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CASE REPORT

X-linked CD40 ligand deficiency in a 1-year-old male Shih Tzu with secondary *Pneumocystis* pneumonia

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Abstract

An approximately 1-year-old male intact Shih Tzu dog was referred to a tertiary facility with a history of progressive tachypnea, increased respiratory effort, and weight loss over a 3-month period that failed to improve with empirical antimicrobial treatment. Upon completion of a comprehensive respiratory evaluation, the dog was diagnosed with severe *Pneumocystis* pneumonia and secondary pulmonary hypertension. Clinical signs resolved and disease resolution was confirmed after completion of an 8-week course of trimethoprim-sulfonamide, 4-week tapering dose of prednisone to decrease an inflammatory response secondary to acute die-off of organisms, a 2-week course of clopidogrel to prevent clot formation, and a 2-week course of a phosphodiesterase-5 inhibitor to treat pulmonary hypertension. Immunodiagnostic testing and genetic sequencing were performed to evaluate for potential immunodeficiency as an underlying cause for the development *Pneumocystis* pneumonia, and identified an X-linked CD40 ligand deficiency.

KEYWORDS

gene mapping, genetic markers, immunodeficiency, infectious diseases, pneumonia, thoracic imaging

1 | INTRODUCTION

Pneumocystis is an opportunistic extracellular yeast-like fungal organism that can be found in low numbers within the pulmonary alveoli in normal individuals but, in immunocompromised domestic animals and humans, it can cause severe and often fatal pneumonia.[1,2] Pneumocystis pneumonia most commonly occurs in human patients with human immunodeficiency virus infection and in those with impaired immunity such as recipients of immunosuppressive drugs for organ transplantation, cancer, and autoimmune disease.[2] In dogs, pneumocystosis has occurred with suspected inherited immunodeficiencies,[3-6] secondary to distemper[7] and with administration of a tyrosine kinase inhibitor.[8] The exact mechanisms of humoral and cell-mediated immunity protective against pneumocystosis in humans and domestic animals still are being elucidated, but over a dozen genes encoding for proteins critical for immune responses have been identified in people.[9] Deficiency of 1 of these proteins, cluster of differentiation 40 ligand (CD40L), is linked to pneumocystosis in experimental murine models[10,11] and in humans,[12] but not in dogs. We describe a case of pneumocystis pneumonia in a young male Shih Tzu dog secondary to a nonsense mutation in *CD40L*.

2 | CASE DESCRIPTION

A 1-year-old male intact Shih Tzu was admitted for evaluation of tachypnea with increased respiratory effort and progressive weight loss of 4 months' duration. The dog was evaluated by several veterinarians over a 3-month period and treated for presumptive bacterial pneumonia using enrofloxacin, erythromycin, amoxicillin-clavulanate, and doxycycline at unknown doses and duration with no improvement in clinical signs. Thoracic radiographs performed 4 weeks before presentation disclosed spontaneous pneumothorax of unknown cause. Thoracocentesis initially improved respiratory rate and effort but respiratory effort worsened a few days after the procedure. Respiratory rate and effort worsened the week before presentation, along with associated progressive lethargy and inappetence. A CBC disclosed severe leukocytosis (white blood cell count 56.05×10^9/L; reference interval, 6-17 $\times 10^9$/L) characterized by a lymphocytosis (8.68×10^9/L; reference interval, 1-4.8 $\times 10^9$/L), monocytosis (2.4×10^9/L; reference interval, 0.2-1.5 $\times 10^9$/L), and mature neutrophilia (44.98×10^9/L; reference interval, 3-12 $\times 10^9$/L). The dog subsequently was referred to the University of Missouri Veterinary Health Center.

On presentation, the dog was quiet, alert, and responsive. The respiratory rate was 116 breaths per minute with increased inspiratory and expiratory effort. Increased bronchovesicular sounds were auscultated bilaterally. The dog was thin, with a body condition score of 3/9. The remainder of the physical examination was within normal limits.

Initial diagnostic testing included 3-view thoracic radiography, echocardiography to assess for pulmonary hypertension, and a CBC, serum biochemical profile and urinalysis before general anesthesia for advanced imaging and respiratory sample collection. Thoracic radiographs (Figure 1A-C) identified a diffuse unstructured interstitial pattern with hyperinflated lung lobes. Echocardiography supported an intermediate probability of pulmonary hypertension with peak tricuspid regurgitation velocity of 4.71 m/s[13] and no evidence of left-sided heart disease. The dog was treated with tadalafil USP (Letco Medical, Alabama) at a dosage of 2 mg/kg PO q24h for pulmonary hypertension to stabilize it for diagnostic testing the next day. It was transitioned to sildenafil (Trupharma, Florida) at a dosage of 2 mg/kg PO q8h after 1 week on the tadalafil because the dog experienced severe priapism. A CBC disclosed severe leukocytosis (42.8×10^3/μL; reference interval, 4.53-14.99 $\times 10^3$/μL) characterized by a mature neutrophilia (31.67×10^3/μL; reference interval, 2.27-10.14 $\times 10^3$/μL) lymphocytosis (4.71×10^3/μL; reference interval, 0.76-4.23 $\times 10^3$/μL), and monocytosis (5.99×10^3/μL; reference interval, 0.11-1.190 $\times 10^3$/μL), and a moderate thrombocytosis (584,000/μL; reference interval, 200,000-500,000/μL). Serum biochemistry identified a mild increase in alkaline phosphatase (270 U/L; reference interval, 12-98 U/L), and hyperphosphatemia (6.7 mg/dL; reference interval, 2.3-5.0 mg/dL) associated with growth, decreased serum creatinine concentration (0.6 mg/dL; reference interval, 0.7-1.4 mg/dL) associated with muscle atrophy, and mild hypocholesterolemia (101 mg/dL; reference, 131-320 mg/dL). No abnormalities were identified on urinalysis.

The next day, thoracic computed tomography (CT; 64-detector row Toshiba Aquilion, Toshiba America Medical Systems, Tustin, California) using ventilator-assisted inspiratory and expiratory breath holds (Engstrom Carestation ventilator, GE Healthcare) with single phase angiography was performed followed by tracheobronchoscopy. Ventilator settings included volume-controlled ventilation with inspired oxygen concentration of 40%, tidal volume 10 mL/kg, respiratory rate of 10 breaths/min, and positive end-expiratory pressure (PEEP) 5 cm H_2O. The PEEP was set to 0 cm H_2O for the expiratory breath hold. Thoracic CT identified diffuse ground glass opacity with mild mediastinal lymphadenomegaly (Figure 2). Endoscopic examination disclosed diffuse hyperemia and thickened, irregular mucosa with accumulation of serous to mucoid exudate in the subsegmental bronchi. Bronchoalveolar lavage (BAL) was performed by instilling 20 mL of warm, sterile saline into the cranial subsegment of the left cranial lung lobe, and the right caudal lung lobe. Fifteen milliliters of BAL was retrieved from each site and pooled for additional testing. The BAL had a total nucleated cell count of 1170/μL. Cytology identified an admixture of macrophages and neutrophils with a moderate number of 5 to 7 μm round, thin-walled structures within the background and within macrophages that contain up to 8 1-2 μm elongate deeply basophilic intracystic bodies consistent with *Pneumocystis* spp cysts. Additionally, 2 to 7 μm long elongated basophilic structures with small central nuclei were present in the background and consistent with *Pneumocystis* trophozoites. An aliquot of BAL was submitted for aerobic and anaerobic bacterial culture and a pan-fungal polymerase chain reaction (PCR; Veterinary Diagnostic Laboratory, Michigan State University, Lansing, Michigan). Pending results, the patient was started on high dose of sulfamethoxazole and trimethoprim oral suspension (Aurobindo Pharmaceuticals, India) at a dosage of 30 mg/kg PO q12h for 8 weeks, a tapering dose of prednisone PO (0.5 mg/kg/d for 20 days, 0.3 mg/kg/d for 3 days, 0.15 mg/kg/d for 3 days, and 0.15 mg/kg q48h for 3 days) to decrease inflammatory response associated with die off of the organisms, and clopidogrel bisulfate (Attix pharmaceuticals, Canada) at a dosage of 2 mg/kg PO q24h for 2 weeks to prevent thromboembolism. Bacterial cultures yielded no clinically relevant growth. The pan-fungal PCR amplicon was further submitted for nucleic acid sequencing. The derived nucleotide sequence was limited to 272 nucleotides that were an 85% match for *Pneumocystis* spp. Unfortunately, further characterization could not be performed because the eluted DNA was consumed in the PCR assay, and the original sample was discarded because of limited freezer space.

The patient was reevaluated 2 weeks after initiation of treatment and showed clinical improvement. An echocardiogram indicated substantial improvement of pulmonary hypertension with an approximated peak tricuspid regurgitation velocity of 3.04 m/s. Sildenafil was discontinued. Clinical signs gradually resolved with treatment over the next few weeks. The dog was returned for a reevaluation 8 weeks later at which time it was still receiving trimethoprim sulfonamide at a dosage of 30 mg/kg PO q12h. Thoracic radiography confirmed near complete resolution of prior lesions (Figure 1D-F). Bronchoalveolar lavage performed by instilling 20 mL of warmed saline blindly via an 8 French sterile red rubber catheter into the airways confirmed cytologic absence of pneumocystis organisms. Additionally at this visit, comprehensive immunodiagnostic testing was performed to investigate for immunodeficiency

syndromes including serum total IgM, IgG and IgA concentrations (Animal Health Diagnostic Center, Cornell University, Ithaca, New York), and flow cytometric assays (Comparative Internal Medicine Laboratory, University of Missouri, Columbia, Missouri) for lymphocyte phenotype (pan T-cell, pan B-cell, CD4 and CD8 markers), CD4+ intracellular cytokine (interleuken-17 and interferon gamma) production, lymphocyte proliferation, and evaluation of CD40 ligand. Controls included a healthy dog and a dog with systemic inflammation associated with sterile nodular panniculitis. A 2 mL sample of EDTA whole blood was shipped the same day in an insulated container with a cool pack to University of Minnesota Canine Genetics Laboratory (St. Paul, Minnesota), and genomic DNA was extracted using a commercial kit (Gentra Puregene Blood Kit, Qiagen Sciences, Germantown, Maryland) to investigate for genetic mutations contributing to immunodeficiency.

Results of serum antibody testing identified serum IgG concentration 670 mg/dL (reference interval, 670-1650 mg/dL), IgA concentration of 35 mg/dL (reference interval, 35-270 mg/dL), and IgM concentration of 189 mg/dL (reference interval, 100-400 mg/dL). Immunodiagnostic testing results (Figure 3A-H) were inconclusive for a specific defect and were not substantially different from results of control dogs. The CD40 ligand activity was assessed utilizing CD40 monoclonal antibody, clone H140a fluorescein isothiocyanate (FITC) purchased from Abnova (Taipei City, Taiwan). The CD40L assay failed to show expression of CD40L in any dog. Additionally, follow-up assays using human blood (data not shown) failed to detect CD40L expression, suggesting the antibody was flawed.

Genetic screening comprised whole genome sequencing (Illumina HiSeq 150 bp paired-end reads) with an average coverage of 18×.

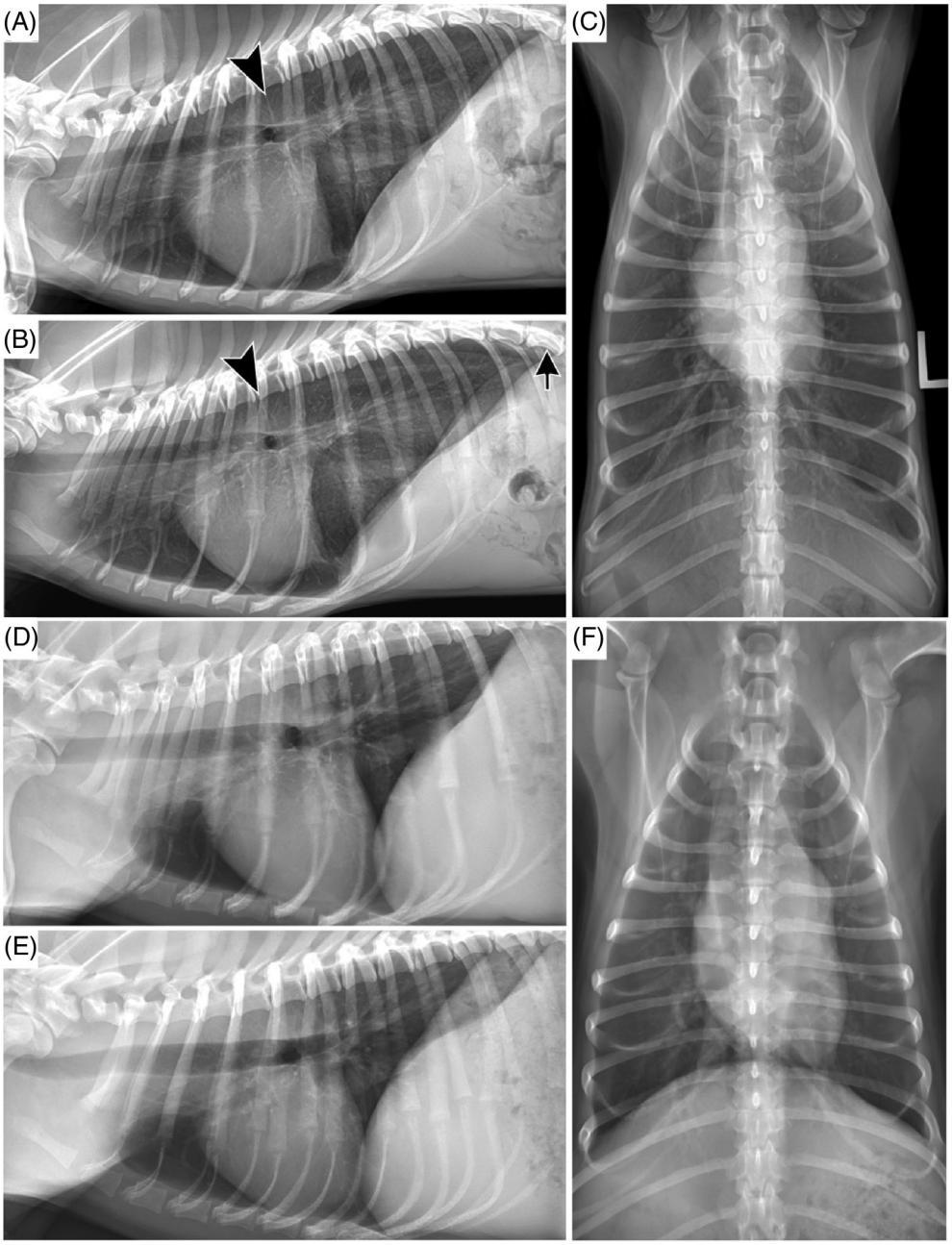

FIGURE 1 Two sets of thoracic radiographs taken 8 weeks apart in a 1-year-old Shih Tzu with pulmonary pneumocystosis. At presentation, right lateral (A), left lateral (B), and ventrodorsal (C) thoracic radiographs show a diffuse unstructured interstitial pattern, thickened pleural fissure lines (arrowheads) and hyperinflated lungs as depicted by the caudal extension of the lung field to the level of L1 (small arrow). Eight weeks later, the pulmonary pattern is near completely resolved on the repeated thoracic examination (D-F)

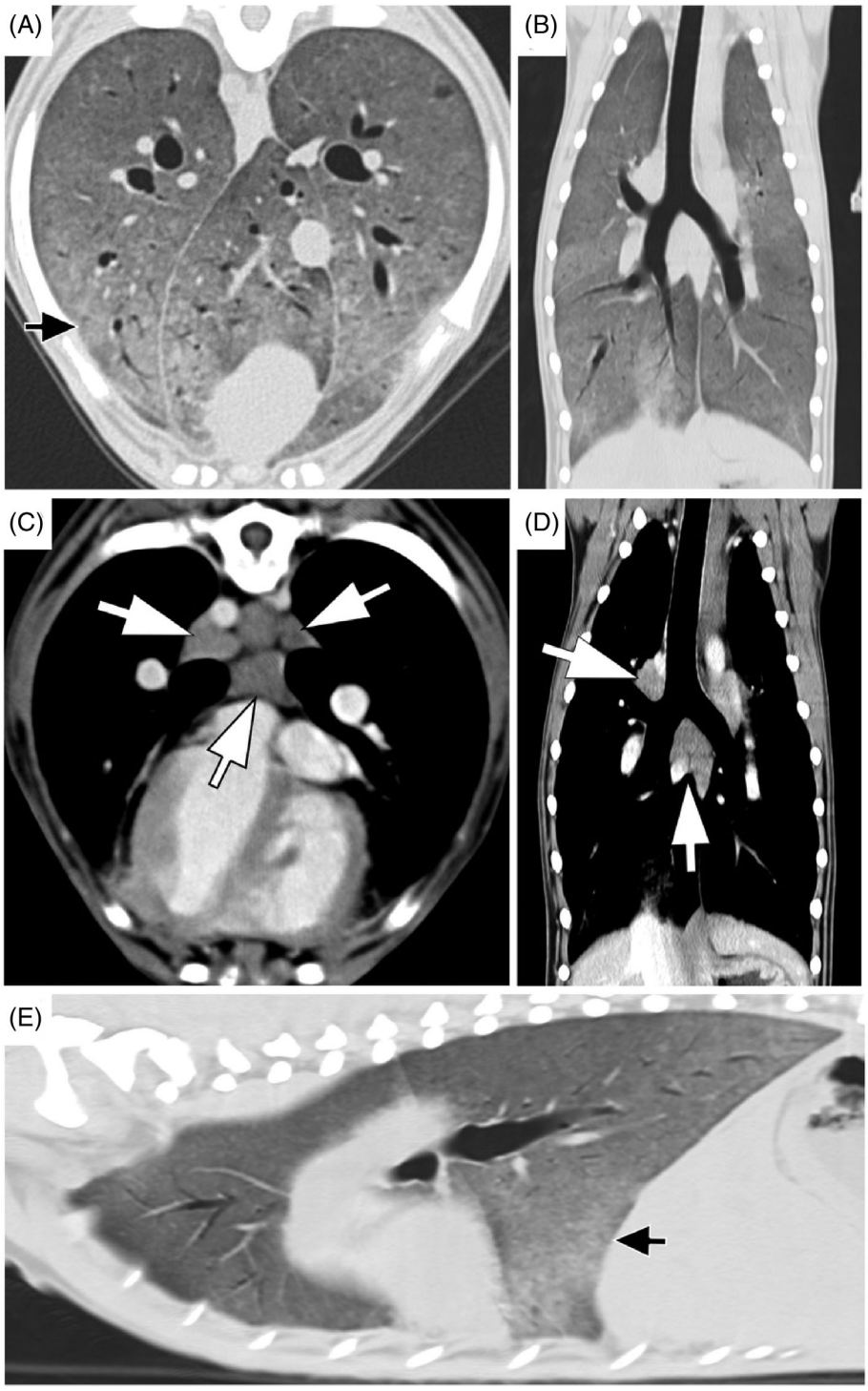

FIGURE 2 Computed tomographic images of a 1-year-old Shih Tzu with pulmonary pneumocystosis. Transverse (A), dorsal (B), and sagittal (E) images reconstructed with a high spatial frequency algorithm show diffuse ground glass opacification of the entire lungs. Overall, the increase in lung density was more severe in the dependent regions (black arrows). Tracheobronchial lymphadenomegaly is highlighted by white arrows on the post contrast transverse (C) and dorsal (D) images, reconstructed with a low (C) and high (D) spatial frequency algorithm

Quality control, mapping, and variant calling were performed using a previously described standardized pipeline, and the CanFam3.1 build of the dog reference genome.[15] Variants were annotated using SnpEff.[16] Loss of function (eg, frameshift, splice site, and premature stop codons) and missense variants predicted to be deleterious by sorting intolerant from tolerant (SIFT)[17] that were present in the patient (heterozygous or homozygous) were filtered against a database of whole genome sequences derived from 496 dogs of 54 breeds to identify those for which no other dog shared the patient's genotype. This internal database did not contain any other Shih Tzus, and the variants that passed internal filtering next were filtered against the variant catalogue of the Dog Biomedical Variant Database Consortium.[18] At the time of the study, this catalogue contained whole genome sequencing variant calls from 813 dogs of 143 breeds, including the Shih Tzu (3 males and 1 female), and wolves. This filtered process resulted in 26 variants (4 homozygous and 22 heterozygous) in which the genotype was unique to the patient (Supplementary File 1). Only 1 variant resided in a gene known to play

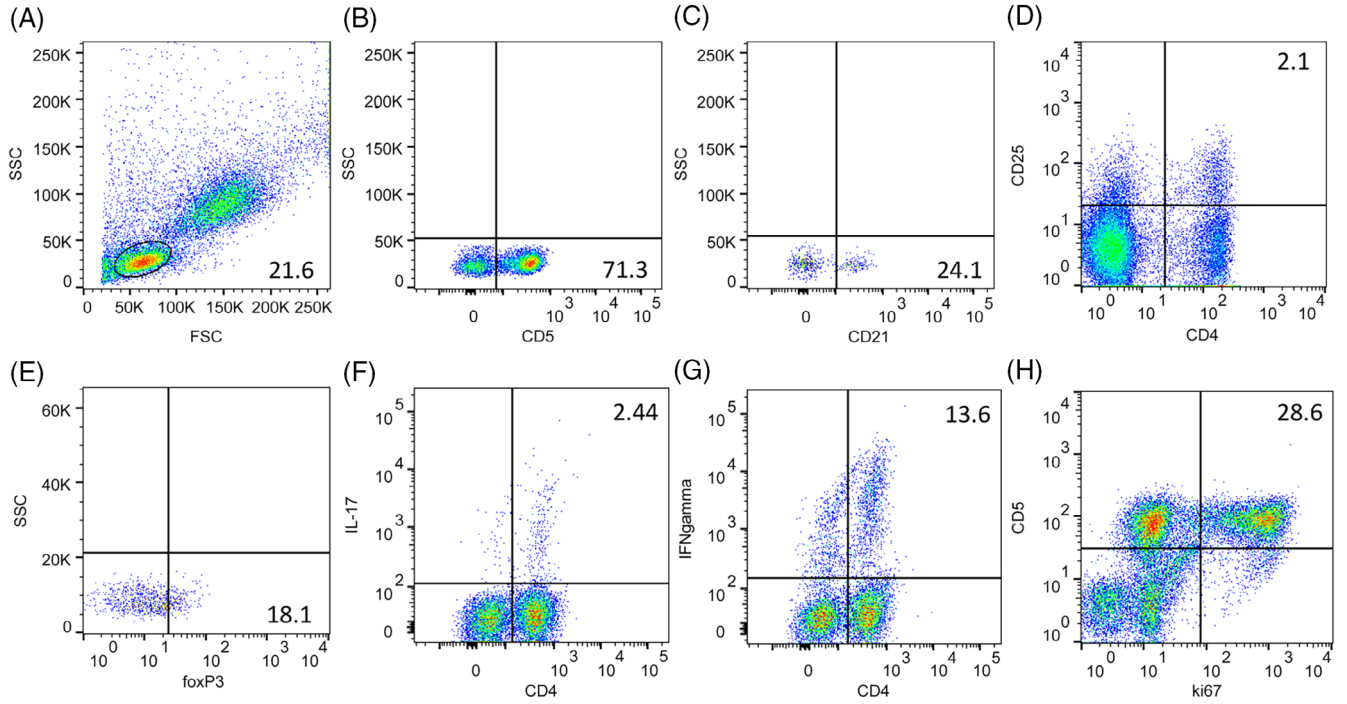

FIGURE 3 A-H, Flow cytometric determination of immune phenotype and function in a 1-year-old Shih Tzu with pulmonary pneumocystosis. Results were similar for a healthy dog and a dog with inflammatory disease (data not shown). The percentage of the lymphocyte population of interest is shown in the respective quadrant of the graphs. A-E, Lymphocyte phenotype was determined by (A) gating on lymphocytes on a FSC versus SSC plot and determining reactivity to specific antibodies including (B) CD5, (C) CD21, (D) CD4+CD25, and (E) (CD4+CD25+) Foxp3+. The percentage of CD4+CD5+ lymphocytes and CD8+CD5+ lymphocytes was 34.8% and 25.8%, respectively (data not shown). T regulatory cells were identified either as CD4+CD25+ lymphocytes (D) or as (CD4+CD25+) Foxp3+ cells (E). The following antibodies were used: CD5, clone YKIX322.3 for pan T cell; CD21, clone CA2.1D6 for pan B cell; CD4-FITC, clone YKIX302.9; CD8-APC, clone YCATE55.9; CD25, clone P4A10; and Foxp3, clone FJK-16S. F-G, Intracellular cytokine production was determined by stimulating whole blood with PMA (0.081 μM) and ionomycin (1.34 μM) in the presence of the protein transport inhibitors brefeldin A (10.6 μM) and monensin (2 μM) and assessing intracellular (F) IL-17 and (G) IFNγ. Antibody clones used were: IL-17, clone eBio17B7; and IFNγ, clone CC302. Lymphocyte proliferation assays were performed as previously described[14] by incubating whole blood in the presence of lipopolysaccharide (0.07 μg/mL) and concanavalin A (0.1 μg/mL) for 4 days. H, Following red blood cell lysis, nuclear antigen ki67 was detected in CD5+ cells as a surrogate marker for cell proliferation, using the canine-reactive ki67 clone SolA15

a role in immunity against *Pneumocystis*, a hemizygous nonsense mutation in *CD40L* (chrX:107018958C>T; ENSCAFP00000027967.3: p.Arg120Ter). The variant was not detected in any other dog in either the internal or consortium database.

To determine the prevalence of this variant in the Shih Tzu breed, we genotyped 64 Shih Tzus (37 males and 27 females) with genomic DNA samples banked at the University of Minnesota Canine Genetics Lab Standard PCR was performed to amplify a 504 bp product that spanned the variant using forward primer 5′ CGTCAATCCGGTAAA GAGGA and reverse primer 5′ TTGTCCCTTCAAGTCCCATC. Conditions consisted of an initial denaturation at 94°C for 15 minutes, 35 cycles with denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final elongation at 72°C for 10 minutes on a Bio-Rad T100 thermal cycler. Genotyping was performed by Sanger sequencing of the PCR products. All dogs tested clear of the variant (homozygous for the reference allele). The 95% confidence interval (binomial distribution) for the variant allele frequency in Shih Tzus (96 alleles from 40 males and 28 females) was 0% to 4%.

Two years after diagnosis of pneumocystis pneumonia, a phone conversation determined that the patient was clinically asymptomatic for respiratory disease.

3 | DISCUSSION

Because pneumocystis can inhabit alveoli without causing pneumonia in immunocompetent individuals, development of pneumocystosis warrants investigation of an underlying cause. Pneumocystosis in the dog of our report prompted investigation of immune defects, culminating in identification of a novel nonsense mutation in *CD40L*. This variant meets the highest level of evidence criteria for pathogenicity, according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines.[19] Namely, it is a loss of function mutation in a for which loss of function is a known mechanism of disease in humans, CD40L null mice have impaired immunity against Pneumocystis, and the variant is absent in a large canine population database. Serving as a costimulatory molecule on

activated CD4+ T cells to facilitate both T cell and B cell adaptive immune responses, CD40L is critical to effectively clear pneumocystis organisms.[10,20] Mutations in *CD40L* in humans (OMIM #300386) are the most common cause of a rare primary immunodeficiency disorder called hyper-IgM syndrome.[20,21] In normal individuals, when CD40L binds CD40 on B cells, B cells produce antibodies starting with IgM, and after class switching, IgG, IgA, and IgE. In patients with CD40L mutations, IgM concentrations are normal to increased, but concentrations of other antibody classes are typically low or absent.[20] In humans, *CD40L* mutations primarily are manifested by recurrent opportunistic infections, but autoimmune disease and neoplasia also may develop.[21] Mutations that result in a premature stop codon occur in 24% of cases with CD40L deficiency and result in a medically severe phenotype (eg, early-onset, frequent, and complicated infections). Long-term outcomes are poor with a 20-year survival rate of 35%.[21] The dog in our study had a nonsense mutation predicted to result in a severe CD40L deficiency. Although it was still alive and doing well at 2 years after diagnosis, the dog is presumed to be at high risk for future infections. The mutation was not detected in other dogs screened, including 68 other Shih Tzus. This finding suggests it is either present at a low allele frequency (<5%) in the breed or is a de novo mutation in the patient.

Although this dog represents the first case of a documented *CD40L* mutation leading to pneumocystosis, CD40L deficiency might have been present in other cases. Defective CD40L impairs T-cell costimulation and leads to a combined cellular (T-cell) and humoral (B-cell) immunodeficiency. This combined immunodeficiency previously has been described in Miniature Dachshunds with pneumocystis pneumonia.[4] It was termed common variable immunodeficiency, but the dogs were not genetically characterized to confirm and refine this diagnosis. Identification of primary immunodeficiencies has been elusive and often incomplete in veterinary medicine. In large part, this is because of limited availability of commercially available immunologic assays in dogs, few research laboratories capable of sophisticated immunologic characterization, requirement for technical expertise, high costs to develop and validate assays, and far fewer canine-specific immunologic reagents as compared with those available for humans and mice. In the dog of our study, after diagnosis of pneumocystosis, immune characterization included commercial quantitation of IgM, IgG, and IgA as well as a number of flow cytometric assays intended to phenotype lymphocytes, investigate deficient cytokine production (IFNγ and IL-17), evaluate the proliferative response of B- and T-cells in response to mitogens and assess cross-reactivity of an anti-CD40L antibody thought to be cross-reactive with canine CD40L. Serum IgM concentration was within the reference range, whereas both IgG and IgA concentrations were at the lower limit of detection of the assay. In a dog with overwhelming and chronic (suspected 3-4 months) pneumocystosis infection, these results are strikingly abnormal because an immunocompetent host would have had a class switch to produce high concentrations of IgG and IgA. No overt differences were noted in T- and B-cell phenotypes or in production of IFNγ and IL-17 compared with control dogs, thus failing to identify another specific immunodeficiency. Control dogs were used because reference ranges for these immunologic assays are not established.

The *CD40L* mutation identified in the dog reported here introduces a premature stop codon early in the gene. The most common consequence of a premature stop codon is lack of production of the protein because of nonsense-mediated decay of mRNA.[22] However, an alternative consequence of a premature stop codon is production of a truncated protein. This outcome has been reported in human patients with hyper-IgM syndrome where CD40L expression appeared normal in immunoassays because the truncated protein could not be distinguished from the normal protein using flow cytometry.[23,24] Diagnosis in those cases required genetic analysis which revealed *CD40L* mutations resulting in a premature stop codon. A limitation of our study is that we were unable to perform the CD40L immunoassays because the anti-human CD40L antibody did not cross-react with canine CD40L. Further testing by our laboratory suggested that the antibody was faulty, because it did not recognize human CD40L in subsequent assays. Genetic analysis confirmed a defect in *CD40L*. However, we cannot definitively conclude whether the dog's variant results in absence of CD40L versus production of a truncated protein. Challenges in identification of decreased CD40L protein underscore the need for genetic testing for definitive confirmation.[23,24]

In conclusion, a nonsense mutation in *CD40L* was identified in a young male Shih Tzu with pneumocystis pneumonia. Although currently no assay is available to assess the expression of CD40L protein in the dog, genetic testing is considered the gold standard for diagnosis of hereditary immunodeficiencies of this type.[25] Thus, when clinical suspicion is high, genetic investigation for a loss of function *CD40L* mutation is recommended and considered definitive for diagnosis.

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CONFLICT OF INTEREST DECLARATION

Eva Furrow is a member of the University of Minnesota Canine Genetics Laboratory. This laboratory offers genetic testing services, and profits from testing go towards research in the laboratory.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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