

Expression levels of *Mycobacterium tuberculosis* antigen-encoding genes versus production levels of antigen-specific T cells during stationary level lung infection in mice

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Introduction

Tuberculosis is a major world disease that kills over 2 million people annually.¹ Although the disease can be successfully treated with chemotherapy, the increasing incidence of cases caused by multidrug-resistant strains of *Mycobacterium tuberculosis* underscores the need for an effective vaccine. Therefore, numerous laboratories are currently engaged in developing a vaccine that is more effective than the currently available vaccine, bacillus Calmette–Guérin (BCG), an attenuated strain of *Mycobacterium bovis*. Needless to say, the rational design of a more efficacious vaccine will be based on knowledge of the *M. tuberculosis* antigens against which immunity

Summary

Mycobacterium tuberculosis lung infection in mice was controlled at an approximately stationary level after 20 days of log linear growth. Onset of stationary level infection was associated with the generation by the host of T helper type 1 (Th1) immunity, as evidenced by the accumulation of CD4 Th1 cells specific for the early secretory antigen (ESAT-6) of *M. tuberculosis* encoded by *esat6*, and for a mycolyl transferase (Ag85B) encoded by *fbpB*. CD4 T cells specific for these antigens were maintained at relatively high numbers throughout the course of infection. The number of CD4 T cells generated against ESAT-6 was larger than the number generated against Ag85B, and this was associated with a higher transcription level of *esat6*. The total number of transcripts of *esat6* increased during the first 15 days of infection, after which it decreased and then approximately stabilized at $10^{6.5}$ per lung. The total number of *fbpB* transcripts increased for 20 days of infection before decreasing and then approximately stabilizing at $10^{4.8}$ per lung. The number of transcripts of *esat6* per colony-forming unit of *M. tuberculosis* fell from 8.6 to 0.8 after day 15, and of *fbpB* from 0.3 to less than 0.02 after day 10, suggesting that at any given time during stationary level infection the latter gene was expressed by a very small percentage of bacilli. Expressed at an even lower level was an *M. tuberculosis* replication gene involved in septum formation (*ftsZ*), indicating that there was no significant turnover of the *M. tuberculosis* population during stationary level infection.

Keywords: antigen-encoding genes; *Mycobacterium tuberculosis*; T cells

needs to be generated and of the level of immunity that is required to cause *M. tuberculosis* infection to resolve. It is known that immunity to tuberculosis is mediated by CD4 T helper type 1 (Th1) cells with the aid of CD8 T cells.^{2,3} It is also known that Th1 CD4 cells are generated against a number of different *M. tuberculosis* antigens of which the early secretory antigen target protein (ESAT-6) encoded by *esat6* (Rv3875), and a mycolyl transferase (Ag85B) encoded by *fbpB* (Rv1886c) are conspicuous examples.^{4,5} It has been shown^{4,5} that mice vaccinated with plasmid DNA expressing ESAT-6 or Ag85B show increased resistance to an *M. tuberculosis* challenge infection. ESAT-6 is considered a dominant antigen because a large proportion of memory T cells acquired by mice

Abbreviations: Ag85B, antigen 85B; APC, antigen-presenting cell; BCG, bacillus Calmette–Guérin; CFU, colony-forming unit; DEPC, diethylpyrocarbonate; ESAT-6, early secretory antigen-6; FCS, fetal calf serum; IFN- γ , interferon- γ ; mAb, monoclonal antibody; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction.

cured of *M. tuberculosis* infection⁶ are specific for a major epitope of this antigen. However, the extent to which ESAT-6-specific T cells might dominate over those specific for Ag85B during the primary immune response to infection appears not to have been formally investigated. Neither has the reason for the antigenic dominance of ESAT-6.

This paper shows that during the generation and maintenance of immunity to airborne *M. tuberculosis* infection in mice, Th1 cells specific for ESAT-6 accumulate and persist in the lungs in larger number than Th1 cells specific for Ag85B. It shows, in addition, that the antigenic dominance of ESAT-6 over Ag85B is associated with a higher level of *esat6* transcription. It also shows that stationary level infection is associated with a very low level of transcription of an *M. tuberculosis* replication gene, *ftsZ*,⁷ indicating that stationary level infection is not associated with a significant turnover of the *M. tuberculosis* population.

Materials and methods

Mice

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and were used in experiments at 12 weeks of age. Infected mice were housed under barrier conditions in a Level III Biosafety Animal Facility, according to guidelines formulated by the Trudeau Institute Animal Care and Use Committee.

Infection

The H37Rv strain of *M. tuberculosis* (Trudeau Mycobacterial Culture Collection no.102) was grown as a suspension culture in Proskauer and Beck medium containing 0.01% Tween-80, and harvested while in log-phase growth, as described previously.⁸ The culture was subjected to two 5-second bursts of ultrasound to break up clumps and diluted appropriately in phosphate-buffered saline (PBS) containing 0.01% Tween-80 for infection via the respiratory route in an aerosol infection chamber, as described previously.⁸ Infection was monitored by measuring changes against time in the total number of *M. tuberculosis* colony-forming units (CFU) in the lungs. This involved killing five mice at the times indicated, homogenizing their lungs, plating serial dilutions of lung homogenates on enriched Middlebrook 7H11 agar, and counting colonies after incubating the plates for 21 days at 37°.

Mycobacterium tuberculosis antigens

The *M. tuberculosis* antigens used to stimulate lung cells to produce interferon- γ (IFN- γ) in the Elispot assay were ESAT-6 (1–20) peptide⁶ and Ag85B (240–260) peptide⁹.

The peptides were purchased from New England Peptide, Fitchburg, MA. ESAT-6 is an early-secreted *M. tuberculosis* protein whereas Ag85B is a member of a family of mycolyl transferases involved in cell wall lipid biosynthesis.¹⁰

Lung cell preparation

Lung cells were harvested as previously described.⁸ Briefly, mice were killed by cervical dislocation, and their lungs were perfused via the right ventricle with PBS containing 10 U/ml of heparin to remove most intravascular leucocytes. The lungs were then perfused with an enzyme cocktail consisting of 150 U/ml of collagenase, 0.2 U/ml of elastase (Roche Applied Science, Indianapolis, IN) and 1 mg/ml of DNase (Sigma, St Louis, MO) in RPMI-1640. The lungs were removed, placed in a dish, diced into small fragments, and the fragments were incubated in the enzyme mixture at 37° for 1 hr. The preparation was then passed through a 60-mesh per square inch stainless screen, and the resulting suspension was triturated with a pipette to break up aggregates. The cells were pelleted and resuspended in red cell lysis buffer (Sigma). They were then washed, passed through a 70- μ m nylon cell strainer (BD Falcon, San Jose, CA), pelleted and resuspended in RPMI-1640 containing 10% fetal calf serum (RPMI-FCS) for counting and analysis.

Elispot

Changes in numbers of *M. tuberculosis*-specific cells in the lungs against time of infection were determined by enumerating changes in the numbers of cells capable of making IFN- γ in response to *M. tuberculosis* antigens in an 18-hr Elispot assay. This was performed with a commercially available Elispot kit (Mouse IFN- γ ELISPOT Set, BD Biosciences, San Jose, CA) according to the manufacturer's instructions using pooled cells from four mice, as described previously.⁸ To supplement the antigen-presenting cells (APC) already present in the lung cell suspension bone marrow-derived dendritic cells prepared as previously described⁸ were added to the cell suspension at a ratio of 5 : 1 lung cells to dendritic cells. Two-fold serial dilutions of the admixture (100 μ l) were added in triplicate to the wells starting at 10⁵ lung cells per well. The wells then received 100 μ l of RPMI-FCS containing no antigen, 2 μ g of ESAT-6 (1–20) peptide, or 2 μ g of Ag85B (240–260) peptide. The number of cells specific for each antigen preparation was calculated by subtracting the number of spots that formed in the absence of added antigen from the number that formed in its presence. The experiment was repeated three times. To determine whether cells that made IFN- γ in the assay were CD4⁺, the lung cell suspension was selectively depleted of CD4 cells magnetically after reacting it with biotin-conjugated

anti-CD4 rat anti-mouse monoclonal antibody (BD Biosciences) and then with streptavidin-coupled magnetic nanoparticles (IMag Streptavidin Particles, BD BioSciences) according to the manufacturer's instructions, as described previously.⁸

Quantification of bacterial gene expression in infected lungs by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Lungs from four or five mice were harvested at progressive times of infection, and snap frozen in liquid nitrogen. To extract total RNA, *M. tuberculosis*-infected lungs were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA) using glass tubes and close-fitting pestles. The homogenized tissue was centrifuged for 5 min at 2000 g and supernatant and pellet were separated. Host lung RNA was extracted from the supernatant, whereas *M. tuberculosis* RNA was extracted from the pellet. The pellet from each whole lung was resuspended in 2 ml Tri reagent (MRC, Cincinnati, OH) in the presence of 20 µl polyacryl carrier molecules (MRC, Cincinnati, OH). The resuspended pellet was subjected to 30–40 s of ultrasound generated by a Braun-Sonic 1510 sonicator set at 400 watts, followed by three cycles of freezing and thawing (liquid nitrogen/warm water) to break open the bacterial cell wall. Following the addition of 0.1 ml of 1-bromo-3-chloropropane (MRC, Cincinnati, OH) samples were centrifuged at 10 000 g at 4° for 15 min for phase separation. The RNA partitioned into the upper aqueous phase and was transferred to a new tube where it was mixed with an equal volume of isopropanol. Following centrifugation the *M. tuberculosis* RNA pellet was washed with 75% ethanol and dissolved in diethylpyrocarbonate (DEPC)-treated distilled water. To remove any contaminating bacterial genomic DNA, RNA samples were digested with RNase-free DNase I (Ambion, Austin, TX) for 1 hr at 37°. RNA samples were then purified on RNeasy mini columns (Qiagen, Valencia, CA), and treated further with DNase I using the Ambion DNA-free kit reagents (Ambion, Austin, TX) to remove any remaining traces of DNA. RNA samples were stored at –70°.

TaqMan primers and probes for *esat6*, *fbpB* and *ftsZ*, were designed with Primer Express Software (PE Biosystems, Foster City, CA). The primers were purchased from Integrated DNA Technologies (Coralville, IA), and probes containing a fluorescent reporter dye (6-carboxy-fluorescein at the 5' end) and a quencher (Black Hole Quencher 1, at the 3' end) were purchased from Biosearch Technologies (Novato, CA). The oligonucleotide sequences for the forward primer, reverse primer, and probe, respectively, were as follows: *esat6*: GTACCAGGGTGTCCAGCA AAA, CTGCAGCGGTTGTTTCAG, and GGGACGCCAC GGCTACCG; *fbpB*: CCTGCGGTTTATCTGCTCGA, TG TAGAAGCTGGACTGCCCG, and AACACCCCGGCGTT

CGAGTGGTACT; and *ftsZ*: GGTTGCTGCAGATGGGA GAT, GGCGAAGTCGACGTTGATTAG, and CTGATGG ATGCTTTCCGTAGCGCC.

Amplicons for each gene were generated by PCR from *M. tuberculosis* RNA using the gene-specific primers described above and were initially analysed by agarose gel electrophoresis to confirm their size. Amplicons were then purified using the WIZARD SV Gel and PCR Clean-up System (Promega, Madison, WI), cloned into the pPCR-Script Amp vector (Stratagene, La Jolla, CA) and their sequences were verified by thermocycler sequencing using a Beckman Coulter CEQ 8000 Genetic Analysis System.

To generate RNA standards for Real-Time RT-PCR analysis, a separate set of purified amplicons containing the T7 phage promoter sequence (incorporated into the forward primer) were used as templates for *in vitro* transcription using the T7-MEGAscript kit (Ambion, Austin, TX). The PCR transcription templates were then removed by digestion with DNase I, and the RNA was purified using RNeasy mini columns followed by further treatment with the DNA-free kit (Ambion, Austin TX) and quantified with the RiboGreen (Molecular Probes, Eugene, OR) assay or by absorbance measurements at 260 nm. To obtain a standard curve, serial dilutions of each transcript were performed to give dilutions ranging from 10⁸ to 10¹ molecules. The dilutions were then subjected to real-time RT-PCR analysis as described below.

For real-time RT-PCR, 0.5–1.0 µg of *M. tuberculosis* RNA was reverse transcribed by using the gene-specific reverse primers described above, and a C. therm. (Carboxythermus hydrogenofomans) polymerase two step RT-PCR kit (Roche, Mannheim, Germany), or the ThermoScript kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR to enumerate *M. tuberculosis* *esat6*, *fbpB* and *ftsZ* amplicons was performed in an ABI-prism 7700 instrument. PCR amplification was performed in a total of 25 µl containing 10 µl cDNA sample, 2.5 µl 10× Taqman Buffer A, 3–9 mM MgCl₂, 200 µM each of dATP, dCTP and dGTP, 400 µM dUTP, 0.1–0.3 µM of each primer, 0.625 U AmpliTaq Gold and 0.25 U AmpErase Uracil N-glycosylase (Perkin Elmer/Applied Biosystems, Foster City, CA). The reaction also contained 0.2 µM of detection probe. Amplification was performed under the following conditions: 2 min at 50° and 10 min at 94° followed by a total of 40 two-temperature cycles (15 s at 94° and 1 min at 60°). The copy number in each sample was calculated according to the formula $N = (Ct - b)/m$, where N is copy number, Ct is the threshold cycle, b is the y -intercept and m is the slope of the standard curve line. Copy number data for *esat6* and *ftsZ* were obtained following analysis of replicate samples from four separate experiments, while *fbpB* data were collected from two of these experiments.

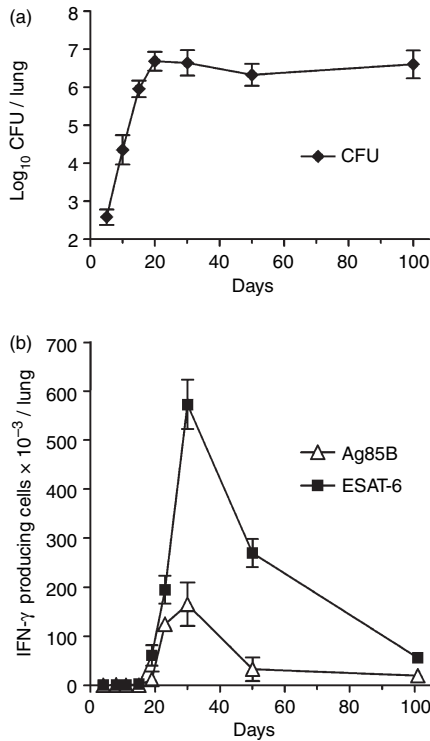


Figure 1. Course of *M. tuberculosis* infection (a) in the lungs of mice infected with approximately 10^2 CFU of *M. tuberculosis* via the airborne route. The *M. tuberculosis* grew progressively for approximately 20 days before further growth was inhibited and the level of infection was stabilized by acquired immunity. The means \pm SD of five mice are shown per time-point. Kinetics of development of acquired immunity (b) in terms of the accumulation in the lungs of T cells capable of making IFN- γ in response to ESAT-6 (1–20) peptide, or Ag85B (240–260) peptide in an 18-hr Elispot assay. The means \pm SD of the number of spots in triplicate wells using pooled cells of four mice are shown per time-point. Identical results were obtained in two separate experiments.

Results

Course of lung infection and generation of ESAT-6- and Ag85B-specific Th1 cells

The progression of lung infection was monitored by enumerating changes against time in the number of *M. tuberculosis* CFU, whereas the host response to lung infection was monitored in a separate group of mice by enumerating changes in the number of *M. tuberculosis*-specific T cells. Figure 1 shows, in agreement with previously published results,⁸ that infection with approximately 10^2 *M. tuberculosis* CFU via the respiratory route resulted in 20 days of log linear *M. tuberculosis* growth in the lungs, after which further growth was inhibited and infection was held at an approximately stationary level of 5×10^6 CFU. Figure 1 shows, in addition, that inhibition of *M. tuberculosis* growth was associated with the accumulation in the lungs of *M. tuberculosis*-specific

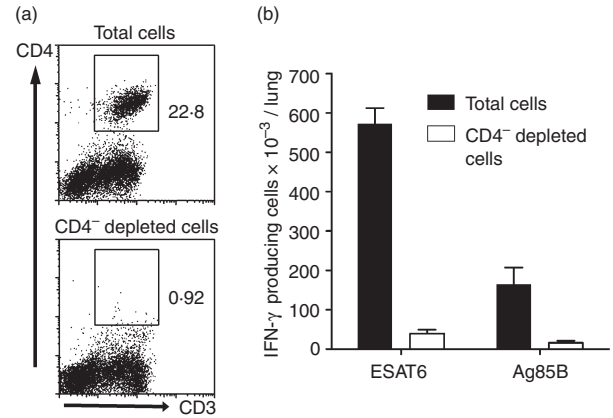


Figure 2. Evidence that cells that made IFN- γ in response to ESAT-6 (1–20) or Ag85B (240–260) peptide as measured with the Elispot assay were CD4 T cells. Flow cytometric analysis (a) showing that magnetic depletion of lung cells treated with biotin-conjugated, anti-CD4 monoclonal antibody and then with streptavidin-coupled magnetic nanoparticles was approximately 95% successful at removing CD4 cells. Depletion of CD4 T cells (b) resulted in loss (open bars) of most cells capable of making IFN- γ (closed bars) in response to ESAT-6 and Ag85B peptide.

T cells, enumerated according to their ability to secrete IFN- γ in response to exposure to ESAT-6 (1–20) peptide, or Ag85B (240–260) peptide in the Elispot assay. Antigen-specific cells began to accumulate in the lungs between days 15 and 18 of infection, reached peak numbers on day 30, and then gradually declined in number until termination of the experiment. At peak accumulation there were 5.7×10^5 ESAT-6-specific cells, compared to 1.65×10^5 Ag85B-specific T cells. Although antigen-specific T cells declined in number after day 30, they remained present in appreciable numbers until day 100 when the experiment was terminated. These results were obtained in two separate experiments and are in close agreement with results that were published earlier.⁸

Most antigen-specific T cells that made IFN- γ in the Elispot assay were CD4-positive, as evidenced by more than a 90% reduction in their number (Fig. 2) after selectively depleting the lung cell suspension of CD4 cells.

Levels of transcription of *esat6*, *fbpB* and *ftsZ* during the course of infection

The foregoing results show that according to the number of ESAT-6- and Ag85B-specific T cells that accumulated in the lungs, ESAT-6 was the more dominant antigen. To determine whether antigenic dominance was associated with a higher level of *esat6* transcription, real-time RT-PCR was employed to determine changes in the copy number of transcripts of each antigen-encoding gene during the course of infection. In addition, changes in the number of transcripts of *ftsZ*, a gene required for

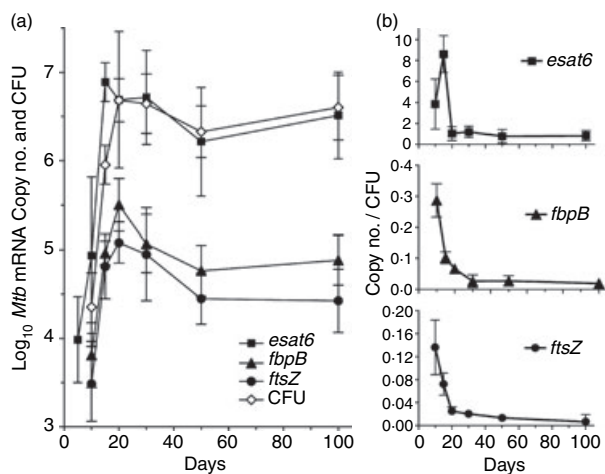


Figure 3. Changes against time of infection (a) in the \log_{10} copy number per lung of mRNA for *esat6*, *fbpB* and *ftsZ*, as quantified by real-time RT-PCR. Changes in the \log_{10} CFU of *M. tuberculosis* are also shown. The number of *esat6* transcripts increased for the first 15 days of infection, and of *fbpB* and *ftsZ* for the first 20 days, after which transcript numbers declined briefly and then approximately stabilized. When transcripts of *esat6*, *fbpB* and *ftsZ* were expressed as copies per CFU of *M. tuberculosis* (b) the number of *esat6* transcripts declined from 8.6 to 0.8 per CFU, of *fbpB* from 0.3 to 0.02 per CFU, and of *ftsZ* from 0.14 to 0.007. Therefore, most *M. tuberculosis* were not transcribing the latter two genes at any one time of infection, particularly after infection became stationary. Means \pm SD for *M. tuberculosis* RNA from lungs of four or five mice per time-point.

M. tuberculosis division, were monitored. The results show (Fig. 3a) that the transcription levels per lung of *fbpB* and *ftsZ* increased in concert with growth of *M. tuberculosis* during the first 20 days or so of infection, after which transcription levels declined before approximately stabilizing at constant levels of $10^{4.8}$ and $10^{4.4}$, respectively. The transcription level of *esat6* peaked on day 15 before declining and then stabilizing at a constant level of $10^{6.5}$. Therefore, number of transcripts of *esat6* per lung was 50-fold higher than the number of transcripts of *fbpB* during the course of stationary level infection.

Reduction and stabilization of the number of transcripts of each gene per lung was associated with a drop (Fig. 3b) in the number of transcripts per CFU of *M. tuberculosis*. Thus after day 15 the number of transcripts of *esat6* per CFU fell from 8.6 to 0.8, while after day 10 the number of transcripts of *fbpB* fell from 0.3 to 0.02, and of *ftsZ* from 0.14 to 0.007. Thus the number of transcripts of *fbpB* declined to approximately 1 per 50 CFU, and of *ftsZ* to approximately 1 per 150 CFU, meaning that at any one time during stationary level infection these genes were not being transcribed by most bacilli.

Discussion

The results of this study show that inhibition of *Mtb* growth in the lungs of mice at day 20 of an airborne

infection was associated with the accumulation in the lungs of CD4 T cells specific for *M. tuberculosis* antigens, as enumerated with the IFN- γ Elispot assay. CD4 T cells began accumulating on days 15–18 of infection, and reached peak number on day 30, at which time the number of T cells specific for ESAT-6 (5.7×10^5) was three- or fourfold more than the number specific for Ag85B (1.65×10^5). Therefore, according to the number of T cells generated, ESAT-6 was dominant as an antigen over Ag85B, a finding in keeping with the conclusion of Andersen and colleagues⁶ that ESAT-6-specific memory T cells are present in dominant numbers in the spleens of mice cured of a primary *M. tuberculosis* infection. After day 30 the number of T cells specific for each antigen slowly declined, but nevertheless remained relatively high until the experiment was terminated at day 100. Because it is known that antigen-specific CD4 cells in the lungs during stationary level infection belong to a replicating population,¹² are produced continuously in absence of the thymus¹² and have an activation phenotype,^{8,12} it is reasonable to assume that the majority of them were generated continuously in the lungs in response to *M. tuberculosis* antigens at sites of infection. It is almost certain, however, that the antecedents of these T cells were generated in draining lymph nodes, and that they reach the lungs after entering the blood via efferent lymph. The interpretation that activated, *M. tuberculosis*-specific CD4 Th1 cells are responsible for inhibiting the growth of *M. tuberculosis* and for stabilizing infection at a stationary level is based on the knowledge that *M. tuberculosis* growth is not inhibited in mice incapable of generating CD4-mediated Th1 immunity^{13,14} and that depleting mice of CD4 T cells during stationary level infection results in a resumption of *M. tuberculosis* growth.¹⁵ Given the evidence that the retention of activated, antigen-specific CD4⁺ T cells requires the continuous presence of antigen,¹⁶ the maintenance of Th1 immunity in an active state almost certainly required the continuous presentation of *M. tuberculosis* antigens during stationary level infection.

According to this study, ESAT-6- and Ag85B-encoding genes were each transcribed at an approximately constant level during the course of stationary level infection. Therefore, it seems reasonable to conclude that these antigens were synthesized at a constant level by the *M. tuberculosis* lung population, and were continuously processed and presented to CD4 T cells by infected APCs. It also seems likely that macrophages were the key APCs involved, because it is within these cells that *M. tuberculosis* almost exclusively resides during infection. The finding that *esat6* was transcribed at a 50-fold higher level than *fbpB* would serve to explain why ESAT-6 is a more dominant antigen than Ag85B. However, the reason for the antigenic dominance of ESAT-6 is more complex than this, because the much smaller

number of transcripts of *fbpB* than of *esat6* per CFU of *M. tuberculosis* means that a much smaller number of macrophages may have been processing and presenting Ag85B. Thus during stationary level infection the number of transcripts of *fbpB* was less than 1 per 50 bacilli, whereas the number of transcripts of *esat6* was approximately one per bacillus. This assumes that mRNA for each gene was present at similar steady-state levels in all bacilli that made up the *M. tuberculosis* population in the lung. Low average steady-state levels of mRNA for protein-encoding genes are a common finding in bacteria.^{17–20} If it is assumed that during stationary infection each macrophage contained 50 bacilli, then, at any one time only one bacillus per macrophage would have been transcribing *fbpB*, whereas essentially all bacilli per macrophage would have been transcribing *esat6*. Under these conditions ESAT-6 would probably be able to out-compete Ag85B for processing and presentation within the same APC. On the other hand, if Mtb gene expression follows stochastic mechanisms^{21–24} then transcripts of both antigen-encoding genes could have been present at more than one copy per bacillus. In this case, all infected macrophages would still have contained some ESAT-6-transcribing bacilli, whereas only a small percentage would have contained bacilli transcribing *fbpB*. Under these conditions ESAT-6 would dominate as an antigen because of its presence in a larger number of APCs. If antigenic dominance were a function of the larger number of macrophages presenting ESAT-6, rather than to a larger number of *M. tuberculosis* bacilli making this antigen, it would serve to explain why a 50-fold higher level of transcription of *esat6* than *fbpB* per lung was associated with only a three- to fourfold larger number of ESAT-6-specific T cells.

It should be remembered that the onset of stationary level lung infection results from inhibition of *M. tuberculosis* growth by host macrophages, and that this is associated with the acquisition of a 'dormancy' transcriptome on the part of *M. tuberculosis* that involves induction of *dosR* (*Rv3133c*) and numerous other genes of the *dosR* regulon,²⁵ including *Rv2626c*, a gene of unknown function, and *Rv2031c*, the alpha-crystallin gene (*acr*), both of which are expressed at high levels. A previous study in this laboratory¹¹ showed that the expression of Th1 immunity against airborne *M. tuberculosis* infection in B6 mice is associated with a significant increase in the level of expression of *Rv2626c* and *acr* per CFU of *M. tuberculosis*. The acquisition of a dormancy transcriptome by *M. tuberculosis* occurs in response to a transcriptional change on the part of infected macrophages²⁶ that results in them acquiring the ability to synthesize inducible nitric oxide synthase and to generate nitric oxide that can act as a signal for *M. tuberculosis* to undergo its protective dormancy transcriptional response.²⁷ As shown here, inhibition of *M. tuberculosis*

growth and its persistence in the lungs in an apparent non-replicating state was associated with an approximately 11-fold decrease in the copy number of *esat6* transcripts per CFU, a 15-fold reduction in the number of *fbpB* transcripts, and a 20-fold reduction in transcript number of *ftsZ*. The function of ESAT-6 is not known but is believed to be essential for *M. tuberculosis* pathogenicity,^{28–30} whereas antigen Ag85B, being a mycolyl transferase, is involved in cell wall lipid biosynthesis.¹⁰ Therefore, synthesis of Ag85B would be needed for *M. tuberculosis* replication. FtsZ is a protein essential for bacterial septum formation^{7,31,32} and therefore for *M. tuberculosis* division. According to this study there were about 5×10^4 transcripts of *ftsZ* per lung and therefore per 5×10^6 CFU of *M. tuberculosis* during stationary level infection. Thus only 5×10^4 bacilli (1% of the population) could each have contained one transcript of *ftsZ*, or only 5×10^3 bacilli could each have contained 10 transcripts. In any case, the very small number of transcripts of this gene per total *M. tuberculosis* population argues against the notion that the *M. tuberculosis* population was turning over to a significant extent during stationary level infection. Thus stationary level infection was not the result of equilibrium between bacterial destruction and growth, but was the result of almost all bacilli being in a state of non-replicating persistence.^{11,33} This would be in keeping with the conclusions recently reached by others,³⁴ who showed that in spite of the resistance of DNA in dead bacilli to degradation, stationary lung infection in mice is associated with the presence of a stationary number of chromosome equivalents of *M. tuberculosis* DNA.

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