Original Articles

Tumor necrosis factor-alpha up-regulation in spontaneously proliferating cells derived from bovine leukemia virus-infected cattle

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Running title*: Up-regulation of TNF-α in spontaneously proliferating PBMCs from BLV-infected cattle*.

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Summary. We previously reported that tumor necrosis factor alpha (TNF-α) was one of the cytokines that contributed to the leukemogenesis caused by bovine leukemia virus (BLV). To determine if the spontaneous cell proliferation observed in the late disease stages, such as persistent lymphocytosis and lymphosarcoma, correlated with the expression level of TNF-α, we analyzed the mRNA expression levels for TNF-α in spontaneously proliferating PBMCs derived from BLV-infected cattle. The mean mRNA expression level for TNF-α was higher in the spontaneously proliferating PBMCs derived from BLV-infected cattle than in non-spontaneously proliferating PBMCs from normal cattle. The TNF-α protein level in the PBMCs was determined by flow cytometric analysis, and it was noted that most of the cells expressing membrane-bound TNF-α in the spontaneously proliferating cells were $CD5^+$ or $sIgM^+$-cells. Additionally, in order to determine if this spontaneous proliferation can be blocked by anti-bovine TNF-α MAb, the spontaneously proliferating PBMCs from a BLV-infected cattle were cultured in the presence of the MAb. The addition of this MAb at the beginning of the 72h-cultivation clearly inhibited spontaneous proliferation of cells in a dose-dependent manner, indicating the direct involvement of TNF-α in the spontaneous proliferation of PBMCs during the late disease stage. These data suggest that an aberrant expression of TNF-α might contribute to the progression of bovine leukosis in animals which develop persistent lymphocytosis of B cells or B-cell lymphosarcoma.

Introduction

The bovine leukemia virus (BLV), which is structurally and functionally related to the human T-lymphotropic virus types (HTLV)-I and II, is the etiological agent of enzootic bovine leukosis [4, 27]. Besides from the difference in host range, a notable difference between the two viruses is that infection by BLV is associated with malignancy of $CD5^+$B-cells, whereas HTLV affects $CD4^+$T-cells, the majority of which harbor the provirus. In cattle, the majority of BLV-infections are clinically inapparent, and are referred to as asymptomatic or aleukemic (AL). After a long latency period, a small fraction of the latently infected individuals developed the terminal disease characterized by persistent lymphocytosis (PL) and lymphosarcoma [4, 30].

In the late disease stage of BLV and HTLV, one of the characteristics is the in vitro proliferation of peripheral blood mononuclear cells (PBMC), when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous lymphocyte proliferation [36]. Spontaneous cellular proliferation in cultures of BLV-positive PBMCs almost exclusively involves B-cells [17]. The spontaneous cell proliferation results in the increase in the number of BLV/HTLV-infected cells. This spontaneous cell proliferation has been associated with the expression of lymphocyte surface molecules such as integrin molecules CD11a, CD18, CD5, major histocompatibility complex (MHC) class I, MHC class II-DP, MHC class II-DQ, MHC class II-DR and interleukin-2 receptor (IL-2R) in HTLV infected patients and BLV-induced PL in cattle [22, 29]. On the other hand, up or down-regulation of cytokine expression are associated with spontaneous proliferation of lymphocytes from HTLV-infected patients

[6, 21]. However, associated cytokines regulating the spontaneous cell proliferation found in the late disease stages of PL and lymphosarcoma, remain unclear.

In previous reports, we indicated that in a sheep model, the expression of tumor necrosis factor alpha (TNF-α) and its receptors were closely associated with disease progression in sheep experimentally infected with BLV [18, 19]. Interestingly, we found conflicting roles of TNF-α in sheep experimentally infected with BLV. The TNF-α mRNA expression was significantly up-regulated in BLV-resistant sheep, while down-regulation of TNF-α was observed in susceptible sheep [18]. In contrast, high level expression of membrane-bound TNF-α on PBMCs of sheep with high BLV load in late phase of infection was observed. Moreover, TNF-α strongly induced the proliferation of PBMCs in the BLV-infected sheep, while mRNA expression for TNF-receptor type I (TNF-RI) was down-regulated in PBMCs from the infected sheep compared to the BLV negative sheep [19]. Although these results suggest that in the early phase of infection, TNF-α may be involved in the elimination of BLV, TNF-α may contribute to the induction of cell proliferation and disease progression in late phase of BLV-infection.

In the present study, we investigated the expression levels of TNF-α mRNA and protein and determined the phenotypes of cells expressing TNF-α among the spontaneously proliferating PBMCs derived from BLV-infected cattle, in order to clarify the role of TNF-α with regard to spontaneous cell proliferation in late phase of BLV-infection.

Materials and methods

Animals

Seventeen adult cattle (16 Japanese black and 1 Holstein-Friesian cattle) were used (Table 1). BLV infection was tested by agar gel immunodiffusion assay using the BLV env glycoprotein gp51 as antigen [26], and was further confirmed by real-time PCR with a primer pair (BLV-LTR256; 5'-GAG CTC TCT TGC TCC CGA GAC -3' and BLV-LTR453; 5'-GAA ACA AAC GCG GGT GCA AGC CAG-3') to amplify BLV long terminal region (LTR) using a LightCycler^{TM} (Roche Diagnostics, Mannheim, Germany) as described previously [31]. The percentage of BLV-infected cells was determined based on viral genome amplification using real-time PCR as described previously [31]. Two animals (Pr. 2747 and Pr. 2750) with lymphosarcoma were diagnosed clinically, and confirmed by microscopic and histological examinations at the Department of Veterinary Pathology, Faculty of Agriculture, Iwate University [16].

PBMCs culture and proliferation assay

PBMCs were isolated from heparinized venous blood of BLV-infected animals and controls by Percoll density gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as described previously [24]. Purified cells were resuspended in RPMI1640 (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) heat-inactivated fetal calf serum (FCS) supplemented with 2mmol/L L-glutamine, 100U/ml penicillin, 100mg/ml streptomycin and 5 x 10^{-5} volume of mercaptoethanol. PBMCs (1 x 10^{5} per well)

from cattle were cultured for 72h in triplicate wells of 96-well culture microplates (Corning, NY, USA) in the absence of exogenous antigens or with concanavalin A (Con A; 5 μg/ml; Sigma, USA) as positive control for induced cell proliferation. Six hours before cells were harvested, 0.5 μCi of [^{3}H]-thymidine (ICN Biochemicals Ins, CA, USA) was added to each well. Cells were harvested onto glass-fiber filters, and the amount of incorporated [^{3}H]-thymidine was determined with a liquid scintillation counter, and expressed as the mean of counts per minute (c.p.m) corresponding to DNA-incorporated radioactivity of triplicate cultures. To confirm spontaneous cell proliferation, [^{3}H]-thymidine was added to all cells at the beginning of culture, and the cells harvested after 6h, which is the time required for [^{3}H]-thymidine incorporation. Animals were regarded as positive for spontaneous cell proliferation if the incorporated radioactivity after the 72h-cultivation was more than 5-fold of that after the 6h-cultivation. BLV-positive animals were divided into two groups; one was BLV-infected but negative for spontaneous cell proliferation (BLV^+SP^-) while the other group was BLV-infected with spontaneous cell proliferation (BLV^+SP^+) based on the proliferation assay results (Table 1). To determine the effect of TNF-α on spontaneous cell proliferation, we also performed proliferation assay using the ID.11-13 (mouse anti-bovine TNF-α MAb, a gift from Dr. Yuichi Yokomizo, National Institute for Animal Health, Tsukuba, Japan) [40]. On the same plate, in parallel, PBMCs were cultured in the presence of increasing concentrations of either the ID.11-13, or isotype-matched control antibody (mouse IgG, Cappel, Cochranville, PA, USA) for 72h followed by 6h-pulsing with [^{3}H]-thymidine. The percentage inhibition

of proliferation was expressed as (c.p.m of cultures with antibody/ c.p.m of control cultures without antibody) x 100.

Real-time RT-PCR

Total RNA was extracted from the freshly separated and 24h-cultivated PBMCs by using the TRIZOLTM reagent according to the manufacturer's protocol (Invitrogen). All samples were treated with deoxyribonuclease I (amplification grade, Invitrogen) to eliminate DNA contamination. Real-time RT-PCR using SYBER Green I was performed by using a LightCycler™ as described previously [20]. The cDNA template was added to a total volume of 20μl containing PCR buffer, oligonucleotide primers (0.2 mM each, final conc.), [either boTNF-α F (5'-AGC CTC AAG TAA CAA GCC-3') and boTNF-α R (5'-TGA AGA GGA CCT GTG AGT-3'), boTNF-β F (5'-ATC AGC AAC TCC CGA CGC-3') and boTNF-β R (5'-GGG GAC CAG GAG GGA ATT GT-3'), boTNF-RI F (5'-CGC CTC TGT CGT CTT AGC AT-3') and boTNF-RI R (5'-TGG AGA CAG GAC TGG AAC TT-3'), or boTNF-RII F (5'-CTC GAC CAG CAG CAC GGA CA-3') and boTNF-RII R (5'-GCG TCT GTG TCC CTC GTG GA-3')], and 2 μl of LightCycler-Fast Start DNA Master SYBER Green I (Roche Diagnostics). The bovine β-actin gene in each sample was also amplified using a primer pair, boβ-actin F (5'-CGC ACC ACT GGC ATT GTC AT-3') and boβ-actin R (5'-TCC AAG GCG ACG TAG CAG AG-3') to check for loading of template cDNAs. To determine whether Tax (a transcriptional activator encoded by the X-region of BLV) was expressed, RT-PCR was performed using same cDNA samples. The

expression of BLV tax mRNA was confirmed by conventional PCR using a thermal cycler (System 9700, Applied Biosystems) with primers BLV tax/rex 4758 (5'-AGG CGC TCT CCT GGC TAC TG-3') and BLV tax/rex 7333 (5'-GGC ACC AGG CAT CGA TGG TG-3') as described previously [31].

Flow-cytometric analysis

To identify the cells expressing TNF-α, single- and dual-color flow cytometric analysis was performed using the following antibodies; Apr.89 (rabbit anti-bovine TNF-α, a gift from Dr. Yuichi Yokomizo), IL-A11 (mouse anti-bovine CD4), CACT105A (mouse anti-bovine CD5, VMRD, Pullman, USA), IL-A51 (mouse anti-bovine CD8), IL-A30 (mouse anti-bovine surface IgM) and BLV3 (mouse anti-BLV p24, VMRD) as described previously [7, 19, 32]. Following 48h-cultivation, cells were harvested and incubated with the optimal concentration of each antibody for 30 min at 4°C. Then, the cells were washed with PBS containing EDTA (0.75 mg/ml), and labeled with isotype-specific secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulins (Immunotech, Marseille, France) or phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulins (Immunotech). Fluorescence of the cells was measured on an EPICS XL, and the data were analyzed by using the SYSTEM IITM software (Beckman Coulter, Miami, FL, USA).

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Student *t*-test.

Differences between groups were considered significant if probability values of $p<0.05$ were obtained. All statistical analyses were performed with the statistical software StatceL (OMS, Saitama, Japan).

Results

Spontaneously proliferating PBMCs in BLV-infected cattle with high provirus load

In the present experiments, the spontaneous cell proliferation was observed in BLV-infected cattle with high percentage of BLV-infected cells (37.9±7.8) and with lymphosarcoma (Pr. 2747 and Pr. 2750). On the other hand, no spontaneous cell proliferation was noted shown in PBMCs derived from cattle with low percentage of BLV-infected cells (0.8±0.3) or BLV-uninfected cattle (Table 1).

Up-regulation of TNF-α and TNF-RII mRNA in spontaneously proliferating PBMCs

In order to investigate the expression levels of the TNFs and TNF receptors in the spontaneously proliferating PBMCs, we used real-time PCR to quantify the expression of TNF-α, TNF-β, TNF-RI and TNF-RII mRNAs in fresh and 24h-cultivated PBMCs isolated from normal and BLV-infected cattle (Fig. 1). TNF-α and TNF-β were low or under detectable levels in PBMCs fleshly isolated from the three groups of cattle. Flesh PBMCs from BLV^+SP^+ animals expressed higher mRNA levels of TNF-RII but not -RI than those from BLV-uninfected cattle (data not shown). As shown in Fig. 1, the TNF-α and TNF-β mRNA levels were increased in spontaneously proliferating PBMCs from the BLV^+SP^+ animals compared to BLV-infected and BLV-negative cattle

which were negative for spontaneous cell proliferation after 24h-cultivation. Similarly, the cells from the BLV^+SP^+ animals expressed higher mRNA levels of TNF-RII but not -RI than those from BLV-uninfected cattle. Thus, these results suggest that up-regulation of TNF-α expression could influence the spontaneous proliferation of cells during the disease progression.

Up-regulated expression of membrane-bound TNF-α

on spontaneously proliferating PBMCs

To determine the proportion of cells expressing membrane-bound TNF-α, we embarked on detecting TNF-α^+ cells among the PBMCs before and after 48h-cultivation by dual-color flow cytometric analysis (Fig. 2). A limited number of cattle were tested, yet as expected, the total percentage of TNF-α^+ cells was higher in BLV^+SP^+ cattle with lymphosarcoma (Pr. 2747 and Pr. 2750) after cultivation (92.9%, 89.9%) than before cultivation (18.2%, 25.3%)(Fig. 2A). The proportion of TNF-α^+-cells among freshly isolated PBMCs from BLV^+SP^+ cattle (18.2%, 25.3%) was higher than that of BLV^+SP^- (10.7%) and uninfected cattle (8.6%), consistent with the observations reported in the previous study (19). Although the total percentage of TNF-α^+-cells among the PBMCs from BLV^+SP^- cattle was also increased after cultivation (28.9%), the magnitude of increase was lower than that of the spontaneously proliferating PBMCs from cattle with lymphoma (92.9%, 89.9%). Although the PBMCs derived from cattle with lymphosarcoma portrayed strong spontaneous cell proliferation, there was no evident difference in the populations of

$CD4^+$, $CD8^+$ and $CD5^+$-cells before and after cultivation. By dual-color flow cytometric analysis, the increase in the proportion of TNF-α^+-cells was mainly due to the increased frequency of the TNF-α^+CD5^+ (56.4%, 54.6%) and TNF-α^+sIgM^+ (48.7%, 48.7%) cells among the spontaneously proliferating cells from the cattle with lymphosarcoma. The expansion of the proportion of TNF-α^+CD4^+ (17.6%, 11.2%) and TNF-α^+CD8^+ (27.6%, 18.5%) cells was also notable in cattle with lymphosarcoma, although there were variations among individuals (Fig. 2B). To further examine the specific expression of TNF-α on BLV-positive cells, we monitored the proportions of TNF-$\alpha^+BLVp24^+$-cells. However, there were no observable differences in the proportion of TNF-$\alpha^+BLVp24^+$-cells from BLV^+SP^+ cattle before and after cell cultivation.

Inhibitory effect of anti-bovine TNF-α antibody against spontaneous proliferation of PBMCs from BLV-infected cattle

To determine if the spontaneous cell proliferation can be blocked by anti-bovine TNF-α MAb, spontaneously proliferating PBMCs from BLV-infected animals, were cultivated in the presence of the MAb. As shown in Fig. 3, the addition of this MAb at the beginning of the 72h-cultivation clearly inhibited spontaneous proliferation of cells in a dose-dependent manner, although there were some individual exceptions. Although the inhibitory effects of the antibody against TNF-α were weaker in cattle exhibiting strong spontaneous cell proliferation (B0.12 and C2.21) than in BLV^+SP^+ cattle, anti-bovine TNF-α MAb at the highest concentration tested (20㎎/ml) inhibited

spontaneous cell proliferation in both groups of cattle. The spontaneous proliferation of PBMCs was not affected in the presence of mouse IgG as a control antibody, indicating the direct involvement of TNF-α in the spontaneous proliferation of PBMCs in BLV-infection.

Discussion

Kabeya *et al* [19] demonstrated that TNF-α strongly induced the proliferative response of PBMCs in sheep with high BLV load in late phase of infection, and that membrane-bound TNF-α expression was up-regulated in PBMCs from the infected sheep compared to the BLV-negative sheep. Most recently, Amills *et al* [1] reported that purified B-cells from BLV-infected cows with PL constitutively expressed TNF-α and TNF-β (lymphotoxin-α) mRNA. These findings suggest that the aberrant expression of TNF-α contribute to progression of BLV-infection. In the present study, to assess this possibility, we monitored the expression of TNF-α and its influence on the spontaneously proliferating PBMCs found in the late disease stages. We found that expression of mRNA for TNF-α and the membrane-bound TNF-α on $CD5^+$-cells or $sIgM^+$-cells from BLV-infected cattle with high provirus load and lymphosarcoma was up-regulated, when compared to those from BLV-infected cattle with low provirus load or normal controls. Additionally, we also found that the spontaneous cell proliferation was inhibited by anti-bovine TNF-α MAb in a dose-dependent manner.

TNF-α and TNF-β have a unique role in regulating the immune response to viral infections after their binding to the specific receptors, TNF-RI and TNF-RII,

respectively [10, 13]. Both TNF-α and TNF-β receptors are co-expressed in various tissues, but majority of the biological responses classically attributed to TNFs, are mediated by TNF-RI. TNF-RII which lacks the intracellular death domain, binds TNFs with higher affinity than TNF-RI and can, on its own, signal proliferation of thymocytes and cytotoxic T cells and inhibit apoptosis, while in combination with TNF-RI, can signal proliferation of mononuclear cells [8, 9, 34, 35]. Interestingly, increased expression of TNF-RII but not TNF-RI have been reported in HTLV-I-infected T cell lines, and the cell lines derived from HTLV-I-infected patients were strongly resistant to TNF-α-mediated apoptosis [39]. We had also previously observed limited TNF-RII expression in a B-cell line (KU-1) and in lymphoma cells derived from BLV-infected cattle [16, 19]. In our present study, we observed increased expression of TNF-RII mRNA in spontaneously proliferating cells isolated from cattle with high provirus load despite there being no difference in the expression levels of TNF-RI mRNA among the different stages of BLV infection. Additionally, we also found that the expression of membrane-bound TNF-α on $CD5^+$-cells and $sIgM^+$-cells (which are BLV-target cells) were up-regulated in PBMCs from BLV^+SP^+ cattle after cultivation compared to that before cultivation. Membrane-bound TNF-α is regarded as a ligand, that induces B-cell activation in virus-induced polyclonal B-cell proliferation [14]. In this respect, it can be postulated that up-regulation of TNF-α and TNF-RII could modulate cellular interaction in an autocrine or paracrine manner, and that direct or indirect effects of TNF-α including cell proliferation or resistance to apoptosis might assist the spontaneous proliferation of cells during the

late disease progression phase, such as in PL and lymphosarcoma.

Earlier, Lal and Rudolf [21] had reported significantly elevated levels of TNF-α and IL-6 in spontaneously proliferating T cells derived from patients with HTLV-I and –II, which is structurally and functionally related to BLV. Nakamura *et al* [25] reported that both the degree of spontaneous proliferation and the production of TNF-α by endothelial-adherent T-cells in patients with HTLV-I-associated myelopathy (HAM) were significantly increased compared to anti-HTLV-I seronegative controls. In the present study, although the IL-6 mRNA expression was under the detectable level (data not shown), similar results were obtained when we measured the TNF-α mRNA and protein expressions in spontaneously proliferating PBMCs derived from BLV-infected cattle. It can be possible that the mechanism by which spontaneously proliferating cells induce the secretion of cytokines might be mediated by transactivation through the HTLV regulatory gene product, *tax*. Tax has been shown to affect cellular gene transcription by acting on proteins such as camp nuclear factor (NF)-kappa B protein [3, 15]. Indeed, a number of reports have indicated aberrant production of several cytokines via HTLV-tax [2, 12, 23, 38]. To determine this possibility, the RNA transcript of BLV-tax gene was analyzed in spontaneously proliferating PBMCs from BLV-infected cattle, however, we could not detect BLV-tax expression in these cells (data not shown). Additionally, in order to determine whether the expression of TNF-α is specifically up-regulated in BLV-infected cells, we also examined the population of membrane-bound TNF-α and BLVp24 expressing cells by flow-cytemetry dual-stain analysis. However, we were also not able to find any

association between BLVp24 expression and up-regulation of membrane-bound TNF-α in spontaneously proliferating cells of BLV-infected cattle with lymphosarcoma. Nevertheless, further studies are required to elucidate this discrepancy.

In the present study, spontaneous cell proliferation was not completely blocked by antibody against TNF-α. It seems likely that the IL-2 might influence the spontaneous cell proliferation as previously reported [37]. Although we observed up-regulated expression of TNF-α in spontaneously proliferating cells derived from BLV-infected cattle, the proliferative response to IL-2 has been reported to be much stronger than that of TNF-α in cattle with PL in the late phase of infection [37]. Since IL-2/CD25 interaction is the main inducer of cell proliferation in BLV infection as well as in HTLV infection [21, 22, 29, 37], we presume that the increased expression of TNF-α may assist the IL-2 induced cell proliferation by anti-apoptosis via TNF-RII. Indeed, in recent years, growing evidence has shown that BLV can interfere with the anti-apoptosis pathway to avoid cell death, and the resistance to apoptosis is speculated to contribute to disease progression [5, 11, 28, 32, 33]. Additionally, Stone *et al* [29] reported that antibodies to lymphocyte surface molecules including MHC-class I, MHC class II, IL-2R, CD5, CD11a and CD18, strongly inhibited spontaneous proliferation of PBMCs from BLV-infected cows with PL. Thus, the increased expressions of surface molecules including membrane-bound TNF-α are also likely to contribute to the inducement of spontaneous cell proliferation in BLV-infected animals.

In summary, we present here an aberrant expression of TNF-α mRNA and

membrane-bound TNF-α in spontaneously proliferating cells derived from BLV-infected cattle. Because clinical and lymphocytes phenotypic changes in BLV-infection are associated with spontaneous proliferation, it is anticipated that spontaneous cell proliferation in vitro could correlate with disease progression. Although the role of TNF-α in the tumorigenesis of BLV-induced PL or lymphosarcoma is still speculative, TNF-α associated spontaneous cell proliferative response through its binding to TNF-RII might contribute to the progression of bovine leukosis in animals which develop persistent lymphocytosis or lymphosarcoma. Further studies are required to clarify the mechanism in more detail and to try and understand why up-regulation of TNF-α could be crucial for the spontaneous cell proliferation.

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Table 1. Tested animals in this study

Cattle No.	Breed[a]	Age (year)	Sex	No. of lymphocyte (/mm^3)	BLV antibody (gp51)	%. of BLV-infected cell[b]	[^{3}H]-thymidine incorporation (c. p. m) non-stimulated 6h[c]	non-stimulated 72h	non-stimulated (-fold)[d]	Concanavalin A 72h	Concanavalin A (S.I)[a]
Controls						[a]					
B2.19	J.B	4	F	8,128	−	ND	226±47	172±20	(0.8)	87,956±5,769	(510)
B2.38	J.B	4	F	6,090	−	ND	227±47	96±26	(0.4)	20,276±1,988	(211)
B2.1	J.B	4	F	4,800	−	ND	154±42	183±104	(1.2)	150,385±305	(823)
B3.17	J.B	3	F	4,548	−	ND	317±80	474±15	(1.5)	95,438±5,368	(201)
503K	J.B	3	F	5,790	−	ND	195±24	205±57	(1.1)	27,664±3,490	(135)
(Mean ± SD)		(3.6±0.5)		(5,871±1,418)			(224±60)	(226±145)	(1.0±0.4)	(76,324±53,572)	(376±289)
BLV$^+$ cattle without SP[a]											
B2.22	J.B	5	F	6,062	+	0.9	274±101	198±28	(0.7)	55,122±3,427	(278)
B0.37	J.B	5	F	7,900	+	0.7	262±56	179±111	(0.7)	69,031±3,824	(386)
F0.15	J.B	6	F	8,400	+	1.2	226±47	185±108	(0.8)	32,931±2,706	(178)
B.2.5	J.B	3	F	5,035	+	1.0	220±103	299±84	(1.4)	51,478±1,952	(172)
590E	J.B	8	F	4,750	+	0.4	186±129	292±79	(1.6)	26,264±3,220	(90)
(Mean ± SD)		(5.4±1.8)		(6,429±1,654)		(0.8±0.3)	(234±35)	(230±60)	(1.0±0.4)	(46,965±17,315)	(221±114)
BLV$^+$ cattle with SP[d]											
C8.27	J.B	7	F	23,600	+	30.3	121±30	2,097±180	(17.3)	88,743±5,660	(42)
C1.9	J.B	4	F	14,516	+	26.0	186±10	5,426±1,126	(29.2)	120,211±6,296	(22)
C2.7	J.B	3	F	19,188	+	41.3	383±76	6,718±117	(17.5)	71,106±2,478	(11)
B0.12	J.B	5	F	20,600	+	36.8	246±52	6,394±76	(26.0)	119,618±2,060	(19)
C2.21	J.B	3	F	22,044	+	39.5	227±44	8,236±857	(36.3)	122,783±6,658	(15)
Pr.2747	J.B	9	F	5,500	+	49.6	488±9	6,484±377	(13.3)	73,966±590	(11)
Pr.2750	H.F	7	F	5,250	+	41.7	609±14	5,875±899	(9.7)	116,134±5,950	(20)
(Mean ± SD)		(5.4±2.3)		(15,814±7,674)[**e]		(37.9±7.8)[**]	(323±177)	(5,890±1,889)	(21.3±9.5)[**]	(101,795±230,556)	(20±11)[**]

[a] J.B, Japanese black; H.F; Holstein-Friesian, SP, spontaneous proliferation; ND, not detected; S.I, Stimulation index were expressed as fold of [^{3}H]-thymidine incorporation by ConA-stimulated PBMCs compared to non-stimulated (media alone) PBMCs.
[b] Percentage of BLV-infected cells was calculated from genomic DNA using real-time PCR as described previously (31).
[c] 6h-cultivation correspond to the cultivation time for [^{3}H]-thymidine incorporation.
[d] Animals were regarded as positive for spontaneous proliferation if the incorporated radioactivity after 72h-cultivation was more than 5 times of that after the 6h-cultivation.
[e] **, $P < 0.01$; Comparisons are between BLV$^+$SP$^+$cattle and Control cattle, or BLV$^+$ SP$^-$ cattle and established with a Student *t* test.

Figure legends

Fig. 1. Quantification of the mRNA expression for TNFs and their receptors in PBMCs by real-time PCR analysis. Total RNA was extracted from fresh and 24h-cultivated PBMCs derived from BLV-negative (*n*=4), BLV^+SP^- (*n*=4) and BLV^+SP^+ (*n*=5) cattle. The results of real time RT-PCR were presented as n-folds of the lowest values obtained from the PBMCs before cultivation.

Fig. 2. Flow cytometric analysis of PBMCs from BLV-negative (503K), BLV^+SP^- (590E) and BLV^+SP^+ (Pr. 2747 and Pr. 2750) cattle. Two-color flow cytometric analysis of uncultivated and 48h-cultivated PBMCs were performed by using combinations of antibodies Apr.89 (anti-TNF-α) and either IL-A11 (anti-CD4) or IL-A51 (anti-CD8) or CACT105A (anti-CD5) or IL-A30 (anti-IgM) or BLV3 (anti-BLV p24). (A) The total percentages of the positive cells within each cell subset. (B) The percentages of TNF-α positive cells within each cell subset.

Fig. 3. Effect of anti-bovine TNF-α antibody on spontaneous cell proliferation. PBMCs from BLV^+SP^+ cattle were incubated with various concentration of anti-bovine TNF-α antibody (0.2, 2 and 20 μg/ml) or control antibody (20 μg/ml). The antibodies were added at the beginning of culture. Cell proliferation was measured as incorporation of tritiated thymidine and expressed as a percentage of the cell proliferation in identical cultures without antibody. Data are means + SD from the triplicate cultures. ND, not done.

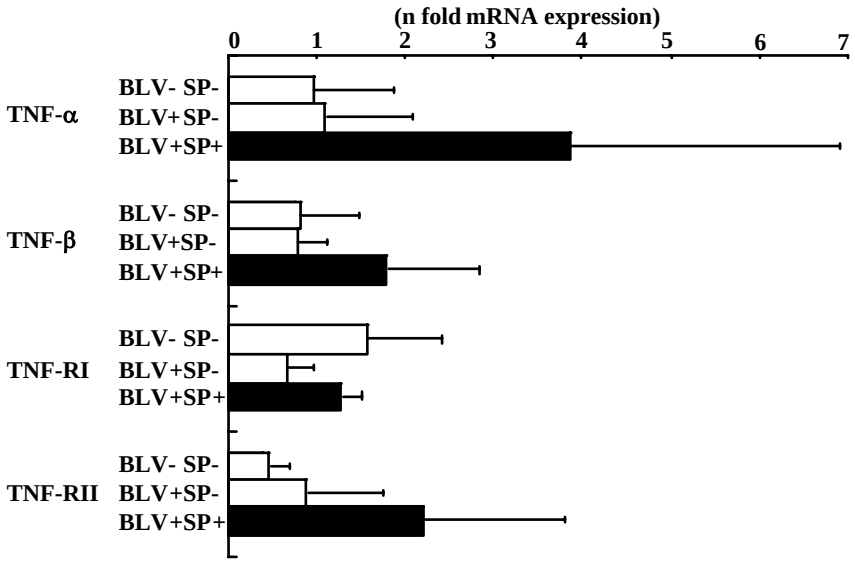
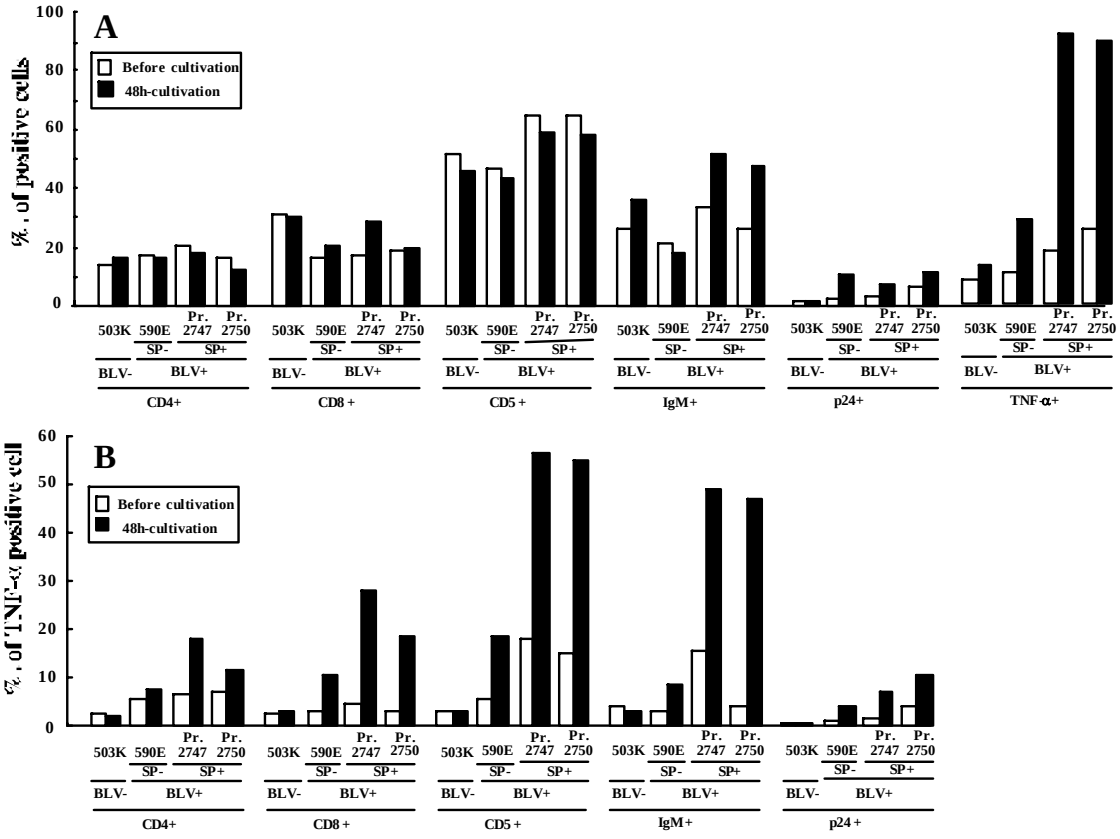

(n fold mRNA expression)
0
1
2
3
4
5
6
7
TNF-α
BLV- SP-
BLV+SP-
BLV+SP+
TNF-β
BLV- SP-
BLV+SP-
BLV+SP+
TNF-RI
BLV- SP-
BLV+SP-
BLV+SP+
TNF-RII
BLV- SP-
BLV+SP-
BLV+SP+

Fig. 1


A
Before cultivation
48h-cultivation
% of positive cells
100
80
60
40
20
0
503K
590E
Pr. 2747
Pr. 2750
SP-
SP+
BLV-
BLV+
CD4+
CD8+
CD5+
IgM+
p24+
TNF-α+

B
Before cultivation
48h-cultivation
% of TNF-α positive cell
60
50
40
30
20
10
0
503K
590E
Pr. 2747
Pr. 2750
SP-
SP+
BLV-
BLV+
CD4+
CD8+
CD5+
IgM+
p24+

Fig. 2

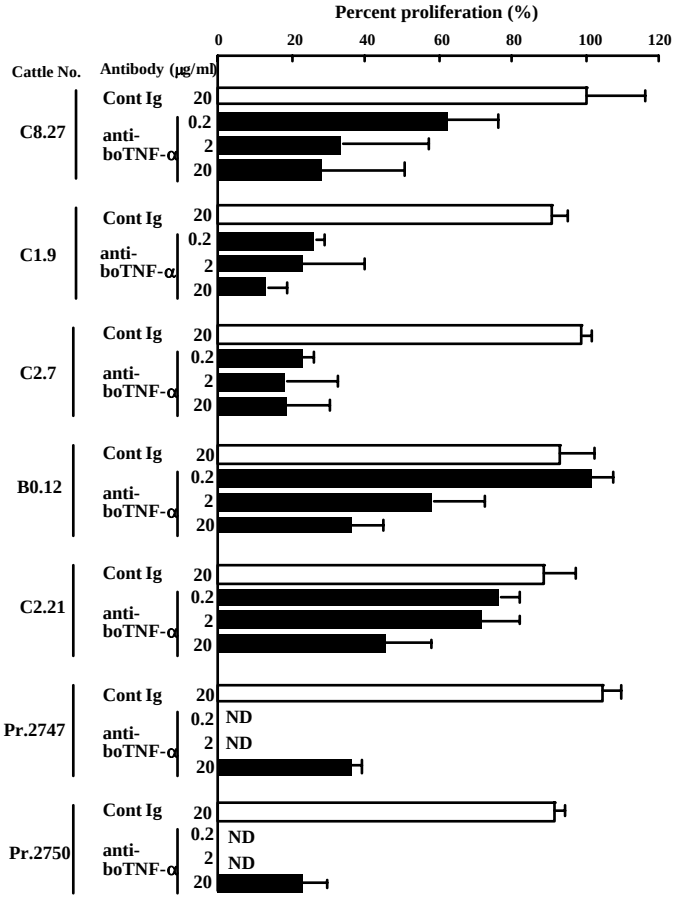

Percent proliferation (%)
0
20
40
60
80
100
120
Cattle No.
Antibody (μg/ml)
C8.27
Cont Ig
20
anti-boTNF-α
0.2
2
20
C1.9
Cont Ig
20
anti-boTNF-α
0.2
2
20
C2.7
Cont Ig
20
anti-boTNF-α
0.2
2
20
B0.12
Cont Ig
20
anti-boTNF-α
0.2
2
20
C2.21
Cont Ig
20
anti-boTNF-α
0.2
2
20
Pr.2747
Cont Ig
20
anti-boTNF-α
0.2
ND
2
ND
20
Pr.2750
Cont Ig
20
anti-boTNF-α
0.2
ND
2
ND
20

Fig. 3