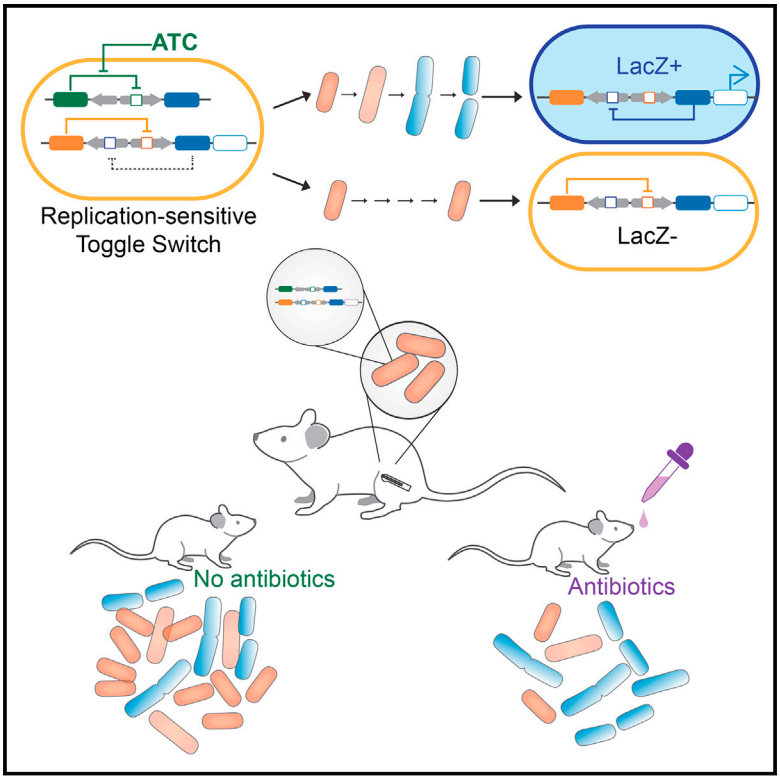
Brief Report

Cell Host & Microbe

Using Engineered Bacteria to Characterize Infection Dynamics and Antibiotic Effects In Vivo

Graphical Abstract



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In Brief

Using *Escherichia coli* engineered with a genetic toggle switch, Certain et al. demonstrate ongoing bacterial replication in a mouse model of chronic infection, even with antibiotic treatment. This finding runs contrary to the expectation that antibiotics preferentially eliminate actively dividing bacteria and highlights the in vivo utility of synthetic biology.

Highlights

- *E. coli* engineered with a genetic toggle switch to monitor bacterial behavior in vivo
- Many bacteria continue to replicate during chronic infection
- Antibiotic treatment enriches for non-dividing bacteria in vitro, but not in vivo
- Non-replicating bacteria do not necessarily confer antibiotic tolerance in vivo

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Brief Report

Using Engineered Bacteria to Characterize Infection Dynamics and Antibiotic Effects In Vivo

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SUMMARY

Synthetic biology has focused on engineering microbes to synthesize useful products or to serve as living diagnostics and therapeutics. Here we utilize a host-derived *Escherichia coli* strain engineered with a genetic toggle switch as a research tool to examine in vivo replicative states in a mouse model of chronic infection, and to compare in vivo and in vitro bacterial behavior. In contrast to the effect of antibiotics in vitro, we find that the fraction of actively dividing bacteria remains relatively high throughout the course of a chronic infection in vivo and increases in response to antibiotics. Moreover, the presence of non-dividing bacteria in vivo does not necessarily lead to an antibiotic-tolerant infection, in contrast to expectations from in vitro experiments. These results demonstrate the utility of engineered bacteria for querying pathogen behavior in vivo, and the importance of validating in vitro studies of antibiotic effects with in vivo models.

Much of our understanding of how antibiotics affect bacteria comes from in vitro work, but we do not always know how well these findings translate into in vivo settings. Recent work using fluorescent reporters has begun to examine the behavior of pathogens in animal models of infection (Claudi et al., 2014; Helaine et al., 2014; Manina et al., 2015; Saliba et al., 2016). Synthetic biology offers a complementary approach, in which synthetic gene circuits can be constructed that register complex inputs, such as a count of multiple events (Friedland et al., 2009) or memory of a much earlier event (Kotula et al., 2014). In this paper, we used *Escherichia coli* engineered with a genetic toggle switch to determine the proportion of bacteria that are actively dividing in a mouse model of chronic infection, so as to characterize infection dynamics and antibiotic effects in vivo, as well as to compare in vivo and in vitro bacterial behavior.

The *E. coli* strain PAS133 carries a bistable genetic toggle switch in the form of an inducible *lacZ* element, such that upon exposure to anhydrotetracycline (ATC) the bacteria switch from lacZ− to lacZ+, and remain lacZ+ even after ATC is removed (Figure 1A) (Gardner et al., 2000; Kobayashi et al., 2004; Kotula et al., 2014; Lee et al., 2016). The toggle switch is based on the cI/Cro system from lambda phage, and at baseline is in the cI (lacZ−) state. Because the switch from lacZ− to lacZ+ requires the concentration of cI protein to fall by dilution, the design of the circuit predicts that response to the ATC trigger requires about three to four cell divisions (Kotula et al., 2014; Shea and Ackers, 1985). Therefore, by treating a population of PAS133 cells with ATC, and then plating on indicator media to distinguish lacZ− from lacZ+ cells, one can determine the proportion of cells that were actively dividing at the time of ATC exposure. We first confirmed this predicted behavior in vitro, and then used it to study the behavior of *E. coli* PAS133 in a mouse model of chronic orthopedic hardware infection.

To confirm in vitro that *E. coli* PAS133 switches from lacZ− to lacZ+ upon exposure to ATC during active growth, but not during stationary phase or halted-growth conditions, we tested the response to ATC under various growth conditions and at various points of the growth curve. As predicted, actively dividing bacteria rapidly switched from lacZ− to lacZ+, but bacteria whose growth had been stopped by low temperatures, lack of nutrients (culturing in saline), or reaching stationary phase did not change from lacZ− to lacZ+ in the presence of ATC (Figures 1B and 1C). Furthermore, bacteria growing more slowly changed to lacZ+ more slowly (Figures S1A and S1B), supporting the claim that the change to lacZ+ state depends on cell division.

We next used this strain to explore the in vitro response of bacteria to antibiotics. Quinolones are more effective in vitro against actively dividing bacteria (Zeiler, 1985), and therefore we predicted that treating with levofloxacin (a quinolone antibiotic) would enrich the bacterial population for non-dividing cells. Indeed, treatment with levofloxacin caused a decrease in the proportion of bacteria that changed to lacZ+ upon exposure to ATC (Figures 1D and 1E). Moreover, the increase in antibiotic tolerance seen as PAS133 approached stationary phase correlated with the decreasing response to ATC (Figure 1C), further validating the toggle switch as a marker of cell division and

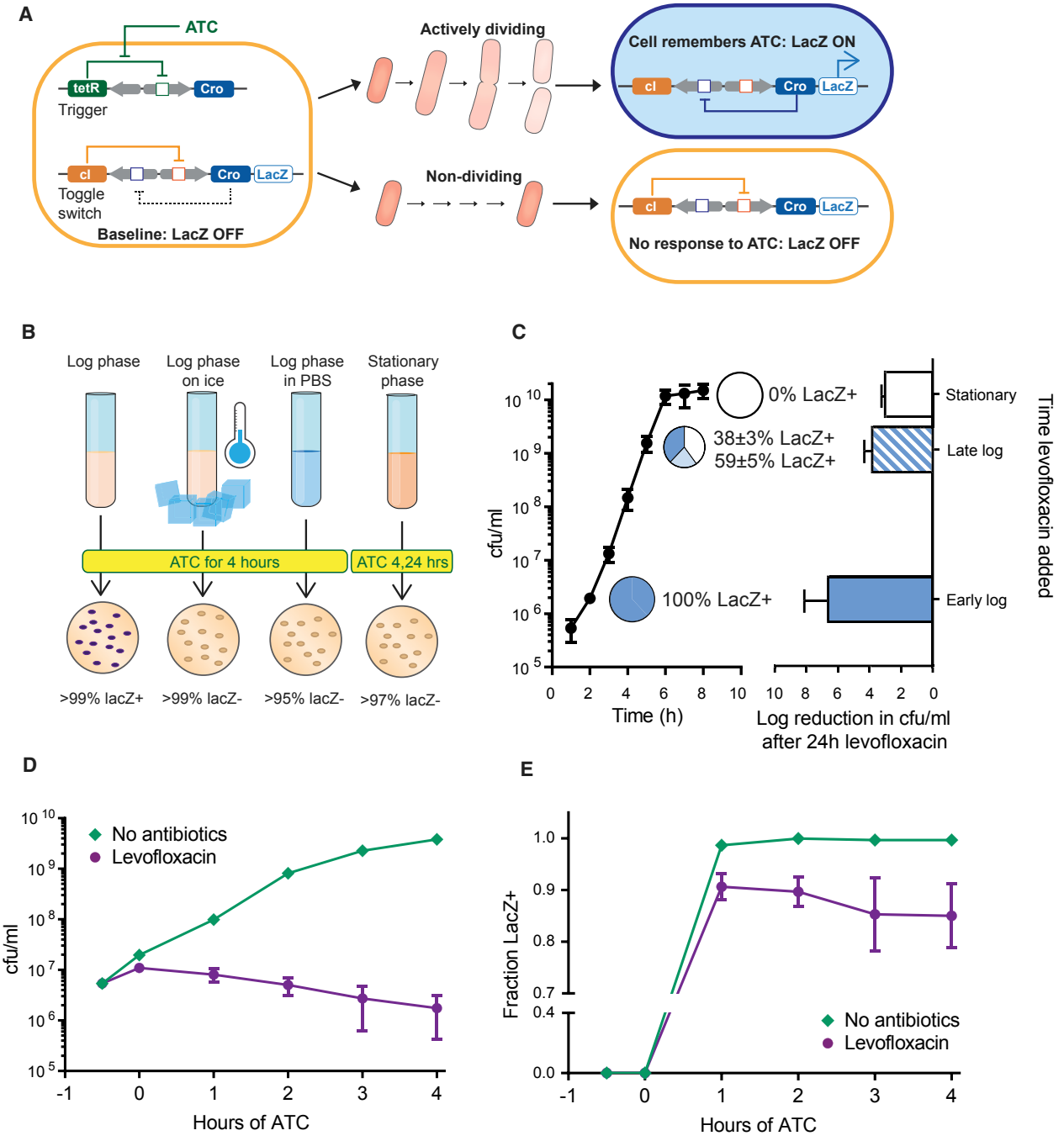

Figure 1. Engineered *E. coli* PAS133 Reports Bacterial Cell Division In Vitro

(A) Diagram of the genetic toggle switch and trigger. Exposure to ATC causes actively dividing *E. coli* PAS133 to switch to the Cro/lacZ+ state; non-dividing *E. coli* PAS133 stay in the cI/lacZ− state.

(B) Schematic of the in vitro experiments. For log-phase bacteria, >7,000 colonies were observed over four independent experiments; two colonies were lacZ−. For stationary-phase bacteria treated with ATC for 4 hr, >6,000 colonies were observed over four independent experiments; three were lacZ+. For stationary-phase bacteria treated with ATC for 24 hr, 350 bacteria were observed (three biological replicates); seven were lacZ+ (2%). For the log-phase bacteria placed on ice, 152 colonies were observed (three biological replicates); none were lacZ+. For the log-phase bacteria in PBS, 601 colonies were observed (six biological replicates); 23 were lacZ+ (3.8%).

(C) Correlation of growth phase with response to ATC (1 hr of treatment at 1 μg/mL) and susceptibility to levofloxacin (24 hr of treatment at 500 ng/mL). Data represent the mean and SD of six biological replicates (two independent experiments each with three biological replicates). The fraction lacZ+ at T = 5 is shown separately for each experiment.

(D and E) Effect of low-dose levofloxacin (50 ng/mL) on bacterial population (D) and response to ATC (E) in vitro; mean and SD of three biological replicates. See also Figure S1.

demonstrating the greater susceptibility of actively dividing bacteria to quinolones.

After confirming the behavior of *E. coli* PAS133 in vitro, we used this strain to determine the growth status of bacteria in a mouse model of chronic orthopedic hardware infection (Figure 2) and compared the in vivo behavior in this infection model to the in vitro results. In humans, orthopedic hardware infections are notoriously recalcitrant, often requiring surgical removal of the infected device, as antibiotics alone do not reliably cure these infections (Tande and Patel, 2014). This refractoriness to treatment is typically explained by two hypotheses: the antibiotic cannot get to the bacteria at adequate concentrations (due to biofilm formation or sequestration inside cells) (Archer et al., 2011; Kalinka et al., 2014; Löffler et al., 2014; Tuchscherr et al., 2016), and/or the bacteria are not actively replicating and are therefore tolerant of antibiotic exposure (Allison et al., 2011; Cohen et al., 2013; Conlon et al., 2016; Grant and Hung, 2013). We used *E. coli* PAS133 to explore this second hypothesis—specifically, we determined the proportion of bacteria in a mouse model of chronic orthopedic hardware infection that were actively dividing, and how treatment with levofloxacin affected this proportion. Though the most common causes of orthopedic infections in humans are staphylococci (Tande and Patel, 2014), *E. coli* can also cause device-associated infections (Zmistowski et al., 2011), and we confirmed histologically that infection with *E. coli* PAS133 causes an inflammatory response in this animal model (Figures S2A and S2B).

Mice had a plastic pin implanted in the femur, and PAS133 was inoculated into the surgical site during surgery (Figure 2A) (Bernthal et al., 2010). Every week, a subset of mice was injected with ATC and then euthanized the following day. Bacteria from the infected legs were plated to determine bacterial burden in the mouse and the fraction of the bacteria that were lacZ+ (Figures 2B–2E). At the beginning of the infection (first 24 hr), all of the bacteria changed from lacZ− to lacZ+ in response to ATC, i.e., all were actively dividing, as in early log-phase growth in vitro. However, by post-operative day four (POD4), the fraction of bacteria that responded to ATC dropped to about half and remained at that proportion for the course of the infection (Figure 2D). This result indicates that by POD4 about half of the bacteria have entered a non-dividing state. Interestingly, the corollary is that many of the bacteria (approximately half of the population) continue to divide, even 6 weeks after the initial infection event. Since the bacterial population at the site of infection is not increasing, this ongoing cell division is presumably balanced by bacterial death or clearance by the immune system. These findings indicate that bacteria in a chronic infection do not mimic stationary phase bacteria in culture, even when the total bacterial population size remains relatively constant.

To determine the effect of antibiotics on the response to ATC in vivo, the infection was allowed to progress for 2 weeks, and then half of the mice were started on levofloxacin. If levofloxacin preferentially kills the actively dividing (ATC-responsive) bacteria, the proportion of bacteria that respond to the ATC trigger should decrease on antibiotic therapy, as we saw in vitro. Levofloxacin treatment did cause a decrease in total number of bacteria recovered from the mouse (median colony-forming units [CFU] 16,000 versus 1,300, p = 0.01 by Mann-Whitney test; Figure 2C). However, the proportion of surviving bacteria that responded to the ATC trigger failed to decrease, and in fact increased from 44% to 65% (p = 0.0004, Mann-Whitney test; Figure 2E). In other words, though mice on levofloxacin had a lower bacterial burden at the site of infection, the antibiotic treatment did not enrich for non-dividing bacteria and instead enriched for actively dividing bacteria, in contrast to the in vitro results (compare Figure 1E with Figure 2E).

One explanation for these results is that a subset of the bacterial population in the mouse has evolved resistance and is therefore better able to replicate in the face of antibiotics. To test this hypothesis, we screened bacteria isolated from the infected mouse legs for antibiotic resistance. We determined that the starting levofloxacin MIC (minimum inhibitory concentration) of *E. coli* PAS133 was 50 ng/mL. We screened 366 *E. coli* colonies from mice treated with levofloxacin for 1–4 weeks (189 lacZ+ and 177 lacZ−), and none showed robust growth at levofloxacin 50 ng/mL (Figure S2C). These results indicate that the emergence of resistance does not explain the enrichment of actively dividing bacteria at an infection site during antibiotic treatment. Additionally, these findings are in contrast to the relative ease with which quinolone resistance emerges in vitro (Lee et al., 2010; Ling et al., 2015).

Our results suggest that levofloxacin treatment does not preferentially kill dividing bacteria in vivo, in contrast to its effects in vitro. It is possible that some of the non-dividing bacteria in the mouse switch into a dividing state, thus becoming susceptible to killing. The heterogeneity in the data is consistent with this hypothesis, as we may be observing different states of a continuously fluctuating ecosystem. It may even be that the reduction in bacterial population by antibiotics is what triggers dormant bacteria to begin dividing, as suggested by our finding that (for time points from POD4 onward) the proportion of lacZ+ bacteria was inversely correlated with bacterial burden in the mouse (Figure 2F). Of note, with a higher dose of levofloxacin we were able to cure the mice (Figure 2G), indicating either that all of the bacteria divide at some point during the antibiotic treatment and the lower dose just wasn't reaching them all, or that the non-dividing bacteria can be easily killed with a slight increase in antibiotics. Either explanation runs counter to the typical perception of chronic infections, in which quiescent, non-dividing bacteria are presumed to be part of the population and to survive high-dose antibiotic treatment. Importantly, our findings indicate that the presence of non-dividing bacteria at an infection site does not necessarily lead to an antibiotic-tolerant infection, in contrast to expectations from in vitro experiments.

An assumption made by this study is that ATC is able to reach all of the bacteria, and therefore the bacteria that fail to change to lacZ+ are non-dividing, not simply unreachable by small molecules. Because in some mice all the recovered bacteria had changed to lacZ+, we are confident that the dose of ATC is adequate to reach the infecting *E. coli*. However, it is still possible that the bacteria that remain lacZ− reflect a sequestered population, rather than a non-dividing one (e.g., in a biofilm). In theory, one could test this hypothesis by doing immunohistochemical staining for the beta-galactosidase enzyme and determining if the lacZ+ bacteria are distributed differently in the tissue from the lacZ− bacteria. Unfortunately, in this mouse model the bacterial burden is too low for this technique to be

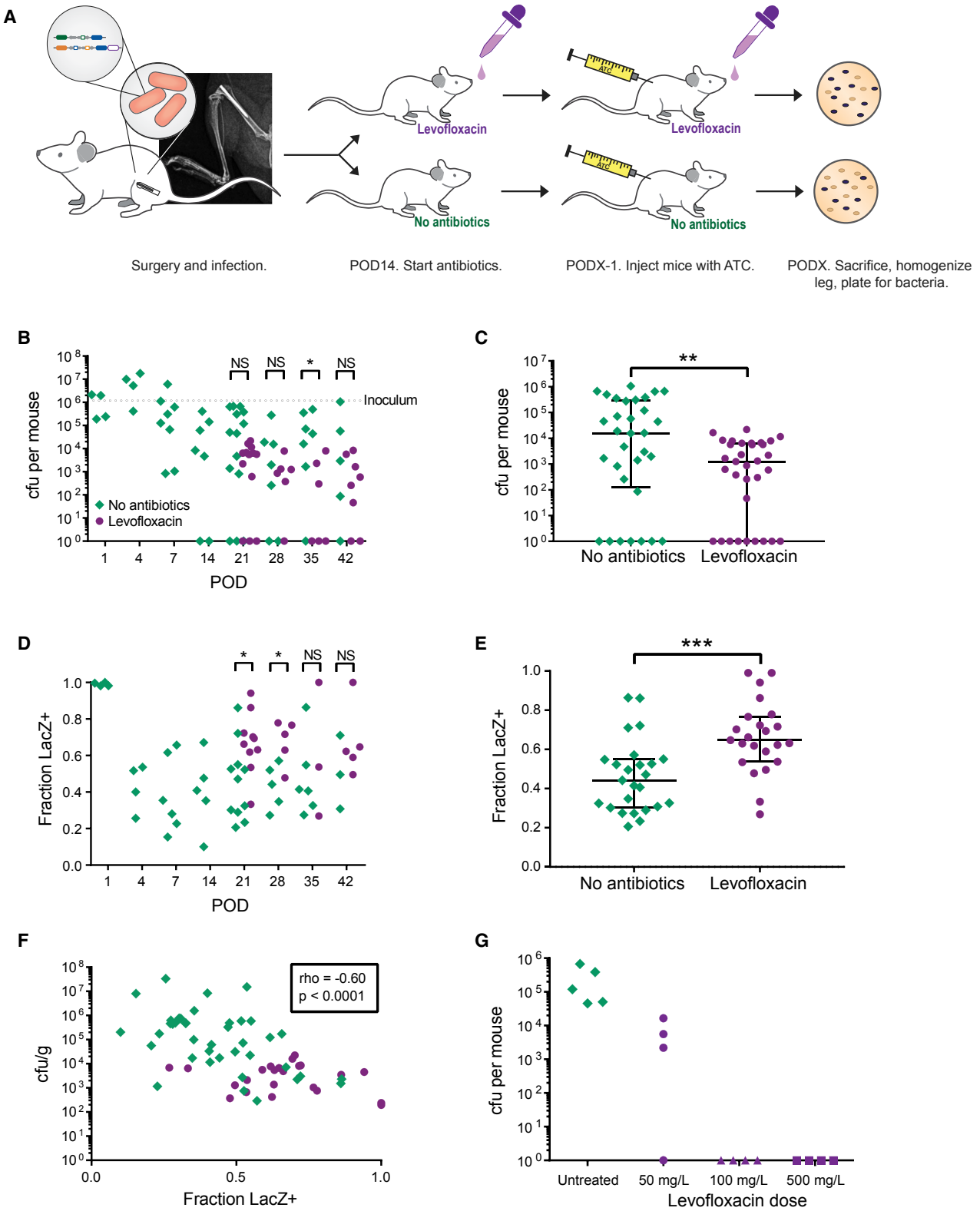

Figure 2. Engineered *E. coli* PAS133 Demonstrates Ongoing Replication in a Mouse Model of Chronic Infection

(A) Schematic of mouse experiments; radiograph shows the location of the pin.

(B–E) Bacterial burden (B and C) and fraction of recovered bacteria that were lacZ+ (D and E) in mice infected with 10^6 CFU *E. coli* PAS133 and injected with 0.08 mg ATC twice on the day prior to sacrifice. (C) and (E) show the combined data for mice sacrificed POD21–POD42.

(legend continued on next page)

useful (too few bacteria per histological section); however, in other animal models of infection it could work well.

Our study demonstrates the value of synthetic biology tools for probing bacterial behavior in vivo and illustrates that in vitro responses to antibiotic treatment do not always predict the in vivo responses. The *lacZ*$^{+/-}$ output of the genetic toggle switch used here is easily determined with standard microbiological techniques, can be engineered in various bacteria, and is adaptable to a variety of animal models of infection to study bacterial replication at the single-cell level. It requires no special equipment, such as a confocal microscope or cell sorter. Much work in synthetic biology to date has focused on engineering diagnostic or therapeutic devices (Braff et al., 2016; Daeffler et al., 2017; Danino et al., 2015; Hwang et al., 2017; Kojima et al., 2016; Riglar et al., 2017; Slomovic et al., 2015). We hope that future work will expand the use of engineered organisms as research tools for exploring the complexities of in vivo settings and the dynamic actions of antibiotics and other therapeutic agents.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2017.08.001.

AUTHOR CONTRIBUTIONS

L.K.C. conceived the project, designed and performed the experiments, and wrote the manuscript. J.C.W. contributed to project conception and edited the manuscript. M.J.P. assisted with the animal experiments and reviewed the manuscript. J.J.C. contributed to project conception, experimental design, and preparation of the manuscript.

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(C) Bacterial burden in mice off (n = 33; 15 males and 18 females) and on (n = 34; 11 males and 23 females) 50 mg/L levofloxacin.
(E) Fraction of recovered bacteria that were lacZ+ in mice off (n = 25; 10 males and 15 females) and on (n = 23; 8 males and 15 females) 50 mg/L levofloxacin. Bars in (C) and (E) indicate median and 25th/75th percentiles. NS, no significant difference; *p < 0.05, **p = 0.005, ***p = 0.0004 by Mann-Whitney test.
(F) Spearman correlation of bacterial burden (CFU/g) with the fraction of *E. coli* PAS133 that were lacZ+ from POD4 onward (n = 63; 18 males and 45 females).
(G) Bacterial burden in mice sacrificed on POD21; treated for 1 week with 50, 100, or 500 mg/L levofloxacin; or left untreated.
See also Figure S2.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Escherichia coli PAS133, created by Pam Silver's lab	Kotula et al., 2014	N/A
Chemicals, Peptides, and Recombinant Proteins		
Anhydrotetracycline hydrochloride	Abcam Biochemicals	Ab145350
Levofloxacin hydrochloride	Enzo Life Sciences	ALX-380-292-G005
Acrylic compound (Objet VeroClear RGD810) for 3D-printed plastic pins	Stratasys	SDS-06119
Experimental Models: Organisms/Strains		
Mouse: CD-1	Charles River Labs	Crl:CD1(ICR)
Other		
Custom K-wire, Titanium 6AL4V, 0.5 mm diameter, 10 mm length	Modern Grinding	N/A
Custom plastic pin, 0.4 mm diameter, 8 mm length, printed on a Stratasys Objet500 Connex 3D printer	Wyss Institute for Biologically Inspired Engineering	N/A
Bio-Gen PRO200 homogenizer	PRO Scientific	01-01200
Multi-Gen 7XL homogenizer probes	PRO Scientific	02-070MGXL-12

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, James J. Collins (jimjc@mit.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacteria

Escherichia coli strain PAS133 and its construction have been described previously (Kotula et al., 2014). This strain, originally isolated from laboratory mouse feces, has been engineered with a bistable genetic toggle switch based on the cI/Cro element from lambda phage. The components were constructed by a combination of PCR, commercial synthesis, and overlap extension PCR, then inserted into *E. coli* TB10 by recombineering. The elements were then transferred into a mouse fecal *E. coli* by P1*vir* transduction. The toggle switch comprises both a reporter element and a trigger element: the reporter element is the *cI*/*cro* operon inserted upstream of *lacZ*; the trigger element is the *tetA* promoter upstream of a second copy of *cro* (Figure 1A). In the ''off'' state, the cI protein is expressed, inhibiting the expression of *cro* and *lacZ*. Exposure to the trigger anhydrotetracycline (ATC) causes an increase in *cro* expression, which in turn represses *cI*, ultimately de-repressing *cro* and allowing expression of *lacZ*, thereby changing the cell from lacZ− to lacZ+. Mechanistically, this strain switches upon repression of new cI protein synthesis by Cro protein expressed from the trigger element, followed by about 8- to 16-fold dilution of the cI protein over about 3-4 cell divisions, which results in a decrease from > 99% cI functional activity to about 20%–40% activity (Ackers et al., 1982; Shea and Ackers, 1985), resulting in Cro expression from the cI/Cro toggle switch. These calculations are consistent with the measurements of switching times reported here and by Kotula et al. (2014), as well as with the observation that switching is not observed in non-growing cells (Figures 1B and 1C). The Cro protein is known to be somewhat unstable in cells, while the cI protein appears to be stable (Pakula et al., 1986). As protein degradation increases in stationary phase (Gottesman, 2003), the reversion from the Cro state to the cI state is not surprising (Figures S1C, S2D, and S2E). *E. coli* were cultured in LB broth at 37°C and plated either on LB plates containing x-gal 60 μg/ml (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), with or without kanamycin 25 μg/ml, or on MacConkey agar.

Experimental Animals

CD1 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in a Harvard University animal facility under a 12 hr light/dark cycle, with ad libitum access to water and standard chow. Mice were purchased at age 7 weeks and underwent surgery at age 8 weeks; all animals were used in scientific experiments for the first time. All mice used in experiments were socially

housed, except for the first three days after surgery, when they were housed individually. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Harvard University Faculty of Arts and Sciences, and all animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Both sexes of mice were used, with the exact numbers of each sex in each experiment indicated below or in the figure legends. Female mice were preferred, due to ease of long-term cohabitation; however, male mice were also included in keeping with new guidelines requiring the use of both sexes in animal experiments. The study was not designed or powered to detect differences in outcome between the sexes, as the focus was on the behavior of the bacteria, not the mice; however, no differences were noted. Mice were assigned to antibiotic treatment groups at the time of surgery. They were not randomized, but rather were assigned to groups (antibiotics versus no antibiotics) such that each group had mice from each day of surgery (for experiments involving more than 12 mice, which took place over multiple days). There was no blinding with regards to treatment group, as researchers were responsible for administering the antibiotic treatment. After recovering from the surgery, the mice appeared healthy throughout the course of the experiment; they had appropriate weight gain, were well-groomed, and moved about the cage without difficulty.

METHOD DETAILS

In Vitro Experiments

Effect of Growth Phase

To characterize the behavior of *E. coli* PAS133 in vitro (Figure 1B), bacteria were grown overnight in LB. For characterization of stationary-phase bacteria, these overnight cultures were used without further adjustment. For characterization of actively dividing bacteria, the overnight culture was diluted 1:1000 in fresh, pre-warmed LB media and incubated with agitation at 37°C for two hours. Response to ATC was tested by adding 1 μg/ml ATC to the bacterial cultures and incubating for four hours. Stationary-phase bacteria were incubated for 24 hr in addition to four hours. Alternatively, log-phase cultures (i.e., those that had been diluted and then cultured for two hours) were placed on ice for 3-4 hr, or re-suspended in phosphate buffered saline (PBS) for 3-4 hr, prior to the addition of ATC.

To correlate growth phase with response to ATC and with susceptibility to levofloxacin (Figure 1C), overnight cultures were diluted 1:10,000 in pre-warmed LB at time 0 and put in a 37°C shaker. Every hour, spot dilutions were made for quantification of cfu/ml. At times 2, 5, and 8 hr, 1 mL samples were removed from the culture (2 samples per culture flask) and transferred to culture tubes. Levofloxacin or ATC was added, to a final concentration of 500 ng/ml or 1 μg/ml, respectively. These treated samples were cultured at 37°C with agitation. After one hour (ATC-treated samples) or 24 hr (levofloxacin-treated samples), they were removed from the incubator. The ATC-treated samples were diluted and plated on LB-kan-x-gal plates for quantification of fraction lacZ+. The levofloxacin-treated samples were centrifuged, media removed, and re-suspended in an equal volume of PBS before making spot dilutions for cfu quantification. For the early-log samples, instead of spot dilutions the entire sample was plated. The degree of killing was calculated by comparing the cfu/ml at the time levofloxacin was added to the cfu/ml after 24 hr of levofloxacin treatment. For the early-log samples, five of six replicates had no remaining bacteria, and therefore for the purposes of calculating the logs of killing we used a value of 0.1 cfu/ml. The remaining sample had about 200 cfu/ml.

Effect of Antibiotic Treatment

To characterize the effect of levofloxacin on the behavior of *E. coli* in vitro (Figures 1D and 1E), overnight cultures were diluted 1:1000 in LB and incubated at 37°C with agitation for 90 min, at which point levofloxacin was added, to a final concentration of 50 ng/ml (at the MIC). Thirty minutes later, ATC was added, to a final concentration of 1 μg/ml. Cells were plated every hour for quantification of cfu/ml and fraction lacZ+.

Effect of Growth Rate

To determine the effect of different growth media on PAS133 (Figures S1A and S1B), overnight cultures were rinsed twice with PBS and resuspended in M9 media with 0.5% casamino acids. They were then diluted 1:1000 in M9-casamino acids with a variable amount of dextrose (0.3, 3, or 30 μM), or in LB. They were grown at 37°C for two hours, then induced with ATC (time 0). To measure the rate of return to the lacZ− state once the bacteria reached stationary phase (Figure S1C), log-phase bacteria were induced with ATC for four hours, then rinsed and resuspended in LB. They were diluted 1:10 at time 0 (resulting concentration 2×10^8 cfu/ml) then monitored for the loss of the lacZ+ state over time.

Determining Minimum Inhibitory Concentration

We determined the MIC of *E. coli* PAS133 by standard liquid media techniques (Wiegand et al., 2008). Briefly, bacteria were grown overnight, then diluted 1:1000 in fresh media containing two-fold serial dilutions of antibiotic. These bacterial cultures were incubated in a 96-well microplate at 37°C, with agitation, for 24 hr and then observed for growth. The lowest concentration that inhibited visible growth was taken as the MIC, which for *E. coli* PAS133 was 50 ng/ml. We screened the bacteria recovered from the mice for antibiotic resistance by growing cultures in a 96-well microplate for ~6 hr and diluting 1:30, and then pinning the diluted cultures onto LB agar plates containing levofloxacin 50 ng/ml. If there were a meaningful increase in MIC then we would expect the bacteria to demonstrate robust growth at this concentration. As a control, we pinned onto plates containing no antibiotic to confirm technique; we pinned first onto the antibiotic plate and then onto the no-antibiotic plate without reloading the pins.

In Vivo Experiments

Surgical Technique

Eight-week-old CD1 mice underwent surgery as outlined by Bernthal et al. (2010). Briefly, mice were anesthetized with isoflurane, prepped and draped for surgery in a sterile fashion, and a 1 cm pin was inserted retrograde into the intramedullary canal. For the purposes of the radiograph in Figure 2A, the pin was made of titanium (manufactured by Modern Grinding, Port Washington, WI). For mice infected with *E. coli*, it was made of biocompatible plastic (3D-printed at the Wyss Institute, Harvard University). The pins were 3D-printed attached to a solid base, with 12 pins per block. Just prior to surgery, the pins were sterilized by immersion of the block in 95% ethanol, then detached from the block using sterile scissors. After pin insertion, the end of the pin was inoculated with 10^6 cfu *E. coli* PAS133. Bacterial counts were confirmed by plating 10^4-fold dilutions of the infecting stock, incubating at 37°C for 24 hr, and counting cfu.

Antibiotic Treatment of Mice

For mice treated with levofloxacin, treatment began on post-operative day (POD) 14 and was administered in the drinking water at a concentration of 50 mg/L (unless otherwise specified). The antibiotic-treated water was changed twice per week. This antibiotic was chosen because it had a low MIC for the target strains and is commonly used to treat human infections with *E. coli* (Osmon et al., 2013). In addition, it has excellent oral bioavailability and is water soluble, facilitating the administration of antibiotics via the drinking water (Marx et al., 2014). To determine the best dose of levofloxacin to use (Figure 2G), we gave four mice levofloxacin 50 mg/L, four mice levofloxacin 100 mg/L, four mice levofloxacin 500 mg/L, and left five mice untreated; all 17 of these mice were female. All the mice treated with 100 mg/L or 500 mg/L were cured; one of the mice treated with levofloxacin 50 mg/L was cured; none of the control mice were cured. We therefore used the lowest concentration of levofloxacin for *E. coli*-infected mice because it reduced the bacterial burden in the mouse but did not cure the infection. Levofloxacin 50 mg/L provides an estimated dose of 10 mg/kg/day (Bachmanov et al., 2002), which is comparable to human dosing.

Administering Anhydrotetracycline

Mice infected with *E. coli* PAS133 were injected with ATC 0.08 mg intraperitoneally (IP) twice on the day prior to sacrifice unless otherwise indicated in the results (Loessner et al., 2009); the doses were given approximately 8 hr apart, and the second dose preceded euthanasia by at least 16 hr. By waiting 16 hr from the second dose, we ensured that there was not enough residual ATC in the tissues to cause the bacteria to switch on the plate. This was validated by injecting uninfected mice (n = 3 female) with ATC, sacrificing 16 hr after the second dose, homogenizing their legs, then mixing the homogenates with log-phase *E. coli* PAS133 prior to plating on indicator media; none of the resulting colonies were lacZ+. In contrast, if we sacrificed mice (n = 4 female) only four hours after a single dose of ATC, homogenized the legs, and mixed the homogenates with log-phase *E. coli* PAS133, then all the colonies were lacZ+. Likewise, if we injected with 0.24 mg ATC x 2, rather than 0.08 mg, sacrificed 16 hr after the second dose, homogenized the legs, then mixed the homogenates with *E. coli* PAS133, about 20% of colonies were lacZ+ (n = 1 male, 1 female; 57 blue cfu out of 252 total for one, 55 out of 265 for the other). We did not wait longer than a day after ATC injection because the lacZ+ state slowly reverts to lacZ− in the mouse (Figure S2D, n = 9 female; Figure S2E, n = 17 female, 14 male). To confirm that the *E. coli* did not to switch to lacZ+ in the absence of ATC treatment, we sacrificed mice (n = 4 males, 3 females) on POD20 without administering ATC; no colonies were lacZ+ (> 1000 cfu observed from 2 mice on levofloxacin and 5 mice not on antibiotics).

Processing of Mouse Tissue

After euthanasia, the mouse's infected hind limb was drenched with iodine then removed at the hip with sterile scissors and forceps. The skin was removed as well as the distal leg (from the mid-tibia down). The entire remaining leg – bone, muscle, cartilage, pin – was morcellated with scissors then homogenized in 3 mL sterile phosphate buffered saline (PBS). The volume of the homogenate (usually about 4.0 ml) was recorded to allow calculation of the total bacterial burden in the mouse leg. Serial dilutions of the homogenate were plated onto LB agar containing 60 μg/ml x-gal, with or without kanamycin, and incubated at 37°C for 24 hr.

To confirm that the bacteria recovered from the mice were PAS133, we screened a subset of the colonies recovered from each mouse for kanamycin resistance and response to ATC. (Kanamycin is the selectable marker in the toggle switch.) We screened 763 colonies from 38 mice, and found 48 colonies from eight mice that were kanamycin sensitive. Data from these eight mice were not included in the results, and we subsequently plated the leg homogenates on LB-x-gal agar containing kanamycin, to avoid skewing the results with contaminating bacteria. We additionally screened 189 kanamycin-resistant lacZ- colonies from 19 mice for response to ATC; all 189 flipped to lacZ+ upon exposure to ATC. For mice from whom fewer than five colonies of bacteria were recovered (n = 5 mice), the fraction that were lacZ+ was not included in the results, as it is too few colonies to reflect an accurate percentage.

Histology

Mice infected with *E. coli* PAS133, or mice that underwent surgery but were inoculated with sterile liquid, were sacrificed on POD23. Their legs were fixed in formalin for 72 hr, then transferred to 70% ethanol and transferred to the Rodent Histopathology Core at Harvard Medical School. There, they were decalcified in EDTA, sliced along the sagittal plane, embedded in paraffin, and sectioned.

QUANTIFICATION AND STATISTICAL ANALYSIS

Initial estimates of how many mice would be needed per group to see a significant difference were based on prior work using this mouse model (Bernthal et al., 2010). All *p*-values are two-sided. For comparing groups, we used the Mann-Whitney test rather than a *t* test, due to small sample size/non-normal distribution. For the in vivo experiments, visual inspection of the data suggested

that there was no time-dependent effect of antibiotics. That is, the bacterial burden of mice on antibiotics was the same regardless of the length of antibiotic treatment; this impression was confirmed by finding no difference by either the Mann-Whitney test or the Kolmogorov-Smirnov test for any pairwise comparison of time-points from POD21 onward for mice within the same treatment group. We therefore combined data for POD21-42 to increase power to detect a difference between treated and untreated mice. Data from each time-point may be seen in Figures 2B and 2D.