学位論文内容の要旨
（Summary of dissertation）

博士の専攻分野の名称　博士（医　学）　氏 名　スン　シン
（Degree conferred: Doctor of Philosophy）　（Name of recipient:　Sun Xin　）

学 位 論 文 題 名
（Title of dissertation）

Studies of roles of *NLRC5* expression in cancers on antigen presentation and host anti-cancer immunity
（癌の抗原提示とホストの抗癌免疫における *NLRC5* 発現の役割の研究）

【**Background and Objectives**】NLRC5 (NLR Family CARD Domain Containing 5), also known as CITA (Class I transactivator), is identified as transcription activator for MHC class I and related genes. CD8+ T cells recognize antigens presented by MHC class I and consist of a critical part of anti-cancer immunity. Although a data mining study has identified *NLRC5* as a target for immune evasion in cancer, the molecular mechanisms are not well defined. In this study, we investigated the role of *NLRC5* expression in cancer in modulating host anti-cancer immunity, and efficacy of anti-PD-1 treatment. We sought to enhance our understanding of the interactions involving NLRC5, cancer, and the immune system, which eventually aimed to help the development of novel cancer therapy.

【**Materials and methods**】To investigate the role of NLRC5 in host anti-cancer immunity, we induced genomic deletion of *Nlrc5* in mouse melanoma B16F10 and mouse breast cancer E0771 cell lines. Furthermore, to investigate whether *NLRC5* is a viable target for development of novel immunotherapy, we developed a novel CRISPR/dCas9-TET1cd based targeted demethylation and activation (TDMa) system to enhance *Nlrc5/NLRC5* expression in mouse melanoma B16F10 and human breast cancer MCF7 cell lines. QRT-PCR and RNA-Seq were performed to determine the mRNA expression levels after knockout of *Nlrc5* or *Nlrc5/NLRC5* activation. Bisulfite sequencing was performed to determine promoter methylation levels. Flow cytometry were performed to determine cancer cell immunogenicity. Subsequently, mouse cell lines were subcutaneously implanted *in vivo* to observe tumor growth. Flow cytometry and immunohistochemistry were performed using *in vivo* tumors to characterize tumor infiltrating lymphocytes (TILs) and tumor cells.

【**Results**】We found that genomic deletion of *Nlrc5* specifically downregulated MHC class I gene expression in B16F10 and E0771 cells. *Nlrc5* deletion promoted E0771 tumor growth *in vivo* by inhibiting CD8+ T cell infiltration and activation through MHC class I. On the other hand, *Nlrc5* deletion did not affect B16F10 tumor growth without stimulation. This is because B16F10 has low basal MHC class I expression and genomic deletion of *Nlrc5* did not have significant effect on B16F10 basal MHC class I levels. However, we found that *Nlrc5* is required for response to anti-PD-1 therapy in B16F10 tumor transplantation model. Based on these results, we performed transcriptome screening and generated the optimal prediction model for prediction of response to anti-PD-1 therapy in melanoma. Furthermore, we induced targeted demethylation and activation of *Nlrc5* promoter in B16F10 and MCF7 cell lines using CRISPR/dCas9 based system (TDM and TDMa systm). Although targeted demethylation of *Nlrc5* (TDM

system) alone was insufficient to significantly augment MHC class I expression and tumor immunogenicity, we found that the expression of MHC class I is robustly augmented by combination of targeted demethylation activation of *Nlrc5* (TDMa system). RNA-Seq analysis showed that TDMa system induced specific augmentation of expression of *Nlrc5* and dependent MHC class I expression. OT-I co-culture analysis showed that the immunogenicity of B16F10 were augmented by targeted demethylation and activation of *Nlrc5*. *In vivo* implantation of these cells showed that tumor growth is impaired by targeted demethylation and activation of *Nlrc5* in C57BL/6 mice, but not in NUDE mice. CD8+ T cell infiltration and activation was augmented by targeted demethylation and activation of *Nlrc5* in B16F10 tumors, but not in CD4+ T cells. Moreover, immunohistochemistry analysis showed that targeted demethylation and activation of *Nlrc5* in B16F10 not only increased the number of TILs, but it also promoted concentration of CD8+ T cells into the tumor center. Furthermore, we showed that targeted demethylation and activation of *Nlrc5* sensitized the response to anti-PD-1 treatment in B16F10. Finally, we showed that CD8+ T cell infiltration and activation were specifically enhanced by anti-PD-1 treatment, but not in CD4+ T cell.

【**Discussion**】Although numerous studies have identified the role of NLRC5 as class I transactivator, most of the investigations were focused its role in the immune system. Few studies investigated the role of NLRC5 in cancers and cancer derived NLRC5 in modulating host anti-cancer immunity. MHC class I is of high priority as target for cancer treatment, there, our research is importance. Here, using both genomic deletion, and targeted demethylation and activation approach, we systemically illustrated the role of *Nlrc5* in modulating immunogenicity through MHC class I and host anti-cancer immunity in melanoma and breast cancer. For genomic deletion of *Nlrc5*, consistent with the previous investigations using *Nlrc5* knockout mice, we found that *Nlrc5* deletion downregulated MHC class I expression levels with or without mIFN-γ stimulation. Our study is the first to characterize the effect of *Nlrc5* in MHC class I antigen presentation pathway to our knowledge. On the contrary, we also induced targeted demethylation and activation of *Nlrc5/NLRC5* in B16F10 and MCF7 using the TDMa system that we developed. A previous study characterized the phenotype of *Nlrc5* overexpression in B16F10 *in vitro* and *in vivo*. Our results further confirmed that activation of *Nlrc5* enhanced the expression of MHC class I and host anti-cancer immunity. In addition, we found that targeted demethylation and activation of *Nlrc5* promoted concentration of CD8+ T cell into the tumor center. Previous studies observed that aggravation of CD8+ T cell into tumor center is correlated with a better prognosis, while the mechanism is left unexplored. Here, we further confirmed that expression of surface MHC class I in cancer is one of the mechanisms that promote CD8+ T cell infiltration into tumor center. Furthermore, we found that targeted demethylation and activation of *Nlrc5* sensitized response of B16F10 to anti-PD-1 treatment. Previous studies have identified that surface MHC class I levels and *NLRC5* expression levels correlates with a good response to immune checkpoint blockade therapy. However, the underlying mechanisms are not well studied. Our results indicate that CD8+ T cell were exclusively activated by anti-PD-1 treatment and targeted demethylation and activation of *Nlrc5* synergistically enhanced CD8+ T cell mediated anti-cancer immunity, highlighting *Nlrc5* as a therapeutic target to synergistically enhance the efficacy of anti-PD-1 treatment.

【**Conclusion**】In conclusion, our research deepened our understanding of the role of *Nlrc5* in host anti-cancer immunity. Our strategy to induce and genotyping genomic deletion is solid and can be applied to other genes and cell lines. We show that the CRISPR/dCas9 mediated targeted demethylation and activation strategy of *Nlrc5* is a viable and novel immunotherapy approach to enhance cancer immunogenicity. For future study, optimization of delivery system to effectively induce targeted demethylation and activation *in vivo* could contribute to development of novel immunotherapy.