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Evaluation of immunological parameters in pit bull terrier-type dogs with juvenile onset generalized demodicosis and age-matched healthy pit bull terrier-type dogs

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Background – Juvenile onset generalized demodicosis (JOGD) is thought to occur due to immunological abnormalities and is over-represented in pit bull terrier-type dogs.

Animals – Twelve pit bull terrier-type dogs with JOGD and 12 age-matched healthy pit bull terrier-type dogs.

Objective – To investigate immunological differences between age-matched healthy and JOGD pit bull terrier-type dogs by flow cytometry, multiplex, molecular and serological assays.

Methods and materials – Flow cytometry quantified B cells expressing MHCII or surface-bound IgG, CD4+ T cells expressing MHCII, CD8 T cells expressing MHCII or CD11a, neutrophil and monocyte markers. Surface expression was quantified by calculating the geometric mean fluorescence index. The Wilcoxon rank sum test was used to compare median results for IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, FOXP3, monocyte chemoattractant protein-1, GM-CSF, KC, IgE, IgA, IgG, IgM, C-reactive protein, lymphocyte, neutrophil and monocyte in the groups. IFN-gamma, IP-10, IL-15, IL-31 and TNF-alpha also were measured; however, insufficient dogs (<5) had values that were in range of the assay to allow for statistical evaluation. Significance was defined as $P < 0.05$.

Results – Serum concentrations of IL-2, IL-18 and MCP-1 were significantly higher ($P = 0.01$, $P = 0.01$, $P = 0.04$) in the JOGD group. Also, IgA median value was significantly higher ($P = 0.002$) in pit bull terrier-type dogs with JOGD. Flow cytometry revealed that T-cell, neutrophil and monocyte markers were not different between groups.

Conclusions – Results suggest an appropriate compensatory immune response by pit bull terrier-type dogs in the JOGD group and do not support the explanation of global immune deficiency in these dogs.

Introduction

Canine demodicosis is a common cutaneous condition caused by the overpopulation of *Demodex canis* mites in hair follicles and sebaceous glands. It can be clinically classified as localized or generalized, as well as juvenile or adult onset forms.[1,2] *Demodex canis* is considered a normal inhabitant of canine skin and when clinical disease occurs, it is thought to be the consequence of a genetically mediated specific immunodeficiency that allows the proliferation of the mites.[3,4] Evidence suggests that the host immune system is responsible for the control of *Demodex* population in canine skin; however, there is a scarcity of information regarding the mechanism of immune response as well as the host–mite relationship. It has been suggested that the efficiency of the host immune system controls the mite population. However, other authors propose that cutaneous immunosuppression occurs during disease rather than preceding the clinical signs and may be a consequence instead of a primary trigger for mite overpopulation.[5–8]

Different investigations suggest that dogs with generalized demodicosis suffer from T-cell exhaustion, usually characterized by low production of supportive/stimulatory cytokines and high levels of suppressive cytokines.[8–10] Demodicosis also has been associated with microsatellite markers linked to dog leucocyte antigen (DLA) class II

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Conflict of interests: Michael Lappin is on the advisory board of one of the sponsors of this project (Nestle Purina PetCare, St. Louis, MO, USA) and is the director of the Center for Companion Animal Studies that received the donations to fund this project. However, the sponsors were not associated with the performance of any of the assays or interpretation of the data.

alleles in boxers, Argentinean mastiffs and mixed breed dogs.[4] This mirrors findings in humans demonstrating that specific human leucocyte antigen (HLA) class I alleles lead to protection or susceptibility to mite overgrowth.[11]

Although observational studies have described immunological abnormalities in dogs with generalized demodicosis, the lack of standardization of age, breed, clinical presentation, disease onset and health status of dogs have rendered results inconclusive.[8]

The aim of this research study was to identify immunological differences through flow cytometry and multiplex, molecular and serological assays, between age-matched pit bull terrier-type dogs, with and without, juvenile onset generalized demodicosis (JOGD).

Methods and materials

Dogs

The study was approved by the Institutional Animal Care and Use Committee at Colorado State University (Protocol 15-5997A). The dog population consisted of a convenience sampling of 24 shelter dogs, up to 14 months of age, with physical characteristics that led the participating veterinarians to classify the dogs as pit bull terrier-type dogs. American pit bull terrier, American Staffordshire terrier, American bulldog, Staffordshire bull terrier and potential crosses amongst these breeds or with other breeds were included as pit bull-type dogs.

The study included 12 dogs with JOGD and 12 age-matched healthy dogs. The pit bull terrier-type dog group was chosen based on a multivariate analysis study that reported some dog breeds are at high risk for the diagnosis of JOGD including the American Staffordshire terrier and Staffordshire bull terrier (odds ratio 35.6 and 17.1, respectively).[1]

Generalized demodicosis was defined as involvement of an entire body region or more than five focal areas or at least two affected paws.[12,13] Demodicosis was confirmed by observation of *Demodex* mites in deep skin scrapings, and JOGD was defined as initial diagnosis of generalized demodicosis up to 18 months old.[1,12]

Exclusion criteria included pregnancy, lactation, significant systemic illness and previous use of systemic glucocorticoids and/or a parasiticidal drug with known activity against *Demodex*. A physical examination, complete blood cell count, serum biochemical panel, faecal flotation and screening sera for *Dirofilaria immitis* antigen and antibodies against *Borrelia burgdorferi*, *Anaplasma* spp. and *Ehrlichia* spp. (SNAP 4DX Plus test®, IDEXX Laboratories: Westbrook, ME, USA) were performed for every dog before enrolment. Dogs that were positive for other infectious agents or with CBC or serum biochemical abnormalities suggesting systemic abnormalities were excluded.

Mite counts

At enrolment, mite counts were determined by deep skin scrapings from four affected areas of pit bull terrier-type dogs in the JOGD group. Scrapings were obtained from a cutaneous area of 1 cm^2. The skin was pinched and released a few times, then an oily scalpel blade applied to the skin until capillary bleeding was observed. The material sampled was smeared out on a microscope glass slide with the addition of mineral oil and covered with a coverslip. Slides were examined immediately after the skin scrapings were performed, under a microscope with ×10 objective. Absolute numbers of each life stage (adults, nymphs, larvae and eggs) and whether the mites were alive or dead were recorded.

Skin lesion extent and severity score

The clinical score was based on the Canine Atopic Dermatitis Extent and Severity Index (CADESI-03),[14] with adaptation for the lesions of generalized demodicosis.[15] Cutaneous lesions were classified as erythema, scales/crusts, comedones/papules/pustules and alopecia. A severity score (0 normal to 6 extremely severe) was assigned for each lesion type at each body region. To determine the extent of skin lesions, the body was divided in nine different areas including the muzzle/chin, face/periocular area, dorsal head/pinnae, cervical area, thoracic area, abdominal area, front legs, hind legs and tail/perianal area. The maximum total possible score was 216.

Impression smears or acetate tape preparations were examined from two different affected areas of each dog to screen for secondary pyoderma. For impression smears, a section of a microscope glass slide was pressed on the skin and stained in Diff Quick®. For acetate tape cytological evaluation, the sticky side of a short piece of single-sided clear acetate tape was impressed on the skin. A drop of the thiazine dye of the Diff Quick® was placed on a microscope slide, and the tape was positioned over it with the sticky side down on the stain. All slides were examined under oil immersion to identify bacteria.

Sample collection

Venous blood (15 mL) was collected from each dog and placed in EDTA-containing tubes or clot tubes. Blood in EDTA was processed immediately as described for flow cytometry, 2 mL was submitted for a complete blood cell count (Veterinary Diagnostic Laboratory, Colorado State University) and 1.6 mL of blood was mixed with 3.4 mL of RNALater® (Thermo Fisher Scientific; Waltham, MA, USA) for quantitative PCR assays (qPCR) for mRNA of selected cytokines. Clotted blood was centrifuged 904.1 ***g*** for 10 min at room temperature and then the sera removed. The blood in RNALater® and sera were aliquoted into plastic tubes and stored at −80°C until assayed.

Serum assays

Prior to storage, sera were submitted for performance of a serum biochemical panel (Veterinary Diagnostic Laboratory). Stored sera were thawed at room temperature and evaluated for total IgA (Canine IgA Test Kit Radial Immunodiffusion Test Kit, Triple J Farms; Bellingham, WA, USA), total IgG (Triple J Farms), total IgM (Triple J Farms), total IgE (IgE Dog ELISA kit, Abcam; Cambridge, MA, USA), IFN-gamma (Quantikine ELISA Canine IFN-gamma, R&D Systems; Minneapolis, MN, USA) and C-reactive protein [CRP] (C-reactive protein (PTX1) Dog ELISA kit, Abcam) using commercially available kits following the manufacturer's instructions. A commercially available fluorescence-based system [Milliplex MAP Canine Cytokine/Chemokine Premixed Magnetic Bead Kit (Cat# CCYTMG-90K-PX13), Millipore/Sigma; Burlington, MA, USA] was used to evaluate for selected cytokines and chemokines in the stored sera including GM-CSF, keratinocyte chemoattractant (KC, CXCL1), IFN-γ-inducible protein 10 (IP-10; CXCL10), IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, monocyte chemoattractant protein-1 (MCP-1) and TNF-alpha.

qPCR for FoxP3, IL-4, IL-13, IL-31 and interferon-γ cytokines

Each stored whole blood sample in RNALater® was thawed at room temperature for approximately 20–30 min, followed by gentle resuspension. The RNA extraction was done using a commercial kit following manufacturer's instructions, including the optional DNase treatment (RiboPure-Blood kit, Invitrogen; Carlsbad, CA, USA). All of the whole blood RNA was stored at −20°C following extraction. Immediately following extraction and prior to storage, 40–80 μL RNA from each of the healthy normal pitbull RNA samples was pooled to make a normal pitbull RNA (NPR) calibrator and 50 μL affected pitbull RNA (DFP) calibrator.

The NPR calibrator total RNA was quantified with a NanoDrop 1000 (Thermo Fisher Scientific). Previously described primers for the canine cytokines IL-13, IL-31, FoxP3 and hypoxanthine phosphoribosyltransferase (HPRT) gene/enzyme were used.[16–18] The primer concentrations were optimized using the NPR calibrator. The template RNA concentration was optimized using the NPR calibrator simultaneously with the primer optimization. The thermal cycling conditions were followed as suggested by the kit manufacturer (Power SYBR Green RNA-C_T 1-Step Kit, Applied Biosystems; Waltham, MA, USA); 48°C for 30 min, 95°C for 10 min, followed by (95°C × 15 s,

60°C × 1 min) for 40 cycles. A melt curve was run after the thermal cycling program to insure there was a single, specific product for each cytokine. Briefly, IL-13 primers were optimized to use 200 nM of the forward primer, 450 nM of the reverse primer. IL-31 was optimized to utilize 450 nM of each primer, whereas FoxP3 and HPRT both utilized 200 nM of each primer. An optimized RNA concentration of 70 ng total RNA per reaction was used with a template volume of 2 μL in a total reaction volume of 20 μl for all four assays. Prior to assay, all case samples were thawed on ice and the total RNA was quantified and diluted to a concentration of 35 ng/μL in 200 μL.

Complementary DNA (cDNA) was synthesized from all dog samples along with the NPR calibrator for use in the assays for IL-4, interferon-γ (IFN-Gamma) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[19,20] For each sample, cDNA was synthesized using 10 μL total RNA in a commercially available kit (SuperScript IV First-Strand cDNA Synthesis System, Invitrogen), following the manufacturer's instructions. The final cDNA product (20 μl) was mixed with RNase-free water to a volume of 100 μL and stored at −80°C.

For the canine cytokines, IL-4, IFN-gamma and GAPDH, the cDNA template volume was optimized to use 5 μL, in a 25 μL total reaction volume using TaqMan 2× Universal PCR Master Mix (Applied Biosystems) with 200 nM of each primer and 100 nM probe. The thermal cycling conditions for all three assays were 10 min at 50°C, 3 min at 95°C, and 40 cycles of 10 sec at 95°C followed by 30 s at 60°C.

The delta Cq of each sample, as well as the NPR calibrator, was calculated in comparison with the reference gene for each assay type (HPRT for IL-13, IL-31 and FoxP3 SYBR green assays; GAPDH for the IL-4 and IFN-gamma TaqMan probe assays).[16–20]

All real-time (RT)-PCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad; Hercules, CA, USA) and CFX Manager™ Software (Bio-Rad) for data analysis. The quantification cycle (Cq) was set at 10 times the standard deviation of the baseline fluorescence. The individual *Demodex*-affected and healthy dog RNA samples were run alongside the NPR and DFP calibrators and a no template control (NTC; RNase-free water in place of RNA template). All samples, calibrators and controls were run in triplicate reactions on all seven assays. The relative quantification method was utilized following the comparative Ct method: (https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/qpcr-education/absolute-vs-relative-quantification-for-qpcr.html).

Flow cytometric assessment of lymphoid and myeloid cell activation

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA collected blood samples using discontinuous density gradient centrifugation (Ficoll-Plaque Plus, GE Healthcare; Uppsala, Sweden). Whole blood was diluted 1:2 with phosphate-buffered saline (PBS) and slowly layered on top of the discontinuous density gradient to prevent mixing and centrifuged at 400 ***g*** for 30 min. PBMCs were then collected and washed with PBS before being diluted in FACS buffer; 2.5×10^5 canine PBMC were added to duplicate wells of a 96-well round bottom plate. Cells were incubated with 5% v/v normal dog serum in FACS buffer for 10 min at RT. For analysis of T-cell activation, the cells were incubated with directly conjugated antibodies to canine CD4 (clone YKIX302.9, conjugated to Pacific Blue, AbD Serotec), canine CD8 (clone YCATE55.9, Alexa 647, AbD Serotec), canine CD5 (clone YKIX322.3, PE, eBioscience; San Diego, CA, USA) and canine MHC Class II (clone YKIX334.2, FITC, eBioscience) or a Rat IgG2a K isotype control (clone 3BR2a, FITC, eBioscience) diluted in FACS buffer for 30 minutes at RT in the dark. For analysis of neutrophils and monocytes, PBMC were immunostained with antibodies to CD11b (clone Mac-1, PeCy7, Beckman Coulter; Indianapolis, IN, USA), CD14 (clone TüK4, APC, Invitrogen; Grand Island, NY, USA), CD4 to determine cell type, and either MHC Class II antibody or isotype, to determine activation status in FACS buffer for 30 min at RT in the dark. For analysis of B cells, PBMC were incubated with directly conjugated antibodies to canine CD21 (clone CA2.1D6, PE, AbD Serotec) to determine cell type, and either MHC Class II and anti-Dog IgG (Rabbit anti-Dog IgG (H + L), Alexa 647, Jackson ImmunoResearch; West Grove, PA, USA) or isotype to determine activation status.

The cells were then washed twice at the completion of immunostaining and fixed in 4% paraformaldehyde (PFA) for 10 min at RT in the dark. Two wash steps were performed before PBMC were resuspended in FACS buffer and transferred to flow tubes for analysis by flow cytometry.

For assessment of T-cell population, lymphocytes were gated initially by forward and side-scatter properties; then, $CD5^+$ cells were gated and separated into $CD5^+CD4^+$ and $CD5^+CD8^+$ populations. B-cell numbers were assessed by initially gating lymphocytes by forward and side-scatter properties and then gating $CD21^+$ cells. To determine neutrophil and monocyte percentages, myeloid cells were initially gated by forward and side-scatter properties; then, neutrophils were classified as $CD11b^+CD4^+CD14^-$, whereas monocytes were classified as $CD11b^+CD14^+$ $CD4^-$. Gates for the analysis of MHC Class II expression (as a marker of cell activation) were based on binding of isotype control antibodies by the respective CD4 and CD8 T-cell, monocyte, neutrophil and B-cell populations.

Statistical evaluation

Descriptive statistics were calculated, and continuous data were expressed as medians and ranges. The Shapiro–Wilk test was used to assess normalcy of data. Because of non-normalcy of variables, the Wilcoxon rank sum test was used to compare median results for cytokine, chemokine, immunoglobulin, lymphocyte, monocyte and neutrophil levels in the JOGD group as compared to the healthy group. Group medians were compared if levels were detectable in at least five dogs from both the JOGD and healthy groups. Although multiple cytokines were compared between groups, this study's intent was to detect differences in the cytokines; therefore, to avoid unnecessarily increasing type 2 errors, adjustments for multiple comparisons were deemed unnecessary.[21,22]

Commercially available software (StataCorp 2015: Release 14, Stata Statistical Software; College Station, TX, USA) was used for all comparisons. Significance was defined as $P < 0.05$.

Results

Signalment

Dog age at the time of enrolment, gender and sexual status are described in Table S1.

Mite counts and clinical evaluation

Total mite counts and total clinical score for each dog with generalized demodicosis are reported in Table 1. All pit bull terrier-type dogs in the JOGD group had been administered antibiotic treatment for at least three days prior to entering into the study. However, all of the dogs still had cytological evidence of secondary pyoderma.

Cytokines/Chemokines

Serum concentrations of IL-2, IL-18 and MCP1 were significantly higher in the JOGD group (Table 2, Figure 1). The concentrations of IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, GM-CSF and KC were not significantly different between the healthy dogs and the JOGD groups (Table 2).

IFN-gamma was not detected in the stored sera from any dog by the commercially available kit. However, IFN-gamma levels were measurable in blood by PCR assay (Table 2).

Due to the limited number of dogs ($n < 5$) with detectable values, group comparisons could not be made for IP-10, IL-15, IL-31 and TNF-alpha. In the group of healthy pit bull terrier-type dogs, only four dogs expressed IL-15

Table 1. Mite burden and lesion clinical severity in pit bull terrier-type with juvenile onset generalized demodicosis

Dogs	Total mite counts (number of eggs, larvae, nymphs and adults)	Total clinical score
D1	156	53
D2	6	51
D3	24	107
D4	503	182
D5	450	166
D6	333	190
D7	221	78
D8	18	73
D9	24	91
D10	54	86
D11	112	136
D12	176	111

values above the level of detection, and none of the dogs showed detectable results for TNF-alpha. In the JOGD group, only four dogs showed detectable values for IP-10. IL-31 was not consistently amplified from the blood samples. Group comparisons could not be performed for these cytokines.

Immunoglobulins, FOXP3, CRP

The IgA median value was significantly higher in the JOGD pit bull terrier-type dogs compared to healthy pit bull terrier-type dogs (Table 3, Figure 1). IgG, IgM, IgE, CRP and FOXP3 concentrations were not significantly different between the two groups of dogs. (Table 3).

Flow cytometry

Flow cytometry revealed numerically higher IgG expression by B cells in pit bull terrier-type dogs with JOGD; however, the results were not statistically different when compared to healthy dogs. Monocytes, neutrophils and T-cell activation markers also were not statistically different between the two groups (Table 4).

Discussion

In our study, we found that IL-2 and IL-18 levels were significantly higher in pit bull terrier-type dogs with JOGD

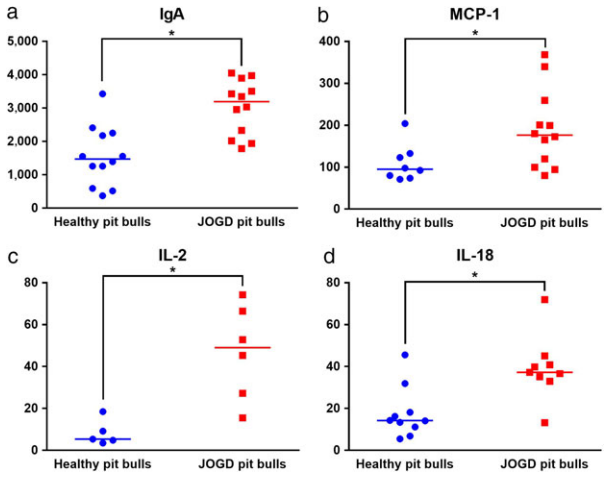

Figure 1. (a) IgA concentrations, (b) MCP-1 concentrations, (c) IL-2 concentrations, (d) IL-18 concentrations in pit bull terrier-type with juvenile onset generalized demodicosis (JOGD) and age-matched healthy pit bull terrier-type.
Horizontal lines indicate the median concentration significantly increased ($P < 0.05$) in pit bull terrier-type with JOGD.

than age-matched controls. These cytokines have been considered to be important regulators of Th1 and Th2 cytokine production. One study reported on the spontaneous development of demodicosis in double knockout STAT6/CD28 −/− mice and the concurrent development of a T helper 1 response.[23] It was suggested that overpopulation of *Demodex* can contribute to severe skin disease when specific immune cell signalling pathways associated with T helper 2 cell activation and function are inhibited. Without significant different IFN-gamma values between the JOGD and healthy dogs in the current study, we were unable to conclude that dogs with demodicosis were mounting a Th1 immune response against the mite.

Data showing that dogs with demodicosis had fewer cells expressing the higher affinity IL-2 receptor CD25 and decreased IL-2 production when compared to control dogs[24] or normal IL-2 expression[25] have been reported. Conversely, other reports suggest an upregulated T helper 2 like response in dogs with first time or reoccurring demodicosis.[25,26] The prior reports included dogs of

Table 2. Cytokine profiles of healthy pit bull terrier-type and age-matched pit bull terrier-type with juvenile onset generalized demodicosis (JOGD)

	Healthy pit bull terrier-type dogs			JOGD pit bull terrier-type dogs			
Immunological markers	n	Median	Range	n	Median	Range	*P*-value
IL-2	5	5.38	3.52–18.47	6	49.04	15.53–74.22	0.0106*
IL-4	5	32.12	30.03–34.9	8	32.18	29.97–35.16	0.1877**
IL-6	7	8.54	2.95–38.11	11	19.71	4.6–60.32	0.1604
IL-7	12	17.18	7.18–67.37	12	41.9	6.49–174.44	0.0647
IL-8	11	2,107.97	984.72–11,536.8	12	5,914.11	1,455–19,699.27	0.1396
IL-10	7	14.21	3.74–37.23	9	16.83	3.09–42.46	0.8738
IL-13	12	34.90	30.83–36.31	12	36.02	34.5–37.5	0.4189**
IL-18	10	14.22	5.5–31.93	9	37.21	13.23–71.95	0.0114*
GM-CSF	9	25.32	6.36–140.96	11	44.19	17.01–80.68	0.1105
Keratinocyte chemoattractant	12	159.07	43.88–1,556.4	12	336.53	73.18–1,672.39	0.2482
Monocyte chemoattractant protein-1	8	95.17	71.23–204.21	12	176.49	80.14–368.42	0.0338*
Interferon gamma	12	34.32	29.64–37.58	12	31.92	30.74–36.47	0.7290**

All cytokine values are in pg/mL; n number of dogs.
*$P < 0.05$.
**P-values stated on delta–delta cq values.

Table 3. Immunoglobulins, C-reactive protein (CRP) and FOXP3 profiles of healthy pit bull terrier-type and age-matched pit bull terrier-type with juvenile onset generalized demodicosis (JOGD)

	Healthy pit bull terrier-type dogs			JOGD pit bull terrier-type dogs			
Immunological markers	n	Median	Range	n	Median	Range	*P*-value
IgA (mg/dL)	12	1472.5	372–3427	12	3192	1785–4051	0.0016*
IgG (mg/dL)	12	25	4–235	12	27	4–181	0.9074
IgM (mg/dL)	12	306	241–354	12	306	235–389	0.4871
IgE (ng/mL)	7	4.35	0.201–66.56	11	8.44	1.72–80.62	0.3416
CRP (ng/mL)	12	9.9	1.29–64.22	12	13.3	3.80–239.95	0.8625
FOXP3	12	33	31.96–35.4	12	32.3	31.25–35.47	0.4189**

n number of dogs.
* $P < 0.05$.
** *P* values stated on delta–delta cq values.

Table 4. Flow cytometric profiles of lymphoid and myeloid cell activation markers of healthy pit bull terrier-type and age-matched pit bull terrier-type with juvenile onset generalized demodicosis (JOGD)

	Healthy pit bull terrier-type dogs			JOGD pit bull terrier-type dogs			
Cell Type	n	Median	Range	n	Median	Range	*P*-value
B Cell IgG	12	2.09	0.91–18.68	12	3.06	1.85–20.17	0.07
B Cell MHC II	12	38.85	16.18–54.04	12	27.12	8.69–81.66	0.73
CD4 + T Cell MHC II	12	5.50	2.40–25.32	12	10.70	3.49–49.97	0.45
CD5 + T Cell MHC II	12	9.70	2.57–35.85	12	20.69	6.24–57.63	0.15
CD8 + T Cell MHC II	12	12.21	3.55–27.74	12	12.53	7.17–58.84	0.25
Monocytes MHC II	12	5.77	1.97–23.98	12	8.36	2.89–14.88	0.39
Neutrophils MHC II	12	1.35	0.88–2.18	12	1.55	0.42–9.50	0.17

All values are presented in percentages (%).

different ages, breeds, clinical presentation (localized versus generalized demodicosis), disease severity and disease chronicity, unlike dogs in the current study. Treatment history, presence of secondary cutaneous infections, immunoassay techniques and statistical analysis also have not been standardized in previous studies. All of these variables may contribute to the discrepant results that are difficult to compare. Moreover, studies of cytokines have proposed that measuring multiple cytokines (cytokine networks) in any given health condition provides more relevant information than the selective measurement of single cytokines.[27]

MCP-1 is a chemokine that regulates migration and infiltration of monocytes / macrophages.[28] Evidence suggests that MCP-1 is a key cytokine mediator in a variety of infectious and inflammatory diseases and may also be useful in assessing disease severity in critically ill dogs.[29] Serum concentrations of MCP-1 were significantly increased in the group of JOGD pit bull terrier-type dogs in comparison with the values observed in healthy pit bull terrier-type dogs, possibly due to the cutaneous inflammation present in all dogs. However, pit bull terrier-type dogs with a more severe clinical disease and larger number of mites did not seem to produce more MCP-1 than pit bull terrier-type dogs with milder clinical demodicosis.

IgA was observed in higher concentrations in pit bull terrier-type dogs with JOGD. It is known that IgA serum levels are positively correlated to age and dogs younger than one year of age do not have a stable production.[30] Thus, total IgA levels would be expected to be higher in our study dogs if they were older. A previous study reported lower concentrations of IgA in dogs of different ages and breeds parasitized with endo- or ectoparasites.[31] Because serum IgA concentrations also are found to vary widely between breeds,[30] meaningful comparisons cannot be drawn. With regard to the role of IgA in ectoparasitism, it is speculated that in human scabies increased secretions of proteases into the skin by *Sarcoptes* mites may in part induce increased levels of IgA in the blood.[32] In human *Demodex* infestation, the main antigen detected by the host immune system is unknown, although there are some publications reporting the detection of lipase and some proteases of the mites.[8,33] Furthermore, human patients with an allele that is associated with increased risk of demodicosis may show higher concentrations of serum IgA.[11] Increased IgA levels in pit bull terrier-type dogs with JOGD likely represent a compensatory response to the *Demodex* infestation; therefore, IgA would not be a primary defect responsible for the mite overpopulation.

Multiplication of *Demodex* mites in humans is suspected to be associated with an aberrant innate immune response that facilitates a humoral immune inflammatory response.[34] However, it is likely that several factors can interfere with the host–mite relationship, as well as with the host reaction to the cutaneous insult caused by the mite.

In the present study, cytokine, chemokine and immunoglobulin concentrations did not seem to correlate with disease severity, but statistical analysis was not performed to assess comparisons between test results and clinical severity. Aside from JOGD pit bull terrier-type dog D4 – that presented with severe clinical disease and the largest number of mites according to the clinical score – the pit bull terrier-type dogs expressing the highest immunological markers levels were not the ones with the

largest mite counts or clinical scores. Thus, the severity of the cutaneous disease may not be directly associated with the expression of certain immunological markers. Observation of these parameters over time would provide better understanding of the role of these cytokines with regard to disease severity and resolution.

One of the limitations of the current study was the low detection level of expression of some cytokines, chemokines and immunoglobulins in several dogs, which prevented statistical comparison of all 12 dogs within each group. This resulted in smaller sample size and a potential increase of type II errors.[35] It also is possible that the inability to assign a value to those individuals that had a level below the detection threshold might have led to a falsely increased median score in either one or both of the groups, which could have resulted in an increase of type I errors.[36] Another limitation was that only blood samples were used for the immunological evaluation of the dogs. Additional skin samples for histopathological and immunohistochemical evaluation of affected areas could have given more information on inflammatory and specific immune responses against *Demodex* mites.[37] Furthermore, all pit bull terrier-type dogs with JOGD had cytological and clinical evidence of secondary pyoderma of varied clinical severity when the samples were collected for analysis. A previous study of human atopic dermatitis and secondary *Staphylococcus aureus* infection in adults reported that elevated colonization of the bacteria may lead to high IL-18 levels.[38] The cutaneous bacterial infection on the dogs' skin also could possibly induce changes in immune responses, making it difficult to determine which changes were related to demodicosis or pyoderma in the dogs described here.

To the best of the authors' knowledge, this is the first age- and breed-matched assessment of immunological profile of dogs with JOGD. Further studies like this are required to determine if the immune response changes are induced by the presence of mites or are a consequence of the mite overgrowth. Also, longitudinal studies assessing the dynamics of immunological parameters associated with mite burden, clinical signs, secondary infection and recovery of disease, would be helpful to determine the role of the immune response in the pathogenesis and resolution of JOGD.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Signalment of pit bull terrier-type enrolled in the study

Résumé

Contexte – La démodécie généralisée juvénile (JOGD) est suspectée liée à des anomalies immunologiques et est sur-représentée chez les chiens de type pit bull terrier.

Sujets – Douze chiens de type pit bull terrier avec JOGD et 12 chiens de type pit bull terrier sains de différents âges.

Objectifs – Etudier les différences immunologiques entre les chiens JOGD et sains de même âge par cytométrie de flux, multiplexe, moléculaire et sérologie.

Matériel et méthode – Les cellules B quantifiées par cytométrie de flux exprimaient MHCII ou des IgG de surface, les cellules T CD4+ exprimant MCHII, les cellules T CD8 exprimant MHCII ou CD11a, marqueurs des neutrophiles et monocytes. L'expression de surface a été quantifiée en calculant l'index de fluorescence moyenne géométrique. Le test de Wilcoxon a été utilisée pour comparer les résultats moyens pour IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, FOXP3, monocyte chemoattractant protein-1, GM-CSF, KC, IgE, IgA, IgG, IgM, la protéine C-réactive, lymphocyte, neutrophile et monocyte dans les groupes. IFN-gamma, IP-10, IL-15, IL-31 et TNF-alpha aussi ont été mesurés; cependant, les chiens insuffisants (<5) avaient des valeurs qui étaient dans les paramètres du test pour permettre une évaluation statistique. La valeur significative était établie comme $P < 0.05$.

Résultats – Les concentrations sériques de IL-2, IL-18 et MCP-1 étaient significativement plus élevées ($P = 0.01$, $P = 0.01$, $P = 0.04$) dans le groupe JOGD. En outre, la valeur médiane était significativement lus élevée ($P = 0.002$) chez les chiens pit bull terrier avec JOGD. LA cytométrie de flux a révélé que les marqueurs de cellules T, neutrophiles et monocytes n'étaient pas différents entre les groupes.

Conclusions – Les résultats suggèrent une réponse immunitaire compensatoire appropriée chez les chiens de type pit bull terriers dans le groupe JOGD et ne supportent pas l'explication du déficit immunitaire global chez ces chiens.

Resumen

Introducción – se cree que la demodicosis generalizada de edad juvenil (JOGD) ocurre debido a anormalidades inmunológicas y está sobrerrepresentada en perros de raza Pitbull Terrier.

Animales – doce perros de raza Pitbull Terrier con JOGD y 12 perros sanos Pitbull terrier de la misma edad.

Objetivo – investigar las diferencias inmunológicas entre perros de raza Pitbull con JOGD y sanos de la misma edad mediante citometría de flujo, múltiplex, pruebas moleculares y serológicas.

Métodos y materiales – cuantificación de linfocitos B por citometría de flujo expresando MHCII o IgG unida a la superficie, linfocitos T CD4+ que expresan MHCII, linfocitos T CD8 que expresan MHCII o CD11a, marcadores de neutrófilos y monocitos. La expresión en la superficie se cuantificó calculando la media geométrica de fluorescencia. La prueba de suma de rangos de Wilcoxon se utilizó para comparar las medianas de expresión de IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, FOXP3, proteína quimiotáctica de monocitos- 1, GM-CSF, KC, IgE, IgA, IgG, IgM, proteína C-reactiva, linfocitos, neutrófilos y monocitos en los grupos. También se midieron IFN-gamma, IP-10, IL-15, IL-31 y TNF-alfa; sin embargo, perros no hubo suficiente perros (<5) con valores dentro del rango de detección de la prueba como para permitir una evaluación estadística. Valores significativos se definieron como $P < 0,05$.

Resultados – Las concentraciones séricas de IL-2, IL-18 y MCP-1 fueron significativamente más altas (P = 0,01, P = 0,01, P = 0,04) en el grupo JOGD. Además, el valor de la mediana de IgA fue significativamente mayor (P = 0,002) en los perros de raza Pitbull Terrier con JOGD. La citometría de flujo reveló que los marcadores de linfocitos T, neutrófilos y monocitos no fueron diferentes entre los grupos.
Conclusiones – los resultados sugieren una respuesta inmune compensatoria apropiada en perros de raza Pitbull Terrier en el grupo JOGD y no apoyan una explicación de inmunodeficiencia global en estos perros.

Zusammenfassung

Hintergrund – Es wird vermutet, dass die juvenile generalisierte Demodikose (JOGD) aufgrund immunologischer Abnormalitäten auftritt und beim Pitbullterrier und –ähnlichen Hunden überrepräsentiert vorkommt.
Tiere – Zwölf Pitbullterrier-artige Hunde mit JOGD und 12 altersgleiche gesunde Pitbullterrier-artige Hunde.
Ziele – Eine Untersuchung der immunologischen Unterschiede zwischen den altersgleichen gesunden und JOGD Pitbullterrier-artigen Hunden mittels Flowzytometrie, Multiplex, molekularen und serologischen Assays.
Methoden und Material – Mittels Flowzytometrie wurden die B Zellen, die MHCII oder Oberflächengebundenes IgG exprimierten, $CD4^+$ T Zellen, die MHCII exprimierten, CD8 T Zellen, die MCHII oder CD11a exprimierten, sowie Neutrophilen- und Monozytenmarker quantifiziert. Die Oberflächenexprimierung wurde mittels Kalkulation des geometrischen durchschnittlichen Fluoreszenzindex quantifiziert. Der Wilcoxon Vorzeichen Rang Test wurde verwendet, um die Medianen Ergebnisse für IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, FOXP3, Monocyten Chemoattractant Protein-1, GM-CSF, KC, IgE, IgA, IgG, IgM, C-reactives Protein, Lymphozyten, Neutrophile und Monozyten in den Gruppen zu vergleichen. IFN-gamma, IP-10, IL-15, IL-31 und TNF-alpha wurden ebenfalls gemessen; es hatten jedoch zu wenige Hunde (<5) Werte, die in der Spannbreite des Assays lagen, um eine statistische Auswertung zu erlauben. Die Signifikanz wurde mit $P < 0,05$ festgelegt.
Ergebnisse – Die Serumkonzentrationen von IL-2, IL-18 und MCP-1 waren in der JOGD Gruppe signifikant höher (P = 0,01; P = 0,01; P= 0,04). Ebenso waren die Medianwerte von IgA bei Pitbullterrier-artigen Hunden mit JOGD signifikant höher (P = 0,002). Die Flowzytometrie zeigte, dass T Zellen, Neutrophile und Monozytenmarker sich in den Gruppen nicht unterschieden.
Schlussfolgerungen – Die Ergebnisse lassen auf eine ausreichende kompensatorische Immunantwort bei Pitbullterrier-artigen Hunden in der JOGD Gruppe schließen und können eine globale Immundefizient bei diesen Hunden nicht erklären.

要約 – 背景 - 若年発症性汎発性ニキビダニ症(JOGD)は、免疫学的異常により生じると考えられ、ピット・ブル・テリア系統の犬で過剰発症している。
被験動物 – JOGD を呈する12頭のピットブルテリア系統の犬及び年齢の対応する12頭の健常なピット・ブル・テリア系統の犬。
目的 – 本研究の目的は、フローサイトメトリー、マルチプレックス、分子および血清学的分析により、年齢の対応した健常およびJOGDピット・ブル・テリア系統の犬の免疫学的差異を調べることである。
方法および材料 – フローサイトメトリーは、MHCIIを発現させたB細胞または表面にIgGを結合させたB細胞、MHCIIを発現するCD4 + T細胞、MHCIIまたはCD11aを発現するCD8 T細胞、好中球および単球マーカーの量を定量した。表面の発現量は、幾何平均蛍光指数を計算することによって定量した。ウィルコキソンの順位和検定を各グループ内のIL-2、IL-4、IL-6、IL-7、IL-8、IL-10、IL-13、IL-18、FOXP3、単球走化性タンパク1、GM-CSF、KC、IgE、IgA、IgG、IgM、C-反応性タンパク質、リンパ球、好中球および単球の中央値結果を比較するために使用した。 IFN-c、IP-10、IL-15、IL-31およびTNF-aも測定した。しかし、不適当な犬(<5)は、統計学的評価を可能にする分析範囲内の値を有していた。有意性はP <0.05として定義した。
結果 – - IL-2、IL 18およびMCP-1の血清濃度はJOGD群で有意に高かった(P = 0.01、P = 0.01、P = 0.04)。また、IgA中央値は、JOGDを有するピット・ブル・テリア系統の犬において有意に高かった(P = 0.002)。フローサイトメトリーは、T細胞、好中球および単球マーカーが群間に差異がないことを明らかにした。
結論 – 結果は、JOGDのピット・ブル・テリア犬による適切な代償性免疫応答を示唆し、これらの犬における全体的な免疫不全の説明を支持しない。

摘要

背景 — 幼年发病的全身性蠕形螨病(JOGD)被认为是由于免疫异常所导致,并且比特斗牛梗很高发。
动物 — 12只JOGD比特斗牛梗和12只年龄匹配的健康比特斗牛梗。
目的 — 通过流式细胞、多重分析、分子和血清学检测方法,研究年龄匹配的健康和JOGD比特斗牛梗犬之间的免疫学差异。
材料和方法 — 流式细胞仪检测并量化表达MHCII或表面结合IgG的B细胞,表达MHCII的CD4+T细胞,表达MHCII或CD11a的CD8T细胞,以及中性粒细胞和单核细胞标记物。通过计算几何平均荧光指数来量化细胞表面表达。Wilcoxon秩和检验用于比较IL-2、IL-4、IL-6、IL-7、IL-8、IL-10、IL-13、IL-18、FOXP3、单

核细胞趋化蛋白-1、GM-CSF、KC、IgE、IgA、IgG、IgM、C-反应蛋白、淋巴细胞、中性粒细胞和单核细胞的中位数结果。还测量了IFN-γ、IP-10、IL-15、IL-31和TNF-α；然而,无效犬只数(<5)在试验允许范围内,不影响统计学评估。显著性为P <0.05。
结果 — JOGD组血清IL-2、IL-18和MCP-1浓度显着升高(P=0.01,P=0.01,P=0.04)。 此外,JOGD比特斗牛梗的IgA中值显著更高(P=0.002)。流式细胞仪显示T细胞、中性粒细胞和单核细胞标志物在各组之间没有差异。
结论 — 结果表明,在JOGD组中,比特斗牛梗具有一定代偿性免疫反应,此结果不能诠释这些犬本身具有的免疫缺陷。

Resumo
Contexto – Acredita-se que a demodiciose juvenil generalizada (DJG) ocorra devido a alterações imunológicas e é observada uma maior ocorrência em cães do tipo pit bull terrier.
Animais – Doze cães do tipo pit bull terrier com DJG e 12 doze cães do tipo pit bull terrier saudáveis de idades semelhantes ao grupo DJG.
Objetivo – Investigar as diferenças imunológicas entre cães saudáveis e cães com DJG do tipo pit bull terrier, de idades semelhantes, por citometria de fluxo e ensaios moleculares e sorológicos.
Métodos e materiais – A citometria de fluxo quantificou as células B expressando MHCII ou IgG ligada à superfície, células T CD4+ expressando MHCII, células T CD8 expressando MHCII ou CD11a, marcadores de neutrófilos e monócitos. A expressão na superfície foi quantificada pelo cálculo da média geométrica do índice de fluorescência. O teste de Wilcoxon foi utilizado para comparar entre os grupos os resultados medianos para IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, FOXP3, proteína quimiotática de monócitos-1, GM-CSF, KC, IgE, IgA, IgG, IgM, proteína C-reativa, linfócitos, neutrófilos e monócitos. IFN-gamma, IP-10, IL-15, IL-31 e TNF-alpha também foram mensurados; entretanto, o número de cães (<5) que obtiveram valores dentro intervalo do ensaio que permitia análise estatística foi insuficiente. O valor de $P < 0.05$ foi considerado significante.
Resultados – As concentrações séricas de IL-2, IL-18 e MCP-1 foram significativamente mais altas ($P = 0.01$, $P = 0.01$, $P = 0.04$) no grupo DJG. Além disso, o valor mediano de IgA foi significativamente ($P = 0.002$) mais alto nos cães do tipo pit bull com DJG. A citometria de fluxo revelou que não havia diferenças significativas entre os grupos para os marcadores de células T, neutrófilos e monócitos.
Conclusões – Os resultados sugerem uma resposta imune compensatória nos cães do tipo pit bull terrier com DJG e não corroboram com o argumento de imunodeficiência global nestes cães.