Opsonic monoclonal antibodies enhance phagocytic killing activity and clearance of *Mycobacterium tuberculosis* from blood in a quantitative qPCR mouse model

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ABSTRACT

Background: Patients with impaired immunity often have rapid progression of tuberculosis (TB) which can lead to highly lethal *Mycobacterium tuberculosis* (MTB) sepsis. Opsonic monoclonal antibodies (MABs) directed against MTB that enhance phagocytic killing activity and clearance of MTB from blood may be useful to enhance TB immunity.
Methods: BALB/c mice were immunized with ethanol-killed MTB (EK-MTB) and MABs were produced and screened by ELISA for binding to killed and live *Mycobacterium smegmatis* (SMEG) and MTB. MAB opsonophagocytic killing activity (OPKA) was examined using SMEG with HL60 and U-937 cells and MTB with U-937 cells. Clearance of MTB from blood was evaluated in Institute of Cancer Research (ICR) mice given opsonic anti-MTB MABs or saline (control) 24 h prior to intravenous infusion with 10^8 CFUs gamma-irradiated MTB (HN878). MTB levels in murine blood collected 0.25, 4 and 24 h post-challenge were assessed by qPCR. MAB binding to peptidoglycan (PGN) was examined by ELISA using PGN cell wall mixture and ultra-pure PGN.
Results: Two MABs (GG9 and JG7) bound to killed and live SMEG and MTB (susceptible and resistant), and promoted OPKA with live MTB. MAB JG7 significantly enhanced OPKA of MTB. Both MABs significantly enhanced clearance of killed MTB from murine blood at 4 and 24 h as measured by qPCR. These opsonic MABs bound to PGN, a major cell wall constituent.
Conclusions: Anti-MTB MABs that promote bactericidal phagocytic activity of MTB and enhance clearance of killed MTB from the blood, may offer an immunotherapeutic approach for treatment of MTB bacteremia or sepsis, and augment treatment of multi-drug resistant (MDR) or extensively drug resistant (XDR) TB.

1. Introduction

Tuberculosis (TB) ranks among the top ten causes of death worldwide and continues to be a major threat to global health [1]. In 2017, there were an estimated 10 million new TB cases worldwide, of which 1.6 million individuals died of the disease [1, 2]. Additionally, an estimated 0.9 million of these new cases were HIV-positive of which 0.3 million (30%) died of TB. In sub-Saharan Africa, many HIV positive individuals are co-infected with TB [2, 3]. In HIV-positive patients, TB is the leading cause of death [2], and many develop MTB bacteremia, sepsis and disseminated disease [4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15], often dying within 30 days of hospitalization [10, 15]. Furthermore, strains of MTB are becoming increasingly resistant to antibiotic therapy, and the emergence of multiple drug resistant (MDR) and extensively drug resistant (XDR) strains have increased the urgency to find new approaches for prevention and treatment of TB [16].

In the pre-antibiotic period, specific antibacterial serum was deemed beneficial in the treatment of pneumococcal pneumonia and sepsis [17, 18]. The development of antibiotics decreased the use of anti-serum therapy until the availability of intravenous immune globulin (IVIG) in the 1980's [19, 20]. Antibodies in IVIG that bound to the bacterial capsule of gram positive bacteria enhanced phagocytosis and provided protection
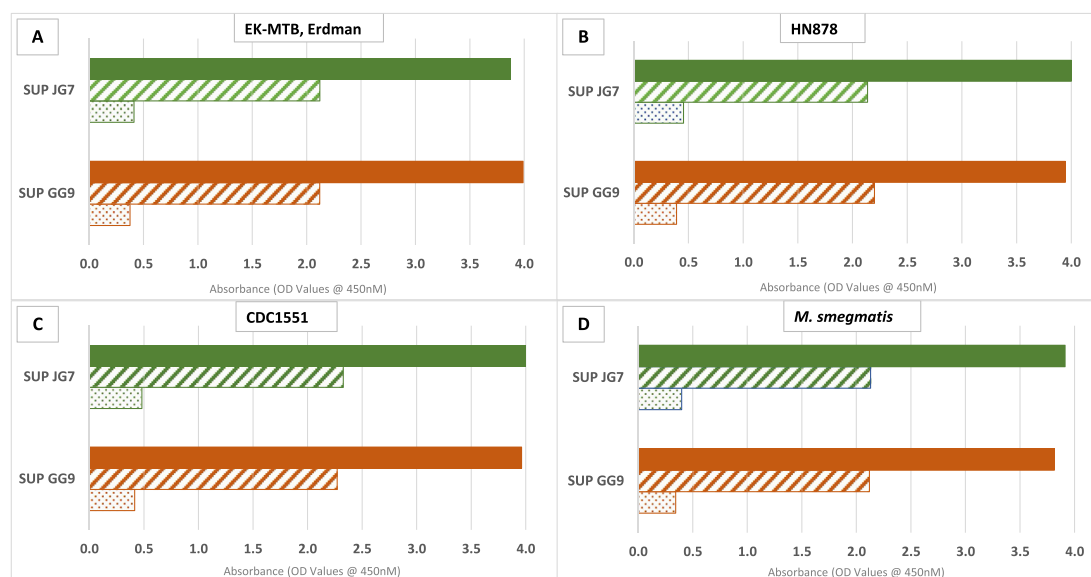

Fig. 1. Binding activities of supernatants from hybridomas JG7 and GG9 to *Mycobacterium tuberculosis* (MTB) and *Mycobacterium smegmatis* (SMEG), evaluated at dilutions 1:10 (solid), 1:100 (diagonal stripes) and 1:1000 (dotted) on fixed mycobacteria at 1×10^5 CFU/well. Panel (A) through (C), respectively, shows binding of supernatant to killed MTB Erdman, HN878 and CDC1551. Panel (D) depicts binding of supernatants to fixed SMEG. OD values for growth media without antibody (negative control) range between 0.046–0.060 (data not shown).

in vivo [19, 20]. In addition, the ability to produce monoclonal antibodies (MABs) that react to specific pathogens and are functionally active has provided highly effective therapeutics with minimal side effects [21]. MTB has a thick waxy capsule which is an important virulence factor enabling survival in the infected host [22, 23, 24, 25, 26] and anti-capsular antibodies may promote phagocytosis and killing of MTB [24]. During infection, MTB bacilli are engulfed by macrophages which subsequently enter phagosomes [27]. Membrane-bound proteins on the mycobacteria-containing phagosomes recruit lysosomes leading to phagosome-lysosome fusion (P-L fusion) forming mature phagolysosomes which kill and digest MTB [28]. Conversely, MTB evades this defense mechanism by persisting in macrophages [29]. Antibodies to MTB have been shown to promote FcγR-mediated macrophage phagocytosis, phagolysosome maturation and MTB killing and therefore may play an important role in phagocytic killing of MTB [30]. While evidence suggests that cell-mediated immunity is the protective immune function against MTB [31], the emergence of MDR and XDR MTB strains coupled with inadequate protection (among adults) from current vaccine strategies [31] has led to a renewed interest in exploring the potential use of antibodies to prevent or treat TB [30]. Evidence suggests that antibodies could play an important role in intracellular killing of MTB [32] and recent studies have demonstrated that opsonic antibodies promote phagolysosome maturation that enhances phagocytic killing of MTB [24, 28, 32].

In this report, we describe novel MABs directed against MTB that promote phagocytosis and killing of mycobacteria (MTB and SMEG) as well as enhance clearance of MTB DNA from the blood in a murine MTB bacteremia model.

2. Materials and methods

2.1. Mycobacterium smegmatis (SMEG)

M. smegmatis ATCC Cat 21701 (ATCC, Manassas, VA, USA) was cultured aerobically in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) overnight to mid logarithmic phase at 37°C with shaking at 250rpm and the resulting suspension used as a surrogate for MTB.

2.2. Mycobacterium tuberculosis (MTB), inactivated laboratory isolates

Ethanol-killed MTB (EK-MTB), strain Erdman, ATCC 35801 (Battelle,

Columbus, OH, USA) was provided at 10^8 CFU/mL (OD 600nM = 1.000). Gamma Irradiated MTB, strains HN878 (NR-14821) and CDC1551 (NR-14820) were obtained from BEI Resources (Manassas, VA, USA) at 8.7×10^{10} CFU/mL.

2.3. Mycobacterium tuberculosis (MTB), live laboratory and clinical isolates

H37Ra ATCC 25177, two susceptible clinical MTB strains (STB1 and STB2), three multidrug-resistant MTB strains (MDR1, MDR2 and MDR3) and two extensively drug-resistant MTB strains (XDR1, XDR2) were used. Strains were obtained from both the National Health Laboratory Service-Tshwane Academic Division (NHLS/TAD) in Pretoria and the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. The standard Ziehl-Neelson acid-fast staining technique was carried out on the cultures and viewed under the microscope for the presence of mycobacteria and the MPT64TB (SD Bioline, South Korea) antigen test was carried out for confirmation of MTB.

2.4. Mice immunizations

Female BALB/c mice 3-4-week-old, Harlan Laboratories (Indianapolis, IN, USA) were obtained for use in this study. Stock EK-MTB (vaccine) was centrifuged at 12000rpm for 5 min at room temperature (RT), washed three times in Phosphate Buffered Saline pH 7.4 (PBS, Fisher Scientific, Pittsburg, PA, USA) and 100μL of the immunogen injected subcutaneously, without adjuvant, into mice on Day 0 at approximately 1×10^5 CFU/mouse. Boosts were given per two protocols: (a) days 7, 16, 27 and 42 with blood samples obtained prior to initiation of the first protocol, and on days 19, 41, and 63 for Mice 1319-1324, and (b) days 14, 29 and 43 with blood samples obtained prior to the initiation of the second protocol and on days 28, 35, 42 and 63 for Mice 1417-1420. Three days prior to fusion, mice were given an intravenous final boost of 1×10^4 bacteria. About 150–200 μL of blood was collected at each bleed. All animal procedures were performed in an AALAC-accredited facility. All procedures were reviewed and approved by the facility IACUC.

2.5. Fusion and hybridoma production

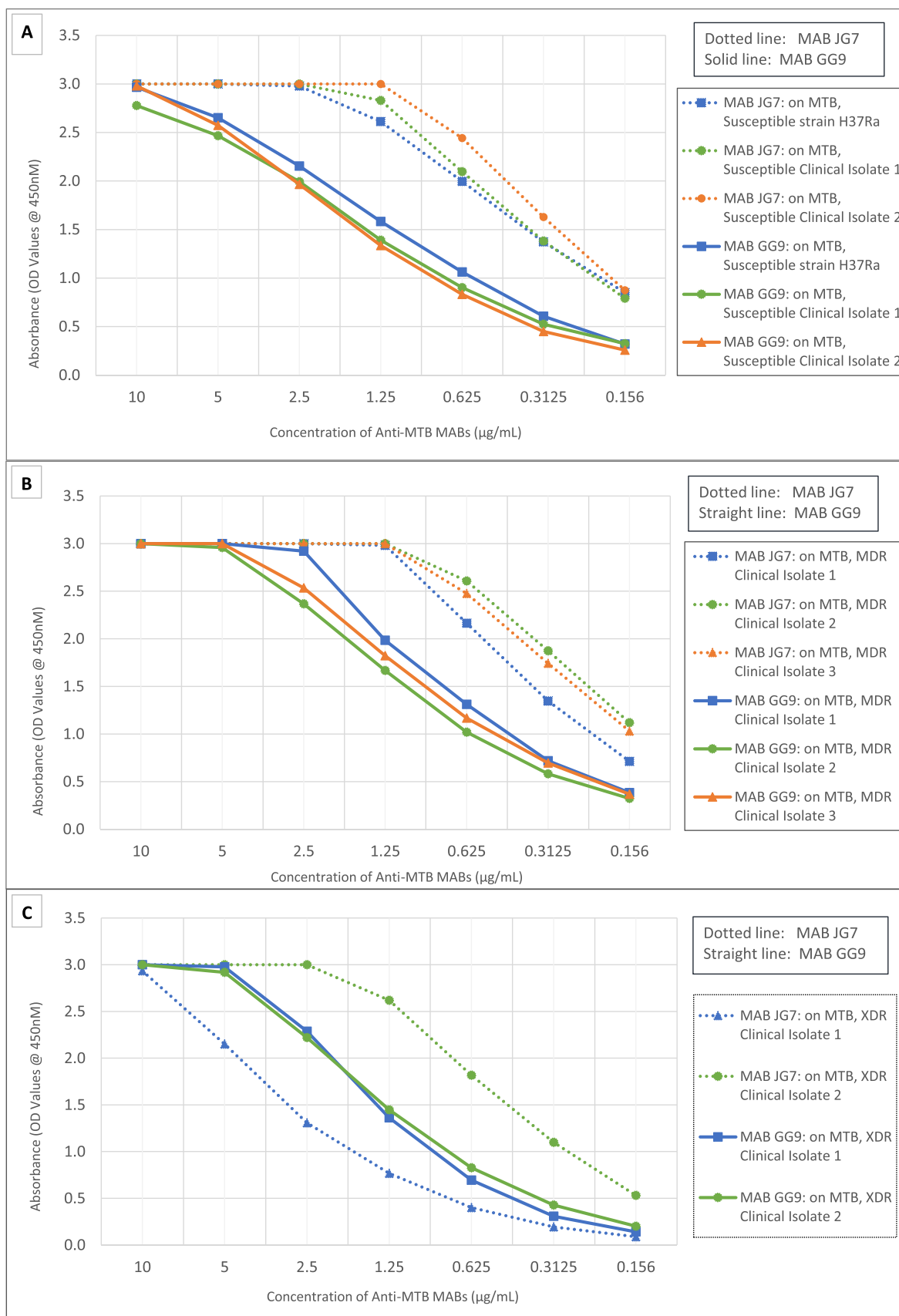Two BALB/c mice (Mice 1323 and 1420 – one from each

Fig. 2. Binding activity of purified anti-*Mycobacterium tuberculosis* monoclonal antibodies (anti-MTB MABs) JG7 and GG9 on fixed MTB at 1×10^5 CFU/well. Panel (A) demonstrates MAB binding to susceptible lab strain H37Ra and clinical isolates 1 & 2; Panel (B) to multidrug-resistant (MDR) clinical isolates 1, 2 & 3; and Panel (C) to extensively drug-resistant (XDR) clinical isolates 1 and 2. Data (expressed as mean) are representative of three individual experiments.
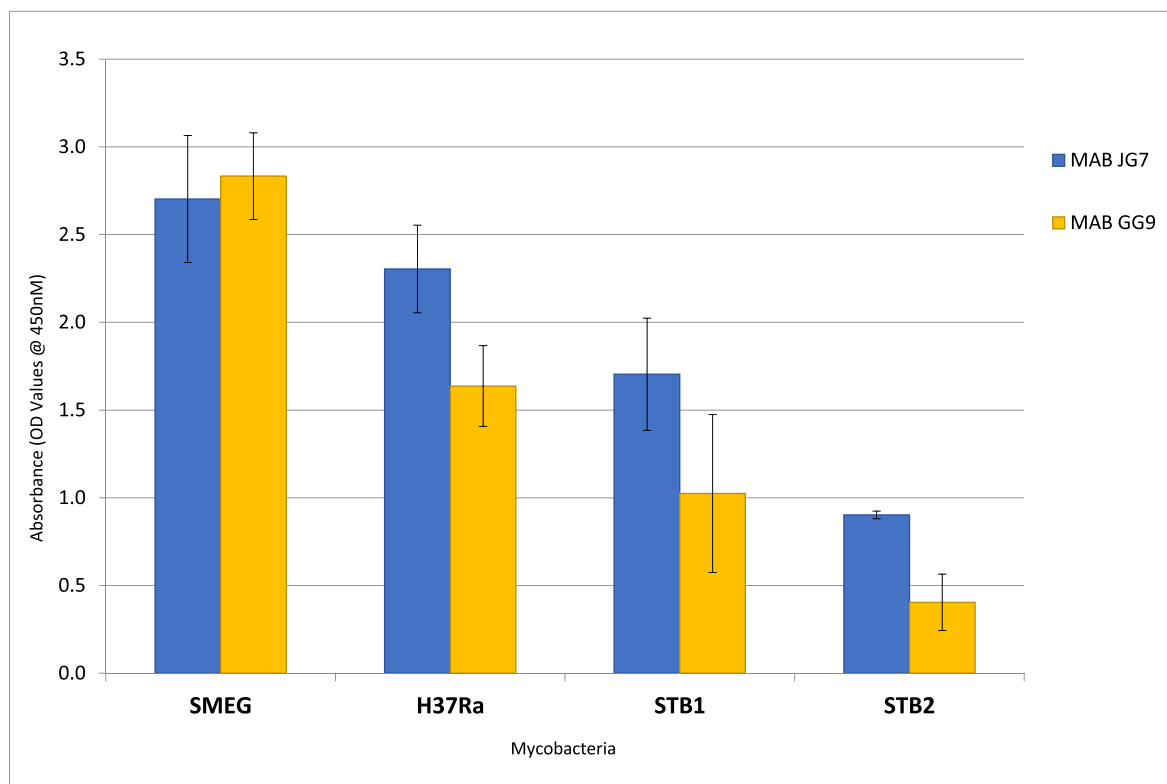
Fig. 3. Binding activity of purified anti-*Mycobacterium tuberculosis* monoclonal antibodies (anti-MTB MABs) GG9 and JG7 to live *Mycobacterium smegmatis* (SMEG) and live susceptible MTB H37Ra (lab strain) and STB1 and STB2 (susceptible clinical isolates) as demonstrated in a Live Bacteria ELISA. Data (expressed as mean ± standard errors; n = 3) are representative of three individual experiments.

immunization protocol) with high anti-MTB serum titers were euthanized [33], and single cell spleen suspensions from each mouse [34] were fused to SP2/0 myeloma cells (Sigma-Aldrich, St. Louis, MO, USA) using standard techniques [34, 35]. Hybridoma supernatants were tested by Enzyme-Linked Immunosorbent Assay (ELISA) for binding to EK-MTB and high producing cells were selected for cloning, tested further for MAB production, and then expanded for supernatant collection, purification by Protein G Column Chromatography [36] and quantification by IgG Capture ELISA [35].

2.6. Detection of antibodies that bind to mycobacteria

2.6.1. Anti-sera ELISA

Serum anti-MTB levels were evaluated using killed MTB. 96-well NUNC™ MaxiSorp ELISA plates (Fisher Scientific, Pittsburg, PA, USA) were coated with EK-MTB in 70% alcohol (Fisher Scientific, Pittsburg, PA, USA) at 100μL per well (1×10^5 CFU) for 18–24 h at room temperature (RT) or up to 2 days at 2–8°C. MTB-coated plates were blocked with 3% Normal Goat Serum (NGS, Southern Biotech, Birmingham, AL, USA) in PBS and subsequently, washed with PBS-0.05% Tween 20 (PBS-T, Fisher Scientific, Pittsburg, PA, USA) using an ELx405 Automated Plate Washer (BioTek, Winooski, VT, USA). Serial dilutions of serum samples were added. Anti-MTB antibodies were detected with Horse Radish Peroxidase (HRP)-Conjugated, gamma-specific, Goat anti-Mouse IgG (Southern Biotech, Birmingham, AL, USA) or HRP-Conjugated, IgG isotype-specific Goat anti-Mouse IgG1, IgG2a, IgG2b, IgG3 (Southern Biotech, Birmingham, AL, USA). TMB Substrate Solution (Fisher Scientific, Pittsburg, PA, USA) was added prior to being quenched with TMB STOP solution (Fisher Scientific, Pittsburg, PA, USA). The absorbance values (450nM) of each well were obtained using a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Pre-immunization sera were used as negative controls.

2.6.2. Fixed ELISA

Hybridoma supernatants were screened for their capacity to bind to killed laboratory MTB isolates: Ethanol-killed Erdman, and Gamma-irradiated HN878 and CDC1551 MTB strains, using procedures described above, excluding the blocking step with 3% NGS.

Purified anti-MTB MABs were screened for their capacity to bind to clinical susceptible, MDR and XDR isolates. MTB suspensions, from either ATCC or BACTEC 960-mycobacterial growth incubation tube (MGIT) stock cultures, were sub-cultured in MGITs at 1:10 dilutions (0.5mL in 5mL) containing Middlebrook 7H9 broth and incubated in the BACTEC 960 machine at 37°C for approximately three weeks, until an optical density (OD600nM) of 0.120–0.150 was reached. The OD was measured using a HELIOS spectrophotometer (Thomas Scientific, Swedesboro, NJ, USA). The MTB strains were then washed three times in PBS (pH 7.2–7.4, tablets from Sigma-Aldrich Life Science, Chemie GmBH) by adding 3mL of the bacterial suspension to 15mL of PBS (1:5 dilution), and centrifuged at 2600rpm for 5 min at RT. The washed bacteria were resuspended in 3mL of PBS (1mL per 96-well plate, per MAB). 96-well NUNC™ MaxiSorp flat-bottom ELISA plates (Sigma-Aldrich life science, Chemie GmbH) were coated with serial dilutions of MTB at 100μL per well and incubated for at least 18 h at RT. The MTB coated plates were washed using an automated plate washer (Biotek, Winooski, VT, USA). Serial dilutions of the MABs were added and incubated for 1 h at RT. The anti-MTB MABs were then detected with IgG1 detection antibody (Southern Biotechnologies, Birmingham, AL, USA). TMB substrate solution (Sigma-Aldrich life science, Chemie GmbH) was added and after 15 min the reaction was quenched with TMB STOP solution (Sigma-Aldrich life science, Chemie GmbH). The absorbance was read at 450nM using the ELx800 reader (Biotek, Winooski, VT, USA).

2.6.3. Live bacteria ELISA for MAB-binding to SMEG

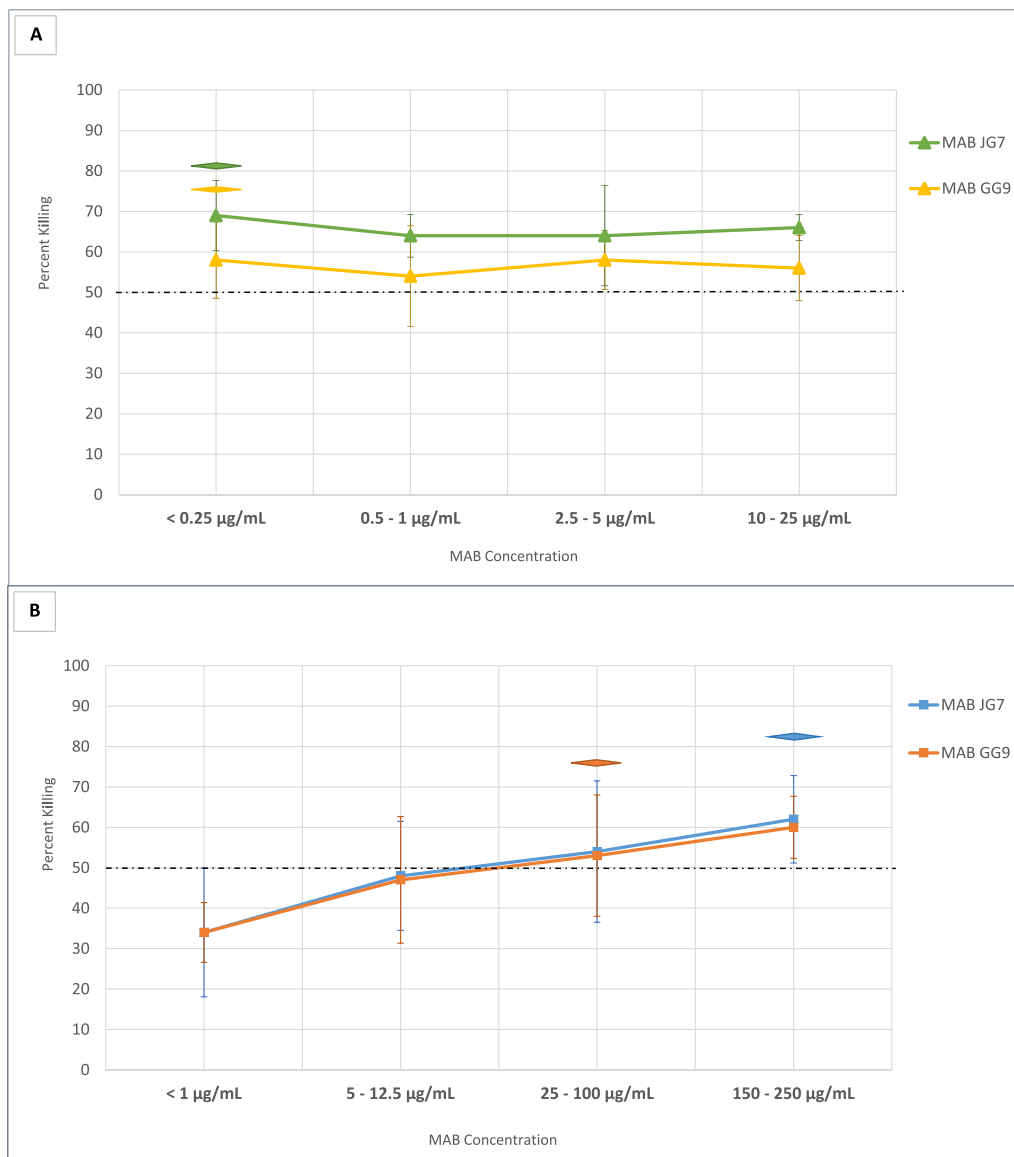The ability of purified anti-MTB MABs to bind to live SMEG was

Fig. 4. Enhanced OPKA of MABs JG7 and GG9 against *Mycobacterium smegmatis* (SMEG) using HL60 granulocytes and C1q (Panel A) occurred at low antibody concentrations (< 0.25μg/mL) and stayed constant when antibody levels were increased over one hundred fold. While MAB JG7 consistently had higher percent killing, the difference did not reach statistical significance. Peak OPKA for both JG7 and GG9 occurred at 0.06μg/mL and were 81% (green icon) and 76% (yellow icon), respectively. In panel B, enhanced MAB OPKA against SMEG using U-937 macrophages (without C1q) was significantly more pronounced at higher antibody concentrations (JG7: $p = 0.0001$, GG9: $p < 0.0001$) and both MABs tracked closely together across all antibody concentrations. Peak OPKA for JG7 and GG9 were 82% (blue icon) at 175μg/mL and 76% (orange icon) at 100μg/mL, respectively.

evaluated to confirm use of SMEG as target mycobacteria in opsonic assays. Overnight culture at mid-log phase was adjusted to 40% transmittance and washed in PBS containing 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA) by centrifugation at 2500rpm for 5 min at RT to remove traces of 7H9 broth, resuspended in PBS with 0.1% BSA and added to round-bottom 96-well plates (VWR, Randor, PA, USA) at 100μL per well, (~10^6 CFU/well). 50μL of diluted MABs (1, 10 and 25 μg/mL) were added on top of SMEG and placed in a 37°C shaker incubator at 100rpm for 1 h. Subsequently, assay plates containing bacteria and MAB were washed three times by centrifugation. After washing, HRP-conjugated isotype-specific goat anti-mouse IgG (Southern Biotechnologies, Birmingham, AL, USA) was added with subsequent incubation at RT for 30 min. The assay plates were washed and TMB Substrate Solution was added prior to quenching with TMB STOP Solution. The assay plates were subsequently centrifuged to pellet the bacteria and 180μL of each sample was then carefully transferred into a NUNC™ Maxisorp 96-well ELISA plate, without disturbing the bacterial pellet, and absorbance at 450nM was determined using a SpectraMax Plus Microplate Reader.

2.6.4. Live bacteria ELISA for MAB-binding to live MTB
The ability of purified anti-MTB MAB JG7 to bind to live MTB was

evaluated. The same bacterial ODs that were used for the fixed assay were used for the live Bacteria ELISAs to confirm binding to live MTB prior to beginning opsonic studies. MTB strains, H37Ra and clinical susceptible MTB, were grown to mid-logarithmic phase and serially diluted in PBS/BSA solution. The dilutions were dispensed into 96-well polypropylene plates (Sigma-Aldrich life science, Chemie GmbH) in 100μL volumes. Various concentrations of JG7 were made from stock, in PBS-T and 50μL of each resulting concentration was dispensed into each well of the plates containing bacteria. The plates were sealed and incubated for 1 h at 37°C in a shaking incubator (Thermostar, BMG Labtech, Ortenberg, Germany) at 250rpm.

After incubation, the plates were centrifuged at 2600rpm for 5–10 min at 25°C. The plates were manually washed twice with PBST/BSA. A 100μL volume of diluted goat anti-mouse IgG1 detection antibody was dispensed into each well of the plates, sealed and incubated in the shaking incubator at 37°C for 30 min at 250rpm. The plates were washed thrice with PBS-T/BSA. After the final wash, the solution was pipetted out completely and 100μL of TMB substrate solution was dispensed into the wells. The plate was incubated in the dark for 15 min at room temperature. One hundred microliters (100μL) of TMB stop solution was added into the wells after the incubation and centrifuged at 2600rpm for
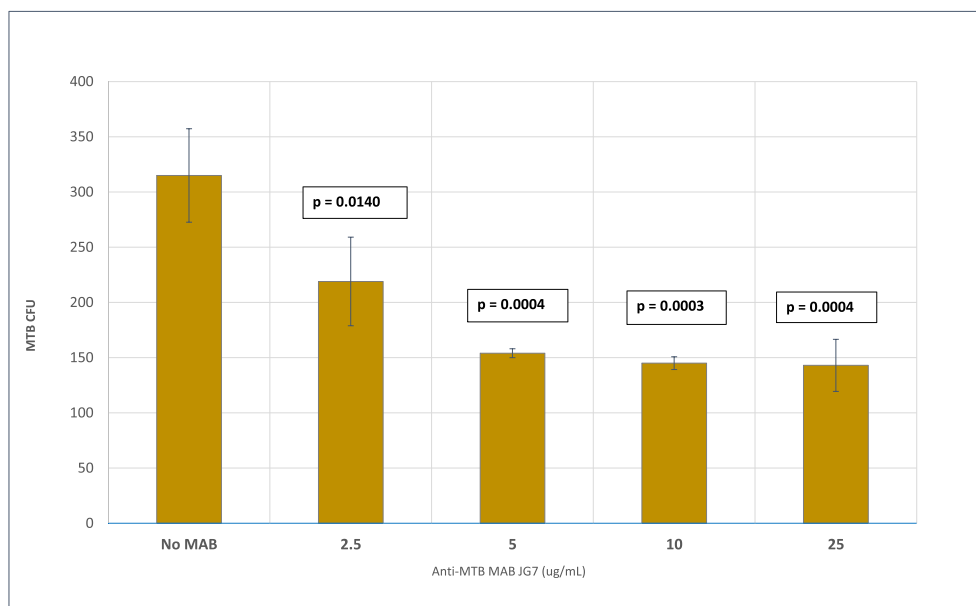
Fig. 5. OPKA of MAB JG7 against live *Mycobacterium tuberculosis* (MTB) clinical isolate STB1, using U-937 macrophages (without C1q) was significantly enhanced at MAB levels 2.5–25 μg/mL. Compared to the control sample wells (without MAB), antibody sample wells had CFU counts that were significantly reduced (p < 0.5) from 315 (No MAB) to 219 (2.5μg/mL), 154 (5μg/mL), 145 (10μg/mL) and 143 (25μg/mL).

5 min at 25°C. A 180μL volume of the supernatant was carefully taken out (without the pellet) per well, and transferred onto corresponding wells of the ELISA NUNC™ Maxisorp flat-bottom plate. The NUNC™ plate was read immediately at either 450nM or 630nM, depending on the TMB STOP solution used.

2.7. Opsonophagocytic killing activity (OPKA)

Two novel anti-MTB MABs were selected for evaluation of their opsonophagocytic activity (OPKA) against SMEG using two effector cell lines: using HL60 cells differentiated to granulocytes and U-937 cells differentiated to macrophages, with MAB concentration ranges of 0.06–25 μg/mL and 0.05–250 μg/mL, respectively. Notably, anti-MTB MAB OPKA against MTB was examined using the U-937 macrophage cell line only.

2.7.1. Granulocytic cell line (against SMEG)

Human leukemia promyelocytic (HL60) cells (Sigma-Aldrich, St. Louis, MO and ATCC, Manassas, VA, USA) were cultured in complete growth medium consisting of RPMI-1640, 10% heat-inactivated fetal bovine serum (FBS; Fisher Scientific, Pittsburg, PA, USA) and 2mM L-glutamine (Fisher Scientific, Pittsburg, PA, USA). Cells were differentiated into granulocytes, using 1.25% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) for five to six days and subsequently diluted to 5×10^7 cells/mL in OP assay medium consisting of DMEM-F12 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10mM HEPES (Sigma-Aldrich, St. Louis, MO, USA). Sextuplet reaction wells were prepared by sequential addition of: 40μL diluted MAB, 40μL differentiated HL60s at 2×10^6 cells/well (5×10^7 cells/mL), 10μL diluted complement component C1q (VWR, Radnor, PA, USA), and 10μL of SMEG at $1–5 \times 10^4$ CFU/mL. The reaction mixture was incubated at 37°C for 4 h in a Max@ 4450 Orbital Shaker (Fisher Scientific, Pittsburg, PA, USA) at 300rpm. Following incubation, HL60 cells were lysed by transferring 10μL from the reaction wells into 190μL of ice cold tissue culture grade water (Fisher Scientific, Pittsburg, PA, USA) containing 0.1% BSA, then incubated on ice at 2–6°C for 30 min. A 100μL aliquot of each sample was plated on 5% sheep blood agar (Fisher Scientific, Pittsburg, PA, USA), incubated for 46–48 h at 37°C, and subsequently enumerated for colony forming units (CFU) using a MiniCount colony counter (IPI, Simi Valley, CA, USA). Bacterial killing activity was defined as the percentage of mean CFU counts in sample wells (with MAB, HL60 and C1q) divided by mean CFU counts in control wells (with HL60 and C1q but without MAB). HL60 cell opsonic activity and bactericidal killing has been previously considered antibody enhanced when the percentage of SMEG killed in the presence of MAB and C1q was ≥50% compared to values from HL60 cells and C1q alone [37]. Three independent HL60 cell opsonic assays were analyzed and OPKA across a range of concentrations was plotted.

2.7.2. Macrophage cell line (against SMEG)

Human histiocytic lymphoma monocytic (U-937) cells (ATCC, Manassas, VA, USA) were seeded into flat bottom tissue culture plates (Fisher Scientific, Pittsburg, PA, USA) at 1×10^6 cells/mL and differentiated for 3 days using 50ng/mL of Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) in complete growth media. The medium was changed after 24 h to complete growth medium - without PMA. U-937 cells grow as a suspension culture of monocytic cells prior to differentiation and become adherent macrophages-like post-differentiation. After differentiation, complete growth medium was replaced with 50μL per well of pre-warmed OP assay medium. Octuplet reaction wells were prepared by addition of 40μL diluted MAB directly into the wells with U-937 cells followed by 10μL of SMEG or MTB at 1×10^5 CFU/well. In previous bacterial studies, complement did not increase U-937 cell activity. The reaction mixture was incubated for 4 h at 37°C with shaking at 300rpm in a Max@ 4450 Orbital Shaker. After incubation, the entire 100μL of reaction mixture in each well was carefully discarded to remove extracellular bacteria. A wash with 200μL of PBS was performed and adherent cells were then lysed to release intracellular bacteria by adding ice cold tissue culture grade water containing 0.1% BSA and incubated on ice for 30 min. Each sample was plated on 5% sheep blood agar, incubated for 46–48 h at 37°C, and CFUs counted. The OP killing data was defined as the percentage of mean CFU counts in sample wells, divided by the mean CFU counts in controls wells, containing no MAB. Three independent U-937 opsonic assays were analyzed and OPKA across a range of concentrations was plotted.

2.7.3. Macrophage cell line (against MTB)

U-937 cells were differentiated same as the method described above for SMEG. Pure cultures of MTB (H37Ra ATCC 25177 and clinical
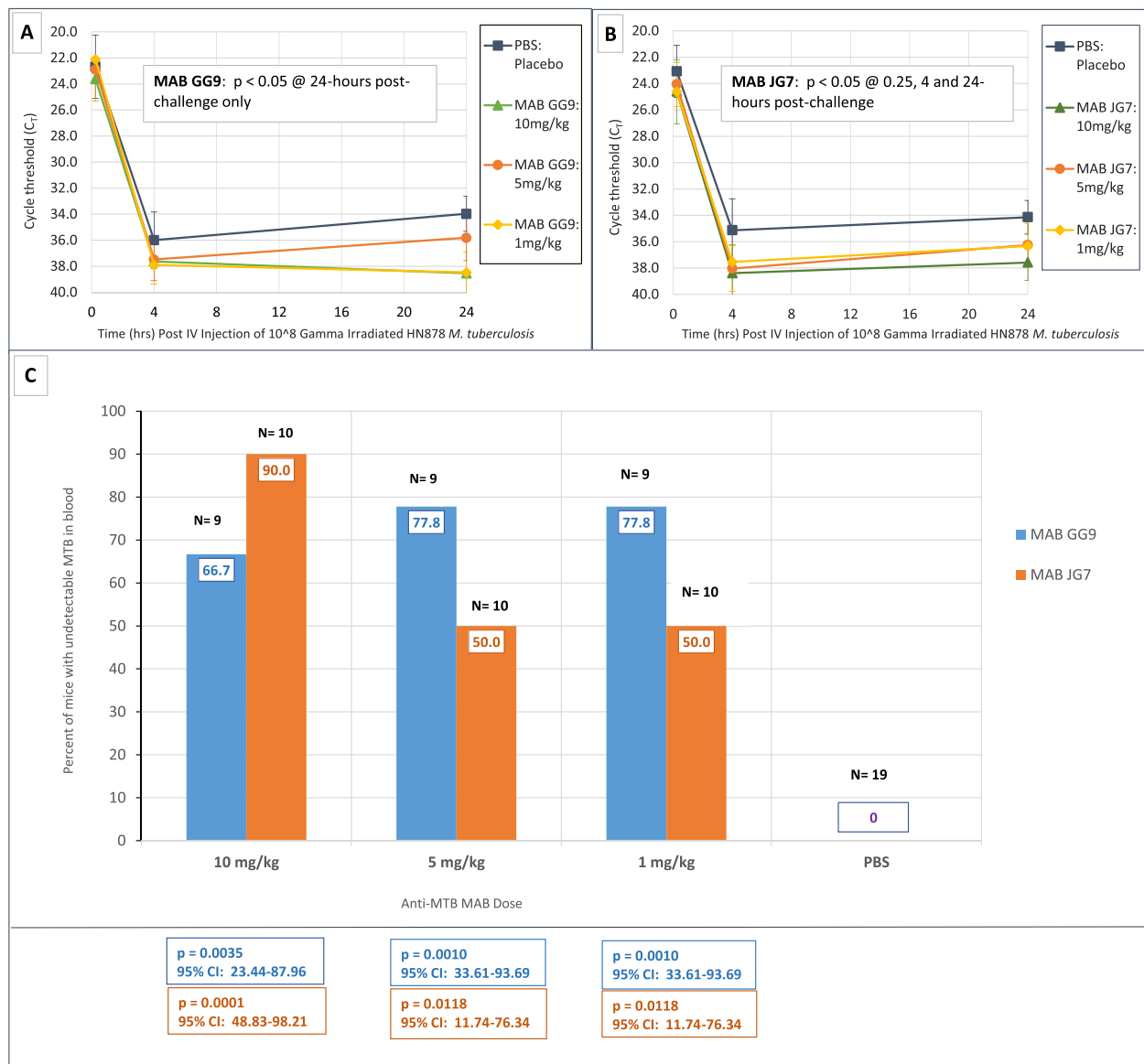
Fig. 6. Using qPCR, rapid clearance of *Mycobacterium tuberculosis* (MTB) in blood was observed in all groups from the *in vivo* study with N = 76 ICR mice. While MAB GG9 (panel A) significantly enhanced blood clearance at 24 h post challenge (1 mg/kg p = 0.0021, 10 mg/kg p = 0.0013), MAB JG7 (panel B) significantly enhanced clearance at all time points (0.25, 4 and 24 h) and at one or more doses. Panel C shows the percentage of mice with undetectable levels of MTB in blood according to qPCR. Statistical significance determined by comparison of MAB-treated vs. PBS-treated blood samples from mice according to no detection (i.e. $C_T = 40$, qPCR) was calculated using the Chi-squared test, with significance threshold set at $p < 0.05$ and 95% confidence intervals shown.

susceptible TB) were grown in MGIT 7H9 broth at 37°C until mid-logarithmic phase (1×10^6 CFU/mL – 1×10^8 CFU/mL). Two bacterial dilutions were used in each assay run (1:100 and 1:1000) in order to assess any differences in phagocytosis based on bacterial load. JG7 was used at concentrations ranging from 0.25–100 μg/mL. A volume of 40μL of JG7, followed by 10μL of either of the two dilutions of MTB was added on top of 50μL of the adherent, differentiated U-937 cells in sextuplet wells per sample. The plate was sealed and incubated for 4 h at 37°C with shaking at 300rpm in an orbital shaker (Thermostar, BMG Labtech, Ortenberg, Germany). After incubation, 90μL of the reaction mixture was discarded from each well on the incubation plate and 190μL of ice cold 0.1% BSA (30% BSA, Sigma-Aldrich Life Science, Chemie GmBH), diluted in sterile tissue culture grade water (Sigma-Aldrich life science, Chemie GmBH) was added to each well to lyse the cells. The plate was incubated on ice for 30 min. 100μL of each sample was plated on Middlebrook 7H10 or 7H11 agar, incubated for 6–10 weeks at 37°C, and subsequently, enumerated for CFUs using the Acolyte 3 colony counter

(Lasec, CT, SA). MAB JG7 OPKA was reported as reduction of CFU in the sample wells containing JG7, compared to the control wells without MAB.

2.8. qPCR for monitoring MTB clearance from murine blood

MABs GG9 and JG7 were evaluated *in vivo* for their capacity to enhance clearance of killed MTB from blood. Female Institute of Cancer Research (ICR) mice weighing approximately 30g (Harlan Laboratories, Indianapolis, IN, USA) were given intraperitoneal injections of 300, 150 and 30μg of MABs in 0.3mL sterile PBS (10, 5 and 1 mg/kg, respectively) 24 h prior to MTB challenge. Control mice received 0.3mL of PBS. All seventy-six mice used in this study were then challenged with intravenous injection of 0.3mL of killed HN878 MTB at 10^8 CFU/mouse. Blood was obtained via retro-orbital sinus at 0.25 and 4 h post-challenge [38], and cardiac bleeds (following euthanasia) at 24 h post-challenge [33]. Approximately 0.2mL of whole blood was placed into K2 EDTA tubes
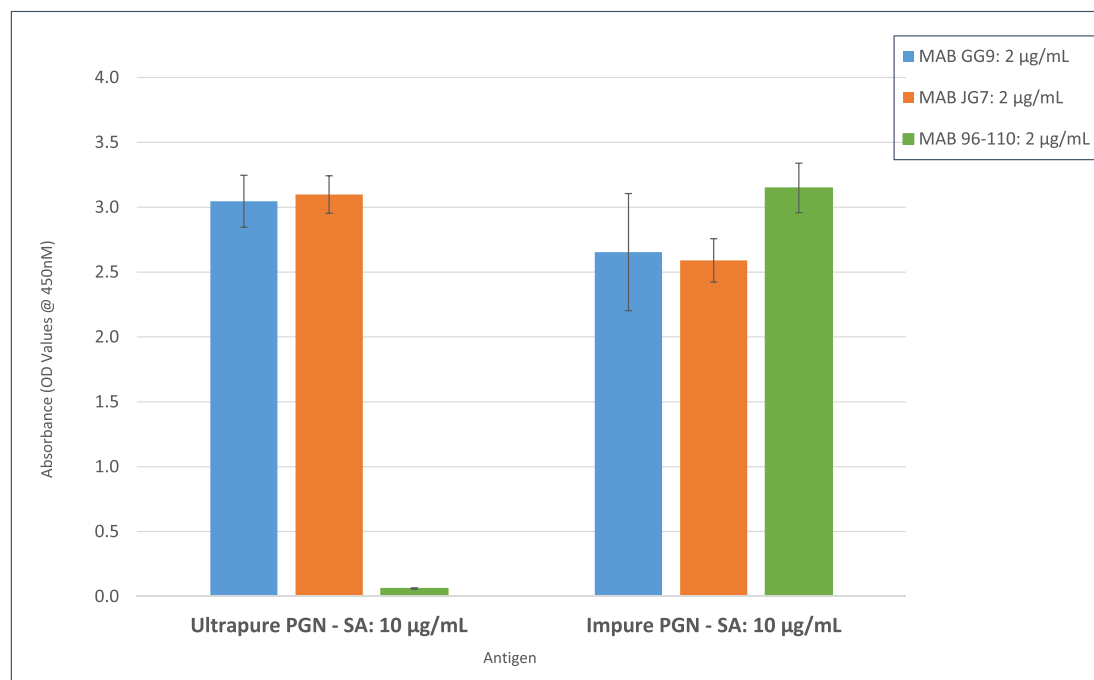
Fig. 7. PGN binding activity of MABs GG9 and JG7 was demonstrated to Ultrapure and Impure PGN, while anti-LTA MAB 96-110 only bound the Impure PGN.

(Becton Dickinson, Sparks, MD, USA), mixed gently, and then 100μL transferred into 1.0mL of PrimeStore MTM® (PS-MTM) (Longhorn Vaccines and Diagnostics, San Antonio, TX, USA). Murine blood specimens were transported overnight at ambient temperature to San Antonio, Texas for qPCR analysis. DNA extraction and multiplex qPCR were performed as described by Daum *et al* [39].

2.9. Binding of anti-MTB MABs and anti-LTA MAB to peptidoglycan

The ability of anti-MTB MABs to bind to Peptidoglycan was evaluated using Ultrapure Peptidoglycan (InvivoGen, San Diego, CA, USA) and Impure Peptidoglycan (InvivoGen, San Diego, CA, USA) both derived from *Staphylococcus aureus* to determine the target epitope for anti-MTB MABs GG9 and JG7. An anti-LTA MAB, 96-110 (Antibody and Immunoassay Consultants, Rockville, MD, USA) was used to confirm the purity of the Ultrapure Peptidoglycan from *S. aureus*. 96-well NUNC™ MaxiSorp ELISA plates (Fisher Scientific, Pittsburg, PA, USA) were coated with Ultrapure and Impure Peptidoglycan at 10μg/mL for 18–24 h at RT. Peptidoglycan-coated plates were washed with PBS-0.05% Tween 20 (PBS-T, Fisher Scientific, Pittsburg, PA, USA) using an ELx405 Automated Plate Washer (BioTek, Winooski, VT, USA). MAB samples were added and incubated for 1 h at RT. Anti-MTB antibodies were detected with HRP-Conjugated, isotype-specific Goat anti-Mouse IgG (Southern Biotech, Birmingham, AL, USA). TMB Substrate Solution (Fisher Scientific, Pittsburg, PA, USA) was added prior to being quenched with TMB STOP solution (Fisher Scientific, Pittsburg, PA, USA). The absorbance values (450nM) of each well were obtained using a SpectraMax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.9.1. Statistical analysis

For *in vitro* studies, results for Live Bacteria ELISA were expressed as mean (±standard deviation) from three individual experiments. In the OPKA assays using HL60 granulocytic cells and U-937 macrophage cells, results were expressed as mean (±standard deviation) from 6 to 12 replicate sets from three individual experiments, with significance threshold set at $p < 0.05$ using the Student t-test. In the *in vivo* studies, results for each treatment group were reported as the mean (qPCR IS-6110) C_T value (±standard error) for ≥ 9 individual mice. The mean C_T values of the MAB treated groups were then compared to the mean C_T

values of the placebo (PBS) treated groups at each timepoint and the differences were considered significant at $p < 0.05$ using the Student t-test. The percentage of animals that had undetectable levels of blood MTB in MAB treated versus placebo (PBS) treated groups were compared and differences analyzed using the Chi-squared test, with significance threshold set at $p < 0.05$ and 95% confidence intervals reported. Results for the Peptidoglycan ELISA were expressed as mean (±standard deviation) from three individual experiments.

3. Results

3.1. Production and Characterization of anti-MTB MABs

Subcutaneous immunization with EK-MTB elicited high ELISA antisera titers to EK-MTB. At Day 63, Mice 1323 and 1420 (each from a different boost protocol) had the strongest immune response within their respective groups with peak OD values of 4.000 and 3.134, respectively. Two hybridomas (Mouse 1323 MAB JG7 and Mouse 1420 MAB GG9) produced from spleen cell fusions, were selected for initial evaluation and demonstrated binding activity to killed MTB strains Erdman, HN878, and CDC1551 and to SMEG (Fig. 1). HN878 and CDC1551 were killed by gamma irradiation and had an intact cell surface without alcohol disruption and the MABs bound well to each strain demonstrating that the targeted epitopes were readily detected by the MABs. In addition, using susceptible MTB lab strain H37Ra and two clinical isolates the fixed MTB ELISA demonstrated good binding to both JG7 and GG9 at MAB concentrations from 10–0.156 μg/mL (Fig. 2, panel A). At 5 and 10 μg/mL, binding activity of JG7 and GG9 were each above OD 2.0 for three MDR clinical isolates (Fig. 2, panel B) and two XDR clinical isolates (Fig. 2, panel C), and their binding activity was similar to that of susceptible strains. In the Live Bacteria ELISA (Fig. 3), the binding profiles of purified MABs JG7 and GG9 were similar for SMEG at 25μg/mL. Both MABs bound to live MTB, however JG7 appeared to bind more efficiently to all MTB strains compared to GG9 and hence was selected for further OPKA studies.

3.2. OPKA of anti-MTB MABs GG9 and JG7 against live SMEG

OPKA in control wells containing complement and HL60 or U-937

cells in the absence of MAB demonstrated minimal reduction in SMEG for both HL60 or U-937 cells. Notably, OPKA was markedly increased with MAB plus complement and HL60 granulocytes, demonstrating greater than 50% OPKA above control wells with complement alone across high and low MAB concentrations ranging between 0.06–25 µg/mL (Fig. 4a). Both MABs promoted mycobacterial phagocytosis and had maximum killing of 81% for JG7 and 76% for GG9, both at 0.06µg/mL with HL60 granulocytes. These antibodies demonstrated good OPKA even at very low MAB concentrations using HL60 granulocytes and C1q (Fig. 4a). There was however a significant difference between the mean percent OPKA using JG7 compared to GG9 ($p < 0.05$) that did not change by increasing the concentration of MAB (Fig. 4a). In contrast, using U-937 macrophage cells, OPKA at antibody concentrations below 25µg/mL was statistically less robust than OPKA between 50 and 150 µg/mL ($p < 0.05$) (Fig. 4b). There was statistical significance ($p < 0.0001$) in the OPKA differences between the HL60 granulocytes and U-937 macrophages at low concentrations (< 1µg/mL) for both MABs JG7 and GG9.

3.3. OPKA of anti-MTB MABs GG9 and JG7 against live MTB

OPKA of MAB JG7 against live MTB clinical isolate STB1, using U-937 macrophages was significantly enhanced at MAB levels 2.5–25 µg/mL (Fig. 5). Compared to the control sample wells (without MAB), antibody sample wells had CFU counts that were significantly reduced ($p < 0.5$) from 315 (No MAB) to 219 (2.5µg/mL), 154 (5µg/mL), 145 (10µg/mL) and 143 (25µg/mL).

3.3.1. Dynamics of blood MTB clearance using qPCR in a mouse model

Compared to PBS-treated mice, opsonic anti-MTB MABs GG9 and JG7 enhanced clearance of MTB in mice given a high dose of killed HN878 bacilli (10^8 CFU/mouse) using MTB DNA quantification by qPCR (Fig. 6a and b). Murine blood specimens collected 0.25 h (15 min) post-challenge were qPCR positive for MTB (mean $C_T = 23.5$; N = 76). At 0.25 h post-challenge, MAB JG7-treated mice showed enhanced clearance of MTB from blood, compared to placebo with significant reduction in mean C_T evident at 1 mg/kg dose ($p = 0.0449$). At 4 h, blood specimens from MAB JG7-treated mice showed significant reduction in mean C_T ($p = 0.0025$, 0.0053 and 0.0232 at 10, 5 and 1 mg/kg doses, respectively) compared to placebo (Fig. 6b), while MAB GG9-treated mice were not statistically different from placebo ($p = 0.1318$, 0.1782, 0.0529) (Fig. 6a). Between 4 and 24 h, reduction in mean C_T was observed only in blood specimens from MAB GG9-treated mice (Fig. 6a). At 24 h post-challenge, mice treated with both MABs had significant reduction in mean C_T: MAB GG9, $p = 0.0013$ and 0.0021 at 10 and 1 mg/kg doses, respectively; while for MAB JG7, $p = 0.0012$ and 0.0258 at 10 and 5 mg/kg doses, respectively, compared to placebo. Notably, mean C_T reduction in MAB JG7-treated mice was statistically significant ($p < 0.05$) at 0.25 (15 min), 4 and 24 h post-challenge, while in MAB GG9-treated mice, only at 24 h post-challenge.

3.3.2. Percent of MAB-treated mice with undetectable levels of MTB in the blood, post-challenge

A significantly greater percentage of mice treated with opsonic anti-MTB MABs GG9 and JG7 given a high dose of killed HN878 bacilli (10^8 CFU/mouse) had undetectable MTB DNA in blood compared to placebo (Fig. 6c). There was no detectable MTB DNA in the blood (qPCR IS-6110 target, $C_T = 40$) at 4 and 24 h post-challenge with killed MTB in 77.8% (7/9 mice), 77.8% (7/9 mice), and 66.7% (6/9 mice) - given 1, 5 and 10 mg/kg of MAB GG9, respectively. MTB DNA was not detected in the blood at 4 and 24 h in 50% (5/10 mice), 50% (5/10 mice), and 90% (9/10 mice) - given 1, 5 and 10 mg/kg, respectively (Fig. 6c). In contrast, none of the control mice given PBS (0%, 0/19 mice) had undetectable MTB in blood at 4 and 24 h post-challenge (qPCR IS-6110, $C_T = 40$). Notably, MAB JG7, at 10 mg/kg dose, significantly enhanced clearance of MTB from blood ($p = 0.0001$, 95% CI: 48.83 to 98.21), while MAB GG9 significantly enhanced clearance of MTB from blood at 1 and 5 mg/kg doses ($p = 0.0010$, 95% CI: 33.61 to 93.69).

3.4. Anti-MTB MABs GG9 and JG7 versus anti-LTA MAB 96-110 binding activity to peptidoglycan

Purified anti-MTB MABs GG9 and JG7 each bound well to both Ultrapure and Impure PGN-SA (ODs range between 2.590 and 3.098) screened by ELISA (Fig. 7). However, the anti-LTA MAB, 96-110, only bound well to Impure PGN-SA (OD 3.149) containing cell wall LTA, and did not bind to Ultrapure PGN-SA (OD 0.061).

4. Discussion

Opsonic antibodies enhance phagocytosis and killing of encapsulated bacteria such as Group B *Streptococcus in vitro*, and enhance protection *in vivo* [19, 20, 21]. Monoclonal antibodies that bind to MTB capsule components, e.g. arabinomannan (AM), promote mycobacterial opsonization, phagocytosis, induced intracellular mycobacterial growth reduction, P-L fusion [24], and prolonged survival in mice [40, 41]. Using THP-1 macrophage cells, Chen *et al.*, showed that enhancement of BCG phagocytosis and reduction in growth rate correlated with AM epitope specific IgG [42]. In addition, studies by Lu and colleagues suggest that antibodies could direct inflammasome activation in macrophages that may contribute to bacterial control [43]. In this study, we demonstrate that IgG MABs directed against MTB promote OPKA *in vitro* and enhance blood clearance of killed MTB *in vivo*. While qPCR can rapidly monitor the removal of MTB DNA from the blood, it does not indicate if the MTB have been killed by the phagocytic cells, but MTB OPKA was shown to be enhanced by MAB JG7.

Immunizing mice with EK-MTB may have altered the capsule exposing deeper cell wall epitopes. Previous studies have shown that cell wall lipoteichoic acid (LTA) antibodies promote phagocytosis and killing of staphylococci and enhanced blood clearance *in vivo* [44, 45]. Data from the studies presented here using highly purified peptidoglycan strongly suggest that the target of MABs GG9 and JG7 is an epitope on peptidoglycan. These anti-peptidoglycan MABs bind to antibiotic sensitive and resistant live MTB and promote opsonophagocytic killing of MTB by U-937 macrophage cells. Kumar *et al.,* observed that not all antibodies to MTB in serum of individuals with TB took part in facilitating opsonization of MTB [32]. It is not clear whether anti-peptidoglycan antibodies are induced in individuals with TB infections.

Both granulocytes and macrophages are important for clearance of microbes from the blood and body tissues. When compared to PBS, MABs GG9 and JG7 enhanced MTB clearance in ICR mice given an intravenous injection of killed HN878. The clearance of killed MTB from murine blood was monitored by qPCR at a distant laboratory. This method of collecting blood, safely transporting specimens at ambient temperature to a distant lab, and analyzing samples using qPCR [39] may be useful for detecting MTB sepsis, determining the level of bacteremia and monitoring clearance of MTB in patients with MTB sepsis.

Immunotherapy may be useful to improve the treatment of bacteremic MTB infections. Antibodies that bind to MDR and XDR MTB, promote bactericidal phagocytic activity and enhance clearance of MTB bacteremia may provide adjunctive therapy for MTB sepsis, and augment treatment with antibiotics for patients with MDR and XDR TB. A larger comprehensive evaluation of these MABs using live MTB and *in vivo* infection models will be critical to determine if these MABs might be effective in treating patients with MTB sepsis or MDR/XDR TB.

Declarations

Author contribution statement

Clara J. Sei, Bong-Akee Shey, Richard F. Schuman, Nimisha Rikhi, Luke T. Daum, P Bernard Fourie, Gerald W. Fischer: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

Clara J. Sei, Bong-Akee Shey, Richard F. Schuman, Nimisha Rikhi,

Luke T. Daum, Kevin Muema, John D. Rodriguez: Performed the experiments; Analyzed and interpreted the data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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