Supplemental methodology

1. PGN purification and testing

The PGN purification methodology and qualitative analysis of PGN end product was described in detail by Langer et al.[1] and is summarized below. PGN was purified from *Bacillus anthracis* vegetative bacteria grown to stationary phase. Throughout the procedure PGN is retained in the insoluble cell wall fraction, which was collected by centrifugation at 15000 x g x10 min in between purification steps. Bacteria were collected by centrifugation and boiled in SDS for initial extraction/solubilization and bacterial killing. The pellet was washed and resuspended in endotoxin-free water and plating overnight tested residual bacterial viability. After confirmation of no viability, the PGN fraction was sequentially subjected to DNase/RNase digestion, hydrofluoric acid (HF, 48%) extraction and proteinase K digestion. Each step was repeated twice and SDS extraction was used to remove recombinant enzymes. The end product was washed extensively to remove SDS, sonicated, and dried in a speed vacuum in pre-weighed endotoxin free microfuge tubes. The PGN was resuspended at 10 mg/mL in endotoxin water for analysis and/or in saline solution for *in vivo* experiments.

For analytical testing, PGN samples were collected after each purification step and analyzed for amino acid content, phosphate content, TLR signaling, inflammatory bioactivity. Overall, we only find aminoacids from the crosslinking stem peptide, residual organic phosphate content was minimal, no TLR2 or TLR4 signaling capacity was observed, and proinflammatory bioactivity through internalization dependent NOD signaling[1]. The final product does not stimulate mouse macrophages, which express abundant TLRs and is part of our purification criteria. Human cells respond optimally when the material is phagocytized and hence the response is sensitive to cytochalasin D, also an analytical criterion.

Quantitative amino acid analysis was performed on PGN samples after acid hydrolysis by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization and HPLC separation according to published methodology[2]. Proteinase K digestion reduced the complexity of aminoacid content and we only identified 3 aminoacids in the end product: alanine, glutamate and diaminopimelic acid, which are the expected aminoacids in the anthrax PGN stem peptide. Aminoacid content confirms the lack of other lipoproteins, potential TLR1/2/6 ligands, as well as lack of toxin components in our final PGN preparation. On selected PGN preparations, the glycan and stem peptide structure was confirmed by mass spectrometry analysis.

PGN phosphate content was assessed after acid hydrolysis as previously described[3]. During purification we calculated that PGN phosphate content was reduced from 0.3 nmoles phosphate/ µg PGN in heat killed bacteria to 0.004 nmoles/µg PGN after HF extraction[1]. Results indicate virtually complete removal of organic phosphate, including lipoteichoic acids that could stimulate TLR2 receptors.

PGN ability to stimulate TLR and TLR4 receptors was investigated using commercial HEK293 reporter cells lines (Invivogen). We used 2 cell lines for PGN testing: HEK-TLR2 stably expressing human TLR receptor and CD14, and HEK-TLR4, stably expressing human TLR4 and CD14. Cell lines were cultured according to manufacturer protocols. Each reporter cell line stably expresses a secreted alkaline phosphatase (SEAP) reporter as well, which is produced by ligand-receptor signaling, either TLR2 or TLR4. The activity of SEAP released in culture media is determined spectrophotometrically through chromogenic conversion of the HEK-Blue Detection Medium (Invivogen) according to manufacturer protocol. Anthrax PGN end product does not stimulate either TLR2 or TLR4 reporter cells with SEAP activity within mean + 2 standard deviation of negative control (endotoxin-free water). During purification we observed that some recombinant enzymes, especially proteinase K, could introduce TLR2 reporter activity in the PGN prep. This agonist activity is removed however by additional SDS extractions at the end of the purification protocol.

Alternatively, endotoxin levels were quantified using commercial LAL assays according to manufacturer protocols. The lack of TLR signaling capacity was further confirmed through stimulation of mouse bone marrow derived macrophages (BMDM). Anthrax PGN does not stimulate mouse BMDM despite abundant expression of TLRs in these cells.

PGN bioactivity was assessed throughout the purification procedure and at endpoint by proinflammatory cytokine induction in primary human monocytes. PGN ability to induce proinflammatory cytokines was lowered by the purification procedure due to the elimination of TLR agonists. The end product induced proinflammatory cytokines in monocytes, and PGN signaling was sensitive to serum opsonization, PGN internalization and lysosomal processing, as we previously reported in multiple studies referenced in text, and consistent with signaling through intracellular NOD sensors.

2. Quantification of coagulation protease-serpin complexes

Complexes formed between coagulation-protease and their circulating serpins were quantified by either commercial assays (TAT) or in house developed ELISA. The in-house developed assays include FXIIa-AT, FXIIa-C1inh, kallikrein-C1inh, FXIa-AT and FVIIa-AT. Antibody sources and/or clones are described in text.

Plates were coated overnight at 4ºC with coating antibodies in carbonate buffer. For AT-containing complexes (FXIIa-AT, FXIa-AT, FVIIa-AT) the coating antibody was directed against the protease component, while for C1inh-containing complexes we coated with the KOK12 monoclonal anti-C1inh. Coating antibody concentration was 2 µg/mL with the exception of the affinity-purified goat anti human FVII polyclonal (R&D Systems), which was used at 0.3 µg/mL.

Coated plates were washed, blocked with 3% BSA /PBS for 2 hours at room temperature, then incubated with standards and unknown samples diluted in sample diluent (PBS + 0.1% Tween-20 + 10 mM EDTA + 5 mM Benzamidine + 0.1mg/mL soybean trypsin inhibitor) for 1 hour at room temperature. As appropriate, complexes were detected with

biotinylated anti-human antithrombin antibody (affinity purified sheep polyclonal, Affinity Biologicals) or 13G11 monoclonal anti-human kallikrein (Pierce) or 9G3 monoclonal anti-human FXII for 1 hour at room temperature, followed by streptavidin-peroxidase for 30 min. Complexes were visualized through peroxidatic conversion of o-phenylenediamine (Sigma) and quantified after extrapolation from a standard curve obtained by *in vitro* activation of lepirudinated pooled normal baboon plasma.

Generation of relative baboon standards: In the absence of analytic standards for baboon protease-serpin complexes we generated relative standards through *in vitro* activation of lepirudinated baboon plasma. Pooled normal baboon plasma was activated *in vitro* with 1 volume of either aPTT reagent (for FXIIa-AT, FXIIa-C1inh, FXIa-AT, kallikrein-C1inh) or PT reagent (for FVIIa-AT) in the presence of 10 mM $CaCl_2$ for 1 hour at 37ºC. Reaction was stop by diluting 5 fold into sample diluent (PBS + 0.1% Tween-20 + 10 mM EDTA + 5 mM Benzamidine + 0.1mg/mL soybean trypsin inhibitor). Activated plasma was centrifuged for 10 min at 15000 x g to remove the particulate material from the aPTT reaction, then aliquoted and stored frozen at -20ºC until use. The amount of protease-serpin complexes generated by 1 hour *in vitro* activation were considered to be 1 unit/mL for each respective complex, and serial standard dilutions were used for each ELISA run. Standards and samples (EDTA anticoagulated plasma collected from the *in vivo* PGN challenge) were further diluted in diluent to reach the optimized dynamic range for each assay.

TAT ELISA was performed according to manufacturer protocol and complexes were extrapolated from a standard curve generated by serial dilution of pre-quantified complexes of human origin available in the kit.

3. PGN effect on contact coagulation initiation in vitro

Purified contact coagulation factors were acquired as zymogens or activated proteases from two commercial sources (Enzyme Research Laboratories or Haematologic Technology

Inc.) and were >95% pure by SDS PAGE analysis. We used two structurally identical chromogenic substrates: S2302 (Diapharma, West Chester, OH) and/or Biophen CS-31(02) (Aniara Diagnostica, West Chester, OH). The substrate mimetic can be proteolyzed by FXIIa, kallikrein and FXIa, which releases the chromophore p-nitroaniline (pNA) measured spectrophotometrically at 405 nm. The observed catalytic conversion was higher for kallikrein than any FXIIa active forms: α-kallikrein>α-FXIIa>β-FXIIa.

Investigations of contact factors autoactivation, transactivation and PGN effect on active contact proteases were performed in HBS (10 mM HEPES, 140 mM NaCl, 4 mM KCl) supplemented with 10-20 μM $ZnCl_2$. Product (pNA) concentrations were calculated from absorbance changes through Lambert's law conversion using the pNA extinction coefficient $9920 M^{-1} cm^{-1}$[4,5]. Initial enzyme velocities were calculated from the linear range of productivity curves and reported as μM pNA/min. In some cases specific activities were calculated by normalizing initial velocities to the amount of enzyme used in the reaction and they are expressed as μM pNA/min/pmole enzyme.

PGN effect on contact pathway autoactivation using a continuous chromogenic assay: Purified plasma FXII and prekallikrein, 200 nM each, were incubated with various amount of PGN in a microtiter plate, followed by addition of the S2302 substrate (0.5-0.6 mM). Changes in optical density at 405 nm (pNA) were continuously monitored for up to 2 hours. No significant changes in pNA generation were observed with either zymogen (figure 2E and supplemental figure 2).

PGN effect on transactivation of contact pathway initiators: Purified plasma FXII and prekallikrein, 200 nM each, were co-incubated in the absence or presence of PGN (1 mg/mL). Transactivation was measured continuously using the S2302 substrate, which is proteolyzed by both factors. Representative productivity traces are shown in supplemental figure 2. We observed only a minimal effect of PGN on zymogen transactivation at this concentrations (supplemental figure 2) leading to a shift to the left of the productivity curve. We did not

calculate productivity rates due to the different catalytic properties of the 2 proteases towards the same substrate.

PGN effect on FXI autoactivation: FXI, 30 nM, was incubated with increasing amounts of PGN and amidolytic conversion of the S2302 chromogenic substrate was monitored continuously as above. No significant FXI autoactivation was observed, indicating that PGN does not bypass FXII to promote FXIa.

PGN effect on contact initiator proteases: PGN effect on the catalytic activities of α-FXIIa, β-FXIIa and α-kallikrein were assessed in a continuous assay using the S2302 chromogenic substrate. Initial velocities were calculated from productivity curves at different protease concentration in the absence or presence of PGN at either 50 or 200 µg/mL. Under these conditions PGN induced a small but consistent improvement of α-FXIIa activity at all concentrations tested, and improves β-FXIIa and α-kallikrein activity at sub-saturating concentrations. In each case, PGN increased the sensitivity of the chromogenic assay (lowered the enzyme concentration for which consistent chromogenic conversion was observed). At constant protease concentration we observed a dose-dependent effect of PGN on each enzyme activity with a strong linear correlation between PGN concentration and initial velocities (Figure 2F). To assess PGN effect on enzymatic activities against physiologic substrates (protease-mediated transactivation) we incubated low amounts of active proteases (either FXIIa or kallikrein, used at 5-10% circulating zymogen concentration) with a constant amount of transactivation substrate (200 nM prekallikrein or FXII zymogen respectively). The transactivation reaction was monitored continuously using S2302 chromogenic substrate (supplemental figure 2). In each case, PGN enhanced the transactivation reactions mediated by active initiating proteases (productivity curves shifted to the left). Altogether these data indicate that PGN improves enzymatic activities of low concentrations of contact pathway initiating proteases that are likely generated during pathway activation *in vivo*.

PGN effect on antithrombin (AT) - mediated regulation of FXIIa: The inhibitory effect of antithrombin on FXIIa enzymatic activity was assessed using the S2302 chromogenic substrate by quantification of residual FXIIa activity at various AT concentrations within physiologic range. Purified AT was obtained from Enzyme Research Laboratories or Haematologic Technologies Inc., and heparin was acquired from Sigma. AT (0-2 μM) was preincubated with a saturating concentration of heparin (200 μg/mL = 40 IU/mL) before incubation with α-FXIIa (100 nM) in a microtiter plate. The reaction was incubated for 30 min at 37ºC in a thermostated plate reader with continuous mixing before addition of the S2302 chromogenic substrate. Residual α-FXIIa activity was quantified from the productivity curves as above (μM pNA/min) and plotted against the AT concentration. AT-heparin IC_{50}, the AT concentration that inhibits half of the α-FXIIa activity, was estimated from the best-fit non-linear curves using Prism (GraphPad Software). The effect of PGN on the AT-heparin regulation of FXIIa activity was assessed by calculating AT-heparin IC_{50} in the absence or presence of PGN (50 μg/mL and 200 μg/mL) and is illustrated in figure 2G.

References:

1. Langer M, Girton AW, Popescu NI, Burgett T, Metcalf JP, Coggeshall KM. Neither Lys- and DAP-type peptidoglycans stimulate mouse or human innate immune cells via Toll-like receptor 2. *PLoS One.* 2018;13(2):e0193207.
2. Cohen SA, Michaud DP. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Anal Biochem.* 1993;211(2):279-287.
3. Gao JJ, Xue Q, Zuvanich EG, Haghi KR, Morrison DC. Commercial preparations of lipoteichoic acid contain endotoxin that contributes to activation of mouse macrophages in vitro. *Infect Immun.* 2001;69(2):751-757.
4. Lottenberg R, Jackson CM. Solution composition dependent variation in extinction coefficients for p-nitroaniline. *Biochim Biophys Acta.* 1983;742(3):558-564.
5. Ivanov I, Matafonov A, Sun MF, Cheng Q, Dickeson SK, Verhamme IM, Emsley J, Gailani D. Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. *Blood.* 2017;129(11):1527-1537.

Supplemental data

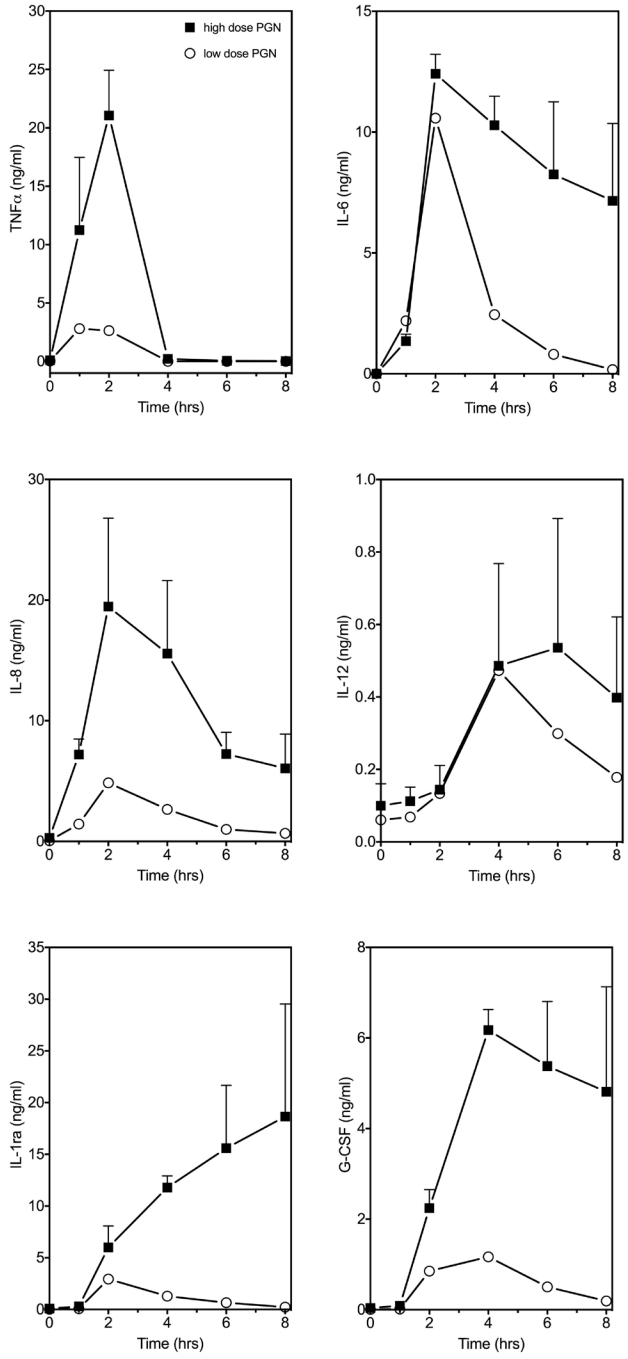

Figure S1. **PGN challenge induces system inflammation in a dose dependent manner**. Time course dynamics of PGN-induced proinflammatory/procoagulant mediators (TNFα, IL6, IL8, IL12) as well as anti-inflammatory regulators (IL-1ra, G-CSF) are depicted.

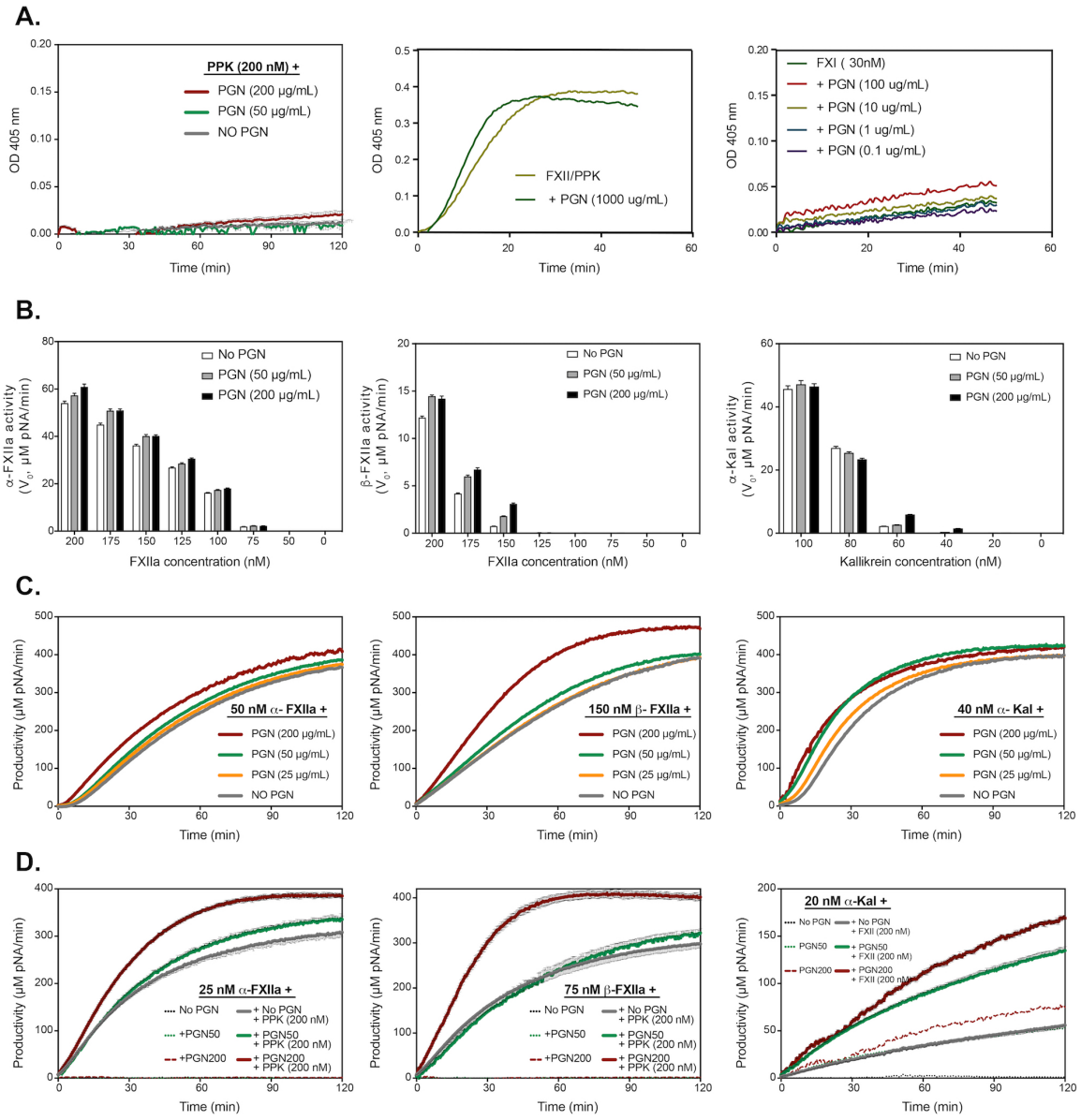

Figure S2. **PGN effect on contact pathway initiation**. (A) PGN does not induce autoactivation of purified contact pathway initiators measured in a continuous assay using the S2302 substrate. Similar to zymogen FXII (figure 2E), PGN does not support autoactivation of plasma prekallikrein (PPK, left panel), and at high concentrations has little effect on the transactivation of zymogen initiators (middle panel). PGN does not support FXI autoactivation (right panel) thus it cannot promote contact pathway propagation in the absence of initiators activation. (B) PGN

enhances active initiators protease activity at low concentrations. Enzymatic function of α-FXIIa (left panel), β-FXIIa (middle panel) and α-kallikrein (a-Kal, right panel) were measured in the absence or presence of PGN (either 50 or 200 µg/mL). Initial rates of chromophore release from the S2302 substrate were calculated from the linear fit of the productivity curves. PGN induces a small increase in the enzymatic activity of initiating proteases, which is more prominent at low enzyme concentrations. (C) At constant enzyme concentration, PGN induces a dose-dependent increase in the enzymatic activity of α-FXIIa (left panel), β-FXIIa (middle panel) and α-Kal (right panel) toward the chromogenic substrate mimetic. Productivity rates (µM pNA generated over time) in the presence of increasing amounts of PGN (1-200 µg/mL) were analyzed and select traces are exemplified (at 25, 50 and 200 µg/mL PGN). The correlation between enzyme activity and PGN concentration for α-FXIIa and α-Kal is shown in figure 2F. (D) PGN effect on the transactivation reactions mediated by initiating proteases was investigated in a continuous chromogenic assay. PGN enhanced the transactivation reaction mediated by α-FXIIa (left panel), β-FXIIa (middle panel) and α-Kal (right panel) in a dose dependent manner. For each reaction, low protease concentration, stated in the figure, were mixed with a constant concentration of transactivation substrate (200 nM prekallikrein or zymogen FXII respectively). Both the initiating protease and the transactivation product cut the chromogenic substrate leading to the left shift in productivity traces. PGN enhanced this shift in a dose dependent manner.

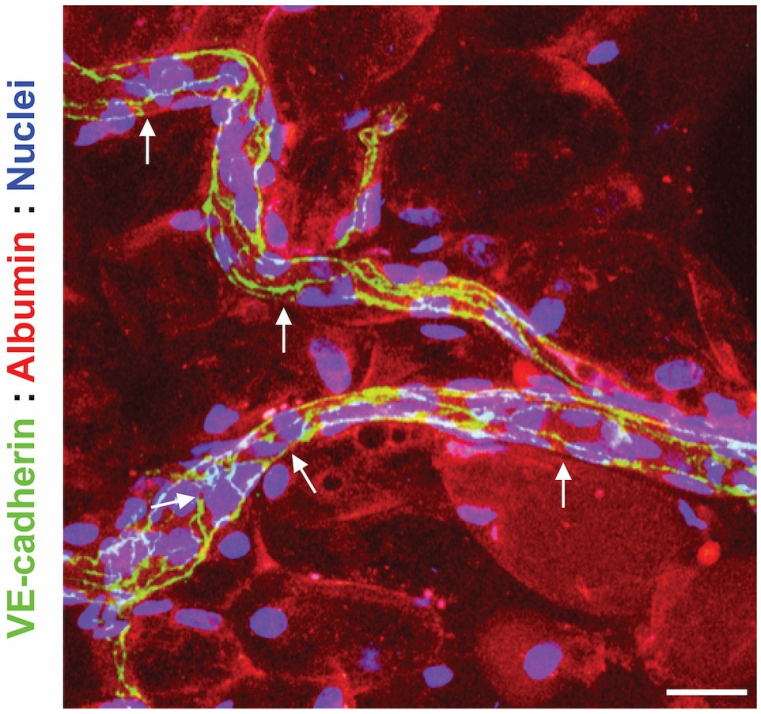

Figure S3. Capillary leakage in the mesentery after 2 hrs PGN challenge.

Paraformaldehyde fixed mesentery was whole-mount stained with antibodies against VE-cadherin (endothelial cell junction marker; green) and TO-PRO3 (nuclei marker; blue). Extravasation of albumin – Alexa Fluor 594 (Invitrogen) injected intravenously in the animal 15 min before the PGN challenge was visualized on the red channel. The image is a maximum-intensity projection of 20 confocal images (Z-stack) spanning approximately 100 μm in depth. Arrows show apparent areas of junction disruption. The fluorescent albumin tracer is observed in the extravascular area and adopts a pericellular distribution. Magnification bar: 20 μm.

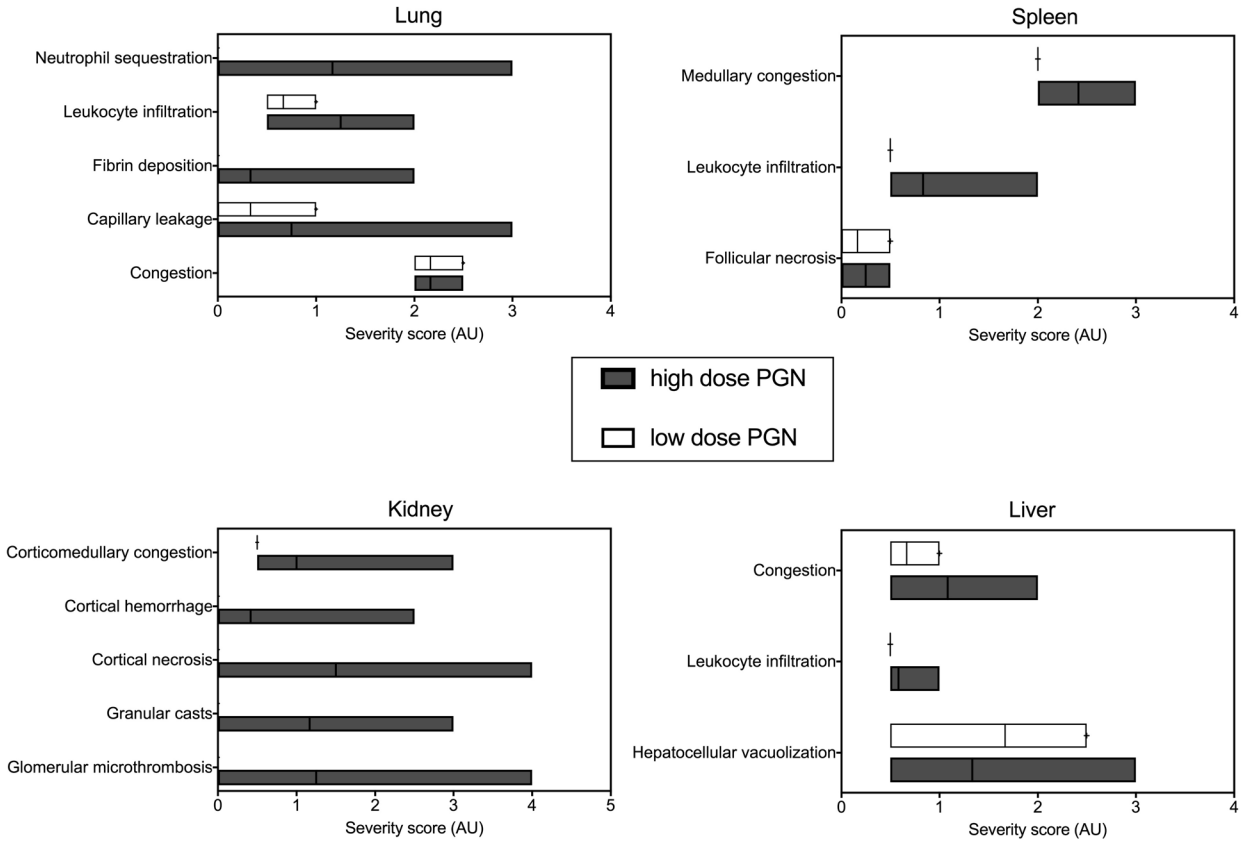

Figure S4. PGN challenge induces multiple organ dysfunctions. Histopathologic changes in peripheral organs in PGN challenged primates were assessed by an experienced veterinary pathologist (S. Kosanke, OUHSC) blinded to the experimental condition. Semiquantitative grading of organ pathology at endpoint was performed on a scale from 0 to 4, with 0 being normal and 4 being severe. Data are represented as min-to-max floating bars with mean highlighted by the internal line (high dose PGN, dark bars, n = 6; low dose PGN, white bars, n = 3).