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Abstract

Bacteria are well known to cause chronic infections in humans by entering dormancy and by developing biofilms. These mechanisms allow bacteria to exhibit antibiotic tolerance and relapse to an active virulent state when the antibiotic treatments are discontinued. During bacterial invasions, the host immune cells secrete special signaling proteins, known as cytokines which orchestrate events leading to human immune response and elimination of bacterial pathogens. However, compared to the well documented activities of cytokines in immune reaction, little is known about the direct effects of cytokines on bacterial cells.

In this study, we focused on granulocyte macrophage colony-stimulating factor (GM-CSF), a cytokine produced by macrophages, T-cells, endothelial cells, and fibroblasts. We chose *Pseudomonas aeruginosa* as a model bacterium. It is an opportunistic pathogenic bacterium and a major cause of nosocomial infections in individuals with compromised immune systems and cystic fibrosis (CF) patients. We show for the first time that GM-CSF can sensitize the persister cells of *P. aeruginosa* PAO1 to multiple antibiotics including ciprofloxacin, tobramycin, tetracycline, and gentamicin. The mucoid variant, *P. aeruginosa* PDO300 was also sensitized by GM-CSF to tobramycin in the presence of alginate lyase. In addition, GM-CSF sensitized the biofilm cells of *P. aeruginosa* PAO1 and PDO300 to tobramycin in presence of biofilm matrix degrading enzymes DNase I and alginate lyase, respectively. In comparison, the normal cells of *P. aeruginosa* and the non-pathogenic *Escherichia coli* K12 persister cells were not affected by GM-CSF.

DNA microarray and qPCR analyses revealed that GM-CSF induced flagella and pyocin associated genes in persister cells of *P. aeruginosa* PAO1, while the same genes in normal cells did not show significant change. Using co-immunoprecipitation (co-IP) and cross-linking, GM-CSF was found to interact with the protein FliC (flagellin). Deletion of *fliC* gene abolished the effects of GM-CSF on *P. aeruginosa* persister cells, which was restored by complementation of the *fliC* gene. Overall, the findings from this study suggest that cytokines have a direct interaction with bacterial cells and disturb their persistence. The results are helpful for understanding bacterial physiology and for developing new persistence control methods.

Keywords: GM-CSF, persister cells, biofilms, *Pseudomonas aeruginosa*, antibiotic tolerance, flagella, pyocins.

**Controlling *Pseudomonas aeruginosa* Persister Cells by Human
Granulocyte Macrophage Colony-Stimulating Factor**

By

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DISSERTATION

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	V
TABLE OF CONTENTS.....	VII
LIST OF TABLES.....	X
LIST OF FIGURES.....	XI
CHAPTER 1 MOTIVATION, HYPOTHESIS, AND OBJECTIVES.....	1
1.1 Motivation.....	2
1.2 Hypothesis and Research objectives.....	3
1.3 References.....	5
CHAPTER 2 LITERATURE REVIEW.....	7
2.1 Bacterial persistence.....	8
2.1.1 Toxin-Antitoxin System.....	9
2.1.2 Controlling bacterial persistence.....	12
2.2 Bacterial biofilms.....	15
2.3 <i>Pseudomonas aeruginosa</i>.....	17
2.4 Immune system.....	22
2.4.1 Cytokine therapy.....	27
2.4.2 Granulocyte Macrophage Colony-Stimulating Factor.....	28
2.4.3 Clinical Significance and Uses of GM-CSF.....	30
2.5 References.....	32
CHAPTER 3 SYNERGY BETWEEN GRANULOCYTE MACROPHAGE COLONY- STIMULATING FACTOR (GM-CSF) AND ANTIBIOTICS PROMOTE THE KILLING OF PERSISTER CELLS OF <i>PSEUDOMONAS AERUGINOSA</i>.....	57
3.1 Abstract.....	58
3.2 Introduction.....	59
3.3 Materials and Methods.....	61
3.3.1 Bacterial strains and growth media.....	61
3.3.2 Effect of GM-CSF on planktonic cells.....	61
3.3.3 Effect of GM-CSF on biofilm cells.....	63
3.3.4 Effect of alginate lyase on GM-CSF diffusion across alginate layer.....	64
3.3.5 Kinetics of bacterial killing during antibiotic treatment.....	65
3.3.6 Statistical Analyses.....	65
3.4 Results.....	66
3.4.1 Identifying the appropriate antibiotic concentrations for persister isolation.....	66

3.4.2 GM-CSF sensitized the planktonic persister cells of <i>P. aeruginosa</i> PAO1 to antibiotics.....	68
3.4.3 GM-CSF is effective against the mucoid strain <i>P. aeruginosa</i> PDO300 in the presence of alginate lyase.....	73
3.4.4 GM-CSF enhanced the killing of biofilm cells.....	74
3.4.5 Alginate lyase allows the diffusion of GM-CSF across alginate layer....	78
3.4.6 GM-CSF is not effective against planktonic normal cells of <i>P. aeruginosa</i> PAO1 and PDO300.....	79
3.4.7 GM-CSF is not effective against the non-pathogenic <i>E. coli</i> K12.....	81
3.5 Discussion.....	82
3.6 Conclusions.....	84
3.7 Acknowledgements.....	84
3.8 References.....	85

CHAPTER 4 INVESTIGATION OF THE EFFECTS OF GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR ON <i>PSEUDOMONAS AERUGINOSA</i>.....	92
4.1 Abstract.....	93
4.2 Introduction.....	94
4.3 Materials and Methods.....	95
4.3.1 Bacterial strains and growth media.....	95
4.3.2 DNA microarray analysis.....	95
4.3.3 Quantitative real-time PCR (qPCR) analysis.....	96
4.3.4 Effect of GM-CSF on the induction of pyocin genes in <i>P. aeruginosa</i> PAO1 persister cells.....	99
4.3.5 Effect of GM-CSF on the mutants of ATP-binding cassette (ABC) and Major facilitator superfamily (MFS) transcriptional regulator.....	101
4.4 Results.....	101
4.4.1 Effects of GM-CSF on gene expression in <i>P. aeruginosa</i> PAO1.....	101
4.4.2 GM-CSF induces pyocin production in persister cells of <i>P. aeruginosa</i> PAO1.....	108
4.4.3 Defect in MFS transporter transcriptional regulator shows lower synergistic activity between antibiotics and GM-CSF compared to wild-type <i>P. aeruginosa</i> PAO1.....	111
4.5 Discussion.....	112
4.6 Conclusions.....	117
4.7 Acknowledgements.....	118
4.8 References.....	118

CHAPTER 5 ROLE OF FLAGELLIN (FlhC) IN INTERACTION BETWEEN GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) AND <i>PSEUDOMONAS AERUGINOSA</i>.....	126
5.1 Abstract.....	127

5.2 Introduction.....	128
5.3 Materials and Methods.....	129
5.3.1 Bacterial strains and growth media.....	129
5.3.2 Isolation of flagella from <i>P. aeruginosa</i> PAO1 cells.....	130
5.3.3 GM-CSF binding with cross-linking and Co-Immunoprecipitation (Co-IP).....	131
5.3.4 Combined effect of GM-CSF, flagella, and outer membrane vesicles on <i>fliC</i> mutant, PAO1 $\Delta fliC$	133
5.3.5 Inhibition of flagellar activities.....	134
5.4 Results.....	136
5.4.1 GM-CSF binds to FliC in <i>P. aeruginosa</i>	136
5.4.2 FliC is important to the effects of GM-CSF on <i>P. aeruginosa</i> persister cells.....	137
5.4.3 GM-CSF sensitized the persister cells of <i>P. aeruginosa</i> strains with both a-type and b-type flagellins to antibiotics.....	140
5.4.4 External addition of flagella and outer membrane vesicles (OMVs) partially restored the activity of GM-CSF on the <i>fliC</i> mutant.....	143
5.4.5 Inhibition of flagellar activity enabled GM-CSF to sensitize the normal cells of <i>P. aeruginosa</i> PAO1 to antibiotic.....	146
5.5 Discussion.....	151
5.6 Conclusions.....	160
5.7 Acknowledgements.....	160
5.8 References.....	160
CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK.....	174
6.1 Conclusions.....	175
6.2 Recommendations for future work.....	178
6.2.1 Exploration of a wide range of microbes for testing GM-CSF.....	178
6.2.2 GM-CSF activity in chronic infections.....	178
6.2.3 Multiple cytokine therapy.....	179
6.2.4 Utilization of STORM as a powerful microscopic tool to visualize GM-CSF binding.....	180
6.3 References.....	180
APPENDICES.....	183
Protocol I Synergistic effects of GM-CSF and antibiotics on planktonic cells.....	184
Protocol II Synergistic effects of GM-CSF and antibiotics on biofilm cells....	185
Protocol III RNA isolation from normal and persister cells after GM-CSF treatment.....	186
Protocol IV Synthesis of cDNA and quantitative polymerase chain reaction (qPCR).....	189
CURRICULUM VITAE.....	191

LIST OF TABLES

Table 2.1 Currently known types of TA systems.....	11
Table 2.2 Some known methods to control bacterial persistence.....	13
Table 2.3 Extracellular virulence factors produced by <i>Pseudomonas aeruginosa</i>	19
Table 2.4 Some major cytokines secreted during pathogenic invasions.....	24
Table 4.1 Primers used for qPCR of persister cells.....	98
Table 4.2 Primers used for qPCR of normal cells.....	99
Table 4.3 Expression fold change of representative genes in <i>P. aeruginosa</i> PAO1 persister cells based on the average of two DNA microarray Runs.....	106
Table 4.4 Comparison of qPCR results with the DNA microarray results for 10 representative genes of <i>P. aeruginosa</i> PAO1 persister cells.....	107
Table 4.5 Comparison of qPCR results with the DNA microarray results for 4 representative genes of <i>P. aeruginosa</i> PAO1 normal cells.....	107
Table 5.1 Expression of some flagella associated genes in normal and persister cells of <i>P. aeruginosa</i> after treatment with GM-CSF.....	153

LIST OF FIGURES

<p>Figure 2.1 Schematic diagram of bacterial persistence. Green cells indicate normal cells, red cells indicate persister cells. (A) Bacterial cells in a culture, (B) Cell lysis of normal cells after antibiotic treatment, (C) Survival of persister cells in presence of antibiotics, which revert to normal state and repopulate due to antibiotic deprivation and favorable conditions for growth.....</p>	9
<p>Figure 2.2 Stages of bacterial biofilm formation on surfaces.....</p>	16
<p>Figure 2.3 Schematic diagram of multiple virulence factors exhibited by <i>Pseudomonas aeruginosa</i>.....</p>	19
<p>Figure 2.4 Difference between normal and cystic fibrosis lung airway.....</p>	21
<p>Figure 2.5 Leukocytes of human immune system.....</p>	23
<p>Figure 2.6 GM-CSF pathway in the immune system.....</p>	29
<p>Figure 3.1 Antibiotic conditions used for the isolation of persister cells provide drug-tolerant bacterial population in both exponential and stationary phase cultures. Antibiotic conditions used for the isolation of persister cells from cultures at exponential phase for (A) <i>P. aeruginosa</i> PAO1 (200 µg/mL ciprofloxacin for 3.5 h), (B) <i>P. aeruginosa</i> PDO300 (200 µg/mL ciprofloxacin for 3.5 h), and (C) <i>E.coli</i> K12 (100 µg/mL ampicillin for 3.5 h), cause biphasic kill curves with a significant decline in killing rate and a dominant drug-tolerant population. Similar biphasic kill curves were observed for antibiotic conditions used for the isolation of persister cells from the cultures at stationary phase for (D) <i>P. aeruginosa</i> PAO1 (200 µg/mL ciprofloxacin for 3.5 h), (E) <i>P. aeruginosa</i> PDO300 (200 µg/mL ciprofloxacin for 3.5 h), and (F) <i>E.coli</i> K12 (100 µg/mL ampicillin for 3.5 h). Cip: ciprofloxacin. Amp: Ampicillin. The samples were tested in triplicate (n=3). Error bars represent SD.....</p>	67
<p>Figure 3.2 GM-CSF sensitized the persister cells of <i>P. aeruginosa</i> PAO1 isolated from exponential phase cultures to antibiotics. The wild-type PAO1 obtained from two different sources were tested including one (A) from Prof. Thomas K. Wood at Pennsylvania State University and another (B) from Prof. Matthew Parsek at University of Washington. The persister cells were isolated from exponential phase cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with 0.17 pM GM-CSF alone for 1 h, followed by additional treatment</p>	

with GM-CSF plus an antibiotic as indicated for 3.5 h (all tested at 200 µg/mL). Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. Tet: tetracycline. Gen: gentamicin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

69

Figure 3.3 GM-CSF sensitized the persister cells of *P. aeruginosa* PAO1 isolated from stationary phase cultures to antibiotics. The persister cells were isolated from stationary phase cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF plus (A) 5 µg/mL ciprofloxacin, and (B) 5 µg/mL tobramycin. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

71

Figure 3.4 Effect of 0.17 pM GM-CSF on *P. aeruginosa* PAO1 persister cells was abolished by anti-GM-CSF. The persister cells were isolated from exponential phase cultures. All samples underwent the same incubation duration. The figure shows the viability of persister cells treated with GM-CSF alone, anti-GM-CSF alone, or GM-CSF neutralized by anti-GM-CSF (2 h incubation) followed by 5 µg/mL ciprofloxacin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

72

Figure 3.5 Alginate lyase is required for the activity of GM-CSF against persister cells of the mucoid strain *P. aeruginosa* PDO300. The persister cells were isolated from exponential phase cultures and GM-CSF was tested at 0.17 pM. The viability of persister cells treated with tobramycin (200 µg/mL) alone, tobramycin along with alginate lyase (50 µg/mL), or tobramycin along with alginate lyase and GM-CSF is shown. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. AL: alginate lyase. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

74

Figure 3.6 GM-CSF enhanced the killing of *P. aeruginosa* PDO300 biofilm cells by tobramycin and alginate lyase. *P. aeruginosa* PDO300 cells in 24 h biofilms were treated with (i) 0.17 pM GM-CSF alone, (ii) 200

µg/mL tobramycin alone, (iii) 0.17 pM GM-CSF and 50 µg/mL alginate lyase, (iv) 200 µg/mL tobramycin and 50 µg/mL alginate lyase, (v) 0.17 pM GM-CSF and 200 µg/mL tobramycin, and (vi) 0.17 pM GM-CSF, 200 µg/mL tobramycin, and 50 µg/mL alginate lyase, for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. AL: alginate lyase. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

76

Figure 3.7 GM-CSF enhanced the killing of *P. aeruginosa* PAO1 biofilm cells by tobramycin and DNase I. *P. aeruginosa* PAO1 cells in early biofilms (4 h after inoculation) were treated with (i) 0.17 pM GM-CSF alone, (ii) 20 µg/mL tobramycin alone, (iii) 0.17 pM GM-CSF and 5 units/mL DNase I, (iv) 20 µg/mL tobramycin and 5 units/mL DNase I, (v) 0.17 pM GM-CSF and 20 µg/mL tobramycin, and (vi) 0.17 pM GM-CSF, 20 µg/mL tobramycin, and 5 units/mL DNase I for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

78

Figure 3.8 GM-CSF diffused across an alginate layer in presence of alginate lyase. The figure shows the Western blotting results of GM-CSF diffusion across 0.3% w/v alginate layer on top of transwell inserts in presence and absence of 100 µg/mL alginate lyase over a period of 0, 1, and 2 h. The bands indicate GM-CSF detected by anti-GM-CSF (primary antibody) followed by anti-mouse IgG (secondary antibody). As a positive control, 50 ng GM-CSF was added during western blotting. Top: Top of the transwell insert, Bottom: Bottom of the 12-well plate.....

79

Figure 3.9 GM-CSF did not sensitize the normal cells of *P. aeruginosa* PAO1 and PDO300 isolated from exponential phase cultures to antibiotics. The total viable cells were obtained from the exponential cultures of wild-type *P. aeruginosa* (A) and its isogenic mucoid mutant PDO300 (B). The cells were treated with GM-CSF alone or with GM-CSF plus antibiotics as indicated for 3.5 h (all tested at 200 µg/mL). The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of normal cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. Tet: tetracycline. Gen: gentamicin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....

80

<p>Figure 3.10 GM-CSF did not sensitize the persister cells of <i>E. coli</i> K12 to antibiotics. The persister cells were isolated from exponential phase cultures by killing the normal cells with 100 µg/mL ampicillin for 3.5 h, and then treated with 0.17 pM GM-CSF alone for 1 h, followed by additional treatment with GM-CSF plus an antibiotic as indicated for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: 2 µg/mL ciprofloxacin. Tob: 70 µg/mL tobramycin. Tet: 20 µg/mL tetracycline. Gen: 200 µg/mL gentamicin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....</p>	82
<p>Figure 4.1 Effect of 0.17 pM GM-CSF on gene expression in <i>P. aeruginosa</i> PAO1. (A) Number and categories of genes that were consistently induced or repressed in two biological replicates of <i>P. aeruginosa</i> PAO1 persister cells. (B) Number and categories of genes that were consistently induced or repressed in two biological replicates of <i>P. aeruginosa</i> PAO1 normal cells.....</p>	105
<p>Figure 4.2 Effects of supernatants of GM-CSF-treated <i>P. aeruginosa</i> PAO1 persister cells. The normal cells harvested from stationary phase cultures of <i>P. aeruginosa</i> PAO1 and PAK were treated with the supernatant collected from the persister cells of <i>P. aeruginosa</i> PAO1 (A), and PA0620::<i>phoA</i> (B) after treatment with 0.17 pM or 0.17 nM GM-CSF for 2 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of PAO1 and PAK cells was determined by counting CFU. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....</p>	109
<p>Figure 4.3 Mutation in PA0620 (R2-pyocin tail fiber gene) reduced the synergistic effects of GM-CSF and antibiotics on persister cells isolated from stationary phase cultures compared to wild-type <i>P. aeruginosa</i> PAO1. The persister cells of PA0620::<i>phoA</i> were isolated from stationary phase cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF alone or with GM-CSF plus antibiotics as indicated for 3.5 h (all tested at 5 µg/mL). The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....</p>	110

Figure 4.4 GM-CSF show marginal reduction in sensitization of the persister cells of ABC (PA0218) and MFS transporter (PA3594) transcriptional regulator mutants to antibiotics. The persister cells were isolated from stationary phase cultures of PA0218:: <i>phoA</i> (ABC transporter transcriptional regulator mutant) and PA3594:: <i>phoA</i> (MFS transporter transcriptional regulator mutant) by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF plus 5 µg/mL ciprofloxacin, and 5 µg/mL tobramycin. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....	112
Figure 4.5 Structure of R2-pyocin. Adapted from Scholl <i>et al.</i> Copyright, 2009, American Society for Microbiology.....	115
Figure 4.6 Multidrug efflux pumps of Gram-negative bacteria. Adapted from Piddock <i>et al.</i> Copyright, 2006, American Society for Microbiology.....	117
Figure 5.1 Co-Immunoprecipitation identified FliC as GM-CSF binding target in <i>P. aeruginosa</i> PAO1. (A) Co-IP eluent of anti-GM-CSF coated beads after incubation with <i>P. aeruginosa</i> PAO1 membrane protein-GM-CSF cross-linking sample using Western blotting. (B) Binding was observed between isolated flagellar fraction from <i>P. aeruginosa</i> PAO1 and GM-CSF, as detected by anti-GM-CSF using Western blotting.....	136
Figure 5.2 Western blotting showed that GM-CSF binding was abolished in the <i>fliC</i> mutant strain, but was recovered with complementation of <i>fliC</i>. The whole cell lysates of wild-type <i>P. aeruginosa</i> PAO1, <i>fliC</i> mutant strain PAO1 $\Delta fliC$, and <i>fliC</i> complemented strain PAO1 $\Delta fliC/pUCPfliC$ were cross-linked with GM-CSF and detected by anti-GM-CSF.....	137
Figure 5.3 GM-CSF sensitized the persister cells of <i>P. aeruginosa</i> PAO1 and PAO1 $\Delta fliC/pUCPfliC$ isolated from stationary phase cultures to antibiotics, while $\Delta fliC$ persister cells did not get sensitized. The persister cells of <i>P. aeruginosa</i> PAO1, PAO1 $\Delta fliC/pUCPfliC$, and PAO1 $\Delta fliC$ were isolated from stationary phase cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF alone and with GM-CSF plus 5 µg/mL ciprofloxacin or tobramycin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p <$	

0.05, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test..... 139

Figure 5.4 Blocking FliC with the anti-FliC antibody reduced the activity of GM-CSF. Persister cells of wild-type *P. aeruginosa* PAO1 were isolated from the stationary cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h. The harvested persister cells were pretreated with or without 10 µg/mL anti-FliC for 1 h, followed by treatment with 0.17 pM GM-CSF and 5 µg/mL tobramycin for 3.5 h. Following the treatment, the viability of persister cells was determined by counting CFU. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test..... 140

Figure 5.5 GM-CSF sensitized the persister cells of a-type flagellin producing *P. aeruginosa* PAK isolated from exponential and stationary phase cultures to antibiotics. The persister cells of *P. aeruginosa* PAK were isolated from exponential phase (A) and stationary phase (B) cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF alone and with GM-CSF plus 5 µg/mL ciprofloxacin or tobramycin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test..... 142

Figure 5.6 GM-CSF binds to both a-type and b-type flagellins. Flagella were isolated from the wild-type *P. aeruginosa* PAO1 possessing b-type flagellins and *P. aeruginosa* PAK possessing a-type flagellins. The flagella were incubated in the presence and absence of GM-CSF with cross-linking using BS3, followed with detection by anti-GM-CSF..... 143

Figure 5.7 Additions of isolated flagella and OMVs partially restores the effects of GM-CSF on the persister cells of the *fliC* mutant PAO1 $\Delta fliC$. The flagella and OMVs were isolated from the wild-type *P. aeruginosa* PAO1. The persister cells of *fliC* mutant PAO1 $\Delta fliC$ were isolated from stationary phase normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with 5 µg/mL tobramycin plus 0.17 pM GM-CSF (i), 0.17 pM GM-CSF and isolated flagella (ii), 0.17 pM GM-CSF and OMVs (iii) or 0.17 pM GM-CSF, isolated flagella, and OMVs (iv), for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p <$

0.001, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....	145
Figure 5.8 BF8 inhibited the motility of <i>P. aeruginosa</i> PAO1. (A) Swimming motility assay of <i>P. aeruginosa</i> PAO1 in 0.3% (w/v) agar plates. (B) Viability of total viable cells of <i>P. aeruginosa</i> PAO1 after treatment with 1, 5, 10, and 20 $\mu\text{g}/\text{mL}$ BF8 for 3.5 h. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....	147
Figure 5.9 GM-CSF sensitized the normal cells of <i>P. aeruginosa</i> PAO1 isolated from stationary phase cultures when flagellar activity was inhibited. Normal cells of <i>P. aeruginosa</i> PAO1 were isolated from stationary phase cultures and pretreated with 5 $\mu\text{g}/\text{mL}$ BF8 (A), or 256 $\mu\text{g}/\text{mL}$ (B) for 3.5 h. The pretreated cells were then treated with 0.17 pM GM-CSF in the absence or presence of 5 $\mu\text{g}/\text{mL}$ ciprofloxacin or 5 $\mu\text{g}/\text{mL}$ tobramycin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of PAO1 cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....	149
Figure 5.10 Reduction in flagellar motor activity due to mutations in <i>motA</i> and <i>motD</i> allows GM-CSF to sensitize the normal cells of <i>P. aeruginosa</i> to antibiotics. The normal cells of motor mutants <i>motA::lacZ</i> and <i>motD::lacZ</i> were isolated from stationary phase cultures and then treated with GM-CSF plus 5 $\mu\text{g}/\text{mL}$ ciprofloxacin (A) or 5 $\mu\text{g}/\text{mL}$ tobramycin (B) for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of normal cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.....	151
Figure 5.11 Structure of a typical Gram-negative bacterial flagellum and its components. Reproduced from Chevance <i>et al.</i> with permission. Copyright, 2008, Nature Reviews Microbiology.....	154
Figure 6.1 A model to explain the observed effects of GM-CSF on <i>P. aeruginosa</i> persister cells. The possible induction of pyocin production and inactivation of transporters are supported by DNA microarray data. Further tests are required to validate this model at the protein and cellular levels.....	177

Dedicated to my beloved parents,

Lata and Sanjay Choudhary

CHAPTER 1

MOTIVATION, HYPOTHESIS, AND OBJECTIVES

1.1 Motivation

Bacterial infections are caused by pathogenic bacteria through multiple-factorial processes involving the increase in bacterial cell population, release of virulence factors, and the availability of host immune factors and antimicrobials ^{1,2}. Infections are initiated when the pathogenic bacteria reach their target site, multiply rapidly, acquire nutrients from the host, survive the attack by the host's immune system, and progress into disease with damage to the host's vital functions ³. Previous studies on bacterial virulence factors, toxins, endotoxins, transmission, and target sites in the past decades led to the discovery of antibiotics for controlling bacterial infections and diseases ⁴. Common modes of antibiotic action on bacteria include the inhibition of cell wall synthesis, protein synthesis, and DNA replication/repair ^{5,6}. Antibiotics are broadly classified into beta-lactams, aminoglycosides, quinolones, sulfonamides, and macrolides, based on their modes of action ⁵.

The development of antibiotics since 1940s drastically reduced bacterial infections and patient fatality rate ⁷. However, due to overuse of antibiotics, strains of bacteria began acquiring antibiotic resistance, which is recognized as a great threat to human health worldwide ⁷⁻⁹. In the North America, nearly 2 million people develop hospital-acquired infections (HAIs) per year and at least 23,000 deaths are caused directly by antibiotic-resistant infections (*Antibiotic Resistance Threats in the United States 2013, CDC report*). Antibiotic resistance also places a huge economic burden on our healthcare system with an annual cost estimated as \$21 to \$34 billion in the U.S. along with more than 8 million additional days of hospital stay (*Antimicrobial Resistance: Global Report on Surveillance 2014, WHO*). With increasing damage to the

healthcare and economy caused by antibiotic resistant infections, there is an urgent need to find novel methods to control persistent bacteria.

1.2 Hypothesis and Research objectives

Cytokines, the signaling protein molecules secreted by immune cells play a significant role in protection against bacterial and viral infections¹⁰. Direct interactions between bacteria and cytokines haven't been studied extensively. Kanangat *et al.*¹¹ reported that intracellular bacterial growth of the pathogens *Staphylococcus aureus*, *P. aeruginosa*, and *Acinetobacter sp.* changed with concentration of IL-1 β , IL-6, and TNF- α . Recently, the use of immunotherapeutic agents like IL-12 and recombinant GM-CSF is being explored for the treatment of drug resistant Tuberculosis (TB) caused by *Mycobacterium tuberculosis*¹². The main focus of our study is the effects of GM-CSF on *P. aeruginosa*. We hypothesize that GM-CSF can sensitize *P. aeruginosa* persister cells to antibiotics through interaction with specific targets in this bacterium. This study has the following aims:

Aim 1 (Chapter 3): To test the effects of GM-CSF on planktonic cells and biofilms of *P. aeruginosa* PAO1, PDO300, and *E. coli* K12 in the presence and absence of representative antibiotics (ciprofloxacin, tobramycin, tetracycline and gentamicin).

The results showed that GM-CSF can sensitize *P. aeruginosa* persister cells to multiple antibiotics. Moreover, after addition of enzymes like alginate lyase and DNase I, GM-

CSF and antibiotics displayed synergistic killing effects on biofilm cells of *P. aeruginosa*.

Aim 2 (Chapter 4): To understand the effects of GM-CSF on gene expression in *P.*

***aeruginosa* PAO1.** DNA microarrays and quantitative real-time PCR were used to identify the genes induced or repressed by GM-CSF. After categorizing the genes according to their functions, potential targets were selected and mutant strains were tested with GM-CSF and antibiotics. The comparative studies between normal and persister cells revealed that treatment with GM-CSF induced pyocin and flagella genes in *P. aeruginosa* persisters, which is not observed in normal cells. Next, to understand how binding of GM-CSF with its targets affects the persister cells of *P. aeruginosa* PAO1, some representative genes found in our microarray studies were characterized. The induced pyocin production after GM-CSF treatment was explored by using R2-pyocin-producing *P. aeruginosa* PAO1, and the R2-pyocin-sensitive strain, *P. aeruginosa* PAK.

Aim 3 (Chapter 5): To characterize the interaction between GM-CSF and potential

cellular targets in *P. aeruginosa* PAO1. The upregulation of flagellar genes by GM-CSF indicated possible role of flagella in the persister control by GM-CSF. Using co-immunoprecipitation (co-IP) and cross-linking experiments, GM-CSF was found to interact with FliC of *P. aeruginosa*. Consistently, deletion of the *fliC* gene abolished the activity of GM-CSF, which was restored in the strain complemented with plasmid-borne *fliC*. The tests with *motA* and *motD* mutants along with motility inhibiting agents indicated that GM-CSF is more effective in interaction with resting flagella.

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CHAPTER 2

LITERATURE REVIEW

2.1 Bacterial persistence

First described by Joseph Bigger in the early 1940s¹, persister cells are small subpopulations of dormant phenotypic variants, which can be found in virtually all bacterial cultures¹. Recent research has shown that persister cells play important roles in intrinsic antibiotic resistance of bacteria². The dormant nature of persister cells allows this subpopulation to survive the attack of essentially all antibiotics³. Balaban *et al.*⁴ demonstrated by microscopic observation of individual bacterial cells grown in microfluidic devices that persisters have a significantly reduced growth. Thus, when an antibiotic therapy is stopped, the surviving persisters can relapse to normal cells, causing chronic infections with recurring symptoms.

Because persisters are phenotypic variants rather than genetic mutants, these cells can revert to normal cells upon inoculation of new culture⁴. Antibiotic tolerance differs from antibiotic resistance as it is not caused by mutations but rather by a small bacterial population existing in a transient, dormant state⁵. Moreover, persisters have the ability to shield themselves from recognition and elimination by the host immune system by hiding in locations like biofilms (e.g. *Pseudomonas aeruginosa*), central nervous system (e.g. *Treponema pallidum*), macrophages or granulomas (e.g. *Mycobacterium tuberculosis*), stomach (e.g. *Helicobacter pylori*), gall bladder (e.g. *Salmonella typhi*), nose (e.g. *Staphylococcus aureus*), etc.⁶. The significant increase in the frequency of persister formation in biofilms can partially explain the biofilm associated tolerance to antibiotics⁷. Given the high level antibiotic tolerance⁷ of persister cells and the pathogenic infections, the ways to control and tackle their emergence and progress need to be explored.

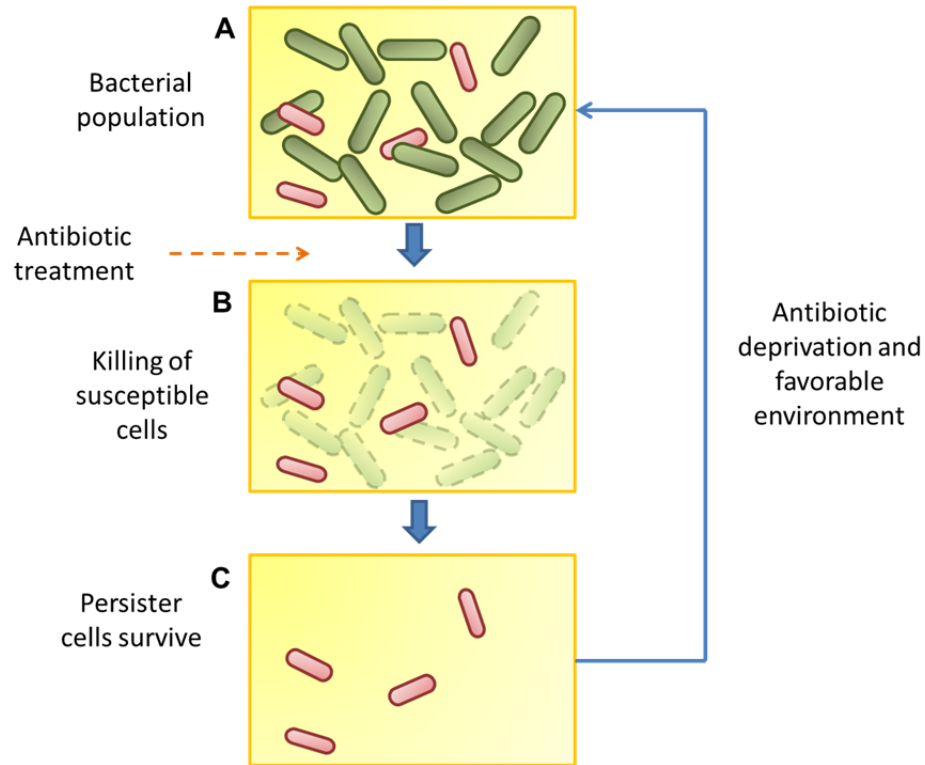


Figure 2.1 Schematic diagram of bacterial persistence. Green cells indicate normal cells, red cells indicate persister cells. (A) Bacterial cells in a culture, (B) Cell lysis of normal cells after antibiotic treatment, (C) Survival of persister cells in presence of antibiotics, which revert to normal state and repopulate due to antibiotic deprivation and favorable conditions for growth.

2.1.1 Toxin-Antitoxin system

Although not fully understood, persister formation has been constantly linked to toxin-antitoxin (TA) modules, which are ubiquitous in bacterial chromosomes and are involved in bacterial stress response⁸. These TA modules encode a “toxin” that disrupts cellular processes and a corresponding “antitoxin” that neutralizes the toxin^{8,9}. Five types of TA systems have been discovered to date and are described in Table 2.1. These TA systems include type I and III, where the antitoxins are RNAs that inhibit the toxin translation or

activity; and type II, IV, and V, where the antitoxins are proteins that inhibit toxin activity, counterbalance toxin activity or inhibit toxin synthesis ¹⁰.

Besides persistence, TA systems are also involved in the regulation of bacterial motility, biofilm formation, and quorum sensing ⁹. Quorum sensing is a process of cell-cell communication that involves regulation of gene expression in response to fluctuations in cell-population density. Autoinducers are the extracellular signaling molecules that are produced during quorum sensing ^{11,12}. At low cell density, these autoinducers are below detectable concentrations, whereas the autoinducers reach a high concentrations at high cell density, leading to their detection and response ¹¹. The production is followed by their detection by receptors existing in bacterial cytoplasm or membrane ¹¹. The detection of autoinducers causes activation of gene expression for group behaviors and further production of autoinducers ^{11,13}. A vast majority of Gram-negative bacteria use acylated homoserine lactones (AHL) as autoinducers, and Gram-positive bacteria use processed oligo-peptides for signaling ^{12,13}. The other mechanisms involved in persister formation include SOS response to DNA damage, nutrient transition, amino acid starvation, oxidative stress, quorum signaling, indole signaling, and other stresses ^{14,15}. A variety of bacterial species produce large quantities of intercellular signal molecules known as indoles, which control bacterial physiological activities like plasmid stability, drug resistance, virulence, biofilm formation, and cell-cycle regulation ¹⁶⁻¹⁸. By understanding the diverse pathways through which bacterial persistence is achieved, potential ways to control persister cells can be found.

Table 2.1 Currently known types of TA systems.

TA system	Toxin	Antitoxin	Mode of Action	Examples	References
Type I	Protein	RNA	Antitoxin, a small antisense RNA, base-pairs with the toxin encoding mRNA.	BsrG/SR4, Hok/Sok, Tisb/IstR, SymER, Ldr/Rdl, Ibs/Sib, Shob/OhsC, Zor/Orz	Jahn <i>et al.</i> ¹⁹ , Gerdes <i>et al.</i> ²⁰ , Vogel <i>et al.</i> ²¹ , Kawano ²² , Fozo ²³ , Fozo ²³ , Fozo ²³
Type II	Protein	Protein	Antitoxin, an unstable protein, binds to the toxin and neutralizes it.	MazE/MazF, RelE/RelB, YefM/YoeB, MqsR/MqsA, HipB/HipA, VapB/VapC.	Engelberg-Kulka <i>et al.</i> ²⁴ , Pederson <i>et al.</i> ²⁵ , Kamada <i>et al.</i> ²⁶ , Wang <i>et al.</i> ²⁷ , Hansen <i>et al.</i> ²⁸ , Zhang <i>et al.</i> ²⁹
Type III	Protein	RNA	Antitoxin, a small antisense RNA, directly binds with the toxin and neutralizes it.	ToxI/ToxN, CptI/CptN, TenpI/TenpN	Fineran <i>et al.</i> ³⁰ , Blower <i>et al.</i> ³¹ , Blower <i>et al.</i> ³¹
Type IV	Protein	Protein	Antitoxin, a protein, interferes with binding of the toxin to its target.	CbtA/CbtB	Masuda <i>et al.</i> ³²
Type V	Protein	Protein	Antitoxin, a protein, cleaves toxin mRNA preventing toxin translation.	GhoS/GhoT	Wang <i>et al.</i> ³³

Consistently, genes encoding for TA modules have been found upregulated in the persister cells of *Escherichia coli*, compared to normal cells, according to the report by Shah et al.³⁴. Deletion of the *hipBA* module led to a sharp reduction in the number of persister cells (by 10-100 fold in stationary cultures)³⁵. Dorr et al.³⁶ reported that knocking out SOS-TA locus *tisAB/istR* significantly reduced the tolerance of persister cells to the antibiotic ciprofloxacin by 10-100 fold. As shown by Jayaraman *et al.*⁶, persister formation is also stochastic in nature and is believed to be a strategy for cells to reserve a small fraction of bacterial population for possible, unavoidable environmental stresses. To date, two types of persister cells have been reported: type I persisters are non-growing cells formed in response to stress factors mostly in stationary phase, while type II persisters are slowly growing persisters formed in exponential phase by phenotypic switch in the absence of stress factors and can revert to normal cells and regrow^{4,37}. Zhang *et al.*³⁸ studied persisters in tuberculosis (TB) and established a Yin-Yang model of persisters and latent infections. The model described reverters as a small population of non-growing or slowly growing persisters in exponential phase which offers resistance during latent infection, while the “stem” persisters are described as small population in stationary phase with the ability to cause reactivation³⁸. The dormancy and antibiotic tolerance of persister cells as well as their capabilities to relapse to normal cells pose a major challenge to the treatment of infectious diseases².

2.1.2 Controlling bacterial persistence

Persisters pose significant hindrance to complete eradication of bacterial infections, due to their ability to survive multiple stress conditions³⁹. Controlling persister cells is

considered as a powerful strategy to reduce or eliminate the occurrence of chronic infections. Some of the methods that have been studied to control bacterial persister cells are discussed in Table 2.2 which includes waking-up persister cells, persister-specific antibiotics, combination therapies, and inducing reactive oxygen species (ROS) production etc.

Table 2.2 Some known methods to control bacterial persistence.

Method	Mechanism	Species	References
Metabolite-enabled killing strategy	Aminoglycosides in combination with specific proton motive force (PMF)-stimulating metabolites.	<i>Escherichia coli</i> , <i>Staphylococcus aureus</i>	Allison <i>et al.</i> ⁴⁰
Antimicrobial peptides	Bacterial membrane disruption.	<i>Escherichia coli</i>	Chen <i>et al.</i> ⁴¹
Stimulating reactive oxygen species (ROS) production	Increasing ROS via an NADH-dependent redox cycling pathway by the antibiotic clofazimine.	<i>Mycobacterium smegmatis</i>	Grant <i>et al.</i> ⁴²
Stimulating reactive nitrogen intermediates (RNI) production	Exogenous nitric oxide (NO) at sublethal concentrations increases RNI accumulation, leading to dispersal of persistent biofilms and improved tobramycin susceptibility.	<i>Pseudomonas aeruginosa</i>	Barraud <i>et al.</i> ⁴³
Protease activating antibiotic	Acyldepsipeptide antibiotic (ADEP4) activates the ClpP protease and causes extensive protein degradation. ADEP4 in combination with Rifampicin or linezolid eradicates persisters.	<i>Staphylococcus aureus</i>	Conlon <i>et al.</i> ⁴⁴
Silver and antibiotics combinations	Silver ions induce formation of hydroxyl radical and increase membrane permeability through	<i>Escherichia coli</i>	Morones-Ramirez <i>et al.</i> ⁴⁵

	disruption of disulfide bond formation and misfolded protein secretion.		
Weak electrochemical currents and antibiotic synergy	Low-level direct currents (DCs) and release of metal ions in presence of an electric field have a bacteriostatic effect and enhance effects of aminoglycoside tobramycin.	<i>Pseudomonas aeruginosa</i>	Niepa <i>et al.</i> ⁴⁶
Persister-specific targeting	Metronidazole (MTZ), an antibiotic that causes DNA damage in persister cells only in hypoxic environments.	<i>Mycobacterium tuberculosis</i>	Lin <i>et al.</i> ⁴⁷
	Pyrazinamide(PZA), an antibiotic enters bacteria through passive diffusion. It is more active against persister cells due to slowed down energy production and efflux pump activities, which leads to accumulation of toxic pyrazinoic acid (POA).	<i>Mycobacterium tuberculosis</i>	Zhang <i>et al.</i> ⁴⁸
	3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate (C10), a chemical kills persister cells and reduces the persister frequency by waking up and reverting them to antibiotic-sensitive cells.	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>	Kim <i>et al.</i> ⁴⁹
Engineered prototypical persister-specific antibiotic	Pentobra, a multifunctional antibiotic that combines membrane activity with protein synthesis inhibition. The antibiotic is engineered by addition of 12 amino acids to tobramycin.	<i>Escherichia coli</i> <i>Staphylococcus aureus</i>	Schmidt <i>et al.</i> ⁵⁰
Triggering persister wake-up by nutrient sources	Addition of mannitol, a carbon source improves the efficacy of aminoglycoside tobramycin by reverting the persister cells to normal metabolically active cells.	<i>Pseudomonas aeruginosa</i>	Barraud <i>et al.</i> ⁵¹
Quorum sensing inhibition	(Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8), a quorum sensing inhibitor reverts antibiotic tolerance of persister cells.	<i>Pseudomonas aeruginosa</i>	Pan <i>et al.</i> ⁵²

In spite of some promising studies and findings to tackle bacterial persister cells, much remains to be understood about the biology of persisters. There are limitations with considering individual persister cells for transcriptomic and proteomic profiling, sensitive diagnosis of latent infections harboring dormant persister cells, establishing relevant models for persistence to conduct mechanistic studies of *in vivo* conditions, analysis of host response to persister-specific targeting, etc. It will be interesting to identify host immune mechanisms during latent infections, and potentially develop useful immunotherapeutic treatments that can alter the microenvironments required for persister survivals and eventually eradicate persister cells.

2.2 Bacterial biofilms

Persister population increases when a culture enters stationary phase or when cells form a biofilm, which is a complex community of cells growing on a surface with the protection of an extracellular matrix secreted by the attached cells⁵³. Biofilms account for 80% of chronic infections in humans diseases⁵³. The biofilm infections are 10 to 1000 times more resistant to antibiotic effects⁵⁴. For example, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* are known to form biofilms which cause chronic infections in patients suffering from cystic fibrosis and infected surgical implants respectively⁷. Biofilm structures are complex with structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substances⁵³. The extracellular polymeric substances (EPS) consist of polysaccharides, and in some cases nucleic acids, proteins, lipids, biosurfactants, bacterial flagella and pili⁵⁵. The biofilm matrix can limit the penetration of antibiotics and immune factors leading to

recalcitrance of infections ². Biofilms are formed on both biotic and abiotic surfaces, making them ubiquitous in nature and potent reservoirs of chronic infections ⁵⁶.

The polymeric substances comprising the extracellular matrix retard the diffusion of antibiotics, making it harder for the antibiotics to reach the target site ⁵³. Moreover, the presence of polysaccharide matrix makes it difficult for the phagocytic cells to engulf the biofilm cells ⁵⁷. Besides these physical protections, the high level of persister formation in nutrient-deficient environment of biofilms acts as a survival strategy towards antibiotics or stress ⁷. As depicted in Figure 2.2, bacterial biofilm development involves five stages: (i) reversible attachment of bacterial cells on the surface, (ii) irreversible attachment by loss of flagellar motility and mediated by exopolymeric substances, (iii) early development of biofilm architecture, (iv) maturation by development of complex biofilm architecture, and (v) dispersal by release of motile cells from the biofilm microcolonies ⁵⁸. Besides flagella, *P. aeruginosa* also uses type IV pili-mediated twitching motility and chaperone usher pathway (CUP) fimbriae in the biofilm formation process ^{59,60}.

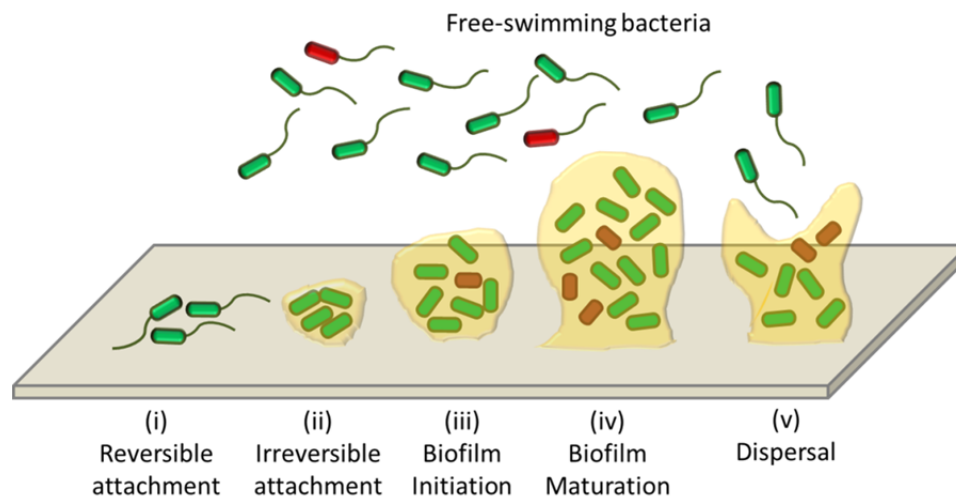


Figure 2.2 Stages of bacterial biofilm formation on surfaces.

The rate of bacterial attachment to a surface and biofilm formation are also affected by the nutrient concentration and type, temperature, pH and ionic strength ⁶¹. Novel approaches to control antibiotic resistant biofilms include: (i) small molecules that interfere with biofilm formation, e.g. D-amino acids on *S. aureus* and *P. aeruginosa* biofilms ⁶², chelators on *S. aureus* biofilms ⁶³, *N*-acetylcysteine on *S. epidermidis* biofilms ⁶⁴, (ii) enzymes to degrade biofilm matrix, e.g. DNase I, Proteinase K and trypsin on *S. aureus* biofilms ^{65,66}, dispersin B on *S. epidermidis* biofilms ⁶⁷, and (iii) surface modification to inhibit biofilm formation, e.g. bactericidal/bacteriostatic coating agents (silver, furanones) ^{68,69}, anti-adhesion coating agents (silica colloids, trimethylsilane plasma) ^{70,71}. Due to high tendency of bacteria to form biofilm under unfavorable conditions, in addition to their resilience towards antibiotics and host immune systems, biofilms pose challenges to the control of bacterial infections.

2.3 *Pseudomonas aeruginosa*

P. aeruginosa is an opportunistic Gram-negative bacterial pathogen, that mainly affects humans with compromised immune systems ⁷². It is a metabolically versatile bacterium and is known to cause a wide range of severe infections ⁷³. These infections include urinary tract *infections*, bacteremia, bone and joint infections, respiratory system *infections*, dermatitis, pneumonia, meningitis, endophthalmitis, endocarditis, septicemia, and malignant external otitis ⁷⁴⁻⁷⁷. Due to significant damage to host tissues, intrinsic antibiotic resistance, and tendency to form biofilms, eradication of *P. aeruginosa* infection is difficult ^{73,78,79}. In addition, the multiple virulence factors of *P. aeruginosa* counteract the host immune defenses and increase the bacterium's competitiveness in

mixed microbial populations^{73,80}. The environmental conditions and the status of host immune system dictate the severity of *P. aeruginosa* infections, which can involve inert colonization, chronic infections, or highly virulent acute infections^{78,81}.

The cell-associated virulence factors like pili, fimbriae, and flagella aid in bacterial adherence to epithelial cells, motility, and invasion⁸². In addition, the presence of outer membrane non-pilus adhesins provides strong binding properties to *P. aeruginosa*^{83,84}. Another virulence factor is lipopolysaccharide, a glycolipid and an endotoxin, which forms the major portion of the outermost membrane of *P. aeruginosa* and acts as strong stimulator of host innate immune responses^{85,86}. Under stress conditions, *P. aeruginosa* converts to a mucoid phenotype, characterized by overproduction of exopolysaccharide alginate, which behaves as a virulence factor by forming a protective layer around the bacteria thereby combating high antibiotic concentrations and heightened immune responses^{87,88}. Apart from the cell-associated virulence factors, *P. aeruginosa* tends to produce extracellular virulence factors including toxins, proteases, hemolysins, and enzymes^{80,89}. Figure 2.3 and Table 2.3 show the multidimensional *P. aeruginosa* virulence factors that determine the pathogenicity of the bacterium and are responsible for causing severe damage to the host.

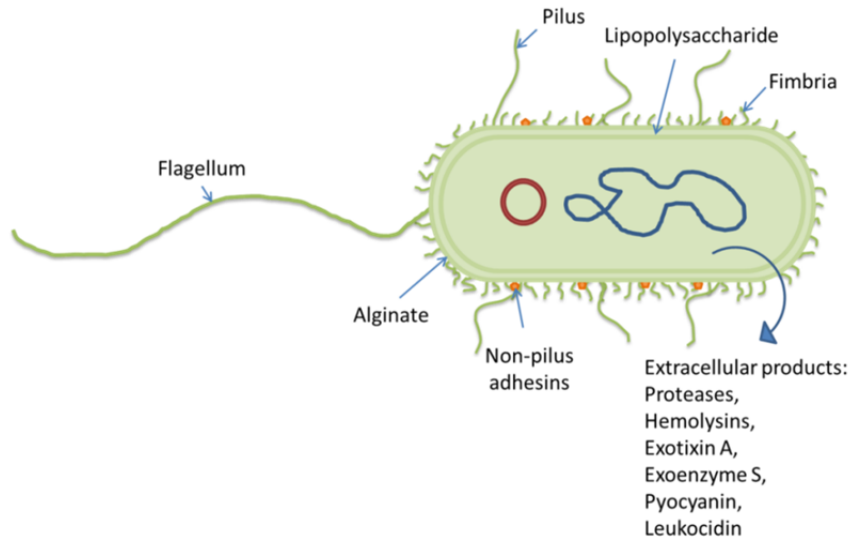


Figure 2.3 Schematic diagram of multiple virulence factors exhibited by *Pseudomonas aeruginosa*.

Table 2.3 Extracellular virulence factors produced by *Pseudomonas aeruginosa*.

Virulence factor	Functions
Exotoxin A	Inhibits host protein synthesis, causes tissue damage and immunosuppression ⁹⁰ .
Exoenzyme S	Mitogenic for T lymphocytes and induces T cell apoptosis ⁹¹ .
Elastase B	Degrades host elastin and collagen found in organs and tissues ⁹² .
Elastase A	Enhances the virulence activity of elastase B and host elastolytic proteases like human leukocyte elastase and human neutrophil elastase ⁹³ .
Protease IV	Degrades biologically important host proteins such as fibrinogen, plasminogen, and immunoglobulin G (IgG) ⁹⁴ .

Alkaline protease	Degrades laminin, a tissue-associated basement membrane protein and causes hemorrhagic host tissue necrosis ⁹⁵ .
Hemolytic phospholipase C	Causes intravascular hemolysis, organ damage, capillary injury, myonecrosis, and allows bacterial escape from intracellular phagolysosomes ⁹⁶ .
Pyocyanin	Interferes with vital host functions like cellular respiration, electron transport, and cell-cycle regulation along with maintaining dominance of <i>P. aeruginosa</i> with its antimicrobial properties ⁹⁷ .
Leukocidin	Damages leukocytes and host tissues ⁹⁸ .

In patients suffering from cystic fibrosis (CF), *P. aeruginosa* converts to the mucoid phenotype influenced by the CF microenvironment, and by overexposure to antibiotics ⁹⁹. Cystic Fibrosis is a severe autosomal recessive disease and is caused by homozygous mutations in the *CFTR* (The Cystic Fibrosis Transmembrane Conductance Regulator) gene, which is involved in transport of negatively charged chloride ions in and out of cells ^{100,101}. Water content is regulated by the transport of chloride ions to produce thin, free flowing mucus ¹⁰². This mucus acts as a protective coating in the lungs, digestive system, and reproductive system. ¹⁰³. The *CFTR* gene is also responsible for the transport of positively charged sodium ions across cell membrane ¹⁰¹. Mutations in the *CFTR* gene lead to overproduction of mucus in the tissues involving the respiratory system, digestive system, reproductive system, and other organs ¹⁰¹. The defects in Na⁺ transport and the failure to secrete Cl⁻ cause abnormal ion transport in CF airway epithelia, leading to depleted airway surface liquid (ASL) volume and persistent mucin secretions ¹⁰⁴.

The abnormal hyperproduction of viscid mucus observed in the lungs of CF patients augment the risk of bacterial infections¹⁰⁵. For example, *Staphylococcus aureus* and *Hemophilus influenzae* are most commonly found in the first decade of life of CF patients, while in the second and third decade, *Pseudomonas aeruginosa* is the most commonly found infectious agent, infecting around 80% of CF patients¹⁰⁵⁻¹⁰⁷. In CF patients, once *P. aeruginosa* forms biofilms, it is extremely difficult to eradicate¹⁰⁸. The mucoid matrix provides increased protection to biofilms and contains a large quantity of alginate exopolysaccharide¹⁰⁹. Fick *et al.*¹¹⁰ found that about 85% of *P. aeruginosa* strains isolated from the lungs of advanced stage CF patients had a mucoid morphology.

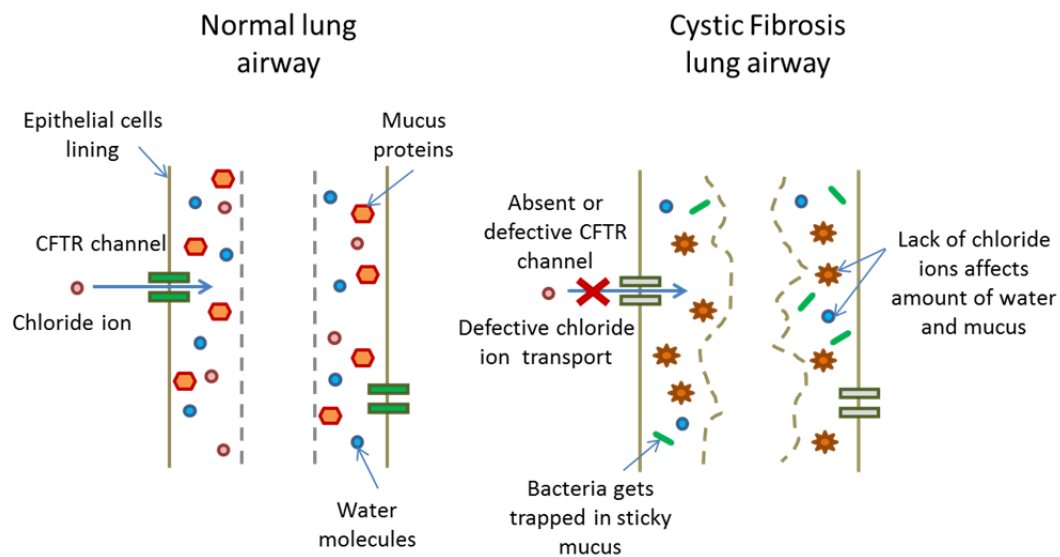


Figure 2.4 Differences between normal and cystic fibrosis lung airway.

Alginate is an *O*-acetylated linear polymer of D-mannuronate and L-guluronate residues, that provides altered biofilm architecture for enhanced attachment to surfaces and higher resistance towards antimicrobial treatments^{88,111}. The current recommendations for eradication of *P. aeruginosa* in respiratory secretions include (i)

intravenous anti-pseudomonal antibiotics, (ii) a regimen of nebulized colistin and oral ciprofloxacin, and (iii) tobramycin solution for inhalation (TSI) ¹¹²⁻¹¹⁴. Nebulized aztreonam lysine has been recently shown as a safe treatment for repeated use to suppress chronic *P. aeruginosa* infections in the lungs of CF patients ¹¹⁵. Azithromycin, a macrolide antibiotic, is regularly used as a treatment of chronic *P. aeruginosa* infections, and has been shown to reduce exacerbations in the lungs of CF patients ^{116,117}. Recently, it was demonstrated that intranasally administered bacteriophages (PAK-P3 and P3-CHA) were effective in treating lung infections caused by *P. aeruginosa* strains ¹¹⁸. Moreover, use of fosfomycin/tobramycin for inhalation (FTI) treatment was introduced as a promising combination antipseudomonal therapy for patients with CF ¹¹⁹. Among the treatments being tested for effective reduction of *P. aeruginosa* infections in CF patients, alternating and combination antibiotic therapies, and new drug delivery options, are being extensively studied for trials ¹⁰⁴. In spite of the ongoing efforts, the limitations to antibiotic dosage, host cell cytotoxicity, and ability of the bacterium to build up resistance, require novel methods to eradicate chronic infections.

2.4 Immune System

During bacterial infection, the human immune system coordinates many types of cells and molecules to eliminate the invading pathogen ¹²⁰. Host innate immunity acts as the first line of defense to block the entry of pathogens and to kill the microbes that successfully penetrate the epithelial barrier ¹²⁰. The innate immune system also activates adaptive immunity, which is more specific against the invading microbe and provides long-term protection by developing antibodies and memory lymphocytes ¹²⁰. During

innate immune response, macrophages and dendritic cells secrete cytokines, which are signaling proteins acting as mediators to attract more immune cells, such as phagocytes¹²¹. Figure 2.5 shows a schematic diagram of hematopoiesis of human immune system depicting differentiation of stem cells into different types of leukocytes.

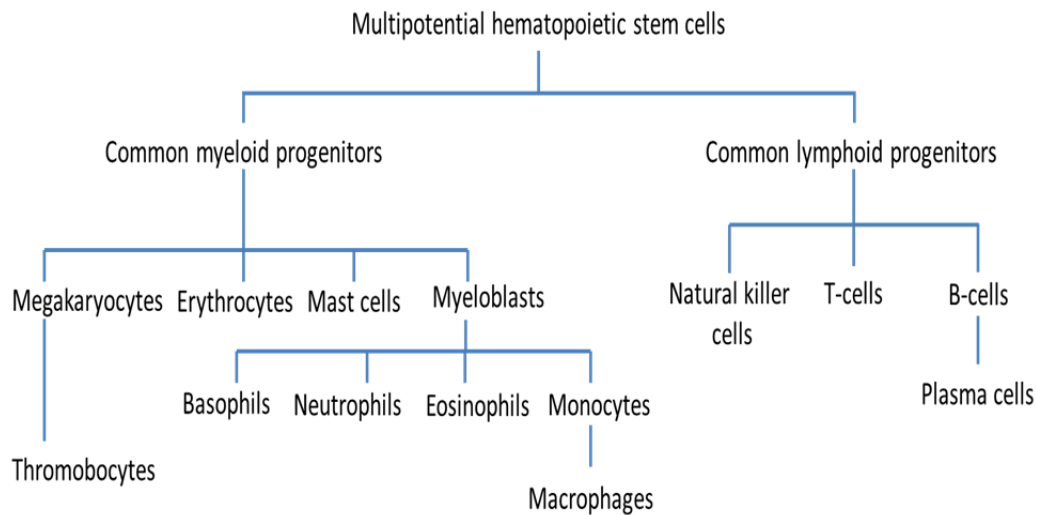


Figure 2.5 Leukocytes of human immune system.

Innate immune cells express pattern recognition receptors (PRRs) to identify pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS), flagellin, peptidoglycan, and lipoteichoic acid molecules, which are essential for survival of the microorganisms¹²². The recognition of pathogens is followed by their elimination by phagocytosis, which involve uptake of pathogens in phagosomes, and micropinocytosis, which involves uptake of macromolecules and extracellular fluid^{121,123,124}. For example, *P. aeruginosa* is recognized by macrophages by PAMPs and undergoes phagocytosis, while enterohemorrhagic *E. coli* produces Shiga toxin that enter host cells via macropinocytosis^{125,126}.

Cytokines are broadly categorized into groups such as lymphokines, chemokines, and interleukins, depending on the cell of secretion, functions, and targets of action ¹²⁷. Cytokines derived from monocytes, are called monokines, while lymphokines are derived from lymphocytes ¹²⁷. Based on their functions, cytokines can be classified into subgroups, including interleukins, tumor necrosis factors, interferons, colony stimulating factors, transforming growth factors, and chemokines ¹²⁸. These cytokines have important functions in regulating the host responses to infections and inflammation ¹²⁸. The bacterial PAMPs, such as lipopolysaccharides (LPS) and flagellin, induce secretion of cytokines, thus mediating immune response ¹²⁹. Some of the major cytokines secreted and actively involved in immune responses during pathogenic infections are shown in Table 2.4.

Table 2.4 Some major cytokines secreted during pathogenic invasions.

Cytokines	Size (kDa)	Source Immune Cells	Functions
Interferons:			
Interferon-α (IFN- α)	19-26	Monocytes/ Macrophages, lymphoblastoid cells, Fibroblasts ^{130,131} .	Antiviral, antiparasitic, antiproliferative ^{130,131} .
Interferon-β (IFN- β)	20	Fibroblasts, epithelial cells ¹³²⁻¹³⁴ .	Antiviral, antiproliferative, induces nerve growth factor production ¹³²⁻¹³⁴ .
Interferon-γ (IFN- γ)	20-25	T-cells, natural killer (NK) cells ^{135,136} .	Immunomodulation, antiviral, antiparasitic, antiproliferative ^{135,136} .
Tumor Necrosis Factors:			
Tumor Necrosis Factor-α (TNF- α)	17	Monocytes/macrophages, T-cells, NK cells, neutrophils, lymphocytes, mast cells ^{137,138} .	Inflammatory responses during pathogenic infections, induces tumor cell line apoptosis ^{137,138} .
Lymphotoxin-α (LT- α, also known as TNF- β)	25	T-lymphocytes ^{139,140} .	Induces lymphocyte proliferation, induces inflammatory responses ^{139,140} .

Colony Stimulating Factors:			
Granulocyte Colony-Stimulating Factor (G-CSF)	20	Monocytes/macrophages, neutrophils, fibroblasts, endothelial cells, stromal cells ^{141,142}	Stimulates proliferation and differentiation of hematopoietic progenitor cells into neutrophils, modulates neutrophil functions ^{141,142}
Macrophage Colony-Stimulating Factor (M-CSF)	70-90	Monocytes, granulocytes, fibroblasts, endothelial cells ^{143,144}	Stimulates proliferation and differentiation of hematopoietic progenitor cells into macrophages, stimulates phagocytic and chemotactic activities of macrophages ^{143,144}
Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF)	14-35	Macrophages, T cells, mast cells, NK cells, endothelial cells, fibroblasts ^{141,142,145,146}	Stimulates stem cells to produce granulocytes and macrophages, enhances antimicrobial activity, oxidative metabolism, and phagocytic activity of neutrophils and macrophages ^{141,142,145,146}
Interleukins:			
Interleukin-1 (IL-1)	17	Monocytes/macrophages, neutrophils, endothelial cells, fibroblasts, keratinocytes ^{147,148}	Induces inflammatory response during infections, stimulates T-helper cells, promotes proliferation of B-cells ^{142,143}
Interleukin-2 (IL-2)	15.4	T-cells, B-cells, NK cells ^{149,150}	Promotes proliferation of T-cells, anti-inflammatory response to microbial infections ^{144,145}
Interleukin-3 (IL-3)	15-17	T-cells, keratinocytes, mast cells, NK cells, endothelial cells, monocytes ¹⁵¹	Connects immune system and hematopoietic system, stimulates differentiation of multipotent hematopoietic stem cells into granulocytes, macrophages, erythroid cells, megakaryocytes, mast cells ¹⁵¹
Interleukin-4 (IL-4)	20	T-cells, mast cells ^{152,153}	Promotes proliferation of T-cells, induces anti-inflammatory response to infections, stimulates activated B-cells ^{152,153}

Interleukin-5 (IL-5)	50-60	T-cells, mast cells ^{151,154}	Proliferation, cell activation, differentiation of eosinophils, promotes generation of T-cells, stimulates activity of B-cells and increases immunoglobulin secretion during infections ^{146,149}
Interleukin-6 (IL-6)	21.5-28	Monocytes/macrophages, T-cells, fibroblasts, endothelial cells ¹⁵⁵⁻¹⁵⁷	Induces inflammatory response during infections, supports B-cells growth ¹⁵⁵⁻¹⁵⁷
Interleukin-7 (IL-7)	17.4	Bone marrow stromal cells and thymic cells, keratinocytes ^{158,159}	Induces proliferation during maturation of B-cells, T-cells, magakaryocytes ^{158,159}
Interleukin-8 (IL-8)	8	Monocytes/macrophages, endothelial cells, epithelial cells ^{157,160}	Neutrophil chemotactic factor, induces phagocytosis ^{157,160}
Interleukin-10 (IL-10)	19	Monocytes, T-cells ¹⁶¹⁻¹⁶³	Induces anti-inflammatory response during infections, enhances B-cell survival and proliferation, inhibits synthesis of proinflammatory cytokines ¹⁶¹⁻¹⁶³
Interleukin-11 (IL-11)	23	Bone marrow stromal cells, mesenchymal cells ^{164,165}	Stimulates lymphocytes growth, modulates antigen-antibody responses ^{164,165}
Interleukin-12 (IL-12)	70	Dendritic cells, macrophages, lymphocytes ^{166,167}	Stimulates growth and function of T-cells, enhances cytotoxic activity of NK cells ^{166,167}
Growth factors:			
Transforming Growth Factor-α (TGF- α)	5-8	Macrophages, pituitary cells, keratinocytes, hepatocytes, platelets ^{168,169}	Multiple cell proliferation, involved in tumerogenesis promotes angiogenesis, ^{168,169}
Transforming Growth Factor-β (TGF- β)	25	Macrophages, lymphocytes, endothelial cells, keratinocytes, leukemia cells ¹⁷⁰⁻¹⁷²	Induces proinflammatory response, inhibits proliferation of T-cells ¹⁷⁰⁻¹⁷²

The table shows examples of major cytokines involved in immunological response during pathogenic infections, and is not an all-inclusive list.

2.4.1 Cytokine therapy

Cytokines play important roles in the regulation of cell proliferation, inflammation, immunity, migration, fibrosis, repair, and angiogenesis¹⁷³. Cytokines are being explored for therapeutic purposes, owing to their multiple actions on target cells at low concentration ranges (e.g. picomolar and femtomolar)¹⁷⁴. Immune-based therapies (IBTs) are designed to improve the immune system and approved cytokine therapies are being used, while clinical trials are being carried out for some cytokine therapies. For example, IFN- α is used against hepatitis B & C, leukemia, malignant melanoma, and Kaposi's sarcoma (KS); IFN- β is used in multiple sclerosis; IFN- γ is used in chronic granulomatous disease, osteoporosis, and cancer; IL-2 is used in renal cell carcinoma and metastatic melanoma; IL-11 is used in post chemotherapy induced thrombocytopenia; G-CSF is used to treat neutropenia and recovery of bone marrow; TNF- α is used in therapeutics of Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis¹⁷⁴.

Active research is being performed on anti-HIV therapy through cytokines and their inhibitors besides clinical use of cytokine therapies¹⁷⁵. The findings from extensive studies and clinical research being carried out on cytokine therapies indicate novel unknown functions of cytokines, which have not been thought of previously. Cytokine pleiotropy and redundancy suggest that cytokine have multiple actions, and difference cytokines can display similar functions¹⁷⁶. Studying every cytokine individually and exploring its functions can give answers and solutions to health issues we have been struggling to find effective cures for. The complex role of cytokines during bacterial pathogenesis needs to be studied and understood further. There have been several studies,

which show that bacterial attachment to epithelial cells induce the release of cytokines¹⁷⁷⁻¹⁷⁹. Porat *et al.*¹⁸⁰ showed that IL- β , IL-2, GM-CSF, and epidermal growth factor (EGF) bind to virulent strains, and also stimulate their growth. Denis *et al.*¹⁸¹ were able to enhance the growth of a virulent strain of *E. coli* by IL-2 and GM-CSF. However, to our knowledge, there haven't been studies showing a direct connection between bacterial persistence and host cytokines, and the effect of cytokines on bacterial persistence has yet to be explored.

2.4.2 Granulocyte Macrophage Colony-Stimulating Factor

Granulocyte macrophage colony-stimulating factor (GM-CSF) is considered as the central regulator of innate immune response and is previously found to reactivate the impaired immune responses in immunocompromised mice or human cells¹⁸²⁻¹⁸⁴. Burgess *et al.*¹⁸⁵ first identified granulocyte macrophage colony-stimulating factor (GM-CSF) in the mouse, by its ability to stimulate the proliferation of mouse bone marrow cells *in vitro*, and to generate colonies of granulocytes and macrophages. GM-CSF is secreted by macrophages in response to microbial infections, and plays important roles in the survival and activation of macrophages, neutrophils, eosinophils, and the maturation of dendritic cells (Figure 2.6)¹⁸⁶. Human GM-CSF contains 127 amino acids, with four cysteine residues, two glycosylation sites; its calculated molecular weight (MW) is 14 kDa¹⁸⁷. The receptor for GM-CSF is CD116, which is expressed on the hematopoietic cells, and is composed of a α chain and β chain^{188,189}. GM-CSF binds to the α chain of CD116 with low affinity, but its binding to the β chain causes dimerization of both α and β subunits^{188,189}. This dimerization increases the binding affinity of GM-CSF to its receptor, leading

to receptor activation and subsequent stimulation of the JAK2 (Janus Kinase 2) signaling pathway¹⁸⁸⁻¹⁹⁰, which controls the production of blood cells from hematopoietic stem cells¹⁹⁰. Increase in the serum level of GM-CSF helps recruit monocytes/macrophages to sites of infection¹⁹¹. Under normal conditions, the level of human GM-CSF in the circulation is below 0.35 pM, but it increases in response to *P. aeruginosa* lipopolysaccharide (LPS), a major component of the outer membrane of this microbe.

86,192

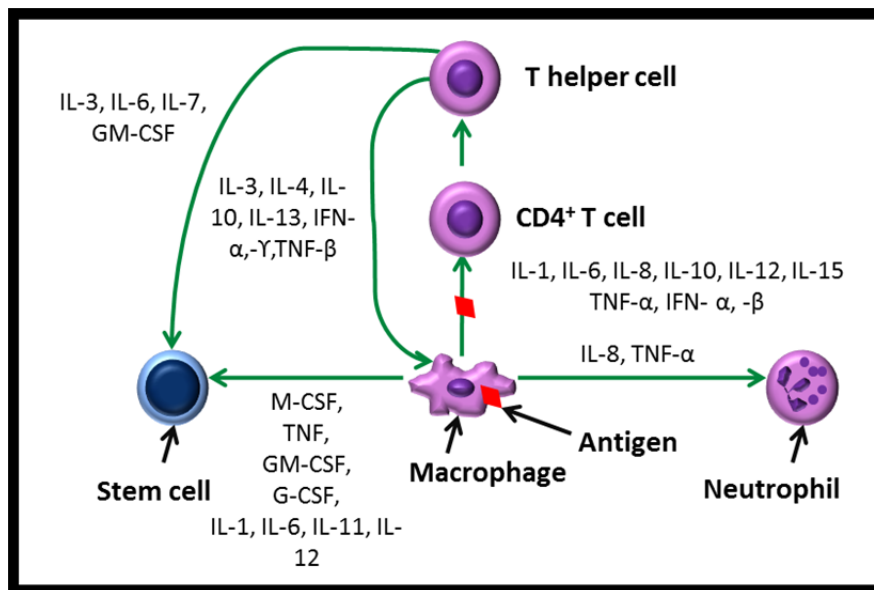


Figure 2.6 GM-CSF pathway in the immune system.

Compared to the well documented studies on cytokine production and the functions of cytokines in stimulating host immune cells, little is known about the direct effects of these immune factors on bacteria. The mechanisms of such phenomenon are not well understood and, to our knowledge, effects of cytokines on antibiotic tolerant persister cells have not been explored. Kanangat *et al.*¹⁹³ studied intracellular growth of

S. aureus, *P. aeruginosa*, and *Acinetobacter sp.* (6×10^6 CFU of each strain) added to monocytic cells primed with low doses (0, 10, 100, and 250 pg) and high doses (1 and 10 ng) of IL-1 β , IL-6, and TNF- α . It was found that at low cytokine doses (10 to 250 pg), the intracellular bacterial growth of all strains decreased; however, as the dose increased to 10 ng, the trend reversed¹⁹³. It was speculated that above the threshold of host cellular activation, the conditions become favorable for survival and replication of the ingested bacteria¹⁹³.

2.4.3 Clinical Significance and Uses of GM-CSF

A major clinical application of GM-CSF is the treatment of neutropenia following chemotherapy, which is often characterized by an abnormally low number of neutrophils, leading to weakening of primary defense against infections by pathogenic microorganisms¹⁹⁴. GM-CSF treatment leads to a significant increase in the total white blood cell count (TWBC), and absolute neutrophil count (ANC)¹⁹⁴. GM-CSF is also known to cause faster neutrophil recovery in patients receiving autologous bone marrow transplantation¹⁹⁵. Besides shortening the period of absolute neutropenia, the use of GM-CSF also led in one study to fewer infections, lowered antibiotic usage, and shorter duration of hospitalization¹⁹⁵. Moreover, Ye *et al.*¹⁹⁶ constructed a cytokine fusion protein consisting of GM-CSF and monocyte chemotactic activating factor (MCAF), which was able to sustain the growth of GM-CSF-dependent cell line, TF1 (a human premyeloid cell line which proliferates in response to cytokines) and TF1 cells were chemotactic for monocytes. The presence of fusion protein inhibited growth of several human tumor cell lines and mediated recruitment of monocytes to the tumor site¹⁹⁶. GM-

CSF was also shown to suppress leukemic cell apoptosis induced by Vp16, a cytotoxic anticancer drug ¹⁹⁷.

Some animal studies indicate that GM-CSF modulates lipid peroxidation and glutathione (GSH) content of the skin wound, thus reducing the lethal irradiation effects on incisional healing and production of cell damaging oxygen radicals ¹⁹⁸. Cytotoxic drug tolerance is enhanced by GM-CSF, permitting drug dose maintenance or increase in desired drug effects. For example, combination therapy with GM-CSF and tiazofurin shows led to a decrease in GTP pools caused by tiazofurin *in vitro*, and was beneficial for refractory leukemia patients ¹⁹⁹. The use of immunotherapeutic agents is being explored for the treatment of drug resistant Tuberculosis (TB) caused by *Mycobacterium tuberculosis* ²⁰⁰. Nambiar *et al.* ²⁰¹ demonstrated that target delivery of GM-CSF to the lungs of immunodeficient mice through the GM-CSF-secreting *Mycobacterium bovis* BCG vaccine strain (BCG:GM-CSF) led to an increase in pulmonary dendritic cell numbers and 10-fold more efficient clearance of *Mycobacterium tuberculosis* H37Rv. Fleischmann *et al.* ²⁰² demonstrated that 90% of polymorphonuclear neutrophils from humans turned phagocytic after exposure to 100 pM GM-CSF for 2 h. These studies suggest that GM-CSF is actively involved in immune response to pathogenic invasion.

In summary, GM-CSF has good therapeutic potential, as demonstrated for its antitumor activities, functions in reconstitution of hematopoietic system, increase in cytotoxic drug tolerance, reduction in infections by pathogens, and enhanced efficacy of antibiotics. More extensive studies on the dosage of GM-CSF, its possible roles depending on the presence or absence of other cytokines, and overall context of the immune response need to be performed to establish a reliable GM-CSF based therapy.

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CHAPTER 3

SYNERGY BETWEEN GRANULOCYTE MACROPHAGE COLONY- STIMULATING FACTOR (GM-CSF) AND ANTIBIOTICS PROMOTE THE KILLING OF PERSISTER CELLS OF *PSEUDOMONAS AERUGINOSA*

3.1 Abstract

Bacterial persister cells are highly tolerant to antibiotics and play important roles in chronic infections. However, the effects of host immune factors on persister cells have not been studied. To bridge this knowledge gap, we investigated the effects of granulocyte macrophage-colony stimulating factor (GM-CSF), a human cytokine, on the viability and persistence of the wild-type strain *Pseudomonas aeruginosa* PAO1, its mucoid mutant *P. aeruginosa* PDO300, and the non-pathogenic *Escherichia coli* K12. GM-CSF was found to sensitize the persister cells of *P. aeruginosa* PAO1 and PDO300 to multiple antibiotics including ciprofloxacin, tobramycin, tetracycline, and gentamicin. For example, after treatment with 0.17 pM GM-CSF for 1 h, $96.2 \pm 5.9\%$ and $79.3 \pm 8.3\%$ of persister cells of two different strains of *P. aeruginosa* PAO1 from exponential phase cultures were rendered sensitive to 200 $\mu\text{g}/\text{mL}$ ciprofloxacin. Significant effects were also observed for the mucoid strain *P. aeruginosa* PDO300, but not for the non-pathogenic *E. coli* K12. In comparison, no such effect was found against the normal cells of *P. aeruginosa* PAO1, PDO300, and *E. coli* K12. GM-CSF was found to significantly sensitize the biofilm cells of *P. aeruginosa* PAO1 and PDO300 to tobramycin in the presence of biofilm degrading enzymes like DNase I and alginate lyase respectively.

3.2 Introduction

Bacterial populations commonly harbor a phenotypically distinct and dormant subpopulation of persister cells which possess high level antibiotic resistance¹. The opportunistic pathogen *Pseudomonas aeruginosa* is a good model system for persister research². It is known to cause respiratory system infections, bone and joint infections, urinary tract infections, dermatitis, gastrointestinal infections, etc. in patients with weakened immune systems due to burn wounds, cystic fibrosis, organ transplants, AIDS, and acute leukemia². In cystic fibrosis patients, *P. aeruginosa* causes chronic infections, despite highly aggressive antimicrobial therapy³⁻⁵. The ability of *P. aeruginosa* to attach to surfaces and form biofilms with increased number of persister cells embedded in a protective extracellular matrix makes the pathogen even more challenging to treat⁶⁻⁸.

The antibiotics regularly used to treat *P. aeruginosa* include aminoglycosides (protein synthesis inhibitors), β -lactams (cell wall synthesis inhibitors), fluoroquinolones (nucleic acid synthesis inhibitors), and polymyxins (membrane disruptors)⁹. Because persisters are resistant to antibiotic treatments, it remains a challenge to eradicate this dormant subpopulation using antibiotics which can only destroy normal cells^{10,11}. When the treatment is stopped, the surviving persister cells revert to normal state and repopulate by actively multiplying^{12,13}. Owing to the survival strategies displayed by pathogenic bacteria through dormancy, antibiotic resistance, and biofilm formation, it is important to develop innovative methods to address these challenges.

Cytokines are signaling proteins produced by the immune cells and play a critical role in protection against bacterial and viral infections¹⁴⁻¹⁶. We were motivated to investigate the interactions between cytokines and bacteria. The pro-inflammatory

cytokines such as IL-1 and TNF- α promote systematic inflammation^{17,18}. In contrast, the anti-inflammatory cytokines such as IL-4, IL-10, and GM-CSF control the pro-inflammatory response and counteract the inflammation effects¹⁹⁻²¹. Granulocyte macrophage colony-stimulating factor (GM-CSF), a cytokine secreted by macrophages, T-cells, mast cells, NK cells, endothelial cells, and fibroblasts is considered as a major regulator governing the maturation of granulocytes and macrophages^{22,23}. GM-CSF also plays a key role in inflammatory and autoimmune diseases²⁴⁻²⁶. The significant roles of GM-CSF during bacterial infections have been well documented²⁶⁻³³.

However, the direct interaction of GM-CSF and bacterial persister cells has not been explored. Thus, we selected GM-CSF as a representative cytokine to investigate its effects on bacterial persister cells. We chose *P. aeruginosa* as the model bacterium because it is a widely used organism for research on persister cells and biofilms^{7,11,34-38}. Effects of GM-CSF on *P. aeruginosa* PAO1 and the mucoid strain PDO300 were compared with GM-CSF introduced either alone, or with an antibiotic, to test synergy. PDO300 is a *mucA22* mutant (due to a single base pair deletion) of *P. aeruginosa* PAO1, which overproduces the exopolysaccharide alginate³⁹. The alginate overproduction by mucoid *P. aeruginosa* makes the biofilms thicker, which hinders the penetration of antibiotics and reduces the phagocytic activity of macrophages⁴⁰. Alginate overproduction also leads to mucoidity, which is commonly seen in late stage cystic fibrosis patients with multidrug tolerant infections⁴¹. The non-pathogenic laboratory strain *Escherichia. coli* K12 was selected in this study to understand if GM-CSF has different activities on pathogenic and non-pathogenic strains.

3.3. Materials and Methods

3.3.1 Bacterial strains and growth media

The bacterial strains used in this study include two *P. aeruginosa* PAO1 strains (obtained from Prof. Thomas. K Wood⁴² at Pennsylvania State University and Prof. Matthew Parsek⁴³ at University of Washington, respectively) an isogenic mucoid mutant PDO300 (*mucA22*), and *E. coli* K12. Overnight cultures of these strains were prepared in Luria Bertani (LB) medium⁴⁴ containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at 37°C with shaking at 200 rpm. Recombinant human GM-CSF was purchased from R&D systems (Minneapolis, MN, USA). The stocks used in this study contained 10 µg/mL GM-CSF, dissolved in phosphate buffer saline (PBS) pH 7.4, supplemented with 0.1% bovine serum albumin (BSA).

3.3.2 Effects of GM-CSF on planktonic cells

The experiments performed on the planktonic cells were conducted with cells harvested from both the exponential and stationary phase (16 h after inoculation) cultures. For stationary cultures, the cells from an overnight culture of in LB medium were collected by centrifuging at 8,000 rpm for 10 min and washed twice with PBS (pH 7.4). The washed cells were resuspended in 25 mL PBS buffer and vortexed gently for 1 min to separate cells. A portion of the sample was used to determine the viability by plating the cells on LB agar plates and counting CFU using the drop plate method, as described previously⁴⁵, while the remainder was used for isolation of persisters by adding 200 µg/mL ciprofloxacin for *P. aeruginosa* PAO1 and PDO300, and 100 µg/mL ampicillin for *E. coli* K12, and incubating at 37°C for 3.5 h with shaking at 200 rpm^{11,46}. After

incubation, the antibiotic was washed away with PBS buffer by centrifuging thrice at 4°C, 8,000 rpm for 10 min each, and vortexed for 1 min after adding PBS. To test the effects of GM-CSF on viability of persister cells, the washed cells were transferred to microcentrifuge tubes, with 1 mL of washed cells in each tube. GM-CSF was added at 0, 0.17, 1.7, and 17 pM. These concentrations were selected because 0.17 pM is the concentration of GM-CSF found in the blood plasma of healthy humans⁴⁷. The amount of BSA (0.1%) was adjusted to be the same for all samples so that the effects of GM-CSF can be studied specifically.

For exponential phase planktonic cells, the experiments were performed with cells harvested from exponential phase subcultures with an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.4. After preparing an overnight culture of the tested strain in 25 ml LB medium, a subculture in LB medium was inoculated to an OD₆₀₀ of 0.01. The subculture was incubated at 37°C with shaking at 200 rpm for 3-4 h, until the OD₆₀₀ reached 0.3 to 0.4. After washing the subculture twice by centrifuging at 8,000 rpm for 10 min with PBS (pH 7.4) and isolating persister cells as described above, sequential treatment was performed for 1 h with 0.17 pM GM-CSF, followed by an antibiotic for 3.5 h. The viability of both normal and persister populations were quantified after treatments for 1 h and 3.5 h using drop plate method as described above. The antibiotics (ciprofloxacin, tobramycin, tetracycline, and gentamicin) used for exponential phase cultures were added at a concentration of 200 µg/mL for both *P. aeruginosa* PAO1 and PDO300. To confirm that any change in the viability of bacterial cells is due to the specific effect of GM-CSF rather than any contaminant, the persister cells of *P. aeruginosa* PAO1 isolated from exponential phase cultures were treated with 0.17 pM GM-CSF in the presence of

different concentrations of anti-GM-CSF antibody (0, 17, and 170 pM). The persister cells underwent treatment with GM-CSF alone, anti-GM-CSF alone, or GM-CSF neutralized by anti-GM-CSF for 2 h. Five $\mu\text{g}/\text{mL}$ ciprofloxacin was then added to all samples, which were incubated for 3.5 h. After washing the cells thrice at 13,200 rpm for 2 min, CFU was counted using drop plate method.

3.3.3 Effect of GM-CSF on biofilm cells

After preparing an overnight culture, each bacterial strain tested for biofilm formation was subcultured to an initial OD_{600} of 0.01 in a petri dish containing 20 mL LB medium and sterile 316L stainless steel coupons (1.75 cm x 1 cm, 0.05 cm thick). The biofilms of *P. aeruginosa* PAO1 and PDO300 were grown for 24 h at 37°C without shaking. After incubation, the coupons were washed by gently dipping in PBS and placed in 12-well plates. There were 7 treatment conditions in total and each condition was tested in triplicate: (i) GM-CSF alone, (ii) antibiotic alone, (iii) GM-CSF and alginate lyase, (iv) GM-CSF and antibiotic, (v) antibiotic and alginate lyase, and (vi) GM-CSF, antibiotic, and alginate lyase. In all the experiments, the concentrations of GM-CSF and alginate lyase were kept at 0.17 pM and 50 $\mu\text{g}/\text{mL}$, respectively. The control samples were supplemented with the same amount of BSA (0.1%) as present in the samples with 0.17 pM GM-CSF. The coupons were incubated at 37°C for 3.5 h. After treatment, each coupon was gently washed with PBS and placed in a test tube containing 3 mL of PBS. The coupons were gently sonicated (B200, Sinosonic Industrial Co., Ltd., Taiwan) for 4 min to release biofilm cells from coupon surface. This condition was confirmed to not kill the cells⁴⁸. After vortexing for 1 min, the cell suspensions were plated on LB agar

plates using drop plate method to count the number of CFU after incubation at 37°C for 24 h as described previously⁴⁵. Similar tests were performed on early (4 h) biofilm cells of *P. aeruginosa* PAO1 by using 5 units/mL DNase I instead of alginate lyase, and a lower concentration of 20 µg/mL tobramycin was used as the early stage biofilms have a significantly smaller bacterial population. To find if alginate lyase and DNase I have any cidal effects on the biofilm cells, alginate lyase at concentrations 10, 50, 100, and 200 µg/mL were tested on 24 h biofilms of *P. aeruginosa* PAO1 and PDO300, while DNase I at 1, 2, 5, and 10 units/mL were tested on 4 h biofilms of *P. aeruginosa* PAO1.

3.3.4 Effect of alginate lyase on GM-CSF diffusion across alginate layer

Polysaccharide alginate layers were developed on transwell inserts (0.4 µm pore size) by adding 100 µL of 0.3% w/v alginate (Sigma-Aldrich, St. Louis, MO, USA) in deionized water to each well. The transwell inserts were kept in a desiccator for 15 min under vacuum to remove water, followed by addition of 50 ng of GM-CSF (R&D systems, Minneapolis, MN) in 100 µL deionized water to each well. For the treatment samples, 100 µg/mL alginate lyase (in 100 µL deionized water) was added along with GM-CSF. Then the transwells were transferred to a 12-well plate with 1 mL deionized water in each well to submerge the transwell insert. GM-CSF was allowed to diffuse at 37°C over 2 h. The samples (10 µL each) were taken from transwell insert (labeled as “top”) and the well underneath the transwell (labeled as “bottom”) at 0, 1, and 2 h to determine the amount of GM-CSF using Western blotting.

For Western blotting, the protein samples were first separated with electrophoresis using 10% acrylamide gels. As a positive control, 50 ng GM-CSF was

loaded. After electrophoresis, the gels were transferred to blotting chambers for Western blotting (to PVDF transfer membranes at 250 mA for 2 h) and GM-CSF was detected using mouse-derived anti-GM-CSF (1:2000 dilution) as the primary antibody and anti-mouse IgG conjugated with alkaline phosphatase (1:20,000 dilution) as the secondary antibody. BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) were used (30 min of incubation with the membranes) to detect the alkaline phosphatase activity of conjugated secondary antibody. This experiment was conducted in triplicate and consistent results were obtained.

3.3.5 Kinetics of bacterial killing during antibiotic treatment

The antibiotic conditions used for isolation of persister cells of *P. aeruginosa* PAO1 , PDO300, and *E. coli* K12 were confirmed by measuring the killing curves over time similar to method described previously^{49,50}. The cells were harvested from exponential and stationary cultures, and treated with 200 µg/mL ciprofloxacin (*P. aeruginosa* PAO1 and PDO300) or 100 µg/mL ampicillin (*E. coli* K12) for 4.5 h. CFU during treatment was determined from samples at every 0.5 h after antibiotic treatment.

3.3.6 Statistical Analyses

The CFU data were analyzed with one-way ANOVA followed by Tukey test using SAS 9.2 software (SAS Institute, Cary, NC, USA). The results with $p < 0.05$ are considered significant.

3.4 Results

3.4.1 Identifying the appropriate antibiotic concentrations for persister isolation

The multidrug tolerant persister cells differ from antibiotic resistant mutants^{49,50}.

Antibiotic resistant mutants are based on acquired genes and have the ability to grow in the presence of antibiotics, while multidrug tolerance is a reversible physiological state without a genetic basis^{51,52}. This latter characteristic enables the subpopulation of persisters to survive killing by antibiotics, and to resume growth when antibiotics are removed, thus reverting to normal physiological stage of antibiotic sensitivity^{51,52}. The presence of the persister population was confirmed for the planktonic cells of *P.*

aeruginosa PAO1, PD300 and *E. coli* at exponential and stationary phases, as shown in Figure 3.1. It was observed that the 3.5 h treatment with high concentration of antibiotics (200 µg/mL ciprofloxacin for *P. aeruginosa* PAO1 and PDO300; 100 µg/mL Ampicillin for *E. coli* 12) resulted in a small group cells that did not respond to the increase in antibiotic treatment duration. Such a biphasic killing curve is a signature of cultures harboring persister cells, which proved that the conditions are effective in isolating persisters.

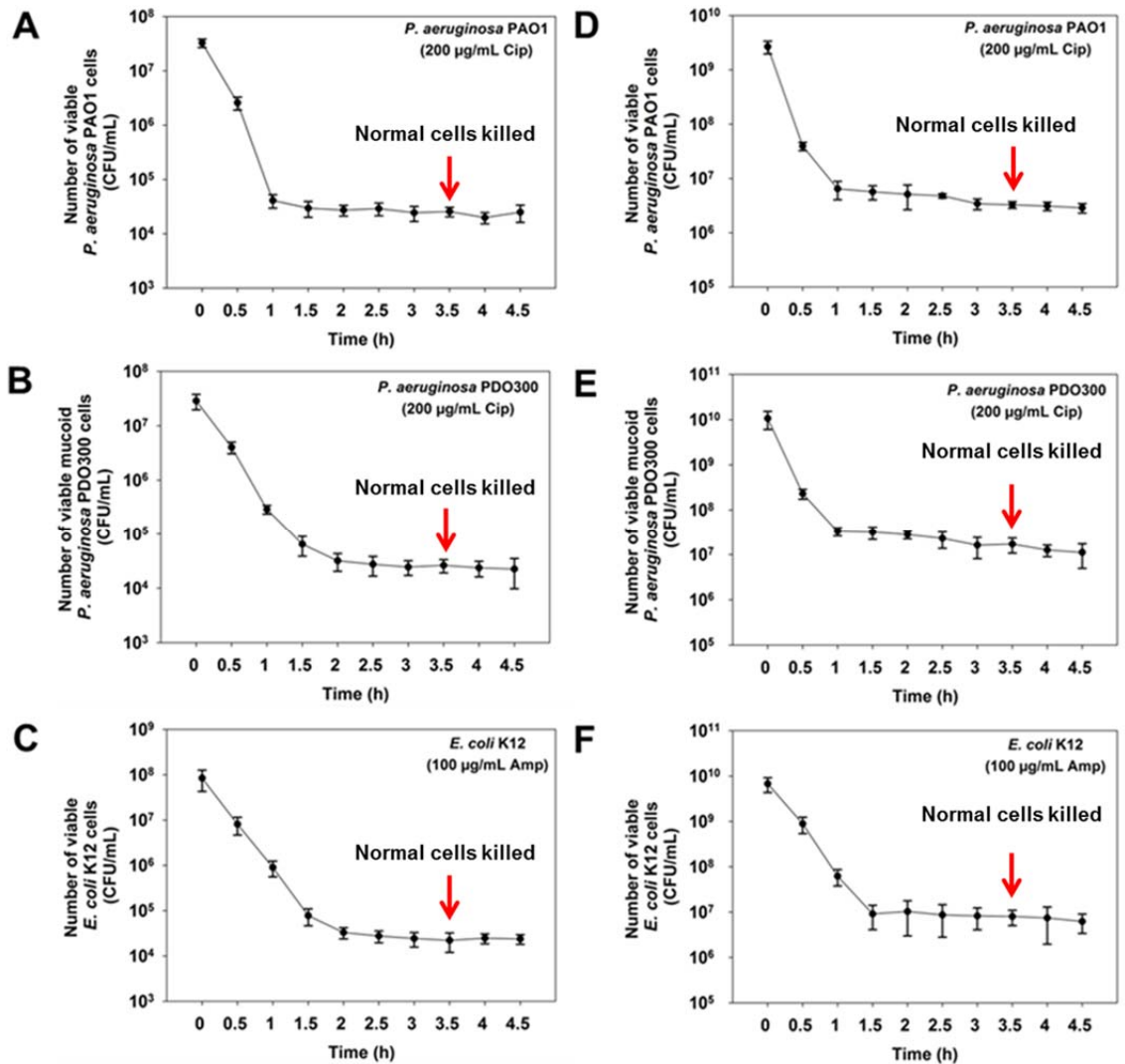


Figure 3.1 Antibiotic conditions used for the isolation of persister cells provide drug-tolerant bacterial population in both exponential and stationary phase cultures.

Antibiotic conditions used for the isolation of persister cells from cultures at exponential phase for (A) *P. aeruginosa* PAO1 (200 µg/mL ciprofloxacin for 3.5 h), (B) *P. aeruginosa* PDO300 (200 µg/mL ciprofloxacin for 3.5 h), and (C) *E. coli* K12 (100 µg/mL ampicillin for 3.5 h), cause biphasic kill curves with a significant decline in killing rate and a dominant drug-tolerant population. Similar biphasic kill curves were observed for antibiotic conditions used for the isolation of persister cells from the cultures at stationary phase for (D) *P. aeruginosa* PAO1 (200 µg/mL ciprofloxacin for 3.5 h), (E) *P. aeruginosa* PDO300 (200 µg/mL ciprofloxacin for 3.5 h), and (F) *E. coli* K12 (100 µg/mL ampicillin for 3.5 h). Cip: ciprofloxacin. Amp: Ampicillin. The samples were tested in triplicate (n=3). Error bars represent SD.

3.4.2 GM-CSF sensitized the planktonic persister cells of *P. aeruginosa* PAO1 to antibiotics

We started this study using the *P. aeruginosa* PAO1 strain⁴² obtained from Prof. Thomas K. Wood at Penn State University. Treatment with 0.17 pM GM-CSF alone did not affect the viability of persister cells isolated (by treatment with 200 µg/mL ciprofloxacin for 3.5 h) from the exponential cultures ($p=0.36$; One-way ANOVA followed by Tukey test (when needed) used throughout this study) (Figure 3.2A). However, the treatment with GM-CSF significantly sensitized the persister cells to antibiotics. For example, treatment with 0.17 pM recombinant human GM-CSF (henceforth GM-CSF) for 1 h sensitized $96.2\pm 5.9\%$ ($p = 0.0002$), $91.3\pm 1.2\%$ ($p < 0.0001$), $61.4\pm 16.6\%$ ($p = 0.0119$), and $47.6\pm 14.9\%$ ($p = 0.0030$) of persister cells to 200 µg/mL of ciprofloxacin, tobramycin, tetracycline, and gentamicin respectively; while these antibiotics alone were found ineffective in killing persister cells ($p > 0.05$) (Figure 3.2A). To test if GM-CSF is also effective against the mucoid strain of *P. aeruginosa*, we also tested another wild-type strain of *P. aeruginosa* PAO1⁴³ obtained from Prof. Matthew Parsek at the University of Washington, so that the isogenic mucoid strain *P. aeruginosa* PDO300⁴³ can be compared. Similar results were obtained for the PAO1 strain from Parsek lab; e.g., treatment with 0.17 pM GM-CSF alone did not affect the viability of persister cells isolated from exponential cultures ($p = 0.37$) (Figure 3.2B), but sensitized the persister cells to antibiotics. For example, treatment with 0.17 pM GM-CSF for 1 h sensitized $79.3\pm 8.3\%$ ($p = 0.0002$), $72.2\pm 12.7\%$ ($p = 0.0012$), and $45.7\pm 7.8\%$ ($p = 0.001$) of persister cells to 200 µg/mL of ciprofloxacin, tobramycin, and tetracycline,

respectively (Figure 3.2B). Treatment with any of these antibiotics alone did not cause significant killing of persister cells ($p > 0.05$ for all).

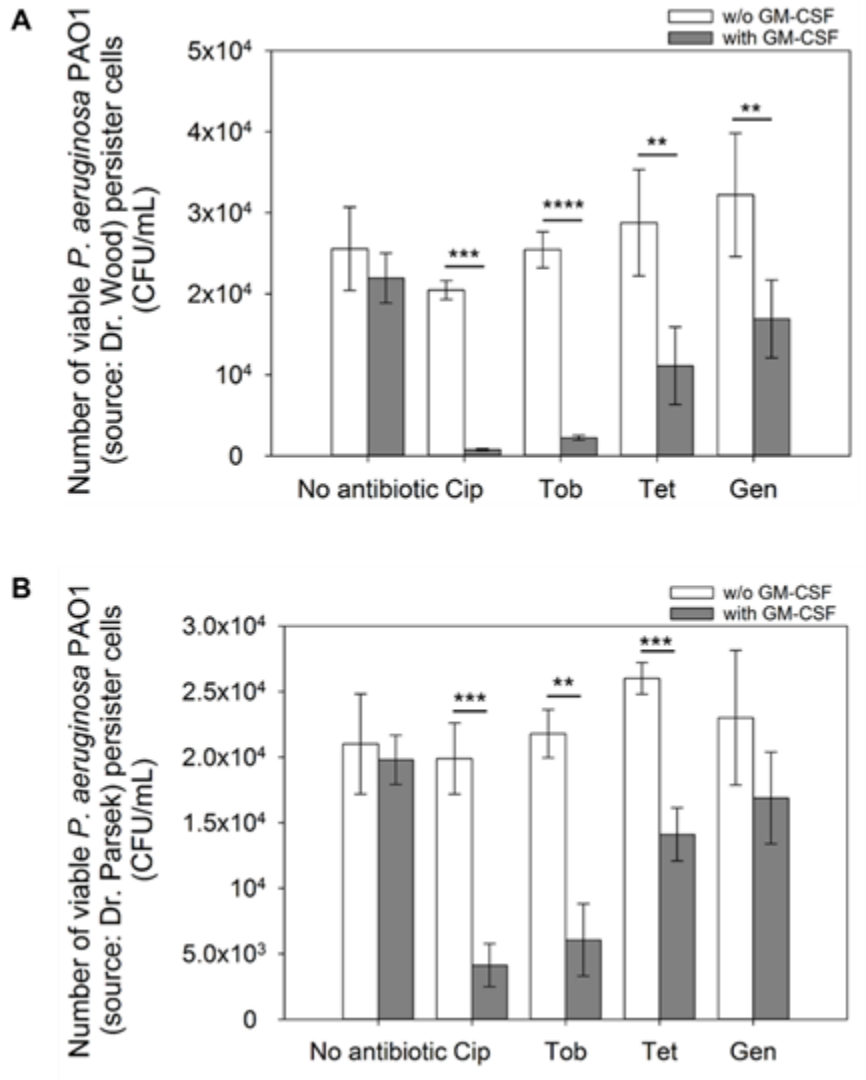


Figure 3.2 GM-CSF sensitized the persister cells of *P. aeruginosa* PAO1 isolated from exponential phase cultures to antibiotics. The wild-type PAO1 obtained from two different sources were tested including one (A) from Prof. Thomas K. Wood at Pennsylvania State University and another (B) from Prof. Matthew Parsek at University of Washington. The persister cells were isolated from exponential phase cultures by killing the normal cells with 200 μ g/mL ciprofloxacin for 3.5 h, and then treated with 0.17 pM GM-CSF alone for 1 h, followed by additional treatment with GM-CSF plus an antibiotic as indicated for 3.5 h (all tested at 200 μ g/mL). Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob:

tobramycin. Tet: tetracycline. Gen: gentamicin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

Since the results were consistent with the other PAO1 strain and an isogenic mucoid strain is available, the PAO1 strain from the Parsek lab (henceforth PAO1) was used for the rest of this study. Moreover, since the persister population is higher in stationary phase, we also tested if GM-CSF is effective against persister cells isolated from stationary phase cultures. The concentrations of antibiotics were also reduced to understand if GM-CSF can sensitize the persister cells to antibiotics at lower concentrations. GM-CSF did not significantly affect the viability of persisters in the absence of an antibiotic (0.17, 1.7, and 17 pM GM-CSF tested; $p > 0.3$ for all conditions), and synergistic effects were observed between GM-CSF and antibiotics in killing PAO1 persister cells isolated from stationary phase cultures. Specifically, treatment with 0.17 pM GM-CSF sensitized $61.5 \pm 14.5\%$ ($p = 0.0003$) and $77.1 \pm 2.0\%$ ($p = 0.0048$) of persister cells to 5 $\mu\text{g}/\text{mL}$ ciprofloxacin, and 5 $\mu\text{g}/\text{mL}$ tobramycin, respectively (Figure 3.3A and B). At a higher concentration of 17 pM, GM-CSF sensitized $74.0 \pm 2.9\%$ ($p = 0.0005$) and $86.5 \pm 1.7\%$ ($p = 0.0002$) of persister cells to 5 $\mu\text{g}/\text{mL}$ ciprofloxacin and 5 $\mu\text{g}/\text{mL}$ tobramycin, respectively (Figure 3.3A and B).

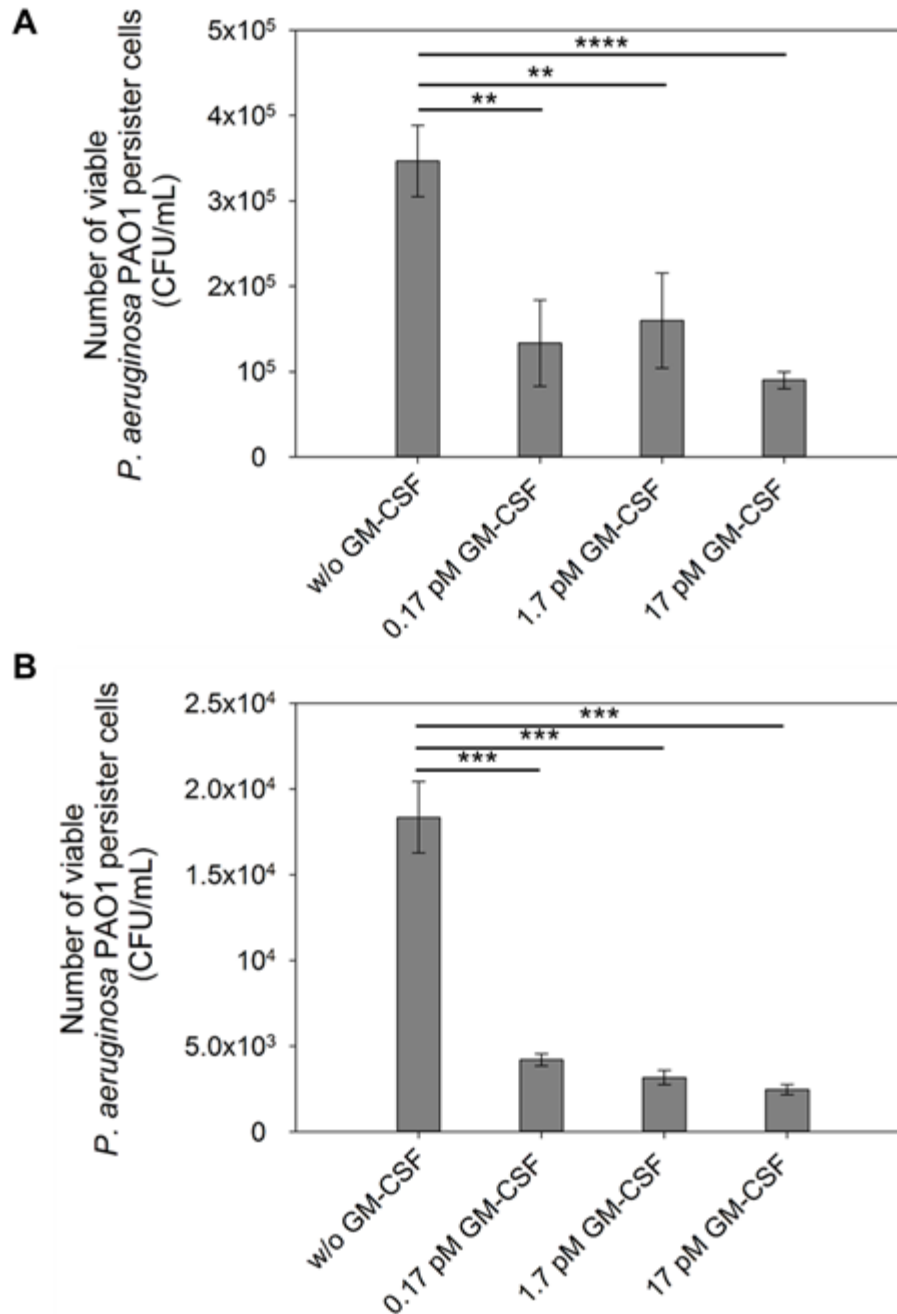


Figure 3.3 GM-CSF sensitized the persister cells of *P. aeruginosa* PAO1 isolated from stationary phase cultures to antibiotics. The persister cells were isolated from stationary phase cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF plus (A) 5 µg/mL ciprofloxacin, and (B) 5 µg/mL tobramycin. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

To confirm that the observed effects were caused by GM-CSF, rather than contaminant in the sample, we also tested the effects in the presence of anti-GM-CSF. As shown in Figure 3.4, addition of anti-GM-CSF abolished the effects of GM-CSF. Thus, the observed effects on persister cells were indeed caused by GM-CSF.

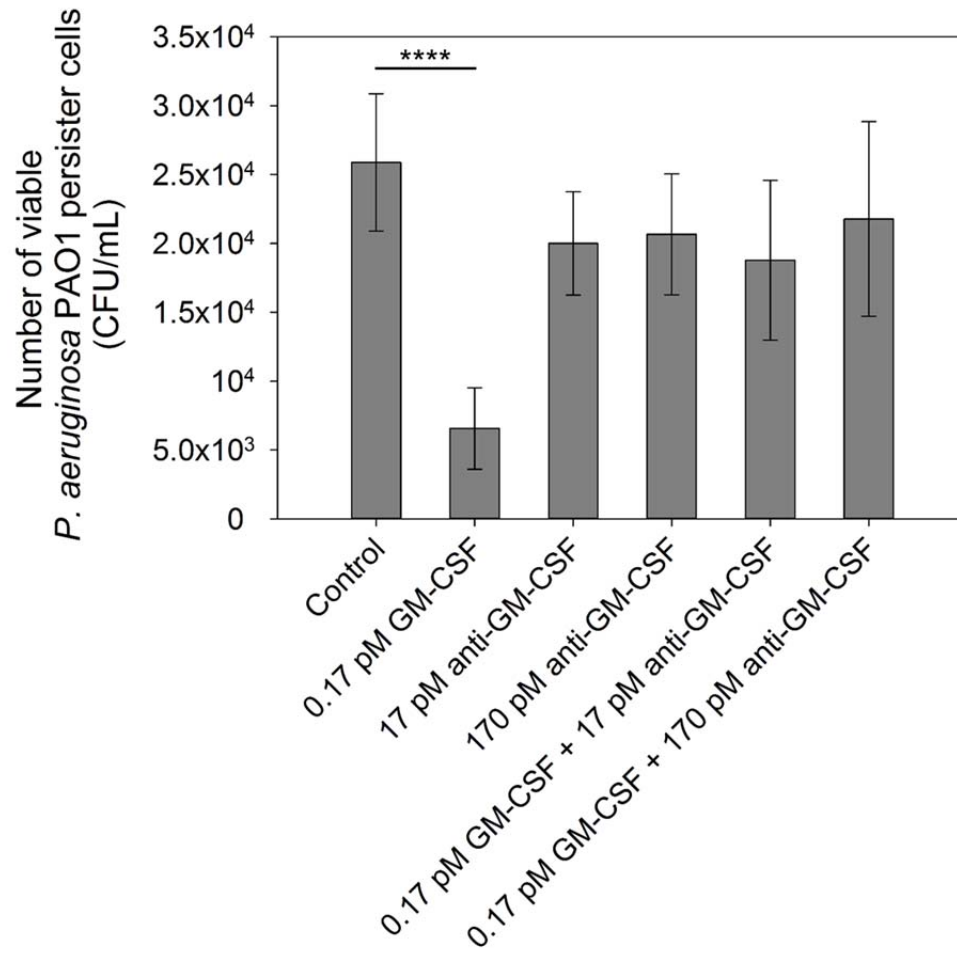


Figure 3.4 Effect of 0.17 pM GM-CSF on *P. aeruginosa* PAO1 persister cells was abolished by anti-GM-CSF. The persister cells were isolated from exponential phase cultures. All samples underwent the same incubation duration. The figure shows the viability of persister cells treated with GM-CSF alone, anti-GM-CSF alone, or GM-CSF neutralized by anti-GM-CSF (2 h incubation) followed by 5 μ g/mL ciprofloxacin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

3.4.3 GM-CSF is effective against the mucoid strain *P. aeruginosa* PDO300 in the presence of alginate lyase

To understand if GM-CSF also affects the mucoid strains of *P. aeruginosa*, the persister cells of *P. aeruginosa* PDO300 (henceforth PDO300) isolated from exponential cultures were tested following the same protocol. Similar to the results of the wild-type PAO1, treatment with 0.17 pM GM-CSF did not change the viability of PDO300 persister cells ($p = 0.77$), but sensitized $40.5 \pm 18.6\%$ ($p = 0.04$) persister cells to 200 $\mu\text{g}/\text{mL}$ tetracycline. The decrease in activities of GM-CSF against PDO300 persister cells is probably due to the presence of its alginate layer since when 50 $\mu\text{g}/\text{mL}$ alginate lyase was added, the killing by 200 $\mu\text{g}/\text{mL}$ tobramycin and 0.17 pM GM-CSF increased to $66.9 \pm 12.4\%$ ($p = 0.0002$) (Figure 3.5).

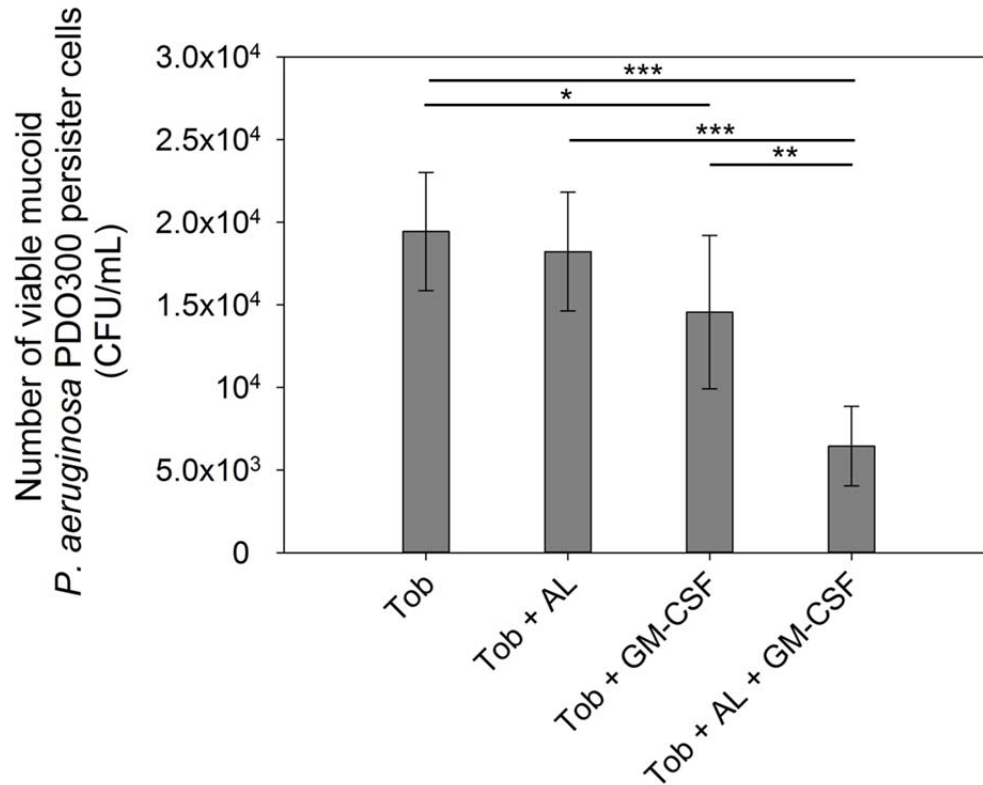


Figure 3.5 Alginate lyase is required for the activity of GM-CSF against persister cells of the mucoid strain *P. aeruginosa* PDO300. The persister cells were isolated from exponential phase cultures and GM-CSF was tested at 0.17 pM. The viability of persister cells treated with tobramycin (200 µg/mL) alone, tobramycin along with alginate lyase (50 µg/mL), or tobramycin along with alginate lyase and GM-CSF is shown. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. AL: alginate lyase. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

3.4.4 GM-CSF enhanced the killing of biofilm cells

To understand if GM-CSF is also effective against *P. aeruginosa* biofilm cells, the 24 h biofilm cells of PAO1 and PDO300 were treated with GM-CSF in the presence and absence of antibiotics. Treatment with 0.17 pM GM-CSF alone did not change the

viability of biofilm cells of either strain. We hypothesized that the presence of biofilm matrix may block or retard the penetration of GM-CSF. Alginate lyase at different concentrations (10, 50, 100, and 200 $\mu\text{g}/\text{mL}$) was tested on *P. aeruginosa* PAO1 and PDO300 biofilms, but alginate lyase had insignificant effect ($p > 0.05$) on the biofilm cells. To test if GM-CSF is more effective if the biofilm matrix is degraded, alginate lyase was added at 50 $\mu\text{g}/\text{mL}$ in addition to 200 $\mu\text{g}/\text{mL}$ tobramycin and 0.17 pM GM-CSF. GM-CSF and alginate lyase did not kill biofilm cells significantly in the absence of antibiotic for both strains; however, co-treatment with 50 $\mu\text{g}/\text{mL}$ alginate lyase, 0.17 pM GM-CSF, and 200 $\mu\text{g}/\text{mL}$ tobramycin killed the PDO300 biofilm cells by $97.2 \pm 0.4\%$ ($p = 0.0002$), corresponding to $61.3 \pm 6.0\%$ ($p = 0.03$) more killing than treatment with tobramycin alone and $57.1 \pm 6.6\%$ ($p = 0.07$) more killing compared to tobramycin and alginate lyase together (Figure 3.6). However, for *P. aeruginosa* PAO1 biofilm cells, addition of alginate lyase did not exhibit synergistic effects between GM-CSF and antibiotics. For example, addition of 0.17 pM GM-CSF and 0.05 mg/mL alginate lyase did not further reduce the viability of biofilm cells compared to treatment with 200 $\mu\text{g}/\text{mL}$ tobramycin alone ($p = 0.82$). This is probably because alginate is not a major component of the extracellular polysaccharide matrix of the wild-type *P. aeruginosa* PAO1, as reported by Wozniak *et al.*⁵³.

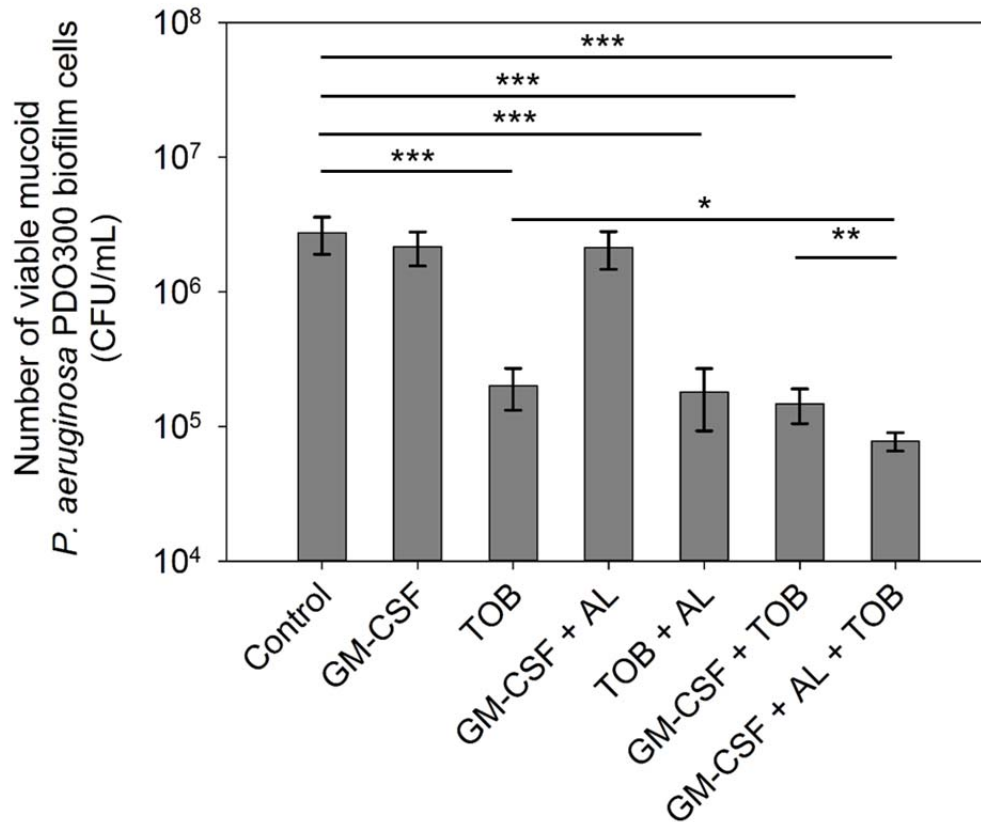


Figure 3.6 GM-CSF enhanced the killing of *P. aeruginosa* PDO300 biofilm cells by tobramycin and alginate lyase. *P. aeruginosa* PDO300 cells in 24 h biofilms were treated with (i) 0.17 pM GM-CSF alone, (ii) 200 µg/mL tobramycin alone, (iii) 0.17 pM GM-CSF and 50 µg/mL alginate lyase, (iv) 200 µg/mL tobramycin and 50 µg/mL alginate lyase, (v) 0.17 pM GM-CSF and 200 µg/mL tobramycin, and (vi) 0.17 pM GM-CSF, 200 µg/mL tobramycin, and 50 µg/mL alginate lyase, for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. AL: alginate lyase. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

The major components of PAO1 biofilms are (i) extracellular DNA which mediates cell to cell interaction, (ii) Pel, a glucose-rich polymer which provides structural scaffold at early stages of biofilm formation, and (iii) Psl, rich in mannose and galactose, and is involved in initial attachment and biofilm maturation⁵⁴. Since early PAO1 biofilms are known to contain a large amount of DNA, we tested if addition of DNase I

could increase the activity of GM-CSF in killing early stage (4 h) PAO1 biofilm cells. DNase I was tested at different concentrations (1, 2, 5, and 10 units/mL) on early PAO1 biofilms and it was found that DNase I had insignificant effects ($p > 0.05$) on the viability of biofilm cells. However, addition of DNase I along with GM-CSF and tobramycin was found to kill *P. aeruginosa* PAO1 biofilm cells significantly. For example, when 5 units/mL DNase I and 0.17 pM GM-CSF were added along with 20 $\mu\text{g/mL}$ tobramycin (lower antibiotic concentration was used since this was tested for early stage biofilms), a total of $99.7 \pm 0.1\%$ ($p = 0.0008$) biofilm cells were killed, corresponding to $83.3 \pm 4.5\%$ ($p = 0.05$) more than that by tobramycin alone and $66.4 \pm 9.1\%$ more than the treatment with tobramycin and DNase I together (Figure 3.7). Collectively, these findings indicate that GM-CSF is also effective against biofilm cells if the biofilm matrix is removed.

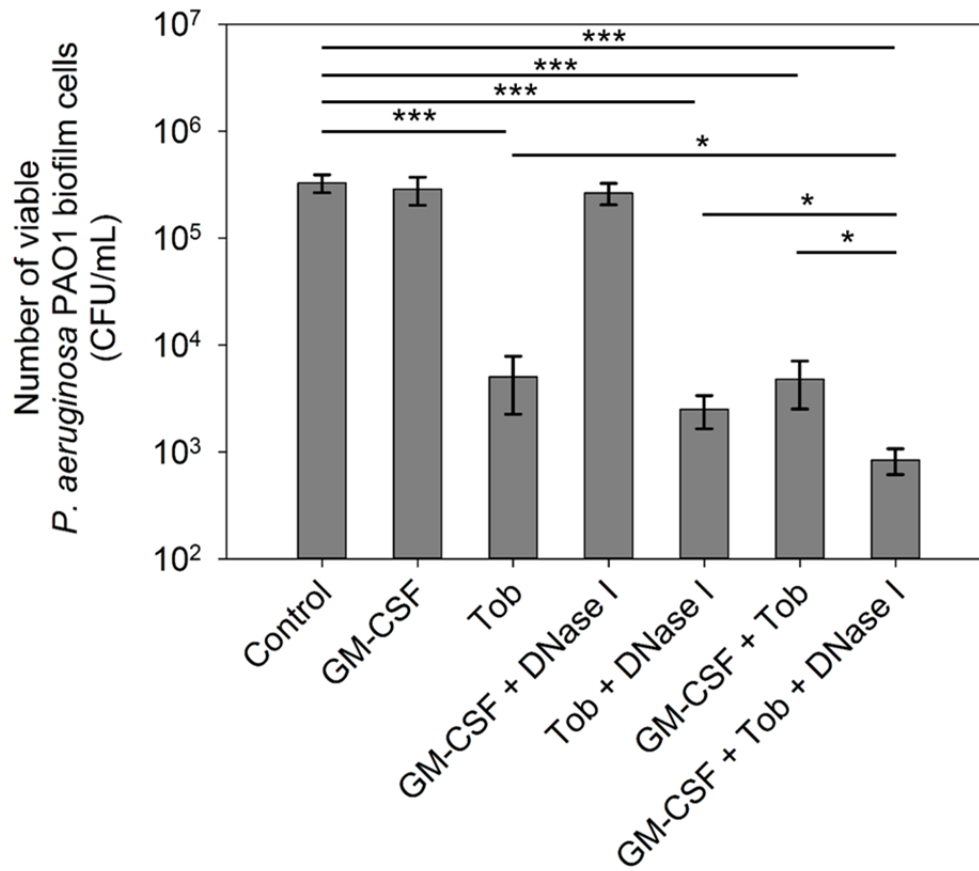


Figure 3.7 GM-CSF enhanced the killing of *P. aeruginosa* PAO1 biofilm cells by tobramycin and DNase I. *P. aeruginosa* PAO1 cells in early biofilms (4 h after inoculation) were treated with (i) 0.17 pM GM-CSF alone, (ii) 20 µg/mL tobramycin alone, (iii) 0.17 pM GM-CSF and 5 units/mL DNase I, (iv) 20 µg/mL tobramycin and 5 units/mL DNase I, (v) 0.17 pM GM-CSF and 20 µg/mL tobramycin, and (vi) 0.17 pM GM-CSF, 20 µg/mL tobramycin, and 5 units/mL DNase I for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

3.4.5 Alginate lyase allows the diffusion of GM-CSF across alginate layer

P. aeruginosa PDO300 biofilm matrix mainly comprises of alginate which can be degraded by alginate lyase^{39,40,53,55-58}. Since addition of alginate lyase along with GM-CSF and tobramycin enhanced the killing of *P. aeruginosa* PDO300 biofilm cells, we

attempted to obtain experimental evidence that the degradation of the alginate layer promotes the diffusion of GM-CSF. As shown in Figure 3.8, for samples without 100 $\mu\text{g}/\text{mL}$ alginate lyase, there was no detectable level of GM-CSF below the alginate layer after 2 h of incubation, indicating that no significant diffusion occurred. In contrast, when alginate lyase was added along with 50 ng GM-CSF, considerable amount of GM-CSF diffused across the alginate later within 1 h. These results indicate that degradation of the alginate layer promotes the diffusion of GM-CSF.

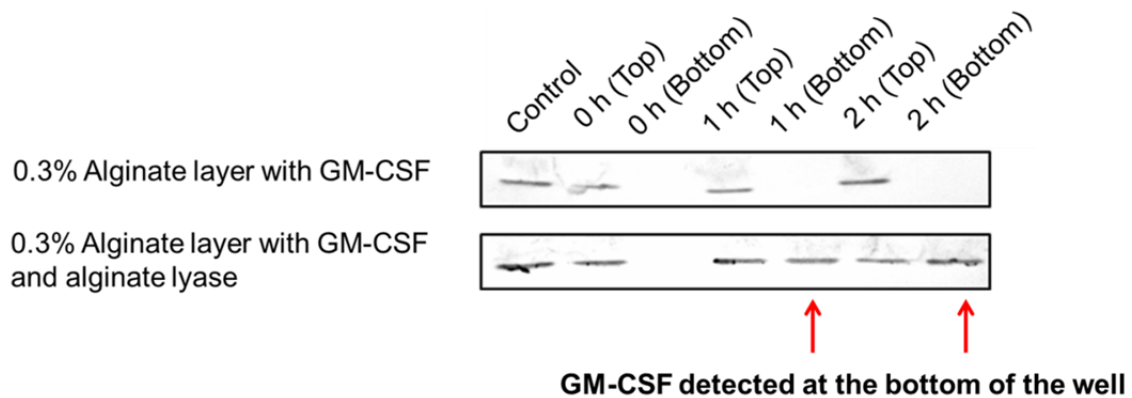


Figure 3.8 GM-CSF diffused across an alginate layer in presence of alginate lyase. The figure shows the Western blotting results of GM-CSF diffusion across 0.3% w/v alginate layer on top of transwell inserts in presence and absence of 100 $\mu\text{g}/\text{mL}$ alginate lyase over a period of 0, 1, and 2 h. The bands indicate GM-CSF detected by anti-GM-CSF (primary antibody) followed by anti-mouse IgG (secondary antibody). As a positive control, 50 ng GM-CSF was added during western blotting. Top: Top of the transwell insert, Bottom: Bottom of the 12-well plate.

3.4.6 GM-CSF is not effective against planktonic normal cells of *P. aeruginosa*

PAO1 and PDO300

To understand if the activities of GM-CSF are specific to persister cells, the total population (without persister isolation, containing more than 99% normal cells) from

exponential phase and stationary phase cultures of PAO1 and PD0300 were also treated with GM-CSF following the same procedure. Similar to persister cells, GM-CSF alone did not affect the viability of PAO1 and PDO300 normal cells ($p > 0.1$ for all conditions tested). However, unlike persister cells, GM-CSF did not exhibit any synergistic effects with antibiotics (ciprofloxacin and tobramycin) in killing normal cells of these two strains ($p > 0.1$ for all conditions tested) (Figure 3.9A and B).

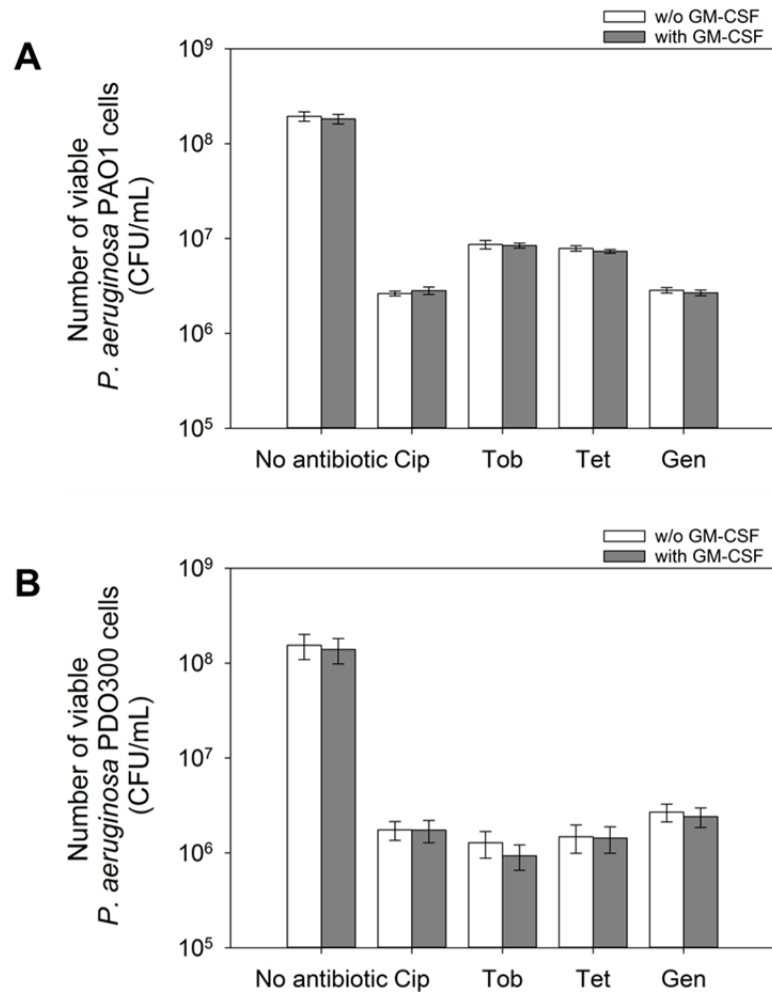


Figure 3.9 GM-CSF did not sensitize the normal cells of *P. aeruginosa* PAO1 and PDO300 isolated from exponential phase cultures to antibiotics. The total viable cells were obtained from the exponential cultures of wild-type *P. aeruginosa* (A) and its isogenic mucoid mutant PDO300 (B). The cells were treated with GM-CSF alone or with GM-CSF plus antibiotics as indicated for 3.5 h (all tested at 200 μ g/mL). The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the

viability of normal cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. Tet: tetracycline. Gen: gentamicin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

3.4.7 GM-CSF is not effective against the non-pathogenic *E. coli* K12

Since GM-CSF is a cytokine in host immune systems, we hypothesized that it has different activities against pathogens and host friendly bacteria. To test this hypothesis, the above experiments were also conducted with the non-pathogenic laboratory strain *E. coli* K12. Unlike *P. aeruginosa* PAO1 and PDO300, GM-CSF did not exhibit significant effect either on the planktonic or biofilm cells of *E. coli* K12. The concentration of each antibiotic that showed 2 log reduction in CFU of normal cells was selected for this test. The results showed that GM-CSF was not effective against *E. coli* K12. For example, treatment with 0.17 pM GM-CSF did not change the susceptibility of *E. coli* K12 persister cells to 2 $\mu\text{g/mL}$ ciprofloxacin ($p = 0.93$) and 70 $\mu\text{g/mL}$ tobramycin ($p = 0.95$) (Figure 3.10). These results indicate that GM-CSF is not effective on the non-pathogenic *E. coli* K12.

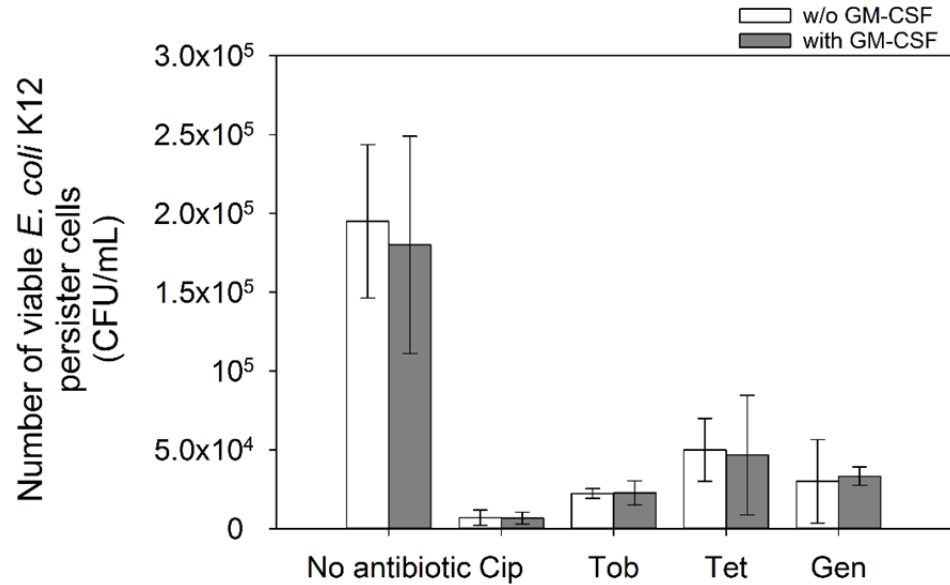


Figure 3.10 GM-CSF did not sensitize the persister cells of *E. coli* K12 to antibiotics.

The persister cells were isolated from exponential phase cultures by killing the normal cells with 100 µg/mL ampicillin for 3.5 h, and then treated with 0.17 pM GM-CSF alone for 1 h, followed by additional treatment with GM-CSF plus an antibiotic as indicated for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: 2 µg/mL ciprofloxacin. Tob: 70 µg/mL tobramycin. Tet: 20 µg/mL tetracycline. Gen: 200 µg/mL gentamicin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

3.5 Discussion

GM-CSF is a vital cytokine for the host to fight invading pathogens, as it has been shown previously in studies that reducing the expression of GM-CSF leads to higher bacterial load and increased mortality of the infected mice^{28,29}. Champsi *et al.*⁵⁹ also showed that during bacterial infections, if the macrophages/monocytes are exposed to GM-CSF, the bactericidal activity of the macrophages/monocytes is enhanced. In addition to the functions in bacterial clearance, it has also been shown previously that in cystic fibrosis (CF) patients, the induced cytokines can assist in the survival of polymorphonuclear

neutrophils (PMNs)⁶⁰. Despite the well documented effects of GM-CSF on immune cells, little is known about the direct effects of GM-CSF on bacterial cells, and the effects on persister cells have not been investigated to date. The data from this study suggest that GM-CSF is effective in sensitizing the persister cells of *P. aeruginosa* towards certain antibiotics. The co-treatment of GM-CSF with ciprofloxacin or tobramycin exhibited strong synergistic effects in killing *P. aeruginosa* persisters while both antibiotics alone are ineffective against persister cells. Such synergy suggests that GM-CSF may have previously unknown functions besides the recruitment and activation of leukocytes.

We observed that, compared to the strong activities of GM-CSF in sensitizing *P. aeruginosa* persister cells, this cytokine is not effective against the non-pathogenic *E. coli* K12. To understand if the effects observed in this study also exist against other bacterial species, GM-CSF can be tested on other pathogenic and non-pathogenic bacteria. Most of the pathogenic Gram-negative bacteria express virulence-related outer membrane proteins, which are required for the bacterial survival within macrophages and under other immune responses⁶¹. Lin *et al.*⁶² demonstrated that an outer membrane protein, OprI serves as the receptor for cationic α -helical antimicrobial peptides (AMPs). It will be interesting to study whether GM-CSF also has an outer membrane protein target on the *P. aeruginosa*.

Compared to the potent activities in sensitizing planktonic persister cells of *P. aeruginosa* PAO1 and PDO300 to antibiotics, DNase I and alginate lyase were required for activities on biofilms of PAO1 and PDO300, respectively. This finding suggests that the biofilm matrix may present a barrier for the penetration of GM-CSF. In cystic fibrosis patients, during prolonged infection and exposure to antibiotics, some non-mucoid strains

of *P. aeruginosa* mutate to convert to mucoid strains, causing alginate overproduction³⁹. Mucoid conversion has been shown to be a hallmark of chronic lung infection in cystic fibrosis patients. The protection of alginate against GM-CSF is consistent with this observation and provides new insights in the pathogenesis of *P. aeruginosa* infections. Our results suggest that developing new delivery strategies to allow GM-CSF to penetrate the extracellular matrix of biofilms might increase the efficacy of antibiotic therapies.

3.6 Conclusions

Overall, we found that GM-CSF has direct effects on bacterial persister cells. The exact mechanism of this new phenomenon needs to be further investigated. With high tolerance to antibiotics, persister cells remain as a major challenge to the treatment of chronic infections. The results of this study suggest that immune factors have previously unknown activities against persister cells. Understanding the importance of each cytokine and knowing the exact mechanism of their actions on bacteria can help understand how bacteria establish chronic infections and pave the way for developing more effective treatments.

3.7 Acknowledgements

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CHAPTER 4

INVESTIGATION OF THE EFFECTS OF GRANULOCYTE MACROPHAGE COLONY- STIMULATING FACTOR (GM-CSF) ON *PSEUDOMONAS AERUGINOSA*

4.1 Abstract

As described in chapter 3, GM-CSF was found to sensitize the persister cells of *P. aeruginosa* PAO1 to antibiotics, while no such effects were observed for normal cells. In this chapter, DNA microarray analysis and qPCR were used to study the effects of GM-CSF on gene expression in normal and persister cells of *P. aeruginosa* PAO1. The DNA microarray results indicated that treatment with 0.17 pM GM-CSF induced the expression of 19 pyocin-related genes, and 18 chemotaxis/motility genes in persister cells. The qPCR results confirmed the gene expression of 10 representative genes in persister cells and 4 representative genes in normal cells, with *rpoD* (encoding for RNA polymerase sigma factor RpoD) as a housekeeping gene. The reduction in viability of R2-pyocin sensitive *P. aeruginosa* PAK strain indicated that GM-CSF induces R2-pyocin-related genes in the persister cells of *P. aeruginosa* PAO1. Approximately 9 genes associated with multidrug efflux pumps were also found to be repressed in persister cells. On testing the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter transcriptional regulator mutants, it was found that MFS transporters probably have a marginal role in GM-CSF activity.

4.2 Introduction

In chapter 3, I reported data showing that the persister cells of *P. aeruginosa* PAO1 were sensitized to antibiotics by GM-CSF. For example, GM-CSF sensitized the persister cells of *P. aeruginosa* PAO1 to ciprofloxacin and tobramycin, while no such effects were observed for normal cells. To understand the distinct effects of GM-CSF on persister and normal cells, these two populations were treated with or without 0.17 pM GM-CSF and the gene expression was studied using DNA microarray analysis, which is a well-established technology for studying gene expression at the genome-wide scale¹⁻³. The microarray technology involves using glass or silicon chips with microscopic DNA spots¹⁻⁴. Each spot has multiple probes for a particular gene¹⁻⁴. By detecting the fluorescent signals of target cDNA from mRNA, the expression level of each gene can be determined^{1,2,4}. DNA microarray allows simultaneous measurement of the mRNA levels of thousands of genes.

The results of DNA microarray analysis were confirmed using quantitative real-time polymerase chain reaction (qPCR). The qPCR is a powerful method for gene expression study. By using the intensity of fluorescence emitted during PCR, it allows the researchers to monitor the abundance of DNA product using PCR reaction⁵⁻⁷. Thus, one can compare the amount of target DNA in different samples. The expression levels of the selected genes were compared between DNA microarray and qPCR results to affirm the effect of GM-CSF on persister and normal cells.

The DNA microarray studies showed that GM-CSF have different effects on normal and persister cells of *P. aeruginosa* PAO1. Specifically, genes associated with

flagella and pyocins were induced, and genes associated with transporters were repressed in persister cells, but not in normal cells.

4.3. Materials and Methods

4.3.1 Bacterial strains and growth media

The bacterial strains *P. aeruginosa* PAO1⁸ (obtained from Prof. Matthew Parsek) and PAK⁹ were used in this chapter. Individual transposon mutants, PA0620::*phoA*, PA0218::*phoA* and PA3594::*phoA* with the transposon insertion IS*phoA*/hah were obtained from the *P. aeruginosa* PAO1 mutant library at University of Washington¹⁰. Overnight cultures of these strains were prepared in Luria Bertani (LB) medium¹¹ containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at 37°C with shaking at 200 rpm. The transposon mutants were grown in LB medium supplemented with 60 µg/mL tetracycline. Recombinant human GM-CSF (E. coli-derived Ala18-Glu144) was purchased from R&D systems (Minneapolis, MN, USA). The stocks used in this study contained 10 µg/mL GM-CSF, dissolved in phosphate buffer saline (PBS) pH 7.4, supplemented with 0.1% bovine serum albumin (BSA).

4.3.2 DNA microarray analysis

The persister cells of *P. aeruginosa* PAO1 were isolated from 60 mL overnight cultures by adding 200 µg/mL ciprofloxacin and incubating at 37°C for 3.5 h with shaking at 200 rpm. The isolated persister cells were washed with PBS and resuspended in 300 mL PBS. These persister cells were supplemented with 0.17 pM GM-CSF (treatment) or the same amount of BSA (0.1%), but no GM-CSF (control). The control and the treatment samples

were incubated at 37°C for 1 h with shaking at 200 rpm. After incubation, the cells were quickly collected by centrifugation at 10,000 rpm for 2 min at 2°C. The supernatant was decanted and the cell pellets were flash frozen in a dry ice-ethanol bath. Then cell pellets were stored at -80°C until RNA isolation. Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA, USA) including on-column DNase treatment, and sent to the DNA microarray facility at SUNY Upstate Medical University (Syracuse, NY, USA) to check on a bioanalyzer before hybridization to GeneChip *P. aeruginosa* Genome Array (Affymetrix, Santa Clara, CA, USA). GeneChip Operating Software (MAS 5.0) was used to identify the differentially expressed genes by signal detection based on Wilcoxon signed rank test and Tukey's biweight. The fold change for each gene was calculated as a ratio of treatment to control signals. In comparison, similar DNA microarray analysis was also performed on the total population from stationary cultures of *P. aeruginosa* PAO1.

4.3.3 Quantitative real-time PCR (qPCR) analysis

To validate the DNA microarray results, the transcriptional levels of nine representative genes were also tested using qPCR, including six induced genes (*flgF*, *priN*, *fliN*, PA0620, PA0633 and PA0640), two repressed genes (*wbpK*, *algA*), and one unchanged gene (*argH*). The gene *rpoD* (RNA polymerase sigma factor RpoD) was selected as housekeeping gene for the qPCR study as described previously¹². For the regular population (no persister isolation), qPCR was performed on two induced genes (*yrfI*, *dnaB*) and two repressed genes (PA0364, PA5548) to confirm the microarray results. The cDNA was synthesized from the isolated RNA of control and treatment samples using iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA, USA). The primers were designed

using OligoPerfect™ Designer (Life Technologies, Grand Island, NY, USA) and Primer blast (NCBI) to obtain products with sizes between 231 and 350 bp, and melting temperatures between 59.5 and 60.3°C. The sequence and the product size for each primer pair are listed in Tables 4.1 and 4.2. The qPCR samples were prepared by mixing cDNA, primers, and iTaq™ Universal SYBR Green Supermix (Biorad, Hercules, CA, USA). The qPCR reactions were performed using an Eppendorf Mastercycler Realplex thermal cycler (Eppendorf, Hauppauge, NY, USA). The qPCR reactions underwent the following cycles: heat activation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C. A melting curve was added after the PCR cycle as a dissociation analysis to confirm if the PCR reaction produced only the desired product. The melting curve was set at 95°C for 15 s, 50°C for 30 s, 20 min hold with temperature gradient, and 95°C for 15 s. After the qPCR reactions, the expression ratios of the selected genes were analyzed using LinReg PCR program (Heart Failure Research Center, Amsterdam, Netherlands).

Table 4.1 Primers used for qPCR of persister cells.

Primer	Sequence	Product size (bp)
<i>rpoD</i> Forward	CGGTCAACCTGAAGGACGAT	253
<i>rpoD</i> Reverse	ACCAGCTTGATCGGCATGAA	
<i>flgF</i> Forward	TCAACCCGAACCTGAAGCAG	273
<i>flgF</i> Reverse	CACCACGCTCAAGTGATTAGC	
<i>fliN</i> Forward	GGATGTGATCCTGGACATCCC	245
<i>fliN</i> Reverse	GCAGCTTCTTGATGCGTTCG	
<i>prtN</i> Forward	ACCGTGGAATTGGTCTACCG	271
<i>prtN</i> Reverse	CTCAGGATGCGATGCTGTCA	
PA0620 Forward	TGCTGTCTCGAACAGTAGCG	268
PA0620 Reverse	AGGAACCTCCAATGTCGCAG	
PA0633 Forward	CAGATCTACGCCCTGGTTCC	253
PA0633 Reverse	TCGTCTCGCCATCTTTCTCG	
PA0640 Forward	CGACATATTCAAGCGAGCCG	240
PA0640 Reverse	AGGTCAGCCCTTTCGATTCC	
<i>recA</i> Forward	TCGGAACATTCTTCCCCTCG	258
<i>recA</i> Reverse	CGATGTCCAGACCCAGGGAG	
<i>wbpK</i> Forward	ACAGGTCGATGCTTCCAAGG	243
<i>wbpK</i> Reverse	CCCCAGGTAGCGACAAATGA	
<i>algA</i> Forward	GTCGTCGATCTGGGACGTG	263
<i>algA</i> Reverse	GGTAGACCTCGCAGTGGTTC	
<i>argH</i> Forward	CAGGAAGACAAGGAACCGCT	231
<i>argH</i> Reverse	GCTGTCTACGCCGTA CT TCA	

Table 4.2 Primers used for qPCR of normal cells.

Primer	Sequence	Product size (bp)
<i>dnaB</i> Forward	CGACGTGGAAACCACTCTGA	347
<i>dnaB</i> Reverse	CGATGGTCATGGCGGTAGAA	
<i>yrfI</i> Forward	CCCGCATGTCCCATTTCAGAT	303
<i>yrfI</i> Reverse	CGATGGACTCCGCCGAATAG	
PA5548 Forward	TATTCTTCGTCGGCGTGACC	300
PA5548 Reverse	TGGTCGGGATGATGATTGCC	
PA0364 Forward	AGCTCTCGGTCTACGGTCTT	303
PA0364 Reverse	GAAAAGCCGTGAAGCACGTT	

4.3.4 Effect of GM-CSF on the induction of pyocin genes in *P. aeruginosa* PAO1 persister cells.

GM-CSF induced a large number of pyocin-related genes in persister cells of *P. aeruginosa* PAO1, which included the pyocin regulatory gene *priN* (induced by 2.8-fold) and R-pyocin (PA0617, PA0619-22, PA0625-30) related genes. Since the R2-pyocins generated by *P. aeruginosa* PAO1 cause cell lysis in the R1-pyocin producing strain *P. aeruginosa* PAK, an experiment was designed to test the effect of GM-CSF on the production of R2-pyocins in *P. aeruginosa* PAO1 persister cells¹³. The planktonic cells were harvested from overnight cultures (16 h after inoculation) of *P. aeruginosa* PAO1 and PAK grown in 30 mL LB medium. After washing the cells with PBS (pH 7.4) by centrifuging at 8,000 rpm for 10 min twice at room temperature, the cells were resuspended in 30 mL PBS and were stored for testing their viability after treatment with

supernatant obtained by treating isolated persister cells of *P. aeruginosa* PAO1 with 0.17 pM and 0.17 nM GM-CSF. To isolate persister cells of *P. aeruginosa* PAO1, the washed 15 mL overnight culture was treated with 200 µg/mL ciprofloxacin for 3.5 h at 37°C, with shaking at 200 rpm. Thereafter, the ciprofloxacin was washed away from the isolated persister cells by centrifugation for three times at 8,000 rpm for 10 min at 4°C and resuspended in 15 mL PBS.

To test the effects of GM-CSF on the R2-pyocin production of *P. aeruginosa* PAO1, the washed cells were transferred to centrifuge tubes, with 5 mL of washed persister cells in each tube. GM-CSF was added at 0.17 pM, and 0.17 nM. All samples included adjusted amount of BSA (0.1%). The samples were incubated at 37°C for 2 h with shaking at 200 rpm. The supernatant for testing the presence of pyocins was collected by centrifugation 13,200 rpm for 5 min. To ensure that the supernatant did not contain any cells, it was filtered using 0.2 µm nylon filter. The sterile supernatants were added at a volume of 100 µL to microcentrifuge tubes with a total volume of 1 mL washed normal cells ($\sim 10^7$ cells) of *P. aeruginosa* PAO1 and PAK, and the samples were incubated at 37°C for 3.5 h with shaking at 200 rpm. After washing the cells three times with PBS, the viability was determined by plating the cells on LB agar plates and counting CFU using the drop plate method as described earlier¹⁴. For comparison, a similar experiment was performed with a *P. aeruginosa* PAO1 strain having a mutation in the gene encoding for R2-pyocin tail fiber (PA0620::*phoA*) to test if GM-CSF treatment on a strain with structurally defective R2-pyocins shows any different effects on the killing of *P. aeruginosa* PAO1 and PAK normal cells.

4.3.5 Effect of GM-CSF on the mutants of ATP-binding cassette (ABC) and Major facilitator superfamily (MFS) transcriptional regulator

The planktonic cells were harvested from stationary phase (16 h after inoculation) cultures of ABC (PA0218:: *phoA*) and MFS (PA03594:: *phoA*) transcriptional regulator strains. The persister cells were isolated as described in chapter 3As described previously in the materials and methods section of Chapter 3 and treated with GM-CSF at 0 and 0.17 pM in the presence and absence of 5 µg/mL ciprofloxacin and tobramycin. The amount of BSA (0.1%) was adjusted to be the same for all samples so that the effects of GM-CSF can be studied specifically. The viability of cells was determined by plating the samples on LB agar plates using drop plate method as described previously ¹⁴.

4.4 Results

4.4.1 Effects of GM-CSF on gene expression in *P. aeruginosa* PAO1

GM-CSF at pM level can sensitize *P. aeruginosa* persister cells to antibiotics. Since there was a difference between the effects of GM-CSF on normal and persister populations, we considered the possibility of GM-CSF affecting the gene expression of these populations in different ways. To better understand this phenomenon, DNA microarrays were used to compare gene expression profiles of *P. aeruginosa* PAO1 persister cells with and without 1 h treatment with 0.17 pM GM-CSF. The results show that a total of 89 genes were induced and 149 genes were repressed by GM-CSF more than 1.5-fold in both biological replicates (Figure 4.1A). The induced genes include 34 genes coding for hypothetical proteins, 19 bacteriophage genes, 10 chemotaxis genes, 8 motility genes, and 18 genes

with other functions (Figure 4.1A). The repressed genes include 61 genes coding for hypothetical proteins, 16 genes related to the transport of small molecules, and 12 genes for transcriptional regulators (Figure 4.1A). Table 4.3 shows the expression fold change of some representative genes in persister cells based on the DNA microarray results of two biological replicates. The gene expression patterns showed effects of GM-CSF on persister cells of *P. aeruginosa* PAO1. First, a group of genes involved in motility and flagella were induced, including *flgBCDEFGHIJK*, *fliACDGMN*, and *cheYZ*. In addition, GM-CSF at 0.17 pM level was found to induce a large number of pyocin genes. For example, the pyocin regulatory gene *priN* was induced by 2.8-fold compared to the GM-CSF free control. A large number of R-pyocin (PA0617, PA0619-22, PA0625-30) and F-pyocin (PA0631-35, PA0636-37, PA640) related genes were also induced. The R and F pyocins are bacteriophages with a tail-like structure¹⁵. Both the R and F-type pyocins can cause cytoplasmic membrane depolarization in bacteria by pore formation¹⁵. The R-type pyocins are comprised of an outer sheath, inner core, a baseplate, and 6 tail fibers. While the F-pyocins are devoid of an outer sheath, they do have a core, baseplate and tail fibers with short or long filaments^{13,15,16}. Pyocin production can be provoked by DNA damage, and it is believed that the pyocinogenic bacteria can gain predominance by producing pyocins to eliminate pyocin-sensitive species in a mixed bacterial population¹⁷.

There were also a large number of genes repressed in response to GM-CSF. For example, *wbpK* (encoding NAD-dependent epimerase/dehydratase), *algAL* (encoding alginate related genes), *phnA* (encoding anthranilate synthase component), and *str* (encoding streptomycin 3-phosphotransferase) were repressed by GM-CSF, suggesting that cell wall mediated protection and antibiotic resistance may be repressed. The qPCR

technique was used to validate the DNA microarray results including 10 representative genes. Consistent results were obtained for 9 of these 10 genes, except for *recA* which was induced by 2.2-fold in DNA microarray data but was not significantly changed according to qPCR results (Table 4.4). For the confirmed genes, *flgF* encodes the flagellar rod protein FlgF, which is a part of a group of proteins that form the rod section of the basal-body assembly of the flagellar motor¹⁸. The rod transmits torque from the motor through the hook to the flagellar filament, resulting in bacterial motility¹⁹. The gene *fliN* encodes a flagellar motor switch protein FliN. FliM and FliN, along with FliG form a motor switch complex which controls flagellar rotation and direction²⁰. The qPCR results showed that treatment with GM-CSF resulted in 3.3- and 8.5-fold induction in transcription of *flgF* and *fliN*, respectively, which is consistent with the DNA microarray results.

The qPCR results also confirmed the induction of pyocin related genes. PA0620, which displayed a 5.2-fold induction in qPCR results, encodes a tail fiber protein of R2-pyocins¹⁵. PA0633 and PA0640 are F2-pyocin proteins, which showed an induction by 6.5- and 4.7-fold, respectively¹⁶. It was also observed that *prtN*, a transcriptional regulatory gene which activates the expression of pyocin genes displayed a 3.3-fold induction in qPCR results. The *prtN* gene product interacts with the DNA sequences in the 5' noncoding regions (P box) of pyocin genes to activate these genes²¹. The induction of these genes suggests that GM-CSF treatment may enhance pyocin production in *P. aeruginosa* PAO1 persister cells, causing them further stress and inducing waking up. Moreover, qPCR also confirmed the repression of *wbpK*, encoding an NAD-dependent epimerase/dehydratase involved in cell envelope biogenesis and catabolism, and *algA*, an

alginate biosynthesis gene (encoding mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase) which produces a precursor for alginate polymerization^{22,23}.

To understand if the effects of GM-CSF on *P. aeruginosa* PAO1 are specific to persister cells, DNA microarrays were also used to study the effects of 0.17 pM GM-CSF on the total population (> 99% are normal cells) of *P. aeruginosa* PAO1 since GM-CSF only sensitized the persister subpopulation to antibiotics. Using the same 1.5-fold change as the cut off ratio, 106 genes were found induced and 39 genes were found repressed in both biological replicates. The induced genes include 44 genes coding for hypothetical proteins, 11 genes associated with the transport of small molecules, 9 genes related to the biosynthesis of cofactors, 9 genes encoding putative enzymes, and 8 genes for transcriptional regulators (Figure 4.1B). Unlike the results of persister cells, there was no induction of motility and phage related genes in normal cells. Among the repressed genes, there were 22 genes coding for hypothetical proteins, 4 genes related to metabolism, 3 genes associated with energy metabolism, and 3 genes encoding for putative enzymes (Figure 4.1B). The microarray data were validated by qPCR involving two induced genes (*yrfI* and *dnaB*), and two repressed genes (PA0364, PA0558) (Table 4.5). These results indicate the GM-CSF has different effects on the gene expression of persister cells and normal cells of *P. aeruginosa* PAO1.

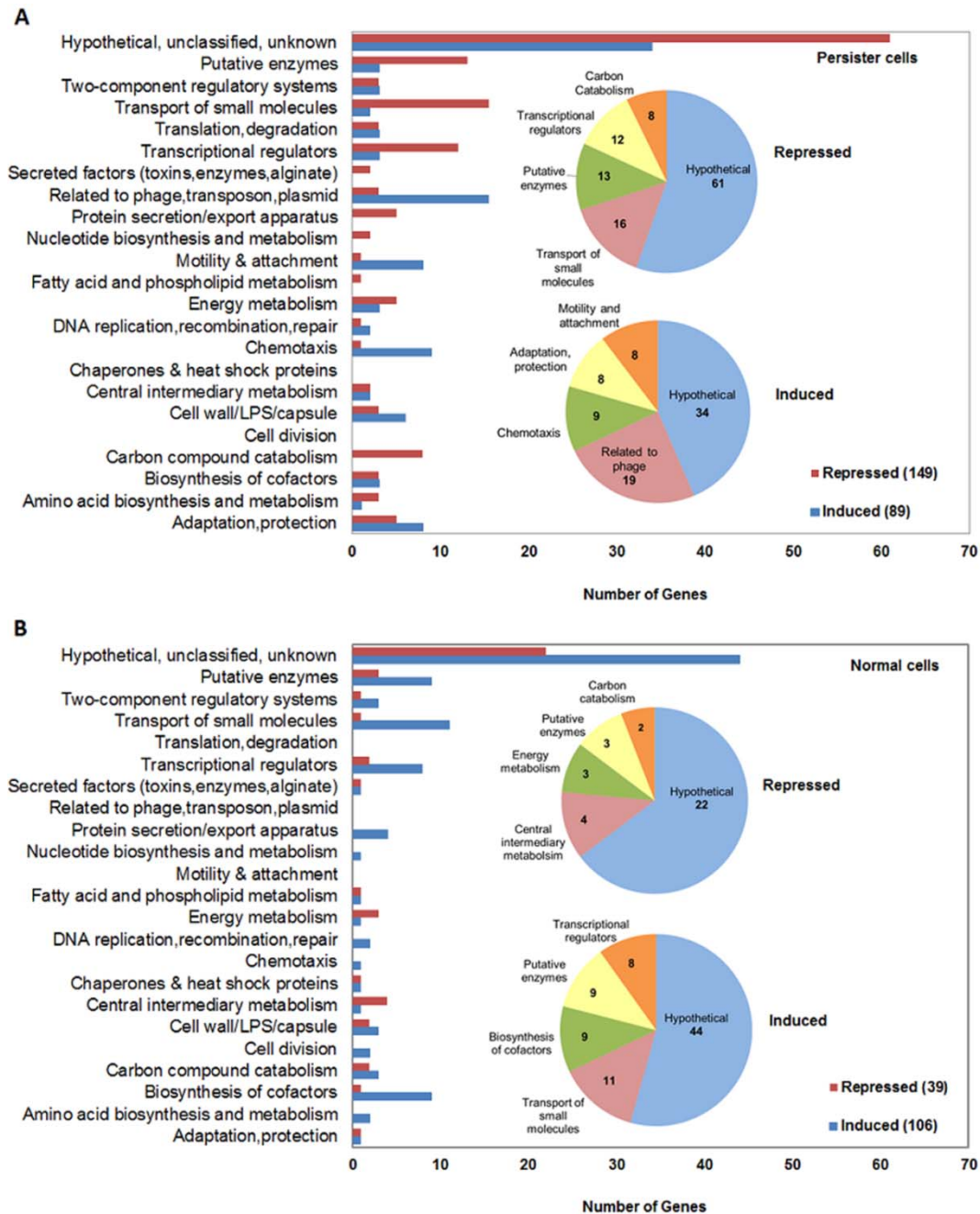


Figure 4.1 Effect of 0.17 pM GM-CSF on gene expression in *P. aeruginosa* PAO1. (A) Number and categories of genes that were consistently induced or repressed in two biological replicates of *P. aeruginosa* PAO1 persister cells. (B) Number and categories of genes that were consistently induced or repressed in two biological replicates of *P. aeruginosa* PAO1 normal cells.

Table 4.3 Expression fold change of representative genes in *P. aeruginosa* PAO1 persister cells based on the average of two DNA microarray runs.

Gene	Expression fold Change (GM-CSF vs. Control)	Functions
<i>flgBFH</i>	+(2.1-2.6)	Motility & Attachment; Cell wall / LPS / capsule
<i>cheYZ</i>	+(2.1-2.2)	Chemotaxis
<i>fliN</i>	+2.3	Motility & Attachment; Chemotaxis; Adaptation,
<i>priN</i>	+2.8	Pyocin regulatory gene
PA0618	+2.8	R-pyocin bacteriophage
PA0619	+2.8	R-pyocin bacteriophage
PA0620	+2.7	R-pyocin bacteriophage
PA0625	+2.8	R-pyocin bacteriophage
PA0633	+3.1	F-pyocin bacteriophage
PA0638	+2.9	F-pyocin bacteriophage
PA0640	+3.1	F-pyocin bacteriophage
PA4593	-15.0	probable permease of ABC transporter
PA0757	-11.5	probable two-component sensor
PA4187	-7.5	probable MFS transporter
PA2408	-5.7	probable ATP-binding component of ABC transporter
<i>phnA</i>	-5.6	anthranilate synthase component I
<i>wbpK</i>	-5	NAD-dependent epimerase/dehydratase
<i>ureA</i>	-4.9	urease gamma subunit
<i>pscR</i>	-4.5	translocation protein in type III secretion
PA1256	-3.9	putative ATP-binding component of ABC transporter
<i>ccmA</i>	-3.7	heme exporter protein CcmA
PA5216	-3.3	iron ABC transporter substrate-binding protein
<i>mmsR</i>	-3.2	transcriptional regulator MmsR
PA4037	-3.0	probable ATP-binding component of ABC transporter
<i>algL</i>	-2.5	poly(beta-D-mannuronate) lyase
<i>algA</i>	-2.5	phosphomannose isomerase / guanosine 5'-diphospho-D-mannose pyrophosphorylase
PA4113	-2.1	probable MFS transporter

Table 4.4 Comparison of qPCR results with the DNA microarray results for 10 representative genes of *P. aeruginosa* PAO1 persister cells.

Gene	Expression fold change (GM-CSF vs. Control)	
	DNA microarray	qPCR
<i>flgF</i>	+2.3	+3.3
<i>fliN</i>	+2.3	+8.5
<i>prtN</i>	+2.8	+3.3
PA0620	+2.7	+5.2
PA0633	+3.1	+6.5
PA0640	+3.1	+4.7
<i>recA</i>	+2.2	No significant change
<i>wbpK</i>	-5.0	-2.1
<i>algA</i>	-2.5	-5.9
<i>argH</i>	No significant change	No significant change

Table 4.5 Comparison of qPCR results with the DNA microarray results for 4 representative genes of *P. aeruginosa* PAO1 normal cells.

Gene	Expression fold change (GM-CSF vs. Control)	
	DNA microarray	qPCR
<i>dnaB</i>	+8.2	+3.0
<i>yrfI</i>	+4.7	+4.3
PA5548	-5.5	-1.7
PA0364	-11.8	-35.5

4.4.2 GM-CSF induces pyocin production in persister cells of *P. aeruginosa* PAO1

R-type pyocins produced by *P. aeruginosa* strains can be categorized into five types termed R1 to R5²⁴. Kohler *et al.*¹³ showed that the R1-pyocin producing *P. aeruginosa* PAK strain is susceptible to the R2-pyocins produced by *P. aeruginosa* PAO1. We found that treatment with GM-CSF induced R-pyocin related genes in *P. aeruginosa* PAO1 including PA0617, PA0619-22, and PA0625-30. Further test showed that, after *P. aeruginosa* PAO1 persister cells were treated with GM-CSF at 0.17 pM and 0.17 nM, the supernatant reduced the viability of normal cells of *P. aeruginosa* PAK by 65.3±14.5% ($p = 0.0201$) and 67.8±9.7% ($p = 0.0132$) respectively compared to the GM-CSF free control (Figure 4.2A). In contrast, no significant difference ($p > 0.1$) was observed for the same treatment of the normal cells of R2-pyocin resistant *P. aeruginosa* PAO1 (Figure 4.2A). Moreover, when the persister cells of PA0620::*phoA*, a deletion mutant of PA0620 encoding for R2-pyocin tail fiber protein, were treated with 0.17 pM and 0.17 nM GM-CSF, and the supernatant was tested on the normal cells of *P. aeruginosa* PAO1 and PAK, no significant change was observed in the viability of cells for both the strains (Figure 4.2B). These results indicate that pyocins are important for the observed phenomenon. To our knowledge, the interaction between persister cells and R2-pyocins has not been explored to date.

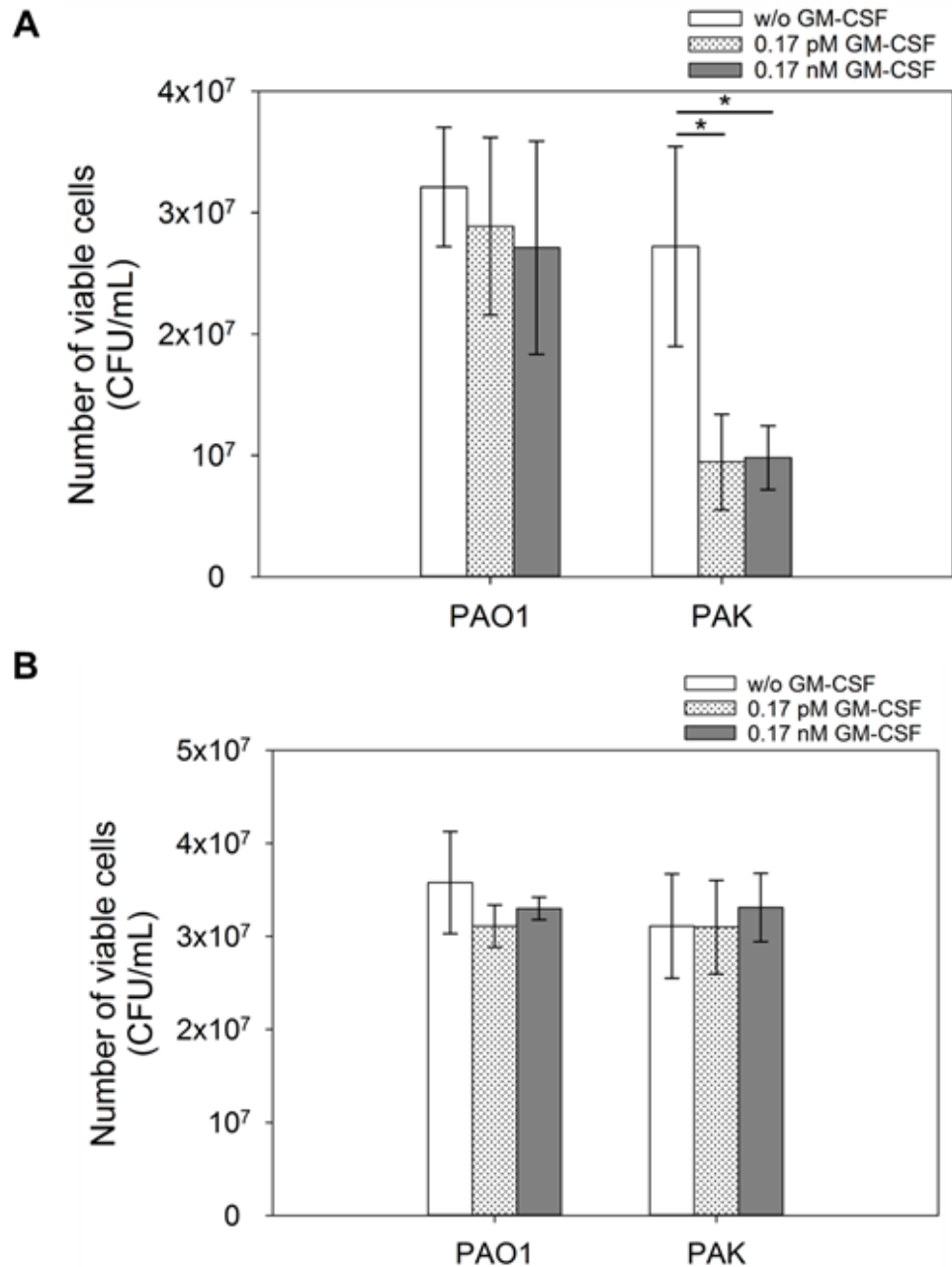


Figure 4.2 Effects of supernatants of GM-CSF-treated *P. aeruginosa* PAO1 persister cells. The normal cells harvested from stationary phase cultures of *P. aeruginosa* PAO1 and PAK were treated with the supernatant collected from the persister cells of *P. aeruginosa* PAO1 (A), and PA0620::*phoA* (B) after treatment with 0.17 pM or 0.17 nM GM-CSF for 2 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of PAO1 and PAK cells was determined by counting CFU. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

To test the synergistic effect of GM-CSF and antibiotics on PA0620::*phoA*, a similar experiment was performed as described in chapter 3, by isolating persister cells from stationary cultures of PA0620::*phoA*, followed by treatment with GM-CSF alone or co-treatment with antibiotics. As shown in Figure 4.3, treatment with 0.17 pM GM-CSF sensitized 57.2±5.6% ($p = 0.0019$) and 55.6±6.3% ($p = 0.0005$) of persister cells to 5 µg/mL ciprofloxacin and tobramycin respectively. In comparison to the wild-type *P. aeruginosa* PAO1, a mutation in the R2-pyocin tail fiber gene reduced the synergistic effects.

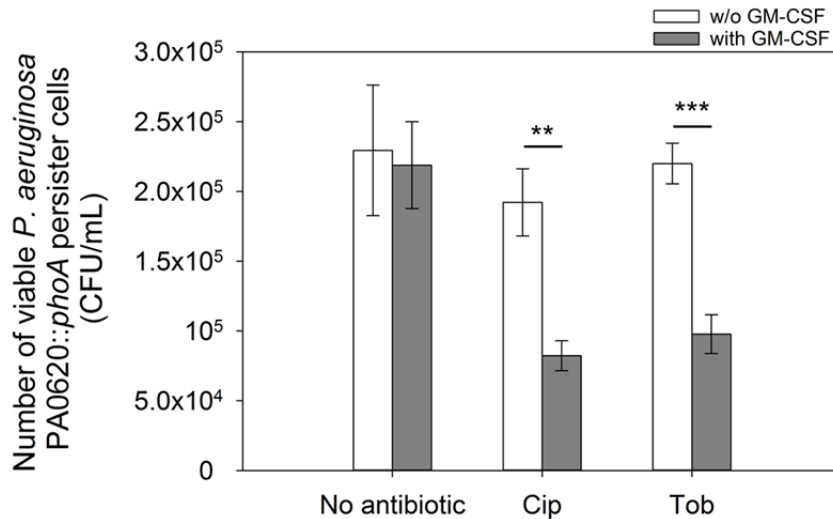


Figure 4.3 Mutation in PA0620 (R2-pyocin tail fiber gene) reduced the synergistic effects of GM-CSF and antibiotics on persister cells isolated from stationary phase cultures compared to wild-type *P. aeruginosa* PAO1. The persister cells of PA0620::*phoA* were isolated from stationary phase cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF alone or with GM-CSF plus antibiotics as indicated for 3.5 h (all tested at 5 µg/mL). The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

4.4.3 Defect in MFS transporter transcriptional regulator shows lower synergistic activity between antibiotics and GM-CSF compared to wild-type *P. aeruginosa*

PAO1

GM-CSF treatment repressed the genes associated with ABC and MFS transporters (e.g. PA2408, PA1256, PA4187, PA4113) in *P. aeruginosa* PAO1 persisters as shown in Table 4.3. ABC transporters utilize the energy of ATP hydrolysis to transport substances including amino acids, ions, peptides, sugars, antibiotics, nutrients, toxins, proteases, polysaccharides, and other hydrophilic molecules across bacterial membranes^{25,26}. MFS transporters use the energy from chemiosmotic gradient of ions instead to carry the substances including simple sugars, metabolites, drugs, amino acids, ions, oligosaccharides, nucleosides, etc. across bacterial membranes^{27,28}. For both the mutant strains PA0218::*phoA* (ABC transporter transcriptional regulator mutant) and PA3594::*phoA* (MFS transporter transcriptional regulator mutant), GM-CSF treatment alone had insignificant effect ($p > 0.1$) on the viability of persister cells similar to that of the wild-type *P. aeruginosa* PAO1. GM-CSF was also found to sensitize both strains to antibiotics. For example, 0.17 pM GM-CSF sensitized the persister cells of MFS transporter transcriptional regulator mutant PA3594::*phoA* to 5 µg/mL ciprofloxacin and tobramycin by 49.2±10.2% ($p = 0.005$) and 38.0±9.0% ($p = 0.0163$) respectively (Figure 4.4). In comparison, ABC transporter transcriptional regulator mutant PA0218::*phoA* persister cells were sensitized by 66.2±41.4% ($p = 0.0677$) and 75.8±16.3% ($p = 0.0025$) (Figure 4.4). The results indicate that MFS transporters have a marginal role in GM-CSF activities on the persister cells of wild-type *P. aeruginosa* PAO1.

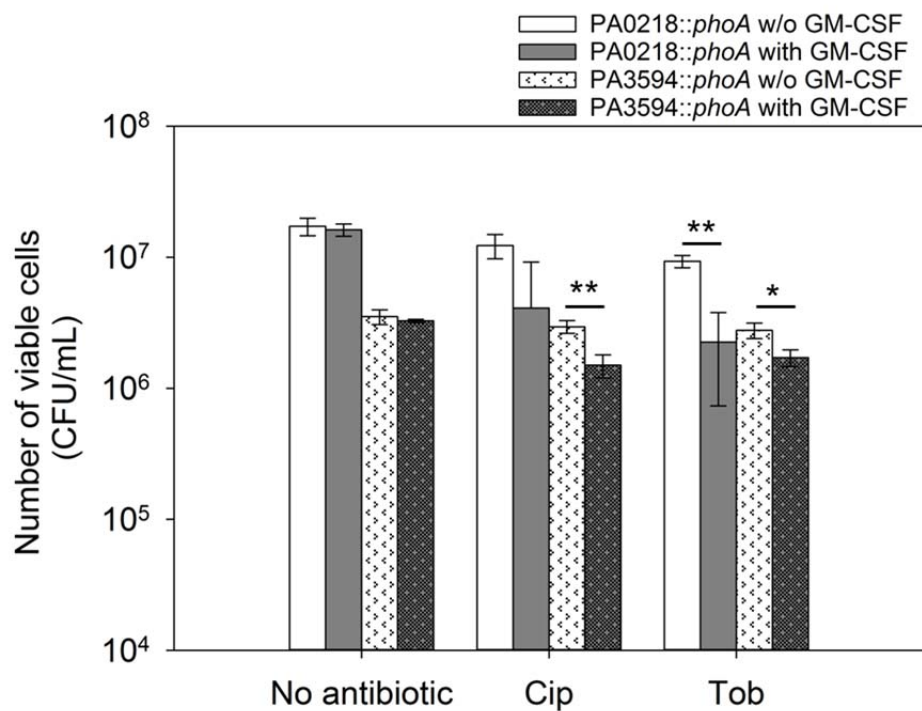


Figure 4.4 GM-CSF show marginal reduction in sensitization of the persister cells of ABC (PA0218) and MFS transporter (PA3594) transcriptional regulator mutants to antibiotics. The persister cells were isolated from stationary phase cultures of PA0218::*phoA* (ABC transporter transcriptional regulator mutant) and PA3594::*phoA* (MFS transporter transcriptional regulator mutant) by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h, and then treated with GM-CSF plus 5 µg/mL ciprofloxacin, and 5 µg/mL tobramycin. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

4.5 Discussion

In the DNA microarray studies, we found that a total of 19 pyocin genes including both R-type and F-type pyocin genes were induced by GM-CSF in *P. aeruginosa* PAO1 persister cells. The up-regulation of R- and F-pyocin genes suggest that GM-CSF treatment may induce the production of bacteriocins. R2, F2-pyocin genes and *prtN* are

also known to be induced by treatment with ciprofloxacin and hydrogen peroxide^{29,30}. Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and hydroxyl radicals are continuously encountered by microorganisms, especially during active infection with an oxidative stress generated by the host defense^{30,31}. Thus, we speculate that GM-CSF might be creating stress on the persister cells. The R-type pyocins have a higher bactericidal activity than F-type pyocins¹⁵.

The lipopolysaccharides found in the outer membrane of *P. aeruginosa* cells and other Gram-negative bacteria act as receptors of R-type pyocins^{13,15,32}. The killing mechanism of R-type pyocins involves membrane depolarization by pore formation^{13,15,32}. The R2-pyocin genes found induced by GM-CSF are involved in the formation of base plate (PA0617), tail fiber (PA0619-21), tail sheath (PA0622), and tail (PA0625-28)³³. The R-type pyocins with five subgroups: R1, R2, R3, R4, and R5 are similar to each other structurally, but differ in receptor specificity³⁴. The R-type pyocins have the ability to rapidly kill target cells by binding to the bacterial cell surface through their tail fiber, followed by contraction of the pyocin sheath and penetration of the core through bacterial membranes^{15,17}. This leads to depolarization of the cytoplasmic membrane as the concentrated intracellular ions begin to leak causing cell death^{15,17}. The tail fiber protein is required for binding to the receptors of sensitive cells and contraction of tail is necessary for bactericidal action^{35,36}.

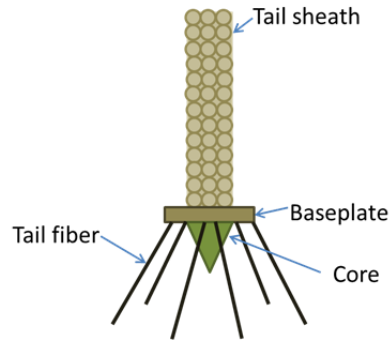


Figure 4.5 Structure of R2-pyocin. Adapted from Scholl *et al.* ¹⁷. Copyright, 2009, American Society for Microbiology.

P. aeruginosa PAO1 produces R2-pyocins that infect R2-pyocin sensitive *P. aeruginosa* strains ^{13,33}. The R2-pyocin producing strain and its next generations are resistant to the produced pyocins ³³. It was observed that when the persister cells of *P. aeruginosa* PAO1 were treated with 0.17 pM and 0.17 pM GM-CSF for 2 h, the supernatant collected after centrifugation exhibited cidal effects on the normal cells of R2-pyocin sensitive *P. aeruginosa* PAK strain. No such effect was observed on the normal cells of R2-pyocin producing *P. aeruginosa* PAO1. These results support the DNA microarray data which show that GM-CSF induces R2-pyocins related genes (PA0617, PA0619-22, PA0625-30) ³³. F-type pyocins can be categorized into three subtypes: F1, F2 and F3 ¹⁶. The induced F2-pyocin genes have structural function in the formation of tail (PA0635) and baseplate (PA0637, PA0638, PA0640) ³⁰. Moreover, the gene *priN* showed an up-regulation by 2.8-fold in two DNA microarray runs, which was confirmed by qPCR analysis (3.3-fold induction). This gene encodes an activator of the R and F-type pyocin genes ²¹.

Besides the insoluble phage-like R- and F-type pyocins, *P. aeruginosa* PAO1 also produces soluble S-type pyocins ¹⁵. S-type pyocins are colicin-like, protease-

sensitive proteins, consisting of two components: (i) a large component, which shows killing activity, and (ii) a small component, which is an immunity protein protecting the host bacteria from killing activity of the large component^{37,38}. The S-type pyocins include S1, S2, S3, and AP41 pyocins possessing DNase activity, S4 pyocin exhibiting tRNase activity, and S5 pyocin with membrane damage activity^{39,40}. However, unlike R- and F-type pyocin genes, the genes associated with S-type pyocins did not show a significant change in expression.

In addition to the induced genes, qPCR also confirmed the repression of *wbpK*, an NAD-dependent epimerase/dehydratase gene involved in cell envelope biogenesis and catabolism, and *algA*, an alginate biosynthesis gene (mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase) which produces a precursor for alginate polymerization²³. King *et al.*⁴¹ postulated WbpK to be a dinucleotide co-factor-dependent 4-reductase enzyme which catalyzes the reduction of UDP-4-keto-D-QuiNAc to generate UDP-D-FucNAc. Moreover, Belanger M. *et al.*⁴² indicated that *wbpK* plays a role in O-antigen biosynthesis. The O-antigens are immunogenic, eliciting a strong antibody response from the infected host⁴². They protect the bacteria from phagocytosis during *P. aeruginosa* infections⁴³.

Repression of *algA* may lead to lowered alginate layer formation, which in turn could reduce protection against host immune defenses and antibiotics⁴⁴. The repression of *algL* indicates possible abnormality in the alginate production as the alginate lyase activity of AlgL is required to degrade mislocalized alginate or mannuronate residues, which may help explain the enhanced killing by antibiotics⁴⁵. In cystic fibrosis patients with *P. aeruginosa* infections, pyocyanin is secreted by this bacterium, which is a blue

redox-active secondary metabolite and generates superoxide and H₂O₂ in the infected lungs^{46,47}. In the pyocyanin biosynthesis pathway, the conversion of chorismate to anthranilate is catalyzed by anthranilate synthase encoded by *phnA* and *phnB* genes⁴⁸. The repression in *phnA* gene by GM-CSF treatment suggests possibly lowered pyocyanin production, which may result in reduced virulence.

Multidrug efflux transporters found in Gram-negative bacteria are known to display a powerful defense mechanism by extrusion of antimicrobial agents and toxins from the bacterial cells, thus facilitating the survival of bacteria in stressful environments^{49,50}. There are five major families of bacterial efflux transporters include: ABC (ATP binding cassette), MFS (major facilitator superfamily), RND (resistance-nodulation-division), MATE (multidrug and toxic efflux), and SMR (small multidrug resistance)^{50,51}. As shown in Figure 4.5, ABC transporters utilize the free energy from ATP hydrolysis to pump out the antibiotics, while all other transporters utilize the proton motive force as an energy source^{51,52}. These multidrug efflux pumps have broad antibiotic specificity and elevation of their expression level due to alterations in physiological or genetic regulations provides high resistance to a variety of antimicrobial agents⁵³⁻⁵⁵.

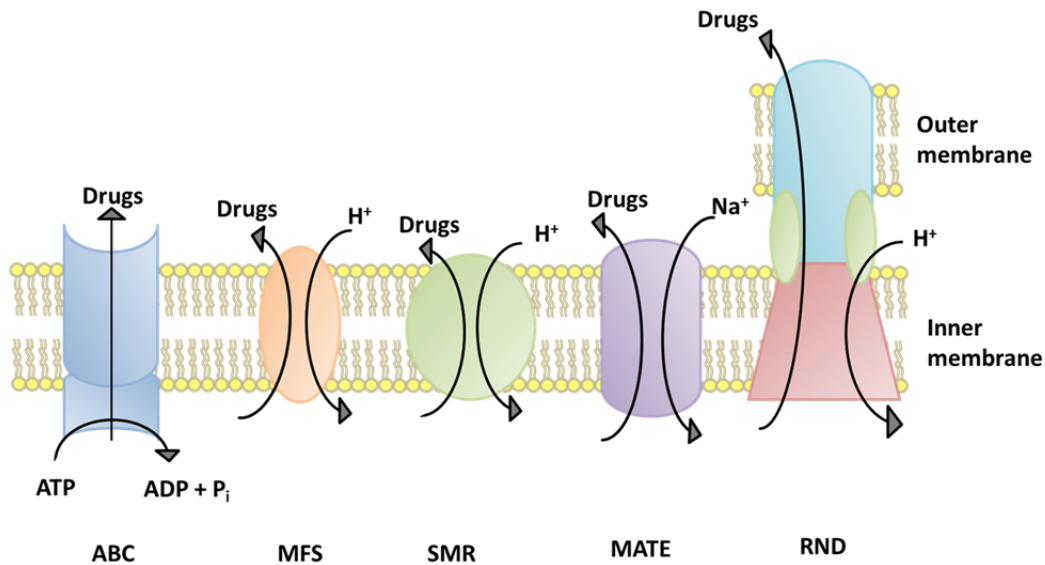


Figure 4.6 Multidrug efflux pumps of Gram-negative bacteria. Adapted from Piddock *et al.* ⁵⁶. Copyright, 2006, American Society for Microbiology.

The repression of genes related to ABC and MFS transporters in *P. aeruginosa* PAO1 persister cells after GM-CSF treatment indicates that GM-CSF possibly plays a role in suppressing the antibiotic resistance systems in the persister cells, rendering them susceptible to antibiotic treatments. Comparison of intracellular level of antibiotics in these mutants and the wild-type *P. aeruginosa* PAO1 in the presence and absence of GM-CSF can give a clarification of the role of GM-CSF on the efflux pumps.

4.6 Conclusions

GM-CSF sensitized the persister cells of *P. aeruginosa* PAO1 to antibiotics. In this chapter, we characterized the changes in gene expression caused by GM-CSF, using DNA microarrays and qPCR. The difference in the expression of genes related to pyocins and transporters in persister cells and normal cells suggest that GM-CSF has profound

effects on persisters. The persister cells showed an induction of 19 pyocin related genes, whereas normal cells showed no significant change in the expression level of these genes. Moreover, 16 genes related to transport of small molecules were repressed in persister cells, while normal cells displayed an induction of 11 genes in this category. The reduced viability of normal cells of *P. aeruginosa* PAK by supernatant of GM-CSF treated *P. aeruginosa* PAO1 persister cells indicates that GM-CSF induced R2-pyocin-related genes.

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CHAPTER 5

ROLE OF FLAGELLIN (FliC) IN INTERACTION BETWEEN GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) AND *PSEUDOMONAS AERUGINOSA*

Dr. Xiangyu Yao constructed *fliC* mutant strain of PAO1 (PAO1 Δ *fliC*) and *fliC* complemented strain (PAO1 Δ *fliC*/pUCP*fliC*); performed co-immunoprecipitation and cross-linking experiments.

5.1 Abstract

Our DNA microarray studies performed on normal and persister cells of the wild-type *P. aeruginosa* PAO1 revealed that 18 bacterial motility associated genes were induced in persister cells by GM-CSF, whereas no such change was observed for the motility genes in normal cells. The protein FliC (flagellin) was identified as the target of GM-CSF binding after performing co-immunoprecipitation and cross-linking. To confirm that interaction with FliC is important the activity of GM-CSF, we tested GM-CSF and antibiotics on the *fliC* mutant strain PAO1 Δ *fliC*, and *fliC*-complemented strain PAO1 Δ *fliC*/pUCP*fliC*. Deletion of *fliC* gene abolished the effects of GM-CSF on *P. aeruginosa* PAO1 persisters, which was restored in PAO1 Δ *fliC* /pUCP*fliC*. Moreover, GM-CSF appeared to be more effective on *P. aeruginosa* cells with resting flagella since the effects were enhanced in normal cells of PAO1 pretreated with inhibitors of flagellar motility, and in the normal cells of the flagellar motor mutants. Thus, the presence of FliC and a suppressed motility, which are generally found in persister cells, appeared to be necessary for GM-CSF binding and associated activities.

5.2 Introduction

Flagellum is a highly complex bacterial organelle that appears as a long, filamentous appendage attached to the cell¹. Flagella are involved in bacterial motility, chemotaxis, and acquisition of essential nutrients¹. It has been shown previously that flagella contribute to the virulence of *P. aeruginosa*¹⁻³. Flagella also mediate cell adherence, colonization, and biofilm formation⁴⁻⁹. Thus it is not surprising that nonopsonic phagocytosis is mediated by bacterial flagella; e.g. macrophages bind to bacterial flagella and subsequently clear the bacteria from the host^{10,11}. The protein flagellin (FliC), is the primary component of flagella. FliC proteins form the filament of flagella of *P. aeruginosa* with a 15-18 nm diameter hollow channel¹²⁻¹⁶. The mammalian host cells detect the flagellin monomers using Toll-like receptor 5 (TLR5), which is involved in both innate and adaptive immune responses¹⁷⁻¹⁹.

As discussed in chapter 4, treatment of PAO1 persisters with GM-CSF induced 10 chemotaxis genes and 8 motility including *flgBCDEFGHIJK*, *fliACDGMN*, and *cheYZ*. The finding that GM-CSF at pM level can sensitize PAO1 persisters to antibiotics suggests that PAO1 may have a binding target of GM-CSF. Previously, Mahdavi *et al.*²⁰ showed that the uptake of cytokines TNF- α and IL-8 by *Neisseria meningitidis* is facilitated by pilus assembly proteins (PilQ and Pile). In another study, Paino *et al.*²¹ identified a bacterial interleukin receptor I (BilRI) on *Aggregatibacter actinomycetemcomitans*, which binds to cytokine IL-1 β . Wu *et al.*²² reported that IFN- γ binds to the *P. aeruginosa* outer-membrane protein OprF and induces quorum sensing and pyocyanin expression.

The co-immunoprecipitation (co-IP) results described in this Chapter verified the interaction between FliC and GM-CSF. Co-IP is a technique for determining the presence or absence of interaction between two proteins *in vitro* by using target protein-specific antibodies to indirectly capture proteins bound to a specific target protein^{23,24}. The GM-CSF-FliC binding was identified when the flagellar fractions isolated from PAO1 were cross-linked with GM-CSF, and co-IP was performed followed by SDS-PAGE analysis.

To further explore the role of FliC in interaction between GM-CSF and *P. aeruginosa*, the gene *fliC* was deleted from PAO1, and both the deletion mutant and complemented strain were compared with the wild-type PAO1 for the effects of GM-CSF. The synergistic effects between GM-CSF and antibiotics were abolished in absence of a functional FliC protein, but were restored when *fliC* was complemented on a plasmid. Further tests were performed on the wild-type *P. aeruginosa* PAO1 after inhibiting the activity of flagella by pre-treatment with inhibitors of protein synthesis and flagellar activity. It was found that when the flagella of normal cells of *P. aeruginosa* PAO1 were made inactive, GM-CSF was able to sensitize them to antibiotics. Since GM-CSF did not exhibit any effects on the normal cells with functional flagella (as discussed in Chapter 3), the inactive/resting flagella are possibly essential for the binding.

5.3. Materials and Methods

5.3.1 Bacterial strains and growth media

P. aeruginosa PAO1²⁵ (obtained from Prof. Matthew Parsek) and PAK²⁶ were used in this study. The mutant strains PAO1 $\Delta fliC$ (*fliC* deletion mutant) and PAO1

$\Delta fliC/pUCPfliC$ (*fliC* complemented strain), were constructed by Dr. Xiangyu Yao in the Ren lab and used to study GM-CSF activity. Individual transposon mutant of the genes *motD* (PA4593::*lacZ*) and *motA* (PA4954::*lacZ*) were obtained from the *P. aeruginosa* PAO1 mutant library (from Manoil lab at University of Washington)²⁷. Overnight cultures of these strains were prepared in Luria Bertani (LB) medium²⁸ containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at 37°C with shaking at 200 rpm. The transposon mutants were grown in LB medium supplemented with 60 µg/mL tetracycline. PAO1 $\Delta fliC$ was cultured in LB medium supplemented with 100 µg/mL gentamicin and 500 µg/mL streptomycin, while PAO1 $\Delta fliC/pUCPfliC$ was cultured in LB medium supplemented with 150 µg/mL carbenicillin. Recombinant human GM-CSF was purchased from R&D systems (Minneapolis, MN, USA). The stocks used in this study contained 10 µg/mL GM-CSF, dissolved in phosphate buffer saline (PBS) pH 7.4, supplemented with 0.1% bovine serum albumin (BSA). (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8) was synthesized previously by Dr. Jiachuan Pan in the Ren lab and stored at 60 mg/mL in absolute ethanol before experiments.

5.3.2 Isolation of flagella from *P. aeruginosa* PAO1 cells

In order to isolate the flagella of the wild-type *P. aeruginosa* PAO1, 100 µL of stationary phase culture with an OD₆₀₀ (optical density at 600 nm) around 2.0 was spread on top of 1.5% agar plates, and grown at 37 °C for 16 h. Approximately 2 mL of PBS was then added on top of the plates and the cells were collected using a spreader. Cells were transferred to microcentrifuge tubes and vortexed vigorously for 30 min. Thereafter, the

samples were centrifuged at a speed of 13,200 rpm for 20 min at 4 °C to remove insoluble proteins and separate the flagella from cell pellet²⁹. When the isolated flagella were used in the tests on cells, 200 µg/mL ciprofloxacin was added to the collected supernatants and the samples were incubated for 3.5 h at 37 °C with shaking at 200 rpm kill all remaining cells, followed by dialysis against PBS (pH=7.4) at room temperature to remove ciprofloxacin. Dialysis was performed using dialysis membranes (Spectrum Labs, Rancho Dominguez, CA, USA) with 3,500 Da as cut-off molecular weight. For testing the effects of sheared flagella, the resulting solution with flagella was sheared with 0.1 mm diameter zirconia/silica beads (Biospec Products, Bartlesville, OK, USA) for 5 min (1 min beating followed by an interval of 20 s) at speed setting 48 (~5000 rpm) (Mini-Beadbeater-1, Biospec Products, Bartlesville, OK, USA).

Flagella were also isolated for tests of GM-CSF binding, by performing dialysis against PBS without any ciprofloxacin instead, followed by addition of MgCl₂ to a final concentration of 100 mM for precipitation of isolated flagella. The resulting supernatant was incubated overnight at 4 °C to precipitate flagella. The precipitate was collected by centrifugation at 13,200 rpm for 20 min at 4 °C and pellet was resuspended in PBS. SDS-PAGE/ SYPRO Ruby and Western blotting were performed to assess the purity of isolated flagella.

5.3.3 GM-CSF binding with cross-linking and Co-Immunoprecipitation (Co-IP)

The inner and outer membrane proteins of *P. aeruginosa* PAO1 were isolated by cell fractionation method demonstrated by Filip *et al.*³⁴ and Portnoy *et al.*³⁵. To cross-link

GM-CSF with *P. aeruginosa* cells, membrane proteins and isolated flagella fraction, 10 μ L of 5 ng/ μ L GM-CSF was incubated with 30 μ L of each sample for 2 h at room temperature, followed by addition of the cross-linker bisulfosuccinimidyl suberate (BS3) to a final concentration of 2.5 mM. The cross-linking reaction was performed at room temperature for 30 min and quenched by 50 mM Tris-Cl (pH 8.0) for 15 min at room temperature. After mixing with Laemmli sample buffer (2 mM β -mercaptoethanol, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 62.5 mM Tris-HCl (pH 6.8))³⁰. The samples were analyzed by SDS-PAGE followed by immunoblotting detection or staining with SYPRO Ruby (Bio-Rad, Hercules, CA, USA). Co-immunoprecipitation was performed using 165 μ L protein A/G magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) with 35 μ g anti-GM-CSF covalently coupled using BS3 and conjugation buffer. Meanwhile, 300 ng GM-CSF was cross-linked with 180 μ L of membrane proteins or isolated flagella. After precleaning the GM-CSF-protein mixture with uncoupled beads for 2 h, immunoprecipitation (IP) was performed on the resulting supernatant by subjecting it to anti-GM-CSF coupled beads for 2 h at room temperature with gentle shaking (50 rpm). The protein A/G beads were washed twice with 500 μ L IP buffer (PBS with 0.05% Tween 20) and once with 500 μ L PBS containing 0.5% Triton X-100, followed by incubation with 50 μ L Laemmli sample buffer for 5 min at 95°C. SDS-PAGE was performed to analyze the samples and proteins were visualized using SYPRO Ruby staining.

For Western blotting, the proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% milk in TTBS, followed by desired primary antibody (anti-GM-CSF, 1:2000; anti-FliC, 1:2000). The secondary antibody incubation was

performed using anti-mouse IgG conjugated with alkaline phosphatase. The signals were developed using BCIP/NBT for detection of the alkaline phosphatase activity of conjugated secondary antibody.

5.3.4 Combined effect of GM-CSF, flagella, and outer membrane vesicles on *fliC* mutant, PAO1 Δ *fliC*

Outer membrane vesicles (OMVs) were tested with GM-CSF and flagella on PAO1 Δ *fliC* to see if OMV can facilitate the transport of GM-CSF and flagella, and thus sensitize the PAO1 Δ *fliC* cells to GM-CSF. As described by Maredia *et al.*³¹, to isolate the OMVs along with outer membrane proteins (OMPs), an overnight culture of *P. aeruginosa* PAO1 grown in LB medium was subcultured to an OD₆₀₀ of 0.01 in 7 mL LB medium and incubated at 37 °C for 8 h with a shaking at 250 rpm. After the first 2 h, 1 µg/mL ciprofloxacin was added to the subculture to induce the production of OMVs. The cells were thereafter removed by centrifugation at 10,000 rpm for 10 min at 4 °C. To avoid contamination of PAO1 cells in the supernatant, it was passed through a 0.2 µm filter. A centrifugal filter unit (EMD Millipore, Billerica, MA, USA) containing a high recovery cellulose membrane with 10 kDa as MWCO was used to retain the OMPs and OMVs but remove the constituents that have a molecular weight below 10 kDa. Up to 12 mL of filtered supernatant was added to the filtration device, followed by centrifugation at 5,000 × g for 20 min. Desalting procedure was followed by addition of 14.8 mL of 100 mM NaCl to the 200 µL of concentrated proteins and centrifugation at 5,000 × g for 5 min. The resultant 200 µL protein solution was given additional 14.8 mL of 10 mM NaCl,

followed by centrifugation at $5,000 \times g$ for 5 min to wash out the contaminating salts. The final concentrated 200 μL protein solution was used for experiments.

In order to test whether OMVs and sheared flagella isolated from wild-type *P. aeruginosa* PAO1 could restore the effects of GM-CSF to sensitize *fliC* mutant persister cells to antibiotics, the persister cells isolated from the stationary phase cultures of PAO1 $\Delta fliC$ were treated with (i) 0.17 pM GM-CSF, (ii) 0.17 pM GM-CSF and 100 μL of sheared flagella, (iii) 0.17 pM GM-CSF and 100 μL of OMVs, (iv) 0.17 pM GM-CSF, 100 μL of sheared flagella, and 100 μL of OMVs, for 3.5 h at 37°C with shaking at 200 rpm. All of the treated samples were supplemented with 5 $\mu\text{g}/\text{mL}$ tobramycin to determine if synergistic effects exist. After treatment, the cells were washed thrice by centrifuging at 13,200 rpm for 2 min, followed by CFU counting using the drop plate method³² to determine the viability of cells.

5.3.5 Inhibition of flagellar activities

P. aeruginosa PAO1 cells were harvested from overnight cultures grown in LB medium after incubation at 37°C for 3.5 h with shaking at 200 rpm. The cells were collected by centrifuging at 8,000 rpm for 10 min and washed twice with PBS (pH=7.4). The washed cells were resuspended in 25 mL PBS buffer and vortexed gently for 1 min to release cells from the aggregates. To inhibit the flagellar activity, the harvested cells were pretreated with 5 $\mu\text{g}/\text{mL}$ BF8 for 3.5 h at 37 °C with shaking at 200 rpm. Before testing the effect of GM-CSF, the remaining free BF8 molecules were washed away by centrifuging twice at 8,000 rpm for 10 min with PBS. To test the effect of GM-CSF, the

washed cells were transferred to microcentrifuge tubes with 1 mL of washed cells in each tube. GM-CSF was added at 0.17 pM, and the amount of BSA (0.1%) was adjusted to be the same in all samples. After treatment at 37°C for 3.5 h with shaking at 200 rpm, the cells were washed thrice by centrifuging at 13,200 rpm for 2 min, followed by CFU counting using drop plate method to determine the cell viability.

Swimming motility assay was performed for normal cells of *P. aeruginosa* PAO1 treated with different concentrations of BF8 (1, 5, 10, 20, and 40 µg/mL) by adding BF8 to the normal cells and incubating for 3.5 h, followed by growing the cells on 0.3% (w/v) agar plates for 12 h at 37 °C as described by Ha *et al.*³³. Furthermore, viability of normal cells of *P. aeruginosa* PAO1 was tested after treatment with BF8 (1, 5, 10, and 20 µg/mL) for 3.5 h at 37 °C with shaking at 200 rpm. To corroborate the results of BF8, another experiment was performed by inhibiting protein synthesis (with 256 µg/mL chloramphenicol for 3.5 h). The normal cells pretreated with BF8 or chloramphenicol were treated with GM-CSF and antibiotics as described above to study the role of flagellar motility in persister control by GM-CSF.

5.4 Results

5.4.1 GM-CSF binds to FliC in *P. aeruginosa*

Since Co-IP is a technique that helps in determining whether two proteins interact or not in physiological conditions *in vitro*, it was performed to find the protein that possibly binds with GM-CSF. Crosslinking was used to covalently link together possible interacting proteins, domains, or peptides by forming chemical bonds between specific amino acid functional groups on biomolecules that occur in close proximity due to their interaction. The co-IP results indicated that GM-CSF binds to the membrane fraction of PAO1 cells (Figure 5.1A). GM-CSF binding was also detected in the isolated flagella of wild-type *P. aeruginosa* PAO1 (Figure 5.1B). Since flagella are anchored to the cell wall and membrane through basal body component of the flagella, FliC can be found in both the external appendage and outer membrane vesicles (OMVs). The Western blotting and SYPRO Ruby staining indicated cross-linked GM-CSF-flagella conjugates.

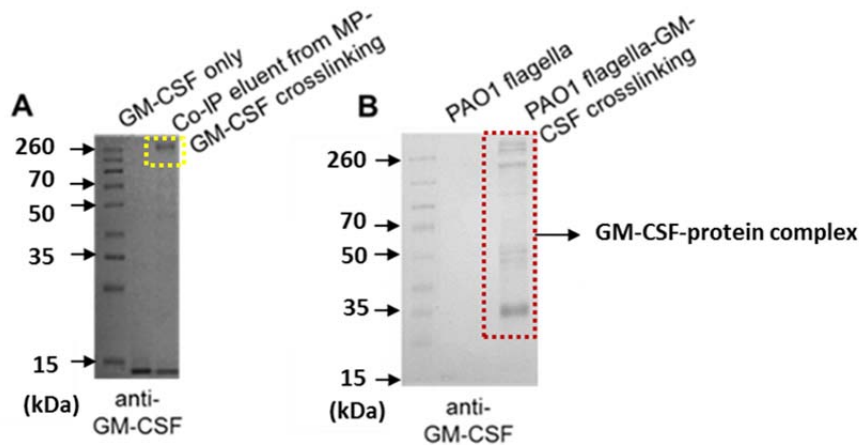


Figure 5.1 Co-Immunoprecipitation identified FliC as GM-CSF binding target in *P. aeruginosa* PAO1. (A) Co-IP eluent of anti-GM-CSF coated beads after incubation with *P. aeruginosa* PAO1 membrane protein-GM-CSF cross-linking sample using Western blotting. (B) Binding was observed between isolated flagellar fraction from *P. aeruginosa* PAO1 and GM-CSF, as detected by anti-GM-CSF using Western blotting.

Comparison between the whole cell lysates wild-type PAO1, PAO1 $\Delta fliC$, and the complemented strain PAO1 $\Delta fliC/pUCPfliC$ verified that FliC is a binding site for GM-CSF as the isolated flagellar portion from PAO1 $\Delta fliC$ did not show a detectable band as observed for the wild-type *P. aeruginosa* PAO1 and PAO1 $\Delta fliC/pUCPfliC$ (Figure 5.2).

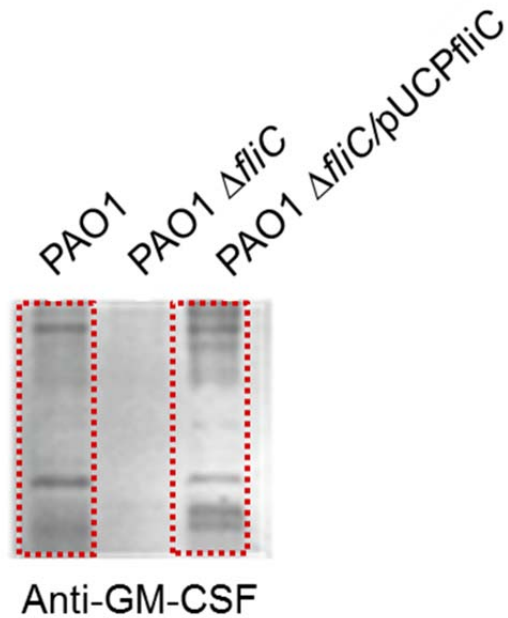


Figure 5.2 Western blotting showed that GM-CSF binding was abolished in the *fliC* mutant strain, but was recovered with complementation of *fliC*. The whole cell lysates of wild-type *P. aeruginosa* PAO1, *fliC* mutant strain PAO1 $\Delta fliC$, and *fliC* complemented strain PAO1 $\Delta fliC/pUCPfliC$ were cross-linked with GM-CSF and detected by anti-GM-CSF.

5.4.2 FliC is important to the effects of GM-CSF on *P. aeruginosa* persister cells

Using co-IP and proteomics, Dr. Xiangyu Yao in the Ren lab discovered that GM-CSF binds to the flagellin (FliC) of *P. aeruginosa* PAO1. The flagellin protein FliC is required for bacterial adhesion and virulence⁸. To verify if FliC is important to the activities of GM-CSF to sensitize *P. aeruginosa* persister cells to antibiotics, we repeated the

experiments described in Chapter 3 with the *fliC* mutant of *P. aeruginosa* PAO1 (PAO1 $\Delta fliC$) and the one with complemented *fliC* (PAO1 $\Delta fliC$ /pUCP*fliC*). As described in Chapter 3, treatment with 0.17 pM GM-CSF sensitized 61.5±14.5% ($p = 0.0003$) and 77.1±2.0% ($p = 0.0048$) of *P. aeruginosa* PAO1 persister cells to 5 µg/mL ciprofloxacin and 5 µg/mL tobramycin respectively (Figure 5.3). Such effects were abolished in the *fliC* mutant since GM-CSF failed to sensitize stationary phase persister cells of the *fliC* mutant to either 5 µg/mL ciprofloxacin (16.5±5.7% ($p = 0.0317$)) or 5 µg/mL tobramycin ($p > 0.05$) (Figure 5.3). Consistently, the activity of GM-CSF was restored when the *fliC* gene was complemented using the plasmid pUCP*fliC*. For example, 0.17 pM GM-CSF sensitized 80.5±4.7% ($p = 0.0004$) and 78.1±5.8% ($p = 0.0002$) of persister cells isolated from stationary phase culture of PAO1 $\Delta fliC$ /pUCP*fliC* to 5 µg/mL ciprofloxacin and tobramycin respectively (Figure 5.3).

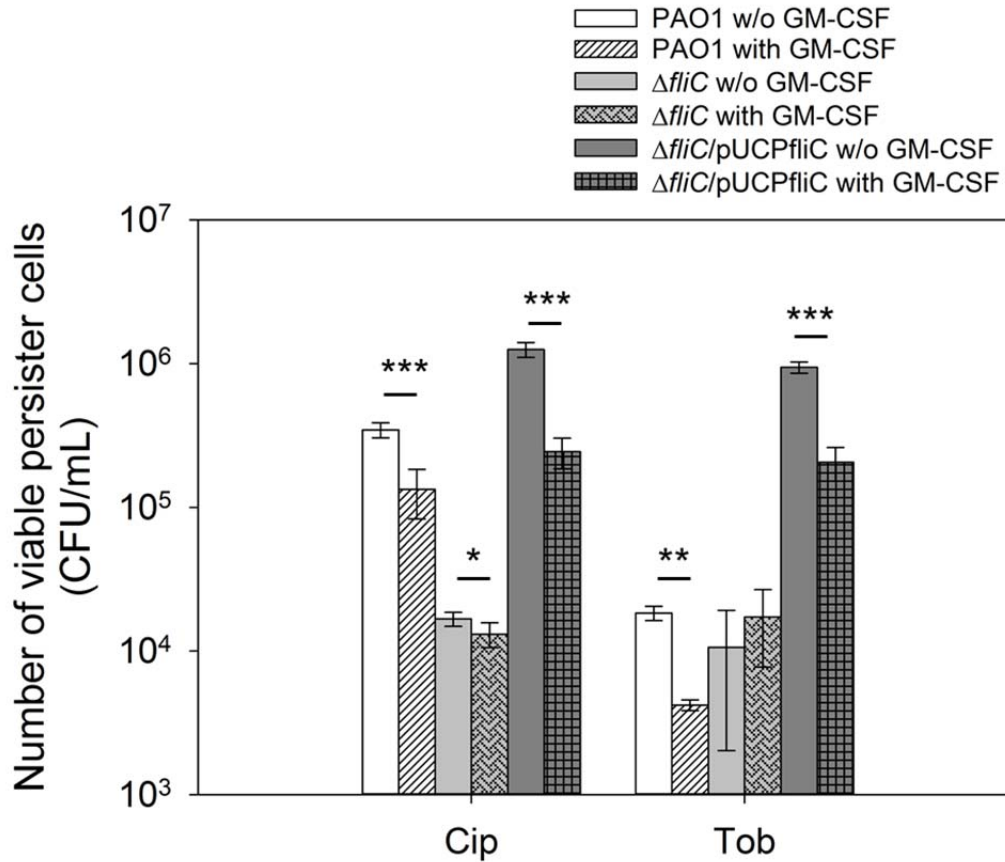


Figure 5.3 GM-CSF sensitized the persister cells of *P. aeruginosa* PAO1 and PAO1 $\Delta fliC/pUCPfliC$ isolated from stationary phase cultures to antibiotics, while $\Delta fliC$ persister cells did not get sensitized. The persister cells of *P. aeruginosa* PAO1, PAO1 $\Delta fliC/pUCPfliC$, and PAO1 $\Delta fliC$ were isolated from stationary phase cultures by killing the normal cells with 200 $\mu\text{g}/\text{mL}$ ciprofloxacin for 3.5 h, and then treated with GM-CSF alone and with GM-CSF plus 5 $\mu\text{g}/\text{mL}$ ciprofloxacin or tobramycin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

The aforementioned results of *fliC* mutant and complemented strain are consistent with the finding that GM-CSF interacts with FliC and indicate that FliC is required for synergy in persister killing by GM-CSF and antibiotics. Consistently, we also found that

the effects of GM-CSF were reduced when the persister cells of *P. aeruginosa* PAO1 were pretreated with 10 µg/mL anti-FliC for 1 h. For example, GM-CSF sensitized 27.0±9.4% ($p = 0.0117$) and 57.7±3.5% ($p < 0.0001$) of persister cells of *P. aeruginosa* PAO1 pretreated with and without 10 µg/mL anti-FliC respectively (Figure 5.4). This observation also supports the presence of GM-CSF-flagella binding.

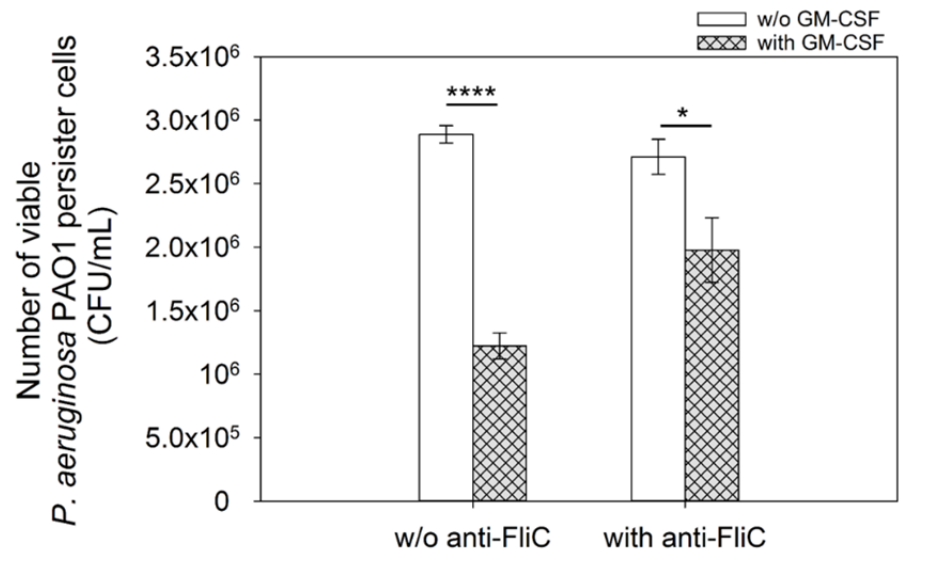


Figure 5.4 Blocking FliC with the anti-FliC antibody reduced the activity of GM-CSF. Persister cells of wild-type *P. aeruginosa* PAO1 were isolated from the stationary cultures by killing the normal cells with 200 µg/mL ciprofloxacin for 3.5 h. The harvested persister cells were pretreated with or without 10 µg/mL anti-FliC for 1 h, followed by treatment with 0.17 pM GM-CSF and 5 µg/mL tobramycin for 3.5 h. Following the treatment, the viability of persister cells was determined by counting CFU. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

5.4.3 GM-CSF sensitized the persister cells of *P. aeruginosa* strains with both a-type and b-type flagellins to antibiotics

The single polar flagella of *P. aeruginosa* strains express either a-type or b-type flagellins (FliC), based on the molecular weights of the flagella subunits encoded by *fliC* and their

antigenicity³⁴⁻³⁷. The a-type flagellin expressed by *P. aeruginosa* PAK is heterogeneous and has a variable molecular weight in the range of 45-52 kDa^{38,39}. The 16 kb genomic island encoding the determinants of the flagellin glycosylation in the strain PAK contains 14 genes, *orfA* to *orfN*, arranged in several putative operons and located between *flgL* and *fliC*³⁸⁻⁴⁰. Schirm *et al.*³⁸ reported that the a-type flagellin is glycosylated with a heterogeneous O-linked glycan attached to Thr189 and Ser260. In comparison, the b-type flagellin expressed by *P. aeruginosa* PAO1 is homogenous and has an invariant molecular weight of 53 kDa³⁶. The PAO1 strain contains only 4 genes, PA1088-PA1091 (PA1088-PA1090 have unknown functions, PA1091 is a homologue of the *rfbC* gene that is 35% identical to the PAK *orfN*) in the same chromosomal position as 16 kb glycosylation island found in a-type flagellin producing PAK strain¹³. Verma *et al.*⁴¹ reported that the b-type flagellin produced by *P. aeruginosa* PAO1 is glycosylated at two serine residues Ser191 and Ser195.

To understand if GM-CSF has different effects on the strains producing a-type and b-type flagellins, we also repeated the experiments using the strain *P. aeruginosa* PAK. GM-CSF also significantly sensitized the persister cells of PAK to antibiotics. For example, 0.17 pM GM-CSF sensitized 85.3±4.3% ($p < 0.0001$) and 80.9±6.8% ($p < 0.0001$) of the persister cells isolated from exponential phase cultures of PAK to 5 µg/mL ciprofloxacin and tobramycin, respectively (Figure 5.5A). Similar effects were observed for stationary phase cultures, with a reduction in 89.3±5.4% ($p = 0.0016$) and 84.1±2.6% ($p < 0.0001$) of persister cells after treatment with 0.17 pM GM-CSF along with 5 µg/mL ciprofloxacin and tobramycin respectively (Figure 5.5B). As observed for PAO1, GM-CSF alone did not affect the viability of PAK persister cells.

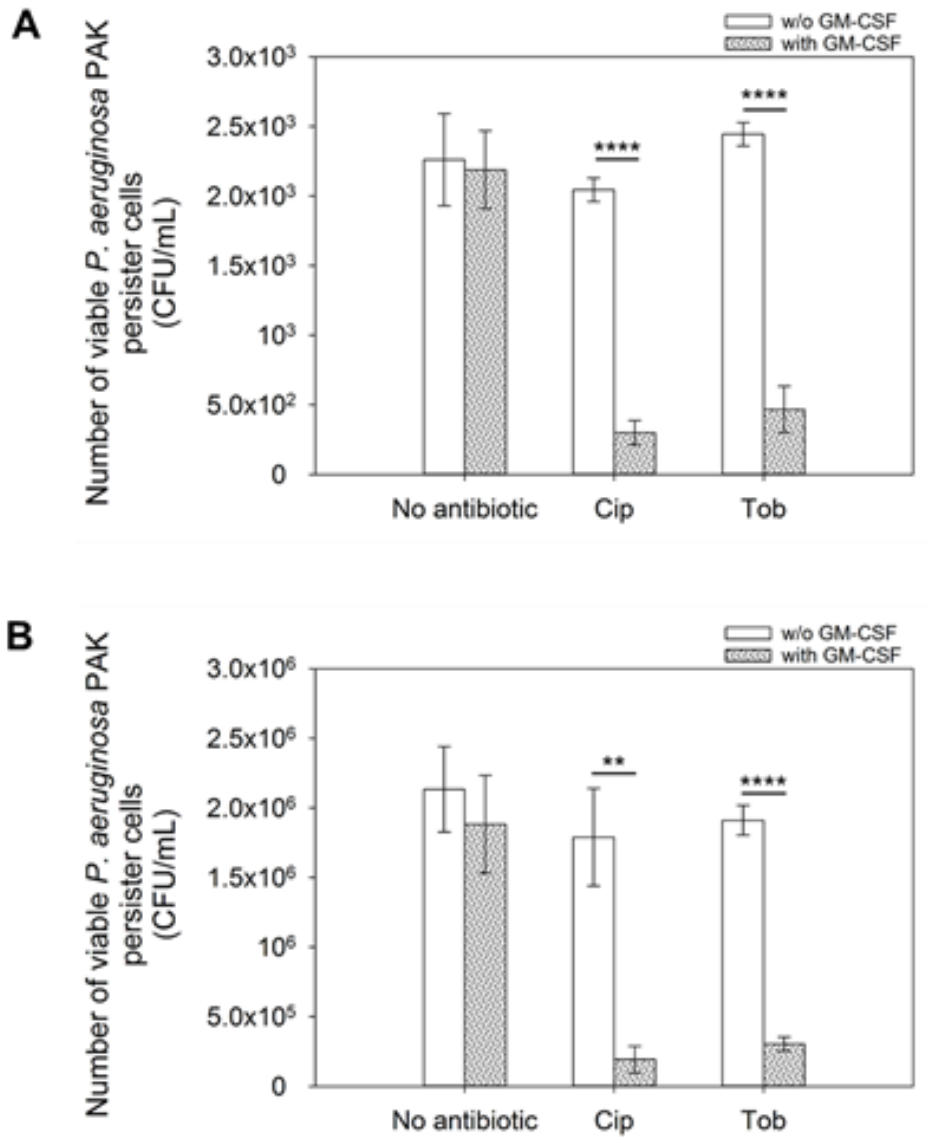


Figure 5.5 GM-CSF sensitized the persister cells of a-type flagellin producing *P. aeruginosa* PAK isolated from exponential and stationary phase cultures to antibiotics. The persister cells of *P. aeruginosa* PAK were isolated from exponential phase (A) and stationary phase (B) cultures by killing the normal cells with 200 $\mu\text{g}/\text{mL}$ ciprofloxacin for 3.5 h, and then treated with GM-CSF alone and with GM-CSF plus 5 $\mu\text{g}/\text{mL}$ ciprofloxacin or tobramycin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

GM-CSF-flagella binding for a-type flagellin was also confirmed by Western blotting using the flagella isolated from *P. aeruginosa* PAK (Figure 5.6). Interestingly, GM-CSF was found to bind more strongly to PAK flagellin than that of *P. aeruginosa* PAO1 (Figure 5.6), suggesting that GM-CSF may have higher binding activity with a-type flagellin.

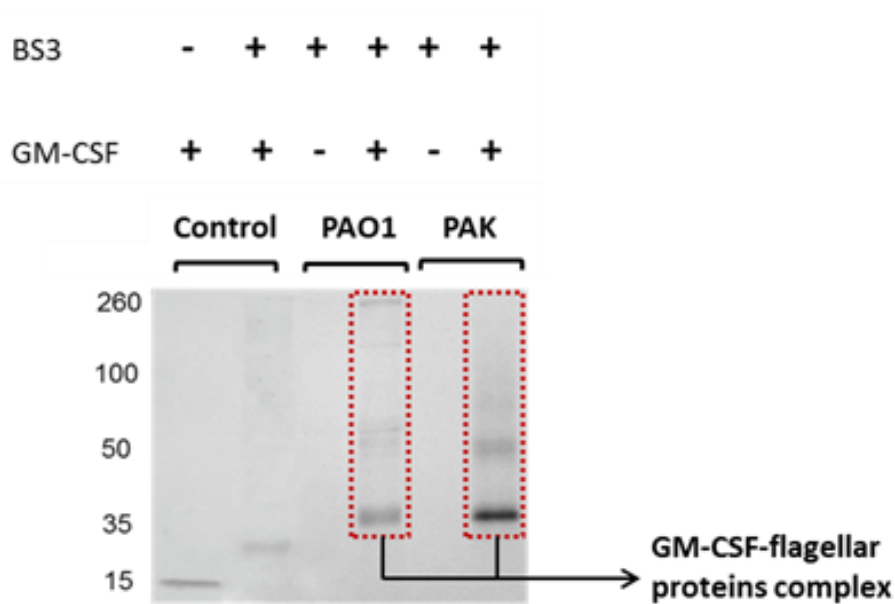


Figure 5.6 GM-CSF binds to both a-type and b-type flagellins. Flagella were isolated from the wild-type *P. aeruginosa* PAO1 possessing b-type flagellins and *P. aeruginosa* PAK possessing a-type flagellins. The flagella were incubated in the presence and absence of GM-CSF with cross-linking using BS3, followed with detection by anti-GM-CSF.

5.4.4 External addition of flagella and outer membrane vesicles (OMVs) partially restored the activity of GM-CSF on the *fliC* mutant

Outer membrane vesicles (OMVs) are produced by Gram-negative bacteria and are known to contain biologically active proteins⁴²⁻⁴⁴. OMVs, composed of outer membrane and periplasmic constituents, act as secretory vehicles for proteins, lipids, and PAMPs⁴⁴⁻

⁴⁸. OMVs formed by bulging outer membrane and subsequent fission, are involved in multiple functions including establishing a colonization niche, virulence, transformation, biofilm formation, mediation of bacterial envelope stress, and modulating host defense and response ^{42,49-52}. As discussed above, mutation of *fliC* abolished the ability of GM-CSF to sensitize *P. aeruginosa* persister cells. We were curious if addition of external flagella along with OMVs and OMPs (outer membrane proteins) of wild-type *P. aeruginosa* PAO1 will show any change. We hypothesized that the OMVs can help deliver flagellins into PAO1 $\Delta fliC$ cells, and restore some of the activity of GM-CSF and antibiotic. However, co-treatment with 0.17 pM GM-CSF, isolated PAO1 flagella, and PAO1 OMVs sensitized $40.0 \pm 12.6\%$ ($p = 0.0011$) of persister cells from stationary culture of PAO1 $\Delta fliC$ to 5 $\mu\text{g}/\text{mL}$ tobramycin (Figure 5.7). The co-treatment reduced the viability of persister cells by $38.6 \pm 12.9\%$ ($p = 0.0016$) compared to GM-CSF treatment alone (Figure 5.7). Even though there was a significant increase in synergy between GM-CSF and antibiotic, when GM-CSF was added to PAO1 $\Delta fliC$ persister cells supplemented with flagella and OMVs, the change was rather minor. Thus the original flagellin seems to be necessary for GM-CSF binding.

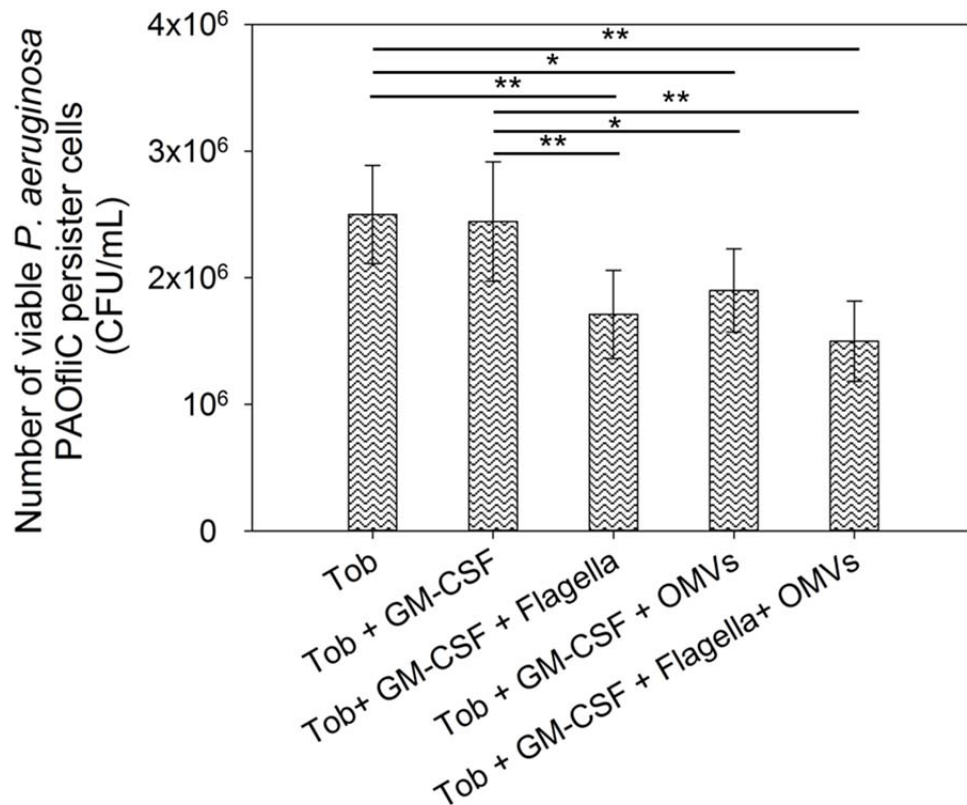


Figure 5.7 Additions of isolated flagella and OMVs partially restores the effects of GM-CSF on the persister cells of the *fliC* mutant PAO1 $\Delta fliC$. The flagella and OMVs were isolated from the wild-type *P. aeruginosa* PAO1. The persister cells of *fliC* mutant PAO1 $\Delta fliC$ were isolated from stationary phase normal cells with 200 $\mu\text{g/mL}$ ciprofloxacin for 3.5 h, and then treated with 5 $\mu\text{g/mL}$ tobramycin plus 0.17 pM GM-CSF (i), 0.17 pM GM-CSF and isolated flagella (ii), 0.17 pM GM-CSF and OMVs (iii) or 0.17 pM GM-CSF, isolated flagella, and OMVs (iv), for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of persister cells was determined by counting CFU. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

5.4.5 Inhibition of flagellar activity enabled GM-CSF to sensitize the normal cells of *P. aeruginosa* PAO1 to antibiotics

GM-CSF induced flagellar genes in PAO1 persister cells, but not normal cells (Chapter 4). Because normal cells have actively rotating flagella, we hypothesize that binding of FliC and GM-CSF is reduced on normal cells, and this inactivation of flagellar motility may enhance the activity of GM-CSF on normal cells of PAO1. To test this hypothesis, (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8), synthesized by a former Ren lab member Dr. Jiachuan Pan, was used to inhibit the flagellar activity of normal cells of *P. aeruginosa* PAO1. After performing swimming assay with different concentrations of BF8 (1, 5, 10, 20, and 40 $\mu\text{g}/\text{mL}$) on normal cells of *P. aeruginosa* PAO1 (Figure 5.8A), and testing the viability of cells after treatment with BF8 (1, 5, 10, and 20 $\mu\text{g}/\text{mL}$) (Figure 5.8B), it was found that up to 5 $\mu\text{g}/\text{mL}$ BF8 can successfully inhibit the swimming activity without any significant effect on cell viability.

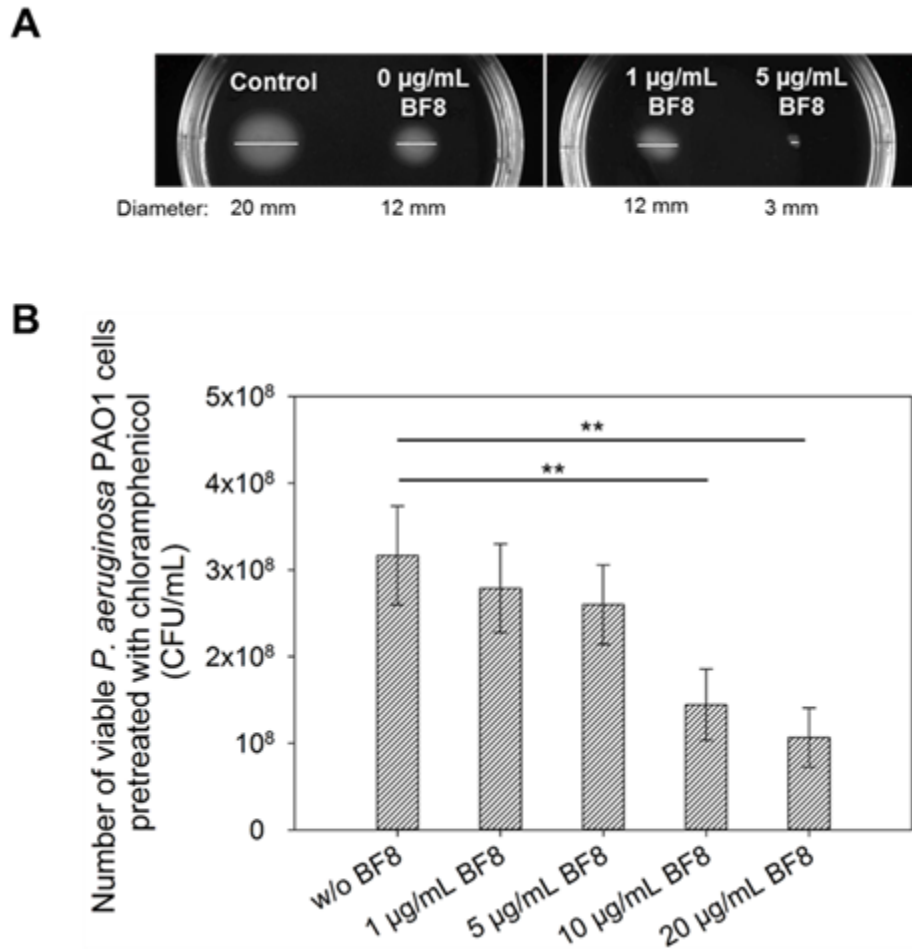


Figure 5.8 BF8 inhibited the motility of *P. aeruginosa* PAO1. (A) Swimming motility assay of *P. aeruginosa* PAO1 in 0.3% (w/v) agar plates. (B) Viability of total viable cells of *P. aeruginosa* PAO1 after treatment with 1, 5, 10, and 20 µg/mL BF8 for 3.5 h. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

Thus, to lower the activity of flagella, normal cells of *P. aeruginosa* PAO1 were pretreated with 5 µg/mL BF8 for 3.5 h, followed by treatment with GM-CSF and antibiotics. Interestingly, GM-CSF sensitized the BF8 pretreated normal cells of *P. aeruginosa* PAO1 to 5 µg/mL ciprofloxacin and tobramycin by 45.2±3.2% ($p = 0.0135$) and 40.3±10.8% ($p = 0.0042$) respectively (Figure 5.9A). No such effects were observed

without BF8 pretreatment, no significant sensitization ($p > 0.05$). To corroborate the results, a similar experiment was performed by pretreating the normal cells of *P. aeruginosa* PAO1 with 256 $\mu\text{g}/\text{mL}$ chloramphenicol (instead of BF8), an antibiotic that interacts with the 50S subunit of ribosome and interferes with the formation of peptide bonds, thus inhibiting protein synthesis^{53,54}. The concentration of chloramphenicol used was the MIC determined in our laboratory. The inhibition of protein synthesis leads to reduced flagellar activity. It was found that GM-CSF sensitized $49.0 \pm 18.0\%$ ($p = 0.0060$) and $43.2 \pm 7.5\%$ ($p = 0.0142$) of chloramphenicol-pretreated normal cells of *P. aeruginosa* PAO1 to 5 $\mu\text{g}/\text{mL}$ ciprofloxacin and tobramycin respectively (Figure 5.9B), compared to the cells without pretreatment. These results indicate that the interaction between GM-CSF and bacterial flagella is important for its activity in synergistic killing of bacterial cells with antibiotics. It also suggests that GM-CSF is more effective in binding to resting flagella.

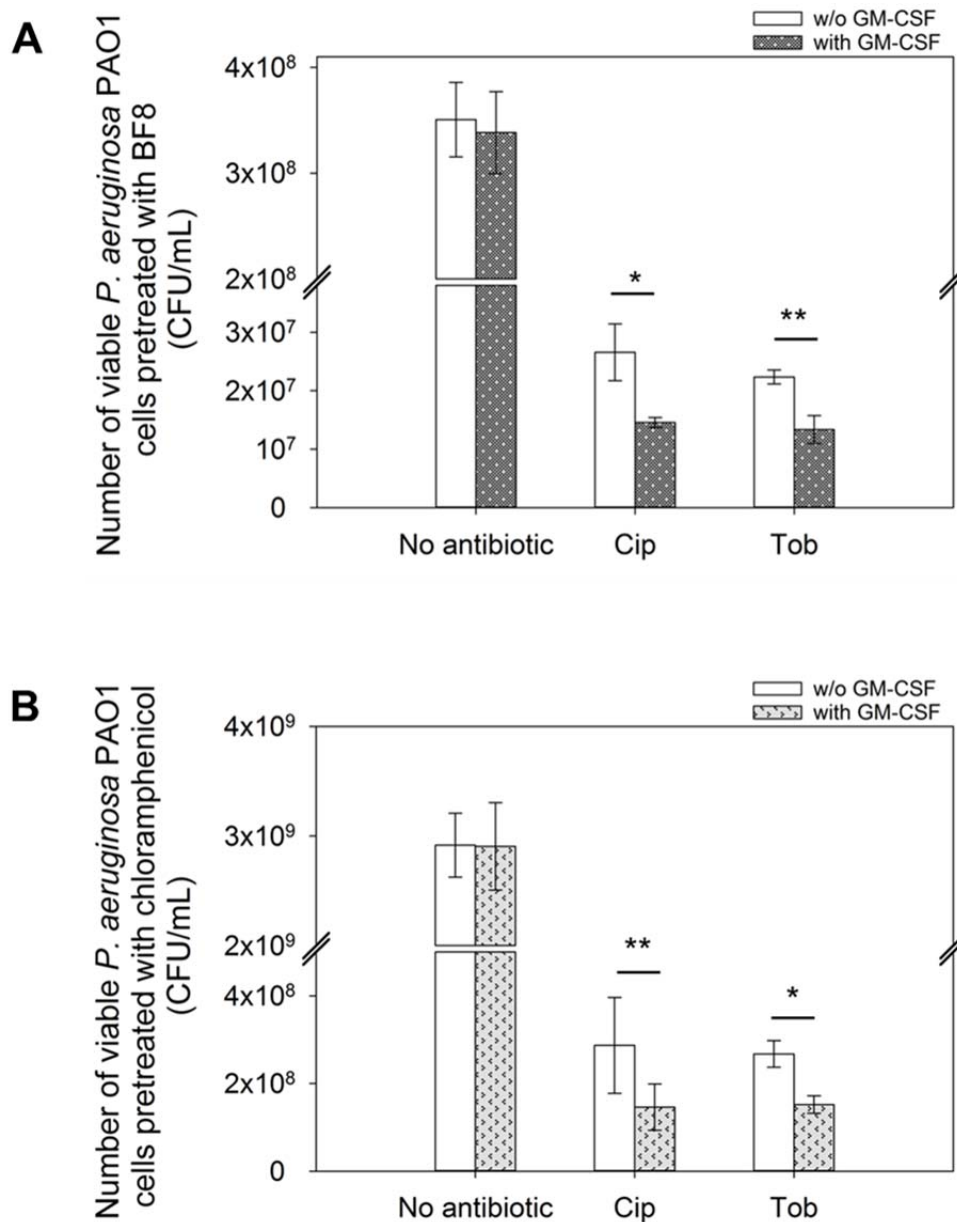


Figure 5.9 GM-CSF sensitized the normal cells of *P. aeruginosa* PAO1 isolated from stationary phase cultures when flagellar activity was inhibited. Normal cells of *P. aeruginosa* PAO1 were isolated from stationary phase cultures and pretreated with 5 $\mu\text{g}/\text{mL}$ BF8 (A), or 256 $\mu\text{g}/\text{mL}$ (B) for 3.5 h. The pretreated cells were then treated with 0.17 μM GM-CSF in the absence or presence of 5 $\mu\text{g}/\text{mL}$ ciprofloxacin or 5 $\mu\text{g}/\text{mL}$ tobramycin for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of PAO1 cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

The flagella based motility is controlled by a complex ion driven motor situated in the bacterial cell envelope⁵⁵⁻⁵⁷. The motor allows the clockwise and anticlockwise rotation of the flagella⁵⁵⁻⁵⁸. *P. aeruginosa* PAO1 contains dual sets of *motA* and *motB* genes (*motAB*), *motC* and *motD* (*motCD*), and an individual *motY* gene, that are involved in the motor functions^{59,60}. We found that GM-CSF was able to sensitize the normal cells in stationary phase cultures of motor mutants *motA::lacZ* and *motD::lacZ* to antibiotics. For example, 0.17 pM GM-CSF sensitized 29.6±6.6% ($p = 0.0063$) and 28.3±13.8% ($p = 0.0475$) of normal cells of *motA::lacZ* to 5 µg/mL ciprofloxacin and tobramycin respectively (Figure 5.10). The other motor mutant *motD::lacZ* also displayed a similar trend with 36.5±5.1% ($p = 0.0076$) and 29.3±9.6% ($p = 0.0169$) of persister cells sensitized by 0.17 pM GM-CSF to 5 µg/mL ciprofloxacin and tobramycin respectively (Figure 5.10). The strains lacking *motA* or *motD* have flagella that are unable to function as efficiently as the wild-type *P. aeruginosa* PAO1. Even though GM-CSF sensitized the normal cells of *motA::lacZ* and *motA::lacZ* significantly, the killing was not strong. This can be explained by the results reported by Doyle *et al.*⁵⁹ which show that mutation in *motA* or *motD* affects swimming motility, but does not abolish it. Kato *et al.*⁶¹ also reported that the insertion mutants of *motA* and *motB* were motile. Collectively, these data further indicate that GM-CSF binds to resting flagella more effectively.

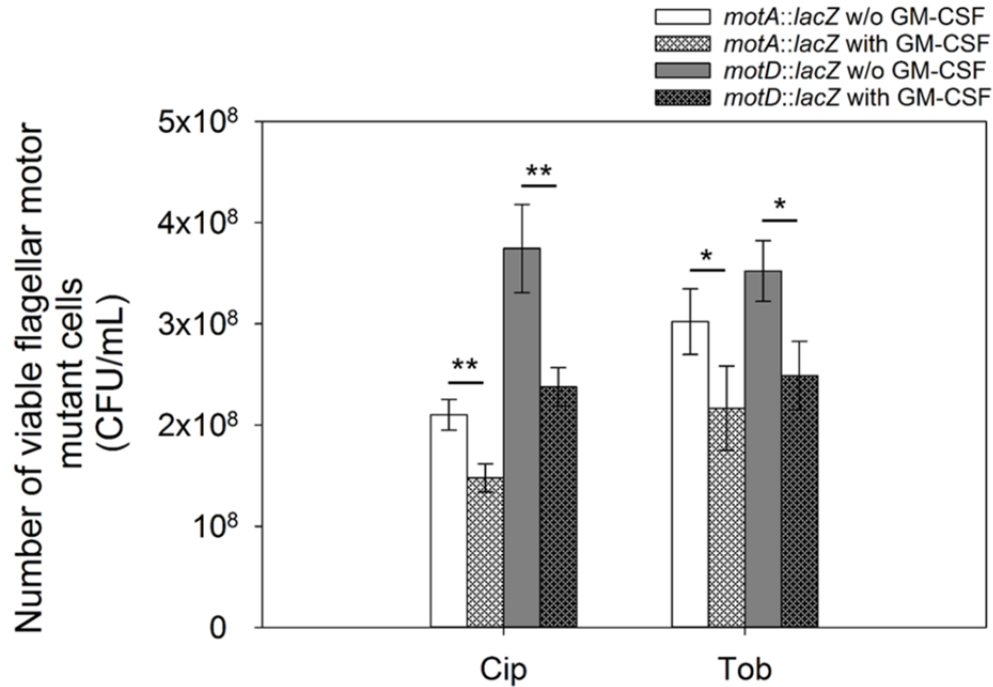


Figure 5.10 Reduction in flagellar motor activity due to mutations in *motA* and *motD* allows GM-CSF to sensitize the normal cells of *P. aeruginosa* to antibiotics. The normal cells of motor mutants *motA::lacZ* and *motD::lacZ* were isolated from stationary phase cultures and then treated with GM-CSF plus 5 µg/mL ciprofloxacin (A) or 5 µg/mL tobramycin (B) for 3.5 h. The amount of BSA (0.1%) was adjusted to be the same for all samples. Following the treatment, the viability of normal cells was determined by counting CFU. Cip: ciprofloxacin. Tob: tobramycin. The samples were tested in triplicate (n=3). Error bars represent SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Tukey test.

5.5 Discussion

Human cytokines play a critical role in protection against bacterial infections and are actively involved in proinflammatory/anti-inflammatory symptoms in the host during infections like fever, swelling, fatigue, etc.⁶²⁻⁶⁵. In Chapter 3, I have shown that recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) can significantly reduce the persistence of *P. aeruginosa* following antibiotic treatments. To initiate an infection, *P. aeruginosa* needs to break the first-line of host defense and

colonize host tissues with its external appendages, such as type IV pili, fimbria, and flagella, along with non-pilus adhesins⁶⁶⁻⁷⁰. Besides other intracellular virulence factors like OMPs and lipopolysaccharide, several extracellular virulence factors including secreted toxins, proteases, hemolysins, and enzymes are involved in the pathogenesis of *P. aeruginosa*^{71,72}. In addition, special spherical structures known as outer membrane vesicles (OMVs), with a diameter around 20-250 nm are released from outer membranes and serve as virulence mediators to carry toxins, proteases, and other proinflammatory molecules like flagellin and peptidoglycan to defend ecological niche against competing bacterial species and leads to release of cytokines which invokes inflammatory host response^{50,73-76}.

The DNA microarray studies on the persister and normal cells of wild-type *P. aeruginosa* PAO1 after GM-CSF treatment indicated an induction of flagella-associated genes in persister cells, whereas the normal cells displayed little or no change in these genes (Table 5.1). Since GM-CSF was able to sensitize only the persister cells to antibiotics but not the normal cells, the difference in flagella in these populations is of interest. Bacterial adhesion mediated by flagella is a critical step of switch from motile to sessile state⁸.

Table 5.1 Expression of some flagella associated genes in normal and persister cells of *P. aeruginosa* after treatment with GM-CSF.

Gene	Expression fold change		Functions
	Normal cells	Persister cells	
<i>flgB</i>	-1.3	+2.1	Basal body rod
<i>flgC</i>	-1.7	+1.7	Basal body rod
<i>flgD</i>	NC	+1.9	Hook cap scaffold
<i>flgE</i>	-1.4	+2.0	Hook
<i>flgF</i>	-1.4	+2.3	Basal body rod
<i>flgG</i>	-1.1	+2.0	Basal body rod
<i>flgH</i>	-0.1	+2.6	Basal body L ring
<i>flgI</i>	NC	+1.7	Basal body P ring
<i>flgJ</i>	NC	+1.7	Flagellum specific muramidase
<i>flgK</i>	NC	+2.0	Hook-filament junctional protein
<i>flgL</i>	+1.3	+1.7	F-pyocin bacteriophage
<i>fliA</i>	-1.2	+2.0	σ_{28} sigma factor
<i>fliC</i>	-0.1	+1.7	Flagellin
<i>fliD</i>	-0.3	+1.7	Filament cap, mucin adhesin
<i>fliG</i>	+0.1	+1.5	Motor/switch, mounted onto MS ring,
<i>fliH</i>	NC	+2.6	Negative regulator of FliI
<i>fliM</i>	-1.2	+1.8	Motor/switch
<i>fliN</i>	-0.1	+2.3	Motor/switch
<i>cheY</i>	NC	+2.1	Switch regulator
<i>cheZ</i>	NC	+2.2	CheY phosphatase
<i>fleN</i>	-1.7	+1.8	Flagellar number regulator

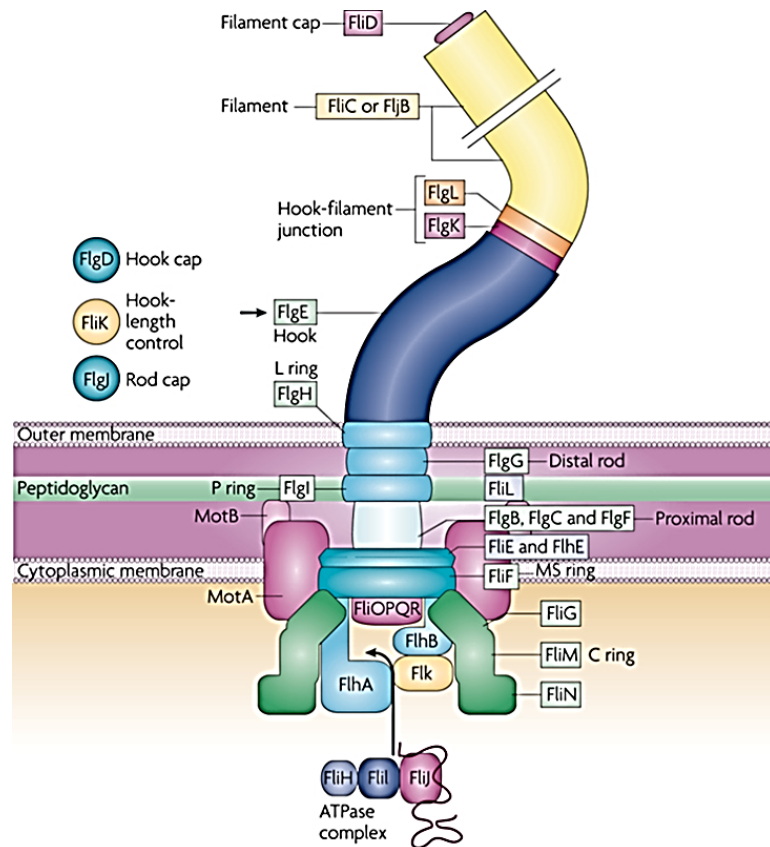


Figure 5.11 Structure of a typical Gram-negative bacterial flagellum and its components. Reproduced from Chevance *et al.*⁷⁷ with permission. Copyright, 2008, Nature Reviews Microbiology.

Figure 5.11 shows a typical bacterial flagellum. In Gram-negative bacteria, the flagellar structure comprises of a basal body which consists of four rings, L, P, MS and C¹. The flagellar genes induced by GM-CSF are involved in flagellar basal body proteins (FlgB, FlgC, FlgF and FlgG), flagellar basal body rod modification protein (FlgD), flagellar basal body L-ring protein (FlgH), flagellar basal body P-ring protein (FlgI), flagellar hook protein (FlgE), flagellar hook associated protein (FlgK) and flagellar rod assembly protein (FlgJ)⁷⁸. The induced genes *fliACDGMN* and *cheYZ* are also associated with the flagellar activities. FliA is a RNA polymerase sigma factor for flagellar operon, induced by 2-fold in persister cells; while *fliC* was also upregulated by 1.7 fold in

persister cells but not changed in normal cells. FliC is a b-type flagellin for *P. aeruginosa* PAO1, which polymerizes to form the filaments of flagella^{41,79}. Flagellin is the structural protein that forms helical chains around the hollow core of the flagellar filament and acts as a virulence factor that elicits host innate immune response^{18,80}. Innate immune response to pathogen-associated molecular patterns (PAMPs) plays a significant role in early defense against pathogens; and the *P. aeruginosa* flagellin is one of the main active PAMPs, which means flagellin can invoke host immune response⁸¹. FliD forms the flagellar capping protein⁸². FliG, FliM and FliN form a motor switch complex, located at the base of the basal body⁸³. The chemotaxis proteins CheY and CheZ interact with this complex, determining the clockwise/counterclockwise direction of the flagellar rotation⁸³. The clockwise rotation of flagellar motor is a result of binding of phosphorylated CheY (CheY-P) to FliM, causing tumbling of the cell^{83,84}. In the absence or at low concentrations of CheY-P, the flagellum typically continues with counterclockwise direction of rotation^{83,84}. The induction of the genes associated with flagellar structure, flagellar motor switch and chemotaxis suggests that GM-CSF may induce cellular motility of persister cells, allowing them to escape the attack of immune cells. Based on previous studies, the genes *flgBCDEFGHIJK*, *fliACDGMN* and *cheYZ* were observed to be down-regulated when *P. aeruginosa* PAO1 was treated with 1 µg/mL ciprofloxacin and 10% sputum from cystic fibrosis patients^{85,86}. The induction of flagellar motility suggests that GM-CSF may disturb the stress response of *P. aeruginosa* and thus sensitize persister cells to antibiotics and other host immune factors.

The genes involved in energy production and flagella synthesis have been previously shown to be downregulated in persister cells compared to exponentially

growing cells⁸⁷. The ability of GM-CSF to induce expression of 18 motility and chemotaxis related genes in persister cells possibly suggests that this cytokine may activate flagellar synthesis in otherwise dormant persister cells. This might lead to partial “wake up” and increase certain cellular activities, thus reducing the antibiotic tolerance. Moreover, in this study we also showed that FliC is required for GM-CSF binding based on co-IP results and the mutant study. Each flagellum of *P. aeruginosa* contains 20,000 units of FliC⁸⁸. There was a drastic decrease in the activity of GM-CSF *fliC* mutant of *P. aeruginosa* PAO1. The finding was further confirmed when the mutation in PAO1 Δ *fliC* was complemented with plasmid-borne *fliC* gene. Consistently, when the persister cells of the wild-type *P. aeruginosa* PAO1 were pretreated with anti-FliC, synergistic effects between GM-CSF and antibiotics in persister killing was reduced significantly. These complementary experiments demonstrated that FliC is required for the activity of GM-CSF in persister control.

Since the normal cells also have flagella but were not sensitized to antibiotics by GM-CSF, we hypothesized that the resting flagella are targeted by GM-CSF. Indeed, when the normal cells pretreated with chloramphenicol (an antibiotic that inhibits protein synthesis) or BF8 (an inhibitor of quorum sensing and motility), GM-CSF sensitized a significant portion of normal cells to antibiotics, although the activity was lower than that observed for persister cells. It will be interesting to study if any subtle changes exist in flagella (e.g. protein modifications) between normal and persister cells. Bacterial flagellar motility enables the host phagocytes to bind and engulf *P. aeruginosa*⁸⁹. The loss in the bacterial motility observed in clinical isolates from chronic infections due to elevated persistence help the bacteria to evade identification and ingestion by phagocytes both *in*

vitro and *in vivo*⁸⁹. The suppressed flagellar motility helps bacteria to escape phagocytosis. The results from our study suggest that GM-CSF, which is a cytokine produced by phagocytes, may help kill persister cells by inducing flagellar expression and triggering them to revert to antibiotic sensitive normal state. It will be interesting to test if similar synergy exists with host produced antimicrobial peptides.

Bacterial flagellum has a basal body, which consists of a motor embedded in the cell envelope comprising an outer membrane ring (L), a periplasmic ring (P), an internal membrane ring (MS), a cytoplasmic ring (C), and a rod that traverses the periplasmic space^{88,90-92}. The basal body also consist of a flagellar motor with two major components named as stator and rotor. The stator made from proteins MotA and MotB, is attached to the peptidoglycan and remains stationary. However, the rotor which consists of FliG protein attached to MS ring with Mot proteins, is involved in the process of torque generation^{88,93-96}. Additionally, a motor switch is also present as a part of basal body structure, consisting of proteins FliG, FliM, and FliN^{56,97}. The switch is responsible for the counterclockwise and clockwise direction of the motor⁹⁸. FliG is primarily involved in torque generation, while FliM and FliN interact with chemotaxis proteins^{99,100}. The transmembrane protein MotA-MotB complex conducts protons from periplasm to cytoplasm for the proton gradient driven motor^{56,88,101,102}. The motor function of wild-type *P. aeruginosa* PAO1 is controlled by *motA*, *motB*, *motC*, *motD*, and *motY* genes^{59,60}. We tested the normal cells of *motA* and *motD* mutant strains of PAO1 with GM-CSF and antibiotics. GM-CSF was able to sensitize 36.5±5.1% ($p = 0.0076$) of persister cells of *motD::lacZ* to ciprofloxacin. Doyle *et al.*⁵⁹ demonstrated that the flagellum of *P. aeruginosa* PAO1 has a complex configuration, and the loss of a function in a motor

protein can be compensated by other existing functional motor proteins. For example, it was reported that in the absence of MotA/MotB, the motility function is taken over by MotC/MotD and vice versa⁵⁹. This complex flagellar motor behavior may explain why GM-CSF could not effectively bind to the flagella of *motA::lacZ* and *motD::lacZ*, which happen to still retain motility function.

GM-CSF-FliC binding is not specific to just b-type flagellin expressed by *P. aeruginosa* PAO1. The cross-linking and cell viability tests on *P. aeruginosa* PAK showed that GM-CSF can also effectively bind to a-type flagellin and exhibit synergistic effects with antibiotics in persister killing significantly. In fact, the heterogeneous a-type flagellin appeared to have a stronger binding to GM-CSF compared to the homogenous b-type flagellin according to the cross-linking experiment. However, the synergy between GM-CSF and antibiotics found to be very similar in *P. aeruginosa* PAO1 and PAK. Thus, the distinct glycosylation sites and number of genes involved did not affect the GM-CSF activity significantly. Since FliC appeared to play a significant role in GM-CSF activity, we introduced isolated flagella of the wild-type *P. aeruginosa* PAO1 and outer membrane vesicles (OMVs) to the *fliC* mutant PAO1 $\Delta fliC$, to see if any uptake of external flagellin by PAO1 $\Delta fliC$ may restore the activity of GM-CSF. Outer membrane vesicle production by *P. aeruginosa* is an important response when this bacterium is exposed to environmental stresses⁴⁸. OMVs are carriers of PAMPs such as LPS and flagellin, outer membrane proteins, and virulence factors⁴⁸. The isolated flagella and OMVs were able to partially restore the activity of GM-CSF in killing persister cells of PAO1 $\Delta fliC$ with antibiotics. However, the change was relatively small. It is possible that externally added flagellin did not affect the intracellular target(s) or pathway(s), as it did

by the original flagellin (FliC) in periplasm, or there might be other pathways affected by binding of GM-CSF to the native flagella. The exact mechanism by which GM-CCSF binds to FliC sensitizes persister cells to antibiotics remains unknown and needs further investigation.

Besides OMVs, rhamnolipids may also help deliver FliC. Rhamnolipids are biosurfactants composed of mono or di-rhamnose linked to 3-hydroxy-fatty acids of different length produced by *P. aeruginosa* during late-exponential and stationary growth phases¹⁰³⁻¹⁰⁶. These glycolipid surface-active molecules possess antimicrobial properties and show activity against a wide range of microbes including Gram-negative and Gram-positive bacteria, fungal species, and viruses¹⁰⁷⁻¹¹¹. Gerstel *et al.*¹¹² reported that rhamnolipids induce shedding of flagellin from flagella of *P. aeruginosa*, leading to recognition of flagellin by epithelial cells and provocation of host immune response. It would be interesting to treat wild-type *P. aeruginosa* PAO1 with rhamnolipids, GM-CSF, and antibiotics together to investigate if any possible rhamnolipid induced shedding of flagellin and consequent changes might occur in the GM-CSF related effects, GM-CSF-flagellin binding, and antibiotic susceptibility of treated cells. Similar experiment can be performed on the *fliC* mutant PAO1 $\Delta fliC$ with rhamnolipids, GM-CSF, antibiotics, and isolated flagella for *P. aeruginosa* PAO1. By exploring new ways to allow GM-CSF to bind efficiently to both persister and normal cells of *P. aeruginosa*, we can potentially improve the activity of GM-CSF in bacterial killing.

5.6 Conclusions

Flagella are important for bacteria to establish acute infections and are thus detected by the host for immune response. Based on co-IP and Western analyses, we found an interaction between the host cytokine GM-CSF and bacterial FliC. We demonstrated that when the *fliC* gene is deleted, GM-CSF failed to sensitize the persister cells to antibiotics. Consistently, complementation of *fliC* restored the activity. We further demonstrated that resting flagella are probably necessary for the activity of GM-CSF. When the flagellar activity was repressed in otherwise motile normal cells of *P. aeruginosa* PAO1, we observed an increase in the activity of GM-CSF. Further understanding of how GM-CSF-flagellin interaction leads to killing of persister cells by antibiotics will help develop novel control strategies to combat antibiotic-tolerant infections.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Conclusions

Bacterial persistence poses significant challenges to the control of bacterial infections, which are responsible for several health and economic repercussions. Understanding bacterial pathogenesis and the host immune response to the bacterial invasion can potentially provide novel strategies to control bacterial persistence. The host immune system produces cytokines, which are essential signaling molecules that mediate inflammatory response during the invasion of pathogens. Numerous studies have been conducted to study the cytokine networks activated in response to various pathogens and the role of individual cytokines in host response. However, there have been few studies on the direct interaction between cytokines and bacteria, and no study has been conducted to investigate the direct interaction between cytokines and persister cells. Here, for the first time, we show an interaction between bacterial persister cells and granulocyte macrophage colony-stimulating factor (GM-CSF), a cytokine secreted mainly by macrophages.

Pseudomonas aeruginosa, an opportunistic Gram-negative bacterial pathogen is known to exhibit multifaceted mechanisms for acquiring persistence and cause chronic infections. GM-CSF was found to sensitize persister cells of *P. aeruginosa* PAO1 to different antibiotics. GM-CSF enhanced antibiotic susceptibility of *P. aeruginosa* PAO1 persister cells isolated from both exponential and stationary phase cultures to antibiotics at different concentrations. When treated with GM-CSF and appropriate extracellular matrix degrading enzymes, biofilm cells of *P. aeruginosa* were also sensitized to antibiotics. Interestingly, the persister cells of non-pathogenic strain *E. coli* K12 did not exhibit the same response to GM-CSF.

In order to understand this phenomenon, we performed DNA microarray studies and qPCR analyses. It was evident from the results that genes related to pyocins were specifically induced in persister cells of *P. aeruginosa* PAO1 after GM-CSF treatment, while normal cells displayed no such changes. Consistent with the induction of pyocin genes, the supernatant collected from the persister cells treated with GM-CSF showed killing activities against the R2-pyocin sensitive strain *P. aeruginosa* PAK. The DNA microarray also indicated an induction in flagellar genes in persister cells, which was not observed in normal cells. We observed that GM-CSF activity reduced on persister cells in the absence of *fliC* gene, and the activity was restored when *fliC* was complemented. The presence of interaction between GM-CSF and flagella was verified by Co-Immunoprecipitation, and crosslinking experiments.

Overall, this study revealed interactions between the pathogenic bacterium *P. aeruginosa* and the human cytokine GM-CSF. Such effects of a cytokine on bacterial persister cells have not been reported previously. The underlying mechanism deserves further study. Figure 6.1 summarizes the major findings of this thesis.

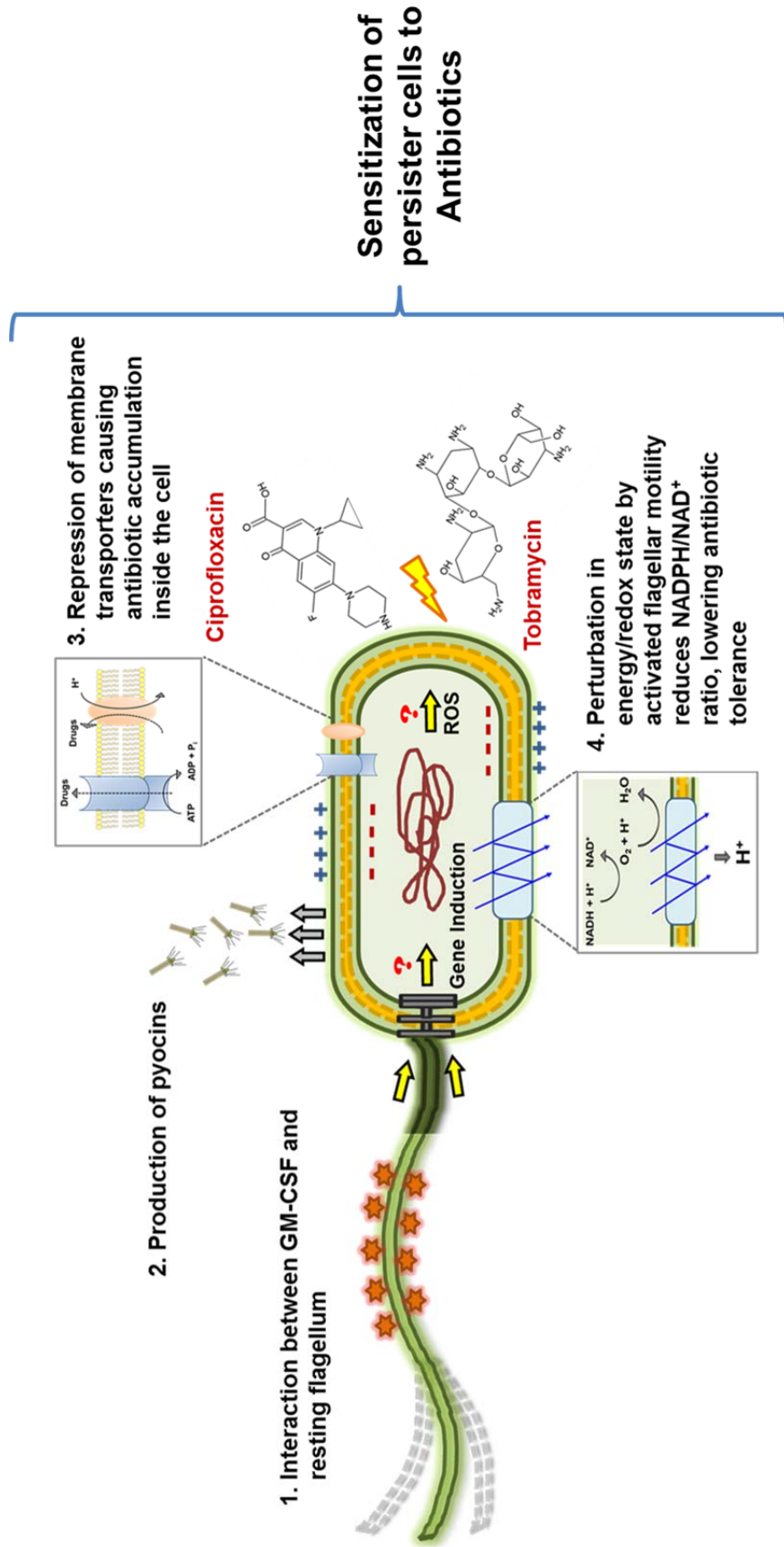


Figure 6.1 A model to explain the observed effects of GM-CSF on *P. aeruginosa* persister cells. The possible induction of pyocin production and inactivation of transporters are supported by DNA microarray data. Further tests are required to validate this model at the protein and cellular levels.

6.2 Recommendations for future work

6.2.1 Effects of GM-CSF on other bacterial species

Our study was primarily focused on the effect of GM-CSF on *P. aeruginosa* PAO1. It would be interesting to study some other Gram-negative pathogens, and also Gram-positive pathogens. Since GM-CSF was found to interact with flagellin, testing if GM-CSF has other binding target in non-flagellated bacterial species will help us understand the spectrum of targeted bacteria. For example, a Gram-negative opportunistic pathogen *Acinetobacter baumannii* is responsible for infections including bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection. It is a good candidate for testing the effects of GM-CSF on non-flagellated Gram-negative opportunistic pathogen¹. *A. baumannii* lacks flagellar structure and is a non-motile bacterium². *Klebsiella pneumoniae*, which is also a Gram-negative pathogenic bacterium lacking flagella and motility, can be tested for GM-CSF effects as well³. Other Gram-negative pathogens such as *E. coli* O157:H7 and *Enterobacter spp.*, displaying peritrichous flagella can give more insight on how GM-CSF can interact with bacterial strains possessing multiple flagella around the entire bacterial surface^{4,5}. Studying the effects of GM-CSF on Gram-positive pathogenic bacteria, such as *Listeria monocytogenes* and *Clostridium difficile* lacking L and P basal body rings in their flagellar structure, may demonstrate differences or similarities between Gram-negative and Gram-positive bacteria in interactions with GM-CSF^{6,7}.

6.2.2 Role of GM-CSF during chronic infections

We found that, in the presence of appropriate enzymes that degrade exopolysaccharide biofilm matrix of *P. aeruginosa* PAO1 and PDO300, GM-CSF is capable of sensitizing

the biofilm cells to antibiotics. However, it would be beneficial to reproduce these effects in cystic fibrosis (CF) airway conditions, which is due to cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction and mucus hyperproduction⁸. The infected CF airways have a complex configuration due to abnormalities caused by altered mucin and lipid composition, increased content of proinflammatory cytokines, impaired mucociliary clearance, dehydrated and infected mucus⁹. Growing *P. aeruginosa* strains in normal and CF conditions with varying mucus concentrations (2.5% mucus for normal condition; 8.0% mucus for CF condition) in the medium as described by Matsui *et al.*¹⁰, and testing clinical isolates for GM-CSF activity can bring us closer to studying animal models¹¹.

6.2.3 Therapies with multiple cytokines

During bacterial invasions, multiple cytokines participate in a cascade of events that lead to host inflammatory response¹². Evaluating the role of individual cytokines, specifically the ones that have stronger influence in directing the inflammatory response, will reveal if there are other cytokines that have direct interaction with bacterial persister cells. If an effective role of cytokines on persistent infections is defined, a multi-cytokine therapy can potentially lead to an advanced control of bacterial persistence.

6.2.4 Utilization of STORM as a powerful microscopic tool to visualize GM-CSF binding.

Stochastic optical reconstruction microscopy (STORM), which is still a relatively new technology, has the ability to determine ultrastructural features of bacterial cells at nanoscopic scale, and provide detailed information via imaging¹³. This high-resolution optical microscopy, allows us to view the position of fluorophores with high accuracy for immunofluorescence imaging¹³. Ideal fluorophores for STORM are required to exhibit brightness with minimal photobleaching in thiol-containing buffers and have a high rate of photoswitching cycles¹⁴. If GM-CSF labeled with a detectable fluorophore can be observed at the binding position on flagella, it will provide the most direct evidence of GM-CSF-flagellin interactions.

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APPENDICES

EXPERIMENTAL PROTOCOLS

Protocol I

Synergistic effects of GM-CSF and antibiotics on planktonic cells.

1. Grow 50 mL overnight culture (*P. aeruginosa* PAO1, PDO300, or *E. coli* K12) in LB medium.
2. Check OD₆₀₀ and wash the cells twice by centrifugation at 8,000 rpm for 10 min.
3. Separate the washed culture in equal volumes in two tubes. Select a tube for isolation of persister cells, and keep another tube for testing the effects of GM-CSF and antibiotics on the total viable cell population.
4. Add 200 µg/mL ciprofloxacin and treat for 3.5 h at 37°C, with shaking at 200 rpm.
5. Wash the antibiotic from isolated persisters thrice by centrifugation at 8,000 rpm for 10 min at 4°C.
6. For both the normal and persister cells population, prepare samples for control and GM-CSF treatment samples (triplicates). Treat the samples without antibiotics for 1 h at 37°C, with shaking at 200 rpm. For the samples with antibiotics, treat for 3.5 h at 37°C, with shaking at 200 rpm.
7. Wash the samples thrice by centrifugation at 13,200 rpm for 2 min.
8. Perform drop plate method for CFU quantification on 1.5% agar plates.

Protocol II

Synergistic effects of GM-CSF and antibiotics on biofilm cells.

1. Grow 25 mL overnight culture (*P. aeruginosa* PAO1, PDO300, or *E. coli* K12) in LB medium.
2. Sterilize 316L stainless steel coupons (1.75 cm × 1 cm, 0.05 cm thick) by soaking in ethanol for 30 min and drying at 50°C for 15 min.
3. Place the sterilized coupons in empty petri dishes and subculture to an OD₆₀₀ of 0.01 containing 20 mL LB medium.
4. Grow the 4 h or 24 h biofilms by incubating at 37°C for 4 h or 24 h without shaking.
5. Wash the coupons gently by dipping in PBS and place in 12-well plates. Prepare control and treatment samples (triplicates) to a final volume of 2 mL PBS, and incubate at 37°C for 3.5 h, without shaking.
6. Wash the coupons gently by dipping in PBS, and transfer the coupons in tubes individually with 3 mL PBS.
7. Sonicate the coupons (B200, Sinosonic Industrial Co., Ltd., Taiwan) for 4 min to disperse biofilm cells from the coupons.
8. Vortex for 1 min, and perform drop plate method to quantify CFU on 1.5% agar plates.

Protocol III

RNA isolation from normal and persister cells after GM-CSF treatment.

Harvesting cells.

1. Grow 60 mL overnight culture of *P. aeruginosa* PAO1 in LB medium.
2. Wash the cells twice by centrifuging at 8,000 rpm for 10 min with PBS.
3. Add 200 µg/mL ciprofloxacin and treat the washed cells for 3.5 h at 37°C, with shaking at 200 rpm for isolating the persister cells.
4. Wash the persister cells thrice by centrifuging at 8,000 rpm for 10 min at 4°C with PBS.
5. Dilute the persister culture 5 times by PBS and divide the diluted culture into 8 tubes. Each tube contains 37.5 mL diluted persisters (4 tubes for control and 4 tubes for treatment).
6. Treat the diluted persister culture with different conditions (0.1% BSA for control samples, and 0.17 pM GM-CSF for treatment samples)
7. Pre cool all the tubes and centrifuges around 0-4°C.
8. Centrifuge the samples at 10,000 rpm for 2 min at 2°C and decant supernatant
9. Flash-freeze the cell pellets in dry ice and store at -80°C until used.

RNA isolation using RNeasy Mini Kit (QIAGEN, Valencia, CA).

▪ Preparation

1. Add 200 µL Zirconia/Silica beads to bead beater tube & cool on ice.
2. RLT Buffer: 10 µL of 2-Mercaptoethanol (βME) per 1 mL RLT (2 mL/sample)

3. RPE Buffer: 8 mL EtOH per 2 mL RPE (4 mL/sample).
4. DNase mix: 45 μ L DNase I stock per 315 μ L RDD Buffer (360 μ L/2 samples).

▪ **Isolation**

1. Add 450 μ L RLT buffer to all the 8 tubes with pellets. After vortexing, combine 2 tubes of control and 2 tubes of treatment. In total, there will be 4 tubes with 900 μ L RLT mixed with cells (2 control samples and 2 for treatment samples).
2. Transfer 900 μ L of RLT with cells in bead beater tubes with zirconia/silica beads and beat the samples for 60s (set timer 6) at speed 48 (~5000 rpm).
3. Centrifuge the samples at 13,000 rpm for 15 s at 4°C. (Keep all centrifugation steps at these settings unless noted)
4. Collect supernatant, and add 445 μ L EtOH.
5. Load 700 μ L of sample in RNeasy column (QIAGEN), and centrifuge
6. Add 350 μ L RW1, centrifuge twice.
7. Add 180 μ L DNase I incubation mix directly onto membrane
8. Incubate at room temperature for 30 min.
9. Add 350 μ L RW1, and centrifuge twice.
10. Add 500 μ L RPE, centrifuge thrice.
11. Add 500 μ L RPE, centrifuge at 13,000 rpm for 2 min
12. Replace collection tube, and centrifuge at 13,000 rpm for 1 min.
13. Place column in a fresh 1.5 collection tube
14. Add 40 μ L RNase-free water, centrifuge for 1 min. Collect the flow through and repeat the step once.

- **Quantification**

1. Measure optical density at 260 nm (OD_{260}) and 280 nm (OD_{280}), using TE as the background.

Yield: OD_{260} of 1.0 = 40 $\mu\text{g/mL}$.

Ratio of OD_{260}/OD_{280} should be higher than 2.0.

2. Check the samples by running them on a 1.4% agarose gel. The samples should have two clear bands (23S at 3.1 kb, 16S at 1.5 kb). Presence of smear patterns at low molecular range indicates RNA contamination.

Protocol IV

Synthesis of cDNA and quantitative polymerase chain reaction (qPCR).

- **cDNA synthesis using Script™ cDNA Synthesis Kit (Biorad, Hercules, CA).**

Components:

mRNA	500 ng
5x iScript Reaction Mix	8 µL
iScript™ Reverse Transcriptase	2 µL
Nuclease-free water	Balance to a final volume of 40 µL

Reaction protocol:

5 min at 25°C.

30 min at 42 °C.

5 min at 85 °C.

Hold at 4 °C.

Quantification:

cDNA yield (µg/mL): $OD_{260} \times 50 \mu\text{g/mL} \times \text{dilution factor}$.

- **qPCR using iTaq™ Universal SYBR Green Supermix (Biorad, Hercules, CA).**

Components

iTaq™ Universal SYBR Green Supermix (2×)	10 µL
Forward and reverse primers (from 10 mM)	0.5 µL each
cDNA template (200 ng/reaction)	5 µL
de-ionized water	4 µL

qPCR reaction protocol:

Heat activation at 95°C for 15 s.

Cycle 1-40:

Denaturation at 95°C for 15 s.

Annealing/extension at 60°C for 1 min.

Melting curve:

95°C for 15 s.

50°C for 30 s.

Hold for 20 min.

95°C for 15 s.

Curriculum Vitae

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EDUCATION

Doctor of Philosophy in Chemical Engineering (*Anticipated*) (August, 2010 – July, 2015)

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Dissertation Topic: Controlling *Pseudomonas aeruginosa* persister cells by Granulocyte Macrophage Colony-Stimulating Factor.

Master of Science in Chemical Engineering (August, 2008 – May, 2010)

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Project Title: Characterization of bacterial species isolated from a bio-filter system for formaldehyde removal in indoor air.

Bachelor of Technology (B. Tech) in Chemical Engineering (August, 2004 – May, 2008)

Nirma Institute of Technology, Nirma University, Ahmedabad, Gujarat, India.

PEER-REVIEWED PUBLICATIONS

- Xiangyu Yao, **Geetika S. Choudhary**, Flaviyana Jerome Irudayanathan, Shikha Nangia, & Dacheng Ren (2015). Human Granulocyte Macrophage Colony-Stimulating Factor Sensitizes *Pseudomonas aeruginosa* Persister Cells to Antibiotics through Interaction with FliC (in preparation).
- **Geetika S. Choudhary**, Xiangyu Yao, Jing Wang, Rebecca A. Bader, & Dacheng Ren (2015). Human Granulocyte Macrophage Colony-Stimulating Factor Enhances Antibiotic Susceptibility of *Pseudomonas aeruginosa* Persister Cells (in submission).
- Stephen Desalvo, Yating Liu, **Geetika Choudhary**, Dacheng Ren, Shikha Nangia, Radhakrishna Sureshkumar (2015). Signaling Factor Interactions with Polysaccharide Aggregates of Bacterial Biofilms. *Langmuir*, 31(6), 1958-1966.
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PATENT APPLICATIONS

- Ren D, **Choudhary G**, and Yao X, 2013. Controlling bacterial persister cells with host immune factor, WO Patent, 2013173828 A1. (Patent Application).

CONFERENCE PRESENTATIONS

- “Controlling bacterial persister cells by Granulocyte Macrophage Colony-Stimulating Factor.” - Poster Presentation at the **Nunan Research Day**, Syracuse, NY. - (April 6, 2015).
- “Controlling bacterial persister cells by Granulocyte Macrophage Colony-Stimulating Factor.” - Poster Presentation at the **112nd ASM General Meeting**, San Francisco, CA. - (June 16-19, 2012).
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RESEARCH EXPERIENCE

Graduate Research Assistant at Syracuse University, Syracuse, NY (August, 2010 - July, 2015)

Thesis title: Controlling *Pseudomonas aeruginosa* persister cells by Granulocyte Macrophage Colony-Stimulating Factor.

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- Investigated the interaction between host immune factors and pathogenic/non-pathogenic bacteria.
- Developed a method to sensitize antibiotic tolerant bacterial cells to antibiotics, and possibly reduce the occurrence of chronic bacterial infections.

Graduate Research Assistant at Syracuse University, Syracuse, NY (August, 2009 – May, 2010)

Project title: Characterization of bacterial species isolated from a bio-filter system for formaldehyde removal in indoor air.

The project was funded by US Environmental Protection Agency through Syracuse Center of Excellence.

- Identified a bacterial strain *Arthrobacter aureescens* TC1 isolated from the roots of indoor plant, which can reduce formaldehyde from the indoor air significantly.
- Developed a bio-filter system which can work as an indoor volatile organic compounds (VOC) removal system.

Senior Undergraduate Student at Nirma University, India (August, 2007 – May, 2008)

Major project title: Determination of molecular weights of proteins present in human blood plasma using gel electrophoresis.

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Industrial training project: Ammonia production and energy consumption.

LABORATORY SKILLS: DNA microarray, DNA sequencing, PCR and Quantitative-PCR, Primer designing, Cloning, Western blotting, SDS-PAGE, Protein purification, DNA electrophoresis, RNA isolation, Fluorescence microscopy, Confocal microscopy, Atomic Force Microscopy, Transmission Electron Microscopy, Flow cytometry, Cell viability tests, Slot blotting, Cell culturing.

TEACHING/MENTORING EXPERIENCE

NSF REM (Research Experience and Mentoring) Mentor at Syracuse University, Syracuse, NY.

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ACADEMIC AND PROFESSIONAL HONORS & AWARDS

- *Graduate Research Assistantship*, Department of Biomedical and Chemical Engineering, Syracuse University, Syracuse, NY.
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