Tryptophan Biosynthesis Protects Mycobacteria from CD4 T-Cell-Mediated Killing


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SUMMARY

Bacteria that cause disease rely on their ability to counteract and overcome host defenses. Here, we present a genome-scale study of Mycobacterium tuberculosis (Mtb) that uncovers the bacterial determinants of surviving host immunity, sets of genes we term “counteractomes.” Through this analysis, we found that CD4 T cells attempt to contain Mtb growth by starving it of tryptophan—a mechanism that successfully limits infections by Chlamydia and Leishmania, natural tryptophan auxotrophs. Mtb, however, can synthesize tryptophan under stress conditions, and thus, starvation fails as an Mtb-killing mechanism. We then identify a small-molecule inhibitor of Mtb tryptophan synthesis, which converts Mtb into a tryptophan auxotroph and restores the efficacy of a failed host defense. Together, our findings demonstrate that the Mtb immune counteractomes serve as probes of host immunity, uncovering immune-mediated stresses that can be leveraged for therapeutic discovery.

INTRODUCTION

Mycobacterium tuberculosis (Mtb), the etiologic agent of tuberculosis (TB), remains one of the world’s major bacterial pathogens. After a century-long decline, the last few decades have seen a resurgence in TB, with an estimated 2 billion people infected and about 1.7 million deaths per year (WHO, 2011). The success of Mtb as a pathogen lies in its adaptation to the human host and its capacity to counteract the many arms of antibacterial immunity (Ernst, 2012). Its ability to survive host defenses is directly responsible for the large reservoir of infected people, whereas its ability to subvert bactericidal mechanisms allows it to replicate in vivo and cause disease (Ehrt and Schnappinger, 2009). Elucidating these mechanisms will help us to understand the complex host-pathogen interface, and targeting these mechanisms is an underutilized therapeutic strategy that can help patients’ immune systems kill Mtb.

In the multifaceted immune response against Mtb, CD4 T cells make up one of the most biologically and epidemiologically important compartments. Humans and mice generally cannot clear Mtb during infection, but both are able to limit bacterial growth, and in the case of immunocompetent humans, prevent disease (Ernst, 2012; Flynn, 2006). This response is dependent on CD4 T cells. The CD4-deficient MHC class II knockout (KO) mice, and other mice that lack CD4 T cells, cannot stop mycobacterial growth and rapidly succumb to disease and death (Cosgrove et al., 1991; Grusby et al., 1991; Mogues et al., 2001; Scanga et al., 2000). In human disease, progressive CD4 T cell loss due to HIV infection also increases the risk for TB disease and death (Pawlowski et al., 2012; Selwyn et al., 1989).

However, crucial as they are for TB immunity, CD4 T cells ultimately fail to sterilize infection. The surviving bacteria remain latent, with the potential to cause disease in the future (Ernst, 2012; Flynn, 2006). The nature of the environment imposed by CD4 T cells, enough to limit growth but not kill Mtb, is not well studied. Reports have shown that the Th1 subset is especially effective in limiting Mtb growth, and cytokines such as IFN-γ and TNF-α are needed in some, but not all, models of CD4 T cell-mediated defenses (Bold et al., 2011; Flynn et al., 1993, 1995; Gallegos et al., 2011; Nandi and Behar, 2011; Scanga et al., 2000). But the exact nature of CD4-mediated stress—the repertoire of antipathogen effectors induced by CD4 T cells—is poorly understood. Knowing how CD4 T cells attempt to kill Mtb and how Mtb survives could identify Mtb vulnerabilities and aid drug discovery efforts.

Here, we profile the mycobacterial genetic requirements for surviving the CD4 response, the CD4 “counteractome.” We compare this with relevant in vitro survival signatures such as acid, oxidative stress, and nutrient starvation, creating...
counteractomes for physical stresses encountered in the host. Using these, we focus on a particular bacterial pathway, tryptophan (Trp) synthesis, and find the host mechanism for inducing amino acid starvation in intracellular mycobacteria. We go on to find a small molecule that targets Trp synthesis, determine the structural and biochemical basis of its activity, and show that it acts together with the host to kill Mtb both in vitro and during a model infection. Together, our findings demonstrate the utility of profiling the pathogen response to host-mediated stresses. We characterize the stresses induced by CD4 T cells and leverage one stress, Trp starvation, to synergize with a small molecule to more effectively treat TB.

RESULTS

Genes Required for Survival during Infection: Virulence Factors

In order to define the set of Mtb genes required for surviving the CD4 T cell response, we infected both wild-type (WT) and MHC class II KO (MHCII−/−) mice with a library of Mtb transposon mutants (Zhang et al., 2012). We injected 10⁶ bacteria intravenously and plated surviving bacteria from infected spleens at 10 days and 45 days after infection (Figure 1A). To identify mutant Mtb in the surviving pools, we deep sequenced transposon junctions to map the insertion site of each mutant. For each time point, we made two comparisons. First, we compared the surviving pool of mutants from WT mice (WT output library) to the inoculating pool (input library), defining genes with a statistically validated decrease in output library as required for growth during infection, commonly known as virulence factors (Figure 1A). Second, we compared the output library from WT mice to the output library from MHCII−/− mice (Figure 1A). In effect, we screened for Mtb mutants with in vivo growth defects that were rescued in the absence of CD4 T cells. We reasoned that genes required for surviving the CD4 T cell response would be required for growth in WT mice, but not required for growth in MHCII−/− mice, which lack CD4 T cells (Figure 1A). Because these genes are responsible for counteracting the effects of CD4 T cells, we refer to this set of genes as Mtb’s CD4 counteractome.

We found 576 genes that were required for growth during infection (Figures 1B and 1C; see Figure S1A available online). These genes had a statistically significant (false discovery rate [FDR], q < 0.01) 10-fold or more decrease in insertion counts across the gene. On day 10, 405 genes were required for growth in vivo, and on day 45, 317 genes were required. A total of 146 genes were required at both time points (Figures 1B and 1C; Table S1). Genes required late but not early could represent mutants that are able to establish infection but unable to sustain long-term in vivo growth. Genes required early but not late could represent mutants that grow slowly in mice. These mutants would be underrepresented at day 10 but catch up by day 45 postinfection. In fact, genes that were required at day 10 but not at day 45 were enriched for loss of insertions (Figure S1B). The average in vitro:in vivo ratio for these genes was 4.3.
compared to the nonrequired average of 1.3 (p < 0.001) (Figure S1C). Although this set significantly overlapped with a previously defined set of genes required for growth in mice (gene set enrichment analysis [GSEA], p < 0.01), it also includes more than 400 newly discovered genes required for in vivo growth (Figure S2A) (Sassetti and Rubin, 2003).

**Genes Required for Surviving CD4-Mediated Stress: The CD4 Counteractome**

To screen for the CD4 T cell counteractome, the set of mycobacterial genes required for counteracting CD4-mediated stress, we searched for genes wherein mutations caused a growth defect in WT mice, but not in CD4-deficient mice. By comparing the WT output libraries to the MHCII−/− output libraries, we found that 58 genes had a statistically validated increase in insertions in MHCII−/− mice compared to WT mice. These genes had at least a 5-fold increase in insertions and a Mann-Whitney U p value of less than 0.05 (Figure 2A; Table S2). Two biochemical pathways, gluconeogenesis and Trp biosynthesis, were enriched in this list, suggesting that CD4 T cells are responsible for inducing a shift in Mtb metabolic demands (Table S2).

The CD4 counteractome could be required for the bacterium’s physiologic changes in response to CD4-driven environmental changes. Thus, the counteractome is a signature that cues us into the nature of CD4-mediated stress. To identify these cues, we selected our Mtb transposon library in a series of in vitro models of immune-driven stress conditions and compared the counteractome signatures. Using the same transposon-mapping technique on our selected library, we tested carbon starvation, amino acid starvation (and isolated Trp starvation), iron depletion, acid stress, and nitrosative stress (Tables S3A, S3B, S3C, and S3D). We created conditionally required gene sets as well as ranked gene lists for each condition, which allowed us to compare gene sets using the running-sum enrichment analysis in the GSEA tool (Subramanian et al., 2005). We found that both nitrosative stress and acid stress had significantly similar profiles to genes required for in vivo growth (Figure 2E, pairwise comparisons of “Tyloxapol pH 4.5,” “pcit pH 4.5,” “DETA-NO,” and “in vivo day 45”). This is consistent with the reactive nitrogen synthase, converts chorismate to anthranilate in the first committed step of Trp biosynthesis. The next enzyme in the pathway, TrpD, converts anthranilate to N-(5′-phosphoribosyl)anthranilate. An Mtb strain carrying a deletion of the trpD gene has been shown to be a Trp auxotroph (Figure 3B) (Parish, 2003; Smith et al., 2001). The trpE deletion strain grew normally in liquid media only when supplemented with 1 mM Trp (Figure 3C). Interestingly, lower amounts of Trp delayed entry into the logarithmic phase of growth, but the growth dynamics from that point onward was similar to WT. Genetic complementation with the trpE gene restored normal growth in media lacking Trp (Figure 3C).

Auxotrophy in Mtb is not always bactericidal (Parish, 2003). Our results suggested that CD4 T cells help starve Mtb of exogenous Trp, so we sought to test the bactericidal potential of blocking endogenous Trp biosynthesis in the face of this exogenous starvation. To do so, we cultured the auxotroph in Trp to both midlog and stationary phase, continued the culture either in the presence or absence of Trp, and measured bacterial survival. The auxotroph was rapidly killed when starved of Trp, suggesting that Trp biosynthesis is an attractive target for a bactericidal drug (Figures 3D and 3E). Interestingly, the trpE deletion strain dies far more rapidly than a trpD deletion (Parish, 2003). The trpE deletion strain has about a 100,000-fold loss of viability at 2 weeks, a level that is reportedly not achieved after 13 weeks of starvation of the trpD deletion strain. This may be due to accumulation of intermediary metabolites or the presence of an as yet undescribed alternative Trp synthesis pathway.

**Trp Auxotrophs Are Hypersusceptible to Macrophages Activated by CD4 T Cells**

During a typical lung infection, Mtb first enters alveolar macrophages, which then form an inflammatory structure known as the granuloma. Upon adaptive immune activation, CD4 T cells enter the granuloma and stimulate the infected macrophages (Ernst, 2012). Our screen results suggested that one of these stimulatory mechanisms imposes a need for bacterial Trp biosynthesis, and we sought to delineate the mechanism by which CD4 T cells exert this need. To do so, we infected thioglycolate-elicited mouse peritoneal macrophages with WT Mtb, the trpE deletion strain and the complemented strain. Over a 5-day infection, the WT and complemented strains increased in colony-forming units (cfu) by about 5-fold, whereas the trpE deletion strain had no measurable growth, demonstrating that Trp biosynthesis is required for growth even in unstimulated macrophages (Figure 4A).

Our mouse findings predicted that the trpE deletion strain would be particularly sensitive to CD4-mediated stress. Indeed, the trpE deletion strain was significantly hypersusceptible to the effects of CD4 T cells in coculture with macrophages (Figures 4A and 4B). By adding CD4 T cells harvested from the spleens of Mtb-infected mice, macrophages were able to kill the auxotroph more effectively. Compared to unstimulated macrophages, CD4 T cell-stimulated macrophages decreased growth of WT Mtb by about 50%, whereas CD4 coculture decreased trpE deletion strain growth by about 80% (Figure 4B). Additionally, the trpE deletion strain was also hypersusceptible to IFN-γ and TNF-α, cytokines secreted upon the arrival of CD4 T cells to an Mtb
lesion (Figures S3A and S3B). These data confirmed our findings in mice and showed that CD4 T cells and the cytokines they secrete demand the need for mycobacterial Trp biosynthesis during infection. Because human macrophages differ from mouse macrophages in their mycobacterial-killing strategies, we tested the growth of our Mtb strains in monocyte-derived

Figure 2. Trp Biosynthesis Is Required during Infection of WT, but Not MHCII−/−, Mice

(A) To search for genes required for surviving the CD4 T cell response, we identified genes that had a statistically validated increase in insertion counts (above the axis).

(B and C) Transposon insertion counts in the regions containing trpE (B) and trpD (C) are illustrated. Histograms represent the number of times insertions were found at each potential insertion site. Both trpE and trpD sustained insertions in our library, but whereas we were unable to retrieve insertions in these genes from WT mice, we were able to recover trpE and trpD mutants from MHC class II KO mice.

(D) We infected WT, trpE KO, and complemented strain Mtb into WT and MHC class II KO mice. Growth of the three strains was determined, confirming the results of our transposon screen. Error bars represent SE.

(E) By comparing gene-requirement signatures, we profiled the similarity of CD4-mediated stress to in vitro models of potential immune-mediated stresses. Each box represents a pairwise comparison between two gene sets, where the larger gene set was ordered by p value and ratio, and the smaller gene set was used by the GSEA preranked tool to search for the enrichment of the second set of genes in the first.

See also Figure S2.
macrophages from human donors. As with previous reports, we observed that IFN-γ alone does not measurably inhibit WT Mtb growth (Fabri et al., 2011). However, whereas the trpE deletion strain grew slightly over the 5-day infection, its growth was inhibited by IFN-γ (Figure 4E).

**IFN-γ Induction of IDO Necessitates Mycobacterial Trp Biosynthesis**

To show that the auxotroph’s hypersusceptibility to IFN-γ was Trp dependent, we added Trp to the media in IFN-γ-treated macrophages, which reversed the IFN-γ hypersusceptibility (Figure S3E). As expected, Trp did not change the bacterial growth inhibitory effect of IFN-γ in WT Mtb, showing that Trp supplementation does not have a general growth effect on Mtb (Figure 4E). Interestingly, Trp supplementation could not restore growth of the auxotroph in unstimulated macrophages to WT Mtb levels. It is possible that the levels of Trp needed to restore WT growth (1 mM in liquid broth) cannot be reached intracellularly, whereas the amount of Trp required to protect from IFN-γ-mediated killing could.

Many intracellular pathogens are natural Trp auxotrophs whose intracellular growth is also inhibited by IFN-γ. Thus, we attempted to determine if similar processes affected Trp availability in Mtb infection. One of the transcriptionally induced genes in response to IFN-γ is a Trp-catabolizing enzyme: indoleamine-2,3-dioxygenase (IDO) (Alberati-Giani et al., 1997). IDO utilizes Trp as a synthetic precursor for kynurenes, immune signaling molecules that help control inflammation (Zelante et al., 2009). In this synthetic process, it also greatly decreases the intracellular Trp pool. IDO is thus required for IFN-γ-mediated growth inhibition of *Chlamydia* and other Trp auxotrophic intracellular pathogens (Ibana et al., 2011; Zelante et al., 2009).

We tested the role of IDO in the *trpE* deletion strain’s hypersusceptibility to CD4 T cell and IFN-γ by either (1) inhibiting IDO in both human and mouse macrophages with a specific chemical inhibitor, 1-methyl Trp (1-MT) (Figures 4C and 4E), or (2) using mouse macrophages derived from IDO KO mice (Figure 4D). In both cases, the hypersusceptibility was reversed. The inhibitor acts on the macrophage rather than the pathogen because it had no effect on bacteria grown in a defined medium (Figure S3D). These data support that CD4 T cells, likely acting through IFN-γ, stimulate intracellular Trp depletion, forcing Mtb to synthesize its own Trp.

**Halogenated Anthranilate Analogs Disrupt Trp Biosynthesis to Kill Mtb In Vitro**

Because Trp is required for intracellular growth in the face of CD4-mediated immunity, compounds that inhibit the bacterial Trp synthesis pathway should synergize with host immunity. We focused on anthranilate analogs, compounds that have been shown to inhibit the synthesis of quorum-sensing molecules in *Pseudomonas aeruginosa* that, like Trp synthesis, also have an anthranilate intermediate (Lesic et al., 2007). We tested a panel of anthranilate analogs for Mtb growth inhibition in the presence and absence of Trp. Two fluorinated anthranilates, 2-amino-5-fluorobenzoic acid (5-FABA) and 2-amino-6-fluorobenzoic acid (6-FABA), had an MIC of 5 μM in liquid broth in the absence of Trp (Figure 5A). The addition of Trp blocked toxicity (Figure 5A). 6-FABA was less active against *Mycobacterium smegmatis* (Msm), with an MIC of 65 μM, but this was, again, blocked by added Trp (Figure S4). As predicted from the
experiments with auxotrophs, 6-FABA was also bactericidal in liquid broth (Figure 5B) with an ~100-fold and ~10,000-fold decrease in cfu compared to the starting inoculum or the untreated control on day 6, respectively.

To determine the likely target of these compounds, we selected for mutants on solid media containing 150 μM 5-FABA or 300 μM 6-FABA. We plated 10^9 bacteria treated with 0.25% ethyl methanesulfonate on 150 μM 5-FABA, which resulted in ~30 resistant colonies. We then sequenced the genome of a resistant clone and found the following polymorphisms: TrpE:F68I, Rv2585c:A154V, Pks13:P568A, and 4359303:A > G (downstream of RD1 deletion). The trpE mutation was surprising because the enzyme converts chorismate into anthranilate, and we had hypothesized that TrpD, which utilizes anthranilate, would be the target of these anthranilate analogs. This led to two competing hypotheses for the mechanism of resistance. The FABA molecules could target TrpE by inhibiting the enzyme at its allosteric site, and the F68I mutation might confer resistance to this targeting. Alternatively, the F68I mutant could be a hypermorphic, and insensitivity to allosteric inhibition by Trp might confer resistance through increased Trp synthesis.

Testing the possibility that the FABAs are inhibitors of TrpE activity was technically challenging. We cloned and purified both WT and the F68I mutant form of TrpE and assayed their activity by measuring the fluorescent product, anthranilate (Baker and Crawford, 1966). Both anthranilate-derivative FABA compounds had fluorescent spectra that overlapped with anthranilate. Therefore, we were unable to assess the possibility of the FABA compounds being inhibitors of TrpE. However, we were able to test the enzymatic properties of both mutant and WT TrpE and address the possibility of a hypermorphic allele. Indeed, we found that the mutant enzyme had a 3-fold increase in in vitro activity compared to the WT enzyme (Figure 5C). Furthermore, the mutant enzyme was ~50 times less sensitive to allosteric inhibition by Trp in vitro (Figure 5D). When we modeled the Mtb TrpE structure using the Serratia marcescens homolog, we found that F68 resides in the allosteric binding pocket of Trp (Figure 5E). Altogether, this suggests that the F68I mutant is a hypermorphic...
allele that is also less sensitive to allosteric inhibition by Trp. The likely increase in Trp production, then, confers resistance to the FABA compounds in the same way that added exogenous Trp does (Figures 3C–3E).

6-FABA Synergizes with IFN-γ to Kill Mtb in Macrophages

If these compounds act through the Trp biosynthetic pathway, they should phenocopy trpE deletion strains during macrophage infection. To test this, we infected macrophages with Mtb and, after 1 day, added 6-FABA and measured bacterial growth by plating for cfu on day 5. At concentrations as low as 10 μM, 6-FABA significantly limited growth by over 10-fold (Figure 6A). The number of cfu at day 5 was lower than at day 1, demonstrating that 6-FABA had bactericidal activity in macrophages. To ensure that activity was not due to the death of macrophages, we tested cytotoxicity and found that 6-FABA did not affect cell viability (Figure S5A).

Because the Trp auxotroph was hypersusceptible to IFN-γ, we hypothesized that 6-FABA’s block of Trp biosynthesis would work in synergy with IFN-γ to kill Mtb in macrophages. To test this, we dosed both IFN-γ (10 U/ml) and 6-FABA (0.2 μM) to a level where each individually had about a 2-fold inhibitory effect on bacterial growth (Figure 6B). Without synergy, we predicted that the combined effect of 6-FABA and IFN-γ would be about 4-fold. Instead, the effect was ~40-fold in mouse and ~9-fold in human macrophages, demonstrating clear synergy (Figures 6C, S5B, and S5C).

6-FABA Inhibits Mtb Growth during Infection

Although many compounds are active in vitro, few have adequate bioavailability to be used in animal infections without considerable modification. Fortunately, however, we found that both 6-FABA and an ester derivative (that is rapidly cleaved to the free acid in the circulation) are absorbed orally and achieve high serum concentrations, though with relatively short half-lives (Figure S6). Moreover, doses of up to 250 mg/kg/day did not result in clinical illness or weight loss during a 5-day tolerability trial. This allowed us to test the activity of these compounds in a mouse model of TB.

We infected mice with 10⁵ aerosolized Mtb bacilli and allowed infection to establish in the lungs for 8 days. We treated mice six times a week with INH (25 mg/kg/day), 6-FABA (200 mg/kg/day), or the ester derivative (200 mg/kg/day) and planned to measure bacterial growth at 2 and 4 weeks after initiating treatment. Unfortunately, prolonged treatment with 6-FABA resulted in 50% death of animals within 4 weeks. Thus, we could only evaluate 2-week efficacy with this compound. Notably, the ester form of 6-FABA was not toxic, even though it releases the same amount of 6-FABA in mouse serum. This suggested to us that toxicity of 6-FABA was due to an as of yet undetermined off-target effect that might be influenced by an interaction with anesthesia used for gavaging Mtb-infected animals (anesthesia was not used for tolerability trial). Importantly, the nontoxic ester derivative allowed us to assess the in vivo efficacy of pharmaceutical Trp.

Figure 5. A Small Molecule, 6-FABA, Targets Mycobacterial Trp Biosynthesis
(A) 6-FABA inhibits the growth of Mtb in 7H9. For 6-FABA inhibition of M. smegmatis growth, please see Figure S4.
(B) To test the bactericidal potential of 6-FABA, WT Mtb was treated with 6-FABA in 7H9, and cultures were plated for cfu at various time points.
(C) WT (black) and mutant TrpE (F68I; blue) were isolated, and enzymatic activity was assessed by measuring chorismate concentration.
(D) The inhibitory effect of Trp on both WT (blue) and mutant (black) TrpE was measured as percent (%) initial velocity of the no-inhibitor control reaction.
(E) Mtb TrpE structure (blue) was modeled based on the homologous enzyme from S. marcescens (beige), showing F68 in the allosteric binding pocket of Trp.

See also Figure S5.
biosynthesis targeting over a longer time period. At 2 weeks after infection, growth in mouse spleens was decreased by greater than 10-fold in both 6-FABA and the ester derivative-treated mice (Figure 6E). 6-FABA also decreased bacterial growth in the lungs, but to a lesser extent (Figure 6D). At 4 weeks after infection, the ester continued to significantly decrease mycobacterial growth in both spleens and lungs (Figures 6F and 6G). The effect of these molecules on decreasing bacterial growth in vivo demonstrates that Trp biosynthesis is a viable target for drug development. Combined with the fact that CD4 T cells are required for optimal killing of Trp auxotrophs (Figure 2D), we have shown that this therapeutic strategy leverages CD4 T cell activity to kill Mtb in vivo.

**DISCUSSION**

CD4 T cells are paramount in the host defense against TB but are insufficient to clear the bacteria from a diseased patient. We found that one of the mechanisms by which bacteria survive host CD4-generated stress is through the production of Trp, thus avoiding starvation and death. Loss or inhibition of the Trp biosynthetic pathway renders Mtb hypersusceptible to IFN-γ-mediated killing within macrophages, both in vitro and during infection (Figure 7).

This observation highlights one of the major mechanisms of host protection against invading organisms: depriving them of key nutrients. Infection triggers host responses that sequester important compounds such as amino acids and iron (Hood and Skaar, 2012; Zhang and Rubin, 2013). Although these nonspecific responses are likely important for the control of poorly adapted invaders, successful pathogens have developed strategies to counteract this.
these defenses, including biosynthesis of key compounds and efficient scavenging of others. Mtb in particular relies upon cholesterol and fatty acid catabolism, gluconeogenesis, and the glyoxylate shunt to survive the host environment (Lee et al., 2013; Marrero et al., 2010; McKinney et al., 2000; Muñoz-Elias and McKinney, 2005; Pandey and Sassetti, 2008).

Trp starvation, through IDO-mediated depletion of cytoplasmic pools of this amino acid, appears to be a mechanism that has the added benefit of leading to kynurenine production and prevention of immunopathology (Desvignes and Ernst, 2009). This clearly benefits the host in blocking the growth of cytoplasmic pathogens. However, it is less obvious that this is due to immune-driven starvation mechanisms (Honda et al., 2000; Pavelka et al., 2003; Price et al., 2011; Tattoli et al., 2012; Zhang and Rubin, 2013).

Although scavenging Trp in MHCII−/− mice allows the Trp deletion strains to survive, it is important to note that these mutants are eventually attenuated even in MHCII−/− mice. Similarly, we showed that the TrpE deletion strain is attenuated in macrophages even before stimulation by CD4 T cells or IFN-γ and TNF-α. So, whereas our data clearly demonstrate the importance of CD4 T cells in demanding bacterial Trp synthesis, it appears that there may also be CD4-independent mechanisms of Trp starvation. Importantly, this suggests that targeting Trp biosynthesis would retain some effectiveness even in patients, such as HIV-positive individuals, who lack a normal CD4 compartment.

Here, we used an improved analytic approach to identify conditionally essential genes using a transposon library. Each gene has multiple potential insertion sites, and most insertion count comparisons sum counts across each gene to compare between conditions. However, in reasonably saturated libraries, treating each potential insertion site as an independent assessment of gene requirement drastically increased the statistical power of such comparisons. Most transposon insertion-sequencing techniques currently use insertion count totals summed across all sites in a gene (Goodman et al., 2009; Griffin et al., 2011). We found that this drastically decreases the power of statistical hypothesis testing and increases the likelihood that a single differential site could make the entire gene look differentially required. We developed a more discriminating method for assessing the conditional requirement of genes in genome-wide bacterial transposon screens. Using an easily scalable nonparametric test to comprehensively compare the distribution of insertion counts (rather than the sums of insertion counts) for Trp points toward a capability to uptake other amino acids. A host of other Mtb amino acid auxotrophs are attenuated for growth in mice; it is possible that this is due to immune-driven starvation mechanisms (Honda et al., 2000; Pavelka et al., 2003; Price et al., 2011; Tattoli et al., 2012; Zhang and Rubin, 2013).
are newly described as required. Furthermore, by searching for 576 genes that were required for growth in vivo, most of which surviving mutant and, thus, improved significantly on the sensi-
across every gene, we were able to assess genetic requirement across a host of different conditions.

In contrast to similar microarray-based screens, deep sequencing allowed us to directly map insertion sites for each surviving mutant and, thus, improved significantly on the sensi-
tivity and specificity of insertion count comparisons. We found 576 genes that were required for growth in vivo, most of which are newly described as required. Furthermore, by searching for mutants rescued for growth in \textit{MHCII}^{-/-} mice, we described 58 genes that were likely required for surviving CD4-mediated host defenses.

One power of this approach is that we can easily compare gene requirements in multiple conditions, essentially using com-
plex profiles to identify critical environmental determinants. We showed that the in vivo environment as a whole, as well as the specific CD4-generated component of that environment, could be understood by comparing multiple gene-requirement profiles. GSEA comparing in vivo profiles to in vitro profiles confirmed that \textit{Mtb}'s infectious niche is characterized by acidic and oxidative stress and that CD4 T cells impose Trp starvation.

Gene-requirement signatures are an effective way to understand both mycobacterial physiology as well as the environments created by components of host immunity. Our study is limited to the determination of the CD4 counteractome, but other counteractomes—those specific to IFN-\gamma, iNOS, CD8 T cells, or any immune system component that can be perturbed in a mouse—will further our understanding of the tug-of-war between host and pathogen. In fact, given that many CD4-mediated mechanisms are IFN-\gamma dependent, we would predict that bacterial strains rescued in the \textit{MHCII}^{-/-} mice, like the \textit{trpE} deletion strain, would also be rescued in IFN-\gamma KO mice. Thus, on a genomics scale, the IFN-\gamma counteractome should look similar to the CD4 counteractome. Further profiling can test such hypotheses and uncover new mechanistic differences between the many arms of host immunity.

Trp biosynthesis is an attractive target for antibiotic development. Small-molecule inhibitors of Trp and other aromatic amino acid biosynthesis exist for other organisms, suggesting that certain enzymes in these pathways might be “druggable,” or suitable for chemical inhibition (Coggins et al., 2003). Furthermore, Trp is an essential amino acid for humans (WHO, 2007). Because hosts get all of their needed Trp from their diets, any cross-species activity of anti-Trp synthesis drugs should be nontoxic (though off-target toxicity remains a possibility).

Helogenated anthranilates were first described as inhibitors of both quorum sensing and growth in \textit{P. aeruginosa} infection (Lesic et al., 2007). We found that two of these compounds, 6-FABA and 5-FABA, had bactericidal activity against \textit{Mtb} at low concentrations, though only in the absence of Trp. Two lines of evidence suggest that Trp biosynthesis pathway is the phys-
ologistic target for both of these compounds. First, normal bacte-
rial growth is restored during \textit{FABA} treatment by the addition of Trp. Second, resistant mutants harbor a hypermorphic enzyme that is resistant to allosteric inhibition by Trp. Together, these provide chemical and genetic confirmation that Trp can rescue the effect of FABA, but it does not prove that FABA inhibits Trp production. Because TrpD utilizes anthranilate as a substrate, we speculated that 6-FABA might be a competitive inhibitor of TrpD. However, it is also possible that 6-FABA could be a substrate of TrpD, resulting in the production of fluorinated inter-
mediates that inhibit Trp biosynthesis or even fluorinated Trp that incorporates into and poisons protein synthesis or function. Our data clearly demonstrate that the bactericidal activity of 6-FABA is due to its negative effect on Trp biosynthesis, and further studies to elucidate the precise mechanism of toxicity should focus on this Trp effect.

The vast majority of antibiotics are effective against organisms grown in vitro. However, one of the most effective antitubercu-
losis drugs, pyrazinamide, is poorly active under most in vitro growth conditions (Tarshis and Weed, 1953; Zhang and Mitchi-
son, 2003). Instead, it relies on the host environment for efficacy. Similar compounds, which target processes critical in the host, could effectively synergize with the host response to infection and have substantial efficacy. Probing CD4-mediated immunity helped us identify a specific facet of the host-derived environ-
ment that could be utilized for bacterial killing. Although we do not yet have a compound that is highly effective during infection, our results provide genetic and chemical validation of the Trp biosynthesis pathway as a target for highly active antibiotics.

**EXPERIMENTAL PROCEDURES**

**Library Generation**

Transposon libraries were created as described previously (Zhang et al., 2012) but plated on 7H10 plates with glycerol, OADC, Tween 80, Cas-amino acids, and Trp. A total of 500,000 mutants were scraped, frozen, and saved for future use.

**Mouse Infections and Harvests**

WT mice (C57BL/6) were obtained from Jackson Laboratory. MHC class II KO mice (Abb H2-Ao1) were obtained from Taconic Farms. Mice were infected with 10^6 bacteria via tail vein injection. At 10 and 45 days postinfection, spleens were harvested and plated for bacteria. For each mouse, 10^9 surviving colonies were scraped, and DNA was extracted for analysis. The protocols, personnel, and animal use were approved and monitored by the Institutional Animal Care and Use Committee. The animal facilities are AAALAC accredited.

**In Vitro Transposon Library Selections**

For acid stress, the library was suspended in liquid media at a starting concentra-
tion of 10^8 cfu/ml and selected in 7H10 with tyloxapol (in place of Tween 80) buffered to pH 6.5 or pH 4.5, and in phosphate-citrate buffer at pH 4.5. Bacteria were plated after 6 days and scraped for DNA prep. For nitrosative stress, the library was suspended in 5 mM DETA-NO for 3 days. Trp starvation and amino acid starvation were measured using libraries that were created either on normal 7H10, or on 7H10 supplemented with 1 mM Trp or 1% Cas-amino acids, respectively. Iron-supplemented libraries were plated on 7H10 with 450 \( \mu \)M and 1.5 mM iron. All primary data can be found in Tables S1, S2, S3, and S4. All the raw data are available in Table S4.

**Statistical Analysis**

For each insertion count comparison, the control libraries were combined using a script that normalized insertion counts to the sequencing run’s total read counts. This combined control library was then used to compare to each experimental library. For each gene, we treated the insertion counts at sites within the middle 90% of the gene as nonparametric distributions and assumed the null hypothesis that the distributions would be the same between conditions. We used a Mann-Whitney U test for hypothesis testing. A p value was thus calculated for each replicate, and a composite p value was generated by using a Bonferroni correction and Fisher’s method. We then generated Benjamini-Hochberg FDRs using the composite p values. Ratios were
calculated by averaging the read counts per gene for all replicates and comparing to the combined control library, after normalizing for total read counts.

GSEA
GSEA was performed using the preranked tool. Genes were stratified by p value (<0.01 or 0.05 and > 0.01 or 0.05) and then ranked by ratio within the strata. For each conditional gene requirement experiment, a ranked list and a conditional essential “calls” gene set were created. Each ranked list was then assessed against each gene set, and familywise error rate was used to generate the p values for significant enrichment.

Construction of trpE Deletion and Complement Strains
The hygromycin-resistance gene was amplified with flanks containing 500 bp regions upstream and downstream (and slightly overlapping with) of trpE. This construct was electroporated into a recombining strain of Mtb containing the plasmid pNIT(kan)-RecET-SacB. Transformations were plated on 7H10 agar with 1 mM Trp. Positive clones were plated on 7H10 agar containing 10% sucrose to counterselect against the recombining plasmid. Deletion of the endogenous locus was confirmed by PCR and by phenotypic tests for auxotrophy. Finally, to complement, the trpE-rv1610 two-gene operon was amplified and cloned, along with an artificial promoter, using multisite gateway into pDE43-MCK, which integrates into the L5 site.

Macrophage Infections
Peritoneal macrophages were stimulated with thioglycollate medium (3%) by intraperitoneal infection, harvested 3–5 days poststimulation, and isolated using CD11b MicroBeads (Miltenyi Biotec). Human monocyte-derived macrophages were made from donated buffy coats. Ficol-separated cells were harvested, and macrophages were isolated with CD14 MicroBeads (Miltenyi Biotec) and grown in GM-CSF (10 ng/ml) for 5 days before use. Cells were infected with Mtb at an moi of 10:1 for 2 hr at 37°C in a shaking incubator for another 1–2 days. Plates were read, and the MIC was determined as the first concentration at which the color changes.

Kinetic Analysis of WT and Mutant Anthranilate Synthase
A master mix containing 100 mM NH4Cl, 10 mM MgCl2, 0.1 mM EDTA, 1 μM of WT or mutant TrpE, 20 μM chorismate, and 20 mM of Tris (pH 9) was made for each reaction to a final volume of 199 μl. Either Trp or 5-fluorotryptophan was also added in 1 μl amounts at concentrations ranging from 0.025 to 24 μM. The readings for fluorescence emission (anthranilate) within the range of 480–512 nM were taken using the POLARstar Omega plate reader.

Alamar Blue Assays
In a 96-well plate, bacterial cultures were started at an OD600 of 0.003. After 480–512 nM were taken using the POLARstar Omega plate reader.

LC-MS Conditions
The analysis was performed on a Sciex Applied Biosystems API4000 triple-quadrupole mass spectrometer coupled to an Agilent 1260 HPLC system. Sample analysis was accepted if the low-level quality control samples were within ±20% of nominal concentration and ±15% for mid- and high-level quality control samples.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.10.045.


