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Data Article

# Data in support of Rap2a GTPase expression, activation and effects in LPS-mediated innate immune response and NF-κB activation

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ABSTRACT

We present here the data to support the understanding of the implication of Rap2a GTPase in LPS-induced innate immune response and NF-κB activation. The data presented are related to molecular tools that were generated, acquired, optimized or validated to investigate Rap2a expression, activation and its effects in mammalian cells including RAW264.7 macrophages and THP-1 monocytes under inflammatory conditions. These data supplement important technical and biological information on immune function of Rap2a in macrophages activated by LPS, recently reported by us (Carvalho et al., 2019) [1].



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[1] Equal contribution.

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Specifications table

| Subject area | Immunology |
|---|---|
| More specific subject area | Innate Immunity, Signal transduction. |
| Type of data | Graph, figure and text. |
| How data was acquired | Using electrophoresis/western blot apparatus (Mini Protean, BioRad) and power supply (PowerPac HC, BioRad), phodocumentation system (UVP PhotoDoc-It), luminometer (Lumicount, Packard), ELISA reader (Multiskan FC, ThermoScientific), real-time PCR (CFX96 Touch™ PCR Detection System, BioRad). |
| Data format | Raw and analyzed. |
| Experimental factors | - Amplification and cloning of RBD of murine RalGDS.<br>- IPTG treatment of bacterial colony harboring pGEX-6p-1-RBD-RalGDS.<br>- Treatment of RAW264.7 macrophages for obtaining cell extracts for affinitiy precipitation of active Rap2a GTPase.<br>- Analysis of Rap2a mRNA levels in siRNA's validation.<br>- TNF ELISA.<br>- Transfection of mammalian cells.<br>- Identity among Rap2 members. |
| Experimental features | - Successful recombinant expression of GST-RBD-RalGDS fusion protein.<br>- Affinity precipitation of activated Rap2a GTPase from cell extracts of RAW264.7 cells.<br>- mRNA expression profile of Rap2a in murine RAW264.7 macrophages and human THP-1 monocytes treated with LPS.<br>- Efficiency of Rap2a knockdown using two distinct siRNAs.<br>- TNF production in siRNA-Rap2a transfected murine macrophages.<br>- Successful transfection to report NF-κB activity in mammalian cells with altered Rap2a amounts.<br>- Rap proteins alignment and identity between human and murine Rap2a. |
| Data source location | Belo Horizonte, MG, Brazil, and New York city, NY, USA. |
| Data accessibility | Data is within this article. |
| Related research article | B.C. Carvalho, L.C. Oliveira, C.D. Rocha, H.B. Fernandes, I.M. Oliveira, F.B. Leão, T.M. Valverde, I.M.G. Rego, S. Ghosh, A.M. Silva. Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression. Mol Immunol, 109, 2019, 27–37 [1]. |

**Value of the data**

- Method description to detect the activation of Rap2a GTPase can be used in further Rap2 studies in different biological questions.
- The first description of Rap2a mRNA expression profile after LPS stimulation of cells of innate immune system.
- The validation of two distinct siRNAs targeting Rap2a could be useful for researchers interested in investigating Rap2a functions in cells.
- First description of gene reporter studies to assess the implication of Rap2a GTPase in NF-κB activity.

## 1. Data

Besides providing methods used for generating molecular tools to investigate Rap2a activation in cells this article supplements with biological data that support the GTPase expression and its implication in Toll-like receptor (TLR)-mediated innate immune response, recently reported by us (Carvalho et al., 2019) [1]. Fig. 1a–d depict the amplification and cloning of the Ras/Rap GTPase binding domain (RBD) of mouse Ral guanine nucleotide dissociation stimulator (RalGDS) from mammalian mouse embryonic fibroblasts (MEFs) and RAW264.7 macrophages. Recombinant expression of GST-RBD-RalDGS and its use in pull-down assays is validated in cell extracts from RAW264.7 macrophages (Fig. 2a–c). Analyses of the mRNA levels of Rap2a GTPase upon LPS or poly-IC stimulation of cells are shown in Fig. 3a–c. Validation of two distinct siRNAs targeting murine Rap2a and their effects are shown in Figs. 4 and 6. ELISA data for TNF in Rap2a siRNA-transfected RAW264.7 macrophages is shown in Fig. 5. The effects of Rap2a in NF-κB activitiy in human THP-1 monocytes upon LPS stimulation is shown (Fig. 7). Aminoacid and tree alignments data showing identity among Rap2 family members, and also among Rap1 members a and b are depicted in Fig. 8a–d.

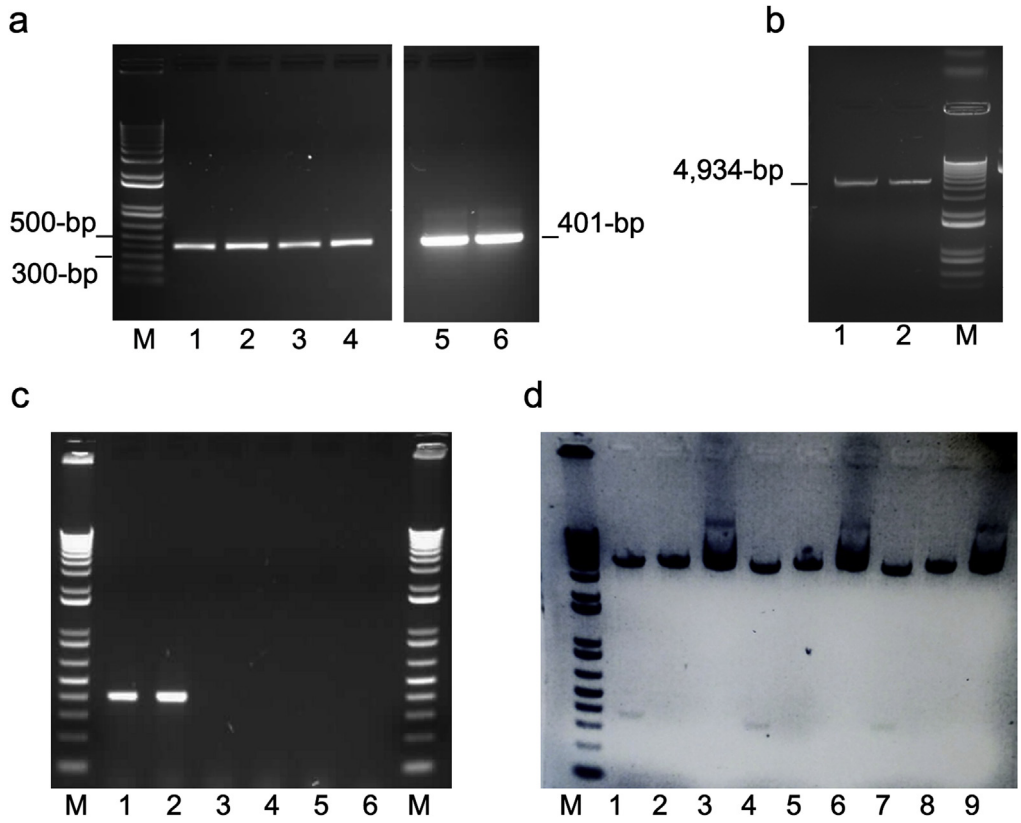


**Fig. 1.** Cloning of Rap2a GTPase binding domain (RBD) from mouse Ral guanine nucleotide dissociation stimulator (RalGDS). (**a**, *left*) Total RNA was obtained from mouse embryonic fibroblasts (MEFs) and RAW264.7 cells and subsequently transcribed into cDNA that was used as template in RT-PCR reactions. PCR reactions were fracionated on 1.2% agarose gel. Expected size of the amplicons: 401-bp. Lanes 1–4: cDNA amplicons from MEFs (1–2) and RAW264.7 cells (3–4). (a, *right*) The band from lane 3 was excised from gel, purified and cloned into a pCR2.1 vector, used to transform Stbl3 *E. coli* cells. Lanes 5–6: PCR from pCR2.1 plasmid containing RBD-RalGDS insert. (**b**) Plasmid pGEX-6P-1 was digested with *Bam*HI and *Eco*RI and purified (lanes 1 and 2), and fractionated on 1% agarose gel. (**c**) RBD-RalGDS inserts that were digested and purified from pCR2.1 plasmids were ligated into *Bam*HI and *Eco*RI sites of pGEX-6P-1 and used to transform Stbl3 cells. Shown is a representative ethidium bromide-stained 1.2% agarose gel of a PCR screening of Stbl3 *E. coli* colonies for the presence of insert where lanes 1 and 2 show positive ones. Lanes 3–6: negative colonies. (**d**) Positive colonies that were then grown in bacteria liquid medium were further processed for plasmid miniprep, followed by digestion with *Bam*HI and *Eco*RI and fractionated on 1.2% agarose gel to reveal for the presence of the insert. Shown is a negative image of the photodocumented gel. Lanes 1, 4 and 7 show the expected insert of 401-bp. Lanes 2, 5 and 8: linearized pGEX-6P-1-RalGDS-RBD plasmids. Lanes 3, 6 and 9: undigested pGEX-6P-1-RalGDS-RBD plasmids. Plasmids were sent for sequencing. Agarose gels in the panels were run in TAE 1X, stained with ethidium bromide and photodocumented. DNA marker (lanes M): 1kb plus DNA ladder (Invitrogen).

## 2. Experimental design, materials and methods

### *2.1. Construction of pGEX plasmid containing the RBD from murine RalGDS*

The Rap2 GTPase binding domain (RBD) of murine Ral guanine nucleotide dissociation stimulator (RalGDS) (GenBank accession no.: NM_009058.2) was cloned from mouse embryonic fibroblasts (MEFs). Briefly, total RNA was extracted from MEFs and subsequently transcribed into cDNA that was used as template in RT-PCR reactions using Q5 High fidelity DNA polymerase (New England Biolabs)

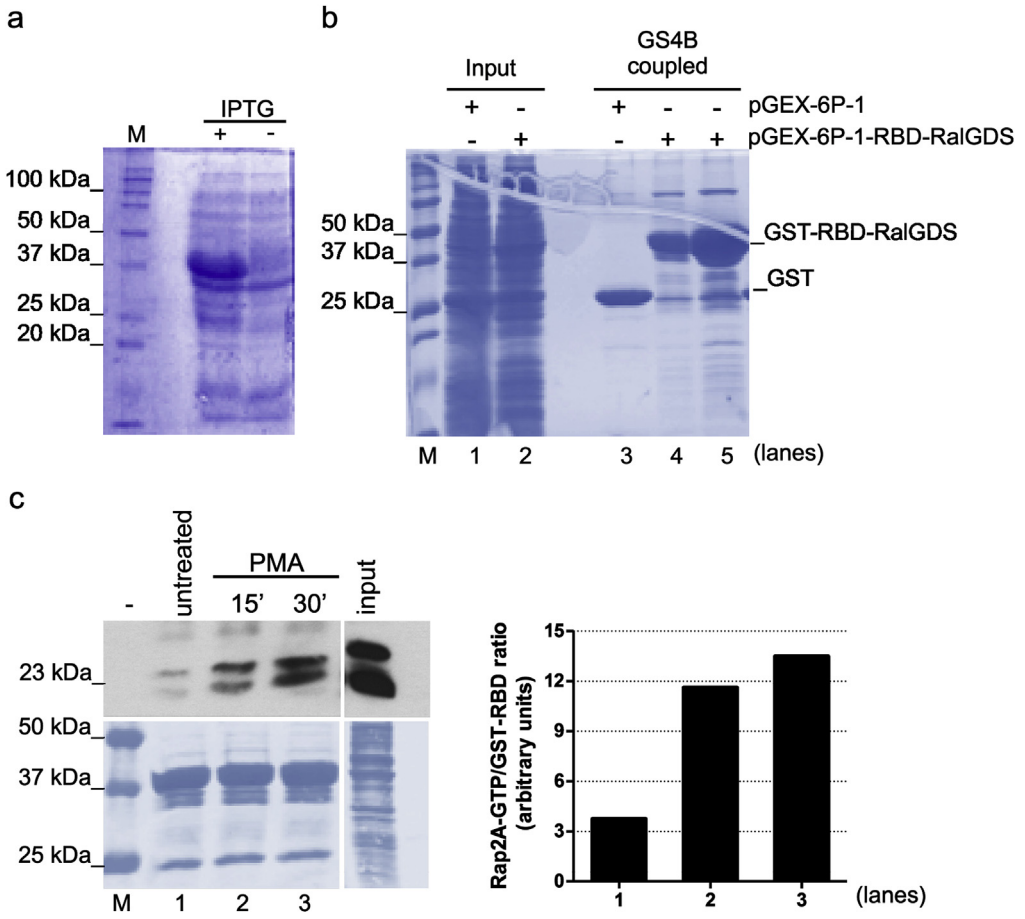


**Fig. 2.** Expression of GST-RBD-RalGDS and validation of affinity precipitation of Rap2a in mammalian cell extracts. (**a**) The expression of GST-RBD-RalGDS was obtained upon induction with 1 mM isopropyl-1-thio-beta-D-galactopyranoside (IPTG) for 2 hours of bacterial cell cultures transformed with pGEX-6P-1-RBD-RalGDS. Bacterial extracts were fractionated onto 10% SDS-PAGE, and followed by coomassie blue staining. (**b**) Coupling of GST-RBD-RalGDS to glutathione sepharose 4B (GS4B) beads. Suspensions of IPTG-induced bacterial cell cultures transformed with pGEX-6P-1 or pGEX-6P-1-RBD-RalGDS were centrifuged, lysed and sonicated. GST or GST-RBD-RalGDS fusion protein were mixed with GS4B. Bacterial cell lysates (lanes 1 and 2) and eluted samples (lanes 3–5) were separated by 10% SDS-PAGE, followed by coomassie blue staining. Two independent bacterial clones of pGEX-6P-1-RBD-RalGDS were used in lanes 4 and 5, respectively. (**c**) RAW264 cells were treated with PMA (100nM) as indicated. The lysates (500 μg) were then incubated with 100μl (~0.5mg) of bacterial lysates containing GST-RalGDS-RBD precoupled to GS4B beads. After washes, the beads mixtures were fractionated on 12% SDS–PAGE, transferred to PVDF membrane, and probed with anti-Rap2A antibody. Anti-Rabbit IgG (H + L), peroxidase conjugated was used as the secondary antibody. The detection was performed with Clarity Western ECL Blotting Substrate (BioRad) and followed by exposure to X-ray film. Densitometrical analysis of the western blots is shown on right where the densitometry values obtained for Rap2a were normalized to GST-RBD-RalGDS values in lanes 1–3. *M*, protein standards (250, 150, 100, 75, 50, 37, 25, 20, 10 KDa).

and the oligonucleotides RalGDS-Bam-Fwd: 5′- TCATGGATCCTCACTGCCTCTCTACA -3′, and RalGDS-Eco-Rev: 5′- TTAGGAATTCGAAGATGCCTTTGGCA -3'. PCR-amplified cDNA fragment of 401-bp comprising the RBD-RalGDS mRNA coding sequence was cloned into pCR2.1 vector, and then a *Bam*HI-*Eco*RI fragment was obtained by digestion, purified and finally subcloned in pGEX-6P-1 vector (GE Healthcare). The authenticity of the RalGDS-RBP insert sequence was confirmed by automated sequencing.

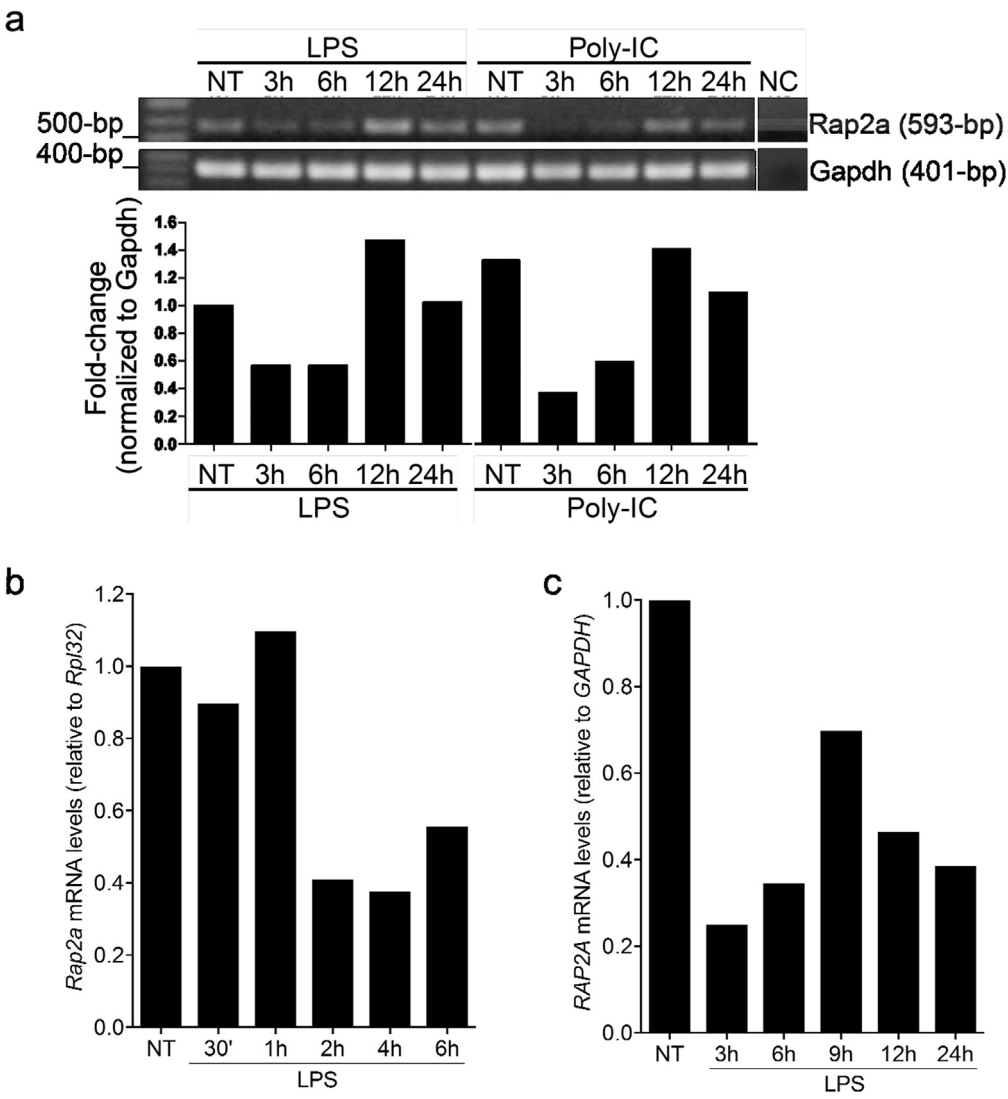


**Fig. 3.** Expression analysis of Rap2a in LPS-treated RAW264.7 macrophages and human THP-1 monocytes. Non-quantitative RT-PCR and quantitative RT-qPCR analyses of Rap2a in RAW264.7 (**a**, **b**) and RAP2A in THP-1 (**c**) cells that were stimulated as indicated. Lower graph in (**a**) shows fold change expression after densitometric analysis of Rap2a:Gapdh ratio. Total RNA was extracted and subsequently transcribed into cDNA that was used as template in RT-qPCR reactions for detection of mRNAs. NT, *not treated*. NC, *PCR negative control.*

### *2.2. Expression and isolation of GST-RalGDS fusion protein*

Procedures were followed as outlined by van Triest et al., 2001 [2] with some minor modifications. An overnight culture of BL21(DE3)pLysS bacteria bearing the plasmid pGEX-6P-1-RBD-RalGDS was diluted 1:50 (v/v) in one liter of LB (10 g/l (w/v) tryptone, 10 g/l (w/v) NaCl, 5 g/l (w/v) yeast extract) containing 50 μg/ml ampicillin and grown for a further hour at 37 °C. Protein synthesis was induced for 2 h with the addition of 1 mM IPTG. Cells were then pelleted (7700× *g* at 4 °C, 10 min) and frozen at −80 °C overnight to break bacterial cell walls. The following day cells were resuspended in 25 ml of

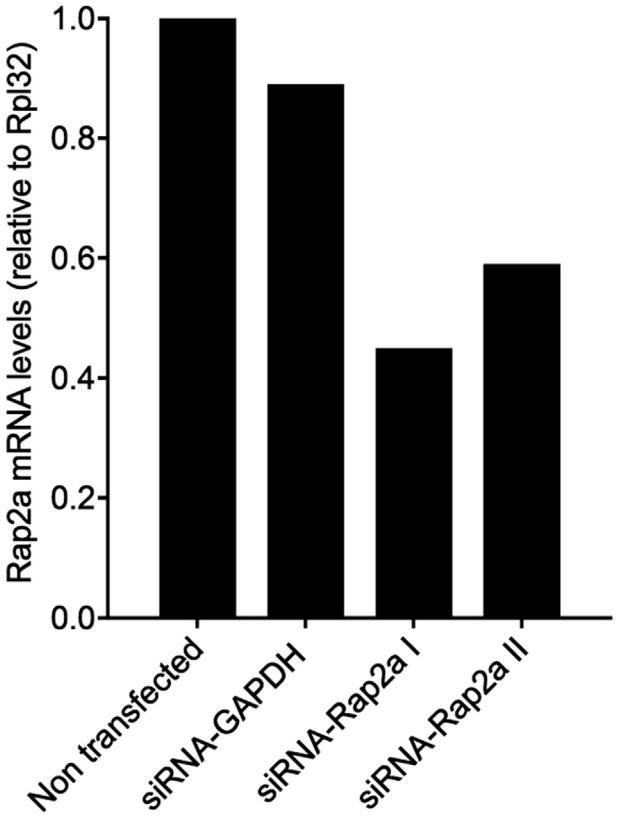


**Fig. 4.** Rap2a mRNA levels are reduced after transfection with two different specific siRNAs. MEFs-SV40 were left untransfected or transfected with siRNA-GAPDH or two different siRNA targetting Rap2a for 24h. Then, cells were lysed to obtain total RNA transcribed into cDNA which was used as template in RT-qPCR reactions for detection of Rap2a.

cold 10 ml of 50 mM Tris—HCl pH 8.0, 20% (w/v) sucrose, 10% (v/v) glycerol, 2 mM dithiothreitol (DTT), 2 mM $MgCl_2$, containing protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, leupeptin 1μg/ml, and aprotinin 2 μg/ml). Cell suspensions were sonicated on ice to shear DNA (10 × 15 sec bursts, at amplitude of 20—30%), and then cell debris was pelleted by centrifugation (12,000× *g* at 4 °C, 60 min). The supernatant was collected and stored in aliquots of 1 ml at −80 °C until use. The presence of GST-RBD-RalGDS fusion protein in the clear was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### *2.3. Affinity precipitation of active GTP-bound Rap2a and immunoblotting*

Bacterial lysates containing GST-RalGDS-RBD were used to determine the relative amount of active GTP-bound Rap2a in cell extracts of mammalian cell cultures. Detergent extracts of whole cells were prepared by solubilization in cold lysis buffer (Tris HCl 50 mM pH 7.4; NaCl 200mM; 2.5mM $MgCl_2$; 50mM NaF; 10% v/v glycerol; 1% v/v nonidet P-40, and protease and phosphatase inhibitors) and centrifuged at 4 °C (10,000× *g*, 10 min). Cell extracts (500μg to 3mg) were incubated with 200μl (~1mg) of bacterial lysates containing GST-RalGDS-RBD pre-coupled to glutathione sepharose 4B beads (30 μl per cell extracts) for 45 min at 4 °C under rotation. The beads were then washed four times in lysis buffer, followed by SDS—PAGE and immunoblotting with anti-Rap2a antibody (Thermo Fisher Scientific, Product # PA5-23298) used at a concentration of 1:500 (v/v).

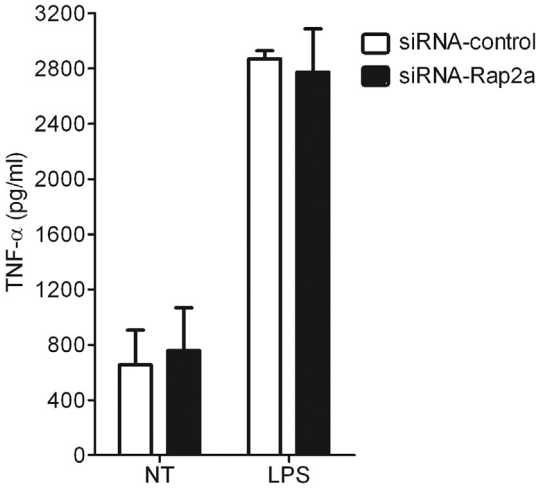


**Fig. 5.** Silencing of Rap2a expression does not impair LPS-induced production of TNF in macrophages. RAW264.7 cells were transfected with siRNA-control or siRNA-Rap2a for 48 hours. Then, cells were stimulated with LPS (100ng/ml) for 8h. To evaluate the production of TNF, ELISA assays were performed from the supernatants collected at the end of stimuli. NT, *non-treated*. Data represent mean ± SD, n = 3.

### 2.4. End-point RT-PCR analysis

After treatment with TLRs agonists, medium was discarded and total RNA was obtained with Trizol following instructions of the manufacturer (Invitrogen). One microgram of total RNA was used to obtain the first strand cDNA in the presence of oligo-$dT_{15}$, dNTPs, RNAsin, and reverse transcriptase (MMLV RT, Promega, Madison, WI, USA). One-tenth of first strand cDNA reaction was used as template in PCR reactions to amplify cDNA fragments corresponding to Rap2a and Gapdh mRNAs. The sequences of the primers used were as follows: Rap2a-F: 5′CGATGCGCGAGTACAAAGTG-3′, Rap2a-R: 5′GCCTAGACGAATCCTGTCCG-3′, annealing at 62 °C, 25 cycles. Gapdh-F: 5′CCTCAACTACATGGTCTAC-3′ and Gapdh-R: 5′CCTTCCACAATGCCAAAGT-3′, annealing at 52 °C, 25 cycles. Levels of the mRNA for the Gapdh were monitored in parallel to normalize the total amount of cDNA in each sample. The PCR products were fractionated onto 1.2% agarose gel, stained with ethidium bromide, visualized in UV transiluminator, and the images captured by a CCD camera in a photodumentation system.

### 2.5. RT-qPCR

The expression of mRNA for human RAP2A in THP-1 cells was analyzed with quantitative PCR performed with iQSybr Master Mix kit (BioRad) in the CFX96 Touch™ Real Time detection system (BioRad). The following primers for human RAP2A and GAPDH were used: RAP2A-FWD, 5′-ATGCGCGAGTACAAAGTGGT-3′ and RAP2A-REV 5′- GCGACGAATCCACCTCGAT-3`; GAPDH-FWD, 5′-ACAGTCAGCCGCATCTTCTT-3′ and GAPDH-REV, 5′- ACGACCAAATCCGTTGACTC-3`. Please refer to the research article “Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression” for the analysis of expression of mRNA for mouse Rap2a (Carvalho et al., 2019) [1].

### 2.6. siRNA validation

Mouse embryonic fibroblasts (MEFs) ($1 \times 10^6$ cells/well) were transfected with control (Sigma® MISSION® siRNA Universal Negative Control #1) and two different Rap2a-siRNA (Ambiom Silencer

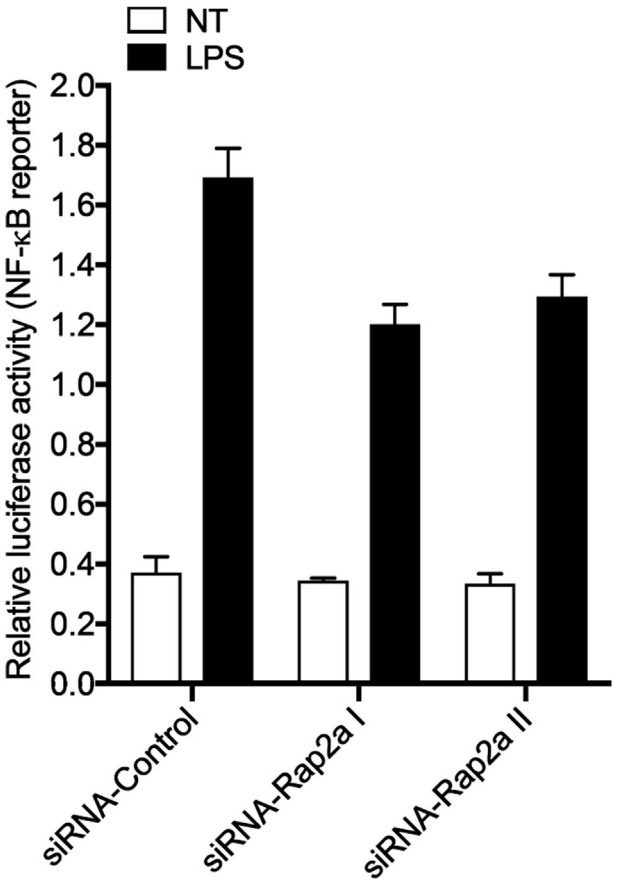


**Fig. 6.** Silencing of Rap2a with different specific siRNAs hampers NF-κB activity. RAW264.7 cells were transfected with siRNA-control or two different siRNA targetting Rap2a along with the reporter plasmids pGL3-E-sel and pRL-TK for 24h and then stimulated with LPS (100ng/ml) for more 24h. NT, *not treated*. At the end of the experiment, luciferase activities were measured in a luminometer. DNA amounts were kept constant between experimental groups. Results are shown as relative firefly luciferase activities normalized to Renilla luciferase activities. Data represent mean ± SD, n = 3.

select®; siRNA-I sequence CCUUCAUUGAGAAAUACGAtt; or siRNA-II sequence GAUGAGCUCUUUGCAGAAAtt) at 100 nM using Lipofectamine2000 transfection reagents (Life Technologies) for 24h. Then, cells were lysed to obtain total RNA transcribed into cDNA which was used as template in RT-qPCR reactions for detection of Rap2a. Please refer to the research article "Both knock-down and over-expression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression" for more data obtained with siRNA Rap2a I.

### *2.7. TNF cytokine assay*

Supernatants from siRNA-control or siRNA-Rap2a (siRNA-I) transfected murine macrophages were harvested and the concentrations of TNF were measured by commercially available ELISA kit, according to the manufacturer's instructions (R&D Systems).

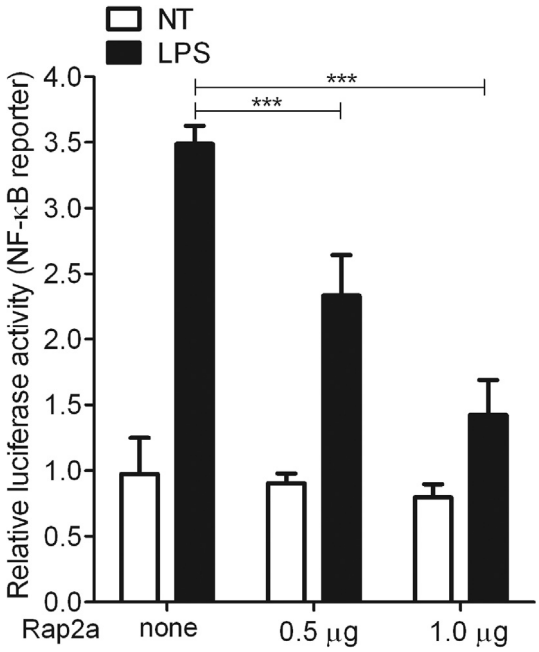


**Fig. 7.** Overexpression of Rap2a impairs NF-κB activity induced through TLR4 in human monocytes. THP-1 cells were transfected with endofree plasmids preparations of the NF-κB luciferase and pRL-TK reporters in combination with different amounts of pFLAG-CMV4 (vector) or pCIneo-Myc-Rap2a as indicated. Twenty-four hours post-transfection cells were stimulated with LPS (1μg/ml) for 8h. NT, *not treated*. Luciferase activities were determined in a luminometer. DNA amounts were kept constant between experimental groups. Results are shown as relative firefly luciferase activities normalized to Renilla luciferase activities. Data represent mean ± SD of independent replicates (n = 3). ***p<0.001 (One-way ANOVA).

### 2.8. Transfection of RAW264.7 and THP-1 cells and analysis of NF-κB activity by gene luciferase reporter

RAW264.7 and THP-1 cells ($2 \times 10^5$/well) were transfected with endotoxin free preparations (GenElute™ Endotoxin-free Plasmid Midiprep Kit, Sigma-Aldrich) NF-κB reporter construct (400ng) and pRL-TK plasmid (100ng). RAW264.7 cells were then co-transfected with control and Rap2a-siRNA I and II at 100 nM using RNAiMax. THP-1 cells were co-transfected with 0.5 or 1.0μg of Myc-Rap2a plasmid per well as indicated in Fig. 7. After one or two-day post-transfections, cells were stimulated with LPS. Cell lysates were harvested with passive lysis buffer (PLB1x, Promega) and assayed for Firefly and *Renilla* luciferase activities with Dual-Luciferase Reporter Assay Kit from Promega. The ratio of firefly luciferase to Renilla luciferase was calculated and the results presented as relative luciferase activity.

### 2.9. Sequence alignments

The Rap protein sequences which accession numbers on GenBank are AAM12628.1 (RAP2A); NP_083795.2 (Rap2a); CAJ18500.1 (Rap2b); AAH50056.2 (Rap2c); NP_663516.1 (Rap1a); NP_077777.1 (Rap1b) were aligned using the ClustalW software. The percentage of similarity was calculated and a tree alignment data was generated from the *in silico* analysis of ClustalW.

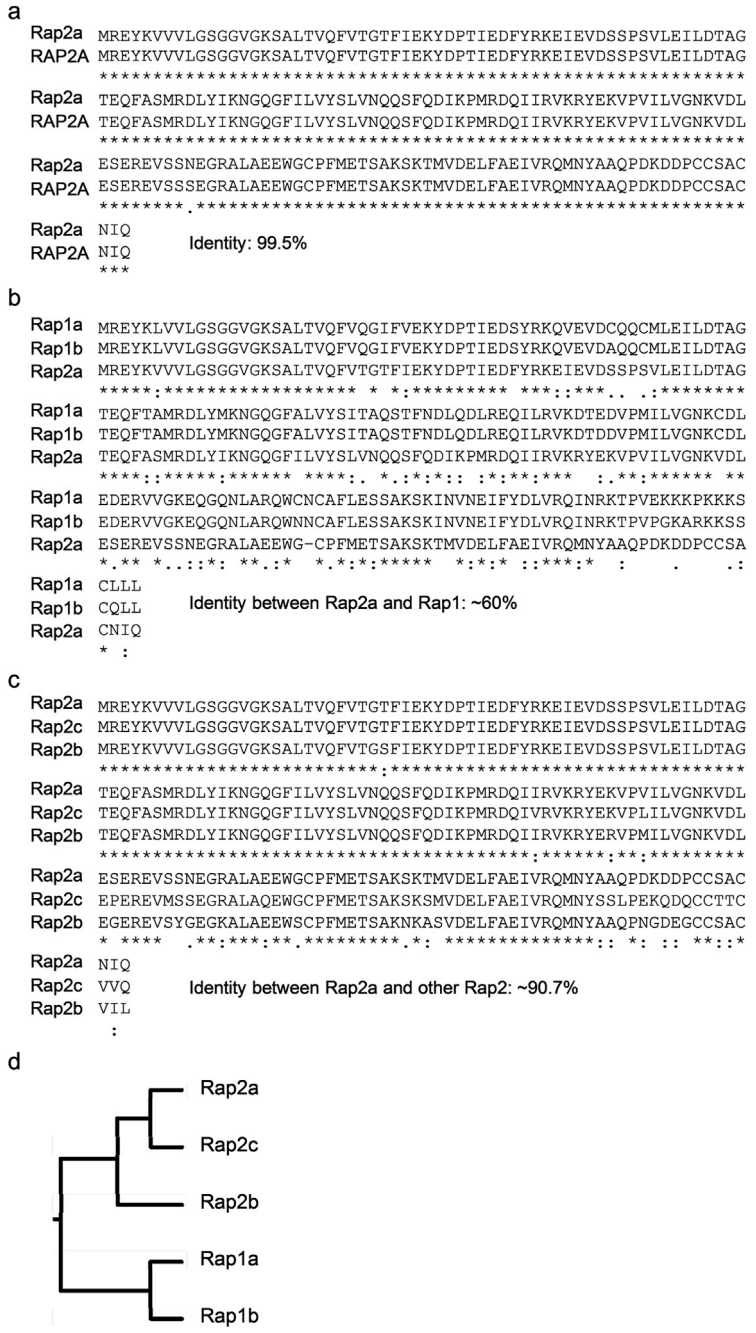


**Fig. 8. Analysis of similarity between Rap proteins.** The aminoacid sequence of murine Rap2a was aligned with human RAP2A (**a**), Rap1 members (**b**), and Rap2b and Rap2c (**c**). Tree alignment data of murine Rap proteins (**d**). Clustal W software was used to calculate the percentage of similarity and to generate tree alignment. Aminoacid sequences were obtained through NCBI (National Center for Biotechnology Information - USA) and the GenBank accession numbers for each protein are: AAM12628.1 (RAP2A); NP_083795.2 (Rap2a); CAJ18500.1 (Rap2b); AAH50056.2 (Rap2c); NP_663516.1 (Rap1a); NP_077777.1 (Rap1b).

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## Transparency document

Transparency document associated with this article can be found in the online version at https://doi.org/10.1016/j.dib.2019.103965.

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[1] B.C. Carvalho, L.C. Oliveira, C.D. Rocha, H.B. Fernandes, I.M. Oliveira, F.B. Leão, T.M. Valverde, I.M.G. Rego, S. Ghosh, A.M. Silva, Both knock-down and overexpression of Rap2a small GTPase in macrophages result in impairment of NF-κB activity and inflammatory gene expression, Mol. Immunol. 109 (2019) 27–37.

[2] M. Van Triest, J. De Rooij, J.L. Bos, Measurement of GTP-bound Ras-like GTPases by activation-specific probes, Methods Enzymol. 333 (2001) 343–348.