MAJOR ARTICLE

Association between the Infectivity of *Mycobacterium tuberculosis* Strains and Their Efficiency for Extrarespiratory Infection

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Extrarespiratory tuberculosis is determined mainly by impaired immunity of the host. The additional role played by bacterial factors in determining whether an infection by *Mycobacterium tuberculosis* disseminates to extrarespiratory sites has not been analyzed in depth. In the present study, we selected patients who were dually infected with 2 *M. tuberculosis* strains but in whom only one of the strains infected extrarespiratory sites, whereas the other strain remained at the respiratory site. We compared the infectivity of respiratory and extrarespiratory strains in a newly designed ex vivo competitive macrophage coinfection assay and in the murine aerosol-infection model. The extrarespiratory strains infected macrophages more efficiently than did the respiratory strains, and a representative extrarespiratory strain also showed higher infectivity in vivo. Our data indicate that, in addition to host immune status, a bacterial factor—the infectivity of a *M. tuberculosis* strain—should be considered in determining the likelihood of extrarespiratory dissemination.

Extrarespiratory tuberculosis (TB) is found in 13.8% of patients with TB at our institution (Hospital General Universitario Gregorio Marañón) and in 5%–20% of patients with TB in different countries [1, 2]. However, the factors that determine extrarespiratory TB remain elusive. Extrarespiratory disease is assumed to occur when a patient has impaired immunity that favors the extrarespiratory dissemination of *Mycobacterium tuberculosis* [2, 3], and the increase in extrarespiratory *M. tuberculosis* infection observed in patients with AIDS is consistent with this assumption [4, 5].

Until recently, the factors that determine the transmission and outcome of TB were considered to be mainly related to the host. Nevertheless, it is becoming clear that, in addition to host factors, bacterial factors are also involved in key aspects of TB [6–9]. Multiple studies have demonstrated that transmission and infection are especially efficient for certain strains [7–12]. The virulence of *M. tuberculosis* has been related to resistance to killing by hydrogen peroxide and organic peroxides and to a failure to induce interferon (IFN)–γ expression, and these relationships have been demonstrated in different strains [13, 14].

Nevertheless, extrarespiratory TB is still mainly considered to be the consequence of host factors. The potential role played by bacterial factors in determining whether an infection remains at the respiratory site or disseminates to extrarespiratory sites has received little attention [5].

In a previous report [15], we described patients who were dually infected with 2 *M. tuberculosis* strains from whom different *M. tuberculosis* strains were isolated

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from respiratory and extrarespiratory sites during the same TB episode. This finding suggests that, for 2 strains infecting the same host, the efficiency for extrarespiratory infection is not equivalent. In the present study, we compared the infectivity of *M. tuberculosis* strains isolated from respiratory and extrarespiratory sites in dually infected hosts, to explore whether bacterial factors play a role in the dissemination of *M. tuberculosis* to extrarespiratory sites.

PATIENTS, MATERIALS, AND METHODS

Patients and* M. tuberculosis *strains. For the analysis of *M. tuberculosis* strains with potential differences in their efficiency for extrarespiratory infection, we selected 3 patients who had different *M. tuberculosis* strains (confirmed by spoligotyping, double repetitive elements polymerase chain reaction, and IS*6110* restriction fragment–length polymorphism analysis) [15] isolated from respiratory (sputum and transtracheal puncture) and extrarespiratory (urine, peritoneal fluid, and lymphadenopathy) sites during the same TB episode (figure 1), a phenomenon that indicates a differing tendency for each of the coinfecting strains to infect extrarespiratory sites.

Competitive macrophage coinfection assay. The general schema of the coinfection assay was to infect macrophages simultaneously with an equivalent proportion (1:1) of 2 different *M. tuberculosis* strains and to analyze, in the same experiment, the infectivity of each strain in relation to the other. The assay was based on the assumption that if one of the coinfecting *M. tuberculosis* strains was more infective, it would compete with the other strain to infect macrophages. If competition occurred, the strain with the higher infectivity would be overrepresented in the bacterial population recovered from the macrophages after infection (analysis of lysates 24 h after infection). We also considered a strain to be highly infective when it showed a higher efficiency for replication in macrophages (analysis of lysates 7 days after infection).

Human monocyte–derived macrophages were cultured from healthy donors as described elsewhere [16] and were infected using procedures described elsewhere [10]. To prepare the coinfection inoculum, each strain was cultured separately, and bacterial suspensions were incubated in a sonicated water bath for 2 min and passed 5 times through a 23-gauge syringe, to disrupt potential aggregates (bacterial clumping was ruled out by microscopy). The bacterial concentration was adjusted to match the 0.5 McFarland turbidity standard. Then, identical volumes of each of the 2 strains to be used in the coinfection assay were mixed to obtain a 1:1 proportion.

This inoculum was added to 24-well plates (Costar) containing human monocyte-derived macrophages, with an MOI of 1 bacterium:1 macrophage (5×10^5 bacteria:5×10^5 macrophages in each well). Infection was allowed to proceed for 2 h, after which the supernatants were thoroughly washed with PBS. Independent sets of infected macrophages were lysed after 24 h and 7 days, to check for competition between the strains to either infect macrophages or replicate in them, respectively. Serial dilutions of the macrophage lysates were plated, and 50 single colonies from the postinfection lysate were genotyped, to determine the relative proportions of the 2 coinfecting strains after passage through macrophages. Deviations from the initial 50% for each of the strains indicated competition between the strains and, therefore, a greater ability of one of the strains to infect and/or replicate in macrophages.

For each experiment, to precisely determine the percentage of each strain in the coinfection inoculum, an aliquot of this inoculum was plated and 50 single colonies were spoligotyped, to determine the actual percentage in the inoculum. This determined percentage (and not the theoretical 50% for each strain that was expected in the inoculum) was used as the reference for comparison with the percentages obtained for the bacterial population recovered from the postinfection lysates.

Genotyping. Single colonies from the lysates were chosen and cultured for 21 days. Bacteria were pelleted, inactivated by heating, and sonicated. Genotyping by spoligotyping or by my-

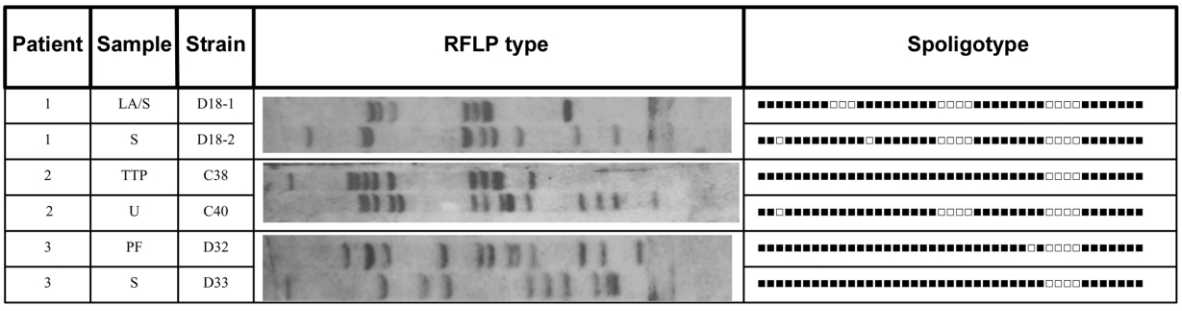

Figure 1. Restriction fragment–length polymorphism (RFLP) types and spoligotypes for the 3 patients dually infected with different *Mycobacterium tuberculosis* strains at respiratory and extrarespiratory sites. For the spoligotypes, black boxes indicate hybridization with the spacers of the directed-repeats region, and white boxes indicate the absence of hybridization. The patient nos., clinical samples, and the no. assigned to each strain are indicated. LA, lymphadenopathy; PF, peritoneal fluid; S, sputum; TTP, transtracheal puncture; U, urine.

cobacterial interspersed repetitive unit–variable-number tandem repeat typing was performed on these crude extracts, as described elsewhere [17].

Murine aerosol-infection model. *M. tuberculosis* strains were grown to midlog phase in Proskauer-Beck medium containing 0.01% Tween 80. Specific pathogen–free C57BL/6 and CB-17 SCID female mice (6–8 weeks old) were obtained from Charles River Laboratories. Airborne infection was done in a Middlebrook apparatus (Glas-Col), as described elsewhere [18]. Three different assays were performed. In the first one, 10 SCID mice were infected to determine the virulence of the different *M. tuberculosis* strains on the basis of the survival of hosts without acquired immunity. SCID mice were killed according to an end-point protocol. In this protocol, the most important parameter in the decision to kill a mouse was weight. Once a mouse lost 35% of its greatest weight, it was killed.

In the second assay, C57BL/6 mice were used to study the evolution of infection on the basis of several factors. Four mice were used for each time point in each experimental group. The numbers of viable bacteria in left lung and spleen homogenates and in bronchoalveolar lavage (BAL) fluid at weeks 3, 9, 18, and 22 were determined by plating serial dilutions and counting bacterial colony formations after 21 days of incubation at 37°C.

In the third assay, C57BL/6 mice were coinfected with respiratory and extrarespiratory *M. tuberculosis* strains by use of a procedure equivalent to that used for infection with single isolates. In this case, the proportion of each of the 2 strains in the inoculum was determined to be equivalent (1:1) by genotyping 50 colonies cultured from the inoculum. Fifty colonies were also genotyped for each time point, to assess the relative proportion of the respiratory and the extrarespiratory strain.

Histometry. The procedures used for histometry have been described elsewhere [18]. Briefly, 2 lobes from the right lung of each C57BL/6 mouse were fixed and embedded in paraffin. For histometry, 5-μm-thick sections from each specimen were stained with hematoxylin-eosin and photomicrographed. The area of each single lesion and the total tissue area on photomicrographs at each time point was determined to obtain histometric values, expressed as the percentage of granuloma-involved areas relative to global tissue areas. To obtain more-objective measurements, sections were evaluated in a blinded fashion by investigators.

Determination of immune response by mRNA quantification. The procedures and primers used for mRNA quantification have been described elsewhere [18]. Briefly, total RNA from the middle right lung lobe of each C57BL/6 mouse was extracted, and cDNA was obtained by reverse transcribing 5 μg of RNA. To evaluate the immune response after infection of mice with the *M. tuberculosis* strains, quantitative analysis of IFN-γ, tumor necrosis factor (TNF)–α, interleukin (IL)–12p40, and inducible nitric oxide synthase (iNOS) mRNAs was performed at weeks 0, 3, 9, 18, and 22, by use of a LightCycler system (Roche Biochemicals). Hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression was analyzed for every target sample, to normalize for efficiencies in cDNA synthesis and mRNA input amounts. Ratios of HPRT mRNA expression to IFN-γ, RANTES, intercellular adhesion molecule 1 (ICAM-1), TNF-α, IL-12p40, and iNOS mRNA expression was obtained for every sample.

Statistical methods. We compared the proportions of strains in macrophage cultures between baseline and 24 h after infection and between 24 h and 7 days after infection by Fisher's exact test (2-sided). Kaplan-Meier survival curves of infected mice were compared by the log-rank test (2-sided). Differences in $\log_{10}$ colony-forming-unit counts and in chemokine or cytokine expression between infected mice were assessed by the Wilcoxon rank-sum test. The relationships between strain, time, and percentage of lung infiltration were analyzed using a linear regression model that included an interaction between time and strain. All statistical analyses were performed using Stata (version 8.2; StataCorp).

RESULTS

Competitive macrophage coinfection assay. Competitive coinfection of macrophages was performed to measure and compare the infectivity of both respiratory and extrarespiratory strains. For all of the patients analyzed (figure 1), the extrarespiratory strains infected macrophages more efficiently in the coinfection assay. For patient 1, the percentage of the extrarespiratory strain, compared with the percentage in the initial coinfection inoculum (44.2%), increased significantly at both the 24-h (72.5%) and 7-day (90.2%) time points; for patient 2, the percentage of the extrarespiratory strain increased at the 24-h time point (72.1% at the 24-h time point vs. 42.9% in the coinfection inoculum); and for patient 3, the percentage of the extrarespiratory strain increased at the 7-day time point (78.4% at the 7-day time point vs. 58.1% in the coinfection inoculum) (table 1). These findings indicate that all of the *M. tuberculosis* strains that showed a tendency toward extrarespiratory dissemination correlated with a higher efficiency for invasion of macrophages, a higher efficiency for replication in macrophages, or both.

Murine aerosol-infection model. To confirm that the increased ability to infect macrophages shown by the extrarespiratory strains corresponded to a true high infectivity, we performed in vivo infection assays using an experimental murine aerosol-infection model. To compare the severities of the infections caused by the *M. tuberculosis* strains studied, we selected representative strains that, in the macrophage coinfection assay, either had demonstrated a high infectivity and a high efficiency for dissemination to extrarespiratory sites (D18-1) or had demonstrated a low infectivity and a low efficiency for dissemination to extrarespiratory sites (D18-2).

Table 1. Proportions of extrarespiratory *Mycobacterium tuberculosis* strains in the ex vivo competitive macrophage coinfection assay.

Patient, extrarespiratory/ respiratory strain	Initial coinfection inoculum	*P* (initial vs. 24 h)	24 h after infection	*P* (24 h vs. 7 days)	7 days after infection
Patient 1, D18-1/D18-2	44.2	.014	72.5	.049	90.2
Patient 2, C40/C38	42.9	.009	72.1	.490	62.8
Patient 3, D32/D33	58.1	.517	50.0	.011	78.4

NOTE. Data are percentages of the extrarespiratory strain, unless otherwise noted. Percentages are indicated for the initial coinfection inoculum and for the postinfection lysates. *P* values are by Fisher's exact test (2-tailed).

Survival was significantly lower for SCID mice infected with the extrarespiratory strain (average survival, 28.8 days), compared with that in SCID mice infected with the respiratory strain (average survival, 37.2 days) (figure 2*A*). In addition to survival in SCID mice, other factors were considered in infected C57BL/6 mice: bacterial concentrations in infected tissues, the development of granulomatous infiltration in the lungs, and expression of cytokines. The number of colony-forming units in infected lung and spleen tissue and in BAL fluid were higher for the extrarespiratory strain (figure 2*B*) at weeks 3, 9, and 18, indicating that this strain had a higher efficiency for infection of tissues and for systemic and bronchogenic dissemination. When a linear regression model was fitted to study the evolution of the respiratory infiltration by both strains, a significant interaction was found between time and strain ($P < .001$), suggesting that the extrarespiratory strain also induced quicker respiratory infiltration (figure 2*C*). Last, with regard to mRNA expression, the extrarespiratory strain induced earlier IL-12p40 and IFN-γ expression and a late increase in iNOS and TNF-α expression, reflecting the higher bacterial concentration and the final increase in the inflammatory response caused by the higher dissemination through the parenchyma [18] (figure 2*D*). RANTES and ICAM-1 expression differed little (data not shown).

To re-create in vivo the clinical situation (2 strains coinfecting the same host), a coinfection assay was performed using the same representative extrarespiratory (D18-1) and respiratory (D18-2) strains. The infectivity of the extrarespiratory strain was superior to that of the respiratory strain, and it led to strong competition between the coinfecting strains; at week 22, the extrarespiratory strain had completely displaced the respiratory strain in both lung and spleen (table 2).

These in vivo data confirmed that our macrophage coinfection assay succeeded in identifying strains with a comparatively higher infectivity. They also confirmed that there is a correlation between in vivo infectivity and a tendency toward extrarespiratory dissemination.

DISCUSSION

Before the arrival of molecular approaches to analysis, differences in TB transmission or outcome were considered to depend exclusively on host factors. It has gradually become understood that certain strains have higher transmissibility or higher infectivity [10, 11], which has gradually led to consideration of the role that bacterial factors play in TB, in addition to that of host factors. Nevertheless, the involvement of bacterial factors in the development of extrarespiratory TB has received little attention [5].

In a previous report [15], we described several patients who were dually infected with 2 genotypically different strains during the same TB episode. Interestingly, the strains showed differing behaviors in dissemination, despite the fact that they were infecting the same host and, therefore, shared equivalent clinical-immunological contexts—in these patients, one of the coinfecting strains remained at the respiratory site, whereas the other succeeded in infecting extrarespiratory sites. The assumption that coinfection and compartmentalization corresponded to a single, active TB episode was based on careful revision of the patients' clinical charts, which indicated that none of them had clinical or radiological findings that suggested a previous TB episode.

The selection of dually infected hosts with compartmentalization of the infection is the only way to guarantee that only differences in bacterial factors are involved in the differing behavior of the strains, precisely because they share the same host. The detection of patients dually infected by 2 strains and with compartmentalization of the infection is a laborious process and has been undertaken only in a small number of studies [19–21].

Our purpose here was to define whether differences in efficiency for invasion of macrophages, efficiency for replication in macrophages, and infectivity in vivo could be found between respiratory and extrarespiratory strains isolated from a single patient. First, we designed an assay to simultaneously and competitively coinfect macrophages with 2 strains. This design guarantees precision in the measurement of infectivity by circumventing biases that stem from using macrophages from different donors for each of the strains. It also allowed us to re-create the in vivo circumstances—that is, 2 *M. tuberculosis* strains coinfecting the same patient. The data we obtained from our macrophage coinfection assay indicated that all of the extrarespiratory strains studied were more efficient

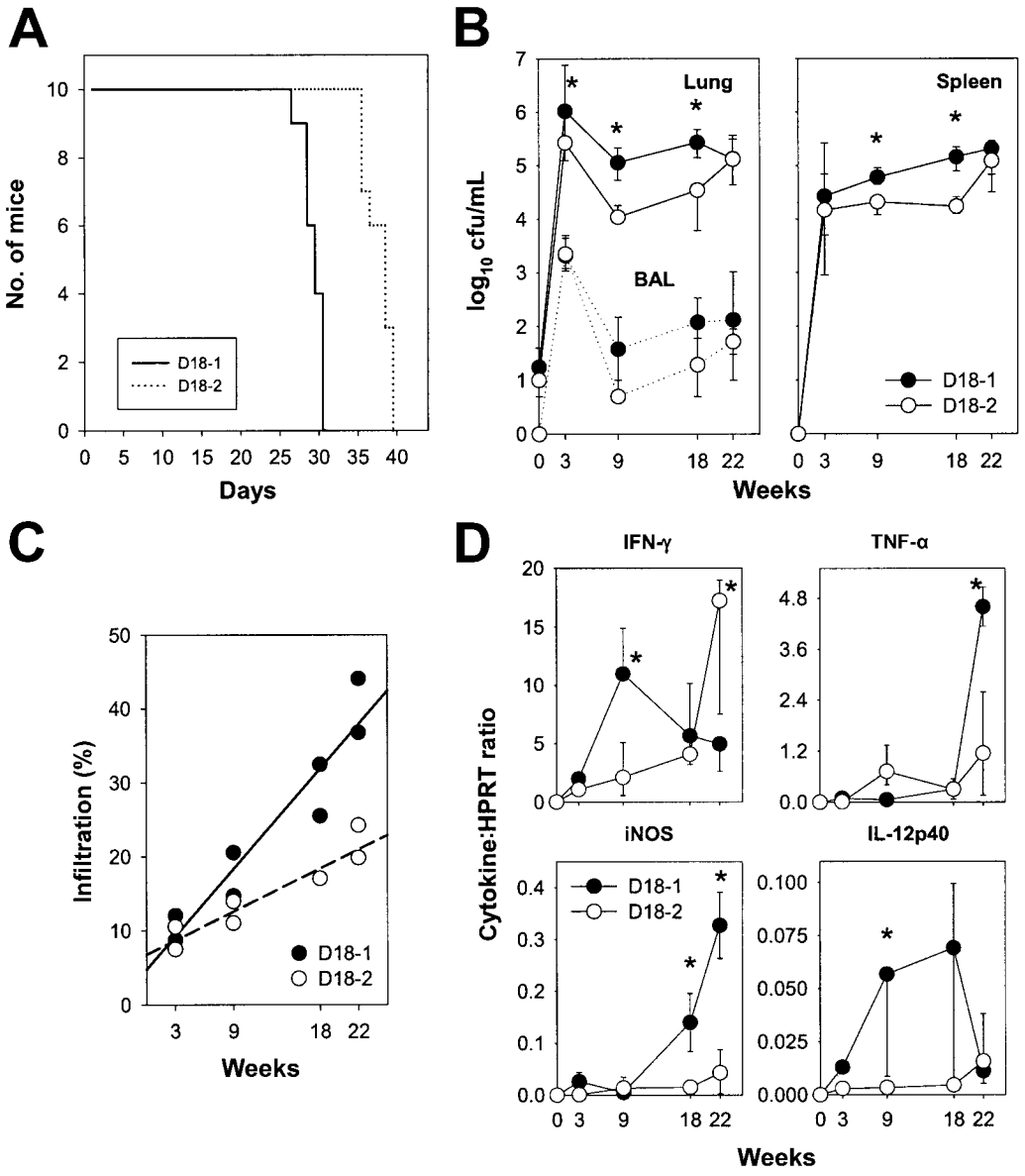

Figure 2. *A,* Survival of SCID mice (10 mice/group) infected by aerosol with either a representative extrarespiratory *Mycobacterium tuberculosis* strain (D18-1) or a representative respiratory strain (D18-2). The difference between the curves is significant ($P < .05$, log-rank test [2-tailed]). *B,* Evolution of the no. of colony-forming units in the lungs *(continuous lines, at left),* spleens *(continuous lines, at right),* and bronchoalveolar lavage (BAL) fluid *(dotted lines, at left)* of infected C57BL/6 mice. Circles show the median $\log_{10}$ colony-forming units and vertical spikes show the minimum and maximum $\log_{10}$ colony-forming units for each strain at each time point for a set of 4 mice. *C,* Evolution of granulomatous infiltration in the lungs of infected C57BL/6 mice. Data are the percentages of granuloma-involved areas relative to global tissue areas in 2 lung lobes from each mouse. Linear regression analysis showed a significant interaction between time and strain ($P < .05$). *D,* Evolution of respiratory expression of mRNA for Th1-related cytokines, tumor necrosis factor (TNF)–α, and inducible nitric oxide synthase (iNOS) in infected C57BL/6 mice. Ratios of hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA expression to that for each chemokine and cytokine for the corresponding sample are shown. Circles show the median ratios and vertical spikes show the minimum and maximum ratios at each time point for the set of mice analyzed. IFN, interferon; IL, interleukin. *$P < .05$, for the difference (Wilcoxon rank-sum test).

for invasion of macrophages or replication in them, compared with the coinfecting respiratory strains.

Second, to measure infectivity in vivo, a representative respiratory strain and a representative extrarespiratory strain were selected for use in a murine aerosol-infection model, in which the severity of the infections caused by these strains could be determined. The course of bacterial concentration in this model has been determined to be a useful tool for determining the virulence of different strains [22, 23]. Our in vivo data indicated lower survival for the mice infected with the extrarespiratory

Table 2. Proportions of a representative extrarespiratory *Mycobacterium tuberculosis* strain (D18-1) and a representative respiratory strain (D18-2) in the in vivo coinfection assay, for 2 infected mice.

Week after infection	Lung				Spleen			
	Mouse A		Mouse B		Mouse A		Mouse B	
	D18-1	D18-2	D18-1	D18-2	D18-1	D18-2	D18-1	D18-2
3	10.35	89.65	35.42	64.58	33.40	66.60	24.40	75.60
9	77.55	22.45	74.40	25.60	71.10	28.90	67.50	32.50
18	67.45	32.55	78.72	21.28	57.15	42.85	49.00	51.00
22	100	0	100	0	100	0	100	0

NOTE. Data are percentages of strains.

strain as well as a higher efficiency of the extrarespiratory strain for infection of tissue and bronchogenic dissemination. The in vivo assay was also performed using a coinfection design that would mirror the clinical situation, and the higher infectivity of the extrarespiratory strain was confirmed in a circumstance in which it infected mice together with the respiratory strain. We selected only a pair of representative strains for the in vivo infection and coinfection assays because of the great number of colonies that should be analyzed and genotyped (400 colonies for each infected mouse). We are aware that further studies using a wider set of strains should be conducted, to test the present findings obtained with representative strains.

Recently, the introduction of new parameters, such as the percentage of granulomatous infiltration in lung tissue and the level of expression of Th1-related cytokine mRNA, has provided new tools with which to better study *M. tuberculosis* strains [18]. Our data clearly showed that the higher bacterial concentration of the extrarespiratory strain induced a higher Th1 response through a higher expression of IL-12p40 and IFN-γ. The higher iNOS expression during the chronic stage of infection in the mice infected with the extrarespiratory strain is consistent with greater respiratory infiltration and, thus, with a greater presence of foamy macrophages, which are the cells that mainly express iNOS during this stage of infection [15]. The same explanation is applicable to the higher TNF-α expression at week 22, because the greater granulomatous infiltration increases the inflammatory response. Interestingly, IFN-γ expression increased significantly at week 22 in the mice infected with the respiratory strain, which could have been a reaction to the reactivation of the infection at this time. Differences in cytokine levels might be the cause or the effect of differing growth.

Apart from the description of bacterial factors involved in extrarespiratory dissemination, our study investigated another relatively unexplored issue—competition between coinfecting *M. tuberculosis* strains. We selected dually infected patients whose infections were strictly compartmentalized, with a single and different *M. tuberculosis* strain at each infected site. This suggests that there is some kind of competition between the coinfecting strains in the host, one that leads to a selection of the strain that is more "adapted" to infecting either the respiratory site or an extrarespiratory site. Our ex vivo macrophage coinfection assay showed that this competition can be observed when 2 *M. tuberculosis* strains are assayed together in the same experiment. It is possible that, apart from competition, the differing results for the strains in the ex vivo assay could also reflect the differing infectivity and growth characteristics of each strain, which definitely have consequences for dissemination or containment.

An objection could be made that the respiratory strain could be detected in mouse spleen and that this finding is disharmonious with the situation in humans, in whom the respiratory strain is confined to the respiratory site. Unfortunately, it is very difficult to infect a mouse and achieve only a local infected focus (i.e., only the lungs), with no systemic spreading. As we have stated elsewhere [24], mice are very tolerant to the presence of *M. tuberculosis,* and thus there is constant bronchial and hematological dissemination. Our experience with the experimental model has shown us that the infecting strain does not spread to the spleen only when it is avirulent (such as H37Ra or certain laboratory mutant strains).

In this sense, what is relevant is that the extrarespiratory strain was more efficient at infecting the spleen than was the respiratory strain, a finding that was supported by data from the in vivo coinfection assay, in which the extrarespiratory strain fully displaced the respiratory strain at week 22. It is surprising that the respiratory strain disappeared from the lung at week 22. The high virulence of the extrarespiratory strain probably induced a marked immune response because of the high bacterial concentration that it can achieve in infected tissue (figure 2*B*). This immune response triggered by the extrarespiratory strain could have caused the disappearance of the less-virulent coinfecting respiratory strain in both the lung and spleen, whereas the extrarespiratory strain, with its higher virulence, was able to remain.

It is remarkable that, at week 3, the respiratory strain had a higher bacterial concentration than did the more-virulent extrarespiratory strain. Nevertheless, after week 3, the percentage of the extrarespiratory strain began to increase, and its repli-

cation efficiency clearly exceeded that of the respiratory strain. This might suggest that the advantage of the extrarespiratory strain is observed only after the initiation of the immune response in mice (i.e., after week 3), because the extrarespiratory strain more efficiently resists the stress that infection generates on the host.

To the best of our knowledge, our study is the first to present several clinical cases in which extrarespiratory infection with *M. tuberculosis* seems to depend on bacterial factors. Strains with a high infectivity corresponded with those with a high efficiency for infection of extrarespiratory sites. This finding encourages further exploration of the bacterial factors involved in the extrarespiratory dissemination of *M. tuberculosis,* to evaluate whether our findings for representative strains can be confirmed using a wider set of strains. Further studies would clarify whether molecular genotyping could be used to target strains with a greater tendency to disseminate to extrarespiratory tissues, in the same way that we are now targeting strains with greater transmissibility or virulence. These may be the first steps toward allowing us to predict the likelihood of extrarespiratory *M. tuberculosis* infection on the basis of the genetic and microbiological features of the *M. tuberculosis* strains involved.

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