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Research Article
Role of Vitamin C on Immune Function Under Heat Stress Condition in Dairy Cows

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Abstract

Objective: The aim of this study is to investigate the effect of seasonal change in thermal environment on leukocytes constituents and effects of vitamin C on immune response under heat stress conditions, in high and low producing dairy cows in Tunisia. **Methodology:** Three experiments were carried out into two different periods: Spring P_1 and summer P_2 using a randomized block design per level milk production, within 72 Holstein cows divided in 3 groups formed by 24 cows. In each group there is 12 high and 12 low producing cows. The first group was used as a control group during P_1, second and third group were tested in P_2 with same rations. Meanwhile, the third group is supplemented with VC in ration (20 g/100 kg live weight). Cow's blood was sampled to determine plasma VC and cortisol concentration. Percentages of leukocyte relative to total counted cells were calculated. Peripheral blood mono-nuclear cell function was evaluated and stimulated with phytohemagglutinin and poke weed and finally, their response in terms of DNA synthesis and IgM secretion was measured. **Results:** As results, VC concentration in P_1 for first group (2.97 and 2.96 mg L^{-1} in HPC and LPC, respectively) was higher relative to concentrations in P_2 for second group (1.77 and 1.88 mg L^{-1} in HPC and LPC, respectively). Plasma VC concentration was effected by the period but not by level milk production ($p>0.05$). However, DNA synthesis in PBMC stimulated with mitogens in second group was decreased in heat stress period ($p<0.001$). Whereas the IgM secretion ($p<0.001$) and the plasma cortisol concentration ($p<0.05$) in the second group was higher compared to the control group. But, DNA synthesis in PBMC and plasma cortisol concentration returned to base line levels in presence of VC in ration of third group in hot temperature and IgM secretion in PBMC of the same group was significantly reduced with the same treatment. **Conclusion:** This study indicates that the effects of high environmental temperatures on the immune response depend on the plasma VC concentration and milk production potential. However, ration supplemented with VC in dairy cows play an important role in immune functions under heat stress condition.

Key words: Vitamin C, heat stress, immune function, dairy cows, leukocytes

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heat stress is probably, the most important factor of the leading causes on the performance of dairy cows. These negative effects of heat stress imply thermal regulatory reactions in order to try to maintain heat balance[1]. These reactions imply reduced feed intake, redistributed blood supply, increased evaporative heat loss via increased respiration and sweating and altered immune function.

Vitamin C (VC) is the generic term for all compounds showing the biological activity of ascorbic acid (AsA)[2]. The VC is an important water soluble vitamin sensitive to heat stress; it is essential for the metabolism of many mammals[3], who can synthesize AsA from glucose in the liver[4]. The majority of VC exists as AsA in the bodies of animals and AsA can be reversibly oxidized to dehydroascorbic acid (DAsA)[2]. On the other hand, Santos *et al.*[5] reported that plasma AsA concentration was not affected by the stage of lactation and they suggested that endogenous AsA production met the VC demand in lactating.

So, heat stress probably affects antioxidative mechanisms and involves the production of Reactive Oxygen Spices (ROS)[6], which induces oxidative stress[7]. However, Harmon *et al.*[8] reported that heat stress decreased the antioxidant-activity of plasma in lactating cows. For instance, the decreases of concentration of AsA in plasma in heat stress conditions has been confirmed in pigs[9], in poultry[10,11] and in birds[12,13]. Oxidative stress plays an important role in ruminant health. Moreover, mastitis is the most devastating disease of dairy animals. Inflammatory reaction accompanying mastitis may be caused by oxidative stress[14]. The decrease in AsA concentration seems to be accompanied by an increase in the levels of lipid hydroperoxide in erythrocytes isolated from dairy cows with acute mastitis. Kleczkowski *et al.*[15] suggested that the severity of clinical signs is proportional with the magnitude of the decrease in plasma VC concentration.

Therefore, the adequacy of VC can be evaluated by analysis of VC or AsA in plasma, serum, leukocytes or urine[16]. Furthermore, VC increases neutrophils protection against oxidative stress induced by free radicals associated with the oxidative burst[17]. In the other hand Goetzl *et al.*[18] suggested that the VC stimulates interferon production, which protects leukocytes responses and could be related to variation of white blood cells types at high ambient temperatures.

Hartmann *et al.*[19] found a significant decrease in leukocytes numbers in the alarm phase and leucocytosis in the resistance phase of heat stress. Lee *et al.*[20] observed that the enhanced ambient temperature evoked leucocytosis in cattle. Koubkova *et al.*[21] described that the proportional distribution of white blood cells changes at high temperatures. Broucek *et al.*[22] found a decrease in neutrophiles and eosinophiles and an increase in lymphocytes and monocytes. High producing cows have more metabolic activity and produce more body heat than low producers; thus, a higher milk yield may increase heat stress if the cause of stress is not mitigated[23,24]. There are many articles confirming, that high-yielding dairy cows are more efficient producers of milk than low-yielding dairy cows[25,26]. However, in view of the mentioned results, it would be interesting to attempt the treatment with VC to prevent the consequences of immune function altering related oxidative stress. Antioxidant nutrition plays an important part in resistance to the many diseases[27-29].

The present field study was undertaken under significant climate changes in a mediterranean climate in North-West of Tunisia, to assess the effects of intense summer heat on the lymphocyte function of high and low producing dairy cows in presence of vitamin C in the ration. The investigation of the relationships among them with vitamin C, lymphocyte function and antibody levels of dairy cows under high ambient temperature in summer was carried out in this study.

MATERIALS AND METHODS

Animals, feeding and management: The study were carried out at the Office des Terres Domaniale BADROUNA dairy farm, Bousalem Jandouba (North-West of Tunisia), which is located 36°0.6' latitude North and 8°0.9' longitude West. Three experiments were conducted in two different periods. Spring (P_1: 15 February-15 March; mean daily THI value 65.62±1.32, no heat stress) and summer (P_2: 1-30 August; mean daily THI value 83.27±1.26, stress conditions). Ambient temperature and relative humidity were measured using a thermo hygrometer (HI 91610C, Hanna instrument, Portugal) Estimation of THI was performed for each test day using the equation described by Kibler[30]. Seventy two multiparious and primipares lactating Holstein Friesian dairy cows (Table 1) were used. They were housed in a covered tie stall barn with straw bedding. Metal roofs covered the stalls.

The diets were typical with those in the region with forage ratio of 59 and 62% in P_1 and P_2, respectively, on Dry Matter (DM) basis. The concentrate (8 kg cow^{-1} day^{-1}) was fed in four equal meals daily. Food and water were available *ad libitum*. Ingredients and chemical composition of basal diet fed to animals during the experiment are reported in Table 2. Diets were defined including ingredients commonly used in North-West of Tunisia. Crude protein and neutral detergent fiber content in diets ranged from 13.9-16% and

Table 1: Description of cows used in experiment during the two periods P_1 and P_2

	P_1		P_2			
	First group		Second group		Third group	
Animals	†HPC	‡LPC	HPC	LPC	HPC	LPC
Cows (No.)	12	12	12	12	12	12
Age (months)	71 (±1)	72.5 (±2)	72.25 (±1)	72 (±1.5)	72 (±1)	71.75 (±1.5)
Live weight (kg)[a]	515.25 (±14)	509 (±20)	517 (±15)	505.9 (±15)	518 (±15)	507 (±17)
§DIM (days)	170 (±17.5)	172 (±17)	171.5 (±16)	170 (±18.5)	170 (±16)	172 (±16)
No. of lactation	3 (±1.5)	2.5 (±1.5)	3 (±1.5)	3 (±2)	3 (±1.5)	3 (±1.5)
Milk yield (kg day^{-1})	36.11 (±1.03)	23.60 (±1.02)	28.56 (±1.90)	19.96 (±1.60)	28 (±2)	19.5 (±1.02)

†HPC: High producing cows, ‡LPC: Low producing cows, [a]Estimated according to Heinrichs *et al.*[31], BW = 4.134×(HG-318.51), standard deviation, §DIM: Days in milk

Table 2: Ingredients and chemical composition of the total mixed ration diet

Items	P_1	P_2
Feed ingredient (DM%)		
Tritical ground green forage	--	18.50
Bersim green forage	--	11.60
Alfalfa forage	13.80	9.00
Oat hay	8.20	6.60
Corn silage	--	16.60
Oat silage	37.60	--
Corn grain grind	11.50	10.90
Soybean meal	8.50	7.80
Barley grain	9.40	9.10
Wheat bran	7.40	7.10
Vitamins A, D and E	0.8	0.80
Mineral	2.0	2.00
Sodium bicarbonate	0.2	--
Calcium phosphate	0.6	--
Chemical composition		
DM (%)	32.3	28.70
CP (DM%)	13.9	16.00
Starch (DM%)	22.8	18.70
NDF from forages (DM%)	76.8	80.40
NDF (DM%)	41.8	39.60
NEL (Mcal kg^{-1})	1.49	1.49
Minerals composition		
Ca (DM%)	0.80	0.80
P (DM%)	0.60	0.50
Fe (DM ppm)	260.10	159.80
Mg (DM%)	0.30	0.30
Cl (DM%)	0.50	0.40
K (DM%)	2.10	1.90
Na (DM%)	0.50	0.30

39.6-41.8% DM, respectively. Supplementation on basal diet as treatment was: Third group: Basal diet defined in P_2+vitamin C (20 g/100 kg live weight). First group and second group were fed by a basal diet (unsupplemented) defined in P_1 and P_2, respectively.

Measurements, sampling and laboratory analysis

Sample collection: At each test day, 55 mL of blood samples was collected from the caudal vein puncture at approximately 1 pm using sterile vacutainers. About 15 mL volume of blood collected into an EDTA tube was kept in an ice bath for a few hours until centrifugation (3000 tours min^{-1} at 4°C) to recover plasma. About 40 mL of heparinized blood were immediately placed in a portable refrigerator after collection and transferred to the laboratory, in order to carry out lymphocyte assays and evaluate IgM secretion.

Differential leukocytes count: From each blood samples collected, smears were prepared using wrights-Giesma method (Fisher Scientific Company) for differential leukocytes profile. Total leukocytes number was determined for each smears blood by light microscope.

Total plasma vitamin C and Cortisol concentrations: Blood plasma was analyzed for a total plasma vitamin C concentration[32]. The total cortisol concentration was determined using the ^{125}I RIA kit (IM 1841).

Lymphocyte proliferation assay: Lymphocyte proliferation assays were performed on isolated Peripheral Blood Mononuclear Cell (PBMC) by density gradient centrifugation. Blood was diluted 1:1 with RPMI-1640 medium containing 25 mM HEPES (Sigma-Aldrich, Tunisie) Supplemented with penicillin (100 U mL^{-1}, Sigma-Aldrich) and streptomycin (0.1 mg mL^{-1}, Sigma-Aldrich). This whole blood suspension was overlaid onto 3 mL of Ficoll-paque (Amersham Pharmacia Biotech) and centrifuged and centrifuged for 20 min at 500×g. The mononuclear cell band was recovered and washed twice in phosphate buffered saline and red blood cells were lysed with tris-ammonium chloride (0.1- 0.8%, v/v). The PBMC was centrifuged for an additional 10 min and resuspended at a concentration of 106 cells mL^{-1} in the RPMI 1640 medium containing 25 mM HEPES.

About 100 µL cell suspensions (PBMC) were added to triplicate wells in 96-well plates and were stimulated with mitogen phytohemagglutinin (PHA) and mitogen pokeweed (PWM) (Sigma Aldrich) or remained unstimulated. The T-lymphocyte phytohemagglutinin (PHA) was added at final

concentration of 2.5 μg mL^{-1} and mitogen poke weed (PWM) was added at final concentration of 1 μg mL^{-1}. A penicillin, streptomycin, L glutamine (2 mM; Sigma Aldrich), HEPES buffer (20 mM, Sigma Aldrich) and fetal bovine serum (10% final concentration; Sigma Aldrich) were supplemented into all wells to the 100 μL of RPMI-1640 culture. The plates were incubated at for 48 h at 39°C in an atmosphere of 95% air and 5% CO_2. About 100 μM of 5-bromo-2-deoxyuridine (BrdU) in 10 μL of RPMI-1640 were added to each well to give a final concentration of 10 μM BrdU. The tissue culture plates was incubated for 18 h and the culture medium was removed by centrifuging the tissue culture plates at 300×g for 10 min. Thereafter, the tissue culture plates were dried at 60°C for 1 h finally lymphocyte proliferation was measured by ELISA using a commercial kit for the measurement of BrdU incorporation during DNA synthesis in proliferating cells. The incubation time with peroxidase-labelled monoclonal anti-BrdU antibody was 90 min. The plates were incubated for 30 min with the substrate before being assessed at 450 nm using a spectrophotometer.

Evaluation of IgM secretion: Details of evaluation of IgM secretion was reported elsewhere[33].

Data analysis: Data were analyzed by SPSS (version 17.0) (23 August, 2008) and effects were considered to be significant at a value of $p<0.05$.

RESULTS AND DISCUSSION

Environmental conditions during the experimental periods: Tunisia has a climate characterized by a hot summer season. However, heat stress usually begins in June and lasts through September[23]. Ambient temperature and humidity are combined to derive the temperature-humidity index THI[30]. In the present study, recorded THI in P_1 of 65.62 (±2.43) indicated absence of heat stress conditions. As expected, heat stress occurred during P_2 with THI values of 83.27 (±1.90). This confirms the reported findings by Silanikove[34] and Pereyra *et al.*[35], which indicate that high producing cows become heat stressed when the THI index threshold for dairy cattle has been above 78. Kadzere *et al.*[36] noted that the THI index values of 70 or less are considered comfortable. But, Thatcher *et al.*[37] reported that lactating cows are considered unstressed when the THI is less than 72.

Effects of heat stress plasma vitamin C concentration and leukocytes number: The results in Table 3 showed a significant ($p<0.001$) decrease in plasma VC concentration in the second group as the values went from 2.97 and 2.96 mg L^{-1} in HPC and LPC (First group), respectively in P_1 to 1.77 and 1.82 mg L^{-1}, respectively in HPC and LPC (second group), in P_2. Some researchers also reported that heat stress decreased plasma vitamin C levels in dairy cows[6,38], in pigs[9] and poultry[11,13], which accords with the present findings. As mentioned, the metabolic demand of VC in dairy cows is influenced by environmental conditions but the plasma VC concentration did not vary according to level of milk production ($p>0.05$).

Heat stressed cows reduce feed intake[1,24]. The plasma VC concentration depends on the synthesis of ascorbic acid (AsA), wherein glucose is the sole precursor of AsA in animal body[39]. The reduction in plasma VC in hot environment in the present study is probably attributed to fall in blood glucose, following the decrease in the amount of dry matter intake and hence the amount of carbohydrates during the summer period (P_2) for second groups of cows.

Lymphocytes, neutrophils, eosinophils and monocytes, relative to total number of leukocytes were significantly affected by period ($p<0.001$). Percentage of lymphocytes was lower ($p<0.001$) for heat-stressed cows in second group (74.1 vs 60.4% and 73.7 vs 65.1% in HPC and LPC, respectively). Percentage of neutrophils increased from P_1 (24.2 and 23.9% in HPC and LPC for first group, respectively) to P_2 (20.2 and 21.0% in HPC and LPC for second group, respectively). Whereas, the percentage of eosinophils and monocytes in HPC and LPC were decreased ($p<0.001$) from P_1-P_2. Similar results were observed by Hartmann *et al.*[19] and

Table 3: Heat stress and level milk production in dairy cows (High and low producing cows) effects on VC concentration and leukocytes percentages

	P_1 (First group)		P_2 (Second group)		Effect		
	†HPC	‡LPC	HPC	LPC	G	P	G×P
VC concentration (mg L^{-1})	2.97^a	2.96^b	1.77^a	1.82^b	ns	***	ns
Lymphocytes (%)	74.10^a	73.70^c	60.40^a	65.10^c	***	***	***
Eosinophils (%)	1.20^a	1.80^a	7.40^a	8.50^a	***	***	***
Neutrophils (%)	24.20^a	23.90^{a,b}	20.20^a	21.00^a	**	***	**
Monocytes (%)	1.70^a	1.60^b	4.90^c	5.60^b	***	***	***

†HPC: High producing cows, ‡LPC: Low producing cows, ***$p<0.001$, **$p<0.01$, Ns: Not significant ($p>0.05$), least squares means on the same row with the same letter are not significantly different ($p>0.05$)

Kamwanja *et al.*[40] who suggested a depression in immune system activity for heat-stressed cows. Furthermore, Broucek *et al.*[22] found a decrease in the percentage of neutrophils and eosinophils and an increase in the percentage of lymphocytes and monocytes. Lee *et al.*[20] noted a tendency of eosinophils count increase in dairy cows exposed to high temperature. But, obtained results are conflicting with those studies where no effectwere reported by Lacetera *et al.*[41]. According to Franci *et al.*[42] the increase in the number of lymphocytes in high temperature conditions is treated by the synthesis of heat shock proteins.

Percentage of leukocytes types was varied according to cistern size. Indeed, the parameter changes of lymphocytes ($p<0.001$) and neutrophils ($p<0.01$) were the most intensive in dairy cows with the highest milk yield. But, the immune response is less sensitive to heat stress in HPC as a percentage of monocytes ($p<0.001$) and eosinophils ($p<0.001$) than LPC.

Circulating leukocytes numbers vary considerably between those mentioned in the present study and those reported in literature. This variation may be described to several factors, in particular to emotional state of animal (stress) at the time of blood sampling and the interaction of the effects of heat stress with others parameters related to the animal (breed, physiological stage and production potential) and diet (composition, nutritional value and distribution mode).

Plasma VC concentration-leukocytes cells relationship: Lymphocyte stimulation is widely used to measure immune competence by stimulation of lymphocytes with mitogens[43] and exposure to infectious agents by stimulation of lymphocytes with specific antigens[44]. In this study, results indicate that summer conditions was associated with depressed cellular immunity as assessed by measuring DNA synthesis in PBMC stimulated with mitogens, an enhanced humoral response as assessed by measuring antibody secretion in PBMC stimulated with PWM and higher concentrations of plasma cortisol. However, DNA synthesis in PBMC stimulated with mitogens (Fig. 1) was decreased in heat stress period for the second group ($p<0.001$). Whereas the IgM secretion (Fig. 2) was higher ($p<0.001$) in P_2 for the second group compared to P_1 for first group cows. There were significant differences between the groups (HPC, LPC). The variations observed were dependent of milk production potential of these dairy cows.

However, the lymphocyte function is more sensitive to heat stress in HPC than LPC. Jain[45] reported that the cause of stress-induced lymphopenia could be due to either lympholysis or a redistribution of cells. Results have demonstrated a significant suppression of lymphocyte

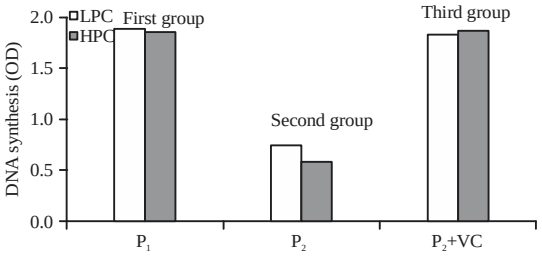

Fig. 1: DNA synthesis in Peripheral Blood Mononuclear Cells (PBMC) isolated from first, second and third cow group. The PBMC were stimulated with phytohemagglutinin (PHA) and poke weed (PWM), Bars with values in different groups (LPC, HPC) that lack a common letter are significantly different (a,b $p<0.001$)

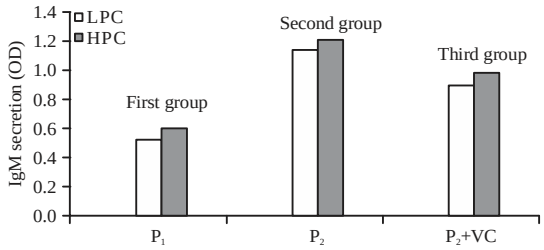

Fig. 2: IgM secretion in Peripheral Blood Mononuclear Cells (PBMC) isolated from first, second and third cows group. Bars with values in different groups (LPC, HPC) that lack a common letter are significantly different (a,b $p<0.001$)

numbers and DNA expression in PBMC stimulated with mitogens (PHA or ConA) in heat stress for second group of dairy cows. Impaired lymphocyte proliferation and increased antibody production could potentially influence immune responses to infectious agents in hot environment. Regnier and Kelley[46] reported that heat exposure suppressed *in vivo* and *in vitro* cellular response and increased antibody production in chickens injected with sheep red blood cells. In the other hand, Amat and Torres[47] observed an increase of the antibody response to sheep red blood cells in rats at high environmental temperature. Recently, it have been reported that summer THI conditions heat stress in dairy cows impair cell-mediated immunity and increase antibody production in dairy cows[33].

Obtained results are conflicting with those studies that indicated that hot weather increased the proliferation of PBMC isolated from cows located in a temperate climate[48]. Conversely, Elvinger *et al.*[49] have reported that the proliferation of bovine lymphocytes after stimulation with PHA was reduced when cells were incubated for 60 h at 42°C. Plasma cortisol concentrations of cows were different in both

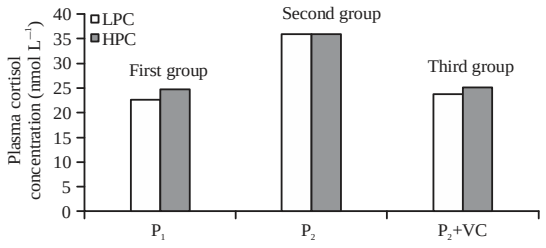

Fig. 3: Concentrations of plasma cortisol isolated from first, second and third cows group, bars with values in different groups (LPC, HPC) are significantly no different (p>0.05)

periods (P_1, P_2). The average concentration of cortisol was increased ($p<0.05$) in heat stressed period for the second group. Blood cortisol concentration did not vary according to production level ($p>0.05$). However, the increase in plasma cortisol in hot environment cows (second group) provides at least a partial explanation for the changes in cell-mediated and humoral immunity and alteration in lymphocyte functions observed in this study as shown in Fig. 3. Besides previous studies carried out on laboratory animals under a large variety of experimental conditions have rapported that cortisol depresses the activity of the immune system and lowers its resistance to diseases.

Younes *et al.*[23] and Segel *et al.*[50] found inhibition of human lymphocyte blastogenesis by cortisol only when suboptimal PHA levels were used. The increase of plasma cortisol level during acute heat stress is attributed to the fact that glucocorticoid hormones have hyperglycemic action through the gluconeogenesis process, thus enhancing glucose formation in heat stressed animals. However, the results of these studies are conflicting, some researchers have reported a decrease or any variation of in cortisol concentration under heat stress conditions[51,52]. Lactating cows supplemented with VC in P_2 showed a decrease in plasma cortisol concentration.

The assessment of these characteristics would evaluate the interaction of adaptive immune responses with heat-associated stress and the nature of ration in dairy cows. However, the presence of VC in ration may play a part in the body's immune response in hot environment. As a result, supplementation of vitamin C (20 g/100 kg live weight) on basal diet in heat stress conditions has enhanced immune response. Indeed, DNA synthesis in PBMC and plasma cortisol concentration for third group returned to baseline levels in presence of VC in ration of dairy cows in P_2 and IgM secretion in PBMC for third group of lactating cows was significantly reduced with the same treatment. Dietary supplementation of vitamin C alleviated the adverse effects of heat stress in dairy cows during the hot dry season. However, several studies are suggesting that cows with mastitis have lower concentrations of vitamin C in the plasma and milk[14,15]. Oxidative stress is associated with some desorders of dairy cows as mastitis, which induce increase of formation free radicals in plasma and milk[53]. Several studies were carried out in other publications and have suggested that some factors contribue to the voluminous production of free radicals in mastitic milk; one of these factors is the increased numbers of cells like neutrophiles, macrophages, lymphocytes, eiosinophiles and various epithelial cells of mammary tissue[54].

CONCLUSION

The present study has demonstrated that plasma VC concentrations may be a useful indicator of animal welfare and may be the more sensitive in response immune. Indeed as depicted by the above results, a large part of the variation in the percentage of white blood cells profiles could be attributed to the VC concentration decreasing in hot environment summer. Moreover, it's necessary to evaluate the level of plasma VC in dairy cows throughout summer. Nonetheless, a more detailed investigation of innate and adaptive immune responses in dairy cows in heat stress period is required to determine whether specific protocols for addition of VC in diet and handling of these animals should be considered to minimize the effect of high ambient temperature on the immune system.

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