INFLUENZA D VIRUSES IN POLYMICROBIAL INFECTIONS

by

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DEDICATION

This work is dedicated to Dr. Steve Arch.

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LIST OF ABBREVIATIONS

AUC: area under the curve

BRD: bovine respiratory disease

BCG: bromocresol green

CFU: colony forming unit

DPI: days post-infection

G.C.: genome copy number

GMT: geometric mean titer

HA: hemagglutinin

HEF: hemagglutinin-esterase fusion protein

HI: hemagglutinin inhibition

IACUC: Institutional Animal Care and Use Committee

IAV: influenza A virus

IBV: influenza B virus

ICV: influenza C virus

IDV: influenza D virus

JFLF: Johnson Family Livestock Facility

LRT: lower respiratory tract

M1: matrix 1 protein

M2: matrix 2 protein

MDCK: Madin-Darby canine kidney (cells)

M. ovipneumoniae: *Mycoplasma ovipneumoniae*

LIST OF ABBREVIATIONS CONTINUED

Mo-IDV: *M. ovipneumoniae*-IDV coinfected cohort

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NA: neuraminidase

NP: nucleoprotein

NS1: nonstructural protein 1

NS2: nonstructural protein 2

PB1: polymerase basic 1

PB2: polymerase basic 2

P3: polymerase 3

PFU: plaque-forming unit

PFU Equiv.: plaque-forming unit equivalents

qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction

r_s: Spearman correlation coefficient

RT-PCR: reverse-transcriptase polymerase chain reaction

Sal-IDV: Saline-IDV monoinfected cohort

SD: standard deviation

SPF: specific-pathogen-free

ST: swine testicular (cells)

URT: upper respiratory tract

WT: wild-type

ABSTRACT

Influenza D viruses (IDVs) comprise *Deltainfluenzaviridae*, the newest genus of the *Orthomyxoviridae* family. This group of viruses primarily infects the upper respiratory tract and causes only mild symptoms. Unlike most influenza viruses, IDVs infect a wide range of ungulates, including cattle, swine, and sheep. In cattle, IDVs are thought to act as an etiologic agent in bovine respiratory disease (BRD), a severe polymicrobial disease primarily impacting calves. In chapters 2-4 of this thesis, we report the first experimental infection of an IDV in sheep with and without a recent *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) bacterial infection. We found no evidence of overt illness in IDV-infected lambs. Our findings suggested that recent *M. ovipneumoniae* infection induced a mild proinflammatory innate immune response that contributed to an enhanced neutralizing antibody response compared to that of *M. ovipneumoniae*-naïve IDV-infected lambs. These findings suggest that although these lambs did not present with clinical symptoms in response to IDV, carriage could contribute to the inflammatory response in sheep experiencing polymicrobial infections.

Influenza infections can confer short-term protection against additional viral pathogens. This process, called heterologous viral interference, is mediated by type I interferon antiviral signaling. This phenomenon predominantly occurs when a host is infected with a mild virus followed by a more severe virus. In humans, heterologous viral interference can result in an attenuation or delay of symptoms associated with the more severe pathogen. Evidence suggests that IDVs infect humans, although there is no evidence that these infections are symptomatic. To gain insight into whether IDVs can reduce the symptoms of influenza A virus (IAV), we performed a series of experiments in mice. In Chapter 5 of this thesis we show that IDV infection can, but does not always, reduce disease associated with IAV. We subsequently sought to identify critical type I interferon signaling events underlaying this phenomenon, but our results remain inconclusive.

CHAPTER ONE

INTRODUCTION

Influenza D Viruses

Influenza viruses are common pathogens which pose a challenge to people and animals around the world. Most influenza infections are asymptomatic or feature mild respiratory illness, and are readily cleared by a healthy immune system.[1–3] A minority of influenza viruses, particularly those associated with near-annual epidemics and rare pandemics, cause infections that result in moderate-to-lethal symptoms and require medical intervention.[1–3] Beyond their direct pathogenic activity, under certain conditions influenza viruses can predispose the host to severe secondary infections.[4,5] This phenomenon was tragically exemplified by the 1918 influenza pandemic, which killed approximately 50 million people, over half of whom are thought to have died not from the primary viral infection, but rather succumbed to secondary bacterial pneumonia.[6,7] The past 100 years of research has identified a multitude of mechanisms through which influenza viruses modulate host susceptibility, including stimulating cytokine production, altering immune cell function, inducing ciliostasis, and causing tissue damage.[7–11] While our understanding of these mechanisms expands every year, we are still unable to effectively predict or prevent these occurrences.

Influenza viruses belong to the *Orthomyxoviridae* family and are subdivided into 4 genera: *Alphainfluenzavirus*, *Betainfluenzavirus*, *Gammainfluenzavirus*, and *Deltainfluenzavirus,* with species influenza As, Bs, Cs, and Ds (IAV, IBV, ICV, IDV). Influenzas are enveloped viruses with 2 (IAV, IBV) or 1 (ICV, IDV) major surface glycoprotein(s) and 1 (IAV, ICV, IDV) or 2 (IBV)

minor surface protein(s). Within the virion, there is a (-)-sense single stranded RNA genome of either 8 (IAV, IBV) or 7 (ICV, IDV) segments.[12] The segmented structure of the influenza genome allows for intra-genus reassortment that can result in abrupt, significant changes in antigenicity (antigenic shift). Most reassorted virions are non-viable or sufficiently antigenically similar to previously-circulating influenza strains that herd immunity contains any outbreak that may occur. Occasionally, an antigenically-distinct reassorted virus efficiently evades preexisting immunity, spreads, and results in a pandemic.[13] This type of abrupt change in antigenicity is associated with inter-species transmission, and therefore primarily impacts humans when the virus has a non-human reservoir host.[13] Among influenzas, only IAV and IDV have non-human reservoirs.[14] To date, all known influenza pandemics have been caused by an IAV, and a large body of research details IAV mechanisms of infectivity and host interactions.[15] Much less is known about IDV.

Discovery and Structure

IDV was first isolated from a domestic pig exhibiting respiratory symptoms in Oklahoma in 2011.[12] Electron micrographs showed that the isolate was an enveloped virus with features consistent with orthomyxoviruses.[12] The isolate failed to act as a neuraminidase (a function of the surface glycoproteins of IAV and IBV) and was instead found to have *O*-acetylesterase activity consistent with that of the surface glycoprotein of ICV.[12] Reverse-transcription polymerase chain reaction (RT-PCR) failed to identify the virus as IAV, IBV, or ICV but *de novo* sequencing confirmed a 7-segmented genome with approximately 50% sequence similarity to known ICVs. As such, this virus was initially thought to represent a novel subtype of ICV and was provisionally denoted as C/OK.[12] In 2013, closely-related viruses (>96% sequence similarity) were isolated from cattle in Oklahoma and Minnesota, enabling a stronger phylogenetic analysis which

suggested that these viruses were divergent from ICVs.[12] This, coupled with failure of these viruses to viably reassort with any ICVs, was sufficient evidence to define a new genus now known as *Deltainfluenzavirus*, the species of which are IDVs.[12]

IDVs have a structure consistent with all influenzas, with a few important differences. IAVs and IBVs have two major surface glycoproteins which fulfill 3 functions. Acting together, these proteins (1) facilitate cellular docking (hemagglutinin, HA) via sialic acids decorating cellular membrane proteins, (2) enable viral fusion with the endosomal membrane (HA) allowing the ribonucleoproteins to escape into the cytosol, and (3) cleave sialic acids (neuraminidase, NA) to allow for the dispersal of progeny virions. ICV and IDV have only one major surface glycoprotein, the hemagglutinin-esterase fusion protein (HEF) which fulfills all 3 of these functions.[12] HAs, NAs, and HEFs must interact with glycans on the cell surface with specificity for particular glycolytic linkages and modifications. While IAV and IBV tropism is restricted to host cells with either α2,6- or α2,3-linked unacetylated sialic acids, ICV and IDV can bind either of these glycolytic linkages, but require that the sialic acid is 9-*O*-acetylated.[12,16,17] Similarly, NAs cleave off the entire sialic acid, whereas HEFs merely deacetylate, leaving behind an intact unacetylated sialic acid residue. IDVs have remarkably heat- and acid-tolerant HEFs, and can maintain 80% of original infectivity even after 30 minute incubation at a pH of 3.0.[18] While this could have implications for host-range or tissue tropism, it remains to be determined whether HEF stability is biologically relevant.

IDVs have one minor surface protein, matrix 2 (M2), which acts as a hydrogen channel that equalizes the pH of the virion with its surroundings.[19] Beneath the host-derived envelope is a scaffolding protein called matrix 1 (M1).[20] Within the core of the virion lies the 7-segmented

genome. As with other influenzas, each segment is stabilized by viral nucleoproteins (NP) which interact with cellular nuclear importins and, together with 3 polymerase subunits (polymerase basic 1, PB1; polymerase basic 2, PB2; polymerase 3, P3) form a ribonucleoprotein complex.[21] Interestingly, despite having only 7 segments, ICVs and IDVs take on the same canonical "7+1" conformation as IAVs and IBVs, where 7 segments surround one internal segment, forming a barrel containing one duplicate segment.[22,23] Finally, the IDV genome encodes 2 non-structural proteins (nonstructural 1, NS1; nonstructural 2, NS2) which play a role in pathogenicity.[22,24]

Host Range, Global Distribution, and Impact

Since its discovery in swine, further characterization of IDV has identified a broad natural host range, including cattle, domestic and feral swine, domestic and wild goats, domestic sheep, dromedary camels, wild Asian buffalo, horses, and humans.[12,20,25–43] Evidence of IDV (serologically or genomically-confirmed) has been established in the United States, Canada, Mexico, Argentina, Ireland, the United Kingdom, Italy, France, Switzerland, Luxembourg, Turkey, Morocco, Togo, Benin, Côte d'Ivoire, Uganda, Ethiopia, Kenya, China, and Japan.[26,28–38,41–59]

Cattle are thought to be the reservoir host and primary vector for IDVs, and IDV seroprevalence is higher in cattle than in other species. In the United States, a serosurvey including 1,992 bovine serum samples from 42 states returned an average seropositivity rate of 77.5% (ranging regionally from 47.7% – 84.6%).[58] These rates are comparable to those observed in France (31% – 70%), Luxembourg (80.2%), and Italy (74% - 92.4%) but higher than those reported in African or Asian countries (<50%).[37,38,41,42,50,60] By comparison, swine seropositivity rates rarely exceed 15%, and ovine rates fall between 2-6%.[20,30,31,34,35,41,42,50,51,54]

There is a paucity of data on human IDV infections, with an initial study suggesting 1.3% prevalence in the general population.[12] Later, a study of cattle handlers in Florida reported 97% seroprevalence in those with occupational exposure to cattle, versus a general population value of 18%, suggesting that bovine contact may account for the majority of human IDV infections.[27] Trombetta and colleagues reported that between 2005 – 2017, 5.1% - 46.0% of randomly-sampled human sera were positive for anti-IDV antibodies, with fluctuations corresponding to regional livestock outbreaks, supporting the importance of spillover.[43] On the contrary, a subsequent study found an unexpectedly low seropositivity rate (4.9%) in a cohort of Italian swine veterinarians, who have high occupational exposure to swine with IDV.[26] These data suggest that horizontal transmission among cattle is common and that other species like sheep, swine, and humans may become infected during discreet zoonotic spillover events, but may be poor vectors relative to cattle.[60,61]

IDV infection is extremely prevalent in cattle in the United States. One study of neonatal calves identified 97% seropositivity, presumably from transferred maternal antibodies.[28] It is hypothesized that after weaning when passive immunity wanes, young calves become particularly susceptible to IDV and many other pathogens. Indeed, lack of antibody transfer is associated with calves developing polymicrobial pneumonia, referred to as bovine respiratory disease (BRD, formerly called shipping fever).[28,62] BRD results in severe and often-lethal pneumonia in pre- and post-weaned calves. There is no fixed pathogen profile in BRD, but infections can feature a mixture of *Mycoplasma bovis*, *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni,* bovine respiratory syncytial virus, bovine herpesvirus 1, parainfluenza type 3 virus, bovine diarrhea virus, bovine coronavirus, ICV, and IDV.[63] Many of these pathogens, including IDV, do

not induce severe illness on their own. Rather, it is hypothesized that bacterial-viral synergism drives BRD disease severity.[55–57,59,64,65] Since influenzas are common actors in coinfections, and since IAVs are known to promote bacterial colonization of the lower respiratory tract (LRT), it is thought that IDVs may play a similar role in BRD.[59]

Transmission and Pathogenesis

In order to understand how IDVs may contribute to BRD and other polymicrobial infections, we must first understand how they circulate in livestock populations. Controlled experiments have demonstrated that cattle can transmit IDVs to other cattle via direct contact and by aerosol, but failed to transmit from cow to ferrets via fomites.[40,66] A recent study of interspecies transmission showed that calves and pigs could contract IDVs originating from either cattle (D/bovine/Texas/72/2017) or swine (D/swine/Oklahoma/1334/2011) by direct inoculation, and that calves could readily acquire a bovine IDV from pigs, but only 1/3 pigs contracted swine IDV from calves.[67] Interestingly, pigs failed to contract bovine-originating IDV from calves, and calves failed to contract swine-originating IDV from pigs.[67] It remains unclear what underpins these transmission dynamics; multiple studies have noted that swine shed less IDV than cattle do, but this loss in interspecies transmission may also be attributed to viral fitness.[12,30,67]

Experimental infections have demonstrated that IDV infection causes mild-to-moderate symptoms in directly-inoculated cattle, and mild symptoms in directly-inoculated and contact-infected swine as well as in aerosol- and contact-infected cattle.[30,40,66–68] Model species like ferrets, mice, and guinea pigs are also asymptomatic during experimental IDV infection, and attempts at mouse-adaptation of the virus have failed.[40,69–71] When present, clinical signs are primarily respiratory and include cough, nasal discharge, mild dyspnea, and elevated breathing rate.[40,66,72]

Tissue tropism varies between species but always includes the upper respiratory tract (URT). Viral replication can occur in the LRT of cattle, swine, mice, and guinea pigs, but is typically asymptomatic in experimental infections, especially in model organisms.[30,40,66,70–72] There is some evidence suggesting that IDVs isolated from swine may be less fit for LRT infection compared to IDVs isolated from cattle, as to date, swine experimentally infected with a swine IDV isolates have never shown evidence of LRT infection, while those infected with a bovine isolate IDVs have.[12,30] Other infection sites include soft palate (common in bovine infections) and intestines (confirmed in rodent models), and transient viremia can occur. [40,66,71,72]

Cellular and Tissue Tropism

Our understanding of IDV host range and cellular tropism is incomplete. Cellular permissivity to influenza infection is determined by several factors including ambient temperature and pH, but IDV cellular entry is strictly restricted to cells expressing 9-*O*-acetylated α2,6- or α2,3-linked sialic acids.[16,17] To overcome the challenges inherent to animal husbandry and the scarcity of *in vivo* IDV-specific probing methods, several groups have utilized tissue microarrays and explants to investigate cellular tropism. These findings suggest that LRT infections may be rare in hosts other than cattle, such as goats, sheep, horses, and swine.[73–75] Moreover, work by Nemanichvili *et al*. generated recombinant HEF from two lineages of IDV, D/660 (bovine) and D/OK (swine) and found that D/660 HEF had higher avidity, but not altered cellular tropism, in cattle nasal epithelial tissue. In this study, the HEF from each virus bound tissue from the URT of swine, sheep, horses, goats, and cattle but bound the LRT in cattle tissue only.[73] Work by the same group suggests that similar to cattle, IDVs can bind camel URT and LRT tissues.[75] Interestingly, this study also found that D/660 and D/OK viruses showed divergent tissue tropism in elephant

and water buffalo, with D/660 exhibiting higher overall avidity as well as tropism for tissue deeper in the respiratory tract than D/OK.[75] A study of sheep explants compared replication of swine and cattle IDVs in tissue of the nasal turbinate, trachea, and lung and found that IDVs of bovine origin replicate more readily in all 3 tissues, and that swine-isolate IDV infection was weak, with modest replication in the lung explant.[74] Staining illustrated that tropism in ovine lung tissue was localized to macrophages, as opposed to the bovine lung tropism which showed localization with type II pneumocytes.[74] Across species, IDV tropism skews towards ciliated cells, predominantly in the URT, however one group reported tropism in non-ciliated cells of a well-differentiated human nasal epithelial cell culture system.[16,73,74,76] Together, these findings indicate that cattle may be an outlier with respect to tissue tropism, which could contribute to their more pronounced symptoms and transmission efficacy as compared to other species. Additional *in vivo* experiments using an array of natural hosts will be required to further explore this possibility.

Role in Bovine Respiratory Disease

IDVs are putative etiological agents in BRD and as such, efforts are underway to understand their contributions risk factors.[55–57,59,64,65] To date, the only two species known to present symptoms after becoming naturally infected with IDVs are cattle and swine.[12,20,28,33,38,44,48,51,52,54,56,60,64] Interestingly, not all cattle and swine become ill from IDV infection and surveillance studies have found IDV genomes in 2.4-5.0% of healthy cattle.[28,55,57,59,77] While studies have reported up to 60% IDV positivity rates in sick cattle, in most studies IDVs are present in less than 10% of sick cattle, and do not represent the most commonly-found pathogen.[28,33,47–49,56,57,59,64,65,77–80] Some studies report a comparable percentage of IDV-positive cattle in symptomatic and asymptomatic study groups, which could suggest that IDV may not be a

significant driving factor in BRD pathogenesis.[57,77] Contrarily, IDV infection status positively correlates with clinical score in BRD cattle.[33,59,64] Moreover, while Nissly *et al*. found that IDV infection does not increase the probability of coinfection by additional pathogens, they and others have shown that IDV shedding is increased in those animals coinfected with additional pathogens versus IDV-monoinfected animals.[59,64,72] Additionally, among IDV-positive cattle, those with BRD symptoms shed more IDV than asymptomatic penmates.[59,64] While none of these data demonstrate that IDVs are causal agents of BRD, they strongly suggest a link between BRD susceptibility and IDV infection.

Environmental variables like crowding, hygiene, and stress play a role in BRD and immunological susceptibility in general, but many virus-specific variables also contribute to the heterogeneous outcomes in studies of natural infection.[81] Zhang et al. found significantly lower IDV positivity rates in lung homogenate samples (representing LRT infection) compared to URT samples from BRD cattle.[56] The same group noted a higher rate of IDV positivity in samples collected by nasal swab than those collected by tracheal washes, although analysis of these two sampling methods are often pooled in studies using convenience sampling methods.[55,56] These observations support the hypothesis that IDV predominantly replicates in the URT, which should be considered during experimental design and when comparing datasets. Since IDVs of different lineages have shown indications of differential tissue tropism, viral heterogeneity over time and across regions also may contribute to inconsistent outcomes. Awareness of these variables can improve our sampling and data handling to help clarify the contribution of IDVs to BRD in natural settings.[75]

Contribution to Coinfections

While BRD has not been induced experimentally, several controlled experiments have addressed the role of IDVs in coinfection models, with mixed results. Two studies in cattle sought to determine whether IDV exacerbated infection by additional respiratory bacterial pathogens associated with BRD.[72,82] In the first, calves were administered IDV or mock and subsequently infected with the opportunistic pathogen *Mannheimia haemolytica* or mock on day 5.[82] Compared to calves that received either IDV alone or *Mannheimia haemolytica* alone, calves infected by both pathogens did not show signs of synergism, however the authors note that this could be due in part to timing, dosage, age of animals, and inoculation route.[82] The second study evaluated simultaneous infection by IDV and *Mycoplasma bovis*, a pathogen found in BRD calves and the causative agent of bovine tuberculosis.[72,83] On day 0 of this study, immunologically naïve calves were infected through an aerosol route with *Mycoplasma bovis* alone, IDV alone, a mixture of both pathogens, or mock.[72] In contrast to the study with *Mannheimia haemolytica,* calves that were coinfected with IDV and *Mycoplasma bovis* showed increased clinical signs and tissue damage compared to those infected with only one pathogen.[72] Compared to animals infected with only one pathogen, coinfected animals began shedding *Mycoplasma bovis* earlier and cleared IDV more rapidly.[72] Although both influenzas and *Mycoplasma* ssp. have been found to promote LRT colonization by opportunistic URT pathogens, in this study coinfection increased *Mycoplasma bovis* LRT infection, but did not alter LRT infection of IDV.[72] These data are in contrast to the results of a mouse study of IDV infection followed 7 days later by *Staphylococcus aureus* infection, in which IDV infection did not exacerbate *Staphylococcus aureus* infection.[69] Together, these studies show diverse outcomes which may depend on pathogen or timing. Administration route may also confound these outcomes; the studies with staggered inoculation times also utilized

methods that bypassed the URT, and no synergism was observed, whereas simultaneous administration by a method that allowed for URT infection (aerosol administration) of *Mycoplasma bovis* with IDV enhanced pathology and clinical presentation.[69,72,82]

As discussed in detail in Chapter 5 of this thesis, influenzas can elicit a host response that inhibits heterologous viral replication, and as a result, viral-viral coinfection dynamics are often divergent from viral-bacterial coinfections. The most recently published IDV coinfection study investigated IDV-IAV coinfection in swine, which are natural hosts to both viruses.[84] Moreover, one retrospective study found that 42.7% of IAV-seropositive feral swine serum samples were also seropositive for IDV, indicating that these pathogens cocirculate in the same swine populations and could therefore simultaneously infect the same animal.[30] To address this possibility and approximate outcomes of such a coinfection, confirmed-seronegative pigs were infected intranasally with an H3N2 IAV, a D/OK IDV, a mix of IAV and IDV, or mock.[84] The authors found that coinfection (IAV + IDV) had no impact on symptoms as compared to IAV-only, but exhibited a differential effect on IAV versus IDV burden. Specifically, IAV burden remained unchanged with or without IDV present, whereas the presence of IAV increased the IDV burden in the URT, but not in the LRT, of infected pigs.[84] The same group observed viral interference using swine testicular epithelial cell model. Interestingly, they found that IAV and IDV stimulate the expression of the same group of cytokines (primarily IFN-β, CCL5, IP-10, and TNF-α), however in this system there is a delayed response to IDV compared to IAV, which was corroborated by others in an *in vitro* experiment conducted in A549 cells.[69,84] Accordingly, while each influenza could induced viral interference against the other, this effect was conferred more quickly by IAV-infected cells than by IDV-infected cells.[84]

Host Antiviral Response

In the classical paradigm, influenza viruses are first recognized subsequent to cellular entry. This recognition usually occurs through the cytosolic RNA pattern recognition receptors RIG-I and MDA5 or by endosomal RNA pattern recognition receptors TLR3, TLR7, and (in some animal species) TLR8.[85,86] A signaling cascade proceeds through adapter proteins like TRIF, MyD88, TRAF3, TRAF6 and MAVS, resulting in the assembly and translocation of transcription factors like IRF3, IRF7, AP1, and NFκB to the nucleus where they induce the expression of Type I (predominantly IFN-β and IFN-αs) and Type III (IFN-λs) interferons, plus proinflammatory cytokines like TNF-α, IL-1, IL-6, IL-4, IL-10, IL-12, IFN-γ, IL-17, Eotaxin, CCL3, CCL4, CXCL12, and hundreds of other interferon-stimulated genes.[87–89] These autocrine- and paracrine-acting proteins potentiate antiviral signaling and innate immune response by either binding interferon/interleukin receptors (IFNAR, IFNGR, IFNLR, IL-6R/gp130, etc.) or acting to recruit, activate, and polarize immune cells like macrophages, monocytes, neutrophils, and lymphocytes.[88,90,91] While these processes facilitate viral clearance, they can also drive disease pathology by inducing significant damage to the surrounding tissue.[89,92–94] Evaluation of immune cell infiltrate, upregulated genes, and overexpressed proteins can give insight into viral mechanisms and potential treatments.

IDV infection is marked by mild local (usually URT) and systemic inflammation facilitated by immune cell infiltrate.[12,20,40,66,69–72,82] To begin to understand the innate immunological response that underlies disease pathogenesis, susceptibility, and outcomes of IDV infections, several groups have utilized quantitative RT-PCR (qRT-PCR) to quantify inflammatory cytokine transcript copy numbers, a few groups have followed up with protein quantification, and one group utilized transgenic luciferase reporter mice to evaluate NFκB-driven inflammatory

responses.[66,71,72] Other groups have approached this problem through the use of *in vitro* experimentation, which allows for use of a wider array of molecular tools and more complex experimental designs.[24,69,76,84,95]

There is good agreeance that early-mid (days 2-4) IDV infection features the recruitment of immune cell populations to upper respiratory tissue or to the lumen of the lungs of cattle, pigs, ferrets, mice, and guinea pigs.[12,20,40,66,69,71,72,84] Bronchoalveolar lavage and tissue sectioning consistently show recruitment of neutrophils, macrophages, and lymphocytes (in order of prevalence) early in infection.[40,69,72] Concordantly, a variety of chemoattractants are upregulated including CCL2, CCL3, CCL4, CCL5, CCL8, CXCL1, CXCL10, Eotaxin-3, and CXCL11.[66,71] An array of cytokines are also expressed, including IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-13, TNF-α, IP-10, and IFN-γ.[66,71,72] IFN-γ expression is consistently high in *in vivo* IDV infections, confirmed in mice and cattle by transcript copy number and protein quantification.[66,71,72] While IFN-γ is known to be produced by Th1-polarized CD4+ T-cells while suppressing Th2 polarization, studies in diverse systems (bovine, mouse, and human nasal epithelial cells) indicate that IDV elicits a mixed Th1/Th2 response, as determined by transcript copy number of polarizing cytokines.[66,71,72,76]

Antagonism of Type I Interferon Response

Surprisingly, type I interferon signaling, which is tightly associated with antiviral response, seems to play a relatively small role in IDV infection (particularly when compared to that of IFN-γ), and an intact type I interferon receptor (IFNAR1/2) is not required for resolution of IDV infection.[71] While dampened or absent in natural hosts, IFN-β response is sometimes present in *in vitro* studies and in studies in model organisms.[66,71,72,84] One study conducted in human

adenocarcinoma (A549) cells showed that IDV infection stimulated the production of IFN-β at both the transcript (24h post-infection) and protein level (48h post-infection), but failed to induce expression of other type I (IFN-α), type II (IFN-γ), or type III (IFN-λ) interferons.[69] This divergence in interferon signaling patterns could be mediated by the NS1 protein. In a study by Nogales *et al*., wild-type mice expressed IFN-β in response to the NS1 protein of IDV (DNS1). In this study, recombinant viruses were constructed by replacing the IAV (A/Puerto Rico/8/1934) NS1 gene (ANS1) with the NS1 from an IDV (D/Oklahoma/1334/2011). While DNS1 failed to stimulate IFN-β expression as robustly as its IAV counterpart, it also could not inhibit upstream activation of the IFN-β promoter to the extent of ANS1, resulting in a net IFN-β production greater than that of BNS1 and CNS1 at 2 and 4 days post-infection in wild-type mice.[24]

Type I interferon signaling is also suppressed by other IDV proteins. A recent study demonstrated that transfection of a plasmid carrying the M1 protein of IDV is sufficient to suppresses host type I interferon response by promoting proteasomal degradation of an important antiviral and inflammatory signaling adapter protein, TRAF6.[95] M1 was not the only protein that inhibited type I interferon signaling; this study found the same net effect after transfection by plasmids containing PB1, P3, HEF, M2, M1, NS1, and NS2, but not PB2 or NP.[24] Together these studies indicate that, like other influenzas, IDVs elicit a type I interferon response from the host but have evolved to suppress it.

Viral Evolution and Taxonomy

The earliest serological evidence for IDV circulation is cattle serum collected in 2003, and evolutionary analysis suggests that IDV entered circulation between 1990-2003.[29,96] In the last 20 years, IDVs have spread around the world rapidly, and are now present in North and South

America, Europe, Africa, and Asia.[12,29,30,32,33,35,37,38,41–45,48,49,51,52,70,77,79,97] There are currently 5 antigenically-distinct lineages in circulation, denoted as D/OK, D/660, D/CA2019, D/Yama2016, and D/Yama2019.[22,80,98–100] Depending on the phylogenetic analysis, a subset of D/OK viruses can be classified as their own lineage denoted D/China, and a cluster of isolates from France and Ireland (sometimes called D/OK-like) may be denoted as D/IM.[66,71,96] Whereas D/OK and D/660 are thought to have originated in the United States and currently circulate worldwide, D/Yama2016 and D/Yama2019 are so far contained in Japan. At the time of writing, the discovery of D/CA2019 was published just 5 months ago, and little is known about this lineage except that antisera against D/CA2019 show broad cross-reactivity against multiple D/OK and D/660 viruses, but antisera against those same D/OK and D/660 viruses do not cross-react strongly with D/CA2019.[98] In IAVs, this type of mixed heterologous neutralization can result in original antigenic sin, and therefore D/CA2019 could plausibly induce a similar effect.

Lineages are determined in part by comparisons of IDV genomes, but phylogenetic relatedness can vary greatly between segments of each IDV, indicating frequent reassortment.[101] For instance, when the first D/Yama2019 virus was sequenced, most segments showed closest relatedness to D/Yama2016, but the M segment (encoding M1 and M2) was most related to D/OK.[99] In addition to reassortment, nucleotide substitutions are abundant in IDVs. One model reports a rate of 1.0-1.7 x 10^{-3} substitutions per site per year in HEF, which is about twice that of the HEF from ICV and about 40% that of the HA from a H1N1 IAV.[101] Interestingly, the ratio of nonsynonymous to synonymous mutations in HEF was found to be higher in swine than in cattle, and codon usage analysis indicates that swine, despite being a nonprimary host, impart the strongest evolutionary pressure on IDVs.[96,101] Genetic shift, caused by reassortment, and genetic

drift, caused primarily by point mutations, can each alter pathogenicity and antigenicity. In IAVs, trans-species reassortants can undergo antigenic shift and result in pandemics. Current research suggests that the HEFs from each IDV lineage are substantially less diverse than HAs from IAVs, making dramatic antigenic shift much less likely for IDVs.[102] On the other hand, *in vitro* work utilizing a random mutation library demonstrated multiple sites on the PB2 gene that, if changed, could result in an almost-10fold increase in polymerase activity, which could dramatically increase pathogenicity in a natural setting.[103]

Surveillance and Zoonotic Potential

It remains unclear to what extent IDV poses a zoonotic threat, and to what extent it promotes BRD. Still, efforts are underway to track and limit its spread. Hemagglutinin inhibition assays (HIs) are the gold standard for identifying neutralizing antibody responses in influenzas, but there is not consensus on the positivity threshold. While the historical titer is ≥ 1:40, some groups report seropositivity as titers ≥ 1:20 or ≥ 1:10 for surveillance purposes.[12,35,42,53] Recently, Saegerman *et al*. used a receiver operating characteristic curve to determine that a titer of 1:10 was sufficient to match the results of a reference virus microneutralization test, although there is still heterogeneity in HI cutoff reporting by others.[46] As an alternative to HI assays, a solid-phase competitive enzyme-linked immunosorbent assay has been developed that can distinguish between antisera for IAV/IBV versus both high and low IDV antisera.[104] IDV genomes can also be detected simultaneously with other pathogens in multiplexed BRD PCR panels, as well as in multiplexed pan-influenza panels.[105,106]

In addition to diagnostics development, IDV-prevention measures are also a focus of research. To date, multiple attempts at vaccination have yielded mixed results.[107,108] Hause *et al*.

administered an inactivated IDV vaccine with a single booster at day 14, then challenged with the same strain of IDV, finding that while calves still became infected when challenged, titers were lower than mock-vaccinated controls.[107] Wan *et al*. used a DNA vaccine approach, using plasmid DNA encoding an in-house-designed "consensus" HEF sequence with high similarity to both D/OK- and D/660-lineage HEFs. The vaccine and 3 boosts were administered to guinea pigs intramuscularly, resulting in full protection from infection by IDVs from D/OK or D/660.[108] This is a clever, and apparently effective approach, given the diversity in antigenicity between different IDV lineages, but it remains to be determined whether this strategy will confer long-lasting protection in natural hosts.

Future Work

Much has been learned about IDVs in the 11 years since their discovery.[12] By adapting existing tools and techniques employed with other influenzas, we have isolated IDVs from diverse animal species around the world, modelled IDV evolution, and gained insight into mechanisms of IDV-host interaction. Moreover, we have begun to untangle the role of IDVs in BRD and are working to understand their potential as a zoonotic threat. Still, many questions remain unanswered. To date, there are no published infection experiments in three of the five most affected species (camels, sheep, and goats) and pathogenesis and transmission dynamics in these species remains unclear. It has been suggested by multiple authors that infection route may be critical in understanding infection dynamics, and that exposure of the soft palate may be important since it is highly permissive, but this remains to be experimentally confirmed.[72,74,82] We have observed mixed responses to coinfections, leaving our understanding of the contribution of IDVs to host susceptibility largely speculative.[72,82] IDVs consistently elicit a type II interferon response, further

investigation of which may provide some insight, as IFN-γ has been implicated in post-IAV secondary infection.[8,11] Gaining a clearer understanding of the innate and adaptive immunological responses to IDV infection may shed light on the nature of polymicrobial infection dynamics in general, in addition to contributing to our ever-growing body of knowledge of influenza pathogenesis

Mycoplasma ovipneumoniae

M. ovipneumoniae is a bacterial parasite commonly found in the upper respiratory tracts of both sick and healthy sheep and goats.[109–115] *M. ovipneumoniae* exists worldwide and is prevalent in the United States; in a 2011 United States Department of Agriculture survey of 453 sheep farming operations, *M. ovipneumoniae* was detected in nasal swabs from ewes at 401 test sites (88.5%).[109] On its own, *M. ovipneumoniae* produces microscopic lesions in the LRT, and induces the recruitment of immune cells to the lungs.[116,117] While typically nonlethal, *M. ovipneumoniae* can cause chronic nonprogressive pneumonia (also called atypical pneumonia and chronic bronchopneumonia) in lambs (2-12 months old), which is associated with reduced growth rate, decreased carcass quality, and lower overall productivity.[111,114,118–125] It is hypothesized that *M. ovipneumoniae* infection predisposes lambs to secondary infection during which, in combination with stress or upon exposure to additional pathogens (commonly *Mannheimia haemolytica*, *Mycoplasma arginini*, *Bibersteinia trehalosi*, parainfluenza 3 virus, or respiratory syncytial virus), pathology can progress to severe or lethal interstitial pneumonia.[116,126–129] Over the past 50 years a consistent effort has been made towards understanding the biological factors that drive *M. ovipneumoniae* virulence and we are now beginning to understand some host-pathogen interactions that contribute to the variance in *M. ovipneumoniae* pathogenesis.

Discovery and Association with Chronic Nonprogressive Pneumonia

M. ovipneumoniae was originally discovered in pneumonic sheep in Queensland, Australia by St. George *et al*.[130] Per Koch's postulates, isolates were used to infect healthy sheep and confirm its pathogenicity, and it was subsequently characterized by Carmichael *et al*.[122,130] Soon after, another strain of *M. ovipneumoniae* was discovered in Victoria, Australia and 6 distinct strains were isolated from sheep in New Zealand, indicating that *M. ovipneumoniae* is both prevalent and heterogeneous.[131,132] Initially, *in vivo* experimentation gave inconsistent results with respect to disease severity (presence, absence, and location of microscopic or macroscopic lesions in the lung; ability to recover *M. ovipneumoniae* at necropsy, etc.).[123,130,133] It was hypothesized that *M. ovipneumoniae* strain and presence of additional pathogens in inoculae (i.e. lung homogenates versus isolate cultures) may be contributing factors. In 1978, Jones, Gilmour, and Rae compared the effect of infection by lung homogenate versus a "sterile" homogenate consisting of irradiated lung homogenate spiked with equal concentrations of pathogens cultured from the original homogenate (*Mannheimia haemolytica, Escherichia coli, and Mycoplasma arginini*).[129] Sheep receiving the sterile homogenate showed only slightly decreased disease severity compared to those with the bona fide homogenate, indicating that controlling for variables like the presence of additional pathogens and strain-related differences can improve experimental reproducibility. The next year, the same group (Gilmour, Jones, and Rae) demonstrated that coinfection by *M. ovipneumoniae* increased chances of *Mannheimia haemolytica* infection when culture isolates were used as inoculae.[117] At the same time, Alley and Clarke found that as a cultured isolate, *M. ovipneumoniae* can produce microscopic lesions typical of chronic nonproliferative pneumonia, but interstitial pneumonia occurs more readily when additional pathogens like *M. haemolytica*, *Neissera* ssp., α-hemolytic streptococci, and *Escherichia coli* are present, as in the context of a

pneumatic lung homogenate.[128] Taken together, these findings support the hypothesis that *M. ovipneumoniae* may modulate host susceptibility to subsequent pathogens, and that strain may play an important role.

Strain Diversity

M. ovipneumoniae is a species of the *Hominis* group in the class *Mollicutes*, and although often compared to *Mycoplasma pneumoniae* (a human-infecting *Mollicutes* in the group *Pneumoniae*), it is most genetically similar to *Mycoplasma hypopneumoniae* (a porcine-infecting *Mollicutes* in *Hominis*).[134,135] Its genome is only 1 million bases long but encodes everything necessary to sustain its life as a predominantly extracellular (occasionally intracellular) parasite.[136–140] While reduced and lacking genes for some metabolic pathways, this genome length is average for a member of *Mollicutes*. Moreover, its protein coding usage (>80%) is average for bacteria, thus leaving room for a high degree of genetic diversity.[139,141–143] Indeed, every investigation of *M. ovipneumoniae* isolate heterogeneity has found that the vast majority of isolates represent different strains.[117,122,144–151] Initial evaluations of strain heterogeneity were based on metabolic rate, genomic variation as determined by bacterial restriction endonuclease DNA analysis, antigenicity as determined through cross-reactivity with or neutralization by antisera, and virulence as determined by ciliostasis in *ex vivo* coculture with ovine trachea or through *in vivo* sheep infections.[132,148,151–156] Modern PCR-based methods offer more accurate quantitation of strain heterogeneity. In a 2019 study, Kamath *et al*. determined that of 207 isolates collected from domestic sheep, 159 (77%) represented distinct strains, without any geographical correlation between those strains recovered more than once.[144] Moreover, all but 3 farming establishments

surveyed had 100% heterogeneity among recovered strains.[144] Given how many strains cocirculate in the same geographic regions, frequent exposure to new strains is inevitable.

Strain diversity is also found not only within flocks, but also within the host. Ionas, Clarke, and Marshall isolated 2 strains of *M. ovipneumoniae* from each of 2 pneumonic sheep.[148] Even with extensive passaging in culture, isolate genomes were very stable (lacking genetic changes as determined by restriction enzyme digest after 400 generations in culture), without evidence of plasmids or horizontal gene transfer, which is puzzling given the prevalence of strain diversity (discussed above) as well as the well-established rapid culture attenuation that *M. ovipneumoniae* undergoes (discussed below).[149] Later, Ionas *et al*. conducted a longitudinal study of lambs in two flocks, and found that nasal colonization is unstable, but after a strain is cleared another distinct strain may recolonize.[157] Interestingly, Buddle *et al*. found that mixing multiple strains together did not exacerbate pathogenesis in a mouse mastitis model, although it is unclear whether this model is a suitable proxy for *in vivo* virulence.[158] Together, these experiments provide strong evidence that *M. ovipneumoniae* strain diversity contributes to immune evasion; when the host develops immune recognition of one strain and clears it, immunological memory is unable to prevent infection by a second strain less than a month later.[157]

<u>Antagonism of Host Innate Immune Response</u>

In a healthy host, ciliated epithelial cells line the lumen of the URT and work to sweep living and nonliving matter up towards the nose and mouth. This so-called mucociliary escalator represents a frontline defense against colonization of the respiratory tract by pathogens. The mucociliary escalator can become compromised and rendered ineffective by either decreased ciliary beating (ciliostasis) or an insufficient density of cilia due to dysregulated epithelial repair.

M. ovipneumoniae targets respiratory epithelium through multiple known mechanisms. Upon docking onto cilia, likely via adhesin homologs protruding from the capsular surface, *M. ovipneumoniae* can induce ciliostasis as well as ciliary clearing, as documented by light and electron microscopy of tissue from experimentally infected lambs and of ovine tracheal ring explants.[133,138,139,156] Notably, Niang *et al*. found that not only did different strains have differing impacts on ciliary beating, but that this also correlated to the strain's capacity to produce H_2O_2.[156] In this study, ciliary clearance coincided with ciliated epithelial cells shedding off of the basement membrane which was subsequently repopulated by nonciliated cells.[156] Interestingly, *M. ovipneumoniae*-infected lambs can also develop autoreactive antibodies against their cilia, which could then be targeted for destruction by the host itself not just during infection, but also after the infection has resolved.[159] By interfering with the mucociliary escalator, *M. ovipneumoniae* may effectively pave the way for secondary pathogens to reach the LRT.

While ciliostasis and ciliary clearing likely contribute to host susceptibility to secondary pathogens, they are also outcomes of epithelial cell pathogen responses including apoptosis and secretion of proinflammatory cytokines. Few have investigated the underlying mechanisms, but so far research indicates that *M. ovipneumoniae* induces epithelial cell apoptosis through increased intracellular reactive oxygen species and suppressed antioxidant activity.[160,161] Together, this results in mitochondrial damage and the induction of apoptosis, a process which can be attenuated through treatment with reactive oxygen species scavengers like N-acetylcysteine prior to *M. ovipneumoniae* infection.[160–162] These studies also indicated that *M. ovipneumoniae* elicits both intrinsic and extrinsic apoptosis, although cross-talk cannot be ruled out.[163] Accordingly, apoptosis could not only be stimulated by live, H_2O_2-producing *M. ovipneumoniae*, but also by purified

capsular polysaccharide, indicating that despite its lack of a cell wall, the surface of *M. ovipneumoniae* is immunogenic.[162]

As described above, upon encountering a pathogen, epithelial cells produce cytokines that recruit or activate immune cells. Bacteria are typically detected by toll-like receptors (TLRs). In addition to apoptosis, *M. ovipneumoniae* capsular polysaccharide exposure induces the upregulation of TLR1, 2, 4, and 6, contributing to the initiation of a signal cascade including adapter proteins MyD88, IRAK1, IRAK2, and IRAK4, TRIF, and TRAF6, and resulting in the phosphorylation of the transcription factors IRF3, NF-κB, and AP-1.[162] Ultimately, this predominantly proinflammatory cascade results in upregulation of IL-1α and IL-1β, IL-13, and IL-12b, and the secretion of TNF-α.[162,164]

When *M. ovipneumoniae* targets epithelial cells, alveolar macrophages act as first responders. Macrophages are a critical immune cell type which serve to phagocytose and destroy pathogens and help to coordinate recruitment, polarization, and activation of additional immune cell populations, and coordinate the initiation of the adaptive immune response. While histological evidence and differential counts from bronchoalveolar lavage fluid indicate robust macrophage recruitment during *M. ovipneumoniae* infection, *in vitro* work suggests that *M. ovipneumoniae* can block mitogenic expansion of mononuclear cells, inhibit phagocytosis, survive inside phagosomes, and induce macrophage apoptosis.[137,140,165–167] A 1983 study by Al-Kaissi and Alley used electron microscopy to monitor sheep alveolar macrophages interacting with *M. ovipneumoniae*, finding that macrophages failed to phagocytose *M. ovipneumoniae* until the addition of *M. ovipneumoniae*-specific antiserum, hypothesizing that high post-infection antibody titers should suffice to facilitate macrophage clearance during natural infection.[165] Because there was no control branch of this

study using naïve serum or specific-antibody-depleted serum, it is unclear whether this was truly antibody-mediated; additional studies have demonstrated the presence of complement receptors on sheep alveolar macrophages, which could have contributed to opsonization and phagocytic uptake.[166] Interestingly, stimulation of normal sheep alveolar macrophages by live or heat-killed *M. ovipneumoniae* promotes heterologous phagocytic killing of *Staphylococcus aureus*, suggesting that this failure to phagocytose occurs downstream of initial pathogen detection, or that *M. ovipneumoniae* actively inhibits this process.[166]

Macrophages normally detect extracellular bacterial pathogens through extracellular TLR recognition of bacterial cell wall components like lipopolysaccharides, peptidoglycans, and lipoproteins. While *M. ovipneumoniae* lacks a cell wall thereby eliminating many common immunostimulatory agents from its surface, it encodes at least one putative lipoprotein homolog (GenBank Accession #WP_157356156), and its surface contains exposed membrane proteins that could potentially act as TLR agonists.[168] In 2020 Bai *et al*. determined that when exposed to purified lipid-associated membrane proteins from *M. ovipneumoniae*, primary mouse peritoneal macrophages signaled through TLR2, leading to IL-1β secretion that was partially dependent on the NLRP3 inflammasome.[169] It remains unclear whether this signaling occurs via TLR2/1, TLR2/6, through a novel dimerization, or through other TLRs, although transcriptomics profiling of *M. ovipneumoniae*-infected Bashbay sheep implicates TLR2/6.[170]

Intracellular detection may also play a role in the initiation of the host response to *M. ovipneumoniae*. It has been shown that following endocytic uptake, *Salmonella enterica, serovar Typhimurium* components including peptidoglycan can be selectively transferred into the cytosol where they are recognized by cytosolic NOD-like receptors.[171] Recent work has shown that in

mouse macrophages, *M. ovipneumoniae* activates NOD2, but not NOD1, and leads to JNK-mediated autophagy.[137] Although detection and phagocytic clearance may be impaired, other macrophage antimicrobial mechanisms remain intact during *M. ovipneumoniae* infection, including antibody-dependent cellular cytotoxicity.[166] As with epithelial cells, *M. ovipneumoniae* induces both intrinsic and extrinsic apoptosis in macrophages, which is accompanied by a proinflammatory response.[140] Much remains to be learned about innate detection of *M. ovipneumoniae* and how it differs from other pathogens.

Antagonism of Host Adaptive Immune Response

As with innate immunity, the adaptive immune response is also stifled by *M. ovipneumoniae* infection and as a result antibody responses are slow to initiate and are not always protective.[157] *In vitro* research has shown that MHC-I and MHC-II become transiently upregulated in response to *M. ovipneumoniae*, followed by downregulation after 24 hours, which could reduce antigen presentation and initiation the adaptive immune response.[166] As is the case in other organisms, the ovine major histocompatibility complex (*OHC*) locus has a high degree of polymorphism. A recent study indicated that *M. ovipneumoniae* susceptibility may be correlated to haplotype, identifying that polymorphisms in the *OHC-II* locus may correlate to disease severity.[172] In this study, the cytokine profile of the susceptible genotype skewed towards a Th1 (proinflammatory) response whereas the resistant sheep expressed more Th2 (immunosuppressive) cytokines.[172] Moreover, two studies have shown that *M. ovipneumoniae*-experienced lymphocytes show poor proliferative capacity in the presence of *M. ovipneumoniae*, likely mediated by a trypsin-cleavable surface protein.[136,173] Each of these mechanisms could result in a compromised adaptive response.

Early serostudies demonstrated wide variability in antigenicity between *M. ovipneumoniae* strains.[148,150,151,155] One study showed that when probed with homologous antisera, samples from 22 sheep each showed a distinct set of recognized antigens.[151] Another study found that within a flock of *M. ovipneumoniae*-infected lambs, the clonality of the antibody response expanded steadily between 2 and 8 weeks post-infection, after which time responses against certain antigens waned.[155] Three antigens appear conserved between studies and strains, which are approximately 22 kDa, 65 kDa, and 110 kDa in size as determined by SDS-PAGE gel migration.[148,150,151,155] While these antigens were never definitively identified, one candidate for the 65 kDa protein is heat-shock protein 70 (HSP70, molecular weight = 66.1 kDa), homologs of which are common throughout both prokaryotic and eukaryotic life.[174,175]

While antigenic diversity between strains of *M. ovipneumoniae* results in natural antibody responses with poor neutralizing power, broadly-neutralizing antibodies could be elicited through vaccination. *M. ovipneumoniae* HSP70 is a highly-conserved membrane-associated protein, making it a vaccine target with the potential to neutralize many strains of *M. ovipneumoniae*.[175,176] Recombinantly-produced HSP70 C-terminal domain, thought to be the predominant antigenic region, was found to be highly reactive with *M. ovipneumoniae* antiserum.[174] Mice immunized using recombinant full-length HSP70 showed a robust mixed Th1/Th2 immune response, rapid T-cell reactivation upon repeat stimulation, and strong growth inhibition of *M. ovipneumoniae* cultures.[176] In the same study, recombinant full-length *M. ovipneumoniae* elongation factor tu produced similar results, indicating that well-conserved surface proteins may serve as viable vaccine targets.[176] Two groups have attempted experimental immunization of domestic lambs using lysed *M. ovipneumoniae* in adjuvant.[136,177] Sheep in each study developed modest protection

against *M. ovipneumoniae*, but one study showed evidence of poor T-cell expansion upon repeat stimulation, indicating that memory responses may be slow or absent.[136,177]

Intrinsic Host Susceptibility

Perhaps the most obvious determinant of susceptibility to and severity of *M. ovipneumoniae* infection is the host genome. While domestic sheep (*Ovis aries*) rarely experience overt disease from *M. ovipneumoniae* infection, closely related species like Bighorn sheep (*Ovis canadensis*) and Argali sheep (*Ovis ammon*) experience high mortality rates upon infection.[170,178] In these highly susceptible species, *M. ovipneumoniae* exposure often eliminates an entire flock. This disparity in responses clearly suggests a role for genetic predisposition. One study indicated that even among domestic sheep, Rambouillet sheep shed significantly less *M. ovipneumoniae* than Suffolk and Polypay.[179] While the underlying mechanisms that drive *M. ovipneumoniae* susceptibility are not well-understood, genetic and transcriptomics analysis has allowed us to compare pathogen responses that may determine outcomes as well as identify possible therapeutic interventions.[170,180] Aside from the polymorphism in *OHC-II* described above, single nucleotide polymorphisms in the gene encoding mannose-binding lectin also correlate to *M. ovipneumoniae* severity among China Merino sheep, indicating that it may be a particularly important opsonin.[172,181] Interestingly, nasal shedding of *M. ovipneumoniae* has been correlated with the presence of single nucleotide polymorphisms near genes involved with immunity and chromosomal remodeling, although the impact of this finding remains unclear.[179]

While genome-level information can inform our understanding of anti-*M. ovipneumoniae* immune response, transcriptomics data affords us a more complete picture. Two transcriptomics studies of *M. ovipneumoniae* infection are published to date: one describing Bashbay (domestic)

sheep response to *M. ovipneumoniae* infection and one comparing Bashbay with Bashbay-Argali hybrid sheep, which are more resilient against *M. ovipneumoniae* than Argali sheep but more susceptible than Bashbay sheep.[170,180] Analysis of Bashbay *M. ovipneumoniae* response indicates that recognition occurs through TLR2/6 or TLR8 (normally involved in detection of endosomal single-stranded RNA), leading to AP-1 and IRF5 activation and expression of proinflammatory genes including IL-1β and RANTES, suggesting a Th1 response.[180] The second experiment identified differences in inflammatory and acute-phase processes between Bashbay and Argali hybrid sheep, but the most differently-regulated transcript was that encoding short palate, lung, and nasal epithelium clone 1 (SPLUNC1), an important actor in host defense and mitigation of airway diseases in humans.[170] Remarkably, the administration of recombinant SPLUNC1 to Argali hybrid sheep reduced the cytokine response and *M. ovipneumoniae* burden to the levels found in Bashbay sheep.[182] Further investigations into the genomic and transcriptomic underpinnings of pathogenesis could uncover additional actionable mechanisms of treatment.

Future Work

Chronic nonprogressive pneumonia remains a worldwide problem in sheep production but our understanding of how *M. ovipneumoniae* infection facilitates severe secondary infection is developing. Technological advances have enabled us to identify viable treatment options and move closer to an effective vaccine, but we still have hindrances to overcome. One major challenge is the lack of a model system, without which *in vivo* work is cumbersome and slow. Moreover, research is somewhat limited by a dearth of sheep-specific reagents. The ability to obtain transcriptomics data is enormously helpful and has the potential to propel the field, however validation of translation and proper function of the encoded proteins is necessary to confirm these

findings. Developing an understanding of a polymicrobial infection like chronic nonprogressive pneumonia, initiated by a relatively benign pathogen like *M. ovipneumoniae* will provide context and insight into how other coinfections progress. These new insights may be beneficial for addressing the problems at hand but could also assist us in preventing the spread of new polymicrobial diseases in the future.

CHAPTER TWO

MATERIALS AND METHODS

Ovine Experiment

Animals and Husbandry

All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Montana State University, protocol# 2019-109. We previously reported the derivation of a SPF sheep flock through established motherless rearing methods using mixed-breed (Rambouillet/Targhee) founders (F0).[183] The SPF flock was maintained under SPF conditions at the Johnson Family Livestock Facility (JFLF) in Bozeman, MT. Of the F1 SPF sheep, 2 rams and 11 ewes were bred and of resulting progeny (F2), 3 ewes and 4 wethers were selected for each experimental group in this study (n = 7 lambs/group).

Four days prior to the study start date, 12-15-week-old lambs were weaned and moved into a temperature-controlled room (15.5 - 16.8 °C) in the JFLF ABSL-2 facility for acclimation. Experimental groups were housed in different rooms, separated by a procedure area. To minimize the possibility of cross-contact or accidental exposures, all personnel showered and donned sterile personal protective equipment before and after entering either room. All protocols were performed on the *M. ovipneumoniae*-naïve group prior to the *M. ovipneumoniae*-inoculated group. Equipment was sterilized between experimental groups and, when possible, separate sets of procedural equipment were used for each group.

Health Monitoring

Lamb health status was monitored daily and combined clinical scores were assigned daily using previously-established metrics.[183] To summarize, lambs were scored in five categories: (1) behavior, (2) appetite, (3) respiratory symptoms, (4) clinical interventions, and (5) body temperature (measured rectally). Each category uses a scale from 0-5 with higher scores indicating a worsening state, giving a maximum possible combined clinical score of 25. Lambs were also weighed every 14 days. Trained animal husbandry staff examined lambs twice daily and monitored them by video feed throughout each day.

M. ovipneumoniae Preparation

M. ovipneumoniae inoculum was grown from a previously-described nasal wash isolate (MSU NW-4) and expanded exactly as described in the original publication. [183] Briefly, inoculum was grown at 37 °C in Mycoplasma broth under microaerophilic conditions. On the day of inoculation, media was removed (10,000 x g, 10 minutes, 4 °C) and cells were resuspended in sterile DPBS. An aliquot of resuspended cells was diluted in sterile FACS buffer (2% fetal bovine serum and 0.1% sodium azide in DPBS) for counting by flow cytometry.[184] Cells were subsequently stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA) at 4 °C for 30 minutes and Absolute Counting Beads (Invitrogen, Carlsbad, CA) were added to solution immediately prior to analysis using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Virus Preparation

Influenza D virus (D/swine/Oklahoma/1334/2011, a kind gift from Dr. Victor Huber) was propagated in embryonated chicken eggs and inoculum titer was determined by plaque assay as previously described with modifications.[76,185] On the day of inoculation, an aliquot of inoculum

was serially-diluted in 1X inoculation media (1X MEM, 0.3% bovine serum albumin, 2 mM HEPES, with penicillin/streptomycin) containing 1 µg/mL TPCK-trypsin (Thermo Fisher Scientific, Waltham, MA). Dilutions were plated in duplicate onto 90-100% confluent monolayers of Madin-Darby Canine Kidney cells (MDCK, ATCC# NBL-2), a kind gift from Dr. Benfeng Lei. After a 1 h infection period at 33 °C, cells were washed and a semisolid overlay was added (1X inoculation media, 1 µg/mL TPCK-trypsin (Thermo Fisher Scientific, Waltham, MA), 0.6% agarose, 100 µg/mL DEAE-dextran (Sigma Aldrich, St. Louis, MO)). Plates incubated for 5 days at 33 °C. Plaques were visualized by tetrazolium dye vital staining by dispensing 0.5 mL dye solution (3 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, Thermo Fisher Scientific, Waltham, MA) in 150 mM NaCl) on top of each overlay as previously described.[186] Cells were incubated with the dye for 60 minutes at 37 °C, 5% CO_2, or until blue puncta developed and plaques were enumerated immediately.

IDV for hemagglutinin inhibition assays was propagated in swine testicular cells (ST, ATCC# CRL-1746, a kind gift from Dr. Feng Li). Upon reaching 60% confluence, cells were inoculated (MOI = 0.1) in a minimal volume of inoculation media at 33 °C, 5% CO_2 for 1h. Cells were then cultured for 5 days at 37 °C, 5% CO_2 in inoculation media with TPCK-trypsin reduced to 0.1 µg/mL. After outgrowth period, media was collected and virus was concentrated by ultracetrifugation over a 30% sucrose cushion at 100,000 x g, 90 minutes, 4 °C. Pellets were resuspended in sterile DPBS and stored at -80 °C until use.

Intranasal Inoculations

Each experimental group consisted of 3 ewes and 4 wethers aged 12 - 15 weeks old. Among the entire cohort were 3 sets of twins, which were distributed evenly across groups. On the first

day of the study lambs were inoculated with either sterile DPBS (n = 7) or 3.5 x 10^8 CFU *M. ovipneumoniae* (n = 7) by instilling 15 mL into each naris and 10 mL orally. On day 28 of the study all lambs were inoculated intranasally with 1.86 x 10^5 PFU egg-grown IDV in DPBS.

Sampling

Nasal and Rectal Swabs

At indicated timepoints, either rectum or both nares were swabbed. Swabs were transported in DPBS (2 mL), vortexed briefly and transport media was aliquoted and stored at -20 °C until use.

Serum Collection

At the stated timepoints, whole blood was collected by jugular venipuncture using BD Vacutainer serum tubes (Becton, Dickson and Company, Franklin Lakes, NJ) and serum was separated by centrifugation at 1,000 x g, 10 m, 4 °C. Aliquots were stored at -20 °C until use.

Fecal Samples

Fecal samples were obtained directly from the rectum and were transported on ice. Samples (80-120 mg) were subsequently homogenized in 500 µL TRIzol™ Reagent (Invitrogen, Carlsbad, CA) using a bead beater, then stored at -80 °C until time of RNA extraction.

Determining Pathogen Burden

M. ovipneumoniae Quantification

D DNA was extracted from nasal swab samples (500 µL) using the DNeasy Ultraclean Microbial Kit (Qiagen, Redwood City, CA) with modifications. Briefly, cells were pelleted at 10,000 x g for 10 minutes, resuspended in lysis buffer, and incubated at 95 °C for 10 minutes prior

to proceeding with the remainder of the manufacturer's protocol. Each sample was eluted in 50 µL of provided elution buffer. *M. ovipneumoniae* burden was determined by qPCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and previously-validated primers.[187] Samples were volume-normalized using 0.5 µL DNA per reaction. Genome copy number was interpolated from a standard curve using a fragment of the *p113* gene (Twist Bioscience, San Francisco, CA, USA; sequence listed in Appendix A). A standard curve was analyzed in each sample plate. Thermal cycling reactions proceeded in 10 µL volumes using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: 3 minute polymerase activation at 98 °C followed by 40 cycles of denaturation for 15 seconds at 98 °C and annealing/extension for 30 seconds at 60 °C.

IDV Quantification

Viral RNA was extracted from nasal and rectal swabs (140 µL) with the QIAamp Viral RNA Mini Kit (Qiagen, Redwood City, CA) as directed, using the provided carrier RNA, and eluted in 60 µL nuclease-free water. Fecal samples were processed exactly as previously described.[188] Blood samples (100 µL) were processed using the PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences™, Cheshire, UK) using 10 µL RNA extract per reaction. Prior to IDV quantification, successful RNA extraction was confirmed using qRT-PCR to probe for pan-eukarya 18S rRNA (Table 2.1). Extractions with $Cq > 25$ were excluded from analysis and extraction was repeated. IDV burden was determined by qRT-PCR using SsoAdvanced Universal Probes Supermix (Bio-Rad

Laboratories, Hercules, CA) and previously-validated FAM-labelled primers targeting the segment encoding PB1 (Table 2.1).[106] To generate a standard curve for interpolation of PFU equivalents, IDV inoculum was serially-diluted in sterile DPBS and RNA was extracted from each dilution as described for nasal and rectal swabs. A plaque assay was performed in parallel as described above to confirm the inoculum concentration. cDNA libraries were generated as described above and the resulting standard curve was analyzed in each sample plate. Thermal cycling reactions proceeded as for *M. ovipneumoniae* except with the following cycling conditions: 30 second polymerase activation at 95 °C followed by 40 cycles of denaturation for 15 seconds at 95 °C and annealing/extension for 30 seconds at 60 °C.

HI Assay

IDV neutralizing antibody titers were determined by HI as previously described with minor modifications.[189] Briefly, serum was treated with receptor destroying enzyme II (Denka Seiken, Tokyo, Japan) at a 3:1 ratio for 18 h at 37 °C, followed by enzyme inactivation at 56 °C for 30 min. Sera were diluted to 0.1X in PBS and stored at -20 °C until use. HI was performed on sera diluted in series from 1:10-1:1280 using a 0.1% suspension of washed chicken red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA) and in the presence of 4 HAUs of ST-grown IDV. The agglutination reaction proceeded for 45 minutes at room temperature in V-bottom plates and titers were read by tilting the plate 90° for 30 seconds. Viral HA activity was determined just prior to test and confirmed with an in-plate dilution series. Tests were performed on two independent days, twice each day. Partially-agglutinated wells were considered agglutinated. Titers are reported as inverse dilution, and sera with titers < 10 were assigned a titer of 5 for statistical analysis and graphical representation.

Acute Phase Protein and L-Lactate Quantification

Total protein was measured by BCA assay as directed (Thermo Fisher Scientific, Waltham, MA). Albumin was determined using a modified bromocresol green (BCG) protocol. Briefly, 50 µL BCG reagent (Eagle Diagnostics, De Soto, TX, USA) was combined with 50 µL serum diluted appropriately in 0.9% saline. Reaction incubated 15 minutes at room temperature before measuring absorbance at 630 nm. Each test plate included a 125 – 1500 µg/mL standard curve using a commercially-available 2 mg/mL bovine serum albumin standard (Thermo Fisher Scientific, Waltham, MA). Serum amyloid A was measured using a commercially available ELISA (PHASE™ RANGE Multispecies SAA ELISA kit, Tridelta Development, Ltd, Maynooth, Co. Kildare, Ireland) using the bovine standard. L-lactate was measured using the EnzyChrom™ L-lactate Assay Kit (BioAssay Systems, Hayward, CA, USA) as directed, scaled to a final volume of 100 µL/reaction. Sera exhibiting gross hemolysis (background-corrected absorbance at 520 nm > 0.5) were omitted from statistical analyses.

Statistical Analyses

Data are presented as means of at least two technical replicates per sample. Unless otherwise stated in figure legend, differences between treatment groups were analyzed using unpaired two-tailed Student's t-test and all data are reported as means ± standard deviation (SD). When multiple comparisons were made, P-values were adjusted by the Bonferroni-Dunn method. Confidence in Spearman's rank-order correlation analyses was expressed using a two-tailed P-value. All analyses were performed using GraphPad Prism 9 software.

Mouse Experiments

Animals

All mouse studies were conducted at Montana State University, which is an accredited by the Association of Assessment and Accreditation of Laboratory Animal Care (accreditation no. 713), and all studies were approved by the IACUC. Wild-type (WT) C57B/JL6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at the Animal Resources Center at Montana State University (Bozeman, MT). *Ifnar1*$^{-/-}$ breeding pairs were generated as previously described, *Ifnar2*$^{-/-}$ (*Ifnar2*$^{tm1(KOMP)Vlc}$) breeding pairs were originally purchased from UC Davis KOMP repository, and *Ifnb1*$^{-/-}$ breeding pairs were a kind gift from Dr. Stefanie Vogel (University of Maryland in Baltimore).[190] Double knockout mice (*Ifnar1*$^{-/-}$*Ifnb1*$^{-/-}$ and *Ifnar2*$^{-/-}$ *Ifnb1*$^{-/-}$) were generated in-house. All single- and double-knockout mice were bred and maintained at the Animal Resources Center at Montana State University (Bozeman, MT). Mice were between 6 and 12 weeks old at the time of initial infection.

Virus Preparation

IDV was propagated exactly as described for sheep experiments (above), except that cell-grown IDV was propagated in MDCK cells (ATCC# NBL-2), a kind gift from Dr. Benfeng Lei. MDCK-grown IDV titers were verified by plaque assay as described for sheep experiments. Influenza A virus (A/California/7/2009; Cal/09) was propagated in embryonated chicken eggs and TCID$_{50}$ was determined as previously described.[191] Egg-grown viruses and allantoic fluid controls were kind gifts from Dr. Victor Huber.

Mouse Inoculations

Nonsurgical intratracheal inoculations were performed under light anesthesia (2.5% inhaled isoflurane, 2% inhaled oxygen) in 100 µL volumes. Mice were inoculated with 1.73 x 10^4 PFUs of IDV and/or 4 x 10^3 PFU IAV unless otherwise specified. Mice were weighed and monitored for indicators of morbidity and mortality daily. Mice exceeding 25% body weight loss or the IACUC-approved morbidity threshold were euthanized by intraperitoneal injection of sodium pentobarbitol (90 mg/kg) and exsanguinated after loss of pedal reflex.

Bronchoalveolar Lavage and Differential Immune Cell Counts

At indicated timepoints, mice were sacrificed as described above. Bronchoalveolar lavage fluid was collected in lavage buffer (3 mM EDTA in DPBS). Live cells were counted using trypan exclusion and cellular differential counts were obtained by staining cytospins with Quick-Diff solution (Siemens; Medical Solutions Diagnostics, Tarrytown, NY, USA).

Determination of Viral Titers

Lavaged lungs were homogenized in lavage buffer, flash-frozen, and stored at -80C. RNA was extracted from thawed lung homogenates using TRIzol™ Reagent (Invitrogen, Carlsbad, CA) and RNA-containing aqueous layer was further processed using EconoSpin® RNA Mini Spin Columns (Epoch Life Science Inc, Missouri City, TX) per the manufacturer's instructions. RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosciences™, Cheshire, UK). Target cDNA was quantified using SsoAdvanced Universal SYBR Green Supermix (*Ifnb1*, *Rpl13a*) or SsoAdvanced Universal Probes Supermix (IDV *PB1*, IAV *M1*, *Gapdh*) (Bio-Rad, Hercules, CA). Primers are listed in Table 2.1. qRT-PCR reactions were performed in 20 µL volumes using a LightCycler® 96 System (Roche; Basel, Switzerland) and

the following cycling conditions: polymerase activation for 30 s at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C.

Statistical Analysis

Unless otherwise stated, mouse experiments were performed in duplicate with at least 3 mice per group per experiment, with one representative experiment shown. All qRT-PCR reactions were performed in duplicate and normalized using the ΔΔCq method (housekeeping genes and normalization timepoints stated in text). Differences between treatment groups were analyzed using unpaired two-tailed Student's t-test and all data are reported as means ± SD. Comparisons were evaluated by Student's t-test, and P-values were adjusted by the Bonferroni-Dunn method when multiple comparisons were made. All analyses were performed using GraphPad Prism 9 software.

Table 2.1. Primer sequences of qPCR and qRT-PCR. *M. ovi*: *M. ovipneumoniae*.

Target (Species)	**Genbank Accession**	**primer (5'->3') (*TaqmanID; **Bio-Rad UniqueAssayID)**	**Source**	**Ref**
PB-1 **(IDV)**	NC_036615.1	F: CAGCTGCGATGTCTGTCATAAG R: ACAAATTCGCAGGGCCATTA P: FAM-AATGGACTTTCTCCTGGGACTGCT-TAMRA	Bio-Rad Laboratories (Hercules, CA, USA)	106
M1 **(IAV)**	NC_026431.1	F: GGACTGCAGCGTAGACGCTT R: CATCCTGTTGTATATGAGGCCCAT P: FAM-CTAAGCTATTCAACTGGTGCACTTGCCA-TAMRA	Bio-Rad Laboratories (Hercules, CA, USA)	192
18S rRNA (Eukarya)	X03205.1	Hs99999901_s1*	Applied Biosystems (Cheshire, UK)	
Gapdh **(Murine)**	NM_008084.3	qMmuCEP0039581**	Bio-Rad Laboratories (Hercules, CA, USA)	
p113 **(*M. ovi*)**	KR021380	F: TCTCCCAGATGATGCTAACC R: TGAAAATCAACTGGTCTAA	Integrated DNA Technologies (Coralville, IA, USA)	187
Ifnb1 **(Murine)**	NM_010510.1	F: CTGGAGCAGCTGAATGGAAAG R: CTTCTCCGTCATCTCCATAGGG	Integrated DNA Technologies (Coralville, IA, USA)	193
Rpl13a **(Murine)**	NM_009438.5	F: CTCTGGAGGAGAAACGGAAGGAAA R: GGTCTTGAGGACCTCTGTGAACTT	Integrated DNA Technologies (Coralville, IA, USA)	194

CHAPTER THREE

PATHOPHYSIOLOGY OF IDV INFECTION IN SPF LAMBS WITH OR WITHOUT PRIOR *M. OVIPNEUMONIAE* EXPOSURE

Introduction

Polymicrobial pneumonias occur frequently in cattle, swine, and sheep, and result in major economic losses in the livestock industry. Curiously, the individual pathogens comprising these complex infections do not always cause overt illness on their own, instead acting as or facilitating infection by opportunistic pathogens.[59,195,196] Some prominent examples include *Mannheimia haemolytica*, *Mycoplasma ssp.* including *Mycoplasma bovis* and *M. ovipneumoniae*, as well as bovine viral diarrhea viruses, bovine coronaviruses, and IDVs.[72,82,83,117,128,196,197] It is hypothesized that the primary pathogen may remodel or suppress the host immune response, thereby facilitating infection by additional pathogens and/or limiting the host's ability to combat the new infections, ultimately resulting in severe illness.[9,11,95,161,166,167] Understanding the immunological processes driving these events is critical for effective prevention and management. Our group has investigated influenza-associated polymicrobial infections and identified several processes by which influenza A virus infection modulates host susceptibility to subsequent bacterial secondary infection.[190,198,199] We subsequently extended these investigations to include IDV-*Staphylococcus aureus* sequential infection using a mouse model.[69] In the current study we utilized a recently-developed SPF sheep flock to investigate the impact of prior *M. ovipneumoniae* infection on IDV pathogenesis in a natural host.

M. ovipneumoniae is a respiratory bacterium commonly detected in healthy and diseased lambs.[109,110,118] *M. ovipneumoniae* infection can induce chronic nonprogressive pneumonia, particularly in 2 to 12 month old lambs.[113,120,128] While its prevalence in healthy domestic lambs demonstrates that *M. ovipneumoniae* infection can be asymptomatic, evidence suggests that asymptomatic carriage may reduce lamb growth rates.[119,127,196,200] Importantly, while domestic sheep tolerate *M. ovipneumoniae* infection, wild sheep species like Rocky Mountain Bighorn sheep (*Ovis canadensis*) and argali (*Ovis ammon*) are highly susceptible to *M. ovipneumoniae* infection, which predisposes them to severe-to-lethal polymicrobial interstitial pneumonia.[125,127,170,196,200,201] The mechanisms through which *M. ovipneumoniae* promotes secondary infection are not fully understood. *Ex vivo* studies have demonstrated that *M. ovipneumoniae* damages tracheal epithelium through the generation of reactive oxygen species, which may increase susceptibility to a secondary pathogen.[156,161] *M. ovipneumoniae* also induces ciliostasis, promotes anti-ciliary autoantibody production, and may compromise the mucociliary escalator and enable the migration of inert upper respiratory tract-resident microbes like *Mannheimia haemolytica* into the lower respiratory tract, which can result in severe interstitial pneumonia.[138,159,201] In the lower respiratory tract, *M. ovipneumoniae* reduces the phagocytic capacity of alveolar macrophages and impairs pathogen clearance, while stimulating proinflammatory cytokine secretion which damages host tissue.[140,161,165,166] *M. ovipneumoniae* may also impair the adaptive immune response as it bears a surface protein that directly inhibits mitogen-stimulated expansion of T-cells and B-cells.[136,173] Each of these mechanisms may confer an immunosuppressed state and contribute to the increased disease severity associated with *M. ovipneumoniae* colonization.

IDVs comprise a recently-identified genus of *Orthomyxoviridae* about which little is known.[12,20] The natural host range is broad, including (in order of known seroprevalence) livestock like cattle (40-95%), swine (6-12%), horses (12%), sheep (2-6%), goats (1-4%), dromedary camels (6-99%, possible cross-reactivity with influenza C virus), as well as wild ungulates including feral swine[30,34] and water buffalo[37]. [12,25,31,34–36,39,41,42,49,59,202] There is also serological evidence of human infection, and experimental infections have been carried out in model organisms of the human respiratory tract (mice, guinea pigs, and ferrets).[20,26,27,43,69–71] In most species, IDV primarily infects ciliated cells of the upper respiratory tract and soft palate.[12,40,74,161] Cellular tropism varies between species due in part to differences in respiratory system glycosylation patterns.[73,74] IDV infections depend on docking of the HEF to extracellular 9-*O*-acetylated sialic acid residues, and as such cellular permissivity is dependent on the presence of these modified glycans.[16,17] IDV infections typically remain subclinical, but metagenomics studies suggest that IDVs may play an etiologic role in a BRD, a severe-to-lethal polymicrobial infection common in cattle.[59,64,65] In cattle, IDV infects both the upper and lower respiratory tract and can cause mild-to-moderate respiratory symptoms, however, respiratory tissue tropism has not been established for most hosts, including sheep. Until now, active IDV infection has never been documented in sheep, but serostudies in the United States, Ireland, France, and West Africa report IDV-neutralizing antibodies in domestic sheep, suggesting that IDV infections in sheep occur commonly across multiple continents.[31,35,39,41,42]

Despite evidence of circulation of both *M. ovipneumoniae* and IDV in sheep, no studies have characterized infections involving both pathogens. Our previous work and the work of others suggests that *M. ovipneumoniae* monoinfection does not cause clinical illness in lambs. To date,

experimental coinfection of IDV with a bacterial pathogen has been limited to two studies in calves and one in mice.[69,72,82] In the current study, we infected SPF lambs with *M. ovipneumoniae* or mock treatment. After four weeks, we infected all animals with IDV to characterize IDV pathogenesis with and without a recent or ongoing *M. ovipneumoniae* infection. We found that on their own, both *M. ovipneumoniae* and IDV infections were asymptomatic. While we observed no significant difference in overt respiratory disease or shedding when *M. ovipneumoniae*-infected versus naïve lambs were inoculated with IDV, we found subclinical (< 40 °C) elevation in rectal temperature as well as elevated serum protein in lambs exposed to both pathogens. Our data also allowed for correlative analysis which suggests that the amount of recent *M. ovipneumoniae* burden may correspond to the degree of early-phase IDV replication and the endpoint IDV-neutralizing antibody titer. Because no causal relationship has been established, these data must be confirmed by additional experiments.

Results

M. ovipneumoniae Infection Elicits Subclinical Symptoms in IDV-Infected Lambs

To assess IDV pathogenesis with and without prior *M. ovipneumoniae* infection, fourteen SPF lambs were divided into two groups housed in separate pens (n = 7 lambs/group). On day 0 (initiation of Phase 1), *M. ovipneumoniae* (3.5×10^8 CFU) or phosphate-buffered saline sham were introduced intranasally. After 4 weeks, all lambs were intranasally inoculated with IDV (1.86×10^5 PFU), resulting in a double-infected group (*Mo*-IDV) and an *M. ovipneumoniae*-naïve group infected only with IDV (sal-IDV) (Figure 3.1A). Lambs were monitored for an additional 22 (sal-IDV) or 23 (*Mo*-IDV) days before necropsy. For clarity, the first four weeks of the study during

which time we evaluated the difference between *M. ovipneumoniae*-inoculated lambs and immunologically naïve saline controls is denoted as phase 1, and the time period after IDV infection, comparing IDV pathogenesis with versus without prior *M. ovipneumoniae* exposure, is denoted as phase 2 (Figure 3.1A). Overall health, respiratory symptoms, demeanor, weight, and rectal temperature were documented throughout the study to identify signs of illness (Table 3.1, Figure 3.1B-C). For reasons deemed unrelated the experimental conditions, one sal-IDV wether (4104) was euthanized 13 days post-IDV after exhibiting signs of abdominal discomfort and anorexia starting 8 days post-IDV. Data collected from this lamb after 7 days post-IDV were omitted from analysis.

As expected based on our previous work, during phase 1 of the study *M. ovipneumoniae*-infected and sham-treated lambs remained asymptomatic, gained weight at the same rate, and showed no significant difference in rectal temperatures (Table 3.1, Figure 3.1B-C).[183] Following IDV infection (phase 2), we observed no clinical manifestations in any lamb, and no difference in growth between groups (Table 3.1, Figure 3.1B). We did find that *Mo*-IDV lambs had significantly higher rectal temperatures versus sal-IDV lambs at both 3 and 8 days post-IDV (Table 3.1, Figure 3.1B-C). While all temperatures remained subclinical (< 40 °C), this finding could indicate that recent *M. ovipneumoniae* infection promotes a mild inflammatory response during subsequent IDV infection.

Necropsy was performed on 5-6 lambs per group at 22 (sal-IDV) or 23 (*Mo*-IDV) days post-IDV. All lambs in both groups were apparently healthy at necropsy, with no abnormalities or signs of pathogen-associated inflammation. Lung tissue featured mild-to-moderate congestion, petechiae, hemorrhage, edema, and/or atelectasis, all of which were deemed agonal by a board-

certified veterinary pathologist. Accordingly, histological evaluation showed no evidence of microscopic lesions, and no evidence of inflammation-associated immune cell recruitment (Figure 3.1D).

Recent *M. ovipneumoniae* Infection Has A Minimal Impact on IDV Shedding

Nasal swabs were collected throughout the study to determine bacterial and/or viral burden. Pathogen burden (*M. ovipneumoniae*: Genome Copies, G.C.; IDV: plaque-forming unit equivalents, PFU Equiv., see Chapter 2 for details) was quantified by qPCR or RT-qPCR using the standard curve method (Figure 3.2A-B and Figure A1.A-B). We found that after intranasal inoculation, *M. ovipneumoniae* shedding varied widely between lambs and over time (Figure 3.2A). Shedding was never detected for two wethers (3625 and 2811). One ewe and one wether (4527 and 3713) were *M. ovipneumoniae*-positive at only one timepoint (day 14 or 20, respectively), and one wether (4305) shed only at the day 14 and day 20 timepoints. Two ewes (3318 and 4205) shed *M. ovipneumoniae* throughout phase 1 (until 1 day prior to IDV inoculation) and were presumably shedding at the time of IDV infection. Of those two, *M. ovipneumoniae* titers dropped sharply at the next collection timepoint (3 days post-IDV) and remained undetectable for the duration of the study (4205) or remained relatively low (3318) until IDV shedding resolved (Figure 3.2B, Figure A1.A).

All lambs were inoculated intranasally with IDV at the start of phase 2 (28 days post-*M. ovipneumoniae*). We found no significant difference in IDV shedding between groups at any discrete collection timepoint and no difference in total shedding as reflected by area under the curve (AUC) (Figure 3.2B, Figure S1A-B). IDV remained undetectable for one ewe from each group (sal-IDV: 4016, *Mo*-IDV: 4527) (Figure A1.A-B). The remaining 6 sal-IDV lambs and 5 of

the remaining 6 *Mo*-IDV lambs shed IDV at 3, 6, 8, and 10 days post-IDV. One *Mo*-IDV ewe (3811) shed only at 6, 8, and 10 days post-IDV. While not significant, *Mo*-IDV lambs showed a decreased IDV burden compared to sal-IDV animals at 6 days post-IDV (sal-IDV = $9.60 \times 10^4 \pm 1.32 \times 10^5$; *Mo*-IDV = $4.71 \times 10^4 \pm 5.55 \times 10^4$; ratio-paired t-test, adjusted P-value = 0.08, Figure 3.2B).

The wide distribution in degree and timing of shedding of both pathogens allowed us to assess whether the amount of *M. ovipneumoniae* shedding correlated to the amount of IDV shedding within the *Mo*-IDV lambs. To test this, we utilized a Spearman's rank-order correlation analysis, which separately ranked lambs by order of their degree of IDV shedding and by order of their *M. ovipneumoniae* shedding, then determine how similar those rankings are between the two rank orders. This test describes both the strength and directionality of the correlation (Spearman's coefficient, r_s; positive correlation: 0 to 1, negative correlation: 0 to -1, with 1 or -1 being perfect correlations) as well as the certainty of that strength (a standard P-value, P). We found a strong positive correlation between total *M. ovipneumoniae* shedding during phase 1 of the study (days 0 – 28 post-*M. ovipneumoniae*) and total IDV shedding but this correlation had weak certainty (r_s = 0.703, P = 0.228 (Figure 3.2C). Interestingly, we found a very strong positive correlation with high certainty between phase 1 *M. ovipneumoniae* shedding and subsequent IDV shedding at 3 days post-IDV (r_s = 0.882, P = 0.014) (Figure 3.2D). While causation cannot be determined, these data suggest that *M. ovipneumoniae* infection is associated with subsequent accelerated early-infection IDV replication.

<u>Total Serum Protein and Acute Phase Response</u>

Increased total protein in serum can occur during prolonged infection due to the generation of acute-phase proteins during an inflammatory response. We collected serum every 7 or 14 days throughout the study and found no difference in total serum protein levels between treatment groups at any serum collection timepoint (Table 3.2, Figure 3.3A). Since the total serum protein response occurs over time and may not be reflected by single-timepoint or even sequential-timepoint comparisons, we also compared total serum protein concentrations from serum collected before versus after the IDV shedding period (1 day prior to IDV and 14 days post-IDV) (Figure 3.1B). Consistent with our earlier findings that recent *M. ovipneumoniae* infection may increase IDV-associated body temperature and early viral replication, we found that *Mo*-IDV lambs also showed an increase in total serum protein concentration after IDV infection, while sal-IDV lambs showed a small decrease in total serum protein during this time-period (*Mo*-IDV: 2.00 ± 1.43 g/dL versus sal-IDV: -0.413 ± 2.27 g/dL; adjusted P-value = 0.035) (Figure 3.3B).

We also measured acute-phase response via serum albumin (a negative acute-phase protein), serum amyloid A (a positive acute-phase protein), and L-lactate (a metabolic marker of critical illness). As expected, each group displayed decreased serum albumin levels following IDV infection (14 days post-IDV), although these were not statistically significant (Table 3.2, Figure 3.3C). Albumin fraction (albumin / total protein) remained largely stable, and no significant differences were observed between groups at any timepoint, indicating relative health (Figure 3.3D). Serum amyloid A levels varied over time and between lambs but were not affected by treatment (Table 3.2).

While it has been used as a prognostic indicator in some species, the predictive power of L-lactate in sheep infection outcomes remains unexplored in a controlled infection setting. To

investigate whether L-lactate was a predictor of IDV-associated pathology, we compared serum L-lactate at 1 day prior to and 6 days post-IDV and found no significant difference between experimental groups at either timepoint (Table 3.2). L-lactate also failed to predict viral load or reflect past (phase 1) or ongoing bacterial burden (correlative data not shown).

IDV Infection Elicits a Neutralizing Antibody Response in SPF Lambs

To date, studies have reported domestic sheep seroconversion against D/OK, D/660, and D/IM lineage IDVs.[31] To investigate the induction of a neutralizing antibody response in sal-IDV and *Mo*-IDV lambs, we evaluated titers by hemagglutinin inhibition assay (HI) (Figure 3.4A). Lambs showed seropositivity beginning 14 days post-IDV, with all but 2 sal-IDV (3525 and 4016) and all but 2 *Mo*-IDV (3318 and 3811) lambs reaching the defined seropositivity threshold (geometric mean titer (GMT) $\geq$ 10; sal-IDV GMT = 21.5; *Mo*-IDV GMT = 17.2). Interestingly, overall titers were diminished at the experimental endpoint (22-23 days post-IDV; sal-IDV GMT = 14.1; *Mo*-IDV GMT = 11.2), when all but 2 sal-IDV (3525 and 3418) and all but 3 *Mo*-IDV (3713, 3811, and 4527) lambs remained above the seropositivity threshold. No IDV-neutralizing antibody response was detected in one IDV-positive wether from the *Mo*-IDV group (3811; HI $\leq$ 5). This lamb shed only low levels of IDV at 6, 8, and 10 DP-IDV (6 DP-IDV: 9.0 ± 17.0 PFU/mL; 8 DP-IDV: 99.0 ± 64.1 PFU/mL; 10 DP-IDV: 86.8 ± 65.2 PFU/mL) and never shed *M. ovipneumoniae,* despite having been inoculated. Conversely, both lambs with sub-detectable IDV shedding (sal-IDV: 4016; *Mo*-IDV: 4527) showed a measurable neutralizing antibody response at either 14 days post-IDV (4527; GMT = 16.8) or 22 days post-IDV (4016; GMT = 7.1). Interestingly, *Mo*-IDV lamb 4527 exhibited the lowest shedding of any lamb with detectable *M. ovipneumoniae* and no detectable IDV, but still produced IDV-neutralizing antibodies. Notably,

these data support serosurveillance data which suggests that in natural IDV infections, sheep mount a neutralizing antibody response, but that HI titers remain lower than those in some other species like cattle and swine.[12,20,30,31,51]

As with the viral and bacterial shedding data, the HI results were heterogeneous, providing an opportunity for correlative analysis. We found a moderate positive correlation between IDV shedding at 3 days post-IDV and neutralizing antibody response at the endpoint of the study (22 or 23 days post-IDV) (r_s = 0.671, P = 0.012). Interestingly, this effect was strongly driven by the *Mo*-IDV lambs (r_s = 0.954, P = 0.031) and was not observed within the sal-IDV group (r_s = 0.031, P = 0.967) (Figure 3.4B). Moreover, we also found a strong positive correlation between phase 1 *M. ovipneumoniae* burden and endpoint IDV neutralizing antibody titers (r_s = 0.817, P = 0.037) (Figure 3.4C). Taken together, these correlative data imply a connection between recent *M. ovipneumoniae* burden, early IDV infection, and neutralizing antibody response. Moreover, differential responses between *Mo*-IDV versus sal-IDV lambs suggest that while subtle, recent *M. ovipneumoniae* infection does in some way modify the immunological response to IDV in SPF lambs.

Discussion

IDV is a recently-discovered pathogen, and so far its pathogenesis has only been studied in a small subset of its natural hosts.[12,30,40,67,72,82,84] The primary goal of this study was to characterize IDV infection in immunologically naïve domestic lambs, which to our knowledge has not been studied previously. Because lambs are commonly transiently colonized by *M. ovipneumoniae* early in life (2 - 12 months), we also sought to establish whether IDV progression is altered in the context of a recent or ongoing *M. ovipneumoniae* infection.[157] Our experimental

design also allowed us to compare our current findings regarding *M. ovipneumoniae* infection to those we obtained with the previous year's flock.[183] Finally, we found variable shedding, acute phase responses, and neutralizing antibody titers from lamb to lamb, which we leveraged to identify correlations between recent *M. ovipneumoniae* burden, early-infection IDV replication, and neutralizing antibody response (Figure 3.2C-D, Figure 3.4B-C).

In phase 1 of this study, lambs were infected intranasally with a cultured *M. ovipneumoniae* isolate (NW-4) or mock-infected with phosphate-buffered saline, whereas in our previous study lambs were infected using fresh nasal washes from confirmed *M. ovipneumoniae*-positive sheep.[183] While all nasal wash-inoculated lambs in our previous study sustained a detectable *M. ovipneumoniae* infection for 12 weeks, only 5 of 7 isolate-inoculated lambs in the current study ever detectably shed *M. ovipneumoniae*. Moreover, 6 of 7 lambs in the current study apparently cleared the infection by week 5 (Figure 3.1A). While lambs in neither study showed overt clinical signs, nasal wash-inoculated lambs may have shown transient respiratory symptoms, although in that study the mock-infected control group exhibited similar behavior so etiology remains inconclusive.[183] It is well-established that individual *M. ovipneumoniae* strains vary widely in virulence and that *M. ovipneumoniae* is readily culture-attenuated, both of which could have contributed to our comparatively early *M. ovipneumoniae* clearance and complete lack of respiratory symptoms.[153,157,159,168]

In phase 2, all lambs were inoculated with IDV. Similar to *M. ovipneumoniae*, IDV shedding was variable, and 2 lambs failed to detectibly shed virus at any point. Our shedding data show that in sheep, IDV maintains a replication pattern similar to influenza D viruses in other species, inducing an acute infection which persists for less than 2 weeks (Figure 3.2B and S1A-

B).[12,30,40,66,70,71] Moreover, this experiment demonstrated that IDV infection can remain subclinical in lambs, even after recent or ongoing *M. ovipneumoniae* infection, and that virus readily sheds during asymptomatic infection (Figure 3.2B and Figure A1.A-B). Although others have observed rectal shedding and viremia in cattle and swine, we found no evidence of either.[30,37,82] To date, there are no reported IDV isolates from small ruminant respiratory samples, although there is one report of IDV recovered from a rectal swab from a goat that was critically-ill with gastric distress.[37] This goat isolate belongs to the same phylogenetic lineage as the IDV used in this study (D/OK) and is closely related to both bovine and swine IDVs, suggesting that some IDVs may contribute to illness (albeit gastric) in sheep under certain circumstances.[37]

IDV is often asymptomatic in cattle, but may predispose its hosts to secondary infections by facultative pathogens, including *Mycoplasma* ssp. This effect was demonstrated in a study by Lion and colleagues which found that in immunologically naïve calves, simultaneous IDV-*M. bovis* infection increased early-infection *M. bovis* nasal shedding and promoted *M. bovis* colonization in the lower respiratory tract compared to calves that were not coinfected with IDV.[72] Moreover, Nissly and colleagues found that among IDV-positive cattle, those coinfected by additional respiratory pathogens had a higher IDV burden than those solely infected with IDV.[64] While etiology remains unclear, these data suggest that IDV may act synergistically with other respiratory pathogens. In this experiment, *M. ovipneumoniae* was introduced first and had cleared for most lambs by the time IDV was introduced, so for most lambs any impact of *M. ovipneumoniae* would be due to recent rather than ongoing infection, and therefore true synergism would not be possible.

Accordingly, in phase 2 we found no difference in clinical symptoms or overall viral shedding between groups. Despite this, we found several indicators that recent *M. ovipneumoniae* infection impacted IDV pathogenesis. For instance, during IDV infection *Mo*-IDV lambs had significantly elevated rectal temperatures compared to the sal-IDV (Figure 3.1C). While these temperatures always remained subclinical, elevated body temperature is a hallmark response to influenza infection in many species. Interestingly, in our study *Mo*-IDV group showed a strong positive correlation between total phase 1 *M. ovipneumoniae* burden and early-infection IDV shedding (3 days post-IDV) (Figure 3.2D). While these data are correlative, one possible explanation is that recent *M. ovipneumoniae* infection expedites the establishment of a subsequent IDV infection. In an immunocompromised host, this might result in a more severe infection, but if the immune system is functioning optimally, as viral burden rises, there is a proinflammatory response which in turn contains the infection to result in asymptomatic carriage, as we observed in these lambs. During an inflammatory response, serum protein levels rise as acute phase proteins are produced. Consistent with our observation of elevated body temperature in *Mo*-IDV lambs during viral infection, we found that *Mo*-IDV total serum protein level was elevated at 14 days post-IDV as compared to 1 day before IDV (Figure 3.3B). Conversely, serum protein levels in sal-IDV lambs fell for all but 1 animal, suggesting that recent *M. ovipneumoniae* infection promoted inflammation during IDV infection whereas IDV alone did not. Collectively, these data suggest the presence of a subclinical immune response to IDV which is enhanced by recent or ongoing *M. ovipneumoniae* infection.

While an innate immunity-driven inflammatory response may hamper an infection, neutralizing antibodies are typically required for complete clearance of a pathogen. Interestingly,

hemagglutinin inhibition assays on serum from naturally-infected sheep tend to show lower neutralizing antibody titers than those from naturally-infected cattle. In a U.S. retrospective study Quast et al. reported more than half of seropositive sheep (defined as HI ≥ 1:40) had titers of 1:40, with a maximum documented titer of 1:320.[31] This is not specific to the United States; in France no reported titers have exceeded 1:160 and in West Africa the maximum reported titer reported to date is 1:80.[35,41,42] By comparison, HI results in 2,400 randomly-sampled Irish cattle reported only 7% of seropositive cattle to have titers as low as 1:40, with over 15% of titers ≥ 1:640-1:10,240.[39] A recent study established that a 1:10 HI cutoff had equivalent specificity and sensitivity to viral microneutralization-based IDV diagnostics and it may therefore be appropriate to consider whether a lower seropositivity cutoff value should be employed for reporting purposes.[46] Herein we used a cutoff value of GMT ≥ 1:10, *i.e.* excluding samples for which any replicate well agglutinated. Mirroring surveillance data, we observed a maximum GMT of 1:67, with a minority of responses exceeding the traditional influenza seropositivity threshold of ≥ 1:40 (sal-IDV = 3/7, *Mo*-IDV = 2/7). Lambs failed to maintain a GMT ≥ 1:40 to the endpoint of the study (22 or 23 days post-IDV), and only 3/6 sal-IDV and 4/7 *Mo*-IDV maintained GMT ≥ 1:10 through the endpoint. These findings are in contrast to 3 separate calf studies, in which animals sustained or increased their titers between the same timepoints.[40,66,72] Notably, no lambs in this study seroconverted at 6 days post-IDV, which diverges from the response reported in intranasally-inoculated feral swine and calves, but was consistent with colostrum-deprived calves as well as calves infected by contact-transmission.[30,40,66]

Overall, we observed low and unstable neutralizing antibody response to IDV which may not be uncommon to sheep. Despite this, we found a correlation between IDV shedding at 3 days

post-IDV with endpoint HI titer (Figure 4B), suggesting that those lambs with higher early-infection viral loads may mount a more robust secondary immune response and the strength of this correlation was almost exclusively driven by the *Mo*-IDV group (r_s = 0.954, P = 0.031). This finding is somewhat surprising because *M. ovipneumoniae* has been shown to directly suppress mitogenic expansion of T-cells and B-cells, which could plausibly result in a delayed or impaired antibody response.[136,173] Interestingly, the lamb with the highest overall *M. ovipneumoniae* burden also had the highest endpoint HI titer (lamb 3318; GMT = 33.6) and *M. ovipneumoniae* burden had a strong positive correlation with endpoint titer, suggesting that recent or ongoing *M. ovipneumoniae* infection may not suppress a heterologous antibody response in lambs (Figure 3.4C). Taken together, our serological data support prior evidence that sheep fail to either mount or maintain a robust neutralizing antibody response against IDV and indicate that this response may also wane more rapidly than with other hosts, but that recent or ongoing *M. ovipneumoniae* infection does not inhibit antibody production or efficacy.

In conclusion, this study confirms that IDV replicates in sheep and elicits a neutralizing antibody response, but that IDV does not confer pathology or strong acute-phase responses even in sheep infected with *M. ovipneumoniae*. Our findings corroborate serosurveillance data that indicate that IDV neutralizing antibody titers appear relatively weak in comparison to other species' responses, suggest that they were sufficient to clear the pathogen within 2 weeks of infection.[31,35,39,41,42] We also noted rapid waning of the antibody response, which could impact serosurveillance study outcomes. Although subclinical, we found multiple indicators that recent *M. ovipneumoniae* infection stimulated an immunological response, featuring elevated body temperature and an increase in total serum protein in response to IDV infection only in lambs

previously infected with *M. ovipneumoniae*. Moreover, we found that at day 3 post-IDV viral titers and endpoint HI titers correlated to prior *M. ovipneumoniae* burden. We posit that on their own, both *M. ovipneumoniae* and IDV are minimally immunostimulatory in sheep, but that *M. ovipneumoniae* infection may prime the lamb immune system to respond more robustly to secondary infection by IDV. In the context of two subclinical infections this may not lead to overt respiratory symptoms, but virulence varies widely between *M. ovipneumoniae* strains, and IDV is not genetically stable, readily reassorts, and its tissue tropism and host range can vary by strain.[24,67,78,101,149,153,157] Changes in any of these parameters could dramatically alter outcomes to coinfection of both of these pathogens in sheep. Finally, recent work has demonstrated that interspecies transmission is possible and suggested a model wherein cattle are the primary host of IDV, but periodic spillover events result in contained outbreaks in other species.[67] Still, it is unclear whether sheep are an intermediate host and whether sheep-adapted IDVs can infect other species. Therefore, it is important not only that IDV surveillance continues, but also that a standardized HI titer cutoff is implemented, and a sheep IDV isolate(s) is obtained and sequenced for reference.

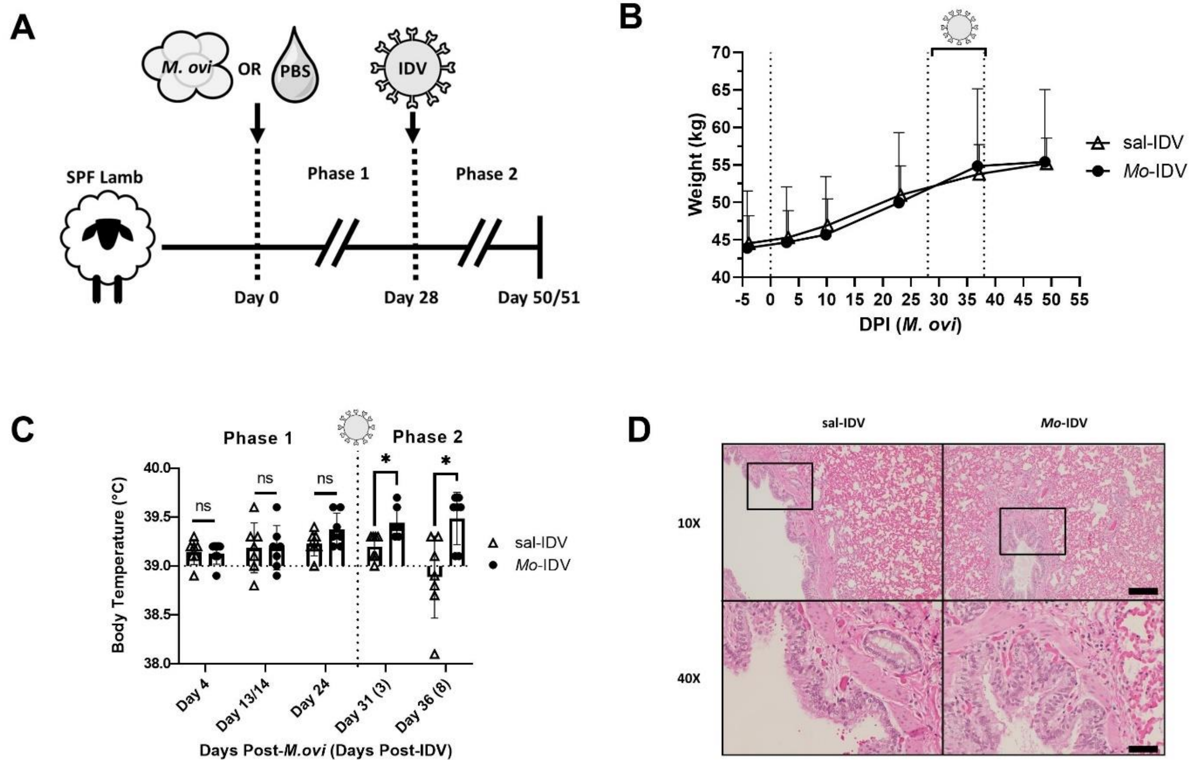

Figure 3.1. IDV with or without recent *M. ovipneumoniae* infection does not induce clinical symptoms in SPF lambs. (A) Schematic representation of experimental design. On day 0, seven lambs were administered *M. ovipneumoniae* (3.5 x 10^8 CFU) or PBS control (n = 7 lambs/group); at day 28 all lambs were inoculated with IDV (D/Oklahoma/1337/2011; 1.86 x 10^5 PFU). (B) Weights and (C) rectal temperatures were monitored throughout the study. (D) Representative histological analysis of distal lungs from each treatment group. Boxes on 10X images denote 40X field. 10X scale bar: 500 µm; 40X scale bar: 100 µm; *M. ovi*: *M. ovipneumoniae*; comparisons evaluated by Student's t-test; * Bonferroni-Dunn-adjusted P < 0.05. Vertical dashed lines denote IDV shedding period, horizontal line indicates average clinically normal lamb rectal temperature (39 °C). Data are represented as mean ± SD.

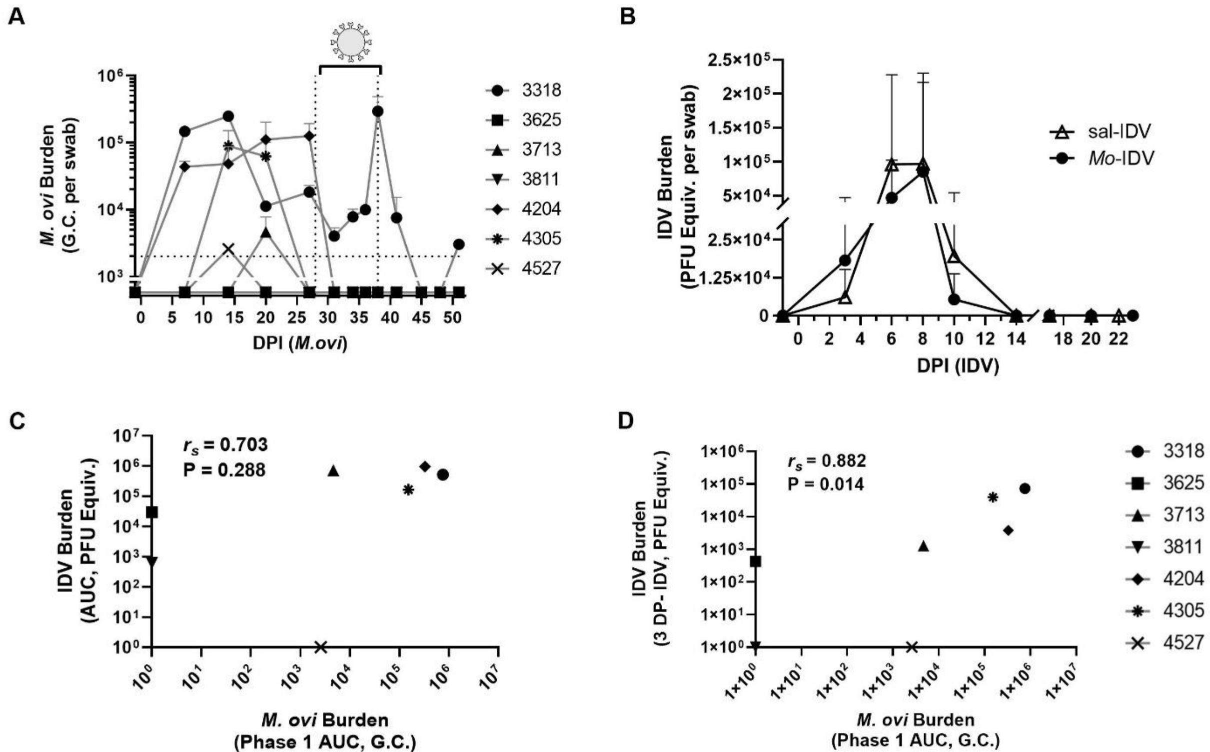

Figure 3.2. Recent *M. ovipneumoniae* infection correlates to early IDV shedding. Nasal shedding was monitored over time for (A) *M. ovipneumoniae*, individual lambs and (B) IDV, mean for each group. (C-D) Scatterplot showing correlation between total *M. ovipneumoniae* burden and (C) total IDV shedding, and (D) IDV shedding at 3 days post-IDV. Dotted horizontal line represents assay detection limit. Phase 1 AUC: area under the curve for phase 1 section of traces in (A); G.C.: enumeration of genome copies by qPCR; PFU Equiv.: enumeration of plaque-forming units by qRT-PCR; r_s: Spearman correlation coefficient; P: P-value. Data presented as mean ± SD.

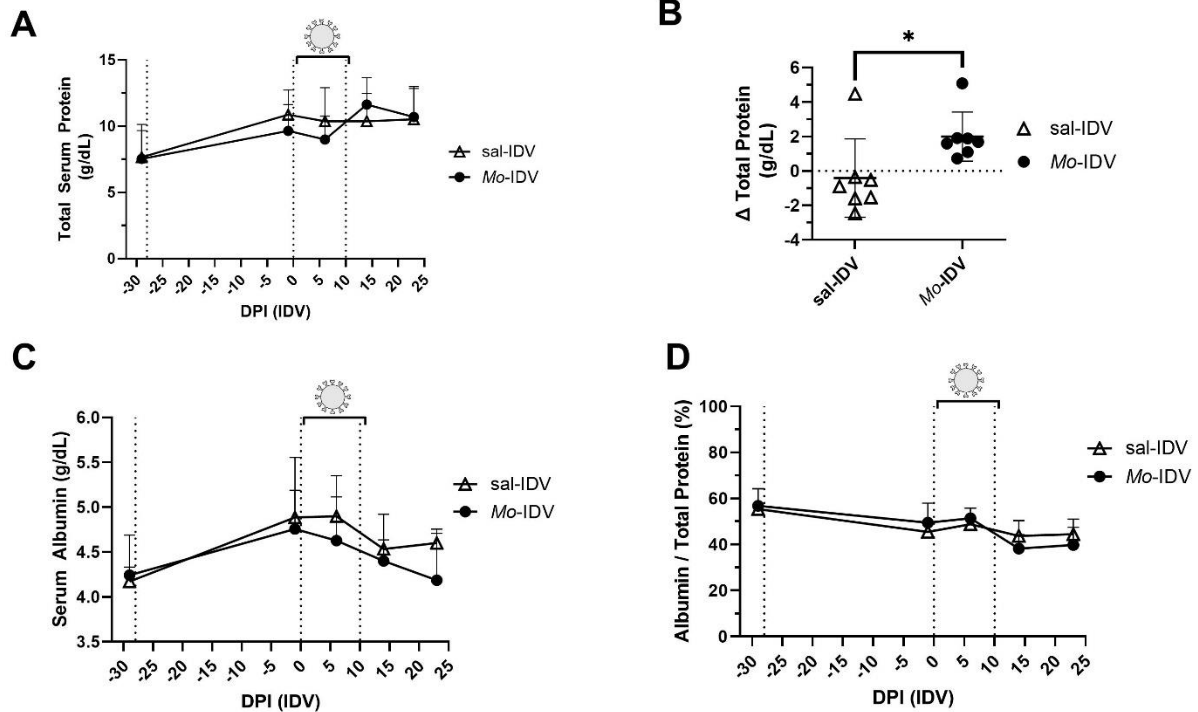

Figure 3.3. Prior infection with *M. ovipneumoniae* has a minimal effect on IDV-associated acute-phase response. (A) Total serum protein was measured throughout duration of study. (B) Change in total serum protein between 1 day prior to IDV infection and 14 days post-infection. (C) Serum albumin concentration were measured throughout duration of study. (D) Ratio of serum albumin:total protein. Vertical dotted lines signify inoculation with *M. ovipneumoniae* or IDV, or the last observed timepoint of IDV shedding, respectively. DPI: days post-infection. Data are presented as mean ± SD. Comparisons were evaluated by Student's t-test. *Bonferroni-Dunn adjusted P < 0.05.

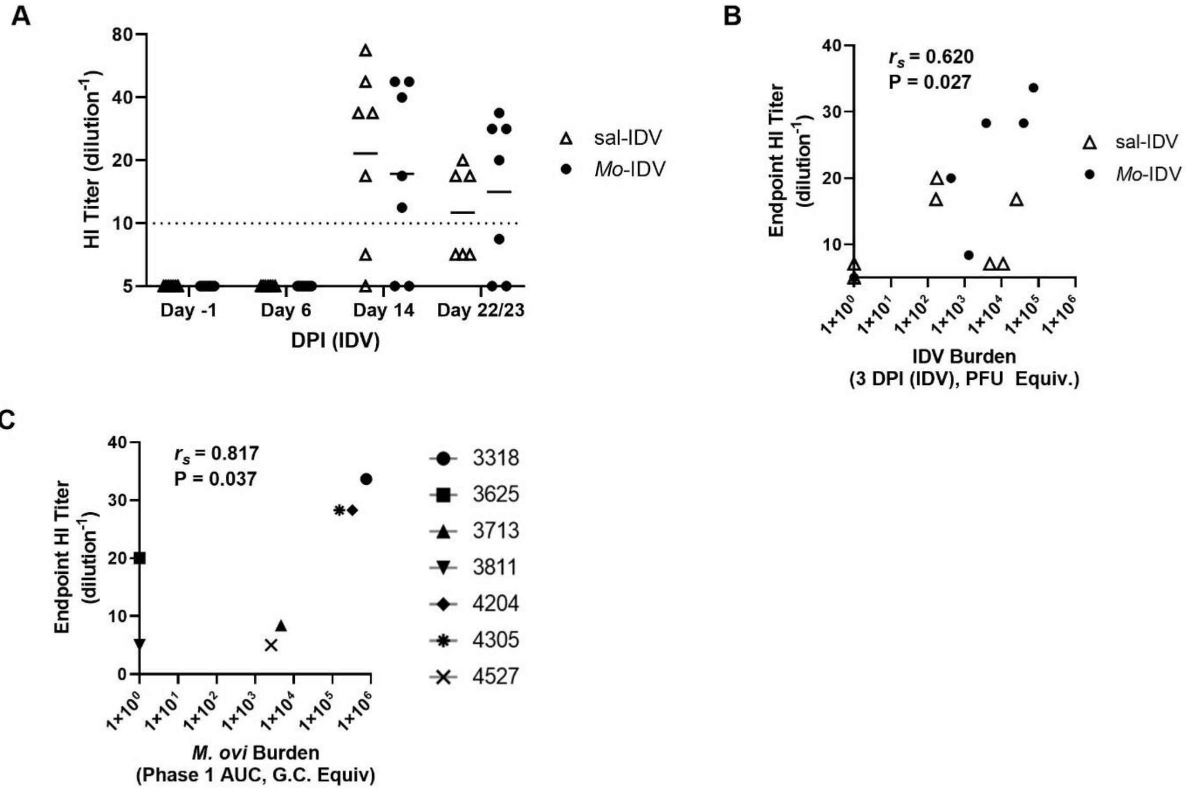

Figure 3.4. IDV antibody response correlates to prior *M. ovipneumoniae* burden and early-IDV-infection viral load. (A) Neutralizing antibody titer by HI assay. (B-C) Correlations between HI titer and (B) viral burden at 3 days post-IDV or (C) total *M. ovipneumoniae* burden during phase 1 (Phase 1 AUC). AUC: area under the curve; r_s: Spearman correlation coefficient; P: P-value. Horizontal line denotes HI cutoff value (10).

Table 3.1. Clinical parameters from each group at indicated timepoints. DPI: days post-infection; N.D.: not done.

	DPI (*M. ovi*)	**DPI (IDV)**	**sal-IDV (SD)**	***Mo*-IDV (SD)**
Attitude	2	-	0 (0)	0.14 (0.38)
	3	-	0.14 (0.38)	0 (0)
	14	-	0 (0)	0.17 (0.41)
	21	-	0 (0)	0.14 (0.38)
	23	-	0 (0)	0.14 (0.38)
	42	14	0 (0)	0.14 (0.38)
Body	2	-	3.29 (0.27)	3.36 (0.24)
	9	-	3.29 (0.39)	3.29 (0.39)
	16	-	3.21 (0.27)	3.07 (0.35)
	23	-	3.36 (0.38)	3.57 (0.45)
	30	2	3.64 (0.38)	3.5 (0.29)
	37	9	3.86 (0.24)	3.79 (0.39)
	44	16	3.42 (0.2)	3.57 (0.35)
Clinical	2	-	0 (0)	0.14 (0.38)
	3	-	0.14 (0.38)	0 (0)
	23	-	0 (0)	0.14 (0.38)
	25	-	0.14 (0.38)	0 (0)
	27	-	0 (0)	0.14 (0.38)
	28	0	0 (0)	0.14 (0.38)
	28	0	0 (0)	0.14 (0.38)
Temperature	4	-	1 (0)	1 (0)
	13	-	N.D.	1 (0.58)
	14	-	1 (0.58)	N.D.
	23	-	1.43 (0.53)	1.29 (0.49)
	24	-	1 (0)	1.29 (0.49)
	31	3	1 (0)	1.29 (0.49)
	36	8	0.33 (0.52)	1.71 (0.49)

Table 3.2. Total serum protein and acute-phase response. Start denotes 1 day prior to *M. ovipneumoniae* infection. All other days relative to IDV infection. Endpoint denotes 22 days post-IDV (sal-IDV) or 23 days post-IDV (Mo-IDV). SAA: serum amyloid A; P: Bonferroni-Dunn adjusted P-value. Comparisons evaluated by Student's t-test.

	timepoint	**sal-IDV**			***Mo* -IDV**			
		mean	median	range	mean	median	range	P
Total Protein (g/dL)	Start	7.66	7.3	6.2 - 8.8	7.6	7.6	6.5 - 8.7	> 0.999
	Day -1	10.8	11	9.1 - 11.7	9.6	9.7	9.1 - 10.1	0.244
	Day 6	10.4	9.9	7.8 - 13.1	9	9.5	6.8 - 10	0.236
	Day 14	10.4	10.3	8 - 13.5	11.6	11.4	10.5 - 14.2	0.581
	Endpoint	10.5	10.7	8.6 - 12.2	10.7	10.5	8.8 - 13	> 0.999
Albumin (g/dL)	Start	4.2	4.2	3.9 - 4.4	4.2	4.3	3.3 - 4.7	> 0.999
	Day -1	4.9	4.9	4.6 - 5.5	4.8	4.8	3.3 - 5.9	> 0.999
	Day 6	4.9	4.9	4.7 - 5.3	4.6	4.8	3.3 - 5.5	> 0.999
	Day 14	4.4	4.4	3.9 - 4.6	4.4	4.4	3.6 - 5.2	> 0.999
	Endpoint	4.6	4.6	4.4 - 4.7	4.2	4.3	3.1 - 5	0.546
SAA (ng/mL)	Day -1	618.5	58.5	3.4 - 2902.2	499.7	42.4	0 - 2936.8	> 0.999
	Day 6	158.4	30.8	0 - 838	667.3	231.2	36.2 - 2793.5	0.587
L-lactate (mM)	Day -1	1.9	2	1.4 - 2.2	1.7	1.8	1.1 - 2.3	0.495
	Day 6	1.8	1.9	0.3 - 3	1.9	1.8	0.8 - 2.8	0.838

CHAPTER FOUR

IDVs IN SHEEP: OUTLOOK

In the preceding study we found indications that recent *M. ovipneumoniae* infection induces a mild inflammatory response in IDV-infected lambs, but this effect has yet to be characterized. We also found a correlation between prior *M. ovipneumoniae* burden, early-infection IDV shedding, and endpoint neutralizing antibody titer. We posit that the immunological activation induced by *M. ovipneumoniae* infection primed lambs for a proinflammatory response to the subsequent IDV infection. Additional data could help elucidate the validity of this hypothesis. In particular, bronchoalveolar lavage of the lambs during active IDV infection would provide information about the kinetics of the cellular infiltrate to the lung as well as allow us to measure cytokine concentrations including IFN-γ, IL-10, IL-6, and TNF-α. As discussed above, IFN-γ is the predominant differentially-expressed cytokine during IDV infection in cattle and mice, but heretofore there are no data on IFN-γ during IDV infection in sheep.[66,71,72] Data on IL-10 could shed light on the state of macrophage activation in the lung, and IL-6 and TNF-α are indicative of a potentially-damaging proinflammatory response. Together cellular and cytokine analysis would elucidate some of the cellular recruitment and activation during IDV infection in sheep.

Tropism in sheep tissues also remains unverified. *Ex vivo* studies of ovine tissues suggest that IDV infections are confined to the URT and submucosal glands.[74] In mice, the gastrointestinal tract can also become infected, and cattle exhibit fecal shedding of IDV in addition to viremia.[40,66,71] While our fecal, rectal, and blood samples were negative for IDV, shedding from

these tissues as well as viremia are transient phenomenon that may not have coincided with our sampling. Aside from tissue tropism, IDV infectivity in immune cell populations has not been profiled in any species, including sheep.[74] More certainty as to viral tropism will foster rational experimental design *in vivo*, *ex vivo*, and *in vitro* and lay a conceptual framework for discrimination between leukocyte activation versus *bona fide* leukocyte infection.

Consistent with serosurveillance data, we noted low neutralizing antibody titers following IDV infection, however it is unclear what antibody isotypes mediated this neutralization, and to what extent each persists. Notably, this response was presumably sufficient to clear the IDV infection, but this could be due to either affinity (class-switched isotypes) or avidity (IgM neutralization). We found that in addition to low overall titers, neutralizing antibodies waned between 14 and 22/23 days post-IDV. In cattle, which have a much stronger neutralizing antibody response to natural IDV infection than sheep, vaccination attempts have given modest results when faced with homologous challenge, introducing the possibility that anti-IDV immunological memory may be weak.[107] So far, there have not been challenge studies with serial IDV infection in an experimental setting, which would provide clearer answers. In humans and mice, B-cells with receptors that have class-switched to IgG, IgE, or IgA are more likely to become plasma cells rather than memory B-cells, but this has not been confirmed in sheep.[203] Determining the predominant isotypes in the IDV neutralizing antibody response could inform our understanding of both antibody specificity and long-term immunity, and in turn help us understand the dynamics of IDV outbreaks.

IDV interspecies transmission is thought to be driven by spillover from cattle to intermediate species (swine, sheep, humans, etc.).[27,43,67] A recent study demonstrated that

interspecies transmission is restricted by viral strain and correlated with the host-of-origin.[67] Analysis of the HEF nucleotide sequences from cattle- and swine-originating strains suggested that evolutionary changes that occur in swine hosts confer higher virulence in cattle.[96] To date, IDV has not been isolated from sheep, therefore we have no information about whether sheep strains exist or what evolutionary changes are conferred in sheep. In fact, we have not yet shown that sheep are capable of intra- or inter-species IDV transmission. Addressing these unknowns would require either consistently monitoring flocks for natural infection, then sequencing isolates from many sheep to determine likely transmission patterns, or a series of controlled transmission experiments. The outcomes of such studies may enable better surveillance and control of transmission.

CHAPTER FIVE

IDV-MEDIATED HETEROLOGOUS VIRAL INTERFERENCE

Introduction

In 1957 Alick Isaacs and Jean Lindenmann published their discovery of a soluble agent capable of inhibiting influenza infection.[204,205] We now know that what Isaacs and Lindenmann observed was influenza-stimulated type I interferon production, which can induce an antiviral state in uninfected cells through autocrine and paracrine signaling. Type I interferon signaling remains a cornerstone in our understanding of the host response to influenzas and other viruses, and recombinant interferons are now used as antiviral therapeutics during viral infections.[206,207] Natural, infection-stimulated interferon production must be carefully choreographed; imperfect timing or concentration can result in either failure to clear a pathogen, or the induction of a catastrophic inflammatory response.[92,93] When appropriately executed, the antiviral state produces heterologous viral interference which protects the host against not just the primary infecting virus, but also against secondary viral infections.[208,209] As a result, in immunocompetent hosts, viral-viral coinfections are often antagonistic; that is, the primary viral infection protects the host against additional viruses, resulting in comparable or milder symptoms than in single infections by either pathogen.[210–212]

While many viruses can induce heterologous viral interference, this phenomenon is driven by the host antiviral response and therefore is dependent on both the virus' capacity to elicit and/or suppress type I interferon production, as well as the host's capacity to mount a type I interferon response. Typically, the *in vivo* type I interferon protein response (predominantly IFN-α subtypes

and IFN-β) requires more than 24 hours to escalate to a detectable level. Then, within a few days, it gives way to the type II interferon response (IFN-γ), which is less potently antiviral.[213] This leaves only a short time during which interferon-mediated viral interference may occur efficiently. Moreover, viruses have an arsenal of host-antagonistic mechanisms which often target the type I interferon signaling cascade.[214,215] For instance, the IAV nonstructural 1 (NS1) protein has been shown to interfere with type I interferon production by inhibiting RIG-I signal transduction in response to detection of the influenza genome, by preventing the interaction between cellular mRNA and its processing machinery to globally downregulate gene expression, and by directly antagonizing interferon stimulated gene products like ZAP thereby weakening the overall antiviral response.[216–218] Therefore, the degree of interferon signaling, and the resulting viral interference, is dependent upon interplay between the host and virus.

Host-intrinsic aberrant type I interferon signaling can also result in weak heterologous viral interference.[219] Children and immunocompromised populations are more susceptible to respiratory viral coinfections than individuals with fully-developed, healthy immune systems.[220–222] Paradoxically, severe primary viral infection itself can lead to sufficiently robust interferon response to induce severe inflammation, thereby damaging the host and ablating viral interference.[92] As such, it tends to be the case that mild primary infections attenuate the symptoms of more severe secondary viral infections, but severe primary infections are exacerbated by the addition of mild secondary viruses.[212,223] For example, a recent *in vitro* study showed that human rhinovirus infection (mild) could attenuate subsequent SARS-CoV-2 (potentially severe) infection in epithelial cells, but this effect was lost if the order was reversed.[224] Interestingly, a similar effect was observed during the 2009 H1N1 influenza pandemic, wherein circulating rhinovirus delayed

the pandemic by several weeks in some parts of Europe.[225] These results have been recapitulated multiple times in *in vivo* mouse experiments demonstrating that protection is dependent on type I interferon signaling, the order of infection, and the severity of each infection.[212,219,223,226] Some evidence – including the work of Isaacs and Lindenmann – suggests that influenzas can also confer protection against a heterologous or heterosubtypic influenza challenge during early infection.[84,227,228]

Little is known about IDV modulation of host susceptibility to secondary viral infection. There is currently only one published study on this topic (published after the work presented in the following chapter was completed).[84] In this study, Guan *et al.* used a mix of *in vitro* and *in vivo* approaches to investigate IAV-IDV coinfection. The *in vitro* branch of this study included variations in the timing and order of infections, finding that viral interference occurred concomitant with and proportional to the IFN-β response elicited by each virus.[84] The *in vivo* branch of the study used a simultaneous coinfection approach, finding that IDV-IAV coinfection limited IDV replication with a minimal impact on IAV.[84] These data are internally consistent and corroborate other *in vivo* and *in vitro* experiments, which suggest that IDV elicits a weaker type I interferon response than IAV, although Guan *et al.* used a somewhat narrow approach specifically centered on type I interferon signaling.[24,40,66,69,72,84,95] While IDV-induced IFN-β expression may not produce as strong of an antiviral effect *in vivo* as it does *in vitro*, subdued IFN-β production reduces the risk of provoking an unchecked inflammatory response, thereby increasing the likelihood of a protective, rather than exacerbative, effect against subsequent IAV infection.

Type I interferon signaling proceeds through interferon α/β receptor (IFNAR), which is a heterodimer of IFNAR1 and IFNAR2. Although it was initially thought that heterodimerization

was strictly required for signal transduction, our current understanding is that IFNAR1 and IFNAR2 can signal together or independently of one another with varying affinity (Table 5.1).[190,229,230] Moreover, it has been shown that differential signaling through IFNAR1 or IFNAR2 elicits distinct host responses and infection outcomes.[190,198,229–232] Work by our group and others demonstrates that IFNAR1/2 signaling is required for an efficient anti-influenza response, and that IFN-β signaling through IFNAR2 is particularly protective against IAV-induced morbidity and mortality.[198,233] Moreover, we found that early-IAV IFN-β induction (day 3) was protective against subsequent bacterial infection, but that mid-late (day 7) IFN-α signaling increased susceptibility.[190] Interestingly, increased susceptibility to bacterial secondary infection 7 days post-influenza was not upheld when using IDV rather than IAV as the primary infectious agent, suggesting fundamental differences in host responses to each influenza.[69]

Since IDV infection results in absent or mild respiratory symptoms with a controlled IFN-β response, comparable to that of rhinovirus, we hypothesized that ongoing IDV infection may attenuate a subsequent IAV infection. To study this, we utilized a suite of transgenic mice with incomplete type I interferon signaling pathways (lacking IFN-β, IFNAR1, IFNAR2, or combinations thereof). We began by characterizing the IFN-β response and viral loads during IDV monoinfection, then moved on to show that IDV can, but does not always, attenuate the weight loss associated with IAV infection in mice. Moreover, it remains unclear what role type I interferon signaling plays in this protection, and to what extent differential IFNAR subunit signaling plays a role.

Results

We first began by characterizing the murine response to IDV using C57/BL6 wild-type mice. As previously documented by us and others, IDV infection did not result in weight loss, clinical symptoms, morbidity, or mortality in wild-type mice (Figure 5.1A).[69,71] Despite this lack of presentation, we detected IDV in the lungs from days 2-7 post-inoculation with a peak at day 4 and a full resolution at day 9 (Figure 5.1B). Moreover, we confirmed our previous observation of neutrophil recruitment the lungs, which was detectable at 3 days post-IDV, and increased until at least day 7 (Figure 5.1C).[69] As discussed in prior sections of this thesis, IFN-β production is subdued during IDV infection in cattle and wild-type DBA/2 mice.[40,66,71,72] In IDV-infected C57/BL6 wild-type mice, we observed an induction if IFN-β expression which was detectable at 3 days post-IDV and increased to 7 days post-IDV (Figure 5.1D).

Since IDV infection stimulated IFN-β expression in this system, we next sought to determine the impact of ongoing IDV infection on IAV pathogenesis. We performed sequential infection experiments wherein on day -4 we inoculated mice with either IDV or PBS mock and after 4 days (day 0), we infected mice with IAV, resulting in 2 groups: IDV-IAV and PBS-IAV. As expected with IAV infections, PBS-IAV and IDV-IAV mice lost weight starting 2 days post-IAV. We found that in some experiments IDV-IAV mice lost less weight than PBS-IAV mice (Figure 5.2A) and protected mice from IAV-associated mortality (Figure 5.2B), suggesting that IDV may interfere with IAV infection. This result was inconsistent and in other experiments, prior IDV infection exacerbated rather than minimized IAV symptoms (Figure 5.2C). To identify the source of this variability, we first investigated whether sporadic lost protection was related to our use of egg-grown IDV inoculum. We conducted an experiment varying the source of the IDV,

along with controls. We used 1.73 x 10^4 PFU of either egg-grown IDV (the original stock used in prior experiments) or IDV grown in MDCK cells. As controls, we used allantoic fluid or PBS. After 4 days of IDV (egg-grown or MDCK-grown) or mock (allantoic fluid or PBS) infection, mice were inoculated with 4000 PFU IAV (the standard dose up until this point). Surprisingly, all groups experienced severe weight loss starting 3 days post-IAV, but this effect was enhanced in the allantoic fluid-IAV, MDCK-grown IDV-IAV, and egg-grown IDV-IAV groups as compared to the PBS-IAV group (Figure 5.2D). This experiment failed to identify a problem with the inoculum that could have resulted in a switch from IDV protection to IDV exacerbation of IAV infection.

We then suspected that this variability could be due to a broad shift in susceptibility that resulted in hypersensitivity to IAV, as we noted that at that time that even PBS-IAV mice were exhibiting more severe symptoms than in prior experiments using inoculae from the same stock. To test this hypothesis, we performed the same sequential IDV-IAV infection with 4 groups in parallel, wherein each group received a different dose of IAV (low = 250 PFU, subclinical = 750 PFU, clinical = 1500 PFU, severe = 4000 PFU). In this experiment, as expected, lower-dose IAV resulted in less weight loss than observed with the higher doses (Figure 5.3A-C). We found that IDV (egg-grown) was protective against the IAV-induced weight loss at low, mid, and clinical doses. Mice receiving IDV followed by the severe dose of IAV showed a delay in weight loss compared to control PBS-IAV mice, but the overall extent of weight loss was comparable between the two groups (Figure 5.2C). Since some protection was restored at lower IAV doses, we subsequently lowered our standard inoculation dose to 1500 PFU (clinical). Unfortunately, as was

the case with the original IAV dosage, IDV-mediated protection was not consistently restored in response to the decreased IAV dosage (Figure 5.3D).

To better understand the role of type I interferon signaling in IDV-induced host response, we deployed a set of single- and double-knockout mice deficient in IFNAR1 (*Ifnar1*$^{-/-}$), IFNAR2 (*Ifnar2*$^{-/-}$), IFN-β (*Ifnb1*$^{-/-}$), both IFNAR1 and IFN-β (*Ifnar1*$^{-/-}$*Ifnb1*$^{-/-}$), or both IFNAR2 and IFN-β (*Ifnar2*$^{-/-}$*Ifnb1*$^{-/-}$) on C57/Bl6 background. Each mouse strain retains some type I interferon signaling, albeit through different signaling interactions each with its own predicted binding affinity (summarized in Table 5.1).[234] This system allows us to gain insight into which specific type I interferon signal transduction events contribute to protection.

We began by characterizing the effect of IDV infection in each strain. We found that in all 4 knockout strains tested (all permutations except *Ifnar2*$^{-/-}$*Ifnb1*$^{-/-}$, which were not available), IDV alone did not induce clinical symptoms, weight loss, morbidity, or mortality, corroborating and extending work by others that found that IFNAR1 was not required for clearance of IDV (Figure 5.4A).[71] Interestingly, we found that when we inoculated with PBS 4 days post-IDV, mice lacking IFNAR1, IFNAR1 and IFN-β, or IFNAR2 and IFN-β exhibited rapid body weight loss over the following 3 days, after which time they fully recovered (Figure 5.4B). Surprisingly, IFN-β-deficient mice with intact IFNAR1/2 receptors showed no response to PBS inoculation at 4 days post-IDV. It has been established that intranasal instillation procedures exacerbate ongoing symptomatic IAV infections in mice, which we confirmed in our lab using our intratracheal administration route (data not shown) but to our knowledge this effect has not previously been observed in asymptomatic infections.[235] These data suggest an important role for mediators outside of IFN-β/IFNAR1/2 signaling that is canonically associated with mediation of antiviral response.

Finally, we attempted to examine the role of type I interferon signaling in protection against IAV that is sometimes conferred by IDV. We found no indication of protection in IFNAR1 or IFNAR2-deficient mice (Figure 5.5A, n = 2 or 1 experiments each, respectively) but we did observe protection in IFN-β-deficient mice (Figure 5.5B, n = 2 experiments). These data are strictly preliminary and the results remain unconfirmed, but it is worth noting that IFN-β-deficient mice also recapitulated the protection observed in wild-type mice when inoculating with IDV followed by PBS. Taken together these data suggest that IFN-β signaling through IFNAR1 or IFNAR2 may be deleterious in the context of IDV infections.

Discussion and Outlook

Little is known about how IDV infections impact subsequent viral infections. In the experiments presented in this chapter we found that IDVs can protect against IAV-associated weight loss, but we found that this result was not consistently reproducible. In an effort to understand this variability, we modulated both the source of IDV (egg-grown versus cell-culture grown) and the dose of IAV (250 – 4000 PFU). We found no evidence that outcomes differed between IDV sources, and although protection was initially restored by reducing the IAV dose to 1500 PFU, we continued to see mixed results with respect to protection. Although it is not shown in this thesis, we also found no impact associated with mouse age, sex, or breeder.

Since the cessation of this project, we have identified several additional possible variables and potentially-fruitful modifications. In this study, we introduced both IDV and IAV intratracheally. Since IDV predominantly infects the URT, intranasal inoculation may be a more appropriate infection route. Importantly, intranasal inoculation is less invasive and can be performed under lighter anesthesia. It is known that intranasal instillation of PBS alone can

exacerbate existing IAV infection, which we recapitulated using our intratracheal method (data not shown).[235] It has been proposed that this is due to liquid rapidly sweeping virus out of damaged URT epithelium into the distal lung.[235] If this is true, results from our interferon knockout mice would suggest a critical role for type I interferon signaling in control of IDV infection in the LRT. It is worth noting that *Ifnb1*$^{-/-}$ mice were the only knockout mice in this study that did not show dramatic weight loss when inoculated with IDV followed 4 days later with PBS, and they were also the only knockout mice in this study for which IDV protected against IAV weight loss. Counterintuitively, in these studies *Ifnb1*$^{-/-}$ mice experienced less weight loss than other type I interferon signaling-deficient mice, suggesting a protective role for IFN-α subtypes that requires intact IFNAR1/2.

Transcriptomics data suggests the IAVs can induce a biphasic inflammatory response in mice, with the first wave occurring between 1-3 days post-IAV and the second at day 7.[89] This is reflected in weight loss curves; in our hands mice typically lose weight from 2-4 days post-IAV (A/California/04/2009), weight loss slows or stalls for 1-2 days, then resumes a second time before mice begin to recover at day 8 or 9. Although mice do not lose weight during IDV infection, data from NF-κB reporter mice suggested modest NF-κB-driven inflammation at 2 and 5 days post-IDV, with minimal inflammation at days 3 and 4.[71] Viral interference is driven by the cytokine milieu in place when the second infection begins. Since both IDV and IAV have undulating, rather than persistent, inflammation during early infection, a small shift in the timing of inflammation could ablate viral interference. Interestingly, in almost all experiments including those where protection was absent, IDV-IAV mice showed either a delay in the first phase of weight loss or less weight loss (sometimes even weight gain) during the first phase as compared to PBS-IAV

mice. While shifting the timing of the inoculations may shed some light on this complex and sensitive system, inter-host variability may continue to stymie these efforts.

In this study, mice with incomplete type I interferon receptors never showed IDV-mediated protection against IAVs. Because wild-type mice showed variability in this protection, and because only 1 or 2 protection studies were conducted using *Ifnar1*$^{-/-}$ or *Ifnar2*$^{-/-}$ mice, we cannot rule out the possibility that they retain the capacity to sustain IDV-IAV viral interference. Interestingly, we saw significant protection in *Ifnb1*$^{-/-}$ mice at days 6-7 post-IAV. These data are puzzling because in the absence of IFN-β, type I interferon signaling relies on IFN-α subtypes, which have a relatively weak interaction with IFNAR1 and IFNAR2.[230] IFN-γ has been shown to be the predominant interferon expressed in response to IDV infection, and is primarily secreted from natural killer cells and T-lymphocytes. It could be the case that IFN-α signaling through an intact IFNAR1/2 receptor is critical for regulation of these cell types, thereby regulating IFN-γ production. This could be addressed through a series of cellular-subset depletion/adoptive transfer experiments.

In conclusion, these studies show that although it does not elicit a strong inflammatory response in most species, and its induction of type I interferons is relatively weak, IDV infection can confer protection against IAV-induced weight loss. Moreover, this work highlights the fine balance required for this effect. Clinical reports of viral coinfection are often mixed, with a trend towards slight exacerbation during viral-viral coinfection.[220,222,236] In most studies, causation is not clear and it is impossible to ascertain whether an immunocompromised host was predisposed to polymicrobial infection, or whether a an otherwise healthy host contracted a primary infection that increased its susceptibility to infection by additional pathogens. Moreover, asymptomatic

infections are rarely diagnosed, and if viral interference occurs to its fullest potential, a host may never become ill or seek medical intervention.

It is currently unclear to what extent IDV is transmitted from human to human, but if IDV suppresses IAV symptoms in a natural setting, this could contribute to asymptomatic carriage and inadvertent spread of IAV. IDV may or may not be a zoonotic threat in its current form, but, like IAV, its genome is unstable and reassortment events are common.[80,101] Among other factors, host response drives influenza evolution.[237] Although IAVs and IDVs by definition cannot reassort, they may act on similar pathways but to different extents, which could push viral evolution towards new targets of antagonism, resulting in a trend towards increased virulence. Given the potential consequences of these possibilities, IDV circulation in humans should be monitored and tracked in order to gain a better understanding of the potential impact on public health.

Table 5.1. Relative signaling strength and mechanisms among knockout mice used in this study.

Genotype	**Remaining Signaling**	**Interaction**[234]
wild-type	IFN-α and IFN-β through IFNAR1/2	Complete
Ifnar1$^{-/-}$	IFN-α and IFN-β through IFNAR2	Incomplete
Ifnar2$^{-/-}$	IFN-β through IFNAR1	Weak
Ifnb1$^{-/-}$	IFN-α through IFNAR1/2	Weak
Ifnar1$^{-/-}$*Ifnb1*$^{-/-}$	IFN-α through IFNAR2	Very Weak
Ifnar2$^{-/-}$*Ifnb1*$^{-/-}$	IFN-α through IFNAR1	Weakest

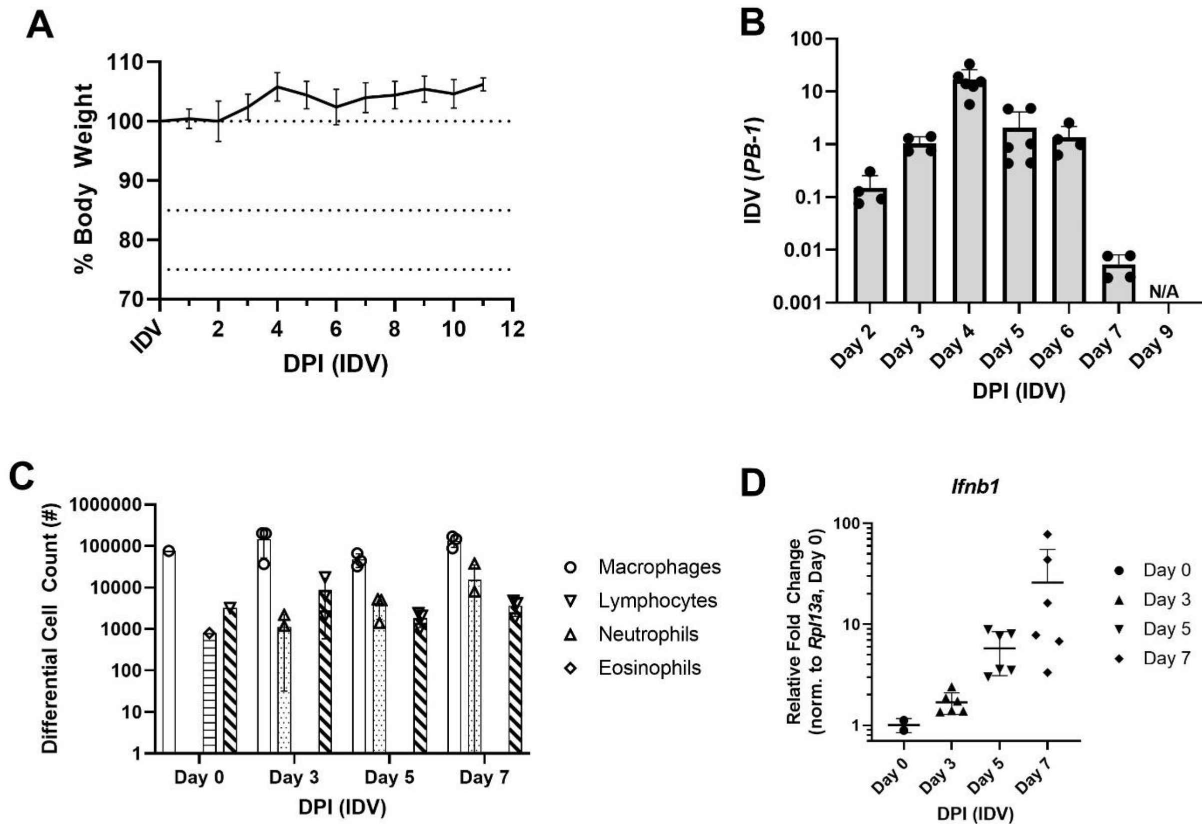

Figure 5.1. IDV infection in C57BL/6 wild-type mice. (A) On day 0 mice were inoculated intratracheally with IDV and body weights were reported as % of weight at day 0 (n = 5 mice). (B) Following inoculation, IDV burden was measured in lung homogenates at indicated days (DPI: days post-infection; relative fold change expressed as $2^{-\Delta\Delta Cq}$ normalized to *Gapdh* and day 3 PB-1 titer; n = 2-3 mice per timepoint). (C) Differential cell counts from bronchoalveolar lavage fluid at indicated timepoints (n = 1 mouse at day 0, n = 3 mice all other timepoints) (D) Quantification of *Ifnb1* expression in lung homogenates at indicated timepoints; expressed as $2^{-\Delta\Delta Cq}$ normalized to *Rpl13a* and day 0 *Ifnb1* expression (same cohort as (C)). Data presented as mean ± SD.

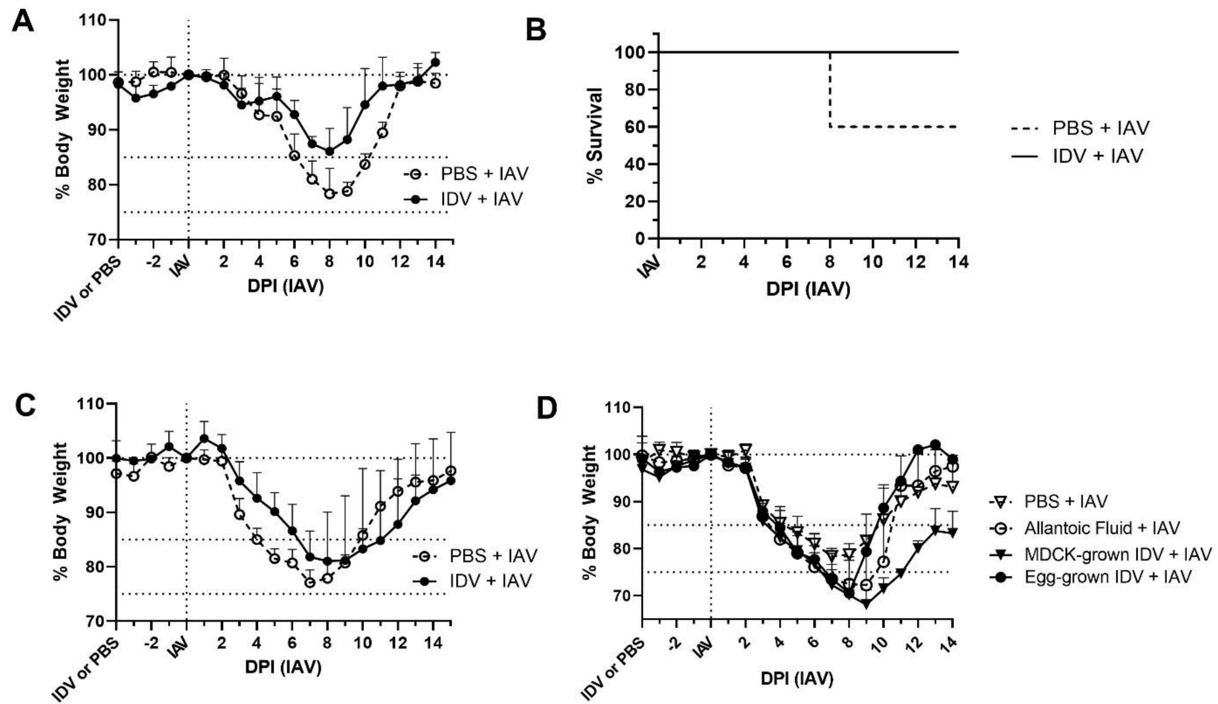

Figure 5.2 IDV infection inconsistently provides protection against IAV-induced weight loss in wild-type mice. Wild-type mice were inoculated with PBS (white circles) or IDV (black circles) on day -4 and challenged with IAV on day 0 (vertical dotted line). (A, C) Body weight (percent of day 0), and (B) survival were monitored daily. (D) Wild-type mice were inoculated with PBS (open triangles, dotted line), allantoic fluid (open circles, dotted line), cell-grown IDV (closed triangles, solid line) or egg-grown IDV (closed circles, solid line) at day 0 followed by IAV at day 0 (vertical dotted line). * Bonferroni-Dunn-adjusted $P < 0.05$ comparing groups using Student's t-test; MDCK: Madin-Darby Canine Kidney cells. Data presented as mean ± SD, $n \geq 4$ mice/group. Experiment in (D) was only performed one time.

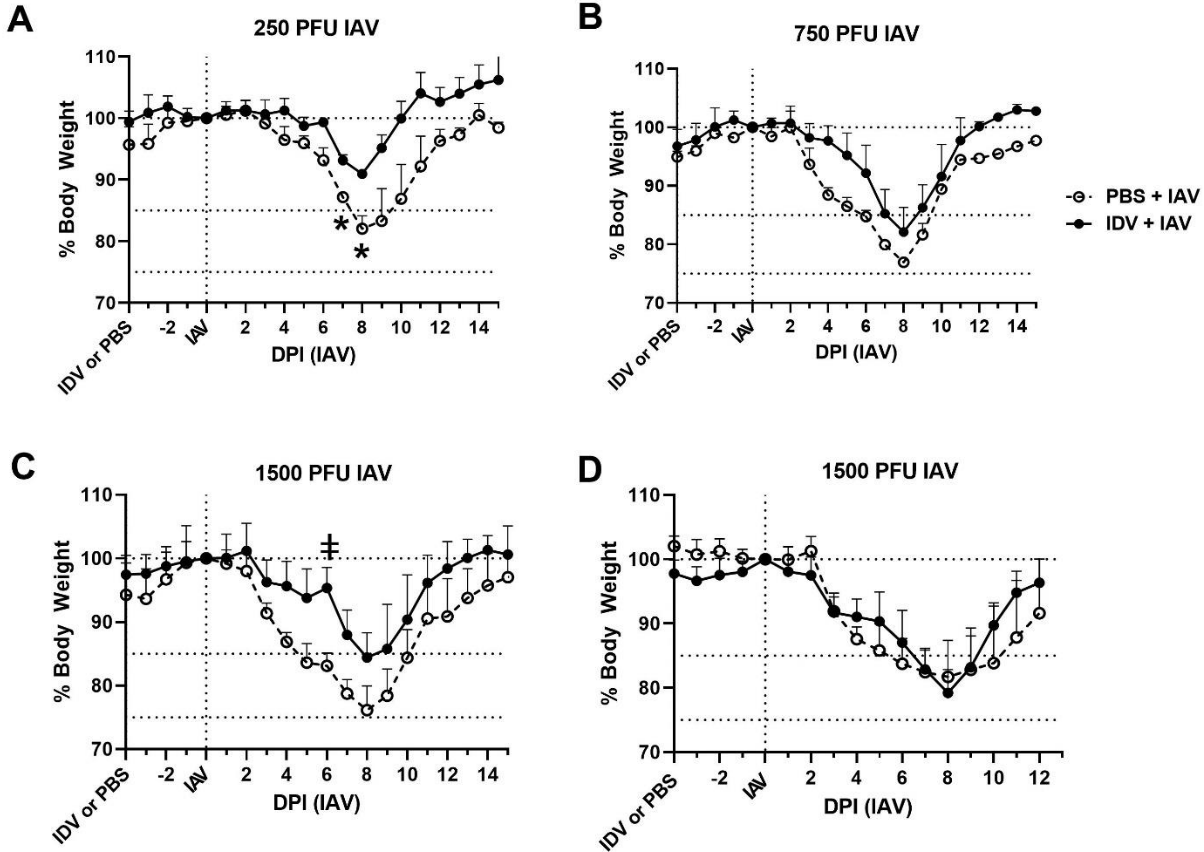

Figure 5.3. **Inconsistency in IDV-mediated IAV protection persists at lower IAV titers.** Body weights of wild-type mice (% of day 0) inoculated with PBS (white circles) or IDV (black circles) on day -4 and challenged with IAV on day 0 (vertical dotted line) using (A) 250 PFU, (B) 750 PFU, or (C-D) 1500 PFU IAV. * Bonferroni-Dunn-adjusted $P < 0.05$; ‡ $P = 0.055$; groups compared using Student's t-test. Experiments for (A) and (B) performed only once, $n \geq 3$ mice for all groups.

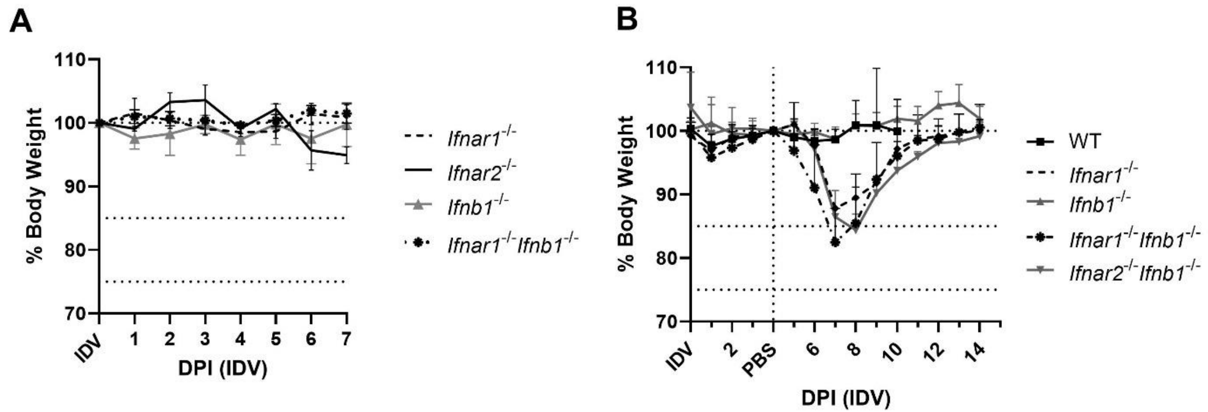

Figure 5.4. **Type I interferon signaling deficiency impacts host response to IDV.** Body weights of (A) *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$, *Ifnb1*$^{-/-}$, and *Ifnar1*$^{-/-}$*Ifnb1*$^{-/-}$ mice (% of day 0) inoculated with IDV at day 0 or (B) Wild-type (WT), *Ifnar1*$^{-/-}$, *Ifnb1*$^{-/-}$, *Ifnar1*$^{-/-}$*Ifnb1*$^{-/-}$, and *Ifnar2*$^{-/-}$*Ifnb1*$^{-/-}$ mice with IDV at day 0 followed by PBS at day 4. * Bonferroni-Dunn-adjusted $P < 0.05$ comparing groups using Student's t-test. $N \geq 3$ mice/group, experiments performed 1-2 times with comparable results.

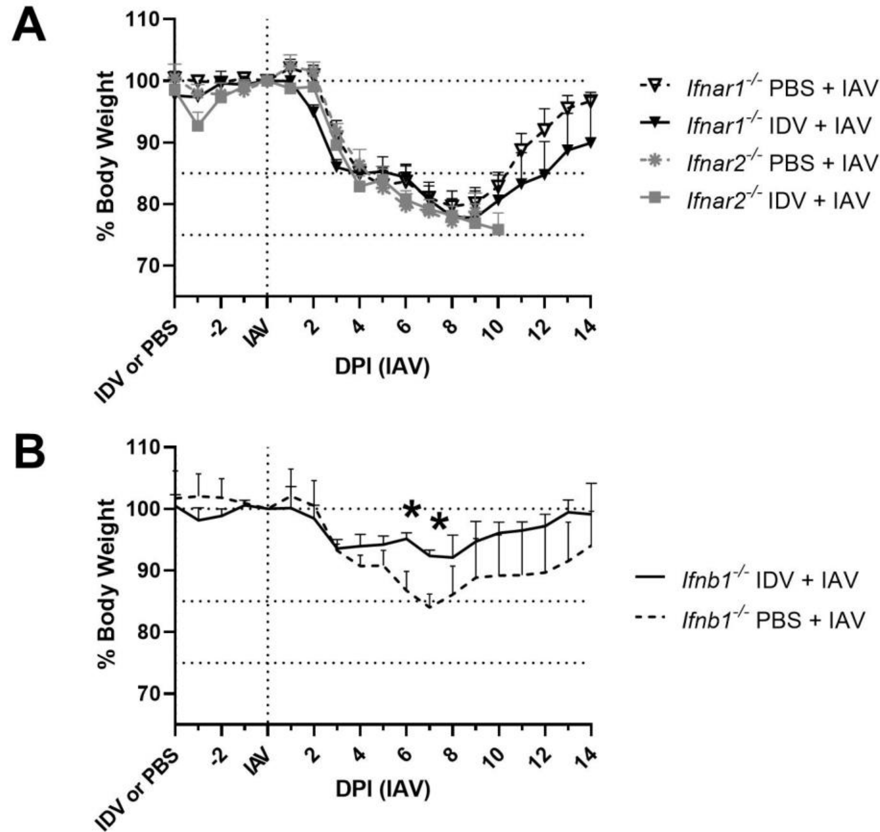

Figure 5.5. **IFN-β signaling is dispensable for IDV protection against IAV weight loss.** Body weights of (A) *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$, and (B) *Ifnb1*$^{-/-}$ mice (% of day 0) inoculated with IDV at day -4 followed by IAV at day 0. * Bonferroni-Dunn-adjusted $P < 0.05$ comparing groups using Student's t-test. $N \geq 3$ mice/group, experiments repeated 1 (*Ifnar2*$^{-/-}$) or 2 (*Ifnar1*$^{-/-}$ and *Ifnb1*$^{-/-}$) times with comparable results.

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APPENDICES

APPENDIX A

P113 FRAGMENT SEQUENCE

M. ovipneumoniae p113 Fragment Sequence (5’->3’):

TAGCTACCAAGTCGACATTCTAGAAATTCTCCCAGATGATGCTAACCAAAATTTTAA

AGTCAAATTTCAAGCTAGCCAAAAATTAGCAAACGGTGACATCGCCAAATCTGACA

TTTATGAACAAGTTGTTTCTTTTGTCAAAGAATCAACTATTTTAATTGCCGAATTTAA

TTTTTCCTTACAAAAAATTACAAGCAGACTTAATCAACAAGTCCAAAATTTAATTTC

TGCTCGAACCGCCAATTTTGCTGATCAAAATTCAGCTACTTCAAATCCAACAGATCC

TAGCACAATTAGACCAGTTGATTTTCAACATGACTTAAGAATTCATAAAGCAAA

APPENDIX B

SUPPLEMENTARY FIGURE

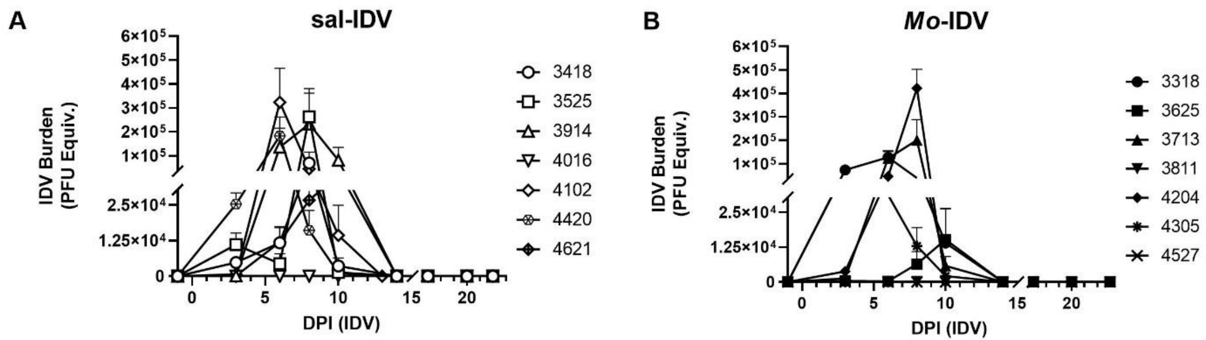

Figure A1. IDV nasal shedding patterns in lambs varies within both groups. IDV burden per nasal swab for individual lambs in (A) the sal-IDV group, or (B) the *Mo*-IDV group. Data presented as mean of technical replicates ± SD. Enumerated by qRT-PCR using the standard curve method.