A novel pathway of rapid TLR-triggered activation of integrin-dependent leukocyte adhesion that requires Rap1 GTPase

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ABSTRACT **Rapid β2-integrin activation is indispensable for leukocyte adhesion and recruitment to sites of infection and is mediated by chemokine- or P-selectin glycoprotein ligand-1–induced inside-out signaling. Here we uncovered a novel pathway for rapid activation of integrin-dependent leukocyte adhesion, triggered by toll-like receptor (TLR)–mediated signaling. TLR2 or TLR5 ligation rapidly activated integrin-dependent leukocyte adhesion to immobilized ICAM-1 and fibronectin. Consistently, in vivo administration of the TLR2-ligand Pam3CSK4 increased integrin-dependent slow rolling and adhesion to endothelium within minutes, as identified by intravital microscopy in the cremaster model. TLR2 and TLR5 ligation increased β2-integrin affinity, as assessed by the detection of activation-dependent neoepitopes. TLR2- and TLR5-triggered integrin activation in leukocytes required enhanced Rap1 GTPase activity, which was mediated by Rac1 activation and NADPH oxidase-2–dependent reactive oxygen species production. This novel direct pathway linking initial pathogen recognition by TLRs to rapid β2-integrin activation may critically regulate acute leukocyte infiltration to sites of pathogen invasion.**

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INTRODUCTION

Leukocyte recruitment into sites of infection and injury has a fundamental role in host protection against pathogens, as well as in the course of inflammatory diseases. Leukocyte infiltration is a multistep process, including the initial selectin–mediated tethering and rolling, chemokine-induced integrin activation, which in turn results in integrin-dependent leukocyte slow rolling and firm adhesion. Finally, leukocytes move slowly to the sites of transendothelial migration in a manner largely dependent on β2-integrins, a process called crawling, before their transendothelial migration (Ley *et al.*, 2007; Chavakis *et al.*, 2009; Hajishengallis and Chavakis, 2013; Zarbock *et al.*, 2011; Herter and Zarbock, 2013; Kolaczkowska and Kubes, 2013).

The interactions of the β2-integrins, leukocyte function–associated antigen-1 (LFA-1; αLβ2; CD11a/CD18) and macrophage-1 antigen (Mac-1; αMβ2; CD11b/CD18), with their endothelial counterreceptors, mainly intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, play a key role in leukocyte adhesion to endothelium (Ley *et al.*, 2007; Chavakis *et al.*, 2009; Hajishengallis and Chavakis, 2013; Zarbock *et al.*, 2011; Herter and Zarbock, 2013; Kolaczkowska and Kubes, 2013). The ability of integrins to promote cell adhesion is mediated by their activation, which depends on changes in their affinity and valency. The affinity of integrins for their ligands is determined by the conformational status of integrin subunits, involving a shift from the bend, low-affinity conformation to the extended, intermediate- and high-affinity conformation (Luo *et al.*, 2007; Abram and Lowell, 2009; Gahmberg *et al.*, 2009). Integrin

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Abbreviations used: BSA, bovine serum albumin; DAP12, DNAX-activating protein of 12 kDa; DPI, diphenyleneiodonium; dsRNA, double-stranded RNA; FcR, Fc receptor; ICAM, intercellular adhesion molecule; LFA-1, leukocyte function–associated antigen-1; LPS, lipopolysaccharide; Mac-1, macrophage-1 antigen; MFI, mean fluorescence intensity; Nox2, NADPH oxidase 2; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; PSGL-1, P-selectin glycoprotein ligand-1; Rac1, Ras-related C3 botulinum toxin substrate 1; Rap1, Ras-related protein 1; ROS, reactive oxygen species; ssRNA, single-stranded RNA; syk, spleen tyrosine kinase; TLR, toll-like receptor.

affinity in leukocytes is regulated by rapid inside-out signaling pathways triggered by chemokines present on the apical endothelial surface and/or ligation of PSGL-1 by endothelial selectins. Chemokine-induced integrin activation is mediated by the small GTPase Rap1 and involves the effector proteins RIAM and RAPL, as well as talin and kindlin-3, which promote the separation of the cytoplasmic tails of the α- and β-subunits and thereby subsequent conformational changes of integrins toward intermediate- or high-affinity conformations (Luo *et al.*, 2007; Abram and Lowell, 2009; Gahmberg *et al.*, 2009; Lefort *et al.*, 2012).

In addition, binding of PSGL-1 to endothelial selectins induces LFA-1 activation, mediated by a pathway that involves Fgr, DAP12, FcRg, and Syk kinase (Sperandio *et al.*, 2003; Zarbock *et al.*, 2008). On the other hand, β2-integrin binding results in the initiation of a signal transduction cascade characterized as outside-in signaling through activation of intracellular protein kinase Src and Syk, further supporting cell adhesion (Zarbock *et al.,* 2008; Abram and Lowell, 2009). Besides the two aforementioned chemokine and selectin ligand-dependent pathways of rapid integrin activation in myeloid cells, resulting in acute adhesion and recruitment of leukocytes, not much is known about integrin activation by further proinflammatory signals that may participate in the initial response to infection and injury.

A critical role in immune responses to invading pathogens is played by toll-like receptors (TLRs), the most extensively studied pattern recognition receptors (PRRs; Kawai and Akira, 2010). TLRs recognize a variety of conserved motifs on pathogens (pathogen-associated molecular patterns [PAMPs]), including lipoproteins (TLR1, TLR2, and TLR6), double-stranded RNA (dsRNA; TLR3), lipopolysaccharide (LPS; TLR4), flagellin (TLR5), single-stranded RNA (ssRNA; TLR7 and TLR8), and DNA (TLR9), enabling pathogen recognition by innate immune cells (Kawai and Akira, 2010). TLR ligation drives signaling events that result in the production of inflammatory mediators, which in turn further fuel both innate and adaptive immune responses (Kawai and Akira, 2010, 2011). The critical involvement of both TLRs and β2-integrins in host defense against pathogens indicates that a possible interplay between TLR and β2-integrin cascades may exist. Indeed, previous work suggested that outside-in integrin signaling interferes with TLR signaling, either promoting or attenuating TLR-dependent responses (Perera *et al.*, 2001; Han *et al.*, 2010). However, the involvement of TLR signaling in β2-integrin activation and leukocyte recruitment in the context of the response to infection and injury is less well studied. In the present study, we screened whether TLR ligation may induce rapid integrin-dependent leukocyte adhesion. Strikingly, we identified TLR2 and TLR5 ligation to activate rapidly β2-integrin affinity and leukocyte adhesion in vitro, whereas TLR2 ligation induced leukocyte recruitment in vivo in the acute mouse cremaster model of inflammation. We demonstrated that TLR-mediated integrin activation required Rap1 GTPase. In addition, TLR-triggered integrin activation via the Rap1 GTPase was mediated by a cascade that involved Rac1 and NADPH oxidase 2 (Nox2)–dependent reactive oxygen species (ROS) production. The pathway described here linking TLRs to acute leukocyte integrin activation and inflammatory cell recruitment may play a major role in the acute response to infection or injury.

RESULTS

TLR2 and TLR5 activation induces β2-integrin–dependent leukocyte adhesion in vitro

Rapid integrin activation is required for integrin-dependent leukocyte adhesion. In an effort to assess whether ligation of TLRs could induce rapid integrin-dependent adhesion, we studied the effect of coincubation with TLR1-9 ligands on leukocyte adhesion to immobilized ligands, using a 20-min adhesion assay. The β2-integrin–dependent adhesion (Smith *et al.*, 1989) of THP-1 myelomonocytic cells to immobilized ICAM-1 was assessed. In addition, THP-1 cell adhesion to fibronectin was studied, which is mediated by both β1- and β2-integrins (Owen *et al.*, 1992; Frieser *et al.*, 1996). As a control, adhesion to immobilized BSA was assessed. Whereas several TLR ligands seemingly stimulated slightly the adhesion of THP-1 cells to ICAM-1 and fibronectin but not to bovine serum albumin (BSA), a significant increase of cell adhesion to ICAM-1 or fibronectin was consistently observed with HKLM, flagellin, and FSL-1, which stimulate TLR2, TLR5, and TLR2/6, respectively (Figure 1, A and B). Moreover, ligation of the same TLRs (TLR2, TLR5, and TLR2/6) induced adhesion of primary human neutrophils to immobilized ICAM-1 (Figure 1C). Therefore in the subsequent studies we used TLR2 and TLR5 ligands to assess the effects of TLR-dependent activation of leukocyte adhesion, whereas the TLR9 ligand ODN2006, which had the weakest effect in stimulating integrin-dependent leukocyte adhesion, was used as a negative control. Together these data unequivocally suggested that acute (within 20 min) TLR2 and TLR5 ligation is capable of inducing rapid integrin-dependent leukocyte adhesion, a property that is traditionally attributed to activation of leukocytes by chemokines or selectin ligands.

TLR2-induced leukocyte adhesion in cremaster muscle venules in vivo

We next tested whether the observed stimulation of leukocyte adhesion by TLR ligation in vitro could be relevant in vivo as well. To address acute leukocyte adhesion in vivo, we used the cremaster model of acute inflammation associated with intravital microscopy analysis. We tested whether systemic injection of the TLR2-agonist Pam3CSK4 into C57BL/6 mice via a carotid artery catheter led to induction of firm leukocyte arrest in exteriorized cremaster muscle venules in vivo. We found that after the first minute postinjection of the TLR2 ligand, leukocyte adhesion efficiency (number of adherent leukocytes/systemic leukocyte count) was significantly elevated compared with leukocyte adhesion efficiency before injection of Pam3CSK4 (Figure 2A). Similarly, the absolute number of adherent leukocytes 1–2 min after Pam3CSK4 injection was significantly higher than the number before Pam3CSK4 injection (Figure 2B and Supplemental Videos S1 and S2), suggesting that signaling via TLR2 can induce rapid firm leukocyte arrest in vivo. We found no significant changes in the leukocyte rolling flux fraction before and 1–2 min after injection of the TLR2 agonist Pam3CSK4 (Figure 2C). In contrast, leukocyte rolling velocities decreased significantly upon stimulation with Pam3CSK4 (Figure 2D), implying that TLR2 ligation induced a transition from rolling to firm leukocyte adhesion. In contrast, systemic injection of the TLR9 agonist ODN1668 had no effect on leukocyte adhesion to endothelial cells (Supplemental Figure S1). Besides the well-established dependence of firm leukocyte arrest on leukocyte β2-integrins (Henderson *et al.*, 2001), previous work also suggested that the transition from rolling to firm leukocyte adhesion is accompanied by intermediate activation of the β2-integrin LFA-1, which has been linked to a reduction in leukocyte rolling velocities in vivo (Zarbock *et al.*, 2008). Taken together, our in vivo findings, supported by our in vitro data, point to a previously unknown rapid activation of β2-integrin–dependent leukocyte adhesion upon TLR2 ligation.

TLR2 and TLR5 ligation activate β2-integrin affinity

The rapid activation of leukocyte adhesion in vitro by TLR2 and TLR5 and in vivo by TLR2 ligation implied alterations in β2-integrin affinity

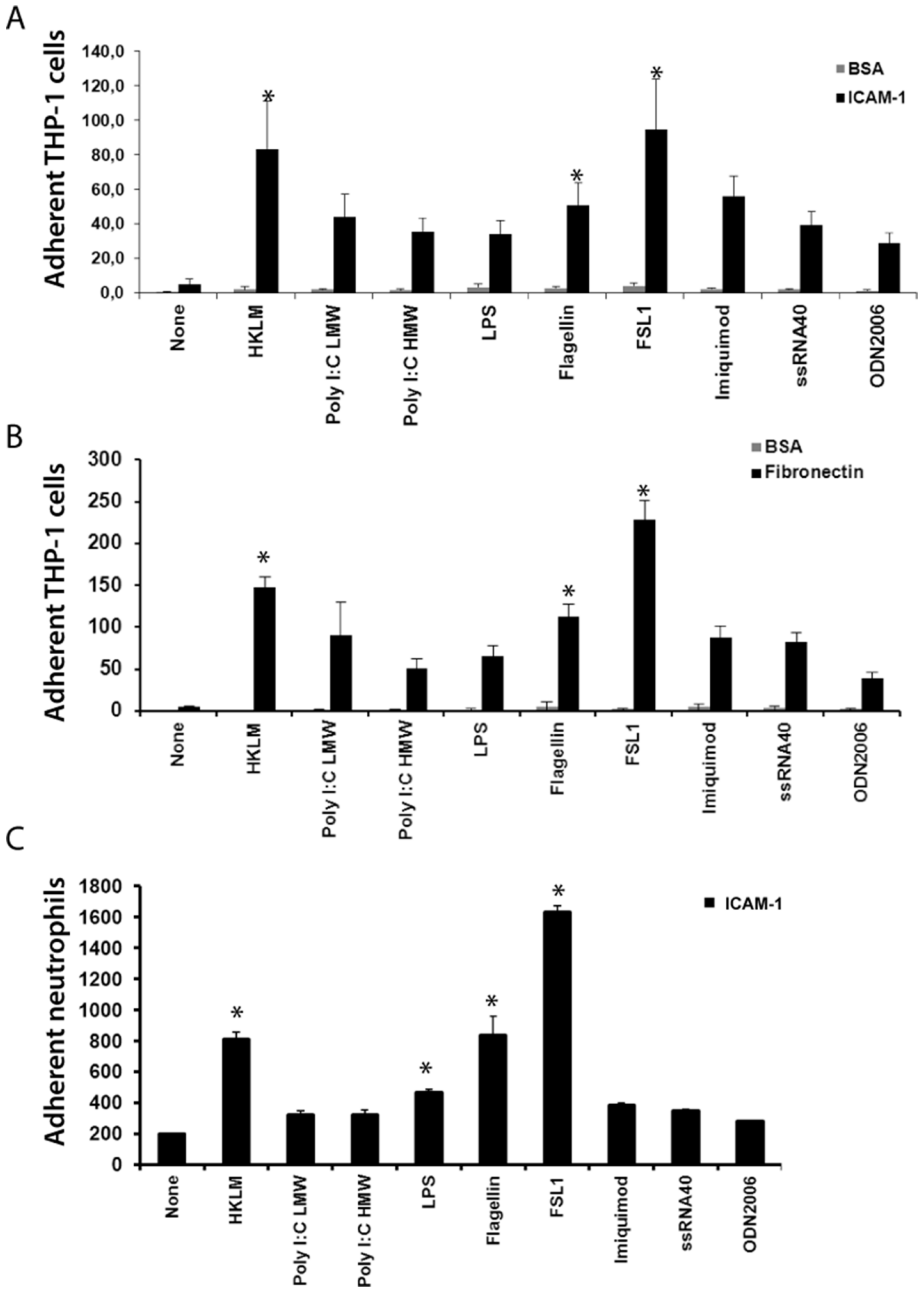

FIGURE 1: Adhesion of THP-1 cells and human neutrophils stimulated with TLR ligands to ICAM-1 or fibronectin. The number of THP-1 cells adherent to immobilized ICAM-1 (black bars; A) or fibronectin (black bars; B) was assessed in the absence or presence of TLR1-9 ligands. Adhesion to BSA (gray bars) was used as control. (C) The number of adherent human neutrophils to immobilized ICAM-1 after treatment with the TLR1-9 ligands. Data are mean ± SEM (n = 3–5 independent experiments). One-way ANOVA with Bonferroni posthoc analysis (asterisk denotes significance of the posthoc test).

rather than changes in their expression upon TLR2 and TLR5 ligation. To this end, we stimulated THP-1 myelomonocytes acutely (20 min) with TLR2 (HKLM) or TLR5 (flagellin) ligands, used TLR9 (ODN2006) ligand as a negative control, and assessed β2-integrin conformational status by flow cytometry using KIM127 and mAb24 antibodies, which recognize the intermediate- and high-affinity conformations on β2-integrins (Stanley *et al.*, 2008; Kuwano *et al.*, 2010). Acute TLR2 and TLR5 activation increased the expression of the KIM127- and mAb24-recognized neoepitopes on β2-integrins (Figure 3, A and B) without affecting β2-integrin expression. In contrast, TLR9 stimulation had no effect on the conformational status of β2-integrins (Figure 3, A and B). Additional experiments demonstrated that the activation of the conformational status of β2-integrins by TLR2 stimulation can be detected as early as 1 min upon treatment with the ligand (Supplemental Figure S2A). Thus TLR2 and TLR5 ligation can rapidly induce the intermediate- and high-affinity conformation of β2-integrins.

Rap1 mediates TLR2- and TLR5-induced β2-integrin activation and leukocyte adhesion

We went on to investigate the signaling pathways that link TLR ligation to β2-integrin affinity activation. Rap1 GTPase is a major player in β2-integrin activation (Luo *et al.*, 2007; Abram and Lowell, 2009; Gahmberg *et al.*, 2009; Lefort *et al.*, 2012). We therefore focused on this pathway. We observed that acute stimulation of TLR2 and TLR5 increased Rap1 activation in THP-1 myelomonocytes, as assessed by studying the levels of GTP-bound Rap1 (Figure 3, C and D). Moreover, Rap1 activation was detected already 1 min after THP-1 cell stimulation with the TLR2 ligand (Supplemental Figure S2B). Furthermore, transfection of THP-1 cells with Rap1a small interfering RNA (siRNA) but not control siRNA (Supplemental Figure S3A) attenuated TLR2- and TLR5-induced cell adhesion to ICAM-1 (Figure 3E) and abolished TLR2- and TLR5-triggered stimulation of β2-integrin affinity, as assessed by studying the exposure of the mAb24-recognized epitope (Figure 3F). On the other hand, Rap1a gene silencing had no effect on β2-integrin surface expression on THP-1 cells (Supplemental Figure S4).

The role of Nox2 and Rac1 in TLR2- and TLR5-dependent β2-integrin activation

Having found that TLR2 and TLR5 ligation induced β2-integrin activation and β2-integrin–dependent leukocyte adhesion in a Rap1-dependent manner, we continued to consider the signaling pathways between TLR ligation and Rap1 activation. ROS production through Nox2 NADPH oxidase activation has been proposed as a downstream event to TLR stimulation (Martinon *et al.*, 2010). To investigate the involvement of ROS production in Rap1 and β2-integrin activation caused by TLR2 and TLR5 ligation, we initially confirmed that treatment with HKLM or flagellin induced ROS production in THP-1 myelomonocytes, as assessed by flow cytometry (Figure 4A). We further studied the effect of Nox2 inhibition in TLR2- and TLR5-induced adhesion of THP-1 cells to ICAM-1. In particular, siRNA-mediated NOX2 gene silencing (Supplemental Figure S3B), but not control siRNA, blocked cell adhesion to ICAM-1 downstream of TLR ligation (Figure 4B) by

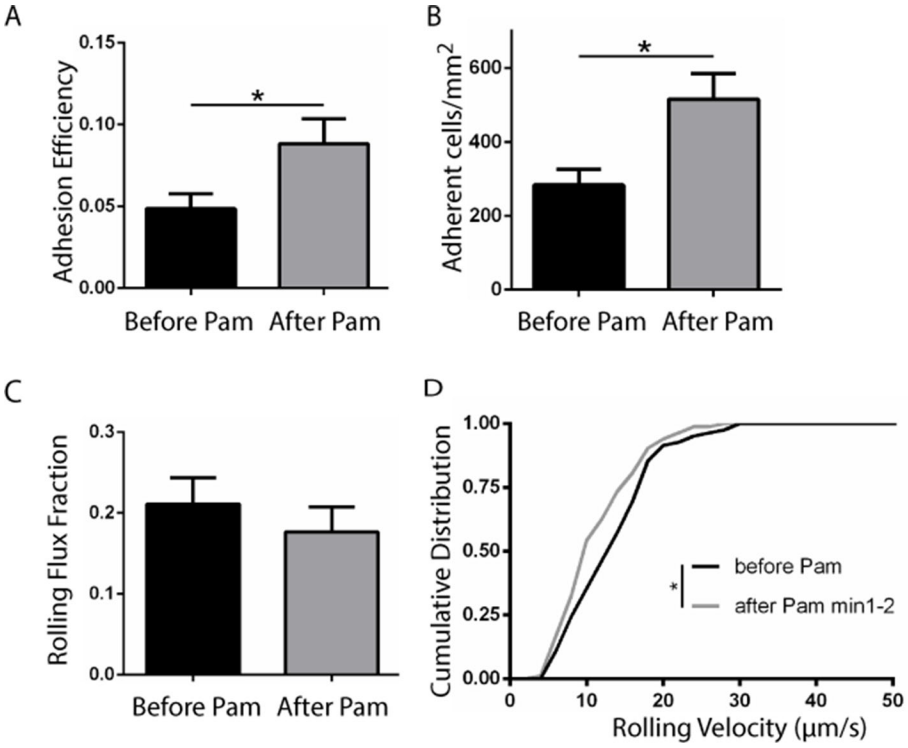

FIGURE 2: Adhesion of inflammatory cells in vivo in the cremaster muscle model is enhanced by Pam3CSK4 administration. (A) Leukocyte adhesion efficiency (percentage of adherent leukocytes per square millimeter relative to the white blood cell count per microliter), (B) number of adherent leukocytes per vessel surface (in square millimeters), (C) leukocyte rolling flux fraction (percentage of rolling leukocytes relative to the number of leukocytes entering the vessel), and (D) leukocyte rolling velocities investigated in exteriorized cremaster muscle venules at baseline conditions (before PAM), as well as 1–2 min after Pam3CSK4 injection (after Pam). Results are shown as mean ± SEM in A–C (*$p < 0.05$, $n = 9$ mice). Leukocyte rolling velocities in D are displayed as cumulative histogram of 82 analyzed leukocyte rolling velocities before and 83 measured rolling velocities 1–2 min after Pam3CSK4 injection. Student's *t* test was used for statistical analysis.

preventing TLR2-induced Rap1 activation (Figure 4, C and D). In addition, we observed that treatment with diphenyleneiodonium (DPI), a NADPH oxidase inhibitor, significantly blocked TLR2- and TLR5-induced cell adhesion to ICAM-1 (Supplemental Figure S5). These data suggest that Nox2-dependent ROS generation is a signaling intermediate between TLR2 and TLR5 ligation and Rap1-dependent β2-integrin activation.

Rac1 is an intermediate in TLR-induced signaling (Shen *et al.*, 2010) and ROS activation (Bäumer *et al.*, 2008). We therefore studied the role of Rac1 in the TLR-dependent pathway of β2-integrin activation. We initially observed that treatment with a Rac1 inhibitor significantly down-regulated TLR2-induced ROS production (Figure 5A). Moreover, Rac1 inhibition abolished THP-1 myelomonocyte adhesion to ICAM-1 after TLR2 and TLR5 stimulation (Figure 5B). Taken together, TLR2- and TLR5-dependent activation of Rap1 GTPase and consequent β2-integrin affinity and β2-integrin–dependent leukocyte adhesion required the activation of Rac1 and Nox2 upon TLR2 and TLR5 ligation.

DISCUSSION

Rapid integrin activation is a critical process in the recruitment of inflammatory cells into sites of microbial infection or tissue injury. Hitherto, chemokines and selectin ligands were known to induce rapid integrin activation, thus promoting the transition from rolling to slow rolling and firm adhesion. Here we establish that TLR2 and TLR5 ligation can induce rapid integrin activation and acute leukocyte adhesion in vitro. In addition, our in vivo findings provided the first evidence that TLR-dependent β2-integrin activation is a rapid process, resulting in the induction of firm leukocyte adhesion within the first minutes after administration of TLR2 agonist. Thus we define a novel pathway in the immediate response to infection or injury by which TLR2 and TLR5 stimulation can mediate acute leukocyte integrin activation and inflammatory cell adhesion. As a result, initial pathogen recognition by TLRs can directly promote β2-integrin–dependent leukocyte arrest and subsequent recruitment and accumulation of inflammatory cells to the infected tissue.

In this study, we identified the effectors downstream of TLR2 and TLR5 in the pathway that leads to β2-integrin activation. We demonstrate that Rac1-mediated Nox2 activation and ROS production upon TLR2 and TLR5 ligation results in the activation of Rap1 GTPase, which, in turn, induces β2-integrin affinity changes promoting leukocyte adhesion. Rac1 plays a central role in TLR-mediated cellular responses (Shen *et al.*, 2010), whereas TLR-dependent Nox2-dependent ROS production (Martinon *et al.*, 2010) is involved in microbial killing, cytokine production, and regulation of cell death pathways in leukocytes (Lam *et al.*, 2010; Lambeth and Neish, 2013). We here expand the palette of acute responses to infection activated by TLR ligation to include TLR-dependent rapid integrin activation and leukocyte adhesion.

Previous reports involved cytohesin-1 and phosphoinositide 3-kinase in LPS-induced monocyte adhesion (Hmama *et al.*, 1999), as well as in integrin-dependent cell adhesion induced by TLR2 stimulation by *Porphyromonas gingivalis* fimbriae (Harokopakis 2005; Harokopakis *et al.*, 2006; Hajishengallis *et al.*, 2009). Here we provide evidence that the TLR/Rac1/Nox2 axis induces β2-integrin–dependent cell adhesion through Rap1 activation.

Besides the cross-talk identified here between TLRs and β2-integrins, there is evidence that β2-integrins may also act as regulators of TLR signaling. Activation of β2-integrins by fibrinogen can inhibit TLR signaling, in a process that depends on interleukin-10, STAT3, SOCS3, ABIN-3, Hes1, and A20 induction (Wang *et al.*, 2010). Moreover, CD11b deficiency in macrophages enhanced TLR-mediated inflammatory responses, affecting survival in murine endotoxemia (Han *et al.*, 2010). Han *et al.* (2010) proposed that TLR4 ligation induced CD11b activation, which in turn inhibited TLRs by promoting degradation of MyD88 and TRIF, suggesting a negative feedback loop. In addition, engagement of Mac-1 in human monocytes and macrophages was shown to down-regulate TLR7/8-dependent inflammatory responses (Reed *et al.*, 2013), whereas it negatively regulated immune responses in TLR9-triggered dendritic cells (Bai *et al.*, 2012). On the other hand, cross-talk between Mac-1 and TLR/interleukin-1 signaling was suggested (Shi *et al.*, 2001; Kagan and Medzhitov, 2006). In addition, Mac-1 may participate in the recruitment of the adaptor protein TIRAP to the

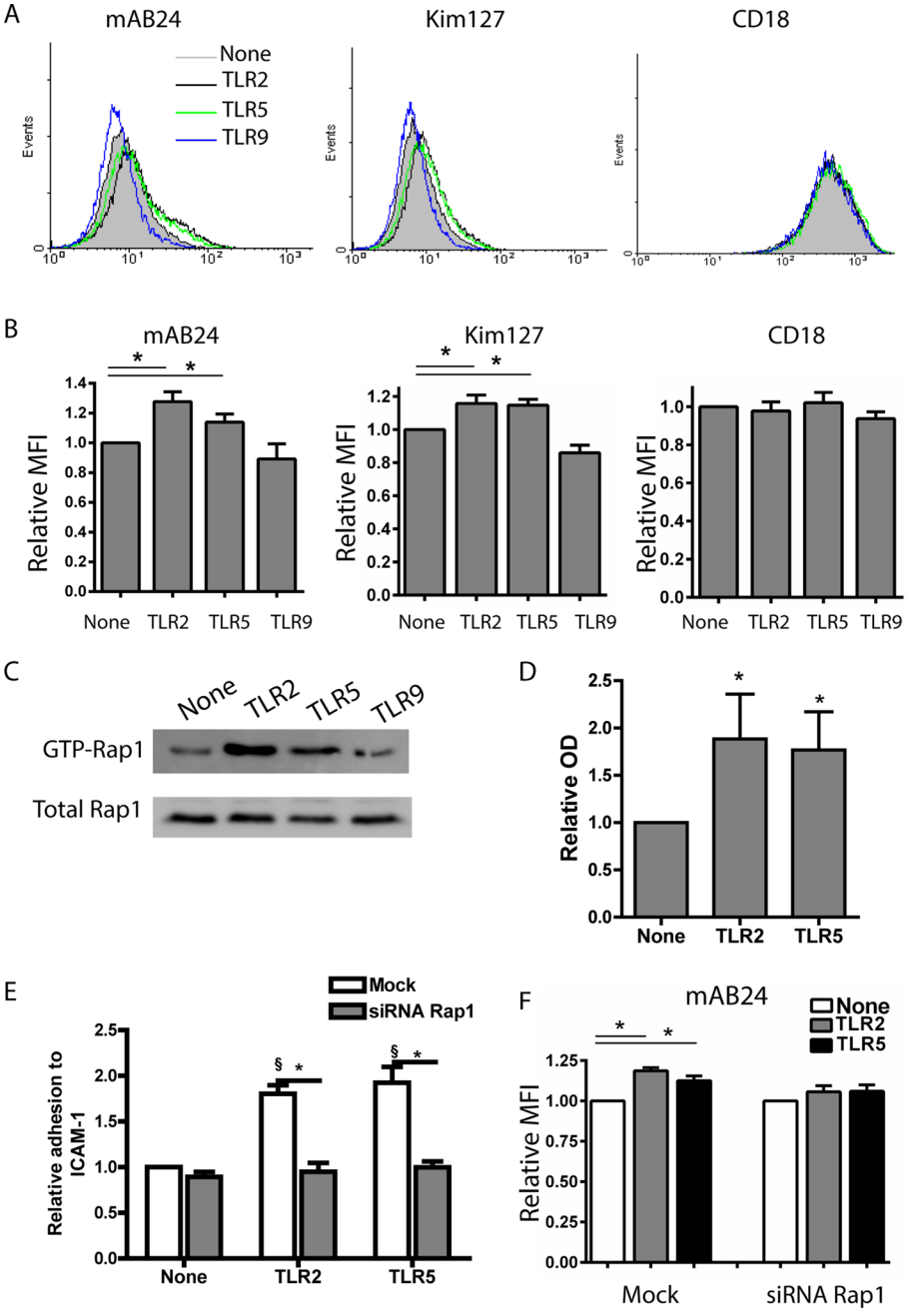

FIGURE 3: TLR2 and TLR5 stimulation activates β2-integrin affinity in a pathway that involves Rap1-GTPase. (A, B) To detect activation-dependent neoepitopes on β2-integrins, THP-1 myelomonocytes were stimulated without or with HKLM (TLR2 ligand), flagellin (TLR5 ligand), or ODN2006 (TLR9 ligand). The conformational status of β2-intergrin was studied by flow cytometry using Kim127 and mAb24 antibodies. The total surface expression of β2-intergrin was analyzed by an anti-CD18 antibody. (A) Representative histograms of fluorescence intensity and (B) relative mean fluorescence intensity (MFI) compared with untreated THP-1 cells ($n = 3$–5 per group). *$p < 0.05$. Student's t test was used for statistical analysis. (C) Rap1-GTP (activated Rap1) levels in THP-1 myelomonocytes treated or not with TLR2 ligand, TLR5 ligand, or TLR9 ligand for 10 min. Total Rap1 protein levels were used as loading control. Data derived from one representative experiment. (D) Densitometric analysis of immunoblots indicating activation of Rap1 by TLR2 and TLR5 ligation ($n = 5$–7 independent experiments). *$p < 0.05$. Student's t test was used for statistical analysis. (E) Adhesion of THP-1 cells transfected with control siRNA (Mock) or siRNA targeting Rap1a to immobilized ICAM-1 in the absence or presence of the indicated ligands. Data expressed as relative adhesion. One-way ANOVA with Bonferroni posthoc analysis (asterisk and section sign [§] denote significance of the posthoc test; the latter symbol indicates comparison with unstimulated cells transfected with control siRNA; $n = 3$ independent experiments). (F) β2-Integrin conformational status in THP-1 cells transfected with siRNA targeting Rap1a in the absence or presence of HKLM (TLR2 ligand) or flagellin (TLR5 ligand) was assessed by flow cytometry using mAb24. Data expressed as relative MFI compared with unstimulated cells ($n = 4$ independent experiments). Data are shown as mean ± SEM. *$p < 0.05$. Student's t test was used for statistical analysis.

cell membrane, a critical event in the initiation of TIRAP-dependent TLR4 signaling (Kagan and Medzhitov, 2006). Furthermore, in mouse dendritic cells, TLR signaling can regulate the expression and trafficking of the β2-integrin subunit CD11c (Singh-Jasuja *et al.*, 2013). Our findings presented here on TLR-dependent integrin activation, in combination with previous reports on regulation of TLR-signaling by integrins, may point to a bidirectional cross-regulation between TLRs and leukocyte integrins in innate immunity.

A limitation of our in vivo study is that we cannot entirely exclude that increased expression of adhesion molecules and/or chemokines in the endothelium in vivo induced by TLR signaling could also contribute to increased leukocyte adhesion to endothelial cells. Activation of the endothelium by TLR ligation could be operative on top of the rapid β2-integrin activation in leukocytes. However, because we observed a very rapid (within 2 min) induction of leukocyte adhesion upon TLR2 ligation in mice and TLR-dependent endothelial cell activation is not a rapid process (Zeuke *et al.*, 2002), we believe that the role of endothelial activation by TLR-signaling in our experimental system is rather unlikely.

In conclusion, our study solidifies the idea that PAMP recognition by TLRs may trigger inflammatory cell infiltration to the site of pathogen invasion or sterile tissue injury via rapid Rap1 GTPase-dependent β2-integrin activation. The TLR-dependent rapid β2-integrin activation may have major importance in leukocyte–endothelial interactions in the context of host defense against pathogens or in sterile inflammatory injury.

MATERIALS AND METHODS

Reagents and antibodies

Human TLR1-9 agonist kit was purchased from InvivoGen (San Diego, CA). DPI, fibronectin, and Ficoll Histopaque (1077 and 1119) were purchased from Sigma-Aldrich (St. Louis, MO). Rac1 inhibitor was purchased from Calbiochem (Darmstadt, Germany).

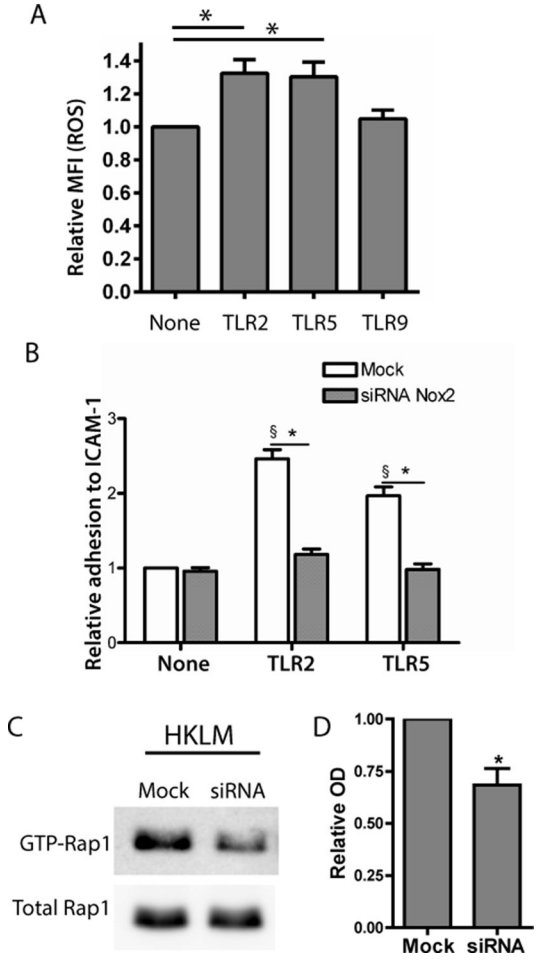

FIGURE 4: Nox2-dependent ROS production mediates TLR-dependent cell adhesion by inducing Rap1 activation. (A) ROS levels in THP-1 cells treated with HKLM (TLR2 ligand), flagellin (TLR5 ligand), or ODN2006 (TLR9 ligand) were assessed by flow cytometry using CellROX green reagent. Data are the mean MFI ± SEM ($n = 4$ independent experiments). *$p < 0.05$. Student's t test was used for statistical analysis. (B) Adhesion of THP-1 cells transfected with control siRNA (Mock) or siRNA targeting Nox2 to immobilized ICAM-1 in the absence or presence of the indicated TLR ligands. Data expressed as relative adhesion. Data are shown as mean ± SEM ($n = 4$ independent experiments). One-way ANOVA with Bonferroni posthoc analysis (asterisk and section sign [§] denote significance of the posthoc test; the latter symbol indicates comparison with unstimulated cells transfected with control siRNA). (C) Rap1-GTP (activated Rap1) levels in THP-1 cells transfected with control siRNA (Mock) or siRNA targeting Nox2 (siRNA) and treated with HKLM (TLR2). Data derived from one representative experiment. (D) Densitometric analysis of immunoblots demonstrating that Nox2 knockdown blocks the TLR2-induced Rap1 activation ($n = 4$ independent experiments). *$p < 0.05$. Student's t test was used for statistical analysis.

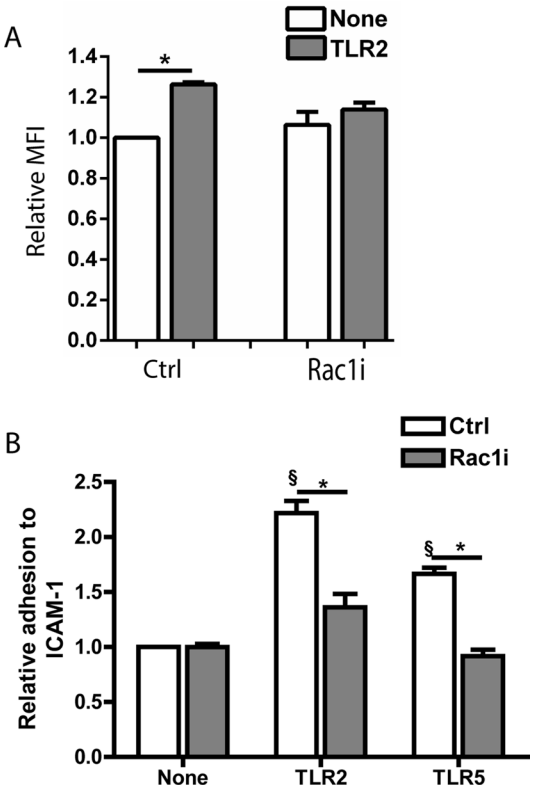

FIGURE 5: Rac1 modulates TLR-dependent leukocyte adhesion via ROS production. (A) ROS levels in THP-1 cells pretreated or not with a Rac1 inhibitor and subsequently stimulated with HKLM (TLR2 ligand) were assessed by flow cytometry using CellROX green reagent. Data are expressed as relative MFI compared with untreated THP-1 cells. Data are shown as mean ± SEM ($n = 3$ or 4 independent experiments). *$p < 0.05$. Student's t test was used for the statistical analysis. (B) Adhesion of THP-1 cells pretreated with dimethyl sulfoxide (Ctrl) or Rac1 inhibitor to immobilize ICAM-1 is shown in the absence or presence of indicated TLR ligands. Data expressed as relative adhesion. One-way ANOVA with Bonferroni posthoc analysis (asterisk and section sign [§] denote significance of the posthoc test; the latter symbol indicates comparison with control-treated unstimulated cells. Data are the mean ± SEM ($n = 3$ independent experiments).

RBC lysis buffer was purchased from eBioscience (San Diego, CA). Human ICAM-1 Fc was purchased from R&D Systems (Minneapolis, MN). Allophycocyanin-conjugated CD18 (MEM-48) antibody and mAb24 antibody recognizing the β2-integrin high-affinity conformation were purchased from Abcam (Cambridge, United Kingdom). Monoclonal Kim127 antibody against β2-integrin intermediate affinity conformation (American Type Culture Collection, Manassas, VA) was previously described (Kuwano *et al.*, 2010).

Intravital microscopy of leukocyte adhesion, rolling, and rolling velocities in the cremaster muscle of the mouse

The cremaster muscle preparation in C57Bl/6 mice (obtained from Janvier Labs, Saint-Berthevin, France) was performed as previously described (Frommhold *et al.*, 2008). Briefly, the scrotum of the mouse was incised, the cremaster muscle exteriorized, additional tissue removed, and the muscle opened through a longitudinal incision and then mounted to a self-customized stage. The cremaster muscle was constantly superfused with warmed superfusion buffer (Ley *et al.*, 1991).

Intravital microscopy was conducted on a BX 51 WI microscope (Olympus, Hamburg, Germany) equipped with a saline immersion objective (Olympus MplanFI/RI; 0.8 numerical aperture) and a charge-coupled device camera (KAPPA CF8 HS). Blood flow velocity was measured using a dual-slit photodiode live measuring device (Circusoft Instrumentation, Hockessin, DE). The recording of the postcapillary venules was conducted for at least 1 min before and up to 3 min after a single injection of Pam3CSK4 or ODN1668 using Virtual-Dub (version 1.9.11). Leukocyte rolling was assessed

as rolling flux fraction (percentage of rolling leukocytes relative to the number of leukocytes passing the vessel), leukocyte adhesion (number of adherent cells per square millimeter), and leukocyte adhesion efficiency (percentage of adherent leukocytes per square millimeter relative to the white blood cell count per microliter). Adherent leukocytes were defined as nonmoving cells or cell displacement less than one cell diameter during 1 min of observation (Sperandio *et al.*, 2006). Leukocyte rolling velocities were measured as averages over a 1-s time window as described using Fiji (Schindelin *et al.*, 2012). Blood counts were determined through Idexx Procyte Dx hematology analyzer (Idexx Europe, Hoofddorp, Netherlands). Counts were acquired before the administration of Pam3CSK4. All experiments were performed in accordance with Bavarian state regulations for animal keeping and experimentation as approved by the responsible authorities of the Regierung of Oberbayern.

Cell adhesion assay

Human neutrophils were isolated from heparinized blood from healthy volunteers. Neutrophils were isolated after Ficoll-Histopaque double-gradient density centrifugation. Erythrocyte lysis was performed by RBC lysis buffer. Human neutrophils or THP-1 myelomonocytic cells to immobilized human ICAM-1 or fibronectin (each 10 μg/ml) was performed as previously described (Chavakis *et al.*, 2003; Sotiriou *et al.*, 2006; Choi *et al.*, 2008a,b). Briefly, 96-well microplates were coated with ICAM-1, fibronectin, or BSA in phosphate-buffered saline (PBS) and then blocked with 3% BSA. Human neutrophils or THP-1 cells were washed twice with RPMI medium with 0.1% BSA and plated onto precoated wells at 37°C for 20 min in the absence or presence of HKLM (TLR2, 10^8 cells/ml), Poly(I:C) (TLR3, 10 μg/ml), Poly(I:C)LMW (TLR3, 10 μg/ml), LPS (TLR4, 100 ng/ml), flagellin (TLR5, 1 μg/ml), FSL1 (TLR6/2, 1 μg/ml), imiquimod (TLR7, 1 μg/ml), ssRNA40 (TLR8, 1 μg/ml), and ODN2006 (TLR9, 5 μM). Wells were then washed three times with PBS, and cells were fixed with 4% paraformaldehyde. Adherent cells were counted under an Axiovert 25 inverted microscope (Zeiss, Jena, Germany).

Rap1 assay

To assess active Rap1 in THP-1 myelomonocytes, a pull-down assay was performed with Rap1 Activation Kit (Millipore, Temecula, CA) according to the manufacturer's instructions. Briefly, cells (5×10^6) were treated or not with TLR2 or TLR5 agonists at 37°C for different time points (up to 10 min) in complete RPMI medium. Ral GDS-RBD agarose was added to each cell extract and incubated for 45 min at 4°C. Precipitates were then washed, resuspended in 40 μl of 2× sample buffer, boiled for 5 min at 100°C, and separated by 12% SDS–PAGE. Rap1 antibody was used to detect active Rap1. Whole-cell lysates were used to assess total levels of Rap1. Optical intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Flow cytometric analysis of β2-integrin affinity

For the analysis of β2-integrin conformation status, THP-1 cells (1×10^6) were coincubated in the absence or presence of TLR2, TLR5, or TLR9 ligands and mAb24 or Kim127 for 20 min at 37°C in RPMI (0.1% BSA) medium. Cells were incubated with anti-mouse Alexa Fluor 488 antibody for 10 min. To detect rapid conformational changes of β2-integrin, THP-1 cells were coincubated with TLR2 ligand, mAb24, and anti-mouse Alexa Fluor 488 for different time points (up to 5 min). Thereafter cells were directly assayed by flow cytometer (FACSCantoII; BD Bioscience, Heidelberg, Germany). Total CD18 levels were quantified using a mouse anti-CD18 monoclonal antibody (clone MEM-48).

ROS measurement

THP-1 myelomonocytic cells (1×10^6) were treated with TLR agonist (TLR2, 5, or 9) at 37°C for 20 min in complete RPMI medium. Cells were washed with complete medium. CellROX green reagent (5 μM; Life Technologies, Darmstadt, Germany) was added to the resuspended cells and incubated at 37°C for 30 min. Cells were washed, and ROS levels were determined by flow cytometry (FACSCantoII).

siRNA-mediated knockdown and inhibitor treatment

THP-1 cells (5×10^5) were transfected with 5 nM siRNA against human Rap1a and gp91phox (NOX2) or control siRNA (Dharmacon, Lafayette, CO) using INTERFERin (Polyplus Transfection, New York, NY) for 48 h. The siRNA-mediated knockdown was studied by Western blot (Supplemental Figure S3). THP-1 cells (5×10^5 or 1×10^6) were also treated with DPI (10 μM) or Rac1 inhibitor (100 μM) at 37°C for 1 h.

Statistical analysis

Statistical analysis was performed using the Student's *t* test or analysis of variance (ANOVA) with Bonferroni posthoc analysis as appropriate. $p < 0.05$ was regarded as significant.

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