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Pigs with Severe Combined Immunodeficiency Are Impaired in Controlling Influenza A Virus Infection

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Abstract
Influenza A viruses (IAV) infect many host species, including humans and pigs. Severe combined immunodeficiency (SCID) is a condition characterized by a deficiency of T, B, and/or natural killer (NK) cells. Animal models of SCID have great value for biomedical research. Here, we evaluated the pathogenesis and the innate immune response to the 2009 H1N1 pandemic IAV (H1N1pdm09) using a recently identified line of naturally occurring SCID pigs deficient in T and B lymphocytes that still have functional NK cells. SCID pigs challenged with H1N1pdm09 showed milder lung pathology compared to the non-SCID heterozygous carrier pigs. Viral titers in the lungs and nasal swabs of challenged SCID pigs were significantly higher than in carrier pigs 7 days postinfection, despite higher levels of IL-1β and IFN-α in the lungs of SCID pigs. The lower levels of pulmonary pathology were associated with the T and B cell absence in response to infection. The higher viral titers, prolonged shedding, and delayed viral clearance indicated that innate immunity was insufficient for controlling IAV in pigs. This recently identified line of SCID pigs provides a valuable model to understand the immune mechanisms associated with influenza protection and recovery in a natural host.

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Introduction

Severe combined immunodeficiency (SCID) is characterized by a life-threatening deficiency of T, B, and/or natural killer (NK) cells. Spontaneous SCID has been discovered in humans, horses, and dogs, and has been artificially created by transgenics in mice and rats [1, 2]. An X-linked SCID phenotype has been artificially induced in pigs through the disruption of the interleukin-2 receptor gamma chain gene (IL2RG) [3, 4], as well as the RAG1 and/or RAG2 genes required for the development of B and T lymphocytes [5, 6]. Pigs from a line of purebred Yorkshires under selection for increased feed efficiency at Iowa State University [7] were affected by naturally occurring SCID [8–10]. These SCID pigs were shown to have decreased numbers of B and T cells [11] and were unable to reject xenografted human cancer cells [9].

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Genetic analyses revealed 2 mutations in the *Artemis* (*DCLRE1C*) gene that were responsible for the naturally occurring SCID in this line of pigs [10]. Mutations in *DCLRE1C* were first linked to human SCID in 2001 [12, reviewed in 13]. Some Artemis-deficient patients were reported to have recurring respiratory infections [14], but none were specifically associated with influenza virus infection. The discovery of SCID in pigs gives rise to new avenues of biomedical research, such as studies to further examine the role of *Artemis* in immunity as well as in cancer and clinical therapy of immunodeficient patients, or leveraged for improvements in xenotransplantation. Development of gene tests for the loci that confer the naturally occurring SCID phenotype may also be valuable to commercial swine producers.

Influenza A virus (IAV) is a segmented, negative-sense RNA virus that is pathogenic in many host species, including humans and pigs [15]. Swine are a natural host of influenza infection and can serve as intermediary hosts in the evolution of influenza viruses of risk to humans [16], representing an ideal model for the study of influenza infection. Clinical signs of influenza in pigs are similar to those observed in humans and are manifested as acute respiratory disease characterized by fever, inactivity, decreased food intake, respiratory distress, coughing, sneezing, conjunctivitis, and nasal discharge [17]. The clinical disease in pigs and humans ranges from clinically unaffected to severe respiratory signs; the variation is due to prior immunity, properties of the virus, and many other contributing health and environmental factors [17]. The disease incubation period is between 1 and 3 days, with rapid recovery beginning 4–7 days after onset in healthy individuals without complicating infections of multiple etiology [17]. A primary virulence mechanism of IAV is disruption of the innate immune response by interfering with the type I interferon response early in infection [18]. However, as early as 5–7 days after infection, compensating innate mechanisms and the adaptive arm of the immune system begin to clear the virus from the respiratory tract. Protective antibody responses can typically be measured within 2 weeks of viral exposure [19].

Although IAV infection has been characterized in SCID mice as an absence of cell-mediated and humoral immune responses postvaccination [20] and prolonged weight loss with failure to clear virus [21], mice are not a natural host for IAV. Furthermore, laboratory-adapted viral strains used in most of these studies may not be relevant to fully virulent circulating strains. Mice susceptible to IAV have a large mutation in the *Mx* gene and fail to synthesize Mx protein, a type I interferon-induced protein that can selectively inhibit IAV replication [22]. Immunocompromised human patients affected with SCID often develop complications after influenza infection [23, 24]; however, the course of influenza infection in humans affected by the genetic defect causing the SCID in this pig model has not been described. Here, we evaluated IAV pathogenesis and the immune response in SCID pigs after inoculation with the 2009 H1N1 pandemic IAV strain (H1N1pdm09) that now circulates globally as a seasonal human strain. H1N1pdm09 is also endemically or sporadically detected in pigs globally [25]. Our objective was to utilize this unique SCID pig model to evaluate the ability of the innate immune response in swine, a natural host for IAV, to defend against acute influenza infection in the absence of the adaptive immune response. As H1N1pdm09 is a pathogen for humans and immunodeficiency is known to be a risk for severe influenza disease in humans [26], this swine SCID model provides an excellent opportunity for evaluating potential therapies for human patients.

Table 1. Experimental design and group assignment according to immune deficiency genotype

Treatment group	Genotype	Challenge strain	*n*
SCID/NC	SCID	None	5
SCID/CH	SCID	H1N1pdm09	6
Carrier/NC	Carrier	None	7
Carrier/CH	Carrier	H1N1pdm09	8

NC, nonchallenged controls; CH, challenged; SCID, severe combined immunodeficiency; H1N1pdm09, pandemic H1N1 A/California/04/2009.

Material and Methods

Study Design

Three SCID-carrier sows were used as the source of SCID pigs in this study. These sows were seropositive for IAV antibodies, indicating natural exposure to IAV prior to start of the study, but were shown to be free of IAV by virus isolation. The SCID carrier sows were artificially inseminated using semen collected from a boar that was a compound heterozygote for the 2 mutations identified in *Artemis* and rescued by bone marrow transfer. All inseminations were within a window of 1 week and all 3 sows farrowed without surgical intervention. In order to obtain seronegative SCID offspring from the 3 carrier sows, piglets were removed from their dams prior to suckling colostrum and fostered onto 4 healthy

Table 2. Primers and PCR conditions used to identify immune-deficient piglets by molecular genotype

SNP name	Allele	Product size	Forward primer sequence	Reverse primer sequence	T, °C	Extension time, s
ASGA-0048074	A	177	TCCTCTGACCAAGCCTCTGT	TCGTCCATGTACCAGAGCCT	56	30
	C	177	TCCTCTGACCAAGCCTCTGT	CGTCCATGTACCAGAGCCG	56	30
ASGA-0048114	A	659	AACCAGTCCCTGACCAACTG	TCCATATTTGTTAAGGGCAGTAATCT	54	30
	G	131	TGCTCAGAGCTTTACATGGATTTAG	GGCCCATGTTGACATAAAGC	54	30
ALGA-0059043	A	643	TCCTCTGCAGGGTTTCAAAG	CAGGGTGTGGGACTTTGTT	54	30
	G	127	TCAGCTTGGGCAGCTAGG	CCACAGGCACATTGATCTTG	54	30
H3GA-0030245	A	576	AGTTGAAATCAAAGTATCCCAA	AACTGTAACAAGCGTCCCTTTCT	55	30
	C	576	AGTTGAAATCAAAGTATCCCAA	AACTGTAACAAGCGTCCCTTTCG	55	30
ALGA-059061	A	593	GGTATTCTCCTCCTCTACCTCT	CTGGATTGGCAGAGGCTCTTTAT	55	30
	G	593	GGTATTCTCCTCCTCTACCTCT	CTGGATTGGCAGAGGCTCTTTAC	55	30
ALGA-0059066	A	475	GAATGGGAGGTGAGTAAGTAAA	CCAGCTGCAAGGGAGACT	55	30
	C	475	GAATGGGAGGTGAGTAAGTAAA	CCAGCTGCAAGGGAGACG	55	30
ALGA-0059080	A	425	AGCATTAAGACTGTGTGTGTGT	GGTCAAAGTCGTGGGTGTGTTT	55	30
	G	425	AGCATTAAGACTGTGTGTGTGT	GGTCAAAGTCGTGGGTGTGTTC	55	30

	ASGA-0048074	ASGA-0048114	ALGA-0059043	H3GA-0030245	ALGA-059061	ALGA-0059066	ALGA-0059080
Location	51153137	51812252	51975024	52066694	52086867	52109172	52174549
h12	C	G	G	C	A	C	A
h16	C	A	G	C	A	C	A
Normal	A	G	A	A	G	A	G

The bottom section of the table shows the locations and genotypes of SNPs for chromosomes that carry normal, h12, and h16 haplotypes, as described previously [10]. T, annealing temperature.

sows from an IAV-negative source herd within 6 h of life. Genomic DNA isolated from tail tissue was used for individual DNA testing to check for the SCID-associated genotype described below.

Twenty-six piglets were weaned at approximately 3 weeks of age and divided into 4 groups according to SCID genotype screening and challenge (Table 1). All pigs were treated with ceftiofur crystalline-free acid (Zoetis, Parsippany, NJ, USA) and enrofloxacin (Bayer Animal Health, Shawnee Mission, KS, USA) at weaning to reduce respiratory bacterial contaminants. Pigs were demonstrated to be free of IAV antibodies and IAV based on nasal swab samples prior to the start of the study. Pigs were housed in biosafety level 2 containment and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center.

At weaning, pigs in the challenge groups were inoculated intranasally with 2 mL of 1×10^6 $TCID_{50}$/mL of A/California/04/2009 (H1N1pdm09). The challenge virus was propagated in Madin-Darby canine kidney (MDCK) cells following standard procedures. Clinical signs of respiratory disease and rectal temperatures were observed daily. At 7 days postinfection (dpi), the pigs were humanely euthanized with a lethal dose of pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) and necropsied. Necropsy samples included bronchoalveolar lavage fluid (BALF), trachea, and right cardiac or affected lung lobe in 10% buffered formalin.

Molecular Determination of SCID Genotype

Allele-specific primer sets were designed for 7 single-nucleotide polymorphisms (SNPs) in the region surrounding the *Artemis* gene using web-based allele-specific PCR (WASP) [27]. PCR cycling conditions included an initial hot start at 95°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at temperatures indicated for each primer pair in Table 2 for 30 s, and elongation at 72°C for the time indicated in Table 2, ending with a final elongation step at 72°C for 5 min. Primer sequences, location, and genotypes for the 7 SNPs for which allele-specific primers were designed are given in Table 2. These SNPs were chosen as they mark specific haplotypes that segregated with SCID phenotype in prior litters [10]. The causative mutations and the 2 haplotypes (h12 and h16) that carry the mutations are described by Waide et al. [10].

Peripheral Lymphocyte Enumeration by Flow Cytometry

Blood was collected by venipuncture into an EDTA-treated vacutainer tube before (0 dpi) and after challenge (7 dpi) to determine the number of lymphocytes in circulation using a single-tube whole blood staining technique as previously described [28], with minor modifications. Briefly, 50 μL of whole blood was stained with a pan-lymphocyte antibody (PG106A; Washington State University, Pullman, WA, USA) and a fluorochrome-conjugated secondary antibody specific to mouse IgM. For enumeration, 50 μL of beads (10^6 beads/mL, Spherotech, Lake Forest, IL, USA)

was added and 1 mL of FACSLyse (BD Biosciences, Franklin Lakes, NJ, USA) to lyse red blood cells and fix leukocytes. Data were acquired using an LSRII with FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software.

Viral Titers in Lungs and Nasal Swabs

Nasal swabs (Nylon Minitip Flocked Dry Swabs; Copan Diagnostics, Murrieta, CA, USA) taken at 0, 1, 3, 5, and 7 dpi were filtered (0.45-mm syringe tip filters; Millipore, Billerica, MA, USA) and plated onto 24-well plates with confluent phosphate-buffered saline-washed MDCK cells for virus isolation, as previously described [29]. For viral titration, 10-fold serial dilutions of virus isolation-positive nasal swabs and BALF samples in serum-free MEM supplemented with 1 μg/mL tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin and antibiotics were plated in triplicate onto phosphate-buffered saline-washed confluent MDCK cells in 96-well plates. The plates were fixed with 4% phosphate-buffered formalin after 48 h of incubation and stained using an anti-IAV nucleoprotein monoclonal antibody and $TCID_{50}$/mL titers were calculated for each sample as previously described [29].

Pathological Examination of the Trachea and Lungs

The percentage of the lung affected with macroscopic lesions typical of IAV infection was evaluated at necropsy, with the percentage of the affected surface calculated based on weighted proportions for each lobe as previously described [30]. Fixed trachea and lung tissue samples were routinely processed and stained with hematoxylin and eosin for histopathologic examination. A veterinary pathologist blinded to the treatment groups evaluated and scored microscopic lesions according to previously described parameters [30]. Individual categorical scores were totaled into a composite score for lung and trachea microscopic lesions. Individual scores were also obtained for IAV-specific antigen immunohistochemical (IHC) staining in lung and trachea tissues, as previously described [30].

IAV Antibody Detection

Serum samples were collected by venipuncture before (0 dpi) and after challenge (7 dpi) and used to detect IAV-specific systemic antibodies. An enzyme-linked immunosorbent assay (ELISA; AI MultiS-Screen Ab Test, IDEXX, Westbrook, ME, USA) was used to detect anti-IAV nucleoprotein antibodies in serum according to the manufacturer's recommendation with modifications to include a suspect threshold. The presence or absence of antibodies was calculated based on the sample to negative (S/N) ratio, and the means of each treatment group were compared. For use in hemagglutination inhibition (HI) assays, sera were heat inactivated at 56°C for 30 min, treated with a 20% suspension of kaolin (Sigma-Aldrich, St. Louis, MO, USA), and adsorbed with 0.5% turkey red blood cells to remove nonspecific hemagglutinin inhibitors and natural serum agglutinins. HI assays were performed with H1N1pdm09 as the antigen and 0.5% turkey red blood cells using standard techniques [31].

Cytokine Assays

Upon collection, an aliquot of BALF was centrifuged at 400 *g* for 10 min to pellet cells and debris. The cell-free BALF was used to determine the concentration of IFN-α, IFN-γ, IL-2, IL-1β, IL-6, IL-8, TNF-α, IL-10, and IL-4 by multiplex ELISA (Aushon Biosystems, Billerica, MA, USA). Samples were analyzed in duplicate and averaged concentrations were used for statistical analysis.

Statistical Analysis

Statistically significant differences were identified by 1-way fixed-effects analysis of variance for each individual parameter: macroscopic lesions, composite microscopic lung and trachea scores, $\log_{10}$-transformed virus titers, mean OD for ELISA, and cytokine levels, with $p \leq 0.05$ considered significant using Prism 6.0 (GraphPad, La Jolla, CA, USA). Variables shown to have significant effects were subjected to pairwise mean comparisons using the Bonferroni test and p values were corrected for multiple comparisons.

Results

SCID Phenotype Confirmed by Enumerating Circulating Lymphocytes

Eleven pigs were shown by DNA testing to have the SNP genotype that predicts a SCID phenotype, and 15 were classified as carriers. The number of lymphocytes in circulation on the day of challenge and at necropsy (dpi 7) was enumerated using flow cytometry to confirm the SCID phenotype in the pigs with a predicted SCID genotype, and the results are shown in Figure 1. Pigs with the SCID genotype displayed typical flow cytometric plots, with means of less than 100 stained lymphocytes per microliter compared to means over 1,000 lymphocytes per microliter in the carriers. These remaining lymphocytes are likely to be primarily NK cells, which have been shown to circulate in these SCID pigs [10, 11].

Virus Replication Was Not Controlled in SCID Pigs

Nasal swabs were collected at 0, 3, 5, and 7 dpi to evaluate viral shedding, and the nonchallenged pigs were negative throughout the study. Virus shedding was similar between SCID and carrier challenged groups until 5 dpi, however SCID pigs continued to shed with significantly higher titers at 7 dpi when compared to carrier pigs ($p < 0.0001$; Fig. 2).

At 7 dpi, virus titers were determined in BALF. IAV was not isolated from the BALF of any nonchallenged pigs. Virus was detected in BALF of 6/6 and 5/8 challenged SCID and carrier pigs, respectively; however, the viral titers in BALF from challenged SCID pigs were significantly higher than those from carrier pigs ($p = 0.0001$; Fig. 3a), similar to the 7 dpi nasal swab virus titers.

Clinical Disease and Lung Pathology

Challenge with H1N1pdm09 resulted in mild apparent clinical signs in both SCID and carrier pigs during the 7-day challenge period. Body temperatures were not significantly different between challenged and nonchal-

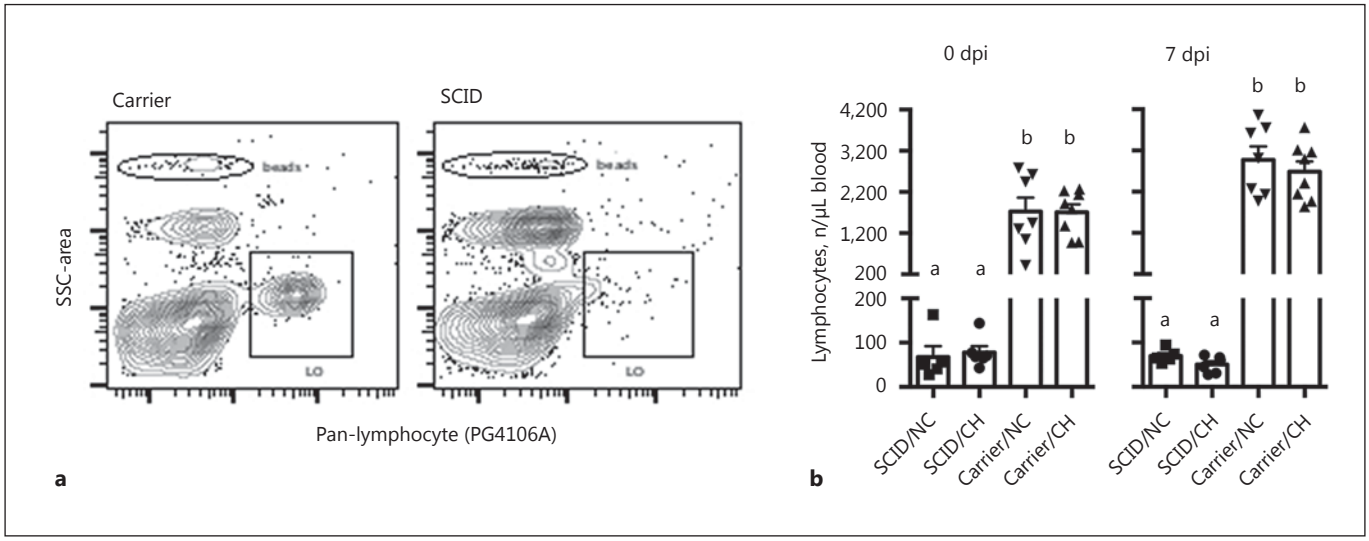

Fig. 1. Enumeration of peripheral lymphocytes by flow cytometry confirmed the SCID phenotype. Peripheral blood was collected and the number of circulating lymphocytes labeled with a pan-lymphocyte antibody (clone PG106A) as described in the Materials and Methods. **a** Representative flow plots of lymphocyte populations (PG106A+) in carrier and SCID pigs. Beads were used to enumerate the number of lymphocytes (LO). **b** The number of lymphocytes in the indicated groups on the day of challenge (0 dpi) and necropsy (7 dpi). Each individual symbol indicates the value for a pig in that respective treatment group, with mean and SEM also indicated. Bars with different letters indicate statistical differences ($p \leq 0.05$). CH, challenged; NC, nonchallenged control.

lenged groups (data not shown), with an exception of lower temperatures observed for carrier/CH pigs compared to nonchallenged and SCID/CH pigs at 5 dpi, perhaps related to a difference in inflammatory response.

Macroscopic lung lesions and microscopic lung and trachea lesions were evaluated at 7 dpi. SCID and carrier pigs challenged with H1N1pdm09 displayed moderate pathologic changes characterized by cranioventral purple-colored consolidation typical of IAV infection. Of note, SCID pigs challenged with H1N1pdm09 displayed a significantly lower percentage of macroscopic lung lesions than carrier/CH pigs ($p = 0.04$; Fig. 3b). Following the same trend, microscopic pneumonia scores were significantly lower for SCID/CH pigs compared to challenged carrier pigs ($p < 0.0001$; Fig. 3c), with SCID/CH pigs having only mild necrotizing bronchiolitis and minimal peribronchiolar lymphocytic cuffing. However, trachea microscopic scores were similar for the 2 challenged groups (Fig. 3e).

In contrast to the lung pathology findings, detection of IAV-specific antigen in lungs and trachea by IHC was significantly higher in challenged SCID pigs compared to carrier pigs ($p < 0.0001$; Fig. 3d, f). The IHC scores were consistent with virus titers in BALF and nasal shedding at 7 dpi, as more virus was detected in challenged SCID pigs compared to challenged carrier pigs.

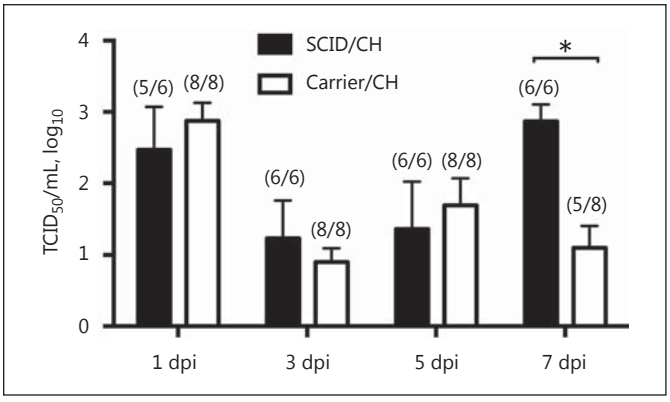

Fig. 2. Virus titers in nasal swabs at 1, 3, 5, and 7 dpi of pigs with SCID or carrier control pigs challenged (CH) with A/California/04/2009. The numbers of infected pigs/total number of pigs are indicated in parentheses. Results are shown as means and SEM of $\log_{10}$ titers. Significant differences (* $p \leq 0.05$) within each dpi are indicated.

Local Cytokine Response following IAV Challenge

To assess differences in the local immune response following IAV infection between SCID and carrier pigs, the levels of 9 cytokines in cell-free lung lavage collected at necropsy (dpi 7) were measured (Fig. 4). Cytokines IL-4,

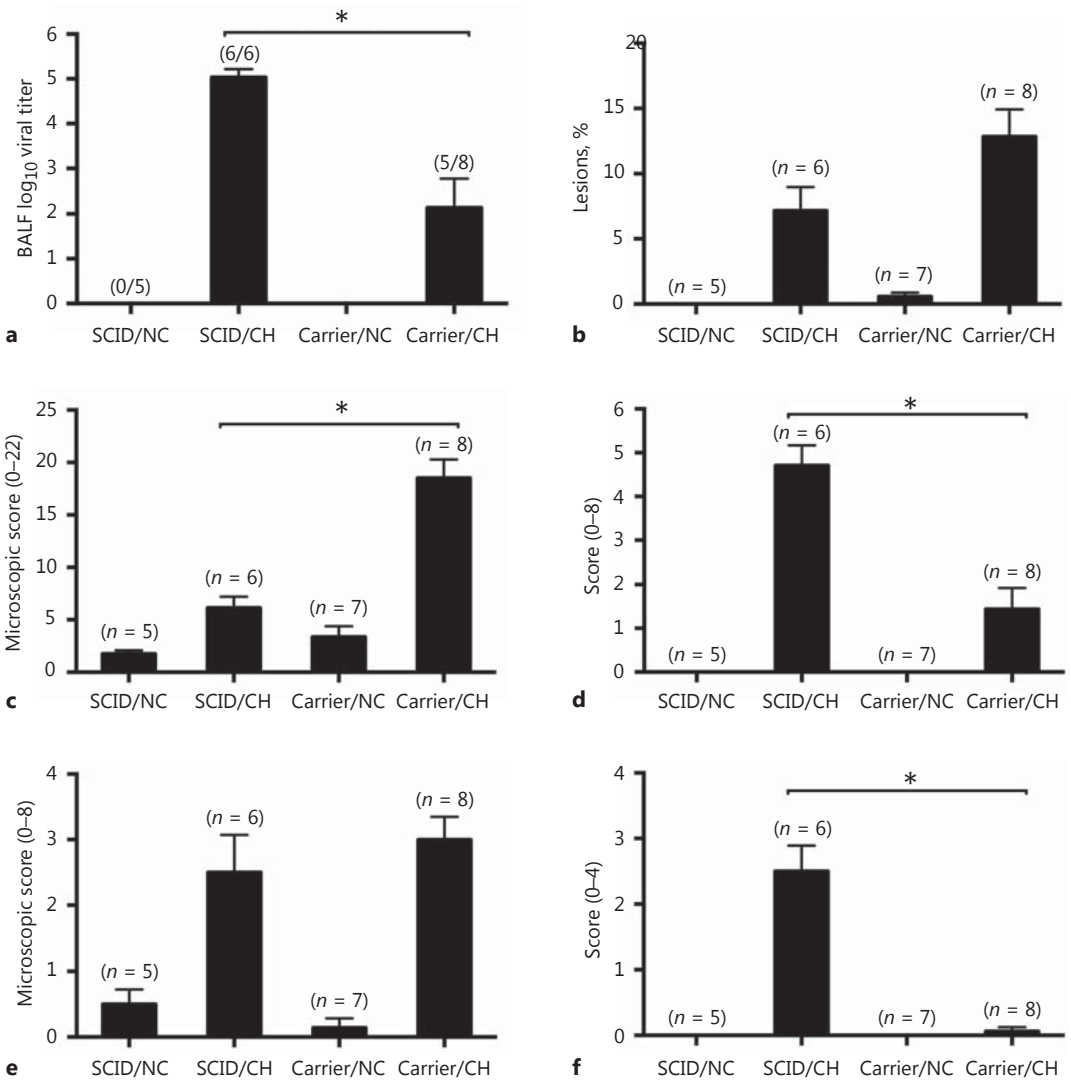

Fig. 3. Influenza effects on the lower respiratory tract differ in SCID pigs. Virus titers in BALF at 7 dpi (**a**), macroscopic pneumonia (**b**), lung microscopic pathology (**c**), IAV antigen detection scores in lungs (**d**), trachea microscopic pathology (**e**), and IAV antigen detection scores in trachea (**f**) in pigs with SCID or carrier control pigs challenged (CH) with A/California/04/2009, and non-challenged controls (NC). The numbers of pigs per group or numbers of infected pigs/total number of pigs are indicated in parentheses. The results are shown as means and SEM. Significant differences (* $p \leq 0.05$) between challenged groups are indicated.

TNF-α, and IL-10 were below the limit of detection (data not shown). There was a significant increase in the amount of IFN-α and IL-1β detected in the lungs of SCID pigs challenged with IAV when compared to levels in the lungs of challenged carrier pigs ($p < 0.05$). The average amount of IFN-α in the lavage collected from the SCID/CH group was 724.9 pg/mL, compared to 9.6 pg/mL in the carrier/CH group, and average levels of IL-1β were 83 pg/mL compared to 16.6 pg/mL for the same respective groups. While there was a trend for increased levels of IFN-γ and IL-6 in the lung lavage collected from challenged SCID pigs, these levels were not statistically different from those of challenged carrier pigs. The levels of IL-2 and IL-8 in the lungs were also not statistically different between the 2 challenged groups.

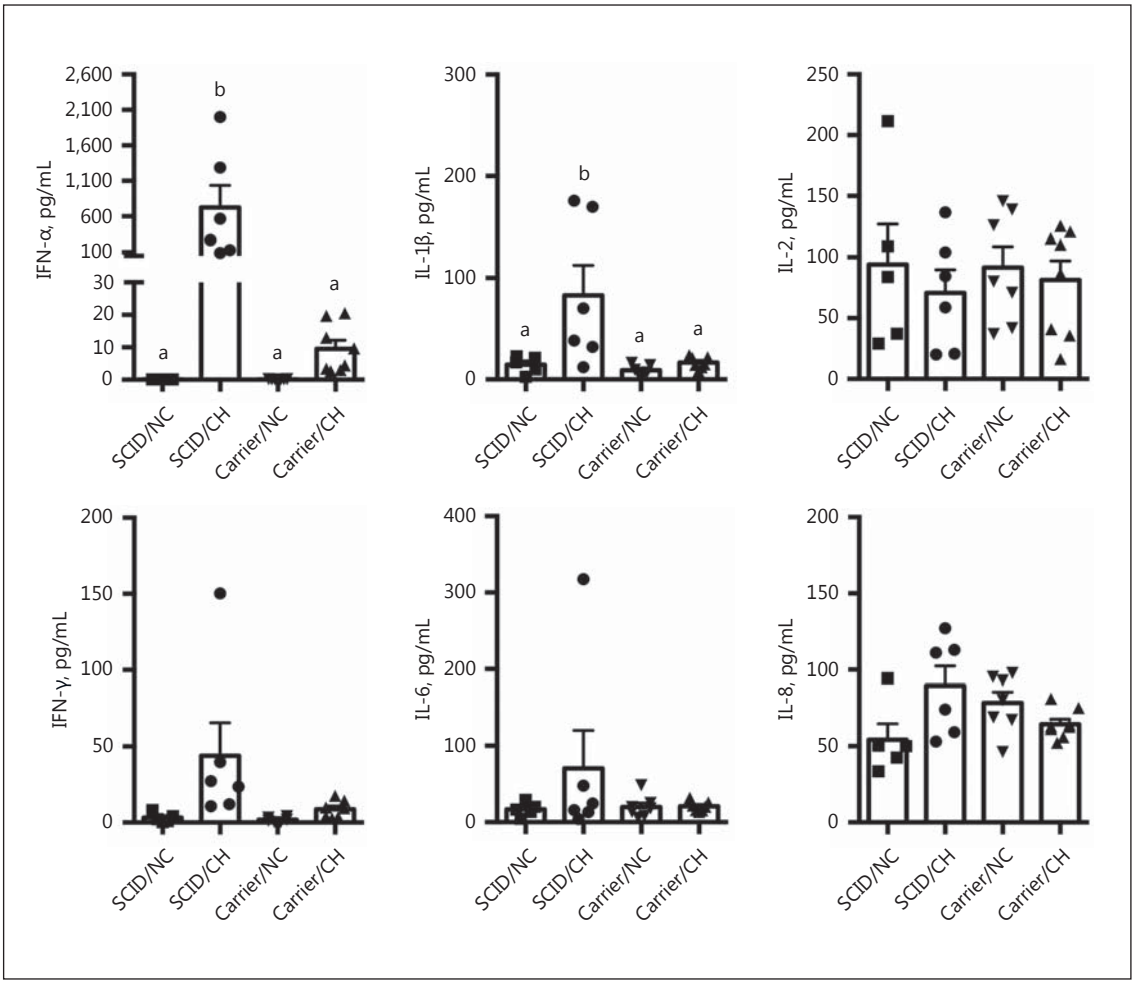

Fig. 4. Levels of IFN-α, IL-1β, IL-2, IFN-γ, IL-6, and IL-8 in the lungs of pigs with SCID or carrier control pigs challenged (CH) with A/California/04/2009, and nonchallenged controls (NC). Lung lavage was collected on day 7 following challenge, and the amount of each indicated cytokine was measured by multiplex ELISA. Each individual symbol indicates the value for a pig in that respective treatment group, with the mean and SEM also indicated. Bars with different letters indicate statistical differences ($p \leq 0.05$).

Induction of IAV-Specific Antibody Response in Carrier but Not SCID Pigs

Serum anti-IAV nucleoprotein antibodies were not detected in challenged SCID pigs. Challenged carrier pigs showed significantly greater levels of detectable nucleoprotein antibodies at 7 dpi compared to the nonchallenged controls and challenged SCID pigs ($p < 0.0001$; Fig. 5). Although the group average was within the suspect range by test standards, 5/8 animals were considered positive or suspects, indicating early specific antibody responses. HI antibody titers were below the detection limit for all pigs (data not shown).

Discussion

Swine influenza is typically an acute, self-limiting respiratory disease that affects animals of all ages and has a short course of infection [17], resembling the clinical onset of IAV infection in humans. Both innate and adaptive immunity are important for the effective protection against influenza virus infection, resolution of clinical signs, and viral clearance [18]. The same immune response factors necessary for elimination of IAV and recovery can also initiate or exacerbate tissue damage as a result of dysregulated immune responses [32]. Here, we assessed the course of IAV infection and the immune re-

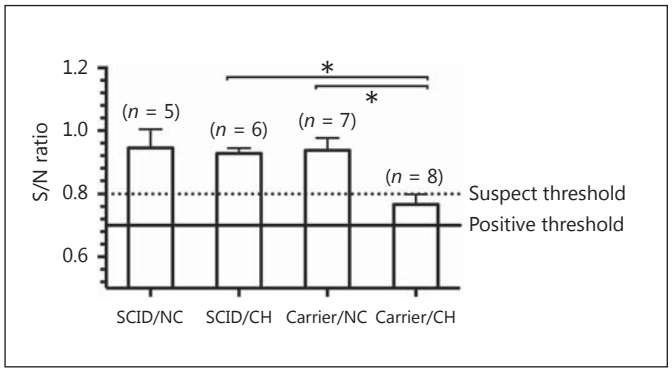

Fig. 5. Serum antibody detection at 7 dpi in pigs with SCID or carrier control pigs challenged (CH) with A/California/04/2009, and nonchallenged controls (NC) by blocking ELISA. Values are shown as mean S/N ratios and SEM, with lower S/N ratios indicative of the presence of IAV-specific antibodies. The cut-off value for a negative sample is indicated by the dotted line, and the maximum value for a sample to be considered positive is indicated by the solid line (positive threshold) with a suspect range between the two. The number of pigs per group is indicated in parentheses. Significant differences (* $p \leq 0.05$) between groups are indicated.

sponse in SCID pigs in which B and T cells were absent or in very low numbers, but NK cell numbers and their in vitro function were unaffected [11, 33]. Our results show that the lack of adaptive immune response in the SCID pigs resulted in delayed viral clearance from the lungs and prolonged viral shedding, despite clinical signs being milder than in carrier controls.

The SCID pigs used here were deficient in B and T cells but retained NK cells and, therefore, did not have the common cytokine receptor γ-chain (γ_c) deficiency that results in X-linked SCID, the most common form of SCID in humans (T–B+NK–) [34]. Genetic causes of SCID are heterogeneous, and X-linked SCID is estimated to constitute 17% of SCID occurrences, while T–B–NK+ cases (primarily caused by Artemis and RAG-1/2 mutations) make up approximately 11% of SCID patients worldwide [35]. Immunocompromised patients, such as individuals affected with SCID or who received bone marrow or organ transplants, are at increased risk of developing severe complications after influenza infection and often show greater mortality and prolonged viral shedding [23, 24]. Mouse models of SCID are primarily based on RAG1/2 knockouts, which result in a similar immune system disfunction as the SCID pigs used here. Thus, these pigs provide an additional model to study the immune response mechanisms involved in the protection and/or resolution of IAV infection, particularly in regards to the adaptive compartment of the immune system. Because swine are natural hosts for IAV, are genetically and anatomically similar to humans [36], and also have an immune genome component that is very similar to humans [37], these SCID pigs represent an improved model to the use of SCID mice for evaluating potential therapies and preventive strategies for human influenza [38].

The recruitment of NK cells to the lungs of aerosol-infected mice was shown to have a minimal role in controlling IAV infection in SCID mouse models, while specific T cells were shown to mediate recovery from infection with a lethal dose of IAV [39]. Our results suggest that innate immunity may not be sufficient to control IAV infection in pigs. Despite the presence of some peribronchiolar lymphocytic cuffing in the SCID pigs, likely NK cells, and previous reports of the involvement of these cells in early response to influenza in swine [40], the SCID pigs showed a trend for increasing virus titers at 7 dpi, when carrier pigs were beginning to clear infection, underscoring the critical role of the adaptive immune response in IAV clearance. The innate immune response may work efficiently to control viral replication in the early stages of influenza infection, but the adaptive immune response is required at later stages to clear the virus and resolve the infection, as shown in RAG2 knockout mice that were deficient in both B and T cells but still produced NK cells [21]. We recently demonstrated that NK cells isolated from healthy SCID pigs are intrinsically functional, as they could be activated by IL-2, or a combination of IL-12 and IL-18, to express higher levels of perforin and to phagocytose human cancer cells [33]. However, it is not known if NK cells in the *Artemis* SCID pig model are functional in vivo, which could explain why implanted human cells are not eliminated in these SCID pigs [9].

Although the SCID pigs consistently showed minimal numbers of circulating B and T lymphocytes [11], levels of IL-2 in their lungs were similar to those of carrier control pigs, which suggests another cell type could be the source of this cytokine. Dendritic cells have been associated with the production of IL-2 in response to stimulation by microbes [41, 42]. Furthermore, epithelial cells of the human airways were also identified as potential sources of IL-2 after exposure to inflammatory stimuli [43].

Interestingly, an inverse relationship between virus load and pathology was observed in the infected SCID pigs at 7 dpi. Infected SCID pigs demonstrated minimal cellular infiltrates through the microscopic evaluation of lungs, suggesting that lymphocyte infiltration and adap-

tive immunity are involved in the immunopathology of influenza. Lung lesions in SCID pigs were milder than in carrier controls and less pronounced inflammation and peribronchiolar cuffing was observed (data included in composite microscopic scores), which could be ascribed to the lack of T cells producing cytotoxic factors in the SCID pigs. In murine models, reduced numbers of CD8+ T cells were associated with attenuation of immune-mediated influenza pneumonia [44], while memory CD4+ T cell response to secondary exposure was associated with lung immunopathology [45]. Because T lymphocytes are essential for IAV clearance from the infected host [46], the SCID pigs were not expected to efficiently eliminate the virus. While healthy carrier pigs had begun to decrease virus titers by 7 dpi, viral titers in SCID pigs remained significantly higher than carriers and increased over previous time points, with no sign of recovery. Type I interferons, including IFN-α, are important for activating innate antiviral mechanisms involved in responses to IAV infection [47]. In SCID pigs there was more IFN-α in the lungs at 7 dpi compared to carrier pigs, and while IFN-α-mediated responses may have contributed to controlling IAV replication, it was not as effective as having an intact adaptive immune system (carrier pigs).

Using a recently identified line of naturally occurring SCID pigs, we demonstrated prolonged viral replication and milder pathology upon influenza infection in the absence of an adaptive B and T cell immune response. Future studies are important to evaluate whether IAV would be eventually cleared if the SCID pigs were followed for a longer time. This SCID pig model could prove beneficial to identify minimal immune factors essential to control IAV infection, using partial immune cell or antibody replacement treatments and to understand the immune mechanisms associated with influenza protection and recovery. Additionally, they may provide a valuable model to study therapeutics for immunodeficient patients infected with influenza.

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Disclosure Statement

USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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