It has been well recognized that bacterial peptidoglycan (PGN) activates cells through the Toll-like receptor 2 (TLR2), which was first shown in 1999 using TLR2-transfected HEK293 and CHO cells (18, 27) and was then confirmed using TLR2-knockout mice (20). Since then, this observation has been supported by almost 200 publications.

However, a recent study by Travassos et al. (25) challenged this established view that PGN is a TLR2-dependent cell activator. Travassos et al. (25) purified PGN from cell walls of several gram-positive and gram-negative bacteria by treatment with sodium dodecyl sulfate (SDS), amylase, trypsin, acetone, and HF. Whereas crude cell wall preparations were active, purified PGN at 1 μg/ml did not activate TLR2-transfected HEK293 cells, while it still activated Nod2-transfected cells. Since many previous studies used commercial PGN, Travassos et al. (25) also repurified commercial *Staphylococcus aureus* PGN by treatment with SDS, and this treatment abolished the ability of this preparation at 1 μg/ml to activate cells through TLR2. Purified PGN at 1 to 10 μg/ml also did not induce interleukin-6 and tumor necrosis factor alpha (TNF-α) secretion in mouse macrophages (25).

Based on these results and on the ability of lipoteichoic acid (LTA) and lipopeptides to activate cells through TLR2, Travassos et al. (25) concluded that PGN is not a TLR2 agonist and that PGN sensing is lost after removal of lipoproteins or LTA from contamination with TA, LTA, or other possible contaminants. We digested our PGN with penicillin as described previously (17). sPGN is a newly synthesized PGN, whose incorporation into the existing cell wall is prevented by penicillin, which results in secretion of sPGN into the medium (17). This sPGN does not have wall teichoic acids (TA) or proteins bound to it, because these modifications occur after incorporation of PGN into the existing cell wall (7, 15, 17, 23, 28).

Chemical analysis of the above iPGN and sPGN, performed as described previously (3, 17), revealed amino sugar and amino acid composition typical for *S. aureus* PGN and similar to the composition of the purified iPGN of Travassos et al. (Table 1). The higher alanine content in sPGN than in iPGN reflects the presence of terminal D-Ala-D-Ala in the peptide of un-cross-linked sPGN (the structure specifically recognized by vancomycin and uniquely present in un-cross-linked sPGN, which is the basis of vancomycin affinity purification of sPGN). iPGN in the cell wall loses the terminal D-Ala during peptide cross-linking.

However, because higher alanine content could also originate from wall TA or LTA contamination, we determined whether alanine in our PGN was an integral part of PGN or came from contamination with TA, LTA, or other possible contaminants. We digested our sPGN with N-acetylmuramyl-L-alanine amidase (which digests the amide bond between N-acetylmuramic acid and L-Ala of PGN and thus separates the peptide from the glycan chain of PGN), and we analyzed the digestion products by mass spectrometry (MS), as described in our recent publication (26). This digestion yielded five peptides (Table 2 in reference 26): AEKAA+G6, AEKAAA+4G, AEKAAA+G4, AEKAAA+G2, and AEKAAA+G5 (with the first two peptides being the most abundant). No other peptides were detected. The peptide peaks for all five peptides were quantified, and the ratio of alanine to lysine was calculated to be 2.9, which is identical to the alanine-to-lysine ratio determined by amino acid analysis of sPGN hydrolysates (Table 1).

These results demonstrate that (i) all alanine detected by quantitative amino acid analysis (Table 1) is present in the PGN stem peptides; (ii) our sPGN is not contaminated with wall TA or LTA which would have contributed additional alanine to our sPGN above the amount present in the PGN.
TABLE 1. Amino sugar and amino acid composition of *S. aureus* PGN (molar ratios to Lys)

<table>
<thead>
<tr>
<th>Component</th>
<th>Theoretical PGN</th>
<th>sPGNa</th>
<th>iPGNb</th>
<th>IPGN (reference 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc/MurNAc</td>
<td>2</td>
<td>1.44</td>
<td>1.39</td>
<td>1.36</td>
</tr>
<tr>
<td>Ala</td>
<td>2–3</td>
<td>2.92</td>
<td>1.69</td>
<td>2.23</td>
</tr>
<tr>
<td>Gia</td>
<td>1</td>
<td>1.03</td>
<td>0.67</td>
<td>1.55</td>
</tr>
<tr>
<td>Lys</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gly</td>
<td>2–6</td>
<td>3.60</td>
<td>3.55</td>
<td>4.13</td>
</tr>
<tr>
<td>Other amino acids</td>
<td>0–1</td>
<td>0.5</td>
<td>0.09</td>
<td>0.7</td>
</tr>
</tbody>
</table>

a Soluble PGN purified by vancomycin affinity chromatography from supernatants of bacteria grown in the presence of penicillin.
b Insoluble PGN purified by treatment with trypsin, DNase, RNase, trichloroacetic acid, acetone, and SDS.
c Insoluble PGN purified by treatment with SDS, amylase, trypsin, acetone, and HF (25).

stem peptide; (iii) all our sPGN is a non-cross-linked PGN (because cross-linked peptides were not detected in the digests), which is consistent with the origin of this sPGN as newly synthesized and secreted from the cells without being cross-linked to the existing PGN in the cell wall; and (iv) our sPGN does not contain fragments of the old cell wall PGN released by autolytic enzymes (which again would have contained cross-linked peptides).

Our sPGN and iPGN were free of any protein contamination because their amino acid composition was typical of *S. aureus* PGN (Table 1) and showed the absence of amino acids found only in proteins and never found in PGN, such as aromatic or sulfur-containing amino acids. Moreover, silver staining of our sPGN at 125 µg/lane on SDS-polyacrylamide gel electrophoresis, which can detect 0.2 ng protein/band, showed no staining (3, 4).

To further confirm the purity of our PGN preparations, we have also hydrolyzed our sPGN and iPGN with HCl (6 N, 5 h, 105°C) and subjected the hydrolysates to MS analysis. As standards, we used individual PGN amino sugars and amino acids and a mixture of these components in the same proportion as in PGN (Table 1), all subjected to the same HCl treatment (to account for any modifications, such as decylation or decomposition of amino sugars). In the standard analysis by matrix-assisted laser desorption ionization-reflection-time of flight MS (performed as described in reference 26), both our sPGN and iPGN hydrolysates showed peaks with correct predicted masses, identical to the peaks present in the mix of standards, with no extraneous peaks. We also analyzed the hydrolysates of sPGN, iPGN, and the standards (dissolved in acetonitrile:H₂O:formic acid, 50:50:0.1) by triple quadrupole/linear ion trap (Q-Trap) MS, in both the positive and negative ionization modes, because some residues or adducts produced by hydrolysis (especially polyglycerol phosphate and polyribitol phosphate hydrolysates) may have low ionization efficiency and may not be readily detected by matrix-assisted laser desorption ionization–MS. Q-Trap-MS analysis also did not detect any glycerol, ribitol, or their adducts in the sPGN and iPGN hydrolysates. These results confirm the purity of our PGN preparations (and lack of contamination with TA and LTA) using two MS methods, which detect all the components of the preparation based on different principles than the detection on an amino acid/amino sugar analyzer shown in Table 1.

Both purified *S. aureus* sPGN and iPGN activated transiently transfected HEK293 cells (18) in a concentration-dependent and TLR2-dependent manner (Fig. 1A to C), consistent with our previous results (18). Only cells transfected with TLR2 plus CD14 (Fig. 1B) or TLR2 alone (not shown) were responsive to PGN, whereas cells transfected with a control vector (Fig. 1A), TLR4 plus CD14 (Fig. 1C), TLR4 alone (not shown), or TLR4 plus CD14 plus MD2 (19) (not shown) were totally unresponsive to sPGN and iPGN. The integrity of TLR4 was confirmed by its responsiveness to purified lipopolysaccharide (LPS) from *Salmonella enterica* serovar Minnesota Re 595 in cells transfected with TLR4 plus CD14 plus MD2 (not shown). sPGN activated TLR2-plus-CD14-transfected cells at 0.1 to 1 µg/ml, whereas iPGN activated these cells at 10 to 100 µg/ml. CD14 by itself (without TLR2) does not confer the responsiveness to PGN in HEK293 cells and is not required for the TLR2-dependent response to PGN, but it enhances TLR2-mediated response to PGN (18). As expected (18), *S. aureus* LTA, purified as previously described (6), also activated cells in a TLR2-dependent manner at concentrations of 0.1 µg/ml and higher (Fig. 1A to C).

Similar TLR2-dependent activation was obtained with iPGN reextracted with hot SDS (2 mg iPGN/ml treated with 8% SDS at 90°C for 30 min, followed by 10 washes with H₂O to remove SDS) (Fig. 1D), a procedure that abolished TLR2-dependent activation of commercial iPGN at 1 µg/ml in the study by Travassos et al. (25). Moreover, reextraction of our *S. aureus* iPGN (2 mg/ml) with 45% phenol-55% water (at 60°C for 60 min, followed by removal of residual phenol from iPGN by six extractions with ethyl ether) did not change its ability to activate TLR2 (Fig. 1D). The phenol-water extraction (8) was selected rather than the butanol extraction (16), because hot phenol-water extraction is more effective in completely removing LTA (and also LPS and other lipids) from bacterial cell walls (8) than the gentler butanol extraction (although it diminished the activity of LTA due to partial removal of alanine). The extracted fraction (which should have contained LTA and was contaminated by lyophilizing) was not active (Fig. 1D).

Because in the study by Travassos et al. (25) purified iPGN at 1 to 10 µg/ml also did not induce TNF-α and interleukin-6 secretion in cultures of mouse macrophages, we compared the TNF-α-inducing capacity of our *S. aureus* iPGN before and after reextraction with hot SDS or phenol-water (as above). Untreated and both SDS- and phenol-water-reextracted iPGN had the same TNF-α-inducing capacity in cultures of a mouse macrophage (RAW264.7) cell line (Fig. 1E), determined by the L929 cell cytotoxicity assay as previously described (11). The extracted water fraction was inactive, further confirming that our iPGN was not contaminated with bioactive LTA. This is not surprising, because trichloroacetic acid and acetone treatments included in our iPGN purification procedure also effectively remove LTA. The PGN-induced cell activation was also not due to endotoxin contamination of our PGN or synergistic stimulation by PGN and LPS, because (i) both sPGN and iPGN were free of any detectable LPS (negative by the *Limulus* lysate assay at <25 pg LPS/mg PGN); (ii) with sPGN being active at 0.1 µg/ml, this corresponds to a possible <2.5 fg LPS/ml, which is more than 4 orders of magnitude lower than the minimum concentration of LPS (100 pg/ml) that activates macrophages (6, 11, 24) or synergizes with muramyl peptides in...
(ii) similar results were obtained when the cells were stimulated in the absence or presence of 5 μg/ml polymyxin B (not shown), a polypeptide that inhibits cell activation by endotoxin, which we and others have shown in numerous publications (11–13).

The PGN-induced cell activation was also not due to contamination of our PGN with proteins, wall TA, or LTA, because chemical and mass spectrometry analysis did not detect them in our PGN (as described above) and because neither wall TA, obtained as described previously (17), nor protein A (major *S. aureus* cell wall protein, obtained as a recombinant protein from Amersham/Pharmacia, Piscataway, NJ) activated mouse macrophages or TLR2-transfected HEK293 cells at 100 μg/ml (not shown). Moreover, this activation was also not due to any possible synergistic activation by PGN and LTA, because this synergism in vitro is evident only for the production of nitric oxide, but not for TNF-α (in contrast to the in vivo effect, which is synergistic for both inducible nitric oxide synthase and TNF-α (2, 14), and because we also could not demonstrate any synergistic effect between PGN and LTA for the activation of mouse macrophage cells or TLR2-transfected HEK293 cells (not shown). In both systems, we could at best show an additive effect, which suggests activation through the same receptor and signal transduction pathway.
To further confirm that the TLR2-dependent responsiveness to our PGN preparations was due to PGN and not due to LTA or some other undetectable contaminant, we next tested whether digestion of PGN with muramidase, an enzyme that hydrolyzes glycosidic bonds between MurNAc and GlcNAc of PGN, would decrease the TLR2-dependent activation by PGN. Muramidase (mutanolysin from Streptomyces globisporus, from Sigma, St. Louis, MO) was used at a substrate: enzyme ratio of 5:1 at 37°C for 16 h, and the entire digestion mixture without further treatment or the digestion mixture after dialysis (against phosphate-buffered saline, 12-kDa cutoff) was tested. The effectiveness of muramidase digestion of sPGN was confirmed by Western blotting as previously described (6) (not shown), and the effectiveness of iPGN digestion was confirmed by measuring a decrease in optical density at 660 nm, which after 6 h of digestion was reduced from 0.50 to 0.03.

The TLR2-dependent activation by sPGN and iPGN was reduced by 87% and 64%, respectively, after muramidase digestion, and was reduced by 95% and 80%, respectively, when muramidase digestion was followed by dialysis to remove low-molecular-weight PGN fragments (Fig. 1F). Dialysis by itself (without muramidase digestion) did not change the TLR2-dependent cell-activating capacity of sPGN and iPGN (Fig. 1F), and muramidase by itself had no stimulatory activity (Fig. 1F). TLR2-dependent cell-activating capacity of sPGN and iPGN, as well as the integrity of these PGNs, was unaffected by digestion with trypsin, DNase, or RNase (not shown), which confirms the specificity of the muramidase.

TLR2-dependent cell activation by LTA was unaffected by dialysis, muramidase digestion, or muramidase digestion followed by dialysis (Fig. 1F). These results demonstrate that the TLR2-dependent cell activation was due to PGN itself and not due to a contamination with LTA or another non-PGN contaminant.

Our results conclusively show that polymeric PGN is a TLR2-dependent cell activator. They also indicate that disaccharide-peptide PGN fragments generated by muramidase digestion are inactive or have greatly reduced activity. Our results with iPGN are consistent with the results of Travassos et al. (25), who showed that purified iPGN does not activate cells at 1 μg/ml. Our purified iPGN at 1 μg/ml did not activate TLR2-transfected cells either, and more than 10 μg/ml of iPGN was required to activate TLR2-transfected cells (Fig. 1B and D). However, our purified sPGN activated TLR2-transfected cells at 0.1 to 1 μg/ml (Fig. 1B). Travassos et al. (25) did not test sPGN equivalent to our preparation but instead used a lysostaphin digest of an iPGN, which has a different peptide structure than our sPGN (it mainly lacks terminal D-Ala-D-Ala) and may have other modifications. Moreover, it is unclear what the efficiency of lysostaphin digestion was, what the size of sPGN fragments was, and how the concentration of this sPGN was determined in the experiments of Travassos et al. (25).

Another explanation for the inability of PGN to activate TLR2 at the concentration that activated Nod2 in the study by Travassos et al. (25) could have been potential damage to the TLR2-activating structure of PGN by their purification procedure. This structure could be required for TLR2 activation but not for Nod activation and, for example, could contain the terminal d-Ala-d-Ala in the PGN stem peptide, which is present in our sPGN and is scarce in iPGN. Travassos et al. (25) did not show that the contaminants that they were removing from the PGN preparations were active, nor did they show what they were.

It should be noted that iPGN is insoluble and, therefore, its molecular weight cannot be determined (but it is >5,000,000, probably ~3 × 10^8), whereas sPGN is soluble and its average molecular weight is ~100,000. Therefore, the effective stimulatory concentrations of iPGN (~10 μg/ml = ~2 nM) and sPGN (~1 μg/ml = ~10 nM) are similar on a per-mol basis, and moreover, on a per-mol basis, they are similar to the effective TLR2-stimulatory concentrations of a lipopeptide, PamCS(K)₄, which is ~10 ng/ml (6 nM) (21, 22), or Nod2- or Nod1-activating concentrations of muramyl dipeptide and other muropeptides, which are ~10 ng/ml (~2 nM) (1, 9, 10).

Therefore, the conclusion whether PGN is recognized by TLR2 at “physiologic concentrations” depends on the definition of a physiologic concentration of a bacterial product in host tissues. Travassos et al. (25) consider PGN concentrations higher than 1 μg/ml “unphysiologic.” By this definition, our sPGN, which activates cells in a TLR2-dependent manner at <1 μg/ml, would be considered a “physiologic” activator. However, the concentrations of PGN encountered by host cells during bacterial infections are likely to be higher at local sites of infection, especially in infections with gram-positive cocci, such as S. aureus, which have a much higher PGN content than gram-negative bacteria. For example, the 50% lethal dose of live S. aureus in an intraperitoneal infection model in mice is 10^9 (5), and this amount of S. aureus bacteria contains 400 μg of PGN. Therefore, half of the mice still recover from infection with local presence of live bacteria containing 400 μg PGN in a peritoneal cavity fluid that is <0.1 ml. Similarly high amounts of bacteria can be present in local abscesses, and the host can recover from such infections. When infections start with low numbers of gram-positive bacteria, such as S. aureus, the bacteria are usually opsonized and efficiently eliminated by phagocytosis (which is not TLR2 mediated) without extensive inflammation. Accumulation of higher numbers of bacteria would start to induce a strong inflammatory response, part of which would be TLR2 mediated. Thus, in infections with gram-positive cocci, which have a very high PGN content, the host cells are often exposed to high concentrations of PGN. Similar numbers of gram-negative bacteria contain much lower amounts of PGN, and therefore, sensing of extracellular gram-negative bacteria by the host may occur primarily through recognition of other molecules, such as LPS and lipoproteins.

We also note, however, that interaction of bacteria with host cells is based on interaction of individual bacterial cells with individual host cells, and in this context considering concentrations of bacterial components in terms of μg/ml is artificial. Particulate matter (such as bacteria or insoluble PGN) interacting with a host cell exposes the host cell surface at the site of interaction to a highly concentrated array of molecules that cannot be measured as an average concentration per ml.

We conclude that the extracellular recognition of S. aureus occurs through interaction of TLR2 with or without CD14 with polymeric PGN and LTA, which is best responsive to large numbers of extracellular bacteria and a high concentration of their products. Intracellular recognition, which occurs
through Nod1/Nod2 (1, 9, 10, 25) is responsive to small numbers of intracellular bacteria or low concentrations of their products.

We are grateful to Carsten J. Kirschning for TLR2, TLR4, and CD14 plasmids; to Kensuke Miyake for the MD-2 plasmid; and to Mu Wang, Ross Cocklin, Derek Janecki, and Chad Walls for performing mass spectrometry analyses.

This work was supported by USPHS grant AI2879 from NIH.

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