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Strain-dependent Effects of PB1-F2 of Triple Reassortant H3N2 Influenza Viruses in Swine.

Running title: Effects of influenza PB1-F2 in pigs.

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Abstract

The PB1-F2 protein of the influenza A viruses (IAV) can act as a virulence factor in mice. Its contribution to the virulence of IAV in swine, however, remains largely unexplored. In this study, we chose two genetically related H3N2 triple reassortant (TR) IAVs to assess the impact of PB1-F2 in viral replication and virulence in pigs. Using reverse genetics, we disrupted the PB1-F2 open reading frame (ORF) of A/swine/WI/14094/1999 (H3N2) [Sw/99] and A/turkey/OH/313053/2004 (H3N2) [Ty/04]. Removing the PB1-F2 ORF led to increase expression of PB1-N40 in a strain-dependent manner. Ablation of the PB1-F2 ORF (or incorporation of the N66S mutation in the PB1-F2 ORF, Sw/99 N66S) affected the replication in porcine alveolar macrophages of only the Sw/99 KO and Sw/99 N66S variants. The Ty/04 KO strain showed decreased virus replication in swine respiratory explants, whereas no such effect was observed in the Sw/99 KO, compared to the WT counterparts. In pigs, PB1-F2 did not affect viral shedding or viral load in the lungs for any of these strains. Upon necropsy, PB1-F2 had no effect on the lung pathology caused by Sw/99 variants. Interestingly, the Ty/04 KO-infected pigs showed significantly increased lung pathology at 3 days post-infection (dpi) compared to pigs infected with the Ty/04 WT strain. In addition, the pulmonary levels of IL-6, IL-8, and IFN-γ were differentially regulated by the expression of PB1-F2. Taken together, these results indicate that PB1-F2 modulates viral replication, virulence, and innate immune responses in pigs in a strain-dependent fashion.

Keywords: PB1-F2, triple reassortant H3N2 influenza viruses, respiratory explants, alveolar macrophages, swine.

Word count: abstract, 244.

Introduction

In pigs, IAV infections bear remarkable resemblance to influenza infections in humans with respect to the clinical presentations and pathological features of the disease. The A/swine/IA/15/30 (H1N1) virus, also known as classical swine H1N1 virus (cH1N1), was the first virus strain isolated and associated with the etiology of swine influenza in this species (Shope, 1931). The cH1N1 viruses were the predominant IAV subtype circulating in North American swine populations for nearly 70 years (Vincent *et al.*, 2008). Since the late 1990's, however, the epidemiology of swine influenza in North American pigs has changed significantly with the introduction of triple reassortant (TR) influenza viruses. Initially introduced as H3N2 TR IAVs, these viruses contain gene segments from human- (HA, NA, and PB1), avian- (PB2 and PA), and classical swine-origin (NS, NP, and M) influenza viruses. Some of these viruses have expanded their host range and caused outbreaks in domestic turkeys in North Carolina, Minnesota, and Ohio in 2003-2004 (Choi *et al.*, 2004). A unique feature shared by TR reassortants is the maintenance of the triple reassortant internal gene (TRIG) cassette, which consists of the human-origin PB1, the avian-origin PA and PB2, and the swine-origin M, NP, and NS gene segments. Currently, TR strains of the H3N2, H1N2, and H1N1 are enzootic in the US swine population (Vincent *et al.*, 2008). There have been at least three independent introductions of human H3N2 subtype viruses (as reassortants with the TRIG cassette) into the swine population, leading to phylogenetic clusters I, II, III and IV (the latter derived from cluster III) (Gramer *et al.*, 2007; Kumar *et al.*, 2011; Nfon *et al.*, 2011; Richt *et al.*, 2003; Webby *et al.*, 2004).

The virulence of IAVs is considered a polygenic trait (Fukuyama & Kawaoka, 2011). One such virulence factor is the PB1-F2, which is an 87-90 amino-acid long protein encoded by an alternate (+1) open reading frame (ORF) within the PB1 gene segment 2 (Chen *et al.*, 2001). Full-length PB1-F2 is expressed by most avian IAVs, but it is truncated in cH1N1s and in human H1N1s isolated after 1950. Truncated versions of PB1-F2 have been also reported in some human H3N2 isolates and in TR swine viruses (Zell *et al.*, 2007). Overall, the effects of PB1-F2 in relation to influenza-host interaction are not completely understood and appear to be cell-type, virus strain, and even host-specific. We have previously shown that restoring the PB1-F2 open reading frame in the context of a 2009 pandemic H1N1 strain has minimal effects in swine; however, the contribution of PB1-F2 to the virulence of IAVs in natural hosts (like birds and swine) has been limited (Marjuki *et al.*, 2010; Pena *et al.*, 2012; Schmolke *et al.*, 2011).

In this study, we compared the effects of PB1-F2 expression, in the background of two TR H3N2 IAVs, with respect to viral replication and cell death using *ex vivo* infected porcine tissues and changes in pathogenicity and host responses in pigs.

Results

Generation of PB1-F2 recombinant influenza viruses. The sequences of the Sw/99 and Ty/04 PB1-F2 proteins are 90 amino acid (aa) long and differ only at 4 aa positions from each other. The Sw/99 differs from Ty/04 at positions S23N, R44K, F57S, and F83S (Fig. S1). The functional roles of these polymorphisms have not been established. The Ty/04 PB1-F2 is 54.4% divergent from the 1918 H1N1 PB1-F2 and shares 74.4% aa sequence identity with the 1968 A/Hong Kong/1/68 (H3N2) pandemic strain and 86.6% with the A/Wuhan/359/95 (H3N2) seasonal strain (Fig. S1).

The Sw/99 or Ty/04 strains were cloned by RG and then the PB1-F2 ORF in each strain was abolished by mutating the ATG start codon to ACG and introducing 2 stop codons downstream of the gene. The strategy maintains the PB1 ORF wild type sequence. Since the Sw/99 virus is of low virulence to pigs, we introduce the N66S mutation in the PB1-F2 as described (Conenello *et al.*, 2007). The viruses used herein are summarized in Table 1. Beyond PB1-F2, there are significant nucleotide and amino acid differences between the Ty/04 and Sw/99 viruses (Table S1); however, in this study we concentrated our efforts on determining the contribution of PB1-F2 for both of these strains and compared the effects on isogenic backgrounds.

The effect of PB1-F2 ORF for polymerase activity in the background of the Sw/99 and Ty/04 polymerase complexes was assessed *in vitro* using a minireplicon assay as previously described (Pena *et al.*, 2011). We found no differences in polymerase activity in the presence or absence of the PB1-F2 ORF in segment 2, suggesting that PB1-F2 does not affect polymerase activity of any of these H3N2 TR strains (not shown).

More recently, the expression of a 12th gene product from influenza viruses has been described which, like the PB1-F2, is expressed from an alternative AUG start codon in segment 2 (Wise *et al.*, 2009). The resulting product is produced from the same PB1 ORF, but lacks the first 39 aa of PB1 and it is termed N40. Removal of the PB1-F2 AUG increases expression of N40 in the context of the strain A/WSN/33 (H1N1) (Tauber *et al.*, 2012). The Sw/99 and Ty/04

97 strains used in this study encode for a potential N40 product and therefore we were interested in
98 determining if ablation of the PB1-F2 ORF would have any effects on its expression. Expression
99 of the influenza virus polymerase complex was detected in cells infected with the isogenic Ty/04
100 and Sw/99 variants (Fig S2). Additional analysis included isogenic viruses from the pandemic
101 strain A/California/04/2009 (H1N1), which encode for either a short 11 aa peptide (WT) or a full-
102 length PB1-F2 ORF (90 aa, knock in or KI) (Pena *et al*., 2012). Our results indicate that the
103 Ty/04 KO and Sw/99 KO strains produce detectable levels of a protein product consistent with
104 N40 (Fig S2, upper panel, lanes 4 and 6, respectively). Densitometry analysis (not shown);
105 however, indicated that the proportion of N40 produced was higher in the Sw/99 KO than in the
106 Ty/04 KO, although the biological significance of such observation remains to be determined. In
107 other recombinants (Sw/99 N66S, Ca/04 WT, and Ca/04 KI), the presence or absence of PB1-
108 F2 had negligible effects on N40 expression. It is interesting to note that the Ty/04 KO
109 consistently produced lesser quantities of the PB1, PB2, and PA subunits despite producing
110 similar or higher levels of NP compared to other variants. Taken together, these results suggest
111 that ablation of PB1-F2 affects N40 expression in a strain-dependent manner.

112 **Strain-dependent effects of PB1-F2 with respect to infectivity in and viability of porcine**
113 **alveolar macrophages (PAMs).** Since PB1-F2 has been shown to induce cell death in immune
114 cells (Chen *et al*., 2001; McAuley *et al*., 2010a) we investigated whether the presence of PB1-
115 F2 affects the infectivity in and the viability of freshly isolated PAMs. The purity of PAM
116 preparations was over 98%, as determined by flow cytometry using anti-porcine CD163
117 antibody (data not shown). Disruption of PB1-F2 expression in Sw/99 (Sw/99 KO) significantly
118 reduced viral yields in PAMs from 6-48 hpi compared to the Sw/99 WT (Fig. 1(a)). Surprisingly,
119 the presence of the N66S mutation (Sw/99 N66S) had detrimental effects on viral replication.
120 Ty/04 WT replicated ~10 fold less in PAMs compared to Sw/99 WT and knocking out PB1-F2
121 (Ty/04 KO) had no effect on virus growth (Fig. 1(b)). The impact of Sw/99 PB1-F2 in PAMs
122 viability, assessed by the XTT method, correlated with the replication of these viruses. Sw/99
123 WT caused more cytotoxicity to PAMs relative to Sw/99 KO and Sw/99 N66S viruses (Fig.
124 2(a,c)). PAMs viability was also reduced upon infection with the Ty/04 isogenic viruses, but
125 disruption of PB1-F2 ORF only impacted apoptotic cell death and not the overall viability as
126 determined by the XTT method (Fig. 2(b,c)). It should be noted that cytotoxicity measured by
127 the XTT assay was only detected when PAMs were infected with an MOI of 10. Although the
128 role of N40 overexpression by the Sw/99 KO strain for virus replication needs further

investigation, the data suggest that PB1-F2 affects the replication and viability of PAMs in a strain-dependent manner.

PB1-F2 enhances replication of TR H3N2 IAV in porcine respiratory explants. An *ex vivo* organ culture model of the pig respiratory tract maintained in an air–liquid interface was used to study the replication of PB1-F2 recombinant viruses in a biologically relevant *in vitro* system. Tissue explants were prepared from nasal turbinates (NTs), trachea, proximal lung (close to the bronchi), and distal lung (close to the alveoli) from a healthy pig. In this system, PB1-F2 (absence or presence or the N66S mutation) had negligible effects on the replication of Sw/99 recombinants (Fig. 3(a-d)). However, the Ty/04 KO had significantly decreased replication in respiratory explants compared to the Ty/04 WT (Fig. 3(e-h)). Collectively, these data indicate strain-dependent effects of PB1-F2 in modulating viral production in swine respiratory tissues.

Deletion of PB1-F2 increases the virulence of Ty/04. We next evaluated the effects of PB1-F2 in the virulence of TR H3N2 IAVs *in vivo*. Three-week old cross-bred pigs were separated in 5 groups (10 pigs/group) and a control group (n=5) and inoculated intratracheally with 10^5 $TCID_{50}$/animal (control animals received sterile media). Five pigs from each group were euthanized at either 1 or 3 dpi (control pigs were sacrificed at 3 dpi). Sw/99 and Ty/04 recombinant viruses showed similar levels of nasal shedding and lung virus load irrespective of the presence of PB1-F2 ORF (or the N66S mutation) (Fig. 4(a-d), and data not shown). At necropsy, the absence of the PB1-F2 ORF or the N66S substitution had no effect in either the macroscopic or microscopic lung pathology (Fig. 4(e),(f), and data not shown) caused by the Sw/99 recombinants. Surprisingly, Ty/04 KO strain showed significantly increased macroscopic and microscopic pneumonia compared to the Ty/04 WT (3dpi) (Fig. 4 (e) and (f)). Contrary to what was expected, the presence of PB1-F2 may decrease the virulence of certain IAVs in pigs. By IHC, we observed that the levels of viral NP antigen correlated with the levels of virus replication observed for each virus (Fig. 5).

PB1-F2 modulates the innate immune response in swine lungs. PB1-F2 strongly influences the early host responses during IAV infection (Le Goffic *et al.*, 2011; McAuley *et al.*, 2007) and therefore we measured the protein levels of ten porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-α, and IFN-γ) in BALF collected from pigs sacrificed at 1 and 3 dpi (Fig 6). Pigs infected with Ty/04 WT had higher levels of IFN-α and IL-1β at 1 dpi than those infected with Sw/99 WT. The profile of other cytokines/chemokines was not altered by the presence or absence of PB1-F2 ORF in these strains (Fig 6(a) and (b) and data not

shown). Increased pulmonary levels of IL-6 were seen at 1 dpi during infection with Sw/99 WT compared to Sw/99 KO, suggesting differential regulation of this cytokine in the presence of PB1-F2 in pigs infected with this virus background (Fig. 6(c)). The N66S mutation resulted in no stimulation of IL-6 and higher stimulation of IL-8 response compared to the Sw/99 WT (Fig 6(c) and (d)). Ty/04 WT and Ty/04 KO strains stimulated IL-8 production at 1 dpi, but only pigs infected with Ty/04 KO showed sustained responses of this cytokine by 3 dpi (Fig 6(d)). Likewise, Ty/04 KO-infected pigs showed higher levels of IFN-γ at 3 dpi compared to pigs from other virus groups, which is consistent with the development of enhanced pneumonia in the former (Fig 6(e)). Collectively, these results indicate that PB1-F2 differentially modulates the local levels of cytokines and chemokines during infection with TR H3N2 in pigs and that the heightened production of IL-8 and IFN-γ pro-inflammatory mediators correlated with the lung pathology developed in Ty/04 KO-infected animals.

Discussion

The phenotypes associated with the presence of PB1-F2 derived from TR H3N2 IAVs have not yet been elucidated. We created PB1-F2 null viruses in the background of two representative lineages of swine-origin North American TR H3N2 viruses: Sw/99 (cluster III) and Ty/04 (cluster IV). These viruses were chosen because they are genetically related and they both circulate in the swine population. Since Sw/99 is of low virulence for pigs (Pena *et al*, unpublished), we studied the effects of not only removing PB1-F2 but also in the context of the virulence marker N66S. Previous studies have shown that removal of the PB1-F2 AUG increases expression of N40 (Tauber *et al.*, 2012). A band consistent with the expression of N40 has been observed in influenza strains of human, avian, and equine origin (Wise *et al.*, 2009). A protein band consistent with N40 is readily detected when PB1-F2 is removed from the Sw/99 background, but only slightly when PB1-F2 is removed from the Ty/04 background. The pandemic Ca/04 strain, which naturally encodes only the first 11 aa of PB1-F2, does not show expression of N40 despite encoding such polypeptide. In our previous report, we showed that restoring PB1-F2 in the Ca/04 background had minimal, although discernible, effects in pigs with respect to virulence (Pena *et al.*, 2012). Our current western blot assay indicates that these differences cannot be attributed to N40 expression because neither the Ca/04 WT, nor the Ca/04 KI strains produce detectable N40. Whether the difference in N40 expression in the Sw/99 and Ty/04 strains could account for some of the differences observed *in vitro* and *in vivo* deserves further investigation beyond the scope of the current report.

IAVs infect alveolar macrophages in a variety of species, including mice, swine, and humans (Reading *et al.*, 2000; Rodgers & Mims, 1982; Seo *et al.*, 2004; Upham *et al.*, 2010; van Riel *et al.*, 2011). In pigs, *in vivo* depletion of alveolar macrophages by chemical treatment unveiled that these cells are indispensable for controlling IAV infection (Kim *et al.*, 2008). PB1-F2 has been suggested to influence virulence by destroying host immune cells such as macrophages (Chen *et al.*, 2001; Lamb & Takeda, 2001; Zamarin *et al.*, 2005). By infecting PAMs *ex vivo* with PB1-F2 recombinant viruses, we found that the presence of PB1-F2 was required for optimal replication of Sw/99, but not Ty/04 (Fig. 1). Likewise, PAMs' viability was affected by the presence of PB1-F2 in the Sw/99 strain (Fig. 2). Differences in apoptotic cell death were detected by TUNEL when PAMs were infected at MOI 2, but this effect was only quantifiable by XTT when PAMs were infected at MOI 10. It remains to be determined whether differences seen with the Sw/99 KO strain are due to lack of PB1-F2 expression or overexpression of N40 or a combination of both. Nevertheless, there was no correlation between the ability of PB1-F2 recombinant viruses to replicate in PAMs and the virulence to pigs. Disruption of PB1-F2 ORF had no effect in Sw/99 virulence as measured by viral shedding, viral lung load, and lung pathology. These results are in agreement with those that showed no correlation between macrophage infectivity and/or cell death *in vitro* and the course of IAV disease in mice (McAuley *et al.*, 2010a; Tate *et al.*, 2010). In the background of Ty/04, PB1-F2 had no effect on viral replication, apoptosis or viability of PAMs, which would also suggest a limited, if any, role of N40 under these conditions.

Expression of full-length PB1-F2 as well as the N66S substitution has been shown to increase replication of certain IAV isolates in tissue culture (McAuley *et al.*, 2010b; Schmolke *et al.*, 2011; Smith *et al.*, 2011). Our results support the hypothesis that PB1-F2 modulates viral replication in a strain-dependent manner. Although Ty/04 PB1-F2 did not affect polymerase activity, higher viral yields were observed in respiratory explants infected with Ty/04 WT relative to Ty/04 KO. This is consistent with expression levels of the polymerase subunits as revealed by western blot. In the backbone of Sw/99 viruses, neither PB1-F2 deletion nor N66S mutation affected viral growth kinetics in this system. These observations also emphasize a minor role of N40 in this system (Fig. 3). The replication of Sw/99 PB1-F2 recombinant viruses *ex vivo* paralleled with the levels of viral shedding in nasal secretions and lung viral load *in vivo*. However, we found no differences between Ty/04 WT and Ty/04 KO regarding viral shedding in the upper respiratory tract and BALF titers, suggesting that results obtained from *ex vivo* and *in vivo* results not always correlate with each other.

PB1-F2 proteins from human H3N2 IAV differ profoundly in their ability to induce inflammatory responses and pathology in lungs (McAuley *et al.*, 2010a) with pro-inflammatory mutations in PB1-F2 mapped to P62L, H75R, Q79R, and S82L (Alymova *et al.*, 2011). The PB1-F2 sequences of both Sw/99 and Ty/04 present conserved residues in these positions, which include a mix of the pro-inflammatory motifs 62(L) and 82(L) as well as non-inflammatory mutations 75(H), 79(Q) (Fig. S1). The PB1-F2 deletion or N66S substitution in the background of Sw/99 did not alter viral-induced pneumonia in pigs. Unexpectedly, disruption of PB1-F2 ORF from the Ty/04 strain significantly aggravated both the microscopic and macroscopic pneumonia caused by this virus at 3 dpi. The degree of pneumonia in Ty/04 KO cannot be explained by improved replication since it replicated to similar levels than Ty/04 WT throughout the respiratory tract. Whether under these conditions N40 plays a role it is not known, however, we would have also expected phenotypic differences using the Sw/99 KO virus, which expresses higher levels of N40 in vitro.

The pro-inflammatory cytokine response is critical for recruiting effector cells to the site of infection. Concomitantly with the development of severe pneumonia in Ty/04 KO-infected pigs, there was a significant increase in the pulmonary levels of IL-8 and IFN-γ at 3 dpi compared to the group Ty/04 WT group. IL-8 is a major chemokine involved in neutrophil recruitment to infection sites. Elevated levels of IL-8 have been implicated in the pathogenesis of viral-induced bronchiolitis in humans (Koh *et al.*, 2007).

IFN-γ is an important immunoregulatory cytokine, which is produced by T cells, NK cells, and macrophages. IFN-γ has pleiotropic effects on various cells of the immune systems and plays an important role in T-cell–mediated lung injury (Bruder *et al.*, 2006). Influenza virus infection induces the local production of IFN-γ in the respiratory tract. Experiments in gene-targeted mice showed that IFN-γ plays no significant role in protection from lethal infection (Graham *et al.*, 1993) and IAV has developed mechanisms to evade its antiviral activity by disrupting intracellular signaling pathways (Uetani *et al.*, 2008). Recently, Le Goffic *et al.* reported that infection with the laboratory-adapted strain A/WSN/1933 (H1N1) in mice resulted in higher levels of IFN-γ relative to infection with an isogenic virus lacking PB1-F2 (Le Goffic *et al.*, 2011). These results are in disagreement with the findings in pigs using a TR H3N2 IAV, in which deletion of PB1-F2 led to enhance IFN-γ concentrations in BALF. The reason for this discrepancy is unclear, but it might be due to differences in viral strains and host systems used. There have been conflicting reports of the role of PB1-F2 in modulating type I interferons, and thus the exact role of PB1-F2 in influencing the host innate immune response remains

unresolved (Dudek *et al.*, 2011; Le Goffic *et al.*, 2010; Varga *et al.*, 2011). It is possible that deregulation of the innate host defense through IL-8 and IFN-γ pathways might have played a role in the exacerbated immunopathology induced by Ty/04 KO virus. Should Ty/04 PB1-F2 have a direct effect in blocking or down-regulating IL-8 and IFN-γ expression, this effect would explain the lessened pulmonary lesions developed in the Ty/04 WT group. These studies are beyond the scope of this work and warrant further investigation. Finally, it must be noted the potential role of PB1-F2 as a contributor of secondary bacterial infections. Studies in mice have shown that a virus encoding the 1918 PB1-F2 ORF led to increased virulence and more severe bacterial pneumonia (McAuley *et al.*, 2007). In our studies, pigs are treated with antibiotic once prior to inoculation with influenza, in order to reduce bacterial contaminants that could obscure the specific effects provided by the virus. Thus, our studies were not designed to establish the role of PB1-F2 in secondary infections. Nevertheless, aerobic bacterial screening with BALF and colony counts were performed to rule out bacterial contribution to pneumonia lesions. We found no evidence of bacterial involvement in disease or pathology. Further research efforts focusing on the modulation of the porcine immune system by PB1-F2, its interaction with host factors, and modulation of secondary bacterial pneumonia should shed light into the molecular mechanisms of PB1-F2 in strain and host-dependent manner.

Material and Methods

Cell lines and virus strains. Human embryonic kidney cells (293-T) were cultured in OptiMEM I (GIBCO, Grand Island, NY) containing 10% FBS and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), L-glutamine and antibiotics. A/swine/Wisconsin/14094/99 (H3N2) (Genbank taxonomy 136472) was a kind gift from Dr. Sagar Goyal, University of Minnesota, Minneapolis, MN. A/turkey/Ohio/313053/04 (Genbank taxonomy ID 533026) was kindly provided by Dr. Yehia Saif, Ohio State University, Wooster, OH. Both viruses were successfully rescued from cloned cDNAs as described below and amplified in either MDCK cells (Sw/99) or embryonated eggs (Ty/04). Sw/99 and Ty/04 bearing mutations in PB1-F2 were generated by RG and are described below. The Sw/99 and Ty/04 WT PB1-F2 gene products were aligned with the 1918 H1N1, 1957 H2N2, and 1968 H3N2 and previously characterized PB1-F2 proteins. Alignment was carried out using ClustalW in MegAlign (Lasergene v.8.1.5., DNAStar, Madison, WI). The A/California/04/2009 (H1N1) WT and knock-in variants (Ca/04 WT and Ca/04 KI, respectively) have been previously described (Pena *et al.*, 2012).

Reverse genetics and mutagenesis. The 8-plasmid-based RG system was used to rescue WT A/swine/Wisconsin/14094/99 (H3N2) (Sw/99). The RG A/turkey/Ohio/313053/04 (H3N2) (Ty/04) has been previously described (Pena *et al.*, 2011). The QuickChange II site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) was used according to manufacturer's protocols to disrupt the PB1-F2 ORF of both Sw/99 and Ty/04, which display a low and high virulent phenotype for pigs, respectively (unpublished). To ensure complete abolition of PB1-F2 translation, we mutated the ATG start codon to ACG and introduced 2 stop codons at amino acid positions 12 (C153G) and 58 (G291A) as previously described by Zamarin *et al.* (Zamarin *et al.*, 2006). The virulence marker N66S was introduced into the Sw/99 virus as described previously (Conenello *et al.*, 2007). RG plasmids and recovered recombinant viruses were prepared as described (Hoffmann *et al.*, 2000) and fully sequenced to confirm their identity.

Western blotting. MDCK cells growing in 12-well plates were infected by the indicated virus strains with MOI of 1. After 1 h incubation at 35°C, cells were washed two times with PBS to remove unattached viruses. After 23 h infection at 35°C, cells were lysed with 2 x Laemmli sample buffer and incubated at 100°C for 10 min. Western blots were performed standard techniques and incubating with different primary antibodies: anti-PB1 (goat) antibody targeting residues 50-100 of PB1 at 1:100 dilution (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PB2 (goat) antibody targeting N-terminus of PB2 at 1:200 dilution (Santa Cruz Biotechnology), anti-PA (rabbit) antibody targeting residues 250-300 of PA at 1:150 dilution (GenScript, Piscataway, NJ), anti-NP (mouse monoclonal) antibody at a dilution 1:1000 (2F4, made in house) and anti-GAPDH (mouse) antibody at a dilution 1:3000 (Santa Cruz Biotechnology). A corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at 1:3000 dilution (Donkey anti-goat IgG-HRP, Donkey anti-rabbit IgG-HRP, Donkey anti-mouse IgG-HRP (Santa Cruz Biotechnology)) was used. Immunoreactive proteins were visualized using West Pico (Pierce, Rockford, IL) enhanced chemiluminescence substrate and autoradiography.

Isolation, infection, and viability of porcine alveolar macrophages (PAMs). PAMs were isolated from 5 healthy pigs as described (Loving *et al.*, 2006). PAMs were seeded at a density of $1x10^5$ cells per well in a 96-well flat-bottom plate with a final volume of 100 μl of PAMs complete media. At 24 h after plating, the media was removed and PAMs were infected with 100 μl of the recombinant viruses diluted in PAMs media at a multiplicity of infection (MOI) of 2 or 10. PAMs viability was measured by the XTT colorimetric method using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. At the indicated time points, 20 μl of XTT working solution (1 mg/ml), prepared immediately

before use, were added to each well and the microplate was incubated for an additional 2 h at 37°C and read using an ELISA plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) with a 450 nm optical filter. PAMs viability was determined as a relative percentage of the mock-treated non-infected control. The infectivity of PB1-F2 recombinant viruses was tested in freshly isolated PAMs seeded on 6-well plates in 3 ml of MEMα supplemented with 10% fetal bovine serum and antibiotics. PAMs were incubated overnight and infected at MOI 2 with MEM without trypsin. Following 1 h adsorption at 37°C, PAMs were washed three times with PBS and the culture was replenished with 3 ml MEMα supplemented with 10% fetal bovine serum and antibiotics. At the indicated time points, 300 μl of supernatant was collected to assess virus replication. Virus yield in PAMs was determined by standard $TCID_{50}$ in MDCK cells.

TUNEL assay in *ex vivo* infected porcine alveolar macrophages. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used to quantify the levels of apoptotic-cell death induced by recombinant viruses in *ex vivo* infected PAMs. Apoptotic cells were detected using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. Briefly, PAMs were infected at MOI of 2 and at 72 hpi cells were fixed with 4% paraformaldehyde and permeabilized in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate. Cells were then incubated in the TUNEL reaction mixture in the dark at 37°C for 60 min followed by detection of DNA strand breaks in apoptotic cells by fluorescence microscopy. A minimum of 600 cells was counted in a blinded-fashion from more than 10 random microscopic fields and the percentage of cell apoptosis was determined.

Virus titration. Viral stocks and virus present in biological samples were titrated on MDCK cells and the $TCID_{50}$/ml was determined by the Reed and Muench method (Reed & Muench, 1938). The endpoint viral titer was determined by an HA assay using 0.5% turkey red blood cells.

Animal Studies. Swine pathogenicity studies were conducted at the National Animal Disease Center in Ames, IA under protocols approved by the USDA- ARS Animal Care and Use Committee. Porcine explants were prepared under protocols approved by the Institutional Animal Care and Use Committee, University of Maryland, College Park. Animal studies adhered strictly to the US Animal Welfare Act (AWA) laws and regulations. Where indicated, animals were humanely euthanized with Beuthanasia®-D (Intervet/Schering-Plough, Summit, NJ) at a dosage of 1 ml/4.5 Kg of body weight.

Three-week-old cross-bred pigs were obtained from a high-health herd free of SIV and porcine reproductive and respiratory syndrome virus. All pigs were treated once, at 11 days prior to inoculation with influenza virus, with Ceftiofur crystalline free acid (5 mg/kg) (Pfizer Animal Health, New York, NY) to reduce bacterial contaminants prior to the start of the experiment. Fifty pigs were randomly divided in five groups (n=10) and housed in separate isolation rooms. Pigs were infected intratracheally with 1×10^5 $TCID_{50}$ of each of the recombinant viruses diluted in 2 ml of MEM using previously described protocols and monitored for signs of disease and virus shedding as described (Pena *et al.*, 2012; Vincent *et al.*, 2010).

Pathologic examination of swine lungs. At necropsy, lungs were removed *in toto* and evaluated to determine the percentage of the lung affected by purple-red, consolidated lesions that are typical of influenza virus infection in pigs. The percentage of the surface affected with pneumonia was calculated based on weighted proportions of each lobe to the total lung volume as previously described (Halbur *et al.*, 1995). Each lung was then lavaged with 50 ml MEM to obtain bronchoalveolar lavage fluid (BALF). A single veterinary pathologist scored all lungs and was blinded to the treatment groups.

Isolation, culture, and infection of porcine respiratory explants. Porcine nasal turbinates (NT), tracheal, and lung explants were prepared as described in detail previously (Pena *et al.*, 2012; Van Poucke *et al.*, 2010). At 24 h of culture, explants were washed with PBS and 10^6 $TCID_{50}$ of recombinant viruses diluted in 500 μl of explants media were deposited in the upper compartment for 1 h at 37°C. Subsequently, explants were washed 3 times with PBS and the culture replenished with 500 μl of explants media. At 24, 48, and 72 hpi, 100 μl of supernatant was removed to assess virus yields.

Histopathology and immunohistochemistry. Tissue samples were collected, fixed and stained as described (Pena *et al.*, 2012) Microscopic lesions were evaluated by a board-certified veterinary pathologist blinded to treatment groups. Scoring of lesions was based on scales adapted from Gauger et al (Gauger *et al.*, 2011). Influenza virus Type A-specific antigen was detected in lung tissues using a previously described immunohistochemical (IHC) method with minor modifications (Pena *et al.*, 2012; Vincent *et al.*, 1997).

Quantification of cytokine/chemokine protein levels in bronchoalveolar lavage fluid. Levels of nine porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, and IFN-γ) in BALF were determined by multiplex ELISA following the manufacturer's

recommendations (SearchLight, Aushon Biosystems). Levels of IFN-α protein were measured by ELISA using F17 monoclonal antibody, K9 MAb and recombinant porcine IFN-α (R&D Systems Inc., Minneapolis, MN) as previously described (Brockmeier *et al.*, 2009).

Statistical analysis. All statistical analyses were performed using GraphPad Prism Software Version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student t-test, whereas multiple comparisons were carried out by analysis of variance (ANOVA). The differences were considered statistically significant at $p < 0.05$.

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561 **Figure Legends.**

562 **Figure 1. Infectivity of PAMs with PB1-F2 viruses.** Virus titers at different hpi from PAMs
563 previously infected with MOI of 2 virus. (A) Sw/99 WT (black squares), Sw/99 KO (open
564 squares), or Sw/99 N66S (black diamonds). (B) Ty/04 WT (black circles), Ty/04KO (open
565 circles). One-way ANOVA followed by Dunnett's post hoc test was used to determine significant
566 differences between the WT and PB1-F2 modified viruses (dashed brackets, $p < 0.05$). Mean ±
567 SD is shown.

568 **Figure 2. Viability of PAMs after infection with influenza viruses.** PAMs were mock-infected
569 or infected with PB1-F2 recombinant viruses at an MOI of 10 and cell viability was measured by
570 the XTT assay at 24, 48, and 72 hpi. Each data point represents the mean ± SD. A) Sw/99
571 viruses, B) Ty/04 viruses. C) PAMs infected at MOI of 2 with the indicated viruses and assayed
572 for apoptotic-cell death using TUNEL assay at 72 hpi. Data is represented as mean ± SD
573 (panels A and B) or each replicate with the mean ± SD (panel C). Letters above data points
574 (panels A and B) indicate statistical relationships. Data with the same letters indicate no
575 statistical difference, whereas data with different letters indicate a statistical difference ($p <$
576 0.05). Dashed brackets in panel C indicate a statistically significant difference ($p < 0.05$).

577 **Figure 3. Replication of PB1-F2 viruses in porcine respiratory explants.** Nasal turbinates (A
578 and E), tracheal (B and F), proximal (C and G), and distal lung (D and H) explants were infected
579 with 10^6 $TCID_{50}$ of each virus and titrated by $TCID_{50}$. Values shown correspond to virus titer
580 (log_{10} $TCID_{50}$/ml) from each replicate. Data is presented as mean ± SD. Dashed brackets
581 indicate $p < 0.05$.

582 **Figure 4. Replication and pathology induced by PB1-F2 recombinant viruses in swine.**
583 Pigs (n=10/group) were infected with 10^5 $TCID_{50}$ of virus and nasal swabs were collected from
584 1-3 dpi to measure virus shedding. Lungs were collected at 1 and 3 dpi (n=5/day) for virus
585 titration and pathological analysis. (A and B) Viral shedding in nasal secretions. (C and D)
586 Pulmonary replication of PB1-F2 isogenic viruses in pigs. Virus titer (log_{10} $TCID_{50}$/ml) in nasal
587 swabs (A and B) or BALF (C and D) from each animal collected at the indicated time points. (E)
588 Percentage of macroscopic lung lesions. (F) Lung histopathologic scores. Data is presented as
589 mean ± SD. Dashed brackets ($p < 0.05$).

590 **Figure 5. Immunohistochemistry of influenza viruses in swine lungs.** A) anti-NP antigen
591 IHC staining in lungs of infected pigs. Values are the mean ± SD IHC scores based on the

592 percent of influenza-positive cells in the airway and lung interstitium. B) Representative IHC
593 slides depicting viral antigen primarily in airway epithelium at 1 and 3 dpi.

594 **Figure 6. PB1-F2 modulates the innate immune response in swine lungs.** Data represents
595 cytokines/chemokines levels (by ELISA) in BALF from each pig in the respective groups. Data is
596 presented as mean ± standard deviation. Dashed brackets ($p < 0.05$).

Table 1. Influenza viruses used in this study.

Viruses	Acronym in main text
A/turkey/Ohio/313053/04 (H3N2)	Ty/04 WT
A/turkey/Ohio/313053/04 (H3N2) PB1-F2 knock-out	Ty/04 KO
A/swine/Wisconsin/14094/99 (H3N2)	Sw/99 WT
A/swine/Wisconsin/14094/99 (H3N2) PB1-F2 knock-out	Sw/99 KO
A/swine/Wisconsin/14094/99 (H3N2) PB1-F2 N66S	Sw/99 N66S

Supplementary Figures

Figure S1. Alignment of triple reassortant H3N2 PB1-F2s used in this study. Sw/99 and Ty/04 PB1-F2 ORFs were aligned to those of the 20th century pandemic strains (1918 H1N1, 1957 H2N2, and 1968 H3N2), and contemporary swine and human IAV strains, and other previously characterized PB1-F2 proteins. Dashed boxes highlight amino acid residues that have been associated with increased virulence in the 1918 and highly pathogenic H5N1 strains: 51(T), 56(V), 66(S), and 87(E). ***** indicate the positions in which pro-inflammatory mutations (P62L, H75R, Q79R, and S82L) have been found in the human H3N2 PB1-F2 lineage. **§** indicates aa differences between Ty/04 and Sw/99 PB1-F2 ORFs.

Figure S2. Effect of presence or absence of PB1-F2 on expression levels of PB1, N40, PB2, PA and NP. MDCK cells were infected with the indicated influenza viruses at an MOI of 1. Cells were lysed at 23 h post-infection and subjected to Western blotting using specific antibodies against PB1, PB2, PA, NP, and the host's GAPDH (used as loading control). WT, wild type virus; KI, PB1-F2 knock in virus; KO, PB1-F2 knock out virus Size of molecular weight markers in kilodaltons (kd) is indicated on the left, whereas the arrows indicate the corresponding viral protein products.

Supplementary Table

Table S1. Sequence identity and amino acid differences between Sw/99 and Ty/04 viruses

Gene segment	nt sequence identity within ORF (%)	aa mutation Sw/99 →Ty/04
HA	96.8	L7F†, V14I†, N6S¥, T10M¥, N31D¥, D53N¥, G78D¥, D101Y¥, K122Q¥, G124F¥, K140R¥, G142E¥, K145N¥, K156N¥, E158N¥, S186G¥, S189R¥, S194N¥, N246S¥, R261Q¥, D275G¥, N49TØ, L55VØ, E57KØ, E177QØ, G184DØ
NA	96.1	T16I, M20I, Y40H, E41D, I51M, I62T, K93N, R143G, K172R, V194I, H197D, E199K, D198N, K221N, R249K, P267T, L269S, R331S, S332F, N336H, N339D, I292T, D399E, D402N, Q432E, E435K
PB2	97.5	D254E, M292I, N456S, A524T, T559S, V560L, M645L, V667I, I731V
PB1	98.2	V113I, G216S, K433R, K578R, N642S
PA	97.7	P28S, D115N, I118V, K256Q, I292V, A448S
NP	97.7	T3S, R31K, R38K, T217V, T402S, R446K
M	97.3	I15V§, T139A§, L181I§, M203I§, A227T§, T239A§
		A28D¶, Y50C¶, K60Q¶, A92V¶
NS	99.2	G51Sœ, T56Aœ, S114Pœ
		S60N∂, A89T∂

† Mutations within signal peptide sequence.

¥ Mutations within HA1 subunit.

Ø Mutations within HA2 subunit.

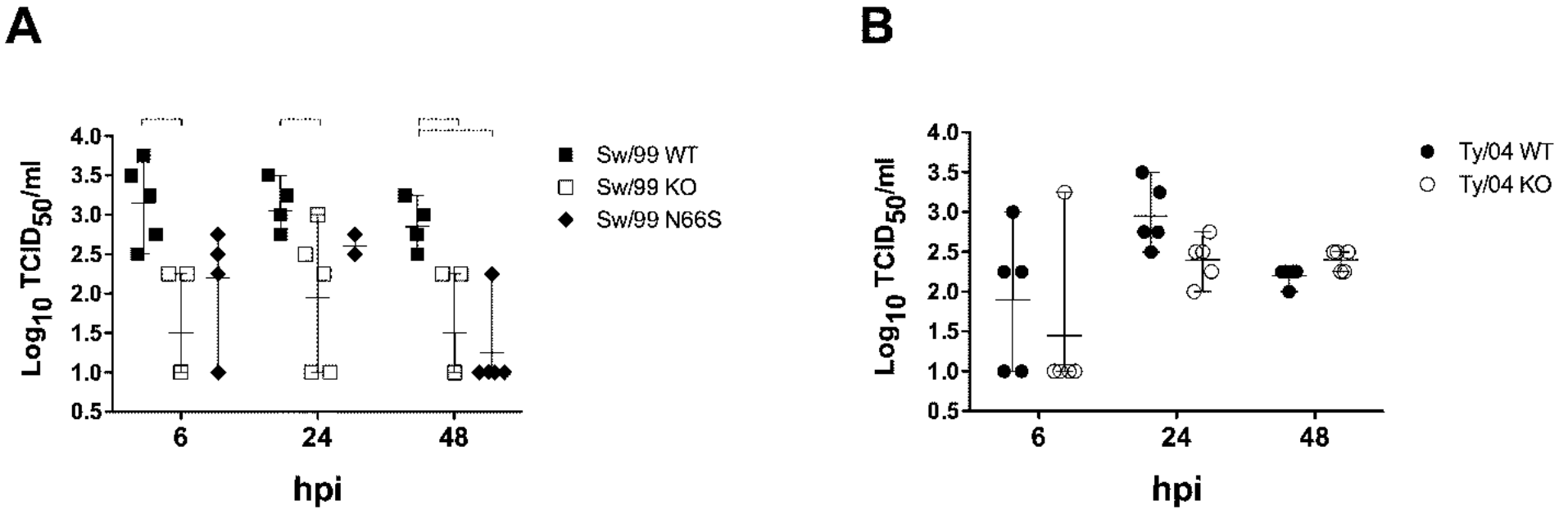
_ Underline indicates mutations in predicted glycosylation sites.

§ Mutations in M1.

¶ Mutations in M2.

œ Mutations in NS1.

∂ Mutations in NS2/NEP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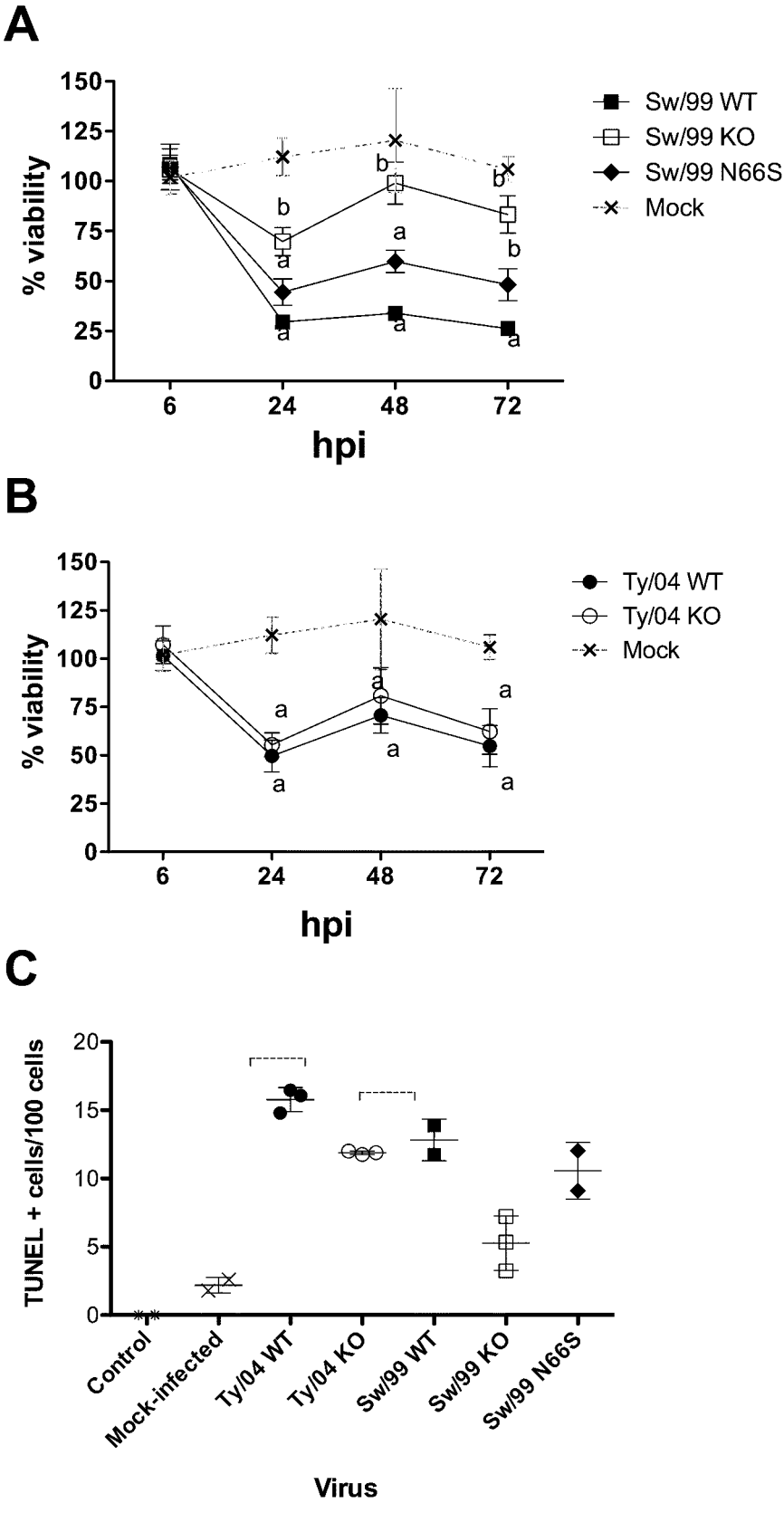
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Ty/04 WT
Ty/04 KO
Mock
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10
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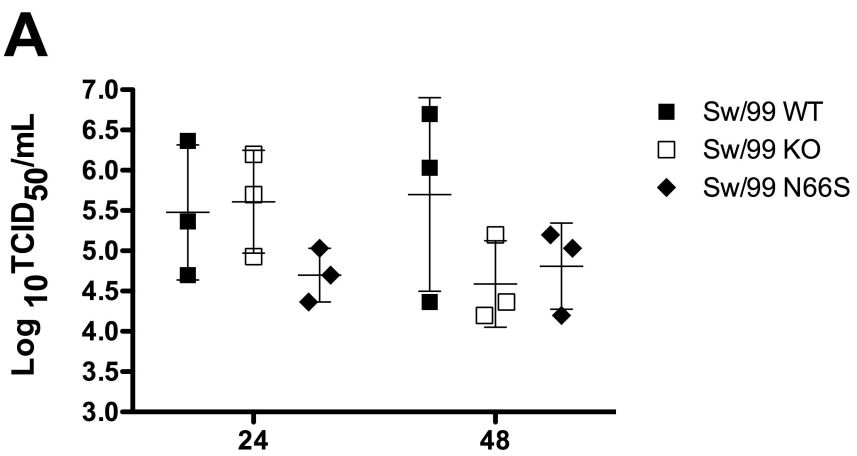
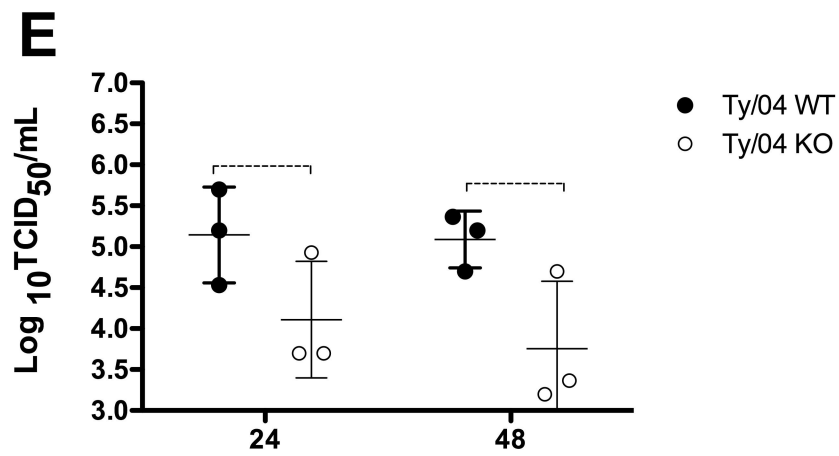
TUNEL + cells/100 cells
Control
Mock-infected
Ty/04 WT
Ty/04 KO
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Virus

Nasal turbinates





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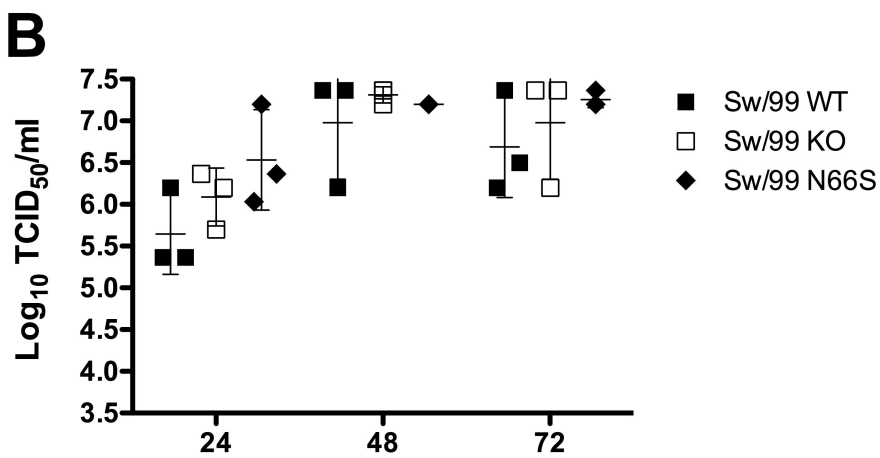
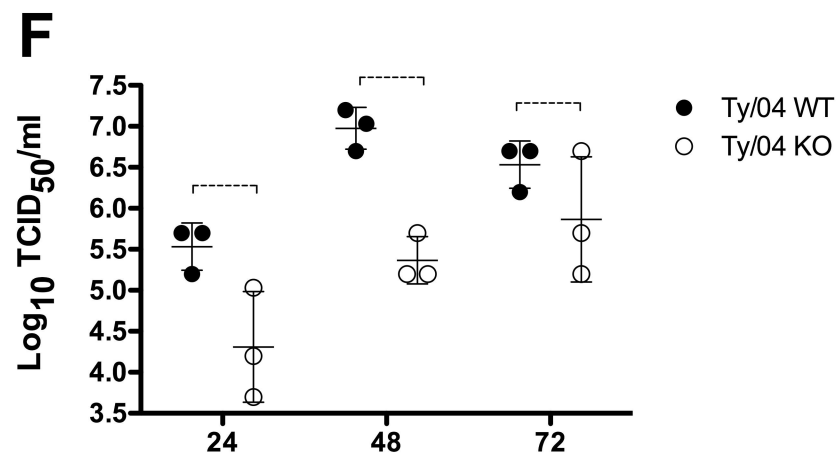
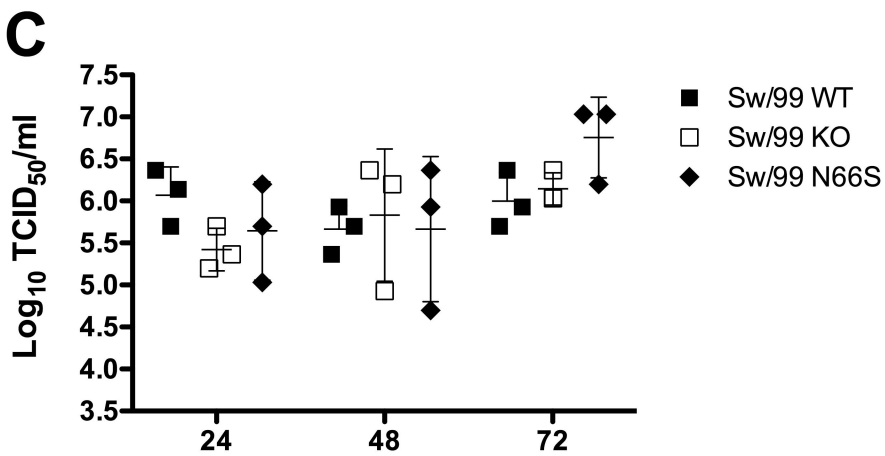
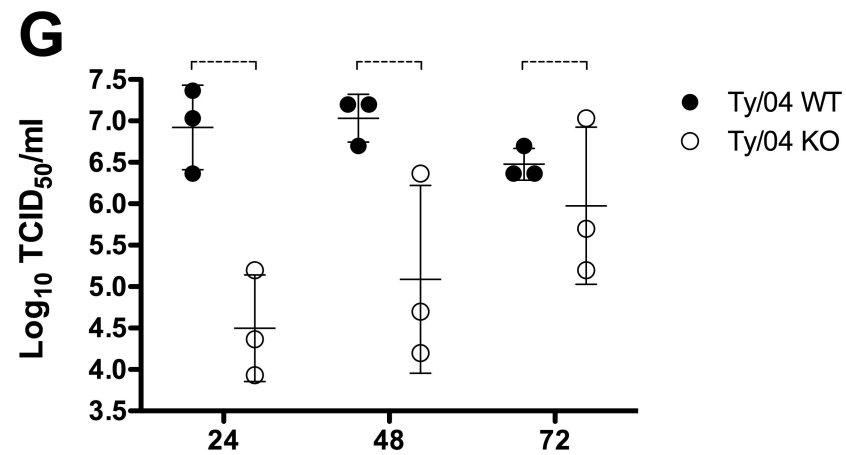


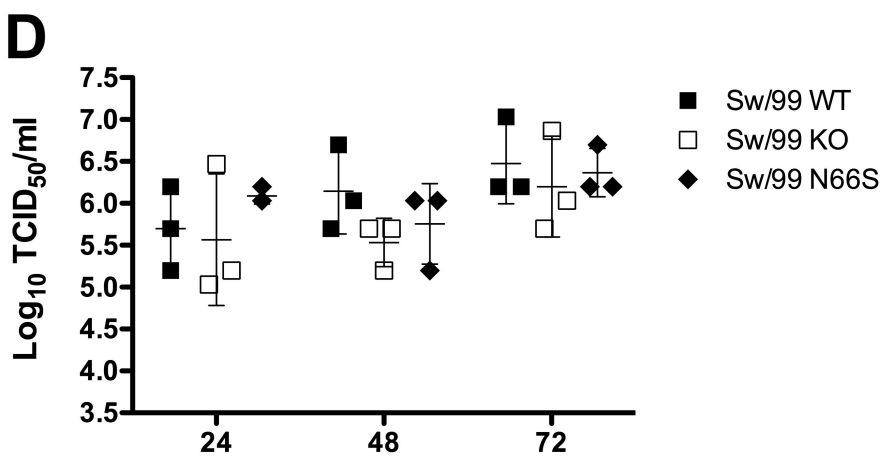


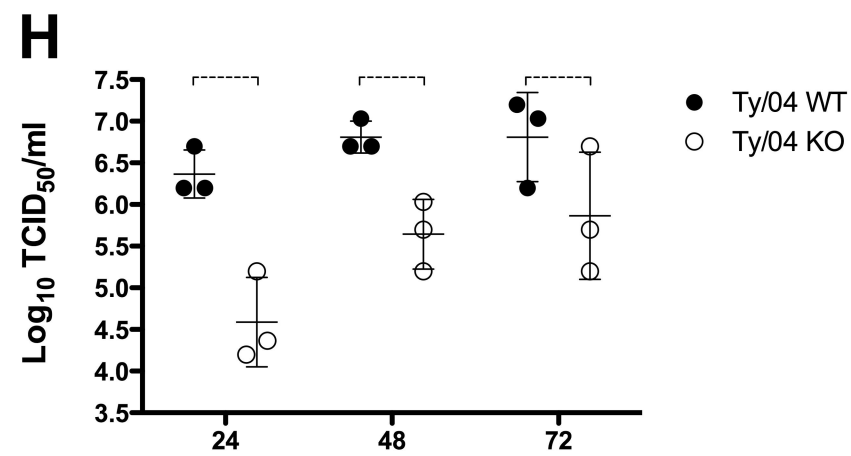
Proximal lungs



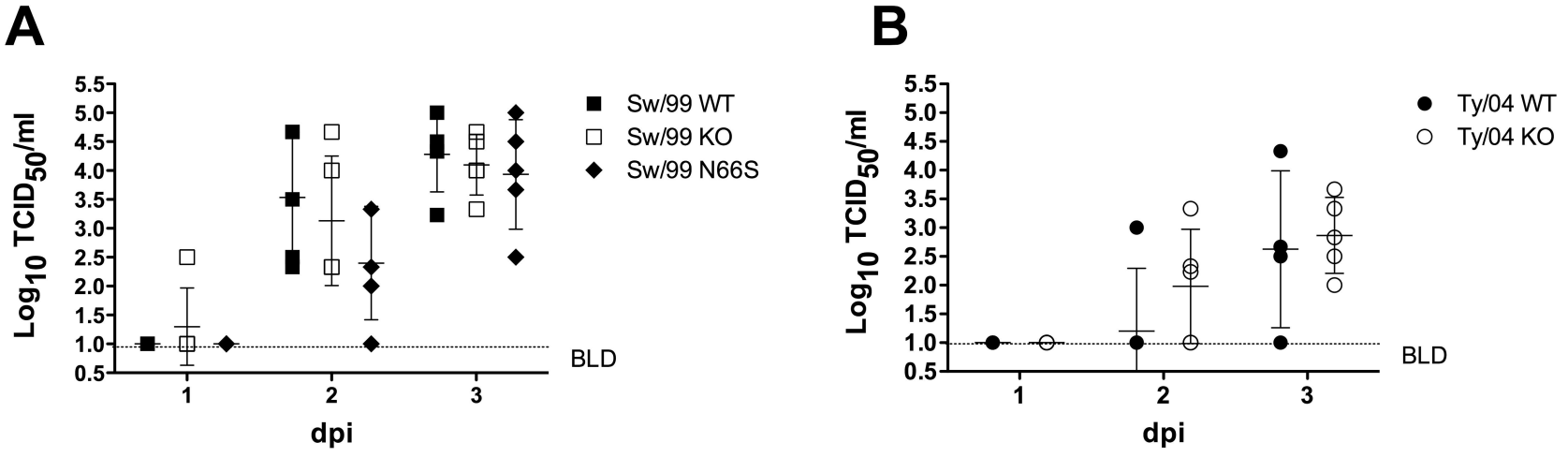


Distal lungs

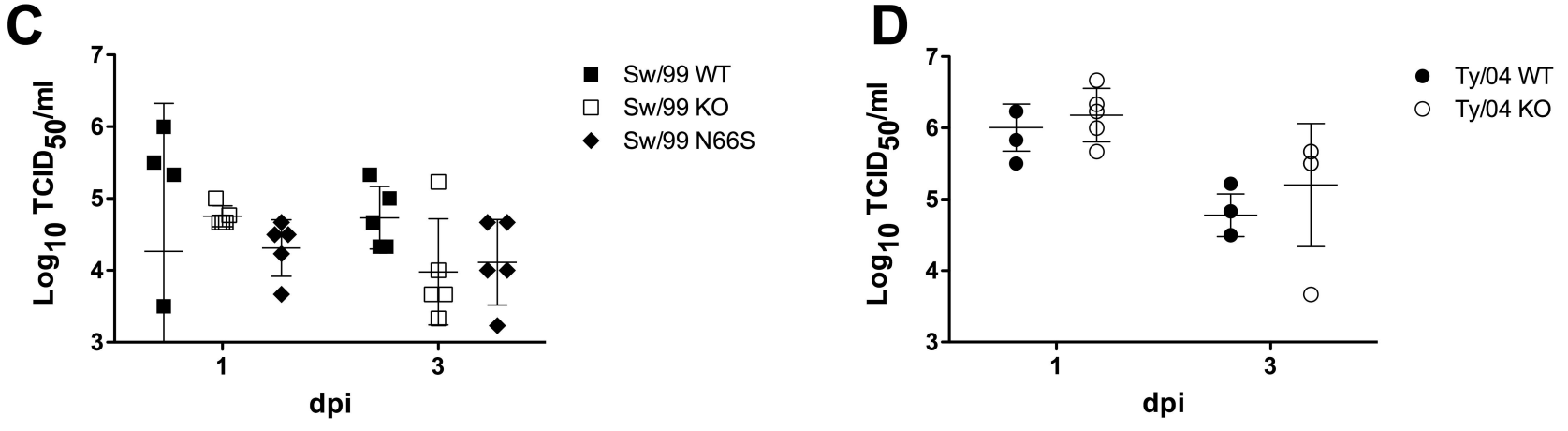




Nasal shedding

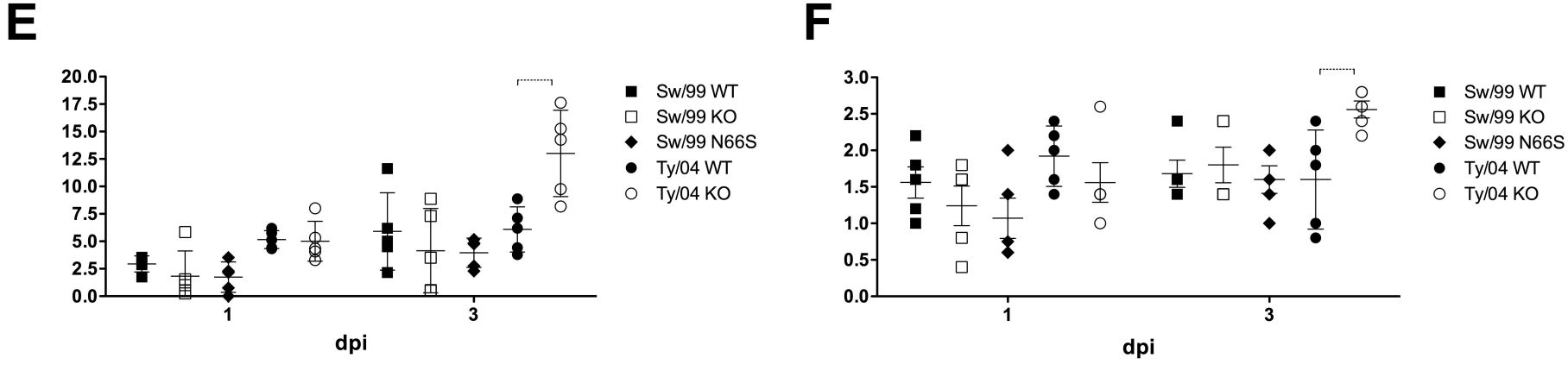


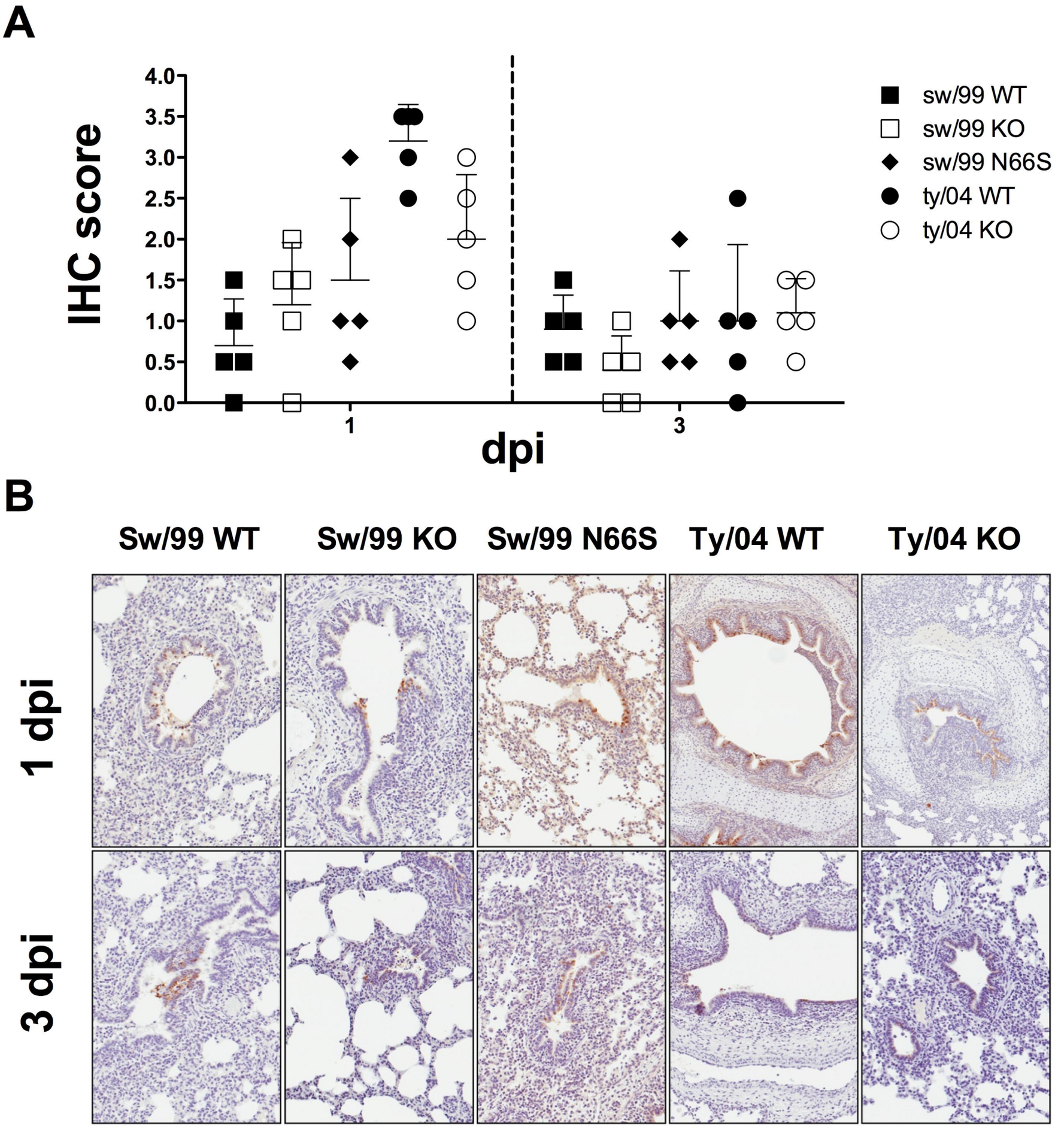
BALF



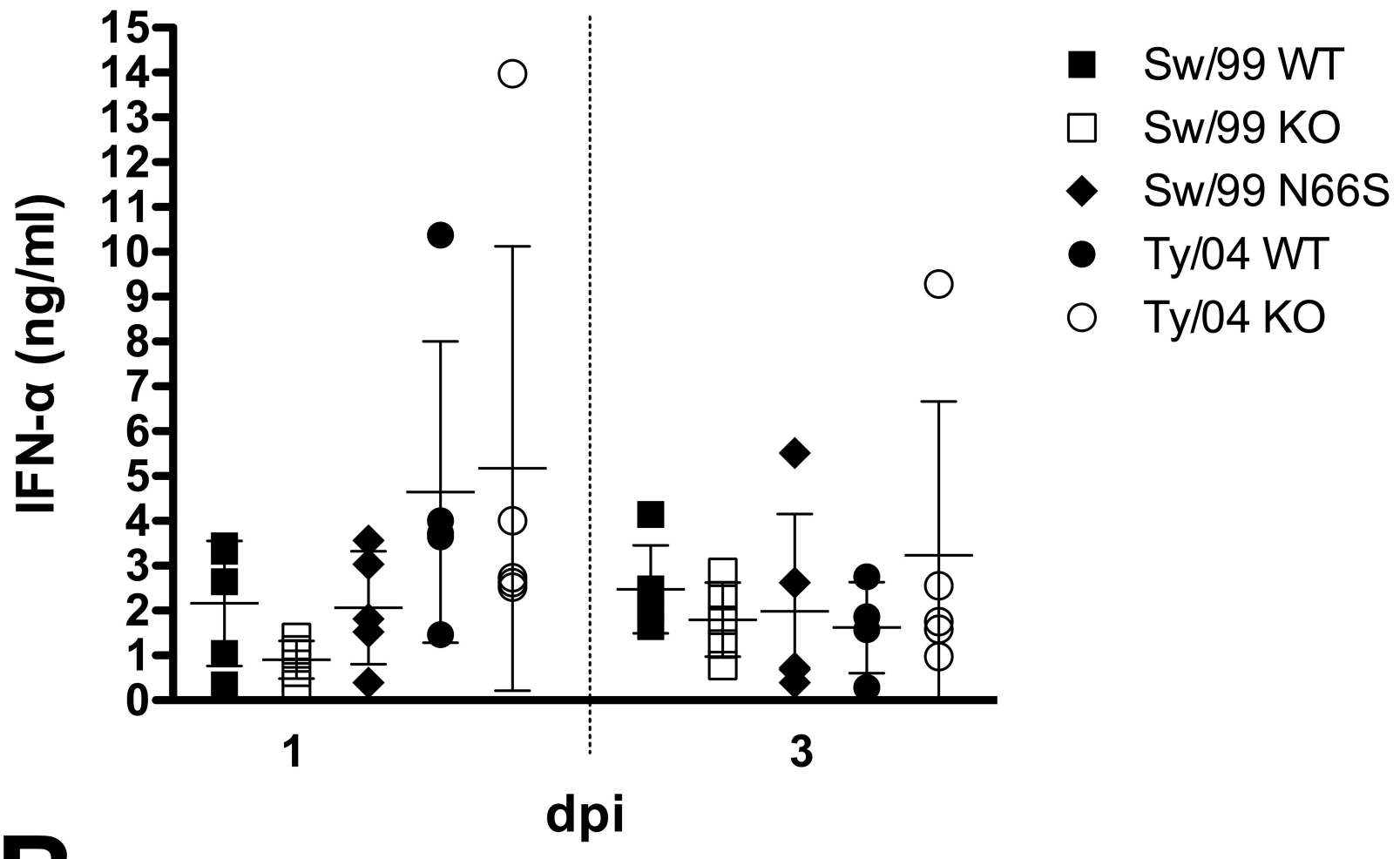
% Macroscopic pneumonia

Histopathology score

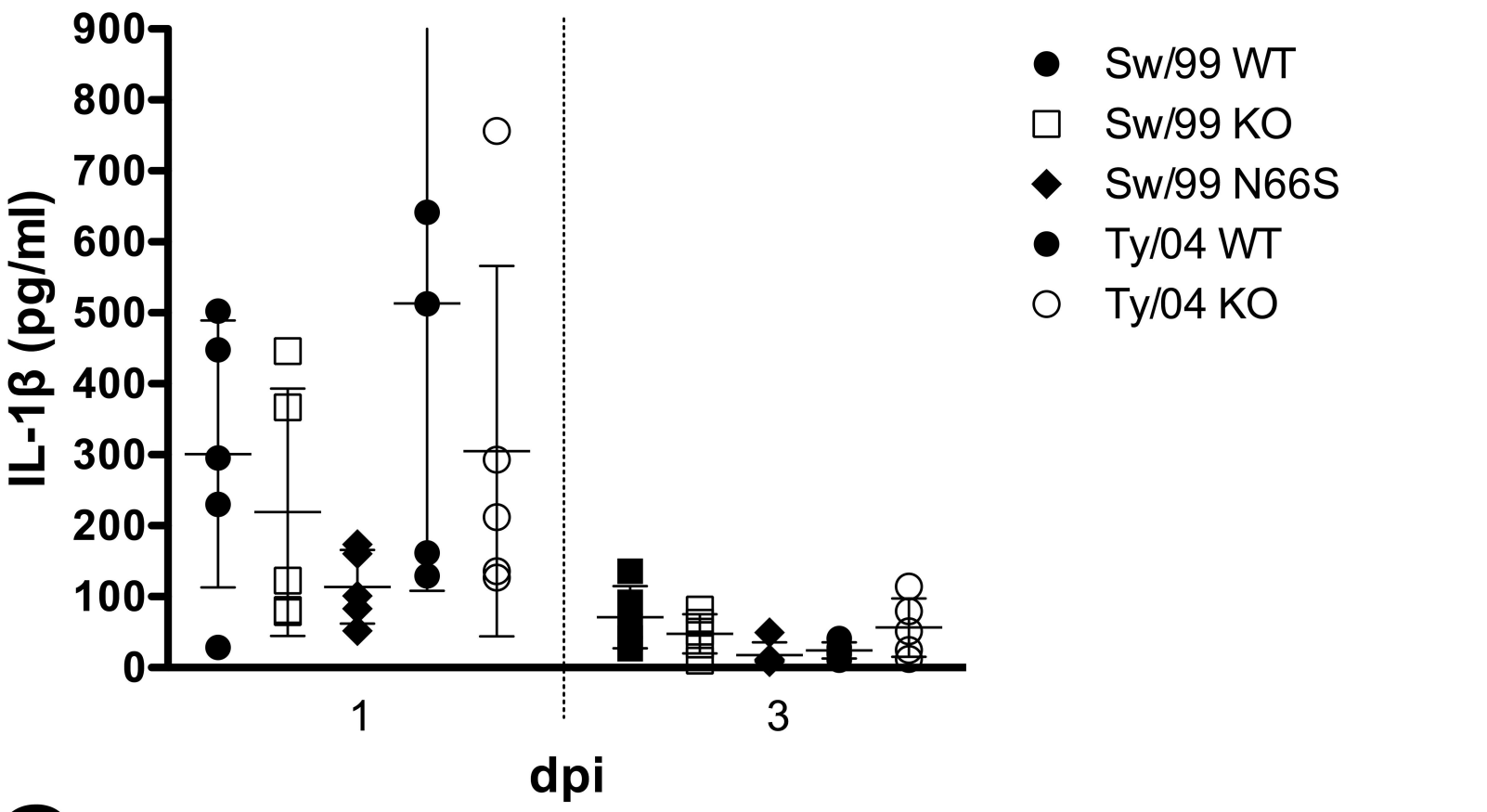



A
IHC score
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
3
dpi
sw/99 WT
sw/99 KO
sw/99 N66S
ty/04 WT
ty/04 KO
B
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Ty/04 WT
Ty/04 KO
1 dpi
3 dpi

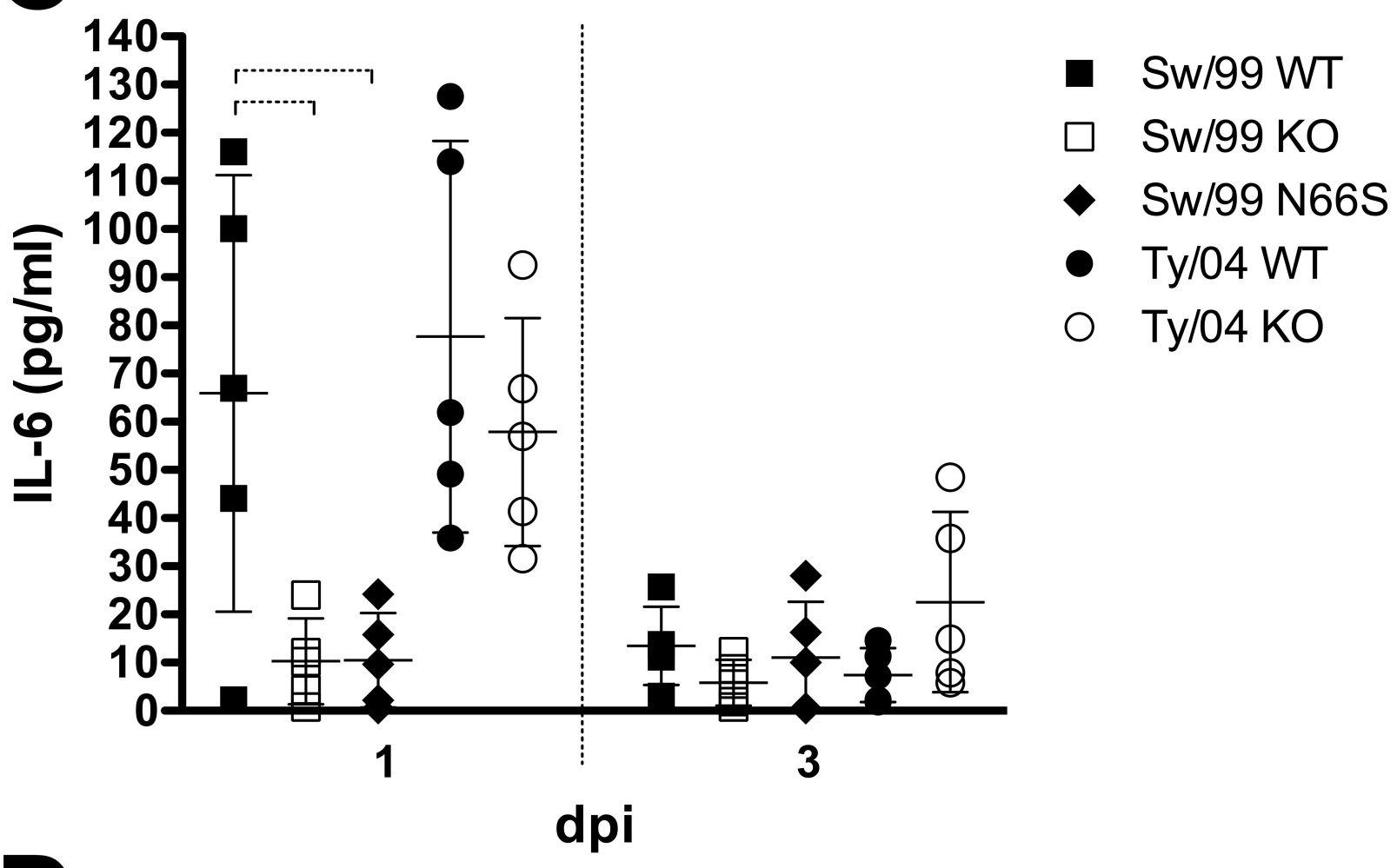
A


IFN-α (ng/ml)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1
3
dpi
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Ty/04 WT
Ty/04 KO

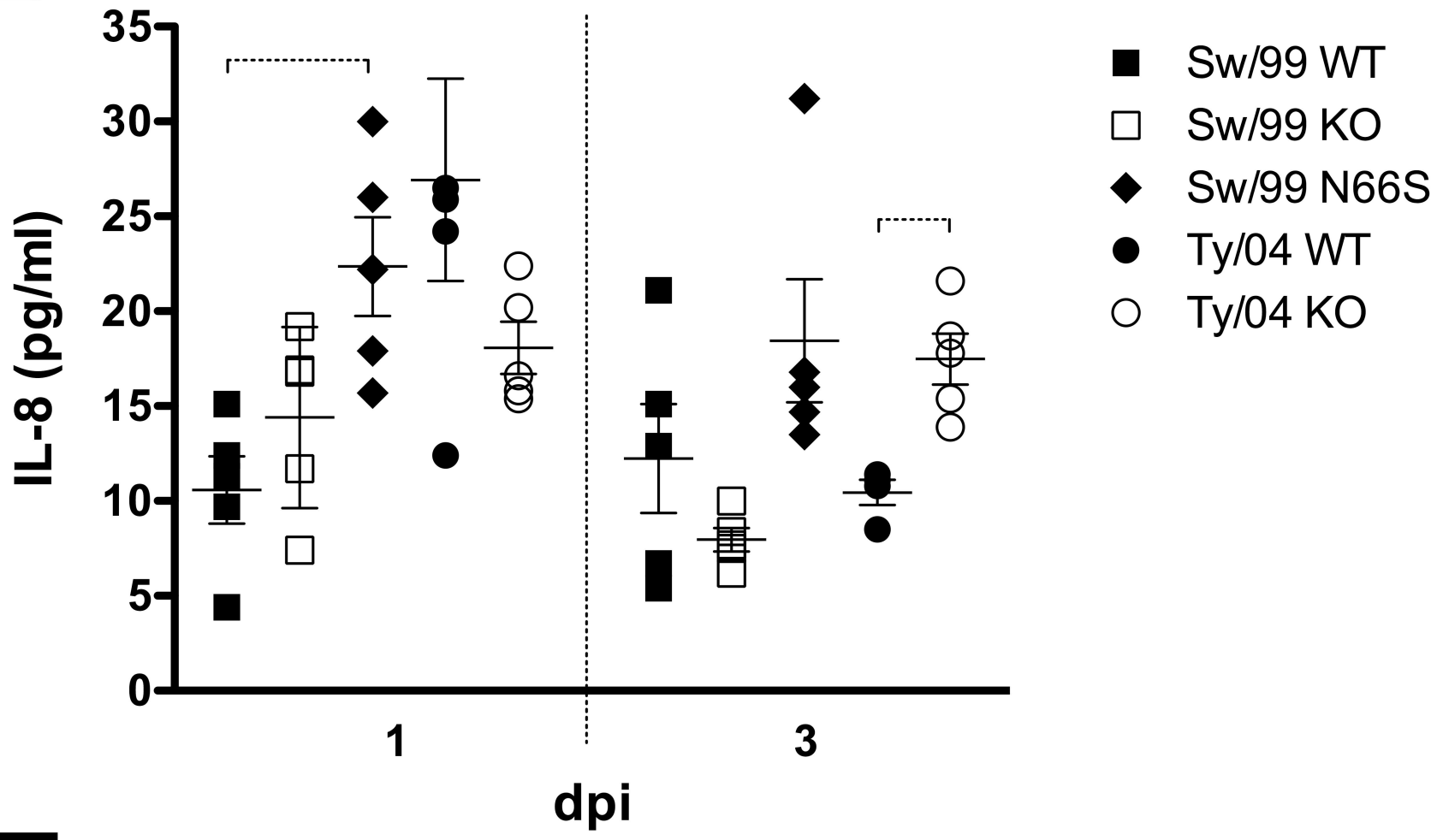
B


IL-1β (pg/ml)
0
100
200
300
400
500
600
700
800
900
1
3
dpi
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Ty/04 WT
Ty/04 KO

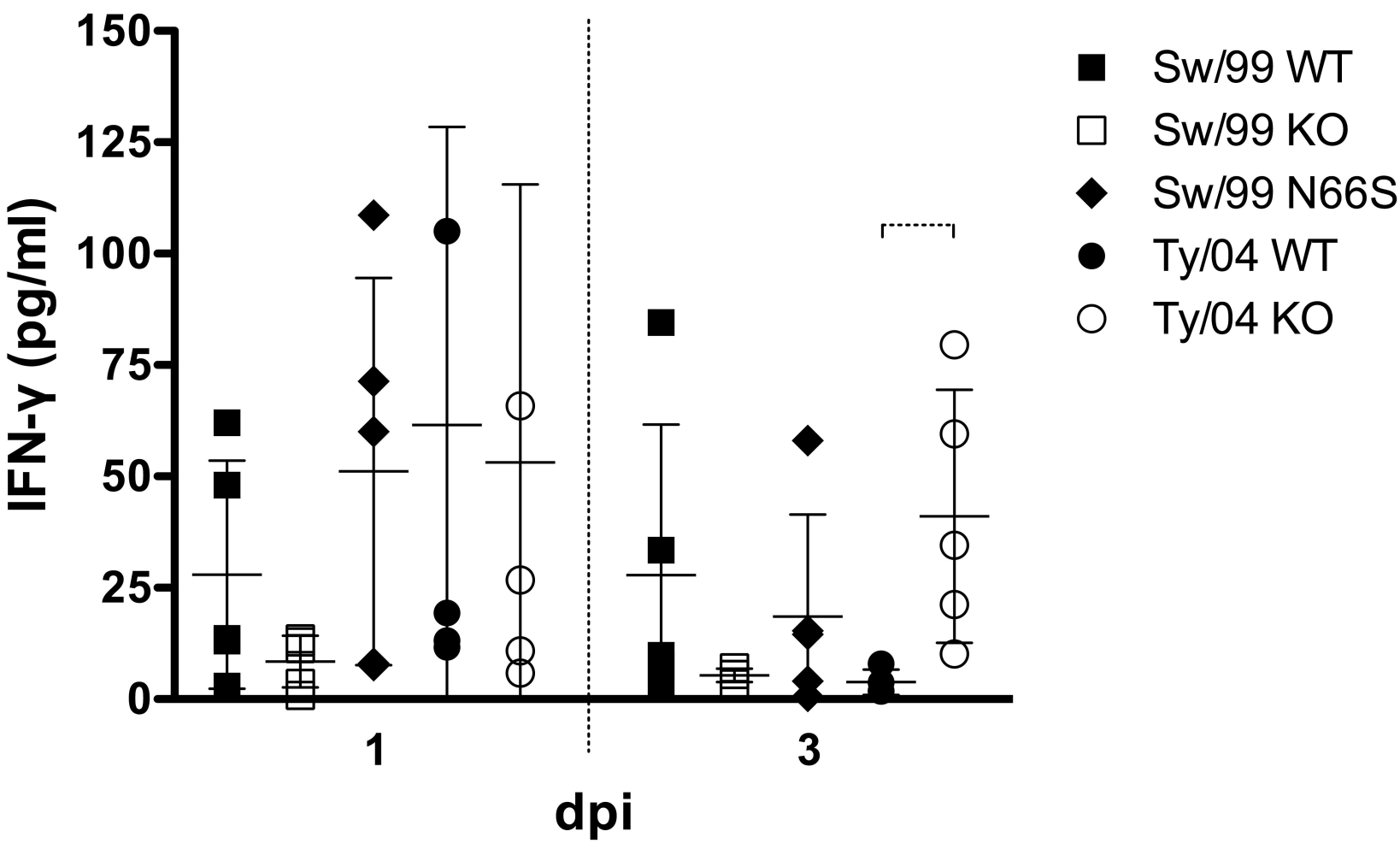
C


IL-6 (pg/ml)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
1
3
dpi
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Ty/04 WT
Ty/04 KO

D


IL-8 (pg/ml)
0
5
10
15
20
25
30
35
1
3
dpi
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Ty/04 WT
Ty/04 KO

E


IFN-γ (pg/ml)
0
25
50
75
100
125
150
1
3
dpi
Sw/99 WT
Sw/99 KO
Sw/99 N66S
Ty/04 WT
Ty/04 KO