

High Performance Cell-Free Wheat Germ Protein Expression System

Application Note

International malaria R&D projects supported by the GHIT Fund of Japan

Abstract

Malaria is still one of the greatest threats to global health, infecting hundreds of millions of people every year despite huge efforts to eliminate the disease. As part of Japan's commitment to global health, the **Global Health Innovative Technology (GHIT) Fund of Japan** was established five years ago to support international product development partnerships for new health care solutions needed in the developing world. The development of new drugs, vaccines, and diagnostic tests for malaria is an important aspect of the work at the GHIT Fund and their investment strategy. Their portfolio also includes malaria-related projects utilizing the wheat germ cell-free protein expression system from CellFree Sciences as further described in this Application Note.

Introduction

The **Global Health Innovative Technology (GHIT) Fund of Japan** (<https://www.ghitfund.org/>) supports international product development partnerships for new health care solutions to fight infectious diseases and poverty in the developing world [1]. Bringing in Japanese innovation, investment, and leadership, the GHIT Fund wants to help reducing the disease burdens of billions of people so that they can have the same levels of prosperity and longevity already common in industrialized countries. In the first five years since establishment in 2013, the GHIT Fund has invested about 132 million USD to support 74 worldwide development partnerships. With about 40.6 million USD, nearly a third of this money had been dedicated to promoting projects related to elimination of malaria.

Despite tremendous progress in many countries on the elimination of malaria [2], about half of the world population is still at risk of infection by mosquito-borne malaria parasites of the *Plasmodium* genus.

According to the WHO [3] ongoing transmission has been observed in 91 countries with the highest malaria case numbers and deaths occurring in sub-Saharan Africa. In their *World malaria report 2017* [3], the WHO lists for 2016 about 216 million malaria cases causing about 445,000 deaths worldwide, often children under the age of five. While we have seen an overall decline in the number of malaria cases since 2010, the WHO observes a substantial increase for malaria cases in the Americas, and to a smaller extent in the Western Pacific and African regions. Therefore, there is a clear need for additional investments in malaria control and to support elimination. Part of this investment must be used to develop new approaches for fighting the disease.

Cell-free protein expression in malaria research

The wheat germ cell-free protein expression system from CellFree Sciences proved very powerful to express malaria proteins during many studies for identifying new vaccine candidates or working on biomarker discovery [4-6]. For example, in a search

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for new antigens for vaccine development, 27 *P. falciparum* pre-erythrocytic antigens were prepared by the wheat germ system to study the immune response after injecting *P. falciparum* radiation-attenuated sporozoites into humans [7]. Similarly, naturally acquired antibody responses were identified using a set of 307 *P. vivax* proteins made in the wheat germ system [8]. Such approaches have been further extended by the expression of a library of 1,827 *P. falciparum* proteins derived from 1,565 genes representing ~30% of the entire *P. falciparum* genome [9, 10]. The wheat germ system enables such studies by making mono-biotinylated proteins for direct use in the PerkinElmer AlphaScreen™ system detecting protein-protein interactions [11]. This is a very forcible approach for the massive analysis of many proteins and patient samples to understand the immune response after malaria infections. These technologies developed by Eizo Takashima and Takafumi Tsuboi at Ehime University [12, 13] have been the starting point for several malaria related product development partnerships supported by the GHIT Fund.

Product development partnerships on malaria

Ehime University and/or CellFree Sciences have actively contributed with our technologies to the following product development partnerships supported by the GHIT Fund.

PATH's Malaria Vaccine Initiative: Accelerating development of vaccines for malaria elimination using a novel clinical target validation approach

Collaboration Partners: Ehime University, CellFree Sciences

Although with GlaxoSmithKline Vaccines' Mosquirix (formally known as RTS,S/AS01) the first recombinant protein-based malaria vaccine has been approved by EMA in July 2015 for active immunization of children aged 6 weeks up to 17 months [14], there is still an urgent need for more malaria vaccines to address parasite diversity and to reach better efficacy than presently possible with Mosquirix alone [15]. Pre-erythrocytic (PE) vaccines could prevent the disease directly after mosquito-to-human transmission and are therefore a preferred

research target. In this project, the wheat germ system had been used to successfully prepare 24 novel PE vaccine candidates on a mg-scale for testing by an accelerated target validation approach. This approach included the preparation of human antibodies against the target proteins to demonstrate their functional activity in several distinct assays and potential use in malaria protection [16]. Still unpublished data at PATH indicate that this project succeeded in identifying new PE vaccine candidates and could provide human antibodies against them for further product development of malaria vaccines.

<https://www.ghitfund.org/impact/portfolio/awpdetail/detail/15>

PATH's Malaria Vaccine Initiative: Accelerating Development of Transmission-Blocking Vaccines for Malaria Elimination Using a Novel Vaccine Candidate
Collaboration Partner: Ehime University

Malaria is a vector-borne disease, where the parasites must transfer from infected *Anopheles* mosquitoes to humans to complete their life cycle. Therefore, blocking the transmission of parasites from humans to mosquitoes could be a perfect intervention to block infection of mosquitoes and thus to prevent later new malaria infections in humans. Different approaches have been taken to develop so-called Transmission-Blocking Vaccines (TBVs), where the laboratory of Prof. Tsuboi at Ehime University had identified the Py75 protein from *P. yoelii* as a possible transmission-blocking antigen. While *P. yoelii* does not infect humans, it is a rodent malaria parasite that allows for whole animal studies in mice. In such experiments, mice immunized with Py75 could no longer transmit the parasite to mosquitoes indicating strong transmission-blocking activity of the antigen [17]. Using different bioinformatics tools and a gametocyte protein database, they were able to find the Py75 homolog Pf75 in *P. falciparum*, the most common malaria parasite found in humans. The partnership intended to validate Pf75 as a TBV candidate to block transmission of *P. falciparum* malaria. Using the wheat germ system, 12 different domains of the Pf75 protein could be produced on a milligram scale. The antigens were used to raise anti-Pf75 antibodies, which could detect the native protein in immunofluorescence assays (IFA) on gametocytes

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and gametes. While also restricting parasite mobility in an exflagellation assay, the anti-Pf75 antibodies failed to achieve at least 80% transmission-blocking activity in Standard Membrane Feeding Assays for testing the infection of mosquitos after a blood meal. Because of the limiting transmission-blocking activity of all anti-Pf75 antibodies, the partners decided not to continue the development of a Pf75-based TBV.

<https://www.ghitfund.org/investment/portfoliodetail/detail/48>

The Walter and Eliza Hall Institute of Medical Research: Development of serological biomarkers as indicators of recent and asymptomatic infections for innovative tools to accelerate malaria elimination

Collaboration Partners: CellFree Sciences, Ehime University, Foundation for Innovative New Diagnostics (FIND)

Notably in the Asia-Pacific and American regions malaria elimination programs must address *P. vivax* as the predominant malaria parasite species. *P. vivax* is more persistent than *P. falciparum* because of its long-lasting dormant stages. So-called hypnozoites can remain in the liver for many months after a malaria infection causing mosquito-independent relapses of the infection and thus driving new transmission cycles. Present diagnostic tests do not allow for direct detection of hypnozoites. However, almost all mosquito-derived *P. vivax* infections result both in a primary, often symptomatic blood-stage infection as well as the formation of a dormant hypnozoite reservoir. Therefore, detecting past infection could be a good indicator for hypnozoites in the liver. The partnership identified and validated in three cohort studies with over 2,500 participants serological markers for past-infection with *P. vivax* within the last 9 months. The resulting panel of 8 serological markers uses antibody responses in patient sera for detecting recent infections with 80% sensitivity and specificity. This panel holds great promises for the development of different diagnostic tests, where the partnership provided Target Product Profiles (TPPs) for a general surveillance test for use in malaria control in low transmission areas, and a diagnostic test to identify hypnozoite carriers for targeted treatment in line with the original objectives

of the project. However, these markers may further be used in the development of other new rapid tests to detect acute and recent infections for routine use in malaria control and therapy.

<https://www.ghitfund.org/impact/portfolio/awpdetail/detail/66>

The University of Florida: Lead optimization of an evolution-proof malaria transmission-blocking vaccine immunogen that is based on a mosquito protein target and effective against both P. falciparum and P. vivax

Collaboration Partner: CellFree Sciences

As outlined above for the TBV project on Pf75, TBVs should be very potent tools to drive the elimination of malaria. TBV development projects are commonly using malaria proteins like the gametocyte proteins Pfs25, Pfs48/45, or Pfs230 [18] for raising high antibody titers. These antibodies are included in the blood meal to later prevent uptake or development of the parasite in the mosquito midgut. This partnership used a different approach by developing a TBV based on the anopheline mosquito midgut-specific alanyl aminopeptidase N (AnAPN1) protein [19]. By focusing on a mosquito protein, this approach avoids the risk that parasites could develop resistance against the invention. Moreover, working with a highly conserved mosquito protein enables blocking the transmission of multiple malaria species as already demonstrated for blocking the transmission of *P. falciparum* and *P. vivax* in both model systems and in the field, via Direct Membrane Feeding Assays in Africa and Southeast Asia [20]. TBVs can only be effective if very high antibody titers can be achieved with well-targeted antibodies against the transmission-blocking domain within the target protein. Therefore, this partnership used detailed protein structure [21] and mapping information to design and test optimized antigens for an AnAPN1-based TBV. These antigens were expressed in the wheat germ system and tested in immunization experiments in combination with different adjuvants (Alhydrogel, GLA-LSQ and Stable Emulsion/SE). The kinetics and magnitude of the humoral responses were confirmed in ELISA assays, where the antisera demonstrated high binding activity to native AnAPN1

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and epitope specificity peptides. The transmission-blocking activity against *P. falciparum* was determined in Standard Membrane Feeding Assays using a model system and again in Direct Membrane Feeding Assays using patient samples from Cameroon. Based on the results from this project, the new antigen denoted as UF6 in combination with the GLA-LSQ adjuvant was proposed for preclinical development of an immuno-focused, mosquito-based pan-malaria transmission-blocking vaccine as further outlined below.

<https://www.ghitfund.org/impact/portfolio/awpdetail/detail/81>

The University of Florida: Process Development and Clinical Manufacturing of an Immuno-focused, Mosquito-based Pan-malaria transmission-blocking vaccine: AnAPN1 v. 2.0

Collaboration Partners: CellFree Sciences, Centre Pasteur du Cameroun (CPC), Hamamatsu Pharma Research, Infectious Disease Research Institute, Ology Bioservices Inc.

This new project initiated in 2018 is a continuation of the forgoing development project of an AnAPN1-based TBV. The previous project identified the very potent UF6 antigen that focuses the immune response to the transmission-blocking epitopes within AnAPN1. This new antigen, in combination with the GLA-LSQ adjuvant demonstrated strong transmission-blocking activity against wildtype *P. falciparum* strains. While still using a UF6 protein made in the wheat germ system as a reference standard, this project will setup process development leading to cGMP production of an untagged version of the UF6 protein in an *E. coli* host. The *E. coli*-produced UF6 antigen will be used to prepare UF6:GLA-LSQ vaccine formulations for testing in mice and non-human primates. Antibodies obtained during the animal studies will be evaluated using immunological and biological assays at the University of Florida and the Centre Pasteur du Cameroon to also include testing the transmission-blocking activity against natural *Plasmodium* strains. This project will be completed by end of March 2020 as a further milestone towards an Investigational New Drug (IND) application for an AnAPN1-based TBV.

<https://www.ghitfund.org/impact/portfolio/awpdetail/detail/122>

PATH's Malaria Vaccine Initiative: A Vaccine to Block Malaria Transmission: Pfs230 Antigen Design and Display

Collaboration partner: Ehime University

As mentioned above, Pfs230 is another prominent candidate protein for the development of an effective TBV [18]. It is a gametocyte surface protein that is required for male gamete exflagellation, interaction with erythrocytes, and male-female gamete fusion. Blood-stage malaria parasites develop into either male or female gametocytes. After ingestion with a blood meal, they differentiate into gametes of both sexes in the mosquito gut. After the gametes emerge from red blood cells, Pfs230 will get exposed to anti-Pfs230 antibodies also present in the blood meal, that may block fertilization of parasites and thus disrupting the life cycle. However, Pfs230 is a very large and complex protein of 3,135 amino acids having multiple domains and disulfide bonds. Previous studies used only for example the N-terminal domain of Pfs230 to demonstrate transmission-blocking activity [22, 23]. However, the expression of full-length Pfs230 remained a complex task that was addressed by this project partnership. Scientists at Ehime University have used the wheat germ system to identify suitable regions within Pfs230 to produce potent TBV antigens for further vaccine development. One of the fragments denoted as Pfs230C1 induced strong transmission-blocking antibodies in mice when it is formulated with Montanide ISA720, Alhydrogel, or SA-1, a novel adjuvant developed by Sumitomo Dainippon Pharma. The partners are also testing whether the immunity can be further augmented when the antigens are displayed as virus-like nanoparticles. New antigen candidates from this ongoing study will later be transferred to a scalable insect cell expression system for large-scale production. For future vaccine development of their lead antigen, the partners will select the most promising antigen configuration and adjuvants formulation, to seek additional funding to initiate manufacture development and IND-enabling studies on a Pfs230-based TBV. It is hoped that the

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Pfs230-based TBV will support elimination of *P. falciparum* which could be responsible for about half of all malaria cases worldwide.

<https://www.ghitfund.org/investment/portfoliodetail/detail/102>

Conclusion

We are indebted to the GHIT Fund for its important contributions to address crucial medical needs in developing countries and encouraging more Japanese entities to participate in such projects. For CFS it is an important part of our contribution to society to participate in projects related to neglected diseases and to support global health. It is satisfying to see that our technology proved useful in the projects described here to drive the development of new malaria vaccines and diagnostic tests.

You can find more information on each of the projects described in here on the GHIT homepage under the links given in the text. Visit the GHIT homepage at <https://www.ghitfund.org> to learn more about their important work, supported projects and new funding opportunities.

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