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Peripheral Blood Gamma Interferon Release Assays Predict Lung Responses and *Mycobacterium tuberculosis* Disease Outcome in Mice[▽]

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Current diagnostic tests for tuberculosis (TB) are not able to distinguish active disease from latent *Mycobacterium tuberculosis* infection, nor are they able to quantify the risk of a latently infected person progressing to active TB. There is interest, however, in adapting antigen-specific gamma interferon (IFN-γ) release assays (IGRAs) to predict disease outcome. In this study, we used the differential susceptibilities of inbred mouse strains to *M. tuberculosis* infection to evaluate the prognostic capabilities of IGRAs. Using lung and blood cultures, we determined that CBA/J, DBA/2, and C3H/HeJ mice (models of heightened risk of progression to active TB) produced less antigen-specific IFN-γ in response to *M. tuberculosis* culture filtrate proteins and early secreted antigenic target-6 than the relatively resistant C57BL/6 mouse strain. Additionally, reduced IFN-γ secretion in supernatants reflected a reduced frequency of IFN-γ-responding cells in the lung and blood and not a specific defect in IFN-γ secretion at the single-cell level. Importantly, detection of antigen-specific IFN-γ from blood cultures accurately reflected lung responses, indicating that blood can be an appropriate test tissue in humans. Furthermore, reduced antigen-specific IFN-γ production and low frequencies of IFN-γ-responding cells from peripheral blood predicted increased risk of TB disease progression across genetically diverse TB disease-susceptible mouse strains, suggesting that similar results may occur in humans. The development of efficacious predictive diagnostic tests for humans would lead to targeted therapy prior to progression to active TB, reducing transmission, incidence, and prevalence rates while maximizing the use of public health resources.

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), infects up to one-third of the world's population (60). The majority (90%) of infected adults control *M. tuberculosis* in a latent state that is clinically silent and is not considered contagious. In contrast, the remaining 10% of *M. tuberculosis*-infected individuals progress to contagious, active TB and may transmit bacilli to others, resulting in significant global morbidity and mortality each year (61). The mechanisms that contribute to *M. tuberculosis* susceptibility and TB disease progression in humans are multifactorial and reflect altered pulmonary immunity due to polygenic interactions, immune status, age, and environmental factors (6, 50). These interactions contribute to the common outcome of active TB, associated with increased growth of *M. tuberculosis* in the lung and detrimental pulmonary inflammation, for which similar disease characteristics have been described in animal models (4, 12, 54).

Although the predisposing mechanisms underlying progression to active TB are not fully understood, we and others (1, 36, 41) propose that the transition from latency to active TB can be detected, quantified, and predicted by peripheral immune responses prior to disease onset. The identification of such biomarkers could then be used to quantify the risk of TB disease progression, allowing targeted treatment of susceptible individuals. Current application of diagnostic tests, including skin tests and gamma interferon (IFN-γ) release assays (IGRAs), cannot distinguish latent *M. tuberculosis* infection from active TB, nor can they assess the risk of TB disease progression (1, 25, 41). Research interest does exist, however, in longitudinal assessment of IGRAs and its adaptation for TB prognosis in humans. Indeed, evidence exists to show that IFN-γ responders stratify into high, middle, and low categories (1), suggesting that IGRA results could also assess the risk of disease progression in humans.

Commercial IGRAs quantify the amounts of antigen-specific IFN-γ in blood culture supernatants (QuantiFERON-TB Gold; Cellestis Ltd., Carnegie, Victoria, Australia) or determine the frequency of IFN-γ-producing blood leukocytes (T-SPOT.TB; Oxford Immunotec, Oxford, United Kingdom) in response to specific mycobacterial peptides. IGRA peptides are synthetic immunogenic mycobacterial antigens from early secreted antigenic target-6 (ESAT-6), culture filtrate protein (CFP) 10, and TB7.7. These proteins are almost exclusively expressed by members of the *M. tuberculosis* complex and not by environmental, opportunistic mycobacteria or the vaccine strain *Mycobacterium bovis* BCG. Therefore, immune responses to these peptides are highly sensitive and specific for *M. tuberculosis* infection (31). Furthermore, ESAT-6 stimulates T-cell responses in mice (3, 46, 62) and humans (45). It is currently unknown whether the magnitude of *M. tuberculosis*

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antigen-specific responses could quantify the risk of TB disease progression or whether antigen-specific IFN-γ from the blood accurately reflects responses within the lung. Interpretation of differential responses in IGRAs may identify individuals with predicted poor outcomes and holds promise for earlier, targeted therapy.

Using immunocompetent inbred mouse strains (C57BL/6, CBA/J, DBA/2, and C3H/HeJ) with known timelines of TB disease progression (30), we modified IGRAs to assess antigen-specific IFN-γ production from the peripheral blood as an immunological predictor of TB disease outcome. All analyses were performed on mice that had no outward signs of TB disease (i.e., normal body weight, normal hair coat and posture, normal socialization, etc.) and stable bacterial loads within the lungs. The results, therefore, did not reflect morbidity-associated inflammatory responses. Our data demonstrate that mouse strains with reduced survival times following *M. tuberculosis* infection (30) failed to sustain or generate a robust antigen-specific IFN-γ response to *M. tuberculosis* CFP and ESAT-6 in both lung and peripheral-blood cultures. Most significant was the fact that low IFN-γ production from blood cells was evident throughout the entire infection period in CBA/J mice, even in the absence of clinical signs and prior to TB disease progression. Furthermore, using short-term infection studies, poor antigen-specific IFN-γ production was identified in two additional TB-susceptible mouse strains. These data indicate that reduced antigen-specific responses ex vivo can predict an increased susceptibility to TB disease progression in mice. Furthermore, these data demonstrate that the total amount of IFN-γ and the frequency of IFN-γ-responding cells in the blood directly reflect immune responses in the lung. These results, therefore, define a simple blood assay as both an indicator of lung-specific immunity and a predictor of disease outcome.

MATERIALS AND METHODS

Mice. Specific-pathogen-free 8-week-old female C57BL/6 and CBA/J mice (Charles River Laboratories, Wilmington, MA) and specific-pathogen-free 8-week-old female C57BL/6J, DBA/2J, and C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in ventilated cages within biosafety level 3 facilities and provided with sterile food and water ad libitum. All protocols were approved by The Ohio State University's Institutional Laboratory Animal Care and Use Committee.

***M. tuberculosis* stocks.** *M. tuberculosis* Erdman (ATCC 35801) was obtained from the American Type Culture Collection (Manassas, VA). Stocks were grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and frozen in 1-ml aliquots at −80°C.

***M. tuberculosis* infection.** Mice were exposed to an aerosol of *M. tuberculosis* Erdman using an inhalation exposure system (Glas-col, Terre Haute, IN) calibrated to deliver 50 to 100 CFU to the lungs of each individual mouse. Confirmation of the *M. tuberculosis* dose and calculation of the *M. tuberculosis* burden throughout the experimental-infection period were performed by plating serial dilutions of partial lung homogenates onto oleic acid-albumin-dextrose-catalase-supplemented 7H11 agar. *M. tuberculosis* CFU were counted after 3 weeks at 37°C and transformed to a $\log_{10}$ scale. The mice were weighed weekly. Mice weighing 20% less than the average for age- and sex-matched noninfected control animals with concurrent signs of disease progression (scruffy hair coats, tachypnea, and social isolation) were excluded from the study.

Lung cell isolation. Single-cell suspensions were obtained from the lungs at specific time points postinfection (58). Briefly, the lungs were perfused through the right cardiac ventricular lumen and pulmonary trunk with 10 ml of phosphate-buffered saline (PBS) containing 50 U/ml of heparin (Sigma, St. Louis, MO) and placed in Dulbecco's modified Eagle's medium (DMEM) (500 ml; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 1% HEPES buffer (Sigma), 10 ml of 100× nonessential amino acid solution (Sigma), 5 ml of penicillin-streptomycin solution (50,000 U penicillin, 50 mg streptomycin; Sigma), and 0.14% mercaptoethanol (complete DMEM). Single-cell suspensions from individual mice were obtained by dicing lung lobes with sterile razor blades, followed by a 30-min incubation at 37°C with 4 ml of complete DMEM containing collagenase XI (0.7 mg/ml; Sigma) and bovine pancreatic DNase (30 μg/ml; Sigma). Six milliliters of complete DMEM was subsequently added to dilute enzymatic activity, and the lung pieces were pressed through sterile 70-μm nylon mesh screens (BD Biosciences, San Jose, CA) to obtain a single-cell suspension. Residual red blood cells were lysed using 2 ml of ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$) for 3 min at room temperature, followed by washing with complete DMEM. Viable cells were counted using trypan blue exclusion and resuspended at working concentrations in complete DMEM.

Blood collection. Two hundred to 300 μl of blood was collected from the right cardiac ventricular lumen with a 22-gauge needle immediately following euthanasia and placed into 20 U of heparin (Sigma) diluted in 10 μl of sterile PBS. The heparinized whole blood was then diluted 1:10 in complete DMEM for use in modified antigen-specific IGRAs. Blood leukocytes for flow cytometry and enzyme-linked immunospot (ELISpot) assays were obtained by lysing red blood cells with two 4-minute room temperature incubations in ACK lysis buffer, separated by washes in 5 to 10 ml of complete DMEM. Isolated blood leukocytes were counted by trypan blue exclusion and resuspended at working concentrations in complete DMEM for ELISpot assays or in fluorescence-activated cell sorter (FACS) buffer (deficient RPMI [Irvine Scientific, Santa Ana, CA] supplemented with 0.1% sodium azide [Sigma]) for 30 min at 4°C to fix cell surface membranes for flow cytometry.

Bone marrow-derived APCs. Bone marrow cells were obtained from the tibiae and femora of age- and sex-matched noninfected C57BL/6 and CBA/J mice. The cells were differentiated into antigen-presenting cells (APCs) using complete DMEM supplemented with 10% conditioned medium derived from GM-EL4 cells, a granulocyte-macrophage colony-stimulating factor-producing clone kindly provided by Arthur A. Hurwitz (National Cancer Institute, NIH, Frederick, MD). The clone was created by a retroviral transfection described previously (22). Bone marrow cells (1×10^6 to 2×10^6) were plated at 37°C in 1 ml of GM-EL4 conditioned medium in sterile 24-well tissue culture plates. An additional 1 ml GM-EL4 conditioned medium was added on day 1. The GM-EL4 conditioned medium was replaced on days 3, 5, and 7. On day 8, the differentiated APCs were briefly centrifuged, and the GM-EL4 conditioned medium was removed. The cells were washed and stimulated for 24 h with 1 ml of 50-pg/ml endotoxin-free recombinant murine IFN-γ (Peprotech, Rocky Hill, NJ) in complete DMEM. On day 9, the APCs were washed in complete DMEM to remove IFN-γ and pulsed for 24 h with 1 ml of the following antigens in complete DMEM: 10 μg/ml ovalbumin (Sigma), 10 μg/ml *M. tuberculosis* CFP, and 5 μg/ml *M. tuberculosis* ESAT-6. All *M. tuberculosis* antigens were obtained or produced from plasmids through Colorado State University's National Institute of Allergy and Infectious Diseases (NIAID), NIH, contract no. HHSN266200400091C. On day 10, the APCs were washed to remove excess antigen, harvested, counted, and resuspended at working concentrations in complete DMEM.

Lung and blood modified IGRAs by enzyme-linked immunosorbent assay. Lung cell suspensions (100 μl) containing 2×10^5 cells or 100-μl aliquots of diluted blood from each individual mouse were cultured in duplicate in 96-well sterile tissue culture plates (BD Falcon Microtest; Fisher Scientific). Antigens (100 μl) in complete DMEM were added to the cultures, resulting in a final volume of 200 μl per well and the following final antigen concentrations: ovalbumin, 10 μg/ml (Sigma); *M. tuberculosis* CFP, 10 μg/ml; *M. tuberculosis* ESAT-6, 5 μg/ml; concanavalin A, 10 μg/ml (Sigma). The cultures were incubated for 72 h at 37°C, and the supernatants were stored at −80°C.

IFN-γ was quantified in lung cell and whole-blood culture supernatants by enzyme-linked immunosorbent assay using antibody pairs (BD Biosciences). High-binding 96-well round-bottom plates (Nunc Maxisorb Immunoplates; Fisher Scientific) were coated overnight with anti-IFN-γ (R4-6A2), and the wells were blocked with 10% bovine serum albumin in PBS or complete DMEM containing heat-inactivated 10% fetal calf serum for 2 h at room temperature. Samples from individual mice or recombinant murine IFN-γ standards (BD Biosciences) were dispensed in duplicate and incubated for 2 h at room temperature. IFN-γ was detected with secondary biotinylated anti-IFN-γ (XMG1.2) for 1 h at room temperature, followed by a 30-min room temperature conjugation with streptavidin-horseradish peroxidase (Invitrogen, Carlsbad, CA). Detection was quantified with 3,3′,5,5′-tetramethylbenzidine substrate (Dako, Carpinteria, CA), and the reactions were stopped with 0.18 M H_2SO_4. Positive samples were quantified by optical density at 450-nm wavelength with 570-nm correction and compared to the linear standard curve (ng/ml).

Lung and blood modified IGRAs by ELISpot assays. At each time point, six mice per inbred strain were euthanized, and their lung cells were pooled in groups of two for CD4 purification using magnetic MACS columns and anti-CD4 beads (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions with one modification. Following lung cell isolation, adherent cells from each individual mouse were removed by incubation in 100-mm sterile tissue culture petri dishes for 1 h at 37°C. CD4 T-cell purity was confirmed by flow cytometry (93.5% ± 1.7% of the purified lung cells were $CD3^+$ $CD4^+$). Purified cells from each pair of mice were serially diluted 1:2 in complete DMEM on precoated, blocked, sterile ELISpot plates according to the manufacturer's instructions (Ready-Set-Go Mouse IFN-γ ELISpot kit; eBioscience, San Diego, CA). At least six serial dilutions were performed for lung cells. Following the serial dilutions, 2.5×10^4 antigen-pulsed bone marrow-derived APCs were added to each well.

Pooled samples from six mice per strain were used for ELISpot assays with blood leukocytes. Following red blood cell lysis, leukocytes were counted by trypan blue exclusion, resuspended in complete DMEM, and serially diluted 1:2 in triplicate on precoated (Ready-Set-Go Mouse IFN-γ ELISpot kit; eBioscience), blocked, sterile ELISpot plates (Millipore, Billerica, MA). At least three serial dilutions were performed for blood leukocytes. Following the serial dilutions, 2.5×10^4 antigen-pulsed bone marrow-derived APCs were added to each well.

For both lung CD4 and blood leukocyte ELISpot assays, the plates were incubated for 36 h at 37°C. IFN-γ spot-forming units were detected and developed according to the manufacturer's instructions (eBioscience; Ready-Set-Go Mouse IFN-γ ELISpot kit). Spots were counted and analyzed using the Immunospot Analyzer (C.T.L., Cleveland, OH). The numbers of antigen-specific IFN-γ spot-forming units were determined by subtracting the number of spots formed in the presence of a negative control antigen (ovalbumin).

Flow cytometry. All conjugated and unconjugated antibodies and isotype controls were purchased from BD Biosciences, including Fc Block (clone 2.462), PerCP-Cy5.5 anti-CD3ε (145-2C11), APC-Cy7 anti-CD4 (GK1.5), PeCy7 anti-CD8 (53–6.7), APC anti-T-cell receptor β chain (TCR-β) (H57597), PerCP anti-CD8 (53–6.7), PeCy7 anti-IFN-γ (XMG1.2), and PeCy-7 rat immunoglobulin G1(κ) isotype control. Analyses were performed on lung cells or blood leukocytes from individual mice, except for groups of six pooled blood samples (see Fig. 3C and D and 5C and D) and three groups of two pooled lung CD4 T cells (see Fig. 5A and B). For surface staining, aliquots of lung cell suspensions were adjusted to 4×10^6 cells/ml and placed immediately into 1 ml of FACS buffer, and blood leukocytes were fixed in 500 μl of FACS buffer for 30 min at 4°C. For intracellular-cytokine staining, separate aliquots of lung cells from individual mice or isolated blood leukocytes from pooled mice were adjusted to 2×10^6 cells/ml in complete DMEM, stimulated with 1 μg/ml of purified anti-CD3ε plus 0.1 μg/ml of purified anti-CD28, and incubated with GolgiStop for 4 h at 37°C according to the manufacturer's instructions for inhibition of cytokine transport (Cytofix/Cytoperm kit; BD Biosciences). After 4 h, the cells were fixed in FACS buffer for 30 min at 4°C.

Following fixation, approximately 5×10^5 to 1×10^6 lung cells or blood leukocytes were incubated with 0.31 μg of Fc Block for 10 to 15 min at room temperature to minimize nonspecific antibody binding. Samples were then incubated with 0.31 μg of fluorescent antibodies in the dark for 20 min at 4°C, followed by three washes in FACS buffer. Lymphocytes were identified according to characteristic forward and side scatter profiles, and 20,000 to 50,000 events were counted within the lymphocyte gate using an LSRII flow cytometer. CD4 T cells were identified based upon CD3 or TCR-β, in combination with CD4 expression. The results were analyzed with FACSDiva software (BD Biosciences). Isotype controls were included for each mouse strain at each time point and used to set gates for analysis.

Statistics. Statistical analyses were performed using Prism 4 software (GraphPad Software, San Diego, CA). Multigroup comparisons were analyzed with one-way analysis of variance (ANOVA) using Dunnett's posttest (for comparison to noninfected control mice within each strain over time) or Tukey's posttest (for comparison of all groups). For pairwise comparisons between strains within time points, Student's *t* test was used. Statistical significance in all analyses was defined as *P* values of <0.05, <0.01, and <0.001. Where necessary, one outlier per strain per time point was identified using Grubb's outlier test (GraphPad QuickCalcs) and removed.

RESULTS

CBA/J mice maintain stable *M. tuberculosis* CFU with no external signs of TB disease. The abilities of inbred mouse strains to survive *M. tuberculosis* infection segregate into TB disease-resistant and -susceptible clusters with known survival times (4, 8, 23, 29, 30, 44, 54, 55). C57BL/6 mice survive *M. tuberculosis* infection into their second year of life and are considered relatively resistant (30, 34, 35, 44). In contrast, CBA/J mice are more susceptible to *M. tuberculosis* infection (30). Infection in CBA/J mice is associated with a persistently elevated *M. tuberculosis* bacterial load in the lung with early death due to overwhelming pulmonary disease (4, 29, 54).

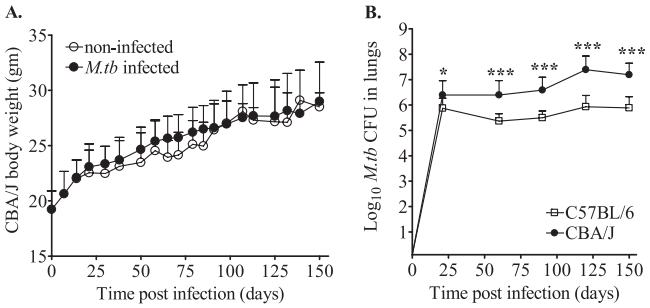

FIG. 1. Asymptomatic *M. tuberculosis* infection in susceptible CBA/J mice. (A) C57BL/6 and CBA/J mice were infected with 50 to 100 CFU of *M. tuberculosis* Erdman by aerosol. At weekly intervals, *M. tuberculosis* (*M.tb*)-infected mice and noninfected age- and sex-matched control CBA/J mice were weighed. The body weight results are the average ± standard deviation (SD) from two independent experiments using 10 to 52 *M. tuberculosis*-infected mice and 4 to 10 noninfected mice per time point. Weights were analyzed by one-way ANOVA with Tukey's posttest. No statistical differences were found. (B) Mice were euthanized at various time points, and *M. tuberculosis* CFU in the lungs were determined. The CFU results are the average ± SD from four independent experiments, each with four or five mice per strain per time point, analyzed by one-way ANOVA with Tukey's posttest. *, $P < 0.05$; ***, $P < 0.001$.

In this study, we analyzed immune responses during a period of *M. tuberculosis* infection when CBA/J mice showed no outward signs of disease and compared the responses to those of the relatively resistant C57BL/6 mouse strain. Throughout the experimental time frame, *M. tuberculosis*-infected CBA/J mice exhibited normal weight gain relative to noninfected age- and sex-matched controls (Fig. 1A). A small proportion (10%) of CBA/J mice developed morbidity between days 90 and 150 postinfection (data not shown), and they were excluded from the study. All C57BL/6 mice appeared clinically healthy and gained weight at the same rate as noninfected age- and sex-matched control mice over 150 days of *M. tuberculosis* infection (data not shown). Despite remaining clinically healthy, CBA/J mice had significantly more *M. tuberculosis* CFU recovered from the lungs than C57BL/6 mice at all time points tested (Fig. 1B).

Increased risk of TB disease progression is associated with reduced antigen-specific IFN-γ production from lung cells. Lung cells from infected C57BL/6 and CBA/J mice were cultured with *M. tuberculosis* CFP or ESAT-6 to determine antigen-specific IFN-γ secretion ex vivo. Lung cells from C57BL/6 mice produced abundant IFN-γ in response to both CFP (Fig. 2A) and ESAT-6 (Fig. 2C), and these responses were sustained throughout the first 150 days of *M. tuberculosis* infection. In contrast, lung cells from CBA/J mice responded with a potent but transient peak of IFN-γ in response to CFP at day 21

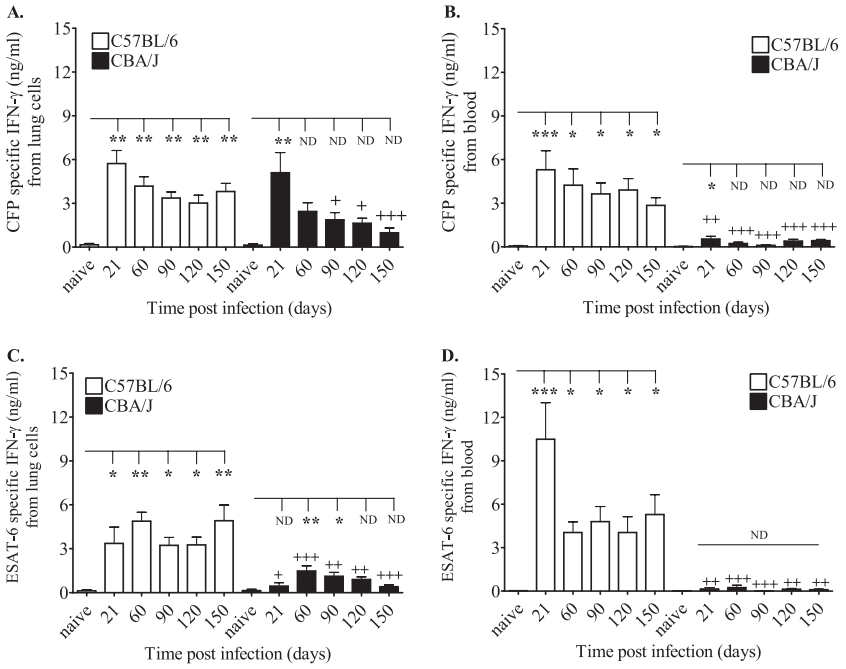

FIG. 2. Antigen-specific IFN-γ production from lung cells and blood. C57BL/6 and CBA/J mice were infected with 50 to 100 CFU of *M. tuberculosis* Erdman by aerosol. Lung cells (2×10^5) (A and C) and 1:10-diluted whole blood (B and D) from noninfected and infected mice at specific time points postinfection were cultured with *M. tuberculosis* CFP (A and B) or *M. tuberculosis* ESAT-6 (C and D). The results are the mean plus standard error of the mean of four (A and B) or two (C and D) independent experiments with four or five mice per strain per time point. The data were analyzed by mouse strain, with comparison to noninfected controls by one-way ANOVA with Dunnett's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ND, no significant difference. Direct pairwise comparisons between C57BL/6 and CBA/J mice at each time point were analyzed by Student's *t* test. +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$. IFN-γ production in response to ovalbumin (negative control) did not exceed 0.12 ± 0.3 ng/ml from lung cells and 0.02 ± 0.07 ng/ml from blood.

postinfection, but this response declined over time (Fig. 2A). As infection progressed beyond day 60, CFP-specific IFN-γ production from CBA/J lung cells was significantly less than that of C57BL/6 mice. ESAT-6 induced a small and delayed peak of antigen-specific IFN-γ at day 60 in CBA/J lung cell cultures (Fig. 2C) that, similar to CFP-specific responses, was not sustained. At all time points tested, ESAT-6-specific IFN-γ production was significantly reduced in lung cell cultures from CBA/J mice compared to C57BL/6 mice (Fig. 2C).

These data demonstrate that predicted TB disease progression and decreased survival in susceptible CBA/J mice following *M. tuberculosis* infection are associated with reduced antigen-specific immune responses throughout chronic, asymptomatic pulmonary infection. Poor antigen-specific IFN-γ production from the primary site of *M. tuberculosis* infection can therefore predict accelerated TB disease progression in mice. Because lung cells from *M. tuberculosis*-exposed or -infected humans are not readily available for testing, we subsequently determined whether blood was an appropriate surrogate tissue for lung immune responses.

Antigen-specific IFN-γ responses from blood are accurate indicators of lung responses. *M. tuberculosis* IGRAs approved for human diagnostics use heparinized whole blood or freshly isolated peripheral blood mononuclear cells cocultured with *M. tuberculosis* antigens. We modified IGRAs for use in *M. tuberculosis*-infected C57BL/6 and CBA/J mice by stimulating diluted heparinized whole blood with the *M. tuberculosis* antigens CFP and ESAT-6. Blood samples were analyzed in parallel with the lung cultures.

Antigen-specific IFN-γ from blood samples generated profiles similar to that observed from isolated lung cells. Blood cell cultures from C57BL/6 mice produced abundant and sustained IFN-γ in response to *M. tuberculosis* CFP (Fig. 2B) and ESAT-6 (Fig. 2D) throughout 150 days of infection. ESAT-6-specific IFN-γ production from blood cell cultures was elevated after 21 days of infection in comparison to the lung but then was comparable between 60 and 150 days (Fig. 2D). Therefore, the data indicate that for the relatively resistant C57BL/6 mouse strain, antigen-specific IFN-γ production from blood cell cultures directly reflects the immune status of the lung and can be used to predict disease outcome.

Minimal IFN-γ was secreted by blood cell cultures from CBA/J mice in response to *M. tuberculosis* CFP (Fig. 2B) and ESAT-6 (Fig. 2D). Similar to what was observed for lung cell cultures, IFN-γ production from blood cultures of CBA/J mice was significantly less than that observed in C57BL/6 blood cultures. Furthermore, comparison of CBA/J lung and blood profiles showed that CFP-specific IFN-γ production in blood cultures was substantially reduced at all time points, and ESAT-6-specific IFN-γ was lower at days 60 and 90 of infection. These results suggest that sequestration of antigen-specific T cells to the lungs may have occurred in CBA/J mice. Therefore, blood cultures from susceptible CBA/J mice reflect lung-specific events to some degree, and in fact, due to the substantially

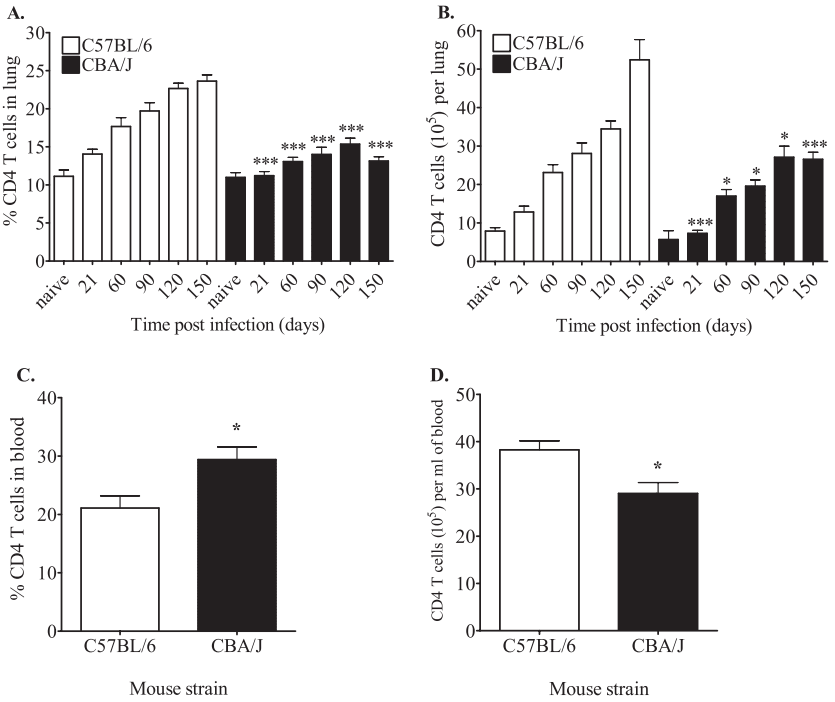

FIG. 3. CD4 T cells in the lung and blood. C57BL/6 and CBA/J mice were infected with 50 to 100 CFU of *M. tuberculosis* Erdman by aerosol. Lung and blood cells were labeled with fluorescent anti-CD3 or anti-TCR-β and anti-CD4 and quantified using flow cytometry. The proportion (A) and absolute numbers (B) of lung CD4 T cells from C57BL/6 and CBA/J mice are shown throughout infection. The proportion (C) and absolute numbers (D) of blood CD4 T cells are shown from day 21 postinfection. The results are the mean plus standard error of the mean (SEM) of four separate experiments (A and B), each with four or five mice per strain per time point. The results shown in panels C and D are the mean plus SEM from three independent experiments. The data were analyzed by pairwise comparisons between strains at each time point using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

reduced responses, blood may be a better predictor of disease outcome than the lung.

It is widely accepted that CD4 T cells are the dominant source of antigen-specific IFN-γ during *M. tuberculosis* infection (20). We therefore determined whether poor antigen-specific IFN-γ production from the lungs and blood of CBA/J mice was associated with inadequate numbers or function of CD4 T cells.

Number and function of CD4 T cells in the lungs and blood. Although primary genetic defects (6), human immunodeficiency virus infection (51), and pharmacologic (63) immunosuppression increase the risk of TB disease progression, most active cases are not attributable to any of these immunodeficiencies (6, 11, 15). Furthermore, many TB patients appear to have intact acquired immune responses and produce IFN-γ in response to *M. tuberculosis* antigens and unrelated antigens (2, 13). In conjunction with mice with genes disrupted (5, 10), these observations suggest that a critical mass of functional CD4 T cells is optimal for *M. tuberculosis* control. To determine whether increased risk of TB disease progression in CBA/J mice was associated with low CD4 T-cell numbers in the lungs or blood, CD4 T-cell numbers were determined. The proportion of CD4 T cells in the lungs of *M. tuberculosis*-infected CBA/J mice was significantly less than those of C57BL/6 mice throughout the 150-day study (Fig. 3A), leading to fewer total CD4 T cells recovered from the lungs (Fig. 3B) and fewer well-analyzed CD4 T cells in each cell culture (data not shown). Although the proportion of CD4 T cells in the blood was increased in CBA/J mice compared to C57BL/6 mice (Fig. 3C), the absolute number of CD4 T cells was less (Fig. 3D) due to fewer leukocytes being recovered from equivalent volumes of blood (data not shown). These data indicate that reduced numbers of lung and circulating CD4 T cells may contribute to poor TB disease outcome.

To determine whether CD4 T cells from CBA/J mice had the potential to respond in an antigen-dependent manner during infection, lung and blood cells were stimulated with anti-CD3 and anti-CD28 for 4 h with GolgiStop, and accumulated intracellular IFN-γ was quantified by flow cytometry. In lung (Fig. 4A) and blood (Fig. 4B) cell cultures, the proportion of CD4 T cells that produced IFN-γ in response to TCR stimulation from CBA/J mice was approximately one-half of that from C57BL/6 mice. The fluorescence intensity of IFN-γ within individual CD4 T cells from the lungs or blood of CBA/J mice was equivalent to that for C57BL/6 mice (data not shown). These data show that IFN-γ responses were not deficient in CBA/J mice on an individual CD4 T-cell basis but that CBA/J mice have fewer CD4 T cells with the potential to respond to TCR-mediated stimulation. Therefore, CBA/J mice generate or maintain a smaller pool of T cells that are capable of secreting IFN-γ. We next determined whether susceptible CBA/J mice had fewer *M. tuberculosis* CFP and ESAT-6 IFN-γ-responding cells than C57BL/6 mice.

Low IFN-γ production results from decreased antigen-specific T cells in CBA/J mice. To confirm that the low levels of antigen-specific IFN-γ produced by lung and blood cells from

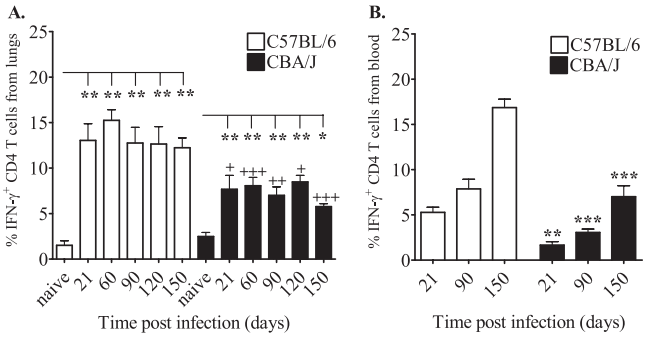

FIG. 4. TCR-stimulated IFN-γ production from lung and blood cells. C57BL/6 and CBA/J mice were infected with 50 to 100 CFU of *M. tuberculosis* Erdman by aerosol. Lung cells (A) or blood leukocytes (B) were stimulated with anti-CD3 and anti-CD28 to cross-link and induce TCR signaling, labeled with fluorescent anti-TCR-β and anti-CD4, permeabilized, and labeled with anti-IFN-γ. Intracellular IFN-γ was quantified by flow cytometry for IFN-γ^+ CD4 T cells. The lung results are the mean plus standard error of the mean from four independent experiments, each with four or five mice per strain per time point, analyzed by strain over time using one-way ANOVA with Dunnett's posttest compared to noninfected mice; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Direct pairwise comparisons between C57BL/6 and CBA/J mice at each time point were analyzed by Student's *t* test. +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$. The blood results are from two experiments with six mice per strain per time point, analyzed by strain over time using one-way ANOVA with Tukey's posttest. **, $P < 0.01$; ***, $P < 0.001$. The average isotype control levels for lung CD4 T cells and blood CD4 T cells were 0.38% and 0.46%, respectively. IFN-γ^+, IFN-γ positive.

CBA/J mice in culture were attributable to reduced numbers of antigen-specific cells in vivo, we performed ELISpot assays and calculated the frequencies of cells capable of secreting IFN-γ in response to *M. tuberculosis* CFP and ESAT-6. As anticipated, CBA/J mice had significantly fewer CD4 T cells that could secrete IFN-γ in response to CFP (Fig. 5A) and ESAT-6 (Fig. 5C) than C57BL/6 mice. Isolated blood leukocytes from CBA/J mice also showed a substantially reduced frequency of IFN-γ responders to CFP (Fig. 5B) and ESAT-6 (Fig. 5D) compared to blood cells from C57BL/6 mice. ELISpot analysis therefore confirmed that CBA/J mice generate or sustain fewer antigen-specific CD4 T cells within the lungs and circulation during *M. tuberculosis* infection. To maintain the integrity of the IGRA ELISpot model, CD4 T cells were not purified from blood samples; however, depletion of CD4 T cells from ex vivo cultures has been shown to nearly abrogate IFN-γ detection (46). These data demonstrate that increased risk of TB disease progression is associated with a reduced precursor frequency of antigen-specific CD4 T cells in the lung and the peripheral blood, providing further evidence that antigen-specific responses in the blood accurately reflect immune events within the lungs.

Reduced antigen-specific IFN-γ production is common among *M. tuberculosis*-susceptible mouse strains. To determine whether reduced antigen-specific IFN-γ can be used as a

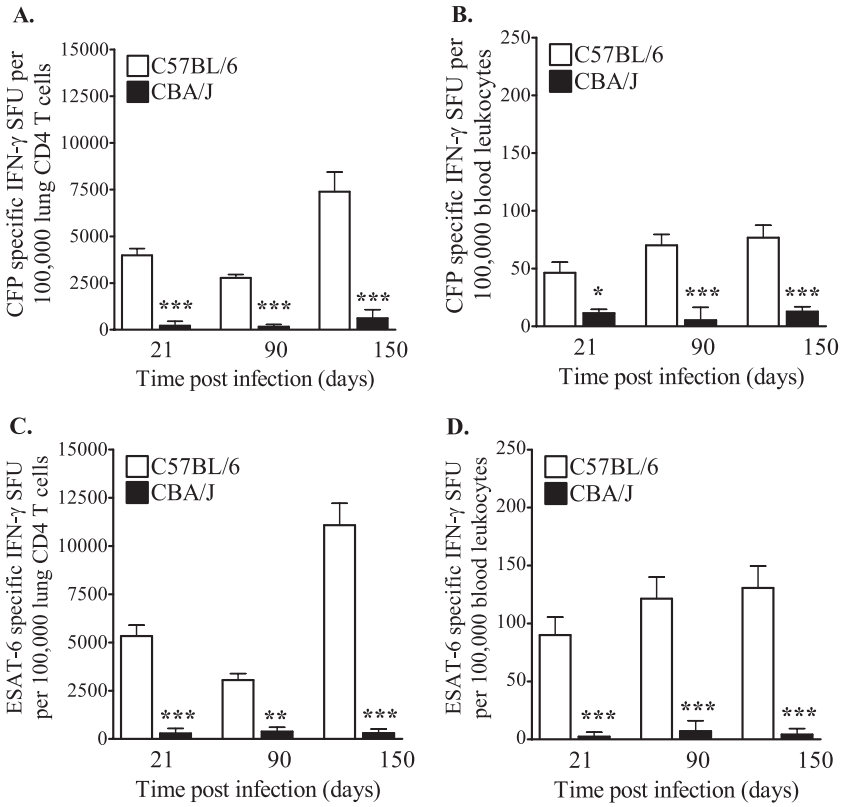

FIG. 5. Frequencies of antigen-specific IFN-γ-producing cells from lungs and blood. C57BL/6 and CBA/J mice were infected with 50 to 100 CFU of *M. tuberculosis* Erdman by aerosol. Purified lung CD4 T cells (A and C) and blood leukocytes (B and D) were stimulated with *M. tuberculosis* CFP (A and B)- or ESAT-6 (C and D)-pulsed bone marrow-derived APCs for 36 h. IFN-γ spot-forming units (SFU) were counted, and the frequency of antigen-specific cells was calculated per 100,000 cells by subtracting spots formed in the presence of negative control antigen (ovalbumin). The results shown are the mean plus standard error of the mean of three (A and C) or two (B and D) independent experiments with six mice per strain per time point analyzed by strain over time using one-way ANOVA with Tukey's posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

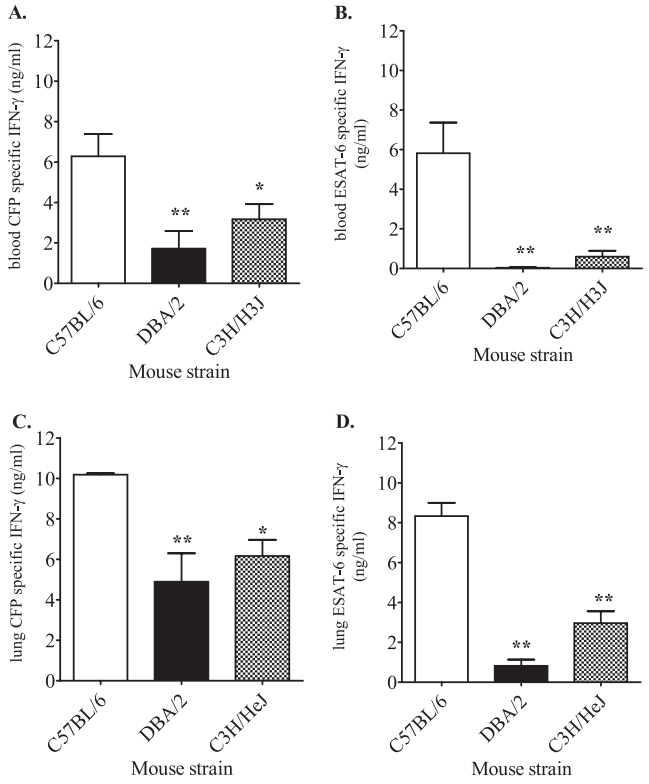

FIG. 6. Antigen-specific IFN-γ from blood and lungs. C57BL/6, DBA/2, and C3H/HeJ mice were infected with 50 to 100 CFU of *M. tuberculosis* Erdman by aerosol. Whole blood (1:10 diluted) (A and B) or 2×10^5 lung cells (C and D) from mice at day 21 postinfection were cultured with *M. tuberculosis* CFP (A and C) or ESAT-6 (B and D), and IFN-γ in the supernatants was quantified. The results are the mean plus standard error of the mean of two independent experiments, each with four or five mice per strain, for blood and one experiment with five mice per strain for lungs. The data were analyzed by one-way ANOVA with Dunnett's posttest. *, $P < 0.05$, and **, $P < 0.01$ compared to C57BL/6 mice. The average IFN-γ production in response to ovalbumin (negative control) was 0.003 ng/ml (blood) and 0.7 ng/ml (lung).

common predictor of TB disease outcome, we analyzed two additional *M. tuberculosis*-susceptible mouse strains (4, 7). Modified IGRAs were performed on lung and blood samples from DBA/2 and C3H/HeJ mice, and the responses were compared to those of C57BL/6 mice. As previously observed in CBA/J mice, low CFP and ESAT-6 IFN-γ responses accurately predicted an increased risk of TB disease progression from both blood (Fig. 6A and B) and lung (Fig. 6C and D) cultures during asymptomatic infection. Blood and lung cultures from DBA/2 and C3H/HeJ mice produced abundant IFN-γ when stimulated nonspecifically with concanavalin A (data not shown), indicating no general defects in IFN-γ production in either mouse strain. These results indicate that reduced antigen-specific IFN-γ production, and responses to ESAT-6 in particular, is a common immunological outcome among susceptible mouse strains during early *M. tuberculosis* infection. These data indicate that blood-derived immunological biomarkers may predict TB disease outcome across genetically disparate individuals.

DISCUSSION

Using several different immunocompetent inbred mouse strains, we have shown that modified IGRAs can successfully identify strains that have shorter survival times in response to infection with *M. tuberculosis.* Mouse strains with accelerated TB disease progression (CBA/J, DBA/2, and C3H/HeJ) produced suboptimal IFN-γ secretion ex vivo from lung and blood cultures in response to the *M. tuberculosis* antigens CFP and ESAT-6. In CBA/J mice, the decreased antigen-specific IFN-γ corresponded to low frequencies of antigen-specific IFN-γ T cells in the lung and blood. Furthermore, antigen-specific responses in peripheral-blood cultures provided a more sensitive indicator of risk for TB disease progression than the lung. These data demonstrate that diagnostic test results based on blood cell culture supernatants (Quantiferon-Gold) or frequency of antigen-specific blood cells (T-Spot.TB) may be adapted for use to predict TB disease outcome in humans infected with *M. tuberculosis.*

Several studies have characterized antigen-specific T-cell responses in the lungs or spleens of *M. tuberculosis*-infected mice (27, 28, 53); however, we believe we are the first to study and report the relationship between peripheral-blood immune responses and that of lung cells in mice. Using murine models with differential TB susceptibilities, we have shown that IFN-γ antigen-specific immune responses in the blood are a sensitive indicator of lung-specific events. Our studies are relevant to humans, for whom the most common source of antigen-specific T cells is the peripheral blood. Very few human or animal studies have compared the lung and blood immune responses during long-term *M. tuberculosis* infection, and to our knowledge, none have correlated the results with disease outcome. A recent study of human TB patients, however, reported that ESAT-6-specific IFN-γ from normalized cell numbers was elevated in the lungs compared to blood (48), indicating that our findings in the murine system can accurately model humans. Furthermore, individual variation in secreted ESAT-6-specific IFN-γ from blood assays has been described in *M. tuberculosis*-infected nonhuman primates. In a study of four animals, two had minimal responses to ESAT-6, and of those, one animal had massive infection with hematogenous spread at necropsy (57). Although lung-specific responses were not compared to those of blood, these studies in nonhuman primates provide additional evidence that low IFN-γ responses to ESAT-6 can be associated with increased susceptibility to TB disease and elevated bacterial burden, similar to our findings in mice. In contrast to our murine models, however, both studies quantified ESAT-6-specific responses during clinical disease, rather than prior to disease onset.

Our studies also demonstrate that we can successfully predict the risks of different mouse strains for developing TB disease using a simple blood IGRA. The potential to predict TB outcome in humans using a simple blood assay could create a powerful tool for the early detection and treatment of susceptible individuals prior to the onset of active TB. Such a strategy would decrease *M. tuberculosis* transmission and lead to a reduction in the global burden of TB. Many clinical research papers (25, 38–40) and reviews (1, 36, 41, 42) support the use of IGRAs to monitor or classify stages of TB disease and to predict disease outcome in humans. Additionally, recent

analysis (1) of previously published data (14) indicated that healthy contacts of TB patients stratify into low, medium, and high IFN-γ ESAT-6 responders, suggesting that a correlation with TB disease progression might exist in humans. Results from large-scale prospective longitudinal studies in humans are not yet available, and currently, it is unknown whether low or high responders progress to active TB.

A number of studies have investigated associations between the TB disease state and the strength of antigen-specific responses. For example, active TB disease has been associated with depressed *M. tuberculosis*-specific IFN-γ responses (49), whereas latently infected individuals and patients with minimal TB disease had increased numbers of ESAT-6-specific IFN-γ-secreting blood cells (43, 56). In contrast, other human studies (19, 21, 25) have associated strong ESAT-6-specific IFN-γ responses with active TB and/or elevated *M. tuberculosis* bacterial load; however, subjects were not assessed prior to disease onset, and it is unclear whether the study participants had ongoing *M. tuberculosis* exposure throughout the follow-up periods. These contradictory findings from human IGRA data may be due to small group sizes or differing methodologies or may indicate that subpopulations of *M. tuberculosis*-susceptible individuals have differential responses to ESAT-6. Larger and longitudinal studies are under way to critically evaluate whether IGRAs can be successfully used to predict TB disease progression in humans (1, 36). Using inbred mouse strains with different susceptibilities to *M. tuberculosis*, our results suggest that TB disease outcome can be predicted by low ESAT-6 antigen-specific IFN-γ secretion ex vivo.

With the inclusion of highly specific *M. tuberculosis* antigens, IGRAs are more sensitive and specific for *M. tuberculosis* infection than the purified protein derivative skin test but cannot yet distinguish latent *M. tuberculosis* infection from active TB disease or predict disease outcome. In these experiments, IGRAs were modified to accommodate the small numbers of lung and blood cells available from mice and ESAT-6 was chosen to model human assays. Complex proteins (CFP) were also included to quantify global CD4 T-cell IFN-γ responses to soluble mycobacterial antigens. The incubation times in our assays were 36 and 72 h, which likely resulted in antigen IFN-γ production from effector rather than memory T cells (26). Our data clearly indicate that purified ESAT-6 was a superior predictor of TB disease susceptibility in our mouse models, with better discrimination between the relatively resistant and susceptible strains than is seen with CFP. Perhaps most intriguing was our finding that in the blood, ESAT-6-specific IFN-γ production could not distinguish *M. tuberculosis*-infected susceptible CBA/J mice from noninfected mice. The same pattern can also be observed in humans, for whom peripheral-blood IGRAs failed to detect small proportions of *M. tuberculosis*-infected individuals (18, 37) and TB patients (45). As our data indicate, the inclusion of a less selective pool of *M. tuberculosis* antigens, such as CFP, may ensure that all *M. tuberculosis*-infected individuals are identified. Nonetheless, our results indicate that poor immune responses to complex (CFP) and single (ESAT-6) antigens can successfully predict the *M. tuberculosis* infection outcome in mice. It is currently unknown how peripheral-blood IGRA responses of *M. tuberculosis*-susceptible inbred mouse strains are affected by vaccination, antibiotic treatment, or immunosuppression. In other animal models, *M. bovis* BCG vaccination of calves (59) and relatively resistant C57BL/6 mice (17) showed decreased ESAT-6-specific IFN-γ blood responses, likely reflecting antigenic loads lower than those of nonvaccinated control animals. Because CBA/J, DBA/2, and C3H/HeJ mice had low circulating ESAT-6 responses during primary *M. tuberculosis* infection, we predict minimal changes due to BCG vaccination, antibiotic treatment, or immunosuppression. Following therapy of *M. tuberculosis*-infected humans, inconsistent responses from IGRAs have been reported, which included maintenance of sustained ESAT-6 responses (9), temporally dependent and discordant results (16), and declining frequencies of antigen-specific cells (43). The immunologic mechanisms underlying these disparities in susceptible humans are currently unknown; however, some of these clinically relevant questions can now be addressed with the use of multiple *M. tuberculosis*-susceptible inbred mouse strains, such as we describe here.

An intriguing finding was that CBA/J mice had an initial robust antigen-specific immune response to CFP antigens within the lung, which subsequently waned as infection progressed. Loss of acquired immunity occurred, despite increased bacterial loads in the lung at all time points tested. These data suggest that CBA/J mice can initially generate a protective immune response, coinciding with control of *M. tuberculosis* growth within the lung, but for reasons currently unknown fail to maintain a sufficient functional pool of antigen-specific IFN-γ-secreting CD4 T cells within the lung. An understanding of this mechanism could provide valuable information about why protective immunity cannot be sustained in a subset of individuals with latent *M. tuberculosis* infection, resulting in reactivation of infection and TB disease.

Increased susceptibility of inbred mouse strains to infection with *M. tuberculosis* has been associated with poor localization of T cells to the lung (4), decreased expression of T-cell adhesion molecules (54), delayed *M. tuberculosis* dissemination (4, 7), and increased interleukin 10 (IL-10) production (53). Poor expression of adhesion molecules may contribute to the reduced number of CD4 T cells localizing to the lungs; however, additional mechanisms must contribute, as the reduction in IFN-γ and antigen-specific cells was not proportional to the reduction in total lung CD4 T cells. Impaired dissemination of *M. tuberculosis* antigens to the regional lymph nodes could also contribute to susceptibility; however, if protective immunity were simply due to slow dissemination and delayed generation of acquired immunity, IFN-γ responses should eventually reach levels equivalent to those in resistant mice, which was not observed. Increased levels of IL-10, an immunosuppressive cytokine, have been shown within the lungs of CBA/J mice during chronic infection with *M. tuberculosis* (53), and the pleotropic effects of IL-10 could certainly contribute to poor antigen-specific IFN-γ production. IL-10 has been shown to down regulate macrophage activation by inhibiting major histocompatibility complex class II recycling, peptide loading, or inhibition of transcription factors (33), all of which could impair the generation or maintenance of antigen-specific T cells capable secreting IFN-γ. Furthermore, overexpression of IL-10 on the relatively resistant C57BL/6 background strain led to reduced antigen-specific IFN-γ secretion and significantly elevated bacterial burdens (53). The exact mechanism through which IL-10 negatively influences chronic *M. tuberculosis* in-

fection is not fully known, but in other mycobacterial infections, blocking IL-10 promoted macrophage activation, proinflammatory cytokine production, and production of reactive nitrogen intermediates, as well as positively influencing vaccination and therapeutic responses (47, 52).

The complex immune response to *M. tuberculosis* likely cannot be explained by one underlying mechanism in susceptible mice or humans; however, the use of genetically disparate mouse strains can mimic the genetic diversity of the human population. These models, therefore, can be used to identify and validate immunologic mechanisms and biomarkers of TB disease progression in humans.

The identification of immunological predictors of TB disease progression has the potential to diminish the global TB disease burden by targeted monitoring of at-risk patients and initiation of treatment prior to disease onset, thus limiting potential *M. tuberculosis* transmission. Adaptation of IGRAs, singly or in combination with additional markers, such as IL-10 and IL-2 (24, 32), to predict outcomes in humans is an important area of ongoing research. For humans, many questions remain, including whether immune responses from peripheral blood truly reflect immune events within the lung and whether the amount or frequency of IFN-γ-responding cells correlates with TB disease progression (31, 36, 41). The use of genetically disparate mouse strains, such as CBA/J, DBA/2, and C3H/HeJ, provides a tool to model different susceptibilities to infection with *M. tuberculosis*, to expand our understanding of immune mechanisms that lead to poor disease outcome, and to identify potential biomarkers that can be used to monitor disease progression in humans.

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