This report should be submitted within 2 weeks after you return to Japan. Please do not change the formatting

（Abroad・Domestic）Internship report form（Student） 2023/11/07 (Year/Month/Day)

Name	Wisa TIYAMANEE
Laboratory	Infectious Diseases
Year (Grade)	D2
Internship institution	Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota
Internship period	Internship period: 10/01/2023 - 10/27/2023 (Departure Date from Sapporo: 09/30/2023, Arrival Date in Sapporo: 10/29/2023)
Purpose	- To broaden the perspective of an immunology research field - To get new idea or inspiration for current research - To survey the working environment for Postdoctoral study

- The reason why you chose this institute

The Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota is the institute that researches the lentivirus's immunotherapeutic strategy that belongs to Retroviridae. My current research is also related to bovine leukemia virus infection, which is a Retroviridae member. However, my research mainly focuses on the pathogenesis of immune exhaustion and finding effective therapeutic countermeasures such as antibody production. I would like to broaden my knowledge about other therapeutic methods for immune suppression, including chimeric antigen receptor (CAR) T-cell therapy. CAR T cell therapy is the specific therapeutic method that directly modifies T lymphocyte construction, which is not similar to antibody therapy that does not change any immune cell construction. This innovation is widespread in the United States and well-developed in this laboratory. Moreover, attending an internship during this period broadened my perspective on my current research activity and could enhance my doctoral research with well-trained experimental skills.

- Result of the activity （about 800 words、provide photos, tables and figures that clearly show the activities during the period）

CAR T cell therapy is one of the most recent cell therapies in immunotherapeutic research. This research group developed CAR T cells for treating Human Immunodeficiency Virus (HIV) infection in mice and nonhuman primate (NHP) models. All CAR T cell experimentally infusion steps usually take

approximately six months. However, this internship has a limited time to one month. Thus, Following the schedule below, they let me join the experiment at the most crucial time.

SUN	MON	TUE	WED	THU	FRI	SAT
10/01 Lab introduction	10/02 - Transduction D1 (virus) - Flow cytometry characterization	10/03 - Transduction D2 (virus) - Preparing Histogel	10/04 - Transduction D3 (virus)	10/05 - Lab meeting #1 - Flow cytometry compensation	10/06 - Transduction D5 cell expansion (virus) - Mouse party (Blood collection, CD4 count)	10/07
10/08	10/09 - Flow cytometry compensation	10/10 - Transduction D9 (virus) - Cytokine assays D1 - RNA extraction, cDNA synthesis - DELFIA killing assays - Migration assays - 40th Annual Symposium on NHP models for AIDS	10/11 - Cytokine assays D2 - qPCR of PD-1 KO gene - 40th Annual Symposium on NHP models for AIDS	10/12 - 40th Annual Symposium on NHP models for AIDS	10/13 - Joint HIV Lab Meeting #1 - 40th Annual Symposium on NHP models for AIDS	10/14
10/15	10/16 - Transduction D1 (Transposon) - Flow cytometry characterization	10/17 - Transduction D2 (Transposon)	10/18 - Transduction D3 (Transposon)	10/19 - Lab meeting #2 (Happy hour) - Mice receival	10/20 - Transduction D5 cell expansion (Transposon) - Mice health check - Joint HIV Lab Meeting #2	10/21 - Mice health check
10/22 - Mice health check	10/23 - Flow cytometry compensation	10/24 - Dual CAR RNAscope D1 Transduction D9 - Cytokine assays D1 - RNA extraction, cDNA synthesis - DELFIA killing assays - Migration assays	10/25 - Dual CAR RNAscope D2 - Cytokine assays D2 - qPCR of PD-1 KO gene	10/26 - Blood collection CD4 count - PBMCs activation (suppression assays) - Progress presentation (Lab meeting #3)	10/27 - Blood collection CD4 count - PBMCs stimulation (suppression assays) - Joint HIV Lab Meeting #3	10/28 Departure
10/29 Arrival Sapporo	10/30	10/31				

All experiments I joined can be grouped into main 3 topics, including CAR T cell production and efficiency test, CAR T cell functionality, and RNAscope results of *in vivo* CAR T cell infusion and histogel from *in vitro* transduction. Detailed activity in each topic will be described below.

- **CAR T cell production and efficiency test**

  The second step of the whole experiment is to test the efficiency of CAR T cell production before infusion into any animal. However, due to the limited time, I didn't have the chance to join the first step, CAR T cell

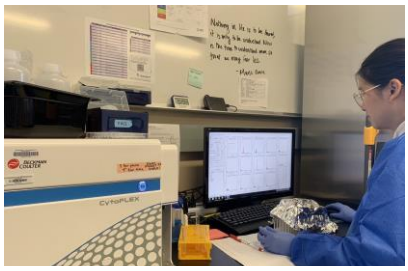

Figure 1 Flow cytometry analysis of PBMCs characteristic before transduction

structure design. My postdoctoral mentor taught me about CAR T cell structure design. In this experiment, they use CAR/CXCR5 T cells with PD1 knocked down because previously, they found that HIV can escape the immune system by accumulating in B cell follicles. Original CAR T cells can't successfully control HIV infection; additional CXCR5 as a follicular homing receptor can bring T cells to the follicle. They try to optimize the CAR/CXCR5 T cells for improved efficacy. Consequently, they were designed to knock down PD1 due to the downregulation of immune functions. Thus, I learned how to make CAR T cells and test their efficacy using a flow cytometer with basic cell culture technique, RNA extraction and cDNA synthesis, and qPCR.

They also compare the CAR T cell transduction method between gamma retrovirus and transposon technique because they would like to reduce the cost-benefit of CAR T cell production. However, gamma retrovirus is the most effective way for CAR T cell production; they have to adjust culture conditions for the best transduction efficacy.

For the first step of the transduction process, they use PBMCs from HIV patients to selectively enhance the expression of CD4+ and CD8+ cells with IL-2 culture medium and transduce cells with gamma retrovirus or transposon technique.

- **CAR T cell functionality**

  The transduction process needs 9 days to completely transduce CAR T cells, change media, check the characteristics of cells, and transfer to expansion wells. After 9-day cultivation, a functionality test was conducted for efficacy confirmation before infusion. The functionality test consisted of many steps, including cytokine production assays, qPCR of the knocked-down gene, DELFIA® cell cytotoxicity assays, and migration assays. All purposes of each functionality test are explained in detail below.

  - <u>Cytokine production assays</u>

    The primary function of this CAR T cell is to control HIV infection that need to produce major immunoregulatory cytokine, including IFN-γ, TNF-α and IL-2. They provide short cultivation of CAR T cells with HIV-infected target cells and measure the cytokine expression via flow cytometry analysis.

- qPCR of knocked-down gene

  To confirm the successfully knocked down immunoinhibitory gene, PD1, they extract RNA from 9-day cultured PBMCs and synthesize cDNA for qPCR.

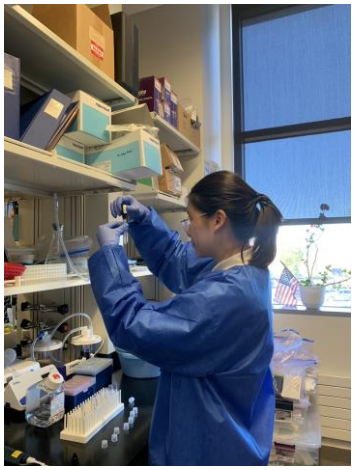

Figure 2 RNA extraction and cDNA synthesis for further qPCR of PD1 expression

- DELFIA® cell cytotoxicity assays

  One of the most critical functionality tests is DELFIA® cell cytotoxicity assays; this method can evaluate the CD8+ T cell function. They conducted short cultivation of CAR T cells with HIV-infected target cells and measured the component releasing during killing activity using a DELFIA® commercial kit.

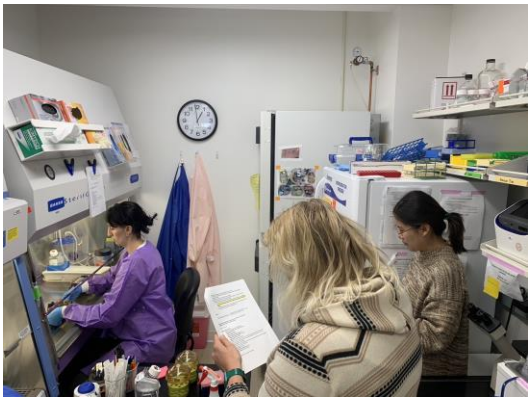

Figure 3 cell cytotoxicity assay for CAR/CXCR5 functionality test

- Migration assay

  The critical factor of homing to follicles of CAR T cells for HIV controlling strategy as described above is migration assay. They test

migration ability using CAR T cell culture with C-X-C motif chemokine ligand 13 (CXCL13) and CXCL12 in the specific "trans-well plate," as CXCL13 is essential for follicle homing.

- **RNAscope result of *in vivo* CAR T cell infusion and histogel from *in vitro* transduction**

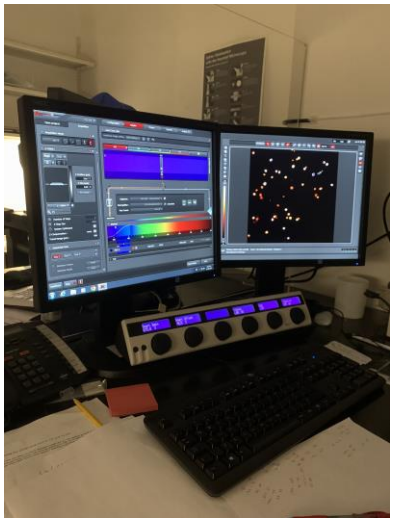

Figure 5 RNAscope software, Leica Application Suite X, as user interface for RNAscope imaging

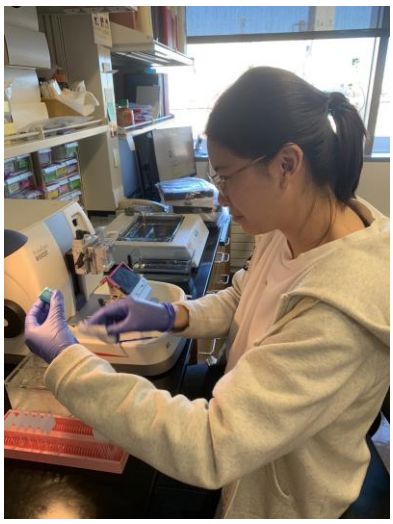

Figure 4 Tissue sectioning and slides preparation for RNAscope imaging

RNAscope is also one of the best choices to visualize the specific cell components in protein levels, which might combined with infectious particle detection.

Thus, from the step before infusion, they also conduct the RNAscope analysis to visualize the successful transduction of the CAR T cell.

Moreover, this assay can confirm the homing ability of engineered CAR/CXCR5 T cells *in vivo* by using tissue sectioning of infused animals.

More than experiment activities, I also have the chance to join many seminars listed in the table above. I can meet a lot of scientists and join the discussion during their presentations to expand my knowledge and perspective.

Figure 6 Joint HIV lab meeting

Finally, during the last week of my internship, I updated my training progress and discussed the usage of some techniques in my current research.

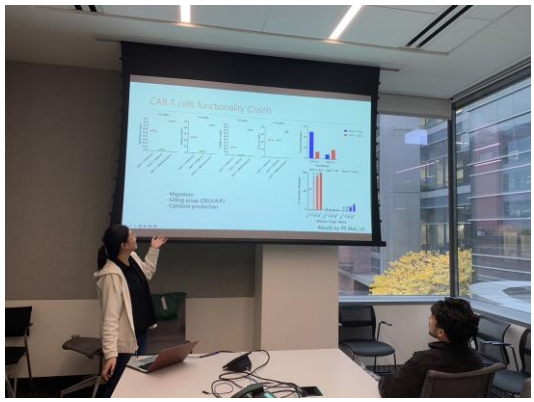

Figure 7 update and progress presentation in this institute

- What do you think the positive impact of the activity will have on your further career path?

After entering the graduate school of Infectious Diseases, Laboratory of Infectious Diseases, I learned about immunopathogenesis of chronic infectious diseases and immunotherapy knowledge is needed for my future career. Thus, I decided to learn other methods of immunotherapy, including CAR T cell therapy, at this institute. The internship abroad in the U.S.A. will improve many soft skills, including scientific explanation, communication, and live adaptation. Moreover, it's essential that we require collaboration from the U.S.A. and Japan that will improve immunology research, whether in my home country or worldwide.

- Advice for your junior fellows

Don't be afraid to think beyond.

For the one who is considering going abroad, please keep in mind that you should think beyond. Consider the best place you would like to go and prepare many plans in case of rejection or no responseat

Extra budgets is the most important.

Money is one of the most critical factors in going abroad, especially in Europe or the United States. Please consider your budget and the living cost of your destination. Moreover, you must prepare more extra money than you estimate, either cash or card, because anything can happen at any time.

The excellent performance gives you more chances

Just a tiny tip for first impression and attitude that they will look at you. If you have excellent performance in their experiment and more specific background knowledge to discuss with other researches without any ego, you will have many chances to receive better responses.

Approval of supervisor	Institution・Official title・Name Laboratory of Infectious Disease, Department of Diseases Control, Faculty of Veterinary Medicine Prof. Satory Konnai

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※3 The Steering Committee for the WISE Program will first confirm the content of this report and report will be forwarded to the Educational Affairs Committee for credits evaluation.

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