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Profiling Antibodies to *Mycobacterium tuberculosis* by Multiplex Microbead Suspension Arrays for Serodiagnosis of Tuberculosis▽

Imran H. Khan,[1,2]*† Resmi Ravindran,[1]† JoAnn Yee,[3] Melanie Ziman,[1] David M. Lewinsohn,[5] Marila L. Gennaro,[6] JoAnne L. Flynn,[7] Celia W. Goulding,[8] Kathryn DeRiemer,[4] Nickolas W. Lerche,[3] and Paul A. Luciw[1,2,3]

Center for Comparative Medicine,[1] Department of Pathology and Laboratory Medicine,[2] California National Primate Research Center,[3] and School of Medicine,[4] University of California, Davis, California 95616; Division of Pulmonary and Critical Care Medicine, Oregon Health and Sciences University, Portland, Oregon 97239[5]; Public Health Research Institute, Newark, New Jersey 07103[6]; Department of Molecular Genetics and Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15261[7]; and University of California, Irvine, California 91679[8]

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Tuberculosis (TB) is a serious global disease. The fatality rate attributed to TB is among the highest of infectious diseases, with approximately 2 million deaths occurring per year worldwide. Identification of individuals infected with *Mycobacterium tuberculosis* and screening of their immediate contacts is crucial for controlling the spread of TB. Current methods for detection of *M. tuberculosis* infection are not efficient, in particular, for testing large numbers of samples. We report a novel and efficient multiplex microbead immunoassay (MMIA), based on Luminex technology, for profiling antibodies to *M. tuberculosis*. Microbead sets identifiable by unique fluorescence were individually coated with each of several *M. tuberculosis* antigens and tested in multiplex format for antibody detection in the experimental nonhuman primate model of TB. Certain *M. tuberculosis* antigens, e.g., ESAT-6, CFP-10, and HspX, were included to enhance the specificity of the MMIA, because these antigens are absent in nontuberculous mycobacteria and the vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin. The MMIA enabled simultaneous detection of multiple *M. tuberculosis* plasma antibodies in several cohorts of macaques representing different stages of infection and/or disease. Antibody profiles were defined in early and latent/chronic infection. These proof-of-concept findings demonstrate the potential clinical use of the MMIA. In addition, the MMIA serodetection system has a potential for mining *M. tuberculosis* open reading frames (about 4,000) to discover novel target proteins for the development of more-comprehensive TB serodiagnostic tests.

Tuberculosis (TB) is a global disease, with about one-third of the world's population infected with the etiological agent, *Mycobacterium tuberculosis* (6). New infections appear at the rate of about 8 million cases per year, and the annual death toll due to TB is placed at about 2 million (6). For effective control of TB, it is critical to identify infected individuals and screen their immediate contacts so that drug treatment can be administered quickly. For diagnosis of *M. tuberculosis* infection, more than one diagnostic test is generally applied (8, 21). The tuberculin skin test (TST) is used extensively in both humans as well as nonhuman primates. Results are variable and subject to interpretation and are thus not consistent (8, 21). The sputum smear test allows direct identification of *M. tuberculosis* and is, therefore, highly specific, but results can be variable (8, 10). Bacterial culture for identification of *M. tuberculosis* infection requires a dedicated microbiology laboratory and is time-consuming (several weeks) (10).

More-specific and -sensitive TB diagnostic tests have been developed by using *M. tuberculosis*-specific antigens and by taking advantage of recent advances in sequencing and annotation of the *M. tuberculosis* genome, which have revealed approximately 4,000 open reading frames (http://genolist.pasteur.fr/TubercuList/). These tests include PCR amplification of an *M. tuberculosis* gene(s) and assays for cell-mediated immunity based on gamma interferon (IFN-γ) release assays (IGRAs) (20). The IGRAs, including QuantiFERON-TB Gold (Cellestis, Victoria, Australia) and the enzyme-linked immunospot-formatted T-SPOT.TB (Oxford Immunotec, Oxford, United Kingdom), measure IFN-γ produced by T cells in whole blood upon stimulation by *M. tuberculosis* antigens. Both PCR and IGRA tests require a sophisticated laboratory and depend on personnel with significant expertise in patient sample handling and processing.

In contrast to the above assays, diagnostic tests for infection based on antibody detection are relatively straightforward. A key drawback is that antibodies to any single *M. tuberculosis* antigen may not be detected in most *M. tuberculosis*-infected individuals (2). Therefore, detection of multiple *M. tuberculosis* antibodies may be more useful. A multiantigen print immunoassay (MAPIA), in which several antigens are printed on a nitrocellulose membrane by microaerosolization, has been used for profiling multiple *M. tuberculosis* antibodies (18, 19). A recent report described the use of a mixture of multiple antigens, selected by the use of a MAPIA (18, 19), for serodetection of *M. tuberculosis* infection in a membrane-based, lateral flow antibody detection method (19). We have used a multiplex microbead immunoassay (MMIA) to profile anti-*M.*

* Corresponding author. Mailing address: Center for Comparative Medicine and Dept. of Pathology and Laboratory Medicine, University of California, Davis, CA 95616. Phone: (530) 752-1245. Fax: (530) 752-7914. E-mail: ihkhan@ucdavis.edu.

† I.H.K. and R.R. contributed equally.

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tuberculosis antibodies in nonhuman primates. Profiles of antibodies against multiple antigens are valuable in TB serodiagnosis and may also be useful in differentiating between disease states (7). Because conventional immunoassays, such as enzyme-linked immunosorbent assays (ELISAs) and Western blotting, detect one antibody at a time, their use in antibody profiling has limits for widespread diagnostic use.

We reasoned that a method allowing determination of profiles of antibodies against multiple *M. tuberculosis* antigens simultaneously would be highly efficient for serodiagnosis of infection. Accordingly, our report describes the development of a novel MMIA for serodiagnosis of *M. tuberculosis* infection based on the Luminex technology (Austin, TX). This robust diagnostic system is based on 100 microbead sets, and each set is identifiable by a unique fluorescence (11, 22). For our study, six individual microbead sets were coated with six *M. tuberculosis* antigens. We report that the MMIA enabled simultaneous detection of antibodies against these antigens in nonhuman primates experimentally infected with *M. tuberculosis.* Because nonhuman primates are vulnerable to TB, an efficient immunoassay with potentially high-throughput testing for TB in thousands of animals in nonhuman primate colonies is highly desirable. In addition, nonhuman primates are a relevant model of TB in humans. Lung pathology, disease progression, and immune correlates of infection are more accurately modeled in nonhuman primates than in the mouse, rabbit, or guinea pig (9). Our report describes proof-of-concept studies that support the use of the multiplex microbead suspension array for defining antibody profiles in *M. tuberculosis* infection and disease in the macaque model. The highly manipulatable macaque model of TB enables rigorous control over experimental infection conditions. Importantly, this work has implications for profiling antibodies against multiple antigens for use in TB serodiagnosis in humans.

MATERIALS AND METHODS

***M. tuberculosis* antigens.** Early secretory antigenic target 6 (ESAT-6; Rv3875), culture filtrate protein (CFP-10; Rv3874), HspX (Rv2031C), MPT53 (Rv2878C), and MPT63 (Rv1926C) were expressed in *Escherichia coli* as recombinant histidine-tagged products and purified to near-homogeneity as previously described (5). These recombinant antigens were characterized for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Peptides representing Ag85B protein were obtained from Genemed Synthesis Inc. (San Antonio, TX). These Ag85B peptides have been previously used as antigens in measuring frequencies of anti-*M. tuberculosis* $CD8^+$ T cells in TB patients (16).

Antigen coupling to microbeads. Microbead sets, individually identifiable by unique fluorescence (Luminex Corp., Austin, TX), were coupled through carbodiimide linkages with each of the five *M. tuberculosis* recombinant antigens and Ag85B synthetic peptides as previously described (12, 13).

Nonhuman primates and plasma samples. Four specific-pathogen-free (SPF) rhesus monkeys (numbers 21200, 21300, 21222, and 21229), housed at the Oregon National Primate Research Center (Beaverton, OR), were experimentally inoculated as previously described (17). Briefly, *M. tuberculosis* strain H37Rv was inoculated by instillation of 1,000 CFU into the right lower lobe of each subject. Disease progression was monitored, and animals were characterized for infection and disease by computer tomography (CT) scanning, chest X-rays, and *M. tuberculosis* culture in bronchoalveolar lavage fluid (17). In these animals, disease progression varied. CT scans revealed early signs of small focal lung lesions in three of four animals at week 4 (21229, 21222, and 21200); lesions became progressively more pronounced at 8 and 12 weeks postinoculation in all four animals. Results from chest X-rays and *M. tuberculosis* cultures from bronchoalveolar lavage fluid followed a similar pattern as for the CT scans with the exception that animal 21203 did not become culture positive during the 12 weeks of monitoring (17). Necropsies were done on these animals, and the specimens were subjected to histopathological examination (17). Plasma samples were obtained from these animals at preinoculation and postinoculation time points as follows: 4 weeks, 8 weeks, and 12 weeks.

For latent and active-chronic infection studies, cynomolgus macaques, housed at the Primate Facility at the University of Pittsburgh (Pittsburgh, PA), were inoculated with *M. tuberculosis* strain Erdman (4). Animals were inoculated with 25 CFU per animal in 2 ml of sterile saline via bronchoscopic instillation into the right caudal or middle lung lobe. Latent infection was characterized as the presence of granulomas and low bacterial loads (compared to active disease) in the lung at necropsy but no radiographic involvement for 4 weeks postinfection or clinical signs of TB for at least 6 months postinfection (4). Samples at several time points, spanning a period over 2 years postinoculation, from two animals representing latent disease were analyzed by MMIA (see Fig. 2B, below). Active-chronic disease was characterized as previously described (4) and was based on evidence of persistent disease, radiographic involvement, persistent culture positivity, or other clinical signs of active disease. Samples at several time points spanning about 8 months to 1 year postinoculation from four active-chronic disease animals were analyzed by MMIA (see Fig. 2B, below). In addition, samples drawn at 1 year postinoculation from five active-chronic infected animals were analyzed (see Fig. 2A, below). Preinoculation plasma samples from four of these animals were used as controls (see Fig. 2A, below). Animal protocols were approved by the Institutional Animal Care and Utilization committees at the University of California, Davis.

TST and Primagam. TST and Primagam assays were performed as previously described (23). For TST, animals were skin tested using mammalian old tuberculin as follows: (i) SPF animals ($n = 20$), *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) inoculated ($n = 9$), and *Mycobacterium avium*-positive monkeys ($n = 10$) were tested once a year; (ii) rhesus macaques (see Fig. 1, below) were tested prior to and 1 month after inoculation with H37Rv; (iii) cynomolgus macaques (see Fig. 2, below), infected with the Erdman strain, were tested by skin test and Primagam assay prior to infection and at various time points during the infection.

Multiplex detection of anti-*M. tuberculosis* antibodies. The MMIA, containing a mixture of six microbead sets, one for each of six coated antigens described above, was used in the analysis of plasma samples, which were diluted 1:200 in Prionex (bio-WORLD, Dublin, OH), as previously described (12). Plasma samples from 20 SPF rhesus macaques provided the baseline (background) median fluorescence intensity (MFI) for the MMIA (see Fig. 1A, below). Positive control plasma samples for MMIA and Western blot analysis (see Fig. 1B, below) were obtained from three naturally infected rhesus macaques (from a TB outbreak at the Tulane National Primate Research Center, Corington, LA; P. J. Didier, personal communication). All three of these animals (EV64, BH33, and BC64) were skin test positive and Primagam positive. In addition, these animals were found positive for TB pneumonia (EV64) and TB lymphadenitis (BH33 and BC64) at necropsy. Additional negative control plasma samples, obtained at the California National Primate Research Center (Davis, CA), were as follows: six serial samples taken over 2 years from 10 SPF animals (control group II) (see Fig. 2A, below), 10 animals infected with a nontuberculous mycobacterium (*M. avium*) (control group III), and 9 animals vaccinated with BCG (control group IV) (see Fig. 2A, below).

Western blotting. Western blot analysis to confirm the detection of antibodies by MMIA against ESAT-6, CFP-10, and HspX was performed as previously detailed (14).

RESULTS

Detection of *M. tuberculosis* antibodies in early stages of infection. Profiles of antibodies against *M. tuberculosis* antigens in four rhesus macaques, at multiple time points postinfection, are shown in Fig. 1A. The MMIA included a mixture of six microbead sets, each coated with one of six antigens: ESAT-6, CFP-10, HspX, MPT53, MPT63, and Ag85B. In each sample, the MMIA was able to simultaneously detect antibodies to all but MPT63. Anti-*M. tuberculosis* antibody profiles produced by the MMIA allowed serodetection of infection in two animals as early as 4 weeks (21222 and 21229), in three animals by 8 weeks (21203, 21222, and 21229), and in all four by 12 weeks (Fig. 1A). Each of the four monkeys contained antibodies against at least two antigens during the 12-week period. One monkey

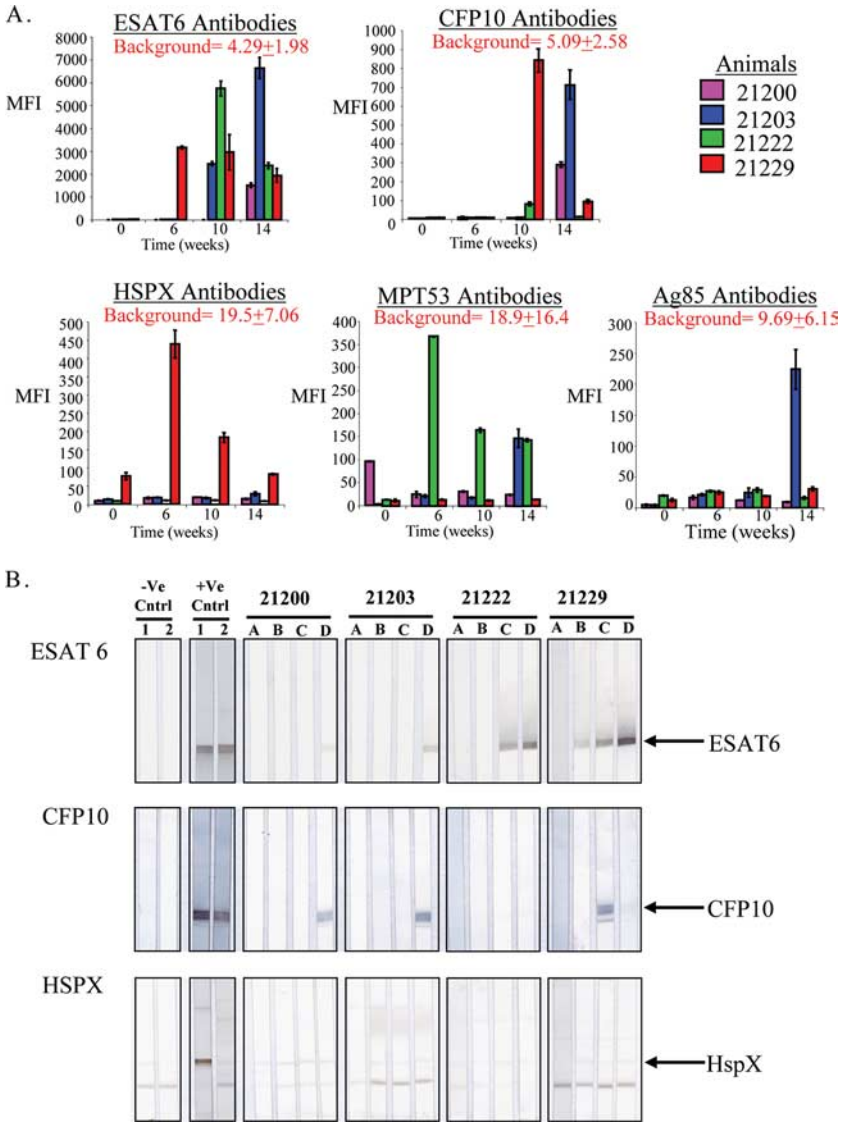
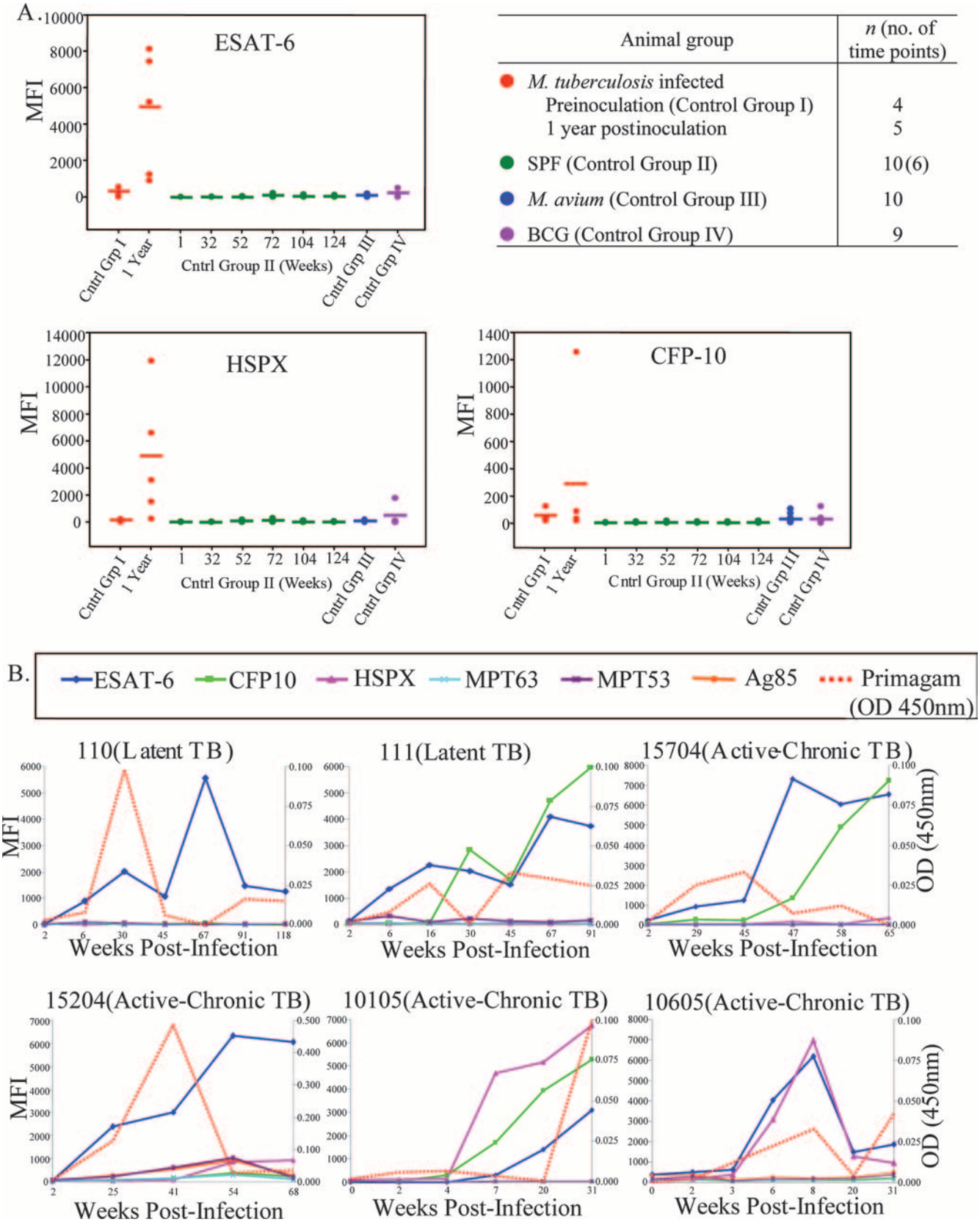


FIG. 1. Analysis of *M. tuberculosis* antibodies in early stages after experimental inoculation of four rhesus macaques. (A) Detection of antibodies to *M. tuberculosis* antigens by MMIA. All bead sets with positive results are shown. Results are shown as the MFI for each microbead set coated with an antigen. The assay background for each microbead set is shown in red (average MFI ± standard deviation), representing the reactivity of each to plasma samples from uninfected rhesus macaques ($n = 20$). (B) Detection of antibodies by Western blotting to ESAT-6, CFP-10, and HspX. Animal numbers are indicated, and samples A, B, C, and D represent 0, 4, 8, and 12 weeks postinoculation. In addition, two negative and two positive control plasma samples (from rhesus macaques naturally infected in a TB outbreak at Tulane National Primate Research Center, LA) were included in the analysis (-Ve Cntrl and +Ve Cntrl, respectively). Positive control animals used for ESAT-6 and CFP-10 are numbers BC64 and EV64 (lanes 1 and 2, respectively), and those for HspX are BH33 and EV64 (lanes 1 and 2, respectively). Antibodies are indicated by arrows. HspX antibodies were only detected in one of two positive control samples and in none of the experimentally infected animals.

(21203) contained antibodies to four antigens (ESAT-6, CFP-10, MPT53, and Ag85B), while another (21229) contained antibodies to three antigens (ESAT-6, CFP-10, and HspX) over the experimental period. Western blot analysis was performed to confirm detection of ESAT-6, CFP10, and HspX antibodies (Fig. 1B). Results obtained by the MMIA correlated with those obtained by Western blotting. However, the multiplex assay detected HspX antibodies that were undetectable by Western blotting (Fig. 1A and B). Antibodies against MPT63 were not detected in experimentally infected animals; the MPT63-coated microbead set detected antibodies in naturally infected rhesus macaques (data not shown).

Antibody profiles to *M. tuberculosis* in latent and active-chronic infection. To test the utility of antibody profiling in latent and active-chronic *M. tuberculosis* infection, cynomolgus macaques infected with *M. tuberculosis* for various lengths of time (4) were studied. In a group of five macaques at 1 year postinoculation, antibodies against *M. tuberculosis* antigens that were clearly detected were as follows: five of five animals were positive for ESAT-6, three of five animals were positive for HspX, and one of five animals was positive for CFP-10 antibodies (Fig. 2A). In preinoculation plasma samples (control group I), anti-*M. tuberculosis* antibodies were not detected (Fig. 2A). In addition, three other control groups (control



Animal group	*n* (no. of time points)
M. tuberculosis infected	
Preinoculation (Control Group I)	4
1 year postinoculation	5
SPF (Control Group II)	10(6)
M. avium (Control Group III)	10
BCG (Control Group IV)	9

FIG. 2. Detection of antibodies to *M. tuberculosis* antigens by MMIA from latent and active-chronic infection samples (cynomolgus macaques). (A) All bead sets with positive results are shown. Plasma samples from five animals experimentally infected with *M. tuberculosis* for 1 year and samples from four of the same animals before inoculation (control group I) are shown as red closed circles. Samples from uninfected animals collected at

groups II, III, and IV) were included in the analysis, all of which were negative for anti-*M. tuberculosis* antibodies (Fig. 2A), demonstrating specificity of the MMIA for serodetection of *M. tuberculosis* infection.

To investigate the kinetics and dynamics of anti-*M. tuberculosis* antibody profiles in latent and active-chronic infection, serial plasma samples from six cynomolgus macaques, experimentally infected with *M. tuberculosis*, were analyzed. Plasma samples from two monkeys were collected from each of three groups at several time points (Fig. 2B): (i) those latently infected for 2 years (numbers 110 and 111), (ii) those chronically infected for 1 year (15204 and 15704), and (iii) those infected for 0.5 year (10105 and 10605). In comparison to controls (control groups I, II, III, and IV) (Fig. 2A), all infected animals contained antibodies to multiple *M. tuberculosis* antigens (except animal 110, in which only ESAT-6 antibody was detected) (Fig. 2B). The Primagam test for cell-mediated immune responses, in contrast, displayed negative or barely detectable results at a number of time points (Fig. 2B). TST results were similarly inconsistent (data not shown). The following observations were made: (i) antibodies against ESAT-6 appeared early (6 to 7 weeks) and in most of the infected animals, (ii) antibodies against CFP-10 (animals 111, 15704, and 10105) and HspX (15204, 10105, and 10605) appeared in fewer animals, and (iii) antibodies against Ag85 and MPT53 were detected in only one animal (15204, at 41 weeks). Antibodies against MPT63 were not detected in any of the above samples.

DISCUSSION

These proof-of-concept studies explored the feasibility of the novel detection system MMIA to test for antibody profiles in *M. tuberculosis* infection. Antibodies against any single TB antigen are expected to be detectable in only some infected individuals (2). A recent report described the use of a mixture of four antigens of *M. tuberculosis* (ESAT-6, CFP-10, MPB83, and TBF-10), selected on the basis of a MAPIA (18, 19), in a membrane-based TB antibody detection test by a lateral flow method (19). We report the use of a MMIA, on the Luminex platform, for potentially studying a large number of samples to profile antibodies against each individual antigen in nonhuman primates infected with *M. tuberculosis*. Conventional immunoassays, e.g., ELISAs, generally detect one analyte per test and are therefore inefficient in generating profiles of antibodies. The MMIA was evaluated for profiling antibodies to six *M. tuberculosis* antigens in plasma samples from four experimentally infected rhesus monkeys that were TST positive at 4 weeks postinoculation (17). CT scanning revealed TB lesions in lungs of these animals (17). Anti-*M. tuberculosis* antibody profiles by MMIA enabled serodetection of infection in two animals (21229 and 21222) as early as 4 weeks and in all four animals by 12 weeks (Fig. 1A). Confirmation by Western blotting, and the fact that MMIA antibodies against different antigens were not detected in preinoculation samples, indicated that antibody profiles generated by MMIA are specific (Fig. 1A). For animal 21203, ESAT-6 antibodies were readily detected at weeks 8 and 12 by MMIA. However, by Western blotting, ESAT-6 antibodies in this animal were only weakly detected at week 12 (Fig. 1B). In addition, the MMIA was able to detect HspX antibodies in one animal (21229), while HspX antibodies were undetectable by Western blotting in any of the four animals (Fig. 1).

The MMIA did not detect antibodies in animals vaccinated with BCG ($n = 9$) or those infected with *M. avium* ($n = 10$) (Fig. 2A). Animals vaccinated with BCG were assumed to have anti-BCG immune responses as previously shown (15). Animals infected with *M. avium* were tested by Primagam assay and were found to contain strong anti-*M. avium* immune responses (reference 15 and data not shown). Experiments with these samples and those from SPF animals further suggest that the MMIA is specific for the detection of anti-*M. tuberculosis* antibodies (against ESAT-6, CFP10, and HspX). These results indicate that anti-*M. tuberculosis* antibody profiles are of value as a diagnostic modality for the detection of *M. tuberculosis* infection.

In about 90% of human cases with exposure to *M. tuberculosis*, either active-chronic or latent infection ensues (1). To obtain antibody profiles representative of these clinical groups, we studied cynomolgus macaques infected with *M. tuberculosis* over a period of about 2 years. In latently infected animals with no overt TB symptoms, the MMIA detected antibody responses at all the time points tested, after the first 2 weeks, to ESAT-6 or CFP-10 or both (animals 110 and 111) (Fig. 2B). In another group, plasma samples from a cohort of five infected animals were collected at 1 year postinoculation (Fig. 2A). These animals had antibodies against ESAT-6, HspX, and CFP-10 (Fig. 2A). Similarly, in a time course analysis, anti-*M. tuberculosis* antibodies could be readily detected in four macaques with active-chronic infection at all time points tested (animals 15704, 15204, 10105, and 10605) (Fig. 2B). In most of the infected animals, ESAT-6 antibodies were the earliest to appear, either alone or in combination with other antibodies. This finding is consistent with previously reported antibody detection by ELISA and the lateral flow method with *M. tuberculosis*-infected nonhuman primates (3, 19). Generally, anti-*M. tuberculosis* antibodies, once generated, remained detectable throughout the period of investigation. In contrast, detection of IFN-γ as detected by the Primagam assay was sporadic (Fig. 2B). These results suggest that the MMIA is a useful modality to study profiles of *M. tuberculosis* antibodies. In this study, correlation of antibody profiles by MMIA with

six time points over more than 2 years are shown as green closed circles ($n = 10$ animals; control group II). Animals infected with *M. avium* ($n = 10$ animals; control group III) and those inoculated with BCG vaccine ($n = 9$ animals; control group IV) are shown in blue and purple closed circles, respectively. (B) Time course analysis of *M. tuberculosis* antibody profiles by multiplex microbead immunoassay. Results for all six bead sets are shown; two samples (110 and 111) were an exception, where the Ag85 bead set was not included in the experiments. Animals 110 and 111 were latently infected for about 2 years. Animals 15704 and 15204 and animals 10105 and 10605 were infected for about 1 year and about one-half year, respectively. Immune responses measured by Primagam assay are shown as the optical density at 450 nm for comparison. The Primagam assay cutoff was an optical density of 0.025.

severity of disease in rhesus macaques (Fig. 1) and cynomolgus macaques (Fig. 2) was not observed.

This report utilized the well-controlled macaque model to demonstrate that profiles of antibodies against *M. tuberculosis* can be studied by MMIA. Infection in macaques was detected as early as 4 weeks postinoculation by this method. These results suggest that antibody profiling in the multiplex format, using multiple *M. tuberculosis* target antigens (the six reported here, plus others selected from 4,000 open reading frames), will afford a useful and efficient serodiagnostic modality for the detection and survey of *M. tuberculosis* infection in nonhuman primates as well as humans. A key advantage of the MMIA is flexibility of the Luminex platform and the potential for testing additional antigens defined by the deciphering of the *M. tuberculosis* genome for TB diagnosis. Because the Luminex platform is adaptable, flexible, and user-friendly, those additional antigens against which antibodies are more consistently detected in *M. tuberculosis*-infected subjects could be easily incorporated into the MMIA. Additionally, the MMIA will be very useful for monitoring antibody responses in TB vaccine studies because of the flexibility of antibody targets and high-throughput capabilities of this technology.

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