Cutting Edge: Primary Innate Immune Cells Respond Efficiently to Polymeric Peptidoglycan, but Not to Peptidoglycan Monomers

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Cutting Edge: Primary Innate Immune Cells Respond Efficiently to Polymeric Peptidoglycan, but Not to Peptidoglycan Monomers

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The cell wall of bacteria induces proinflammatory cytokines in monocytes and neutrophils in human blood. The nature of the stimulating component of bacterial cell walls is not well understood. We have previously shown polymeric peptidoglycan (PGN) has this activity, and the cytokine response requires PGN internalization and trafficking to lysosomes. In this study, we demonstrate that peptidoglycan monomers such as muramyl dipeptide and soluble peptidoglycan fail to induce robust cytokine production in immune cells, although they activate the nucleotide-binding oligomerization domain proteins in transfected cell models. We further show that lysosomal extracts from immune cells degrade intact peptidoglycan into simpler products and that the lysosomal digestion products activate the nucleotide-binding oligomerization domain proteins. We conclude that naive innate immune cells recognize PGN in its polymeric form rather than monomers such as muramyl dipeptide and require PGN lysosomal hydrolysis to respond. These findings offer new opportunities in the treatment of sepsis, especially sepsis arising from Gram-positive organisms. The Journal of Immunology, 2011, 186: 3841–3845.

Peptidoglycan (PGN), a component of the cell wall of all bacteria, is a polymer made of alternating N-acetyl glucosamine and N-acetyl muramic acid with a short stem peptide composed of alanine, glutamic acid/glutamine, and diaminopimelic acid (DAP)/lysine covalently linked to the N-acetyl muramic acid. Two or more peptidoglycan strands are cross-linked by covalent linkages between the stem peptides of each strand (1). The monomeric components of PGN include muramyl dipeptide (MDP), which consists of muramic acid linked to Ala-Glu and the DAP- or Lys-containing stem peptides.

Studies on the biological activity of PGN have been confounded by bacterial cell wall impurities, including lipoteichoic acid and triacetylated lipopeptides that stimulate TLR2 (2). PGN itself was thought to be recognized by TLR2 (3), but later studies revealed that the recognition was due to contaminants in the PGN preparations (4, 5). Subsequently, two members of the nucleotide oligomerization domain (NOD)-like receptor family were found to detect PGN monomers, as portions of the PGN stem peptide containing DAP (recognized by NOD1), or MDP (recognized by NOD2) (6–9). The notion of NOD recognition of PGN monomers is largely based on studies of transfected embryonic kidney cells overexpressing the NOD receptors (6–8). With the discovery of NOD function as PGN monomer sensors, it was thought that the PGN had no biological activity as a polymer. Indeed, polymeric PGN was shown to be inert toward innate immune cells (4, 5, 10) and intact intestinal epithelial cells (6), although there is evidence to the contrary (11–13).

The notion that immune cells recognize PGN monomers introduces the following conundrum: how do water-soluble monomeric components enter immune cells? Accordingly, elaborate models have been invoked by which monomeric PGN fragments are delivered to immune cells, as follows: uptake of monomeric PGN fragments in clathrin-coated pits (11, 14), delivery of monomeric PGN fragments via bacterially-secreted vesicles (16), surface-expressed NOD proteins where monomeric fragments can be detected (17), or NOD ligands generated by bacterial hydrolytic enzymes and shed from growing bacteria [reviewed in (18)]. These models are based on transformed cell lines that may have different processes than naive immune cells.

To resolve these controversies, we purified PGN from the Gram-positive sporulating bacteria Bacillus anthracis such that the material lacks detectable TLR2 contaminants and has insignificant levels of endotoxin and minimal protein contamination (11, 12). We showed that the pure preparation of PGN induced production of proinflammatory cytokines in human innate immune cells (12), which required PGN internalization and lysosomal trafficking (11).

In the current study, we compared intact polymeric PGN with monomeric MDP and soluble PGN (sPGN) and found the PGN polymer to be a much more potent inducer of pro-

Abbreviations used in this article: DAP, diaminopimelic acid; MDP, muramyl dipeptide; M-TriDAP, N-acetyl-muramyl-L-Ala-g-D-Glu-mDAP; NOD, nucleotide-binding oligomerization domain; PGN, peptidoglycan; sPGN, soluble PGN.

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inflammatory cytokines in human innate immune cells. To our knowledge, we demonstrate for the first time that lysozymic extracts obtained from human innate immune cells have the ability to degrade the PGN into soluble components, and that the lysosome-generated products of PGN hydrolysis activate the NOD proteins. Our findings support a new model of PGN recognition in which polymeric PGN is internalized, traffics to the lysosome, and is degraded into simpler products that are efficiently recognized by the intracellular NOD receptors.

Materials and Methods

Cells

HEK293 cell lines stably expressing vector only (HEK-Blue-Null1, HEK-Blue-Null2), or human Nod1 (HEK-Blue-hNOD1) or human Nod2 (HEK-Blue-hNOD2) genes and secreted alkaline phosphatase on a NF-κB promoter were purchased from InvivoGen (San Diego, CA). The cells were cultured, according to the manufacturer’s recommendations. Heparinized peripheral blood obtained by venipuncture was cultured, as described (11, 12).

Preparation of B. anthracis peptidoglycan

Peptidoglycan was isolated from B. anthracis ΔSterne strain that lacks the capsule and toxins, as described previously (11, 12). FITC labeling of PGN was performed, as described (11). The material lacks detectable endotoxin, lipopeptides, or other TLR stimuli (11, 12).

Flow cytometry analysis

Detection of cytokines and chemokines produced by brefeldin A-treated blood cells was performed by intracellular cytokine staining, as described (11). A total of 200,000 cells per sample was analyzed.

Lysosome preparation

Cells were resuspended in 0.25 M sucrose, lysed, and centrifuged 6,000 × g. The supernatants were centrifuged 100,000 × g for 60 min. The pellet was lysed in 50 mM sodium citrate buffer (pH 5.5), 2 mM DTT, and 0.5% Triton X-100. Preliminary experiments established that lysosomal enzymes were most active at pH 5.5. A total of 20-50 μg lysosomal extract was incubated with 10 μg FITC-PGN for 24 h at 37°C, followed by centrifugation at 20,000 × g for 10 min. The supernatants were analyzed using a luminescence spectrometer (PerkinElmer, Waltham, MA). For experiments measuring NOD activation in lysosome-treated PGN, the cells were sonicated to avoid detergent.

Liquid chromatography and mass spectroscopy analysis of lysosomal products

Samples were acid hydrolyzed with 6 N HCl for 3 h at 105°C. The acid-hydrolyzed sample was dried, redissolved in 10 mM ammonium acetate and 80% acetonitrile, and injected onto a ZIC pHILC column (150 × 2.1 mm, 5 μm, 200 Å; The Nest Group, Southborough, MA). The material was eluted with an acetonitrile gradient, and the eluted peaks were analyzed and quantitated by mass spectroscopy (Bruker Daltonics HCT Ultra Ion trap in positive mode). The elution time and mass spectra of standard amounts of purified muramic acid were determined in preliminary experiments.

NOD activation

HEK293 cell lines were treated with appropriate ligands for 24 h. NF-κB activation was detected by measuring secreted embryonic alkaline phosphatase spectrophotometrically at 630 nm.

Statistical analysis

All statistical analyses were performed using GraphPad Prism. Statistical significance was determined by the nonparametric Mann–Whitney U test, and p values <0.05 were considered statistically significant.
FIGURE 3. PGN is degraded by lysosomal enzymes of monocytes and neutrophils. A, Lysosomal extracts prepared from monocytes or neutrophils were incubated with 10 μg FITC-PGN for 24 h, and FITC in the supernatant was measured. Results shown are from three independent experiments performed with lysosomal extracts prepared from three different donors. B, Unlabeled PGN was incubated with lysosomal extracts obtained from neutrophils. The supernatants of these reactions were hydrolyzed with 6 N HCl for 3 h at 105˚C, followed by HPLC-mass spectroscopy analysis. Results from one representative experiment are shown. *p < 0.05.

Results
Polymeric PGN is a more potent stimulant of innate immune cells than MDP and/or sPGN
We compared the ability of monomeric MDP versus polymeric PGN to induce cytokine production in primary human blood cells and in Nod-transfected HEK293 cells. Human peripheral blood cells were treated with 10 μg/ml MDP or 10 μg/ml intact PGN, and cytokine production was measured, as previously described (11, 12). As shown in Fig. 1A, only ~13% monocytes produced TNF-α in response to monomeric MDP in contrast to 42% monocytes that produced TNF-α in response to polymeric PGN. Eighteen percent of monocytes became TNF-α+ with 12-h MDP incubation, and a higher dose (50 μg/ml) did not improve the response at 6 or 12 h. A similar trend was observed for IL-8 production in neutrophils, as follows: 4% neutrophils produced IL-8 in response to MDP versus nearly 60% in response to PGN (Fig. 1B). The data from several experiments using polymeric PGN and a variety of commercial PGN monomeric forms, including N-acetylmuramyl-l-Ala-γ-D-Glu-mDAP (M-TriDAP), MDP, and N-glycolylMDP, are shown in Fig. 1C. In contrast, HEK293 cells stably expressing NOD2 and a NF-kB reporter gene responded robustly to the monomeric forms of PGN, but failed to respond to polymeric PGN as measured by NF-kB activation (Fig. 1D).

Because pure MDP may not truly represent naturally occurring sPGN fragments, we digested intact PGN with mutanolysin (an enzyme isolated from Streptomyces having lysozyme activity) to generate sPGN degradation products. We then measured TNF-α production in monocytes or NF-kB activation in the Nod-transfected HEK293 cells treated with digested or intact PGN. We found that intact PGN was a better stimulator of TNF-α production than sPGN (Fig. 2A). In contrast, sPGN was able to activate NF-kB in HEK293 cells overexpressing either NOD proteins (Fig. 2B, 2C). Together, these data show that monomeric components of PGN are poor stimulants of innate immune cells, but potent stimulants of artificial models in which NOD receptors are overexpressed. In contrast, polymeric PGN is capable of efficiently stimulating immune cells.

PGN is digested by lysosomal extracts
We have previously shown that lysosomotropic agents block cytokine production in innate immune cells responding to PGN. The observation suggests that lysosomal activity is required for cytokine production in innate immune cells exposed to intact polymeric PGN. To determine whether lysosomes from innate immune cells had the capacity to degrade intact PGN, we labeled PGN with FITC. The FITC covalently bound to PGN was completely recoverable by low-speed centrifugation. We reasoned that digestion of the PGN disaccharide backbone would render a fraction of the FITC to become water soluble and thus present in the supernatant of a lysosomal digest. For this experiment, the FITC-labeled PGN was incubated with lysosomal extracts from monocytes and neutrophils, and PGN hydrolysis was determined by the appearance of water-soluble fluorescein in the supernatant. We found (Fig. 3A) that the lysosomal fraction of both monocytes and neutrophils was able to solubilize FITC-PGN and that the activity was sensitive to heat inactivation. We verified that the PGN disaccharide backbone was degraded by assaying for muramic acid in the lysosomal reactions. In this study, the lysosome-generated PGN hydrolytic products were acid hydrolyzed and monitored by HPLC and mass spectrometry. We found that muramic acid was detected only in the supernatants of lysosomal reactions incubated with PGN and not in the controls (Fig. 3B). These data establish that PGN is hydrolyzed to water-soluble products by the lysosomal enzymes of innate immune cells.

Neutrophil lysosomal extracts degrade PGN to generate NOD ligands
Because lysosomes can degrade PGN into soluble moieties, we asked whether PGN fragments generated by the lysosome fraction contained a ligand for NOD1 or NOD2. HEK293

FIGURE 4. Degradation of PGN by lysosomes generates NOD ligands. A total of 20 μg PGN was incubated with lysosomal extracts obtained from neutrophils or buffer alone. HEK293 cells stably expressing human NOD1 (HEK293-Nod1) or NOD2 (HEK293-Nod2) were treated with the supernatants of these reactions for 24 h at 37˚C. NF-kB activation was determined spectrophotometrically at 630 nm. Similar results were obtained in three additional experiments. *p < 0.05.
cells stably expressing 

\texttt{Nod1} or \texttt{Nod2} were treated with the supernatants of lysosome-digested PGN or with PGN incubated with buffer alone or the lysosomal fractions alone as controls. We found that PGN digested by the lysosomal fraction of neutrophils was able to stimulate both \texttt{NOD1-} and \texttt{NOD2}-expressing HEK293 cells, indicated by NF-\kappaB activation. Untransfected HEK293 cells failed to respond (data not shown). In accordance with the above data, no NOD activation was observed when we used heat-inactivated lysosomal extracts to degrade PGN (Fig. 4).

\textbf{Discussion}

Although PGN is a well-characterized cell wall component of bacteria, whether immune cells recognize and respond to PGN and how they respond is controversial. It has been well documented that transformed cell lines expressing NOD receptors respond to the water-soluble monomeric moieties that make up PGN. In this study, we show that MDP and other bona fide ligands weakly induce the production of TNF-\alpha in primary naive monocytes and IL-8 in primary naive neutrophils, whereas the intact, polymeric PGN is a more potent inducer of these proinflammatory molecules. Because PGN, but not monomeric versions of PGN or mutanolysin-solubilized PGN fragments were able to induce cytokine production in monocytes, we conclude that innate immune cells efficiently recognize and respond to PGN only in its polymeric form, but the cells must process it intracellularly.

These data are in contrast to current models of monomeric PGN recognition by innate immune cells (14, 15) and indeed to rather abundant literature showing that murine innate immune cells respond vigorously to various monomeric fragments of PGN. We note that many such studies showing responses to PGN monomers manipulate the responder mouse macrophages in some way prior to stimulation, as follows: pretreatment of macrophages with LPS (19) or cytosolchin A (20); direct transfection of MDP into macrophages (21); or the studies rely exclusively on \textit{Nod}-transfected HEK293, HL-60, HT-29, Caco-2, or HEK293 T cells (22). We found that a 12-h pretreatment of bone marrow–derived mouse macrophages or a 3-h pretreatment of human blood with LPS (1 ng/ml) caused the cells to be essentially equally responsive to monomeric MDP and polymeric PGN. LPS pretreatment might cause the responding cells to express a transporter of MDP or DAP-containing stem peptides or alter a signaling cascade (23) to enable a response to these monomeric components. However, our data in this study establish that primary unmanipulated innate immune cells in blood do not respond well to the PGN monomers like MDP and sPGN, whereas they respond vigorously to the PGN polymer.

Our earlier report (11) showed that polymeric PGN bound some blood cells (B cells, neutrophils, and monocytes), but not others (T cells). The observation suggests that some immune cells express an extracellular receptor capable of binding PGN. We found (data not shown) that only 7% of HEK293 cells bind polymeric PGN, suggesting that they fail to respond in part because they lack the PGN receptor.

The nature of the NOD ligand(s) derived from lysosomal digestion of PGN is not known. The monomeric components MDP (6) and DAP-containing peptides (24) have been isolated from mutanolysin-digested PGN derived from \textit{Neisseria meningitidis} and \textit{Bacillus subtilis}. Clearly, lysosomes of innate immune cells express lysozyme (25), suggesting the monomers previously identified might be natural products of innate immune cell lysosomal digestion of PGN. We speculate that the need for PGN endocytosis and/or lysozyme digestion and/ or NOD expression levels might be mechanism(s) by which intestinal epithelial cells ignore the copious quantities of PGN to which they are exposed (26).

We conclude that the polymeric nature of PGN is required for naive immune cells to efficiently phagocytose it and traffic it to the lysosome. We provide direct evidence that lysosomes derived from innate immune cells are able to degrade intact PGN, and show that lysosomal degradation generates a NOD ligand in the process. Our model in which intact PGN rather than PGN monomers is phagocytosed and digested in the lysosome is also supported by a recent study performed in murine macrophages using PGN from \textit{Staphylococcus aureus} (13). These findings provide a novel mechanism on how human monocytes and neutrophils in peripheral blood recognize and respond to PGN.

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\textbf{Disclosures}

The authors have no financial interests of conflict.

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