

ABSTRACT: Inflammatory myopathies (IMs) occur relatively frequently in dogs, and, with the exception of masticatory muscle myositis (MMM), have not been characterized. This study analyzed the distribution and types of cellular infiltrates in 21 cases of generalized IM, 3 cases of focal IM (MMM), and 1 case with features of both generalized and focal IM, using a panel of monoclonal antibodies to cell surface markers. In generalized IM, mononuclear cells showed an endomysial and perimysial distribution with invasion of non-necrotic fibers similar to human IM. T lymphocytes with T-cell receptor (TCR) $\alpha\beta$ predominated. Distinct differences were seen in MMM including prominent B-cell infiltration, dendritic cells and macrophages in greater numbers than T cells, and numerous T cells with TCR $\gamma\delta$. Thus, generalized IM and MMM appear to be distinct diseases with different mechanisms. Canine generalized IM may be an important animal model for human IM.

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CANINE INFLAMMATORY MYOPATHY: ANALYSIS OF CELLULAR INFILTRATES

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Inflammatory myopathies (IMs) refer to a heterogeneous group of disorders characterized by infiltration of inflammatory cells into muscle.¹⁴ Etiological classifications include immune-mediated and infectious types.³⁰ The main types of immune-mediated IM are polymyositis (PM), in which inflammation is limited to muscle, dermatomyositis (DM) with extramuscular manifestations, and inclusion-body myositis with distinct pathological features. In addition to the clinical presentation, the distribution of infiltrating cells, their phenotypes, and the autoaggressive behavior of the mononuclear inflammatory cells differentiate these disorders.¹⁴ In PM, inflammatory cells, including CD8⁺ T cells and macrophages, are concentrated in the endomysium and surround and

invade non-necrotic fibers. In DM, the inflammatory cells are concentrated in the perimysium, where they are either perivascular or are scattered diffusely; B cells and CD4⁺ cells predominate.

Inflammatory cells have not been analyzed previously in canine IM. Canine IMs may be classified as focal or generalized based on the clinical presentation and muscle biopsy specimens. A unique form of focal inflammatory myopathy, masticatory muscle myositis (MMM), is relatively well characterized.^{15,37,43,45} Masticatory muscle type 2M fibers have been shown to differ both histochemically³⁷ and biochemically⁴² from muscle fibers found in the limbs. These differences may account in part for the selective clinical involvement of masticatory muscles and the sparing of limb muscles in MMM. Another focal IM, extraocular myositis, has also been described.⁷

Scattered case reports document the occurrence in dogs of generalized IM associated with various infectious agents including *Toxoplasma gondii*,^{5,17} *Neospora caninum*,¹² *Ehrlichia canis*,⁶ and *Hepatozoon canis*.²⁸ However, idiopathic polymyositis^{27,39,44} and breed-associated dermatomyositis¹⁸ have been documented less well, and the distribution and types of

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Abbreviations: CK, creatine kinase; CLAW, canine leukocyte antigen workshop; DM, dermatomyositis; H&E, hematoxylin and eosin; IM, inflammatory myopathy; MHC, major histocompatibility complex; MMM, masticatory muscle myositis; PM, polymyositis; TBS, Tris-buffered saline; TCR, T-cell receptor
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cellular infiltrates in these canine disorders are not known. With the availability of reagents to study cell surface leukocyte antigens in the dog,^{1,8,32-36} infiltrating mononuclear cells can now be characterized. This report describes the distribution and types of infiltrating cells in 25 cases of canine IM using a panel of monoclonal antibodies against 22 cell surface antigens of canine leukocytes.

METHODS

Clinical Cases. Fresh frozen limb (biceps femoris, vastus lateralis, or triceps brachii) and masticatory muscle (temporalis or masseter) biopsy specimens ($n = 38$) from 25 dogs with histologically confirmed inflammatory myopathy and 2 control dogs without cellular infiltrations or other pathological abnormalities were selected from the tissue archives of the Comparative Neuromuscular Laboratory at the University of California, San Diego. A diagnosis of a focal IM was made if clinical signs were restricted to a single muscle group, the masticatory muscles in MMM. If clinical signs involved limb muscles as well as other muscle groups, a diagnosis of generalized IM was made. Data were analyzed as to age, breed, gender, serum creatine kinase (CK) concentration, and diagnosis.

Characterization of Cellular Infiltrations. Using routine hematoxylin and eosin (H&E) staining on fresh frozen muscle biopsy sections (8 μ m) from confirmed cases of IM, distributions of cellular infiltrates were classified according to Engel et al.¹³ as follows:

1. Perimysial: diffusely scattered cells in the perimysium.
2. Perivascular: a compact collection of cells around a perimysial or endomysial blood vessel.
3. Endomysial, focal: a compact collection of cells that focally surrounds and may invade non-necrotic muscle fibers.
4. Endomysial, diffuse: infiltrating cells scattered diffusely or distributed in small groups.

Cell accumulations were subjectively graded as follows: 0, no positive cells; +, small numbers (<5) of cells; ++, moderate numbers (5-10) of cells; and +++, large numbers (>10) of cells. All interpretations were performed by the same investigator throughout the study to minimize interpersonal variations. Other pathological changes evaluated included the presence of central nuclei, and endomysial and perimysial fibrosis.

Immunocytochemical Procedures. Serial 8- μ m cryostat sections of unfixed muscle were cut in the transverse plane and picked up on commercial adhesive-coated slides. The sections were dried for 30 minutes at room temperature and were then incubated with a panel of mouse monoclonal antibodies against canine leukocyte antigens (T cells, B cells, dendritic cells, macrophages, and major histocompatibility complex [MHC] class II).^{1,8,32-36} Control sections were treated with a feline-specific monoclonal antibody.

Monoclonal antibodies, their specificities, and their immunoglobulin subclasses are shown in Table 1. Many of the monoclonal antibodies specific for canine leukocyte antigens were developed and characterized prior to the First International Canine Leukocyte Antigen Workshop (CLAW)^{8-11,34,35}; these antibodies were also clustered at the CLAW. More recently developed monoclonal antibodies specific for canine CD1a and CD1c^{32,36} have been characterized by cell and tissue distribution, and by immunoprecipitation. Monoclonal antibodies specific for canine T-cell receptor TCR/CD3 complex^{32,33,35} were developed by immunization of mice with affinity-enriched TCR/CD3 complexes. These antibodies were characterized by tissue distribution, immunoprecipitation, and reactivity with cloned recombinant canine CD3 ϵ and TCR β . Monoclonal antibodies specific for canine CD80 and CD86 were developed by immunization with a canine T-cell line (CLGL-90) and screening with mouse L-cells transfected with canine CD80 and CD86.^{47,48} Cross-reactivity of canine-specific monoclonal antibodies with equivalent human leukocyte antigens has been limited to human CD11b and CD18. In the feline, cross-reactivity of canine monoclonal antibodies has been observed for feline CD11b, CD11d, CD18, and CD21. The monoclonal antibody clone CD3-12, specific for a conserved cytoplasmic epitope of CD3 ϵ ,^{26,29} is reactive in all species tested to date (canine, feline, equine, bovine, ovine, porcine, and human).

Avidin-biotin immunoperoxidase assay was performed as previously described.¹ Briefly, all primary antibodies were diluted 1:10 in 20 mM Tris-buffered saline (TBS) at pH 7.6. All washes and reagent dilutions with the exception of the final development step were performed in TBS. Sections were blocked with 10% horse serum for 20 minutes, treated with primary antibody (1 hour at room temperature), and incubated with biotinylated horse anti-mouse immunoglobulin G (1:400; Vector Laboratories, Burlingame, CA) containing 1% (v/v) normal dog serum at room temperature for 30 minutes and

Table 1. Monoclonal antibodies used for leukocyte classification.

Canine antigen	Clone	Isotype	Leukocyte expression
CD1c	CA13.9H11	IgG1	DC, B(A), B(PB)
CD3	CA17.2A12	IgG1	T, T(NK1)
CD3 ϵ	CD3.12		T, T(NK1)*
CD4	CA13.1E4	IgG1	MHC class II-restricted cells, T, tissue macrophages, granulocytes
CD8 α	CA9.JD3	IgG2a	MHC class I-restricted cells, T(C)
CD8 β	CA15.4G2	IgG1	MHC class I-restricted cells, T(C)
CD11a	CAQ11.1B11	IgG1	All leukocytes
CD11b	CA16.3E10	IgG1	Granulocytes, monocytes, NK cells, subsets of T and B cells, most macrophages
CD11c	CA11.6A1	IgG1	Monocytes, DC, pulmonary macrophages, NK, granulocytes, subsets of T and B cells
α D (CD11d)	CA11.8H2	IgG1	Macrophages (splenic red pulp, medullary cord in LN, BM macrophages, some dendritic cell populations)
CD18	CA1.4E9	IgG1	All blood leukocytes
CD18	CA16.3C10	IgG1	All blood leukocytes
CD21	CA2.1D6	IgG1	B, follicular DC
CD45	CA12.10C12	IgG1	Leukocyte common antigen
CD45RA	CA4.1D3	IgG1	Restricted isoform of CD45 (naive CD4 T cells, resting activated CD4 T cells, CD8 T cells, B cells, mast cells, NK cells)
CD45RA	CA21.4B3	IgG1	As above
CD80	CA24.5D4	IgG1	DC(R), macrophages, T(A), B(A)
CD86	CA24.3E4	IgG1	DC(R), macrophages, T(A), B(A)
Thy-1	CA1.4G8	IgG1	Connective tissue, fibroblasts, stromal cell lines; peripheral T cells, subpopulations of DC, eosinophils, monocytes
MHC II	CA2.1C12	IgG1	MHC class II-restricted cells, T(R), T(A)
TCR $\alpha\beta$	CA15.8G7	IgG1	T-cell receptor composed of α and β
TCR $\gamma\delta$	CA20.8H1	IgG2a	T-cell receptor composed of γ and δ
Feline CD4	FE1.7B12	IgG1	Control, no canine specificity

B, B lymphocytes; B(A), activated B-lymphocyte subpopulation; B(PB), peripheral blood B cells; DC, dendritic cells; DC(R), resting dendritic cells; MHC, major histocompatibility complex; T, T lymphocytes; T(A), activated T lymphocyte; T(R), resting T lymphocytes; T(C), MHC class 1-restricted cells; cytotoxic T lymphocytes; T(NK1), NK1 T lymphocytes.

*Recognizes a conserved peptide sequence in the cytoplasmic domain of CD3 ϵ .

streptavidin–peroxidase reagent (1:400; Zymed, South San Francisco, CA) for 20 minutes. Subsequently the sections were incubated with 0.01% hydrogen peroxide with 0.05% diaminobenzidine in 100 mM TBS (pH 7.6) for 30 minutes. The sections were then rinsed, dehydrated, and mounted with Permount (Fisher Scientific, Fairlawn, NJ).

Characterization of Cellular Infiltrates. Reactions were considered positive for a surface antigen if a rim of reaction product was observed around the cell.² Similar to the procedure used for definition and grading of cellular infiltrates, mononuclear cell subsets were defined as perimysial, perivascular, or endomysial (focal or diffuse).

Serological Assays. Serum samples (from the 25 dogs with IM and 2 control dogs) were evaluated for the presence of circulating antibodies against masticatory muscle type 2M fibers^{43–45} or sarcolemmal proteins.^{27,44} The 2M antibody assay has previously been established as a reliable immunocytochemical test for the diagnosis of MMM in dogs and the methodology

previously described.⁴⁵ Using the same methodology, staining for sarcolemmal antibodies was also evaluated.

RESULTS

Clinical Cases. A diagnosis of generalized IM was made in 21 dogs with clinical signs of generalized weakness, stilted gait, generalized muscle atrophy, and in some cases regurgitation or dysphagia. Muscle biopsies had inflammatory cells in all cases confirming the diagnosis. Although several breeds were affected without a gender predilection, 6 of the 21 dogs with generalized IM were Boxers. Age of onset ranged from less than 1 year to 9 years. Serum CK concentrations ranged from 172 to 10,872 IU/L (normal <200 IU/L). Two of the 21 dogs initially diagnosed with IM were diagnosed with lymphoma with infiltration into muscle on repeat muscle biopsy several months following the diagnosis of IM. One of the 21 dogs was distinct as it had autoantibodies against the sarcolemma by direct and indirect immunostaining (not shown). Three of 21 dogs had significantly positive serum antibody titers against *Neos-*

pora caninum and their IM was therefore associated with an infectious disease. A diagnosis of MMM was made in 3 dogs with clinical signs restricted to the muscles of mastication including muscle atrophy or swelling, inability to open the jaw, or restricted jaw mobility. Biopsies from a masticatory muscle were inflammatory in all cases and circulating antibodies against masticatory muscle type 2M fibers were detected in all three dogs. An overlap syndrome with generalized clinical signs, cellular infiltrates into both masticatory and limb muscles, and circulating and fixed antibodies against masticatory muscle type 2M fibers was identified in one dog.

Histopathology. Pathological changes in muscle from all the IM groups and subclasses were similar, although they varied in severity. These included multifocal, mixed populations of mononuclear inflammatory cells of variable degree and with endomysial (focal and diffuse) and perimysial distributions (Fig. 1A,B), and invasion of non-necrotic fibers (Fig. 1C). A perivascular pattern of cellular infiltration (Fig. 1D,E) was evident in the perimysium. In the endomysium, where cellular infiltration was marked, it was more difficult to discern whether the infiltration accumulated around blood vessels. Perivascular infiltration was most prominent in MMM and less so in other IMs. Various stages of myonecrosis were common. Endomysial fibrosis was prominent in limb muscles of chronic IM (Fig. 1B) and in masticatory muscles of MMM. Perimysial fibrosis was most prominent in MMM (Fig. 1F). Muscle fibers with central nuclei were commonly found in limb muscles at all stages of generalized IM, but were infrequent in masticatory muscles in MMM. Cellular infiltrates were not observed in muscles from either of the two control dogs.

Immunohistochemistry. Lineages of inflammatory cells were identified in the biopsies based on combinations of antibody against cell-surface proteins. All leukocytes were identified by an antibody against CD45 (leukocyte common antigen). T cells were identified by antibodies against CD3, CD4, CD8, TCR $\alpha\beta$, and TCR $\gamma\delta$. B cells were identified with anti-CD21. Dendritic cells were identified with antibodies against CD1c, CD11c, MHC II, CD80, and CD86. Macrophages were highlighted by staining with CD11b, α D (CD11d), and CD11c. Eosinophils were identified by staining with antibodies against CD11a,b,c and Thy-1.

Normal Canine Limb and Masticatory Muscle. Leukocytes were present in normal canine skeletal muscle only in very small numbers (not shown). T

cells were difficult to find in either normal limb or normal masticatory muscles. Dendritic cells and macrophages were relatively more abundant but still rare in both muscles and were found mostly in the perimysium and endomysium. Dendritic cells and endothelial cells were positive for MHC II. Eosinophils were present in low numbers and with distributions similar to T cells. T cells and eosinophils were present in the perimysium in limb muscle but in the endomysium in masticatory muscle. B cells were not found in any muscle.

Generalized Inflammatory Myopathy. Identification of inflammatory cells in generalized canine IM is shown in Figure 2. Dendritic cells and macrophages were the predominant cell populations having an endomysial, perimysial, and in some cases perivascular distribution. T cells were also present in similar distributions. CD8⁺ T cells were present in greater numbers than CD4⁺ cells and had predominantly T-cell receptor (TCR) $\alpha\beta$ usage with only few TCR $\gamma\delta$ ⁺ cells in a small number of cases. An exception was found in one dog where TCR $\gamma\delta$ ⁺ cells composed 60–80% of the T-cell population. B cells were not identified in any of the muscle specimens. Vascular adventitia, endothelial cells, and cellular infiltrates within the perimysium and endomysium stained intensely for MHC II.

An important difference was observed between generalized IM and IM associated with an infectious disease. In cases associated with positive serum antibody titers against *Neospora caninum*, there was a marked increase in the dendritic cell population compared to generalized IMs of noninfectious origin. T cells were also more abundant than in IM alone. Populations of macrophages and other leukocytes were similar to those of other IM biopsies. No apparent differences were identified between muscle biopsies from dogs with generalized IM and IM associated with neoplasia.

Focal IM (Masticatory Muscle Myositis). Important differences were identified in the proportion of different cell types between dogs with MMM compared to generalized IM (Fig. 3). Although T lymphocytes were present in similar numbers and distributions in MMM and generalized IMs, CD4⁺ T lymphocytes were present in greater numbers than CD8⁺ lymphocytes in MMM. Although $\alpha\beta$ T cells were most frequent, numerous $\gamma\delta$ T cells were present in all cases of MMM. In contrast to generalized IMs, B lymphocytes were present in biopsies from all cases of MMM and in the masticatory muscle of the overlap case. The large numbers of macrophages and dendritic cells, smaller numbers of other

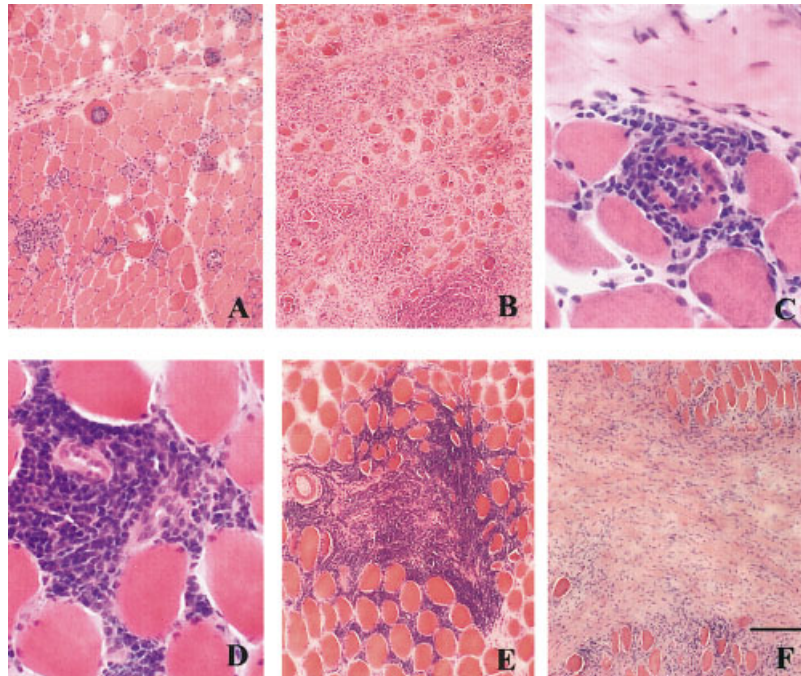


FIGURE 1. Fresh frozen muscle biopsy sections from a dog with generalized inflammatory myopathy (IM) (A)–(C) and a dog with masticatory muscle myositis (MMM) (D)–(F). Mononuclear cell infiltrates had an endomysial and perimysial distribution (A, B) in generalized IM with invasion of non-necrotic fibers (C). Perivascular distributions of inflammatory cells (D, E) and perimysial fibrosis (F) were common in MMM [H&E stain; bar = 100 μ m for (A), (B), (E), and (F), and 50 μ m for (C) and (D)].

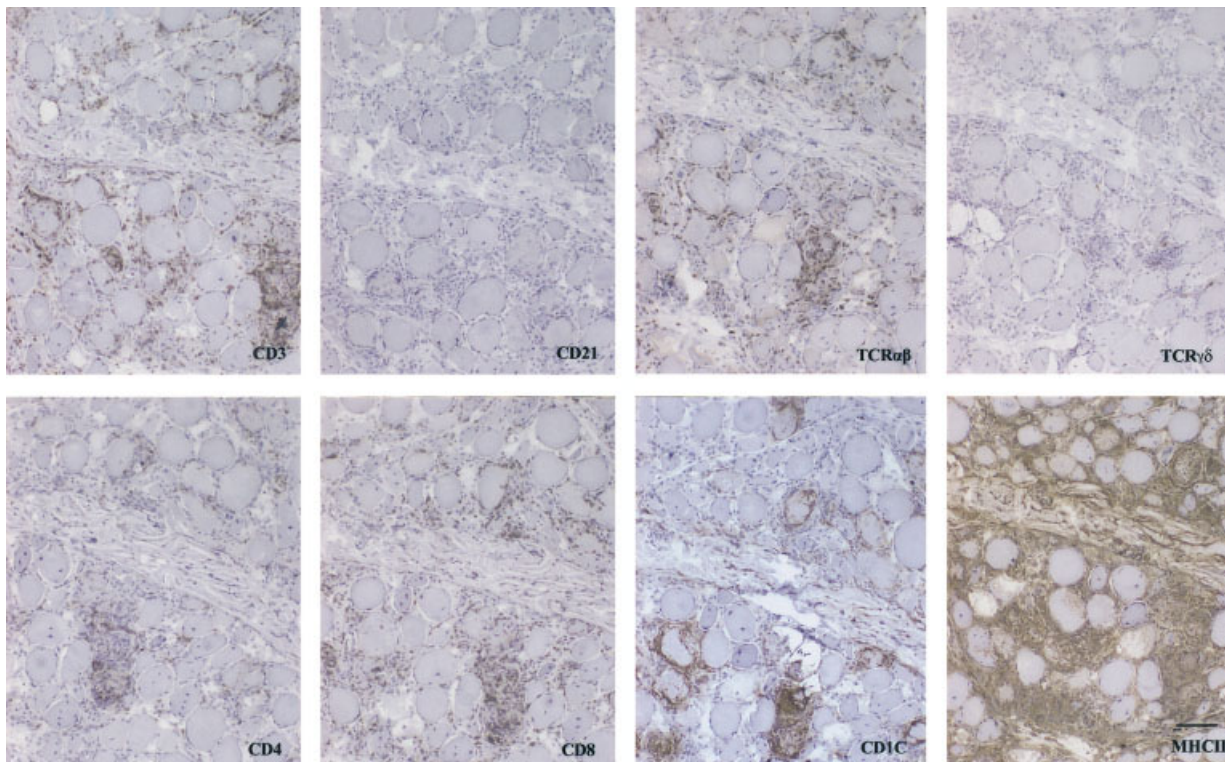


FIGURE 2. Fresh frozen limb muscle biopsy sections from a dog with generalized IM. T lymphocytes (CD3) were present in the cellular infiltrates with CD8⁺ T cells in greater numbers than CD4⁺ T cells, and predominantly TCR $\alpha\beta$ usage. Rare T cells used TCR $\gamma\delta$. B cells (CD21) were not detected. Dendritic cells (CD1c) were present in greater numbers than T cells. Intense staining for MHC II was present in the vascular adventitia, endothelial cells, and cellular infiltrates within the endomysium and perimysium (avidin–biotin immunoperoxidase stain; bar = 100 μ m for all panels).

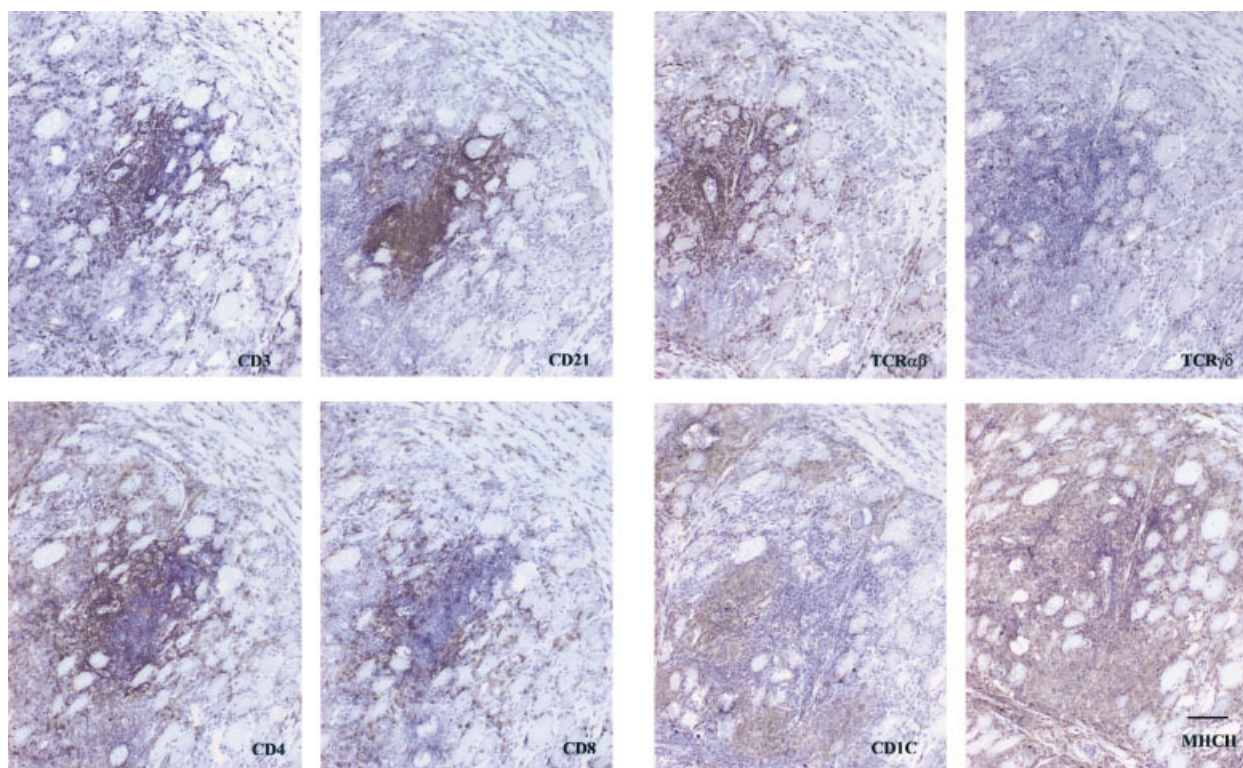


FIGURE 3. Significant differences were present in fresh frozen muscle biopsy specimens in dogs with MMM compared to generalized IM. Infiltrations of T cells (CD3) were composed of greater numbers of CD4⁺ cells than CD8⁺ cells. Although TCRαβ predominated, several T cells were positive for TCRγδ in all cases. Staining for B cells (CD21) was striking in all cases. Similar to generalized IM, large numbers of dendritic cells (CD1c) and intense staining for MHC II were present (avidin–biotin immunoperoxidase stain; bar = 100 μm for all panels).

leukocytes, and intense staining for MHC II were similar in MMM and generalized IM.

Serological Assays. All dogs with a presumptive diagnosis of MMM were positive for circulating antibodies against masticatory muscle type 2M fibers. With the exception of one dog with an overlap syndrome of MMM/IM, such antibodies were not detected in any of the dogs with generalized IM. Muscle from one dog with generalized IM showed labeling of the sarcolemma or basal lamina in the absence of primary antibody, indicating endogenous immunoglobulin at this location. In this dog, CD8⁺ T cells were present in greater numbers than CD4⁺ T cells. B cells were not detected. Macrophages, dendritic cells, and other leukocyte populations were present in numbers similar to generalized IM.

DISCUSSION

As previously shown for human IMs,^{2,13} canine generalized IMs are characterized by mononuclear cell infiltrates having an endomyisial, perimysial, and perivascular distribution with invasion of non-necrotic and necrotic muscle fibers. Dendritic cells

and macrophages are the predominant cell populations, which could be indicative of a chronic condition. Similar to human polymyositis, CD8⁺ T cells were present in greater numbers than CD4⁺ cells with invasion of non-necrotic fibers. Although it was not possible to differentiate IMs associated with neoplasia from the general population of IM dogs by analysis of infiltrating cells, it was possible to differentiate IMs having an infectious etiology, as in those cases dendritic cells were markedly increased over T cells. In peripheral tissue such as muscle, dendritic cells identify and ingest pathogens through receptors that recognize features common to microbial surfaces. An infection triggers migration of dendritic cells to regional lymph nodes where they lose phagocytic function and act as potent antigen-presenting cells. Dendritic cells are particularly effective at inducing immune responses in naive T cells due to the abundant MHC II/peptide complexes on their surface and the presence of costimulatory molecules (CD80/CD86) required for naive T-cell activation.

Autoantibodies against an unidentified sarcolemmal or basal lamina antigen may differentiate true immune-mediated PM cases and represent the equiv-

alent of the human PM group. In the one dog with the sarcolemmal or basal lamina staining pattern, CD8⁺ T cells predominated. The sarcolemmal staining pattern has been previously reported in canine inflammatory myopathies^{27,44} and warrants further investigation.

Histological and phenotypic analysis of masticatory muscle biopsies from dogs with MMM showed a pattern of infiltrating cells that differed significantly from that of dogs or humans with IM. Although the clinical phenotypes are different, MMM had some histological and immunophenotypic similarities to human DM.¹⁴ Similar to human DM, in MMM perivascular collections of inflammatory cells were prominent in the perimysial connective tissue compared to the predominantly endomysial distribution found in IM. Furthermore, masticatory muscles from the 3 dogs with MMM and the 1 dog with an overlap syndrome contained multifocal clusters of B lymphocytes, also identified in human DM. This finding is of interest because MMM is associated with the production of autoantibodies specifically against masticatory muscle type 2M fibers. The pathological significance of these autoantibodies is not yet known; however, they have proven to be a reliable diagnostic marker for the disease.^{39,44,45} Finally, in MMM, CD4⁺ T cells were present in greater numbers than CD8⁺ cells, also typical of human DM and different from IM.

Another important difference between MMM and generalized IM is the presence of numerous TCR $\gamma\delta$ ⁺ cells. The majority of CD8⁺ T cells in human cases of IM have the most common T-cell receptor, the $\alpha\beta$ T-cell receptor.^{20,40} The relatively uncommon $\gamma\delta$ T-cell receptor has been reported in one human case of PM.²² Similar to the dogs with MMM, inflammation in the human patient was very responsive to steroids.

The role of $\gamma\delta$ T cells in local immunoregulation has not been fully elucidated, but has been most completely studied in the skin and gastrointestinal tract.¹⁹ Through mostly unknown mechanisms, $\gamma\delta$ T cells may aid in the prevention of systemic immune responses that could be detrimental at interfaces with the external environment by either downregulation or upregulation.¹⁹ Although the canine masticatory muscles are not directly at an interface, they are near the oral cavity and ear canals, potential routes of entry of infectious agents. TCR α -deficient mice, which lack TCR $\alpha\beta$ cells, were found to produce high levels of class-switched, T-cell-dependent autoantibodies.⁴⁶ TCR $\gamma\delta$ cells have been shown to induce immunoglobulin class-switching in humans,²⁴ and can promote germinal-center development in infected TCR β -deficient mice.³⁸ In the cases

of MMM, autoantibody production has been documented, and multifocal clusters of B cells resembling lymphoid nodules have been recognized in muscle biopsy sections. With the exception of the one dog with an overlap syndrome, neither B cells nor significant populations of $\gamma\delta$ T cells were present in dogs with generalized IM.

TCR $\gamma\delta$ ⁺ cells have been shown to accumulate in granulomas in leprosy³¹ and leishmaniasis²⁷ and in synovial cells in rheumatoid arthritis.²³ Some $\gamma\delta$ T cells recognize heat-shock proteins (HSPs) expressed by mycobacteria and stressed mammalian cells.^{4,16} HSPs have been identified in muscle of human patients with IM.²¹ In addition, viral,²⁵ bacterial superantigen,^{3,41} and bacterial⁹ antigens can activate $\gamma\delta$ T cells. These studies highlight the association of $\gamma\delta$ T cells with infectious diseases. The pathophysiological role of $\gamma\delta$ T cells in canine MMM warrants further study.

In conclusion, this study has documented histological and cellular similarities between human and canine generalized IM, supporting a role for canine IM as an animal model for the study of human IMs. This study has also documented important differences between immune responses in canine IM and MMM, suggesting the presence of immunologically distinct microenvironments between masticatory muscles and limb muscles. As in human IM, canine IMs are a heterogeneous group of disorders and further studies are warranted to define the full clinical spectrum and pathogenesis.

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