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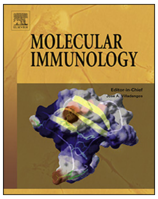

Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression

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ABSTRACT

Small Ras GTPases are key molecules that regulate a variety of cellular responses in different cell types. Rap1 plays important functions in the regulation of macrophage biology during inflammation triggered by toll-like receptors (TLRs). However, despite sharing a relatively high degree of similarity with Rap1, no studies concerning Rap2 in macrophages and innate immunity have been reported yet. In this work, we show that either way alterations in the levels of Rap2a hampers proper macrophages response to TLR stimulation. Rap2a is activated by LPS in macrophages, and although putative activator TLR-inducible Ras guanine exchange factor RasGEF1b was sufficient to induce, it was not fully required for Rap2a activation. Silencing of Rap2a impaired LPS-induced production of IL-6 cytokine and KC/Cxcl1 chemokine, and also NF-κB activity as measured by reporter gene studies. Surprisingly, overexpression of Rap2a did also lead to marked inhibition of NF-κB activation induced by LPS, Pam3CSK4 and downstream TLR signaling molecules. We also found that Rap2a can inhibit the LPS-induced phosphorylation of the NF-κB subunit p65 at serine 536. Collectively, our data suggest that expression levels of Rap2a in macrophages might be tightly regulated to avoid unbalanced immune response. Our results implicate Rap2a in TLR-mediated responses by contributing to balanced NF-κB activity status in macrophages.

1. Introduction

Innate immune system is a set of a variety of cells and molecules, including toll-like receptors (TLRs) for pathogen recognition that are mainly expressed in macrophages, forming a part of a complex whole for the organism's defense. Upon recognition, TLRs prompt intracellular activation of critical downstream signaling pathways that eventually culminates the production of proinflammatory cytokines. Properly controlled activation of NF-κB and mitogen-activated protein kinases (MAPKs) is a result of coordinated recruitment of intracellular signaling molecules and cascade events involving mainly adaptor molecule MyD88, kinases IRAK1/4, ubiquitin ligase TRAF6, and TAK1 and IKK's complex (Kawasaki and Kawai, 2014). This is imperative for governing the transcriptional activation of an array of genes encoding proinflammatory cytokines, including TNF and IL-6 (Beutler, 2009). Given its complexity, it is likely that cellular components other than from innate immune system pathway participate in regulating the production of inflammatory mediators.

Ras small GTPases are critical molecular switches that connect extracellular signals to intracellular signaling pathways initiated by membrane receptors, such as growth factor receptors and G protein-coupled receptors (Simanshu et al., 2017). When shifting between active GTP-bound and inactive GDP-bound form, Ras-small GTPases coordinate several cellular functions as proliferation, cell differentiation, apoptosis, migration, adhesion, and gene expression (Sandí et al., 2017; Wen et al., 2018; Stankiewicz et al., 2015; Muñoz-Félix et al., 2016; Xu et al., 2016)

The subfamily of Ras-like small GTPases contains numerous members including classical Ras (H-, K-and N-Ras), R-Ras, TC21/R-Ras2, MRas/R-Ras3, DexRas1/RasD1, RalA/B, Rheb, Rit, Rin, Rap1 and Rap2,

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and atypical κB-Ras1 and κB-Ras2 (Goitre et al., 2014; Fenwick et al., 2000). Despite considerable structural, sequence and biochemical similarities, these proteins are found differentially expressed in tissues and play multiple and divergent roles, including regulation of immunity and inflammation (Johnson e Chen, 2012).

Macrophages in innate immune system have implicated distinct requirements for Ras-like small GTPases, including Rap1 (Katagiri et al., 2000; Caron et al., 2000; Tang et al., 2014). Rap1 is capable of regulating the activation of the integrin αMβ2 in response to LPS and other inflammatory mediators in macrophages (Caron et al., 2000). Expression of B-cell activating factor (BAFF) in macrophages stimulated with LPS is regulated, in part, through Epac1-mediated Rap1 activation (Moon et al., 2011). Pathogen associated molecular patterns (PAMPs) recognition by TLR2 and TLR5 in monocytes triggers inflammatory cell infiltration to the site of pathogen invasion or sterile tissue injury via Rap1 GTPase-dependent β2-integrin activation (Chung et al., 2014). In addition, through RasGRP3-dependent activation, Rap1 is implicated in Toll-like receptor (TLR)-triggered inflammatory response by limiting IL-6 cytokine production in macrophages (Tang et al., 2014).

Rap2, the closest relative of Rap1 and sharing ˜60% identity, had been long thought to be functionally analogous to Rap1. Contrary to Rap1 function, however, Rap2 was initially shown unable to reverse Ras-induced transformation of fibroblasts (Jimenez et al., 1991). To date, specific roles for Rap2 in a number of cellular processes have been reported (Bruurs and Bos, 2014; Wang et al., 2017; Kong et al., 2007; Meng et al., 2018). Biological functions of Rap2 in macrophages and inflammation, however, have remained largely unexplored. In addition, despite a number of guanine exchange factors implicated in the activity for Rap2 (De Rooij et al., 1999, 2000; Yaman et al., 2009; Gao et al., 2001), a few have been characterized in inflammatory responses (Scott et al., 2016; Silva et al., 2018 - submitted). Among reports associating Rap2 proteins with immunity, it has recently been demonstrated that microtubule BCR receptor polarization is impaired in Rap2c depleted B lymphocytes (Wang et al., 2018). Furthermore, the involvement of Rap2 proteins in neutrophil migration and adhesion has been described (Gera et al., 2017; Jenei et al., 2006).

Therefore, data is lacking to date that has investigated specific roles for Rap2 molecules in macrophages. In this work, we asked if Rap2a plays a role in the regulation of TLR-triggered response of macrophages. To wealth of our knowledge, our results show for the first time that Rap2a is activated upon LPS stimulation of macrophages. In addition, we found that cytokine production and NF-κB activation were impaired in macrophages silenced of Rap2a. Strikingly, overexpression of the GTPase also inhibited NF-κB by interfering in critical intracellular molecules engaged in TLR pathway. Our findings provide evidence that implicate Rap2a GTPase in the well-studied TLR signaling pathway to NF-κB and suggest that dysregulation of Rap2a expression in an inflammatory context may significantly alter the status of NF-κB activation.

2. Material and methods

2.1. Cell cultures

Human HEK293 cells were cultured in DMEM medium supplemented with 10% FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). RAW264.7 and RAW-κB-luc macrophages were cultured in DMEM supplemented with 10% FBS.

2.2. Generation of bone marrow derived macrophages (BMDMs)

Protocols for animal handling was approved by CEUA-UFMG (nos. 069/2014 and 047/2017). Femurs from eight to 12 week-old C57BL/6 (provided by CEBIO-UFMG) and RasGEF1b-KO mice (Silva et al., 2018 – submitted) were used to isolate bone marrow cells. Briefly, bone marrow cells were plated in non-treated tissue 10-cm^2 plates and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated FBS (Gibco Invitrogen), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Invitrogen), 2-mercaptoethanol as well as 30% (v/v) L929 cell conditioned medium. Culture medium was replaced by fresh medium every 2–3 days and adherent macrophage monolayers were obtained within seven days. Differentiated bone marrow-derived macrophages (BMDMs) were harvested by gently resuspending the cells from the dishes using cold PBS and seeded onto cell cultures plates for the experiments.

2.3. Cell treatments

When indicated, cells were stimulated with 100 ng/ml or 1μg/ml of LPS O55: B5, 100 μg/ml poly-IC, 100 ng/ml Pam3CSK4 and PMA at 100 nM. All reagents were purchased from by Sigma-Aldrich, USA.

2.4. Quantitative RT-qPCR and RT-PCR

Total RNA was extracted using TRIzol® according to the manufacturer's instructions (Life Technologies, USA). Total RNA was reverse transcribed using MMLV reverse transcriptase (Life Technologies). Quantitative PCR was performed using iQSybr Master Mix kit (BioRad) in the CFX96 Touch™ Real Time detection system (BioRad). Oligonucleotide primer sequences were either obtained from the PrimerBank public database (Wang et al., 2012) or designed through freely available Primer3 website. Oligonucleotide synthesis was carried by Integrated DNA Technologies (IDT Inc., USA), and the following primers were used: Rap2a-FWD, AATACGACCCCACCATCGAG and Rap2a-REV ACCTTCTCATACCGCTTCACG; Rap2b-FWD, 5`-GCTCACC GTGCAGTTCGTAA-3` and Rap2b-REV, 5`-GCTGTAGACGAGAATGAA GCC-3`; Rap2c-FWD, 5`-ATGAGGGAATACAAGGTAGTGGT-3` and Rap2c-REV, 5`-ACTTCGATCTCTTTGCGGTAGA-3`; IL-6-FWD, 5`- TAG TCCTTCCTACCCCAATTTCC-3` and IL-6-REV, 5`- ACGACCAAATCCGT TGACTC-3`; TNF-α-FWD, 5`- ACAGAAAGCATGATCCGCG-3`and TNF-α-REV, 5`- GCCCCCCATCTTTTGGG-3`; Nos2-FWD, 5`-GCTTGCCCCAA CAGGAGAAG-3` and Nos2-REV, 5`- GCTGCCCGGAAGGTTTGTAC-3`; Rpl32-FWD, 5′-GCTGCCATCTGTTTTACGG-3′ and Rpl32-REV, 5′-TGA CTGGTGCCTGATGAACT-3′. Where indicated, expression data obtained in the quantitative RT-PCR were normalized to those of the reference gene *Rpl32* (Large Ribosomal Subunit Protein L32). The data were analyzed according to the comparative method of the cycle threshold, according to the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.5. Purification of GST-RalGDS RBD domain and Rap2a activity assay

Procedures used in this work for the generation of the active probes and pull-down of Rap-GTP have been described previously (Van Triest et al., 2001). Briefly, RalGDS RBD domain sequence was amplified from mouse embryonic fibroblasts (MEFs) and cloned into plasmid pGEX-6P-1 (see Fig. 1a–d of Carvalho et al., 2019). Active probes of GST-RalGDS RBD were expressed from the transformation of plasmids pGEX-6P-1-RalGDS-RBD into IPTG-induced BL21 bacteria (see Fig. 2a–c of Carvalho et al., 2019). Pull-down assays for active Rap2a were carried out from cell extracts obtained from RAW264.7 cells or BMDMs. After treatments, cells were washed with cold PBS and lysed in cold lysis buffer (Tris HCl 50 mM pH 7.4; NaCl 200 mM; 2.5 mM $MgCl_2$; 50 mM NaF; 10% v/v glicerol; 1% v/v nonidet P-40, and protease and phosphatase inhibitors) and centrifuged at 4 °C. Purified GST-RalGDS RBD domain previously incubated with beads coated with glutathione sepharose were then used to pull-down Rap-GTP proteins from cell extracts. After several washes with lysis buffer, the purified extract was fractionated by immunoblotting with antibody specific for Rap2a detection.

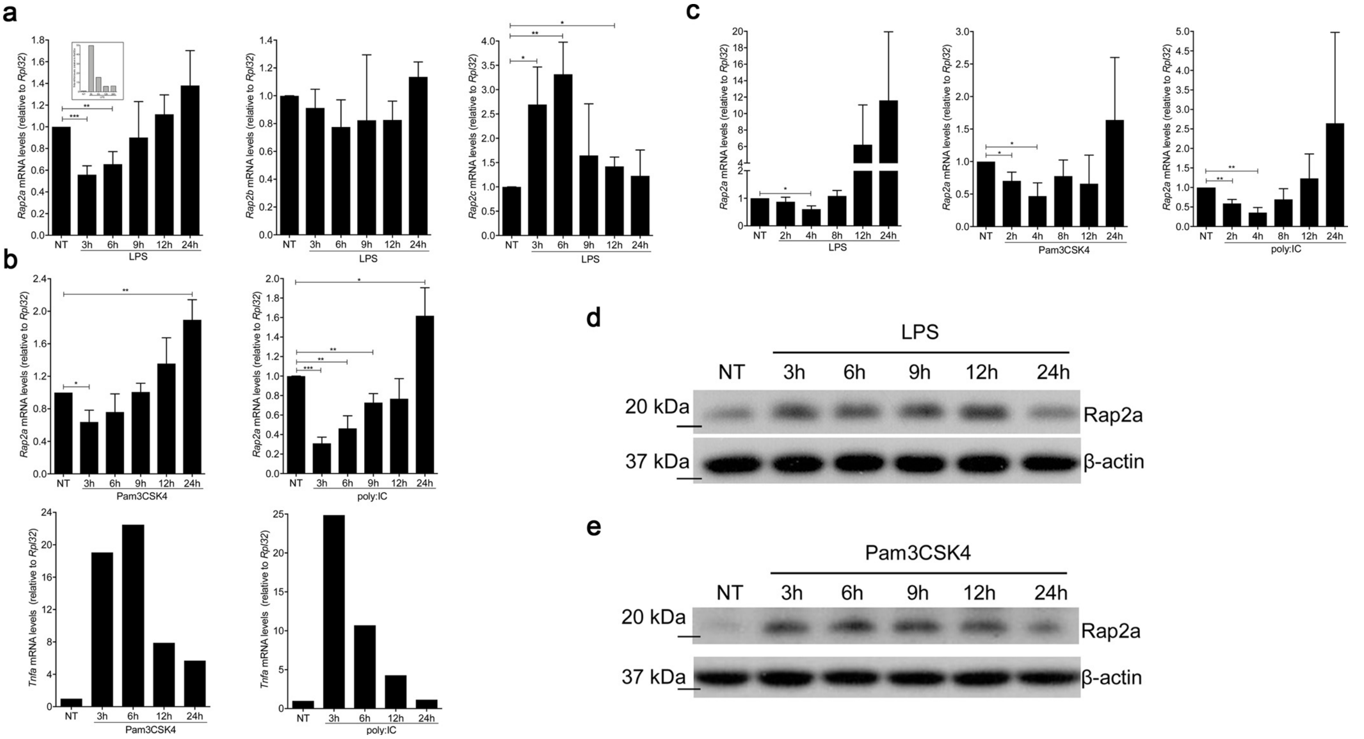

Fig. 1. Macrophages differentially express Rap2 GTPases mRNA levels; Rap2a protein levels increase upon TLR stimulation. (**a**, **b**) RAW264.7 cells or (**c**) BMDMs were stimulated with LPS (100 ng /ml) or Pam3CSK4 (100 ng /ml) or poly: IC (100 μg /ml) for different time intervals. At the end of the stimulations, total RNA was extracted and subsequently transcribed into cDNA which was used as template in RT-qPCR reactions for detection of Rap2a, Rap2b, Rap2c or TNF mRNAs. NT, *non-treated*. Inset graph in "a", *TNF expression in LPS-treated samples*. Data represent mean ± SD, n = 3. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's unpaired t-test). (**d, e**) Cell lysates of RAW264.7 cells harvested at the indicated time points were immunoblotted with anti-Rap2a antibody. Equal loadings of cell extracts were assessed by immunoblotting with anti-beta-actin.

2.6. Immunoblotting

Cells were washed with cold PBS and then lysed with specific buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 10 mM beta-glycerolphosphate, 0.1 mM EDTA a pH 8.0, 10% glycerol, 1% Triton X-100) containing protease inhibitors (1 mM PMSF, 2 mM Orthovanadate Sodium, 1 μg/ml Pepstatin, 2 mg/ml Aprotinin, 1 μg/ml Leupeptin). Protein concentration was determined by Bradford colorimetric assay (Bio-Rad) using bovine serum albumin (BSA) as standard. Total protein extracts were fractionated on 12% SDS-PAGE, followed by transfer to PVDF membranes (Millipore). The membranes were blocked and incubated with the following antibodies to the following antigens: anti-Rap2a (ThermoFisher Scientific); anti-Myc; anti-Flag M2 (Sigma); anti-phospho-AKT (S473); anti-phospho-ERK1/2 (Thr202/Tyr204); anti-AKT; anti-ERK1/2 (all from Cell Signaling); and mouse anti-beta actin (Sigma-Aldrich). After incubation with primary antibodies and washings, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, followed by detection with enhanced chemiluminescent substrate according to the manufacturer's protocol (Clarity® ECL, BioRad).

2.7. Plasmids

pRL-TK and pGL3-promoter plasmids were provided by Promega. pFLAG-CMV4 supplied by Sigma. pcDNA3.1-Flag-RasGEF1b purchased from Genescript (clone ID: OMu11992). pCIneo-Myc-Rap2a kindly provided by Ken-ichi Kariya (University of Ryukyus, Japan). pGL3-E-selectin; pFLAG-TLR2, -TLR3, -TLR4; pcDNA-TAB1; pFLAG-TAK1; pFLAG-Mal / TIRAP kindly provided by Bryan Williams (Cleveland Clinic, USA). pFLAG-IKKα, -TRAF6, -p65 and pHA-IKKα kindly provided by Daniel Mansur (UFSC, Brazil). pcDNA3-CD14 and pFLAG-CMV1-hMD-2 kindly provided by Douglas Golenbock (University of Massachusetts, USA).

2.8. Transient transfections

RAW264.7 cells (2×10^5/well) were transfected with endotoxin free preparations (GenElute™ Endotoxin-free Plasmid Midiprep Kit, Sigma-Aldrich) of NF-κB reporters construct (400 ng), pRL-TK plasmid (100 ng), and control and Rap2a-siRNA (Ambiom Silencer select®, sequence CCUUCAUUGAGAAAUACGAtt) at 100 nM using RNAiMax or Lipofectamine transfection reagents (Life Technologies). Stimuli were performed 48 h after transfections. HEK293 cells (1×10^5/per well) growing in 24-well plates were transiently transfected with NF-κB constructs luciferase reporter plasmids (400 ng) and 100 ng pRL-TK using Polyethylenimine "MAX" MW 40,000 (PEI MAX 40K) from Polysciences, Inc., USA. Where indicated, pGL3-E-selectin was replaced by pGL3-promoter in the amount of 400 ng. The plasmids for TLR pathway molecules overexpression were co-transfected with the reporter plasmids when indicated in the figures. Plasmids for overexpression of the Mal / TIRAP molecules; TRAF6; IKKα; IKKβ; and p65 were transfected in the amount of 500 ng per well. TAK1 and TAB1 were transfected at 250 ng each per well. TLR2 and TLR3 were transfected in amounts of 50 ng per well and TLR4, CD14 and MD2 in the amounts of 30 ng, 30 ng and 5 ng respectively per well. The Rap2a plasmids were transfected in different quantities per well as indicated in the figures. After transfections and stimuli (when indicated) cells were lysed with passive lysis buffer (PLB1x, Promega). The lysates were processed in accordance with the Dual-Luciferase Reporter Assay Kit from Promega protocol and measurements were performed in Lumicount-Packard luminometer. The ratio of firefly luciferase to Renilla luciferase was calculated and the results presented as relative luciferase activity.

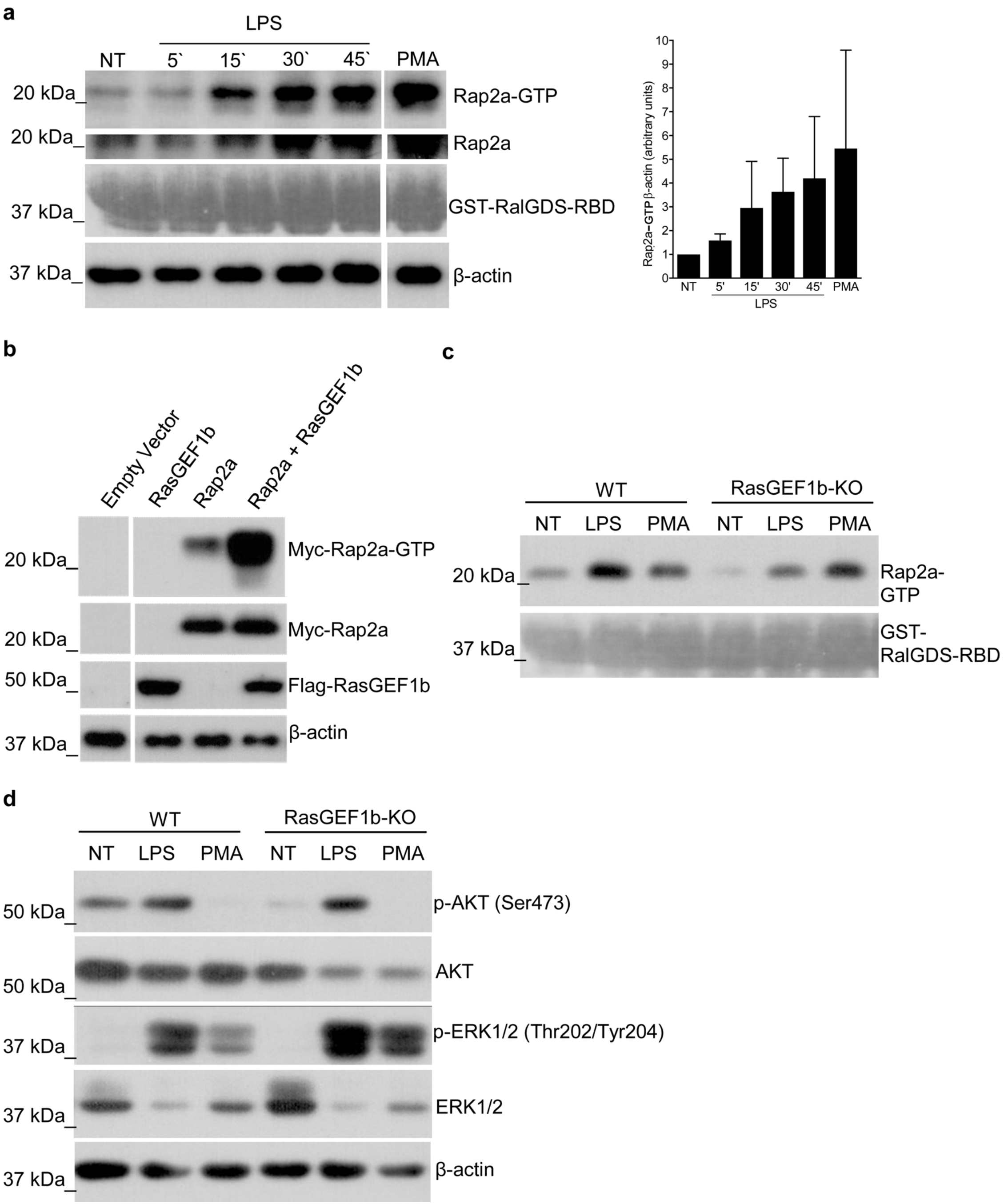

Fig. 2. LPS activates Rap2a in macrophages. (**a**) RAW264.7 cells were stimulated with LPS (1 μg/ml) or PMA (100 nM) as indicated. Lysates were harvested and used in pull-down assay followed by immunoblotting specific for Rap2a. PonceauS staining was performed for GST-RalGDS-RBD detection. Representative western blot is shown. Densitometrical analysis is shown on the right. The data represent mean ± SD of two biological replicates. (**b**) HEK293 cells were transfected with pFLAG-CMV4 (empty vector) or pcDNA3.1-Flag- RasGEF1b along with pCIneo-Myc-Rap2a as indicated. After 24 h, cells were lysed and protein extracts were processed as in "a". Immunoblottings using anti-Myc, anti-Flag and anit-β-actin were carried out using total cell extracts. (**c**, **d**) Wild type BMDMs (WT) or RasGEF1b knockout BMDMs (RasGEF1b-KO) were stimulated with LPS (1 μg/ml) or PMA (100 nM). Cells were lysed and processed as in "a". (**c**) Rap-GTP molecules were captured from the lysates by pull-down assay and then used in specific immunoblotting for Rap2a. PonceauS staining was performed for GST-RalGDS-RBD detection. (**d**) Total extracts were used in the immunoblotting of phospho-AKT (p-AKT), AKT, phospho-ERK (p-ERK), ERK and β-actin. NT, *non-treated*. n = 2 for all experiments described above.

2.9. ELISA

KC/Cxcl1 and IL-6 concentrations in cell cultures supernatants were determined using commercially available ELISA kits, according to the manufacturer's instructions (R&D Systems).

2.10. Statistical analyses

Statistical analyses were performed using Prism 5.0 software (GraphPad). Results are given as mean ± SD where indicated. Comparisons between two groups were done using Student's t-test. Multiple comparisons were done with ANOVA. Statistical significance was determined as *p < 0.05; **p < 0.01; ***p < 0.001.

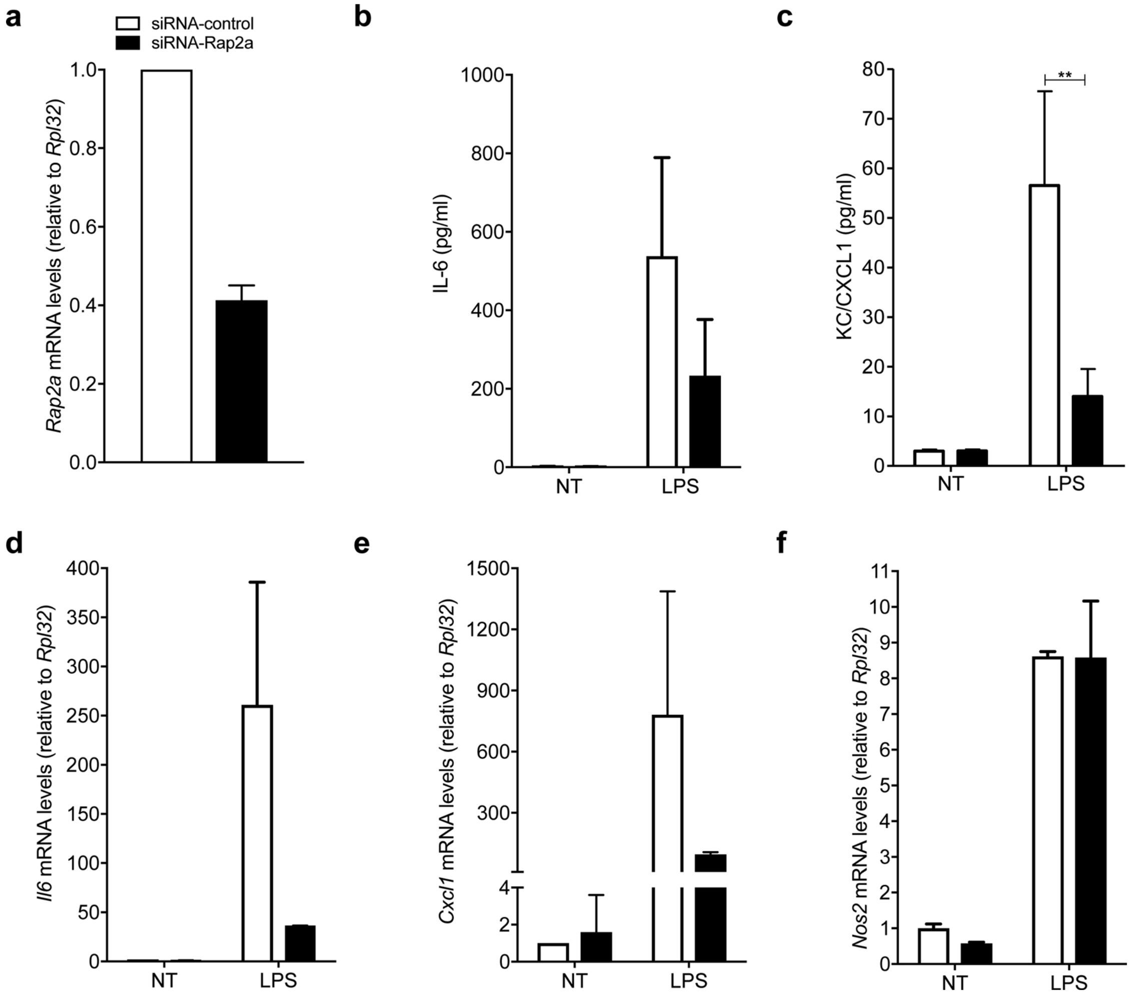

Fig. 3. Silencing of Rap2a expression impairs LPS-induced expression and production of inflammatory mediators in macrophages. (**a-f**) RAW264.7 cells were transfected with siRNA-control or siRNA-Rap2a for 48 h. Then, cells were either (**a**) lysed to obtain total RNA or (**b-f**) stimulated with LPS (100 ng /ml) for 8 h. To evaluate the production of (**b**) IL-6 or (**c**) KC/Cxcl1, ELISA assays were performed from the supernatants collected at the end of stimuli. NT, *non-treated*. Expression analyses of (**a**) Rap2a, (**d**) IL-6, (**e**) KC/Cxcl1, and (**f**) iNOS were evaluated by means of RT-qPCR reactions. Data represent mean ± SD, n = 2 for RT-qPCRs and n = 3 for ELISAs. ** $p < 0.01$ (Student's t-test).

3. Results

3.1. TLRs agonists induce changes in the expression of Rap2a in macrophages

In general, molecules implicated in intracellular pathways in immune system undergo changes in their expression and activity upon antigen recognition by the cell. However, studies are lacking that have examined the expression of members of the Rap2 GTPases in the immune response. Therefore, we evaluated the mRNA levels of Rap2a, b and c in RAW264.7 macrophages upon treatment with TLR agonists. The mRNA levels of Rap2a and Rap2c, but not Rap2b, are significantly altered upon treatment with LPS (Fig. 1a). While Rap2c is upregulated, the expression of Rap2a is slightly downregulated up to 6 h post-stimulation. This occurs in a transient manner as seen by a recovery after 9 h and 12 h. After 24 h post-stimulation, the levels returned greater than those detected in control untreated cells. Additional endpoint PCR analysis of Rap2a amplicons confirmed diminished expression after 3 h and 6 h with LPS or poly-IC (an agonist of TLR3) treatments (see Fig. 3a of Carvalho et al., 2019). Because of the numerous Ras family small GTPases, it is important to know which specific small GTPase regulates a given cellular process. Therefore, we focused on Rap2a to better examine its expression, as well as its activation and its potential role in the innate immune response mediated by TLRs. Rap2a mRNA levels are significantly reduced after stimulation with other TLRs agonists in RAW264.7 cells and primary bone marrow derived macrophages (BMDMs) (Fig. 1b, c). Of note, the expression of TNF confirms the responsiveness of cells in the experiments (Fig. 1a, b). None expressive changes in Rap2a mRNA levels were seen at earlier time points upon LPS treatment (see Fig. 3b of Carvalho et al., 2019). We also examined Rap2a mRNA levels in human monocytes (THP-1 cells) treated with LPS, and similar results were obtained upon LPS stimulation at earlier time points (see Fig. 3c of Carvalho et al., 2019). Strikingly, in contrast to mRNA levels, Rap2a protein levels are increased after LPS treatments of macrophages, and this was sustained up to 12 h (Fig. 1d). Similar results were obtained upon Pam3CSK4 treatment (Fig. 1e). Why do TLR agonists induce such opposite alterations in mRNA and protein levels is intriguing, but it clearly suggests that by undergoing changes in its expression, Rap2a may play an important role in innate immunity mediated by TLR2 and TLR4 pathways.

3.2. Rap2a is activated in LPS-treated macrophages independent of TLR-inducible RasGEF1b

Like other Ras-like small GTPases, Rap2a cycles between an inactive GDP-bound and an active GTP-bound form to regulate specific molecular events in signaling transduction pathways. Therefore, we assessed whether Rap2a undergoes activation in macrophages stimulated with

LPS. To this, RAW264.7 macrophages were treated with LPS and cell extracts obtained to assess the GTP-bound Rap2a. Despite untreated cells contain detectable levels of Rap2a-GTP, the result (Fig. 2a) shows that LPS induced robust activation of Rap2a in macrophages after 15 min post-treatment. We also examined and determined the activation of Rap2a in the cells treated with phorbol-12- myristate-13-acetate (PMA) because Rap1 has been shown to be activated by the mitogen in macrophages (Caron et al., 2000).

A previous biochemical study has suggested that GTP exchange on recombinant purified Rap2a is uniquely stimulated by the guanine nucleotide exchange factors RasGEF1a and RasGEF1b (Yaman et al., 2009). As we have recently reported (Silva et al., 2018), RasGEF1b expression in macrophages is markedly dominant over the RasGEF1 members (Silva et al., 2018). Therefore, we confirmed by overexpression studies that RasGEF1b has the ability to directly induce Rap2a activation in cells (Fig. 2b). No GTP-bound Rap2a levels were detected in cells transfected with RasGEF1b only, suggesting that HEK293 cells may express low levels of Rap2a. We next assessed the activation of Rap2a in LPS-treated or PMA-treated BMDMs devoid of RasGEF1b and compared with wild-type cells. We found that LPS treatment leads to robust increase in GTP-bound Rap2a in wild-type macrophages, but this was only reduced in cells devoid of RasGEF1b (Fig. 2c). Interestingly, GTP-bound Rap2a induced by PMA in the knock-out cells was as similar as seen in wild-type cells. Assessment of downstream effector pathways to Rap1/2 GTPases in RasGEF1b-KO cells indicates that AKT phosphorylation is unaffected, although an increase is seen in the phosphorylated ERK1/2 (Fig. 2d). Collectively, these results indicate that TLR stimulation of macrophages with LPS leads to robust activation of Rap2a, and that RasGEF1b contributes partially.

3.3. Silencing of Rap2a expression in macrophages impairs LPS-induced expression and production of IL-6 and KC/Cxcl1

A major outcome of macrophages to TLR activation is the production of inflammatory mediators. Therefore, to determine whether Rap2a plays any immune function in cytokines production, we knocked-down its expression by delivering specific siRNA into RAW264.7 cells, and further treated with LPS. Upon validation of a commercially available siRNAs specific to Rap2a (see Fig. 4 of Carvalho et al., 2019), we determined that the expression was significantly reduced in cells transfected with a specific siRNA (Fig. 3a). We then examined the production of acute phase cytokine IL-6, and the chemokine KC/Cxcl1. Interestingly, the production of IL-6 and KC/Cxcl1 was significantly reduced in LPS-treated cells where Rap2a was knocked-down (Fig. 3b, c). In contrast, TNF production was similar between siRNA control and siRNA Rap2a transfected cells (see Fig. 5 of Carvalho et al., 2019). The mRNA levels of the cytokine and chemokine were also impaired after LPS stimulation in cells silenced of Rap2a (Fig. 3d, e). In contrast, the mRNA levels of iNOS were comparable between siRNA control and siRNA Rap2a transfected cells (Fig. 3f).

3.4. Knock-down or overexpression of Rap2a affects NF-κB activation

Because IL-6 and KC/Cxcl1 expressions are mainly regulated by NF-κB (Tanaka et al., 2014; Amiri e Richmond, 2003), the above observations prompted us to investigate the effects of Rap2a silencing in the regulation of the activity of the transcription factor in macrophages. To this, we transfected RAW264.7 macrophages with siRNA-Rap2a and a plasmid encoding a luciferase regulated by NF-κB, and further treating them with LPS. The result (Fig. 4a) shows that cells transfected with the siRNA of Rap2a exhibit reduced NF-κB activation in comparison to cells transfected with siRNA-control. Similar effects were observed when using a distinct siRNA sequence (see Fig. 6 of Carvalho et al., 2019). To rule out potential technical artifacts due to reporter plasmid transfections, we also carried out the experiment in RAW-κB-luc cells that harbor an NF-κB reporter in a stable manner. We confirm that NF-κB activity was reduced in cells transfected with siRNA-Rap2a and treated with LPS (Fig. 4b). Because Rap2a knockdown reduced NF-κB activation in LPS-treated cells, we reasoned that by forcing Rap2a expression in cells would lead to an increase in NF-κB activity. Therefore, we transfected RAW264.7 cells with NF-κB luciferase reporter, with or without Rap2a encoding plasmid, followed by treatment of cells with LPS. Surprisingly, Rap2a reduced the activation of NF-κB (Fig. 4c). Similar results were obtained with human THP-1 monocytes treated with LPS (see Fig. 7 of Carvalho et al., 2019). We next sought to better clarify this phenomenon by co-transfecting the NF-κB reporter with TLR2, TLR3, and TLR4, CD14, MD2 and Rap2a encoding plasmids into

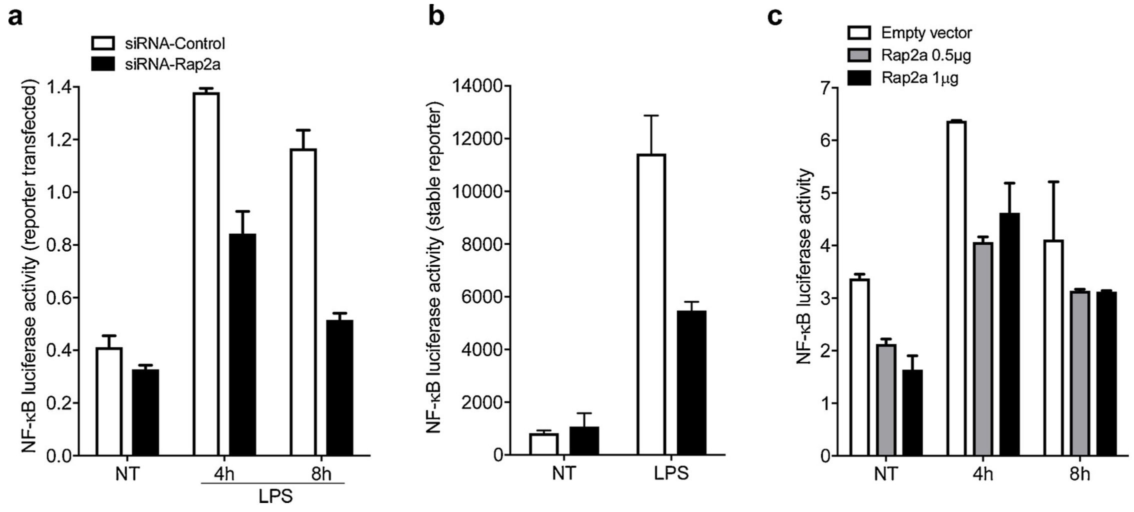

Fig. 4. Both depletion and overexpression of Rap2a hampers normal NF-κB activity induced by LPS in macrophages. (**a**) RAW264.7 cells were transfected with siRNA-control or siRNA-Rap2a along with the reporter plasmids pGL3-E-sel and pRL-TK for 48 h, and then stimulated with LPS (100 ng/ml) as indicated. (**b**) RAW264.7-κB-luc cells were transfected with siRNA-control or siRNA-Rap2a for 48 h and then stimulated with LPS (100 ng/ml) for 8 h. (**c**) RAW264.7 cells were transfected with the reporter plasmids pGL3-E-Sel and pRL-TK along with pFLAG-CMV4 (empty vector) or increasing amounts of pCIneo-Myc-Rap2a. Twenty-four hours post-transfection the cells were stimulated with LPS (100 ng/ml) as indicated. At the end of the experiments, luciferase activities were measured in a luminometer. DNA amounts were kept constant in the experiments. Results are shown as relative firefly luciferase activities normalized to (**a**, **c**) Renilla luciferase activities or (**b**) to the total amount of proteins in the lysates. Data represent mean ± SD, n = 2.

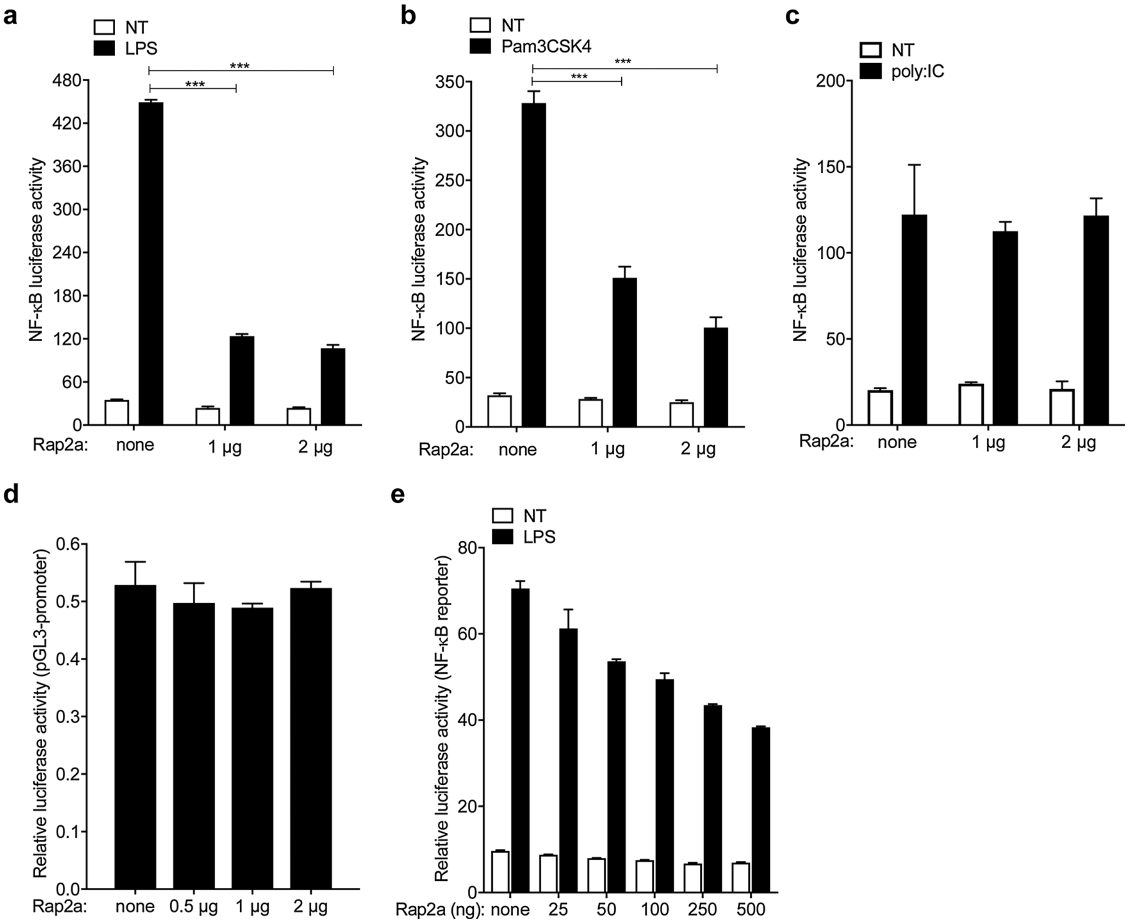

Fig. 5. Overexpression of Rap2a impairs NF-κB activity induced through TLR4 and TLR2, but not TLR3. HEK293 cells were transfected with (**a**, **e**) endofree plasmids preparations of MD2, CD14 and TLR4, (**b**) TLR2, or (**c**) TLR3 along with the NF-κB luciferase and pRL-TK reporters in combination with different amounts of pFLAG-CMV4 (vector) or pCIneo-Myc-Rap2a in indicated. Twenty-four hours post-transfection cells were stimulated with (**a**, **e**) LPS (1 µg/ml) or (**b**) Pam3CSK4 or (**c**) poly:IC for 18 h. (**d**) HEK293 cells were transfected with plasmids pGL3-promoter and pRL-TK in combination with different amounts of pCIneo-Myc-Rap2a in as indicated. Twenty-four hours post-transfection, cell extracts were harvested. Luciferase activities were determined in a luminometer. DNA amounts were kept constant in all experiments. Results are shown as relative firefly luciferase activities normalized to Renilla luciferase activities. Data represent mean ± SD of three independent experiments (n = 3).

HEK293 cells, and further treating the cells with the TLR ligands. Stimulation of cells with any TLR ligands augmented luciferase reporter activity, whereas coexpression of Rap2a potently suppressed TLR2 and TLR4-induced luciferase NF-κB reporter (Fig.5 a, b). Interestingly, poly-IC stimulation of cells in the presence of Rap2a did not inhibit reporter transactivation (Fig. 5c). To rule out the possibility that enforced expression of Rap2a unscrupulously disrupts any cellular steps to eventual luciferase synthesis, we transfected HEK293 cells with pGL3-promoter that express the luciferase in a constitutive manner. We found that luciferase activity remained largely unaltered in the increasing amounts of Rap2a (Fig. 5d). To provide additional evidence of Rap2a on inhibiting the LPS-induced NF-κB activity, we examined the effects of Rap2a when transfected at low amounts. The result (Fig. 5e) indicates that as little as 25 ng of transfected Rap2a are sufficient to reduce NF-κB activity.

One of the key mechanisms implicated in the regulation of NF-κB transcriptional activity in response to inflammatory stimuli is the phosphorylation of NF-κB at Ser536 of the p65 subunit. Since our results indicated that Rap2a inhibits NF-κB activation upon LPS stimulation, we examined whether Rap2a could affect p65 phosphorylation. As shown in Fig. 6, western-blot analysis indicates that co-transfection of 1 or 2 µg of Rap2a with TLR4-CD14-MD2 plasmids complex in HEK293 cells markedly inhibited p65 Ser536 phosphorylation after 30 and 60 min of LPS stimulation.

We next went to determine whether Rap2a inhibits NF-κB activation induced by downstream signaling molecules that are critical in TLR4 signaling. To this, we transfected HEK293 cells with NF-κB-luc reporter gene and TLR adapter Mal/TIRAP, TRAF6, TAK1/ TAB1, IKK-α, IKK-β, or NF-κB p65 subunit in the presence of empty or Rap2a encoding plasmids. By comparing the maximum activation of NF-κB induced by the expression of the adapter molecule, ubiquitin ligase or the kinases we found that NF-κB activity was reduced to up 2.5-fold by increasing amounts of Rap2a (Fig. 7a–e). Interestingly, Rap2a exhibited a more potent inhibitory effect on Mal/TIRAP and TRAF6-induced NF-κB activation. In contrast, Rap2a failed to inhibit NF-κB activity induced directly by the monomer p65(RelA) (Fig. 7f, g), suggesting that Rap2a interferes in NF-κB activation by affecting upstream signaling events to p65(RelA).

4. Discussion

In the present work, we report for the first time that Rap2a GTPase is activated by LPS and plays a role in macrophages response to TLR4 activation. Silencing of Rap2a in cells disturbed the expression and production of important inflammatory mediators. We also found that knock-down of Rap2a in macrophages led to impairment of NF-κB activity. Thus, we expected that overexpression of the GTPase should enhance the activation of the transcription factor. Surprisingly,

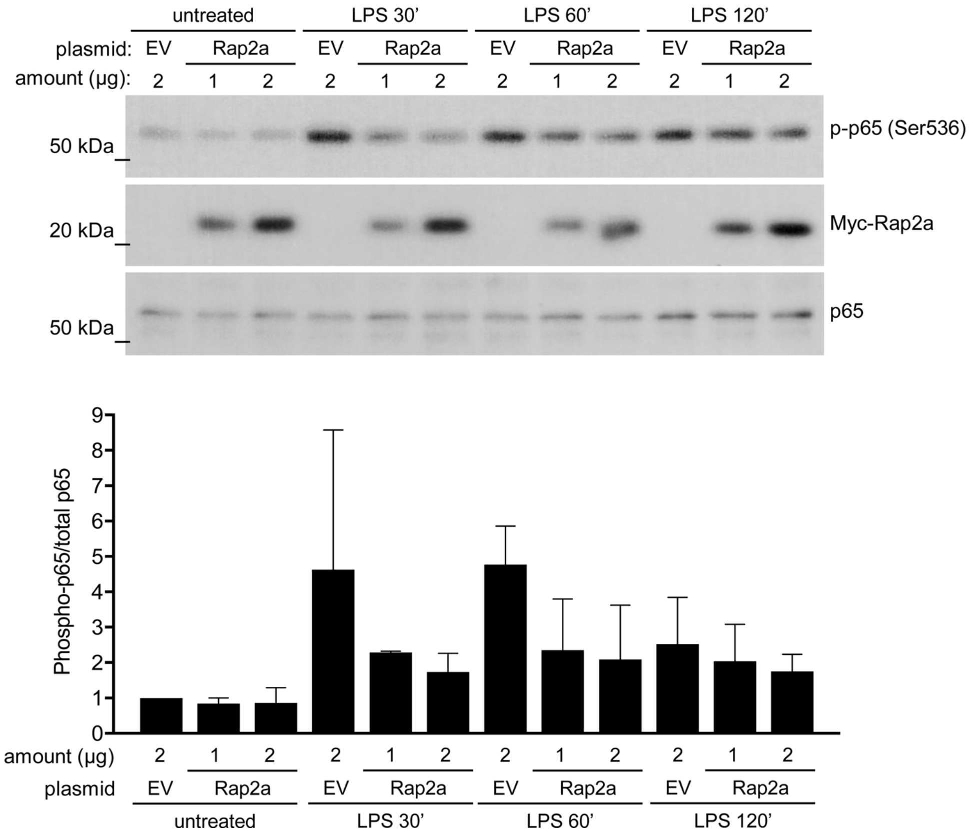

Fig. 6. Effects of Rap2a on p65(RelA)-Ser536 phosphorylation in the TLR4 pathway after LPS stimulation. HEK293 cells were transfected with endofree plasmid preparations of MD2, CD14 and TLR4 along with empty vector (EV) or different amounts of pCIneo-Myc-Rap2a as indicated. Twenty-four hours post-transfection cells were stimulated with LPS for different time intervals as indicated. Cells lysates were harvested and used in immunoblotting for detecting phospho-p65 (p-p65); Myc-Rap2a and total p65. DNA amounts were kept constant in all conditions by the transfecting with empty vector pFLAG-CMV4. Densitometrical analysis of the western blots is shown below western blots where the densitometry values obtained for p-p65 were normalized by p65 values. The data represent mean ± SD of two biological replicates.

however, we found that Rap2a significantly inhibited NF-κB activation.

It is generally expected that overexpression and knockdown of a functionally gene should lead to opposite cellular responses and phenotypes. The implication for Rap2a in innate immune response induced through TLR2 or TLR4 is supported by our data because either silencing or overexpression of Rap2a hampered NF-κB activity in cells exposed to LPS or Pam3CSK4 stimulation, and also by critical signaling components of TLR pathway. Our data suggest that the interference on NF-κB activation provoked by Rap2a may vary depending upon the TLR signaling components involved, in addition to that macrophages may be more sensitive to alterations when Rap2a levels are disturbed. This observation can be speculated at the light of the transient nature of the mRNA levels of Rap2a and the protein levels that are sustained in macrophages treated with Pam3CSK4 and LPS. These remarkable differences seen between the expression of Rap2a mRNA and protein levels suggest that Rap2a protein expression is regulated at the post-transcriptional level during macrophage activation by TLR2 or TLR4 pathways.

It is not unusual to observe similar phenotypes for cells where proteins whose levels are both increased or silenced (Manzini et al., 2014; Wang et al., 2014). It is possible that overexpression of Rap2a may result into formation of unbalanced protein complexes, by sequestering or trapping critical proteins associated with intracellular events to NF-κB activation and inhibiting in turn its normal function.

The notion that LPS (and other PAMPs) activate members of the Ras superfamily is not fully clarified. Ras can be activated by CpG in macrophages, and it is implicated in signaling initiated by the TLR ligand as an early event by associating with TLR9 and promoting IRAK1/TRAF6 complex formation, and MAPK and NF-κB activation (Xu et al., 2003). Rap1 has been shown to be a target of RasGRP3 in TLRs signalling in macrophages. Besides, the GEF may regulate IL-6 production via activating Rap1a (Tang et al., 2014).

Our study expands our current knowledge of Ras-like small GTPase molecules involved in TLR-mediated responses in macrophages. We found that steady-state levels of Rap2a mRNA and protein undergo alterations after stimuli with TLR agonists. Although statistically significant, the effect of LPS-induced early reduction on Rap2a mRNA levels in macrophages is modest, not achieving a reduction of more than 2-fold, which could be interpreted as of questionable biological importance. However, the level of reduction in gene expression does not exclude the relevance of the observed effect, since alterations in the mRNA levels in macrophages used in this study is very consistent among the different agonists tested. More important, we found that the protein levels of Rap2a in cell extracts from macrophages treated for extended time intervals with LPS or Pam3CSK4 agonists did not correlate straight with the mRNA levels, being intriguing and also challenging to search for the mechanisms involved. One possible explanation for these interesting findings is that TLR2 or TLR4 pathways may regulate Rap2a protein levels at the posttranscriptional events during macrophage activation. These observations suggest that perturbations in Rap2a mRNA levels caused in response to treatment with TLR agonists in macrophages may lead to critical biological changes in the translation of its own mRNA. An interesting study has found similar findings to our observations by demonstrating that Gfi1 mRNA levels (an important molecule implicated in myeloid differentiation) decrease during monocyte differentiation; however, that protein levels increase significantly due to lower proteasomal degradation (Marteijn et al., 2007).

Several important regulators of NF-κB activity and immune system signaling pathway have their expression modulated in inflammation so they may play their functions. The expression of A20 protein is rapidly induced after activation of NF-κB (Verstrepen et al., 2010). SIRPα and also RasGRP3 are highly abundant in macrophages, and their levels are significantly reduced following treatment with inflammatory TLR agonist. This indicates that a well-controlled and tightly decrease for some molecules might be critical for macrophage activation (Kong et al., 2007; Tang et al., 2014).

Interestingly, levels of active Rap2a in RAW264.7 macrophages

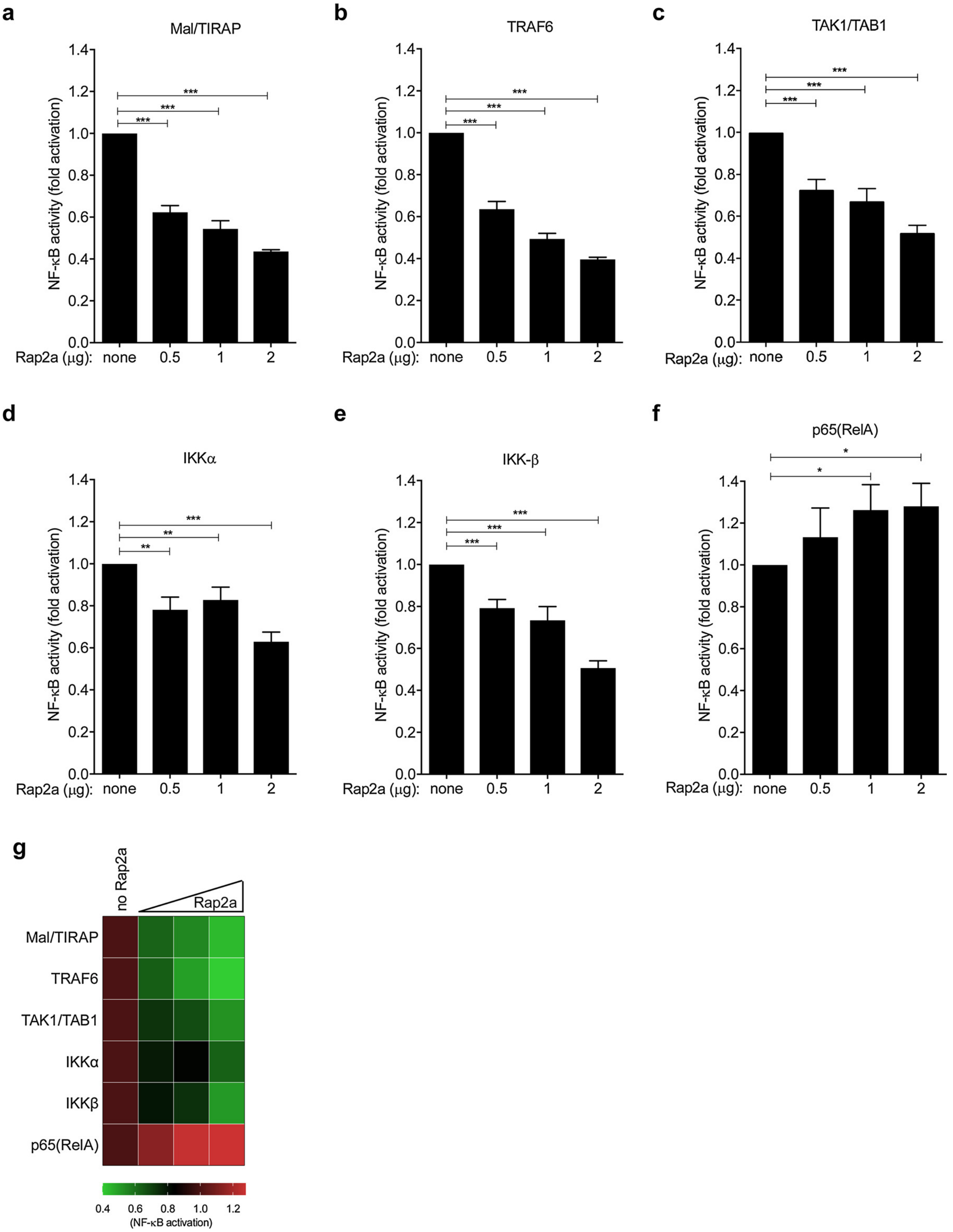

Fig. 7. Rap2a Inhibits TLR-Induced NF-κB Activation upstream of p65(RelA). HEK293 cells were transfected with NF-κB luciferase reporter, (**a**) Mal/TIRAP, (**b**) TRAF6, (**c**) TAK1/TAB1, (**d**) IKKα, (**e**) IKKβ, and (**f**) p65(RelA), along with increasing amounts of myc-tagged Rap2a. Twenty-four hours post-transfection, cell extracts were obtained and analyzed for NF-κB-dependent luciferase activity. Data represent mean ± SD of independent replicates (n = 3). (**g**) Representative heat-map analysis of NF-κB fold activation induced by signaling components of NF-κB pathway in the absence (maximum activation) or presence of increasing amounts of Rap2a.

following PMA treatment are higher than the observed for LPS stimuli. Rap1 is a PMA-activated molecule in different cell types, such as platelets and lymphocytes (Franke et al., 2000; Eppler et al., 2017). PMA is a PKC activator, a kinase involved in signal transduction of several pathways related to cell growth and proliferation (Wu-Zhang and Newton, 2013), indicating that further studies on Rap2 proteins in PMA-activated pathways may be promising and reveal new implications for these GTPases.

It is not clearly defined to date the degree of substrate specificity of GEFs for the Rap1a/b and Rap2a/b/c isoforms in cellular systems or in vivo, as many GEFs have been proposed. Given the numerous GEFs and their partner GTPase, a systematic study will be thus required to determine the GEF and/or GEFs that regulate Rap2a activation in macrophages in response to TLR stimulation. RasGEF1b in HEK293 cells can directly activate Rap2a, and reduced active Rap2a is seen in BMDMs lacking RasGEF1b upon LPS stimulation. Biochemical approaches indicated that RasGEF1b acts as GEF specific to Rap2a (Yaman et al., 2009). Therefore, it is very likely that additional GEFs will act on the activation of Rap2a induced by LPS or contributes with RasGEF1b. EPAC2 was recently demonstrated as Rap2a activator in neuroendocrine cells (Emery et al., 2017), and could be a putative GEF to be investigated in Rap2a-related inflammatory pathways.

Ras small GTPases have been implicated in the production of molecules related to the immune system, most of them related to leukocyte motility and adhesion and chemokine production (Bokoch, 2005; Yu et al., 2015). To our knowledge, our work is pioneer by demonstrating the interference of Rap2a protein in the production of inflammatory mediators and NF-κB activity in macrophages. Thus, the relevance of our findings provokes further investigation by examining the function of Rap2a with additional TLR agonists.

Because Rap2a failed to inhibit the cellular response to poly-IC, we speculate a selective role for Rap2a in regulating TRIF-independent pathway, suggesting a potential role in signaling pathways triggered by pathogen associated molecular patterns other than viral dsRNAs.

There are important questions that remain to be answered. How does Rap2a protein affect NF-κB activation? Why do Rap2a silencing and overexpression results to the identical hampered NF-κB activation phenotype? Overexpression experiments have demonstrated that atypical κB-Ras GTPases could inhibit NF-κB activation (Chen et al., 2003; Fenwick et al., 2000; Tago et al., 2010). The major difference between κB-Ras1 and κB-Ras2 and other Ras proteins is the lack of COOH-terminal membrane attachment sequences (CAAX sequences, where “A” is any aliphatic amino acid and “X” is any amino acid) and the presence of alanine or leucine at position 13 (instead of glycine) and leucine at position 65 (instead of glutamine) (equivalent to positions 12 and 61, respectively, in H-Ras) (Valencia et al., 1991; Macara et al., 1996; Fenwick et al., 2000). These alterations render known Ras proteins into a deregulated, active, GTP-bound conformation and underlies their oncogenicity (Lowy and Willumsen, 1993; Winkler et al., 1997). In the case of Rap2a, though presence of Glycine at position 12, there is a threonine at position 61. Moreover, at the CAAX motif, the second amino acid is non-aliphatic, i.e., charged polar. The observation that κB-Ras1 and 2 harbor V12 and Q61 that render the protein constitutively bound to GTP, and thus maintaining in an activated state, it is possible that GTP-bound Rap2A activated by inflammatory stimuli is a mechanism by which NF-κB activation might be inhibited.

Our present study has some limitations. Because of near 90% aminoacid sequence identity among Rap2 members, besides about 60% with Rap1 (see Fig. 8 of Carvalho et al., 2019), one could assume these molecules to serve redundant roles in cells. Experiments with individual silencing or knockout mice may increase our understanding of Rap2 function, as well as the specific functions performed each Rap2a, b or c in macrophages. Additionally, potential off-target effects might complicate the use of siRNA specific for Rap2a. It will be required further studies to assess the expression of Rap2 members and their functions in cells where Rap2a is knocked-down.

A number of speculations could arise for the unanticipated signaling role of Rap2a in macrophage activation by TLRs. Rap2 can enhance MAP4K4-induced activation of JNK, and it is suggested as putative effector of Rap2 (Machida et al., 2004). Rap2 activates MAP4K4 and is required for regulation of mechanotransducers target genes CTGF, CYR61, and ANKRD1 (Meng et al., 2018). Moreover, orally delivered siRNA targeting macrophage Map4k4 has been shown to suppress systemic inflammation by regulating the production of inflammatory mediators (Aouadi et al., 2009). One would speculate whether MAP4k4 is a putative effector of Rap2a in mediating cytokines production in TLR-activated macrophages.

In summary, we show in this study that both increase and decrease in the expression of a single GTPase can lead to inhibition of NF-κB in macrophages and contribute to cytokines production through targeting distinct gene signaling and thereby associating with different biological consequences. Our data suggest that although GTPases serve as key signal transducers of cellular responses by mainly regulating adhesion and cytoskeleton dynamics, their expression in cells under inflammatory conditions might be finely tuned to prevent NF-κB dysregulation. Modulation of Rap2a GTPase, for example, may decrease inflammatory responses through suppression of proinflammatory signaling pathways. Collectively, our work provides novel insight into the complexity and biological importance of small Ras GTPases regulation in TLR-mediated inflammation, especially through TLR4.

Contributions

B.C.C. carried out most experiments; L.C.O., C.D.R., T.M.V., I.M.G.R., contributed with plasmids and gene reporter studies; H.B.F., I.M.O., F.B.L. contributed BMDMs preparations, ELISA and RT-qPCR; S.G. helped to supervise the findings of this work, and contributed new reagents and tools. A.M.S. originally conceived this study, supervised the findings of this work and wrote the final manuscript with input from all authors.

Declarations of interest

None.

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