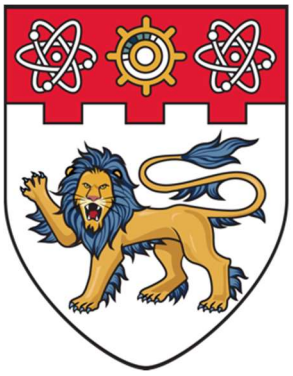

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

Mechanisms of *Enterococcus faecalis*-mediated Immunomodulation

Kao Hsien-Neng Patrick

SCHOOL OF BIOLOGICAL SCIENCES

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Kao Hsien-Neng Patrick

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Kao Hsien-Neng Patrick

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Mechanisms of *Enterococcus faecalis*-mediated immunomodulating effects

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Summary

Enterococci are a major cause of hospital-acquired infections. Family members *Enterococcus faecalis* and *Enterococcus faecium* predominate, frequently found as a component of chronic, polymicrobial infections alongside other bacterial species including *Staphylococcus aureus*. This challenge for the medical system is made even more acute by the acquisition of extensive antibiotic resistance by these strains, necessitating the development of alternate treatment strategies. One approach is the use of targeted therapies which modulate the host's own immune response in order to improve disease outcomes. This study explores interactions between cells of the innate immune system and *E. faecalis*, with a focus on identifying both effective responses deployed by these cells to eliminate the bacteria, as well as specific immunomodulating mechanisms utilized by *E. faecalis* to evade clearance.

I first established an *in vitro* model to profile murine neutrophil responses upon *E. faecalis* infection, both mono- and polymicrobial infections alongside *S. aureus*. This identified the capacity of *E. faecalis* to avoid a number of classic neutrophil antimicrobial pathways, including degranulation and the formation of neutrophil extracellular traps (NETs), while the production of reactive oxygen species (ROS) was strongly induced. Notably, in co-infection with potent NET-inducer *S. aureus*, *E. faecalis* both reduced *S. aureus*-induced NETosis and promoted *S. aureus* survival. In contrast, in intracellular ROS assays *S. aureus* co-infection reduced overall ROS

production and thus protected *E. faecalis* from neutrophil-mediated killing. This points to the possible collaboration mechanisms underpinning the persistence of chronic, polymicrobial infection with these two bacterial strains.

I also aimed to identify bacterial factors that may contribute to *E. faecalis* chronic infection, specifically genes that are involved in macrophage immunomodulation. *E. faecalis* was reported to suppress LPS-induced NF-κB activation, so a transposon library screening was conducted to identify the mutants that failed to reach a similar level of activity reduction. This screen identified 47 potential contributory genes, including genes linked to compound transporting, metabolism regulation, and hypothetical proteins with unknown functions. Additionally, a number of the genes were determined to be components of the shikimic acid pathway. This metabolism pathway found across all domains of life is responsible for the production of phenolic and extracellular ROS. Our further studies showed that shikimate pathway promoted *E. faecalis*-driven cytotoxicity in macrophages, likely by inducing apoptosis and thus reduced the overall immune activity such as cytokine production.

Collectively, these findings provide clues behind the high correlation of *E. faecalis* with *S. aureus* in persistent polymicrobial infections. It furthermore illustrated key mechanisms utilized by *E. faecalis* to subvert the antimicrobial mechanisms of both murine neutrophils and macrophages directly. These findings have important implications for our understanding of *E. faecalis* host-pathogen interactions, most importantly through the identification of potential targets which may serve to interrupt *E. faecalis* immune evasion.

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Abbreviations

ABC transporter	**ATP-binding cassette transporter**
BHI	**brain heart infusion**
BMDM	**bone marrow-derived macrophage**
CD	**cluster of differentiation**
CFU	**colony forming unit**
ChEPI	**chemical entities of biological interest**
CitHis	**citrullinated histone**
CLR	**C-type lectin receptor**
CR	**complement receptor**
CSF	**colony-stimulating factor**
DAMP	**damage-associated molecular pattern**
DCFDA	**dichlorofluorescin diacetate**
DMEM	**Dulbecco's modified eagle medium**
DNA	**deoxyribonucleic acid**
DNase	**deoxyribonuclease**
eDNA	**extracellular deoxyribonucleic acid**
EDTA	**ethylenediaminetetraacetic acid**
E. faecalis	***Enterococcus faecalis***
E. faecium	***Enterococcus faecium***
ELISA	**enzyme-linked immunosorbent assay**
Em	**emission**
Ex	**excitation**
FBS	**fetal bovine serum**
Fc	**fragment crystallizable**

FSC	**forward scatter**
HBSS	**Hank's balanced salt solution**
HCl	**hydrogen chloride**
HMDB	**human metabolome database**
HPI	**hours post infection**
IACUC	**institutional animal care and use committee**
ICAM	**intercellular adhesion molecule**
IL	**Interleukin**
LAP	**LC3-Associated Phagocytosis**
LC3	**microtubule-associated protein 1A/1B-light chain 3**
LDH	**lactate dehydrogenase**
LPS	**lipopolysaccharides**
M1	**classically activated macrophage**
M2	**alternative activated macrophage**
MACS	**magnetic-activated cell sorting**
MFI	**mean fluorescence intensity**
MOI	**multiplicity of infection**
MPO	**myeloperoxidase**
MyD88	**myeloid differentiation factor 88**
NADPH	**nicotinamide adenine dinucleotide phosphate**
NE	**neutrophil elastase**
NET	**neutrophil extracellular trap**
NF-κB	**nuclear factor kappa light chain enhancer of activated B cells**
NLR	**Nod-like receptor**

NMS	**normal mouse serum**
n.s.	**not significant**
O.D.	**optical density**
PAD4	**protein arginine deiminase 4**
PBS	**phosphate-buffered saline**
pH	**potential of hydrogen**
PRR	**pathogen recognition receptors**
PS	**phosphatidylserine**
PTS	**phosphotransferase**
RAW cell line	**Ralph and William's cell line**
RIG	**retinoic acid-inducible gene**
ROS	**reactive oxygen species**
S. aureus	***Staphylococcus aureus***
SD	**standard deviation**
SEAP	**secreted embryonic alkaline phosphatase**
SSC	**side scatter**
SSTI	**skin and soft tissue infection**
TBHP	**tert-Butyl hydroperoxide**
TLR	**toll-like receptor**
TNF-α	**tumor necrosis factor alpha**
TSB	**tryptic soy broth**
VEGF	**vascular endothelial growth factor**
WT	**wildtype**

Chapter 1: Background and Significance

1.1 Motivation

Enterococci, a family of bacteria that exist ubiquitously in the animal gastrointestinal tract (GIT), environment, and food products [1], have emerged as one of the leading causes of healthcare-associated infections [2]. While enterococci mostly live harmlessly with healthy hosts, they may cause various infectious diseases in immunocompromised patients [3]. Among them, *Enterococcus faecalis* and *Enterococcus faecium* are the two microbes most frequently isolated from infection sites, which consist of urinary tract infection, bloodstream infection, wound infection, endocarditis, and more [4]. While *E. faecium* is responsible for more vancomycin-resistant infections, both *E. faecalis* and *E. faecium* share various mechanisms of resistance, either intrinsic or acquired, against a wide array of antibiotics, which result in limited therapeutic options and hinder recovery [5]. With the resistance against drugs like vancomycin and ampicillin spreading throughout the world, it is urgent to develop alternative strategies against enterococcal infection [6, 7].

Enterococcal infections are widely considered opportunistic, often resulting from contamination rather than active invasion, with relatively limited virulence factors and toxins compared to pathogens like *Staphylococcus aureus*. However, enterococci

also exhibit various evasion mechanisms to either avoid immunosurveillance or escape immune responses that follow **(Fig. 1.1)** [8]. These immune evasion mechanisms promote enterococcal colonization and contribute to persistence, which is often associated with chronic and polymicrobial infections and make the diseases more complicated for the treatments. That said, under optimal circumstances, the immune system can effectively eliminate enterococci and protect the host from enterococci-mediated damages [9, 10]. Developing therapeutic options that utilize the efficiency of the immune system, including but not limited to vaccination, is one of the promising strategies in the battle against microbes with antibiotic resistance [11, 12]. To achieve such a goal, we must gain more insights regarding immune responses tailored against enterococci infection as well as evading mechanisms used by these pathogens.

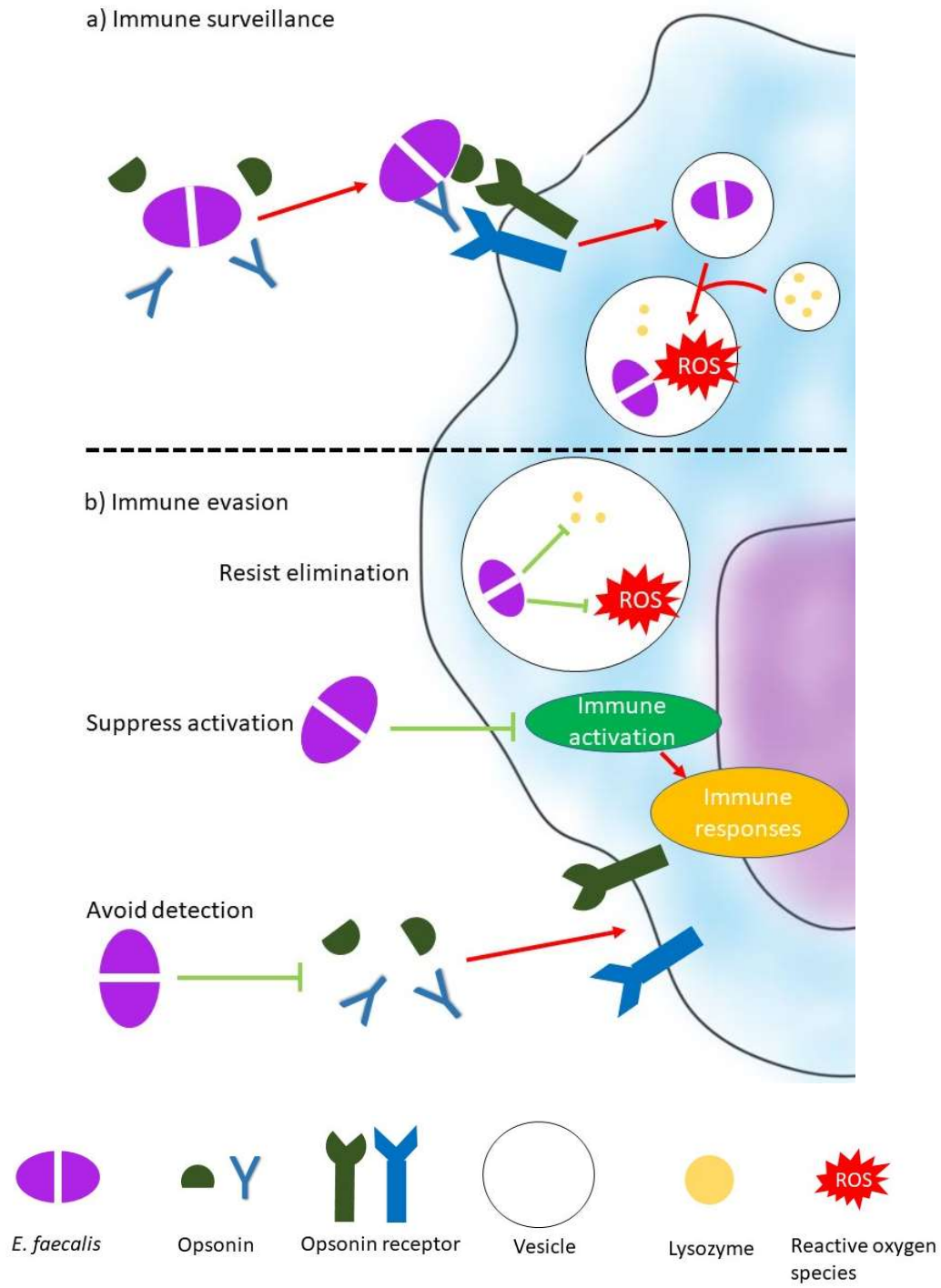

Figure 1.1 Immune evasion mechanisms facilitated by *E. faecalis*

a) The immune system produces opsonins, such as complement and antibodies, to recognize and label invading microbes for phagocytic engulfment, which is followed by intracellular killing. b) *E. faecalis* has mechanisms to avoid being detected by opsonins. The production of certain molecules, either surface-bound or secrete, can suppress immune activation induced by *E. faecalis*. Internalized *E. faecalis* can tolerate lysozyme and ROS to survive intracellularly for a period of time.

In this thesis, the immune responses against *E. faecalis*, one of the leading causes of human enterococcal infections, were studied. This includes the responses driven by neutrophils and macrophages upon *E. faecalis* infection, either alone or in the presence of other microbes, in an attempt to identify effectors that are responsible for bacterial clearance. Immunomodulating mechanisms facilitated by *E. faecalis* were also investigated, which contribute to the suppression of immune responses and promote colonization of pathogens. These work fills some existing knowledge gaps of how *E. faecalis*-mediated colonization is often associated with chronic and polymicrobial infections and provide some insights regarding promising targets to develop immunity-based therapeutic strategies.

1.2 *Enterococcus faecalis* interaction with host tissues and environment

Before emerging as a medical threat and one of the leading causes of healthcare-associated infections [13], enterococci were identified as members of commensal bacteria that have lived with hosts peacefully since the terrestrialization of animals [14]. Since then, enterococci have adapted to colonize a variety of hosts including insects, birds, mammals like humans, and even some aquatic hosts, primarily in their

digestive organs [14, 15]. However, host tissues are challenging environments posing multiple threats to the residing microbes. Here we discuss the strategies utilized by enterococci, with emphasis on *E. faecalis*, to persist and thrive in mammalian tissues.

1.2.1 Commensal *E. faecalis*

E. faecalis has been identified as part of the microbiome in the human oral cavity [16], urinary tract [17], and gastrointestinal tract [14, 15, 18]. These are all mucosal tissues, which means they are covered with mucus containing antimicrobial substances. Given the high turnover rate of mucin [19], the first challenge that surface residents must overcome is staying attached to host tissues. *E. faecalis* facilitates mucosal invasion through the expression of aggregation substance in *ex vivo* experiments [20]. The idea that *E. faecalis* can invade the mucus layer is further supported by *in vivo* models, in which *E. faecalis* can get in close proximity to the intestinal epithelium and attach directly to the epithelial surface [21]. The colonization of host tissues is further secured by the formation of biofilm [21, 22], overcoming the environmental challenges and stay within the niche. Biofilm bacteria also trigger weaker immune responses compared to planktonic cells, potentially contributing to better survival at tissue surface [23, 24].

Another trait that contributes to the commensalism of *E. faecalis* is its ubiquity in

the environment, especially in food materials and products [1, 25]. *E. faecalis* can even be isolated from the first meal of many infants: human breast milk [26, 27]. This finding could explain why a high percentage of infants are colonized by enterococcus (60-80%) [28, 29]. *E. faecalis* in milk may be beneficial to infants by stabilizing the gut microbiome community either through inhibiting detrimental microbes or promoting a probiotic population [27, 30]. In addition to regulating the population of microbes, *E. faecalis* can also attenuate the virulence of other pathogens, including suppressing the hyphae and biofilm formation of *Candida albicans* and attenuating the adherence of *Listeria monocytogenes* to epithelial cells [31, 32]. Furthermore, *E. faecalis* can directly interact with host mucosal immune components, inducing IgA production without inducing local inflammation and promoting gut homeostasis by increasing IL-10 secretion [33, 34]. In short, *E. faecalis* is beneficial to GIT health as a member of commensal on many levels.

1.2.2 Opportunistic *E. faecalis*

Despite its beneficiary role of *E. faecalis* to its human host, the ubiquity of *E. faecalis* can also lead to medical challenges in immunocompromised individuals. For example, cross-transmission among residents in healthcare units can be frequent, which may contribute to the spread of antibiotic resistance determinants [35, 36].

This is an even more serious issue considering the fact *E. faecalis* has long clonal persistence and high clonal diversity [37]. In addition, if individuals have open wounds that are slow to heal, self-contamination of *E. faecalis* from commensal niches to wound sites can also occur [38]. Another route of contamination is through medical devices. Mechanical ventilation was reported to be a source of transmission for *E. faecalis*, making respiratory tract infection a common problem in patients undergoing such treatment [39]. Peritoneal dialysis, on the other hand, is associated with enterococcal peritonitis, facilitating the translocation of intestinal *E. faecalis* to the peritoneal cavity that may eventually lead to systemic infection and inflammation [40, 41]. Two characteristics contribute to these medical device-related infections. One is the ability for *E. faecalis* to adhere to abiotic surfaces, which can render such devices a reservoir for persistence of the bacteria [42]. The other is the resistance *E. faecalis* possess against many disinfecting treatments such as heat [43], ultraviolet radiation [44], and disinfectants [43, 45, 46], increasing the risk of contaminated equipment.

In general, infections caused by *E. faecalis* are usually opportunistic in nature instead of initiated by active invasion of the host. However, once *E. faecalis* enters host tissues, it will face overwhelming pressure from the immune system. In order to colonize and survive, *E. faecalis* has to develop strategies to evade immune

surveillance and tolerate immune responses. In the next section we will go through the basics of immunity, and how *E. faecalis* exploits the system to persist in infected tissues.

1.3 *E. faecalis* interaction with host immune system

The primary goal of the immune system is to prevent pathogens from entering and damaging the host. The immune system orchestrates multiple strategies in order to achieve the goal. In this section, we will discuss these defensive strategies, and briefly describe the known evasion mechanisms utilized by *E. faecalis*. More detailed immunomodulating and survival mechanisms mediated by *E. faecalis* regarding specific immune responses and effector cells will be reviewed in more detail in the introductory sections of the following chapters.

1.3.1 Barrier defense

The first innate defense mechanism that most microbes encounter is the barrier defense, which blocks foreign bodies from entering the host altogether **(Fig. 1.2)**. Among many barriers, skin is one of the most extensive. Consisting of epidermis, dermis, and subcutaneous layers, skin can protect the host from not only microbe

invasion, but also ultraviolet radiation and water loss [47]. In addition to forming an intact physical barrier by connecting keratinocytes with tight junctions, the epidermal layer is also covered with lipids containing a substantial amount of antimicrobial peptides (AMP), preventing microbes from colonizing and overpopulating on the surface [48]. Beneath the epidermis, a thin basal membrane consisting of extracellular matrix (ECM) components including collagen, perlecan, nidogen, and laminin, serves as an extra layer of barrier to prevent the invasion of microbes [49]. Within and under the epidermis, residential immune cells such as Langerhans cells, mast cells, and macrophages are positioned to rapidly detect and react to microbial dysbiosis or invasion [47]. Another natural barrier, the mucous membrane, has loosely similar structure to skin, with a luminal layer of non-living substance that often contains AMPs, with an underlying layer of specialized epithelial cells joined by tight junctions, and another underlying layer of basal membrane, which altogether act to limit microbial penetration. However, mucosal barriers are more diverse given they cover tissue where the exchange and intake of foreign substances are more frequent, such as lung and intestine; thus, a more permeable mucosal surface is needed. Instead of tough keratin and epidermis at the surface, mucosal barriers use an AMP-containing mucus layer to limit microbial colonization and penetration [50, 51]. Beneath the epithelium, two compartments, the basement membrane and

interstitial matrix, combine as a thick layer of ECM that stands between the mucosal epithelium and submucosa [52]. Similar to skin tissue, residential immune cells are also spread throughout the mucosal epithelium and interstitial matrix to help regulate luminal microbiota communities [53]. Despite the fact that the physical defense of mucosal barriers is weakened by its higher permeability, mucosal immunity possesses IgA, an immunoglobulin that can be secreted through mucus, as an additional weapon to prevent or remove invading microbes [54]. Both the mucosal epidermis and the mucus layer are shed and renewed regularly, and this high turnover rate acts as another mechanism to inhibit microbial colonization [19, 55]. With such an arsenal, un-adapted pathogens would struggle to persist on the surface and be even less likely to penetrate the barrier and invade the tissue.

While *E. faecalis* is part of GIT commensal microbiota, there are many scenarios where this microbe can translocate from its natural niche to other tissues to cause infections **(Fig. 1.3)**. As mentioned previously, *E. faecalis* is well-adapted to colonize mucosal tissues by being able to attach to the epithelium in the GIT [21]. While *E. faecalis* is not considered to be part of skin microbiota [56, 57], it is often isolated from wound tissues including surgical sites and diabetic foot ulcers [58, 59]. Several ECM components, such as laminin and collagens, can be used by *E. faecalis* for attachment [60, 61], indicating that once the skin barrier is damaged by other

factors, *E. faecalis* can more easily adhere to the surface. Attachment to collagen can be further enhanced under an alkaline environment [62], such is the case in wound tissues [63]. Once *E. faecalis* colonizes the epithelial surface, it utilizes mechanisms to translocate through physical barriers into deeper tissues [64]. Potential mechanisms for such invasion may include decorating the bacterial surface with specific polysaccharides [65], producing or activating enzymes that break down tight junctions and basal membranes [66, 67], or entering host cells directly [68].

Penetrating the barrier defense, however, is just the start of challenges those invading pathogens must overcome. Once the barrier is breached, damaged cells start to release danger-associated molecular patterns (DAMP), which include a large amount of alarmin that can act as a chemoattractant [69, 70]. Residential immune cells also start to detect the type of invasion, and release corresponding cytokines to shape the environment, initiating the next step of immune responses [71, 72].

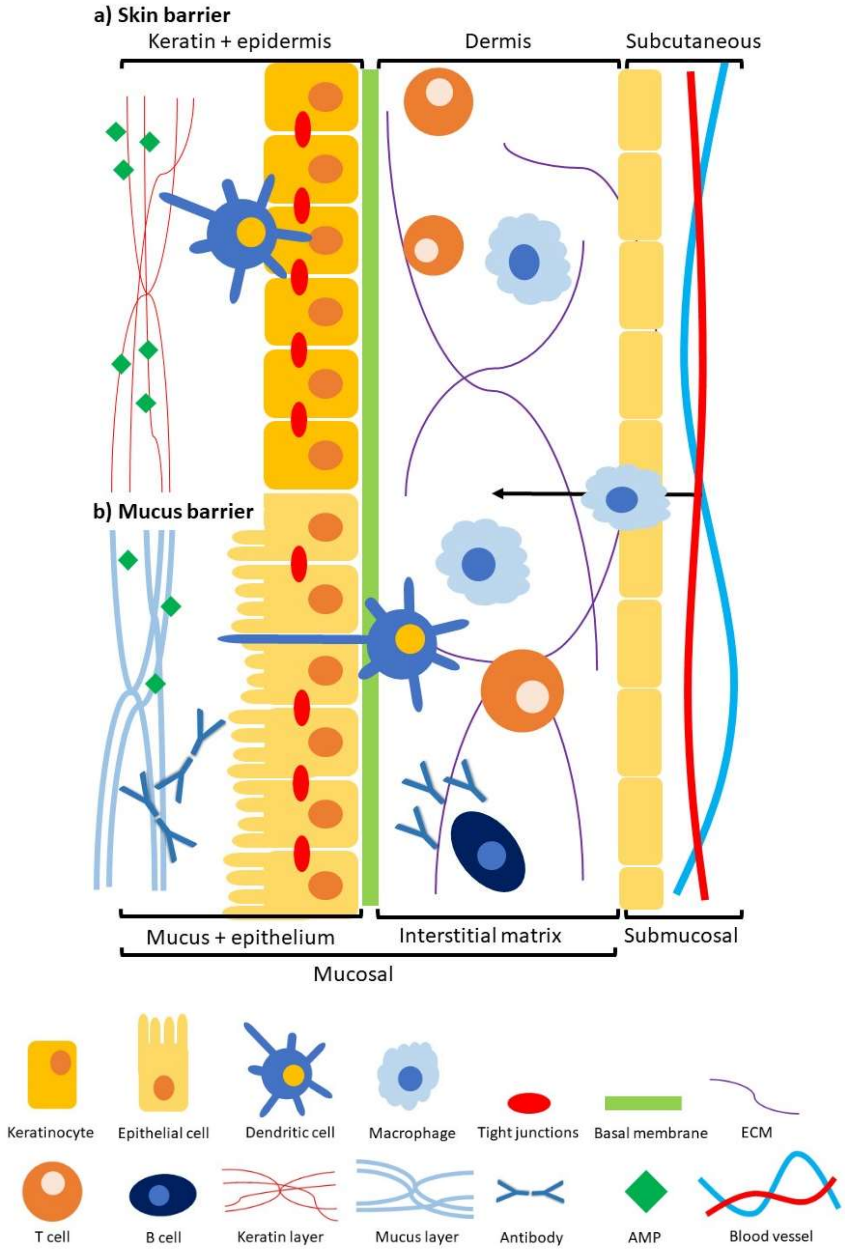

Figure 1.2 Barrier defense at the surface of the host body

Barrier defense usually starts with a layer of AMP-containing extracellular matrix such as keratin (a) or mucin (b), secreted by specialized epithelial cells underneath. In addition, mucus contains IgA to bind or neutralize microbes. Epithelial cells are connected together by tight junctions. This layer of epithelium together with the basal membrane form a physical barrier to prevent microbes from entering host tissues. Another layer of ECM made up of collagen, laminin, and nidogen presents below the basal membrane contains tissue residential immune cells such as macrophages and lymphocytes. Upon infection additional leukocytes are typically recruited from the blood vessel at the submucosal, infiltrating the infection sites.

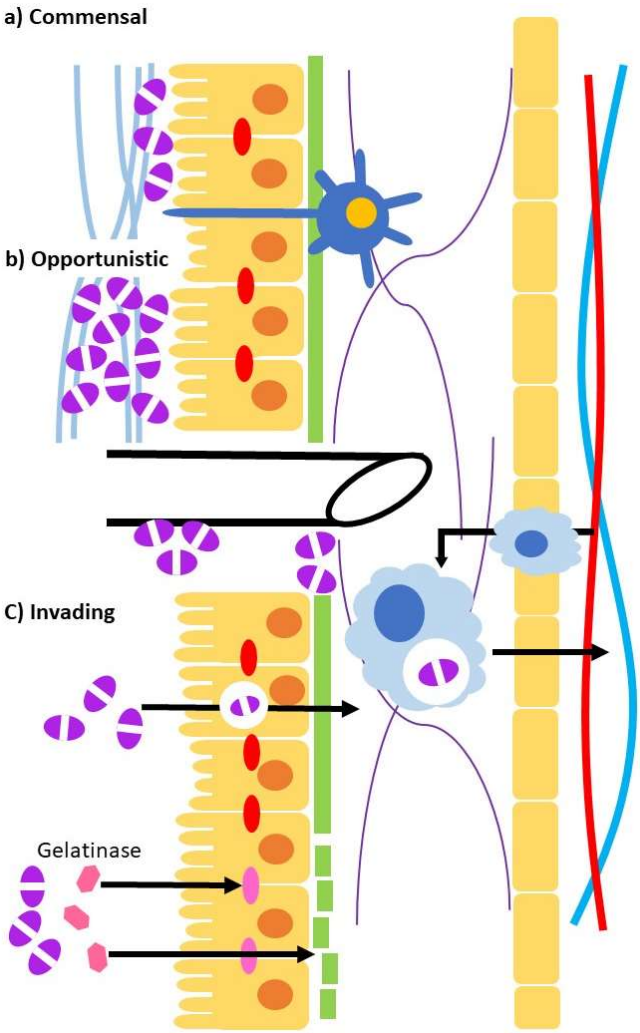

Figure 1.3 *E. faecalis* transition from commensal microbe to invading pathogen
a) Under the healthy state where the barriers are intact, *E. faecalis* penetrates the mucus layer and attaches to the epithelium for colonization. *E. faecalis* may form biofilm and avoid triggering immune responses. b) *E. faecalis* translocation often takes place upon distress at the surface barrier. Overgrowth of *E. faecalis* during antibiotic treatment and inflammation can increase the likelihood for *E. faecalis* to translocate from surface to tissue. Damaged barrier defenses, either by mechanical injury or inflammation, can allow *E. faecalis* to attach to ECM beneath the epithelium to initiate colonization. c) Several potential invading mechanisms facilitated by *E. faecalis* have been identified in *in vitro* models. By surviving intracellularly within enterocytes or phagocytes, *E. faecalis* may invade host tissues locally or systemically. Secreted gelatinase produced by *E. faecalis* can degrade tight junctions and basal membranes, allowing *E. faecalis* to pass through barrier defense. See Figure 1.2 for image legend.

1.3.2 Infiltrating leukocytes

In response to infection, various immune cells will be recruited from circulation or the bone marrow to the infection site in order to contain and eliminate invading microbes. This infiltration is carefully orchestrated, with specific cell types following a specific temporal order, performing designated functions. Immune cell recruitment has to be so well-controlled partly because reactions from immune cells are not only lethal to microbes, but they can also be harmful to the host tissue if left unchecked. In addition, the immune responses are responsible for tissue regeneration, so different phenotypes of immune cells at each stage facilitate almost opposite functions, with the initial inflammatory stage causing collateral tissue damage and limiting cell regeneration, and the resolution stage promoting tissue repair and enhanced cell proliferation [73-75]. Unregulated immune responses not only impede recovery but could lead to damages more severe than the ones caused by pathogens [76, 77].

Granulocytes include neutrophils, eosinophils, and basophils, and are usually some of the first responders to a variety of infections, arriving within hours to days [73-75, 78]. This family of cells is sensitive to environmental stress, DAMP, cytokines, as well a wide array of microorganisms. The primary goal of these cells is to rapidly

eliminate the invading agents, so they carry pre-formed granules, which contain AMPs that are effective against pathogens smaller in size, as well as cytotoxins that could cause damage to larger microorganisms like helminths [79]. Recently, granulocytes were also identified to present antigen to lymphocytes under controlled conditions to promote adaptive immunity, a different immune function that contributes to the fight against infections [80]. On the other hand, granulocytes rely heavily on opsonins like complement and antibodies to recognize microbes, which provides another mechanism for regulation since different cell types are responsive to different types of antibodies [81, 82].

Despite the significant role of granulocytes in combatting pathogens, little is known how *E. faecalis* triggers granulocytes, particularly neutrophils, since eosinophil and basophil are generally not responsive towards bacteria. It has long been known that neutrophils can effectively eliminate *E. faecalis* in an opsonin-dependent manner [83], yet the specific effectors that mediate *E. faecalis* clearance are unclear. However, *E. faecalis* has mechanisms to avoid opsonin detection, such as avoiding opsonin deposition by decorating or masking surface molecules or simply degrading complement factors [84-87]. Understanding the responses and clearance mechanisms mediated by neutrophils against *E. faecalis* could provide hints for optimized therapeutic strategies. In **chapter 2** of my thesis, I illustrate the responses

mediated by neutrophils upon *E. faecalis* infection, including the production of reactive oxidative species (ROS), profile of degranulation, and induction of neutrophil extracellular traps.

Closely following the migration of neutrophils during infection is the infiltration of monocytes [73-75]. Monocytes are a family of myeloid cells present in the bone marrow and circulating in the bloodstream. While monocyte can differentiate into many functional subsets, namely dendritic cells and macrophages, one function is shared by all of them: a phagocytic response that acts as their primary tool to contain and remove pathogens [88]. Once a microbe is engulfed, monocyte-derived dendritic cells and macrophages degrade the foreign cell body and present fragments of antigen on the surface. This antigen presentation promotes the activation and maturation of adaptive immune cells, leading to specific immune responses and long-term immune memory [89]. However, presenting antigen alone is not enough information to guide adaptive immunity, and monocyte-derived cells, especially macrophages, are also responsible for shaping the local environment and guiding immune responses according to the stage of infection. To provide such thorough guidance, macrophages can further polarize into various subsets that mediate different stages of immune responses through the production of a vast array of cytokines [90, 91]. Given the central role of macrophages in directing immune

responses, there is no surprise that disorder of macrophage regulation can lead to detrimental outcome [92]. Microorganisms that invade human tissues have also developed strategies to interfere with macrophage activation. Whether it is avoiding detection, directly kill macrophages, or even altering macrophage activation, a wide range of microbes target macrophages to promote microbial survival rate and chances to colonize host tissues [93], and *E. faecalis* is one of them. *E. faecalis* uses many strategies to evade macrophage-mediated immune responses, as described in **section 1.1** and **figure 1.1**. However, the underlying mechanisms for many *E. faecalis*-mediated immunomodulating mechanisms remain unclear. In **chapter 3** of this thesis, I identify genes that are predicted to be involved in the reduction of macrophage activation and further investigate some potential pathways that are responsible for such interference.

Lastly, adaptive immunity, represented by lymphocytes like T cells and B cells, kicks in in response to infection [73-75]. It takes weeks to educate lymphocytes stationed in lymph nodes upon infection of unprecedented pathogens. However, memory cells, either from circulation or resident in the tissue, are quickly recruited to the site of infection just within days [94]. And even though lymphocytes are not recruited as robustly as some of the innate leukocytes, they can tailor the immune responses targeting invading pathogens by producing specific cytokines and

antibodies [95].,Class-switching of antibodies can determine the cell types to be activated or recruited. For instance, IgG mostly binds to neutrophils while eosinophils are more responsive to IgE, and this sophisticated regulation directs the immune system to only employ the most effective mechanisms in microbe clearance [81, 82]. On the other hand, the cytokine profile determined by lymphocytes can modify the phenotype of involved cells, either to enhance microbicidal activity or to resolve local inflammation and prepare for tissue repair [75].

Due to the wide range of antibiotic resistance possessed by enterococci, immunotherapy such as vaccination appeared to be one of the promising alternative strategies [96, 97]. However, more studies are needed regarding the virulence and pathogenesis of enterococci, as well as their interaction with the immune system, in order to develop effective vaccines with critical bacterial targets and ideal class-switching [98-100].

1.3.3 Tissue recovery

After the threat of pathogens is cleared and inflammation resolved, the immune system turns its focus to tissue repair, which includes fibrosis, angiogenesis, and re-epithelialize [75, 101]. The efficiency of tissue repairing is highly correlated with the balance of immune responses and requires rapid elimination of invading microbes,

removal of tissue debris, and termination of inflammation, which again highlight the importance of regulatory mechanisms of each immune reaction [102]. Lingering colonization of bacteria, for instance *E. faecalis* infection at wound sites, would slow down the re-epithelization [103], and immune responses such as NETosis could also hinder the healing process [104]. However, this is beyond the scope of this thesis, despite it is a topic strongly relevant to clinical practice and worth future investigation.

1.4 Thesis outline and aims

Given that many or most *E. faecalis* infections are polymicrobial in nature, I investigated the mechanisms utilized by *E. faecalis* to evade immunosurveillance mediated by innate immune cells. This dissertation is focusing on two specific aims:

Aim 1: To characterize the neutrophil responses upon *E. faecalis* infection in single- and multi-species infections. I first establish a neutrophil-mediated killing assay that is efficient in removing *E. faecalis*, and profile the triggered neutrophil responses. I also expose neutrophils to multi-species infection, mixing *E. faecalis* and *S. aureus*, to investigate the impact of the polymicrobial community in immune

responses and the potential pathways that are interfered with. To understand the contribution of polymicrobial infection to bacterial persistence as well as potential clinical relevance, I study bacterial clearance both *in vitro* and *in vivo*. The findings regarding this part of the work are described mainly in **chapter 2**.

Aim 2: Previously, our lab characterized an immunomodulating effect by *E. faecalis*, that is able to suppress *E. coli*-mediated macrophage activation and that correlates with augmented *E. coli* growth in mixed-species infection [105]. To identify genes that contribute or participate in the immunomodulation of macrophage activity, I conduct genetic screen to identify genes involved in suppressing effects of *E. faecalis* against LPS-induced NF-κB activity in macrophages using an *E. faecalis* OG1RF transposon library and study the basic physiology of shortlisted mutants. I then investigate the responsible bacterial pathways and the mechanisms interfering with host responses. I also look into the potential bacterial effectors inducing the altered macrophage phenotypes. Identified mutants and their phenotypes are described in **chapter 3**.

Overall, the findings of this thesis provide insights regarding the virulence of *E. faecalis*, especially in polymicrobial and chronic infections. Such understanding could identify the optimal immune responses that are effective in *E. faecalis* clearance, which will be summarized and discussed in **chapter 4**. Elucidating the most effective

immune responses and the mechanisms used by microbes to interfere with the immune responses will allow us to develop alternative or enhanced therapeutic options against *E. faecalis*. Strategies of collaborating with the immune system, instead of administration of antibiotics, could not only avoid the current obstacles of antibiotic resistance but also prevent the emergence of resistance against this type of treatment.

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CHAPTER 2: Immunomodulating effects of polymicrobial infection on neutrophil responses

2.1 Introduction

2.1.1 Enterococci in polymicrobial infections

Originally recognized as harmless commensal microbiota, enterococci have emerged to pose a major threat in terms of healthcare associated infections, and in particular with widespread vancomycin resistance are now considered one of the top crises by the US CDC [1]. Among enterococcal infections, *Enterococcus faecalis* and *Enterococcus faecium* are two leading causes, with as high as 80% of *E. faecium* possessing vancomycin-resistance [1, 2]. Enterococci are highly associated with polymicrobial infections, isolated from infection sites with other causative microbes [3]. In studies performed between 2009 to 2018, 14.6-39% of enterococcal bloodstream infections were found to be polymicrobial, with these mixed infections associated with longer hospital stays, longer intensive care unit stay, higher incidents of septic shock, and more in-hospital mortality [4-7]. Orthopedic infections (including implant infections and septic arthritis) have also been strongly correlated with polymicrobial infections, with 28-54% of enterococcal prosthetic joint infections found to be polymicrobial, with shorter onset of initial symptoms after implantation and higher failure rate than monomicrobial infections [8-11]. Focusing on *E. faecalis*,

two studies conducted in Europe from 2005 to 2016 indicated 80% of the *E. faecalis*-involved orthopedic infections and 100% of spinal infections are polymicrobial in nature [12, 13].

Enterococci are one of the leading pathogens involved in skin and soft tissue infections (SSTIs), notably in surgical site infections and diabetic foot ulcers [14]. Specifically, in 2001, Enterococcus species were reported as the causative agent for 10.3 - 24.9% of SSTIs around the world, and in the top three causes in all countries under study [15]. Within the genus, *E. faecalis* and *E. faecium* are the most frequently isolated from SSTI [15-17]. Interestingly, while more *E. faecium* isolates are vancomycin-resistant, *E. faecalis* are responsible for a higher number of infections, accounting for ~5% of all surgical site infections in the US and Europe [18, 19]. Another type of SSTIs, diabetic lower limb infection, is also strongly associated with *E. faecalis* infection, comprising 13.2 - 45.2% of total studied cases and the most frequently isolated bacteria in lower limb bone infection in diabetics [20-24].

While the majority (> 80%) of diabetic foot infections are polymicrobial in nature [23, 24], this trend is taken to the extreme with *E. faecalis*, with less than 1% of *E. faecalis*-involved infections being mono-species [23]. Polymicrobial infection tends to be associated with two traits, firstly more severe diabetic foot infections, which lead to the vast majority of amputations in this population (77.8 - 100%) [25, 26].

Secondly, 64.5% of these polymicrobial diabetic wound infections are associated with biofilm-producing *E. faecalis*, with the isolated strains from these infections also exhibiting higher and broader resistance against antibiotics [26]. Biofilm formation has long been associated with chronic wound infection, acting as the primary impediment to wound recovery, due to intrinsic resistance against both natural immune responses and therapeutic antimicrobial treatment [27, 28]. In the initial stage of biofilm formation, *E. faecalis* needs to attach to a surface, and an open wound provides a perfect platform with enriched fibrinogen and collagen, facilitating otherwise commensal *E. faecalis* to colonize these sites [29, 30]. The ability to produce and form biofilm, including molecules that are responsible for adherence to host tissue or aggregation are the most commonly identified virulence factors of enterococcal wound infection [29, 31].

2.1.2 Virulence factors contributed by polymicrobial communities

Due to the prevalence of polymicrobial communities in wound infections, there is a pressing need to develop *in vitro* and *in vivo* models to study their impact and virulence factors. *In vivo* wound infection models involving polymicrobial biofilms established in mice and pigs which are accurately mimic human clinical observations, where polymicrobial biofilm-associated infection in both species exhibits prolonged

recovery time and impaired resolution of inflammation, with enhanced antimicrobial tolerance observed in the murine model compared to single species infections [32, 33] . Mechanisms that promote virulence in polymicrobial infections include metabolite exploitation, immune modulation, niched optimization, and virulence induction [34].

E. faecalis possesses all these mechanisms, making it especially suitable and versatile in multispecies microbe communities [35].For example, by exporting the non-proteinogenic amino acid L-ornithine, *E. faecalis* facilitates biosynthesis of *Escherichia coli* iron acquisition machinery, promoting the growth of *E. coli* in iron-limited environment [36]. *E. faecalis* can also manipulate the immune system to promote the growth of co-infecting *E. coli* in the urinary tract and peritoneal cavity [37, 38]. *E. faecalis* also exhibits synergistic biofilm production when co-cultured with *Staphylococcus aureus* and *Corynebacterium*, facilitating the establishment of a niche for all involved microbes to avoid immune surveillance and antibiotic penetration [39]. Most concerningly, horizontal transfer of vancomycin resistance genes from *E. faecalis*, (but not *E. faecium*), to *S. aureus* has been reported *in vitro*, suggesting the high overlap between vancomycin-resistant *S. aureus* and vancomycin-resistant enterococci could result from this transferability [40]. However, while extensive (and continuing) studies have examined the interactions among microbes in these

polymicrobial communities, the response from the immune system when facing multiple pathogens at the same time is still poorly understood.

2.1.3 Homeostasis and migration of neutrophils

The innate immune system, comprising physical barriers, chemical mediators and immune cells represents a diverse defense arsenal against infection. Among leukocytes, neutrophils are the most abundant, comprising ~50% of total peripheral white blood cells and acting as the primary infiltrating cells to the site of stress [41]. However, under normal, homeostatic conditions only 1-2% of total body neutrophils reside in the blood, with the remainder reside in the bone marrow or within tissues, playing a variety of roles and contributing to cross-talk between other cell types [42]. This highly regulated homeostasis is necessary due to the double-edge sword nature of neutrophil, which not only possesses numerous toxic compounds that could cause collateral damage to the host tissue during pathogens clearance but are also linked to pathogenicity of autoimmune diseases [43-45].

The first step in regulation of the neutrophil response starts with the recruitment of neutrophil. Neutrophils are produced in the bone marrow, where they mature and are released to the circulation at a controlled rate. Different subpopulations of neutrophil will then either stay in the circulation, migrate into tissues, or return to

the bone marrow [42, 46]. During infection, by expressing P- and E-selectin, endothelial cells of the blood vessel in close proximity to the infection site trigger neutrophils to roll on endothelium instead of being carried rapidly past by the flow of blood. A rolling neutrophil is then be arrested by the interaction between integrins and various ligands like intercellular adhesion molecules (ICAMs) or collagen, promoting transmigration and are eventually guided to site of distress [47, 48].

2.1.4 Recognizing environmental and microbial cues by neutrophils

Upon arrival at the injury site, by interpreting various damage- or pathogen-associated molecular patterns (DAMPS/PAMPs), in addition to various cytokines or chemokines produced by tissue resident macrophages or epithelial cells, neutrophils are able to respond accordingly. For example, sites of hypoxia may recruit neutrophils to promote angiogenesis by producing vascular endothelial growth factor (VEGF) or other related growth factors [49]. During sterile inflammation, DAMPs released by dying epithelial cells trigger toll-like receptor (TLR) 7/8/9, which in turn activates MyD88 and induce neutrophils to produce reactive oxygen species which may kill surrounding cells [50]. Neutrophils have also been linked to the chronic airway inflammation that leads to asthma, with associated with stimuli such as cigarette smoke, cold air, and air pollution although the underlying mechanism is unclear [51,

52].

Neutrophils identify microbes by a wide array of pathogen recognizing receptors (PRRs). One of the most well-known family of PRRs are the TLRs, which consist of extracellular surface receptors (TLR 1, 2, 4, 5, and 6) that primarily detect bacterial cell wall molecules and flagellin, and intracellular endosomal receptors (TLR 3, 7, 8, and 9) that detect nucleic acids [53]. Other families such as cytosolic nod-like receptors (NLRs), glycan detecting C-type lectin receptors (CLRs), and anti-viral retinoic acid-inducible gene (RIG)-like helicases are also expressed in neutrophils, making neutrophil not only capable of distinguishing different classes of pathogens, but also whether the pathogens are infecting intracellularly or extracellularly. This discrimination is crucial for neutrophils to activate tailored antimicrobial mechanisms and to limit excessive tissue damage [54]. Humoral immunity further promotes the elimination of pathogens by neutrophils, allowing neutrophils to effectively capture and kill microbes that are bound by opsonins such as immunoglobulins and complement factors, with Fcγ receptors (FcγRII and FcγRIII) recognizing IgG and complement receptors (CR1, CR3, C3aR, and C5aR) binding to C3a, C3b and C5a [55-57].

2.1.5 Killing mechanisms of neutrophils

By producing different chemokines and cytokines, neutrophils can shape their environment, either towards a more pro- or anti-inflammatory state and recruit or crosstalk with other cell types [46, 58]. Critically, neutrophils possess a range of killing functions to eliminate invading pathogens. In general, three main mechanisms of killing have been identified, which are phagocytosis, degranulation, and neutrophil extracellular traps [42].

Phagocytosis is one of the most described anti-microbial responses, where phagocytes like neutrophils undergo drastically cytoskeleton rearrangement resulting in microbe engulfment upon contact and containment within single-membrane phagosomes [59]. Phagosomes fuse with lysosomes, resulting in the production of oxygen radicals and the acidification of the compartment to induce bacterial killing [60, 61]. Besides classical phagocytosis, several alternative mechanisms may take place, including LC3-associated phagocytosis (LAP), in which phagosomes are decorated with LC3. LAP performs similar bactericidal activities to classical phagocytosis, however, the decoration of LC3 accelerates the recruitment of lysosome to enhance the degradation of phagosomal contents as well as killing efficiency. [62]. Some pathogens can escape the phagosome into cytoplasm, where

another antimicrobial mechanism called autophagy is activated to re-capture the cytosolic microbes into double-membraned autophagosomes, which will also fuse with lysosome to degrade the cargo inside it [63, 64].

Other than contact-based phagocytosis and intracellular killing, neutrophils have several types of granules which contain anti-microbial compounds including proteases, defensins, and peroxidases [65]. These granules are pre-synthesized at the different stages of myelopoiesis, ready for released into the extracellular environment upon stimulation, and play many roles in inflammatory responses [66]. For example, gelatinase granules degrade vascular basement membrane and promote transmigration of neutrophils to infected tissues [67]. However, this response can also induce extensive tissue damage and thus is highly regulated [66, 68].

A special type of cell death was described in 1996, showing phenotypes different from apoptosis such as decondensed chromatin and non-degraded organelles [69]. Further observations defined this specific programmed cell death as neutrophil extracellular traps (NET) formation, with chromatins released to form a web-like scaffold co-localized with concentrated antimicrobial compounds like neutrophil elastase (NE) and myeloperoxidase (MPO) [70]. NETs mediate extracellular killing against bacteria [71], fungi [72], and parasites [73]. Under circumstances in which

NETs cannot kill invading pathogens, the NET structure may still serve as a physical barrier to prevent dissemination of the microbes [74]. In addition to directly eliminating or neutralizing pathogens, NETs can also initiate or resolve inflammation, guiding the course of immune responses [75]. A broad array of microbes including bacteria, viruses, fungi, and parasites are reported to induce NETs, and many signaling pathways such as autophagy and inflammasome have been identified to participate in the induction of NETosis [70, 76, 77]. Pathogens that cannot be engulfed by neutrophils, either by being too large or possessing anti-phagocytosis proteins, are more likely to trigger NETs, a decision determined by Dectin-1 signaling pathway [78, 79]. Indeed, cells that undergo phagocytosis are inhibited from forming NETs, with NE and MPO translocated to phagosomes instead of to the nucleus [78, 80]. Other cellular mediators like reactive oxygen species (ROS) and calcium ion influx to cytoplasm are also involved in NET induction, yet these mechanisms are also generic immune responses that also participate in phagocytosis and degranulation and are not always be essential to NET formation [81-84]. Another molecule that has been associated with NET formation is peptidylarginine deiminases 4 (PAD4), an enzyme that citrullinates histones to promote de-condensation of chromatin, thus inducing NET [85]. However, studies have shown conflicted results regarding whether NET formation is dependent on PAD4 [86, 87], leaving precise understanding of the

regulation of NET still unknown. Besides bacterial stimulation and cellular factors, interactions between neutrophil and other immune factors like platelets, antibodies, and immune complexes also play a role in the release of NETs, either stimulating NETs on their own or enhancing microbe-induced NETs [88-90]. Excessive formation and dysregulation of NETs can damage host tissues similar to degranulation, but it poses additional threats such as the exposure of autoantigens in this inflammatory environment, potentially increasing the risk to autoimmune diseases [91]. The structures of aggregated NETs formed by multiple neutrophils can also clot blood vessels and lead to thrombosis [91]. Excessive NET formation also delays wound closure by inducing inflammation and occupying debris-processing macrophages, which leads to prolonged exposure to foreign antigens [92, 93]. This negative effect of NETosis in wound healing is particularly concerning in diabetic individuals, given that high glucose environment prepares neutrophil to release NET structure more robustly [94]. To avoid such pathogenic outcomes, NETs are strictly regulated. For instance, Siglec-9, a receptor that triggers inhibitory responses in neutrophils, recognizes host glycans such as poly sialic acids and suppresses NET formations [95]. In addition, the host clears NET structures rapidly, with degradation by DNase, opsonization by the complement system, and engulfing by macrophages, even without pro-inflammatory cytokine productions [96]. Such mechanisms have been

hijacked by microbes to evade NET-mediated killing, either by mimicking host glycans on their surface or directly take glycans from host cells to trigger inhibiting receptors, as well as secreting nucleases to degrade the chromatin scaffold of NET [97-99].

There exists extensive literature on the various anti-microbial mechanisms of neutrophils, which are that are highly regulated and effective against specific pathogens, however, our understanding of neutrophil responses when facing multiple bacterial species is very limited. Here, I first characterized the neutrophil responses triggered by mono-species infection of *E. faecalis*. I next examined co-infection of neutrophils with *S. aureus* and *E. faecalis*. Finally, I evaluated neutrophil-mediated killing in co-infection *in vitro* and *in vivo* to understand its impact on multispecies infection and persistence.

2.2 Results

2.2.1 Neutrophils control *E. faecalis* infection through phagocytosis and intracellular ROS production

Neutrophil-mediated *E. faecalis* killing is opsonin-dependent [57, 100], so I first tested whether normal mouse serum can opsonize *E. faecalis*. Incubating *E. faecalis* suspensions in normal mouse serum (NMS) collected from naïve mice formed aggregated particles that was not found in the HBSS-treated group, indicating there are natural antibodies recognizing *E. faecalis* in NMS **(Fig. 2.1A)**. All of the following neutrophil infection experiments were conducted in the presence of freshly harvested mouse serum for opsonization required to promote neutrophil-mediated inhibition of *E. faecalis* growth. In the presence of NMS, neutrophils significantly reduced the CFU of *E. faecalis* compared to the *E. faecalis* grown in the absence of neutrophils **(Fig. 2.1B)**. To identify the mechanism used by neutrophils to control *E. faecalis* infection, cytochalasin B and D were added in the medium to block polymerization of actin filaments, preventing neutrophils from undergoing phagocytosis. The addition of cytochalasin restored the CFU of *E. faecalis* **(Fig. 2.1B)**, indicating phagocytosis is the primary antimicrobial mechanism against *E. faecalis*.

Opsonized *E. faecalis* can be phagocytosed by neutrophils followed by intracellular ROS induction; however, the level of ROS production does not necessarily correlate with neutrophil-mediated killing [101, 102]. To first evaluate the role of ROS in the inhibition of *E. faecalis* in our infection model, we pretreated neutrophils with diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase, to prevent intracellular ROS production [103] before infection with *E. faecalis*. Compared to an approximately 80% reduction in *E. faecalis* CFU by vehicle-treated neutrophils, ROS inhibition resulted in only a 50% reduction in CFU **(Fig. 2.1C)**. I further measured intracellular reactive oxygen species (ROS) using the DCFDA Cellular ROS Detection Assay Kit. DCFDA measures oxidative stress, and by pre-incubating neutrophils with this chemical before infection with bacteria, I could specifically detect the intracellular ROS production within neutrophils. I infected neutrophils with *E. faecalis* at a multiplicity of infection (MOI) of 10 and observed a strong production of intracellular ROS to a level close to the positive control group that was treated with 55 µM of tert-butyl hydrogen peroxide (TBHP) **(Fig. 2.1D)**. By contrast, when neutrophils were pretreated with DPI, ROS production was significantly reduced **(Fig. 2.1D, E)**, indicating that ROS is involved in neutrophil-mediated antimicrobial function against *E. faecalis*.

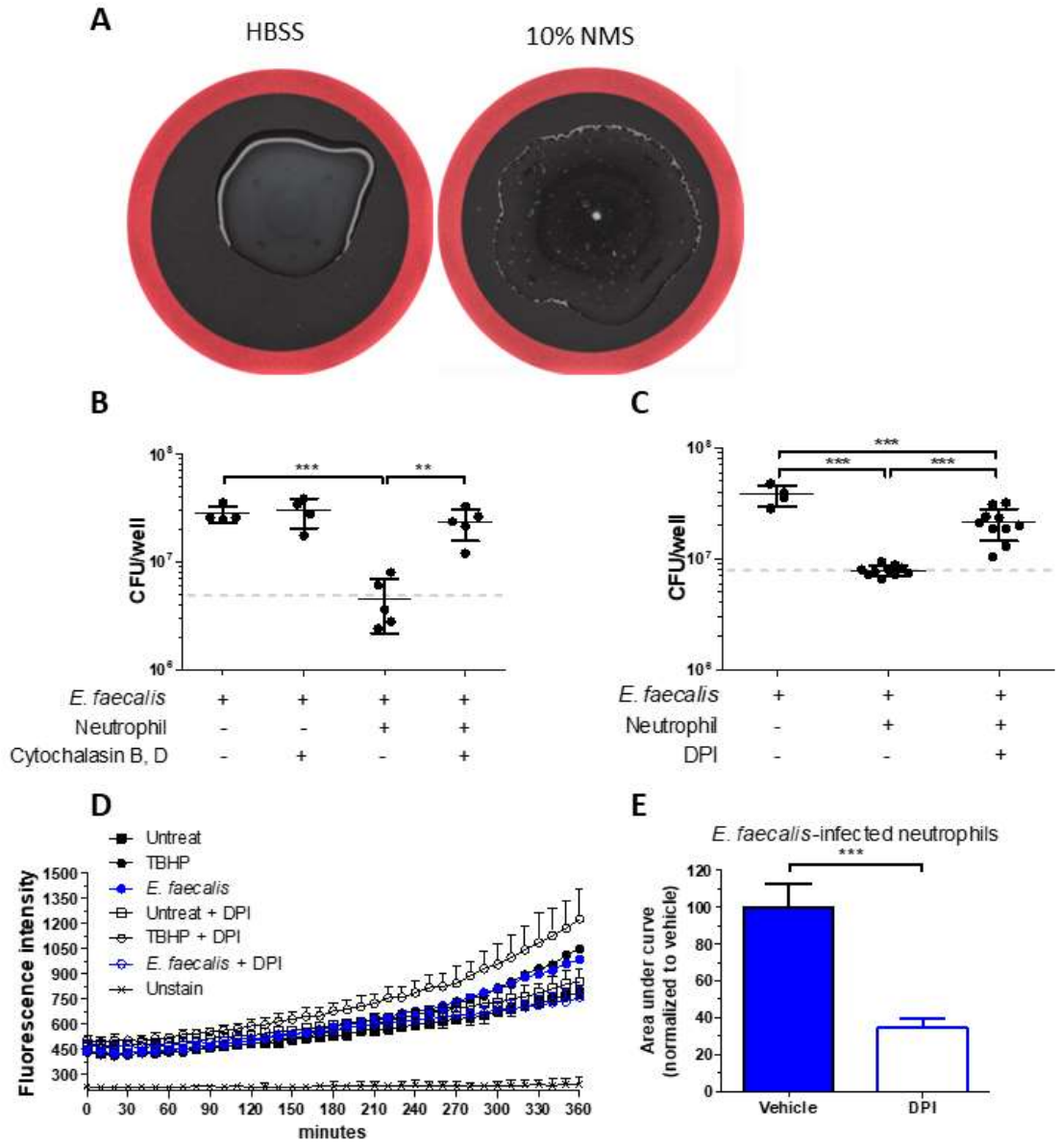

Figure 2.1 Neutrophils control *E. faecalis* infection through phagocytosis and intracellular ROS production

(A) Aggregation test of normal mouse serum with *E. faecalis* suspension. HBSS with (right) or without (left) 10% normal mouse serum was incubated with *E. faecalis* bacterial suspension for 30 minutes at room temperature. Aggregated particles suggest the presence of binding antibodies in the serum. (B-C) Bacterial CFU after incubated for 4 hours in the presence of neutrophils treated with indicated compounds. (B) *E. faecalis* infection with neutrophils for 4 hours in the presence of cytochalasin B and D (30 µM each). (C) Neutrophils pretreated with 20 µM of diphenyleneiodonium chloride (DPI) for 30 minutes before infected with *E. faecalis* for 4 hours. (D-E) Intracellular ROS production by neutrophils with or without DPI

pretreatment upon *E. faecalis* infection was recorded in time-lapse and one representative of 3 independent experiments is shown with TBHP used for positive control (D). Area under curve was compiled from 3 independent experiments to compare the overall production (E).All analyses are presented as mean ± SD. One-way ANOVA (B, C) or Student *t* test (E) was used to analyze statistical differences. **= $p < 0.01$, *** = $p < 0.001$.

2.2.2 *E. faecalis* reduces *S. aureus*-induced NETosis by interfering with histone citrullination

Given that neutrophils were able to clear *E. faecalis* in the presence of mouse serum **(Fig 2.1B)**, I next investigated the possible role of NET formation. NETosis is crucial in *S. aureus* clearance, and in some cases the presence of antibody is required [71, 89], which suggests the opsonin-dependent clearance of *E. faecalis* could be mediated by NETosis. However, the potential of *E. faecalis* to stimulate NETs is not known. The phenotype of NETosis is often characterized with three hallmarks: decondensed chromatin, the release of extracellular DNA (eDNA), and the association of granular compounds such as neutrophil elastase or myeloperoxidase with eDNA [104]. To evaluate NETosis induction, all three markers were analyzed by immunofluorescent microscopy. After *E. faecalis* infection, neutrophil nuclei exhibited ring-like shape with multiple lobes, similar to the resting state in uninfected controls **(Fig. 2.2A, 2.2C)**. By contrast, nuclear morphology became undefined after *S. aureus* infection, exhibiting significant chromatin de-condensation **(Fig. 2.2A, 2.2C)**. The level of extracellular DNA measured by Sytox Orange, a non-permeable DNA dye, showed similar results, with *S. aureus* infection leading to intense staining while the *E. faecalis* infected group was comparable to untreated control **(Fig. 2.2B, 2.2E)**.

Furthermore, extracellular neutrophil elastase, detected in non-permeabilized samples, and which is associated with eDNA, also showed similar results in that it was detectable after infection by *S. aureus* but not by *E. faecalis* **(Fig. 2.2A, 2.2D)**. With all three markers showing the lack of NETosis phenotype observed upon *E. faecalis* infection, I concluded that *E. faecalis* does not induce NETosis in mouse neutrophils, hence the neutrophil-mediated *E. faecalis* clearance I observed was also not mediated by NETs.

Besides the single infections of either *E. faecalis* or *S. aureus*, I also examined the response of neutrophil upon mixed infection of *E. faecalis* and *S. aureus* at ratio of 1:1, infecting neutrophils with 10^6 of *S. aureus* and 10^6 of *E. faecalis*. Surprisingly, not only did *E. faecalis* fail to induce NETosis by itself, but the presence of *E. faecalis* also mildly reduced the level of NETosis formation stimulated by *S. aureus* **(Fig. 2.2C, 2.2D,2.2E)**. In order to determine the mechanism by which *E. faecalis* reduced *S. aureus*-mediated NET formation, I started to investigate pathways that are known to regulate NET formation, to identify those that were affected by *E. faecalis* co-infection. One of the regulation steps for NETosis is the peptidylarginine deiminase 4 (PAD4)-driven citrullination of histones, which helps the de-condensation of chromatin [85]. PAD4 activity has been shown to be essential in NETosis induced by LPS, H_2O_2, PMA, and *Shigella flexneri* [87, 94], so I evaluated the level of histone

citrullination to determine whether co-infection interfered with this pathway. With Immunofluorescence (IF) imaging I observed that *E. faecalis* by itself did not stimulate histone citrullination **(Fig. 2.3A, 2.3B)**, which is not surprising considering *E. faecalis* did not promote NET formation. By contrast, *S. aureus* infection resulted in large amount of citrullinated histone, which is also consistent with the induced NETosis I observed **(Fig. 2.3A, 2.3B)**. Finally, co-infection with *E. faecalis* reduced the induction of histone citrullination by *S. aureus* **(Fig. 2.3A, 2.3B)**. The reduction was only partial, similar to the reduction of NETosis observed in **Figure 2.2.** Collectively, these results further demonstrate that *E. faecalis* does not stimulate NETosis. Instead, the presence of *E. faecalis* interferes with PAD4 activity and leads to reduced NETosis formation triggered by *S. aureus*. However, it was unclear whether the reduction of NETosis by *E. faecalis* was the result of an active suppressing mechanism from *E. faecalis* or simply due to the fact neutrophils underwent phagocytosis and are therefore unable to form NETs [78].

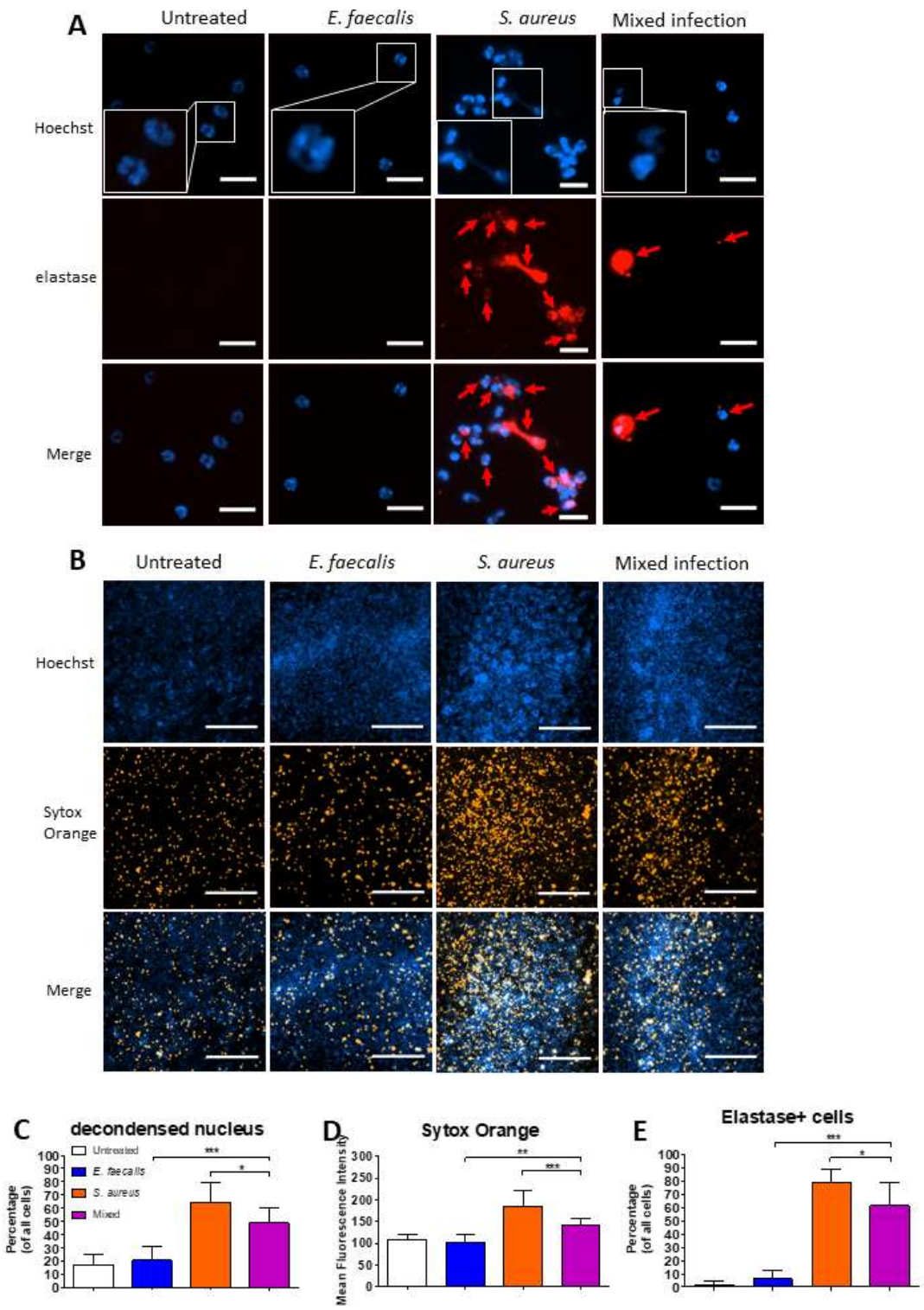

Figure 2.2 NETosis is triggered by *S. aureus* but not *E. faecalis*

(A-B) NET staining. Cells, after 4 hours of infection with MOI 10 of *E. faecalis*, MOI 10 of *S. aureus*, or both bacteria at MOI 10, were fixed with 4% paraformaldehyde. Externalized elastase (red arrows) and DNA were stained. Scale bar of (A) is 20 µm and (B) is 200 µm. (A) Elastase-releasing neutrophils (red) and total neutrophils (blue)

were counted to determine the percentage of NET-forming neutrophils and compiled into (E). Morphology of nucleus was also visually compared to identify neutrophils with decondensed chromatin and organized into (C). (B) Extracellular DNA was stained with Sytox Orange, a non-permeable DNA dye, under low-power field. Co-localization of pre-stained DNA (blue) and extracellular DNA (orange) can be observed (white). Mean fluorescent intensity was analyzed with ImageJ and quantified into (D). (C-E) All analysis were compiled from three independent experiments and presented as mean ± SD. One-way ANOVA was used to analyze statistical differences. *= $p < 0.05$,**= $p < 0.01$, *** = $p < 0.001$

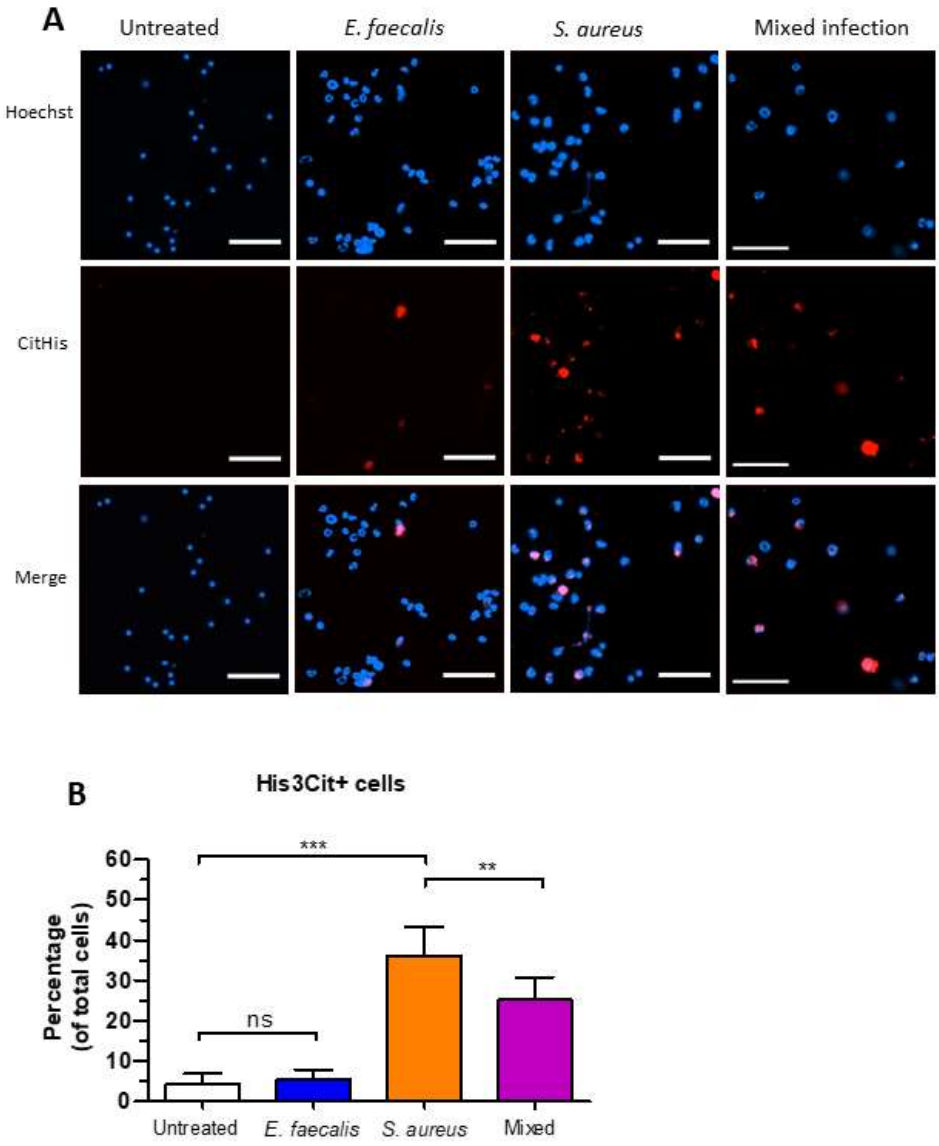

Figure 2.3 Histone citrullination is reduced in the presence of *E. faecalis*

(A-B) Cells were fixed and permeabilized before staining of citrullinated histone 3. (A) Neutrophils expressing citrullinated histone 3 (red) and total neutrophils (blue) were counted to determine the level of histone citrullination and compiled into (B). Analysis were compiled from three independent experiments and presented as mean ± SD. One-way ANOVA was used to analyze statistical differences. ** = $p < 0.01$. Scale bars represent 50 µm.

2.2.3 *E. faecalis*-mediated NETosis reduction is independent of ROS production, autophagy, and cell adhesion

In collaboration with PAD4-driven histone modification, there are many other factors which participate in the process of NETosis, the most studied being NADPH oxidase and subsequent ROS production. ROS, either produced intracellularly or extracellularly by neutrophils, can positively regulate NETosis [81, 82], so I hypothesized that *E. faecalis* may interfere with *S. aureus*-induced ROS production to reduce NETosis. To test the hypothesis, I used a cellular ROS detection assay to evaluate intracellular ROS production. In human neutrophils infection model, *S. aureus* induces two types of NETosis, a rapid induction which is ROS-independent, and the subsequent NETosis induction that can be partially suppressed when ROS is inhibited [71]. To our surprise, there was no intracellular ROS detected in neutrophils infected with *S. aureus* throughout the tested course **(Fig. 2.4A, 2.4B)**, suggesting that *S. aureus*-induced NETosis only relies on extracellular ROS production. Consistent with our observation that *E. faecalis* triggered intracellular ROS production **(Fig. 2.1D, 2.1E)**, here ROS was again highly produced by neutrophils upon *E. faecalis* infection **(Fig. 2.4A, 2.4B)**. Furthermore, ROS production in mixed infection with 10^6 of *S. aureus* and 10^6 of *E. faecalis* was reduced comparing to *E.*

faecalis single infection, suggesting that *S. aureus* can suppress *E. faecalis*-induced ROS production **(Fig. 2.4A, 2.4B)**. Based on these findings, I concluded that *S. aureus*-induced NETosis was not associated with the production of intracellular ROS from mouse neutrophils, and thus *E. faecalis* did not reduce NET formation by interfere with this specific host mechanism.

Another alternative trigger of NETosis is autophagy, which acts as secondary trigger for ROS stimulation [72, 83, 105]. Autophagy is characterized by the accumulation of LC3 onto phagosomes or autophagosomes, which then promotes the recruitment of endosomes and lysosomes for degradation of the compartment's contents. Using immunofluorescence imaging, LC3 appears as a cloudy pattern within resting cells and speckled pattern within autophagy-activated cells. Thus, by counting the cell population with LC3 foci, I can evaluate the level of autophagy in the population [106]. In our preliminary data, I observed that *S. aureus* infection induced a high percentage of the cells expressing LC3 foci, which was correlated with NET formation **(Fig. 2.4C, 2.4D)**. *E. faecalis* infection did not induced significant LC3 foci or autophagy compared to uninfected neutrophils **(Fig. 2.4C, 2.4D)**. In macrophages, *E. faecalis* can inhibit the activation of autophagy [107]. However, such suppression was not observed in mixed infection where 10^6 of *S. aureus* and 10^6 of *E. faecalis* were added to infect neutrophils, with most cells still formed LC3 foci **(Fig.**

2.4C, 2.4D), suggesting that *E. faecalis* did not inhibit autophagy in neutrophils, or that the inhibition is not strong enough to inhibit *S. aureus*-induced autophagy.

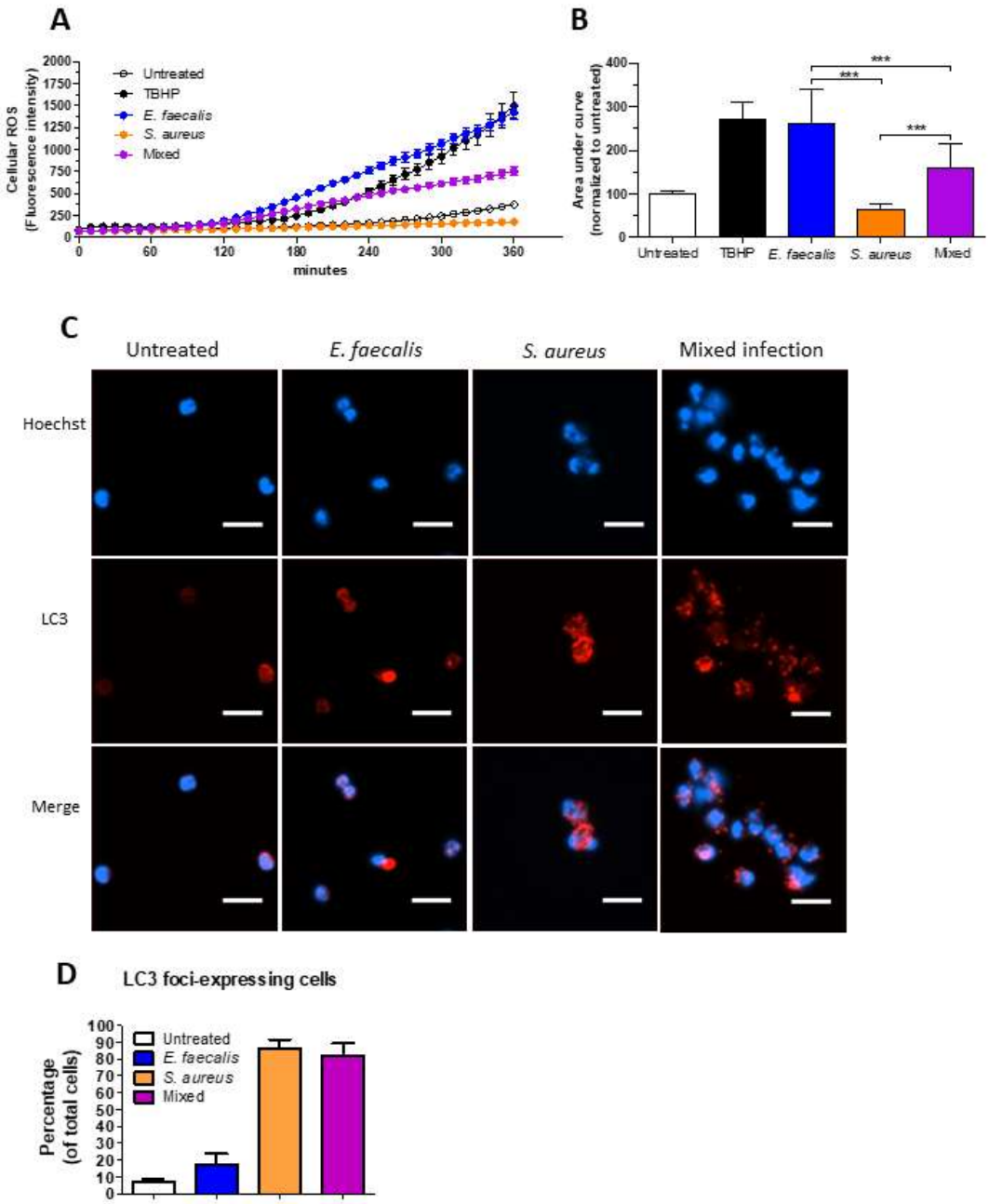

Figure 2.4 The reduction of NET formation in mixed infection was not due to inhibited ROS production or autophagy

(A-B) Intracellular ROS production upon bacterial infection was recorded in time-lapse and a representative of 3 independent experiments was shown with TBHP used for positive control (A). Area under curve was compiled from 3 experiments to compare the overall production (B). The data were analyzed with One-way ANOVA. *= $p < 0.05$, *** = $p < 0.001$. (C-D) Autophagy was determined with staining of LC3. (C-D) LC3 was spontaneously presence in neutrophils, distributed throughout cytoplasm. LC3 accumulated to form foci upon *S. aureus*- or mixed-infections, which

was recognized as activated autophagy (C) and the percentage of autophagy-activating neutrophils was calculated (D).

In an *in vitro* model, *Acinetobacter baumannii* can inhibit PMA-induced NET formation by blocking the adherence of neutrophils to the surface [108]. The phenotype of such suppression is characterized by low cell adherence to the plate surface *in vitro*, and reduced CD11a and increased CD11b expression on the cell surface. CD11a and CD11b are both components for different types of integrins, forming LFA-1 and Mac-1, respectively. Integrins are important to cell adherence and migration. To determine whether *E. faecalis* may be suppressing NET formation through a similar mechanism, I performed experiments to investigated how single and mixed infection affected surface levels of CD11a and CD11b by flow cytometry four hours after incubation. Upon infection of *E. faecalis*, *S. aureus*, or both, the surface level of CD11a on neutrophils showed no significant differences between untreated group and any infected group **(Fig. 2.5A, 2.5B)**, indicating that the role of this molecule may be limited in our model. Even though the surface level of CD11a was significantly higher in *S. aureus*-infected group than *E. faecalis*-infected group, no reduction was observed in mixed-infection group **(Fig. 2.5A, 2.5B)**. All neutrophils expressed CD11b, and the level of CD11b slightly increased upon bacterial infection **(Fig. 2.5C)**. However, only when infected with *E. faecalis* did neutrophils show significant increase of surface CD11b **(Fig. 2.5D)**. Finally, to determine the adherence of neutrophils upon *S. aureus* infection, the attached neutrophils after 4 hours of

infection were detached by EDTA for cell counting. Both *E. faecalis* and *S. aureus* infection led to increased attachment for neutrophils compared to the untreated group **(Fig. 2.5E)**. The number of the attached neutrophils in the mixed infection group was comparable to *S. aureus* single species infection, indicating that the presence of *E. faecalis* did not interfere with neutrophil adherence, thus this is not the mechanism used by *E. faecalis* to reduce NETosis **(Fig. 2.5E)**. Taken together, these data allow us to exclude ROS suppression, autophagy inhibition, and adhesion prevention as mechanisms responsible for *E. faecalis*-mediated suppression of NET formation.

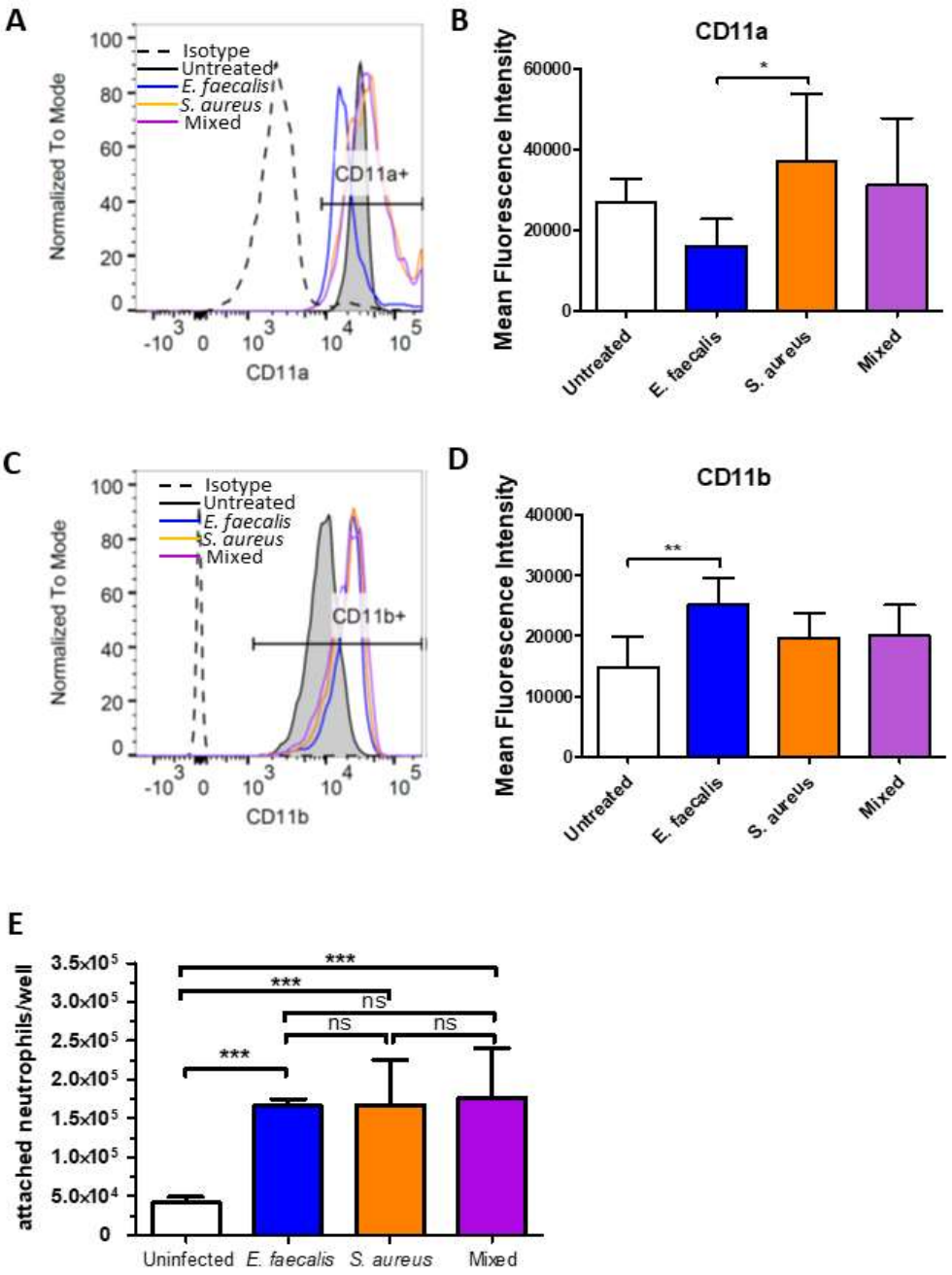

Figure 2.5 Attachment of neutrophils and integrin surface expression were comparable in mixed infection comparing to *S. aureus* infection

(A, C) After gated on neutrophils ($CD45^+$, $Ly6G^+$), expression levels of CD11a and CD11b were analyzed by flow cytometry after 4 hours of incubation. (B) and (D) are compiled mean fluorescent intensity among different conditions. 3-5 biological repeats were performed through 3 independent experiments. (E) Attached cells were collected and counted by cell counter after being infected for four hours. Graph was presented as mean ± SD with 3-5 independent experiments. All data were analyzed with One-way ANOVA. *= $p < 0.05$, **= $p < 0.01$, *** = $p < 0.001$.

2.2.4 Neutrophil-mediated killing against either *E. faecalis* or *S. aureus* was less effective in mixed infection

NET formation plays an important role in neutrophil-mediated *S. aureus* clearance *in vitro* [71]. Since I observed reduced NET formation in *E. faecalis* plus *S. aureus* mixed infection compared to *S. aureus* single species infection **(Fig. 2.2C, 2.2D,2.2E)**, I hypothesized that neutrophil-mediated killing against *S. aureus* may also be compromised when NET formation was reduced. Using a neutrophil-mediated bactericidal assay, I determined the killing efficiency against *S. aureus* and *E. faecalis* in single and mixed infections. CFU retrieved from the culture in the presence of neutrophils showed a small increase of *S. aureus* in mixed species infection comparing to single species infection, however the difference did not reach statistical significance **(Fig. 2.6A)**. In the absence of neutrophils, a slight decrease of *S. aureus* in mixed culture was observed, so I normalized the recovered CFU to that of the bacteria only groups. With this normalized analysis, I could see that mixed infection, with 10^6 of *S. aureus* and 10^6 of *E. faecalis*, promoted the viability of *S. aureus* from neutrophil-mediated killing **(Fig. 2.6B)**, presumably as a result of reduced NETosis in the mixed infection.

Unexpectedly, neutrophil-mediated killing against *E. faecalis* was also decreased in

the mixed infection compared to mono-species infection. The average CFU retrieved from *E. faecalis* single infections was below 7 million and from mixed infection was 12 million **(Fig. 2.6A)**, and the average viability of *E. faecalis* increased from 26% in single infection to 45% in mixed infection **(Fig. 2.6B)**. One explanation for higher *E. faecalis* CFU in the presence of *S. aureus* could be due to the suppression of intracellular ROS production by *S. aureus* in the mixed infection **(Fig. 2.4A, 2.4B)**. Another possibility for the impaired antimicrobial function could be due to cytotoxicity. *S. aureus* infection induced high level of neutrophil death, while *E. faecalis* showed even lower level of cytotoxicity than untreated group **(Fig 2.6C)**. In mixed-species infection, however, the level of cell death was lower than *S. aureus* single infection **(Fig 2.6C)**. The lower level of cell death in mixed-species infection correlated with higher CFU of *S. aureus*, indicating the form of neutrophil cell death induced by *S. aureus* is mostly NETosis instead of simply being killed by *S. aureus*. These data also suggest that *E. faecalis* is resistant to NET-mediated killing since I can measure NET release in the mixed infection, yet *E. faecalis* survival increased despite of the presence of NETs **(Fig. 2.2A, 2.2D)**. With these observations, I concluded that mixed *E. faecalis* and *S. aureus* infection promotes the persistence of both *S. aureus* and *E. faecalis*, potentially due to altered immune responses such as reduced NETosis and suppressed ROS production. This increased *S. aureus* survival was further

supported by *in vivo* wound infection model, in which an excisional wound was created on the back of each mouse prior to infection with indicated inoculum [109]. Unpublished data from our lab shows that single species infection with *S. aureus* colonizes the wound tissue much better than *E. faecalis*, resulting in a several hundred folds difference in recovered CFU. Based on that observation, I used different inoculum for *S. aureus* and *E. faecalis* in order to better balance the infection outcome **(Fig. 2.6D**, inoculum indicated by horizontal gray bars**)**. The bacterial load of *S. aureus* in wound tissue after 24 hours was more than 10-fold greater in mixed infection compared to single infection while *E. faecalis* CFU only increased slightly **(Fig. 2.6D)**.

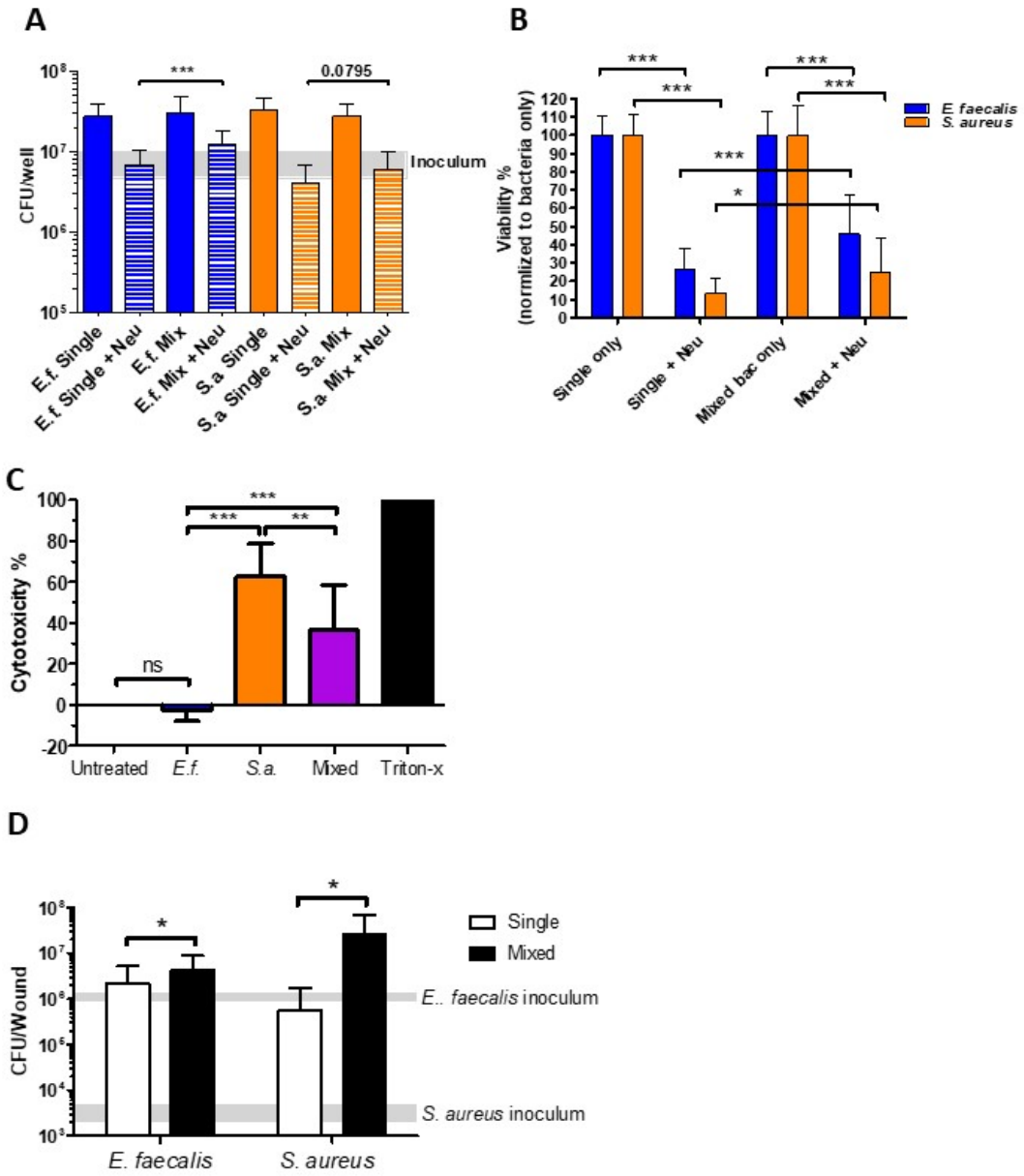

Figure 2.6 Mixed infection promotes bacterial survival both *in vitro* and *in vivo*

(A-B) Bacterial survival was determined after *in vitro* incubation for four hours with neutrophil by CFU enumeration and viability, respectively. (A) CFU of *E. faecalis* were presented in blue bar and *S. aureus* in red. The striped bars represent bacteria incubated with neutrophils, and the grey shade indicates inoculum. (B) Viability of bacteria was calculated by normalizing to corresponding groups without neutrophils. Analysis was compiled from 6 independent experiments and presented as mean ± SD. (C) Cell death of neutrophils was measured by ATPlite assay. Neutrophils incubated with indicated treatments for 4 hours and supernatants were collected for ATP detection. For each independent experiment, untreated group was used as 0% of

cell death and triton-x group was used for 100%. Data shown are a combination of 3 independent experiments were performed with total of 7 biological replicates. (D) CFU of bacteria retrieved from mouse wound tissues 24 hours after infection were counted and compared between single and mixed infection, with the grey shades indicating inoculum of each microbe. 3 independent animal experiments were repeated with total 10 to 12 mice per group. Data were analyzed with One-way ANOVA (C) or Student t test (A, B, D). *= $p < 0.05$, **= $p < 0.01$, *** = $p < 0.001$.

2.2.5 *S. aureus* and *E. faecalis* induce similar neutrophil degranulation profile in single and mixed infections

In addition to performing phagocytosis to kill pathogens intracellularly or undergoing NET formation to trap invading microbes, neutrophils are also equipped with various granules that contains different sets of antimicrobial compounds, as described in the introduction session **(2.1.5)** [65, 110]. To further characterize the neutrophil responses to infection, I used the same *in vitro* infection assay to run experiments to assess the degranulation profile within 4 hours after infection. Degranulation can be detected and distinguished by flow cytometry because specific markers are expressed on cell surface when granules are fused with cell membrane [111]. CD63, a marker for azurophil granules, was significantly increased after any type of infection, with especially high levels in the presence of *S. aureus* **(Fig. 2.7A)**. Similarly, CD15, a marker for specific granules, showed a slight increase after any type of infection **(Fig. 2.7B)**. Finally, CD14 and CD16 were used as indicators of secretory granule release. While the level of CD14 did not change significantly among all groups, the surface expression of CD16 was reduced after any type of infection compared to the untreated group **(Fig. 2.7C, D)**. No significant alteration was observed in any of the markers of degranulation except CD63 in mixed infection

group compared to either *E. faecalis* or *S. aureus* single species infection **(Fig. 2.7A-D)**. Together, these initial results showed very similar degranulation profiles stimulated by *S. aureus*, *E. faecalis*, and mixed infection, with a strong release of azurophil granules, a slight release of specific granules, and no release of secretory granules.

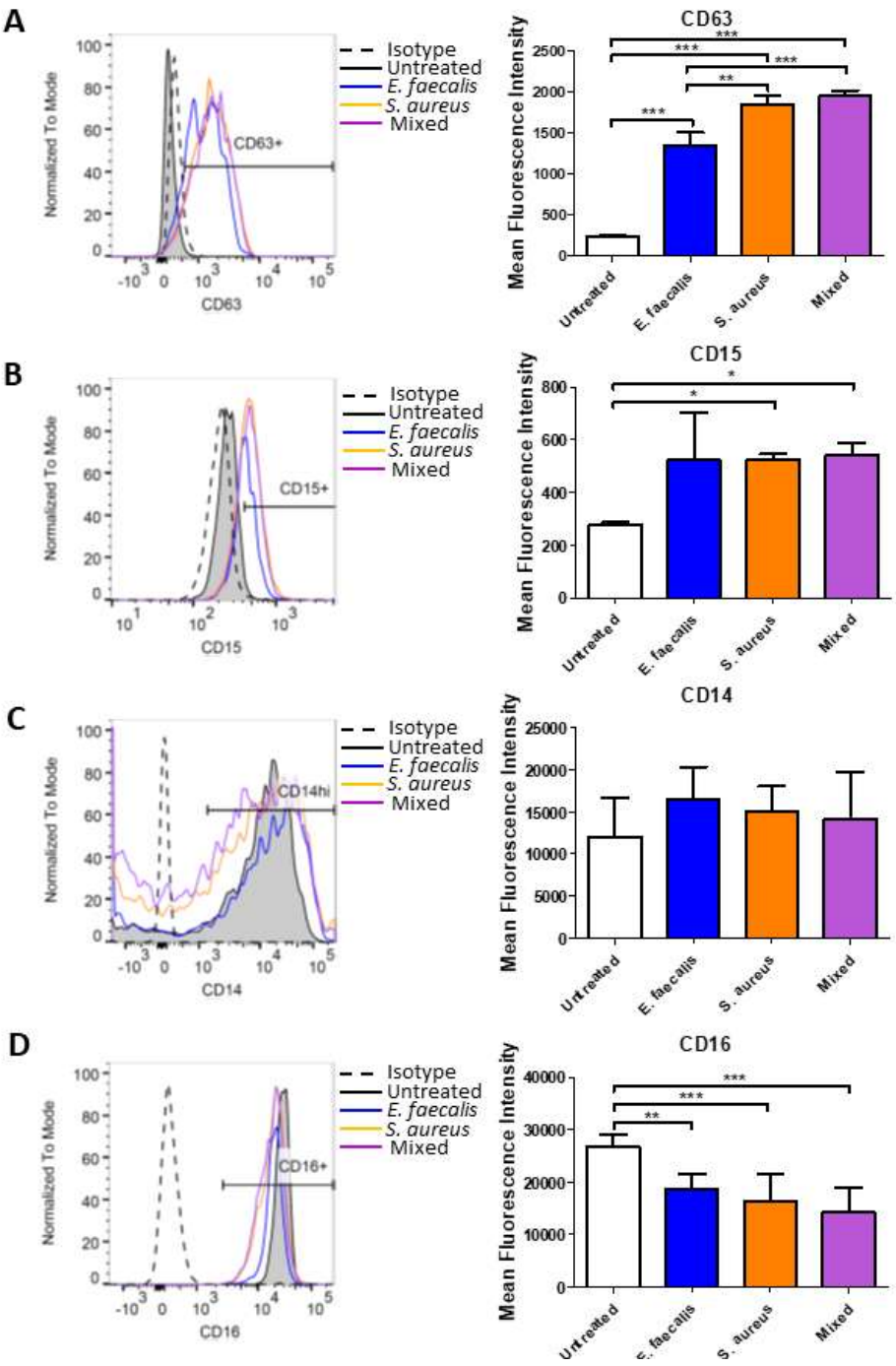

Figure 2.7 Degranulation patterns are similar among all infected conditions, showing no suppression in mixed infection

(A-D) Degranulation of neutrophils was evaluated by surface level of granule markers. Surface levels of CD63 (A), CD15 (B), CD14 (C), and CD16 (D) on neutrophils (gated on $CD45^+$, $Ly6G^+$) were analyzed by flow cytometry 4 hours after indicated treatments. Histogram (left) and mean fluorescent intensity (right) of each marker is shown. 3-5 biological repeats collected through 3 independent experiments were analyzed with One-way ANOVA. *= $p < 0.05$, **= $p < 0.01$, *** = $p < 0.001$.

2.2.6 In mixed-ratio infection, the phenotype of neutrophils resembled the single-species infection of the species at higher inoculum

Previously I showed that neutrophils exhibit pathogen-specific responses, namely NETosis for *S. aureus* **(Fig. 2.2)** infection and intracellular ROS production for *E. faecalis* infection **(Fig. 2.4)**, and each of these responses was reduced in mixed-species infection. To understand whether one of the two co-infecting species had a dominant impact on the impairment of neutrophil bactericidal activity, we reduced one species in the inoculum by a factor of ten and assessed bacterial CFU post infection. For ROS production, lowering the *E. faecalis* inoculum to MOI 1 resulted in delayed and lower ROS levels, which was still significantly decreased in the presence of *S. aureus* MOI 10 **(Fig. 2.8A, B)**. By contrast, when *S. aureus* was outnumbered by *E. faecalis* 1:10, ROS production in co-infection was comparable to the *E. faecalis* single-species infection **(Fig. 2.8C, D)**. These results show that neutrophils respond to *E. faecalis* with intracellular ROS production, which can be reduced in a *S. aureus*-dose-dependent manner. Together these data suggest that *S. aureus*-mediated reduction of ROS, which is normally induced by *E. faecalis* and contributes to its inhibition, can promote *E. faecalis* survival during co-infection.

Similarly, *E. faecalis*-mediated NETosis reduction was lost when its ratio in the

inoculum was reduced to MOI 1 **(Fig. 2.8E)**. Reducing the *S. aureus* MOI to 1 resulted in fewer neutrophils undergoing NETosis as assessed by decondensed chromatin, as compared to MOI 10 (~50% to 30%, respectively), and this number was significantly reduced during co-infection with *E. faecalis*, to levels comparable to *E. faecalis* single-species infection **(Fig. 2.8F)**. These data show that *E. faecalis* interferes with *S. aureus*-induced NETosis during co-infection, which explains why co-infection with *E. faecalis* results in increased *S. aureus* survival.

Lastly, the CFU of *E. faecalis* and *S. aureus* were measured to evaluate the antimicrobial functions of neutrophils in mixed-ratio infection. When infected neutrophils with *E. faecalis* at MOI 1 with *S. aureus* at MOI 10, I observed that only *E. faecalis* CFU significantly increased in the co-infection, whereas *S. aureus* CFU remained comparable between single- and mixed-species infections **(Fig. 2.9A, B)**. By contrast, when we infected neutrophils with *E. faecalis* at MOI 10 and *S. aureus* at MOI 1, *S. aureus* CFU were significantly increased compared to the single-species infection and *E. faecalis* CFU remained unchanged **(Fig. 2.9C, D)**. Together, these results suggest that phenotypes of neutrophil are determined by the predominant species, and the pathogen-specific response focus on eliminating the predominant species while favoring the survival of the less abundant inoculating species.

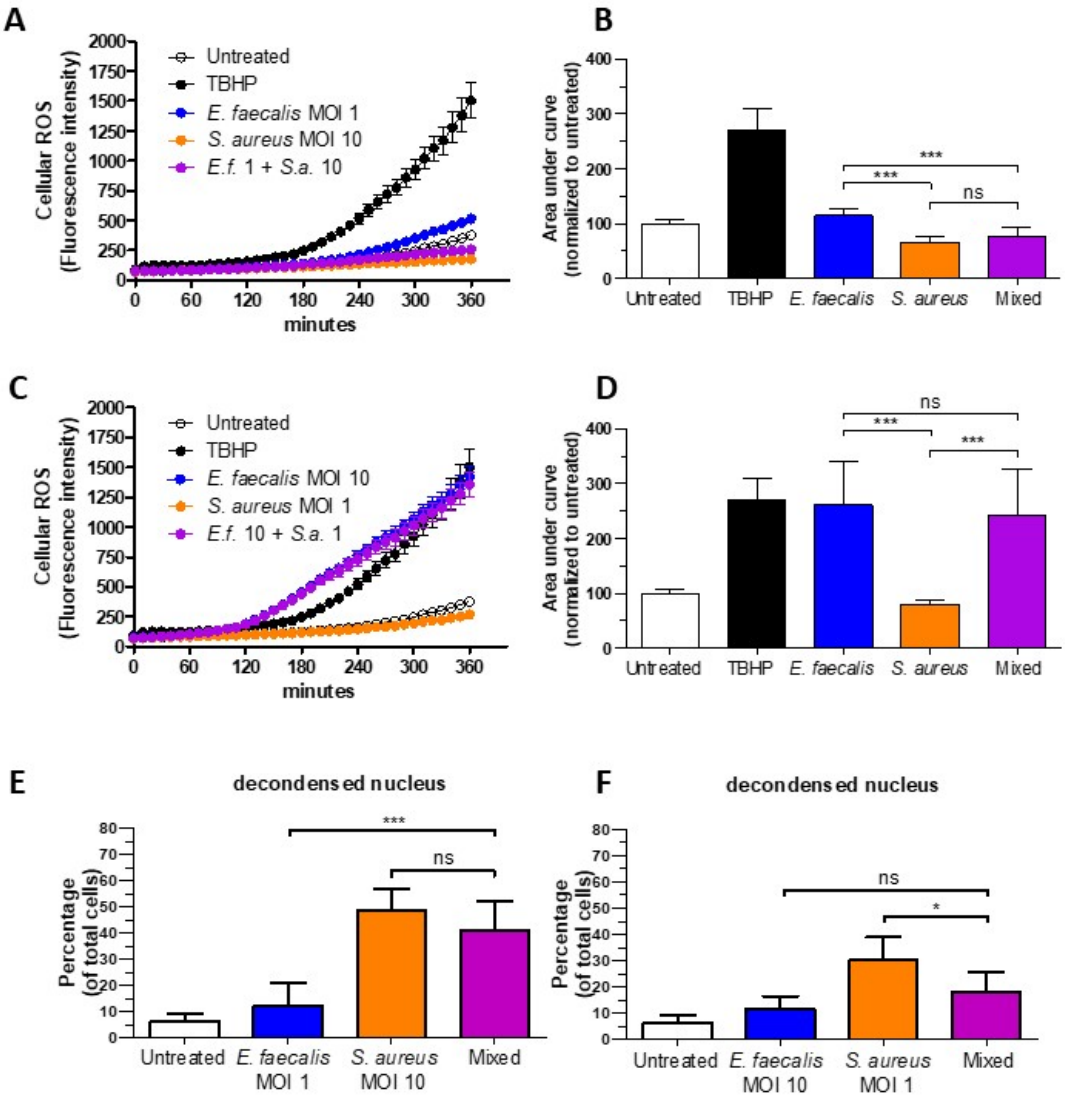

Figure 2.8 Neutrophil phenotypes in mixed-ratio infection resembled the single-species infection with higher inoculum

(A-D) Intracellular ROS production upon mixed-ratio bacterial infection was recorded in time-lapse and a representative of 3 independent experiments was shown with TBHP used for positive control (A, C). Area under curve was compiled from 3 experiments to compare the overall production (B, D). (E-F) Morphology of neutrophil nucleus was examined to evaluate the level of NETosis after 4-hour incubation with different inoculum of E. faecalis and/or S. aureus. (E) MOI 1 of *E. faecalis* and MOI 10 of *S. aureus* were used for infection. (F) MOI 10 of *E. faecalis* and MOI 1 of *S. aureus* were used for infection. All quantified results are presented with means ± SD. Statistics was analyzed with one-way ANOVA and differences were considered significant for * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ns = not significant.

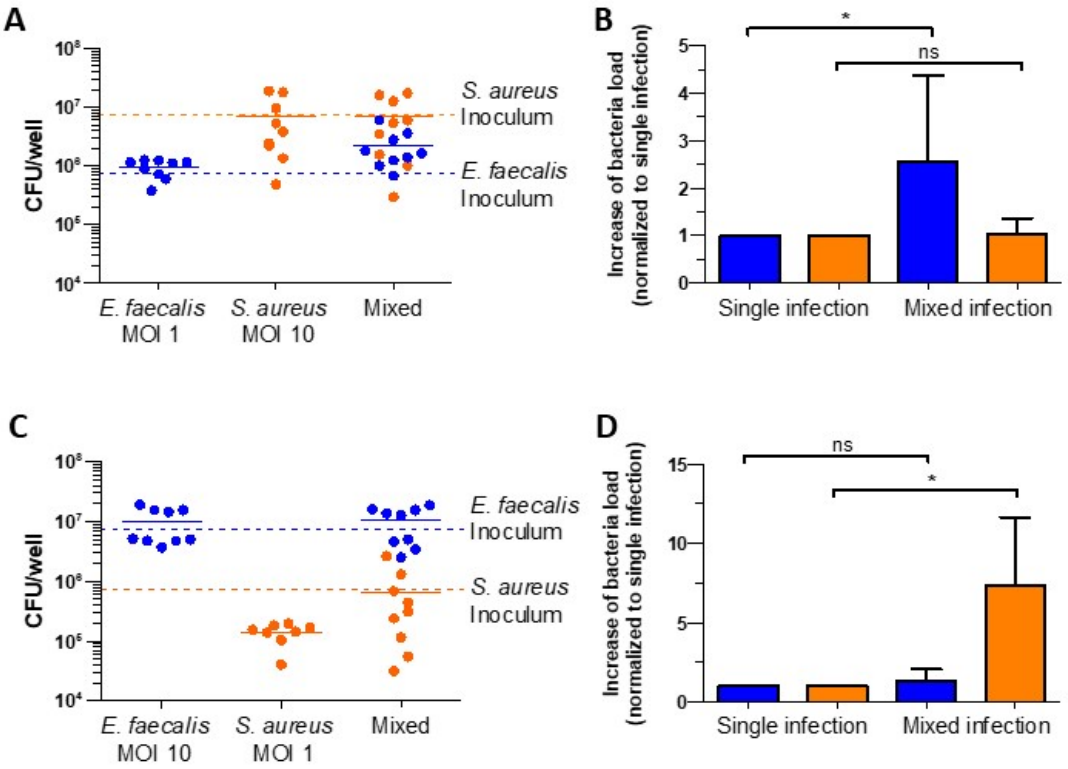

Figure 2.9 Mixed-species infection results in impaired neutrophil-mediated inhibition, favoring the less abundant inoculating species

(A-B) CFU (A) and fold-change (B) of E. faecalis (MOI 1), S. aureus (MOI 10), or mixed-infection (MOI E. faecalis 1 + MOI S. aureus 10). (C-D) CFU (C) and fold-change (D) of E. faecalis (MOI 10), S. aureus (MOI 1), or mixed-infection (MOI E. faecalis 10 + MOI S. aureus 1). (A,C) Horizontal bars represent CFU means and dotted lines to indicate bacterial inoculum. (B,D) Bars represent means ± SD for fold change relative to each single-species infection. Differences between mixed- and single-species infection were analyzed by paired t-test within each species and differences were considered significant for * $p<0.05$. ns = not significant.

2.3 Discussion and conclusion

Understanding how the immune system responds to multi-species bacterial communities is a crucial first step towards the development of therapeutic strategies. This is particularly pressing given the high prevalence of polymicrobial infections within chronic wound sites, such as diabetic lower limb ulcers [22, 23]. Here I show that neutrophils respond differently to different pathogens. Upon *S. aureus* infection, for instance, neutrophils underwent NETosis with hallmarks like decondensed chromatin, extracellular DNA, and associated elastase **(Fig. 2.2)**. In contrast, *E. faecalis* infection did not induce any NETosis, and instead promoted a large amount of intracellular ROS production in the neutrophils **(Fig. 2.4)**. When neutrophils face two microbes at the same time, however, specific responses such as NETosis and intracellular ROS production were both compromised compared to single infections. The compromised responses may have led to impaired clearance against microbes, potentially contributing to the virulence of this polymicrobial infection. This type of interactions among multiple microbes and host defense system has not been well-studied in the current literature, but further understanding could lead to novel treating strategies.

While *E. faecalis* induced strong intracellular ROS production, it did not induce

significant NET formation, which is surprising considering the central role of ROS in NET formation [81, 82]. Neutrophils undergoing phagocytosis will translocate elastase and myeloperoxidase, both are important for NET formation, to the phagolysosome and sequestered from neutrophil nucleus, leading to prevention of NETosis [112]. ROS induced upon *E. faecalis* infection may have a similar spatial separation, only concentrated or produced within the phagosomes, thus unable to contribute to NETosis. This compartmental organization could also explain the *E. faecalis*-mediated interference of *S. aureus*-induced NETosis, containing crucial enzymes from inducing NET. However, more studies are required to understand whether the phagocytosis of *E. faecalis* is responsible for the impaired NETosis response or the formation of NET is actively suppressed by *E. faecalis*. One potential active mechanism for *E. faecalis* to reduce *S. aureus*-induced NETosis is by degrading the eDNA structure with nuclease, which is reported to be utilized by Streptococci [113]. *E. faecalis* can express several types of exo- and endonucleases, together with the fact that eDNA is an important ingredient of *E. faecalis* biofilm [114], suggesting that *E. faecalis* could degrade eDNA to free itself. However, no reports indicate that *E. faecalis* secretes nucleases to extracellular environment, making the theory of *E. faecalis* escape NETosis through self-produced nucleases unlikely. Coincident with reduced NET formation, I also observed lower level of histone citrullination,

suggesting PAD4 activity may be the mechanism compromised by *E. faecalis,* which eventually leads to reduction of NETosis. Studies have shown *S. aureus* infection can potentially activate PAD4 [115, 116], but it is unclear whether PAD4 is essential for *S. aureus*-induced NETosis, so it would be helpful to first determine if *S. aureus*-induced NET is PAD4-dependent before investigating mechanisms of *E. faecalis*-mediated PAD4 inhibition. We may also gain insights into infection dynamics by infecting neutrophils with *E. faecalis* and *S. aureus* in a time-staggered manner to investigate the dynamic among cells and two microbes. For instance, if neutrophils are exposed to *S. aureus* first and pre-form NETs before *E. faecalis* infection, it could allow even better survival of *E. faecalis* and potentially promote *E. faecalis* biofilm formation by enhancing microbe attachment. Another piece of information that could be useful is the spatial distribution of each microbe. However, only partial of the fluorescence bearing *E. faecalis* shows strong signal, making the comparison of localization unreliable.

Neutrophil-mediated clearance against *E. faecalis* is poorly studied, with only the crucial role of opsonin having been addressed, where the presence of antibodies or complement are essential for effective bactericidal activity [57, 117]. Whether neutrophils kill *E. faecalis* primarily via intracellular or extracellular mechanism is unclear due to the lack of investigation. In this study, I studied neutrophil responses

such as degranulation, intracellular ROS production, and NET formation upon *E. faecalis* infection as a first step in understanding *E. faecalis*-induced neutrophil activation, as none of this work had been done previously. Among all of the phenotypes I examined, only intracellular ROS production was correlated with *E. faecalis* killing, where single species infection with *E. faecalis* induced strong intracellular ROS production and efficient killing by the neutrophils, and mixed infection showed reduced reaction for both ROS production and bacterial clearance. Another interesting observation regarding ROS production is that *S. aureus* was capable of suppressing *E. faecalis*-induced intracellular ROS induction. Several *S. aureus*-derived factors like SaeR/S-regulated factors and lipoic acids have shown suppressive effects towards ROS productions in neutrophils and macrophages [118, 119]. In addition, NETosis may also interfere the intracellular ROS accumulation by inhibiting phagocytosis and emphasizing on producing ROS extracellularly [81, 112]. Either way, identification of the underlying mechanisms *S. aureus*-mediated reduction of intracellular ROS could provide targets for therapies in polymicrobial infections. In our initial tests, the neutrophil degranulation profiles upon infection did not show distinct patterns in either *E. faecalis* or *S. aureus* single infections or in the mixed infection **(Fig. 2.7)**. In addition to repeating the experiments for more conclusive results, additional markers for each type of granules can be used in order

to validate the results. Lastly, *E. faecalis* appear to tolerate NET-mediated killing in mixed infection, despite the effective elimination of *S. aureus* by the same NETs. Resistance to NET-mediated killing could explain the prevalence of *E. faecalis* in chronic and polymicrobial wound infections given NETosis can be triggered by several types of microbes or even spontaneously upon tissue damage. It is unclear whether *E. faecalis* achieve that by degrading DNA scaffold to escape, as is the case for Streptococci [120], being insensitive to anti-microbial compounds in NET structure, or avoiding being trapped all together. Future directions should determine the mechanism by which of *E. faecalis* avoids NET-mediated killing and whether NETosis, despite not capable of killing *E. faecalis*, can prevent dissemination of this microbe.

Using an *in vivo* model of wound infection, I observed an increased *S. aureus* bacteria load in mixed infection comparing to single infection, which is consistent with the results of *in vitro* model **(Fig. 2.6)**. To better align the *in vivo* model with *in vitro* findings, future investigation regarding the levels of NETosis within the wound tissue under mixed infection comparing to single infection should be performed. In addition, the impact of this early increase in bacterial CFU on later timepoints and chronic, persistent wound infection remains to be determined. It is possible that polymicrobial infection, and associated synergistic effects, could promote persistence or even dissemination, so an examination of CFU at extended timepoints and at

other body sites is warranted. Moreover, it is unclear whether the *in vivo* phenotypes I observed are due to suppression of specific neutrophil responses in the same way as *in vitro* models. Quantifying NET formation at wound and phagocytosis by infiltrating neutrophils could provide more insights on why I observe a synergistic increase in both *E. faecalis* and *S. aureus* CFU in wound infections. However, the pathways required for clearance of microbes and for wound healing can sometimes conflict with each other. NETosis has been reported to delay wound closure in sterile conditions, and treatment of DNase to remove NET structure enhances wound healing [94]. *E. faecalis* has been reported to associate with improvement of diabetic foot ulcer [24], but also a delay in wound healing in mouse models of wound infection[109], raising the question of how the potential self-protective role of NET suppression relates to both wound healing and the progression of polymicrobial infection. In addition, it is unclear whether human neutrophils will respond to *E. faecalis* the same way as mouse neutrophils respond, and it will be interesting to repeat the assays performed in this study with primary human cells. *E. faecalis* also possesses many other immunomodulating effects and are associated with chronic infections despite under inflamed tissues, which will be described more in **Chapter 3**, where we focus on the interactions between *E. faecalis* and macrophages. Macrophages are the second wave of infiltrating cells that orchestrate more

sophisticated immune responses until the inflammation is resolved. Understanding evading mechanisms utilized by *E. faecalis* to escape both neutrophils and macrophages challenges will give us a big picture of how *E. faecalis* exploit innate immunity to eventually establish persistent infection.

2.4 Material and Method

2.4.1 Bacterial strains and cultures

Staphylococcus aureus strain USA 300 was grown in tryptic soy broth (TSB), and *Enterococcus faecalis* OG1RF was grown in brain heart infusion (BHI) medium. To prepare the bacterial inoculum, a single colony was picked and grown in 5 ml of liquid broth for 16-20 hours at 37°C, ambient air conditions. *S. aureus* cultures were washed and adjusted in PBS to $O.D._{600\ nm}$= 1.5 (5 x 10^8 CFU/ml) and *E. faecalis* to $O.D._{600\ nm}$= 0.5 (3 x 10^8 CFU/ml) before diluted to indicated CFU or multiplicity of infection (MOI).

2.4.2 Colony forming units (CFU) enumeration

Bacterial suspension was collected with careful pipetting, and serially diluted 10-fold in PBS to achieve 10^7 -dilution. To measure colony forming units (CFU), TSB agar plates were used for *S. aureus* alone and BHI agar plates for *E. faecalis* alone. For mixed culture of *S. aureus* (USA300) and *E. faecalis* (OG1RF), MRSAII selective plate (BIO-RAD, #63757) was used for *S. aureus* and BHI plates with 25µg/ml of rifampicin for *E. faecalis*.

2.4.3 Aggregation test

Bacterial suspension was normalized to $O.D._{600\ nm}$= 0.5 (3 x 10^8 CFU/ml), and 100 µl of bacterial suspension was mixed with either HBSS or HBSS with 10% of NMS. The mixture was spread on Streptex/Wellcogen Disposable Card (Thermo Scientific, #R30368601) and incubated on shaker for 30 minutes at room temperature. Pictures were taken to check for aggregated particles.

2.4.4 Mouse neutrophil isolation

Bone marrow from C57BL/6 mice were used for neutrophil collection. Tibia and femur of the hind limbs were collected to flush the marrow with ice cold phosphate-buffered saline (PBS). To purify neutrophil from bone marrow cells, magnetic-activated cell sorting (MACS) technique was performed by using LS column (Miltenyi Biotec, # 130-042-401) and mouse neutrophil isolation kit (Biolegend, #480058) following the manufacturer's instructions. In short, the cells were suspended in sorting buffer and labeled with biotin-antibody cocktail for 15 minutes on ice. After being washed and resuspended, cells were incubated with streptavidin-conjugated nanobeads for another 15 minutes on ice before adding to the LS column installed on magnetic separator and the flowthrough would be collected as purified neutrophils. In general, 4-6 x 10^6 neutrophils were collected from one mouse. After isolation,

neutrophils were rested in Hank's balance salt solution (HBSS) with 10% of mouse serum at 37°C for 30 to 60 minutes before further stimulations or infections.

2.4.5 *In vitro* infection assay

Assay was modified from previously described model [71]. In short, plates for infection assays were coated with 10% fetal bovine serum at 4°C for overnight. The next day bacteria were pre-incubated in HBSS with 10% of mouse serum for 30 minutes on ice before being washed and normalized in HBSS to indicated MOI. After resting, neutrophils were seeded to each well in HBSS with 10% of mouse serum (8 x 10^5/well for 24-well plate and 1 x 10^5/well for 96-well plate). Neutrophils were then infected with indicated bacteria in the same volume of HBSS. The culture was incubated in HBSS with final concentration of 5% mouse serum at 37 °C before proceeding to different readouts: 4 hours incubation before plating bacteria in bacterial killing assays and bioimaging assays, 6 hours incubation in plate reader for ROS detection assay, or indicated time before staining of neutrophil for flow cytometry. For heat-inactivated serum, the serum was inactivated in a 55 °C water bath for 30 minutes before adding to HBSS and served as incubation medium of infection assay.

For ROS inhibition, neutrophils were pretreated with 20 μM of DPI (Sigma-Aldrich,

#D2926-10MG) for 30 minutes at 37°C before the cell suspension was washed in PBS and seeded to each well. The cells were then rested at 37°C for another 30 minutes followed by *E. faecalis* infection. For phagocytosis inhibition, cytochalasin B and D (30 µM each, abcam #ab143482 and #ab143484) were added to neutrophils prior to seeding the cells for an hour of resting at 37°C, followed by *E. faecalis* infection.

2.4.6 Cytotoxicity assay

For cytotoxicity assay, ATPlite 1step Luminescence Assay System (PerkinElmer, #6016736) was used to detect ATP released upon cell death. Supernatants of cells with indicated treatments were collected and stored at -80°C until detection. Samples were thawed and assay was performed according to the manufacturer's instructions. In short, 100 µl of the supernatants were added to 100 µl of the assay reagent in white-well plate. The plate was kept in dark and left on a shaker for 5 minutes at room temperature before being detected by plate reader (Tecan Infinite® 200 PRO spectrophotometer) for the intensity of luminescence. For the calculation of percentage, untreated group was used as 0% cell death while cells incubated with 0.1% triton-x were considered 100% cell death.

2.4.7 Immunofluorescence microscopy

For immunofluorescence staining, cells were seeded in either glass-bottom plates or wells with cover slides inside. After indicated stimulation, the cells were fixed with 4% paraformaldehyde (Biolegend, #420801) for 10 minutes at room temperature. For the staining of citrullinated histone 3 and autophagy marker LC3B, cells were then permeabilized with 10-minute incubation of 0.1% triton-x in PBS. After carefully washing with PBS, the samples were incubated with 2% bovine serum albumin in PBS for 1 hour at room temperature to block the nonspecific binding. Samples were then incubated overnight at 4 ^{o}C with primary antibody (neutrophil elastase, Abcam #ab68672; LC3B, Abcam #ab48394; citrullinated histone 3, Abcam #ab5103; all were used at 1 : 500 dilution). On the second day, samples were incubated with secondary antibody for 1 hour at room temperature in dark. Finally, Hoechst 33342 (100 ng/ml) was used to stain the nucleus for 15 minutes at room temperature. Images were taken with Carl Zeiss Axio Observer and cells in each field of view were manually counted. For each sample, at least 10 images were taken and over 100 cells were counted to a single data.

2.4.8 Extracellular DNA staining and reactive oxidative species (ROS) production

For extracellular DNA staining, neutrophils were pre-stained with Hoechst 33342 (100 ng/ml) for 45 minutes in dark at 37 ^{o}C after being purified from bone marrow. The cells were then washed and co-cultured with bacteria in the presence of 500 nM of Sytox Orange (ThermoFisher #S11368). After 4 hours of incubation at 37°C, cells were washed and fixed for microscopy. One image per sample were taken at low power field and were then analyzed with ImageJ, by which the mean fluorescence intensity of the Sytox Orange signal was calculated.

To detect ROS production, DCFDA / H2DCFDA – Cellular ROS Assay Kit (Abcam # ab113851) was used according to manufacturer's instructions. Neutrophils were stained by DCFDA for 45 minutes after collection. Cells were seeded into black-wall glass-bottom 96-well plates (ibidi #89626) and placed at plate reader (Tecan Infinite® 200 PRO spectrophotometer) immediately after addition of stimuli. Placed in plate readers set at 37 ^{o}C with Ex/Em of 485/535, the signals were recorded for 6 hours with 10 minute-interval. Time-lapse data was analyzed by Graphpad Prism 9 and the area under curve was calculated with Excel.

2.4.9 Cell attachment

To detect cell attachment, neutrophil first underwent *S. aureus* or mixed infection for 4 hours as stated previously (2.4.4). Non-attaching cells were removed along with supernatant before 5 nM of EDTA in PBS was added to the wells and the plate was incubated for 5 minutes at 37 ^{o}C. Each well was then gently pipetted to detach the remaining neutrophils and 10 µl of cell suspension was used for cell count by countless II FL (Invitrogen).

2.4.10 Flow cytometry

Flow cytometer Fortessa X was used to evaluate the surface expression of degranulating markers and integrins. To stain the cells, around 8 x 10^5 neutrophils were collected after indicated stimulation and washed with 1 ml of PBS and stained with fixable viability dye (1 : 1000, reconstituted according to manufacturer's instructions) (ThermoFisher) for 30 minutes at 4 ^{o}C in dark. Cells were then washed with 1 ml of 2% FBS in PBS and incubated with Fc blockers (50 ng/ml) (Biolegend) for 15 minutes to avoid nonspecific binding. Cocktail of antibodies (CD45, Ly6G, CD11a, CD11b, CD63, CD15, CD14, CD16, details listed on Appendix table 1) recognizing indicated markers were then added and incubated with cells for 30 minutes at 4 ^{o}C in dark. Cells were ready for flow cytometry after one more wash with PBS. Data

analysis was performed with FlowJo, version 10.

2.4.11 *In vivo* infection assay

In vivo infection assay was performed following a previously established model [109]. Bacteria were cultured overnight and normalized in PBS to prepare an inoculum of 10^6 CFU per mouse for *E. faecalis* and is 5 x 10^3 CFU per mouse for *S. aureus*. *E. faecalis* : *S. aureus* ratio in mixed species infection was 200:1. Mice were first anesthetized with ketamine and xylazine . Hair on the dorsal skin was shaved and further removed with hair removal gel. After disinfecting with 70% ethanol, a single wound was created on the dorsal skin by 6-mm biopsy punch. 10 µl of bacterial inoculum was added to the wound and let to airdry for 3 minutes. A piece of adhesive dressing (Tegaderm^{TM} 3M) was applied to seal the wound. Whole operation was under heat lamp to keep mice from hypothermia. 24 hours post infection, a 1 x 1 cm piece of the wound tissue was collected and homogenized in PBS. Homogenates were then diluted and plated to agar plates for overnight incubation, and colonies were counted to determine viable bacteria.

2.4.12 Statistical analysis

The software GraphPad Prism was used for statistical analysis. Differences between groups with parametric distributions were analyzed using the One-way

ANOVA when analyzed among more than 2 groups and Student's 2-tailed *t* test when analyzed between 2 groups. Data are presented with mean ± SD. *P* values of 0.05 or less were considered statistically significant.

2.4.13 Ethics statement

All the animal procedures were approved by Institutional Animal Care and Use Committee (IACUC) in Nanyang Technological University (AUP# A19061) and performed accordingly.

2.5 Contribution

The study of this project was initiated by me, kindly approve by Prof Kimberly, as well as all the experimental design, operation, data collection, and analysis. Immunofluorescence staining protocol was established by Dr. Tay Wei Hong and mouse infection model established by Dr. Chong, Kelvin Kain Long. I was responsible with the adaptation and optimization for each assay, with assistance from Choo Pei Yi for imaging and Wong Jun Jie for mouse infections. Prof Christine Wong provided countless invaluable advice along the way to make this project happen.

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Chapter 3: Immunomodulating effects mediated by *E. faecalis* to reduce macrophage activation

3.1 Introduction

3.1.1 Immune functions of macrophages

Macrophages are some of the most versatile cells of our immune system, playing central roles in pathogen detection, immunomodulation, and tissue recovery [1]. There are two distinct lineages of macrophages. The first type are tissue-resident macrophages, including Kupffer cells in the liver and Langerhans cells in the skin, and macroglia in the central nervous system. Tissue-resident macrophages are sentinels that detect tissue damage and microbe invasion, and they respond by producing cytokines and promoting inflammation, which in turn recruit other immune cells [2]. The other lineage is monocyte-derived macrophages, which are in the same family as monocytes and interstitial dendritic cells [3]. Monocytes in circulation are recruited to inflamed tissues and differentiate into macrophages to help contain infection as well as process captured antigens to stimulate adaptive immunity [4]. While these cells are derived from entirely different lineages, both possess similar functions including phagocytosis to either eliminate invading pathogens or scavenge cellular debris [1].

One of the most well-studied functions of macrophages is phagocytosis, by which

macrophages can engulf microbes, dead host cells, and even lipid droplets [5, 6]. To effectively phagocytose various compounds and respond accordingly, macrophages are equipped with a wide array of receptors to recognize pathogens, apoptotic cells, and opsonins. Together, these signals trigger actin polymerization and cytoskeleton restructuring to internalize targets [7]. After phagocytosis, the phagosome fuses with a series of vesicles such as early endosome, late endosome, and lysosome. Each vesicle provides specific anti-microbial compounds to drive intracellular killing, making this the primary mechanism for macrophages to eliminate microbes [8, 9]. Upon phagocytosis, V-ATPase on the phagosome membrane starts to pump protons from the cytosol into the lumen, acidifying the environment. Phagosomes continue to recruit early and late endosomes, which further accumulate V-ATPases and gradually reduce the pH inside the vesicle to as low as 4.5. Late endosomes also provide enzymes like cathepsins and hydrolases to degrade proteins. Eventually, phagosomes fuse with lysosomes to become phagolysosomes, which possess extreme microbicidal functions that result from a combination of the low pH environment, various hydrolytic enzymes (cathepsin, hydrolase, lysozyme, and lipase), and reactive oxygen species produced by a series of proteins (NADPH oxidase, superoxide dismutase, and myeloperoxidase) [10, 11].

3.1.2 Diverse immune profiles driven by polarized macrophages

Besides phagocytosis, another broadly studied function of macrophages is cytokine production. Macrophages can secrete a wide range of cytokines, triggering various responses ranging from eliminating bacteria and inhibiting proliferation of damaged cells, to removal of cell debris and promoting wound recovery. Macrophages achieve this level of versatility by differentiating into specialized subsets depending on environmental cues, which is a reversible process [12]. Classically activated macrophages, often called M1, are specialized in microbial clearance with enhanced phagocytosis capacity and production of pro-inflammatory cytokines. During infection, macrophages recognize invading microbes through receptors including toll-like receptors (TLR), which then through a complex signaling pathway activate the cell by translocating NF-κB from cytosol to nucleus, inducing expression of cytokines such as TNF-α and polarization toward M1-like phenotype [13]. In addition, M1 macrophages can assemble inflammasomes to produce and secrete active forms of IL-1β and IL-18, shaping the inflammatory environment [14]. In addition to specific cytokine profiles and immune responses, the metabolism in M1 macrophages is also changed. M1 macrophages enhance aerobic glycolysis and attenuate their respiratory chain, which allows enhanced reactive oxygen species

(ROS) production [15]. On the other hand, alternatively activated macrophages, also known as the M2 subset, generally possess anti-inflammatory responses. Triggered by IL-4, IL-13, or apoptotic cells, M2 subsets can promote wound healing and tissue repair by removing cell debris and inducing cell proliferation or even angiogenesis [12, 16]. The metabolism of M2 macrophages depends strongly on oxidative metabolism, which is less energy-productive in a short time, but more sustainable than aerobic glycolysis [15]. Polarization of macrophages can be manipulated by environmental cues that change metabolism, which has been used to develop therapeutic strategies for atherosclerosis and cancers [17, 18]. Bacteria can exploit macrophage polarization in order to resist intracellular killing and promote intracellular survival or even replication. For instance, Salmonella induces M2 polarization by secreting the effector protein SteE to reduce antimicrobial activity and persist intracellularly, while *Listeria*, a professional intracellular microbe, favors M1 polarization by amplifying signal of TLR4 to promote its own phagocytosis [19].

3.1.3 *E. faecalis* resistance against phagocytosis-mediated killing

Enterococcus faecalis is a common member of the human gut microbiota from early life, living in healthy hosts without inducing inflammation [20, 21]. Even when colonizing in urinary tract instead of the gastrointestinal tract, this microbe can evade

immunosurveillance and avoid inflammation [22]. Furthermore, *E. faecalis* can persist in infected tissues in the presence of inflammation for an extended time, indicating it possesses immunomodulating effects to tolerate or attenuate immune responses [23-26]. The tolerance can be seen by the fact that this microbe can survive inside phagocytes after being engulfed from hours to days despite successful activation of neutrophils or macrophages [27, 28]. *E. faecalis* achieve prolonged intra-macrophage survival by possessing multiple defenses against phagocyte-derived killing mechanisms including the acidified phagosome, enzymatic activity of lysozyme, and oxidative stress as mentioned in previous paragraph. First, without disrupting V-ATPase recruitment to phagosome, *E. faecalis* can survive under acidic environments because of its high resistance of its cell membrane to acid stress, maintaining the membrane integrity under pH 2.5 to 4 [29, 30]. *E. faecalis* is also durable to lysozyme-mediated killing, yet the underlying mechanism remains unclear [31, 32]. *E. faecalis* can endure phagocyte-mediated oxidative burst by several different mechanisms. In the absence of heme, *E. faecalis* lacks catalase activity, making it insensitive to NADPH-mediated oxidative burst [33]. *E. faecalis* can also produce manganese-containing superoxide dismutase, which disrupts hydroperoxide-mediated killing and protects cells under aerobic conditions [34]. Other antioxidative enzymes are found in *E. faecalis*, which neutralize free radicals

and superoxide, promoting intracellular survival [35]. Besides resistance to oxidative stress, *E. faecalis* can reduce production of ROS to enhance the intracellular viability [36] or suppress autophagy, which is known to contribute to ROS and nitric oxide production [29]. Another less understood mechanism that contributes to tolerance against macrophage intracellular killing is mediated by a P-type ATPase in *E. faecalis*, partly by exporting zinc to avoid zinc overload but also increase resistance against oxidative stress and lysozyme, showing the rich arsenal possessed by this microbe to endure macrophage clearance [37].

3.1.4 *E. faecalis*-mediated immunomodulating functions in macrophages

In addition to direct suppression of microbicidal mechanisms, *E. faecalis* can inhibit NF-κB activation and cytokine production [38-40] and polarize macrophages to IL-10-producing phenotype [41]. Another potential mechanism to interfere with immune response is cytotoxicity [42]. Upon sensing target cells, some strains of *E. faecalis* encode and produce cytolysin, which forms pores on target cell membranes to kill either eukaryotic or prokaryotic cells [43-45]. Our lab previously demonstrated an additional immunomodulating effect where *E. faecalis* reduces the NF-κB activity induced by LPS in macrophages, and this immunomodulating effect also protects the co-infecting *E. coli* in mouse CAUTI model [46]. However, the underlying mechanisms

and bacterial factors responsible for reduced NF-κB activity are unclear.

In this chapter, a genetic screen was conducted using a transposon library to identify the *E. faecalis* genes involved in - macrophage immunomodulation. I identified 47 genes which showed weaker macrophage suppressing effects comparing to the wildtype parental strain, many of which were associated with impaired acidification or cytotoxicity; however, no single phenotype was strongly correlated with suppression. Multiple genes that participate in shikimate pathway were among the shortlist, suggesting metabolites from this pathway are involved in immunomodulation. While the specific immunomodulatory factors remain unclear, I identified the shikimate pathway as a potential novel suppressing mechanism that allows immune evasion, contributing to virulence of *E. faecalis* during infection.

3.2 Results

3.2.1 *E. faecalis* suppresses LPS-induced NF-κB activation with involvement of multiple genes

Before designing high-throughput screen to identify genes involved in the suppression of macrophage activation by *E. faecalis*, we first investigated macrophage phenotypes under different bacteria loads in order to identify the optimal condition for the assay. When infecting the RAW-Blue cell line, which enables screening for NF-κB activation using an NF-κB-responsive secreted alkaline phosphatase (SEAP) reporter, *E. faecalis* at high MOI (100 and 50) exhibited NF-κB suppressive effects in the presence of NF-κB activation induced by LPS **(Fig. 3.1A)**. I also used a primary cell line derived from mouse bone marrow to confirm the immunomodulatory phenotype. TNF-α secretion was used as an orthogonal indicator of macrophage activation since NF-κB activation leads to TNF-α transcription and production. In supernatants, the level of TNF-α decreased when infected with high MOI of *E. faecalis*, which correlated with NF-κB activity in RAW-Blue cells, and together indicating that *E. faecalis* infection results in reduced macrophage activation **(Fig. 3.1B)**. To confirm that the reduction in immune activity resulted from

suppression instead of cell death, the level of cytotoxicity was measured by the lactate dehydrogenase (LDH) assay, which detects extracellular LDH released upon cell death. Under MOI 100, the there was no significant difference in the level of cytotoxicity between the infected group and control group **(Fig. 3.1C)**, indicating that *E. faecalis* has suppressing effects on macrophage activation. To further investigate the mechanism of *E. faecalis* macrophage suppression, I conducted a genetic screen to identify genes that are responsible for the suppression RAW-Blue macrophage activation using a nearly saturating transposon library in the same strain we were using in our studies, OG1RF [47]. On day 0, RAW-Blue cells were seeded in 96-well plates and allowed to adhere overnight. The next day, 20 µl of each bacterial mutant strain grown to stationary phase in 96 well plates (roughly equal to MOI 120) was added to cell culture, along with 100 ng/ml of lipopolysaccharide (LPS), for 6 hours. Several doses of bacterial suspension were tested, and 20 µl was chosen after confirming that this amount did not induce significant cytotoxicity **(Fig. 3.1D)**. Supernatants were then collected for SEAP quantification as a measure of NF-κB activity **(Fig 3.1E)**. 677 weakly-suppressing mutants, which resulted in NF-κB activities higher than the mean of wildtype plus 3 times of standard deviation were subjected to the assay, this time in biologically independent triplicate to remove false positives. This resulted in the removal of 444 false positives. Mutants with impaired growth in

the assay conditions would also display a non-suppressing phenotype, so the shortlist was further confirmed by testing growth rates **(Fig 3.1F)** in DMEM to exclude growth deficient mutants, which resulted in the further elimination of 81 of mutants. From there, all 152 shortlisted mutants underwent secondary screening. The mutants were precisely normalized to specific multiplicities of infection (MOI) of 100 for detection of immunomodulating effects using the SEAP assay **(Fig 3.2)**. Growth rates of these mutants were also tested after being normalized to optical density $(OD)_{600\ nm}$ of 0.1 **(Fig 3.2)**. After secondary screening, and the further elimination of 76 of genes due to growth defects and 29 whose suppressive effect could not be validated, 47 genes were predicted to be involved in suppressing LPS-induced NF-κB activation **(Table 3.1)**. The list consisted of genes responsible for diverse functions, including transporting cargo and mediating metabolism, as well as some hypothetical proteins with no known functions. With such a wide array of genes and functions, it was difficult to predict a single suppressing mechanism. To further narrow this gene list, several potential mechanisms were chosen for testing to understand how *E. faecalis* suppresses macrophage activity.

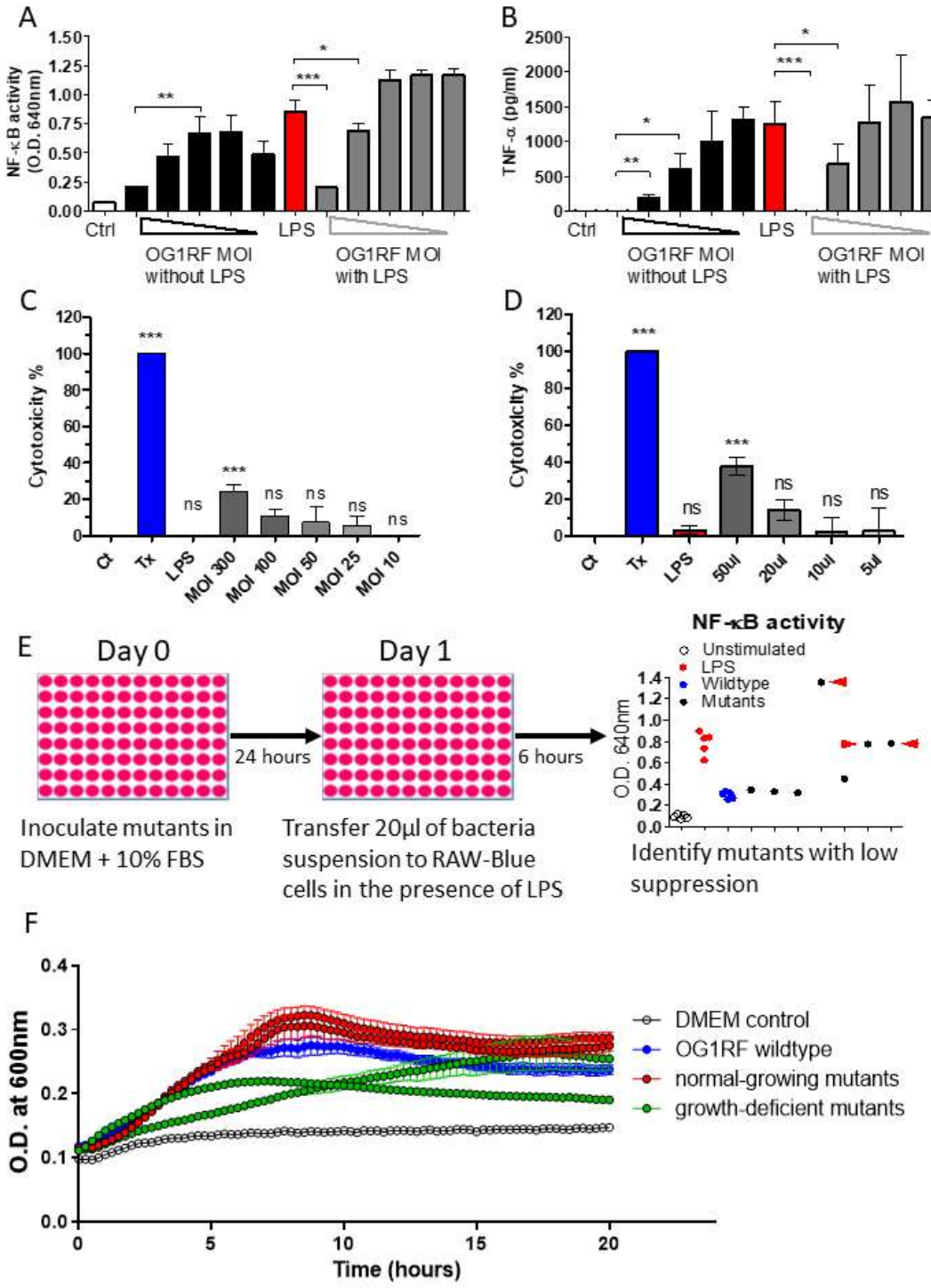

Figure 3.1 Transposon library screen and validation to identify *E. faecalis* genes involved in the suppression of macrophage activation

(A-B) Macrophage responses upon 6 hours of infection by OG1RF, an *E. faecalis* strain, and LPS stimulation. Final concentration of LPS was 100 ng/ml while MOI of *E. faecalis* used from highest to lowest were 100, 50, 25, 10, and 1. Black bars are infection with bacteria only, and gray bars are stimulation with both LPS and *E.*

faecalis. The graph is representative of 3 biologically independent experiments. NF-κB activity of RAW-Blue cells was evaluated by SEAP assay (A), and TNF-α secretion by BMDM was detected by ELISA for supernatant (B). (C-D) Cytotoxicity as a result of infection in our models was evaluated by LDH assay. After macrophages were incubated with wildtype OG1RF at various MOI (C) or with overnight bacterial suspension (D) for 6 hours, supernatants were collected for test. Triton-x (Tx) was added to induce 100% cell death. Indicated statistical differences were compared for each group with the control (Ct) group. (E) Workflow of primary screening. Mutants were used for infection to test their ability to suppress LPS-induced activation, and those with SEAP signals higher than wildtype + 3 standard deviations were identified as potential targets. Among those, approximately 150 mutants did not show growth deficiency and were carried on for secondary screening. (F) Growth curves of shortlisted mutants were compared with wildtype (blue). The normal-growing mutants (red) were selected for secondary screening while the grow-deficient mutants (green) were ruled out. All graphs represent the combined results of 3 independent experiments. *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Student t-test was used to compare between the two groups.

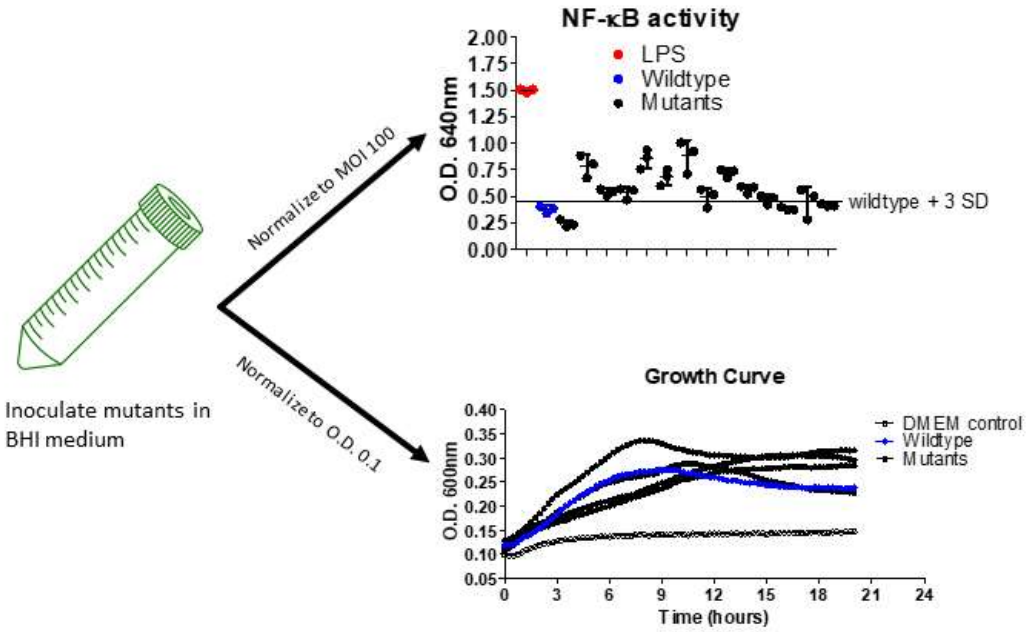

Figure 3.2 Workflow of the secondary screening

152 shortlisted mutants went through the secondary screening with proper normalization of the bacterial overnight cultures. This screening included detection of suppressing effects against LPS-induced macrophage activation by SEAP assay and measurement of growth rate of mutants by illustrated growth curves. Mutants that gave rise to a SEAP signal that was higher than the mean plus 3 standard deviations of wildtype OG1RF, and that also had no growth deficiency, were validated to be non-suppressing mutants. All experiments were performed with 3 technical replicates.

Gene Code	Gene Product	Weak acidification
OG1RF_10013	adenylosuccinate synthase	+
OG1RF_10021	PTS system mannose/fructose/sorbose transporter subunit IID	+
OG1RF_10199	L-lactate dehydrogenase	+
OG1RF_10329	thioesterase	-
OG1RF_10418	hypothetical protein	-
OG1RF_10427	ABC superfamily ATP binding cassette transporter, membrane protein	-
OG1RF_10514	DNA-directed RNA polymerase sigma subunit RpoN	+
OG1RF_10682	beta-phosphoglucomutase	-
OG1RF_10956	dihydrodipicolinate synthase	-
OG1RF_10967	hypothetical protein	-
OG1RF_11022	ABC superfamily ATP binding cassette transporter, binding protein	+
OG1RF_11023	ABC superfamily ATP binding cassette transporter, membrane protein	+
OG1RF_11036	E1-E2 family cation-transporting ATPase	-
OG1RF_11131	ABC superfamily ATP binding cassette transporter, ABC protein	-
OG1RF_11161	DHH family protein	+
OG1RF_11270	hypothetical protein	-
OG1RF_11280	shikimate dehydrogenase	-
OG1RF_11283	chorismate synthase	-
OG1RF_11300	transketolase	+
OG1RF_11329	formate acetyltransferase	+
OG1RF_11391	signal peptidase I	-
OG1RF_11450	penicillin-binding protein 1B	-
OG1RF_11602	putative calcium-transporting ATPase	+
OG1RF_11622	DNA (cytosine-5-)-methyltransferase	-
OG1RF_11636	GTP diphosphokinase	+
OG1RF_11669	superoxide dismutase	-
OG1RF_11677	ABC superfamily ATP binding cassette transporter, ABC protein	-
OG1RF_11766	multidrug ABC superfamily ATP binding cassette transporter, ABC protein	-
OG1RF_11854	metal transport repressor protein CzrA	-
OG1RF_11923	hypothetical protein	-
OG1RF_12024	spermidine/putrescine ABC superfamily ATP binding cassette transporter, ABC protein	-
OG1RF_12033	recombinase D	-
OG1RF_12043	dithiol-disulfide isomerase	-

OG1RF_12058	exonuclease SbcC	-
OG1RF_12066	hypothetical protein	-
OG1RF_12113	ABC superfamily ATP binding cassette transporter, membrane protein	-
OG1RF_12190	hypothetical protein	-
OG1RF_12284	sulfate transporter/STAS domain protein	-
OG1RF_12385	serine/threonine protein phosphatase 1	-
OG1RF_12388	formylmethionine deformylase	+
OG1RF_12401	PTS system mannose/fructose/sorbose transporter subunit IID	-
OG1RF_12446	succinyl-diaminopimelate desuccinylase	-
OG1RF_12459	isochorismatase transposase	-
OG1RF_12507	1,4-dihydroxy-2-naphthoate octaprenyltransferase	-
OG1RF_12536	sensor histidine kinase	-
OG1RF_12565	citrate (Pro-3S)-lyase	-
OG1RF_12567	[citrate [pro-3S]-lyase] ligase	-

Table 3.1 Genes predicted to be involved in *E. faecalis*-mediated immunomodulating effects

Mutants labeled as weak acidification (+) do not acidify the growth media as much as the wildtype parental OG1RF. Specifically, weak acidification was defined as a supernatant pH that is less than 80% of the acidification caused by wildtype OG1RF.

3.2.2 Suppression is not positively correlated with a single bacterial phenotype

Acidic environments can have mixed effects on immune cells, suppressing some immune responses while enhancing others [48-50]. During our primary transposon screening, I observed that many of the mutants that failed to suppress macrophage activation also failed to turn DMEM yellow after overnight culture, suggesting these mutants either did not grow in the medium or that they did not acidifying the growth media by some other mechanism. After excluding the growth deficient mutants **(Fig 3.2)** during secondary screening, I tested the ability of all shortlisted mutants for to acidify the culture environment. I found that 12 out of 47 shortlisted mutants exhibited acidification below 80% compared to the wildtype, suggesting pH reduction may contribute the macrophage suppressing mechanisms **(Fig. 3.3A, Table 1)**. However, there was no strong correlation based on R-squared values below 0.3 between acidification and suppression, indicating there must be other factors involved **(Fig. 3.3B)**. The interaction between acidosis and immune suppression was further pursued by my colleague, in which she discovered that the acidified medium, either due to *E. faecalis* conditional medium or the addition of organic acids, result in the suppression of NK-κB activation.

Cytotoxicity is another possible explanation for reduced NF-κB activity, since non-viable cells would not respond to LPS activating signal in the first place, leading to false positive *E. faecalis* mutants that appear to suppress macrophage activation, but in reality are killing the macrophages. While no significant cytotoxicity was observed when macrophages were infected with MOI 100 of *E. faecalis* **(Fig. 3.1.C)**, it did show on average around 10% of cells were dead, which may partly contribute to the reduced immune activity. Some strains of *E. faecalis* secrete cytotoxic effectors such as hemolysin [42]; however the OG1RF strain that I use in this study does not encode this hemolysin (cytolysin). Nonetheless, there may be other cytotoxic factors produced by *E. faecalis* which could induce cell death, resulting in reduced activation of macrophages. Previously, the LDH assay was used to evaluate cytotoxicity. However, considering that the LDH assay may only detect later stages of cell death when the membrane becomes compromised, it was possible the cells were already dying but had yet to release intracellular LDH. To distinctly evaluate the cytotoxicity of all 47 shortlisted mutants, the infection was increased to MOI 300 to enhance cytotoxicity while keeping the same incubation time at 6 hours. At this higher MOI, wildtype OG1RF induced cell death at a level of >15%, whereas a 34 of the 47 shortlisted mutants exhibited cell death below 10%, indicating that *E. faecalis*-induced cell death in macrophages could be one of the mechanisms that participate

in reduced NF-κB activity **(Fig. 3.3A, 3.3C)**. Nonetheless, the correlation between cytotoxicity and suppression was still weak, with R-squared values below 0.3, suggesting it is also not the sole factor **(Fig. 3.3C)**. There was also no correlation between acidification and cytotoxicity with R-squared value below 0.1 **(Fig. 3.3D)**, indicating that the observed cytotoxicity was not specifically induced by a low pH environment. Altogether, these results indicate the previously observed immune-modulating effect in macrophages is caused by multiple factors.

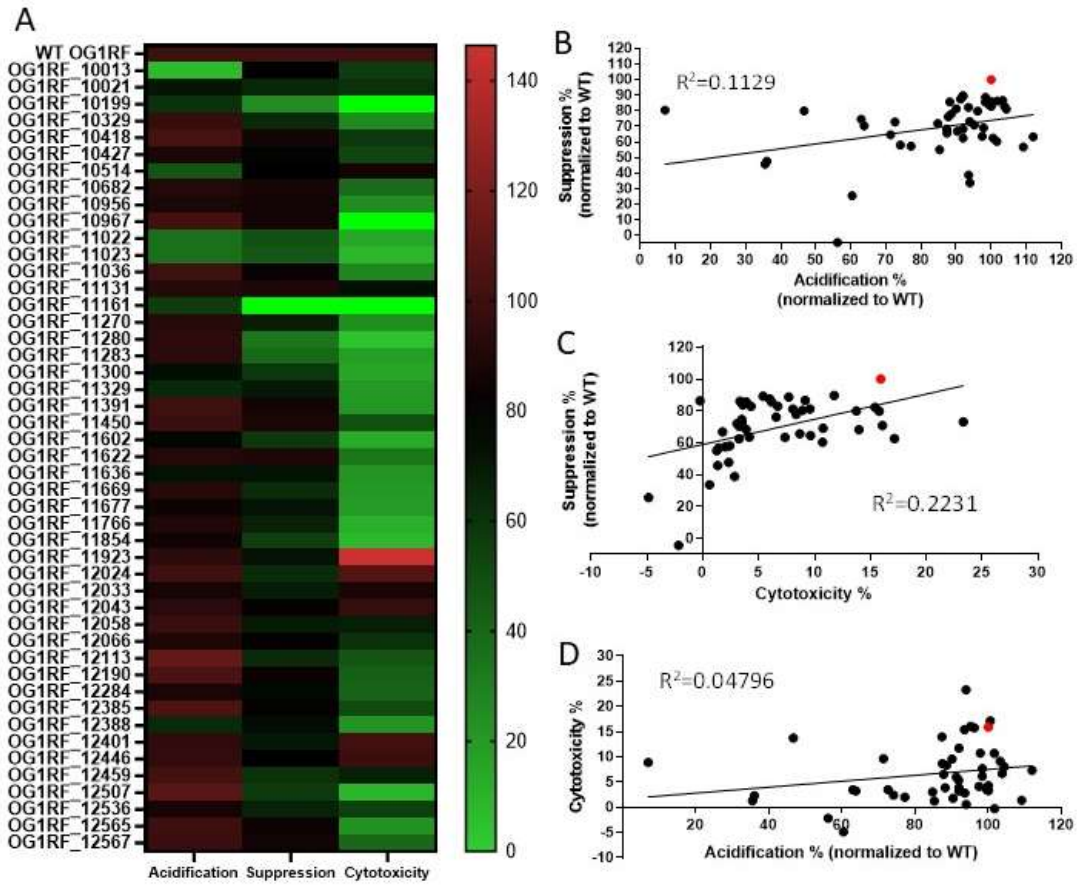

Figure 3.3 Comparison and correlations of acidification, cytotoxicity, and immune suppressive phenotypes among OG1RF wildtype and transposon mutants

(A) Heat map of the level of acidification, NF-κB suppression, and cytotoxicity for each transposon mutant. All data were normalized to wildtype (set to 100%, with the color black indicating 80%. Only mutants with suppressing effects below 90% compared to wildtype were shortlisted and shown in this figure. (B-D) Correlation between the indicated phenotypes. The red dot indicates wildtype, while each black dot represents a mutant. Each data is the mean of 3 independent biological replicates. (B) Correlation between acidification and suppression. Both were normalized to wildtype. (C) Correlation between cytotoxicity and suppression. Cytotoxicity was normalized, using positive control (triton-x) as 100% and negative control (untreated) as 0%. (D) Correlation between cytotoxicity and acidification.

3.2.3 Genes of the shikimate pathway contribute to *E. faecalis* cytotoxicity over macrophages, potentially through apoptosis induction

Among the predicted gene from screening, three participate in shikimate pathway **(Table 3.1:** OG1RF_11280, 11283, 12459**)**, marking this pathway as a promising target to further understand the *E. faecalis*-mediated macrophage suppressing effect. The shikimate pathway is a seven-step metabolism that converts phosphoenolpyruvate and erythrose 4-phosphate to chorismate, with an important intermediate metabolite of shikimate, hence the name [51]. Chorismate can then be further converted to various products, including hormones, vitamins, and aromatic amino acids [52] **(Fig. 3.4A)**. Direct administration of shikimate and its derivatives have also shown suppressing effects on activation of macrophages and a protective effect in murine colitis model by reducing inflammatory mediators [53, 54]. However, most of the reports are investigating the role of shikimate metabolites in diet supplements, leaving the potential of this pathway as an immune-evading mechanism utilized by pathogens unexplored. To determine the role of the shikimate pathway in the immunomodulating effect, mutants with insertions on the main operon of the shikimate pathway were collected to evaluate their suppressing effects **(Fig. 3.4B)**. Two other mutants exhibited weaker suppressing effects (OG1RF_11281 and 11285),

while the genes responsible for aromatic amino acid biosynthesis (OG1RF_11284 and 11287) showed comparable suppression levels to wildtype, suggesting the responsible products were not downstream of aromatic amino acid metabolism **(Fig. 3.4A)**.

Suppressing effects of shikimate pathway-related mutants were tightly correlated with cytotoxicity. For instance, mutants exhibiting strong suppression also induced higher cell death, similar to the wildtype phenotype **(Fig. 3.4C)**, indicating this pathway might be involved in *E. faecalis*-mediated cytotoxicity. By contrast, pH reduction in the bacterial culture supernatants was comparable between mutants and wildtype OG1RF **(Fig. 3.4D)**, suggesting the shikimate pathway did not contribute to immunosuppression through acidosis.

To confirm the cytotoxicity caused by *E. faecalis* in RAW-Blue macrophages and the role of shikimate pathway in it, the viability of BMDM following *E. faecalis* was determined by flow cytometry, which allowed us to identify compromised membrane and should be able to detect stressed cells even at the early stage **(Fig. 3.5A)**. After infection at MOI 100 with wildtype *E. faecalis* for 6 hours either with or without LPS, only 20% of the BMDM exhibited intact membrane, while mutants with insertion in the shikimate dehydrogenase (OG1RF_11280) reduced macrophage membrane disruption (increased membrane integrity), leaving around 60% of the cells with

intact membrane integrity **(Fig. 3.5B)**. This observation in BMDM is consistent with cytotoxicity determined by LDH assay in RAW-Blue macrophages, in which higher cytotoxicity was observed after infection with wildtype *E. faecalis* and which was lesser upon infection with the shikimate dehydrogenase (OG1RF_11280) mutant **(Fig. 3.4C)**. Surprisingly, the production of TNF-α within live BMDM was not suppressed by *E. faecalis* infection **(Fig. 3.5C, 3.5D)**, contradicting previous ELISA results in RAW-Blue macrophages **(Fig. 3.1B)**. This contradiction suggests that the *E. faecalis*-mediated reduction of TNF-α in infected RAW-Blue supernatants was entirely due to cell death.

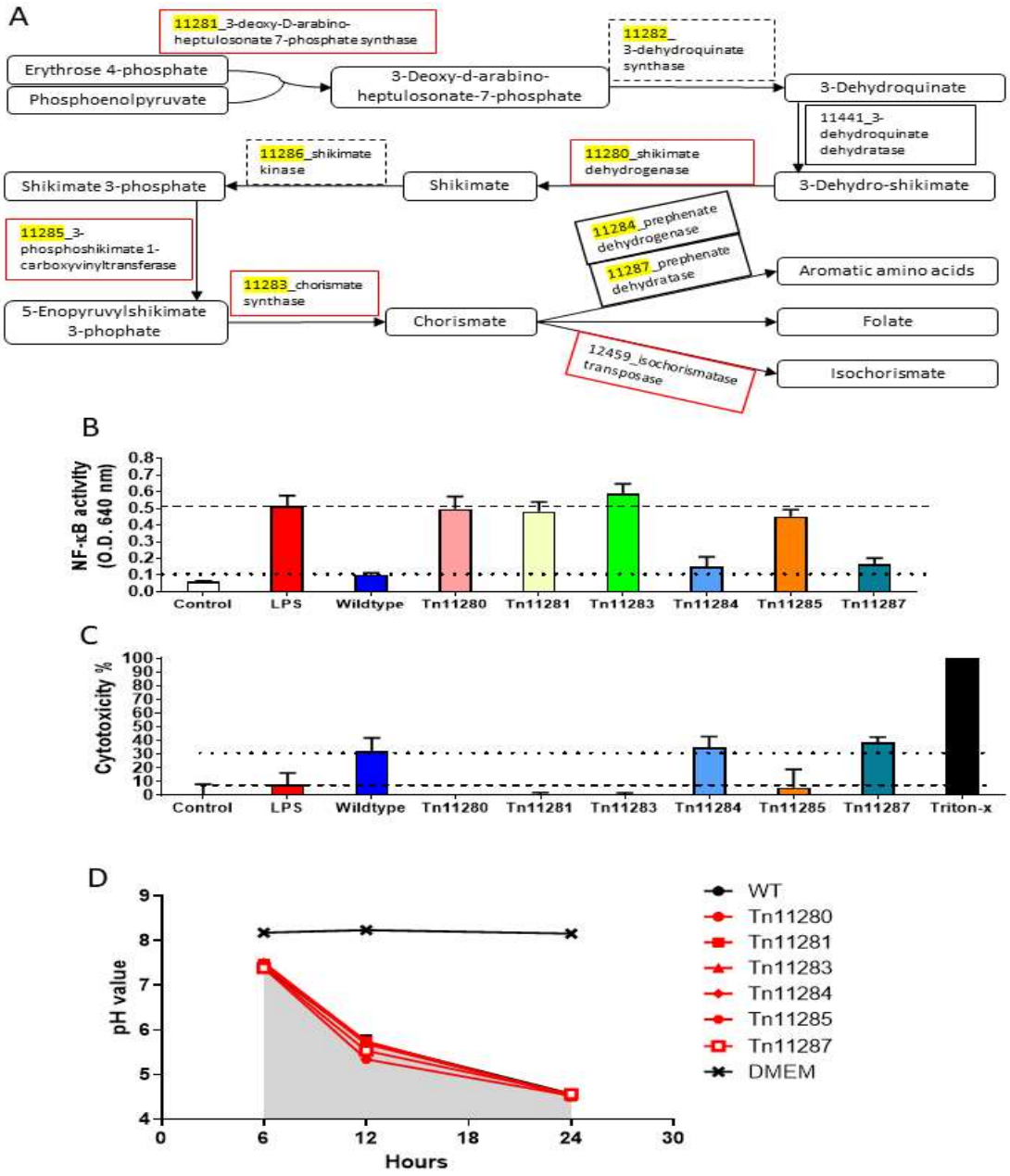

Figure 3.4 Contribution of the shikimate pathway to *E. faecalis*-mediated macrophage suppression cytotoxicity, and pH

(A) Shikimate pathway. Black boxes are the substrates/products that participate in shikimate pathway. The number in the boxes indicates the gene codes, and the yellow highlighted ones are in the same operon. Red boxes are the genes/enzymes predicted to immunomodulation. Dashed boxes are mutants not found in our transposon library. (B-D) Phenotypes of shikimate operon. At MOI 300, the NF-κB suppression (B), cytotoxicity (C), and acidification (D) of wildtype and mutants with disrupted genes in shikimate pathway were evaluated. (D) The gray area indicates the area under the curve of wildtype.

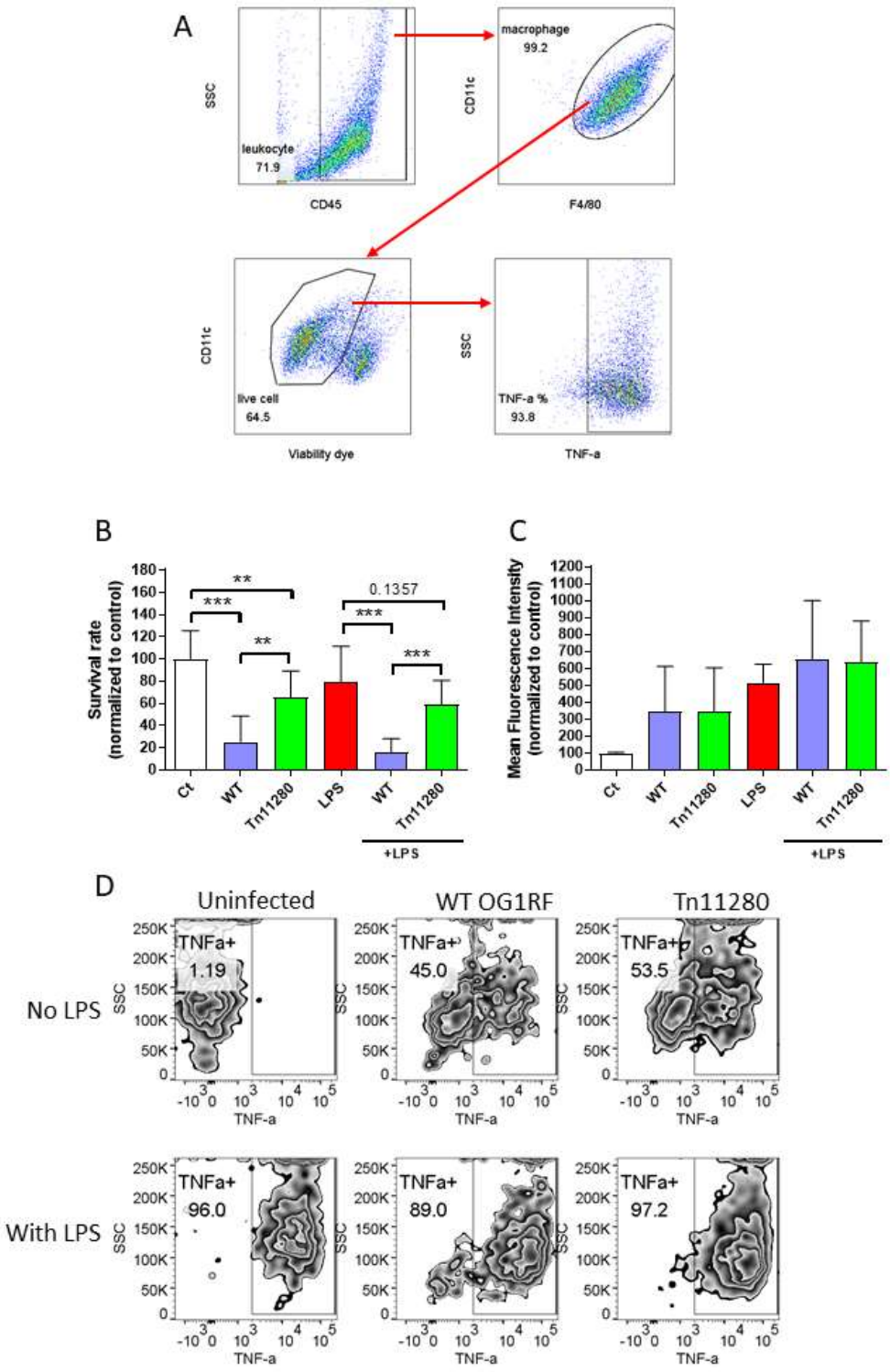

Figure 3.5 *E. faecalis*-mediated cytotoxicity in BMDM

(A) Gating strategy for live BMDM. The first gate was on $CD45^+$ cells (leukocytes), followed by a gate on $F4/80^+CD11c^+$ macrophages before analyzing for viability. TNF-α production was only measured in live cells. (B-D) Viability and TNF-α production levels were compiled from 3 independent experiments. (B) Viable population of

infected cells was measured in percentage of macrophages and normalized to the untreated group of the same batch. Production of TNF-α was measured with mean fluorescent intensity normalized to the untreated group (C) or percentage of TNF-α expressing cells (D). ** = $p < 0.01$, *** = $p < 0.001$.

Cell death comes in many shapes and forms. In addition to necrosis, which is usually induced by external physical disruption. Most mammalian cells undergo apoptotic programmed cell death to avoid activating inflammation and to avoid exposing self-antigens to immune system[55]. Identifying the cell fate upon *E. faecalis* infection could help us to understand the potential following responses. For instance, inducing pyroptosis and necroptosis would both enhance inflammation, release damage-associated molecular patterns (DAMPs) and pro-inflammatory cytokines following cell lysis, as well as alert nearby tissues of pathogen invasion [56]. By contrast, apoptosis avoids triggering inflammation by trapping intracellular contents in apoptotic bodies and recruits phagocytes for rapid clearance of the apoptotic cells [57]. To distinguish between different cell death pathways, flow cytometry was used to determine cell sizes and surface markers of the infected RAW-Blue cells, which is indicative of different death pathways. Membrane integrity was evaluated with a viability dye that only enters and accumulates intracellularly when the cell membrane is compromised, which happens during late apoptosis as well as in any other type of cell death. Annexin V, on the other hand, binds to phosphatidylserine, which is usually expressed on the inner side of the cell membrane but is flipped to the outside during early apoptosis and acts as a marker for recognition by phagocytes. Cells with single positive signals for annexin V

(annexin V +, viability -) indicate early apoptosis, whereas double positive cells (annexin V +, viability +) suggest late apoptosis or non-specific cell death **(Fig. 3.6A)**. At 4 hours post-infection, wildtype *E. faecalis* reduced the live cell population by around 20% comparing to uninfected cells **(Fig. 3.6B)**, again indicating *E. faecalis*-mediated cytotoxicity. Mutation in the shikimate dehydrogenase (OG1RF_11280) showed significantly lower cytotoxicity, with a slightly lower apoptotic population, than wildtype infected cells **(Fig. 3.6B)**. Regardless of the treatments, cells with positive signal of annexin V, whether at early or late stage, showed smaller cell size determined by forward scatter signals **(Fig. 3.6A)**. This is consistent with previous understanding that apoptotic cells shrink in size while pyroptotic and necroptotic cells undergo swelling before cell lysis [55]. Together, our data suggest that wildtype *E. faecalis* induced macrophage cell death by inducing apoptosis, and this mechanism is facilitated by the shikimate pathway.

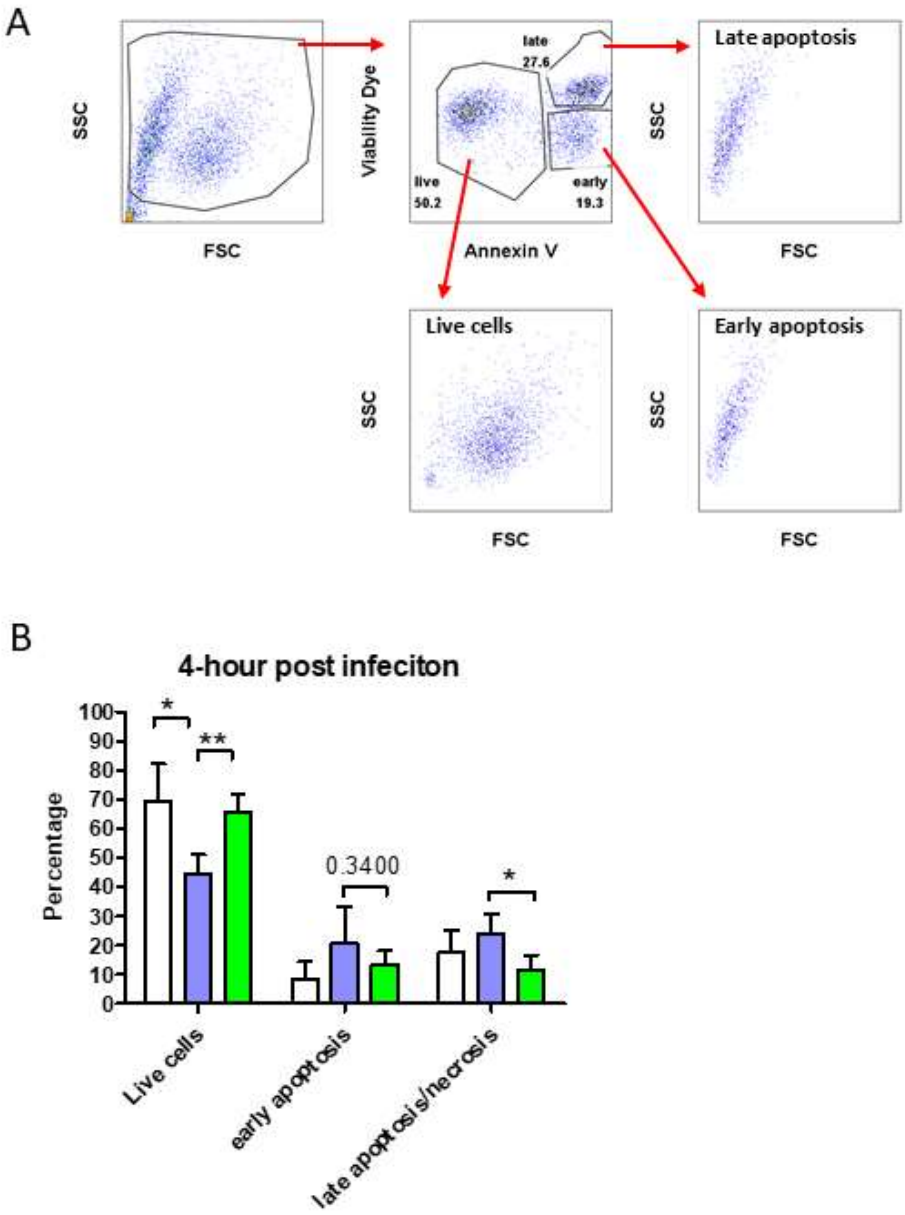

Figure 3.6 *E. faecalis*-induced apoptosis in RAW-Blue cells

(A) Gating strategy for live cells, early apoptosis, and late apoptosis. First, exclude cell debris with FSC/SSC gating. Double negative cells are live cells. Annexin V^+ and viability dye^- cells are early apoptosis, with membrane that lost asymmetry but is still intact. Double positive cells are late apoptosis, with disrupted membrane. FSC/SSC of each population was shown to compare the size of each population. (B-C) Quantification of apoptotic cell population at 3- and 4-hour post-infection. Bio-replicates of 3-4 per test group were collected through 3 independent experiments. *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.2.4 Cyanidin 3-O-glucosides and *p*-coumaric acid are not responsible for *E. faecalis*-mediated macrophage immunomodulation

To identify *E. faecalis* shikimate pathway-related factors that are responsible for macrophage cytotoxicity, supernatants of wildtype and the shikimate dehydrogenase (OG1RF_11280) mutant were collected after overnight culture and subjected to mass spectrometry analysis **(Fig. 3.7)**. Compounds that have a lower abundance in supernatant of mutant compared to wildtype (fold-change < $\log_2$ -5 and < $\log_2$ -10) and which also reached statistical significance (p-value >-$\log_{10}$ 2) were noted **(Table 3.2)**. Since the compounds were determined by molecular mass, some of the masses have multiple molecular candidates with decorations at different sites, making the confirmation difficult. In addition, the majority of the compounds on the list are not directly connected to shikimate pathway. Instead, they are likely factors present in the culture medium, such as sodium salts and several types of amino acids.

Compounds that are connected to quinate (highlight in pink in **Table 2**), a pre-shikimate derivative, were ruled out given the mutations impacting post-shikimate processing were identified to be involved in the cytotoxicity **(Fig. 3.4)**. Given the central role of shikimate pathway in phenolic compound metabolism, chemicals with aromatic rings (highlight in yellow in **Table 3.2**) were prioritized since it's more likely

these are direct products from this pathway.

Among the shortlisted compounds, two contain p-coumaric acid structure (code HMDB 38065 and CHEBI 57768) and one is a derivative of cyanidin 3-glucoside (CHEBI 31442, HMDB 37974, or HMDB 41409) **(Table 3.2)**. P-coumaric acids and cyanidin 3-glucoside are both derived from products of shikimate pathway and also are reported to induce apoptosis in mammalian cell lines, suggesting a potential cytotoxic effect against macrophages [58, 59]. A preliminary test with the three chemicals, including p-coumaric acid and two forms of cyanidin 3-glucoside (kuromanin chloride and peonidin 3-O-glucoside chloride), were conducted for their cytotoxicity in RAW-Blue cells and suppressing effect against LPS-induced NF-κB activity. P-coumaric acid did not induce cytotoxicity nor suppression of NF-κB activity, indicating its derivatives are less likely to be the cytotoxic effectors **(Fig. 3.8A, 3.8B, 3.8C, 3.8D)**. Kuromanin and peonidin 3-O-glucoside, at high concentration (400 μM), lead to enhanced signals in the cytotoxic assay **(Fig. 3.8A and 3.8B)**. However, although kuromanin reduced NF-κB activity in the absence of LPS **(Fig. 3.8C)**, both cyanidin derivatives failed to suppress LPS-induced activation **(Fig. 3.8D)**. Similar results were seen in TNF-α secretion, where neither kuromanin chloride nor peonidin 3-O-glucoside chloride reduced the cytokine level in supernatant **(Fig. 3.8E, 3.8F)**, suggesting cyanidin derivatives may also not be responsible for *E. faecalis*-mediate

cytotoxicity. The strong signal of cytotoxicity assay was most likely due to the intense color of the chemicals themselves, which made culturing medium dark purple before colorization of the experimental assay **(Fig. 3.8G)**. This negative outcome is not too surprising, given that p-coumaric acid structure and cyanidin 3-glucoside are both derived from aromatic acids. Aromatic amino acids are produced through prephenate dehydrogenase (OG1RF_11284) and prephenate dehydratase (OG1RF_11287), neither were predicted to be involved in the observed phenotype **(Fig. 3.4B, 3.4C)**.

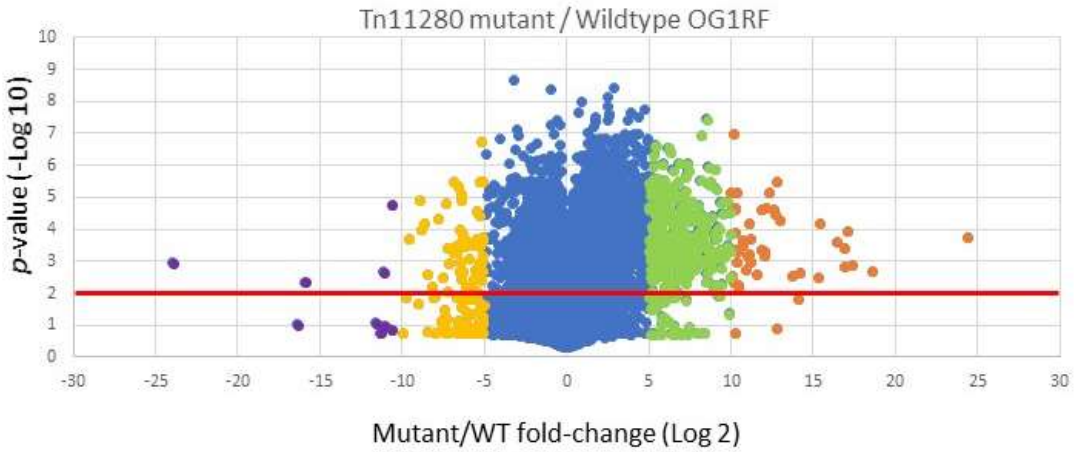

Figure 3.7 Differential expression of secreted compounds by wildtype *E. faecalis* and shikimate dehydrogenase mutant

Compounds in supernatants of wildtype and shikimate dehydrogenase (OG1RF_11280) mutant were analyzed by mass spectrometry. X-axis indicated the fold-change of each compound between mutant and wildtype, and Y-axis indicated the significance. Compounds reduced in supernatants of mutant are labeled purple ($<\log_2^{-10}$) and yellow ($>\log_2^{-10}$, $<\log_2^{-5}$). Compounds increased in supernatants of mutant are labeled orange ($>\log_2^{10}$) and green ($<\log_2^{10}$, $>\log_2^{5}$). Red line indicates p-value = 0.01. Compounds with p value below 0.01 and fold-change $<\log_2^{-5}$ were shortlisted as candidates.

	fold-change < log_2^{-10}		fold-change < log_2^{-5}		
Compound	0.60_779.5406m/z	0.70_218.0438m/z	2.16_475.1208m/z	0.83_242.0260m/z	1.51_445.0185m/z
Potential chemicals	HMDB38065	CHEBI:62955	CHEBI:48491	CHEBI:34598	CHEBI:10048
			CHEBI:69536		
			HMDB29528		
			HMDB37341		
			HMDB37342		
			HMDB39746		
			HMDB39747		
			HMDB39748		
			HMDB41215		
	fold-change < log_2^{-5}				
Compound	0.60_431.3150m/z	0.68_338.1031m/z	1.52_428.0602m/z	1.53_422.0512m/z	0.60_779.3574m/z
Potential chemicals	CHEBI:15574	CHEBI:2906	CHEBI:209807	CHEBI:34953	CHEBI:80570
	CHEBI:3863	CHEBI:48926	HMDB15426	CHEBI:82008	
	CHEBI:47812	HMDB04662			
	CHEBI:5633	HMDB33374			
	CHEBI:66759	HMDB39733			
	CHEBI:67729				
	CHEBI:68041				
	CHEBI:71824				
	CHEBI:80533				
	CHEBI:81016				
	CHEBI:81367				
	CHEBI:83036				
	CHEBI:87783				
	CHEBI:8913				
	HMDB11560				
	HMDB11590				
	HMDB12458				
	HMDB30066				
	HMDB34403				
	HMDB36249				
	HMDB60127				
	HMDB60128				
	fold-change < $log2^{-5}$				
Compound	0.68_320.0913m/z	1.21_308.0032m/z	2.32_536.1116m/z	0.70_200.0331m/z	
Potential chemicals	CHEBI:20596	CHEBI:82040	CHEBI:31442	CHEBI:15961	
	CHEBI:57768		HMDB37974	CHEBI:31692	
	CHEBI:65727		HMDB41409	CHEBI:37525	
				CHEBI:72710	
				HMDB03484	
				HMDB11185	
				HMDB60483	
	fold-change < $log2^{-5}$				
Compound	1.51_481.1193m/z	0.47_353.0469m/z	2.40_136.0603m/z	0.86_260.0366m/z	
Potential chemicals	CHEBI:72951	CHEBI:30235	CHEBI:28187	CHEBI:85917	
		CHEBI:58453	CHEBI:28330		
		CHEBI:81695	CHEBI:60904		
			HMDB29389		

Table 3.2 Compounds with lower abundance in supernatants of the shikimate dehydrogenase (OG1RF_11280) mutant comparing to wildtype OG1RF

Chemicals are coded according to Chemical Entities of Biological Interest (ChEBI) or Human Metabolome Database (HMDB). Compounds related to quinate are highlighted in pink, and remaining compounds containing phenol rings are highlighted in yellow.

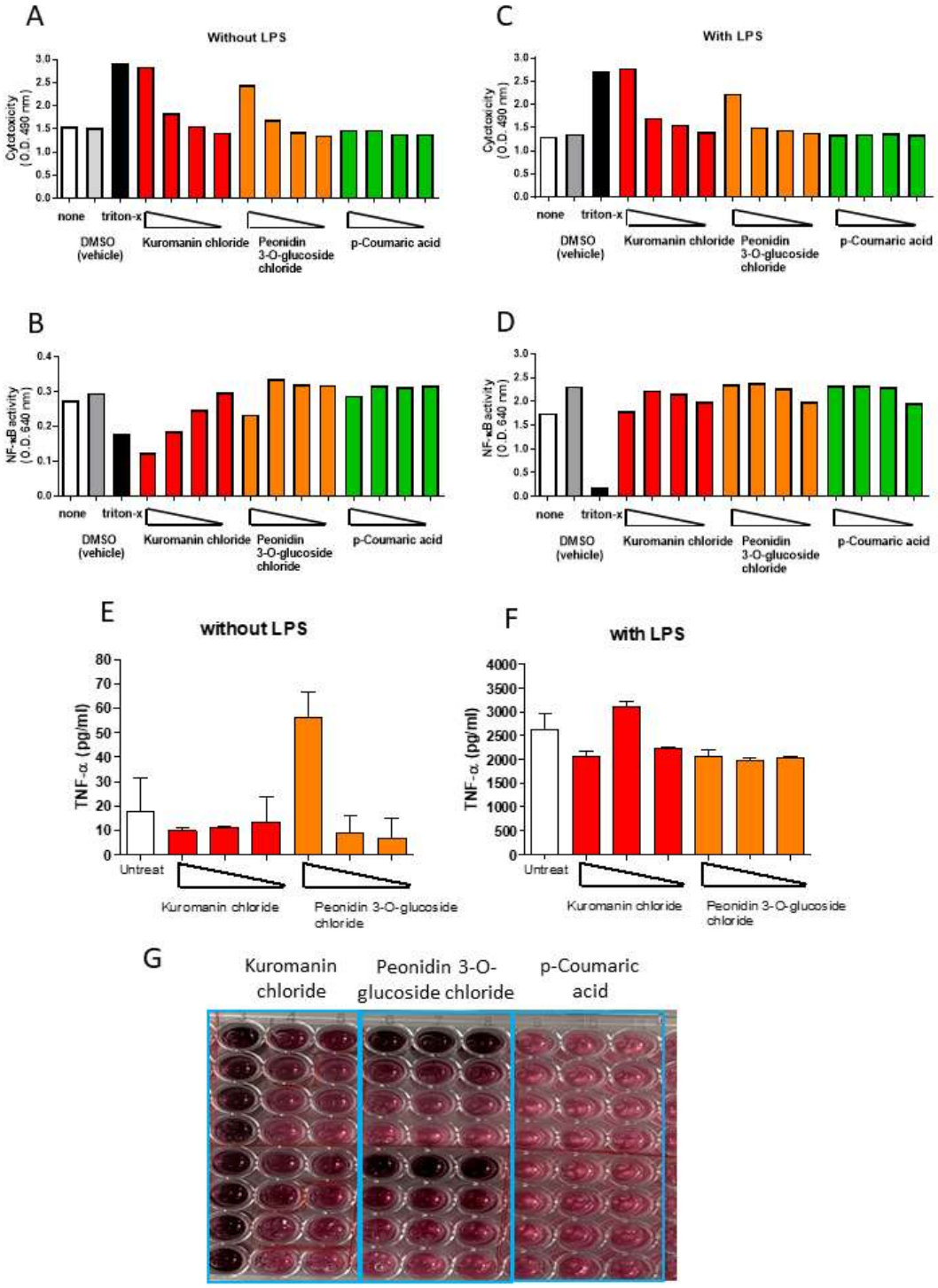

Figure 3.8 Chemical-induced suppression of macrophage activation

(A-D) Cell viability and NF-κB activity were analyzed for chemical-treated RAW-Blue cells with or without LPS (100 ng/ml). Final concentrations of each chemical are 400 µM, 100 µM, 50 µM, 10 µM. (A-B) Cytotoxicity and NF-κB activity in the absence of LPS. (C-D) Cytotoxicity and NF-κB activity in the presence of LPS. This experiment was done once with 4 technical replicates. (E-F) TNF-α secretion by RAW-Blue cells upon

chemical stimulation with or without LPS (100ng/ml). Concentration of chemicals used were 400 µM, 100 µM, 50 µM. Two independent experiments were conducted. (G) Visual record of stimulating culture in the presence of indicated chemicals.

3.2.5 Mutations in the shikimate pathway impair *E. faecalis*-produced extracellular superoxide

An interesting candidate that may be responsible for macrophage cytotoxicity could be *E. faecalis*-derived extracellular superoxide, which is not detectable by mass spectrometry. *E. faecalis* utilizes membrane-associated demethylmenaquinone, an aromatic compound synthesized from chorismate and isochorismate, to produce superoxide and eventually generate hydroxyl radicals [60]. *E. faecalis*-mediated oxidative stress, including superoxide, hydrogen peroxide, and hydroxyl radicals can damage mammalian cell DNA, leading to apoptosis [61]. Extracellular superoxide produced by *E. faecalis* can also activate macrophage cyclooxygenase-2 (COX-2), which further contributes to oxidative stress that causes nearby cell death [62]. To evaluate the production of extracellular superoxide overtime, bacteria were grown in DMEM with the addition of 20 µM DCFDA, a probe that generates highly fluorescent products once oxidized [63]. While all bacteria tested can oxidize the environment, wildtype OG1RF as well as mutants with mutations at prephenate dehydrogenase (OG1RF_11284) or prephenate dehydratase (OG1RF_11287) showed higher level of superoxide production than the rest **(Fig. 3.9A, 3.9B)**, indicating that both prephenate dehydrogenase and prephenate dehydratase are not involved in

superoxide production. This is not surprising considering prephenate dehydrogenase and prephenate dehydratase are involved in turning chorismate into aromatic amino acids instead of isochorismate **(Fig. 3.4A)**. By contrast, mutations at shikimate dehydrogenase (OG1RF_11280), phospho-2-dehydro-3-deoxyheptonate aldolase (OG1RF_11281), chorismate synthase (OG1RF_11283), and 3-phosphoshikimate 1-carboxyvinyltransferase (OG1RF_11285) significantly reduced superoxide production **(Fig. 3.9A, 3.9B)**.

The pattern of superoxide production **(Fig. 3.9B)** by the shikimate pathway mutants was consistent the pattern of cytotoxicity **(Fig. 3.4C)**, suggesting the *E. faecalis*-produced superoxide is likely responsible for the macrophage cell death. To evaluate whether the secreted superoxide is enough to induce cytotoxicity, I filtered the supernatant of bacterial culture 6 hours after incubation and cultured RAW-Blue cells in such conditioned mediums for 24 hours before collecting the cells for viability staining. However, none of the conditioned media resulted in significant cell death **(Fig. 3.9C)**. This could be due to the unstable nature of superoxide, or that the concentration of superoxide in the medium is not enough to cause cell death.

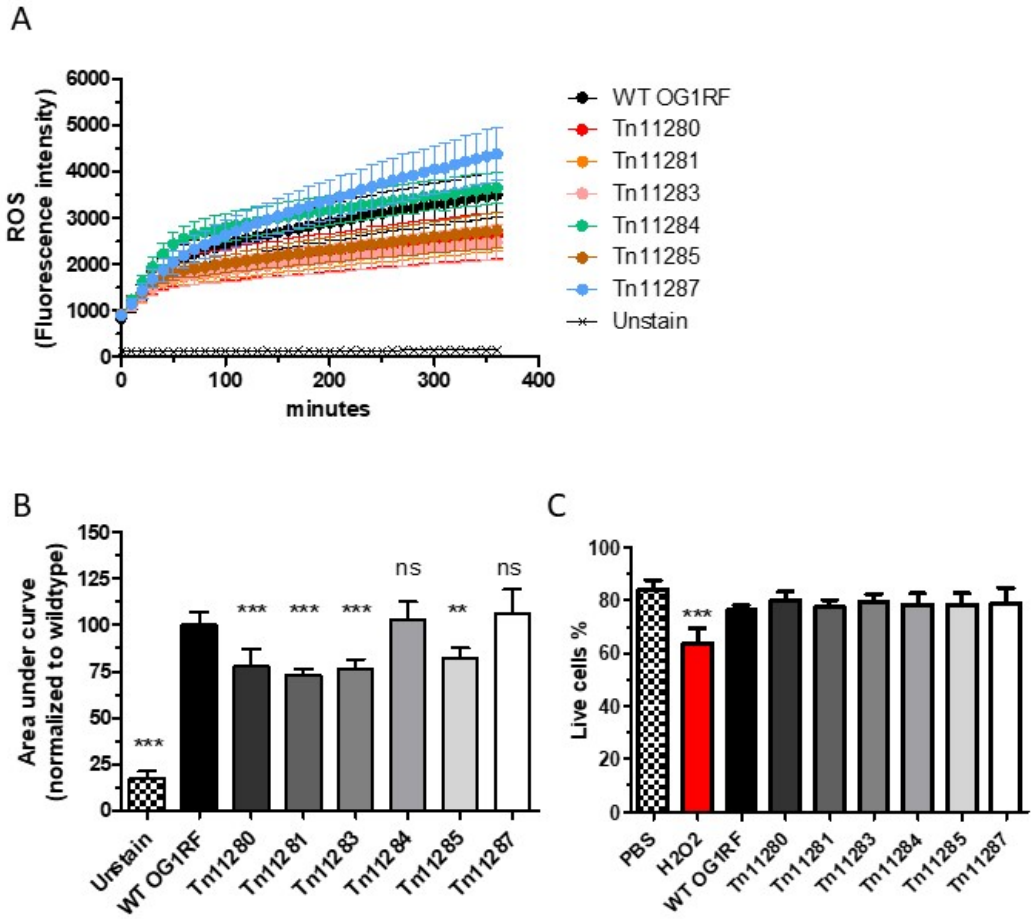

Figure 3.9 The shikimate pathway is involved in the production of superoxide by *E. faecalis*

(A-B) Superoxide production by wildtype OG1RF and transposon mutants. *E. faecalis* was cultured in DMEM with 20 µM of DCFDA and the level of fluorescence was detected for 6 hours (A) and the area under curve for each bacterial culture was calculated to show overall production (B). The statistical differences of each group compared to wildtype OG1RF is indicated. (C) The percentage of live macrophages after 24 hours incubation with bacterial conditioned medium was determined by flow cytometry. 150 μl of indicated medium and 50 µl of fresh DMEM were added to each well. The statistical differences of each group comparing to PBS group were indicated. All results were acquired from 3 independent experiments. One-way ANOVA was used for statistical analysis. ** = $p < 0.01$, *** = $p < 0.001$.

3.2.6 *E. faecalis* exhibits persistent intracellular survival and potential proliferation within macrophages

Since the supernatant of *E. faecalis* was sufficient to induce significant cytotoxicity, alternative routes may be involved. One of the potential routes to accumulate a high concentration of cytotoxic effectors could be through intracellular proliferation. Our preliminary data showed that the CFU of intracellular *E. faecalis* increased 4 hours after being engulfed by macrophages, indicating potential intracellular proliferation **(Fig. 3.10A)**. Moreover, around 5 % of macrophages were observed to contain over 20 *E. faecalis* cells (green) 4 hours post infection **(Fig. 3.10B)**. Within macrophages with only few *E. faecalis*, the microbes still retained the proliferation dye signals, indicating non-proliferating phenotype. In contrast, *E. faecalis* within the macrophages with more intracellular *E. faecalis* had little to no dye signals (red arrow), suggesting these were proliferated bacteria **(Fig. 3.10B)**. However, only around 5% of macrophages showed evidence of *E. faecalis* intracellular replication within them, suggesting there are unknown factors, either from the host side or the bacterial side, that contribute to this phenotype.

The intracellular environment in macrophages can be shaped by various cues to become more or less potently bactericidal, and this could directly impact the

intracellular survival of *E. faecalis*. In general, macrophages exposed to IFN-γ, a cytokine mostly produced by T helper cells, will be primed or activated for stronger microbial killing [64-66]. Surprisingly, when I polarized macrophages with IFN-γ (M1, red line) before introducing *E. faecalis*, the intracellular survival of the bacteria was better compared to unpolarized (M0, blue line) and anti-inflammatory phenotype (M2, green line) macrophages **(Fig. 3.11A)**. A similar outcome was observed when IFN-γ was added to activate macrophages after *E. faecalis* was engulfed, where the IFN-γ stimulated macrophages (red line) caused poorer clearance and allowed more intracellular *E. faecalis* to survive compared to the un-stimulated group (blue line) **(Fig. 3.11B)**. These preliminary data suggest *E. faecalis* survive better under an inflamed environment, yet the underlying mechanism is unknown. Other studies have similar observations in clinical samples and *ex vivo* experiments, showing *E. faecalis* population is positively correlated with inflamed tissue [25, 67]. With more understanding of the mechanisms facilitate by *E. faecalis* to improve survival and to alter the immune landscape, we should be able to identify druggable targets to interrupt the microbial survival strategies and resolve chronic infections. The intracellular survival of *E. faecalis* is now being further pursued by my colleague.

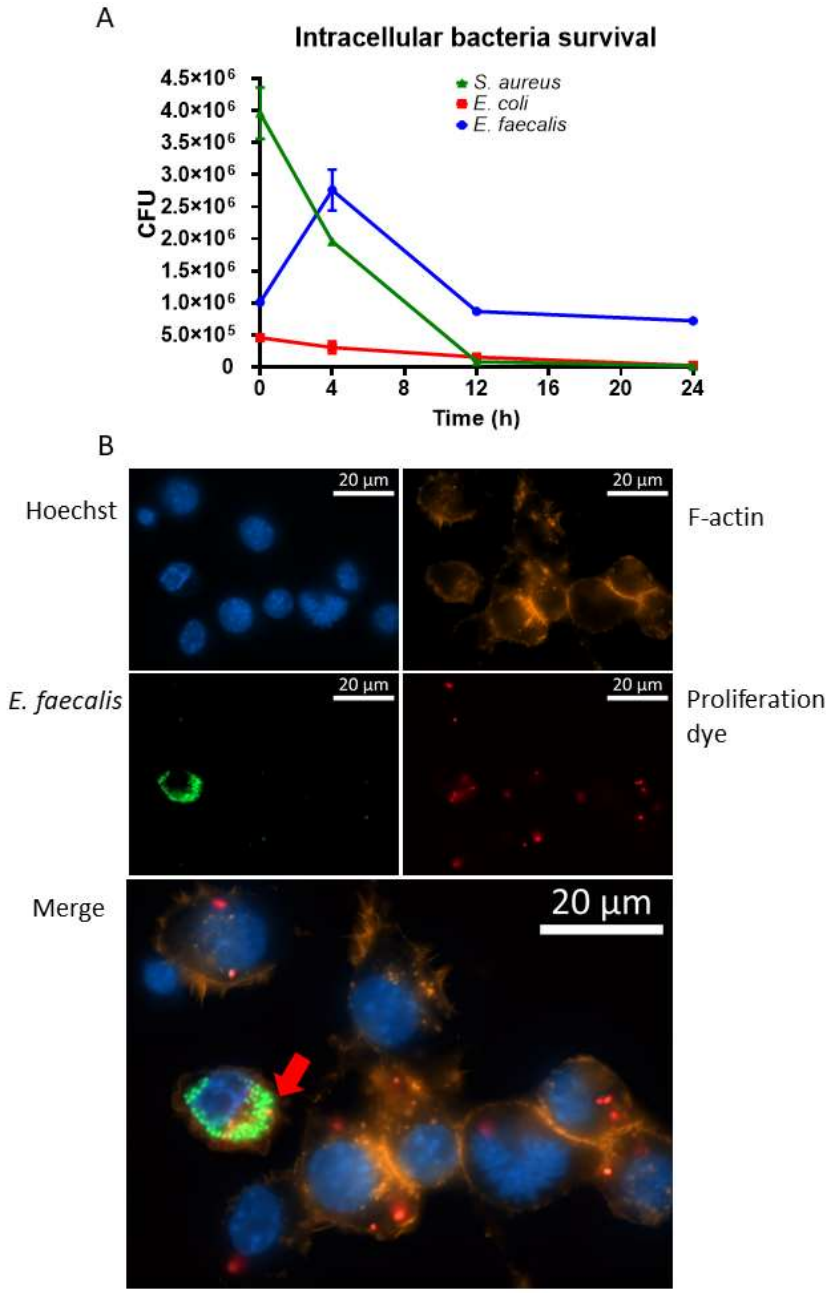

Figure 3.10 *E. faecalis* survive and proliferate intracellularly

(A) CFU of bacteria after engulfment by RAW-Blue cells for the indicated amount of time. Cells were incubated with bacteria for 1 hour before changing to an antibiotic-containing medium in order to remove extracellular bacteria. Cells were further incubated for the indicated amount of time before being lysed to collect and count intracellular CFU. One experiment with three technical replicates was performed. (B) Fluorescent microscope images of the intracellular distribution of *E. faecalis* within RAW-Blue cells. RAW-Blue cells were stained by the nucleus (blue) and the F-actin (orange). GFP-expressing *E. faecalis* was used and pre-stained with proliferation dye (red). For both experiments, MOI 10 of bacteria was used for infection.

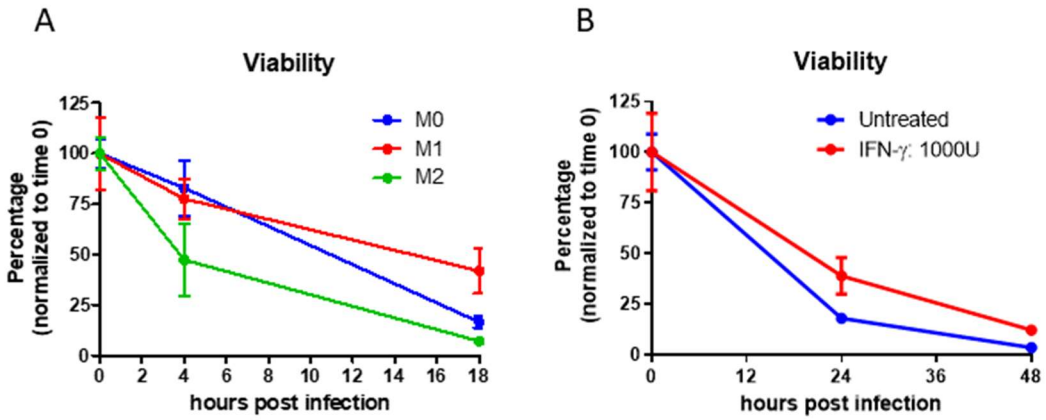

Figure 3.11 Classically activated macrophages exhibited poorer intracellular clearance against *E. faecalis*

(A) Intracellular survival of *E. faecalis* in differently polarized macrophages. Bone marrow-derived macrophages were polarized for 24 hours before being infected with *E. faecalis*. After the indicated time, macrophages were lysed and intracellular CFU was plated and counted. (B) Intracellular survival of *E. faecalis* in the presence of IFN-γ. RAW-Blue cells were treated with IFN-γ after engulfed *E. faecalis* for the indicated amount of time before the cells were lysed to collect and count intracellular CFU of *E. faecalis*. For all data, one experiment with three technical replicates were performed.

3.3 Discussion and conclusion

Enterococci have been identified by the United States CDC as a serious threat, largely due to the emergence of vancomycin resistance. With therapeutic options getting more limited, it is crucial to have a better understanding of these microbes' pathogenic mechanisms in order to develop alternative therapeutic strategies. Here I first conducted a transposon screen to identify bacterial factors that contribute to immunomodulating effects of *E. faecalis* and investigated their underlying mechanisms. Results of the screen revealed 47 genes predicted to be involved in the suppression of NF-κB activation in macrophages **(Table 3.1)**, with phenotypes like acidification of the environment and cytotoxicity likely to participate in such suppressing effects **(Fig. 3.3A)**. Cytotoxicity, more specifically apoptosis-inducing cytotoxicity regulated by the shikimate pathway **(Fig. 3.6C)**, was identified to play a role in suppression. However, specific mechanisms remain unclear.

From the library screening, the shikimate pathway stood out among the candidates because I hit multiple genes in the pathway. With 10 genes in this pathway (8 of which can be found in our library), 3 were predicted to be involved in immunomodulation based on our transposon screen data. During further investigation, two more genes in shikimate pathway showed weaker suppressing effects, indicating 5 out of 8 genes tested in this pathway are involved in macrophage

immunosuppression. These additional identified genes indicate that although the screening missed some hits, it efficiently identified potential pathways which can then be further evaluated. Thus, the current list of mutants attenuated for macrophage suppression should be followed up by future studies. Mutants with associated genes available our transposon library, such as genes in the same operon or same pathway, should be collected and tested for their suppressing effects. Six genes on the list are annotated as hypothetical proteins, making the identification of pathways more difficult. Nonetheless, with current information, we should be able to identify more participating pathways and potential bacterial factors.

The *in vitro* model used in this study used MOI 100 for infection, which may not be the best mimic of initial bacterial colonization. Other studies usually use around MOI 1 to 15 in infection assays of epithelial cells [68, 69]. Clinical infection sites are likely to have a more dynamic leukocyte infiltration and tissue level homeostasis, making the relevance of *E. faecalis*-mediated acidification of the local environment and cytotoxicity difficult to predict. Furthermore, under such a stressed environment with drastically different nutrient profiles as well as host defense mechanisms, the gene expression of *E. faecalis* alters rapidly and drastically. For instance, within 30 to 60 minutes of being cultured in blood, massive alterations in metabolism-related gene expression are observed, indicating an adjustment to different nutritional

conditions [70]. During subdermal colonization, genes in *E. faecalis* that are involved in metabolism and nutrient acquisition are also differentially expressed within 2 hours, and further alterations were observed at 8-hour post-infection, indicating how strong can the environment influence bacterial physiology [71]. In both cases, genes participating in the seven-step shikimate pathway were downregulated, most likely in response to environmental changes, which makes the role of this pathway in actual infection unclear. In order to investigate the relevance of the suppressing effects we observed, the dynamic of the infection should be illustrated in *in vivo* model with a detailed time course analysis.

Nonetheless, this study demonstrated that the shikimate pathway is a clear candidate involved in *E. faecalis*-mediated immunomodulating effects. This pathway is crucial for microbial viability and tolerance during infection, indicating that it contributes to pathogen virulence [72, 73]. Furthermore, metabolites produced through this pathway are predicted to attenuate inflammation and promote intestinal homeostasis, exhibiting a protective role in inflammatory bowel diseases [74-76]. With five genes in this pathway (OG1RF 11280, 11281, 11283, 11285, 12459) identified to exhibit weaker macrophage suppression, this pathway may contribute to long-term colonization through the synthesis of isochorismate/quinone, instead of aromatic amino acids or folate. All of the shikimate pathway mutants with weakened

immunomodulatory phenotypes coincidentally showed reduced cytotoxicity **(Fig. 3.4C)** but similar acidification comparing to wildtype **(Fig. 3.4D)**, indicating that cytotoxicity is the primary reason why of the pathway gives rise to an immunosuppressive phenotype. Also, to fully investigate the contribution of the shikimate pathway to macrophage suppression, clean knockout mutants and complementation strains should be prepared to confirm the observed phenotypes. Since this pathway is only found in microorganisms and plants but not in humans, future tests to target the participating enzymes to either directly kill the bacteria, which is a strategy under study, or to inhibit immune evasion could be helpful in developing novel therapeutic strategies [77, 78].

A functional *E. faecalis* shikimate pathway is necessary for apoptosis induction in macrophages, but I was not yet able to identify the responsible bacterial product. While many compounds showed reduced expression when the shikimate pathway was interrupted **(Table 3.2)**, those compounds tested so far did not exhibit cytotoxic nor immune suppressing potency.

Fourteen of the shortlisted genes are subunits of transporters, including families like phosphotransferase (PTS) system transporters and ATP-binding cassette (ABC) transporters. Transposon insertions in four of the transporters weakened acidification, suggesting that these may contribute to the suppressing effects through

acidosis. However, the cargo of these transporters are not known, making the investigation of underlying mechanisms challenging. While ion transporters are involved in tolerance against macrophage-mediated killing [37], no reports exist addressing the relationship between transporters and immunomodulation. It would be interesting to understand the role of these transporters and their interaction with other identified pathways. With these transporters potentially locate at the surface of bacterial membrane, they could be ideal targets for new therapeutic drugs.

In summary, with the observation of *E. faecalis*-mediated suppression against LPS-induced NF-κB activity in macrophages, I conducted a transposon library screen to identify *E. faecalis* factors responsible for the immunomodulation **(Fig. 3.12)**. Mechanisms like acidosis and cytotoxicity contribute to the reduced immune activity, while other yet-to-be-defined pathways may also participate in this inhibitory phenotype. The shikimate pathway was identified to contribute to cytotoxicity, instead of immunosuppression, likely by inducing apoptosis of macrophages; nonetheless, resulting in a reduced inflammatory response in the presence of *E. faecalis*.

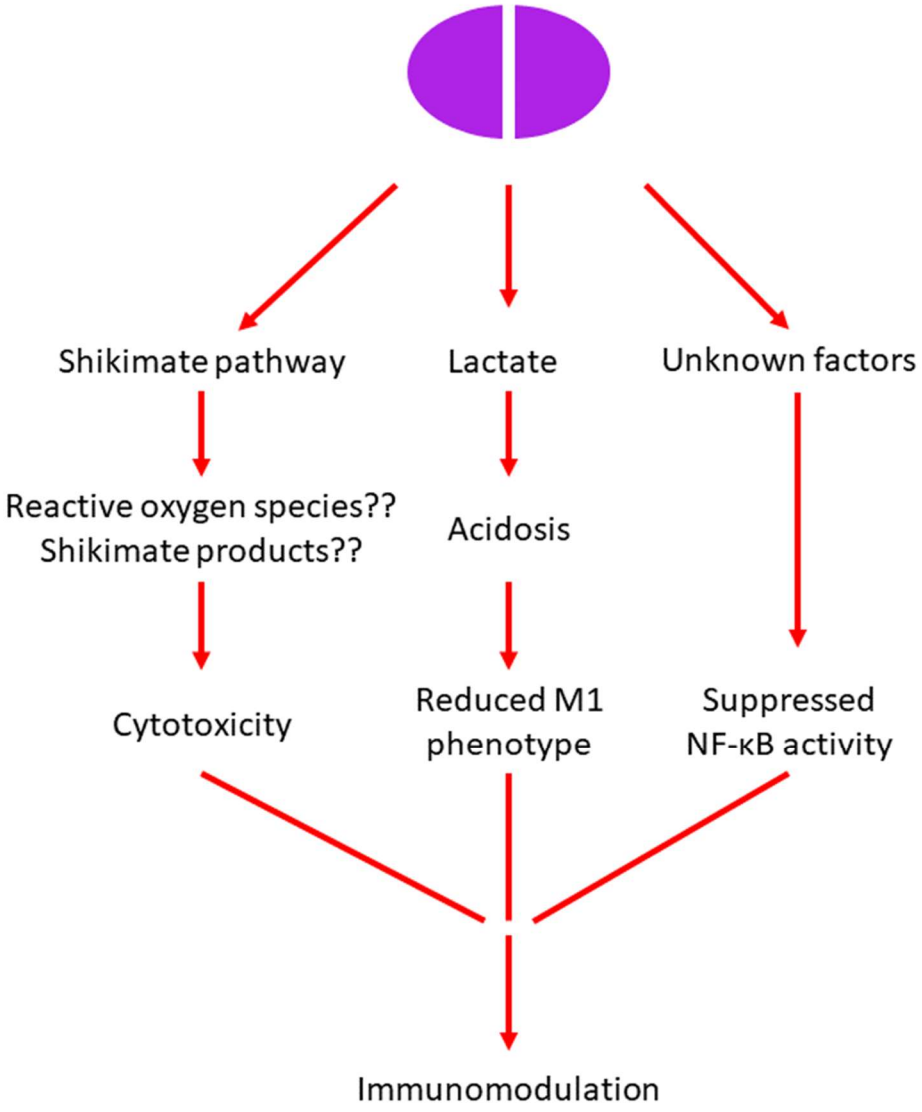

Figure 3.12 Mechanisms of *E. faecalis*-mediated immunomodulation identified in this study

3.4 Material and Method

3.4.1 RAW-Blue cells and BMDM culture

RAW-Blue cells (Invivogen^{TM}, USA), derived from RAW 264.7 macrophages, are reporter cells that were used to detect the NF-κB activity and evaluate the suppressing effects. These cells were transfected with plasmid which produces secreted embryonic alkaline phosphatase (SEAP) upon NF-κB activation. To maintain the expression of the reporter plasmid, cells were cultured in DMEM supplemented with 10% FBS under antibiotic pressure with zeocin (200 µg/ml).

Bone-marrow derived macrophage were differentiated from hematopoietic stem cells collected from C57BL/6 bone marrow, cultured in DMEM supplemented with 10% FBS and additional M-CSF (25 ng/ml, Biolegend) for 7 to 10 days with fresh medium supplied every 3-4 days before checking the purity by flow cytometry. The cells were considered differentiated macrophage when over 90% of total live cells were $CD45^{+}$, $F4/80^{+,}$ and $CD11c^{+}$.

3.4.2 Bacteria culture and normalization, detection of growth rate and acidification

OG1RF was chosen as the strain of *E. faecalis* used in this study. The transposon

library used for screening was also constructed in OG1RF background [47]. *E. faecalis* was grown in brain-heart infusion (BHI) medium overnight before infecting macrophages or running for assays. The overnight suspensions were normalized to 0.5 at O.D. 600 nm as 3 x 10^8 CFU/ml and diluted to infect macrophages with indicated multiplicity of infection (MOI).

To determine the growth rate of each strain, *E. faecalis* mutants were first normalized before being inoculated in colorless DMEM supplemented with 10% FBS and detected with O.D. 600 nm every 15 minutes for 20 hours by plate reader TECAN M200 set at 37^{o}C. Growth curves were drawn, and the mutants that failed to reach a plateau similar to wildtype were excluded.

To evaluate the acidification activities, 10^8 CFU of *E. faecalis* was cultured in 5 ml of DMEM supplemented with 10% FBS, and the pH value was measured by pH meter (Satorius, Docu-pH) after incubated at 37^{o}C for indicated hours.

3.4.3 Primary screening workflow

For screening, 10^5/well RAW-Blue cells were seeded in 96-well plates overnight for attachment and washed with PBS once on the day of infection. Mutants from transposon library were inoculated and grew in 200 µl/well DMEM with 10% FBS for 24 hours. After thorough pipetting, 20 µl of bacterial suspension was added to cells

for 6-hour stimulation in the presence of LPS (100 ng/ml). Mutants with lower suppressing effects (higher NF-κB activity) than wildtype OG1RF were shortlisted for validation and secondary screening.

3.4.4 SEAP assay, ELISA, and cytotoxic assay

To evaluate immune activation, 10^5/well macrophages were seeded in 96-well plates for overnight adherence. *E. faecalis* at indicated MOI was added to each well, with or without LPS, and cells were stimulated for 6 hours. NF-κB activity was evaluated by the level of SEAP in the supernatant, which was measured by overnight incubation with QUANTI-Blue reagent according to the manufacturer's instruction. Biolegend cytokine ELISA deluxe kit was used to detect the secreted level of TNF-α within supernatant. Assays were performed according to the manufacturer's instructions.

Cytotoxicity was determined by the level of lactate dehydrogenase (LDH) released to supernatant by LDH cytotoxicity detection kit (TaKaRa). Following the manufacturer's instruction, 100 µl of supernatant was collected to mix with working reagent, incubated at room temperature for 5 minutes before stopping reaction by 1 M HCl. Results were detected by O.D. 490nm, and cells treated with 0.1% triton-x were used as 100% cytotoxicity while untreated cells were considered as no cell

death induced.

3.4.5 Flow cytometry intracellular staining and apoptosis assay

To evaluate the level of viability and TNF-α production in macrophages, 2 x 10^5 RB cells/well were seeded in 24-well plates or 10^5 RB cells/well in 96-well plates. Wildtype OG1RF and transposon mutant with disrupted shikimate dehydrogenase (OG1RF_11280) were used to infect macrophages at MOI 100 without or with LPS (100 ng/ml) for 6 hours before macrophages were collected and stained for flow cytometry analysis. Cells were first gated on macrophage (CD45+, F4/80+ and CD11c+) population before further analysis. For intracellular staining of cytokine production, brefeldin A (Biolegend) was added at the beginning of stimulation to accumulate cytokines intracellularly. Cells were fixed and permeabilized accordingly to assay recommended procedure (Biolegend). All the antibodies used were purchased from Biolegend and details are listed in Appendix table 1, and fixable viability dye from ThermoFisher was used for viability evaluation. Apoptosis was determined by the staining of both annexin V-APC (Biolegend) and viability dyes (ThermoFisher) after cells were infected for indicated time. Samples were acquired with BD Fortessa X20 flow cytometer. Data were analyzed with FlowJo software version 10.

3.4.6 Mass spectrometry detection and analysis

OG1RF E. faecalis and mutants were cultured for 24 hours at 37^{o}C in DMEM + 10% FBS. Supernatants were filtered through a 0.2 µm filter and frozen at -80^{o}C. Samples were then lyophilized for 1 day by FreeZone freezer dryer (Labconco) and sent to Singapore Phenome Center (SPC, LKC medical school) for mass spectrometry profiling. The results were analyzed and compounds at a lower level (fold-change < log2 -5) in mutant supernatants that reached statistical significance ($p<0.01$) were shortlisted.

3.4.7 Chemical stimulation assay

Kuromanin chloride, peodinidin 3-O-glucose chloride, and p-coumaric acid were dissolved in DMSO. Indicated concentrations were used to stimulate RAW-Blue cells for 6 hours at 37^{o}C and supernatants were collected for cytotoxicity and immune activity detection.

3.4.8 Superoxide production

To detect superoxide production, DCFDA / H2DCFDA – Cellular ROS Assay Kit (Abcam # ab113851) was used. Bacterial suspension was normalized to 5 x 10^{7} CFU/ml and resuspend in colorless DMEM with 10% FBS. 20 µM of DCFDA was added

before and bacteria were seeded into black-wall glass-bottom 96-well plates (ibidi #89626) and placed at plate reader (Tecan Infinite® 200 PRO spectrophotometer) immediately. Placed in plate readers set at 37 ^{o}C with Ex/Em of 485/535, the signals were recorded for 6 hours with 10 minute-interval. Time-lapse data was analyzed by Graphpad Prism 9 and the area under curve was calculated with Excel.

3.4.9 Intracellular bactericidal assay

5 x 10^5 RAW-Blue cells/well were seeded in 6-well plates and incubated overnight to allow attaching to the surface. After washing, 5 x 10^6 *E. faecalis* were added to the macrophages and centrifuged (200g, 5 minutes) to promote cell-bacteria contact. The plates were incubated under cell culture condition for 1 hour to allow internalization of *E. faecalis* before the removing supernatant and washing the wells to get rid of extracellular bacteria. Medium with antibiotics (vancomycin + gentamicin = 10 µg + 150 µg/ml) and indicated cytokines was added to the wells to kill remaining extracellular bacteria. Macrophages were lysed with 0.1% triton X at indicated time points and the lysates were collect to plate on BHI agar for CFU counting. All recombinant cytokines were purchased from Biolegend (mouse carrier-free).

3.4.10 Statistical analysis

For most statistical analyses and heat map, the software GraphPad Prism 9 was used. Primary screening results and mass spectrometry results were processed and analyzed by Excel Microsoft.

3.5 Contribution

This project was initiated by Dr. Soumili Bhaduri Tagore and further established by Dr. Brenda Yin Qi Tien. I designed and analyzed all the experiments including the screening using the transposon library developed and provided by Dr. Gary Dunny [47]. Except for mass spectrometry, all the experiments were performed by me. For mass spectrometry, samples were prepared by me before being sent to Singapore Phenome Center (SPC, LKC medical school) for mass spectrometry profiling.

3.6 Reference

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Chapter 4: Conclusion and future perspectives

4.1 Summary of Key Findings

In this thesis, I studied the interactions between *E. faecalis* and innate immune cells, and specifically asked how *E. faecalis* may benefit by modulating these interactions. Here I highlight some of the key findings of our work that have advanced knowledge in this field.

In **Chapter 2**, I investigated the neutrophil-mediated responses upon *E. faecalis* infection and how the involvement of extra coinfecting microbe, in this case *S. aureus*, impacts neutrophil functions, and three discoveries stood out:

1) *E. faecalis* induced a large amount of intracellular ROS production by neutrophils, and this is correlated to neutrophil-mediated killing against *E. faecalis* **(Fig. 2.4)**. The current understanding of neutrophil responses against *E. faecalis* is very limited, starting with why opsonization is crucial in the bactericidal activity in clearance of *E. faecalis* [1, 2]. Our findings indicate that intracellular ROS may be the main neutrophil effector responsible for *E. faecalis* clearance. With experiments like evaluating the level of phagocytosis between opsonized and non-opsonized condition as well as using phagocytosis inhibitor to determine the importance of intracellular killing, I should be able to pinpoint more specifically the specific mechanisms facilitate *E. faecalis* killing.

2) Mixed infection of *E. faecalis* and *S. aureus* interfered with neutrophil-mediated killing and led to better bacterial survival. Polymicrobial infections are getting increasing attention and are known to impede wound healing compared to single species infections [3]. However, the studies are mostly focusing on the interactions among microbes and on the resistance against antibiotics, rather than considering the multi-way interaction between different species *and* the host immune response [4, 5]. Our results show how polymicrobial infections impact immune responses, for instance how *E. faecalis* impairs intracellular ROS induced by *S. aureus* **(Fig. 2.4)**. The presence of *E. faecalis* also reduced *S. aureus*-triggered NETosis **(Fig. 2.2)**. The impaired immune responses were correlated with compromised neutrophil-mediated killing **(Fig. 2.6)**, suggesting that the polymicrobial community composition can contribute to virulence by interfering with immune responses. Our *in vivo* model findings support the idea that polymicrobial infection and immune suppression promotes overall bacterial burden. Further investigation, for instance determining the level of NET structures in wound tissues, could help us to better understand whether the increased bacterial load is indeed due to impairment of immune responses, as the *in vitro* models suggested.

3) *E. faecalis* did not trigger NET formation from neutrophils and was resistant to *S. aureus*-induced NETosis killing. The potential of *E. faecalis* to trigger NETosis has

been poorly studied, with only one publication suggesting *E. faecalis* can induce NETosis when the neutrophils are incubated in cow milk [6]. Our observations indicated that *E. faecalis* did not induce NETosis, at least in our model in which neutrophils encountered planktonic microbes in the presence of opsonin **(Fig. 2.2)**. Furthermore, while S. aureus induced NET formation under mixed infection, *E. faecalis* viability was actually enhanced **(Fig. 2.6)**. There is still much to learn about the interaction between NETs and *E. faecalis*, and there are multiple future avenues that can be explored, which will be further elaborated in **future perspectives (4.2.1)**.

In **Chapter 3**, I studied the immunomodulating effect of *E. faecalis* towards LPS-induced macrophage activation and identified genes that may contribute to immunosuppression via a transposon library screen, and the three main findings are:

1) A list of genes predicted to participate in *E. faecalis*-mediated immunomodulation. Components of *E. faecalis* such as lipoteichoic acids have shown to suppress NF-κB activity, however, it is unclear how live *E. faecalis* modulate immune responses [7, 8]. The genes I shortlisted have a wide variety of functions including nutrient transport and metabolism regulation **(Table 3.1)**, suggesting live bacteria may have multiple mechanisms leading to immunomodulating effects. By studying the role for each of these genes in immunomodulation, we should identify pathways that are involved.

2) Immunomodulation of macrophages appears to be a multi-factor process, including processes of acidification and cytotoxicity. Acidosis interferes with immune responses [9], and this mechanism is best described in tumor environments [10], but not in the context of microbe-host interactions. Our lab has identified that *E. faecalis*-produced lactate acidifies the environment, which then contributes to suppressed macrophage activation [Brenda Tien, unpublished data from Kline lab], which is supported by this screening result with lactate dehydrogenase being shortlisted as well as a quarter of predicted genes participate in acidosis **(Table 3.1, Fig. 3.3)**. On the other hand, some strains of *E. faecalis* cause cytotoxicity through hemolysin [11] as an alternative mechanism to modulate immune responses. While hemolysin is not encoded in the *E. faecalis* OG1RF genome used in this study, cytotoxicity was a major factor with 70% of the predicted genes contributed to inducing cell death in macrophages **(Fig. 3.3)**. However, cytotoxicity was only observed with high bacterial inoculum (MOI 300). The route of which cytotoxic agents are delivered with such high bacterial density is unclear, and this will be further speculated in **future perspectives (4.2.2)**.

3) The shikimate pathway contributed to *E. faecalis*-mediated cytotoxicity toward macrophages. The shikimate pathway is responsible for production of phenolic compounds [12-14] and has been associated with immune suppressions [15, 16]. In

our study, an intact shikimate pathway facilitated cytotoxicity against macrophages **(Fig. 3.6)**. While the cytotoxic effector has not been identified, extracellular ROS produced by *E. faecalis* is one of the candidates that could induce apoptosis of mammalian cells [17]. By correlating ROS production and cytotoxicity in future work and neutralizing ROS with antioxidants, we should be able to confirm the role of *E. faecalis*-produced ROS in cytotoxicity. Investigating whether intracellular *E. faecalis* is associated with compromised macrophages, and whether this association is also shikimate pathway-dependent could provide some insights regarding pathogenicity of *E. faecalis*. I will discuss this more in **future perspectives (4.2.2)**.

4.2 Future perspectives

4.2.1 Interactions between NETosis and *E. faecalis*

***E. faecalis* biofilm versus planktonic neutrophil interactions**

One key discovery our work is that planktonic *E. faecalis* do not induce neutrophils to undergo NETosis **(Fig. 2.2)**. Pathogen can trigger NETosis by a number of mechanisms, but NETosis can be better triggered by microbes that are too large to be engulfed, such as fungi in their hyphal form [18]. Further, specific stimuli like microbial extracellular nucleic acids also promotes NET formation [19]. It is therefore notable that the extracellular matrix of *E. faecalis* biofilms, at least based on *in vitro* data, is primarily composed of extracellular DNA (eDNA) [20, 21]. However, *E. faecalis* biofilms are reported to be less immunostimulatory than planktonic *E. faecalis* [22, 23]. It is thus an open question of whether *E. faecalis* biofilms and their associated eDNA matrix trigger NETosis. Given the importance of biofilm in the virulence of *E. faecalis* [24], it is of interest to determine whether their biofilm eDNA can trigger NETosis, and if not, why not? NETosis has functions beyond bacterial clearance and can also act as a physical barrier to prevent bacterial dissemination [25]. If *E. faecalis* biofilms do in fact trigger NETosis, NETosis inhibitors or PAD4 knockout mice may be used to prevent NET formation and evaluate the role of NETs in containing *E. faecalis*

dissemination beyond the initial site of infection, such as bloodstream.

Potential contribution of NETs to *E. faecalis* pathogenicity

While in our study *S. aureus*-induced NETosis did not exhibit potent bactericidal activity against *E. faecalis*, the NET formation could still play a role in controlling the dissemination of *E. faecalis*. On the other hand, NETosis can be a double-edged sword that promotes the formation of biofilm structure under certain situations, by providing a source of the eDNA that often comprises the biofilm matrix. Indeed, this has been demonstrated for *Streptococcus mutans* and *S. aureus*, such that NETosis leads to an increase size of vegetations in infective endocarditis [26, 27]. Furthermore, neutrophils undergo NETosis at wound tissue even without microbial invasion, and this activation is more prominent in diabetic individuals, meaning that NET structures may be present in chronic wounds even before infection, and may therefore promote infection when pathogens subsequently colonize [28]. Given the resistance of *E. faecalis* against NETosis, a pre-formed NET could be a perfect platform for *E. faecalis* to attach and start the development of biofilm. Investigating the interaction between *E. faecalis* biofilm and NETosis could provide more insights into the dynamic of wound infection.

4.2.2 Harnessing knowledge of *E. faecalis*-host immune interactions to

develop antimicrobial strategies

With the current knowledge of the immune responses against *E. faecalis*, as well as the understanding of *E. faecalis*-mediated immunomodulating effects, this work has potential implications for several host-based therapeutic strategies that could be considered for future development, coupled with or in lieu of antibiotic treatments:

1) Polymicrobial community-specific antibiotic treatments. Neutrophils can effectively eliminate single-species infection [29]. However, when facing multiple pathogens, the efficiency of neutrophil-mediated killing is reduced **(Fig. 2.6)**. Therefore, one possible strategy for mixed species infections is to administrate specific antibiotics to focus on removing a single pathogen that is contributing most to synergistic disease, instead of using broad-spectrum antibiotics which may have adverse effects on the normal healthy microbiota. Once the microbial community is simplified or cleared of the most problematic pathogens, it may be possible to induce neutrophils and other immune cells to better battle the remaining microbes more effectively. For instance, *S. aureus* and *E. faecalis* triggered different neutrophil responses, leading to impaired clearance against either bacterium **(Fig. 2.6)**. On the other hand, *E. coli* can induce NETosis and is susceptible to NETosis-mediated killing [30], similar to *S. aureus*. When facing infections consist of *E. faecalis*, *S. aureus*, and

E. coli, it is possible that by removing *E. faecalis* with specific antibiotics, the immune system will be able to effectively eliminate the remaining *S. aureus* and *E. coli* through NETosis-mediated killing. However, given that all these microbes are known pathogens that inflict diseases in human, rapid removal by antibiotics is still the main strategy, and more studies are needed before such targeted approaches can be considered applicable in practice. Nonetheless, immunotherapies of the future that are designed to promote immune clearance of pathogens must factor in this complexity given that infections are often polymicrobial in nature.

2) Inhibiting the shikimate pathway to promote immune-driven bacterial clearance. The *E. faecalis* shikimate pathway contributed to cytotoxicity, inducing cell deaths in macrophages **(Fig. 3.6)**. This cytotoxic effect may contribute to immune evasion by *E. faecalis* and lead to chronic or recurrent infections. While the shikimate pathway is not essential for *E. faecalis* survival, inhibitors targeting this pathway such as glyphosate [31] and marein [32] could suppress the cytotoxicity caused by *E. faecalis* and allow immune system to perform better microbial clearance. Considering that shikimate pathway does not exist in mammalian cells [33], a specific drug targeting the activity of this metabolism should be harmless to the patients.

4.3 Final reflections

Overall, we have begun to elucidate the interactions between host immune cells and *E. faecalis*, in both mono- and poly-microbial settings. This work provides mechanistic insight for both how *E. faecalis* can modulate the immune response to promote polymicrobial infections, and how *E. faecalis* confers cytotoxic effects to reduce immune activation. These findings also pave the way for future studies regarding biofilm-NET interaction and intracellular survival of *E. faecalis*, as well as provide clues for developing therapeutic strategies.

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Antibody				
Target	**Application**	**Clone**	**Brand**	**Working dilution**
CD11a	**Flow cytometry**	**M17/4**	**Biolegend**	**1:100**
CD11b	**Flow cytometry**	**M1/70**	**Biolegend**	**1:100**
CD11c	**Flow cytometry**	**N418**	**Biolegend**	**1:100**
CD15 (SSEA-1)	**Flow cytometry**	**MC480**	**Biolegend**	**1:100**
CD14	**Flow cytometry**	**Sa14-2**	**Biolegend**	**1:100**
CD16	**Flow cytometry**	**S17014E**	**Biolegend**	**1:100**
CD45	**Flow cytometry**	**30-F11**	**Biolegend**	**1:100**
CD63	**Flow cytometry**	**NVG-2**	**Biolegend**	**1:100**
Citrullinated Histone 3	**Immunofluorescence**	**polyclonal**	**abcam**	**1:500**
F4/80	**Flow cytometry**	**BM8**	**Biolegend**	**1:100**
LC3B	**Immunofluorescence**	**polyclonal**	**abcam**	**1:500**
Ly6G	**Flow cytometry**	**1A8**	**Biolegend**	**1:100**
Neutrophil elastase	**Immunofluorescence**	**polyclonal**	**abcam**	**1:500**
TNF-α	**Flow cytometry**	**MP6-XT22**	**Biolegend**	**1:100**
CD4	**Cocktail for neutrophil isolation (negative selection)**		**Biolegend**	**1:50**
CD5				
B220				
CD11c				
CX3CR1				
F4/80				
CD117				
CD244.2				
TER-119				

Appendix Table 1. Antibodies used in the study

Gene Code	SEAP signal (mean ± SD)	pH value (mean ± SD)	Cytotoxicity % (mean ± SD)
Wildtype OG1RF	0.29±0.09	5.84±0.36	17.69±3.55
OG1RF_10013	0.40±0.27	7.81±0.09	9.06±1.97
OG1RF_10021	0.60±0.04	6.32±0.02	10.59±1.92
OG1RF_10199	0.93±0.06	6.61±0.01	0.93±0.83
OG1RF_10329	0.53±0.02	6.07±0.03	5.40±2.49
OG1RF_10418	0.46±0.05	6.22±0.03	10.16±2.70
OG1RF_10427	0.56±0.06	5.92±0.64	8.94±3.37
OG1RF_10514	0.59±0.16	7.07±0.04	15.05±3.84
OG1RF_10682	0.46±0.03	6.44±0.04	7.43±4.07
OG1RF_10956	0.42±0.06	6.06±0.09	4.66±1.64
OG1RF_10967	0.35±0.06	5.78±0.06	0.67±0.97
OG1RF_11022	0.67±0.08	7.28±0.02	2.91±1.84
OG1RF_11023	0.69±0.06	7.29±0.02	1.27±0.46
OG1RF_11036	0.56±0.09	6.12±0.02	2.24±4.48
OG1RF_11131	0.37±0.01	5.66±0.04	10.74±2.04
OG1RF_11161	1.18±0.09	6.72±0.03	0.88±0.87
OG1RF_11270	0.55±0.12	5.65±0.12	5.26±2.78
OG1RF_11280	0.85±0.03	5.72±0.08	0.97±1.26
OG1RF_11283	0.80±0.15	5.73±0.09	2.41±1.52
OG1RF_11300	0.66±0.04	6.53±0.02	2.64±0.98
OG1RF_11329	0.55±0.10	6.52±0.03	4.63±3.16
OG1RF_11391	0.53±0.06	6.29±0.01	4.64±4.33
OG1RF_11450	0.40±0.03	6.12±0.04	5.45±5.12
OG1RF_11602	0.85±0.09	6.47±0.02	2.16±1.92
OG1RF_11622	0.36±0.06	6.22±0.03	4.40±2.63
OG1RF_11636	0.68±0.08	6.56±0.03	3.20±2.65
OG1RF_11669	0.60±0.09	5.66±0.04	2.38±3.23
OG1RF_11677	0.71±0.03	6.32±0.05	2.19±2.59
OG1RF_11766	0.76±0.07	6.21±0.03	1.32±1.87
OG1RF_11854	0.83±0.11	6.31±0.01	1.28±2.56
OG1RF_11923	0.51±0.03	5.61±0.08	22.67±1.40
OG1RF_12024	0.81±0.02	6.27±0.02	18.06±2.38
OG1RF_12033	0.75±0.09	6.08±0.04	15.66±3.48
OG1RF_12043	0.46±0.07	5.44±0.20	17.81±4.81
OG1RF_12058	0.72±0.04	6.32±0.04	11.62±1.73
OG1RF_12066	0.44±0.05	5.71±0.05	11.64±4.19
OG1RF_12113	0.79±0.11	6.06±0.04	8.84±3.23
OG1RF_12190	0.57±0.04	6.22±0.02	7.76±2.24
OG1RF_12284	0.46±0.10	5.77±0.02	4.92±3.31
OG1RF_12385	0.44±0.03	6.20±0.01	9.11±2.12
OG1RF_12388	0.38±0.06	6.75±0.01	5.04±3.30
OG1RF_12401	0.72±0.05	5.90±0.03	10.09±12.02
OG1RF_12446	0.46±0.06	5.55±0.14	14.73±2.11
OG1RF_12459	0.83±0.05	6.25±0.03	11.00±0.77
OG1RF_12507	0.86±0.09	5.84±0.01	3.65±2.45
OG1RF_12536	0.62±0.08	5.78±0.04	9.82±3.27
OG1RF_12565	0.42±0.06	6.29±0.04	5.36±4.20
OG1RF_12567	0.54±0.03	6.31±0.01	6.10±0.86
Without bacteria	1.16±0.26 (LPS-treated)	8.14±0.11	0.92±1.27

Appendix Table 2. Raw data of the immunosuppression, acidification, and cytotoxicity phenotypes from the shortlisted mutants