

Galectins and collectins expression are increased in *Haemonchus contortus*-infected corriedale sheep

Aumento da expressão gênica de colectinas e galectinas em ovinos corriedale infectados por *Haemonchus contortus*

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Abstract

Galectins and collectins are proteins classified in the lectin family that have the ability to recognize molecular patterns associated with pathogens. Studies on cattle have demonstrated high expression of these proteins during infection with gastrointestinal nematodes. The aim of this study was to investigate whether the level of *Haemonchus contortus* infection would alter the expression of galectins (Gal11 and Gal14) and collectins (SPA and CGN) in sheep. Twelve Corriedale sheep exposed to natural infection with nematodes were divided into two groups: group 1 (G1, n = 7) and group 2 (G2, n = 5), with low and high parasite burdens, respectively, based on fecal egg counts and abomasal parasite counts. The fecal egg counts and abomasal parasite counts were significantly different ($p < 0.05$) between the groups. Galectin and collectin gene expression was observed in all sheep abomasal samples. However, animals with lower infection levels showed lower expression of the genes Gal14, SPA and CGN ($p < 0.05$). Expression of lectins was associated with the abomasal *H. contortus* burden, thus suggesting that these proteins may have a role in controlling of this infection.

Keywords: Innate immunity, lectins, helminths, small ruminants, PCR.

Resumo

Colectinas e galectinas são proteínas da família das lectinas que possuem a capacidade de reconhecer padrões moleculares associados aos patógenos. Estudos em bovinos têm demonstrado a alta expressão dessas proteínas durante a infecção por nematoides gastrintestinais. O objetivo deste estudo foi investigar se o nível de infecção de *Haemonchus contortus* altera a expressão de colectinas (SPA e CGN) e galectinas (Gal11 e Gal14) de ovinos. Doze ovinos da raça Corriedale expostos a infecção natural com nematoides foram separados em dois grupos: grupo 1 (G1, n=7) com menor grau de parasitismo; e grupo 2 (G2, n=5) com maior grau, a partir da contagem do número de parasitos recuperados do abomaso e OPG. A contagem de OPG e de parasitos recuperados do abomaso dos grupos G1 e G2 apresentaram diferença estatística ($p < 0,05$). A expressão dos genes de colectinas e galectina foi observada em todas as amostras de abomaso dos ovinos, porém animais com menor grau de infecção apresentaram menor expressão dos genes de Gal14, SPA e CGN ($p < 0,05$). A expressão de lectinas foi associada ao número de *H. contortus* encontrados no abomaso de ovinos, indicando um possível papel dessas proteínas no controle da infecção.

Palavras-chave: Imunidade inata, lectinas, helmintos, pequenos ruminantes, PCR.

Introduction

Haemonchosis is responsible for low performance and can cause severe disruption to sheep production chain in many tropical and subtropical regions, where it is endemic. Selection of sheep that are

genetically resistant to gastrointestinal nematodes is an alternative control method (KASSAI & SRETER, 1992), which is based on the animal's ability to acquire immunity and limit the establishment of the parasite, thereby reducing productivity losses.

Host immunity against helminth infections is dependent on innate and acquired immune responses (STEAR & WAKELIN, 1998) and the interaction of these responses confers protection by inducing

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eosinophilic inflammation, mastocytosis and activation of Th2 responses, with increased expression of interleukin (IL) -4, IL-5 and IL-13 in target tissues and production of IgE and IgA (MACKINNON et al., 2009, 2010). In contrast, animals susceptible to infection develop Th1 responses, with secretion of cytokines such as IL-2, interferon-gamma (IFNG) and tumor necrosis factor (GILL et al., 2000).

In addition to Th1 or Th2-triggered responses to helminth antigens, other components may influence the host's ability to control parasitism. Recognition proteins such as lectins are involved in the immune response against nematodes (MEEUSEN et al., 2005). In particular, members of the galectin and the C-type lectin family have been identified in the abomasum of cattle and sheep infected with *Ostertagia ostertagi* and *Cooperia oncophora* (DUNPHY et al., 2000; LI & GASBARRE, 2009; LI et al., 2010; ROBINSON et al., 2011). Collectins belong to a mammalian superfamily of the lectin C-type protein subgroup with the ability to recognize pathogen-associated molecular patterns (PAMPs) found on the surface of microorganisms (KILPATRICK, 2002). These proteins inhibit the infectious process through neutralization, agglutination and complement activation, they also act as immunomodulators of allergic and inflammatory responses and apoptosis (DEC & WERNICKI, 2006; HÅKANSSON & REID, 2000). Galectins are defined through recognition of β -galactoside, by means of a calcium-independent carbohydrate recognition domain (BARONDES et al., 1994), through the immune response involved in regulating cell differentiation, modulation of cell adhesion and activation, and chemotaxis (LIU, 2005).

These mechanisms are not fully understood in nematode-ruminant interactions, but some hypotheses have been suggested. In primary infection of cattle with *O. ostertagi*, increased expression of collectin, conglutinin and CL-46, in the abomasal mucosa has been observed, along with increased protein regulation of the full complement C3, complement factor B and complement factor I. This suggests that there is activation of the alternative complement pathway and early reactivation in secondary infections (LI et al., 2010). Another response mechanism observed in *H. contortus*-infected sheep is characterized by abundant secretion of galectin-11 in the abomasum, increased mucus production (DUNPHY et al., 2000) and alteration of mucus viscosity, thus providing an unfavorable environment for parasite survival (ROBINSON et al., 2011).

The nature of nematode contact with components of the innate immune system of ruminants is decisive in determining the resistance or susceptibility of the host. The present study investigated whether the gene expression of galectins (Gal11 and Gal14) and collectins (SPA and CGN) in sheep differed between groups with different levels of *H. contortus* infection, thereby contributing towards better understanding of the innate immune response against gastrointestinal helminths, based on the premise that lectins are pattern recognition molecules of the cell surface of parasites.

Material and Methods

Animals

Twelve 18-month-old Corriedale sheep (both males and females) were used in the experiment. The animals were exposed to natural infection with helminths at the Embrapa Pecuária Sul research farm, in Bagé (31° S 54° W), Rio Grande do Sul, Brazil. After 12 months

of monitoring, the animals were slaughtered to recover parasites and obtain abomasal mucosa samples. All procedures followed were in accordance with the Brazilian legislation, as per Resolution no. 1000/2012 of the Federal Council of Veterinary Medicine and were approved by our institution's ethics committee for animal use (March 29, 2011). The animals were divided into two groups: low (G1, n = 7) and high (G2, n = 5) levels of *H. contortus* parasitism. The division was based on fecal egg counts (FEC) and abomasal parasite counts, after performing ascending hierarchical multivariate cluster analysis, using Euclidean distance as the algorithm for the complete-link method (JOHNSON & WICHERN, 1998), in order to obtain an estimate of the animals' profile in terms of their degree of parasitism (V-Statistics software 6.0).

Fecal samples were collected directly from the rectum in order to count the number of nematode eggs per gram of feces (FEC) (UENO & GONÇALVES, 1998). Blood samples were taken by means of jugular venipuncture and collected in tubes containing 10% ethylenediaminetetraacetic acid (EDTA) for hematocrit determination and white blood cell counts (JAIN, 1993). The abomasal samples were stored in stabilizing solution (RNALater®, Applied Biosystems, USA) at -70 °C for gene expression quantification and analyses.

RNA extraction and cDNA synthesis

Total RNA was obtained from approximately 50 to 100 mg of tissue that was homogenized in liquid nitrogen and homogenized in 1 mL of TRIzol reagent (Invitrogen, USA), following the manufacturer's recommendations. The RNA quality was confirmed by means of electrophoresis on 1.5% agarose gel, stained with Sybr Gold (Invitrogen, USA) and the RNA concentration was estimated using the Quant-iT™ RNA assay kit in a Qubit fluorometer (Invitrogen, USA). The RNA samples were treated with DNase and incubated for 15 min to remove any genomic DNA residue. cDNA was obtained by means of the reverse transcription reaction (RT) using the SuperScript® III reverse transcriptase kit (Invitrogen, USA), Oligo (dT) 12-18 oligonucleotides and RNase inhibitors, following the conditions described by the manufacturer.

Real-time PCR

Seven different oligonucleotide primers were used, along with a Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control and six target genes: two collectins (CGN and SPA), two galectins (Gal14 and Gal11) and two cytokines (interleukin-4 (IL-4) and IFNG) (Box 1). Primers for CGN and SPA were designed based on data from GenBank, and the remainder were based on literature: galectins 11 and 14 (ROBINSON et al., 2011), IL-4 (HEIN et al., 2010) and IFNG (LACROUX et al., 2006). Due to the absence of data in the literature regarding collectin sequences in sheep, oligonucleotides were designed based on bovine collectin mRNA sequences in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The amplified fragments were subjected to sequencing and the results were used to design sheep collectin gene expression primers by using the Primer Express software, version 3.0 (Applied Biosystems, USA). The specificity

Box 1. Sequences of primers used in the qPCR reaction and their GenBank accession number.

Gene	Oligonucleotides	GenBank
GAPDH		
GAPDH-R	5'- ATGGTCTACATGTTCCAGTATGATTCC-3'	AF272837
GAPDH-F	5'- TGACGAGCTTCCCGTTCTCT -3'	
Conglutinin		
CGN-R	5'- GAGAATGAGGCCGTGACACA -3'	KF130969
CGN- F	5'- ACCTCCCCTCCGTGGAGAT -3'	
Surfactant protein A (SP-A)		
SP-A F	5'- CTGCCTGCTGATGAACACATT-3'	KF130970
SP-A R	5'- GCATGTCAAGGTGCTTGGTGTA-3'	

and identifications of oligonucleotides were double-checked using the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The GoTaq qPCR kit (Promega, USA) was used to amplify the sequences, in accordance with the manufacturer's instructions. Each sample was tested as duplicates, with 100 ng of cDNA per reaction and 10 pmol of each specific primer, in a final volume of 20 µl. The PCR reaction was performed in the 7500 Real-Time PCR apparatus (Applied Biosystems, USA). Samples with a positive reaction were detected by means of fluorescence emission at each amplification cycle and were subsequently analyzed by using the Sequence Detection software, version 2.0.3 (Applied Biosystems). For gene expression analysis, the average values of duplicate readings were used. Results, expressed as CT values, indicated the number of PCR cycles necessary for signal amplification to reach detection threshold levels (SDS v.1.3, Applied Biosystems). Gene expression quantification was performed using the Δ CT method (Δ CT = target CT gene minus CT endogenous gene control), with GAPDH as the endogenous control, and was expressed as relative values normalized against an arbitrary factor of 20 (ROBINSON et al., 2011).

Statistical analysis

Differences between groups were compared by means of the Mann-Whitney test and correlations between variables using the Spearman correlation test, a P-value <0.05 was considered significant (SPSS v. 17, IBM, USA).

Results

Significant differences between the two groups were observed in relation to FEC counts and parasite counts. Mucosal eosinophil counts, hemoglobin, hematocrit and plasma protein values did not differ between the groups (Table 1). *H. contortus* was the only species found in the abomasum of these animals.

Gene expression of galectins and collectins was observed in all sheep abomasal samples. However, animals with lower infection had lower gene expression levels of Gal14, SPA and CGN than shown by those in the high infection group ($p < 0.05$) (Figure 1).

Table 1. Mean \pm standard deviation values of parasitological and hematological traits of Corriedale sheep with lower (G1) and higher (G2) *Haemonchus contortus* parasitism level.

Traits	Groups		p†
	G1	G2	
Faecal egg count (FEC)	0 \pm 0	680.0 \pm 831.9	0.05
Abomasal <i>H. contortus</i> burden (L4 and L5)	10.6 \pm 8.3	59.2 \pm 27.7	0.01
Mucosal eosinophil count (mm ²)	24.9 \pm 5.4	25.1 \pm 3.9	0.91
Total leukocyte count (x10 ³ /µL)	5857.1 \pm 1444.4	4920.0 \pm 1435.1	0.29
Hematocrit (%)	31.4 \pm 1.3	30.4 \pm 2.3	0.40
Total serum protein (g/dL)	7.1 \pm 0.3	6.7 \pm 0.6	0.28

†A p-value <0.05 was considered significant.

H. contortus infection levels did not affect the gene expression levels of Gal11, IL-4 or IFNG.

Gal14 gene expression levels and abomasal parasite burden were negatively correlated, while there were strong positive correlations among IFNG, Gal14, SPA and IL-4 (Table 2). Within-group testing found strong positive correlations between G1/G1 SPA and IL-4 ($r = 0.893$; $p = 0.012$); G1/G1 IL-4 and IFNG ($r = 0.929$; $p = 0.007$); G2/G2 abomasal parasite burden and Gal14 ($r = 1.00$; $p = 0.017$); and G1/G1 eosinophil count and CGN ($r = 0.982$; $p = 0.0004$).

Discussion

All the animals, exposed to natural infection, were similar in age, breed, health program and supplementary feeding system. They were kept in the same paddock and were subjected to repeated gastrointestinal nematode challenge and infections. The overall low FEC and abomasal parasite burden was a consequence of anthelmintic treatment performed two weeks before slaughter, because of extreme FEC counts. As a consequence, the number of parasites in both

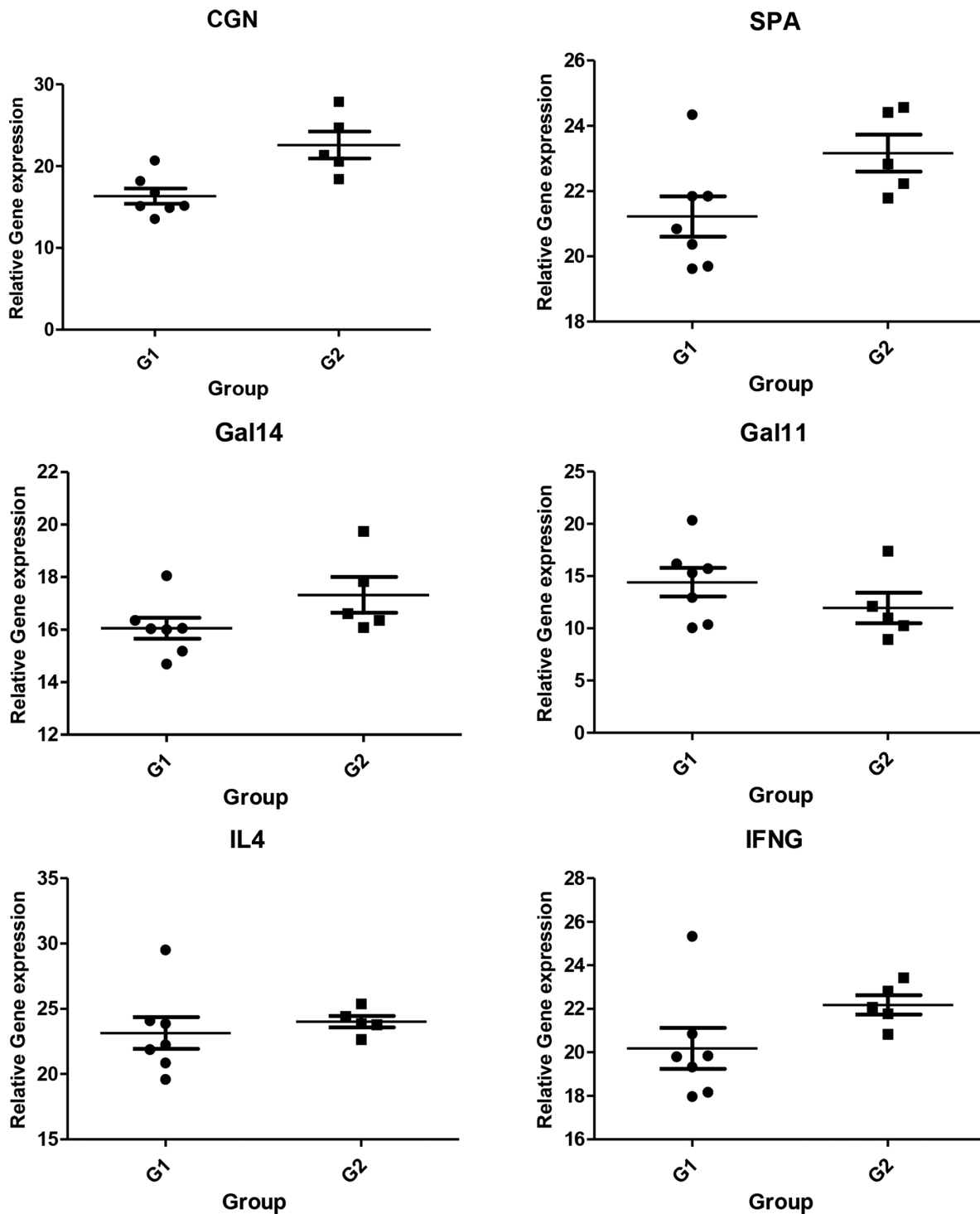


Figure 1. Conglutinin (CGN), surfactant protein A (SPA), galectins-14 (Gal14) and 11 (Gal11), interleukin 4 (IL4) and interferon gamma (IFNG) relative gene expression in low (G1) and high (G2) *Haemonchus contortus* infected Corriedale sheep.

groups decreased, and this intervention might explain the absence of other nematode species. Nonetheless, significant differences between parasitism levels were detected between the two groups.

There were no differences in hematological and biochemical parameters between the groups, which may have been due to the improvement of physiological conditions after anthelmintic treatment. A steady leukocyte count response in cases of

gastrointestinal nematode infection, especially regarding abomasal eosinophil counts, was observed by Amarante et al., (2005) and Terefe et al., (2007) when studying sheep that were resistant and susceptible to *H. contortus* infection. This was seen because mobilization of effector cells is important in the early days of infection (TEREFE et al., 2009). This argument may also be an explanation for our findings, since the sheep had chronic infection.

The sheep with high *H. contortus* infection levels showed higher conglutinin (CGN), surfactant protein A (SPA) and galectin 14 (Gal14) gene expression levels than shown by the sheep with low *H. contortus* infection levels (Table 2). Similar behavior was observed in studies on cattle experimentally infected with *O. ostertagi* and *C. oncophora* (LI & GASBARRE, 2009; LI et al., 2010), in which collectin-46, conglutinin and galectin-11 gene expression levels increased in animals after repeated infection or during the course of infection, revealing that tissue response is a dynamic process and the peak of the expression of these and other genes was detected between the 14 -28th day post infection. In our study, it was possible to measure the variation in expression in sheep kept under natural pasture conditions but in low magnitude and the the lack of correlation of other genes and tissue eosinophils may be related to the moment of the host response and infection phase.

Conglutinin and SPA appear to have an indirect effect on the immune response. SPA is capable of limiting inflammation and tissue damage during bacterial or fungal infection (GIANNONI et al., 2006; MADAN et al., 2010), as demonstrated in *Mycoplasma pneumoniae*-infected mice, in which SPA inhibited the binding of eosinophils to bacteria and therefore reduced the release of eosinophil peroxidase protein, which was implicated in the killing of pathogens (LEDFORD et al., 2012). In *Aspergillus fumigatus* infection, SPA inhibited the binding of IgE to basophils and prevented the release of histamine (MADAN et al., 1997). In contrast, *A. fumigatus* infected SPA-deficient knockout mice (-/-), showed protein pulmonary eosinophilia and increased tissue damage (MADAN et al., 2005), after SPA had been administered. These events emphasize that SPA can perform both functions as a pro-inflammatory or anti-inflammatory agent and, perhaps for this reason, there were high positive correlations between SPA and IL-4 and between SPA and IFNG in *H. contortus*-infected sheep.

The conglutinin studied here had, up until now, been regarded a bovine-specific protein. The present study provides the first description of an association between *H. contortus* infection levels and conglutinin and SPA gene expression levels, in sheep. The action of conglutinin is not restricted to antimicrobial activity: it can also serve as an anti-inflammatory agent for maintaining symbiosis and

preventing abomasum inflammation (HANSEN et al., 2002, 2003). Interestingly, we observed a strong correlation between CGN and eosinophil counts at low *H. contortus* infection levels, thus suggesting that, like SPA, these collectins may act by inhibiting the release of inflammatory substances, even though it is not known which collectins are regulated during abomasal infection. However, because of the high similarity of the CL-43, CL-46 and conglutinin sequences in cattle, the primer CGN annealed a conserved collectin sequence.

Unlike collectins, it has been suggested that Gal14 has a direct effect on nematodes, since high correlation was found between these protein levels and the abomasal parasite burden, in both between and within-group analyses. This galectin is considered to be an eosinophil-specific protein (DUNPHY et al., 2002): it is abundantly present in the mucus of the gastrointestinal tract after parasite challenge (ROBINSON et al., 2011) and has a strong ability to promote adhesion and alter mucus properties during infection (YOUNG et al., 2009). Interestingly, no strong correlation between eosinophil counts and Gal14 gene expression levels was observed, thus coinciding with the results found by Robinson et al., (2011) in sheep after experimental infection with *H. contortus*. High expression of Gal14 may be a consequence of the exposure period, since Gal14 becomes more sensitive to eosinophil degranulation after repeated infections (KEMP et al., 2009).

The gene expression of IL-4 and IFNG showed no difference between the groups. The high correlation observed between IL-4 and IFNG confirms that both of these cytokines can be elicited in the same environment during gastrointestinal nematode infection, as also described by Meeusen et al. (2005) and by Pernthaner et al. (2006). Understanding the factors that lead to polarization and differentiation of Th1 and Th2 responses in gastrointestinal nematode infections in ruminants is complex (LI et al., 2010; ROBINSON et al., 2011). Polarization is not as important as the speed at which these responses become established, such that animals that are resistant to nematodes are able to adjust more quickly to the Th2 response (TEREFE et al., 2007).

In conclusion, we have demonstrated that *H. contortus*-infected sheep showed induction of gene expression for conglutinin, surfactant protein A and galectin-14, and that the group with the highest degree of infection also showed the highest gene expression for these proteins. From these results together, it was possible to determine that the abomasal *H. contortus* count in sheep is associated with the expression of collectins and Gal14, may participate in the host defense against the gastrointestinal nematode infection. Lectins can act as pro- or anti-inflammatory markers according to different interactions at different moments of the infection. However, these results constitute merely an initial step towards identifying the role of galectins and collectins in sheep gastrointestinal nematode infections.

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Table 2. Correlation between cytokine gene expression, collectins and abomasal *Haemonchus contortus* counts.

Parameters	r	p†
<i>H. contortus</i> burden		
Gal14	-0.608	0.036
IL4		
SPA	0.818	0.001
IFNG	0.748	0.005
IFNG		
SPA	0.790	0.002
Gal14	0.755	0.005
Eosinophil count	-0.691	0.013
SPA		
Gal14	0.622	0.031

†A p-value <0.05 was considered significant.

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