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Serum levels of innate immunity cytokines are elevated in dogs with metaphyseal osteopathy (hypertrophic osteodytrophy) during active disease and remission

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Abstract

Metaphyseal osteopathy (MO) (hypertrophic osteodystrophy) is a developmental disorder of unexplained etiology affecting dogs during rapid growth. Affected dogs experience relapsing episodes of lytic/sclerotic metaphyseal lesions and systemic inflammation. MO is rare in the general dog population; however, some breeds (Weimaraner, Great Dane and Irish Setter) have a much higher incidence, supporting a hereditary etiology. Autoinflammatory childhood disorders of parallel presentation such as chronic recurrent multi-focal osteomyelitis (CRMO), and deficiency of interleukin-1 receptor antagonist (DIRA), involve impaired innate immunity pathways and aberrant cytokine production. Given the similarities between these diseases, we hypothesize that MO is an autoinflammatory disease mediated by cytokines involved in innate immunity. To characterize immune dysregulation in MO dogs we measured serum levels of inflammatory markers in 26 MO and 102 control dogs. MO dogs had significantly higher levels (pg/ml) of serum Interleukin-1beta (IL-1β), IL-18, IL-6, Granulocyte-macrophage colony stimulating factor (GM-CSF), C-X-C motif chemokine 10 (CXCL10), tumor necrosis factor (TNF), and IL-10. Notably, recovered MO dogs were not different from dogs during active MO disease, providing a suggestive mechanism for disease predisposition. This is the first documentation of elevated immune markers in MO dogs, uncovering an immune profile similar to comparable autoinflammatory disorders in children.

Keywords

Canine; Hypertrophic osteodystrophy; Innate immunity; Autoinflammatory; Cytokines

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1. Introduction

Metaphyseal osteopathy (MO) is an inflammatory bone disease characterized by metaphyseal necrosis, hemorrhage and inflammation (Joiner and Montgomery, 2011). The disease affects young dogs during the peak of their growth and diminishes after closure of the growth plates (Grondalen, 1976; Munjar et al., 1998). Clinically, dogs present with non-specific multisystemic signs of inflammation including fever, ocular and nasal discharge, skin pustules, diarrhea, hematochezia, vomiting, vulvovaginitis, and inflammation involving the airways (Abeles et al., 1999; Safra et al., 2012). The hematological and serum biochemical findings of MO are non-specific but often indicate inflammation (Safra et al., 2012). Radiographs reveal lytic sclerotic lesions in the meta-physes of multiple long bones, which on metaphyseal biopsies demonstrate neutrophilic inflammation, hemorrhage and necrosis (Franklin et al., 2008; Miller, 2001).

Treatment of MO consists of non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids, to decrease immune activation and inflammation. Individuals that respond poorly to NSAIDs often respond favorably to immunosuppressive doses of corticosteroids (Abeles et al., 1999; Safra et al., 2012). Finally, relapses during the growth period are common, especially in dogs with multi-organ involvement (Safra et al., 2012). The overall picture supports an idiopathic chronic inflammatory disease similar to immune-mediated steroid-responsive meningitis arteritis (Biedermann et al., 2016), granulomatous meningoencephalitis (Park et al., 2013), immune-mediated polyarthropathy (Foster et al., 2014), and inflammatory bowel disease (Maeda et al., 2016) in dogs.

The underlying etiology of MO remains unknown. Several mechanisms have been proposed, including nutritional, infectious and vaccine induced causes, although no substantial evidence supporting these theories has been identified (Meier et al., 1957; Munjar et al., 1998; Safra et al., 2012). A hereditary component for MO is evidenced by predisposition of specific breeds such as Great Danes, Boxers, German Shepherd Dogs, Irish Setters, and Weimaraners (Harrus et al., 2002; Miller, 2001; Munjar et al., 1998). MO in litter-mates and closely related dogs has been reported for Weimaraners (Abeles et al., 1999; Safra et al., 2012), Australian Kelpies (Greenwell et al., 2014) and Irish Setters (Brown, 2007).

In children, chronic recurrent multifocal osteomyelitis (CRMO) is a complex autoinflammatory disease that closely resembles MO in its clinical presentation (Ferguson and Laxer, 2015; Sharma and Ferguson, 2013). CRMO patients typically present with recurrent episodes of inflammation involving the bone, skin, and occasionally the intestines. The inflammation is sterile and autoantibodies are absent, suggesting a dysregulation of innate immunity pathways (Chitu et al., 2009). A form of CRMO with a known molecular basis is deficiency of interleukin-1 receptor antagonist (DIRA). Due to over-signaling of IL-1, DIRA patients experience systemic inflammation, sterile lytic bone lesions and pustulosis (Moghaddas and Masters, 2015). A naturally occurring model to CRMO is the chronic multi-focal osteomyelitis (cmo) mouse. Due to a homozygous mutation in the proline-serine-threonine phosphatase interacting protein 2 (Pstpip2), cmo mice develop sterile multifocal osteomyelitis and extramedullary hematopoiesis with occasional inflammation of the skin (Chitu et al., 2009). The human analog of Pstpip2 is a

phosphatidate phosphatase gene named Lipin 2, defects of which also cause CRMO. CRMO is marked by a pro-inflammatory cytokine profile with elevated serum levels of IL-1β, IL-6 and TNF (Stern and Ferguson, 2013).

Given these findings, we hypothesize that MO is an autoinflammatory disorder characterized by increased levels of cytokines involved in innate immunity, similar to CRMO patients and cmo mice. To test this hypothesis, we performed a prospective descriptive study of serum levels of inflammatory markers in MO dogs and controls.

2. Materials and methods

2.1. Dogs

26 MO cases (9 Irish Setters and 17 Weimaraners, Table S1 in the online version, at http://dx.doi.org/10.1016/j.vetimm.2016.08.003) were included on the basis of signalment; clinical signs of pyrexia, lethargy, and ostealgia; and radiographic evidence of MO as evaluated by a board-certified veterinary radiologist (EGJ). Complete medical records and copies of the diagnostic radiographs were required for inclusion. Information obtained from the medical records consisted of signalment, clinical signs, vaccination history, treatment, response to treatment, and relapse episodes. Eight of the dogs were in remission (4 puppies and 4 adults), defined by absence of clinical signs and observed levels of activity appropriate to age. Individuals were assessed to be in remission if they had an unremarkable physical examination documented in the medical record, and had no gait abnormalities as assessed through review of video material and owner interviews. Participation of dog owners was solicited with announcements posted on the Weimaraner Club of America and Irish Setter Club of America websites, by direct communication with dog breeders, owners, attending veterinarians of MO cases, and via the Veterinary Information Network. 102 control dogs free of MO (according to medical records) were included, consisting of 34 unaffected Irish Setter dogs, 52 laboratory mixed-breed dogs (Marshall Farms USA, Inc.), and 16 mixed-breed shelter dogs. Dogs of both sexes and of various ages were included (Table S1 in the online version, at http://dx.doi.org/10.1016/j.vetimm.2016.08.003).

2.2. Serum markers

Whole blood samples were collected in serum collection tubes (red or tiger top). Clotted blood was separated by centrifugation and the serum was aspirated and stored in 1.5 ml eppendorf tubes at −20° C until the time of analysis. Each sample was analyzed once. Serum levels of 13 cytokines (GM-CSF, IFN-γ, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, CXCL10/IP-10, KC-like, CCL2/MCP-1and TNF), were determined using MILLIPLEX MAP Canine Cytokine/Chemokine Magnetic Bead Panel by EMD Millipore (Billerica Massachusetts, USA). The multiplex includes cytokines of interest that were previously implicated in autoinflammatory disorders, and was validated for canine serum. The samples were incubated according to protocol and reading was performed using a Bio-Plex 200 Multiplex System at the UC Davis Human Immune Monitoring Core. Serum levels of canine IL-1β were evaluated because of its involvement in CRMO, and were determined by ELISA (RayBiotech, Inc. Norcross, Georgia, USA), following the kit protocol. Cytokine functions

are summarized in Table S2 in the online version, at http://dx.doi.org/10.1016/j.vetimm.2016.08.003.

2.3. Statistical analysis

Shapiro-Wilk test for normal distribution of serum levels of immune markers (pg/ml) was performed for each of the markers. Since none of the marker levels (including log-transformed levels) were normally distributed, the Wilcoxon rank-sum (Mann–Whitney) test was used to compare marker levels between MO affected and unaffected dogs. Analyses of affected *versus* unaffected dogs were conducted for all dogs combined, as well as stratified by sex, breed, and age group (adult, puppy). Logistic regression was used to estimate odds ratios (OR), defined as the relative odds of being affected *versus* non-affected, and displaying 95% confidence intervals. Marker levels were log-transformed for the regression analysis. Models were adjusted for age (puppy/adult), sex (female/male), and remission status (yes/no). Data were statistically analysed using Stata (StataCorp. 2013. Stata Statistical Software: Release 13.1. College Station, TX: StataCorp LP.)

3. Results and discussion

All confirmed MO dogs (11 females and 15 males) had their first episode during accelerated growth of long bones, between the ages of 7–18 weeks. Clinical signs consisted of ostealgia (n = 26), pyrexia (n = 24), depression (n = 24), anorexia (n = 22), diarrhea (n = 16), vulvovaginitis (n = 7), pustules (n = 5), hematochezia (n = 4), vomiting (n = 4), abnormal lung sounds (n = 4), sore jaw (n = 4), ocular (n = 4) and nasal (n = 3) discharges. Twenty dogs were vaccinated within 30 days prior to a MO episode, and 10 dogs experienced 2–8 relapses.

Six dogs responded to initial treatment with NSAIDs (carprofen (Rimadyl; Zoetis, USA or Vetprofen; Vetoquinol USA, 4.4 mg/kg (2 mg/lb), q 24 h for 7 days (n = 4); firocoxib) (Previcox; Merial USA, 5 mg/kg (2.27 mg/lb), q 24 h for 7 days (n = 1); meloxicam (Metacam; Boehringer Ingelheim Vetmedica, USA) 0.1 mg/kg (0.045 mg/lb), q 24 h for 7 days (n = 1)), and 10 dogs responded to initial treatment with prednisone (makers not specified), 0.75–1.5 mg/kg (0.34–0.68 mg/lb), q 12 h for 7 days, followed by a tapering dose or as needed. Ten dogs were NSAID-resistant, and all of them responded positively to a change of treatment to prednisone.

The results of the statistical analysis comparing serum levels of cytokines between MO dogs and controls are summarized in Table 1 and Fig. S1 in the online version, at http://dx.doi.org/10.1016/j.vetimm.2016.08.003. Compared with control dogs, MO dogs had significantly higher levels (pg/ml) of serum Interleukin-1beta (IL-1β), IL-18, IL-6, Granulocyte-macrophage colony stimulating factor (GM-CSF), C-X-C motif chemokine 10 (CXCL10), Tumor necrosis factor (TNF), and IL-10. The increased levels of pro-inflammatory cytokines regulating innate immunity in MO dogs are comparable to previously described cytokinopathies in autoinflammatory diseases (Moghaddas and Masters, 2015).

Unexpectedly, when MO dogs in remission (n = 8) were compared to MO dogs with active disease (n = 18), there were no significant differences in serum values of cytokines between the two groups. Dogs were considered in remission if they were no longer experiencing episodes of MO as adults (n = 4; duration of remission was between 9 and 36 months) or responded to therapy and had no clinical evidence of MO as puppies (n = 4; duration of remission was between 1 and 3 months). Curiously, dogs in remission had equal or slightly higher (but not statistically significant) medians of inflammatory cytokines compared to dogs with active disease. Non-significant differences in the medians of marker values (pg/ml) were observed for GM-CSF (remission = 35.15, active disease = 20.76), IL-6 (remission = 57.49, active disease = 23.56), IL-18 (remission = 53.26, active disease = 33.15), and IL-1β (remission = 34.67, active disease = 86.79).

Prior to this finding, it was presumed that dogs in remission will have intermediate or basal levels of inflammatory cytokines, reflecting response to treatment and/or signaling a resolution of the disease (Robinson et al., 2013). Moreover, similar findings of significantly increased levels of IL-18 have been documented in patients with autoinflammatory macrophage activation syndrome (MAS), irrespective of disease activity (Canna et al., 2015). This finding provides new information with regard to “predisposition” to MO and highlights a possible pathophysiological mechanism involving constitutive overexpression of pro-inflammatory cytokines. Additional studies are needed to examine the relation between overexpression of pro-inflammatory innate cytokines in dogs predisposed to MO and manifestation of disease.

The only marker with intermediate levels in dogs in remission was IL-1β. Overall, IL-1β levels were increased in MO dogs, similarly to what observed in cmo mice with comparable sterile inflammation in bones, intestine and skin (Ferguson and Laxer, 2015). Elevated levels of IL-1β are not constantly detected in dogs with inflammatory diseases, such as pyometra, gingivitis-stomatitis, pyelonephritis, and neoplasia. (Christian Prachar et al., 2013). The elevated serum level of IL-1β during active disease and remission suggests that MO differs from these other inflammatory conditions in dogs.

IL-18 was also elevated in MO dogs, and this is consistent with high levels of IL-1β, as both are activated by caspase-1 and share a downstream proinflammatory pathway (Man and Kanneganti, 2016). Additionally, MO dogs had elevated levels of CXCL10, similar to what observed in cmo mice (Chitu et al., 2009). CXCL10, also termed Interferon gamma-induced protein 10, is a pro-inflammatory chemokine produced by innate immune cells such as monocytes and neutrophils as well as by osteoclasts in models of increased bone absorption (Yim et al., 2016).

CRMO patients and cmo mice have elevated levels of TNF (Stern and Ferguson, 2013); similarly, MO dogs had elevated levels of TNF when compared to controls. However, additional larger-scale studies are needed to confirm this finding. Serum from MO dogs had elevated levels of IL-6, an acute phase inflammatory cytokine that is elevated in serum from CRMO patients (Hofmann et al., 2016). IL-6 is involved in bone inflammation, and elevated levels are found in dogs with idiopathic immune-mediated polyarthropathy (Foster et al., 2014).

IL-10, an important anti-inflammatory cytokine that inhibits secretion of proinflammatory cytokines and acts to maintain balanced immune responses (Glocker et al., 2011), was elevated in MO dogs. Elevated levels of IL-10 are documented in a number of autoinflammatory disorders such as juvenile idiopathic arthritis in humans (Walters et al., 2016), inflammasome-derived macrophage activation syndrome in humans (Canna et al., 2014), and IL-18 dependent pyrin-inflammasome and autoinflammatory disease in mice (Kim et al., 2015).

An additional marker with increased levels in MO dogs is GM-CSF. It mediates inflammatory processes by regulating the proliferation and activation of hematopoietic granulocytes and macrophages (Ling et al., 2014). It has been implicated in other inflammatory-mediated diseases affecting bone, such as rheumatoid arthritis (Van Nieuwenhuijze et al., 2015), as well as childhood autoinflammatory syndrome periodic fever, aphothous stomatitis, pharyngitis and cervical adenopathy (Ling et al., 2014).

Control dogs were pets living in homes (n = 34), laboratory dogs (n = 52), and shelter dogs (n = 16). When serum levels of cytokines were compared between the 3 groups of control dogs, they were not significantly different (data not shown). Shelter dogs, with high exposure to infectious agents and stressful environments, were not different from laboratory dogs or dogs residing in homes. This suggests that at baseline, non-MO dogs have low levels of pro-inflammatory cytokines, regardless of potential unidentified inflammatory stimuli. In contrast, MO dogs (pets in homes, n = 26) with active disease and in clinical remission had significantly higher levels of innate immunity pro-inflammatory cytokines.

Increased inflammatory cytokines in MO dogs during remission provide a novel insight into a possible predisposition to MO. Future evaluation of innate immune cells from MO dogs during active disease and remission will provide additional data to expand on these results as well as studies designed to identify the aberrant molecular pathways leading to MO episodes. Roles for suppressors of cytokine signaling (SOCS) proteins, especially SOCS3 that regulates responses induced by IL-6 and IL-10 (Yoshimura et al., 2007), could be evaluated in the future as potential etiopathological pathways and as targets for alternative, more precise modules of therapy. In addition, cytokine antagonism serves as highly effective specific therapy in a subset of autoinflammatory conditions with increased cytokine activity (Dinarello et al., 2012), and could be evaluated in the future for cases of MO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Comparison of serum levels of cytokines (pg/ml) between MO dogs and controls.

Markers	Controls (N = 102)	MO (N = 26)	Wilcoxon rank- sum	Logistic regression	
	Median (range)	Median (range)	*p*-value	OR (95% CI)	*p*-value
IL-1β	5.04 (294.91)	62.05 (524.07)	*<0.0001*	1.87 (1.37–2.65)	*<0.0001*
CXCL10	4.16 (73.27)	30.87 (90.72)	*<0.0001*	1.85 (1.85–5.2)	*<0.0001*
IL-6	6.27 (26.85)	30.8 (81.45)	*0.004*	1.43 (1.13–1.84)	*0.004*
TNF	1.85 (22.76)	5.97 (27.5)	*0.01*	1.21 (0.93–1.57)	0.12
IL-10	1.87 (358.64)	1.87 (187.98)	*0.01*	1.42 (1.02–1.99)	*0.03*
IL-18	29.53 (4396.04)	45.02 (11715.69)	*0.01*	1.38 (1.06–1.83)	*0.01*
GM-CSF	5.1 (1022.88)	22.29 (1272.3)	*0.02*	1.28 (1.03–1.62)	*0.03*
IFN-g	5.16 (23.03)	5.16 (0.00)	*0.94*	1.51 (0.43–4.26)	*0.44*
KC-Like	1264 (3474.3)	1174 (4563.68)	*0.94*	0.67 (0.38–1.14)	*0.14*
IL-8	7317 (49822.51)	6965 (75625.14)	*0.69*	0.68 (0.32–1.2)	*0.23*
IL-7	4.16 (7995.6)	30.87 (2961)	*0.07*	1.18 (0.97–1.43)	*0.09*
IL-2	1.89 (11085.26)	1.89 (3051.7)	*0.48*	1.13 (0.88–1.41)	*0.3*
IL-15	48.49 (19804.18)	50.13 (14609.26)	*0.62*	1.28 (0.98–1.69)	*0.07*
MCP-1	271.01 (3167.86)	146.2 (1541.26)	*0.25*	0.85 (0.58–1.25)	*0.41*

OR: Odds Ratios, CI: confidence intervals, Range—the difference between the highest and lowest values. Significant values (<0.05) are italicized.

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