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# Dok proteins are recruited to the phagosome and degraded in a GP63-dependent manner during *Leishmania major* infection

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## Abstract

Three adaptor molecules of the Dok family, Dok-1, Dok-2 and Dok-3 are expressed in macrophages and are involved in the negative regulation of signaling in response to lipopolysaccharide and various cytokines and growth factors. We investigated the role and the fate of these proteins following infection with *Leishmania major* promastigotes in macrophages. The protozoan parasite *L. major* causes cutaneous leishmaniasis and is known for its capacity to alter host-cell signaling and function. *Dok-1/Dok-2<sup>-/-</sup>* bone marrow-derived macrophages displayed normal uptake of *L. major* promastigotes.

Following *Leishmania* infection, Dok-1 was barely detectable by confocal microscopy. By contrast, phagocytosis of latex beads or zymosan led to the recruitment of Dok-1 to phagosomes. In the absence of the *Leishmania* pathogenesis-associated metalloprotease GP63, Dok-1 was also, partially, recruited to phagosomes containing *L. major* promastigotes. Further biochemical analyses revealed that similar to Dok-1, Dok-2 and Dok-3 were targets of GP63. Moreover, we showed that upon infection with wild-type or  $\Delta gp63$  *L. major* promastigotes, production of nitric oxide and tumor necrosis factor by interferon- $\gamma$ -primed *Dok-1/Dok-2<sup>-/-</sup>* macrophages was reduced compared to WT macrophages. These results suggest that Dok proteins may be important regulators of macrophage responses to *Leishmania* infection.

## Keywords

- Leishmania;
- Metalloprotease GP63;
- Dok proteins;
- Macrophage;

- Phagosome
- 

## 1. Introduction

Phagocytosis is initiated through the activation of one or more receptors expressed on the surface of macrophages following the binding of ligands exposed on the particle surface. This process involves a complex series of events, initiated by receptor clustering, which causes the activation of multiple intracellular signaling cascades. These events lead to membrane and cytoskeletal rearrangements and to the internalization of the particle into an intracellular compartment, the phagosome [1].

The Dok family members are adaptor proteins involved in signaling downstream of tyrosine kinases. They contain a pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain, and a COOH-terminal region containing multiple tyrosine phosphorylation sites and PXXP motifs which serve as potential docking sites for SH2- and SH3-domain containing proteins, respectively, [reviewed in 2]. Dok-1, Dok-2, and Dok-3, the three members of Dok family proteins expressed in myeloid cells, are involved in the negative regulation of responses downstream a variety of receptors. The current model for Dok-mediated inhibition of signaling proposes that upon tyrosine phosphorylation elicited by receptor stimulation, Dok proteins are recruited to the plasma membrane in a PH domain-dependent manner, where they recruit inhibitory effector molecules [3]. Dok-1 and Dok-2, the two family members with highest homology, exhibit functional redundancy, whereas Dok-3 presents structural and functional differences with Dok-1 and Dok-2 isoforms [2]. Dok-1, Dok-2, and Dok-3 seem to cooperatively regulate several macrophage responses. Indeed, Dok-3 has been reported to play a non redundant role compared to Dok-1 and Dok-2 in the suppression of macrophage proliferation and in the inhibition of lipopolysaccharide (LPS)-induced responses [4], [5],[6], [7] and [8]. Moreover, the three Dok proteins are involved, although by different mechanisms, in the inhibition of the Ras/MAPK signaling pathway [9], [10], [11] and [12]. One of the most important events for phagocytic ingestion and phagosomal maturation involves actin remodeling [13]. Both Dok-1 and Dok-2 have docking sites for signaling molecules that have been implicated in the regulation of the actin-cytoskeletal reorganization such as Nck, Crk-L and

Cas [14], [15], [16] and [17]. Hence, Dok proteins might participate in the actin assembly process triggered during phagocytosis in macrophages. Consistent with the involvement of Dok in phagocytosis, Dok-3 has been shown to undergo rapid tyrosine phosphorylation in response to engagement of FcγRI [18] and we recently found that Dok-1 is recruited to the phagosome [19].

The protozoan parasite *Leishmania* is responsible for a spectrum of human diseases collectively termed leishmaniases. Promastigote forms of this parasite are transmitted to humans by infected sand flies and are taken up by macrophages, where they ultimately differentiate into amastigotes. One mechanism used by promastigotes to establish infection within macrophages is the inhibition of phagosolysosome biogenesis [20] and [21]. In addition, *Leishmania*-infected macrophages are characterized by an impaired responsiveness to various activating signals [22], [23], [24], [25], [26], [27], [28] and [29]. Recent studies revealed that the glycosylphosphatidylinositol-anchored metalloprotease GP63 plays a key role in the ability of promastigotes to modulate phagolysosomal properties and to alter the host cell signal transduction machinery by cleaving various signaling molecules [30] and [31].

Because Dok proteins are present in the phagosome proteome [19] and [32], we hypothesized that they might play a role during phagocytosis. To gain insight into Dok proteins functions, we investigated their subcellular localization during the phagocytosis of *Leishmania major* promastigotes and their involvement in the host response to parasite infection. We provide evidence that Dok proteins are cleaved in a GP63-dependent manner and contribute positively to nitric oxide (NO) and tumor necrosis factor (TNF) production in response to *Leishmania* infection.

## 2. Materials and methods

### 2.1. Parasites

Wild-type (WT), GP63-deficient ( $\Delta gp63$ ) and add-back ( $\Delta gp63 + gp63$ ) *L. major* (Seidman clone A2) [33] were kindly provided by Dr. W.R. McMaster. Promastigotes were grown at 26 °C in M199 medium supplemented with 20% heat inactivated fetal bovine serum (FBS), 100 μM hypoxanthine, 40 mM HEPES, 5 μM hemin, 3 μM biopterin, 1 μM biotin and antibiotics (*Leishmania* medium). For infections, promastigotes were used in the late

stationary phase of growth as described [34] and metacyclic promastigotes were purified as described [35].

## 2.2. Mice

129/Sv *Dok-1<sup>-/-</sup>Dok-2<sup>-/-</sup>* mice were previously described [5]. 129/Sv mice were obtained from Jackson Laboratories. Three to 4-wk old mice were used in this study. All mice were maintained in our pathogen-free animal facilities (INRS-Institut Armand-Frappier, Laval, Canada). Mice were manipulated in strict accordance to protocol 0910-01, approved by the *Comité Institutionnel de Protection des Animaux* of the INRS-Institut Armand-Frappier. This protocol respects guidelines on good animal practice provided by the Canadian Council on animal care.

## 2.3. Antibodies and reagents

Antibodies directed against the C-terminal region and PTB domain of murine Dok-1 were produced in rabbits using a glutathione S-transferase (GST) fusion protein encompassing amino acids (aa) 252–482 and aa 138–296 of murine Dok-1 respectively. Immune sera were first adsorbed on GST-sepharose 4B beads and anti-Dok-1 antibodies were immunoaffinity purified on GST/Dok-1 fusion protein coupled to sepharose beads as described [36]. Anti-Dok-3 antibodies (kindly provided by A. Veillette) have been described [18]. Anti-Dok-2 (#3914), anti-phospho-ERK12 and anti-ERK1/2 were from Cell Signaling Technology. Anti- $\beta$  actin antibodies were from Sigma-Aldrich. For cytometry analysis, the following antibodies were used: anti-F4/80 Fluorescein isothiocyanate (FITC) (AbD Serotec), anti-CD44 PE (eBioscience); anti-Gr1 Biotin (eBioscience), anti-Mac-1 FITC, anti-CD80 PE (eBioscience), 2.4G2 FITC and streptavidin-PerCP (BD Bioscience).

## 2.4. Bone marrow-derived macrophages (BMM) preparation

BMM were obtained by extracting the femurs and tibias from 3- to 4-wk-old female mice as described previously [34]. Briefly, cells were grown at 37 °C for 24 h in 5% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine supplemented with 10% heat-inactivated FBS, 10 mM HEPES (pH 7.4), and antibiotics in the presence of 15% (v/v) L929 cell-conditioned medium as a source of colony-stimulating factor-1. After 18 h, non-adherent cells were

transferred to new plate. On day 7, adherent cells were collected, cultured for a further 18 h in absence of L929 cell-conditioned medium and used in subsequent experiments.

## 2.5. Flow cytometry

Cells were washed twice with fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline (PBS) + 1% FBS + 0.01% NaN<sub>3</sub>) and stained with antibodies. Viable cells were analyzed on a FACSCalibur cytometer (Becton Dickinson) and FACS analysis was performed using CellQuest software or FCS Express software (De Novo software).

## 2.6. Confocal microscopy and immunofluorescence

To synchronize phagocytosis assays, macrophages were incubated with particles (beads, zymosan or parasites) at a particle-to-cell ratio of 15:1 for 10 min at 4 °C and 10 min at 37 °C. Excess particles were removed by several thorough washes with DMEM and phagocytosis was triggered by transferring the cells to 37 °C for the indicated time points before processing for microscopy. Macrophages were fixed, permeabilized using 0.1% Triton X-100, and non-specific surface binding were blocked using 1% bovine serum albumin (BSA), 2% goat serum, 6% milk, and 50% FBS. For immunostaining, cells were labeled with the appropriate combinations of antibodies and secondary antibodies (anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 568 conjugates, Molecular Probes). DRAQ5 (Biostatus) was used to visualize cell nuclei. All coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates). Analysis of protein localization was performed using Zeiss LSM780 system equipped with 30 mW 405 nm diode laser, 25 mW 458/488/514 argon multiline laser, 20 mW DPSS 561 nm laser and 5 mW HeNe 633 nm laser mounted on Zeiss Axio Observer Z1 and operated with Zen 2011 software (Zeiss). We used a Plan-APOCHROMAT 63× oil DIC 1.4NA objective for our observations and images were acquired via sequential acquisition.

## 2.7. Phagosome preparation and isolation

Phagosomes were isolated from an immortalized BMM cell line (LM-1) as previously described [\[19\]](#). Briefly, adherent macrophages were incubated with 3 μm magnetic beads (Spherotech) diluted in 1/50 in 10 ml complete medium at

37 °C for 15 min, 1 or 2 h. Cells were resuspended in purification buffer (5 mM EDTA pH 8.0 and PBS, with a final pH of 7.2), lysed by aspiration through a 22 G needle, and then transferred into microfuge tubes. Phagosomes were isolated with a magnet.

## 2.8. Phagocytosis and survival assays

Red fluorescent polystyrene beads (3.0 µm, Sigma-Aldrich, Oakville, Ontario, Canada) were incubated in 10 µg/ml BSA in PBS overnight at 4 °C. The beads were then washed 2 times in PBS and resuspended in PBS. Rabbit anti-BSA antibodies (Sigma-Aldrich), were added to a final dilution of 5 µg/ml and incubated for 1 h at room temperature. The beads were then washed 2 times in DMEM and used immediately. Macrophages were plated in 6-cm dishes at  $2 \times 10^6$  cells/well. Media was changed to serum-free media prior to adding the beads. Opsonized beads were added to macrophages (15:1 ratio) and incubated at 37 °C in 5% CO<sub>2</sub> for the indicated time points. Cells were washed with ice-cold PBS and were detached with a trypsin (0.25%)-EDTA (0.53 mM) solution (ATCC, Manssas, VA, USA) 5 min at 37 °C. Harvested cells were washed 2 times with FACS buffer and analyzed by flow cytometry. *L. major* promastigotes were opsonized with C5-deficient mouse serum. BMM were incubated with promastigotes at ratio of 15:1 for the indicated time points at 37 °C. Excess parasites were removed with ice-cold PBS. Cells were stained with Wright-Giemsa (HEMA 3, Fisher Scientific Co) and processed for microscopy to quantify parasite internalization. Results are based on at least 100 cells chosen by blinded scoring in triplicates. Survival of parasites in BMM was determined at 2, 24, 48 and 72 h post infection. Briefly, after a 2 h chase, non-internalized parasites were removed by two washes in serum-free medium and cells were either fixed immediately with methanol (2 h timepoint) or incubated for the aforementioned time points. Infection levels were assessed by microscopic examination of infected cells upon Giemsa staining. Survival is described as the number of parasites per 100 infected macrophages. Results are based on at least 300 cells chosen by blinded scoring, performed in triplicates.

## 2.9. Quantitation of TNF and determination of nitrite

Adherent BMM ( $3 \times 10^5$ /well in 24 wells plate) were stimulated with IFN-γ 100 U/ml for 18 h. Cells were washed twice and infected with *L. major* promastigotes at a parasite-to-macrophage ratio of 15:1. Supernatants

were harvested after 24 h of culture. TNF production was evaluated using a mouse TNF ELISA kit (eBioscience). The amounts of nitrite released were determined by mixing equal volumes (100  $\mu$ l) of culture supernatants and Griess reagent [37]. Nitrite concentrations were determined by comparing the absorbance values of the test samples with a standard curve generated by serial dilution of 200  $\mu$ M sodium nitrite.

#### 2.10. Protein purification and *in vitro* degradation assays

The pGEX-4T3-Dok-1-PTB and pGEX-4T3-Dok-1-C-term plasmids were kindly provided by Dr. Jacques Nunès (Institut de Cancérologie et d'Immunologie de Marseille, France). GST fusion proteins were purified using glutathione sepharose beads (GE Healthcare). For *in vitro* cleavage assays, 1  $\mu$ g of either GST- Dok-1-PTB or GST- Dok-1-C-term was added to  $30 \times 10^6$  L.

*major* promastigotes (WT,  $\Delta$ *gp63*,  $\Delta$ *gp63* + *gp63*, and WT with 2 mM 1,10-phenanthroline) in 200  $\mu$ l *Leishmania* medium, and incubated for 1 h at 37 °C. After incubation, parasites were discarded, SDS loading buffer containing 2 mM 1,10-phenanthroline was added, and samples were boiled at 100 °C for 6 min.

#### 2.11. Western blot analysis

For *Leishmania* infection, BMM were infected with parasites at a parasite-to-cell ratio of 15:1. After the indicated time at 37 °C, cells were washed twice with ice-cold DMEM and lysed with 1% Nonidet P-40 containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, and 1 mM EGTA in the presence of inhibitors of proteases and phosphatases (10  $\mu$ g/ml leupeptin and aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>). Immunoblotting was performed, as described previously [38]. For  $\lambda$  protein phosphatase treatment (New England Biolabs), cell extracts were prepared without phosphatase inhibitors and 800 unit of phosphatase was added to 1  $\mu$ g of extract for 1 h at 30 °C in presence of MnCl<sub>2</sub>.

#### 2.12. Statistical analysis

An unpaired *t* test was performed using GraphPad software to assess whether the differences between control and infected groups were significant or not. P values <0.05 were considered significant.

### 3. Results



### 3.1. Dok-1 and Dok-2 do not modulate FcγR-mediated phagocytosis or Leishmania internalization

Dok-1 and Dok-2 control several responses of macrophages through the negative regulation of ERK1/2 [2]. Given that activation of ERK1/2 is essential for FcγR-mediated phagocytosis, we hypothesized that Dok-1 and Dok-2 may negatively regulate this process. To examine whether FcγR-mediated phagocytosis is influenced by Dok-1 and Dok-2, we used flow cytometry to compare the capacity of WT and Dok-1/Dok-2-deficient BMM to internalize IgG-coated latex beads (Fig. 1A). The proportions of phagocytic cells containing one, two or more beads were identical for WT and *Dok-1/Dok-2*<sup>-/-</sup> BMM at each time point tested ( Fig. 1A–B). It is important to note that in the absence of Dok-1 and Dok-2, macrophages from bone marrow developed normally under the influence of L929 cell-conditioned media since similar numbers of BMM were recovered from WT and Dok-1/Dok-2-deficient mice and the cell surface expression of CD11b, CD32/16, CD44, Gr-1, F4/80 and CD80 was identical ( Fig. S1). Together these results indicate that Dok-1 and Dok-2 do not modulate the phagocytic capacity of macrophages initiated through FcγR. We next asked whether phagocytosis mediated through other receptors than FcγR may be controlled by Dok-1 and Dok-2. To this end, we used *L. major* promastigotes opsonized with normal mouse serum to infect BMM from WT and *Dok1/Dok2*<sup>-/-</sup> mice. Under these conditions, the predominant receptors that mediate the entry of *Leishmani*promastigotes are the mannose receptor and the complement receptor CR1 and CR3 [reviewed in Ref. [39]]. No difference in the number of internalized parasites was observed between WT and *Dok1/Dok2*<sup>-/-</sup> BMM ( Fig. 1C). Entry of *L. major*promastigotes induced the activation of ERK1/2 ( Fig. 1D). However, activation of ERK1/2 following the entry of the parasite was not modulated by Dok-1 and Dok-2. ( Fig 1D). These results indicate that Dok-1 and Dok-2 are not required for the internalization of *L. major* promastigotes in BMM. Collectively, our data show that Dok-1 and Dok-2 are dispensable for phagocytosis through the receptors studied.

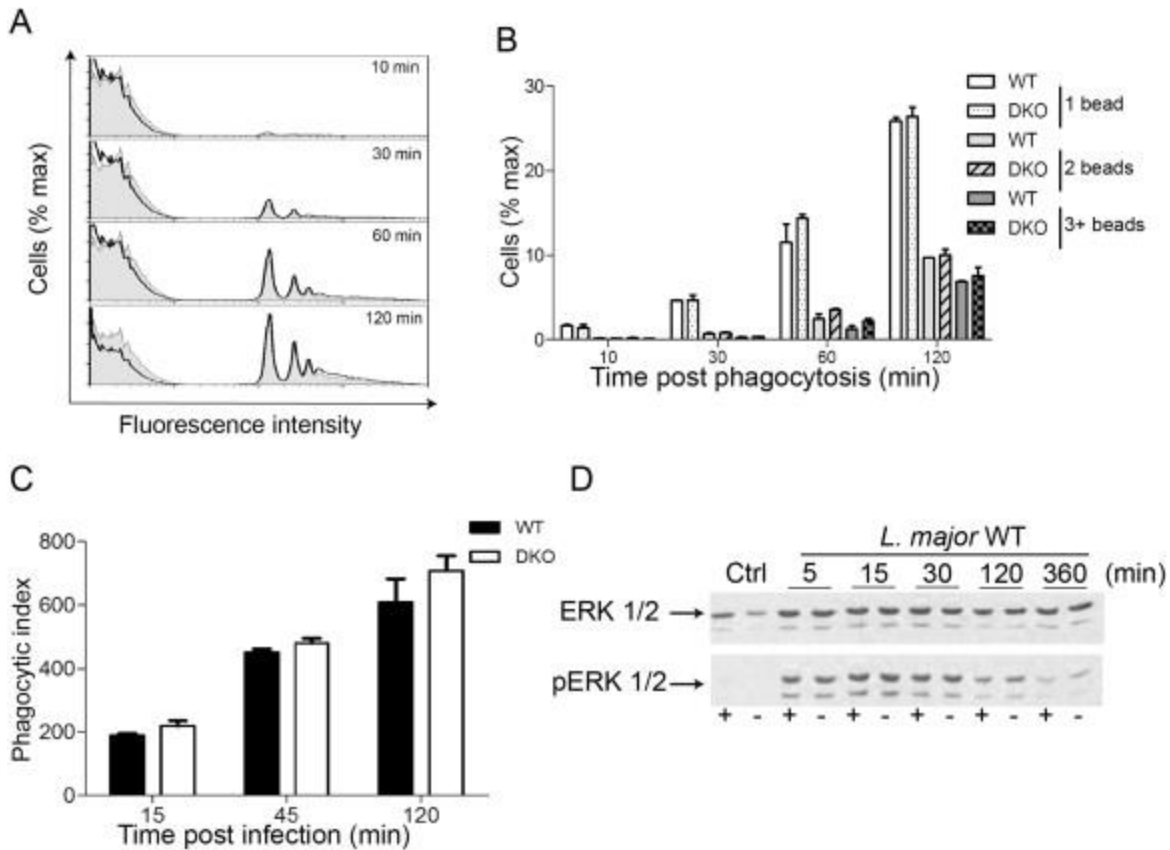


Fig. 1.

Dok-1 and Dok-2 do not control uptake of *L. major* promastigotes or IgG coated beads (A) Representative histograms of BMM from WT (empty histogram) and *Dok-1/Dok-2*<sup>-/-</sup> (filled histograms) mice following ingestion of IgG-coated fluorescent latex beads at the indicated time points. (B) Quantification of phagocytic capacity. Graph is representative of 3 independent experiments and error bars represent  $\pm$ SE. Data shown includes the percentage of cells ingesting one, two, and three and more beads (% phagocytic cells). (C) Adherent WT and *Dok-1/Dok-2*<sup>-/-</sup> (DKO) BMM were incubated with WT *L. major* promastigotes at a parasite-to-cell ratio of 15:1, for the indicated time points. Phagocytic index was determined by counting the number of parasites per 100 macrophages. Graph is representative of 3 independent experiments and error bars represent  $\pm$ SE. (D) BMM from WT (+) and *Dok-1/Dok-2*<sup>-/-</sup> (-) mice were incubated with WT *L. major* promastigotes as in (C). Total cell extracts were used to perform Western blot assays by using antibodies against phospho-ERK1/2. Anti-anti-ERK1/2 antibodies were used as loading controls. Data are representative of at least three experiments.

Figure options

### 3.2. Dok proteins are recruited to phagosomes

We previously identified Dok-1 as a component of the latex beads-containing phagosomes in a macrophage cell line [19]. To investigate whether in primary cells Dok-1 and the other members of the Dok family also associate with phagosomes, we performed confocal microscopy and cellular fractionation to localize Dok-1, Dok-2, and Dok-3 during the phagocytosis of latex beads and zymosan. As shown in Fig. 2A–B, Dok-1 is distributed throughout the cytoplasm, and in few areas near the plasma membrane of BMM. Only background staining was visible in Dok-1/Dok-2-deficient BMM, used as a negative control for the anti-Dok-1 antibody (Fig. 2A–B). Similar to Dok-1, Dok-3 had a cytoplasmic localization (Fig. 2 A–B). After 15 min of phagocytosis of latex beads or zymosan, Dok-1 was enriched in the phagosomal membrane (Fig. 2A–B). Similarly, Dok-3 rapidly associated with phagosomes (Fig. 2A–B). The maximal association occurred after 30 min where 60% and 70% of the phagosomes were positive for Dok-1 and Dok-3 respectively (data not shown and Fig. 2A–B). Dok proteins remained associated with phagosomes for at least 2 h (Fig. 3A–B). The antibodies available against Dok-2 did not allow its specific detection by confocal microscopy. To further analyze the association of Dok proteins to the phagosome, we prepared lysates from magnetic bead phagosomes purified at different time points after initiation of phagocytosis and performed Western blot analysis with anti-Dok-1 and Dok-2 antibodies. As shown in Fig. 2C, Dok-1 and Dok-2 followed a similar kinetics of recruitment to the magnetic beads-containing phagosomes. Altogether, these data demonstrate that the 3 members of the Dok family expressed in macrophages, Dok-1, Dok-2, and Dok-3, are recruited early to phagosomes and remain present throughout the maturation process.

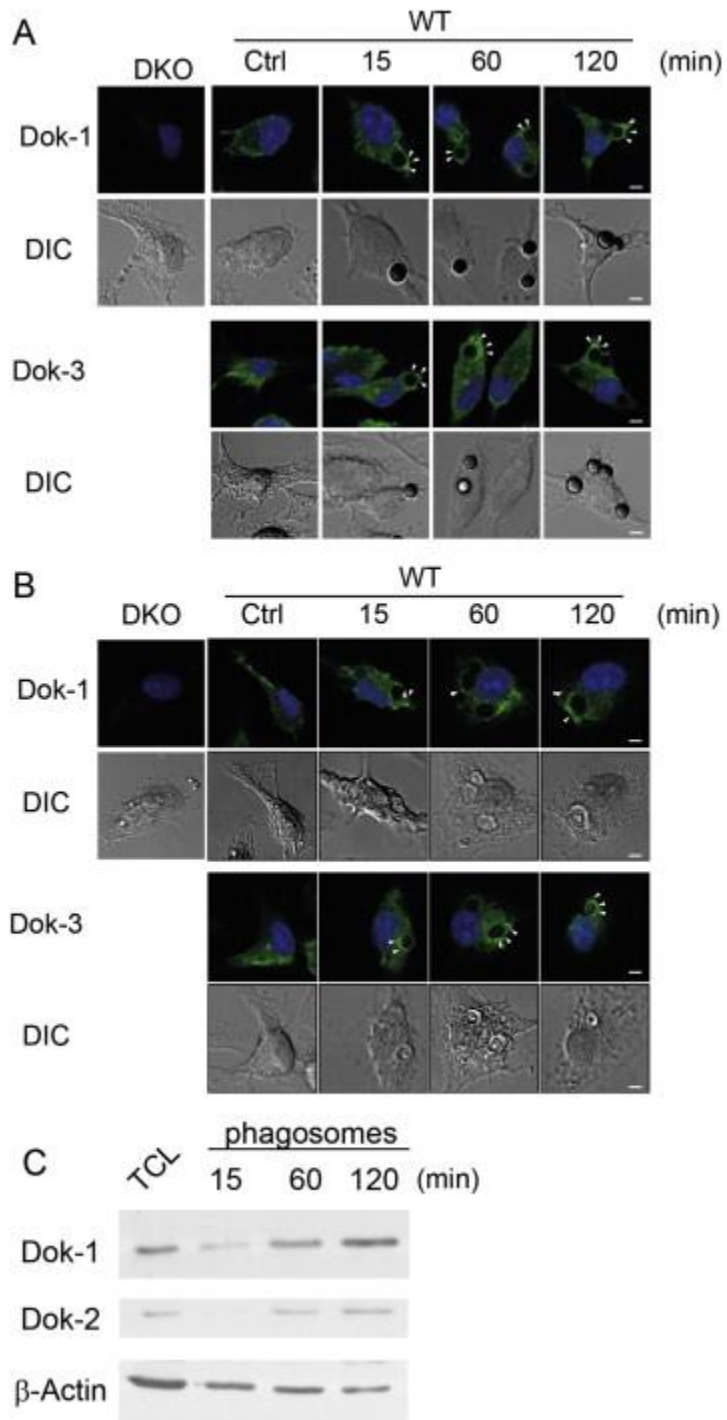


Fig. 2.

Dok proteins are recruited to phagosomes (A–B) BMM were left unstimulated (Ctrl) or allowed to internalize latex beads (A) or zymosan (B) for different time points at a bead-to-cell ratio of 15:1. Cells were fixed and stained with DNA marker DRAQ5 (blue) and with anti-Dok-1 C-terminus and anti-Dok-3 antibodies (green) for immunofluorescence confocal microscopy. *Dok-1/Dok-2*<sup>-/-</sup> (DKO) BMM were used as negative control for

staining. The recruitment of Dok-1 and Dok-3 to phagosomes is shown by arrowheads. (C) Dok-1, Dok-2 proteins are present in phagosome lysates from LM-1 macrophages. Total cell lysates ( $10^5$  cells equivalent) (TCL) or phagosome extracts ( $1.5 \times 10^6$  cells equivalent) were analyzed by Western blot with anti-Dok-1 C-terminal, anti-Dok-2 and anti- $\beta$ -actin antibodies as indicated.

Figure options

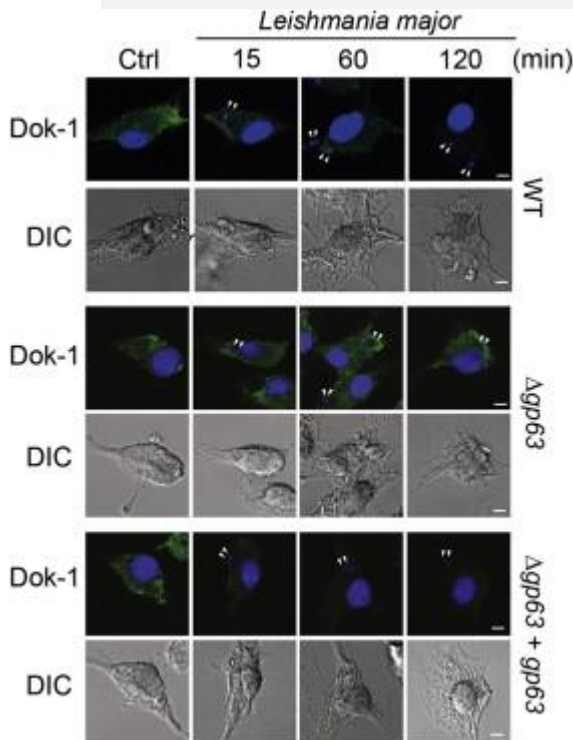


Fig. 3.

Dok-1 is down-modulated in the presence of GP63 Adherent WT BMM were incubated with WT,  $\Delta gp63$ , or  $\Delta gp63 + gp63$  *L. major* promastigotes at a parasite-to-cell ratio of 15:1, for the indicated time points. Immunostaining with anti-Dok-1 (C-Ter) antibodies (green) and DNA marker DRAQ5 (blue) was performed in non-infected (Ctrl) and *L. major*-infected BMM. White arrowheads show phagosomes containing *L. major*.

Figure options

### 3.3. Dok proteins are cleaved in a manner dependent of the GP63 metalloprotease during *Leishmania major* infection

*Leishmania* promastigotes inhibit phagolysosome biogenesis and function by remodeling the phagosomal compartments [21] and [31]. We therefore analyzed whether Dok proteins are recruited to the phagosome and whether they are modulated during *Leishmania* infection. Surprisingly, following *Leishmania* infection, Dok-1 was barely detectable by confocal microscopy ( Fig. 3). Decrease in Dok-1 levels occurred as early as 15 min

following *L. major* infection. GP63, an abundant surface metalloprotease of *Leishmania*, cleaves several molecules involved in the regulation of various pathways of the macrophage [31], [40], [41], [42], [43] and [44]. To determine whether GP63 was responsible for the reduction of Dok-1 levels, we infected BMM with  $\Delta gp63$  *L. major* promastigotes. The levels of Dok-1 were unaltered upon infection with  $\Delta gp63$  *L. major* but were barely detectable when cells were infected with the add-back ( $\Delta gp63 + gp63$ ) *L. major* promastigotes ( Fig. 3). This result indicates that GP63 is responsible for the down-modulation of Dok-1 in BMM infected with *L. major* promastigotes. Of note, although recruitment of Dok-1 to phagosomes containing  $\Delta gp63$  *L. major* was sometimes visible, it was less pronounced than for latex beads or zymosan-containing phagosomes. To analyze whether Dok-2 and Dok-3 are also substrates of GP63, we performed Western blot analysis following infection with WT or  $\Delta gp63$  *L. major* promastigotes. As shown in Fig. 4A, the bulk of Dok-1 and Dok-2 was cleaved within 15 min of infection when GP63 was present. A Dok-1 cleavage product of 40 kDa, which was clearly detected by antibodies directed against the PTB domain of Dok-1, accumulated as the infection progressed. It should be noted that the C-terminus of Dok-1, encompassing amino-acids residues 252–482, was almost completely degraded following *Leishmania* infection since Dok-1 was only faintly detected by Western blotting ( Fig. 4A) or by confocal microscopy using antibodies directed against this C-terminal region of Dok-1 ( Fig. 3). In BMMs Dok-3 migrates on SDS-PAGE as two major diffuse bands of approximately 56 and 60 kDa. Infection by WT or  $\Delta gp63$  promastigotes caused a clear retardation in the electrophoretic mobility of Dok-3 ( Fig. 4B) rendering difficult to assess the cleavage of Dok-3 by GP63. The mobility shift was likely due to serine/threonine phosphorylation and tyrosine phosphorylation since it was abolished by treatment with  $\lambda$  protein phosphatase, a Ser/Thr and Tyr phosphatase and only partially with Tyr or Thr/Ser protein phosphatase (data not shown and Fig. 4B). *In vitro* phosphatase treatment revealed clearly that the apparent molecular weight of Dok-3 was reduced in macrophages infected with WT but not with  $\Delta gp63$  *L. major* ( Fig. 4B). The migration of Dok-3 was similar when cells were infected with WT or ( $\Delta gp63 + gp63$ ) parasites but different from cells infected with  $\Delta gp63$  parasites and not infected control. This result indicates that infection with the GP63 add-back parasites ( $\Delta gp63 + gp63$ ) restored the ability of the  $\Delta gp63$  mutant to cleave Dok-3 ( Fig. 4C). Similarly, the ability to cleave Dok-1 and Dok-2 was regained when cells were infected with the add-



back mutant ( $\Delta gp63 + gp63$ ) promastigotes ( Fig. 4C). These results demonstrate that cleavage of Dok-1, Dok-2, and Dok-3 observed during *Leishmania* infection is GP63-dependent. To analyze whether Dok cleavage by GP63 was dependent of other host proteins, we incubated *in vitro* purified GST fusion proteins encompassing the C-terminal domain (GST-C-ter) or the PTB domain of Dok-1 (GST-PTB) with WT,  $\Delta gp63$ , and  $\Delta gp63 + gp63$  parasites. Only GST-Dok-1 C-ter fusion protein was degraded in presence of an active GP63 ( Fig. 4D). These data support the hypothesis that GP63 cleaves the C-terminal domain of Dok-1 directly ( Fig. S1).

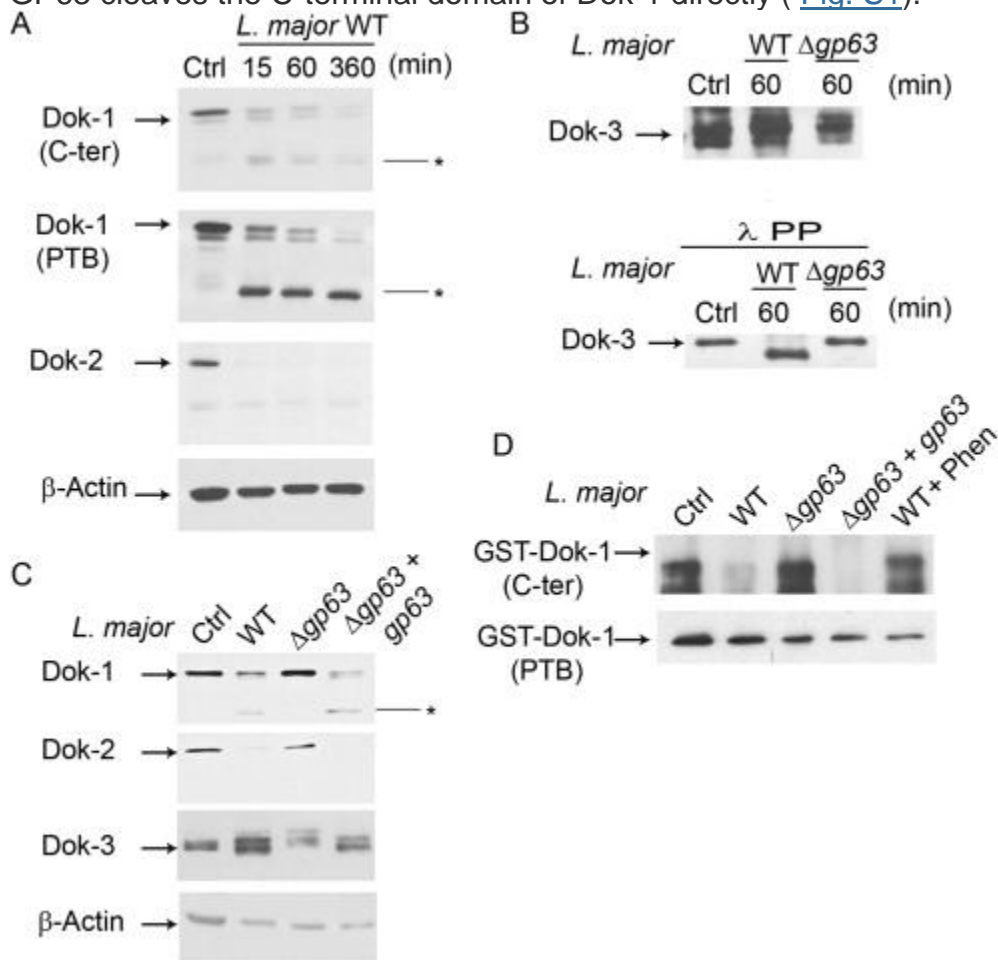


Fig. 4.

Dok-1, Dok2 and Dok-3 proteins are cleaved by GP63 BMM from WT mice were incubated with WT,  $\Delta gp63$ , or  $\Delta gp63 + gp63$  *L. major* promastigotes at a parasite-to-cell ratio of 15:1, for the indicated time points. Non-infected cells were used as control (Ctrl). Total cell extracts were used to perform Western blot analysis using antibodies against Dok-1 C-terminal (C-Ter), Dok-1 PTB (PTB), Dok-2, Dok-3 and  $\beta$ -actin as a loading control (A and C). Cell extracts were left untreated (top panel) or treated with  $\lambda$  protein

phosphatase (APP, bottom panel) before loading and were immunoblotted with anti-Dok-3 antibodies (B). \* indicates the GP63-cleaved product of Dok-1. Data are representative of at least three experiments. (D) GST-Dok-1 C-terminal (GST-C-ter) and GST-Dok-1 PTB (GST-PTB) fusion proteins were incubated for 1 h with media (Ctrl), *L. major* WT,  $\Delta gp63$ ,  $\Delta gp63 + gp63$  promastigotes or with WT parasite in presence of with 1,10-phenanthroline (WT + Phen). Levels of GST fusion proteins were assessed by Western blot analysis with anti-GST antibodies (top panel) or anti-Dok1 PTB domain antibodies (bottom panel).

[Figure options](#)

### 3.4. Dok proteins regulate positively TNF and NO production induced by *Leishmania* infection in IFN- $\gamma$ activated macrophages

Previous studies revealed that Dok-1 and Dok-2 negatively regulate TNF and NO release in LPS-treated macrophages [6]. To investigate whether GP63-dependent cleavage of Dok has an impact on macrophage responses to *Leishmania*, we compared NO and TNF production by control and *Dok-1/Dok-2*<sup>-/-</sup> BMM upon infection with WT or  $\Delta gp63$  *L. major* promastigotes. Entry of promastigotes into naive macrophages occurs in the absence of a proinflammatory response and NO secretion [45]. By contrast, in IFN- $\gamma$ -primed macrophages, *Leishmania* promastigotes induce the secretion of TNF and NO [37] and [45], which is required for parasite killing [37]. Surprisingly, in response to *L. major*, TNF and NO (assessed by measuring nitrite) release was reduced in IFN- $\gamma$ -primed BMM isolated from *Dok-1/Dok-2*-deficient mice compared to WT mice. This result indicates that Dok-1 and Dok-2 positively regulate TNF and NO production induced by *Leishmania* infection in IFN- $\gamma$ -primed BMM ( Fig. 5A–B). However, Dok-mediated up-regulation of NO and TNF release in response to *Leishmania* infection was independent of GP63 ( Fig. 5A–B).



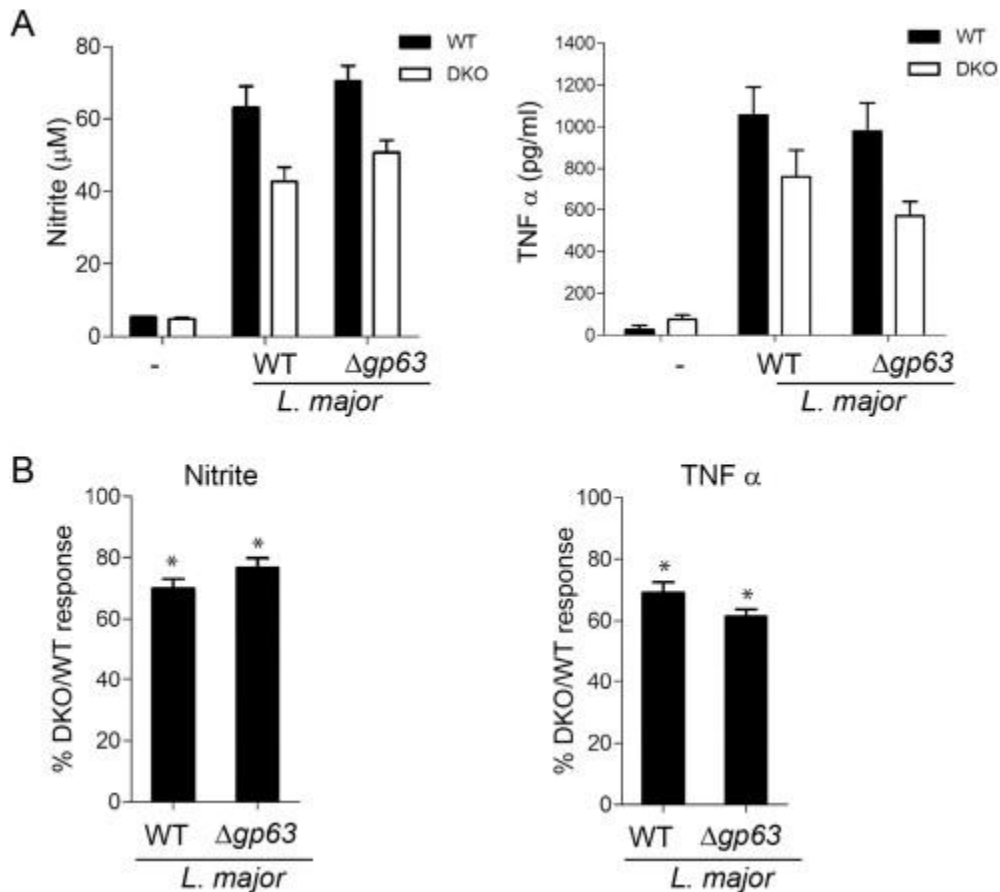


Fig. 5.

NO and TNF secretion is reduced in *Dok-1/Dok-2*<sup>-/-</sup> BMM (A–B) IFN- $\gamma$  primed BMM from WT or *Dok-1/Dok-2*<sup>-/-</sup> (DKO) mice were left unstimulated (-) and incubated with either WT or  $\Delta$ gp63 *L. major* promastigotes in a parasite-to-cell ratio of 15:1. Supernatants of stimulated BMM were evaluated for TNF and nitrites production after 24 h. (A) The experiment shown is representative of 3 independent experiments performed in triplicate. (B) Graphs represent the average of 3 independent experiments and are expressed as percentage of *Dok-1/Dok-2*<sup>-/-</sup> BMM responses compared to WT BMM responses to eliminate variability between experiments. Statistical significance was determined with an unpaired Student's *t* test.\* indicates  $p < 0.05$ .

[Figure options](#)

### 3.5. Dok-1 and Dok2 do not modulate promastigote survival in BMM

Upon their internalization by macrophages, *Leishmania* promastigotes inhibit phagolysosome biogenesis. Since Dok proteins associate to phagosomes, we tested the impact of Dok on the survival of *Leishmania* in macrophages in absence of IFN- $\gamma$  mediated effects. Metacyclic promastigotes were used to infect

WT and Dok1/Dok2-deficient BMM. The absence of *Dok-1* and *Dok-2* had no influence on the survival of parasite during the first 3 days post-infection ( [Fig. 6](#)).

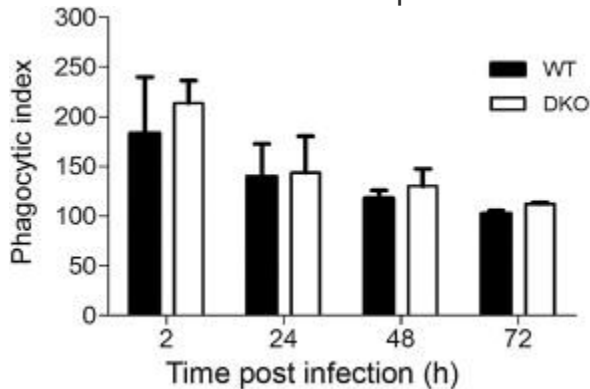


Fig. 6.

Dok-1 and Dok-2 do not regulate survival of *L. major* promastigotes in BMM Adherent WT and *Dok-1/Dok-2*<sup>-/-</sup> (DKO) BMM were incubated with WT *L. major* promastigotes at a parasite-to-cell ratio of 15:1, for the indicated time points. Phagocytic index was determined by counting the number of parasites per 100 macrophages. Values are means  $\pm$  SE from 3 separate experiments and are represented by the error bars.

[Figure options](#)

#### 4. Discussion

Dok-1, Dok-2 and Dok-3, the three Dok proteins expressed in myeloid cells, are essential for myeloid homeostasis and are key modulators of cytokine and LPS responses through negative regulation of ERK1/2 signaling [\[6\]](#). In this study, we analyzed the fate and the involvement of Dok proteins in phagocytosis and following *L. major* infection in BMM.

Phagocytosis triggers signaling pathways that lead to the recruitment of Dok proteins to the phagosome. The signals that target Dok to the membrane might be delivered by various phagocytic receptors including the mannose receptor and the complement receptors CR1 and CR3. Dok-1 and Dok-2 PH domains bind *in vitro* to mono phosphoinositide species, mainly phosphatidylinositol 5-phosphate (PI5P) and 4-phosphate and to polyphosphoinositide species including phosphatidylinositol 3,4,5-triphosphate, 3,4-diphosphate and 4,5-diphosphate albeit with less affinity [\[3\]](#) and [\[46\]](#). These phospholipids are present in the membrane at different time points during phagosomal maturation and might provide anchoring sites for Dok proteins at the phagosome [\[47\]](#). Interestingly, PI5P, which binds to Dok PH domain with the highest affinity, is potentially generated at the late phagosome maturation stage [\[47\]](#).

Dok binding partners that are recruited to the nascent phagosome might be involved in different aspects of the phagocytotic process, such as cytoskeletal remodeling, fission, or phagosomal maturation. In particular, SHIP recruitment to the phagosome might be, at least in part, mediated by Dok proteins since the three Dok-1, Dok-2 and Dok-3 have been shown to bind SHIP-1 after FcR signaling [48], [49] and [50]. Therefore we propose that membrane localization of Dok proteins following phagocytosis allowed the docking of signaling molecules at the phagosome. Additional work will be required to determine which binding partners play a role in Dok-mediated signaling at the phagosome and the consequence of these interactions on phagosome function.

The three Dok proteins are *in vivo* targets of the GP63 protease. GP63 is a virulence factor [33] that cleaves several host molecules to modulate macrophage responses to infection. These include signaling molecules, transcription factors, and components of the macrophage translation and membrane fusion machineries [40], [41], [44],[51] and [52]. How GP63 accesses and cleaves host cell substrates is poorly understood. The presence of GP63 in exosomes released by *Leishmania* points to a potential mechanism for the transport of this protease from the phagosome to the host cell cytoplasm [53]. This scenario is in agreement with the ability of this protease to degrade host molecules outside parasitophorous vacuoles. Indeed, previous studies indicated that GP63 exits the phagosome and colocalizes with macrophage membrane microdomains [40] thereby giving access to various host cell substrates. In the case of Dok proteins, their recruitment to membranes through their PH domain may facilitate their cleavage by GP63. However, we cannot exclude the possibility that GP63 mediates indirectly the cleavage of Dok proteins. Future studies will address this issue.

The determination of the potential cleavage sites [54] and the size of the cleavage products detected with domain-specific antibodies, led us to predict the most likely positions of the *in vivo* Dok cleavage by GP63 following *Leishmania* infection ( Fig. S2). GP63-dependent cleavage should let an intact PH and PTB domains for Dok-1 and Dok-2 and therefore should not affect their membrane localization. We cannot predict what will be the effect of GP63 cleavage on the membrane localization of Dok-3 since the two potential cleavage sites are either in the PH or in the C-terminus domain. The deletion of most of the C-terminal part of Dok-1 and Dok-2 after GP63-dependent cleavage

will likely prevent the recruitment of Dok-binding partners to the phagosomal membrane.

The association of Dok proteins with phagosomes suggests that these molecules may participate in phagolysosome biogenesis or phagosome function, possibly through the modulation of phosphotyrosine signaling [32]. Elucidating the impact of Dok proteins on the biology of phagosomes is clearly an important issue that will deserve further investigation. Indeed, such knowledge is expected to contribute to our understanding of the impact of Dok cleavage in a GP63-dependent manner on the functionality of the vacuoles in which *Leishmania* parasites establish infection. It is also possible that Dok proteins participate in phagosome signaling that is linked to the detection of pathogens and the activation of appropriate innate immune responses [55]. For instance, toll-like receptors recruited to phagosomes signal the presence of pathogen-derived molecules. Dok-1, Dok-2 and Dok-3, which were previously shown to act downstream of toll-like receptor 4 [6], could be part of the platforms of regulators that assemble on phagosomes and that are linked to innate immune sensors present on those organelles [55]. Our finding that Dok-1 and Dok-2 proteins modulate TNF and NO production induced by *Leishmania* infection is consistent with this potential role, and highlights the contribution of Dok signaling in the host response to *Leishmania*. Intriguingly, Dok-1 and Dok-2 appear to act positively on these responses which contrasts with their negative regulatory role in most macrophage responses studied so far [2]. However, this effect is independent of GP63 raising the possibility that Dok-1/Dok-2 portions deleted by GP63 are not necessary for the regulation of TNF and NO responses. In conclusion, the finding that Dok proteins are recruited to phagosomes points towards a novel role for this family of signaling molecules in the biology of phagosomes. The targeting of Dok proteins by an intracellular pathogen such as *Leishmania* may thus be relevant in this context. To address this issue, future studies will be aimed at determining the role of Dok proteins during experimental leishmaniasis. Recently, it has been reported that Epstein–Barr virus is directly involved in the down-modulation of Dok-1 expression [56]. Therefore, inactivation of Dok protein might be a common strategy used by several pathogens for their survival in the host.

#### Conflict of interest

The authors have no financial conflicts of interest.

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