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Short Communication

Anti-MSP11 IgG inhibits *Plasmodium falciparum* merozoite invasion into erythrocytes *in vitro*

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ABSTRACT

Merozoite surface proteins (MSPs) are considered as promising blood-stage malaria vaccine candidates. MSP3 has long been evaluated for its vaccine candidacy, however, the candidacy of other members of MSP3 family is insufficiently characterized. Here, we investigated *Plasmodium falciparum* MSP11 (PF3D7_1036000), a member of the MSP3 family, for its potential as a blood-stage vaccine candidate. The full-length protein (MSP11-FL) as well as the N-terminal half-MSP11 (MSP11-N), known to be unique among the MSP3 family members, were expressed by wheat germ cell-free system, and used to raise antibodies in rabbit. Immunoblot analysis of schizont lysates probed with anti-MSP11-N antibodies detected double bands at approximately 40 and 60 kDa, consistent with the previous report thus confirming antibodies specificity. However, inconsistent with previously reported merozoite's surface localization, immunofluorescence assay (IFA) revealed that MSP11 likely localizes to rhoptry neck of merozoites in mature schizonts. After invasion, MSP11 localized to parasitophorous vacuole and thereafter in Maurer's clefts in trophozoites. Anti-MSP11-FL antibody levels were significantly higher in asymptomatic than symptomatic *P. falciparum* cases in malaria low endemic Thailand. This reconfirmed that anti-MSP11 antibodies play an important role in protection against clinical malaria, as previously reported. Furthermore, in vitro growth inhibition assay revealed that anti-MSP11-FL rabbit antibodies biologically function by inhibiting merozoite invasion of erythrocytes. These findings further support the vaccine candidacy of MSP11.

Antibodies are key effectors of protective immunity against *Plasmodium falciparum* malaria as evidenced by passive immunization experiments in which total IgGs from malaria-immune adults were used to treat patients with severe malaria [1]. These findings provide a rationale that an effective asexual blood-stage malaria vaccine is achievable by inducing humoral immune response. Since cytophilic subclasses of human antibodies against MSP3 were observed to significantly associate with reduced risk of clinical malaria [2], MSP3 has been considered a promising blood-stage vaccine candidate. However, a recent phase 2b clinical trial of MSP3-based malaria vaccine in combination with Glutamate-Rich Protein (GLURP), resulted in low vaccine efficacy (14%) [3]. Nonetheless, an additional member of MSP3 multigene family, which consists of MSP3, MSP6, MSP11, MSP3.3, MSPDBL1 and MSPDBL2, was identified as a target of naturally occurring antibodies in Papua New Guinea and Senegal [4,5]. We also recently reported that elevated antibody levels to MSP3 and MSPDBL1 in sera of residents of a malaria hyper-endemic village in Uganda associated with clinical protection [6]. This clearly implies that further evaluation of the other MSP3 family members for their candidacy as novel blood-stage vaccines is a logical priority.

Since MSP3 family harbors a highly conserved C-terminal secreted polymorphic antigen associated with merozoites (SPAM) domain,

Abbreviations: ADCI, antibody dependent cellular inhibition; GIA, growth inhibition activity; WGCFS, wheat germ cell-free system

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antibodies against one member have been shown to be cross-reactive across the family. Importantly, these "cross-reactive" human antibodies inhibited parasite growth in an in vitro antibody dependent cellular inhibition (ADCI) assay [5]. However, it remains unclear whether antibodies specific to individual MSP3 family members contributed to the ADCI activities. We then reported that anti-MSPDBL1 rabbit antibodies raised against recombinant full-length MSPDBL1 inhibited erythrocyte invasion without cross-reactivity to the other five MSP3 family members [7], demonstrating that anti-MSPDBL1 antibody alone has an invasion inhibitory effect independent of cellular components. However, identification of individual vaccine candidacy not by the "cross-reactive" effects of the other MSP3 family members still remains elusive. Recently, a combination of human IgG3 antibody responses against MSP11 (PF3D7_1036000) with three other MSPs was reported to have strong association with protection against clinical malaria [8]. In addition, erythrocyte binding MSP11 peptides inhibit merozoite invasion in vitro [9]. These characteristics of MSP11 prompted us to conduct this study to further investigate MSP11 as a potential blood-stage vaccine candidate.

To characterize MSP11, we generated full-length and N-terminal recombinant proteins by wheat germ cell-free system (WGCFS) (CellFree Sciences, Matsuyama, Japan) [10], for antibody production and other immunoassays. N-terminal region of MSP11 is known to be unique among the MSP3 family members. The GST-fused full-length MSP11 (MSP11-GST-FL) expression product (Fig. 1A lane 1: arrowhead) and AcTEV protease (Invitrogen, Carlsbad, CA, USA) purified proteins (MSP11-FL) (lane 2: arrow) were resolved on an SDS-PAGE as indicated. C-terminal his-tagged MSP11-FL-His and MSP11-N-His (MSP11-N) proteins were also synthesized and purified using an Ni-Sepharose column (GE Healthcare, Camarillo, CA). Purified MSP11-FL-His and MSP11-N proteins resolved at the expected molecular weights (Fig. 1A; dashed arrows in lane 3 and 4 respectively). To generate MSP11 antisera, 250 μg of purified recombinant MSP11-FL (Fig. 1A lane 2) or MSP11-N (Fig. 1A lane 4) was used with Freund's complete adjuvant to subcutaneously immunize and raise antibodies in Japanese white rabbit (Kitayama Labes, Ina, Japan) as previously described [15]. The animal work was conducted by Kitayama Labes in compliance with the guidelines based on "Charter for Laboratory Animal Welfare" (Japanese Society for Laboratory Animal Resources).

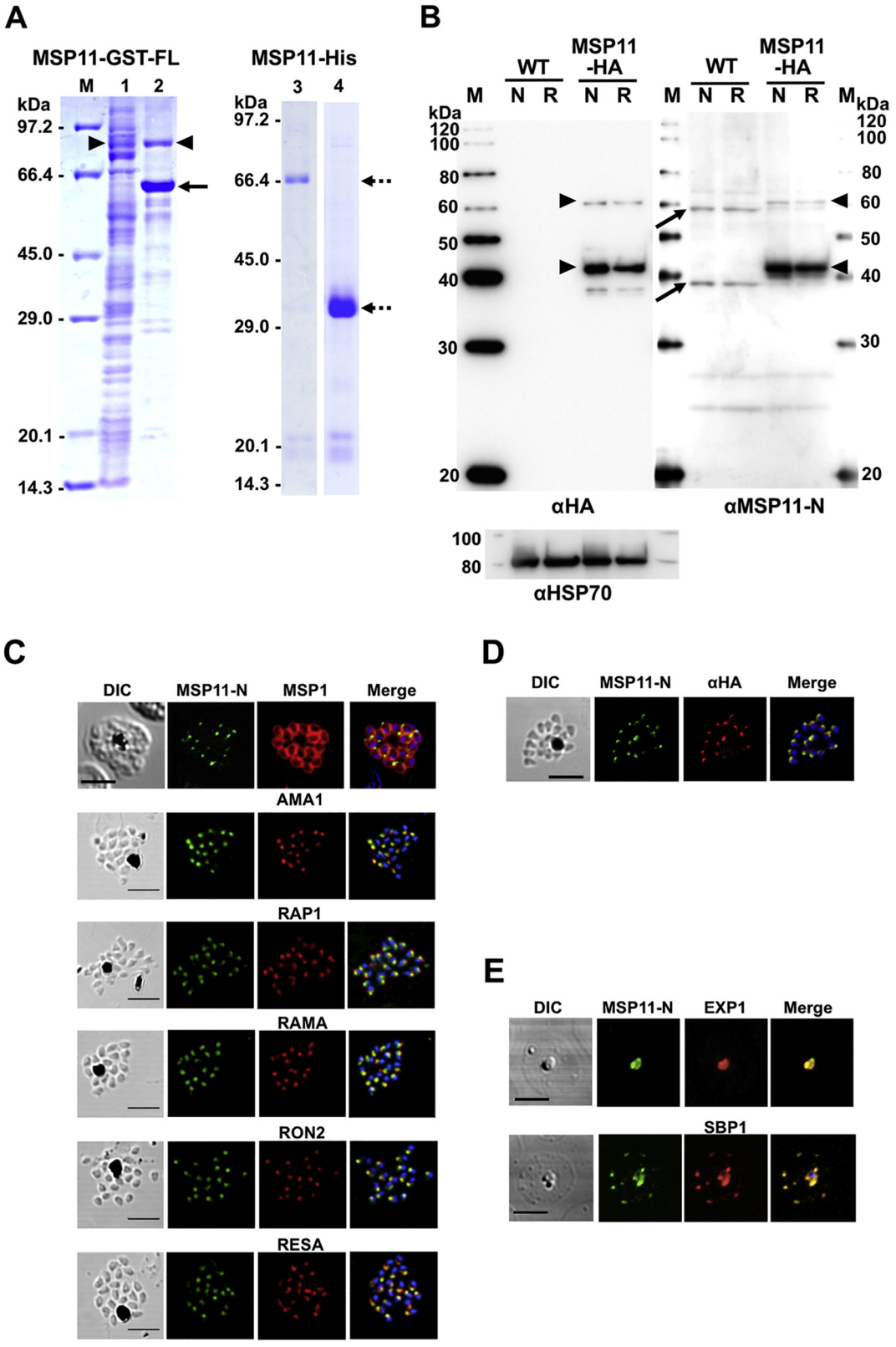
The reactivity and specificity of anti-MSP11-N polyclonal antibodies was examined by Western blot analysis (Fig. 1B) using approximately 10^6 schizont-rich *P. falciparum* 3D7 (WT) parasite extracts per lane, under non-reducing (lane N) and reducing (lane R) conditions. Double bands at approximately 40 and 60 kDa (arrows) were detected consistent with the bands of previously reported MSP11 [4] (Fig.1B right panel, WT lanes N and R). To further confirm the specific reactivities of the anti-MSP11-N antibodies, we used a transgenic parasites harboring C-terminal 3 × HA-tagged MSP11 by a single crossover recombination [11]. The transgenic parasites harbored plasmid expressing human dihydrofolate reductase allowing their maintenance under WR99210 drug pressure. Immunoblot analysis of the MSP11-HA parasite extracts using both anti-HA rat monoclonal antibody (mAb; clone 3F10, Roche, Mannheim, Germany) (Fig. 1B left panel, MSP11-HA) and anti-MSP11-N antibodies (Fig. 1B right panel, MSP11-HA) revealed double bands of MSP11 (arrowheads) at slightly higher molecular weight in agreement with the size of 3 × HA-tag used. These results indicated that the rabbit anti-MSP11-N antibodies specifically recognized MSP11.

In order to reconfirm the surface localization of the MSP11 in merozoites [4,5], immunofluorescent assay (IFA) was performed on mature schizonts. However, contrary to the previous reports of merozoite surface localization of MSP11 [4,5], the fluorescent signals of anti-MSP11-N were detected as a punctate pattern in each merozoite (Fig. 1C) suggesting MSP11 localization in apical organelles, such as microneme, rhoptry, and dense granule. To confirm this, we assessed MSP11 in mature schizont of MSP11-HA transgenic parasites that were double stained with anti-MSP11-N and anti-HA. Fig. 1D clearly indicated a complete overlap of the punctate staining patterns with both antibodies, confirming that the punctate localization in each merozoite is the MSP11. To determine in which apical organelle the MSP11 localized, double labelling IFA was performed using each apical organelle specific antibodies and anti-MSP11-N antibodies. As shown in Fig. 1C, MSP11-N signals closely overlapped with those of anti-RON2 (Fig. 1C, 5th panels) than other apical organelle markers. We calculated the co-localization intensity correlation by Pearson's correlation coefficients (r) between signals of MSP11-N and those of other apical organelle markers by using Zen 2010 software (Carl Zeiss MicroImaging). MSP11-N showed tighter co-localization with RON2 (r = 0.90) than with AMA1 (r = 0.75), RAP1 (r = 0.71), RAMA (r = 0.73) and RESA (r = 0.77). Using immunoelectron microscopy (IEM) we attempted to investigate the precise localization of MSP11. However, even after multiple attempts, we could not obtain clear IEM images (data not shown). Nonetheless, signals of anti-MSP11-N in ring stage parasites overlapped with those of anti-exported protein 1 (EXP1); a parasitophorous vacuole marker (Fig. 1E, upper panels). In trophozoite stage, MSP11 was localized in the infected erythrocyte cytoplasm as patchy pattern that overlapped SBP1 signals; a Maurer's clefts marker (Fig. 1E, lower panels) [12]. Rabbit pre-immune serum didn't show any signal on both WT and MSP11-HA parasites (data not shown). These findings suggest that MSP11 is stored in rhoptry neck of merozoites prior to egress from erythrocytes, is translocated to the parasitophorous vacuole after merozoites invasion of erythrocytes, and thereafter exported to the Maurer's clefts. Because there is no *Plasmodium* export element (PEXEL) motif found in the sequence, our results suggest that MSP11 is a novel PEXEL-negative exported protein [13].

The merozoite surface localization of MSP11 was first reported by Pearce et al. [4] using rabbit antibodies raised against full-length MSP11 ([aa] 25–405) recombinant protein expressed using an *Escherichia coli* system. The merozoite surface localization of the MSP11 in that report may have been due to cross-reactivity of the antibody with other MSP3 family members. Singh et al. [5] also reported the merozoite surface localization of MSP11 using human antibodies that were affinity purified using E. coli-produced recombinant protein specific to the unique region of MSP11 ([aa] 60–122). Based on the IFA images presented in the Singh et al. [5], we could not make clear assessment whether the surface localization is merozoite specific or not because there was no bright field image provided. Moreover, Western blot data shown as single band around 40 kDa [5] was inconsistent to our results (Fig. 1B) and that of Pearce et al. [4]. Therefore, the definitive apical organellar localization of MSP11 in merozoite awaits further investigation by IEM.

In natural *P. falciparum* infection, MSP11 is a target of naturally acquired protective antibodies in residents of malaria hyper-endemic areas [4,5]. Here, we set to offer preliminary insight into whether full-length MSP11 is similarly a target of protective immunity against malaria even in low transmission areas such as South East Asia. To investigate this, human serum samples from Thailand were obtained from asymptomatic *P. falciparum* carriers (Asy: n = 17), symptomatic *P. falciparum* malaria patients (Sym: n = 22), and malaria naïve individuals (Nor: n = 10) as described in our study [14]. Ethics statements of the study was described [14]. Measurements of antibodies by enzyme-linked immunosorbent assays (ELISAs) against C-terminal His-tagged recombinant MSP11-FL (Fig. 1A, lane 3) in the serum samples were performed as described [15]. Sera of Asy showed significantly higher reactivity to MSP11-FL than those of Sym ($P < 0.01$, Kruskal-Wallis test followed by Dunn's multiple comparisons test) and Nor ($P < 0.001$). However, there was no difference between Sym and Nor groups (Fig. 2A). Recently, Kana et al. [8] reported that a combination of IgG3 antibody responses against MSP11 and three MSPs (Pf12, MSP3.3, MSP2) was strongly associated with protection against clinical malaria. These results suggest that the MSP11 is a potential target of protective humoral immunity.

To assess the potential mode of action of anti-MSP11-FL, we



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evaluated the in vitro growth inhibition activity (GIA) of rabbit anti-MSP11-FL [15,16]. When tested at a final concentration of 20, 10, and 5 mg/ml, anti-MSP11-FL antibodies inhibited invasion by 20.4% ± 2.1%, 12.4% ± 1.9%, and 7.2% ± 2.6%, respectively (mean ± SEM; Fig. 2B). Anti-EBA175_R3-5 (PF3D7_0731500) (47.8% ± 3.6%) and anti-GST (7.1% ± 1.8%) rabbit antibodies at a final concentration of 20 mg/ml served as positive and negative controls, respectively. Anti-MSP11-FL antibodies GIA exhibited a dose dependent response and was significantly higher than that of the anti-GST antibody at 20 mg/ml (Kruskal-Wallis test with Dunn's multiple-

Fig. 1. (A) SDS-PAGE analysis of the proteins expressed by the wheat germ cell-free system. Lane labels represent: protein molecular weight marker (M), total reaction mixture of GST-fused MSP11-FL (lane 1), GST-fused MSP11-FL (lane 1: arrowhead), AcTEV protease purified MSP11-FL (lane 2: arrow), Affinity purified MSP11-FL-His (lane 3: dashed arrows) and MSP11-N-His (lane 4: dashed arrows). (B) Western blot analyses using antibodies against HA-tag or MSP11-N. Proteins were extracted under non-reducing (lane N) and reducing (lane R) conditions. Arrows represent MSP11 signals in WT parasite extracts. Arrowheads represent MSP11-HA signals in MSP11-HA parasite extracts. Bottom panel (αHSP70) was probed with anti-PfHSP70 monoclonal antibody as a quantitative parasite protein marker [17]. Protein molecular weight marker (lanes M). (C) Subcellular localization of MSP11 in schizonts by indirect immunofluorescence assay (IFA). Infected blood smears were stained with rabbit anti-MSP11-N antibodies and co-stained with mouse anti-$MSP1_{19}$ as merozoite surface marker [7], mouse anti-AMA1 as microneme marker [10], mouse anti-RAP1 as rhoptry body marker [17], mouse anti-RAMA as rhoptry body membrane marker [18], mouse anti-RON2 as rhoptry neck marker [19], or mouse anti-RESA mAb as dense granule marker [19]. DIC; differential interference contrast image. Merge; merged image including DAPI stained nucleus. Bar = 5 μm. (D) Subcellular localization of MSP11 in MSP11-HA transgenic *P. falciparum* schizont by IFA. MSP11-N; rabbit anti-MSP11-N polyclonal antibodies, αHA; rat anti-HA mAb (1:100) [20]. Bar = 5 μm. (E) Subcellular localization of MSP11 in ring (upper pictures) and trophozoite (lower pictures) stages by IFA. MSP11-N; rabbit anti-MSP11-N polyclonal antibodies, EXP1; mouse anti-EXP1 polyclonal antibodies, SBP1; mouse anti-SBP1 ([aa] 258–337) polyclonal antibodies. Bar = 5 μm. Additional details are described in Supplementary Materials and methods.

comparison post-hoc test, $P < 0.05$).

Since human antibodies against cross-reactive C-terminal SPAM domain of MSP11 had significant ADCI activity in vitro [5], we sought to determine the extent to which cross-reactive antibodies against other MSP3 members potentially affected the GIA of the rabbit anti-MSP11-FL antibodies (Fig. 2B). To this end, six recombinant SPAM domains of the MSP3 family members [5] were expressed and purified as described [7]. Western blot analyses of the rabbit anti-MSP11-FL antibody (Fig. 1A, lane 2) showed that strong reactivity against SPAM domain of MSP11 (Fig. 2C, MSP11) was observed followed by mild reactivity against MSP3.3 and MSPDBL1 (Fig. 2C). To quantify those reactivities, densitometry of the positive signals was performed by ImageJ 1.52a. The signal densities to MSP3.3 and MSPDBL1 showed a relatively low intensity of 5.5% and 0.9% respectively, when compared to that of MSP11 (Fig. 2C). We previously reported that anti-MSPDBL1 rabbit antibodies at a final concentration of 20 mg/ml had significant GIA, however, the GIA reduced to the negligible levels at 5 mg/ml or less concentrations [7], suggesting that the observed 0.9% relative reactivity against MSPDBL1 had negligible effect of the MSP11 GIA. In contrast, no cross-reactivities against the other antigens was detected (Fig. 2C). These evidences suggest that the GIA observed with anti-MSP11-FL mostly targeted the functions of MSP11 and negligibly targeting MSP3.3 and MSPDBL1, and that the GIA observed was majorly by antibodies specific to MSP11.

Overall, we identified that MSP11 of *P. falciparum* was localized not on the surface [4,5] but likely in the rhoptry neck of merozoites. After the invasion, MSP11 localized to parasitophorous vacuole and thereafter to the Maurer's clefts in mature parasite. Anti-MSP11 antibody levels were significantly higher in Asy than Sym in malaria low endemic Thailand, reconfirming that anti-MSP11 antibodies play an important role in protection against clinical malaria as reported in malaria high endemic area [5]. These findings indicate that MSP11 is immunogenic in humans, and anti-MSP11 antibodies may contribute, at least in part, to naturally acquired protective immunity. Furthermore, anti-MSP11-FL rabbit antibodies significantly inhibit in vitro parasite growth, independent of cellular components. More importantly, among MSP3 family members, anti-MSP11 IgG has specific but not "cross-reactive" GIA as similarly observed with MSPDBL1. Therefore, MSP11 is a potential blood-stage vaccine candidate antigen against falciparum malaria.

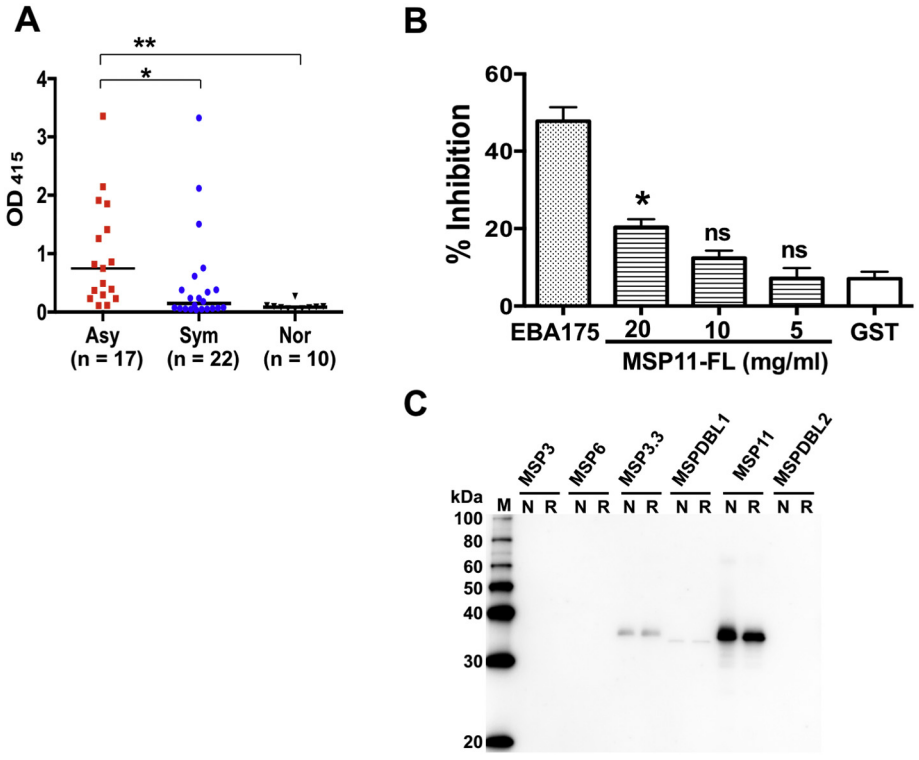

Fig. 2. (A) Human sera from malaria endemic area in Thailand recognize MSP11 in ELISA. Probing of MSP11-FL with sera from asymptomatic *P. falciparum* carriers (Asy), symptomatic *P. falciparum* patients (Sym), and malaria naïve individuals (Nor). The number (n) of samples analyzed is shown in parentheses. OD; optical density. Average OD values of two independent measurements are shown as scatter dot plots. Horizontal bar designates the median number of mean OD values in each group. P values were calculated by Kruskal-Wallis test followed by Dunn's multiple-comparison test. Statistical significance; *$P < 0.01$, **: $P < 0.001$. (B) Anti-MSP11-FL rabbit antibodies have invasion-inhibitory activity *in vitro*. The GIA of the anti-MSP11-FL antibodies was tested in a one-cycle GIA. Anti-EBA175 region 3–5 (EBA175) and anti-GST (GST) antibodies were used as positive and negative controls, respectively. The error bars represent the standard errors of the means of three independent experiments in triplicate. Kruskal-Wallis test followed by Dunn's multiple-comparison test was used to compare percentage inhibition between anti-GST and anti-MSP11 antibodies at 20, 10 and 5 mg/ml IgG final concentration. *; statistical significance ($P < 0.05$). (C) Cross-reactivity of anti-MSP11-FL rabbit antibodies against recombinant MSP3 family members. Six recombinant SPAM domains of MSP3 family members were MSP11, MSP3, MSP6, MSP3.3, MSPDBL1, and MSPDBL2. The proteins were separated on SDS-PAGE under non-reducing (lane N) and reducing (lane R) conditions, and Western blot analysis was performed with anti-MSP11-FL rabbit antibodies (1:500). M; protein molecular weight marker. Additional details are described in Supplementary Materials and methods.

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2018.10.012.

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