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Supplementary Information for

Borrelia burgdorferi peptidoglycan is a persistent antigen in patients with Lyme arthritis

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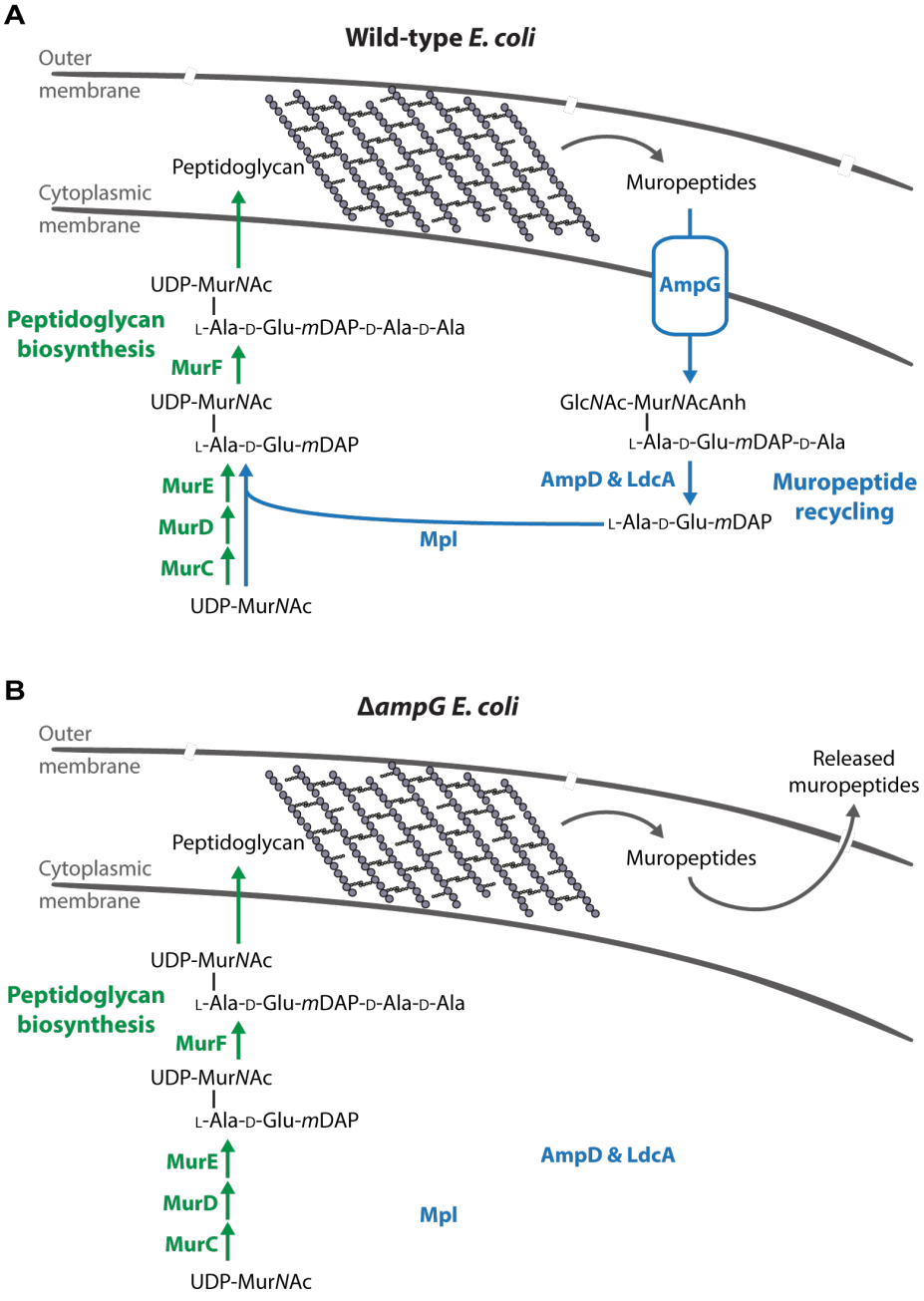

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E. coli protein	*B. burgdorferi* homolog	BLASTP E-values
AmpG	None	N/A
AmpD	None	N/A
LdcA	None	N/A
Mpl	BB_0817 (MurC)	$6x10^{-27}$
MurC	BB_0817 (MurC)	$6x10^{-54}$

Fig. S1. Fate of muropeptides in wild-type and Δ*ampG* mutant strains of *Escherichia coli*. (*A*) Schematic showing the pathway involved in recycling the peptide moiety of the peptidoglycan (PG) of wild-type *E. coli* (and other Gram-negative bacteria) into the cytoplasmic PG biosynthetic pathway. (*B*) Schematic showing that the majority of muropeptides generated during normal PG turnover are released into the environment in a Δ*ampG* mutant of *E. coli*. (*C*) Sequence homology search analysis showing that *B. burgdorferi* lacks apparent homologs of PG recycling proteins found in *E. coli*. Homology searches were performed using BLASTP's default parameters and a query sequence retrieved from NCBI's RefSeq database for each of the *E. coli* K-12 MG1655 proteins shown.

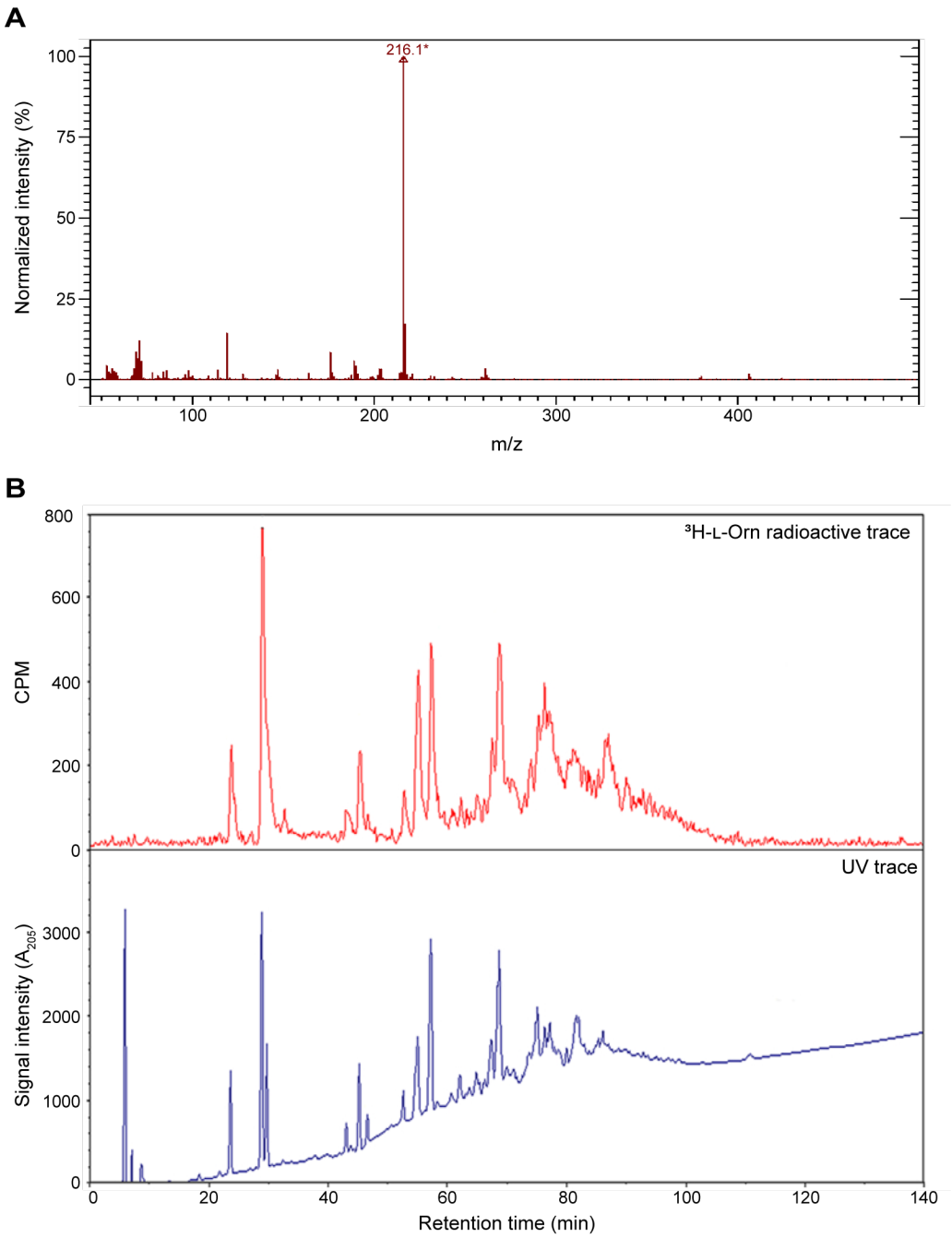


Fig. S2. Verification of the presence of ornithine in PG^{Bb}. (*A*) Electron impact mass spectrum (normalized intensity versus mass-to-charge ratio (m/z)) showing the presence of Orn in PG^{Bb}. Gas chromatography (GC) coupled to mass spectrometry (MS) proved the identity of *N*-pentafluoropropionyl ornithine isopropylester obtained from the hydrolysat (4N HCl, 100 °C, 16 h) of PG^{Bb} by the characteristic cyclic imminium fragment-ion at 216 m/z (shown by the asterisk), in agreement with data of the authentic standard substance. (*B*) Analysis of radiolabeled PG^{Bb}. Shown are chromatograms of cellosyl-digested PG^{Bb} labeled with ^{3}H-L-Orn. Muropeptides were detected by UV absorbance or liquid scintillation in counts per minute (CPM).

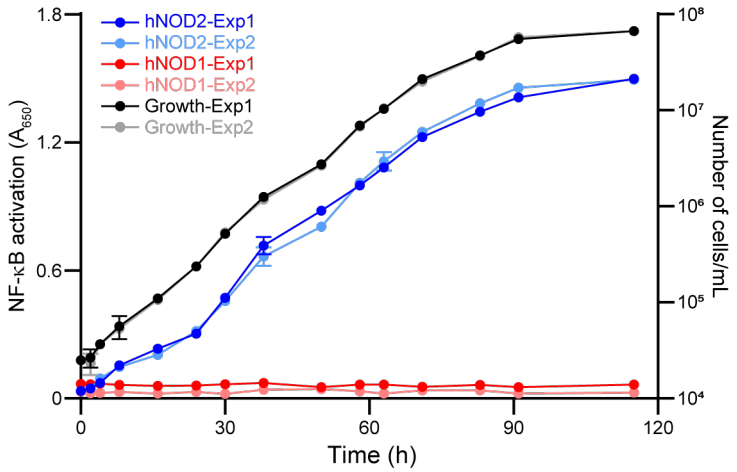
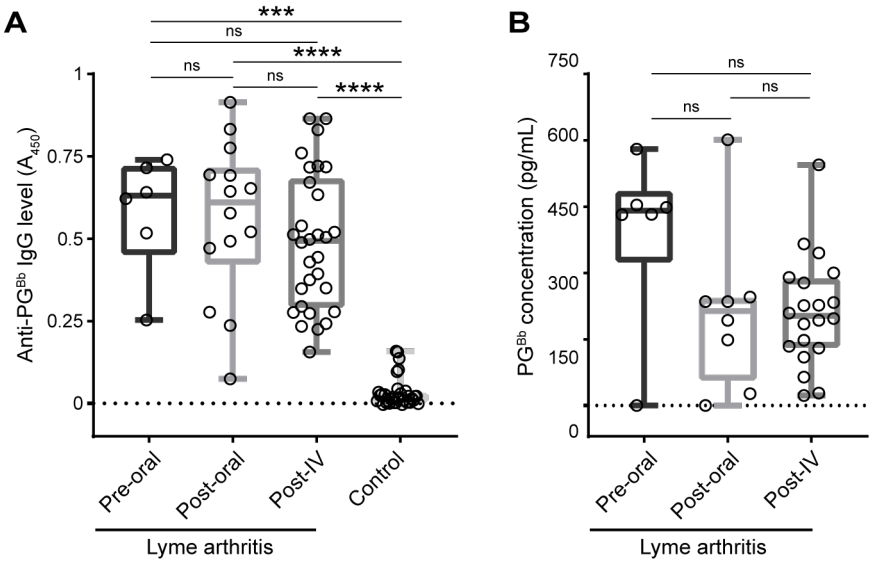


Fig. S3. Time-course analysis of muropeptide accumulation in the culture medium during *B. burgdorferi* growth. Batch cultures of *B. burgdorferi* were monitored for growth and muropeptide release using human NOD2 and NOD1 reporter cells (hNOD1 and hNOD2). At each time point (dots), a fraction of two independent cultures was removed, the density determined in cells/mL using a counting chamber (black and grey lines) and supernatants collected, processed, and assayed for hNOD1 or hNOD2 activation by monitoring NF-κB activity. Replicate cultures were analyzed in tandem (Exp 1 and 2) and each sample was analyzed in triplicate. Data points are the mean ± standard deviation for each experiment. Note that the black line largely covers the grey line because of their overlap.



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Patient code	Treatment stage	PCR test		Anti-PG^{Bb} IgG level (A_{450})		PG^{Bb} concentration (pg/mL)
		Serum	*Joint fluid*	*Serum*	*Joint fluid*	
1	Post-IV	-	-	0.1360	0.3485	196.5678
2	Post-IV	-	-	0.2635	0.5200	290.0891
3	Post-oral	-	-	0.4825	0.5215	235.4267
4	Post-IV	-	-	0.2530	0.4985	192.7818
5	Post-IV	-	-	0.1580	0.2950	147.8163
6	Post-IV	-	-	0.3665	0.5050	365.4844
7	Post-IV	-	-	0.1635	0.4295	226.4551
8	Post-oral	-	-	0.2545	0.4925	234.7741
9	Pre-oral	+	+	0.1565	0.2530	ND**
10	Pre-oral	-	-	0.0825	0.7150	453.4818
11	Post-IV	-	-	0.2620	0.4885	299.9957
12	Post-IV	-	-	0.4225	0.7180	544.4852
13	Post-oral*	+	+	0.1810	0.6535	245.4398
14	Post-IV	-	-	0.1610	0.5115	208.9477
15	Post-IV	-	-	0.1505	0.5390	345.3268
16	Post-IV	-	-	0.1610	0.5130	184.3923
17	Pre-oral	+	+	0.3755	0.7400	579.9464
18	Post-IV	-	-	0.2570	0.3940	64.423
19	Post-IV	-	-	0.1950	0.6340	227.0843
20	Pre-oral	+	+	0.2805	0.6410	448.2324
21	Post-IV	-	-	0.0240	0.2345	22.63914
22	Pre-oral	-	-	0.4135	0.6220	432.8897
23	Post-IV	-	-	0.1715	0.3505	232.8273
24	Post-IV	-	-	0.1960	0.2425	134.2467
25	Post-oral	-	-	0.3925	0.5780	192.7818
26	Post-IV	-	-	0.1120	0.3755	27.92979
27	Post-oral	-	-	0.3800	0.6440	148.652
28	Post-oral	-	-	0.0375	0.2370	ND**
29	Post-IV	-	-	0.2895	0.4445	130.1027
30	Post-IV	-	-	0.4220	0.6715	277.4131
31	Post-oral	-	-	0.3745	0.6945	601.4136
32	Post-IV	-	-	0.1720	0.2780	109.4105
33	Pre-oral	+	-	0.1640	0.5170	431.638
34	Post-oral	-	-	0.1495	0.2770	26.4551

*Patient received 3 weeks of doxycycline (oral).
** No PG^{Bb} could be detected.

Fig. S4. Analysis of human Lyme arthritis synovial fluids based on treatment stage. (*A*) Box plot showing the levels of anti-PG^{Bb} IgG in synovial fluid samples of Lyme arthritis (LA) patients based on treatment stage, i.e., before oral antibiotic treatment (pre-oral, n = 6), after oral antibiotic treatment (post-oral, n = 14) or after both oral and intravenous antibiotic treatment (post-IV, n = 30) as determined by ELISA (see Fig. 2*A*). Included are control synovial fluid samples from patients suffering from a torn ACL (n = 1) or other forms of arthritis (n = 32). A Kruskal-Wallis test on the four groups resulted in $p < 0.0001$.

Kruskal-Wallis test followed by a Dunn's post-hoc pairwise test revealed that the anti-PG^{Bb} IgG level of all LA sample groups (stage (pre-oral, post-oral or post-IV antibiotic) was statistically significant different from the control group (**** indicates $p < 0.0001$ whereas *** indicates $p < 0.001$). In contrast, there was no significant difference (ns) in anti-PG^{Bb} IgG levels between the different LA sample groups (adjusted $p > 0.99$). (*B*) Box plot showing the concentration of PG^{Bb} in LA samples organized by treatment stage: before oral antibiotic treatment (n = 6), after oral antibiotic treatment (n = 8) or after both oral and intravenous antibiotic treatment (n = 20) based on a competitive ELISA using anti-serum raised against PG^{Bb}. Only patients with available synovial and serum samples were considered in this assay. Kruskal-Wallis test comparing the three groups yielded $p = 0.1$. (*C*) Table showing the results of PCR, anti-PG^{Bb} IgG and PG-concentration analyses for the serum and synovial fluid samples of 34 LA patients at different treatment stages of disease. See Methods for details.

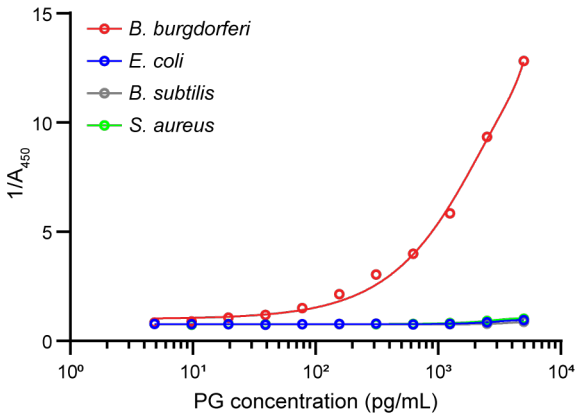

Fig. S5. Specificity and sensitivity of rabbit anti-serum raised against *B. burgdorferi* PG (anti-PG^{Bb}) using a competitive ELISA. PG^{Bb}-coated plates were incubated with titrations of known concentrations of PG purified from *B. burgdorferi*, *E. coli, Staphylococcus aureus*, and *Bacillus subtilis* that were pre-incubated with fixed amount of anti-PG^{Bb} serum. Inverse spectrophotometric absorbance values are graphed as a function of PG concentrations (in pg/mL). A third-order polynomial equation (lines) was used to calculate the amount of PG in patient samples.

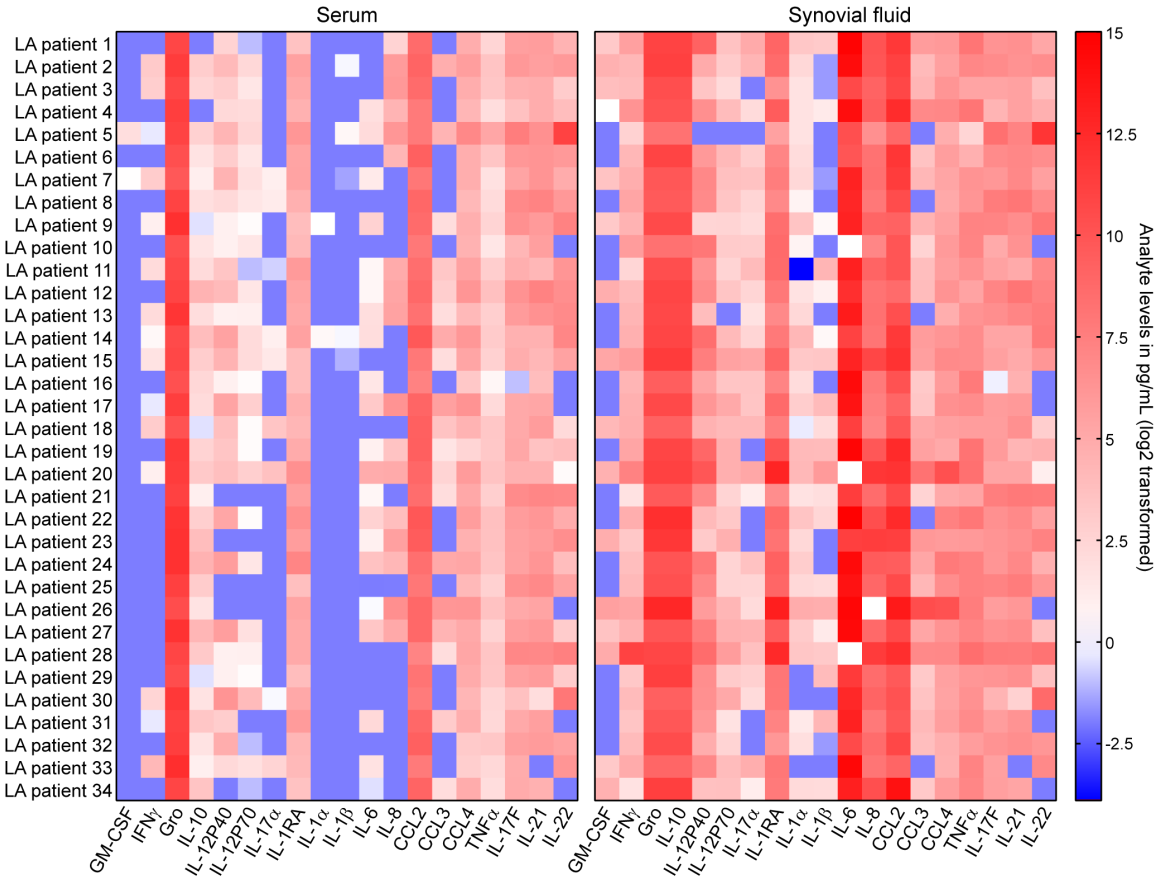

Fig. S6. Pairwise comparison of cytokine profiles in serum and synovial fluid samples of LA patients using Luminex bead arrays. Heat map values are the mean of replicate values in pg/mL of each analyte, followed by $\log_2$ transformation.

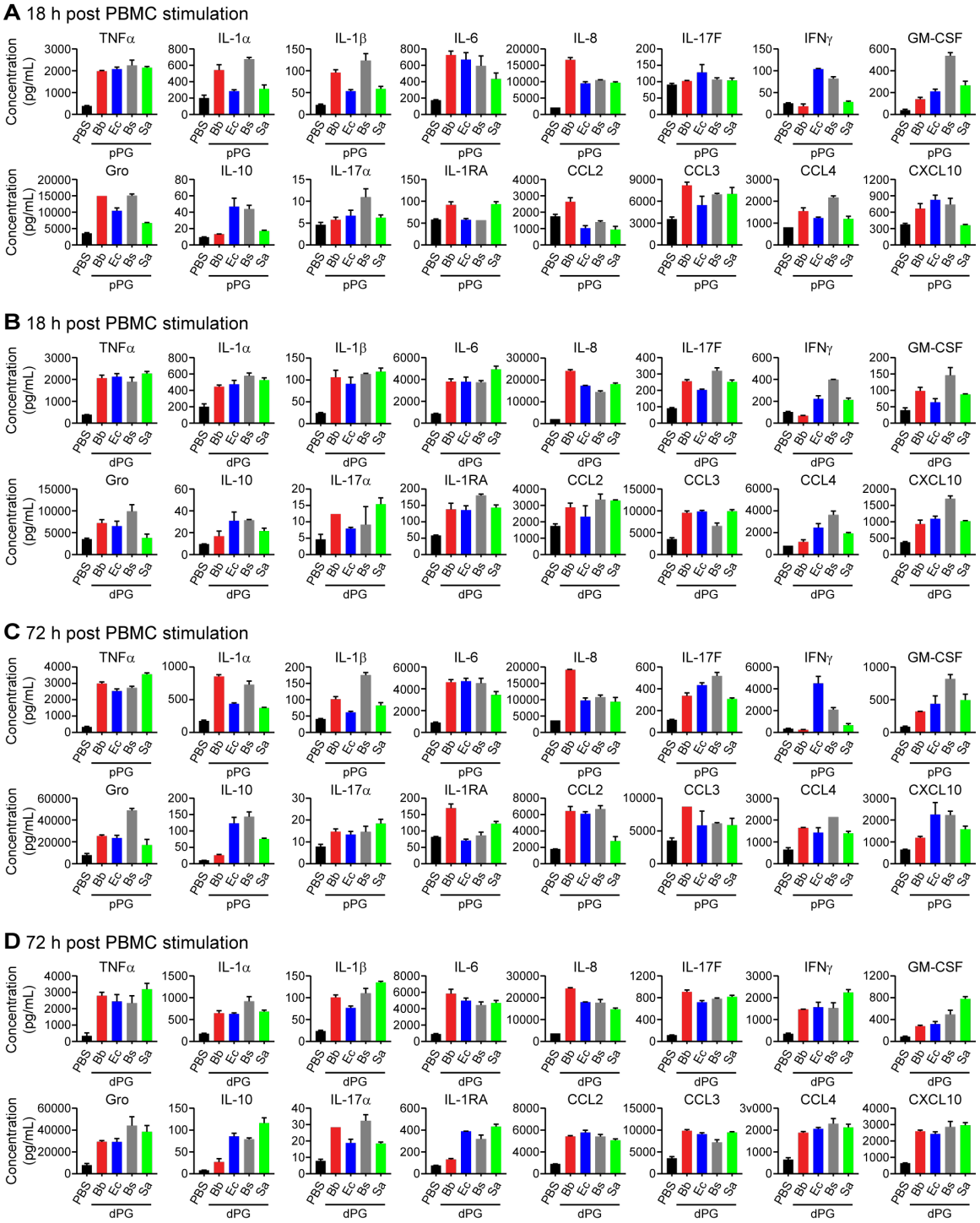

Fig. S7. In vitro stimulation of cytokine production in human peripheral blood mononuclear cells (PBMCs) by different types of PG. (*A*) Levels of indicated analytes secreted by control human PBMCs stimulated for 18 h by PBS (control) or 100 µg/mL of polymeric(pPG) isolated from *B. burgdorferi* (Bb), *E. coli* (Ec), *B. subtilis* (Bs), or *S. aureus* (Sa). (*B*) Same as (*A*) except that stimulation was with mutanolysin-digested (dPG) PG. (*C*) Same as (*A*) but after 72 h of stimulation. (*D*) Same as (*B*) but after 72 h of stimulation.

Table S1. Analysis of muropeptides produced by mutanolysin digestion of *B. burgdorferi* peptidoglycan

Peak	Muropeptide	Mean peak percent area (+/- SD) [Ŧ]	Mass (neutral from m/z)		RT (min)	CPM per area
			Measured	Theoretical		
1	Mur*N*Ac(r)-L-Ala-D-Glu-L-Orn-Gly	3.99 (0.36)	666.3134	666.3072	23.47	2.17
2	Glc*N*Ac-Mur*N*Ac(r)-L-Ala-D-Glu-L-Orn-Gly	9.37 (0.57)	869.3881	869.3866	28.70	2.99
3	Hex*N*Ac-Glc*N*Ac-Mur*N*AcAnh-L-Ala-D-Glu-L-Orn-Gly	3.69 (0.23)	1052.4354	1052.4398	42.90	3.63
4	Glc*N*Ac-Mur*N*AcAnh-L-Ala-D-Glu-L-Orn-Gly (isomer of 7)	2.01 (0.31)	849.3560	849.3604	45.10	2.13
5	Glc*N*Ac-Mur*N*Ac(L-Ala-D-Glu-L-Orn-Gly)-Glc*N*Ac-Mur*N*Ac(r)-L-Ala-D-Glu-L-Orn-Gly	0.93 (0.10)	1718.7708	1718.7469	46.45	1.52
6	Mur*N*Ac(r)-L-Ala-D-Glu-L-Orn-Gly-D-Ala-L-Orn-Gly-D-Glu-L-Ala-Mur*N*Ac(r)	1.11 0.05	1385.6420	1385.6409	52.48	3.77
7	Glc*N*Ac-Mur*N*AcAnh-L-Ala-D-Glu-L-Orn-Gly (isomer of 4)	6.47 (1.45)	849.3598	849.3604	54.48	0
8	Mur*N*Ac(r)-L-Ala-D-Glu-L-Orn-Gly-D-Ala-L-Orn-Gly-D-Glu-L-Ala-Mur*N*Ac(r)-Glc*N*Ac	1.32 (1.03)	1588.7210	1588.7203	54.82	9.49
9	Glc*N*Ac-Mur*N*Ac(r)-L-Ala-D-Glu-L-Orn-Gly-D-Ala-L-Orn-Gly-D-Glu-L-Ala-Mur*N*Ac(r)-Glc*N*Ac	11.41 (0.42)	1791.8010	1791.7997	57.18	4.60
10	Glc*N*Ac-Mur*N*AcAnh-L-Ala-D-Glu-L-Orn-Gly-D-Ala-L-Orn-Gly-D-Glu-L-Ala-Mur*N*Ac(r)-Glc*N*Ac (isomer of 11)	3.13 (0.12)	1771.7735	1771.7782	68.60	5.30
11	Glc*N*Ac-Mur*N*AcAnh-L-Ala-D-Glu-L-Orn-Gly-D-Ala-L-Orn-Gly-D-Glu-L-Ala-Mur*N*Ac(r)-Glc*N*Ac (isomer of 10)	1.69 (0.37)	1771.7735	1771.7750	76.27	5.65

1 - 11	all known	45.10 (1.98)	-	-	-	-
*	Unknown	6.58 (0.24)	906.3449		29.55	0
	monomers (total)	56.50 (3.41)			-	-
	monomer anhydro	26.89 (2.99)			-	-
	dimers (total)	43.50 (3.41)			-	-
	dimers anhydro	12.76 (0.90)			-	-
	Glycan strand length (anhydro chain ends)	33.27 (2.57)			-	-
	degree of cross-linkage	21.75 (1.70)			-	-
	% peptides in cross-links	43.50 (3.41)			-	-

Analysis was performed by high performance liquid chromatography (HPLC) and mass spectrometry.
Numbering and peak identity corresponds to the HPLC peak numbers in Fig. 1.
RT: Retention Time, CPM/Area: Radioisotope counts per minute divided by the peak area.
* Unidentified peak that does not contain L-Orn.
[T] Means and standard deviations were calculated from three independent experiments.
Note: Peaks 4 and 10 could not be distinguished from peaks 7 and 11, respectively (by radioisotope trace).