# Tables

## Table S1. Cytokine analysis at 4 h, 2 d, and 7 d post-intranasal infection with G636.

| Cytokine[a,b] | WT | | | *tlr4* mutant | | |
|---|---|---|---|---|---|---|
| **4 h** | $10^5$ G636 | $10^8$ G636 | Mock | $10^5$ G636 | $10^8$ G636 | Mock |
| IL-23 | 23.26 (10.14) | 38.53 (6.36) | 10.67 (7.09) | 22.57 (13.26) | 32.61 (3.61) | 15.90 (15.90) |
| IL-1α | 58.57 (18.27) | 323.11 (75.42)$^{\#,\$}$ | 2.5 (0.43) | 2.77 (0.47) | 429.19 (90.22)$^{\#,\$}$ | 1.49 (0.13) |
| IFN-γ | 0.77 (0.36) | 5.13 (1.30)$^{\#,\$}$ | 0.00 (0.00) | 0.23 (0.23) | 4.34 (0.39)$^{\#,\$}$ | 0.00 (0.00) |
| TNF-α | 2219.55 (214.72) | 14888.73 (311.27)$^{\#,\$}$ | 108.38 (66.66) | 49.51 (6.47) | 12802.47 (3073.56)$^{\#,\$}$ | 12.82 (3.58) |
| MCP-1 | 0.00 (0.00) | 65.73 (15.07)$^{\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 65.57 (8.23)$^{\#,\$}$ | 0.00 (0.00) |
| IL-12p70 | 0.00 (0.00) | 1.43 (1.43) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-1β | 9.50 (1.95) | 46.26 (10.54)$^{\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 56.58 (12.58)$^{\#,\$}$ | 0.00 (0.00) |
| IL-10 | 0.00 (0.00) | 4.18 (4.18) | 0.00 (0.00) | 0.00 (0.00) | 2.04 (2.04) | 0.00 (0.00) |
| IL-6 | 910.41 (172.71) | 7885.57 (1645.56)$^{\#,\$}$ | 18.19 (5.67) | 8.09 (2.69) | 6334.72 (1218.67)$^{\#,\$}$ | 2.70 (2.70) |
| IL-27 | 0.00 (0.00) | 85.90 (13.54)$^{\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 42.77 (24.83)$^{\$}$ | 0.00 (0.00) |
| IL-17A | 1.05 (0.74) | 8.36 (2.46)$^{\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 4.66 (0.39)$^{\#,\$}$ | 0.00 (0.00) |
| IFN-β | 0.00 (0.00) | 29.75 (17.23)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| GM-CSF | 96.65 (13.37)$^{*,\#}$ | 55.42 (7.19)$^{\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 47.41 (6.96)$^{\#,\$}$ | 0.00 (0.00) |
| **2 d** | | | | | | |
| IL-23 | 19.93 (9.61) | 27.05 (8.76) | 44.09 (21.87) | 26.04 (11.37) | 11.35 (5.56) | 72.50 (36.30) |
| IL-1α | 1.14 (0.19) | 157.28 (58.45)$^{*,\#,\$}$ | 1.13 (0.20) | 2.00 (0.50) | 25.68 (6.15) | 19.64 (18.02) |
| IFN-γ | 2.20 (0.98) | 212.26 (54.96)$^{*,\#,\$}$ | 0.00 (0.00) | 3.70 (2.48) | 4.96 (2.48) | 0.00 (0.00) |
| TNF-α | 1.85 (0.82) | 592.26 (154.11)$^{*,\#,\$}$ | 1.03 (0.60) | 19.79 (6.79) | 52.20 (8.86) | 5.76 (4.31) |
| MCP-1 | 0.00 (0.00) | 160.30 (14.27)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 33.52 (6.37)$^{\#,\$}$ | 0.00 (0.00) |
| IL-12p70 | 0.00 (0.00) | 46.17 (17.63)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-1β | 1.06 (1.06) | 12.35 (2.80)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-10 | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-6 | 0.00 (0.00) | 1179.63 (323.29)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 20.45 (3.83) | 4.29 (4.29) |
| IL-27 | 0.00 (0.00) | 137.76 (81.43)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-17A | 0.42 (0.42) | 25.31 (10.22)$^{*,\#,\$}$ | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IFN-β | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| GM-CSF | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| **7 d** | | | | | | |
| IL-23 | 41.43 (14.43) | 30.14 (19.51) | 20.66 (20.66) | 9.07 (5.32) | 29.77 (12.87) | 12.69 (12.69) |
| IL-1α | 1.05 (0.18) | 41.50 (23.66) | 1.62 (0.92) | 5.54 (4.27) | 4.60 (2.50) | 1.79 (0.85) |
| IFN-γ | 0.00 (0.00) | 53.36 (40.45)$^{\$}$ | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| TNF-α | 0.00 (0.00) | 93.32 (36.14)$^{*,\#,\$}$ | 0.00 (0.00) | 3.17 (3.17) | 2.41 (1.54) | 0.00 (0.00) |
| MCP-1 | 0.00 (0.00) | 14.83 (14.83) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-12p70 | 0.00 (0.00) | 1.54 (1.54) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-1β | 0.00 (0.00) | 1.43 (1.43) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-10 | 0.00 (0.00) | 6.84 (6.84) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-6 | 0.00 (0.00) | 232.41 (182.45) | 0.00 (0.00) | 5.19 (5.19) | 16.72 (16.72) | 0.00 (0.00) |
| IL-27 | 0.00 (0.00) | 26.87 (26.87) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IL-17A | 0.00 (0.00) | 13.67 (11.48) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| IFN-β | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |
| GM-CSF | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) |

[a]Mean pg/ml (SEM) from two independent experiments at each timepoint is displayed.

[b]$^{*}P < 0.05$ relative to *tlr4* mutant at same inoculum; $^{\#}P < 0.05$ relative to mock in same mouse strain. $^{\$}P < 0.05$ relative to $10^5$ inoculum in same mouse strain. Two-way ANOVA, Tukey's test for multiple comparisons. Significant differences are also highlighted in green.

**Table S2. MICs for *A. baumannii* strains G636 and G654.**

| Antibiotic | G636 (Resistant/Sensitive) | G654 (Resistant/Sensitive) | Clinical Breakpoint[a] |
|---|---|---|---|
| **Imipenem** | >256 µg/ml (Resistant) | >256 µg/ml (Resistant) | 2 µg/ml |
| **Ampicillin** | >256 µg/ml (N/A) | >256 µg/ml (N/A) | N/A[c] |
| **Ciprofloxacin** | >256 µg/ml (Resistant) | >256 µg/ml (Resistant) | 1 µg/ml |
| **Levofloxacin** | 32 µg/ml (Resistant) | 128 µg/ml (Resistant) | 2 µg/ml |
| **Colistin** | 1 µg/ml (Intermediate)[b] | 8 µg/ml (Resistant) | 2 µg/ml[b] |
| **Polymyxin B** | 2 µg/ml (Intermediate)[b] | 4 µg/ml (Resistant) | 2 µg/ml[b] |
| **Tigecycline** | 2 µg/ml (N/A)[c] | 1 µg/ml (N/A)[c] | N/A[c] |
| **Gentamicin** | >256 µg/ml (Resistant) | 2-4 µg/ml (Sensitive) | 4 µg/ml |
| **Apramycin** | 16 µg/ml (N/A)[c] | 16 µg/ml (N/A)[c] | N/A[c] |

[a]Clinical breakpoints are according to the Clinical and Laboratory Standard Institute (CLSI) M100 Performance Standards for Antimicrobial Susceptibility Testing 30th Edition (122).

[b] A "sensitive" breakpoint is not available for colistin or polymyxin B from the CLSI. Strains with MICs of less than or equal to 2 µg/ml are considered to have "intermediate resistance."

[c]The clinical breakpoint has not been defined for ampicillin, tigecycline, and apramycin by the CLSI.

**Table S3. Plasmids and strains used in this study.**

| Plasmid or Strain | Description[a] | Source[b] |
|---|---|---|
| Plasmids | | |
| pEX18Tc | Precursor plasmid used for generation of pEX18Ap; Tet$^r$ | (114) |
| pKD4-Apr | Source for apramycin cassette for mutant generation; Apr$^r$ | (115) |
| pEX18Ap | Plasmid background used for generation of *A. baumannii* mutants; Apr$^r$ | This study |
| pEX18Ap::G636 *invL*KO | Plasmid used for mutation of *invL* in G636; Apr$^r$ | This study |
| pUC18T-miniTn7T-Apr | Vector used for genetic complementation at the mTn7 site; Apr$^r$ | (116) |
| pUC18T-miniTn7T-Apr::G636 *invL*KO comp | Plasmid used for complementation of the Δ*invL* mutant; Apr$^r$ | This study |
| PB-FLuc+GFPd2 | Plasmid source for *gfp* cassette; Amp$^r$ | [b] |
| pUC18T-miniTn7T-Apr::*gfpd2* | Expression vector; Apr$^r$ | This study |
| pRK2013 | Helper plasmid for mobilization of non-self-transmissible plasmids; Kan$^r$ | (123) |
| pTNS2 | T7 transposase expression vector; Amp$^r$ | (124) |
| Strains | | |
| *E. coli* | | |
| Stellar | *mrr-hsdRMS-mcrBC* and *mcrA*; Host strain for cloning | TaKaRa |
| HB101 | F- *mcrB mrr hsdS*20(rB- mB-) *recA*13 *leuB*6 *ara*-14 *proA*2 *lacY*1 *galK*2 *xyl*-5 *mtl*-1 *rpsL*20 *glnV*44 λ-; Host strain for pRK2013 | Promega |
| EC100D | F - mcrA Δ(*mrr-hsdRMS-mcrBC*) φ80*dlacZ*ΔM15 Δ*lacX*74 *recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*)7697 *galU galK* λ- *rpsL nupG pir* +(DHFR); Host strain for pTNS2 | Fisher |
| *A. baumannii* | | |
| G636 | 2018 *A. baumannii* respiratory isolate (Strain 3689) | [c] |
| G636 Δ*invL* | G636 *invL* mutant | This study |
| G636 *invL*$^+$ | G636 *invL* mutant complemented | This study |
| G636 Δ*bap* | G636 *bap* mutant | This study |
| G636 Δ*ata* | G636 *ata* mutant | This study |
| G636 Δ*fhaBC* | G636 *fhaBC* mutant | This study |
| G636-*gfp* | G636 expressing *gfpd2* | This study |
| G654 | 2020 *A. baumannii* respiratory isolate (Strain 6919) | [c] |
| *Ab*19606 | 1948 *A. baumannii* urinary isolate | (125) |
| *S. aureus* | | |
| Newman | 1952 osteomyelitis isolate | (126) |
| *K. pneumoniae* | | |
| TOP52 | 2006 cystitis isolate | (127) |

[a]Tet, tetracycline; Apr, apramycin; Amp, ampicillin; Kan, kanamycin.

[b]PB-FLuc+GFPd2 was a gift from Jordan Green (Addgene plasmid # 127190; http://n2t.net/addgene:127190; RRID: Addgene_127190).

[c]Strains G636 and G654 were collected by the CDC-funded Georgia Emerging Infections Program's (EIP) Multi-site Gram-Negative Surveillance Initiative (MuGSI) and kindly provided by Sarah Satola.

**Table S4. Primers used in this study.**

| Primer | Sequence |
|---|---|
| 5' pEX18 marker swap | ACACGGTGCCTGACTGCGTTAGC |
| 3' pEX18 marker swap | ATGGAAGCCGGCGGCACC |
| 5' Apr for pEX18Ap | GAGGTGCCGCCGGCTTCCATGATCCTCAGCCAATCGACTGGC |
| 3' Apr for pEX18Ap | AACGCAGTCAGGCACCGTGTGATTCCCTTTGTCAACAGCAATGG |
| 5' pEX18Tc | ATGCCTGCAGGTCGACTCTAGAGG |
| 3' pEX18Tc | GCAAGCTTGGCACTGGCCGT |
| 5' F1 G636 *invL*KO | ACGGCCAGTGCCAAGCTTGCGGCAATGTCTCAAATAAAAAATTTAACTC |
| 3' F1 G636 *invL*KO | TGAGATCCGCTATTATTACTTCCAG |
| 5' F2 G636 *invL*KO | AGTAATAATAGCGGATCTCATGCTTCTTTTTTTAGAGTTGTGTTCC |
| 3' F2 G636 *invL*KO | TAGAGTCGACCTGCAGGCATAAAATAACCGCATAGCCAGCTTGAGC |
| 5' G636 *fdeC*KO Comp | GCATGAGCTCACTAGTGGATCCGAGATTAAGACTTTACTTGGCATACACC |
| 5' F1 G636 *bap*KO | ACGGCCAGTGCCAAGCTTGCAGAAGCGGCTGGCAATGTCACG |
| 3' F1 G636 *bap*KO | TCAAGCACCGGTGCATACTGACC |
| 5' F2 G636 *bap*KO | CAGTATGCACCGGTGCTTGAGGTGGTAACACTACAATTCAGATTGACC |
| 3' F2 G636 *bap*KO | TAGAGTCGACCTGCAGGCATTCCATAAATGAATTTGCCATTTTCTTGAATCTG |
| 5' F1 G636 *ata*KO | ACGGCCAGTGCCAAGCTTGCTAAGTCGGTCTGGCTATTCGCC |
| 3' F1 G636 *ata*KO | TGATGACGTTGAGAAAAAAGCTAATGCAGG |
| 5' F2 G636 *ata*KO | CTTTTTTCTCAACGTCATCAAAAACTTCTCAGACAAATACCGAACTCAACG |
| 3' F2 G636 *ata*KO | TAGAGTCGACCTGCAGGCATCGTGATCAATTTCTTCTGTAAGCGAATCTTTTTGC |
| 5' F1 G636 *fhaBC*KO | ACGGCCAGTGCCAAGCTTGCTTAAAATTTTAAAGCAGTTTGATGAGCC |
| 3' F1 G636 *fhaBC*KO | CAGAATTGTACGTATAAGAACTTTATTTTACAC |
| 5' F2 G636 *fhaBC*KO | TTCTTATACGTACAATTCTGTAACAATGAAAAATGCACATGCGG |
| 3' F2 G636 *fhaBC*KO | TAGAGTCGACCTGCAGGCATCTTTATTGGTACCCTGATTGCG |
| 3' G636 *fdeC*KO Comp-His6 v2 | TCAGTGGTGATGGTGATGATGATTACCATTTGAACAGTTTGGATCTATTCC |
| Tn7 linear Fwd-His6 | CATCATCACCATCACCACTGAAAGCTTGGGCCCGGTACCTC |
| Tn7 linear Rev | GGATCCACTAGTGAGCTCATGC |

| | |
|---|---|
| 5' d2EGFP for pUC18T-mTn7 | AGAAAGAGGAGAAATACTAGATGGTGAGCAAGGGCGAGG |
| 3' d2EGFP for pUC18T-mTn7 | GAGGTACCGGGCCCAAGCTTCTACACATTGATCCTAGCAGAAGC |
| 5' pUC18T-mTn7 for d2EGFP | AAGCTTGGGCCCGGTACCTCG |
| 3' pUC18T-mTn7 for d2EGFP | CTAGTATTTCTCCTCTTTCTCTAGTAATTGTTATCC |

568

569

**Figure S1.** Intracellular *A. baumannii* are detectable in BALF at early timepoints in the chronic respiratory infection model. Groups of C3H/HeJ (*tlr4* mutant) mice were intranasally inoculated with $10^5$ G636, and BALF was collected at 4 hpi (A), 2 dpi (B), and 7 dpi (C) and either treated with 50 µg/ml colistin or mock-treated. Following, bacterial CFU in the treated (intracellular; IC) and mock treated (total) BALF, as well as in the remaining lungs following BALF collection, were enumerated by serial dilution plating. The horizontal line represents the mean, and the SEM is indicated by error bars. Shown are the results from at least two independent experiments. C3H/HeJ (*tlr4* mutant) mice were infected with G636 expressing *gfp*, and, at these same timepoints, BALF was collected, and host cells were isolated and stained with DAPI (blue) and phalloidin (red). Intracellular bacteria were identified by microscopy at 4 hpi (D) and 2 dpi (E). Shown are representative images from independent samples from at least two biological replicates. Scale bar = 5 µm.

**Figure S2.** The chronic respiratory infection model results in lung pathology during infection. Groups of C3H/HeJ (*tlr4* mutant) mice were inoculated with $10^5$ G636 or mock-inoculated with PBS, and at 4 hpi (A), 2 dpi (B), 7 dpi (C), 14 dpi (D), and 21 dpi (E), lungs slices were prepared and H&E stained. Shown are representative images from each timepoint. Lung slice scale bar: 1000 µm; Inset scale bar: 100 µm.

**Figure S3.** The chronic respiratory infection model does not cause goblet cell hyperplasia or fibrosis. Groups of C3H/HeJ (*tlr4* mutant) mice were inoculated with $10^5$ G636 or mock-inoculated with PBS, and at 4 hpi, 2 dpi, 7 dpi, 14 dpi, and 21 dpi, lungs slices were prepared, H&E stained, and scored for goblet cell hyperplasia (A) and fibrosis (B). The mean is shown on the graph, and the SEM is indicated by error bars. Unpaired Student's *t*-test.

**Figure S4.** Testing of G636 adhesin mutants in the chronic respiratory infection model reveals a potential role for InvL in bacterial persistence. Groups of C3H/HeJ (*tlr4* mutant) mice were intranasally inoculated with $10^5$ G636, G636 Δ*bap*, G636 Δ*ata*, G636 Δ*fhaBC*, and G636 Δ*invL*. 1 (A) and 14 (B) dpi, mice were sacrificed, and CFU in the lungs were quantified. Each data point represents an individual mouse, the horizontal line represents the mean, and the SEM is indicated by error bars. Shown are results from single experiments for each strain.

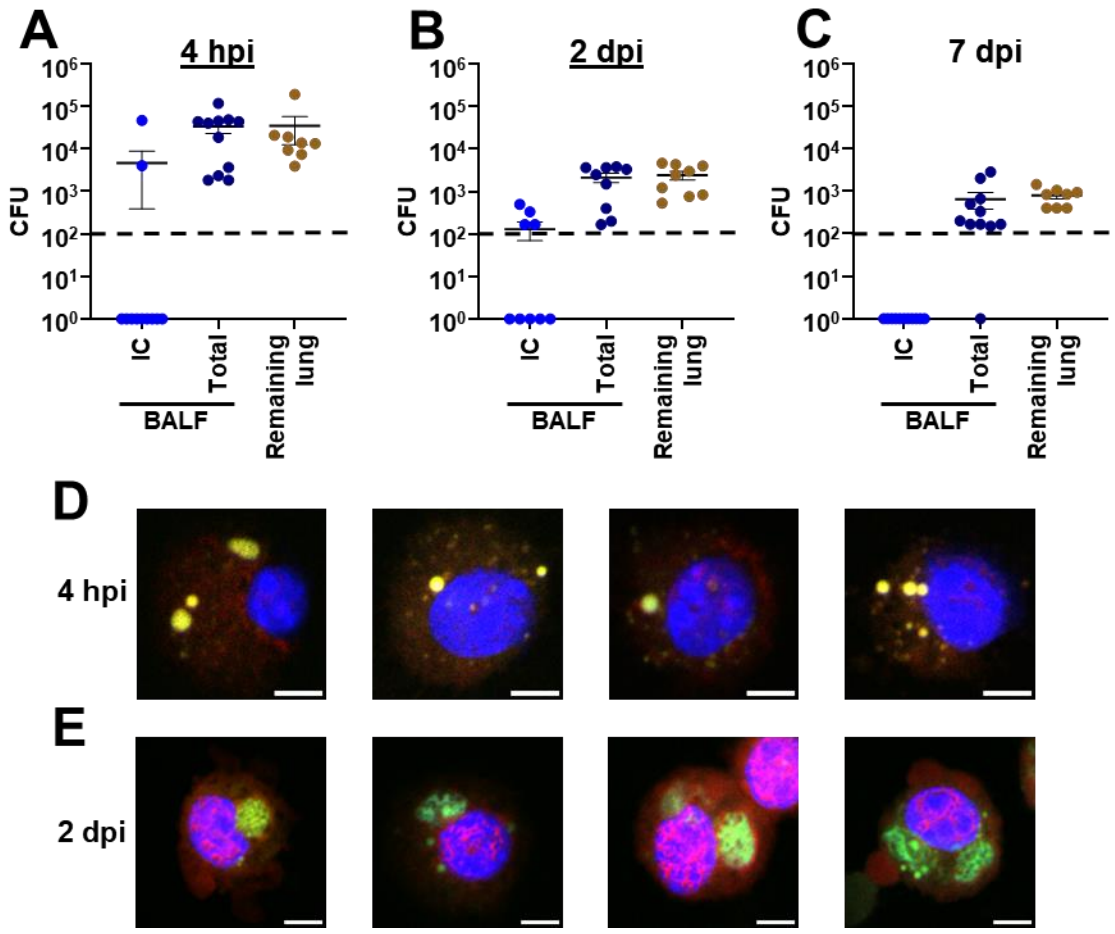
**Figure S5.** The chronic respiratory infection model can be used to study outcomes of antibiotic treatment. Groups of C3H/HeJ (*tlr4* mutant) mice were infected with $10^5$ G636 (A, C) or $10^5$ G654 (B, D) and sacrificed at 1, 3, and 5 dpi (long-term). Additionally, groups of C57Bl/6 mice were infected with $10^9$ G636 (A, C) or $10^9$ G654 (B, D) and sacrificed at 24 hpi (acute). Mice in both infection models were treated intraperitoneally treated with PBS or 5 mg/kg colistin (col) every 8 h (A, B) or PBS or 100 mg/kg imipenem (im) every 12 h (C, D) with all treatments beginning 4 hpi. At each timepoint, CFU were quantified in the lungs. Shown are the results from at least two

independent experiments, each data point represents an individual mouse, the horizontal line represents the mean, and the SEM is represented by error bars. *$P < 0.05$; Mann-Whitney *U* test.

**Figure S6.** *S. aureus* secondary infection sometimes causes *A. baumannii* dissemination to the spleen and kidneys in the chronic respiratory infection model. C3H/HeJ (*tlr4* mutant) mice were intranasally inoculated with $10^5$ G636. At 14 days post-*A. baumannii* infection, groups of mice were either not inoculated (untreated), inoculated with PBS (mock-infected), infected with *S. aureus*, or infected with *K. pneumoniae*. Subsequently, on days 15 (A and B) and 16 (C and D) post-*A. baumannii* infection (1 and 2 days post-secondary infection), groups of mice were sacrificed, and *A. baumannii* CFU were quantified in the spleen (A and C), and kidneys (C and D). Each data point represents an individual mouse, the horizontal line represents the mean, and the SEM is indicated by error bars. Shown are results from at least 2 independent experiments. Significant differences were not detected; Kruskal-Wallis *H* test with Dunn's test for multiple comparisons.

**Figure S7.** Ongoing *A. baumannii* pneumonia alters bacterial numbers following secondary infection with *S. aureus* and *K. pneumoniae*. C3H/HeJ (*tlr4* mutant) mice were either intranasally inoculated with $10^5$ G636 14 days prior to infection with *S. aureus* or *K. pneumoniae* (+*Ab*) or not infected prior to *S. aureus* or *K. pneumoniae* infection (-*Ab*). At 14 days post-*A. baumannii* infection, groups of mice were infected with *S. aureus* or *K. pneumoniae*. 1 and 2 dpi with *S. aureus* (A, B, and C) or *K. pneumoniae* (D, E, and F), mice were sacrificed, and these bacteria were quantified in the lung (A and D), spleen (B and E), and kidneys (C and F). Each data point represents an individual mouse, the horizontal line represents the mean, and the SEM is indicated by error bars. Shown are results from at least 2 independent experiments. *$P < 0.05$; Mann-Whitney *U* test.

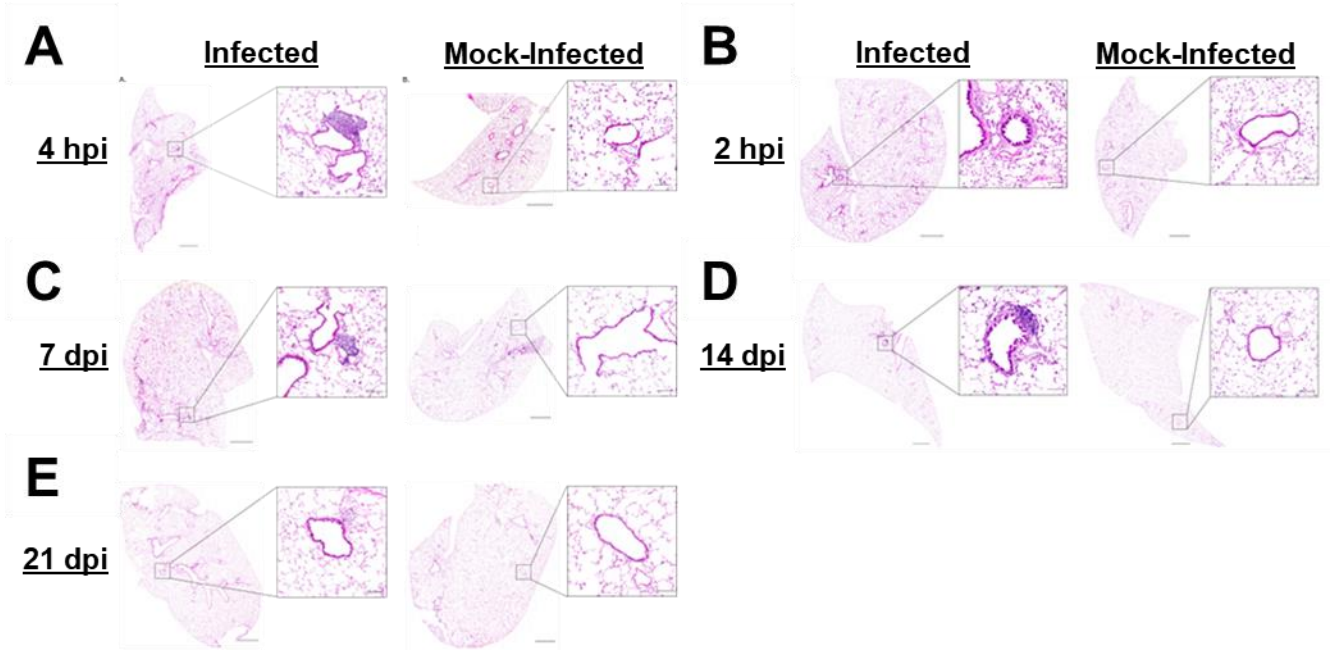
699 Figure S1:



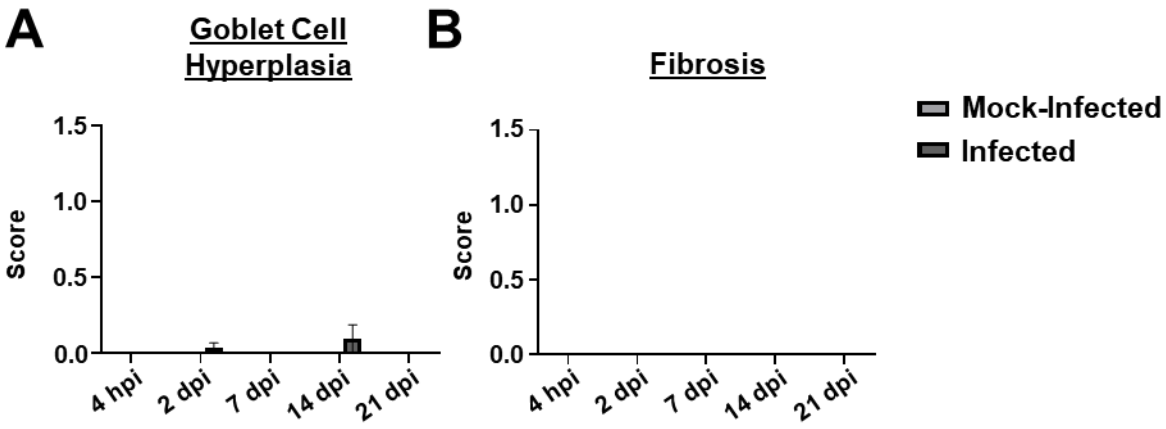

700

701

Figure S2:



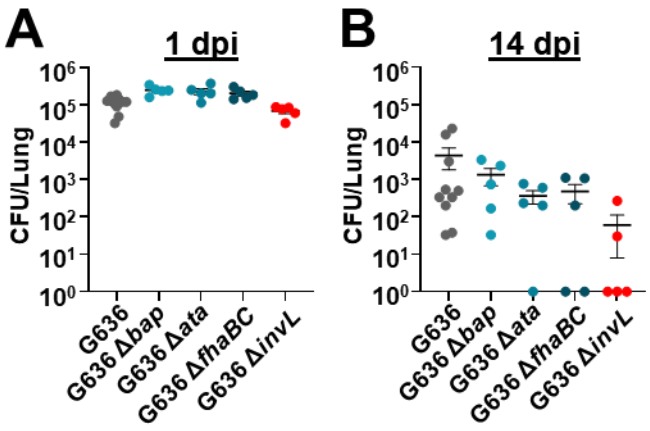
705 Figure S3




706

707

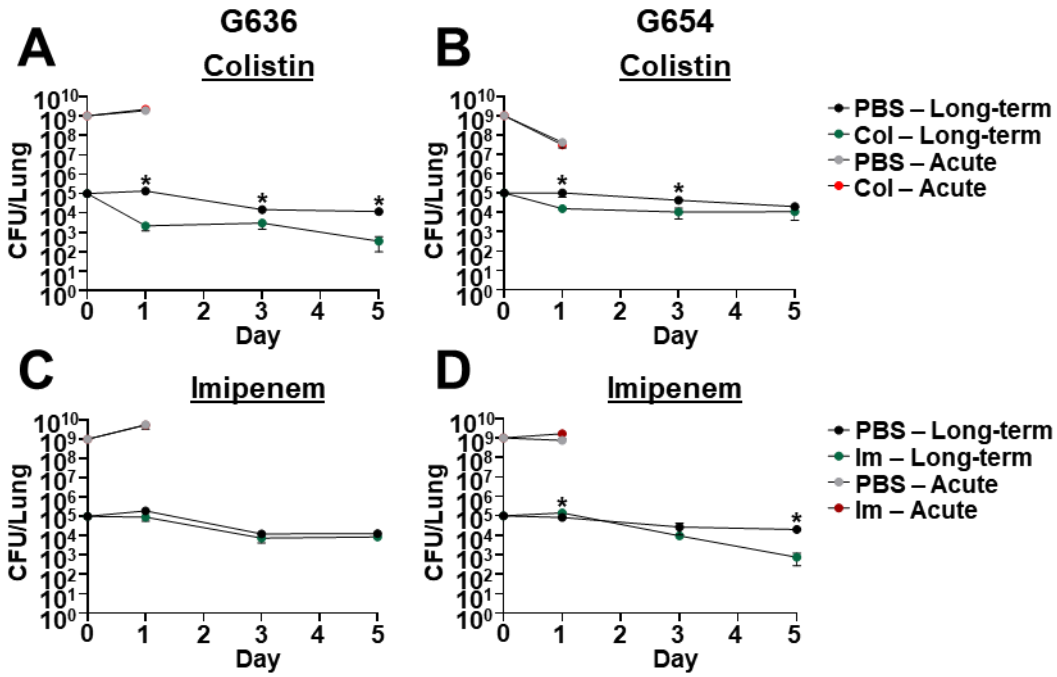
708 Figure S4:




709

710

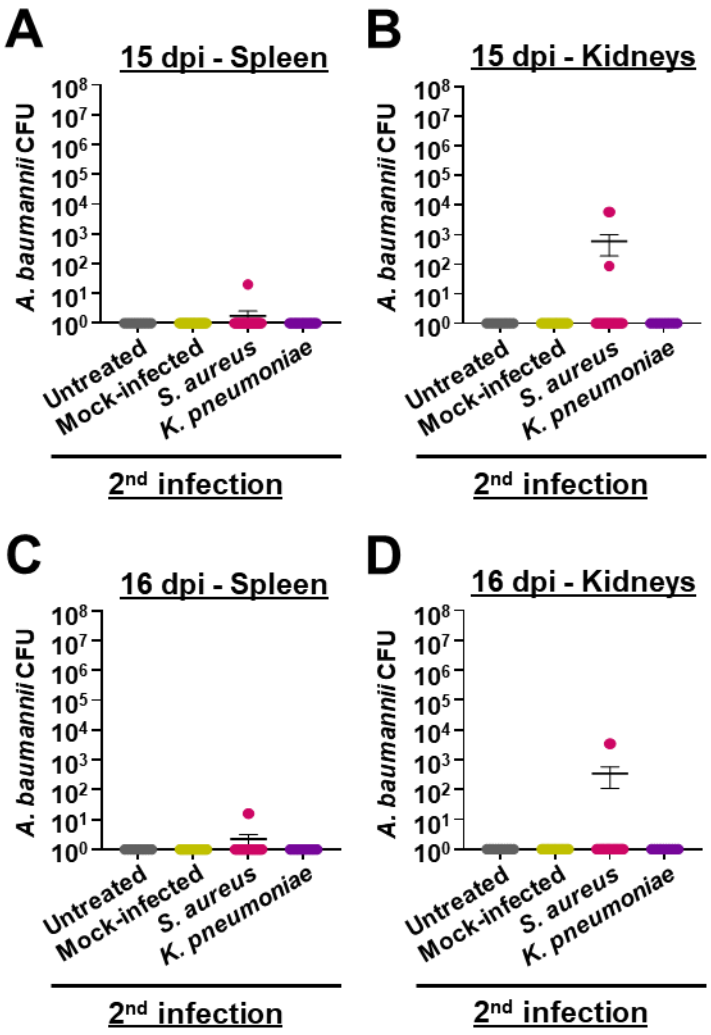
711 Figure S5




712

713

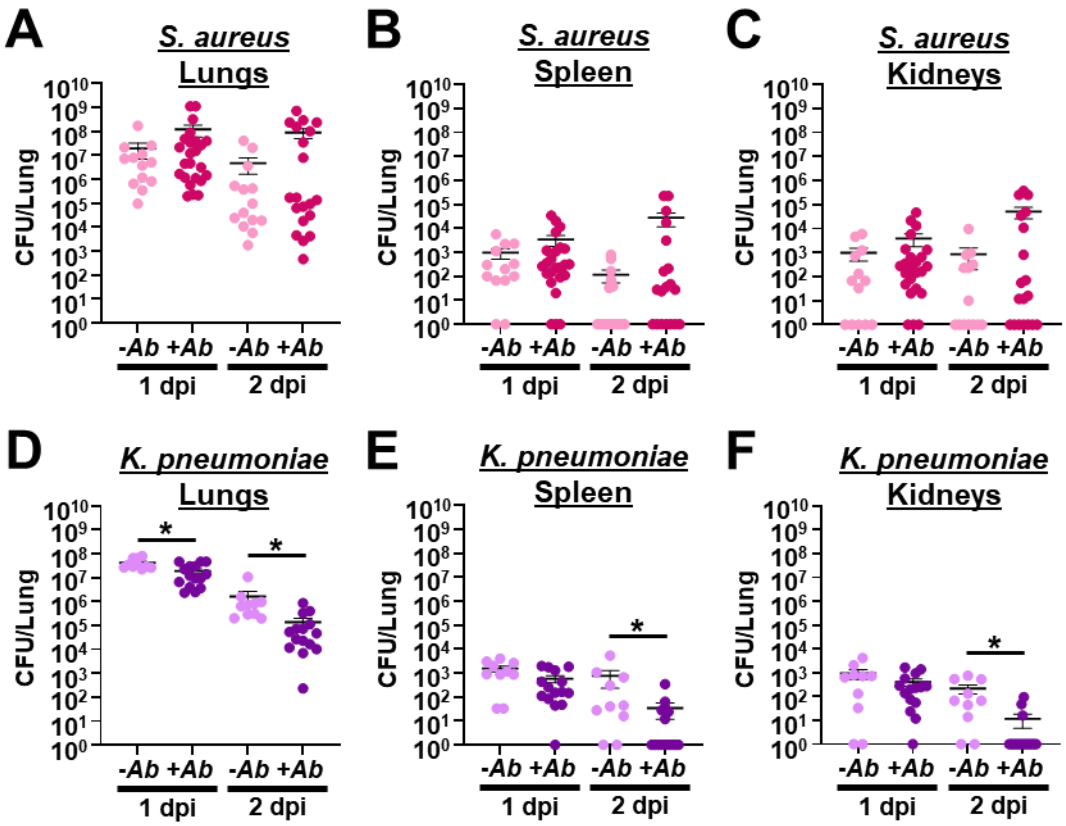
714 Figure S6




715

716

717 Figure S7




718

719