Differential role of Dok1 and Dok2 in TLR2-induced inflammatory signaling in glia

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Abstract

Accumulating evidence continues to underpin the role of the innate immune system in pathologies associated with neuroinflammation. Innate immunity is regulated by pattern recognition receptors that detect pathogens, and in the case of Gram-positive bacteria, binding of bacterial lipopeptides to toll-like receptor (TLR)2 is emerging as an important mechanism controlling glial cell activation. In the present study, we employed the use of the synthetic bacterial lipoprotein and a selective TLR2 agonist, Pam3CSK4, to induce inflammatory signaling in microglia and astrocytes. The adaptor proteins, downstream of kinase (Dok)1 and Dok2, are known to have a role in negatively regulating the Ras-ERK signaling cascade, with downstream consequences on pro-inflammatory cytokine expression. Data presented herein demonstrate that TLR2 enhanced the tyrosine phosphorylation of Dok1 and Dok2 in astrocytes and microglia, and that knockdown of these adaptors using small interfering RNA robustly elevated TLR2-induced ERK activation. Importantly, TLR2-induced NF-κB activation, and IL-6 production was exacerbated in astrocytes transfected with Dok1 and Dok2 siRNA, indicating that both Dok proteins negatively regulate TLR2-induced inflammatory signaling in astrocytes. In contrast, Dok1 knockdown attenuated TLR2-induced NF-κB activation and IL-6 production in microglia, while Dok2 siRNA failed to affect TLR2-induced NF-κB activity and subsequent cytokine expression in this cell type. Overall, this indicates that Dok1 and Dok2 are novel adaptors for TLR2 in glial cells and importantly indicates that Dok1 and Dok2 differentially regulate TLR2-induced pro-inflammatory signaling in astrocytes and microglia.

Introduction

The innate immune system represents the earliest defense against bacterial molecules, orchestrating protection via an inflammatory response to invading pathogens. This system is tightly regulated by a complex mechanism involving pattern recognition receptors (PRRs) that recognize molecular signatures of microbes known as pathogen-associated molecular patterns (PAMPs), in addition to endogenous damage-associated molecular patterns (DAMPs) that are released by injured tissue (Vezzani et al., 2011). Toll-like receptors (TLRs) belong to the family of signaling PRRs, and a large body of evidence indicates that TLR ligands induce central nervous system (CNS) inflammation via production of cytokines, nitric oxide and chemokines (Lehnardt, 2010; Lehnardt et al., 2007). In the case of Gram-positive bacteria, components of the bacterial cell wall interact with TLR2, a receptor ubiquitously expressed on immune cells but also identified on cells of the CNS (Aravalli et al., 2008; Babcock et al., 2006; Bsibsi et al., 2002). TLR2 continues to emerge as a player in CNS diseases and data link TLR2 activation with multiple sclerosis (MS) (Farez et al., 2009), Parkinson's disease (PD) (Kalinderi et al., 2013) and Alzheimer's disease (AD) (Wang et al., 2011). The best known TLR2 ligands are bacterial lipoproteins and peptidoglycans (Akira, 2003), although the exact molecular motif required for binding has not been identified. Once activated, TLR2 recruits the adaptor myeloid differentiation factor 88 (MyD88) (Medzhitov et al., 1998), resulting in the activation of transcription factors, such as nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs), and the subsequent induction of genes encoding interferons (IFNs) and pro-inflammatory cytokines (Moynagh, 2005).

The downstream of kinase (Dok) family members are adaptor proteins that function as modulators of protein tyrosine kinase (PTK) signaling. Dok adaptors constitute a family of at least seven members and continue to emerge as an expanding group of insulin receptor substrates-related signaling molecules, consisting of amino-terminal

Abbreviations: AD, Alzheimer's disease; CNS, central nervous system; DAMPs, damage-associated molecular patterns; Dok, downstream of kinase; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GM-CSF, granulocyte macrophage colony stimulating factor; HBSS, Hanks' balanced salt solution buffered; IFN, interferon; Mal, MyD88-adaptor like; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony stimulating factor; MS, multiple sclerosis; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; PAMPs, pathogen-associated molecular patterns; PD, Parkinson's disease; PH, pleckstrin homology; PPRs, pattern recognition receptors; PTK, protein tyrosine kinase; SH, Src homology; siRNA, small interfering RNA; SLAM, signaling lymphocyte activation molecule; TIR, Toll-interleukin-1 receptor; TLR, Toll-like receptor; TRAM, TRIF-related adaptor molecule; TRIF, Toll-interleukin-1 receptor (TIR)-domain-containing adaptor-inducing IFN-β.

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pleckstrin homology (PH) and PTB domains (Carpino et al., 1997). Dok1 is a common substrate of many PTKs, and once tyrosine phosphorylated, Dok1 and its closest homologue, Dok2, recruit multiple Src homology (SH)2-containing molecules such as p120 rasGAP (Jones and Dumont, 1998). Dok1 and Dok2 are involved in a wide array of immunoreceptor signaling pathways, including CD200 receptor (Zhang and Phillips, 2006), signaling lymphocyte activation molecule (SLAM) (Latour et al., 2001) and TLR4 (Shinohara et al., 2005). Evidence indicates that upon tyrosine phosphorylation, both Dok1 and Dok2 bind Ras-GTPase-activating protein, thereby acting as negative regulators of Ras-extracellular signal-regulated kinase (ERK) activation (Yamanashi et al., 2000). Dok1 and Dok2 are preferentially expressed in immune cells, but evidence of their expression in non-hematopoietic cells suggests non-immunological functions (Berger et al., 2010; Smith et al., 2004). Furthermore, the precise stage at which Dok1 and Dok2 impact downstream signaling is unclear (Smith et al., 2004).

Given previous reports linking TLR2 with neuroinflammatory disorders, in addition to evidence that Dok1 and Dok2 are novel adaptors for TLR4 signaling in macrophages (Shinohara et al., 2005), we were particularly interested to explore the role of Dok1 and Dok2 in mediating TLR2-induced inflammatory signaling in glial cells. We demonstrate that Dok1 and Dok2 are novel signaling adaptors for TLR2 in both microglia and astrocytes and that these adaptors differentially regulate downstream inflammatory signaling in glia in response to TLR2 stimulation. This study thus identifies novel targets for regulating the innate immune response in neuroinflammatory disorders.

Results

Engagement of TLR2 on astrocytes and microglia induces phosphorylation of Dok1 and Dok2 and recruitment of RasGAP

We initially characterized the expression of TLR2, Dok1 and Dok2 by PCR and immunohistochemistry in isolated astrocytes, microglia and neurons. Astrocytes and microglia express TLR2, Dok1 and Dok2 mRNA (Fig. 1A) and protein (Fig. 1B). Dok1 mRNA expression and Dok1 immunoreactivity is markedly greater than Dok2 in astrocytes (Fig. 1A and B). Detectable expression of TLR2, Dok1 and Dok2 mRNA was also determined in neurons (Fig. 1A). We next determined if the synthetic TLR2 agonist, Pam3CSK4, had a regulatory role on endogenous TLR2, Dok1 and Dok2 expression in microglia and astrocytes. TLR2 stimulation time-dependently enhanced relative TLR2 mRNA expression in astrocytes (Fig. 1C) and microglia (Fig. 1D), as determined by PCR. The increases were maximal at 4 h (300-fold in astrocytes; 87-fold in microglia), but the increases persisted for at least 24 h (25-fold in astrocytes; 23-fold in microglia). However, Pam3CSK4 had no effect on relative Dok1 and Dok2 mRNA expression in astrocytes (Fig. 1E) or microglia (Fig. 1F), indicating that TLR2 activation is not associated with an increase in the relative expression profiles of both Dok proteins. Next, to address the role of Dok1 and Dok2 in TLR2-induced signaling, we examined tyrosine phosphorylation of the Dok proteins in glia. Anti-phosphotyrosine immunoblot and co-immunoprecipitation analysis revealed that Dok1 and Dok2 are time-dependently tyrosine phosphorylated in both astrocytes (Fig. 1G) and microglia (Fig. 1H) following incubation with the synthetic TLR2 agonist, Pam3CSK4. Interestingly, the tyrosine phosphorylation of Dok1 and Dok2 occurred after 10 and 60 min in astrocytes, respectively (Fig. 1G). In microglia, tyrosine phosphorylation of Dok1 and Dok2 occurred after 30 and 10 min, respectively (Fig. 1H). Furthermore, analysis determined that tyrosine phosphorylation of Dok1/2 after Pam3CSK4 treatment co-immunoprecipitated with p120 rasGAP (Fig. 1G and H). This indicates that although TLR2 stimulation enhances tyrosine phosphorylation of both Dok proteins within 60 min, the biochemical responses of the two proteins differ in both cell types.

TLR2 stimulation enhances ERK and NF-κB in glia

To temporally characterize TLR2-induced inflammatory signaling in glia, we next assessed the impact of Pam3CSK4 treatment on two signal mediators of cytokine production, ERK and NF-κB. Pam3CSK4 promoted the time-dependent increase in ERK phosphorylation in astrocytes (Fig. 2A) and microglia (Fig. 2B). Similarly, TLR2 stimulation time-dependently enhanced NF-κB activation in astrocytes (Fig. 2C) and microglia (Fig. 2D), as determined by the electrophoretic mobility shift assay (EMSA) and phosphorylation of IκB proteins, which regulate NF-κB sequestration in the cytoplasm (Clement et al., 2008). This indicates that TLR2 stimulation robustly enhances ERK and NF-κB signaling in microglia and astrocytes.

Dok1 and Dok2 are negative regulators of ERK signaling upon Pam3CSK4 treatment in glia

To understand the role of the Dok1 and Dok2 proteins in TLR2-induced inflammatory signaling in glia, we employed the use of Dok1 and Dok2 siRNA. The efficiency of Dok1 and Dok2 knockdown in astrocytes (Fig. 3A) and microglia (Fig. 3B) was assessed by western blotting. The quantitative data obtained from Western immunoblotting indicated that there was 38% and 46% reduction in Dok1 and Dok2 expression in astrocytes, respectively, relative to a non-target control siRNA (Fig. 3A). Similarly, Dok1 and Dok2 expression was reduced by 59% and 40% in microglia, respectively, relative to a non-target control siRNA (Fig. 3B). The effect of Dok knockdown on the activation of ERK was determined upon Pam3CSK4 treatment in both astrocytes and microglia. Pam3CSK4 time-dependently enhanced ERK phosphorylation in astrocytes transfected with non-target siRNA, with mean significant phosphorylation observed at 60 min post-TLR2 exposure (Fig. 3C). Transfecting astrocytes with Dok1 and Dok2 siRNA prior to Pam3CSK4 treatment significantly enhanced ERK phosphorylation, and the enhancement persisted for 60 min (Fig. 3C). Interestingly, the TLR2-induced time-dependent increase in ERK phosphorylation was lost following Dok1 and Dok2 siRNA transfection, suggesting that the effect of knockdown alters the profile of the effect of TLR2 activation. Similarly, Pam3CSK4 time-dependently enhanced ERK phosphorylation in microglial cells transfected with non-target siRNA, with mean significant phosphorylation observed at 10 min post-TLR2 exposure (Fig. 3D). Again, Pam3CSK4-induced ERK phosphorylation was robustly enhanced and sustained in Dok1 and Dok2 siRNA-treated microglia (Fig. 3D). Overall, this indicates that Dok1 and Dok2 are essential adaptors in the negative regulation of ERK upon TLR2 stimulation.

Dok1 and Dok2 are differential regulators of NF-κB upon Pam3CSK4 exposure in glia

We next assessed the impact of Dok1/2 knockdown on TLR2-induced phosphorylation of IκB-α and activation of NF-κB. First in astrocytes, Pam3CSK4 exposure (20 min) enhanced phospho-IκB-α expression and NF-κB DNA binding activity (Fig. 3E). Importantly, Pam3CSK4-induced IκB-α phosphorylation, and NF-κB DNA binding activity was exacerbated in astrocytes transfected with both Dok1 and Dok2 siRNA, indicating that both Dok proteins negatively regulate TLR2-induced NF-κB activity in astrocytes. Microglia robustly responded to Pam3CSK4 exposure in terms of IκB-α phosphorylation and NF-κB DNA binding activity (Fig. 3F). Interestingly, Dok1 knockdown significantly attenuated TLR2-induced phosphorylation of IκB-α and NF-κB activation in microglia, while transfection of Dok2 siRNA failed to impact Pam3CSK4-induced phospho-IκB-α expression and NF-κB activation in this cell type. This indicates that Dok1 and Dok2 differentially regulate TLR2 signaling to NF-κB in astrocytes and microglia.

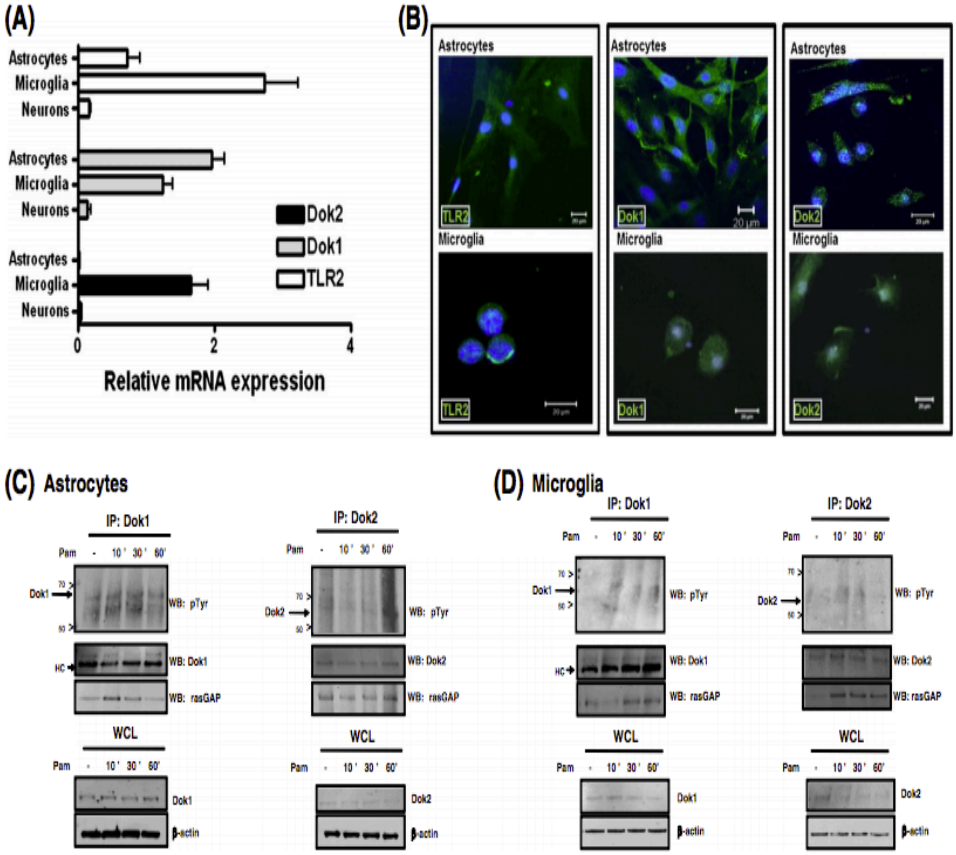


Fig. 1. TLR2, Dok1 and Dok2 are expressed in glia, and Dok1/2 phosphorylation is induced by TLR2 activation. (A) cDNA was generated from primary mouse microglia, astrocytes and neurons and assayed by quantitative real-time PCR for levels of TLR2, Dok1 and Dok2 mRNA. The expression level of each gene was normalized relative to expression of 18S rRNA. (B) Primary astrocytes and microglia were cultured on coverslips coated with poly-l-lysine, fixed, mounted in anti-fade medium with DAPI and visualized using confocal microscopy. Images are representative of three independent experiments. Scale bars are 20 μm. (C) Astrocytes and (D) microglia were treated with Pam3CSK4 (100 ng/ml; 1–24 h), cDNA generated and assayed by quantitative real-time PCR for levels of TLR2 mRNA. (E) Astrocytes and (F) microglia were treated with Pam3CSK4 (100 ng/ml; 24 h), and cDNA was generated and assayed by quantitative real-time PCR for levels of Dok1 and Dok2 mRNA. (G) Astrocytes and (H) microglia were treated with Pam3CSK4 (100 ng/ml; 10–60 min) and Dok1 and Dok2 immunoprecipitates (IP), or whole cell lysates (WCL) were subjected to western immunoblotting using Dok1, Dok2, phosphotyrosine (pTyr), p120 rasGAP and β-actin antibodies. The position of the immunoglobulin heavy chain (HC) is indicated. The bottom panels represent average data quantified by densitometry of immunoblots, expressed as percentage tyrosine phosphorylation. Data are presented as the mean ± SEM and are triplicate determinations from 6 animals (A, C, D, E, F), or are representative of data obtained from 9 to 12 animals (B, G, H). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with vehicle-treated cells.

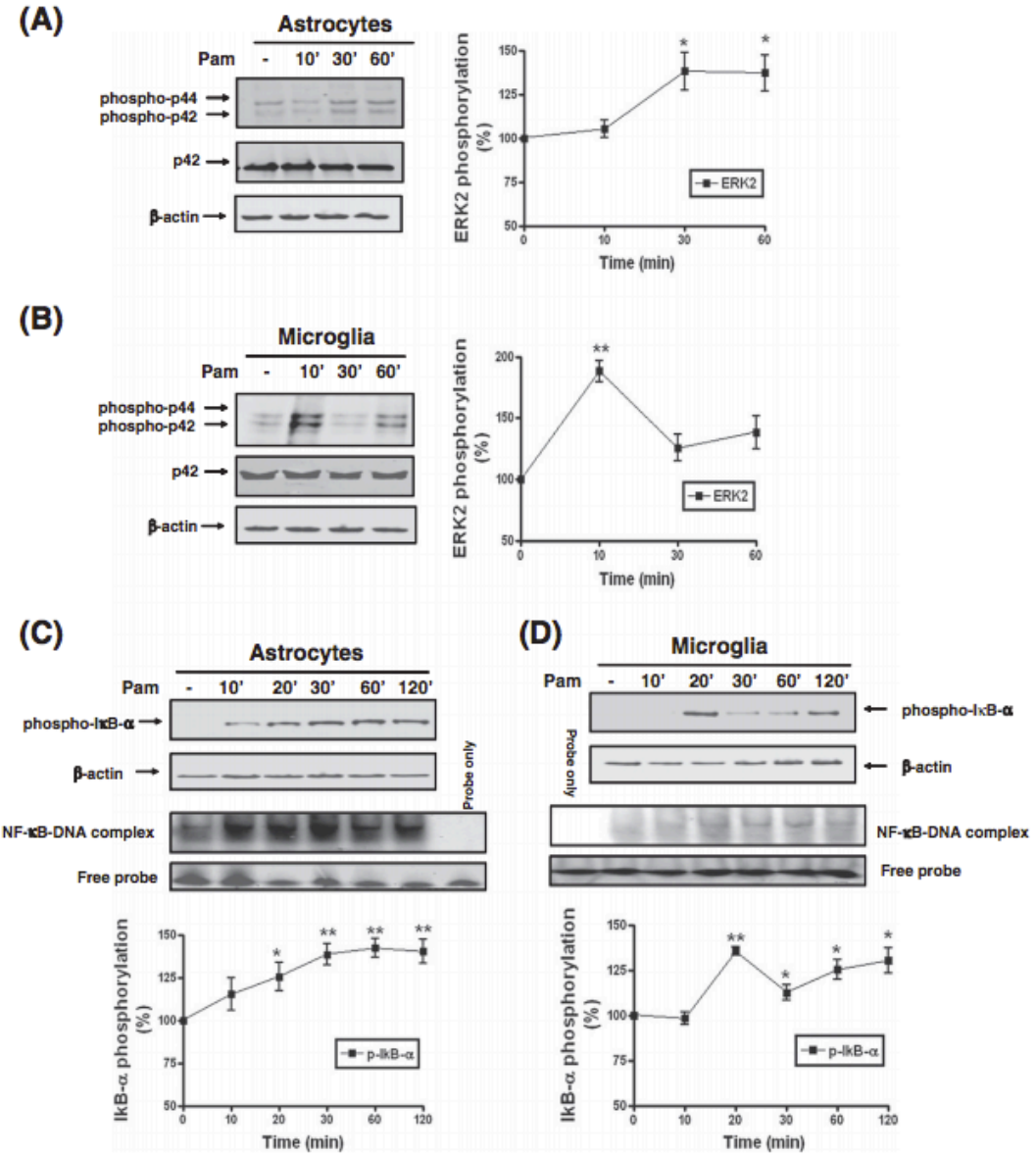
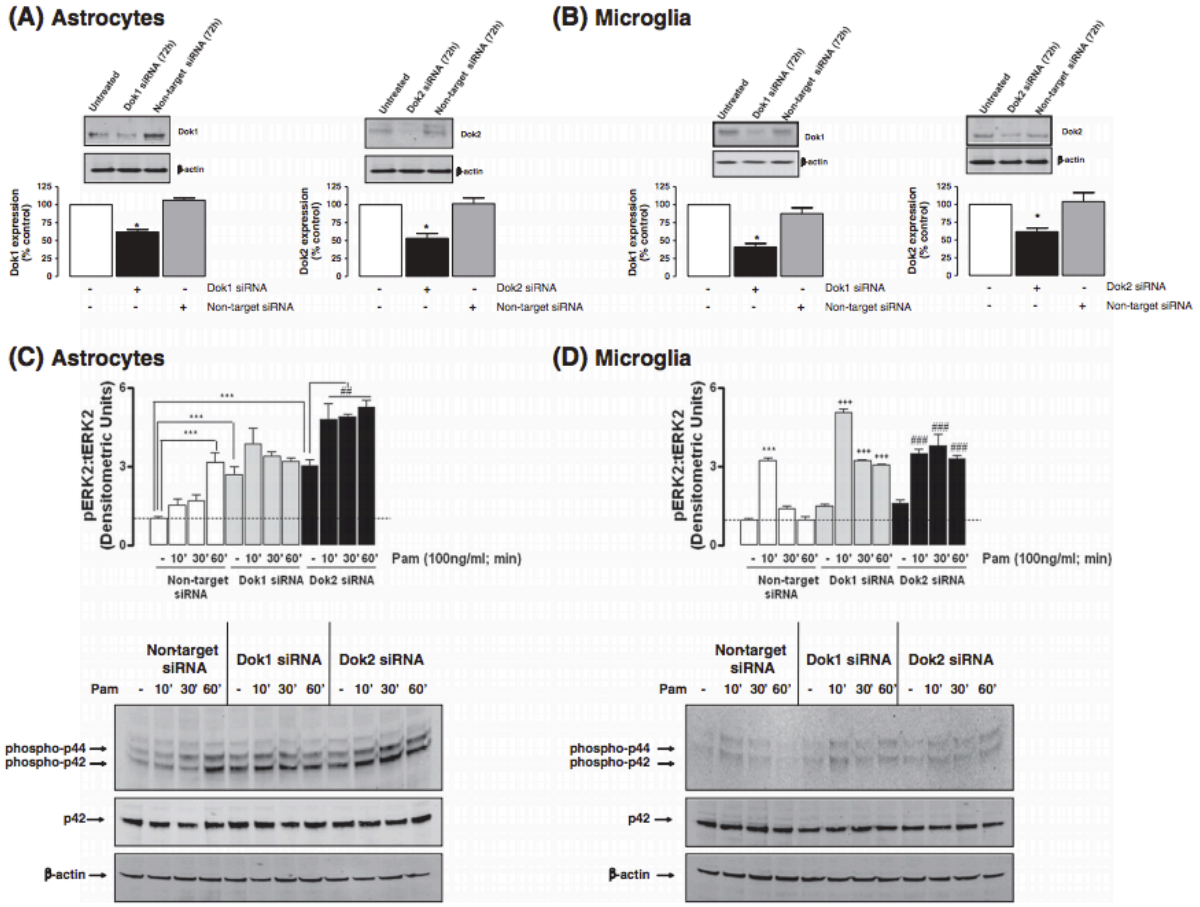


Fig. 2. TLR2 signals to ERK and NF-κB in glia. Primary mouse (A) astrocytes and (B) microglia were treated with Pam3CSK4 (100 ng/ml; 10–60 min). Cell lysates were prepared and subsequently subjected to western immunoblotting using anti-phospho-ERK, anti-ERK2 and anti-β-actin antibodies. Primary mouse (C) astrocytes and (D) microglia were treated with Pam3CSK4 (100 ng/ml; 10–120 min), cytosolic fractions were prepared and subjected to western immunoblotting using anti-phospho-IκB-α and anti-β-actin antibodies. Nuclear fractions were prepared and assessed for NF-κB activity by gel mobility shift assay. Positions of the NF-κB complex and free probe are indicated. Data are mean ± SEM and are representative of data obtained from 12 animals. *$p < 0.05$ and **$p < 0.01$ compared with vehicle-treated cells.

Dok1 and Dok2 differentially regulate TLR2-induced IL-6 production in glia

To determine the role of Dok1 and Dok2 proteins in TLR2-induced cytokine production in astrocytes and microglia, we again employed the use of Dok1 and Dok2 siRNA and assessed the production of IL-6 following Pam3CSK4 exposure. Pam3CSK4 significantly enhanced IL-6 expression at both protein (Fig. 4A) and mRNA (Fig. 4B) levels in astrocytes, with mean maximal stimulatory effects on protein (Fig. 4A) and mRNA (Fig. 4B) expression levels observed at 8–24 and 4 h, respectively. The transfection of astrocytes with Dok1 and Dok2 siRNA prior to Pam3CSK4 treatment significantly enhanced IL-6 expression (Fig. 4C), mirroring the data presented in Fig. 3. Similarly, the exposure of microglia to Pam3CSK4 significantly enhanced IL-6 expression at both protein (Fig. 4D) and mRNA (Fig. 4E) level in microglia, with significant effects on protein (Fig. 4D) and mRNA (Fig. 4E) observed at 24 and 4 h, respectively. Importantly, Dok1 knockdown significantly attenuated TLR2-induced IL-6 production in microglia, while Dok2 siRNA failed to impact Pam3CSK4-induced IL-6 production in microglial cells (Fig. 4F). Similarly Dok1 and


(A) Astrocytes
(B) Microglia
Untreated
Dok1 siRNA (72h)
Non-target siRNA (72h)
Dok2 siRNA (72h)
Dok1
Dok2
β-actin
Dok1 expression (% control)
Dok2 expression (% control)
Dok1 siRNA
Dok2 siRNA
Non-target siRNA
(C) Astrocytes
(D) Microglia
pERK2:tERK2 (Densitometric Units)
Pam (100ng/ml; min)
Non-target siRNA
Dok1 siRNA
Dok2 siRNA
phospho-p44
phospho-p42
p42
β-actin

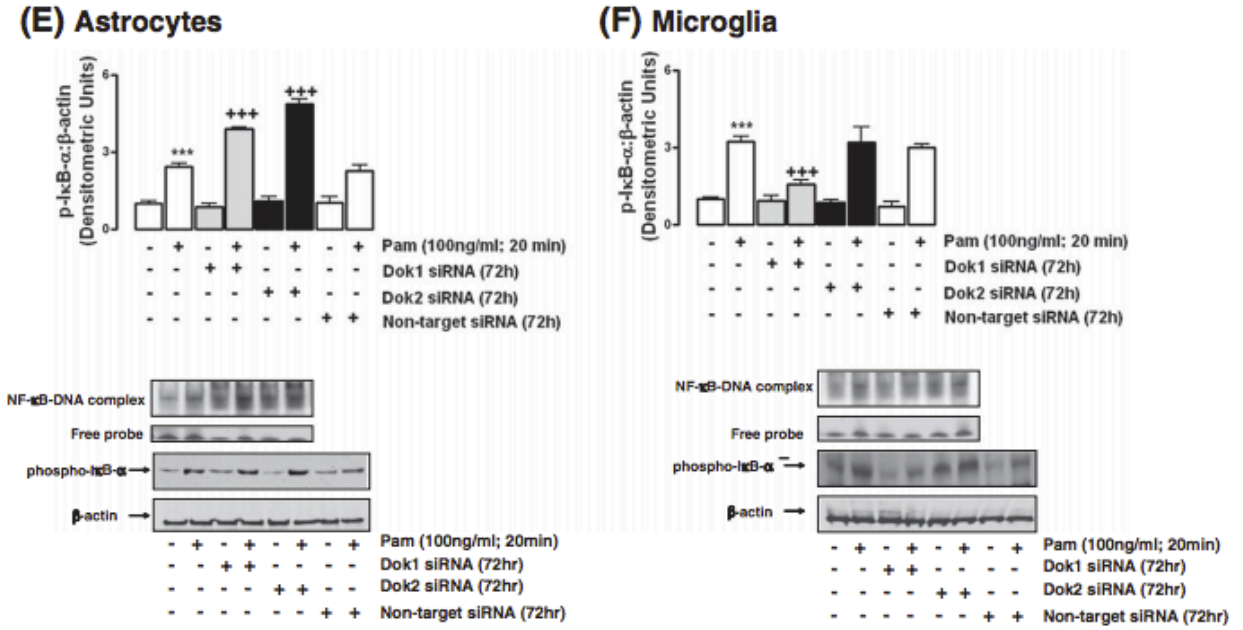


Fig. 3. Dok1 and Dok2 are adaptors associated with TLR2 signaling to ERK and NF-κB in glia. Primary mouse (A) astrocytes and (B) microglia were transfected with non-targeting siRNA and ON-TARGET plus siRNA targeting mouse Dok1 and Dok2. After 72 h, cell lysates were prepared and subjected to western immunoblotting using anti-Dok1, anti-Dok2 and anti-β-actin antibodies. Primary (C) astrocytes and (D) microglia were transfected with non-targeting siRNA and Dok1/Dok2 siRNA. After 72 h, cells were treated with Pam3CSK4 (100 ng/ml; 10–60 min), lysates were prepared and subjected to immunoblotting using anti-phospho-ERK, anti-ERK2 and anti-β-actin antibodies. (E) Astrocytes and (F) microglia were transfected with non-targeting siRNA and Dok1/Dok2 siRNA. After 72 h, cells were treated with Pam3CSK4 (100 ng/ml; 20 min), and cytosolic fractions were subjected to western immunoblotting using anti-phospho-IκB-α and anti-β-actin antibodies. Nuclear fractions were prepared and assessed for NF-κB activity by gel mobility shift assay. All data are mean ± SEM and are representative of data obtained from 12 animals. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with vehicle-treated cells (A, B, E, F) and untreated control siRNA-transfected cells (C, D). $^{+++}p < 0.001$ compared with untreated Dok1 siRNA-transfected cells (D) and non-transfected cells treated with Pam3CSK4 (E, F). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with untreated Dok2 siRNA-transfected cells (C, D).

Dok2 knockdown differentially regulated TNF-α production in microglia and astrocytes (Supplemental Fig. 1), but supernatant concentrations of IL-1β were at or below detectable levels (data not shown). Overall, this supports the data presented in Fig. 3, indicating that Dok1 and Dok2 differentially regulate TLR2 inflammatory signaling in astrocytes and microglia.

Discussion

In this study, we identify Dok1 and Dok2 as novel adaptors in TLR2-induced inflammatory signaling in microglia and astrocytes. We demonstrate that the engagement of TLR2 on astrocytes and microglia induces the rapid phosphorylation of Dok1 and Dok2, and these adaptors are intrinsically associated with ERK and NF-κB signaling and consequently with downstream cytokine production.

There is growing interest in the role of TLRs in the CNS with compelling evidence that several members of the TLR superfamily are expressed in cells of the CNS, particularly glia. We examined the relative expression of TLR2 on astrocytes, microglia and neurons, and our data indicating higher expression on microglia and astrocytes are supported by findings from other groups (Bowman et al., 2003; Henry et al., 2008). Cumulative data from TLR2$^{-/-}$ mice support the diverse role for this receptor in the CNS. Indeed, evidence demonstrates an early role for this receptor in the glial response to axonal injury (Babcock et al., 2006), neonatal brain development (Du et al., 2011), neuronal damage (Hoffmann et al., 2007), cognitive impairment (Vollmar et al., 2010), experimental autoimmune encephalomyelitis (Farez et al., 2009) and the regulation of microglial response to viral infection (Aravalli et al., 2005). A role for TLR2 in disease pathogenesis has been supported by numerous reports of TLR2 polymorphisms linked to diseases like AD and PD (Kalinderi et al., 2013; Wang et al., 2011), which are characterized by neuroinflammatory changes. These findings highlight the relevance of this innate immune receptor to neuroinflammatory processes.

TLR2 (like all TLRs apart from TLR3) signals via MyD88 and another adapter MyD88-adaptor like (Mal), which, by cooperating with MyD88, regulates a complex series of signaling events, ultimately resulting in an increased expression of pro-inflammatory cytokines, chemokines and effector molecules (Fitzgerald et al., 2001). Both the ERK and the NF-κB activation are known downstream signaling events following ligation of TLR2 (Beaulieu et al., 2011), and signaling via both cascades is perturbed following TLR2 knockout (Anand et al., 2011). Data presented herein indicate that Pam3CSK4 enhanced ERK and NF-κB activation in microglia and astrocytes in 10–30 min, with the kinetics of this response mirroring data described elsewhere (Dye et al., 2007; Shinohara et al., 2005). Indeed, temporal regulation of these pathways depends on the specific receptor involved, with transient and sustained modes of activation determined depending on the existence of diverse modulators of the pathways including macrophage migratory inhibitory factor and calcium and calmodulin-binding proteins (Agell et al., 2002; Lue et al., 2006).

Studies conducted in mice lacking Dok1 and Dok2 indicate an indispensable role in the negative regulation of ERK activation in various hematopoietic cells (Yamanashi et al., 2000; Yasuda et al., 2004), with distinct consequences on cellular activation (Mihrshahi et al., 2009; Zhang et al., 2004). Indeed, the stimulation of macrophages from mice lacking Dok1 and Dok2 with LPS (Shinohara et al., 2005) and M-CSF (Yasuda et al., 2004) induced elevated ERK activation, which was associated with hyperproduction of TNF-α (Shinohara et al., 2005) and cell proliferative capacity (Yasuda et al., 2004). In support of this, *in vitro* evidence in U937 macrophages indicates that Dok knockdown is associated with enhanced IL-8 production (Mihrshahi et al., 2009) while mast cell degranulation and TNF-α production is associated with CD200 receptor-Dok-ERK signaling (Zhang et al., 2004). Dok1 and Dok2 are

expressed in neutrophils, T and B cells (Mashima et al., 2009), as well as macrophages, and mice lacking Dok1 and(or) Dok2 proteins display significant defects in immune responses and immune cell development (Shinohara et al., 2005; Yasuda et al., 2007). Importantly, our data indicate that both Dok1 and Dok2 are expressed in cells of the CNS, and this is supported by evidence demonstrating expression of both Dok proteins in brain (Grimm et al., 2001; Smith et al., 2004). It is also noteworthy that Dok1 expression was comparable in microglia and astrocytes, while Dok2 mRNA, but not protein, expression was higher in microglia when compared to astrocytes. Inconsistency in correlations between mRNA and protein expression has been shown (Guo et al., 2008), and further experiments will determine if this represents endogenous autoregulatory roles of Dok proteins in glial cells. Furthermore, the differential expression of Dok1 and Dok2 in astrocytes may reflect cross talk between signaling mechanisms controlling the expression pattern of each protein, as demonstrated elsewhere (Mihrshahi and Brown, 2010).

A significant finding here is that Dok1 and Dok2 negatively regulate ERK signaling in primary glial cells, indicating a novel modulatory role for Dok adaptors in glia. Using microscopy and real-time PCR, we determined that both microglia and astrocytes express Dok1 and Dok2. We also determine important functional roles for both proteins in glial cell signaling to NF-κB and pro-inflammatory IL-6 production, in addition to ERK regulation. This indicates that the functional role of Dok1 and Dok2 is conserved across hematopoietic/immune and CNS cells.

Anti-phosphotyrosine immunoblot analysis determined that both Dok1 and Dok2 were tyrosine phosphorylated following TLR2 activation in astrocytes and microglia and furthermore co-immunoprecipitated with p120 rasGAP in each cell type assessed. This is consistent with the previous data which demonstrated that Dok1 and Dok2 were rapidly tyrosine phosphorylated and co-immunoprecipitated with p120 rasGAP following TLR4 (Shinohara et al., 2005) and CD200R (Mihrshahi et al., 2009) activation in macrophages. In the present study, tyrosine phosphorylation of Dok1 and co-immunoprecipitation with p120 rasGAP occurred at 10 and 30 min in astrocytes and microglia, respectively, whereas tyrosine phosphorylation of Dok2 and co-immunoprecipitation with p120 rasGAP occurred at 60 and 10 min in astrocytes and microglia, respectively. Interestingly, it has been determined that cross talk between Dok1- and Dok2-mediated signaling exists, with evidence that Dok1 has the proclivity to negatively regulate Dok2 signaling (Mihrshahi and Brown, 2010); this cross talk provides a plausible mechanistic basis for the different temporal effects observed in astrocytes and microglia.

We show that Dok1 and Dok2 differentially regulated TLR2-induced IL-6 production in glia. In astrocytes, Dok1 and Dok2 knockdown enhanced TLR2-induced IL-6 expression and, in parallel, increased IκB-α phosphorylation. In microglia, Dok1 knockdown, but not Dok2 knockdown, attenuated TLR2-induced IL-6 expression and IκB-α phosphorylation. The data indicate that IκB-α sequestration of NF-κB in the cytoplasm is the likely cellular target that exerts the differential control, though the precise mechanistic basis remains to be delineated. Shinohara and colleagues (2005) reported that Dok1 and Dok2 were irrelevant to TLR2 signaling in murine bone marrow-derived macrophages, specifically in relation to ERK phosphorylation. Hence, data presented herein and elsewhere highlight distinct functions of these closely related proteins that are cell specific and governed by the activating stimuli. In support of this, differential roles for the Dok adaptors have been reported in other cells. Thus, the expression of Dok1, but not Dok2, is decreased following the exposure of macrophages to LPS (Shinohara et al., 2005), while the expression of Dok2, but not Dok1, is increased following treatment with M-CSF (Suzu et al., 2000). Similarly in platelets, the patterns of thrombin-induced Dok1 and Dok2 phosphorylation are distinct (Hughan and Watson, 2007).

Dok1 and Dok2 are highly related in structure (Mashima et al., 2009) and are typical docking proteins, with amino-terminal PH and PTB domains, followed by regions rich in SH2 and SH3 domains (Carpino et al., 1997). The precise stage at which Dok1 and Dok2 intervene in receptor signaling is unknown as is the underlying mechanism of their action, but it is interesting to speculate that Dok1 and Dok2 may be functional regulators of Toll-interleukin-1 receptor (TIR) adaptors, including MyD88, Mal, TIR-domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). However, the differential impact of Dok1 and Dok2 on TIR adaptor expression and downstream signaling in astrocytes and microglia remains to be mechanistically dissected. Indeed, TLR4, but not TLR9, TLR3 or TLR2 employs Dok1 and Dok2 as signaling intermediates in macrophages (Shinohara et al., 2005). In addition to the ERK mitogen-activated protein kinase (MAPK) pathway, Dok1 and Dok2 couple downstream to c-Jun N-terminal kinases, p38 and protein kinase B pathways (Shinohara et al., 2005; Van Slyke et al., 2005), and the relevance of this to downstream inflammatory signaling in glia will be the focus of further studies.

In summary, this study demonstrates that Dok1 and Dok2 are novel adaptors for TLR2 in microglia and astrocytes, and that both Dok1 and Dok2 differentially regulate TLR2-induced inflammatory signaling in both glial cell types (Fig. 5). The stimulation of TLR2 on astrocytes and microglia induced the rapid phosphorylation of Dok1 and Dok2 and recruitment of RasGAP, with downstream consequences on ERK and NF-κB activation and subsequent cytokine production. Specifically, our findings highlight that Dok1 represents a novel anti-inflammatory target in microglial cells, and negative regulation of this protein ameliorates pro-inflammatory signaling in microglia. A large body of evidence continues to link TLR2 signaling with neuroinflammatory conditions, and therefore Dok1 and Dok2 offer new targets for potential therapeutic strategies in conditions where microglial and astrocytic activation trigger excessive inflammatory changes.

Experimental methods

Preparation of primary cultures

Primary microglia and astrocytes were prepared from the whole brain of 1-day-old C57/BL6 mice as previously described (Downer et al., 2010) in accordance with the guidelines laid down by the local ethics committee (Trinity College Dublin). Briefly, dissected whole brains were chopped, added to DMEM (Invitrogen, Dublin, Ireland), triturated, passed through a sterile mesh filter (40 μm) and centrifuged (2000 ×*g* for 3 min at 20 °C). The pellet was resuspended in DMEM and plated onto T25 flasks. Cells were grown at 37 °C in a humidified environment (5% CO_2/95% air). DMEM-containing macrophage colony stimulating factor (M-CSF) (20 ng/ml; R&D Systems, UK) and granulocyte macrophage colony stimulating factor (GM-CSF) (10 ng/ml; R&D Systems, UK) were changed after 1, 5 and 8 days. At day 14, the non-adherent microglia were obtained by shaking, samples were centrifuged (2000 ×*g* for 5 min at 20 °C) and the microglia-enriched pellet was resuspended in DMEM. Microglia were plated (1×10^5 cells/ml) on 13-mm-diameter coverslips coated with poly-L-lysine (Sigma, UK). Astrocytes were isolated from mixed glia at days 10–14 by removing non-adherent cells and harvesting by trypsinization (0.25% Trypsin–0.02% EDTA). Cells were centrifuged (2000 ×*g* for 5 min at 20 °C) and the astrocyte-enriched pellet resuspended in DMEM. Astrocytes were plated (2×10^5 cells/ml) on 6- or 24-well plates and treated 24 h later.

Primary neurons were derived from from the whole brain of 1-day-old C57/BL6 mice. Brains were harvested in Hanks' balanced salt solution buffered (HBSS) with 1 mM sodium pyruvate and 1 mM HEPES, pH 7.4. Samples were minced using a scalpel and dissociated by incubating the tissue with papain (15 U/ml; Sigma, UK) for 30 min at 37 °C before triturating in Neurobasal medium (Invitrogen, Dublin, Ireland) supplemented with B27 (2%), penicillin (100 U/ml), streptomycin (100 U/ml) and glutamine (0.5 mM; all Invitrogen). Undissociated brain tissue was removed by passing the cell suspension through a 40-μm cell strainer (Thermo Fisher Scientific, Dublin, Ireland). Dissociated neurons were centrifuged (200 ×*g* for 3 min at room temperature), and the pellet was resuspended in supplemented Neurobasal medium. Viable neurons were plated (2×10^5 cells/ml) on 13-mm-diameter coverslips coated

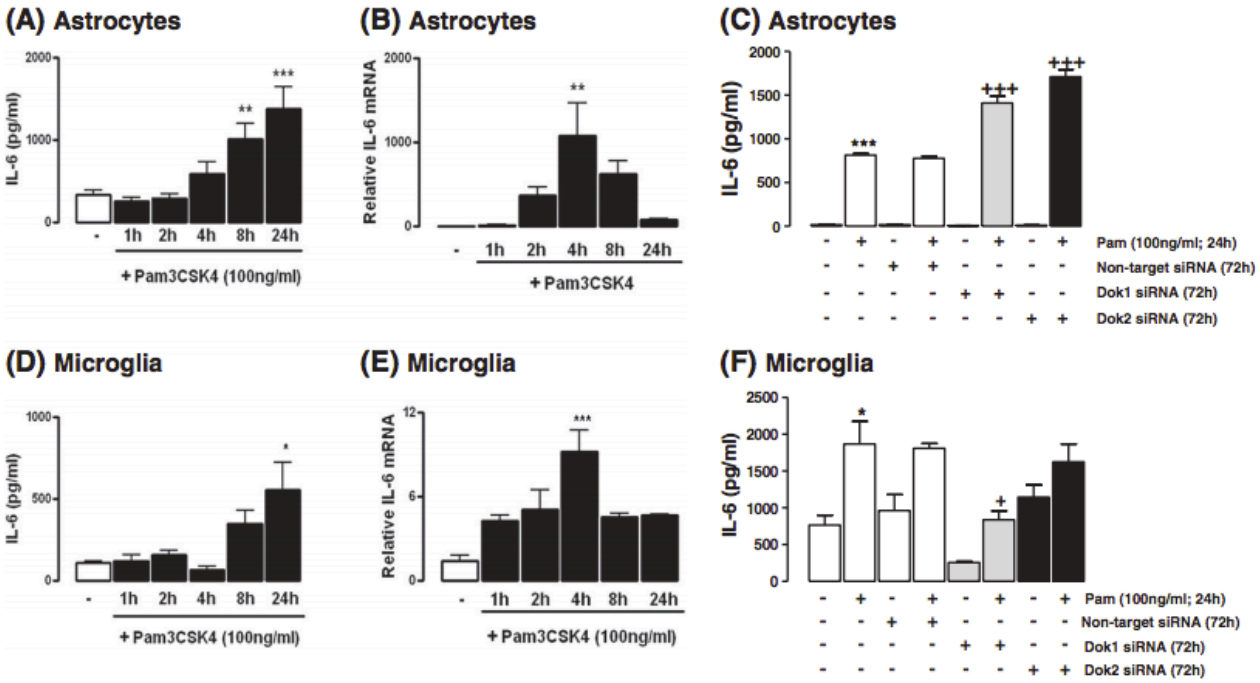


Fig. 4. Dok1 and Dok2 differentially mediate TLR2-induced cytokine production in glia. Primary mouse (A) astrocytes and (D) microglia were treated with Pam3CSK4 (100 ng/ml; 1–24 h). Supernatants were analyzed for IL-6 production using sandwich ELISA. cDNA was generated from primary mouse (B) astrocytes and (E) microglia treated with Pam3CSK4 (100 ng/ml; 1–24 h) and assayed by quantitative real-time PCR for levels of IL-6 mRNA. The expression level of IL-6 was normalized relative to expression of 18S rRNA. Primary (C) astrocytes and (F) microglia were transfected with non-targeting siRNA and ON-TARGET plus siRNA targeting mouse Dok1 and Dok2. After 72 h, cells were treated with Pam3CSK4 (100 ng/ml; 24 h), and supernatants were analyzed for IL-6 production using sandwich ELISA. Data are presented as the mean ± SEM of triplicate determinations from 12 animals (A, C, D, F), or are triplicate determinations from 9 animals (B, E). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with vehicle-treated cells. $^{+}p < 0.05$ and $^{+++}p < 0.001$ compared with non-transfected cells treated with Pam3CSK4.

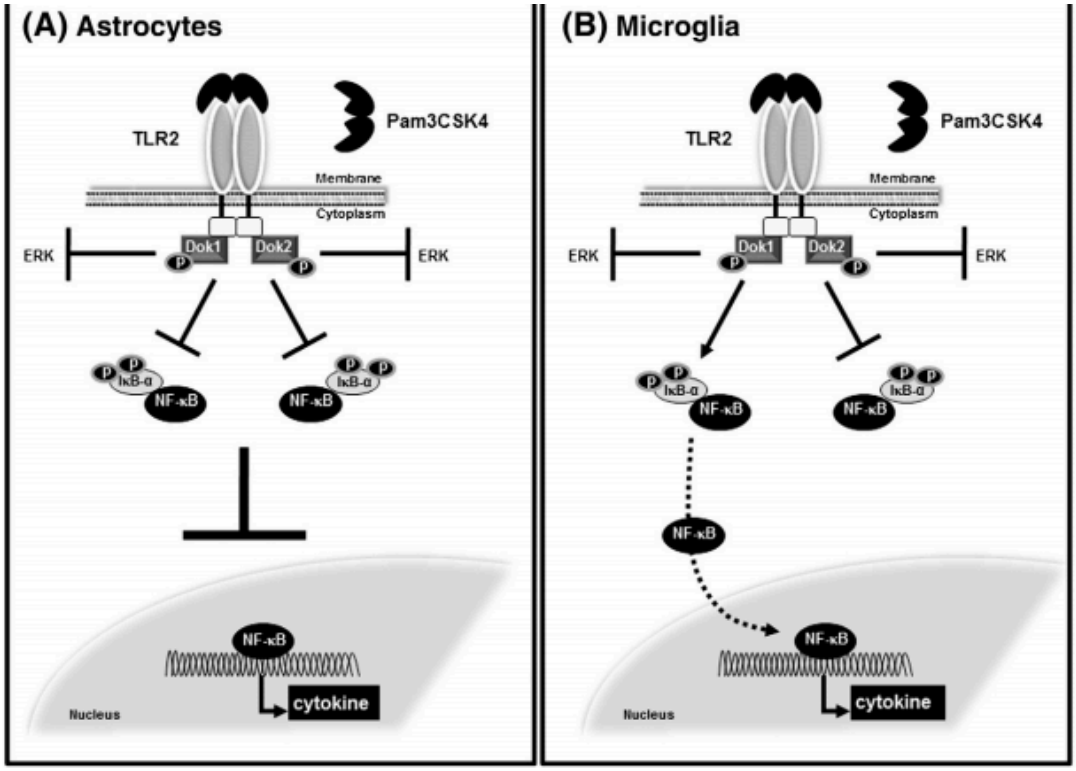


Fig. 5. Schematic representation of the mechanism by which Dok1 and Dok2 mediate TLR2-induced signaling in astrocytes and microglia. (A) In astrocytes, both Dok1 and Dok2 act as classic negative regulators of TLR2-induced ERK phosphorylation, NF-κB activation and pro-inflammatory cytokine expression. (B) In microglia, both Dok1 and Dok2 act as negative regulators of TLR2-induced ERK phosphorylation. However, Dok1, but not Dok2, promotes IκB-α phosphorylation, NF-κB DNA binding activity and pro-inflammatory cytokine production. *Pointed arrows*, activation; *blunt arrows*, inhibition.

with poly-L-lysine (Sigma, UK) and incubated in a humidified atmosphere containing 5% CO_2/95% atmosphere at 37 °C. One-half of the plating medium was replaced with fresh pen/strep-free medium after 5 days.

RNA interference

Custom ON-TARGET plus smart pool small interfering RNA (siRNA) targeting mouse Dok1 (GenBank accession number NM_010070), Dok2 (GenBank accession number NM_010071) were purchased from Dharmacon (Lafayette, CO). Primary astrocytes and microglial cells were transfected with Dok1 and Dok2 siRNA (5 nM) using DharmaFECT 1 transfection reagent (Dharmacon). After 72 h (38–59% Dok knockdown), cells were pre-treated with Pam3CSK4 (100 ng/ml) for the indicated times. A control siRNA duplex containing at least 4 mismatches of any mouse gene (ON-TARGET plus Non-targeting siRNA) was used in parallel experiments. Effective Dok1 and Dok2 knockdown was determined using confocal microscopy and western blotting.

Electrophoretic mobility shift assay (EMSA)

Microglia (1×10^5 cells/ml) and astrocytes (2×10^5 cells/ml) were seeded in 24- or 6-well plates, respectively. Cells were treated with Pam3CSK4 (100 ng/ml; 10–120 min) or transfected with Dok1 and Dok2 siRNA (5 nM) for 72 h prior to treatment with Pam3CSK4 (100 ng/ml; 20 min). Nuclear extracts were generated as described previously (Downer et al., 2011). Nuclear protein (10 μg) was incubated with LiCor IRDye 700-labeled oligonucleotide containing the NF-κB binding site 5-AGTTGAGGGGACTTTCCCAGGC-3′ (κB site underlined) according to the manufacturer's instructions (LiCor Biosciences, Lincoln, NE). All incubations were subjected to electrophoresis on a 4% native polyacrylamide gel for 2 h at 110 V and subsequently analyzed and images captured using the Odyssey infrared imaging system.

Western immunoblotting

Microglia (1×10^5 cells/ml) and astrocytes (2×10^5 cells/ml) were seeded in 24- or 6-well plates, respectively. Cells were treated with Pam3CSK4 (100 ng/ml; 10–120 min) or transfected with Dok1 and Dok2 siRNA (5 nM) for 72 h. Following Dok1 and Dok2, knockdown cells were either left untreated or treated with Pam3CSK4 (100 ng/ml) for time points ranging from 10 to 60 min. Cells were washed in ice-cold PBS before being lysed on ice for 10 min in 150 μl of lysis buffer (20 mM HEPES, pH 7.4, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, pepstatin A (5 μg/ml), leupeptin (2 μg/ml) and aprotinin (2 μg/ml)). Cell lysates were centrifuged (13,000 ×g for 15 min at 4 °C). The supernatant was mixed with SDS–PAGE sample buffer (0.125 Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 1% β-mercaptoethanol, and 0.0025% (w/v) bromophenol blue). Proteins were transferred to nitrocellulose membrane (Sigma, UK) and blocked for 2 h in 5% dried milk or BSA. Membranes were incubated overnight at 4 °C with mouse monoclonal phospho-ERK antibody (1:1000 in 5% dried milk; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal phospho-IκB-α antibody (1:1000 in 5% dried milk; Cell Signaling Technology Inc., Danvers, MA) or rabbit polyclonal Dok1 and Dok2 antibodies (1:500 in 5% BSA; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and incubated with anti-mouse or anti-rabbit IRDye Infrared secondary

antibody (1:5000 in 5% dried milk; Licor Biosciences, Lincoln, NE) for 1 h in the dark at room temperature. The membranes were washed, and immunoreactive bands were detected using the Odyssey Infrared Imaging System (Licor Biosciences). Membranes were stripped and incubated with mouse monoclonal anti-β-actin antibody (1:10,000; overnight at 4 °C; Sigma, UK) or mouse monoclonal ERK2 antibody (1:500 in 5% dried milk; Santa Cruz Biotechnology, Santa Cruz, CA). Molecular weight markers were used to calculate molecular weights of proteins represented by immunoreactive bands. Densitometry was performed using ImageJ software, values were normalized for protein loading relative to levels of β-actin or total ERK2 and data were expressed as densitometric units representing the ratio of density of the target protein to β-actin or total ERK2. No significant changes were observed in β-actin or ERK2.

Co-immunoprecipitation analysis

Microglia (1×10^5 cells/ml) and astrocytes (2×10^5 cells/ml) were seeded in 24- or 6-well plates. Cells were treated with Pam3CSK4 (100 ng/ml; 10–60 min), washed with pre-chilled PBS and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 0.5% (v/v) IGEPAL, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (leupeptin (25 μg/ml), aprotinin (25 μg/ml)) for 30 min on a rocker at 4 °C. Lysates were centrifuged at 16,000 ×*g* for 10 min at 4 °C. Supernatants were removed to new tubes, and protein concentrations were determined. Aliquots (30 μl) were retained for western immunoblotting, whereas the remaining supernatant was incubated with mixing for 30 min at 4 °C with rabbit IgG (1 μg) and protein A/G-agarose beads (10 μl). Samples were centrifuged (1000 ×*g* for 10 min at 4 °C) to pellet non-specifically bound proteins. Supernatants were removed to fresh pre-chilled tubes. Samples were incubated overnight with anti-Dok1 and anti-Dok2 antibodies (1 μg) at 4 °C with rocking. This was followed by the addition of protein A/G-agarose beads (20 μl/sample). Incubations were placed at 4 °C with rocking for 5 h. Immunoprecipitates were collected by centrifugation (1000 ×*g* for 10 min at 4 °C), and the beads were washed 4 times with lysis buffer (500 μl) lacking the protease inhibitor mixture. The beads were resuspended in 2× SDS–PAGE sample buffer (50 μl) and incubated for 30 min at room temperature. Samples were centrifuged (16,000 ×*g* for 2 min) and supernatants were boiled at 100 °C for 5 min and separated by gel electrophoresis. Nitrocellulose blots were blocked for 2 h in 5% dried milk or BSA and membranes were incubated overnight at 4 °C with antibodies to phosphotyrosine (1:1000; Millipore, Cork, Ireland), Dok1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), Dok2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and p120 rasGAP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and incubated with anti-mouse or anti-rabbit IRDye infrared secondary antibody (1:5000 in 5% dried milk; Licor Biosciences, Lincoln, NE) for 1 h in the dark at room temperature. The membranes were washed, and immunoreactive bands were detected using the Odyssey Infrared Imaging System (Licor Biosciences). Densitometry of tyrosine phosphorylation bands was performed using ImageJ software.

Cytokine analysis in culture supernatants

Primary microglia (1×10^5 cells/ml) and astrocytes (2×10^5 cells/ml) were seeded in 24- or 6-well plates, respectively. Cells were treated with Pam3CSK4 (100 ng/ml; 1–24 h) and supernatants assayed for interleukin-6 (IL-6) concentration by ELISA according to manufacturer's instructions (Duoset, R&D Systems, Abingdon, UK). In siRNA experiments, cells were transfected with Dok1, Dok2 or control siRNA (5 nM; 72 h) prior to treatment with Pam3CSK4 (100 ng/ml; 24 h) and assessment of IL-6 in supernatants.

Quantitative real-time PCR

RNA was extracted from untreated neurons, microglia and astrocytes, and from microglia and astrocytes treated with Pam3CSK4 (100 ng/ml; 1–24 h), using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc., Geschäftsführer Germany). The concentration of RNA was determined using a UV/Vis spectrophotometer. cDNA synthesis was performed on 1 μg RNA using a High Capacity cDNA RT Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Equal amounts of cDNA were used for RT-PCR amplification. Real-time PCR primers were delivered as "Taqman® Gene Expression Assays" containing forward and reverse primers, and a FAM-labeled MGB Taqman probe for each gene (Applied Biosystems). Primers used were as follows: TLR2, Dok1, Dok2 and IL-6 (Taqman® gene expression assay no. Mm00442346_m1, Mm00438532_m1, Mm00438538_m1 and Mm00446190_m1, respectively). cDNA (1:4 dilution) was prepared and real-time PCR performed using Applied Biosystems 7300 Real-time PCR System. cDNA was mixed with qPCR™ Mastermix Plus (Applied Biosystems) and the respective gene assay in a 25-μl volume (10 μl of diluted cDNA, 12.5 μl Taqman® Universal PCR Mastermix, 1.25 μl target primer and 1.25 μl 18S). Eukaryotic 18S rRNA was used as an endogenous control, and expression was conducted using a gene expression assay containing forward and reverse primers and a VIC-labeled MGB Taqman probe (#4319413E; Applied Biosystems, USA). Samples were run in duplicate, and 40 cycles were run as follows: 10 min at 95 °C and for each cycle, 15 s at 95 °C and 1 min at 60 °C. Gene expression was calculated relative to the endogenous control, and analysis was performed using the $2^{-\Delta\Delta CT}$ method. In all experiments, no change in relative 18S rRNA expression between treatment groups was observed.

Confocal microscopic analysis

Primary astrocytes and microglia were seeded (1×10^5 cells/ml) on 13-mm-diameter coverslips coated with poly-L-lysine (Sigma, UK) and grown for 24 h. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and blocked with 10% chicken serum (Vector Laboratories, Peterborough, UK) for 2 h. Cells were treated overnight at 4 °C with rabbit polyclonal Dok1 and Dok2 antibodies (1:200 in 5% chicken serum; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed and incubated with chicken anti-rabbit Alexa488 secondary antibody (1:1000 in 5% chicken serum; Invitrogen, Dublin, Ireland) and DAPI (1.5 μg/ml) in PBS, washed and mounted (Vectashield; Vector Laboratories, Peterborough, UK). In the case of TLR2 immunostaining, cells were fixed and blocked with 10% goat serum (Vector Laboratories, Peterborough, UK) for 2 h and incubated overnight at 4 °C with FITC anti-mouse TLR2 antibody (1:500 in 5% goat serum; Hycult Biotech, Cambridge, UK) and DAPI (1.5 μg/ml). Samples were viewed by confocal microscopy (Zeiss, Hertfordshire, UK). Negative control experiments were performed by replacing the primary antibody with isotype controls (Santa Cruz Biotechnology, Santa Cruz, CA) and by using equal gain settings during acquisition and analysis.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) as appropriate. When analysis by ANOVA indicated significance ($P < 0.05$), the post hoc Student Newman–Keuls test was used. Data are expressed as means ± standard errors of the mean (SEM).

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