Supplementary Information

Dynamic Pneumococcal Adaptations Support Bacterial Growth and Inflammation During Coinfection with Influenza

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Tn-seq Sample Preparation and Illumina Sequencing

Genomic DNA from the frozen pellets from each of the three time points (pre-selection (inoculum) (t_1) and post-selection (after infection) at 12 h (t_2) or 24 h (t_3) pbi) was digested overnight at 37°C with MmeI (NEB), the 5' phosphate group was removed with Calf intestinal alkaline phosphatase (NEB) after which DNA was extracted with the Geneaid Small Fragment DNA kit and dissolved in H_2O. An adapter was ligated with T4 DNA ligase (NEB) onto the overhang left by MmeI after which a PCR was performed with the adapter-ligated samples as template. One primer was complementary to the mini-transposon inverted repeat sequence and one primer was to the adapter (Table S1). The resulting PCR product was 140 bp in length and was amplified with the following parameters: 95°C for 30 sec, 26 cycles of 10 sec at 95°C, 25 sec at 55°C and 45 sec at 72°C, 1 cycle of 10 min at 72°C, and held at 4°C. The PCR product was purified using the Agencourt AMPure XP kit, dissolved in H_2O and sequenced in rapid run mode on an Illumina HiSeq 2000 according to the manufacturers protocol (Illumina). A 6-nucleotide barcode sequence was included with the adapter so that harvested libraries coud be multiplexed. Following 30 sequencing cycles, raw data is extracted, split into different samples based on the 6-nucleotide-barcode sequences and stripped from the barcode and four nucleotides of the adapter sequence. This resulted in 5-15x10^6 pneumococcal specific reads per flow cell lane.

Fitness Calculations

Fitness calculations were performed as previously described (1, 2). Following sequencing, reads were mapped to the D39 genome using Bowtie (3). Bowtie parameters (-m_1–n_1–best) were set so that reads could contain a single mismatch but were only allowed if they mapped to a unique location. If mapping to multiple sites was possible, the read was excluded from the analyses.

Approximately 8% of the reads had to be discarded because they could be mapped to multiple sites such as endogenous transposon related genes or other repeated sequences (6%) or could not be mapped to anywhere and were categorized as junk sequences (2%). Insertions that mapped to a location within the first 5% or the last 10% of a gene were removed from the analysis to minimize the influence of truncated functional genes. On average, 250 reads were mapped per insertion/time point. Only insertions with >15 reads in the inoculum were included in the analyses because insertions with a low number of reads slightly fluctuate over time and can influence the data disproportionately. The data were normalized to the total number of sequenced reads per time point (normalization factors were between 0.92 and 1.06). Fitness was calculated as the change in the number of reads at a specific location over time (see Main Text). Following fitness calculations, the values were normalized against a set of 'neutral' genes. These genes have no fitness effect and consist of pseudo genes and degenerate transposon related sequences. The same factor was then used to normalize the remaining dataset and make all fitness values relative to the WT D39 background. The normalization factors used for all datasets were small and were between 0.98 and 1.09. Each insertion was used to calculate the average fitness and standard deviation of the gene. A weighted average was used to control for fitness deviations due to insertions with small numbers of reads (<50 reads). This resulted in a small increase in replicate correlation and lower standard deviation.

To determine *in vivo* fitness and account for random loss of mutants during inoculation, the same proportion of insertion mutants that disappeared during *in vivo* selection were removed from the total number of insertions for each gene. The resulting set of insertions was then reanalyzed and the fitness recalculated. The resulting fitness (W_i, see Main Text) for each gene represents the growth rate per generation, which enables direct comparisons between experiments. To determine which genes differed with statistical significance, the fitness in influenza-infected mice was compared to the fitness in PBS-infected mice. Statistical significance was established when (i) fitness was composed of at least four data points, (ii) fitness deviated by at least 20%, and (iii) a one sample *t*-test with Bonferroni correction had a p-value less than 0.05.

Table S1: Tn-seq Primers: Primers and amplification cycle used to sequence the 140 bp Tn-seq region. One primer was complementary to the mini-transposon inverted repeat sequence and one to the adapter.

Tn-seq Primers	**Primer Sequence (5'-3')**	**Amplification Cycle**
P1-M6-MmeI	CAAGCAGAAGACGGCATACGAAGACCGGGGACTTATCATCCAACCTGT	95°C (30sec), [95°C (10sec), 55°C (25sec), 72°C (45sec) x25], 72°C (10min), 4°C (hold)
ADPT-Tnseq-PCR	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	

Generation of Single-gene Deletion Bacterial Mutants

Primers and cycling conditions used to generate single-gene deletion mutants (D39Δ*cbiO1*, D39Δ*purD*, D39Δ*1414*, D39Δ*1098*, and D39Δ*proB*) by SOEing PCR (4, 5) are in Table S2 and S3. Primers and cycling conditions used to confirm ERM resistance cassette insertion and target locus deletion in the knockout bacteria are in Table S3.

Table S2: Primers and Cycling Conditions used to Generate SOEing Regions for Locus Deletion. Forward (F) and reverse (R) primers and cycling conditions used to amplify the erythromycin (ERM) resistance cassette and regions flanking the D39 locus targeted for deletion with overhangs complementary to ERM resistance cassette (Regions A and B). In the Region A and B primer sets, the sequences in black are complementary to D39 genomic DNA and the sequences in red are complementary to ERM resistance cassette DNA.

Target Locus	Product		Primer Sequence (5'-3')	Amplification Cycle
SPD0058 1.26kb : base 55644-56906	Region A	F	CGCTTTGATTGCAGAATACTTCACAGC	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (80sec) x40], 72°C (7min), 4°C (hold)
		R	GTTTGCTTCTAAGTCTTATTTCC TCCTCAACCTCTTTTGCGAATTATTTACC	
	Region B	F	GAGTCGCTTTTGTAAATTTGG AGATATAAGAATAACGCGCCGTAGTCGC	
		R	CGACATTTTCAAACTCGTAAGTGAGG	
SPD1098 2.17kb : base 1127189-1129354	Region A	F	GCCAAAAGAACCACCTGATAGCC	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (90sec) x40], 72°C (7min), 4°C (hold)
		R	GTTTGCTTCTAAGTCTTATTTCC TTTTAGTCTCCTTTTCCGAATATTCTC	
	Region B	F	GAGTCGCTTTTGTAAATTTGG TGGCAAAATTAAAAATTGATGTAAATGATTTAC	
		R	GCCAAATCAAGGATGGACTCG	
SPD1414 1.23kb : base 1433092-1434318	Region A	F	CCAACTGCAAAGACACCAGGAACG	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (90sec) x40], 72°C (7min), 4°C (hold)
		R	GTTTGCTTCTAAGTCTTATTTCC AAAACCTCCTATTTTCGAACATTTATTC	
	Region B	F	GAGTCGCTTTTGTAAATTTGG TTTTCTGTAGAAATGGGGCTATCTTTTGC	
		R	CCGATACGGTGAACATAACTCTCAGG	
SPD2047 840bp : base 2023773-2024612	Region A	F	GCTATTATTTTCTTGCATATTACATTGG	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (90sec) x40], 72°C (7min), 4°C (hold)
		R	GTTTGCTTCTAAGTCTTATTTCC AGCTTATCCTCTAGCTCACTTTCTGTC	
	Region B	F	GAGTCGCTTTTGTAAATTTGG TATGATTTTGGGGCGTTATATCCCAGGG	
		R	CTCTATCAACCAATGAGATTCCATCTCC	
SPD0822 1.1kb : base 840495-841604	Region A	F	GCTGGTTGGCATAAGAGTGC	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (120sec) x40], 72°C (7min), 4°C (hold)
		R	GTTTGCTTCTAAGTCTTATTTCC AGTCTCACTCCGATTATTCATATTTATCC	
	Region B	F	GAGTCGCTTTTGTAAATTTGG AGGTAAACTATGGTGAGTAGAC	
		R	GCTGTGGGATTCAATGTGC	
	ERM	F	GGAAATAAGACTTAGAAGCAAAC	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (90sec) x40], 72°C (7min), 4°C (hold)
		R	CCAAATTTACAAAAGCGACTC	

Table S3: Primers and Cycling Conditions used to Generate Knockout Bacteria by SOEing PCR and Confirm Locus Deletion. Forward (F) and reverse (R) primers and cycling conditions used to recombine regions A, B, and ERM resistance cassette from Table S2 by SOEing PCR to delete single target loci. D39 was transformed with the product of SOEing PCR, and genomic DNA of the knockout bacteria were confirmed to lack the target locus and contain the ERM resistance cassette (primers and cycles in 'Confirmation of Locus Deletion').

SOEing PCR to Delete Target Locus			
Locus		**Primer Sequence (5'-3')**	**Amplification Cycle**
SPD0058	F	CGCTTTGATTGCAGAATACTTCACAGC	[94°C (30sec), 50°C (30sec), 72°C (3.25min) x34], 72°C (5min), 4°C (hold)
	R	CGACATTTTCAAACTCGTAAGTGAGG	
SPD1098	F	GCCAAAAGAACCACCTGATAGCC	[94°C (30sec), 50°C (30sec), 72°C (3.50min) x34], 72°C (10min), 4°C (hold)
	R	GCCAAATCAAGGATGGACTCG	
SPD1414	F	CCAACTGCAAAGACACCAGGAACG	[94°C (30sec), 50°C (30sec), 72°C (3.50min) x34], 72°C (10min), 4°C (hold)
	R	CCGATACGGTGAACATAACTCTCAGG	
SPD2047	F	GCTATTATTTTCTTGCATATTACATTGG	[94°C (30sec), 48°C (30sec), 72°C (3.50min) x34], 72°C (5min), 4°C (hold)
	R	CTCTATCAACCAATGAGATTCCATCTCC	
SPD0822	F	GCTGGTTGGCATAAGAGTGC	[94°C (30sec), 50°C (30sec), 72°C (4min) x34], 72°C (10min), 4°C (hold)
	R	GCTGTGGGATTCAATGTGC	
Confirmation of Locus Deletion			
Locus		**Primer Sequence (5'-3')**	**Amplification Cycle**
SPD0058	F	GCCCTTGCTGCTGGTATCGTGG	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (60sec) x40], 72°C (7min), 4°C (hold)
	R	GGCTTGACAATGGTGTCAACCG	
SPD1098	F	GCTGTAGGAAGTTTTGCTTTCGG	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (60sec) x40], 72°C (7min), 4°C (hold)
	R	CCGATTGGAGTTCCAGAGATTGG	
SPD1414	F	GCAATCTTTTGTTTGGGCTTATCGG	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (60sec) x40], 72°C (7min), 4°C (hold)
	R	GCTGGAATCAAAGAAAAACCAGC	
SPD2047	F	CGGATGTTTCTTTGACGATTGAAGATGGC	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (60sec) x40], 72°C (7min), 4°C (hold)
	R	CCCTAGAGGATCTAGACCAGCTGTTGGC	
SPD0822	F	CGTCAAATCGTTTCTGC	95°C (2min), [95°C (40sec), 48°C (30sec), 72°C (60sec) x40], 72°C (7min), 4°C (hold)
	R	GCCATTGTTTCTGGGTACG	

Pathogenicity of Single-gene Deletion Bacterial Mutants

Table S4 summarizes the log_{10} changes and statistical comparisons in lung bacteria, bacteremia, and viral load during naïve infection and IAV-coinfection with each of the knockout bacteria, compared to WT D39. The change in percent survival and significant differences in Kaplan-Meier survival curves during naïve infection and IAV-coinfection with each of the knockout bacteria compared to WT D39 is also summarized in Table S4.

Table S4: Summary of Pathogenicity of Knockout Bacteria. The log_{10} change (Δ) in the levels of lung bacteria, blood bacteria, and lung virus from Balb/cJ mice infected with PBS (mock) or 75 $TCID_{50}$ PR8 followed 7 d later by 10^6 CFU of WT D39 or the indicated knockout bacteria. Comparisons were made between average log_{10} bacteria or virus at 4 h pbi and 24 h pbi. Significance of the analysis of variance (ANOVA) using a Dunnett correction for multiple comparisons (to WT D39) on raw values is indicated in bold. The percent increase in survival of animals (groups of 10) infected with PBS (mock) or PR8 followed 7 d later by 10^6 CFU of indicated bacteria compared to WT D39. Significant differences in Kaplan-Meier survival curves by the log rank test are indicated in bold. Significance is *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001.

Bacteria	Δ Lung Bacteria (log_{10})				Δ Blood Bacteria (log_{10})				Δ Virus (log_{10})		Survival	
	PBS + Bacteria		PR8 + Bacteria		PBS + Bacteria		PR8 + Bacteria		PR8 + Bacteria		PBS	PR8
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h		
D39Δ1414	**-0.61****	-2.71	**-0.94****	**-0.83****	-3.79	-5.87	-1.65	**-4.97****	0.02	0.43	**100%****	**80%****
D39ΔcbiO1	**-1.36****	-5.86	**-2.05****	**-2.11****	-2.31	-6.56	-1.38	**-2.35****	0.15	0.29	**90%****	**40%*****
D39ΔpurD	**-0.74****	-0.47	**-0.44****	**-0.87***	-1.49	-1.28	-0.40	**-2.64****	-0.83	0.26	**100%****	**60%*****
D39Δ1098	**-0.59****	-2.17	**-0.76****	**-0.78****	-3.78	-3.51	-1.69	**-2.25****	-0.83	-0.93	**100%****	**90%****
D39ΔproB	**-1.07****	-1.70	**-1.08****	**-0.86***	-3.21	-6.56	-1.86	**-2.98****	-0.04	-0.19	**40%****	**0%†****

† Mean survival time at 7 d pbi

In vitro Growth of Single-gene Deletion Bacterial Mutants following Metabolic Starvation

Fig S1 shows the growth of each knockout bacteria and WT D39 in cultures supplemented with lung homogenate supernatants from PBS- or IAV-infected mice, following 5 h of metabolic starvation.

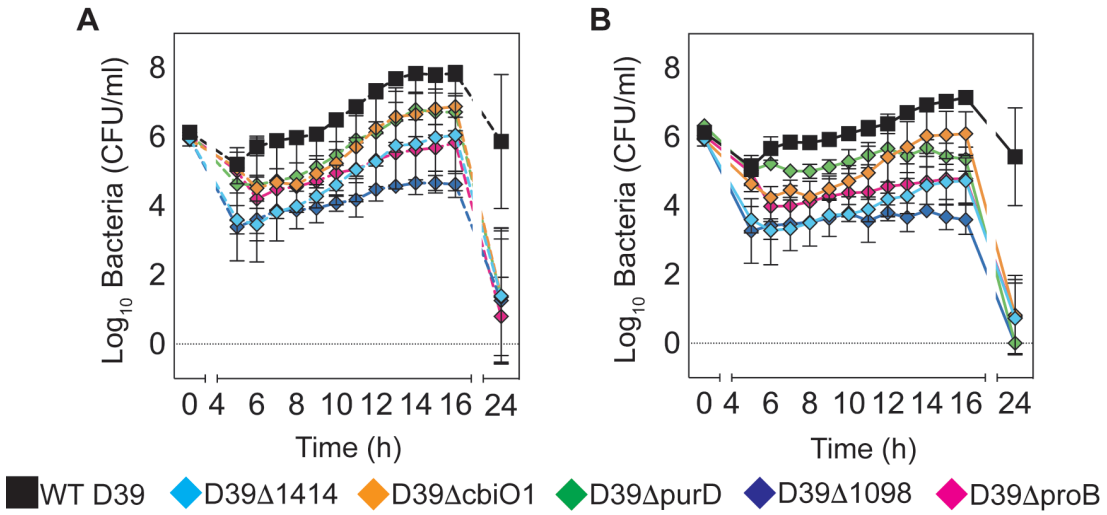

Fig S1: ***In vitro*** **Growth following Metabolic Starvation.** Bacteria were grown at 37°C in 1 ml of PBS for 5 h, then the cultures were supplemented with 0.5 ml lung homogenate supernatants from mice infected with PBS (mock) (Panel A) or 75 $TCID_{50}$ PR8 (Panel B). Cultures were sampled hourly (50 µl) for the following 11 h of culture, and then at 24 h of culture. Samples were serially diluted in PBS and plated on TSA (WT) (squares) or TSA-ERM (diamonds) plates. Bacterial titers were normalized to the total volume. From hours 7-16 of culture, each knockout bacteria was significantly lower than WT D39 ($p<0.05$) after the addition of lung supernatant from mock infected mice, except for D39Δ*purD* at 11 hours (Panel A). Also, from hours 7-16 of culture, each knockout bacteria was significantly lower than WT D39 ($p<0.05$) after the addition of supernatant from IAV-infected mice, except for D39Δ*cbiO1*, D39Δ*purD*, and D39Δ*proB* at 12 h (Panel B).

Kinetics of Pulmonary Cytokines, Chemokines, and Immune Cells during Naïve infection and IAV-Coinfection with Single-gene Deletion Bacterial Mutants

The absolute log_{10} picograms (pg) of all measured cytokines and chemokines in PBS- and IAV-infected animals are in Fig S2-S4. A heat map of the fold change in cytokines and chemokines is in Fig 5 of the main text. Pulmonary immune cells, quantified by flow cytometry according to the gating scheme in Fig S5, are shown in Fig S6. Additional cell kinetics are shown in Fig 6 of the main text.

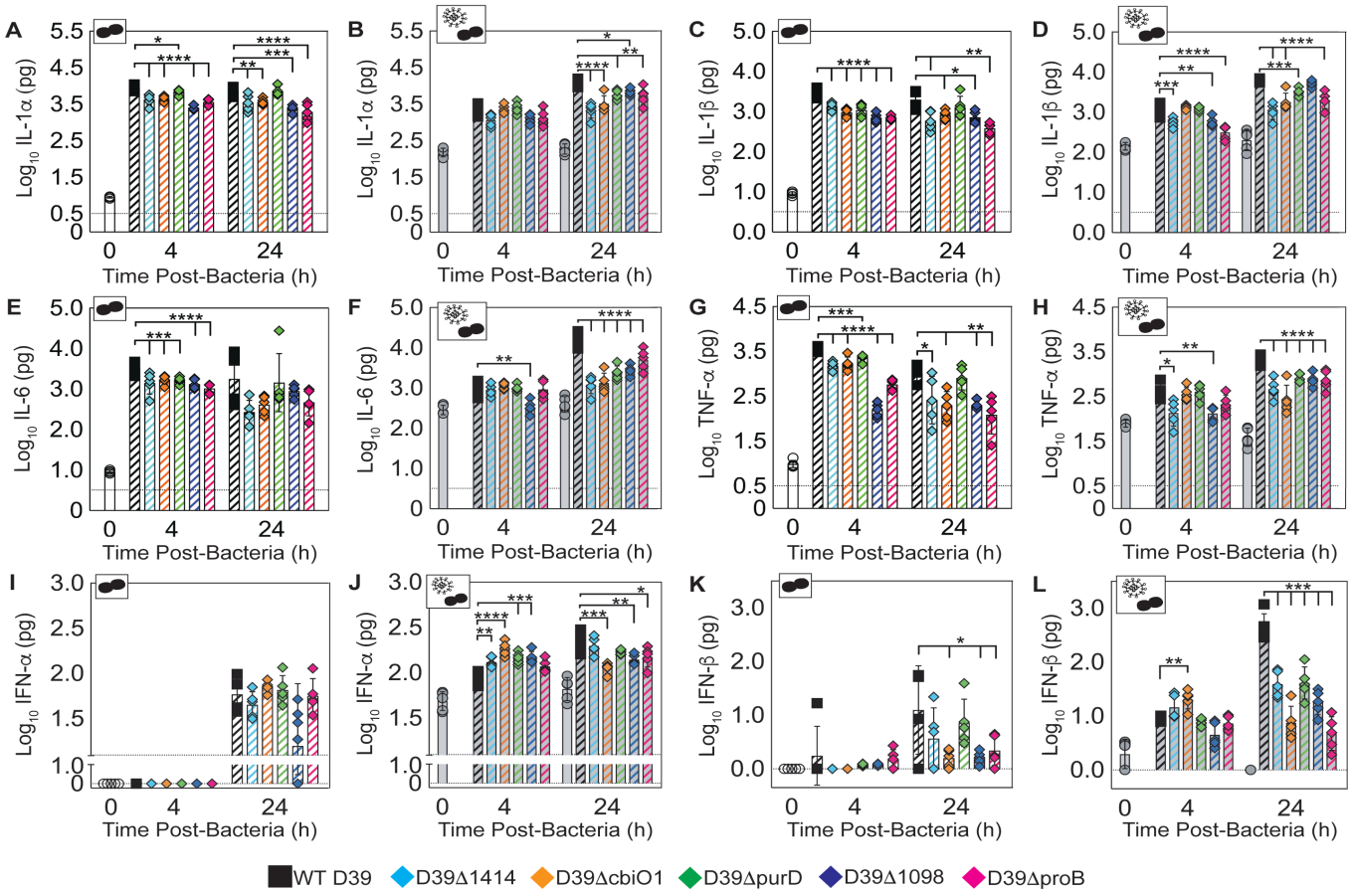

Fig S2: Pulmonary Cytokine Kinetics. Kinetics at 4 h and 24 h pbi of IL-1α (A-B), IL-1β (C-D), IL-6 (E-F), TNF-α (G-H), IFN-α (I-J), and IFN-β (K-L) from mice infected with PBS (mock) (Panels A, C, E, G, I, K) or 75 $TCID_{50}$ PR8 (Panels B, D, F, H, J, L) followed 7 d later with 10^6 CFU of the indicated bacteria. Each symbol (circles, squares, or diamonds) represents a single mouse, and the bars are the geometric mean ± standard deviation (SD). Mice were either uninfected (open white), influenza-infected only (solid grey), bacteria-infected (open hashed, colored), or influenza-bacteria coinfected (solid hashed, colored). Cartoons indicating infection status of study group (bacteria alone or virus plus bacteria) are in the upper left corner of each graph. The dashed line indicates the lower limit of detection (LOD), undetectable amounts are plotted at 0 log_{10} pg, and values extrapolated by 4PL analysis appear between 0 log_{10} pg and the LOD. Signficance is indicated as *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001. Additional cytokines are shown in Fig S3 and a heat map of cytokines and chemokines is in Fig 5 (Main Text).

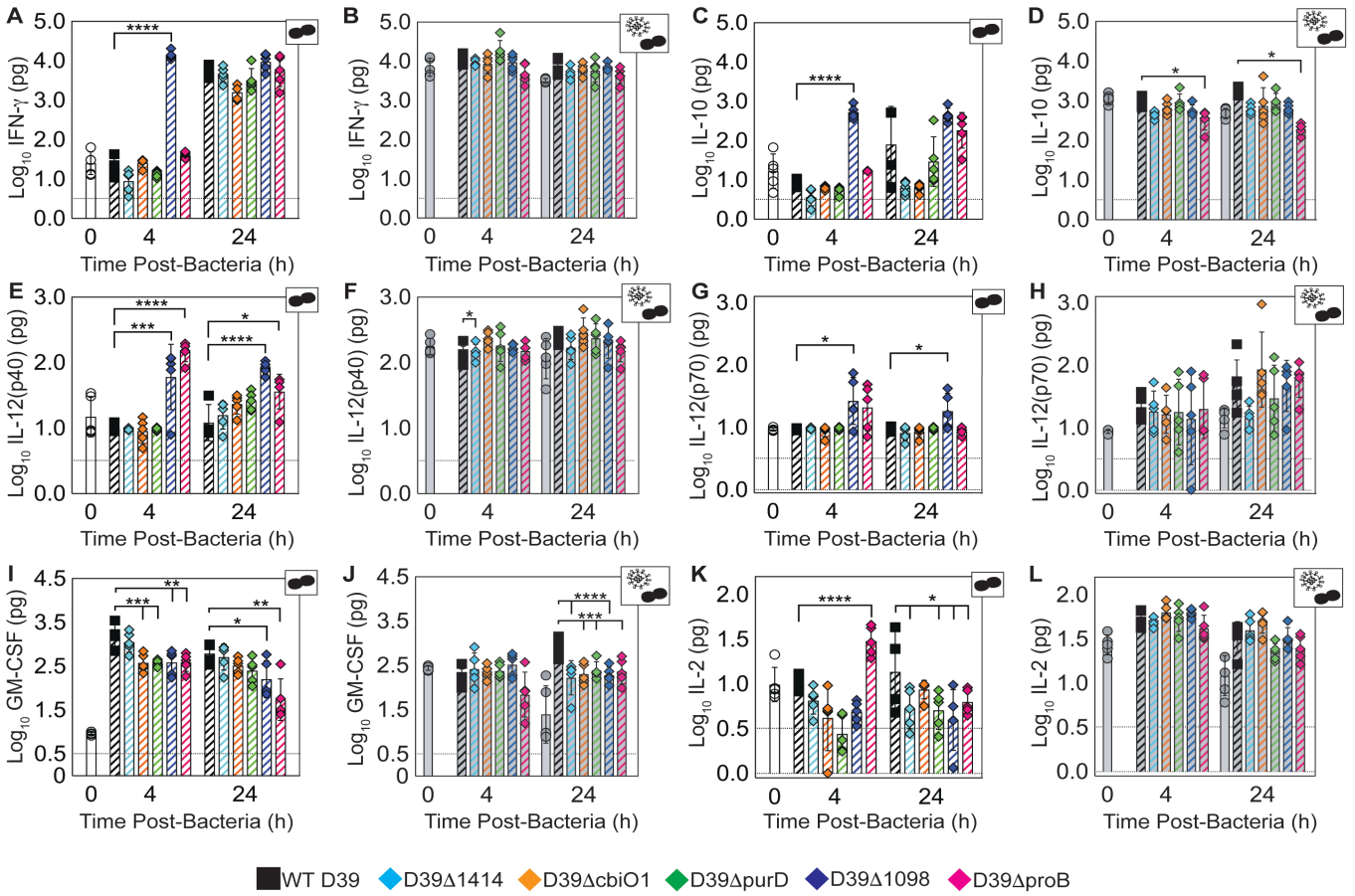

Fig S3: Additional Cytokine Kinetics in the Lung. Kinetics at 4 h and 24 h pbi of IFN-γ (A-B), IL-10 (C-D), IL-12(p40) (E-F), IL-12(p70) (G-H), GM-CSF (I-J), and IL-2 (K- L) from mice infected with PBS (mock) (Panels A, C, E, G, I, K) or 75 $TCID_{50}$ PR8 (Panels B, D, F, H, J, L) followed 7 d later with 10^6 CFU of the indicated bacteria. Each symbol (circles, squares, or diamonds) represents a single mouse, and the bars are the geometric mean ± standard deviation (SD). Mice were either uninfected (open white), influenza-infected only (solid grey), bacteria-infected (open hashed, colored), or influenza-bacteria coinfected (solid hashed, colored). Cartoons indicating infection status of study group (bacteria alone or virus plus bacteria) are in the upper right corner of each graph. Dashed line indicates the lower limit of detection (LOD), undetectable amounts are plotted at 0 log_{10} pg, and values extrapolated by the 4PL analysis appear between 0 log_{10} pg and the LOD. Signficance is indicated as *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001. Additional cytokines are shown in Fig S2 and a heat map of cytokines and chemokines is in Fig 5 (Main Text).

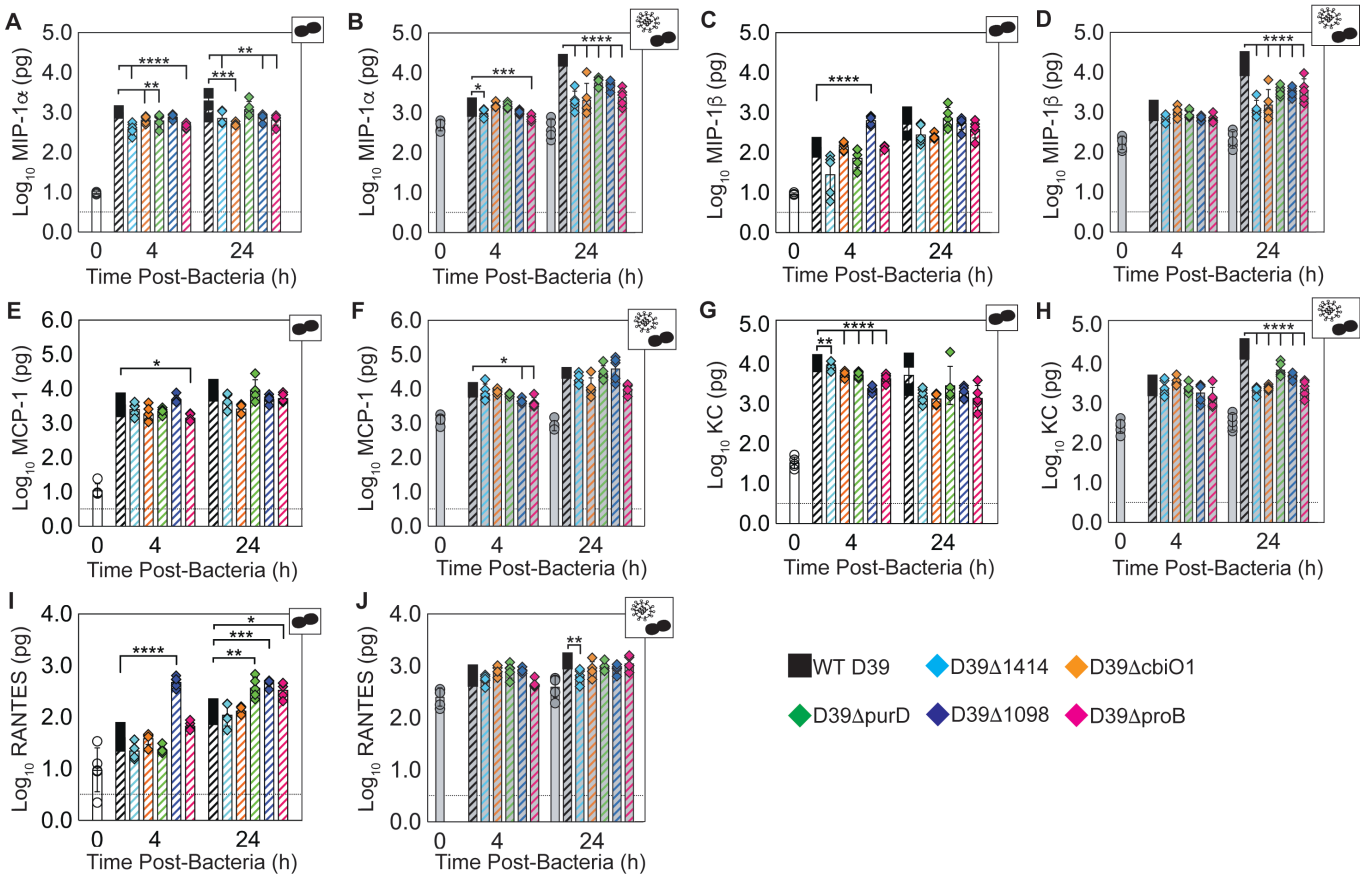

Fig S4: Pulmonary Chemokine Kinetics. Kinetics at 4 h and 24 h pbi of MIP-1α (A-B), MIP-1β (C-D), MCP-1 (E-F), KC (G-H), and RANTES (I-J) from mice infected with PBS (mock) (Panels A, C, E, G, I) or 75 $TCID_{50}$ PR8 (Panels B, D, F, H, J) followed 7 d later with 10^6 CFU of the indicated bacteria. Each symbol (circles, squares, or diamonds) represents a single mouse, and the bars are the geometric mean ± standard deviation (SD). Mice were either uninfected (open white), influenza-infected only (solid grey), bacteria-infected (open hashed, colored), or influenza-bacteria coinfected (solid hashed, colored). Cartoons indicating infection status of study group (bacteria alone or virus plus bacteria) are in the upper right corner of each graph. The dashed line indicates the lower limit of detection (LOD). Signficance is indicated as *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001. A heat map of cytokines and chemokines is in Fig 5 (Main Text).

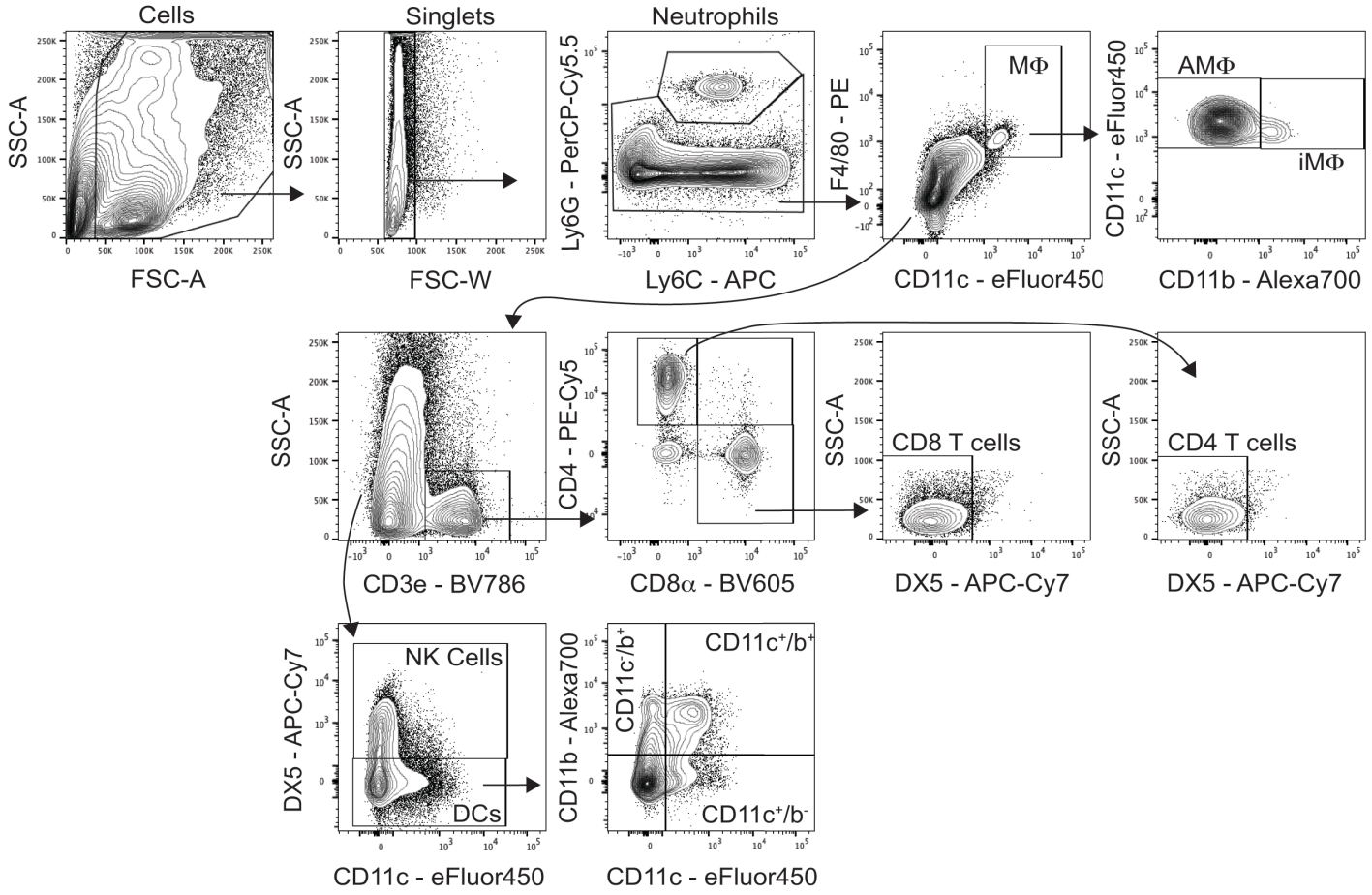

Fig S5: Flow Cytometry Gating Scheme for Lung Cell Analysis. Live cells were first gated on forward scatter (FSC-A) and side scatter (SSC-A) then as singlets. Neutrophils ($Ly6G^{hi}$) were then gated and excluded from remaining parent populations. Macrophages (MΦ) were gated as $CD11c^{hi}F4/80^{hi}$ with alveolar macrophages (AMΦ) sub-gated as $CD11b^{-}$ $MHC\text{-}II^{low}$ and inflammatory macrophages (iMΦ) as $CD11b^{+}MHC\text{-}II^{mid/hi}$. Following MΦ exclusion, T cells were gated as $CD3e^{+}$ and subgated into $CD8^{+}$ T cells ($CD3^{+}CD8^{+}CD4^{-}DX5^{-}$) and $CD4^{+}$ T cells ($CD3^{+}CD8^{-}CD4^{+}DX5^{-}$) populations. Of the cells that were $CD3e^{-}$, natural killer (NK) cells were gated as $CD3^{-}DX5^{+}$ and dendritic cells (DC) as $CD3^{-}DX5^{-}$. DCs were subgated as $CD11c^{+}CD11b^{-}$ DCs, $CD11c^{+}CD11b^{+}$ DCs, and $CD11c^{-}CD11b^{+}$ DCs.

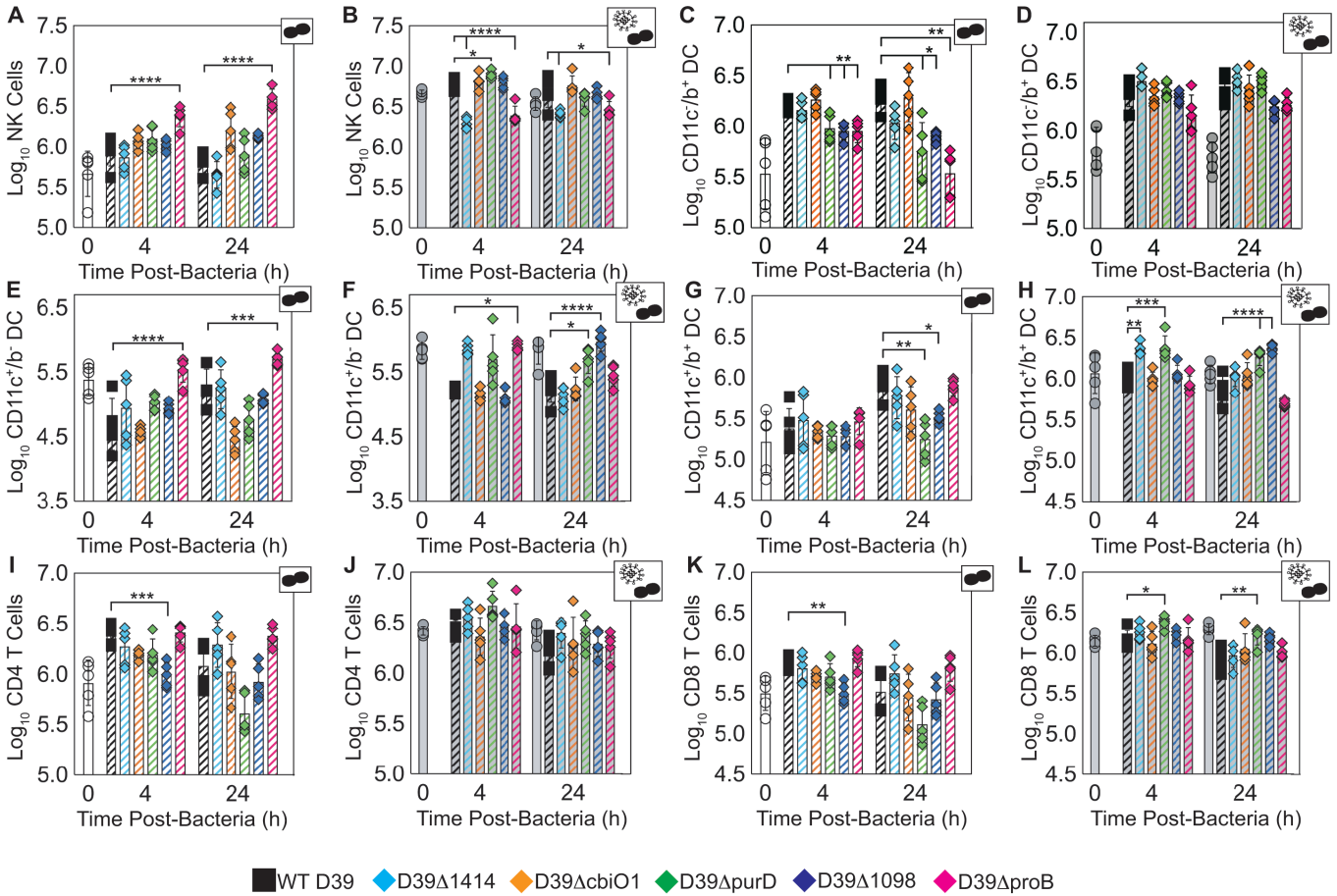

Fig S6: Additional Pulmonary Immune Cell Kinetics. Kinetics at 4 h and 24 h pbi of NK cells (A-B), DCs (C-H), and T cells (I-L) from mice infected with PBS (mock) (Panels A, C, E, G, I, K) or 75 $TCID_{50}$ PR8 (Panels B, D, F, H, J, L) followed 7 d later with 10^6 CFU of the indicated bacteria. Each symbol (circles, squares, or diamonds) represents a single mouse, and the bars are the geometric mean ± standard deviation (SD). Mice were either uninfected (open white), influenza-infected only (solid grey), bacteria-infected (open hashed, colored), or influenza-bacteria coinfected (solid hashed, colored). Cartoons indicating infection status of study group (bacteria alone or virus plus bacteria) are in the upper right corner of each graph. Signficance is indicated as *p<0.05, **p<0.01, ***p<0.005, ****p<0.0001. Additional cells are shown in Fig 6 (Main Text) and the flow cytometry gating scheme is in Fig S5.

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