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Homodimeric Granzyme A Opsonizes *Mycobacterium tuberculosis* and Inhibits Its Intracellular Growth in Human Monocytes via Toll-Like Receptor 4 and CD14

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Mycobacterium tuberculosis (Mtb)-specific γ9δ2 T cells secrete granzyme A (GzmA) protective against intracellular Mtb growth. However, GzmA-enzymatic activity is unnecessary for pathogen inhibition, and the mechanisms of GzmA-mediated protection remain unknown. We show that GzmA homodimerization is essential for opsonization of mycobacteria, altered uptake into human monocytes, and subsequent pathogen clearance within the phagolysosome. Although monomeric and homodimeric GzmA bind mycobacteria, only homodimers also bind cluster of differentiation 14 (CD14) and Toll-like receptor 4 (TLR4). Without access to surface-expressed CD14 and TLR4, GzmA fails to inhibit intracellular Mtb. Upregulation of Rab11FIP1 was associated with inhibitory activity. Furthermore, GzmA colocalized with and was regulated by protein disulfide isomerase AI (PDIA1), which cleaves GzmA homodimers into monomers and prevents Mtb inhibitory activity. These studies identify a previously unrecognized role for homodimeric GzmA structure in opsonization, phagocytosis, and elimination of Mtb in human monocytes, and they highlight PDIA1 as a potential host-directed therapy for prevention and treatment of tuberculosis, a major human disease.

Keywords. CD14; granzyme A; mycobacteria; PDIA1; TLR4.

Currently, one quarter of the world's population is infected with *Mycobacterium tuberculosis* (Mtb), the pathogen that causes tuberculosis (TB) [1]. The rise of multidrug-resistant TB has accelerated efforts to investigate novel treatments and vaccines against this disease, and new investigations are uncovering new host-pathogen interactions [2, 3]. *Mycobacterium tuberculosis* can efficiently infect alveolar macrophages, which in turn phagocytize and degrade this organism. However, Mtb has also developed escape mechanisms by which macrophages are no longer able to neutralize the pathogen [4, 5]. Macrophages possess canonical surface receptors cluster of differentiation 14 (CD14) and Toll-like receptors (TLRs), such as TLR2 and TLR4 [6]. The most studied ligand for TLR4 is lipopolysaccharide (LPS), but other known pathogens, including Mtb, can be sensed by this receptor [7]. Lipopolysaccharide is known to attach to the LPS-binding protein (LBP) and dock onto TLR4 and CD14 receptors for subsequent internalization and intracellular signaling through MyD88 and Trif [8, 9]. Macrophages then activate a proinflammatory response, which leads to the production of cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, and interferons [7]. This inflammatory phenotype has been studied as a plausible source of cytokine in bacterial sepsis models [10–13].

Granzyme A (GzmA) is a serine protease found within the cytotoxic granules of CD8 T, natural killer (NK), and γδ T cells [10]. In the context of TB, GzmA has been shown to be secreted from these cells, but its protective role has not been elucidated [13]. Each GzmA monomer has an active site with the trypsin-like catalytic triad formed by Ser195, and the 2 monomers are joined by a disulfide bond at Cys93 [14]. Granzyme A is best known for its proapoptotic role associated with perforin and for leading to mitochondrial disruption and cell death through its enzymatic activity [15–17]. However, other groups have shown that physiologic concentrations of GzmA can instead induce activation of human macrophages without evidence of cell death [18]. Moreover, GzmA can directly, and without the need of perforin, (1) stimulate macrophages to produce inflammatory cytokines such as TNF and IL-1β [18], (2) synergistically potentiate LPS inflammatory response [19], (3) and inhibit the growth of intracellular mycobacteria within infected

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macrophages enzymatically independent [20, 21]. Moreover, GzmA does not act directly on the pathogen, and instead it seems to potentiate activation of phagocytes. Granzyme A, but not GzmB, levels in the supernatant correlate with mycobacterial growth inhibition; therefore, GzmA was pursued for its inhibitory effects [21].

In this study, we elucidate an important mechanism by which GzmA controls Mtb infection. We show that homodimeric GzmA, but not the monomer, inhibits mycobacterial growth in a CD14- and TLR4-dependent fashion. We demonstrate that GzmA binds mycobacteria, TLR4 as well as CD14, such that mycobacteria opsonization leads to phagocytosis and directs its neutralization through enhancement of phagolysosome fusion. Finally, we demonstrate that surface protein disulfide isomerase A1 (PDIA1) converts GzmA homodimer into monomer and that PDIA1 inhibition conversely enhances GzmA-mediated protection.

METHODS

For more details (including detailed protocols and reagent numbers), see Supplemental Methods section.

Granzyme A (GzmA) Purification and Measurement of GzmA Enzyme Activity

Recombinant GzmA was purified using an updated protocol as previously reported [22]. Benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterase assays and calculation of specific activity were adapted from [20, 22].

Human Samples and Monocyte Isolation

Human monocytes were isolated from healthy donors as previously described [20]. Once thawed, monocytes were purified using EasySep Human Monocyte Isolation Kit (Catalog number 19359; STEMCELL Technologies).

Preparation of Mycobacteria for Infection

Connaught strain Bacillus Calmette-Guérin (BCG) or Mtb H37Rv were grown to mid-logarithmic phase in Middlebrook 7H9 media supplemented with 10% albumin, dextrose, and catalase. Multiplicity of infection (MOI) was 3 for BCG and 1 for Mtb.

Mycobacterial Growth Inhibition Assay

Primary CD14 + monocytes were plated and infected as described previously [22, 23]. The percentage inhibition was calculated as follows: 100–100 × (disintegration per minute [DPM] from treated wells/DPM from untreated wells).

Expression and Purification of Human Recombinant Protein Disulfide Isomerase A1

Purification was performed as previously described [23].

Confocal Microscopy and Stimulation Emission Depletion Experiments

For confocal and stimulation emission depletion (STED) microscopy experiments, cells were incubated on coverslips pretreated with Cell-Tak, incubated in primary and secondary antibodies with respective washes. Cells were then visualized using a Leica SP8 TCS STED 3 times.

Quantitative Shotgun Proteomics

Human monocytes were incubated with or without 200-nM GzmA and infected or not with BCG (MOI = 3) overnight. After incubation, cells were then washed thrice with phosphate-buffered saline (PBS), and the pellet was lysed using lysis buffer from EasyPep Mini MS Sample Prep Kit and analyzed as previously described [24].

Antibody Neutralization Studies

Monocytes were pretreated with anti-CD14 at 10 μg/mL (Catalog number MAB3832; R&D Systems, Minneapolis, MN), anti-TLR4 at 10 μg/mL (Catalog number AF1478; R&D Systems), anti-TLR2 at 10 μg/mL (Catalog number MAB2616; R&D Systems), or isotype control at 10 μg/mL (Catalog number MAB002; R&D Systems) for 30 minutes before GzmA treatment and infection.

Immortalized Mouse Macrophage Studies

Immortalized mouse macrophage (iMac) cells were cultured and used as previously described [25, 26]. Cells were then used in the same manner as human monocytes in the mycobacterial growth inhibition assay (MGIA).

Granzyme A Opsonization of Mycobacteria

A mycobacteria BCG-green fluorescence protein (GFP) aliquot was incubated alone, with 200 nM of GzmA (wild type [WT], S195A, or C93S), or with 200 nM of recombinant Trans-Sialidase (rTS) for 1 hour at 37°C. Bacteria were then washed 3 times with PBS solution and resuspended in primary anti-HIS and secondary antimouse antibodies.

Phagocytic Score

Monocytes were treated or not with GzmA, after which BCG-GFP was used to infect cells for 2 hours. The phagocytic score was measured as previously described [25].

Immunoprecipitation Experiments

Monocytes were infected or not with mycobacteria and treated or not with GzmA (WT or C93S) for 16 hours. Cells were then washed thrice with PBS to remove serum proteins and lysed in immunoprecipitation (IP) lysis buffer. Proteins were incubated in 25 μg/mL anti-GzmA antibody and mixed gently for 6 hours at 4°C. Membranes were then probed using anti-CD14 and anti-TLR4.

Bacillus Calmette-Guérin-Green Fluorescence Protein Protection Experiments and Kinetics

After simultaneous treatment with GzmA and infection with mycobacteria, monocytes were stained. Previous and subsequent steps were performed with standard flow cytometry washes and centrifugation to remove excess reagents. Next, cells were incubated with anti-GzmA antibody conjugated with phycoerythrin (PE) (Catalog number 558904; BD Biosciences, San Diego, CA). A flow cytometer was then used to analyze BCG-GFP and GzmA-PE positive cells.

Protein Disulfide Isomerase A1 Inhibitors

PACMA-31 was used to pretreat monocytes at 200 nM in media for 30 minutes, after GzmA treatment and infection. Similarly, rutin was used to pretreat monocytes for 30 minutes at 50 μM in media.

RESULTS

Expression and Characterization of Monomeric Form of Granzyme A

We recently expressed enzymatically inactive GzmA (GzmA-S195A) and demonstrated that catalytic activity is unnecessary for GzmA-mediated inhibition of intracellular mycobacterial growth in the MGIA [20]. To investigate the biochemical properties of GzmA required for growth inhibition, we substituted cysteine 93 with a serine to disrupt the disulfide bond critical for homodimeric formation [14]. As demonstrated by both silver stain and Western blot (WB) under nonreducing conditions, mutation of this key residue produces only the monomer of GzmA (Figure 1*A* and *B*). Using GzmA-specific substrate, we demonstrated that homodimerization was not required for esterase activity (Figure 1*C*). As previously shown, GzmA-S195A inhibits intracellular replication of mycobacteria in the MGIA (Figure 1*D*). However, GzmA-C93S loses its inhibitory activity. We also performed a mixing experiment with the same concentration of GzmA-WT, GzmA-C93S, or both GzmA-WT and GzmA-C93S, and the results suggest that monomeric GzmA can partially inhibit the protective effects of GzmA-WT. (Supplementary Figure 1*A* and Figure 1*E* show raw data as DPM as label incorporates into residual live mycobacteria.)

CD14 and Toll-Like Receptor 4 Surface Receptors Are Important in the Granzyme A-Mediated Inhibition in Human Monocytes

Multiple reports have shown that GzmA retains biological activity independent of enzymatic activity [19, 20]. It has also been reported that neutralizing antibody against the CD14 receptor reversed GzmA-induced TNF after LPS costimulation [19]. We next determined whether CD14 neutralization would reverse GzmA-mediated mycobacterial inhibition. As observed in Figure 2*A*, GzmA-WT and GzmA-S195A lost their inhibitory activity in cells pretreated with α-CD14, indicating that GzmA/CD14 interactions are necessary for GzmA-specific inhibitory effects. The CD14 receptor is known to interact with TLRs, including TLR2 and TLR4 [6, 26]. Similar to our studies targeting CD14, neutralization of TLR4 reversed GzmA-mediated mycobacterial BCG growth inhibition (Figure 2*B*), whereas neutralization of TLR2 did not (Figure 2*C*). More importantly, neutralizing antibodies against CD14 and TLR4 alone did not alter bacterial burdens within infected monocytes, providing further evidence that GzmA-directed interactions with these receptors are important for mycobacterial inhibitory effects (Supplementary Figure 2*A*).

Monocytes Require CD14 and Toll-Like Receptor 4 for Granzyme A-Mediated Inhibitory Effects

To further assess the role of CD14 and TLR4 in the GzmA-mediated inhibition, iMac cells were studied [27, 28]. Similar to our human monocytes results, GzmA-WT and GzmA-S195A were able to modestly inhibit mycobacterial growth in iMac-WT cells. In contrast, GzmA-C93S did not (Figure 2*D*). Furthermore, iMac cells deficient in either CD14 or TLR4 were incapable of inhibiting BCG after treatment with GzmA-WT, confirming the antibody neutralization studies (Figure 2*E*).

Granzyme A Stably Binds to Mycobacteria

It has been shown that GzmA can stably bind to *Pseudomonas aeruginosa*, *Neisseria meningitidis*, and *Escherichia coli* [19]. To investigate whether GzmA binds to mycobacteria, His-tagged GzmA variants were analyzed for their binding to bacteria (Supplementary Figure 3*A*). Both GzmA-WT and S195A bound mycobacteria (Figure 3*A*), indicating that esterase activity is not required for mycobacterial binding. We were surprised to find that monomeric GzmA-C93S was able to bind mycobacteria, indicating that binding to mycobacteria is not sufficient for inhibitory activity (Figure 3*A*). As a negative control, recombinant His-tagged trans-sialidase [29] was unable to bind mycobacteria (Figure 3*A*).

Granzyme A Acts as an Opsonin-Enhancing Mycobacterial Phagocytosis

We next investigated whether the addition of GzmA before, during, or after mycobacterial infection most impacted mycobacterial growth. As indicated in Figure 3*B*, maximal inhibition was observed when GzmA was added before and/or during infection; however, addition of GzmA to already infected cells did not mediate inhibition. Consistent with opsonization of bacteria by complement proteins [30], after 2 hours of infection, GzmA-treated cells had a higher phagocytic score compared to untreated cells (Figure 3*C*). In addition, quantitative shotgun proteomic analysis found that Rab11-FIP1, a protein critical for optimal phagocytosis that regulates TLR4

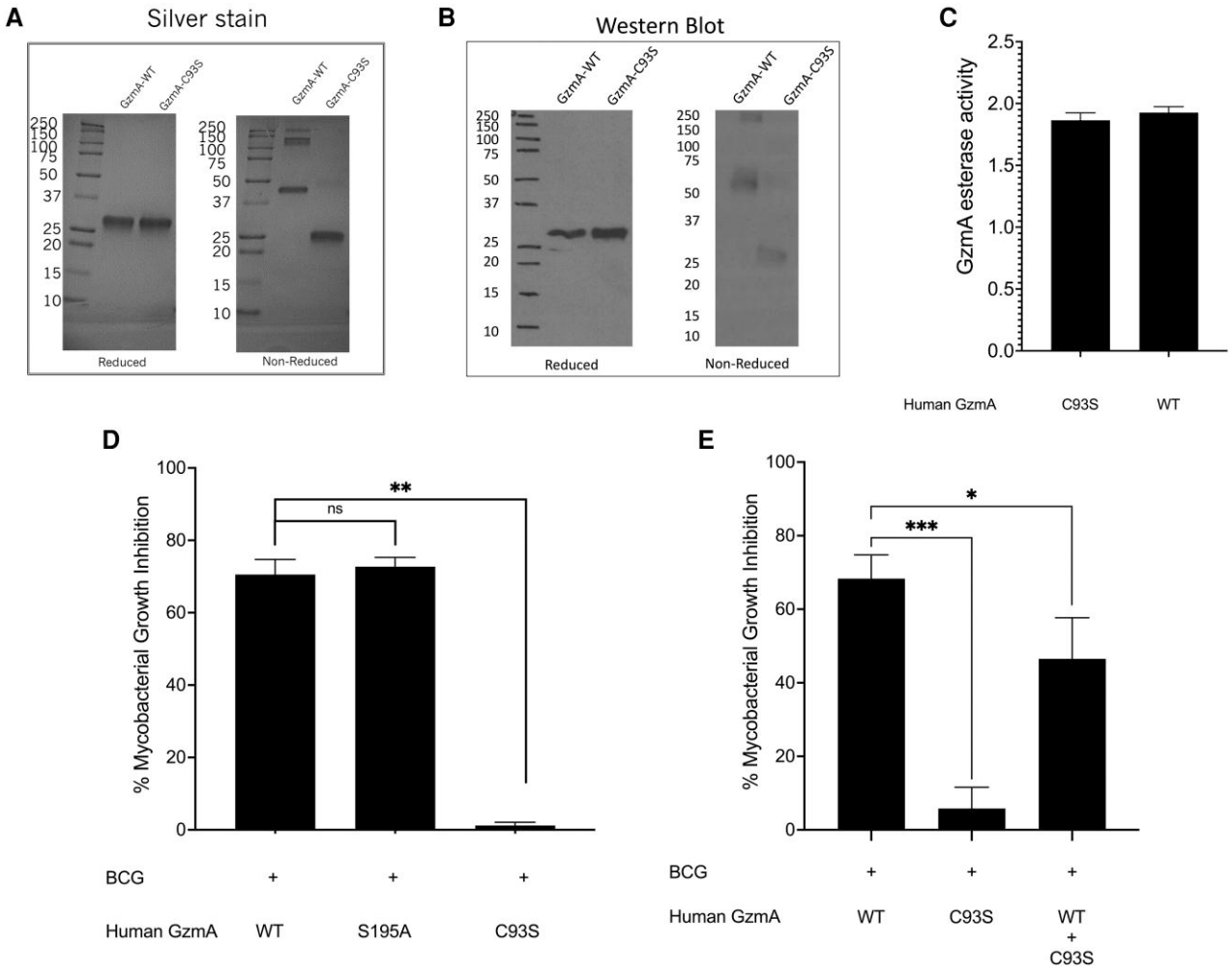

Figure 1. The granzyme A (GzmA)-C93S variant forms only monomers, and GzmA-monomer cannot inhibit the intracellular mycobacterial growth. (*A*) and (*B*) The GzmA-wild type (WT), but not GzmA-C93S, can form homodimers (54 kDa) under nonreducing conditions as shown by silver stain and anti-GzmA Western blot. (*C*) The GzmA-C93S retains enzymatic activity as measured by the benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) cleavage assay (n = 3 independent experiments, mean ± standard deviation). (*D*) The GzmA-WT and GzmA-S195A inhibit intracellular mycobacterial replication, whereas GzmA-C93S does not (n = 8 subjects from 3 independent experiments; mean ± standard error of the mean [SEM]; Wilcoxon matched-pairs signed-rank test). (*E*) Mixing experiment combining GzmA-WT and GzmA-C93S shows partial inhibition of GzmA-WT activity (n = 6 subjects from 3 independent experiments; mean and SEM; Wilcoxon matched-pairs signed-rank test) (***, $P < .0001$; **, $P < .001$; *, $P < .01$; ns, not significant).

internalization [31], was upregulated in GzmA-treated and infected cells (Figure 3*D*).

Granzyme A-Opsonized Mycobacteria Are Limited in Intracellular Replication Capacity

Given GzmA binding to mycobacteria, we tested whether GzmA-WT versus PBS opsonized BCG, then washed of non-bounded protein, would affect intracellular replication of phagocytosed mycobacteria within monocytes (summary schematic in Supplementary Figure 3*B*). Although inhibition was modest compared to standard assay, GzmA-WT opsonized BCG had restricted replication (Figure 3*E*). These data suggest that CD14 and TLR4 favor internalization of mycobacteria for neutralization. To further study GzmA-inhibitory effects, cells were infected with GFP-BCG. When GzmA was detected in monocytes, these cells had the lowest BCG-GFP burden. Overall, a 78% reduction in mycobacterial growth area under the curve was seen (Supplementary Figure 3*C*).

CD14 and Toll-Like Receptor 4 Stably Bind to Granzyme A

Because GzmA inhibitory activity depends on CD14 and TLR4, we investigated whether GzmA could form stable interactions with these receptors by IP. Protein lysates were generated from human cells treated with either GzmA-WT or GzmA-C93S and then infected with BCG. Immunoprecipitation using an anti-GzmA antibody was used to identify proteins associated with GzmA, specifically, CD14 and TLR4. We found that GzmA-WT, but not monomeric GzmA-C93S, bound to CD14 (Figure 4*A*). We also found that TLR4 bound to GzmA-WT, but not

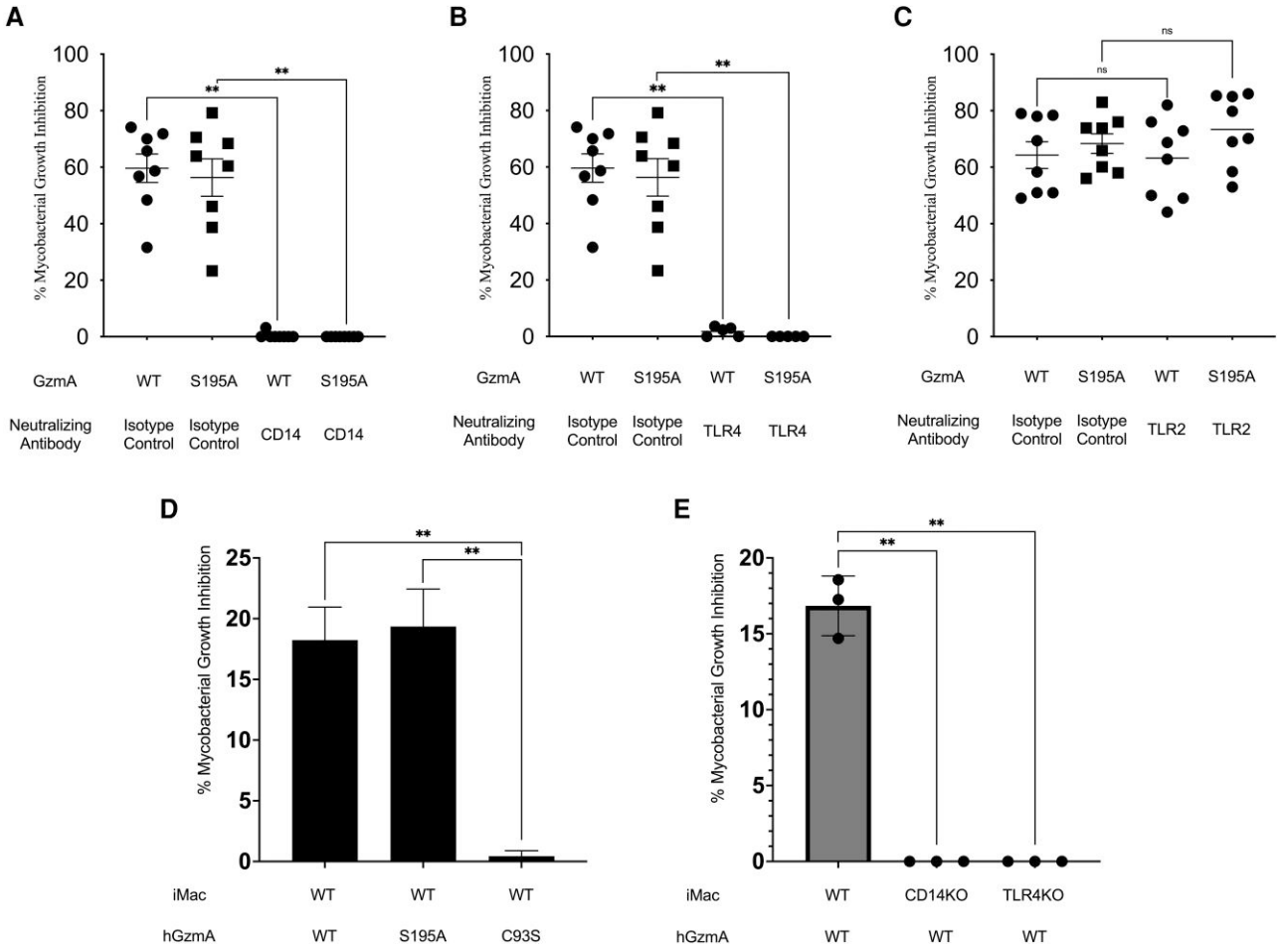

Figure 2. CD14 and Toll-like receptor (TLR) 4 receptors are important for granzyme A (GzmA)-mediated inhibition of intracellular mycobacterial Bacillus Calmette-Guérin growth. (*A*) Neutralization of CD14 receptor by neutralizing antibody abolishes GzmA-wild type (WT) and GzmA-S195A inhibitory activities (n = 8 subjects from 3 independent experiments; mean ± standard error of the mean [SEM]; Wilcoxon matched-pairs signed-rank test). (*B*) The TLR4 was neutralized using neutralizing antibody similar to (*A*) (n = 8 subjects from 3 independent experiments; mean and SEM; Wilcoxon matched-pairs signed-rank test). (*C*) Neutralization of TLR2 does not reverse GzmA-WT or GzmA-S195A inhibitory function (n = 8 subjects from 3 independent experiments; mean and SEM; Wilcoxon matched-pairs signed-rank test). (*D*) The GzmA-WT and GzmA-S195A, but not GzmA-C93S, induced inhibition of mycobacteria within immortalized mouse macrophage (iMac)-WT mouse cells (n = 3 independent experiments; mean and standard error of the mean [SEM]; Student *t* test). (*E*) The iMac-WT responding to GzmA-WT mediates intracellular mycobacterial inhibition within iMac-WT, but not CD14KO and iMac TLR4KO cells (n = 3 independent experiments; mean and SEM; Student *t* test) (**, $P < .001$; ns, not significant).

GzmA-C93S (Figure 4*B*). The interaction between GzmA and CD14/TLR4 occurred in both BCG-infected and uninfected cells. Cell lysates were immunoprecipitated with isotype control antibody, and then they were probed for CD14 and TLR4 WB as controls (Supplementary Figure 4*A* and *B*).

Granzyme A Opsonization of Mycobacteria Results in Enhanced Phagolysosome Fusion

To understand the downstream effects of GzmA opsonization on phagocytosed mycobacteria, monocytes were infected with BCG-expressing GFP, treated with GzmA, and stained with LAMP1. LAMP1 has been studied as marker of late phagosome-lysosome fusion to assess colocalization of mycobacteria within the phagolysosome [32]. Our studies showed a greater degree of colocalization between LAMP1 and BCG-GFP after GzmA-WT treatment compared with untreated controls or GzmA-C93S (see Figure 4*C* and representative figures in Supplementary Figure 4*C*–*F*). In these figures, it can be appreciated that BCG-GFP colocalizes more with LAMP-1 after treatment with GzmA-WT, compared with untreated infected cells or infected cells treated with GzmA-C93S.

Granzyme A Treatment Has Identical Effects on *Mycobacterium tuberculosis*-Infected Human Monocytes

Although BCG closely mimics the infection kinetics and can elicit a similarly strong cellular response as Mtb, it was important to verify that our studies could be generalizable to fully virulent Mtb. Thus, human monocytes were infected with Mtb

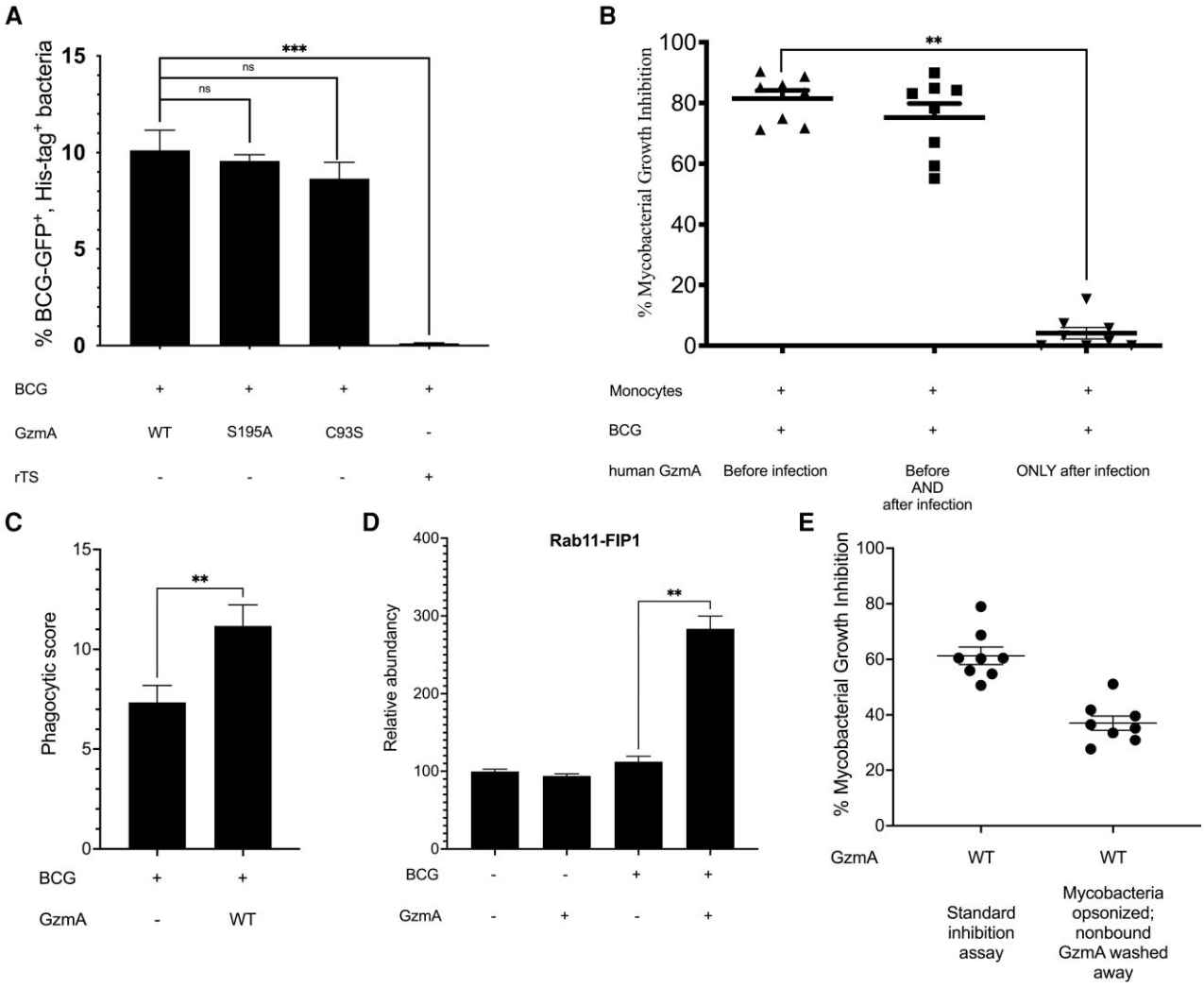

Figure 3. Granzyme A (GzmA) binds to Bacillus Calmette-Guérin (BCG), and its opsonization enhances monocyte inhibitory and phagocytic capacity. (*A*) Results demonstrating that GzmA-wild type (WT), GzmA-S195A, and GzmA-C93S bind to BCG, whereas negative control protein rTS cannot (n = 3 independent experiments; mean and standard error of the mean [SEM]; Student *t* test). (*B*) The GzmA needs to be added to cells before and/or during infection to induce inhibitory activity (n = 8 subjects from 3 independent experiments; mean and SEM; Wilcoxon matched-pairs signed-rank test). (*C*) The GzmA-WT enhances phagocytosis of BCG-green fluorescence protein (GFP) compared to untreated cells at 2 hours postinfection (n = 3 independent experiments; mean and SEM; Student *t* test). (*D*) Rab11-FIP1 protein is upregulated in GzmA-treated and BCG-infected cells as measured by quantitative shotgun proteomics (n = 4 subjects from 4 independent experiments; mean and standard deviation). (*E*) The GzmA-WT opsonizes BCG, and after its excess is removed it continues to inhibit the intracellular replication of the pathogen, whereas GzmA-C93S cannot (n = 8 subjects from 3 independent experiments; mean and SEM) (***, $P < .0001$; **, $P < .001$; ns, not significant).

and treated with GzmA-WT, S195A, and C93S. Similar to the effects of GzmA on intracellular BCG, both enzymatically active and inactive homodimeric GzmA inhibited Mtb, whereas monomeric GzmA did not (Figure 4*D*). Neutralization of CD14 and TLR4 also reversed GzmA-mediated mycobacterial growth inhibition. Thus, our studies are generalizable to virulent Mtb, strengthening the biological significance of our findings.

Protein Disulfide Isomerase A1 Cleaves and Colocalizes With Granzyme A

Previous study showed that PDIA1 is upregulated in human monocytes after mycobacterial infection (31% compared to control) and significantly more in cells infected and treated with GzmA (90% compared to control) [20]. Based on previous reports showing that PDIA1 can cleave disulfide bonds and is found on the surface of human cells [33, 34] and in human plasma [35], and from our findings that GzmA-C93S cannot inhibit mycobacteria, we next investigated the effects of PDIA1 on GzmA. First, we incubated GzmA-WT with PDIA1 and observed the monomeric band at 27 kDa, which was not present when heat-inactivated PDIA1 was used (Figure 5*A*). As demonstrated in Figure 5*B*, anti-GzmA antibody recognized the 27-kDa protein, demonstrating the GzmA monomer. To assess colocalization, cells were analyzed by immunocytochemistry, and as indicated in Figure 5*C* (orange regions), there was

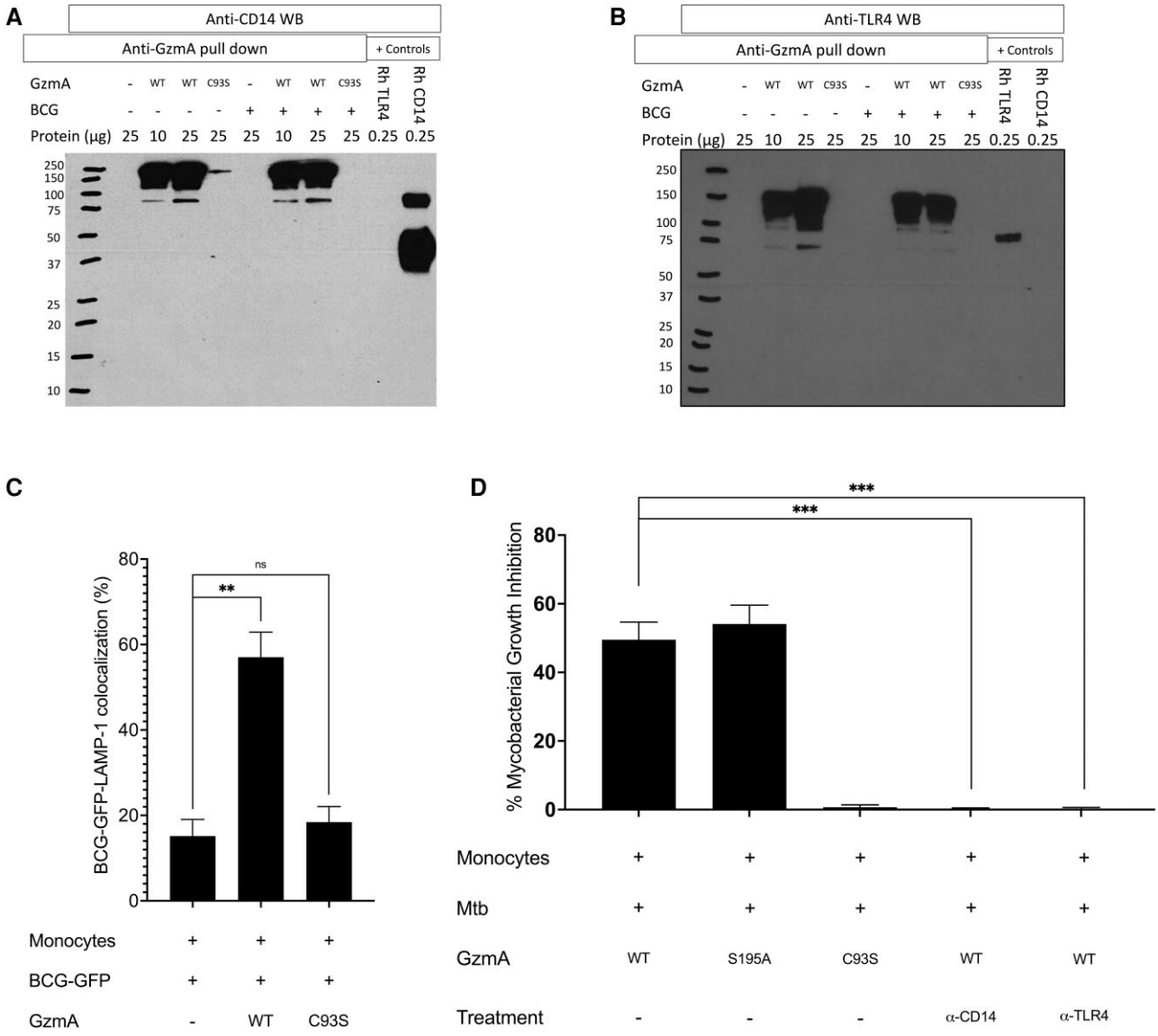

Figure 4. Granzyme A wild type (GzmA-WT), but not GzmA-C93S, interacts with CD14 and Toll-like receptor 4 (TLR4), kinetics of GzmA action, evidence of enhanced phagolysosome fusion, and translation of key findings to virulent *Mycobacterium tuberculosis* (Mtb) model of infection. (*A*) and (*B*) Different amounts of protein lysates made from Bacillus Calmette-Guérin (BCG)-infected human monocytes were incubated with α-GzmA to perform immunoprecipitation of bound proteins. These proteins were then probed for CD14 (*A*) or TLR4 (*B*), which showed that GzmA-WT binds to CD14 and TLR4, whereas GzmA-C93S does not (representative figures from 2 independent experiments). (*C*) The BCG-green fluorescence protein (GFP) colocalizes with LAMP1 after GzmA-WT treatment, demonstrating that opsonized mycobacteria enter the phagolysosome leading to better neutralization. The GzmA-C93S fails to direct mycobacteria into the phagolysosome (quantification of integrated density from the confocal microscopy experiments for the colocalization of mycobacteria and LAMP1 marker analyzed: values are mean ± standard deviation of ∼40 cells per each experiment, from 2 independent experiments). (*D*) All components of GzmA effects shown with BCG-infected cells (shown in Figures 1 and 2) were also confirmed with Mtb-infected monocytes (n = 6 subjects, from 2 independent experiments; mean and standard error of the mean; Wilcoxon matched-pairs signed-rank test) (***, $P < .0001$; **, $P < .001$; ns, not significant).

significant overlap between GzmA and PDIA1. Because the resolution of traditional immunocytochemistry is limited (∼200 nm), STED microscopy was used (∼50-nm resolution) (Supplementary Figure 5*A*) [34]. Using software deconvolution, we obtained images that showed the overlap of GzmA and PDIA1 as seen in orange in Figure 5*D*. Quantification showed a high degree of colocalization (75%) between GzmA and PDIA1, whereas a low degree was found with ATP5H, a mitochondrial protein previously found to be upregulated by GzmA but of unknown importance [20] (Figure 5*E*). Representative images are shown in Supplementary Figure 5*B* and *C*.

Protein Disulfide Isomerase A1 Inhibitors Enhance Granzyme A-Mediated Inhibition of Intracellular Mycobacteria

Because PDIA1 cleaves GzmA-WT into a monomer that lacks inhibitory activity, we tested whether PDIA1-specific inhibitors

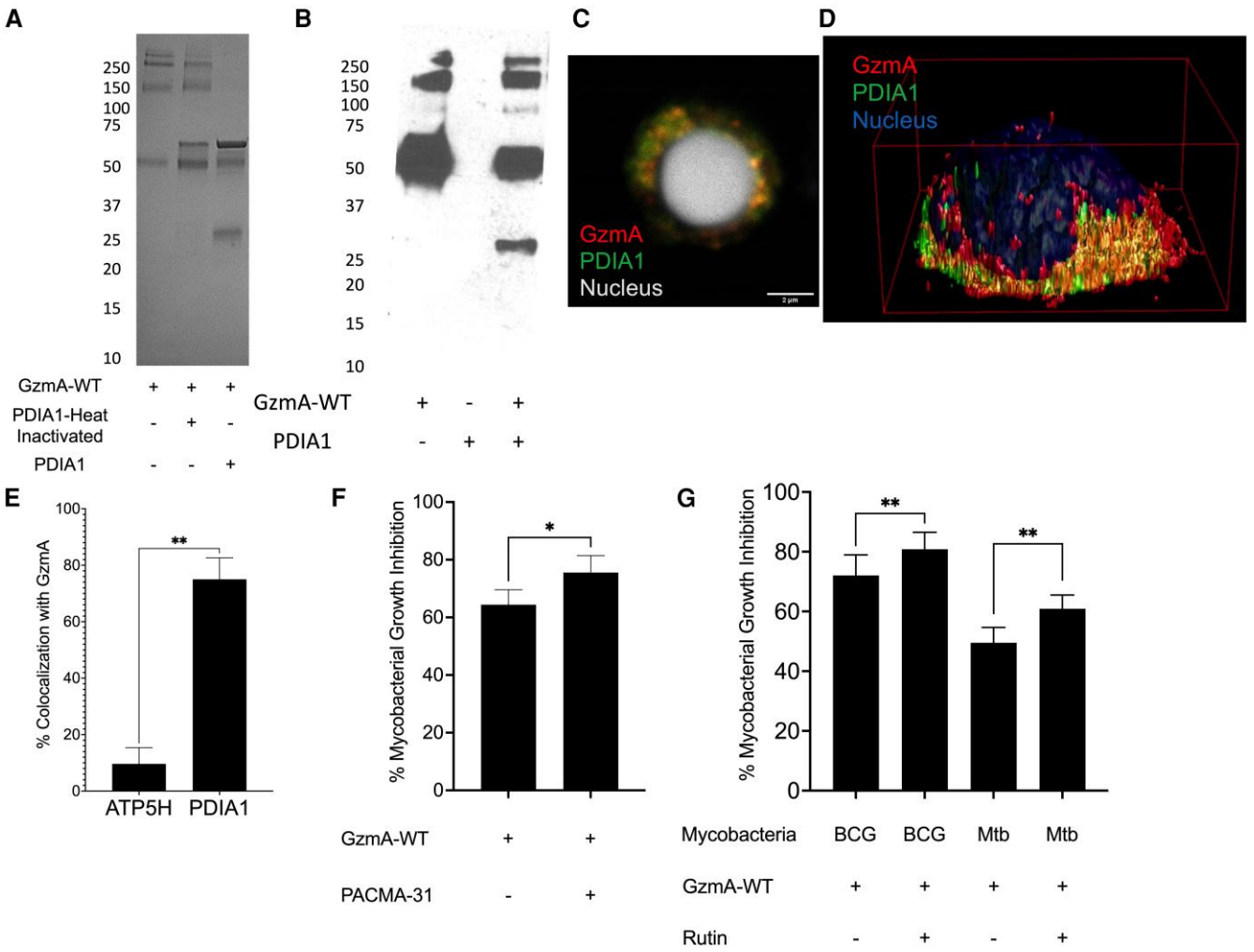

Figure 5. Protein disulfide isomerase A1 (PDIA1) cleaves granzyme A (GzmA) homodimers into monomers, and PDIA1 colocalizes with GzmA after treatment. (*A* and *B*) Silver-stained and anti-GzmA Western blot showing results from incubating heat-inactivated or fresh PDIA1 with GzmA-wild type (WT) and the appearance of GzmA monomer at 27 kDa. (*C*) Confocal microscopy image of human monocytes infected with mycobacteria and treated with GzmA, and its colocalization with PDIA1. (*D*) Deconvoluted stimulation emission depletion images showing visual colocalization of GzmA and PDIA1 (representative figure from 3 independent experiments). (*E*) Percentage of colocalization by quantifying objective Pearson correlation for GzmA and ATP5H (control) or PDIA1 (data from 3 independent experiments; mean ± standard deviation). (*F*) Cell-permeable inhibitor PACMA-31 (200 nM) enhances GzmA-inhibitory activity (n = 8 subjects from 3 independent experiments; mean and standard error of the mean [SEM]; Wilcoxon matched-pairs signed-rank test). (*G*) Similar to (*F*), but cell-impermeable inhibitor rutin (50 μM) was used and showed GzmA enhancement in Bacillus Calmette-Guérin (BCG) and *Mycobacterium tuberculosis* (Mtb)-infected monocytes (n = 8 subjects from 3 independent experiments; mean and SEM; Wilcoxon matched-pairs signed-rank test) (**, $P < .001$; *, $P < .01$; ns, not significant).

would enhance GzmA-WT inhibitory activity. Cell-permeable PDIA1 inhibitor PACMA-31 did not influence intracellular bacterial burden when given alone (Supplementary Figure 5*D*), but only when it was combined with GzmA-WT did it enhance inhibition (Figure 5*F*). Reports have shown that in addition to endoplasmic reticulum (ER) localization, cells can express PDIA1 on their surface [33, 34]. Flow cytometry confirmed that human monocytes express PDIA1 protein on the cell surface versus isotype control (Supplementary Figure 5*E*). To further identify the location of PDIA1 effects, we used the cell-impermeable PDIA1 inhibitor rutin and found that it also enhanced GzmA-mediated inhibition, presumably by limiting the proposed turnover of GzmA-WT into monomer (Figure 5*G* and Supplementary Figure 5*F*). The enhancement of GzmA-mediated inhibition was seen with both BCG and Mtb infections. Thus, the moderate PDIA1-enhancing effects on GzmA-mediated mycobacterial growth inhibition occur at the monocyte cell surface and within the cytoplasm.

DISCUSSION

Because GzmA's enzymatic activity is not necessary for the inhibition of intracellular mycobacteria, a biochemical approach was taken to investigate homodimerization as a key determinant of protection. In this study, we demonstrate that homodimerization is required for the inhibition of intracellular pathogen growth. To the best of our knowledge, this is the first time that GzmA's monomeric function was interrogated. Moreover, proteomic studies found upregulation of PDIA1 expression after mycobacterial infection [20]. Given the PDIA1 disulfide bond-breaking capacity, we found that PDIA1 converts GzmA homodimer into monomer (Figure 5*A* and *B*) and PDIA1 inhibitors (Figure 5*F* and *G*) moderately enhance GzmA inhibitory effects on mycobacteria. Given PDIA1's presence in the plasma of human samples [35] and on the cell surface of cells [33, 34], we found PDIA1 to be expressed on the cell surface of human monocytes. This suggests that use of PDIA1 inhibitors may be an attractive avenue to enhance GzmA effects without intracellular off-target effects, and additional experiments are needed to identify optimal drug delivery systems and the specific active components of GzmA required for mycobacterial inhibition. Moreover, there is evidence of higher thrombotic events in active TB patients compared to the general population [36]. Thus, the use of PDIA1 inhibitors could function as both antithrombotic as well as host-directed therapies to enhance immune responses against Mtb.

We found that both CD14 and TLR4 are important for the inhibition of mycobacteria (Figure 2*A* and *B*). Toll-like receptor 4 has been shown to enhance CD14- and LBP-triggered signaling in monocytes, leading to the production of IL-1β, IL-6, IL-8, and TNF [37]. The iMac studies confirmed the importance of CD14 and TLR4 for GzmA-mediated inhibition (Figure 2*D* and *E*). The more modest GzmA-mediated inhibition observed with iMac cells could be explained by the use of human GzmA with mouse cells. Further studies will investigate the relative concentration and affinity differences of surface receptors present among cell types and their relationship to inhibition.

Based on previous reports, it seems that GzmA strongly binds to some Gram-negative bacteria [19]. We found that both homodimer and monomer versions bind to BCG (Figure 3*B*). Thus, the GzmA protein structure can bind to components of the bacteria (likely the outer mycolic acid layer), and future studies will investigate which cell wall components are necessary for this interaction. Moreover, it is possible that GzmA homodimer interacts with multiple CD14 and TLR4 receptors, whereas GzmA monomer cannot, and future structural biology experiments will further investigate this. Similarly, we demonstrate by IP that GzmA stably binds to complexes including CD14 and TLR4, and future experiments will better delineate this interaction. Taken together, our data demonstrate that GzmA homodimer opsonizes mycobacteria for phagocytosis in parallel with the upregulation of Rab11-FIP1 (Figure 6). Recent data indicate that Rab11-FIP1 aids in the recycling of endocytic vesicles during an incoming phagocytic event [38], and that phagocytosis is severely impaired in Rab11-FIP1 knockout (KO) cells [31]. Thus, our data suggest that GzmA-opsonized mycobacteria signals through TLR4 and CD14, and Rab11-FIP1 increases the phagocytic process, enhancing the degradation of the mycobacteria within the phagolysosome. To the best of our knowledge, this is the first report that indicates a GzmA-mediated opsonization effect against a pathogen, and future research will investigate the implications against other microbes. Thus, we propose that when $\gamma_9\delta_2$ T cells as well as other immune cells (eg, αβ T cells and NK cells) secrete GzmA at the site of infection (eg, lungs for TB), GzmA opsonizes Mtb for easier destruction. Future work will need to determine whether GzmA directly enhances mycobacteria uptake by binding to a specific receptor or enhances affinity of a mycobacterial surface molecule for binding to TLR4 and/or CD14. The higher degree of colocalization of LAMP1 and mycobacteria highlights the restoration of phagolysosome fusion induced by GzmA (Figure 4*C*). Previous studies showed that ER stress response and ATP-producing proteins are important components of the GzmA-mediated intracellular pathogen degradation process, and future studies will investigate whether TLR4 and CD14 signaling triggers these antimicrobial properties. A recent study found that Rv3463 interacts through the TLR4 pathway, which leads to enhancement of phagolysosomal fusion and pathogen clearance [32]. Another study showed that GzmA-triggered inflammatory responses were abrogated in TLR4KO macrophages or WT cells pretreated with TLR4 inhibitors [13]. Recent studies have also shown the role of TLR4 in aiding the phagocytosis of pathogens and its

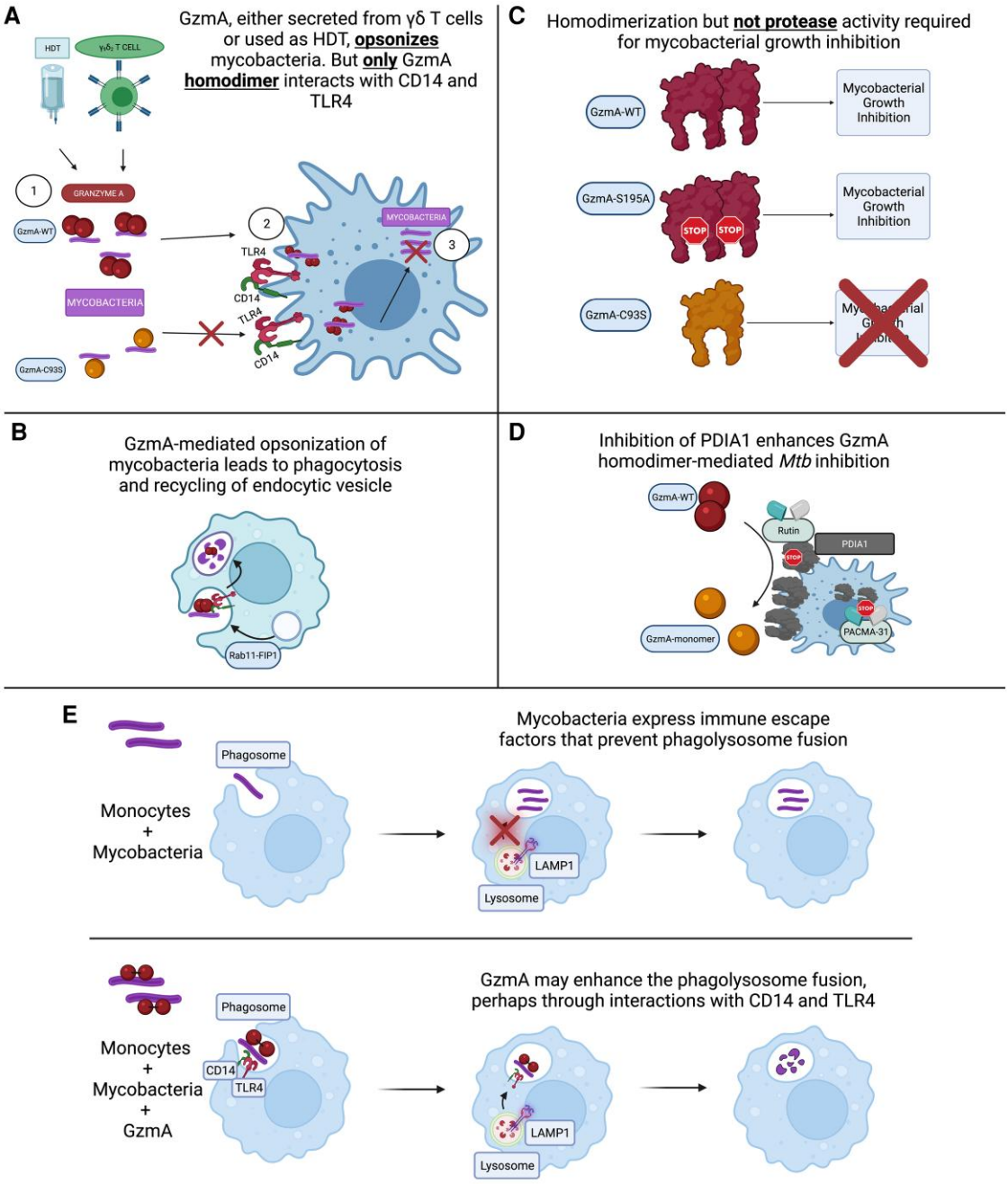

Figure 6. (*A*) Summary figures depicting our understanding of granzyme A (GzmA) in the inhibition of intracellular *Mycobacterium tuberculosis* (Mtb) growth. The GzmA either used as host-directed therapy (HDT) or secreted by $\gamma_9\delta_2$ T cells opsonizes mycobacteria. Only GzmA-wild type can interact with Toll-like receptor 4 (TLR4) and CD14 leading to inhibition. (*B*) The GzmA-treated cells have enhanced phagocytosis and Rab11-FIP1 protein aids in the recycling of the endocytic vesicle. (*C*) Diagram showing that homodimeric GzmA is feature necessary for mediating mycobacterial growth inhibition. Monomeric GzmA cannot inhibit mycobacterial growth. (*D*) Protein disulfide isomerase A1 (PDIA1) converts GzmA homodimer into monomer, and its inhibition potentiates GzmA-inhibitory activity. (*E*) Homodimeric GzmA-treated cells show enhancement of phagolysosome fusion (figure created with BioRender.com).

redirection in TLR4-containing endosomes [39]. Our results extend these earlier observations and indicate that GzmA opsonizes mycobacteria and redirects the pathogen into a direct phagolysosomal maturation dependent on TLR4 and CD14 that leads to efficient neutralization of the pathogen. Limitations of our interpretations include whether our theorized pathway is the only one involved or whether other unknown mechanisms further activate monocytes for mycobacterial clearance.

Our studies did not investigate the in vivo effects of GzmA in humans because of the need to use Good Manufacturing Practices material for clinical studies. Separately, we have not

investigated the effects of GzmA in vivo in mice because of the known differences between human and murine GzmA, both at the sequence and functional levels. Furthermore, there are the following functional redundancies among different mouse granzymes: there is a larger family of proteins found in mice (5 in humans and 8 in mice); there are different substrate specificities between mouse and human GzmA; mouse GzmA is cytotoxic compared to human GzmA; and there is a lack of increased susceptibility in GzmA$^{-/-}$ mice challenged with Mtb [13, 40]. Future research will investigate GzmA in vivo treatments in Mtb-infected nonhuman primates, humanized mice, and humans. However, our key findings were reproducible across models of attenuated BCG and virulent Mtb infection of monocytes.

CONCLUSIONS

In conclusion, GzmA homodimer opsonizes mycobacteria to form strong interactions with CD14 and TLR4 that leads to phagocytosis. This event likely enables cells to direct the incoming infection for better degradation. The use of PDIA1 inhibitors are attractive candidates as host-directed therapies that would enhance the antimicrobial properties of GzmA, and future studies should investigate humanized in vivo therapeutic potential of these strategies to lower tuberculosis burden worldwide.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Experiments were planned and executed by VR with the oversight of DFH. KRP and GRK contributed to the confocal microscopy and stimulation emission depletion experiments. CSE helped with the planning and support of all experiments. MC and NP helped with the protein disulfide isomerase A1 experiments. MDG and IZ helped with the CD14 and Toll-like receptor 4 experiments. SS, HLC, and DAF helped with the shotgun proteomics experiments. All authors reviewed and approved the final version of this manuscript.

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Potential conflicts of interest. All authors declare that they have no conflict of interest.

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