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HOST RESPONSE AND INFLAMMATION

Surfactant Protein A Enhances the Degradation of LPS-Induced TLR4 in Primary Alveolar Macrophages Involving Rab7, β-arrestin2, and mTORC1

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ABSTRACT Respiratory infections by Gram-negative bacteria are a major cause of global morbidity and mortality. Alveolar macrophages (AMs) play a central role in maintaining lung immune homeostasis and host defense by sensing pathogens via pattern recognition receptors (PRR). The PRR Toll-like receptor (TLR) 4 is a key sensor of lipopolysaccharide (LPS) from Gram-negative bacteria. Pulmonary surfactant is the natural microenvironment of AMs. Surfactant protein A (SP-A), a multifunctional host defense collectin, controls LPS-induced pro-inflammatory immune responses at the organismal and cellular level via distinct mechanisms. We found that SP-A post-transcriptionally restricts LPS-induced TLR4 protein expression in primary AMs from healthy humans, rats, wild-type and SP-A$^{-/-}$ mice by further decreasing cycloheximide-reduced TLR4 protein translation and enhances the co-localization of TLR4 with the late endosome/lysosome. Both effects as well as the SP-A-mediated inhibition of LPS-induced TNF-α release are counteracted by pharmacological inhibition of the small GTPase Rab7. SP-A-enhanced Rab7 expression requires β-arrestin2 and, in β-arrestin2$^{-/-}$ AMs and after intratracheal LPS challenge of β-arrestin2$^{-/-}$ mice, SP-A fails to enhance TLR4/lysosome co-localization and degradation of LPS-induced TLR4. In SP-A$^{-/-}$ mice, TLR4 levels are increased after pulmonary LPS challenge. SP-A-induced activation of mechanistic target of rapamycin complex 1 (mTORC1) kinase requires β-arrestin2 and is critically involved in degradation of LPS-induced TLR4. The data suggest that SP-A post-translationally limits LPS-induced TLR4 expression in primary AMs by lysosomal degradation comprising Rab7, β-arrestin2, and mTORC1. This study may indicate a potential role of SP-A-based therapeutic interventions in unrestricted TLR4-driven immune responses to lower respiratory tract infections caused by Gram-negative bacteria.

KEYWORDS LPS, TLR4, innate immunity, lung, surfactant protein A

Lower respiratory tract infection by Gram-negative bacteria is a major cause of global morbidity and mortality (1, 2). Emerging antibiotic resistance of Gram-negative bacteria causing hospital-acquired (3), ventilator-associated (3, 4), and community-acquired pneumonia (5) is highly concerning. Accordingly, additional therapeutic and prophylactic approaches are indispensable, including the continuing functional characterization of endogenous lung defense molecules for their ability to induce and regulate antibacterial immune responses.

Under steady-state conditions, alveolar macrophages (AMs) account for 90–95% of resident leukocytes in the alveolar space, promoting immune tolerance to innocuous antigens (6). Further, during pulmonary infection, AMs are key players in directing the

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equilibrium between host defense against pathogens and the restriction of tissue damage either directly caused by pathogens or indirectly by the immune response (7, 8). The functional phenotype of AMs is dominated by pulmonary surfactant, a lipid-protein complex that mediates extrauterine breathing and modulates lung immune responses (9, 10). The pulmonary collectin surfactant protein (SP)-A, which constitutes the major protein component of surfactant (9), modulates immune responses of the lung partially by direct and indirect effects on AMs. SP-A-deficient mice (SP-A$^{-/-}$) exhibit increased susceptibility to infection, reduced survival, reduced macrophage activation, and inability to combat infection after pulmonary challenge with a broad variety of clinically relevant pathogens, specifically Gram-negative bacteria (11–13), as well as to isolated lipopolysaccharide (LPS) (14, 15), their invariant virulence factor. In these models, SP-A promotes pulmonary immune homeostasis through partially understood mechanisms including a considerable number of immune-instructive functions on AMs activation and deactivation.

Toll-like receptors (TLR) are key initiators of innate and adaptive immune responses to infection (16). An immediate response to Gram-negative bacteria is important to fight infection but requires tight control to avoid excessive activation with subsequent sepsis-induced organ failure (17). TLR4, in concert with MD-2 and CD14, provides the recognition of LPS (18–20). The activation of TLR4 by LPS triggers internalization of the receptor-ligand complex which is directed for lysosomal degradation or endocytic recycling (21). The relative expression level of TLR4 belongs to numerous endogenous control mechanisms known to regulate the TLR4 responsiveness to LPS (22). Earlier data demonstrated that SP-A limits TLR4 expression *in vitro* and *in vivo* in distinct organs and cell types (23–27). However, the regulatory pathways involved in SP-A-mediated restriction of LPS-induced TRL4 expression in primary AMs are largely unknown.

Small *Rab* (Ras related in brain) GTPases are pivotal regulators of phagocytic, endocytic, and exocytic trafficking in eukaryotic cells and regulate receptor trafficking and signaling (28). An increasing number of stimuli, including soluble mediators, bacterial virulence factors, and microorganisms, have been shown to affect Rab protein expression correlated to distinct immune functions (28). Several studies emphasized the role of Rab-regulated TLR4 trafficking and signaling in RAW264.7 macrophages, mouse peritoneal and bone marrow derived macrophages, mouse models and human monocytes (29–31). We have previously shown that SP-A enhances Rab7 protein expression that was functionally correlated to enhanced lysophagosomal pathogen delivery in rat AMs (32). However, the regulation of Rab-mediated TLR4 trafficking in primary lung-specific immune cells is only beginning to be understood and a potential impact of SP-A on TLR4 trafficking and signaling by regulating Rab GTPases in AMs remains to be addressed.

The multifunctional adaptor and signaling proteins β-arrestin1 and β-arrestin2, originally characterized in the context of desensitizing 7-transmembrane receptors, scaffold numerous proteins involved in regulating inflammation (33, 34). β-arrestin2 acts as a negative regulator of LPS-induced TLR4 activation through various mechanisms (34–38). We previously demonstrated that SP-A enhances the expression of β-arrestin2 in primary rat AMs and that β-arrestin2 is required for SP-A-mediated attenuation of TLR4 signaling in a murine model of intratracheal LPS challenge (26). The possible involvement of β-arrestin2 in SP-A-mediated TLR4 degradation pathways has not been investigated.

Recently, the mechanistic target of rapamycin (mTOR), an evolutionary highly conserved serine/threonine kinase central to metabolic control and polarization of eukaryotic cells (39), has been implicated in TLR4 degradation pathways (40), proliferative renewal of murine AMs (41), cellular metabolism and accumulation of AMs (42), and in β-arrestin2-reduced pro-inflammatory signaling in human bronchial epithel cells (43). However, the lung-specific surfactant-derived regulation of mTORC activity in AMs and the functional consequences thereof are unknown.

In this study, we aimed at understanding the SP-A-mediated restriction of TLR4 levels in AMs more detailed and found that SP-A post-translationally limits LPS-induced TLR4 expression in primary AMs from humans, rats, and mice by enhancing TLR4 degradation

engaging Rab7, the pivotal regulator of late endocytic trafficking, the multifunctional scaffold and signaling protein β-arrestin2, and the metabolic hub mTORC1.

RESULTS

SP-A inhibits LPS-induced TLR4 protein expression in human AMs. An increasing number of studies demonstrated that SP-A limits TLR4 expression in distinct cell types and at the organismal level functionally associated with anti-inflammatory consequences (23–27). The effect of SP-A on TLR4 expression in primary human AMs from healthy volunteers has not been validated so far. We therefore initially determined the effects of SP-A on constitutive and LPS-induced TLR4 protein expression in freshly isolated human AMs obtained by bronchoalveolar lavage from healthy individuals by Western blotting analysis. Stimulation of human AMs with LPS (100 ng/ml) significantly increased the expression of TLR4 protein compared with control after 1 h ($P < 0.05$) and 2 h ($P < 0.05$), respectively (Fig. 1A). Whereas SP-A alone (40 μg/ml, 1 h) did not affect the constitutive expression of TLR4, pretreatment of the cells with SP-A prior to LPS markedly restricted LPS-induced TLR4 expression after 1 h and significantly inhibited LPS-induced TLR4 expression after 2 h ($P < 0.01$) (Fig. 1A).

SP-A inhibits LPS-induced TLR4 expression by enhancing TLR4 degradation. To determine whether the SP-A-mediated decrease in TLR4 reflects reduced TLR4 protein synthesis or enhanced TLR4 degradation, primary AMs from humans, rats, wild-type and SP-A$^{-/-}$ mice were treated with the translation inhibitor cycloheximide (CHX) in the absence or presence of LPS or SP-A prior to LPS. Stimulation of rat AMs with low-dose LPS at concentrations of 1 and 10 ng/ml slightly but not significantly enhanced TLR4 protein expression (data not shown).

LPS-induced TLR4 expression (Fig. 1B and C) was impaired by pretreatment of the cells with CHX (50 μg/ml, 30 min) and further decreased to baseline levels by SP-A in AMs from humans ($P < 0.01$) (Fig. 1B) and rats ($P < 0.05$) (Fig. 1C). To determine the effect of SP-A on TLR4 mRNA expression, rat AMs were left untreated or were treated with LPS (100 ng/ml), SP-A (40 μg/ml), or SP-A prior to LPS and quantitative RT-PCR was performed after 2 and 6 h. While SP-A did not affect basal TLR4 mRNA, LPS slightly enhanced the expression of TLR4 mRNA. Preincubation of the cells with SP-A, however, did not affect LPS-enhanced TLR4 mRNA expression after 2 h, further supporting a post-transcriptional pathway of SP-A-mediated restriction of TLR4 protein expression (Fig. 1D).

SP-A$^{-/-}$ mice are more susceptible to pulmonary LPS challenge than wild-type mice (14, 15). To determine whether TLR4 levels are constitutively higher in AMs from SP-A$^{-/-}$ mice and the LPS/CHX effect is more prominent AMs from wild-type and SP-A$^{-/-}$ mice were treated with the CHX in the absence or presence of LPS or SP-A prior to LPS. LPS-induced TLR4 expression (Fig. 1E and F) was impaired by pretreatment of the cells with CHX (50 μg/ml, 30 min) and further decreased to baseline levels by SP-A in AMs from wild-type mice ($P < 0.05$) (Fig. 1E) and SP-A$^{-/-}$ mice ($P < 0.05$) (Fig. 1F), suggesting that SP-A enhances the degradation of LPS-induced TLR4 in AMs from both genotypes to a comparable degree.

The lack of endogenous SP-A results in increased TLR4 levels on bronchoalveolar cells. To determine whether the enhanced susceptibility of SP-A$^{-/-}$ mice toward LPS challenge correlates with TLR4 levels *in vivo*, SP-A$^{-/-}$ and wild-type mice were oropharyngeally challenged with LPS or PBS (control) and TLR4 levels on bronchoalveolar lavage (BAL) cells were compared after 24 h. The data demonstrate that the basal TLR4 levels after PBS application were slightly enhanced in SP-A$^{-/-}$ mice compared with wild-type mice and LPS treatment significantly enhanced TLR4 levels on BAL cells from both wild-type and SP-A$^{-/-}$ mice compared with the corresponding genotype given PBS ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 1G). TLR4 levels were higher on BAL cells from SP-A$^{-/-}$ mice challenged with LPS compared with those on BAL cells from WT mice ($P < 0.05$) (Fig. 1G). The results suggest that the lack of endogenous SP-A results in increased TLR4 levels *in vivo*, which may account for the enhanced susceptibility of SP-A$^{-/-}$ mice toward LPS.

Rab7 is critically involved in SP-A-enhanced degradation of LPS-induced TLR4. The activation of TLR4 by LPS triggers internalization of the receptor-ligand complex which

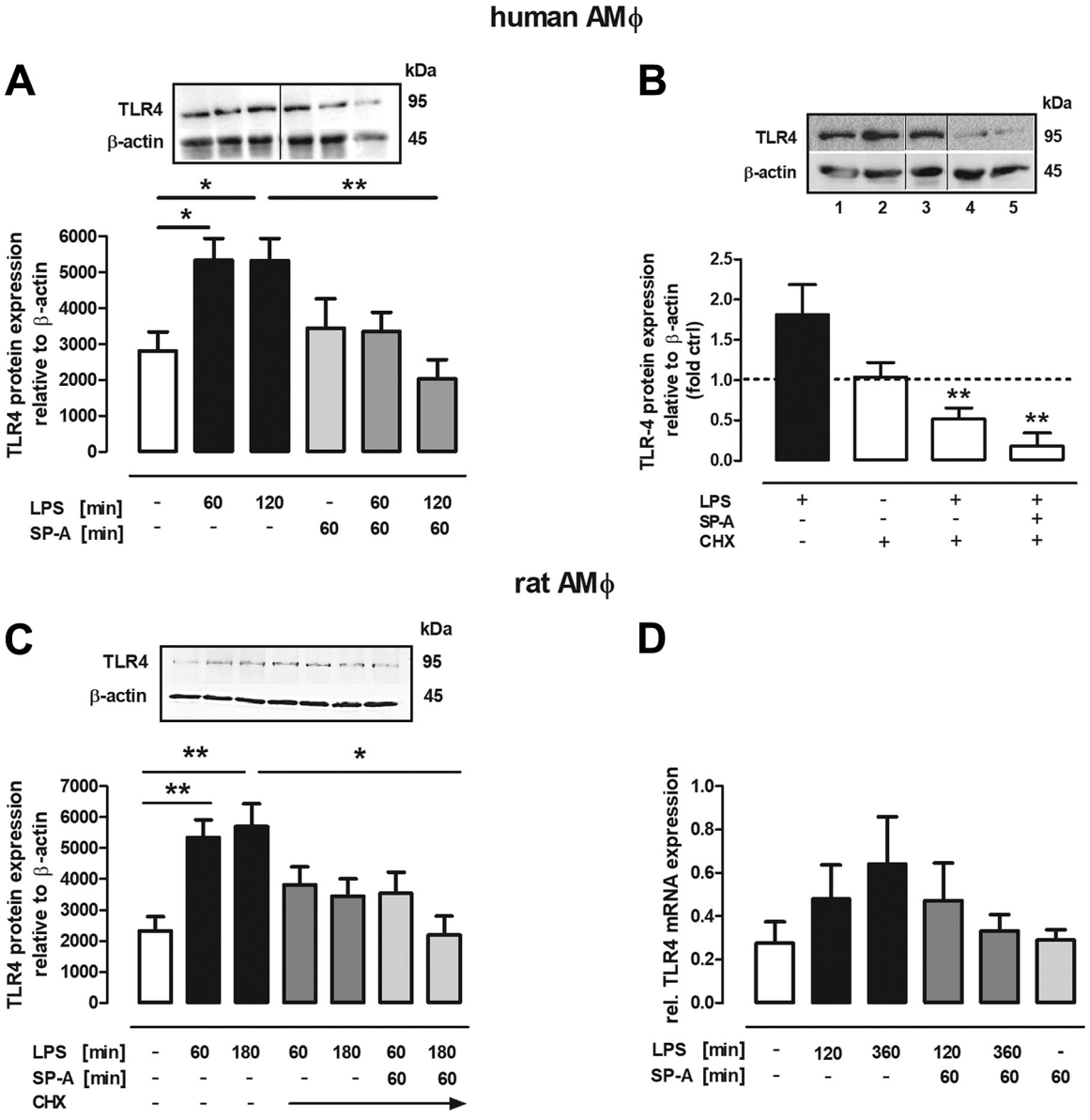

FIG 1 (Continued)

is directed toward lysosomal degradation or endocytic recycling (21). Several studies have demonstrated a crucial role of Rab GTPases in TLR4 trafficking and signaling (29–31). In rat AMs, SP-A transiently enhances functionally active Rab7b and Rab7 protein expression, the latter effect being associated with enhanced phagosome delivery of *E. coli* (32).

To investigate whether Rab7 is involved in SP-A-enhanced degradation of LPS-induced TLR4, AMs were treated with LPS or SP-A prior to LPS in the absence or presence of cell-permeable Rab7 blocking peptides and TLR4 protein levels were determined by Western blotting analysis. LPS-induced TLR4 was significantly inhibited by SP-A ($P < 0.05$) (Fig. 2A and B) and treatment of AMs with Rab7 blocking peptides (20 μg/ml, 15 min) antagonized the SP-A-enhanced degradation of LPS-induced TLR4 protein levels in AMs from rats ($P < 0.05$) (Fig. 2A) and wild-type mice ($P < 0.05$) (Fig. 2B). The results obtained from subsequent confocal microscopy analyses and their quantification revealed that SP-A enhanced the co-localization of TLR4 with the late endosome/lysosome marker LysoTracker in AMs from wild-type

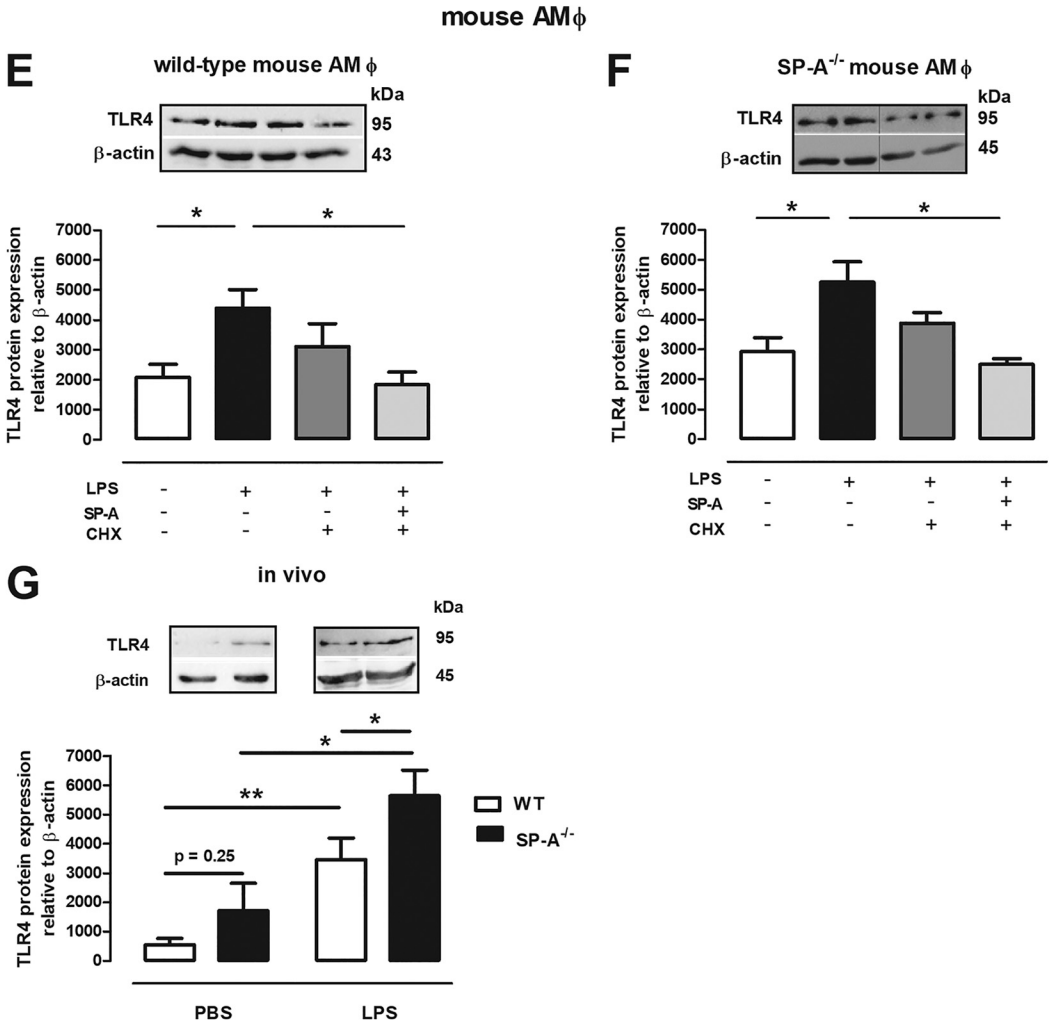

FIG 1 SP-A decreases LPS-induced TLR4 protein expression in human alveolar macrophages (AMs). (A) Primary human AMs from healthy volunteers were left untreated or were treated with LPS (100 ng/ml), SP-A (40 μg/ml) or SP-A prior to LPS for the times indicated. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of at least nine independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$. (B and C) SP-A decreases LPS-induced TLR4 protein expression in primary AMs from humans and rats independent of translational inhibition. (B) Human AMs were left untreated (lane 1), or were treated with LPS (100 ng/ml, 60 min) (lane 2), cycloheximide (CHX) (50 μg/ml, 30 min) (lane 3), CHX followed by LPS (lane 4), or CHX followed by SP-A (40 μg/ml, 60 min) prior to LPS (lane 5). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of two to four independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). ** $P < 0.01$ versus LPS. (C) Primary rat AMs were left untreated or were treated with cycloheximide (CHX; 50 μg/ml, 30 min), followed by the treatment with SP-A (40 μg/ml), LPS (100 ng/ml) or SP-A prior to LPS for the times indicated. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Data of at least five independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$. (D) SP-A does not inhibit LPS-enhanced TLR4 mRNA expression. Primary rat AMs were left untreated or were treated with LPS (100 ng/ml), SP-A (40 μg/ml) or SP-A (40 μg/ml) prior to LPS (100 ng/ml) for the times indicated and quantitative reverse transcriptase PCR was performed. Data of five independent experiments were normalized to GAPDH mRNA (mean $\pm$ SEM). (E and F) SP-A decreases LPS-induced TLR4 protein expression in primary AMs from wild-type and SP-A$^{-/-}$ mice independent of translational inhibition. Primary AMs from (E) wild-type mice and (F) SP-A$^{-/-}$ mice were left untreated or treated with LPS (100 ng/ml), or CHX (50 μg/ml, 30 min), followed by LPS (100 ng/ml, 60 min), or followed by SP-A (40 μg/ml, 60 min) prior to LPS (100 ng/ml, 60 min). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of at least four independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). * $P < 0.05$. (G) TLR4 levels are higher on BAL cells from SP-A$^{-/-}$ mice challenged with LPS compared with wild-type mice. Western blot analysis of TLR4 expression in BAL cells from wild-type (n =5) and SP-A$^{-/-}$ mice (n = 4) 24 h after intratracheal challenge with 5 μg/kg BW of LPS or PBS (control). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of four to five independent experiments were normalized to β-actin and quantified by t test or one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$.

mice ($P < 0.001$) (Fig. 2C and D) which was antagonized ($P < 0.001$) by Rab7 blocking peptides. To figure out whether these findings were reproducible in the complete absence of endogenous SP-A, AMs from SP-A$^{-/-}$ mice were analyzed for TLR4 expression and localization by Western blotting and confocal microscopy, respectively, as described. LPS-induced TLR4 was significantly inhibited by SP-A ($P < 0.05$) (Fig. 2E) and treatment of AMs with Rab7 blocking peptides (20 μg/ml, 15 min) antagonized the SP-A-enhanced degradation of LPS-induced TLR4 protein levels in AMs from SP-A$^{-/-}$ mice ($P < 0.05$) (Fig. 2E). The results obtained from subsequent confocal microscopy analyses and their quantification revealed that SP-A enhanced the co-localization of TLR4 with the late endosome/lysosome marker LysoTracker in AMs from SP-A$^{-/-}$ mice ($P < 0.001$) (Fig. 2F and G), which was antagonized ($P < 0.001$) by Rab7 blocking peptides. The combined data suggest that Rab7 is critically involved in SP-A-enhanced degradation of LPS-induced TLR4 protein expression in AMs from rats, wild-type and SP-A$^{-/-}$ mice.

SP-A-enhanced degradation of LPS-induced TLR4 is associated with a Rab7-dependent decrease in TNF-α release. Previous data demonstrated that SP-A reduces LPS-induced TNF-α release *in vitro* and *in vivo* (14, 44–48). To ascertain whether Rab7 is required for SP-A-mediated inhibition of LPS-induced TNF-α release, AMs from wild-type and SP-A$^{-/-}$ mice were treated with LPS, or SP-A prior to LPS in the absence or presence of Rab7 blocking peptides and TNF-α release was determined by ELISA. The basal TNF-α release by AMs from SP-A$^{-/-}$ mice was higher compared with wild-type mice (121 $\pm$ 23 versus 7.5 $\pm$ 3.8 pg/ml, $P < 0.01$). Supporting previous data (14), LPS significantly enhanced the release of TNF-α ($P < 0.001$) which was inhibited by SP-A ($P < 0.01$) in AMs from both genotypes (Fig. 2H and I). The SP-A-mediated inhibition of LPS-enhanced TNF-α release was partially counteracted in AMs from wild-type mice (Fig. 2H) and was antagonized ($P < 0.05$) by Rab7 blocking peptides (20 μg/ml, 15 min) in AMs from SP-A$^{-/-}$ mice (Fig. 2I), suggesting that Rab7 is distinctly implicated in SP-A-mediated restriction of LPS-induced pro-inflammatory activity.

β-arrestin2 is required for SP-A-enhanced degradation of LPS-induced TLR4 protein expression. The cytosolic scaffold and signaling protein β-arrestin2 negatively regulates LPS-induced TLR4 activation *in vitro* and *in vivo* (34–38). We previously demonstrated that SP-A enhances the protein expression of β-arrestin2 in primary rat AMs and that β-arrestin2 is required for an SP-A-mediated attenuation of TLR4 signaling in a murine model of intratracheal LPS challenge (26). To evaluate whether β-arrestin2 is also involved in SP-A-enhanced degradation of LPS-induced TLR4, AMs from β-arrestin2$^{-/-}$mice were treated with LPS, SP-A, or SP-A prior to LPS and TLR4 protein levels were determined by Western blotting analysis. Whereas LPS significantly enhanced the expression of TLR4 ($P < 0.05$) in both β-arrestin2$^{-/-}$ AMs (Fig. 3A) and wild-type mice AMs (Fig. 3B), SP-A failed to increase the degradation of LPS-induced TLR4 in β-arrestin2$^{-/-}$ AMs (Fig. 3A) compared with wild-type mice AMs (Fig. 3B). Supporting these findings, as shown by confocal microscopy analyses and their quantification, SP-A failed to increase the co-localization of TLR4 with the late endosome/lysosome in AMs from β-arrestin2$^{-/-}$ mice (Fig. 3C and D). To determine whether SP-A-enhanced degradation of LPS-induced TLR4 requires β-arrestin2 *in vivo*, BAL cells from β-arrestin2$^{-/-}$ mice and wild-type mice were analyzed for TLR4 expression 5 h after intratracheal application of LPS or LPS plus SP-A. The analysis of BAL inflammatory cellular composition revealed no significant differences between both genotypes and treatment (Fig. 3E). Whereas SP-A inhibited the LPS-induced TLR4 expression in BAL cells from wild-type mice by approximately 37%, SP-A failed to inhibit TLR4 expression in BAL cells from β-arrestin2$^{-/-}$ mice (Fig. 3F). Together, the experiments suggest that β-arrestin2 is required for SP-A-enhanced degradation of LPS-induced TLR4 in the murine lung.

β-arrestin2 is required for SP-A-enhanced Rab7 expression in the presence of LPS. SP-A transiently enhances the protein levels of functionally active Rab7 in rat AMs (32). To determine whether β-arrestin2 is required for SP-A-enhanced Rab7 expression, AMs from β-arrestin2$^{-/-}$ and wild-type mice were treated with SP-A, LPS, or both for the indicated time periods. Rab7 protein levels were determined by Western blotting analysis. Both SP-A and LPS significantly enhanced Rab7 protein expression in AMs

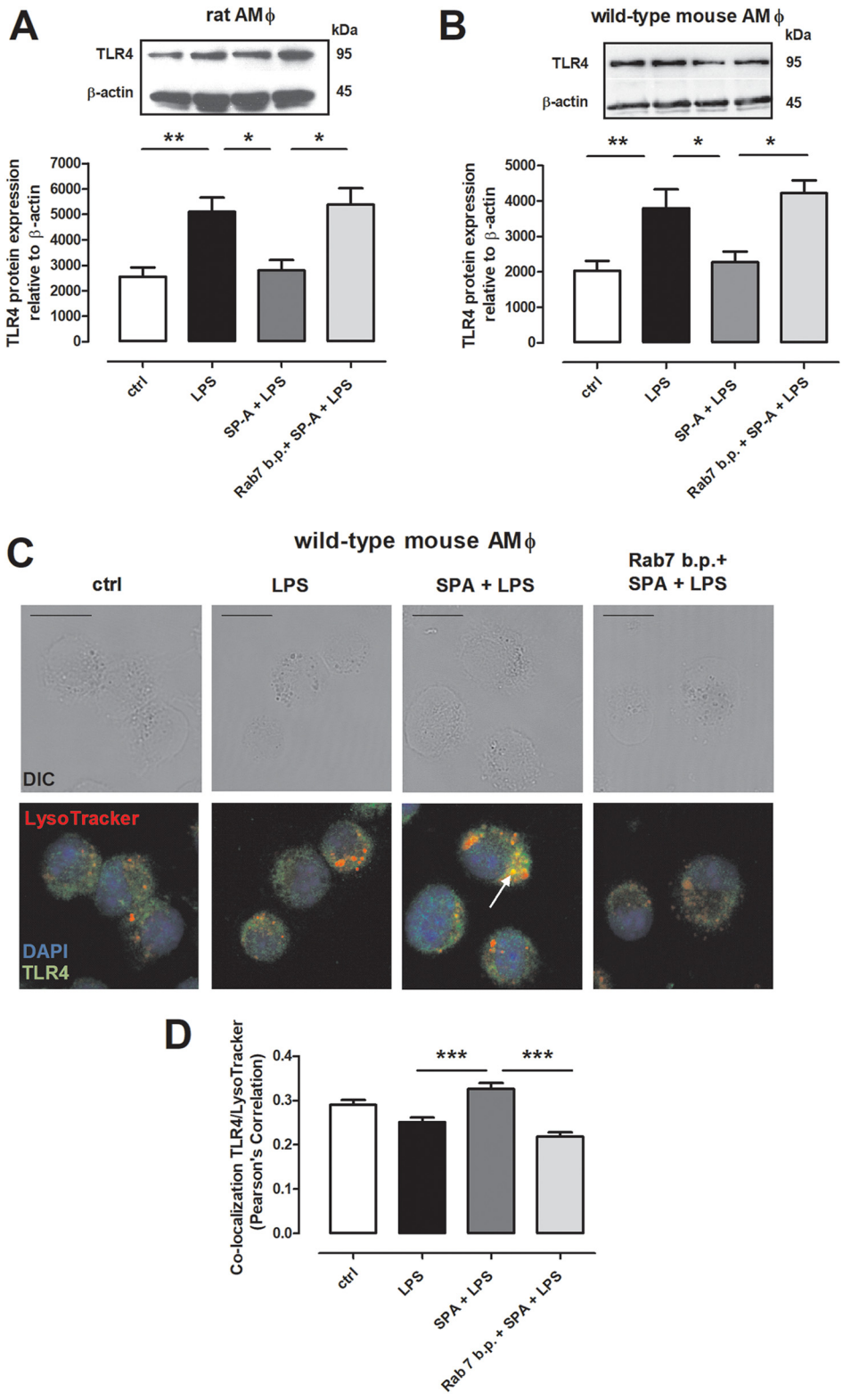

FIG 2 (Continued)

from wild-type ($P < 0.05$) (Fig. 4A) and β-arrestin2$^{-/-}$ mice ($P < 0.05$) (Fig. 4B), demonstrating that β-arrestin2 is not required for Rab7 enhancement by either ligand alone.

Stimulation of the cells with both ligands, SP-A prior to LPS, did not synergistically enhance Rab7 expression in AMs from wild-type mice (Fig. 4A) but reduced Rab7

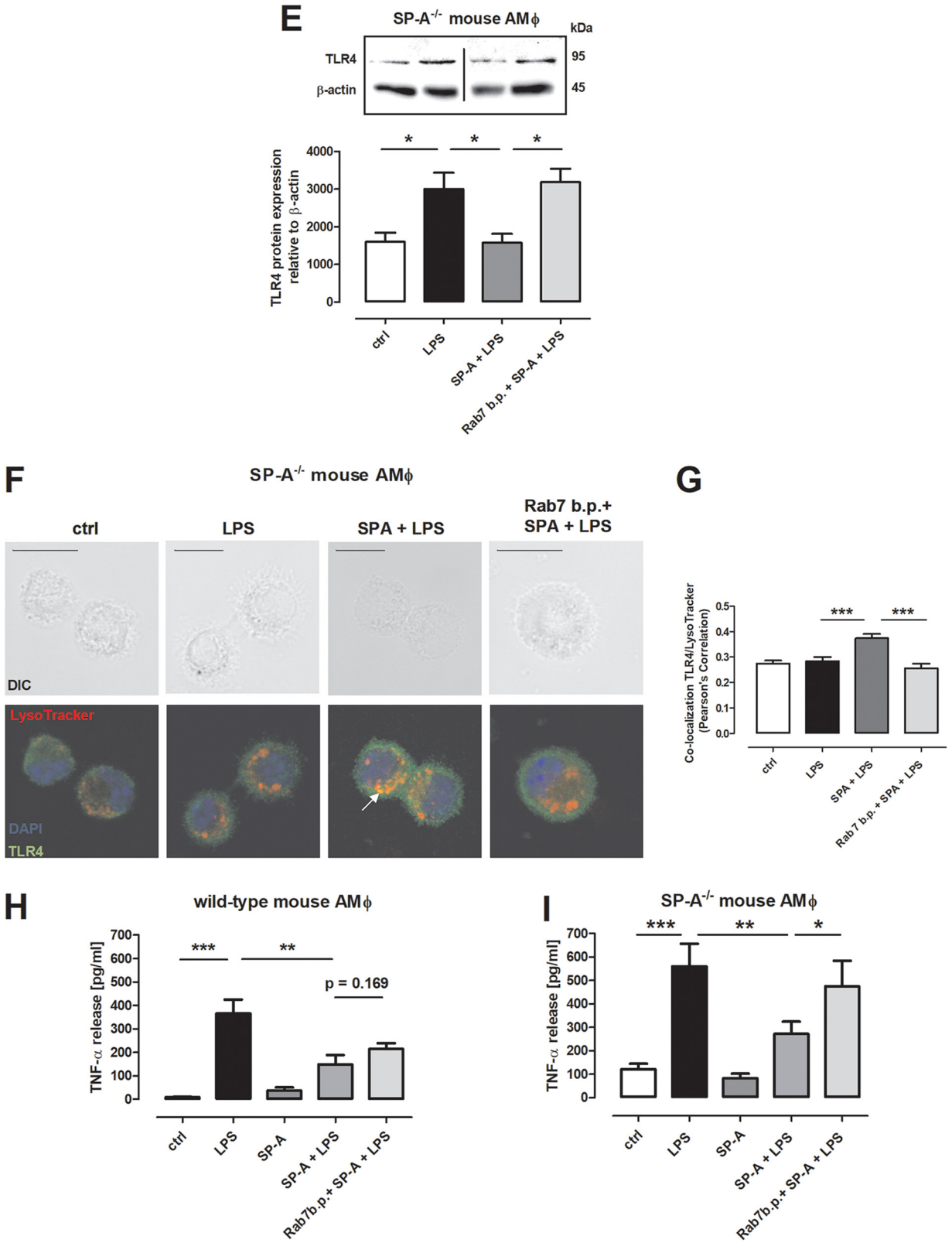

FIG 2 Inhibition of Rab7 counteracts SP-A-enhanced degradation of LPS-induced TLR4. Primary rat AMs (A) were left untreated or were treated with cell permeable Rab7 blocking peptide (20 μg/ml, 15 min), LPS (100 ng/ml, 60 min) or SP-A (40 μg/ml, 60 min) prior to LPS (100 ng/ml, 60 min). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of at least five independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$. Primary AMs from wild-type mice (B) were

(Continued on next page)

expression to baseline levels in AMs from β-arrestin2$^{-/-}$ mice ($P < 0.05$) compared with wild-type mice AMs (Fig. 4B). β-arrestin1/2 double-deficient mice are neonatal lethal due to lung immaturity including decreased surfactant generation and significantly lowered SP-A expression (49). Therefore, to evaluate the role of β-arrestin2 in the regulation of SP-A, SP-A protein expression was determined by Western blotting in lung tissue homogenates from β-arrestin2$^{-/-}$ and wild-type mice. The basal expression of the monomeric forms of SP-A (~25 and ~35 kDa) were reduced by approximately 58% ($P < 0.05$) in lung tissue from β-arrestin2$^{-/-}$ mice compared with wild-type mice (Fig. 4C).

SP-A-induced mTORC1 activation is required for SP-A-enhanced degradation of LPS-induced TLR4 protein expression. The mTORC1 pathway, which is central in regulating cell growth and anabolic and catabolic processes (50), has recently been implicated in TLR4 degradation pathways (40). Eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) is a canonical mTORC1 substrate (51). Supporting previous studies employing bone marrow derived macrophages and RAW264.7 macrophages (52), we found that IL-4, serving as positive control, induced a significant and rapamycin-sensitive phosphorylation of 4E-BP1 at Ser^{65} in primary rat AMs (Fig. 5A). To define whether SP-A can activate mTORC1, rat AMs were treated with SP-A in the absence or presence of rapamycin and the phosphorylation of 4E-BP1 at Ser^{65} was determined by Western blotting analysis. SP-A significantly enhanced the phosphorylation of 4E-BP1 at Ser^{65} after 10 min ($P < 0.001$) and 60 min ($P < 0.001$) (Fig. 5B) in a rapamycin-sensitive manner, implicating that SP-A-induced phosphorylation of 4E-BP1 at Ser^{65} is mTORC1-dependent.

To determine whether SP-A-induced mTORC1 activation is involved in TLR4 degradation, rat AMs were treated with SP-A, rapamycin, or both and TLR4 expression was determined by Western blotting analysis. Whereas SP-A did not affect basal TLR4 expression compared with untreated AMs, rapamycin treatment resulted in an increase of basal TLR4 expression which was further enhanced by the addition of SP-A ($P < 0.01$) (Fig. 5C).

In line with previous studies employing bone marrow derived macrophages and RAW264.7 macrophages (40, 52), respectively, we found that LPS (100 ng/ml) induced the phosphorylation of 4E-BP1 at Ser^{65} in primary AMs after 60 min ($P < 0.01$) without an additive effect after stimulation with SP-A prior LPS (Fig. 5D). Pretreatment of the cells with rapamycin inhibited LPS- and SP-A/LPS-induced 4E-BP1 Ser^{65} phosphorylation by 41% ($P < 0.05$) and 40%, respectively (Fig. 5D).

To determine whether SP-A-induced mTORC1 activation is required for SP-A-enhanced degradation of LPS-induced TLR4 expression, rat AMs were treated with LPS or SP-A prior to LPS in the absence or presence of rapamycin. Rapamycin treatment counteracted the SP-A-mediated inhibition of LPS-induced TLR4 protein expression ($P < 0.01$) (Fig. 5E), suggesting that SP-A-activated mTORC1 is required for SP-A-enhanced degradation of LPS-induced TLR4 protein expression.

Comparing mTORC1 activation induced by SP-A, LPS, or both in AMs from wild-type and SP-A$^{-/-}$ mice revealed no significant differences in basal and ligand-induced activation in AMs from both genotypes (Fig. 5F). In contrast, SP-A- and SP-A/LPS-induced

FIG 2 Legend (Continued)
treated as in (A). Representative TLR4 and β-actin Western blots are shown. Data of at least five independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean ± SEM). * $P < 0.05$, ** $P < 0.01$. Inhibition of Rab7 counteracts SP-A-enhanced TLR4/lysosome co-localization. SP-A increases TLR4/lysosome co-localization in AMs. (C) Representative confocal images for TLR4 localization in primary AMs from wild-type mice left untreated or treated with Rab7 blocking peptide (20 μg/ml, 15 min), LPS (100 ng/ml, 60 min), or SP-A (40 μg/ml, 60 min) prior LPS (100 ng/ml, 60 min). Images are representative of three independent experiments with at least 70 cells per condition. Scale bars, 10 μm. (D) Pearson's correlation coefficient values ± SEM for co-localization of TLR4 and LysoTracker. *** $P < 0.001$. (E) Primary AMs from SP-A$^{-/-}$ mice (E) were treated as in (A and B). Representative TLR4 and β-actin Western blots are shown. Data of at least five independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean ± SEM). * $P < 0.05$. (F) Representative confocal images for TLR4 localization in primary AMs from SP-A$^{-/-}$ mice left untreated or treated with Rab7 blocking peptide (20 μg/ml, 15 min), LPS (100 ng/ml, 60 min), or SP-A (40 μg/ml, 60 min) prior LPS (100 ng/ml, 60 min). Images are representative of three independent experiments with at least 70 cells per condition. Scale bars, 10 μm. (G) Pearson's correlation coefficient values ± SEM for co-localization of TLR4 and LysoTracker. *** $P < 0.001$. (H and I) Inhibition of Rab7 counteracts SP-A-mediated restriction of LPS-induced TNF-α release. Primary AMs from wild-type (H) and SP-A$^{-/-}$ mice (I) were left untreated or treated with LPS (100 ng/ml, 120 min), SP-A (40 μg/ml, 60 min), or both in the absence or presence of Rab7 blocking peptide (20 μg/ml, 15 min). TNF-α ELISA was performed on cell-free supernatant of four to five independent experiments and quantified by one-way ANOVA with Newman-Keuls posttest. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

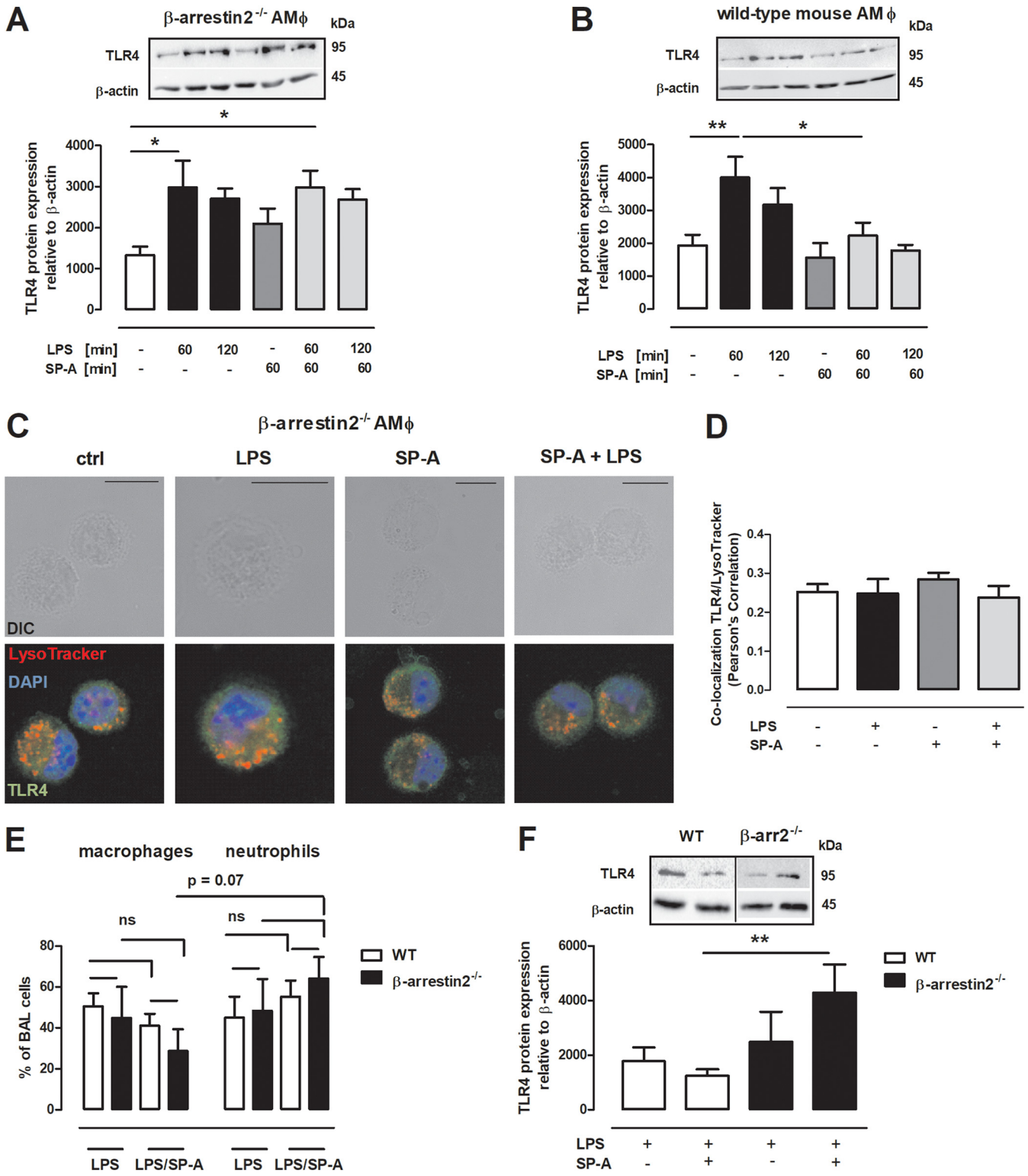

FIG 3 SP-A fails to increase degradation of LPS-induced TLR4 in β-arrestin2$^{-/-}$ AMs. Primary AMs from β-arrestin2$^{-/-}$ mice (A) and wild-type mice (B) were left untreated or were treated with LPS (100 ng/ml), SP-A (40 μg/ml), or both for the times indicated. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of five to seven independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean ± SEM). *$P < 0.05$, ** $P < 0.01$. SP-A does not enhance TLR4/lysosome co-localization in AMs from β-arrestin2$^{-/-}$ mice. (C) Representative confocal images for TLR4 localization in primary AMs from β-arrestin2$^{-/-}$ mice that were left untreated or were treated with LPS (100 ng/ml, 60 min), SP-A (40 μg/ml, 60 min) or both. Images are representative of three independent experiments with at least 70 cells per condition. Scale bars, 10 μm. (D) Pearson's correlation coefficient values ± SEM for co-localization of TLR4 and LysoTracker. (E) Differential BAL cell count from wild-type and β-arrestin2$^{-/-}$ mice 5 h after intratracheal challenge with 2.5 μg/kg BW of

(Continued on next page)

mTORC1 activation in AMs from β-arrestin2$^{-/-}$ was significantly reduced ($P < 0.05$ and $P < 0.01$, respectively) compared with AMs from wild-type mice and markedly inhibited compared with AMs from SP-A$^{-/-}$ mice (Fig. 5F).

DISCUSSION

Given the global burden of lower respiratory tract infections caused by increasingly drug resistant Gram-negative bacteria (1, 2), endogenous lung defense molecules may provide a perspective for the development of additional new therapeutic approaches to treating Gram-negative infections. The multifunctional host defense collectin SP-A provides immune protection against numerous respiratory pathogens, promotes pathogen clearance, limits inflammation, and activates mechanisms that induce tissue repair (9, 10). Specifically, SP-A has a key role in restricting TLR4-mediated inflammatory signaling and maintaining immune homeostasis in Gram-negative infection (11, 13, 53, 54) or LPS challenge of the lung (14, 15, 26). Although TLR4 is critical for eradicating Gram-negative respiratory pathogens (55, 56), excessive TLR4-driven inflammatory responses can cause organ damage or fatal consequences like sepsis (17). Thus, TLR4 is intrinsically subject to multiple levels of endogenous control mechanisms covering synthesis, trafficking, signaling, and degradation (22). The present study demonstrates a role of SP-A in the degradation of LPS-induced TLR4 and delineates proteins involved.

Consistent with earlier studies using human AMs (57, 58), we found that LPS enhances the expression of TLR4 protein and, for the first time, demonstrates that SP-A restricts LPS-induced TLR4 protein expression in human AMs from healthy volunteers. The latter observation is supported by previous data demonstrating that SP-A attenuates TLR4 expression and associated functional consequences in distinct organs and cell types (23–27). For example, intestinal TLR4 protein expression, pro-inflammatory cytokines, and mortality are significantly decreased upon oral administration of SP-A in a rat model of experimental necrotizing enterocolitis (23). SP-A protein is expressed in the central nervous system of rats in a model of autoimmune encephalomyelitis and increases with progression of the disease and, *in vitro*, SP-A significantly decreases LPS-induced TLR4 protein expression and pro-inflammatory cytokine release in human astrocytes and microglia (24). Likewise, the LPS-induced increase in TLR4 protein expression is significantly enhanced in intestinal epithelial cells from SP-A/D-deficient mice compared with those from wild-type mice and is attenuated by preincubation of the cells with SP-A or SP-D (25). We previously demonstrated that SP-A reduces LPS-enhanced TLR4 protein expression in primary rat AMs (26). In neonatal mouse ileum, SP-A reduces TLR4 and pro-inflammatory cytokine mRNA (27). In contrast, supporting our current findings in human AMs, SP-A does not affect basal TLR4 surface expression on 5-day old human monocyte-derived macrophages and, also under basal conditions, has very little effect on TLR4 mRNA expression during monocyte differentiation into macrophages (48). The combined data indicate that SP-A can restrict LPS-induced TLR4 expression ubiquitously in the body. Although the major site of SP-A synthesis is the lung, SP-A gene and/or protein are expressed in numerous extrapulmonary sites, predominantly in mucosal tissues (59), further pointing at a more general capacity of this multifunctional collectin in host defense.

The mechanisms of SP-A-mediated TLR4 expression restriction are unknown. In this study, within a short-term observation period, SP-A limits the expresssion of TLR4 protein independent of translational inhibition and does not affect LPS-induced TLR4 mRNA expression, suggesting a post-transcriptional and post-translational regulation of TLR4 by SP-A. Indeed, LPS-enhanced TLR4 expression was reduced by pretreatment of the cells with cycloheximide and further decreased to baseline levels by SP-A in

FIG 3 Legend (Continued)
LPS or 2.5 μg/kg BW of LPS plus 5 mg/kg BW of SP-A. n = 3-4 mice per genotype. (F) Exogenous SP-A fails to increase degradation of LPS-induced TLR4 in β-arrestin2$^{-/-}$ mice. Western blot analysis of cytosolic TLR4 expression in BAL cells from wild-type and β-arrestin2$^{-/-}$ mice 5 h after intratracheal challenge with 2.5 μg/kg BW of LPS or 2.5 μg/kg BW of LPS plus 5 mg/kg BW of SP-A. Equal amounts of cytosolic fractions were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown.

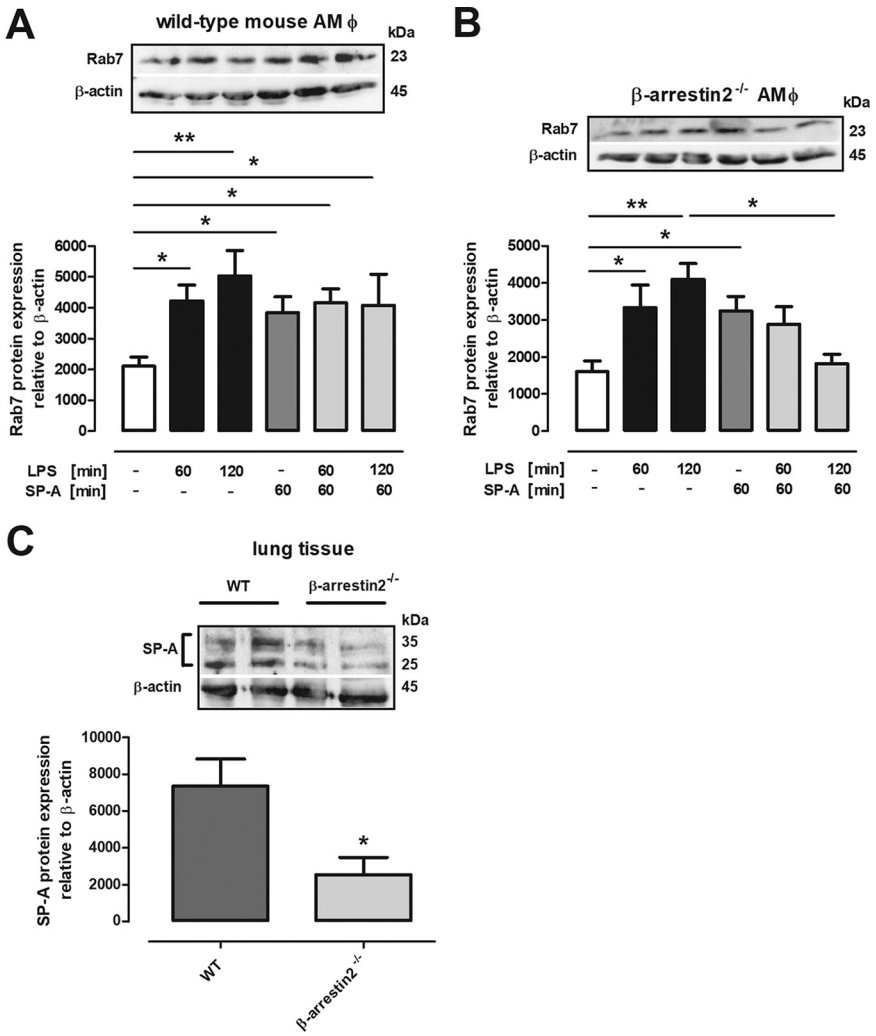

FIG 4 β-arrestin2 is required for SP-A-enhanced Rab7 expression in the presence of LPS. Primary AMs from wild-type mice (A) and β-arrestin2$^{-/-}$ mice (B) were left untreated or were treated with LPS (100 ng/ml), SP-A (40 μg/ml), or SP-A prior to LPS for the times indicated. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for Rab7 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of at least four (A) and at least five (B) independent experiments were normalized to β-actin and quantified by one-way ANOVA with Newman-Keuls posttest (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$. (C) The expression of SP-A in lung homogenates from β-arrestin2$^{-/-}$ mice is reduced. Constitutive SP-A protein expression in lung homogenates from wild-type and β-arrestin2$^{-/-}$ mice. Representative SP-A and β-actin Western blots are shown. Data of two independent experiments with four mice for each genotype were normalized to β-actin and quantified by Student's *t* test (mean $\pm$ SEM). * $P < 0.05$.

human and rat AMs and in AMs from wild-type and SP-A-deficient mice, strongly suggesting that SP-A enhances the degradation of LPS-induced TLR4 in AMs from distinct mammalian species and genotypes. In addition, in SP-A$^{-/-}$ mice, TLR4 levels were increased after pulmonary LPS challenge, suggesting that the lack of endogenous SP-A may account for the enhanced susceptibility of SP-A$^{-/-}$ mice toward LPS.

Rab7 and β-arrestin2 are required for SP-A-enhanced degradation of LPS-induced TLR4 expression. The degradation of TLR4 requires the delivery of TLR4 into lysosomes via an endocytic trafficking pathway (21, 60). An increasing number of studies demonstrate a central role of Rab GTPases, which generally regulate receptor endocytosis, their trafficking through the endosomal pathway and targeting to lysosomes or to endocytic recycling (28), in TLR4 trafficking and signaling (29–31, 61–66). For example, upon LPS stimulation, Rab10 promotes continuous replenishment of TLR4 to the plasma membrane of

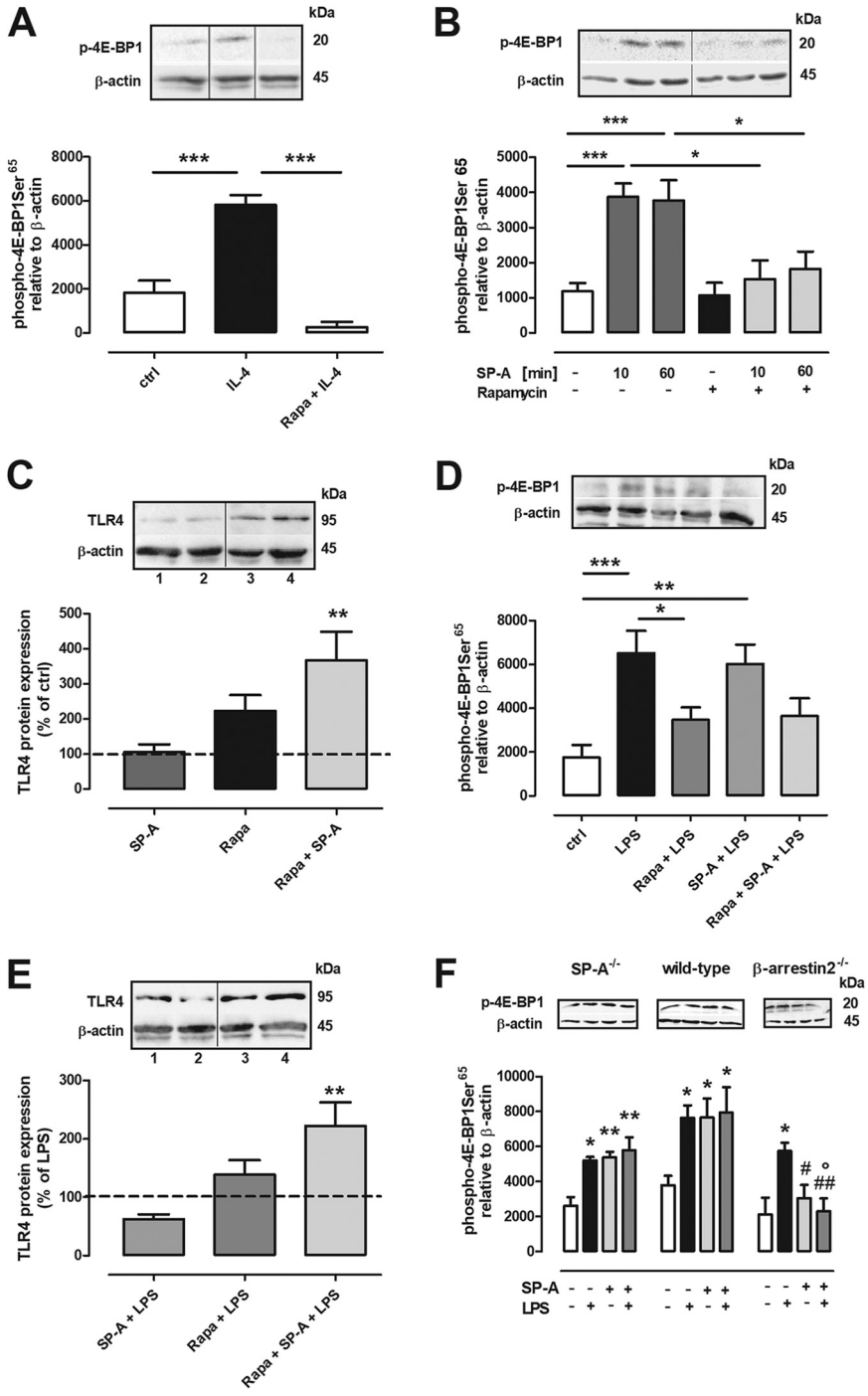

FIG 5 SP-A-induced mTORC1 activation is required for SP-A-enhanced TLR4 degradation. (A) IL-4 enhances 4E-BP1 phosphorylation in primary rat AMs. Primary rat AMs were left untreated or were treated with IL-4 (0.5 μg/ml, 60 min) in the absence or presence of rapamycin (20 nM, 60 min). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for phospho-4E-BP1Ser^{65} and β-actin. Representative phospho-4E-BP1Ser^{65} and β-actin Western blots are shown. Data of at least three independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). *** $P < 0.001$. (B) SP-A enhances 4E-BP1 phosphorylation at Ser^{65}. Primary rat AMs were left untreated or were treated with rapamycin (20 nM, 60 min) followed by SP-A (40 μg/ml) for the times indicated. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for phospho-4E-BP1Ser^{65} and β-actin. Representative phospho-4E-BP1Ser^{65} and β-actin Western blots are shown. Data of at least five independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). *** $P < 0.001$, * $P < 0.05$. (C) Rat AMs were left untreated (dashed line 1, control), or were treated with SP-A (40 μg/ml, 60 min, lane 2), rapamycin (20 nM, 60 min, lane 3), or rapamycin

(Continued on next page)

RAW264.7 cells and enhances LPS-induced lung injury when overexpressed in macrophages (30). Rab11a is essential for TLR4 and Toll-receptor-associated molecule, TRAM, trafficking to *E.coli*-enriched phagosomes in human monocytes and controls interferon regulatory factor 3 activation from this compartment (31). Rab21 regulates LPS-induced pro-inflammatory responses by promoting TLR4 endosomal trafficking and downstream signaling activation in murine bone marrow-derived macrophages and human monocytes (63). Overexpression of Rab26 downregulates TLR4 cell membrane expression in pulmonary microvascular endothelial cells (64). Rab1a upregulates surface expression of TLR4 following LPS stimulation in bone marrow-derived macrophages and Rab1a activity in AMs from septic patients is positively associated with respiratory dysfunction and severity of sepsis (66).

In the present study, Rab7 was critically implicated in SP-A-enhanced TLR4 degradation in primary AMs, as cell-permeable Rab7 blocking peptides antagonized the SP-A-enhanced degradation of LPS-induced TLR4 protein and counteracted the SP-A-enhanced TLR4/lysosome co-localization. Functionally, the SP-A-mediated inhibition of LPS-induced TNF-α was reversed by Rab7 blocking peptides, suggesting that Rab7, by enhancing TLR4 degradation, is critically involved in anti-inflammatory effects of SP-A on LPS activity.

Rab7 is fundamental for lysosomal biogenesis and for trafficking and degradation of several signaling receptors (67). We have previously shown that SP-A transiently enhances the expression of functionally active Rab7 in rat AMs which correlated to enhanced lysosomal delivery of *E. coli* in these cells (32). Comparable to our current findings, Rab7b, that controls trafficking between endosomes and the *trans*-Golgi network (68), has been shown to negatively regulate TLR4 signaling by promoting TLR4 lysosomal degradation in RAW264.7 cells (29). Rab7b is upregulated in the rat brain following ischemic brain damage, and functionally, Rab7b overexpression improves neurological outcome assocoiated with suppressed expression of TLR4 mRNA and protein as well as pro-inflammatory mediators (65). Furthermore, *Trichuris suis* soluble products induce Rab7b expression associated with reduced pro-inflammatory responses through suppression of TLR4 signaling and surface expression (62). The combined data demonstrate that both Rab7 and Rab7b, which cannot be defined as Rab isoforms as their identity is limited to 50% (68), can regulate TLR4 trafficking, signaling, and degradation.

The cytosolic scaffold and signaling protein β-arrestin2 negatively regulates LPS-induced TLR4 activation *in vitro* and *in vivo* (35–38) through various mechanisms. By investigating lung-specific functions of β-arrestin2 in response to LPS, we previously found that SP-A enhances the protein expression of β-arrestin2 in primary rat AMs and

FIG 5 Legend (Continued)
prior to SP-A (lane 4). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of at least four independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). ** $P < 0.01$ compared with control. (D) Rat AMs were left untreated or were treated with LPS (100 ng/ml) or SP-A (40 μg/ml) prior to LPS in the absence or presence of rapamycin. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for phospho-4E-BP1Ser^{65} and β-actin. Representative phospho-4E-BP1Ser^{65} and β-actin Western blots are shown. Data of at least four independent experiments were normalized to β-actin and quantified by one-way ANOVA with Newman-Keuls posttest (mean $\pm$ SEM). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. (E) Rat AMs were treated with LPS (100 ng/ml, 60 min, dashed line 1), SP-A (40 μg/ml, 60 min, lane 2), rapamycin (20 nM, 60 min, lane 3), or both prior to LPS (100 ng/ml, lane 4). Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for TLR4 and β-actin. Representative TLR4 and β-actin Western blots are shown. Data of at least four independent experiments were normalized to β-actin and quantified by one-way ANOVA with Bonferroni's posttest (mean $\pm$ SEM). ** $P < 0.01$ compared with LPS. (F) SP-A fails to increase 4E-BP1 phosphorylation at Ser^{65} in AMs from β-arrestin2$^{-/-}$ mice. AMs from wild-type, SP-A$^{-/-}$ and β-arrestin2$^{-/-}$ mice were left untreated or were treated with LPS (100 ng/ml), SP-A (40 μg/ml), or SP-A prior to LPS. Equal amounts of whole cell lysates were subjected to SDS-PAGE and immunoblotted for phospho-4E-BP1Ser^{65} and β-actin. Representative phospho-4E-BP1Ser^{65} and β-actin Western blots are shown. Data of at least four independent experiments were normalized to β-actin and quantified by one-way ANOVA with Dunnett's or Bonferoni's posttest (mean $\pm$ SEM). * $P < 0.05$, ** $P < 0.01$ compared with untreated AMs of the same genotype. # $P < 0.05$, ## $P < 0.01$ compared with AMs from wild-type mice treated with SP-A or SP-A plus LPS; ° $P < 0.05$ compared with AMs from SP-A$^{-/-}$ mice treated with SP-A plus LPS.

that β-arrestin2 is required for the SP-A-mediated attenuation of TLR4 signaling in a murine model of intratracheal LPS challenge (26). In that study, β-arrestin2 was critically involved in SP-A-mediated limitation of LPS-induced co-localization of TLR4 with early endosomes in primary AMs (26). In the present study, we found that SP-A fails to enhance degradation of LPS-induced TLR4 both in β-arrestin2$^{-/-}$ AMs and after intratracheal LPS challenge of β-arrestin2$^{-/-}$ mice, suggesting that β-arrestin2 is also required for SP-A-enhanced TLR4 degradation in the murine lung.

Previous studies demonstrated a pivotal role of β-arrestins1 and 2 in surfactant protein synthesis which is required for lung maturation (49, 69). Beta-arrestin1/2 double-deficient mice are neonatal lethal due to lung immaturity including decreased surfactant generation and significantly lowered SP-A expression (49). A similar phenotype including reduced surfactant protein expression was described for mice deficient in endothelial SAM and SH3 domain containing protein 1 (Sash1) that die perinatally (69). In that study, Sash1 was shown to interact with β-arrestin1 to induce alveolar epithelial cell maturation and subsequent surfactant synthesis (69). Compared with wild-type AMs, the expression of β-arrestin2 is significantly decreased in AMs from SP-A$^{-/-}$ mice (26) and, as shown in this study, SP-A protein expression is significantly reduced in lung tissue from β-arrestin2$^{-/-}$ mice, further supporting the hypothesis that SP-A and β-arrestin2 may be mutually regulated in the lung. Comparably, the complement protein C1q, which is structurally homologous to SP-A in its molecular assembly and collagen tail (9), is greatly reduced in bone marrow-derived macrophages from β-arrestin2$^{-/-}$ mice compared with wild-type mice (70). It is therefore likely that β-arrestin2 indirectly promotes anti-inflammatory responses by driving SP-A expression.

In the present study, we found that Rab7, which was reduced in AMs from β-arrestin2$^{-/-}$ mice, was required for SP-A-enhanced degradation of LPS-induced TLR4. Therefore, a decreased expression of Rab7 in β-arrestin2$^{-/-}$ AMs may account for reduced trafficking of LPS-induced TLR4 to the degradation pathways. It is becoming increasingly evident that β-arrestins and Rabs act in concert to dictate intracellular trafficking (71). The questions of interest are whether Rabs and β-arrestins functionally affect each other, whether β-arrestins bind to Rabs to modulate their activity (71) and, related to this study, whether β-arrestin2 can directly modulate GTP loading of Rab7. So far, proteomic analyses of proteins that interact with β-arrestins point to a direct interaction of small GTPases-related proteins with β-arrestins (72).

SP-A-induced mTORC1 activation is required for enhanced TLR4 degradation. Recent data demonstrated that mTORC1 has an essential role in proliferative renewal of murine AMs (41) and that mTOR is necessary to regulate cellular metabolism and accumulation of murine AMs (42). The signals, however, that provide surfactant-derived adjustment of mTORC activity in AMs under basal and infectious conditions are not yet identified. In the present study, both LPS, used as a positive control, and SP-A activated mTORC1 in primary rat AMs as indicated by increased phosphorylation of the mTORC1 downstream target 4E-BP1, infering that SP-A, comparable to LPS, may coordinate metabolic changes during AMs activation. Functionally, SP-A-mediated mTORC1 activation was required for SP-A-enhanced degradation of LPS-induced TLR4, but was apparently not involved in basal TLR4 degradation. The SP-A-enhanced degradation of LPS-induced TLR4 was abrogated in AMs from β-arrestin2$^{-/-}$ mice and the SP-A-induced activity of mTORC1 was significantly lower in AMs from β-arrestin2$^{-/-}$ mice compared with AMs from SP-A$^{-/-}$ mice, suggesting that β-arrestin2 is critically involved in mTORC1 activation by SP-A. Comparable to this finding, ghrelin-induced mTORC1 was shown to be inhibitable by β-arrestin 2 siRNA in the adipocyte-like cell line, 3T3-L1 (73).

The combined findings infer that SP-A targets an mTORC-mediated TLR4 degradation pathway. Recent mechanistic studies on the role of mTORC in TLR4 degradation revealed that the metabolic regulator late endosomal/lysosomal adaptor, Lamtor5, regulates mTOR-mediated TLR4 degradation by counteracting LPS-induced mTORC1 activation resulting in the initiation of the autolysosomal program and finally in enhanced TLR4 degradation (40). Comparably, transmembrane BAX inhibitor motiv-containing 1

was identified to mediate TLR4 degradation via the multivesicular body-lysosomal pathway (74). However, the mechanistic pathways that mediate SP-A-driven regulation of basal and ligand-induced TLR4 expression comprising the metabolic hub mTORC1 in AMs remain to be identified.

Together, our findings support a critical role for SP-A in the restriction of LPS-induced TLR4 expression and signaling in AMs by enhancing TLR4 degradation. This study may indicate a potential role of SP-A-based therapeutic interventions in unimpeded TLR4-driven immune responses to lower respiratory tract infections caused by Gram-negative bacteria.

MATERIALS AND METHODS

Primary AMs from healthy adult volunteers. The study is listed in the German register for clinical trials (www.DRKS.de; identifier DRKS00016932) and was approved by the Ethics Committee of the University of Lübeck (Ref. 15-194). Healthy adult volunteers, aged $\geq$ 18 years, gave written and verbal informed consent according to ICH/GCP (Good Clinical Practice) standards.

Exclusion criteria were history of immunosuppression, systemic corticosteroids during the previous month or antibiotic therapy within the last 2 months, asthma or chronic obstructive lung disease stage GOLD III or GOLD IV, respiratory tract infection in the previous month, previous or active tuberculosis, diabetes mellitus, pregnancy, or lactation. Data from 12 healthy adult volunteers (10 male, 2 female) mean age 30 (18–64) years are presented.

All study subjects underwent a flexible fiberoptic bronchoscopy according to current German guidelines with intravenous (Midazolam, Propofol) and local (Lidocaine) anesthesia at the physicians' discretion (75). The bronchoscope was wedged into a subsegmental bronchus of the middle lobe and BAL was performed in 15 $\times$ 20 ml fractions of warmed sterile 0.9% sodium chloride and retrieved by gentle suction. Because of possible contamination, the recovery of the first lavage fraction was discarded. The remaining fractions were pooled, washed two times by centrifugation at 400 $\times$ *g* for 10 min with Dulbecco's phosphate-buffered saline (PAA Laboratories, Pasching, Austria). The cells were resuspended in RPMI 1640 (PAN-Biotech, Aidenbach, Germany) and AMs were purified by adherence for 90 min at 37°C and 5% CO_2.The purity of macrophages on Diff-Quick-stained cytospin slides was 92.9 $\pm$ 1.4%, and the cell viability as determined by trypan blue dye exclusion was $>$ 98%.

Tumor-free lung tissues from surgical specimens of three lung cancer patients who underwent pneumectomy or lobectomy were used for extraction of alveolar macrophages. The study was approved by the Ethics Committee of the University of Lübeck (Ref. 15-163A and 17–137). The mean age of the donors, one male and two female patients, was 75 years (73–78) at time of surgery. The cells were isolated as described (76).

Primary AMs from rats and mice. Primary AMs were obtained from pathogen-free male Sprague-Dawley rats, from C57BL/6J and C57BL/6 wild-type mice (Charles River Laboratories, Sulzfeld, Germany), SP-A$^{-/-}$ mice (77) and β-arrestin2$^{-/-}$ mice (78). Gender matched mice used (6–12 weeks old) were maintained at the Research Center Borstel animal facility, a constant-temperature facility with 12 h controlled light/dark cycles, under specific pathogen-free conditions.

Animal care and experiments were approved by the Schleswig-Holstein Ministry of Energy, Agriculture, the Environment, Nature and Digitalization. The intratracheal challenge of mice and isolation of mouse and rat AMs from BAL fluid was performed as previously described (26).

LPS extraction and reagents. The smooth LPS from *Salmonella friedenau* strain H909 was extracted by the phenol/water method, purified, lyophilized, and transformed into the triethylamine salt form (79). All other reagents (except as noted) were obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) or Sigma (Steinheim, Germany).

SP-A purification. SP-A purification from bronchoalveolar lavage of patients with alveolar proteinosis was modified as descibed previously (26). In brief, the lavage fluid was treated with butanol to extract SP-A, and the resulting pellet was sequentially solubilized in octylglucoside and 5 mM Tris-buffered water (pH 7.4). To reduce endotoxin contamination, SP-A was treated with polymyxin B agarose beads. SP-A preparations were tested for the presence of bacterial endotoxin using the *Limulus* amebocyte lysate assay (Pyroquant Diagnostic, Mörfeld-Walldorf, Germany). All SP-A preparations used contained $<$ 0.2 pg endotoxin/μg SP-A.

Stimulation of AMs from humans, rats and mice. Human AMs (1 $\times$ 10^6 cells/500 μl), rat AMs (1 $\times$ 10^6 cells/500 μl) or AM from SP-A$^{-/-}$, β-arrestin2$^{-/-}$ and C57BL/6J wild-type mice (2 $\times$ 10^5 cells/500 μl) were seeded in 24-well plates (Nunc, Wiesbaden, Germany) and allow to attach for 90 min at 37°C in a 5% CO_2 atmosphere. The medium was changed, and the cells were left untreated or treated with SP-A (40 μg/ml) or LPS (100 ng/ml), a combination thereof, or IL-4 (0.5 μg/ml, PeproTech, Hamburg, Germany) for indicated times at 37°C in the presence of 0.2% heat-inactivated fetal bovine serum (PAA Laboratories, Pasching, Austria). In separate experiments, AMs were pretreated with cycloheximide (50 μg/ml, 30 min; Sigma), Rab7 blocking peptide (20 μg/ml, 15 min, Santa Cruz Biotechnology, Heidelberg, Germany), or rapamycin (20 nM, 60 min; Calbiochem Sigma, Molsheim, France) before the addition of SP-A, LPS, SP-A plus LPS, or IL-4. Under these conditions cell viability was $>$ 95%. After stimulation, cells were washed with 500 μl cold PBS, scraped off, and lysed in Laemmli buffer for 30 min to prepare whole cell lysates.

TNF-α ELISA. Secreted TNF-α was quantified in cell-free supernatants of treated AMs from wild-type and SP-A$^{-/-}$ mice by BD OptEIA sandwich ELISA (BD Biosciences, San Diego, CA) according to the manufacturer's protocol.

RNA isolation, reverse transcription, and quantitative RT-PCR. For quantitative real-time PCR, total RNA was extracted from rat AMs poststimulation using the innuPREP RNA minikit 2.0 (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions. The concentration and purity of isolated RNA were determined with the DeNovix DS-11+ spectrophotometer (DeNovix, Wilmington, DE). Total RNA was reverse transcribed into cDNA using Oligo(dT)$_{12-18}$ and Superscript III reverse transcriptase (Invitrogen/Thermo Fisher Scientific, Karlsruhe, Germany). Target genes were quantified by quantitative real-time PCR using the 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. TLR4 mRNA levels were normalized to GAPDH mRNA in the same sample and are expressed in arbitrary units. Primers used are as follows:

GAPDH-for TGATTCTACCCACGGCAAGTT;
GAPDH-rev:TGATGGGTTTCCCATTGATGA;
TLR4-for ATCATCCAGGAAGGCTTCCA;
TLR4-rev GCTGCCTCAGCAAGGACTTCT.

Relative expression of target gene mRNA was calculated by ΔC_T compared with the indicated housekeeping gene.

Western blotting. Western analysis was performed on whole cell lysates and cytosolic fractions from human, rat and mice AMs and on lung tissue homogenates from mice. Protein content was measured by the bicinchoninic acid reagent (Interchim, Montlucon, France). Defined amounts of the lysates were separated on SDS-PAGE and transferred to nitrocellulose membrane. Membranes were incubated with anti-TLR4 (rabbit polyclonal, 1:200, Santa Cruz), anti-β-arrestin2 (mouse monoclonal, 1: 200, Santa Cruz), anti-β-actin (mouse monoclonal, 1:200, Santa Cruz), anti-Rab7 (rabbit monoclonal, 1:1000, Cell Signaling Technology, Danvers, MA), anti-SP-A (rabbit polyclonal, 1:500, GeneTex, Irvine, CA), or anti-phospho-4E-BP1Ser^{65} (rabbit polyclonal, 1:1000, Cell Signaling) Abs. Goat anti-rabbit IgG-HRP (1:2000, Santa Cruz) or donkey anti-mouse IgG-HRP (1:2000, Cell Signaling) served as secondary Abs. Immunoreactive proteins were visualized using the ECL Western blotting detection system (Bio-Rad Laboratories GmbH, Feldkirchen, Germany), band intensity was quantified by analysis with ImageJ 1.42 (NIH), and data were normalized to β-actin levels.

Protein isolation from lung homogenates. Lungs were dissected from C57BL/6J and β-arrestin2$^{-/-}$ mice and snap-frozen in liquid nitrogen. Deep frozen lungs were homogenized with mortar and pestle. An aliquot of 30 mg of lung powder was transferred into RIPA buffer (Thermo Fisher Scientific, Waltham, MA) containing Protease Inhibitor (Thermo Fisher Scientific). Protein concentration in the supernatant was determined with the bicinchoninic acid reagent (Interchim).

Confocal microscopy. AMs from wild-type, SP-A$^{-/-}$ and β-arrestin2$^{-/-}$ mice were seeded at 1×10^5 cells/well on 8-well Lab-Tek II chamber slides (Nunc) and allowed to adhere for 90 min at 37°C in a 5% CO_2 atmosphere. After treatment, the cells were incubated with LysoTracker Red (100 nM, Molecular Probes, Eugene, OR), fixed with ice-cold (–20°C) 2% paraformaldehyde, permeabilized with 0.25% Triton X-100, blocked with 10% BSA/PBS and incubated with anti-TLR4 (1:50, mouse monoclonal, Santa Cruz). Alexa Fluor 488 goat anti-mouse IgG (1:500, Molecular Probes/Invitrogen) served as secondary Ab. Cell nuclei were counterstained with 4′,6-diamidin-2-phenylindol (Invitrogen). Samples were analyzed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Bensheim, Germany). Confocal images were acquired with the Leica TCS NT software and assembled using Adobe Photoshop 10.0.

LPS mouse models. Animal experiments were approved by the Schleswig-Holstein Ministry of Energy, Agriculture, the Environment, Nature and Digitalization (AZ 312–72241-123-3 and AZ 101-9/19). All mice were housed under pathogen-free conditions with an inverted 12-h light/dark cycle and had free access to food and water. Gender matched β-arrestin2$^{-/-}$ mice and C57BL/6J wild-type mice (age 6–12 weeks) were intratracheally challenged with 2.5 μg/kg BW of LPS or 2.5 μg/kg BW of LPS plus 5 mg/kg BW of SP-A using a small Animal Laryngoscope-Model LS-2 and a MicroSprayer (Penn-Century, PA, USA). For intratracheal applications, the animals were anesthetized by i. p. injection of ketamin (100 mg/kg BW, WDT, Garbsen, Germany) and xylazine (10 mg/kg BW, Bayer AG, Leverkusen, Germany) mixture. Mice were euthanized after 5 h by i. p. injection of a lethal dose of pentobarbital sodium and BAL was performed. In a second approach, gender matched SP-A$^{-/-}$ mice and C57BL/6J wild-type mice (age 8 to 12 weeks) were used. The application of PBS and LPS (5 μg/kg BW) was performed oropharyngeally under 5 Vol% Sevoflurane anesthesia (SevoFlo 100%, Ecuphar, Greifswald, Germany). Mice were kept under spontaneous breathing and regained consciousness after less than 1 min. Mice were euthanized after 24 h by i.p. injection of a lethal dose of pentobarbital sodium and BAL was performed.

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