

Assessment of Molecular Signalling Mechanisms for Eosinophilia in Rottweilers

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ABSTRACT

Rottweilers are predisposed to eosinophilic diseases, including hypereosinophilic syndrome. The immunopathogenesis of idiopathic eosinophilia is poorly characterised in dogs and man. Studies in people have suggested cytokines, particularly interleukin (IL)-5, play a role in instigating and perpetuating eosinophilia. This study sought to establish whether differences in gene expression, and concentration of selected, cytokines and chemokines were associated with eosinophilia in Rottweilers. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assays were used to quantify messenger ribonucleic acid (mRNA) encoding cytokines IL-4, -5, -10, -12p19, -12p35, -12p40, -18, interferon gamma (IFN- γ) and chemokines eotaxin-2 and -3 from peripheral blood mononuclear cell (PMBC) samples obtained from healthy dogs (breeds other than Rottweiler) with normal eosinophil blood counts (n = 5) and Rottweilers with normal (n = 6), mildly increased (n = 7) and high (n = 3) eosinophil blood counts. Quantification of plasma IFN- γ and IL-5 was performed using commercially available canine-specific enzyme-linked immunosorbent assays ELISAs. Cytokine mRNA was measurable in all samples, although eotaxin-2 and -3 were not detected. No significant differences in gene expression of any cytokine were found between groups (based on eosinophil count or breed). No significant difference in plasma IL-5 or IFN- γ concentration was present between groups. In conclusion, there were no significant differences in cytokine mRNA profiles or plasma IL-5 and IFN- γ levels between Rottweilers with increased eosinophil counts and Rottweiler and non-Rottweiler dogs with normal eosinophil counts.

Keywords: Cytokines; Dogs; IL-5; IFN- γ ; Hypereosinophilic Syndrome

1. Introduction

Eosinophils play an important role in innate immunity, inflammation and homeostatic host responses in dogs [1]. Eosinophil granules contain highly cationic proteins with cytotoxic and pro-thrombotic properties, and these cells are known to incite endothelial damage [1]. In particular, eosinophils can produce substances such as platelet activating factor that can alter bronchial and vascular smooth muscle tone, modify vascular permeability and stimulate chemotaxis of other inflammatory cells [1,2]. Eosinophils respond to different stimuli, particularly parasitic infection, where their cellular products can be protective to the host [3]. However, exuberant release of cellular products by eosinophils can contribute to tissue damage, particularly in the respiratory and gastrointestinal tracts [4,5].

Idiopathic eosinophilic diseases (or eosinophilic infiltration in the absence of an identifiable parasitic or allergic trigger) have been described in multiple breeds such

as German shepherd dogs, Cavalier King Charles spaniels, Siberian Huskies, Alaskan Malamutes and Rottweilers [3-11]. In many reports, Rottweilers appear to be overrepresented with idiopathic eosinophilic disease [4]. It has been postulated that Rottweilers may have an exaggerated response to allergic stimuli or parasite exposure [4,12].

Studies in people with eosinophilic disorders have highlighted the role of cytokines, particularly interleukin (IL)-5, in mediating eosinophil release [13,14]. IL-5 forms part of the type-2 (Th2) cytokine profile, and is essential for eosinophil maturation, differentiation and egress from the bone marrow [1,2]. In people with eosinophilic disorders, exaggerated release of IL-5 by reactive eosinophils has been demonstrated *in vivo* [5]. The chemokines eotaxin-2 (CCL24) and eotaxin-3 (CCL26) are also able to induce chemotaxis and release of reactive oxygen species in eosinophils via interaction with chemokine receptor CCR3 [15-18]. It is unknown what

cellular signalling drives the production of hypereosinophilic syndrome (HES) and other eosinophilic disease in dogs, or why Rottweilers are predisposed.

The aim of this study was to establish if there are definable differences in expression of mRNA encoding cytokines and eotaxins, or in plasma concentrations of IL-5 and IFN- γ in Rottweilers with variable eosinophil counts and healthy dogs of other breeds.

2. Materials and Methods

2.1. Animals

The study was conducted with full Murdoch University Animal Ethics Committee approval, based on NHMRC guidelines, with signed owner consent obtained. Dogs were identified upon presentation to Murdoch University Veterinary Hospital, as well as from Rottweilers identified from a haematological survey of breeding kennels performed previously (Mansfield, unpublished observations 2004). Each dog had a full physical examination, with particular focus on the detection of ectoparasites and evidence of allergic skin disease. Dogs with eosinophilia were further assessed for common aetiologies via faecal floatation, repeat haematology, heartworm antigen testing, skin scraping and an anthelmintic trial. Further tests, such as radiography, ultrasonography, adrenocorticotrophic (ACTH)-stimulation tests, fine-needle aspiration of affected organs, bone marrow biopsy or endoscopy, were performed on an individual basis, determined by the animals' degree of eosinophilia, physical examination findings and localising clinical signs.

2.2. Collection and Processing of Material

Whole blood was obtained via cephalic or jugular venipuncture into a 2.5 mL EDTA container and three 4.5 mL lithium heparin containers. A complete blood count (CBC) was performed using an automated haematology analyser (Advia, Siemens, Germany) and confirmed by a manual differential. Heparinised blood samples were processed by a 1:1 dilution with physiological saline followed by density gradient centrifugation (Lymphoprep, Axis-Shield, Norway), to isolate peripheral blood mononuclear cells (PBMC). The pelleted PBMC were washed, resuspended in freezing medium (foetal calf serum with 10% dimethyl sulfoxide) and stored in liquid nitrogen until shipped on dry ice for RNA isolation.

2.3. RNA Isolation

Total RNA was isolated from PBMC using a Macherey-Nagel NucleoSpin RNA II Isolation Kit (ABgene, Epsom, UK). Further deoxyribonuclease (DNase) digestion of the RNA solution was performed using RQ1 Ri-

bonuclease-Free DNase (Promega Corporation, Southampton, UK), as per the manufacturer's instructions. Quantity and quality of resulting RNA was assessed by automated gel electrophoresis (Experion, Bio-Rad Laboratories, Hemel Hempstead, UK) prior to storage at -70°C .

2.4. Primer and Hydrolysis Probe Design

Primers and probes for chemokines and cytokines were designed using Primer 3 [19] and MFold, from the canine specific GenBank sequences for IL-12p35 (accession number: U49085) and IL-12p19 (accession number: XM_538231) as described by Peters *et al.* (2004) (**Table 1**) [19]. IL-4, -5, -10, -12p19, -12p35, -12p40, -18, interferon (IFN)- γ , eotaxin-2 and eotaxin-3 specific assays have previously been validated in dogs [20-24]. Assays for potential housekeeper genes glyceraldehyde-3-phosphate dehydrogenase (G3PDH), hydroxymethylbilane synthase (HMBS), hypoxanthine phosphoribosyl-transferase 1 (HPRT1), ribosomal protein L13a (RPL13A), ribosomal protein S18 (RPS18), succinate dehydrogenase complex, subunit A (SDHA), TATA box binding protein (TBP), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein and zeta polypeptide (YWAZ) were performed as described by Peters *et al.* (2007) [24] (Supplementary **Table A1**).

2.5. Quantitative RT-PCR

Synthesis of complimentary DNA (cDNA) was performed using 500ng of random hexamers with the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI), according to the manufacturer's instructions. Quantitative RT-PCR was performed using HotStarTaq Master Mix (Qiagen Ltd., Crawley, UK), with sample incubations performed in an iCycler IQ (Bio-Rad Laboratories Ltd., Hercules, CA) during which fluorescence data were collected. The absence of genomic contamination of the RNA samples and nuclease-free water was confirmed prior to the reverse transcriptase reactions and none of the samples showed evidence of amplifiable genomic DNA with the G3PDH qPCR assay.

In order to determine the most appropriate housekeeper genes for the study, all 8 potential genes were quantified in all cDNA samples. A mean cycle threshold (Ct) value was calculated for each PBMC sample using the two measured Ct values. The mean Ct value was converted to a relative copy number value using the $E^{\Delta\text{Ct}}$ method (E: reaction efficiency) with ΔCt values calculated relative to the sample with the largest Ct (fewest gene copies). The geNorm VBA applet for Microsoft Excel was used to determine the level of stability of the test genes [25].

Relative expression data for each of the gene cytokine/chemokine targets was calculated using the gBase applet for Microsoft Excel (<http://medgen.ugent.be/qbase/>), normalised against the 3 most stable housekeeper genes. The sample with the fewest gene copies (latest Ct value) was given a relative copy number of 1 and all other samples were given values relative to this sample. These relative copy number results were used for the statistical testing and presentation in graphical form.

2.6. Quantification of IFN- γ and IL-5 Using ELISA

IFN- γ and IL-5 concentrations were measured in heparinised plasma samples with canine-specific ELISA kits for IFN- γ (R&D Systems, Minneapolis, USA) and IL-5 (USCN Life Science Inc., USA), according to manufacturer's instructions.

2.7. Statistical Analysis

Dogs were categorised as clinically healthy Rottweilers with normal eosinophil counts ("RottL"; $\leq 1.0 \times 10^9/L$); clinically healthy adult Rottweilers with mild increase in eosinophil counts ("RottM"; $1.0 - 2.0 \times 10^9/L$); Rottweilers that had a moderate to severe increase in eosinophil counts ($\geq 2.0 \times 10^9/L$) ("RottH"); and dogs of other breeds with normal eosinophil counts ("N").

For each cytokine, a Kruskal-Wallis analysis of variance and Mann Whitney *U*-test were performed to examine differences in relative copy numbers obtained in the plasma samples between the groups RottH, RottM, RottL and N, between RottL and N, and between RottH + RottM + RottL and N, as well as between RottH + RottM and RottL and N. As the results were not normally distributed (using a Kolmogorov-Smirnov test), non-parametric testing was used to examine differences between the groups for each cytokine. The results of each group for IL-5 and IFN- γ concentrations were compared using unpaired *t*-tests with unequal variances. For all analyses, differences were considered significant when $P < 0.05$.

The correlation coefficient was calculated to investigate the relation between IL-5 concentration and mRNA.

3. Results

3.1. Animals

There were a total of 21 dogs recruited for the study. There were 6 clinically healthy Rottweilers with normal eosinophil counts ("RottL"), 7 clinically healthy adult Rottweilers with increased eosinophil counts ("RottM"), 3 Rottweilers with hypereosinophilic disease ("RottH"); and 5 healthy dogs of other breeds with normal eosino-

phil counts ("N"). The other breeds included 1 English springer spaniel, 2 Whippets, 1 Greyhound and a Labrador crossbreed dog. The mean age of Rottweilers in groups RottM and RottH was 2.5 years (range 1 to 4 years), compared to a mean age of 3.27 years (range 1 to 6 years) of dogs in RottL and N respectively. There were four males (three entire) and one female (neutered) in N, four females (all entire) and two males (both neutered) in RottL, five females (one neutered) and two males (both entire) in RottM, and one neutered male and two females (one neutered, one entire) in RottH. The majority of Rottweilers were breeding animals kept in kennel situations.

3.2. Quantitative RT-PCR

Transcripts of cytokines IL-4, -5, -10, -12p19, -12p35, -12p40, -18, and IFN- γ could be quantified in all of the samples, but mRNA encoding eotaxins-2 and -3 were not detected in any sample. All samples were positive for the housekeeper genes tested. RPL13A, SDHA and YWAZ were selected as the most stably expressed genes for use in normalisation of the expression data (**Table 1**).

Plots of relative copy numbers for IL-5 and IFN- γ are shown in **Figures 1** and **2**, respectively. There were no significant differences in mRNA encoding between the groups for any cytokine. Despite a tendency for IFN- γ mRNA expression to be lower in RottH/RottM versus RottL/N, this did not reach statistical significance ($P = 0.062$).

3.3. Quantification of IFN- γ by ELISA

The trend for decreased IFN- γ mRNA expression in

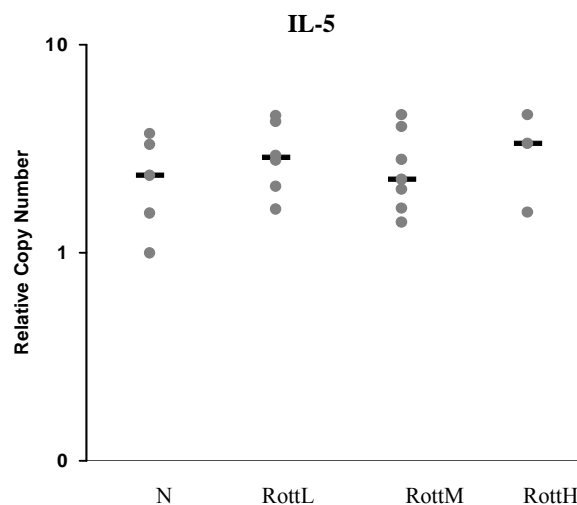


Figure 1. Plots of the relative copy number from QRT-PCR assays for IL-5. The horizontal line corresponds to the median value of that group. There were no significant differences in the transcription of mRNA encoding IL-5 between the groups.

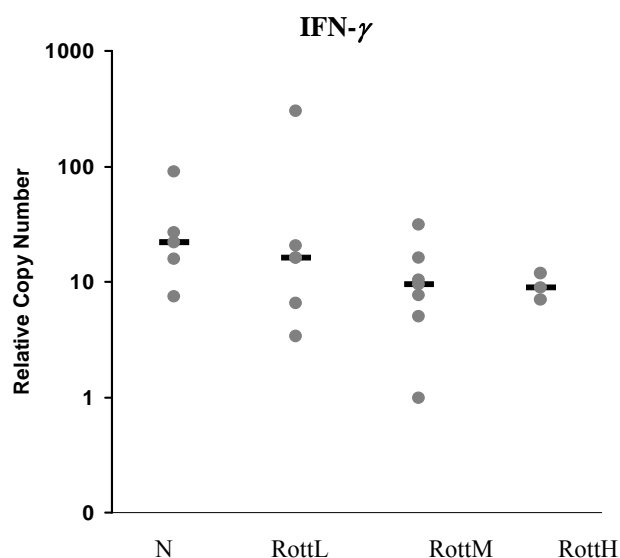


Figure 2. Plots of the relative copy number from QRT-PCR assays for IFN- γ . The horizontal line corresponds to the median value of that group. There were no significant differences in the transcription of mRNA encoding IFN- γ between the groups, although there was a tendency for IFN- γ mRNA expression to be lower in the diseased group versus the control dogs.

Rottweilers with increased eosinophil count did not correlate with changes in the plasma concentration of IFN- γ . The majority of dogs had IFN- γ concentrations below the minimum detectable level of the assay (**Figure 3**). Only 3/7 dogs from group RottM had measurable IFN- γ concentrations (16.45, 48.71, and 51.47 pg/mL). There were no significant differences between normal and diseased animals in the concentration of plasma IFN- γ .

3.4. Quantification of IL-5 by ELISA

Plasma IL-5 was detectable in 11 dogs. The median concentrations of IL-5 concentrations detected in RottH + RottM versus RottL+N were 14.325 pg/mL and 0.0 pg/mL respectively. The median concentrations of RottL + RottM + RottH versus non-Rottweiler dogs were 11.36 pg/mL and 14.09 pg/mL respectively. There were no significant differences between RottH + RottM versus RottL + N, or between breeds (**Figure 4**).

3.5. Correlation of Quantification of IL-5 and IFN- γ mRNA and Protein Quantified by ELISA

In this study, plasma IL-5 and IFN- γ measured by ELISA

Table 1. Results of quantification of transcripts for IL-4, IL-5, IL-10, IL-12p19, IL-12p35, IL-12p40, IL-18, and IFN- γ : All results were expressed relative to the sample with the fewest number of gene copies for each target (not always the same sample for different genes).

Sample type	Absolute eosinophils	IL-4	IL-5	IL-10	IL-12p19	IL-12p35	IL-12p40	IL-18	IFN- γ
N	0	1.0	3.8	2.6	1.8	2.1	1.8	1.0	15.7
N	0.2	9.4	1.0	3.0	2.1	1.0	3.4	1.9	22.0
N	0.3	9.4	2.4	4.3	2.0	3.2	5.5	3.8	7.5
N	0.1	24.7	1.6	1.0	1.0	1.4	1.0	3.9	90.5
N	0.1	6.2	3.3	3.4	3.2	1.5	5.3	3.4	26.9
RottL	0.3	20.9	4.3	1.2	3.3	1.2	5.1	1.4	6.6
RottL	0.5	8.4	2.8	2.3	2.2	3.7	4.7	5.3	301.8
RottL	0.6	4.9	1.6	2.3	1.5	2.0	3.8	2.4	16.1
RottL	1	12.0	4.6	1.9	2.2	1.5	2.1	5.4	16.1
RottL	0.6	5.9	3.0	2.3	2.6	1.5	2.4	3.1	3.4
RottL	0.7	10.2	2.1	2.0	1.5	1.0	7.3	4.0	20.7
RottM	0.9	17.1	1.6	3.7	2.0	1.8	3.3	2.9	10.4
RottM	0.7	11.4	2.3	3.2	3.6	2.1	5.8	3.4	5.1
RottM	0.8	6.0	2.8	1.3	2.9	1.3	5.3	5.4	1.0
RottM	0.9	11.3	4.6	1.7	3.2	1.4	5.4	3.7	16.3
RottM	1.1	5.7	2.0	1.7	2.0	1.3	5.6	2.5	7.6
RottM	1.2	9.0	4.1	2.3	2.1	5.2	2.0	2.7	31.7
RottM	0.7	29.3	1.4	3.4	2.0	2.1	2.9	3.0	9.5
RottH	2	72.9	3.4	4.6	2.6	2.8	2.2	2.5	7.0
RottH	2.3	2.0	1.6	2.9	2.3	1.3	5.9	3.5	9.0
RottH	3.6	174.9	4.6	3.2	1.5	2.3	3.3	6.5	11.9

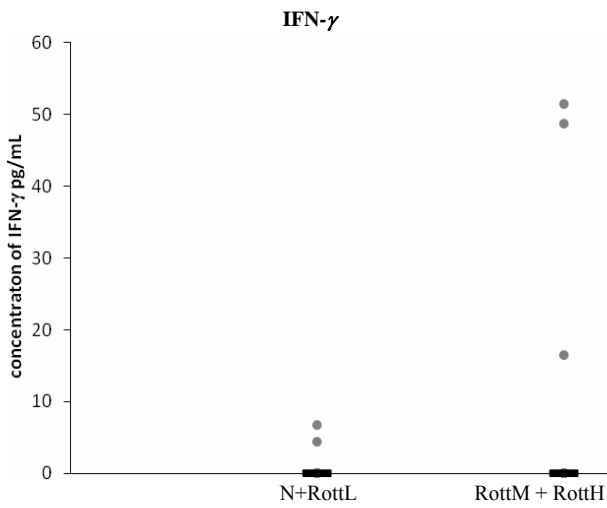


Figure 3. Plot of the concentrations of serum IFN- γ determined by ELISA. The horizontal line corresponds to the median value of that group. There were no significant differences between normal and diseased animals in the concentration of plasma IFN- γ quantified by ELISA.

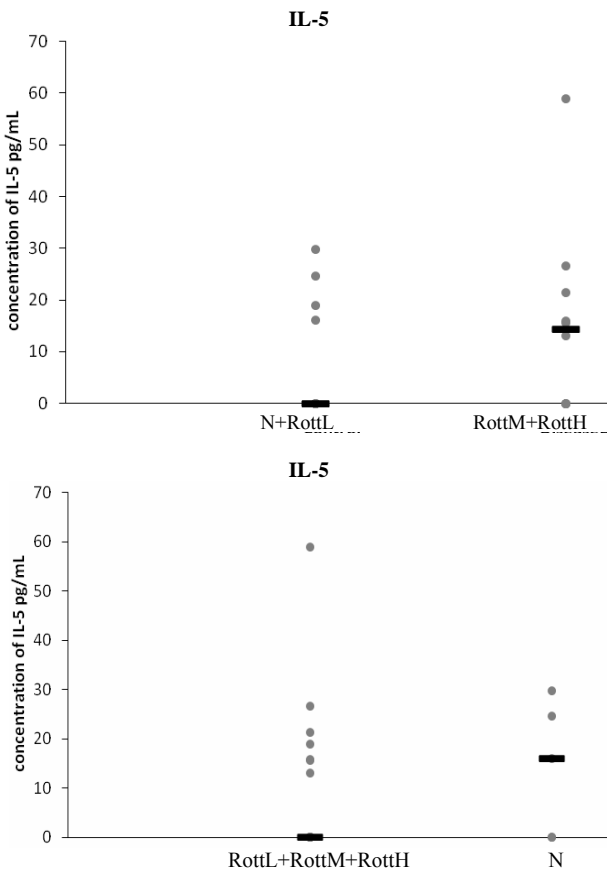


Figure 4. Plots of serum IL-5 concentrations quantified by ELISA. The horizontal line corresponds to the median value of that group. There were no significant differences between normal and diseased animals in the concentration of plasma IL-5 quantified by ELISA, or between Rottweilers and non-Rottweiler dogs.

was poorly correlated with mRNA encoding IL-5 and IFN- γ quantified through RT-PCR. The correlation coefficients for IL-5 and IFN- γ were 0.1101 and -0.108, respectively.

4. Discussion

To our knowledge, this is the first study reporting quantification of mRNA encoding cytokines in dogs with peripheral blood eosinophilia.

IFN- γ is a pro-inflammatory cytokine that is predominantly produced by type 1 (Th1) cells and natural killer cells upon activation by IL-12 [1]. Decreased IFN- γ , and by association down-regulation of the Th1 response, may be observed with eosinophilia in people? [26]. This observation suggests that IFN- γ is unlikely to play a major role in HES, and therefore should be expressed at lower levels in dogs with eosinophilia than in those without. However, as there was no difference in IFN- γ , as determined by mRNA or ELISA, the role of IFN- γ in canine eosinophil physiology remains unknown.

Increased IL-5 plasma concentrations have been demonstrated in a proportion of people diagnosed with HES [27]. However, this production is unrelated to aetiology (neoplastic vs. idiopathic) and is thought to be a consequence of the eosinophils themselves rather than the cause of the eosinophilia [1,2,13,27].

The lack of significant differences in mRNA and protein concentrations of IL-5 between groups in our study could be due to factors such as time of sampling and the type of samples collected. Circulating IL-5 concentrations have been observed to rise approximately two to four weeks preceding peripheral eosinophilia in some people [27], whilst episodic increases in IL-5 concentrations have been noted in others [27]. Therefore, serial sampling may have proved more useful in determining IL-5 trends in this study. It is also possible that blood concentration of IL-5 does not reflect tissue concentrations, and measurement of IL-5 in bone marrow or intestinal tissue may have produced different results. Such a level of invasive diagnostic sampling was not possible in this population of dogs. The small number of affected dogs assessed, the lack of serial sampling, and the variable clinical presentations were also factors that could have contributed to a lack of obvious differences in cytokine profiles.

It is also possible that eosinophilia in Rottweilers does not occur in response to IL-5-mediated mechanisms. Monoclonal expansion of the eosinophils themselves, independent of cytokine stimulation, has been described in people [5]. Investigation into other cytokines that stimulate eosinophil production, such as IL-3 or granulocyte-macrophage colony stimulating factor (GM-CSF) may shed more light on the immunopathogenesis of eosinophilia in dogs.

There has been a focus on the development of anti-IL-5 therapy in the management of eosinophilic disorders in man [27]. Studies assessing two different anti-IL-5 antibodies; SCH55700 (Schering-Plough, Deerfield, ILL) and mepolizumab (GlaxoSmithKline, London, UK) demonstrated effective and prolonged control of peripheral eosinophilia in people, regardless of underlying cause, disease variant, or baseline IL-5 concentration [14,27]. Although significantly increased IL-5 concentrations in our eosinophilic dogs would have lent weight to use of anti-IL-5 antibodies, normal baseline IL-5 concentrations should not preclude further investigation into its use. Additional studies are needed to determine safety, efficacy, optimal dosing, as well as observation for rebound eosinophilia in the canine model.

Eotaxins serve as chemoattractants mainly for eosinophils via inducing processes such as actin polymerization [15,16,28]. Eotaxins are produced by various tissue sources such as bronchial epithelial and intestinal cells and increased plasma eotaxin concentrations have been documented in people with allergic disease amongst others [29-32]. There is minimal information regarding plasma detection of eotaxins in dogs, with most veterinary studies reporting the detection and quantification of eotaxin-2 and -3 in tissue samples [17,21,33]. Inability to detect expression of eotaxin mRNA in our cohort of dogs with eosinophilia was unexpected. Furthermore, daily fluctuations in canine plasma eotaxin concentrations have not been investigated. As previously, the small number of dogs assessed, and the type of sample assessed may also have influenced the results.

5. Conclusion

In summary, there was no significant difference in cytokine mRNA expression (IL-4, -5, -10, -12p19, -12p35, -12p40, -18, and IFN- γ) and quantification via ELISA of IL-5 and IFN- γ in blood between Rottweilers with varying degrees of eosinophilia compared to Rottweiler with normal eosinophil counts and health dogs of other breeds. Additional genetic, molecular, cytokine and chemokine studies including larger numbers of affected dogs are warranted before accurate conclusions can be made about the pathogenesis of eosinophilic disease in Rottweilers.

6. Conflicts of Interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Supplementary Table A1. Primer and probe sequences used for quantitative RT-PCR.

Primer Set	Product Length	Forward Primer	Reverse Primer	Probe
		(5'-3')	(5'-3')	(5'-3')
G3PDH	90	TCAACG-GATTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	TexasRed-CAGGGCTGCTTTAACTCTG GCAAAGTGA-BHQ-2
HMBS	112	TCACCATCGGAGCCATCT	GTTCCACCACGCTCTTCT	FAM-CAGCATCATAGGGTTCTCCCGC-B HQ-1
HPRT1	123	CACTGGGAAAACAATGCAGA	ACAAAGTCAGGTTA-TAGCCAACA	FAM-TGCTGGTAAAAGGACCCTCG-BH Q-1
RPL13A	87	GCCGGAAGGTTGTAGTCGT	GGAGGAAGGCCAGGTAATTC	FAM-TGTGAAGGCATCAACATTCTGGC A-BHQ-1
RPS18	116	TGCTCATGTGGTATTGAGGAA	TCTTATACTGGCGTGGATTCTG	FAM-CACTGAGGATGAGGTGGAACGTGT G-BHQ-1
SDHA	92	GCCTGGATCTCTTGATGGA	TTCTTGGCTCTTATGCGATG	FAM-TGTCGTGGTGTATTGCACTGTGCA- BHQ-1
TBP	96	CTATTCTTGGTGTGCAT-GAGG	CCTCGGCATTCAGTCTTTTC	FAM-TCGCTAAGAAGCGTGTACTGGGGA TG-BHQ-1
YWHAZ	94	CGAAGTTGCTGCTGGTGA	TTGCATTTCCTTTTGCTGA	FAM-AGGGATTGTGGATCAGTCACAACA AGC-BHQ-1
IL-4	123	GCTCCAAAGAACACAAGCGA	CATGCTGCTGAGGTTCCCTGT	Texas Red-TGCAGAGCTGCTACTGTACTG CGGC-ELLE
IL-5	158	GACTGGTGGCAGAGACCTTGA	CGTGGGCAGTTTGGTTCTTC	FAM-CGAACTTGGCTGATAGGCGATGG-B HQ-1
IL-10	101	CGACCCAGACATCAAGAACC	CACAGGAAGAAATCGGTGA	FAM-TCCCTGGGAGAGAAGCTCAAGACC C-BHQ-1
IL-12p19	108	GTGATCCCCAAGGACTCAAA	GCTCCCCTGTGAAAATGTCT	FAM-AAAGGATCCACCAGGGCCTGG-BH Q-1
IL-12p35	146	CGTCAGCAACACGCTTCA	AACTCTCATTGTTAATTC-CAG	FAM-CCAGCACAGTGGAGGCCTGC-BHQ- 1
IL-12p40	109	CAGCAGAGAGGGTCA-GAGTGG	ACGACCTCGATGGGTAGGC	FAM-TGGAGTGTGAGGAGGCAGTGC-B HQ-1
IL-18	139	TTAAAGCGGAAAGTGAT-GAAGG	TCGGGCATATCCTCAAATACA	Texas Red-GAAATTTGAACGAC CAAGTCTCTTCG-ELLE
IFN-γ	113	TCAACCCCTTCTCGCCACT	GCTGCCTACTTGGTCCCTGA	FAM-CCCCACCCGAACCTCTTCCT-BHQ-1
Eotaxin-2	95	AAGGCAGGAGTGGTCTTCAC	GCCTCTATGTTCTTCAT-CAACTTCT	FAM-TGTGGCGATCCCCAGCTGCA-BHQ- 1
Eotaxin-3	128	TCTGCTGCTTCCAATATAGCC	TGCACAGACTTTATGGCCTTT	FAM-AGCTGCTCCCAGCAGGCTGTGATA T-BHQ-1