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Simultaneous Host-Pathogen Transcriptome Analysis during *Granulibacter bethesdensis* Infection of Neutrophils from Healthy Subjects and Patients with Chronic Granulomatous Disease

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Polymorphonuclear leukocytes (PMN) from patients with chronic granulomatous disease (CGD) fail to produce microbicidal concentrations of reactive oxygen species (ROS) due to mutations in NOX2. Patients with CGD suffer from severe, life-threatening infections and inflammatory complications. *Granulibacter bethesdensis* is an emerging Gram-negative pathogen in CGD that resists killing by PMN of CGD patients (CGD PMN) and inhibits PMN apoptosis through unknown mechanisms. Microarray analysis was used to study mRNA expression in PMN from healthy subjects (normal PMN) and CGD PMN during incubation with *G. bethesdensis* and, simultaneously, in *G. bethesdensis* with normal and CGD PMN. We detected upregulation of antiapoptotic genes (e.g., XIAP and GADD45B) and downregulation of proapoptotic genes (e.g., CASP8 and APAF1) in infected PMN. Transcript and protein levels of inflammation- and immunity-related genes were also altered. Upon interaction with PMN, *G. bethesdensis* altered the expression of ROS resistance genes in the presence of normal but not CGD PMN. Levels of bacterial stress response genes, including the ClpB gene, increased during phagocytosis by both normal and CGD PMN demonstrating responses to oxygen-independent PMN antimicrobial systems. Antisense knockdown demonstrated that ClpB is dispensable for extracellular growth but is essential for bacterial resistance to both normal and CGD PMN. Metabolic adaptation of *Granulibacter* growth in PMN included the upregulation of pyruvate dehydrogenase. Pharmacological inhibition of pyruvate dehydrogenase by triphenylbismuthdichloride was lethal to *Granulibacter*. This study expands knowledge of microbial pathogenesis of *Granulibacter* in cells from permissive (CGD) and nonpermissive (normal) hosts and identifies potentially druggable microbial factors, such as pyruvate dehydrogenase and ClpB, to help combat this antibiotic-resistant pathogen.

Chronic granulomatous disease (CGD) is a primary immunodeficiency caused by mutations in genes encoding $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$, components of NOX2, an NADPH-oxidase enzyme complex that produces superoxide anions (1–3). Cells from patients with CGD have decreased NOX2-dependent superoxide anion production, resulting in abnormally low phagosomal concentrations of antimicrobial H_2O_2 and myeloperoxidase-dependent hypohalous acids (e.g., hypochlorite). Patients with CGD typically require prophylactic antibacterial and antifungal therapy to prevent frequent life-threatening infections characteristic of CGD. CGD patient cells also have altered signaling and transcriptional regulation as well as dysregulated production of inflammatory mediators (4), which likely contribute to the life-threatening granulomas also associated with CGD. The great diversity of mutations in cells from patients with CGD, particularly in $gp91^{phox}$, results in phenotypic differences, including survival (3), that complicate the study of these patients.

Common pathogens in CGD patients include *Staphylococcus aureus*, members of the *Burkholderia cepacia* complex, *Serratia marcescens*, *Nocardia*, and *Aspergillus*. However, less common microbes can also cause serious morbidity and mortality in CGD patients (1). *Granulibacter bethesdensis*, an emergent Gram-negative pathogen, has been cultured from at least 9 CGD patients and has been associated with 2 fatalities in CGD patients in North America and Europe (5–8; E. L. Falcone, J. R. Petts, M. B. Fasano, B. Ford, W. Nauseef, J. F. Neves, M. L. Tierce, M. de la Morena, D. E. Greenberg, C. S. Zerbe, A. M. Zelazny, and S. M. Holland, submitted for publication). The clinical presentation of *Granulibacter* infection generally includes fever, weight loss, fatigue, severe lymphadenitis, ascites, and bacteremia. However, in some subjects, long-term seropositivity prior to the isolation of the pathogen has been noted (7). Serologic testing of larger cohorts of CGD patients suggests that the observed prevalence of *G. bethesdensis* infections in CGD may be underestimated (9). Although members of the *Acetobacteraceae* were not previously thought to be human pathogens, infections have also been reported for two species of *Acetobacter* (10, 11), two species of *Asaia* (12, 13), and

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TABLE 1 Human subject demographics and gp91^{phox} CGD mutations

Expt	Subject	Age of subject (yr)	Gender of patient	Mutation[a]	Superoxide production (nM/million PMN/60 min)[b]	Medication(s)
Microarray	CGD	39	Male	c.296_306 del CCTTTCATAAA, ins TCC = p.Thr99llefsX21	1.79	Cefdinir, itraconazole, dicloxacillin, oxycodone-acetaminophen (Percocet), loratadine (Claritin), omeprazole (Prilosec), loperamide (Imodium)
Microarray	CGD	24	Male	c.676 C>T = p.Arg226X	0.97	Dapsone, sulfamethoxazole-trimethoprim (Bactrim), fluticasone-salmeterol (Advair), prednisone
Microarray	CGD	24	Male	c.676 C>T = p.Arg226X	0.8	Ciprofloxacin, itraconazole, prednisone, sulfamethoxazole-trimethoprim
Microarray	CGD	24	Male	c.458 T>G = p.Leu153Arg	4.17	Itraconazole, sulfamethoxazole-trimethoprim
Microarray	Normal	37	Male	ND[c]	ND	ND
Microarray	Normal	33	Male	ND	ND	ND
Microarray	Normal	26	Male	ND	ND	ND
Microarray	Normal	22	Male	ND	ND	ND
PPMO	CGD	24	Male	c.676 C>T = p.Arg226X	0.97	Amoxicillin-clavulanic acid (Augmentin), sulfamethoxazole-trimethoprim, posaconazole
PPMO	CGD	25	Male	c.80–83 del TCTG = p.Val27GlyfsX33	0.57	Itraconazole, ondansetron (Zofran), omeprazole, sulfamethoxazole-trimethoprim
PPMO	CGD	16	Male	c.676 C>T = p.Arg226X	0.32	Posaconazole, sulfamethoxazole-trimethoprim, levofloxacin, omeprazole, mesalamine, prednisone
PPMO	Normal	56	Male	ND	ND	ND
PPMO	Normal	73	Male	ND	ND	ND
PPMO	Normal	56	Female	ND	ND	ND
PPMO	Normal	66	Female	ND	ND	ND
PPMO	Normal	60	Female	ND	ND	ND

[a] Mutations as reported in reference 3.
[b] PMA-induced superoxide production by neutrophils as determined by cytochrome *c* reduction (3). The normal range is 226 ± 3 nM/million PMN/60 min.
[c] ND, not determined.

three species of *Roseomonas* (14–16), mostly in immunocompromised patients or those with vascular access lines. *Acidomonas methanolica* has also been reported to cause a *Granulibacter*-like disease in at least 2 CGD patients (17; Falcone et al., submitted).

G. bethesdensis remains the only member of the *Acetobacteraceae* for which Koch's postulates have been fulfilled. CGD mice are significantly more susceptible to infection by *G. bethesdensis* than are normal mice, yet viable *G. bethesdensis* bacteria have been recovered from wild-type mouse spleens at >76 days postinfection (5), suggesting considerable resistance of this organism to the intact murine immune system. Previous studies demonstrated that *G. bethesdensis* is resistant to human serum complement; the LL-37 human cathelicidin peptide; and other oxygen-independent antimicrobial factors still present in polymorphonuclear leukocytes (PMN) from CGD patients (CGD PMN) (18), monocytes, and monocyte-derived macrophages (19). Normally, PMN apoptosis contributes to the resolution of inflammation (20), and CGD PMN exhibit retarded apoptosis and increased proinflammatory activity *in vitro* (21, 22). In contrast to many pathogens that promote apoptosis following their phagocytosis by PMN (23), *G. bethesdensis* significantly delays both constitutive and Fas-induced PMN apoptosis (18), although the mechanisms contributing to this phenomenon remain unclear.

To elucidate host responses to this pathogen, we performed transcriptional profiling of CGD PMN and PMN from healthy subjects (normal PMN) infected with *G. bethesdensis*. Simultaneously, we examined microbial responses to normal and CGD PMN using a custom microarray to help identify key molecules involved in microbial pathogenesis and potential targets for antimicrobial drugs.

MATERIALS AND METHODS

Preparation and culture of cells. Blood was obtained after informed consent and/or assent was obtained from subjects enrolled in studies under National Institute of Allergy and Infectious Diseases Institutional Review Board (IRB)-approved protocols 05-I-0213 and 93-I-0119 or NIH Clinical Center IRB-approved protocol 99-CC-0168. Demographic, mutational, and medication data about CGD patients and healthy subjects participating in this study are shown in Table 1. For the microarray experiments, CGD patients were male (mean age, 27.8 years; range, 24 to 39 years) and had no history of *G. bethesdensis* infection, and healthy male subjects (mean age, 29.5 years; range 22 to 37 years) were used as controls. Demographic information for peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO)-mediated gene knockdown experiments are also shown in Table 1 for CGD patients (mean age, 21.7 years; range, 16 to 25 years) (100% male) and five normal subjects (mean age, 62.2 years; range 56 to 73 years) (60% female).

Serum and Percoll-isolated PMN were isolated from venous blood as previously described (24). PMN purity in these experiments averaged 96.3% ± 2.9% (mean ± standard deviation [SD]). *G. bethesdensis* strain NIH1.1 (strain CGDNIH1^T) was grown to mid-log phase at 37°C in yeast extract-peptone-glucose (YPG) medium, as previously described (18). Assays were performed essentially as described previously (25), with slight modifications, as follows: 24-well Costar plates were blocked with 1% human serum albumin (HSA; Talecris Biotherapeutics, Research Triangle Park, NC) at 37°C for 1 h and then rinsed twice with 150 mM NaCl. One

milliliter cultures contained 10% subject autologous serum in RPMI 1640 (Gibco) supplemented with 25 mM HEPES (pH 7.2) with or without 1×10^7 PMN and with or without 1×10^8 *G. bethesdensis* bacteria. Upon addition, infections were synchronized by spinning the plates for 8 min at 500 × *g*, and samples were collected immediately (time zero) or after further incubations of 1, 4, and 24 h at 37°C in a 5% CO_2 incubator. Samples were harvested by spinning at 5,000 × *g*, and supernatants were collected for cytokine analysis as described below. Cells were lysed in 600 μl of RLT buffer (Qiagen, Valencia, CA) and frozen at −80°C for batch extraction.

Simultaneous extraction of bacterial and eukaryotic RNAs. RLT cell lysates were homogenized by using the QIAshredder system, and neutrophil and bacterial RNAs were simultaneously extracted by using an AllPrep DNA/RNA 96 kit (Qiagen) with on-column DNase I treatment. RNA was quantitated spectrophotometrically, and quality was determined by using the Agilent 2100 Bioanalyzer Pico kit (Agilent Technologies, Santa Clara, CA). Bacterial RNA quantities in mixed RNA samples were determined as described previously (26), except that the *G. bethesdensis* DNA gyrase subunit B gene (GbCGDNIH1_0321) was used as the constitutive gene. Genomic DNA contamination was measured in RNA samples via TaqMan using primers for the *clpB* gene (GenBank accession number YP_745624) (forward primer CGGTCCTGAAGGAAATTG AGTCT, probe 6-carboxyfluorescein [FAM]-CGGCGAAGTCATTCT-MGB, and reverse primer TCCAACCAGTGTGTGCATCTC) and was <0.03% of the total nucleic acids (average threshold cycle [C_T] value of >37).

Human GeneChip target preparation. Five nanograms of RNA sample was concentrated to 5 μl and processed by using the WT-Ovation Pico RNA amplification system (Nugen Inc., San Carlos, CA) according to the manufacturer's instructions. Each RNA sample was spiked with a mixture of four *Bacillus subtilis* poly(A)-tailed mRNAs to monitor cDNA synthesis and amplification during target preparation. Amplified single-stranded cDNAs were purified with a modified QIAquick 96-well protocol (Qiagen) (27). Sample quantity was measured by using the SpectraMax Plus^{384} instrument (Molecular Devices, Sunnyvale, CA). Single-stranded single primer isothermal amplification (SPIA) cDNA quality was determined by using an Agilent 2100 bioanalyzer. Fragmentation and labeling of 5 μg of double-stranded cDNA by using FL-Ovation cDNA biotin module V2 (Nugen Inc.) was performed according to the manufacturer's protocol.

Bacterial GeneChip target preparation. A Life Technologies (Carlsbad, CA) MessageAmp II-Bacteria kit was used, as recommended by the manufacturer, to amplify an average of 0.3 μg of *G. bethesdensis* and human neutrophil RNAs. RNA was concentrated to 5 μl, denatured for 10 min at 70°C, polyadenylated by using *Escherichia coli* poly(A) polymerase for 15 min at 37°C, and reverse transcribed for 2 h at 42°C by using ArrayScript and a poly(dT) primer with a T7 tail. Second-strand synthesis was performed immediately after first-strand cDNA synthesis for 2 h at 16°C. Purified cDNA was mixed with 75 nmol of biotin-16-UTP (Roche Applied Science, Mannheim, Germany) and concentrated to 18 μl. Biotin-labeled complementary pathogen-host RNA was amplified at 37°C overnight in a 40-μl *in vitro* transcription reaction mixture. The resulting cRNA was purified by using an RNeasy 96 kit (Qiagen). All cRNA was incubated in 1× fragmentation buffer to generate 20 to 100 base fragments according to the manufacturer's protocol (Life Technologies).

Microarray analysis of RNA. DNA microarray analysis followed a three-factor experimental design with two host types (normal or CGD PMN), culture of bacteria or PMN in autologous serum alone or together, and four time points (0, 1, 4, and 24 h), as described above. Labeled bacterial targets were hybridized to a custom Affymetrix GeneChip, RMLchip3a520351; washed; and imaged as previously described (28). For human targets, hybridization, fluidics, and scanning were performed according to standard Affymetrix protocols, and HG-U133 Plus 2.0 chips were used. GeneChip Operating Software (GCOS; v1.4) was used to convert the image files to cell intensity data files (cel files). All cel files, representing individual samples, were normalized by using the scaling method within GCOS and a scaled target of 500 to produce the analyzed cel files (chp files) along with the report files. The cel files were evaluated with Partek Genomics Suite software (v 6.3, build number 6080414; Partek Inc., St. Louis, MO) and quantile normalized. Of the 128 host and pathogen samples, 5 were determined to be outliers by using the Pearson dissimilarity hierarchical clustering algorithm within the Partek Genomics Suite and were excluded from the following analyses. An analysis of variance (ANOVA) was performed in Partek to obtain multiple-test-corrected *P* values using the false discovery rate (FDR) method at a 0.05 significance level, and data were combined with fold change values, signal above background, and call consistency, as calculated by using custom Microsoft Excel templates for each comparison using all samples. For gene lists in the supplemental material, ambiguous probe sets (e.g., x_at and s_at probe sets) and probe sets without annotation were removed, and genes with multiple probe sets were the filtered by using Excel so that only a single probe set per gene remained. Gene Set Enrichment Analysis (GSEA) (29, 30) was used to analyze pathogen array data at all time points by comparing expression by *G. bethesdensis* in medium to that in medium with either normal or CGD PMN. A total of 99 gene sets were defined by using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, 54 of which passed through the gene set size filter (>10 and <500 genes). Gene sets were considered significantly enriched with an FDR of ≤0.25.

Peptide-mediated antisense knockdown of genes in *G. bethesdensis*. PPMOs (kindly provided by Sarepta Therapeutics) were designed to target mRNA of *acpP* (GbCGDNIH1_2209 [5′-ACTTCGCTCAT-3′]) and *clpB* (GbCGDNIH1_1803 [5′-CCATGTCCCTA-3′]). A scrambled oligonucleotide (5′-TCTCAGATGGT-3′) was used as a control. PPMOs were coupled to a membrane-penetrating carrier peptide (RFFRFFRFFRXB, where X is 6-aminohexanoic acid and B is β-alanine), as previously described (25). To determine whether gene products were essential for survival *in vitro*, 5×10^5 CFU/ml of saline-washed mid-log-phase bacteria were incubated in YPG medium for up to 2 days with or without 40 μM the indicated PPMO. Viability was determined by plating of dilutions onto YPG agar, as previously described (25). To determine if bacterial genes were required for bacterial survival in PMN, bacteria were preincubated with 40 μM the indicated PPMOs or buffer for 60 min prior to inoculation of PMN cultures in RPMI 1640 supplemented with 25 mM HEPES (pH 7.2) with or without autologous sera and incubation at 37°C in 5% CO_2. After the indicated time points, PMN were lysed, and CFU were enumerated as described previously (25).

PMN lysates and Western blotting. Freshly isolated normal PMN (5×10^6 PMN per condition in RPMI 1640 with 10 mM HEPES in 24-well plates) were cultured as described above for 24 h at a multiplicity of infection (MOI) of 0, 1, or 10 with *G. bethesdensis*, which had been preopsonized in human serum for 20 min at 37°C and washed twice with 150 mM NaCl. Plates were precoated with 10% autologous serum for 1 h at 37°C before washing three times with Hanks balanced salt solution without divalent cations [HBSS(−)]. Freshly isolated PMN (5×10^6) or PMN harvested from wells via Cellstripper (Mediatech Inc., Manassas, VA) at 24 h were pelleted at 13,000 × *g* for 1 min at 4°C. PMN lysates were prepared essentially as described previously (31), except that the protease buffers used contained 7.8 mM Pefabloc SC-AEBSF [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride] (Roche, Indianapolis, IN) and one-half of a cOmplete Mini protease inhibitor tablet (Roche) in 3.5 ml Tris-buffered saline per 5×10^6 cells. Detergent-soluble supernatants were assayed for protein content by using the Bio-Rad detergent-compatible protein assay and boiled in Invitrogen NuPAGE lithium dodecyl sulfate sample buffer with 5% β-mercaptoethanol. Twenty-eight micrograms of protein was run per lane of 1-mm/10-well NuPAGE 4 to 12% Bis-Tris gels and resolved in morpholineethanesulfonic acid (MES)-SDS running buffer. Standard Western blotting procedures were performed, using bovine serum albumin (BSA) as a blocking agent for nitrocellulose membranes. The primary antibodies used were mouse anti-caspase-8 (Novus Biologicals, Littleton, CO), rabbit anti-XIAP (Sigma, St. Louis, MO), and mouse anti-β-actin (BioLegend, San Diego, CA). Where indi-

cated, blots were stripped with Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL) and reprobed. ImageJ (NIH) was used for densitometry analysis of specific bands in the blot images. Band density is represented as a ratio relative to the band density of the 24-h sample at an MOI of 0 (for caspase-8 and β-actin) or relative to that of the 0-h sample at an MOI of 0 (XIAP).

Cytokine analyses. Cytokine levels in supernatants were measured by using the Bio-Rad 27-plex human cytokine panel kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations and analyzed by using Bioplex Manager v5.0.

Microarray data accession number. The data sets supporting the results of this article are available in the NCBI Gene Expression Omnibus (32) under accession number GSE55849 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55849).

RESULTS AND DISCUSSION

Microarray analysis of normal and CGD PMN. To reduce variation, this study focused on the most common form of CGD, i.e., male patients with X-linked mutations in gp91^{phox}, as well as age- and sex-matched healthy control subjects. RNA was isolated following 0, 1, 4, and 24 h of incubation of normal or CGD PMN with and without *G. bethesdensis* and analyzed as described in Materials and Methods. Despite the fact that CGD patients were taking a spectrum of drugs to manage their individual clinical needs (Table 1), the mRNA levels of only 71 genes were significantly different between uninfected CGD PMN and normal PMN at baseline, with 47 being ≥2-fold lower and 4 being higher in CGD PMN (Table 2; see also the supplemental material). Previous studies reported differences in mRNA and protein expression levels in CGD PMN compared to those in normal PMN (33, 34) and monocytes (35). For example, the levels of Toll-like receptor 5 (TLR5), which detects bacterial flagellin, have been reported to be both significantly increased (33) and decreased (34) in CGD PMN, whereas we detected no significant difference between normal and CGD PMN at the mRNA level. Although not a focus of the present study, our data suggest that larger subject groups are necessary to determine whether reported differences are indeed intrinsic to CGD or merely reflect the variation in patient clinical conditions, treatments, genotypes, or other factors typical of small studies of human subjects with rare diseases.

To identify genes and pathways that were altered in PMN in response to *G. bethesdensis*, we first expressed the values for the 1-, 4-, and 24 h time points relative to those at 0 h and determined the numbers of genes whose expression changed significantly during culture using criteria described in Materials and Methods (see Data File S1 in the supplemental material). In addition to the differences at time zero mentioned above, we identified 162, 186, and 635 genes that were differentially expressed between normal and CGD PMN in the presence of *G. bethesdensis* at 1, 4, and 24 h, respectively (see Data File S1 in the supplemental material).

There were a large number of genes whose expression levels changed in culture in the absence of bacteria. We employed Venn analysis to compare *Granulibacter*-infected to noninfected PMN (Fig. 1A; see also Data File S2 in the supplemental material). Subsets of genes whose expression was altered only during culture in the absence of bacteria (class 1) could be differentiated from genes whose expression was altered in either the presence or the absence of bacteria (class 2) and those genes whose expression was altered only in the presence of *G. bethesdensis* (class 3). We next compared the class 3 (infection-associated) genes from both normal and CGD PMN (Fig. 1B). This analysis revealed genes whose expression is altered significantly only in normal PMN (class A), in both normal and CGD PMN (class B), or only in CGD PMN (class C). Class A likely represents reactive oxygen species (ROS)-dependent processes and those related to decreased bacterial viability, class B represents the PMN response to bacteria independent of bacterial viability, and class C represents the genes whose expression is altered to contend with continued bacterial viability, as CGD PMN are unable to clear *Granulibacter* (see Data File S2 in the supplemental material).

To help identify cellular functions that were altered during infection, we used Ingenuity Pathway Analysis (IPA) to further analyze class A, class B, and class C genes at each time point, and the top 5 significantly altered canonical pathways in each class are shown in Fig. 1C. Although not always in the top 5 pathways shown, death receptor signaling and NF-κB signaling genes were consistently overrepresented. Various cytokine networks also appeared to be altered, including interleukin 10 (IL-10), IL-17, and others. Upstream Regulator Analysis, a component of the IPA suite, utilizes observed transcriptional outputs to predict the regulatory inputs that might account for them. We focused on class B genes to identify the common response of PMN to *G. bethesdensis*. At all time points, there was a predicted activation of the NF-κB complex as well as activation by TLR9, lipopolysaccharide (LPS), gamma interferon (IFN-γ), and tumor necrosis factor (TNF). In addition to the upstream regulators predicted at all time points, at 1 h, triggering receptor expressed on myeloid cells 1 (TREM1), a potent immunoregulator of myeloid cells (36), was likely activated in both normal and CGD PMN, as was nuclear protein 1 (NUPR1). At later time points, there was a suggestion of activation due to the production of and autocrine stimulation by numerous cytokines, including IL-1, IL-8, IL-17, and CCL5, among others, some of which were shown to be produced at the protein level (see below). Further studies will be required to determine the dominant signaling pathways that are activated during infection.

Over time in culture, we identified a larger set of genes whose expression differed between normal and CGD PMN alone (possibly reflecting differences in rates of apoptosis) and in the presence of *G. bethesdensis*. Class A genes (Table 3), upregulated primarily in normal PMN but not in CGD PMN, included dual-specificity phosphatase 16 (DUSP16) (increased 21- to 35-fold), which is acetylated by the *Mycobacterium tuberculosis* Eis protein, resulting in the suppression of certain host responses (37). Class C genes, altered primarily in CGD but not normal PMN, included several factors that might represent an attempt to control microbial growth, such as hepcidin (HAMP) (increased 3-fold), an antimicrobial peptide and hormone that decreases bioavailable iron in the circulation, and sphingomyelin synthase 2 (SGMS2) (increased 23- to 72-fold) which is important for PMN killing of *Cryptococcus* (38). It is unclear whether these differences reflect the continued survival of *Granulibacter* in CGD PMN and its killing by normal PMN or whether they reflect different host responses to *Granulibacter*.

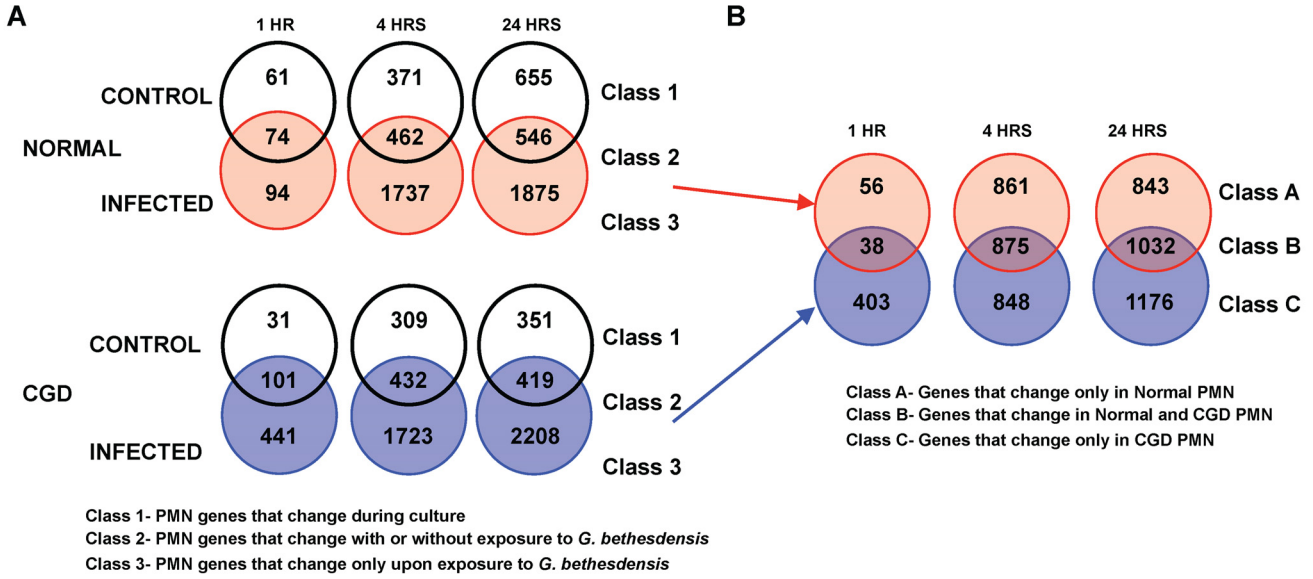
Apoptosis-related gene expression by normal and CGD PMN following infection with *G. bethesdensis*. Although phagocytosis of microbes often accelerates PMN apoptosis (23), a small subset of bacteria, including *Anaplasma*, *Francisella*, *Neisseria*, *Mycobacterium*, and *Granulibacter*, delay PMN apoptosis (18, 39–42). It has been conjectured that inhibition of apoptosis may enhance microbial dissemination during infection (43). *Granulibacter* inhibited both constitutive and Fas-induced PMN apoptosis at

TABLE 2 Genes whose transcript levels were significantly different (>2-fold) at baseline between normal and CGD PMN[a]

Gene	Probe set	Gene name (common name/family)	Fold change in expression (CGD PMN/normal PMN)
LCN2	212531_at	Lipocalin 2	3.7
GPR84	223767_at	G-protein-coupled receptor 84	3.6
LOC100292701	229264_at	Hypothetical protein LOC100292701	3.2
FCER1G	204232_at	Fc fragment of IgE, high-affinity I, receptor for; gamma polypeptide	2.4
RASGRP1	205590_at	RAS guanyl-releasing protein 1 (calcium and diacylglycerol regulated)	−2.1
ETS1	224833_at	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	−2.1
RPL14	219138_at	Ribosomal protein L14	−2.1
LOC439949	232001_at	Hypothetical protein LOC439949	−2.1
CAMK4	241871_at	Calcium/calmodulin-dependent protein kinase IV	−2.2
RBM18	225236_at	RNA-binding motif protein 18	−2.2
KLRK1	1555691_a_at	Killer cell lectin-like receptor subfamily K, member 1	−2.3
RPL36A	201406_at	Ribosomal protein L36a	−2.3
GZMB	210164_at	Granzyme B	−2.4
IQGAP2	203474_at	IQ motif-containing GTPase-activating protein 2	−2.5
TC2N	1553132_a_at	Tandem C2 domains, nuclear	−2.5
OXNAD1	227686_at	Oxidoreductase NAD-binding-domain-containing 1	−2.5
CD28	206545_at	CD28 molecule	−2.6
RCAN3	226272_at	RCAN family member 3 (calcineurin inhibitor)	−2.7
PDCD6	1568762_at	Programmed cell death 6 pseudogene	−2.7
IL7R	226218_at	Interleukin 7 receptor	−2.8
CCL5	204655_at	Chemokine (C-C motif) ligand 5 (RANTES)	−2.9
LAT/SPNS1	211005_at	Linker for activation of T cells/spinster homolog 1 (*Drosophila*)	−2.9
S1PR5	230464_at	Sphingosine-1-phosphate receptor 5	−2.9
RORA	236266_at	Retinoic acid receptor-related orphan receptor A	−2.9
RPS5	200024_at	Ribosomal protein S5	−3.0
CD247	210031_at	CD247 molecule	−3.2
CNPY4	227313_at	Canopy 4 homolog (FGF signaling regulator 4)	−3.3
KIAA1683	232433_at	KIAA1683	−3.3
SNHG1	224610_at	Small nucleolar RNA host gene 1 (non-protein coding)	−3.6
CD6	1566099_at	CD6 molecule	−3.7
CD3G	206804_at	CD3g molecule, gamma (CD3-TCR complex)	−3.8
KLRB1	214470_at	Killer cell lectin-like receptor subfamily B, member 1	−3.9
KLF12	227261_at	Kruppel-like factor 12	−4.1
SNRPN	228370_at	Small nuclear ribonucleoprotein polypeptide N	−4.1
CCR6	206983_at	Chemokine (C-C motif) receptor 6 (MIP3α receptor)	−4.3
BEX5	229963_at	Brain expressed, X-linked 5	−4.5
IL2RB	205291_at	Interleukin 2 receptor beta	−4.6
ICOS	210439_at	Inducible T-cell costimulator	−4.7
NELL2	203413_at	Neural EGFL-like 2 (protein kinase C binding)	−4.9
PRRT1	229890_at	Proline-rich transmembrane protein 1 (dispanin, interferon-induced transmembrane protein)	−5.5
KCNA3	207237_at	Potassium voltage-gated channel, shaker-related subfamily, member 3	−5.6
GCNT4	220831_at	Glucosaminyl (*N*-acetyl) transferase 4, core 2 (beta-1,6-*N*-acetylglucosaminyltransferase)	−5.9
NR3C2	205259_at	Nuclear receptor subfamily 3, group C, member 2 (mineralocorticoid receptor)	−6.2
PLXDC1	219700_at	Plexin domain-containing 1	−7.2
KCNA6	205616_at	Potassium voltage-gated channel, shaker-related subfamily, member 6	−8.9
MAP2K5	216435_at	Mitogen-activated protein kinase kinase 5	−21.8

[a] The difference in expression is expressed as the fold difference of expression in CGD PMN relative to normal PMN. FGF, fibroblast growth factor; TCR, T-cell receptor.

least in part due to delayed caspase-3 activation (18). To further explore the possible mechanistic basis for delayed apoptosis of PMN following infection by *G. bethesdensis*, we analyzed genes associated with the regulation of apoptosis, as depicted in the heat map shown in Fig. 2A. Several proapoptotic genes were significantly downregulated after infection of CGD and normal PMN, for example, *MCL1*, *SHIP-1*, *CAPN1* (*Calpain-1*), cytochrome *c* (*CYCS*), *CASP2*, *CASP3*, *CASP8*, and *CASP9*, whereas the antiapoptotic genes *XIAP* and *CSF-3* were upregulated. To confirm some of these results at the protein level, immunoblotting was performed. The apoptosis initiator caspase-8 was upregulated in the absence of bacteria over time, but this upregulation was reduced by 50 to 60% in the presence of *G. bethesdensis* (Fig. 2A and B; see also Fig. S1 in the supplemental material). In contrast, in the absence of bacteria, the protein levels of the inhibitor of apoptosis XIAP decreased by 40% in PMN over 24 h but were maintained or increased in the presence of *G. bethesdensis* (Fig. 2B; see also Fig. S1 in the supplemental material). The low-molecular-weight bands at 24 h are likely cleavage fragments of XIAP resulting from caspase activation during apoptosis (44). Interestingly, β-actin, which was previously shown to be degraded during apoptosis of PMN (45), showed less degradation in the presence of *G. bethes-*



C

	1 hr	4 hr	24 hr
Class A (Normal)	1 Role of JAK family kinases in IL-6-type Cytokine Signaling (7%) 2 Telomerase Signaling (3%) 3 Oncostatin M Signaling (6%) 4 Acute Phase Response Signaling (2%) 5 Mouse Embryonic Stem Cell Pluripotency (2%)	1 Gα12/13 Signaling (12%) 2 Signaling by Rho Family GTPases (9%) 3 ERK5 Signaling (15%) 4 Actin Nucleation by ARP-WASP complex (13%) 5 Regulation of Actin-based motility by Rho (12%)	1 RhoA Signaling (11%) 2 Tryptophan degradation to 2-Amino-3-carboxymuconate (17%) 3 Actin Nucleation by ARP-WASP complex (12%) 4 Amyotrophic lateral sclerosis signaling (9%) 5 Actin Cytoskeleton Signaling (7%)
Class B (Common)	1 Role of IL-17A in Psoriasis (14%) 2 Role of IL-17A in Arthritis (3%) 3 IL-17A Signaling in Airway Cells (3%) 4 UDP-N-acetyl-D-glucosamine Biosynthesis (6%) 5 Airway pathology in Chronic Obstructive Pulmonary Disease (9%)	1 Death Receptor Signaling (18%) 2 IL-10 Signaling (15%) 3 NF-Kβ Signaling (11%) 4 IL-17A Signaling in Airway Cells (15%) 5 Role of IL-17A in Arthritis (16%)	1 NF-Kβ Signaling (15%) 2 TNFR2 Signaling (29%) 3 Macropinocytosis signaling (20%) 4 Production of NO and ROS by Mϕ (12%) 5 TWEAK Signaling (26%)
Class C (CGD)	1 ERK5 Signaling (10%) 2 Actin Cytoskeleton Signaling (5%) 3 Assembly of RNA Polymerase II complex (11%) 4 Death Receptor Signaling (9%) 5 fMLP Signaling in Neutrophils (6%)	1 T Helper Cell Differentiation (14%) 2 β-alanine Degradation I (20%) 3 Paxillin Signaling (9%) 4 Role of JAK family kinases in IL-6-type Cytokine Signaling (18%) 5 CNTF signaling (12%)	1 PPARα/RXRα activation (11%) 2 EIF2 signaling (10%) 3 Sphingomyelin Metabolism (19%) 4 Integrin Signaling (10%) 5 Renal Cell Carcinoma Signaling (13%)

FIG 1 Identification of PMN genes and pathways altered by *G. bethesdensis*. (A) Venn analysis was used to identify subsets of genes whose expression was altered significantly in PMN at the indicated time points compared to that at time zero. The numbers in each case refer to unique genes associated with each set. Class 1 contains genes that were altered only during culture without bacteria, class 2 contains genes that were altered irrespective of the presence of *G. bethesdensis*, and class 3 contains genes altered only in infected PMN. (B) Infection-specific genes (class 3) from both host types were subjected to secondary Venn analysis to identify genes whose expression was altered only by normal PMN (class A), those whose expression was altered significantly by both normal and CGD PMN (class B), and those whose expression was altered only by CGD PMN (class C). (C) Ingenuity Pathway Analysis was used to identify canonical pathways that were altered in PMN in response to *G. bethesdensis* infection at the indicated time points. The values in parentheses indicate the percentage of the total number of genes related to the indicated pathway that were altered under each condition.

densis (Fig. 2B; see also Fig. S1 in the supplemental material). We hypothesize that delayed PMN apoptosis in the presence of *Granulibacter* (18) is explained, in part, by these transcriptional changes, some of which are also reflected at the protein level (summarized in Fig. 2C). Delayed apoptosis of permissive CGD PMN may allow bacterial adaptation prior to delivery to macrophages (via efferocytosis), in which *Granulibacter* further proliferates and may persist (19). Alternatively, it is possible that delayed apoptosis of PMN may be a host-protective response to decrease dissemination of bacteria during infections; however, further studies are required to differentiate between these possibilities.

Inflammatory responses of *G. bethesdensis*-infected normal and CGD PMN. Cytokine and chemokine production by PMN coordinates the acute inflammatory response and has been shown in some cases to differ between normal and CGD PMN (4). It was previously shown that although *G. bethesdensis* was internalized by PMN, it was a poor stimulator of NOX2 (18) and induced lower concentrations of a variety of cytokines in monocytes and monocyte-derived macrophages than did *E. coli* (19). We examined mRNA expression levels of a selection of cytokines and chemokines and their receptors (Fig. 3A). Significant induction of a spectrum of proinflammatory genes in response to bacteria was observed. To validate the microarray findings, we measured protein concentrations in culture supernatants using a Luminex-based bead assay. As shown in Fig. 3B to G, protein accumulation in culture supernatants paralleled the observed induction of mRNA. In several cases, e.g., monocyte chemotactic protein 1 (MCP1; CCL2) (Fig. 3C), IP-10 (CXCL10) (Fig. 3D), and RANTES (CCL5) (Fig. 3F), larger amounts of protein were detected in CGD PMN cultures both at rest and following exposure to bacteria. Interestingly, in the case of CCL5, CGD PMN expressed lower levels of mRNA at baseline, suggesting either the production of protein from preformed stores in PMN or posttranscriptional regulation. For several important genes, e.g., IL-8 (CXCL8), the microarrays lacked

TABLE 3 Genes whose expression levels were significantly altered by incubation of PMN with *Granulibacter* at 1, 4, and 24 h relative to those at time zero[a]

Class and probe set	Gene	Description	Fold change in expression					
			Normal PMN			CGD PMN		
			1 h	4 h	24 h	1 h	4 h	24 h
A (normal PMN)								
244080_at	LOC100287039	Hypothetical protein LOC100287039	39	35	71			
1563505_at	DUSP16	Dual-specificity phosphatase 16	26	41	21		9	
222293_at	CADM4	Cell adhesion molecule 4	6	9	9			
B (shared)								
207850_at	CXCL3	Chemokine (C-X-C motif) ligand 3	24	64	112	18	77	106
1564630_at	EDN1	Endothelin 1	21	10	42	20	18	44
205220_at	GPR109B	G-protein-coupled receptor 109B	7	11	8	5	5	5
1555011_at	ZFYVE16	Zinc finger, FYVE domain-containing 16	6	23	14	7	12	4
223346_at	VPS18	Vacuolar protein sorting 18 homolog (*Saccharomyces cerevisiae*)	5	11	3	5	5	3
213560_at	GADD45B	Growth arrest and DNA damage inducible, beta	5	10	9	4	5	3
230328_at	LOC100292228	Hypothetical protein LOC100292228/RRN3 RNA polymerase I transcription factor homolog (*S. cerevisiae*)	3	8	8	2	4	3
214972_at	MGEA5	Meningioma-expressed antigen 5 (hyaluronidase)	−3	−3	−4	−2	−2	−4
C (GCD PMN)								
227038_at	SGMS2	Sphingomyelin synthase 2				34	74	23
1553286_at	ZNF555	Zinc finger protein 555				23	40	55
224277_at	MOP-1	MOP-1				21	12	11
1553364_at	PNPLA1	Patatin-like phospholipase domain-containing 1	16	10		17	12	8
209674_at	CRY1	Cryptochrome 1 (photolyase-like)				4	5	3
224959_at	SLC26A2	Solute carrier family 26 (sulfate transporter), member 2			4	4	18	17
201479_at	DKC1	Dyskeratosis congenita 1, dyskerin				4	2	2
235324_at	SFRS3	Splicing factor, arginine/serine-rich 3				4	5	3
220491_at	HAMP	Hepcidin antimicrobial peptide				3	3	3
224820_at	FAM36A	Family with sequence similarity 36, member A			2	3	2	4
225643_at	MAPK1IP1L	Mitogen-activated protein kinase 1-interacting protein 1-like				2	2	3
224835_at	GPCPD1	Glycerophosphocholine phosphodiesterase GDE1 homolog (*S. cerevisiae*)			−3	2	2	−3
200010_at	RPL11	Ribosomal protein L11				−2	−2	−2
38241_at	BTN3A3	Butyrophilin, subfamily 3, member A3				−2	−3	−2
223440_at	C16orf70	Chromosome 16 open reading frame 70				−3	−2	−2
235415_at	RPRD2	Regulation of nuclear pre-mRNA domain-containing 2				−3	−3	−2
205488_at	GZMA	Granzyme A (granzyme 1, cytotoxic-T-lymphocyte-associated serine esterase 3)		−4		−3	−9	−5
227134_at	SYTL1	Synaptotagmin-like 1				−4	−7	−8
226018_at	C7orf41	Chromosome 7 open reading frame 41				−5	−4	−5

[a] Genes identified by Venn analysis as being expressed in infected normal PMN only, both infected normal and CGD PMN, or only infected CGD PMN at all time points.

high-quality probe sets, although we detected a nearly 4-log increase in the IL-8 protein concentration in supernatants upon exposure of both normal and CGD PMN to *G. bethesdensis*. Protein concentrations for an additional 17 cytokines, chemokines, and soluble receptors are presented in Fig. S2 in the supplemental material. Paracrine and autocrine activation of PMN by some of these secreted factors was evident in the IPA upstream activator analysis described above and likely contributes to the cellular responses seen here.

Transcriptome analyses of *G. bethesdensis* in normal and CGD PMN. To better understand microbial stress responses and adaptation to human PMN, we prepared RNA from cultures of *G. bethesdensis* grown alone and in the presence of either normal or CGD PMN and analyzed pathogen mRNA expression using a custom oligonucleotide array developed for *G. bethesdensis* (6). Previous studies using identical culture conditions showed that *Granulibacter* is internalized by PMN in a complement-dependent manner, and although normal PMN reduce *Granulibacter* CFU by ~50% at 24 h, CGD PMN slow the growth of the bacteria but do not kill them. Importantly, we detected no significant differences in gene expression between bacteria incubated in the presence of normal serum and those in the presence of CGD serum, suggesting that the antibiotics and drugs that were present in the CGD subjects were not contributing significantly to the experimental outcome. We found that several genes were so rapidly induced that their largest change occurred during sample processing (likely during the 8-min centrifugation step used to synchronize the interaction of bacteria with host cells). For example, at time zero (following synchronization), the raw normalized fluorescence of catalase (GbCGDNIH1_1969) (Fig. 4A) increased 15-fold from a fluorescence value of 201 ± 50 (mean ± SD) for *G. bethesdensis* in normal serum to a value of 3,061 ± 214 in the

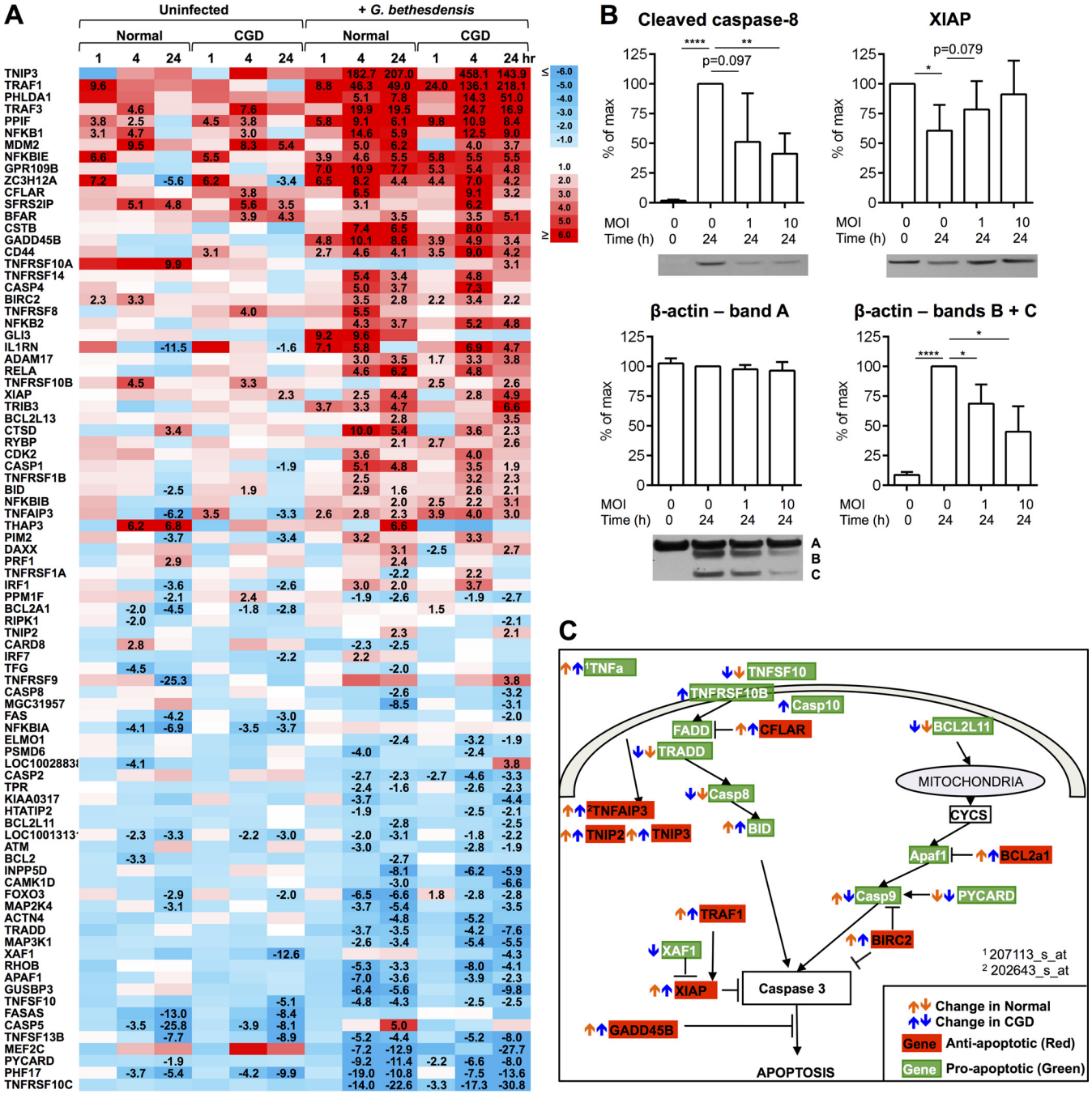


FIG 2 Regulation of PMN apoptosis by *G. bethesdensis*. (A) Heat map of genes related to apoptosis. Colors indicate the fold change observed independent of measured significance under the indicated conditions (see the key). Under conditions where a statistically significant difference between time zero and the indicated time points was detected, the fold change is shown as black text. (B) Lysates were generated from normal PMN at time zero or 24 h after incubation with preopsonized *G. bethesdensis* cells at an MOI of 0, 1, or 10 bacteria per PMN and immunoblotted for caspase-8 and XIAP. All blots were stripped and reprobed for β-actin. A representative blot for each protein is shown, along with summary densitometry results for all 4 donors, expressed as the mean percentage of the maximum signal ± SD, and statistical significance is indicated with asterisks (*, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$). (C) Model of alterations in gene expression that might contribute to the observed delay of PMN apoptosis induced by *G. bethesdensis*. For TNF-α and TNFAIP3, the probe sets used are indicated.

presence of normal PMN. In CGD serum, the catalase values were 210 ± 70, versus 99 ± 22 in the presence of CGD PMN. These results are in agreement with previous studies of *Escherichia coli* gene expression in response to normal and CGD neutrophils (46). Catalase peroxidase (GbCGDNIH1_1677) and peroxiredoxin (GbCGDNIH1_0680) displayed similar expression patterns (Fig. 4B and C). Several stress response genes, for example, *groEL* and *groES*, were upregulated rapidly in normal but not CGD PMN (Fig. 4D and E), while others, such as the one encoding the small heat shock protein (GbCGDNIH1_1358), were upregulated by

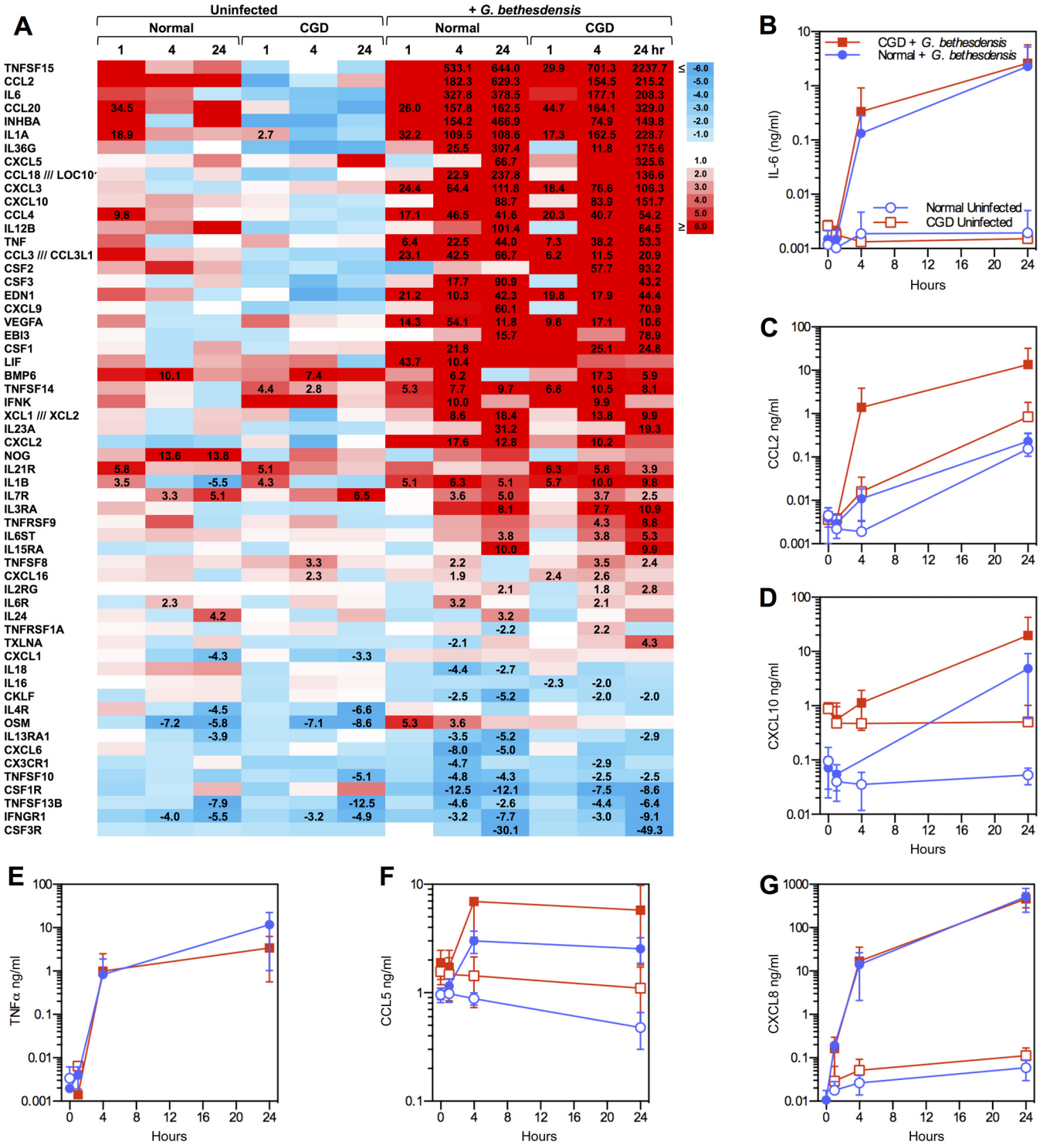


FIG 3 Inflammatory genes are induced by PMN at the mRNA and protein levels. (A) Heat map of cytokines, chemokines, and receptors whose expression was altered significantly under at least two of the conditions tested. Significant fold changes are shown as text. (B to G) Protein concentrations of the indicated cytokines, in mean nanograms per milliliter ± SD, detected in culture supernatants from normal PMN and CGD PMN that were either uninfected or infected with *G. bethesdensis*. The key shown in panel B applies to panels B to G.

Granulibacter in the presence of either PMN type (Fig. 4F). Possibly in response to PMN lactoferrin-mediated iron sequestration, the bacteria upregulated genes involved in iron acquisition upon incubation with PMN (Fig. 4I). Together, these findings validate a key difference between microbial responses to ROS-generating normal PMN and those to ROS-defective CGD PMN while demonstrating similar responses to other host processes not thought to depend on ROS generation (e.g., iron deprivation).

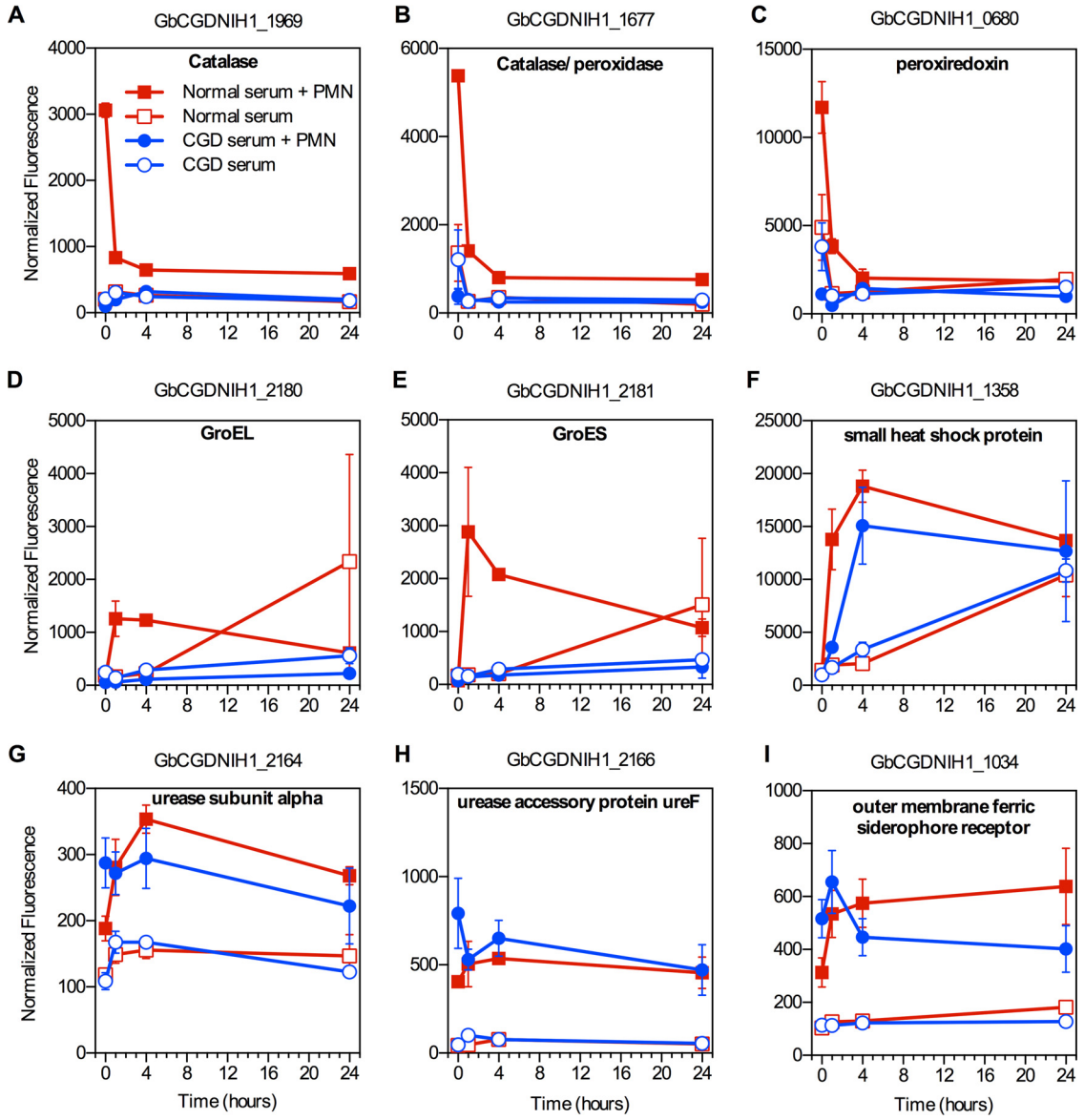


FIG 4 Alterations in *G. bethesdensis* gene expression upon exposure to normal or CGD PMN. Normalized raw fluorescence values from the custom DNA array at 0, 1, 4, and 24 h of culture are shown as means ± standard errors of the means. Representative genes involved in antioxidant defenses (A to C), the stress response (D and E), acid tolerance (G and H), and iron acquisition (I) are shown for comparison of expression levels in serum alone to those in the presence of serum and PMN from normal donors and CGD donors. The key shown in panel A applies to all panels.

Given these extremely rapid responses to recognized innate immune defenses of PMN, we chose to perform a global analysis of *G. bethesdensis* gene expression relative to that for the serum control at each time point instead of relative to that for the samples at time zero, as had been done for host cells. First, we used Gene Set Enrichment Analysis (GSEA) to identify pathways that were significantly altered upon incubation of bacteria with PMN. Ninety-nine gene sets based on functional categories defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) were assembled, and 45/99 were filtered out of the analysis by using a minimum gene set size of 10 and a maximum gene set size of 500, leaving 54/99 gene sets for GSEA. Gene sets achieving an FDR *q* score of 0.25 or lower are listed in Table 4. The expressions of most gene sets were similarly altered by bacteria in the presence of either normal or CGD PMN, notably the ribosome (gbe03010) and one-carbon pool by folate (gbe00670) associated with the absence of PMN (downregulated with PMN), suggesting that adaptation to intracellular persistence by *Granulibacter* may involve a less active metabolic state. The pentose phosphate pathway (gbe00030), which generates NADPH and pentoses from glucose, was downregulated in normal but not CGD PMN. Glycerophospholipid metabolism (gbe00564) increased only for *G. bethesdensis* in CGD PMN at 24 h. Together, these results suggest that although *G. bethesdensis* may be more metabolically active in CGD PMN than in normal PMN, the overall response may indicate that intracellular access to nutrients is severely limited compared to that in serum alone.

Given that not all genes are mapped to KEGG gene sets and that

TABLE 4 GSEA analysis of *G. bethesdensis* transcriptomes over time for comparison to those in the absence of PMN

KEGG gene set	Description	Enrichment[a]							
		Normal PMN				CGD PMN			
		0 h	1 h	4 h	24 h	0 h	1 h	4 h	24 h
gbe00010	Glycolysis/gluconeogenesis								
gbe00020	Citrate cycle (TCA cycle)		↓	↓		↓		↓	
gbe00030	Pentose phosphate pathway	↓	↓	↓					
gbe00051	Fructose and mannose metabolism								
gbe00061	Fatty acid biosynthesis	↓	↓	↓	↓		↓	↓	↓
gbe00190	Oxidative phosphorylation		↓	↓		↓	↓	↓	
gbe00230	Purine metabolism			↓	↓			↓	↓
gbe00240	Pyrimidine metabolism	↓	↓	↓	↓	↓		↓	↓
gbe00250	Alanine, aspartate, and glutamate metabolism	↓		↓	↓	↓		↓	↓
gbe00260	Glycine, serine, and threonine metabolism	↓	↓	↓	↓			↓	
gbe00270	Cysteine and methionine metabolism		↓		↓	↓			↓
gbe00290	Valine, leucine, and isoleucine biosynthesis		↓	↓	↓	↓			↓
gbe00300	Lysine biosynthesis	↓	↓	↓	↓	↓	↓	↓	
gbe00310	Lysine degradation								
gbe00330	Arginine and proline metabolism		↓						
gbe00340	Histidine metabolism								
gbe00350	Tyrosine metabolism								
gbe00362	Benzoate degradation								
gbe00380	Tryptophan metabolism								
gbe00400	Phenylalanine, tyrosine, and tryptophan biosynthesis		↓	↓				↓	
gbe00480	Glutathione metabolism								
gbe00500	Starch and sucrose metabolism								
gbe00520	Amino sugar and nucleotide sugar metabolism								
gbe00540	Lipopolysaccharide biosynthesis								
gbe00550	Peptidoglycan biosynthesis		↓	↓	↓		↓	↓	
gbe00564	Glycerophospholipid metabolism								↑
gbe00620	Pyruvate metabolism		↓	↓				↓	
gbe00624	Polycyclic aromatic hydrocarbon degradation								
gbe00627	Aminobenzoate degradation								
gbe00630	Glyoxylate and dicarboxylate metabolism		↓		↓		↓		
gbe00640	Propanoate metabolism		↓	↓	↓		↓	↓	↓
gbe00650	Butanoate metabolism		↓	↓		↓		↓	
gbe00670	1-carbon pool by folate	↓	↓	↓	↓	↓	↓	↓	↓
gbe00680	Methane metabolism		↓				↓		
gbe00730	Thiamine metabolism								
gbe00770	Pantothenate and coenzyme A biosynthesis							↓	
gbe00790	Folate biosynthesis								
gbe00860	Porphyrin and chlorophyll metabolism								
gbe00900	Terpenoid backbone biosynthesis		↓	↓					
gbe00910	Nitrogen metabolism	↓	↓	↓	↓			↓	↓
gbe00970	Aminoacyl-tRNA biosynthesis	↓	↓	↓	↓	↓		↓	
gbe01100	Metabolic pathways	↓	↓	↓	↓	↓	↓	↓	
gbe01110	Biosynthesis of secondary metabolites		↓	↓	↓	↓	↓	↓	
gbe01120	Microbial metabolism in diverse environments		↓	↓	↓	↓	↓	↓	
gbe02010	ABC transporters								
gbe02020	Two-component system								
gbe03010	Ribosome	↓	↓	↓	↓	↓	↓	↓	↓
gbe03018	RNA degradation	↓			↓	↓		↓	↓
gbe03030	DNA replication								
gbe03060	Protein export	↓	↓			↓			
gbe03070	Bacterial secretion system								
gbe03410	Base excision repair		↓			↑			
gbe03430	Mismatch repair								
gbe03440	Homologous recombination					↑			

[a] ↑, FDR *q* value of ≤0.25 for enrichment correlating with the presence of PMN; ↓, FDR *q* value of ≤0.25 for enrichment correlating with the absence of PMN.

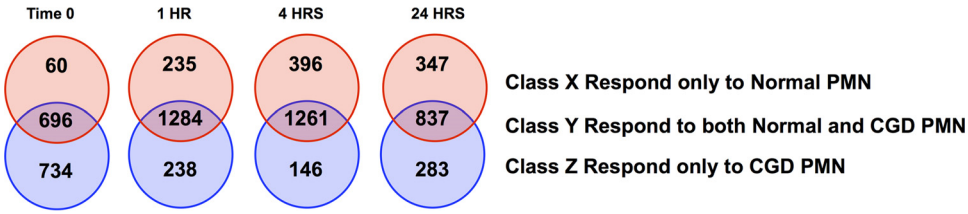


FIG 5 Identification of *Granulibacter* genes that respond to normal PMN, both normal and CGD PMN, or only CGD PMN. Shown is Venn analysis of unique genes (the numbers of genes belonging to each set are indicated) whose expression was significantly altered at each time point by the presence of the indicated type of PMN. *G. bethesdensis* genes whose expression was altered only in the presence of normal PMN formed class X, genes whose expression was altered in response to both normal and CGD PMN formed class Y, and genes whose expression was altered only in the presence of CGD PMN formed class Z.

a large proportion of gene sets were excluded from GSEA because of their small size (<10 genes), we also subjected the data to Venn analysis. Numbers of genes that were significantly different between serum and serum-plus-PMN conditions at each time point for each host are shown in Fig. 5, and gene identifications are listed in Data File S3 in the supplemental material. Venn analysis of class X (significantly different between *Granulibacter* cells cultured with normal PMN compared to those cultured in serum alone), class Y (common to both normal and CGD PMN), and class Z (different only for bacteria in CGD PMN compared to serum) was used to identify genes in each class whose expression was consistently different at 3 out of 4 time points.

Direct comparisons of incubations of *Granulibacter* cells with either normal PMN or CGD PMN across the 0-, 1-, 4-, and 24-h time points indicated that the expression levels of only 91, 107, 64, and 11 *G. bethesdensis* genes, respectively, were significantly different in CGD compared to normal PMN (see Data File S4 in the supplemental material). As expected, transcripts encoding catalases and GroES/EL, among others, were expressed at significantly lower levels in CGD PMN than in normal PMN. Genes that were expressed at higher levels by *Granulibacter* in CGD PMN than in normal PMN included the genes encoding outer membrane protein A (OmpA; GbCGDNIH1_0359), hemagglutinin-related protein (GbCGDNIH1_1474), and several others (see Data File S4 in the supplemental material).

Bacterial responses to host cells are an important aspect of microbial pathogenesis and may provide specific targets for future antimicrobial therapies. In general, microbes must first contend with immediate physicochemical stresses exerted by host defenses and then adapt through metabolic reprogramming to permit proliferation and persistence in their new niche (47, 48). Both normal and CGD PMN contain a variety of antimicrobial factors to prevent microbial survival, including preformed oxygen-independent antimicrobial polypeptides (e.g., defensins and cathelicidins), enzymes (e.g., elastase), and nutrient-sequestering proteins (e.g., lactoferrin, neutrophil gelatinase-associated lipocalin) (49). It is expected that many of the class Y genes (responsive to both types of PMN) reflect these stressors. In contrast, due to mutations in the superoxide anion-producing NADPH oxidase NOX2, the CGD phagosome is deficient in rapidly induced chemically reactive products such as superoxide anions, hydrogen peroxide, and hypohalous acids (2). As discussed above, when bacteria were incubated with normal but not CGD PMN, rapid upregulation of bacterial genes involved in protection against ROS (e.g., catalase [GbCGDNIH_1969] and peroxidase [GbCGDNIH_1677]) was observed.

ClpB is essential for *G. bethesdensis* survival in PMN. Among the many stress-related genes whose expression was altered by *G. bethesdensis* in response to normal and CGD PMN, we noted early induction of ClpB (GbCGDNIH1_1803) (Fig. 6A). The protein encoded by *clpB* is a protein aggregate-disrupting chaperone that has been shown to be a virulence factor in a variety of pathogens, including *Francisella tularensis* (50), *Leptospira interrogans* (51), and *Enterococcus faecalis* (52). To date, our efforts to genetically manipulate *G. bethesdensis* using standard molecular biology approaches have failed. We report here for the first time the use of antisense interference in *Granulibacter* using peptide-coupled antisense phosphorodiamidate morpholino oligomers (PPMOs). PPMOs are thought to act through steric blockade of ribosome binding to or translation of mRNA (B. L. Geller and D. E. Greenberg, unpublished data). We prepared PPMOs targeting *clpB* and *acpP* (GbCGDNIH1_2209), a gene previously shown to be essential in other Gram-negative microbes, to confirm that the peptide used was capable of delivering antisense oligonucleotides to *Granulibacter*. As shown in Fig. 6B, a PPMO with a scrambled-sequence oligomer (Scr PPMO) had no effect on bacterial growth in medium over 2 days compared to that in control cultures. In contrast, the *G. bethesdensis* AcpP PPMO inhibited growth in medium, while the ClpB PPMO had no effect (Fig. 6B). We next tested whether knockdown of these genes altered bacterial survival in the presence of serum and PMN. Although there was no effect of the Scr PPMO on the viability of *G. bethesdensis* in either normal or CGD PMN, PPMOs targeting either *acpP* or *clpB* reduced the viability of *G. bethesdensis* in PMN for both host types (Fig. 6C). That *clpB* knockdown changes the bacteriostatic effect of CGD PMN into a bactericidal effect suggests that *clpB* plays an essential role in microbial defenses against oxygen-independent PMN antimicrobial factors and that *clpB* is essential for survival of *Granulibacter* in PMN.

***G. bethesdensis* metabolism as an antibiotic target.** In addition to responses to host defenses, microbial adaptation to an intracellular existence within host cells requires reprogramming of basic metabolism. Microbes attempt to counteract nutrient-sequestering host factors such as iron-binding lactoferrin and zinc-binding calprotectin through the production of trace element acquisition systems such as GbCGDNIH_1034 (outer membrane ferric siderophore receptor) and the upregulation of specific transporters and heme oxygenase (GbCGDNIH1_0284). Interestingly, several genes involved in copper resistance were upregulated; for example, the copper-exporting ATPase (GbCGDNIH1_2088) was strongly upregulated in normal PMN, and CutA (GbCGDNIH_1018), a periplasmic divalent cation tolerance protein which is involved in copper resistance, was upregulated in both PMN types.

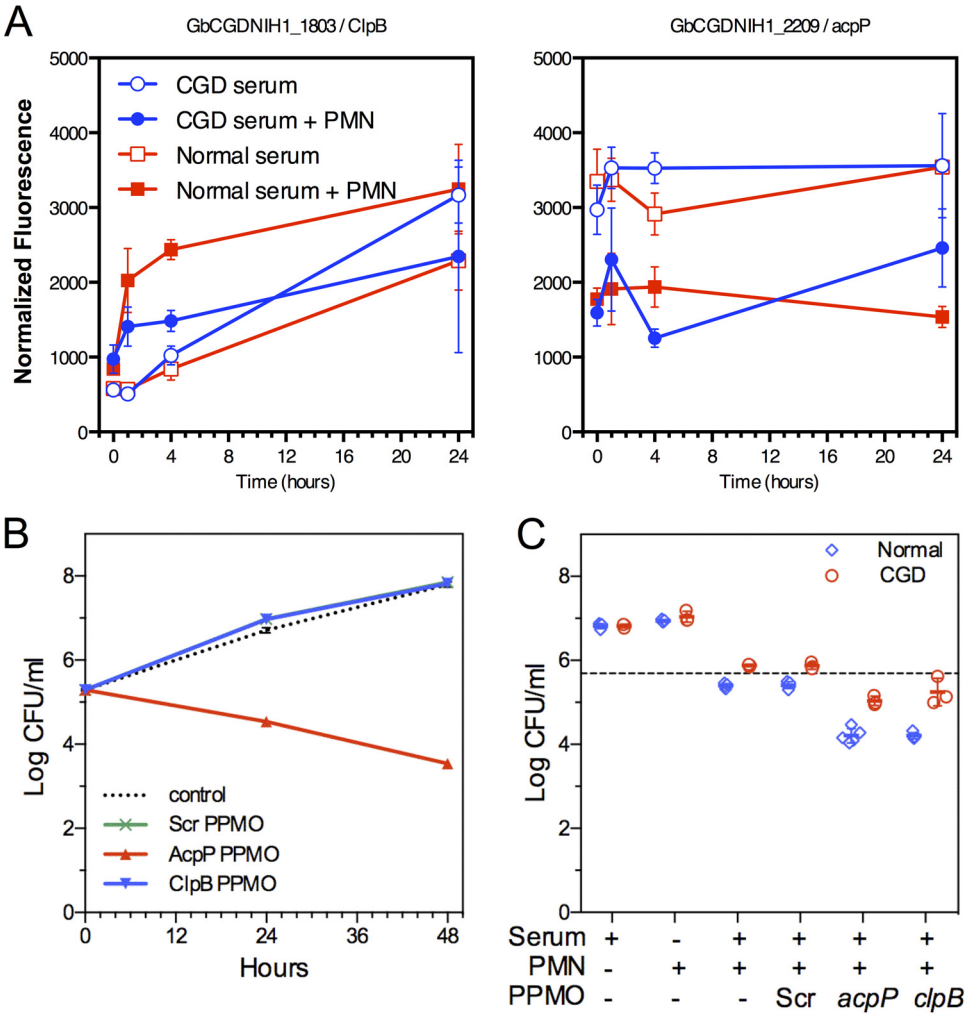


FIG 6 PPMO knockdown of *clpB* and *acpP* expression in *G. bethesdensis*. (A) Normalized fluorescence values (means ± standard errors of the means) for ClpB and AcpP probe sets. (B) *G. bethesdensis* was grown in simple medium as described in Materials and Methods, without and with 40 μM PPMOs possessing a scrambled sequence (Scr PPMO) or sequences targeting *acpP* (AcpP PPMO) or *clpB* (ClpB PPMO). Results shown are means ± SD ($n = 3$ independent experiments). (C) Surviving CFU of *G. bethesdensis* following 24 h of incubation with the indicated PPMO in the presence or absence of serum or normal or CGD PMNs. Individual results as well as means ± SD are shown for 5 normal and 3 gp91^{phox} CGD subjects, and the inoculum at time zero (MOI of ~1:1) is depicted as a dotted line.

Less is known, however, of the carbon and nitrogen sources of pathogens in host cell compartments such as the phagosome or the cytoplasm and the alterations to intermediary metabolism during intracellular existence. Studies to date suggest that, at least during the time frame of these studies, *Granulibacter* resides in a membrane-bound phagosomal compartment. GSEA indicated a significant downregulation of numerous metabolic pathways by *Granulibacter* in both normal and CGD PMN (Table 4). Downregulation of ribosomes, a hallmark of the stringent response, was uniformly observed in *Granulibacter* cells in the presence of PMN. Interestingly, several metabolic enzymes were strongly upregulated, suggesting that they might be important for intracellular metabolism. Isoamylase (GbCGDNIH_0916) and α amylase (GbCGDNIH_0752) were both strongly upregulated by *Granulibacter* in CGD PMN but less so in normal PMN. Some components of one-carbon (C_1) metabolism (e.g., methanol dehydrogenase and formate dehydrogenase [GbCGDNIH_2385]) are upregulated, as are enzymes involved in three-carbon (C_3) metabolism, including pyruvate dehydrogenase (PDH) E1 (GbCGDNIH_1183). Based on the finding of elevated PDH expression levels, we tested the antibacterial activity of triphenylbismuthdichloride (TPBC), an inhibitor of PDH (53). *Granulibacter* cells were sensitive to TPBC, especially in the absence of serum, and could be killed intracellularly as well (Fig. 7). Although further studies are required to establish efficacy in mouse infection models, these data demonstrate the importance of pyruvate dehydrogenase during intracellular survival of *G. bethesdensis*. Previously, transcriptional responses of conidia (spores) and hyphae of *Aspergillus fumigatus*, an opportunistic fungal pathogen in CGD and other diseases, in response to normal and CGD PMN were examined (54). *Granulibacter* shares with *Aspergillus* a rapid response to the oxidative environment of normal but not CGD PMN with the upregulation of catalases and peroxidases. Both microbes attempt to acquire iron, either for metabolic function or as an additional antioxidant factor, and both organisms appear to significantly alter their metabolism during their adaptation to growth or survival

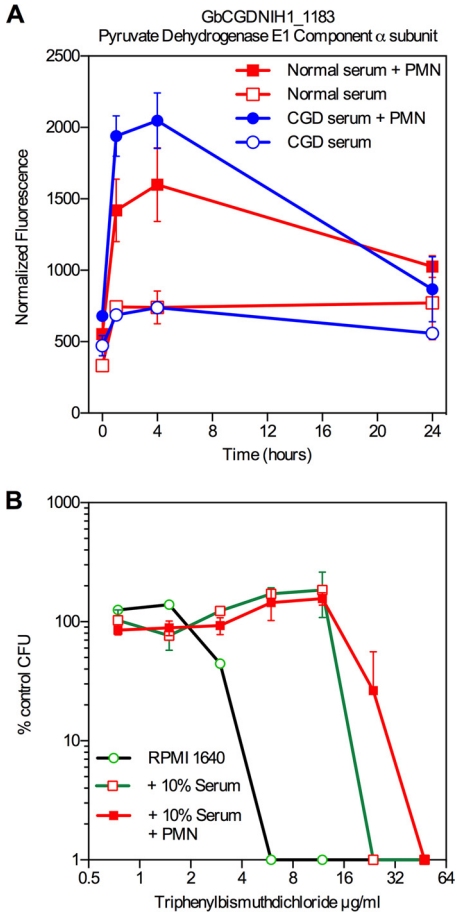


FIG 7 Pyruvate dehydrogenase as an antimicrobial target. (A) Normalized fluorescence values from the microarray experiments for the pyruvate dehydrogenase E1 α subunit (GbCGDNIH1_1183), shown as means ± standard errors of the means. (B) Antimicrobial activity of triphenylbismuthdichloride against *G. bethesdensis* in HEPES-buffered RPMI alone, in the presence of 10% human serum, and in the presence of serum with PMN relative to the control (bacteria alone) after 24 h of incubation ($n = 3$ to 6 normal donors or experimental replicates).

in PMN. In the presence of PMN, *A. fumigatus* upregulated isocitrate lyase and the glyoxylate shunt of the tricarboxylic acid (TCA) cycle (54), and similar adaptations have been observed for *Candida* and *Mycobacterium* during intracellular growth in macrophages (55). In *Granulibacter*, however, there was a downregulation of isocitrate lyase (GbCGD1_0486), and the expression of malate synthase (GbCGD1_2371) was not altered during intracellular growth, suggesting that C_2 compounds (e.g., acetate), the main substrates for the glyoxylate shunt, may not be as important to the intracellular persistence of *Granulibacter*.

Finally, as a consequence of the failure of CGD PMN to kill *Granulibacter*, several genes associated with bacterial replication were altered. *maf* (GbCGDNIH1_1029), which inhibits septum formation, was downregulated most in CGD PMN than under other conditions, while a septum formation initiator (GbCGDNIH1_1182) was downregulated in normal PMN and upregulated in CGD PMN, suggesting that *Granulibacter* proliferates in CGD PMN. These regulators of microbial growth may be ideal targets for PPMO-mediated knockdown in future studies.

Simultaneous systems-level analysis of both the host and pathogen during infection *in vitro* has great potential to elucidate the dynamic processes that govern which entity will ultimately survive the interaction. Although this study identified several microbial factors for further evaluation as antimicrobial targets, further work will be required to determine whether these will be useful clinically. Future studies will attempt to determine whether or not ClpB plays an important role during infections *in vivo* and whether or not TPBC can prevent or cure *Granulibacter* infections.

Granulibacter shares with several other known intracellular pathogens a phenotype of causing delayed neutrophil apoptosis as well as decreased stimulation of host defenses compared to other microbes. Ongoing studies are examining the persistence of *Granulibacter*, particularly in macrophages, and its subcellular trafficking during infection, with the working hypothesis being that metabolic adaptations of this microbe to slow intracellular growth contribute to its apparent long-term survival in human patients with CGD without causing overt clinical disease.

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