Cellular Immune Response and Abomasum worm burden in Goats Vaccinated with HC58cDNA Vaccine against *H. contortus* Infection

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ABSTRACT

Vaccination with DNA vaccines derived from adult *H. contortus* induces significant level of protection against homologous infection in goat. To date however, mechanism of protection is not well understood, especially in goat. In this study, HC58 DNA vaccinated goats were artificially infected with 5, 000 dose of infective *H. contortus* L_3 (third larval stage), and cellular immune responses and abomasum worm burden examined. The results showed that peripheral $CD4^+$, $CD8^+$ T and B lymphocytes for nematode challenged Groups 1, 2 and 4 increased subsequent to L_3 infection compared to negative control Group 3. Likewise, the mean eosinophil and lymphocyte counts increased substantially after vaccination and L_3 challenge. On the contrary, circulating neutrophil and white blood cells reduced under similar experimental conditions in goats carrying an equal L_3 nematode burden. These findings suggested that regulation of *H. contortus* expulsion in goat is a complex mechanism orchestrated by $CD4^+$ and $CD8^+$T cells, recruitment of eosinophil and lymphocytes and inclined towards development of Th_2 responses.

Keywords: *Haemonchus contortus*; goat; HC58DNA vaccine; cellular immune responses.

1.0 INTRODUCTION

H. contortus is an abomasal nematode that parasitizes cattle, sheep and goat causing major production losses. Debilitating infection with this parasite is most commonly seen in young animals, and resistance to infection develops, with exposure in older shoats. Majority of vaccination studies suggest that DNA vaccines contribute to the control of *H. contortus* infection (Knox and Smith, 2001). However, the mechanism(s) of immunity induced by these vaccines against *H. contortus* is not well understood, especially in goat.

Depletion studies in sheep have shown that $CD4^+$ T lymphocytes play pivotal roles in mediating resistance to *H. contortus* (Gill *et al.,* 1993). Resistance to *H. contortus* infection is associated with increased eosinophils and generation of anti-Haemonchus antibodies (Amarantea et al., 1999; Balic *et al.,* 2002; Balic *et al.,* 2006). Although systemic cellular immune responses have often been studied, the results are contradictory and inconsistent (Smith, 1993; Sinski *et al.,* 1995). Studies focusing on the cellular immune responses are not only relevant for monitoring development of resistance against *H. contortus* infection in goat, but also appropriate for assessing responses to candidate protective antigens for use in vaccination programmes.

Previously we reported characterization of HC58 cDNA, a putative cysteine protease from *H. contortus* and purification of recombinant HC58 protein (Muleke *et al.*, 2006). The present study was undertaken to further investigate worm burden and cellular immune responses in goats vaccinated with HC58DNA vaccine and subsequently challenged with L_3 larvae of *H. contortus* parasite.

2.0 METHODS AND MATERIALS

2.1 Parasites and infective larvae

H. contortus strain used was originally obtained from Nanjing, Jiangsu Province, China and maintained by serial passage in helminth-free goats for the last few years in the department. Feces were collected over two weeks period and cultured at (26^0C) to yield third stage infective larvae (L_3) according to the Manual of Veterinary Parasitological Laboratory Techniques (Anonymous, 1987). Larvae used for challenge infection were less than 1 months old and were stored in water at concentrations of 2500 ml^{-1} at 4^0C until used.

2.2 Construction of recombinant pET-28a/HC58cDNA

Recombinant HC58 protein was constructed as described (Muleke *et al.,* 2006). Purified HC58cDNA PCR product was cloned into pMD-18T cloning vector (Takara Biotechnology). *E. coli* $DH_{5\alpha}$ was transformed with recombinant pMD-18T/HC58cDNA using competent calcium chloride treated cells as described by Sambrook *et al.,* (2002). Recombinant pMD-18T/HC58cDNA was digested with *BamH* Ⅰ and *Hind*Ⅲ restriction enzymes for identification and purified with "Takara DNA agarose gel purification kit, Ver.2", as per manufacturer instructions. Purified HC58cDNA fragment was ligated to pET-28a vector DNA previously linearized with identical enzymes. *E. coli* BL_{21} (DE3) was transformed with recombinant pET-28a/HC58cDNA for construction

of HC58 DNA vaccine.

2.3 Construction of HC58 DNA vaccine

The recombinant pET28a/HC58cDNA was digested with *BamH*Ⅰ and *Hind*Ⅲ restriction enzymes and HC58DNA product directionally cloned into pVAX1 vector (Invitrogen, life technologies) previously linearized with identical enzymes. Recombinant pVAX1/HC58DNA was digested with *BamH*Ⅰ and *Hind*Ⅲ restriction enzymes followed by gel electrophoresis for identification purposes and the HC58DNA verified by sequencing (Shanghai Casarray Co., LTD, Shanghai, China). *Escherichia coli* $DH_{5\alpha}$ (JM109) was transformed with recombinant pVAX1/HC58DNA using calcium chloride treated cells as described (Sambrook *et al*., 2002), and cultured in Luria Bartini media for 8 h at 37ºC. The HC58 DNA was eluted with the Qiagen Plasmid DNA Mid Kit (Qiagen) as per manufacturer instructions and stored at -20^0C until used for vaccination.

2.4 Vaccination trials

Sixteen local goats (8-10 months old) were raised indoors under nematode-free conditions. The animals were allocated into four experimental groups of four goats each and matched for sex and body weight. Experimental groups were treated as follows: group 1 was administered with 200μg of HC58 DNA vaccine (3μg/μl) constituted in 1ml of PBS pH 7.4. The 1ml injection volume was equally divided between two injections sites, alternating between thigh and shoulder muscles. Group 2 was administered with 10mg of recombinant HC58 protein (1mg/ml) emulsified with complete Freund's adjuvant (Sigma, UK), administered subcutaneously. A booster dose was repeated with the protein suspended in the incomplete Freund's adjuvant (Sigma, UK) after two weeks period. Group 3 served as unvaccinated negative (–ve) control that was not challenged with L_3, but mock-vaccinated with 1ml of PBS pH 7.4. Group 4 was the unvaccinated positive (+ve) control, challenged with L_3 infection and mock-vaccinated with 1ml of PBS pH 7.4. Animals were run in two adjacent pens, one of group 1, 2 and 4 and the other of group 3. Vaccinated animals received two vaccinations at 2 week intervals, 28 and 14 days before challenge, with 5000 infective *H. contortus* L_3 larvae which was given two (2) weeks after the final injection.

2.5 Abomasum worm counts

At 35 days post infection goats were killed humanely and abomasum worm counts determined. The abomasa were removed, scrapped and washed with warm 0.85% NaCl saline solution to detach any adhering worms from both abomasal content and mucosal digests. Adult male and female worms, developing stages and degenerates were retrieved and processed for worm counting according to the Manual of Veterinary Parasitological Laboratory Techniques (Anonymous, 1987). Parasites were counted and sorted according to their sexes.

2.6 Determination of CD4⁺, CD8⁺ and B lymphocyte levels

Heparinized blood (0.2mg/ml) was collected by jugular venipuncture for $CD4^+$, $CD8^+$T and B lymphocyte analysis on 0, 14, 28 and 63 days of the study. Erythrocyte lyses was performed on pelleted blood cells with pre-warmed ammonium chloride pH 7.4 at 37^0C, followed by three washes in PBS pH 7.4. Isolated cells were re-suspended to a concentration of 1×10^6 cells in 100μl volume of PBS pH7.4 containing 2% FCS (fetal calf serum). Cells were centrifuged at 1500rpm for 5 min and the media was removed. PE-conjugated monoclonal antibodies to CD4 (GC50A1) or CD8 (BAQ111A) or CD2 (MUC2A) or B lymphocytes (B-B2) (BAQ44A) (Caltag Laboratories, USA) were added, respectively. Cells and the antibodies were suspended in 1ml PBS pH 7.4 and placed in the dark for 15 min. Unbound antibodies were removed by washing with 2%FCS in PBS pH7.4. The fluorescein labeled secondary antibody (goat anti-mouse IgG1, FITC, 488mm, fluorochrome conjugate antibody (M32101) (Caltag Laboratories, USA) was diluted 1/40 with 2% FCS in PBS pH 7.4. A 10-μl of diluted secondary antibody was added to the cells. The cells and antibodies were mixed and incubated on ice in the dark for 15 min. Cells were then washed once in 2% FCS in PBS and again in FACS buffer (5% FCS and 1% sodium azide in PBS). After the final wash, cells were re-suspended in 500μl of FACS buffer and 1% paraformaldehyde (500μl) in PBS. Stained cells were analyzed with the FACScan flow cytometer equipped with CellQuest software (Becton Dickinson, Mountain View, CA).

2.7 Total blood cell count

Heparinized blood (0.2mg/ml) for total blood cell count was collected into evacuated glass tubes (Medage, China) on 0, 14, 28 and 63 days of the study. Differential cell count was performed with the automated electronic cell Coulter Counter (HYCEL DZANA Model 5, France).

2.8 Statistical analysis

Statistical analysis was performed with the SPSS statistical package (SPSS for Windows 11.5, SPSS Inc., Chicago, IL, USA). Differences between groups were tested with the One-Way ANOVA Duncan test. Differences were considered significant when $P < 0.05$. Correlations were tested with Spearman's rank correlation coefficient.

3.0 RESULTS

3.1 Abomasum worm count

The mean (±SD) abomasum worm counts for Group 1 and 2 were 532.3±143.5 and 622.5±71.3 respectively, (P<0.05), as opposed to 741.5±241.5 of group 4, translating to worm burden reductions of 28.3% and 16.1% respectively (Table 1). The ratio of female to male worms retrieved from Group 1 and 2 was ~1.1. While the reduction of female and male worms for Group 1 were 32.3 and 23% as opposed to those for Group 2 of 18.8 and 12.5%, respectively (Table 1).

3.2 The percentage of CD4$^+$, CD8$^+$T and B lymphocytes

The percentage of CD4$^+$ T cells for Group 3 provided the background reference values. Group 1 and 2 had equivalent proportions of CD4$^+$ T cells to the control Group 3 and 4 respectively, before L$_3$ challenge at 0d (Table 2). However, after L$_3$ challenge percentage of CD4$^+$ T cells increased tremendously to peak values of 20-22% for vaccinated Groups 1 and 2, until end of the study (at 63d) (Table 2). The percentage of CD4$^+$ T cells for Group 3 and 4 was not significantly different during the study (Table 2). No correlation was detected between abomasum worm count or egg out put (data not shown) and percentage of CD4$^+$ T lymphocytes.

Vaccinated groups had significantly (P<0.05) higher percentage of CD8$^+$ T cells compared to Group 3 and 4, before L$_3$ challenge (at 0d and 14d of the study) (Table 2). Notably, there was a drastic increase in percentage of CD8$^+$ T lymphocytes for Group 1 from 4.72±5.77 to 10.89±3.76, Group 2 from 2.82±1.79 to 8.79±4.28 and Group 4 from 3.23±0.54 to 15.36±2.60 as from 28d (after L$_3$ challenge) until the end of study (63d) (Table 2). No correlation was detected between abomasum worm counts or egg output (data not shown) and percentage of CD8$^+$T lymphocytes.

Analysis of percentage of B cells revealed a large difference between the four study groups (Table 2). In overall, percentage of B cells recorded for vaccinated Groups 1 and 2 were significantly higher than Group 3 and 4, respectively (P<0.05) (Table 2). Unlike CD4$^+$ and CD8$^+$T cells, a significant correlation was detected between percentage B cells and abomasum worm count (r = 0.473, $P < 0.05$).

3.3   Total Blood Cell Count

3.3.1 Eosinophils

The mean eosinophil count for Group 1 (26.3%) and Group 2 (16.5%) differed significantly (P < 0.05) from that of Group 3 and 4 that was fairly low (1~2.3%) during the study. The blood eosinophil count for Groups 1, 2 and 4 increased drastically after L$_3$ challenge infection until end of the trial (Fig. 1). A positive correlation was detected between eosinophil count and percentage of CD4$^+$T cells (r = +0.45; P < 0.05) and a negative correlation between eosinophil number and egg output (data not shown) (r= -0.534; P < 0.05).

3.3.2 Lymphocytes

Lymphocyte level varied in the four experimental groups (Fig. 2). In overall, there were fewer lymphocytes in Groups 1, 2 and 4 before L$_3$ challenge infection. The lymphocyte population increased significantly in these nematode challenged groups after infection in relation to Group 3 (P<0.05) (Fig. 2).

3.3.2 White blood cells

There were more white blood cells in Groups 1, 2 and 4 before L$_3$ challenge infection. The white blood cell level in these groups dropped significantly after L$_3$ challenge at (28d) compared to Group 3 (P <0.01) (Table 2). The mean (±SD) white blood cells for Group 1 in the range (15.150~22.900) and Group 2 (13.725~20.500) was high compared to either Group 3 or 4 in the range (12.795~19.050) respectively, (P < 0.05)

3.3.3 Neutrophils

Like the white blood cells, there were more neutrophils in Groups 1, 2 and 4 prior to L$_3$ challenge infection followed by a sharp reduction in the neutrophil population after L$_3$ challenge in relation to Group 3 (Fig. 3). Unlike white blood cells, no significant difference was detected between neutrophils and other parameters between the four groups.

3.3.4 Red blood cells

The mean (±SD) red blood cells for Group 3 and 4 were in the range 1.178~1.385 and 1.093~1.738 respectively, during the study. Concomitant red blood cell population for Group 1 and 2 were in the range 0.758~2.030 and 0.910~1.808, respectively (Table 2). These values were all within the normal range for healthy goats (Beriajaya and Copeman, 2006).

3.3.5 Hemoglobin level

Overall, there was more hemoglobin in L$_3$ challenged groups before infection. The hemoglobin level in Groups 1, 2 and 4 dropped enormously after nematode challenge infection from 113 to 43.75g/L, 109 to 51.25g/L and 106 to 51.0g/L respectively, in relation to Group 3 (P<0.05). No significant change was detected in the amount of hemoglobin for Group 3 during the study (Table 2).

4.0 DISCUSSION

In this study, we describe the potential of HC58 DNA vaccine of *H. contortus* to induce cellular immunity against homologous challenge in goat. The results indicated there was a significant increase of circulating $CD4^+$, $CD8^+$ T and B lymphocytes for Groups 1, 2 and 4 as from 28d onwards (after L_3 challenge). Groups 1 and 2 had significantly more plasma IgG and IgA ($p<0.05$) (data not shown), that overlapped and paralleled throughout the study, concurring with the elevated eosinophils following nematode challenge infection (at 28d) in relation to Group 3. However, protection level was low and failed to match the enormous immune response, as evidenced by significantly low worm burden reductions (~28%). This observation suggested vaccination with HC58 DNA vaccine induced Th2 responses accompanied with low protection level in goat. Our findings agree with those of secondarily infected sheep that also had low or non-protective Th2 based responses (Schallig, 2000), suggesting the ratio of different cytokines could be vital for the final outcome of Th2 responses in goat

A positive correlation was detected between %$CD4^+$ T cells and eosinophils in the study (r=+0.45; $P < 0.05$), suggesting eosinophil recruitment was probably dependent on cytokines produced by $CD4^+$ Th2 cells. More eosinophils were found in nematode challenged goats and correlated negatively with egg output (data not shown)(r= - 0.534; $P < 0.05$), implying eosinophil cells could play a role in the rejection of *H. contortus* parasite. Antigens produced by helminths are thought to be strong inducers of reaginins and hemocytotropic IgG elements which, together with eosinophils are responsible for antibody-dependent and cell-mediated cytotoxicity (ADCC) (Butterworth, 1984).The low population of eosinophils in Group 3 suggested these cells could play a role or have their numbers stimulated as a byproduct of the immune process.

Evidence suggests that B cells are activated by infection for rapid Th2 responses in *Trichuris muris* infected mice (Blackwell and Else, 2001). The authors of this mice model proposed that B cells act through accessory antigen presenting cells or via antibody production. In the present study, L_3 challenge infection stimulated B cell production, coincident with elevated serum IgG and IgA antibodies (data not shown) for Groups 1 and 2. Reports for animals where peripheral cellular immune responses have been studied are contradictory and inconsistent. This is probably due to re-circulating parasite-specific cells in the blood from mucosal predilection sites of *H. contortus* parasite, suggesting that monitoring of immune events at local tissue sites during parasite infection may provide a better insight for cellular responses in goat.

In overall, vaccinated animals had significantly more lymphocytes after L_3 challenge in comparison to control Group 4. This observation concurred with findings in sheep vaccinated against *H. contortus* (Schallig and Van Leeuwen, 1997). It is suggested that sheep naïve to *H. contortus* infection respond with increased lymphocyte cells against L_3 challenge accompanied by low susceptibility to experimental infections (Torgerson and Lloyd, 1993; Schallig and Van Leeuwen, 1997). The elevated lymphocytes for vaccinated goats, notably after L_3 challenge suggested these cells could be involved in expulsion of the parasite.

The reduction effect of hemoglobin by *H. contortus* parasite is well documented (Soulsby, 1982), and was also observed following nematode challenge in Groups 1, 2 and 4 in the present study. However, there was no proportional relationship between abomasum worm burden and hemoglobin level for individual groups. This suggested that hemoglobin values could only provide a useful aid to group diagnosis of haemonchosis, but was not a reliable indicator of worm burdens for goat. In conclusion, the outcome of the present vaccination trial suggested the mechanism of *H. contortus* expulsion in goat is a complex process that still requires further investigation.

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Legend of Tables

Table 1: Mean (±SD) abomasal worm count for vaccinated (HC58 DNA-Group 1 and HC58 recombinant protein-Group 2) and non-vaccinated [Groups 3 (negative control) and 4 (positive control)] goats. Groups 1, 2 and 4 were challenged with 5000 *Haemonchus contortus* L$_3$. *=significant difference (p < 0.05). F/M= female to male worm ratio.

Group	Female	Male	Total	F/M	Female worm reductions	Male worm reductions	Total worm reductions
1(n=4)	283.5±84.34	248.75±61.15	532.25±143.54	1.13	32.3%	23.0%	28.3%*
2(n=4)	339.75±30.54	282.75±49.24	622.5±71.29	1.20	18.8%	12.5%	16.1%*
3(n=4)	0	0	0	0	0	0	0
4(n=4)	418.5±124.24	323±117.90	741.5±241.45	1.29	--	--	--

Table 2: The mean (±SD) %CD4+, %CD8+ T, %B lymphocytes, white blood cells, red blood cells and hemoglobin level for vaccinated (HC58 DNA-Group 1 and HC58 recombinant protein-Group 2) and non-vaccinated [Groups 3 (negative control) and 4 (positive control)] goats. Groups 1, 2 and 4 were challenged with 5000 *Haemonchus contortus* L$_3$ at 28d. In each row, results with different superscripts are significantly different (p < 0.05).

Cell type	Group	Days of Experiment			
		0	**14**	**28**	**63d**
%CD4+T lymphocytes	1	10.53±1.58[b]	20.56±8.72[a]	18.19±12.50[a]	18.42±7.30[a]
	2	9.96±1.35[b]	16.66±6.69[a]	20.52±10.90[a]	15.27±8.63[ab]
	3	11.50±2.43[a]	11.44±1.30[a]	11.35±1.33[a]	11.04±3.26[a]
	4	10.75±2.20[b]	10.07±2.07[b]	10.36±1.2[b]	17.21±2.2[a]
%CD8+T lymphocytes	1	2.33±0.77[c]	2.49±0.81[c]	4.72±5.77[b]	10.89±3.76[a]
	2	2.14±1.09[b]	3.49±3.10[b]	2.82±1.79[b]	8.79±4.28[a]
	3	1.19±0.34[c]	2.22±3.35[b]	2.16±0.15[b]	2.88±0.86[a]
	4	2.03±0.57[c]	1.94±0.71[c]	3.23±0.54[b]	15.36±2.60[a]
%B cells	1	13.69±2.98[c]	15.60±5.14[c]	35.76±14.5[b]	44.94±13.43[a]
	2	14.66±3.21[c]	22.40±5.09[b]	25.22±13.5[b]	38.81±13.15[a]
	3	10.02±2.76[c]	12.86±2.76[c]	19.61±4.74[b]	22.79±6.39[a]
	4	12.30±3.38[c]	10.77±3.19[c]	14.09±3.82[b]	28.45±7.28[a]
White Blood cells (x10^9/L)	1	16.89±3.98[b]	20.53±8.9[ac]	22.90±9.46[a]	15.15±2.37[b]
	2	17.93±6.76[a]	20.50±7.67[ac]	18.13±6.76[a]	13.73±3.79[b]
	3	19.05±2.71[a]	18.65±3.12[a]	18.93±6.78[a]	17.45±7.36[a]
	4	17.80±6.11[a]	17.95±8.08[ac]	18.89±6.55[a]	12.79±4.53[b]
Red Blood cells (x10^12/L)	1	1.108±0.50[b]	0.978±0.32[b]	0.758±0.75[c]	2.030±0.50[a]
	2	0.910±0.20[b]	0.818±0.19[b]	1.710±1.38[a]	1.808±0.40[a]
	3	1.385±0.56[a]	1.330±0.52[a]	1.178±0.62[b]	1.333±0.41[a]
	4	1.093±0.23[b]	1.288±0.51[b]	1.123±0.48[b]	1.738±0.38[a]
Hemoglobin (g/L)	1	113.0±5.77[b]	103.3±11.9[b]	98.8±20.96[b]	43.8±5.73[a]
	2	109.0±16.9[b]	104.3±16.87[b]	104.8±18.22[b]	51.3±31.33[a]
	3	111.5±7.18[a]	119.0±8.24[a]	112.3±25.42[a]	117.7±21.6[a]
	4	106.0±15.4[b]	109.5±10.7[b]	112.7±36.5[b]	51.0±30.25[a]

Legend of Figures

Fig. 1: The (mean ± SD) eosinophil for goats vaccinated with HC58 DNA vaccine in group 1 compared to groups 2, 3 and 4. Goats were vaccinated at 0d, 14d (short arrow) and subsequently challenged with L_3 larvae at 28d (long arrow). *=significance at P<0.05.
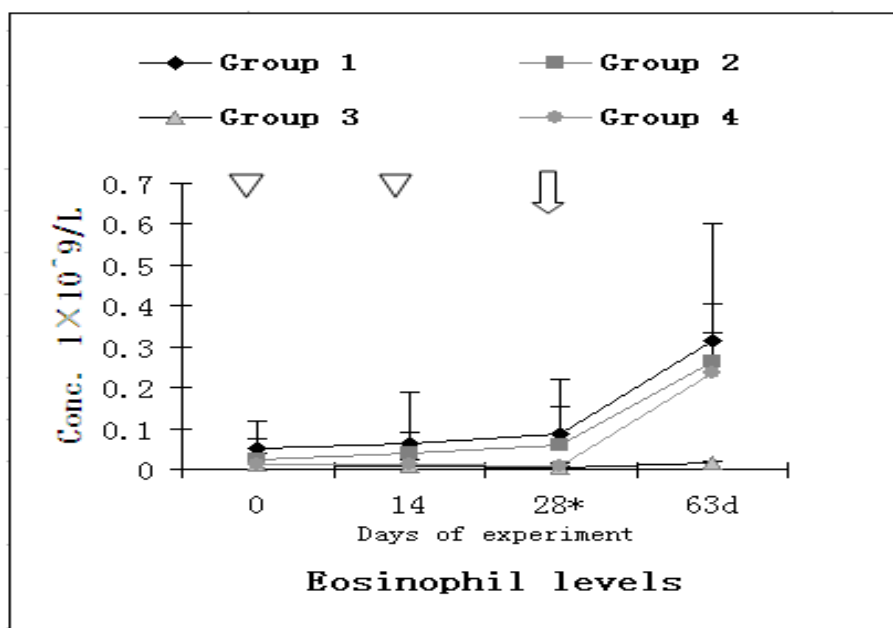
Fig. 2: The (mean ±SD) lymphocytes for goats vaccinated at 0d, 14d (short arrows) and subsequently challenged with L$_3$ larvae at 28d (long arrow). *=significance at P<0.05.
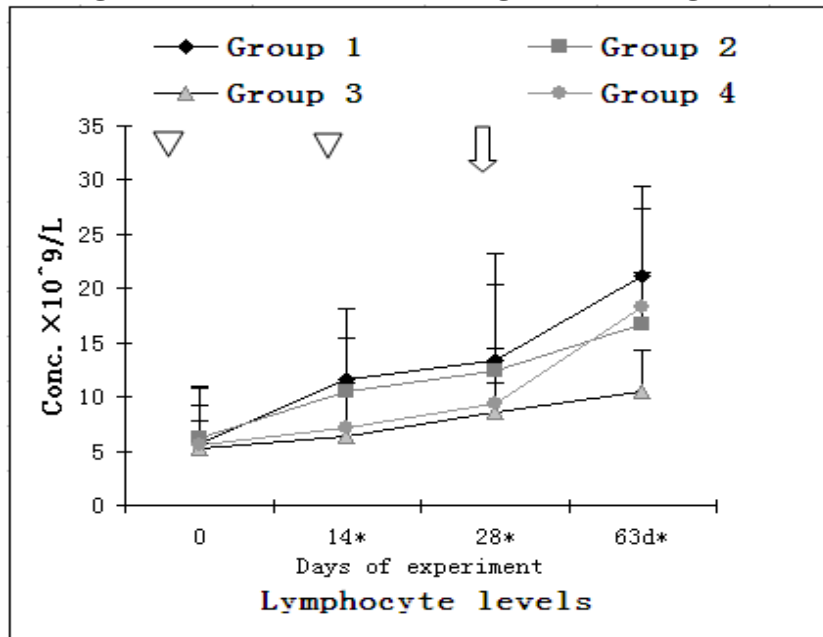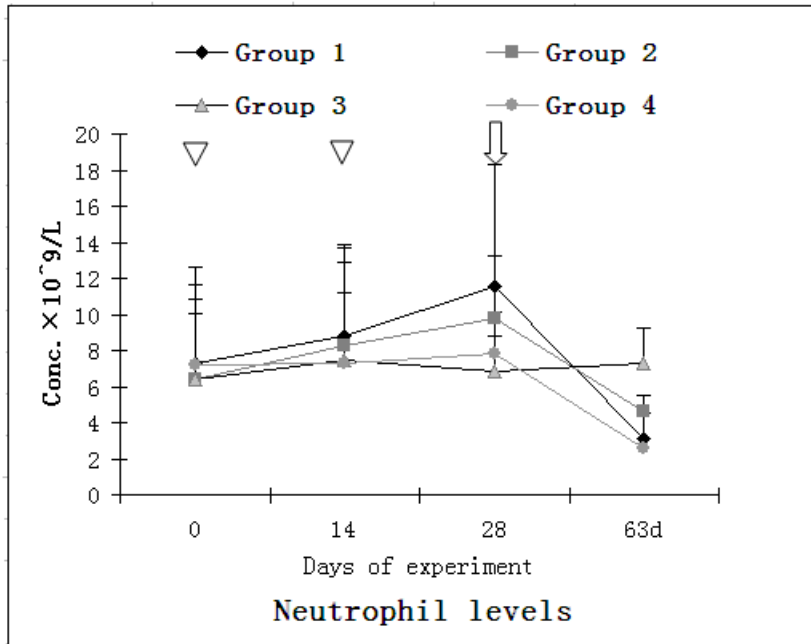

Fig. 3: The (mean ±SD) neutrophils for goats vaccinated at 0d, 14d (short arrows) and subsequently challenged with L$_3$ larvae at 28d (long arrow).

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