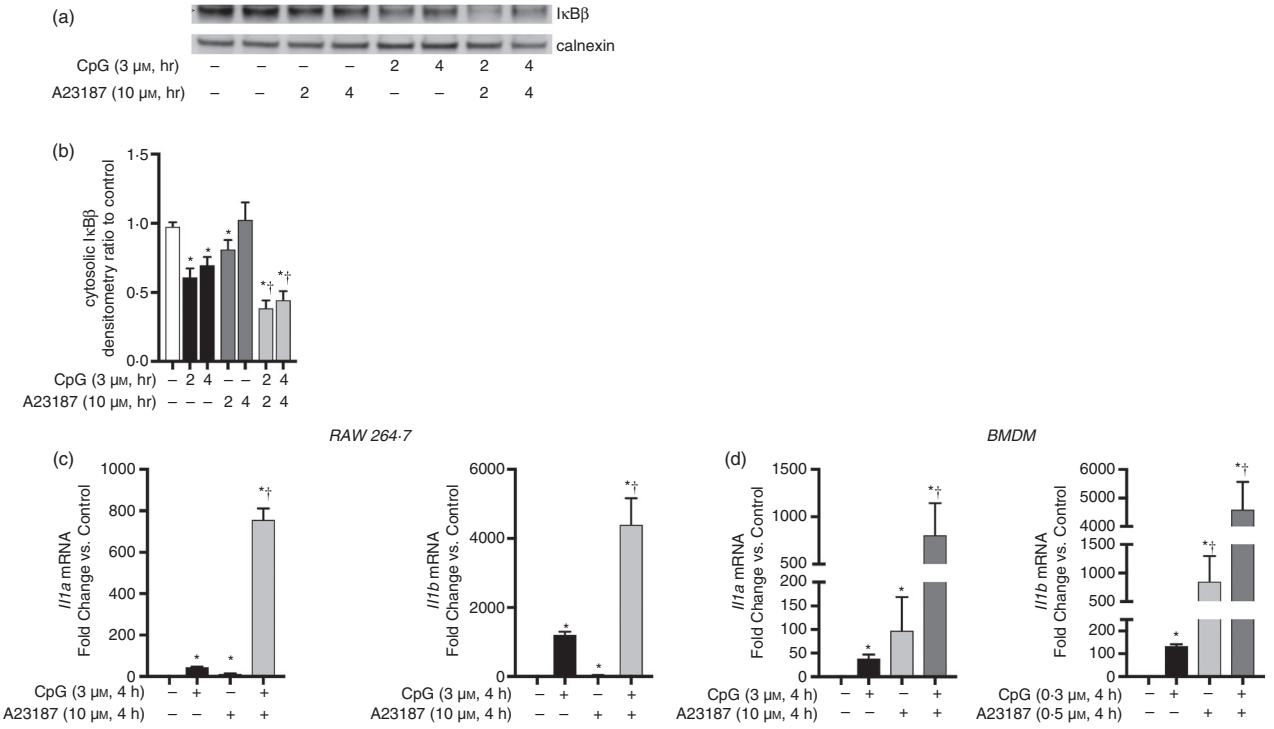

Figure 6. Concurrent exposure to CpG-ODN and A23187 accelerates IκBβ degradation, and increases IL1α and IL1β expression. (a) Representative Western blot of whole-cell lysates from RAW 264.7 lysates exposed concurrently to calcium ionophore A23187 (10 μM, 0–4 hr) and CpG-ODN (3 μM, 0–4 hr) with calnexin shown as the loading control. (b) Densitometry ratio to control of IκBβ in whole-cell lysates from RAW 264.7 following concurrent exposure to calcium ionophore A23187 and CpG-ODN. Values shown as means ± SEM; n = 6/time point. *$P < 0{\cdot}05$ versus unexposed control. †$P < 0{\cdot}05$ versus CpG-ODN exposed. (c) Fold-change in IL1α and IL1β mRNA expression in RAW 264.7 following concurrent exposure to calcium ionophore A23817 (10 μM, 4 hr) and CpG-ODN (3 μM, 0–4 hr). Values shown as means ± SEM; n = 6/time point. *$P < 0{\cdot}05$ versus unexposed control. †$P < 0{\cdot}05$ versus CpG-ODN exposed. (d) Fold-change in IL1α and IL1β mRNA expression in bone marrow-derived macrophages (BMDMs) following concurrent exposure to calcium ionophore A23817 (0·5 μM, 4 hr) and CpG-ODN (0·3 μM, 0–4 hr). Values shown as means ± SEM; n = 6/time point. *$P < 0{\cdot}05$ versus unexposed control. †$P < 0{\cdot}05$ versus CpG-ODN exposed.

with previous reports demonstrating a relationship between intracellular calcium and other TLR-mediated innate immune signalling pathways, including TLR4.[61–71] Exposure to CpG-ODN alone causes decreased mitochondrial Ca^{2+} uptake,[92] which could affect intracellular calcium availability. We did observe an increase in signal from the cell-permeable calcium indicator (Fig. 5a,b). These mechanisms alone may explain why calcium chelation with EGTA-AM and calcineurin inhibition with FK-506 attenuate CpG-ODN-stimulated IL1 expression (Fig. 5c–f). Whether other causes of calcium dyshomeostasis, including store-operate calcium entry and second messenger-operated calcium entry, are mechanistically active after CpG-ODN exposure deserves further study. Previous reports have demonstrated that intracellular calcium affects CpG-ODN-induced gene expression, but whether this effect was mediated in part though cytosolic signalling was not investigated.[93] We believe that our findings are particularly relevant for understanding the role of TLR9-mediated innate immune signalling and the pathogenesis of sterile inflammation. Mitochondrial stress and injury are known to trigger pro-inflammatory NFκB activation via endosomal TLR9 binding mitochondrial CpG-rich DNA.[3–9] The ability of CpG-rich mitochondrial DNA to trigger TLR9 activation has led to the appropriate use of CpG-ODN for the study of multiple disease states where mitochondrial stress and injury are believed to play a pathogenic role.[51–56] These include atherosclerosis,[10–14] ischaemia-reperfusion,[11,15–19,94] infection/sepsis,[20–37] and various autoimmune diseases.[38–50] Due to the direct link between mitochondrial stress and intracellular calcium dyshomeostasis,[84–86] the effect of intracellular calcium on CpG-ODN-mediated innate immune signalling is relevant to the study of these disease states associated with sterile inflammation.

An additional mechanistic contribution is the recognition that intracellular calcium plays an important role in CpG-ODN-mediated innate immune signalling and the kinetics of IκBβ degradation. Previous studies have demonstrated that intracellular calcium has a specific effect on IκBβ degradation. The inhibitory function of IκBβ depends on phosphorylation of two c-terminal serine residues.[95–98] Importantly, increased cytoplasmic calcium is associated with increased calcineurin activity.[99]

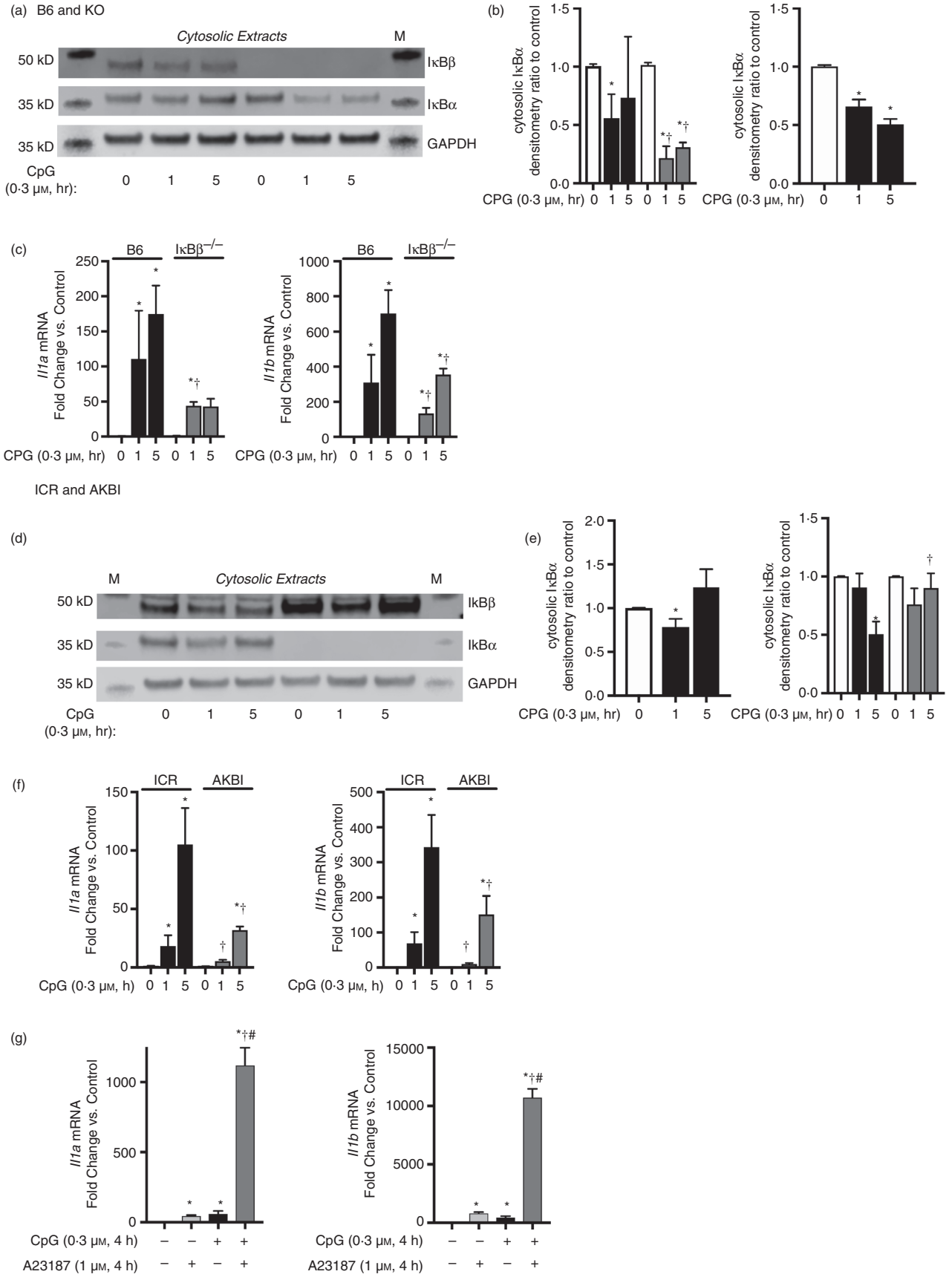
(a) B6 and KO
Cytosolic Extracts
M
50 kD
35 kD
35 kD
IκBβ
IκBα
GAPDH
CpG (0·3 μM, hr):
0 1 5 0 1 5
(b)
cytosolic IκBα densitometry ratio to control
CPG (0·3 μM, hr) 0 1 5 0 1 5
(c)
B6
IκBβ−/−
Il1a mRNA Fold Change vs. Control
Il1b mRNA Fold Change vs. Control
CPG (0·3 μM, hr) 0 1 5 0 1 5
ICR and AKBI
(d)
M
Cytosolic Extracts
M
50 kD
35 kD
35 kD
IkBβ
IkBα
GAPDH
CpG (0·3 μM, hr):
0 1 5 0 1 5
(e)
cytosolic IκBα densitometry ratio to control
CPG (0·3 μM, hr) 0 1 5
CPG (0·3 μM, hr) 0 1 5 0 1 5
(f)
ICR
AKBI
Il1a mRNA Fold Change vs. Control
Il1b mRNA Fold Change vs. Control
CpG (0·3 μM, h) 0 1 5 0 1 5
(g)
Il1a mRNA Fold Change vs. Control
Il1b mRNA Fold Change vs. Control
CpG (0·3 μM, 4 h) – – + +
A23187 (1 μM, 4 h) – + – +

Figure 7. IκBβ/NFκB signalling mediates CpG-ODN-induced IL1α and IL1β expression. (a) Representative Western blot of cytosolic extracts from WT and IκBβ$^{-/-}$ bone marrow-derived macrophages (BMDMs) exposed to CpG-ODN (0·3 μM, 0–5 hr) with GAPDH shown as the loading control. (b) Densitometry ratio to control of IκBα and IκBβ in cytosolic extracts from WT and IκBβ$^{-/-}$ BMDMs following exposure to CpG-ODN. Values shown as means ± SEM; n = 4/time point. *$P < 0·05$ versus unexposed control. †$P < 0·05$ versus WT CpG-ODN exposed. (c) Fold change in IL1α and IL1β mRNA expression in WT and IκBβ$^{-/-}$ BMDMs following CpG-ODN exposure (0·3 μM, 0–5 hr). Values shown as means ± SEM; n = 4/time point. *$P < 0·05$ versus unexposed control. †$P < 0·05$ versus WT CpG-ODN exposed. (d) Representative Western blot of cytosolic extracts from WT and AKBI BMDMs exposed to CpG-ODN (0·3 μM, 0–5 hr) with GAPDH shown as the loading control. (e) Densitometry ratio to control of cytosolic IκBα and IκBβ in cytosolic extracts from WT and AKBI BMDMs following exposure to CpG-ODN. Values shown as means ± SEM; n = 4/time point. *$P < 0·05$ versus unexposed control. †$P < 0·05$ versus WT CpG-ODN exposed. (f) Fold-change in IL1α and IL1β mRNA expression in WT and AKBI BMDMs following CpG-ODN exposure (0·3 μM, 0–5 hr). Values shown as means ± SEM; n = 4/time point. *$P < 0·05$ versus unexposed control. †$P < 0·05$ versus WT CpG-ODN exposed. (g) Fold-change in IL1α and IL1β mRNA expression in AKBI BMDMs following concurrent exposure to calcium ionophore A23187 (1 μM, 4 hr) and CpG-ODN (0·3 μM, 4 hr). Values shown as means ± SEM; n = 3/time point. *$P < 0·05$ versus unexposed control. †$P < 0·05$ versus AKBI CpG-ODN exposed. #$P < 0·05$ versus AKBI A23187 exposed.

Calcineurin has been shown to inactivate IκBβ through dephosphorylation of the c-terminal serine residues that dictate its inhibitory activity.[72] These previous reports have established an important mechanistic link between intracellular calcium, IκBβ and NFκB activity. Our report demonstrates that these mechanisms are relevant to TLR9-mediated innate immune signalling.

Lastly, we demonstrate a mechanistic link between CpG-ODN-mediated TLR9 innate immunity, calcium-sensitive IκBβ/NFkB signalling, and the expression of IL1α and IL1β. It is not unanticipated that the CpG-ODN-mediated IL1α and IL1β expressions are NFκB dependent (Fig. 3). Building on this observation, we show that CpG-ODN IL1α and IL1β expression is sensitive to intracellular calcium, with expression being significantly attenuated by calcium chelation and calcineurin inhibition (Fig. 5), and significantly increased with ionophore exposure (Fig. 6). Finally, we demonstrate that calcium-dependent IκBβ/NFκB signalling is sufficient for IL1α and IL1β expression (Fig. 7g), and that CpG-ODN and calcium ionophore exposure synergistically and significantly increase IL1α and IL1β expression (Fig. 7g). These findings have therapeutic implications for various disease states associated with mitochondrial injury/stress, sterile inflammation and IL1 expression.[1,2]

TLR9 was first identified as the receptor for bacterial DNA.[8] Through TLR9-mediated innate and adaptive immune responses, the host organism mounts a defense against these offending pathogens. However, many endogenous ligands activate TLR9 signalling. These include alarmins such has HMGB1 and mitochondrial DNA.[2] Thus, shared signalling underlies the pathophysiology of this 'sterile inflammation' that occurs in the absence of microbial stimulation, and the response to microbial challenge. It has been argued that the cost−benefit ratio of the inflammatory response to endogenous ligands is much lower than that of the response to microbial challenge.[1] While the inflammatory response helps clear microbial challenge and assist in tissue repair, chronic inflammation can result in tissue damage and ongoing injury. With sterile inflammation, the innate immune activation may not confer benefit to the host organism as the inciting stimulus is not threatening. Thus, attenuating innate immune activity in the setting of sterile inflammatory stimulus represents a potential therapeutic target to improve the cost−benefit ratio of this response. Understanding the mechanisms underlying sterile inflammation may reveal therapeutic targets to limit this ongoing tissue injury.

There are many limitations to the current study. We have evaluated the transcriptional regulation of TLR9-mediated IL1α and IL1β expression. However, we did not evaluate NLRP3 inflammasome activation, which is a critical step in IL1β secretion and activity. Of note, calcium flux can activate the NLRP3 inflammasome.[100] Consequently, further studies are needed to assess how targeting calcium flux after TLR9 stimulation would affect inflammasome activity. Furthermore, we did not assess IL1α and IL1β protein expression in this set of experiments. While our findings regarding the transcriptional activation of IL1α and IL1β protein expression remain valid, more work needs to be done to determine the functional outcomes of altered IL1α and IL1β protein expression. We did not measure intracellular calcium flux in these experiments. However, we used doses of EGTA-AM, FK-506 and A23187 previously reported to affect RAW 264.7 cells, and performed robust dose−response experiments. Furthermore, we only evaluated this pathway in murine macrophages. It is not known whether these findings are relevant to other cells.

The role of TLR9-mediated IL1 expression is increasingly recognized in the pathogenesis of diseases associated with sterile inflammation.[1–4,101] Given the strength of these findings, targeting IL1 activity has been proposed as a therapeutic intervention to limit inflammatory injury.[2] We believe that our data support targeting calcium dyshomeostasis to limit injury associated with sterile inflammation. The role of the mitochondria in maintaining intracellular calcium homeostasis is well known, and mitochondrial injury leads to increased intracellular calcium levels.[84–86] Our findings demonstrate that when

paired with the release of unmethylated CpG-rich DNA, increased intracellular calcium acts as a second stimulatory event that converges on TLR9 innate immune signalling. These converging signals result in a synergistic increase in IL1α and IL1β expression. These findings are critically important as pre-clinical models have demonstrated the limitations of targeting IL1 signalling.[102] Our results raise the possibility of targeting calcium dyshomeostasis or IκBβ/NFκB signalling to attenuate injury associated with sterile inflammation. In fact, these mechanisms may underlie the protective effect of tacrolimus on ischaemia-reperfusion.[103] Furthermore, specifically targeting the TLR9-mediated IκBβ/NFκB signalling pathway may be particularly effective due to the role of IL1 in recruiting neutrophils to the site of injury.[1] By targeting this mechanism, it may be possible to attenuate IL1α and IL1β expression, and the degree and duration of sterile inflammation. It is possible that therapeutically manipulating this pathway may result in shifting the cost−benefit ratio of the sterile inflammatory response so it is beneficial to the host.

Funding

This work was supported by NIH grant R01HL132941 to CJW.

Disclosure

The authors declare no potential financial or ethical conflicts of interest.

Author contributions

RD, LN, SM, CJW conception and design of research; RD, LN, SM, CJW performed experiments; RD, LN, SM, CJW analysed data; RD, LN, SM, CJW interpreted results of experiments; CJW drafted the manuscript; CJW prepared figures; RD, LN, SM, CJW edited and revised the manuscript; RD, LN, SG, SM, CJW approved the final version of the manuscript.

References

1 Rock KL, Latz E, Ontiveros F, Kono H. The sterile inflammatory response. *Annu Rev Immunol* 2010; **28**:321–42.
2 Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* 2010; **10**:826–37.
3 Grazioli S, Pugin J. Mitochondrial damage-associated molecular patterns: from inflammatory signaling to human diseases. *Front Immunol* 2018; **9**:832.
4 Fang C, Wei X, Wei Y. Mitochondrial DNA in the regulation of innate immune responses. *Protein Cell* 2016; **7**:11–6.
5 Shen H, Kreisel D, Goldstein DR. Processes of sterile inflammation. *J Immunol* 2013; **191**:2857–63.
6 Kawai T, Akira S. Signaling to NF-kappaB by toll-like receptors. *Trends Mol Med* 2007; **13**:460–9.
7 Sparwasser T, Miethke T, Lipford G, Erdmann A, Hacker H, Heeg K *et al.* Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. *Eur J Immunol* 1997; **27**:1671–9.
8 Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H *et al.* A toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**:740–5.
9 Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**:499–511.
10 McCarthy CG, Wenceslau CF, Goulopoulou S, Ogbi S, Baban B, Sullivan JC *et al.* Circulating mitochondrial DNA and toll-like receptor 9 are associated with vascular dysfunction in spontaneously hypertensive rats. *Cardiovasc Res* 2015; **107**:119–30.
11 Hirata Y, Kurobe H, Higashida M, Fukuda D, Shimabukuro M, Tanaka K *et al.* HMGB1 plays a critical role in vascular inflammation and lesion formation via toll-like receptor 9. *Atherosclerosis* 2013; **231**:227–33.
12 Fuster-Matanzo A, Llorens-Martin M, Hernandez F, Avila J. Role of neuroinflammation in adult neurogenesis and Alzheimer disease: therapeutic approaches. *Mediators Inflamm* 2013; **2013**:260 925.
13 Sorrentino R, Morello S, Chen S, Bonavita E, Pinto A. The activation of liver X receptors inhibits toll-like receptor-9-induced foam cell formation. *J Cell Physiol* 2010; **223**:158–67.
14 Kim JS, Park DW, Lee HK, Kim JR, Baek SH. Early growth response-1 is involved in foam cell formation and is upregulated by the TLR9-MyD88-ERK1/2 pathway. *Biochem Biophys Res Commun* 2009; **390**:196–200.
15 Xie L, He S, Kong N, Zhu Y, Tang Y, Li J *et al.* Cpg-ODN, a TLR9 agonist, aggravates myocardial ischemia/reperfusion injury by activation of TLR9-P38 MAPK signaling. *Cell Physiol Biochem* 2018; **47**:1389–98.
16 Chinnery HR, McLenachan S, Binz N, Sun Y, Forrester JV, Degli-Esposti MA *et al.* TLR9 ligand CpG-ODN applied to the injured mouse cornea elicits retinal inflammation. *Am J Pathol* 2012; **180**:209–20.
17 Watanabe A, Hashmi A, Gomes DA, Town T, Badou A, Flavell RA *et al.* Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via toll-like receptor 9. *Hepatology* 2007; **46**:1509–18.
18 Krogmann AO, Lusebrink E, Steinmetz M, Asdonk T, Lahrmann C, Lutjohann D *et al.* Proinflammatory stimulation of toll-like receptor 9 with high dose CpG ODN 1826 impairs endothelial regeneration and promotes atherosclerosis in mice. *PLoS ONE* 2016; **11**:e0146326.
19 Bamboat ZM, Balachandran VP, Ocuin LM, Obaid H, Plitas G, DeMatteo RP. Toll-like receptor 9 inhibition confers protection from liver ischemia-reperfusion injury. *Hepatology* 2010; **51**:621–32.
20 Meng X, Sun W, Ren Y, Xiao Y, Zhao P, Lu W *et al.* Protective role of surface toll-like receptor 9 expressing neutrophils in local inflammation during systemic inflammatory response syndrome in mice. *Mol Immunol* 2017; **90**:74–86.
21 Zhao X, Shen Y, Hu W, Chen J, Wu T, Sun X *et al.* DCIR negatively regulates CpG-ODN-induced IL-1beta and IL-6 production. *Mol Immunol* 2015; **68**(Pt C):641–7.
22 Christensen LB, Woods TA, Carmody AB, Caughey B, Peterson KE. Age-related differences in neuroinflammatory responses associated with a distinct profile of regulatory markers on neonatal microglia. *J Neuroinflammation* 2014; **11**:70.
23 Hoang M, Potter JA, Gysler SM, Han CS, Guller S, Norwitz ER *et al.* Human fetal membranes generate distinct cytokine profiles in response to bacterial Toll-like receptor and nod-like receptor agonists. *Biol Reprod* 2014; **90**:39.
24 De Nardo D, De Nardo CM, Nguyen T, Hamilton JA, Scholz GM. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *J Immunol* 2009; **183**:8110–8.
25 Ravindran C, Cheng YC, Liang SM. CpG-ODNs induces up-regulated expression of chemokine CCL9 in mouse macrophages and microglia. *Cell Immunol* 2010; **260**:113–8.
26 Liu M, Liu T, Bordin A, Nerren J, Cohen N. Activation of foal neutrophils at different ages by CpG oligodeoxynucleotides and *Rhodococcus equi*. *Cytokine* 2009; **48**:280–9.
27 El Kebir D, Jozsef L, Pan W, Wang L, Filep JG. Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. *J Immunol* 2009; **182**:4386–94.
28 Ribes S, Ebert S, Czesnik D, Regen T, Zeug A, Bukowski S *et al.* Toll-like receptor prestimulation increases phagocytosis of *Escherichia coli* DH5alpha and *Escherichia coli* K1 strains by murine microglial cells. *Infect Immun* 2009; **77**:557–64.
29 Knuefermann P, Baumgarten G, Koch A, Schwederski M, Velten M, Ehrentraut H *et al.* CpG oligonucleotide activates toll-like receptor 9 and causes lung inflammation in vivo. *Respir Res* 2007; **8**:72.
30 Ivanov S, Dragoi AM, Wang X, Dallacosta C, Louten J, Musco G *et al.* A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood* 2007; **110**:1970–81.
31 Wei L, Hong Z, Jiang Z, Guofu D, Hongwei C, Liangxi W *et al.* Decreased intracellular TLR9 confers hyporesponsiveness of RAW264.7 cells to subsequent CpG ODN challenge. *Int Immunopharmacol* 2006; **6**:935–46.
32 Martin-Armas M, Simon-Santamaria J, Pettersen I, Moens U, Smedsrod B, Sveinbjornsson B. Toll-like receptor 9 (TLR9) is present in murine liver sinusoidal endothelial cells (LSECs) and mediates the effect of CpG-oligonucleotides. *J Hepatol* 2006; **44**:939–46.

33 Lee S, Hong J, Choi SY, Oh SB, Park K, Kim JS *et al.* CpG oligodeoxynucleotides induce expression of proinflammatory cytokines and chemokines in astrocytes: the role of c-Jun N-terminal kinase in CpG ODN-mediated NF-kappaB activation. *J Neuroimmunol* 2004; **153**:50–63.

34 Fernandez S, Jose P, Avdiushko MG, Kaplan AM, Cohen DA. Inhibition of IL-10 receptor function in alveolar macrophages by toll-like receptor agonists. *J Immunol* 2004; **172**:2613–20.

35 Dalpke AH, Schafer MK, Frey M, Zimmermann S, Tebbe J, Weihe E *et al.* Immunostimulatory CpG-DNA activates murine microglia. *J Immunol* 2002; **168**:4854–63.

36 Chuang TH, Lee J, Kline L, Mathison JC, Ulevitch RJ. Toll-like receptor 9 mediates CpG-DNA signaling. *J Leukoc Biol* 2002; **71**:538–44.

37 Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M *et al.* Cutting edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells. *J Immunol* 2001; **167**:3555–8.

38 Mortezagholi S, Babaloo Z, Rahimzadeh P, Namdari H, Ghaedi M, Gharibdoost F *et al.* Evaluation of TLR9 expression on PBMCs and CpG ODN-TLR9 ligation on IFN-alpha production in SLE patients. *Immunopharmacol Immunotoxicol* 2017; **39**:11–8.

39 Luan JJ, Xing GQ. Pathogenesis of antimicrobial peptides LL-37 and CpG-ODN in ANCA associated vasculitis. *J Nephrol* 2017; **30**:63–71.

40 Rahman MJ, Rahir G, Dong MB, Zhao Y, Rodrigues KB, Hotta-Iwamura C *et al.* Despite increased type 1 IFN, autoimmune nonobese diabetic mice display impaired dendritic cell response to CpG and decreased nuclear localization of IFN-activated STAT1. *J Immunol* 2016; **196**:2031–40.

41 Lee TP, Huang JC, Liu CJ, Chen HJ, Chen YH, Tsai YT *et al.* Interactions of surface-expressed TLR-4 and endosomal TLR-9 accelerate lupus progression in anti-dsDNA antibody transgenic mice. *Exp Biol Med (Maywood)* 2014; **239**:715–23.

42 Steinhagen F, McFarland AP, Rodriguez LG, Tewary P, Jarret A, Savan R *et al.* IRF-5 and NF-kappaB p50 co-regulate IFN-beta and IL-6 expression in TLR9-stimulated human plasmacytoid dendritic cells. *Eur J Immunol* 2013; **43**:1896–906.

43 Gilboa-Geffen A, Wolf Y, Hanin G, Melamed-Book N, Pick M, Bennett ER *et al.* Activation of the alternative NFkappaB pathway improves disease symptoms in a model of Sjogren's syndrome. *PLoS ONE* 2011; **6**:e28727.

44 Tadema H, Abdulahad WH, Lepse N, Stegeman CA, Kallenberg CG, Heeringa P. Bacterial DNA motifs trigger ANCA production in ANCA-associated vasculitis in remission. *Rheumatology (Oxford)* 2011; **50**:689–96.

45 Zhang Y, Lee AS, Shameli A, Geng X, Finegood D, Santamaria P *et al.* TLR9 blockade inhibits activation of diabetogenic $CD8^+$ T cells and delays autoimmune diabetes. *J Immunol* 2010; **184**:5645–53.

46 Hurtado PR, Jeffs L, Nitschke J, Patel M, Sarvestani G, Cassidy J *et al.* CpG oligodeoxynucleotide stimulates production of anti-neutrophil cytoplasmic antibodies in ANCA associated vasculitis. *BMC Immunol* 2008; **9**:34.

47 Lartigue A, Courville P, Auquit I, Francois A, Arnoult C, Tron F *et al.* Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus. *J Immunol* 2006; **177**:1349–54.

48 Tolusso B, Fabris M, Di Poi E, Assaloni R, Tomietto P, Ferraccioli GF. Response of mononuclear cells to lipopolysaccharide and CpG oligonucleotide stimulation: possible additive effect in rheumatoid inflammation. *Ann Rheum Dis* 2003; **62**:284–5.

49 Sacher T, Knolle P, Nichterlein T, Arnold B, Hammerling GJ, Limmer A. CpG-ODN-induced inflammation is sufficient to cause T-cell-mediated autoaggression against hepatocytes. *Eur J Immunol* 2002; **32**:3628–37.

50 Ichikawa HT, Williams LP, Segal BM. Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J Immunol* 2002; **169**:2781–7.

51 Peng W, Cai G, Xia Y, Chen J, Wu P, Wang Z *et al.* Mitochondrial dysfunction in atherosclerosis. *DNA Cell Biol* 2019; **38**:597–606.

52 Yang M, Linn BS, Zhang Y, Ren J. Mitophagy and mitochondrial integrity in cardiac ischemia-reperfusion injury. *Biochim Biophys Acta Mol Basis Dis* 2019; **1865**:2293–302.

53 Yu H, Kalogeris T, Korthuis RJ. Reactive species-induced microvascular dysfunction in ischemia/reperfusion. *Free Radic Biol Med* 2019; **135**:182–97.

54 Supinski GS, Schroder EA, Callahan LA. Contemporary review in critical care medicine: mitochondria and critical illness. *Chest* 2019; **157**:310–22.

55 Kozlov AV, Lancaster JR Jr, Meszaros AT, Weidinger A. Mitochondria-meditated pathways of organ failure upon inflammation. *Redox Biol* 2017; **13**:170–81.

56 Smallwood MJ, Nissim A, Knight AR, Whiteman M, Haigh R, Winyard PG. Oxidative stress in autoimmune rheumatic diseases. *Free Radic Biol Med* 2018; **125**:3–14.

57 Breda CNS, Davanzo GG, Basso PJ, Saraiva Camara NO, Moraes-Vieira PMM. Mitochondria as central hub of the immune system. *Redox Biol* 2019; **26**:101 255.

58 Banoth B, Cassel SL. Mitochondria in innate immune signaling. *Transl Res* 2018; **202**:52–68.

59 West AP. Mitochondrial dysfunction as a trigger of innate immune responses and inflammation. *Toxicology* 2017; **391**:54–63.

60 Jin HS, Suh HW, Kim SJ, Jo EK. Mitochondrial control of innate immunity and inflammation. *Immune Netw* 2017; **17**:77–88.

61 Buyck JM, Verriere V, Benmahdi R, Higgins G, Guery B, Matran R *et al.* aeruginosa LPS stimulates calcium signaling and chloride secretion via CFTR in human bronchial epithelial cells. *J Cyst Fibros* 2013; **12**:60–7.

62 Martin L, Pingle SC, Hallam DM, Rybak LP, Ramkumar V. Activation of the adenosine A3 receptor in RAW 264.7 cells inhibits lipopolysaccharide-stimulated tumor necrosis factor-alpha release by reducing calcium-dependent activation of nuclear factor-kappaB and extracellular signal-regulated kinase 1/2. *J Pharmacol Exp Ther* 2006; **316**:71–8.

63 Luo SF, Wang CC, Chien CS, Hsiao LD, Yang CM. Induction of cyclooxygenase-2 by lipopolysaccharide in canine tracheal smooth muscle cells: involvement of p42/p44 and p38 mitogen-activated protein kinases and nuclear factor-kappaB pathways. *Cell Signal* 2003; **15**:497–509.

64 Chen BC, Hsieh SL, Lin WW. Involvement of protein kinases in the potentiation of lipopolysaccharide-induced inflammatory mediator formation by thapsigargin in peritoneal macrophages. *J Leukoc Biol* 2001; **69**:280–8.

65 Park YC, Jun CD, Kang HS, Kim HD, Kim HM, Chung HT. Role of intracellular calcium as a priming signal for the induction of nitric oxide synthesis in murine peritoneal macrophages. *Immunology* 1996; **87**:296–302.

66 Ohmori Y, Hamilton TA. Ca^{2+} and calmodulin selectively regulate lipopolysaccharide-inducible cytokine mRNA expression in murine peritoneal macrophages. *J Immunol* 1992; **148**:538–45.

67 Chen YJ, Lin-Shiau SY. The role of endoplasmic reticular Ca(2+) stores in cell viability and tumor necrosis factor-alpha production of the murine macrophage RAW 264.7 cell line. *J Biomed Sci* 2000; **7**:122–7.

68 Feske S, Okamura H, Hogan PG, Rao A. Ca^{2+}/calcineurin signalling in cells of the immune system. *Biochem Biophys Res Commun* 2003; **311**:1117–32.

69 Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 1998; **392**:933–6.

70 Jennings C, Kusler B, Jones PP. Calcineurin inactivation leads to decreased responsiveness to LPS in macrophages and dendritic cells and protects against LPS-induced toxicity in vivo. *Innate Immun* 2009; **15**:109–20.

71 Kim Y, Moon JS, Lee KS, Park SY, Cheong J, Kang HS *et al.* Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. *Biochem Biophys Res Commun* 2004; **314**:695–703.

72 Biswas G, Anandatheerthavarada HK, Zaidi M, Avadhani NG. Mitochondria to nucleus stress signaling: a distinctive mechanism of NFkappaB/Rel activation through calcineurin-mediated inactivation of IkappaBbeta. *J Cell Biol* 2003; **161**:507–19.

73 Biswas G, Tang W, Sondheimer N, Guha M, Bansal S, Avadhani NG. A distinctive physiological role for IkappaBbeta in the propagation of mitochondrial respiratory stress signaling. *J Biol Chem* 2008; **283**:12 586–94.

74 Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 2002; **298**:1241–5.

75 Tergaonkar V, Correa RG, Ikawa M, Verma IM. Distinct roles of IkappaB proteins in regulating constitutive NF-kappaB activity. *Nat Cell Biol* 2005; **7**:921–3.

76 Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes Dev* 2004; **18**:2195–224.

77 Sen R, Smale ST. Selectivity of the NF-{kappa}B response. *Cold Spring Harb Perspect Biol* 2010; **2**:a000257.

78 Scheibel M, Klein B, Merkle H, Schulz M, Fritsch R, Greten FR *et al.* IkappaBbeta is an essential co-activator for LPS-induced IL-1beta transcription in vivo. *J Exp Med* 2010; **207**:2621–30.

79 Rao P, Hayden MS, Long M, Scott ML, West AP, Zhang D *et al.* IkappaBbeta acts to inhibit and activate gene expression during the inflammatory response. *Nature* 2010; **466**:1115–9.

80 McKenna S, Butler B, Jatana L, Ghosh S, Wright CJ. Inhibition of IkappaBbeta/NFkappaB signaling prevents LPS-induced IL1beta expression without increasing apoptosis in the developing mouse lung. *Pediatr Res* 2017; **82**:1064–72.

81 Butler B, De Dios R, Nguyen L, McKenna S, Ghosh S, Wright CJ. Developmentally regulated innate immune NFkappaB signaling mediates IL-1alpha expression in the perinatal murine lung. *Front Immunol* 2019; **10**:1555.

82 Li J, Ma Z, Tang ZL, Stevens T, Pitt B, Li S. CpG DNA-mediated immune response in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2004; **287**:L552–8.

83 Yi AK, Krieg AM. CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. *J Immunol* 1998; **160**:1240–5.

84 Granatiero V, De Stefani D, Rizzuto R. Mitochondrial calcium handling in physiology and disease. *Adv Exp Med Biol* 2017; **982**:25–47.

85 Marchi S, Patergnani S, Missiroli S, Morciano G, Rimessi A, Wieckowski MR *et al.* Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. *Cell Calcium* 2018; **69**:62–72.

86 Bravo-Sagua R, Parra V, Lopez-Crisosto C, Diaz P, Quest AF, Lavandero S. Calcium transport and signaling in mitochondria. *Compr Physiol* 2017; **7**:623–34.

87 Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protocols Immunol* 2008; Chapter **14**: Unit 14 1.

88 Kearns JD, Basak S, Werner SL, Huang CS, Hoffmann A. IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. *J Cell Biol* 2006; **173**:659–64.

89 Beg AA, Sha WC, Bronson RT, Baltimore D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev* 1995; **9**:2736–46.

90 McKenna S, Gossling M, Bugarini A, Hill E, Anderson AL, Rancourt RC *et al.* Endotoxemia induces IkappaBbeta/NF-kappaB-dependent endothelin-1 expression in hepatic macrophages. *J Immunol* 2015; **195**:3866–79.

91 McKenna S, Wright CJ. Inhibiting IkappaBbeta-NFkappaB signaling attenuates the expression of select pro-inflammatory genes. *J Cell Sci* 2015; **128**:2143–55.

92 Shintani Y, Drexler HC, Kioka H, Terracciano CM, Coppen SR, Imamura H *et al.* Toll-like receptor 9 protects non-immune cells from stress by modulating mitochondrial ATP synthesis through the inhibition of SERCA2. *EMBO Rep* 2014; **15**:438–45.

93 Huang E, Showalter L, Xu S, Czernliecki BJ, Koski GK. Calcium mobilizing treatment acts as a co-signal for TLR-mediated induction of Interleukin-12 (IL-12p70) secretion by murine bone marrow-derived dendritic cells. *Cell Immunol* 2017; **314**:26–35.

94 Mariero LH, Torp MK, Heiestad CM, Baysa A, Li Y, Valen G *et al.* Inhibiting nucleolin reduce inflammation induced by mitochondrial DNA in cardiomyocytes exposed to hypoxia and reoxygenation. *Br J Pharmacol* 2019; **176**:4360–72.

95 McKinsey TA, Chu ZL, Ballard DW. Phosphorylation of the PEST domain of IkappaBbeta regulates the function of NF-kappaB/IkappaBbeta complexes. *J Biol Chem* 1997; **272**:22 377–80.

96 Tran K, Merika M, Thanos D. Distinct functional properties of IkappaB alpha and IkappaB beta. *Mol Cell Biol* 1997; **17**:5386–99.

97 Link E, Kerr LD, Schreck R, Zabel U, Verma I, Baeuerle PA. Purified I kappa B-beta is inactivated upon dephosphorylation. *J Biol Chem* 1992; **267**:239–46.

98 Kerr LD, Inoue J, Davis N, Link E, Baeuerle PA, Bose HR Jr *et al.* The rel-associated pp40 protein prevents DNA binding of Rel and NF-kappa B: relationship with I kappa B beta and regulation by phosphorylation. *Genes Dev* 1991; **5**:1464–76.

99 Biswas G, Adebanjo OA, Freedman BD, Anandatheerthavarada HK, Vijayasarathy C, Zaidi M *et al.* Retrograde Ca^{2+} signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk. *EMBO J* 1999; **18**:522–33.

100 Horng T. Calcium signaling and mitochondrial destabilization in the triggering of the NLRP3 inflammasome. *Trends Immunol* 2014; **35**:253–61.

101 Piantadosi CA. Mitochondrial DNA, oxidants, and innate immunity. *Free Radic Biol Med* 2020; **S0891-5849**:31575–8.

102 Benjamin JT, Moore DJ, Bennett C, van der Meer R, Royce A, Loveland R *et al.* Cutting edge: IL-1alpha and not IL-1beta drives IL-1R1-dependent neonatal murine sepsis lethality. *J Immunol* 2018; **201**:2873–8.

103 St Peter SD, Moss AA, Mulligan DC. Effects of tacrolimus on ischemia-reperfusion injury. *Liver Transpl* 2003; **9**:105–16.