Protective efficacy of a IL-12-expressing baculoviral malaria vaccine

Mitsuhiro Iyori[1], Andrew M. Blagborough[2], Katarzyna A. Sala[2], Hidesato Nishiura[1]

Kentaro Takagi[1] and Shigeto Yoshida[1]

[1]Laboratory of Vaccinology and Applied Immunology, Kanazawa University School of Pharmacy, Kanazawa, Japan

[2]Department of Life Sciences, Imperial College London, London, UK

*Correspondence should be addressed to S.Y.

Laboratory of Vaccinology and Applied Immunology, Kanazawa University School of Pharmacy. First Natural Science Building 1A219, Kakuma-machi, Kanazawa 920-1192, Japan. Tel: +81-76-234-4463 Fax: +81-76-234-4464 E-mail: shigeto@p.kanazawa-u.ac.jp

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Conflict of Interest Statement

S.Y. is an inventor listed on the international patent application WO/2007/091624 entitled "Novel Viral Vector." This does not alter the authors' adherence to all of the journal's

policies on the sharing of data and materials. All other authors declare no conflict of interest.

Abstract

Interleukin-12 (IL-12) plays an important role in antigen-specific adaptive immunity against malaria sporozoites, and this requirement allows for a new approach to developing an effective malaria vaccine. In the present study, we examined whether IL-12 could enhance protective efficacy of a baculovirus-based malaria vaccine. For this aim, a baculoviral vector expressing murine IL-12 (mIL-12) under the control of CMV promoter (BES-mIL-12-Spider) and a baculoviral vector expressing *Plasmodium falciparum* circumsporozoite protein (PfCSP) with post-transcriptional regulatory element of woodchuck hepatitis virus (BDES-sPfCSP2-WPRE-Spider) were generated. BES-mIL-12-Spider produced bioactive IL-12 which activates splenocytes, resulting in induction of IFN-γ. When co-immunized with BES-mIL-12-Spider and BDES-sPfCSP2-WPRE-Spider, the mouse number for high IgG2a/IgG1 ratios as well as the geometric mean in this group were both increased as compared with those of the other groups, indicating a shift toward a Th1-type response following immunization with BES-mIL-12-Spider. Finally, co-immunization of BES-mIL-12-Spider with BDES-sPfCSP2-WPRE-Spider had a higher protective efficacy (70%) than immunization with BDES-sPfCSP2-WPRE-

Spider alone (21%) against challenge with transgenic *Plasmodium berghei* sporozoites expressing PfCSP. These results suggest that co-administration of IL-12 expressing baculoviral vector, instead of IL-12 plasmid DNA, with viral-vectored vaccines provides a new feasible vaccine platform to enhance Th1-type cellular immune responses against malaria parasites.

Keywords: Interleukin-12, Interferon-gamma, Baculoviridae, malaria vaccines, *Plasmodium falciparum*, PfCSP, Transgenic

Introduction

Baculovirus is widely used as a gene-delivery vector for vaccine antigens. Currently, several protein-based vaccines have been commercialized by using the baculovirus expression system (BES). Cervarix®, a human papillomavirus vaccine based on the BES platform, protects against cervical cancer; it was approved by the EMA in 2007 and by the US Food and Drug Administration (FDA) in 2009 (GlaxoSmithKline Biologicals) (1). Another BES-based vaccine, Provenge®, is used against human prostate cancer and was approved by the FDA in 2010 (Dendreon Corporation) (1). Additionally, Protein Sciences Corporation produces the BES-based vaccine, FluBlock™, which is an influenza vaccine with a recombinant hemagglutinin of influenza virus subtypes A and B that was approved by the FDA in 2013 (1). Thus, the BES platform has powerful proven potential for developing various subunit vaccines. We previously developed a baculovirus dual expression system (BDES) that drives malaria antigen expression by a dual promoter consisting of baculovirus-derived polyhedrin and mammal-derived cytomegalovirus (CMV) promoters (2-10). This system can induce strong anti-malarial immunity in animal models and has been shown to be an effective malaria vaccine platform for

targeting all parasite life stages (2-10). Recently, we successfully developed an enhanced "BDES-Spider" vaccine platform, which expresses a decay-accelerating factor on the surface of the viral envelope, resulting in a higher transduction efficacy in the presence of complement both *in vitro* and *in vivo* [submitted manuscript].

The sequence for the post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) is a strong cis-acting RNA element located in the viral 3′ untranslated region (3′ UTR). WPRE improves gene expression at the post-transcriptional level by modifying RNA polyadenylation (11). Insertion of the WPRE sequence into the 3′ UTR of transgenes in an influenza DNA vaccine induced strong antigen expression in host cells, followed by a high level of protective immunity (12). The WPRE also significantly boosted baculovirus-mediated transgene expression in mammalian cells (13, 14). Thus, modification of the BDES vaccine with the WPRE sequence may be a viable option for developing novel malaria vaccines with improved expression of antigen, and subsequently, efficacy. To this end, we modified a BDES-based *Plasmodium falciparum* circumsporozoite protein (PfCSP) vaccine with the insertion of the WPRE sequence, producing a new vaccine candidate called BDES-sPfCSP2-WPRE-Spider.

Interleukin (IL)-12 is a heterodimeric pro-inflammatory cytokine that induces proliferation, enhancement of cytotoxicity and of cytotoxic mediator expression, and the production of cytokines, particularly interferon-γ (IFN-γ) (15). In the protective immune response against malaria, IL-12 is required for antigen-specific adaptive immunity against malaria sporozoites (16). Furthermore, IL-12 has an important role in the protection mediated by immunization with radiation-attenuated parasites, where it activates effector/memory $CD8^+$ T cells through their induction from conventional $CD8\alpha^+$ dendritic cells in the liver (17). We have also demonstrated that local immunization into the liver via gene gun with malaria DNA vaccines containing the IL-12 gene is effective at conferring protective immune responses in a mouse model (18). Therefore, incorporating IL-12 to the BES platform may be another new approach for developing an more effective malaria vaccine.

Here, we constructed a viral-vectored vaccine expressing murine IL-12 (mIL-12) based on a BES platform, called BES-mIL-12-Spider, and assessed the ability of this vaccine to produce bioactive IL-12 and to induce IFN-γ secretion by murine splenocytes. We also used the newly developed BDES-sPfCSP2-WPRE-Spider vaccine together with

the BES-mIL-12-Spider vector to co-immunize mice, and we conducted challenge infection studies using a transgenic rodent malaria parasite expressing native PfCSP in place of its murine homolog to evaluate protective efficacy. Finally, we examined how the humoral immune response was modified by the BES-mIL-12-Spider vector.

Materials and Methods

Vaccine construction and functional analysis

For the construction of a gene cassette expressing the sPfCSP2-VSV-G TM fusion protein under dual promoter control with the WPRE sequence, the *wpre* sequence was synthesized by Genscript (Piscataway, NJ, USA); it included a *Hind* III site at the 5′-flanking region, a *wpre* sequence (Accession number: M18752; range: 1093–1681), and an *Xho* I site at the 3′-flanking region. The vector was digested with *Hind* III and *Xho* I and then ligated to a *Hind* III/*Xho* I-cut pENTR-D-sPfCSP2-G2 vector (pENTR-D-sPfCSP2-G2-WPRE). The pENTR-D-sPfCSP2-G2 and pENTR-D-sPfCSP2-G2-WPRE vectors were used as donor vectors for Gateway® Cloning. For the construction of the pENTR-CMV-sSP163-mIL-12-EcoRI vector, the mIL-12 p40 and p35 single-chain genes were amplified from the pcDNA-mIL12p40p35 parent vector (19) by performing PCR with the pmIL12p40-F1 forward primer and the pEntry-R1 reverse primer, and the product was then ligated into a pENTR-TOPO vector. The resulting vector, pENTR-mIL12-EcoRI, was digested with *Not* I and *Eco* RI, and the resulting fragment of a *Not* I*/Eco* RI-cut pENTR-CMV-sSP163 vector was inserted into the pENTR-mIL12-EcoRI

vector. This vector, pENTR-CMV-sSP163-mIL-12-EcoRI, was used as a donor vector for Gateway® Cloning. For the baculovirus transfer vector generation and subsequent production of recombinant viral vaccines, pFast-polh-EGFP-p10-hDAF-G-Piggy-D-sPfCSP2(R) (pFast-sPfCSP2-Spider), pFast-polh-EGFP-p10-hDAF-G-Piggy-D-sPfCSP2-WPRE(R) (pFast-sPfCSP2-WPRE-Spider), pFast-GL3-Spider, and pFast-mIL12-Spider were generated by LR clonase reactions using LR clonase II (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) with the above donor vectors and destination vectors according to the manufacturer's instructions. Recombinant bacmids were generated by Tn7-mediated transposition of the gene cassettes in pFast vectors (pFast-sPfCSP2-Spider, pFast-sPfCSP2-WPRE-Spider, pFast-GL3-Spider, and pFast-mIL-12-Spider) using the Bac-to-Bac system (Thermo Fisher Scientific) according to the manufacturer's instructions. Amplification and purification of the baculoviruses were performed as described previously (8, 9).

Immunoblotting

HEK293A cells were transfected with 500 ng of pFast-D-sPfCSP2-Spider or pFast-D-sPfCSP2-WPRE-Spider. After 48 h, the cells were lysed with loading buffer containing 2% 2-mercaptoethanol, boiled for 5 min, and then subjected to 12% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes, and then probed with either an anti-PfCSP mouse mAb (2A10). Blots probed with an anti-mouse IgG secondary Ab conjugated to IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA) were visualized using an Odyssey infrared imager (LI-COR, Lincoln, NE). An anti-GAPDH mouse mAb (5A12, Wako, Osaka, Japan) was used as a loading control to the same membrane.

Indirect fluorescent assay

For indirect fluorescent assays, HEK293A cells (10^4 cells/well) were transduced with purified baculoviruses at multiplicities of infection (MOIs) of 100. After incubation with the viruses for 48 h, the cells were fixed and permeabilized by using IC Fixation Buffer and Permeabilization Buffer (eBioscience, San Diego, CA, USA) and then stained with

Alexa Fluor 488-conjugated anti-PfCSP monoclonal antibody (mAb) (2A10) and biotinylated anti-mIL-12 p40 mAb (C17.8; Biolegend, San Diego, CA, USA) followed by staining with streptavidin conjugated to allophycocyanin (Thermo Fisher Scientific). The eight-well chambers were mounted with a drop of Vectashield containing 4,6′-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). An LSM710 inverted laser scanning microscope (Carl Zeiss, Tokyo, Japan) with 20× objectives was used for image acquisition.

Enzyme-linked immunosorbent assay (ELISA)

For the detection of bioactive mIL-12, HepG2 cells (2.5×10^5 cells/well) in a 24-well plate were transduced with baculoviruses at MOIs of 100 or transfected with pFast-mIL-12-Spider formulated by Lipofectamine LTX. After 24 h, the cell supernatants were collected, and their concentrations of mIL-12 p40 were assessed using a Mouse IL-12/IL-23 (p40) ELISA MAX™ standard kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. Meanwhile, splenocytes were dissected and purified from a

normal BALB/c mouse, and the cells (5×10^5 cells/well) were incubated with the above supernatants in a 96-well plate for 24 h. The concentration of murine IFN-γ in each of the supernatants was assessed by an IFN-γ ELISA MAX™ Standard kit (Biolegend) according to the manufacturer's instructions.

For evaluation of the anti-PfCSP antibody (Ab) response, sera from immunized mice were collected from tail blood samples taken 2 weeks after the last immunization. PfCSP-specific Ab levels were quantified by ELISA. EIA/RIA plates (Corning Inc.; Corning, NY, USA) pre-coated with 0.4 μg/well of *Escherichia coli*-produced rPfCSP were blocked with 1% bovine serum albumin in PBS and incubated with serial dilutions of sera from the immunized or control mice. Titers of the total IgG, IgG1, IgG2a, and IgG2b specific for the above antigens were detected using horseradish peroxidase (HRP)-conjugated anti-mouse IgGs as described previously (8). Endpoint titers were expressed as the reciprocal of the last dilution that gave an optical density at 414 nm of 0.15 U above the values of the negative controls (< 0.1). All mice used were seronegative prior to immunization.

PfCSP-Tc/Pb sporozoite challenge

Balb/c mice were immunized intramuscularly two or three times at 3-week intervals with 10^8 pfu of the viral vectored vaccines. The method used for sporozoite challenge infections, which involved infected mosquito bites, was performed as described previously (8). Briefly, *Anopheles stephensi* mosquitoes were infected with PfCSP-Tc/Pb, which are transgenic *Plasmodium berghei* parasites expressing PfCSP instead of *P. berghei* CSP. Starved infected mosquitoes were allowed to feed on the abdomen of each mouse for 15 minutes. All mosquitoes were subsequently examined by dissection midguts and salivary glands post-bite. Mosquitoes that had a blood spot in the midgut were deemed to have fed, whereas presence of sporozoites in the salivary glands was used to identify infected mosquitoes. All animal care and handling procedures were approved by the Animal Care and Use Committee of Kanazawa University (No. 22118-1). In the UK, all procedures were performed in accordance with the UK Animals (Scientific Procedures) Act (PPL 70/8788) and approved by Imperial College AWERB. All efforts were made to minimize suffering in the animals.

Statistical analyses

A two-tailed Fisher's exact probability test was performed to determine statistical differences in the protective efficacies of the vaccines using SPSS software (version 19, Chicago, IL, USA). All other statistical analyses, such as the Mann-Whitney U-test for two-groups comparison and the Kruskal-Wallis test with Dunn's correction for multiple comparison, were performed using Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Aiming to enhance PfCSP expression in transduced cells, we introduced the WPRE sequence after the PfCSP-VSV-G open reading frame in a transfer vector of BDES, pFast-D-sPfCSP2-Spider (Fig. 1A). This sequence is a noncoding RNA sequence that acts to increase mRNA stability and its extranuclear transport to the cytoplasm (12). The resulting plasmid, pFast-D-sPfCSP2-WPRE-Spider, induced PfCSP expression levels in COS-7 cells that were 3.3-fold higher than those induced by the WPRE sequence-lacking construct (Fig. 1B, 1C).

To add mIL-12 expression to the BES vaccine, we constructed a baculovirus expressing a single chain fragment of mIL-12 p35 and p40 under the control of a CMV promoter, named BES-mIL-12-Spider (Fig. 1A). Transduction studies with BDES-sPfCSP2-WPRE-Spider and BES-mIL-12-Spider revealed that HEK293A cells were able to express PfCSP and mIL-12, respectively (Fig. 1D). The PfCSP was distributed in not only the cytoplasm but also the cell membrane, while mIL-12 was detected only in the cytoplasm (Fig. 1D). HepG2 cells transduced with BES-mIL-12-Spider expressed and secreted mIL-12 in the supernatant, as did those transduced with a mIL-12-expressing

plasmid; however, mIL-12 was not detected in either the cells transduced with BDES-sPfCSP2-WPRE-Spider or the control cells (Fig. 2A). The mIL-12-containing supernatants were able to induce interferon (IFN)-γ expression in murine splenocytes (Fig. 2B), indicating that the secreted mIL-12 was bioactive.

Two weeks after a three-dose immunization with BDES-sPfCSP2-Spider and/or BES-mIL-12-Spider, or BDES-sPfCSP2-WPRE-Spider, the mice were challenged via natural feeding by PfCSP-Tc/Pb-infected mosquitoes. Sterile protection was determined by the complete absence of infection at 14 days post-infection. Significant protective efficacy was observed in the mice immunized with BDES-sPfCSP2-Spider plus BES-mIL-12-Spider (80%) but not in those immunized with BDES-sPfCSP2-WPRE-Spider alone (40%) or BDES-sPfCSP2-Spider alone (10%), compared with the control mice (Table 1). We next performed a two-dose immunization with BDES-sPfCSP2-WPRE-Spider vaccine in the presence or absence of BES-mIL-12-Spider. The challenge infection study showed significant protective efficacy in the mice immunized with BDES-sPfCSP2-WPRE-Spider plus BES-mIL-12-Spider (70%) or with BES-mIL-12-Spider (50%) but not in those immunized with BDES-sPfCSP2-WPRE-Spider alone (21%) (Table 2).

These results indicate that the BES-mIL-12-Spider vector can enhance the BDES vaccine-mediated protective immunity through a synergistic effect via the induction of IL-12.

The sera of the immunized mice described in Table 1 were collected just before infection to examine the Ab responses against PfCSP and to determine if BES-mIL-12-Spider modulated the Ab response. The geometric mean of total Ab endpoint titers after the last immunization with BDES-sPfCSP2-WPRE-Spider alone (137,795) or with BDES-sPfCSP2-Spider plus BES-mIL-12-Spider (115,367) was each markedly, but not significantly, higher than that following immunization with BDES-sPfCSP2-Spider alone (81,317) (Fig. 2A). In all groups, immunization with BDES vaccines elicited not only IgG1 Ab responses but also IgG2a and IgG2b Ab responses (Fig. 2B–2D). The mouse number for high IgG2a/IgG1 ratios (>1) in the group of BDES-sPfCSP2-Spider plus BES-mIL-12-Spider as well as the geometric mean in this group were both increased as compared with those of the other groups (Fig. 2E), indicating a shift toward a Th1-type response following immunization with BES-mIL-12-Spider. However, individual mice

mice with high IgG2a/IgG1 ratios did not directly correlate with high levels of protective efficacy within these experiments (Fig. 3).

Discussion

IL-12 activates natural killer (NK) cells to produce IFN-γ and induces the differentiation of Th1 cells from naïve T cells (20). Th1 and Th2 cells are each able to induce Ab responses against vaccine antigens, but the resulting immunoglobulin subclasses differ. In mice, Th2-type $CD4^+$ T cell activation results in IgG1 production, whereas IgG2a induction requires Th1 cell activation (21). Our vaccine construct, BES-mIL-12-Spider, successfully expressed a bioactive mIL-12 *in vitro*, and immunization with the combination of BES-mIL-12-Spider and the malaria sporozoite vaccine BDES-sPfCSP2-Spider modified the Ab response against PfCSP toward the induction of higher IgG2a/IgG1 ratio in mice.

Baculoviruses are known to activate bone marrow-derived dendritic cells (DCs) to induce the expression of activated cell-surface markers and to produce various kinds of cytokines (IL-6, IL-12p70, and TNF-α) (22). Baculovirus-activated DCs also induce IFN-γ production from NK cells and T cells (22). A recent study reported that while wildtype mice could produce IFN-γ and experienced protection against *Plasmodium yoelii* after a single immunization with irradiated sporozoites, IL-12p40$^{-/-}$ mice were

neither able to produce IFN-γ nor were protected by this immunization protocol (23). We observed that BES-mIL-12-Spider could induce IL-12 expression *in vitro*; the resulting IL-12 may, directly or through the induction of IFN-γ, result in Th1 cell activation and the subsequent production of Th1-associated subclasses of IgG, such as IgG2a, in mice.

This study shows that the induction of recombinant IL-12 elicited effective (and enhanced) protective immunity within a anti-sporozoite vaccine against transgenic rodent malaria parasites expressing the CSP of *P. falciparum*. This finding is in agreement with previous mouse model work demonstrating that IL-12 plays important roles in irradiated sporozoite-induced protective immunity against rodent malaria (16). We found that co-immunization with BDES-sPfCSP2-Spider and BES-mIL-12-Spider resulted in a significant improvement (80% protection) over immunization with BDES-sPfCSP2-Spider alone ($p = 0.005$ using a χ^2 test). Our recent studies showed that $CD8^+$ memory T cells against PfCSP were not induced in mice by co-immunization with BDES and BES-mIL-12-Spider (data not shown), and the current study indicates that the Ab responses were not correlated with protection (Fig. 3); both of these findings are consistent with previous work (8). As the Abs raised by BDES vaccines were able to effectively inhibit a

sporozoite invasion by transgenic *P. berghei* (8), these Abs might help neutralize the parasites, but the precise mechanism of this protection, in terms of the mode of action, remains unclear.

Currently, the immune effectors for protective pre-erythrocytic immunity remain poorly understood, but one possible mechanism relies on Th1 immune response. In *P. berghei* and *P. yoelii* models, Th1 responses induced by vaccination with sporozoites have been shown to confer strong protection against malaria challenge (24, 25). In monkey models, IFN-γ-secreting $CD4^+$ T cells play an important role in the protective immunity against malaria infection (26). In regards to the RTS,S vaccine, Th1 cytokine-expressing $CD4^+$ T cells were induced by the immunization, which was associated with protection against malaria infection in a human challenge model (27). Thus, the induction of Th1 responses by co-immunization with BES-mIL-12-Spider may allow modification of the vaccine-induced protective immune response during immunization.

In summary, the novel vaccine vector BES-mIL-12-Spider has strong immunomodulatory properties that enhance the vaccine-induced protective immunity against malaria. These findings introduce a new vaccine concept for designing viral-

vectored vaccines against malaria. Further investigation will be required to examine and overcome issues related to potentialassociated side effects, such as autoimmune responses.

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Figure legends

Fig. 1. Schematic representation of BDES constructs and the expression of proteins. (A) Schematic representation of the BDES constructs. BDES-sPfCSP2-WPRE-Spider: The post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) was positioned after the *pfcsp* gene to enhance its expression. BES-mIL-12-Spider: Mouse *il-12 p35* and *il-12 p40* genes were cloned in the BES vector. (B) Western blotting analysis of HEK293A cells transfected with pFast-D-sPfCSP2-Spider (WPRE (−)) or pFast-D-sPfCSP2-WPRE-Spider (WPRE (+)). (C) The antigen signals (anti-FLAG Ab) from the blot shown in (B) were normalized with those of anti-GAPDH Ab. Means ± S.E.M are shown (N = 6, from two independent studies). Comparison between groups was assessed by the Mann–Whitney *U*-test. $^{**}p < 0.01$, compared with WPRE (−). (D) HEK293A cells were transduced with BDES-sPfCSP2-WPRE-Spider (WPRE) and/or BES-mIL-12-Spider (mIL-12) at the indicated MOI for 24 h, and then permeabilized cells were intracellularly stained with anti-PfCSP mAb (green) and anti-mIL-12 mAb (red). Bars, 100 μm. Nucleic acids are visualized with DAPI (blue).

Fig. 2. Assessment of IL-12 expression and bioactivity. (A) HepG2 cells were transduced with either BDES-sPfCSP2-WPRE-Spider or BES-mIL-12-Spider or were transfected with a plasmid encoding the *mil-12* gene (plasmid). After 24 h, the mIL-12 p40 concentration in each of the supernatants was analyzed by ELISA. (B) The supernatants of the samples described in (A) were incubated with naïve splenocytes for 24 h. Concentrations of secreted IFN-γ are shown (n = 3). Between-group differences

were assessed by the Kruskal–Wallis test with Dunn's correction for multiple comparisons. $^{**}p < 0.01$, compared with naïve splenocytes (PBS).

Fig. 3. Subclass analysis of anti-PfCSP humoral immune responses. The sera of mice immunized with BDES-sPfCSP2-Spider (Spider), BDES-sPfCSP2-WPRE-Spider (WPRE), or BDES-sPfCSP2-Spider plus BES-mIL-12-Spider (Spider + IL-12) were collected just before the challenge infection study and examined for their Ab responses against PfCSP by ELISA (n = 10). Plots of the total IgG Ab titer (A), IgG2a Ab titer (B), IgG2a Ab titer (C), IgG2b Ab titer (D), and IgG2a/IgG1ratio (E) are shown. Bars and dots indicate the geometric mean and individual values, respectively. White and black symbols represent the protected and non-protected mice, respectively.