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Lipopolysaccharide-induced binding of receptor CD300b to Toll-like receptor 4 alters signaling to drive lethal cytokine responses that enhance septic shock

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SUMMARY

Receptor CD300b is implicated in regulating the immune response to bacterial infection by an unknown mechanism. Here, we identified CD300b as an lipopolysaccharide (LPS)-binding receptor and determined the mechanism underlying CD300b augmentation of septic shock. *In vivo* depletion and adoptive transfer studies identified CD300b-expressing macrophages as the key cell type augmenting sepsis. We showed that CD300b, and its adaptor DAP12, associated with Toll-like receptor 4 (TLR4) upon LPS binding, thereby enhancing TLR4-adaptor MyD88- and TRIF-dependent signaling that resulted in an elevated pro-inflammatory cytokine storm. LPS engagement of the CD300b-TLR4 complex led to the recruitment and activation of spleen tyrosine kinase (Syk) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). This resulted in an inhibition of the ERK1/2 protein kinase- and NFκB transcription factor-mediated signaling pathways, which subsequently led to a reduced interleukin 10 (IL-10) production. Collectively, our data describe a mechanism of TLR4 signaling regulated by CD300b in myeloid cells in response to LPS.

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AUTHORS CONTRIBUTIONS

O.H.V., Y.M., K.K., D.H.M. and J.E.C. planned the research, analyzed and interpreted data; O.H.V., Y.M., M.P., H.N.L., and L.T. did the experiments; O.H.V., and C.F.Q. analyzed and interpreted histological data; J.S. and P.Y. helped with the CLP experiments; O.H.V., K.K. and J.E.C. wrote the manuscript; all authors participated in editing the manuscript.

INTRODUCTION

The innate immune system is the first line of host defense against invading pathogens (Iwasaki and Medzhitov, 2015). Lipopolysaccharide (LPS), present in gram-negative bacteria membranes, causes strong immune responses following detection by Toll-like receptor 4 (TLR4) on immune cells (Iwasaki and Medzhitov, 2015). Activation of immune cells, including macrophages (Macs) and dendritic cells (DCs), results in the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and IL-12, and clearance of infectious organisms. Concordantly, IL-10, an anti-inflammatory cytokine, is induced to limit the immune response thereby minimizing host tissue damage (Saraiva and O'Garra, 2010). Excess immune cell activation leads to a more severe immunopathology, such as septic shock and, subsequently, death (Hotchkiss et al., 2013; Iwasaki and Medzhitov, 2015).

Commonly, TLR4-dependent LPS recognition is initiated by LPS binding to CD14 (Wright et al., 1990) with subsequent transfer to the TLR4 and lymphocyte antigen 96 (MD-2) complex (Shimazu et al., 1999). This leads to activation of intracellular signaling pathways mediated by the major TLR-adaptor proteins MyD88 and TRIF (Iwasaki and Medzhitov, 2015). These adaptor molecules promote signaling via the p38, Jun, ERK1/2 and TBK1-IKKε protein kinase cascades leading to the activation of transcription factors such as NFκB, AP-1 and IRF3, which promote the expression of cytokine-encoding genes. Other immune receptors, such as TREM1 (Bouchon et al., 2001), TREM2 (Turnbull et al., 2006), CD209 (Nagaoka et al., 2005), CD11b (Ling et al., 2014), human (h)CD300a (Nakahashi-Oda et al., 2012), mouse (m)CLM4 (Totsuka et al., 2014), and mCD300b (CLM7) (Yamanishi et al., 2012) have been reported to modulate the innate immune response to LPS-associated bacterial infections. Yet the precise mechanism(s) for this regulation remain to be elucidated.

The CD300 receptor family is composed of type I transmembrane proteins with a single immunoglobulin V (IgV)-like extracellular domain that can transmit either activating or inhibitory signals (Borrego, 2013). The orthologous mouse family has a variety of names, including CMRF-like molecules (CLM) (Borrego, 2013), but for simplicity in this report we use the human nomenclature for both species. CD300b predominantly expressed on myeloid cells, contains a short intracellular tail and gains activation potential by association with the immunoreceptor tyrosine-based activating motif (ITAM)-bearing adaptor molecule, DAP12 (Yamanishi et al., 2008). We have previously shown that CD300b functions as an activating receptor by recognizing phosphatidylserine (PS) to promote the phagocytosis of apoptotic cells (AC) via the DAP12 signaling pathway (Murakami et al., 2014). In addition, antibody (Ab) cross-linking of CD300b promotes the release of inflammatory cytokines from mast cells (Yamanishi et al., 2008) and *Cd300lb*$^{-/-}$ mice are less prone to LPS-induced lethal inflammation than wild-type (*WT*) mice (Yamanishi et al., 2012), suggesting that CD300b regulates inflammatory responses by as yet to be determined mechanism.

Here, we identified LPS as a ligand for CD300b and showed that CD300b expression enhanced endotoxemic- and peritonitis-induced lethality, which correlated with an increased pro-inflammatory cytokine response (TNFα and IFNγ) and reduced levels of IL-10. We

identified Macs and their reduced IL-10 production as primary causes of this enhanced disease susceptibility. We showed that LPS binding induced the interaction of CD300b with TLR4 and CD14, resulting in the recruitment of DAP12, and the protein kinases Syk and PI3K to the receptor complex in Macs. In turn, Syk-PI3K kinase activation led to the dissociation of TLR4-adaptor molecules MyD88-TIRAP from the complex and promoted the phosphorylation of the AKT protein kinase, leading to a limited production of IL-10 via AKT-mediated inhibition of the ERK1/2 and NFκB signaling cascades. Furthermore, CD300b activated the TLR4-TRIF-IRF3 pathway, resulting in enhanced IFN-β production. Collectively, these findings described an LPS-induced signaling complex consisting of CD300b-DAP12-TLR4-Syk-PI3K that effectively amplified both the TLR4-MyD88- and TLR4-CD14-TRIF-mediated inflammatory responses, which led to an increased mortality from sepsis. These data identified CD300b and DAP12 as important molecules regulating the TLR4 pathway, and provides novel targets for clinical intervention to regulate LPS-induced TLR4 signaling.

RESULTS

CD300b binds LPS *in vitro*

To study the role of CD300b in LPS-induced lethal inflammation, we first determined if CD300b can bind LPS. Using surface plasmon resonance (SPR), we found that both mouse and human CD300b-Fcγ proteins bound to LPS (Figures 1A and 1B). Other CD300 family members, mCD300f-Fcγ, mCD300a-Fcγ, and mCD300d-Fcγ, or the control protein, NITR-Fcγ, failed to bind LPS (Figure 1C). Similarly, pull-down assays demonstrated that mCD300b-Fcγ protein, but not other related proteins, including those that contained the same Fcγ-domain, co-immunoprecipitated with LPS (Figure 1D). We next measured the kinetics of LPS interaction with monomeric mCD300b protein, the well-known LPS-sensor mCD14, and a control protein, mLAIR1. The dissociation constants (K_D) for the binding of LPS to mCD300b and mCD14 were 6.39 x 10^{-6} M and 8.5 x 10^{-7} M, respectively (Figures S1A and S1B). No binding to mLAIR1 was observed (Figure S1C). To examine the nature of the interaction of LPS with CD300b, we compared the inhibition of mCD300b binding to immobilized LPS (serotype B111:B4), after pre-incubating mCD300b with three of the most common *Escherichia coli* (*E.coli*)-LPS serotypes (B55:B5, B111:B4 and B127:B8) used to study LPS-induced endotoxemia. Unlike mCD14 binding preferences (Gangloff et al., 2005), we found each of these serotypes to be equivalent in inhibition of mCD300b binding, suggesting that the variable repeating oligosaccharide domain (O-Ag) is not a major determinant in the interaction with mCD300b (Figures S1D–S1F and S1J). Two LPS chemotypes (Ra-LPS, Re-LPS), consisting mainly of lipid A and residual carbohydrates, more potently inhibited LPS binding to mCD300b (Figures S1G, S1H and S1J); moreover, lipid A, consisting only of two phosphorylated glucosamine residues and its long chain fatty acids, almost completely inhibited the LPS-mCD300b interaction (Figures S1I and S1J), indicating that lipid A was the major determinant for interacting with mCD300b.

To determine if CD300b-expressing cells recognized LPS, we generated mCD300b-, mCD300b-DAP12-, mCD300d-FcRγ-, mCD300f-, and EV-expressing L929 cell lines and incubated them with fluorescein isothiocyanate (FITC)-labeled LPS derived from *E. coli* or

Salmonella minnesota (*S. minnesota*); DAP12 (Yamanishi et al., 2008) and FcRγ (Izawa et al., 2007) were previously established as signaling adaptors for mCD300b and mCD300d, respectively. We found that only cells expressing mCD300b or mCD300b-DAP12 bound LPS (0.5 – 1 h; Figures 1E and 1F). The LPS binding correlated with the level of CD300b surface expression; DAP12 was not required for CD300b surface expression or LPS binding (Figures S2A–S2C). Next, we assessed the specificity of LPS recognition by testing additional TLR or nucleotide-binding oligomerization domain receptor (NOD) ligands, and found that mCD300b did not recognize ligands for TLR2 (PAM3CSK4), TLR3 (Poly(I:C)), or NOD2 (MDP), further indicating that CD300b binding to LPS was specific (Figures S2D and S2E). Competition experiments, utilizing unlabeled LPS from either *E. coli* or *S. minnesota*, showed a concentration-dependent inhibition of FITC-labeled LPS binding to mCD300b-DAP12-expressing L929 cells; no inhibition was observed for cells treated with the TLR2 ligand, Zymosan A (Figure 1G). Given that LPS is found in bacterial membranes, we next tested if CD300b recognized bacteria. We incubated FITC-labeled *E. coli* with L929 cell lines expressing mCD300b, mCD300b-DAP12, mCD300d-FcRγ, mCD300f, or EV, and found that only those expressing mCD300b bound FITC-labeled *E. coli* (Figures S2F and S2G). These findings demonstrate that LPS was a ligand for CD300b.

CD300b exacerbates the pathogenesis of lethal endotoxemia and septic peritonitis

As CD300b bound LPS *in vitro*, we next assessed the importance of the CD300b-LPS interaction *in vivo*, by studying its role in LPS-induced inflammation. Administration of a toxic dose of LPS resulted in 100% mortality in *WT* mice by 65 h (Figure 2A). In contrast, only about 50% of *Cd300lb*$^{-/-}$ mice had succumbed by the 168 h end point, suggesting that CD300b contributes to LPS-induced mortality (Figure 2A), in agreement with a previous report (Yamanishi et al., 2012). Histopathologic analyses of the lungs from LPS-treated animals revealed that, in comparison to *Cd300lb*$^{-/-}$ mice, *WT* animals displayed a more severe inflammatory state associated with a greater influx of inflammatory cells, increased alveolar and interstitial edema, greater alveolar-capillary membrane thickening, and more hemorrhages (Figure 2B). *Cd300lb*-deficiency did not completely protect mice from inflammatory damage, as shown in tissues from PBS-treated mice (Figures 2B and S2H). Analyses of serum cytokine concentrations revealed that *WT* mice had increased amounts of TNFα and IFNγ, and somewhat higher concentrations of IL-6, IL-12, and the MCP-1 chemokine compared to sera from *Cd300lb*$^{-/-}$ mice (Figure 2C). Moreover, sera from *Cd300lb*$^{-/-}$ mice had higher concentrations of IL-10, suggesting that CD300b augments the lethal effect of endotoxemia, at least in part, by inhibiting IL-10 production (Figure 2C).

Although lethal endotoxemia reflects several aspects of sepsis, it does not recapitulate the replication and dissemination of bacteria. To assess a potential protective function of CD300b in sepsis, we subjected *WT* and *Cd300lb*$^{-/-}$ mice to cecal ligation puncture (CLP). We found that *WT* mice were more susceptible to CLP-induced mortality than *Cd300lb*$^{-/-}$ mice (Figure 2D). Furthermore, lung tissue samples from CLP-treated *WT* mice displayed more extensive histopathological changes than seen in tissues of *Cd300lb*$^{-/-}$ mice (Figure 2E). Also, *Cd300lb*-deficiency did not completely protect mice from inflammatory damage as shown in tissues from sham-treated mice (Figures 2E and S2I). The changes seen in lungs of *WT* and *Cd300lb*$^{-/-}$ mice with CLP were quite similar to those seen in LPS-treated mice

of both genotypes (Figures 2B and 2E). Assessment of the bacterial load in the blood, peritoneal cavity, lung, spleen and liver after CLP showed a higher level of bacterial burden in *WT* mice than in *Cd300lb*$^{-/-}$ animals (Figures S2J–S2N). Concentrations of TNFα, IFNγ and IL-12 in sera of *Cd300lb*$^{-/-}$ mice were lower (at most time points) than amounts for *WT* animals, while concentrations of IL-10 were higher (Figure 2F). Thus, *Cd300lb*$^{-/-}$ mice treated with LPS or CLP were characterized by having reduced mortality, decreased bacterial burden, reduced pro-inflammatory cytokine concentrations and higher IL-10 levels in comparison to *WT* mice (Figures 2 and S2), indicating that CD300b exacerbates the pathogenesis of lethal endotoxemia and septic peritonitis.

CD300b amplifies lethal endotoxemia by dampening IL-10 production

Our results suggested that CD300b amplified inflammatory responses triggered by bacterial infection via IL-10 inhibition. To determine if IL-10 was involved in regulating CD300b-induced inflammatory responses, we injected anti-IL-10 or a control Ab into *WT* and *Cd300lb*$^{-/-}$ mice 2 h before treatment with LPS. IL-10 neutralization diminished the survival advantage of *Cd300lb*$^{-/-}$ over *WT* mice, whereas treatment with the control Ab had no effect on the survival of *Cd300lb*$^{-/-}$ mice (Figure 3A). Histopathologic changes in the lungs were more severe in *WT* and *Cd300lb*$^{-/-}$ mice treated with anti-IL-10 Ab than the control Ab-injected *Cd300lb*$^{-/-}$ animals (Figure 3B). Serum concentrations of TNFα, IFNγ, and IL-12, but not of IL-6 or MCP-1, were higher in anti-IL-10 Ab-treated *Cd300lb*$^{-/-}$ mice, reaching amounts similar to those of anti-IL-10 Ab-injected *WT* mice (Figure 3C). IL-10 concentrations in anti-IL-10-injected *WT* and *Cd300lb*$^{-/-}$ mice were similar and considerably lower than in control Ab-injected *WT* and *Cd300lb*$^{-/-}$ mice (Figure 3C). These findings indicate that CD300b-dependent dampening of IL-10 production augments LPS-induced changes in histology and inflammatory cytokine expression.

Expression of CD300b on macrophages augments lethal inflammation

Various immune cells have been implicated in regulating sepsis, including Macs, DCs, neutrophils and NK cells (Iwasaki and Medzhitov, 2015). CD300b was broadly expressed at the mRNA and protein levels among myeloid but not lymphoid cell populations (Figures S3A and S3B). Based on previous findings (Bouchon et al., 2001; Huang et al., 2009; Totsuka et al., 2014), we hypothesized that CD300b-expressing Macs account for the increased mortality of septic mice. To test that, we depleted Macs from *WT* and *Cd300lb*$^{-/-}$ mice using dichloromethylene biphosphate (Cl_2MBP)-encapsulated liposomes (Totsuka et al., 2014) before injection of LPS. Cl_2MBP-liposomes selectively depleted Macs with no effect on neutrophil or DC numbers in the peritoneal cavity, lung or spleen of either *WT* or *Cd300lb*$^{-/-}$ mice (Figures S3C–S3F). The removal of Macs completely negated the survival advantage of *Cd300lb*$^{-/-}$ mice, and enhanced the mortality rate of *WT* animals (Figure 4A). Injection of PBS-liposomes had no effect on *WT* or *Cd300lb*$^{-/-}$ mice survival (Figure 4A). Histopathologic analyses showed that *WT* and *Cd300lb*$^{-/-}$ mice injected with Cl_2MBP-liposomes or *WT* animals receiving PBS-liposomes developed more severe pathologic changes in their lungs compared to *Cd300lb*$^{-/-}$ mice injected with PBS-liposomes (Figure 4B). These results demonstrate that are critical for the protection of *Cd300lb*$^{-/-}$ mice from septic shock. The fact that serum concentrations of IFNγ, IL-6 and IL-12 were higher and reached the same amounts in both Cl_2MBP-treated *Cd300lb*$^{-/-}$ and *WT* mice suggests that

Macs were not the sole source and, indeed, may not be the primary source of these cytokines (Figure 4C). In contrast, TNFα concentrations were reduced following the Macs depletion in *WT* mice to levels similar to those observed in either Macs-depleted or non-depleted *Cd300lb*$^{-/-}$ mice, suggesting that Macs were the primary source of TNFα. IL-10 concentrations in sera from Macs-depleted *Cd300lb*$^{-/-}$ mice were considerably lower than those in non-depleted *Cd300lb*$^{-/-}$ mice (Figure 4C), indicating that Macs were the major source of IL-10 in *Cd300lb*$^{-/-}$ mice. Interestingly, the survival of *WT* and *Cd300lb*$^{-/-}$ mice depleted of neutrophils or NK cells, using an anti-Ly6G Ab or anti-NK1.1 Ab, respectively, was prolonged (Figures S3G and S3I). In these cases, however, the survival was likely due to a reduction in the level of pro-inflammatory cytokines, rather than changes in IL-10 production, as the depletion of these cell types did not result in a reduction of IL-10 serum concentrations (Figures S3H and S3J). These results also implied that neutrophils and NK cells are not a source of IL-10. Collectively, our data suggest that CD300b-expressing Macs failed to control lethal inflammation due to their decreased IL-10 production.

CD300b expression by macrophages shifts the balance toward inflammation in lethal endotoxemia

To examine further the role that CD300b-expressing Macs play in amplifying lethal inflammation, we first compared cytokine secretion by LPS-treated bone marrow-derived macrophages (BMMs) or dendritic cells (BMDCs) from *WT*, *Cd300lb*$^{-/-}$ and *Cd300lf*$^{-/-}$ mice. LPS-treated BMMs from *Cd300lb*$^{-/-}$ mice produced higher concentrations of IL-10, and markedly lower amounts of TNFα, and IL-12 when compared to BMMs from *WT* or *Cd300lf*$^{-/-}$ animals (Figure 5A and data not shown). Although there was a difference in IFNγ production, the amount of IFNγ produced by Macs was very low, indicating that Macs are not a source of this cytokine (Figure 5A). These results corroborate the *in vivo* data, and show that CD300b regulates IL-10 secretion by LPS-treated Macs. Intriguingly, unlike BMMs, BMDCs expressed lower levels of CD300b and TLR4 (Figures S3A, S3B and S4A), and LPS-treated *Cd300lb*$^{-/-}$ BMDCs produced TNFα, IL-6 and MCP-1 at similar concentrations to BMDC from *WT* mice, but lower amounts of IL-10 and IL-12 (Figure S4B). No IFNγ induction was observed in LPS-treated BMDCs from *WT* or *Cd300lb*$^{-/-}$ mice (Figure S4B). The fact that CD300b expression by Macs but not DCs altered the balance of cytokine production toward a more pro-inflammatory state supported our hypothesis that CD300b-expressing Macs amplified the effects of endotoxins. We further tested this hypothesis by transferring BMMs from *WT* or *Cd300lb*$^{-/-}$ mice into *WT* or *Cd300lb*$^{-/-}$ animals prior to LPS injection. Strikingly, transfer of *Cd300lb*$^{-/-}$ BMMs, but not those from *WT* animals, improved the survival of *WT* mice from 0 to 65%, and *Cd300lb*$^{-/-}$ mice from 50 to 95%, in correlation with increased IL-10 serum concentration and decreased TNFα amounts (Figures 5B and 5C). To assess if Macs-mediated IL-10 production contributed to the differential susceptibility to lethal peritonitis in *WT* versus *Cd300lb*$^{-/-}$ mice, we transferred BMMs from *Cd300lb*$^{-/-}$ *IL-10*$^{-/-}$ mice into *WT* or *Cd300lb*$^{-/-}$ animals prior to LPS injection. Transfer of *Cd300lb*$^{-/-}$ *IL-10*$^{-/-}$ BMMs impaired the survival of *WT* and *Cd300lb*$^{-/-}$ mice, and correlated with an enhanced serum concentration of pro-inflammatory cytokines (Figures 5B and 5C). These findings strongly suggested that CD300b expression on Macs enhanced the production of pro-inflammatory cytokines while reducing IL-10 levels, thus amplifying LPS-induced mortality.

CD300b regulates TLR4-CD14-MyD88 complex assembly and IL-10 production

LPS is a well-defined inducer of TLR4 signaling. LPS stimulation of Macs promotes the production of IL-10 as a feedback mechanism to inhibit the pro-inflammatory cytokine response (Siewe et al., 2006). To study if LPS binding to CD300b influenced LPS-induced TLR4 signaling, we first assessed the co-localization of CD300b and TLR4 in unstimulated or LPS-treated *WT* BMMs. We found that while a small portion of CD300b co-localized with TLR4 in unstimulated cells, LPS treatment greatly enhanced the co-localization between CD300b and TLR4 (Figures S4C and S4D; the specificities of the anti-CD300b and anti-TLR4 Abs were validated using *Cd300lb*$^{-/-}$ *and TLR4*$^{-/-}$ BMMs).

To further examine the ability of mCD300b, mTLR4, and mCD14 proteins to physically associate we performed co-immunoprecipitation analyses utilizing the recombinant monomeric proteins, and specific anti-CD300b, anti-TLR4 and anti-CD14 Abs (as evidenced by genotype-specific Ab detection of the proteins in cell lysates from *Cd300lb*$^{-/-}$, *TLR4*$^{-/-}$ or *Cd14*$^{-/-}$ BMMs) (Figure 6A). We found that mCD300b formed a complex with mTLR4 in the absence of LPS, which was enhanced in the presence of LPS (Figure 6B). Of note, no association with mCD14 was observed with LPS, unless a cross-linking reagent was present (Figure 6B), in agreement with previous findings (Akashi et al., 2003; da Silva et al., 2001; Muroi et al., 2002). Next, we examined the physical association between endogenous CD300b and TLR4 in unstimulated or LPS-treated *WT* BMMs by co-immunoprecipitation. In agreement with our confocal and recombinant protein data, some CD300b associated with TLR4 and CD14 in unstimulated cells, which was further enhanced upon LPS treatment (Figures 6C and 6D). Furthermore, we observed that most CD300b was bound to TLR4 upon continued LPS-stimulation (data not shown).

To further corroborate that LPS binding to CD300b induced an association with TLR4 and CD14, we blocked LPS binding to CD300b on *WT* BMMs with an anti-CD300b Ab (Figures S4E and S4F). We found that anti-CD300b Ab treatment inhibited the assembly of the TLR4-CD300b-DAP12 complex, while the addition of anti-CD14 or anti-IgG isotype control Ab failed to block the complex formation (Figure 6E), suggesting that the CD300b-TLR4 complex formation was mediated by LPS binding to CD300b. Treatment with either anti-CD300b or anti-CD14 Abs interfered with LPS binding to *WT* BMMs, and had a cumulative effect was observed when both Abs were used (Figures S4E and S4F), in line with a reduction of LPS binding to BMMs from *Cd300lb*$^{-/-}$ or *Cd14*$^{-/-}$ mice (Figures S4G and S4H). Ab-mediated blocking of LPS binding to CD300b or CD14 inhibited pro-inflammatory cytokine production (Figure S4I), while only LPS blocking to CD300b alone increased the production of IL-10 (Figure S4I). These findings indicated that LPS recognition by both receptors occurred simultaneously resulting in a pro-inflammatory cytokine production, while LPS binding to CD300b induced the CD300b-TLR4 complex formation leading to a reduced IL-10 production.

We further validated the LPS-induced association between CD300b and TLR4 by examining complex assembly in BMMs treated with apoptotic cells (AC), a source of phosphatidylserine (PS), which we previously identified as a CD300b ligand (Murakami et al., 2014). The interaction between CD300b and TLR4 in the presence of AC alone was similar to that of unstimulated cells, indicating that AC do not trigger the association

between CD300b and TLR4 (Figure S5A), even though they stimulate the recruitment of DAP12 to CD300b resulting in efferocytosis and an enhanced IL-10 production (Figures S5B and S5C). As might be expected, exposure of Macs to both AC and LPS reduced the level of efferocytosis compared to that seen with AC alone and the level of CD300b-TLR4 complex formation was also decreased compared to LPS-treated BMMs, suggesting that individual ligand binding was exclusive and dictated the function of CD300b (Figures S5A and S5B).

Given that MyD88 and TIRAP are primary adaptor proteins utilized for TLR4-LPS-induced signaling, we examined if MyD88 and TIRAP were recruited to the CD300b-TLR4 complex. LPS treatment induced an early and transient recruitment of MyD88 and TIRAP reaching a maximum at 0.5 h (~ 3-fold over time 0 h) after LPS stimulation (Figures 6C and 6D). Most of MyD88-TIRAP disassociated from the complex during prolonged LPS stimulation (Figures 6C and 6D). We found an incremental recruitment of DAP12 to the CD300b-TLR4 complex in response to LPS, which reached a maximum binding after 2 h (8-fold over time 0 h) and remained elevated during prolonged LPS stimulation (Figures 6C and 6D). Following the DAP12 recruitment, phosphorylated Syk (pSyk) and PI3K (pPI3K) were recruited to the receptor complex (Figures 6C and 6D). Furthermore, the kinetics of DAP12, pSyk, and pPI3K binding to the receptor complex inversely correlated with the presence of MyD88 and TIRAP (Figures 6C and 6D). TLR4 pull-down experiments using *Cd300lb*$^{-/-}$ BMMs demonstrated that CD300b was necessary for DAP12, pSyk, and pPI3K recruitment, and the displacement of MyD88-TIRAP from the complex, as without CD300b no DAP12, and only small amounts of pSyk or pPI3K were recruited to the TLR4 complex, while MyD88 as well as TIRAP remained associated with the receptor complex (Figure 6D). We further investigated the mechanism of MyD88-TIRAP displacement and found that the activation of Syk and PI3K was required for MyD88 dissociation from the CD300b-TLR4 complex (Figures S5F and S5G). These findings suggest that the CD300b-mediated activation of the Syk-PI3K signaling cascade was required for the coordinated displacement of MyD88-TIRAP from the receptor complex, likely via a PI3K-mediated decrease in the level of PtdIns(4,5)P_2, a required docking site for MyD88-TIRAP binding to the TLR4 complex (Kagan and Medzhitov, 2006).

To assess how DAP12-initiated signals intersect with the TLR4 signaling pathway, we studied the activation kinetics of various downstream signaling molecules in LPS-treated BMMs from *WT*, *Cd300lb*$^{-/-}$, and *Cd300lf*$^{-/-}$ mice. We found that in *WT* and *Cd300lf*$^{-/-}$ BMMs, phosphorylation was increased for protein kinases MEK1/2 (~ 2-fold) and ERK1/2 (~ 5-fold) by 0.5 h and returned to baseline by 1 h after LPS stimulation, whereas in *Cd300lb*$^{-/-}$ BMMs, elevated levels of phosphorylation of MEK1/2 (~ 2-fold) and ERK1/2 (~ 4-fold) were observed until 2 h (Figure 6F). In contrast, the activation of p38 and JNK were similar in BMMs from *WT*, *Cd300lb*$^{-/-}$, and *Cd300lf*$^{-/-}$ mice (Figure 6F), indicating that the pathways utilizing these kinases were not overly affected by *Cd300lb*-deficiency. In *WT* and *Cd300lf*$^{-/-}$ BMMs, but not in *Cd300lb*$^{-/-}$ BMMs, we observed an incremental increase in phosphorylation of AKT, a PI3K substrate, reaching a maximum intensity 2 h after LPS treatment (Figure 6F). The activation kinetics of AKT correlated with a reduced activation of the MEK1/2-ERK1/2 and NFκB signaling pathways (Figure 6F). Indeed, inhibition of PI3K activation in LPS-treated *WT* BMMs resulted in a reduced AKT and enhanced ERK1/2

phosphorylation (Figure S5E), indicating that the ERK1/2 pathway was timely regulated by CD300b-PI3K-AKT-mediated signaling. In agreement, we found that NFκB phosphorylation was not sustained in LPS-treated *WT* and *Cd300lf*$^{-/-}$ compared to *Cd300b*$^{-/-}$ BMMs (Figure 6F). LPS-treatment did not modulate the expression levels of any of the analyzed proteins (Figure 6G).

Previous findings (Banerjee et al., 2006; Chanteux et al., 2007) suggest that ERK1/2 activation promotes the production of IL-10. Given that the activation kinetics of ERK1/2 coincided with the early and enhanced production of IL-10, we studied if ERK1/2 was required for the IL-10 production in LPS-treated BMMs. We found that the pharmacological inhibition of ERK1/2 resulted in a reduction of ERK1/2 phosphorylation and the inhibition of IL-10 production, whereas treatment using a p38 inhibitor had no inhibitory effect on IL-10 production (Figures 6H and 6I). We further assessed if ERK1/2 inhibition affected the production of pro-inflammatory cytokines by LPS-treated BMMs and found that ERK1/2 activation was required for TNFα and IL-6, but not for IL-12 (Figure S5H), in line with previous findings (Kim et al., 2004). In agreement, pharmacological inhibition of Syk or PI3K activity resulted in an enhanced ERK1/2 phosphorylation and elevated production of IL-10 (Figures S5D and S5E).

Previous findings suggest that DAP12 is a critical regulator of TLR4 internalization, and consequently TRIF-IRF3 signaling, which leads to IFN-β production (Zanoni et al., 2011). Therefore, we assessed if CD300b-DAP12 plays a role in regulating TLR4-CD14-TRIF-IRF3 signaling. We examined IFN-β secretion, and found that LPS-treated BMMs from *WT* mice produced higher concentrations of IFN-β compared to BMMs from *Cd300lb*$^{-/-}$ animals (Figure S5I). Furthermore, in LPS-treated *WT* BMMs, phosphorylation of TBK1 and IKKε, two signaling molecules leading to the induction of IFN-β expression, was increased ~ 10-fold and ~ 6-fold, respectively, after 0.5 h and remained elevated until 2 h after LPS stimulation, whereas in *Cd300lb*$^{-/-}$ BMMs LPS treatment resulted in a reduced phosphorylation of TBK1 and IKKε (~ 3-fold and ~ 2-fold, respectively) (Figure S5J). Since LPS-induced IFN-β production requires the activation of the transcription factor, IRF3, we also assessed the activation of IRF3. In *WT* BMMs, IRF3 phosphorylation was increased (~ 5-fold) by 1 h and returned to baseline by 2 h after LPS stimulation, whereas in *Cd300lb*$^{-/-}$ BMMs, phosphorylation of IRF3 was reduced throughout the entire time course (Figure S5J). These findings indicate that CD300b through its association with DAP12 plays a positive role in regulating TLR4-CD14-TRIF signaling in Macs. Taken together, we provided evidence that CD300b played a role in regulating the TLR4-CD14-TRIF-IRF3 signaling pathway, thereby mediating IFN-β production, highlighting CD300b as a potential mediator influencing both the TLR4-MyD88 and TLR4-CD14-TRIF signaling cascades. Our data demonstrated that the CD300b-TLR4-DAP12-Syk-PI3K signaling complex limits the activation of the MEK1/2-ERK1/2-NFκB pathway in Macs, thereby dampening IL-10 production, which likely potentiates lethal inflammation.

DISCUSSION

Sepsis is a systemic inflammatory syndrome caused by infection that results in tissue damage, multisystem organ failure and, subsequently, death (Cohen, 2002). Despite the

availability of antibiotics, current therapies to treat sepsis remain ineffective and all clinical trials based on neutralization of specific inflammatory cytokines have failed, highlighting the need for alternative treatments (Wenzel and Edmond, 2012). Interestingly, strategies designed to normalize several components of the pro-inflammatory cytokine storm through targeting surface receptors have been proposed (Clatworthy and Smith, 2004).

Previous findings suggest a role for CD300b in regulating LPS-induced inflammation (Yamanishi et al., 2012), but neither the CD300b ligand nor the mechanism by which CD300b regulates the inflammatory response have been identified. Here, we showed why and how CD300b functions as a crucial mediator in the pathogenesis of severe gram-negative bacterial infections. We identified LPS as a ligand for CD300b, with a K_D = 6.39 x 10^{-6} M, which was about one order of magnitude lower than that for mCD14 (K_D = 8.5 x 10^{-7} M). Our reported affinity for mCD14 is higher than the value reported previously (K_D = 2.3 x 10^{-6} M; Shin et al., 2007), likely due to different LPS employed in these studies; since CD14 recognizes the variable O-Ag structure of LPS (Gangloff et al., 2005), one would expect its K_D for LPS to vary depending on the LPS subtype. Reported K_D values for mTLR4 and mMD-2 are 1.4 x 10^{-5} M and 2.3 x 10^{-6} M, respectively (Thomas et al., 2002; Shin et al., 2007). However, one should take note that biochemical measurements, like our SPR analysis, do not necessarily reflect the avidity of these receptors when interacting with LPS at the cells surface, due to the fact that receptors are often multimeric (e.g., CD300 receptors are most likely dimers; Martínez-Barriocanal et al., 2010), differ in expression levels, and ligand binding often induces receptor clustering.

We demonstrated that this CD300b recognition of LPS by Macs contributed to the pathogenesis of lethal inflammation through CD300b-mediated regulation of the TLR4 signaling, ultimately resulting in tempered IL-10 production and consequently a lethal prolonged pro-inflammatory cytokine response. IL-10 is known to be protective in the LPS- and CLP-models of sepsis by decreasing pro-inflammatory cytokine levels (Howard et al., 1993; van der Poll et al., 1995). Indeed, we showed that LPS-treated *WT* mice produced less IL-10 when compared to *Cd300lb*$^{-/-}$ mice, and neutralization of endogenous IL-10 impaired the survival advantage of *Cd300lb*$^{-/-}$ mice, which coincided with increased concentrations of TNFα, IL-12, and IFNγ. IL-10 is produced by Macs, DC, B cells and T cells, and to a lower extent by neutrophils and NK cells (Saraiva and O'Garra, 2010). Our *in vivo* depletion and adoptive transfer experiments identified Macs as the key population amplifying the lethal effect of endotoxemia by reducing IL-10 levels. Decreased IL-10 production by CD300b-expressing Macs lead to an enhanced pro-inflammatory cytokine response, likely as the result of heightened activation of T cells, NK cells and other cell types. Indeed, both in *WT* and *Cd300lb*$^{-/-}$ mice, depletion of NK cells or neutrophils led to enhanced survival from lethal endotoxemia that correlated with lower levels of pro-inflammatory cytokines. In contrast to BMMs, LPS-treated BMDCs from *WT* mice produced higher levels of IL-10 than those from *Cd300lb*$^{-/-}$ mice. While we cannot completely rule out DCs as contributing to LPS-induced lethality, their involvement is likely less significant, at least in regards to CD300b function in regulating lethal inflammation. DCs, unlike Macs, expressed relatively low levels of CD300b and TLR4 mRNA and protein, and they are found in lower numbers in the peritoneal cavity and different tissues. In addition, there are reasons to believe that the same receptors may differentially regulate LPS-responses in Macs and DCs, as CD11b has

been shown to regulate the TLR4-LPS signaling response in DCs but not in Macs (Ling et al., 2014). Our future studies will address the LPS-induced function of CD300b in DCs and other cell types.

We defined the mechanism of CD300b-mediated regulation of IL-10 production, and highlight the role of the CD300b-DAP12-Syk-PI3K complex in TLR4 signaling. DAP12 plays an important role in mediating bacterially-induced inflammation by functioning either as an activating (Turnbull et al., 2005) or inhibitory molecule (Hamerman et al., 2005) depending on the severity of the disease. Also, while TREM1 (Bouchon et al., 2001) and TREM2 (Turnbull et al., 2006), two DAP12-associated receptors, were identified as either enhancing or inhibiting the inflammatory response, the question has remained open as to what extent other DAP12-associated receptors can regulate septic shock (Turnbull and Colonna, 2007). We demonstrated that in response to LPS, CD300b associated with TLR4 at the cell surface, leading to the formation of a CD300b-DAP12-TLR4-Syk-PI3K signaling complex, and the dissociation of MyD88-TIRAP from the complex. The precise mechanism by which DAP12, Syk and PI3K regulate the displacement of MyD88-TIRAP and thereby the assembly of the receptor complex remains to be determined. One possibility, implied by our data, was that PI3K recruited to and activated in the complex could phosphorylate PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, thereby reducing PtdIns(4,5)P_2 levels and facilitating the dissociation of MyD88-TIRAP from the receptor complex. Above all, our findings showed that LPS-induced formation of this signaling complex resulted in the augmentation of lethal inflammation by limiting the ERK1/2-mediated IL-10 production via the Syk-PI3K-AKT signaling cascade, and highlighted CD300b as a key player in modulating septic shock. It remains to be seen if CD300b can directly or indirectly cooperate with other receptors, such as TREM1 and/or TREM2, which could lead to a synergistic enhancement or reduction in the pathogenesis of lethal inflammation.

TYROBP/FcγR-deficient BMMs show impaired internalization of TLR4, and consequently lack signaling via the TRIF-IRF3 pathway, which is activated after endocytosis and leads to IFN-β production (Zanoni et al., 2011). Therefore, we examined if *Cd300lb*$^{-/-}$ BMMs had an impairment in the TLR4-TRIF signaling pathway, and found that LPS-treated BMMs from *WT* mice produced higher IFN-β concentrations compared to *Cd300lb*$^{-/-}$ BMMs. In support of this, we found an elevated activation of the TBK1-IKKε-IRF3 signaling cascade, indicating that CD300b-DAP12 amplified the TLR4-CD14-TRIF pathway leading to IFN-β production. Previous findings by Zanoni *et al.* suggest a role for Syk signaling in regulating TLR4 endocytosis and TRIF signaling. In this regard, we showed that association of Syk and PI3K to the TLR4 complex upon LPS treatment required DAP12 via its association with CD300b, indicating that CD300b-DAP12 regulated the endocytosis of TLR4 and thereby TRIF-IRF3 signaling via the recruitment of Syk and PI3K. The findings of Zanoni *et al.* further suggest that at low concentrations of LPS, CD14 is critical for the TLR4-MyD88-dependent TNFα response. Therefore, it might be that the role of CD300b as an LPS-sensor is most critical during severe bacterial infection, wherein the limitation of pro-inflammatory cytokine levels due to IL-10 production may be more detrimental than the potential harm that these cytokines may cause. This relates to the observation that at high LPS concentrations the contributing role of CD14 (Zanoni et al., 2011) may be dispensable

(Figure S6). In sum, our findings showed that CD300b-DAP12 regulates the TLR4-MyD88 as well as the TBK1-IKKε-IRF3 signaling cascade.

The ability to associate with and regulate the function of other receptors may be a common property of CD300 family members. In addition to the data reported here showing CD300b regulation of TLR4 signaling, we have recently shown that CD300f associates with and regulates IL-4 receptor α-mediated responses by augmenting IL-4 and IL-13-induced signaling (Moshkovits et al., 2015). Other CD300 family members have been shown to modulate LPS-induced responses, although not through the direct recognition of LPS. Ab cross-linking of mCD300f, known to bind PS (Tian et al., 2014) and not LPS (this study), can augment cytokine production induced by LPS in bone marrow-derived mast cells while suppressing cytokine production induced by other TLR agonists (Izawa et al., 2007). In contrast to the *Cd300lb*$^{-/-}$ mice reported here, *CLM4*$^{-/-}$ mice are more susceptible to CLP (Totsuka et al., 2014). CLM4, a mouse CD300 family member with no human homologue, associates with FcRγ and Syk upon LPS stimulation leading to enhanced VLA-4-mediated adhesion to VCAM-1. In the absence of CLM4, migration of inflammatory monocytes is inhibited, thereby promoting peritonitis. How the effects of LPS on CD300b and CLM4 are coordinated remains to be elucidated.

Previous studies of sepsis in both humans and mice report an accumulation of AC, particularly from lymphocytes and DCs (Hotchkiss et al., 2013). We previously characterize CD300b as a PS-binding phagocytic receptor promoting efferocytosis via DAP12 signaling (Murakami et al., 2014), suggesting that the CD300b-DAP12 complex plays a role in maintaining cellular homeostasis. In this study, we identified an important mechanism that governs TLR4 signaling in Macs whereby CD300b-DAP12-Syk-PI3K association with TLR4 upon LPS binding resulted in lower IL-10 production. We proposed that under physiological conditions CD300b does not form a robust complex with TLR4 and functions as a receptor that supports homeostasis through efferocytosis by Macs (Figure S6). However, as infections become more acute, the excess amount of endotoxin present shifts the function of CD300b to a receptor that responds to bacterial assault. LPS suppressed CD300b-mediated efferocytosis and anti-inflammatory cytokine production, and stimulated the association of CD300b-DAP12-Syk-PI3K with TLR4; this lead to the displacement of MyD88-TIRAP, which likely resulted in the inhibition of its signaling, and blocked the production of IL-10 (Figure S6). In agreement with Zanoni *et al.,* our data showed that CD300b activated the TLR4-CD14-TRIF-IRF3 signaling pathway, resulting in enhanced IFN-β production. Thus, LPS acted as a molecular switch to temporarily dispense CD300b-mediated efferocytosis, an anti-inflammatory function (Henson and Bratton, 2013), to one that heightened the pro-inflammatory cytokine response (Figure S6). Our data identified CD300b as an LPS-recognizing receptor that regulated TLR4 signaling, thus controlling the balance between pro- and anti-inflammatory cytokine secretion during severe bacterial infection, and highlights CD300b as a potential therapeutic target for clinical intervention to manage septic shock in humans.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted using female *WT*, *Cd300lb*$^{-/-}$, *Cd300lb*$^{-/-}$ *IL-10*$^{-/-}$ and *Cd300lf*$^{-/-}$ C57BL/6 mice in a pathogen-free environment according to NIAID Animal Care and Use Committee guidelines.

Cell culture, transfection and infection

L929 and HEK293T cells were transfected with empty vector (EV) or various CD300 plasmids using PolyJet (Signagen). For infection experiments, L929 cells were infected using lentivirus particles from different CD300 family members and protamine sulfate. Cells were selected with puromycin and clonal cell lines were obtained as described in Supplemental Experimental Procedures.

Surface plasmon resonance (SPR)

Fcγ-chimeric or recombinant protein interactions with biotinylated LPS (InvivoGen) was performed as previously described (Murakami et al., 2014) and measured using the BIAcore T100 SPR instrument as detailed in Supplemental Experimental Procedures.

Cell based TLR-ligand binding assays

TLR-ligand binding assays with different CD300b-expressing L929 cells or BMMs from *WT*, *Cd300lb*$^{-/-}$, *and Cd14*$^{-/-}$ mice were performed by flow cytometry as detailed in Supplemental Experimental Procedures.

Competition assays

FITC-labeled TLR4 (LPS)-ligand competition and Ab blocking experiments were performed as described in Supplemental Experimental Procedures.

Cecal Ligation Puncture (CLP) and lethal endotoxemia

CLP and lethal endotoxemia were performed as described in Supplemental Experimental Procedures.

Measurement of cytokines and chemokines

Measurement of pro- and inflammatory cytokines was performed using the Cytometric Bead Array (BD Biosciences) and LEGENDplex mouse inflammation kit (BioLegend) as described in Supplemental Experimental Procedures.

Histological analysis

All tissue samples were stained with hematoxylin and eosin (H&E) or anti-F4/80 Ab as described in Supplemental Experimental Procedures.

In vivo depletion of macrophages

In vivo depletion of Macs, mice were i.v. injected with 200 μl PBS-liposomes (control) or dichloromethylene biphosphate (Cl_2MBP)-liposomes (Encapsula NanoSciences) 24 h before the induction of lethal endotoxemia as described (Totsuka et al., 2014).

Neutralization of endogenous IL-10

For *in vivo* neutralization of IL-10, mice were i.p. injected with 1 mg of anti-IL-10 (clone JES5-2A5, BioxCell) or control Ab (clone HRPN, BioxCell) 2 h before the induction of lethal endotoxemia.

Differentiation of bone marrow-derived macrophages and DCs

Bone marrow cells were isolated from *WT*, *Cd300lb*$^{-/-}$, *Cd300lb* $^{-/-}$ *IL-10*$^{-/-}$, *Cd300lf*$^{-/-}$ or *TLR4*$^{-/-}$mice and differentiated into BMMs or DCs for 7 days as described in Supplemental Experimental Procedures.

Adoptive transfer of bone marrow derived macrophages

Mice were i.v. injected with 2 x 10^6 of *WT*, *Cd300lb*$^{-/-}$, or *Cd300lb*$^{-/-}$ *IL-10*$^{-/-}$ BMMs 24 h before the induction of lethal endotoxemia.

Extract preparation, immunoprecipitation and western blot analysis

BMMφ extract preparation and immunoprecipitation experiments were performed as previously described (Tian et al., 2014) and are further detailed in Supplemental Experimental Procedures.

Confocal microscopy and flow cytometry

Co-localization and expression studies of CD300b and TLR4 on Macs or other immune cells were performed using anti-TLR4-Alexa647 Ab. Cells were fixed with methanol:acetic acid (95%:5%), blocked with 10% BSA and incubation with an anti-CD300b-Alexa488 or anti-IgG isotype control Abs. Cells were visualized using an confocal microscope (Zeiss) as described as described in Supplemental Experimental Procedures.

Phagocytosis of apoptotic cells

Efferocytosis of apoptotic thymocytes was performed as previously described (Tian et al., 2014) and is detailed in Supplemental Experimental Procedures.

Statistical analysis

Endpoint studies of mice subjected to lethal endotoxemia or CLP were analyzed by using Kaplan-Meir survival curves and the log-rank test (GraphPad Prism Software, version 6.0). The statistical significance was assessed using ANOVA with Bonferroni post-test, or by the two-tailed unpaired Student *t*-test (GraphPad). Data are presented as mean ± SEM, unless stated otherwise. Alpha level was set to 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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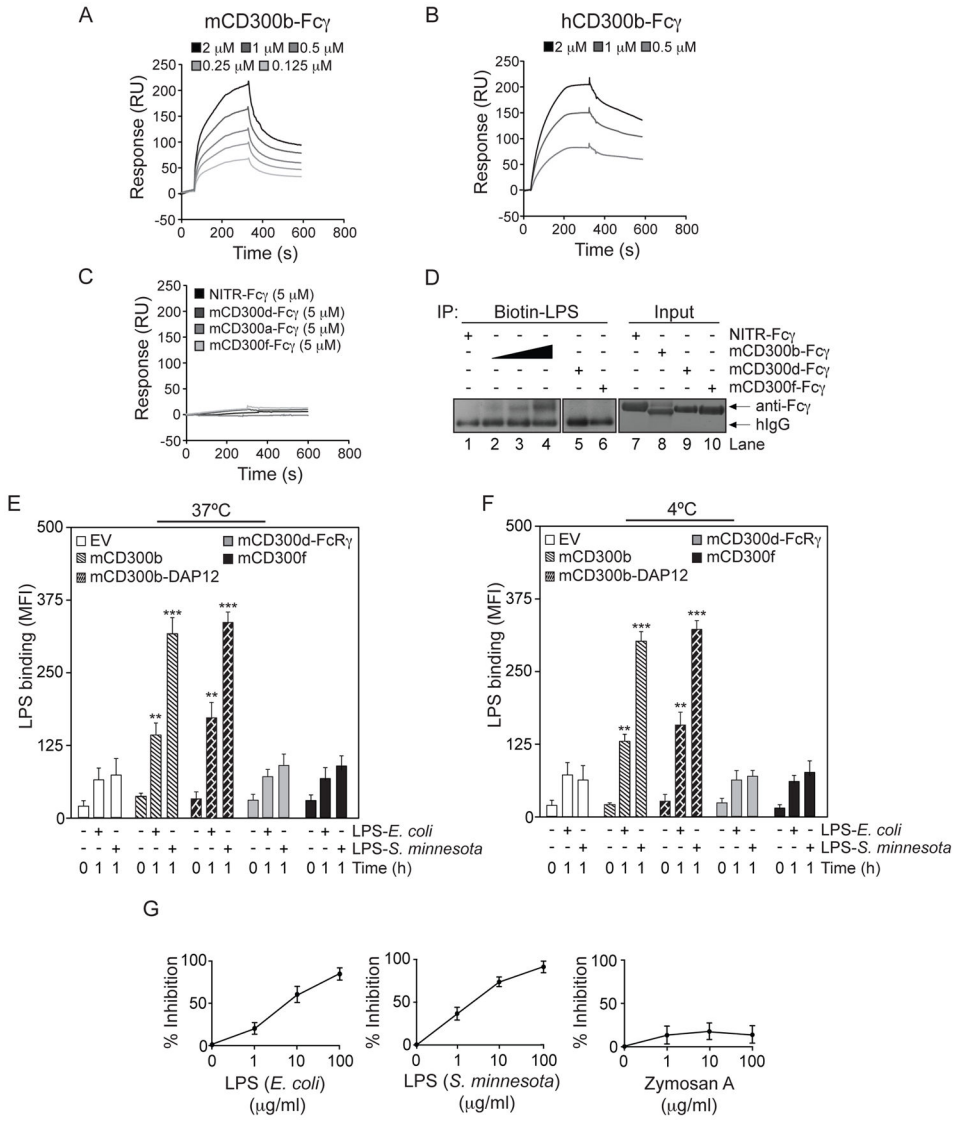

Figure 1. LPS is a ligand for CD300b

(A–C) Sensorgrams of mCD300b-Fcγ (A), hCD300b-Fcγ (B), mCD300d-Fcγ, mCD300a-Fcγ, mCD300f-Fcγ, and NITR-Fcγ (C) protein binding to immobilized LPS (*E.coli* 0111:B4) over the indicated times. Binding was initiated at 60 s and the dissociation phase begun at 240 s and is expressed in resonance units (RU).

(D) Streptavidin pulldown assays determining the binding of mCD300b-Fcγ [2, 5 and 10 μg (lane 2–4)], or 10 μg of mCD300d-Fcγ, mCD300f-Fcγ or NITR-Fcγ proteins to biotin-conjugated LPS (2 μg). Bound protein was determined by immunoblotting using an anti-human IgG Fcγ-specific Ab. hIgG indicates human Ig heavy chain.

(E–F) mCD300b- (▧), mCD300b-DAP12- (▩), mCD300d-FcRγ- (■), mCD300f- (■), EV-expressing L929 cells (□) were incubated with FITC-labeled LPS from *E. coli* or *S. minnesota* (10 μg/ml) for 1 h at 37°C (E) or 4°C (F). Binding was analyzed by flow cytometry and expressed as mean fluorescence intensity (MFI).

(G) mCD300b-DAP12-expressing L929 cells were incubated with FITC-labeled LPS from *E. coli* (10 μg/ml) for 1 h, and mixed with increasing concentrations of unlabeled LPS from *E. coli, S. minnesota* or the TLR2 ligand Zymosan A (negative control). Samples analyzed by flow cytometry and MFI values from reactions without unlabeled *E. coli* LPS were considered as the maximum binding level (0 % Inhibition). Data in (A–D) are a representative of three experiments. Error bars represent SEM (E–G); **p≤0.01, and ***p≤0.001. See also Figures S1 and S2.

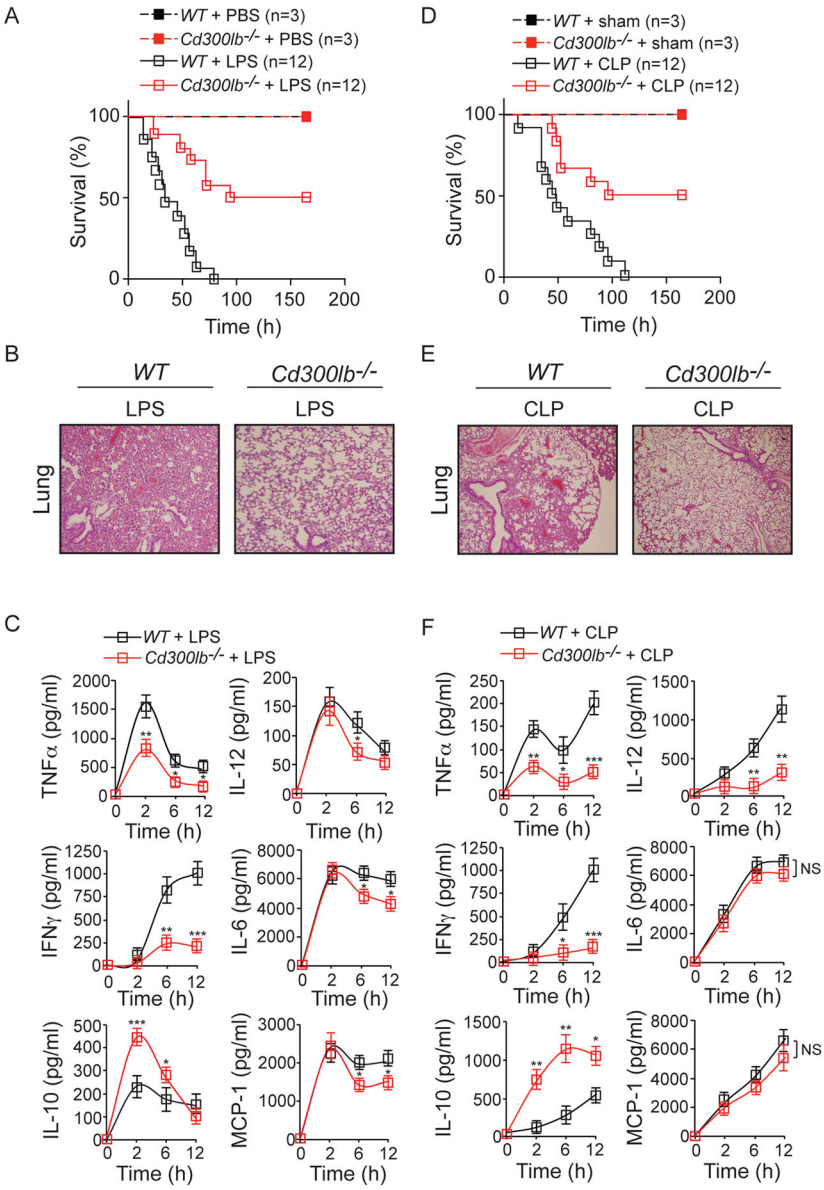

Figure 2. CD300b augments the pathogenesis of lethal endotoxemia and septic peritonitis

(A) *WT* and *Cd300lb*$^{-/-}$ mice (n = 12) were injected i.p. with a toxic dose of LPS (37 mg/kg) or the diluent control PBS (n = 3). Survival was monitored every 6 h for 7 days.

(B) H&E staining of lung tissues from PBS- and LPS-treated *WT* and *Cd300lb*$^{-/-}$ mice.

(C) Serum cytokine concentrations measured from PBS- and LPS-treated *WT* and *Cd300lb*$^{-/-}$ mice.

(D) *WT* and *Cd300lb*$^{-/-}$ mice (n = 12) were subjected to cecal ligation puncture (CLP) or laparotomy without ligation and puncture (sham-control, n = 3). Survival was monitored every 6 h for 7 days.

(E) H&E staining of lung tissues from CLP-treated *WT* and *Cd300lb*$^{-/-}$ mice.

(F) Serum cytokine concentrations measured from CLP-treated *WT* and *Cd300lb*$^{-/-}$ mice.

Data in (B) and (E) are a representative from 5 mice per group. Graphs in (C) and (F) show

mean values ± SEM from 5 mice per group, NS, not significant; *p≤0.05, **p≤0.01, and ***p≤0.001. See also Figures S2.

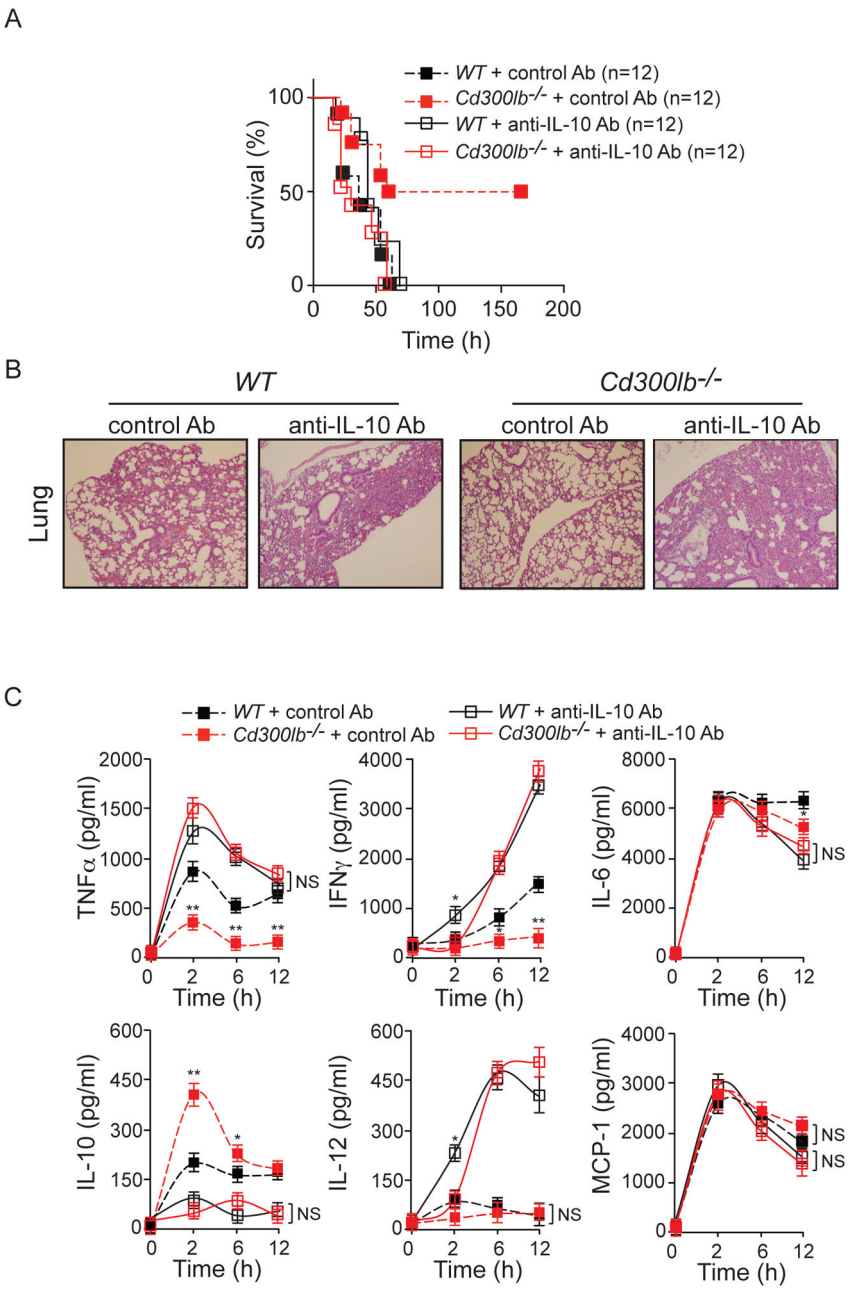

Figure 3. Neutralization of IL-10 augments the pathogenesis of lethal endotoxemia and septic peritonitis

(A) Anti-IL-10- or control Ab-treated *WT* and *Cd300lb*$^{-/-}$ mice (n = 12) were i.p. injected with a toxic dose of LPS (37 mg/kg). Survival was monitored every 6 h for 7 days.

(B) H&E staining of lung tissues from anti-IL-10- or control Ab-treated *WT* and *Cd300lb*$^{-/-}$ mice after LPS-treatment. Data are a representative from 5 mice per group.

(C) Serum cytokine concentrations measured from anti-IL-10- or control Ab-treated *WT* and *Cd300lb*$^{-/-}$ mice after LPS treatment. The graphs in (C) show mean values ± SEM from 5 mice per group, NS, not significant; *p≤0.05, and **p≤0.01.

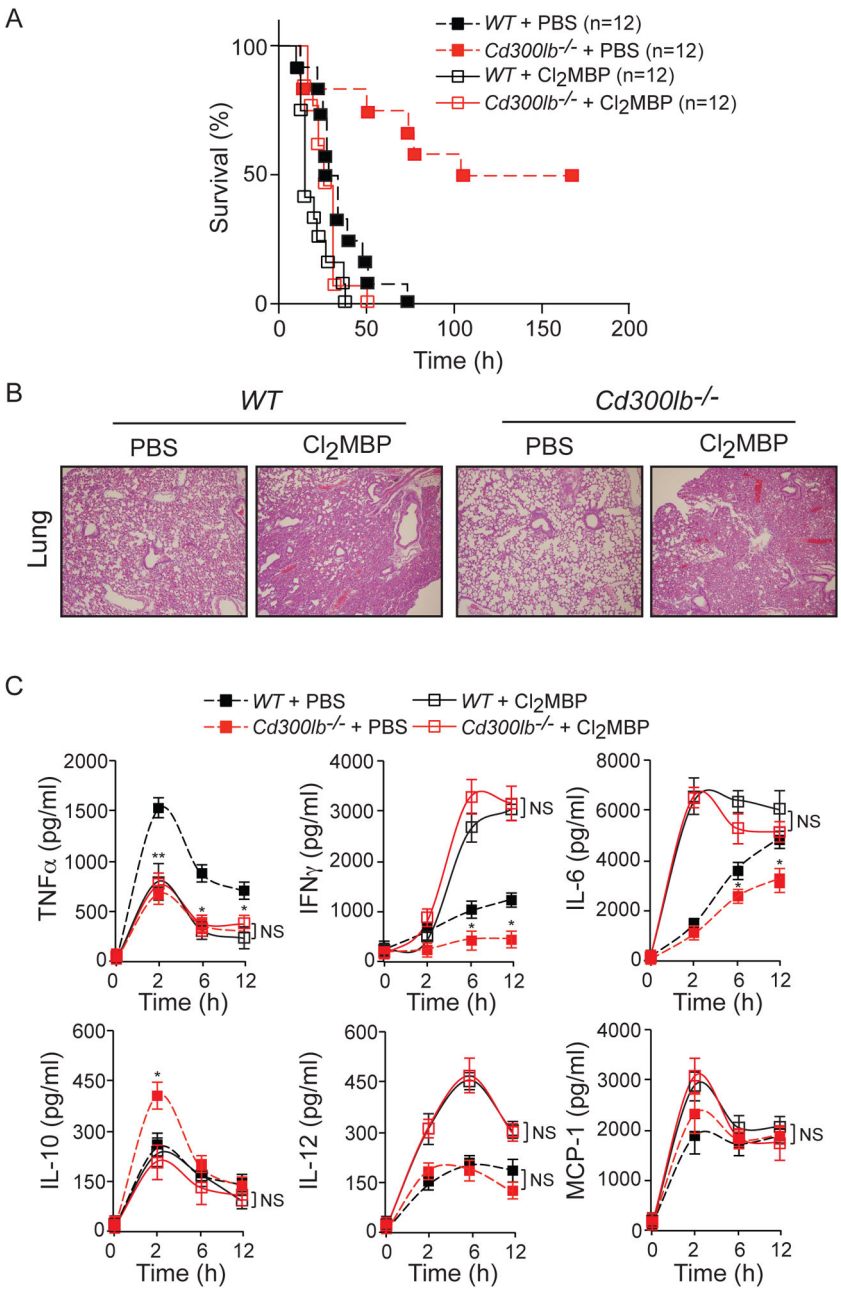

Figure 4. CD300b-expressing macrophages are responsible for augmenting the pathogenesis of LPS-induced lethal endotoxemia

(A) Liposome-encapsulated PBS- or dichloromethylene biphosphate (Cl_2MBP)-treated *WT* and *Cd300lb*$^{-/-}$ mice (n = 12) were i.p. injected with a toxic dose of LPS (37 mg/kg). Survival was monitored every 6 h for 7 days.

(B) H&E staining of lung tissues from PBS- and Cl_2MBP-liposome-injected *WT* and *Cd300lb*$^{-/-}$ mice after LPS treatment. Data are a representative from 5 mice per group.

(C) Serum cytokine concentrations measured from PBS- and Cl_2MBP-liposome-injected *WT* and *Cd300lb*$^{-/-}$ mice after LPS treatment. The graphs in (C) show mean values ± SEM from 5 mice per group, NS, not significant; *p≤0.05, and **p≤0.01. See also Figures S3.

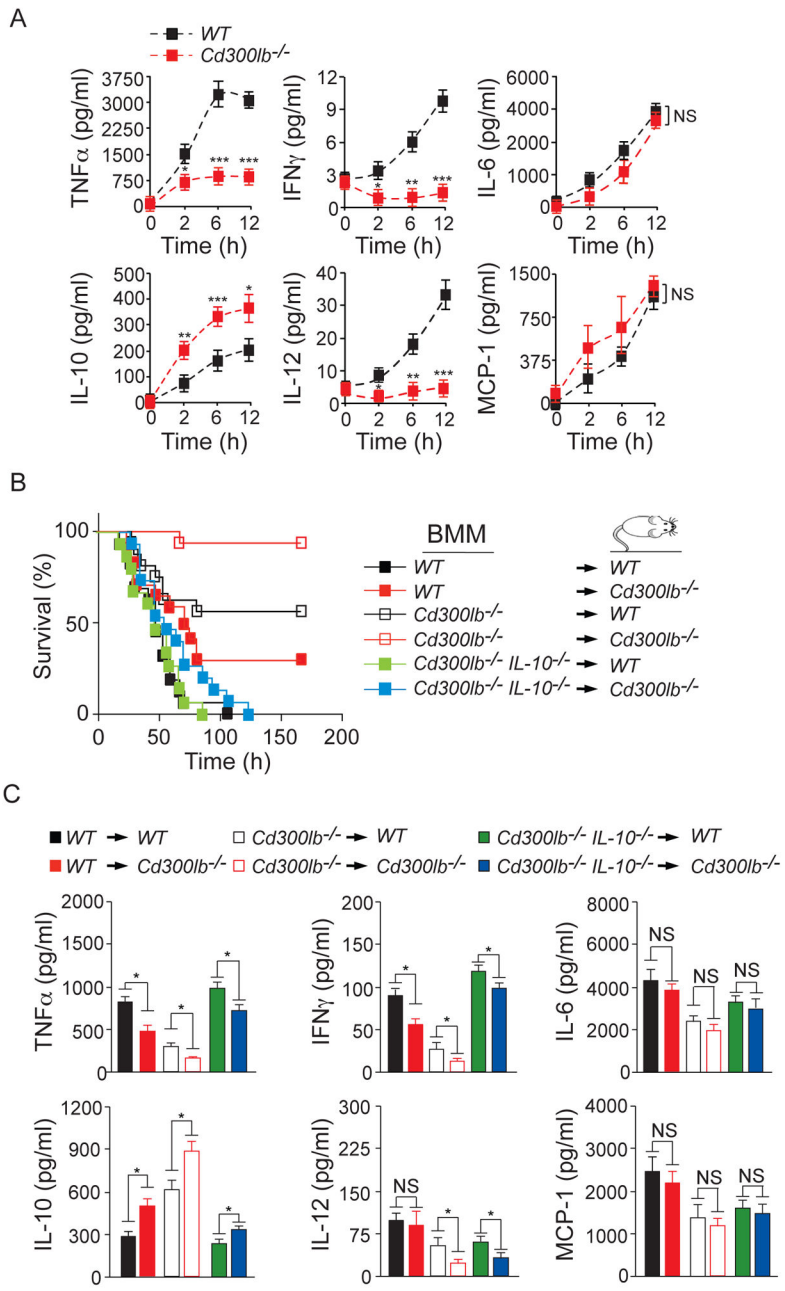

Figure 5. CD300b-expressing BMMs increase the severity of lethal endotoxemia by altering the levels of cytokines produced

(A) BMMs from *WT* or *Cd300lb*$^{-/-}$ mice were stimulated with 2 μg/ml of LPS for various lengths of time. Cytokine levels were assessed by flow cytometry. No differences in cytokine levels between diluent control-treated *WT* and *Cd300lb*$^{-/-}$ BMMs was observed (data not shown).

(B) BMMs from *WT*, *Cd300lb*$^{-/-}$, or *Cd300lb*$^{-/-}$ *IL-10*$^{-/-}$ mice were intravenously transferred into *WT* or *Cd300lb*$^{-/-}$ animals (n = 15) 24 h prior to i.p. injection with a toxic dose of LPS (37 mg/kg). Survival was monitored every 6 h for 7 days.

(C) Serum cytokine concentrations measured from *WT*, *Cd300lb*$^{-/-}$, or *Cd300lb*$^{-/-}$ *IL-10*$^{-/-}$ BMMs-injected *WT* or *Cd300lb*$^{-/-}$ animals 2 h post LPS treatment. The graphs show mean values + SEM from three independent experiments (A) or ± SEM from 5 mice per group (C), NS, not significant; *p≤0.05, **p≤0.01, and ***p≤0.001. See also Figures S4.

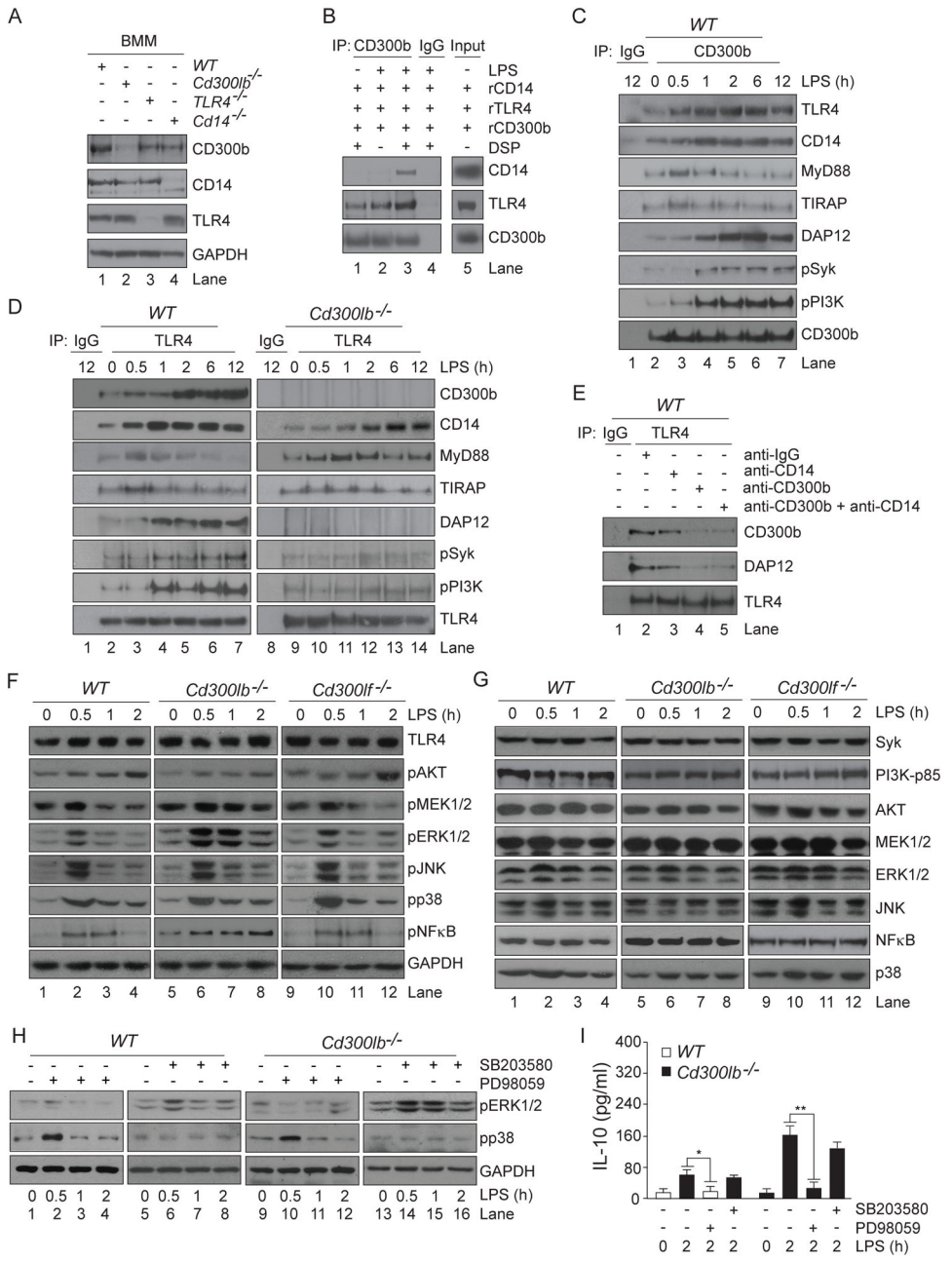

Figure 6. LPS binding by CD300b promotes the association with TLR4-MyD88-TIRAP complex and dampens the production of IL-10 via DAP12-Syk-PI3K recruitment

(A) BMMs from *WT*, *Cd300lb*$^{-/-}$, *Cd14*$^{-/-}$ or *TLR4*$^{-/-}$ mice were lysed and analyzed by immunoblotting with the indicated Abs. GAPDH was used as a loading control.

(B) Purified mCD300b, mTLR4, and mCD14 proteins were incubated in the presence or absence of LPS (2 μg/ml), followed by the addition of dithiobissuccinimidyl propionate (DSP) where indicated. Samples were immunoprecipitated with anti-CD300b or anti-IgG isotype control Ab and analyzed by immunoblotting with the indicated Abs.

(C–D) BMMs from *WT* or *Cd300lb*$^{-/-}$ mice were stimulated with LPS (2 μg/ml) for various lengths of time. Reactions were immunoprecipitated with anti-CD300b (C), anti-TLR4 (D)

or anti-IgG isotype control Ab (C–D), and then analyzed by immunoblotting with the indicated Abs.

(E) BMMs from *WT* were pretreated for 12 h with anti-IgG isotype control Ab, anti-CD300b Ab, anti-CD14 Ab or both anti-CD300b and anti-CD14 Abs before the addition of LPS (2 μg/ml). Cell lysates were immunoprecipitated with anti-TLR4 or anti-IgG isotype control Ab and samples were analyzed by immunoblotting with the indicated Abs.

(F–G) BMMs from *WT*, *Cd300lb*$^{-/-}$, or *Cd300lf*$^{-/-}$ mice were stimulated with LPS (2 μg/ml) for various lengths of time and cell lysates were analyzed for the levels of phosphorylated (F) or total protein expression (G) by immunoblotting with the indicated Ab.

(H) BMMs from *WT* or *Cd300lb*$^{-/-}$ mice were treated with p38 inhibitor SB203580 (10 μM) and/or the ERK1/2 inhibitor PD98059 (25 μM) for 1 h prior to stimulation with LPS (2 μg/ml) for additional 2 h. The levels of p38 and ERK1/2 phosphorylation were analyzed by immunoblotting with the indicated Ab.

(I) IL-10 cytokine levels in the culture medium from *WT* or *Cd300lb*$^{-/-}$ BMMs-treated with SB203580 or PD98059 as determined by flow cytometry. Data in (A–H) are a representative of three experiments. Error bars represent SEM from three experiments (I), *$p \leq 0.05$, and **$p \leq 0.01$. See also Figures S4 and S5.