Supplementary Information Inventory

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Current models for tolerance in TB. Related to Figure 7. The figure depicts two models for the localization of tolerant bacteria in the context of necrotic (caseous) tubercular granulomas. The upper granuloma in the left lung (right side of drawing) opens into the bronchial tree, allowing bacteria to escape the lung and be transmitted via aerosol. It is this type of lesion from which bacteria can be cultured from sputum. The other two granulomas, while still necrotic, do not connect with the bronchial passages (i.e. they are "closed") and do not contribute to sputum burdens. In the classical model of tolerance, closed lesions are presumed to be hypoxic and it is this condition that is thought to induce a uniformly tolerant dormancy state (Barry et al., 2009). Alternative models of tolerance implicate a variety of stresses including nutrient limitation, DNA damage, nitric oxide, as well as the existence of a stochastic population of persister cells that contribute to tolerance. Such models are more agnostic about the placement of the tolerant bacteria, which might reside in all different granuloma types, both within macrophages and in the caseum (Burgos et al., 2008; Connolly et al., 2007; Dhar and McKinney, 2007; Sacchettini et al., 2008; Warner and Mizrahi, 2006). Both models implicate slowly growing and/or metabolically inactive cells in the generation of tolerance.

Figure S2. Toxicity and/or efficacy of DMSO, streptomycin (STM), pyrazinamide (PZA), and MOX in the larval model. Related to Figure 1. (A) To determine the maximum amount of DMSO that could be used to facilitate dissolution of drugs for addition to fish water, the effects of DMSO alone on survival of uninfected larvae was tested. Twenty larvae were placed in each of four concentrations of DMSO at one dpf and monitored daily for survival thereafter.

1% DMSO did not significantly impact survival when compared to 0% DMSO (*P*=0.73, Log-rank test), while 2% and 3% DMSO reduced survival (*P*<0.0001, Log-rank test).

(B) Survival curves of uninfected larvae soaked in fish water alone (UNT) or in the presence of 388 μM RIF, 62.3 μM MOX, 1442 μM EMB, or 290 μM INH (see also **Table S2)**.

(C-E) STM treatment of larvae. (C) Reminiscent of its toxicity in humans, we could not establish a STM MEC due to toxicity in the larvae. Survival of uninfected larvae left untreated (UNT) or in the presence of 110 or 1100 μM STM added starting 2dpf. *P*<0.0001, UNT vs. 1100 μM STM. Log-rank test. N=15 per group. (D and E) Larvae infected with 250 Mm were left untreated for 24 hours prior to adding STM (0, 110 or 1100 μM) for an additional 42 hours. Representative fluorescence images (D) and bacterial burdens as determined by FPC (E) are shown. Bars represent median FPC. Significance testing by Kruskal-Wallis test with Dunn's post test comparing each treatment group to the untreated control. ***, *P* <0.001. The minimum concentration that reduced bacterial burdens at 42 hours post treatment (1100 μM) was associated with 100% mortality only a few hours later (Panel C).

(F and G) PZA, to which Mm is innately resistant (Rastogi et al., 1992, see also **Table S1**), fails to reduce bacterial burdens even at toxic doses. (F) Larvae infected with 500 Mm were immediately soaked in INH (290 μM), left untreated (UNT), or treated with PZA (200, 400, 800, or 1600 μM). Bacterial burdens were assessed by FPC at 4 dpt. Bars represent mean $\log_{10}$FPC. Bacterial burdens of PZA treated embryos were not significantly different from untreated (One-way ANOVA with Dunnett's post-test comparing treated groups to untreated control). (G) Percent survival of larvae in (F) at the time of imaging at 4 dpt. Numbers within each bar represents $n_{final}/n_{initial}$. Survival of embryos treated with 1600 μM PZA was not significantly different from that of untreated embryos (Fischer's exact test).

(H) Dose-dependent efficacy of MOX, and its superior activity compared to EMB. Three-dpf larvae (N=10 per group) were infected with 300 CFU Mm and treatment started the following day with varying concentrations of MOX or a single high dose of EMB (shown in μM). Bacterial burdens were assessed five dpt. Bars represent mean $\log_{10}$FPC. 248 μM MOX had greater activity than 1442 μM EMB (P=0.0213, Student's t-test), consistent with its greater antitubercular potency in humans (Conde et al., 2009).

Figure S3. Fluorescent Pixel Count (FPC) as an accurate measure of relative infection burden in logarithmic and stationary phase bacteria. Related to Figure 1. (A and B) Larvae infected with varying doses of (A) green-fluorescent or (B) red-fluorescent Mm were imaged and bacterial burdens quantified by FPC. Data arranged in order of decreasing FPC (bar) with corresponding embryo image shown underneath. (C and D) Larvae were infected with variable doses of Mm and the infection allowed to progress for five days. Three individual larvae with variable infection burdens (light, medium and heavy) were then mounted in 1% agarose and imaged in dorsal, ventral, left and right lateral orientations. (C) Fluorescence images: top row = left lateral view; second row = dorsal view; third row = right lateral view, and bottom row = ventral view. (D) FPC measurements of the images shown in (C). (E) Correlation between mean $\log_{10}$ FPC and mean $\log_{10}$ CFU counts from populations shown in Figure 1C and 1D. Pearson's Coefficient of Correlation (r) = 0.9341, P= 0.0659. (F) To determine the detection sensitivity of stationary phase Mm, light (left panel) and heavy (right panel) inocula of Mm (OD_{600} of 1.8) were injected into 30 hpf larvae which were imaged immediately at the same settings used in (C). By fluorimetry, stationary phase Mm/p*msp12::gfp* expresses ~ 20% of the fluorescence of logarithmic phase bacteria, yet are still readily visualized by microscopy.

Furthermore, FPC analysis counts fluorescent pixels above a user defined threshold, regardless of intensity. Therefore, all bacteria will count equally, independent of their level of GFP expression and thereby of their growth phase.

Figure S4. INH tolerance in axenic culture. **Related to Figure 4 and Figure 5.** A) INH-tolerance is induced by exposure to INH. Approximately 3000 *M. marinum* were incubated in 19, 58, or 174 μM INH (representing 0.33-, 1- and 3-fold the in vitro MIC) and survival assessed at times shown. Mean percent survival +/- SEM of triplicate samples are plotted. Stippled line and lower limit of y-axis indicate upper and lower limits of detection, respectively. At 174 μM two of three samples were sterilized by 120 hours, and thus are below the limit of detection of 1 CFU. By 72 hours cultures containing 19 μM INH had grown above the limit of detection (3000 CFU). Representative colonies arising from all cultures were verified to be INH-sensitive. B) Bacterial density does not alter tolerance. J774 cells infected with Mm for 96 hours were lysed and the lysates diluted 10 and 100-fold in 7H9 medium. Undiluted and diluted lysates were treated with INH and RIF for 48 hours. C) INH tolerance of stationary phase Mm. Single cell suspensions (see **Experimental Procedures**) of Mm were isolated from broth-grown log and stationary phase cultures and were treated with 174 μM INH or left untreated prior to enumeration of CFU at the time points shown.

Figure S5. Assessment of intramacrophage growth rates using the unstable plasmid, pBP10. Related to Figure 5.

A) In vitro stability of pBP10 in Mm in the absence of antibiotic selection. The total number of Mm, as determined by CFU enumeration, accounting for serial dilutions in continuous log-phase

cultures. Bacteria were grown in 25 ml flasks, standing (static) or in 50 mL conical tubes in a roller drum (rotating). B) The frequency of plasmid bearing Mm/pBP10 as determined by plating on 7H10 agar with and without 20 μg/mL kanamycin. C) The absolute numbers of all bacteria (Total, circles) and those retaining pBP10 (KanR, squares) in static culture. D) Frequency of plasmid bearing Mm/pBP10. Mean of triplicate cultures shown with error bars representing SEM. E) Estimates of Mm generation times during intracellular growth in THP-1 and J774A.1 cell lines. The growth rate (r), death rate (δ) and Cumulative Bacterial Burden (CBB) were calculated as described in **Supplementary Experimental Procedures**. The generation time was calculated by ln(2)/*r*.

Figure S6. Specificity of VER and Mtb Rv1258c in regulating intramacrophage tolerance. Related to Figure 6.

(A) VER does not reduce stationary phase Mm tolerance to INH or RIF. Logarithmic (LOG) and stationary phase (STAT) Mm cultures were treated for 48 hours with 174 μM INH, 1.21 μM RIF, or neither drug (UNT), and in the presence or absence of 81.4uM VER. (B and C) Attenuated mutants develop tolerance. J774 macrophages were infected with Mm strains M ("WT"), RD1-6 ("R") and KK33 ("E"), which are wild-type, or defective for the ESX-1 and Erp virulence determinants, respectively for two or 96 hours. (B) Intracellular growth attenuation of mutant bacteria. Statistical testing performed using one way ANOVA with Dunnett's post test: **, $P<0.01$. (C) Percent Survival of macrophage-released bacteria after an additional 48 hours in 174 μM INH, 1.21 μM RIF, or left untreated (UNT). Statistical testing was performed for INH and RIF cohorts separately, in each case using one way ANOVA with Bonferroni's post test for selected multiple comparisons. *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$. (D) Antibiotic

susceptibility of Rv1258c mutants in logarithmic and stationary phase culture. Logarithmic (LOG) or 6-week old stationary (STAT) phase cultures of CDC1551 ("1551"), JHU1258c-715 ("M1") and JHU1258c-833 ("M2") were incubated in the presence or absence of 1.21 μM RIF for 48 hours prior to enumeration on 7H10 agar.

Figure S7. Macrophage-induced antibiotic tolerance persists after bacteria resume extracellular growth. Related to Figure 6. Mm was used to infect J774 macrophages for 2 hours (black bars) or 96 hours (white and gray bars) prior to being released by macrophage lysis. The lysates were then immediately subjected to 48 hours treatment with 174 μM INH, 1.21 μM RIF, or left untreated (black and white bars), or were allowed to resume growth in the lysate for 120 hours prior to the 48 hour antibiotic treatment (gray bars). (A) Percent survival of bacteria after 48 hours antibiotic treatment relative to pretreatment. Error bars represent SEM. Significance testing was performed using one-way ANOVA for each treatment separately (UNT, INH or RIF). Multiple comparisons were made with Dunnett's post-test using the 96 hour macrophage group (white bars) as the reference group; *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$. (B) Graph shows growth of released bacteria (after 96 hours growth in macrophages) in the lysate, with t=0 representing the CFU count at time of macrophage lysis.

SUPPLEMENTAL TABLES

Antibiotic	Molecular Weight	Mtb MIC (μg/ml)	Mm MIC (μg/ml)	Minimum Effective Concentration (MEC)[1]			
				Fold MIC	(μg/ml)	(μM)	Solvent
Isoniazid	137.14	<0.025-0.2[a]	8.0[d]	5x	40	290	H_2O
Rifampicin	822.95	<0.0625-0.5[a]	0.32[d]	1000x	320	388	DMSO[1%]
Ethambutol	277.23	1-4[a]	8.0[d]	50x	400	1442	H_2O
Moxifloxacin	401.43	0.06-1[b]	1.0[e]	25x	25	62.3	DMSO[1%]
Streptomycin	728.69	<0.5-2.0[a]	8.0[d]	100x	800	1098[2]	H_2O
Pyrazinamide	123.11	6-60[a]	R^+	NA	>200	>1600	H_2O

Table S1. In vitro MIC of antituberculous drugs and their MEC in zebrafish larvae. Related to Figure 1. [1]MEC was defined as the lowest concentration tested that normalized host survival, as shown for INH, EMB, MOX, and RIF (**Figure 1A**). [2]STM was highly toxic **(Figure S2F)** and so no MEC was determined, however bacterial burdens were shown to be reduced **(Figure S2G-H)** at this concentration. MICs reported in [a](Heifets, 1991), [b](Gillespie and Billington, 1999), [c](Kremer et al., 2000), and [d](Cosma et al., 2006). [e]MIC was determined by the agar dilution method as described in the **Supplemental Experimental Procedures**. R^+, innate resistance (Rastogi et al., 1992), although contradictory results have been reported using a non-standard pH (Silcox and David, 1971).

	Mock infected (see Figure S2B-E)				*M. marinum*-infected (see Figure 1A)			
	-Drug[a]	+Drug[a]	HR[b]	*P*[c]	-Drug[a]	+Drug[a]	HR[b]	*P*[c]
Rifampicin	9 (15)	9 (15)	0.57 [0.21-1.6]	0.2781	8 (10)	13 (10)	0.042 [0.010-0.18]	<0.0001
Moxifloxacin	11 (15)	11 (15)	0.23 [0.049-1.1]	0.0693	8 (10)	12 (10)	0.040 [0.009-0.17]	<0.0001
Ethambutol	12 (15)	12 (15)	1.4 [0.37-5.0]	0.6468	9 (10)	12 (10)	0.15 [0.041-0.53]	0.0037
Isoniazid	10 (45)	9 (45)	4.6 [2.4-8.7]	<0.0001	9 (10)	13 (10)	0.036 [0.008-0.16]	<0.0001

Table S2. Summary statistics and significance testing for survival of treated and untreated larvae. Related to Figure 1 and Figure S2B-E. [a]Data reported are median survival days (number of larvae) in the absence and presence of drug. [b]Hazard Ratio [95% confidence interval] of treated larvae relative to untreated. [c]Results of Log-rank test comparing survival of treated vs. untreated larvae. For survival of uninfected larvae, results shown are from four independent experiments, and thus the *P* values can be interpreted relative to $\alpha = 0.05$. For survival of infected larvae, RIF and MOX groups were compared to a single UNT control, which contained 1% DMSO, while the EMB and INH groups were compared to a single UNT control lacking DMSO. Thus the *P* values can be interpreted relative to $\alpha = 0.025$ to account for multiple comparisons. All statistical analyses performed using GraphPad Prism v. 5.01.

Figure	Strain	Time point	UNT	INH	RIF	MOX
4D	WT-Mm M	2 hr	490 ± 54	0.07	0.3 ± 0.1	1.4 ± 0.2
		96 hr	222 ± 27	16 ± 0.8	6.2 ± 0.6	6.7 ± 1.4
4E	WT-Mtb H37Rv	2 hr	7500 ± 1180	0.57	0.57	-
		96 hr	595 ± 49	1.4 ± 0.1	1.7 ± 0.04	-

Table S3. Summary of the percent survival data shown Figure 4. Related to Figure 4. The percent survival was calculated relative to the starting number of bacteria at the beginning of treatment. Numbers are the means of triplicate wells +/- SEM.

Figure	Strain	Condition	UNT	INH	RIF	MOX
5B	WT	UNT	1050 ± 380	18 ± 1.0	6.0 ± 1.5	-
		DEX	510 ± 67	25 ± 2.3	12 ± 2.0	-
5D	Mm/pBP10	Kan^S	928 ± 364	14 ± 2.1	5.3 ± 1.3	12 ± 2.3
		Kan^R	122 ± 16	4.0 ± 1.1	0.7 ± 0.2	1.6 ± 0.5

Table S4. Summary of the percent survival data shown in Figure 5. Related to Figure 5.

The percent survival was calculated relative to the starting number of bacteria at the beginning of treatment. Numbers are the means of triplicate wells +/- SEM.

Figure	Strain	Condition	Timepoint	UNT	INH	RIF	INH+RIF
6A	WT Mm	UNT	2 hr	157 ± 15	0.3 ± 0.03	0.2 ± 0.03	
			96 hr	155 ± 18	19 ± 1.2	2.3 ± 0.6	
		VER	96 hr	127 ± 9.7	1.2 ± 0.07	0.3 ± 0.03	
6B	WT Mm	UNT	2 hr	199 ± 22	0.3 ± 0.04	0.3 ± 0.04	
			96 hr	254 ± 7.4	21 ± 1.0	2.7 ± 0.2	
		RES	96 hr	251 ± 30	4.4 ± 0.7	0.3 ± 0.02	
6C	CDC1551	UNT	2 hr	940 ± 26	0.09 ± 0.007	3.7 ± 0.2	
			96 hr	1990 ± 45	0.5 ± 0.05	12 ± 2.0	
			144 hr	958 ± 38	0.5 ± 0.06	7.6 ± 0.4	
		VER	96 hr	1360 ± 150	0.5 ± 0.04	4.8 ± 0.2	
			144 hr	774 ± 27	0.4 ± 0.05	2.7 ± 0.2	
6D	CDC1551		2 hr	796 ± 45	0.2 ± 0.03	4.2 ± 0.4	
			96 hr	2690 ± 130	0.8 ± 0.1	13 ± 0.5	
	M1		2 hr	859 ± 10	0.3 ± 0.04	6.5 ± 0.9	
			96 hr	2810 ± 215	1.8 ± 0.05	2.5 ± 0.6	
	M2		2 hr	1630 ± 435	0.2 ± 0.03	5.3 ± 0.3	
			96 hr	2980 ± 219	1.9 ± 0.2	2.6 ± 0.2	
6G	WT Mm	UNT	2 hr	1030 ± 221	8.2 ± 1.4	17 ± 1.9	2.8 ± 0.8
			96 hr	67 ± 4.6	24 ± 2.5	23 ± 5.4	16 ± 1.4
		VER	96 hr	50 ± 5.3	12 ± 1.8	12 ± 1.1	7.3 ± 1.5

Table S5. Summary of the percent survival data shown in Figure 6. Related to Figure 6. The percent survival was calculated relative to the starting number of bacteria at the beginning of treatment. Numbers are the means of triplicate wells +/- SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Bacterial Growth Media

Mm was cultured in Middlebrook 7H9 broth (Difco) supplemented with 0.5% BSA, 0.005% Oleic Acid, 0.2% glucose, 0.2% glycerol, 0.085% sodium chloride, and 0.05% Tween-80, or on Middlebrook 7H10 agar (Difco) supplemented with 0.5% BSA, 0.005% Oleic Acid, 0.2% glucose, 0.2% glycerol, and 0.085% sodium chloride. Mtb was cultured in Middlebrook 7H9 broth, or on 7H10 agar supplemented with enriched OADC enrichment (BBL). Media were supplemented with 20 μg/ml kanamycin and 50 μg/ml of hygromycin when appropriate to select for plasmids.

MIC assays

MICs were determined as follows: Individual plates of 7H10 agar (supplemented as described above) were prepared with two-fold dilutions of the antibiotic being tested. Approximately 10^4 Mm CFU were spotted in triplicate onto each plate and incubated at 33°C. The MIC was defined as the lowest concentration that prevented growth after 8-11 days. Alternately, MICs were determined by inoculating round bottom Costar 96-well plates with 100 μl drug-supplemented medium with ~10^4 CFU Mm and incubating for 6-8 days, prior to reading OD_{590} using a Tecan GENios Pro microplate reader. The MIC was defined as the lowest concentration that prevented growth.

Determination of bacterial growth rates using the unstable plasmid, pBP10

Mm strain M was transformed with pBP10, and colonies were selected by growth on 7H10 medium with 20 µg/ml kanamycin, yielding strain Mm/pBP10. For in vitro log-phase cultures, Mm/pBP10 was grown in the absence of kanamycin under both static and rotating conditions to obtain different doubling times. The cultures were diluted daily to maintain continuous log-phase growth. For stationary-phase cultures, Mm/pBP10 was grown in the absence of kanamycin for 20 days. Plasmid loss was determined by CFU by plating on 7H10 with and without kanamycin. The segregation constant (*s*) was determined as previously described (Gill et al., 2009). Briefly, the growth rate and segregation constant of Mm was determined by using the following equations:

$$N(t)=N(0)e^{rt}$$

$$f(t)=f(0)e^{-rst}$$

Where *N*=the total number of bacteria, *r*=population growth rate, *t*=time in days and *f*=the frequency of plasmid-bearing bacteria at time *t*. *r* and *rs* was estimated by fitting regression lines through plots of *ln[N(t)]* and *ln[f(t)]* versus *t* (referred to as *SlopeN* and *Slopef*). *N* and *f* were estimated by CFU on plates with and without kanamycin. The value for *s* was calculated by dividing the estimate for *rs* by the estimate for *r*. The segregation constant *s* for Mm was 0.112 ± 0.001 (SEM). The in vitro determined segregation constant was then used to calculate the growth (*r*) and death rate (δ) of Mm during intracellular growth in THP1 and J774A.1 cell lines. The growth rate and death rate of Mm in THP1 and J774A.1 cell lines were determined by using the following equations:

$$r(t)=[SlopeN - SlopeP] / s$$

$$\delta(t)=r(t) - SlopeN$$

SlopeN and *SlopeP* were estimated from plots of *ln[N(t)]* and *ln[P(t)]* versus *t* (*P* is the number of bacteria carrying the plasmid). The number of dead bacteria for each time interval was determined using the equation:

$$D(t_f)=D(t_s) + \delta N(t_s)[e^{(r-\delta)(t_f-t_s)} - 1] / (r - \delta)$$

where *D(t)* is the total number of bacteria killed at that interval. The total cumulative bacterial burden (CBB) was determined by adding *N(t)+D(t)*.

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