Genome-wide characterization of virulence-related and antibiotic-resistance related hypothetical proteins in Pseudomonas aeruginosa
(銅綠假單胞菌毒力相關和抗生素耐藥性相關假設蛋白的全基因組特徵)

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Background of research

Multidrug-resistant Pseudomonas aeruginosa is a critical threat to public health worldwide

P. aeruginosa is one of the most commonly-isolated nosocomial pathogen, accounting for 10% of all hospital-acquired infections worldwide, including Hong Kong (1). The emergence and spread of various multidrug-resistant *P. aeruginosa* strains have posed alarming challenges to public health worldwide. On the World Health Organization (WHO) list of antibiotic-resistant "priority pathogens" –12 families of bacteria that pose the greatest threat to human health, carbapenem-resistant *P. aeruginosa* is ranked the second place with critical priority. Public demand for new antibiotics is enormous, yet drug development pipelines of the pharmaceutical industry started to run dry with limited targets available for inventing new bactericidal antibiotics. Despite great advances in understanding molecular pathogenesis, effective ways of overcoming antibiotic resistance have not surfaced (2). As novel targets for treating *P. aeruginosa* infections are urgently needed, better understanding of the pathogenesis mechanism is a prerequisite for discovering such therapies. Research in my lab and collaborators has led to the identification of a variety of new virulence factors and mechanisms. Tuning their expression and developing inhibitors are key for the development of strategies to control *Pseudomonas* infections (3). My lab's long-term goals are directed at identifying new virulence factors and investigating the molecular pathogenesis in this bacterium.

Multiple transcription factors (TFs) control P. aeruginosa virulence

P. aeruginosa develops at least 9 pathways to exert its virulence, including quorum sensing (QS) (4), Type VI (T6SS) (5) and Type III secretion systems (T3SS) (6), biofilm (7), motility (8), antibiotic resistance (9), and siderophores (10), stringent response (SR) and persistence (11), and oxidative stress resistance (12). Our recent studies have elucidated a group of key QS TFs (13), including VqsR , VqsM , AlgR , CdpR , and RpoN (14), suggesting that they form a complicated regulatory network. These transcriptional factors not only strictly regulate QS, but also play significant roles in other virulence-related pathways. We performed genome-wide characterizations of protein-DNA binding patterns by performing ChIP-seq and profiled the regulons by integrating all RNA-seq and microarray data together for the tested TFs to displayed profound crosstalk (13). Our recent study has mapped an integrated regulatory network, *Pseudomonas aeruginosa* genomic regulatory network (PAGnet), comprising the interactive regulons of 20 TFs (13). This network revealed a complex crosstalk among virulence-related TFs and clearly depicted the complicated network regulating *P. aeruginosa* pathogenicity. The development of inhibitors against these newly identified master regulators may lead to the discovery of novel drugs targeting *P. aeruginosa*. Recently, we used a high-throughput systematic evolution of ligands by exponential enrichment (HT-SELEX) assay to explore the DNA binding profiles for all TFs in *P. aeruginosa*. We have obtained 182 TFs among all 371 TFs and obtained robust enrichment of 198 sequence motifs describing binding specificities for distinct TFs (15).

Hypothetical proteins in the *P. aeruginosa* genome

P. aeruginosa PAO1 is one of the first bacterial strains that was sequenced from a clinic isolate in 1950s. The genome is 6.3 Mbp long that includes 5708 ORFs, including 89.4% coding regions, and 0.4% stable RNAs. Among these ORFs, there are 2194 hypothetical proteins with 1701 operons (HPs, 38.5%) without almost any experimental data on their biological functions, making them dark matters in the genome (Fig. 1A). Recently, we have characterized that an HP (PA3880, AnvM) is involved in regulation of QS and host immune response(16). Most of HPs are well conserved in other bacterial species, suggesting they have

similar functions. Given that *P. aeruginosa* is one of the best-studied bacterial pathogens with mature genetic approaches, it provides a perfect paradigm for exploring functions and regulatory mechanisms of HPs in many bacterial species.

23 VRHPs are involved in virulence

Our previous RNA-seq results showed many HPs showed high level of expression in the wild type PAO1, indicating the potential important role of HPs in *P. aeruginosa* (Fig. 1B). In our preliminary results, we generated 1000 mutants of HPs to characterize their functions. Combined phenotypic screening with RNA-seq results, we identified a group of virulence-related HPs (VRHPs) and antibiotic-resistance-related HPs (ARHPs) (Fig. 1C). Bioassay using the lux-reporter was employed to determine the signals of QS system and bioassay screening results showed that 9 HPs positively regulated the QS system (Fig. 2A-C). ΔPA0392 and ΔPA2229-30 showed less production of *las* system autoinducer (3OC-HSL). Similarly, the deletion of PA1093, PA2318 and PA2627-28 inhibited the signals of *rhl* system (C4-HSL) and the complemented strains recovered the signals. ΔPA1550, ΔPA1788, ΔPA2229-30, ΔPA3016 and ΔPA4691-92 decreased the production of *pqs* system molecule.

We also found that PA3352, PA3623, PA4128 and PA4463-65 were essential for the swarming motility in PAO1 (Fig. 2D). We used Congo Red assay to detect the EPS production, which is an essential component of biofilm and shelters PAO1 form antibiotics and the immune system from host cells. ΔPA0392, ΔPA0446, ΔPA0862, ΔPA2627-28 and ΔPA3352 showed a brighter phenotype than the wildtype, implying that these mutants increased the production of EPS, while ΔPA2229-30 decreased the production of EPS (Fig. 2E). Biofilm formation assay using crystal violet staining showed that ΔPA0862, ΔPA3016 produced more biofilm than the wild type, while ΔPA3350 and ΔPA3352 had a significant decrease on biofilm formation (Fig. 2F). In addition, 8 HP mutants (ΔPA0038, ΔPA3764, ΔPA2229-30, ΔPA2627-28, ΔPA3939, ΔPA4128, ΔPA4463-65 and ΔPA4852) displayed less green supernatants than the WT, while the supernatant of ΔPA0309 was greener than the WT, indicating that these 9 HPs were critical for the production of pyocyanin (PYO) (Fig. 2G)

18 ARHPs are involved in resistance against multiple antibiotics

We performed MIC screening of five antibiotics, including meropenem, trimethoprim, ciprofloxacin, polymyxin B and tetracycline. A total of 18 HPs were identified as important regulators in antibiotic-resistance (Figure 3). ΔPA0043, ΔPA3998, ΔPA4577, ΔPA3352, ΔPA4463, ΔPA4169. ΔPA1005, ΔPA0566 and ΔPA2141 showed a two-fold reduction in resistance of meropenem. ΔPA3998, ΔPA0566, ΔPA3352, ΔPA4577, ΔPA3350, ΔPA4463, ΔPA4963, ΔPA1005 and ΔPA4918 had higher MIC against trimethoprim. Five HP mutants showed lower resistance against ciprofloxacin, while ΔPA2720 had increased resistance. Two and four HP mutants displayed decreased resistance against polymyxin B and tetracycline, respectively. Besides, ΔPA3317, ΔPA2720 and ΔPA3615 showed higher resistance against polymyxin B than WT.

Taken together, we have characterized 23 novel virulence-related HPs (VRHPs) and 18 antibiotic-resistance related HPs (ARHP), and their complementation strains validated these phenotypes. Based on these promising preliminary results, we plan to comprehensively profile all regulons for these 37 VRHPs and ARHPs and elucidate their regulatory mechanisms and network (Fig. 4) via achieving the following 3 objectives.

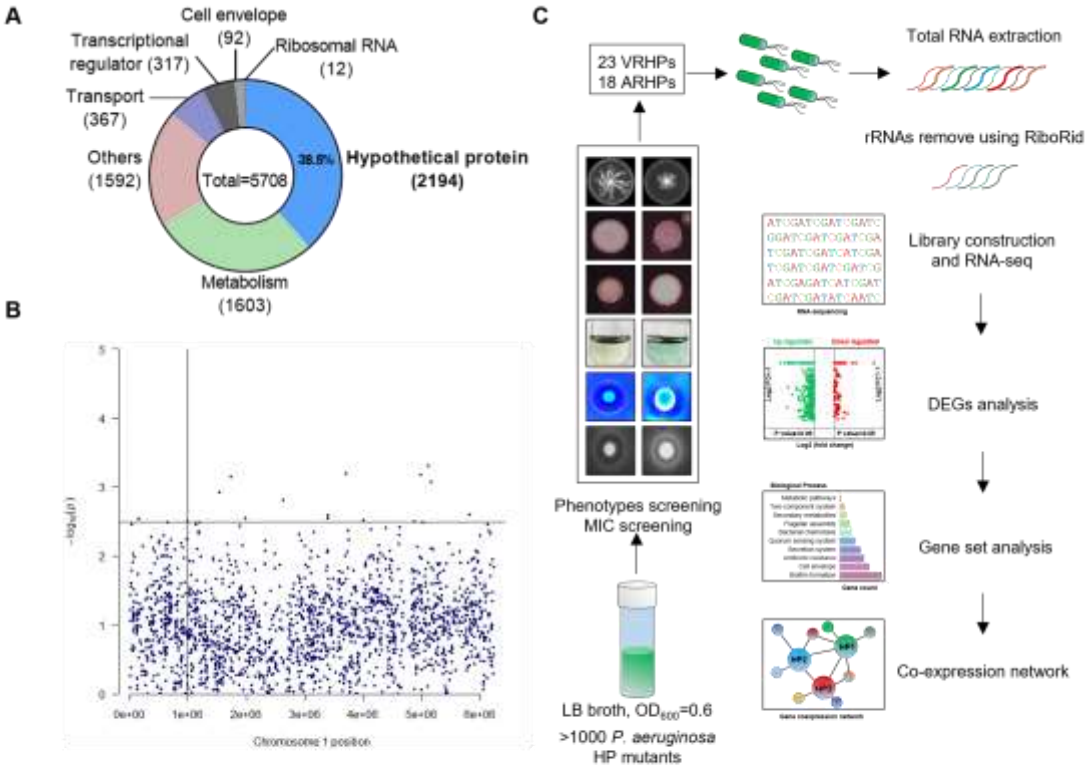

Figure 1. Hypothetical proteins in the *P. aeruginosa* genome. A. HP genes account for 38.5% in *P.* aeruginosa genome. **B.** HPs distribution and expression in *P. aeruginosa* PAO1. **C.** Pipeline of identification of 23 VRHPs and 18 ARHPs and mapping virulence-related and antibiotic-resistance related regulatory network in *P. aeruginosa*

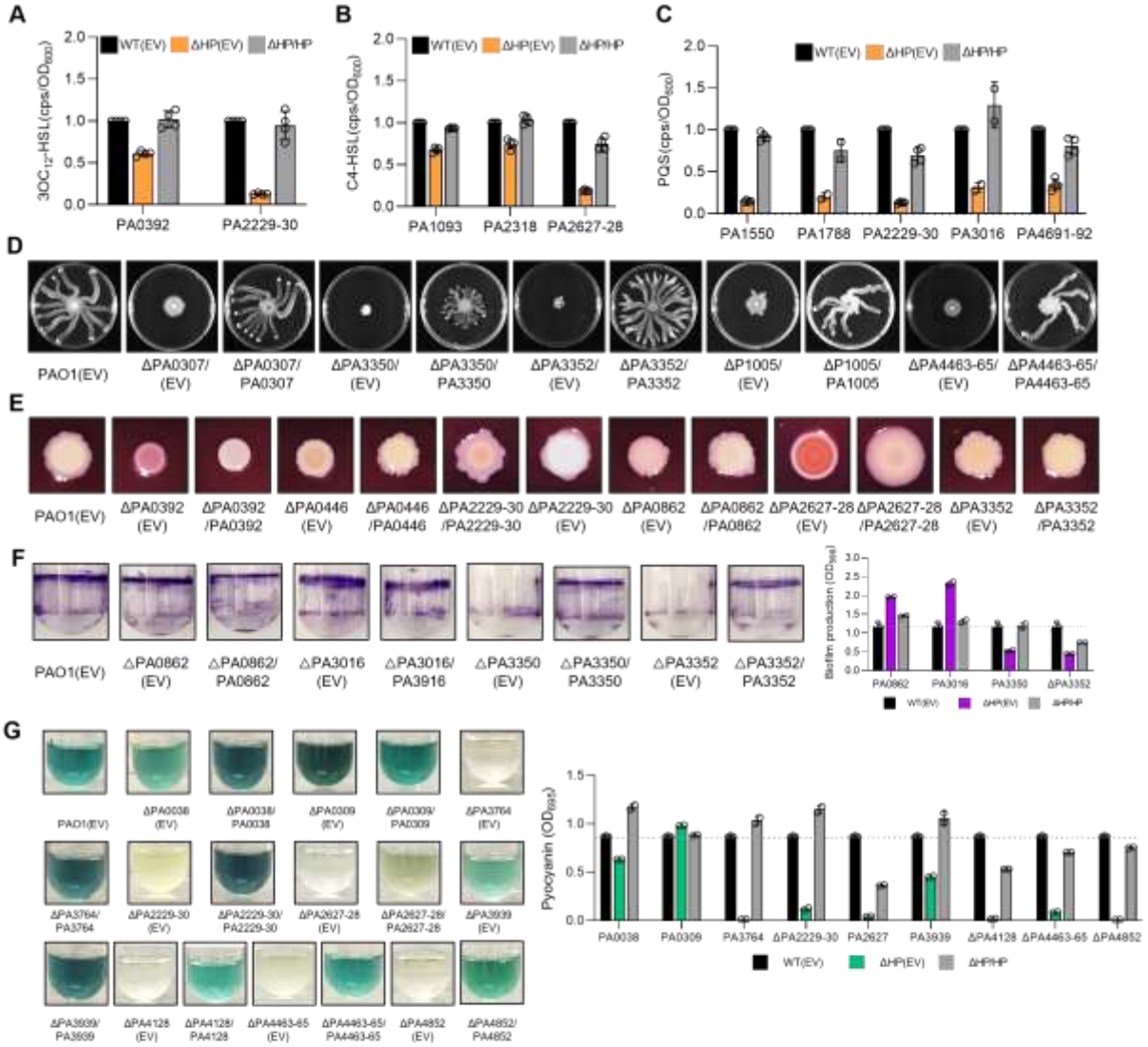

Figure 2. 23 VRHPs regulate QS system and phenotypes. A. Two VRHPs regulate Las system. **B.** Three VRHPs regulate Rhl system. **C.** Five VRHPs regulate Pqs system. **D.** Five VRHPs regulate swarming motility. **E-F.** Six VRHPs regulate EPS and four VRHPs regulate biofilm formation. **G**. Nine VRHPs regulate pyocyanin production.

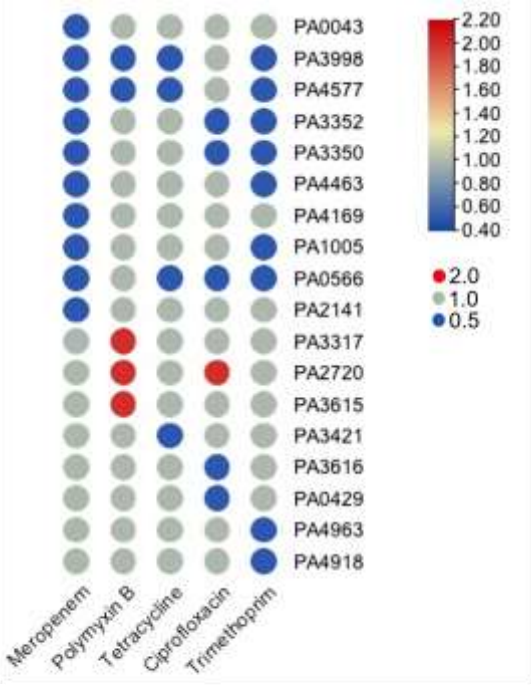

Figure 3. 18 ARHPs regulate multiple antibiotic-resistance of PAO1. Dot color represent the fold change of minimum inhibitory concentration compared to the wild type PAO1.

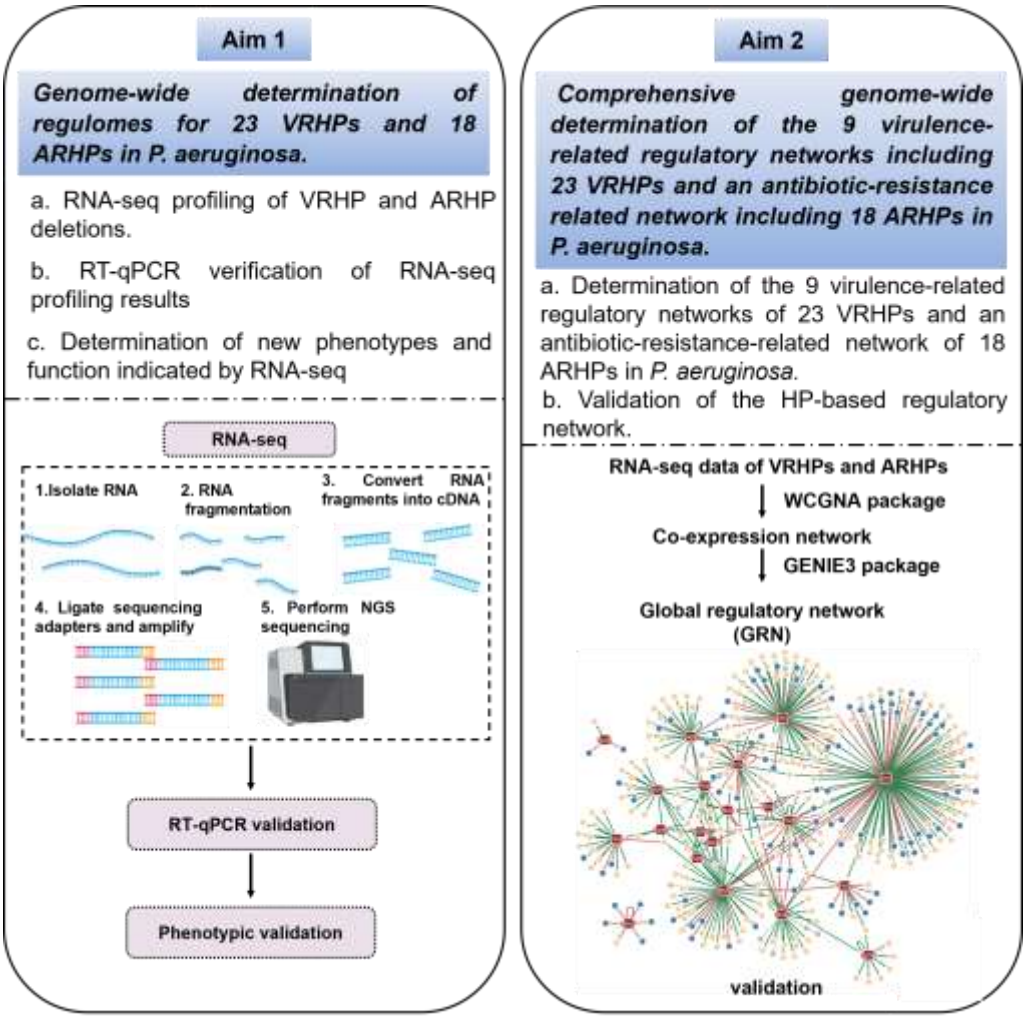

Figure 4. Summary of aims and study design

d) A maximum of two non-text A4 pages of attached diagrams, photos, carts and tables etc.

Key reference

1. Silar P. Peroxide accumulation and cell death in filamentous fungi induced by contact with a contestant. Mycological research. 2005;109(2):137-49.
2. Cantin AM, Hanrahan JW, Bilodeau G, Ellis L, Dupuis A, Liao J, et al. Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. Am J Respir Crit Care Med. 2006;173(10):1139-44.
3. Shao X, Xie Y, Zhang Y, Liu J, Ding Y, Wu M, et al. Novel therapeutic strategies for treating Pseudomonas aeruginosa infection. Expert Opinion on Drug Discovery. 2020:1-21.
4. Lee J, Zhang L. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein Cell. 2015;6(1):26-41.
5. Ho BT, Dong TG, Mekalanos JJ. A view to a kill: the bacterial type VI secretion system. Cell Host Microbe. 2014;15(1):9-21.
6. Hauser AR. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat Rev Microbiol. 2009;7(9):654-65.
7. Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, et al. Gene expression in Pseudomonas aeruginosa biofilms. Nature. 2001;413(6858):860-4.
8. Coleman SR, Blimkie T, Falsafi R, Hancock REW. Multidrug Adaptive Resistance of Pseudomonas aeruginosa Swarming Cells. Antimicrob Agents Chemother. 2020;64(3).
9. Chen L, Yang L, Zhao X, Shen L, Duan K. Identification of Pseudomonas aeruginosa genes associated with antibiotic susceptibility. Sci China Life Sci. 2010;53(10):1247-51.
10. Little AS, Okkotsu Y, Reinhart AA, Damron FH, Barbier M, Barrett B, et al. Pseudomonas aeruginosa AlgR Phosphorylation Status Differentially Regulates Pyocyanin and Pyoverdine Production. mBio. 2018;9(1).
11. Moradali MF, Ghods S, Rehm BH. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front Cell Infect Microbiol. 2017;7:39.
12. Lan L, Murray TS, Kazmierczak BI, He C. Pseudomonas aeruginosa OspR is an oxidative stress sensing regulator that affects pigment production, antibiotic resistance and dissemination during infection. Mol Microbiol. 2010;75(1):76-91.
13. Huang H, Shao X, Xie Y, Wang T, Zhang Y, Wang X, et al. An integrated genomic regulatory network of virulence-related transcriptional factors in Pseudomonas aeruginosa. Nat Commun. 2019;10(1):2931.
14. Shao X, Zhang X, Zhang Y, Zhu M, Yang P, Yuan J, et al. RpoN-Dependent Direct Regulation of Quorum Sensing and the Type VI Secretion System in Pseudomonas aeruginosa PAO1. Journal of bacteriology. 2018;200(16).
15. Wang T, Sun W, Fan L, Hua C, Wu N, Fan S, et al. An atlas of the binding specificities of transcription factors in Pseudomonas aeruginosa directs prediction of novel regulators in virulence. Elife. 2021;10.
16. Zhang Y, Zhou CM, Pu Q, Wu Q, Tan S, Shao X, et al. Pseudomonas aeruginosa Regulatory Protein AnvM Controls Pathogenicity in Anaerobic Environments and Impacts Host Defense. Mbio. 2019;10(4).
17. Kang H, Gan J, Zhao J, Kong W, Zhang J, Zhu M, et al. Crystal structure of Pseudomonas aeruginosa RsaL bound to promoter DNA reaffirms its role as a global regulator involved in quorum-sensing. Nucleic Acids Res. 2017;45(2):699-710.
18. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.
19. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
20. Zhang Y, Zhou C-m, Pu Q, Wu Q, Tan S, Shao X, et al. *Pseudomonas aeruginosa* regulatory protein AnvM controls pathogenicity in anaerobic environments and impacts host defense. MBio. 2019;10(4):e01362-19.
21. Zhao J, Yu X, Zhu M, Kang H, Ma J, Wu M, et al. Structural and Molecular Mechanism of CdpR Involved in Quorum-Sensing and Bacterial Virulence in Pseudomonas aeruginosa. PLoS Biol. 2016;14(4):e1002449.

22. Pu QQ, Gan CP, Li RP, Li Y, Tan SR, Li XF, et al. Atg7 Deficiency Intensifies Inflammasome Activation and Pyroptosis in Pseudomonas Sepsis. J Immunol. 2017;198(8):3205-13.
23. Kannan S, Audet A, Huang H, Chen LJ, Wu M. Cholesterol-rich membrane rafts and Lyn are involved in phagocytosis during pseudomonas aeruginosa infection. J Immunol. 2008;180(4):2396-408.
24. Drannik AG, Pouladi MA, Robbins CS, Goncharova SI, Kianpour S, Stampfli MR. Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. Am J Resp Crit Care. 2004;170(11):1164-71.