Lipid moieties on lipoproteins of commensal and non-commensal staphylococci induce differential immune responses

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Lipoproteins (Lpp) of Gram-positive bacteria are major players in alerting our immune system. Here, we show that the TLR2 response induced by commensal species Staphylococcus aureus and Staphylococcus epidermidis is almost ten times lower than that induced by non-commensal Staphylococcus carnosus, and this is at least partially due to their different modifications of the Lpp lipid moieties. The N terminus of the lipid moiety is acylated with a long-chain fatty acid (C17) in S. aureus and S. epidermidis, while it is acylated with a short-chain fatty acid (C2) in S. carnosus. The long-chain N-acylated Lpp, recognized by TLR2–TLR1 receptors, silences innate and adaptive immune responses, while the short-chain N-acylated Lpp, recognized by TLR2–TLR6 receptors, boosts it.

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Bacterial lipoproteins (Lpp) possess a lipid moiety at their N-terminus that enables their anchorage to the bacterial membranes. The maturation and processing of Lpp involve three enzymes: diacylglycerol transferase (Lgt), lipoprotein signal peptidase (Lsp), and an apolipoprotein N-acyltransferase (Lnt). In *Escherichia coli*, all three enzymes are essential and localized in the cytoplasmic membrane. In Gram-positive bacteria that lack the outer membrane, Lpp are anchored in the outer leaflet of the cytoplasmic membrane where they play a supportive role in uptake of nutrients, acquisition of essential ions, and maintaining metabolic activity and bacterial survival in infection. In Gram-positive bacteria, Lpp control pathogenicity and immunity.

Lpp can be separated into two functional units: the protein part is responsible for the metabolic function, while the lipid moiety anchors the protein in the membrane. However, this lipid structure, unique to bacteria, also acts as a "danger signal" that alerts the innate immune system via activation of pattern recognition receptors (PRR). Thus, Lpp and lipopeptides represent important microbe-associated molecular patterns (MAMPs) that are recognized by the innate immune system, which represents the first line of defense against invasive infectious pathogens. The essential receptor for recognition of Lpp or synthetic lipopeptides is the Toll-like receptor (TLR)-2. The lipid structure, namely the degree of acylation at the lipid moiety, can be discriminated by additional receptors that form heterodimers with TLR2: triacylated Lpp are recognized by the TLR2/TLR1 heterodimer and diacylated Lpp by the TLR2/TLR6 heterodimer.

The signal transduction from Lpp-bound TLR2 (TLR2 or TLR6) heterodimer to the activation of the nuclear factor NF-kappaB involves a cascade of phosphorylation events. The TIR domain-containing adapters MyD88 and TRIF are the first interaction partners and central adapter molecules in TLR2–TLR1/6-mediated signaling which is mediated by downstream activation of IRAK and subsequent translocation of NF-kappaB into the nucleus. This enables transcription and synthesis of proinflammatory cytokines and chemokines.

Since the number of acyl residues at the lipid moiety was shown to be crucial for their recognition via TLR2/1 or TLR2/6, it is important to know which bacteria produce di- or triacylated Lpp. To date, it is known that Gram-negative bacteria and also some high-GC Gram-positive bacteria such as mycobacteria and streptomycetes produce triacylated Lpp. These bacteria encode an apolipoprotein N-acyltransferase (Lnt) homologous to that in *E. coli*. The membrane-bound Lnt acylates the free N terminus of the cysteine residues, thus forming the triacylated lipid moiety. However, in low GC Gram-positive bacteria (*Firmicutes phylum*), the situation is much less clear because up to now, an enzyme with apolipoprotein N-acyltransferase function (e.g., Lnt) has not been identified.

Indeed, in certain bacterial species such as *Mycoplasma*, only diacylated Lpp are produced, such as the widely used macrophage-activating lipopeptide-2 (MALP-2) and *Staphylococcus aureus*, there was also no Lnt homolog identified, and it was assumed that in this bacterial species, Lpp are only diacylated like in *Mycoplasma*. However, Kurokawa and colleagues show that in *S. aureus*, the 33-kDa Lpp SitC, which is considered a model Lpp because of its abundance in staphylococci, is triacylated and acts as a TLR2 ligand. In principle, it should not matter whether the Lpp are di- or triacylated, because TLR2/1 or TLR2/6 heterodimers enable recognition of both Lpp types and, in both cases, this triggers NF-kappaB activation. However, recent reports have provided evidence that there are profound differences in the local immune response depending on whether the skin is exposed to di- or triacylated Lpp. Exclusively diacylated Lpp were capable of suppressing immune responses through interleukin-6 (IL-6)-dependent induction of granulocytic and monocyctic myeloid-derived suppressor cells. However, since these in vivo observations were mainly based on experimental treatments using synthetic Lpp, we asked whether such effects could also be mediated by commensal or noncommensal bacteria.

Here, we demonstrate that Lpp of the two commensal staphylococcal species, namely *S. aureus* and *Staphylococcus epidermidis*, tether the N terminus of S-diacyl-glycerol cysteine residue of the lipid moiety with a long-chain acyl group (heptadecanoyl fatty acid). This lipid structure dampens the immune response. In the noncommensal species, *Staphylococcus carnosus*, the N terminus of the lipid moiety carries only a short-chain (acytetyl) fatty acid, which induces a stronger innate and adaptive immune response.

**Results**

*S. carnosus* and *S. aureus* differ in immune stimulatory activity. When MM6 cells were stimulated with different staphylococcal strains and species, we observed important differences in their immune stimulatory potential. Notably, the *S. carnosus* parent strain TM300 induced a >4x higher TNF-α production than the three *S. aureus* strains USA300FJ2, HG003, and SA113 (Fig. 1a). Since, on the one hand, MM6 cells are responsive to a broad panel of agonists for PRR (e.g., TLR2, TLR4, TLR5, or Nod) and to adjuvants derived thereof, the high stimulatory activity of *S. carnosus* could result from recognition of a multitude of MAMPs. On the other hand, MM6 is unresponsive to TLR3, TLR7, TLR8, or TLR9 ligands and it is, therefore, unlikely that bacterial nucleic acids are responsible for the observed differences in immune stimulation. We, therefore, hypothesized that chemical differences in Lpp or peptidoglycan, which are recognized by TLR2 and Nod2, respectively, lead to differential immune recognition. Considering that TLR2 is a surface receptor and, thus, easily accessible and that peptidoglycan needs to gain access to its cytoplasmic receptors, Lpp were deemed the most likely candidates. To identify the MAMP responsible for the difference in immune stimulation, we repeated the experiment in HEK293 and TLR2-transfected HEK293 cells with the same stimulatory conditions. Untransfected HEK293 cells were not responsive to stimulation. However, in HEK-TLR2, we obtained the same pattern as that observed in MM6 cells: *S. carnosus* induced >10x higher IL-8 production than the three *S. aureus* strains and *S. epidermidis* O47 (Fig. 1b). These results indicate that TLR2-active Lpp are responsible for the differences in immune stimulatory activity. To verify this assumption, SitC, an abundant Lpp in staphylococci was expressed with a His tag and purified from both species and used for immune stimulation.

**SitC from *S. carnosus* shows a higher TLR2 response than that of *S. aureus*.** Previously, SitC has been described as one of the most prominent Lpp in *S. aureus*. It was originally referred to SitC because its protein sequence shares 77% identity to SitC of *S. epidermidis* where it was originally described as an iron transporter. Later, it turned out that in *S. aureus*, SitC is involved in manganese (Mn) transport and it was also referred to as MntC. The sitC gene was fused to a 3′ His tag, cloned into a xylose-inducible vector pTX-sitC-his, and expressed in *S. carnosus* TM300 and *S. aureus* SA113. From these two strains, SitC was extracted from the membrane fraction and purified via Ni-NTA chromatography. The two SitC lipoprotein preparations display a single band at 36 kDa in SDS-PAGE (Fig. 2a). As we express the same Lpp in various strains/species, we expect that folding and stability are the same. Equal amounts of purified SitC-his from both strains were used to stimulate HEK-TLR2 cells at varying conditions.
concentrations (50, 100, and 250 ng/ml). Both SitC-his Lpp induced TLR2-mediated IL-8 secretion in a dose-dependent manner (Fig. 2b). However, SitC-SC (isolated from *S. carnosus*) induced almost tenfold higher IL-8 production than SitC-SA (isolated from *S. aureus*). Importantly, the synthetic Lpp, P2C Pam$_2$CSK$_4$, a synthetic dipalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins, and P3C (Pam$_3$CSK$_4$, synthetic tripalmitoylated lipopeptide) (0.5 μg) also displayed an almost tenfold difference in IL-8 induction. In these synthetic Lpp, the diacyl-glycerol residue is palmitoylated. The only difference lies in the modification of the N terminus of the S-(diacyl-glycerol) cysteine residue: P3C is N-palmitoylated, while P2C is unmodified. These results are a first hint that the marked difference in TLR2 activation between *S. aureus* and *S. carnosus* could originate from species-specific differences in the lipidation structures of the Lpp.

To confirm the results obtained in HEK-TLR2 to more relevant cell systems, we investigated cytokine profiles of human cells. Using monocyte-derived dendritic cells (MoDC), we observed similar differences as in HEK-TLR2. Induction of the proinflammatory cytokines IL-6 and TNF and the Th1-polarizing cytokine IL-12p40 was higher when MoDC were stimulated with SitC-SC than with SitC-SA (IL-12p40: threelfold, IL-6: fivefold, and TNF: 10-fold increase); similarly, the synthetic lipopeptide P2C was more potent than P3C (Fig. 2c). The results in MM6 cells show the same tendency, e.g., higher secretion of TNF with SitC-SC stimulation than with SitC-SA (Fig. 2d). The results confirm that Lpp of *S. carnosus* and *S. aureus* differ in their immune stimulatory potential. Differences in the lipid structure could account for the differences in cytokine secretion levels. Most likely, Lpp in *S. carnosus* are diacylated, while those of *S. aureus* are triacylated. Notably, stimulation with the synthetic Lpp resulted in analogous differences in immune stimulation. We concluded that *S. carnosus* lacks the amide-bound fatty acid at the N terminus of cysteine as in P2C. If this was the case expression of the *E. coli*-specific apolipoprotein N-acyltransferase gene, *lnl* in *S. carnosus* should decrease immune stimulation to the levels seen with *S. aureus*.

Cloning of the *E. coli*-specific *lnl* in *S. carnosus* decreased TLR2-dependent signaling. The *E. coli*-specific *lnl* gene supplied with a 3′ strep tag was cloned into *S. carnosus* using the pCX-Int-strep vector in which *lnl* expression is repressed by glucose but induced by xylose. In *E. coli*, Lnt is an integral membrane protein with a periplasmic loop containing the active site. Western blot (alpha-strepAB) results confirmed that in *S. carnosus*, Lnt (mass 57 kDa) was localized in the membrane fraction and could only be detected when the transformed cells were grown in the presence of xylose (0.25%) but not glucose or in both cases in the vector control without *lnl* insert *S. carnosus* (pCX30) (Fig. 3b). Notably, in the *S. carnosus*, mutant expression of Lnt was low but this is also the case in *E. coli* where its abundance was calculated with ~100–200 molecules/cell. The most important result was that *S. carnosus* (pCX-Int) induced ten times less TNF-α and IL-8 production than *S. carnosus* wild type and *S. carnosus* (pCX30) (Fig. 3a). Thus, expression of Lnt reduced the immune stimulatory effect to the level seen with the *S. aureus* strains. Moreover, SitC purified from *S. carnosus* (pCX-Int), termed as SitC-SClnt, also triggered tenfold less IL-8 and TNF-α production compared to SitC-SC upon stimulation of MM6 and HEK-TLR2 cells (Fig. 2b, d). This result supported the hypothesis that the N-acylation of Lpp in *S. aureus* strains prevents TLR2-dependent immune stimulation when compared to Lpp of *S. carnosus*, in which we assumed the N terminus to be unmodified. To prove this hypothesis, the lipid structure of purified SitC extracted from *S. carnosus* and *S. carnosus* (pCX-Int) was analyzed.

Lpps from *S. carnosus* are aminoacylated with a short-chain fatty acid (C2) while those from *S. carnosus* (pCX-Int), *S. aureus*, and *S. epidermidis* with a long-chain fatty acid (C17). In order to demonstrate that the immune stimulatory activity is directly related to N-acylation of Lpp, we analyzed their lipidation by mass spectrometry (MS) and tandem mass spectrometry (MS/MS). The representative structures of the staphylococcal Lpp determined in this study are summarized in Fig. 4. First, the His-tagged SitC protein isolated from *S. carnosus* (SitC-SC) was subjected to SDSPAGE and in-gel trypsin digestion. The resulting digest was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) MS and nanoflow liquid chromatography (nLC)–MS. The MALDI mass spectrum of the digest of SitC-SC shows ions with 14-Da mass differences between m/z 1058.8 and 1184.8 (Supplementary Fig. 1a), which
can correspond to either diacyl (between 32:0 and 39:0; total numbers of carbon atoms and double bonds in their fatty acyl groups) or triacyl (between 31:0 and 38:0) lipopeptides. The accurate mass values obtained by LC–MS unexpectedly determined that they were triacyl forms (Supplementary Fig. 2). To obtain direct evidence of N-acetylation of the triacylated SitC-SC, we then performed MS/MS analyses. nLC–MS/MS spectra of each major lipopeptide ion demonstrated that the amino group of SitC-SC was acetylated (2:0) (Supplementary Fig. 3). The MS/MS spectra also allowed us to determine that the sn-1 position was O-acetylated with fatty acids of various lengths (between 15:0 and 20:0), while the sn-2 position in S-glycerolipids was exclusively O-pentadecanoylated (15:0), which is consistent with the composition of membrane lipids in S. aureus. Next, we analyzed the SitC protein prepared from S. carnosus (pCX-int) (pTX30-sitC-his). The MALDI TOF mass spectrum of SitC-SCInt shows ions between m/z 1297.0 and 1381.0 corresponding to triacyl (between 47:0 and 55:0) lipopeptides (Supplementary Fig. 1b). Further, MS/MS analyses demonstrated that the N terminus of SitC-SCInt was heptadecanoylated at the amino group and O-acetylated with similar fatty acids to those in SitC-SC, as shown in Supplementary Fig. 4. Note that no ion corresponding to N-acetyl forms could be detected by MALDI TOF MS although one would expect to detect a mixture of both the lipidations in the bacterium. Then, we confirmed our previous results on lipidation in S. aureus and S. epidermidis, the tagged SitC protein expressed in both S. aureus (SitC-SA) and S. epidermidis (SitC-SE) were triacylated structures with varying lengths of acylation (Supplementary Fig. 1c, d). The most abundant lipidation forms for SitC-SCInt, SitC-SA, and SitC-SE were 49, 51, and 53, respectively (Supplementary Fig. 1b–d), which probably reflects the difference in membrane lipid compositions of these three strains. To further understand whether Lpp in S. carnosus are generally N-acetylated, we performed LC–MS/MS analysis of another Lpp, Lpl1, whose amino acid sequence is significantly different from that of SitC. Lpl1 is the first Lpp encoded by the tandem lpl-gene cluster in S. aureus USA300. We analyzed the S. aureus Lpl1-his protein expressed by S. carnosus (Lpl1-SC) and that in S. aureus (Lpl1-SA) by LC–MS/MS. The accurate mass measurement and MS/MS analysis of Lpl1-SC provided unequivocal evidence that the N terminus of Lpl1-SC was also N-acetylated (2:0) and that the S-glyceryl moiety was O-acetylated with fatty acids (one of 17:0–21:0 at sn-1 and 15:0 at sn-2), which closely resembles the lipidation of SitC-SC (Supplementary Fig. 5). For Lpl1-SA, MS, and MS/MS, results of the in-gel digests revealed that the N-terminal lipopeptide of Lpl1-SA was N-long-chain-acylated...
acylation at the activation (Fig. 5). HEK293 cells constitutively express low levels of endogenous TLRs including TLR1 and TLR6 but lack the expression of endogenous TLR2, TLR4, as well as other associated molecules including CD14 and MD2. When we compared wild-type HEK293 cells and the TLR-transfected cells, the wild-type cells were unresponsive to any Lpp ligand, and stimulation of TLR1- and TLR6-transfected cells was negligible. By contrast, TLR2-transfected cells were highly responsive to P2C and SitC-SC and to a lesser extent to P3C and SitC-SA ligands. This finding indicates that there is sufficient endogenous expression of TLR1 and TLR6 in wild-type HEK293 cells to allow heterodimer formation with TLR2 (Fig. 5). Upon overexpression of TLR1 in TLR1 and TLR2 cotransfected cells, one would expect that recognition of P3C and SitC-SA would be strongly improved; however, P2C and SitC-SC remained superior to the triacylated ligands. We assume that the endogenous expression of TLR6 is sufficient to overrule TLR1 expression. Finally, TLR6 and TLR2 cotransfected cells were highly responsive to P2C and SitC-SC and displayed only very limited activity when exposed to P3C and SitC-SA. Again, this suggested that overexpression of TLR6 overrules TLR1/TLR2 responses. The results indicated that P2C and SitC-SC induce nearly comparable and high activation of TLR2/TLR6 heterodimers and that the short-chain N-acylated SitC-SC (the N terminus is only acetylated) behaves like the N-unmodified P2C (diacylated lipopeptide). Apparently, the short N-acetyl residue in SitC-SC is too small to function as a TLR2/1 agonist. The results further imply that TLR6/2 ligands dominate over TLR1/2 agonists with respect to induction of TLR2 signaling. The subsequent question was whether SitC-SC and SitC-SA also differ in their impact on the adaptive immune response.

**Short-chain N-acetylated SitC-SC triggered a TLR2/6 response while long-chain N-acylated SitC-SA a TLR2/1 response.** HEK293 cells were transfected with pFLAG-CMV-1 expressing TLR1, TLR2, or TLR6 alone or in combination and were stimulated with SitC-SC, SitC-SA, P2C, and P3C, respectively. The chemokine IL-8 was used as a readout of the TLR-induced NF-κB activation (Fig. 5). HEK293 cells constitutively express low levels of endogenous TLRs including TLR1 and TLR6 but lack the expression of endogenous TLR2, TLR4, as well as other associated

![Diagram](image)

**Fig. 3** Expression of *E. coli*-specific *int* gene in *S. carnosus*. (a) Plasmid construct: in pCX-int-strep, the *E. coli*-derived *int* carries a strep-tag sequence and two 3’ stop codons; its expression is xylose inducible and glucose repressible. (b) Western blot with membrane fractions generated from *S. carnosus* (pCX-int-strep) and *S. carnosus* (pCX30) cultivated in B medium supplemented with either 0.25% glucose (causes *int* repression) or 0.25% xylose (causes *int* induction); Lnt was targeted with α-strep-tag antibody; M, size standard

![Diagram](image)

**Fig. 4** The N-acylated and N-long-chain-acylated triacyl structures of staphylococcal Lpp. The N-acylation (a) is detected in *S. carnosus*, while the N-long-chain acylation (b) is found in *S. aureus*, *S. epidermidis*, and *S. carnosus* (pCX-int). Representative structures of each type are shown. The long-chain acylation at the α-amino group of Lpp varies in length in *S. aureus* and *S. epidermidis*. In contrast, only heptadecanoyl group is detected in *S. carnosus* (pCX-int). The sn-1 and sn-2 positions are O-acylated with various lengths of fatty acids (15:0–21:0) and exclusively O-pentadecanoic acid (15:0) in all the staphylococcal Lpps examined in this study.

**SitC-SC is a more potent inducer of Th1 responses than SitC-SA.** To assess the effect of the TLR2-active component in SitC on T cell responses, we analyzed secretion of the T cell-derived cytokine IFNγ in PBMC stimulated with MoDC pretreated with the TLR2 ligands, e.g., SitC derived from *S. carnosus* (SitC-SC) or *S. aureus* (SitC-SA) or the synthetic TLR2 agonists P3C, P2C, or nonstimulatory, monoacylated P1C (Pm-Dhc-CSK4, synthetic monopalmitoylated lipopeptide) as control. Figure 6a shows the results obtained by ELISPOT analysis. Notably, in the presence of triacylated Lpp (SitC-SA and P3C), the IFNγ response was lower than with diacylated P2C and the N-acylated SitC-SC.

![Diagram](image)
levels of IFN-γ (SitC-SA) or from $S.\ carnosus$ (pCX-SA) and P3C. MoDC were stimulated with SitC protein, TLR2-active lipoproteins or TLR2-active lipoproteins plus P1C controls. For TCF of mice infected with both strains, on day 2, neutrophil viability was reduced in TCF of mice infected with $S.\ carnosus$ compared to $S.\ carnosus$ (pCX-SA) compared to $S.\ carnosus$ (pCX-SA) (Fig. 7c); an effect that was even more pronounced on day 6 after infection (Fig. 7c). In line with this observation, TNF-α concentrations were increased in TCF of mice infected with $S.\ carnosus$ compared to $S.\ carnosus$ (pCX-SA) on day 2 (Fig. 7b), but neutrophil numbers on day 6 were comparable, suggesting that neutrophil numbers were not related to CFU numbers. Therefore, neutrophil viability and cytokine release were determined in TCF from mice infected with both strains. On day 2, neutrophil viability was reduced in TCF from mice infected with $S.\ carnosus$ compared to $S.\ carnosus$ (pCX-SA) compared to $S.\ carnosus$ (pCX-SA) (Fig. 7d). IL-6 release was similar on day 2, but higher in TCF from mice infected with $S.\ carnosus$ compared to mice infected with $S.\ carnosus$ pCX-SA on day 6 (Fig. 7e). Thus, the unmodified or short-chain (acetyl) modified N terminus of $S.\ carnosus$ Lpp induced tremendous TNF release in recruited neutrophils, increased cell death, and impaired bacterial killing in implant-associated infections.

To confirm that IFN-γ secretion originates from Th1 cells, we next retrieved to MoDC cocultures with purified total CD4+ T cells. MoDC were stimulated with SitC protein, TLR2-active lipoproteins (P2C, P3C), or P1C and cocultured with autologous CD4+ T cells for 5 days. Approximately fivefold higher secretion levels of the Th1 lead cytokine IFN-γ were detected in the supernatants of CD4+ T cells stimulated with MoDC challenged with SitC-SC when compared to SitC-SA (Fig. 6b). Similarly, P3C elicited higher levels of IFN-γ secretion when compared to P2C. By contrast, secretion of IL-17A was comparable in SitC-SC and SitC-SA-stimulated cultures but secretion levels remained low ($≤105$ pg/ml) and IL-17 was nearly absent in conditions with P3C and P2C.

Finally, we separated naive from memory CD4+ T cell fractions to investigate whether one of these subpopulations is more prone to respond to SitC or TLR2 ligands (Fig. 6c). IFN-γ responses were measured in supernatants from cocultures with MoDC stimulated with SitC-SC, SitC-SA, or P3C and P2C or unstimulated with P1C controls. CD4+ memory T cells were highly responsive to SitC-SC but SitC-SC only elicited very low levels of IFN-γ in naive T cells (12-fold difference) (Fig. 6d). The response to SitC-SA was significantly lower in both memory and naive cell fractions (6- and 10-fold difference to SitC-SC, respectively). Of note, in contrast to SitC, naive and memory T cell fractions were equally responsive to P3C and P2C (Fig. 6d).

Altogether, SitC-SC is superior to SitC-SA in regard to promoting a Th1 response; this is particularly reflected by its ability to trigger an IFN-γ response in CD4+ memory T cells. Lipid modifications in SA might, thus, suppress this type of T cell memory, which is generally regarded as protective.

**Discussion**

The skin is the organ most exposed to the environment; at the same time, it harbors highly diverse bacterial communities belonging to four major phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. Among the Firmicutes, staphylococci occupy a special position as many species are commensals of the skin of humans and animals. Each of the various areas within our skin, e.g., the moist, sebaceous, or dry sites represent niches for different staphylococcal species. It is apparent that host factors, immunological imprintment, and habits shape the composition of these microbial communities; vice versa, microbes present on the skin strongly impact the functions of human immunity. It was the aim of this study to investigate the reciprocal influence between the microbiota and the host immune response.

Here, we investigated the immune response of representatives of three different staphylococcal species: *S. aureus*, *S. epidermidis*, and *S. carnosus*. *S. epidermidis* is the most common clinical isolate derived from the cutaneous microbiota. Formerly, it was regarded as a harmless commensal but due to its biofilm-forming ability, it emerged as a frequent cause of foreign-body-associated chronic infections. *S. aureus* is also a skin commensal and its important niche is the anterior nares from where it can be isolated in more than one-third of the population. However, in contrast to *S. epidermidis*, *S. aureus* is equipped to produce an arsenal of toxins that promote acute and sometimes fulminant infections. Despite this battery of toxins, *S. aureus* is found on healthy human skin, acting as a commensal rather than a pathogen. In contrast to *S. aureus* and *S. epidermidis*, *S. carnosus* has the least pathogenic potential and it is not a commensal of human skin; its original habitat remains undefined although it was speculated that it might originate from marine fish.

Comparing the immune response of the two human skin commensals (*S. aureus* and *S. epidermidis*) with the non-commensal species *S. carnosus*, we were stricken by the fulminating immune response against *S. carnosus*, which induced almost...
10-times more IL-6 with MM6 than *S. aureus*. At that time, it was not clear which of the MAMPs was responsible for this effect: RNA, DNA, peptidoglycan, Lpp, and all possible combinations were in question. Since *S. aureus* lipidation of Lpp by the phosphatidylglycerol: prolipoprotein diacylglyceryl transferase (Lgt) is crucial for immune signaling, we hypothesized that an altered structure of its lipid moiety might be responsible for this surprising phenomenon. Considering that Lpp are TLR2 agonists, we carried out stimulation studies with HEK- and HEK-TLR2 cells in the presence and absence of *S. carnosus* or *S. aureus* (SitC-SA or SitC-SC) or with synthetic TLR2 ligands (P3C, P2C, or P1C) as controls. After 2.5-h stimulation, HEK- and HEK-TLR2 cells were added to freshly thawed autologous PBMC. IFN-γ-secreting cells were quantified by ELISPOT after overnight incubation. The results are displayed as means of the enzymatic activity of n = 6 independent donors ± SEM (upper panel). The two-tailed Student’s t test was used to calculate significance (*P < 0.05). ELISPOT images from one representative experiment are shown in the lower panel. (b) MoDC were left unstimulated or stimulated with SitC derived from either *S. carnosus* (SitC-SA) or *S. aureus* (SitC-SC) or with synthetic TLR2 ligands (P3C, P2C, or P1C) as control (1 μg/ml). Total CD4+ T cells were added to stimulated MoDC, MoDC/T cell cocultures incubated for 5 days before IFN-γ and IL-17 secretion was quantified in the supernatants by ELISA. Donors with strong reactivity in the unstimulated and PIC conditions were excluded from the analysis. The graph depicts the results obtained from n = 6 independent donors. The Wilcoxon matched-pairs signed rank test was used for statistical analysis (*P < 0.05). (c) Sorting scheme for obtaining CD4+ naive (CD45RA+CD45RO−) and memory (CD45RA−CD45RO+) T cell fractions. (d) Sorted naive and memory T cell fractions were cocultured with MoDC in the presence and absence of SitC-SA, SitC-SC, or P3C, P2C, and PIC (0.1 μg/ml). After 5 days, T cell-derived IFN-γ secretion was analyzed in the cellular supernatants. The graphs depict the results obtained from 12 independent donors as mean values ± SEM. Statistical analysis was performed using the Wilcoxon test (*P < 0.05; **P < 0.01).
was the case, one could expect that expression of the *E. coli*-specific Int gene in *S. carnosus* should decrease the immune stimulatory activity of Lpp to the level found in *S. aureus*. Our data corroborated this hypothesis (Fig. 1a), suggesting that the enzymatic modification by an Int analog might represent an important prerequisite for enabling commensalism on human skin and mucosal surfaces.

The next step was to analyze the structure of the lipid moiety of SitC-SA, SitC-SC, SitC-SCInt, and SitC-SE (*S. epidermidis*). It turned out that the lipid moiety of SitC-SA, SitC-SE, and of SitC-SCInt was almost identical. The two diacylglyceryl groups were acylated with long-chain fatty acids (C17 and C15, respectively) and the N terminus of *S*-(diacyl-glyceryl) cysteine residue was also acylated with a long-chain fatty acid (C17). This result indicates that *S. aureus* and *S. epidermidis*, the two commensals, possess an Lnt-like enzyme that catalyzes the same reaction as Lnt in *E. coli*.

In some *S. aureus* strains, diacyl Lpp was mainly produced at pH 5.5 and 6.0 and in stationary growth phase. However, we found in the 16-h cell culture of USA300 only triacylated SitC and Lpl1. A possible reason for the pH effect in *S. aureus* could be the expression of the gene encoding the apolipoprotein N-acyltransferase-like enzyme is induced only under certain conditions. Once the gene is identified, this question will be addressed in more detail in a human skin model. Besides triacylated, diacylated, and the short-chain N-acetylated Lpp, there was also a N-acetyl-S-monoacyl-glyceryl-cysteine (named the lyso structure) identified in low-GC Gram-positive *Enterococcus faecalis*, *Bacillus cereus*, *Streptococcus sanguinis*, and *Lactobacillus bulgaricus*. Recently, the gene leading to the lyso structure has been identified in *Enterococcus faecalis* and *Bacillus cereus* and was referred to as lipoprotein intramolecular transacylase (*lit*). It was proposed that Lit transfers a fatty acid from the diacylglycerol moiety to the alpha-amino group of the lipidated cysteine. What do we know about other commensal bacteria such as *Propionibacterium* and *Corynebacterium*? Both genera belong to a branch of high % GC Gram-positive bacteria. *Corynebacterium glutamicum* has also a *E. coli*-type Lnt. In propionibacteria, it is unknown, however, that they may also have the *E. coli*-type Lnt.

The most interesting finding, however, was that the N terminus of *S*-(diacyl-glyceryl) cysteine residue in SitC-SC also turned out to be modified, albeit not by a long-chain N-acylation but rather by N-acetylation; all other structural elements were identical to those in SitC-SA and SitC-SCInt. This type of N-acetylation has, so far, only been described in some *Bacillus* species including *B. subtilis*, *B. licheniformis*, and *B. halodurans*; but it was not detected in *B. cereus*, an environmental bacterial species. The N-acetylation of Lpp in *S. carnosus* and the mentioned *Bacillus* species suggest that this reaction is catalyzed by a different enzyme, which can be designated as an apolipoprotein N-acyltransferase (Lat). However, both the enzyme and the acetyl donor are unknown to date. Comparison of immune activation mediated by SitC-SC and P2C was almost indistinguishable, independent of the cell systems used. Thus, SitC-SC behaves like a diacylated Lpp and both SitC-SC and P2C represent strong TLR2/6 agonists. Of note, this further implies that the N-acetylation in SitC-SC is most likely too short to allow TLR2/1 specificity and subsequent activation.

Differential activation of TLR2 and its co-receptors influences the cytokine secretion and antigen presentation capacity of innate immune cells. The differences observed in MoDC activation were related to a more powerful induction of IL-6, TNF, and IL-12p40 by SitC-SA than by SitC-SC (Fig. 2c). These cytokines are not only important correlates of TLR2-mediated MoDC activation but they are also important co-stimuli that enhance and shape the adaptive immune response, in particular Th cell differentiation. As proposed by Blander and Medzhitov in 2006, the efficiency of antigen presentation via MHCII relies on the presence of TLR ligands within the phagocytosed matter. In the present case, the intrinsic TLR2 activity of the bacterial lipoprotein SitC enables its phagosomal categorization as a microbial antigen and consecutively drives phagosome maturation and antigen presentation. Furthermore, differential recognition via TLR1 or TLR6 is a fine-tuning element in the regulation of the
phagocyte response. It is, therefore, not unexpected that differences in lipid modifications of bacterial proteins that affect the strength and quality of TLR2 activation have direct impact on the efficacy of antigen presentation and subsequent T cell activation. Here, this is demonstrated by the failure of SitC-SA to trigger T cell responses equivalent to those seen with SitC-SC (Fig. 6).

It is known that Th1 and Th17 responses are protective against S. aureus and that both Th1 and Th17 cells are important for bacterial clearance. Furthermore, it has been proposed that DC-derived cytokines such as IL-6, TNF, IL-1β, TGF-β, and IL-23 are implicated in Th17 cell differentiation. Here, we observed that SitC-SC was superior to SitC-SA in triggering a Th1 cell response but there was no evidence for differential induction of Th17 responses (Fig. 6b). Interestingly, an earlier report shows that induction of Th17 responses by MoDC requires engagement of FcγR IIIa by bacterial immune complexes (IC) and TLR2 only amplifies this response. The absence of IC formation in our experimental setting might explain the overall low induction of IL-17 secretion from CD4+ T cells.

Notably, the effect of the SitC preparations on the T cell response can be attributed to stimulation of TLR2 and its co-receptors and, additionally, to the presentation of the antigenic peptides derived from the lipoproteins (SitC-SC and SitC-SA) by MoDC. When autologous CD4+ T cells were stimulated with MoDC pulsed with SitC-SC, T cell-derived secretion of IFN-γ (a cytokine specific for Th1 responses) was significantly higher than that with SitC-SA (Fig. 6b). Assuming that lipid modifications will neither affect processing and presentation of the antigen nor the epitopes recognized by T cells, the silencing of co-stimulatory innate immune signals, in particular, the suppression of the Th1-inducing cytokine IL-12 achieved by lipid modifications in SA, represents an important immune evasion mechanism that counteracts the generation of protective T cell responses. This effect was also confirmed in the context of T cell stimulation in PBMC where co-stimulation via SitC-SC and P2C was more effective than that delivered by SitC-SA and P3C (Fig. 6a). Importantly, with the synthetic lipopeptides, this trend was also reproducible in MoDC cocultures with purified T cells but—in contrast to the lipoproteins—the results remained statistically not significant. This may be due to any of three possible reasons: (1) TLR2 itself is not an endocytosis-promoting scavenger receptor. Thus, the stimulatory potential of the synthetic lipopeptides is limited to activation of surface TLR2. By contrast, the potency of the lipoproteins most likely results from additional endosomal TLR activation, as well as antigen processing and presentation to T cells. (2) PBMC contain a broad variety of TLR2-responsive innate immune cells that could compensate for the missing signals in MoDC. (3) T cell stimulation via the TCR and direct co-stimulatory activation of T cell-derived TLR2 might further result in important synergistic effects that cannot be achieved by stimulation with synthetic TLR2 agonists. Well in line with our findings, this effect could be more pronounced on memory T cells when compared to naive T cells (Fig. 6d).

Although S. carnosus alerts the immune response, it also leads to cell damage. In the murine tissue cage infection model, we show that the unmodified or short-chain (acetyl) modified N terminus of S. carnosus Lpp induced neutrophil cell death and tremendous amounts of TNF. Whether neutrophil death resulted from TLR2 signaling upon recognition of S. carnosus Lpp or high levels of TNF as shown in neutrophil, apoptosis needs to be investigated in further studies. As a consequence of the increased neutrophil death induced by S. carnosus, higher CFU were detected in the tissue cage fluid after perioperative infections. On the other hand, S. aureus-infected neutrophils can undergo apoptosis and other types of cell death.

Altogether, in our experimental conditions, the induction of a T cell response to Lpp is influenced by its inherent TLR2 activity. Notably, diacylated Lpp such as P2C, or the N-acylated SitC-SC were much more efficient than the triacylated Lpp including P3C, SitC-SA, and SitC-SC/II in mediating the activation of antigen-presenting cells such as MoDC. As a consequence, increased formation of Th1 cells in response to SitC-SC-stimulated MoDC is due to stronger activation of MoDC rather than differences in T cell recognition of the specific antigen (SitC). These results corroborate the importance of innate immune stimulation in shaping adaptive immunity. They imply that modification of lipid structures diminishes innate support of T cell responses and, thus, antibacterial defense. In return, out data suggest that this suppressive action enables host adaptation, persistence, and bacterial commensalism. We can, however, only speculate that Lpp-driven imprinting of innate immunity might even impede the development of protective vaccine-induced immunity.

In conclusion, here, we show that the commensal and non-commensal staphylococcal species differ markedly in immune stimulatory activity. The underlying reasoning lies, at least partially, in the altered lipid structure of Lpp. In the commensal staphylococcal species, the N terminus of the lipid moiety was found to be modified by a long-chain fatty acid (C17), while the non-commensal S. carnosus lipid moiety was modified by a short-chain (C2) fatty acid. The long-chain fatty acid at the N terminus decreased both the innate and adaptive immune responses, thus resulting in immune evasion. We interpret our results as an indication that the commensal species S. aureus and S. epidermidis are well adapted to the immune system, while S. carnosus has not adapted and is, therefore, recognized as foreign and consequently cleared by a fulminant immune response. The molecular basis for the host adaptation in the two commensal species would be an apolipoprotein N-acyltransferase, a Lnt-like enzyme, which has, so far, not been identified. Remarkably, only small structural alterations in the lipid moiety of Lpp hold the balance between immune tolerance and defense. Nevertheless, there is still much to learn on how structural alterations of MAMPs imprint the immune system. While adaptation to the immune system plays an important role in colonization of skin and mucosa, we should not forget that other factors might also promote commensalism. We particularly think of adhesion proteins, capsular polysaccharide, polysaccharide intercellular adhesion (PIA), secreted immune evasion factors, the repertoire of two-component sensor signal transduction systems, etc.

**Methods**

**Ethic statement.** The use of human peripheral blood mononuclear cells (PBMC) fromuffy coats obtained from the German Red Cross South transfusion center (Frankfurt am Main, Germany) was approved by Ethics committee of the Medical Faculty of the Goethe University Frankfurt/Main (Approval #154/15). C57BL/6 mice (janvierLabs, France) were kept under specific pathogen-free conditions in Animal facility of the DBM, University Hospital Basel. Perioperative infections of 13-week-old female mice were performed according to the review board of the Kantonale Veterinaeramt Basel-Stadt (permit no. 1710).

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this study and listed in Supplementary Table 1. E. coli strain BL21 was cultivated aerobically in Luria Broth (LB) medium with shaking at 37 °C. S. aureus strains and S. epidermidis O47, a biofilm-forming clinical isolate, were aerobically grown in Tryptic Soy Broth (TSB) at 37 °C. To express the gene cloned into the plasmid with inducible xylose promoter, the medium was supplied with 0.25–0.5% xylose in either basic medium. BM without glucose (1% soy peptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% K2HPO4, pH 7.4), or TSB (TSB: 1.7% tryptone, 0.3% phytone, 0.5% NaCl, 0.25% K2HPO4, and 0.25% glucose) at 37 °C. To prevent the loss of plasmids during bacteria replication, the media were supplemented with tetra-cycline (25 μg/ml) for pTX plasmid system or chloramphenicol (10 μg/ml) for pCX and pUC18 plasmid system.
Construction of Int-expressing plasmid. The int gene from E. coli Bl 21 was amplified by using a pair of primers. The forward primer with BamHI cleavage site (underlined) and reverse primer with EcoRI cleavage site (italic) were used to amplify the previous study18 with a slight modification. Briefly, the inverse primer comprised the StrepTagII coding sequence (italic sequence), two stop codons (bold sequence), and a XbaI site (underlined sequence) (ttttagctttataataataggtcggctttcattagtacg). The forward primer was annealed by using a pair of primers. The forward primer with BamHI cleavage site (underlined) and reverse primer with EcoRI cleavage site (italic) were used to amplify the previous study18 with a slight modification. Briefly, the inverse primer comprised the StrepTagII coding sequence (italic sequence), two stop codons (bold sequence), and a XbaI site (underlined sequence) (ttttagctttataataataggtcggctttcattagtacg). The forward primer was

Puriﬁcation of SitC-his from various staphylococcal clones. The puriﬁcation of lipoprotein SitC-his from the membrane of S. aureus SA113 and S. carnosus (pTX30-sitC-his), S. carnosus (pCX-ini) (pTX30-sitC-his), and S. epidermidis O47 was carried out to purify the previous study18 with a small modiﬁcation. Brieﬂy, the clone was precultivated aerobically at 37 °C in 3 l of B medium without glucose until OD600nm 0.5 was reached, and then the culture was supplemented with 0.5% xylose for 1 h induction of sitC expression. The bacterial cells were harvested by centrifugation at 4000 × g at 4 °C for 20 min and washed two times with Tris buffer (20 mM Tris, 100 mM NaCl, pH 8.0). Then, the pellet was resuspended with 100 ml of Tris buffer containing protease inhibitor tablet (Merck, Darmstadt, Germany), DNAse (10 µg/ml), and lysozyme (30 µg/ml) and incubated at 37 °C for 3 h to disrupt the cell wall. After the ultracentrifugation (235,000 × g for 45 min at 4 °C), membrane proteins were dissolved overnight at 6 °C with Tris buffer containing 0.25% Triton X100 and 20 mM imidazole), subsequently the beads were washed two times with the same buffer containing 500 mM imidazole. SitC-his was concentrated via centrifugal ultraﬁltration with a molecular mass cutoff of 10 kDa (Sartorius AG, Göttingen, Germany). Puriﬁed SitC was veriﬁed by SDS-PAGE and Coomassie blue staining, and the concentration of puriﬁed SitC was determined using anti-Strep Tag antibody (Abcam, Cambridge, England) at the dilution 1:5000.

Western blot. Bacteria were harvested by centrifugation at 5000 × g for 10 min at 4 °C and washed two times with 20 ml Tris buffer (pH 8.0). The cell pellets were again dissolved in Tris buffer containing protease inhibitor tablet (Merck, Darmstadt, Germany) and incubated with lysozyme (30 µg/ml) at 37 °C for 30 min. The membrane proteins were subsequently extracted following the above description, subsequently dissolved in SDS running buffer, and separated in SDS-PAGE. The membrane fraction was detected by SDS-PAGE using anti-Strep Tag antibody (Abcam, Cambridge, England) at the dilution 1:5.000.

Nanoﬂow liquid chromatography (nLC)-tandem mass spectrometry (MS/MS). The in-gel digests were analyzed by a nLC/MS/MS consisted of a nanoflow pump with an autoinjector (Easy nLC 1000, Thermo Fisher Scientiﬁc, San Jose, CA, USA), a ﬁtless electrospary C4 column (100 µm i.d. × 100 mm length; Nikkilo Technik, Tokyo, Japan) with a diode array detector (Q-Exactive, Thermo Fisher Scientiﬁc, San Jose, CA, USA). The method will be described in detail elsewhere. Brieﬂy, the nLC was performed at a ﬂow rate of 300 nl/min using a gradient elution of 0–100% acetonitrile in 0.1% aqueous formic acid solution. The eluate was electrosprayed into the spectrometer which was operated in a data-dependent mode, so that it was automatically switched between MS and MS/MS acquisition. Full scans for surveying precursor ions were acquired with a mass resolution of 70,000 at m/z 400. Each of the 10 most intense peaks in a survey scan was isolated within a 4.0-m/z window and fragmented by higher-energy collisional dissociation with a normalized collision energy of 30. MS/MS spectra were acquired with a mass resolution of 17,500 at m/z 400. The ﬁxed mass shift value for MS/MS was 100.

HEK-TLR2 stimulation assay. Human embryonic kidney (HEK 293) cells, stably transfected with the human TLR2 gene, were purchased from Invitrogen. The cultivation was performed following the previous study18. For the bacterial stimulation, HEK-TLR2 cells were seeded with 5 × 105 cells/200 µl/well into 96-well cell culture plates and incubated at 37 °C with 5% CO2. After 1-day incubation, the number of HEK-TLR2 cells was counted for the stimulation test. Prior to stimulation, bacteria were grown in TSB for 6 h. The cells were harvested and washed three times with DMEM/F before measuring the OD600 in DMEM/F. To calculate bacterial dosage (MOI, multiplicity of infection), bacteria were set to calculate the distinct OD/CFU. For S. aureus USA300 and SA113, OD600 of 1.0 equates to 1 × 108 CFU/ml, whereas for E. coli O47, OD600 equates to 0.4 and 0.2 × 106 CFU/ml, respectively. The final bacterial dosage (MOI 10) was suspended in 50 µl of the HEK-TLR2 medium for the stimulation. Bacteria were unable to grow due to antibiotic supplementation in stimulation medium. For the stimulation assays with puriﬁed proteins, the proteins were diluted in the HEK-TLR2 medium into three different amounts (10, 20, and 50 ng into 200-µl medium containing around 1 × 106 HEK-TLR2 cells prior to application for the test. Two synthetic lipopeptides, P3C (Pam3CSK4) and P2C (Pam2CSK4), were purchased from EMC (Tübingen, Germany), dissolved in water, and used for 100 ng for the above volume stimulation test, equivalent to the concentration of 500 ng/ml. Stimulation was carried out for 18 h. The supernatants were collected and stored at −20 °C until use.

For expression of TLR1/TLR2 and TLR6/TLR2 heterodimers in HEK293 cells, HEK293 cells were seeded at 5 × 105/200-µl RPMI (Gibco, Life science, Darmstadt, Germany) containing 10% fetal calf serum (FCS) (Sigma-Aldrich Chemie GmbH, Munich, Germany), 1% penicillin/streptomycin, and 1% γ-glutamine (both from Biochrom, Berlin, Germany) and incubated overnight at 37 °C, 5% CO2. The next day, the medium was replaced with 150-µl OptiMEM (Gibco, Life science, Darmstadt, Germany). Transient transfection was performed by complexing 0.25-µl lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) with plasmid (pFLAG-CMV-1) coding TLR1, TLR2, or TLR6. The total amount of plasmid DNA in the 50-µl OptiMEM medium used per well for transfection was 100 ng for single TLR1/2 or 60 ng of TLR6 plus 10 ng of TLR2 plasmid only to avoid recognition of Lpp by TLR2 only. Lipofectamine (LI) alone and untransfected HEK293 cells were used as controls. After overnight incubation, cells were washed and incubated with 100 ng/ml SitC-SA, SitC-SC, P2C, or P3C. After 18 h, supernatants were collected and stored at −20 °C.

MonoMac6 stimulation assay. MonoMac6 (MM6), a human monocytic leukemia cell line, was obtained from DSMZ with number AAC 124 (Braunschweig, Germany) and cultured as previously described17. For the stimulation assay, MM6 cells were seeded 1 × 104 cells/1 ml/well into 24-well cell culture plates and incubated for 24 h before adding the 10% bacterial suspension of either 50 µM E. coli O47 or S. aureus SA113 and S. aureus O47. For the stimulation assays with puriﬁed SitC, three concentrations were applied (50, 100, and 250 ng); as control, P3C and P2C were applied at a concentration of 200 ng, MM6 stimulation was carried out for 4 h; subsequently, the supernatants were collected and stored at −20 °C until they were used for ELISA.

Generation of monoocyte-derived dendritic cells. Buffy coats of healthy donors were obtained from German Red Cross South transfusion center (Frankfurt am Main, Germany). Informed consent was obtained from all donors. The protocol for this study was approved by the Ethics Committee of the Medical Faculty of the Goethe University Frankfurt/Main (Approval #154/15). PBMC were isolated by Pancoll gradient centrifugation (PAN-Biotech, Aidenbach, Germany). Monoocytes were isolated by positive selection with anti-CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity was analyzed by ﬂow cytometry on a FACS LSRII (BD Biosciences, Heidelberg, Germany) with anti-human CD14 V450 (BD Biosciences, Heidelberg, Germany) and ranged from 90% to 99%. The remaining PBMC were frozen in RPMI 1640 supplemented with 20% FCS and 2% DMSO (both from Sigma-Aldrich, Munich, Germany) for subsequent isolation of autologous T cells. Autologous monocytes were seeded at a density of 1.5 × 106 cells/ml in RPMI 1640 (Gibco, Life science, Darmstadt, Germany), supplemented with 10% FCS (Sigma-Aldrich Chemie GmbH, Munich, Germany), 1% penicillin/streptomycin, 1% γ-glutamine and 1% HEPES buffer (all from Biochrom, Berlin, Germany), 50 µM 2-Mercaptoethanol (Sigma-Aldrich, Munich, Germany), 50 ng/ml human GM-CSF, and 50 ng/ml human IL-4 (both from Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were incubated at 37 °C and 5% CO2, medium exchanged after 3 days, and cells were harvested on day 6. Differentiation of monocytes into MoDC was veriﬁed by ﬂow cytometric analysis using anti-CD14 V450, anti-CD38 APC, anti-CD11c-PE, and anti-HLA-DR-PerCP-Cy5.5 (all from BD Biosciences, Heidelberg, Germany) for a CD14+, HLA-DR+, CD11c+, and CD83+ phenotype.
P3C, or nonstimulatory P1C. CD4+ T cells were added to MoDC at a ratio of 5:1, respectively, or stimulated with 0.1 or 10 ng/ml LPS. To determine the endotoxin level, the limulus amebocyte lysate (LAL) assay (Cambrex, West Sacramento, CA) was performed according to the manufacturer’s instructions. MoDC and cocultures were incubated at 37 °C and 5% CO2 for the indicated time periods.

Detection of cytokines. ELISA. Human cytokine secretion was measured in cell supernatants using the BD OptEIA ELISA kits for IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFNγ, and TNFα. ELISA kits were performed according to the manufacturer’s instructions. ELISPOT. For ELISPOT assays, MoDC/T cell cocultures were performed in 96-well Multiscreen HTS IP plates (0.45 µm, clear, Merck Chemicals GmbH, Darmstadt, Germany), coated with anti-INF-γ capture antibody (BD Biosciences, Heidelberg, Germany) overnight at 4 °C. Following blocking of the plate for 2 h at room temperature with culture medium, cells were seeded and stimulated as described above. After 16 h of incubation, cells were discarded and wells were washed with sterile water two times followed by washing with PBS/0.05% Tween20 (Sigma-Aldrich Chemie GmbH, Munich, Germany) three times. Biotinylated anti-human IFNγ detection antibody (BD Biosciences, Heidelberg, Germany) was added in PBS/0.1% FCS and plates incubated for 2 h. After washing in PBS/0.05% Tween20, alkaline phosphatase (AP)-conjugated Streptavidin (BD Biosciences, Heidelberg, Germany) was added 1:1000 in PBS/10% FCS followed by incubation for 1 h. Development of the plate was performed with the AP conjugate substrate kit (Bio-Rad Laboratories GmbH, München, Germany); the reaction was stopped by washing with water and the plate dried overnight. Spots and enzymatic activity were quantified with an iSpot FluoroSpot Reader System (AID, Strassberg, Germany).

Endotoxin testing. Purified SttC-SA and SttC-SC were tested for endotoxin contamination using the Endosafe-PTS System (Charles River, USA). Endotoxin levels for 1 µg/ml protein solutions were <0.005 EU/ml for SttC-SA and 0.006 EU/ml for SttC-SC.

Statistical analysis. Unpaired two-tailed Student’s t test or analysis of variance (one-way ANOVA) was used to compare the difference of means. Statistical analysis was performed using SPSS v.19. For the Luminex analysis in MoDC, Wilcoxon matched- pairs signed rank test as a nonparametric version of the dependent t test was applied. For in vivo experiments, mouse values are shown as mean value ± s.d. of each group. Mann–Whitney test was used for comparison of CFU, leukocyte number and viability, TNFα, and IL-6 concentrations in TCF from mice infected with S. carnosus and Int. The significant level was set as: not significant (ns); P > 0.05; * P < 0.05; ** P < 0.01; *** P < 0.001.

Data availability. All the relevant data supporting the findings of the study are available in this article and its Supplementary Information files, or from the corresponding author upon request.

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