higher immunopositivity for programmed cell death ligand 1 (11). Given the difference in therapeutic efficacy according to histologic subtype, our observations based on a thorough analysis of primary and matched metastases may have clinical significance.

Our data suggests that the presence, even at a low percentage, of the SOL subtype in resected primary tumors should raise suspicion for the presence and/or predominance of the SOL subtype in metastatic tumors; this may, in turn, affect the choice of chemoimmunotherapies. ■

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Yusuke Takahashi, M.D., Ph.D.
Memorial Sloan Kettering Cancer Center
New York, New York
and
Keio University School of Medicine
Tokyo, Japan

Takashi Eguchi, M.D., Ph.D.
Memorial Sloan Kettering Cancer Center
New York, New York
and
Shinshu University School of Medicine
Matsumoto, Japan

Shaohua Lu, M.D., Ph.D.
Memorial Sloan Kettering Cancer Center
New York, New York
and
Fudan University
Shanghai, China

Robert J. Downey, M.D.
David R. Jones, M.D.
William D. Travis, M.D.
Prasad S. Adusumilli, M.D.
Memorial Sloan Kettering Cancer Center
New York, New York

ORCID ID: 0000-0002-1699-2046 (P.S.A.).

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Differentially Culturable Tubercule Bacilli Are Generated during Nonpulmonary Tuberculosis Infection

To the Editor:

Mycobacterium tuberculosis (*Mtb*) is a major global cause of death from infectious disease (1). Rarely contagious, extrapulmonary tuberculosis (EPTB) is neglected in tuberculosis (TB) control strategies. Nonetheless, EPTB contributes significantly to the burden of disease, is frequently difficult to diagnose, and inflicts significant morbidity (2).

After the establishment of infection in the host, it is believed that heterogeneous populations develop, distinguishable by specific growth requirements (3–5), metabolic features (6, 7), and differential susceptibility to antimicrobial agents (3, 8). The application of limiting

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dilution method to enumerate *Mtb* in liquid media has previously revealed that human TB sputum was dominated by *Mtb* populations that could not be detected by standard methods but required addition of culture supernatant (CS) obtained from actively growing *Mtb* or recombinant Rpf (resuscitation-promoting factor) (3, 4). The importance of Rpf in recovery of these bacteria was further supported by control experiments with Rpf-depleted or Rpf-inactivated culture supernatants (3, 9). However, a recently published study demonstrated the higher complexity of mycobacterial populations in sputum, including bacilli growing only in 7H9 supplemented with CS or Rpf-deficient CS, those growing in 7H9 medium only (nonplateable), and those growing on standard 7H10 agar plates (plateable) *Mtb* bacilli, collectively designated as differentially culturable tubercule bacteria (DCTB) (4, 5). Furthermore, it was proposed that relative proportions of these populations depended on patient $CD4^+$ T cell counts (4), and it is unknown whether other host factors may hold influence. DCTB can be generated *in vitro* by application of combined stresses (10) or treatment with antimicrobial agents (11).

There is accumulating experimental evidence to suggest the presence of DCTB has ramifications for clinical and treatment outcomes. CS- and Rpf-dependent *Mtb* bacilli were apparently enriched in treated patents and showed high tolerance to killing by antimicrobials, including rifampicin (3), isoniazid, and streptomycin (8). Furthermore, the eradication of CS-dependent *Mtb* prevented relapse in a Cornell mouse model (12). Finally, the application of recombinant Rpf or Rpf-containing CS improved time to positivity in a substantial number of sputum samples and often enhanced bacterial recovery (4, 13). Although three independent studies demonstrated the presence of Rpf- or CS-dependent bacilli in sputum samples (3, 4, 13), it remains unclear whether similar bacilli are commonly generated in EPTB. An understanding of DCTB populations in EPTB has important implications for diagnosis and treatment. Therefore, we examined a broad range of extrapulmonary samples to answer: *1*) Are CS-dependent bacilli present in EPTB? *2*) Can we identify host factors that may modulate CS-dependent populations?

We used previously developed methodology and assessed the number of *Mtb* bacilli recoverable on agar plates (colony-forming units [cfu]), in liquid 7H9 medium (most probable number counts on 7H9 medium [MPN_7H9]), or in 7H9 medium supplemented with CS (most probable number counts in culture supernatant [MPN_CS]) (3). The study was approved by the National Research Ethics Service Committee East Midlands Leicester (07/Q2501/58).

Forty-one patients were recruited before the onset of chemotherapy; 18 were culture positive for *Mtb*, one patient having two separate positive samples (Figure 1). Most patients were from Indian subcontinent backgrounds (83%), and the remainder were black African (17%); 67% were male, 17% were infected with HIV, and 82% were vitamin D deficient (cutoff, <30 nmol/L). Samples were obtained from varying anatomical locations, including colonic tissue, vertebral bone, abscess pus, and lymph nodes, and Ziehl-Neelsen stained within 24 hours after collection. No bacilli were detected using this protocol in any samples before cultivation, which fits with the typically paucibacillary nature of EPTB disease (14).

We detected substantial differences in DCTB counts and relative DCTB proportions, with bacillary counts ranging over $\log_{10}$ 0.69 to 6.7 for MPN_CS, over $\log_{10}$ 0.69 to 4.5 for MPN_7H9, and over $\log_{10}$ 1 to 3.9 for cfu counts (Figure 1). In most samples (11 of 19), the MPN_7H9 counts were lower than MPN_CS and cfu counts. In 52% (10 of 19) of samples, CS-dependent *Mtb* were dominating populations, including three samples that produced *Mtb* cultures only with CS supplementation. Interestingly, in 6 out of 19

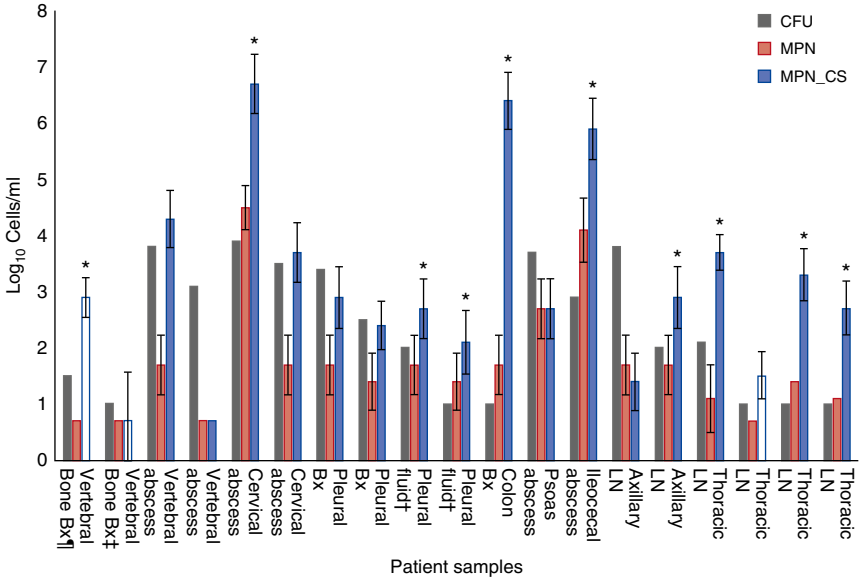

Figure 1. Mycobacterial subpopulations present in extrapulmonary tuberculosis samples. The error bars indicate the 95% confidence intervals for the most probable number (MPN) values. Owing to small sample volumes and the requirement for invasive sampling, only one cfu assay technical replicate per sample was possible. Volumes of liquid samples were in range of 1 to 10 ml. The lymph node (LN) sample sites (from left to right) are left axillary LN; left axillary LN; intrathoracic lymph node (ILN) obtained via mediastinoscopy; ILN station 7; ILN station 4R; ILN station 4R. The MPN assay limit of detection was 5 cells /ml. White bars: samples recovered only with culture supernatant (CS) supplementation. *Samples with dominating CS-dependent populations. ‡Two samples obtained from the same patient separated by 10 days. ¶The cfu assay limit was 10 cfu/ml. Bx = biopsy; cfu = colony-forming units; MPN_CS = MPN with supplementary CS; MPN_7H9 = MPN with standard 7H9 media.

samples (32%), there was no significant difference between cfu and MPN_CS counts (taking into consideration limit of confidence), and, in two samples, plateable *Mtb* exceeded those grown in liquid media.

The isolated *Mtb* strains were acid-fast positive and diverse, with eight (42%) of isolates belonging to the Delhi/Central Asian Strain lineage, four (21%) East African-Indian, two (11%) Haarlem, one (5%) Beijing, one Turkish, one S-type, and one H37Rv-like, as determined by mycobacterial interspersed repetitive units–variable number of tandem repeats typing (15). We found no relationship between MPN_CS counts and strain lineage ($P = 0.45$; Kruskal-Wallis test). Examination of host factors elucidated a significant correlation ($P = 0.04$) between the host peripheral lymphocyte counts (PLC) and MPN_CS bacillary counts. However, additional experiments are required, because multivariate regression analysis to adjust for confounding was precluded by the sample size. No other host parameters, including HIV, diabetes mellitus, or vitamin D status, were significantly associated with DCTB. Furthermore, there was also no correlation between MPN_CS counts and C-reactive protein levels, neutrophil or monocyte counts, or the monocyte:lymphocyte ratio. All patients responded appropriately to antituberculous therapy, and by the end of June 2017, 83% of patients had completed treatment according to World Health Organization definitions (16); treatment outcome for 17% of patients could not be evaluated because they returned to home countries.

CS-dependent DCTB were isolated from a wide range of anatomically and physiologically distinct sites (Figure 1), indicating that CS-dependent and Rpf-dependent bacilli are not exclusive to sputum. Rather, their formation is an important manifestation of *Mtb* and host interactions, as previously demonstrated in a mouse model (17). Whether DCTB population distributions do differ between pulmonary and extrapulmonary disease is unknown. We identified CS-dependent DCTB populations in only 52% of EPTB samples, considerably less than detected in sputum studies (3, 4), although the absence of a comparator group prevents clarification of whether a true difference exists.

The significant positive correlation demonstrated between the PLC and MPN_CS bacillary counts supports the contention that the cellular host immune response is a major factor determining population proportions of CS-dependent bacilli (4, 17). Differences in the effectiveness of individual host immune responses and consequently mycobacterial stress may explain the interhost differences in CS-dependent DCTB populations. We examined certain host factors, such as vitamin D deficiency and diabetes mellitus, but they did not influence CS dependency in our study. Although this pilot study was underpowered for multivariate analysis, these factors may impact DCTB populations and should be examined in future studies. At this stage, we also cannot conclude whether the presence of DCTB may influence treatment outcomes; additional studies focused on duration of treatment and its correlation with DCTB should be conducted.

In conclusion, we demonstrated CS-dependent DCTB are common in EPTB, their proportion potentially modulated by the host immune response. The PLC represents a candidate biomarker of host CS-dependent DCTB population burden, potentially replacing highly laborious growth assays and the need for repeat sampling, difficult in EPTB. The relationship between DCTB populations and treatment outcome should be studied. Host biomarkers may represent an easy way to measure the dynamics of populations during treatment. Immunomodulators have been previously proposed as a tool for reduction of persisters (18) and may abrogate DCTB formation. Future studies should examine whether host factor modulation can minimize DCTB generation. ■

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Andrew Rosser, M.B. B.S.
Manish Pareek, Ph.D.
University of Leicester
Leicester, United Kingdom
and
University Hospitals Leicester National Health Service Trust
Leicester, United Kingdom

Obolbek Turapov, Ph.D.
University of Leicester
Leicester, United Kingdom

Martin J. Wiselka, Ph.D.
University Hospitals Leicester National Health Service Trust
Leicester, United Kingdom

Galina V. Mukamolova, Ph.D.
University of Leicester
Leicester, United Kingdom

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Exaggerated Increase in Pulmonary Artery Pressure during Exercise in Adults Born Preterm

To the Editor:

Information on the long-term cardiopulmonary sequelae in former preterm infants now entering adulthood remains limited (1). Arrested vascularization and augmented vasoreactivity contribute to the development of pulmonary hypertension (PH) in the weeks and months after premature birth (2). PH typically resolves in those who survive to school age (3), but the long-term consequences of arrested lung development resulting from prematurity and potential development of PH in those surviving to adulthood remains completely unknown. The normal age-dependent increase in pulmonary artery pressure, coupled with an augmented pressure response during exercise and/or ascent to high altitude, may predispose preterm individuals to PH as adults. Thus, the need for identifying the long-term consequences of prematurity, including development of PH, remains important. Although the majority of PH diagnoses are based on either echocardiographic findings or right heart catheterization at rest, adults with a history of preterm birth may have an abnormal increase in pulmonary arterial pressure during exercise and/or with hypoxia despite normal resting pulmonary arterial pressure. Some results of these studies have been previously reported in abstract form (4, 5).

In this study, we examined pulmonary arterial pressure in preterm individuals, with and without bronchopulmonary dysplasia (BPD), at rest and during exercise, breathing either room air or a hypoxic gas mixture (12% O_2, balance N_2). Preterm adults with BPD (BPD; $n = 18$, 6 female, 27.3 $\pm$ 1.9 wk gestational age, 1.06 $\pm$ 0.26 kg gestational weight), adults without BPD (PRE; $n = 15$, 5 female, 28.7 $\pm$ 2.2 wk gestational age, 1.16 $\pm$ 0.43 kg gestational weight), and full-term healthy adults (CONT; $n = 20$, 7 female, $>$37 wk gestational age) 18–31 years of age volunteered for the study. On visit 1, spirometry, lung volumes, and D_{LCO} were obtained. On visit 2, cycle ergometry exercise was used to identify peak oxygen consumption ($\dot{V}O_2$peak) and power output while breathing room air. Data from visits 1 and 2 have been previously published for this cohort (6). On visit 3, subjects exercised while breathing room air for 4 minutes each at 25%, 50%, 75%, and 90% of their previously determined peak power output, with a 10-minute break between workloads. After a 60-minute break from exercise, subjects then exercised while breathing 12% O_2 for 4 minutes each at 25%, 50%, and 75% of their previously determined normoxic peak power output. One ultrasound technician with 30 years of stress echo experience made measurements of tricuspid regurgitation peak velocity to calculate pulmonary artery systolic pressure (PASP), as before (7). Right ventricular outflow tract obstruction and pulmonic valve stenosis were ruled out in all subjects. Cardiorespiratory data at rest and during exercise from visit 3 have been previously published for this cohort (6).

BPD and PRE exercised at lower absolute workloads compared with CONT, so PASP data are plotted as a function of absolute $\dot{V}O_2$ such that pulmonary blood flow is comparable across groups (Figures 1 and 2). Resting PASP was similar between all groups breathing either room air or 12% O_2 for 10 minutes (Figure 2). There was no evidence of resting PH in PRE and BPD in either normoxia or hypoxia (Figures 1 and 2). The slope relating PASP to $\dot{V}O_2$ was greater in PRE compared with BPD and CONT in normoxia and in hypoxia (Figure 2). There were no significant differences between PRE and BPD with respect to D_{LCO} at rest (percentage predicted) or $\dot{V}E/\dot{V}CO_2$ at 90% of $\dot{V}O_2$peak (data not shown, $P > 0.05$, one-way ANOVA). There were no differences between PRE and BPD with respect to need for ventilation, prenatal and/or postnatal steroids, umbilical and/or central lines, or transfusions (data not shown, Fisher's exact test, $P > 0.05$).

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