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Research Article
Cytokine Expression Studies in Natural Cases of Bovine Lymphadenopathies

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Abstract

Background: The present study was undertaken to investigate the expression of Th1 and Th2 cytokines and to correlate the expression of these cytokines with pathological changes associated with natural cases of specific and non-specific lymphadenopathies in bovines. **Materials and Methods:** The study included 32 cases of bovine lymphadenopathies presented for necropsy examination to the Department of Veterinary Pathology, GADVASU, Ludhiana, India. Samples from affected lymph nodes and visceral organs showing lesions were collected for cytology, histopathology, immunohistochemistry, ELISA and PCR. **Results:** There was variable expression of Th1 and Th2 cytokines in specific and non-specific lymphadenopathies in bovines. In lymphoma the expression of both Th1 and Th2 cytokines was decreased. There was increased expression of IFN-γ in bovine tuberculosis and strong IL-10 expression in Johne's disease. In non-specific conditions, there was continuation of both Th1 and Th2 responses in acute lymphadenitis, along with increased Th2 expression in cases of hyperplasia, hemorrhagic and chronic lymphadenitis. **Conclusion:** The present study concluded that analysis of cytokine expression and pathological lesions in the lymph nodes of bovines are of paramount importance in diagnosis of specific and non-specific lymphadenopathies.

Key words: Bovine lymphadenopathies, bovine tuberculosis, hyperplasia, immune response, lymphadenitis, lymph nodes, Th1 and Th2 cytokines

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Lymphadenopathy refers to a condition in which lymph nodes become abnormal in size, number or consistency. This can be appreciated grossly by two basic changes; either there may be increase in size of lymph nodes associated with lymphadenitis, lymphoid hyperplasia, hyperplasia of monocyte/macrophage system, primary neoplasia and metastatic neoplasia or decrease in size due to lymphoid atrophy, lymph node degeneration and lymph node hypoplasia[1]. Lymphadenopathies may be associated with certain specific and non-specific conditions like tuberculosis[2-4], paratuberculosis[5,6], theileriosis[7,8] and lymphosarcoma[2].

Lymph nodes are composed of T-cells and B-cells, which play an important role in provoking immune response through Th1 (cell mediated) or Th2 (humoral immune responses) cytokines. The Th1 cells produce interleukin (IL-2), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) whereas, Th2 cells produce IL-4, IL-5, IL-10 and IL-13[9]. The IFN-γ is mainly responsible for their pro-inflammatory effects and it stimulates phagocytosis[10,11], oxidative burst[12,13] and intracellular killing of microbes[14-16]. In addition, it also upregulates expression of class I and class II Major Histocompatibility Complex (MHC) molecules[17-20] on a variety of cells, thus stimulating antigen presentation to T-cells.

There is meager information on cytokine responses of lymph nodes in cattle and buffaloes. Generally lymph nodes are examined to diagnose neoplasms but lymph nodes should also be examined to identify the characteristic pathological lesions that occur in certain infectious diseases like tuberculosis. So, the present study was undertaken to investigate the Th1 and Th2 cytokines profiles and to correlate the expression of these cytokines with pathological lesions associated with natural cases of specific and non-specific lymphadenopathies in bovines.

MATERIALS AND METHODS

Present study was conducted on 32 cases of bovine lymphadenopathies prseted for necropsy examination to the Department of Veterinary Pathology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. Tissue samples of affected lymph nodes and the visceral organs showing lesions were collected fixed in 10% formalin for routine histopathological and imunohistochemical staining, whereas, another part was kept at -20°C for cytokine estimation. In addition, impression smears were prepared from affected lymph nodes and stained with Wright's technique for cytological examination. Lymph nodes collected from three animals showing no obvious pathological lesions served as control. The frozen lymph node samples were thawed, homogenized in 1 mL PBS and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and stored at -20°C till further use. Cytokine estimation was perfomed using commercially available IL-2, IL-4, TNF-α, INF-γ and IL-10 ELISA kits (Table 1). The concentrations of the protein were estimated by using Pierce™ BCA protein assay kit. The values of cytokines were expressed as Mean±SD and Student's t-test was applied to calculate the level of significance.

RESULTS AND DISCUSSION

In the present study, the lymphadenopathies of bovines were diagnosed and classified into neoplastic and non-neoplastic conditions on the basis of cytopathology. With the help of immunohistochemistry using specific markers for T-cells (CD3, CD4 and CD8) and B-cell (p27), 2 cases of lymphomas were further characterized as T-cell and B-cell lymphoma. Non-neoplastic lymphadenopathies included 9 cases of specific diseases including; 1 case of bovine tuberculosis, 2 cases of Johne's disease and 6 cases of theileriosis, whereas, 21 cases of non-specific lymphadenopathies comprised of 9 cases of chronic lymphadenitis, 6 cases of hyperplasia, 4 cases of acute lymphadenitis and 2 cases of hemorrhagic lymphadenitis. Bovine tuberculosis, Johne's disease and theileriosis were further confirmed by immunohistochemistry, conventional PCR, real time PCR as well as nested PCR using specific primers and later confirmed by gene sequencing. Cytokine expression was also studied in affected lymph nodes in cases of lymphoma, tuberculosis, Johne's disease, lymph node hyperplasia, acute lymphadenitis, chronic lymphadenitis and hemorrhagic lymphadenitis (Table 2).

Cytokine expression in lymphoma cases revealed decrease in Th1 and Th2 cytokine profile (Fig. 1) as compared to the control. There was significant decrease in IL-2 and IL-4 expression which is in agreement with the previous findings[21]. Similarly reduced expression of IFN-γ, IL-2 and IL-4 mRNA in $CD4^+$ T-cells from bovine leukemia virus-infected

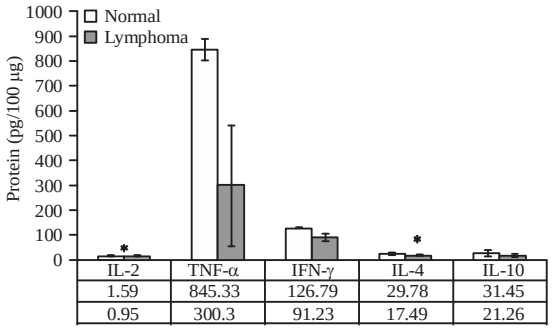
Table 1: ELISA kits used for estimation of various cytokines in affected lymph nodes

Cytokines	ELISA kit
IL-2	Raybio® bovine IL-2 ELISA kit
IL-4	Thermo scientific bovine IL-4 screening kit
IL-10	Cusabio ELISA kit
INF-γ	Thermo scientific bovine INF-γ ELISA kit
TNF-α	Raybio® bovine TNF-α ELISA kit

Table 2: Cytokine values in different disease conditions of bovine lymphadenopathies

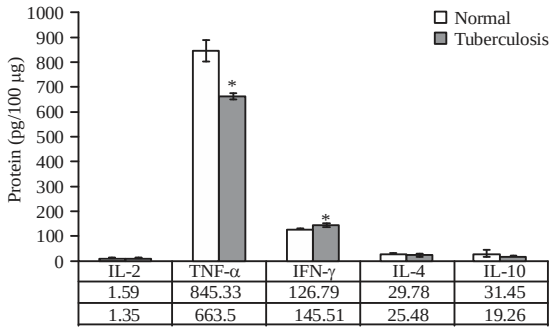
	Th1 cytokine (pg/100 µg protein)			Th2 cytokine (pg/100 µg protein)	
Disease condition	IL-2	TNF-α	IFN-γ	IL-4	IL-10
Normal lymph nodes	1.59 ±0.30	845.33±42.25	126.79±1.54	29.78±5.11	31.45±13.34
Tuberculosis	1.35±0.14	663.50±12.72*	145.51±2.48*	25.48±5.10	19.26±2.05
Johne's disease	5.21±3.50	707.65±130.60	177.30±10.33	55.00±7.07	91.92±53.85
Lymphoma	0.95±0.10*	300.30±242.25	91.23±16.95	17.49±1.93*	21.26±7.27
Acute lymphadenitis	15.71±10.90	943.22±456.68	136.56±42.80	60.66±73.84	49.82±25.35
Chronic lymphadenitis	3.56±2.03	757.78±576.19	110.13±33.20	25.81±10.48	32.42±19.58
Hemorrhagic lymphadenitis	0.97±1.37	401.50±37.47*	100.59±10.94	29.52±5.38	39.44±3.93
Hyperplasia of lymph nodes	4.70±2.97	439.28±255.51	108.51±32.15	29.98±9.18	46.96±24.20

*Significant at p≤0.05



IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
0.95	300.3	91.23	17.49	21.26

Fig. 1: Th1 and Th2 cytokines expression in lymph nodes in lymphoma



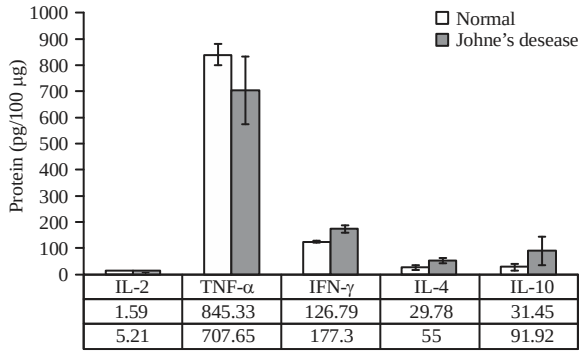
IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
1.35	663.5	145.51	25.48	19.26

Fig. 2: Th1 and Th2 cytokines expression in lymph nodes in bovine tuberculosis

cows with persistent lymphocytosis has been documented previously[22]. The cytokine expression of IL-2, IFN-γ and IL-4 was significantly reduced in the high grade lymphoma as reported earlier[23]. The IL-2 secreted by Th1 cells plays a central role in the activation of T-cell mediated immune responses. The IL-2 has also been reported to play an essential role in antitumor immunity[24]. Further, soluble IL-2 receptor (IL-2R) is a physiologic inhibitor of IL-2[25] and the IL-2R is elevated in patients with malignant and non-Hodgkin's lymphoma[26,27]. In the present study, both the Th1 and Th2 cytokine levels were decreased, which might be associated with the progression tumor to a higher grade.

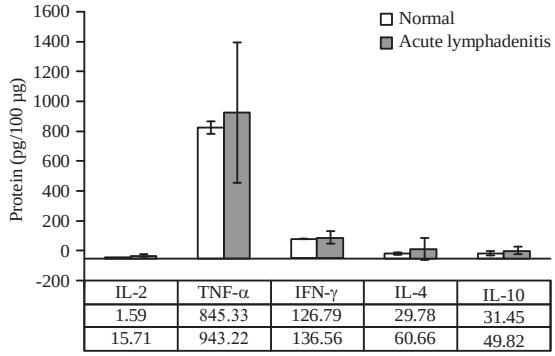
In case of tuberculosis there was significant increase in INF-γ, along with a significant decrease in TNF-α (Fig. 2). Cytokines play an important role in mycobacterial immunity and suppression of IL-4, IL-10 and TNF-α have been reported as compared to that of normal, whereas, there was no change in INF-γ expression[3]. By comparing cytokine expression in tuberculous tissues a reduction in IL-4 and IL-10 had been documented, which suggests that there was a predominant pro-inflammatory immune response to the mycobacteria in lung and lymph node that helped in killing infected cells and extracellular bacteria. *Mycobacterium bovis* infected lymph nodes showed significantly higher expression of INF-γ and TNF-α as reported earlier[28]. Higher IFN-γ gene expression suggested that localized T-cells were primed by macrophages, which in turn produced cytokine leading to production of activated macrophage having mycobactericidal activity. The IFN-γ helps in the activation of macrophages, which further caused accumulation of inflammatory cell, formation of tuberculous granuloma and fibrosis. Further a constant activation of the immune system is required either to limit the mycobacterial growth or to regulate the inflammatory response to ensure survival of the host.

Significant increase in expression of IFN-γ and IL-4 and a non-significant increase in IL-2 and IL-10 levels was observed in cases of paratuberculosis (Fig. 3). In the present study increased expression of Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cytokines was in cases of paratuberculosis as documented earlier[6,29]. Whereas, decrease in TNF-α in cases of paratuberculosis reported in the present study is contrary to the earlier reports[30]. In addition, higher IFN-γ gene expression suggested that localized T-cells were primed by macrophages to produce cytokines that lead to production of activated macrophage having mycobactericidal activity as stated earlier. The IFN-γ regulates growth and differentiation of T-cells, activation of macrophages and hinders IL-4-induced B-cell growth[31]. Immunomodulatory cytokines, IL-10 though



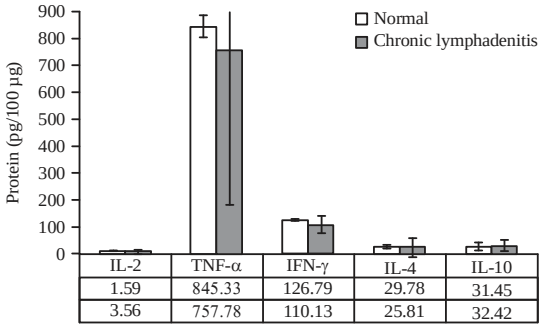
IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
5.21	707.65	177.3	55	91.92

Fig. 3: Th1 and Th2 cytokines expression in lymph nodes in Johne's disease



IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
15.71	943.22	136.56	60.66	49.82

Fig. 4: Th1 and Th2 cytokines expression in lymph nodes in acute lymphadenitis



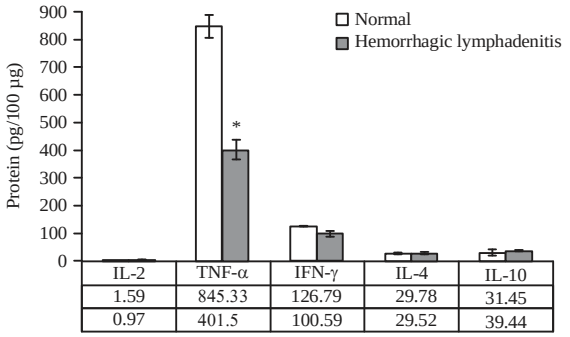
IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
3.56	757.78	110.13	25.81	32.42

Fig. 5: Th1 and Th2 cytokines expression in lymph nodes in chronic lymphadenitis

detrimental in early stages of infection is also required to regulate long term inflammatory responses. The marked upregulation of these cytokines in the present study may be due to the presence of stimulated lymphocytes and potential interplay between many of the cytokines. In the cases of paratuberculosis in the present study, there was a mixed Th1 and Th2 response and a strong induction of IL-10 expression. The mixed expression of cytokines may suppress the effective killing of *M. avium* subsp. *paratuberculosis* and mycobacteria may evade the immune responses of the host[6].

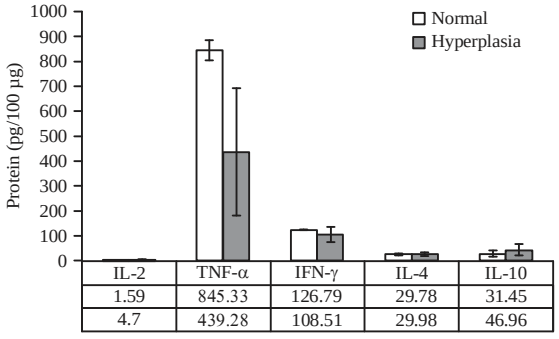
In cases of acute lymphadenitis, an increase in IL-2, IL-4, IL-10, TNF-α and INF-γ was observed, however, this increase was statistically non-significant (Fig. 4). Leukocytosis is a common feature of acute inflammatory reaction, especially in bacterial infection. Neutrophils are the main component of innate immunity due to their phagocytic and microbicidal activities. In addition to being phagocytes, neutrophils generate a wide range of inflammatory mediators, which participate in both innate and adaptive immune response[32]. Further, activated neutrophils express MHC class II molecules, which help in the presentation of the antigen to T-cells and activation and proliferation of T-cells. Activated neutrophils synthesize pro-inflammatory cytokines which can eventually lead to the activation of both neutrophils and other cells of the immune system[33]. Again, during infection, there is recruitment and differentiation of the circulating inflammatory monocytes into macrophages, thus maintaining a homeostatic status with the resident macrophages in the affected tissues. The activated macrophage or M1 phenotype produce a large amount of pro-inflammatory cytokines which eliminate the invading microorganisms and further activate the adaptive immune response[34]. In addition, either apoptosis or polarization of the macrophages into alternatively activated M2 phenotypes occurs in order to check the excessive inflammatory response and protects the host from excessive injury and promote wound healing[35]. During inflammation and infection, the immunomodulatory cytokine, IL-10 acts as a double-edged sword which has both beneficial as well as detrimental effects. The IL-10 and IL-4 exhibit an anti-inflammatory role by preventing production of cytokines and other pro-inflammatory functions by macrophages. During infection, the anti-inflammatory components of the immune system help in regulation of the pro-inflammatory cytokine response. Therefore, in the present study, levels of both Th1 and Th2 cytokines were increased.

In cases of chronic lymphadenitis, there was an increase in the levels of IL-2 and IL-10 and decrease in levels of IL-4, TNF-α and INF-γ (Fig. 5). During inflammatory responses, activated lymphocytes secrete IL-2, which activates a variety of immune cells including lymphocytes and macrophages[36], whereas, IL-10 is secreted by $CD4^+$ and $CD8^+$ T-cells[37]. In the present study, decrease in the levels of IL-4 was observed, which may be due to the inhibitory effect of IL-10 on Th2 cells. The results of the present study indicated that due to increase in IL-10 production, the function of Th1 lymphocytes was impaired which resulted in decreased level of IFN-γ.



IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
0.97	401.5	100.59	29.52	39.44

Fig. 6: Th1 and Th2 cytokines expression in lymph nodes in haemorrhagic lymphadenitis



IL-2	TNF-α	IFN-γ	IL-4	IL-10
1.59	845.33	126.79	29.78	31.45
4.7	439.28	108.51	29.98	46.96

Fig. 7: Th1 and Th2 cytokines expression in lymph nodes in hyperplasia of lymph nodes

Hemorrhagic lymphadenitis cases revealed significant decrease in TNF-α (Fig. 6). Hemorrhage produces alterations in macrophage, T ($CD4^+$ and $CD8^+$) and B-cell function. Hemorrhage triggers $CD4^+$ cells, with collateral restriction of the Th1 subpopulation. Blood loss due to hemorrhages has a suppressive effect on macrophages functions and generation of Th1 cytokines. This is mediated through the increased production of IL-10 produced by $CD4^+$ T-cells in post-hemorrhagic period[38].

The lymph node hyperplasia cases revealed decrease in TNF-α and INF-γ and increase in IL-2, IL-4 and IL-10 as compared to the control (Fig. 7). An increase in IL-10 mRNA in skin and lymph nodes with epidermal hyperplasia in mice has been documented earlier[39]. Histopathology of the lymph nodes showed lymphocytic hyperplasia with congestion and hemorrhage. Thus, as stated above hemorrhages appeared to activate $CD4^+$, Th2 cells, with concomitant suppression of the Th1 subpopulation[38]. In the present study, in the lymph node hyperplasia cases there was increased production of IL-4 and IL-10. Cytokines, such as IL-10 and IL-4 exhibit an anti-inflammatory role by preventing production of cytokines and other pro-inflammatory functions by macrophages. The IL-4 activates the macrophages alternatively to M2 phenotypes[35] whereas, IL-10 is an immunoregulatory cytokine responsible for disease progression and it suppresses the Th1 response[37].

CONCLUSION

The present study concluded that in lymphomas cases cell mediated immune response (Th1) was involved, which helped in tackling the cancerous cells. Whereas, in specific diseases like tuberculosis and Johne's disease, pro-inflammatory cytokines i.e., the Th1 cytokines promotes the killing of infected cells and extracellular bacteria whereas, anti-inflammatory cytokines i.e., Th2 cytokines down regulates the pro-inflammatory response to control tissue damage. In non-specific lymphadenopathies of bovines, pro-inflammatory immune response is regulated by anti-inflammatory components of the immune system.

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