Plexin-A4–semaphorin 3A signaling is required for Toll-like receptor– and sepsis-induced cytokine storm

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Plexins and semaphorins are ligand–receptor pairs that serve as guidance molecules in the nervous system and play some roles in immunity. Plexins are similar to the Toll-like receptors (TLRs) in their evolutionary conservation from flies to mammals. By studying plexin-A4–deficient (Plxna4−/−) innate immune cells, in this study we show a novel influence of plexin-A4 on TLR signaling. Plxna4−/− cells exhibit defective inflammatory cytokine production upon activation by a spectrum of TLR agonists and bacteria. Plexin-A4 is required for TLR-induced activation of the small guanosine triphosphate hydrolase (GTPase) Rac1 (ras-related C3 botulinum toxin substrate 1). Rac1 activation is accompanied by JNK (c-Jun N-terminal kinase) and NF-κB activation, culminating in TLR-induced binding of NF-κB and AP-1 to the promoters of inflammatory cytokines. Plxna4−/− mice are remarkably resistant to TLR agonist–induced inflammation and polymicrobial peritonitis caused by cecal ligation and puncture. Administration of a ligand of plexin-A4, Sem3A (semaphorin 3A), exacerbates the cytokine storm caused by TLR agonists and bacterial sepsis. TLR engagement can induce Sem3A expression, thus completing an autocrine loop. These findings expand the role of plexins to TLR signaling and suggest plexin-A4 and Sem3A as new intervention points for treating sepsis.

The functions of plexins and their ligands, semaphorins, have been extensively studied in the central nervous system (CNS). They represent two large families of molecules that can transduce signals essential for the regulation of neuronal repulsion and attraction, cell shape, motility, and cell–cell interactions (Tran et al., 2007). In addition to their roles in the CNS, the diverse functions of plexins and semaphorins have also been identified in cardiac development (Toyofuku et al., 2004b), vascularization and angiogenesis (Gu et al., 2003a), and tumorigenesis (Neufeld and Kessler, 2008). More recent data strongly indicate a role for these molecules in the immune system (Suzuki et al., 2008). For example, plexin-A1 is expressed by DCs and regulates DC interaction with T cells to affect adaptive immunity (Wong et al., 2003; Takegahara et al., 2006). Plexin-C1 is also found on DCs, although its role is less defined and much higher expression of plexin-A4 in myeloid cells relative to lymphoid cells (Yamamoto et al., 2008), the role of plexin-A4 in cells of thymocytes and a role for this protein in the control of intrathymic migration of these cells from cortical to medullary region (Choi et al., 2008). Therefore, plexins are involved in diverse functions in the immune system.

Plexin-A4 belongs to plexin-A type group (Tran et al., 2007) and serves as a guidance cue molecule in sensory and sympathetic neurons (Yaron et al., 2005) and hippocampal mossy fibers (Suto et al., 2007). One recent study identified plexin-A4 as a negative regulator in T cell activation (Yamamoto et al., 2008). T cells lacking plexin-A4 (Plxna4−/−) exhibited hyperproliferative responses upon stimulations in vivo and in vitro. However, given the much higher expression of plexin-A4 in myeloid cells relative to lymphoid cells (Yamamoto et al., 2008), the role of plexin-A4 in cells of...
myeloid lineage such as macrophages and DCs needs to be elucidated.

The innate immune system constitutes the first line of defense by rapidly detecting invading pathogens and nonmicrobial danger signals through the pattern recognition receptors. Several classes of pattern recognition receptors have been identified; the best-characterized are the Toll-like receptors (TLRs; Iwasaki and Medzhitov, 2004; Akira et al., 2006). TLR family members are localized either on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or in endosomal compartments (TLR3, TLR7, TLR8, and TLR9) to detect a multitude of pathogen-associated molecular patterns. TLR activation leads to the direct interactions of the TLR Toll–IL-1 receptor (TIR) domain with a cytoplasmic TIR–containing adaptive molecule such as MyD88 (myeloid differentiation primary response gene 88), TIR domain–containing adapter-inducing IFN-β (TRIF), TRIF-related adaptor molecule, or TIR domain–containing adapter protein. Activation of MyD88-dependent signaling pathway results in the activation of IL-1 receptor–associated kinases, the ubiquitin ligase TRAF6 (TNF receptor–associated factor 6), TAK1 (TGFβ-activated kinase 1) complex, NF-κB transcription factor, and mitogen-activated protein kinases (MAPKs). TRIF-dependent type I IFN requires a cascade involving the adaptor TRAF3, the kinase TBK1, the IKK-ε (inhibitor of κB kinase ε), and the transcription factor IRF3 (IFN-regulatory factor 3; Akira et al., 2006).

In this study, we demonstrate that plexin-A4 in macrophages is required for optimal cytokine production upon TLR stimulation and bacterial challenge. Plexin-A4 deficiency resulted in compromised activation of NF-κB, c-Jun N-terminal kinase (JNK), and a small guanosine triphosphate hydrolase (GTPase), Rac1 (ras-related C3 botulinum toxin substrate 1). The critical role of plexin-A4 in innate inflammatory responses was confirmed in models of septic shock and polymicrobial peritonitis. Sema3A (semaphorin 3A) serves as a ligand for Plexin-A4 and cytokine production in a plexin-A4–dependent manner. This study extends the role of plexins as regulators of the TLR pathway.

RESULTS
Plexin-A4 is expressed by myeloid and monocytes
Plexin-A4 is highly expressed in the nervous system and functions as an axon guidance factor during neuronal development (Yaron et al., 2005; Suto et al., 2007). To investigate the role of plexin-A4 in the immune system, we analyzed the Plxna4 messenger RNA (mRNA) expression level in different immune subpopulations. Cells of the lymphoid lineage (T, B, and NK cells) showed no detectable expression, whereas cells of the myeloid lineage, including macrophages and myeloid DCs (mDCs), expressed relatively high levels of the gene with the exception of plasmacytoid DCs (pDCs), which expressed a low level of the gene (Fig. 1 A). The highest level of Plxna4 mRNA was observed in peritoneal macrophages. Plexin-A4 protein was detected on the surface of WT but not of Plxna4−/− peritoneal macrophages, as determined by immunofluorescence staining (Fig. 1 B) and flow cytometry analysis (Fig. 1 C).

Because of the selective expression of plexin-A4 in myeloid cells, we sought to delineate the role of plexin-A4 in functions that are well studied in cells of this lineage. Previous work has shown that plexin-A1 on DCs mediates the activation of T cells (Wong et al., 2003; Takegahara et al., 2006). In contrast, plexin-A4 is not involved in DC antigen presentation because OVA323–339–specific OTII CD4+ T cell proliferation was comparable when stimulated with either WT or Plxna4−/− BM–derived DCs (BMDCs) that have been loaded with either OVA323–339 peptide or whole OVA protein (Fig. S1).

Next we evaluated the role of plexin-A4 in type I IFN production by pDCs. WT and Plxna4−/− pDCs isolated from the BM generated comparable amounts of IFN-α in response to stimulation with TLR7 (R837) or TLR9 (B-type CpG oligodeoxynucleotide [CpG-B]) agonists. This indicates that plexin-A4 does not play a role in the antigen-presenting function of mDCs or in type I IFN production by pDCs during TLR stimulation (Fig. S2).

Plexin-A4 is required for TNF and IL-6 production in macrophages
We next examined the role of plexin-A4 in cytokine production in response to TLR activation. Plxna4−/− and WT mice showed no difference in the percentage or absolute number of peritoneal macrophages, as determined by the analysis of a cyto- spin sample (Fig. S3, A–C). A variety of TLR agonists were used to stimulate naive peritoneal macrophages, including Pam3Cys (TLR2), polyinosinic-polyricidylic acid (poly(I:C); TLR3), LPS (TLR4), R837 (TLR7), and CpG-B (TLR9). During TLR stimulation with these diverse agonists, Plxna4−/− peritoneal macrophages generated a significantly lower amount of TNF and IL-6 mRNA and protein compared with similarly treated WT controls (Fig. 1, D and E). In addition to peritoneal macrophages, Plxna4−/− BM–derived macrophages (BMMs; Fig. S4 A) and BMDCs (Fig. S4 B) exhibited defective TNF and IL-6 production upon TLR stimulation when compared with their WT counterparts. Statistical analyses indicate significant differences between similarly treated WT and Plxna4−/− cells. These findings show that plexin-A4 is required for TLR–initiated proinflammatory cytokines.

We also tested the role of macrophage plexin-A4 in response to bacterial challenge. Both Gram–positive (Listeria monocytogenes and Staphylococcus aureus) and –negative (Escherichia coli and Salmonella typhimurium) bacteria were used to stimulate peritoneal macrophages. In all cases, bacteria–challenged Plxna4−/− macrophages produced significantly reduced levels of TNF and IL-6 mRNA and protein when compared with WT macrophages (Fig. 2, A and B). These data suggest that plexin-A4 is important in the bacteria-induced, inflammatory cytokine response.

Plexin-A4 mediates the activation of NF-κB and JNK but not ERK (extracellular signal–regulated kinase) or p38
NF-κB and MAPK signaling pathways control the production of inflammatory cytokines during macrophage activation.
in response to TLR agonists and microbial pathogens (Vallabhapurapu and Karin, 2009). The defective production of TNF and IL-6 by Plxna4−/− macrophages prompted us to investigate the function of plexin-A4 in these signaling pathways. After LPS stimulation, naive WT peritoneal macrophages exhibited enhanced phosphorylation of p65, JNK, p38, and ERK1/2 MAPKs as expected. In contrast, Plxna4−/− macrophages showed an abrogation of p65 phosphorylation and significantly reduced JNK phosphorylation but no change in ERK1/2 and p38 phosphorylation (Fig. 3 A). In accordance with defective p65 phosphorylation during LPS stimulation, Plxna4−/− macrophages showed a defect in IκB phosphorylation compared with WT macrophages (Fig. 3 A). As specificity controls, Plxna4−/− macrophages were not defective in response to TNF, anti-CD40, and IFN-γ treatment. The phosphorylation of MAPKs upon TNF or anti-CD40 treatment (Fig. 3 B) and of STAT1 upon IFN-γ treatment (Fig. 3 C) was not altered in Plxna4−/− macrophages. This indicates that plexin-A4 specifically mediates TLR activation. Additionally, Plxna4−/− peritoneal macrophages showed no defect in Akt phosphorylation in response to TLR activation by Pam3Cys and LPS stimulation (Fig. 3 D). These findings indicate that plexin-A4 deficiency leads to a TLR-specific defect in NF-κB and JNK activation. Furthermore, this defect is not caused by decreased TLR expression because the mRNA levels of Thr2, Thr3, Thr4, Thr7, or Thr9 were similar between WT and Plxna4−/− peritoneal macrophages (Fig. S5). The primer sets used in the RT-PCR analyses are described in Table S2.
Therefore, we examined whether plexin-A4 regulates the activation of small GTPases during TLR activation. A small GTPase pull-down assay was used in which the binding domain of the downstream effector of a particular GTPase was used to pull down the GTP-bound and thus activated form of that GTPase. LPS-stimulated WT macrophages displayed an increase in the GTP-bound form of Rac1 and Cdc42 when compared with unstimulated controls (Fig. 4 A). LPS-stimulated Plxna4−/− macrophages exhibited a dramatic reduction in the quantity of GTP-bound Rac1 when compared with WT cells, whereas GTP-bound Cdc42 and RhoA were indistinguishable. Thus, plexin-A4 affects the activation of Rac1, which is known to promote NF-κB activation and cytokine production in TLR-stimulated macrophages (Arbibe et al., 2000; Sanlioglu et al., 2001). In support of the importance of Rac1 in TLR-stimulated gene expression, a specific Rac1 inhibitor, NSC23766 (Gao et al., 2004), was used and found to significantly attenuate TNF and IL-6 production in WT peritoneal macrophages stimulated by various TLR agonists (Fig. 4 B). However, NSC23766 showed no inhibitory effect on cytokine production by TLR-activated Plxna4−/− macrophages. This is compatible with the conclusion that Rac1 activation is significantly attenuated in Plxna4−/− macrophages such that a Rac1 inhibitor has no further suppressive effect on these cells.

We next tested whether impaired NF-κB and JNK activation in Plxna4−/− macrophages causes defective binding of the downstream transcription factors, p65 and c-Jun, to the promoter regions of Tnfa and Il6. Chromatin immunoprecipitation (ChIP) assays were performed by using specific anti-p65 and anti-phospho-c-Jun antibodies. Appropriate primer sets were designed that flanked the well-defined binding sites of κB and c-Jun at the promoter regions of Tnfa and Il6 (Galvez et al., 2009; Leng et al., 2009). 1 h after stimulation with poly (I:C) or LPS (Fig. 3 E) or Pam3Cys or CpG (Fig. 3 F), WT macrophages showed enhanced binding of p65 and c-Jun at both Tnfa and Il6 promoters, which correlated with the increased expression of these inflammatory genes. Conversely, Plxna4−/− macrophages showed significantly less p65 and c-Jun binding at these promoters compared with similarly treated WT controls. These data show that plexin-A4 mediates TLR-induced NF-κB and JNK activation to cause the chromosomal activation of cytokine promoters.

Plexin-A4 causes the activation of the small GTPase Rac1 but not RhoA and Cdc42

Several members of the plexin family such as plexin-B1 (Swiercz et al., 2002) and plexin-A1 (Turner et al., 2004) mediate their downstream effects through the activation of the Rho family of small GTPases such as RhoA, Rac1, and Cdc42. More importantly, Rac1 serves an essential role in NF-κB activation and cytokine production in response to either TLR2 (Arbibe et al., 2000) or TLR4 (Sanlioglu et al., 2001) agonists. Therefore, we examined whether plexin-A4 regulates the activation of small GTPases during TLR activation. A small GTPase pull-down assay was used in which the binding domain of the downstream effector of a particular GTPase was used to pull down the GTP-bound and thus activated form of that GTPase. LPS-stimulated WT macrophages displayed an increase in the GTP-bound form of Rac1 and Cdc42 when compared with unstimulated controls (Fig. 4 A). LPS-stimulated Plxna4−/− macrophages exhibited a dramatic reduction in the quantity of GTP-bound Rac1 when compared with WT cells, whereas GTP-bound Cdc42 and RhoA were indistinguishable. Thus, plexin-A4 affects the activation of Rac1, which is known to promote NF-κB activation and cytokine production in TLR-stimulated macrophages (Arbibe et al., 2000; Sanlioglu et al., 2001). In support of the importance of Rac1 in TLR-stimulated gene expression, a specific Rac1 inhibitor, NSC23766 (Gao et al., 2004), was used and found to significantly attenuate TNF and IL-6 production in WT peritoneal macrophages stimulated by various TLR agonists (Fig. 4 B). However, NSC23766 showed no inhibitory effect on cytokine production by TLR-activated Plxna4−/− macrophages. This is compatible with the conclusion that Rac1 activation is significantly attenuated in Plxna4−/− macrophages such that a Rac1 inhibitor has no further suppressive effect on these cells.

Plexin-A4−/− mice exhibit reduced cytokine storm and are protected from lethal challenge with LPS and poly(I:C)

We next evaluated the physiological relevance of these findings. We first determined the number of immune cells in the Plxna4−/− mice. No apparent defect was detected for all immune cell types tested, including mDCs, pDCs, neutrophils, monocytes, macrophages, CD4+ or CD8+ T cells, B cells, and NK cells (Fig. S6 and Table S1). Thus, any physiological difference observed between WT and Plxna4−/− mice is not caused by a change in immune subpopulations.

Because Plxna4−/− macrophages showed decreased TNF and IL-6 production during TLR stimulation in vitro, we sought to determine the in vivo role of plexin-A4. Mice were injected intraperitoneally with either LPS (12.5 mg/kg
Figure 3. Plexin-A4 is required for LPS-induced activation of NF-κB and JNK but does not affect TNF, CD40, or PI3K–Akt signaling pathways. Peritoneal macrophages were isolated from WT and Plxna4−/− mice by peritoneal lavage. (A) Immunoblot analysis of NF-κB and MAPK signaling molecules was performed with WT and Plxna4−/− macrophages left untreated (0) or treated for 15, 30, or 60 min with 1 µg/ml ultrapure LPS. Densitometric analysis was performed to quantify the immunoblots based on three independent experiments (bar graphs). Results are expressed as mean ± SD. *, P < 0.05 compared with WT macrophages. (B) Macrophages were treated with 20 ng/ml recombinant mouse TNF or with 5 µg/ml anti-CD40 after 1 ng/ml TNF priming for 3 h. TNF priming is required to up-regulate CD40 expression (Lich et al., 2007). Immunoblotting was performed. (C) Macrophages were treated with either 5 µg/ml Pam3Cys or 1 µg/ml LPS. Akt phosphorylated at Ser473 was detected by immunoblotting. The results shown in B–D are representative of three independent experiments.

(E and F) ChIP assay of the binding of p65 or c-Jun at the promoter regions of Tnfa and Il6 gene in WT and Plxna4−/− peritoneal macrophages left untreated (−) or treated with 10 µg/ml poly(I:C), 1 µg/ml ultrapure LPS, 5 µg/ml Pam3Cys, or 4 µg/ml CpG for 1 h. The results shown in E and F are representative of three independent experiments and are expressed as mean ± SD. *, P < 0.05 compared with similarly treated WT peritoneal macrophages.
peritonitis model that closely replicates the nature and course of clinical sepsis (Hubbard et al., 2005). Acute sepsis results in a high mortality rate, which is primarily caused by the overzealous production of proinflammatory cytokines. WT mice with cecal punctures showed an 80% mortality rate as early as 3 d after CLP (Fig. 6 A). However, \textit{Plxna4}$^{-/-}$ mice were significantly protected from CLP-induced lethality in sepsis ($P = 0.0285$). Thus, the absence of plexin-A4 provided a clear survival benefit. We also examined inflammatory cytokine levels in the peritoneal lavage, serum, and lung homogenate before and after CLP-induced peritonitis. At 4 and 24 h after CLP, \textit{Plxna4}$^{-/-}$ mice produced significantly reduced levels of inflammatory cytokines such as TNF, IL-1$\beta$, IL-6, IL-12p70, CCL2, and CCL3 in the peritoneal lavage fluid (Fig. 6 B), serum (Fig. 6 C), and lung homogenate (Fig. 6 D) compared with WT mice. Therefore, plexin-A4 enhances the septic inflammatory response and promotes a cytokine storm in a peritonitis model.

Plexin-A4 is required for LPS-induced activation of small GTPase Rac1.

(A) WT and \textit{Plxna4}$^{-/-}$ peritoneal macrophages were left untreated (0) or treated for 15 or 30 min with 1$\mu$g/ml ultrapure LPS. GTP-bound Rac1 and Cdc42 were affinity precipitated using Rac1/Cdc42-binding domain of PAK fused with GST. GTP-bound RhoA was affinity precipitated using RhoA-binding domain of rhotekin fused with GST. Bound proteins were analyzed by immunoblotting. Total Rac1, Cdc42, and RhoA in cell lysate were used as loading controls. Densitometric analysis was performed to quantify the immunoblots based on three independent experiments (bar graphs). Results are expressed as mean $\pm$ SD. *, $P < 0.05$ compared with WT macrophages. (B) Peritoneal macrophages isolated from WT and \textit{Plxna4}$^{-/-}$ mice were pretreated with or without (medium) a Rac1 inhibitor, 200$\mu$M NSC23766 for 1 h, followed by stimulation with 5$\mu$g/ml Pam3Cys, 10$\mu$g/ml poly(I:C), 1$\mu$g/ml ultrapure LPS, 10$\mu$g/ml R837, or 4$\mu$g/ml CpG for 4 h. TNF and IL-6 were measured by ELISA. The results shown are representative of three independent experiments and are expressed as mean $\pm$ SD.

Plexin-A4 does not affect macrophage phagocytosis and bacteria killing

Another essential factor that contributes to host survival during acute sepsis is efficient phagocytosis and killing of invading bacteria (Matsukawa et al., 2000). The dramatic survival benefit in \textit{Plxna4}$^{-/-}$ mice prompted us to investigate the function of plexin-A4 in macrophage phagocytosis and bacteria killing. 24 h after CLP surgery, there were similar levels of bacterial loads in

Plxna4$^{-/-}$ mice are resistant to septic inflammation induced by cecal ligation and puncture (CLP)

To further evaluate the role of plexin-A4 during in vivo bacterial infection, we used a well-defined CLP-induced polymicrobial peritonitis model that closely replicates the nature and course of clinical sepsis (Hubbard et al., 2005). Acute sepsis results in a high mortality rate, which is primarily caused by the overzealous production of proinflammatory cytokines. WT mice with cecal punctures showed an 80% mortality rate as early as 3 d after CLP (Fig. 6 A). However, \textit{Plxna4}$^{-/-}$ mice were significantly protected from CLP-induced lethality in sepsis ($P = 0.0285$). Thus, the absence of plexin-A4 provided a clear survival benefit. We also examined inflammatory cytokine levels in the peritoneal lavage, serum, and, lung homogenate before and after CLP-induced peritonitis. At 4 and 24 h after CLP, \textit{Plxna4}$^{-/-}$ mice produced significantly reduced levels of inflammatory cytokines such as TNF, IL-1$\beta$, IL-6, IL-12p70, CCL2, and CCL3 in the peritoneal lavage fluid (Fig. 6 B), serum (Fig. 6 C), and lung homogenate (Fig. 6 D) compared with WT mice. Therefore, plexin-A4 enhances the septic inflammatory response and promotes a cytokine storm in a peritonitis model.

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the peritoneal lavage, blood, and lung homogenate from WT and \( \text{Plxna4}^{-/-} \) mice, indicating that Plexin-A4 is not required for bacterial clearance (Fig. 7 A). WT and \( \text{Plxna4}^{-/-} \) peritoneal macrophages also did not differ in the in vitro phagocytosis of GFP-expressing \( \text{E. coli} \) at different time points (Fig. 7, B and C) or multiplicities of infection (MOIs; 10 or 100; Fig. 7 D). Furthermore, there was no difference in the bacterial killing capacity of WT and \( \text{Plxna4}^{-/-} \) peritoneal macrophages, as similar numbers of \( \text{E. coli} \) were recovered from macrophages of both genotypes after the extracellular bacteria were killed by antibiotics and the \( \text{E. coli} \)-loaded macrophages were incubated for different time periods (Fig. 7 E). These data indicate that Plexin-A4 does not play a role in bacterial phagocytosis and killing in macrophages.

Sema3A enhances LPS-induced cytokine production in a Plexin-A4–dependent manner

Previous studies in the CNS have identified Sema3A and Sema6A as ligands for Plexin-A4 (Yaron et al., 2005;...
To assess whether these two molecules are plexin-A4 ligands in the immune system, we first analyzed Sema3a and Sema6a mRNA expression levels in immune sub-populations (Fig. S7). The highest level of Sema3a mRNA was observed in cells of the lymphoid lineage such as T, B, and NK cells. Myeloid and monocytic cells also expressed Sema3a but at a lower level. Sema6a mRNA was expressed at a relatively low level in all immune subpopulations that we analyzed. The level of Sema6a was nearly 100× lower than that of Sema3a when normalized to the Actb signal.

Figs. 1, 3, and 4 show that optimal LPS-stimulated cytokine production in macrophages requires intact plexin-A4 expression. One possible explanation for this observation is that LPS promotes the production and/or secretion of Sema3A and/or Sema6A in macrophages, which then engage plexin-A4 to activate macrophages through an autocrine loop. To examine this possibility, peritoneal macrophages were treated with either LPS (Fig. 8A) or other TLR agonists, including Pam3Cys, poly(I:C), R837, and CpG-B (Fig. S8). All TLR agonists resulted in significantly elevated Sema3a but not Sema6a mRNA levels. Furthermore, immunoblot analysis of the supernatant after 2 or 4 h of LPS treatment showed the induced appearance of proteolytic-processed Sema3A (65 kD; Fig. 8B), as observed by others (Adams et al., 1997; Catalano et al., 2006; Lepelletier et al., 2006). This suggests that TLR stimulation of macrophages can induce Sema3A expression, which might engage plexin-A4 in a signaling loop that amplifies inflammatory signals and promotes cytokine production.

To functionally assess whether Sema3A or Sema6A serves as a ligand for plexin-A4 in macrophages, WT or Plxna4−/− peritoneal macrophages were stimulated with increasing concentrations of Sema3A or Sema6A in the absence or presence of LPS. These ligands were expressed as chimeric proteins consisting of the human IgG1 Fc fragment fused to either
Sema3A or Sema6A. The IgG, Fc fragment alone was used as a negative control. Neither Sema3A nor Sema6A alone induced TNF and IL-6 production in macrophages. In the presence of a suboptimal concentration of LPS (50 ng/ml), both Tnfα and Il6 mRNA were elevated (Fig. 8 C). The addition of soluble Sema3A (Fig. 8 C, left, open bars) enhanced LPS-induced Tnfα and Il6 mRNA in WT peritoneal macrophages in a dose-dependent manner. In contrast, Sema6A (Fig. 8 C, right, open bars) or IgG Fc control had no effect. This enhancement of LPS-responsiveness was absent in Pkxa4−/− macrophages (Fig. 8 C, closed bars). A similar pattern was detected for TNF and IL-6 proteins (Fig. 8 D). The absence of a functional effect of Sema6A agrees with its low expression in different immune populations (Fig. S7, right) and the failure of LPS to induce its expression (Fig. 8 A). These data indicate that Sema3A engagement enhances cytokine production by macrophages but only when LPS is present. This suggests the possibility that both LPS and Sema3A are required for optimal intracellular signaling and eventual cytokine production in macrophages.

To delineate the separate contribution of LPS and Sema3A to macrophage signaling, we tested the responses of WT, Pkxa4−/−, and Myd88−/− macrophages to LPS alone, Sema3A alone, or LPS + Sema3A. MyD88 was used as a surrogate for TLR because it is the common adaptor of multiple TLRs. We used Rac1 activation as a measurement of macrophage activation because it represents a proximal response in the plexin pathway and it is also required during TLR activation. WT macrophages stimulated by LPS or Sema3A alone showed elevated GTP-bound Rac1 when compared with unstimulated cells, whereas the two stimuli together caused the highest level of activated Rac1 (Fig. 8 E). In contrast, Pkxa4−/− macrophages stimulated by LPS or Sema3A did not exhibit elevated GTP-Rac1 levels. This suggests that plexin-A4 is not only required for signals that emanate from the binding of Sema3A but also required for LPS engagement of TLR. Similarly, neither Sema3A nor LPS increased GTP-Rac1 in Myd88−/− macrophages. These findings indicate that the dual engagement of TLR and plexin-A4 by their respective ligands is required for optimal macrophage signaling. In composite, these data reveal a complex interplay between TLR and plexin-A4 signaling. LPS engagement of TLR induces Sema3A expression to cause autocrine activation of plexin-A4. However, LPS activation also requires the engagement of plexin-A4 by Sema3A for optimal intracellular signaling and vice versa.

The aforementioned experiments were performed in cell culture with highly enriched macrophage preparations; thus, it was important to assess the physiological relevance of these findings. To address this, we assessed whether Sema3A engagement of plexin-A4 could synergistically exacerbate CLP-induced septic inflammation. A single peritoneal injection of Sema3A or control IgG Fc at a dosage of 25 µg/kg body weight was administrated to WT and Pkxa4−/− mice 1 h before CLP procedure. We measured cytokine production in the peritoneal lavage 4 h after CLP. Sema3A-pretreated mice had significantly higher levels of inflammatory cytokines than IgG Fc–treated controls in WT mice but not in Pkxa4−/− mice (Fig. 8 F). Collectively, these in vitro and in vivo results suggest that plexin-A4 binding by Sema3A synergizes with TLR engagement by its agonists to amplify innate inflammatory responses. Strategies to reduce Sema3A engagement of plexin-A4 should be beneficial in controlling an adverse inflammatory response associated with endotoxin shock or sepsis.

**DISCUSSION**

The multimember TLR family was first discovered in *Drosophila melanogaster* and shown to play an important role in host defense to fungal pathogens (Lemaitre et al., 1996). Since then, it is widely recognized that members of this large family are key regulators and receptors that recognize pathogen products ranging from peptides, sugars, and lipids to nucleic acid moieties to initiate innate immunity and inflammatory responses. In parallel, the plexin family was initially discovered as important guidance molecules in the invertebrate CNS but now has expanded its impact to the vasculature, cardiac development, angiogenesis, cancer growth, and immunity (Gu et al., 2003a; Toyoftuku et al., 2004b; Neufeld and Kessler, 2008; Suzuki et al., 2008). In the immune system, several plexin members are found to be involved in cell–cell interaction. For example, plexin-A1 on DCs significantly affects the ability of DCs to activate T lymphocytes (Wong et al., 2003; Takegahara et al., 2006), whereas plexin-C1 on DCs has a slight effect in the same pathway (Walzer et al., 2005). Plexin-B1 on T cells interacts with CD100 expressed by B cells, causing B cell activation (Granziero et al., 2003). However, none of the plexin molecules have been shown to regulate TLR signaling. We now show the novel intersection of the plexin-A4 and TLR pathways through the intracellular activation of Rac1, JNK, and NF-κB. This intersection results in chromosomal changes at the promoters of proinflammatory genes, followed by a pancytokine inflammatory response in response to TLR stimulation and bacterial challenge. It cumulates in a cytokine storm that precipitates endotoxin shock induced by TLR agonists and sepsis induced by bacterial infection. Furthermore, the plexin-A4 ligand Sema3A together with endotoxin can synergistically induce cytokine production in a plexin-A4– and Myd88–dependent manner. Finally, TLR engagement can induce Sema3A expression, thus completing an autocrine loop. Collectively, these data reveal a pivotal role of plexin-A4 in TLR-induced macrophage cytokine production through activation of Rac-1, NF-κB, and JNK. This study significantly expands the repertoire of functions mediated by plexins to include TLR activation.

Plexin’s engagement can result in strong signal transduction within the cells (Tran et al., 2007). The cytoplasmic domains of plexins have two highly conserved regions that share homology with GTPase-activating proteins that are known to stimulate the intrinsic GTPase activities of G proteins and thus regulate the Ras superfamily of small GTPases. This structural similarity suggests that plexins might signal through the Ras family of GTPases. Indeed, plexin-B1 exhibits GTPase-activating protein activity for R-Ras, which is essential for Sema4D-mediated repulsive axon guidance signaling (Oinuma et al., 2004).
Figure 8. Sema3A enhances LPS-induced cytokine production in a plexin-A4–dependent manner. (A and B) WT peritoneal macrophages were left unstimulated (−) or were stimulated with 1 µg/ml LPS for 2 or 4 h. Sema3a and Sema6a mRNA (A) and Sema3A protein (B) were analyzed by RT-PCR and immunoblotting, respectively. *, P < 0.05 compared with unstimulated macrophages. (C and D) WT and Plxna4−/− peritoneal macrophages were stimulated or not with 50 ng/ml LPS for 4 h in the absence or presence of either Sema3A-Fc or Sema6A-Fc fusion proteins (20 or 100 ng/ml). TNF and IL-6 mRNA (C) and protein (D) were measured by RT-PCR and ELISA, respectively. 100 ng/ml of the IgG Fc fragment was used as negative control. *, P < 0.05 compared with unstimulated macrophages. (E) Peritoneal macrophages isolated from WT, Plxna4−/−, and Myd88−/− mice were stimulated with 1 µg/ml LPS alone, 100 ng/ml Sema3A-Fc alone, or both LPS and Sema3A-Fc for 30 min. Rac1 pull-down assay was performed. Total Rac1 in cell lysate was used as control. Densitometric analysis was performed to quantify the immunoblots based on two independent experiments (bar graphs). *, P < 0.05 compared with similarly treated WT macrophages. (F) WT and Plxna4−/− mice (n = 4 per group per experiment) were pretreated with either Sema3A-Fc or IgG Fc control proteins at a dosage of 25 µg/kg body weight 1 h before CLP procedure. Inflammatory cytokines in peritoneal lavage collected 4 h after CLP were determined by ELISA. *, P < 0.05 compared with peritoneal lavage from WT mice pretreated with Sema3A-Fc. The results shown in A–F are representative of two independent experiments and are expressed as mean ± SD.
In addition, the Rho family of small GTPases, which is a subgroup of Ras superfamily, has been tightly linked to plexin-mediated signaling (Tran et al., 2007). Plexin-A1 interacts with and depends on Rac1 for its activity in the nervous system (Turner et al., 2004). Plexin-B1 activates RhoA and mediates Sema4D-induced axonal growth cone collapse (Swierzcz et al., 2002). Therefore, the Rho family of small GTPases plays an important role in plexin-mediated signal transduction.

The Rho family of small GTPases has also been linked to many aspects of the immune system, including hematopoietic stem cell regulation (Gu et al., 2003b) and B cell development and signaling (Walmsley et al., 2003), as well as DC-induced T cell activation (Benvenuti et al., 2004). Importantly, Rac1, a well-studied member of the Rho subfamily, has been linked to NF-κB activation and cytokine production in response to either TLR2 (Arbibe et al., 2000) or TLR4 (Sanlioglu et al., 2001) agonists. The finding that plexins can cause the activation of Rho GTPases prompted us to investigate whether plexin-A4 imposed its effects on NF-κB activation through Rho GTPases. TLR stimulation can cause an increase in GTP-bound (activated) Rac1 and Cdc42 in macrophages, which is then necessary for the activation of NF-κB and the production of downstream cytokines. Our results show that plexin-A4 deficiency abolished LPS-induced Rac1 activation, whereas Cdc42 activation was left intact. Thus, Rac1 serves as a signal transducer downstream of plexin-A4 and modulates NF-κB and JNK activation in macrophages through TLRs. The role of Rac in TLR activation was verified by the use of a pharmacologic Rac1 inhibitor that dampened TLR-induced TNF and IL-6 production in macrophages.

Whereas it is generally believed that TLR engagement by TLR agonists is able to fully activate immune cells, our study identifies another requirement during this process involving the engagement of membrane plexin-A4 by Sema3A. During TLR activation, plexin-A4 binds to its soluble ligand, Sema3A, which triggers Rac1, leading to the activation of NF-κB and JNK. In the absence of TLR stimulation, plexin-A4 activation by Sema3A failed to induce cytokine production, indicating that plexin-A4–mediated signal is not sufficient for the induction of cytokine production. In the absence of plexin-A4, TLR signaling is significantly attenuated but not abolished, indicating that TLR signaling is amplified by plexin-A4 activation but is not initiated by this activation.

A previous study has shown an inhibitory role of plexin-A4 in mouse T cell activation (Yamamoto et al., 2008), and Sema3A has been reported to suppress human T cell proliferation (Lepelletier et al., 2006). The opposite roles of the Sema3A–plexin-A4 signaling axis in innate versus adaptive immune response mimic other signaling systems such as TRAF3, which serves as an inhibitor in lymphocytes but an activator in macrophages (He et al., 2007). Our findings show that although plexin-A4 is most highly expressed by monocyte and myeloid cell lineages and significantly reduced on lymphoid cells, Sema3A is highly expressed by lymphoid cells with reduced expression by myeloid/monocytic cell types. Thus, it is possible that during an early inflammatory response, the smaller amount of Sema3A produced by myeloid and monocytic cells together with TLR ligands is sufficient to activate the higher density of plexin-A4 on macrophage membrane. Later when T cells are recruited, the higher amounts of Sema3A expressed by activated T cells then serve as a feedback loop to down-regulate T cell functions, which express less plexin-A4 receptors. In addition to these issues, neuropilin, the ligand–binding subunit of Sema3A receptor complex in the cardiovascular and nervous system (Gu et al., 2003a), may also participate in Sema3A–plexin-A4–mediated immune functions. Future investigation is required to assess its role in Sema3A–mediated suppression and activation of T cells and macrophages, respectively.

Our finding shows that Sema6A does not affect inflammatory cytokine production in macrophages. There are several ways to explain why a bona fide ligand such as Sema6A engages plexin-A4 in the nervous system but not macrophages. First, plexins identified in one cell type/organ do not always functionally interact with the same semaphorins in different cells/tissues. For example, Sema3A interacts with plexin-A1 in the nervous system but with Sema6D during cardiac morphogenesis (Toyofuku et al., 2004a). Second, immunostimulation may only induce the expression of some semaphorins but not others. Our data show that LPS induces the production of Sema3A but not Sema6A by macrophages. The induced Sema3A synthesis by macrophages can act in an autocrine fashion via plexin-A4 to promote inflammatory cytokine production. Third, different semaphorins mediate different immunological functions. Our findings suggest that Sema3A but not Sema6A promotes LPS-induced cytokine production by macrophages in a plexin-A4–dependent manner. However, we cannot rule out the possibility that Sema6A may be required for other immune functions such as cell migration that depend on plexin-A4. For example, the binding of DC–derived plexin-A1 and T cell–derived Sema6D is important during DC–stimulated T cell activation (Takegahara et al., 2006; O’Connor et al., 2008), whereas plexin-A1–dependent entry of DCs into lymphatics depends on Sema3A but not Sema6D (Takamatsu et al., 2010). Finally, plexin may form a signaling complex with other molecules such as neuropilin or DAP12 to mediate divergent semaphorin-initiated signal transduction.

In summary, the biological function of plexin-A4 in the activation of a pan-cytokine response has broad clinical implications. New antiinflammatory therapeutic methods are needed in the treatment of immune–related diseases such as sepsis, rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus. The hallmark of sepsis in the acute phase is an exacerbated production of proinflammatory cytokines and chemokines, leading to the cytokine storm. Although these inflammatory mediators are essential in providing an immediate host defense, their overzealous production can be deleterious to the host if left uncontrolled. Considering that sepsis is a leading cause of death worldwide that has little effective treatment, there is an urgent need for new therapies targeting this disease. Our study suggests a critical role of plexin-A4 in mediating the production of proinflammatory cytokines.
which affects host survival in models of sepsis. Therefore, the interaction of plexin-A4 and Sema3A presents a new therapeuetic target for antiinflammatory and antisepsis treatment.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. Plxna4−/− mice were provided by M. Tessler-Lavigne (Stanford University, Stanford, CA; Yaron et al., 2005) and were backcrossed for nine generations onto the C57BL/6 background. Myd88−/− mice were provided by S. Akira (Osaka University, Suita, Osaka, Japan). OTII mice were obtained from M. Croft (La Jolla Institute of Allergy and Immunology, La Jolla, CA). Mice were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill approved all experimental procedures.

Reagents. Pam3Cys, poly(l-C), ultrapure LPS, imiquimod (R837), and mouse CpG-B (ODN 1826) were purchased from InvivoGen. LPS (E. coli 0111:B4) used for in vivo injections was purchased from Sigma-Aldrich. Recombinant human Sema3A-Fc, human Sema6A-Fc, and human IgG1 Fc (C-14), Cdc42 (B-8), and RhoA (26C4) were purchased from Santa Cruz Biotechnology, Inc. Antibody specific for mouse plexin-A4 has been previously described (Sato et al., 2007) and was provided by F. Suto (Tohoku University, Aoba-ku, Sendai, Japan). In ChIP analysis, the antibodies specific for NF-κB p65 (A) and c-Jun phosphorylated at Ser63 were purchased from Santa Cruz Biotechnology, Inc. and Cell Signaling Technology, respectively. In assays for GTP-bound Rac1, Cdc42, and RhoA, glutathione-Septarose beads conjugated with either GST-Pak1 PBD (for GTP-bound Rac1 and Cdc42) or GST-rhotekin RBD (for GTP-bound RhoA) were provided by K. Burridge and L. Sharkey of K. Burridge’s laboratory (University of North Carolina at Chapel Hill, Chapel Hill, NC).

Peritoneal macrophage isolation and in vitro stimulation. Total peritoneal cells were harvested from the peritoneal cavities of naive WT or Plxna4−/− mice with two 10-ml cold sterile PBS washes. Lavage was pooled from mice in the same group. RBCs were lysed in ammonium chloride buffer (150 mM NH4Cl, 10 mM NaHCO3, and 1 mM EDTA-tetrasodium). Cyto- spins were prepared and stained with Diff-Quick solutions, and the number of peritoneal macrophages was determined and cultured in RPMI 1640 supplemented with 10% FCS, 2 mM l-Gln, 100 U/ml penicillin, and 100 µg streptomycin. Macrophages were plated in 96-well cell culture plate (2 × 105/well) and incubated for 2 h at 37°C. Nonadherent cells were removed, and adherent cells were washed with complete RPMI 1640 medium, followed by stimulation with various reagents. In some experiments, cells were pretreated with 200 mM NSC23766 for 1 h, followed by stimulation with TLR agonists.

Bacterial infection in vitro. L. monocytogenes (43251) and S. typhimurium (14028) were obtained from the American Type Culture Collection. E. coli strain LF82 was provided by R. Balfour Sartor (University of North Carolina at Chapel Hill). S. aureus strain R6N390 has been previously described (Labeauzera-Rey et al., 2007) and was provided by M. Gabriela Bowden (Texas A&M University, College Station, TX). All bacterial strains were grown to the late exponential phase. The number of viable bacteria used in each experiment was calibrated by colony counting. Bacteria were added to peritoneal macrophages at an MOI of 40 for 1 h at 37°C, and then cells were treated with 100 µg/ml gentamicin to kill extracellular bacteria and were further incubated for various time periods (S. typhimurium for 1 h; L. monocytogenes, E. coli, and S. aureus for 3 h).

ELISA. Cytokines generated by in vitro cultured peritoneal macrophages were quantified using the ELISA set for mouse TNF-α and IL-6 (BD) and mouse IFN-β ELISA kit (PBL InterferonSource). For the in vivo experiments, concentrations of TNF-α, IL-1β, IL-6, IL-12p70, CCL2, and CCL3 were measured in cell-free peritoneal lavage fluid, serum, and lung homogenate using a customized mouse cytokine 6-plex panel (BioLegend) and a Luminex Bio-Plex 200 system (Bio-Rad Laboratories).

Immunoblotting. Electrophoresis of proteins was performed by using the NuPAGE system (Invitrogen) according to the manufacturer’s protocol. In brief, peritoneal macrophages stimulated with LPS or left unstimulated were collected and lysed with buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, and protease inhibitor cocktail). Proteins were separated on a NuPAGE gel and were transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 10% milk proteins in 1× PBS and 0.1% Tween 20 and then probed with the primary antibodies. Appropriate horseradish peroxidase-conjugated secondary antibodies were used, and proteins were detected using the enhanced chemiluminescent reagent (Thermo Fisher Scientific).

ChIP assay. Resident peritoneal macrophages were stimulated with various TLR agonists for 1 h, and the ChIP assay was performed as described previously (Wen et al., 2008). Real-time PCR was performed using the primers described in Table S3.

Detection of GTP-bound Rac1, Cdc42, and RhoA. The assays for GTP-bound Rac1, Cdc42, and RhoA were performed as described previously (Noren et al., 2000). In brief, peritoneal macrophages were stimulated with 1 µg/ml LPS, 100 ng/ml Sema3A-Fc alone, or both. Cells were lysed, and GTP-bound Rac1 and Cdc42 were affinity precipitated using Rac1/Cdc42-binding domain of PAK (PBD) fused with GST. GTP-bound RhoA was affinity precipitated using RhoA-binding domain of rhokin (RB-D) fused with GST. Bound proteins were resolved on a NuPAGE gel and were transferred onto nitrocellulose membranes. Membranes were immunoblotted with the appropriate primary antibodies against Rac1, Cdc42, or RhoA.

Experimental sepsis induced by CLP. CLP surgery was performed on mice as previously described with minor modifications (Wen et al., 2008). In brief, mice were anesthetized with an intraperitoneal injection of 8 mg of 2,2,2-trichloroethanol (Avertin; Sigma-Aldrich). Under sterile surgical conditions, a 1-cm midline incision was made to the ventral surface of the abdomen, and the cecum was exposed. The cecum was partially ligated at its base with a 3.0 silk suture and punctured two times with a 21-gauge needle. Sham-operated mice were subjected to a similar laparotomy without ligation and puncture. Serum and cell-free peritoneal lavage fluid were collected for colony counting. Bacteria were added to peritoneal macrophages at an MOI of 25 µg/kg 1 h before CLP procedure.

Statistical analysis. Statistical analysis was performed with Prism 4 (GraphPad Software, Inc.) for Macintosh. In survival experiments, a log-rank test was used to test for significance. For all other experiments, results were presented as the mean ± SD, and the unpaired Student’s t test (one tailed) was applied to evaluate significance. P-values <0.05 were considered statistically significant.

Online supplemental material. Fig. S1 shows that plexin-A4 does not affect TLR-induced IFN-β production by BMDCs. Fig. S2 shows that plexin-A4 does not affect TLR-induced TNF-α production by pDCs. Fig. S3 shows that plexin-A4 does not affect the number of peritoneal macrophages. Fig. S4 shows that plexin-A4 is required for TLR-induced cytokine production
in BMMs and BMDCs. Fig. S5 shows that plexin-A4 does not affect TLR mRNA expression in peritoneal macrophages. Fig. S6 shows that plexin-A4 does not affect immune cell composition in the spleen. Fig. S7 shows the expression of Sema3a and Sema6a mRNA in different immune cells. Fig. S8 shows that TLR agonists induce an increase in Sema3a mRNA level in peritoneal macrophages. Table S1 shows the quantification of different immune cell populations in the spleens from seven WT and six Phox−/− naive mice. Table S2 shows the sequences of RT-PCR primers. Table S3 shows the sequences of primers for the CHIP assay. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101138/DC1.

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