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Original Article

Effect of immunosuppressive drugs on cytokine production in canine whole blood stimulated with lipopolysaccharide or a combination of ionomycin and phorbol 12-myristate 13-acetate

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Abstract

A pharmacodynamic assay has been previously developed to monitor ciclosporin treatment in dogs by assessing inhibition of cytokine transcription after whole blood stimulation with 12-myristate 13-1 acetate and ionomycin (PMA/I). In this study, whole blood stimulation with either PMA/I or lipopolysaccharide (LPS) was used to assess the effect of multiple drugs (azathioprine, ciclosporin, mycophenolate, leflunomide and prednisone) after a 7-day treatment course on production of cytokines measured with a multiplex assay in healthy dogs (n = 4 for each treatment). Interleukin-10 (IL-10), interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) were significantly activated by PMA/I stimulation and IL-6, IL-10 and TNFα by LPS stimulation, in the absence of immunosuppressive drugs. After ciclosporin treatment, IL-10, IFNγ and TNFα production was significantly reduced after stimulation with PMA/I compared to pre-treatment. After prednisone treatment, TNFα production was significantly reduced after stimulation with PMA/I or LPS compared to pre-treatment. No significant change was observed after treatment with azathioprine, leflunomide or mycophenolate. This methodology may be useful to monitor dogs not only treated with ciclosporin, but also with prednisone or a combination of both. Further studies are needed to assess the use of this assay in a clinical setting.

Keywords: Canine, cytokines, immune monitoring, immunosuppressive drug, whole blood stimulation.

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Introduction

Immunosuppressive drugs are regularly used in dogs for a wide range of diseases, but monitoring is typically limited to clinical response and adverse effects (Whitley & Day 2011). A pharmacodynamic assay has been developed and validated in healthy dogs and is currently used to assess the amount of immune suppression in dogs receiving ciclosporin therapy. This assay utilizes quantitative real-time polymerase chain reaction on blood stimulated with 12-myristate 13-1 acetate and ionomycin (PMA/I) (Riggs *et al.* 2013). Previous pharmacodynamic studies have shown Interferon gamma (IFNγ) and interleukin 2 (IL-2) to be consistently inhibited using high doses of oral ciclosporin (10 mg/kg PO q12 h) and inconsistently inhibited using a lower dose (5 mg/kg PO q24 h) in healthy dogs(Archer *et al.* 2011; Fellman *et al.* 2016). Whole blood stimulation has the potential to be used to assess the effect of other

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immunosuppressive drugs, as shown in rats and humans (Liu *et al.* 2009; Ai *et al.* 2013).

The aims of this study were to (1) determine which of the IFNγ, IL-2, IL-6, IL-10 and tumour necrosis factor alpha (TNFα) are reliably and significantly increased after whole blood stimulation with PMA/I or LPS; and (2) determine the effect of five oral immunosuppressive medications (prednisone; azathioprine; ciclosporin; mycophenolate mofetil; and leflunomide) on cytokine production in healthy dogs.

Material and methods

Healthy, purpose-bred, adult Walker hounds were deemed clinically normal without significant abnormalities on physical examination, complete blood count, serum biochemistry profile, urinalysis, faecal flotation and heartworm testing. Study protocols and animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee (MSU-IACUC #14-077).

Each immunosuppressive treatment was randomized for each dog and administered for 7 days (with blood collected on day 8, 2 h after morning drug administration) with a minimum 3-week washout between treatments (dosage listed in Table 1). Blood was collected via jugular sampling with a 20-gauge needle before and after treatment and transferred to sodium heparin tubes. All cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS (#L3137) stimulation was performed in a sterile 24-well plate with dilution of 0.5 mL of whole blood in 1 mL Roswell Park Memorial Institute 1640 (RPMI) medium (#R8758) at a final concentration of 10 ng/mL. PMA/I stimulation was performed in a sterile 48-well plate without dilution of 1.2 mL of whole blood with PMA (#P8139) and ionomycin (#IO634) at final concentrations of 12.5 ng mL^{-1} and 0.8 μmol L^{-1}, respectively. The same volume of RPMI was used for control samples. Plates were incubated for 5 h at 37°C and 5% CO_2. Samples were centrifuged for 10 min at 2000 *g* and supernatant collected and stored at −80°C until analysis.

Cytokines were measured in duplicate in a multiplex assay validated for dogs (Milliplex®, CCYTOMAG-90K) following manufacturer's instructions. Standard curves consisted of six to seven dilutions in duplicates after subtraction of blanks. Two quality controls ensured adequate performance of the plates. Minimum detectable concentrations for this assay are 3.5 pg mL^{-1} for IL-2, 3.7 pg mL^{-1} for IL-6, 8.5 pg mL^{-1} for IL-10, 18 pg mL^{-1} for IFNγ and 6.1 pg mL^{-1} for TNFα. Samples with a value within the range of the standard curves were quantified and samples with values lower than the lowest standard were given that value (9.7 pg mL^{-1} for IFNγ and 12.2 pg mL^{-1} for the other analytes). No samples were over the range of the standard curves. Cytokines were measured over three consecutive days and all samples for a given dog (before and after treatment, stimulated and unstimulated) were run on the same plate to avoid inter-assay variability. Cytokine production for each activator was assessed by comparing cytokine concentration pre- and post-stimulation in untreated dogs (n = 13). Drug effect was assessed by comparing cytokine production after stimulation before and after 7 days of treatment (n = 4 for each drug before and after treatment).

All statistical analysis was performed using a commercial software (Stata Statistical; StataCorp LLC. 2017. Release 15. College Station, TX). Visual assessment of histograms indicated that data were not normally distributed. Accordingly, non-parametric tests were used in the analysis of the data. To test for carry-over effects, Kruskall-Wallis tests were conducted for each cytokine and activator combination to determine if activated pre-treatment cytokine values differed among the drug groups. Wilcoxon Signed Rank tests were used to determine if pre-treatment cytokine values were different for each cytokine and activator combination. A P = 0.05 was used to determine statistical significance.

Finally, a rank transformation was implemented together with mixed effects linear regression accounting for individual dog effect to compare each cytokine and activator combination before and after treatment to overcome the distributional assumptions (Iman & Conover 1979). Significant P-value was determined with the Holm-Bonferroni method to account for multiple hypothesis tests (Holm 1979).

Table 1. Effect of five different immunosuppressive drugs on cytokine production after activation with PMA/I[†] (a) or LPS[‡] (b).

a)

	IFNγ			IL-10			TNFα		
	Pre[§]	Post[¶]	*P*-value	Pre[§]	Post[¶]	*P*-value	Pre[§]	Post[¶]	*P*-value
Azathioprine	1040.1	1239.3	0.312	192.8	270.6	0.783	192.8	270.6	0.518
2 mg/kg q24 h	[57.4–5649.0]	[9.7–3725.6]		[15.9–753.0]	[12.2–506.9]		[15.9–753.0]	[12.2–506.9]	
Ciclopsorin	2035.2	9.7	**<0.001***	354.3	12.2	**<0.001***	2086.8	12.2	**<0.001***
10 mg/kg q12 h	[47.0–4556.7]	[9.7–9.7]		[47.1–555.8]	[12.2–12.2]		[487.0–3308.8]	[12.2–13.5]	
Leflunomide	654.6	1438.3	0.198	193	252.5	0.443	1141.8	2316.4	0.087
4 mg/kg q24 h	[9.7–2868.3]	[57.6–4975.2]		[12.2–740.9]	[12.2–697.8]		[216.9–4885.7]	[1009.2–5477.3]	
Mycophenolate	1127.2	678.1	0.788	538.0	338.6	0.093	2160.9	1753.0	0.857
10 mg/kg q12 h	[644.2–4185.6]	[311.3–3538.7]		[87.2–828.2]	[12.2–501.3]		[682.2–2644.5]	[813.1–2956.9]	
Prednisone	1008.0	44.5	0.017	142.4	43.4	0.031	1575.0	395.3	**0.013***
2 mg/kg q24 h	[9.7–2831.0]	[9.7–333.9]		[12.2–155.3]	[12.2–155.3]		[322.6–4605.0]	[52.4–1269.0]	

b)

	IL-6			IL-10			TNFα		
	Pre[§]	Post[¶]	*P*-value	Pre[§]	Post[¶]	*P*-value	Pre[§]	Post[¶]	*P*-value
Azathioprine	590.1	505.9	0.283	397.7	419.4	0.327	2882.9	2421.5	0.484
2 mg/kg q24 h	[345.8–1449.0]	[239.3–1132.2]		[233.5–557.7]	[332.3–645.6]		[1640.2–6532.2]	[1695.4–4349.0]	
Ciclopsorin	525.2	598.7	1	343.1	284.0	0.115	3096.4	3236.4	0.600
10 mg/kg q12 h	[503.3–712.6]	[373.9–827.7]		[301.3–734.9]	[206.6–442.9]		[2273.4–5370.9]	[1495.7–3714.0]	
Leflunomide	510.1	355.4	0.025	397.0	365.4	0.234	2282.5	2281.8	0.633
4 mg/kg q24 h	[297.7–1112.1]	[267.0–463.5]		[350.3–706.5]	[278.6–458.4]		[1390.2–4183.3]	[1053.1–3127.0]	
Mycophenolate	645.9	561.5	0.732	414.6	496.7	0.779	2643.7	3024.8	0.567
10 mg/kg q12 h	[479.8–869.0]	[463.6–831.7]		[367.8–678.3]	[315.1–733.2]		[2488.6–4633.2]	[2028.8–6295.5]	
Prednisone	397.5	276.6	0.083	242.6	278.3	0.128	2290.5	1123.0	**0.003***
2 mg/kg q24 h	[301.8–572.1]	[207.4–446.2]		[230.4–283.5]	[250.9–541.9]		[1630.0–3250.0]	[1043.0–1588.0]	

All results are given as median cytokine concentration in pg/mL and range. Bold value indicates significance of *P*-value. [†]Ionomycin and 12-myristate 13-1 acetate.[‡]Lipopolysaccharide.[§]Pre-treatment concentration.[¶]Post-treatment concentration.*Statistically significant difference between pre- and post-treatment concentration after Holm-Bonferroni correction.

Table 2. Baseline cytokine production after 5 h of incubation (37°C and 5% CO_2) without or with activation using either PMA/I[†] or LPS[‡] in 13 healthy dogs. Results are given as median cytokine concentration in pg/mL and range.

Cytokine	PMA/I[†]					LPS[‡]				
	UA[§]	A[¶]	n[‖]	Fold**	*P*-value	UA[§]	A[¶]	n[‖]	Fold**	*P*-value
IL-2	12.2 [12.2 -66.9]	12.2 [12.2–60.0]	3	1.0 [0.5–1.0]	0.25	12.2 [12.2–12.2]	12.2 [12.2–12.2]	0	N/A	N/A
IL-6	12.2 [12.2–65.0]	12.2 [12.2–79.7]	3	1.0 [0.7–1.4]	0.75	12.2 [12.2–18.9]	539.7 [301.8–1449.0]	13	41.9 [24.7–118.8]	**<0.001**
IL-10	12.2 [12.2–42.3]	322.2 [12.2–753.0]	12	26.4 [1.0–61.7]	**<0.001**	85.9 [12.2–165.7]	304.3 [230.4–734.9]	13	3.8 [2.6–24.9]	**<0.001**
IFNγ	9.7 [9.7–53.7]	1755.5 [9.7–5649.0]	12	59.0 [1.0–528.4]	**<0.001**	58.7 [9.7–119.9]	47.0 [9.7–243.2]	10	1.0 [0.1–4.8]	0.42
TNFα	12.2 [12.2–12.2]	1837.0 [322.6–4885.7]	13	150.6 [26.4–400.5]	**<0.001**	12.2 [12.2–12.2]	2899.4 [1630.0–6532.2]	13	237.7 [133.6–535.4]	**<0.001**

Bold value indicates significance of *P*-value [†]12-myristate 13-1 acetate and ionomycin.[‡]Lipopolysaccharide.[§]Un-activated.[¶]Activated.[‖]Number of dogs with measurable cytokine concentration after activation (maximum 13 dogs).** Fold increase between pre- and post-stimulation samples. *n*: number of dogs with measurable cytokine concentration after stimulation.

Results

No drug carry over effects were noted (results not shown). Cytokine production after stimulation with LPS or PMA/I is summarized in Table 2; IL-6, IL-10 and TNFα were significantly increased after LPS stimulation and IL-10, IFNγ and TNFα after PMA/I stimulation. No significant increase in IL-2 was noted.

A significant decrease in TNFα production was noted after treatment with ciclosporin and prednisone after PMA/I stimulation and with prednisone after LPS stimulation (Table 1). Significant decreases in IFNγ and IL-10 productions were also noted with ciclosporin (PMA/I stimulation). Cytokine production inhibition is reported in Table S1.

Discussion

We identified which cytokines reliably increased production with either PMA/I or LPS stimulation in our conditions; PMA/I stimulation is commonly used to activate lymphocytes whereas LPS is used to activate monocytes (Rossol *et al.* 2011). Although LPS can activate T cells (induction of adhesion), monocytes and macrophages are the main cell types producing cytokines after LPS activation (Zanin-Zhorov *et al.* 2007). Monocytes/macrophages are the main source of IL-6 and TNFα and lymphocytes of IL-2 and IFNγ (Turner et al. 2014). Our findings are consistent with this, with an increased production of IL-6 after LPS stimulation and increased production of IFNγ after PMA/I stimulation. Both IL-10 and TNFα have an increased production after stimulation with either PMA/I or LPS, similar to findings in human macrophages (Agbanoma *et al.* 2012). IL-10 production by macrophages after LPS stimulation was also noted in the same study.

The absence of IL-2 in the supernatant after 5 h of stimulation was unexpected as increased IL-2 mRNA after 5 h of PMA/I stimulation and intra-cellular IL-2 after 6 h have been documented in dogs (Fellman *et al.* 2011; Riggs *et al.* 2013). Increased production of IL-2 is also reported in rat whole blood culture after 4 h (Ai *et al.* 2013). To exclude degradation after storage, the experiment was repeated and IL-2 measured immediately but remained undetectable. Explanations for this unexpected result include either a lack of sensitivity of the Milliplex® multiplex assay used to detect IL-2 or a delay between mRNA production, as mRNA was shown to be increased in a previous study and mRNA translation (Riggs *et al.* 2013). Similarly, the intra-cellular IL-2 detected by fluorescence-activated cell sorter in another study might be released at a later stage in the supernatant (Archer *et al.* 2011). Finally, post-translational modification could also account for the lack of IL-2 detection. A lack of activation is deemed

unlikely in view of the increase in other cytokines post-stimulation with PMA/I. Because IL-2 was not detectable upon stimulation with either activator in the present study, it was not further assessed.

Treatment dosage was chosen accordingly to reported immunosuppressive dosages for each drug and previous experiments (Archer *et al.* 2014; Archer 2017). Inhibition >50% of IL-10 and IFNγ after PMA/I stimulation was observed in the ciclosporin treated samples, as previously reported (Archer *et al.* 2011). Interestingly, the same pattern was consistently seen with prednisolone, but not with the three other drugs tested. These results highlight that the precise concentration to achieve immunosuppression with azathioprine, leflunomide and mycophenolate in an individual dog is unknown. Higher dosage, longer treatment duration or possibly a different evaluation method (such as lymphocyte proliferation) are likely needed to identify changes indicative of immune suppression.

TNFα production was inhibited in all dogs treated with ciclosporin or prednisolone after PMA/I stimulation, but only in dogs treated with prednisolone after LPS stimulation. Ciclosporin specifically targets T lymphocytes, whereas prednisolone affects most cells including lymphocytes and monocytes (Coutinho & Chapman 2011; Archer *et al.* 2014). A previous study reported an increase in TNFα mRNA after *in vitro* treatment with ciclosporin and activation with phytohaemagglutin (PHA) (Kobayashi *et al.* 2007). Several reasons can explain this discrepancy including measurement of mRNA rather than protein concentration, the use of different activators (PHA), post-translational controls, duration of incubation (24 h) and different conditions (use of peripheral blood mononuclear cells, PBMC). The decrease in TNFα with ciclosporin and prednisolone observed in our study is consistent with findings in other species (Flores *et al.* 2004; Ai *et al.* 2013). The absence of TNFα inhibition with LPS stimulation in dogs treated with ciclosporin compared with prednisolone emphasizes two aspects. Firstly, LPS stimulates cytokine secretion specifically in monocytes independently from lymphocytes, whereas ciclosporin targets lymphocytes only (Zanin-Zhorov *et al.* 2007; van Dooren *et al.* 2013). Secondly, prednisolone not only affects lymphocytes, but also monocytes, which can explain the inhibition of TNFα secretion after LPS stimulation (Whitley & Day 2011).

Limitations of this study include the small number of healthy dogs included and the fact that these results need to be confirmed in diseased dogs. Whole blood was used rather than separated PBMC, because similar cytokine profiles after stimulation have been reported with both methods, there is less manipulation of blood which means less risk of contamination or cell activations, blood is a more physiological surrogate than PMBC and finally repeatability has been reported to be higher with whole blood than PBMC (Thurm & Halsey 2005; van Dooren *et al.* 2013; Duffy *et al.* 2017).

Conclusion

In summary, immune suppression with ciclosporin and prednisolone at the dosage used in this study can be assessed with cytokine concentrations in whole blood stimulated with either LPS or PMA/I after 7 days of treatment. Ciclosporin and prednisolone do not have the same inhibition profile after LPS stimulation, which suggests that the use of different stimulators should be used to assess the effect of different drugs. However, further studies are needed to confirm these preliminary findings in dogs with clinical disease and to assess the effect of the use of concurrent treatments with ciclosporin and prednisolone on cytokine production.

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Conflict of interest

None.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guideline page, have been adhered to and the study protocols and animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee (MSU-IACUC #14-077). The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

Contribution

JRSD was involved in study conception and design, acquisition of data, interpretation of data, manuscript preparation and submission. TMA was involved in study conception and design, interpretation of data and manuscript revising. LN was involved in acquisition of data and manuscript revising. SF was involved in data analysis and interpretation. CM CSM instead of CM was involved in study conception and design, data interpretation and manuscript revising. All authors have read and approved the final manuscript.

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Supporting information

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

Table S1. Cytokines inhibition after PMA/I[†] or LPS[‡] activation following a week of oral immune-suppressive treatment.