Serum amyloid P and IgG exhibit differential capabilities in the activation of the innate immune system in response to *Bacillus anthracis* peptidoglycan.1

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Running title: SAP supports responses to DAP-type PGN

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List of abbreviations:

- Allophycocyanin (APC), R-1-(6-(R-2-carboxypyrrolidin-1-yl)-6-oxohexanoyl)pyrrolidine-2-carboxylic acid (CPHPC), diaminopimelic acid (DAP),
macrophage inflammatory protein-1α (MIP1α), pathogen-associated molecular patterns (PAMPs), pattern recognition receptors (PRRs), peptidoglycan (PGN), phosphatidylinositol 3-kinase (PI3-kinase), serum amyloid P (SAP), terminal complement complex (TCC), Ultra low IgG fetal calf serum (FCS).
Abstract

We showed that human IgG supported the response by human innate immune cells to peptidoglycan (PGN) from *Bacillus anthracis* and PGN-induced complement activation. However, other serum constituents have been shown to interact with peptidoglycan, including the IgG-like soluble pattern recognition receptor serum amyloid P (SAP). Here, we compared the ability of SAP and of IgG to support monocyte and complement responses to PGN. Utilizing *in vitro* methods, we demonstrate that SAP is superior to IgG in supporting monocyte production of cytokines in response PGN. Like IgG, the response supported by SAP was enhanced by phagocytosis and signaling kinases such as Syk, Src and phosphatidylinositol 3-kinase that are involved various cellular processes including Fc receptor signaling. Unlike IgG, SAP had no effect on the activation of complement in response to PGN. These data demonstrate an opsonophagocytic role for SAP in response to PGN that propagates a cellular response without propagating the formation of the terminal complement complex.
Introduction

Infection by *Bacillus anthracis* via the inhalation route shows a high rate of mortality in humans. We proposed that the mortality in these cases is due to sepsis (1), given the attending bacteremia (2) and signs of inflammation (3, 4) in the terminal stages of infection. The pathophysiology of sepsis by any infectious agent is propagated by pathogen-associated molecular patterns (PAMPs), which are components of pathogens that are recognized by host pattern recognition receptors (PRRs). In sepsis, the large amount of PAMPs in the blood triggers an amplified and dysregulated response by the host’s innate immune system. In the absence of effective medical intervention, the consequences of such a massive response leads to a cytokine storm, disseminated intravascular coagulation, complement activation, immune suppression, organ failure and eventually death. PGN triggers sepsis pathophysiology following *in vivo* challenges (5, 6). However, the mechanisms by which PGN induces this damaging pathophysiology are not understood.

PGN is composed of a disaccharide backbone that is highly cross-linked by a short stem peptide. In *B. anthracis*, the stem peptide is composed of L-alanine, D-glutamic acid, diaminopimelic acid (DAP) and D-alanine. Most Gram-positive bacteria substitute lysine for DAP in the stem peptide. Our studies on human responses to PGN indicate a model in which PGN is opsonized by human serum factors to allow binding and uptake by Fcγ receptors on innate immune cells (7, 8). Following uptake, PGN is digested in phagolysosomes to monomeric components that stimulate cytoplasmic receptors NOD1 and 2 (7). We showed that human IgG can act as a serum opsonin, binding PGN and permitting uptake by Fcγ receptors (9).
Recently, serum amyloid P (SAP) was shown to bind to PGN of the lysine type and support phagocytosis of *Staphylococcus aureus* whole bacteria by neutrophils via Fc\(\gamma\) receptors (10). SAP is a member of the pentraxin family of proteins that also includes the acute phase proteins C-reactive protein (CRP) and pentraxin-3. Unlike the other pentraxins, SAP is constitutively present in human serum at concentrations of between 30-50 \(\mu\)g/ml. Similar to IgG, SAP has been demonstrated to interact with components of the complement pathway and with Fc\(\gamma\) receptors (11-16).

In this study, we investigated the relative contributions of SAP and IgG in supporting the innate immune responses to DAP-containing *B. anthracis*-derived PGN. Utilizing *in vitro* assays, we found that SAP was superior to IgG in supporting monocyte cytokine production in response to PGN while IgG was superior to SAP in supporting complement activation. These findings shed light on the role of human opsonins in mediating immune responses to PAMPs such as PGN during septicemia.
Results

The anti-PGN titer is not limiting in monocyte cytokine responses to PGN

Our previous studies had demonstrated that IgG was capable of supporting monocyte and neutrophil pro-inflammatory responses to PGN and all tested human sera contained anti-PGN antibodies, albeit at different titers (9). We therefore hypothesized that if IgG is the sole supporter of monocyte activation in response to PGN, then the extent of the cytokine response should correlate with the titer of anti-PGN IgG.

To test our hypothesis, we first titered donors via ELISA using PGN coated wells for capturing anti-PGN antibodies, and measured the absorbance at various dilutions of human serum. By using a direct ELISA for anti-PGN activity, we identified two donors containing high anti-PGN IgG titers (NHS#2 and 6) and two donors containing low titers (NHS#5 and 15) (Figure 1A). Paired PBMC samples were stimulated with PGN in the presence of the four sera. Production of TNFα by CD14+ monocytes was measured using intracellular staining and flow cytometry (Figure 1B). Although the presence of the different human sera supported the TNFα response, we observed no difference in TNFα production by CD14+ monocytes despite the difference in anti-PGN titers. These data suggest that while IgG is able to support the TNFα response, IgG is not the sole opsonin in human serum able to do so.

SAP binds to purified B. anthracis PGN in a calcium dependent manner and enhances monocyte TNFα production in response to PGN

An earlier study established that SAP was able to opsonize the lysine type PGN (Lys-type PGN) present in Staphylococcus aureus (10). SAP interaction with the S....
aureus Lys-type PGN and subsequent recognition by Fcγ-receptors supports neutrophil binding and phagocytosis of live S. aureus (10). To assess the ability of SAP to bind to the DAP-type B. anthracis PGN we incubated human serum from healthy donors with PGN (100 μg), washed away non-binding proteins and subsequently immunoblotted for eluted SAP or IgG. We observed that, indeed, SAP was capable of binding B. anthracis PGN and this binding was dependent on calcium (Figure 2A). As a control, we observed no effect of EGTA on the binding of IgG to PGN (Figure 2B). These data show that SAP binds the DAP-type B. anthracis PGN in a calcium dependent manner, as it does the Lys-type S. aureus-derived PGN. We also tested the ability of SAP and of IgG to support monocyte TNFα production. We found that both proteins were able to support TNFα responses in monocytes (Figure 2, C and D). We observed a maximal response for SAP opsonized PGN at 40 μg/ml SAP, and we observed no saturation within the tested concentration range. The IgG supported response was maximal and saturable at 200 μg/ml. All tested concentrations are within circulating blood concentrations in humans and are thus physiologically relevant.

SAP is superior to IgG in supporting monocyte activation in response to PGN

To compare the relative ability of SAP and IgG to support PGN-induced cytokine production, we stimulated paired PBMCs with PGN in the presence of 1% fetal calf sera (FCS) supplemented with either recombinant SAP or IgG for 12 hours. As shown in Figure 3, A-C, we were able to detect a significantly greater percentage of TNFα-positive monocytes stimulated with PGN in the presence of recombinant SAP compared to unopsonized PGN or to IgG opsonized PGN. Not only did more cells produce TNFα in
the presence of recombinant SAP, but the amount of TNFα produced appeared greater as indicated by the fluorescence intensity (Figure 3B). The percentage of TNFα-producing monocytes in response to PGN from a number of similar experiments are shown in Figure 3C. These results indicate that SAP is superior to IgG at supporting PGN-driven TNFα production by monocytes.

We focused on TNFα production as a measure of monocyte activation to compare the relative contributions of SAP and IgG. Nevertheless, we previously identified other cytokines and chemokines that were secreted during whole blood stimulation with PGN (17). Therefore, we investigated the ability of SAP and IgG to support the production of a second representative inflammatory cytokine, IL-6, and the monocyte chemokine macrophage inflammatory protein 1α (MIP-1α) by PBMCs in response to PGN.

Following PBMC stimulation with PGN for 12 hours we observed a superior increase in both IL-6 and MIP-1α in PBMCs stimulated with PGN in the presence of SAP compared to IgG (Figure 3D, E). In line with our TNFα data, these results indicate that SAP is a better supporter of PBMC cytokine and chemokine production in response to PGN compared to IgG.

SAP-induced response to PGN requires phagocytosis and involves Syk, Src and PI3 kinase signaling

Our earlier data showed that PGN-IgG immune complexes required Fc receptor-mediated internalization and lysosomal digestion to elicit a monocyte response. Like IgG, SAP has been shown to bind both human and mouse Fcγ receptors (11, 13). We therefore hypothesized that, like IgG, SAP-mediated monocyte responses to PGN would
be dependent on phagocytosis and involve Fc\(\gamma\)R-dependent signaling pathways. A signaling cascade involving Src family kinases, Syk kinase and phosphatidylinositol 3-kinase (PI3-kinase) promotes the cytoskeletal rearrangements necessary for Fc\(\gamma\) receptor-mediated phagocytosis (18). To assess the role of these signaling mediators involvement in SAP-mediated monocyte responses to PGN, we pretreated PBMCs with the Syk inhibitor piceatannol, the Src inhibitor PP2, the PI3 kinase inhibitor LY294002, or cytochalasin D, a phagocytosis inhibitor. Cells were stimulated for 6 hours with PGN in media containing FCS alone or supplemented with SAP or IgG. We observed that SAP-supported TNF\(\alpha\) production was significantly blocked by inhibition of all three kinases (Figure 4A). These results were similar to IgG-supported responses (Figure 4B), known to use Src, Syk and PI3 kinase (18). These data are consistent with the notion that SAP and IgG rely on a similar Fc\(\gamma\)R signaling cascade to support monocyte responses to PGN, although the results do not establish Fc\(\gamma\)R involvement.

**SAP-induced PGN responses are independent of complement**

Our earlier data showed that PGN was able to activate the complement cascade after IgG opsonization (19). Likewise, SAP was shown to interact with components of the complement pathway (15, 16). We therefore tested whether SAP, like IgG, can support PGN-induced activation of the complement cascade and formation of the C5b-9 terminal complement complex (TCC). For these studies, we generated SAP-depleted serum using DNA-cellulose and found that the depletion process resulted in the partial depletion of C1q (data not shown). We therefore tested SAP-depleted or mock-depleted sera that was reconstituted or not with C1q to its normal level found in circulation (60
μg/ml). The depleted serum was then incubated with PGN and complement activation progressing to TCC was determined using our previously established ELISA protocol (19). We found (Figure 5A) that SAP-depleted serum was able to support PGN-triggered complement activation as well as the control serum, regardless of the depleted C1q. Adding back recombinant SAP or C1q did not change the result. We also used R-1-(6-(R-2-carboxypyrrolidin-1-yl)-6-oxohexanoyl)pyrrolidine-2-carboxylic acid (CPHPC), an inhibitor of SAP (20), to test the effect of SAP inhibition on complement activation in the presence of all serum constituents. SAP binding assays conducted as described in Figure 2 show that CPHPC reduces SAP binding to PGN in a dose-dependent manner (Figure 5B). We then measured TCC formation in serum in the presence or absence of 50 µM CPHPC. We found that CPHPC had no effect on PGN-induced TCC formation (Figure 6C) despite robustly inhibiting SAP-PGN interaction. These findings show that, although SAP binds PGN, it cannot support activation of complement, unlike IgG (19).
Discussion

PGN opsonization by serum factors is important in initiating immune responses to this bacterial PAMP. Here, we investigated the relative ability of SAP and of IgG to promote innate cellular and humoral immune responses to the DAP-type PGN of *B. anthracis*. Our findings reported here indicate that SAP functioned as a serum opsonin of DAP-type PGN and that SAP was superior to IgG in promoting cellular proinflammatory responses in monocytes. However, unlike IgG, SAP failed to support PGN-induced complement activation and formation of the biolytic TCC complex. These findings add to the growing body of evidence that SAP can contribute to the response of the host to systemic infection.

Previous findings showed that SAP could bind to and opsonize the Lys-type *S. aureus*-derived PGN. We utilized *in vitro* assays to evaluate the ability of SAP to bind to the DAP-type *B. anthracis*-derived PGN and to support monocyte activation by PGN. The response supported by SAP was sensitive to cytochalasin D treatment, thus showing a requirement for phagocytosis. Phagocytosis is also required for IgG supported PGN responses (7-9). This is in agreement with a model whereby PGN requires internalization and lysosomal digestion for a response by innate immune cells via NOD1/2 recognition (7).

Our earlier studies on IgG as a PGN opsonin showed that human Fcγ receptors were able to participate in PGN uptake (9, 19). We used Fcγ receptor blocking antibodies to test the involvement of Fcγ receptors in recognizing SAP-opsonized PGN. In our hands, the blocking antibodies we tested did not prevent SAP-opsonized PGN uptake by monocytes despite blocking IgG mediated events. Similar results have been observed in
studies investigating SAP-mediated phagocytosis and cellular adherence (21). SAP- and IgG-opsonized particles engage the Fcγ receptors at somewhat different sites (12). Specific sites at which commercial Fc blocking antibodies bind Fcγ receptors are not clear but it is likely that the antibodies were screened based on IgG blocking and not SAP blocking, possibly explaining their failure to prevent SAP-mediated internalization. Pharmacologic inhibition of kinases known to be required for Fcγ receptor signaling show that both IgG- and SAP-opsonized PGN are sensitive to the same set of kinase inhibitors (Figure 4). While these kinases are not exclusively involved in FcγR signaling, the data is consistent with FcγR involvement by SAP-opsonized PGN. Further studies in cellular models expressing defined receptors as opposed to primary cells used here are required to establish or eliminate FcγR-mediated internalization.

We found that SAP, in the absence of other human serum constituents, is better than IgG at supporting monocyte activation in response to PGN as determined by TNFα production. Furthermore, our multiplex analysis of IL-6 and MIP-1α indicates that SAP is superior at supporting PBMC secretion of other proinflammatory proteins, at least in vitro. The superior ability of SAP over IgG at supporting PGN responses may be due to the carbohydrate component of the PGN molecule. IgG shows a modest affinity for carbohydrate antigens (22) while SAP exhibits high affinity for carbohydrates such as LPS and PGN (10, 23). It may be that in serum, SAP displaces weaker affinity immunoglobulins from this PAMP to better promote cellular innate responses.

Complement is a critical component of innate immune responses to pathogens in the blood and is known to play an important role in sepsis pathophysiology (24-26). We showed that PGN primarily triggers the classical pathway of complement activation with
additional input from the lectin-dependent pathway (19). However, pentraxin-mediated formation of the terminal complement complex C5b-9 was reported to be inefficient relative to IgG (27). Here, we investigated the formation of TCC as a measure of SAP-opsonized PGN to promote complement cascade activation and progression. We found that, unlike IgG (19), neither depletion nor inhibition of SAP had any effect on PGN-induced TCC formation indicating that SAP does not support formation of the TCC. Nevertheless, these data do not exclude SAP interaction with upstream complement components to enhance opsonization of PGN. In fact a recent report shows that SAP interacts with multiple upstream components of the complement cascade (28). Consistent with this latter study we found that depletion of SAP also led to partial depletion of C1q. It is possible that SAP acts as immunomodulatory molecule to drive innate cellular responses at the cost of limiting complement-mediated events, although this requires further investigation.

These findings have led us to refine our previous model in which IgG interacts through Fcγ receptors and complement activation to induce a pro-inflammatory state in monocytes. We now add SAP to this model, as an opsonin that works preferentially to modulate PGN-induced inflammation through phagocytosis and subsequent intracellular processing, to drive the activation of monocytes. While IgG supports the activation of complement in response to PGN, SAP does not support the formation of terminal complement complexes. However, the interplay between SAP and IgG immune functions have yet to be elucidated in an in vivo model and are required to understand the contribution of SAP to sepsis pathophysiology induced by PGN.
Materials and Methods

Materials

Allophycocyanin (APC)-conjugated mouse anti-human TNFα (clone: MAb11), APC-conjugated mouse IgG1 isotype control (clone: P3.6.2.8.1), PE-Cy7-conjugated mouse anti-human CD14 (clone: 61D3), PE-Cy7-conjugated mouse IgG1 isotype control (clone: P3.6.2.8.1), and Brefeldin A were purchased from eBioscience (San Diego, CA). Rabbit monoclonal anti-human SAP (clone: EP1018Y) and rabbit monoclonal anti-human IgG (clone: EPR4421) were purchased from Abcam (Cambridge, MA). Recombinant human-SAP was purchased from BioLegend (San Diego, CA). Human IV immune globulins (IgG) was used as a source of purified human IgG and kindly provided by Grifols Therapeutics (Los Angeles, CA). Ultra low IgG fetal calf serum (FCS) was purchased from Gibco (Waltham, MA). Single-stranded DNA cellulose was acquired from Sigma-Aldrich (St. Louis, MO). Custom Milliplex human cytokine/chemokine magnetic bead kit was purchased from MilliporeSigma (Burlington, MA).

Preparation of Bacillus anthracis PGN

Bacillus anthracis Delta Sterne PGN was prepared using non-pyrogenic labware and endotoxin free water for all solutions as previously described (8, 9). Briefly, 4x500 ml cultures of vegetative bacteria were collected after overnight growth in Trypticase™ Soy Broth and subsequently washed once with endotoxin-free water. The cultures were collected by centrifugation at 15,000 x g for 10 min at 4°C and then resuspended and boiled three times in 8% UltraPure SDS (Invitrogen). Following the final boil, the material was treated twice with DNase/RNase for 30 minutes at room temperature, and
was followed with washes with endotoxin-free water and centrifugation as described above. Hydrofluoric acid was added to the material overnight at 4°C in order to remove any teichoic acid residues. Following hydrofluoric acid treatment, the material was washed and re-suspended in Proteinase K solution overnight at 50°C. Proteinase K digestion was conducted twice followed by washing and subsequent sonication. The now purified PGN was then boiled in 8% SDS, washed and dried. PGN was weighed and re-suspended in endotoxin-free water at 20 mg/ml.

**Anti-PGN titer assay**

ELISA plates were coated at room temperature overnight with 200 μg/ml PGN in carbonate buffer pH 9.6. Following the overnight incubation, plates were washed and blocked with 4% BSA in PBS. Serum from human donors was diluted in blocking buffer and incubated with PGN-coated wells for 1 hour at room temperature. Plates were washed and incubated with 0.5 mg/ml HRP-conjugated mouse anti-human IgG (BD Biosciences) diluted in 1% BSA/PBS for 1 hour at room temperature. The plates were washed and developed with 100 μl/well chromogenic o-phenylenediamine (Sigma-Aldrich) solution for 5-20 minutes followed by acid stop. Absorbance was read at 492 nm with wavelength correction at 650 nm.

**Pulldown assay**

Serum pretreated or not with 2.5 mM EGTA was incubated with PGN in the presence of a protease and phosphatase inhibitor cocktail (Sigma-Aldrich) for 1 hour at 4°C. The opsonized PGN was collected by centrifugation at 20,000 x g for 5 minutes at 4°C.
PGN was washed 3 times with PBS and recovered by centrifugation. PGN was resuspended in NuPAGE LDS sample buffer under reducing conditions (Life Technologies) and bound proteins were eluted by heating for 10 minutes at 70°C. The eluted proteins and PGN were separated by centrifugation as above and the supernatant was saved for analysis.

PAGE and Western Blotting

Equal volumes of eluates from control and experimental conditions were run on NuPAGE Bis-Tris gels according to manufacturer’s protocol (Life Technologies). For Western Blotting, separated proteins were transferred to polyvinylidene difluoride membranes in NuPAGE transfer buffer containing 20% v/v methanol according to the manufacturer’s instructions (Life Technologies). Membranes were washed, blocked for 1 hour with 5% w/v dry milk in TBS containing 0.1% Tween-20, washed again and incubated with primary antibodies overnight at 4°C. Following overnight incubation, membranes were washed, incubated with secondary antibodies for 1 hour at room temperature, washed again and imaged on the Odyssey Imager (Li-Cor Biotechnology). Membranes were stripped and re-probed when necessary.

PBMCs isolation and culture

Peripheral blood from healthy donors was collected by venipuncture into heparinized vacutainers according to a protocol approved by the Oklahoma Medical Research Foundation Internal Review Board. PBMCs were isolated via density gradient centrifugation using Histopaque®-1077 (Sigma-Aldrich) according to manufacturer’s
protocol. PBMCs were washed and reconstituted in RPMI containing FCS (1% v/v) and Glutamax-1 (2 mM). The cells were cultured in the presence of recombinant SAP, IgG, or whole human serum. Cells were stimulated as described in the Results section. Brefeldin A (3.0 μg/ml) was added to inhibit cytokine secretion and Polymyxin B (10 μg/ml) was used to inhibit any LPS contamination. In some experiments, cytochalasin D (15 μM), piceatannol (50 μM), PP2 (1 μM), or LY294002 (50 μM) were added 45 minutes prior to PGN stimulation and kept throughout the experiment.

Flow cytometric analysis

TNFα production in monocytes was assessed by stimulating 4.5-9.0 x 10^5 PBMCs with PGN (10 μg/ml) in 100 μl for 6 or 12 hours at 37°C in the presence of 1% FCS supplemented with either recombinant SAP, IgG, or human serum. Cells were then washed in PBS and blocked with 10% human AB serum (Sigma-Aldrich) in PBS. Cells were fixed, permeabilized and stained with anti-CD14 and anti-TNFα antibodies. Stained cells were then analyzed on a FACSCelesta flow cytometer (BD Biosciences) where a minimum of 5000 events identified by forward and side scatter as monocytes were collected per sample. Paired isotype and unstained controls were used to set up the gating thresholds for each antibody.

Multiplex analysis

The production of IL-6 and MIP-1α was assessed by stimulating 4.5-9.0 x 10^5 PBMCs with PGN (10 μg/ml) in 100 μl for 12 hours at 37°C in the presence of 1% FCS supplemented with either recombinant SAP, IgG, or human serum. The cultures were
collected and centrifuged at 500 x g to remove cells. Supernatants were harvested and stored at -80°C until they could be processed according the manufacturer’s protocol (MilliporeSigma). Processed samples were then analyzed using the BioPlex 200 (Bio-Rad).

Depletion of SAP from human serum

SAP was depleted from fresh serum as previously described (14). Briefly, fresh human serum was incubated twice with DNA cellulose on ice for 1 hour intervals. After incubation, the depleted serum was sterile filtered and stored at -80°C.

Terminal complement complex assay

Pooled SAP-depleted serum or whole serum was diluted once with GVB++ reagent buffer containing 0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.025%NaN₃, and 2 mM CaCl₂. In some experiments, depleted serum was reconstituted with recombinant SAP (40 μg/ml), or SAP was inhibited with 50 µM CPHPC (R-1-(6-(R-2-carboxypyrrolidin-1-yl)-6-oxohexanoyl)pyrrolidine-2-carboxylic acid, Cayman Chemical). Complement activation was initiated with PGN (10 μg/ml). Samples were incubated for 30 minutes at 37°C, then reactions were stopped with 10 mM EDTA. Terminal complement complex formation was assessed using the ELISA method previously described (19).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism software. For all studies, means between groups were analyzed using either a one-way ANOVA, a two-way
ANOVA or a repeated-measures two-way ANOVA with a Bonferroni posttest. A $p$ value of $\leq 0.05$ was considered significant.
Figure 1. Monocyte responses to PGN are not influenced by IgG titer. (A) Serum from 8 individual donors was analyzed for IgG titers as described in the methods section. From these data we identified 2 high titer donors (open shapes) and 2 low titer donors (black-filled shapes) Data shown represents mean ± SEM of sera tested in quintuplicate at each concentration. (B) Paired PBMCs were stimulated with PGN in the presence of serum from either the high or low titer donors. Monocyte responses to PGN were assayed by intracellular staining for TNFα. Titration curve was conducted in quintuplicate. Data shown represents mean ± SD of 3 separate donors. Unless connected by line, significance shown compares to cells stimulated PGN in the presence of FCS only. *p < 0.05, **p < 0.01, ***p < 0.001 Two-way ANOVA with Bonferroni posttest.

Figure 2. SAP binds *B. anthracis* PGN in a calcium-dependent manner and enhances monocyte TNFα production in response to PGN. Human serum was pretreated with or without EGTA and then incubated with PGN for 1 hour at 4°C. PGN was then pelleted by centrifugation and washed. Bound proteins were eluted in LDS sample buffer by heating. (A) Samples were then subjected to PAGE and immunoblotting for SAP. (B) Membranes were then stripped and analyzed for IgG via Western Blot. Images are representative of 3 human donor sera. Subsequently, PBMCs were stimulated with or without PGN (10 μg/ml) in the presence of: 1% FCS, or 1% FCS supplemented with recombinant SAP (1, 5, 10 or 40 μg/ml) (C) or IgG (50, 100, 200 or 400 μg/ml) (D) for 12 hours. Cells were then harvested and stained for CD14 and TNFα. Flow cytometry
was utilized to assess the percent of CD14 cells that produced TNFα. Graphs show mean ± SD of 3 independent donors.

Figure 3. SAP supports better monocyte TNFα responses compared to IgG. PBMCs were stimulated for 12 hours with PGN in the presence of 1% FCS alone or 1% FCS supplemented with either recombinant SAP (40 μg/ml) or IgG (200 μg/ml). (A) Representative plots and gating strategy used to identify TNFα producing CD14+ monocytes. (B) Representative histogram overlay comparing PGN-induced TNFα fluorescence in CD14+ monocytes in the presence of FCS, FCS supplemented with IgG, or FCS supplemented with recombinant SAP. (C). TNFα graph represents mean ± SD of 4 individual donors. (D) Multiplex analysis of IL-6 and MIP-1α concentrations in the supernatant of PBMCs stimulated as described above. Unless connected by line, significance shown compares to cells stimulated PGN in the presence of FCS only. **p < 0.01, ****p < 0.0001, two-way ANOVA (C) or repeated measures two-way ANOVA (D,E) with Bonferroni posttest.

Figure 4. SAP and IgG signaling overlap during PGN stimulation. PBMCs were pretreated for 45 minutes with either cytochalasin D (Cyto, 15 μM), piceatannol (Pic, 50 μM), PP2 (1 μM), LY294002 (LY, 50 μM), DMSO (D) or left untreated. Cells were then stimulated for 6 hours with PGN in the presence of FCS supplement with or without 40 μg/ml SAP (A) or 200 μg/ml IgG (B). Cells were harvested and stained for CD14 and TNFα. Data shown represent mean ± SD of 2 independent donors. *p < 0.05, ** p < 0.01, **** p < 0.0001, ANOVA with Bonferroni posttest.
Figure 5. SAP does not influence PGN-induced formation of the terminal complement complex. (A) Pooled serum from three donors was either unmanipulated (C), mock depleted (Mock) or depleted of serum amyloid P (SAP-D). C1q normalized SAP-depleted serum was reconstituted with or without recombinant SAP and incubated with PGN for 30 minutes at 37°C. Formation of the terminal complement complex was assayed by ELISA. (B) Serum SAP was inhibited with increasing amounts of CPHPC for 30 minutes at room temperature prior to PGN interaction. SAP pulldown was performed as in Figure 2. (C) Serum SAP was inhibited with 50 µM CPHPC for 30 minutes before PGN-dependent complement activation. Formation of the terminal complement complex was assessed by ELISA as in A. Data shown is representative of 3 independent experiments.
Figure 1

A

Absorbance (A590/As30)

0  0.5  1.0  1.5  2.0  2.5

0  2  4  6  8  10

Normal Human Serum (%)

B

CD14+ monocytes (% TNFα)

0  20  40  60  80

Serum # 1  2  5  6  15

No stimulation
PGN (10 μg/ml)

n.s.
Figure 2
Figure 3

A

B

C

D

E

Figure 4
Figure 5

A

![Graph A](image)

No stimulation
PGN (10 μg/ml)

B

![Image B](image)

C

![Graph C](image)
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