Immunomodulatory effects of porcine interleukin-18 on a modified live vaccine immune response against swine influenza virus

Matthew Allan Kappes

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Immunomodulatory effects of porcine interleukin-18 on a modified live vaccine immune response against swine influenza virus

by

Matthew Allan Kappes

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
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Iowa State University
Ames, Iowa
2009
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<th>Description</th>
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<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
<td>Interkeukin-1β converting enzyme (caspase-1)</td>
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<tr>
<td>ANT3</td>
<td>Adenine nucleotide translocator 3</td>
<td>Interferon consensus sequence-binding protein</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein–1</td>
<td>Interferon α/β (Type I interferons)</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
<td>Interferon-γ (Type II interferon)</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell mediated immunity</td>
<td></td>
</tr>
<tr>
<td>CPSF</td>
<td>Cleavage and polyadenylation specificity factor</td>
<td></td>
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<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
<td>Interleukin – 12</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive [CD4+CD8+]</td>
<td>IL-18 binding protein</td>
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<td>FMDV</td>
<td>Foot-and mouth disease virus</td>
<td>IL-18 receptor complex</td>
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<td>F/T</td>
<td>Freeze / Thaw</td>
<td>Intranasal</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
<td>Matrix</td>
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<tr>
<td>huIL-18</td>
<td>Human IL-18</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
<td></td>
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<tr>
<td>MDA-5</td>
<td>Melanoma differentiation-associated gene 5</td>
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<td>Abbreviation</td>
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<tr>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MLV</td>
<td>Modified live virus</td>
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<tr>
<td>NA</td>
<td>Neuraminidase</td>
<td></td>
</tr>
<tr>
<td>NADC</td>
<td>National Animal Disease Center</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
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<td>NLRP3</td>
<td>NLR family, pyrin domain containing 3</td>
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</tr>
<tr>
<td>NS2</td>
<td>Non-structural 2</td>
<td></td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear export protein</td>
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<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
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<tr>
<td>PA</td>
<td>Polymerase acidic</td>
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<tr>
<td>p.c.</td>
<td>Post challenge</td>
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<td>PAM</td>
<td>Pathogen-associated molecular patterns</td>
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<td>PB1</td>
<td>Polymerase basic 1</td>
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<tr>
<td>PB2</td>
<td>Post inoculation</td>
<td></td>
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<tr>
<td>rIL-18</td>
<td>Recombinant IL-18</td>
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<tr>
<td>PKR</td>
<td>Protein kinase RNA-regulated</td>
<td></td>
</tr>
<tr>
<td>PRDC</td>
<td>Porcine respiratory disease</td>
<td></td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
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<tr>
<td>rpIL-12</td>
<td>Recombinant porcine IL-12</td>
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<tr>
<td>rpIL-18</td>
<td>Recombinant porcine IL-18</td>
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<tr>
<td>RIG-I</td>
<td>Retinoic-acid-inducible gene I</td>
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<td>SIV</td>
<td>Swine influenza virus</td>
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<tr>
<td>SP</td>
<td>Single positive [CD4+ or CD8+]</td>
<td></td>
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<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Tissue culture infectious dose 50%</td>
<td></td>
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<tr>
<td>T&lt;sub&gt;H1&lt;/sub&gt;</td>
<td>T helper 1</td>
<td></td>
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<td>T&lt;sub&gt;H2&lt;/sub&gt;</td>
<td>T helper 2</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
<td>VDAC1</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
<td>vRNP</td>
</tr>
<tr>
<td>TRAF-6</td>
<td>TNFR-associated factor-6</td>
<td>WHO</td>
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I would like to sincerely thank Dr. Marcus Kehrli for the opportunity he has given me; for his continual leadership, patience, and enthusiasm to teach. I would also like to thank my mentors Drs. James Roth, Amy Vincent, Kelly Lager, and Mark Ackermann for their help, dedication, and patience over the years. Without your guidance and support, your willingness to teach, and your dedication to the development of young researchers such as myself, this thesis would not have been possible. I have been blessed to have so many wonderful mentors in my graduate education. To Michelle Harland and Dr. David D. Michael, I could not thank you enough; your efforts and advice have been invaluable in my education. I wish to also thank Dr. Wenjun Ma, Dr. Ratree Platt, Dr. Kay Faaberg, Dr. Janice Ciacci-Zanella, Dr. Eraldo Zanella, Dr. Laura Miller, Dr. Crystal Loving, Sarah Pohl, Deborah Adolphson, Ann Vorwald, Brian Brunelle, and Robert Schaut for their assistance and advice. To my family and friends for their continual support and encouragement, thank you.
ABSTRACT

Current swine influenza vaccines fail to protect against the broad range of strains circulating within the United States. To induce effective broad cross-protection against influenza, strong humoral and cell-mediated immune responses are needed. However, animals are often vaccinated at a young age when they exhibit a host of dysregulated or insufficient immune responses including biased T cell polarization and immature inflammatory responses. Porcine neonates have diminished expression of IL-18 in their respiratory tract, a major site of infection in young animals. Initially named interferon-γ inducing factor (IGIF), IL-18 is most widely known for its potent ability to up-regulate expression of interferon-γ (IFN-γ), resulting in a phenotypic T\textsubscript{H}1 driven T cell polarization.

For many infections, such as influenza, deficiencies in IL-18 expression have been shown to result in deleterious effects on the development of protective immune responses. Insufficient expression of IL-18 during neonatal development in swine may result in a reduced expansion of a T\textsubscript{H}1 immune response and may result in improper immunological polarization in response to vaccination. Based on findings by Kim et al. (2001), two amino acid conversions were introduced (E41A & K88A) to porcine IL-18 by PCR primer site directed mutagenesis. Synonymous mutations to human IL-18 (huIL-18) by Kim et al. (2001, 2002) resulted in 4-fold increases in biological activity and extended half-life. Replication-defective adenoviruses expressing either the wild type porcine IL-18 or the mutated form was generated. Additionally, to create a succinct swine influenza modified live virus (MLV) vaccine expression rIL-18 protein, the mutated form of IL-18 (mutIL-18) was inserted into the truncated region of the NS1Δ126 gene of an attenuated H3N2 modified live swine
influenza virus. The properties of biological expression and biological activity of both wild type IL-18 and mutated IL-18 were assessed *in vitro* and *in vivo*. Replication-defective adenovirus expression vectors were confirmed to express high levels (>10ng/mL) of wild type and mutated forms of IL-18. The rIL-18 expressing constructs (MLV+mutIL-18, MLV+Ad5wtIL-18, and MLV+Ad5mutIL-18) were administered as single dose influenza vaccines. The immunomodulatory effects of wild type and mutated forms of IL-18 were evaluated for their efficacy to enhance the immune protection afforded by the MLV vaccine alone in response to heterosubtypic influenza challenge (H1N1). The MLV+Ad5mutIL-18 vaccination group resulted in significantly higher antibody titers post challenge compared to the MLV alone. Additionally, both groups receiving either mutIL-18 construct (MLV+mutIL-18, MLV+Ad5mutIL-18) exhibited significantly lower viral shedding post challenge, lower viral replication in the lungs, and reduced microscopic lung lesion scores at time of necropsy. Flow cytometric analysis of circulating lymphocyte populations revealed significant differences in cell populations between IL-18/non-IL-18 groups and adenovirus and non-adenovirus groups. CD4+CD8+ double positive (DP) and γ/δ T cells were the main cell populations activated upon influenza vaccination and heterosubtypic challenge. Results indicate that, along with rIL-18 administration, the type of expression vector (adenovirus or influenza) plays a significant role in determining cytokine expression and responding T cell populations.
CHAPTER 1 INTRODUCTION

1. General Introduction

Young animals are known to exhibit impaired or insufficient immune functions such as decreased production of inflammatory mediators and an impaired ability to formulate a phenotypic T\textsubscript{H}1 based T cell response (Sarzotti et al., 1996; Siegrist, 2001; Suen et al., 1998). The immunologically altered state of young animals makes them particularly vulnerable to developing insufficient or unprotective immune responses following infection or vaccination. Specifically, pigs have been shown to exhibit a significant reduction in interleukin (IL)-18 expression in the mucosal epithelium for months after birth (Muneta et al., 2002). In that study, the mucosal epithelial cells were additionally unable to up-regulate interferon-\(\gamma\) (IFN-\(\gamma\)) expression upon concanavalin A (Con A) stimulation. However, treatment of the same cells by exogenous IL-18 resulted in IFN-\(\gamma\) production. Therefore, the administration of exogenous cytokines may help restore correct immune function and, in conjunction with vaccines, may help generate enhanced immune responses.

Influenza is an acute respiratory illness often accompanied with secondary bacterial infections in severe cases (Brundage, 2006). Swine influenza is a common component contributing to the porcine respiratory disease complex (PRDC) (Baskerville, 1981) and was ranked the second leading cause of productivity losses by swine producers (Holtkamp et al., 2007). To protect against a broad range of influenza viruses, next generation influenza vaccines will need to elicit a strong adaptive immune responses comprising both cell mediated and humoral immune elements. While pre-existing antibody is the only known
mechanism to achieve sterilizing immunity to influenza (Gerhard, 2001), mutations (drift) and reassortment events (shift) result in swiftly changing antibody recognized epitopes. Inversely, T helper and cytotoxic T cells typically recognize more static internal influenza proteins possessing conserved epitopes (Doherty et al., 1997; Doherty et al., 2006; Flynn et al., 1999; Thomas et al., 2006). Recovery from established influenza infection is primarily mediated by cellular immune responses (Graham and Braciale, 1997; Topham et al., 1996). Studies with IL-18 deficient (IL-18 −/−) transgenic mice have demonstrated the need for adequate IL-18 expression for normal recovery from influenza infection. IL-18 −/− transgenic mice infected with influenza A virus resulted in (a) a higher rate of viral replication, (b) significantly elevated viral titers, (c) significantly lower IFN-γ production, and (d) lower activity levels of NK cell-mediated cytolysis (Billaut-Mulot et al., 2000; Denton et al., 2007; Dinarello, 1999; Foss et al., 2001; Liu et al., 2004; Takeda et al., 1998). Further research showed IL-18 is required for optimal production of cytokines IFN-γ, TNFα, and IL-2 from CD8+ T cells during the course of influenza infection (Denton et al., 2007). Consequently, administration of IL-18 in conjunction with influenza vaccination may result in a more robust CMI response conveying stronger protective immunity.

Cytokine administration for therapeutic immunomodulation has been able to restore correct immune function (Pertmer et al., 2001; Ridge et al., 1996; Siegrist, 2001) or enhance protective immunity (Zuckermann et al., 1998). However, a short half-life in vivo is a particular problem concerning the use of recombinant cytokine protein for therapeutic purposes. To evaluate the immunomodulatory effects of recombinant porcine IL-18 in conjunction with vaccine administration, IL-18 viral expression vectors were generated from a replication-defective adenovirus and a modified live swine influenza virus. Furthermore, to
generate an enhanced immunomodulatory agent, two amino acid conversions were introduced to wild type porcine IL-18 (E41A & K88A). Mutations were completed by PCR site directed mutagenesis, with which two charged amino acids critical for binding to the inhibitory IL-18 binding protein (IL-18BP) were converted to alanine (Figure 1.1). Previous research introducing synonymous mutations to human IL-18 (huIL-18) resulted in enhanced biological activity and extended half-life (Kim et al., 2001).

Properties of biological expression and biological activity of recombinant IL-18 (rIL-18) were evaluated both in vitro and in vivo. To assess the immunomodulatory effect of rIL-18 on the immune response to influenza vaccination, both wtIL-18 and mutIL-18 expression vectors were administered in conjunction with a previously described modified live swine influenza virus vaccine (Tx98NS1Δ126) (Richt et al., 2006; Solorzano et al., 2005). Vaccination groups were evaluated in response to heterosubtypic challenge [H1N1 IA04]. Flow cytometric analysis post vaccination, pre challenge, and post challenge identified CD4+, CD8+, and γ/δ T cell populations expressing CD25, IFN-γ, and IL-10 [as activation, Th1, and Th2 markers respectively]. Results indicate the co-administration of rIL-18 with the modified live influenza virus vaccine conveys a stronger level of protection against heterosubtypic challenge compared to the modified live influenza vaccine alone.

2. Thesis Organization

This thesis is organized in the alternative format. In the first chapter, after the general introduction, a review of pertinent literature is presented. Chapters two and three are the authors own work formatted for publication in Veterinary Immunology and
**Immunopathology.** General conclusions are discussed in Chapter 4. Citations are displayed at the end of each chapter in separate bibliographies.

The main author contributed to the development and planning of research, conducted experiments, evaluated data, and authored the primary manuscript. Dr. Wen-jun Ma generated the mutated IL-18 cDNA construct and the influenza MLV containing the mutated IL-18 cDNA construct. Dr. Jürgen Richt assisted in the generation of the mutated IL-18 cDNA construct and the generation of the influenza MLV containing the mutated IL-18 cDNA construct. Drs. Amy Vincent and Kelly Lager aided in the planning and execution of experiments. Dr. James Roth contributed to the planning and execution of experiments and to the writing of the manuscript. Dr. Ratree Platt conducted the flow cytometry CMI study and evaluated the flow cytometry data. Dr. Michael Murtaugh provided the wild type porcine IL-18 cDNA plasmid. Dr. Marcus Kehrli conceived of the experimental concept, contributed to the planning and execution of experiments, aided in the evaluation of data, and to writing of the manuscript.

### 3. Literature Review

#### 3.1 Interleukin-18

**Properties of IL-18**

IL–18 was first identified as a soluble protein from the sera of mice pretreated with *Propionibacterium acnes* which markedly increased interferon-γ (IFN-γ) protein production in resting splenic nonadherent cells upon administration (Nakamura et al., 1993). Since the
initial discovery, IL-18 has been cloned and characterized from many species including mice (Okamura et al., 1995), humans (Ushio et al., 1996), and swine (Muneta et al., 2000). Currently, the full genetic sequence of IL-18 has been identified from at least 14 species (NIH, 2009). IL-18 is secreted by a variety of cells including macrophages, dendritic cells, T cells, neutrophils, Kupffer cells, and epithelial cells (Akira, 2000; Fortin et al., 2009; Nakanishi et al., 2001; Stoll et al., 1998). IL-18 expression can be activated by TLR signaling (Akira et al., 2001), induced by IFN-γ via ICSBP (interferon consensus sequence-binding protein) and AP-1 (activator protein-1) pathways (Kim et al., 2000), by inflammasome activation (Stasakova et al., 2005), or by autocrine signaling through the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway (Fortin et al., 2009). In the presence of IL-12, IL-18 induces strong Th1 type immune responses (Denton et al., 2007; Liu et al., 2004; Nakamura et al., 1993; Takeda et al., 1998), primarily through the induction of IFN-γ expression by T cells and natural killer cells (Dinarello, 1999; Yoshimoto et al., 1997). As a potent inducer of IFN-γ expression, IL-18 is characterized as a member of the Th1-inducing cytokine family along with IFN-γ, IL-2, IL-12, and IL-15 (Dinarello, 1999).

IL-18 is expressed as a biologically inactive precursor protein. The 192 amino acid pro protein (24kDa) lacking an exportation signal sequence is stored in intracellular vesicles until activation. Upon stimulation of the inflammasome complex, IL-1β cleaving enzyme (caspase-1 or ICE) is cleaved into the active form. Mature caspace-1 is than able to cleave pro-IL-18 at the Asp-X site of amino acid position 35. Cleavage at position 35 results in a biologically active 18kD mature IL-18 protein (Ghayur et al., 1997; Gu et al., 1997; NIH,
2009). Cleavage by caspase-3 at Asp71-Ser72 or Asp76-Asn77 on either pro or active IL-18 protein results in a biologically inactive protein (Akita et al., 1997). IL-18 neutrophil stimulation activates cleavage of both pro and active IL-18 forms into a variety of products, possibly by serine proteases elastase and cathepsin G (Gracie et al., 2003), but the biological significance of those products is yet unknown.

IL-18 activity results from the signaling cascades activated from binding to the IL-18 receptor complex (IL-18R). IL-18R is 60-100kD in size and shares 35% sequence identity with IL-1RI (Dinarello, 1999; Kojima et al., 1998). IL-18R is comprised of an IL-18R binding chain α (IL18R1 or IL1Rrp) and a IL18Rβ signaling chain (formerly IL18RAP or IL1AcPL) (Dinarello, 1999). Affinity of IL-18 for the IL-18Rα chain is reported at a (K_d) of 25nM, which is a relatively low for the known picomolar activity range of IL-18 (Thomassen et al., 1998). However, after IL-18 binds to IL-18Rα, the IL18Rβ signal chain is quickly recruited to form a high affinity heterodimeric complex, able to activate downstream intracellular signaling cascades (Dinarello, 1999). Amino acid residues Lys79, Lys84, and Asp98 on huIL-18 were found to be biologically important for the induction of IFN-γ even though these residues are not involved in IL-18Rα receptor binding (Kato et al., 2003).

Further assessment identified the residues as important amino acids for the binding of IL-18Rβ to the IL-18/IL-18Rα complex, though IL-18β could not bind to IL-18 or the IL-18Rα alone (Kato et al., 2003). These results indicate the IL-18Rα and IL-18Rβ chains bind to different locations on the IL-18 protein, but interact to cause a change in ligand affinity as the heteromeric complex is formed.
Once bound to IL-18, the IL-18R complex signals through the IRAK (IL-1R-activating Kinase) (Dinarello, 1999; Kojima et al., 1998), TRAF-6 (TNFR-associated factor-6), NF-κB (Kojima et al., 1998), MyD88 (Adachi et al., 1998), and p38 mitogen activated protein kinase (MAPK) pathways (Fortin et al., 2009; Gracie et al., 2003). Most notably, IL-18 signaling up-regulates the expression of IFN-γ, but also results in activation of a variety of other antiviral, antimicrobial, and antifungal associated immune functions (Akira, 2000; Biet et al., 2002; Fortin et al., 2009; Foss et al., 2001; Liu et al., 2004; Pirhonen et al., 1999; Zhang et al., 1997). Specifically, IL-18 activates T cells to synthesize IFN-γ, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor–α (TNFα), IL-1β, and IL-8 (Dinarello, 1999; Kohno et al., 1997; Puren et al., 1998; Ushio et al., 1996). IL-18 also potentiates the expression of Fas ligand (Ohtsuki et al., 1997) resulting in enhanced NK and CTL cell cytotoxicity and apoptotic killing of infected cells (Akira, 2000).

**IL18 and IL12 act synergistically to induce T_{H1} immune responses**

Both IL-12 and IL-18 are characterized as prototypical T_{H1} cytokines for their potent abilities to up-regulate IFN-γ protein expression. In many models, research has shown IL-12 or IL-18 cytokines produce negligible quantities of IFN-γ protein individually, but act synergistically to induce high levels of IFN-γ production when administered together (Denton et al., 2007; Dinarello, 1999; Kohno et al., 1997; Liu et al., 2004; Robinson et al., 1997; Takeda et al., 1998; Yoshimoto et al., 1998). However, IL-12/IL-18 co-induction may
not always be needed to elicit high quantities of IFN-γ. Kehrli (2003) was able to induce high IFN-γ concentrations utilizing IL-18 treatments alone in a bovine model (Kehrli, 2003).

IL-12 acts not only to activate the IFN-γ pathway via STAT3/STAT4 signaling but also acts to up-regulate IL-18R on primed T cells (Yoshimoto et al., 1998). As the inhibitory IL-18BP competes with IL-18R for IL-18 binding, up-regulating IL-18R on primed T cells results in an increased sensitivity to IL-18 and thus stronger induction of IFN-γ expression. On anti-CD40 primed B-cells, IL-18 in the presence of IL-12 induces production of IFN-γ and inhibits IgE and IgG1 subclass production (Yoshimoto et al., 1997). IL-18/IL-12 co-administration has also been shown to induce subclass switching to the complement activating IgG2 isotype (Moran et al., 1999).

IL-18 and IL-12 act to up-regulate IFN-γ expression by different signaling pathways. IL-18 activates the IFN-γ promoter at the AP-1 site, whereas IL-12 acts at a STAT4 initiation site (Dinarello, 1999). AP-1 activation via IL-18 signaling is alone sufficient for IFN-γ transcription, while STAT4 signaling needs a secondary signal such as anti-CD3 or anti-CD28 (Dinarello, 1999). When IL-18 and IL-12 are present, both activation sites are stimulated, a mechanism by which IL-18/IL-12 co-induction acts synergistically to enhance IFN-γ expression.

**Interleukin-18 binding protein**

IL-18BP was first isolated as a protein product binding to an IL-18-agarose column from the urine of healthy subjects (Novick et al., 1999). One of the major regulatory elements of IL-18 signaling, IL-18BP competes with IL-18R to bind IL-18 protein. IL-18BP
abolishes biological activity of IL-18 upon binding (Novick et al., 1999). In COS7 cells, rIL-18BP treatment abolished IL-18 inducted expression of IFN-γ and IL-8 (Novick et al., 1999).

**Figure 1.1 Chemical structure comparison of mutated IL-18 amino acid conversions**

![Chemical structure comparison of mutated IL-18 amino acid conversions](image)

- **Human IL-18**: AA 42
- **Porcine IL-18**: AA 41

**Figure 1.1.** PCR primer site directed mutagenesis was utilized to introduce two amino acid conversions, E41A & K88A, into wild type porcine IL-18. Mutations are synonymous to mutations introduced to human IL-18 (E42A & K89A) by Kim et al. (2001).

IL-18BP is not a variant of the IL-18R and shares no significant homology with the IL-18R, making IL-18BP unique among soluble cytokine receptors (Novick et al., 1999).

Most soluble cytokine receptors are variants of the cell surface receptor lacking the transmembrane domain. Conversely, IL-18BP shares a unique identity to a family of secreted proteins belonging to the immunoglobulin superfamily (Novick et al., 1999). Because of the differences between IL-18R and IL-18BP, mutations introduce to the IL-18/IL-18BP interface were able to abolish IL-18BP binding and enhance biological activity.
of huIL-18 (Kim et al., 2002; Kim et al., 2001). Research showed site-specific mutations altering charged residues E42A or K89A (Figure 1.1) of recombinant human IL-18 (rhuIL-18) resulted in a 2-fold increase in biological activity. A E42A / K89A double mutant additionally exhibited a 4-fold increase in biological activity and was rendered fully resistant to IL-18BP neutralization (Kim et al., 2001).

3.2 Unique attributes of the porcine immune system

The porcine immune system is unique compared to most other species. Porcine lymph nodes exhibit an inverted lymph node structure compared to most other animals, as the medulla is external to the cortex (Binns, 1982). The recirculation of lymphocytes directly entering into the blood from the lymph node capillaries instead of efferent lymph node tubules is also unique (Carr et al., 1994; Nayeem et al., 1997; Pescovitz et al., 1994; Yang and Parkhouse, 1996). Additionally, the porcine immune system possesses unusually high \(\gamma/\delta\) and CD4\(^+\)CD8\(^+\) double-positive (DP) T cell populations (Carr et al., 1994; Nayeem et al., 1997; Pescovitz et al., 1994; Yang and Parkhouse, 1996). While the use of swine as a research model has greatly increased in recent years due to the interest in swine-to-human xenograft transplantation, their similarities to human physiology, and the elevated populations of \(\gamma/\delta\) and CD4\(^+\)CD8\(^+\) DP T cells; characterization of the porcine immune system, is lacking in comparison to human and murine models.
T-cell populations

T cells can be categorized partially based upon αβ and γδ T cell receptors (TCR). αβ-TCR\(^+\) T cells can be loosely divided into cytotoxic verses T helper categories, and T-helper cells further into \(T_H^1\), \(T_H^2\), and \(T_H^{17}\). NK T and regulatory T cells have also been identified but are poorly understood. γδ T cells are a T cell subclass for which mechanisms and functions are still incompletely understood. γδ T cells are believed to play a role in a variety of immune functions including T cell regulation, immunosurveillance, T cell tolerance, antigen-dependent and antigen-independent detection of pathogens, T helper like function, cytotoxic effects, APC like activation of B cells, antibody class switching, and are largely associated with the mucosal immune system (Fujihashi et al., 1996; Hayday et al., 2000; Horner et al., 1995; King et al., 1999; Rivas et al., 1989; Takamatsu et al., 2006; Wen et al., 1998).

Neonatal pigs have at least four α/β T cell populations in [CD4\(^-\)CD8\(^-\), CD4\(^+\)CD8\(^{lo}\), CD4\(^+\)CD8\(^{lo}\), CD4\(^-\)CD8\(^{hi}\)] (Yang and Parkhouse, 1996). Characteristically, single positive (SP) T cells CD4\(^+\) (CD4\(^+\)CD8\(^-\)) and CD8\(^+\) (CD4\(^-\)CD8\(^{hi}\)) are largely categorized based upon their expression of cytokines and cellular functions.

γδ T cells were formerly classified as ‘null’ cells due to their lack of conventional T or B cell markers. Subsets are not well defined but have been suggested to include CD2\(^-\)CD4\(^-\)CD8\(^-\), CD2\(^+\)CD4\(^-\)CD8\(^{lo}\), and CD2\(^+\)CD4\(^-\)CD8\(^-\) (Takamatsu et al., 2006; Yang and Parkhouse, 1996). At least 12 γδ TCR\(^+\) thymocyte subclasses have been identified in prenatal and postnatal swine based on cell surface markers (Sinkora et al., 2005), some describing different developmental stages of the γδ TCR\(^+\) thymocyte.
γ/δ T cells are found in high numbers in peripheral blood and lymphoid tissues of sheep, cattle, and pigs (Nayeem et al., 1997; Takamatsu et al., 2006; Thome et al., 1993; Yang and Parkhouse, 1996) and preferentially home to extra-lymphoid epithelial-associated tissues (Nayeem et al., 1997). They are known to be able to recognize target antigens in an MHC independent manner (Takamatsu et al., 2006), which may allow them to respond in a shorter period following infection. γ/δ T cells are involved in the innate and acquired immune functions at the mucosal surface. A considerable deficiency in neutrophil infiltration to the lung and marked reduction in IgA production is reported in γ/δ-deficient transgenic mice (Fujihashi et al., 1996; King et al., 1999). Moreover, subsets of γ/δ T cell have been reported to conform to the Th1/Th2 paradigm in both cytokine production and function (Wen et al., 1998). Additionally, γ/δ T cells possess cytotoxic effects [CD3⁺CD4⁻ CD8⁻] in an antigen specific manner (Rivas et al., 1989), and aid in B cell stimulation and antibody isotype switching (Horner et al., 1995).

γ/δ T cells are the predominant TCR⁺ cell class in young pigs, found in high numbers both in the peripheral blood and in lymphoid tissues (Holtmeier et al., 2004; Yang and Parkhouse, 1996). The elevated prevalence of CD4⁺CD8⁺ T cells in the resting peripheral T cell population is unique to ungulates (Pescovitz et al., 1994). γ/δ T cells are most abundant at neonatal age in swine and reduce as the pig reaches adulthood (Nayeem et al., 1997). Unlike γ/δ T cells, porcine CD4⁺CD8⁺ DP T cell numbers increase with age and have been identified as a major class of memory T cells within the pig (Pescovitz et al., 1994).
Deficiencies of the neonatal immune system

Neonatal animals are particularly vulnerable to pathogens due to their immunologically naïve state. The neonatal immune system is known to display a host of dysregulated or insufficient immune functions including inflammatory, innate, and Th1/Th2 T cell polarization responses (Forsthuber et al., 1996; Pertmer et al., 2001; Ridge et al., 1996; Sarzotti et al., 1996). Neonates exhibit an impaired ability to formulate a phenotypic T\textsubscript{H}1 immune response, but the exact mechanism(s) is yet unknown (Forsthuber et al., 1996; Pertmer et al., 2001; Ridge et al., 1996; Sarzotti et al., 1996; Siegrist, 2001; Siegrist et al., 1998; Suen et al., 1998). IL-18 expression in porcine neonates is markedly diminished at the mucosal surfaces in young piglets (Muneta et al., 2002). As IL-12 in the absence of IL-18 is a poor inducer of T\textsubscript{H}1 T cell development, insufficient constitutive expression of IL-18 during the neonatal development period may contribute to the reduced induction of a T\textsubscript{H}1 based immune response in swine neonates. Due to the deficiencies of the neonatal immune system, infections and vaccinations that occur within the first stages of life may result in insufficient or unprotective acquired immune responses.

3.4 Overview of swine influenza

General background of swine influenza

The influenza type A virus is an enveloped negative stranded RNA virus composed of eight genomic segments. Influenza belongs to the Orthomyxoviridae viral family and is 80-120nm in size (Heinen, 2003). Classification of influenza A viruses are based upon two antigenically important glycoproteins, hemagglutinin and neuraminidase. Currently there
have been 16 hemagglutinin and 9 neuraminidase subtypes identified (Fouchier et al., 2005; Olsen et al., 2006a; Skehel, 2009; Webster et al., 1992). In humans, influenza infection is typically characterized as an upper respiratory infection resulting in high fever, myalgia, headache, non-productive cough, sore throat, and rhinitis (WHO, 2003), with similar symptoms in swine. Influenza virus is transmitted by large droplet aerosols like coughing or sneezing or by direct transmission via physical contact with a contaminated surface or infected individual. In the United States, the WHO estimates direct and indirect losses due to seasonal influenza infection in humans to be between $71-$167 billion annually (WHO, 2003). Worldwide, it is estimated that 3 to 5 million severe influenza infections will develop annually resulting in 250,000 to 500,000 deaths (WHO, 2003). Infected individuals will typically be contagious from one or more days prior to onset of symptoms until 5-7 days after onset (Prevention, 2007). Four pandemics have occurred within the past century dating back to the 1918 H1N1 Spanish flu pandemic, which is believed to have caused up to 50 million deaths (de Wit and Fouchier, 2008). Pandemics resulted from introductions of the H2N2 subtype in the 1957 Asian flu pandemic and H3N2 subtype in the 1968 ‘Hong Kong’ flu pandemic (de Wit and Fouchier, 2008; Keen, 1995) and a novel H1N1 swine like influenza virus in 2009 (WHO, 2009).

Influenza epidemics and pandemics in humans have been noted for centuries (Anon., 1890) but had not been correctly attributed to any specific etiology until first isolated from samples of infected swine in 1931 (Shope, 1931b). Swine influenza was initially documented clinically during the 1918 pandemic and has been circulating in the swine population since that time (Koen, 1919; Shope, 1931a).
In the swine industry, seasonal swine influenza infection was ranked the second leading cause of productivity losses by swine producers (Holtkamp et al., 2007). Close relationships exist between swine and human influenza viruses. Introduction of human influenza viruses into the swine population dates back to at least the 1918 pandemic where it is believed the human H1N1 virus crossed over into swine (Reid et al., 1999; Shope, 1931a). At least three other human introductions to swine populations have occurred since that time (Richt et al., 2003). Due to the uncommon physiology of swine respiratory epithelia, pigs are considered a potential mixing vessel capable of genetic reassortment (genetic shift) between multiple influenza lineages. Porcine upper airway epithelia have been shown to express both avian (α 2,3) and human (α 2,6) receptor linkages, enabling swine to be infected by influenza viruses from avian, human, and swine lineages (Ito et al., 1998; Ito and Kawaoka, 2000; Landolt et al., 2006; Massin et al., 2001; Neumann and Kawaoka, 2006; Stern and Tippett, 1963; Suzuki et al., 2000). Both the 1957 and 1968 human pandemic viruses contained genomic segments from an avian lineage (Kawaoka et al., 1989). However, fully avian parental viruses were never isolated from the human population during either pandemic, indicating reassortment may have occurred in an intermediate host (Webster, 1972; Zhou et al., 1999). In 1998 many novel influenza A viruses of a new subtype, H3N2, were detected as a quickly spreading infection in the United States swine herds. The novel viruses were initially characterized as double reassortant and triple reassortant genotypes composed from swine, human, and avian influenza viruses (Webby et al., 2000). Triple reassortant H3N2 viruses contained the hemagglutinin (HA), neuraminidase (NA), and polymerase basic 1 (PB1) genomic segments from human lineage; the nucleoprotein (NP), matrix (M), and non-structural (NS) segments from the classical swine influenza viruses, and polymerase basic 2
(PB2) and polymerase acidic (PA) segments from avian viruses (Webby et al., 2000; Zhou et al., 1999). Prior to the identification of the double and triple reassortant strains, the epidemiology of circulating swine influenza viruses had remained moderately constant for the previous 80 years as mostly drifting classical H1N1 strains (Olsen, 2002). In the human population, numerous sporadic human infections of (H1) triple-reassortant swine influenza viruses have been detected since 2005 (Shinde et al., 2009). In March 2009, a large-scale outbreak of a novel influenza A H1N1 triple reassortant virus, most closely related to swine influenza viruses, occurred in North America (Dawood et al., 2009; Kerr, 2009). The outbreak of the novel H1N1 virus into the naïve human population has resulted in 53,685 confirmed infections in over 70 countries and over 302 deaths resulting in a stage 6 pandemic as of June 2009 (Kerr, 2009; WHO, 2009).

**Viral replication**

The influenza A virus genome is comprised of eight segments encoding 10 to 11 known protein products; hemagglutinin (HA), neuraminidase (NA), polymerase basic 1 and 2 (PB1, PB2), polymerase acidic (PA), nucleoprotein (NP), the non-structural (NS) segment coding for non-structural 1 (NS1) and non-structural 2 or nuclear export protein (NS2 or NEP respectively) proteins, and the matrix (M) segment coding for matrix 1 (M1) and matrix 2 (M2) proteins. The 11th protein, PB1-F2, is a non-structural +1 frameshift alternative reading frame on the PB1 genomic segment. PB1-F2 is not present in all influenza A viruses and is not required for viral replication or survival. Genomic segments range from 890 to approximately 2340 base pairs in length for a total genomic size of ~14 Kb.
Initiation of viral replication begins with the association of PB1, PB2, PA and ribonucleoprotein (vRNP) to form the viral replication complex. The error-prone RNA replication complex transcribes multiple copies of the viral genome while viral RNA segments are translated by host machinery into protein products. HA, NA, and M2 protein products are transported to the cellular surface by the Golgi apparatus and become anchored the plasma membrane (Baigent and McCauley, 2003). At the start of viral packaging, genomic segments become closely associated with helical nucleoproteins resulting in vRNP complexes prior to being positioned below the membrane anchored HA, NA, and M2 proteins (Keen, 1995). The M1 protein associates around the inside of the virion prior to budding, becoming chemically bound to the vRNP segments. M2 functions as an integral membrane protein on the influenza virion, forming an ion channel necessary for membrane fusion and viral entry into the cell. Two glycoproteins HA and NA form the only protruding elements on the outside of the virion and thus compose the major antigenic elements to host viral defenses. As M1 and vRNPs aggregate below the anchored proteins the virion ‘buds’ from the surface. Sialylated progeny virions are cleaved by NA enzymatic activity and released into the extracellular space (Baigent and McCauley, 2003).

**Mechanism of infection and virulence factors of influenza virus**

Influenza viruses gain entry into the cell by receptor-mediated endocytosis facilitated by the viral surface ligand HA. HA must be cleaved by a trypsin-like protease into two segments, HA₁ and HA₂ (Hampson and Mackenzie, 2006), prior to entry into the cell. Once bound, the influenza virus is encapsulated by an endosome. The rapid drop in endosomal pH
results in a conformational change of the HA protein, initiating viral opening. HA binds to the glycosylated sialic acid receptor in either an α-2,3 (avian) or α-2,6 (human) linkage configuration (Rogers and Paulson, 1983). Sialic acid receptor linkages are a major determinant of host specificity. Due to the requirement of trypsin-like proteases for HA cleavage influenza infection is restricted to the respiratory and intestinal epithelia where trypsin like proteases are produced by a variety of cell types (Hampson and Mackenzie, 2006). The exception is highly pathogenic avian influenza (HPAI), which possess multiple basic residues at the HA cleavage site allowing trypsin independent cleavage and systemic influenza infection (de Wit and Fouchier, 2008). Antibody mediated responses to the HA glycoprotein can effectively neutralize influenza infectivity by blocking the receptor mediated entry into the host cell (Skehel, 2009). However, common antibody recognized epitopes rapidly evolve in response to immunological pressure to evade pre-existing protective antibody driven responses. Just recently, highly conserved epitopes on the HA protein have been identified which have broad neutralizing abilities (Ekiert et al., 2009; Sui et al., 2009). If proven successful across a wide range of circulating strains, neutralizing antibodies with broad cross-protection against multiple influenza subtypes would effectively diminish the impact of influenza to human and animal health.

The second major surface glycoprotein, NA, functions to cleave sialic acid motifs from the viral surface facilitating release of progeny viruses. NA sialidase activity promotes infectivity in three main ways: (a) by cleaving newly formed virions from sialic acid residues on the cell surface, (b) by preventing newly released virions from aggregating to each other, and (c) by promoting viral penetration of sialic acid rich mucin which protects the respiratory epithelium (Bhatia and Kast, 2007). To date there are nine NA subtypes identified (Olsen et
The NA protein acts as a host determinant by its substrate specificity (α2,3 or α2,6) and by pH specificity [airway versus intestinal epithelia] (Kobasa et al., 1999; Takahashi et al., 2001). The targets for NA enzymatic cleavage must match the linkages of the HA receptor specificity. In order for influenza to infect a host cell, the HA specificity must match the host sialic acid linkages (α2,3 or α2,6) expressed on the host cell surface. If the NA enzymatic cleavage does not recognize the same linkages, the virion will not be able to be removed from the host cell surface upon budding. The pH at which NA enzymatic activity is functional is a major host determinant between mammalian and avian influenza viruses. While influenza is typically an upper respiratory infection in mammalian species, influenza infection is typified as a gastrointestinal infection in avian species. The lower pH of the gut has been shown to inactivate NA function of most human and swine influenza isolates, while avian influenza viruses retained NA enzymatic activity (Takahashi et al., 2001).

Other viral proteins also play important roles in host specificity and virulence, such as the PB1, PB2, and PA proteins. The polymerase proteins PB1, PB2, and PA have all been implicated as strong influencers of host range specificity and virulence (Baigent and McCauley, 2003). Specific amino acid alterations on the PB2, PB1, and PA proteins are known to affect host specificity, virulence, and transcription efficiency (Hatta et al., 2001; Kawaoka et al., 1989; Okazaki et al., 1989). Amino acid substitutions to elements of the polymerase complex can result in viral attenuation or alteration in transcription rate in a temperature sensitive manner (Giesendorf et al., 1986; Kawaguchi et al., 2005; Murphy et al., 1997). Temperature sensitivity can play a role in host specificity and determine the
equilibrium between vRNA (+) and alternative plus-sense RNA (cRNA) transcription rates (Dalton et al., 2006).

The influenza polymerase complex aids in the evasion of host immune recognition indirectly by introducing genetic polymorphisms to produce variable progeny viruses, an event referred to as genetic drift (Hampson and Mackenzie, 2006). Genetic mutations causing amino acid substitution can alter the antigenic sites of important epitopes, resulting in antigenic drift. Drifting influenza viruses are the chief cause of annual variance in circulating strains [epidemics], forcing the continual need to update influenza vaccines. Acute changes to the influenza genome are typically a product of reassortment events, known as genetic shift, resulting in a dramatically altered influenza virus. Reassortant viruses can potentially emerge when two or more differing strains infect the same cell, allowing the genomic segments to be exchanged and packaged in a variety of combinations. Reassortment events in intermediate hosts who’s physiology allows the infection of influenza viruses from multiple species, such as swine, have a greater potential to generate novel viruses exceedingly well fit to replicate in naïve populations (Webby et al., 2000). Such viruses introduced into a naïve population possessing little to no pre-existing protective immunity often results in widespread infection with elevated morbidity and mortality; as seen by the 1957 and 1968 pandemic human-avian reassortant strains (Webster, 1972; Zhou et al., 1999) and the 1998 emergence of the H3N2 double (human & swine) and triple (human, swine, & avian) reassortant viruses in the US swine population (Olsen, 2002; Olsen et al., 2006b; Richt et al., 2003; Webby et al., 2000). Whole influenza viruses can directly cross the species barrier, but such events are not commonly detected.
Influenza encodes two non-structural proteins, NS1 and PB1-F2, capable of regulating host cellular functions. PB1-F2 acts to induce cell death by permeating the mitochondrial membrane (Chanturiya et al., 2004; Chen et al., 2001). PB1-F2 is encoded by a +1 frameshift alternative open reading frame on the PB1 genomic segment encoding for a conserved 87 amino acid protein (Chanturiya et al., 2004; Chen et al., 2001). PB1-F2 preferentially locates to the mitochondria where it permeates the membrane to monovalent cations, chloride, and divalent ions (Chanturiya et al., 2004). The diffusion of ions and chloride results in a loss of membrane potential and the release of cytochrome c (Zamarin et al., 2005). Protein-protein interactions between PB1-F2 and adenine nucleotide translocator 3 (ANT3) or mitochondrial membrane voltage-dependent anion channel 1 (VDAC1) are believed to sensitize the cell to apoptotic signaling (Zamarin et al., 2005). PB1-F2 expression is not essential to viral infectivity or replication. Some influenza viruses do not encode a functioning PB1-F2 product, particularly in older swine isolates (Chen et al., 2001). However, newer SIV isolates typically express the PB1-F2 fragment. PB1 is the only influenza gene with an altered Kozak sequence lacking either an A or G at the -3 position relative to the start codon, suggesting ribosomal scanning initiates translation (Chen et al., 2001).

The second and more abundant non-structural protein, NS1, is a multifunctional protein required for survival in interferon α/β (IFN-α/β) competent systems. NS1 is a splice variant of the eighth genomic segment of influenza, sharing a portion of the 5’ terminal and the 3’ poly (A) region with NS2 (Lamb and Lai, 1980). NS1 encodes a 217-278 amino acid protein with an RNA-binding N-terminal domain and a C-terminal effector domain. The NS1 protein functions in three main capacities: (1) inhibition of post-transcriptional
processing, (2) modulation of host and viral translation, and (3) suppression of host viral defenses.

NS1 utilizes both the RNA binding N-terminal and effector C-terminal domains to inhibit post-transcriptional processing of mRNA in many ways: (a) it inhibits the exportation of mRNA from the nucleus by binding the poly(A) regions of mRNA (Fortes et al., 1994; Qiu and Krug, 1994). (b) NS1 binds to the 30kD subunit of CPSF (cleavage and polyadenylation specificity factor), a critical component of the 3’ processing machinery, effectively blocking 3’ cleavage and subsequent polyadenylation of pre-mRNAs (Nemeroff et al., 1998). (c) NS1 also binds to the purine rich stem-bulge structures of the U6 snRNA, a required component of the spliceosome (Fortes et al., 1994; Qiu et al., 1995) inhibiting post transcriptional processing of pre-mRNAs.

NS1 also alters the translational process within the cell to decrease host mRNA translation while up-regulating vRNA translation. By binding eIF4GI, a required component for cap-dependent translation, NS1 facilitates vRNA preferential transcription (Aragon et al., 2000). Binding of eIF4GI by NS1 serves two purposes: (1) sequestering eIF4GI inhibits the use by cellular components for host mRNA translation and (2) NS1 binds vRNAs via the 5’ UTR along with eIF4GI to deliver the two to ribosomes resulting in an enhanced translational rate of viral proteins (de la Luna et al., 1995). Along with the sequestration of eIL4GI, influenza infection induces hypophosphorylation of eIF4E resulting in the loss of the eIF-4F complex that is required for cap-dependent translation (Feigenblum and Schneider, 1993). By the N-terminal RNA binding domain, NS1 binds dsRNA and ssRNA to limit the detection by RNA-regulated Protein kinase (PKR). Detection of viral RNA by PKR activates host viral defenses including the phosphorylation of eIF2α, which decreases the initiation.
rate of translation (Lu et al., 1995). NS1 inhibits the activation of PKR by sequestering dsRNA and ssRNA, aiding to the aversion of detection by host defenses. NS1 is also known to bind the viral replication complex (Marion et al., 1997) and the cellular multifunctional protein nucleolin, involved in ribosome synthesis (Murayama et al., 2007); but the effects of these interactions are not clearly known.

NS1 suppresses cellular immune defenses by (a) by sequestering dsRNA and ssRNA, limiting detection by PRR receptors, (b) inhibiting immune signaling by binding signaling components, and (c) reducing the translation of host mRNA. By blocking the processing and exportation of cellular mRNA, NS1 is effectively able to suppress the host antiviral response by inhibiting the expression of antiviral genes. The C-terminal effector domain of NS1 also binds to the dsRNA sensor, retinoic-acid-inducible gene I (RIG-I), which inhibits activation of the IFN-α/β pathway by restricting the RIG-I/IPS-1 mediated IRF-3 signaling cascade (Mibayashi et al., 2007). In addition, by binding ssRNA, NS1 is able to resist detection by RIG-I, further inhibiting RIG-I activation of IFN-α/β and IL-6 expression (Pichlmair et al., 2006). As mentioned previously, NS1 binds both dsRNA and ssRNA to limit the detection by host cellular defenses. Pattern recognition receptors on the cellular surface and the intracellular matrix such as PKR, melanoma differentiation-associated gene 5 (MDA-5), RIG-I, and TLRs (Specifically 7 and 8) all recognize dsRNA and ssRNA to up-regulate host immune responses. By sequestering dsRNA and ssRNA, NS1 inhibits the detection by these PRRs, preventing activation of signaling pathways such as OAS (2′-5′ oligo (A) synthetase)/RNase L, PKR, NF-kB, NLRP3, IRF-3, and IRF-7 (Garulli and Castrucci, 2009; Geiss et al., 2002; Lu et al., 1995; Mibayashi et al., 2007; Min and Krug, 2006; Siren et al., 2006).
Pertinent to this study is the previous research characterizing the H3N2 triple reassortant modified live influenza virus (*A/swine/Texas/4199-2/98*). Multiple recombinant influenza viruses were generated expressing carboxyl-terminal NS1 truncations of variable length (Solorzano et al., 2005). The full-length NS1 gene encoding a 219aa protein was truncated to various lengths using restriction enzyme sites, leaving the NS1 gene intact. Three virus constructs were generated encoding carboxyl-truncated NS1 proteins of 73, 99, and 126 amino acids in length (NS1Δ73, NS1Δ99, NS1Δ126 respectfully) (Solorzano et al., 2005). All other segments of the influenza genome were unaltered. Initial findings demonstrated carboxyl-truncations decreased the capacity of Tx98 to prevent IFN-α/β expression. Multicycle growth properties and interferon α/β inhibition assays in conjunction with live animal trials showed carboxyl-truncations to the NS-1 protein conveyed a level of attenuation inversely dependent on the intensity of interferon α/β inhibition. Surprisingly, the highest level of attenuation resulted from the shortest NS1 truncation [NS1Δ126 > NS1Δ99 > NS1Δ73> wtNS1] as measured by macroscopic lung lesion scores and virus titer at day 5 post challenge in pigs (Solorzano et al., 2005). Due to its replicative growth properties and attenuation rate *in vivo*, Tx98 NS1Δ126 was further investigated in subsequent trials as a potential influenza MLV vaccine candidate (Richt et al., 2006; Vincent et al., 2007). In live animal trials, administration of the H3N2 NS1Δ126 MLV administered intranasally provided adequate protection from homologous and homosubtypic challenge but only provided limited protective immunity from heterosubtypic challenge (Vincent et al., 2007). The studies contained herein continues on this line of research utilizing the previously described Tx98NS1Δ126 MLV in conjunction with recombinant porcine IL-18 (rpIL-18) administration to evaluate the potential for broad cross protection.
against the challenge by a heterosubtypic triple reassortant swine influenza virus [H1N1 (A/swine/Iowa/00239/2004)]

**Protective immunity against influenza infection**

Influenza is an acute respiratory illness often accompanied with secondary bacterial infections in severe cases (Brundage, 2006). Swine influenza is a common component contributing to the porcine respiratory disease complex (PRDC) (Baskerville, 1981). While current commercial swine influenza vaccines adequately protect against homologous and homosubtypic influenza viruses, they often fail to elicit protective immunity to heterosubtypic influenza strains. While pre-existing antibody is the only known mechanism to achieve sterilizing immunity to influenza (Gerhard, 2001), mutations (drift) and reassortment events (shift) result in rapid changes to antibody recognized epitopes. Inversely, cell mediated immune responses typically recognize more static internal influenza proteins possessing conserved epitopes (Doherty et al., 1997; Doherty et al., 2006; Flynn et al., 1999; Thomas et al., 2006).

During an influenza infection, CD4+ T cells primarily function to promote a high-quality antibody response (Doherty et al., 2006). CD4+ T cells respond to viral peptides on MHC II molecules by induction of cytokine release. The cytokines have many functions including activating inflammatory pathways, stimulating CD4+ and CD8+ proliferation, and aiding in the differentiation of T and B cells (Moran et al., 1999). In an adoptive-transfer murine model, naïve CD4+ TCR-transgenic cells exhibited proliferative responses 3 weeks past viral clearance in the host (Jelley-Gibbs et al., 2005), indicating that antigen presentation
following influenza infection occurs long after viral clearance. The ultimate nature of the CD4+ proliferative response was dependent upon the stage of infection at time of transfer. Early adoptive transfers at the height of infection resulted in a strong proliferative response resulting in a large primary effector pool, contracting significantly into a small memory CD4+ T cell population after viral clearance (Jelley-Gibbs et al., 2005). Conversely, adoptive transfer of naïve CD4 T cells at or after the time of viral clearance resulted in a pedestrian proliferative response but sustained a higher ultimate number of CD4+ T cells surviving as memory T cells.

During influenza infection, CD8+ T cells’ main effector function is clearance of virus infected cells by Fas (CD69) mediated or perforin and granzyme mediated cytotoxicity (Moran et al., 1999; Topham et al., 1997). Antigen specific memory CD8+ T cells to are maintained in high numbers a month or more post primary influenza infection (Flynn et al., 1999) in a murine model. Primary effector antigen specific CD8+ T cells were mostly cleared by day 15 post primary infection of influenza virus (Flynn et al., 1999).

Recovery from influenza infection is primarily mediated by cellular immune responses (Graham and Braciale, 1997; Topham et al., 1996). The dominant adaptive immune effector functions at the respiratory epithelial layer are secretory IgA (S-IgA) antibodies and antigen specific CTLs (Tamura and Kurata, 2004). While IgG antibodies enter the respiratory tract by diffusion at the alveolar epithelia from the serum (Tamura and Kurata, 2004), IgA antibodies are transported to the mucosal surface by transepithelial transport in dimeric form (Tamura and Kurata, 2004). It is theorized IgA antibodies can bind to intracellular antigen as it is transported through the epithelial cells (Murphy et al., 2008). Upon reinfection with influenza, S-IgA and IgG antibodies form virus-immunoglobulin
complexes, facilitating neutralization and removal of influenza virus. Cytokine signaling contributes significantly to antibody isotype class switching. The complement-activating IgG2 isotype is the major form generated by B cells in a T\textsubscript{H}1 environment, whereas classic T\textsubscript{H}2 cytokines typically stimulates the production of the IgG1 non-complement-activating isotype (Moran et al., 1999).

Studies with IL-18 deficient (IL-18 \textsuperscript{−/−}) transgenic mice have demonstrated the necessity for adequate IL-18 expression to generate optimal protective immunity to influenza infection. IL-18 \textsuperscript{−/−} transgenic mice infected with influenza A virus resulted in (a) viral titers reaching maximum at earlier time points, (b) significantly elevated virus titers, (c) significantly lower IFN-\textgamma production, and (d) lower activity levels of NK cell-mediated cytolysis (Billaut-Mulot et al., 2000; Denton et al., 2007; Dinarello, 1999; Foss et al., 2001; Liu et al., 2004; Takeda et al., 1998). Further research has additionally demonstrated that IL-18 is required for optimal production of cytokines IFN-\textgamma, TNF\alpha, and IL-2 by CD8\textsuperscript{+} T cells during the course of influenza infection (Denton et al., 2007).

Broad cross-protection to multiple subtypes of influenza is known to result following live influenza infection. Cross-protection, or heterosubtypic immunity, is not completely understood but is believed to be the effect of CD4\textsuperscript{+} T-cells, of memory CD8\textsuperscript{+} T-cells targeting the conserved internal proteins presented by MHC class I on the host cell surface, and by S-IgA and IgG virus neutralization and antibody cross-linking (Benton et al., 2001; Graham and Braciale, 1997; Liang et al., 1994; Moran et al., 1999; Nguyen et al., 2000; Schulman and Kilbourne, 1965; Tamura and Kurata, 2004; Tamura et al., 2005; Topham et al., 1996). CD8\textsuperscript{+} T cells tend to target the more conserved influenza proteins NP and M (Doherty et al., 1997; Doherty et al., 2006; Flynn et al., 1999; Thomas et al., 2006), allowing
broader protection from varying strains and subtypes. CD8+ T cells from mice immunized with H1N1 influenza A virus rapidly proliferated in response to intranasal challenge with an H3N2 virus that shares the same immunodominant epitope of the NP protein (Flynn et al., 1998; Flynn et al., 1999). B cell deficient mice naïve to influenza have 50-100X greater susceptibility to a lethal influenza virus challenge than do wild-type mice. However, after priming mice with a sublethal dose, B cell +/- mice exhibited an enhanced resistance to lethal virus infection compared to wild type controls (Graham and Braciale, 1997), suggesting memory CMI responses are efficient mechanisms for influenza viral clearance.

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CHAPTER 2 CHARACTERIZATION OF MUTATED PORCINE INTERLEUKIN-18 PROTEIN EXPRESSED IN A REPLICATION-DEFICIENT ADENOVIRAL VECTOR

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Abstract

Interleukin (IL)-18 is a pleiotropic cytokine participating in a variety of immune functions dictated by the surrounding immunological environment. Characteristically, IL-18 acts to produce a strong cell mediated immune (CMI) response by activating type II interferon signaling; stimulating NK cells, T cells, B cells, and professional APCs to elicit a phenotypic T\(_{\text{H}1}\) immune response. Due to the strong induction of CMI by IL-18, it may prove to be an advantageous cytokine for the therapeutic enhancement of immune responses to vaccination or treatment of disease. The use of cytokines for therapeutic immunomodulation or enhancement of protective immunity has been shown to be efficacious. To generate an enhanced immunomodulatory agent, wild type porcine IL-18 cDNA was mutated at two amino acid positions, E41A and K88A, to generate an IL-18 with increased biological activity. Mutated and wild type forms of IL-18 were used to generate
replication-defective adenoviral expression vectors. High quantities of rIL-18 (>10ng/mL) were generated by adenoviral expression vectors in vitro. Examination of biological activity was inconclusive in vitro and in vivo. Administration of rIL-18 adenoviral vectors to pigs resulted in a consistent up-regulation of IFN-γ in nasal wash samples for two days following inoculation, though protein levels were near the limits of the assay. Serum IFN-γ levels did not produce consistent results regardless of administration route or treatment.
1. Introduction

Through the course of evolution, innate and adaptive immune systems have developed to protect against viral, bacterial, fungal, and parasitic infections. Prophylactic treatments such as vaccinations further reduce the morbidity and mortality associated with many diseases. However, rapidly evolving pathogens and, at times, the development of unprotective or inappropriate immune responses continues to provide challenges to the human and animal health industries. Inappropriate or unprotective immune responses can develop as a consequence of host genetics, stage of immunological development, pathogen derived immunomodulatory agents, or other environmental factors (Fallon and Alcami, 2006; Forsthuber et al., 1996; Muneta et al., 2002; Pertmer et al., 2001; Ridge et al., 1996; Siegrist, 2001). For example, the neonatal immune system exhibits a range of dysregulated or insufficient immune responses including pro-inflammatory pathways and T cell polarization (Forsthuber et al., 1996; Pertmer et al., 2001; Ridge et al., 1996; Sarzotti et al., 1996). In neonates, T cells display an impaired ability to polarize towards a phenotypic Th1 based immune response (Breathnach et al., 2006; Forsthuber et al., 1996; Pertmer et al., 2001; Sarzotti et al., 1996; Siegrist, 2001; Siegrist et al., 1998; Suen et al., 1998).

Therapeutic treatments, such as cytokine administration, have been able to restore correct immune function (Pertmer et al., 2001; Ridge et al., 1996; Siegrist, 2001) or enhance protective immunity (Zuckermann et al., 1998). However, a short half-life in vivo is a particular problem concerning the therapeutic administration of recombinant cytokine protein. A plausible solution is to deliver cytokines by vectored gene delivery, capable of extended cytokine expression. Engineered replication-competent and replication-deficient
adenoviral expression systems have been shown to be safe and effective (Brockmeier et al., 2009; Croyle et al., 2008; Mayr et al., 1999; Mayr et al., 2001; Phillpotts et al., 2005; Stephenson, 1998; Walter et al., 2001; Xiang and Ertl, 1999). The use of adenoviruses as expression vectors is well established in over 200 clinical trials, two licensed vaccines, and numerous studies including HIV, malaria, FMD, yellow fever, rabies, ebola, and influenza virus (Croyle et al., 2008; Li et al., 2007; Mayr et al., 1999; Mayr et al., 2001; Patterson et al., 2009; Phillpotts et al., 2005; Stephenson, 1998; Tang et al., 2009; Tucker et al., 2008; Walter et al., 2001; Xiang and Ertl, 1999; Yang et al., 1994). Adenoviruses are well suited for mucosal administration, including oral and intranasal routes, able to elicit high IgA antibody titers and effective CD8+ T cell responses (Croyle et al., 2008; Phillpotts et al., 2005; Tang et al., 2009; Tucker et al., 2008; Walter et al., 2001; Xiang and Ertl, 1999). Specific to veterinary medicine, adenoviral vectors have been utilized to deliver avian influenza antigens to vaccinate poultry (Tang et al., 2009), to deliver E2 glycoproteins to protect against Venezuelan equine encephalitis virus (Phillpotts et al., 2005), and efficacious induction of immunity against foot-and-mouth disease virus (FMDV) upon adenoviral-mediated delivery of the FMDV capsid (P1-2A) or the viral 3C protease (Mayr et al., 1999; Mayr et al., 2001).

Interleukin (IL)-18 may prove to be a particularly useful cytokine for therapeutic immunomodulation. Initially named interferon-γ inducing factor (IGIF), IL-18 is most widely known for its potent ability to up-regulate interferon-γ (IFN-γ) in the presence of IL-12, resulting in phenotypic Th1 T cell differentiation and proliferation (Nakamura et al., 1993). Acting at the cellular level, IL-18 stimulates macrophages, NK cells, professional antigen presenting cells (APCs), T cells and B cells, and is able to up-regulate a host of
antiviral, antibacterial, and antifungal associated responses (Kohno et al., 1997; Moran et al., 1999; Yoshimoto et al., 2000; Yoshimoto and Nakanishi, 2006; Yoshimoto et al., 1997).

IL-18 is highly regulated at the transcription and protein level. IL-18 is encoded as a biologically inactive precursor (pro-IL-18) requiring cleavage by Interleukin-1β converting enzyme (Caspase-1 or ICE) to become biologically active. Furthermore, constitutively expressed IL-18 binding protein (IL-18BP) acts to neutralize biologically active IL-18 upon binding (Novick et al., 1999). However, unlike most other soluble cytokine receptors, IL-18BP is not a variant of the IL-18 receptor (IL-18R) and shares no significant homology with IL-18R (Novick et al., 1999). Due to the differences in binding sites between IL-18R and IL-18BP, amino acid conversions introduced within the IL-18/IL-18BP binding interface of human IL-18 (huIL-18) has been reported to yield a longer half-life and enhanced biological activity (Kim et al., 2002; Kim et al., 2001).

To evaluate if synonymous mutations to a porcine IL-18 would result in similar increases in biological activity, a comparable mutated IL-18 construct was generated from porcine IL-18 cDNA (Genbank accession number U68701). Using PCR-primer site directed mutagenesis, two amino acid conversions (E41A and K88A) were introduced at critical amino acid sites for IL-18BP neutralization. For each rIL-18 construct, two different Kozak sequences were used (GCCACCAATGG/E or GCCGCCGCCCATGG [F]) to evaluate optimal expression of rIL-18 (wtIL-18E, wtIL-18F, mutIL-18E, and mutIL-18F). Using a replication-defective adenovirus expression system (AdEasy™ XL System (Stratagene, La Jolla, CA)), adenovirus vectors expressing wtIL-18 or mutIL-18 were generated. Recovered rIL-18 adenoviral isolates were sequenced to confirm desired mutations and Kozak sequences (Figure 2.2). Adenoviral vectors were evaluated for biological expression of recombinant IL-
18 (rIL-18) and the resulting IL-18 was assessed for biological activity both in vitro and in vivo.

2. Materials and Methods

2.1 Cells

AD-HEK-293 cells were cultured in Dulbecco's Modified Eagles Medium high glucose 1X with 4.5g/L D-glucose, L-Glutamine, 110mg/L sodium pyruvate, supplemented with 2-10% fetal bovine serum, 100 units/L of penicillin, 100 µg/L of streptomycin, and 2.5 µg/L of amphotericin B, streptomycin sulfate, and amphotericin B Antimycotic; all from Gibco (Marketed by Invitrogen, Grand Island, NY).

Peripheral blood mononuclear cells were isolated from porcine whole blood by Vacutainer CPT™ sodium citrate separation tubes or by percoll method (Ulmer et al., 1984) treated with 2X acid citrate dextrose (ACD). PBMCs were sustained in RPMI 1640 media containing L-glutamine and 25mM HEPES, supplemented with 10% fetal bovine serum, 100 units of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B Antimycotic; all from Gibco (Marketed by Invitrogen, Grand Island, NY).

2.2 Mutagenesis of porcine IL-18

Dual point mutations were introduced into a porcine wild type IL-18 encoding cDNA construct by PCR primer site-directed mutagenesis. Two sets of PCR primers were designed
to individually introduce amino acid conversions at positions 41 (E to A) and 88 (K to A). Mutations and full-length sequence fidelity were confirmed by sequencing (Figure 2.1). The mutIL-18 plasmid was previously generated within our lab by Ma et al. (unpublished data).

2.3 Addition of Kozak initiation sequence to rIL-18 cDNA

PCR primers were used to incorporate two different Kozak sequences (GCCACCATGG (E) or GCCGCCATGG (F)) to each form of IL-18, generating four cDNA constructs (wtIL-18E, wtIL-18F, mutIL-18E, and mutIL-18F). Restriction enzyme cutting sites were also introduced directly outside the ORF to be utilized for directional cloning in downstream procedures.

Forward 5’ primer: E

5’ATCATTACAGATCTGCCACCATGGCTGCTGAACCGGAAG3’

Bgl II Kozak IL-18 Sequence

Forward 5’ primer: F

5’ATCATATTAGATCTGCCGCCCATGGCTGCTGAACCG3’

Bgl II Kozak IL-18 Sequence

Reverse Primer: R

5’ATACTCATCTCGAGCTAGTTCTTGTTTGAACAGTGAACATTATAG3’

Xho I Stop IL-18 Sequence
2.4 Generation of replication defective adenoviruses (wt & mutIL-18)

Adenoviral expression vectors were constructed using the AdEasy™ XL System (Stratagene, La Jolla, CA) as described in the AdEASY™ vector system manual (v1.4 Q BIOgene, Carlsbad, CA). In short, cDNA constructs were directionally cloned into a transfer vector (pShuttle), introducing a promoter from the immediate early (IE) region of the cytomegalovirus (CMV) and a simian virus 40 polyadenylation signal. pShuttle-CMV-IL-18 constructs were then transformed into One Shot® Top10® electrocompetent cells (Invitrogen, Grand Island, NY) for large DNA preparations. Transformed cells were screened by antibiotic resistance and positive colonies were further screened for retention of correct insertion by restriction enzyme digestion and DNA gel electrophoresis. Preparations of positive isolate colonies were subsequently grown in 5mL LB/ kanamycin, DNA purified, and resulting plasmids linearized by RE digestion prior to transformation into RecA+ BJ5183-AD-1 electroporation competent cells, pre-transformed with the replication defective human adenovirus-5 vector plasmid pAdEasy-1 (huAd5ΔE1ΔE3). Positive recombinants were screened and prepared as stated previously prior to transformation into XL10-Gold™ ultra-competent cells for large DNA preparations. Selected colonies were prepared using a SNAP Midiprep Kit (Invitrogen, Grand Island, NY). DNA from the SNAP preparations was lipotransfected into HEK293 cells per manufacturer’s protocol for Lipofectamine 2000 (Invitrogen, Grand Island, NY). Isolated, well developed plaques were collected following the procedure outlined in the AdEasy Adenoviral Vector System manual. Several rpHuAd5 vector clones were selected for each IL-18 construct and sequenced to confirm correct insertion, Kozak sequence, and desired porcine IL-18 sequence.
2.5 Virus

Low passage replication deficient adenovirus isolates expressing wtIL-18 and mutIL-18 were propagated in specialized AD-HEK-293 cells genetically altered to support replication. For use as inocula, adenovirus isolates were purified and concentrated by double CsCl density gradients as described in the AdEasy™ vector system manual (v1.4 Q BIOgene, Carlsbad, CA). In short, 40 T150 cell culture flasks (Corning, Corning, NY) were inoculated per virus. Samples were collected when CPE had reached 100%. Media was consolidated and spun at 300 rcf for 10 min to obtain a single cell pellet, discarding the supernatant. Pellets were freeze/thawed three times to lyse cells and release the virus. Discontinuous gradients (1.4-1.2 CsCl sp gr) were prepared in 50mL ultracentrifuge tubes. Samples were overlaid on top of discontinuous gradient and centrifuged at 100,000 rcf for 90 minutes. Concentrated adenovirus was collected by aspiration of the adenoviral band and immediately overlaid on previously prepared continuous gradients (1.4-1.2 CsCl sp gr) and spun at 100,000 rcf for 20-24 hours. Resulting concentrated adenoviruses were desalted by dialysis in 10mM Tris (pH 8.0), 2mM MgCl₂, 4% sucrose buffer. Purified adenoviral isolates were titered by tissue culture infectious dose 50% [TCID₅₀/mL] as described in the AdEasy™ vector system manual. Adenoviral inoculums were diluted to 1.0 X 10⁹ TCID₅₀/mL in sterile PBS shortly before administration.

2.6 In vitro biological expression of rIL-18

To confirm rIL-18 expression by adenoviral vectors, AD-HEK-293 cells were inoculated as an approximate MOI of 200 and allowed to progress for various time points.
Prior to rIL-18 measurement, samples were freeze/thawed a total of three times for optimal release of intracellular rIL-18 (data not shown). rIL-18 protein levels of freeze/thawed (F/T) supernatants were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) for swine IL-18 (Invitrogen, Grand Island, NY) per manufacturer’s instructions.

2.7 In vitro biological activity assay

Biological activity of rIL-18 was evaluated by porcine PBMC responsiveness to rIL-18 treatment as measured by IFN-γ up-regulation. rIL-18 preparations were generated in AD-HEK-293 cells at varying media concentrations. Resulting supernatants were F/T 3X prior to centrifugation at 300 rcf for 5 minutes to remove large cellular debris.

Concentrated rIL-18 supernatants were prepared by inoculating 10 nearly confluent T150 flasks (AD-HEK-293) per adenoviral construct. When cells reached a CPE of 90-100% samples were F/T a total of three times to release intracellular rIL-18 protein. Samples were spun at 300 rcf for 5min and resulting supernatants were vacuum filtered (0.2μm) to remove large cellular debris. 100kD and 10 kD (Millipore Corp., MA, USA) centrifugal filter units were used to further filter and concentrate the rIL-18 supernatant. Measurements of resulting rIL-18 concentrates were performed in duplicate using a swine IL-18 ELISA per manufacturer’s instructions.

Biological activity assays were performed at a range of PBMC concentrations per cm², PBMC isolation methods, co-stimulatory agents, and rIL-18 preparation methods to find the optimal parameters. rIL-18 stimulated PBMCs were incubated at 39°C, 5% CO₂ for the duration of each time point. Samples were F/T a total of three times to release intracellular
IFN-\(\gamma\) prior to IFN-\(\gamma\) protein quantitation. The IFN-\(\gamma\) protein concentration present in each PBMC supernatant was measured in duplicate by swine IFN-\(\gamma\) ELISA per manufacturer’s instructions (Invitrogen, Grand Island, NY).

A single wild type rIL-18 expressing adenovirus construct was used as a wild type control. Based on preliminary analysis, the wtIL-18 construct Ad5+wtIL-18K:E containing the E Kozak sequence displayed the highest biological activity and was therefore chosen. The wild type rIL-18 construct (Ad5+wtIL-18K:E) was previously generated by Kehrli et al. (unpublished data). A replication defective adenovirus null vector (Ad5B), expressing a \(\beta\)-galactosidase \(\alpha\) gene fragment was used as an adenoviral control vector (Moraes et al., 2001).

2.8 Virus titration

Titration of adenovirus samples was calculated as the tissue culture infectious dose 50\% (TCID\(_{50}\)) as described in AdEASY™ vector system manual (v1.4 Q BIOgene, Carlsbad, CA). In short, 1X10\(^4\)AD-HEK-293 cells were plated per well in a 96-well plate and were allowed to adhere for 1 hour. 10-fold serial dilutions of each adenovirus sample from 10\(^{-1}\) to 10\(^{-11}\) were administered at 100\(\mu\)L per well in a replicate of eight. Plates were incubated at 37\(^\circ\)C and 5\% CO\(_2\) for 12 days. On day 12, plates were scored for CPE in each row and TCID\(_{50}\) calculated by the formula:

\[
\text{TCID}_{50}/\text{mL} = 10^{1+d(s-0.5)} \times 10, \quad d=\log_{10} \text{(dilution)}, \quad s= \text{sum of ratios}
\]
2.9 In vivo biological activity assay

Comparison of biological activity of wild type IL-18 verses mutated IL-18 constructs were evaluated in vivo by intramuscular (IM) and intranasal (IN) routes of administration. Thirty pigs were delivered at two weeks of age and were randomly separated into five groups of five after being treated with a single dose of EXCEDE™ (Ceftiofur Crystalline) per manufacturer’s recommend dose. Pigs were housed for two weeks prior to start of study. Treatment groups and routes of administration were structured as described in Table 2.1.

Table 2.1 In vivo rIL-18 biological activity assay treatment group outline

<table>
<thead>
<tr>
<th>Group</th>
<th>Room</th>
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<th>Treatment Group</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
<td>Ad5+wtIL-18K:E - IN route</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>5</td>
<td>Ad5+wtIL-18K:E - IM route</td>
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<tr>
<td>3</td>
<td>2</td>
<td>5</td>
<td>Ad5+mutIL-18K:E3 - IN route</td>
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<tr>
<td>4</td>
<td>2</td>
<td>5</td>
<td>Ad5+mutIL-18K:E3 - IM route</td>
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<tr>
<td>5</td>
<td>3</td>
<td>5</td>
<td>Vector Control (Ad5B) - IM route</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>5</td>
<td>Negative Sham: IM</td>
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</tbody>
</table>

Adenovirus stocks purified by double CsCl gradients were diluted to an approximate titer of 1.0 X 10⁹ TCID₅₀/mL in sterile PBS for use as animal inocula. Sterile PBS was used as the sham inoculum. Inocula were administered in 2 mL doses either intranasally (IN), by slowly dripping into the nose, or intramuscularly (IM). Nasal washes and serum samples were collected from day 0 to 5 days post vaccination (dpv). Measurement of IFN-γ protein levels within nasal wash and serum samples were evaluated by swine IFN-γ ELISA (Invitrogen, Grand Island, NY).
3. Results

3.1 Generation of mutated porcine IL-18 by site-specific mutagenesis

Mutations to wild type porcine IL-18 cDNA: Two amino acid conversions were introduced into the wild type porcine IL-18 gene at amino acid positions 41 and 88. Glutamic acid (E) at position 41 and lysine (K) at position 88 were converted to alanine by site directed mutagenesis. Both glutamic acid and lysine possess hydrophilic, charged R groups which are predicted by computer modeling to be involved in binding to the inhibitory IL-18BP (Kim et al., 2002). Alteration at these positions to alanine induced extended half-life and heightened biological activity in human IL-18 (Kim et al., 2001). Sequencing confirmed desired mutations in the mutIL-18 cDNA (Fig. 2.1).

![Figure 2.1 Amino acid alterations: mutIL-18 vs. wtIL-18](image)

Fig. 2.1. Sequence analysis of site-directed mutagenesis to porcine IL-18 cDNA at amino acid positions 41 and 88.

Addition of differing Kozak sequences per rIL-18 cDNA construct: Two different Kozak sequences were used (GCCACCATGG [E] or GCCGCGCCCATGG [F]) for each form of IL-18 to evaluate optimal expression of rIL-18. Forward primers arbitrarily designated E (38mer) or F (39mer) were used with a single IL-18 reverse primer (R) to
introduce desired Kozak sequences into wtIL-18 and mutIL-18 genes by PCR. The four resulting cDNA constructs [wtIL-18K:F, wtIL-18K:E, mutIL-18K:E, and mutIL-18K:F] were sequenced to confirm correct Kozak sequences (Fig. 2.2).

Figure 2.2 Kozak sequence comparison: mutIL-18 E & F, wtIL-18 E & F

![Kozak Sequence Comparison](image)

Fig. 2.2. Sequence analysis of Kozak sequences E & F for both mutated and wild type rIL-18 cDNA plasmids.

3.2 Generation of rIL-18 adenoviral vectors

Generation of replication-defective rIL-18 adenoviral vectors: Adenoviral vectors expressing either wild type or mutated forms of IL-18 were generated using the AdEasy™ XL System (Stratagene, La Jolla, CA). Recombinant wild type and mutated forms of IL-18 of each Kozak sequence were directionally cloned into the pShuttle-CMV plasmid, introducing a promoter from the immediate early (IE) region of the cytomegalovirus (CMV) and a simian virus 40 polyadenylation signal to induce high level expression. rIL-18 constructs were combined with the replication defective human adenovirus-5 plasmid, pAdEasy-1 (huAd5ΔE1ΔE3), by homologous recombination in RecA+ BJ5183-AD-1 electroperoration competent cells. Resulting plasmids were lipotransfected into AD-HEK-293 cells genetically modified to support the replication of the adenoviral vectors. Several clones of each adenoviral construct [Ad5+mutIL-18K:E, Ad5+mutIL-18K:F, Ad5+wtIL-18K:E,
Ad5+wtIL-18K:F] were collected from well developed plaques following the procedure outlined in the AdEasy adenoviral vector system manual. Selected clones were sequenced to confirm correct insertion, Kozak sequences, and sequence homology. Ad5+mutIL-18K:F1 & F2, clonal isolates from the homologous recombination step, are shown to possess a single frameshift nucleotide insertion 12 base pairs upstream of the termination codon. To evaluate the differences between mutIL-18 and wtIL-18 in downstream assays, a single wtIL-18 adenoviral construct [Ad5+wtIL-18K:E] was used.

3.3 In vitro expression of rIL-18 in vitro

**Conformation of adenoviral-mediated rIL-18 expression:** Eight mutIL-18 adenoviral isolates (Ad5+mutIL-18K:E 1-4 & Ad5+mutIL-18K:F 1-4) were evaluated in relation to a single wild type rIL-18 adenoviral construct (Ad5+wtIL-18K:E) to assess biological expression of rIL-18. AD-HEK-293 cells were inoculated and the adenovirus infection was allowed to progress for selected time points. At the completion of each time point, cells were F/T 3X to release intracellular IL-18 protein prior to measurement of supernatants by swine IL-18 enzyme-linked immunosorbent assay (ELISA).

Pharmacokinetic studies have found the half-life (t₁/₂) of human IL-18 to be 35 hours (Robertson et al., 2006) and 16 hours for murine IL-18 (Hosohara et al., 2002). Based upon the previous pharmacokinetic studies, a 12-hour time point was chosen to account for a possible shorter half-life of porcine IL-18.
In three independent trials, expression of rIL-18 protein was confirmed from all adenoviral isolates except for the negative control (Fig. 2.3). All rIL-18 positive isolates produced at or above 500 pg/mL and up to 1000 pg/mL of rIL-18 protein under the conditions tested. Analysis between all groups, for all time points, by Two-way ANOVA analysis showed no statistical differences between groups (P>0.05).

**Kozak sequence and rIL-18 expression levels:** The Kozak sequence is defined in relation to the adenine nucleotide of the start codon (+1), ranging from -9 to +4 with a general consensus sequence of (gcc)gccRccAUGG (Kozak, 1984, 1987); R is defined as a purine. Changes from a ‘strong’ to a ‘weak’ Kozak consensus sequence can result in greater than 20 fold differences in the level of protein translated (Iida and Masuda, 1996; Kozak, 1984, 1986, 1987).
To design an optimal rIL-18 expression vector, two characteristically ‘strong’ Kozak sequences were used with each form of IL-18 to evaluate optimal expression. The Kozak sequence designated [F] is comprised of an extra (gcc) nucleotide sequence at positions -9 to -7, and a guanine nucleotide at position -3, to make a theoretically stronger Kozak sequence compared to [E] (De Angioletti et al., 2004; Kozak, 1987). To evaluate if the difference in Kozak sequence significantly affected the rate of protein translation, the biological expression data was reevaluated based upon Kozak sequence (Fig. 2.4). Results are reported as group means ± SE from three independent trials. Adenoviral vectors containing the [F] Kozak sequence did express slightly higher rIL-18 protein on average from 12-36 hours post inoculation (p.i.). However, the differences in expression levels between E and F Kozak groups were not found to be significant [Two-way ANOVA (P>0.05)].
3.4 Biological activity of rIL-18 in vitro

Primary biological activity assays were conducted utilizing rIL-18 protein generated under typical inoculation conditions (18mL per 75cm²) yielding 0.2-0.3 ng/mL rIL-18 protein concentrations. The highest rIL-18 expressing adenovirus constructs for each Kozak group [E (Ad5+mutIL-18K:E3) and F (Ad5+mutIL-18K:F2)] was chosen as test isolates. Adenoviral isolates Ad5+wtIL-18K:E and Ad5B were used as the wild type rIL-18 control and null vector control respectively. Concanavalin A (Con A) was used as a positive control (0.5-2.5μg/mL). rIL-18 treated PMBCs or whole blood yielded no measureable interferon-γ (IFN-γ) protein from adenoviral supernatants. In all trials the positive control induced measureable IFN-γ protein expression up to 160 ng/mL in PBMCs and 18 ng/mL in whole blood. Conclusions from the first trials, along with previous published findings, suggested higher rIL-18 protein levels are needed to induce a measureable IFN-γ response in vitro.

Low volume rIL-18 preparations and co-stimulatory agents: Previous studies evaluating the biological activity of rIL-18 reported concentrating rIL-18 protein to high quantities (≥100ng/mL) prior to the evaluation of biological activity (Muneta et al., 2000; Nagaya et al., 2004), as well as the use of co-stimulatory agents anti-CD3, Con A, IL-2, or IL-12 (Munder et al., 1998; Muneta et al., 2000; Nagaya et al., 2004; Nakamura et al., 1993; Ushio et al., 1996; Yoshimoto et al., 1998). To generate higher yeilding rIL-18 products, the volume of media used to generate the rIL-18 supernatants was reduced greater than 3 fold. Reduction in inoculation volumes yeilded appreciably higher rIL-18 quantities (Fig. 2.5).
**Figure 2.5 Concentration of rIL-18 yielded by reduced volume culturing conditions**

![Bar chart showing concentration of rIL-18](chart.png)

Fig. 2.5. To generate higher yielding IL-18 supernatants, inoculation media volumes were reduced >3-fold (5ml per 75 cm²). Supernatants from low volume preparations were measured in duplicate by swine IL-18 ELISA. Values are reported as IL-18 protein (pg/mL) concentrations in the vertical axis by adenoviral isolate.

Biological activity of rIL-18 was reevaluated with the low volume rIL-18 preparations in the presence of co-stimulatory agents Con A and recombinant porcine IL-12 (rpIL-18) (Fig. 2.6). PBMCs were obtained from four pigs by Vacutainer CPT™ separation tubes. PBMCs were diluted to final concentrations of approximately 4.0 X 10⁵ PBMC/mL or 2.6 X 10⁵ cells/cm² in Con A (1 μg/mL) / rpIL-18 (180ng/mL) media and 0.5mL added to each well. Low volume preparations of Ad5B, Ad5+wtIL-18K:E, and Ad5+mutIL-18K:E3 were administered at 0.25mL per respective well, resulting in final rIL-18 concentrations of Ad5B (0 ng/mL), wtIL-18 (3.6 ng/mL), or mutIL-18 (3.7 ng/mL). Plain media (0.25mL RPMI 1640) was used for the Con A/rpIL-12 media control wells. Samples were F/T 3X prior to measuring in duplicate by swine IFN-γ ELISA.
Figure 2.6 Biological activity evaluation of rIL-18 (Con A/rpIL-12 co-stimulation):
Low volume protein preparations

![Graph showing IL-18 production](image)

Fig. 2.6. Production of IFN-γ (pg/mL) by PBMCs treated with low volume rIL-18 preparations. PBMCs were stimulated in the presence Con A (1 μg/mL) and rpIL-18 (180ng/mL) co-stimulation. PBMC supernatant samples were measured in duplicate by swine IFN-γ ELISA. Values are reported as mean ± SE from 4 pigs.

While the wtIL-18 treatment group failed to induce IFN-γ protein expression past background levels at any time point, the Con A/rpIL-12 control group exhibited a highly elevated concentration of IFN-γ at the 48 hour time point. The appreciable increase in the mean IFN-γ protein concentration at 48 hours in the Con A/rpIL-12 media control group can be attributed to a single sample, while all others were at or below 6.0 pg/mL IFN-γ. Only the mutIL-18 treatment group induced expression of IFN-γ above background consistently across time points past 12 hours.

**Concentration of rIL-18 by molecular weight exclusion columns:** To account for the possible unknown activity of virus and cellular products in the supernatant, a series of filtration steps were developed to purify and concentrate rIL-18 supernatants based on size
exclusion. Experimental procedures were developed from previous studies on recombinant porcine IL-18 conducted by Muenta et al. (Muneta et al., 2000).

For each adenoviral isolate to be tested, AD-HEK-293 monolayers totaling 600 cm$^2$ were inoculated. At 100% CPE, cell supernatants were F/T 3X and subsequently centrifuged at 300 rcf for 5 minutes. Resulting supernatants were filtered (0.2μM) to remove high molecular weight cellular material not removed by the initial centrifugation. Samples were then passed through 100kD centrifugal filtration units (Millipore Corp., MA, USA) to remove virus and cellular products larger than ~100kD. Finally, 10kD centrifugal filtration units were used to concentrate the rIL-18 10-fold and remove low molecular weight compounds (<10kD). Resulting rIL-18 concentrates were measured in duplicate by swine IL-18 ELISA (Fig. 2.7-2.10).

Evaluation of concentrated rIL-18 protein preparations for biological activity was conducted under three experimental conditions, (a) no co-stimulation, (b) Con A (2.5μg/mL) co stimulation, or (c) rpIL-12 (100ng/mL) co-stimulation. PBMCs were isolated from four pigs by CPT and were aliquoted at 1.0 X 10$^6$ PBMC/cm$^2$ or 2.0 X 10$^6$ PMBC/well. Time points were plated separately and collected at 24 hour intervals for five days.
Fig. 2.7-2.10. Large volume preparations of Ad5B, Ad5+wtIL-18K:E, Ad5+mutIL-18K:E, and Ad5+mutIL-18K:F were concentrated 10-fold by centrifugal molecular weight exclusion columns. Resulting IL-18 (pg/mL) concentrations from the concentrated protein preparations were evaluated by swine IL-18 ELISA.

Biological activity of concentrated rIL-18 preparations in the absence of co-stimulation failed to induce measureable IFN-γ production in all but one of the four pigs (data not shown). The single responding subject did not produce any detectable IFN-γ
protein until 96 hours post vaccination (<0.074ng/mL). Conversely, Co-stimulation with Con A at 2.5μg/mL resulted in a complete exhaustion of all PBMC samples by 24 hours; exhibiting deteriorating levels of IFN-γ concentrations as time points progressed past the initial measurement (data not shown).

**Figure 2.11 Biological activity assessment of rIL-18 (rpIL-12 co-stimulation): Concentrated protein preparations**

![Graph showing IFN-γ production over time for different groups.](image)

Fig. 2.11. Production of IFN-γ (pg/mL) by PBMCs treated with concentrated rIL-18 preparations. PBMCs were stimulated in the presence rpIL-18 (100ng/mL) co-stimulation. PBMC supernatant samples were measured in duplicate by swine IFN-γ ELISA. Values are reported as mean ± SE from 4 pigs.

IFN-γ induced expression by rIL-18 treatments in the presence of 100ng/mL rpIL-12 were detectable to quantities greater than 1.5 ng/mL as measured by swine IFN-γ ELISA (Fig. 2.11). The mutIL-18 treatment groups exhibited elevated IFN-γ protein concentrations compared to the wtIL-18 treatment group. However, the Ad5B vector control group also produced elevated levels of IFN-γ production when assessed in relation to wtIL-18 past 48 hours.
Figure 2.12  IFN-γ expression in relation to the negative control group (Ad5B) by concentrated rIL-18 protein preparations (rpIL-12 co-stimulation)

Fig. 2.12. IFN-γ (pg/mL) concentration of rIL-18 treatment groups in relation to the negative control group (Ad5B). At each time point, each measurement was subtracted from the Ad5B measurement per respective pig and time point. Corrected values were reevaluated by calculating the mean of four pigs for each treatment group. Differences between all treatment groups by time point are statistically significant [Two-way ANOVA (P<0.05)].

As seen in Figure 2.11, the standard error of mean values was high regardless of time point or treatment. This was the result of a high degree of variability between pigs. However, the relative responses to each treatment group from individual pigs were consistent. As the high degree of variability was most notably between pigs and not between treatment groups, each sample measurement was subtracted from their negative control group per respective pig per time point to control for the variability between pigs (ex. [Pig 1 24h Ad5+mutIL-18K:F2] – [Pig 1 24h Ad5B]). The means ± SE (n=4) of the adjusted values are reported in Figure 2.12. Both mutIL-18 treatment groups (Ad5+mutIL-18K:F2 & Ad5+mutIL-18K:E3) exhibited higher IFN-γ concentrations in comparison to either the negative control group or the wild type rIL-18 treatment groups. Differences between
adjusted rIL-18 groups by time point were statistically significant (P>0.05) as calculated by Two-way ANOVA analysis.

3.5 Biological activity of adenoviral mediated rIL-18 expression in vivo

Figure 2.13  In vivo biological activity assessment: Serum IFN-γ protein levels by treatment group

To evaluate the biological activity of rIL-18 in vivo, wild type [Ad5+wtIL-18K:E] and mutated [Ad5+mutIL-18K:E3] rIL-18 expressing adenovirus constructs were compared by intramuscular and intranasal routes of administration in four week old pigs (treatment outline Table 2.1). Nasal wash and serum samples were collected at 24 hour intervals from
the day of inoculation to five days p.i.. IFN-γ protein concentrations were measured in the nasal wash and serum samples from each pig by swine IFN-γ ELISA (Fig. 2.12-2.13).

**Figure 2.14** *In vivo* biological activity assessment: Nasal wash IFN-γ protein concentrations by treatment group

![Graph showing IFN-γ protein concentrations](image)

Days p.i. by treatment group (n=5)
- **Ad5+wL18K:E - IM**
- **Ad5+mlL-18K:E3 - IM**
- **Ad5B - IM**
- **Ad5+wL18K:E - IN**
- **Ad5+mlL-18K:E3 - IN**
- **Sham inoculum - IM**

Fig 2.13. Adenoviral inocula prepared by double CsCl gradients were administered by intranasal (IN) or intramuscular (IM) route. Concentrations of IFN-γ protein (pg/mL) from nasal wash samples were measured by swine IFN-γ ELISA. Values are reported as group means ± SE (n=5).

**IFN-γ expression in response to treatment in vivo:** The quantity of IFN-γ protein detected within the serum samples were generally below the limits of the assay (Fig. 2.12). Positive measurements for IFN-γ protein within serum samples were sporadic and inconclusive regardless of administration route or treatment.

Induction of IFN-γ protein production was more evident within nasal washes. IFN-γ protein detected in samples from nasal washes produced consistent results in both rIL-18
treatment groups administered by IM and IN (Fig. 2.14). Groups receiving rIL-18 adenoviral vectors (Ad5+wtIL-18K:E IM & IN, Ad5+mutIL-18K:E3 IM & IN) displayed a general increase in IFN-\(\gamma\) protein levels for the two days after inoculation followed by a steep decline in IFN-\(\gamma\) levels on day three p.i.. Levels of IFN-\(\gamma\) protein were at or below the limits of detection of the swine IFN-\(\gamma\) ELISA. Control groups also exhibited detectable levels of IFN-\(\gamma\) production.

4. Discussion

Previous research conducted by Kim et al. (2001) on huIL-18 as shown site-specific mutations altering charged residues E42A and K89A resulted in increased biological activity. The amino acid conversions rendered the mutated huIL-18 protein fully resistant to neutralization by IL-18BP, extending the half life and enhancing the biological activity of IL-18 4-fold (Kim et al., 2001). To evaluate if homologous mutations to porcine IL-18 would result in similar increases in biological activity, the amino acid conversions E41A & K88A were introduced by PCR-primer site directed mutagenesis to a wild type porcine IL-18 cDNA construct (Fig. 2.1).

WtIL-18 and mutIL-18 genes were placed under the control of a CMV promoter and strong Kozak sequences prior to insertion into the replication defective adenoviral expression vectors. Testing by swine IL-18 ELISA at various time points confirmed biological expression of IL-18 protein by all adenovirus+ rIL-18 test isolates (Fig. 2.3-2.5). The negative control vector Ad5B, containing a LacZ insertion (Moraes et al., 2001), failed to induce detectable levels of IL-18 protein in vitro. Adenoviral isolates expressing rIL-18 were
shown to produce high concentrations (>10 ng/mL) of rIL-18 protein when cultured in reduced volumes (Fig. 2.5).

Two Kozak sequences were used for each form of IL-18 to evaluate optimal expression (Fig. 2.2 & 2.4). A Kozak sequence is defined in relation to the adenine nucleotide of the start codon (+1), ranging from -9 to +4, with the general consensus of (gcc)gccRccAUGG (Kozak, 1984, 1987). Alterations to the Kozak consensus can produce a ‘strong’ to ‘weak’ initiator sequence resulting in greater than 20 fold difference in protein expression (Iida and Masuda, 1996; Kozak, 1984, 1986, 1987). Although both Kozak [E] & [F] are classified as strong initiator sequences, Kozak [F] is comprised of an extra (gcc) sequence at positions -9 to -7, and a guanine nucleotide at position -3, making a theoretically stronger Kozak sequence (De Angioletti et al., 2004; Kozak, 1987). To evaluate expressional differences between Kozaks [E] and [F], in vitro biological expression of mutIL-18 isolates was reviewed based upon Kozak sequence (Fig. 2.4). The ‘stronger’ Kozak sequence [F] exhibited slightly higher expression of rIL-18 in relation to Kozak [E], although not by a statistically significant margin.

Biological activity of rIL-18 protein was measured as the capacity to induce IFN-γ expression upon administration to swine PBMCs. In vitro, primary trials utilizing low concentrations of rIL-18 (<1 ng/mL) in the absence of co-stimulatory agents were unable to induce detectable IFN-γ protein expression. However, the addition of co-stimulatory agents Con A and rpIL-12 with higher yielding low volume rIL-18 preparations resulted in detectable levels of IFN-γ production by porcine PBMCs (Fig. 2.6). Only the mutIL-18 treatment group (Ad5+mutIL-18K:E3) induced IFN-γ protein levels appreciably above the negative controls consistently throughout the experiment. The Con A/rpIL-12 media control
group also displayed a considerably elevated concentration of IFN-γ at the latest time point (48 hours). The aberrant elevation in the IFN-γ concentration of the Con A/rpIL-12 media control group can be attributed to a single sample (140pg/mL) at 48 hours, while all others within that group were less than 6.0 pg/mL.

Similar findings were noted in biological activity assays utilizing concentrated rIL-18 preparations and rpIL-12 co-stimulation. High variance in measurements was the result of a large variability in the response to stimulation between pigs. PBMC samples from different pigs responded similarly to each treatment group, but displayed a high degree of variability in the level of sensitivity to stimulation between pigs. To control for the variability between test subjects, the respective negative control was subtracted from each sample (Ex. [Pig1-24h Ad5+mutIL-18K:F2] – [Pig1-24h Ad5B]) (Fig. 2.12). Figure 2.12 shows both mutIL-18 treatment groups resulted in the generation of appreciably elevated levels of IFN-γ protein expression compared to the negative control group and the wtIL-18 treatment group [Two-way ANOVA analysis (P<0.05)] (Fig. 2.11 & 2.12). As noted previously, the AD5+mutIL-18K:F2 isolate contains a frameshift insertion 13 bp upstream of the stop codon. The effects of the frameshift mutation are unknown but may be the cause of the reduction in biological activity compared to the Kozak [E] isolate (Ad5+mutIL-18K:E3).

The biological activity trials have indicated the response to treatment groups have a high degree of animal to animal variability. A genetic component, health status, or unidentified environmental factor may play significant role in response to treatment in the in vitro biological activity assay. Additional trials are needed to determine definitively the biological activity of the rIL-18 isolates. The establishment of protocols to generate rIL-18 samples of higher concentration would presumably aid in the testing of biological activity.
Repeated measures from the same blood sample may be needed to account for inherent variability between PBMCs from different subjects. The removal of viral or cellular products while maintaining a high concentration of rIL-18 protein would help ensure accurate measurements of biological activity of rIL-18 proteins.

While the removal of viral and cellular components was attempted by filtration prior to protein concentration, compounds between 10kD and 100kD MW remained and were concentrated 10-fold. Furthermore, adenoviral titers of concentrated protein samples were between $1.0 \times 10^9$ to $5.6 \times 10^9$ TCID$_{50}$/mL. Adenoviruses are able to infect lymphocytes and monocytes (Segerman et al., 2006; van der Veen and Lambriex, 1973). Even though test isolates are replication defective, infection alone may cause up-regulation of some immunological pathways. The adenovirus control group Ad5B was able to illicit some level of IFN-γ protein expression when PBMCs were co-stimulated with rpIL-12 or con A/rpIL-12. The induction of IFN-γ expression by Ad5B is concurrent with a previous study (Hartman et al., 2007). It was reported a similar LacZ expressing adenovirus was able to up-regulate Jak/STAT, MAPK, TLR, and apoptotic-related pathways as well as induce a MyD88 mediated IFN-γ response in vivo (Hartman et al., 2007).

In vivo, IFN-γ levels detected in serum and nasal wash samples are below the limits of the ELISA assay (~2 pg/mL). Further research is required to confirm current findings. With an estimated molecular weight of 17kD, low molecular weight centrifugal columns (10kD) could be utilized to concentrate IFN-γ samples 4 to 6 fold. Condensing sample volumes 4 to 6 fold would elevate IFN-γ protein concentrations in the nasal washes to levels within the limits of the ELISA.
Adenoviruses are well suited for mucosal administration, including oral and intranasal routes, able to elicit high IgA antibody titers and effective CD8+ T cell responses (Croyle et al., 2008; Phillpotts et al., 2005; Tang et al., 2009; Tucker et al., 2008; Walter et al., 2001; Xiang and Ertl, 1999). Intranasal administration of rIL-18 expressing adenoviruses did result in consistent, albeit low, IFN-γ induced expression following a time dependent pattern. Previous studies expressing IFN-α or IL-18 by similar adenoviral vectors in vivo also reported steep declines in expressed protein levels on or before day 3 post vaccination (Brockmeier et al., 2009; Walter et al., 2001).

The generation of a mutated IL-18 cDNA construct was confirmed by sequencing. Utilizing a CMV promoter and strong Kozak initiator sequences resulted in high levels of rIL-18 expression by both wtIL-18 and mutIL-18 adenoviral expression vectors. In vitro, biological activity of mutIL-18 isolates were shown to be appreciably higher than the negative control or wtIL-18 treatment groups. However, the biological activity of the wtIL-18 protein was consistently lower than expected. Future research may need to focus on developing methods to concentrate and purify the rIL-18 protein preparations. Previous literature utilized rIL-18 concentrations ≥100ng/mL to display biological activity (Liu et al., 2000; Munder et al., 1998; Ushio et al., 1996; Yamamoto et al., 2004). Generation of high concentration rIL-18 preparations would allow the evaluation of biological activity at standardized dilutions, correlating IFN-γ induction to rIL-18 concentrations in a dose dependent manner. While current findings need further evaluation, results indicate an increase in biological activity by the mutated IL-18 over the wild type porcine IL-18 as measured in vitro. In vivo the low levels of IFN-γ within the nasal swabs did not suggest an elevation in biological activity of mutIL-18 over the wild type form.
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CHAPTER 3 VECTOR-MEDIATED DELIVERY OF MUTATED INTERLEUKIN-18 WITH A MODIFIED LIVE SWINE INFLUENZA VACCINE CONVEYS ENHANCED PROTECTION AGAINST HETERO SUBTYPIC CHALLENGE

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Abstract

Current influenza vaccines effectively elicit protective immunity against antigenically similar strains of the same subtype, but not against the broader class of subtypes currently circulating. Broad cross-reactive immunity (heterosubtypic immunity) has been attributed to both humoral and cell mediated immune responses. However, at young ages, animals exhibit dysregulated immune functions, as compared to adults, including reduced expression of inflammatory mediators and diminished T_{H1} T cell response. Expression of an important T_{H1} pro-inflammatory cytokine, IL-18, is significantly diminished at the mucosal epithelium in young piglets. IL-18 is a critical signaling component for the development of a strong CMI response and effective viral clearance during influenza infection. Here we report the characterization of the immunomodulatory effects of recombinant porcine IL-18 expression
on the immune response to a modified live virus (MLV) swine influenza vaccine. Two amino acid conversions were introduced to wild type porcine IL-18 to generate a mutated analog exhibiting increased biological activity. Wild type and mutated IL-18 expression vectors were generated from a replication defective adenovirus and a modified live influenza virus. Vaccinations were administered as a single dose MLV influenza vaccine by intranasal route. Upon challenge by a heterosubtypic strain, the mutated IL-18 vaccination groups exhibited lower viral shedding, lower pulmonary viral titers, slightly reduced macroscopic lung lesion scores, and significantly elevated antibody responses. Flow cytometric analysis revealed significant differences in responding T cell populations between vaccination groups and expression vectors. Results indicate the expression vectors along with administration of IL-18 can contribute significantly to activate T cell responses.
1. Introduction

Influenza is an important human and animal pathogen typically causing high rates of morbidity in a diverse range of avian and mammalian hosts. In the northern hemisphere, influenza infection is characterized by seasonal epidemics occurring annually during the winter months. In mammals, influenza infection manifests as a mild to severe respiratory infection transmitted by large droplet aerosols or direct contact. In 2001, influenza associated diseases were ranked the seventh leading cause of death in the human population within the United States (Anderson and Smith, 2003). Worldwide, it is estimated 3 to 5 million severe cases of influenza infection will develop annually resulting in 250,000 to 500,000 deaths (WHO, 2003).

Influenza viruses evolve rapidly due to its segmented genome and error prone replicase in response to immunological pressure. The rapid changes to the major antigenic sites of influenza virus requires current vaccines continually be updated. Current influenza vaccines generally elicit protective immune responses to antigenically similar strains of the same subtype. However, antigenically distinct viruses of numerous subtypes currently circulate in human and swine populations. Even with current vaccine strategies, it is estimated seasonal influenza costs the US economy between $71-$116 billion annually (WHO, 2003). In the swine industry, influenza related disease was ranked as the second leading cause of economic losses for swine producers (Holtkamp et al., 2007). Broad cross-reactive immunity against multiple influenza subtypes (heterosubtypic immunity) has been noted following infection by live influenza viruses (reviewed in (Tamura et al., 2005). Due to the enhanced protective immune responses to live infection, attenuated modified live virus
(MLV) vaccines elicit better CMI responses than inactivated killed vaccines and tend to
induce stronger heterosubtypic immunity (Liang et al., 1994; Schulman and Kilbourne, 1965;
Tamura et al., 2005).

Vaccination against swine influenza virus is often completed at a young age in swine.
However, the neonatal immune system exhibits a host of dysregulated or insufficient immune
responses including pro-inflammatory pathways and biased of T cell polarization (Forsthuber
et al., 1996; Pertmer et al., 2001; Ridge et al., 1996; Sarzotti et al., 1996). In neonates, T cell
differentiation often results in a Th2 biased response due to an impaired ability to polarize T
cells to the Th1 phenotype (Breathnach et al., 2006; Forsthuber et al., 1996; Pertmer et al.,
2001; Sarzotti et al., 1996; Siegrist, 2001; Siegrist et al., 1998; Suen et al., 1998). Expression
of IL-18, an important cytokine involved in pro-inflammatory responses and T cell
polarization towards a Th1 response, is known to be markedly diminished in the respiratory
mucosal epithelium during the first months of a pig’s life (Muneta et al., 2002). The mucosal
surface of the porcine respiratory tract is a primary site of infection in young piglets. IL-18
knock out studies in mice have demonstrated the need for IL-18 in order to generate
protective responses against influenza infection (Billaut-Mulot et al., 2000; Denton et al.,
2007; Foss et al., 2001; Liu et al., 2004; Takeda et al., 1998). In IL-18 deficient (IL-18 –/–)
mice, influenza virus titers reached maximum levels earlier in the course of infection, had
significantly higher virus titers, significantly reduced IFN-γ induction, and lower activity
levels of natural killer cell-mediated cytolysis (Billaut-Mulot et al., 2000; Denton et al.,
2007; Dinarello, 1999; Foss et al., 2001; Liu et al., 2004; Takeda et al., 1998). Additionally,
IL-18 maturation is inhibited upon influenza infection by the NS1 protein (Egorov et al.,
1998).
The NS1 protein is involved in a variety of functions to evade host antiviral responses. NS1 inhibits mRNA nuclear export by binding of the poly(A) tail of mRNAs, hinders the polyadenylation of host mRNAs, and prevents pre-mRNA splicing by U6 spliceosome snRNA binding (Fortes et al., 1994; Nemeroff et al., 1998; Qiu et al., 1995). NS1 inhibits type I interferon activation via its carboxyl-terminal domain, preventing activation of signaling pathways such as 2’-5’ oligo (A) synthetase/RNase L, PKR, NF-kB, and IRF-3 (Geiss et al., 2002; Lu et al., 1995; Mibayashi et al., 2007; Min and Krug, 2006; Siren et al., 2006). Generating various N-terminal mutations to the NS1 protein, Stasakova et al. (2005) showed the NS1 protein inhibits the activation of the inflammasome complex resulting in a 5-fold reduction of biologically active IL-18 and 10-50 fold reduction in biologically active IL-1β (Stasakova et al., 2005). Inflammasome activation and subsequent induction of the ASC/caspase-1-dependent pathway is required for establishment of CD8+ T-cell responses and B-cell secretion of IgA antibodies at the mucosal surface (Petrilli et al., 2007; Stasakova et al., 2005), both critically important to mucosal immunity. NS1 is believed to inhibit inflammasome activation by binding ssRNA and dsRNA to reduce detection by PRR receptors such as TLR3, TLR7 and RIG-I, although exact mechanisms are still being characterized.

To evaluate the immunomodulatory effect of IL-18 administration in conjunction with a one dose modified live swine influenza vaccine, viral vectors were generated to express recombinant IL-18 (rIL-18). Previous work by Kim et al. (2001) on human IL-18 has shown the mutation of two amino acids involved in IL-18 binding to the inhibitory IL-18 binding protein (IL-18BP) resulted in extended half-life and a 4-fold increase in biological activity (Kim et al., 2001; Kim et al., 2000). To evaluate if homologous mutations to porcine
IL-18 would result in similar findings, a porcine mutIL-18 homolog was generated from wild type porcine IL-18. For use in vaccines, replication-defective adenovirus expression vectors delivering either wild type IL-18 (Ad5+wtIL-18) or mutated IL-18 (Ad5+mutIL-18) were co-administered with a H3N2 triple reassortant modified live swine influenza virus (Garcia-Sastre et al., 1998; Richt et al., 2006; Solorzano et al., 2005; Vincent et al., 2007) containing a 93 amino acid carboxyl-terminal truncation of the NS1 gene [Tx98NS1\(\Delta\)126 MLV]. To generate a single component influenza vaccine which expresses IL-18, the mutIL-18 cDNA construct was inserted into the truncated region of the NS1 [\(\Delta\)126] gene of this virus. The resulting recombinant modified live virus [MLV+mutIL-18] was confirmed to express rIL-18 using a swine IL-18 ELISA. Here we report the immunomodulatory properties of rIL-18 in vivo when co-administered in one of two replication defective adenovirus vectors (MLV+Ad5wtIL-18 or MLV+Ad5mutIL-18) or when given as a single virus construct in the form of the MLV+mutIL-18. Efficacy of IL-18 as an adjuvant to influenza vaccination was evaluated in a heterosubtypic swine influenza virus challenge (H1N1 IA04) model in young pigs.

2. Materials and Methods

2.1 Mutagenesis of porcine IL-18

Dual point mutations were introduced into a porcine wild type IL-18 encoding cDNA construct by PCR primer site-directed mutagenesis. Two sets of PCR primers were designed to individually introduce amino acid conversions at positions 41 (E to A) and 88 (K to A).
Mutations and full-length sequence fidelity was confirmed by sequencing (Fig. 2.1). The mutIL-18 plasmid was previously generated by Ma et al. (unpublished data).

2.2 Generation of Tx98NS1Δ126 MLV containing mutIL-18

Live influenza A virus Tx98NS1Δ126 (MLV) (A/Swine/Texas/4199-2/98) was generated by reverse genetics as previously described (Solorzano et al., 2005). All genomic segments of the Tx98NS1Δ126+mutIL-18 recombinant influenza virus were unaltered except for the NS1Δ126 genetic segment. The NS1 gene of Tx98NS1Δ126+mutIL-18 was generated by reverse genetics incorporating the pro form of mutIL-18 to the 3’ end of the NS1Δ126 gene fragment. The Tx98NS1Δ126+mutIL-18 (MLV+mutIL-18) recombinant virus was recovered by the same reverse genetic techniques as the MLV (Solorzano et al., 2005).

2.3 In vitro expression of rIL-18

Expression of rIL-18 by MLV+mutIL-18 was confirmed by swine IL-18 enzyme-linked immunosorbent assay (ELISA) (Invitrogen, Grand Island, NY) and by immunofluorescence assay (IFA). Characterization of expression by adenoviral vectors containing rIL-18 was described previously (Kappes et al., 2009).

Immunofluorescence assay (IFA): Microtiter plates were seeded with low passage MDCK cells and allowed to grow to confluency. Cell sheets were inoculated with MLV, MLV+mutIL-18, or with plain media as a cell culture control in triplicate. At 24 hour post inoculation (p.i.), cell sheets were washed with sterile phosphate buffered saline (PBS) three
times prior to treatment with 10 μg/mL goat anti-porcine IL-18 and incubated for one hour. Wells were again washed with sterile PBS three times, treated with fluorescein isothiocyanate (FITC)-labeled donkey anti-goat antibody, and incubated for 1 hour. Cell sheets were washed a final time with sterile PBS prior to reading.

**Swine IL-18 ELISA:** In four independent trials, 90-100% confluent low passage MDCK cells were inoculated at an approximate multiplicity of infection (MOI) of 25 and incubated at 37°C, 5% CO₂ for the duration of each time point. Samples were collected every 12 hours for two days. Just prior to use, samples were freeze/thawed (F/T) a total of 3 times to maximize the release of intracellular rIL-18. Measurement of rIL-18 protein was performed in duplicate using a swine IL-18 ELISA (Invitrogen, Grand Island, NY) per manufacturer’s instructions.

### 2.4 In vitro assessment of rIL-18 biological activity

Biological activity of rIL-18 expressed protein was assessed by using porcine peripheral blood mononuclear cell (PBMC) responsiveness to rIL-18 treatments, as measured by secretion of IFN--γ into PBMC culture supernatants. Porcine PBMCs were isolated by Vacutainer CPT™ tubes or by density gradient separation over a percoll density gradient (1.084 sp gr) from whole blood collected with 2X acid citrate dextrose (ACD) as anticoagulant (Ulmer et al., 1984). Multiple trials were performed utilizing selected co-stimulatory agents and rIL-18 preparation methods. rIL-18 stimulated PBMCs were cultured with RPMI 1640 media and incubated at 39°C, 5% CO₂ for the duration of each time point. Samples were freeze/thaw a total of three times to release intracellular IFN--γ.
Measurements of IFN-γ in cell lysate supernatants was performed in duplicate using a swine IFN-γ ELISA per manufacturer’s instructions (Invitrogen, Grand Island, NY).

2.5 Hemagglutination Inhibition Assay

Sera samples were prepared by heat inactivation of complement by incubation at 56°C for 30 minutes followed by incubation at room temperature with a 20% kaolin suspension for 30 minutes to remove nonspecific inhibitors. Finally, sera were incubated with a 0.5% (v/v) turkey red blood cells (RBC) solution for 20 min at room temperature. MDCK propagated Tx98 and IA04 influenza A stocks were diluted to an HA=8 for use as standard antigen in HI tests. Each treated serum sample was transferred in triplicate as a 1:10 dilution to a 96-well microtiter plate and 2-fold serial dilutions were performed. Standard HI tests were performed for each sample.

2.6 Virus titration

Bronchoalveolar lavage fluid (BALF), nasal swab samples, and influenza inoculum samples were titrated by TCID_{50} as described by Reed and Muench (Muench, 1938) on MDCK cells grown in 96-well plates. Adenovirus inocula were titrated as described in (AdEASY™ vector manual, v1.4 Q BIOgene, Carlsbad, CA) by TCID_{50}. In short, AD-HEK-293 cells were plated in 96-well plates and allowed to adhere for 1 hour. Ten fold serial dilutions of each adenovirus sample from 10^{-1} to 10^{-11} per 100 μL were prepared and plated in replicates of eight. Plates were incubated at 37°C and 5% CO_{2} for 12 days. On day 12, plates
were scored for highest dilution exhibiting CPE in each row and the TCID<sub>50</sub> calculated by the formula:

\[
\text{TCID}_{50}/\text{mL} = 10^{1+\text{d}(s^{-0.5})} \times 10, \text{ where } d=\log_{10}(\text{dilution}), s= \text{ sum of ratios}
\]

2.7 rIL-18/influenza MLV Animal Study outline

Forty-eight three week old pigs were treated with ceftiofur crystalline antibiotic (EXCEDE™, Pharmacia) per manufacturer’s recommend dose and randomly separated into six groups of eight to be housed for one week prior to primary inoculation. All pigs were confirmed seronegative for SIV by hemagglutination inhibition assays to H1N1 and H3N2 antigen and free from detectable influenza infection by virus isolation from nasal swabs on day 0 post vaccination. At day 0 post vaccination, adenovirus and influenza inocula were prepared by dilution in sterile PBS just prior to use. Inocula were administered or co-administered intranasally by slowly dripping 2 mL of each inoculum into the nasal cavity of each pig as specified in table 3.1.

Table 3.1 Swine influenza MLV vaccine animal study (rIL-18/MLV): group outline

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Description</th>
<th>Name</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>Adeno 5 w/ mutSwIL-18 Insertion</td>
<td>Ad5mutIL-18</td>
<td>IA04</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Tx98NS1Δ126 + Adeno 5 w/ wtSwIL-18 Insertion</td>
<td>MLV+Ad5wtIL-18</td>
<td>IA04</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Tx98NS1Δ126 + Adeno 5 w/ mutSwIL-18 Insertion</td>
<td>MLV+Ad5mutIL-18</td>
<td>IA04</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Tx98NS1Δ126 w/ mutSwIL-18 insertion</td>
<td>MLV+ml-18</td>
<td>IA04</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>Tx98NS1Δ126</td>
<td>MLV</td>
<td>IA04</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>Wild type Tx98</td>
<td>wtTx98</td>
<td>IA04</td>
</tr>
</tbody>
</table>

Back titration of adenovirus and influenza inocula yielded approximately 1 X 10<sup>9</sup> TCID<sub>50</sub>/mL and 1X10<sup>6.5</sup> TCID<sub>50</sub>/mL respectfully. At day 65 post vaccination, all subjects were challenged with a heterologous H1N1 wild-type influenza A strain IA04.
(A/Swine/Iowa/00239/2004) by intranasal inoculation at a dose of 1X10^{6.83} TCID_{50}/mL. Pigs were observed for clinical manifestation of disease for 5 days after challenge (dpc). Rectal temperatures were recorded from day -2 to +5 dpc. Nasal swabs were obtained for 0, 2, 4, 6, 65, 68, 70 dpv and serum samples collected on 0, 2, 4, 6, 14, 21, 28, 49, 56, 65, 70 dpv. PBMCs were collected using Vacutainer CPT™ tubes containing sodium citrate (BD Diagnostics, Franklin Lakes, NJ) on days 14, 28, 49, 56, and 70 dpv. Virus isolation and virus titration assays was performed on nasal swabs for all days collected. Hemagglutination inhibition assays to H3N2 antigen were performed for all days serum was collected and to H1N1 antigen for 0, 65 and 70 dpv. Pigs were euthanized on day 70 post vaccination (5 days post challenge) and necropsy performed. Bronchoalveolar lavage fluid (BALF) (50 mL MEM) was collected for virus titration as described in Vincent et al. 2007. Samples of lung tissue, trachea and the tracheobronchial lymph node were fixed in 4% buffered formalin for histopathological evaluation. The presence of macroscopic lung lesions for all seven lung lobes was examined and scored.

2.8 Cells

MDCK cells were cultured in Minimal essential medium (MEM) supplemented with 5% heat inactivated fetal bovine serum, 100 units of penicillin, 100 µg of streptomycin, and 0.25/mL µg of amphotericin B, 1X L-Glutamine, 1X MEM Vitamins (Gibco, Grand Island, NY) and 0.25 mg/L gentamicin sulfate for maintenance. MDCK cells were transferred to influenza infection media containing Minimal essential medium (MEM) supplemented with 4% bovine serum albumin Fraction V, supplemented with antibiotics and nutrients as stated
above, for propagation of influenza virus and in vitro influenza assays. TPCK [L-
(tosylamido-2-phenyl) ethyl chloromethyl ketone] – Trypsin was added at a 1:1000 dilution
to influenza infection media just prior to use to cleave the HA viral glycoprotein and
facilitate viral entry into MDCK cells.

AD-HEK-293 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM)
high glucose 1X with 4.5 g/L D-glucose, L-Glutamine, and 110 mg/L sodium pyruvate,
supplemented with 5% heat inactivated fetal bovine serum, and antibiotics plus antimycotics
as described above for maintenance of cell line. For use in adenoviral assays, heat
inactivated fetal bovine serum was reduced to 2% (v/v), all else held constant.

Peripheral blood mononuclear cells used for in vitro assessment of IL-18 biological
activity and in ex vivo flow cytometry assays were obtained by venous puncture and cultured
in RPMI 1640 media containing L-glutamine and 25mM HEPES, supplemented with 10%
fetal bovine serum, and antibiotics plus antimycotics as described above.

2.9 Virus

Influenza A viruses wtTx98 H3N2 (wtTx98) (A/Swine/Texas/4199-2/98),
Tx98NS1Δ126 (MLV) (A/Swine/Texas/4199-2/98), Tx98NS1Δ126+mSwIL-18
(MLV+mutIL-18) (A/Swine/Texas/4199-2/98) and wtIA04 H1N1
(A/swine/Iowa/00239/2004) were propagated in the allantoic cavity of 7-10 day old
embryonated chicken eggs. Allantoic stocks of each virus were diluted in sterile PBS to 1 X
10^{6.5} TCID50/mL on the day of administration for use as inoculums.
Low passage replication-defective adenovirus isolates expressing wtIL-18 [Ad5+wtIL-18] and mutIL-18 [Ad5mutIL-18] were propagated in specialized AD-HEK-293 cells genetically altered to support replication. For use as inoculums, adenovirus isolates were purified and concentrated by double CsCl density gradients as described in the (AdEasy™ vector manual v1.4 Q BIOgene, Carlsbad, CA). In short, 40 T150 cell culture flasks (Corning, Corning, NY) were inoculated per virus. Samples were collected when CPE had reached 100%. Media was consolidated and spun at 300 rcf for 10 min to obtain a single cell pellet, discarding the supernatant. Pellets were F/T three times to lyse cells and release the virus. Discontinuous gradients (1.4-1.2 CsCl sp gr) were prepared in 50mL ultracentrifuge tubes. Samples were overlaid on top of discontinuous gradient and centrifuged at 100,000 rcf for 90 minutes. Concentrated adenovirus was collected by aspiration of the adenoviral band and immediately overlaid on previously prepared continuous gradients (1.4-1.2 CsCl sp gr) and spun at 100,000 rcf for 20-24 hours. Resulting concentrated adenoviral isolates were desalted by dialysis in 10 mM Tris (pH 8.0), 2 mM MgCl₂, 4% sucrose buffer. Purified adenoviruses were titered by tissue culture infectious dose 50% (TCID₅₀/mL) as described in the AdEasy™ vector system manual. Adenoviral inoculums were diluted to 1.0 X 10⁹ TCID₅₀/mL in sterile PBS shortly before administration.

Influenza A viruses wtTx98 H3N2 (A/Swine/Texas/4199-2/98), Tx98NS1Δ126 (MLV) (A/Swine/Texas/4199-2/98), and wtIA04 H1N1 (A/swine/Iowa/00239/2004) were propagated in MDCK cells with influenza infection media for use in CMI flow cytometry assays. An aliquot of each virus was UV inactivated by 840 mJoules over the course of 270 seconds in three successive 90-second intervals, approximately 2-3 seconds between intervals. Both live and UV-inactivated viruses were aliquoted and stored at -80°C until use.
2.10 Cell Mediated Immunity (CMI) assay

Influenza A viruses wtTx98, MLV, and wtIA04 viruses were prepared as previously described and diluted to 3 X 10^6 TCID₅₀/ml in RPMI++ (RPMI 1640 (Gibco marketed by Invitrogen, Grand Island, NY) with 15% fetal bovine serum (Atlanta biologicals, Norcross, GA) and antibiotics plus antimycotics as described above.

Peripheral blood mononuclear cells were obtained as previously described on days 28, 49, 56, 70 dpv. Vacutainer CPT tubes were centrifuged at 1500xg for 30 minutes at room temperature (~25°C) within 2 hours following blood collection. Buffy coats were collected into 15 mL conical centrifuge tubes (Falcon, BD Labware, Franklin Lakes, NJ). PBS was added to a final volume of 10 mL and samples were centrifuged at 300 rcf for 10 minutes. Discarding the supernatants, the wash step was repeated once. Each PBMC pellet was resuspended with 2 mL RPMI++ and mixed well. The cell suspensions were counted and 10⁷ PBMC were diluted with RPMI++ to a final volume of 2 mL (5x10⁶ cells/ml final concentration).

Two hundred µl of each PBMC suspension with ~5 X 10⁶ cells/mL were added to each of 6 wells of a 96-well flat-bottomed tissue culture microtiter plate (Falcon cat, BD Labware, Franklin Lakes, NJ). One well received 50 µL RPMI++ as non-antigen stimulated control and another well received 50 µl Con A solution at 5 µg/ml final concentration (as a positive control). Duplicate wells received 50 µL of live Tx98wt, Tx98Δ126, and IA04wt viruses or UV-inactivated Tx98wt or IA04wt viruses. The plates were incubated in 5% CO₂ at 37°C.
Four days after activation, PBMCs were transferred to round-bottomed tissue culture microtiter plates (Falcon cat, BD Labware, Franklin Lakes, NJ). Brefeldin A (50 µl) (BD Biosciences Pharmingen, San Diego, CA) was added to the samples at the concentration recommended by the manufacturer. The plates were incubated for an additional 4 hours. All antibodies used in the staining were previously titrated for their optimum concentrations in the same PBMC setting (data not shown). All washing steps were performed by centrifugation at 400xg for 1 minute. The PBMC wells were washed once with 100 µl PBS++ (PBS with 0.5% bovine serum albumin, and 0.1% sodium azide) and the supernatants were removed. The primary antibody mix for surface antigens (50 µl) was added to all PBMC wells except the secondary antibody control wells and incubated at room temperature (RT, 25°C) for 15 minutes. The monoclonal antibody (mAb) mix consisted of mouse anti-swine CD4 (IgG2b, cat# 74-12-4), mouse anti-swine CD8 (IgG2a, cat# 76-2-11), mouse anti-swine γδ TCR-FITC (IgG1, cat# PGBL22A, direct conjugated with fluorescein isothiocyanate), mouse anti-swine CD25-PE (IgG1, cat# PGBL25A, direct conjugated with phycoerythrin) in PBS++. All primary mAb were purchased from VMRD, Inc., (Pullman, WA). The direct conjugation of fluorochromes was performed by Chromaprobe (Maryland Heights, MO). The primary antibody mix was removed after incubation and cells were washed twice with 100 µL PBS++. The secondary antibody mix for surface antigens (50 µL) was added to all PBMC wells included the secondary antibody control wells and incubated at room temperature for 15 minutes. The secondary antibody mix consisted of goat anti-mouse IgG2b-PE-Cy-7 and goat anti-mouse IgG2a-PE-TR in PBS++. The secondary antibody mix was removed after incubation and the cells were washed three times with 100 µL PBS++. 
For intracellular staining, the PBMC were treated with 100 μL BD cytofix-cytoperm solution (BD Biosciences Pharmingen, San Diego, CA) at room temp for 30 minutes and washed once with BD perm-wash solution (BD Biosciences Pharmingen, San Diego, CA). The primary monoclonal antibody (mAb) mix consisted of rabbit anti-swine IFN-γ (polyclonal Ab, cat# AT-3072) and mouse anti-swine IL-10 biotin (IgG1, cat# AT-3075), both from MBL International (Woburn, MA), in BD perm-wash solution (50 μL) was added to all wells except the secondary antibody control wells and incubated at RT for 15 minutes. The intracellular primary antibody mix was removed after incubation and cells were washed twice with 100 μL BD perm-wash solution. Goat anti-rabbit IgG-AF700 (Alexa Fluor 700, Invitrogen, Molecular Probes, Eugene, OR, cat #A21038) and streptavidin-APC-Cy7 (eBioscience, Inc., San Diego, CA, cat #10-4317-82) (50 μL) in BD perm-wash solution was added to all wells included the secondary antibody control wells and incubated at RT for 15 minutes. Cells were washed three times with 100 μL BD perm-wash solution. Ultrapure formaldehyde (Polyscience, Warrington, PA) 1% solution in PBS (250 μL) was added to all wells and the cells were transferred to flow tubes (Falcon cat # 352008, BD Labware, Franklin Lakes, NJ) and kept in the dark at 4°C until flow cytometric analysis. The Canto (Beckman Coulter, Inc. Fullerton, CA) flow cytometer was used to analyze the samples at the Cell and Hybridoma Facilities, Iowa State University.
3. Results

3.1 Generation of mutated porcine IL-18 by site-specific mutagenesis

Two amino acid conversions were introduced into the wild type porcine IL-18 gene at amino acid positions 41 and 88 by site-directed mutagenesis. Sequencing confirmed desired mutations in the porcine mutIL-18 cDNA (Fig. 2.1).

Figure 3.1 Amino acid alterations between mutIL-18 and wtIL-18

<table>
<thead>
<tr>
<th>Wild type Porcine IL18</th>
<th>Mutated Porcine IL18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe Gly Lys Leu</td>
<td>Pro Lys Leu Ser</td>
</tr>
<tr>
<td>TTT GGC AAG CTT GAA CTT AAG CTC TCA</td>
<td></td>
</tr>
<tr>
<td>Phe Gly Lys Ala Pro Lys Leu Ser</td>
<td></td>
</tr>
<tr>
<td>TTT GGC AAG CTT GCT CTT AAA CTC TCA</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3.1. Sequence analysis of site-directed mutagenesis to porcine IL-18 cDNA at amino acid positions 41 and 88.

3.2 Expression of rIL-18 in vitro

Figure 3.2-3.5 illustrates IL-18 expression of IL-18 as a result of the MLV+mutIL-18 mediated IL-18 expression in MDCK cells by immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA); no IL-18 staining was detected in negative controls by either method. Both wtTx98 and MLV inoculation groups failed to produce measureable IL-18 expression at any time point. MLV+mutIL-18 treatment group was confirmed by IL-18 ELISA to express detectable levels of rIL-18 up to ~100 pg/mL.
Fig. 3.2-3.4. MDCK cells were inoculated with either plain media (cell culture control), MLV (influenza virus control), or MLV+mutIL-18 virus. Cells were stained by a goat anti-porcine IL-18 Ab, followed by an FITC labeled secondary anti-goat Ab. Green staining indicates presence of IL-18 protein.
3.3 Biological Activity

*In vitro* biological activity of wild-type rIL-18 (wtIL-18) and mutated IL-18 (mutIL-18) were assessed by interferon-γ (IFN-γ) induction of rIL-18 treated porcine PBMCs. A variety of parameters such as rIL-18 preparation and concentration, co-stimulatory agents, PBMC isolation methods, and PBMC densities was evaluated in an attempt to optimize the biological activity assay (Chapter 2). Further research is required to determine the biological activity of wtIL-18 and mutIL-18 proteins. Results and Biological activity trials are described in Chapter 2.
3. Serological responses to vaccination

**Figure 3.6** Hemagglutination inhibition (HI) titers [H3N2] by day post vaccination

**Hemagglutination Inhibition (HI) Titers:** All pigs were serologically negative to H3N2 and H1N1 antigens by HI assay on day 0 post vaccination and remained negative to H1N1 antigen for the duration of the study. The negative control treatment group (Ad5mutIL-18) remained negative to H3N2 & H1N1 throughout the study. HI titers reached their highest levels by days 22 to 28 post vaccination for all groups. By day 49 post vaccination, the MLV+Ad5mutIL-18 sustained a significantly higher antibody response compared to the Ad5B control group (Table 3.2). By day 56 post vaccination, both mutIL-18 vaccination groups [MLV+AD5mutIL-18 & MLV+mutIL-18] were significantly higher than the negative control group. HI titers for wtTx98 reached significantly higher levels in relation to all other treatment groups by day 14 post vaccination and remained significantly higher for the remainder of the study. The antibody response as a result of heterosubtypic
challenge was measured at days 3 and 5 post challenge (Fig. 3.6, Table 3.2). By day 5 post challenge, all three treatment groups expressing rIL-18 exhibited significantly higher antibody titers as compared to the negative control group, while the MLV vaccination alone did not. The MLV+Ad5mutIL-18 HI titers were also significantly higher than the MLV and the negative control groups by day 5 post challenge.

**Figure 3.7 Nasal swab samples influenza viral titers [TCID$_{50}$] post vaccination**

![Graph showing nasal swab samples influenza viral titers](image)

Fig. 3.7. Values are reported as the Log$_{10}$ transformed [TCID$_{50}$/mL] group means ± SE influenza viral titers of nasal swab samples collected on days 2, 4, and 6 post vaccination. Virus titers on day 0 of the study were below detectable limit of the assay for all groups (data not shown).

**Viral shedding:** Viral shedding from vaccination and control groups was calculated by titration of nasal swab sample collected on days 2, 4, and 6 post vaccination (Fig. 3.8, Table 3.3) and days 3 and 5 post challenge (Fig. 3.8, Table 3.3). No influenza virus was detected in nasal swabs on day 0 of the study (data not shown). Mean values of viral shedding from the wtTx98 group were approximately 1 X 10$^4$ TCID50/mL on days 2 and 4 post vaccination, recovering to less than 1 X 10$^1$ TCID50/mL by day 6. No influenza virus
was detected in the nasal swabs of the negative control group at any time point prior to challenge. MLV vaccination produced low level viral shedding on days 2 and 4 post vaccination (≤$10^{0.54}$ TCID$_{50}$/mL). A single measureable titer was detected for vaccination group MLV+mutIL-18 on day 4 post vaccination [$10^{0.69}$ TCID$_{50}$/mL] and MLV+Ad5wtIL-18 vaccination group on day 2 post vaccination [$10^{0.31}$ TCID$_{50}$/mL]. No viral shedding was detected at any day prior to challenge for MLV+Ad5mutIL-18. All vaccination groups were negative by day 6 except the wild-type control (wtTx98).

Figure 3.8 Nasal swab samples influenza viral titers [TCID$_{50}$] post challenge

![Graph showing viral titers](image)

Fig. 3.8. Values are reported as the Log$_{10}$ transformed [TCID$_{50}$/mL] group means ± SE influenza viral titers of nasal swab samples collected on days 3 and 5 post challenge.

The evaluation of viral shedding after challenge was measured on days 3 and 5 post challenge (Fig. 3.8, Table 3.3). By day 3 post challenge, all pigs were positive to a mean group titer of approximately $1 \times 10^3$ TCID$_{50}$/mL. Both MLV+Ad5mutIL-18 and wtTx98 treatment groups exhibited significantly (P<0.05) lower viral shedding than the MLV+Ad5wtIL-18 treatment group on day 3 post challenge. By day 5 post challenge, all vaccination groups and the wtTx98 control group were significantly (P<0.05) lower than the
negative control group. The MLV+Ad5mutIL-18 vaccination group also resulted in significantly lower (P<0.05) viral shedding than the MLV vaccination group by 5 days post vaccination.

**Figure 3.9 BALF viral titer**

Fig 3.9 BALF (50mL MEM) was collected at time of necropsy. Influenza titers [TCID\textsubscript{50}/mL] from each pig were Log\textsubscript{10} transformed. Group means ± SE of the Log\textsubscript{10} transformed data are reported. Differences between groups were statistically significant [One-way ANOVA (P<0.0001)]

**BALF viral titters:** BALF samples were obtained at time of necropsy on day 5 post challenge. The negative control group exhibited significantly higher viral titers than all other vaccination groups (P<0.0001). All three rIL-18 expressing treatment groups resulted in lower BALF virus loads than the negative control group, the MLV vaccination group or the wtTx98 group (Figure 3.9, Table 3.3), although not by a statistically significant margin.
Table 3.2  Hemagglutination inhibition (HI) titers [H3N2 antigen] by treatment group

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>0 ±0.00</th>
<th>+6 ±0.00</th>
<th>+14 ±0.00</th>
<th>+22 ±0.00</th>
<th>+28 ±0.00</th>
<th>+49 ±0.00</th>
<th>+56 ±0.00</th>
<th>Post Challenge ±0.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5mutIL18</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
</tr>
<tr>
<td>MLV+Ad5wtIL-18</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>19.4 ±5.19 B</td>
<td>18.1 ±4.80 B</td>
<td>18.1 ±4.00 B</td>
<td>11.3 ±2.27 A</td>
<td>7.5 ±2.07 A</td>
<td>9.7 ±1.32 B</td>
</tr>
<tr>
<td>MLV+Ad5mutIL-18</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>23.1 ±8.52 B</td>
<td>19.7 ±6.30 B</td>
<td>23.8 ±6.90 B</td>
<td>14.1 ±5.40 B</td>
<td>14.0 ±5.42 B</td>
<td>17.5 ±6.49 B</td>
</tr>
<tr>
<td>MLV+mutIL-18</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>27.8 ±8.15 B</td>
<td>18.8 ±6.40 B</td>
<td>23.3 ±5.90 B</td>
<td>12.5 ±4.14 A</td>
<td>16.0 ±3.97 B</td>
<td>15.0 ±3.74 B</td>
</tr>
<tr>
<td>MLV</td>
<td>0.0 ±0.00 A</td>
<td>0.0 ±0.00 A</td>
<td>19.4 ±6.47 B</td>
<td>15.9 ±5.60 B</td>
<td>19.1 ±6.10 B</td>
<td>9.1 ±3.56 A</td>
<td>8.8 ±2.81 A</td>
<td>12.8 ±3.11 B</td>
</tr>
<tr>
<td>wtTx98</td>
<td>0.0 ±0.00 A</td>
<td>3.4 ±1.72 A</td>
<td>105.6 ±39.1 C</td>
<td>131.4 ±69.00 C</td>
<td>117.0 ±40.00 C</td>
<td>83.1 ±37.60 C</td>
<td>82.0 ±40.52 C</td>
<td>101.9 ±31.35 C</td>
</tr>
</tbody>
</table>

1 Values are reported as group mean ± SE of HI titers. Mean results labeled as zero were below the detectable limit of the assay. Differences between groups were significant [Two-Way ANOVA (P < 0.001)].

Table 3.3  Quantitation of viral shedding and pulmonary viral titers [TCID50/mL]

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>0 ±0.00</th>
<th>+6 ±0.00</th>
<th>+14 ±0.00</th>
<th>+22 ±0.00</th>
<th>+28 ±0.00</th>
<th>+49 ±0.00</th>
<th>+56 ±0.00</th>
<th>Post Challenge ±0.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5mutIL18</td>
<td>0.00±0.00 A (0/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>2.87±0.16 A B (8/8)</td>
<td>3.37±0.13 (8/8)</td>
<td>3.73±0.13 (8/8)</td>
<td>5.29±0.20 (8/8)</td>
<td></td>
</tr>
<tr>
<td>MLV+Ad5wtIL-18</td>
<td>0.31±0.21 A (1/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>3.33±0.17 B (8/8)</td>
<td>1.31±0.21 A B (7/8)</td>
<td>0.77±0.38 A (3/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLV+Ad5mutIL-18</td>
<td>0.00±0.00 A (0/8)</td>
<td>0.69±0.33 B (3/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>2.79±0.22 A (8/8)</td>
<td>1.02±0.22 B C (7/8)</td>
<td>0.62±0.41 A (2/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLV+mutIL-18</td>
<td>0.00±0.00 A (0/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>2.91±0.19 A B (8/8)</td>
<td>1.54±0.38 A B (6/8)</td>
<td>0.62±0.41 A (2/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLV</td>
<td>0.54±0.36 A (2/8)</td>
<td>0.23±0.23 A B (1/8)</td>
<td>0.00±0.00 A (0/8)</td>
<td>2.96±0.17 A B (8/8)</td>
<td>1.62±0.21 A (8/8)</td>
<td>1.66±0.71 A (4/8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wtTx98</td>
<td>3.95±1.33 (8/8)</td>
<td>3.96±0.29 (8/8)</td>
<td>0.62±0.43 B (2/8)</td>
<td>2.75±0.08 A (8/8)</td>
<td>0.29±0.20 C (2/8)</td>
<td>1.08±0.41 A (4/8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Values are reported as means ± SE of Log10 transformed TCID50 influenza viral titers. Mean results labeled as zero were below the detectable limit of the assay. Significance between group means by Two-Way ANOVA analysis is significant (P<0.0001). Treatment groups not connected by the same letter are significantly different from each other (P<0.05) by multivariate Heterogeneous Compound Symmetry residual analysis. 
2 Significance is reported between group means by One-Way ANOVA analysis (P<0.0001). Treatment groups not connected by the same letter are significantly different from each other (P<0.05) by Tukey HSD Multiple Comparison test.
Figure 3.10 Mean rectal temperatures (°C) of adenovirus vaccination groups post challenge

Figure 3.11 Mean rectal temperatures (°C) of non-adenovirus vaccination groups post challenge

Fig. 3.10-3.11. Rectal temperatures were recorded from days 0 to 5 post challenge. Values are reported as group means ± SE. Differences between groups were statistically significant [Two-way ANOVA analysis (P<0.05)]

Febrile response to heterosubtypic challenge: Rectal temperatures were recorded between days 0 to 5 post challenge (Fig. 3.10-3.11, Table 3.4). A significant difference between groups is reported (p<0.05) by Two-way ANOVA analysis. As noted in table 3.4, the MLV+mutIL-18 vaccination group had significantly lower mean rectal temperatures on day 3 post challenge compared to the negative control group.

Table 3.4 Rectal temperatures days 0-5 post challenge by group

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Days post Challenge 0</th>
<th>Days post Challenge 1</th>
<th>Days post Challenge 2</th>
<th>Days post Challenge 3</th>
<th>Days post Challenge 4</th>
<th>Days post Challenge 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5mSwIL18</td>
<td>38.5 ±0.3 A</td>
<td>38.5 ±0.2 A</td>
<td>38.6 ±0.2 A</td>
<td>39.2 ±0.1 A</td>
<td>38.7 ±0.1 A</td>
<td>38.6 ±0.2 A</td>
</tr>
<tr>
<td>Txd126+Ad5wtSwIL18</td>
<td>38.9 ±0.1 A</td>
<td>39.2 ±0.2 A</td>
<td>39.1 ±0.1 A</td>
<td>38.9 ±0.2 A</td>
<td>38.7 ±0.2 A</td>
<td>38.0 ±0.3 A</td>
</tr>
<tr>
<td>Txd126+Ad5mSwIL18</td>
<td>38.7 ±0.1 A</td>
<td>38.3 ±0.1 A</td>
<td>38.8 ±0.2 A</td>
<td>38.5 ±0.3 A</td>
<td>38.5 ±0.1 A</td>
<td>38.1 ±0.2 A</td>
</tr>
<tr>
<td>Txd126 w/ mSwIL18</td>
<td>38.6 ±0.2 A</td>
<td>38.5 ±0.2 A</td>
<td>38.4 ±0.2 A</td>
<td>38.1 ±0.2 B</td>
<td>38.4 ±0.2 A</td>
<td>38.1 ±0.2 A</td>
</tr>
<tr>
<td>Tx98 w/126</td>
<td>38.8 ±0.2 A</td>
<td>39.1 ±0.1 A</td>
<td>38.9 ±0.2 A</td>
<td>38.8 ±0.1 A</td>
<td>38.3 ±0.1 A</td>
<td>38.6 ±0.1 A</td>
</tr>
<tr>
<td>Tx98 wt</td>
<td>38.8 ±0.1 A</td>
<td>38.5 ±0.2 A</td>
<td>38.9 ±0.2 A</td>
<td>38.7 ±0.1 A</td>
<td>38.4 ±0.2 A</td>
<td>38.2 ±0.1 A</td>
</tr>
</tbody>
</table>

1 Values are reported as group means ± SE (N=8). Differences between group means were statistically significant [Two-Way ANOVA (p<0.01)]. Levels not connected by the same letter are significantly different from each other (P<0.05) as calculated by residue compound symmetry family wise comparison.
Fig. 3.12-3.15. Lungs were removed in toto and macroscopic lung lesions of each individual lobe were scored as a percent surface area of pneumonic lung lesions. As seen in figures 3.14 & 3.15, macroscopic lung lesions are plum colored consolidated areas noted on all lobes of the lung.
**Figure 3.16** Macroscopic lung lesion score [% surface area pneumonic lesions]

![Graph showing macroscopic lung lesion scores by treatment group](image)

Fig. 3.16. Macroscopic lung lesions were scored as a percent surface area exhibiting pneumonic lung lesions on each lobe. Total weighted averages of the surface area were calculated for each lung. Values are reported as group means ± SE. Differences between groups were not statistically significant (P>0.05) as calculated by One-way ANOVA analysis.

**Macroscopic lung Lesions:** At time of necropsy, each lung was removed *in toto* and each lobe of the lung was scored as a percent surface area of the lung afflicted with pneumonic lesions. Typical lesions resulting from SIV infection are characterized by consolidated, plum-colored areas on the lung lobes, as seen in Figures 3.14 & 3.15. A weighted average of the percent total surface area for each lung was calculated (Fig. 3.16, Table 3.5).

<table>
<thead>
<tr>
<th>Macroscopic lung lesion scores1</th>
<th>[% surface area weighted average]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Group</td>
<td>Mean</td>
</tr>
<tr>
<td>Ad5mutIL-18</td>
<td>5.6 ±0.7</td>
</tr>
<tr>
<td>MLV+Ad5wtIL-18</td>
<td>9.6 ±0.8</td>
</tr>
<tr>
<td>MLV+Ad5mulIL-18</td>
<td>7.7 ±1.0</td>
</tr>
<tr>
<td>MLV+mutIL-18</td>
<td>7.4 ±1.5</td>
</tr>
<tr>
<td>MLV</td>
<td>9.5 ±1.4</td>
</tr>
<tr>
<td>wtTx98</td>
<td>12.1 ±2.6</td>
</tr>
</tbody>
</table>

1 Means ± SE are reported. Differences between groups are not statistically different [One-way ANOVA (p>0.05)]
Cell mediated immunity (CMI) flow cytometry assay: PBMC samples were collected at 4, 7, 8, and 10 weeks post vaccination via Vacutainer CPT™ tubes for use in the CMI flow assay (Fig. 3.17-3.22). PBMCs were diluted in 96-well cell culture plates to a final concentration of \(1 \times 10^6\) PBMC/well and stimulated \textit{ex vivo} with either live wtTx98, wtIA04, Tx98NS1Δ126 MLV, or UV inactivated wtTx98 or wtIA04 viruses 96 hours after antigen addition, cells were fixed and fluorescently labeled by mAb for cell surface markers [CD4, CD8, \(\gamma/\delta\), and CD25] as well as IFN-\(\gamma\) and IL-10 to evaluate responses to \textit{ex vivo} stimulation by homologous and heterosubtypic influenza viruses. CD25, the IL-2 receptor \(\alpha\) chain, was used as an activation marker. IFN-\(\gamma\) and IL-10, \(T_h1\) and \(T_h2\) type cytokines respectively, were chosen to evaluate the phenotypic cytokine expression of the various T-cell populations at each respective time point. Data from weeks 7 and 8 were averaged to obtain an estimate of T cell response. Expression indexes (EI) were calculated for each treatment group, at each time point, for each simulating antigen. EIs are a dividend between the product of the percent positive cells and the respective mean florescence intensity of stimulated cells divided by the product of the percent positive cells and the respective mean florescence intensity of the unstimulated cells.

Response to H3N2 antigen: CD25 a T cell activation marker for flow cytometric analysis. CD4+, CD8+, and non T cell populations failed to up-regulate CD25+ expression above baseline following antigen stimulation (Fig. 3.21). Prior to challenge, the wtTx98 vaccination control group exhibited significantly higher CD25 expression than all other vaccination groups in the CD4+CD8+ T cell population. However, By 1 week post challenge, the MLV+mutIL-18 vaccination group exhibited the highest CD25+ expression in CD4+CD8+ T cells and was significantly higher than the negative control group (Fig. 3.21).
Similarly, by one week post challenge, the MLV+mutIL-18 vaccination group was the highest inducer of CD25+ on \(\gamma/\delta\) T cells and was significantly higher than both the MLV vaccination group and the negative control group (Fig. 3.21).

IFN-\(\gamma\) expression surprisingly was significantly diminished by all three rIL-18 vaccination groups in CD8+ T cells by one week post challenge (Fig. 3.18). IFN-\(\gamma\) expression by the PBMCs from the wtTx98 vaccination control group tended to be higher than all other vaccination groups regardless of T cell population or time point. By one week post challenge, the MLV+mutIL-18 group also exhibited significantly higher IFN-\(\gamma\) expression in \(\gamma/\delta\) T cells compared to the negative control group or either of the adeno+IL-18 vaccination groups [MLV+Ad5wtIL-18 & MLV+Ad5mutIL-18].

Data from both the H3N2 and H1N1 antigen stimulation sets indicate the expression vector itself also contributes significantly to the type of T cell population responding as well as the phenotypic response. As seen in the IL-10 expressing CD4+CD8+ and CD8+ T cell subsets one week post challenge [H3N2 antigen stimulation] (Fig. 3.17) and in the CD8+ IFN-\(\gamma\) expressing T cell population 7/8 weeks post vaccination [H1N1 antigen stimulation] (Fig. 3.20); all adenoviral groups including the negative control group were significantly elevated in comparison to non-adenovirus groups regardless of MLV co-administration or wtIL-18 versus mutIL-18 expression.

*Response to H1N1 antigen:* CD25+ expression was not appreciably elevated above the baseline by any group above or at any time point in response to stimulation by H1N1 antigen. By 7/8 weeks post vaccination, all three adenoviral groups (Ad5mutIL-18, MLV+Ad5wtIL-18, MLV+Ad5mutIL-18) exhibited significantly higher IFN-\(\gamma\) expression by
CD8+ T cells, compared to non-adenovirus groups (Fig. 3.20). Vaccination with MLV+Ad5mutIL-18 resulted in significantly higher IL-10 expression in CD4+ SP, CD4+CD8+ DP, CD8+, and γδ T cell populations in comparison to all other vaccination groups except for MLV+Ad5wtIL-18 group by one week post challenge in response to live H1N1 antigen stimulation. Additionally, the MLV+Ad5wtIL-18 vaccination group, along with MLV+Ad5mutIL-18, induced significantly higher IL-10 expression in CD4+ and CD4+CD8+ DP T cell populations compared to all other vaccination groups by one week post challenge when stimulated with live H1N1 antigen.
Figure 3.17 CMI assay: IL-10 EI [Live wtTx98 H3N2] by group
NADC SIV 1\textsuperscript{st} test results (week 4 post-vaccination)

NADC SIV 2\textsuperscript{nd} & 3\textsuperscript{rd} tests combined results (pre-challenge)

NADC SIV 4\textsuperscript{th} test results (1 week post-challenge)

\[
\text{IL-10 EI} = \frac{(\% \text{IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10}) \text{of stimulated cells}}{(\% \text{IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10}) \text{of unstimulated cells}}
\]

Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwil–18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98A126 w/ mSwil–18 Insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98A126 + Ad5Blue w/ mSwil–18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98A126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98A126 + Ad5Blue w/ mSwil–18 Insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure 3.18  CMI assay: IFN-γ EI [Live wtX98 H3N2] by group
NADC SIV 1\textsuperscript{st} test results (week 4 post-vaccination)

NADC SIV 2\textsuperscript{nd} & 3\textsuperscript{rd} tests combined results (pre-challenge)

NADC SIV 4\textsuperscript{th} test results (1 week post-challenge)

\[
\text{IFN}_\gamma\text{ EI} = \frac{\text{(%IFN}_\gamma\text{ positive cells x mean fluorescent intensity of IFN}_\gamma\text{) of stimulated cells}}{\text{(%IFN}_\gamma\text{ positive cells x mean fluorescent intensity of IFN}_\gamma\text{) of unstimulated cells}}
\]

Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwkL-18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98Δ126 w/ mSwkL-18 Insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwkL-18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98Δ126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwkL-18 Insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure 3.19  CMI assay: IL-10 EI [Live wtIA04 H1N1] by group
NADC SIV 1\textsuperscript{st} test results (week 4 post-vaccination)

NADC SIV 2\textsuperscript{nd} & 3\textsuperscript{rd} tests combined results (pre-challenge)

NADC SIV 4\textsuperscript{th} test results (1 week post-challenge)

\[ \text{IL-10 EI} = \frac{(\% \text{IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10}) \text{ of stimulated cells}}{(\% \text{IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10}) \text{ of unstimulated cells}} \]

Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

| Gr. 1 | Ad5Blue w/ mSwIL-18 Insertion | Gr. 4 | Tx98A126 w/ mSwIL-18 Insertion |
| Gr. 2 | Tx98A126 + Ad5Blue w/ wtSwIL-18 Insertion | Gr. 5 | Tx98A126 |
| Gr. 3 | Tx98A126 + Ad5Blue w/ mSwIL-18 Insertion | Gr. 6 | Tx98 wt |
**Figure 3.20  CMI assay: IFN-γ EI [Live wtIA04 H1N1] by group**

**NADC SIV 1st test results (week 4 post-vaccination)**

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean IFNγ Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

**NADC SIV 2nd & 3rd tests combined results (pre-challenge)**

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean IFNγ Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

**NADC SIV 4th test results (1 week post-challenge)**

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean IFNγ Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

**IFNγ EI =**

\[
\text{IFNγ EI} = \frac{\% \text{IFNγ positive cells} \times \text{mean fluorescent intensity of IFNγ}}{\% \text{IFNγ positive cells} \times \text{mean fluorescent intensity of unstimulated cells}}
\]

of the same subset of the same animal

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwLL−18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98A126 w/ mSwLL−18 insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98A126 + Ad5Blue w/ mSwLL−18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98A126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98A126 + Ad5Blue w/ mSwLL−18 Insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure 3.21  CMI assay: CD25 E1 [Live wtTx98 H3N2] by group

NADC SIV 1\textsuperscript{st} test results (week 4 post-vaccination)

NADC SIV 2\textsuperscript{nd} & 3\textsuperscript{rd} tests combined results (pre-challenge)

NADC SIV 4\textsuperscript{th} test results (1 week post-challenge)

CD25 \text{EI} = \frac{\text{% CD25 positive cells} \times \text{mean fluorescent intensity of CD25 of stimulated cells}}{\text{% CD25 positive cells} \times \text{mean fluorescent intensity of CD25 of unstimulated cells}}

\text{of the same subset of the same animal}

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

Gr. 1  Ad5Blue w/ mSwL–18 Insertion
Gr. 2  T98A126 + Ad5Blue w/ mSwL–18 Insertion
Gr. 3  T98A126 + Ad5Blue w/ mSwL–18 Insertion
Gr. 4  T98A126 w/ mSwL–18 Insertion
Gr. 5  T98A126
Gr. 6  T98 wt
Figure 3.22  CMI assay: CD25 EI [Live wtIA04 H1N1] by group

NADC SIV 1st test results (week 4 post–vaccination)

NADC SIV 2nd & 3rd tests combined results (pre–challenge)

NADC SIV 4th test results (1 week post–challenge)

\[
\text{IFN}_{\gamma} \text{ EI} = \frac{\% \text{IFN}_{\gamma} \text{ positive cells} \times \text{mean fluorescent intensity of IFN}_{\gamma}}{\% \text{IFN}_{\gamma} \text{ positive cells} \times \text{mean fluorescent intensity of IFN}_{\gamma}} \text{ of unstimulated cells of the same subset of the same animal}
\]

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwl–18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98Δ126 w/ mSwl–18 Insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwl–18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98Δ126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwl–18 Insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
The CD25 CMI flow cytometric analysis for the 10th week post vaccination (~1 week post challenge) grouped by antigen is displayed in Figure 3.23. The CD4+CD8+ DP T cell and the γ/δ T cell populations are the major activated T cell groups responding to H3N2 antigen by up-regulating the CD25+ EI for all treatment groups other than the negative control group. DP T cells and γ/δ T cell T cells expressing CD25 only responded to significant levels when stimulation by the Tx98 antigens (live, UV killed, or the MLV antigen stimulation groups). Reactions to H1N1 IA04 viruses (either live or UV inactivated) were not detected for CD25 positive cells.

[Figure 3.24] Stimulation with Tx98 wt antigen (live and UV inactivated) and wtIA04 antigen induced IL-10 expression to significant levels in multiple vaccination groups. Both CD4+ and CD4+CD8+ T cell populations expressing IL-10 responded in highest intensities to the heterosubtypic IA04 challenge virus for both MLV+Ad5wtIL-18 and MLV+Ad5mutIL-18 vaccination groups. IL-10 expressing cells in CD4+, CD4+CD8+, CD8+ T cell populations and all T cell subsets stimulated with any Tx98wt antigen (Live, UV inactivated, or MLV) were below baseline for the MLV+mutIL-18 group. The wtTx98 vaccination control group displayed significantly high levels of IL-10 production by γ/δ T cells responding to Tx98 antigen stimulation (either live, UV inactivated, or MLV).

[Figure 3.25] CD4+ and CD4+CD8+ T cell populations from the MLV+Ad5wtIL-18 and MLV+Ad5mutIL-18 vaccination groups resulted in modest increase in IFN-γ EI in response to Tx98 (live or UV inactivated) and live IA04 viruses. γ/δ and non-T cells from the MLV+mutIL-18 treatment group displayed elevated levels of IFN-γ expression in response to stimulation ex vivo by Tx98 virus (live, UV inactivated, MLV). Every T cell
population except the CD8+ T cell pool of the wtTx98 treatment group resulted in elevated IFN-γ EI levels in response to stimulation with Tx98 antigen (live, UV inactivated, MLV).

Figure 3.23  CMI Assay: CD25 EI by antigen: 10 weeks post vaccination
NADC SIV 4th test antigen comparison (1 week post-challenge)

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Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

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<tr>
<th>Ag 1</th>
<th>Live wtTx98 H3N2</th>
<th>Ag 3</th>
<th>Live wTAD4 H1N1</th>
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<th>Live T98Δ126</th>
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<tr>
<td>Ag 2</td>
<td>Killed wtTx98 H3N1</td>
<td>Ag 4</td>
<td>Killed wTAD4 H1N1</td>
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Live wtTx98 H3N2 and Killed wtTx98 H3N1 treatments showed a significant decrease in CD25 expression compared to the Ad5Blu w/mlv - 18 Insertion and Ad5Blu w/mlv - 18 Insertion treatments, respectively. The T98Δ126 treatments also showed a significant decrease in CD25 expression compared to the Ad5Blu w/mlv - 18 Insertion treatment.
Figure 3.24 CMI Assay: IL-10 EI by antigen: 10 weeks post vaccination

NADC SIV 4th test antigen comparison (1 week post-challenge)

Student’s t-test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p < 0.05)
Figure 3.25  CMI Assay: IFN-γ Ei by antigen: 10 weeks post vaccination

NADC SIV 4th test antigen comparison (1 week post-challenge)

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Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

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<tr>
<td>Live SIV H3N2</td>
<td>Ag 3</td>
<td>Live wtA04 H1N1</td>
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4. Discussion

Current swine influenza vaccines effectively induce immunological protection against antigenically similar strains of the same subtype, but not from other subtypes of circulating influenza viruses. Due to their segmented genome and error prone replicase, influenza viruses rapidly mutate to evade the narrow protective immunity conveyed by current swine influenza vaccines. Broad cross-reactive immunity or heterosubtypic immunity can be elicited following infection by live influenza viruses (Quan et al., 2007), although the exact underlying mechanisms are not well understood. It is believed the induction of heterosubtypic immunity involves cell mediated immunity targeting conserved internal influenza proteins and cross reactive S-IgA and IgG antibodies neutralizing virus and generating virus-antibody complexes (Benton et al., 2001; Graham and Braciale, 1997; Liang et al., 1994; Moran et al., 1999; Nguyen et al., 2000; Schulman and Kilbourne, 1965; Tamura et al., 2005; Topham et al., 1996). Due to the enhanced protective immune responses to live infection, attenuated modified live virus (MLV) vaccines elicit better CMI responses than current inactivated killed vaccines and tend to induce stronger heterosubtypic immunity (Liang et al., 1994; Schulman and Kilbourne, 1965; Tamura et al., 2005). A modified live influenza virus (Tx98NS1Δ126) has been previously described as an effective swine influenza vaccine against homologous and homosubtypic challenge (Richt et al., 2006). Intranasal administration of the MLV was proven to be an effective route of vaccination as a single dose, but was shown to convey little protection to heterosubtypic challenge (Vincent et al., 2007).

Vaccination against swine influenza is often administered at a young age in swine. In young animals, formulation of phenotypic T_{H1} immune responses are impaired due to their
immunologically naïve state (Breathnach et al., 2006; Forsthuber et al., 1996; Pertmer et al., 2001; Ridge et al., 1996; Sarzotti et al., 1996; Siegrist, 2001; Siegrist et al., 1998; Suen et al., 1998). Vaccination at this stage of life may result in a biased T\textsubscript{H}2 immune response that is not fully protective. Studies have shown in some cases that therapeutic treatments, such as cytokine administration, are able to restore correct immune function (Pertmer et al., 2001; Ridge et al., 1996; Siegrist, 2001) or enhance protective immunity (Zuckermann et al., 1998).

IL-18 is an important cytokine involved in the development of strong CMI response and in the clearance of influenza virus during infection (Akira, 2000; Biet et al., 2002; Billaut-Mulot et al., 2000; Denton et al., 2007; Dinarello, 1999; Pirhonen et al., 1999). In the presence of IL-12, IL-18 induces a strong T\textsubscript{H}1 immune response, primarily by stimulating IFN-\(\gamma\) production and by activating CTL, NK cells, and APCs (Akira, 2000; Biet et al., 2002; Dai et al., 2006; Fortin et al., 2009; Kojima et al., 1998; Pirhonen et al., 1999; Yoshimoto et al., 1997; Zhang et al., 1997). IL-18 expression is known to be significantly diminished in mucosal epithelial cells of young pigs for months after birth (Muneta et al., 2002). However, administration of exogenous IL-18 to the mucosal epithelial cells of young pigs restored IFN-\(\gamma\) induction even when Con A stimulation was unable to (Muneta et al., 2002). We theorized the administration of IL-18 via viral expression vectors in conjunction with the previously described swine influenza MLV vaccine (Tx98NS1\(\Delta\)126) would enhance protective immunity against influenza infection. By the addition of IL-18, it was believed the immunomodulatory effect would result in a stronger cell mediated immune response eliciting enhanced heterosubtypic infection in young swine.

In response to vaccination, the MLV+Ad5mutIL-18 vaccination group resulted in enhanced protection from heterosubtypic immunity. As seen in Table 3.2, the
MLV+Ad5mutIL-18 vaccination group maintained antibody titers significantly higher than the negative control out to day 56 post vaccination. By 5 days post challenge, the MLV+Ad5mutIL-18 vaccination group exhibited elevated antibody titers significantly higher than both the MLV and negative control groups (Fig. 3.6 & Table 3.2). Additionally, MLV+mutIL-18 was the only vaccination group not to shed influenza virus post vaccination (Fig. 3.7). By day 3 post challenge, viral shedding from the mutIL-18 vaccination groups [MLV+mutIL-18 & MLV+Ad5mutIL-18] were significantly lower than the MLV+Ad5wtIL-18 vaccination group. By day 5 post challenge, the MLV+Ad5mutIL-18 vaccination group resulted in significantly lower viral shedding compared to the MLV vaccination group and negative control group (Fig. 3.8).

While not statistically significant, the mutIL-18 vaccination groups also resulted in lower pulmonary viral titers and reduced macroscopic lung lesion scores compared to the MLV vaccination group, the negative control, and the wtTx98 control groups. It should be noted the wtTx98 control group resulted in the highest macroscopic lung lesion scores in response to heterosubtypic challenge while the naïve negative control group exhibited the lowest pneumonic lung lesion scores. Post challenge, it would be expected the wtTx98 vaccination group would have resulted in lowest pneumonic lesions, as wild type influenza infection elicits protective immune responses upon recovery from infection, and the unvaccinated control group would theoretically have resulted in the highest pneumonic lung lesion scores due to an absence to pre-existing immunity. However, the inverse was observed. Similar findings of enhanced pneumonic lung lesions developing as a consequence of heterosubtypic challenge have been previously reported in our lab (Vincent et al., 2008). The mechanism underlying this outcome is unknown. However, a possible
mechanism may be indicated by the correlation of lesion scores and antibody titers. The wtTx98 group induced HI titers 5 to 10 fold greater than the MLV vaccine groups, while the negative control group did not result in detectable HI titers. Enhanced pneumonic lesions may result as a consequence of antibody cross-linking. In some circumstances when high quantities of soluble or membrane bound antigens are generated in the presence of reactive antibodies (particularly IgG and IgM), substantial cross-linking can form complexes which may induce severe inflammation and cell necrosis (Murphy et al., 2008). The correlation to HI antibody titers and lung lesion scores supports this theory. Enhanced lung lesions may also be due to a ‘by-stander’ effect of highly reactive cytotoxic T cells resulting in the death of neighboring cells. Further research will be needed to elucidate the function of enhanced lung lesion scores resulting from heterosubtypic challenge.

To evaluate the phenotypic response of T cell populations in response to MLV vaccination and heterosubtypic challenge, six color flow cytometric analysis was used to characterize the T cell populations. By one week post challenge, the MLV+mutIL-18 vaccination group induced significant increases in CD25+ expression (CD4+CD8+) and IFN-γ expression (g/δ T cells, non T cells) in response to stimulation by H3N2 antigen, although responses to the H1N1 antigen was marginal. Conversely, the MLV+Ad5mutIL-18 vaccination group induced significantly elevated IL-10 expression indexes [CD4+, CD4+CD8+, CD8+, and g/δ T cells] by one week post challenge in response to H1N1 antigen (Fig. 3.19). The MLV+Ad5wtIL-18 vaccination also induced significantly higher IL-10 levels in CD4+ and CD4+CD8+ T cells in response to H1N1 antigen (Fig. 3.19). The reactivity to H1N1 antigen in the CMI assay by MLV+Ad5wtIL-18 and MLV+Ad5mutIL-18
groups indicates T cells from those vaccination groups recognize and respond to viruses of other subtypes (H1N1).

Results also indicate the viral vector significantly contributes to the phenotypic response generated by T cells in certain cases. As seen in the CD4+CD8+ and CD8+ T cell subsets one week post vaccination [IL-10 EI, H3N2 antigen stimulation] (Fig. 3.17) and in the CD8+ T cell population 7/8 weeks post vaccination [IFN-γ EI, H1N1 antigen stimulation] (Fig. 3.20); a clear demarcation existed between the EIs of adenoviral vaccination groups and non-adenoviral vaccination groups regardless of MLV co-administration or form of IL-18 expressed (wild type or mutated). While adenoviral vectors are replication-defective, infection of cells alone may be enough to up-regulate cellular immune responses. The fact that the negative control vector reacted to an almost identical EI expression level as the MLV+adenovirus groups when stimulation with influenza antigen suggests, in those instances, the result of adenovirus administration overrode the immune reaction of the influenza MLV vaccination itself. Such results indicate careful consideration may be needed when choosing the expression vector in order to generate the desired immune response upon administration.

Innate activation of the adaptive arm of the immune system is partly dependent upon recognition of pathogens by PRRs such as toll-like receptors, NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) expressed on the cell surface or within the cytoplasm to detect pathogens by pathogen associated molecular patterns (PAMPs) (Akira et al., 2006). Recognition of pathogens by PRRs activate various cellular immune pathways (Petrilli et al., 2007), and are critical to the induction of proper pro-inflammatory responses. Upon stimulation of pro-inflammatory pathways, inflammasomes are activated. Inflammasomes
are caspase-1 activating multi-protein complexes. The activation of caspase-1 cleaves IL-1β, IL-18, and IL-33 pro proteins into their biologically active forms (Petrilli et al., 2007). Inflammasome activation and subsequent induction of the ASC/caspase-1-dependent pathway is required for the establishment of CD8+ T-cell response and the B-cell secretion of IgA antibodies at the mucosal surface (Petrilli et al., 2007; Stasakova et al., 2005).

The influenza protein NS1 is able to suppress caspase-1 activation, pro-IL-18 maturation, and caspase-1 dependent apoptosis (Stasakova et al., 2005). Using a similar NS1 truncated influenza MLV virus (PR8/NS1-125) Stasakova et al. showed that NS1 was able to effectively inhibit IL-1b and IL-18 release from infected human macrophages resulting in 5-fold reduction biologically active IL-18 release and 10-50 fold reduction in biologically active IL-1β release (Egorov et al., 1998; Stasakova et al., 2005).

While attenuated, the MLV in this study has been previously shown to be an effective single dose MLV swine influenza vaccine against homologous and homosubtypic infection when administered intranasally (Richt et al., 2006; Vincent et al., 2007). However, the MLV was unable to elicit protective heterosubtypic immunity (Vincent et al., 2007). As previously mentioned, young animals are known to have impaired abilities to formulate TH1 T cell responses and young pigs have severely diminished IL-18 expression (Muneta et al., 2002; Sarzotti et al., 1996; Siegrist, 2001; Suen et al., 1998). Additionally, the N-terminal of the NS1 protein inhibits release of biologically active IL-18 5-fold (Stasakova et al., 2005). For these reasons, it is theorized the co-administration of IL-18 with swine influenza vaccines will result in a superior influenza vaccine. Based upon previous studies by Kim et al. (2001) two amino acid mutations (E41A & K88A) were introduced by PCR primer site directed mutagenesis in order to generate an enhanced immunomodulatory agent with extended half-
life and increased biological activity. The immunomodulatory effects of IL-18 on the immune response of swine influenza MLV vaccination was evaluated in vivo. Results indicated the addition of Ad5mutIL-18 in conjunction with the MLV resulted in superior protection to heterosubtypic challenge.

The vaccination group MLV+Ad5mutIL-18 maintained significantly elevated antibody titers post vaccination and resulted in significantly higher mean antibody titers in response to heterosubtypic challenge (Fig. 3.6, Table 3.2). The MLV+Ad5mutIL-18 group was the only vaccination group to not shed influenza virus post vaccination (Fig. 3.7, Table 3.3). Additionally, vaccination by MLV+Ad5mutIL-18 resulted in significantly lower viral shedding post challenge than the MLV vaccination group by day 5 post challenge (Fig. 3.8, Table 3.3).

In the Flow cytometric analysis, the MLV+mutIL-18 effectively up-regulated CD25 and IFN-γ expression in CD4+CD8+ and γ/δ T cell subclasses in response to stimulation by H3N2 antigen at one week post challenge. The MLV+Ad5mutIL-18 vaccination group was the only group to significantly enhance expression of any marker in response to stimulation with H1N1 antigen. While the MLV+Ad5mutIL-18 vaccination group exhibited strong responses to heterosubtypic viral antigen post challenge, surprisingly the response to H1N1 induced strong up-regulation of IL-10, a phenotypic Th2 cytokine. IL-10 is largely recognized for its functions as an anti-inflammatory cytokine that inhibits IFN-γ expression and acts as a negative regulator to Th1 T cell immune responses in murine and human models (Couper et al., 2008; Ejrnaes et al., 2006). However, during the course of influenza infection antigen specific effector T cells are known to express high levels of IL-10 (Sun and Metzger, 2008). It has been shown the inhibition of IL-10 signaling during influenza
infection results in enhanced damage to the lung (Sun and Metzger, 2008). Moreover, it has been shown in NK cells that IL-10 can act to enhance IFN-γ production and cytotoxicity of NK cells when co-administered with IL-18 (Cai et al., 1999). Therefore, the up-regulation of IL-10 in response to stimulation by heterosubtypic antigen may be the result of a protective immune function resulting from influenza infection. Further study to evaluate the correlation between macroscopic lung scores and the level of IL-10 protein in the lungs may prove to yield some insight into the possible mechanisms of enhanced damage to the lung resulting from heterosubtypic influenza challenge.

Results show co-administration of mutIL-18 expressing adenoviral vector with an attenuated swine influenza MLV vaccine resulted in superior protection to heterosubtypic infection as measured by viral shedding, viral replication in the lung, HI titers post vaccination and post challenge, macroscopic lung lesions, and by flow cytometric analysis. MLV+Ad5mutIL-18 was the only vaccination group to significantly respond to stimulation by heterosubtypic viral antigen (Fig. 3.19). Conversely, the MLV+mutIL-18 vaccination group was the highest inducer of CD25 and IFN-γ expression in response to stimulation by homologous viral antigen post challenge, but did not respond in kind to stimulation with heterosubtypic antigen (Fig. 3.21, 3.18). Further research to evaluate the effect of the expression vectors on the immune response would be interesting. The induction of IL-10 in response to heterosubtypic viral antigen at one week post challenge was only noted by the adenoviral vaccination groups MLV+Ad5mutIL-18 [CD4+, CD4CD8+, CD8+, γ/δ] and MLV+Ad5wtIL-18 [CD4+, CD4CD8+].
Acknowledgements

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CHAPTER 4 GENERAL SUMMARY

Discussion

Recombinant porcine IL-18 expression vectors were generated to evaluate the immunomodulatory effect of IL-18 on the immune response to an attenuated swine influenza MLV vaccine. Both wtIL-18 and mutIL-18 expression vectors were confirmed to express 0.1-10 ng/mL rIL-18 protein depending on the conditions tested and the expression vector. Expression of rIL-18 by adenoviral vectors resulted in 5-10 fold greater quantities of rIL-18 protein than the influenza mutIL-18 isolate under similar culturing conditions in vitro.

Biological activity of wtIL-18 and mutIL-18 was not definitively confirmed. Initial trials utilizing low concentrations of rIL-18 protein and no co-stimulatory agents failed to induce any IFN-\(\gamma\) expression. Co-stimulating PMBCs with Con A and/or rpIL-12 resulted in detectable levels of IFN-\(\gamma\) production by treatment groups. However, the unexpected activity of negative controls and the apparent variability in responsiveness to treatment between pigs complicated the determination of the true biological activity between treatment groups.

The biological activity trials have indicated the response to treatment groups between pigs displays a high degree of variability. A genetic component, health status, or unidentified environmental factor may play a significant role in response to treatment in the \textit{in vitro} biological activity assay. Additional trials are needed to determine definitively the biological activity of the rIL-18 isolates. The establishment of protocols to generate rIL-18 samples of higher concentration would presumably aid in the testing of biological activity. Repeated measures from the same PBMC samples may be needed to account for inherent variability between PBMCs from different subjects. The removal of viral or cellular products while
maintaining a high concentration of rIL-18 protein would help ensure accurate measurements of biological activity by rIL-18 proteins.

While the removal of viral and cellular components was attempted by filtration prior to protein concentration, compounds between 10kD and 100kD MW remained and were concentrated 10-fold. Furthermore, adenoviral titers in the concentrated adenoviral preparations samples were between $1.0 \times 10^9$ to $5.6 \times 10^9$ TCID$_{50}$/mL after filtration and concentration. Adenoviruses are able to infect lymphocytes and monocytes (Segerman et al., 2006; van der Veen and Lambriex, 1973). Even though adenoviral isolates are replication defective, infection alone may cause up-regulation of some immunological pathways. The adenovirus control group Ad5B was able to illicit some level of IFN-$\gamma$ protein expression when PBMCs were co-stimulated with rpIL-12 or con A/rpIL-12. The induction of IFN-$\gamma$ expression by Ad5B is consistent with a previous study (Hartman et al., 2007). It was reported a similar LacZ expressing adenovirus was able to up-regulate Jak/STAT, MAPK, TLR, and apoptotic-related pathways as well as induce a MyD88 mediated IFN-$\gamma$ response in vivo (Hartman et al., 2007). Adenoviruses are well suited for mucosal administration, including oral and intranasal routes, able to elicit high IgA antibody titers and effective CD8+ T cell responses (Croyle et al., 2008; Phillpotts et al., 2005; Tang et al., 2009; Tucker et al., 2008; Walter et al., 2001; Xiang and Ertl, 1999). Intranasal administration of rIL-18 expressing adenoviruses did result in consistent, albeit low, IFN-$\gamma$ induced expression in nasal wash samples following a time dependent pattern. Previous studies expressing IFN-$\alpha$ or IL-18 by similar adenoviral vectors in vivo also reported steep declines in expressed protein levels on or before day 3 post vaccination (Brockmeier et al., 2009; Walter et al., 2001).
IL-18 expression vectors were administered as part of a single dose swine influenza MLV vaccination. Previous research had shown the swine influenza MLV vaccine (Tx98NS1Δ126) was able to elicit protective immunity to homologous and homosubtypic challenge but failed to protect against heterosubtypic infection (Richt et al., 2006; Vincent et al., 2007). Results indicate co-administration of mutIL-18 expressing adenoviral vectors with the attenuated swine influenza MLV vaccine resulted in superior protection to heterosubtypic infection. MLV+Ad5mutIL-18 vaccination exhibited the strongest protective immunological responses to heterosubtypic challenge as measured by viral shedding, viral replication in the lung, HI titers post vaccination and post challenge, macroscopic lung lesions, and by flow cytometric analysis. The MLV+Ad5mutIL-18 was the only vaccination group to significantly respond to stimulation by heterosubtypic viral antigen (Fig. 3.19). Conversely, the MLV+mutIL-18 vaccination group was the highest inducer of CD25 and IFN-γ expression in response to stimulation by homologous viral antigen, but did not respond in kind to stimulation with heterosubtypic antigen (Figure 3.21, 3.18). Further research to evaluate the effect of the expression vectors on the immune response would be interesting.

The induction of IL-10 in response to heterosubtypic viral antigen at one week post challenge was only noted by the adenoviral vaccination groups MLV+Ad5mutIL-18 [CD4+, CD4CD8+, CD8+, γ/δ] and MLV+Ad5wtIL-18 [CD4+, CD4CD8+]. This may be a product of the enhance level of rIL-18 expression, or it may be due to the adenovirus itself. It would be interesting to evaluate the protein concentration of IL-10 and IFN-γ in BALF samples and see if IL-10 protein concentrations correlate directly to reduced pneumonic lung lesions in response to heterosubtypic immunity. If a direct correlation is found, it may indicate a
possible mechanism for the enhanced pneumonic lung lesions in response to heterosubtypic challenge.

References


APPENDIX A  WTIL-18 AND MUTIL-18 SEQUENCE ANALYSIS

Appendix A.1 wtIL-18/ mutIL-18 cDNA sequence alignment

Figure A.1  Sequence alignment of wtIL-18 and mutIL-18 cDNA constructs
Appendix A.2 wtIL-18/mutIL-18 amino acid alignment

Figure A.2 wtIL-18/mutIL-18 amino acid alignment

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</table>
Appendix A.3 Sequence assessment of adenoviral gene insertions

Figure A.3 Sequence assessment of adenoviral gene insertions
Appendix A.4 Chemical structure comparison of human versus porcine mutIL-18 amino acid conversions

Kim et al. (2001, 2002) identified two amino acids by computer modeling as important residues for the binding of IL-18 to IL-18BP. Mutations to these sites inverting the charged residues (E42K or K89E) resulted in mixed results. While the K89E mutation resulted in a 95% reduction in biological activity, the E42K resulted in 2-fold biological activity (Kim et al, 2002). Converting E42 and K89 to alanine (E42A and K89A) resulted in a 4-fold increase in biological activity and rendered fully resistant to neutralization by IL-18BP. To evaluate if synonymous mutations would result in enhanced biological activity, a mutated IL-18 was generated by PCR primer site directed mutagenesis (E41A & K88A) from a wild type porcine cDNA. Human IL-18 contains an valine (V6) at amino acid position 6 which is not found in the wild type porcine IL-18 cDNA construct.

Figure A.4 Chemical structure comparison of human versus porcine mutIL-18 amino acid conversions

Mutated IL-18 amino Acid substitutions

- Human IL-18: AA 42
  - Porcine IL-18: AA 41

- Human IL-18: AA 89
  - Porcine IL-18: AA 88
APPINDIX B  ADDITIONAL MATERIALS

Appendix B.1 Evaluation of IFN-γ production at various Con A concentrations

To evaluate the effect of Con A concentration on IFN-γ production by porcine PBMCs, titration of Con A concentrations were performed on PMBCs from three sows (age unknown, NADC animal services) (Fig. 2.6). Approximately 5.5 X 10^5 PBMCs per cm^2 were plated in 0.5mL supplemented RPMI 1640 stimulated with 0.0-2.5 μg/mL Con A (final concentration). Samples were collected after a 24 hour incubation period and IFN-γ levels measured in duplicate by swine IFN-γ ELISA. Con A induced expression of IFN-γ was found to plateau as increasing concentrations of ConA past 1.0-2.0 μg/mL.

Figure B.1  Determination of sub-optimal concentrations of Concanavalin A

Fig. A 2.5. IFN-γ production analysis of PBMCs stimulated with increasing concentrations of Con A. PBMCs stimulated with various concentrations of Con A were incubated at 39°C/ 5% CO2 for 24 hours. PBMC supernatants were measured in duplicate by swine IFN-γ ELISA. Values are the result of a single trial of 3 pigs.
Table B.1 Evaluation of IFN-γ production at various Con A concentrations

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>Final Conc./mL</th>
<th>Media</th>
<th>Con A Stock</th>
<th>Total Count / mL</th>
<th>Vol / well</th>
<th>PBMC/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5mg/mL</td>
<td>250μL</td>
<td>250μL</td>
<td>Pig 1</td>
<td>21.14 x 10^6</td>
<td>197μL</td>
<td>4.16 x 10^5</td>
</tr>
<tr>
<td>2.0mg/mL</td>
<td>300μL</td>
<td>200μL</td>
<td>Pig 2</td>
<td>17.1 x 10^6</td>
<td>24.4μL</td>
<td>4.17 x 10^5</td>
</tr>
<tr>
<td>1.5mg/mL</td>
<td>350μL</td>
<td>150μL</td>
<td>Pig 3</td>
<td>22.2 x 10^6</td>
<td>18.8μL</td>
<td>4.17 x 10^5</td>
</tr>
<tr>
<td>1.0mg/mL</td>
<td>400μL</td>
<td>100μL</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>0.5mg/mL</td>
<td>450μL</td>
<td>50μL</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.0mg/mL</td>
<td>500μL</td>
<td>0μL</td>
<td></td>
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</tbody>
</table>

PBMC  Isolation from whole blood (Animal Services, NADC) from 3 donor swine was performed via the percoll PBMC protocol [previous work, Dr. Kehrli]. Isolated PBMCs were resuspended into 1mL media (as described above) and CBCs were performed using the Hemavet hemacytomoter. Dilutions were performed to obtain a cell count of 4.17 x 10^5 PBMC/well. Desired cell counts were calculated based on previous work (Dr. Kehrli) using a 96-well format [200,000 PBMC/well]. Corrections for surface area differences were made. Each set of concentrations were set up in duplicate [per pig] so that different assays could be run (1F/T to 3F/T). See further explanation below.

Duplicates  F/T 3X: Samples frozen while in the plate 3X, upon the third thaw IFNγ levels measured via a swine Interferon-g ELISA (INVITROGEN);

F/T 0X: Repeated aspiration of media within individual wells will resuspend PBMCs. Resuspended PBMCs+Media will be moved to individually labeled steril 1.5mL tubes to be spun at 1500 RPM [200RCF] for 10 minutes to pellet PBMCs. Resulting media was aspirated off and stored within individual tubes, to be assessed for IFNγ levels via a swine Interferon-g ELISA (INVITROGEN) and than properly stored at -80C. Pelleted PBMCs were individually lysed and total RNA extracted via the Total RNA MagMax extraction kit. Extracted RNA may be assessed via a IFNγ qPCR assay for transcriptional levels of IFNγ mRNA.
Appendix B.2 Concentrated rIL-18 biological activity assay: IFN-γ (pg/mL) per time point by pig

Table B.2  Concentrated IL-18 biological activity data per pig:

<table>
<thead>
<tr>
<th>Biological Activity Assay: IFN-γ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment groups: Concentrated adenoviral preparations</td>
</tr>
<tr>
<td>Treatment groups: Concentrated adenoviral preparations</td>
</tr>
<tr>
<td>Co-stimulation: rpIL-12 (100ng/mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Timepoint</th>
<th>Pig 1</th>
<th>Pig 2</th>
<th>Pig 3</th>
<th>Pig 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5B</td>
<td>24 h</td>
<td>32.87</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ad5B</td>
<td>48 h</td>
<td>347.70</td>
<td>1.59</td>
<td>0.00</td>
<td>2.53</td>
</tr>
<tr>
<td>Ad5B</td>
<td>72 h</td>
<td>1160.32</td>
<td>73.12</td>
<td>5.07</td>
<td>0.00</td>
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<tr>
<td>Ad5B</td>
<td>96 h</td>
<td>1174.03</td>
<td>185.33</td>
<td>45.93</td>
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<tr>
<td>Ad5B</td>
<td>120 h</td>
<td>1145.02</td>
<td>351.64</td>
<td>264.86</td>
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<tr>
<td>Ad5+wtIL-18K:E</td>
<td>24 h</td>
<td>25.98</td>
<td>0.00</td>
<td>0.00</td>
<td>0.25</td>
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<tr>
<td>Ad5+wtIL-18K:E</td>
<td>48 h</td>
<td>276.27</td>
<td>12.68</td>
<td>7.63</td>
<td>4.49</td>
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<td>Ad5+wtIL-18K:E</td>
<td>72 h</td>
<td>384.77</td>
<td>34.52</td>
<td>16.13</td>
<td>9.52</td>
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<tr>
<td>Ad5+wtIL-18K:E</td>
<td>96 h</td>
<td>513.66</td>
<td>21.58</td>
<td>8.68</td>
<td>4.36</td>
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<tr>
<td>Ad5+wtIL-18K:E</td>
<td>120 h</td>
<td>500.19</td>
<td>79.63</td>
<td>85.80</td>
<td>7.42</td>
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<tr>
<td>Ad5+mutIL-18K:F2</td>
<td>24 h</td>
<td>49.37</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ad5+mutIL-18K:F2</td>
<td>48 h</td>
<td>891.29</td>
<td>5.52</td>
<td>11.59</td>
<td>0.09</td>
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<td>Ad5+mutIL-18K:F2</td>
<td>72 h</td>
<td>1639.77</td>
<td>16.71</td>
<td>6.69</td>
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<tr>
<td>Ad5+mutIL-18K:F2</td>
<td>96 h</td>
<td>3034.33</td>
<td>88.92</td>
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<td>2404.15</td>
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<td>Ad5+mutIL-18K:E3</td>
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<td>114.99</td>
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<td>1725.82</td>
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<td>Ad5+mutIL-18K:E3</td>
<td>72 h</td>
<td>3098.56</td>
<td>67.13</td>
<td>297.75</td>
<td>20.58</td>
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<td>Ad5+mutIL-18K:E3</td>
<td>96 h</td>
<td>3401.10</td>
<td>243.08</td>
<td>107.34</td>
<td>11.77</td>
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<tr>
<td>Ad5+mutIL-18K:E3</td>
<td>120 h</td>
<td>6650.70</td>
<td>630.59</td>
<td>151.23</td>
<td>5.67</td>
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Appendix B.3 Interleukin-18/MLV vaccination evaluation in response to heterosubtypic challenge: animal study experimental outline

Experimental Outline

Effect of Interleukin-18 on Vaccination Efficacy of Influenza Tx98NS1Δ126 MLV

ACUC Protocol 3846 IBC Protocol 0273, 0310, 0311

Table B.3  IL-18/influenza MLV vaccination animal study: group outline

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Description</th>
<th>ID</th>
<th>Challenge</th>
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<tbody>
<tr>
<td>1</td>
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<td>Adeno 5 w/ mSwIL-18 Insertion</td>
<td>AdSmutIL-18</td>
<td>IA04</td>
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<tr>
<td>2</td>
<td>8</td>
<td>Tx98NS1Δ126 + Adeno 5 w/ wtSwIL-18 Insertion</td>
<td>MLV+Ad5wtIL-18</td>
<td>IA04</td>
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<tr>
<td>3</td>
<td>8</td>
<td>Tx98NS1Δ126 + Adeno 5 w/ mSwIL-18 insertion</td>
<td>MLV+Ad5mutIL-18</td>
<td>IA04</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Tx98NS1Δ126 w/ mSwIL-18 insertion</td>
<td>MLV+mIL-18</td>
<td>IA04</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>Tx98NS1Δ126</td>
<td>MLV</td>
<td>IA04</td>
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<tr>
<td>6</td>
<td>8</td>
<td>Wild type Tx98</td>
<td>wtTx98</td>
<td>IA04</td>
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</tbody>
</table>

11/13/08 60 pigs delivered to NADC - 48 pigs to Barn 3A. 8 per room, 4 per crate X 2. Administer EXCEDE per label dose and tag.
11/20/08 Collect serum to confirm seronegativity to influenza virus and a nasal swab sample per pig. Intranasal administration of 2 mL inoculum to respective groups
11/22/08 Nasal swab and bleed
11/24/08 Nasal swab and bleed
11/26/08 Nasal swab and bleed
12/4/08 Bleed for serum
12/11/08 Bleed for serum
12/18/08 Bleed for serum and PBMC harvest
1/8/09 Bleed for serum and PBMC harvest
1/15/09 Bleed for serum and PBMC harvest
1/22/09 Rectal temperature.
1/23/09 Rectal temperature.
1/24/09 Challenge (all groups) with IA04 wild-type strain (A/swine/Iowa/00239/2004). Pre-challenge: nasal swab, rectal temperature, and serum collection.
1/25/09 Rectal temperature. Observe for clinical manifestation of infection.
1/26/09 Rectal temperature. Observe for clinical manifestation of infection.
1/27/09 Nasal swab and rectal temperature. Observe for clinical manifestation of infection.
1/28/09 Rectal temperature. Observe for clinical manifestation of infection.
1/29/09 Serum Collection, nasal swab, PBMC collection, and rectal temperature. Observe for clinical manifestation of infection. Necropsy.

Primary Inoculum:
Influenza: A dose of 2 mL \( \sim 1.0 \times 10^{6.5} \) TCID\(_{50}\)/mL of egg derived wtTx98 H3N2 (A/Swine/Texas/4199-2/98), Tx98NS1Δ126 (A/Swine/Texas/4199-2/98), or Tx98 NS1Δ126+mutIL-18 insertion (A/Smoke/Texas/4199-2/98) were administered intranasally to the respective groups.

Adenovirus: A dose of 2 mL \( \sim 1.0 \times 10^9 \) TCID\(_{50}\)/mL of CsCl concentrated/purified Ad5+wtSwIL-18 insertion or Ad5+mSwIL-18 insertion were administered to respective groups intranasally.

Challenge:

Influenza: A dose of 2 mL \( \sim 1.0 \times 10^{6.83} \) TCID\(_{50}\)/mL (A/swine/Iowa/00239/2004) virus administered intranasally to all treatment groups.
APPENDIX C FLOW CYTOMETRY DATA ANALYSIS: CELL
MEDIATED IMMUNITY ASSAY

Figure C.1 CMI Assay: CD25 E1 by group 4 weeks post vaccination
NADC SIV 1st test results (week 4 post-vaccination)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean CD25 Expression Index (E1)</th>
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<tbody>
<tr>
<td>Live mT108 HBE2</td>
<td><img src="image1.png" alt="Graph" /></td>
</tr>
<tr>
<td>Killed mT108 H3H2N2</td>
<td><img src="image2.png" alt="Graph" /></td>
</tr>
<tr>
<td>Live mT108 H3H1N1</td>
<td><img src="image3.png" alt="Graph" /></td>
</tr>
<tr>
<td>Killed mT108 H3H1N1</td>
<td><img src="image4.png" alt="Graph" /></td>
</tr>
<tr>
<td>Live T98A126</td>
<td><img src="image5.png" alt="Graph" /></td>
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</tbody>
</table>

CD25 E1 = \( \frac{\% \text{CD25 positive cells} \times \text{mean fluorescent intensity of CD25}}{\text{of stimulated cells}} \)
CD25 E1 = \( \frac{\% \text{CD25 positive cells} \times \text{mean fluorescent intensity of CD25}}{\text{of unstimulated cells}} \)

of the same subset of the same animal

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwiL-18 insertion</th>
<th>Gr. 4</th>
<th>Tx98A126 w/ mSwiL-18 insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98A126 + Ad5Blue w/ mSwiL-18 insertion</td>
<td>Gr. 5</td>
<td>Tx98A126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98A126 + Ad5Blue w/ mSwiL-18 insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
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</tbody>
</table>
Figure C.2  CMI Assay: CD25 EI by antigen 4 weeks post vaccination

NADC SIV 1st test antigen comparison (4 weeks post-vaccination)

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Mean CD25 Expression Index (EI)</th>
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<tbody>
<tr>
<td>Ad5/shL16/w</td>
<td>All PBMC</td>
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<td>Ad5/shL16/w</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26</td>
<td>All PBMC</td>
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<tr>
<td>T-89A.26</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26 w/mShW-18</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26 w/mShW-18</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26</td>
<td>All PBMC</td>
</tr>
<tr>
<td>T-89A.26</td>
<td>All PBMC</td>
</tr>
</tbody>
</table>

Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Ag 1</th>
<th>Live wtx98 H3N2</th>
<th>Ag 3</th>
<th>Live wtIA04 H1N1</th>
<th>Ag 5</th>
<th>Live Tx98a126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 2</td>
<td>Killed wtx98 H3N2</td>
<td>Ag 4</td>
<td>Killed wtIA04 H1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.3  CMI Assay: IFN-γ EI by group 4 weeks post vaccination

NADC SIV 1st test results (week 4 post-vaccination)

IFN-γ EI = \frac{\text{Number of positive cells} \times \text{mean fluorescent intensity of IFN-γ}}{\text{Number of stimulated cells}}

\text{of stimulated cells}

\text{of unstimulated cells}

\text{of the same subset of the same animal}

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 1</td>
<td>Ad5Blue w/ mSwL-18 Insertion</td>
<td>Gr. 4</td>
<td>Tx98Δ126 w/ mSwL-18 insertion</td>
</tr>
<tr>
<td>Gr. 2</td>
<td>Tx98Δ126 + Ad5Blue w/ wtSwL-18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98Δ126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwL-18 insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure C.4  CMI Assay: IFN-γ EI by antigen 4 weeks post vaccination

NADC SIV 1st test antigen comparison (4 weeks post-vaccination)

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)
Figure C.5 CMI Assay: IL-10 EI by group 4 weeks post vaccination

NADC SIV 1st test results (week 4 post-vaccination)

IL-10 EI = \frac{(\text{number of IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10}) \text{of stimulated cells}}{(\text{number of IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10}) \text{of unstimulated cells}}$

of the same subset of the same animal

Student's t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

Gr. 1  Ad5Blue w/ mSwil-18 insertion          Gr. 4  Tx98Δ126 w/ mSwil-18 insertion
Gr. 2  Tx98Δ126 + Ad5Blue w/ wtSwil-18 insertion  Gr. 5  Tx98Δ126  
Gr. 3  Tx98Δ126 + Ad5Blue w/ mSwil-18 insertion  Gr. 6  Tx98 wt
Figure C.6  CMI Assay: IL-10 EI by antigen 4 weeks post vaccination

NADC SIV 1st test antigen comparison (4 weeks post-vaccination)

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Mean IL-10 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>All PBMC</td>
<td>CD4</td>
</tr>
</tbody>
</table>

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p < 0.05)

<table>
<thead>
<tr>
<th>Ag 1</th>
<th>Live wtTx98 H3N2</th>
<th>Ag 3</th>
<th>Live wtIA04 rH1N1</th>
<th>Ag 5</th>
<th>Live Tx98s126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 2</td>
<td>Killed wtTx98 H3N2</td>
<td>Ag 4</td>
<td>Killed wtIA04 rH1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.7  CMI Assay: CD25 EI by group 7/8 weeks post vaccination

NADC SIV 2nd & 3rd tests combined results (pre-challenge)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean CD25 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live wtX98 HN2</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
<tr>
<td>Killed wtX98 HN2</td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>Live wtA4 hH1N1</td>
<td><img src="image3" alt="Graph" /></td>
</tr>
<tr>
<td>Killed wtA4 hH1N1</td>
<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>Live T98Δ126</td>
<td><img src="image5" alt="Graph" /></td>
</tr>
</tbody>
</table>

CD25 EI = (% CD25 positive cells x mean fluorescent intensity of CD25) of stimulated cells / (% CD25 positive cells x mean fluorescent intensity of CD25) of unstimulated cells of the same subset of the same animal

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwIL-18 Insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98Δ126 + Ad5Blue w/ wtSwIL-18 Insertion</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwIL-18 insertion</td>
</tr>
<tr>
<td>Gr. 4</td>
<td>Tx98Δ126 w/ mSwIL-18 insertion</td>
</tr>
<tr>
<td>Gr. 5</td>
<td>Tx98Δ126</td>
</tr>
<tr>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure C.8 CMI Assay: CD25 EI by antigen 7/8 weeks post vaccination

NADC SIV 2\textsuperscript{nd} & 3\textsuperscript{rd} tests combined antigen comparison (pre–challenge)

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Mean CD25 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All PBMC</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Ag. 1</th>
<th>Live wtTx98 H3N2</th>
<th>Ag. 3</th>
<th>Live wtA04 rH1N1</th>
<th>Ag. 5</th>
<th>Live Tx98a126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag. 2</td>
<td>Killed wtTx98 H3N2</td>
<td>Ag. 4</td>
<td>Killed wtA04 rH1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[Bar charts showing data]
Figure C.9  CMI Assay: IFN-γ EI by group 7/8 weeks post vaccination

NADC SIV 2^nd & 3^rd tests combined results (pre-challenge)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean IFNγ Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live wtX39 H3N2</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
<tr>
<td>Killed wtX39 H3N2</td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>Live wtA04 rH3N1</td>
<td><img src="image3" alt="Graph" /></td>
</tr>
<tr>
<td>Killed wtA04 rH3N1</td>
<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>Live Tx98A126</td>
<td><img src="image5" alt="Graph" /></td>
</tr>
</tbody>
</table>

\[
\text{IFNγ EI} = \frac{\%\text{IFNγ positive cells} \times \text{mean fluorescent intensity of IFNγ}}{\text{of stimulated cells}} \times \frac{\%\text{IFNγ positive cells} \times \text{mean fluorescent intensity of IFNγ}}{\text{of unstimulated cells}}
\]

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwiL-18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98A126 w/ mSwiL-18 insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98A126 + Ad5Blue w/ mSwiL-18 Insertion</td>
<td>Gr. 3</td>
<td>Tx98A126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98A126 + Ad5Blue w/ mSwiL-18 insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure C.10  CMI Assay: IFN-γ EI by antigen 7/8 weeks post vaccination

NADC SIV 2nd & 3rd tests combined antigen comparison (pre-challenge)

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Ag 1</th>
<th>Live wtX98 H3N2</th>
<th>Ag 3</th>
<th>Live wtA04 H1N1</th>
<th>Ag 5</th>
<th>Live Tx98s126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 2</td>
<td>Killed wtX98 H3N2</td>
<td>Ag 4</td>
<td>Killed wtA04 H1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.11  CMI Assay: IL-10 EI by group 7/8 weeks post vaccination

NADC SIV 2\textsuperscript{nd} & 3\textsuperscript{rd} tests combined results (pre–challenge)

\[ IL-10 \text{ EI} = \frac{\text{CIL-10 positive cells x mean fluorescent intensity of IL-10} \text{ of stimulated cells}}{\text{CIL-10 positive cells x mean fluorescent intensity of IL-10} \text{ of unstimulated cells of the same subset of the same animal}} \]

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ad5Blue w/ mSwL18 Insertion</td>
<td>4</td>
<td>Tx98α126 w/ mSwL18 insertion</td>
</tr>
<tr>
<td>2</td>
<td>Tx98α126 + Ad5Blue w/ wtSwL18 Insertion</td>
<td>5</td>
<td>Tx98α126</td>
</tr>
<tr>
<td>3</td>
<td>Tx98α126 + Ad5Blue w/ mSwL18 insertion</td>
<td>6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure C.12  CMI Assay: IL-10 EI by antigen 7/8 weeks post vaccination

NADC SIV 2nd & 3rd tests combined antigen comparison (pre-challenge)

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Mean IL-10 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All PBMC</td>
</tr>
<tr>
<td>A538 &amp; w / m,w/m-10 insertion</td>
<td>B</td>
</tr>
<tr>
<td>T98a126 + A538 &amp; w / m,w/m-10 insertion</td>
<td>B</td>
</tr>
<tr>
<td>T98a126 + m,w/m-10 insertion</td>
<td>B</td>
</tr>
<tr>
<td>T98a126 + w / m,w/m-10 insertion</td>
<td>B</td>
</tr>
<tr>
<td>T98a126</td>
<td>B</td>
</tr>
<tr>
<td>T98 wt</td>
<td>B</td>
</tr>
</tbody>
</table>

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Live wtTx98 H3N2</th>
<th>Ag</th>
<th>Live wtA04 H1N1</th>
<th>Ag</th>
<th>Live T98a126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag  1</td>
<td>Live wtTx98 H3N2</td>
<td>Ag  3</td>
<td>Live wtA04 H1N1</td>
<td>Ag  5</td>
<td>Live T98a126</td>
</tr>
<tr>
<td>Ag  2</td>
<td>Killed wtTx98 H3N2</td>
<td>Ag  4</td>
<td>Killed wtA04 H1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.13  CMI Assay: CD25 EI by group 10 weeks post vaccination

NADC SIV 4th test results (1 week post-challenge)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean CD25 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live wtX98 HBN2</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Killed wtX98 HBN2</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Live wtA04 rh111</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Killed wtA04 rh111</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>Live T98α126</td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

CD25 EI = 
\[
\frac{\text{(% CD25 positive cells x mean fluorescent intensity of CD25) of stimulated cells}}{\text{(% CD25 positive cells x mean fluorescent intensity of CD25) of unstimulated cells}}
\]

of the same subset of the same animal

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSvIL-18 Insertion</th>
<th>Gr. 4</th>
<th>T98α126 w/ mSvIL-18 insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>T98α126 + Ad5Blue w/ mSvIL-18 Insertion</td>
<td>Gr. 5</td>
<td>T98α126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>T98α126 + Ad5Blue w/ mSvIL-18 insertion</td>
<td>Gr. 6</td>
<td>T98 wt</td>
</tr>
</tbody>
</table>
Figure C.14  CMI Assay: CD25 EI by antigen 10 weeks post vaccination

NADC SIV 4th test antigen comparison (1 week post-challenge)

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Mean CD25 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5818 w/ m.Sw1-18 Insertion</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>T898A1.26 w/ m.Sw1-18 Insertion</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>T898A1.26 + Ad5818 w/ m.Sw1-18 Insertion</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>T898A1.26 w/ m.Sw1-18 Insertion</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>T898A1.26</td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>T988A126</td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Ag 1</th>
<th>Live wtX98 H3N2</th>
<th>Ag 3</th>
<th>Live wtIA04 rH1N1</th>
<th>Ag 5</th>
<th>Live T988A126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 2</td>
<td>Killed wtX98 H3N2</td>
<td>Ag 4</td>
<td>Killed wtIA04 rH1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.15  CMI Assay: IFN-γ EI by group 10 weeks post vaccination

NADC SIV 4th test results (1 week post-challenge)

<table>
<thead>
<tr>
<th>Ag</th>
<th>IFN-γ Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live wtX98 H3N2</td>
<td></td>
</tr>
<tr>
<td>Killed wtX98 H3N2</td>
<td></td>
</tr>
<tr>
<td>Live wtAd04 H1N1</td>
<td></td>
</tr>
<tr>
<td>Killed wtAd04 H1N1</td>
<td></td>
</tr>
<tr>
<td>Live T98Δ126</td>
<td></td>
</tr>
</tbody>
</table>

\[
\text{IFN}_\gamma \text{ EI} = \frac{\text{%IFN}_\gamma \text{ positive cells} \times \text{mean fluorescent intensity of IFN}_\gamma}{\text{%IFN}_\gamma \text{ positive cells} \times \text{mean fluorescent intensity of IFN}_\gamma \text{ of unstimulated cells of the same subset of the same animal}}
\]

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwIL-18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98Δ126 w/ mSwIL-18 insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwIL-18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98Δ126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98Δ126 + Ad5Blue w/ mSwIL-18 insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure C.16 CMI Assay: IFN-γ EI by antigen 10 weeks post vaccination

NADC SIV 4th test antigen comparison (1 week post-challenge)

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Mean IFNγ Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>All PBMC</td>
<td>A</td>
</tr>
<tr>
<td>CD4</td>
<td>A</td>
</tr>
<tr>
<td>CD4+CD8</td>
<td>A</td>
</tr>
<tr>
<td>CD8</td>
<td>A</td>
</tr>
<tr>
<td>gd</td>
<td>A</td>
</tr>
<tr>
<td>Non T</td>
<td>A</td>
</tr>
</tbody>
</table>

Student's t test for each pair comparison: Levels not connected by same letter are significantly different from each other (p < 0.05)

<table>
<thead>
<tr>
<th>Ag 1</th>
<th>Live wtTx98 H3N2</th>
<th>Ag. 3</th>
<th>Live wtA04 rh1N1</th>
<th>Ag. 5</th>
<th>Live Tx98s126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag. 2</td>
<td>Killed wtTx98 H3N2</td>
<td>Ag. 4</td>
<td>Killed wtA04 rh1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure C.17 CMI Assay: IL-10 EI by group 10 weeks post vaccination

NADC SIV 4th test results (1 week post-challenge)

<table>
<thead>
<tr>
<th>Ag</th>
<th>Mean IL-10 Expression Index (EI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live wt8138 H3N2</td>
<td><img src="chart1" alt="Live wt8138 H3N2" /></td>
</tr>
<tr>
<td>Killed wt8138 H3N2</td>
<td><img src="chart2" alt="Killed wt8138 H3N2" /></td>
</tr>
<tr>
<td>Live wt604 H1N1</td>
<td><img src="chart3" alt="Live wt604 H1N1" /></td>
</tr>
<tr>
<td>Killed wt604 H1N1</td>
<td><img src="chart4" alt="Killed wt604 H1N1" /></td>
</tr>
<tr>
<td>Live Tx98a126</td>
<td><img src="chart5" alt="Live Tx98a126" /></td>
</tr>
</tbody>
</table>

**IL-10 EI** = \( \frac{\text{IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10} \text{ of stimulated cells}}{\text{IL-10 positive cells} \times \text{mean fluorescent intensity of IL-10} \text{ of unstimulated cells of the same subset of the same animal}} \)

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Gr. 1</th>
<th>Ad5Blue w/ mSwil–18 Insertion</th>
<th>Gr. 4</th>
<th>Tx98a126 w/ mSwil–18 insertion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr. 2</td>
<td>Tx98a126 + Ad5Blue w/ wtSwil–18 Insertion</td>
<td>Gr. 5</td>
<td>Tx98a126</td>
</tr>
<tr>
<td>Gr. 3</td>
<td>Tx98a126 + Ad5Blue w/ mSwil–18 insertion</td>
<td>Gr. 6</td>
<td>Tx98 wt</td>
</tr>
</tbody>
</table>
Figure C.18 CMI Assay: IL-10 Ei by antigen 10 weeks post vaccination

NADC SIV 4th test antigen comparison (1 week post-challenge)

Student’s t test for each pair comparison:
Levels not connected by same letter are significantly different from each other (p<0.05)

<table>
<thead>
<tr>
<th>Ag 1</th>
<th>Live wtX98 H3N2</th>
<th>Ag 3</th>
<th>Live wtIA04 H1N1</th>
<th>Ag 5</th>
<th>Live Tx98a126</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 2</td>
<td>Killed wtX98 H3N2</td>
<td>Ag 4</td>
<td>Killed wtIA04 H1N1</td>
<td></td>
<td></td>
</tr>
</tbody>
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