Summary of the dissertation

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The title of the doctoral dissertation

Research on disease progression and immunoinhibitory pathway using sheep model of persistent bovine leukemia virus infection

（牛伝染性リンパ腫ウイルス持続感染羊モデルにおける病態進行および免疫抑制因子に関する研究）

Summary

Persistent infection with bovine leukemia virus (BLV) remains a significant concern for the global cattle industry due to its high prevalence and oncogenic potential. BLV is the causative agent of enzootic bovine leukosis (EBL), B-cell lymphoma that develops in a subset of infected animals. Although the majority of infected cattle remain asymptomatic, approximately 30% of infected cattle develop persistent lymphocytosis (PL), and fewer than 5% ultimately progress to malignant lymphoma. Despite extensive surveillance programs, effective vaccines or antiviral treatments are not currently available. A major limitation in BLV control strategies is the inability to accurately identify animals at risk of malignant state of disease. BLV is phylogenetically related to human T-cell lymphotropic virus 1 (HTLV-1), and both viruses share mechanisms of viral persistence and oncogenesis. Following infection, BLV integrates its proviral DNA into the host genome. Each infected B cell carries a unique integration site, which serves as a molecular marker of clonality. During early infection, viral gene products such as *tax* promote proliferation of infected cells, resulting in polyclonal expansion. However, malignant progression is characterized by the emergence of a dominant clone harboring a specific integration site. Therefore, while proviral load (PVL) quantifies total viral burden, it does not distinguish between benign polyclonal expansion and clonal dominance associated with tumorigenesis. To address this limitation, this thesis employed the experimentally infected sheep model of BLV. Sheep develop lymphoma with higher frequency and shorter latency period than cattle, while preserving key pathological and immunological features of natural infection. This model provides a controlled system for longitudinal analysis of viral kinetics and immune regulation.

The first objective of this thesis in chapter I was to evaluate whether quantitative assessment of proviral clonality could serve as an early predictive marker for lymphoma development. To achieve this, the RAISING-CLOVA (rapid amplification of integration site without interference of genomic DNA contamination followed by clonality value analysis) method was applied to sequential blood samples collected from BLV-infected sheep. This technique amplifies the provirus-host junction region and calculates a clonality value (Cv) reflecting the degree of clonal expansion. Longitudinal analysis demonstrated that Cv increased

prior to clinical lymphoma onset in animals that subsequently developed tumors. In several cases, Cv elevation preceded detectable changes in PVL, indicating that clonal dominance emerges before overt increase in viral burden. In contrast, animals that maintained low Cv did not develop lymphoma, even when PVL remained elevated. Integration site sequencing further confirmed that identical proviral insertion sites were detected in both peripheral blood and tumor tissues in lymphoma-bearing sheep, supporting systemic single-clonal proliferation. These findings indicate that clonality analysis provides superior prognostic resolution compared with PVL quantification alone.

Beyond viral kinetics, BLV infection profoundly affects host immune function. Chronic antigen stimulation induces T-cell exhaustion, characterized by sustained expression of inhibitory receptors known as immune checkpoints. Among these, programmed cell death 1 (PD-1) and its ligand PD-ligand 1 (PD-L1) play central roles in suppressing T-cell effector function. Chapter II addressed the lack of immunological reagents for sheep by molecular characterizing ovine PD-1 and PD-L1. Complementary DNA encoding ovine PD-1 and PD-L1 was cloned, and structural analysis revealed conserved inhibitory motifs consistent with other mammalian species. Functional assays demonstrated that anti-bovine PD-L1 monoclonal antibodies (mAbs) could block ovine PD-L1 expressing cells and capture ovine PD-L1 in peripheral blood mononuclear cells (PBMCs).

Chapter III expanded the investigation to additional inhibitory pathways, focusing on T-cell immunoglobulin and mucin domain 3 (TIM-3). The elevated expression of PD-L1 on B cells and TIM-3 on T cells were observed in BLV-infected sheep. Functional blockade of these pathways enhanced cytokine responses, and combined inhibition demonstrated additive effects. These results indicate that BLV infection induces a classical exhaustion phenotype involving multiple checkpoint receptors and that immune dysfunction is reversible under experimental conditions.

Collectively, this thesis integrates molecular diagnosis and immunopathological analysis to provide a comprehensive understanding of BLV disease progression. The work establishes clonal dominance as the defining early even in lymphoma development and validates RAISING-CLOVA as a robust prognostic tool. Simultaneously, it demonstrates that BLV-induced immune exhaustion is mediated by conserved inhibitory pathways, highlighting potential avenues for immunotherapeutic intervention. The significance of this research lies in its translational implications. Early identification of high-risk animals through clonality analysis could enable targeted herd management strategies, reducing viral transmission and economic losses. Furthermore, characterization of immune checkpoint pathways in the sheep model provides a foundation for developing immunomodulatory approaches analogous to checkpoint blockade therapies used in human oncology. By bridging virology, molecular oncology, and immunology, this thesis advances both the scientific understanding and practical management of BLV infection.

In conclusion, this thesis redefines the diagnostic paradigm of BLV infection by shifting focus from viral burden to clonal architecture and immune regulation. The findings contribute essential insights into viral oncogenesis, immune exhaustion, and the development of predictive biomarkers, establishing the sheep model as a valuable system for future investigations into BLV pathogenesis and therapeutic innovation.