

Effects of bovine lactoferrin on the immune system and the intestinal microflora of adult dogs

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Eighteen Beagle dogs were used to evaluate the effects of bovine lactoferrin (bLF) on immune function and faecal microbial populations. The study comprised three feeding periods, each lasting four weeks. After an initial control Period 1, six dogs per group were supplemented with 0, 120 and 1800 mg bLF/kg dry diet, respectively (Period 2). In Period 3 dogs received again control diets. Peripheral blood mononuclear cell subsets, lymphocyte proliferative response to concanavalin A, phytohaemagglutinin and pokeweed mitogen and plasma IgA and IgG concentrations were analysed. The faecal concentrations of aerobic and anaerobic bacteria, *Escherichia coli*, *Clostridium perfringens*, *Lactobacillus* spp. and *Bifidobacterium* spp. were determined by cultural methods. Supplementation of bLF increased the number of monocytes, T cells and cytotoxic T cells in the blood and the proliferative response of peripheral blood mononuclear cells. The leukocyte counts were not affected, except monocytes that increased after the supplementation with bLF. Plasma immunoglobulin concentrations were unchanged by treatment. Dogs supplemented with bLF tended to have lower faecal concentrations of *E. coli* and *Clostridium perfringens*. In conclusion, bLF seems to alter indices of the cellular immune response and faecal microbial populations of healthy adult dogs.

Keywords: lactoferrin; diet; dog; immune system; microflora

1. Introduction

Several milk proteins have specific functions like enzyme and enzyme inhibitor activities, nutrient binding properties, mediator, hormone and growth factor activities and immunomodulatory effects. One of these bioactive compounds in milk is lactoferrin (LF). It is not only found in milk but also in other body secretions including blood, saliva, tears, urine, seminal fluid, and bile (Baveye et al. 1999). Lactoferrin is also stored in specific granules of polymorphonuclear granulocytes from which it is released during inflammation (Cramer et al. 1985). It has a wide variety of functions in biological systems and is considered as an important innate defence protein because of its antimicrobial (Chierici 2001) and immunomodulatory effects (Baveye et al. 1999). Probably lactoferrin's best-known role is iron binding. Belonging to the transferrin family, lactoferrin consists of a single polypeptide chain folded into two globular lobes, each with one iron binding site (Levay and Viljoen 1995). The growth of many microorganisms is inhibited by iron deprivation. Besides this bacteriostatic effect, direct bactericidal action has been

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demonstrated (Arnold et al. 1981). Immunofluorescence studies have shown that LF binds directly to the surface of microorganisms (Arnold et al. 1977) and damages the outer membrane of gram-negative bacteria causing the release of lipopolysaccharides (Ellison et al. 1988, 1990). Orally administered LF is effective to suppress proliferation of various bacteria and bacterial translocation in the intestine of different animal models (Teraguchi et al. 1995a, 1995b). Proteolytic activity in the gastrointestinal tract does not abolish its activity, since the pepsin-liberated peptides of LF have potent activity. Peptides generated by pepsin cleavage of human and bovine LF were more effective against *Escherichia coli* than undigested LF (Bellamy et al. 1992). Orally administered bovine LF (0, 60 or 120 mg bLF/d) to healthy adult dogs did not show an effect on the faecal microflora except for a reduction of *Streptococcus* spp. (Pope et al. 2006).

In addition to the antibacterial properties, LF has been reported to modulate the immune system in a variety of ways, notably by regulating the production of pro-inflammatory cytokines (Machnicki et al. 1993; Brock 1995). A direct interaction between LF and immune cells could be shown and oral administration of LF or lactoferricin had a protective effect in various infection models (Tomita et al. 2002). The results suggest that effects of oral LF are mediated by modulation of the immune system and the intestinal microflora.

The aim of this study was to examine if bovine lactoferrin exerts effects on the immune system and the faecal microflora of healthy, adult dogs.

2. Materials and methods

2.1. Animals and diet

Eighteen beagles (11 females and seven males, mean age 2.5 years, mean BW 13.6 ± 2.0 kg) were included in this study. A commercially prepared extruded dry diet (Mera[®]Dog Reference, Kevelaer-Wetten, Germany) was fed to the dogs (Table 1). Bovine lactoferrin (bLF) was incorporated after dilution with water under vacuum to a final concentration of 0, 120 and 1800 mg bLF/kg diet. Fresh drinking water was offered *ad libitum*.

Table 1. Chemical composition of dog food (Mera[®]Dog Reference*, Kevelaer-Wetten, Germany) containing different amounts of bovine lactoferrin[#].

	Bovine lactoferrin (bLF) [mg/kg diet]		
	0	120	1800
Dry matter [%]	92.9	93.5	92.6
Crude protein [%]	24.7	24.7	25.5
Crude ash [%]	6.1	6.1	6.2
Ether extract [%]	14.5	14.0	13.8
Crude fibre [%]	1.9	2.0	2.1

*Ingredients: Corn, poultry meat meal, greaves meal, poultry fat, beet fibre, fish-meal, linseed, brewer's yeast, dehydrated egg, minerals; [#]Analysed on an air-dry basis.

2.2. Experimental design

The experiment consisted of three periods each lasting four weeks. In the first four weeks (Period 1) all dogs obtained the control diet. From week five to eight (Period 2), Groups 1, 2 and 3 received 0 mg, 120 mg, and 1800 mg bLF/kg dry diet, respectively. In Period 3

(week 9–12) all dogs obtained again the control diet. Dogs were allotted to one of the three treatments by age and gender. All procedures were approved by the ethic committee according to the Austrian animal protection law.

2.3. Sample collection

At the end of each period blood was collected by venipuncture into an evacuated tube. EDTA blood was used for white blood cell counts, immunoglobulin determination and for flow cytometric analysis, heparin blood for the lymphocyte proliferation test. A fresh faecal sample was collected and immediately processed to investigate the potential impact on the gut flora.

2.4. Determination of white blood cells and faecal microbiology

Determination of white blood cells (WBC) was performed with a Beckman Coulter Counter ZBI®. Microbial populations were determined after serial dilutions (10^{-1} to 10^{-9}) of faecal samples. Agar plates were incubated at 37°C either aerobically for 24 h for total aerobic bacteria (Schaedler agar, Oxoid, Hampshire, UK) and *E. coli* (MacConkey agar, Oxoid, Hampshire, UK), or anaerobically for 48 h for total anaerobic bacteria (Plate count agar, Sigma-Aldrich, Steinheim, Germany), *Lactobacillus* spp. (Man-Rogosa-Sharp agar, Sigma-Aldrich, Steinheim, Germany), *Cl. perfringens* (Tryptose Sulfite Cycloserin agar, Sigma-Aldrich, Steinheim, Germany) and *Bifidobacterium* spp. (Tomato Juice agar, Becton Dickinson and Company, Sparks, USA).

2.5. Proliferation of peripheral blood mononuclear cells

Mitogenic proliferative responsiveness of peripheral blood mononuclear cells (PBMC) to concanavalin A (ConA), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) was determined in whole blood samples to mimic *in vivo* conditions following a modification of a previously described method (Kim et al. 2000). Briefly, heparinised whole blood was diluted 1:10 with RPMI-1640 (Sigma-Aldrich, Steinheim, Germany) containing 10% inactivated foetal calf serum (FCS) and 1% penicillin-streptomycin (Sigma-Aldrich, Steinheim, Germany). The diluted samples were cultured in the presence of 10 µg/ml ConA, 10 µg/ml PHA, 5 µg/ml PWM (Sigma-Aldrich, Steinheim, Germany), or medium only. After 72 h at 37°C in a humidified incubator under 5% CO₂ atmosphere, cultures were pulsed with [³H] thymidine (10 µCi/ml) (Perkin Elmer, Boston, USA). After another 24 h cells were harvested on glass fibre filters (Nunc™, Roskilde, Denmark) and counted in a Tri-Carb® liquid scintillation counter (Packard instrument Co., Meriden, USA). All assays were performed in quadruplicate and stimulation indices were calculated as follows: (³H] thymidine incorporated by stimulated cells minus [³H] thymidine incorporated by unstimulated cells)/[³H] thymidine incorporated by unstimulated cells.

2.6. Flow cytometry

Single colour flow cytometry was used to determine possible changes in lymphocyte subsets. PBMC were isolated by whole blood lysis using a lysis buffer (89.86 g NH₄Cl, 10.01 g KHCO₃ and 0.0372 g Titriplex III per litre). Two millilitres of blood were incubated at 37°C for 4 min with 20 ml of pre-heated lysis buffer. Cells were washed in phosphate buffered saline (PBS) with 3% FCS (FACS buffer) and centrifuged at 400 g at

4°C. After re-suspension, 50 µl aliquots were incubated with the primary antibody. The specificity, clone, immunoglobulin concentration and reactivity of the antibodies employed are listed in Table 2. After 30 min of incubation at 4°C, the cells were washed twice with FACS buffer. After centrifugation, the cells were re-suspended and incubated with the secondary antibodies (goat anti mouse IgG1-FITC, Southern biotechnology, Birmingham, AL, USA, or rat IgG2a FITC, Serotec, Raleigh, NC, USA) for 30 min in the dark at 4°C. Appropriate negative controls were included to correct for background fluorescence. Data were expressed as the percentage of positive-staining cells corrected for cells stained non-specifically with the secondary antibody.

Table 2. Monoclonal primary antibodies* used within the lactoferrin study.

Antibody	Specificity	Dilution	Cell phenotype
CA13.1E4	CD5	1:100	T-cells
CA15.4G2	CD4	1:10	T-helper
CA2.1D6	CD8β	1:20	T-cytotoxic
YK1X322.3	CD21	1:100	B-lymphocytes

*Serotec, Raleigh, NC, USA.

2.7. Measurement of serum immunoglobulins (IgA and IgG)

Capture enzyme-linked immunosorbent assays (ELISA) were used to quantify the total concentrations of plasma IgA and IgG. Commercially available ELISA-test kits (IgA, IgG-ELISA QuantitationKit, Bethyl Laboratories Inc, Montgomery, USA) were used according to the manufacturer's instructions. Polyvinyl microtitre plates (Nunc™, Wiesbaden, Germany) were coated overnight with capture antibody (Sheep anti dog IgG or goat anti dog IgA). Diluted samples and standards were filled in the wells in duplicate. After an incubation period, the wells were loaded with horseradish peroxidase (HRP) conjugated antibody (HRP-conjugated sheep anti dog IgG or goat anti dog IgA). As substrate, TMB (3,3',5,5'-Tetramethylbenzidine) was used. The reaction was stopped with 2 M sulphuric acid. The optical density was measured with a spectrophotometer at 450 nm (Bio-Rad, Hercules, USA) and the concentrations were calculated automatically by comparison with the standard curves (Microplate Manager® 4.0, Biorad, Hercules, USA).

2.8. Statistics

All values represent the mean $\pm$ standard error of the mean (SEM). Data were analysed by Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA). To compare examined blood and microbiological parameters of dogs before and after LF supplementation a repeated measure analysis of variance (ANOVA) was conducted. Scheffé post hoc tests were performed if significance was found between treatments. The probability value used to identify significance was 0.05 ($p \leq 0.05$).

3. Results

Body weight and general health were not affected by treatments. Diets were readily consumed by the dogs. Peripheral WBC counts ($9.3\text{--}11.6 \cdot 10^9/\text{l}$) did not change due to the treatment (Table 3). The number of neutrophils and lymphocytes were not different before

Table 3. Distribution of white blood cells in dogs supplemented with different amounts of bovine lactoferrin (bLF) after 4, 8 and 12 weeks (Periods 1, 2 and 3, respectively).

	Group 1						Group 2						Group 3					
	Period			Period			Period			Period			Period			Period		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	bLF [mg/d]			bLF [mg/d]			bLF [mg/d]			bLF [mg/d]			bLF [mg/d]			bLF [mg/d]		
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM
	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value
Total WBC [$10^9/l$]	10.3	11.6	11.4	10.6	9.94	9.3	10.6	9.94	9.3	10.2	10.6	10.1	10.2	10.6	10.1	10.2	10.6	10.1
Neutrophils [%]	57.3	56.1	63.9	60.3	56.4	58.9	60.3	56.4	58.9	57.9	54.1	58.6	57.9	54.1	58.6	57.9	54.1	58.6
Neutrophils [$10^9/l$]	5.88	6.73	7.31	6.35	6.73	5.41	6.35	6.73	5.41	5.91	5.77	5.97	5.91	5.77	5.97	5.91	5.77	5.97
Lymphocytes [%]	30.7	33.2	26.6	29.3	29.8	31.1	29.3	29.8	31.1	30.2	34	31.7	30.2	34	31.7	30.2	34	31.7
Lymphocytes [$10^9/l$]	3.14	3.72	3.03	3.11	3.39	2.92	3.11	3.39	2.92	3.08	3.54	3.16	3.08	3.54	3.16	3.08	3.54	3.16
Monocytes [%]	5.17	4.03	3.82	6.15 ^a	6.82 ^a	4.12 ^b	6.15 ^a	6.82 ^a	4.12 ^b	4.03 ^a	4.48 ^a	3.47 ^b	4.03 ^a	4.48 ^a	3.47 ^b	4.03 ^a	4.48 ^a	3.47 ^b
Monocytes [$10^9/l$]	0.55	0.47	0.44	0.65 ^a	0.81 ^a	0.39 ^b	0.65 ^a	0.81 ^a	0.39 ^b	0.41	0.49	0.36	0.41	0.49	0.36	0.41	0.49	0.36
	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM
	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value	p value

Means in the same row with unlike superscript letters differ significantly ($p < 0.05$).

and after supplementation of bLF. The percentages of monocytes decreased in Groups 2 and 3 after Period 3 (week 5–8). The absolute count of monocytes also decreased in Group 2 after Period 3.

The distribution of lymphoid cell markers is summarised in Table 4. The CD5 surface marker was used as a general T-cell marker. Dogs supplemented with 120 mg bLF had a higher proportion of CD5⁺ cells after Period 2 than in Period 1 or 3. The percentage of CD5⁺ lymphocytes was 82.3% before supplementation (Period 1), 86.4% after supplementation (Period 2) and 84.9% after Period 3. The CD4 and CD8 surface markers identify helper T cells and cytotoxic T cells. All groups showed a significantly increased proportion of helper T cells and Group 3 exhibited also more cytotoxic T cells after supplementation of 1800 mg bLF. The proportion of CD21⁺ cells representing the B cells did not change throughout the study. The mean range was between 13.6% and 16.6%.

Lactoferrin supplementation did not affect plasma concentrations of IgA or IgG. A significant decrease of IgG ($p = 0.03$) in Period 3 was observed in Group 1.

In Group 3, the lymphocyte proliferation in response to ConA was significantly higher ($p = 0.04$) with a stimulation index of 24.5 after feeding 1800 mg bLF than in Period 1 with 19.0% and Period 3 with 14.7%. In response to PHA and PWM, no differences between groups were detected.

Total anaerobes and total aerobes averaged 10 and 9 colony forming units (cfu) log₁₀/g fresh faeces across treatments. Their concentration was not affected by bLF supplementation. Dogs from Group 2 (120 mg bLF/kg diet) showed a reduced concentration of *E. coli* in Period 2 (6.17 cfu log₁₀/g faeces) and Period 3 (5.97 cfu log₁₀/g faeces) compared to Period 1 (7.68 cfu log₁₀/g faeces). Similarly, faecal concentrations of *Cl. perfringens* decreased after supplementation with 120 mg bLF/kg diet from 8.44 cfu log₁₀/g faeces to 7.87 cfu log₁₀/g faeces. After the Period 3 the number was still significantly lower (7.93 cfu log₁₀/g faeces). The same tendencies were observed after supplementation of 1800 mg bLF/kg diet. No changes were found for faecal concentrations of *Lactobacillus* spp. and *Bifidobacterium* spp. The mean count was between 8.46 and 10 cfu log₁₀/g faeces.

3. Discussion

Lactoferrin is an iron-binding protein but also a modulator of various immunological responses and can be protective against infections (Yamauchi et al. 1998). This study indicates that oral administration of bLF to healthy adult dogs increased the percentage of CD5⁺ cells and in Group 3 (1800 mg bLF/kg diet) also the percentage of CD8⁺ cells. Furthermore, in this group a higher stimulation index in lymphocyte proliferation was found. The number and percentage of monocytes decreased after Period 3 in Groups 2 and 3. In another study in dogs, orally administered bLF (60 or 120 mg/d) had no effects on the differential blood count (Pope et al. 2006), probably due to the lower dosage. Similar observations as described here for dogs were made in mice (Artym 2003). Administration of LF in the drinking water to immunocompromised mice led to a higher content of CD3⁺ and CD4⁺ cells in the spleen and enhanced lymphocyte proliferation after stimulation with ConA and PWM. The total number of white blood cells, lymphocytes and neutrophils was not influenced and monocytes remained unchanged. In another study in mice CD8⁺ cells in the blood were increased after treatment with bLF (Iigo et al. 1999). In contrast, supplementing bLF to sheep did not cause any changes in the percentage of CD4⁺ and CD8⁺ cells and in the ConA induced lymphocyte proliferation of isolated PBMC (Wong et al. 1998). *In vitro*, it was shown that LF up regulates the expression of

Table 4. Lymphocyte subsets, stimulation indices of lymphocytes proliferation and plasma immunoglobulins of dogs supplemented with different amounts of bovine lactoferrin (bLF) after 4, 8 and 12 weeks (Periods 1, 2 and 3, respectively).

	Group 1					Group 2					Group 3				
	Period			SEM	p value	Period			SEM	p value	Period			SEM	p value
	1	2	3			1	2	3			1	2	3		
	bLF [mg/d]					bLF [mg/d]					bLF [mg/d]				
0	0	0			0	120	0			0	1800	0			
<i>Lymphocyte subsets [%]</i>															
CD5 ⁺	82.2	84.6	83.3	1.09	0.59	82.3 ^a	86.4 ^b	84.9 ^b	0.81	0.02	79.7	84.3	82.6	0.81	0.10
CD4 ⁺	47.7 ^a	50.7 ^b	50.9 ^b	1.67	0.02	47.3 ^a	51.4 ^b	52.3 ^b	1.82	0.01	40.6 ^a	48.5 ^b	46.2 ^b	2.45	0.03
CD8 ⁺	20.7	21.7	21.3	0.81	0.95	19.2	20.1	20.7	1.16	0.53	16.6 ^a	20.7 ^b	20.0 ^b	1.23	0.04
CD4 ⁺ /CD8 ⁺	2.34	2.43	2.48	0.13	0.34	2.68	2.72	2.65	0.19	0.87	2.72	2.55	2.59	0.27	0.27
CD21 ⁺	15.1	15.4	14.7	1.09	0.95	13.6	16.6	15	1.42	0.17	14.5	16	14.8	0.53	0.85
<i>Lymphocyte proliferation [SI]*</i>															
ConA	38.6	36	25.3	8.09	0.29	31.3	23.9	30.2	3.81	0.73	19.0 ^{ab}	24.5 ^b	14.7 ^a	3.36	0.04
PHA	37.6	50.9	32.2	12.6	0.43	22.2	24.9	32.1	3.37	0.44	19.6	24.1	18.4	2.87	0.74
PWM	32.4	37.8	23.2	8.91	0.45	21.5	22.4	27.2	3.03	0.73	13.4	17.3	13.2	2.15	0.94
<i>Plasma immunoglobulins [mg/ml]</i>															
IgA	1.57	1.63	1.41	0.13	0.47	1.48	1.23	1.21	0.23	0.27	1.48	1.27	1.39	0.1	0.55
IgG	21.15 ^b	23.1 ^b	14.2 ^a	2.00	0.03	17.7	17.1	15.4	1.13	0.45	17.8	17.2	20.3	1.62	0.32

*SI, stimulation index; calculated as follows: ([³H] thymidine incorporated by stimulated cells minus [³H] thymidine incorporated by unstimulated cells)/[³H] thymidine incorporated by unstimulated cells. Means in the same row with unlike superscripts differ significantly (*p* < 0.05).

CD4 antigen in Jukart-lymphoblastic T-cells through the stimulation of the mitogen-activated protein kinase (Dhennin-Duthille et al. 2000). After incubation with human bLF, CD4⁺CD8⁻ lymphocytes acquired the CD4⁺ phenotype, characteristic for T-helper lymphocytes (Zimecki et al. 1991). The increase of monocytes after bLF supplementation observed in this study might indicate an enhanced response in the innate immune system due to a higher phagocytic and antigen presenting capacity.

Though many *in vitro* and *in vivo* studies found a regulatory effect on the immune response and protective effects against infections and septic shock by LF, complete elucidation of the cellular and molecular mechanisms has not yet been achieved (Legrand et al. 2004). On different mammalian cells like monocytes, lymphocytes and liver cells receptors for LF were identified. They bind LF more or less specific and are key effectors for cell signalling, endocytosis and/or nuclear targeting (Suzuki and Lonnerdal 2002). Lactoferrin is likely to regulate lymphocyte maturation and activation through binding to a 105 kDa receptor (Mazurier et al. 1989).

Another functional arm of the immune system responding to pathogens and other foreign intrusions is the humoral immune system. The plasma concentrations of IgA and IgG were not reacting to LF ingestion. This is in accordance with another study in dogs (Pope et al. 2006). Also in other animal models, effects on the IgG concentration were not observed (Debbabi et al. 1998).

The faecal samples from dogs fed 120 mg bLF/kg diet (Group 2) had lower concentrations of *E. coli* and *Clostridium perfringens*. The total number of anaerobic and aerobic bacteria and the concentrations of bifidobacteria and lactobacilli did not change. Lactoferrin is known to inhibit the growth of many gram-positive and gram-negative bacteria. The bacteriostatic effects of LF are to some extent due to its ability to sequester environmental iron. Lactoferrin can bind to the outer membrane of gram-negative bacteria, causing the release of lipopolysaccharides (Kim et al. 2004). When milk-fed mice were inoculated with *Cl. ramosum*, the addition of bLF to the diet resulted in a significant decrease of clostridia concentrations in the faeces (Teraguchi et al. 1995b). The mechanisms involved in bactericidal activity of LF are not definitively elucidated. The dose dependent release of lipopolysaccharides (LPS) indicates damaging of the outer membrane of gram-negative bacteria. Transmission electron microscopy documented that *E. coli* cells, cultured with LF and lysozyme, become enlarged and hypodense. This suggests that killing is caused by osmotic damage (Ellison and Giehl 1991). A reduction in the number of potentially pathogenic clostridia due to bLF in the gut may be beneficial to the dog because this organism can exert harmful effects on the host.

Pope et al. (2006) supplemented 60 and 120 mg bLF/d to adult female dogs. Neither the examined ileal nor the faecal microbes changed in response to the treatment except for the *Streptococcus* spp. In the current study higher concentrations of bLF were used, which might explain the observed effect on the faecal concentrations of *Clostridium* spp. and *E. coli*. Human and bovine LF promoted growth of bifidobacteria *in vitro* and *in vivo* (Liepke et al. 2002; Kim et al. 2004). Orally administered LF has been shown to promote the establishment of a bifidobacteria predominant flora in human flora associated mice and human full-term infants (Hentges et al. 1992; Roberts et al. 1992; Wharton et al. 1994). The growth-stimulatory activity of LF in these species may be related to the presence of LF binding proteins on the surface of bacterial cells. Such cell surface receptors for LF are believed to contribute to bacterial growth by facilitating the uptake of iron ions. However, in this study, supplementation of bLF did not increase faecal concentrations of bifidobacteria or lactobacilli.

In conclusion, orally administered bLF had significant effects on some cellular immune functions in dogs and lowered the faecal concentrations of *E. coli* and *Clostridium perfringens*. Because of the individual variability of the immunological indices and microbial populations measured, further and more detailed studies of LF effects are required. Challenge studies may allow determining whether these changes in blood immune cells are beneficial to the host.

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