# A porcine model for polymicrobial respiratory infections with swine influenza virus and *Staphylococcus aureus*

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# ABSTRACT

Influenza A virus (IAV) is a significant problem worldwide, and respiratory disease is further complicated by secondary bacterial infection. The emergence of highly pathogenic strains of IAV in conjunction with the increase of antibiotic-resistant bacteria threatens human health. A large-animal model effective for study of polymicrobial infection comparable to humans must therefore be developed. IAV has been studied extensively in small animals, including mice, rats and ferrets. However, these species frequently require IAV adaptation, reducing the capacity of these models to adequately represent human infection. Furthermore, species commonly used lack likeness to humans in both the presentation of symptoms and in lethality of infection. However, pigs are naturally susceptible to unadapted IAV and are considered to be the 'mixing vessel' for the recent pandemic IAV virus. Pigs are also susceptible to infection with *Staphylococcus aureus*, the most commonly isolated bacteria from IAV-infected human adults. Therefore, the use of pigs in the study of polymicrobial respiratory infections would be ideal for characterizing a host immune response comparable to humans, as well as for the development of diagnostics and therapeutics. Using this novel model, we determined that pigs are susceptible to *Staphylococcus aureus*, swine IAV, and polymicrobial infection. Furthermore, we showed that IAV infection predisposes pigs to *Staphylococcus aureus* pneumonia, and this susceptibility is dependent on day post-IAV infection.

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# TABLE OF CONTENTS

## List of Figures

## List of Tables

## List of Abbreviations

| | |
|---|---|
| Influenza A virus | IAV |
| Methicillin-resistant *Staphylococcus aureus* | MRSA |
| Panton-Valentine Leukocidin | PVL |
| Community-acquired pneumonia | CAP |
| Hemagglutinin | HA |
| Neuraminidase | NA |
| Interferon | IFN |
| Interleukin | IL |
| Tumor necrosis factor | TNF |
| Dendritic cell | DC |
| Cytotoxic T cell | $T_c$ |
| Helper T cell | $T_h$ |
| Madin-Darby Canine Kidney | MDCK |
| Alveolar macrophages | AM |
| Swine influenza virus | SIV |
| Polymorphonuclear leukocytes | PMN |
| Colony forming units | CFU |
| Plaque forming units | PFU |
| Cluster of Differentiation | CD |
| Days post infection | DPI |

# Chapter 1. Introduction

Influenza A virus (IAV) is estimated to cause 200,000 hospitalizations and more than 36,000 deaths annually in the United States (90, 91). Seasonal IAV epidemics result in economic loss, greater than $87 billion annually, despite preventative efforts (50). Morbidity and mortality rates associated with IAV substantially increase due to secondary bacterial infection (89). A national survey conducted in 2003 by the Center for Disease Control and Prevention regarding influenza found *Staphylococcus aureus* to be the most commonly isolated secondary bacterial pathogen in adults and second only to *Streptococcus pneumoniae* in children (64). Additionally, *Staphylococcus aureus* was isolated from human tissues following the 1918-1919 "Spanish flu" pandemic (52) and the 1957 IAV epidemic (66), which suggests the high mortality rates associated with these outbreaks were partly due to *Staphylococcus aureus* coinfection. In the 2003-2004 and 2006-2007 IAV seasons, methicillin-resistant *Staphylococcus aureus* (MRSA) led to a 33% fatality rate in previously healthy individuals infected with IAV (1, 28, 36). Influenza enhances bacterial pneumonia and the disease severity is exacerbated during coinfection (73).

# Chapter 2. Review of Literature

## *STAPHYLOCOCCUS AUREUS*

*Staphylococcus aureus* is a Gram-positive coccus capable of causing a variety of diseases in humans and animals. This versatile bacterium commonly colonizes epithelial surfaces and leads to diseases that include subcutaneous abscesses, toxic shock syndrome, food poisoning, and pneumonia. This pathogen's ability to cause an array of diseases is enabled by its arsenal of virulence factors, exotoxins, and enzymes (56). *Staphylococcus aureus* produces several enterotoxins, including alpha and beta toxins, and toxic shock syndrome toxin–1. These factors activate lymphoctyes and induce proliferation of immune cells, which may give rise to an overproduction of cytokines, leading to septic shock (26). Hemolysins are secreted virulence factors capable of lysing host cells by the formation of pores within host cell membranes (92). One such hemolysin, Panton-Valentine Leukocidin (PVL), is composed of two subunits, F and S, which are transferred by bacteriophages and encoded by the genes *lukF-PV* and *lukS-PV* (24). The PVL subunits bind to membranes of polymorphonuclear leukocytes (PMN) to activate the release of radical oxygen species and cytokines. Pore formation by PVL and subsequent opening of calcium channels finally result in PMN death (53). The degranulation of PMN following PVL pore formation can lead to epithelial cell necrosis, increased inflammatory response, and subsequent development of severe pneumonia (9, 53).

The USA300 and USA400 pulse field types of *Staphylococcus aureus* possess PVL and methicillin resistance and are the most common isolates obtained from community-acquired pneumonia (CAP) patients (37, 53). Community acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) frequently infects healthy individuals without risk factors for acquisition of antibiotic-resistant bacteria, which causes greater concern during IAV pandemics due to its

associated virulence among IAV-infected patients. Although CA-MRSA typically causes skin and soft tissue disease, many isolates produce PVL (60, 99). Most cases of necrotizing pneumonia due to PVL positive-MRSA are preceded by influenza-like symptoms (53, 68, 106). Patients suffering from influenza are thought to be susceptible to fatal necrotizing pneumonia caused by PVL-positive MRSA due to airway bleeding and leukocytopenia (27).

In addition to virulence factors contributing to pathogenesis, many strains of *Staphylococcus aureus* are antibiotic resistant, which compromises the treatment of infection. *Staphylococcus aureus* acquires methicillin resistance by way of staphylococcal chromosomal cassette (SCC) (37). The diagnosis of MRSA infections remains a problem and the most useful therapy options are antibiotics, which are now inadequate due to the lack of efficacy against methicillin resistant strains. Additional therapies, such as intravenous immunoglobulin therapy and PVL neutralization, designed to supplement antibiotic treatment must be studied in an *in vivo* model to evaluate efficacy (106). However, the limitations of small animal models have hindered the evaluation of the efficacy of toxin-suppressing agents in conjunction with antibiotic therapy (68). Furthermore, there is conflicting evidence as to whether PVL is directly responsible for pathogenesis and if PVL affects humans and animals in a similar fashion (40). Therefore, the development of an effective animal model is critical to developing methods to control MRSA induced CAP.

**INFLUENZA A VIRUS**

Influenza A virus (IAV) belongs to the *Orthomyxoviridae* family and contains eight segments of single-stranded RNA, which encode 11 proteins (55). Hemagglutinin (HA) and neuraminidase (NA) are surface glycoproteins, which are used to represent the subtype of IAV. They are also the major antigenic determinates, with HA mediating viral entry by binding to

sialic acid receptors on host cell surfaces (77). These glycoproteins are also the means by which subtypes of IAV are classified, i.e. H1N1, H3N2. Viral RNA is packaged and bound by the nucleoprotein (NP). Matrix protein M1 provides structural support and facilitates viral budding, whereas M2 is an integral membrane protein with ion channel activity (62). The nonstructural protein, NS1 is a potent virulence factor, as it acts as an immunosuppressive agent by preventing the release of type I interferons and inhibiting dendritic cell (DC)-induced T cell proliferation, whereas NS2 assists in the export of NP and viral RNA (25). The viral polymerase is made of the protein complex PB2, PB1, and PA. The recently discovered protein, PB1-F2, localizes to mitochondria where it signals cell death by apoptosis (103, 105). All pandemic strains of IAV, including the 1918, 1957, and 1968 viruses, possess an intact PB1-F2, which is believed to be responsible for the high mortality rates (16). The exception being the recent pandemic H1N1 IAV strains, which have a truncated PB1-F2 protein (101). Influenza A/Puerto Rico8/34 virus with intact PB1-F2 was capable of inducing apoptosis in human immune cells, whereas the PB1-F2 open reading frame knock out virus had become attenuated (15). Additionally, PB1-F2 knockout viruses reduced the virulence of the virus in a mouse model (104), suggesting that PB1-F2 is a virulence determinant. A recent study showed that a synthetic c-terminus peptide from 1918 influenza virus H1N1 PB1-F2 protein induced a strong pro-inflammatory response in mice and promoted secondary bacterial pneumonia (43). Infection of mice with this PB1-F2 mutant virus resulted in decreased weight loss and mortality, and decreased numbers of macrophages, PMN, and lymphocytes in the bronchoalveolar lavage (BAL) fluid compared with infection with wild type strain with intact PB1-F2 (43). Although the mechanisms of this are unclear, it has been suggested that the effects of PB1-F2 on macrophages would debilitate and delay adaptive immunity (16).

## HOST RESPONSE TO PULMONARY INFECTION

Proinflammatory cytokines released during pulmonary infection recruit and activate innate immune cells (107). Cytokines such as interferon (IFN)-α/β/γ, interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, exhibit antiviral activity against IAV (35, 75, 95, 96). However, mice deficient in TNF receptor 1 exhibit reduced mortality from H5N1 influenza, suggesting that TNF may also play a role in morbidity (86). Interleukin-1, IL-6, and TNF-α contribute to clearance and pathogenesis of *Staphylococcus aureus* (102). Additionally, IFN-γ treatment protects primary human endothelial cells against infection and severe injury by virulent clinical *Staphylococcus aureus* isolates (7). Nonetheless, IFN-γ depletion results in increased numbers of infiltrating PMN to the lungs of *Staphylococcus aureus* infected mice (54). The production of proinflammatory cytokines IL-6, IL-1, and TNF-α may lead to lung degeneration following infection with IAV (85, 97). These studies emphasize the complexity of unraveling the mechanisms involved in the host response to polymicrobial disease.

*Staphylococcus aureus* adheres to extracellular matrix proteins such as fibronectin, laminin, collagen/β-integrins, and intercellular adhesion molecule (ICAM)-1 (21, 30, 51). β-integrins and ICAM-1 are expressed by leukocytes and epithelial cells respectively, to allow leukocytes to roll along the endothelial layer and mediate cell adhesion during inflammation (47). The leukocytes are recruited to respiratory epithelium by the production of IL-8, which is induced during both *Staphylococcus aureus* and IAV infection (32, 35). Leukocyte function, particularly DC, may be enhanced by surfactant protein D, a pattern recognition molecule secreted by type II alveolar epithelial cells, which acts to enhance bacterial antigen processing by opsonizing infectious bacteria (11, 79). Upon infection with IAV, epithelial cells will present

viral antigen by upregulating MHC class I to alert cytotoxic T cells (Tc) (100). Investigation of the primary site of infection, respiratory epithelium, and its immune response, is paramount to understanding polymicrobial infection.

DC are antigen-presenting cells which utilize MHC class I and II complexes to present antigen (3, 13). DC produce high levels of IFN-α, TNF-α, IL-10, IL-6 and IL-12 when infected with virus (8, 29, 83). Plasmacytoid DC secrete copious amounts of type I interferons during IAV infection (41). Chemokine receptor CCR7 during mycobacterial infection (4), and L-selectin during migration along vascular epithelium (47). DC are capable of inducing an IFN-γ, IL-4, IL-2 response by T cells in order to polarize T cells to helper T cell (Th) type 1, type 2 or Tc pathways (8, 13). Staphylococcal superantigens nonspecifically stimulate CD8 memory T cells (17), while toxins act as mitogens inducing T cell proliferation leading to increased cytokine profiles and increased pathology. Depletion of CD8 T cells or IFN-γ reduces lung pathology in *Staphylococcus aureus* infected mice (54). However, the effect of lymphocyte depletion on viral replication and lung pathology during dual infections remains to be determined. Finally, IL-10, secreted by leukocytes, may be detrimental during influenza infection by limiting development of Th17 cells (45) and may impair host defense against bacteria (94). Depleted mice had increased levels of the chemokine monocyte chemotactic protein (MCP)-1 in BAL fluid. Mice deficient in IL-15 had higher lung colony-forming units (CFU) and were more susceptible to pulmonary infection with *Staphylococcus aureus* than wild-type mice, suggesting a critical role for natural killer cells in clearance of this pathogen (78). Interestingly, infection with IAV results in upregulation of toll-like receptor (TLR) 2 and complement 5a receptor (C5aR) on human PMN (39). The same study suggested a decrease in PMN survival when stimulated with IAV and then incubated with *Staphylococcus aureus*. The

aforementioned cytokines and chemokines associated with these populations may be responsible for the increase in pathology during polymicrobial infection.

## HOST SUSCEPTIBILITY TO POLYMICROBIAL INFECTION

Influenza A virus infection is considered to increase host susceptibility to secondary bacterial infections by altering the respiratory epithelium. Primary infection with IAV significantly increases the ability of *Staphylococcus aureus* to adhere to monolayers of Madin-Darby Canine Kidney (MDCK) cells compared with uninfected controls (20). Cells infected with IAV express different receptors than uninfected cells, which augment the binding affinity of *Staphylococcus aureus* (70). Tissue damage to the trachea by IAV increases secondary bacterial adhesion, and exposes extracellular matrix proteins within the lung, which may increase binding of *Staphylococcus aureus* (21, 58). Pretreatment of *Staphylococcus aureus* with the basement membrane element, fibrinogen, increases bacterial adhesion to IAV-infected cells compared to untreated *Staphylococcus aureus*, which suggests fibrinogen acts as a link between *Staphylococcus aureus* and receptors present on viral infected cells (20). Fibrinogen also contributes to secondary infectivity of Group A streptococcus (34, 69). Complement-activated endothelial cells have increased binding by *Staphylococcus aureus*, although bacterial surface-bound complement reduced adherence (19). Finally, IAV is also theorized to upregulate platelet-activating factor receptor (PAFr), which potentiates additional bacterial adherence in the lung (44, 72).

Staphylococcal proteases may prime viral hemagglutinin (HA) for enhanced viral infection, which could explain the increased lung pathology during dual infection (82, 87). Interestingly, the ability of bacterial proteases to cleave viral HA contributes to the increased viral activation and aggravated respiratory pneumonia in humans and mice (42, 88). The

*Staphylococcus aureus*-derived plasminogen activator staphylokinase is thought to be implicated in HA cleavage (71). Unfortunately, *Staphylococcus aureus* adherence is independent of viral HA and NA, indicating that these glycoproteins are not receptors for *Staphylococcus aureus*.

Influenza A virus is theorized to impair the function of host cells, such as macrophages and PMN, which are essential to the control of pulmonary infection (14). During influenza infection, PMN and monocytes are recruited to the lung by an upregulation of adhesion molecules (61). A mouse model of recombinant influenza virus bearing the 1918 HA and NA glycoproteins in the genetic background of the human A/Texas/36/91 infection showed that increased mortality is due to the increased number of alveolar macrophages (AM) and PMN and increased influx of cytokines and chemokines in lung tissues. Depletion of AM before a sublethal infection resulted in uncontrolled viral replication and increased mortality in mice (93). During polymicrobial infection with IAV and *S. pneumoniae,* PMN, although functionally impaired by IAV, are still heavily recruited by cytokines and chemokines released by the infected epithelium and activated by overexpression of granulocyte colony-stimulating factor (80). In cecal-ligation puncture mice, depletion of PMN during lung infection with *Staphylococcus aureus* resulted in decreased survival due to decreased pulmonary bacterial clearance and increased cytokine secretion (65). Macrophages are also recruited to the lung during IAV infection, but do not contribute to bacterial clearance (57), and their overwhelming presence in the lung induces alveolar epithelial cell apoptosis and extensive tissue damage by release of TNF-related apoptosis-inducing ligand to remove intracellular pathogens (31).

## *STREPTOCOCCUS PNEUMONIAE*

*Streptococcus pneumoniae* was the most commonly isolated secondary pathogen from pediatric influenza patients in the 2003-2004 influenza season (64). This bacterium commonly colonizes mucosal surfaces and with a myriad of virulence factors, this human pathogen leads to a variety of diseases such as otitis media, meningitis, and pneumonia (48). The high mortality rates associated with the 1918-1919 "Spanish flu" pandemic are thought to be due in part to severe pneumococcal coinfection (52). Primary infection with IAV increases adherence of *S. pneumoniae* to type II alveolar epithelial cells *in vitro* (5). IAV upregulates PAFr, which enhances pneumococcal adhesion to respiratory epithelial cells *in vitro* (18). However, utilization of a competitive agonist of PAFr in a mouse model highlighted the variability of pneumoccal virulence, as well other mechanisms used by pneumococci during adhesion and evasion (44). DNA microarray analysis of co-infected mouse lungs suggests that PAF and related molecules are still crucial in the development of pneumonia (72). IAV further contributes to secondary pneumococcal pneumonia by decreasing tracheal mucociliary clearance of bacteria (63). Macrophages and PMN are essential to the control of pulmonary infection (14), but IAV is theorized to be able to impair host cell function. IAV depresses PMN response, which may be related to decreased bactericidal activity and reduced bacterial clearance due to impaired phagocytic ability, intracellular generation of reactive oxygen species, and respiratory burst (2, 46). Type I IFN are secreted as part of antiviral defense, yet they impair the host's ability to clear secondary bacterial pathogens by hindering the release of chemoattractants needed for PMN function (76). Decreased PMN function in the lungs, in conjunction with excessive IL-10 production, may lead to an immunosuppressive state that predisposes the host to secondary bacterial pneumonia by *S. pneumoniae* during primary IAV infection (80, 94). Although

lymphocyte or PMN depleted mice infected with *S. pneumoniae* did not have altered bacterial counts compared with controls (81), PMN depletion did contribute to increased mortality (65).

Unlike for *Staphylococcus aureus*, there is an efficacious vaccine approved for pediatric use against *S. pneumoniae*. This 7-valent pneumococcal vaccine was effective in preventing secondary *S. pneumoniae* infection both in normal and severe pandemic influenza seasons, due to herd immunity (67). Therefore, it is crucial to establish an appropriate animal model for *Staphylococcus aureus*, as there are fewer means by which to control this pathogen compared with *S. pneumoniae*.

## ANIMAL MODELS

Development of appropriate animal models to study the mechanisms of pathogenesis may provide important information for disease prevention, diagnosis and treatment. Mice have traditionally been used to study IAV and bacterial co-infection. Although mice co-infected with *S. pneumoniae* and IAV suffer from more severe bronchopneumonia than mice with a single infection (74), disease synergy is not evident in the mouse model of *Staphylococcus aureus* and influenza (23). Furthermore, the IAV strains used in mouse co-infection studies are adapted to replicate effectively within the mouse, limiting the likeness of this model to human infection. Interestingly, cotton rats (*Sigmodon hispidus*) do not require IAV adaptation to succumb to infection, which induces respiratory tract lesions and clinical signs similar to humans (59). Upon infection with IAV and *Staphylococcus aureus,* the cotton rat exhibits signs of synergistic disease, with greater hypothermia, reduced recovery of pathology, and increased induction of cytokines (10).

Despite some success as polymicrobial models, rodents are limited in terms of biological and immunological similarity to humans (6, 49). In humans, healthy individuals are generally able to recover from IAV-related illness within 7 days (95), whereas the infection in rodents is often fatal. However, the pig is naturally susceptible to infection by both human strains of IAV (84) and *Staphylococcus aureus* (22, 38). Furthermore, cells within the porcine respiratory tract display receptors for human influenza viruses, making pigs ideal to study human influenza illness (33). Also, a temporal correlation has been established in swine between pro-inflammatory cytokines in BAL fluids, lung virus titers, PMN infiltration and clinical signs (96, 98). The pig possesses several similarities to human physiology and has been a successful model of human disease pathogenesis (12). Pigs, therefore, have the potential to serve as a model for co-infection with these pathogens.

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# Chapter 3. Pilot: An intranasal polymicrobial respiratory infection model for influenza A virus and *Staphylococcus aureus* in nursery pigs

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Running title: Pig model of post-influenza *Staphylococcus aureus* pneumonia

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**ABSTRACT**

Pneumonia due to secondary bacterial infections enhances the burden of influenza on society, healthcare and individuals. Pigs are naturally susceptible to both IAV and *Staphylococcus aureus* and have respiratory physiology and immune response comparable to humans. In this study, we first established an intranasal dose of methicillin-resistant *Staphylococcus aureus* (MRSA) that would induce respiratory symptoms without causing mortality in conventionally reared nursery pigs. Second, we established a polymicrobial model with an intranasal IAV infection followed by secondary intranasal MRSA inoculation. Using this novel model, we showed an increase in severity of interstitial pneumonia by MRSA in the presence of IAV. A pronounced influx of T-lymphocytes in the bronchoalveolar lavage (BAL) fluid on D5 and increased mRNA expression of pro-inflammatory (IL-1β, IL-6, TNF-α) and immunoregulatory (IL-10, IFN-γ) cytokine genes were evident in dual infected animals compared to single infected. Transcriptome analysis suggested that key immunoprotective cytokine transcripts in the lungs were downregulated in dual infected animals on D11. Together, these results provide novel insights into IAV-MRSA synergism and suggest a previously under appreciated role of IAV in hospital/community-acquired MRSA infection. Further, our intranasal challenge model in pigs is the first of its kind, and affords a platform for studying pathogenesis, biomarkers for initiating antibiotic therapy, and outcome of vaccination/treatment regimens in polymicrobial respiratory infections.

## INTRODUCTION

Influenza A (IAV) is estimated to cause more than 36,000 deaths and 200,000 hospitalizations in the United States every year (37, 38). Additionally, IAV epidemics result in substantial economic drain, largely due to missed work-days and loss of lives (24). The morbidity and mortality rates associated with IAV can substantially increase with secondary bacterial infection (36). *Staphylococcus aureus* is the most commonly isolated secondary bacterial pathogen in adults, with the 77% of isolates from influenza patients also being methicillin resistant (29). Methicillin-resistant *Staphylococcus aureus* (MRSA) was responsible for a 33% fatality rate in previously healthy individuals infected with IAV during the 2003-2004 and 2006-2007 seasons (1, 11, 17).

IAV infection is thought to increase host susceptibility to secondary bacterial infection by inducing innate immune cell dysfunction and damaging respiratory epithelium. IAV has been shown to depress PMN response, by reducing phagocytic ability, intracellular generation of reactive oxygen species, and respiratory burst, which likely impairs bactericidal activity and bacterial clearance (2, 21). During polymicrobial infection with IAV and *Streptococcus pneumoniae,* functionally impaired PMN are still heavily recruited by cytokines and chemokines released by infected epithelium and activated by overexpression of granulocyte colony-stimulating factor (33). Macrophages are also recruited to the lung during IAV infection, and in addition to contributing to reduced bacterial clearance (26), their overwhelming presence in the lung induces alveolar epithelial cell apoptosis and extensive tissue damage (13). Although it is generally accepted that IAV enhances bacterial pneumonia and exacerbates disease severity during coinfection (30), reliable animal models are lacking to directly test this hypothesis, to

identify early biomarkers for initiating antibiotic therapy, and to predict the outcomes of vaccination/treatment strategies.

Mice have traditionally been used to study IAV and bacterial co-infection. Although mice co-infected with *S. pneumoniae* and IAV suffered from more severe bronchopneumonia than single infection (31), disease synergy was not evident in the mouse model of *Staphylococcus aureus* and IAV (9). Furthermore, the IAV strains used in mouse co-infection studies are adapted to replicate effectively within the mouse, limiting the likeness of this model to human infection. However, cotton rats (*Sigmodon hispidus*) do not require adaptation of the virus to succumb to infection (27), and exhibit signs of synergistic disease (4). Although rodents are successful as a polymicrobial model, this success is limited in terms of biological and immunological similarities to humans (3, 23). However, the pig is naturally susceptible to infection by both human strains of IAV (34) and *Staphylococcus aureus* (8, 18). Furthermore, the pig possesses several similarities to human respiratory physiology and has been a successful model of human disease pathogenesis (5). We have developed a staggered intranasal challenge model of post-influenza MRSA pneumonia in conventionally reared nursery pigs. By using this novel model, we showed that disease severity, immune cell and pro-inflammatory cytokine influx were enhanced while key immunoprotective cytokines were downregulated in dual infected pigs compared to IAV or MRSA alone.

## MATERIALS AND METHODS

**Animals.** Conventionally reared nursery pigs, approximately 10-20 kg in bodyweight, were obtained from the Swine breeding facility at Virginia Polytechnic Institute and State University Center for Molecular Medicine and Infectious Disease animal facility, Blacksburg, VA. All procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Virginia Tech.

**Viruses.** Two strains of recent triple reassortant cluster IV influenza A viruses, designated A/Swine/Minnesota/1145/2007 (H3N2) (SIV 1145) or A/Swine/Minnesota/761/2007 (H3N2) (SIV 761) originally obtained from the Veterinary diagnostic laboratory, University of Minnesota were utlized. Virus stocks were prepared and titrated in Madin-Darby Canine Kidney (MDCK) cells upon receipt. Prior to inoculation, each virus was diluted in sterile Dulbecco's Modified Eagle's minimum essential Medium (DMEM) to $5x10^7$ plaque forming units (PFU)/mL.

***Staphylococcus aureus.*** The community-acquired MRSA isolate NRS123 was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) and is known to carry the Panton-Valentine leukocidin (PVL) gene. To prepare bacterial inoculum, 50 mL of trypticase soy broth (TSB) was inoculated with a single pure colony of MRSA grown overnight on Trypitcase soy agar plates. The bacteria were grown at 37° C for 18 h with shaking. Bacterial pellets were washed with PBS and resuspended to 1 x $10^8$ CFU/0.5 mL using absorbance at 600 nm. Test animals received 0.5 mL of bacterial suspension in each nostril as the infection dose.

**Experimental protocol.** All pigs were acclimated for 2 days before the start of the experiment. Pre-inoculation blood samples were drawn after intramuscular injection of tiletamine plus zolazepam (Telazol, Fort Dodge Animal Health; Forth Dodge, IA) at 6 mg/kg bodyweight. Subsequently, groups of three pigs were infected intranasally with either strain of IAV on D0. Each pig received 1.5 mL of $5x10^7$ PFU/mL of IAV in each nostril. One group (n=3) of pigs received equal volume of PBS to serve as uninoculated controls. Three days post infection with influenza (D3), animals were infected intranasally with $1x10^8$ CFU of MRSA or PBS, resulting in the following experimental groups of 3 pigs each: Sham, MRSA only, SIV 1145, SIV 761, and SIV 1145 + MRSA and SIV 761 + MRSA.

Animals were monitored twice daily for changes in rectal temperature and clinical score (0- no clinical signs, 1- mild clinical signs, 2- moderate clinical signs, 3- severe clinical signs). On D5, 1 pig from each of the 6 groups was humanely euthanized by the intravenous injection of sodium pentobarbital (Fatal-Plus®, Vortech Pharmaceuticals; Dearborn, MI) at 0.22 mL/kg bodyweight. The remaining animals were euthanized on D11.

Bronchoalveolar lavage (BAL) was collected immediately after euthanasia. Fifty mL of cold 0.3% EDTA in PBS was injected into the right and left anterior lobes of the lung using a sterile catheter and the fluid was aspirated immediately. Blood was collected from the anterior vena cava into EDTA tubes (BD Vacutainer; Franklin Lakes, NJ). Lung, spleen, kidney and lymph tissue samples were collected and immediately frozen in liquid nitrogen and stored at -80°C until processed. Lung samples were collected in 10% neutral buffered formalin and processed for histology.

**Cytokine mRNA analysis.** The expression of selected cytokine mRNA was determined using quantitative real time (RT) PCR analysis. Tissues (lung, lymph node, and kidney) were

homogenized and total RNA was isolated using the RNeasy mini kit (Qiagen Sciences; Germantown, MD) according to manufacturer's instructions. RNA from each sample was used for making cDNA and then amplified using RT-PCR system (Applied Biosystems; Foster City, CA). Porcine specific primers were created using Primer Express® Software v3.0 (Applied Biosystems; Foster City, CA). The relative concentratoin of pro-inflammatory (IL-1β, IL-6, TNF-α) and immunoregulatory (IL-10, IFN-γ) cytokine mRNA transcripts in the tissues of infected animals were determined (Table 3-1).

**Microarray analysis.** Lung tissue RNA was isolated as above and subjected to microarray analysis using the Affymetrix GeneChip® Porcine Genome Array at the Virginia Bioinformatics Institute, Blacksburg, VA. Analysis was conducted comparing dual-infected animals from the SIV 1145 and MRSA group and respective single infected and sham groups.

**Flow cytometry.** Flow cytometric analysis of cell surface markers in BAL and blood was performed with monoclonal primary antibodies specific for porcine antigens. Fifty μL of 1 x $10^6$ cell suspension were prepared in FACS wash (0.05% sodium azide in PBS) buffer. Cells were incubated with primary antibodies CD8-α, CD4, CD14, MHC II, and CD11b (VMRD; Pullman, WA) for 1 h on ice. The cells were washed and incubated with respective fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen, USA) for 30 min on ice. The cells were washed and fixed in 100 μL of 1% paraformaldehyde. Percentages of cells resulting from immunolabelling were determined using a 6-color FacsCalibur flow cytometer (BD biosciences; San Jose, CA) and analyzed using Flowjo software v7.6.1 (Tree star Inc.; Ashland, OR).

**Statistical analysis**. Data were analyzed in GraphPad Prism v5.0c (GraphPad Prism Software, Inc.) using a 2-way analysis of variance comparing treatments and day post infection. Significance was declared at $P < 0.01$.

## RESULTS

***Staphylococcus aureus* induces clinical respiratory symptoms in nursery pigs.** Groups of two pigs were intranasally infected 1 x $10^6$, 1 x $10^8$, or 1 x $10^8$ CFU of MRSA with CFU of MRSA on D0. Animals infected with 1 x $10^8$ CFU showed an increase in body temperature compared with other doses (Figure 3-1, A), but there was no difference in percent body weight change between treatments (Figure 3-1, B). However, animals infected with 1 x $10^8$ CFU showed significantly more respiratory distress 24 and 48h following infection when compared with all other treatments, ($P > 0.01$). Animals infected with 1 x $10^8$ CFU exhibited frequent sneezing, coughing and nasal mucous secretion.

**Polymicrobial intranasal challenge model induced pneumonia in pigs**. Pigs were infected on D0 with IAV and on D3 with MRSA. Animals were monitored daily and all the dual-infected pigs reacted with pyrexia (Figure 3-2), sneezing, nasal discharge, coughing and lethargy. At necropsy, mild to moderate interstitial pneumonia and/or petechial hemorrhages were noticed in the lung parenchyma of infected animals (Figure 3-3). Lesions in SIV761-infected animals were fewer compared with SIV1145-infected animals. Dual-infected animals showed more severe pneumonia along with evidence of secondary bacterial infection compared with either of the SIV-only infected animals. Sham-treated animals showed no signs of infection.

**Proinflammatory cytokine response is augmented in dual-infected pigs.** Real-time RT-PCR analyses revealed an increased expression of pro-inflammatory (IL-1β and IL-6) cytokine genes in the lungs of dual-infected animals compared with sham and single-infected animals (Figure 3-

4). Cytokine expression profiles differed between the lung and lymph node, as well as and over the course of the infection period. On D5, inflammatory cytokine expression increased in the lung of dual-infected animals compared to sham and singular infections, but was comparable to sham by D11 (Figure 3-5). Furthermore, cytokine increases differed with SIV strains. SIV 761 appeared to induce proinflammatory cytokine production on D5 more strongly within the lung of dual infected animals compared to SIV 1145. Overall, the proinflammatory cytokine expression in the lung of dual infected animals superseded those in MRSA and SIV only infected animals, thereby explaining in part, the more severe pneumonia in dual infected pigs.

**Increased influx of lymphocytes in BAL reflects increased pathology in lungs of dual infected animals.** Lymphocyte profiles of the BAL fluid showed a marked decrease in the number of lymphocytes in MRSA-infected pigs, but an increase in SIV1145 only and SIV1145 + MRSA as compared to control animals (Figure 3-6). Specifically, the percentages of $CD8^+$ T cells rose from 5.5% in SIV only, to 24.97% in SIV + MRSA. Percentages of $CD4^+$ T cells increased from 5.19% in SIV only to 18.30% in SIV + MRSA. Interestingly, analysis of blood lymphocyte profiles indicated an increase in lymphocyte percentages in MRSA-infected animals but a marked decrease (lymphopenia) in both SIV1145-only and dual-infected animals as compared to sham (Figure 3-7).

**Gene expression in the lung parenchyma determines the outcome of post influenza susceptibility to secondary bacterial infection.** Microarray analyses of lung tissue (Table 1) highlight the fact that dual infection with SIV and MRSA reduced the expression of key protective genes compared to sham and MRSA only infected animals. On D11, infection with

MRSA alone resulted in increased expression of certain chemokines, pro-inflammatory cytokines and adhesion factors, and complement components. On the other hand, SIV infection resulted in a decreased expression of the same chemokines, pro-inflammatory cytokines, and adhesion factors in the lung of infected animals. Interestingly in dual infected animals, the effect of SIV on chemokine, cytokine, and adhesion factor expression superseded the effect of MRSA on these genes.

## DISCUSSION

There is no available cure for IAV, although vaccination and anti-virals such as neuraminidase inhibitors can reduce the duration of symptoms. The mainstay treatment for IAV complicated by pneumonia includes administration of antibacterial agents against pneumococci and staphylococci (10). At present, C-reactive protein (6) and procalcitonin (14, 15, 19) are the only predictive markers available for the differentiation of viral and bacterial pneumonia. The decision to commence antibacterial therapy is based on clinical judgment in most cases. Furthermore, there are no reliable animal models to study viral-bacterial synergy in post-influenza pneumonia, although a cotton rat model was proposed recently (4). But, the cotton rat model suffers from the same limitations as the mouse model, in that rodents are not natural IAV hosts and they do not represent the ideal model to study the pathogenesis of lung lesions, fever or respiratory distress. In fact, mice and cotton rats do not show an increase in body temperature, or PMN infiltration in the lungs, and the infection is frequently lethal. On the other hand, SIV is one of the rare respiratory viruses of swine that can cause acute respiratory disease on its own (44) and has remarkable similarities to human influenza. Pigs are natural hosts to MRSA, and commonly succumb to post-influenza pneumonia. In this study, pigs infected with MRSA at 1 x $10^8$ CFU showed significantly greater respiratory distress compared with lower doses, yet the infection did not have an adverse affect on weight gain or survival. Therefore, we hypothesized pigs may serve as an excellent animal model for the study of post-influenza pneumonia.

Pigs co-infected with SIV and MRSA exhibited disease signs consistent with viral-bacterial synergy, which included high fever, more severe pneumonia, increased induction of proinflammatory cytokines, and increased immune cell infiltration when compared to infection with SIV or MRSA alone. This study contributed to our understanding of polymicrobial infection

in a large animal model and therefore may prove crucial in the design of preventive and treatment strategies for influenza and associated complications.

Proinflammatory cytokines released during pulmonary infection recruit and activate innate immune cells to combat viral pathogens (45). However, the production of proinflammatory cytokines (IL-6, and IL-1, and TNF-α), while aiding innate immunity, leads to lung degeneration following infection with IAV (35, 42). Furthermore, a mouse model of recombinant influenza virus demonstrated that increased mortality compared to control mice is due to the increased number of alveolar macrophages and PMN and increased influx of cytokines and chemokines in lung tissues (39). A temporal correlation between increased pro-inflammatory cytokines in lung lavage fluids of SIV infected pigs and lung virus titers, PMN infiltration and clinical signs has been established (41, 43).

In the cotton-rat model of post-influenza pneumonia by *Staphylococcus aureus* (4), early indicators of exacerbated disease coincided with higher pulmonary mRNA levels for IL-1β, IL-6, IL-10 and IFN-γ, supporting the idea that these may contribute to disease severity. Our data also supports the concept that proinflammatory cytokines might play a role in increased lung pathology in dual infections. However, causal inferences from correlation studies are difficult to establish. It is possible that direct damage to the respiratory epithelium due to virus replication could increase secondary bacterial invaders. Further, lung damage and subsequent presence of fibrinogen may increase susceptibility to secondary Staphylococcal adhesion to IAV infected cells (7). In this study, the increased expression of IL-10 in dual infected pigs compared to single infected and sham animals is consistent with an earlier report of increased susceptibility to secondary bacterial infection due to IL-10 expression, reported to impair host defense against bacteria (40). The cytokine profiles during influenza infections in pigs and humans have

remarkable similarities. Several studies of natural influenza in humans have confirmed the importance of IFN-α, TNF-α, and IL-6 (12, 16, 32).

The increase in T cell population in SIV infection is consistent with an earlier report (20). During IAV infection, PMN and monocytes are recruited to the lung by an upregulation of adhesion molecules in the lung (28). The cytokines and chemokines associated with these populations (45) may be responsible for the influx of T cells from the blood and into the lung, thus the reversal in trend. The influx of T cells in dual infected animals further confirms that the pig is an appropriate model, with disease condition in dual infected animals more severe than single infected animals.

Microarray analysis of lung tissue from pigs infected with MRSA exhibited increased expression of lipoprotein lipase, a protein associated with infections and macrophage function (22), regardless of SIV status but only increased matrix metalloproteinase (MMP), involved in tissue remodeling (25), expression in the absence of SIV infection. Further, evaluation of the expression of lipoprotein lipase and MMP1 during dual infections will provide key insights into the role of these genes in lung recovery post infection, onset of pathology, and development of pneumonia.

Our results demonstrate that pigs are a good large animal model for *Staphylococcus aureus* intranasal infection with significantly respiratory distress 24 and 48h following infection. Furthermore, the porcine is a successful model for post-influenza pneumonia, as dual-infected animals showed more severe pneumonia along with evidence of secondary bacterial infection compared with either of the SIV-only infected animals. The increased clinical signs and lung pathology in dual-infected animals are indicative synergistic disease that may have resulted from an increase in proinflammatory and immunoregulatory cytokines. Future experiments will

expand on this pilot study to evaluate the extent of polymicrobial infection. This model serves as a useful tool to identify disease factors and for the development of preventive strategies against bacterial infection in IAV-infected patients.

## TABLES

### Table 3-1. Primer and probe sequences from 5’ to 3’ end

| Gene | Forward primer | Reverse primer |
|---|---|---|
| GAPDH | 5’ ACATGGCCTCCAAGGAGTAAGA 3’ | 5’ GATCGAGTTGGGGCTGTGACT 3’ |
| TNF-α | 5’ TGCCTCAGCCTCTTCTCCTT 3’ | 5’ TGAGACGATGATCTGAGTCC 3’ |
| IL-6 | 5’ CTGGCAGAAAACAACCTGAACC 3’ | 5’ TGATTCTCATCAAGCAGGTCTCC 3’ |
| IL-1β | 5’ GTGATGGCTAACTACGGTGACAA 3’ | 5’ CTCCCATTTCTCAGAGAACCAAG 3’ |
| IL-10 | 5’ CGGCGCTGTCATCAATTTCTG 3’ | 5’ CCCCTCTCTTGGAGCTTGCTA 3’ |
| IFN-γ | 5’CGATCCTAAAGGACTATTTTAATGCAA 3’ | 5’ TTTTGTCACTCTCCTCTTTCCAAT 3’ |

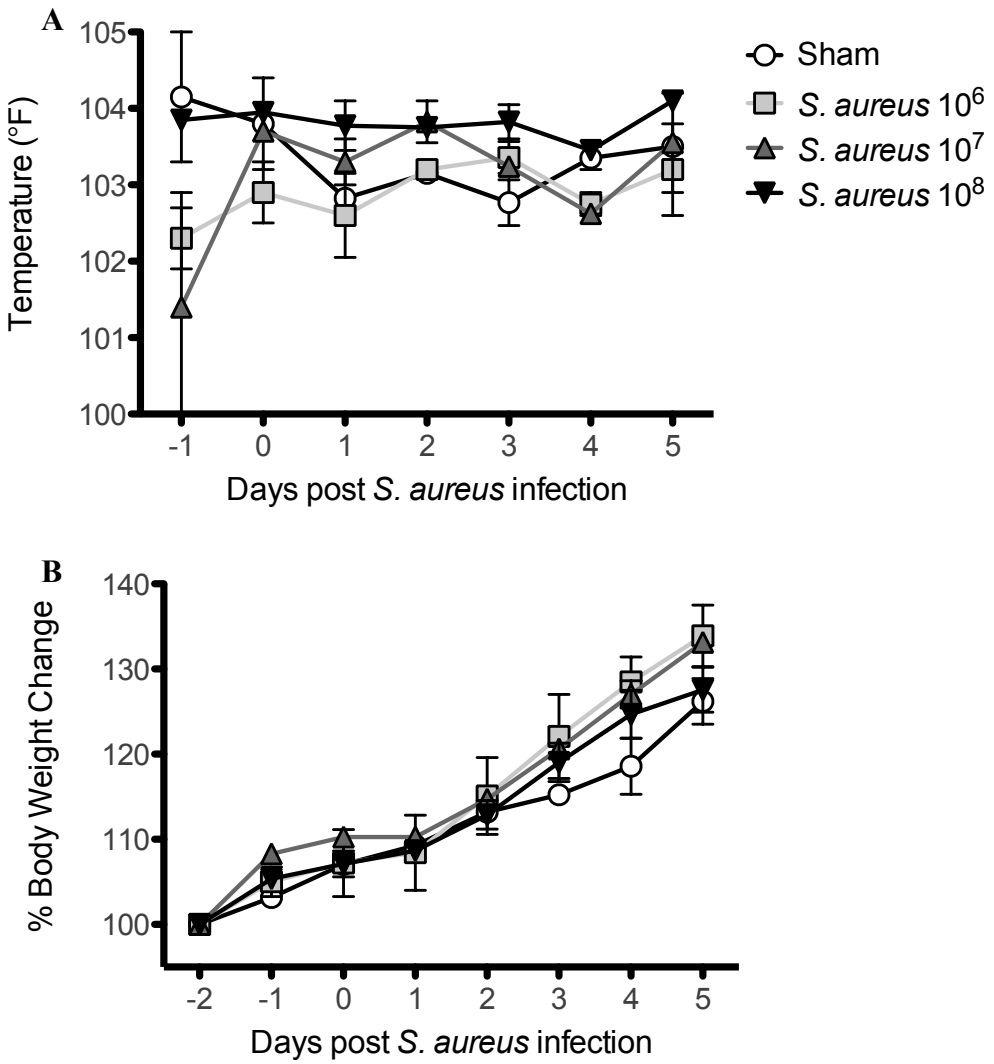
**Table 3-2. Microarray analysis of lung tissue 11 days post infection**

| Gene Expression | Compared to Sham | | | Compared to SIV | |
|---|---|---|---|---|---|
| | SA | SIV | Dual | SA | Dual |
| Chemokines (CCL2, 28, CXCL2, 3, 14, CCR1, 5, AMCP II) | + | - | - | + | + |
| Pro-inflammatory cytokines and Adhesion factors (IRF-7, IFI30, IL1RN, IL1 alpha, beta, IL13RA1, IL8, SELL, VCAM 1) | + | - | - | + | N |
| Complement components (C2, C4, C7, C9, C1R, C1Q-A, B, C) | + | N | - | + | N |
| Lipoprotein lipase | + | N | + | + | + |
| Heat shock protein (hsp70 related) | + | N | + | N | + |
| MMP (1 and 7) | + | N | N | + | N |
| Lipocalin-1 | N | + | N | N | + |

(+) Indicates up regulation; (-) indicates down regulation; and (N) indicates no change in gene expression.

**Figure 3-1. Pathogenesis of different MRSA doses.**

(A) Body temperature, (B) percent body weight change, and (C) respiratory score over the first 5 days of disease. Groups of two animals were infected with each dose. Days post-infection are indicated on the *X*-axis, body temperature, respiratory score, or percent body weight change are plotted on the *Y*-axis. The respiratory score represents the sum of respiratory disease scores, graded on a 0–1–2–3 scale. Error bars represent the standard deviation. Stars indicate a *P* value of < 0.01 for the marked data point compared to all others.



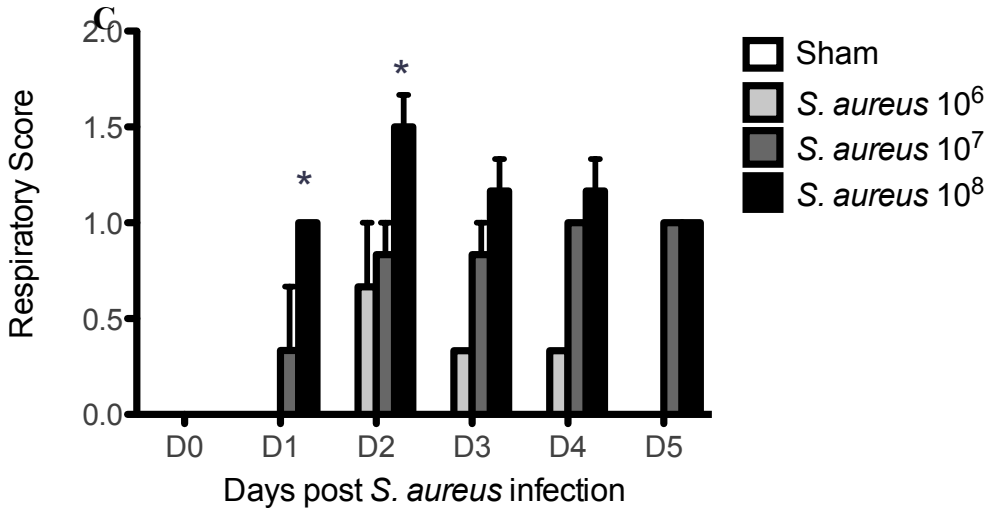
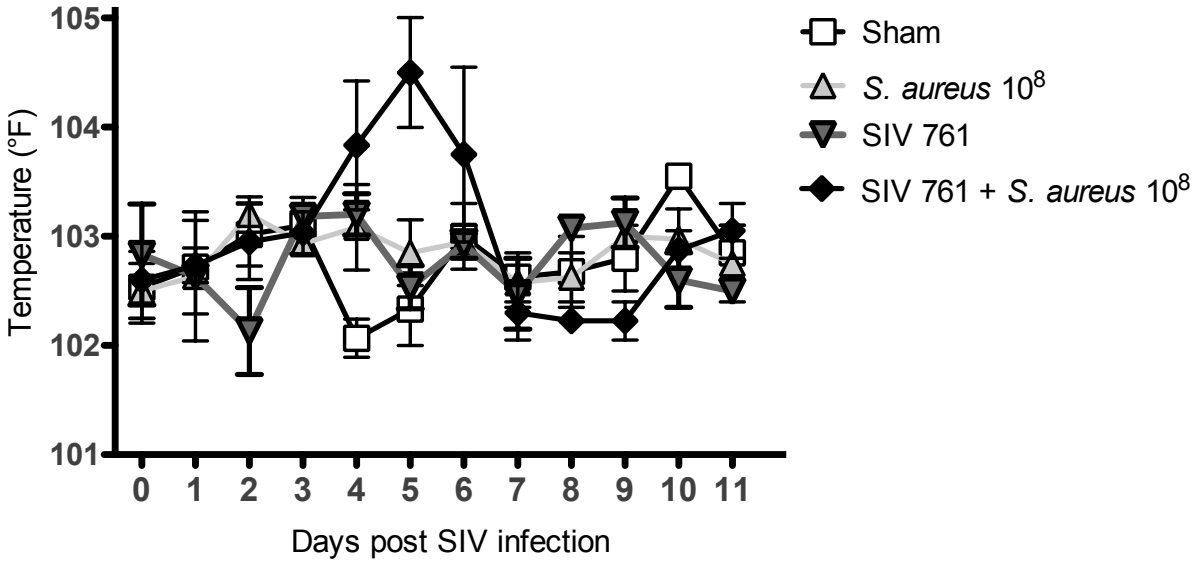

C
Respiratory Score
2.0
1.5
1.0
0.5
0.0
*
*
D0
D1
D2
D3
D4
D5
Days post S. aureus infection
Sham
S. aureus 10^6
S. aureus 10^7
S. aureus 10^8

**Figure 3-2. Rectal temperature during SIV and MRSA infection.**

(A) SIV761, (B) SIV1145 over the first 11 days of disease. Groups of three animals were infected on D0 with each virus and *Staphylococcus aureus* on D3. Day post SIV-infection is indicated on the *X*-axis, and body temperature is plotted on the *Y*-axis. Error bars represent the standard deviation.

**A**




**B**

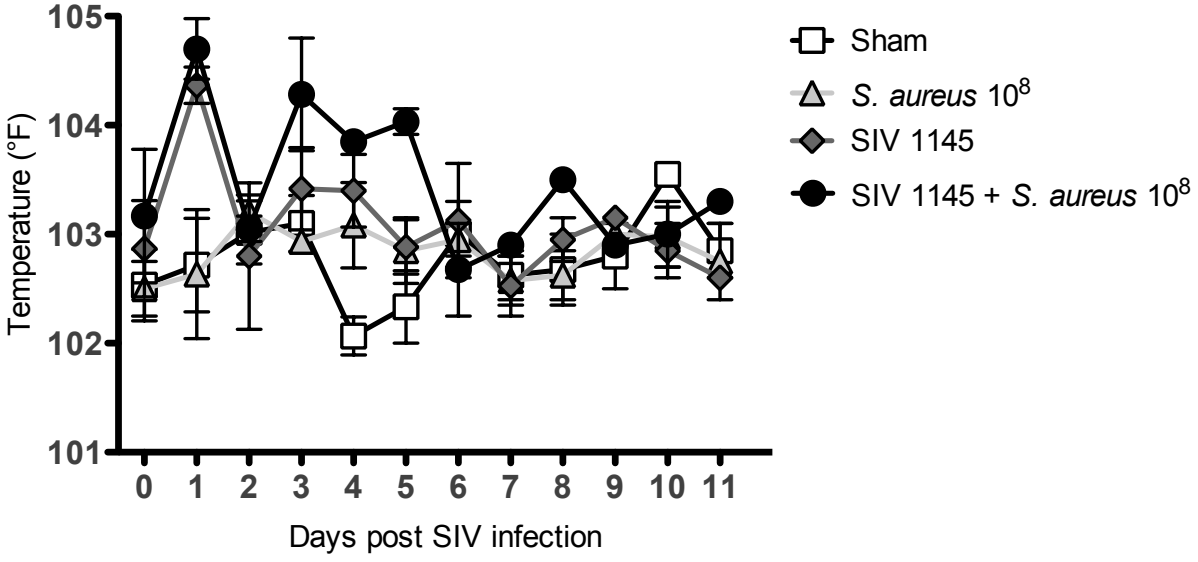


**Figure 3-3. Gross lesions in the lungs of SIV and MRSA infected pigs.**

(A) Sham-infected, (B) SIV761 only, (C) SIV1145 only, (D) SIV761 + MRSA, and (E) SIV1145 + MRSA. Two animals of each group were sacrificed eleven days after SIV infection. Lungs were photographed on D11 at necropsy. A dorsal view of a representative lung is shown for each treatment.

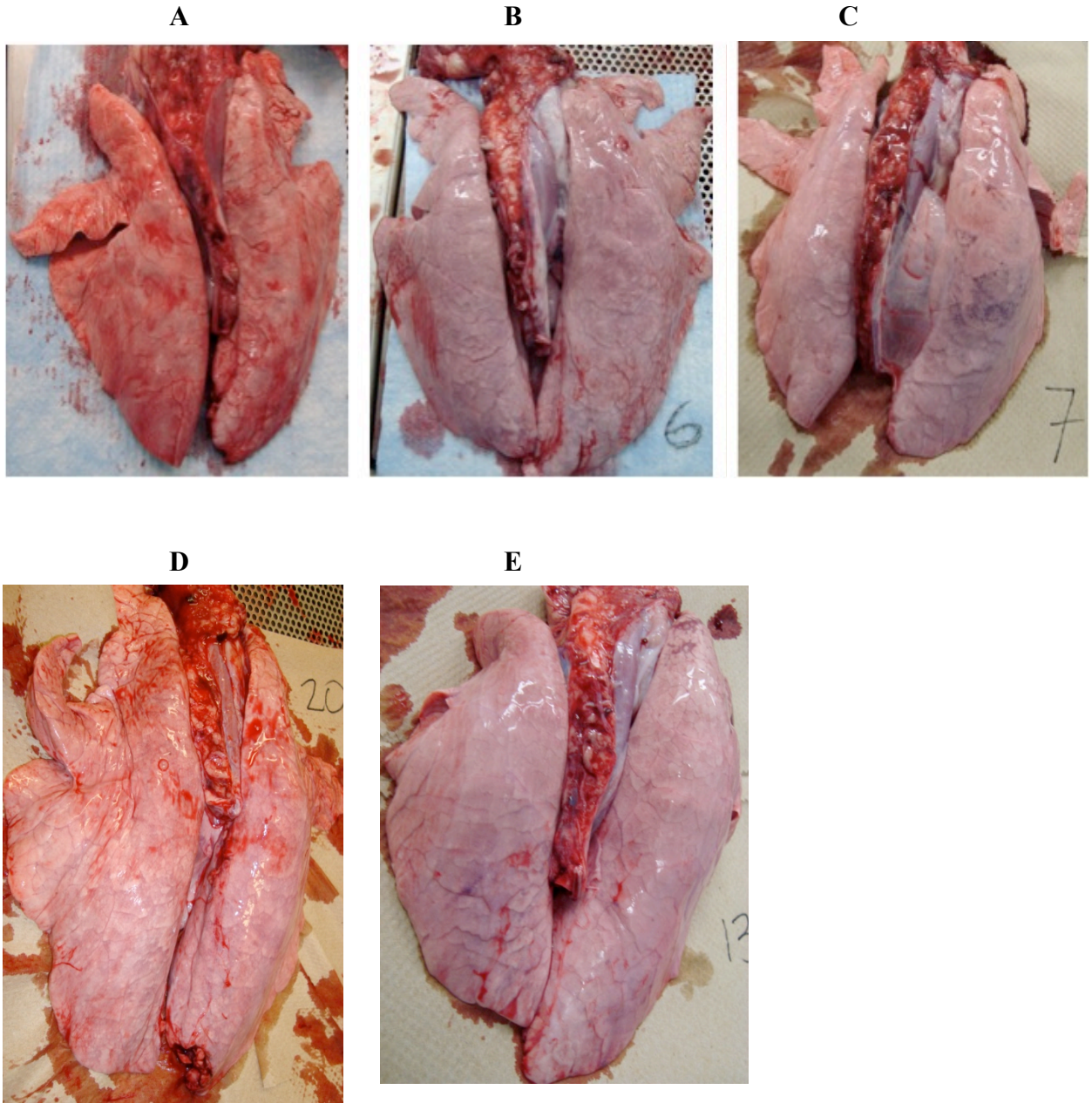


**Figure 3-4. Cytokine expression on D5.**

(A) IL-6, (B) IL-1β, (C) TNF-α, (D) IL-10, and (E) IFN-γ cytokine gene expression by quantitative RT-PCR. One animal from each treatment group was harvested on D5. RNA was collected from homogenized lung, lymph, and spleen tissue.

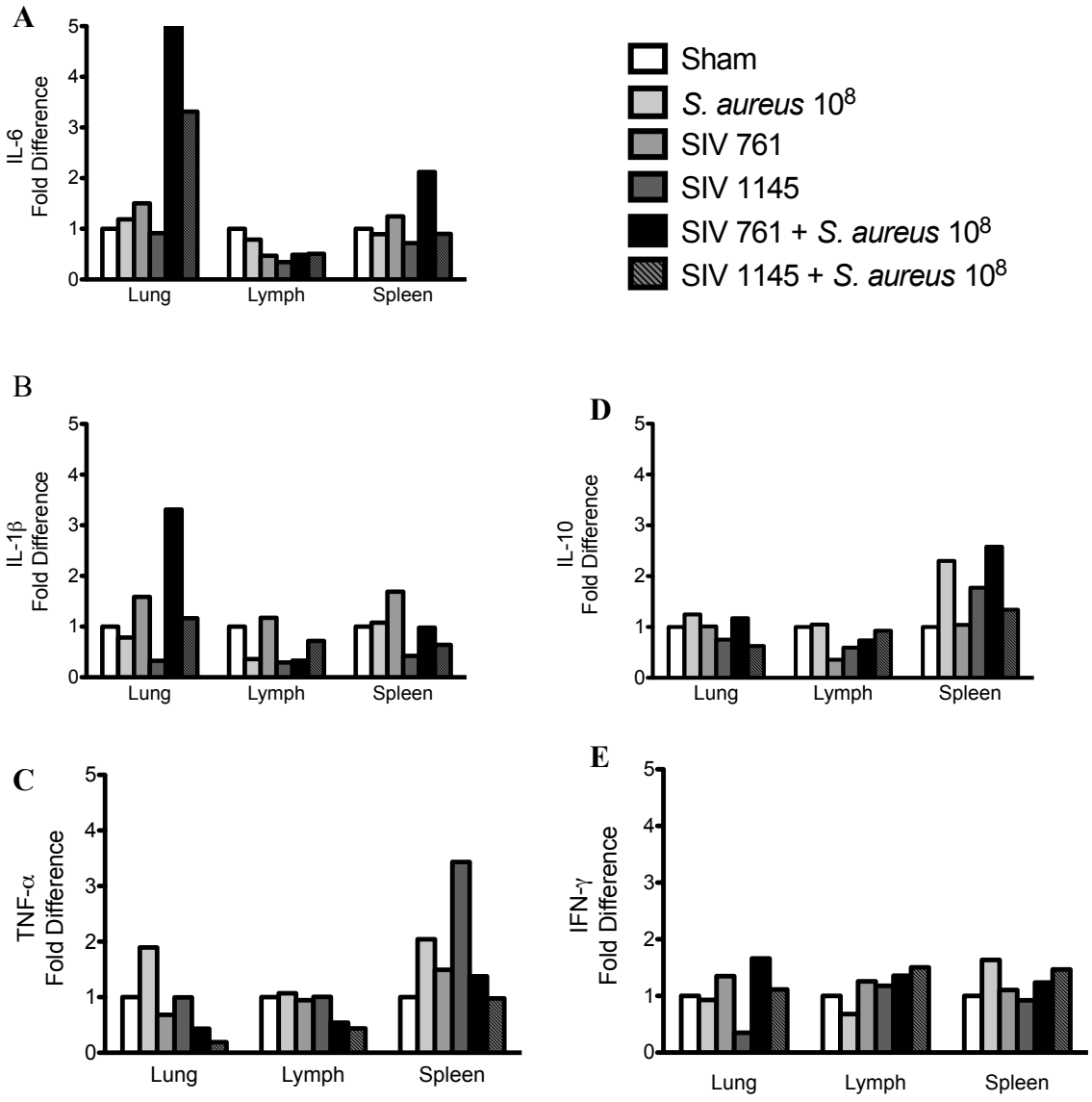


**Figure 3-5. Cytokine expression on D11.**

(A) IL-6, (B) IL-1β, (C) TNF-α, (D) IL-10, and (E) IFN-γ cytokine gene expression by quantitative RT-PCR. Two animals from each treatment group were harvested on D11. RNA was collected from homogenized lung, lymph, and spleen tissue. Each value represents the average of two animals, and error bars indicate the standard deviation.

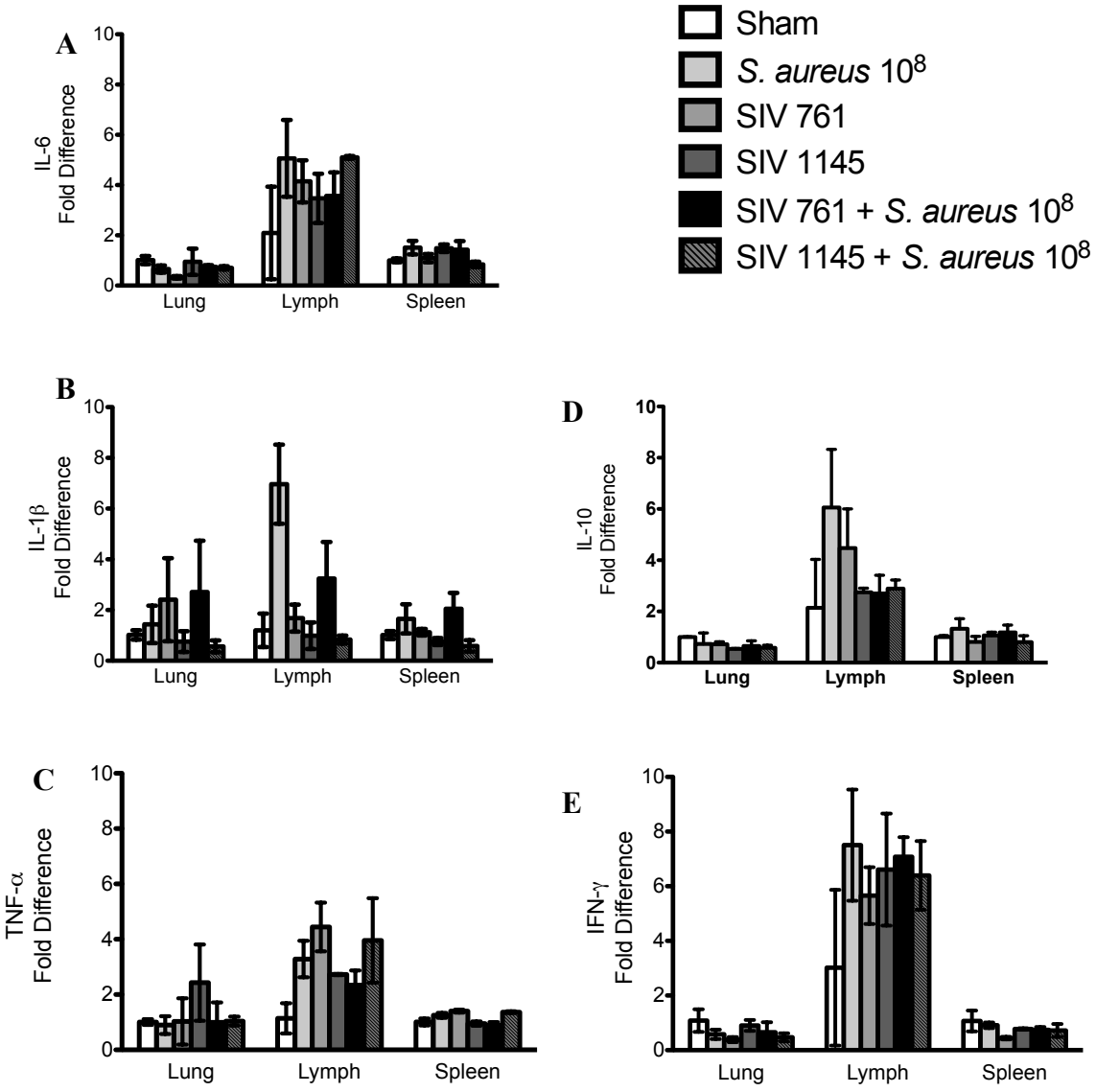


**Figure 3-6. BAL Lymphocyte profiles during SIV and MRSA infection.**

(A) Sham-infected, (B) MRSA only, (C) SIV1145 only, and (D) SIV1145 + MRSA. One animal of from group was sacrificed on D5, BAL fluid collected, and analyzed by flow cytometry for percentages of CD4 and CD8 positive cells within the lymphocyte gate.

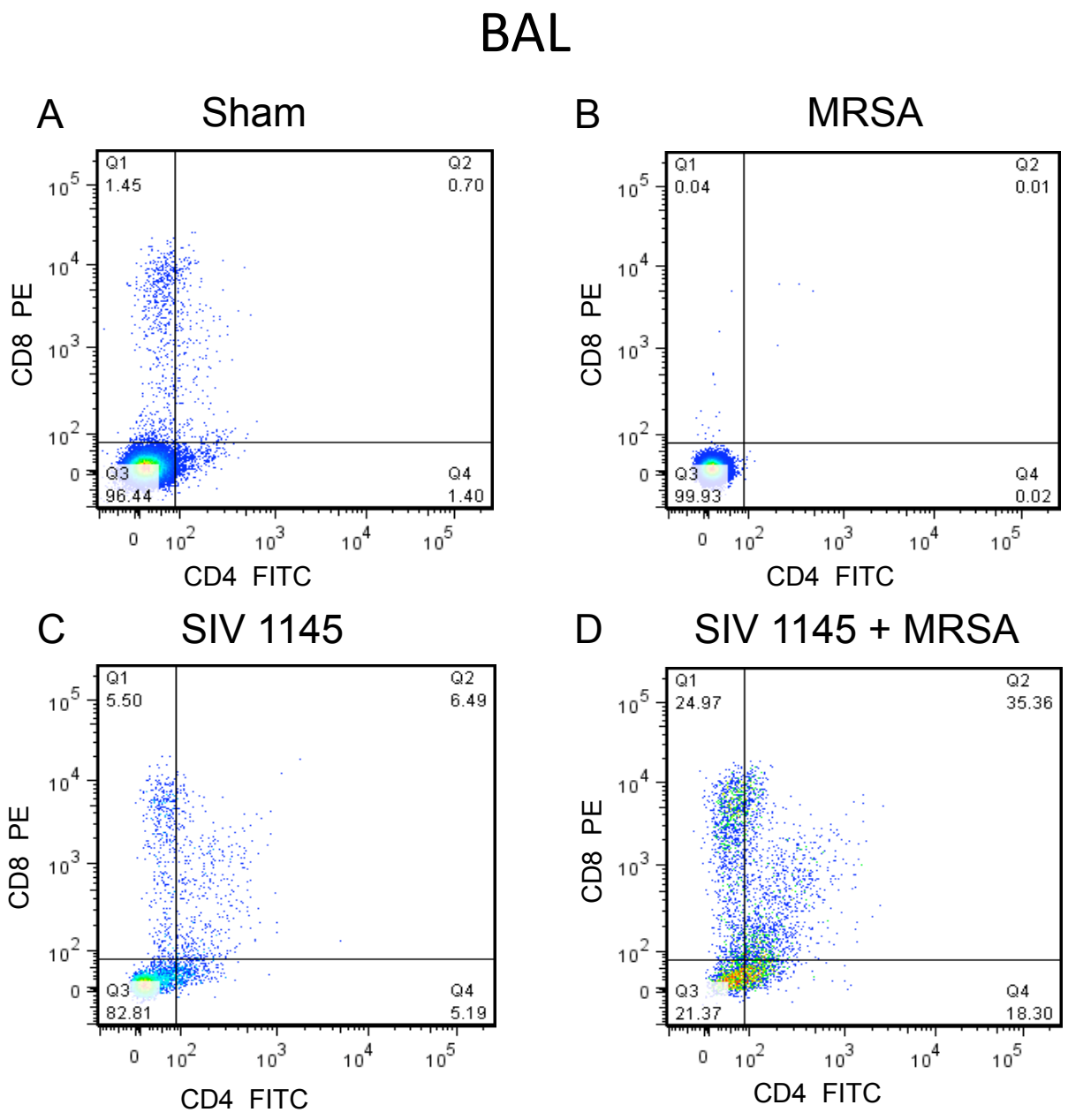
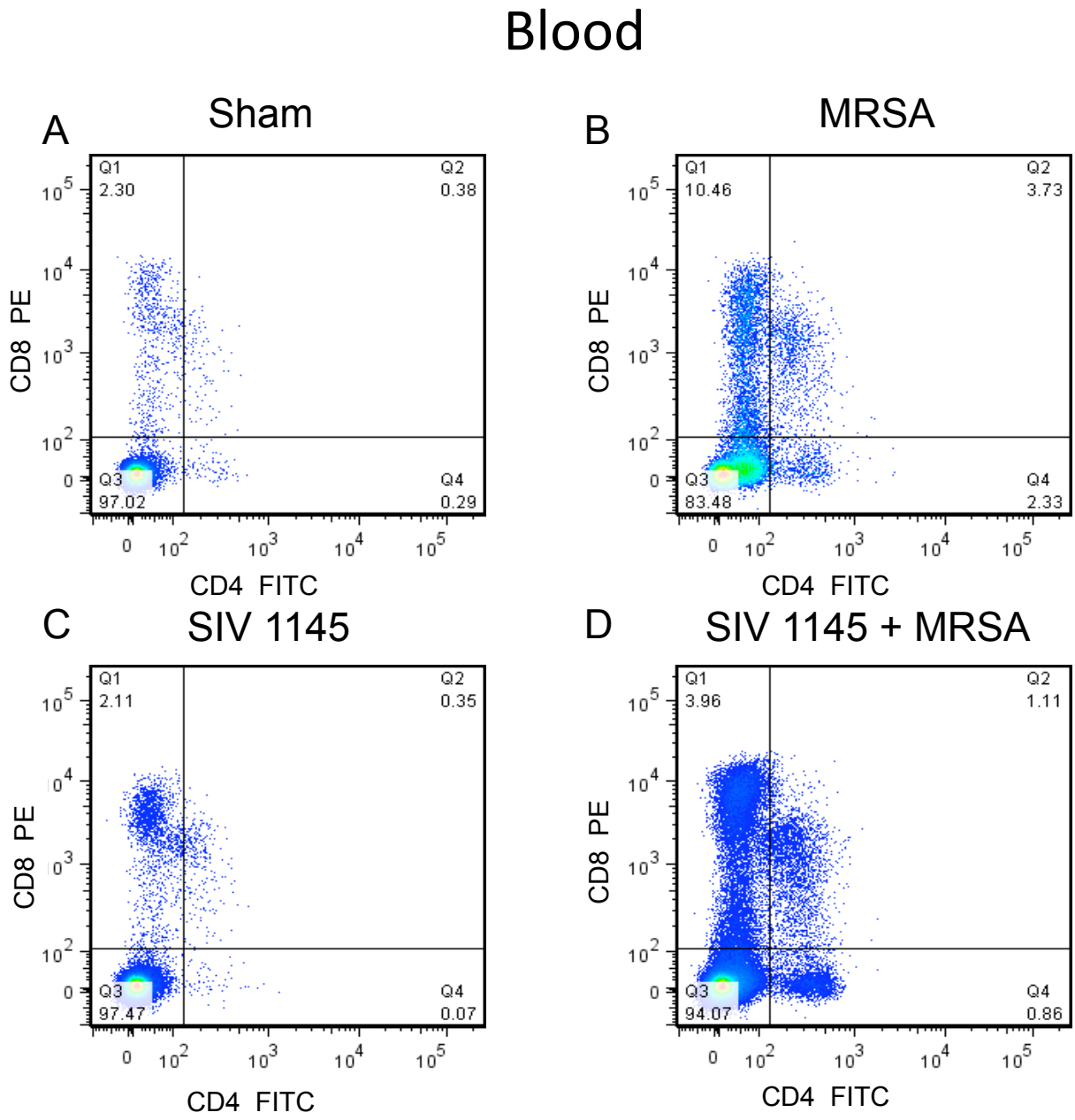


**Figure 3-7. Blood lymphocyte profiles during SIV and MRSA infection.**

(A) Sham-infected, (B) MRSA only, (C) SIV1145 only, and (D) SIV1145 + MRSA. One animal of from group was sacrificed on D5, blood collected, and analyzed by flow cytometry for percentages of CD4 and CD8 positive cells within the lymphocyte gate.



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# Chapter 4. A time course for increased susceptibility to *Staphylococcus aureus* respiratory infection post-influenza in a swine model

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Running title: Time course of post-influenza *Staphylococcus aureus* pneumonia in swine

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**ABSTRACT**

Bacterial super-infections following influenza A virus (IAV) are predominant causes of morbidity and mortality. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) and highly virulent IAV strains enhances the importance of understanding the mechanisms of viral-bacterial synergy. Development of an appropriate animal model to study the increased susceptibility to secondary *Staphylococcus aureus* may provide important information regarding disease pathogenesis. Pigs are natural hosts to both IAV and *Staphylococcus aureus* and have respiratory physiology and immune response comparable to humans, making them an ideal model to examine polymicrobial infections. To establish a time course of susceptibility to *Staphylococcus aureus* after IAV infection, nursery pigs infected intranasally with 1 x $10^7$ $TCID_{50}$ influenza A/Swine/Minnesota/1145/2007 (H3N2) virus for 3, 4, 5 or 6 days (D) were challenged with 1 x $10^8$ CFU of MRSA. Single-pathogen control animals were utilized in this study and mock-infected pigs received 1 mL of phosphate buffered saline. Dual-infected animals were harvested 48 hours following bacterial challenge. We found that lung pathology peaked in dual-infected animals harvested on D6 post-IAV infection, while it decreased over time in IAV only animals. The bacterial CFU in the lung was highest in dual-infected animals harvested on D5 post-IAV infection, and decreased over time. Flow cytometric analysis of bronchoalveolar lavage fluid indicated differences between treatments. These results demonstrate that the intranasal challenge model in nursery pigs to understand the pathogenesis of IAV and *Staphylococcus aureus* co-infection is appropriate.

## INTRODUCTION

Influenza A virus (IAV) infections in humans are generally mild and not often fatal; however, morbidity and mortality significantly increase with bacterial super-infections (35, 37). *Staphylococcus aureus* commonly causes pneumonia in influenza patients, and methicillin resistant strains currently account for 20-40% of all community acquired pneumonia (CAP) (11). With the increasing prevalence of antibiotic-resistant bacteria and emergence of highly virulent IAV strains and with the recent pandemic, development of an appropriate animal model is critical.

The majority of animal studies have utilized adapted strains of IAV, which induce fatal respiratory disease atypical of human infection (16, 20). Pigs are naturally susceptible to IAV, and have been suggested as vessels for reassortment of human and avian IAV because of the presence of both types of sialic acid receptors (3, 13). Swine are an ideal model for the study of IAV because the distribution pattern of viral attachment in respiratory tract tissues is closely mirrored to that in humans (39). Furthermore, pigs are susceptible to *Staphylococcus aureus* within the respiratory tract (10, 12). Previous IAV challenge studies in humans describe the duration of viral shedding to last between 2-4 days post-infection, during which respiratory symptoms, such as coughing and fever, peaked before resolving within a week (5, 14). IAV infected mice are most susceptible to secondary *S. pneumoniae* infection after the viral load peaks (17, 31). Mice infected with *S. pneumoniae* prior to IAV infection did not differ from control, whereas secondary inoculation 3, 5, or 7 days post IAV resulted in one hundred percent mortality (17). However, a successful time course for *Staphylococcus aureus* and IAV infection has yet to be established in an animal model. After the demonstration that synergistic disease was seen when IAV infection preceded *Staphylococcus aureus* challenge, we sought to determine the

optimal timing necessary to achieve this effect. A time course was developed to examine the susceptibility to *Staphylococcus aureus* infection after 3, 4, 5 or 6 days of IAV infection in nursery pigs. Evaluation of this model confirmed the suitability of the pig for study of IAV-MRSA coinfection.

## MATERIALS AND METHODS

**Animals.** Thirty-four **c**onventionally reared nursery pigs, approximately 10-20 kg in bodyweight, were obtained from the swine breeding facility at Virginia Polytechnic Institute and State University Center for Molecular Medicine and Infectious Disease animal facility, Blacksburg, VA. Animals were SIV-free, which was confirmed by antibody titers prior to this study. All procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Virginia Tech.

**Viruses.** A strain of recent triple reassortant cluster IV influenza A virus, designated A/Swine/Minnesota/1145/2007 H3N2 (SIV 1145) originally obtained from the Veterinary diagnostic laboratory, University of Minnesota was employed. Virus stock was prepared and titrated in Madin-Darby Canine Kidney (MDCK) cells upon receipt. Prior to inoculation, each virus was diluted in sterile Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM) to 1 x$10^7$ $TCID_{50}$ per 1.5 mL.

***Staphylococcus aureus.*** The community-acquired MRSA isolate NRS123 was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) and known to carry the Panton-Valentine leukocidin (PVL) gene. To prepare bacterial inoculum, 50 mL of trypticase soy broth (TSB) was inoculated with a single pure colony of MRSA grown overnight on Trypitcase soy agar plates. The bacteria were grown at 37° C for 18 h with shaking. Bacterial pellets were washed with PBS and resuspended to 1 x $10^8$ CFU per mL using absorbance at 600 nm. Test animals received 0.5 mL of bacterial suspension in each nostril as the infection dose.

**Experimental protocol.** All pigs (n=34) were acclimated for 2 days before the start of the experiment. Additionally, animals were treated for 3 days with Lincomycin at 11 mg/kg bodyweight to reduce incumbent bacterial load prior to experimental IAV infection. Pre-inoculation blood samples were drawn after intramuscular injection of Tiletamine + Zolazepam (Telazol, Fort Dodge Animal Health; Forth Dodge, IA) and Xylazine (Lloyd Laboratories, Lloyd Inc. Shenandoah, IA), each at 4.4 mg/kg bodyweight. Animals were tested seronegative for IAV prior to experimentation. Subsequently, pigs were infected intranasally with SIV on D0 (n=28). Each pig received 1.5 mL of 1 x $10^7$ $TCID_{50}$/ 1.5 mL of SIV in each nostril. MRSA only-infected animals (n=4) were harvested 48h post infection. Mock-infected pigs (n=4) received equal volume of PBS to serve as uninoculated controls. To establish a time course of susceptibility to *Staphylococcus aureus*, animals (n=4 per) were challenged with 1 x $10^8$ CFU of MRSA on D3, 4, 5 or 6 following primary IAV infection and harvested 48 h following secondary infection (i.e. on D5, 6, 7, or 8).

Animals were monitored twice daily for changes in rectal temperature and clinical score (0– no clinical signs, 1– mild clinical signs, 2– moderate clinical signs, 3– severe clinical signs). Nasal swabs were taken daily for quantification of viral shedding. Dual infected animals were harvested 48 hours following bacterial challenge. Animals were humanely euthanized by the intravenous injection of sodium pentobarbital (Fatal-Plus®, Vortech Pharmaceuticals; Dearborn, MI) at 0.22 mL/kg bodyweight.

Bronchoalveolar lavage (BAL) was collected immediately after euthanasia. Fifty mL of cold 0.3% EDTA in PBS was injected into the right and left anterior lobes of the lung using a sterile catheter and the fluid was aspirated immediately. Blood was collected from the anterior vena cava into EDTA tubes (BD Vacutainer; Franklin Lakes, NJ). Lung, spleen, and mediastinal

lymph node tissue samples were collected and flash frozen in liquid nitrogen and stored at -80° C until processed. Lung samples were collected in 10% neutral buffered formalin and processed for histology.

Following euthanasia, tissue from lung, spleen and mediastinal lymph nodes was stored in 20 mL of cold PBS. Homogenates were used directly for bacterial cultures. Quantification of methicillin resistant staphylococcal colony counts was done by 10-fold dilutions on mannitol salt agar with cefoxitin (United States Pharmacopeial, Inc., Rockville, MD). Isolates were confirmed as the MRSA inoculate by positive Gram stain, catalase, coagulase, and PCR analysis for the presence of the PVL gene (Table 4-1), data not shown. Virus titers were determined for nasal swabs, blood and lung tissue by 10-fold serial dilutions on MDCK cell monolayers as previously described (17).

**Lung Pathology.** Lungs were removed immediately after euthanasia and examined for pathologic alterations. A pathologist experienced in respiratory disease pathogenesis examined lungs from each pig and scored by region on a severity scale– Right, and left caudal region (1-5), Right and left cranial and middle regions (1-10), Accessory lobe (1-5). The overall lesion score was determined by the degree of inflammation, necrosis, and interstitial pneumonia.

**Flow cytometry.** Flow cytometric analysis of cell surface markers in BAL was performed with monoclonal primary antibodies specific for porcine antigens. Fifty μL of $10^6$ cell suspension were prepared in FACS wash (0.05% sodium azide in PBS) buffer. Cells were incubated with primary antibodies CD8-α, CD4, 2B11, CD21, CD14, MHC II, (VMRD; Pullman, WA) and CD80 (LifeSciences, Inc.; Seattle,WA) for 1 h on ice. The cells were washed and incubated with

respective fluorochrome-conjugated isotype specific secondary antibodies (Invitrogen, USA) for 30 min on ice. The cells were washed and fixed in 100 μL of 1% paraformaldehyde. Percentages of cells resulting from immunolabelling were determined using a 6-color FacsCalibur flow cytometer (BD biosciences; San Jose, CA) and analyzed using Flowjo software v7.6.1 (Tree star Inc.; Ashland, OR).

**Statistical analysis**. Data were analyzed using the Proc Glimmix procedure in SAS v.9.2 (SAS Institute Inc.; Cary, NC). *P* values were generated for paired comparisons of treatments with Tukey's tests. Significance was set to $P < 0.05$.

Analyses of lesion scores, as well as BAL cells positive for CD4, CD8, 2B11, CD21, CD80, CD14, and MHCII were conducted using the chart below. Comparisons of SIV-only versus dual-infected animals were made within each harvest day. These effects in MRSA-only and dual-infected animals were compared in a model that contained the effects of treatment and residual. Tukey's tests were performed to determine significant effects of treatment.

| Effect | Degrees of freedom | Fixed/Random | Denominator degrees of freedom |
|---|---|---|---|
| Type | 1 | F | 20 |
| Harvest Day | 3 | F | 20 |
| Type*Harvest Day | 3 | F | 20 |
| Residual | 20 | R | |

Analyses of body temperature were conducted using the chart below. Comparisons of SIV-only versus dual-infected animals were made within each harvest day.

| Effect | Degrees of freedom | Fixed/Random | Denominator degrees of freedom |
|---|---|---|---|
| Treatment | 1 | F | 5 |
| Pig (Treatment) | 5 | R | |
| Day | 7 | F | 35 |
| Treatment*Day | 7 | F | 35 |
| Residual | 35 | R | |

## RESULTS

**Polymicrobial intranasal challenge model in pigs.** IAV infection consistently caused significant increase in body temperature 24-48 h after infection ($P < 0.05$) (Figure 4-1), as well as sneezing, nasal discharge, coughing and lethargy. Animals infected with MRSA on D5 post-IAV infection showed a second significant increase in body temperature ($P < 0.05$). At necropsy, sham-infected animals showed no signs of infection and there were no significant differences compared to pigs infected with MRSA only (Figure 4-2). However, dual-infected animals showed more disperse areas of red hepatization throughout the lung as compared with single-infected animals. There was a significant effect of treatment ($P = 0.03$). Pair-wise comparison by Tukey's tests indicated dual-infected animals inoculated with MRSA on D4 had a significantly greater lesion score compared with MRSA-only infected animals ($P = 0.02$) (Figure 4-2). Macroscopic pathology of lesions decreased over time in IAV only animals. Mild to moderate interstitial pneumonia and/or petechial hemorrhages were seen in the lung parenchyma of infected all IAV animals (Figure 4-3). Edema and congestion were particularly evident in the caudal lobes of animals inoculated with MRSA on D4 and 5 post-infection with SIV.

**SIV infection predisposes pigs to bacterial infection.** Sham, MRSA only and SIV only animals did not have recoverable MRSA CFU in the lung, lymph node or spleen. However, animals infected with SIV prior to MRSA inoculation had viable MRSA in the lung 48 h post infection. Animals infected with MRSA on D3 had greatest recoverable CFU per mL. Recoverable MRSA CFU decreased steadily in dual infected animals (Figure 4-4).

**Leukocyte profiles differ between treatments.** BAL was collected directly following euthanasia and analyzed by flow cytometry. The amount of CD4, CD8, 2B11, CD21, CD14, or MHC II positive (+) cells in the BAL did not change over time in SIV-infected animals. However, there was a significant decrease in the number of CD80+ cells on D5 post SIV compared with D8 with a *P* value of $< 0.05$ (Figure 4-5). The number of CD4+ cells was significantly affected by treatment in MRSA-only compared with dual-infected animals (*P* value $< 0.05$). Tukey's tests revealed differences between dual D4 and 5 ($P < 0.09$) and between MRSA only and dual D5 ($P < 0.08$); no other pair-wise comparisons were significant. There was also a significant decrease ($P < 0.05$) in the number of CD4+ cells between animals harvested on D4 compared with D5 (Figure 4-6A). The numbers of CD8+ cells were not affected by treatment (Figure 4-6B). The number of 2B11+ cells increased significantly in D5 and D6 dual-infected animals compared with MRSA-only infected animals, and there was a significant increase in 2B11+ cells in animals harvested D5 and D7 ($P < 0.05$) (Figure 4-6C). Dual-infected animals on D6 showed a significant ($P < 0.05$) increase in the number of CD21+ cells compared with D3 and D4 (Figure 4-6D). The number of CD80+ cells was significantly greater in animals harvested on D5 and D6 compared with those harvested on D8 ($P < 0.05$) (Figure 4-7A). However, the numbers of CD14+ or MHCII+ cells were not significantly affected by treatment (Figure 4-7B and C respectively). Additionally, there was no difference in the expression of any markers in dual-infected animals compared with SIV-only infected animals.

## DISCUSSION

IAV infection establishes an environment in the respiratory tract that allows for an aggravated response to secondary bacterial infection. Single infected animals did not display increased pathology compared to sham, whereas dual infected animals had bacterial pneumonia. This study was also effective in establishing the period in which IAV infected pigs are most susceptible to secondary *Staphylococcus aureus* infection.

All pigs infected with SIV exhibited a significant increase in body temperature following inoculation compared to sham and MRSA only infected animals. This data is comparable with the human IAV symptoms and in direct contrast with rodent models, which show a significant drop in body temperature following IAV infection (5, 22). Fever has been correlated with PMN infiltration into the lung, as well as of IFN-α, TNF-α, IL-1 and IL-6 production in response to SIV infection (38). Following viral clearance, the anti-inflammatory cytokine, IL-10 is produced to reduce inflammation. Previous research suggests that the secretion of IL-10 due to IAV infection predisposes mice to secondary bacterial infection due to the inhibitory effects of this cytokine (37). We are currently investigating whether this may in fact be the case in the porcine model.

Lung lesion scores were greatest in IAV infected animals inoculated with MRSA on D4 (Figure 4-2), directly following the resolution of fever (Figure 4-1). However, MRSA was unable to establish an infection without prior SIV infection (Figure 4-4). MRSA appears to capitalize on a state of predisposition created by primary SIV infection. This is evident by the increase in bacterial load in dual infected animals when compared with MRSA only infected animals (Figure 4-4). The fact that MRSA was unable to manifest an infection without prior SIV

infection, apparent by the lack of pathology (Figure 4-3), is consistent with previous polymicrobial models (17).

The increase in pathology of dual infected animals compared to single and mock infected animals may be due to several factors. MRSA is capable of causing tissue damage during infection, thereby contributing to the exacerbated damage of lung parenchyma in dual infected animals compared with those only infected with SIV (Figure 4-3). It is also plausible that the increase in pathology in dual infected animals is due to effector functions of immune cells. PMN and alveolar macrophages (AM) are credited with inducing cell damage within lungs infected with IAV and *S. pneumoniae*, by the extensive release of proinflammatory cytokine (36). The increase in CD80+ cells in the BAL of dual-infected animals compared with SIV-only suggests an influx of AM (Figure 4-7). The high bacterial load within the lung in IAV infected animals inoculated with MRSA on D3 may be responsible for the increased number of CD80+ cells. Dendritic cells, which display CD80, are capable of inducing an IFN-γ, IL-4, IL-2 response by T cells in order to polarize T cells to helper T cell ($T_h$) type 1, type 2 or cytotoxic T cell pathways (2, 4, 23). In this study, treatment had a significant affect on the overall number of T cells, 2B11+, within the lung. However, numbers of CD4+ or CD8+ cells were not affected by treatment. This suggests that other subtypes of T cells are affected by treatment. One such subtype, $T_h17$ cells have been shown to be affected by IAV (18). Further investigation into T cell profiles must be done to understand cellular response during polymicrobial infection.

Interestingly, the similarity in immune cells profiles between dual-infected and SIV-only infected animals suggests that the immune response is primarily dictated by SIV. Previous research has suggested that innate immune cell impairment during IAV infection creates a predisposed state, optimal for secondary bacterial infection (17, 19, 26). The analysis of

proinflammatory cytokines within the lung will help to explain the increased pathology of dual-infected animals, and explain possible differences in immune cell function.

These results highlight the days directly following fever resolution as when pigs are most susceptible to *Staphylococcus aureus* infiltration. The Center for Disease Control and Prevention suggests that those presenting Influenza-like symptoms should remain home for at least 24 hours after fever resolution, without fever-reducing medication (27). This recommendation is supported by this study, as SIV-infected pigs were most susceptible to secondary MRSA 1-2 days after fever resolution, and 3-4D post-SIV infection.

## TABLES

### Table 4-1. Primer and probe sequences from 5’ to 3’ end

| Gene | Forward primer | Reverse primer |
|---|---|---|
| lukPVS | 5’ AGCAATGAGGTGGCCTTTC 3’ | 5’ GGGGGTAATTCATTGTCTG 3’ |

**Figure 4-1. Rectal temperature during SIV and MRSA infection.**

Twenty-eight animals were intranasally infected on D0 with SIV1145, four animals were infected with MRSA, and six were sham-infected with PBS. Three, four, five or six days following SIV infection, groups of four animals were inoculated with MRSA. Stars indicate significance ($P < 0.05$) for the marked data point compared with temperature on D0. Error bars represent the standard error.

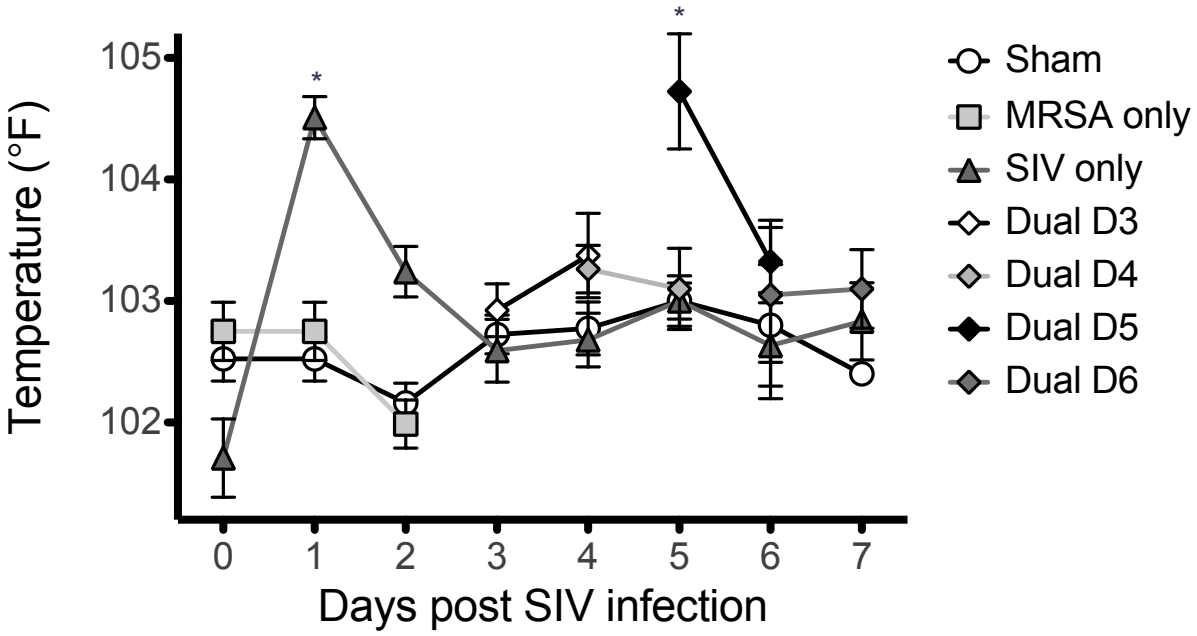


**Figure 4-2. Macroscopic lung pathology.**

The pathology score represents the sum of lesion scores, graded by region: right and left caudal region (1-5), right and left cranial and middle regions (1-10), and accessory lobe (1-5). Average lesion score is depicted for MRSA only (n=4), SIV only (n=4) and dual-infected animals (n=4). Star indicates a *P* value of < 0.05 for MRSA compared with animals dual-infected on D4. Error bars represent the standard error.

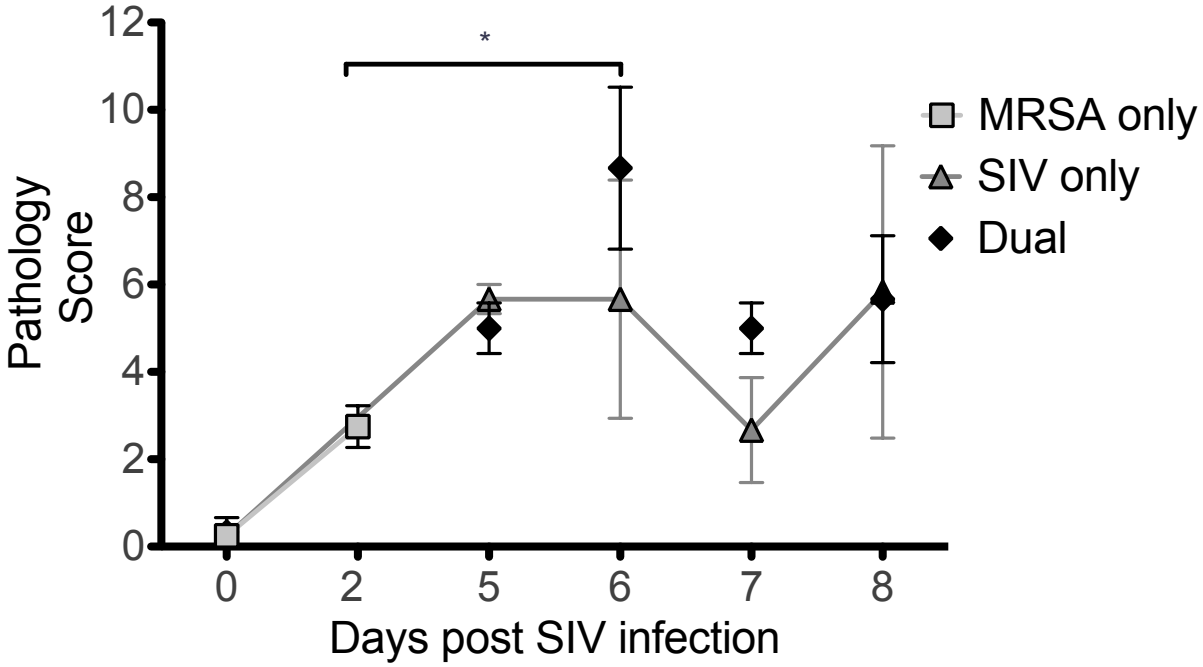


**Figure 4-3. Macroscopic lesions in SIV and MRSA infected pigs.**

(A) Sham-infected, (B) MRSA only (C), SIV only (D), and SIV+MRSA groups: Dual 3 (E), Dual 4 (F), Dual 5 (G), Dual 6. Lungs were photographed at necropsy, and are representative of treatment.

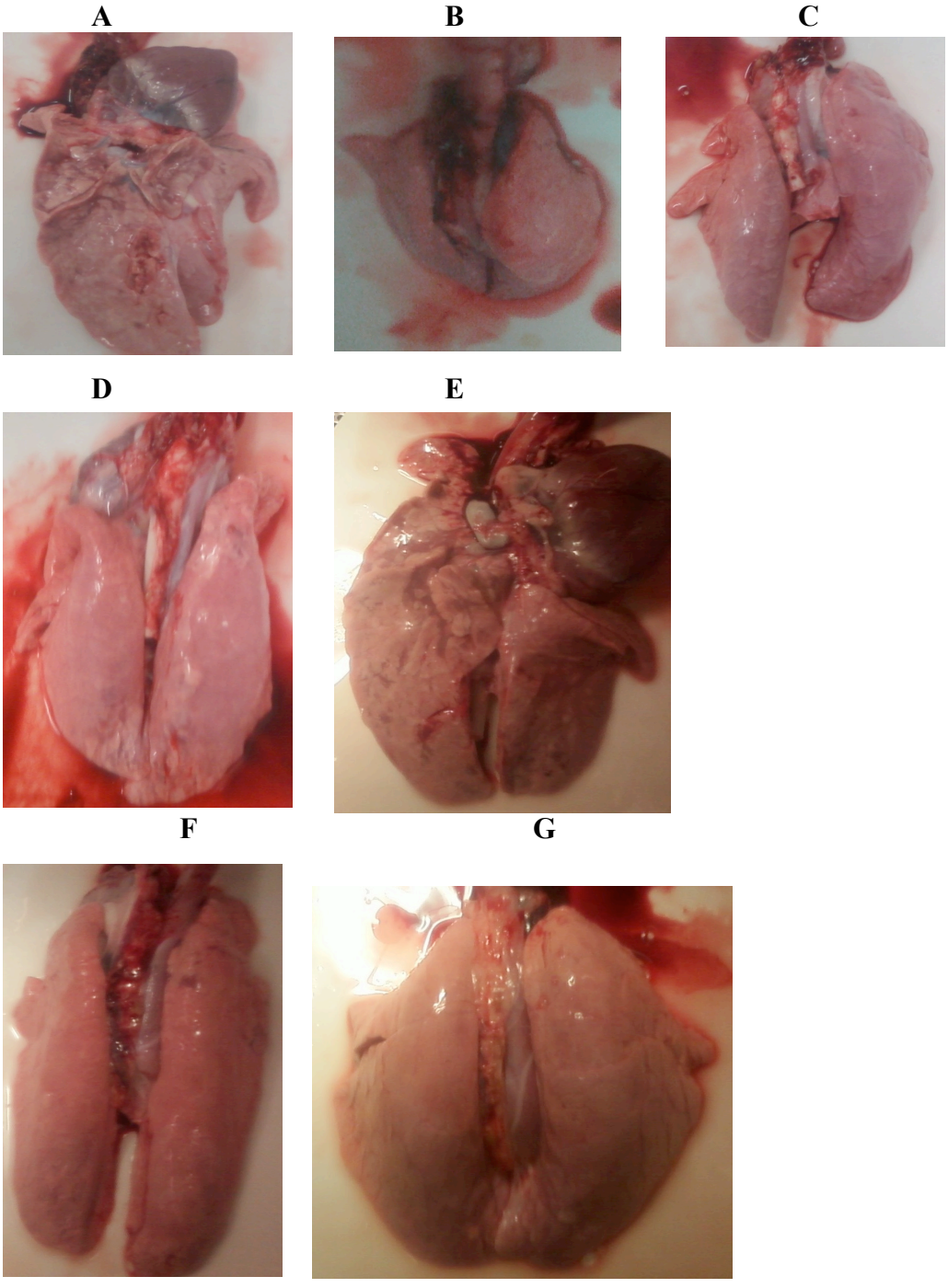


**Figure 4-4. Bacterial load of MRSA in the lung.**

Tissue from lung was homogenized, serially diluted and plated on media selective for methicillin resistant staphylococci. Additional biochemical tests selected for PVL+ MRSA. Average CFU per mL is depicted for sham (n=4), MRSA only (n=4), SIV only (n=4) and dual infected animals (n=4). Error bars represent the standard error.

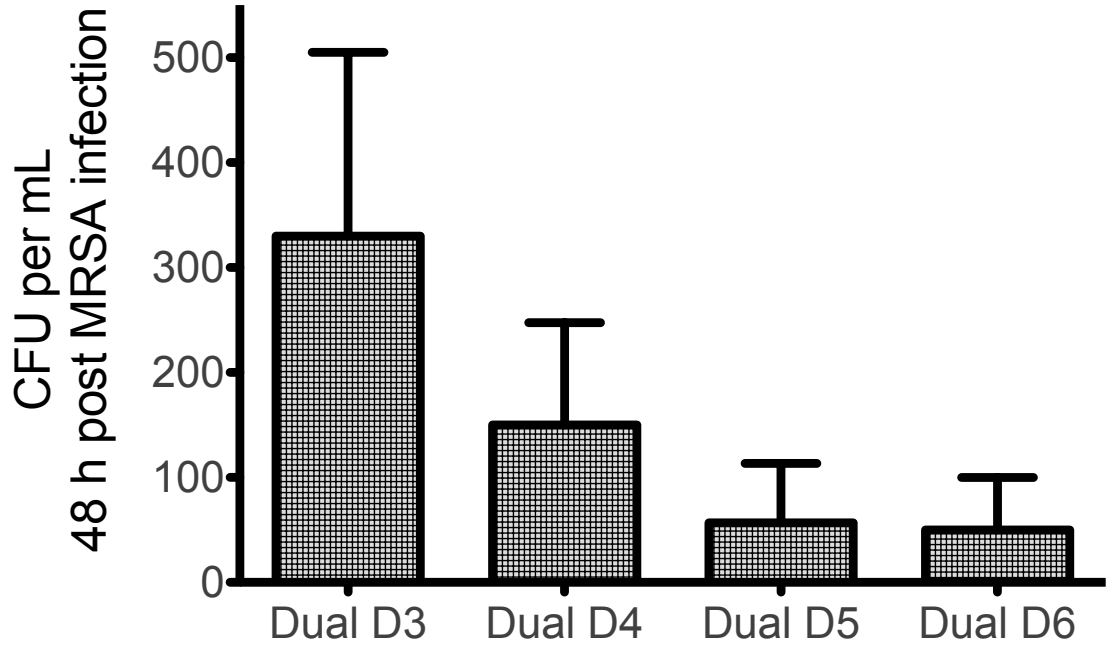
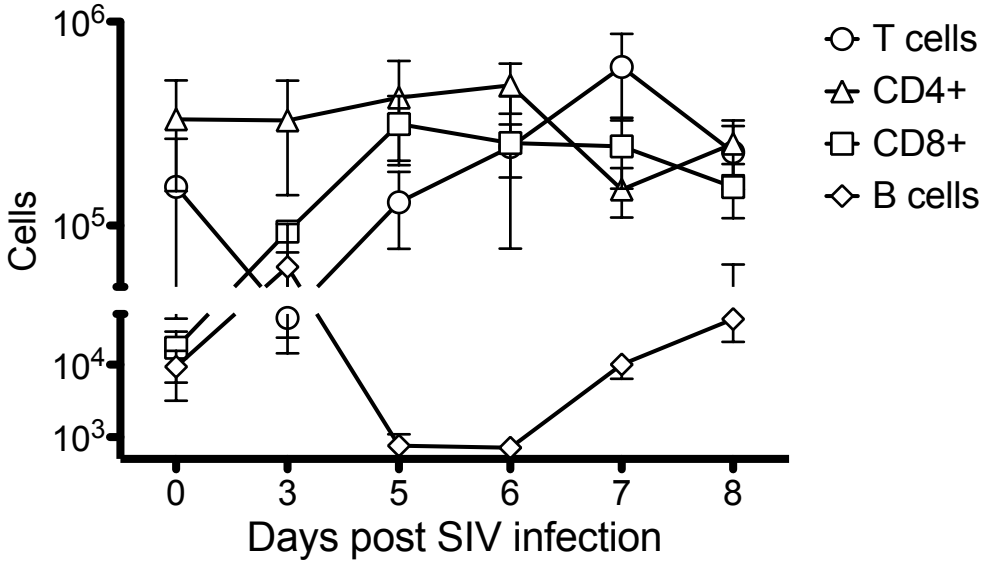


**Figure 4-5. Leukocyte profiles during SIV infection.**

(A) Mix 1: CD4+, CD8+, 2B11+, and CD21+ (B) Mix 2: MHCII+, CD14+, and CD80+ cells within the lymphocyte gate are shown. BAL was collected from SIV only infected animals on day of harvest (n=3). Stars indicate significance ($P < 0.05$) for selected data points along the line for MHCII+ cells. Error bars represent the standard error.

**A**



**B**

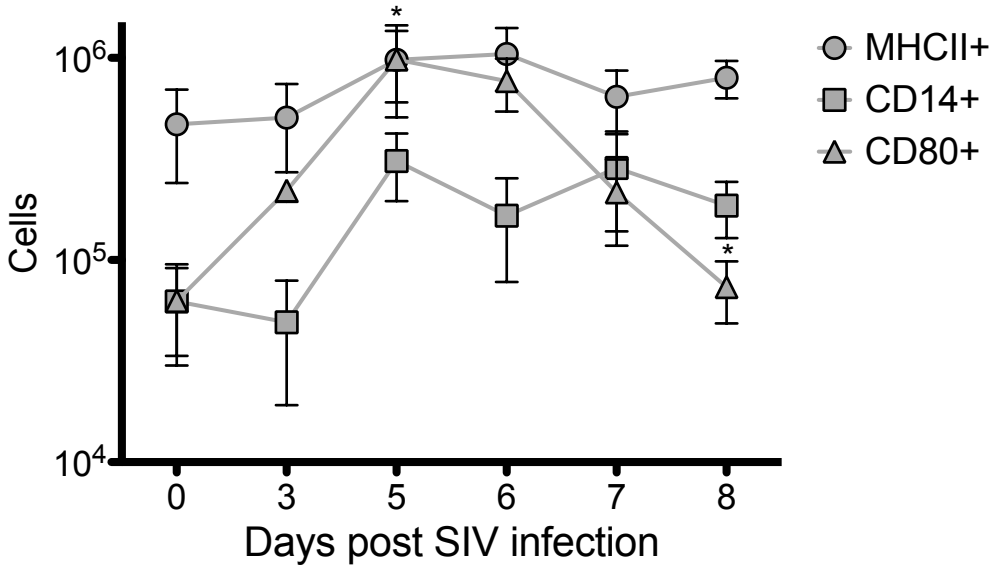
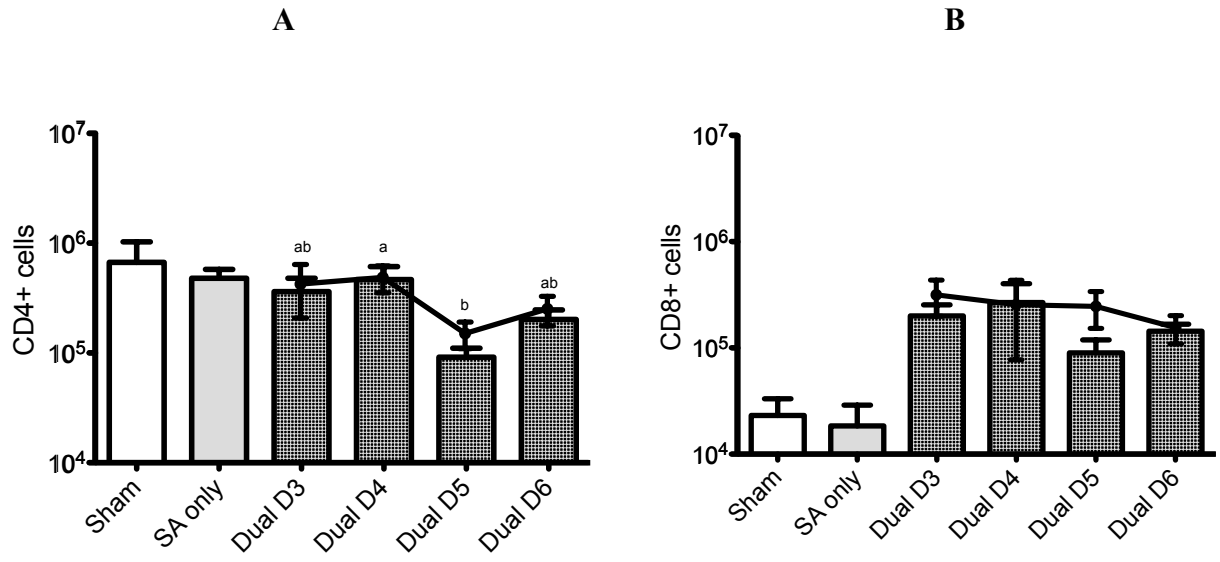
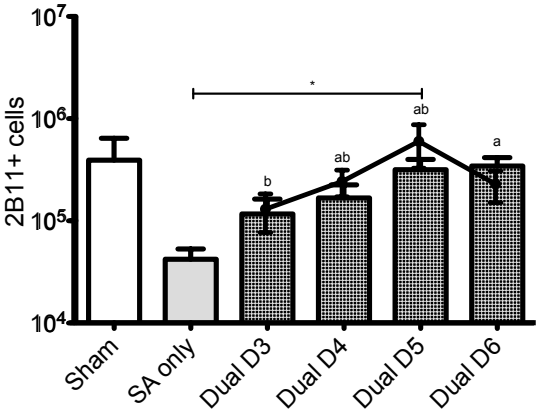

MHCII+
CD14+
CD80+
Cells
$10^6$
$10^5$
$10^4$
0
3
5
6
7
8
Days post SIV infection
*
*

**Figure 4-6. Leukocyte profiles during SIV and MRSA infection.**

Mix 1: (A) CD4+, (B) CD8+, (C) 2B11+, and (D) CD21+ cells within the lymphocyte gate are shown. BAL was collected from sham (n=4) MRSA only, and dual-infected animals 48h post-MRSA infection (n=4). There was an overall effect of treatment ($P < 0.05$) for CD4+, 2B11+, and CD21+ cells. Star indicates significance across treatments ($P < 0.05$). The black line indicates positive cells from SIV only infected animals harvested on the same day as the dual-infected animals (error bars indicate +/- standard error). Letters indicate differences in all SIV-infected animals ($P < 0.05$).



**D**


2B11+ cells
$10^7$
$10^6$
$10^5$
$10^4$
*
b
ab
ab
a
Sham
SA only
Dual D3
Dual D4
Dual D5
Dual D6

**E**

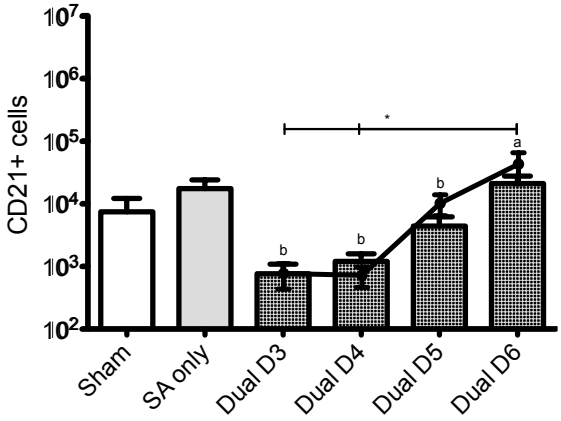
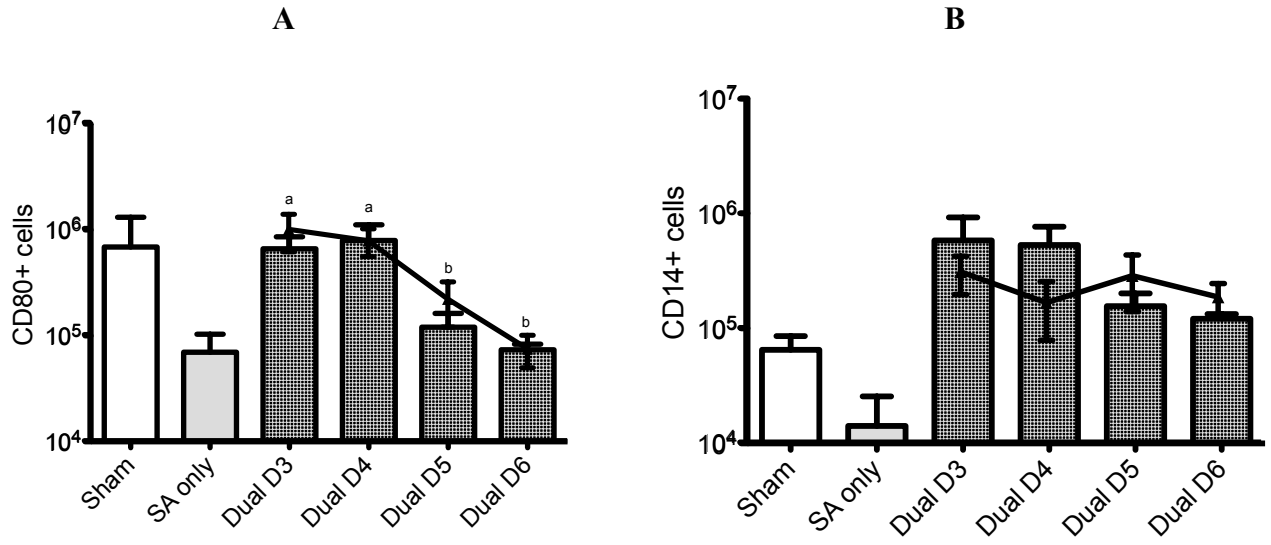
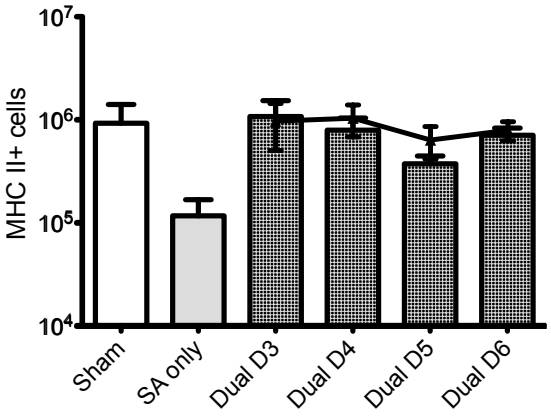

CD21+ cells
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
*
b
b
b
a
Sham
SA only
Dual D3
Dual D4
Dual D5
Dual D6

**Figure 4-7. Leukocyte profiles during SIV and MRSA infection.**

Mix 2: (A) CD14+, (B) CD80+, and (C) MHCII+ cells within the lymphocyte gate are shown. BAL was collected from sham (n=4) MRSA only, and dual-infected animals 48h post-MRSA infection (n=4). There was an overall effect of treatment ($P < 0.05$) for CD80+ cells. Star indicates significance across treatments ($P < 0.05$). The black line indicates positive cells from SIV only infected animals harvested on the same day as the dual-infected animals (error bars indicate +/- standard error). Letters indicate differences in all SIV-infected animals ($P < 0.05$). The black line indicates positive cells from SIV only infected animals harvested on the same day as the dual-infected animals.



**C**


MHC II+ cells
$10^7$
$10^6$
$10^5$
$10^4$
Sham
SA only
Dual D3
Dual D4
Dual D5
Dual D6

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# Chapter 5. Conclusions

The development of a model for polymicrobial infection is an important step towards achieving a large animal model capable of reproducing the heightened mortality seen in human epidemiologic studies. In this thesis, we show that the pig exhibited disease signs consistent with viral-bacterial synergy. Therefore, the pig may prove crucial in the design of prevention and treatment strategies for IAV and associated complications. Additionally, this model exemplifies the intricate factors that contribute to the aggravation of IAV by secondary pathogens.

Pigs co-infected with SIV and MRSA suffered from more substantial disease condition, which included high fever, more severe pneumonia, increased induction of proinflammatory cytokines, and increased immune cell infiltration when compared to infection with SIV or MRSA alone. Thus, the model will be useful for the study of polymicrobial disease, because the degree and duration of illness can be controlled, as evident by the time course. The time course highlighted the predisposed state following resolution of fever as the time in which pigs are most susceptible to secondary *Staphylococcus aureus* infection. By better understanding how IAV affects immune cell function and cytokine expression, therapies can be developed to prevent secondary *Staphylococcus aureus* infections and the increased morbidity and mortality associated with them.

Limitations of this model concern the lack of reagents available for characterizing the host immune cells in swine. As of yet, there are no commercially available antibodies specific for PMN for use in flow cytometry. However, a recent study successfully utilized 6D10 and 2B2 surface antigens to distinguish porcine PMN from macrophages (25). During pulmonary

infection, PMN are recruited to the lung along with macrophages as part of the innate immune response (24). Mice depleted of PMN succumb to fatal *Staphylococcus aureus* pulmonary infection (28). Primary IAV infection rendered mice incapable of a resistance to secondary *S. pneumoniae*, likely due to the suppression of bactericidal functions (19). This study further showed that PMN isolated from IAV-infected mice were unable to effectively produce radical oxygen species in response to bacterial infection. In contrast, human PMN simultaneously infected with IAV and *Escherichia coli* elicited greater hydrogen peroxide production than did either pathogen alone, and IAV dramatically increased phagocytosis of *E. coli* (9). Therefore it is important to examine PMN function in the porcine model to credit its likeness to human infection. Flow cytometry can be used to determine oxidative burst by porcine PMN to aid in assessment of bactericidal function (34).

Real-Time PCR analysis of cytokine production within spleen, lung and lymph tissues provides an excellent starting point from which to study porcine host response. However, it is difficult to distinguish which cells within the organ are responsible for cytokine expression. *In vitro* studies of specific cell groups would help to characterize the host immune response, and provide possible targets for treatment. Due to the lack of porcine cell lines available for studying respiratory epithelial response (30), methods have been developed to isolate and characterize primary porcine lung epithelial cells for use in culture (32-34). Two human cell lines, A549 and Beas-2b were used to evaluate pulmonary epithelial cell susceptibility to intracellular *Staphylococcus aureus* infection (Figure A-3). These lines were chosen based on previous studies evaluating host response to IAV (1, 7, 15). Following primary IAV with secondary intracellular *Staphylococcus aureus* infection would provide insight to the stages of

polymicrobial infection. The host response could be evaluated by examining extracellular matrix proteins and cytokines over time.

DC are antigen presenting cells capable of inducing strong T cells responses (2, 4). Castiglioni et al. suggest reactivation of memory CD8 T cells against IAV requires antigen be presented by bone marrow derived DC in the draining lymph nodes (6). The role of DC should also be considered in the polymicrobial model, as priming with viral HA peptide and the TLR2 agonist, Pam3CysK, lead to successful specific memory CD4 T cell proliferation and IL-2 and IL-17 production (8). The ability of DC to induce alloreactive T cell proliferation is diminished with high doses of IAV, which may contribute to the lymphocytopenia observed during acute IAV infection (21), as well as increased susceptibility to secondary bacterial infection. The role of DC in pulmonary *Staphylococcus aureus* infection is not well understood. Therefore, as an aside to this thesis, DC were derived from primary porcine bone marrow cells using recombinant porcine IL-4 and GM-CSF, and the expression of DC markers, MHC II, CD80, and CD14 verified by flow cytometry (Figure A-4). DC were stimulated for 6 h with bacterial cell wall components: peptidoglycan (PGN), lipopolysaccharide (LPS), lipoteichoic acid (LTA), in addition to supernatant from overnight culture of *Staphylococcus aureus* (Figure A-2). Real-Time PCR quantified mRNA expression of pro-inflammatory (IL-1β, IL-6, TNF-α) and immunoregulatory (IL-10, IFN-γ) cytokines, and showed that porcine bone marrow derived DC successfully respond to stimuli. Additional research is needed to reveal how porcine DC respond to IAV infection, as well as polymicrobial infection with live *Staphylococcus aureus*.

Conventionally reared pigs are not pathogen-free, as opposed to laboratory rodents. Swine are natural carriers of a multitude of bacteria, including antibiotic resistant strains, which are often transmitted by veterinary and laboratory staff (29). Although pretreatment with

antibiotics can reduce incumbent bacterial load, the immune response in the polymicrobial model may be affected by the presence of memory lymphocytes in the lung. Further characterization of T cell populations could help to identify a memory response aiding in the clearance of *Staphylococcus aureus*.

This animal model may prove helpful in the study of predisposed state induced by IAV, and the ability of opportunistic *Staphylococcus aureus* to cause pneumonia. Supplementing the animal model with primary *in vitro* data can help identify targets for intervention in human disease.

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# Appendix A. Supporting Data

**Figure A-1. Analysis of *Staphylococcus aureus* dose curve in pigs**

Nursery pigs, 7 weeks of age, (n=2) were infected intranasally with $10^6$, $10^7$, or $10^8$ CFU of *Staphylococcus aureus* on day (D) 0, and harvested on D5. Sham animals received PBS. Animals were monitored twice daily for rectal temperature (a) respiratory score (b) 0 – no clinical signs, 1 – mild clinical signs, 2 – moderate clinical signs, 3 – severe clinical signs, and changes in percent initial bodyweight (c).

**A**

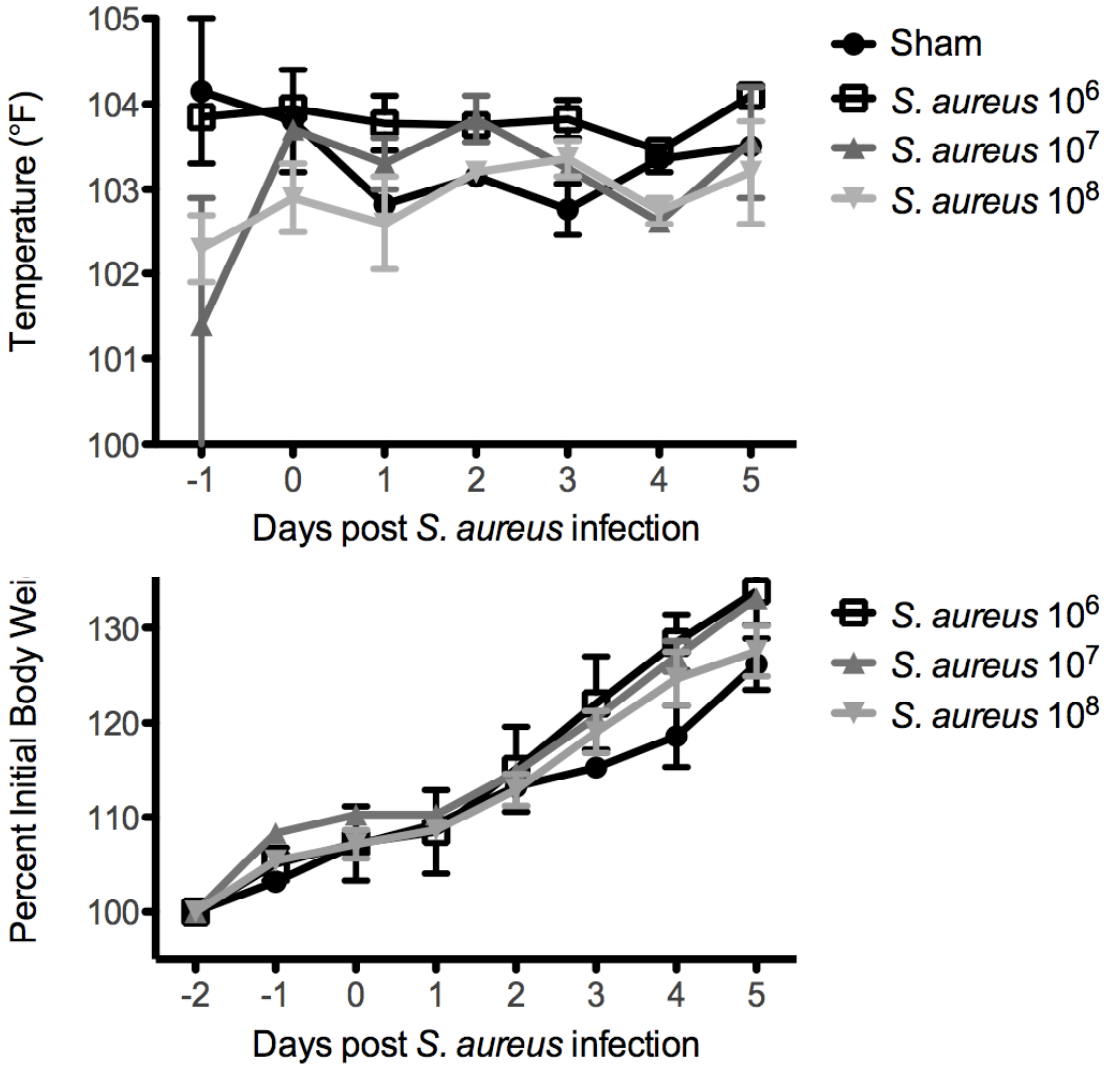


**Figure A-2. Stimulation of porcine bone marrow derived dendritic cells**

DC were harvested and seeded in 6 well dishes at 1.4 x $10^6$ cells per well and stimulated with lipteichoic acid (LTA), peptidoglycan (PGN), lipopolysaccharide (LPS), and supernatant from overnight culture of *Staphylococcus aureus*, diluted 1:10 or 1:100 in PBS. After 6 h, mRNA was isolated, and expression was measured by Real Time-PCR using porcine specific primers.

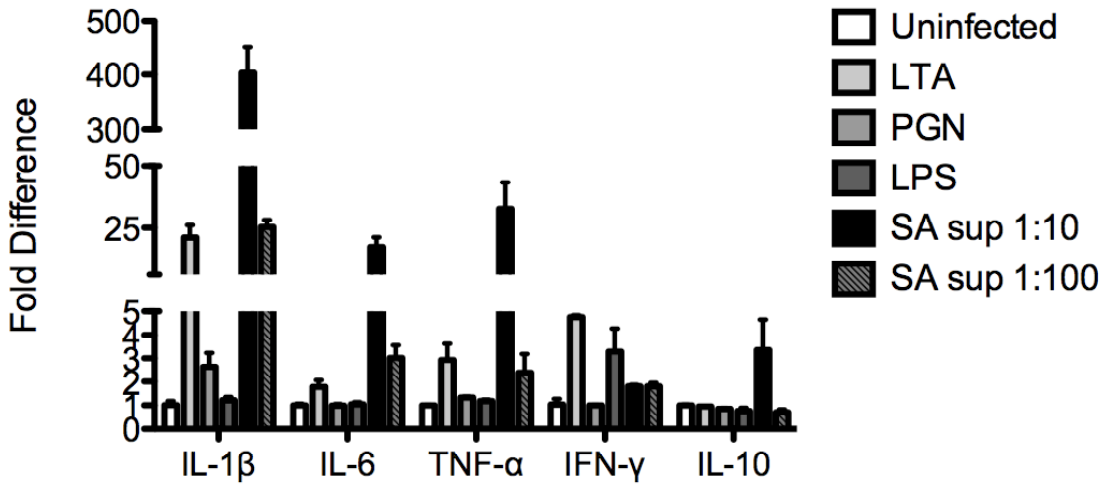


**Figure A-3. Intracellular infection of human respiratory cells**

Human alveolar (A549) and bronchiolar (Beas-2b) epithelial cell lines were seeded in 6 well dishes at 1 x $10^5$ cells per well. Cells were infected with *Staphylococcus aureus* diluted in PBS to different multiplicities of infection (MOI). After 2 h, extracellular bacteria was killed using lysostaphin, media containing gentamicin was added to wells, and cells were incubated for 3 h at 37° C. Following incubation, lysates were collected and serially diluted by a factor of 10 for enumeration of intracellular bacteria.

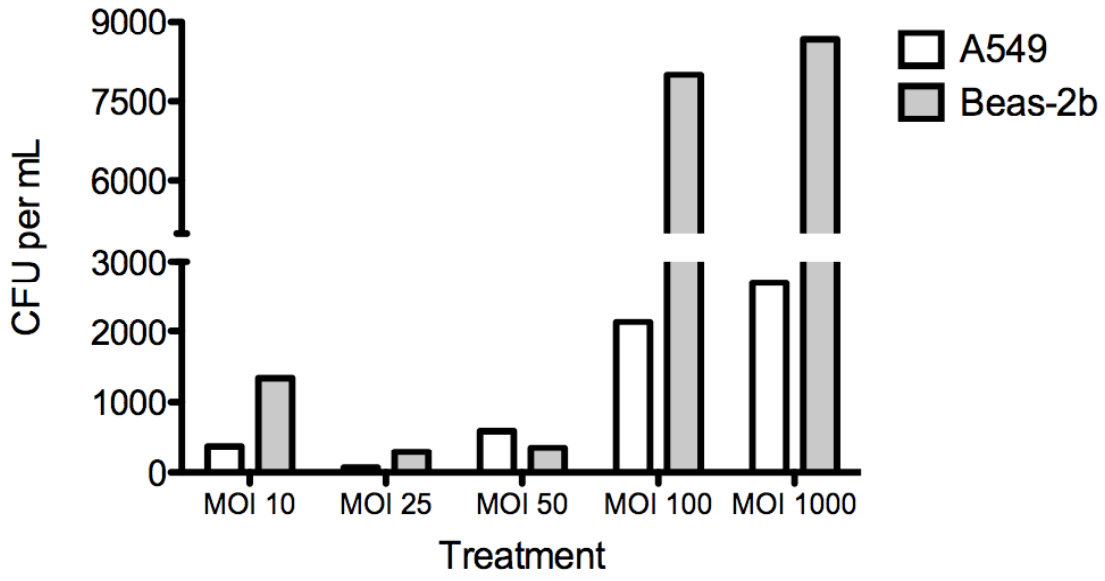
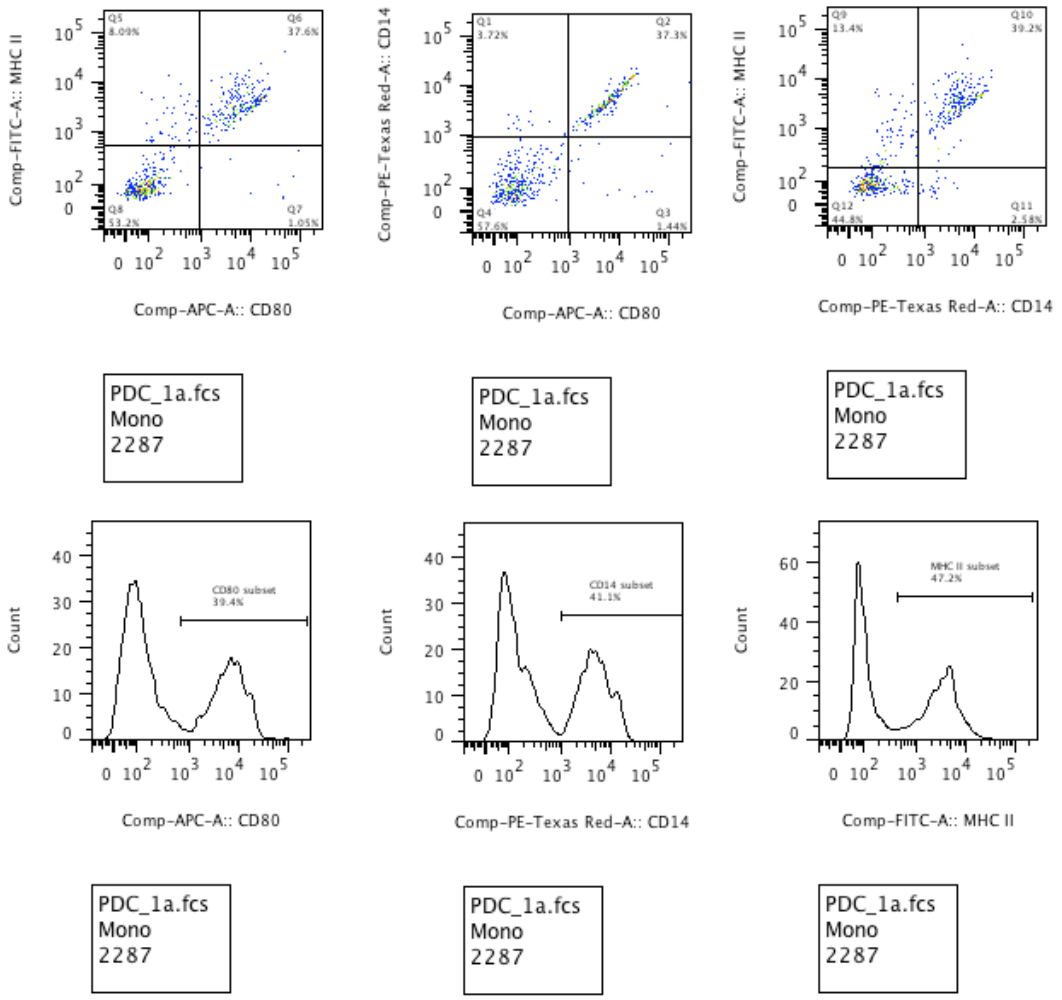


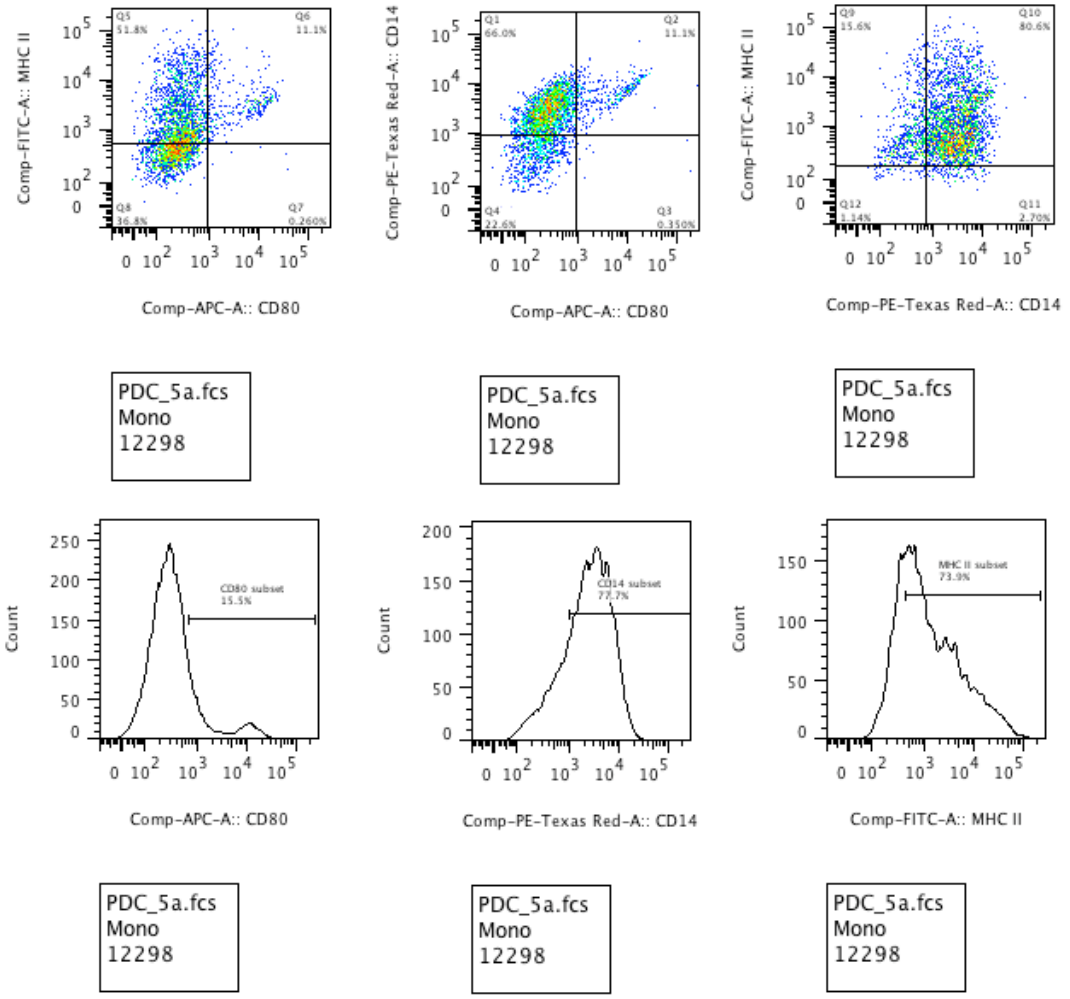
**Figure A-4. Porcine bone marrow derived DC**

Porcine bone marrow cells were collected from the sternum, and seeded in large petri dishes at 1.5 x $10^7$ cells per dish for the purpose of deriving DC. The cells cultured without cytokines (a) or with exogenous recombinant porcine IL-4 and GM-CSF for 7D (b). On the seventh day of culture, cells were collected and stained with MHC II, CD14, and CD80 antibodies for evaluation by flow cytometry. Cells cultured in the presence of cytokines had DC morphology and an increase in cells positive for DC markers compared with unstimulated cells.

**A**



**B**


Comp-FITC-A:: MHC II
Comp-APC-A:: CD80
Q5 11.8%
Q6 11.1%
Q8 16.8%
Q7 0.260%
PDC_5a.fcs
Mono
12298
Comp-PE-Texas Red-A:: CD14
Comp-APC-A:: CD80
Q1 66.0%
Q2 11.1%
Q4 22.6%
Q3 0.350%
PDC_5a.fcs
Mono
12298
Comp-FITC-A:: MHC II
Comp-PE-Texas Red-A:: CD14
Q9 15.6%
Q10 80.6%
Q12 1.14%
Q11 2.70%
PDC_5a.fcs
Mono
12298
Count
CD80 subset
15.5%
Comp-APC-A:: CD80
PDC_5a.fcs
Mono
12298
Count
CD14 subset
77.7%
Comp-PE-Texas Red-A:: CD14
PDC_5a.fcs
Mono
12298
Count
MHC II subset
73.9%
Comp-FITC-A:: MHC II
PDC_5a.fcs
Mono
12298

## Table A-1. Microarray analysis of lung tissue

RNA was isolated from lung homogenates of animals harvested on D11 post-SIV infection. The analysis was conducted using the Affymetrix GeneChip® Porcine Genome Array at the Virginia Bioinformatics Institute, Blacksburg, VA. In short, labeled cRNA targets derived from the mRNA of experimental samples were hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it was possible to infer the abundance of each mRNA species represented. Analysis was conducted comparing dual-infected animals from the SIV 1145 and MRSA group and respective single infected and sham groups. Genes that had a 2-fold difference in expression from housekeeping genes were considered significant. Genes meeting these criteria were subsequently grouped into categories based on biological relevance and function.

| **Chemotactic Factors** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene Name** | **Abbreviation** | **MRSA vs Sham** | **MRSA vs Dual** | **Dual vs SIV** | **SIV vs Sham** | **MRSA vs SIV** | **Dual vs Sham** |
| Chemokine | CXCL14 | 2.45 | 2.93 | | | 3.68 | |
| Chemokine | CXCL14 | | 2.48 | | | 3.01 | |
| chemokine (C-C motif) ligand 19 | CCL19 | | 2.85 | | | 2.25 | -2.11 |
| chemokine (C-C motif) ligand 2 | CCL2 | | 2.25 | | | 2.39 | |
| Chemokine (C-C motif) ligand 28 | CCL28 | | | 2.30 | | | 2.08 |
| chemokine (C-C motif) ligand 4 | CCL4 | | | | | | -2.01 |
| chemokine (C-C motif) ligand 5 | CCL5 | | | | | | -2.22 |
| chemokine (C-C motif) receptor 1 | CCR1 | | 2.10 | | | 2.60 | |
| chemokine (C-C motif) receptor 5 | CCR5 | | 2.13 | | | | |
| chemokine (C-C motif) receptor-like 2 | CCRL2 | | | | | | -2.00 |
| chemokine (C-X-C motif) ligand 2 | CXCL2 | | 2.07 | 2.50 | -3.36 | 5.17 | |
| chemokine (C-X-C motif) ligand 2 | CXCL2 | | | | -2.51 | 2.48 | |
| chemokine (C-X-C motif) ligand 9 | CXCL9 | | | | | | -2.23 |
| alveolar macrophage-derived chemotactic factor-II | AMCF-II | 4.11 | 2.53 | | | | |
| alveolar macrophage-derived chemotactic factor-II | AMCF-II | 3.41 | | | | | |
| **AVERAGE Chemotactic Factors** | | **3.32** | **2.42** | **2.40** | **-2.94** | **3.08** | **-1.42** |
| **Standard Deviation** | | **0.84** | **0.34** | **0.14** | **0.60** | **1.04** | **1.72** |

| **Interleukins/adhesion factors** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene Name** | **Abbreviation** | **MRSA vs Sham** | **MRSA vs Dual** | **Dual vs SIV** | **SIV vs Sham** | **MRSA vs SIV** | **Dual vs Sham** |
| interferon regulatory factor 7 | IRF7 | | 2.50 | | | 2.20 | |
| interferon, gamma-inducible protein 30 | IFI30 | | 2.16 | | | 2.25 | |
| interleukin 1 receptor antagonist | IL1RN | 2.28 | | | | 2.35 | |
| interleukin 1 receptor antagonist | IL1RN | 2.13 | | | | 2.13 | |
| interleukin 1, alpha | IL1A | 2.13 | | | -2.01 | | |
| interleukin 1, beta | IL1B | | 2.13 | | | 2.13 | |
| interleukin 13 receptor, alpha 1 | IL13RA1 | | | | | 2.11 | |
| interleukin 8 | IL8 | | 6.36 | | -5.86 | 7.89 | -4.72 |
| Selectin L | SELL | | 4.03 | | | 2.91 | -3.29 |
| Selectin L | SELL | | | | | | -2.13 |
| vascular cell adhesion molecule | VCAM1 | | | | | 2.23 | |
| **AVERAGE interleukins/adhesion factors** | | **2.18** | **3.44** | | **-3.94** | **2.91** | **-3.38** |
| **Standard Deviation** | | **0.09** | **1.81** | | **2.72** | **1.88** | **1.30** |

| **Complement factors** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene Name** | **Abbreviation** | **MRSA vs Sham** | **MRSA vs Dual** | **Dual vs SIV** | **SIV vs Sham** | **MRSA vs SIV** | **Dual vs Sham** |
| complement C4 | SBAB-514B12.5 | | 2.20 | | | 2.04 | |
| complement component 1, q subcomponent, A chain | C1QA | | | | | | -2.48 |
| complement component 1, q subcomponent, B chain | C1QB | | | | | | -2.10 |
| complement component 1, q subcomponent, C chain | C1QC | | | | | | -2.07 |
| complement component 1, r subcomponent | C1R | | 2.22 | | | 2.27 | |
| complement component 2 | SBAB-707F1.3 | | 2.25 | | | 3.66 | |
| Complement component 7 | C7 | 2.04 | 2.57 | | | 2.28 | |
| Complement component C9 | LOC100037951 | 3.20 | 2.55 | | | 4.06 | |
| **AVERAGE complement factors** | | **2.62** | **2.36** | | | **2.86** | **-2.22** |
| **Standard Deviation** | | **0.82** | **0.18** | | | **0.92** | **0.23** |

| **Lipoprotein lipase** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene Name** | **Abbreviation** | **MRSA vs Sham** | **MRSA vs Dual** | **Dual vs SIV** | **SIV vs Sham** | **MRSA vs SIV** | **Dual vs Sham** |
| Lipoprotein lipase | LPL | 13.74 | -2.75 | 16.11 | | 13.74 | 9.51 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| lipoprotein lipase | LPL | 4.38 | -2.00 | 11.79 | | 5.90 | 8.75 |
| lipoprotein lipase | LPL | 3.46 | | 8.46 | | 5.86 | 8.46 |
| **AVERAGE lipoprotein lipase** | | **7.19** | **-2.38** | **12.12** | | **8.50** | **8.91** |
| **Standard Deviation** | | **5.69** | **0.53** | **3.84** | | **4.54** | **0.55** |

**Glutathione related**

| Gene Name | Abbreviation | MRSA vs Sham | MRSA vs Dual | Dual vs SIV | SIV vs Sham | MRSA vs SIV | Dual vs Sham |
|---|---|---|---|---|---|---|---|
| Glutathione peroxidase 2 | GPX2 | | -2.91 | | | | 2.81 |
| glutathione S-transferase | LOC396850 | | -3.78 | | | | 3.51 |
| glutathione S-transferase | GSTA2 | -5.06 | -2.79 | | -2.50 | | |
| glutathione S-transferase | LOC396850 | | | | 2.35 | | |
| glutathione S-transferase /// similar to glutathione S-transferase /// glutathione S-transferase | GSTA2 /// LOC100156585 /// LOC396850 | -2.04 | -2.50 | 2.41 | | | |
| similar to glutathione S-transferase, alpha 3 | LOC100154154 | | -17.88 | | 2.01 | | |
| **AVERAGE Glutathion related** | | **-3.55** | **-5.97** | **2.41** | **0.62** | | **3.16** |
| **Standard Deviation** | | **2.14** | **6.67** | **#** | **2.71** | | **0.49** |

**Heat shock**

| Gene Name | Abbreviation | MRSA vs Sham | MRSA vs Dual | Dual vs SIV | SIV vs Sham | MRSA vs SIV | Dual vs Sham |
|---|---|---|---|---|---|---|---|
| heat shock protein 70.2 | SBAB-707F1.4 | 2.31 | | 2.10 | | | |
| Heat shock protein 70.2 | SBAB-707F1.4 | 2.22 | | 2.10 | | | |
| heat shock 105kDa/110kDa protein 1 | HSPH1 | | | | | | 2.06 |
| heat shock protein 70.2 | SBAB-707F1.4 | | | | | | 2.22 |
| Heat shock protein 70.2 | SBAB-707F1.4 | | | | | | 2.06 |
| **AVERAGE heat shock** | | **2.27** | | **2.10** | | | **2.11** |
| **Standard Deviation** | | **0.07** | | **0.00** | | | **0.09** |

**MMP**

| Gene Name | Abbreviation | MRSA vs Sham | MRSA vs Dual | Dual vs SIV | SIV vs Sham | MRSA vs SIV | Dual vs Sham |
|---|---|---|---|---|---|---|---|
| matrix metallopeptidase 1 (interstitial collagenase) | MMP1 | 2.39 | 2.50 | | | 2.36 | |
| matrix metallopeptidase 7 (matrilysin, uterine) | MMP7 | 8.28 | 6.11 | | | 8.28 | |
| **AVERAGE MMP** | | **5.34** | **4.30** | | | **5.32** | |
| **Standard Deviation** | | **4.16** | **2.55** | | | **4.19** | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| cytochrome P450 3A29 | CYP3A29 | | 2.20 | | | | |
| cytochrome P450 CYP3A88 | CYP3A88 | | 2.89 | | | | |
| Cytochrome P450 CYP3A88 | CYP3A88 | | 2.69 | | | | |
| **AVERAGE MMP** | | | **2.60** | | | | |
| **Standard Deviation** | | | **0.35** | | | | |

| **Miscellaneous** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Gene Name** | **Abbreviation** | **MRSA vs Sham** | **MRSA vs Dual** | **Dual vs SIV** | **SIV vs Sham** | **MRSA vs SIV** | **Dual vs Sham** |
| haptocorrin | LOC396873 | 6.73 | 5.43 | | | 5.90 | |
| lipocalin 1 (tear prealbumin) | LCN1 | | -32.00 | 2.99 | 10.70 | | |
| cathepsin K | CTSK | 2.23 | | | | | -4.23 |
| NK-lysin | NKL | -2.31 | | | -2.20 | | |
| membrane-associated protein 17 | MAP17 | 3.51 | | | | | |

**Table A-2. Standard error for least squares means for polymicrobial time course**

| Study | Effect | Standard Error |
|---|---|---|
| **Temperature** (Dual3SIV5) | Tmt | 0.21-0.24 |
| | day | 0.25 |
| | Tmt*day | 0.33-0.38 |
| **Temperature** (Dual4SIV6) | Tmt | 0.11-0.12 |
| | day | 0.19-0.21 |
| | Tmt*day | 0.25-0.29 |
| **Temperature** (Dual5SIV7) | Tmt | 0.13-0.15 |
| | day | 0.26 |
| | Tmt*day | 0.35-0.40 |
| **Temperature** (Dual6SIV8) | Tmt | 0.07-0.08 |
| | day | 0.18 |
| | Tmt*day | 0.24-0.28 |
| **Pathology** (MRSA and Dual) | Tmt | 1.51 |
| **Pathology** (SIV and Dual) | Type | 0.89-1.03 |
| | Harvest Day | 1.37 |
| | Type*Harvest Day | 1.79-2.06 |
| **CFU** (MRSA and Dual) | Tmt | 107.03 |
| **CD4** (SIV) | Tmt | 1.46 |
| **CD8** (SIV) | Tmt | 1.06 |
| **2B11** (SIV) | Tmt | 1.33 |
| **CD21** (SIV) | Tmt | 0.20 |
| **CD80** (SIV) | Tmt | 2.02 |
| **CD14** (SIV) | Tmt | 0.96 |
| **MHCII** (SIV) | Tmt | 3.13 |
| **CD4** (MRSA and Dual) | Tmt | 0.96 |
| **CD4** (SIV and Dual) | Type | 0.52-0.60 |
| | Harvest Day | 0.80 |
| | Type*Harvest Day | 1.05-1.21 |
| **CD8** (MRSA and Dual) | Tmt | 0.68 |
| **CD8** (SIV and Dual) | Type | 0.44-0.50 |
| | Harvest Day | 0.67 |
| | Type*Harvest Day | 0.87-1.01 |
| **2B11** (MRSA and Dual) | Tmt | 0.59 |
| **2B11** (SIV and Dual) | Type | 0.48-0.55 |
| | Harvest Day | 0.73 |
| | Type*Harvest Day | 0.96-1.11 |
| **CD21** (MRSA and Dual) | Tmt | 0.04 |
| **CD21** (SIV and Dual) | Type | 0.03-0.04 |

| | | |
|---|---|---|
| | Harvest Day | 0.05 |
| | Type*Harvest Day | 0.07-0.08 |
| **CD14** (MRSA and Dual) | Tmt | 1.87 |
| **CD14** (SIV and Dual) | Type | 0.86-0.99 |
| | Harvest Day | 1.31 |
| | Type*Harvest Day | 1.72-1.98 |
| **CD80** (MRSA and Dual) | Tmt | 1.72 |
| **CD80** (SIV and Dual) | Type | 0.97-1.11 |
| | Harvest Day | 1.47 |
| | Type*Harvest Day | 1.93-2.23 |
| **MHCII** (MRSA and Dual) | Tmt | 2.41 |
| **MHCII** (SIV and Dual) | Type | 1.38-1.59 |
| | Harvest Day | 2.14 |
| | Type*Harvest Day | 2.76-3.18 |

**Table A-3. Detection of *lukS-PV* gene in methicillin resistant staphylococci**

Lung homogenates were cultured on mannitol salt agar + cefoxitin to select for methicillin resistant staphylococci. Isolates compared to original infectious PVL+ MRSA strain by positive Gram stain, catalase, coagulase, and presence of *lukS-PV* gene.

| Treatment | Sham (n=4) | MRSA only (n=4) | SIV only (n=4) | Dual 3 (n=4) | Dual 4 (n=4) | Dual 5 (n=4) | Dual 6 (n=4) |
|---|---|---|---|---|---|---|---|
| Methicillin resistant | 3/4 | 1/4 | 3/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| Gram stain | 3/4 | 1/4 | 3/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| Catalase | 3/4 | 1/4 | 3/4 | 4/4 | 4/4 | 4/4 | 4/4 |
| Coagulase | 0/4 | 0/4 | 0/4 | 4/4 | 2/4 | 1/4 | 1/4 |
| *lukS-PV* | 0/4 | 0/4 | 1/4 | 4/4 | 2/4 | 1/4 | 1/4 |

# Appendix B. Detailed Protocols

### Culture of *Staphylococcus aureus* for Infection

Purpose: Growth of *Staphylococcus aureus* for infection of pigs.

Reagents:
*Staphylococcus aureus* cultures
Disposable cuvettes
Spectrophotometer
Phosphate Buffered Saline (PBS)
1 mL syringe

Procedure:

1. Inoculate 100 ml TSB using one colony of *Staphylococcus aureus* from overnight culture on TSA.
2. Place TSB in shaker at 37°C overnight (15-18 hrs).
3. Prepare serial dilutions of overnight culture in 15 ml sterile conical tubes.
4. Take OD readings at 600nm of serial dilutions to determine concentrations of overnight culture of *Staphylococcus aureus* based on Standard Curve.
5. Transfer 50 ml of overnight TSB culture into 50 ml conical tube.
6. Centrifuge at 3000 rpm for 10 min at 4°C.
7. Remove supernatant into 20% Lysol container and resuspend bacterial pellet in 10 ml PBS. Bring volume up to 50 ml.
8. Centrifuge at 3000 rpm for 10 min at 4°C.
9. Repeat wash.
10. Resuspend bacterial pellet at appropriate concentration (infectious dose).
    a. Use PBS as diluent for infection of pigs.
11. Prepare syringes with bacterial inoculation.
12. Intranasally infect bacterial inoculation into properly sedated experimental pigs.

**CFU determination in Porcine Tissues**

Purpose: To dilute bacterial cultures for determining CFU/ml.

Reagents:
96-well plate
Multi-channel pipettor and tips
Reagent Reservoir
PBS
TSB plate

Procedure:

1. Homogenize tissue in PBS for 1 min.
2. Prepare 96-well plate by adding PBS to well in the following amounts:
    a. Row A: 100 µL
    b. Row B: 200 µL
    c. Row C-H: 225 µL
3. Add 100 µL of homogenate to Row A (1:2 dilution), mix
4. Transfer 50 µL from Row A to Row B (1:10 dilution),
    a. (make sure to clip tips before mixing and transferring 25 µL to next well).
5. Transfer 25 µL from Row B to Row C ($1x10^2$)
    a. (make sure to clip tips before mixing and transferring 25 µL to next well).
6. Continue transferring 25 µL down the plate to obtain 10 fold dilutions ($1x10^3$ in Row D through $1x10^7$ in row H)
7. Plate appropriate dilutions by dropping 3 x 25 µL drops onto blood plate.
    a. ex. If original culture has $10^9$ bacteria, plate dilutions $10^7$ and $10^6$ (~2.5 and 25 bacteria per drop respectively)
8. Incubate blood plate overnight at 37°C.
9. Count number of colonies in 3 drops:
    a. (colony count/3) x 40 x final dilution = CFU/ml

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Final Dilution |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 100 µL PBS + 100 µL homogenate | | | | | | | | | | | | 1:2 |
| B | 200 µL PBS + 50 µL bacteria from row A | | | | | | | | | | | | 1:10 |
| C | 225 µL PBS + 25 µL bacteria from row B | | | | | | | | | | | | $10^2$ |
| D | 225 µL PBS + 25 µL bacteria from row C | | | | | | | | | | | | $10^3$ |
| E | 225 µL PBS + 25 µL bacteria from row D | | | | | | | | | | | | $10^4$ |
| F | 225 µL PBS + 25 µL bacteria from row E | | | | | | | | | | | | $10^5$ |
| G | 225 µL PBS + 25 µL bacteria from row F | | | | | | | | | | | | $10^6$ |
| H | 225 µL PBS + 25 µL bacteria from row G | | | | | | | | | | | | $10^7$ |

**MDCK Plaque Assay for Influenza Virus**

Purpose: To measure influenza virus titer as plaque forming units/mL.

Reagents:

1.8% Agar (Sigma) in sterile dd $H_2O$

2x Minimal Essential Medium Eagle (MEM)

- 100 mL 10x MEM (Gibco)
- 20 mL 4% BSA
- 30 mL $NaHCO_3$
- 10 mL antibiotics
- 10 mL L-glutamine
- 330 mL sterile dd $H_2O$

Staining reagent

- 270 mL of 37% Formaldehyde
- 10 g Crystal Violet
- 100 mL Ethanol
- 50 mL Acetic acid
- 580 mL dd $H_2O$

6 well plates

PBS + a/a

1.5 mL microcentrifuge tubes

1mg/ml TPCK-trypsin

Procedure:

1. Day(s) before infection.
    a. Seed 6 well plates at $10^6$ cells/well of MDCK cells – will need to form monolayer prior to infection.
2. Heat 1.8% Agar in microwave for a minute, mix well, heat again until solution is liquid, then put it into 50˚C water bath.
    a. Put 2x MEM medium in 37˚C water bath.
3. Make serial dilutions of the virus in sterile 1.5 mL microcentrifuge tubes using PBS + a/a.
4. Wash cell monolayer with PBS + a/a twice, aspirate the second wash buffer off just before inoculating the virus.
5. Infect cells with 0.4 mL of viral dilution.
6. Incubate the plate at 4˚C for 15 min.
7. Incubate in 37˚C $CO_2$ incubator for 45 min.
    a. Rotate plate at 20-25 min intervals.
8. Aspirate unadhered virus.
9. Wash the cell 2x with PBS.
    a. Leave the second wash in the plate while preparing overlay.
10. Prepare agar overlay by diluting the 2x MEM 1:1 with 1.8% Agar.
    a. Keep agar bottles on a foam rack to prevent the overlay medium from cooling too quickly.
11. Add 1 mg/mL TPCK-trypsin to overlay medium to reach 1 ug/mL.

a. ex. 40 µL of TPCK-trypsin in 40 mL overlay medium.

12. Aspirate PBS off infected MDCK cells.
13. Add 2 mL of overlay medium per well.
14. Let the agar solidify completely.
15. Incubate in 37˚C $CO_2$ incubator for 3 days.
16. Remove agar overlay.
17. Stain each well with 2 mL staining reagent for 5 min.
18. Rinse plate with tap water.
19. Enumerate plaques.

**Intracellular Infection of Human Respiratory Cells *in vitro***

Purpose: To intracellularly infect lung epithelial cells with *Staphylococcus aureus*.

Reagents:
*Staphylococcus aureus* culture
Human alveolar epithelial cell line (A549 cells)
Lysostaphin (Sigma L7386 5 mg) [dilute in 2.5 mL sterile $H_2O$ aliquot]
Gentamicin solution
PBSE + 0.05% Trition X-100
TSB
PBS

Procedure:

1. Day before infection
    a. Seed 6 well plates at $10^6$ cells/well of A549 cells.
    b. Inoculate TSB with *Staphylococcus aureus* for overnight culture (15-18 h).
2. Change media on cell culture to a/a free media
    a. Rinse with PBS before adding a/a media
3. Add $10^8$ CFU/well of *Staphylococcus aureus* to appropriate wells.
    a. Wash overnight *Staphylococcus aureus* culture in PBS 2x and resuspend in media at $10^8$ after taking OD600 reading on spectrometer.
4. Incubate at 37° C for 2 hours.
5. Remove media from wells, rinse 1x with PBS.
6. Add 50 μL (100μg) lysostaphin in 1 mL a/a free media per well and incubate at 37° C.
7. Remove lysostaphin and add 3 mL a/a free media + gentamicin (1 μg/mL)
8. Incubate at 37° C.
9. To plate CFU post infection:
    a. Transfer 1 mL sterile 1.7 mL tube. Remove remaining media, rinse 2x with PBS.
    b. Add 0.5 mL PBSE + 0.05% Triton X-100.
    c. Nutate at RT 2-3 min. Scrape.
    d. Transfer lysate to sterile 1.7 mL tube.
    e. Sonicate 5 min at 4° C.
    f. Plate microtiter dilutions, as well as neat lysates and media.

**Isolation of Porcine Bone Marrow Cells and Dendritic Cell Culture**

Purpose: To derive porcine dendritic cells from bone marrow.

Reagents:

β-mercaptoethanol (B-ME) solution:

Add 17.5 μL B-ME to 5 mL water. Store at 4°C no longer than 2 weeks.

cRPMI

10 mL Hepes buffer (1M)
500 μL Gentamicin
100 μL of B-ME solution
50 mL Fetal Porcine Serum (FPS) – Heat Inactivated
5 mL L-glutamine
Bring up to 500 mL with RPMI-1640
*store at 4° C no longer than one month

Recombinant porcine IL-4 (0.1 μg/μL) (R&D Systems, Inc.)

Recombinant porcine GM-CSF (0.1 μg/μL) (R&D Systems, Inc.)

Cell stripper solution

Procedure:

1. Isolate bone marrow from porcine femur and sternum using 14 G syringe, trocar, knife and handsaw using 0.3% EDTA in PBS + gentamicin into 50 mL tubes.
    a. Remove as much excess tissue before cutting bones open.
    b. Keep tubes on ice.
2. Shake vigorously for 15 min.
3. Pass through nylon mesh into new 50 mL tube prior to first spin using disposable pipette.
4. Centrifuge 8 min, 1400 rpm, 4° C.
5. Lyse RBC using ACK lysing buffer.
    a. Resuspend pellet (5-10 mL depending on size of pellet).
    b. Hold in 37° C water bath until color darkens.
6. Add 25 mL cRPMI to each tube.
7. Centrifuge.
8. Wash pellet in 0.3% PBSE + gentamicin.
    a. Resuspend in 10 mL PBSE.
    b. Pass through 70 μm filter.
9. Centrifuge 10 min, 2000 rpm, 4° C.
10. Repeat wash, passing through 40 μm filter.
11. Resuspend in 5 mL PBSE, taking an aliquot for counting.
    a. Bring volume up to 40 mL, centrifuge.

Derivation of DC

1. Resuspend the pelleted cells to approximately 1.5 x $10^7$ cells/mL in cRPMI for culture in large petri dishes.
2. Add GMCSF and IL-4 at 0.2 μL per mL of media.

3. Place plate into humidified incubator at 37° C.
4. Culture cells for 3 days. They may then be harvested and used for other procedures as required.
    a. Cells must be ‘fed’ new medium on day 3.
    b. Remove plate from incubator to biosafety cabinet and allow cells to settle for approximately 10 min.
    c. Carefully remove 1ml medium from each well, care is required to avoid disturbing the cells.
    d. Add 1.5ml fresh, pre-warmed medium to each well and re-culture the DC for required time up to 7 days.

**Cryopreservation of Porcine Dendritic Cells**

Purpose: To freeze bone marrow-derived DC for long term storage.

Reagents:
Cryogenic vials (pre-labeled)
Freezing container with isopropanol
Growth media
Freezing media (Growth Media + 20% FPS + 10% DMSO)

Procedure:

1. To be completed after 6-7 days of culture.
2. Remove non-adherent cells/medium from the wells and place into a 50 mL conical tube, store on ice.
3. Wash the Petri dish 2x with PBS.
4. Add cell stripper solution (6 ml/Petri dish) to the remaining adherent cells, place the plate in incubator at 37°C with 5% $CO_2$, incubate 10-15 min.
5. Place plate in biosafety cabinet.
6. Triturate each well containing DC vigorously to remove cells from plate surface. Pool these with the cells collected in step 1.
    a. NOTE: not all cells will come off plate, but a large majority should, this is normal
7. Centrifuge the cells at 1000 rpm for 10 min at 4° C.
8. Resuspend cell pellet at $1x10^7$ cells/mL in growth media.
9. Add equal amount of freezing media.
10. Transfer 0.5-1 ml of cell suspension per cryogenic vial.
11. Place vials in freezing container in -80° C overnight.
12. Transfer vials to liquid nitrogen storage.

**Isolation of Porcine Lymphocytes from Whole Blood**

Purpose: To isolate lymphocytes cells from whole blood samples for use in functional assay or culture.

Reagents:
EDTA
0.3% EDTA in PBS (PBSE)
Ficoll-paque
Sterile dd$H_2O$
2x RPMI

Procedure:

1. Collect 50 mL blood using EDTA (10% of 2x citrate) or 10% of 40 mM EDTA.
2. Transfer blood into 50 mL centrifuge tubes.
3. Spin at 2000 rpm for 30 min at 15° C with brake turned off.
4. Remove 5 mL buffy coat layer using 10 mL pipette in the smallest possible volume with the least amount of RBC and transfer into a 50 mL centrifuge tube containing 20 mL PBSE. Then layer over 12.5 mL ficoll-paque in a 50 ml centrifuge tube.

    - Pick up 25 ml of cells in 25 ml pipette.
    - Tip tube so that ficoll-paque almost reaches front edge of the tube, and carefully place one drop of cells just in front of the ficoll. Tip the tube so the cells run onto the ficoll, and then slowly add the remaining cells into the tube. As you add more cells, bring the tube back to a near vertical position.

OR for smaller volumes of blood:

- Take off 5 mL of buffy coat and mix with 5 mL PBSE then layer over 5 mL ficoll- paque in a 15 mL centrifuge tube.

NOTE: Do one way or the other, depending on volume of blood.

5. Centrifuge for 30 min at 1300 rpm, 25° C with brake off.
6. Take off mononuclear cell band and add to 20 mL PBSE in a 50 mL centrifuge tube with a 10 mL pipette. Try not to pick up much ficoll.
7. Resuspend in PBSE to 45 mL, and spin 10 min at 1000 RPM, 15°C.
    a. If RBC are present in pellet: Resuspend cells in 5 mL PBSE. Add 10 mL dd$H_2O$, pipette for 18 sec, then add 10 mL 2x RPMI and mix.
    b. Top off tube with PBSE, then spin 10 min at 1000 RPM, 4° C. May repeat if necessary.
8. Resuspend cells in 10 mL PBSE, take and aliquot for counting while cells are pelleting 5 min, 1000 rpm 4° C. Bring volume to 45 mL for centrifuge.
9. Resuspend to use concentration in appropriate medium (approx. 10 mL medium).

**RNA Isolation from Porcine Tissues – Qiagen Kit**

Purpose: To isolate RNA for analysis in TAQMAN assay.

Reagents:
Qiagen RNA Isolation Kit
β-mercaptoethanol (B-ME)

Procedure:

1. Homogenize 30 mg of tissue for 1 min in 650 µL lysis buffer (10 µL B-ME/mL Lysis Buffer).
2. Store up to one week at -20° C.
3. Follow protocol for isolation of RNA from tissue.
4. Spin down tubes for 3 min, 3000 rpm, brake off.
    a. Transfer 600 µL RNA to 600 µL 50% ETOH in 1.7 ml tubes.
    b. Mix by pipetting up and down 5-6x.
    c. Transfer onto column.
    d. Centrifuge 14000 rpm 20 sec.
    e. Dump flo-through.
    f. Add remaining sample to column.
    g. Centrifuge.
    h. Dump flo-through.
    i. Pipet 350 µL Buffer RW1 onto column.
    j. Centrifuge 15 sec at 14000 rpm.
    k. Dump flo through.
    l. Premix 10 µL DNASE stock 1 (2.7 Kunitz units/µL with 70 µL buffer RDD per sample).
    m. Add 80 µL DNASE I mix onto column.
    n. Incubate 30 min RT.
    o. Add 350 µL Buffer RWL onto column.
    p. Centrifuge 15 sec.
    q. Dump flo-through.
    r. Transfer column to new collection tube.
    s. Add 500 µL RPE Buffer.
    t. Centrifuge.
    u. Dump flo-thru.
    v. Repeat wash.
    w. Spin dry 2 min 14000 rpm.
    x. Transfer column to new 1.7 ml collection tube.
    y. Add 40 µL RNase free $H_2O$.
    z. Centrifuge 800 × g for 2 min.
5. OD Reading of RNA using Nanodrop.
6. Based on OD reading dilute RNA (0.2 µg/µL) for making cDNA.

**Detection of PVL genes in *Staphylococcus aureus***

Purpose: To detect PVL genes in *Staphylococcus aureus* isolates using PCR and gel electrophoresis.

Reagents:
- TSA plates
- TSB
- Sterile 1.7 mL microcentrifuge tubes
- Sterile PBS
- Sterile dd $H_2O$
- PVL *lukS* (or PVL *lukF*) forward and reverse primers
- 2 % Agarose Gel
  - 4g agarose
  - 200 ml TBE buffer
  - microwave to boil
  - 20 µL SYBR safe DNA gel stain

Procedure:

DNA Isolation

1. Culture bacterial isolates on TSA plates, incubate 37° C overnight.
2. Suspend one overnight colony in TSB, incubate 37° C overnight.
3. Transfer 1 mL to sterile 1.7 mL microcentrifuge tube.
4. Centrifuge 14000g, 4° C for 5 min.
5. Decant supernatant, add 500 µL sterile PBS, suspend pellet by vortexing.
6. Centrifuge.
7. Decant supernatant, suspend bacterial pellet in 500 µL sterile dd $H_2O$, vortex well, short spin.
8. Secure lid of microcentrifuge tube with cap, then boil bacterial pellet with the heat block at 105° C for 20 min.
9. After boiling, centrifuge.
10. Take OD readings using Nanodrop.
11. Store at -20° C.

PCR Master Mix 1x (µL)

| | |
|---|---|
| 5x Buffer | 10 |
| dNTP's (25 mM) | 4 |
| Forward Primer (5 µM) | 2 |
| Reverse Primer (5 µM) | 2 |
| $MgCl_2$ (25 µM) | 2 |
| Taq – Promega | 0.25 |
| $H_2O$ | 24.75 |
| Total Volume | 45 |

PCR Program

| | | |
|---|---|---|
| Step 1 | 94 ° C | 3 min |
| Step 2 (30x) | 94 ° C | 30 sec |
| | 54 ° C | 30 sec |
| | 72 ° C | 1 min |
| Step 3 | 72 ° C | 5 min |
| Step 4 | 4 ° C | ∞ |

1. Aliquot 45 µL master mix per sample into PCR tubes.
2. Add 5 µL DNA.
3. Run PCR.

Gel electrophoresis

1. Use freshly made 2% agarose gel.
2. Mix 5 µL loading dye with samples.
3. Add 40 µL sample per well.
4. Add 5 µL 100 bp DNA ladder.
5. Run gel around 100 v.

**FACS Staining**

Purpose: To identify cell types by FACS.

Reagents:
Primary antibody
Secondary antibody
FACS Wash (PBS + 2% FCS + .05% NaAz)
1% Formaldehyde in PBS
20 mL PBS
0.55mL of 36.5% Formaldehyde

Procedure:

1. Add 100 µL of $10^6$ cells per well in a 96 well plate
2. Centrifuge at 1200 rpm, 3 min, 4° C. Flick out liquid.
3. Resuspend in 50 µL of FACS wash (unstained control) or 50 µL of primary antibody.
4. Incubate for 1 hour at 4°C.
5. Centrifuge. Flick out liquid.
6. Wash 3x with 200 µL of FACS wash.
7. Add secondary antibody in 50 µL.
8. Incubate for 30 minutes at 4° C.
9. Wash 3x in FACS wash.
10. Resuspend in 1% PBS formaldehyde.
11. Analyze by flow.

**Isolation of Porcine Splenocytes**

Purpose: To isolate cells from spleen for use as positive control in flow cytometry.

Reagents:
0.3% EDTA in PBS (PBSE)
ACK lysis buffer
"old media" – media that is no longer good for use in culture, but contains FBS

Procedure:

1. Collect spleen tissue in 50 mL tube containing PBSE; keep on ice.
2. Mash through a cheesecloth over a funnel into another 50 mL tube using a 5 cc plunger – top off tube with PBSE.
    a. May use scissors to help cut tissue.
3. Centrifuge at 1000 rpm for 25 min at 15° C.
4. Carefully decant supernatant.
5. Add 5-10 mL ACK lysis buffer.
6. Incubate for 5-10 min at 37° C.
7. Top off tube with old media.
8. Centrifuge at 1000 rpm for 10 min, 15° C.
9. Resuspend in 5 mL PBSE, take an aliquot for counting, centrifuge.
10. Resuspend in FACS wash for flow cytometry.

**Isolation of Primary Porcine Lung Epithelia**

Purpose: To isolate primary porcine lung epithelial cells for growth in culture.

Reagents:
Balanced Salt Solution (BSS) pH 7.4
137 mM NaCl
5 mM KCl
0.7 mM $Na_2HPO_4$
10 mM HEPES
5.5 mM glucose
PBSE + Gentamicin
Small airway growth medium (SAGM)
Fibronectin (2 ug/$cm^2$) and collagen (8 ug/$cm^2$) solution
6-well transwell inserts
Coat overnight with fibronectin and collagen prior to isolation and store at 4° C
5% DMEM/F12

Trypsin/Elastase Digestion:
150 mg trypsin type I
0.641 mg elastase
30 ml BSS

Procedure:
1. Remove pulmonary lobe and place in sterile beaker containing sterile COLD PBSE with gentamicin and transport back to lab on ice.
2. In sterile hood, remove visible bronchioles and chop lung tissue into pieces of 0.6 mm thickness.
3. Wash 3 times with BSS (balanced salt solution) over 100 μm nylon gauze.
**(preincubate at 4°C for 1.5 h prior to enzymatic digestion)
4. Incubate tissue with Trypsin – elastase digestion 37°C, 30 min
**(Mince partially digested tissue in 40% FBS in DMEM/F12 medium)
5. Titurate for 5 minutes by pipetting slowly up and down.
6. Filter cell suspension through 40 μm cell strainer.
7. Incubate filtrate with a 5% DMEM/F12 on tissue culture treated plastic petri dishes in humidified incubator for 90 minutes to let macrophages attach onto plastic surface.
8. Transfer non-adhered cells and supernatant onto transwell inserts coated with fibronectin (2 ug/$cm^2$) and collagen (8 ug/$cm^2$) suspended in SAGM with 1% FBS at $8 \times 10^5$ cells/$cm^2$.
9. Change media in transwell at 24 hours (rinse transwell with HBSS to remove as much debris/RBC as possible).

**Alternative options**:
Alternative digestion protocol (used for BMEC)
a. Trypsin – collagenase solution for 15-20 minutes at 37°C.

b. Remove, spin, resuspend pellet in media, reapply solution to tissue for another 15 min, continue to step 5 with tissue.

Before step 8 clean cells on percoll gradient:

1. Layer non-adherent on Percoll density gradient and centrifuge at 25 × g for 20 minutes.
2. Collect cell layer at the interface of the two gradients and wash 4x with BSS to remove Percoll.