Published in final edited form as:
Cell Host Microbe. 2009 January 22; 5(1): 35–46. doi:10.1016/j.chom.2008.12.002.

RP105 facilitates macrophage activation by mycobacterial lipoproteins and viable *Mycobacterium tuberculosis*

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Summary

RP105, a Toll-like receptor (TLR)–like molecule, is reported to facilitate B cell activation by the TLR4-agonist LPS but to limit LPS-induced cytokine production by antigen-presenting cells. We show that RP105 positively regulates macrophage responses to *Mycobacterium tuberculosis* (Mtb). Mtb-infected RP105$^{-/-}$ mice exhibited impaired pro-inflammatory cytokine responses associated with enhanced bacterial burden and increased pathology in the lung. The Mtb 19 kDa lipoprotein induced TNF release in a manner dependent on both TLR2 and RP105, and macrophage activation by Mtb lacking mature lipoproteins was not facilitated by RP105. Thus, mycobacterial lipoproteins were identified as RP105 agonists. RP105 physically interacted with TLR2, and both RP105 and TLR2 were required for optimal macrophage activation by Mtb. Our data identify RP105 as an accessory molecule for TLR2 in sensing mycobacterial lipoproteins, and as part of the receptor complex for Mtb ligands.

Introduction

Toll-like receptors (TLRs) recognize conserved motifs of microbial molecules and initiate signal transduction leading to the induction of innate and acquired immune responses (Medzhitov and Janeway, 2000; Takeda et al., 2003). Agonists have been identified for most members of the TLR family, and the signaling events that result from TLR activation have been extensively studied (Kawai and Akira, 2005). However, such studies only incompletely mimic the interaction of host cells with pathogens, which are complex immunogens with various biochemical components that may engage several receptors at once. Interactions

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between members of the TLR family have been demonstrated. TLR2, for example, forms heterodimers either with TLR1 or with TLR6 to discriminate between di- or tri-acylated lipopeptides (Takeda et al., 2003). Engagement of different intracellular adaptors results in a certain degree of signal specificity downstream of TLRs (Kawai and Akira, 2005). Furthermore, TLRs can work in conjunction with accessory proteins. For example, CD36 facilitates recognition of di-acylated lipoproteins by the TLR2/6 complex (Hoebe et al., 2005).

Mycobacterium tuberculosis (Mtb) engages a multitude of surface receptors on macrophages, leading to uptake (Ernst, 1998), cell activation and establishment of an adaptive immune response (Flynn and Chan, 2005). TLR2, TLR9 and TLR4 are involved in host cell activation by mycobacteria and their products (Bafica et al., 2005; Means et al., 1999; Underhill et al., 1999), yet results addressing the importance of individual TLRs in host resistance in *in vivo* models of tuberculosis are conflicting (Doherty and Arditi, 2004; Bafica et al., 2005; Holscher et al., 2008). Cooperative activation of receptors may shape the host immune response to Mtb, as suggested by the observation that TLR2 and TLR9 act synergistically to mediate resistance to Mtb infection (Bafica et al., 2005).

RP105 (radioprotective 105 kDa) is a TLR-like protein that is expressed by B-lymphocytes, macrophages and dendritic cells (DCs) (Blumenthal et al., 2005; Divanovic et al., 2005; Fugier-Vivier et al., 1997; Miura et al., 1996; Miyake et al., 1995). Phylogenetic analyses placed RP105 and TLR4 into the same subfamily (Divanovic et al., 2005). Similar to TLR4, which is associated with the extracellular protein MD-2, surface expression of RP105 depends on its association with the soluble MD-2 homolog, MD-1 (Miyake et al., 1998; Nagai et al., 2002). RP105 contains the conserved extracellular leucine-rich repeat domain typical for members of the TLR family (Miyake et al., 1995) but lacks the intracellular Toll/IL-1-receptor-(TIR-) domain, which is essential for TLR-signaling. Instead, RP105 contains only 6–11 intra-cytoplasmic amino acid residues and may associate with other signaling molecules to mediate intracellular signal transduction (Kimoto et al., 2003).

The role of RP105/MD-1 in cellular activation has been studied with focus on the TLR4 agonist lipopolysaccharide (LPS) as the stimulating agent. The *ex vivo* proliferation of B cells from RP105-deficient ($^{-/-}$) and MD-1$^{-/-}$ mice was blunted compared to wild type (WT) cells; *in vivo*, a reduction in LPS-driven antibody production has been reported (Nagai et al., 2002; Ogata et al., 2000). In contrast, in macrophages and DCs, RP105 was shown to inhibit LPS-induced TLR4-mediated activation as demonstrated by elevated levels of inflammatory cytokines in cultures of LPS-stimulated RP105$^{-/-}$ cells. Similarly, injection of RP105$^{-/-}$ mice with LPS led to increased TNF concentration in serum, along with increased endotoxicity (Divanovic et al., 2005). Studies with transfected HEK293 cells revealed that the RP105/MD-1 complex interacts directly with TLR4/MD-2 and inhibits LPS binding without binding LPS directly (Divanovic et al., 2005; Tsuneyoshi et al., 2005).

Little is known about RP105 involvement in TLR4-independent activation of host cells. Responses to synthetic TLR2 agonists, such as the lipopeptides Pam_3CSK_4 and MALP-2, have been reported to be impaired in RP105$^{-/-}$ B cells, however, in RP105$^{-/-}$ macrophages and DCs, TNF production in response to these TLR2 agonists was not different from that in WT cells (Nagai et al., 2005). Cell-specific differences seem to determine the different roles for RP105 in mediating cell activation. This hypothesis is supported by the finding that in B cells cross-linking of RP105 by a specific antibody induced proliferation (Miyake et al., 1994), whereas the same antibody failed to activate macrophages or DCs (Divanovic et al., 2005; Kimoto et al., 2003).

Information about the role of RP105 during infectious processes *in vivo* is limited. RP105$^{-/-}$ mice were used to investigate LPS-induced systemic inflammation. These studies indicated that RP105 is involved in dampening inflammatory responses during LPS-induced sepsis (Divanovic et al., 2005; Nagai et al., 2005). RP105$^{-/-}$ mice were also reported to control infection with *Leishmania major* better than WT mice (Divanovic et al., 2007).

We demonstrate that infection of primary mouse macrophages with Mtb led to increased surface expression of RP105 and MD-1. RP105 and MD-1 were required for optimal release of IL-12p40, TNF, IL-10 and RANTES by macrophages following Mtb infection. We provide evidence that RP105 enhances TLR2 signaling in Mtb infected macrophages: (i) the Mtb 19 kDa lipoprotein, a TLR2 agonist, stimulated macrophages in an RP105-dependent fashion; (ii) RP105/MD-1 enhanced TLR2-dependent IL-12p40 and TNF production by Mtb-infected macrophages; and (iii) RP105 co-patched with aggregated TLR2 in the plasma membrane, and both receptors co-immunoprecipitated, indicating physical interactions between TLR2 and RP105. Furthermore, RP105 was found to be important for early cytokine responses during an Mtb infection, and modulated mycobacterial replication and pathology in lungs of chronically infected mice.

Results

Microbial stimulation increases RP105 and MD-1 surface expression by macrophages

Flow cytometry was used to measure cell surface expression of RP105 by murine bone marrow-derived macrophages (BMM) after stimulation with viable Mtb. While at 4 h after stimulation RP105 surface expression was unaltered (not shown), a dose–dependent increase in RP105 expression, compared to unstimulated cells, was detected 18 h after exposure to Mtb (Figure 1A and C). The elevated RP105 surface expression was preceded by an increase in mRNA expression (Figure 1 E) and did not require TLR2 (Figure S1A).

MD-1 is associated with RP105 and is essential for RP105 surface expression (Miyake et al., 1998; Nagai et al., 2002). We therefore also analyzed expression of MD-1 by macrophages. Surface expression of MD-1 was enhanced after Mtb stimulation (Figure 1B and D) similar to that of RP105. However, MD-1 mRNA levels were not different from unstimulated cells (Figure 1F), suggesting a posttranscriptional mechanism for increased MD-1 surface expression, e.g. re-distribution from an intracellular pool. In RP105$^{-/-}$ BMM lower amounts of MD-1 were detected on the surface than in WT cells (Figure S1B), but the fold-increase in MD-1 surface expression upon Mtb stimulation was similar to WT cells and occurred independently of TLR2 (Figure S1A).

RP105 is required for optimal activation of macrophages by Mtb and contributes to host resistance against Mtb infection

To test if RP105 is involved in Mtb-induced macrophage activation, we examined responses of WT and RP105$^{-/-}$ BMM to Mtb. Uptake and intracellular survival of Mtb were similar in both genotypes (Figure S2). Infection of WT BMM with live Mtb induced expression of genes encoding inflammatory cytokines (TNF, IL-6, IL-10, IL-12p40 and IFNβ), chemokines (IP-10, RANTES, MCP-1, KC [mouse homolog of GRO-α), and inducible nitric oxide synthase (iNOS) (Figures 2A and S3). In Mtb-infected RP105$^{-/-}$ macrophages, IL-12p40 mRNA levels were decreased by 60–70% compared to those in WT macrophages (Figure 2A). In contrast, nine other genes showed similar mRNA levels in WT and RP105$^{-/-}$ BMM following Mtb challenge (Figures 2A and S3).

IL-12p40 protein levels were reduced by 50% in RP105$^{-/-}$ BMM compared to WT BMM (Figure 2B), consistent with reduced IL-12p40 mRNA levels in these cells. Moreover, although

similarly expressed at the mRNA level, TNF, IL-10 and RANTES protein amounts were reduced in supernatants of Mtb-infected RP105$^{-/-}$ BMM (Figure 2B). These data show that RP105 contributed to transcriptional and post-transcriptional regulation of Mtb-induced expression of inflammatory genes.

In contrast to Mtb-induced activation, stimulation of RP105$^{-/-}$ macrophages with LPS, or the TLR2 agonist Pam_3CSK_4, and peptidoglycan did not lead to reduced levels of TNF, IL-12p40, IL-10 or RANTES (Figure 3A).

To assess the role of RP105 in resistance and inflammatory responses to Mtb infection *in vivo*, we analyzed systemic IL-12p40 concentrations, bacterial loads, and pulmonary pathology in RP105$^{-/-}$ and WT mice infected with a low dose of virulent Mtb via the aerosol route. RP105$^{-/-}$ mice had 50% lower IL-12p40 serum levels during the acute phase of infection at days 7 and 21 (Figure 2C). With the decline of serum-IL-12p40 in WT mice at later time points, these differences subsided. IL-12p40 in RP105$^{-/-}$ mice did not accumulate to the levels observed in WT animals at 3 weeks post infection. Bacterial loads in lungs of RP105$^{-/-}$ mice were similar at day 7 post infection but elevated compared to WT mice at 21, 56 and 85 days post infection with significant differences at days 56 and 85 (Figure 2D). This was observed in two independent experiments. Survival of RP105$^{-/-}$ mice was not compromised within the duration of the experiment (85 days).

RP105$^{-/-}$ and WT mice showed an effective granulomatous response in lung tissue (Figure 2F), and the mononuclear infiltrate (macrophages, foamy macrophages, lymphocytes, isolated polymorphonuclear cells) was similar. However, at days 21 and 56 RP105$^{-/-}$ mice had more numerous and larger inflammatory lesions in their lungs than WT mice (Figure 2E and F). Especially at day 56, in the chronic phase of infection, the ratio between macrophage infiltrated areas and lymphocyte foci in the same lesion was higher in RP105$^{-/-}$ mice than in WT controls and lesions in RP105$^{-/-}$ mice appeared less organized than in WT animals (Figure 2F). A similar trend of exacerbated pathology and elevated bacterial burden in the chronic phase of infection in lungs of RP105$^{-/-}$ mice was seen after infection with a high dose of Mtb (Figure S4). However, accumulation of IL-12p40 in serum followed a different kinetic than after low dose infection and IL-12p40 levels were not found to be significantly different during the acute phase (Figure S4). Such loss of early differences in immune responses dependent on the infectious dose has been reported previously in a *Leishmania major* infection model (Divanovic et al., 2007) and suggests that the detection of RP105-dependent immune responses might be obscured under conditions of high initial pathogen burden.

Mtb lipoproteins activate macrophages in an RP105-dependent manner

In the cell wall of Mtb, peptidoglycan is covalently linked to arabino-galactan, which at its distal ends is esterified with mycolic acids (mycolyl-arabino-galactan peptidoglycan [mAGP]). Lipoproteins are located in the cell wall, and the outer layer of the mycobacterial cell envelope contains free glycolipids and lipids, which can be extracted with chloroform-methanol (Brennan and Nikaido, 1995; Young and Garbe, 1991). To identify which sub-cellular structure of Mtb confers RP105-dependent macrophage activation, BMM were stimulated with four different isolated mycobacterial cell envelope components: (i) purified mAGP, (ii) a chloroform-methanol extracted lipid fraction of whole cells, (iii) purified phosphatidyl inositol mannosides (PIM) and (iv) purified 19 kDa lipoprotein, an extensively studied immunomodulatory antigen of Mtb. There were no significant differences in TNF release between WT and RP105$^{-/-}$ macrophages after stimulation with mAGP, the lipid extract and the PIM preparation (Figure S5), suggesting that these cell wall constituents activate macrophages independently of RP105. TNF responses in TLR2$^{-/-}$ macrophages were abolished in response to total lipid and PIM preparations, while mAGP-induced TNF

production was largely independent of TLR2, consistent with previous data (Fortune et al., 2004).

Mycobacterial lipoproteins have been implicated in activating innate immune responses and it has been shown that the 19 kDa lipoprotein of Mtb is a potent TLR2 agonist (Brightbill et al., 1999; Thoma-Uszynski et al., 2001). Stimulation with the Mtb 19 kDa lipoprotein and a synthetic tri-palmitoylated hexadecapeptide, which contains the N-terminal amino acid sequence of the 19 kDa lipoprotein, resulted in significantly reduced TNF levels in both RP105$^{-/-}$ and TLR2$^{-/-}$ macrophages compared to WT macrophages (Figure 3B and C). Thus, the 19 kDa lipoprotein induced TNF production in an RP105- and TLR2-dependent manner. Of note, phosphorylation of p38, JNK and IκB-α signaling events involved in TNF gene and protein expression, was unimpaired in RP105$^{-/-}$ BMM after stimulation with the 19 kDa lipoprotein and the synthetic lipopeptide, similar to stimulation with Pam_3CSK_4 (Figure 3E). In splenic B cells from RP105$^{-/-}$ mice, upregulation of CD69 and CD86, surface proteins indicative of cellular activation, was reduced compared to WT B cells after stimulation with the synthetic lipopeptide (Figure S6). Taken together, these data identified the 19 kDa lipoprotein of Mtb as an RP105 agonist and raised the question if other RP105 agonists were present in Mtb.

An Mtb mutant lacking the 19 kDa lipoprotein (Stewart et al., 2005) was not different from WT Mtb in that it required RP105 for full induction of TNF release from BMM (not shown). This suggested that Mtb expresses other RP105 agonists, which might also be lipoproteins. To test this hypothesis, an Mtb strain with a mutated copy of the *lspA* gene (Δ*lspA*), which encodes the lipoprotein signal peptidase was used. Inactivation of LspA in Mtb has been demonstrated to interrupt maturation of lipoproteins, including the 19 kDa lipoprotein, at the stage of signal peptide cleavage (Banaiee et al., 2006; Sander et al., 2004). However, the fatty acid and mycolic acid composition as well as the overall architecture of the Mtb cell envelope remained unaltered in Mtb lacking LspA (Sander et al., 2004). WT and RP105$^{-/-}$ macrophages were infected with either wild type Mtb, the Δ*lspA* strain or the mutant strain reconstituted with the wild type allele (Δ*lspA* comp.). Compared to wild type Mtb, the Δ*lspA* strain elicited marginally reduced TNF levels in WT macrophages at 24 h post stimulation, which is in agreement with previous data (Banaiee et al., 2006). This suggests that other structural components of Mtb can partially compensate for the loss of mature lipoproteins in this mutant in inducing TNF. As expected, the TNF responses to wild type Mtb and complemented *lspA* mutant were significantly reduced in RP105$^{-/-}$ macrophages (Figure 3D). In contrast, infection with the Δ*lspA* strain resulted in similar TNF levels in WT and RP105$^{-/-}$ macrophages (Figure 3D). This demonstrates that Mtb lacking mature lipoproteins induced TNF production independently of RP105 and suggests a role for RP105 in signaling the presence of mature Mtb lipoproteins.

RP105 interacts with TLR2

Because bacterial lipoproteins have been shown to activate cells via TLR2, we investigated if RP105 could interact with TLR2. The plasma membrane distributions of fluorescently labeled RP105 and TLR2 were visualized by confocal microscopy (Figure 4A). Both are integral membrane proteins that showed a largely uniform distribution pattern, and this was not detectably altered by stimulation with heat-killed Mtb (Figure 4A, and not shown). Fluorescent labeling and imaging of CD14 and MHCII showed that other membrane proteins exhibited similar distribution patterns (Figure 4A, not shown for MHCII). To determine if TLR2 and RP105 associate, TLR2 was aggregated at the cell surface with antibodies, and the effects on the distribution of RP105, CD14, and MHCII were analyzed. Incubation of BMM on ice with a specific primary antibody against TLR2, followed by cross-linking with a secondary antibody, led to formation of TLR2 patches in the cell membrane (Figure 4B). After fixation, cells were co-stained for RP105, CD14, and MHCII. RP105, but not CD14 or MHCII,

redistributed with patched TLR2 (Figure 4B and not shown for MHCII). Fluorescence intensity line scans acquired over a distance of approximately 35 μm along the periphery of cells demonstrated almost complete overlap of the TLR2 and RP105, but not CD14, specific signals (Figure 4C). These data suggest a close association between TLR2 and RP105, rather than non-specific co-aggregation of all membrane proteins. Co-patching of RP105 and TLR2 was observed in unstimulated and Mtb-stimulated macrophages (Figure 4B and not shown). Analyses of $TLR2^{-/-}$ and $RP105^{-/-}$ macrophages showed specificity of the staining and demonstrated unaltered expression and distribution of TLR2 and RP105 if either collaboration partner was not expressed (Figure 4D).

We next examined if TLR2 physically interacted with RP105. Ba/F3 cells transfected with RP105/MD-1 and TLR2 as well as RAW264.7 macrophages were used. Flow cytometry confirmed surface expression of endogenous RP105 and TLR2 by RAW264.7 macrophages and successful transfection of Ba/F3 cells (not shown). RP105 and TLR2 bi-directionally co-immunoprecipitated in both experimental settings (Figure 4E and F) indicating a physical association between both receptors. In contrast an isotype-matched control antibody did not precipitate RP105 or TLR2 (Figure 4E and F). These data demonstrate a physical interaction between RP105 and TLR2 and suggest that RP105/MD-1 and TLR2 are present in heteromeric complexes in the cell membrane of macrophages.

RP105/MD-1 cooperates with TLR2 in macrophage activation by Mtb

RP105 lacks an intracellular TIR domain and may require a signaling-competent partner (Kimoto et al., 2003) to participate in macrophage activation. Because RP105 interacted physically with TLR2, we tested if RP105 cooperates with TLR2 to trigger Mtb-induced macrophage activation. RP105/MD-1 can form a complex with TLR4/MD-2 and negatively regulate LPS-induced activation of macrophages and DCs (Divanovic et al., 2005). Therefore, we also investigated if TLR4 is involved in RP105-dependent responses to Mtb. TNF and IL-12p40 levels were significantly reduced in supernatants from $RP105^{-/-}$ and $TLR2^{-/-}$ cells (Figure 5A). TLR2 deficiency had a more extensive impact on cytokine production than RP105 deficiency, indicating that Mtb triggers TLR2-dependent pathways leading to cytokine production, which are independent of RP105. Cytokine production by $TLR2^{-/-}$ cells was dramatically reduced but not abolished and the additional lack of RP105 in $RP105/TLR2^{-/-}$ cells did not result in further decreased cytokine levels. These data suggest that RP105 required the presence of TLR2 to contribute to Mtb-induced macrophage activation. In contrast, TNF and IL-12p40 levels in supernatants from Mtb-infected $TLR4^{-/-}$ macrophages were similar to those of WT cells (Figure 5A) and in macrophages lacking both TLR4 and RP105 cytokine levels were reduced to those measured in $RP105^{-/-}$ cells. Thus, Mtb induced TNF and IL-12p40 production independently of TLR4 and the interaction of RP105 with TLR4 (Divanovic et al., 2005) did not appear to affect Mtb-induced cytokine responses of macrophages.

To further investigate the cooperation of RP105 and TLR2, WT and $RP105^{-/-}$ BMM were pre-incubated with an inhibitory anti-TLR2 antibody (Meng et al., 2004) and infected with Mtb. The Mtb-induced TNF release was significantly inhibited by the anti-TLR2 antibody in both WT and $RP105^{-/-}$ macrophages (WT 65% inhibition, P=0.024; $RP105^{-/-}$ 62% inhibition, P=0.035), compared to BMM without antibody (Figure 5B). The IL-12p40 response was inhibited by 57% in WT cells (P=0.029) in the presence of the anti-TLR2 antibody. In $RP105^{-/-}$ BMM, the anti-TLR2 antibody inhibited IL-12p40 release somewhat, but this was statistically significant in only 2 out of 6 experiments (Figure 5B). Thus, in the absence of RP105, cytokine release in response to Mtb required, at least in part, TLR2. These data support that Mtb-induced RP105-dependent responses are triggered via TLR2 but that Mtb also stimulates TLR2-dependent responses that are RP105-independent.

A blocking antibody against murine RP105 is not available. However, an inhibitory antibody against MD-1 had previously been used to demonstrate a requirement for MD-1 in RP105-mediated modulation of LPS-driven B cell activation (Nagai et al., 2002). To investigate if MD-1 was required for the Mtb-induced cytokine release in macrophages, WT BMM were exposed to Mtb in the presence of the inhibitory anti-MD-1 or an isotype control antibody. Mtb-induced IL-12p40 and TNF release of WT BMM was inhibited by 60% (P=0.028) and 53% (P=0.012) in cultures treated with the anti-MD-1 antibody (Figure 5C). In contrast, in parallel cultures of Mtb-infected RP105$^{-/-}$ macrophages, release of IL-12p40 and TNF was not impaired by the anti-MD-1 antibody (Figure 5C). Expression of MD-1 on RP105$^{-/-}$ BMM was reduced, but not abolished, compared to that on WT BMM (Figure S1B). Because inhibition of residual MD-1 did not affect IL-12p40 and TNF release by RP105$^{-/-}$ BMM, we conclude that MD-1 contributes to the Mtb-induced cytokine response only in the presence of RP105. TLR2$^{-/-}$ BMM expressed RP105 and MD-1 at levels similar to those in WT BMM (Figure S1B). The inhibitory anti-MD-1 antibody did not significantly reduce the Mtb-induced TNF and IL-12p40 release by TLR2$^{-/-}$ BMM (Figure 5D) indicating that RP105/MD-1 only contributed to Mtb-induced cytokine responses if TLR2 was also expressed. Altogether, these data suggest that the RP105/MD-1 complex cooperates with TLR2 to activate macrophages in response to Mtb infection and is required for a maximal cellular response.

Discussion

Mycobacteria and their subcellular components stimulate TLR signaling in host cells leading to transcriptional activation, release of inflammatory mediators, and anti-microbial responses (Bafica et al., 2005; ; Means et al., 2001; Reiling et al., 2002; Shi et al., 2005; Thoma-Uszynski et al., 2001; Underhill et al., 1999). In this study we demonstrated that RP105 was required for optimal Mtb-induced cytokine release *in vitro* and *in vivo*. In contrast to its role as inhibitor of LPS-induced macrophage and DC activation, RP105 participates in signaling that lead to transcriptional activation of some inflammatory genes, e.g. IL-12p40, and post-transcriptional regulation of others, such as TNF, IL-10 or RANTES, in macrophages stimulated with Mtb.

RP105 lacks the intracellular TIR domain that is required for TLR-signal transduction via adapters such as MyD88. Therefore, RP105 is thought to associate with a co-receptor, which can transduce a signal into the cell (Kimoto et al., 2003). We provide three lines of evidence that the RP105/MD-1 complex cooperates with TLR2 to enhance recognition of Mtb and subsequent immune cell activation: First, RP105 redistributed with cross-linked TLR2 in the plasma membrane of macrophages and both receptors co-immunoprecipitated in two different cell types. The receptor complex was observed in uninfected macrophages indicating a constitutive interaction of RP105/MD-1 and TLR2. Similarly, TLR2/TLR1- and TLR2/TLR6-heteromers have been described to pre-exist in the cell membrane, independently of their respective specific agonists (Triantafilou et al., 2006).

Second, the Mtb 19 kDa lipoprotein and tri-palmitoylated hexadecapeptide, two confirmed TLR2 agonists (Thoma-Uszynski et al., 2001) (Figure 3B), stimulated macrophage and B cell activation in an RP105-dependent manner. In contrast, RP105 was dispensable for macrophage responses to the tri-palmitoylated TLR2/1 agonist Pam_3CSK_4 and the TLR2-dependent lipid and glycolipid preparations of Mtb (Figures 3A and S5) (Nagai et al., 2005). The palmitoylation-patterns of the cysteine residues in Pam_3CSK_4 and the synthetic N-terminal peptide of the 19 kDa lipoprotein are identical. This suggests that the peptide/protein part of lipoproteins is important for RP105 agonist activity. Similarly, the amino acid sequence of lipoproteins can contribute to the specificity of recognition by TLR2/TLR1 and TLR2/TLR6 heteromers (Buwitt-Beckmann et al., 2005). The challenge at hand will be to determine which peptide feature confers RP105-agonist-properties to Mtb lipoproteins. Furthermore, it remains to be elucidated if such agonists bind directly to RP105/MD-1 or if RP105/MD-1 regulates

their recognition by TLR2 and potentially other receptors. Perhaps RP105 provides a level of specificity to innate immune recognition of microbial lipoproteins by TLR2.

Third, for optimal production of IL-12p40 and TNF in response to Mtb, both RP105/MD-1 and TLR2 had to be present on macrophages, and RP105/MD-1 required TLR2 to enhance Mtb-induced cytokine responses. TNF and IL-12p40 levels released from Mtb infected $TLR2^{-/-}$ and $RP105/TLR2^{-/-}$ macrophages were lower than those from $RP105^{-/-}$ cells and the RP105-independent TNF response to Mtb was partially TLR2-dependent. Thus, not all Mtb-induced TLR2-dependent responses require RP105. TLR9 in synergy with TLR2 mediated IL-12p40 production by Mtb-stimulated macrophages and both receptors collaborated in conferring resistance to Mtb (Bafica et al., 2005). Also, TLR9-dependent activation of $RP105^{-/-}$ DCs and B cells by oligonucleotides (CpG) was unimpaired (Divanovic et al., 2005; Nagai et al., 2005). Therefore, the residual IL-12p40 production of Mtb-stimulated $RP105^{-/-}$ and $TLR2^{-/-}$ macrophages observed in our studies might have resulted from TLR9 driven signals.

Taken together, our data suggest that RP105/MD-1 forms a heteromeric complex with TLR2. Similar to the accessory proteins CD36 and CD14, which enhance TLR2 signaling in response to diacylglycerides and triacylated lipopeptides respectively (Hoebe et al., 2005; Nakata et al., 2006), the RP105/MD-1 complex augments cellular responses to Mtb and mycobacterial lipoproteins.

Knowledge about the role of RP105 in mediating host immune responses is limited. $RP105^{-/-}$ mice have been reported to exhibit impaired antibody production in response to LPS, heat-killed *Escherichia coli*, and synthetic di- and tri-acylated lipopeptides (Nagai et al., 2005). LPS-challenged $RP105^{-/-}$ mice have also been shown to exhibit elevated TNF levels in the serum, along with accelerated and amplified signs of endotoxicity (Divanovic et al., 2005). Dermal infection of $RP105^{-/-}$ mice with *Leishmania major* led to attenuated disease, with more rapid control of the parasite burden (Divanovic et al., 2007). Here, we have provided evidence that, during infection with live, virulent Mtb, RP105 is required to optimally induce early systemic cytokine responses, such as IL-12p40 production and contributes to control of bacterial replication and lung pathology.

Although moderate, the increased susceptibility of $RP105^{-/-}$ mice to Mtb infection is unexpected. Others and we have shown that $TLR2^{-/-}$ deficient mice controlled mycobacterial replication similar to WT mice after infection with a low dose of Mtb (Bafica et al., 2005; Holscher et al., 2008; Reiling et al., 2002; Shi et al., 2005). However, in one case increased pathology in lungs of $TLR2^{-/-}$ mice was observed (Bafica et al., 2005) and another study described elevated bacterial loads five months post infection in $TLR2^{-/-}$ mice (Drennan et al., 2004). Besides its pro-inflammatory functions, prolonged TLR2 signaling induced by Mtb and mycobacterial structures has been shown to inhibit MHCII expression, antigen processing and IFNγ-responsiveness in macrophages (Fortune et al., 2004; Gehring et al., 2003; Noss et al., 2001; Pai et al., 2004). Although the net contributions of these divergent TLR2 functions *in vivo* remain to be determined, such lack of inhibition of T cell-activation and -effector mechanisms in $TLR2^{-/-}$ mice might lead to control of Mtb infection. Further studies are required to determine if, perhaps, RP105 contributes to some (e.g. inflammatory cytokine induction) but not all (e.g. inhibition of antigen presentation and IFNγ-responsiveness) TLR2-dependent signaling events, which could account for the increased susceptibility of $RP105^{-/-}$ mice to Mtb infection. The agonist-specificity of the RP105-contribution to macrophage activation by TLR2 agonists provides a first hint towards a selective involvement of RP105 in TLR2 dependent events. In addition, it appears that RP105 affects non-canonical TLR signaling, because the diminished TNF release in response to the TLR2-agonists 19 kDa lipoprotein and synthetic decahexapeptide was accompanied by indistinguishable patterns of

phosphorylation of MAP kinases and IκB-α in RP105$^{-/-}$ and WT macrophages. Thus, RP105 might be involved in inducing alternative regulatory mechanisms of TNF production, such as activation of Bmx (bone marrow tyrosine kinase in chromosome X) (Palmer et al., 2008), TACE (TNF-α converting enzyme) (Black et al., 1997; Moss et al., 1997), or HMGB1 (high-mobility group box protein 1) signaling (Andersson et al., 2000). In addition, we speculate that in a complex *in vivo* setting RP105 might modulate not only TLR2 signaling but may also participate in TLR2-independent immune responses during infection with Mtb, potentially by enhancing signaling of other receptors. Further insight into additional functions of RP105 might help understand some of the differences observed in RP105$^{-/-}$ and TLR2$^{-/-}$ mice.

In conclusion, RP105 is important for mediating Mtb-induced cellular immune responses by facilitating recognition of mycobacterial lipoproteins. The identification of RP105 as an accessory molecule for TLR2 and potentially other signaling receptors implies an unexpected role for the RP105/MD-1 complex in innate immune recognition that goes beyond LPS recognition.

Experimental Procedures

Bacteria, antibodies, reagents

Mtb H37Rv was from Dr. R. North (Trudeau Institute). The *lspA* (Rv1539) knockout was previously reported (Banaiee et al., 2006). To control for polar effects of the *lspA* deletion, the null mutant was complemented with *lspA* and the two flanking genes, *ansA* and Rv1540, on an integrative plasmid yielding strain Δ*lspA::lspA (*Δ*lspA comp)*. In one construct, a GC deletion was introduced into *lspA*, at base pair 386, to cause premature termination of LspA at codon 132 yielding strain Δ*lspA::lspA*$_{mut}$ (Δ*lspA*). Complemented clones were confirmed by immunoblotting for processing of MPT83 lipoprotein (Banaiee et al., 2006).

Mtb lacking the 19 kDa lipoprotein gene (Rv3763) and the complemented mutant were kindly provided by Dr. S.M. Newton and Dr. R.J. Wilkinson (Stewart et al., 2005). Synthetic tri-acylated lipopeptide (Pam_3CSK_4), synthetic tri-palmitoylated hexadecapeptide, containing the N-terminal amino acids of the 19 kDa lipoprotein, and peptidoglycan were from EMC Microcollections; purified 19 kDa lipoprotein from H37Rv, mycolyl-arabino-galactan-peptidoglycan (mAGP), total lipid extract and phosphatidylinositol mannosides (PIM1/2) were obtained from Colorado State University via the TB Vaccine Testing and Research Materials Contract; LPS (*Salmonella enterica*, serovar friedenau H909) was a gift from Dr. H. Brade, Research Center Borstel, Germany; inhibitory anti-mouse TLR2 (T2.5), mouse IgG1, anti-mouse RP105 (RP/14), inhibitory anti-mouse MD-1 (MD113), anti-mouse CD14 (Sa2-8), anti-mouse MHCII (M5/114.15.2), rat IgG2b, rat IgG2a were from eBioscience.

Mice

RP105$^{-/-}$ mice have been described previously (Ogata et al., 2000). TLR2$^{-/-}$ mice were from Dr. S. Akira (Takeuchi et al., 1999). RP105/TLR2$^{-/-}$ and RP105/TLR4$^{-/-}$ mice were obtained by crossing RP105$^{-/-}$ mice with TLR2$^{-/-}$ or TLR4$^{-/-}$ mice. Double-deficiency was confirmed by PCR. Bone marrow from double KO mice was kindly provided by Dr. C. L. Karp and Dr. S. Divanovic. C57BL/6 WT mice were from Jackson Laboratory.

Cellular responses of primary mouse macrophages

Bone marrow cells from 10 week-old mice were differentiated into macrophages for 7d in DMEM with 20% L-cell medium, 10% FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, 10 mM HEPES. BMM were infected as described (Ehrt et al., 2001) or were stimulated with bacterial components as indicated. Lipid components of Mtb were layered onto cell culture plates using

isopropanol (Rao et al., 2005). After complete evaporation of the solvent macrophages were seeded into coated wells.

For inhibition of cytokine release, BMM were incubated for 1h with antibodies as indicated before stimulation. Cytokine and chemokine concentrations in supernatants were determined by ELISA (IL-12p40, BD OptEIA; R&D Duo Set all others). For PCR total RNA was isolated post-stimulation and reverse transcribed using oligo-dT nucleotides. For quantitative TaqMan PCR (ABIPrism 7900HT, Perkin Elmer; LightCycler 480, Roche) sequences of gene specific primer pairs and probes were as described (Shi et al., 2005; Shi et al., 2003); sequences for GAPDH, IL-12p40, MD-1 and RP105 primers and probes are available upon request. Gene expression was normalized to expression of GAPDH ($2^{(\text{crossing point GAPDH-crossing point gene})}$).

For immunoblots cell lysates were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Protein detection was performed using anti-p38, anti-phospho-p38, anti-phospho-JNK and anti-phospho-IκB-α antibodies (Cell Signal Technologies), a secondary IRDye-800-conjugated donkey anti-rabbit antibody and the Odyssey Infrared Imaging System (LI-COR Biosciences).

Expression of surface markers

BMM were left untreated or stimulated with Mtb (multiplicity of infection [MOI] 1 or 10) for 18 h. Cells were stained with phycoerythrin (PE) conjugated specific anti-RP105 or anti-MD-1 antibodies or an Alexa647-coupled anti-TLR2 antibody. Matched isotype antibodies were used as controls. Splenic B cells were prepared by CD43-negative sorting (AutoMACS) and stimulated as indicated for 24 h. Cells were stained with biotinylated anti-CD86 (GL-1) and biotinylated anti-CD69 (H1.2F3) antibody (eBioscience), followed by PE-conjugated streptavidin (Southern Biotech). Data were analyzed using WinMDI 2.8 and FlowJo softwares.

Microscopy

BMM were seeded into 1.5 mm glass bottom dishes (gift of Dr. Tim McGraw, Weill Cornell Medical College) and left untreated or stimulated with heat-killed Mtb for 2 h at 37°C. Cells were fixed and permeabilized with 3.3% paraformaldehyde (PFA) + saponin, blocked with 5% FBS, incubated with anti mouse-RP105 or rat IgG2a and subsequently incubated with Alexa546-conjugated goat anti-rat antibody (Molecular Probes).

To stain TLR2 and RP105, cells were treated with 10% mouse serum and incubated at 4°C with anti-TLR2 or matched IgG1 antibody. Each was pre-labeled with Alexa488-conjugated Fab fragments (Zenon, Molecular Probes). Cells were washed, fixed with 3.3% PFA and stained for RP105 expression as described above. For patching experiments, macrophages were washed, blocked with 10% mouse serum and incubated at 4°C with anti-TLR2 or IgG1 antibody pre-labeled with Alexa488-conjugated Fab fragments. Crosslinking was achieved by incubation with a goat-anti-mouse IgG $F(ab')_2$ for 3 h on ice. After PFA fixation, cells were counterstained for RP105, CD14 or MHCII.

A Zeiss LSM510 laser scanning confocal microscope (63x/1.4 numerical aperture oil objective) was used for image acquisition. Fluorescence images of all samples with similar stainings within one experiment were acquired under identical conditions, and images are displayed at the same contrast so that they are directly comparable. Images were processed using LSM510 software (low pass filter, funnel 3×3; identical parameters for brightness and contrast for samples stained with the same antibody). Line scans of approximately 35 μm along peripheries of cells were obtained using the “profile” function.

Immunoprecipitation (IP)

Antibodies against mouse RP105 (RP/14), mouse TLR2 (CB225; Wistar rats immunized with RAW264.7 cells emulsified in zymosan, specificity for TLR2 confirmed via FACS using WT, TLR2−/−, TLR1−/−, TLR6−/−, RP105−/− BMM [Kobayashi et al. unpublished]), or isotype-matched rat IgG2a were immobilized on NHS-activated Sepharose beads (GE Healthcare Sciences) and used for IP. Precipitates were separated by SDS-PAGE and blotted onto PVDF membrane (Millipore) and immunobloted using a polyclonal rabbit anti-mouse RP105 antibody (rabbits immunized with oligo-peptide corresponding to aa 651–661 of mRP105 [KYFLRWKYQHI]; specificity confirmed by Immunoblot) and a rat anti-mouse TLR2 antibody (1–10; IgG2a; Wistar rats immunized with GST-tagged cytoplasmic portion of mTLR2; specificity confirmed by Immunoblot) followed by alkaline phosphatase-coupled secondary goat antibodies (AmericanQualex); NBT and BCIP solutions (Promega) were used for development.

Mouse infections

Ten weeks old mice were infected with Mtb using an aerosol chamber (Glas-Col Inc.) by nebulizing 5 ml bacterial single cell suspension in PBS at ~2×10^8 bacilli/ml (low dose; inoculum sizes 175±98 CFU per lung, determined by plating lung homogenates 24h post infection) or ~2×10^9 bacilli/ml (high dose; inoculum sizes 5600±2600 CFU per lung). Lungs were homogenized in PBS and plated for CFU at indicated times. The upper left lobe was fixed in 10% formalin and lung sections were stained with hematoxylin and eosin (H&E). IL-12p40 concentrations in serum were determined by ELISA (Beckton Dickinson). All animal studies were approved by the Institutional Animal Care and Use Committee, Weill Cornell Medical College.

Statistical analyses

Raw data of experimental groups were analyzed by two-tailed *t* test. mRNA levels and cytokine concentrations are represented as mean ± standard deviation (sd) of triplicates. For depiction of results of multiple experiments, data are represented as means of values of individual experiments ± standard error (se). To measure the inhibitory effect of antibodies in multiple experiments, cytokine concentrations under different culture conditions were normalized to control conditions and represented as means ± standard error (se).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Senad Divanovic and Dr. Christopher L. Karp for providing mouse bone marrow, reagents, and helpful discussions. We are grateful to Dr. William A. Muller for evaluation of the histopathology and Jennifer L. Small for help with microscopy experiments. We thank Diana Dou and Toshiko Odaira for taking care of mice, and Aimee Beaulieu, Dr. Andrea Cerutti and Dr. Carl Nathan for critical review of this manuscript. This work was supported in part by the Stony Wold-Herbert Fund, Inc. (A.B.), NIH HL68525 (S.E.), AI46097 (J.D.E.), and the I.T. Hirschl Trust (S.E.).

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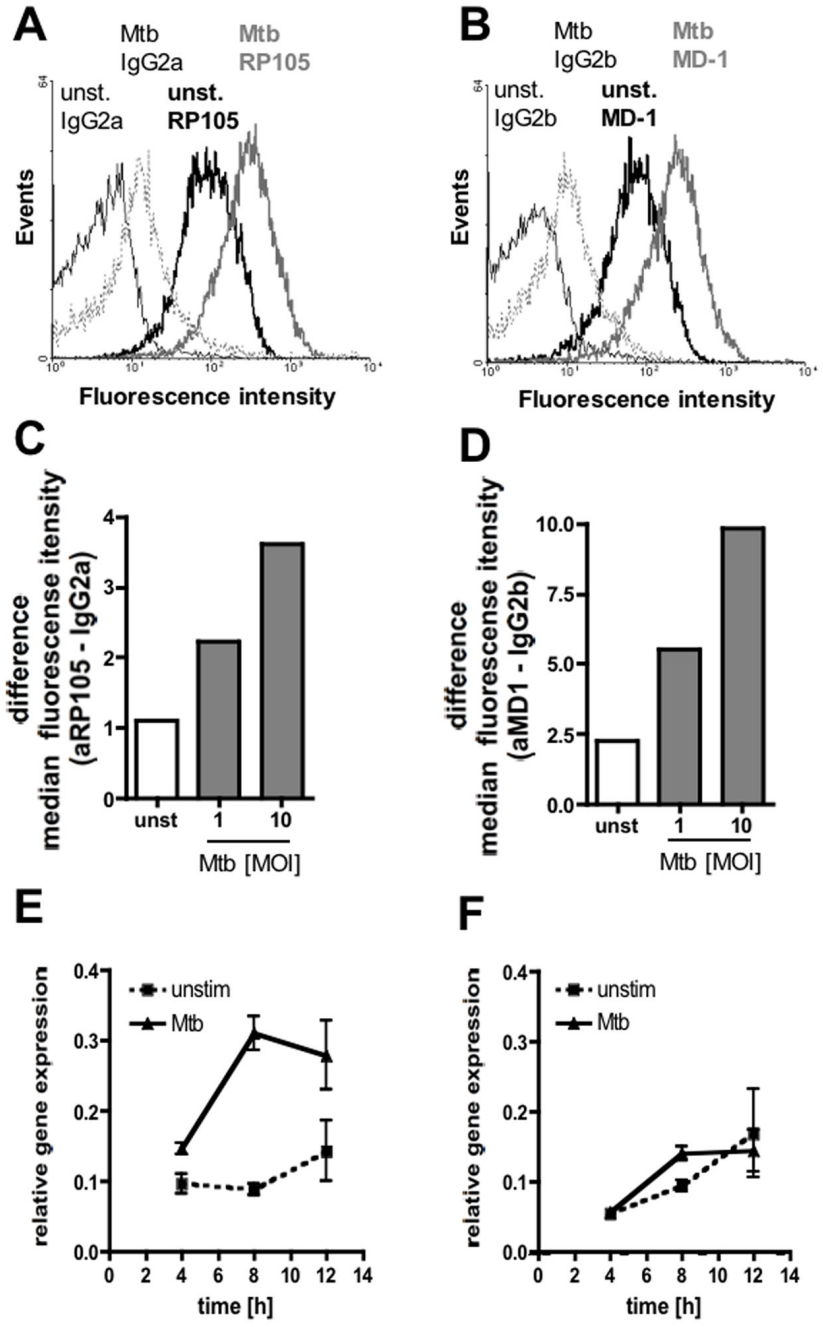

Figure 1.

Mtb-induced up-regulation of RP105 and MD-1 expression by macrophages. Expression of RP105 (**A**) and MD-1 (**B**) by BMM, unstimulated (unst.) or infected with Mtb (MOI 10; 18 h). Data are from 1 experiment representative of 3. Mtb-induced increase in surface expression of RP105 **(C)** and MD-1 **(D)** by macrophages is dose dependent. Median fluorescence intensities (MFI) for isotype control stainings were subtracted from MFI for specific antibody stainings. One representative experiment of 2 is depicted. RP105 **(E)** and MD-1 **(F)** mRNA levels normalized to GAPDH from Mtb-stimulated and unstimulated BMM at indicated times. Data represent means of triplicates ± sd. Results from 1 experiment out of 2 are shown.

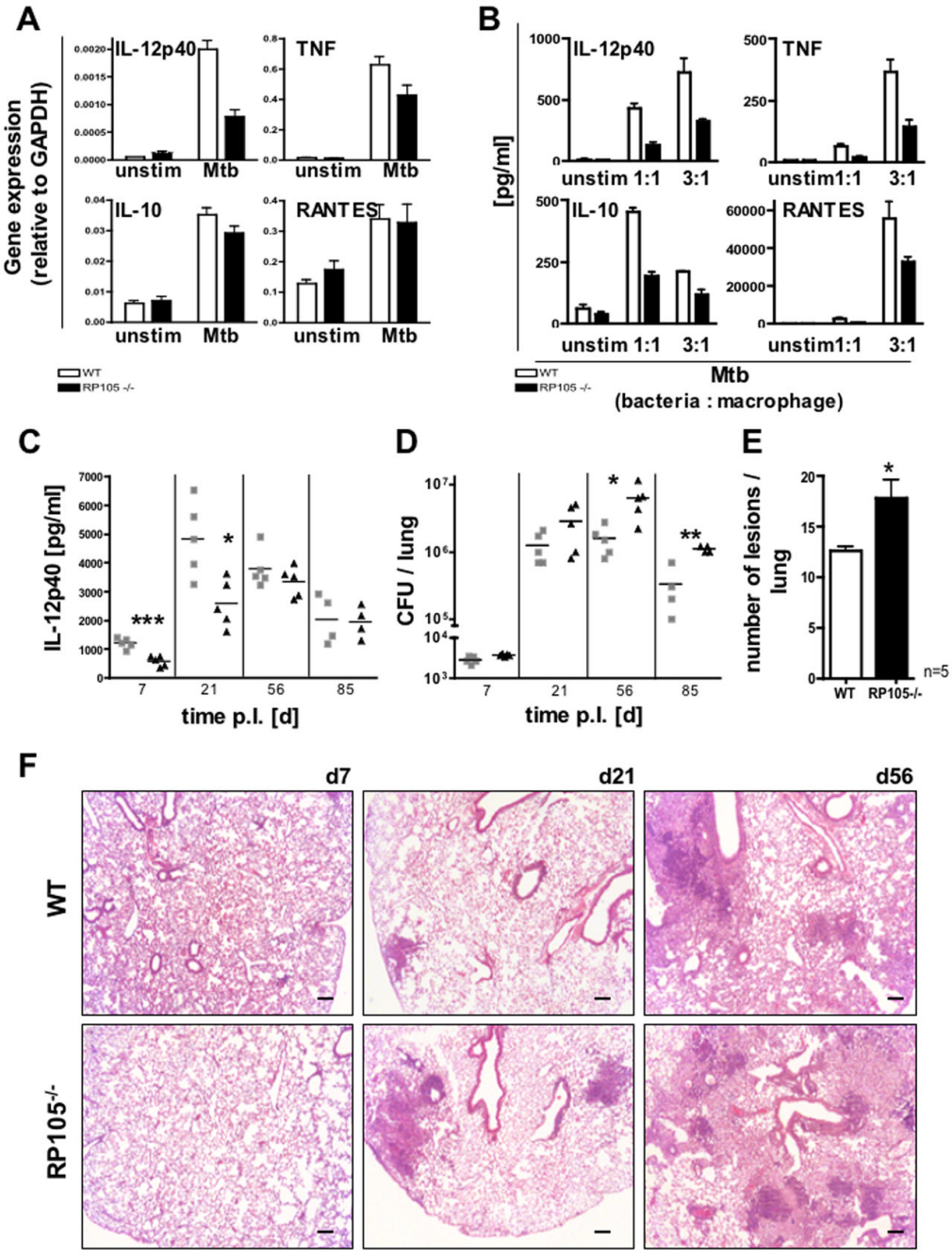

Figure 2.

RP105 is required for optimal macrophage activation in response to Mtb infection and participates in control of Mtb infection *in vivo*. (**A**) mRNA levels in BMM stimulated with Mtb (MOI 3, 4 h) or unstimulated. Data represent means of triplicates ± sd. Comparison of raw data of three independent wells by unpaired *t* test showed a significant difference in IL-12p40 mRNA expression (**P*=0.0032), but no differences for TNF, IL-10, and RANTES. One representative experiment out of 2 is shown. (**B**) Cytokine and chemokine release by Mtb-stimulated WT and RP105$^{-/-}$ macrophages (MOI 1 or 3, 24 h). Data represent means ± sd of 3 parallel cultures in a single experiment and are representative of 3 independent experiments. **(C)-(F)** Infection of WT and RP105$^{-/-}$ mice with a low dose of Mtb (175 ± 98 CFU/lung).

(**C**) IL-12p40 concentrations in serum at indicated times post infection (p.i.). Means are indicated. Groups were compared by unpaired *t* test. **P*<0.05, ***P*<0.002, ****P* ≤ 0.0005. Results of 1 experiment out of 2 are shown. (**D**) Colony forming units (CFU) in lungs monitored over time. Means are indicated. Groups were compared by unpaired *t* test. **P*<0.05, ***P*<0.002. Results of 1 experiment out of 2 are shown. (**E**) Number of lesions per lung section on day 56. Means of the number of lesions in one lung section of 5 mice per group ± se are shown. Groups were compared by unpaired *t* test. **P*=0.0239. (**F**) Exacerbated pathology in lungs of Mtb infected $RP105^{-/-}$ mice. Tissue sections were stained with hematoxylin and eosin. Representative sections from 1 mouse per genotype out of 5 are shown; 2 independent experiments were performed. (Bar = 100 μm).

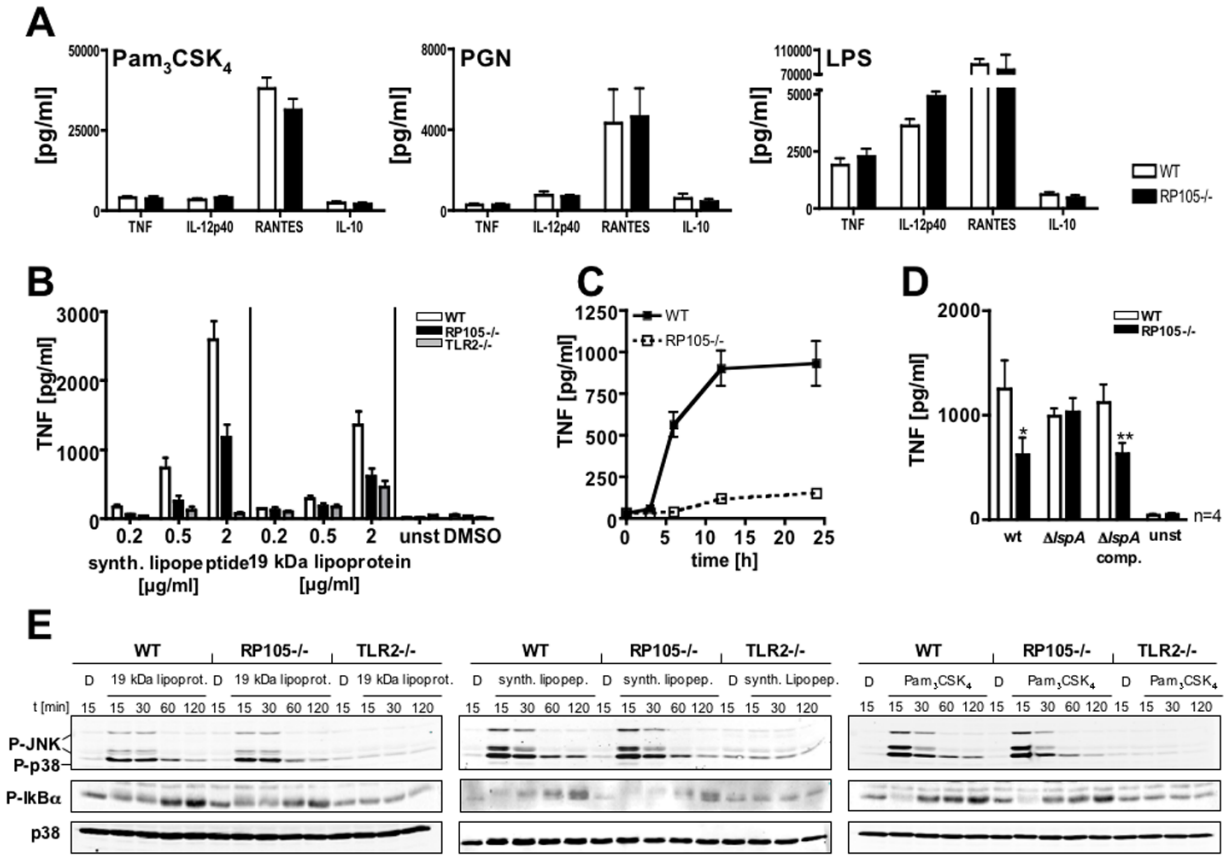

Figure 3.

Mtb lipoproteins activate macrophages in an RP105-dependent fashion. (**A**) Cytokine responses of WT and RP105$^{-/-}$ macrophages after 24 h stimulation with Pam_3CSK_4 (10 ng/ml), peptidoglycan (PGN, 10 μg/ml), or LPS (10 ng/ml), respectively. Means ± sd of triplicate wells of 1 representative out of 3 (Pam_3CSK_4, LPS) or 2 (PGN) experiments are shown. Of note, the minor enhancement of cytokine production seen in RP105$^{-/-}$ cells, might be due to a low amount of TLR2-dependent agonists in the LPS preparation; heterologous TLR stimulation has been shown to overcome RP105-mediated inhibition of TLR4 stimulation (Divanovic et al., 2005). (**B**) TNF concentrations in supernatants of WT, RP105$^{-/-}$, and TLR2$^{-/-}$BMM after stimulation with the 19 kDa lipoprotein of Mtb or the synthetic lipopeptide (resembling the 16 N-terminal amino acids of the 19 kDa lipoprotein) for 24 h. Raw data of 3 independent wells were compared by unpaired *t* test. Data are means ± sd of triplicate wells of 1 experiment representative of 4. (**C**) TNF concentrations in culture supernatants of WT and RP105$^{-/-}$ after stimulation with the synthetic lipopeptide (1 μg/ml). Data are means ± sd of 5 independent wells of one experiment representative of 2. (**D**) TNF produced by WT and RP105$^{-/-}$ macrophages infected with wild type Mtb, Δ*lspA* or Δ*lspA* comp. (MOI 3) was measured after 24 h. Data represent means ± se of 4 independent experiments (performed in triplicates). Paired *t* test was used for comparison. *P<0.05; **P<0.005; ***P<0.0001. (**E**) Immunoblot analyses of p38-, JNK-, and IκB-α-phosphorylation (P) on lysates of WT, RP105$^{-/-}$, and TLR2$^{-/-}$ BMM after stimulation with 19 kDa lipoprotein, synthetic lipopeptide (both 1 μg/ml), Pam_3CSK_4 (10 ng/ml), or treatment with DMSO (D) for indicated times. Total amounts of p38 were detected as control for equal loading. Data represent 1 out of 3 independent experiments.

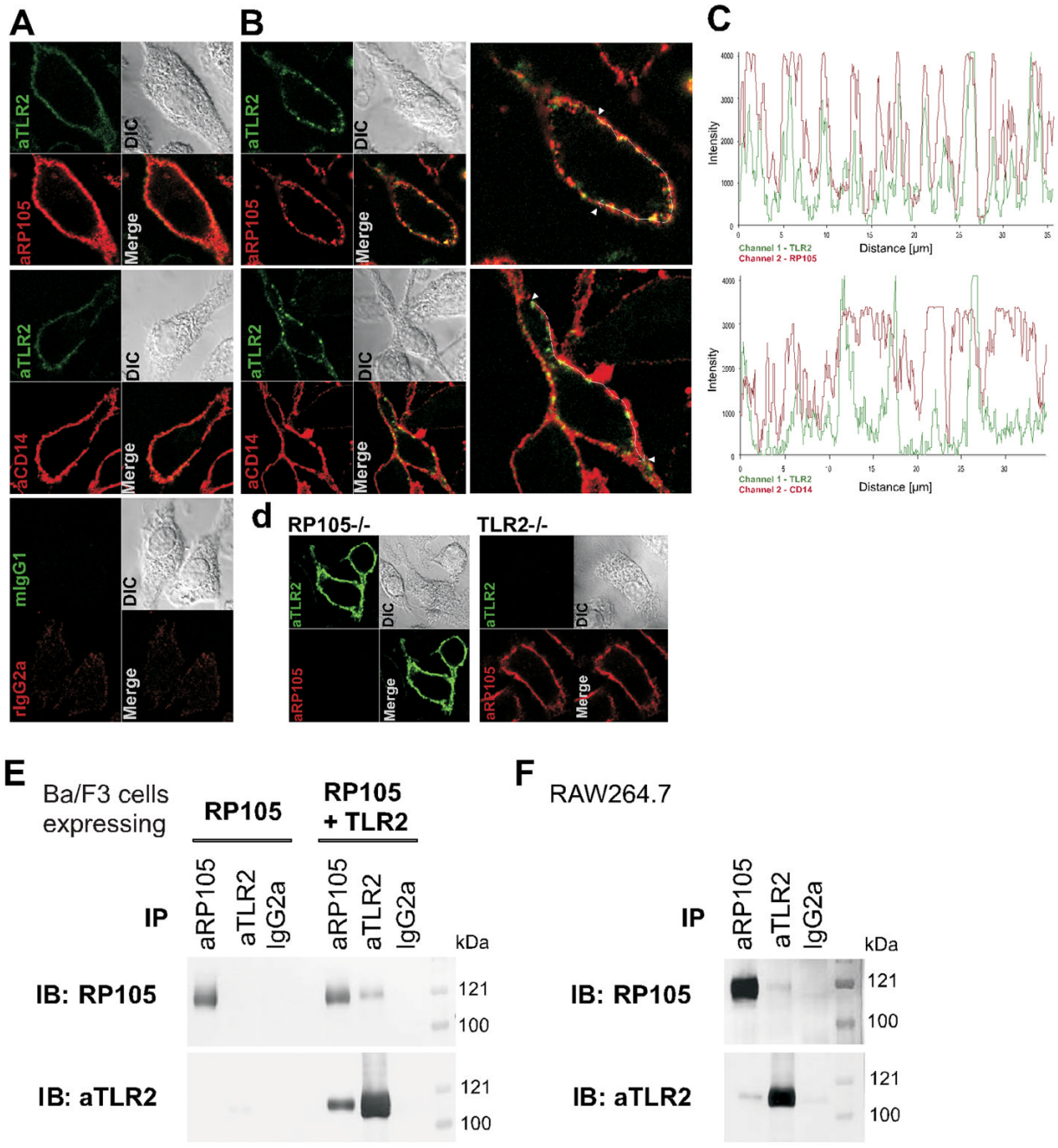

Figure 4.

RP105 and TLR2 co-localize and associate in the cell membrane. (**A**) WT BMM were incubated with heat killed Mtb for 2 h and stained with anti-TLR2 or mIgG1 isotype control antibodies that were specifically labeled with Alexa488-conjugated Fab fragments. After PFA fixation cells were incubated with anti-RP105 or anti-CD14 antibody or matched isotype control (rIgG2a) followed by an Alexa547 conjugated anti-rat secondary antibody. Samples were analyzed by confocal microscopy (DIC = differential interference contrast in transmitted light images; Merge = overlay of images acquired in different fluorescence channels). (**B**) WT macrophages were incubated with heat killed Mtb for 2 h and stained with anti-TLR2 or IgG1 isotype control antibodies that were specifically labeled with Alexa488-conjugated Fab

fragments. Patching was achieved by crosslinking of the anti-TLR2 or mouse IgG1 antibodies with an anti-mouse antibody. After fixation cells were co-stained for RP105 or CD14 expression as described under (A). Data shown are representative for 4 (RP105) and 2 (CD14) independent experiments. Enlarged overlay images are depicted on the right. White arrowheads mark start and end points of line scans for acquisition of fluorescence profiles depicted in panel (C). (**C**) Fluorescence profiles for TLR2 (green) and RP105 or CD14 (red) specific staining acquired along the periphery of the cell (approximately 35 μm) as indicated by the thin white line (arrowheads mark start and end point in enlargement in panel (B)). (**D**) $RP105^{-/-}$ and $TLR2^{-/-}$ BMM were stained for RP105 or TLR2 as described for WT cells (A) and subject to confocal microscopy. (**E, F**) Immunoprecipitation (IP) and immunoblot (IB) of lysates of (**E**) Ba/F3 cells stably transfected with RP105/MD-1 or RP105/MD-1 and TLR2 together and (**F**) RAW264.7 macrophages. Co-IP demonstrating association of RP105 and TLR2 was assessed by IB using anti-RP105 and anti-TLR2 antibodies. An isotype-matched rat IgG2a antibody and Ba/F3 cells transfected with RP105/MD-1 but not TLR2 were used as controls. One representative of 3 independent experiments is shown.

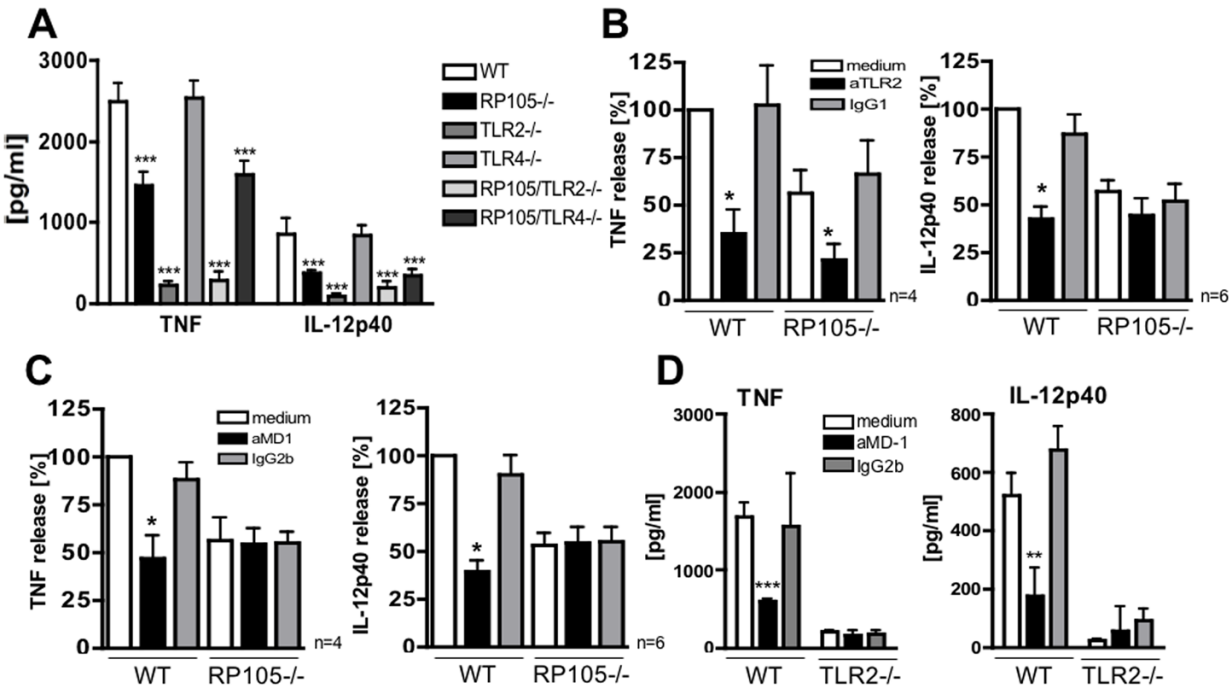

Figure 5.

RP105/MD-1 cooperates with TLR2. (**A**) WT, RP105$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, RP105/TLR2$^{-/-}$ and RP105/TLR4$^{-/-}$ BMM were infected with Mtb (MOI 3). Cytokine concentrations in culture supernatants were determined 24 h post infection. Data are means ± sd from 2 independent experiments each performed in triplicates. Raw data of independent wells of knock-out cells were compared to WT cells by unpaired *t* test. ***P<0.0001 (**B**) WT and RP105$^{-/-}$ BMM were pre-incubated with an anti-TLR2 antibody (1–2 μg/ml) or isotype-matched control antibody (IgG1) for 1 h and infected with Mtb (MOI 3). Cytokine release was measured after 24 h (TNF) and 48 h (IL-12p40). Four (TNF) and 6 (IL-12p40) independent experiments performed in triplicates were compared by normalizing to control conditions in WT macrophages (no antibody added), means ± se are shown. Raw data were compared by paired *t*-test. *P<0.05. (**C**) Concentrations of IL-12p40 and TNF in culture supernatants of WT and RP105$^{-/-}$ BMM that were infected with Mtb (MOI 3) after pre-incubation (1 h) with an inhibitory anti-MD-1 antibody (1–2 μg/ml) or isotype-matched control antibody (IgG2b). Four (TNF) and 6 (IL-12p40) independent experiments, each performed in triplicates were compared by normalizing to control conditions in WT BMM (no antibody added), means ± se are shown. Raw data were compared by paired *t* test. *P<0.05. (**D**) Concentrations of IL-12p40 and TNF in supernatants of WT and TLR2$^{-/-}$ BMM, pre-incubated with an anti-MD-1 or matched isotype control (IgG2b) antibody, after infection with Mtb (MOI 3). Raw data of 3 independent wells were compared to control conditions (medium) by unpaired *t* test. Data points are means ± sd of triplicate wells of 1 experiment representative of 3. **P=0.0088, ***P=0.0005. Cytokine levels in unstimulated macrophage cultures were below the detection limit and were not significantly influenced in the presence of antibody (not shown).