NRCPD-OUAVM Joint Research Report

Date: 2024.5.23
Project no: 2023-joint-21

1. Principal investigator

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2. Project title:

Establishment of DiCre parasite lineages to study essential aspartyl peptidases of *Babesia*

3. Collaborating research group members at NRCPD

Name: Masahito Asada
Position: Associate Professor

4. Research period (in mm/dd/yyyy, and total number of years)

01/04/2023 -31/03/2024, one year

5. Purposes and objectives

The primary objective of the 2023 NRCPD OUAVM Joint Research Proposal was to advance our previous collaborative efforts from 2019 and 2022. In these earlier projects, we successfully developed a transgenic *Babesia bovis* parasite lineage expressing the DiCre recombinase, which enables rapamycin-controlled excision of loxP-flanked DNA sequences. This technology facilitates the functional analysis of essential parasite genes, previously demonstrated in model species such as *Toxoplasma gondii* and *Plasmodium falciparum*. The 2023 proposal aimed to leverage the stable DiCre parasite lineages of *Babesia divergens/ bovis* to perform conditional knockouts of aspartyl proteases BdASP3a (023140) and BdASP3b (006490) of the C clade of apicomplexan aspartyl proteases, which are analogous to plasmepsins X/IX for *Plasmodium falciparum* and thus represent the potential master regulators involved in invasion and egress during Babesia's asexual blood stages. Project aimed at phenotypical characterization of the ASP3a/b deleterious phenotypes, with the aim to validate the critical roles of these enzymes in the erythrocytic lifecycle of Babesia. This work builds up on our preliminary findings with plasmepsin X/IX and TgASP3 specific inhibitor 49c, supporting Bd/BbASP3 as promising drug targets for Babesia control.

The individual objectives of this 2023 joint project were as follows:

(i) verification of the Cre/lox recombination in novel DiCre Babesia lineages using episomal loxP sites holding plasmids
(ii) use of the Dicre transgenic *Babesia divergens/ bovis* lineages to create conditional knock-outs(iKO) of BdASP3a/b proteases
(iii) validation of BdASP3a/b as essential and druggable proteases
(iv) phenotypization - determination of their specific roles in invasion/egress of host erythrocytes during Babesia RBC cycle
(v) biochemical characterization of recombinantly expressed BdASP3a/b

By achieving these objectives, we aimed to advance our understanding of Babesia parasites and the associated disease, ultimately contributing to the development of improved therapeutic strategies.

6. Outline of research process

In line with the proposed plan, we utilized the extended year of the project to obtain cloned populations of DiCRE *Babesia bovis* and *Babesia divergens*. During our work on this project and the visit of Dr. Sojka to NRCPD-OUAVM between October 6-16, 2023, we improved the design and prepared novel version of the DiCre holding cassette for the integration into the *Babesia bovis* genome using homology-based recombination. This construct was designed in such a way that by making only two changes in the homologous regions, we could use the same plasmid/DiCre cassette for integration into the genome of *Babesia divergens,* a related *Babesia* sensu stricto species that is of relevance for us back in the Czech laboratory at IoP BC CAS. PCR confirmed the correct integration of plasmids into parasite genomes, and expression of DiCre recombinase subunits was confirmed via quantitative PCR. Additionally, control GFP/mCHERRY holding plasmid vectors were prepared for episomal control of LoxP site excision by the DiCre recombinase in both *Babesia* species and the Cre/lox excisions are currently being confirmed by transfection of these plasmids to *B. bovis* / B. *divergens* DiCre lineages. The final plasmid for BdASP3 iKO containing the floxed *bdasp3* gene sequence was designed and prepared. The ASP3 iKO plasmid transfection will be performed upon the successful Cre/lox episomal excision tests. Resulting iKO BdASP3a/b lineages will serve for the induction of *bdasp3a/bbasp3b* gene excision by rapamycin treatment and subsequent phenotypic observations will be performed.

The work was parallelly supported by the CAS/JSPS (2021-23) joint mobility project. This enabled one member of the Czech team. Ms. Pavla Šnebergerová (PhD student pf Dr. Sojka) visited NRCPD in Obihiro for a short internship of two months during October-November 2023. Oppositely, the PhD student of Dr. Asada, Atefe Fathi completed a 7-week long internship supported by the JSPS as part of the project at IoP BC CAS, Ceske Budejovice, Czechiam in November-December 2023. During both exchange visits/internships the students primarily focused on generating stable lines of *B. bovis* parasites that express the dimerizable Cre-recombinase (DiCre). Both visiting students further acquired practical skills related to the design and preparation of loxP holding plasmid cassettes and the

preparation of transgenic *Babesia* and subsequent analysis of the resulting ASP3 deleterious phenotype. Additionally, and the associated biochemical and immunostaining work as a part of the biochemical characterization of babesia ASP3 enzymes. Furthermore, we completed the characterization of the two *BdASP3* isoenzymes recombinantly expressed as C-terminal 6x(His) tagged proteins in insoluble form in *E. coli* DE3. This is in contrast with previous plans of expression of these isoenzymes in baculovirus infected insect cells as this method was successful, but we had been observing issues with insufficient recombinant protein yields and their overall instability. The *E. coli* expressed ASP3 proteins were further purified and refolded via step-down dialysis from 8 M urea denaturing buffers and served as active proteases in kinetic assays with fluorescent peptidyl substrates. Inactive mutants of both enzymes were also prepared for large-scale production to obtain protein 3D structures via protein crystallography. All the listed results obtained in 2023 will be part of a forthcoming publication in a high impact factor journal by the end of 2024, with P. Šnebergerová as the first, and Dr. Sojka as the last and corresponding author.

Additionally, our findings were presented at several international conferences in 2023. Dr. Sojka made two active contributions at the 15th International Symposium on Ticks and Tick-borne Diseases in Weimar, Germany, presenting on 'Proteasome inhibitors effectively combat ticks as well as transmitted pathogens' and 'Clade C aspartyl proteases in *Babesia* divergens and their validation as novel drug targets.' Furthermore, at the 12th General Meeting of the International Proteolysis Society in Singapore, our team presented a poster on 'Deciphering the roles of *Babesia* Plasmepsin X/IX homologues and their validation as druggable targets.' Dr. E.F.G. Cubillos also presented at the Fourth International Babesiosis Meeting at Yale School of Medicine, New Haven, CT, USA.

7. Outline of research achievements

- we successfully obtained cloned populations of DiCRE *Babesia bovis* and *Babesia divergens*
- we PCR confirmed their integration to the parasite genome
- we confirmed the expression of DiCre recombinase
- we prepared lox-flanked GFP/mCHERRY holding plasmid vectors for episomal control of LoxP site excision by the DiCre recombinase in both *Babesia* species
- we designed and prepared the final plasmid for BdASP3 iKO using the DiCre approach
- we transfected the parental *B. divergens*/*B. bovis* DiCre lines with the recipient DNA plasmid containing the loxP-flanked bdasp3 gene sequence
- we prepared dASP3a/b DiCRE lineages by serial dilutions
- we verified the Cre/lox excision and perform phenotyping
- In parallel, we successfully expressed BdASP3a/b isoenzymes as C-terminal 6x(His)-tagged proteins in *E. coli* and we are using them for 3D structure studies using protein crystallography

8. Publication of research achievements

By the end of 2023, we published our first joint publication: Cubillos, E. F. G., Snebergerova, P., Borsodi, S., Reichensdorferova, D., Levytska, V., Asada, M., Sojka, D., & Jalovecka, M. (2023). Establishment of a stable transfection and gene targeting system in *Babesia divergens*. *Frontiers in cellular and infection microbiology*, *13*, 1278041. https://doi.org/10.3389/fcimb.2023.1278041).

Attach reference materials as necessary.