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Persistence of the bacterial pathogen *Granulibacter bethesdensis* in Chronic Granulomatous Disease monocytes and macrophages lacking a functional NADPH oxidase[1]

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Abstract

Granulibacter bethesdensis is a Gram-negative pathogen in patients with Chronic Granulomatous Disease (CGD), a deficiency in the phagocyte NADPH oxidase. Repeated isolation of genetically identical strains from the same patient over years, and prolonged waxing and waning seropositivity in some subjects, raises the possibility of long-term persistence. *G. bethesdensis* resists killing by serum, CGD polymorphonuclear leukocytes (PMN), and antimicrobial peptides, indicating resistance to non-oxidative killing mechanisms. While *G. bethesdensis* extends the survival of PMN, persistent intracellular bacterial survival might rely on longer-lived macrophages and their precursor monocytes. Therefore, we examined phagocytic killing by primary human monocytes and monocyte-derived macrophages (MDM). Cells from both normal and CGD subjects internalized *G. bethesdensis* similarly. *G. bethesdensis* stimulated superoxide production in normal monocytes, but to a lesser degree than in normal PMN. Normal but not CGD monocytes and MDM killed *G. bethesdensis* and required *in vitro* treatment with interferon-γ (IFN-γ) to maintain this killing effect. Although *in vitro* IFN-γ did not enhance *G. bethesdensis* killing in CGD monocytes, it restricted growth in proportion to CGD PMN residual superoxide production, providing a potential method to identify patients responsive to IFN-γ therapy. In IFN-γ-treated CGD MDM, *G. bethesdensis* persisted for the duration of the study (7 days) without decreasing viability of the host cells. These results indicate that *G. bethesdensis* is highly resistant to oxygen-independent microbicides of myeloid cells, requires an intact NADPH oxidase for clearance, and can persist long-term in CGD mononuclear phagocytes, likely relating to the persistence of this microorganism in infected CGD patients.

Introduction

Granulibacter bethesdensis, a member of the *Acetobacteraceae* family, is a human pathogen that causes infection in patients with Chronic Granulomatous Disease (CGD), a deficiency in the NADPH oxidase (1, 2). This Gram-negative, rod-shaped bacterium has been isolated from at least 6 CGD patients in the United States and Spain (3–5). *G. bethesdensis* can cause recurrent infections in a CGD patient over several years that may be the result of reinfection with different bacterial strains or reactivation of the same strain (3, 4). This organism displays a high degree of resistance to host defenses as evidenced by isolation from spleens of wild-type mice 76 days after infection and from excised lymph nodes of a CGD patient 5 months after the onset of symptoms and unresponsiveness to antimicrobial treatment (3). The increasing numbers of clinical infections caused by *G. bethesdensis* as well as other

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species of acetic acid bacteria (6–12) underscore the need for a better understanding of the microbial pathogenesis of the *Acetobacteraceae* family.

Previous work has demonstrated that *G. bethesdensis* is serum-resistant and internalized by human peripheral blood polymorphonuclear leukocytes (PMN) in a complement-dependent manner (13). CGD PMN were incapable of killing *G. bethesdensis*, suggesting that this organism is resistant to non-oxidative killing mechanisms. PMN are short-lived cells that undergo apoptosis and efferocytosis by macrophages for inflammation resolution (14). Delayed PMN apoptosis and defective macrophage efferocytosis are observed in CGD (15, 16). Given that *G. bethesdensis* persists for 24 hours in CGD PMN, in contrast to ~50% clearance of the organism by normal PMN in the same time frame (13), we hypothesized that long-term intracellular bacterial survival in the host might rely on longer-lived macrophages and precursor monocytes. Here we show for the first time that normal monocytes and monocyte-derived macrophages (MDM) internalize *G. bethesdensis* and kill it in an NADPH oxidase-dependent manner while this organism persists and proliferates in CGD monocytes and MDM. Resistance of *G. bethesdensis* to NADPH oxidase-independent myeloid host defenses likely plays an important role in long-term infections observed in CGD patients.

Materials and Methods

Ethics statement and cell isolation

Blood samples were obtained after informed consent from healthy and CGD donors enrolled at the NIH Clinical Center as defined in NIH institutional review board-approved protocols. Human polymorphonuclear leukocytes (PMN) were isolated from citrated peripheral blood as previously described (17). For the monocyte isolation, every 10 ml of citrated peripheral blood was diluted 1:3 in HBSS(−) and layered on 15ml of 53% percoll (diluted in PBS without Ca^{2+}, Mg^{2+}). PBMC layers were collected and cells washed twice in HBSS(−). Monocytes were isolated with CD14 antibody-coupled magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Monocyte purity (~85–95%) was confirmed using CD14 surface staining and flow cytometry as well as differential staining and light microscopy. In some cases, $CD14^+$ cells were further differentiated into monocyte-derived macrophages (MDM) by culturing in RPMI 1640 containing 2mM L-glutamine, 10mM HEPES, pH 7.2, 10% autologous serum, and 50 ng/ml M-CSF (Invitrogen, Carlsbad, CA) for 7 days. Media was replaced every 3–4 days. Where indicated, monocytes and MDM were treated with 65 units IFN-γ/ml (eBioscience, San Diego, CA) or PBS control for 2 days. Serum was collected from BD serum separator tubes according to the manufacturer's instructions and used fresh or snap frozen and stored at −80°C.

Bacterial strains and cultures

G. bethesdensis NIH1.1 was cultured for 2 days and subcultured for 1 day to mid-log phase in YPG medium (13). *Escherichia coli* TOP10 was grown for 1 day and subcultured for 3 hours to mid-log phase in Lysogeny broth. Washed bacteria were enumerated by OD 600nm using a NanoDrop® ND-1000 (Grace Scientific, LLC, Clarksburg, MD).

Bacterial internalization assays

Cytospins—Freshly isolated monocytes (2.5×10^5/condition) were infected with *G. bethesdensis* for 1 hr at the indicated multiplicity of infections (MOIs) in 96-well plates. Plates had been precoated with 10% autologous serum for at least 1 hr at 37ºC and washed three times with HBSS(−). Cells were incubated with no serum, autologous serum, or autologous serum incubated for 30 min at 56°C, which inactivates complement. Heat-inactivated serum was centrifuged at 10,000 × g for 10 min at 4°C to pellet protein

aggregates. Plates were centrifuged at 362 × g for 8 min at 4ºC to synchronize phagocytosis. Cells were removed with Cellstripper™ (Mediatech, Inc., Manassas, VA), subjected to cytospin, and differentially stained with HARLECO Hemacolor stain set (EMD Chemicals, Billerica, MA). Each sample was imaged and intracellular bacteria scored in a blinded manner.

Confocal and epifluorescence microscopy—3×10^5 or 5×10^5 freshly isolated monocytes were adhered to 12 mm or 18 mm ethanol-washed cover glasses in 24-well or 12-well plates, respectively. *G. bethesdensis* was labeled with pHrodo™ Red succinimidyl ester (Invitrogen) according to the manufacturer's instructions. pHrodo-labeled bacteria were incubated with cells at the indicated MOIs and time points in the presence of serum or heat-treated serum (as described above) in RPMI 1640 containing 25 mM HEPES, pH 7.2. Plates were centrifuged at 362 × g for 8 min at 4ºC to synchronize phagocytosis. Cells were washed with cold PBS twice, fixed with 4% PFA for 5 min at RT in the dark, and washed with cold PBS or HBSS twice. Cover glasses were mounted on glass slides using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA), or 1 μg/ml DAPI was added to the last wash prior to mounting, and imaged using a Leica SP5 X-WLL confocal microscope or a Leica Episcope with a DFC360FX camera (Leica Microsystems). For kinetic analyses, at least 100 cells were scored for the presence of internalized pHrodo bacteria using Imaris software (Bitplane, South Windsor, CT).

Luminol-enhanced chemiluminescence

Bacterial activation of PMN or monocyte luminol-enhanced chemiluminescence was measured at the indicated MOIs in an assay composed of PMN or monocytes at 1×10^6/ml in RPMI 1640 medium with 10% autologous serum, 25mM HEPES (pH 7.4), and 50 μM luminol essentially as previously described (13).

Bacterial killing assays

Freshly isolated PMN, freshly isolated monocytes, or 2-day old monocytes or MDM treated with 65U IFN-γ/ml or PBS control were infected with *G. bethesdensis* (pre-opsonized with serum) in 96-well plates for the indicated times and at the indicated MOIs. Plates were pre-coated with 10% autologous serum as described above. At each time point, a final concentration of 0.5% saponin was added per well. Plates were incubated on ice for 10 min before shearing the samples 10 times through a 28½-gauge needle to lyse phagocytes. Samples were diluted in saline, spread on YPG agar plates, and incubated 3–4 days at 37°C. In some cases, MDM were grown in 35 mm petri dishes, treated with IFN-γ for 2 days, and infected with pre-opsonized *G. bethesdensis* for 1 week before harvesting with Cellstripper™. Cells were then subjected to cytospin, differentially stained, and imaged via light microscope.

Cytokine analysis

Freshly isolated monocytes (1×10^5/200 μl/well) or MDM (5×10^4/200 μl/well) were not infected or infected with *G. bethesdensis* or *E. coli* at an MOI of 1 or 0.25, respectively, in 96-well plates (pre-coated with 10% autologous serum as described above) in the presence of 10 % autologous serum for 24 hours. Supernatants were collected and frozen until analysis. Thawed supernatants were diluted 1:3 and cytokines measured using a Bio-plex Pro 17-plex Human Cytokine Assay Kit analyzed on a Bio-plex system 200 running Bio-plex Manager (Version 5.0) according to the manufacturer's instructions (Bio-rad, Hercules, CA).

XTT viability assay

Freshly isolated monocytes (2×10^5/200 μl/well) were infected with *G. bethesdensis* at the indicated MOIs in 96-well plates (pre-coated with 10% autologous serum as described above) in the presence of autologous serum for 24 hrs before addition of 50 μl XTT reagent (1 mg/ml XTT, 25 μM phenazine methosulfate). After 4 hrs, the absorbance was measured at 450 nm with a Beckman Coulter DTX 880 Multimode Detector and the background OD of media alone was subtracted to give corrected OD.

Data analyses

Flow cytometry was performed on a BD FACS Canto™ and the data analyzed using FlowJo (Tree Star, Inc., Ashland, OR). All other data was graphed and analyzed statistically using GraphPad Prism (version 5, GraphPad Software, La Jolla, CA). For statistical tests, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Results

Internalization of opsonized G. bethesdensis by CD14$^+$ monocytes

Serum contains opsonins, including heat-stable antibodies and heat-labile complement proteins, which coat microbes and can trigger their uptake by phagocytes. We tested whether normal CD14$^+$ monocytes internalize *G. bethesdensis* and whether this process was serum-dependent. Most bacteria were internalized in the presence of serum after 1 hour at multiplicity of infections (MOIs) of 10:1 (Fig. 1A) and 20:1 (data not shown) as assessed by light microscopy. Little internalization occurred in the absence of serum and there was about a 50% reduction in internalization by normal monocytes incubated with heat-treated serum. Confocal microscopy of normal monocytes incubated with *G. bethesdensis* labeled with pHrodo, a dye that fluoresces more intensely at an acidic pH, also demonstrated greater internalization of bacteria in the presence of serum but not heat-treated serum (Fig. 1B). CGD monocytes internalized a similar amount of bacteria as normal monocytes in the presence of fresh serum ($p=0.114$, Mann Whitney test) and less so in the presence of heat-treated serum ($p=0.081$, Mann Whitney test) (Fig. 1A). CGD monocytes followed a similar time course of pHrodo-bacteria internalization as normal monocytes, with maximal uptake by 30 minutes post-infection (Fig. 1C). Maximal internalization at 30 minutes was also confirmed by scoring the number of pHrodo-labeled bacteria per cell (data not shown). Little to no internalization occurred in the absence of serum for either cell type (data not shown). These results show that monocytes internalize *G. bethesdensis* and this is partially dependent on a temperature-sensitive serum component for normal monocytes.

G. bethesdensis activation of the NADPH oxidase in CD14$^+$ monocytes

The phagocyte NADPH oxidase (NOX2), the enzyme complex defective in CGD, is activated by microbes and their products, and normally generates superoxide that is transformed into a variety of microbicidal reactive oxygen species (ROS) like hydrogen peroxide and hypohalous acids (18). Previous studies showed that *G. bethesdensis* was less effective at activating normal PMN NOX2 than *E. coli* (13). We measured normal monocyte superoxide production in response to *G. bethesdensis* and *E. coli* using luminol-enhanced chemiluminescence. A dose-dependent luminol-enhanced chemiluminescence response occurred when normal monocytes were incubated with *G. bethesdensis* (Fig. 2A and 2B). This response was dampened in the absence of serum (data not shown) suggesting that phagocytosis of *G. bethesdensis* or phagocyte interaction with serum components such as complement is at least partially required for the generation of superoxide in monocytes. *E. coli* was equivalently stimulatory compared to *G. bethesdensis* (Fig. 2B), but the kinetics of

the response differed (Fig. 2A). The relative lag in response to *G. bethesdensis* (Fig. 2A) mirrored the kinetics of internalization (Fig. 1C).

In comparison to PMN, monocytes generated a weaker response to *G. bethesdensis* and *E. coli* (Fig. 2C and 2D). This difference in respiratory burst has been previously shown for PMN and monocytes stimulated with *E. coli*, PMA, or zymosan (19–22). It should also be noted that *E. coli* induced a biphasic response in monocytes and PMN (Fig. 2A and 2C). Based on experiments where the stimulus was mixed in with the cells versus gently added, it was determined that the first peak was due in part to mechanical stimulation during initial stimulus addition while the second peak occurred as a direct result of the stimulus interacting with the phagocytes (data not shown). Taken together, *G. bethesdensis* induces a respiratory burst in normal monocytes and to a greater extent in normal PMN.

NADPH oxidase-dependent killing of G. bethesdensis by CD14$^+$ monocytes

Stimulation of a respiratory burst in monocytes has been linked to killing of internalized organisms such as *Staphylococcus aureus* (23). Given that *G. bethesdensis* induces superoxide in monocytes, we hypothesized that monocytes would also be able to kill *G. bethesdensis*. Normal monocytes killed *G. bethesdensis* in a dose-dependent manner after 24 hours of infection (Fig. 3A). In contrast, CGD monocytes were incapable of killing *G. bethesdensis* and, moreover, provided a suitable environment for growth of the organism (Fig. 3A). To exclude outgrowth of extracellular bacteria, gentamicin protection assays were performed. Although qualitatively similar results were obtained 1 hour post-infection, with or without gentamicin, almost complete killing occurred in both normal and CGD monocytes by 24 hours after gentamicin washout, indicating that this organism is sensitive to gentamicin internalized by monocytes prior to washing (data not shown). A comparison of PMN and monocytes isolated from the same normal donors showed that both cell types killed similar amounts of *G. bethesdensis* (Fig. 3B). Therefore, monocytes require the NADPH oxidase for the killing of *G. bethesdensis* and exhibit bactericidal activity on par with PMN.

Treatment of monocytes with interferon-gamma (IFN-γ) significantly enhances their microbicidal activities (24–26), possibly through the upregulation of NOX2 expression (27, 28). We tested whether treatment of normal monocytes with IFN-γ for two days altered their ability to kill *G. bethesdensis*. While normal monocytes cultured for two days without IFN-γ lost their ability to kill, IFN-γ treatment maintained the killing abilities of normal monocytes (Fig. 4A). Similar amounts of *G. bethesdensis* were recovered when the bacteria were incubated with PBS control or IFN-γ in the absence of monocytes (data not shown). *G. bethesdensis*-stimulated normal monocytes pre-treated with IFN-γ produced significantly more superoxide, as measured by chemiluminescence, compared to monocytes not receiving IFN-γ (data not shown), suggesting a link between induced superoxide and enhanced antibacterial activity after IFN-γ treatment. These data confirm that IFN-γ treatment is important for the maintenance of *in vitro* monocyte bactericidal activity and may augment phagocytic killing *in vivo* (29, 30).

IFN-γ stimulates some CGD monocytes to control G. bethesdensis growth

Since IFN-γ maintains normal monocyte killing of *G. bethesdensis*, we next tested the capacity of IFN-γ-treated CGD monocytes to kill *G. bethesdensis*. When considered as a group, monocytes from CGD patients were responsive to IFN-γ treatment as indicated by significantly reduced bacterial burdens compared to CGD monocytes that had not received the cytokine (Fig. 4A). Interestingly, the magnitude of the monocyte response to IFN-γ varied considerably from patient to patient. Previous studies indicated that IFN-γ treatment can enhance superoxide production in CGD monocytes if there are detectable levels of

residual superoxide at baseline (29, 31). Moreover, enhanced monocyte superoxide production accompanied bactericidal activity against *S. aureus* for a CGD patient receiving *in vivo* IFN-γ treatment (29). Given these previous studies, we predicted *a priori* a positive relationship between residual PMN ROS production (32) and monocyte killing of *G. bethesdensis*. Thus, we employed a one-tailed pvalue to test this prediction. A Spearman correlation demonstrated a positive relationship (r = 0.442) between reduction in bacterial burden and CGD neutrophil residual superoxide that was statistically significant (one-tailed p-value = 0.044) (Fig. 4B). The genetic background of these CGD patients is described in Table I. Understanding the mechanism of IFN-γ-induced bactericidal activity of CGD monocytes could prove a powerful diagnostic tool to determine the likelihood that IFN-γ treatment will benefit specific CGD patients.

G. bethesdensis infection does not affect monocyte survival

We next assessed the impact of *G. bethesdensis* infection on monocyte viability using an XTT assay, which measures the reduction of XTT to an orange formazan product by metabolically active cells. No significant difference was detected between normal and CGD monocytes either uninfected or incubated for 24 hrs with *G. bethesdensis* at MOIs of 0.5:1, 1:1, and 10:1 in the presence of serum (Fig. 5), indicating that monocytes remain viable in the presence of *G. bethesdensis*.

G. bethesdensis persistence in CGD monocyte-derived macrophages

Peripheral blood monocytes are precursors to macrophages, supplying tissues during steady-state conditions or in the setting of infection (34). Culturing $CD14^+$ monocytes in the presence of macrophage colony-stimulating factor (M-CSF) for 1 week results in a macrophage-like phenotype (35). While normal monocyte-derived macrophages (MDM) enabled growth of *G. bethesdensis*, treatment of these cells for 2 days with IFN-γ generated MDM capable of controlling *G. bethesdensis* growth in a dose-dependent manner (Fig. 6A). CGD MDM were significantly less effective in controlling *G. bethesdensis* growth either in the absence or presence of IFN-γ (Fig. 6B). We confirmed via confocal microscopy that normal and CGD MDM internalized similar amounts of bacteria at various MOIs in the presence of serum after 1 hour (data not shown).

Macrophages are highly phagocytic cells that support the persistence of several bacterial pathogens such as *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Brucella* species (36–38). Given that normal MDM were incapable of controlling *G. bethesdensis* growth at the MOIs tested after 24 hours, unless supplemented with IFN-γ, we examined the survival of these bacteria after 1 week of co-culture with MDM. IFN-γ-treated normal MDM infected for 1 week killed *G. bethesdensis* at a MOI of 0.25 bacteria per host cell (Fig. 7A and 7B). However, IFN-γ-treated CGD MDM maintained a favorable environment for *G. bethesdensis* survival (Fig. 7B and 7C). No decrease in viability, as assessed by LDH assay and XTT assay, was observed for normal or CGD MDM infected with *G. bethesdensis* for 1 week compared to non-infected MDM incubated for the same time period (data not shown). Based on these findings, the CGD macrophage may contribute to the persistence of this bacterium.

G. bethesdensis induces cytokine release from normal and CGD monocytes and MDM

Pro-inflammatory cytokines initiate host responses during infection, whereas anti-inflammatory cytokines play a significant role during the resolution phase once infection has been contained. Increased pro-inflammatory and decreased anti-inflammatory cytokine production have been reported for activated CGD cells compared to activated normal cells in some settings (16, 39) whereas in others, the opposite trend was observed (40). We measured cytokine concentrations in supernatants from normal and CGD monocytes and

MDM cultured with *G. bethesdensis* or *E. coli* for 24 hours. Both bacteria induced pro-inflammatory and anti-inflammatory cytokine release from all cell types tested with *E. coli* eliciting significantly greater responses than *G. bethesdensis* (Table II). No significant differences could be detected between non-infected normal and CGD cell cultures. However, CGD monocytes produced significantly more cytokine than normal monocytes when infected with either bacterium while MDM responses were similar in magnitude for normal versus CGD. There was little to no production of IL-1β (for MDM), IL2, IL-4, IL-5, IL-7, IL-12p70, IL-13, and GM-CSF (data not shown). Taken together, the lower cytokine responses of monocytes and MDM to *G. bethesdensis*, compared to *E. coli*, may help *G. bethesdensis* persist in these cells.

Discussion

Granulibacter bethesdensis is a bacterial pathogen that can infect patients with CGD. Isolation of genetically indistinguishable strains from individual subjects over 2–3 years, despite periods without clinically evident infections, may reflect either reinfection or reactivation of a latent infection. Persistence of bacteria has been suggested for a number of human diseases including Legionnaires' disease (*Legionella pneumophila*) (41), tuberculosis (*Mycobacterium tuberculosis*) (42), and brucellosis (*Brucella* species) (43). *G. bethesdensis* shares several attributes with these microbes that may explain its ability to survive in the host for a long period of time: (1) resistance to killing by non-oxidative host defenses, (2) replication and survival inside host cells and (3) promotion of host cell survival.

The natural reservoir for *G. bethesdensis* and the mode of entry into the human host are unknown. Regardless of the site of entry, *G. bethesdensis* must resist a variety of innate immune host defenses to establish infection. *G. bethesdensis* is resistant to killing by human serum and the cathelicidin cationic antimicrobial peptide (13). Serum resistance has been linked to increased virulence in other Gram-negative bacilli such as *L. pneumophila* strain UH1 (44) and O-antigen-positive *B. abortus* strains (45), and many others. Virulence factors also enable *L. pneumophila* (46, 47), *M. tuberculosis* (48), and *Brucella* species (49) to resist killing by a variety of cationic antimicrobial peptides that compose a major first line of host defenses.

G. bethesdensis may also come into contact with innate immune phagocytes. One of the first cellular responders recruited to the site of bacterial infection is the neutrophil. *In vitro* studies show that healthy neutrophils internalize *G. bethesdensis* in a complement-dependent manner and kill ~50% of input bacteria after 24 hours of infection at an MOI of 1 (13). CGD neutrophils internalize *G. bethesdensis* normally but fail to kill the organism and instead control its growth, highlighting the importance of the NADPH oxidase in *G. bethesdensis* killing by PMN. Survival of this bacterium in neutrophils may be further enhanced by the ability of *G. bethesdensis* to inhibit neutrophil apoptosis (13), which would normally aid in the containment and clearance of the microbe by macrophages. Although the neutrophil may play an important role in survival of *G. bethesdensis*, it is relatively short-lived (~5–8-hour half-life in circulation (50)) compared to other innate immune phagocytes like monocytes (~3-day half-life in circulation (51)) and macrophages (~30-day half-life (52)).

Monocytes are recruited to the site of infection and undergo differentiation to macrophages within the infected tissue, which also houses resident macrophages. We demonstrate in this study that monocytes from healthy and CGD donors internalize *G. bethesdensis*. Although normal PMN internalization of *G. bethesdensis* relies almost entirely on a temperature-sensitive serum component (13), normal monocyte internalization only partially depends on a heat-labile serum component. This difference in phagocytic mechanism may be explained by the differential expression of Fc receptors on the two cell types (53) or the wider

repertoire of receptors (*e.g.,* scavenger receptors (54)) on monocytes. Differential expression or activation of phagocytic receptors, as well as pattern recognition receptors (PRRs) (55, 56), on PMN and monocytes may also explain the difference in relative magnitude of the respiratory burst in these two cell types, and in response to *E. coli* versus *G. bethesdensis*. For instance, PRRs dectin-1 and dectin-2 on inflammatory PMN and monocytes/macrophages have been shown to differentially contribute to fungal particle association and subsequent ROS production (57). The same study also showed that serum opsonization of zymosan or *Candida albicans* was important for interaction with PMN, but less so for monocytes/macrophages.

One downstream effect of phagocytosis and PRR activation is the production of cytokines, which contribute to the inflammation and resolution phases of infection. In this study, we show that *G. bethesdensis* induces cytokine release from normal and CGD monocytes and macrophages, but to a significantly lesser extent than *E. coli*. Suppressing cytokine responses can be a microbial evasion mechanism as in the case of *L. pneumophila*, which requires a type II secretion system to dampen cytokine release from and permit growth within infected monocytes (58), although such mechanisms have yet to be described for *Granulibacter*. Our laboratory is currently characterizing structural differences between *G. bethesdensis* and *E. coli* endotoxin that may also contribute to the observed differences in cytokine secretion.

Similar to neutrophils from normal subjects, freshly isolated and IFN-γ-treated normal monocytes kill similar amounts of input bacteria after 24 hours of infection at an MOI of 1. However, unlike CGD neutrophils (13), freshly isolated CGD monocytes allow a two- to threefold expansion of *G. bethesdensis* over 24 hours, demonstrating that these cells provide a favorable environment for survival of the bacterium. Though it does not induce killing, IFN-γ treatment of CGD monocytes significantly limits *G. bethesdensis* outgrowth, and this effect correlates with the amount of residual ROS in CGD neutrophils. In comparison to neutrophils and monocytes, MDM are less successful in exerting bactericidal activity against *G. bethesdensis* even with the help of IFN-γ. Our studies demonstrate that 4 normal IFN-γ-treated MDM are required to control the growth of a single bacterium after 24 hours of infection and only modest killing occurs by 1 week post-infection. This inability to kill *G. bethesdensis* is more pronounced in CGD MDM where IFN-γ could not significantly control intracellular replication of the organism up to 1 week after infection.

These data suggest that the CGD macrophage is a potential niche for *G. bethesdensis* growth and survival *in vivo*. The macrophage as a niche for bacterial replication and persistence is not unprecedented. Alveolar macrophages internalize *L. pneumophila* via conventional complement-mediated phagocytosis (59) or coiling phagocytosis (60) into phagosomes that permit bacterial replication and evade lysosomal fusion (61). This suitable environment for *L. pneumophila* replication may allow the organism to survive for a long period of time. Moreover, *L. pneumophila* inhibits macrophage apoptosis by blocking the effects of pro-apoptotic Bcl2 family members (62). Latency of the organism in patients from the 1976 Philadelphia epidemic of Legionnaires' disease has been suggested, where specific IgM antibodies persisted for 2 years after initial infection (41).

Latent infection by *M. tuberculosis* in one third of the human population has been reported (42). Infected macrophages play a central role in latency by forming granulomas with antigen-specific T cells that inhibit the growth of *M. tuberculosis*, while the bacterium resists macrophage bactericidal killing (63). Like *L. pneumophila*, *M. tuberculosis* also inhibits phagosomal maturation and replicates within macrophages (64). Additionally, its virulence factors are critical for the inhibition of macrophage apoptosis (65) and pyroptosis (66). Lastly, O-antigen-positive *Brucella* species also prevent phagosome-lysosome fusion

inside macrophages (67) and inhibit macrophage apoptosis (68), which likely prolongs the bacterium's intracellular survival. Cases of chronic brucellosis have been described as lasting from several months to several years after acute infection (43, 69). Taken together, the macrophage centrally contributes to the persistence of bacteria that cause chronic disease in humans.

G. bethesdensis causes recurrent infections in CGD patients for years and can be recovered from normal mouse spleens up to 76 days post-inoculation (3, 4). Like the other persistent bacterial pathogens mentioned above, *G. bethesdensis* resists killing by serum and cationic antibacterial peptides, replicates within CGD macrophages, and does not compromise the viability of infected macrophages. *G. bethesdensis* resists some oxygen-independent microbicides but it may not be impervious to all non-oxidative killing mechanisms. Though the NADPH oxidase is required for *G. bethesdensis* clearance, it is possible that the products of the NADPH oxidase interact with and activate oxygen-independent bactericidal systems.

Interestingly, all of the aforementioned bacteria have slow rates of division with *G. bethesdensis* doubling every 5–6 hours, *L. pneumophila* every 3 hours (70), *M. tuberculosis* every 24 hours (71), and *Brucella* species every 2.5–3.5 hours (72), which contrasts to *E. coli*'s faster doubling time of 0.5–1.5 hours (73). This trait could be important for the maintenance of an intact host phagocyte and bacterial replication niche, which might otherwise lyse if overwhelmed with too many intracellular bacteria. It remains to be determined if *in vitro* survival of G*. bethesdensis* in macrophages correlates to persistence of *G. bethesdensis in vivo*.

The intracellular nature of *G. bethesdensis* infection may pose a challenge for clinical treatment. *G. bethesdensis* is innately multidrug resistant and therapy of infected individuals requires aggressive antimicrobial treatments and sometimes surgical intervention (4). Ensuring the efficacy of antimicrobial therapies may require that they can penetrate infected phagocytes as has been shown for the treatment of CGD, tuberculosis, and legionellosis (74). IFN-γ is critical for activating bactericidal mechanisms in macrophages infected with *L. pneumophila* (75, 76) and *M. tuberculosis* (77), and may prove an effective therapy for *in vivo* treatment of infections with these organisms (78, 79). IFN-γ may also be an effective treatment for some CGD patients infected with *G. bethesdensis*, as was shown for CGD patients infected with *S. aureus* (29). We show for the first time that *in vitro* IFN-γ-enhanced control of *G. bethesdensis* outgrowth in CGD monocytes correlates with higher residual superoxide levels in PMN from the same CGD patients. Since higher residual PMN superoxide correlated with increased survival in a large CGD cohort (32), this *in vitro* finding may be a useful test to identify patients that are likely to respond to IFN-γ therapy and those who might not.

Given the high morbidity associated with IFN-γ therapy as well as the high cost, this finding has important clinical implications. It is likely that IFN-γ boosts superoxide levels in CGD phagocytes with a certain threshold of residual superoxide leading to enhanced control of bacterial burden. However, we cannot rule out that IFN-γ stimulates ROS-independent bactericidal or bacteriostatic pathways, such as the downregulation of transferrin receptors that limits iron availability required for the growth of some organisms (24). IFN-γ also plays a role in vitamin D-dependent upregulation of antimicrobial peptides and induction of autophagy (33); however, we found no contribution of vitamin D in the control of *G. bethesdensis* burden (data not shown). It will be important to establish further correlations between *in vitro* and *in vivo* IFN-γ efficacy to help identify specific CGD patients who will respond to treatment. Finding novel therapies to treat chronic intracellular infections, especially in immunocompromised populations, is crucial given that many persistent bacterial pathogens are resistant to antibiotics.

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The views expressed here are those of the authors and not necessarily those of the U.S. Government.

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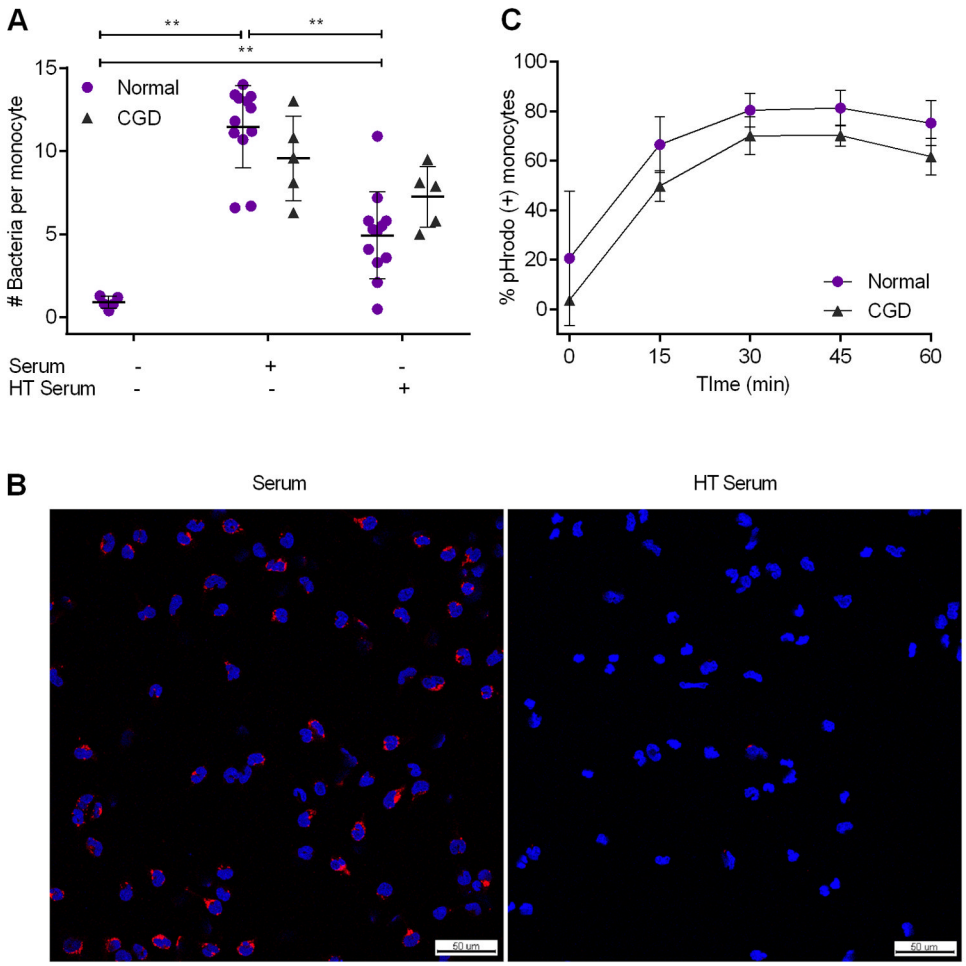

FIGURE 1.

Serum-dependent internalization of *G. bethesdensis* by $CD14^+$ monocytes. (**A**) $CD14^+$ monocytes from normal (n=5–12) and CGD (n=5) donors were incubated with *G. bethesdensis* at an MOI of 10 bacteria per host cell for 1 hour in the absence of serum, presence of autologous serum or heat-treated (HT, 56°C, 30 min) serum as described in methods (mean ± SD, non-parametric t-tests where ** = $p \leq 0.01$). Internalization of bacteria by CGD monocytes in the absence of serum was not determined. (**B**) Confocal microscopy imaging of normal $CD14^+$ monocytes incubated with pHrodo-labeled *G. bethesdensis* (red) at an MOI of 10 bacteria per host cell for 1 hour in the presence of autologous serum or heat-treated serum. Nuclei are labeled with DAPI (blue). Bar length = 50 μm. (**C**) $CD14^+$ monocytes from normal (n=3) and CGD (n=3) donors were incubated with pHrodo-labeled *G. bethesdensis* at an MOI of 1 bacterium per host cell for the indicated time points in the presence of autologous serum. Epifluorescence images were scored and data represented as mean ± SD.

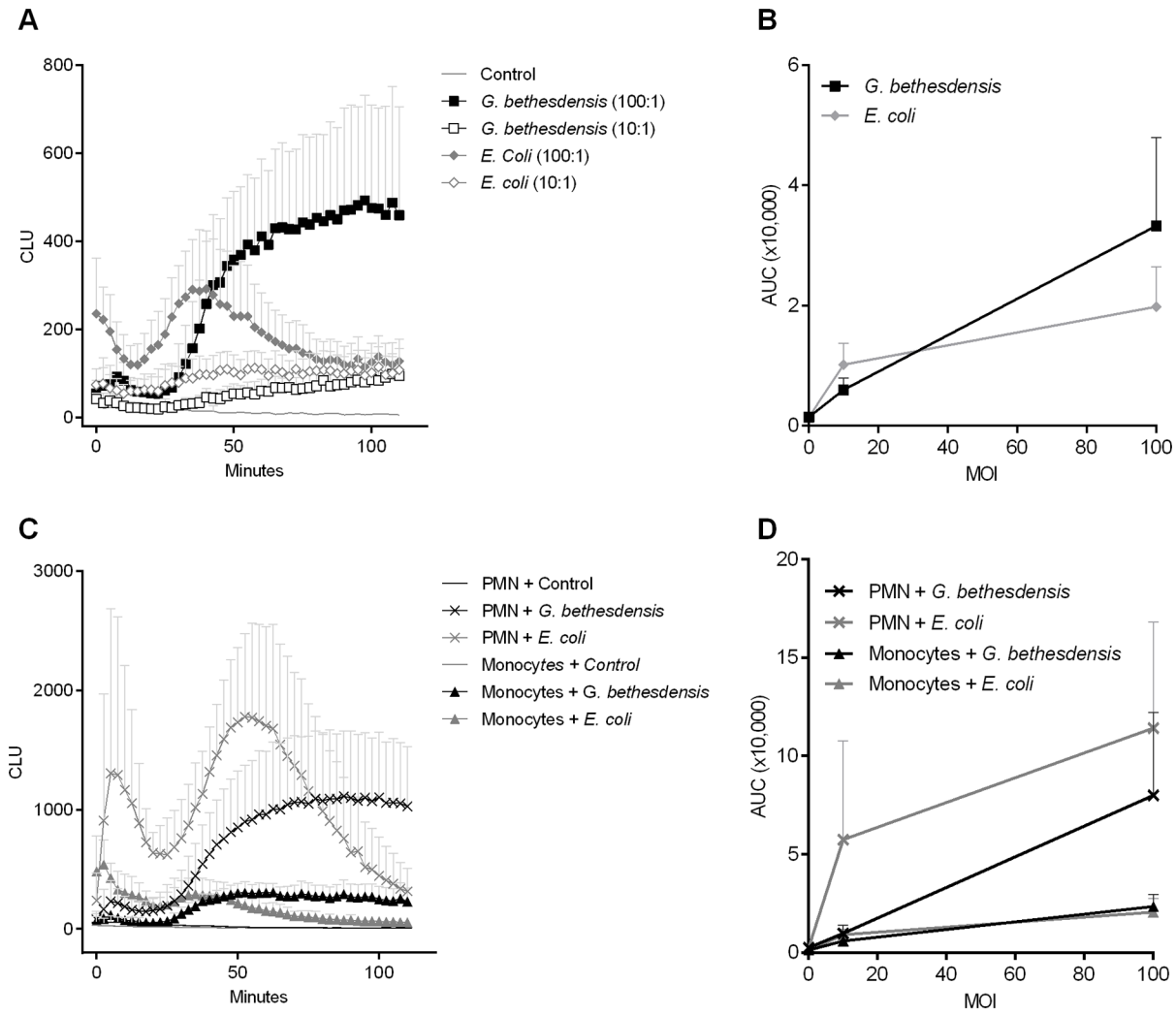

FIGURE 2.

G. bethesdensis induces oxidative burst activity in normal CD14$^+$ monocytes. (**A**) Luminol-enhanced chemiluminescence was measured in monocytes exposed to control buffer, *G. bethesdensis* or *E. coli* at MOIs of 100 and 10 bacteria per host cell in the presence of autologous serum. Data are mean + SD for 7 donors. (**B**) Data from (A) are represented as area under the curve (AUC). (**C**) Luminol-enhanced chemiluminescence was measured in PMN and monocytes, isolated from the same donors (n=6), exposed to control buffer, *G. bethesdensis* or *E. coli* at an MOI of 100 bacteria per host cell in the presence of autologous serum. Data are mean + SD. (**D**) AUC data for PMN and monocytes, from the same donors (n=6), exposed to bacteria at MOIs of 100 (shown in (C)) and 10 bacteria per host cell in the presence of autologous serum.

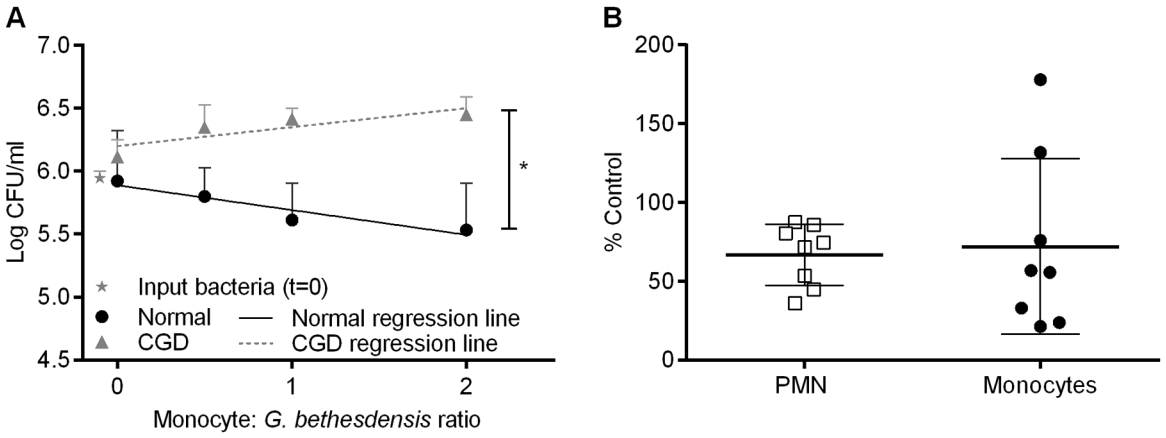

FIGURE 3.

Freshly isolated normal but not CGD CD14$^+$ monocytes kill *G. bethesdensis.* (**A**) CD14$^+$ monocytes from normal (n=9) and CGD (n=3) donors were incubated with *G. bethesdensis* at MOIs of 2, 1, and 0.5 bacteria per host cell in the presence of autologous serum for 24 hours. Data are represented as mean + SD. Linear regression analysis was conducted to assess differences in slope of lines (* = $p \leq 0.05$) for normal versus CGD. (**B**) PMN and monocytes isolated from the same normal donors (n=8) were incubated with *G. bethesdensis* at an MOI of 1 bacteria per host cell in the presence of autologous serum for 24 hours. Data are represented as % control input (mean ± SD).

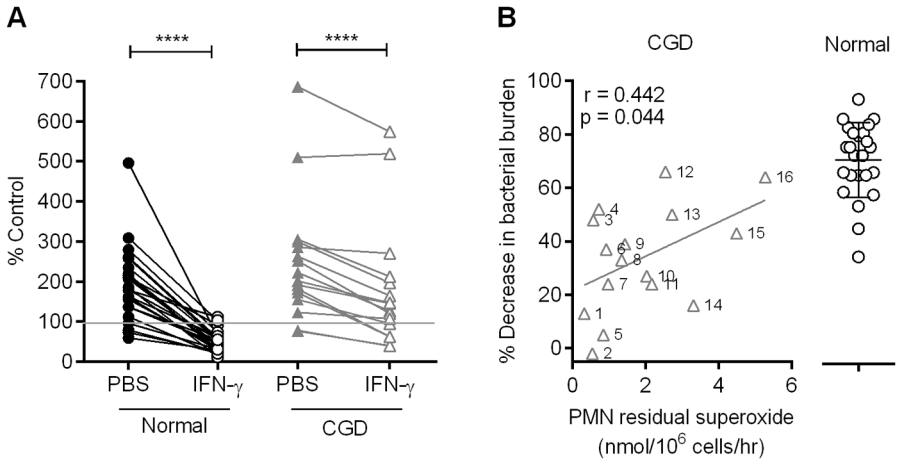

FIGURE 4.

IFN-γ maintains microbicidal activity of normal monocytes and stimulates monocytes from some CGD subjects to control *G. bethesdensis* growth. (A) Monocytes from normal (n=24) and CGD (n=16) donors were treated with PBS control or 65U IFN-γ/ml for 2 days before infection with *G. bethesdensis* for 24 hours at an MOI of 1 bacterium per host cell in the presence of autologous serum. Data are represented as % control input. Significance testing of PBS control versus IFN-γ treatment was by Wilcoxon paired t-test (**** = $p \leq 0.0001$). (B) Percentage decrease in bacterial burden after IFN-γ treatment relative to PBS control for all normal and CGD monocytes in panel (A). Percentages for CGD monocytes are plotted against residual superoxide in PMN from the same CGD patients. The relationship of the two variables was assessed using a Spearman correlation and the best fit (Y = 6.409X + 21.59) is depicted.

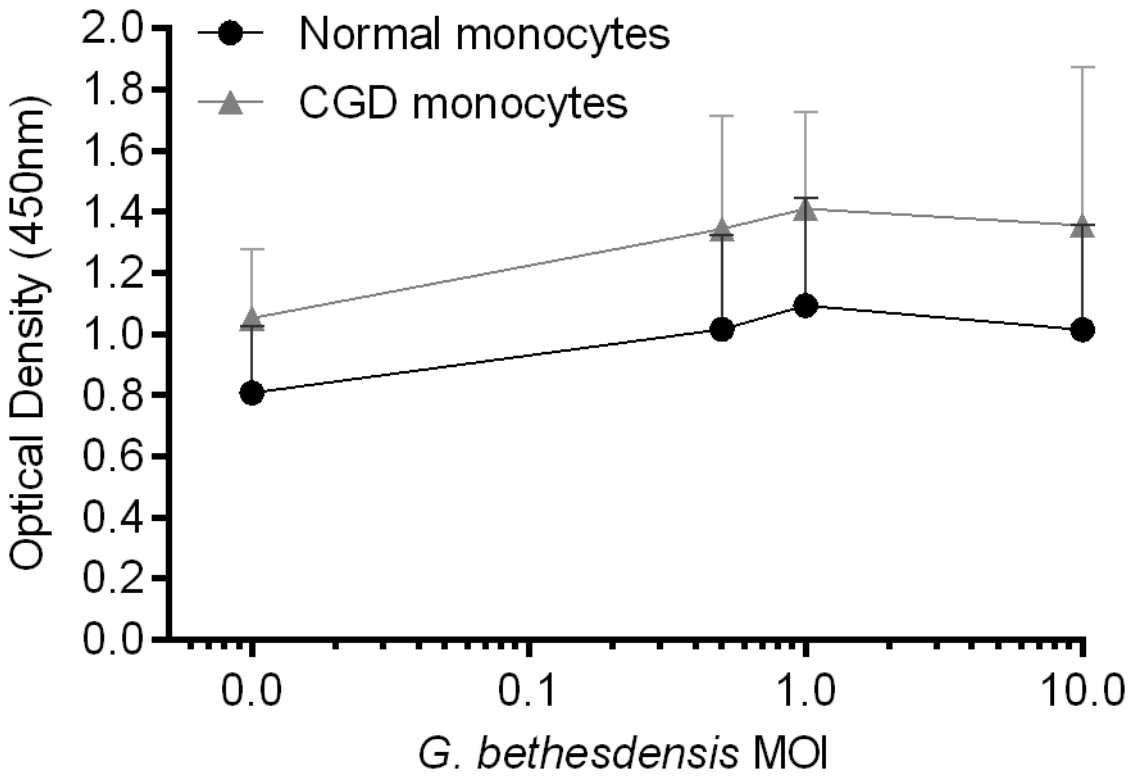

FIGURE 5.

G. bethesdensis infection does not alter monocyte survival. Monocytes from normal (n=6) or CGD (n=3) donors were incubated alone or with *G. bethesdensis* at MOIs of 0.5, 1, or 10 bacteria per host cell in the presence of autologous serum for 24 hours. Cell viability was assessed using an assay that measures conversion of XTT to an orange formazan product by metabolically active cells (mean optical density + SD).

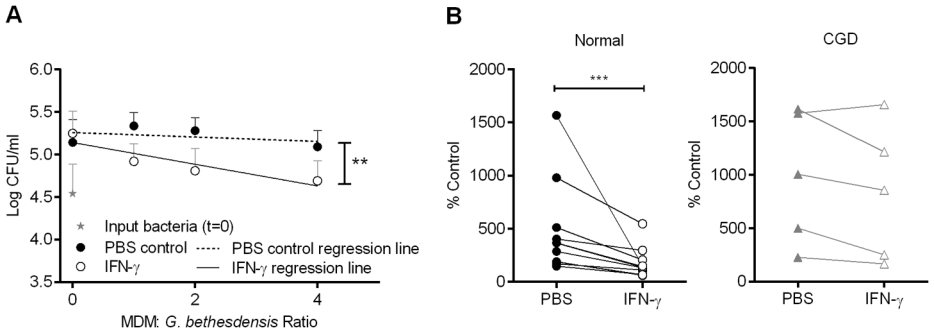

FIGURE 6.

Normal but not CGD MDM control *G. bethesdensis* growth after 24 hours of infection. (**A**) Normal MDM were incubated ± 65 U IFN-γ/ml for 2 days before co-culture with *G. bethesdensis* for 24 hours at the indicated MOI in the presence of autologous serum. Data are presented as mean + SD (n=11 donors). Linear regression analysis was conducted to assess differences in slope of lines (** = $p \leq 0.01$) for PBS control versus IFN-γ treatment. (**B**) MDM were treated as in (A) at an MOI of 0.25 bacteria per host cell. Data are % control input for 11 normal donors and 5 CGD donors. Wilcoxon paired t-test was used to compare PBS control versus IFN-γ treatment (*** = $p \leq 0.001$).

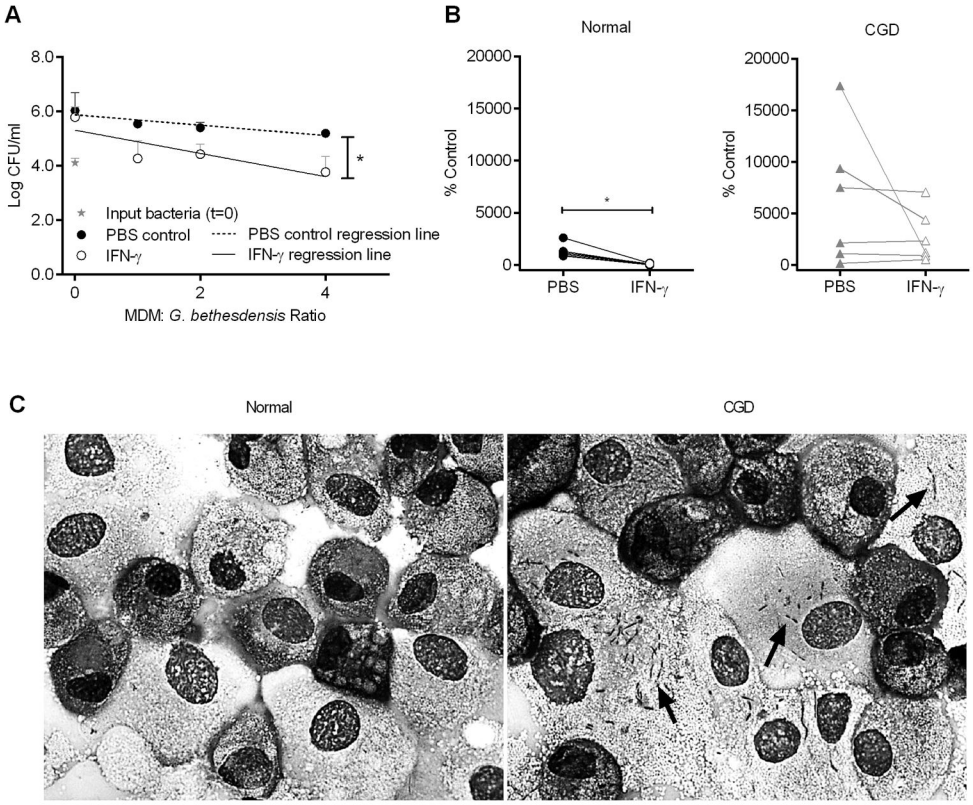

FIGURE 7.

G. bethesdensis persists inside CGD MDM for 1 week. (**A**) Normal MDM were incubated ± 65 U IFN-γ/ml for 2 days before co-culture with *G. bethesdensis* for 1 week at the indicated MOI in the presence of autologous serum. Data are presented as mean + SD (n=6 donors). Linear regression analysis was conducted to assess differences in slope of lines (* = $p \leq 0.05$) for PBS control versus IFN-γ treatment. (**B**) MDM were treated as in (A) at an MOI of 0.25 bacteria per host cell. Data are % control input for 6 normal donors and 6 CGD donors. Wilcoxon paired t-test was used to compare PBS control versus IFN-γ treatment (* = $p \leq 0.05$). (**C**) IFN-γ-treated normal and CGD MDM infected for 1 week with *G. bethesdensis* at an MOI of 1 bacterium per host cell. Black arrows indicate the presence of bacteria.

Table I

CGD patient subtype and specific mutation

CGD Patient	Gene affected	DNA mutation	Protein mutation	Mutation type
1	gp91	c.676 C>T	p.Arg226X	Nonsense
2	gp91	c.483+1 G>C	Del exon 5	Splice
3	gp91	c.80-83 del TCTG	p.Val27GlyfsX33	Frameshift
4	gp91	c.742 dup A	p.Ile248fsX37	Frameshift
5	gp91	c.868 C>T	p.Arg290X	Nonsense
6	gp91	c.676 C>T	p.Arg226X	Nonsense
7	gp91	c.676 C>T	p.Arg226X	Nonsense
8	gp91	c.675-1 G>A	Del exons 6 & 7	Splice
9	p47	Not determined	Not determined	Not determined
10	gp91	c.909 C>A	p.His303Gln	Missense
11	gp91	c.388 C>T	p.Arg130X	Nonsense
12	p47	c.75_76 del GT	p.Tyr26fsX26	Frameshift
13	gp91	c.868 C>T	p.Arg290X	Nonsense
14	p47	c.75_76 del GT	p.Tyr26HisfsX26	Frameshift
15	p47	Not determined	Not determined	Not determined
16	p47	c.75_76 del GT	p.Tyr26HisfsX26	Frameshift

Table II

Cytokine profiles of normal and CGD monocytes and MDM infected with *G. bethesdensis* or *E. coli* for 24 hours

Monocytes

	Normal (n = 7)			CGD (n = 4)			P-values			
Cytokine[a]	**(I) Control**	**(II) *G. bethesdensis***	**(III) *E. coli***	**(IV) Control**	**(V) *G. bethesdensis***	**(VI) *E. coli***	**II vs. III[c]**	**V vs. VI[c]**	**II vs. V[d]**	**III vs. VI[d]**
IL-1β	103 (52)	608 (126)	5129 (2502)	74 (84)	1088 (462)	14244 (3620)	*	0.13	**	*
IL-6	278 (180)	5404 (2619)	13462 (4781)	2605 (3102)	26699 (8989)	31512 (18983)	*	ns	**	*
IL-8	15089 (7437)	22252 (3276)	27901 (6308)	9794 (8272)	20904 (1738)	27254 (5620)	0.08	0.13	ns	ns
IL-10	1 (1)	38(47)	127 (94)	9(13)	539 (147)	528 (469)	*	ns	**	**
IL-17	29 (14)	76 (13)	111 (12)	37 (33)	93 (11)	134 (13)	*	0.13	0.11	*
G-CSF	10 (4)	217 (160)	662 (360)	73 (108)	2310 (895)	1938 (1495)	*	ns	**	*
IFN-γ	82 (45)	437 (119)	815 (208)	177 (187)	714 (145)	1154 (130)	*	0.13	*	**
CCL-2	1587 (1390)	3004 (950)	1435 (1047)	2334 (2218)	2990 (794)	873 (569)	*	0.13	ns	ns
CCL-4	1016 (601)	3974 (1582)	7047 (2495)	2275 (2216)	5155 (485)	7634 (3947)	*	ns	ns	ns
TNF-α	101 (87)	2613 (2178)	11868 (5249)	223 (241)	6814 (6075)	39407 (12606)	*	0.13	*	**

MDM

	Normal (n=5-7)			CGD (n=4-5)			P-values			
Cytokine[b]	**(I) Control (n=7)**	**(II) *G. bethesdensis* (n=7)**	**(III) *E. coli* (n=5)**	**(IV) Control (n=5)**	**(V) *G. bethesdensis* (n=5)**	**(VI) *E. coli* (n=4)**	**II vs. III[c]**	**V vs. VI[c]**	**II vs. V[d]**	**III vs. VI[d]**
IL-6	2 (2)	84 (95)	1890 (1904)	4 (2)	60 (78)	2834 (1912)	**	*	ns	ns
IL-8	185 (117)	1885 (1604)	6667 (4561)	436 (418)	3264 (3083)	11548 (6601)	*	0.06	ns	ns
IL-10	2 (2)	10 (11)	214 (182)	5 (3)	13 (10)	940 (747)	*	*	ns	0.11
G-CSF	2 (1)	6 (4)	81 (48)	3 (1)	8 (4)	184 (137)	**	*	ns	ns
IFN-γ	4 (5)	35 (32)	151 (85)	20 (15)	47 (35)	198 (91)	*	*	ns	ns
CCL-2	547 (398)	972 (880)	1318 (862)	1979 (1104)	2865 (1931)	2602 (1862)	ns	ns	*	ns
CCL-4	139 (210)	902 (1077)	6330 (10096)	136 (71)	1111 (1476)	8350 (6852)	ns	0.11	ns	ns
TNF-α	3 (2)	118 (114)	5502 (6627)	5 (4)	115 (132)	8274 (6333)	**	*	ns	ns

[a] Cytokines from 5×10^5 monocytes/ml infected at an MOI of 1 are represented as mean (SD) (pg/ml).

[b] Cytokines from 2.5×10^5 MDM/ml infected at an MOI of 0.25 are represented as mean (SD) (pg/ml).

[c] Statistical comparisons by exact paired nonparametric Wilcoxon signed rank test,* =$p \leq 0.05$, ns = not significant.

[d] Statistical comparisons by exact unpaired nonparametric Mann-Whitney test, *=$p \leq 0.05$, **=$p \leq 0.01$, ns = not significant.