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ARTICLE

The N-terminal peptide moiety of the *Mycobacterium tuberculosis* 19 kDa lipoprotein harbors RP105-agonistic properties

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Summary: RP105-agonistic properties of synthetic lipopeptides mimicking the N-terminus of the *M. tuberculosis* 19kDa lipoprotein are conferred by a motif of polar amino acids

Abstract

Radioprotective 105 kDa (RP105, CD180) is a member of the Toll-like receptor (TLR) family that interacts with TLR2 and facilitates recognition of mature lipoproteins expressed by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. In this study, we used synthetic lipopeptide analogs of the *M. tuberculosis* 19 kDa lipoprotein to define structural characteristics that promote RP105-mediated host cell responses. A tripalmitoylated lipopeptide composed of the first 16 N-terminal amino acids of the *M. tuberculosis* 19 kDa lipoprotein induced RP105-dependent TNF and IL-6 production by macrophages. Di- and tripalmitoylated variants of this lipopeptide elicited an equivalent RP105-dependent response, indicating that while the lipid moiety is required for macrophage activation, it is not a determinant of RP105 dependency. Instead, substitution of two polar threonine residues at positions 7 and 8 with nonpolar alanine residues resulted in reduced RP105 dependency. These results strongly suggest that the amino acid composition of the *M. tuberculosis* 19 kDa lipoprotein, and likely other mycobacterial lipoproteins, is a key determinant of RP105 agonism.

KEYWORDS

innate immunity, lipopeptide, macrophage, mycobacteria, Toll-like receptor (TLR)

1 | INTRODUCTION

The *Mycobacterium tuberculosis* genome encodes over 90 confirmed and predicted lipoproteins as both cell wall-bound and secreted molecules, with functions in cell adhesion and invasion, signaling and transmembrane transport.[1] Acylation of lipoproteins is facilitated by the lipobox that serves as a recognition site for enzymes involved in the lipoprotein biosynthesis pathway. The lipobox consists of three amino acids followed by a universally conserved cysteine residue to which fatty acids are covalently attached.[2] Fatty acids (collectively referred to here as acyl chains) contained within mycobacterial lipoproteins include, but are likely not limited to, palmitic, oleic, and tuberculostearic acid.[3] Mycobacterial lipoproteins are triacylated, and contain two ester-bound acyl residues attached to the conserved N-terminal cysteine residue forming a diacylglyceryl moiety, and an additional amide-bound acyl chain attached to the amino group.[2]

Multiple Toll-like receptors (TLR) have roles in the host recognition of *M. tuberculosis*, including TLR2,[4–6] TLR4,[7] TLR8,[8,9] and TLR9.[10–12] *M. tuberculosis* lipoproteins such as the 19 kDa lipoprotein are well-known TLR2 agonists.[13] TLR2 recognizes lipoproteins in conjunction with TLR1[14] and TLR6,[15] which initiates conserved intracellular signaling events that result in the activation of a conserved host defense program, including the expression of inflammatory cytokines.[16] Synthetic lipopeptides have been instrumental in defining the current paradigm of coreceptor involvement in the TLR2-mediated recognition of lipoproteins.[13,17] Triacylated lipopeptides such as Pam_3CSK_4 are recognized by TLR2 in conjunction with TLR1,[13] whereas diacylated lipopeptides such as Pam_2CSK_4 are recognized in conjunction with TLR6.[17] Aside from an extensive number of functional studies, this paradigm is supported by X-ray crystallography studies confirming the

Abbreviations: BMDM, bone marrow-derived macrophage; LRR, leucine-rich repeat; Pam_2C, S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteinyl; Pam_3C, N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteinyl; RP105, Radioprotective 105 kDa; TIR, Toll-Interleukin-1-Receptor; TLR, Toll-like receptor

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TLR2/TLR1 complex with cocrystallized Pam_3CSK_4,[14] as well as the TLR2/TLR6 complex with cocrystallized Pam_2CSK_4.[15] However, systematic manipulation of the structural properties of synthetic lipopeptides has demonstrated that some lipopeptides elicit cellular responses outside of the TLR1/TLR6 tri-/diacylation paradigm.[18] This highlights the limitations of the current model in accommodating the complexity of naturally occurring ligands.

RP105 (CD180) is a TLR family member expressed by B cells, macrophages, and dendritic cells.[19] It is most closely related to TLR4 in terms of structure of its extracellular domain.[20] Surface expression and functions of RP105 rely on association with MD-1,[21] a homolog of the TLR4 accessory molecule, MD-2.[22] However, RP105 is distinct from other TLRs in its lack of the conserved intracellular Toll-interleukin-1 receptor (TIR) domain, which is required for initiation of canonical TLR signaling.[23,24] Thus, RP105 has been proposed to require a signaling-competent partner to contribute to host cell activation. In myeloid cells, RP105 physically and functionally interacts with both TLR4 and TLR2.[20,25]

We previously identified RP105 as a positive regulator of proinflammatory cytokine responses in *M. tuberculosis*- and *M. bovis* bacille Calmette Guerin (BCG)-infected macrophages.[25,26] Mice deficient in RP105 displayed elevated bacterial burden and pathology in the chronic phase of *M. tuberculosis* infection.[25] *M. tuberculosis* Δ*lspA* mutant bacteria lack mature lipoproteins and did not induce an RP105-dependent response in macrophages. This indicated that mature mycobacterial lipoproteins are RP105 agonists. Indeed, the *M. tuberculosis* 19 kDa lipoprotein, a known TLR2/TLR1 agonist,[13] activated macrophages in an RP105-dependent manner.[25] Functional and physical interactions of RP105 with TLR2 in the context of mycobacterial infection[25] suggest that RP105 may function as a coreceptor for TLR2. However, how RP105 contributes to recognition of bacterial lipoproteins is currently unknown. To address this, we set out to characterize lipopeptide properties that confer RP105 agonism.

2 | MATERIALS AND METHODS

2.1 | Reagents

The synthesis of peptides and lipopeptides was carried out by fully automated solid-phase peptide synthesis and Fmoc/tBu chemistry on chlorotrityl resins. To generate lipopeptides, the peptide resin was elongated with the unusual amino acid Fmoc-S-(2,3-dihydroxy-2(RS)-propyl)-cysteine (Fmoc-Cys(Dhc))[27] followed by esterification on solid phase with palmitic acid. After Fmoc-de-protection, the lipopeptides were either cleaved from the resins and side chain deprotected or further modified by *N*-palmitoylation to obtain triple-chain lipopeptides. N-Acyl single-chain lipopeptides were prepared by coupling Fmoc-Cys(Dhc) to the resin-bound peptide followed by Fmoc de-protection and palmitoylation. For stimulation assays, lipopeptides were heated briefly to 40°C, vortexed, and sonicated for 30 s. Working stocks were prepared in DMSO. Pam_3CSK_4, Pam_2CSK_4, and ultrapure LPS from *Escherichia coli* strain 0111:B4 were obtained from InvivoGen (San Diego, CA, USA). *M. tuberculosis* 19 kDa lipoprotein was purified as described previously.[26,28,29] Limulus amebocyte lysate assays (Lonza, Basel, Switzerland) demonstrated 4 ng endotoxin units per mg of the 19 kDa lipoprotein in all samples tested. The inhibitory anti-mouse TLR2 T2.5 antibody and matched mouse IgG1 isotype control were from InvivoGen.

2.2 | Mice

Rp105$^{-/-}$[25] and *Tlr1*$^{-/-}$[13] mice have been described previously. C57BL/6 mice were used as wild-type (WT) controls. *Tlr2*$^{-/-}$ mice were obtained by crossing of *Tlr2*$^{-/-}$*Rp105*$^{-/-}$ mice[25] with C57BL/6 mice. RP105 expression and TLR2 deficiency in subsequent generations were confirmed by PCR and flow cytometry. All mice were bred under specific pathogen-free conditions and were age- and sex-matched for all experiments. All animal procedures adhered to the guidelines of the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes and were approved by The University of Queensland Animal Ethics Committee (DI/571/12; DI/555/15).

2.3 | Primary macrophage culture

Bone marrow from 8 to 12 wk old mice was differentiated in DMEM supplemented with 20% L929 cell-conditioned media, 10% FBS (Hyclone/Bovogen), 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES (all Life Technologies) for 6 days. Cells were harvested using 1 mM EDTA/PBS and seeded in DMEM supplemented with 10% L929 cell-conditioned media, 10% FBS (Hyclone/Bovogen), 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES. Cytokine analyses were performed on cell culture supernatants of 0.2×10^6 cells per genotype stimulated for 4 h in triplicate (per condition) in 48-well plates.

2.4 | Macrophage stimulation assays

Unless stated otherwise, synthetic *M. tuberculosis* 19 kDa lipoprotein-based lipopeptides were used at 500 nM and Pam_3CSK_4 was used at 10 ng/ml. LPS was used at 100 EU/ml. Purified *M. tuberculosis* 19 kDa lipoprotein was used at 1 μg/ml. Antibodies were added 1 h prior to cell stimulation. Cytokine concentrations in cell culture supernatants were measured using ELISA (TNF DuoSet, R&D Systems; IL-6 OptEIA, BD Biosciences).

2.5 | Statistical analyses

Comparisons of two groups were conducted using two-tailed paired Student's *t* test; 95% confidence interval. Comparisons of more than two groups were conducted using one-way ANOVA followed by Dunnett's multiple comparisons test. P values < 0.05 were considered statistically significant. GraphPad Prism 7 was used to create all graphs and perform all statistical analyses.

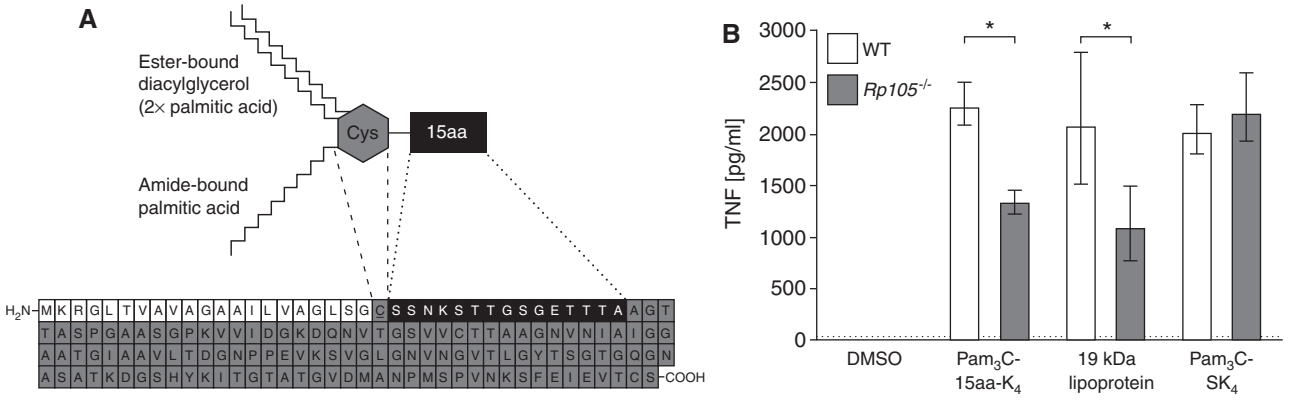

FIGURE 1 **A synthetic lipopeptide analog of the *M. tuberculosis* 19 kDa lipoprotein as a model to investigate RP105 agonistic properties.** (A) The *M. tuberculosis* 19 kDa lipoprotein is 159 amino acids in length (full sequence shown). During lipoprotein synthesis, the N-terminal signal peptide (white) is cleaved, exposing a universally conserved cysteine residue (gray, underlined) to which fatty acid chains are covalently linked during acylation. In this study, a synthetic lipopeptide was used to investigate the structural requirements for RP105-dependent macrophage activation. This lipopeptide consisted of the first 16 amino acids of the N-terminus (black), acylated with 2 ester-bound and one amide-bound palmitoyl residues identically to the motif in Pam_3CSK_4. (B) WT and *Rp105*$^{-/-}$ BMDMs were stimulated for 4 h with 1 μg/ml *M. tuberculosis* 19 kDa lipoprotein, 500 nM $Pam_3CSSNKSTTGSGETTTA\text{-}K_4$, or 10 ng/ml Pam_3CSK_4. TNF concentrations in culture supernatants were determined by ELISA. The dotted line represents ELISA detection limit. Results are expressed as mean ± SEM of 4 independent experiments, each performed in triplicates per condition. WT and *Rp105*$^{-/-}$ cells were compared using Student's *t* test. $^*P < 0.05$. 15aa = SSNKSTTGSGETTTA

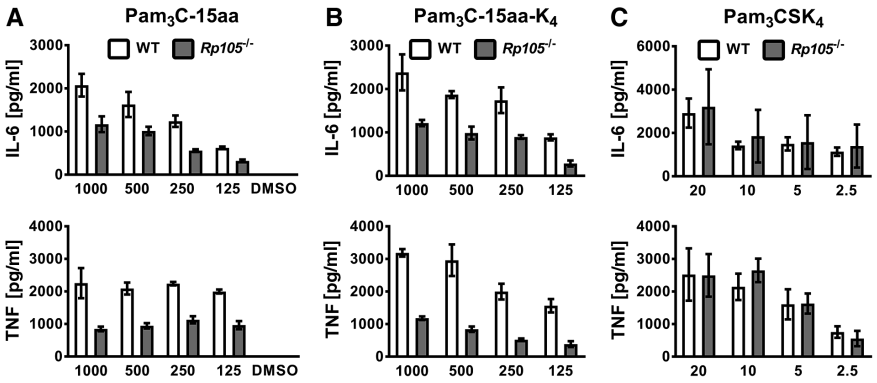

FIGURE 2 **Synthetic lipopeptides induce RP105-dependent TNF and IL-6 production by macrophages across a range of concentrations.** WT and *Rp105*$^{-/-}$ BMDMs were stimulated for 4 h with the indicated concentrations of (A) $Pam_3CSSNKSTTGSGETTTA$, (B) $Pam_3CSSNKSTTGSGETTTA\text{-}K_4$, or (C) Pam_3CSK_4. Unstimulated cells depicted in panel A received DMSO as solvent control and apply to all panels. IL-6 and TNF concentrations in culture supernatants were determined by ELISA. Results are means ± SD of triplicates of one representative of two independent experiments

3 | RESULTS

3.1 | A synthetic RP105-agonistic lipopeptide based on the N-terminus of the *M. tuberculosis* 19 kDa lipoprotein

We have previously reported that a tripalmitoylated hexadecapeptide containing the 16 N-terminal amino acids of the *M. tuberculosis* 19 kDa lipoprotein ($Pam_3CSSNKSTTGSGETTTA$; Figure 1A) induces RP105-dependent cytokine release by macrophages.[25] To improve solubility, four C-terminal lysine residues were added to this RP105 agonist. Figure 1B shows that RP105 agonism was retained after this alteration, with robust RP105-dependent macrophage activation similar to the native *M. tuberculosis* 19 kDa lipoprotein (Figure 1B). Both $Pam_3CSSNKSTTGSGETTTA$ and the lysine-containing $Pam_3CSSNKSTTGSGETTTA\text{-}K_4$ activated macrophages in an RP105-dependent manner across a dose range (Figure 2A, B). Importantly, Pam_3CSK_4 induced an equivalent response in WT and *Rp105*$^{-/-}$ macrophages, confirming that this TLR2/TLR1 agonist activates macrophages independent of RP105 (Figures 1B and 2C).[25] These observations encouraged the use of the synthetic $Pam_3CSSNKSTTGSGETTTA\text{-}K_4$ to interrogate lipopeptide features that confer RP105 agonism.

3.2 | Di- and triacylated synthetic lipopeptide analogs exhibit RP105-agonistic properties

Systematic structural alterations of synthetic lipopeptides have been employed successfully to define TLR2 coreceptor requirements.[18,30] The observation that Pam_3CSK_4, which carries an identical lipid moiety to $Pam_3CSSNKSTTGSGETTTA\text{-}K_4$, activates macrophages independent of RP105 (Figure 1B) led us to hypothesize that the acylation pattern may not define RP105

TABLE 1 Properties of lipopeptides used in this study

Name	Lipid moiety palmitoylation (linkage/s)	Sequence (divergence from SSNKSTTGSGETTTA in bold)	Molecular weight (kDa)	Figure
Pam_3CSSNKSTTGSGETTTA-K_4	Tri (ester + amide)	CSSNKSTTGSGETTTA-**K_4**	2.834	1–5
Pam_3CSSNKSTTGSGETTTA	Tri (ester + amide)	CSSNKSTTGSGETTTA	2.322	2
Pam_2CSSNKSTTGSGETTTA-K_4	Di (ester)	CSSNKSTTGSGETTTA-**K_4**	2.596	3
Pam_1CSSNKSTTGSGETTTA-K_4	Mono (amide)	CSSNKSTTGSGETTTA-**K_4**	2.179	3
Pam_3CSSNKSTTGSGE	Tri (ester + amide)	CSSNKSTTGSGE	1.946	4
Pam_3CSSNKSTTGSGE-K_4	Tri (ester + amide)	CSSNKSTTGSGE-**K_4**	2.459	4
Pam_3CSK_4-SSNKSTTGSGETTTA	Tri (ester + amide)	C**SK_4-SSNKSTTGSGETTTA**	2.938	4
Pam_3CSSNKS**AAGA**GETTTA-K_4	Tri (ester + amide)	CSSNKS**AA**G**A**GETTTA-K_4	2.757	4
Pam_3CSSNKS**AAGS**GETTTA-K_4	Tri (ester + amide)	CSSNKS**AA**GSGETTTA-**K_4**	2.773	4,5
Pam_3CSSNKST*t*GSGETTTA-K_4	Tri (ester + amide)	CSSNKST**t**GSGETTTA-**K_4** (D-threonine substitution)	2.833	4

agonism. To examine the role of acylation in RP105-dependent macrophage activation, the SSNKSTTGSGETTTA-K_4 peptide was synthesized to carry either zero (SSNKSTTGSGETTTA-K_4), one (Pam_1CSSNKSTTGSGETTTA-K_4), two (Pam_2CSSNKSTTGSGETTTA-K_4), or three (Pam_3CSSNKSTTGSGETTTA-K_4) palmitoyl residues (Table 1). Macrophage TNF release induced by the non- or monoacylated peptides did not exceed the assay detection limit. In contrast, the di- and tripalmitoylated peptide variants induced robust, RP105-dependent TNF responses (Figure 3A). These observations underpin that the ester-bound diacylglyceryl motif that has been shown to interact with TLR2 in studies examining Pam_2CSK_4[15] and Pam_3CSK_4,[14] is essential for macrophage activation by the synthetic lipopeptide variants, and we confirmed TLR2 dependency using *Tlr2*$^{-/-}$ macrophages (Figure 3B). Moreover, an inhibitory antibody raised against the extracellular domain of mouse TLR2[31] blocked macrophage TNF release elicited by equimolar concentrations of Pam_3CSK_4 and Pam_3CSSNKSTTGSGETTTA-K_4 (Figure 3C). This suggests similar modes of engagement of TLR2 by these RP105-dependent and RP105-independent lipopeptides. Thus, while TLR2 dominates macrophage activation elicited by Pam_3CSK_4 and the RP105-agonistic synthetic lipopeptides, RP105 agonism in the synthetic lipopeptides analyzed here was independent of the presence or absence of the ester-bound acyl chain.

3.3 | Polar amino acids confer RP105-agonistic properties

As RP105-dependent macrophage activation did not seem to be dominated by the acylation pattern of RP105 agonistic synthetic lipopeptides, we next focused on the contribution of the peptide moiety. Truncated versions of the RP105-agonistic lipopeptide with or without the addition of a C-terminal lysine motif to enhance solubility (Pam_3CSSNKSTTGSGE, Pam_3CSSNKSTTGSGE-K_4, Table 1) exhibited RP105 dependency similar to Pam_3CSSNKSTTGSGETTTA-K_4 (Figure 4A). Previous studies have demonstrated that FSL-1 (10 amino acids) or MALP-2 (14 amino acids) are not RP105 agonists,[25,32] yet the 12 amino acid Pam_3CSSNKSTTGSGE is an RP105 agonistic lipopeptide. This suggested that the composition, but not necessarily the length, of the peptide moiety determines RP105 agonism.

Amino acids near the N-terminus in Pam_2CSK_4 interact with TLR6 via hydrogen bonding.[15] To investigate whether specific N-terminal amino acids conferred RP105-agonism to the synthetic lipopeptides, a four-lysine motif was inserted at the N-terminus. The resulting lipopeptide, Pam_3CSK_4-SSNKSTTGSGETTTA (Table 1) retained RP105-agonistic capacity (Figure 4B), indicating that RP105-dependent macrophage activation by this RP105 agonist is not determined by the first 6 amino acids of the N-terminus.

We next focused our attention on the polar threonine residues in positions 7 and 8, which were only replaced by serine residues by the N-terminal lysine insertion (Figure 4B), thus retaining polar amino acids in these positions. Substitution of the threonines in positions 7 and 8 with alanine (Pam_3CSSNKS **AAGS**GETTTA-K_4, Table 1) diminished RP105 dependency of this lipopeptide so that TNF and IL-6 production in *Rp105*$^{-/-}$ macrophages did not differ statistically from the non-RP105 agonist Pam_3CSK_4 (Figure 4C). Substitution of serine for alanine at position 10 (Pam_3CSSNKS **AAGA**GETTTA-K_4, Table 1) or replacement of the threonine at position 8 with its D-stereoisomer (Pam_3CSSNKST***t***GSGETTTA-K_4) to change the secondary structure of the peptide moiety[33] had no further effect on RP105 dependency (Figure 4C). These results indicate that RP105 agonism in the synthetic lipopeptides was at least partially dependent on the presence of polar amino acids in positions 7 and 8. Of note, substitution of the threonines in positions 7 and 8 with alanine did not alter the reliance on TLR1 (Figure 5). Taken together, the data presented in this study suggest that the lipopeptide characteristics conferring RP105 agonism are distinct from those that engage TLR2 and TLR1.

4 | DISCUSSION

Systematic manipulation of RP105-agonistic synthetic lipopeptides revealed that RP105-dependent macrophage activation was preserved in di- and triacylated lipopeptide variants, setting RP105 agonism apart from the paradigm of TLR1- and TLR6-dependent

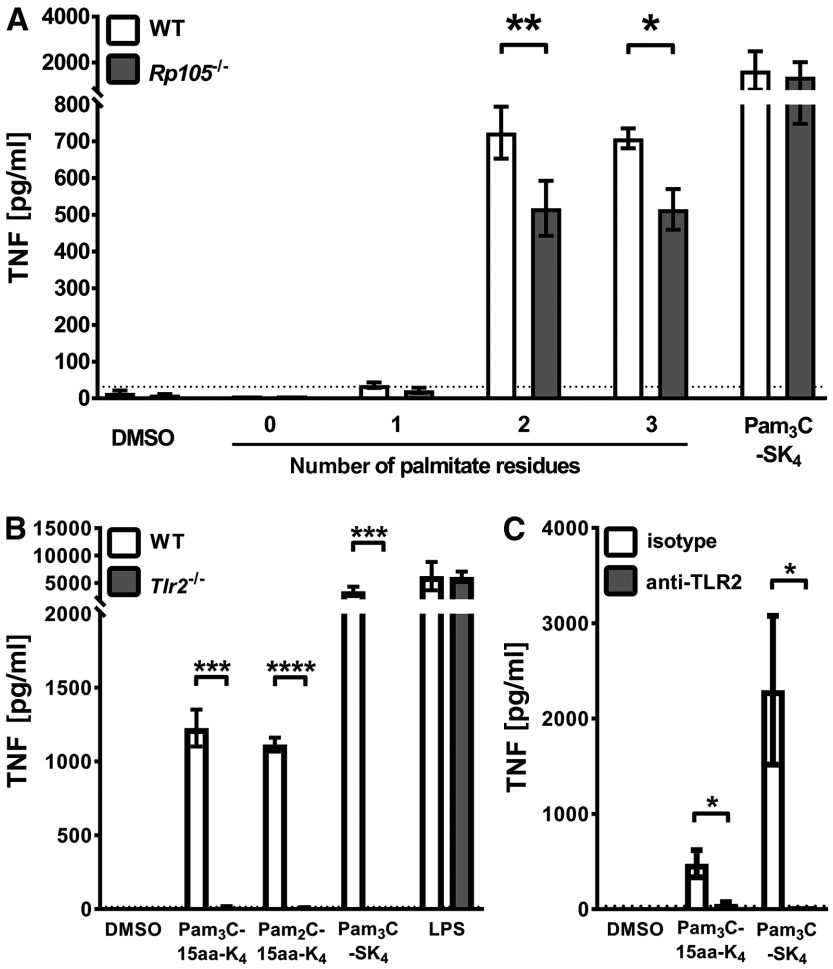

FIGURE 3 **Di- and triacylated lipopeptide analogs of the *M. tuberculosis* 19 kDa lipoprotein are RP105 agonists.** (A) WT and *Rp105*$^{-/-}$ BMDMs were stimulated for 4 h with 500 nM SSNKSTTGSGETTTA-K_4, Pam_1CSSNKSTTGSGETTTA-K_4, Pam_2CSSNKSTTGSGETTTA-K_4, and Pam_3CSSNKSTTGSGETTTA-K_4. Pam_3CSK_4 (10 ng/ml) served as control. (B) WT and *Tlr2*$^{-/-}$ BMDMs were stimulated for 4 h with 500 nM Pam_3CSSNKSTTGSGETTTA-K_4 or Pam_2CSSNKSTTGSGETTTA-K_4, 10 ng/ml Pam_3CSK_4 or 100 EU/ml LPS. TNF concentrations in culture supernatants were determined by ELISA. The dotted line represents ELISA detection limit. Results are means ± SEM of triplicates of three independent experiments. (C) WT BMDMs were treated with 2 μg/ml anti-TLR2 (T2.5) antibody or IgG1 isotype control antibody for 1 h prior to stimulation with Pam_3CSSNKSTTGSGETTTA-K_4 (12.5 nM) or Pam_3CSK_4 (10 ng/ml, 6.6 nM) for 4 h. DMSO served as solvent control. TNF concentrations in culture supernatants were determined by ELISA. The dotted line represents ELISA detection limit. Results are means ± SEM of three independent experiments, each performed in duplicates. The two groups in each experiment were compared using Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. 15aa = SSNKSTTGSGETTTA

recognition of lipopeptides. Instead, polar threonine residues within the peptide moiety contribute to RP105-mediated macrophage activation. Yet, this feature does not appear to affect dependency on TLR2 and TLR1. Interactions of amino acids contained in lipopeptides with TLR2 coreceptors are not unprecedented. Crystallographic studies have revealed interaction of amino acids within Pam_2CSK_4 with the leucine-rich repeat (LRR) domain of TLR6.[15] Moreover, TLR6 dependency of MALP-2 can be modulated by addition of positively, negatively, or uncharged amino acids.[18]

Partial ablation of RP105 agonism after substitution of polar amino acids in positions 7 and 8 within the peptide moiety suggests that there are additional unidentified motifs within this peptide that contribute to RP105 agonism. Furthermore, the remaining 122 amino acids of the native *M. tuberculosis* 19 kDa lipoprotein may contain motifs that similarly contribute to its RP105 agonism. O-glycosylation of threonine-rich motifs near the N-terminus of the native 19 kDa lipoprotein contribute to its biological functions,[34–36] and it remains to be determined whether posttranslational modifications shape RP105 agonism in native proteins. Nevertheless, direct comparison of the synthetic lipopeptide and the native *M. tuberculosis* 19 kDa lipoprotein in our study suggests that the 16 N-terminal amino acids of the 19 kDa lipoprotein are a dominant contributor to RP105-mediated macrophage cytokine production.

The *M. tuberculosis* 19 kDa lipoprotein is a known TLR2/TLR1 agonist,[13] in addition to being an RP105 agonist.[25] The RP105-agonistic synthetic lipopeptides used in this study were fully dependent on TLR2 to activate macrophages, whereas RP105 deficiency only partially diminished macrophage cytokine production. This likely reflects additional contributions of TLR2/1 and/or TLR2/6 complexes, and we confirmed TLR1 dependency for the triacylated lipopeptide

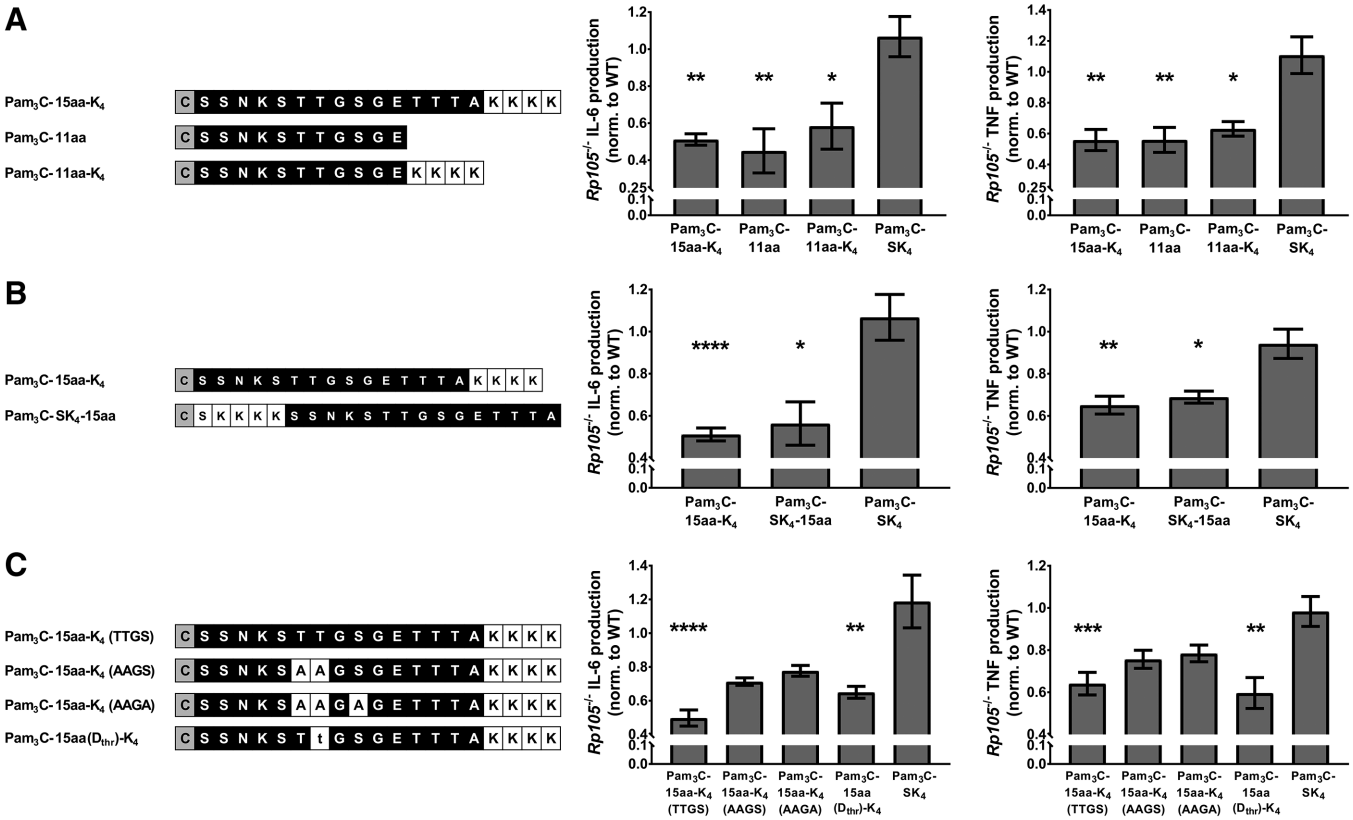

FIGURE 4 Replacement of polar amino acids at positions 7 and 8 resulted in partial reduction of RP105 dependency. WT and *Rp105*$^{-/-}$ BMDMs were stimulated for 4 h with (A) 500 nM Pam_3CSSNKSTTGSGE or Pam_3CSSNKSTTGSGE-K_4; (B) 500 nM Pam_3CSSNKSTTGSGETTTA-K_4 or Pam_3CSK$_4$-SNKSTTGSGETTTA; or (C) 500 nM Pam_3CSSNKS**AAGA**GETTTA-K_4, Pam_3CSSNKS**AAGS**GETTTA-K_4, or Pam_3CSSNKST***t***GSGETTTA-K_4. Pam_3CSK$_4$ (10 ng/ml) served as control. TNF and IL-6 concentrations in culture supernatant were measured by ELISA. TNF and IL-6 concentrations in cultures of *Rp105*$^{-/-}$ BMDMs was normalized to cytokine concentrations in WT BMDM cultures. Results show means ± SEM of 3–5 independent experiments, each performed in triplicates per condition. One-way ANOVA with Dunnett's multiple comparisons test was used to compare RP105 dependency of lipopeptide variants to Pam_3CSK$_4$-induced IL-6 and TNF production. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. 15aa = SSNKSTTGSGETTTA

variants independent of their RP105-agonistic properties. TLR2 and RP105 coimmunoprecipitate, and colocalize on the macrophage cell surface.[25] It is worth noting that the synthetic RP105 agonistic lipopeptides used in this study contained palmitic acid (C16), whereas the diacylglyceryl residues of the native *M. tuberculosis* 19 kDa lipoprotein consist of both palmitic acid and tuberculostearic acid (C19).[3] This has genuine biological implications as fatty acid chain length influences the ability of TLR2 to engage with lipopeptide acyl chains.[30] However, our data demonstrate that RP105 agonism is preserved in di- as well as tripalmitoylated lipopeptides, supporting the notion that the nature of the acyl chains is unlikely to determine RP105 agonism.

While RP105 has no known lipid-binding domains, MD-1 possesses a lipid-binding pocket.[37] It has been hypothesized that in a putative TLR2/RP105 complex, the lipid-binding pocket of TLR2 may align with that of MD-1 when two RP105 molecules are arranged in a "head-to-head" orientation.[38,39] However, our observation that RP105 agonism is preserved in di- and triacylated synthetic lipopeptides suggests that a bridging of TLR2 and MD-1 by accommodating the diacylglyceryl moiety and amid-bound acyl chains, respectively, is unlikely a defining mechanism of engagement. As the lipid-binding pocket in MD-1 can accommodate bulky lipids such as the LPS precursor lipid IVa,[37] it is tempting to speculate that the lipid portion of mycobacterial lipoproteins and synthetic RP105 lipopeptide agonists could be bound by MD-1. This may underlie the observation that RP105 deficiency did not affect canonical TLR signaling via NF-κB and mitogen-activated protein kinases, as well as TNF protein expression, in macrophages stimulated with the RP105-agonistic lipopeptide or live mycobacteria.[25,26] Instead, RP105 directed TNF trafficking and targeted release from macrophages via the activation of Bruton's tyrosine kinase and the class I phosphoinositide 3-kinase p110δ.[26] We have previously attempted to assess the requirement for MD-1 and RP105 for host cell binding using biotinylated lipopeptides, albeit with inconclusive results (data not shown). This may not be surprising in light of the functional contributions of RP105/MD-1, TLR1, and TLR2 to host cell activation by the synthetic lipopeptides. Analyses at single molecule resolution may be required to assess whether RP105/MD-1 and TLR1 interact with TLR2 in the same or distinct receptor complexes and reveal how polar amino acids in the N-terminal peptide sequence of the 19 kDa lipoprotein engage the RP105/MD-1 complex, be it in the presence or absence of TLR2.

Our previous work suggested that *M. tuberculosis* carries RP105 agonistic lipoproteins in addition to the 19 kDa lipoprotein.[25] Amino

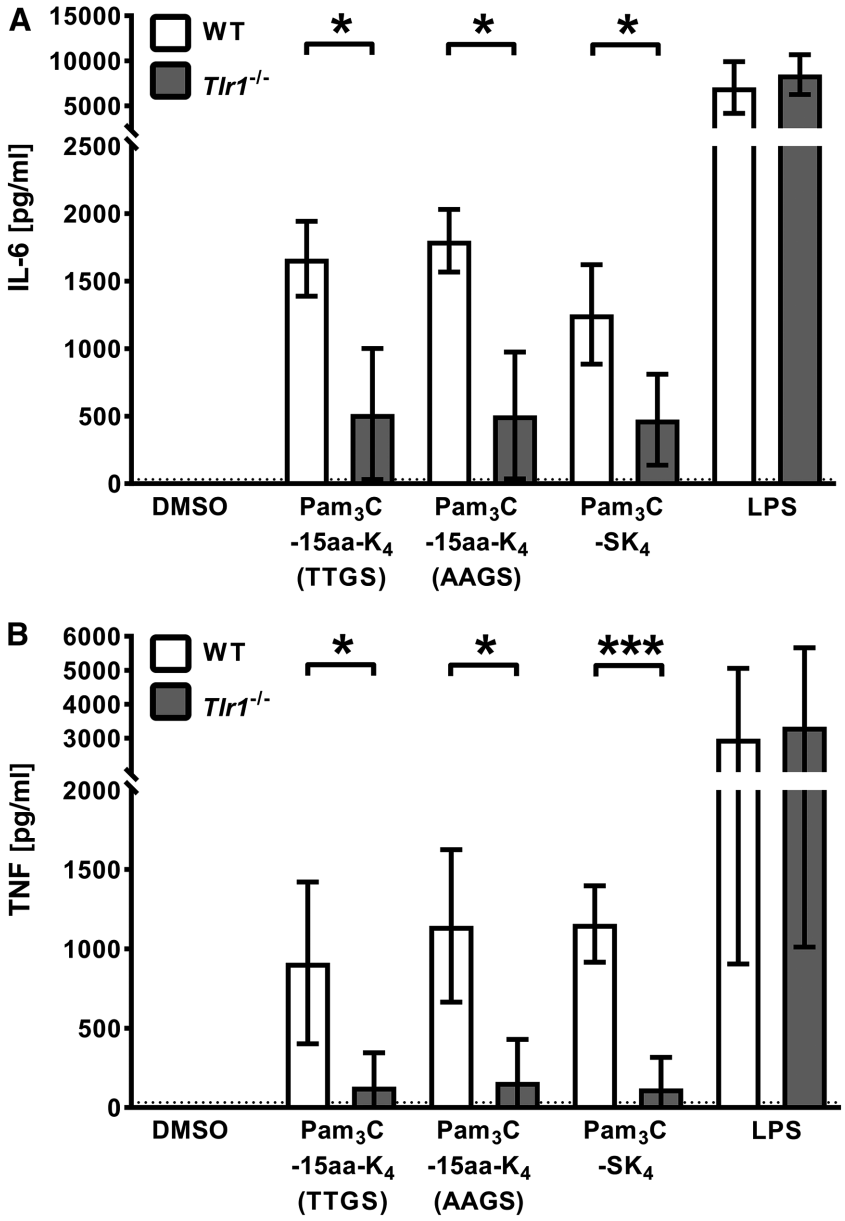

FIGURE 5 **RP105-agonistic triacylated lipopeptides stimulate macrophages dependent on TLR1**. WT and *Tlr1*$^{-/-}$ BMDMs were stimulated for 4 h with 500 nM Pam_3CSSNKSTTGSGETTTA-K_4 or Pam_3CSSNKS**AAGS**GETTTA-K_4. DMSO, Pam_3CSK_4 (10 ng/ml), and LPS (100 EU/ml) served as controls. (A) IL-6 and (B) TNF concentrations in culture supernatants were determined by ELISA. The dotted line represents ELISA detection limit. Results are means ± SEM of four independent experiments, each performed in triplicates. WT and *Tlr1*$^{-/-}$ cells were compared using Student's *t* test. $^*P < 0.05$; $^{***}P < 0.001$. 15aa = SSNKSTTGSGETTTA

acid sequence alignments of 99 confirmed *M. tuberculosis* lipoproteins[1] relative to their N-terminal conserved cysteine residue indicate at least 10 *M. tuberculosis* lipoproteins with two polar amino acids at positions 7 and 8 (data not shown). These are compelling candidates for identification of further RP105-agonistic lipoproteins in *M. tuberculosis*. Aside from innate immune recognition of microbial pathogens, RP105 has functions in autoimmune disease[40] and obesity-associated inflammation.[41] This strongly suggests that, like other TLRs,[42] RP105 recognizes endogenous ligands in addition to those derived from pathogens. While putative endogenous RP105 ligands remain to be identified, initial insights have been gained from the observation that RP105 agonists may differ from endogenous TLR4 lipid ligands such as palmitic and stearic acid.[41] Identification and characterization of RP105 ligands, endogenous or pathogen-derived, are central to our understanding of the physiological roles of RP105/MD-1 in health and disease.

AUTHORSHIP

A.B. conceived the study and designed experiments with T.S. and K.H.W. T.S. performed experiments and analyzed data with A.B. K.H.W. generated synthetic lipopeptide variants and contributed to data interpretation. M.L., K.D., and A.G.B. contributed essential research materials. T.S. and A.B. wrote the manuscript. All authors read, revised, and approved the manuscript.

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DISCLOSURE

K.H.W. is CEO of EMC microcollections GmbH.

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