Studies on the immune status of calves with chronic inflammation and thymus atrophy

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ABSTRACT. The thymus is a primary lymphoid organ where the primary T cell repertoire is generated. Thymus atrophy is induced by various conditions, including infectious diseases, glucocorticoid treatment, and poor breeding management. Cattle with thymus atrophy tend to exhibit weak calf syndrome, a condition in which approximately half of neonates die shortly after birth. Calves with thymus atrophy that survive the first month typically contract chronic inflammatory diseases. In this study, we analyzed the populations of the peripheral blood mononuclear cells and thymocytes in calves with thymus atrophy. In addition, we evaluated polarization of master gene and cytokine mRNA expression in peripheral blood CD4+ cells in the calves. The population of CD4+CD8- cells in thymus of the calves with thymus atrophy was lower than that of control calves. IL10 mRNA expression in peripheral blood CD4+ cells of calves with thymus atrophy was significantly lower than that of control calves. TBX21 mRNA expression in peripheral CD4+ cells of thymus atrophy calves was tended to be higher than that of the control group. In addition, FOXP3 mRNA expression in peripheral CD4+ cells of the thymus atrophy calves was tended to be lower than that of the control calves. Thymus atrophy calves exhibited chronic inflammatory disease leading, in severe situations, to conditions such as pneumonia with caseous necrosis. These severe inflammatory responses likely are due to decreases in IL10 mRNA expression, impairing control of macrophages, one of the main cell fractions of natural immunity.

KEYWORDS: calf, CD4+ cell, chronic inflammation, inhibition of interleukin 10, thymus atrophy

The thymus is a primary lymphoid organ and serves as the location where the primary T cell repertoire is generated [35]. These T cells then migrate from the thymus to the periphery, where these cells, termed recent thymic emigrants, play an important role in cellular immunity. After regression with age, thymopoiesis is reduced and thymic function decreases and replaced with adipose tissue [8, 19, 29, 34, 37]. Thymic function has been evaluated indirectly by analysis of the naïve T cell phenotype in the periphery and by measurement of thymus size [11, 12]. In a previous study, we showed that bovine thymic function is highly variable, changing with growth stage, gender, and environmental factors such as air temperature and ultraviolet irradiation [13].

It is also known that thymus atrophy in cattle is induced by a variety of conditions, including infectious diseases such as bovine viral diarrhea virus, glucocorticoid treatment of metabolic disease, shock, stress, and inflammatory disease [3, 9, 27, 36]. Furthermore, low body weight in Japanese black (JB) calves with weak calf syndrome at neonatal stages has been described histologically as a variable condition of thymus atrophy, with approximately half of neonates dying shortly after birth [33]. Intraterine growth retardation is associated with decreased immunity in calves suffering from this syndrome. In addition, at 5 weeks after birth, the numbers of CD8+ cells and γδ T cells in the peripheral blood of JB calves with thymus atrophy were significantly lower than those of normal calves [23]. The data suggested that some part of cellular immunity is concerned with the alteration of T cell function in calves with thymus atrophy. Calves that survive to 1 month of age with thymus atrophy contract chronic inflammatory diseases such as severe pneumonia, diarrhea, and arthritis as a result of drastic decreases in passive immunity [2]. In the periphery, CD4+ T cells have important roles in cellular immunity. Therefore, we analyzed the concentrations in CD4+ cells of mRNAs encoding cytokines and master transcription factors that contribute to the function and polarization of CD4+ cells. Cytokine stimulation and antigen presentation induce peripheral naïve T cells to differentiate into subpopulations such as Th1, Th2, Th17, or Treg [39]. In addition, TBX21 (also as known as T-bet), GATA3, RORC (also as known as RORγT), and FOXP3 are master transcription factors for Th1, Th2, Th17 and Treg cells, respectively [6, 14, 25].

In fact, we have dissected approximately one hundred JB calves ranging from neonates to 1-year-olds and observed severe

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pneumonia with caseous necrosis and various states of thymus atrophy in half of these animals (data not shown). However, the immune status of thymus atrophy calves that contract chronic and severe inflammatory diseases remains poorly studied. In the present work, we analyzed peripheral blood mononuclear cell and thymocyte populations in calves with thymus atrophy. In addition, we evaluated polarization of master gene expression and cytokine mRNA expression in peripheral blood CD4⁺ cells in calves with thymus atrophy.

**MATERIALS AND METHODS**

**Animals and sample collection**

Peripheral blood (PB) was collected into sterile tubes containing sodium heparin. The subjects were JB calves (n=15, 1–9 months old) obtained from local farms in the Miyazaki and Kagoshima prefectures, Japan. Twelve calves presented with chronic inflammatory diseases such as pneumonia, diarrhea, and/or arthritis. The thymuses of these calves were very small and thin, consistent with atrophied status (thymus atrophy group, n=12). Three calves had no chronic inflammatory diseases (control group, n=3). The calves’ attending veterinarian provided medical history by filling out forms; notably, calves that had received glucocorticoids treatment were excluded from the study. All calves were sacrificed by electric shock following sedation by intravenous injection with a combination of xylazine (0.2 mg/kg) and sodium pentobarbital (15 mg/kg). Thymus were removed and weighed after necropsy. The study design and protocol were reviewed approved by the Institutional Animal Care and Use Committee of the University of Miyazaki, Japan (Approval No. 2015-006 and 2021-006). All applicable national, and/or institutional guidelines for the care and use of animals were followed.

**Histology and immunohistochemical staining (IHC)**

Each formalin-fixed thymus was dehydrated through ethanol and toluene before being embedded in paraffin. To examine the general histological structures, sections were cut at 4-μm thicknesses, mounted on slides, and stained using hematoxylin-eosin. The thymus for IHC were mounted in OCT embedding compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) on Cryomold (Sakura Finetek Japan Co., Ltd.), frozen on dry ice, and then stored at −80°C. Cryostat sections were stained with monoclonal antibodies (mAbs), as described below, using the indirect immunoperoxidase technique [38]. Briefly, sections (8-μm thick) were air-dried on slides and fixed with ice-cold acetone for 10 min. To block nonspecific binding, the sections were rehydrated in phosphate-buffered saline (PBS) and incubated with 10% normal horse serum in PBS for 30 min. The sections then were stained with mAbs for 60 min and washed three times with PBS. After incubation with the secondary antibody (biotin-labeled horse anti-mouse IgG; Vector Labs, Burlingame, CA, USA), endogenous peroxidase was quenched with 0.3% H₂O₂ in methanol for 30 min followed by incubation with ABC complex (Vector Labs) for 15 min. After the sections were rinsed three times in PBS, the reaction was made visible with metal-enhanced diaminobenzidine (DAB; Pierce, Rockford, IL, USA). All IHC staining was performed at room temperature in a moist chamber. Control staining, in which the primary antibody was replaced with PBS, was performed in parallel. No positive staining was observed in the control slides (data not shown).

**Lymphocyte subset analysis and antibodies used in this study**

For lymphocyte subset analysis, we performed direct immunofluorescence labeling methods as described below [13]. Thymic cell suspensions were prepared by mincing in ice-cold PBS containing 0.5% bovine serum albumin and 0.05% sodium azide (BSA-PBS) and then using a cell strainer to remove any residual tissue fragments. The peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation with Ficoll-paque plus (GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, UK). Red blood cells were removed using NH₄Cl lysis buffer. For immunofluorescence assays, thymic cell suspensions and PBMCs were suspended in BSA-PBS. Viable cells, at densities ranging from 1 × 10⁵ to 1 × 10⁷, were incubated with fluorescein labeled mAbs (as described below) at 4°C for 60 min. The stained cells were washed three times with BSA-PBS and resuspended in BSA-PBS containing propidium iodide (1 μg/ml; Sigma Aldrich). Relative immunofluorescence intensities were determined by multicolor FACS with a FACS Canto² system (Becton Dickinson, Franklin Lakes, NJ, USA). Absolute white blood cell (WBC) numbers were counted using a poch-100iV Diff (Sysmex, Kobe, Japan). The percentage of lymphocytes in WBCs was measured by Giemsa staining of blood smears. The numbers of cell in each subset were calculated via the following formula: cell densities (cells/μl)=relative population from FACS ×percentage of lymphocytes in WBC from smears ×WBC numbers.

Anti-CD4 (200-fold dilution, ILA11A; Washington State University Monoclonal antibody center) and anti-CD8 (200-fold dilution, CC63; BioRad Laboratories, Inc., Hercules, CA, USA) mAbs were used for IHC and for staining PBMCs. Anti-γδ (200-fold dilution, GB21A; Washington State University Monoclonal antibody center) and anti-MHC class II (200-fold dilution, TH14B; Washington State University Monoclonal antibody center) were used for staining PBMCs. For fluorescent labeling of mAbs, FITC labeling kit-NH₂ (Dojindo Laboratories, Kumamoto, Japan), HiLyte™ Fluor 555 labeling kit-NH₂ (Dojindo Laboratories), and HiLyte™ Fluor 647 labeling kit-NH₂ (Dojindo Laboratories) were used according to manufacturer’s instruction.

**Isolation of peripheral CD4⁺ cells by magnetic cell sorting system (MACS) and preparation of mRNA**

Peripheral CD4⁺ cells were sorted using MACS® LS Columns (Miltenyi Biotec K.K., Tokyo, Japan) on Midi MACSTM Separator (Miltenyi Biotec K.K.). The cell sorting by MACS was performed according to manufacturer’s instructions. After separation of CD4⁺ cells, total RNA was extracted using the RNeasy® plus Mini Kit (QIAGEN, Tokyo, Japan) according to manufacturer’s instructions. Analysis of cytokine and polarization mRNA expression in peripheral blood CD4⁺ cells using real-time PCR:
Real-time PCR primers pairs were designed using Oligo 7 software (Molecular Biology Insights, Colorado Springs, CO, USA) and are presented in Table 1. Real-time PCR conditions consisted of reverse transcription at 42°C for 5 min; initial PCR activation at 95°C for 10 sec; 40 cycles of 95°C for 5 sec, 57°C for 30 sec, and 72°C for 30 sec; and a dissociation curve. Real-time RT-PCR was performed using the One Step TB Green™ PrimeScript™ PLUS RT-PCR Kit (Takara Bio, Inc., Kusatsu, Japan) according to the manufacturer’s protocol. The real-time PCR assays were performed on a QuantStudio® 3 Real-Time PCR Instrument (Thermo Fisher Scientific K.K., Tokyo, Japan). The mRNA expression level of each target gene was normalized against that of the housekeeping gene GAPDH (encoding glyceraldehyde phosphate dehydrogenase). GAPDH expression levels did not differ significantly among samples, indicating that this locus could be used as a reference gene for real-time PCR. Data were analyzed by the QuantoStudio™ Design & Analysis software (Thermo Fisher Scientific K.K.).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Data were analyzed using EZR [15]. The Mann-Whitney U test was used determine significant differences between control and thymus atrophy groups. We considered $P<0.05$ as significant and $P<0.1$ as a tendency.

RESULTS

Clinical symptoms and thymus size (Table 2)

Thymus weight of the thymus atrophy group was $58.0 ± 17.7$ g, which was significantly lower than that of the control group ($444.5 ± 17.8$ g; $P<0.01$). The ages of calves did not differ significantly between the groups (control group: $5.7 ± 0.8$ months; thymus atrophy group: $5.3 ± 0.6$ months). In the thymus atrophy group, the calves exhibited chronic pneumonia with caseous necrosis. In addition, arthritis and chronic diarrhea were observed in four and two of the thymus atrophy-group calves, respectively.

Histological observation of thymus

Thymus of control calves were observed to consist of thymic lobes comprising medulla and cortex, as shown in Fig. 1A. However, in thymus atrophy calves, large areas of connective and adipose tissues were observed in the thymus, and distinct medullary and cortical regions were not observed.

### Table 1. Primer pairs used for real-time PCR

| Gene symbol | Primer | Sequences (5′- 3′) | Product size | Accession number |
|-------------|--------|--------------------|--------------|-----------------|
| GAPDH       | F      | GTTCACCGCCACAGTCAAGGCAGAG  | 130          | NM_001034034   |
|             | R      | ACCACATCAGCAGACACACCATCAC  |              |                 |
| IL10        | F      | GGCCTGACATCAAGGAGACC     | 103          | NM_174088      |
|             | R      | CTCTGTTCAGCGAGGACGA      |              |                 |
| IL4         | F      | ATCAAAACGCTGAAATCCCTC    | 142          | NM_173921      |
|             | R      | TCCGTAGAGTACGCTAAGGCTT   |              |                 |
| IL17A       | F      | TCCACGGCAATAGGGACCC      | 196          | NM_001008412   |
|             | R      | GCCACGACATCCTTCGGA       |              |                 |
| TGFB1       | F      | AACAATTCTCTGGCCATACCTC   | 196          | M36271         |
|             | R      | GCAGAGACTTGTCATCC       |              |                 |
| IFNG        | F      | TGATTCAAATTCCCGGGAT      | 108          | NM_174086      |
|             | R      | TCTTCCGCTTTCTGAGGT      |              |                 |
| TBX21       | F      | TTGCGCTTAAACACCCTTG     | 163          | NM_00192140    |
|             | R      | TCCACCAGCCAGCCTTCCAC     |              |                 |
| RORC        | F      | GCTGCAAAGAAGCCACCCACC   | 117          | NM_001083451   |
|             | R      | GAAGAAGCCCTGTGCCCCCTCA  |              |                 |
| GATA3       | F      | CTCCGCCACTCTACATGGAC     | 120          | NM_001070804   |
|             | R      | GGCCCTACCGAGTTCCGTA      |              |                 |
| FOXP3       | F      | CCCAGGGCTCTTACTACTGCTA   | 170          | NM_00104593    |
|             | R      | CTCCAGAGATTGCACCACCTCC   |              |                 |

### Table 2. The general data of the calves

| Group                      | Thymus weight (g) | WBC ($×10^{4}$/ml) | Age (months) | Medical history                                                                 |
|----------------------------|-------------------|--------------------|--------------|---------------------------------------------------------------------------------|
| Control (n=3)              | 444.5 ± 17.8      | 0.93 ± 0.22        | 5.3 ± 0.6    | They all have never taken medical treatment for inflammatory disease, but bone fracture in accident made them given up feeding. |
| Thymus atrophy (n=12)      | 58.0 ± 17.7*      | 1.30 ± 0.30        | 5.7 ± 0.8    | Chronic inflammatory disease made them all given up feeding. All of them were affected chronic pneumonia. Four of them were affected arthritis. Two of them were affected chronic diarrheal. |

* Shows ($P<0.01$) vs. control.
cortical regions could not be identified within the thymic lobes (Fig. 1B). Many CD4+ cells (Fig. 1C) and CD8+ cells (Fig. 1E) were observed in the thymic lobes of control calves. On the other hand, CD4+ cells (Fig. 1D) and CD8+ cells (Fig. 1F) were observed diffusely in the thymus atrophy calves.

**Lymphocyte subsets in thymus**

The population of CD4+CD8+ cells in the thymus atrophy calves was tended to be lower than that of the control group ($P=0.06$), as shown in Fig. 2. In addition, there were no significant differences in the populations of CD4+ cells and CD8+ cells were seen between the control and thymus atrophy calves.

**PBMC subsets**

The number of CD8+ cells in the thymus atrophy calves was tended to be lower than that in the control group ($P=0.06$), as shown in Fig. 3. In addition, there were no significant differences in the numbers of CD4+ cells, γδ T cells, or MHC class II+ cells between the control and thymus atrophy calves.

**Expression of cytokine mRNA and master genes of T cell polarization**

The purity of CD4+ cells in all samples after MACS was $>95$. *IL10* mRNA expression in the peripheral CD4+ cells of the thymus atrophy calves was significantly ($P<0.01$) lower than that of control group, as shown in Fig. 4. In addition, *IL4* mRNA expression in the peripheral CD4+ cells of the thymus atrophy calves was tended to be lower than that of control group ($P=0.06$). However, there were no significant differences in the levels of the *IFNG*, *IL4*, *IL17A*, and *TGFB1*-encoding mRNAs between the control and thymus atrophy groups.

*TBX21* (also as known as T-bet) mRNA expression in peripheral CD4+ cells of thymus atrophy calves was tended to be higher than that of the control group ($P<0.1$). In addition, *FOXP3* mRNA expression in the thymus atrophy calves was tended to be lower than that of the control calves ($P=0.1$). There was no significant difference in *GATA3* and *RORC* (also as known as RORγT) mRNA expressions between the control and thymus atrophy groups, as shown in Fig. 5.

Fig. 1. Histological observation of normal thymus and thymus atrophy calves. Thymus of control calves were observed to have thymic lobes consisting of medulla and cortex (A). However, in the thymus atrophy calves, large areas of connective and adipose tissues were observed in the thymus, and medullary and cortical regions could not be identified within the thymic lobes (B). Many CD4+ cells (C) and CD8+ cells (E) were observed in the thymic lobes of control calves. On the other hand, CD4+ cells (D) and CD8+ cells (F) were observed diffusely in the thymus atrophy calves. Co: cortex, M: medulla. Bar=100 μm.
DISCUSSION

Thymus atrophy in neonatal JB calves with weak calf syndrome has been described previously [23, 33]. Notably, the thymus in those animals exhibited abnormal histological structures and the decreased numbers of thymocytes in neonates [33]. Furthermore, the relative populations of CD8+ cells and γδ T cells in the PB were significantly decreased at 2 and 5 weeks of age [23]. Those reports suggested that insufficient immunity in these calves may have led to the onset of infectious disease. However, no published studies have examined the immune status of thymus atrophy calves that have contracted chronic infectious diseases such as pneumonia. The typical symptom of chronic pneumonia calves is severe caseous necrosis in the pulmonary lobes; these lung lesions harbored Mycoplasma bovis, Pasteurella multocida, and/or Mannheimia haemolytica [4, 10, 20, 21, 28]. In the present study, we attempted to elucidate the immune status in thymus atrophy calves suffering from chronic inflammatory diseases.

The thymus weight in calves with thymus atrophy was significantly lower than that of control calves. Thymus size is relevant to animal age, because the thymus regresses with sexual maturation [8, 19, 37]. In the present study, we compared the structures and sizes of thymus between groups of calves of the same age. Histological observation of atrophied thymus revealed that thymus lobes were small with well-developed connective and adipose tissue. In addition, cortical and medullary structures were not identifiable in the thymus lobes of calves with thymus atrophy. IHC showed that CD4+ cells and CD8+ cells were distributed diffusely in the lobes of thymus from these animals. The relative population of CD4+CD8+ cells in the thymus atrophy calves was tended to be lower than that of the control group (P=0.06, right lower).
In the PB, the absolute number of CD8+ cells in thymus atrophy calves was tended to be lower than that in normal calves. In addition, the numbers of γδ T cells, CD4+ cells and MHC class II+ cells in PB did not differ significantly between groups. A previous study reported decreased relative populations of CD8+ cells and γδ T cells in the PB in thymus atrophy calves in calves aged 5 weeks or less [23]. The low number of CD8+ cells in the PB of thymus atrophy calves might be relevant to the low number of cells emigrating from the thymus to the periphery. However, lymphocytes also can proliferate in the periphery, which may explain why the relative number of lymphocytes does not differ significantly between groups [13, 23].

As shown in Fig. 6, Th1 cells promote inflammation and inhibit proliferation of Th2 via interferon gamma (IFN-γ). Th2 cells inhibit inflammation via IL-10 and inhibit proliferation of Th1 via IL-4 [1, 5]. Th17 promote inflammation via IL-17 [17]. Treg cells inhibit inflammation via IL-10 and induce proliferation of Th17 via transforming growth factor beta (TGF-β) [18]. In our results, IL10 mRNA expression in thymus atrophy calves was significantly lower than that in control calves. IL-10 is a potent anti-inflammatory cytokine that inhibits production of proinflammatory cytokines (IFN-γ and tumor necrosis factor alpha (TNFα)) by Th1 cells [7, 25]. IL-10 restrains immune responses to pathogens and microbial flora and prevents associated pathologies [22].
According to the results of TBX21 (also as known as T-bet), and FOXP3 mRNA expressions of CD4+ cells in thymus atrophy groups, acceleration of Th1 cells and inhibition of Treg cells might be occurred. In addition, inhibition of IL10 expression in CD4+ cells might be restraining the activation of macrophages, a process that plays important roles in natural immunity and antigen presentation to CD4+ helper T cells [30]. In preliminary results, we have found that the caseous necrotic tissue seen in calves with severe chronic pneumonia contains many macrophages and neutrophils (manuscript in preparation). These macrophages accumulate IL8 mRNA to levels hundreds of times higher than do normal macrophages (data not shown), which probably leads to the migration of high numbers of neutrophils into the inflamed pulmonary lobe.

In conclusion, thymus atrophy calves exhibited chronic inflammatory disease leading, in severe situations, to conditions such as pneumonia with caseous necrosis. These severe inflammatory responses likely are due to decreases in IL10 mRNA expression, impairing control of macrophages, one of the main cell fractions of natural immunity. Further analysis of the immunological mechanism of chronic inflammatory disease in calves is expected to contribute to improved treatment and prevention of these conditions.

CONFLICT OF INTEREST. The Authors declare no conflicts of interest.

ACKNOWLEDGMENT. The authors are very grateful to the Ito Foundation (R2-34 and R3-69) for its funding assistance.
Fig. 5. Expression of master polarization gene mRNAs in peripheral CD4+ cells in normal thymus (C, n=3) and thymus atrophy (TA, n=6) calves. TBX21 (also as known as T-bet) mRNA expression (left upper) in peripheral CD4+ cells of thymus atrophy calves tended to higher than that of the control group. In addition, FOXP3 mRNA expression (left lower) in the thymus atrophy calves tended to lower than that of the control calves. There were no differences in GATA3 and RORC (also as known as RORγT) mRNA expressions (right upper and lower, respectively) between the control and thymus atrophy groups.

Fig. 6. A diagram of the pathways whereby peripheral CD4+ T cells regulate inflammatory responses via cytokines: inhibition of Interleukin-10 (IL-10) production causes chronic inflammatory disease in thymus atrophy calves. Th1, Th2, Th17, and Treg cells are involved in inflammatory regulation via cytokine expression. IL-10 produced by Th2 and Treg cells inhibits inflammation. On the other hand, IL-17 produced from Th17 cells and interferon gamma (IFN-γ) produced by Th1 cells promote inflammation. IL-4 produced by Th2 cells and IFN-γ produced by Th1 cells prevent the differentiation of naïve T cells into Th1 and Th2 cells, respectively. Transforming growth factor beta (TGF-β) promotes the differentiation of naïve T cells into Th17 cells, resulting in the accumulation of Th17 cells. The present study showed that IL10 mRNA expression in the peripheral CD4+ cells was significantly lower in thymus atrophy calves compared to that in control calves. In addition, promotion of TBX21 (also as known as T-bet) and inhibition of FOXP3 mRNA expression in the thymus atrophy calves might causes excessive inflammation. Together, these data suggested that decreased IL-10 production and impaired Treg cell promote severe chronic inflammatory disease in thymus atrophy calves.
