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Requirement of TLR4 and CD14 in dendritic cell activation by Hemagglutinin B *from Porphyromonas gingivalis*

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Abstract

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium that is one of the causative agents of chronic adult periodontal disease. Among the potential virulence factors *of P. gingivalis* are the hemagglutinins. Recombinant Hemagglutinin B (rHagB) from *P. gingivalis* has been shown to activate the immune system by inducing specific antibodies that protect against experimental periodontal bone loss following *P. gingivalis* infection. Since different microbial products can stimulate dendritic cells (DC) through Toll-like receptors (TLRs), subsequently leading to T cell activation and antibody production, we wanted to investigate the immunostimulatory effect of rHagB on DC and the role of TLR signaling in this process. Using an endotoxin free rHagB preparation, our results show that stimulation of murine bone marrow-derived DC with rHagB leads to upregulation of the costimulatory molecules CD86 and CD40, activation of p38 and ERK MAP kinases, transcription factors NF-κB, CREB and IRF-3 and the production of IL-6, TNF-α, IL-12p40 and to a lesser extent IL-10 and IFN-β. This activation process was absolutely dependent on TLR4 and CD14. While upregulation of CD86 was independent of the adaptor molecule MyD88, CD40 upregulation and optimal cytokine (IL-6, TNF-α, IL-12p40, IL-10 and IFN-β) production required both MyD88 and TRIF molecules. These results are of importance since they are the first to provide insights into the interaction of rHagB with DC and TLRs. The information from this study will aid in the design of effective vaccines strategies against chronic adult periodontal disease.

Keywords

Hemagglutinin B; Dendritic cells; Toll-like receptors; *Porphyromonas gingivalis*

1. Introduction

Porphyromonas gingivalis, a Gram-negative anaerobic coccobacilli, has been implicated in the etiology of adult periodontitis (Lamont and Jenkinson, 1998; Slots et al., 1986; Socransky et al., 1998). This disease is characterized by a chronic inflammatory process of the tissues supporting the teeth, which ultimately causes resorption of alveolar bone. In addition to

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periodontal disease, *P. gingivalis* infection has been associated with a number of systemic disorders such as cardiovascular disease, atherosclerotic complications in hemodialysis patients and preterm low birth weight babies (Beck et al., 1996; Craig, 2004; Craig et al., 2007; Offenbacher et al., 1996). Thus, an understanding of the immune interactions between *P. gingivalis* or its components with the host is of outmost importance for the development of means to protect against *P. gingivalis* infection.

Several virulence factors have been described for *P. gingivalis*, including fimbriae, hemagglutinins, lipopolysacchride (LPS) and cysteine proteases known as gingipains (Duncan et al., 1993; Hirose et al., 1996; Katz et al., 2000; Kuramitsu, 1998; Lamont and Jenkinson, 1998; Oleksy et al., 2002; Potempa et al., 2000; Zhang et al., 1999). Hemagglutinins are non-fimbrial surface expressed adhesins that are thought to mediate the attachment of the bacteria to the host tissue, as well as to agglutinate erythrocytes (Nelson and Cox, 2005). Currently, hemagglutinins A, B, C, D and E (HagA, B, C, D and E, respectively) have been cloned and described (Lepine and Progulske-Fox, 1996; Progulske-Fox et al., 1989, 1995), and much focus in the last few years has been on HagB due to its potential as a vaccine candidate. The basis for this idea comes from several studies that show that HagB can induce an immune response that is protective against *P. gingivalis* infection and alveolar bone loss (Dusek et al., 1993, 1994; Katz et al., 1999; Yang et al., 2002; Zhang et al., 2003, 2004, 2005a,b). The exact mechanism of how HagB exerts its protective effect has not yet been determined.

Dendritic cells (DC) are the antigen-presenting cells per excellence as they link the innate and adaptive arms of the immune system (Reis e Sousa, 2001). Although both macrophages and DC play similar roles in participating in an innate immune response, each cell type has distinct functions. Macrophages are more programmed to recruit other cell types to the inflammatory site, while DC are more effective in developing T cell response (Jang et al., 2008). DC are the only cell type that can take up antigens at inflammatory sites and migrate to the secondary lymphoid tissue to active naive T cells. The activation of DC is characterized by an upregulation in the expression of costimulatory molecules and by the production of inflammatory cytokines (Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Revy et al., 2001; Steinman and Hemmi, 2006). Both of these signals as well as antigen presentation are required for an optimal T cell response.

Toll-like receptors (TLRs) are pattern recognition receptors that act as sensors for conserved microbial components. TLRs are part of the innate immune system but their involvement exerts important consequences on the ensuing adaptive host response (Barton and Medzhitov, 2002; O'Neill, 2006; O'Neill et al., 2003; Takeda and Akira, 2005). Expression of TLRs is detected in different host cells, including DC. Several microbial components have been identified as ligands for specific TLRs. The lipopolysaccharide (LPS) from enteric bacteria is a well-characterized TLR4 agonist. TLR2 can heterodimerize with TLR1 or TLR6 and recognize triacylated or diacylated lipoproteins, respectively (Hajjar et al., 2001). Double and single stranded RNA and DNA are agonists of TLR3, 7 or 8 and 9, respectively. Once DC are activated via TLRs, recruitment of adaptor molecules and phosphorylation events take place, leading to the activation of several signaling pathways that results in the expression of gene products encoding costimulatory molecules, inflammatory mediators and cytokines. Two independent TLR signaling pathways have been well characterized, a MyD88 dependent pathway, which is utilized by all TLRs known except TLR3 and a MyD88 independent pathway (TRIF dependent), which is utilized by TLR3 and TLR4. Studies have suggested that signaling via the MyD88 dependent pathway usually mediates a T helper type 1 (Th-1) response, while a Th-2 response is induced when the adaptor molecule MyD88 is absent, although this is not always the case (Kaisho et al., 2002; Schnare et al., 2001).

Both LPS and fimbriae from *P. gingivalis* have been shown to signal through TLR2 (Asai et al., 2005; Asai et al., 2001; Hajishengallis et al., 2006; Hirschfeld et al., 2000; Pulendran et al., 2001); however, no studies have determined whether TLRs are involved in HagB stimulation of DC. Since some bacterial and viral hemagglutinins have been shown to induce an immune response by stimulation through TLR signaling pathway (Banus et al., 2008; Bieback et al., 2002) and because HagB is a major virulence factor of *P. gingivalis*, it is important to determine its effect on DC and to understand the immune interaction between TLR signaling and HagB as this process can influence the outcome of the immune response. In the current study, we set out to dissect the requirement for TLRs and adaptor molecules as well as the signaling pathways influencing the activation of DC by HagB. The acquired knowledge will be valuable in the future development of protective vaccines or therapeutics against infection with the periodontal pathogen *P. gingivalis*.

2. Material and Methods

2.1. Mice

C57BL/6 wild type (WT), TLR2$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$ and TRIF ^{Lps2} mice were bred and maintained in an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham. The original TLR2$^{-/-}$, TLR4$^{-/-}$ and MyD88$^{-/-}$breeding pairs were obtained under a Materials Transfer Agreement from Dr. Shizuo Akira (Osaka University, Osaka, Japan). The original TRIF^{Lps} breeding pairs were obtained from the Jackson Laboratories (Bar Harbor, ME). The CD14$^{-/-}$ mice were a kind gift from Dr. John Kearney at this same institution. Female mice were 7–10 weeks of age when used in the studies. All experiments were done according to the guidelines of the National Institutes of Health. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

2.2. Purification of rHagB

rHagB was purified as previously described (Yang et al., 2002; Zhang et al., 2003, 2004) with some modifications. Briefly, rHagB was purified from the soluble fraction of the lysate of *Eschericia coli* JM109 expressing the *hagB* gene under the control of a *lac* Z promotor. The cultures were induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h. The soluble fraction of the bacterial lysate was initially denatured using 6 M urea prior to application to a His-bind resin column (Novagen, Madison, WI), according to the manufacturer's instructions. However, prior to elution, the bound rHagB was washed with 0.5% sodium deoxycholate in binding buffer in order to eliminate any contaminating LPS bound to the protein. This method reduced the endotoxin level by > 90% as determined by the Limulus amebocyte Lysate (LAL) assay (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) (data not shown). The eluted rHagB was then dialyzed against 0.5 M NaCl plus 20 mM HEPES solution, concentrated using a centriprep spin filtration system (Amicon, Millipore Corporation, Bedford, MA) and then passed through 0.2 μ,M HT Tuffryn® low protein binding, non-pyrogenic membrane Acrodisc® syringe filters (Pall corporation, Ann Arbor, MI) to sterilize. The purity of rHagB was confirmed by SDS-PAGE and by Western blot probed with Penta.His™ HRP Conjugate kit (Qiagen Inc., Valencia, CA, USA) or rabbit anti-HagB antibody (a kind gift from Dr. Ann Progulske-Fox at University of Florida, Gainesville). The concentration of rHagB was determined using the bicinchoinic acid (BCA) protein determination assay (Pierce, Rockford, IL). The final preparation of rHagB contained a negligible amount of endotoxin (0.0016 ng/μg rHagB protein), as determined by the LAL assay.

2.3. Generation of dendritic cells

Bone marrow-derived dendritic cells (DC) were generated as previously described (Inaba et al., 1992, 1998). Briefly, the femurs and tibias of mice were flushed with ice cold PBS to

remove the bone marrow. Erythrocytes were lysed using M-Lyse buffer (R&D Systems, Minneapolis, MN) and washed cells were suspended in RMPI-1640 media supplemented with 10% heat-inactivated fetal calf serum, pencillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), β-mercaptoethanol (50 μM), sodium pyruvate (1 mM), sodium bicarbonate (1.5 mg/ml) and HEPES (25 mM). The bone marrow cells were cultured in 24-well plates at a density of 10^6 cells/ml/well and incubated at 37°C in a 7.5% CO_2 environment. Recombinant GM-CSF (R&D Systems, Minneapolis, MN) was added to the cultures at a final concentration of 20 ng/ml. Culture media and GM-CSF were replaced on days 2 and 4. Additional culture media with GM-CSF were added on day 6 and cells were harvested on day 7. This protocol routinely yielded > 80% $CD11c^+$ cells as determined by flow cytometry.

2.4. Dendritic cells stimulation

DC (2×10^5/well) were cultured in 96-well plates in supplemented RPMI-1640 culture media at 37°C in a humidified 7.5% CO_2 incubator. Cultures were stimulated with various concentrations of rHagB (10, 20 or 40 μg/ml, see Results) or with *E. coli* K12 LPS (100 ng/ml) (InvivoGen, San Diego, CA) as a positive control. Unstimulated cultures served as the negative control. DC were harvested from one set of cultures following 16 h of stimulation for assessment of the level of expression of costimulatory molecules by flow cytometry. To determine cytokine production by ELISA, culture supernatants were harvested from a second set of cultures at 24 h post stimulation.

To assess the involvement of different signaling pathways in cell activation, DC were cultured in 24-well plates (2.5×10^6 /well) and incubated with rHagB (40 μg/ml) for 10, 30, 60 or 120 min or with *E. coli* LPS (100 ng/ml) for 60 min in a humidified 7.5% CO_2 incubator at 37°C.

The role of specific cell signaling molecules involved in the cytokine response to rHagB was determined by culturing DC (2×10^5/well) in 96-well plates for 2 h at 37°C with one of the following inhibitor (10 μM); U0126 [an inhibitor for ERK1/2 phosphorylation that acts by inhibiting the kinase activity of MEK (Favata et al., 1998)], InSolution™ SB 203580 [an inhibitor that blocks p38 kinase activity, but not its phosphorylation (Tong et al., 1997)], SN50 Cell-Permeable Inhibitor Peptide [an inhibitor of the nuclear translocation of NF-κB (Lin et al., 1995)], InSolution™ GSK3 Inhibitor IX [a reversible ATP competitive inhibitor of GSK3α/β (Meijer et al., 2003)] (Calbiochem Biosciences Inc., LaJolla, CA) followed by stimulation with rHagB (40 μg/ml). Both non-stimulated and non-treated cells served as controls. Culture supernatants were harvested 24 h after stimulation and the levels of cytokines were analyzed as described below.

To ensure that trace amount of endotoxin did not contribute to observed responses, our rHagB preparation was further subjected to the effects of boiling for 30 min, proteinase K (5μg/ml) digestion at 37°C for 2 h (Fermentas Inc., Glen Burnie, MD, USA) and then boiled for 5 min to ensure proteinase K degradation, or polymyxin B sulphate (PMB) (10 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at RT. These preparations and appropriate controls were then used to stimulate DC and the culture supernatants were harvested after 24 h and assessed for TNF-α levels by ELISA. To determine whether these treatments caused degradation of rHagB, equivalent amounts of untreated and treated rHagB samples were analyzed by SDS-PAGE on a 12.5% Tris-HCl gel followed by (a) staining the gel with Gelcode® blue stain reagent (Pierce, Rockford, IL) or (b) electrotransferring the proteins onto immobilon-P transfer membranes (Millipore, Bedford, MA) that were incubated with Penta.His^{TM} HRP Conjugate kit (Qiagen Inc., Valencia, CA, USA), followed by detection of the bands using ECL Western blotting detection reagents (Amersham Bioscience, England).

2.5. Cytokine ELISA and flow cytometry

Culture supernatants were assessed for the levels of TNF-α, IL-6 and IL-23 (p19/p40) (eBioscience, San Diego, CA), IL-10 and IL-12p40 (R&D Systems), and IFN-β (PBL InterferonSource, Piscataway, NJ) by ELISA, according to the manufacturers' instructions. For detection of the expression of costimulatory molecules, DC were harvested and stained with fluorescent-labeled antibodies against CD11c, CD80, CD86 and CD40 or appropriate isotype controls (eBioscience) in PBS buffer supplemented with 2% bovine serum albumin and 0.1% sodium azide for 40 min on ice. Cells were washed twice with buffer. Samples were acquired using a FACSCaliber (BD Bioscience, San Jose, CA) and analyzed using CellQuest software (BD Bioscience).

2.6. Preparation of whole cell lysates and Western blot analysis

Following stimulation, DC were harvested at various times (see Results), washed twice with cold PBS, and then lysed for 10 min on ice in radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, Lake Placid, NY) supplemented with 1 mM phenylmethylsulphonyl flouride, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitor cocktail tablets (Complete, Mini, EDTA-free, Roche Applied Science, Indianapolis, IN, USA) and 5 μM microcystin-LR (Alexis Biochemicals, San Diego, USA). The cell lysates were then transferred to tubes, incubated for an additional 20 min on ice, and then centrifuged (15,000 rpm) for 15 min at 4ºC. The supernatants were collected and stored at −20°C until analyzed.

For Western blot analysis, equivalent amounts of protein from whole cell lysates were analyzed by SDS-PAGE on a 12.5% Tris-HCl gel followed by electrotransfer onto immobilon-P transfer membranes (Millipore, Bedford, MA). The membranes were then incubated with specific antibodies to the phosphorylated form of p38 (Thr180/Tyr182), ERK1/2 (p44/42, Thr202/Tyr204), SAPK/JNK (Thr183/Tyr185), CREB (Ser133), NF-κB p65 (Ser536), GSK3α/β (Ser21/9), Akt (Ser473), IκBα (Ser32) or IRF-3 (Ser396) (Cell Signaling Technology, Beverly, MA). To detect equal loading of samples, blots were also probed with antibodies to either total p38, GSK3β or IRF-3 (Cell signaling Technology, Beverly, MA). Bands were detected by using a HRP-linked anti-rabbit IgG antibody followed by ECL Western blotting detection reagents (Amersham Bioscience, England).

2.7. Statistical analysis

The data were subjected to an unpaired ANOVA, followed by post-hoc analysis with the Tukey-Kramer multiple comparison test using the GraphPad InStat Version 3.0a (GraphPad Software, San Diego, CA). Differences between groups were considered significant at the level of $P < 0.05$.

3. Results

3.1. Activation of dendritic cells by rHagB

Activation of DC is the initial step in mediating an adaptive immune response since it provides signals for naive T cells to proliferate and differentiate. Therefore, to determine if rHagB can activate DC, bone marrow-derived DC from C57BL/6 mice (WT) were stimulated with 10, 20 or 40 μg/ml of rHagB for 24 h, and culture supernatants were assessed for cytokine production by ELISA. A dose-dependent production of the proinflammatory cytokines TNF-α, IL-6 and IL-12p40 was observed following stimulation of DC with rHagB (Fig. 1A). Since the p40 subunit is common to both IL-12 and IL-23 cytokines (Oppmann et al., 2000), we next assessed the supernatants for the presence of IL-23p19. No IL-23p19 was observed (data not shown) indicating that the p40 was likely due to IL-12. The anti-inflammatory cytokine IL-10 was also induced in a dose-dependent manner (Fig. 1B); however, the quantity of IL-10 produced was

relatively low compared to the pro-inflammatory cytokines, indicating that rHagB induced mainly a pro-inflammatory response by DC.

In addition to cytokine production, rHagB induced the upregulation of the costimulatory molecules CD86 and CD40 in a dose-dependent fashion (Fig. 1C). No upregulation of CD80 was detected, which was in agreement with previous findings showing that the HagB specific antibody response is not altered in $CD80^{-/-}$ mice (Zhang et al., 2004). These results suggested that CD80 does not play a role in responses towards rHagB. Since 40 μg/ml of rHagB induced the optimal DC activation, this concentration was used in the subsequent experiments.

3.2. Signaling pathways involved in rHagB activation of DC

Activation of DC by microbial products involves the participation of signaling molecules and transcription factors. Therefore, to delineate the signaling pathways involved in rHagB activation of DC, we determined whether this activation involved MAP kinases and/or Akt/GSK3 pathways. We found that rHagB phosphorylates p38, ERK1/2, Akt and GSK3α/β but not JNK MAP kinase (Fig. 2A and 2B). We also observed phosphorylation of the transcription factor CREB (Fig. 2C), a downstream target of p38, ERK1/2 or Akt (Deak et al., 1998; Du and Montminy, 1998; Kato et al., 2007; Xing et al., 1998) and of NF-κB p65 and IκBα (Fig. 2C). While phosphorylation of p38, ERK1/2 and CREB peaked at 30 min, Akt, GSKα/β, NF-κB p65 and IκBα phosphorylation was more pronounced at 60 min, indicating that following rHagB stimulation of DC, MAP kinase activation occurs earlier than activation of Akt/GSK3 or NF-κB.

3.3. Signaling pathways involved in rHagB induced cytokine response

We next determined the involvement of various signaling molecules in cytokine production by rHagB stimulated DC using specific inhibitors of p38, ERK1/2, NF-κB and GSK3. DC were pre-incubated with each specific inhibitor, stimulated with rHagB and supernatants were then assessed for cytokine production.

Pre-incubation of DC with SB203580, an inhibitor of p38, had no effect on the production of TNF-α or IL-6, but showed a slight increase in IL-12p40 and a decrease in IL-10, although the differences were not significant (Fig. 3). Conversely, when DC were stimulated with LPS, inhibition of p38 activity resulted in a significant decrease in TNF-α production (~ 35.9%). Inhibition of ERK1/2 by U0126 did not have a significant effect on the cytokines induced by rHagB stimulation, whereas a 33% decrease was seen in the amount of TNF-α produced by LPS stimulated DC as compared to non-inhibited cells.

Blocking nuclear translocation of NF-κB with 10 μM SN50 peptide did not affect cytokine production by rHagB or LPS stimulated DC (Fig. 3). However, a severe abrogation of cytokine production was observed, ranging from ~ 81 to 95% depending on the cytokine assessed, with 25 μM of the SN50 peptide (data not shown). Abrogation of cytokine production was also seen with SN50 peptide in LPS stimulated DC cultures with a reduction ranging from ~ 25 and 53% (data not shown).

While inhibition of GSK3α/β did not affect TNF-α or IL-6 production by either rHagB or LPS stimulated DC, it strongly influenced IL-10 production, resulting in a ~ 400% (~ 3.3 fold) and ~ 390% (~ 2.9 fold) increase in rHagB and LPS stimulated DC, respectively (Fig. 3). The increase in IL-10 was accompanied by ~ 31% and 40% decrease in IL-12p40 production in cultures stimulated with rHagB and LPS, respectively. These results are in agreement with those of others demonstrating that inhibition of GSK3 signaling results in an increase in IL-10 and a decrease in IL-12p40 production (Martin et al., 2005;Ohtani et al., 2008;Rodionova et al., 2007). Overall, the differential regulation of cytokine production by rHagB and LPS

suggests that each antigen exerts its unique influence on the different signaling pathways that ultimately lead to cytokine production.

3.4. Activity of rHagB and LPS contamination

Since we purified rHagB from a lysate of *E. coli* expressing the *hagB* gene, it was imperative to rule out that any stimulatory effect seen was not due to LPS contamination. The results of the Limulus amebocyte lysate reaction assay revealed a concentration of approximately 0.0016 ng of endotoxin/μg of rHagB protein. When this amount of LPS was used to stimulate DC cultures, no cytokine production was seen (data not shown). To further ensure that the amount of endotoxin detected had no effect, rHagB was boiled for 30 min, degraded with proteinase K for 2 h or treated with polymyxin B (PMB) for 15 min. A significant decrease (~ 71%) in TNF- α cytokine production was detected when rHagB was boiled prior to stimulation (Fig. 4A). Treatment of rHagB with proteinase K resulted in a complete abolishment of the cytokine response, whereas treatment of rHagB with PMB had no effect on the response (Fig. 4A). Conversely, pretreatment of LPS with PMB prior to stimulation of DC abrogated the cytokine response (~ 75% decrease), whereas boiling or proteinase K treatment resulted in an increase or no effect on the stimulatory activity of LPS, respectively (Fig. 4A). Analysis of the rHagB preparation by SDS-PAGE and Western blot after the various treatments revealed partial degradation and/or dimerization and complete degradation of rHagB (running at ~ 49 kDa) by boiling or proteinase K treatment, respectively (Fig. 4B and 4C). As expected, PMB treatment had no effect on rHagB as compared to the untreated rHagB preparation. These results demonstrate that the stimulatory activity of rHagB was not due to LPS contamination, but rather due to the activity of the protein itself.

3.5. Requirement of TLR4, MyD88 and TRIF for cytokine production by rHagB

Next, we determined whether the activation induced by rHagB is mediated via TLR signaling. The requirement of TLR2 and TLR4 was assessed by determining the production of cytokines by DC derived from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice following stimulation with rHagB. DC from TLR2$^{-/-}$ and WT mice produced similar levels of TNF-α and IL-6 (Fig. 5A). However, no cytokine production was detected in cultures derived from TLR4$^{-/-}$ mice, indicating that rHagB is a TLR4 agonist. Since TLR4 signaling can occur through the MyD88 and TRIF pathways, we examined cytokine production by MyD88$^{-/-}$ and TRIF^{Lps2} DC. DC lacking either MyD88 or TRIF showed a significant reduction in pro-inflammatory cytokine production (Fig. 5B), thus indicating HagB signals through both the MyD88 and TRIF pathways.

Interestingly, DC from TLR2$^{-/-}$ mice produced significantly higher levels of IL-12p40 than WT DC following stimulation with rHagB (Fig. 5A). Since IL-10 and IL-12 are counter-regulatory cytokines, we compared the levels of IL-10 in DC derived from all deficient mice examined to determine if a difference in IL-10 levels could explain the observed differences in the levels of IL-12p40. Our results show that the levels of IL-10 produced by WT and TLR2$^{-/-}$ cells were not significantly different (Fig. 5C). Therefore, the increase in IL-12p40 was not due to a decrease in IL-10 production by TLR2$^{-/-}$ DC. No IL-10 was detected in rHagB stimulated DC cultures derived from TLR4$^{-/-}$, MyD88$^{-/-}$and TRIF^{Lps2} mice.

3.6. Activation of p38, ERK1/2 MAPK, the Akt/GSK3 pathway and NF-κB by rHagB stimulated DC is dependent on TLR4

Since activation of MAP kinase, GSK3 and NF-κB results in the expression of numerous genes involved in the activation of DC and optimal cytokine responses, we next assessed if the abolished cytokine response observed in TLR4$^{-/-}$, MyD88$^{-/-}$ and TRIF^{Lps2} DC was due to a lack in the activation of one of the above signaling molecules following rHagB stimulation. Therefore, we first compared the phosphorylation of p38 and ERK1/2 from lysates of TLR2$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$ and TRIF^{Lps2} DC to that of WT. The phosphorylation kinetics

for p38 and ERK1/2 in TLR2$^{-/-}$ and WT DC lysates were similar and reached maximum at 30 min post rHagB stimulation (Fig. 6A). No phosphorylation was detected in the lysates of DC from TLR4$^{-/-}$ mice. Interestingly, lysates from MyD88$^{-/-}$ and TRIF^{Lps2} DC showed similar kinetics to lysates from WT DC. Similar results were seen with the phosphorylation of Akt (Fig. 6B) and CREB (Fig. 6C). Taken together, these results indicate that a defect in the activation of p38, ERK1/2, Akt or CREB did not account for the diminished cytokine response observed by rHagB stimulated MyD88 and TRIF deficient DC.

However, upon examining GSK3α/β, no phosphorylation was detected in lysates from TLR4$^{-/-}$ and MyD88$^{-/-}$ DC, while phosphorylation kinetics in lysates from TRIF^{Lps2} DC were comparable to that from WT DC, suggesting that rHagB mediated inhibition of GSK3 was dependent on MyD88 pathway rather than the TRIF pathway (Fig. 6B). In addition, a decrease in the phosphorylation of GSK3β was also seen in lysates from TLR2$^{-/-}$ DC, suggesting that the increase in IL-12p40 production detected in cultures from TLR2$^{-/-}$ DC may be due to an increase in the activity of GSK3, as it has been previously shown that GSK3 induces IL-12 production (Martin et al., 2005;Ohtani et al., 2008;Rodionova et al., 2007).

No activation of NF-κB was observed in lysates of TLR4$^{-/-}$ DC, whereas activation kinetics of NF-κB were similar in DC lysates derived from WT, TLR2$^{-/-}$ and MyD88$^{-/-}$ mice, where maximum phosphorylation of IkBα and p65 occurred at 60 min following stimulation with rHagB (Fig. 6C). However, the TRIF^{Lps2} cell lysates showed a weak phosphorylation at 10 min following stimulation, which was sustained for the 2 h experimental period (Fig. 6C). Moreover, no degradation of IκBα was observed (data not shown). These results suggest that TRIF and MyD88 may have differential roles in the activation of NF-κB in HagB stimulated DC. These results further prove that the suboptimal cytokine response in MyD88$^{-/-}$ DC was not due to a defect in the activation of NF-κB.

3.7. Upregulation of CD86 is dependent on TLR4 and TRIF signaling, while CD40 expression requires both MyD88 and TRIF in rHagB activated DC

Since upregulation of costimulatory molecules is a necessary secondary signal for an effective DC-T cell response, we next assessed the upregulation of CD86 and CD40 on rHagB stimulated DC derived from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. Upregulation of CD86 and CD40 was seen with TLR2$^{-/-}$ DC, while no upregulation was noticed with TLR4$^{-/-}$DC (Fig. 7A). Similar to TLR4$^{-/-}$ DC, TRIF^{Lps2} DC showed no upregulation of CD86 and CD40 (Fig. 7B, C and D). This finding was in agreement with previous data showing that the adaptor molecule TRIF is required for upregulation of costimulatory molecules (Hoebe et al., 2003b;Kaisho et al., 2001;Yamamoto et al., 2003). Stimulation of DC with LPS resulted in a similar response. Interestingly, although MyD88$^{-/-}$ DC stimulated with rHagB showed a similar level of CD86 expression as WT DC, the expression of CD40 following stimulation was markedly reduced and was not significantly different from unstimulated MyD88$^{-/-}$ DC (Fig. 7B, C and D). Moreover, we also noticed a reduction in the level of CD40 expression when MyD88$^{-/-}$ DC were stimulated with LPS, although the level of expression was still significantly higher than that seen with unstimulated MyD88$^{-/-}$ DC. These results suggest that the MyD88 adaptor molecule, or a molecule downstream of MyD88, might be playing a major role in regulating CD40, but not CD86 expression.

3.8. Activation of IRF-3 requires TLR4 and TRIF, while production of IFN-β is also dependent on MyD88 in rHagB activated DC

In the case of TLR4 signaling, the activation of IRF-3, production of IFN-β and upregulation of costimulatory molecules occur downstream of the TRIF dependent pathway (Hoebe et al., 2003a,b; Hoshino et al., 2002; Weighardt et al., 2004; Yamamoto et al., 2003). Since TLR4 and TRIF are involved in rHagB activation of DC, we next determined the role of the IRF-3

transcription factor and of IFN-β in the activation of DC, and whether the reduced expression of CD40 in MyD88$^{-/-}$ DC was in part due to a defect in the production of IFN-β. rHagB induced IRF-3 activation in lysates from DC derived from TLR2$^{-/-}$ and MyD88$^{-/-}$ mice, but not from those derived from TLR4$^{-/-}$ or TRIF^{Lps2} mice (Fig. 8A). This was consistent with the results from others (Hoebe et al., 2003a,b; Hoshino et al., 2002; Weighardt et al., 2004; Yamamoto et al., 2003), as well as to our own data with LPS stimulated DC (Fig. 8A). Furthermore, this response correlated with IFN-β production, as no IFN-β was detected in cultures of DC derived from TLR4$^{-/-}$ or TRIF^{Lps2} mice (Fig. 8B). However, IFN-β production was almost abolished in MyD88$^{-/-}$ DC cultures stimulated with rHagB and was not significantly different from that seen with unstimulated MyD88$^{-/-}$ DC cultures. This decrease in IFN-β production correlated with the low upregulation of CD40 in MyD88$^{-/-}$ DC following rHagB stimulation (Fig. 7C and 7D). Since LPS stimulated MyD88$^{-/-}$ DC cultures expressed higher levels of IFN- β production (Fig. 8B) and of CD40 expression than unstimulated MyD88$^{-/-}$ DC cultures (Fig. 7C and 7D), these results suggest that an insufficient production of IFN-β may account for the low CD40 upregulation seen in MyD88$^{-/-}$ DC.

3.9. CD14 is necessary for the immunostimulatory activity of HagB

CD14 is a glycosyl-phosphatidylinositol GPI-anchored cell membrane receptor that upon LPS stimulation is thought to deliver the LPS-LPS binding protein complex to TLR4 (Akashi et al., 2003; Wright et al., 1990). Therefore, we next wanted to assess the role of CD14 in the ability of rHagB to stimulate DC. Stimulation of DC from CD14$^{-/-}$ mice with rHagB failed to induce the production of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12p40 (Fig. 9A), or the production of the anti-inflammatory cytokine IL-10 (Fig. 9B). Moreover, stimulation of CD14$^{-/-}$ DC with rHagB did not result in an upregulation in the expression of costimulatory molecules CD86 or CD40 (Fig. 9C, D and E) or in the phosphorylation of p38, ERK1/2, CREB, Akt, GSK3 or the activation of NF-κB or IRF-3 (data not shown). In addition, the production of IFN-β was completely abolished (data not shown). These results demonstrate that CD14 is absolutely required for rHagB activation of DC. This result was in contrast to that seen with LPS stimulated DC where a comparable level of activation was seen with DC derived from WT and CD14$^{-/-}$ mice, in terms of cytokine production (Fig. 9A and B), costimulatory molecules (Fig. 9C, D and E) or activation of the indicated signaling molecules (data not shown). In our experiments, the media used was supplemented with serum, which is rich with soluble CD14 (Bazil et al., 1989). Upon stimulation of DC with serum free media, the response of LPS stimulated DC derived from CD14$^{-/-}$ was reduced (data not shown). This agrees with other studies that have proposed that CD14 participates in loading of LPS to the TLR4-MD2 complex (Akashi et al., 2003); however, signal transduction was not absolutely dependent on membrane bound (mCD14), since LPS signaling can still occur in mCD14 independent manner (Gangloff et al., 2005; Perera et al., 1997). These results suggested that, unlike LPS, which can be delivered to TLR4 independently from mCD14, rHagB absolutely requires mCD14 to signal through TLR4.

4. Discussion

In the present study, we investigated the immunostimulatory activity of HagB from *P. gingivalis* on murine bone marrow-derived DC. Our results demonstrate that rHagB is a TLR4 agonist and that mCD14, MyD88 and TRIF are critical participants of the TLR4 signaling complex. Furthermore, rHagB stimulation of DC resulted in the activation of Akt and the p38 and ERK1/2 MAP kinases. Although the signaling pathways implicated in the cytokine response of murine macrophages to rHagB had been previously described (Zhang et al., 2005a), our current results with DC differ from those obtained with macrophages, thus underlying the uniqueness of each cell type (Argueta-Donohue et al., 2008; Jang et al., 2008; Pompei et al., 2007; Siegemund et al., 2007; Werling et al., 2004). The dissimilarities observed

were; (i) the relative amount of IL-12p40 and IL-10 produced by each cell type, (ii) the differential requirements of p38, ERK or NF-κB in the production of pro- and anti-inflammatory cytokines, and (iii) the induction of phosphorylated JNK in macrophages, but not in DC. The differences between DC and macrophages in response to rHagB can perhaps be explained by the variability in the engaged signaling mechanism(s). Werling et al. showed that an increase in IL-10 production and variations in MAP kinase activation could be due to differential level of TLR expression on DC and macrophages (Werling et al., 2004). In addition, differences in the nuclear translocation of NF-κB could explain the variations among the two cell types (Argueta-Donohue et al., 2008).

TLR2 and TLR4 are expressed in periodontal tissues of patients with periodontitis, hence, implicating their potential participation in the inflammatory response to periodontal pathogens (Mori et al., 2003). Whereas previous studies have shown the involvement of TLR2 and CD14 in the inflammatory response to *P. gingivalis*, its LPS and fimbriae components (Asai et al., 2001, 2005; Burns et al., 2006; Hajishengallis et al., 2006; Wang and Ohura, 2002), the role of TLR4 remains unclear since studies have seen similar inflammatory responses, bone resorption and delay in bacterial clearance in $TLR4^{-/-}$ and wild type mice after *P. gingivalis* infection (Burns et al., 2006). However, it is critical to understand that the response to the whole pathogen can differ from that mediated by its antigenic components. In this regard, each antigen, depending on its own specificity and its interaction with the host cell, will initiate a unique response. Evidently, our results highlight this phenomenon that is not exclusive of *P. gingivalis*, as attested by investigations with *Mycobacterium* and *Francisella* species (Asai et al., 2001, 2005; Ashtekar et al., 2008; Bulut et al., 2005; Burns et al., 2006; Cole et al., 2007; Duenas et al., 2006; Hajishengallis et al., 2006; Hong et al., 2007; Katz et al., 2006; Li et al., 2006; Ogawa et al., 2002; Pulendran et al., 2001). Since *P. gingivalis* seems to induce an immune response mainly through TLR2, the immunodominant antigen of this pathogen could be its LPS or fimbriae, but not HagB.

Previous studies have shown that microbial molecules not structurally related to LPS have the capacity to interact with TLR4 and induce the activation of immune cells (Aosai et al., 2006; Ashtekar et al., 2008; Bulut et al., 2005; Kurt-Jones et al., 2000). We demonstrate in the present study that HagB, like LPS, is a TLR4 agonist; however, the response induced by each of these antigens is not the same. While rHagB stimulation was completely abrogated in $CD14^{-/-}$ DC, LPS signaling was intact since LPS can utilize both mCD14 and sCD14. The complete dependency of rHagB on mCD14 and its inability to utilize sCD14 suggests that mCD14 might participate or even associate with the TLR4 complex to activate DC. This dependency on mCD14 has been shown with *P. gingivalis* fimbriae, but not with *P. gingivalis* LPS (Hajishengallis et al., 2006; Wang and Ohura, 2002), suggesting that the difference in the requirements for membrane versus soluble CD14 by an antigen could be due to structural differences. In addition, rHagB, but not LPS, failed to induce the phosphorylation of JNK and the upregulation of the costimulatory molecule CD80. These events can be linked since Lim et al. has shown that LPS induced CD80 upregulation involved the activation of JNK MAP kinase, and that blocking JNK prevents CD80 upregulation (Lim et al., 2005). We also observed a differential regulation of TNF-α production with p38 and ERK1/2 inhibitors in cultures stimulated with rHagB or LPS. Our results differ somewhat from those observed by others regarding LPS responses following ERK1/2 inhibition (Ardeshna et al., 2000; Arrighi et al., 2001; Nakahara et al., 2004); however, the disparities may be due to differences in cell types, dose of inhibitor or type of inhibitor used. The fact that LPS signaling is held as the standard for TLR4 immune responses should not preclude the possibility that other TLR4 agonist molecules will induce a different immune pattern. Indeed, as shown in the present study, two structurally distinct microbial molecules signaling through TLR4 induce a different outcome.

We have demonstrated for the first time that both MyD88 and TRIF are indispensable for the optimal pro- and anti-inflammatory cytokine response by rHagB stimulated DC. The defect in MyD88$^{-/-}$ or TRIF^{Lps2} DC to induce cytokines was not due to a defect in the activation of MAP kinase (Kawai et al., 1999, 2001), although NF-κB may play a partial role in the abrogated cytokine response of TRIF^{Lps2} DC, since its activation was diminished compared to WT DC. Moreover, the phosphorylation kinetics of p38, ERK1/2 and NF-κB were similar in WT and MyD88$^{-/-}$ DC. This is in contrast to the findings of others showing that TLR4 signaling causes the activation of MAP kinases and NF-κB in two phases, an early TRIF dependent phase followed by a late MyD88 dependent phase (Hoebe et al., 2003a; Kawai et al., 2001; Yamamoto et al., 2003). In the case of LPS, the delayed response in MyD88$^{-/-}$ cells was attributed to the time required for the TRIF pathway to initiate IRF-3 activation, followed by TNF-α production, which in turn acts on the TNF receptor to induce a delayed NF-κB activation (Covert et al., 2005). However, the similarity in kinetics between rHagB stimulated WT and MyD88$^{-/-}$DC suggests that rHagB signaling through MyD88 and TRIF may occur in two independent or simultaneous signaling events. Our results further suggest that while MyD88 and TRIF are of equal importance in HagB signaling through TLR4 for cytokine production, they have different roles in the activation of NF-κB or the inhibition of GSK3, which are more dependent on TRIF and MyD88, respectively. The selective requirement for one adaptor molecule over the other by a TLR4 agonist has been reported previously. Mata-haro et al. showed that the TLR4 agonist monophosphoryl lipid A (a lower toxicity derivative of LPS) preferentially utilized the TRIF dependent pathway (Mata-Haro et al., 2007). Other studies from our laboratory have shown that the induction of IL-6 and IL-12p40 production by *F. tularensis* DnaK via TLR4 was more dependent on MyD88 than TRIF (Ashtekar et al., 2008).

We have also shown that the upregulation in CD40 expression was completely abolished in stimulated TRIF^{Lps2} DC and that no activation of IRF-3 or production of IFN-β was seen, which is in agreement with findings of others (Hoebe et al., 2003b; Hoshino et al., 2002; Kaisho et al., 2001; Weighardt et al., 2004). However, examination of rHagB or LPS stimulated MyD88$^{-/-}$ DC indicated that rHagB, unlike LPS, did not lead to IFN-β production or upregulation of CD40. Furthermore, even though both rHagB and LPS stimulated MyD88$^{-/-}$ DC showed a reduction in IFN-β levels compared to WT DC (~ 80%), LPS stimulated MyD88$^{-/-}$ DC were still capable of CD40 upregulation. These results suggest that the activation of IRF-3 alone is not sufficient for the induction of CD40 upregulation in MyD88$^{-/-}$ DC, but rather the amount of IFN-β available to the cell since LPS stimulated MyD88$^{-/-}$ DC were producing significantly more IFN-β than unstimulated controls. Hoshino et al. showed that type I interferon signaling through STAT-1 is required for the upregulation of CD40 and that in the absence of the IFN-α/β receptor, CD40 expression was abrogated (Hoshino et al., 2002). Therefore, our results indicate that IFN-β production is partially dependent on MyD88. Whether the requirement for MyD88 to produce IFN-β is a direct or an indirect effect through the activation of another pathway is still to be determined.

In conclusion, we have demonstrated for the first time that HagB is a TLR4 agonist and that the signaling events and the resulting response induced by this antigen are unique and different from that exerted by LPS. Our results extend the findings of others showing the importance of studying different cell types and the uniqueness of their response to a particular antigen. Lastly, we have expanded the current knowledge regarding the *P. gingivalis* HagB antigen, providing information that will aid in designing better therapeutic and preventive methods against periodontitis and the systemic consequences of this disease process.

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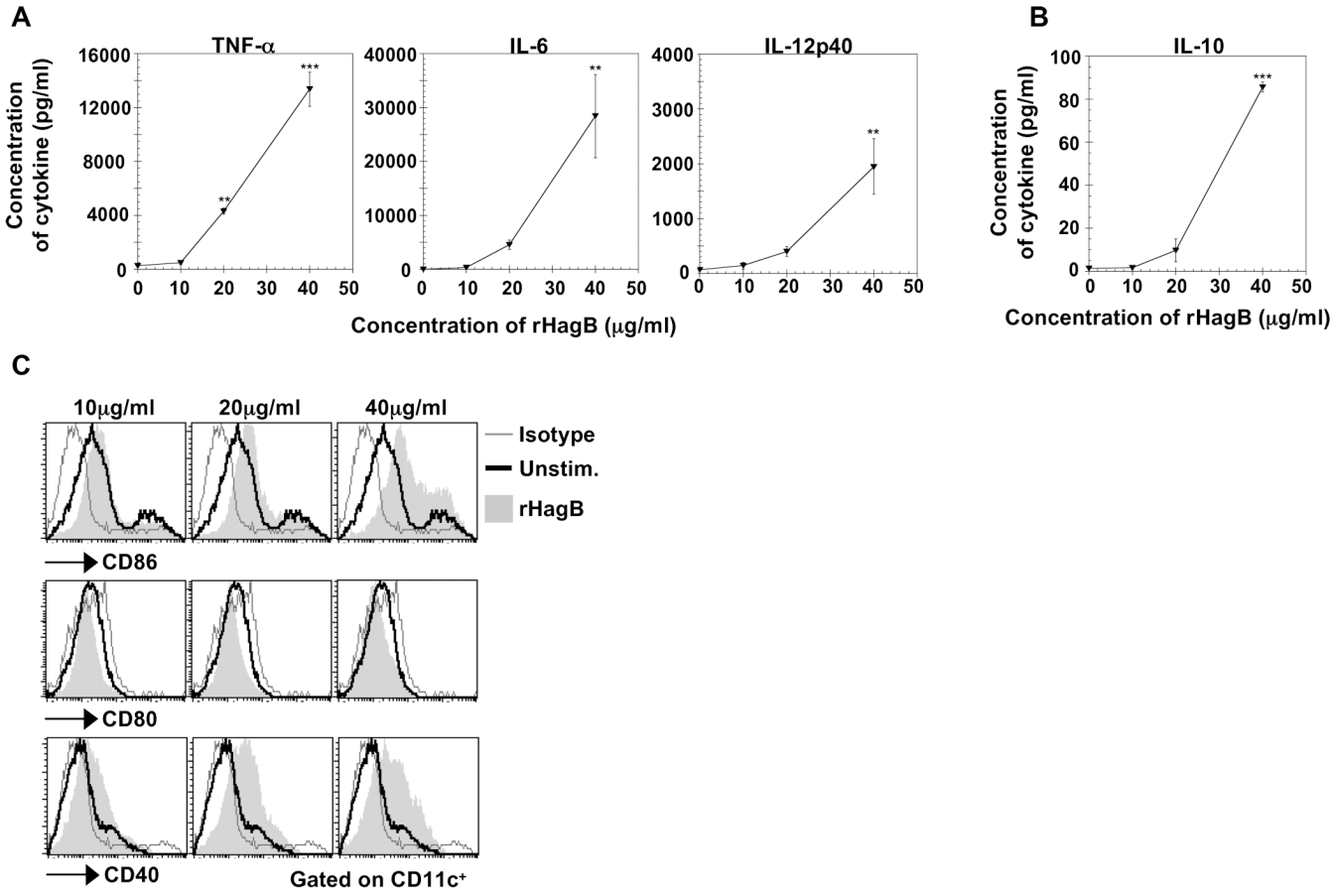

Fig. 1.

Stimulation of DC with rHagB results in the production of cytokines and the upregulation of costimulatory molecules. Bone marrow-derived DC (2×10^5) from WT mice were either unstimulated (0 μg/ml; negative control) or stimulated with 10, 20 or 40 μg/ml rHagB. Culture supernatants were harvested 24 h post-stimulation and assessed for the levels of the pro-inflammatory cytokines TNF-α, IL-6 and IL-12p40 (A) and the anti-inflammatory cytokine IL-10 (B) by ELISA. Results are expressed as the mean ± standard error of triplicate cultures from one of three independent experiments. ***, ** Significant differences at $P < 0.001$ and $P < 0.01$, respectively, compared to unstimulated cultures. (C) DC (2×10^5) from WT mice were stimulated with 10, 20 and 40 μg/ml rHagB for 16 h (shaded histograms) or left unstimulated as negative controls (thick lines). Cells were harvested and stained with fluorescent-labeled antibodies against CD11c, CD80, CD86, CD40 or matched isotype controls (thin lines). Histogram plots were gated on $CD11c^+$ cells. Results represent one of three independent experiments.

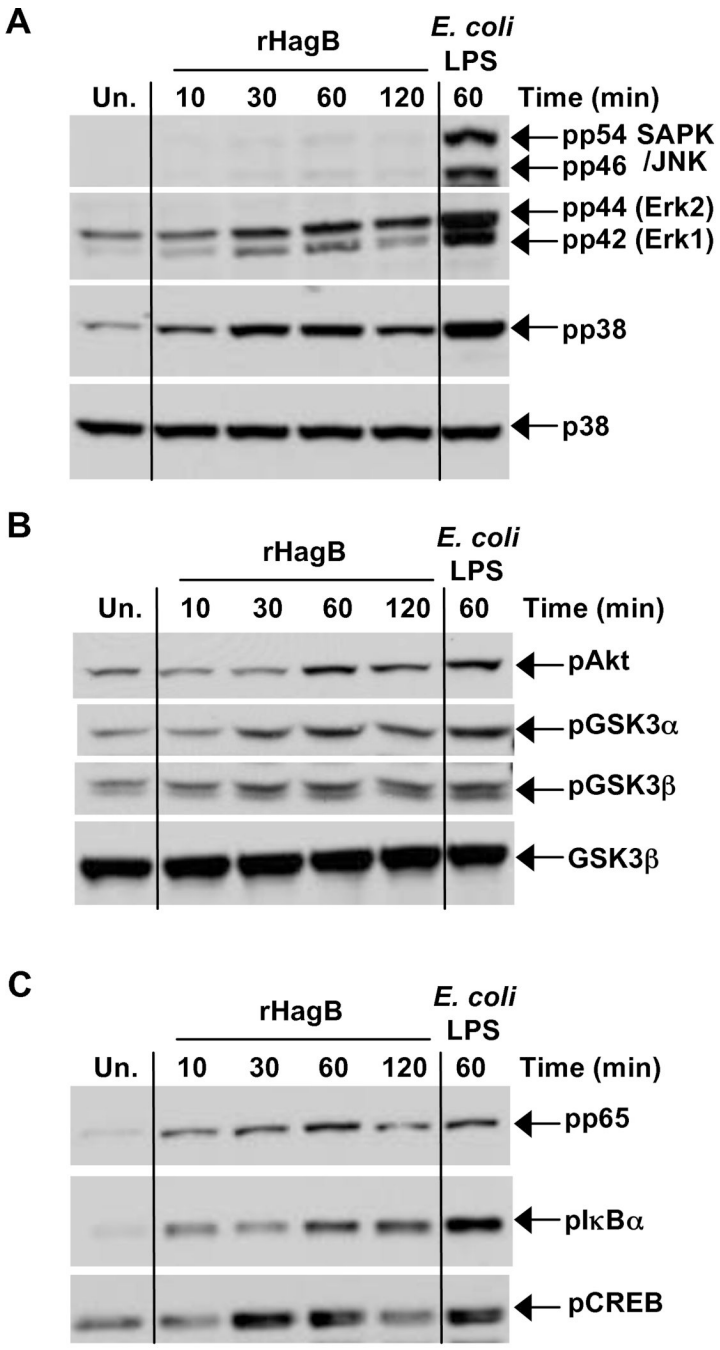

Fig. 2.

Signaling pathways activated following rHagB stimulation of DC. DC were stimulated with 40 μg/ml rHagB for 10, 30, 60 or 120 min. Following stimulation, cells were lysed and whole cell lysates were assessed for phosphorylation of (A) JNK, ERK1/2 and p38, (B) Akt and GSK3α/β and (C) NF-κBp65, IκBα and CREB by Western blot. Total p38 (A) and GSK3β (B) were used as loading controls. Unstimulated DC (far left lane) or DC stimulated with 100 ng/ml *E. coli* K12 LPS for 60 min (far right lane) were used as controls. Results represent one of three independent experiments.

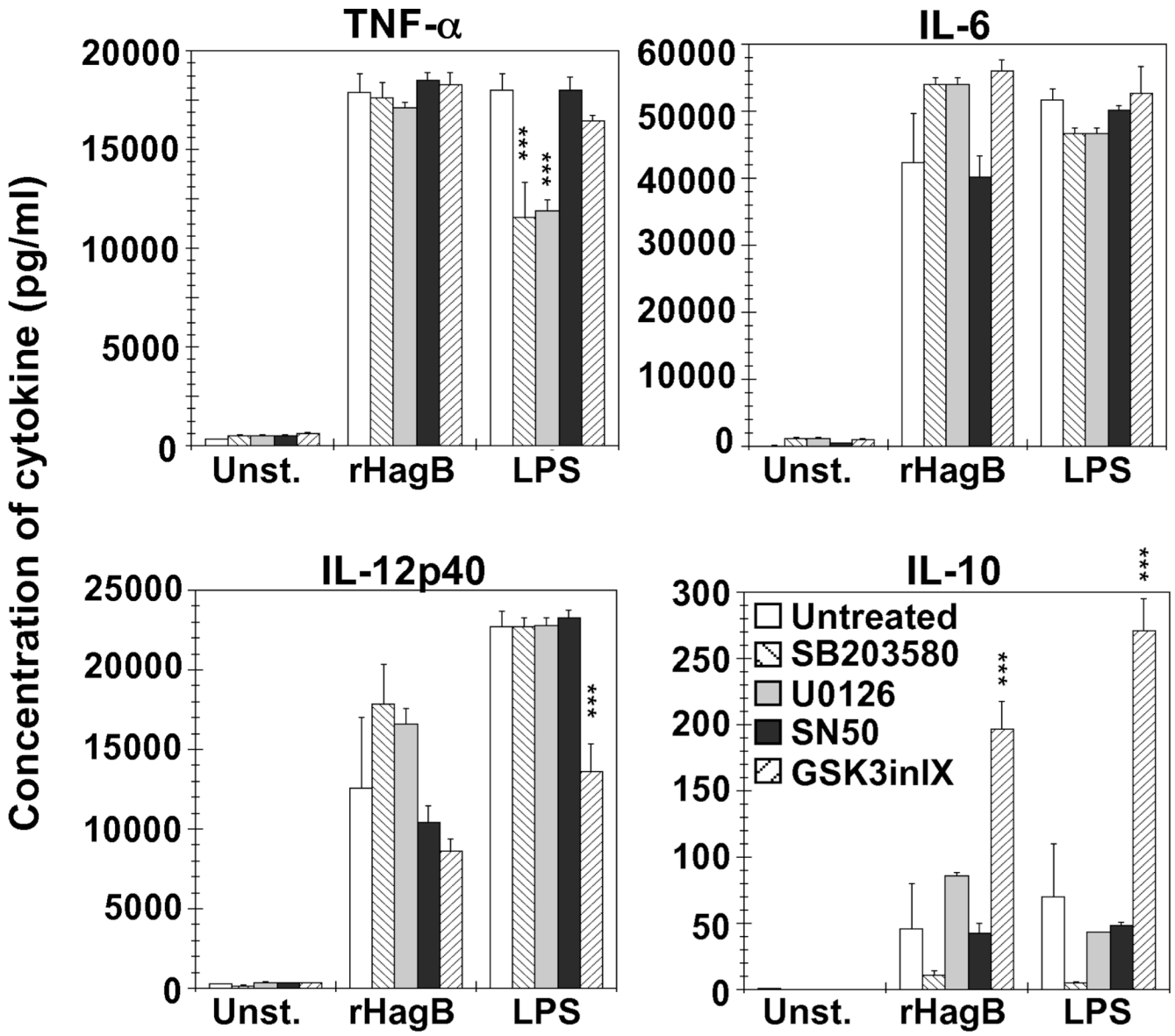

Fig. 3.

Cytokine production by DC stimulated with rHagB is dependent on more than one signaling pathway, while GSK3 signaling regulates IL-10. DC (2×10^5) from WT mice were treated with 10 μM of U0126 (left striped bar), SB203580 (grey bar), NF-κB SN50 (black bar), or GSK-3 Inhibitor IX (right striped bar) (ERK1/2, p38, NF-κB, or GSK3 specific inhibitors, respectively) for 2 h. Untreated cells were used as a negative control (white bars). The cultures were then stimulated with 40 μg/ml rHagB, 100 ng/ml *E. coli* K12 LPS, or left unstimulated. Culture supernatants were harvested 24 h post-stimulation and assessed for the production of TNF-α, IL-6, IL-12p40 and IL-10 by ELISA. Results are expressed as the mean ± standard error of triplicate cultures from one of two independent experiments. *** Significant differences at $P < 0.001$, compared to untreated cultures stimulated with rHagB or LPS.

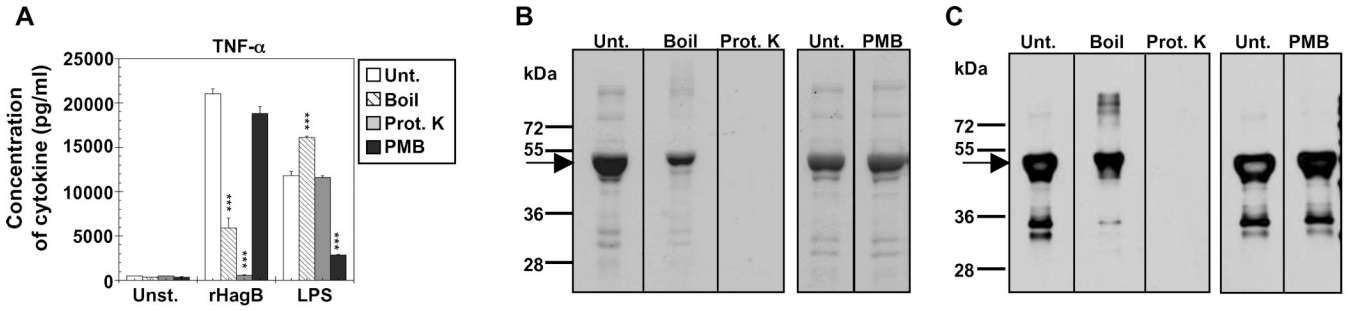

Fig. 4.

Activity of rHagB is not due to endotoxin contamination. (A) DC from WT mice (2×10^5) were stimulated with 40 μg/ml rHagB, 100 ng/ml *E. coli* K12 LPS, or left unstimulated. Prior to stimulation, samples were untreated (white bars), boiled for 30 min (striped bars) or treated with proteinase K (grey bars) or polymyxin B (PMB) (black bars). Culture supernatants were harvested 24 h post-stimulation with rHagB or LPS and assessed for TNF-α production. Results are expressed as the mean ± standard error of triplicate cultures from one of three independent experiments. *** Significant differences at $P < 0.001$ compared to untreated cultures stimulated with rHagB or LPS. (B) Stained SDS-PAGE and (C) Western blot probed with specific HRP conjugated antibody against Penta.His for equivalent amounts of untreated and treated rHagB protein samples. Arrows represent rHagB protein band that runs at ~ 49 kDa. Results represent one of three independent experiments.

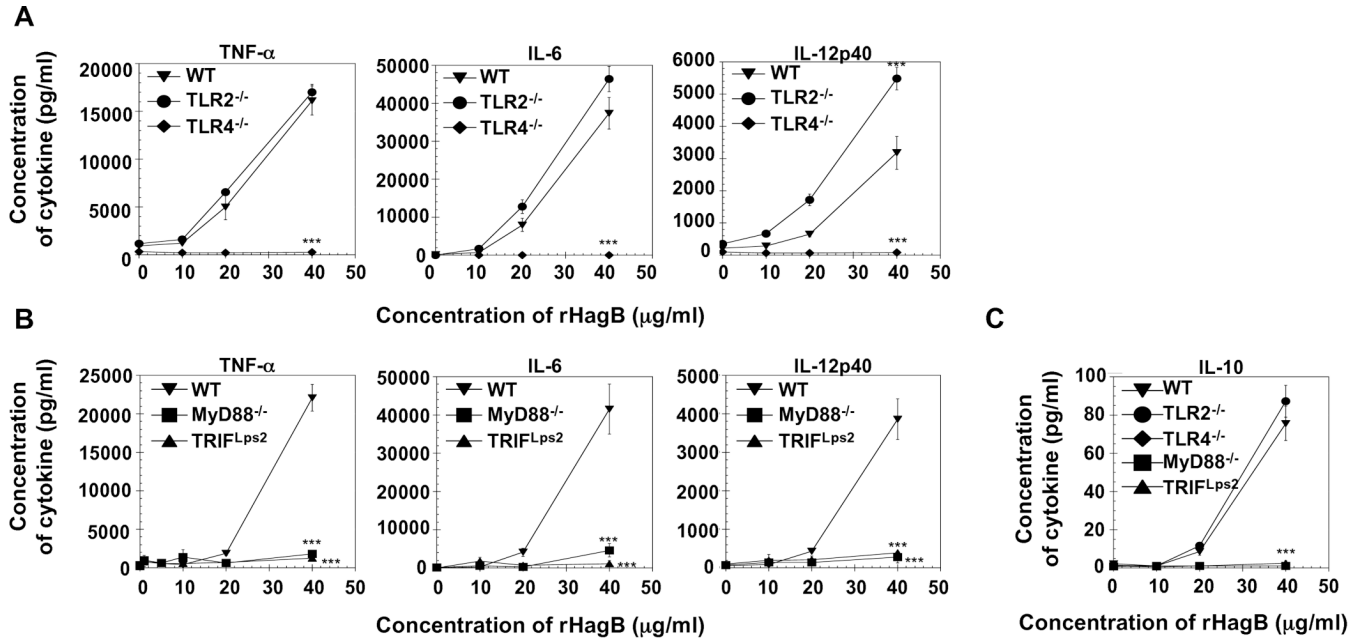

Fig. 5.

Cytokine production by rHagB activated DC is mediated through TLR4 signaling and requires both MyD88 and TRIF. DC (2×10^5) from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (A, C) or WT, MyD88$^{-/-}$ and TRIF ^{Lps2} mice (B, C) were stimulated with 10, 20 or 40 μg/ml rHagB for 24 h. Culture supernatants were then harvested and assessed for TNF-α, IL-6, IL-12p40 (A, B) and IL-10 production (C) by ELISA. Results are expressed as the mean ± standard error of triplicate cultures from one of four independent experiments. *** Significant differences *at* $P < 0.001$ compared to WT cultures stimulated with rHagB.

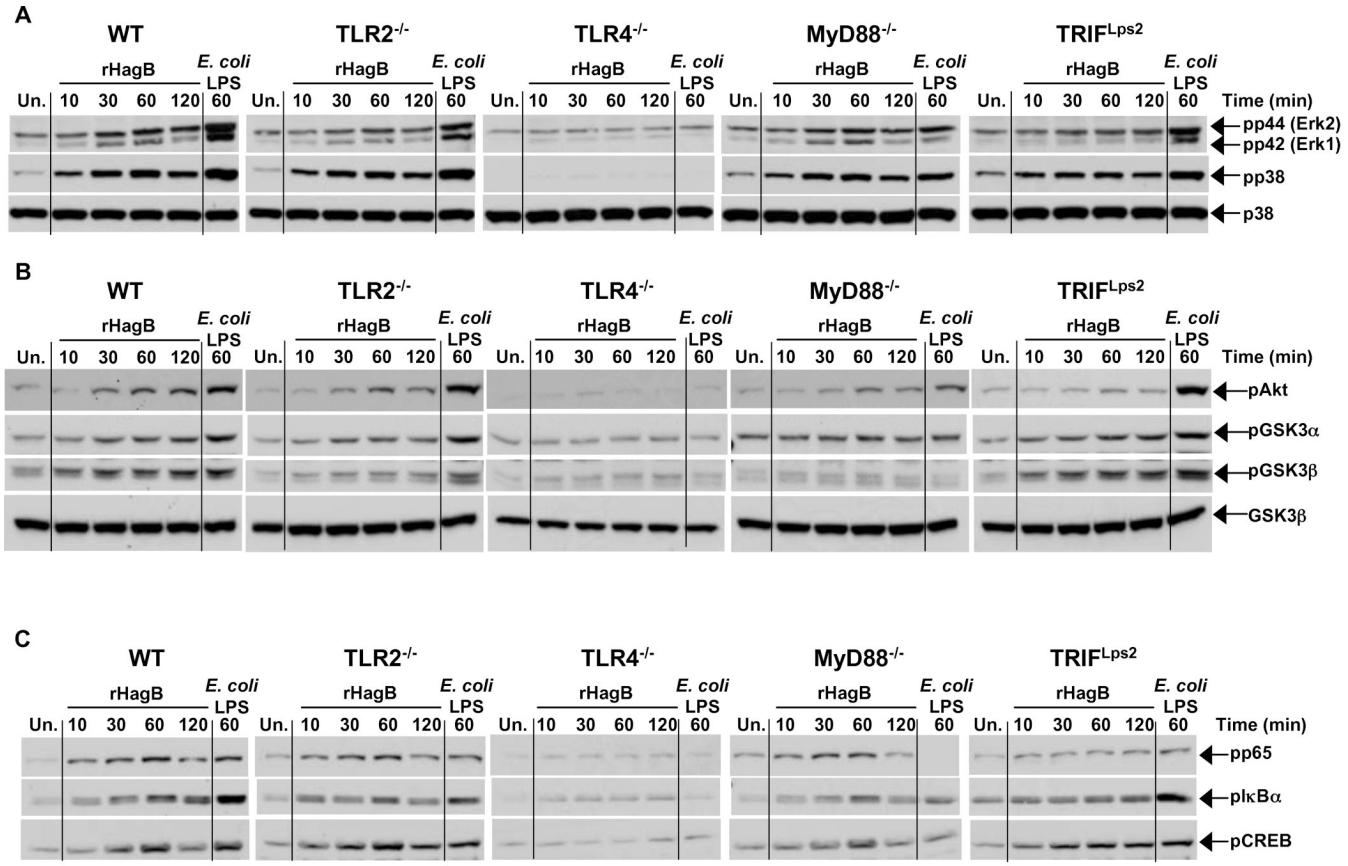

Fig. 6.

Phosphorylation of p38, ERK1/2, Akt/GSK3 and activation of NF-κB and CREB by rHagB stimulated DC is dependent on TLR4. DC from WT, TLR2$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$ and TRIF^{Lps2} mice were stimulated with 40 μg/ml rHagB for 10, 30, 60 or 120 min. Following stimulation, cells were lysed and whole cell lysates were assessed for (A) ERK1/2 and p38 and (B) Akt and GSK3α/β (C) p65 NF-κB (Ser 536), IκBα and CREB phosphorylation by Western blot. Total p38 (A) and GSK3β (B) were used as loading controls. Unstimulated DC (far left lanes) or DC stimulated with 100 ng/ml *E. coli* K12 LPS for 60 min (far right lanes) were used as controls. Results are representative of two independent experiments.

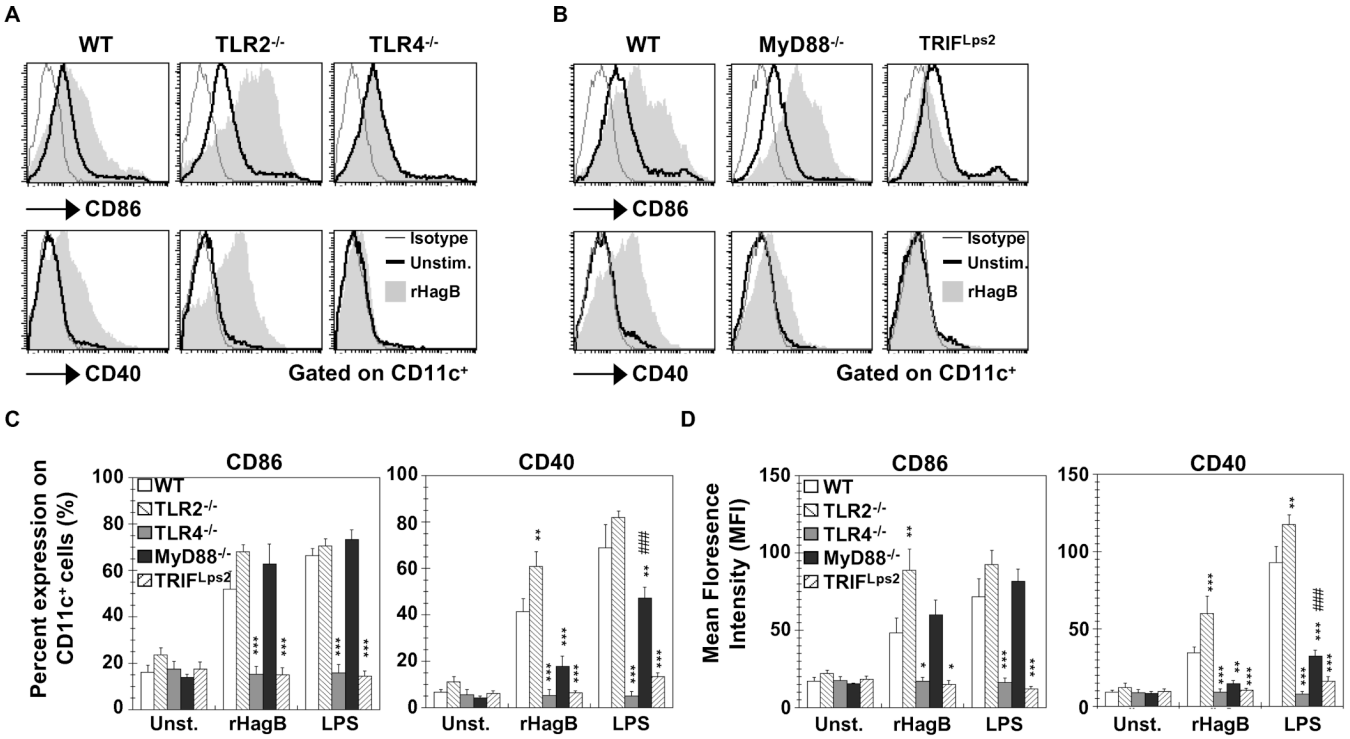

Fig. 7.

Upregulation of CD86 and CD40 with rHagB stimulation is mediated through TLR4 and TRIF signaling. DC (2×10^5) from WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (A, C and D), or WT, MyD88$^{-/-}$ and TRIF ^{Lps2} mice (B, C and D) were stimulated with 40 μg/ml rHagB (shaded histograms), 100 ng/ml *E. coli* K12 LPS or left unstimulated (thick lines) for 16 h. Cells were harvested and stained with fluorescent-labeled antibodies to CD11c, CD86, CD40 or matched isotype controls (thin lines). Histogram plots were gated on CD11c$^+$ cells. Data in (C) and (D) are expressed as the percentage of CD86 and CD40 positive cells and the mean florescence on CD11c$^+$, respectively. Results are expressed as the mean ± standard error of four independent experiments. ***, ** and * Significant differences at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively, compared to WT cultures stimulated with rHagB or LPS. ### Significant differences at $P < 0.001$ compared to unstimulated MyD88$^{-/-}$ cultures.

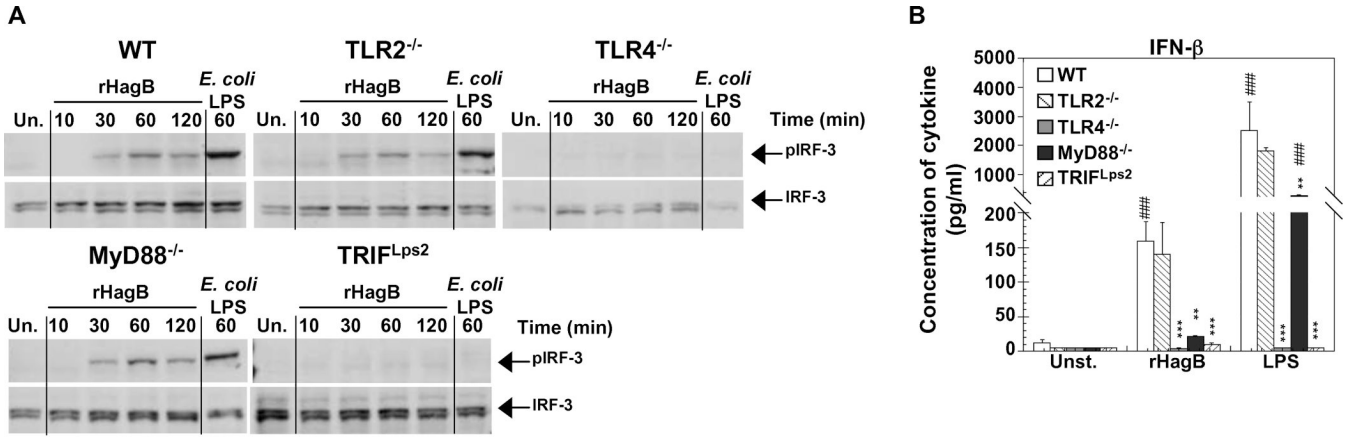

Fig. 8.

Phosphorylation of IRF-3 and production of IFN-β by rHagB stimulated DC is dependent on TLR4 signaling and the adaptor molecule TRIF. (A) DC from WT, TLR2$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$ and TRIF ^{Lps2} mice were stimulated with 40 μg/ml rHagB for 10, 30, 60 or 120 min. Following stimulation, cells were lysed and whole cell lysates were tested for phosphorylation of IRF-3 by Western blot. Total IRF-3 was used as a loading control. Unstimulated DC (far left lanes) or DC stimulated with 100 ng/ml *E. coli* K12 LPS for 60 min (far right lanes) were used as controls. Results are representative of two independent experiments. (B) DC (2×10^5) were stimulated with 40 μg/ml rHagB, 100 ng/ml of *E. coli* K12 LPS or left untreated. Culture supernatants were harvested 24 h post-stimulation and assayed for IFN-β production by ELISA. Results are expressed as the mean ± standard error of duplicate cultures from three independent experiments. *** and ** Significant differences at $P < 0.001$ and $P < 0.01$, respectively, compared to WT cultures stimulated with rHagB or LPS. ### Significant differences at $P < 0.001$ compared to control unstimulated cultures.

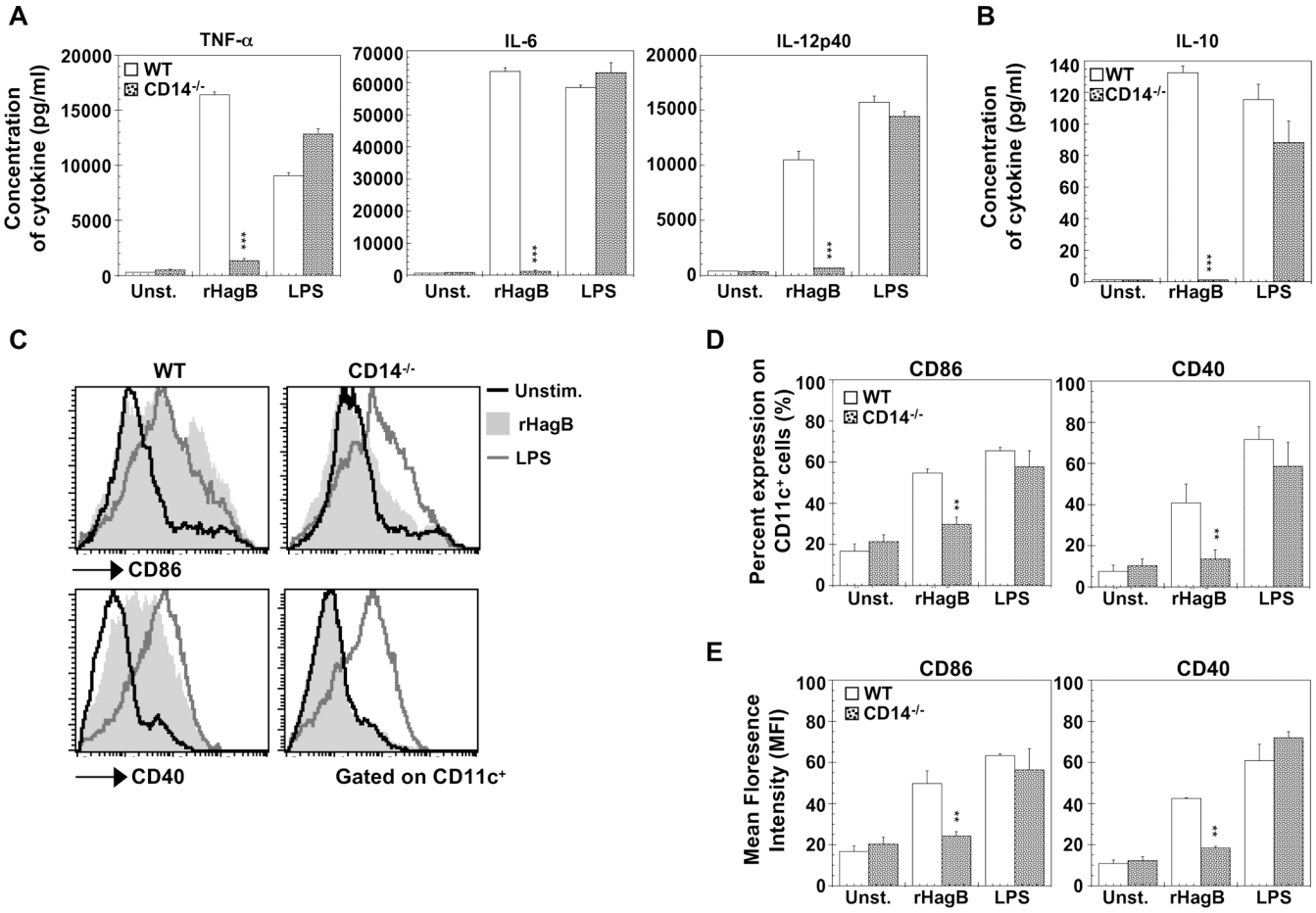

Fig. 9.

CD14 is required for rHagB to activate DC. DC (2×10^5) from WT (open bars) and $CD14^{-/-}$ (grey dotted bars) mice were stimulated with 40 μg/ml rHagB (shaded histograms), 100 ng/ml of *E. coli* K12 LPS (grey lines) or left untreated (black lines). (A and B) Culture supernatants were harvested 24 h post-stimulation and assayed for levels of TNF-α, IL-6, IL-12p40 and IL-10 by ELISA. Results are expressed as the mean ± standard error of triplicate cultures from one of three representative experiments. *** Significant differences at $P < 0.001$ compared to WT cultures stimulated with rHagB. (C, D and E) Cells were harvested 16 h post-stimulation and stained with fluorescent-labeled antibodies against CD11c, CD86 and CD40. Histogram plots were gated on $CD11c^+$ cells (C) and bar graphs represent the percentage expression of CD86 and CD40 on $CD11c^+$ cells (D) or the mean florescence intensity on $CD11c^+$ cells (E). Results are expressed as the mean ± standard error of three independent experiments. ** Significant difference at $P < 0.01$ compared to WT cultures stimulated with rHagB.