Peptidoglycan from *Bacillus anthracis* Inhibits Human Macrophage Efferocytosis in Part by Reducing Cell Surface Expression of MERTK and TIM-3

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ABSTRACT

Bacillus anthracis peptidoglycan (PGN) is a major component of the bacterial cell wall and a key pathogen-associated molecular pattern (PAMP) contributing to anthrax pathology, including organ dysfunction and coagulopathy. Increases in apoptotic lymphocytes are a late-stage feature of anthrax and sepsis, suggesting there is a defect in apoptotic clearance. Here, we tested the hypothesis that *B. anthracis* PGN inhibits the capacity of human monocyte-derived, tissue-like macrophages (MΦ) to efferocytose apoptotic cells. Exposure of $CD206^{+}CD163^{+}$ MΦ to PGN for 24h impaired efferocytosis in a manner dependent on human serum opsonins but independent of complement component C3. PGN treatment reduced cell surface expression of the pro-efferocytic signaling receptors MERTK, TYRO3, AXL, integrin αVβ5, CD36 and TIM-3, whereas TIM-1, αVβ5, CD300b, CD300f, STABILIN-1 and STABILIN-2 were unaffected. Soluble forms of MERTK, TYRO3, AXL, CD36, and TIM-3 were increased in PGN-treated supernatants, suggesting involvement of proteases. ADAM17 is a major membrane-bound protease implicated in mediating efferocytotic receptor cleavage. ADAM17 inhibitors TAPI-0 and Marimastat abolished TNF release, indicating effective protease inhibition, modestly increased cell-surface levels of MerTK and TIM-3 but only partially restored efferocytic capacity by PGN-treated MΦ. We conclude that human serum factors are required for optimal recognition of PGN by human MΦ and that *B. anthracis* PGN inhibits efferocytosis in part by reducing cell surface expression of efferocytic receptors.

KEY WORDS: *Bacillus anthracis,* efferocytosis, peptidoglycan, MerTK, ADAM17

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax infection, is a large, spore-forming and toxin-producing gram-positive organism that typically infects herbivorous animals. Humans can be infected through inhalation, ingestion or cutaneous exposure to spores. Accidental inhalation of spores by humans can lead to rapid and highly fatal infection[1]. Latent spores migrate to and germinate in the mediastinal lymph nodes, followed by progression to fulminant infection characterized by high levels of circulating bacteria and sepsis-like features[2]. Bacterial sepsis is a leading cause of mortality in the United States, and has limited treatment options[3]. Accumulation of uncleared apoptotic lymphocytes is a known hallmark of sepsis[4], and is accompanied by increased levels of circulating nucleosomes[5]. Circulating nucleosomes can be released from uncleared apoptotic cells that progress to secondary necrosis[6], promoting micro- and macro-vascular leakage[7] and contributing to organ dysfunction[8].

Peptidoglycan (PGN) is a major component of the cell wall in Gram-positive bacteria and has been shown to be a significant factor in anthrax-mediated sepsis, contributing to vascular and organ damage and mortality in non-human primate models[9, 10]. Response to PGN was initially thought to be Toll-like receptor 2 (TLR2)-dependent[11]; however, recent work has challenged this view and showed that impurities from lipoteichoic acid (LTA) result in TLR-2 activation[12]. Further work elucidated that highly purified PGN preparations lacking LTA are recognized intracellularly by Nucleotide-binding Oligomerization Domain (NOD), not at the surface by TLR's[13]. An additional controversy is how human immune cells recognize and respond to PGN. An abundance of literature using rat or mouse cells showed that PGN is recognized at the cell surface via TLR2; however, more recent work using human serum factors challenge these early findings and instead show that human monocytes recognize polymeric PGN optimally in the presence of human serum opsonins[14, 15].

The clearance of apoptotic cells, termed efferocytosis, is thought to be mediated by tissue-resident or secondary lymphoid organ macrophages (MΦ)[16-18]. Highly efferocytic MΦ tend to express an M2-like, or alternative activation program[19, 20], and in tissues these cells express CD206 and CD163[21]. Highly efferocytic MΦ can be modeled *in vitro* by polarization with IL-10, IL-4 and/or dexamethasone[22, 23]. Defects in efferocytosis can promote inflammation in sepsis by increasing the levels of circulating nucleosomes and other host damage-associated molecular patterns (DAMPs), which get released by uncleared apoptotic cells that progress into secondary

necrosis[24]. The act of efferocytosis also imparts the efferocytosing MΦ with anti-inflammatory properties that actively dampen inflammation[25].The recognition of apoptotic cells is a complex process involving both tethering receptors that bind directly to phosphatidylserine on apoptotic cells or those that require soluble phosphatidylserine-binding bridge proteins[26]. Currently, there are at least twelve well-established receptors that signal for apoptotic cell engulfment including TYRO3, AXL, MERTK, integrins αVβ3 and αVβ5, CD300b, CD300f, STABILIN-1 and 2, TIM-1 and -3, and BAI-1[27]. Of these, TYRO3, AXL, and MERTK require GAS6, Protein S or GAL-3, and integrins αVβ3 and αVβ5 require MFGE8; the remaining seven receptors directly bind to apoptotic cells[27, 28].

Mechanisms regulating efferocytosis by human MΦ are poorly understood. We previously showed that *Bacillus anthracis* Edema Toxin, an adenylate cyclase that induces supraphysiologic levels of cAMP, impairs human M2-like MΦ efferocytosis through inhibition of Rac1 activation and altered phosphorylation of proteins involved in actin reorganization[29]. Efferocytic receptors are thought to be regulated at the cell surface by metalloproteases, and previous work has shown that TYRO3, AXL, MERTK, TIM-1 and TIM-3 can be cleaved from the cell surface of mouse and human MΦ[30]. Loss of these receptors has been shown to correlate with various disease phenotypes and may be useful as biomarkers in multiple diseases[31, 32]. ADAM17 is a key metalloprotease responsible for cleaving more than 80 substrates, including TNF and MERTK, and has been implicated as a regulator of efferocytosis [33, 34]. Herein, we demonstrate that recognition of *Bacillus anthracis* PGN by human MΦ is enhanced by human serum factors and that PGN inhibits human MΦ efferocytosis in part by promoting receptor shedding of MERTK and TIM-3.

METHODS

Differentiation of monocytes to M2-like MΦ

On Day 0 human mononuclear cells were isolated from fresh buffy coats using Lympholyte (Cedarlane labs, Burlington, Canada) according to the manufacturer's protocol (see **Supplemental Figure 1** for timeline of the assay). Following isolation, PBMC were counted and plated to 100mm petri dishes (Corning, NY, USA, 100mm x 20mm non-treated tissue culture dishes, Cat#: 430591) at $4x10^6$ cells/mL in 25mL per dish in IMDM medium with no other additives. Cells were allowed to adhere for 1hr at 37°C in 5% CO_2, followed by gentle removal of media and replacement with 20mL/dish of complete medium (Iscove's Modified Dulbecco's Medium with glutamate, 10% hiFBS, 5mM PenStrep antibiotic) supplemented with 50ng/mL M-CSF

(PeproTech, Rockyhill NJ, USA, Cat#: 300-25). On Days 2 and 5, half of the media was exchanged with fresh media containing 100ng/mL M-CSF to replenish spent M-CSF (final M-CSF concentration was 50ng/mL). On Day 6 MΦ were polarized to a tissue-like/alternative phenotype by adding 10nM dexamethasone and culturing for 24hrs (Dexamethasone, Sigma Aldrich, St. Louise, MO, USA, Cat#: D4902). All polarized MΦ expressed CD206 and CD163, as we previously published[29].

PGN Treatment

On Day 7, 24hrs after polarization with dexamethasone, MΦ were re-plated to 12-well Nunc UpCell plates (Thermo Fisher Scientific, Waltham, MA, USA, Cat#: 150200). Briefly, 3mL trypsin/EDTA solution (Sigma Aldrich, Louise, MO, USA, Cat#: T4049-100) was added to each petri dish and incubated at 37°C in 5% CO_2 for 10 min to detach cells. After detachment, 10mL complete medium (containing 10%FBS) was added to neutralize trypsin activity. MΦ were pelleted at 1200 rpm for 5 min, counted and re-plated to the UpCell plates at $0.5x10^6$ cells/mL. After re-plating, MΦ were incubated for 16 or 24hrs with 10µg/mL highly purified *Bacillus anthracis* PGN[13] (same isolation batch was used for all experiments), in media supplemented with 50ng/mL M-CSF and various serum additives as follows: 10% heat-inactivated fetal bovine serum (hiFBS), 10% heat-inactivated human serum (hi huS), 10% non-hi huS, or 10% non-hi huS + 200ug/mL Compstatin). Human serum used for all conditions was from the same lot of pooled human AB serum (Fisher Scientific, Waltham, MA, Cat#: BP2525-100). For heat inactivation, serum was placed in a 56°C water bath for 30 min and stored in -20°C until use. Compstatin was purchased from Selleck chemical, Sylvanville TX, USA, Cat#: S8522. For ADAM17 inhibition, MΦ were treated with 10µg/mL PGN and concomitantly with 30µM of TAPI-0 or Marimastat (Tocris, McKinley Place, MN, USA, cat#'s: 5523, 2631).

Preparation of Apoptotic Neutrophils

On the morning of Day 8, human neutrophils (PMN) were isolated by immunomagnetic negative selection from healthy donor buffy coats (EasySep Direct Human Neutrophil isolation kit, Cat#:19666). After isolation and counting, PMN were resuspended to $5x10^6$ cells/mL in IMDM without additives, labeled with eFluor670 according to manufacturer's protocol (Thermo Fisher, Cat#: 64-0840-90), then exposed to 500 mJ/cm^2 UV irradiation for 1.5 min to induce apoptosis. Irradiated PMN were then placed in a 37°C incubator with 5% CO_2 for 4h. After 4hrs, an aliquot of PMN was stained with Annexin-V and PI (Annexin-V Apoptosis Detection kit, eBiosciences,

Cat#:88-8005-74) and assessed for the frequency of Annexin V^+/PI^- early apoptotic PMN by flow cytometry. All apoptotic cell preparations contained ≥70% early apoptotic cells.

Efferocytosis Assay

On Day 8, labeled apoptotic PMN were co-incubated with MΦ to assess efferocytosis. Prior to adding apoptotic PMN, supernatants from cultured MΦ were replaced with 0.5 mL/well of IMDM without serum. The collected supernatant was stored at -80°C until use for detection of secreted cytokines. After 4hrs, irradiated e-fluor670-labeled PMN were added to UpCell plate-containing MΦ at a 5:1 apoptotic PMN:MΦ ratio and incubated for 1hr ($2.5x10^6$/mL PMN:$0.5x10^6$/mL MΦ). After 1hr, UpCell plates were gently washed two times using 1mL PBS per wash to remove unbound PMNs. MΦ were rested at room temperature for 10 min. to allow detachment from UpCell plates. To ensure complete removal, wells were washed once with 1 mL PBS.

Flow Cytometry

MΦ ($0.5x10^6$/tube) were stained for flow cytometry in 5mL round-bottom polystyrene tubes (Corning, NY, USA, Cat#: 352058). After pelleting the cells at 1200rpm for 5 min, they were incubated for 10 min at room temperature with a combined Fc block solution (2μL Human Fc block [BD Biosciences, San Jose, CA, USA] + 3μL heat-inactivated human AB serum [Fisher Scientific, Waltham, MA, Cat#: BP2525-100] + 5uL Brilliant Stain buffer [Thermo Fisher, Waltham, MA, Cat#: 00-4409-42]) per tube. Primary antibodies were then added and, after gentle mixing, incubated for 25 min on ice. After washing the cells with FACSWash (2% FBS, 1mM EDTA in PBS), unconjugated antibodies were detected with secondary PE-conjugated antibody for 20 min on ice. After staining all samples were washed with 2mL FACS Wash and fixed in 1% paraformaldehyde in 0.15M NaCl, pH 7.4 until analysis. Data was collected using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar, Ashland, OR, USA, version 10.7.1). Number of events recorded were: 20,000/treatment for receptors and 40,000/treatment for efferocytosis assay. Antibodies and clones included in the efferocytosis cocktail were PE-conjugated anti-human CD11b (BioLegend, San Diego, CA, USA, clone ICRF44), APC-Cy7-conjugated anti-human CD163 (BioLegend, GHI/61), PerCp-Cy5.5-conjugated anti-human CD206 (BioLegend, 15-2), AlexaFluor-488-conjugated anti-human CD64 (BioLegend, 10.1), and BV421-conjugated antihuman CD66b (BD Biosciences, G10F5). For detecting surface receptor expression, all efferocytic receptors were PE-labeled as follows: DTK/TYRO3 (R&D Systems, clone 96201), AXL (R&D Systems, 108724), MERTK (R&D

Systems, 125518), αVβ3 (R&D Systems, 23C6), or αVβ5 (R&D Systems, P5H9), CD300f (R&D Systems, UP-D2), CD36 (BioLegend, 5-271). Additional receptors were only available unconjugated and purchased from R&D Systems: anti-human CD300b (polyclonal goat IgG, cat#: AF2879) and stained with secondary antibody (PE-conjugated anti-goat IgG, cat#: F0107). Anti-human STABILIN-1 and anti-human STABILIN-2 primary antibodies (both polyclonal sheep IgG, cat#: AF3825, cat#: AF2645) were stained with a PE-conjugated secondary antibody (polyclonal anti-sheep IgG, cat#: 5-001-A). Receptor expression was quantified using mean fluorescence intensity (MFI) for each respective treatment group.

Measurement of soluble analytes

Supernatants from the PGN-treated MΦ (24hrs in non-hi huS) were tested for soluble analytes (TYRO3, AXL, MERTK, CD36, CD366 (TIM-3), TNF, IL-10 and GAS-6) using a custom Procarta 8-plex Luminex assay from Thermo fisher (Waltham, MA, USA). Samples were diluted 1:10 in assay buffer and tested according to the manufacturer's protocol. Results were read on a BioPlex 200 instrument (BioRad, Hercules, CA, USA).

Data analysis

All data are presented as mean ± SEM, with the number of independent donors indicated in the figure legends. Results were analyzed by: one-way or two-way ANOVA, or a mixed-comparisons analysis followed by a Dunnett's or Sidak's multiple-comparison post-hoc test ($p < 0.05$), a paired t-test, or a non-linear fit model as indicated in the figure legends. Graphs and statistics were generated in GraphPad Prism (GraphPad Prism, version 9.2.0, San Diego, CA, USA). * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.0001$.

RESULTS

Non-complement human serum factors enhance human MΦ recognition of *Bacillus anthracis* PGN

To evaluate whether human serum opsonins are important for MΦ responses to peptidoglycan (PGN), we tested primary human MΦ for their capacity to produce cytokines in response to PGN in the presence of various serum conditions. We evaluated TNF and IL-10 production following exposure to 10µg/mL purified *B. anthracis* PGN in the presence of the following: heat-inactivated bovine serum (hi FBS), heat-inactivated human serum (hi huS), non-inactivated human serum (non-hi huS), and non- inactivated human serum pre-treated with Compstatin, a specific inhibitor of C3b-mediated opsonization (non-hi huS+Comp). We observed negligible production of TNF

and IL-10 in PGN-treated MΦ in the presence of hiFBS; however, in the presence of human serum PGN induced large increases in both TNF and IL-10 concentrations in supernatants (**Figure 1A**).

Bacillus anthracis PGN inhibits human MΦ efferocytosis of apoptotic PMN

We next tested whether exposure of M2-like MΦ to PGN impacts their capacity to efferocytose human apoptotic PMN. MΦ were pre-treated with PGN for 24hrs in the presence of the various serum conditions shown in Figure 1, then co-incubated with primary apoptotic PMNs for one hr, followed by assessment of efferocytosis using flow cytometry. Consistent with a human serum opsonin requirement for optimal recognition of PGN by human MΦ, efferocytosis was modestly impaired in the presence of hiFBS but profoundly impaired in the presence of human serum conditions (**Figure 1B, C**). Efferocytosis was slightly reduced in the presence of hi huS compared to non-hi huS (32 ± 4.9 SEM vs, 56.3 ± 5.2 SEM; p=0.0005), but inhibition of C3 had no effect (non-hi huS+Comp, 55.6 ± 5.4 SEM; p=0.99), indicating that the effect of heat inactivation is due to denaturation of opsonins other than C3 (**Figure 1D**). We also performed a dose-response assay to find the 50% inhibitory concentration (IC_{50}) of PGN on efferocytosis using MΦ from three representative donors **(Supplemental Figure 2)**. After extrapolating the IC_{50}, PGN elicited similar responses in both non-hi huS and non-hi huS + Compstatin in its capacity to inhibit efferocytosis (IC_{50} = 1.46µg/mL vs 1.49µg/mL), reinforcing the lack of contribution of complement component C3 in our system.

PGN treatment reduces cell surface expression of efferocytic signaling receptors through proteolytic cleavage

Next, we investigated the cell surface expression of key efferocytosis signaling receptors after 10µg/mL PGN treatment for 24hrs in the presence of hiFBS, hi huS and non-hi huS. A total of thirteen efferocytosis receptors were measured, and significant changes in MFI were found for six of them, including TYRO3, AXL, MERTK, Integrin αVβ5, TIM-3, and CD36 (**Figure 2A-D**). The remaining seven receptors were expressed at low levels or were unchanged following PGN treatment (**Supplemental Figure 3A-D**). All of the significantly reduced cell surface receptors except integrin αVβ3 have been shown in other settings to be regulated via receptor cleavage[35]. Next, we measured the levels of soluble receptors in culture supernatants following PGN treatment and found increased levels of five of six downregulated receptors, demonstrating their cell surface cleavage (**Figure 3A, C**). Additionally, the soluble bridge protein GAS6, as well as TNF and IL-10, were all elevated in culture supernatants of PGN-treated MΦ. (**Figure 3B, C**). Although we observed an inverse correlation between TNF and percentage of MΦ containing

apoptotic PMN (**Figure 3D**), inhibition of TNF failed to prevent the PGN-induced efferocytic defect (**Supplemental Figure 4**). Overall, PGN reduced the cell surface expression of six key efferocytosis receptors on human M2-like MΦ and increased their soluble forms, suggesting involvement of membrane-bound proteases.

Blockade of ADAM17 abolished TNF release but only modestly preserved efferocytic receptor expression and efferocytic capacity

A Disintegrin And Metalloproteases (ADAMs) are membrane-bound metalloproteases that act as sheddases to cleave various receptors from the cell membrane[36]. In particular, ADAM17 is one of the well-studied ADAM family members and is known to cleave a multitude of receptors including TYRO3[37], AXL[38], MERTK[39], CD36[40] and TIM-3[41]. Regulation of ADAM17 activity is complex, and is thought to be dependent on its localization within the cell, specifically its trafficking within the cell[42, 43]. Using two small molecule inhibitors of ADAM17, TAPI-0 and Marimastat, we evaluated if blocking ADAM17 could restore efferocytosis and receptor expression. Though TAPI-0 and Marimastat follow a similar mechanism of inhibition, by chelating zinc ions in the active site, TAPI-0 is not membrane-permeable and inhibits ADAM17 at the cell surface, while Marimastat can permeate cellular membranes[44]. First, we titrated TAPI-0 (1, 30, 60μM, IC_{50} = 0.1μM) and found no difference among the three conditions (**Supplemental Figure 5**). Next, we tested either inhibitor alone, or in combination, and observed partial increases in MERTK and TIM-3 and a minor, though statistically significant increase of efferocytosis (**Figure 4A, B**). We also tested supernatants from the same assays for efficacy of ADAM17 inhibition by monitoring levels of TNF. Following treatment with PGN, supernatants from cultures containing ADAM17 inhibitors showed substantially reduced TNF levels when either inhibitor was used alone, and TNF was undetectable in nearly all donors when used in combination (**Figure 4C)**, indicating robust ADAM17 inhibition.

DISCUSSION

While *Bacillus anthracis* produces toxins that enable the pathogen to replicate to high numbers[45], PGN is an important factor that contributes to late sepsis-like pathology[9, 10]. During sepsis and other forms inflammation, there is an accumulation of uncleared apoptotic cells and host DAMPs including nucleosomes[46, 47], suggesting potential defects in efferocytosis. We previously showed that *B. anthracis* edema toxin can inhibit efferocytosis by human M2-like MΦ[29]. In the present study, we investigated the effect of PGN on human MΦ efferocytosis. Herein, we report that PGN decreases efferocytosis in a manner that is dependent on the presence of human serum. We also provide evidence that PGN recognition is mediated by a process independent of complement, as

PGN-induced TNF production was equivalent in the presence of hi huS compared to non-hi huS. In contrast, efferocytosis was slightly less efficient in the presence of hi huS, and this could reflect denaturation of efferocytic bridge proteins present in human serum, C1q, or both, as efferocytosis was unaffected by inhibition of the complement component C3. These findings agree with previous reports showing that other human immune cells, including monocytes and neutrophils, generally require human serum opsonins for effective responses to PGN[14, 48, 49], supporting a requirement for internalization of polymeric PGN for eliciting responses from human myeloid cells. However, human MΦ did not require C3, which differs from human PMNs[50].

To investigate potential mechanisms leading to defective efferocytosis, we measured cell-surface expression of twelve key receptors that have been shown to signal for apoptotic cell engulfment. Of these receptors, eleven comprise multiple families: TAM (TYRO, AXL, MERTK), TIM (TIM-1, TIM-3), Integrin ($\alpha V\beta 3$, $\alpha V\beta 5$), CD300 (CD300b, CD300f), and Stabilin (STABILIN-1, STABILIN-2), as well as the thrombospondin receptor, CD36. Remarkably, the cell surface expression levels of six of these receptors were significantly reduced following 24hrs of exposure to *B. anthracis* PGN (TYRO3, AXL, MERTK, TIM-3, and CD36), suggesting that downregulation of these receptors might be responsible for PGN-mediated defects in our model. Though BAI-1 is considered to be an efferocytosis signaling receptor in mice[51], a recent publication failed to find expression of this receptor at the protein or RNA level in human MΦ; therefore, we excluded this receptor from our initial screening[52].

One known mechanism of receptor regulation, common to multiple efferocytosis receptors, is enzyme-mediated receptor shedding. In humans, ADAM17 is known to cleave multiple efferocytosis receptors, resulting in their soluble forms[30]. These soluble receptors can prevent efferocytosis in two ways: i) they act as decoy receptors to bind phosphatidylserine sites to inhibit macrophage receptor binding and ii) they directly reduce MΦ binding to apoptotic cells due to lower efferocytic receptor expression. Consistent with *B. anthracis*-induced PGN-induced metalloprotease activity, we observed significant increases in the soluble forms of TYRO3, AXL, MERTK, CD366/TIM-3, and CD36, demonstrating that *B. anthracis* PGN induces their cleavage. We also noted significant increases in two pleotropic cytokines, TNF and IL-10. This differs from results by Michalowski et al, who showed that exposure of unpolarized human MΦ to lipopolysaccharide (LPS) resulted in TNF-dependent defective efferocytosis and opposing secretion profiles of TNF and IL-10[53]. Our work revealed that TNF was not responsible for *B. anthracis* PGN-induced defective efferocytosis by human M2-like MΦ. This was evident from data

showing that both ADAM17 inhibition, where TNF release was essentially abolished, and TNF neutralization failed to substantially restore efferocytosis in PGN-exposed MΦ. Michalowski et al. pretreated non-polarized MΦ with LPS for an extended period of time (120hrs), resulting in much higher TNF levels; thus, differences between the studies may be attributable to differing MΦ activation states and differences in TNF levels.

To block ADAM17, we used two pharmacologic inhibitors, TAPI-0 and Marimastat, and demonstrated partial restoration of TIM-3 and MERTK cell surface expression levels that was accompanied by a partial increase to efferocytosis. Both TAPI-0 and Marimastat are broad-spectrum inhibitors, affecting ADAM17 as well as several matrix metalloproteases (MMPs 1,3,9 and13). Despite good coverage of multiple MMPs and nearly complete ablation of ADAM17-mediated TNF release, we did not see a return to baseline efferocytosis. Therefore, this study suggests the existence of additional mechanisms for downregulation of these receptors and for *B. anthracis* PGN-mediated impairment of efferocytosis, some of which may be transcriptional.

Further studies are required to determine whether other bacterial PGNs can inhibit the capacity of human MΦ to engulf apoptotic cells. PGNs are present in the cell walls of both Gram+ and Gram^{-} bacteria, though the PGN layer is much thicker in Gram+ species. There is structural heterogeneity among bacterial PGNs that depend on variations in their stem peptides and post-synthetic modifications of their glycan strands[54] that results in variable biologically-active breakdown products that in turn alter downstream signaling and functional outcomes[55-57].

In summary, we report that PGN from *Bacillus anthracis* inhibits efferocytosis by human M2-like MΦ and that human serum is important for eliciting maximal responsiveness. We also demonstrate that PGN-mediated defects are partially due to receptor cleavage of MERTK and TIM-3 by ADAM17. We speculate that other mechanisms in addition to metalloprotease-mediated shedding of efferocytic receptors contribute to the capacity of *B. anthracis* PGN to inhibit human MΦ efferocytosis, and these are currently under investigation in our laboratory. Overall, these data show that bacterial factors can affect the capacity of MΦ to efferocytose and provide a new explanation for the accumulation of uncleared apoptotic cells in the context of late stage anthrax infection.

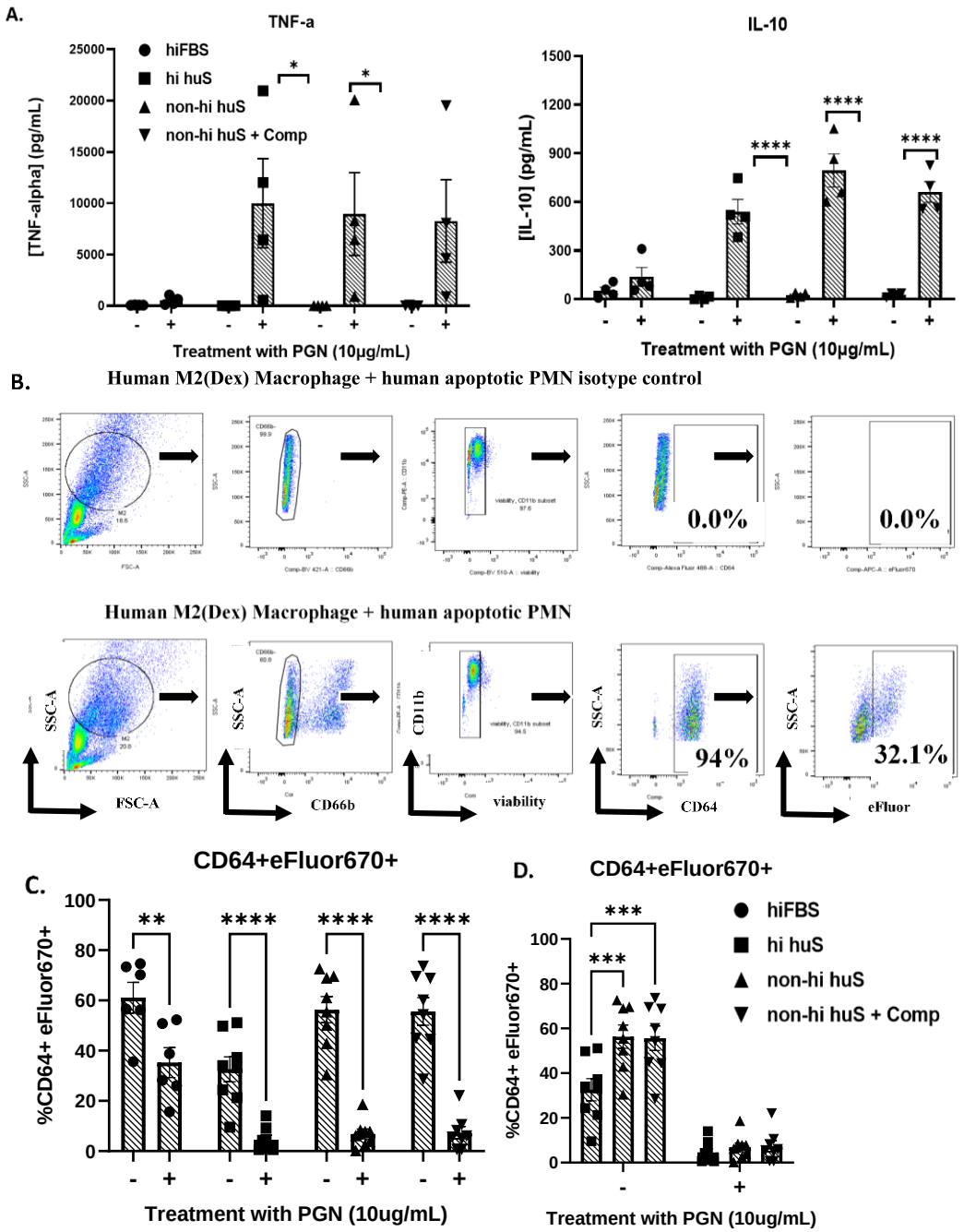

Figure 1: Bacillus anthracis peptidoglycan (PGN) inhibits efferocytosis in the presence of human serum. (A) ELISA for TNF and IL-10 (24hr-treated MΦ, in various serum conditions) from 4 independent donors. (**B)** Gating strategy for flow cytometric evaluation of efferocytosis **(C)** Percentage of CD64+ MΦ containing apoptotic eFluor670-labeled PMN from the indicated serum conditions in the presence of absence of 10ug/mL PGN from ≥5 independent donors analyzed by two-way ANOVA with Sidak's multiple comparison. **(D)** Alternative display of human serum condition data from Figure 1C to demonstrate hihuS has lower efferocytosis compared to the other human serum in untreated MΦ. In addition, inhibition of efferocytosis by PGN did not change with the serum's condition.

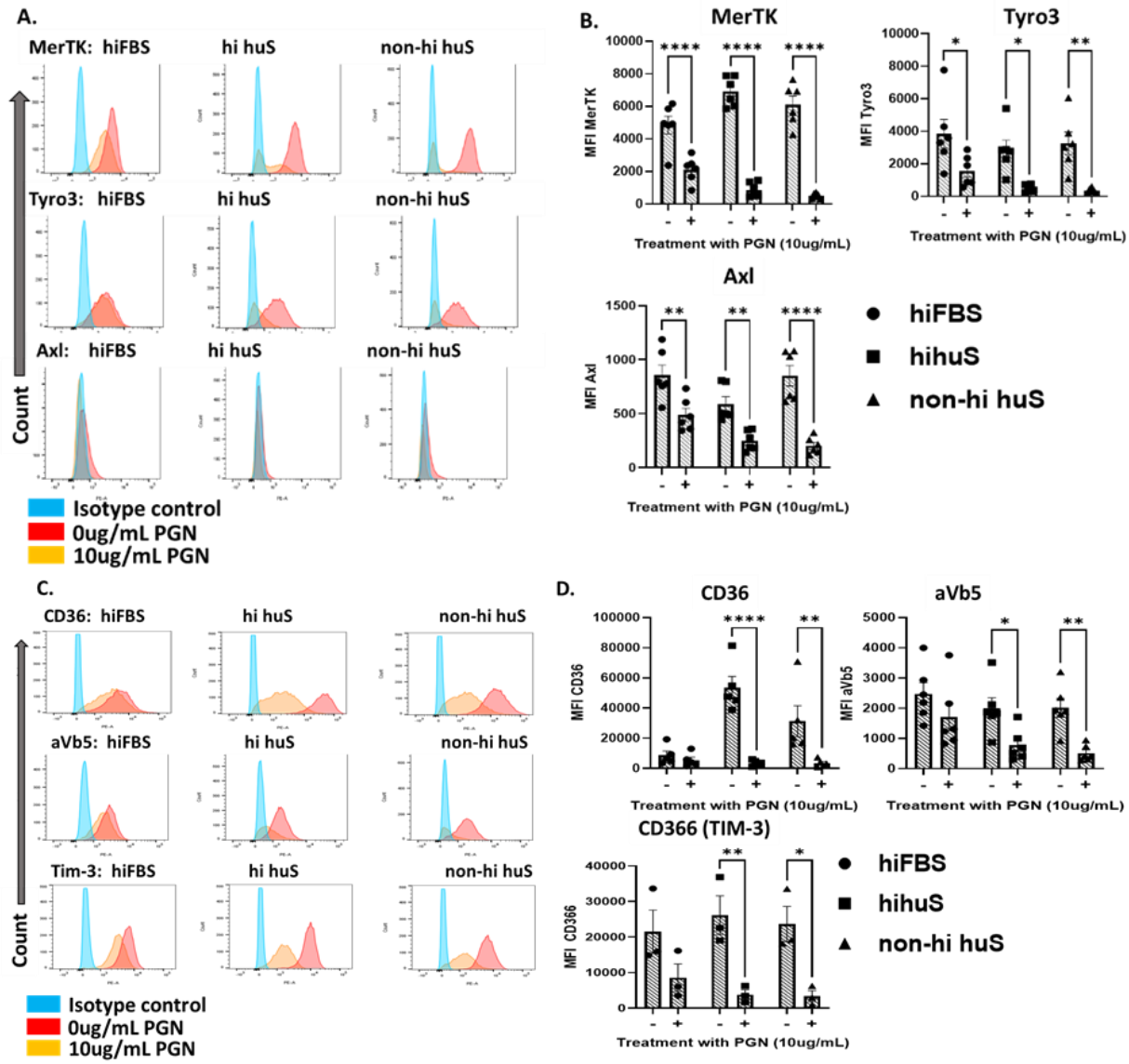

Figure 2. Human M2-like MΦ reduce efferocytic receptor expression in response to PGN. (A, C). Representative histograms of receptor expression. **(B, D)** MFI of efferocytosis receptors from the indicated serum conditions from ≥3 independent donors analyzed by two-way ANOVA with Sidak's multiple comparison.

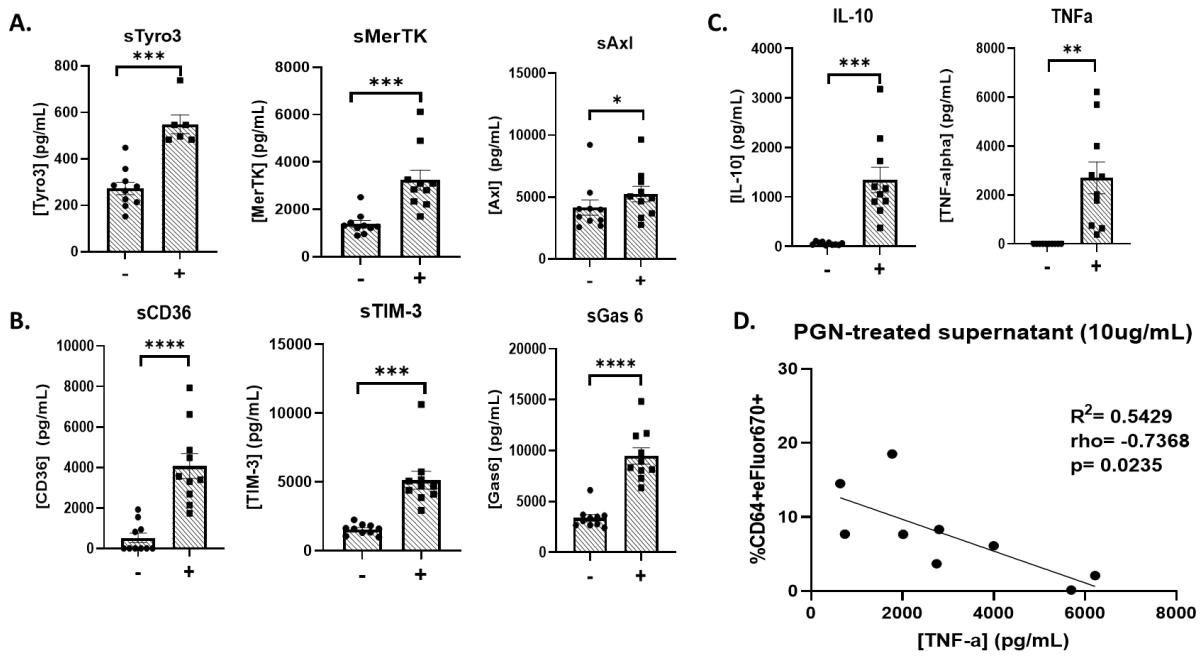

Figure 3. PGN-treated supernatants have increased levels of soluble efferocytic receptors. A) Concentrations of soluble TAM family members. **B)** Concentrations of other soluble efferocytic receptors. **C)** Concentrations of IL-10 and TNF. **D)** Correlation between %efferocytosis (from same donors in Figure 1A) and concentrations of TNF. All analytes were measured from 24hr PGN-treated supernatants in the non-hi huS serum condition from 9 independent donors. All analytes were measured by Luminex assay and analyzed by paired t-tests. Panel D shows a Pearson correlation.

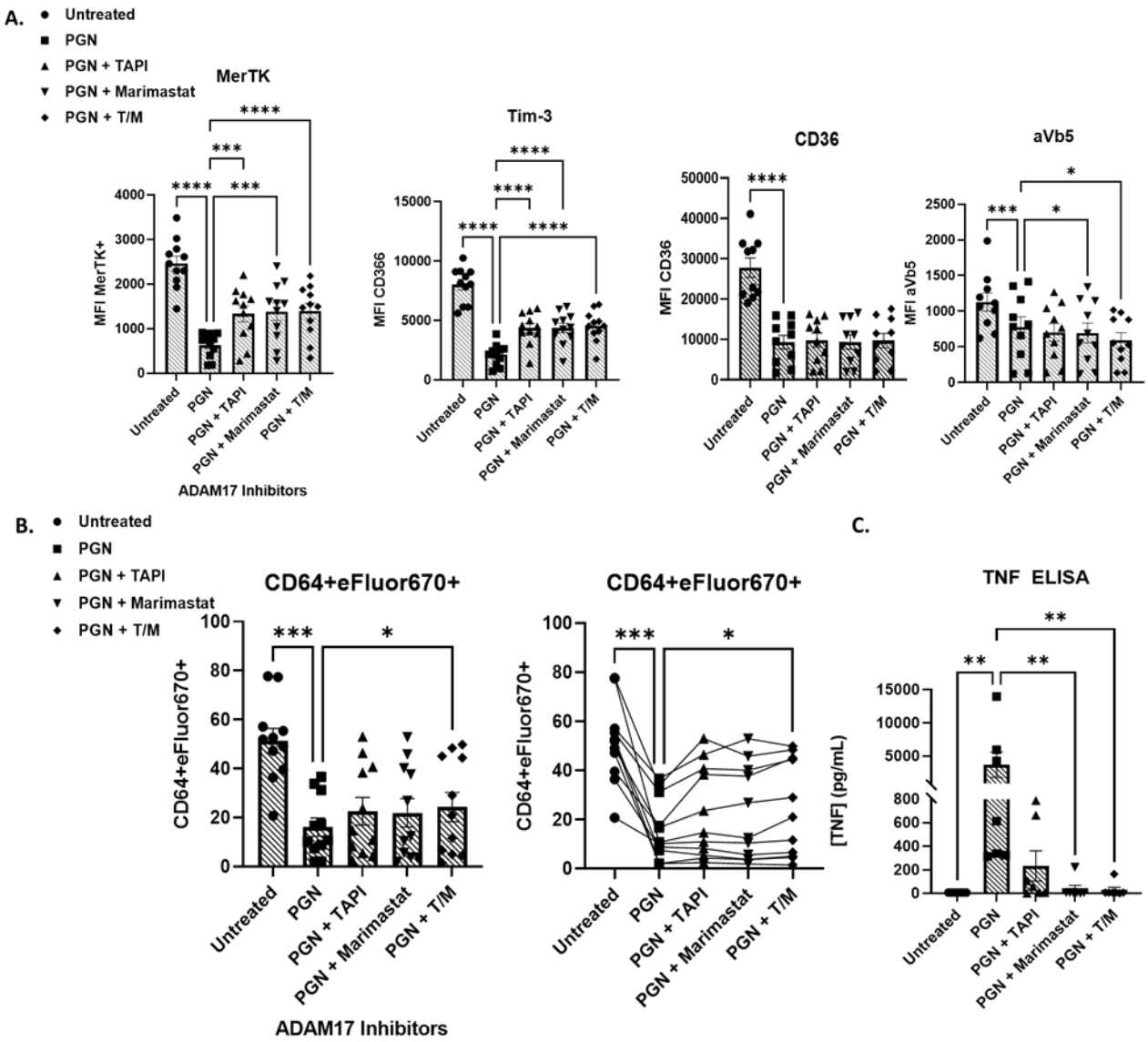

Figure 4: Pharmacological inhibition of ADAM17 partially restores surface receptor expression and efferocytic capacity. A) Levels of soluble efferocytosis receptors following 16hrs of treatment with 10µg/mL PGN alone or in combination with ADAM17 inhibitors. **B)** Mean % efferocytosis (left) or an alternative display of the data showing the same donor tracked across each treatment group (right). **C)** TNF ELISA from donor supernatants. Data are from ≥ 7 independent donors after 16hr pre-treatment with 10µg/mL PGN in the non-hi huS serum condition. A and B are paired data from same donor s (n=11).

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AUTHOR CONTRIBUTIONS

JSM, ZP, and ADF conceived, designed, and supervised the experiments. JSM, ZP and JZ collected and analyzed the data. NP contributed to the reagents and materials (*B. anthracis* PGN isolation and validation of purity). JAJ and NR contributed reagents and materials (healthy donor recruitment and blood collection). CLD and CL isolated PMN and monitored apoptosis by flow cytometry. JSM, ZP and ADF wrote the manuscript. All authors revised the manuscript and approved the final version.

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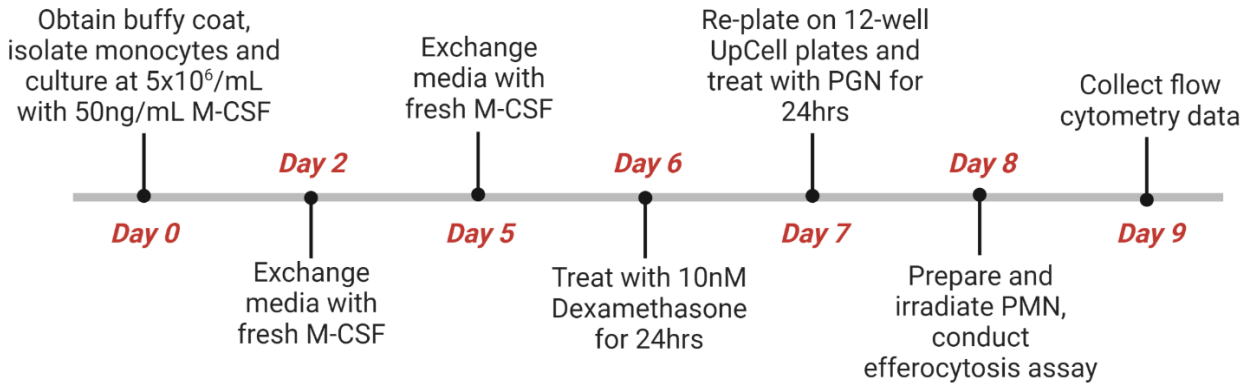
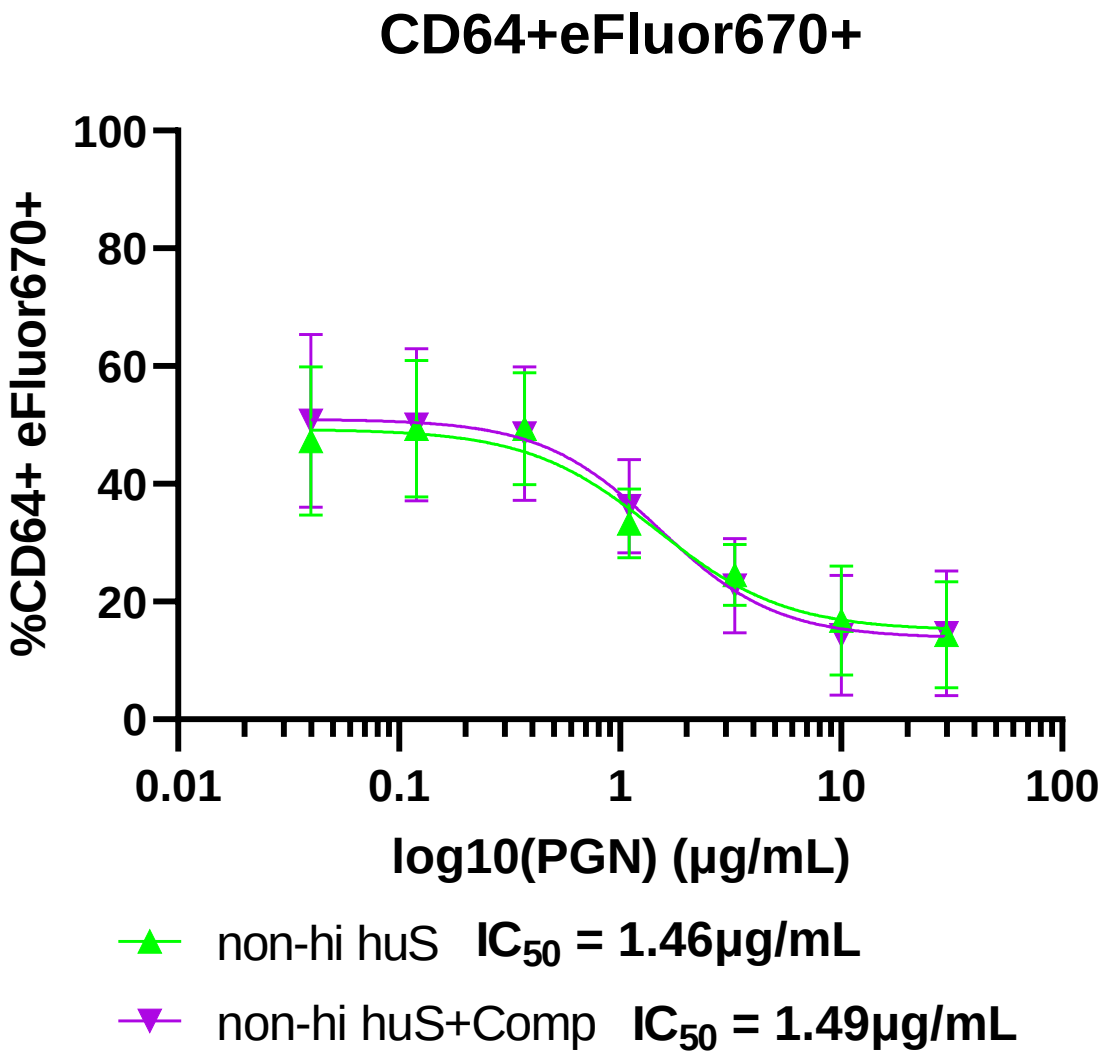
CONFLICTS OF INTEREST

The authors have no conflicts to disclose.

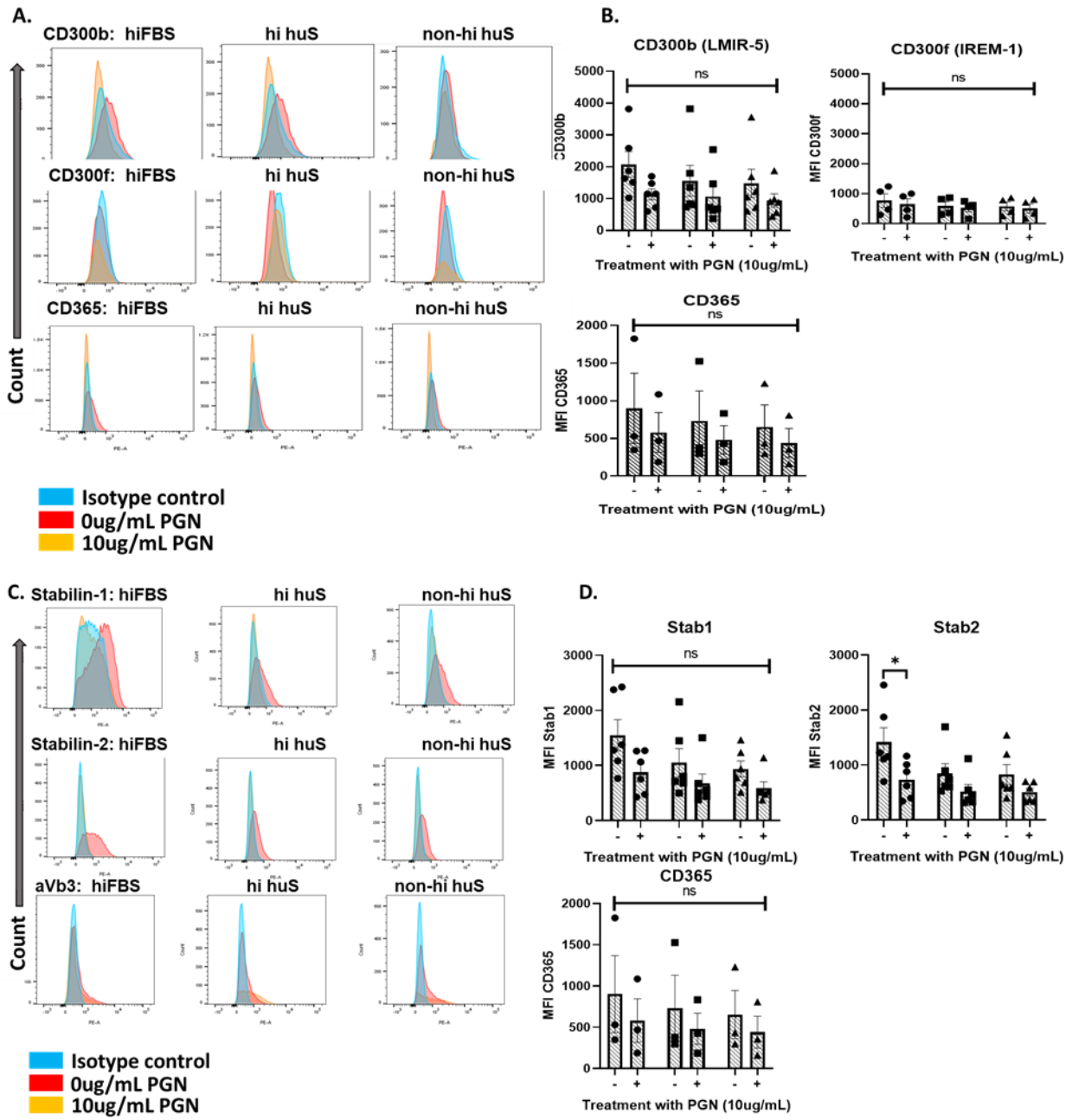
SUPPLEMENTAL FIGURES



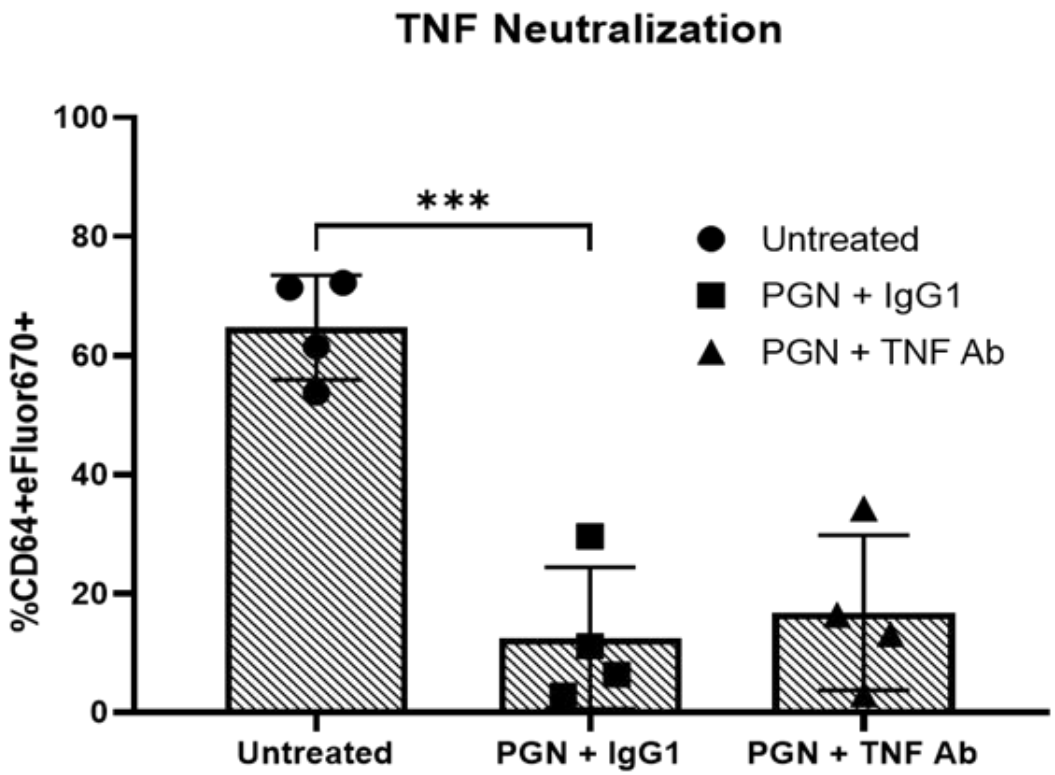
Supplemental Fig 1: Overview of experimental setup. On Day 0 MΦ were isolated from buffy coat donors, adhered to plates for 1h, and cultured with M-CSF. On Day 2 and Day 5 half of the media was replaced with fresh complete media supplemented with 2x M-CSF to ensure differentiation. MΦ were polarized with Dexamethasone on Day 6, and 24hrs later, were re-plated onto 12-well UpCell plates and treated with various treatments depending on the experiment. On Day 8 PMN were isolated, irradiated, and co-cultured for MΦ for 1hr and subsequently stained for flow cytometry. PGN-treated supernatant was removed prior to PMN co-culture. On Day 9 stained samples were run on the LSRII and data was analyzed using Flow Jo. Figure was created using Biorender, Template (9 Segments, Horizonal), by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.



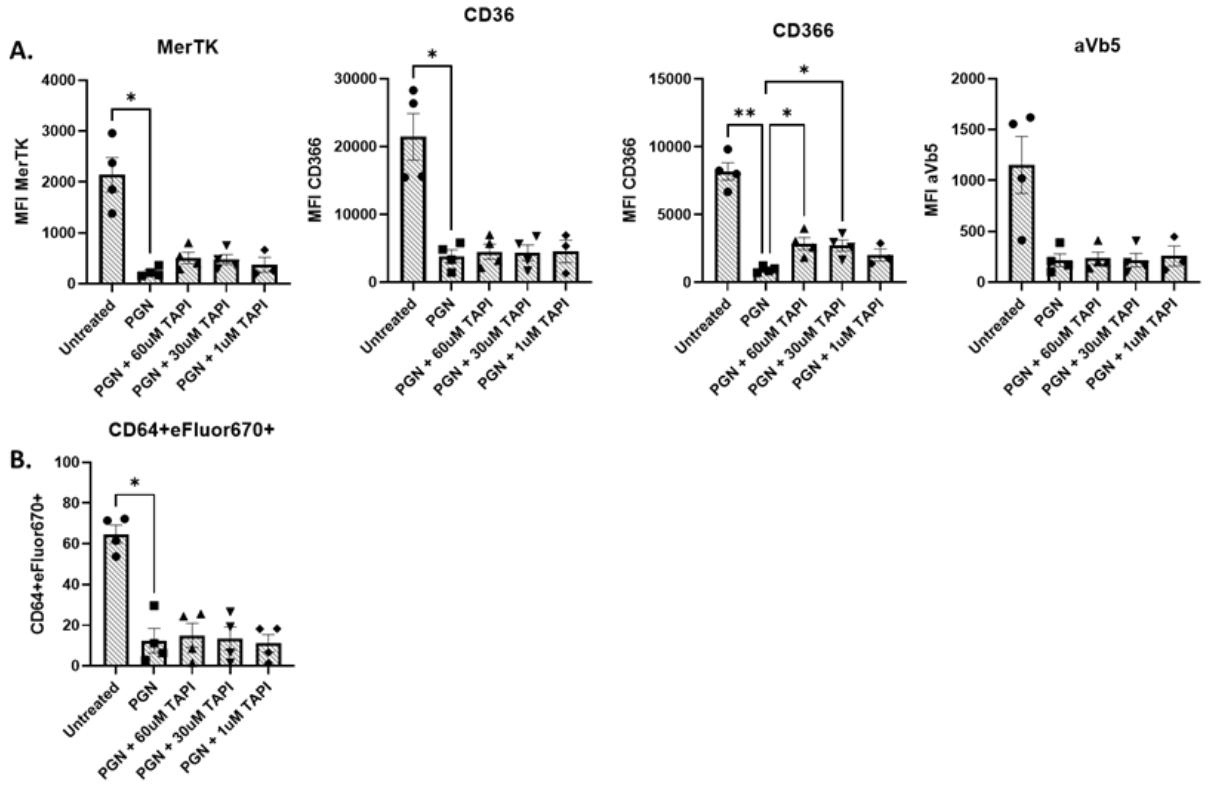
Supplemental Fig 2: Dose response of PGN on MΦ efferocytosis from hihuS or non-hi huS conditions from 3 independent donors (30µg/mL-0.04µg/mL). Data was log(10)-transformed and graphed using a non-linear fit model in Prism. The dose-response response curve was used to calculate IC_{50}.



Supplemental Fig 3: Efferocytosis receptors not affected by PGN treatment in non-hi huS in human M2-like macrophages. **(A, C)** Representative histograms of receptor expression**. (B, D)** MFI of efferocytosis receptors from the indicated serum conditions from ≥3 independent donors treated with 10µg/mL PGN for 24hrs and analyzed by two-way ANOVA with Sidak's multiple comparison.



Supplemental Fig 4: TNF neutralization does not rescue PGN-induced defects in efferocytosis. Percentage of CD64+ MΦ containing apoptotic eFluor670-labeled PMN treated with 6µg/mL of either an IgG isotype control (β-Galactosidase, InvivoGen cat#: bgal-mab1) or human anti-TNF (IgG1, InvivoGen, Cat#: htnf-mab1) antibody in the presence of 10ug/mL PGN for 24 hrs. 4 independent donors analyzed by one-way ANOVA with Dunnet's multiple comparison.



Supplemental Fig 5: (A) MFI of efferocytosis receptors from the non-hihuS condition with various concentrations of TAPI-0 (60, 30, 1μM). from ≥3 independent donors treated with 10μg/mL PGN. **(B)** Percentage of CD64+ MΦ containing apoptotic eFluor670-labeled PMN from the non-hi huS condition in the presence of absence of 10ug/mL PGN and various doses of TAPI-0 from ≥3 independent donors. Data was analyzed by two-way ANOVA with Sidak's multiple comparison.