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A Common Genetic Variant in TLR1 Enhances Human Neutrophil Priming and Impacts Length of Intensive Care Stay in Pediatric Sepsis

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Polymorphonuclear leukocytes (PMN; or neutrophils), essential innate immune cells, recognize danger signals through receptors on their surface, including TLRs. Upon receptor ligation, PMN may undergo priming, a sequential activation process involving shape change, integrin activation, limited granule exocytosis, and low-level reactive oxygen species (ROS) generation. Some of these ROS are formed in intracellular endocytic compartments and are required for activation of intracellular signaling cascades that permit a greatly enhanced cellular response upon exposure to a secondary stimulus (1, 2).

PMN priming was initially perceived as a beneficial phenomenon as primed PMN undergo further activation at sites of infection and are very effective at killing pathogens. However, there is a growing recognition that PMN priming can also be detrimental as activated PMN can potentiate hyperinflammation and subsequent host tissue damage, a leading cause of mortality in patients with sepsis (3).

Our laboratory has extensively investigated PMN priming by endotoxin (1) and TNF-α (4), ligands for TLR4 and the TNF receptors, respectively. However, there is limited literature on PMN priming through other pattern recognition receptors, including TLR2, which has been implicated in several diseases, including tuberculosis (5), leprosy (6, 7), and sepsis (8). TLR2/1 and TLR2/6 heterodimers recognize several stimuli, including triacylated and diacylated lipopolysaccharides, respectively, from bacteria, mycobacteria, and fungi. TLR2, TLR1, and TLR6 are expressed on PMN (9), and a limited number of studies have reported that TLR2/1 and TLR2/6 agonists induce PMN activity. In 1990, Seifert et al. (10) published that PMN exposed to 30–200 μg/ml Pam3CSK4, a specific TLR2/1 agonist, released lysozyme and generated superoxide anion, as measured by the reduction of ferricytochrome c. Sabroe et al. (11) expanded on this finding and demonstrated that 10–10,000 ng/ml Pam3CSK4 increased CD11b surface expression and induced IL-8 secretion. Similarly, PMN treated with 10 ng/ml macrophage-activating lipopeptide-2, a TLR2/6 agonist, or 1–10 μg/ml Pam3CSK4 underwent shape change, displayed enhanced phagocytic capacity, and secreted IL-8 and MIP-1β (12). Finally, Pam3CSK4 and macrophage-activating lipopeptide-2 were shown to delay PMN apoptosis by annexin V staining (13). Notably, these studies reported no differences in signaling pathways downstream of TLR2/1 and TLR2/6 (14), nor any significant donor variation in TLR2 ligand-induced cellular responses.

Our laboratory became interested in neutrophil responses to TLR2 agonists after finding complex and unexpected interactions between TLR2 ligation and the NADPH oxidase 2 using a murine...
model of systemic inflammation elicited by injection of a TLR2 agonist (15, 16). In the current study, we sought to further define human PMN-priming responses to TLR2 ligands in vitro/ex vivo. In contrast to previously published studies, we observed that FSL-1, a TLR2/6 ligand, primes PMN isolated from all donors, but that Pam3CSK4 primes PMN isolated from one subset of donors to a significantly greater extent than PMN isolated from another subset of donors. PMN priming was evidenced by ROS generation, MAPK activation, integrin activation, secondary granule exocytosis, and cytokine release. We determined that the disparity in Pam3CSK4 priming responses correlates with a common single-nucleotide polymorphism (SNP) in TLR1 (rs5743618). PMN from donors with at least one SNP-containing allele have enhanced TLR1 surface expression and prime robustly in response to Pam3CSK4, suggesting that this SNP affects TLR1 trafficking and signaling. As a mechanism for this alteration in trafficking, we demonstrated by immunoprecipitation/immunoblotting that PMNs with the SNP displayed enhanced association between TLR1 and the endoplasmic reticulum (ER) chaperone gp96, previously demonstrated to have a role in TLR trafficking (17, 18). Importantly, this SNP has previously been shown to increase the risk of circulatory dysfunction (8) and mortality (19) in adult patients signanantly, this SNP has previously been shown to increase the risk of pediatric sepsis and demonstrated that patients with the TLR1 SNP displayed enhanced association between TLR1 and the endoplasmic reticulum (ER) chaperone gp96, previously demonstrated to have a role in TLR trafficking (17, 18). Importantly, this SNP has previously been shown to increase the risk of circulatory dysfunction (8) and mortality (19) in adult patients and the endoplasmic reticulum (ER) chaperone gp96, previously demonstrated to have a role in TLR trafficking (17, 18). Importantly, this SNP has previously been shown to increase the risk of circulatory dysfunction (8) and mortality (19) in adult patients and the endoplasmic reticulum (ER) chaperone gp96, previously demonstrated to have a role in TLR trafficking (17, 18).

Materials and Methods

Materials

Dextran was purchased from Pharmacolos (Holboel, Denmark) and Hypaque–Ficoll from GE Healthcare (Piscataway, NJ). HBSS, Dulbecco’s PBS, and RPMI 1640 were obtained from Mediatech (Manassas, VA). Human serum albumin was from Talcric Biotherapeutics (Durham, NC). BSA, dextrose, and BSA were from Fisher Scientific (Pittsburgh, PA). FSL-1 and Pam3CSK4 were obtained from InvivoGen (San Diego, CA), TNF-α was from R&D Systems (Minneapolis, MN), and IMLF was from Santa Cruz Biotechnology (Santa Cruz, CA). Lucigenin, superoxide dismutase (SOD), and cytochrome c were purchased from Sigma–Aldrich (St. Louis, MO), Amplex UltraRed, OxyBURST Green BSA, and protein A and G Dynabead immunoprecipitation kits were from Life Technologies (Grand Island, NY). Anti-CD11b was obtained from BD Pharmingen (San Diego, CA), anti-active CD11b (clone CBRM1/5) from BioLegend (San Diego, CA), and anti-CD66b from ABD Serotec (Raleigh, NC), and anti-CD63 from the Developmental Studies Hybridoma Bank was developed under the auspices of the National Institute of Child Health and Human Development and maintained in University of Iowa (Iowa City, IA). Anti-TLR1 allopregnanolone was purchased from R&D Systems (Minneapolis, MN), anti-TLR6 (CD286) PE from BioLegend, and anti-TLR2 (CD282) FITC from BD Pharmingen. Anti-TLR1, anti-TLR2, and anti-gp96 for immunoprecipitation and immunoblotting were from Abcam (Cambridge, MA), and MAPK Abs were from Cell Signaling Technology (Danvers, MA). Secondary Abs for immunoblotting and flow cytometry were from Bio-Rad (Hercules, CA) and Jackson ImmunoResearch Laboratories (West Grove, PA), respectively. ELISA Abs (including streptavidin–HRP) were from R&D Systems, with the exception of the IL-8 Abs, which were from BD Pharmingen (Franklin Lakes, NJ). Additional reagents were obtained from Sigma–Aldrich.

Isolation and stimulation of human PMN and monocytes

Human PMN and monocytes were isolated from heparin-treated venous blood by standard techniques, and PMN were purified by sequential dextran sedimentation, Hypaque–Ficoll density-gradient centrifugation, and hypotonic lysis of erythrocytes (20). PMN were resuspended in HBSS with calcium and magnesium with 1% human serum albumin and 0.1% dextrose, unless otherwise noted, and were used for experimentation within 10 min of isolation. Using this method, PMN purity was ＞95%. Following Hypaque–Ficoll density-gradient centrifugation, PBMCs were removed and plated in RPMI 1640 with 10% autologous serum at a concentration of 3 × 10^6 cells/ml. Following a 1-h incubation at 37°C, cells were washed three times with Dulbecco’s PBS to remove lymphocytes before fresh RPMI 1640 with 10% autologous serum (with or without a stimulus) added to wells containing adherent monocytes. Unless otherwise stated, cells were stimulated with 100 ng/ml FSL-1, 1 μg/ml Pam3CSK4, or 1 ng/ml TNF-α.

Measurements of NADPH oxidase activity

For lucigenin-ECL (LUC-CL) assays, 200 μl suspension containing a final concentration of 2.5 × 10^5 PMN/ml, 100 μM lucigenin, and stimulus was added per well of a 96-well plate. Each condition was performed in triplicate. For priming experiments, IMLF (1 μM final concentration) was added to the wells up to 30 min. Chemiluminescence was quantitated as relative light units using a kinetic assay with readings every 30 s or 1 min for 0–60 min on a FLUOstar Omega or CLARIOstar (BMG Labtech, Cary, NC).

Amplex UltraRed assays for quantitation of H2O2 generation were performed by adding 100 μM PMN suspension containing 1 × 10^5 PMN/ml to duplicate wells of a 96-well plate with Amplex UltraRed (100 μM final concentration) and excess streptavidin–HRP. PMN were stimulated, as described immediately prior to loading. NADPH oxidase 2 activity was expressed as relative fluorescence units with readings every 60 s for 90 cycles on a FLUOstar Omega. In some wells, IMLF was injected at cycle 30. For each plate, a H2O2 standard curve (0.5–10 μM) was performed and the amount of H2O2 generated was extrapolated. Our laboratory has previously tested the capacity of Amplex UltraRed to be taken up into endosomes upon cellular stimulation; thus, this assay can be used to measure both intracellular and extracellular H2O2 generation (21). For an additional specific measurement of intracellular, endocytic ROS, freshly isolated PMN were stimulated for 0 or 60 min in suspension at 37°C in the presence of OxyBURST green BSA (100 μg/ml). At the specified time points, cells were immediately placed on ice and analyzed for fluorescence on a BD LSR II (BD Biosciences, Sparks, MD).

Extracellular superoxide generation was measured by ferricytochrome c reduction. A total of 4.5 × 10^5 PMN in the presence of 40 μM cytochrome c was added to each well of a 96-well plate in a final volume of 200 μl. PMN were stimulated immediately prior to loading. SOD (50 μg/ml) was added to a duplicate set of wells. Readings were taken every 60 s for 30 cycles, IMLF was added to the wells, and readings were taken for an additional 30 cycles on a FLUOstar Omega. The total nmol superoxide/ml was calculated as the SOD-inhibitable reduction of cytochrome c.

Flow cytometry

To assess cell surface markers of activation and granule exocytosis, freshly isolated PMN were stimulated for 30 min at 37°C. In a subset of experiments, PMN were treated with 10 μM control (SB 204274) or p38 (SB 203580) inhibitor from Santa Cruz Biotechnology (Santa Cruz, CA) for 10 min at room temperature prior to stimulation. Surface expression of TLR1, TLR2, and TLR6 was assayed in freshly isolated, unstimulated PMN. PMN were centrifuged and resuspended in blocking buffer (PBS with 2% nonfat dry milk and 4% normal goat serum) for 20 min on ice, then incubated in the presence of primary Abs for 1 h on ice. PMN were centrifuged and resuspended in 150 μl blocking buffer containing FITC-conjugated secondary Ab diluted 1:1000 and incubated on ice for 30 min. PMN were washed with 3 ml ice-cold PBS and resuspended in PBS for analysis using a BD FACScan (BD Biosciences). Neutrophils (＞98% purity following purification) were gated based on forward and side light scatter, and data were analyzed using FlowJo 7.6.4 software (Tree Star, Ashland, OR).

Analysis of MAPK phosphorylation and total TLR1 by gel electrophoresis and immunoblotting

PMN (2 × 10^5/ml) were incubated in the presence of agonist for the specified time points. After incubation, cells were pelleted and lysed in lysis buffer (100 mM Tris, 150 mM NaCl, 2 mM MgCl2, 1% Triton X-100, 1 mM PMSF, 2% leupeptin/pepsatin A) for 45 min at 4°C. Lysates were centrifuged at 14,000 rpm for 7 min at 4°C, placed in tubes with sample buffer, and heated to 100°C for 3 min. Samples were resolved in an Any KDa gel (Bio-Rad) by SDS-PAGE and then transferred to a nitrocellulose membrane. Membranes were blocked with 3% BSA in TBS with 0.1% Tween 20 and then incubated with primary Abs against phosphorylated or total MAPKs (p38, ERK1/2, or JNK), β-actin, or TLR1. Secondary Abs were conjugated to HRP or fluorophores and detected by chemiluminescence or fluorescence, respectively. Blots were scanned on an Image Station 400MM Pro (Carestream Health, Rochester, NY), and bands were quantitated using ImageQuant TL software (Sunnyvale, CA).
PMN and monocyte supernatant collection and cytokine quantification by ELISA

Freshly isolated PMN or monocytes (2.5 × 10^6/ml) were treated as indicated. PMN were gently tumbled and monocytes were plated at 37°C for 24 h. Following the incubation, PMN were pelleted, and PMN and monocyte supernatants were frozen at −80°C. For cytokine quantification, 96-well NUNC Maxisorp microplates were coated with Abs against IL-6, TNF-α, IL-1β, or TGF-β in carbonate buffer (100 mM NaHCO_3, 34 mM Na_2CO_3, pH 9.6) overnight at room temperature. Wells were blocked with PBS containing 1% BSA and 5% sucrose for 1 h. Standards and samples (thawed supernatants) were diluted in assay diluent (PBS with 0.1% BSA), loaded into duplicate wells, and incubated for 2 h at room temperature. Biotinylated Ab against the target cytokine was added and allowed to incubate for the period and temperature as indicated. Tetramethylbenzidine was added to the wells for 10–30 min for color development, and 0.5 M H_2SO_4 was added to stop the reaction. All incubations were performed at room temperature, and samples were rinsed three times with wash buffer (PBS with 0.05% Tween 20) between each step. Absorbance at 450 nm was measured on a FLUOstar Omega.

SNP selection, DNA isolation, and genotyping

The Exome Variant Server (http://evs.gs.washington.edu/EVS/) was used to identify SNPs in the exons of TLR1. Only two SNPs were noted to have the frequency we would observe in European/American populations, rs5743618 (S602I) and rs4833095 (N248S). To sequence these SNPs, DNA was extracted from isolated PBMCs from consenting healthy adults using a QIAamp DNA mini kit (Qiagen, Venlo, Limburg, the Netherlands). Primers flanking these SNPs were ordered from Integrated DNA Technologies (Coralville, IA). The forward and reverse primers for the rs5743618 (S602I) SNP are 5′−CCCCGAAAAGTATAGAGAACGAC−3′ and 5′−CAGTAAACTGGGAAGATTTCCTTTCG−3′, respectively. The forward and reverse primers for the rs4833095 (N248S) SNP are 5′−TTGTTGGTCCCTCCACAACAAACAA−3′ and 5′−CGAACACATCGTGCAGACT−3′, respectively. For both primers, an annealing temperature of 58°C was used. PCR products were sequenced by Functional Biosciences (Madison, WI) or the Next Generation Sequencing Core at University of Texas Southwestern Medical Center as previously described (22).

Annexin V analysis of PMN apoptosis

Freshly isolated PMN were treated at a concentration of 2.5 × 10^6/ml. PMN were tumbled at 37°C for 24 h. Following this incubation, PMN were pelleted, resuspended in 100 μl 1X annexin V binding buffer, and immediately placed on melting ice. Following the addition of 5 μl annexin V-FITC and 5 μl propidium iodide to each tube, tubes were gently mixed and incubated in the dark for 15 min. A total of 100 μl 1X annexin V binding buffer was then added to each tube, and tubes were analyzed immediately on a BD FACSscan (All buffers and dyes were provided in the Annexin V FITC Apoptosis Detection Kit II from BD Pharmingen.).

Immunoprecipitation

Five hundred micrometers of postnuclear supernatant from cells lysed in lysis buffer (20 mM imidazole, 2 mM EGTA, 100 mM NaCl, 1% Triton X-100, 1 mM PMSF, 2% leupeptin/pepsatin A) was rotated with Ab-conjugated Dynabeads for 1 h at room temperature, according to the manufacturer’s protocol. Beads were washed extensively, followed by elution of bound proteins. Eluted samples were heated at 70°C for 10 min and immediately used for SDS-PAGE and immunoblotting, as described previously, to detect immunoprecipitated proteins.

Pediatric septic shock cohort

DNA samples were obtained from a multicenter database of children with septic shock, previously described in detail (23). Children ≥10 y of age admitted to the PICU and meeting pediatric-specific consensus criteria for septic shock were eligible for enrollment. After informed consent from parents or legal guardians, serum samples were obtained within 24 h of initial presentation to the PICU with septic shock. Clinical and laboratory data were collected daily while in the PICU. PMN were blocked with PBS containing 1% BSA and 5% sucrose for 1 h. Standards and samples were diluted in assay diluent (PBS with 0.1% BSA), loaded into duplicate wells, and incubated for 2 h at room temperature. Following the addition of 5 μl biotinylated Ab against the target cytokine, PMN were immediately placed on melting ice. Following the addition of 5 μl streptavidin-HRP for 20 min, all incubations were performed at room temperature, and samples were rinsed three times with wash buffer (PBS with 0.05% Tween 20) between each step. Absorbance at 450 nm was measured on a FLUOstar Omega.

Results

To explore whether TLR2 agonists prime human PMN, NADPH oxidase 2 activity was evaluated by LUC-CL in PMN treated with no agonist, FSL-1 (a TLR2/6 agonist), Pam3CSK4 (a TLR2/1 agonist), or TNF-α (as a positive control for priming) for 30 min, and then exposed to iMLF as a secondary stimulus. The ROS formed during the 30-min exposure to only the priming stimulus (TLR ligand or TNF-α) are referred to as direct ROS, whereas ROS generated after exposure to the priming and secondary stimuli are referred to as primed ROS. Although there were minor differences in magnitude, PMN from all donors generated both direct and primed ROS in response to FSL-1. However, PMN from only half of the donors generated direct and primed ROS following Pam3CSK4 treatment (Fig. 1). Donors whose PMN generated a >5-fold increase in ROS by LUC-CL when primed with Pam3CSK4 as compared with control cells will henceforth be referred to as TLR2/1 high primers, whereas donors whose PMN generated a <5-fold increase will be referred to as TLR2/1 low primers. TNF-α, used as a positive control for priming, led to direct (data not shown) and primed (Fig. 1D) ROS generation in PMN from all donors.

To determine whether the minimal response observed in the TLR2/1 low primers could be overcome with higher concentrations of ligand, we primed cells from TLR2/1 high and low primers with 0, 1, 3, or 10 μg/ml Pam3CSK4 for 30 min; added iMLF as a secondary stimulus; and measured ROS generation by LUC-CL. There was a dose-dependent increase in primed ROS generation in both TLR2/1 high and low primers, but the difference between the two groups remained highly significant at all agonist concentrations (Supplemental Fig. 1). This argues against the idea that PMN from TLR2/1 low primers merely have a higher threshold dose that is needed to achieve priming responses of similar magnitude to PMN from TLR2/1 high primers. Rather, the data suggest that there is an intrinsic difference in the two subgroups of donors that determines their responsiveness to TLR2/1 agonists.

Statistics

MAPK data were analyzed by two-way ANOVA to compare treatments at each time point. Additional comparisons of neutrophil functional output and phenotype data were made by Student t tests. Statistical analyses were performed with GraphPad PRISM 5.0 (GraphPad Software, La Jolla, CA) and SAS 9.3 (Cary, NC). For the sepsis database genotype comparisons, ANOVA tests were conducted to investigate whether there were significant differences in continuous clinical outcomes among three genotype groups of GG, GT, and TT. The χ^2 or Fisher’s exact tests were used for binary outcomes. Bonferroni corrections were used to adjust the effect of multiple comparisons. Student t tests were used to examine whether there were significant differences in continuous clinical outcomes between GG and (GT + TT). Stepwise linear and logistic regression analyses were conducted to investigate whether genotype was significantly associated with continuous or binary clinical outcomes after controlling the confounding effects of gender, age, and race.

Study approval

Neutrophil and monocyte studies were performed in accordance with a protocol approved by the Institutional Review Board for human subjects at University of Iowa or University of Texas Southwestern Medical Center, and written informed consent was received from all participants prior to inclusion in the study. The pediatric study protocol was approved by the Institutional Review Boards of each participating institution, and written informed consent was obtained from legal guardians prior to inclusion in the study.
To verify these initial LUC-CL findings, and to comprehensively and quantitatively evaluate oxidant generation elicited by Pam3CSK4 or FSL-1 priming, we measured ROS generation by several additional methods. Intra- and extracellular H2O2 generation was measured quantitatively (Amplex Ultra Red) and confirmed that FSL-1 led to direct (Fig. 2A) and primed (Fig. 2B) H2O2 generation in cells from all donors, whereas Pam3CSK4 induced significantly greater direct and primed H2O2 generation in PMN from TLR2/1 high primers than low primers. In fact, TLR2/1 low primers stimulated with Pam3CSK4 did not have greater H2O2 generation than under unstimulated conditions. The division of the donors into TLR2/1 high and low primers using this quantitative methodology was identical to the division observed using the nonquantitative LUC-CL assay.

As we have previously demonstrated that intracellular, direct ROS serve as necessary signaling mediators for PMN priming (2), we measured intracellular endocytic ROS generation (OxyBURST green BSA). Significantly, Pam3CSK4 led to greater intracellular ROS generation in PMN from TLR2/1 high primers than low primers. In fact, TLR2/1 low primers stimulated with Pam3CSK4 did not have greater H2O2 generation than under unstimulated conditions. The division of the donors into TLR2/1 high and low primers using this quantitative methodology was identical to the division observed using the nonquantitative LUC-CL assay. As we have previously demonstrated that intracellular, direct ROS serve as necessary signaling mediators for PMN priming (2), we measured intracellular endocytic ROS generation (OxyBURST green BSA). Significantly, Pam3CSK4 led to greater intracellular ROS generation in PMN from TLR2/1 high primers than low primers, whereas PMN from all donors had the same responsiveness to FSL-1 (Fig. 2C). No extracellular superoxide generation was noted in response to any of the priming stimuli alone, as measured by the reduction of ferricytochrome c (data not shown), but there was enhanced primed extracellular superoxide production in response to Pam3CSK4 by PMN from TLR2/1 high primers compared with low primers (Fig. 2D). Considered in combination, these assays demonstrate that FSL-1 and TNF-α prime intracellular and extracellular ROS generation in PMN from all donors, whereas Pam3CSK4 differentially primes ROS generation in PMN from discrete donor subsets. We postulate that these intracellular endosomal ROS serve a primary role to initiate proinflammatory signaling cascades, including activation of MAPK pathways.

**TLR2/1 high primers activate MAPK signaling**

We have previously demonstrated that MAPK signaling cascades are activated in PMN primed with endotoxin or TNF-α (1, 4, 24). To quantify MAPK activation downstream of TLR2/1 and TLR2/6 ligation, we treated PMN with FSL-1 or Pam3CSK4 and blotted for phosphorylated p38 MAPK, ERK1/2, and JNK1 from the PMN lysate. FSL-1 elicited significant p38 MAPK and ERK1/2 phosphorylation in PMN from all donors (Fig. 3). In contrast, Pam3CSK4 treatment induced phosphorylation of p38 MAPK and ERK1/2 in TLR2/1 high primers, but no enhancement of phosphorylation in TLR2/1 low primers, indicating concordance between activation of NADPH oxidase activity and MAPK activation. Similar trends were seen with JNK1 phosphorylation (Fig. 3C), although not significant. Results were unchanged when total levels of p38, ERK1/2, or JNK1, respectively, were used for normalization (data not shown).

**Integrin activation and partial granule mobilization occur only in primers in response to Pam3CSK4**

MAPK activation has been linked to mobilization of intracellular stores of proteins to the cell surface; thus, surface upregulation of these proteins serves as another functional phenotype of neutrophil
To analyze this phenotype, we studied cell surface expression of critical proteins involved in the neutrophil inflammatory response and in neutrophil activation. FSL-1 elicited upregulation of CD11b, a β2-integrin, on the surface of PMN from all donors. In contrast, Pam3CSK4 only upregulated CD11b expression on PMN from TLR2/1 high primers with no change in CD11b density seen in TLR2/1 low primers as compared with unstimulated cells (Fig. 4A). This pattern was also observed when quantifying surface density for the active conformation of CD11b, which promotes PMN adhesion to the endothelium and subsequent cell extravasation (Fig. 4B). Secondary, or specific, granules may be partially mobilized as a component of priming to aid PMN movement from the vascular space to sites of infection. We found up-regulation of CD66b on the cell surface (a marker of secondary granules) in all donors upon stimulation with FSL-1, but only PMN from TLR2/1 high primers treated with Pam3CSK4 (Fig. 4C). No changes in the expression of CD63, an azurophilic granule marker, were seen in any groups or treatments (Fig. 4D). This served as our negative control, as we would not expect azurophilic granules to be mobilized in response to a priming stimulus alone. Considered in combination, these data suggest that mobilization of intracellular stores of proteins to the cell surface as a component of neutrophil priming is most likely connected to ROS generation and MAPK activation, and does not occur in TLR2/1 low primers in response to Pam3CSK4.

**Integrin activation and granule exocytosis are downstream of p38 signaling**

To determine whether integrin activation and secondary granule exocytosis occur downstream of p38 MAPK signaling, we pretreated PMN with a rapidly diffusing p38 inhibitor or an inactive analog control prior to exposing them to a TLR2 agonist. Following treatment with the p38 inhibitor, we observed a significant decrease in both integrin activation (Fig. 5A) and secondary granule exocytosis (Fig. 5B) in all PMN treated with FSL-1 and in PMN from TLR2/1 high primers treated with Pam3CSK4. As expected, the p38 inhibitor had no effect on TLR2/1 low primers stimulated with Pam3CSK4 as there is no activation of p38 in this population (Fig. 5). These results indicate that integrin activation and secondary granule exocytosis following TLR2 stimulation occur downstream of p38 MAPK signaling.

**PMN and monocytes from TLR2/1 high primers have enhanced cytokine secretion following Pam3CSK4 stimulation**

One mechanism by which innate immune cells orchestrate the inflammatory response is through the production and release of cytokines. We measured cytokine secretion by PMN stimulated with FSL-1 or Pam3CSK4 for 24 h. PMN have been shown to secrete biologically significant levels of proinflammatory IL-8 and MCP-1 and anti-inflammatory IL-1Ra (25). In response to stimulation with Pam3CSK4, PMN from TLR2/1 high primers generated significantly greater amounts of IL-8 and IL-1Ra, but not MCP-1, than PMN from TLR2/1 low primers (Fig. 6), whereas PMN from all donors displayed similar levels of cytokine production in response to stimulation with FSL-1. As monocyte cytokine production is often an order of magnitude greater than production generated by PMN, we also investigated monocyte secretion of pro- and anti-inflammatory cytokines. Interestingly, the pattern of cytokine production by monocytes was similar to the PMN responses to Pam3CSK4. Monocytes from donors that had high PMN priming responses secreted more IL-8, IL-6, and, TNF-α, but not IL-1Ra or MCP-1, than monocytes from TLR2/1 low primers.
primers (Fig. 6). No differences in cytokine production were observed between monocytes from these donors following FSL-1 treatment. These results suggest that TLR2/1 stimuli induce greater cytokine generation in phagocytes from TLR2/1 high primers than low primers, and thus, TLR2/1 high primers may generate an overall greater inflammatory response.

**Pam3CSK4 prolongs the lifespan of PMN from TLR2/1 high primers**

Based on our cytokine data following extended stimulation with TLR2 ligands and published literature demonstrating prolonged PMN lifespan during inflammation in vivo and in vitro (13, 26–28), we investigated whether FSL-1 and Pam3CSK4 delay PMN apoptosis. Minimal numbers of apoptotic PMN were observed at 6 h poststimulation (data not shown), but ~50% of control (non-stimulated) cells were apoptotic at 24 h postisolation (Fig. 7). FSL-1 treatment significantly prolonged the lifespan of PMN from all donors. However, Pam3CSK4 extended the lifespan of PMN from TLR2/1 high primers to a greater extent than PMN from TLR2/1 low primers as evidenced by fewer apoptotic cells at 24 h poststimulation. Thus, the increase in cytokine generation by TLR2/1 high primer PMN in response to Pam3CSK4 could be in part to prolonged PMN lifespan.

**TLR1 SNP determines PMN priming responses**

We predicted that the intrinsic difference in PMN TLR2/1 priming phenotypes and PMN and monocyte cytokine production could be due to a polymorphism in TLR1. Using the Exome Variant Server (University of Washington), we identified two high-frequency nonsynonymous SNPs, rs5743618 (1805G/T) and rs4833095 (743A/G), in the exonic regions of TLR1. These SNPs cause amino acid changes S602I and N248S, respectively. We sequenced these sites in 43 healthy adult donors after determining the priming phenotype of the donors by LUC-CL. All donors that were classified as TLR2/1 low primers were homozygous for the G allele at site 1805G/T, whereas all TLR2/1 high primers were either heterozygous or homozygous for the T allele (Table I). These data strongly indicate that rs5743618 is the causative SNP determining PMN responsiveness to TLR2/1 ligands. For the second SNP sequenced, 743A/G, the G allele was not present in TLR2/1 low primers, but was present in only 77% of the high primers. These data suggest that rs4833095 is not the causative SNP, but rather, is in strong linkage disequilibrium with the causative SNP.

**TLR1 surface expression is increased on PMN from TLR2/1 high primers**

TLR1_1805G/T is located at the cytoplasmic side of the transmembrane region of TLR1, and cell surface trafficking has recently been demonstrated to be altered by this SNP in several cell types (29). Thus, we predicted that TLR1 trafficking to or from the plasma membrane may be affected in neutrophils. Using flow cytometry, we observed that TLR1, but not TLR2 or TLR6, expression was significantly higher on the surface of PMN from TLR2/1 high primers than low primers (Fig. 8A).
To establish whether the increased surface expression of TLR1 on PMN from TLR2/1 high primers was due to higher total cellular levels of TLR1 protein, we blotted for TLR1 in PMN lysate from TLR2/1 high and low primers. Notably, total cellular TLR1 protein levels were not different between the groups (Fig. 8B). This suggests that altered trafficking of TLR1 to or from the PMN surface, rather than dysregulated synthesis of TLR1, leads to the differential priming phenotypes observed between TLR2/1 high and low primers.

**TLR2/1 high primers have enhanced association of TLR1 with the ER chaperone protein gp96**

In view of our data suggesting impaired trafficking as a potential mechanism, and the existing literature identifying gp96 as a critical ER chaperone for several TLR family members in other innate immune cell types (18, 30), we investigated the association between this chaperone and TLR1 in our two donor populations. By immunoprecipitation, we observed a significantly increased association of gp96 with TLR1 in TLR2/1 high primers as compared with TLR2/1 low primers (Fig. 9A, 9B, 9D). After immunoprecipitation for TLR1, we found a 6.6 (± 2.06)-fold increase in the abundance of gp96 by immunoblotting in neutrophils from TLR2/1 high primers as compared with TLR2/1 low primers using paired samples (n = 4). In the TLR1 immunoprecipitates that were blotted for TLR2, there was 2.29-fold greater abundance of TLR2 in TLR2/1 high primers. Although this fold increase did not reach statistical significance, all four paired samples showed greater TLR1–TLR2 association in the TLR2/1 high primer PMN. Considered in combination, the altered PMN functional responses demonstrated in TLR2/1 low primers appear to result from impaired association with the ER chaperone gp96, leading to diminished cell surface expression.
Based on our data and published literature suggesting that the rs5743618 SNP is linked to outcomes in a number of inflammatory and infectious diseases (5, 7, 8, 19, 31–34), we sequenced samples from a pediatric sepsis database (35). Among 140 children admitted with sepsis or septic shock with positive bacterial cultures from any normally sterile site, 40 patients were homozygous for the G allele at site 1805G/T, 53 patients were heterozygous (G/T), and 47 patients were homozygous for the T allele. These data were analyzed to determine whether genotype was related to the clinical outcomes of complicated course (35), maximum organ failure, PICU free days, and PICU LOS. There was no association between these clinical outcomes and genotype in this cohort (Supplemental Table I). In a subanalysis, we focused exclusively on patients with positive bacterial blood cultures. In this subpopulation, patients who were homozygous for the T allele had significantly increased PICU LOS, compared with those who were homozygous for the G allele (Fig. 10A). Moreover, in separate analyses combining the GT and TT populations (based on our in vitro finding that a single T allele conferred enhanced neutrophil activation), patients with the genotype GG had a significant reduction in PICU LOS compared with the combined GT and TT group (Fig. 10B). There were no statistically significant differences between the genotypes in terms of the outcomes of complicated course or maximum organ failure, although there was a trend toward increased organ failure in the TT genotype (2.25 ± 1.1) in comparison with the GG genotype (1.75 ± 1.3). Similarly, the TT patients had a 25% incidence of complicated course versus a 6.2% incidence in the GG genotype patients. We hypothesized that the impact of this SNP would be most notable in patients with Gram-positive bacterial sepsis and thus analyzed Gram-positive bacteremia with sepsis and Gram negative separately. Although there were no significant differences between the genotypes due to the low number of patients in each subgroup, the trend toward prolonged PICU LOS in the TT genotype existed for both Gram-positive and Gram-negative bloodstream infections.

**Neutrophil priming responses in vitro do not differ between GT and TT genotypes**

The donor pool used for all of our initial in vitro assays of PMN priming had only a single donor homozygous for the T allele. In view of the data obtained from genotyping of the pediatric sepsis database, and a subset of the published literature suggesting that the TT genotype might be phenotypically distinct from the GT genotype (19), we evaluated neutrophil NADPH oxidase priming in a geographically distinct and more racially diverse donor pool. Consistent with data from the initial donor pool, PMN from donors with the GG genotype had minimal/no priming of the respiratory burst after incubation with Pam3CSK4, followed by stimulation with fMLF, as measured by LUC-CL. Notably, PMN from donors that were heterozygous or homozygous for the T allele had nearly identical priming responses (Supplemental Fig. 2). PMN from all donors displayed priming in response to stimulation with FSL-1 (data not shown).

### Discussion

PMN priming occurs in vitro and in vivo in response to various stimuli. Primed PMN have enhanced responsiveness to secondary stimuli, which may be beneficial or detrimental depending on the context. Although primed PMN may eradicate pathogens more efficiently, dysregulated PMN activity or recruitment may lead to excessive oxidant generation and protease release and subsequent host tissue injury in settings such as sepsis (3).

The current study evaluated PMN priming responses to TLR2 agonists and provided evidence of three novel related findings. First, in contrast to previous studies (10, 11, 14) and in contrast to our findings with other TLR ligands (1), we demonstrated that TLR2/1 ligation does not prime PMN from all donors. Fifty percent of donors display direct ROS generation in response to Pam3CSK4 and enhanced responsiveness to subsequent stimulation, whereas the other half have minimal to no priming response. (We referred to these donor subsets as TLR2/1 high primers or TLR2/1 low primers, respectively.) In contrast, TLR2/6 ligation by FSL-1 primes PMN from all donors to a similar extent. Second, we showed that TLR2/1 high primers contain at least one copy of a common SNP (rs5743618, 1805G/T) in exon 4 of TLR1 that is not present in TLR2/1 low primers. In a second distinct donor pool, we demonstrated that the presence of a single T allele is sufficient to confer neutrophil priming responses in vitro. And finally, pediatric sepsis and septic shock patients with a positive blood culture and with a copy of the SNP associated with neutrophil priming had prolonged PICU LOS. Due to its role in modulating PMN and monocyte inflammatory function, we suggest that this SNP may significantly impact, and could possibly predict, patient risk and outcome during severe inflammatory conditions.

**TLR1 site 1805G/T has previously been linked to patient risk and outcome in several diseases.** The 1805T allele (consistent with our TLR2/1 high primers) has been shown to be protective against pyelonephritis (31), Candidemia (32), tuberculosis (5), and extension of inflammatory bowel disease (33). Conversely, the 1805T allele has been associated with a higher incidence of *Chlamydia trachomatis* infection (34) and leprosy (7), and in-

### Figure 8

**TLR1 expression is significantly increased on the surface of PMN from TLR2/1 high primers compared with TLR2/1 low primers, although total cellular proteins levels are similar.** (A) PMN surface expression of TLR1, TLR2, and TLR6 was quantified by flow cytometry and adjusted to an IgG control. Mean + SEM, n = 7 per group. **p < 0.01, Student t test. (B) Total TLR1 protein in whole PMN lysates was quantified by gel electrophoresis and immunoblotting in TLR2/1 low primers and TLR2/1 high primers. Mean + SEM, n ≥ 5 per group.
creased circulatory dysfunction and mortality in adult sepsis patients (8, 19). Together, these studies provide evidence that the 1805T allele can confer susceptibility to certain diseases and protection against others, giving insight into why both the 1805G and 1805T alleles remain in the human population in high frequency. The high frequency of both alleles is not unexpected as inflammation is evolutionarily necessary and beneficial in certain infectious diseases; however, the extent of inflammation required to restore immunological homeostasis is dependent on the disease context. The major 1805G allele occurs at high frequencies in European, European American, and some Middle Eastern populations, whereas the derived 1805T allele is more frequent in Asian and African populations (7, 36, 37).

The mechanism by which TLR1 site 1805G/T influences innate immune responses has not yet been fully elucidated. Notably, site 1805G/T is located at the cytoplasmic side of the transmembrane domain of TLR1, where it is not likely to affect ligand recognition or binding. In 2007, Johnson et al. (7) reported that monocytes from 1805G homozygous donors lacked TLR1 surface expression by flow cytometry and fluorescent microscopy; however, monocytes from all donors had similar levels of intracellular TLR1. Similarly, a study by Uciechowski et al. (5) revealed that individuals homozygous for 1805G had no surface expression of TLR1 on monocytes, granulocytes, or lymphocytes by flow cytometry. In contrast to these studies, we observed detectable TLR1 surface expression on PMN from donors homozygous for the 1805G allele (TLR2/1 low primers), although the levels were significantly below those of PMN from heterozygous donors. This low-level surface expression is consistent with our data showing that Pam3CSK4-stimulated monocytes and whole blood from homozygous 1805G donors generate less TNF-α and IL-6, respectively, than monocytes and whole blood samples from donors with at least one copy of 1805T (7, 37). Similarly, HEK 293T cells transfected with the TLR1_1805G allele have significantly less NF-κB activation than cells transfected with TLR1_1805T following exposure to Pam3CSK4 (37). Consistent with previous studies, we observed for the ER chaperone gp96 in TLR1 trafficking in neutrophils. Our data suggest that PMN from donors with the 1805T allele display significantly enhanced association of the gp96 chaperone with TLR1 and significantly greater TLR1–TLR2 association. We are currently seeking to determine whether association with this chaperone is required for conformational stability, as has been shown for TLR9 (17). Moreover, the mechanisms regulating heterodimer formation and balance of TLR2/1 versus TLR2/6 in each cell type are currently unknown, and under investigation in our laboratory.

Previous studies have shown that Pam3CSK4-stimulated monocytes and whole blood from homozygous 1805G donors generate less TNF-α and IL-6, respectively, than monocytes and whole blood samples from donors with at least one copy of 1805T (7, 37). Similarly, HEK 293T cells transfected with the TLR1_1805G allele have significantly less NF-κB activation than cells transfected with TLR1_1805T following exposure to Pam3CSK4 (37). Consistent with previous studies, we observed for the ER chaperone gp96 in TLR1 trafficking in neutrophils. Our data suggest that PMN from donors with the 1805T allele display significantly enhanced association of the gp96 chaperone with TLR1 and significantly greater TLR1–TLR2 association. We are currently seeking to determine whether association with this chaperone is required for conformational stability, as has been shown for TLR9 (17). Moreover, the mechanisms regulating heterodimer formation and balance of TLR2/1 versus TLR2/6 in each cell type are currently unknown, and under investigation in our laboratory.

Several endoplasmic reticulum chaperones for TLR proteins have been demonstrated to regulate TLR trafficking (29, 30). A specific role for these ER chaperones in neutrophils had not been previously studied. In the current study, we demonstrate a likely role

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**FIGURE 9.** Increased association between TLR1 and gp96 in TLR2/1 high primers. (A) Immunoprecipitation of TLR1 followed by immunoblotting for gp96 demonstrates increased association in TLR2/1 high primers, n = 4, *p < 0.05. (B) Immunoprecipitation of gp96 followed by immunoblotting for TLR1 demonstrates increased association in TLR2/1 high primers, n = 3, ***p < 0.0001, t tests. (C) Immunoprecipitation of TLR1 followed by immunoblotting for TLR2 shows a trend toward increased association in the TLR2/1 high primers, n = 4. (D) Representative immunoblots.

**FIGURE 10.** Septic patients homozygous for the 1805T allele with positive blood cultures had significantly longer stays in the PICU. (A) Correlation between individual genotypes and PICU LOS in patients with positive bacterial blood cultures and sepsis, *p < 0.05 for GG versus TT paired comparison, p = 0.053 for ANOVA. (B) Comparison between PICU LOS and GG versus GT + TT combined, *p < 0.05. (C) Comparison between genotypes and PICU LOS in septic patients with Gram-positive bacteremia. (D) Comparison between PICU LOS and genotype in septic patients with Gram-negative bacteremia.
that monocytes from homozygous 1805G donors secreted less TNF-α, as well as IL-6 and IL-8, than monocytes from heterozygous donors. To our knowledge, for the first time, we also showed that PMN from donors homozygous for TLR1_1805G have minimal priming responses to Pam3CSK4 compared with heterozygous donors. This was evidenced by significantly less ROS generation, MAPK signaling, degranulation, and granulocyte production. Importantly, PMN play prominent roles in the pathogenesis of several diseases impacted by TLR1_1805G/T, including pyelonephritis (41), candida infections (42), tuberculosis (43), inflammatory bowel disease (44), leprosy (45), and sepsis (46). Thus, we predict that the degree of PMN activation through TLR2/1, as a consequence of TLR1 genotype, significantly affects patient susceptibility and/or outcome for several diseases.

Further evidence for the importance of this SNP in human disease was generated by our analysis of the frequency of each of these genotypes in a pediatric sepsis database. Our finding that patients with one or two copies of this SNP had prolonged stay in a PICU after sepsis with positive blood culture demonstrates the importance of understanding host genetic susceptibility. In the early years of treating sepsis, the mortality from septicaemia was a consequence of the initial septic shock phase of disease. As modern intensive care practice has evolved, the early mortality from sepsis has declined, and both morbidity and mortality result from multi-organ system dysfunction that stems from the host inflammatory response to the pathogen (47). Appropriate termination of host inflammation is critical to successful recovery. Our data suggest that under some conditions exuberant neutrophil and monocyte inflammatory activation delays recovery, as suggested by longer requirements for intensive care unit admission. Host differences in innate immune responses most likely have a major impact on outcomes from sepsis and other systemic inflammatory diseases. Better understanding of these basic immune mechanisms will likely improve outcomes.

In recent murine studies, Pam3CSK4 was demonstrated to boost immunogenicity to a trivalent influenza vaccine and a Leishmania (TRYP Ag) vaccine (48, 49). This enhanced protection was thought to be due to stimulation of the innate immune system and production of a cytokine mediator that enhances T cell responses. Our research has significant implications for the usefulness of Pam3CSK4 as a potential human vaccine adjuvant. In this study, we have demonstrated that Pam3CSK4 induces a much stronger innate immune response in human cells with at least one copy of the TLR1_1805T allele compared with those with two copies of the TLR1_1805G allele. Thus, the presence or absence of this allele could have a significant impact on the effectiveness of a human vaccine utilizing Pam3CSK4 as an adjuvant due to varied degrees of innate immune stimulation.

In summary, this study defined a novel mechanism of action for TLR1 SNP rs5743618 in modulating PMN priming responses. As the extent of PMN priming can impact the severity and outcome of several global diseases, it is important to understand how this SNP affects PMN activation. Our better understanding of the basic biology of leukocyte responses and the impact of host genetic variability on these responses is required to define the next therapeutic interventions. As we look forward to an era of personalized medicine, we predict that patient genotyping for this SNP could be used to predict patient risk in heterogeneous diseases such as sepsis, and thus, could inform patient treatment.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. Pam₃CSK₄ primes dose-dependent ROS generation in PMN from TLR2/1 high and TLR2/1 low primers, but to significantly different extents. LUC-CL was used to measure the generation of ROS following 30 min of priming with increasing concentrations of Pam₃CSK₄ then secondary stimulation with fMLF. Mean + SEM, n ≥ 4 per group. ***p < 0.001, Student’s t tests.
Supplemental Figure 2. Both GT and TT genotypes display similar priming of NADPH oxidase activity, whereas GG donors do not prime in response to TLR2/1 stimulation in a second donor pool. ROS generation was measured by LUC-CL as RLUs in PMN primed with no agonist or 1μg/ml Pam3CSK4 for 30 min and then stimulated with fMLF. (A) Combined tracings showing primed ROS production in PMN from half of donors in response to Pam3CSK4 priming. Mean ± SEM. (B) Peak RLUs (mean + SEM) immediately following addition of fMLF as shown in (A), and under no priming conditions, n = 8 GG, 4 GT, and 4 TT. ***p < 0.0001. P values were calculated for GT and TT donors by Student’s t tests as compared to GG.
## Supplemental Tables

### Supplemental Table I. Clinical outcomes by genotype among pediatric patients with severe sepsis or septic shock and any positive culture.

<table>
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<th>GG (N = 40)</th>
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<th>TT (N = 47)</th>
<th>All (N = 140)</th>
<th>p-value</th>
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<td>No</td>
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<td>35 (74.47%)</td>
<td>103 (73.57%)</td>
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<td>Yes</td>
<td>12 (30.00%)</td>
<td>13 (24.53%)</td>
<td>12 (25.53%)</td>
<td>37 (26.43%)</td>
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<td><strong>Max Organ Failure</strong></td>
<td>2.150 ± 1.312</td>
<td>2.000 ± 1.359</td>
<td>1.957 ± 1.122</td>
<td>2.029 ± 1.263</td>
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<tr>
<td><strong>PICU Length of Stay</strong></td>
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Statistical significance was determined by ANOVA^a.