Ga(III) Nanoparticles Inhibit Growth of Both TB and HIV and Release of IL-6 and IL-8 in Co-Infected Macrophages

Seoung-ryoung Choi[1], *Bradley E. Britigan*[1,2,3] *and Prabagaran Narayanasamy*[1,*]

[1]Department of Pathology and Microbiology, [2]Department of Internal Medicine, College of Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68198 and [3]Research Service, VA Medical Center-Nebraska Western Iowa, Omaha, Nebraska 68105.

Corresponding author: p.narayanasamy@unmc.edu

Abstract: Treatment of individuals co-infected with human immunodeficiency virus-1 (HIV) and *Mycobacterium tuberculosis* (*M.tb*) is challenging due to prolonged treatment requirements, drug toxicity and emergence of drug resistance. Mononuclear phagocytes (MP; macrophages) are one of the natural reservoirs for both HIV and *M.tb*. Here, the treatment of HIV and *M.tb* co-infection was studied by preloading human macrophages with MP-targeted gallium (Ga) nanoparticles to limit subsequent simultaneous infection with both HIV and *M.tb*. Ga nanoparticles provided sustained drug release for 15 days and significantly inhibited the replication of both HIV and *M.tb*. Addition of Ga nanoparticles to MP already infected with *M.tb* or HIV resulted in a significant decrease in these infections, but was of lesser magnitude than with nanoparticle preloading of the MP. In addition, HIV/*M.tb* co-infected macrophages loaded with Ga nanoparticles showed reduced secretion of IL-6 and IL-8 for up to 15 days after drug loading. Ga nanoparticles also reduced IL-6 and IL-8 secretion by ionomycin and LPS induced macrophages, likely by modulating the IKK-β/NF-κB pathway. Delivery of Ga nanoparticles to macrophages is a potent long acting approach for suppressing HIV/*M.tb* co-infection of macrophages in vitro and sets the stage for the development of new therapeutic approaches for these important infections.

Human immunodeficiency virus-1 (HIV) and *Mycobacterium tuberculosis* (*M.tb*) are two major infectious agents that cause high mortality worldwide. *M.tb,* the causative agent of tuberculosis (TB), is one of the leading causes of death in the world. In 2015, the World Health Organization (WHO) estimated that 9 million people developed TB and 1.5 million died from it. Of the 9 million people who developed TB, 13% were also HIV positive.(1) Furthermore, HIV-associated TB infections and death in the world are increasing in frequency.(2)

Numerous challenges to the treatment of the HIV/TB co-infected patient have emerged. Multi-drug resistant TB and extensively drug resistant TB strains are a growing problem and often occur in the setting of co-existent HIV infection.(3) In addition, treatment of TB associated with HIV requires prolonged multi-drug treatment regimens that interact with some anti-retroviral drugs, increasing the potential for drug toxicity.(4) Thus, an urgent need exists for simplified, long acting and effective regimens to treat HIV/TB co-infection.(5)

In the pathogenesis of *M.tb* infection, the bacillus invades and multiplies intracellularly within monocytes and macrophages. The primary initial target of the bacillus is alveolar macrophages. There the bacilli replicate until their growth is restricted by activation of the macrophages by interferon-γ and other factors released from T-cells.(6, 7) The infection is contained within the lungs by forming granulomas comprised of *M.tb* infected macrophages, dendritic cells and T-cells.

After years of dormancy, *M.tb* can begin to multiply and cause reactivation disease if host immune function decreases. HIV infection amplifies the risk for developing active TB by decreasing T cell-mediated immunity, resulting in reactivation of latent *M.tb* infection. HIV

infection accelerates the rupture of granules, releasing active *M.tb* that is transported by dendritic cells to the lymph nodes.(6)

Both HIV and *M.tb* target and replicate in macrophages, in turn weakening human immunological functions. Intracellular replication of *M.tb* or HIV eventually leads to cell death and extracellular release of the pathogen.(8) Maintenance of the active infection within the host requires the ability of the infecting pathogen to continually establish infection in newly arriving susceptible cell types. Failure to do so would be expected to result in termination of active infection.

Macrophages are located in various tissues and play important roles in immunity by engulfing pathogens, eliminating apoptotic cells and recycling nutrients. Upon encountering bacteria and other inflammatory stimuli, macrophages secrete pro-inflammatory cytokines like tumor necrosis factor (TNF), IL-1, IL-6, IL-8 and IL-12. Some cytokines are potent pyrogens that activate and recruit other cells to sites of microbial invasion/infection. Forming of granulomas is a pathologic hallmark of host response to *M.tb* infection. Several cytokines have been identified in granulomas from BCG infection. Here, IL-6 is involved in the pathological functioning of *M. tb* infection.(9) IL-8 also controls granuloma formation which follows leukocyte influx on *M. tb* infection. High levels of IL-8 are observed in *M. tb* infected human tissue, plasma, pleural fluid and bronchoalveolar lavage fluid. In addition in *in vivo* studies, pretreatment of anti-IL-8 inhibited mycobacterial granuloma formation.(10) Cytokines also play a pivotal role in maintaining granulomas with the aid of $CD4^+$ T cells.(11) However, some cytokines enhance HIV replication in macrophages.(12)

To survive in humans and to be pathogenic, *M.tb* has to be able to acquire critical nutrients. Among these nutrients is iron (Fe), whose acquisition is required for the survival of mycobacteria residing in human macrophages.(13) (14, 15) Iron's ability to undergo redox cycling between ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states allows it to function as an electron transporter in many enzymatic systems, including those involved in DNA replication and cellular energy production.(16) Thus, blocking Fe acquisition by *M.tb* is a potential way to reduce the growth of *M.tb* within macrophages.(17)

Gallium (Ga) is a trivalent cationic element with many features that are similar to Fe, making it largely indistinguishable from Fe to many biologic systems. Ga can interact with biologically important proteins that are involved in Fe metabolism, both interfering with Fe acquisition mechanisms and the function of Fe-dependent enzymes, including catalases, Fe superoxide dismutase and ribonucleotide reductase. Insertion of Ga(III) into the active site of these normally Fe-centered enzymes renders them inactive, as, in contrast to Fe, Ga(III) cannot be reduced to Ga(II) in biological systems.(18) $Ga(NO_3)_3$ is a FDA-approved treatment for hypercalcemia associated with cancer and concentrates in activated macrophages.(19) We previously demonstrated that Ga disrupts Fe acquisition by mycobacteria and iron metabolism, leading to inhibition of the growth of these and other intracellular pathogens.(14, 20-24)

Development of antimicrobial nanoparticles that target macrophages has great potential advantages in drug delivery and clinical efficacy in the inhibition of intracellular pathogens. This strategy was applied to develop a macrophage-targeted nanoparticle to inhibit virus replication.(25, 26) Nanoparticles targeting MDM containing conventional antiretroviral therapy have been reported to lengthen the activity and efficacy of the existing therapies against HIV-1 infection with less toxicity.(27, 28)

In a recent study, we showed that a long acting Ga nanoformulation inhibited the growth of both HIV and *M. smegmatis* residing within monocyte-derived macrophages (MDMs) by releasing Ga(III) over 15 days after the cells were loaded with the drug.(8) No significant cytotoxicity resulting from these Ga nanoparticles was observed.(8) The results encouraged us to explore the potential of Ga nanoparticles for the treatment of HIV and *M.tb* co-infection (Figure **1**). Furthermore, we studied the impact of these Ga nanoparticles on cytokine release by macrophages infected with *M.tb* and HIV-1.

Materials and Methods

Ethics Statement. The human cell samples were purchased from and provided in an anonymized fashion by University of Nebraska Medical Center (UNMC) Department of Pharmacology and Experimental Neuroscience cell core facility. Experiments with human samples are performed in full compliance with the regulations of the National Institutes of Health. All participants involved in this study provided informed written consent. The methods were carried out in accordance with the approved UNMC ethical guidelines. Institutional Review Board (IRB) # 162-93-FB approved all experimental protocols with the title 'Leukapheresis of normal donors for use in studies of disease pathogenesis and therapy'.

Preparation and characterization of gallium nanoparticles. Gallium(III) *meso* tetraphenylporphyrine chloride (GaTP) was purchased from Frontier Scientific (Logan, Utah, USA). Nanoparticles were formulated, manufactured using high pressure homogenizer and characterized by DLS and SEM as per our previously reported procedure.(8, 29)

Cytotoxicity assay of GaNP. Cytotoxic effects of GaNP were determined on THP-1 macrophages using resazurin reduction assay. 3×10^5 THP-1 cells/well in a 48 well plate (0.75×10^6 THP-1 cells/well, 24-well plate) were placed and differentiated in the presence of PMA (7.5 ng/mL) for 24 hours. The THP-1 macrophages were loaded with GaNP (25, 100, 300, 500 µM), washed after 24 hours and placed in culture with RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum. After 24 hours or 15 days, the cells were washed thoroughly with PBS (5 times) and fresh media was added. 40 µL of a resazurin solution (0.15 mg/mL in DPBS, 100 µL for 24-well plate) was added to each well. After incubation at 37 °C for 3 hour, fluorescence intensities were measured at 560 nm excitation/590 nm emission.

***M. tuberculosis* (H37Ra) and HIV-1 co-infection of human MDM.** Human monocytes were prepared and purified by counter-current centrifugal elutriation from normal human donors were purchased from the UNMC Department of Pharmacology and Experimental Neuroscience cell core using an institutional IRB approved protocol. All samples were provided in a de-identified fashion.

To induce differentiation into MDM, the monocytes (0.75×10^6 cells/well/mL in a tissue treated 24 well plate) were incubated in DMEM media that was supplemented with 10% heat-inactivated pooled human serum (Innovative Biologics, Herndon, VA, USA), 50 µg/mL gentamicin (Mediatech Inc., Manassas, VA) to prevent replication of extracellular bacteria, 10 ng/mL MCSF (BioLegend, San Diego, CA), and 10 mM sodium pyruvate (Mediatech Inc., Manassas, VA) at 37 °C in 5 % CO_2 humidified atmosphere. Half the media was replaced on the 5^{th} day of incubation and then every 2 days thereafter until day 10 of incubation. At the 10^{th} day, the differentiated MDM were treated with DMEM supplemented with 1% human serum. On day 11, MDMs were incubated with GaNP (300 µM) for 24 h at 37°C in 5% CO_2 humidified

atmosphere. The drug loaded-MDMs were washed with PBS and incubated for up to an additional 15 days prior to infection to evaluate the long-acting potential of the Ga nanoparticles. Drug treated-MDMs were infected at desired time points (1, 5, 10, or 15 days post-drug loading) with *M.tb* (H37Ra) by incubating them, for 4 h (MOI = 1) in media lacking gentamicin. For studies of HIV infection only, macrophages were incubated with HIV-1_{ADA} (MOI = 0.01) for 24 h. For studies of HIV-1/*M.tb* co-infection, the cells were incubated with HIV-1_{ADA} (MOI = 0.01) for 24 h after 4 h infection with *M.tb* (MOI = 1) at days 1, 5, 10, 15 following drug treatment (again using media without gentamicin). After infection, MDMs were washed with PBS to remove extracellular *M.tb* and/or HIV and the cells were cultured in the same media containing gentamicin as described above to block extracellular replication of *M.tb*.

Determination of *M.tb* growth residing in macrophages after treatment. Drug treated-MDMs or control MDMs were infected with only *M.tb,* only HIV-1, or both *M.tb* and HIV-1 and lysed for analysis of *M.tb* CFU in macrophages 2 days after infection(s). After removing the media from the wells, iced sterile water (300 µL) was added to the wells followed by incubation on ice for 10 min. The MDMs were treated with 1.2 mL of lysis buffer containing 55% of 7H9 broth, 20% of the 0.25% SDS and 25% of the 20% BSA in PBS. The lysed cells were centrifuged at 14,000 x g for 15 min and the pellets were resuspended in 200 µL of PBS, serial diluted in sterile PBS and plated onto 7H11 agar plates. *M.tb* CFU were then counted after 3 weeks.

***M.tb* (H37Ra) or HIV-1 growth inhibition in infected THP-1 macrophages by Ga nanoparticles.** THP-1 macrophages (0.75 x 10^6 cells/well/mL in a tissue treated 24 well plate) were infected with either H37Ra strain (MOI = 1, 4 hour incubation) or HIV-1 (MOI = 0.01, 24 hour incubation) in RPMI1640 media that was supplemented with 10% heat-inactivated fetal bovine serum (no antibiotics were used). After washing with PBS buffer, the cells were treated

with GaNP (300 µM) in media (1% FBS) for 24 hours. Extracellular nanoparticles were removed by washing the cells with PBS buffer three times. The treated cells were lysed at day 3 and 5 following infection to isolate H37Ra and growth of *M.tb* was monitored by determining *M.tb* CFU as described above. For determination of HIV-1 growth, media were saved at day 4 and 5 and reverse transcriptase activity was assayed as described in the manufacture's protocol.

Determination of *M.tb* growth residing in THP-1 macrophages treated with $Ga(NO_3)_3$ or $Fe(NO_3)_3$. THP-1 cells (7.5×10^5 per well) were differentiated in RPMI 1640 containing 1% fetal serum, PMA (7.5 ng/mL), 10 mM sodium pyruvate, 50 µg/mL gentamicin, 0.75% $NaHCO_3$ and 10 mM HEPES (pH 7.0) at 37°C in 5% CO_2 humidified atmosphere for 24 hours. After washing with PBS buffer twice, THP-1 cells were treated with 300 µM GaNP, $Ga(NO_3)_3$ or a combination of GaNP and $Fe(NO_3)_3$ in RPMI 1640 media for 24 h. The growth of *M.tb* was determined as described above. In brief, drug treated-THP-1 macrophages were infected at desired time points (5, 10, or 15 days post-drug loading) with *M.tb* (H37Ra, MOI = 1) for 4 h in media without gentamicin. In a case of combination treatment of GaNP and $Fe(NO_3)_3$, $Fe(NO_3)_3$ was continuously added to the culture media until lysis for CFU counting.

Determination of HIV growth in macrophages after infection. For determination of the magnitude of HIV infection of MDM, MDM-containing wells infected with only HIV-1 or both HIV-1 and *M.tb* (MOI = 1) were incubated for 11 days at 37 °C, replacing the DMEM every 2 days. The cells were harvested at day 11 post-infection and stored at –80°C until Reverse Transcriptase (RT) activity determination. The RT assay (EnzChek) was performed as described in the manufacture's protocol.

194 **Quantitation of cytokines**. Culture supernatants were analyzed using a multiplex kit from Life
195 Technologies to assess levels of the human cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5,
196 IL-6, IL-8, IL-10, TNF-α) according to the manufacturer's instructions. In brief, the 96 well
197 filter plate was pre-wetted, and then 25 µL of the diluted bead suspension was added to each well
198 and washed twice. 100 µL of samples and standards were added to each well. The plate was
199 incubated at room temperature for 2 h on a shaker. Following washing, biotinylated detection
200 antibody was added and incubated for 1 h. After washing, streptavidin-RPE was added to each
201 well and incubated for 0.5 h. The plate was again washed, resuspended in 100 µL of the washing
202 buffer, and read on the Luminex xMAP® system (Bio-Rad, AtheNA Multi-Lyte®, USA). All
203 samples were run in triplicate and standards were run in duplicate.

204 **Whole cell extracts and Western blot analysis.** MDMs cultured in 6-well plates were washed
205 with ice-cold PBS buffer and cell proteins were extracted in Ripa buffer containing Halt™
206 protease inhibitor cocktail (Pierce Biotechnology, IL, USA) and Pierce™ Phosphatase inhibitor
207 (Pierce Biotechnology, IL, USA). The lysates were collected and centrifuged at 4 °C at 12,000 x
208 g for 10 min. The supernatants were collected and BCA assay was performed to determine
209 protein concentration. Protein samples were electrophoresed under denaturing conditions using
210 4-20% polyacrylamide gels (Bio-Rad, USA). After transferring to a PVDF membrane, the
211 membranes were analyzed with goat polyclonal IKKβ antibody (Santa Cruz Biotechnology, CA,
212 USA), rabbit monoclonal NF-κB1 and phosphor-Akt antibodies (Cell Signaling Technology,
213 MA, USA). The bound antibody was detected using horseradish peroxidase-conjugated goat
214 anti-rabbit IgG (Novex, MD, USA) or rabbit anti-goat IgG (Santa Cruz Biotechnology, CA,
215 USA) and developed with a chemiluminescent substrate (Pierce Biotechnology, IL, USA). Image
216 J was used for densitometric analysis of membrane blots.

Statistical analysis. Statistical analysis was performed using Student's t- test. Data are represented as mean ± SEM. Data are considered significant at $p < 0.05$.

Results and Discussion

In order to optimize macrophage-utilized drug delivery, various physical properties of Ga nanoparticles were tested to increase the encapsulating potential, prolong drug release and minimize cytotoxicity. This resulted in the development of gallium nanoparticles (GaNP) using water-insoluble gallium tetraphenyl porphyrin (GaTP) and p407 pluronic polymer using a high-pressure homogenization technique.(8) Excellent drug loading (up to 48%) was achieved and the SEM image showed a rod like shape (Figure **1**). The potential for cytotoxicity of various concentrations (25 ~ 500 μM) of GaNP on THP-1 macrophages was assessed for up to 15 days post loading of the cells with the nanoparticles (Figure **2** and **S1**). No toxicity, as assessed by resazurin reduction, was detected. Uptake of Ga nanoparticles by MDMs was observed and led to sustained Ga release for 15 days.(8)

Pathogenesis of infection with *M.tb* and HIV requires the ongoing infection of uninfected host cells migrating to the site of infection. Interruption of this process by increasing resistance of uninfected cells to infection should terminate infection. Therefore, we assessed the susceptibility of MDMs pretreated with Ga nanoparticles 1 day, 5 days, 10 days and 15 days earlier to infection with *M.tb* and/or HIV compared to untreated MDMs. These studies were designed to test the duration of protective efficacy of the gallium nanoparticle against the two pathogens. Defining the duration of Ga drug availability after drug loading of macrophages is also critical to

a potential strategy of using drug-loaded macrophages to deliver Ga drugs to sites of infection.

GaNP decreases the growth of *M. tuberculosis* in MDMs and THP-1 macrophages. We assessed anti-mycobacterial activities of GaTP and GaNP on human MDMs infected with *M.tb*. MDMs were incubated with GaTP or GaNP for 24 h, washed and then placed back into culture. At defined time points after exposure to GaTP or GaNP (1, 5, 10, or 15 days), the cells were incubated with *M.tb* (H37Ra) for 4 hours, washed free of extracellular bacteria, and then placed back in culture. After an additional 2 days of incubation, the MDM were lysed and *M.tb* CFU were determined. Even only 1 day post drug loading, the free drug GaTP showed minimal inhibitory effect on *M.tb*, with CFU similar to that of untreated control MDM (Figure **3A**). In contrast, GaNP exhibited significant inhibition (more than 10 fold compared to GaTP) on the growth of *M.tb*, even when infection of the MDM occurred as long as 15 days after drug loading. CFU detected in drug treated and control MDM infected at 10 days post drug treatment and beyond were less than those infected with *M.tb* following drug exposure (Figure **3A**). GaNP results, which are consistent with our prior data with *M. smegmatis*,(8) suggest that incorporation of Ga into MDM-deliverable nanoparticles leads to sustained release of Ga into the intracellular compartment at a level sufficient to inhibit growth of *M.tb* up to 15 days post drug loading of the MDM.

Iron (Fe) is a critical nutrient for the survival of mycobacteria residing in human macrophages.(13) In earlier studies, gallium nitrate shown to significantly inhibit intracellular mycobacterial growth. This appeared to occur via disruption of bacterial iron metabolism, as addition of exogenous iron to the media decreased the antimicrobial effect of gallium nitrate.(24) We compared gallium nitrate activity with GaNP. Five days after incubation of the macrophages

with the two drugs, both gallium nitrate and GaNP showed similar reduction in *M.tb* growth (Figure **S3**). However, GaNP continued to limit bacterial growth for 15 days after macrophage drug loading (Figure **3A**). To provide insight into the role of iron limitation in GaNP-mediated inhibition of *M.tb* growth in MDM, GaNP in the presence and absence of $FeNO_3$ were added to THP-1 macrophages, followed by *M.tb* infection. Interestingly, we did not see any difference in bacterial growth (Figure **S2**). The observed result might be due to higher intracellular concentrations of Ga achieved with GaNP, differences in timing of *M.tb* infection and gallium treatment compared to the prior study, effect of the nanoparticle among possible explanations. A detailed mechanistic study will need to be carried out.

HIV growth increases in the presence of *M.tb*/HIV co-infection of MDMs: In preparation for extending studies of GaNP to MDM that were co-infected with *M.tb* and HIV, we determined the effect of co-infection of the MDM on the growth rates of *M.tb* and HIV-1. The growth of *M.tb* residing within the infected MDMs *in vitro*, as assessed by CFU, was not significantly different in the presence of HIV over 2 days (Figure **4A**). This lack of apparent difference may be due to the fact that *M.tb* grows slowly and here, our determinations were made after only two days, possibly masking a difference in growth at a later stage of co-infection. In contrast, *in vitro* co-infection of MDMs with *M.tb* (H37Ra) and HIV-1 increased the levels of HIV-1 up to 2 fold, as assessed by reverse transcriptase (RT) assay, over the course of 11 days in culture (Figure **4B**). The viability of MDMs were decreased by infection with *M.tb* and HIV, but the cells survived up to 15 days as viewed by inverted phase microscopy.

Ga(III) nanoparticle inhibited the growth of *M.tb* and replication of HIV-1 residing in MDMs. As shown in Figure **3**, GaNP treatment of MDMs resulted in decreased growth of *M.tb* when infection was initiated up to 15 days after drug loading occurred. HIV-1 replication

requires Fe-dependent protein ribonucleotide reductase, NF-κB, CDK9, eIF5a and ABCE1 and recent work from our lab has shown the ability of Ga to inhibit HIV replication in MDM.(8) Therefore, we investigated the antimicrobial activities of GaNP on MDMs co-infected with HIV-1 and *M.tb* (Figure **5**).

Consistent with results from the experiments shown in Figure **3**, GaNP showed long-acting inhibitory activity against *M.tb, i.e.* significant *M.tb* growth inhibition was observed up to 15 days after incubation of the MDM with GaNP.

Consistent with the earlier data from our lab(8), MDMs treated with GaNP that were co-infected with *M.tb* and HIV-1 exhibited significantly reduced replication of HIV (2 fold) compared to non-treated MDMs. In contrast, free drug (GaTP) did not have a significant inhibitory effect (Figure **5B**). As with *M.tb*, HIV-1 growth inhibition persisted for 15 days post loading of the MDM with GaNP (Figure **5B**). Interestingly, GaNP reduced the growth of *M.tb* residing in MDMs by 10 fold compared to non-treated MDMs while a 2-fold growth decrease of *M.tb* in co-infected MDMs was observed. This finding may indicate that *M.tb* and HIV are competing over Ga uptake under the limited Ga environment, resulting in less GaNP-induced killing of *M.tb*.

The effect of GaNP administered after THP-1 macrophages were infected with *M.tb* and/or HIV was also investigated. GaNP treatment resulted in significant reduction of bacterial growth, with 61% and 70% compared to their positive controls at day 3 and 5 post infection, respectively (Figure **3B**). HIV growth was also inhibited by 25% and 37% at day 4 and 5, respectively, by GaNP post treatment (Figure **3C**). The magnitude of effect of GaNP on *M.tb* load in the macrophages appears to be greater with GaNP pretreatment (Figure **3A**) versus post treatment (Figure **3B** and Figure **3C**). Inherently, GaNP pretreatment appeared to decrease the initial HIV

and *M.tb* infection of the cells compared to control (Figure **3**). Thus, it seems possible that GaNP is able to both block initial infection and further replication of *M.tb* and HIV, but is unable to decrease the burden of infection of a cell beyond that when GaNP treatment begins.

Secretion of cytokines by MDMs co-infected with HIV-1/*M.tb*. Once exposed to *M.tb*, a host immune defense response is initiated by producing a variety of molecules, including cytokines and, chemokines, and by developing a T-cell mediated immune response.(30) These T-cells produce interferon-γ (IFN-γ), which plays an important role in activating macrophages to produce reactive oxygen and nitric oxide species and release other cytokines and chemokines.(6) IFN-γ, TNF-α and IL-12 appear to be key cytokines in controlling *M.tb* infection in mice and humans.(31, 32)

In parallel to our initial studies examining the effect of macrophage loading with GaNP on growth of *M.tb* (Figure **3**), we also determined the effect of GaNP loading on the release of IL-6 and IL-8 from *M.tb* infected MDMs. Interestingly, GaNP reduced the release of IL-6 and IL-8 from the *M.tb* infected MDMs (See supporting Information) in parallel to reducing the number of *M. tb*. CFU (Figure **3**).

Co-infection of macrophages with *M.tb* and HIV may also alter the profile of cytokines produced by macrophages. In order to investigate this possibility, the cytokine profile of culture supernatants from MDMs co-infected with HIV and *M.tb*, were analyzed using a Luminex system. As seen in Figure **6**, of 10 cytokines assessed, two major interleukins (IL-6 and IL-8) were detected in high quantity, with trace amounts of IL-1β, TNF-α, IFN-γ, MCSF and IL-4 (See supporting Information). No significant amount of TNF-α (<3 pg/mL) was detected, which is in good agreement with other studies on HIV/TB co-infection.(33) Also, TNF-α released by

329 macrophages facilitates macrophage apoptosis in response to *M.tb*.(34-36) Patel *et.al*(33) also
330 observed that HIV infection reduced macrophage apoptosis in response to *M.tb* due to reduced
331 production of TNF-α.

332 It has been suggested that IFN-γ and TNF-α can serve as excellent biomarkers for the diagnosis
333 of TB.(37) Recently, IL-6 was also suggested as a potent biomarker in mycobacterial infections
334 (H37Rv, H37Ra, *M. smegmatis*) of mouse peritoneal macrophages.(38) Although the reported
335 study was conducted with murine macrophages *in vitro*, the high level of IL-6 release is
336 consistent with a human macrophage study.(39) As shown in Figure **6A**, a significantly higher
337 amount of IL-6 was found in culture supernatants from MDM co-infected with HIV and *M.tb*
338 compared to uninfected MDM, indicating that HIV infection does not influence IL-6 release by
339 *M.tb*-infected MDMs, supporting the potential for the use of IL-6 as a biomarker in
340 mycobacterial infection of HIV co-infected patients.

341 Interestingly, IL-6 production by MDMs loaded with GaNP for 5 and 10 days prior to infection
342 with *M.tb* and HIV was 6 fold less than control cells that were not treated with Ga (Figure **6A**).
343 When infection occurred at day 15 post-GaNP loading, the levels of IL-6 in treated or non-
344 treated MDMs were lower compared to those infected at day 5 and 10 post drug loading (Figure
345 **6A**). This was likely at least in part due to a decrease in viability of macrophages with longer
346 time in culture. However, GaNP treated MDM still showed a 6-fold lower level of IL-6 release
347 compared to the non-GaNP treated control (Figure **6A**). In contrast to results with GaNP, MDM
348 that received free drug (GaTP) (Figure **6A**) or nanoparticles not containing GaTP (not shown)
349 exhibited IL-6 responses similar to the non-Ga treated control cells.

We also found that HIV/*M.tb* co-infected MDM production of IL-8 was 20-fold higher than uninfected control MDM, regardless of how many days they were in culture prior to infection (Figure **6B**). IL-8 is a pro-inflammatory chemokine produced by monocytes, macrophages, endothelial cells and other types of cells. Its main role is to recruit neutrophils to sites of infection, as well as T lymphocytes and monocytes.(40) The main sources of IL-8 are monocytes and macrophages infected with pathogens, including mycobacteria.(41) Although higher levels of IL-8 along with IL-6 are found in pleural fluid in TB or HIV/TB patients, there are no significant differences in pattern or level of cytokines between two groups.(42) Recently, Krupa and *et. al*. suggested in their study that IL-8 can bind to *M.tb* and this association may enhance the immune response in patients with TB.(43) These studies are in good agreement with our *in vitro* study of IL-6 and IL-8 secretions in response to *M.tb*/HIV infection of MDM.

Similar to IL-6, GaNP treatment also significantly reduced the secretion of IL-8 by HIV/TB co-infected MDM (Figure **6B**). This effect was maximal in the cells that were infected at day 15 post loading with GaNP (Figure **6B**), where IL-8 production was equal to the uninfected MDMs.

The above data show that GaNP decreases *M.tb*/HIV induced IL-6 and IL-8 production, which could be by inhibiting *M.tb* and/or HIV replication in MDMs. However, it is also possible that the GaNP has an effect on the ability of MDM to produce IL-6 and/or IL-8 by altering an MDM signaling pathway needed for production of these cytokines. Therefore, the effect of GaNP on MDM release of IL-6 and IL-8 in response to lipopolysaccharide (LPS, 10 μg/ml), ionomycin (1 μM), and MCSF (10 ng/ml, See **SI**) was investigated. Interestingly, GaNP was able to significantly inhibit MDM IL-6 and IL-8 production in response to LPS and ionomycin (Figure **7A**). We also observed changes in the regulation of IKK-β/NF-κB by GaNP (Figure **7B**). By western blot we observed the increased expression of IKK- β in the presence of GaNP. In

general, IKK-β is a NF-κB inhibitor and accordingly, we observed the down regulation of NF-κB by western blot. We did not see any changes in phosphoAkt and pP38 expression by western blot with GaNP (Figure **S10** and **S11**). These results are consistent with the possibility that GaNP decreases IL-6 and IL-8 by targeting IKK-β signaling pathway.

In general, IL-6 and IL-8 production appear to have a negative effect on host defense against *M.tb*.(9, 44, 45) IL-6 promotes *M.tb* growth and inhibits production of TNF-α and IL-1β that are needed for maximal killing of *M.tb*.(9, 45) Similarly, IL-8 increases inflammation and granuloma formation of *M.tb*.(44) Therefore, GaNP mediated inhibition of both IL-6 and IL-8 could further enhance host defense against *M.tb* beyond the drug's ability to inhibit growth of the organism through disruption of Fe metabolism. Ga-NP is able to reduce the *M. tb* growth for longer period independently and when it is co-infected with HIV. In addition, it is also able to regulate the secretion of both IL-6 and IL-8 by the MDMs, likely by interfering in the IKKβ-NFκB cell signaling pathway.

In summary, the effect of GaNP loading of human MDM on *in vitro* co-infection of these macrophages with HIV-1 and *M.tb* was studied. Up to 15 days post-loading with the drug, GaNP was able to reduce the growth of both *M.tb* and HIV-1. Given what is known to date about the mechanism of action of Ga, this is most likely due to disruption of iron metabolism critical to pathogen growth/survival, although this could not be confirmed in the present work.

HIV-1/*M.tb* co-infected MDMs *in vitro* exhibited increased production of IL-6 and IL-8, but negligible amounts of TNF relative to uninfected macrophages. GaNP treated MDMs showed reduced IL-6 and IL-8 production. This could be due to inhibition of *M.tb*/HIV infection and/or

GaNP-mediated modification of macrophage signaling pathway(s) that result in production of these two cytokines.

Development of Ga (III)-based drugs that target human macrophages may be a potential approach for the treatment of co-infection of HIV-1/*M.tb* and reduction of IL-6 and IL-8 generation. A similar approach could allow targeting of organisms present in other cellular reservoirs. In addition, improvement of drug loading, cell targeting, encapsulation efficiency and sustained drug release will also be important for optimizing treatment.

Acknowledgement

This work was supported in part by a Nebraska Research Initiative award to PN and VA Merit Review award to BEB.

Disclosures

The authors declare no competing financial interest

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Legends

Figure 1. Scheme for testing antimicrobial activities of gallium (III) in *M.tb* and HIV-infected infected MDMs.

Figure 2. Toxicity of Ga nanoparticle (GaNP) against THP-1 macrophages. In a 24 well plate, 0.75×10^6 cells/well were seeded. The wells were treated with various concentrations of GaNP for 24 hours, and then the wells were washed with PBS buffer thoroughly. The GaNP-treated macrophages were further incubated for additional 15 days with changing media for every 2 day and viability was determined using resazurin reduction assay.

Figure 3. Antimicrobial activities of gallium (III). (A) MDMs were incubated with GaTP or GaNP for 24 hours, following which the cultures were washed free of extracellular drug. At days 1, 5, 10, and 15 following drug treatment, these MDM, as well as control MDM that had not been exposed to drug, were then infected with *M.tb* (H37Ra, MOI = 1) for 4 hours and then the *M.tb* allowed to grow for 2 days. *M.tb* (B) and HIV-1 (C) growth inhibition in infected THP-1 macrophages by Ga nanoparticles. THP-1 macrophages were infected with H37Ra strain (MOI = 1) or HIV-1 (MOI = 0.01) and then incubated with GaNP for 24 hours. Growth inhibition was monitored over time by determining CFU for *M.tb* and RT assay for HIV-1. Ga was used in the form of Gallium (III) tetraphenylporphyrin (GaTP, 300 μM) or GaTP encapsulated nanoparticles (GaNP, 300 μM). Data represents the mean ± SEM of triplicate (n = 3). Statistical differences were determined using Student's *t* test: * $p < 0.05$ compared with non-drug treated control. *** $p < 0.001$, **** $p < 0.0001$.

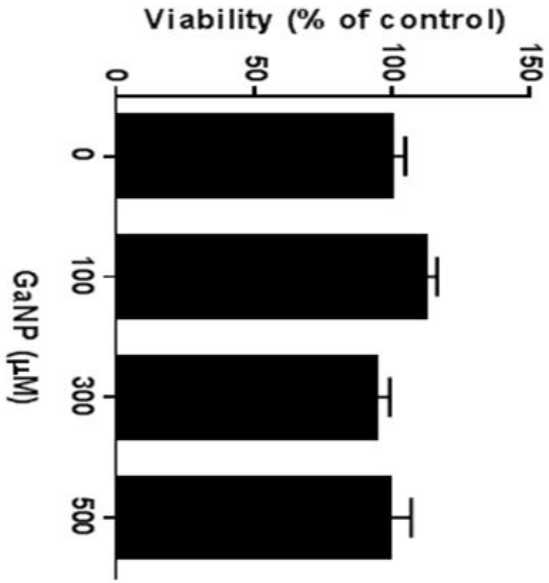
Figure 4. Quantitation of the growth of *M.tb* and the replication of HIV in MDMs co-infected with HIV-1/*M.tb*. A) *M.tb* growth was determined by CFU, B) HIV-1 quantitation in human MDMs was determined by Reverse-Transcriptase assay. MDMs were infected with HIV-1 (MOI = 0.01), *M.tb* (H37Ra, MOI = 1), or co-infected with HIV-1 and *M.tb* at Day 8, 13 and 18 following differentiation. The co-infected MDMs and HIV-infected MDMs were further incubated for 11 days with changing medium every 48 h. Supernatants from HIV-1/*M.tb* co-infected

cells were harvested at day 11 for analysis of RT. Data represents the mean ± SEM of triplicate (n = 3). Statistical differences were determined using Student's *t* test: *p < 0.01 compared with HIV (+) in (B).

Figure 5. Antimicrobial activities of gallium (III) in HIV-1/*M.tb* co-infected MDMs. Antimicrobial activities were determined by (**A**) CFU for *M.tb* and (**B**) RT assay for HIV-1. Gallium (III) tetraphenylporphyrin **(**GaTP, 300 μM) and gallium (III) tetraphenylporphyrin encapsulated nanoparticles (GaNP, 300 μM) treated MDMs were infected with both HIV-1 and *M.tb* (H37Ra) at days 5, 10, and 15 following drug treatment of the MDMs. Data represents the mean ± SEM for n = 3 or 6. Statistical differences were determined using Student's *t* test: *p < 0.01 compared with non-drug treated control in (A) and (B).

Figure 6. Analysis of cytokines present in supernatants from HIV-1/*M.tb* co-infected macrophages. A) IL-6, B) IL-8. MDMs were co-infected with HIV-1 and *M.tb* (H37Ra) at Day 5, 10 and 15 following MDM incubation with (gallium (III) tetraphenylporphyrin **(**GaTP, 300 μM) and gallium (III) tetraphenylporphyrin encapsulated nanoparticles (GaNP, 300 μM)). Following infections, supernatants were analyzed for the presence of cytokines released from infected MDM day 11 after infection. Data represents the mean ± SEM for n = 3 or 6. Statistical differences were determined using Student's *t* test: *p < 0.05 compared with HIV-1 and TB positive control in (A) and (B).

Figure 7. A). Release of IL-6 and IL-8 by MDMs in the presence of LPS (10 μg/ml) or ionomycin (1 μM), compared with GaNP treated MDMs exposed to the same agents. Pre-loading with GaNP significantly reduced IL-6 (*p < 0.01) and IL-8 (*p < 0.05) generation induced by LPS and ionomycin. B). Western blot analysis of IKK-β and over-expression of IKK-β was observed in presence of GaNP. C). Western blot analysis of NF-κB and observed down regulation in the presence of GaNP, confirming the relation with IKK-β. Statistical differences were determined using Student's *t* test: n = 3, *p values compared with non-drug treated MDMs.



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