Bacterial Peptidoglycan as a Driver of Chronic Brain Inflammation

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REVIEWS

Review

Bacterial Peptidoglycan as a Driver of Chronic Brain Inflammation

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Peptidoglycan (PGN) is a cell wall component of both Gram-positive and Gram-negative bacteria. Signature fragments of PGN are proinflammatory through engagement of pattern recognition receptors (PRR) on resident tissue cells and circulating leukocytes. Despite its abundance in the gut microbiota, there is limited recognition that PGN could contribute to chronic neuroinflammation. This review highlights current insights into the roles of PGN as a determinant of brain inflammation, notably in multiple sclerosis (MS) and its experimental autoimmune encephalomyelitis (EAE) models. Recent studies demonstrate PGN in blood of healthy adult humans. PGN amplifies autoimmune pathology via activation of innate immune cells. Novel uptake routes through (altered) gut mucosa by myeloid leukocyte subsets promote PGN transport to the brain.

Highlights

All bacteria require PGN as a major component providing structural rigor. Pattern recognition receptors from several families determine inflammatory versus inhibitory actions of PGN signature fragments.

PGN is a determinant in setting innate immune parameters and brain function.

In animal models and in tissue from live and postmortem MS brain tissue donors, PGN can be detected in phagocytic cells. In EAE models, PGN promotes inflammation, downstream of NOD receptors. Macrophages, dendritic cells, and neutrophils likely transport PGN from the mucosa to the brain.

The clinical implications of PGN as a central element of the gut–brain axis include the development of novel biomarker assays for monitoring of disease activity and treatment, as well as novel interventions involving immunotherapeutics, dietary intervention, and biotics.

Peptidoglycan (PGN): A Macromolecule Overlooked in Brain Physiology and Pathology

PGN is a complex macromolecule that is an integral component of the cell wall of Gram-positive and -negative bacteria. Adding to a century of research, novel biology on PGN biosynthesis, degradation, and contributions to inflammation versus normal physiology [1–4] is emerging (Table 1, Key Table). PGN is an essential building block of all bacterial cell walls, providing structure and resistance to osmotic pressure (Box 1 and Figure 1). PGN and its fragments are strongly proinflammatory, signaling through Toll-like receptors (TLR), NOD-like receptors (NLR), and specialized PGN recognition proteins (PGLYRP1–4) (Box 2 and Figure 2). Muramyl dipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine) is a widely studied signature motif of PGN.

Despite its ubiquitous nature and potential pathogenic actions, as a macromolecular structure PGN is under-recognized and occasionally confused with the connective tissue component proteoglycan. While lipopolysaccharide (LPS) is restricted to Gram-negative bacteria, PGN is present in both Gram-positive and -negative species and accounts for 4–80% of bacterial dry weight. Estimates suggest that the healthy adult human gut contains 2–5 × 10^{13} bacteria, equivalent to 10–100 g of PGN. This immense bacterial antigen burden does not provoke local or systemic inflammation, which emphasizes the highly efficient containment mechanisms underpinning PGN containment within the gut and limiting systemic exposures.

Individual human hosts experience varying intensity and quality of PGN exposure over time. These are affected by gut barrier integrity controlling translocation of microbial components to the systemic circulation and immune system, by bacterial strain-associated PGN variation, and by post-translational modifications such as PGN acetylation, which alters receptor binding and sensitivity to lytic enzymes, notably lysozyme. In addition to the gut microbiome, other epithelial mucosal surfaces have distinct microbiomes that can act as local and systemic sources of PGN.

Of note, host cells can be exposed to intact PGN or its associated fragments in a variety of ways, including live intact bacteria, release of fragments during PGN biosynthesis, fragments

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Key Table

Table 1. Physiological and Pathological Functions of Peptidoglycan (PGN)[a]

Function	Features	Refs
Bacterial structure	PGN is essential to cell wall structure of both Gram-positive (up to 70 layers) and Gram-negative bacteria. Due to the complex nature of PGN and requirement for collective actions of at least three degrading enzymes, it persists within phagocytes such as macrophages, dendritic cells, and neutrophils.	[40–78]
Infection	PGN and its fragments exert robust inflammatory effects, including induction of MHC expression, co-stimulatory molecules, and T cell polarizing cytokines, which are the signals 1–3 required for T-helper cell activation by antigen-presenting cells.	[40–78]
Physiological innate immunity	PGN signaling through NOD1 drives systemic innate immunity and governs lifespan of circulating granulocytes.	[1,2]
Crohn's disease	Genetic risk strongly influenced by variation in molecules involved in PGN recognition, signaling, phagocytosis, and autophagy: NOD1 and 2, ATG16L1.	Summarized in several reviews cited
Autoinflammation	Monogenetic variation in PGN recognition molecules underlies inflammatory disease in Muckle-Wells and Blau syndrome.	Summarized in several reviews cited
Rheumatoid arthritis	Phagocytes within inflamed joints contain PGN, expressing HLA/MHC-II and producing cytokines.	[5]
Multiple sclerosis (MS)	PGN is present in phagocytes in inflammatory infiltrates in MS brain and in non-human primates with MS-like EAE.	[16,23–26]
Brain development and normal behavior	Peptidoglycan recognition protein 2 is expressed during early postnatal brain development and affects development and behavior. PGLYRP2 is the single family member with enzymatic action.	[6,7]
Behavioral dysfunction	Exposure of pregnant mice to the peptidoglycan-teichoic acid complex of bacterial cell wall/PGN induces transcription factor FoxG1 in the fetal cortex and neuroproliferation via TLR2 and abnormal cognitive development in postnatal life.	[8]
Longevity, ageing	Genetic variation in PGN recognition molecules affects life longevity in *Drosophila* fruit flies.	[4]
Sleep	PGN and its fragments contribute to sleep regulation.	[3]

[a]The evidence for the distinct roles of PGN is variable in depth and scope.

Glossary

M-cells: located in the gut epithelium overlying the secondary lymphoid organs called Peyer's patches. The M refers to the microfold structure of their membrane, promoting antigen transport from the gut to the immune cells dwelling in the Peyer's patch. They are specialized in phagocytosis and transcytosis of large molecules, particulate antigen, and microbes (commensals and pathogens).

Specific pathogen-free (SPF): (or specified pathogen-free); animals in experimental facilities are regularly assessed for the absence of a specified list of pathogens, typically between 20 and 50 pathogens in rodents. Hence, they live in a very clean and microbiologically well-defined, but not sterile, environment. In contrast, germ-free animals do live in a sterile environment and do not have microbiota. However, this does not mean that they are antigen-free, since the irradiated sterile food they consume contains a wide spectrum of microbial compounds, including PGN.

and remnants during normal bacterial turnover, infection and intracellular replication, phagocytosis and destructive processing, or proteolytic processing for antigen presentation in the context of HLA-II or HLA-I (to CD4+ T-helper cells or CD8+ cytotoxic cells, respectively).

In the past 20 years, understanding of innate immunity expanded tremendously through the discoveries of several families of pathogen recognition receptors (PRRs). Most relevant to PGN biology are TLR, NLR, and the four PGN recognition proteins (PGLYRP a.k.a. PGRP). These receptors are secreted, expressed on the cell surface membrane, displayed on the endosomal membrane, or monitor the intracellular presence of PGN-associated fragments. Moreover, they can form homo- or heterodimers and may have enzymatic activity (e.g., PGLYRP2). Differential profiles of PRR are expressed by a wide variety of stromal cell types (e.g., gut epithelium) and immune cells (e.g., macrophages, dendritic cells, B cells, and neutrophils). PGN biology in mammalian hosts has been investigated largely in relation to bacterial infection, gut inflammation, and as a target for antibiotic therapies. Moreover, a body of work in

Box 1. Peptidoglycan Structure and Lytic Enzymes

PGN is a polymer of β(1-4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), crosslinked by short peptides, present in the cell walls of virtually all bacteria [41]. The peptides have alternating L- and D-amino acids, with L-lysine as the third amino acid (Lys-type PGN) in Gram-positive cocci or meso-diaminopimelic acid (m-DAP) (DAP-type PGN) in Gram-negative bacteria and Gram-positive bacilli (see Figure 1 in main text) [41]. The Lys-type PGN peptides are usually crosslinked through an 'interpeptide bridge' of various lengths and amino acid composition. The DAP-type PGN peptides are usually directly crosslinked, but m-DAP can be modified (e.g., by de-amidation in *Bacillus subtilis*).

PGN forms a large macromolecule surrounding the entire bacterium, in which polysaccharide chains run circumferentially around the cell [42]. In Gram-positive bacteria the PGN layer is 20–50 nm thick and comprises up to 40–80% of dry weight of the cell. Gram-negative bacteria have tenfold less PGN, with a 2.5–4 nm thick single layer located in the periplasm below the outer membrane [43], which makes it inaccessible on the cell surface. However, Gram-negative bacteria continuously remodel PGN, of which up to 40–70% is released as soluble fragments [44].

In Gram-positive bacteria, PGN is covalently substituted with teichoic acids and often other polysaccharides, proteins, and lipoproteins. In Gram-negative bacteria PGN's m-DAP is covalently bound to the outer membrane murein lipoprotein Lpp (Braun lipoprotein) and PGN-associated outer membrane lipoprotein Pal (BioCyc; http://biocyc.org/). Thus, the host is exposed to PGN covalently decorated with various other bioactive molecules.

Mammals have three PGN hydrolases (see Figure 1 in main text). Lysozyme hydrolyzes the β-(1,4)-glycosidic bond between MurNAc and GlcNAc residues. *N*-deacetylation, *N*-glycosylation, or *O*-acetylation of PGN glycans often confers resistance to lysozyme. Bacteria may also produce lysozyme inhibitors [45]. *N*-acetylmuramoyl-L-alanine amidase, coded by PGLYRP2 (peptidoglycan recognition protein 2), hydrolyzes the amide bond between the MurNAc and L-Ala, which separates peptides from the polysaccharide chains [46,47]. PGLYRP2 is produced in the liver and secreted into blood. β-hexosaminidase hydrolyzes the bond between terminal *N*-acetyl hexosamine residues of host glycoproteins, glycolipids, and proteoglycans, and also terminal GlcNAc of bacterial PGN (BioCyc; http://biocyc.org/) [48,49].

Bacteria have multiple PGN hydrolases (endopeptidases, carboxypeptidases, *N*-acetylglucosaminidases, lysozymes, and lytic transglycosylases, see Figure 1 in main text) able to hydrolyze every bond in PGN, although all enzymes are not found in every species [50]. Bacterial PGN hydrolases are likely the main sources of biologically active PGN fragments in the host, and release of PGN fragments is greatly enhanced by antibiotics.

the past 30 years identified PGN within phagocytic cells infiltrating diseased organs, such as rheumatic joints [5].

Very recent work emphasizes functions of PGN in brain inflammation, notably in MS and its animal models in different species (EAE). Importantly, in rodent and fly models, PGN also plays physiological roles in the regulation of neutrophil numbers, longevity, brain development, sleep, and in normal versus aberrant behavior (Table 1).

Diaz Heijtz reviews recent insights into the actions of PGN in mouse brain development and altered behaviors of offspring by manipulation of PGN by germfree housing and by genetic knockout of PGLYRP2 [6,7]. These findings complement studies demonstrating that PGN (bacterial cell wall preparation) crosses the placenta to engage TLR2 on fetal cells and activates the transcription factor FoxG1 to promote proliferation of neurons in the fetal cortex. Exposure to bacterial cell walls *in utero* correlates with abnormal cognitive behavior in the offspring [8]. Intense recent debate about whether the human placenta harbors a microbiota underscores the technical challenges of detecting low-level microbial compounds in the high biomass matrix of human host tissues [9].

This review evaluates the contributions of PGN to brain inflammation in MS and its animal models (Figure 3) and provides a medical perspective on these findings.

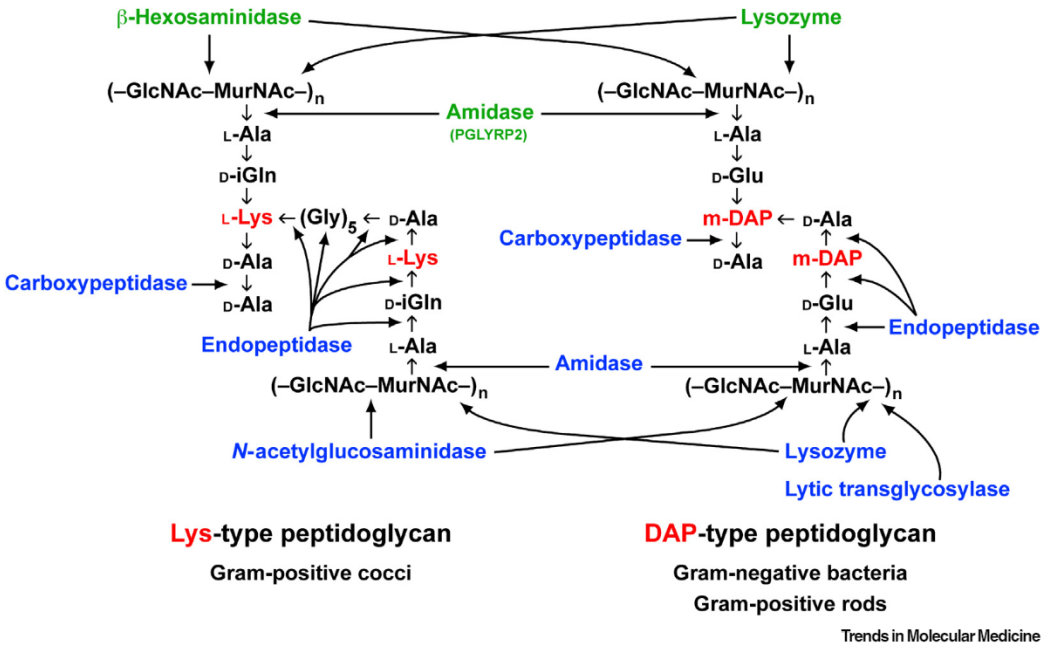

Figure 1. Structure of Lys-type PGN and DAP-type PGN and Bonds Hydrolyzed by Mammalian and Bacterial PGN Hydrolases. Structures of polymeric Lys-type PGN from *Staphylococcus aureus* (characteristic of Gram-positive cocci) and DAP-type PGN from *Escherichia coli* (characteristic of Gram-negative bacteria and Gram-positive rods) are shown in black with L-Lys and m-DAP in red. Mammalian (green) and bacterial (blue) PGN hydrolases, which generate PGN fragments for recognition by host pattern recognition molecules, are shown with the bonds that they hydrolyze indicated by arrows. Lysozyme is an endo-muramidase, whereas lytic transglycosylase has exo-lytic activity releasing 1,6-anhydroMurNAc-containing disaccharide peptides from the end of the glycan strand. Abbreviations: GlcNAc, *N*-acetylglucosamine; m-DAP, meso-diaminopimelic acid; MurNAc, *N*-acetylmuramic acid; PGLYRP2, peptidoglycan recognition protein 2; PGN, peptidoglycan.

Involvement of PGN in Chronic Brain Inflammation

There is no evidence for a primary causal role in the development of MS involving individual bacterial pathogens or bacterial components derived from physiological microbiota-containing sites such as the gut. Nonetheless, MS progression and activity, including relapse frequency and accompanying central nervous system (CNS) damage, are influenced by infections and other sources of systemic inflammation [10]. The popularized concept of 'leaky gut promotes leaky brain' includes the notion that many different insults to gut barrier integrity promote translocation of bacterial compounds, including PGN, across the gut epithelium.

A study in 2015 [11] (see also Commentary with graphical abstract [12]) identified a novel pathway across the gut epithelium for PGN and for soluble protein antigen administered *per os*. Calcium-phosphorus nanoparticles present in the human and mouse gut transport these compounds through **M-cells** (see Glossary) (microfold) overlying the lymphoid Peyer's patch. Interestingly, PGN at these sites supplies a suppressive signal through programmed cell death 1 (PD1)PD1 ligand (PDL1) negative checkpoint regulators.

The observation that PGN is present in human arthritic joints [5] sparked the hypothesis that PGN is similarly transported into the CNS by trafficking phagocytic cells in MS and EAE models. Here, the assumption is that sites of inflammation attract leukocytes, of which some will contain PGN as acquired from the mucosa by direct sampling from the gut and by translocation of live bacteria or their components.

Box 2. Peptidoglycan Recognition

Initially, Toll-like receptor 2 (TLR2) and CD14 were identified as PGN receptors [41]. Subsequently, this activity was attributed to lipopeptide and lipoprotein contamination, because lipoprotein-deficient *Δlgt* mutants did not activate cells [51]. However, other evidence still indicates that PGN activates TLR2: PGN from *Δlgt* mutant has ~1/3 of cell-activating capacity [52], PGN fragments bind TLR2 [53], TLR2 binding was modeled *in silico* [54], and PGN colocalizes with TLR2 [55].

NOD1 and NOD2, members of NLR family, are cytoplasmic PGN receptors (see Figure 2 in main text) that sense L-Ala-D-Glu-*m*-DAP in DAP-type PGN (NOD1) [56,57] or muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine, MDP) (NOD2) [56,58,59] in both Lys- and DAP-type PGN. NOD1 is expressed in many tissues and cell types, whereas NOD2 is highly expressed in immune cells and less in epithelial and other cells. Activation of NOD1 and NOD2 induces secretion of cytokines, chemokines, and antibacterial peptides [60,61] (see Figure 2 in main text).

NLRP3 (cryopyrin), another member of the NLR family, is activated by PGN, expressed mainly in macrophages, and affects inflammation and cell survival in the CNS [23]. The NLRP3 inflammasome detects danger signals such as uric acid and ATP released from damaged cells. GlcNAc released from PGN induces dissociation of hexokinase from mitochondria, which triggers NLRP3, resulting in activation of caspase 1 and release of IL-1β [48] (see Figure 2 in main text).

Mannose binding lectin (MBL), present in the serum, binds mannose and/or GlcNAc-containing polymers, including PGN, and activates the lectin pathway of complement [62,63]. Activated complement components opsonize microorganisms and are chemotactic and proinflammatory (see Figure 2 in main text).

Mouse RegIIIβ and RegIIIγ and human HIP/PAP are lectins secreted from Paneth cells in the intestine and bind PGN's GlcNAc [64–66]. RegIIIγ is bactericidal for Gram-positive bacteria only, whereas RegIIIβ also binds GlcNAc in lipopolysaccharide and is bactericidal for both Gram-positive and -negative bacteria [66].

Peptidoglycan recognition proteins (PGLYRPs) are specific for MurNAc-peptides [41] (see Figure 2 in main text). PGLYRP2 is PGN amidohydrolase (see Figure 1 in main text) present in serum and induced in epithelial cells. PGLYRP1, PGLYRP3, and PGLYRP4 are bactericidal [67–69] and expressed in neutrophils and eosinophils (PGLYRP1), epithelial cells (PGLYRP3, PGLYRP4) [41,45], and brain [7]. PGLYRPs modulate inflammation in the intestine, skin, lungs, and joints and protect from colitis (all PGLYRPs) [70,71], atopic dermatitis (PGLYRP3, PGLYRP4) [72], and psoriasis-like inflammation (PGLYRP2) [73] in mice. However, PGLYRP1 is proinflammatory and promotes asthma and skin inflammation [72–75], whereas PGLYRP2 promotes arthritis [76]. PGLYRPs also maintain beneficial intestinal microbiome that protects mice from colitis [70,71,77].

Two recent studies now suggest that tonic PGN levels can be measured in blood [13,14]. Using an immunoassay based on a monoclonal antibody recognizing MDP, healthy human volunteers from two continents were sampled: in 75% of donors, MDP plasma concentrations ranged between 0.16 and 1 μg/ml of plasma and in 22%, between 4 and 5 μg/ml. Donors at extreme ends of the spectrum had either undetectable MDP or very high levels (4–5 μg/ml). Plasma MDP levels were stable over several days [13]. These interesting data are in need of confirmation, since the concentration estimates appear high. But in general support of PGN being present in the circulation, Molinaro *et al.* [14] demonstrated that trace amounts of PGN in serum are critical to NOD-dependent cytokine responses evoked by endoplasmic reticulum stress. These findings provide important new insights and, consequently, soluble PGN/MDP in blood could also be considered an antecedent step to PGN presence in the brain. It is incompletely known how soluble PGN and derived fragments traverse a fully functional blood–brain barrier and this is currently intensely investigated in mouse models. Modifications of PGN, particularly with adducts, greatly expands the repertoire of receptors that promote migration of these particles into and across epithelial and endothelial barriers. Intravenously administered PGN contributes to acute neuroinflammation in animal models, but this review focuses on chronic brain inflammation in MS and its animal models.

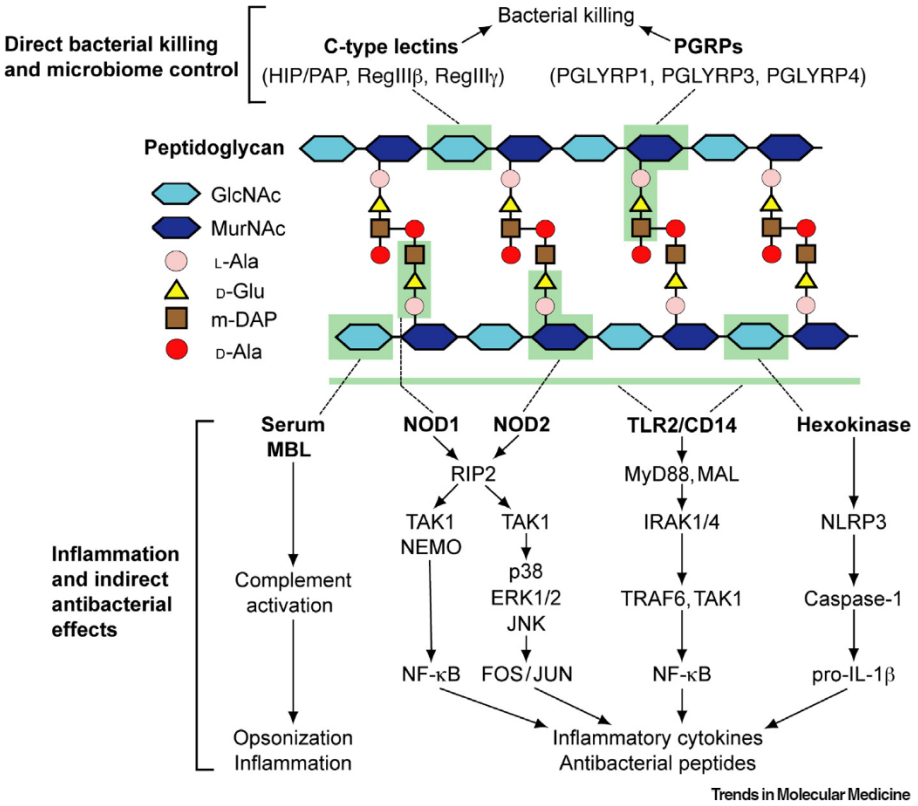

Figure 2. Specificities and Functions of Mammalian PGN Recognition Molecules. Schematic structure of DAP-type PGN is shown in the center. Soluble bactericidal proteins (top), C-type lectins (RegIIIβ, RegIIIγ, HIP/PAP) and PGRPs (peptidoglycan recognition proteins), mediate the direct killing of bacteria and control of the microbiome. Inflammation and indirect antibacterial effects (bottom) are generated through activation of complement by serum MBL and activation of PGN receptors and their signal transduction pathways in host cells. The minimal PGN structures recognized by the indicated pattern recognition molecules are shaded in green. NOD1 and NOD2 activate RIP2, which activates TAK1 and NEMO, which lead to the activation of transcription factors NF-κB and FOS/JUN. TLR2/CD14 complex activates adaptors MyD88 and MAL, which activate IRAK1/4➔TRAF6 and TAK1➔NF-κB pathway. These pathways induce production of inflammatory cytokines and antibacterial peptides. GlcNAc in PGN inhibits hexokinase, which is released from mitochondria and activates NLRP3 inflammasome, which activates caspase-1 to cleave pro-IL-1β into mature IL-1β. Abbreviations: ERK1/2, extracellular signal-regulated kinases 1 and 2; FOS/JUN, heterodimer of c-Fos and c-Jun; GlcNAc, *N*-acetylglucosamine; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; JNK, JUN N terminal kinase; MAL, myelin and lymphocyte protein; MBL, mannose binding lectin; m-DAP, meso-diaminopimelic acid; MurNAc, *N*-acetylmuramic acid; MyD88, myeloid differentiation primary response 88; NEMO, NF-κB essential modulator; NLR, NOD-like receptor; NLRP3, LRR and PYD domains-containing protein 3; NOD, nucleotide-binding oligomerization domain; p38, p38 MAP kinase; PGN, peptidoglycan; PGRP or PGLYRP, peptidoglycan recognition protein; RIP2, receptor-interacting protein-2; TAK1, MAP kinase kinase kinase 7; TLR2, Toll-like receptor 2; TRAF6, TNF receptor-associated factor 6.

MS is a chronic inflammatory neurological disease, manifesting in young adults, with prevalence in the Western world of one in 1000. Proving a definitive autoimmune basis of this severely disabling disease has been elusive, as no antigens driving pathogenic adaptive T and B cell responses have been established beyond reasonable doubt. However, advanced genetics demonstrate that most of the currently 200 identified SNPs, in addition to the main genetic risk factor MHC/HLA (33 loci), notably HLA-II presenting antigenic peptides to CD4+ T-helper cells, relate to immune responses, including those of T and B cells. A very recent systems biology approach suggests that within an individual patient, the genetic risk may involve T cells, B cells, myeloid cells, or in susceptibility of the brain tissue itself [15]. MS immunotherapies that limit inflammation and leukocyte trafficking or eliminate CD20+ B cells reduce disease relapses and perhaps progression.

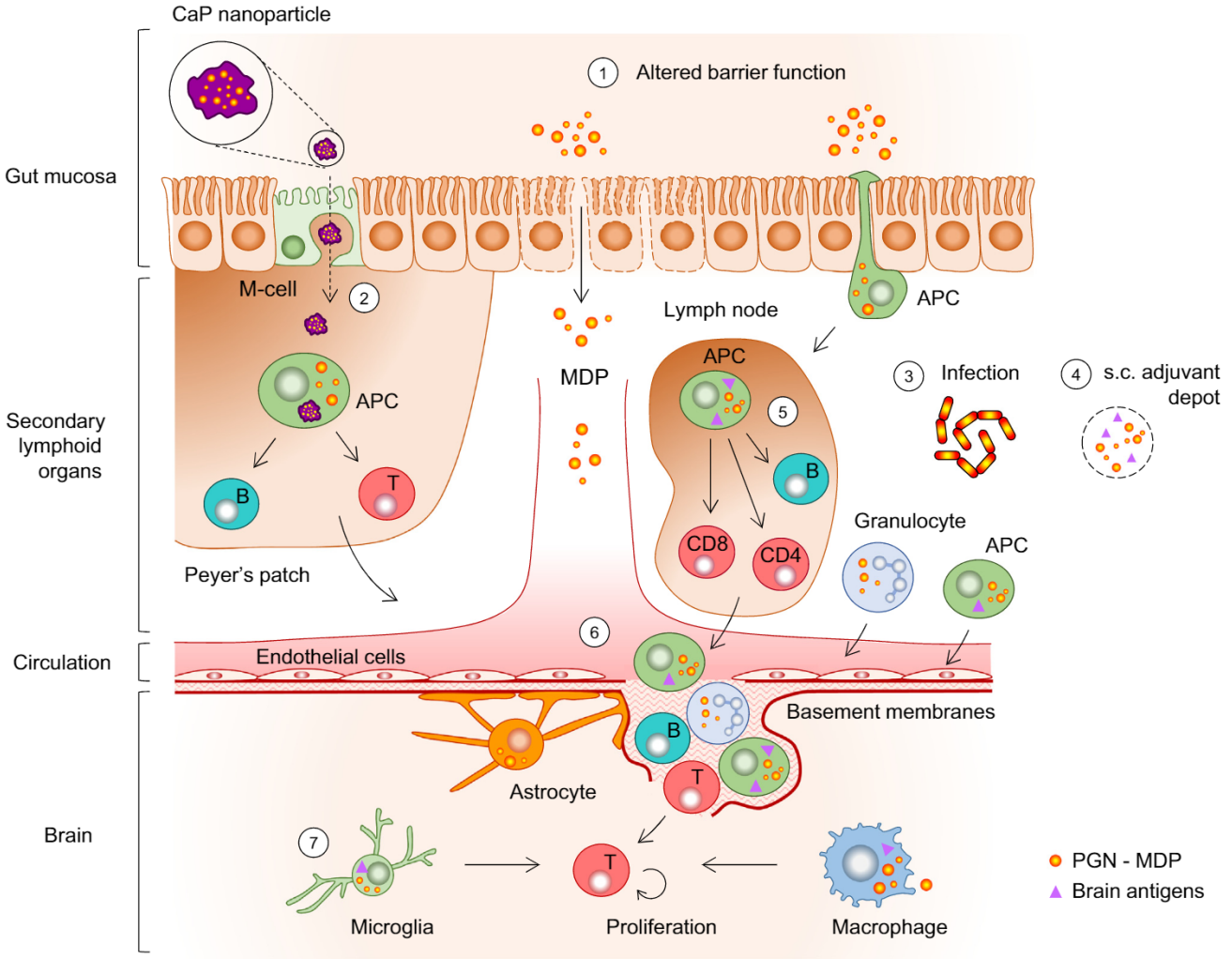

Figure 3. Conceptualization of Peptidoglycan (PGN) Contributions to Brain Inflammation across Anatomical Sites. (1) Altered barrier function promotes translocation of live bacteria and components, including lipopolysaccharide (LPS)and PGN. Phagocytic antigen-presenting cells (APCs) such as macrophages, neutrophilic granulocytes, and dendritic cells can sample gut content by projections extending between epithelial cells. PGN/muramyl dipeptide (MDP) can be detected at stable levels in plasma in healthy donors [13]. (2) Naturally occurring CaP nanoparticles containing PGN and other gut components interact with M-cells (microfold) specialized for transporting compounds into underlying Peyer's patches as secondary lymphoid organs. M-cells selectively express peptidoglycan recognition protein 1 (PGLYRP1) [85]. (3) In addition to the gut microbiota as a perpetual source of high microbial biomass, PGN can also originate from bacterial infection. (4) In experimental autoimmune encephalomyelitis (EAE) models, myelin proteins are injected with adjuvant. (5) APC containing PGN travel from the periphery (e.g., mucosa or sites of infection) to secondary lymphoid organs, where they interact with B cells, T-helper cells (CD4), and cytotoxic T cells. PGN promotes innate and adaptive immunity by inflammatory cytokine induction [e.g., interleukin (IL)-1beta, IL-18]. (6) APC can travel onward from their first secondary lymphoid organ base to next draining sites (e.g., from Peyer's patch to mesenteric lymph node). (7) Macrophages/microglia, neutrophils, and probably also astrocytes contain PGN in non-human primate EAE and multiple sclerosis. This chart is based on Figure 6 in Visser *et al.* [25], extended with recent findings, including the upper left part inspired by the Commentary on Powell *et al.* [12].

In MS, blood–brain barrier integrity is severely impaired in lesions with inflammatory activity, as visualized by contrast-enhancement on magnetic resonance images. Neuropathologists employ *in situ* detection of blood components such as albumin as a measure of barrier dysfunction. Hence, soluble PGN and its fragments can in principle directly access lesion areas, at the same concentrations as found in plasma.

Microbial Molecule Detection in the CNS

The CNS is widely regarded to be sterile in the absence of disease, although the effects of immune disruption are unclear in terms of brain microbial and related product diversity or quantity. Most microbes and/or their products are assumed to enter the CNS by crossing the blood–brain or blood–choroid plexus barriers. Several studies demonstrate bacterial (Proteobacteria)-encoded RNA and DNA sequences as well as PGN and other bacterial proteins in brains from humans [16,17], non-human primates [18], rodents [19], and other species [20] (and correction in [21]).

Studies detecting bacterial RNA or DNA in the CNS are controversial due to potential bacterial contamination during preparation, species variation, and autopsy times, leaving the existence of CNS microbiota unresolved [22]. For the most part, bacterial molecules in brain were detected intracellularly within glia based on microscopic analyses of immunodetection and *in situ* hybridization. Nonetheless, bacterial molecules (e.g., PGN, DNA, RNA) are widely recognized triggers for innate immune responses, including inflammasomes [23].

Among patients with HIV/AIDS and other disorders, the profile of infectious agents and PGN presence was investigated in pathologically normal and abnormal autopsied and surgically resected brain tissues [18]. Deep sequencing of cerebral white matter-derived RNA revealed bacterially encoded 16s RNA sequences in all brain specimens. Bacterial rRNA was detected in white matter glial cells by *in situ* hybridization and PGN immunoreactivity was localized principally in glia in human brains.

PGN was immunodetected in phagocytes within demyelinating lesions from MS patients and in non-human primate models of MS [24,25] and was found concentrated in lesions [26]. 16s rRNA sequencing revealed that Proteobacteria was the dominant phylum with restricted diversity in cerebral white matter from MS compared with non-MS patients. Both the MS and non-MS groups showed 1200–1400 bacterial genomes/cm^3. RNAseq analyses showed a predominance of nongut-derived Proteobacteria in progressive MS patients' white matter, associated with increased inflammatory gene expression, relative to a broader range of bacterial phyla in relapsing-remitting MS patients' white matter [26]. PGN and RNA polymerase beta subunit immunoreactivities were detected in brains from all patients. PGN immunodetection was correlated with CNS demyelination in MS, based on luxol fast blue staining, and with inflammatory gene transcript levels in MS brains. The differences in brain tissues in terms of both bacterial RNA diversity and PGN expression levels between clinical groups highlights the possibility that bacteria-derived nucleic acids and proteins might influence disease mechanisms. Indeed, MDP is an established ligand of NOD2 [linked to receptor-interacting serine/threonine-protein kinase 2 (RIPK2)] and the NLRP3 inflammasome in human microglia [27], which can influence demyelination [28].

In a 2019 deep sequencing study [17], presence of PGN was also assessed in resected brain tissues from 30 live patients, including two specimens taken at different time points from a single patient with MS. Importantly, this approach excludes technical concerns on postmortem delay and causes of death, which could affect PGN burden. Several MS brain samples of these living donors contained PGN. Thus, the presence of PGN within demyelinating lesions could promote or enhance local neuroinflammation with ensuing demyelination. In MS, blood–brain barrier integrity is severely impaired in lesions with inflammatory activity, as visualized by gadolinium-enhancing lesions on magnetic resonance images.

PGN in Animal Models for MS

EAE is the collective term for a wide range of MS animal models, in guinea pigs, rats, mice, and non-human primates, including rhesus and cynomolgus macaques and marmosets. Induction can be active [peripheral immunization with myelin proteins or peptides in strong adjuvants like Complete Freund's Adjuvant (CFA)], passive by adoptive transfer of T cells and/or antibody, and spontaneous by transgenic single or double expression of T cell receptors and B cell receptors specific for myelin antigen, mostly epitopes of myelin oligodendrocyte glycoprotein (MOG). EAE is a widely used model for investigating MS pathogenesis and treatment, as well as for more fundamental questions on immunology and inflammation, such as the biology regarding T-helper 1 (Th1), Th17, and regulatory T cells. Although EAE has directly enabled the development of relatively effective MS therapies (e.g., the anti-VLA4 antibody, natalizumab), it is also a contentious model, as it is not always predictive of disease mechanisms in humans or effective therapies. Studies in immunologically naïve 8–10-week-old **specific pathogen-free (SPF)**-bred mice led to the dogma that induction of this autoimmune-mediated inflammatory disorder requires the presence within the inoculum of an adaptive immune trigger, such as a myelin protein (MBP, PLP, MOG) as an antigenic stimulus, in conjunction with an innate immune stimulus , most often heat-killed *Mycobacterium tuberculosis* particles. The large majority of studies use the strong bacterial adjuvant CFA, with *M. tuberculosis* dispersed in a mineral oil [Incomplete Freund's Adjuvant (IFA)], to relay innate immune activation signals to antigen-presenting cells.

Since PGN is able to promote arthritis in rodents, the question arose whether PGN by itself could support EAE induction. Indeed, in a proof of principle experiment, purified PGN (soluble or particulate) can fully replace the intact complete mycobacteria particles in mouse EAE driven by the MOG35-55 peptide [29]. These notions were extended elegantly by demonstrating that PGN signaling through NOD1 and NOD2, but not through TLR2, engages RIPK2 (a.k.a. RIP2 and RICK) in dendritic cells infiltrating the CNS [30]. Furthermore, an MDP-neutralizing monoclonal antibody limits mouse EAE, supporting the notion that endogenous PGN contributes to brain inflammation [13].

Recent influential studies showed that the immune system of pathogen-experienced wild mice provides a better reflection of human immunity than that of young adult SPF rodents [31,32]. The importance of the gut microbiota in development of this matured immunity is further underscored by the creation of 'wildling' mice, where SPF-embryos are transplanted into wild mice with a natural microbiota [32]. These wildling mice better predict success and failure of immunotherapy for use in humans.

In contrast to the situation in immunologically naïve SPF-bred mice, and much more alike to the human condition, primates have a mature immune system that has been educated by lifelong interaction with microbes from the environment or from within the host (herpes- and polyomaviruses, gut microbiota) [33]. Accordingly, in contrast to SPF-rodents, in outbred, adult (over 2 years of age), conventionally reared non-human primates, common marmosets (*Callithrix jacchus*), for example, sensitization with only the adaptive stimulus (e.g., synthetic MOG34-56 peptide) admixed in IFA suffices for robust disease induction.

The induction of autoimmunity without the normally requisite innate immune stimulation within the antigen-adjuvant inoculum may be explained by activation of strongly autoreactive effector memory T cells in the immune repertoire. These effector memory T cells reacted with a mimicry epitope shared between an encephalitogenic peptide from MOG (residues 34–56) and an antigen encoded by the UL86 open reading frame of cytomegalovirus (CMV residues 981–1003). T cell

Clinician's Corner

PGN is widely abundant at mucosal and epithelial surfaces but is at the highest density in its primary source, the gut microbiota. A broad panel of lytic enzymes (e.g., lysozyme) and receptors (e.g., NOD1/2, PGLYRP1-4) protect the host against undue immune reactions elicited by intact PGN and its proinflammatory fragments.

Genetic variation in PGN receptors is associated with increased risk of Crohn's disease (e.g., NOD1/2, ATG16L1, PGLYRPs), ulcerative colitis, psoriasis, and Parkinson's disease (e.g., PGLYRPs) and rare autoinflammatory syndromes such as Blau's and Muckle-Wells disease. The chemically atypical PGN of *Borrelia burgdorferi* drives chronic inflammation in Lyme's disease [78].

In addition to its roles in inflammation and infection, PGN now appears to play critical roles in mammalian host physiology, including mouse brain development and calibration of human neutrophil counts.

Microglia and astrocytes express surface and intracellular receptors for PGN and its fragments, via which PGN relays signals modulating function of these glia cell types in normal physiology and disease.

There is a huge untapped potential for PGN-based biomarkers in pathophysiology, diagnostics, and treatment monitoring in a wide range of diseases, as well as ageing. Emerging technologies allow sensitive detection of PGN fragments in intact tissues, fluids from different anatomical compartments, and flow cytometer-sorted subsets of phagocytes (macrophages, neutrophils, subsets of dendritic cells). Levels of PGN soluble receptors can also be informative (e.g., PGLYRP1 in atherosclerosis cohorts [79–81]).

From a therapeutic point of view, administration of probiotic bacterial strains containing anti-inflammatory PGN structures is effective in animal model studies [82]. Limiting inflammatory activity of naturally occurring PGN relies on avoidance and treatment of bacterial infection and notably on improvement of gut barrier function, for instance with selazalimide, anti-

activation involved presentation of the mimicry epitope MOG40-48 via MHC-E molecules by B cells infected with a murine Epstein Barr virus-like γ1-herpesvirus (CalHV3) as the antigen-presenting cell type [33,34].

These observations highlight the novel concept that brain-targeting autoimmunity in human MS is promoted by lifelong dealing with the gut microbiota, requiring a reactive state of the primate immune system. Brain-targeting autoimmunity is elicited once the immune system encounters brain antigens released from idiopathic myelin damage or its physiological turnover, in the context of genetic predisposition for MS (notably HLA-II and 200 SNPs). In this scenario, PGN could act as a microbe-associated molecular pattern co-driving activation of such effector memory T cells.

inflammatory diet, IL-22 cytokine therapy, and postbiotic compounds produced by the gut microbiota.

Proinflammatory cytokines produced downstream of PGN exposure and pyroptosis can be countered by immunotherapeutic biologicals neutralizing IL-1β (canakinumab, rilonacept), IL-1α (bermekimab), blocking the IL-1 receptor (anakinra), or neutralizing IL-18 [83]. Finally, small molecule inhibitors of RIPK2 are under development [84].

Could PGN Contribute to Loss of Immune Regulation in MS?

In MS, the cause of the myelin damage from which autoantigens are released is unknown. Myelin damage *per se* rarely triggers autoimmunity, as exemplified in stroke. Apparently, there are protective mechanisms in place that prevent autoimmune induction, for example, regulatory T cell activity, central and peripheral tolerance, and interaction of self-associated molecular patterns expressed on myelin with inhibitory C-type lectin receptors (CLR) on myelin capturing phagocytic cells [35]. Evidently, additional stimuli are needed to disrupt this multipronged protection against autoimmune disease. PGN may provide one such stimulus by its putative transfer into the brain by CNS-infiltrating macrophages and neutrophils [25], or in soluble form directly from the circulation. Interestingly, the outcome of the pathogenic process seems related to the number and type of cells that bring PGN into the CNS and the presence of digesting enzymes in these cells, such as lysozyme and PGLYRP2. The acute, large, and destructive lesions that are characteristic of EAE in rhesus macaques contain extensive infiltrates of polymorphonuclear phagocytic cells containing PGN colocalized with PGLYRP2 immunoreactivity [25]. In marmosets with EAE, the brain shows significantly higher numbers of PGN and PGLYRP2 immunopositive mononuclear phagocytes in comparison with non-EAE animals, although their frequencies were substantially lower than in the brains of rhesus macaques with EAE [25]. Another difference between these non-human primate models with EAE was the PGN-containing phagocyte type in the brain, which were mainly neutrophils and dendritic cells in rhesus macaques with EAE but macrophages in marmoset EAE, recapitulating observations in MS. The neuropathology in rhesus macaques with EAE resembles acute demyelinating encephalomyelitis, with acute and fulminant disease development. The paucity of cells expressing PGN-digesting enzymes, such as lysozyme and PGLYRP2 in marmoset EAE brains, may underlie long-term intracellular PGN persistence, promoting chronic inflammatory EAE pathology.

The notion that antigens originating from gut microbiota can exert profound detrimental effects in autoimmune-mediated inflammatory pathology, such as in MS and its closest animal model (marmoset EAE), raises the question whether disease-mitigating effects can be achieved by modification of gut microbiota composition. In this context it is an intriguing observation that modification of a dietary supplement given to marmosets reduced EAE incidence as well as spinal cord pathology associated with marked changes in all compartments along the gut–immune–brain axis [36]. Eight dizygotic twins were divided over two dietary treatment groups, receiving either a yogurt-based supplement or a water-based supplement for 2 months. The gut microbiota of healthy marmosets was typed for the first time and also assessed during the experiment. The change in diet did not affect the microbiota much and shifts in its composition only became more evident weeks after immunization for EAE induction. It can be hypothesized that differential load of translocated PGN and its fragments is one of the factors contributing to loss of immune regulation.

Concluding Remarks

In less than a decade, appreciation of PGN biology has evolved from infection and gut microbiota, to brain inflammation, and beyond to include, potentially, brain development and behavior, as well as (*Drosophila*) lifespan in relation to gut dysbiosis [4]. Opening another window on physiological brain function, two intriguing studies [37,38] provide evidence in *Drosophila* for a relationship in flies between neuronal innate immunity, synaptic vesicle stabilization, and presynaptic homeostatic plasticity. PGN recognition protein-LC (PGRP-LC) and Tak1 (Map3K) seem to have central roles in these processes. It is suspected that an endogenous ligand engages PGRP-LC, but undue access of PGN fragments to the CNS might interfere in physiological synaptic function.

We anticipate that major fundamental insights into PGN biology will emerge, stimulated by rapid innovation in detection technologies (e.g., immunoassays, advanced mass spectrometry) for PGN in blood and other fluids such as the cerebrospinal fluid, deep-sequencing of microbiota populations, and synthetic chemistry for high density antigen arrays [39] (see Outstanding Questions). For MS and selected other chronic brain diseases, quantitative measures of PGN and its moieties could potentially contribute to predicting disease relapse and progression, as well as monitoring of current (immune)therapies. New interventions, including dietary approaches, fecal microbiota transfer/transplantation, and novel biologicals can also be assessed for improvement versus deterioration of gut barrier function.

Finally, the adaptive immune mechanisms targeting PGN also warrant further investigation (e.g., [40]). Antibody responses against the stem peptide, the interpeptide bridge, and epitopes composed of peptide in juxtaposition with glycan structures, demand a deeper characterization. Hence, we hypothesize that epitope specificity in relation to antibody isotype and subclass will provide valuable insights into gut bacterial niche formation, barrier (dys)function, and immune exclusion preventing PGN translocation.

Outstanding Questions

How can physiological functions of PGN driving immune set points, mouse brain development, and behavior, mechanistically be reconciled with its strong inflammatory activities?

Do circulating MDP/PGN levels correlate meaningfully with insults to gut barrier function? Can variation in these levels be translated into clinically actionable biomarker assays?

Can soluble PGN receptors and lytic enzymes be utilized in useful biomarker assays?

Can identification of PGN-types with distinct biological functions (e.g., pro- versus anti-inflammatory action, induction of the regulatory PD1–PDL1 negative checkpoint regulator) inform on pathogenesis and, in the future, even on prognosis and treatment monitoring?

Do soluble versus cell-transported PGN differentially contribute to brain inflammation?

Are PGN signaling, processing, and intracellular transport of fragments (e.g., transporters of the SLCA15 family) the same or distinct for microglia and astrocytes versus systemic antigen-presenting cells, including macrophages, dendritic cells, and stromal cells, like in epithelia?

Do antibody patterns against PGN reflect previous exposure (B cell memory response) and inform on establishment of physiological versus perturbed gut microbiota?

Could antibody neutralization of PGN fragments be developed as short-term or chronic therapy to limit inflammatory disease activity, including those affecting brain and spinal cord?

Can RIPK2 inhibitors and cytokine neutralization by biologicals limit DNA damage driven by PGN signaling?

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