Wall teichoic acid is a pathogen-associated molecular pattern of *Staphylococcus aureus* that is recognized by langerin (CD207) on skin Langerhans cells

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Summary

Staphylococcus aureus is a major cause of skin and soft tissue infections and aggravator of the inflammatory skin disease atopic dermatitis (AD). Langerhans cells (LCs) initiate a Th17 response upon exposure to *S. aureus*, which contributes to host defense but also to AD pathogenesis. However, the molecular mechanisms underlying the unique pro-inflammatory capacities of *S. aureus* remain unclear. We demonstrate that human LCs directly interact with *S. aureus* through the pattern-recognition receptor langerin (CD207), which specifically recognizes the conserved β-*N*-acetylglucosamine (GlcNAc) modifications of wall teichoic acid (WTA) that are not expressed by other staphylococcal species. The WTA glycoprofile strongly influences the production of Th1- and Th17-polarizing cytokines by LCs. Specifically, β-GlcNAc activates LCs, whereas co-decoration of WTA with α-GlcNAc through the enzyme TarM, uniformly present in the AD-associated CC1 lineage, attenuates LC immune activation. Our findings provide important mechanistic insights into the role of *S. aureus* in inflammatory skin disease.

Keywords

Langerhans cell, langerin, CD207, *Staphylococcus aureus*, wall teichoic acid, glycosylation, atopic dermatitis, pathogen-associated molecular pattern

Introduction

Staphylococus aureus is a common colonizer of the skin and nasal cavities but is also a major cause of skin disease. *S. aureus*-associated skin and soft tissue infections (SSTIs) are an important cause of disease and hospitalization and account for over 10 million cases each year in the US alone (Hersh et al., 2008). *S. aureus* is also an aggravator of the inflammatory skin disease atopic dermatitis (AD, eczema), which affects up to 20% of children and 3% of adults worldwide (Nutten, 2015). AD-affected skin is characterized by a disturbed composition of the microbiota with low species diversity and dominance of *S. aureus* (Totte et al., 2016). In particular, *S. aureus* strains of the CC1 lineage are overrepresented in AD isolates and were proposed to have particular yet-unidentified features that enable colonization of AD skin (Geoghegan et al., 2017).

When the skin barrier is breached by pathogens such as *S. aureus* a coordinated immune response is initiated. This response involves both resident and recruited immune cells as well as secreted antimicrobial factors. Important resident immune cells of the skin epidermis are Langerhans cells (LCs). These cells fulfill a gatekeeping function by sensing evolutionary conserved pathogen-associated molecular patterns (PAMPs) on invading pathogens through pattern recognition receptors (PRRs). In response, LCs can phagocytose invaders and initiate adaptive immune responses by activating skin-resident immune memory cells or naïve immune cells in the lymph nodes (Kissenpfennig et al., 2005, Seneschal et al., 2012). LCs are equipped with a diverse set of PRRs, including the LC-specific C-type lectin receptor (CLR) langerin (CD207) (Valladeau et al., 2000).

Wall teichoic acid (WTA) is an evolutionarily conserved and dominant component of the *S. aureus* cell wall. It plays an important role in nasal colonization, *S. aureus*-induced endocarditis, beta-lactam resistance and phage-mediated horizontal gene transfer (Winstel et al., 2015, Weidenmaier et al., 2005, Brown et al., 2012, Xia et al., 2011, Winstel et al., 2013). In the majority of *S. aureus* lineages, WTA is composed of a glycopolymer backbone of 20 - 40 ribitol-phosphate (RboP) subunits modified by *N*-acetylglucosamine (GlcNAc) and

D-alanine residues. The GlcNAc modifications can be either α-O-linked or β-O-linked to the anomeric C4 of the RboP subunits by the glycosyltransferases TarM and TarS, respectively (Xia et al., 2010, Brown et al., 2012). Overall, several *S. aureus* WTA glycoprofiles can be discriminated: WTA β-GlcNAcylation is conserved in almost all *S. aureus* strains, whereas WTA α-GlcNAcylation is only present in about one third of the *S. aureus* isolates, and a small selection of isolates have no WTA glycosylation at all (Li et al., 2015, Winstel et al., 2015). In addition, WTA of *S. aureus* lineage ST395 is composed of a glycerol-phosphate (GroP) backbone modified by *N*-acetylgalactosamine (GalNAc) instead of the common RboP-GlcNAc WTA (Winstel et al., 2013).

WTA glycosylation is an important determinant in host-pathogen interactions through both innate and adaptive immune recognition. For example, opsonizing antibodies interact with WTA GlcNAc to initiate complement deposition and subsequent phagocytic uptake of *S. aureus* (Kurokawa et al., 2013). Innate immune component interacting with WTA include the scavenger receptor SREC-1, which is important for attachment of *S. aureus* to the nasal epithelium, and mannose-binding lectin, which initiates deposition of complement C4 on *S. aureus* (Baur et al., 2014, Park et al., 2010). The interactions with these innate receptors are not influenced by the anomeric configuration of WTA GlcNAc.

S. aureus can enter the epidermis through a damaged stratum corneum, which is an important predisposing determinant for AD (Nakatsuji et al., 2016). Epicutaneous exposure of LCs to *S. aureus* initiates Th17 responses, which contribute to host defense but also to AD pathogenesis (Igyártó et al., 2011, Kobayashi et al., 2015). Previous studies indeed show that *S. aureus*-exposed LCs induce T cell proliferation (van der Aar et al., 2013). However, the molecular interaction between LCs and *S. aureus* and the functional consequences have received little attention. Therefore, the aim of this study was to investigate the interaction between *S. aureus* and human LCs at a molecular and functional level.

We demonstrate an important role of the PRR langerin in sensing the *S. aureus* cell wall component WTA. Langerin specifically recognizes β-GlcNAc modifications on *S. aureus* WTA, which contributes to LC activation and production of Th1- and Th17-polarizing cytokines. The selectivity of langerin for β-GlcNAc also explains the lack of binding to other non-AD associated staphylococcal species. Interestingly, simultaneous decoration of WTA with α-GlcNAc impairs langerin interaction and attenuates the functional response of LCs, implying that *S. aureus* can modulate immune detection and subsequent inflammation in the epidermis. In conclusion, we identify WTA as a pathogen-associated molecular pattern of *S. aureus*, which is recognized by langerin on LCs.

Results

Langerin is a receptor for *S. aureus* on human LCs

To determine whether LCs and *S. aureus* interact directly, we incubated primary LCs isolated from human skin with GFP-expressing *S. aureus*. LCs from four different donors bound *S. aureus* in a dose-dependent manner (Figure 1A). The levels at which the interaction was saturated varied between the donors from approximately 40% (donor 1) to 80% (donor 3) of *S. aureus*-positive LCs. To investigate the nature of interacting receptors on LCs, we pre-incubated LCs with mannan, a ligand for many CLRs. Depending on the bacteria-to-cell ratio, *S. aureus* binding was reduced by 35-70% compared to non-blocking conditions in all donors (Figure 1A). Similarly, the interaction was inhibited by approximately 35% by pre-incubation of the LCs with the monosaccharide GlcNAc. As langerin is a mannan- and GlcNAc-specific CLR that is exclusively expressed on LCs, we investigated whether langerin would be involved in interaction with *S. aureus*. Indeed, pre-incubation with an anti-langerin blocking antibody reduced binding of (*spa* and *sbi*-deficient) *S. aureus* in donors 3 and 4 by 25-50% compared to control, depending on the infective dose (Figure 1A). To confirm involvement of langerin in the interaction between *S. aureus* and LCs, we introduced langerin in THP1 cells. Transduction of langerin, but not of empty vector (EV), conferred *S. aureus* binding to THP-1 cells, which could be completely inhibited by addition of mannan or anti-langerin blocking antibody (Figure 1B). Although it was previously demonstrated that *S. aureus*-exposed LCs initiate T cell proliferation (van der Aar et al., 2013), the functional response of LCs was not assessed. Therefore, we incubated MUTZ-3-derived LCs (muLCs), a well-established cell model for human LCs (Masterson et al., 2002, Santegoets et al., 2006), with *S. aureus* and measured muLC activation through expression of co-stimulatory molecules and cytokine production after 24 hours. Indeed, muLCs upregulated expression of co-stimulatory molecules CD80 and CD86 and produced significant amounts of IL-8 and IL-12p70 in a dose-dependent response to *S. aureus* (Figure 1C). Together, these data demonstrate that LCs respond to *S. aureus* and that langerin is an important innate receptor for *S. aureus* on human LCs.

Langerin recognizes *S. aureus* in a *tarS*-dependent manner through the conserved WTA β-GlcNAc epitope

To further investigate langerin interaction with staphylococci, we tested binding of a FITC-labeled trimeric construct of the extracellular domain of human langerin (langerin-FITC) to a broader collection of *S. aureus* strains (18 strains from 11 different clonal complexes (CCs)) as well as several coagulase-negative staphylococci (CoNS) (Figure 2A). Langerin-FITC bound to 16 out of 18 (89%) tested *S. aureus* strains but to none of the tested CoNS species, indicating that langerin interacts with a ligand that is specific for and highly conserved in *S. aureus*. The three tested S*. aureus* strains that showed no or low-level binding of langerin-FITC (ED133, Lowenstein and PS187; Figure 2A), differ from the other tested *S. aureus* strains in the structural composition of WTA. ED133 and Lowenstein completely lack WTA GlcNAcylation, whereas PS187 belongs to the ST395 lineage that expresses GroP-GalNAc WTA (Li et al., 2015, Lee et al., 2015, Winstel et al., 2013). Given the high density of WTA on the *S. aureus* surface and apparent correlation between langerin interaction and WTA structure, we hypothesized that WTA GlcNAc modifications are likely candidates for the interaction with langerin.

To test this, we assessed binding of langerin-FITC to a panel of *S. aureus* knockout strains, which lack glycosyltransferases TarM and TarS required to modify WTA with α-GlcNAc and β-GlcNAc, respectively. Loss of both glycosyltransferases (Δ*tarMS*) reduced langerin-FITC binding to *S. aureus* to background levels (Figures 2B and S1BC), demonstrating that WTA GlcNAc is the target for langerin. To investigate whether langerin specifically recognized either α-GlcNAc or β-GlcNAc, we tested the individual TarM and TarS knockout strains as well as Δ*tarMS* complemented with either *tarM* or *tarS* on an expression plasmid (Δ*tarMS* p*tarM* and Δ*tarMS* p*tarS*). Langerin-FITC only bound to *S. aureus* strains that express β-GlcNAc, whereas α-GlcNAc was dispensable for binding (Figures 2B and S1BC). Similarly, langerin-FITC binding to *S. aureus* strains 82086 and PS66, which are naturally deficient for WTA α-GlcNAc, was completely abrogated in isogenic Δ*tarS* strains (Figure 2C). These

results show that langerin interacts with *S. aureus* in a *tarS*-dependent manner and provide the first demonstration of an anomeric-specific interaction of a human innate receptor with a Gram-positive surface polysaccharide.

Although α-GlcNAc is not the target of langerin, its presence or absence influences the level of langerin-FITC binding: mutant strains lacking *tarM* (Δ*tarM* and Δ*tarMS* p*tarS*) showed significantly increased binding compared to wild-type (Figures 2B and S1BC). Enhanced binding is likely due to loss of shielding by α-GlcNAc, since chemical analysis of the WTA composition by Kurokawa *et al*. suggests that Δ*tarM* WTA does not have increased β-GlcNAcylation (Kurokawa et al., 2013).

Since *S. aureus* expresses many human-specific factors (Koymans et al., 2016), we also investigated the interaction with murine langerin-FITC, which has 76% identity with the human langerin-FITC construct (Hanske et al., 2017). Binding of murine langerin-FITC to *S. aureus* was detectable, however binding levels were 10 to 100-fold lower than human langerin (Figure S1A), suggesting the langerin-*S. aureus* interaction displays a certain degree of species-specificity.

Altogether, these data show that human langerin interacts with *S. aureus* in a *tarS*-dependent manner through the conserved β-GlcNAc WTA modification and that the level of interaction is influenced by the simultaneous modification with α-GlcNAc.

S. aureus-induced production of Th1/Th17 polarizing cytokines by LCs is influenced by the WTA glycoprofile

Given the importance of langerin for interaction between *S. aureus* and LCs, we investigated whether distinct WTA GlcNAc glycoprofiles influenced the LC response at the level of co-stimulatory molecules and cytokine expression. Stimulation of muLCs for 24h with γ-irradiated wildtype *S. aureus* (WT) induced upregulation of activation markers CD80, CD83 and CD86 (Figure 3A). Stimulation with β-GlcNAc deficient *S. aureus* (Δ*tarS*) reduced expression of these markers compared to WT, whereas stimulation with α-GlcNAc deficient *S. aureus* (Δ*tarM*) enhanced expression. MuLCs secreted significant levels of IL-6, IL-8 IL-

12p70, IL-23p19 and TNFα (Figure 3B), but not anti-inflammatory cytokine IL-10 (not shown), in response to *S. aureus*. Especially IL-8, IL-12p70 and IL-23p19 were highly produced. Similar to the activation markers, stimulation of muLCs with *S. aureus* Δ*tarS* reduced IL-6, IL-8, IL-12p70, IL-23p19 and TNFα secretion compared to WT, whereas stimulation with *S. aureus* Δ*tarM* significantly enhanced secretion of these cytokines (Figure 3B). Interestingly, the level of activation matched the interaction levels of recombinant langerin-FITC to *S. aureus* WT, Δ*tarM* and Δ*tarS* strains (Figure 2B). These data suggest that the previously described Th17-polarizing response initiated by LCs in response to *S. aureus* is strongly influenced by detection of WTA β-GlcNAc. Moreover, the presence of WTA α-GlcNAc attenuates the LC response to *S. aureus*.

Discussion

Despite the emerging role of LCs in *S. aureus*-mediated skin inflammation, the molecular interaction between LCs and *S. aureus* has not been clarified. We identify WTA as a PAMP of *S. aureus*, of which the β-GlcNAc modifications are recognized by langerin on LCs. This interaction contributes to LC activation and production of Th1- and Th17-polarizing cytokines. Importantly, the extend of LC activation is strongly influenced by the WTA glycoprofile and suggest the possibility for immune evasion. Although several innate receptors have been described for *S. aureus* WTA, langerin is the first human innate receptor to discriminate between the α-GlcNAc and β-GlcNAc modifications.

Similar to lipopolysaccharide (LPS) of Gram-negative bacteria, WTA is an abundant evolutionarily conserved feature on the surface of Gram-positive bacteria and essential for maintenance of cell wall architecture and bacterial physiology. Therefore, it is advantageous for the host to recognize such structures in a timely manner through PRRs that are strategically localized at mucosal surfaces. In the case of *S. aureus*, a common colonizer of skin but also a major cause of skin disease, recognition of *S. aureus* WTA by langerin on epidermal LCs could be key to maintaining skin homeostasis. Since bacterial physiology is severely affected without WTA (Weidenmaier and Lee, 2016), bacteria have evolved other ways to regulate WTA expression and composition. First of all, WTA density depends on expression of *tarH*, which encodes the energy generating component of the WTA ABC transporter TarGH. Importantly, the amount of WTA correlates to the ability of *S. aureus* to induce skin abscess formation (Wanner et al., 2017). Second, studies on lytic podophage infection of *S. aureus* suggest careful regulation of WTA GlcNAcylation (Li et al., 2015). In support of this, *in silico* genome analysis identified *tarM* as part of the GraRS regulon (Falord et al., 2011), which is known to control resistance to cationic antimicrobials, oxidative stress and growth at high temperatures. Whether and how conditions during skin colonization affect WTA density or GlcNAcylation remains to be determined and will require the development of analytical tools that can accurately and immediately analyze the WTA glycoprofile.

We show that LCs respond to *S. aureus* with production of a Th1/Th17-polarizing cytokine profile. These data corroborate finding by others, who have demonstrated that LCs internalize *S. aureus* and subsequently polarize T cells towards Th17 (Igyártó et al., 2011, Kobayashi et al., 2015, van der Aar et al., 2013, Iwamoto et al., 2017). We now elucidate that detection of *S. aureus* WTA β-GlcNAc is of critical importance for the induced response. LCs and langerin were previously implicated in host defense against various other pathogens. LCs internalize and break down HIV-1 viral particles in a langerin-dependent manner to prevent infection of deeper layers of the mucosa (de Witte et al., 2007, Ribeiro et al., 2016). Langerin has also been identified as a major receptor for fungal pathogens on LCs through recognition of mannose and beta-glucan structures (de Jong et al., 2010). The Gram-negative bacterium *Yersinia pestis* is the only other bacterium known to interact with langerin through its LPS core oligosaccharide (Yang et al., 2015). We hereby identify *S. aureus* as a new langerin-interacting pathogen.

As is the case for many *S. aureus* virulence factors, we observe a certain degree of species specificity for the interaction between langerin and WTA (Koymans et al., 2016). Despite the high homology between human and mouse langerin (66% identity), the binding levels of mouse langerin were approximately 50-fold lower compared to human langerin. This corroborates previous studies that reported differences in ligand specificity of these orthologs (Hanske et al., 2017). It has become increasingly clear that mouse and human skin immunology can be very different, varying from altered receptor specificities to unique cell types and associated functions. This limits the translation of data obtained from mouse studies to humans. The different ligand specificities and thereby *S. aureus* WTA binding potential of human and mouse langerin are yet another example of this.

S. aureus is a driver of AD disease progression, which is mediated by LCs (Kobayashi et al., 2015). Interestingly, *CD207*, the gene encoding for langerin, was identified in genome-wide association studies as an AD susceptibility locus (Paternoster et al., 2015, Cai et al., 2017). Curiously, polymorphisms in a putative enhancer region of CD207, which are suggested to

enhance expression of langerin, were protective for AD. Our data now functionally link langerin to *S. aureus*. One might hypothesize that enhanced langerin expression results in enhanced bacterial recognition, which helps to eradicate *S. aureus* and prevents the development of chronic inflammation as observed in AD. However, an alternative explanation could be that *S. aureus* exploits langerin interaction to intentionally elicit inflammation to perturb the skin barrier and release nutrients. Since *S. aureus* is largely resistant to host defenses but most of the other commensals are not, this could explain the strong association between *S. aureus* and AD and the described driver function of *S. aureus* in AD disease progression. This suggests that langerin could play an important role in sensing and regulating skin microbiota homeostasis.

Although *S. aureus* is associated with AD in general, the CC1 lineage is overrepresented in isolates from AD skin (Geoghegan et al., 2017). This lineage was suggested to have particular yet-unidentified features that enable colonization of and proliferation in AD skin. Interestingly, all CC1 strains are *tarM*-positive (Winstel et al., 2014). Therefore, CC1 strains have the potential to regulate their WTA glycoprofile, balancing the inflammatory status of the skin and gaining an advantage to colonize AD skin. Therefore, our data provide molecular insight into the association between AD and *S. aureus* from two different angles: on the immunological side we show how langerin and LCs are involved in the immune response to *S. aureus*, while on the microbiological side the involvement of langerin could explain the association of *S. aureus* but not CoNS species with AD, and possibly also the overrepresentation of *tarM*-bearing CC1 strains in AD.

In conclusion, we identify WTA as a PAMP on *S. aureus* and pinpoint langerin as a molecular trigger for *S. aureus*-induced skin immune responses. Our findings may have implications for our deeper understanding of the association of *S. aureus* with skin inflammation and can help in the development of new targeted treatment strategies for the treatment of *S. aureus*-associated skin and soft tissue infections and inflammatory skin diseases.

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Declaration of Interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Langerin is a receptor for *S. aureus* on human LCs

(A) Binding of *S. aureus* to isolated primary human LCs. LCs from donors 1 and 2 were incubated with GFP-expressing *S. aureus* Newman and LCs from donors 3 and 4 with GFP-expressing *S. aureus* Newman Δ*spa*Δ*sbi* and binding was assessed by flow cytometry. The interaction was blocked by addition of mannan (10 µg/ml), GlcNAc (50 mM) or anti-langerin blocking antibody (20 µg/ml, donors 3 and 4 only).

(B) Binding of *S. aureus* to THP1-langerin cells. Human langerin-transduced or empty vector (EV)-transduced THP1 cells were incubated with GFP-expressing *S. aureus* Newman Δ*spa*Δ*sbi*. The interaction was blocked by addition of mannan (10 µg/ml) or anti-langerin blocking antibody (20 µg/ml). Within each ratio, THP1-langerin was compared to the other conditions.

(C) Expression of co-stimulatory molecules CD80 and CD86 and production of cytokines IL-8 and IL12p70 by muLCs after 24h incubation with γ-irradiated *S. aureus* USA300.

All data are represented as geometric mean fluorescent intensity or mean concentration + SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 2. Langerin recognizes *S. aureus* in a *tarS*-dependent manner through the conserved WTA β-GlcNAc epitope

Binding of recombinant human langerin-FITC to (A) 18 wild-type *S. aureus* strains (11 different clonal complexes, indicated above the bars) and a selection of coagulase-negative staphylococcal species (CoNS); (B) *S. aureus* USA300 wild-type (WT) and WTA biosynthesis mutants Δ*tarMS*, Δ*tarM*, Δ*tarS*, Δ*tarMS* p*tarM* and Δ*tarMS* p*tarS*; and (C) two representative *S. aureus* strains (82086 and PS66) that naturally lack *tarM* and their isogenic Δ*tarS* mutants. All strains were grown to mid-exponential phase and incubated with 5 µg/ml langerin-FITC or no langerin-FITC (background). Binding was assessed by flow cytometry. Data are represented as geometric mean fluorescence intensity + SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figure S1.

Figure 3. *S. aureus*-induced production of Th1/Th17 polarizing cytokines by LCs is influenced by the WTA glycoprofile

(A) Expression of co-stimulatory molecules CD80 and CD86 and maturation marker CD83 and (B) production of cytokines IL-6, IL-8, IL12p70, IL23p19 and TNFα by muLCs. muLCs were incubated 24h with γ-irradiated *S. aureus* USA300 wild-type (WT), Δ*tarM* and Δ*tarS*. muLCs stimulated with WT *S. aureus* were compared to the unstimulated control, whereas muLCs stimulated with Δ*tarM* and Δ*tarS* were compared to their respective WT controls within the same ratio. Data are represented as geometric mean fluorescence intensity or mean concentration + SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Nina M. van Sorge (nsorge3@umcutrecht.nl).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

Human skin tissue was collected from otherwise healthy donors undergoing corrective breast or abdominal surgery. This study, including the tissue harvesting procedures, were approved by the Medical Ethics Review Committee of the Academic Medical Center.

Bacterial culture

S. aureus, *S. capitis*, *S. carnosus*, *S. epidermidis*, *S. lugdunensis*, *S. pseudintermedius*, *S. saprophyticus* and *S. simulans* strains were grown overnight at 37°C with agitation in 5 ml Todd-Hewitt broth (THB; Oxoid). For *S. aureus* strains that were plasmid complemented THB was supplemented with 10 µg/ml chloramphenicol (Sigma Aldrich). A fresh 5 ml THB culture was inoculated by 150 µl overnight culture and grown to an optical density at 600 nm (OD_{600nm}) of 0.4 for *S. capitis* and to OD_{600nm} 0.6-0.7 for all other bacteria, which corresponds to mid-exponential growth phase.

Cell culture and differentiation

MUTZ-3 cells (human, male; ACC-295, DSMZ) were cultured in a 12-well tissue culture plates (Corning) at a density of 0.5-1.0x10^6 cells/ml in MEM-alpha (Gibco) with 20% fetal bovine serum (FBS, Hyclone, GE Healthcare), 1% GlutaMAX (Gibco), 10% conditioned supernatant from renal carcinoma cell line 5637 (ACC-35, DSMZ), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C with 5% CO_2. We obtained MUTZ-3 derived Langerhans cells (muLCs) by differentiation of MUTZ-3 cells for 10 days in 100 ng/ml Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF, GenWay Biotech), 10 ng/ml Transforming Growth Factor-beta (TGFβ; R&D Systems) and 2.5 ng/ml Tumor Necrosis

Factor-alpha (TNFα; R&D Systems) as described previously (Masterson et al., 2002, Santegoets et al., 2006). The phenotype of differentiated muLCs was verified by surface staining of CD34 (clone 581, BD Biosciences), CD1a (clone HI149, BD Biosciences) and CD207 (clone DCGM4, Beckman Coulter) using the respective antibodies and analysis by flow cytometry (FACSVerse, BD Biosciences). Data were analyzed using FlowJo 10 (FlowJo, LLC).

THP1 cells (human, male; TIB-202, ATCC) transduced with a lentiviral langerin construct or empty vector (EV) were cultured in RPMI (Lonza) supplemented with 5% FBS (Biowest), 1% GlutaMAX 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C with 5% CO_2.

METHOD DETAILS

Isolation of primary human Langerhans cells

Human Langerhans cells were isolated as described previously (Ribeiro et al., 2016). In short, skin grafts were obtained using a dermatome (Zimmer) and incubated in medium supplemented with Dispase II (1 U/ml, Roche Diagnostics) after which epidermal sheets were separated from the dermis and cultured for three days. After incubation, migrated LCs were harvested and further purified using a Ficoll gradient (Axis-shield). Isolated LCs were routinely 90% pure (CD1a+ Langerin+) and were frozen in Iscoves Modified Dulbeccos's Medium (IMDM, Thermo Fischer Scientific) supplemented with 20% FBS and 10% DMSO. Before use, LCs were thawed by drop-wise addition of cold IMDM with 10% FBS, washed twice and incubated in IMDM with FBS for 2 hours at 37°C with 5% CO_2 to recover.

Creation of GFP-expression *S. aureus*

To create GFP-expressing bacteria, *S. aureus* Newman wildtype and *S. aureus* Newman Δ*spa*Δ*sbi* were transformed as described previously with pCM29, which encodes superfolded green fluorescent protein (sGFP) driven by the *sarA*P1 promoter (Pang et al., 2010, Schenk and Laddaga, 1992). In short, competent *S. aureus* were electroporated with pCM29 isolated from *E. coli* DC10B with a Gene Pulser II (BioRad; 100 Ohm, 25uF, 2.5kV).

After recovery, bacteria were selected on TH agar supplemented with 10 µg/ml chloramphenicol. A single colony was grown in THB with 10 µg/ml chloramphenicol under the usual growth conditions. Bacterial expression of GFP was verified by confocal laser scanning microscopy (Leica SP5, Leica).

Gamma-irradiation of *S. aureus*

Gamma-irradiated stocks of *S. aureus* strains were made by harvesting cultures in mid-exponential growth phase by centrifugation (4,000 rpm, 8 min), which were concentrated 10x in phosphate-buffered saline (PBS; Lonza) with 17% glycerol (VWR), frozen at -70°C and exposed to 10 kGy of γ-radiation (Synergy Health, Ede, The Netherlands). Loss of viability of *S. aureus* was verified by plating of the irradiated bacteria. A non-irradiated aliquot that underwent the same freezing procedure was used to determine the concentration of colony forming units (CFU) of the irradiated stocks.

Lentiviral transduction

A TrueORF sequence-validated cDNA clone of human CD207 (OriGene Technologies) was amplified by PCR using Phusion polymerase (Thermo Fisher) and primers hLangerin-Fw and hLangerin-FLAG-Rv (IDT). The PCR amplicon was cloned in a BIC-PGK-Zeo-T2a-mAmetrine;EF1A construct by Gibson assembly (NEB) according to the manufacturer's instructions. The langerin-encoding vector and an empty vector (EV) control were introduced into THP1 cells by lentiviral transduction, as described by Van de Weijer *et al*. (van de Weijer et al., 2016). In short, lentivirus was produced by HEK293T cells (CRL-3216, ATCC) in 24-well plates using standard lentiviral production protocols and third-generation packaging vectors. After 3-4 days the supernatant containing the viral particles was harvested and stored at -70ºC to kill any remaining cells. Approximately 50,000 THP1 cells were transduced by spin infection (1000xg, 2 h, 33°C) using 100 µl supernatant supplemented with 8 µg/ml polybrene (Santa Cruz Biotechnology). Complete medium was added after centrifugation and cells were selected three days post-infection by 100 µg/ml zeocin (Gibco).

Cellular expression of langerin was verified by antibody staining of langerin (DCGM4, Beckman Coulter) and measured using flow cytometry.

Bacterial binding assays

To test binding of bacteria to cells, 10^5 LCs, THP1-EV or THP1-langerin were incubated with GFP-expressing *S. aureus* Newman or GFP-expressing *S. aureus* Newman Δ*spa* Δ*sbi* at bacteria-to-cell ratios from 1 to 8 in TSM buffer (2.4 g/L Tris (Roche), 8.77 g/L NaCl (Sigma Aldrich), 294 mg/L $CaCl_2$·2H2O (Merck), 294 mg/L $MgCl_2$·6H2O (Merck), pH=7.4) with 0.1% bovine serum albumin (BSA; Merck) for 30 minutes at 4°C. Binding was blocked by 15 minutes pre-incubation with 10 µg/ml mannan (Sigma Aldrich), 50 mM GlcNAc (Serva) or 20 µg/ml anti-langerin blocking antibody (10E2, Sony Biotechnology). Cells were washed once with TSM 1% BSA, fixed in 1% formaldehyde (Brunschwig Chemie) and measured by flow cytometry. We assessed bacterial binding by LCs or THP1 cells as the percentage of GFP-positive cells.

Production of recombinant langerin extracellular domains

The extracellular domains of truncated human langerin (residues 148–328) and mouse langerin (residues 150–331) were recombinantly expressed from codon-optimized constructs containing a C-terminal TEV cleavage site followed by a Strep-tag II cloned into pUC19 and pET30a (EMD Millipore) expression vectors as described previously (Hanske et al., 2017). Recombinant human and murine ECDs were insolubly expressed in *E. coli* BL21(DE3), solubilized in 6 M guanidinium hydrochloride in 100 mM Tris (pH 8) with 1 mM DTT, refolded by dialyisis against Tris-buffered saline (pH 7.5) containing 10 mM $CaCl_2$ and purified via mannan-coupled sepharose beads (Sigma Aldrich). Bound protein was eluted with Tris-buffered saline (pH 7.5) containing 5 mM EDTA. Protein concentrations were determined by A280 nm using the calculated molar extinction coefficients of 56,170 M^{-1} cm^{-1} for the human langerin ECD and 56,170 M^{-1} cm^{-1} for the murine ECD. The proteins were fluorescently labeled with fluorescein isothiocyanate (FITC, Thermo Fisher) by adding slowly 100 µL of the dye solution (1 mg/ml in DMSO) to 2 ml of a 2 mg/ml protein solution in HEPES-buffered

saline (pH 7.2) containing 20 mM D-mannose (Sigma Aldrich) and 5 mM $CaCl_2$. After stirring for 90 min at room temperature, the reaction was quenched by addition of 50 mM ethanolamine (pH 8.5, Sigma Aldrich). Unreacted dye molecules were removed by buffer exchange using a Zeba spin column (Thermo Fisher) and active protein was purified over mannan affinity column as described above. All chemicals used for the production of recombinant langerin extracellular domains were obtained from Carl Roth if not indicated otherwise.

Langerin binding assay

Bacteria in mid-exponential growth phase were harvested by centrifugation (4,000 rpm, 8 minutes) and resuspended at OD_{600nm} 0.4 in TSM buffer with 0.1% BSA. Bacteria were incubated with 1-50 µg/ml recombinant langerin-FITC (human or mouse) for 30 minutes at 37°C with agitation, washed once with TSM 1% BSA, fixed in 1% formaldehyde and analyzed by flow cytometry. We determined langerin-FITC binding of the bacteria using the geometric mean signal in the FITC channel.

muLC stimulation

We stimulated $5x10^4$ muLCs with *S. aureus* USA300 WT, USA300 Δ*tarM* or USA300 Δ*tarS* at bacteria-to-cell ratios of 0, 1, 10 and 50 in IMDM with 10% FBS. After 24 hours, supernatants were collected by centrifugation (300xg, 10 min, 4°C) and stored at -150°C until further analysis, and cells were washed once in PBS 0.1% BSA. Expression levels of the activation and maturation markers CD80, CD83 and CD86 were determined by flow cytometry using antibody clones 2D10 (CD80), HB15e (CD83) and IT2.2 (CD86) (Sony Biotechnology) and their corresponding isotype controls (BD Biosciences).

Cytokine assays

The IL-8 and IL12p70 concentrations were initially determined by ELISA (Sanquin and Thermo Fisher, respectively) according to the manufacturer's instructions. Concentrations of

IL-6, IL-8, IL-10, IL-12p70, IL-23p19 and TNFα were determined by Luminex xMAP assay (Luminex Corporation), performed by Multiplex Core Facility UMC Utrecht, The Netherlands.

QUANTIFICATION AND STATISTICAL ANALYSIS

We analyzed flow cytometry data using FlowJo 10 (FlowJo LLC). Statistical analyses were performed using Graphpad Prism 7.02 (GraphPad Software). We used unpaired two-tailed *t*-tests for comparisons between two groups and one-way ANOVAs with a common control group followed by Dunnett's test for comparisons between multiple groups. The human and mouse langerin concentration curves were tested using a two-way ANOVA followed by Tukey's test for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

Fig.1

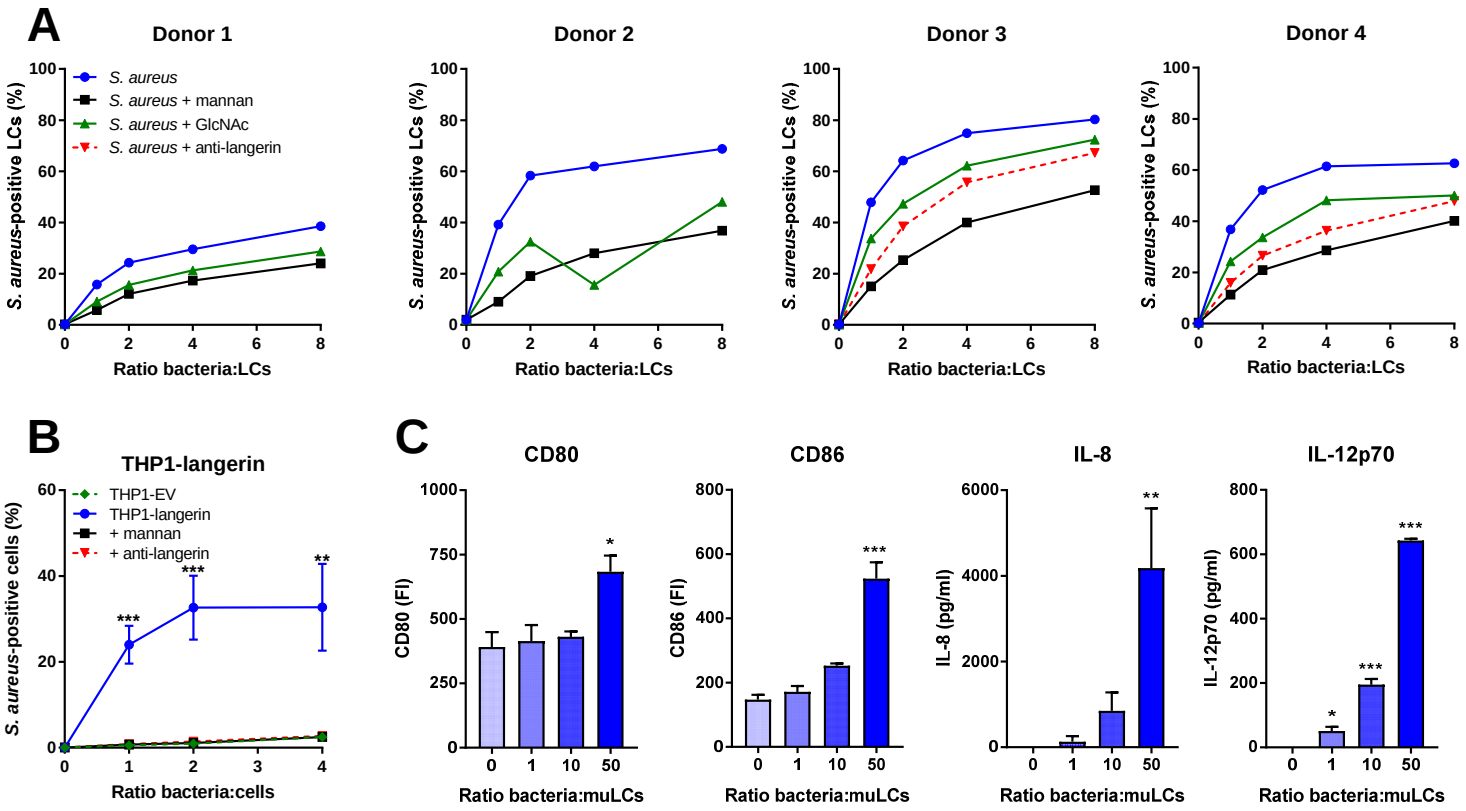
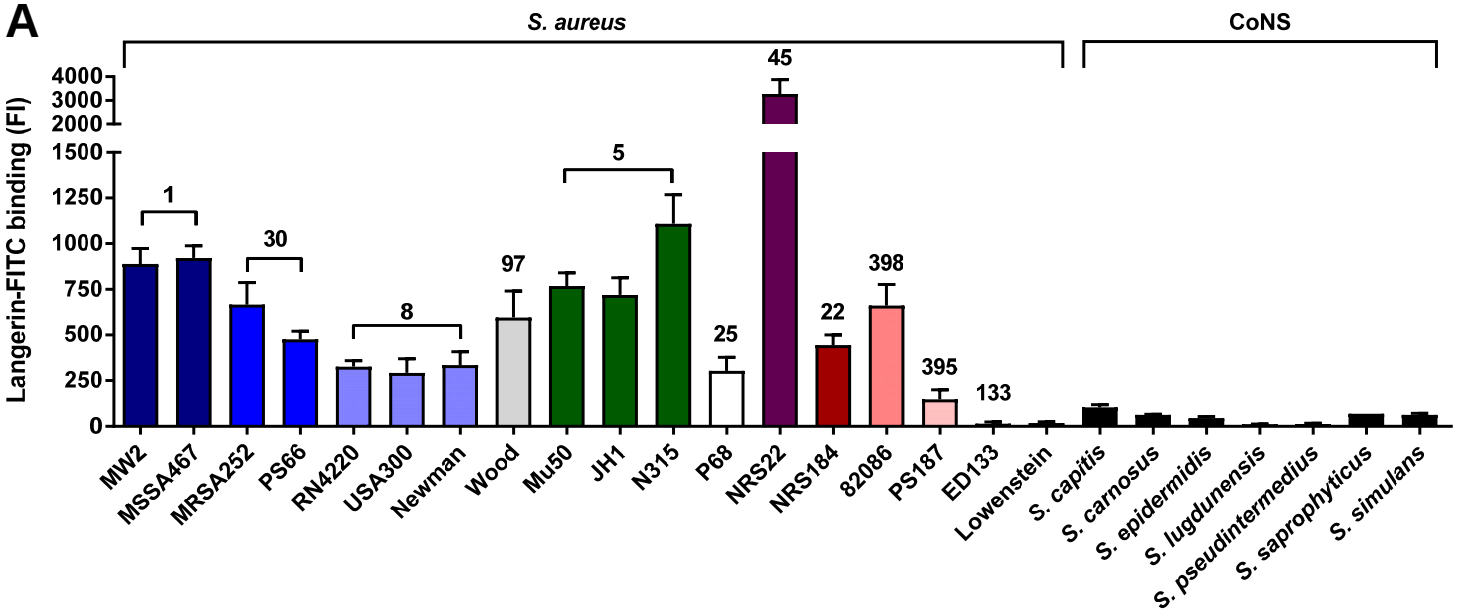
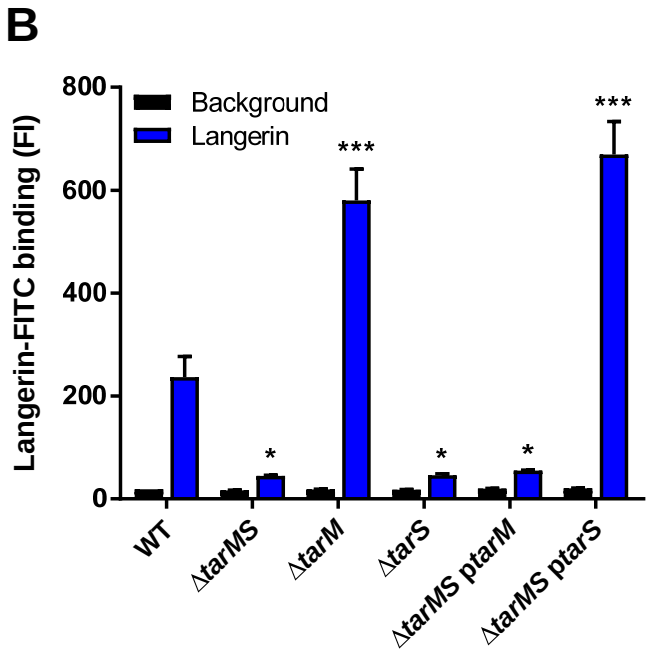

A
Donor 1
Donor 2
Donor 3
Donor 4
S. aureus
S. aureus + mannan
S. aureus + GlcNAc
S. aureus + anti-langerin
S. aureus-positive LCs (%)
Ratio bacteria:LCs
B
THP1-langerin
THP1-EV
THP1-langerin
+ mannan
+ anti-langerin
S. aureus-positive cells (%)
Ratio bacteria:cells
C
CD80
CD86
IL-8
IL-12p70
CD80 (FI)
CD86 (FI)
IL-8 (pg/ml)
IL-12p70 (pg/ml)
Ratio bacteria:muLCs

Fig.2


A
S. aureus
CoNS
Langerin-FITC binding (FI)
4000
3000
2000
1500
1250
1000
750
500
250
0
1
30
8
5
97
45
25
22
398
395
133
MW2
MSSA467
MRSA252
PS66
RN4220
USA300
Newman
Wood
Mu50
JH1
N315
P68
NRS22
NRS184
82086
PS187
ED133
Lowenstein
S. capitis
S. carnosus
S. epidermidis
S. lugdunensis
S. pseudintermedius
S. saprophyticus
S. simulans


B
Langerin-FITC binding (FI)
800
600
400
200
0
Background
Langerin
*
***
*
*
***
WT
ΔtarMS
ΔtarM
ΔtarS
ΔtarMS ptarM
ΔtarMS ptarS

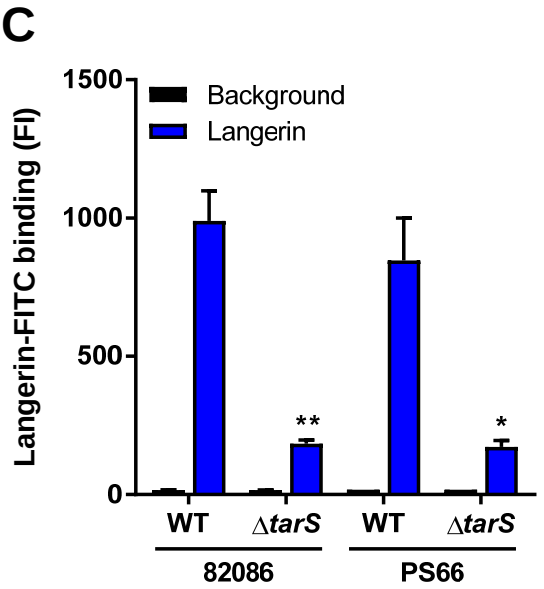
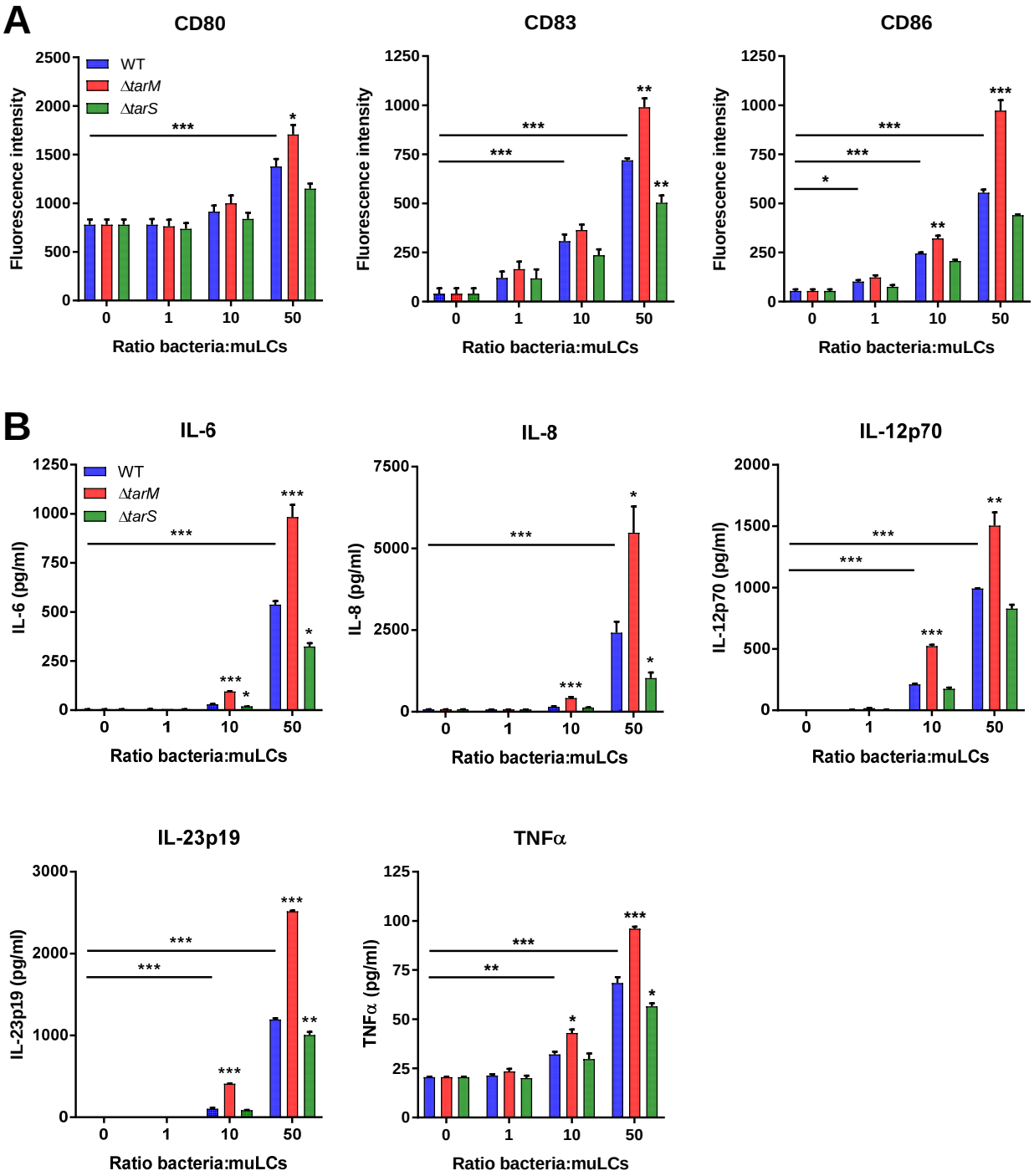

C
Langerin-FITC binding (FI)
1500
1000
500
0
Background
Langerin
**
*
WT
ΔtarS
WT
ΔtarS
82086
PS66

Fig.3


A
CD80
CD83
CD86
WT
ΔtarM
ΔtarS
Fluorescence intensity
Ratio bacteria:muLCs
B
IL-6
IL-8
IL-12p70
IL-23p19
TNFα
IL-6 (pg/ml)
IL-8 (pg/ml)
IL-12p70 (pg/ml)
IL-23p19 (pg/ml)
TNFα (pg/ml)