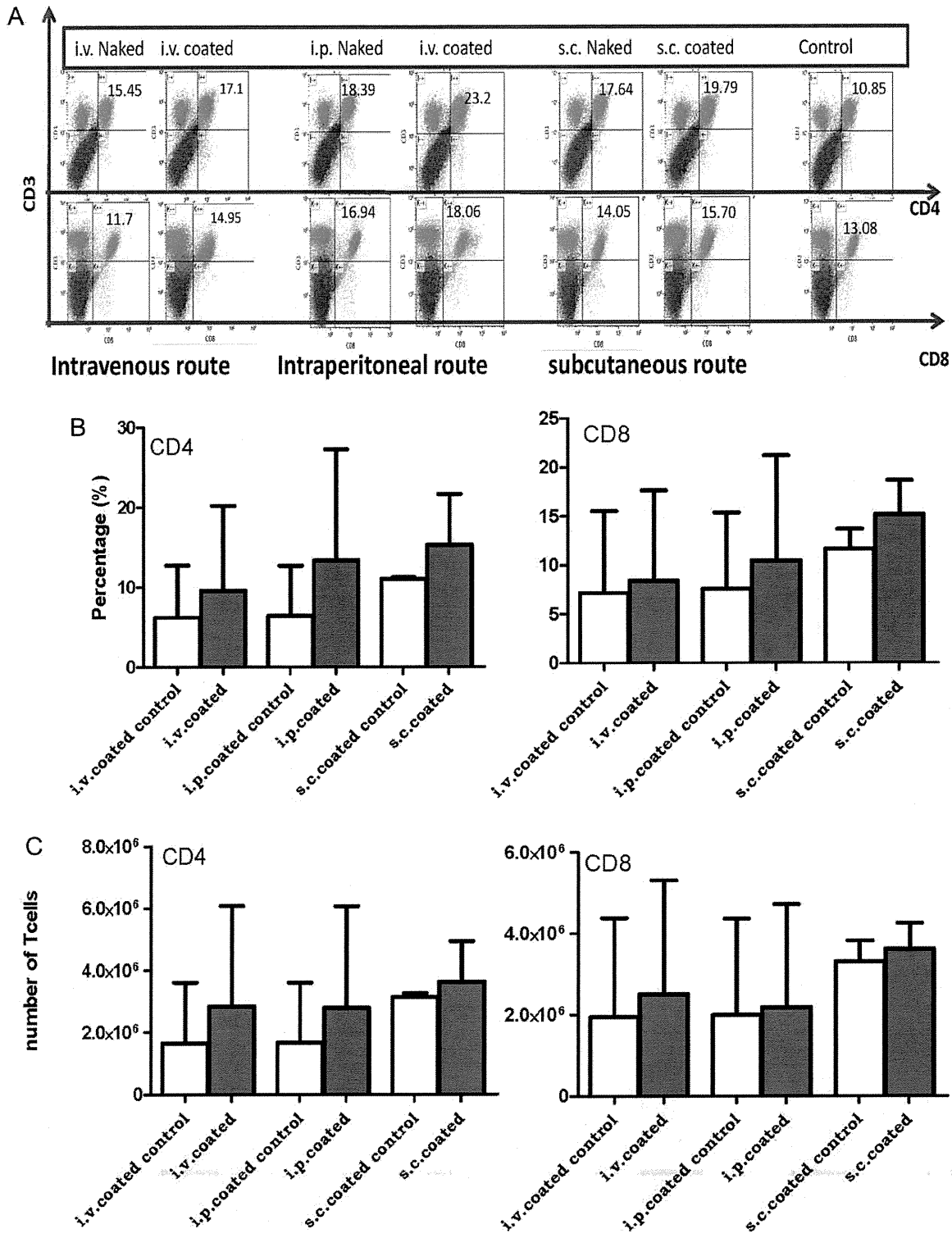

Fig. 3. Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cell subpopulation. Mice were immunized either with nanoparticle coated or naked MSP-1 C-terminus plasmid or PBS. Two weeks after the last immunization, 2 mice from each group were sacrificed, their spleens removed and splenocytes were labeled with CD3-APC, CD4-FITC and CD8-PE cell surface markers.(A) Representative figure of proportion $CD4^+/CD3^+$ (green) $CD8^+/CD3^+$ (orange) from first experiment. (B and C) Stimulatory effect of coating on $CD4^+$ and $CD8^+$ T cell subpopulation in the spleen from second experiment. Mice were immunized either with NP-coated DNA or -coated blank plasmid. Two weeks after the last immunization, 2 mice from each group were sacrificed, their spleens removed and splenocytes were labeled with CD3-APC, CD4-FITC and CD8-PE cell surface markers and the T cell populations were determined by flow cytometry. Each bar representing mean ± SD taken from two different experiments. (C) Percentage of $CD4^+$ and $CD8^+$ T cells. (D) Absolute number of $CD4^+$ and $CD8^+$ T cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

to coated group in the previous experiments. In contrast, 50% of mice immunized sub-cutaneously with NP-coated plasmid survived from lethal *P. yoelii* challenge (Fig. 1B). To determine the antigen stimulatory effect of nanoparticle coating on antibody production, IgG and its subclass were measured in the serum collected 2 weeks after the last boost. Group of mice immunized by i.v. and i.p., with coated DNA plasmid showed high antibody titre as compared to the group of mice immunized with coated blank plasmid. However, no significant increase of antibody in the group of mice immunized s.c. route (Fig. 2A and D). Similar results were obtained using native crude antigen (Fig. S2). Flow cytometric analysis was performed on splenocytes by staining with surface molecules (CD3-APC, CD4-FITC and CD8-PE), and the percentage of $CD4^+$ and $CD8^+$ T cells was higher in the spleen of mice from the coated group as compared coated blank plasmid group across all the route administration (Fig. 3B). Furthermore, analysis of the absolute number of $CD4^+$ and $CD8^+$ T cells from the splenocyte of the mice vaccinated with coated DNA plasmid showed an increase across the three routes of administration (Fig. 3C).

3.2.1. On cytokine production

Four (4) cytokines (IL-4, IL-10, IL-12p40 and IFN-γ) were estimated from cultured splenocyte supernatants and pooled sera from each group of immunized mice. Significant differences were observed between the coated and coated-control groups from both the splenocyte cultured supernatants and pooled sera, across all the three routes of administration (Figs. 4A and B). IL-4 levels in mice immunized with coated vaccine by i.p. route, consistently showed significantly higher levels in both supernatant ($p = 0.02$) and serum ($p = 0.001$), respectively, as compared to coated blank plasmid immunized mice. Also IFN-γ ($p = 0.005$) and IL10 ($p = 0.001$) were significantly higher in the cultured splenocytes supernatant. Mice immunized with coated plasmid by s.c. route showed consistently significant elevation of IL-12p40 levels in both the serum and the supernatant ($p = 0.0006$ and $p = 0.01$, respectively) as compared to coated blank plasmid immunized mice. Analyses by Pearson rank test showed a positive correlation between protection and IL-12p40 cytokines levels in both serum ($r = 0.92$ and $p = 0.0083$) and supernatant ($r = 0.90$ and $p = 0.0065$) and IL-4 in the cultured splenocyte supernatant ($r = 0.84$ and $p = 0.018$). These results suggest that the most significant production of Th1 and Th2 cytokines from antigen reactive T cells were induced by i.p. followed by i.v. route of administration, while s.c. immunization activated only one of the Th1-type cytokines (IL-12p40).

3.2.2. On cytokine production of splenocytes stimulated with crude antigen (ELISPOT)

To evaluate the functional activity of splenocytes, ELISPOT assay was also performed to detect number of cells producing either IFN-γ or IL-4 in response to crude *P. yoelii* merozoite antigen. Number

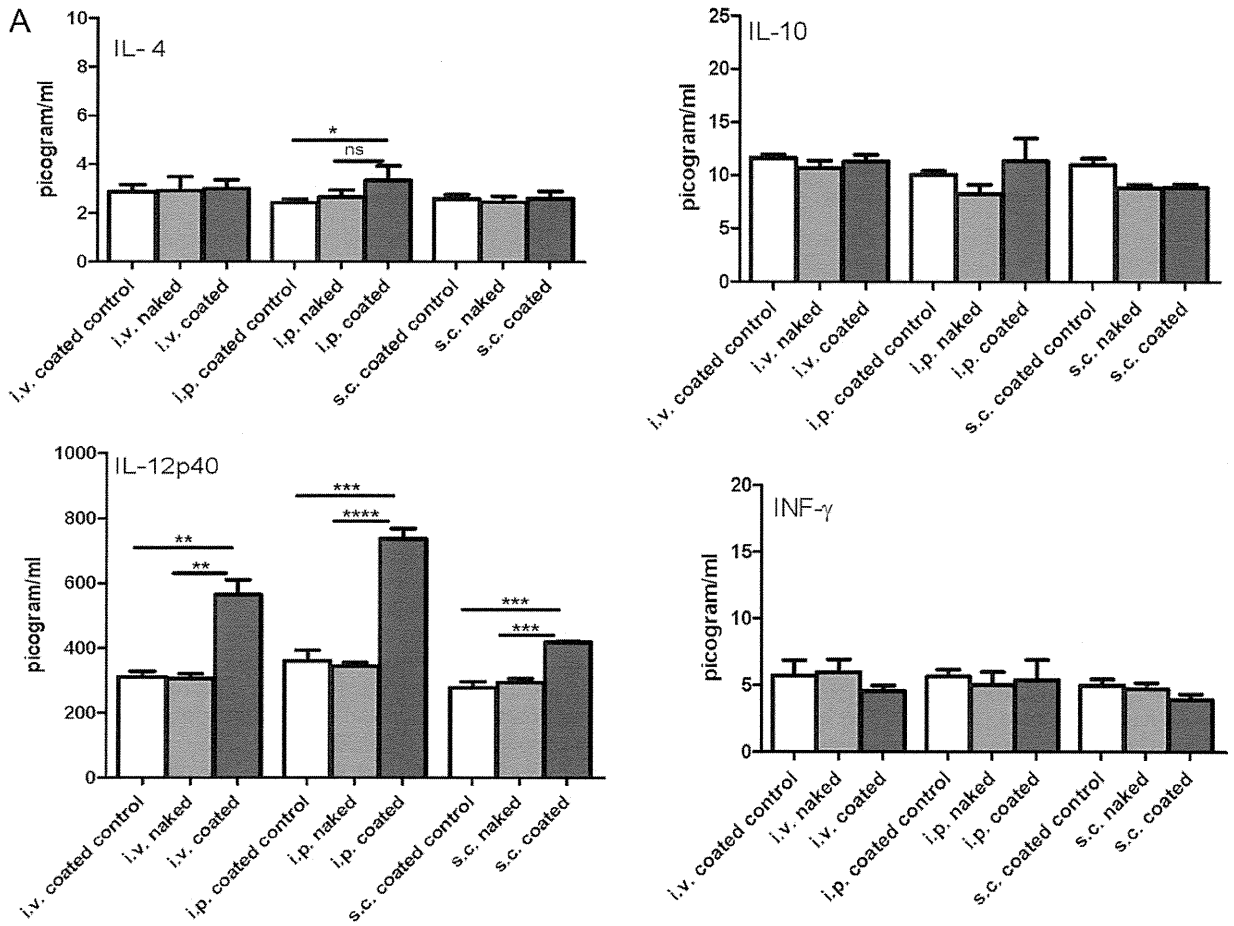

Fig. 4. Cytokine levels from pooled sera and cultured splenocytes supernatant from immunized mice from first and experiment. (A) Cytokine levels form pooled sera collected from mice in each group 2 weeks after the last immunization. (B) Cytokine levels in the splenocyte cultured supernatants: 2 weeks after the last immunization, 2 mice from each group were sacrificed, their spleens removed and splenocytes were prepared, cultured for 48 h and supernatant were collected. All cytokines were assayed using Procarta® Mouse Cytokine Plex kit. Each bar represents the mean ± SD of cytokine levels in quadruplicate wells. Comparison between coated and naked was done by student test. Statistical significance was designated as $p < 0.05$ ($^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$).

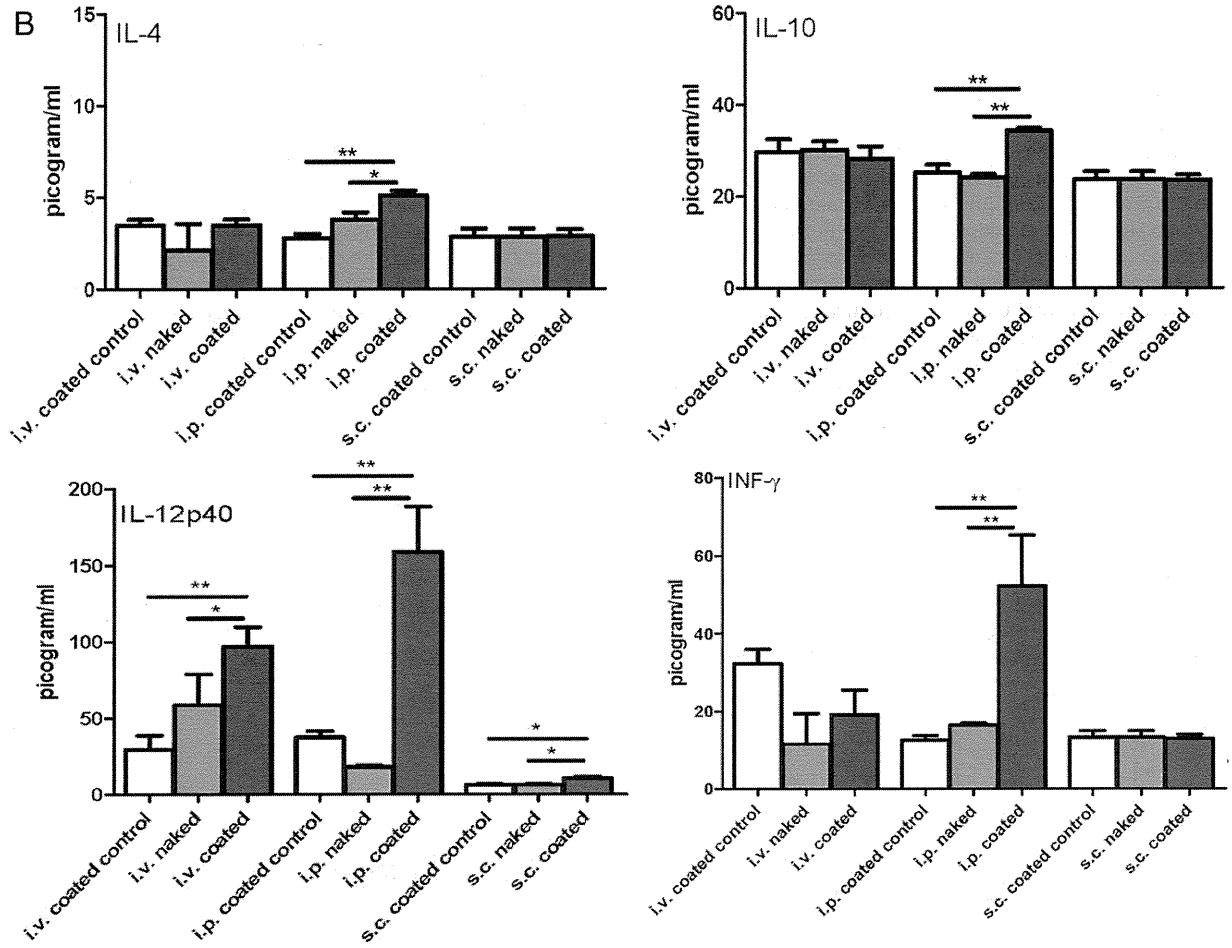

Fig. 4. *Continued.*

of IFN-γ-producing splenocytes in the coated group was more as compared to naked or control groups. However, the number IL-4-producing splenocytes was observed to be higher in the antigen stimulated coated group from coated-control groups (Fig. 5A and B). Pearson rank test showed a positive correlation between survival rate and INF-γ-producing splenocytes detected by ELISPOT assay ($r = 0.89$; $p = 0.0074$).

4. Discussion

DNA vaccines are yet to proceed beyond the phase two trials due to limited induction of desired immune response in humans. Efforts to identify methods of enhancing immune response of plasmid DNA vaccination have been tried [23,40–43] and among them is the development of delivery systems; which include delivery route and NP formulation [31,44,45]. Enhancing the immunogenicity of MSP-1; one of the leading malaria blood stage vaccine candidates [46], will be a very important step towards defeating malaria scourge. In this study, we have used two approaches (use of NP formulation and different routes of administration) to evaluate immunogenicity of C-terminus fragment of MSP-1. We have demonstrated the ability of the nanoparticle formulation used in this current study to enhance the immune response against *P. yoelii* lethal challenge across different routes of administration. It was observed that group of mice immunized with naked plasmid, developed high parasitaemia and was not able to control the parasite growth, and eventually died as compared to those mice immunized with NP-coated plasmid DNA in two different experiments.

Blood-stage malaria vaccine development is aimed at inducing high-titre growth-inhibitory antibodies against merozoite antigens involved in erythrocyte invasion [12,46]. High levels of protection against blood stage malaria were observed with elevated levels of IgG1 and IgG2a [47,48] and partial protection was observed when IgG1 and IgG2b predominates [30,47,49]. Here, the NP formulation enhanced the generation of antibody levels in the NP-coated plasmid DNA group. It was clearly shown that mice immunized with the NP-coated DNA plasmid produced high levels of IgG and its subtype antibodies (Fig. 2A–C). A significant difference of IgG subtypes (IgG2a and IgG2b) between NP-coated and naked plasmid DNA group in mice immunized by i.p. and i.v. routes of administration was observed (Fig. 2B and C) and this correlated with protection as previously reported [30,50,51].

It was reported that antibodies are required to reduce parasitaemia and $CD4^+$ T cells are required to achieve complete protection, probably to provide T cell help for antibody production [17]. Our data showed that, percentage of $CD4^+$ and $CD8^+$ T cells was high in mice immunized with coated plasmid DNA than those immunized with naked, across all the three routes of administration (Fig. 3). This is consistent with the low parasitaemia observed in mice from the coated group. We observed that survival rate in the group of immunized mice were strongly correlated (Pearson rank test) with IgG ($r = 0.62$; $p = 0.03$), IgG1 ($r = 0.59$; $p = 0.04$), IgG2a ($r = 0.7$; $p = 0.006$), and IgG2b ($r = 0.71$; $p = 0.01$) antibodies

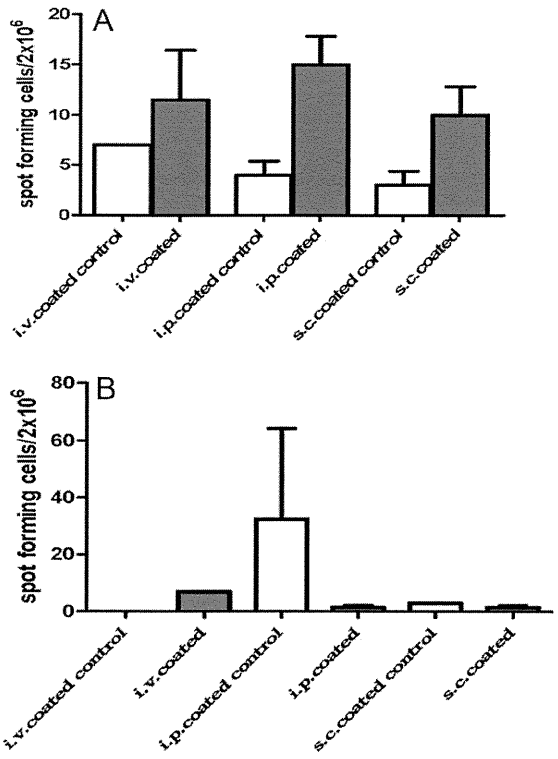

Fig. 5. Stimulatory effect of nanoparticle coating on antigen specific IL-4 and IFN-γ producing T cells in spleen as determined by ELISPOT. Splenocytes were prepared and cultured with *P. yoelii* crude antigen. Spot number evaluation was done automatically using an immunospot-image analyzer. 2×10^6 splenocyte/well were incubated 48 h in vitro with *P. yoelii* antigen or medium. (A) IFN-γ producing T cells, (B) IL-4 producing T cells in spleen. Each bar represents mean ± SD of spots number in duplicate wells.

estimated by ELISA. On the other hand, low antibody response in s.c. vaccinated mice, may explain the partial protection observed, although the T cells proportion in this group appeared to be higher than both the i.p. and i.v. vaccinated groups (Fig. 3). It was reported that antibodies are required to reduce parasitaemia in the first 12 days after challenge, and $CD4^+$ T cells are required to achieve complete protection [17], probably to provide T cell help for antibody production. It is interesting that the mice immunized with coated DNA plasmid by s.c. delivery route in the second experiment did not show elevated levels of anti-MSP-1 IgG and subtypes but exhibited 50% survival (Fig. 1A) and the survived mice developed high parasitaemia during the first 2 weeks post-infection although some were able to clear their parasitaemia afterwards (Fig. 1A).

Various studies have shown that, with the same formulation, route of injection influenced the immune response [35,52,53]. In the larger number of studies that have evaluated DNA-based immunization, few have directly compared the immune responses generated by different routes of delivery. Effective translation of this approach to human clinical setting could be tasking and perhaps controversial, particularly by IP route. IP vaccination is predominantly used in veterinary medicine due to the ease of administration compared with other parenteral methods and induction of protective immune response in several type of animals such as fish [54] chicken [55] and pigs [56,57]. Also, Lue et al. [58] reported that Ip vaccination of human subjects with tetanus toxoid induces specific immune response. Thus, we assumed that this formulation may work as well in mucosal route of delivery system, because it was reported that the peritoneal cavity contains a major reservoir of self-replenishing cells that play a significant role in the mucosal immune response [59,60]. Currently, modification to this formulation to fit into clinical setting is ongoing. Eventually, this would be feasible if the *Plasmodium falciparum* counterpart of MSP-1 is used and all the immunological parameters rival or better than the reported ones. However, in human IV routes are used to administer drugs [61] and vaccine [58].

It is believed that clearance of blood stage infection required Th1 and Th2 types of immune response. The observed complete protection against lethal challenge in immunized mice in the coated group of i.v. and i.p. routes of delivery may be partly explained by the contributory effect of IL-12p40, which may have induced a Th1 immune response. This effect is observable in s.c.-vaccinated group with low antibody production but with partial protection. It was reported that, IL-12p40 appears to be critically linked to or to act through IFN-γ production, thereby allowing an early and sustained Th1 response and is shown to be required for the production of protective IgG2a antibody [33,62–64]. However, a number of observations indicate that exposure to IL-4 is essential for the priming of Th2-type effector T cells [65]. These results suggest that enhanced Th1 and Th2 cytokines were induced by i.p. followed by i.v. route of administration, while marginally by s.c. immunization.

Nanoparticles are considered efficient immune–potentitor and antigen–carrier to DC [66,67] and γ-PGA nanoparticle induced DC activation by acting as a potent vaccine adjuvant as well as an efficient antigen carrier to DC [68]. This NP-coated MSP-1 formulation predominantly was taken up by DC and which started to produce IL-12 abundantly, essential for the priming of Th1-type effector T cells. Also, our data showed that NP coating is a promising strategy for potent induction of antigen specific immune responses. We have already reported the enhancing effect of nanoparticle coating for the MSP-1 DNA vaccination model, however, the level of protection and antibody production were not so higher as the current study [30]. One of the possible reasons might be the use of different vector named VR1020 that had been approved by FDA for human vaccine use. The major advantage of this vector is described elsewhere (Vical Incorporated, San Diego, CA).

In summary, vaccination with NP-coated MSP-1 C-terminus plasmid using three different routes of delivery was evaluated, and i.p. and i.v. delivery routes showed complete protection against lethal challenge with significant increase in levels of IgG and its subtypes and also of both Th1 and Th2 type cytokines production. On the other hand, in s.c. vaccinated group, mice showed about 50% protection and marginal levels of specific antibody. These findings, therefore, underscore the relevance of DNA vaccination and could be contributory to the current approaches to meet our desired goal of defeating malaria and other infection diseases.

Acknowledgements

We would like to thank Dr Shibata, H. for technical assistance and Vical incorporated for providing the vector VR1020 (Vical, San Diego, CA, USA). MSC is a graduate student under the GCOE program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.09.031.

References

[1] Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature 2005;434(March (7030)):214–7.
[2] Abdullah S, Adazu K, Masanja H, Diallo D, Hodgson A, Ilboudo-Sanogo E, et al. Patterns of age-specific mortality in children in endemic areas of sub-Saharan Africa. Am J Trop Med Hyg 2007;77(December (Suppl. 6)):99–105.

[3] Carneiro I, Roca-Feltrer A, Griffin JT, Smith L, Tanner M, Schellenberg JA, et al. Age-patterns of malaria vary with severity, transmission intensity and seasonality in sub-Saharan Africa: a systematic review and pooled analysis. PloS One 2010;5(2):e8988.
[4] Nosten F, White NJ. Artemisinin-based combination treatment of falciparum malaria. Am J Trop Med Hyg 2007;77(December (Suppl. 6)):181–92.
[5] Lengeler C, Snow RW. From efficacy to effectiveness: insecticide-treated bed nets in Africa. Bull World Health Organ 1996;74(3):325–32.
[6] Jha P. Reliable mortality data: a powerful tool for public health. Natl Med J India 2001;14(May–June (3)):129–31.
[7] Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, et al. A world malaria map: *Plasmodium falciparum* endemicity in 2007. PLoS Med 2009;6(March (3)):e1000048.
[8] Anderson T. Mapping the spread of malaria drug resistance. PLoS Med 2009;6(April (4)):e1000054.
[9] Hu J, Chen Z, Gu J, Wan M, Shen Q, Kieny MP, et al. Safety and immunogenicity of a malaria vaccine, *Plasmodium falciparum* AMA-1/MSP-1 chimeric protein formulated in montanide ISA 720 in healthy adults. PLoS One 2008;3(4):e1952.
[10] Nyarango PM, Gebremeskel T, Mebrahtu G, Mufunda J, Abdulmumini U, Ogbamariam A, et al. A steep decline of malaria morbidity and mortality trends in Eritrea between 2000 and 2004: the effect of combination of control methods. Malaria J 2006;5:33.
[11] Feachem R, Sabot O. A new global malaria eradication strategy. Lancet 2008;371(May (9624)):1633–5.
[12] Kang Y, Calvo PA, Daly TM, Long CA. Comparison of humoral immune responses elicited by DNA and protein vaccines based on merozoite surface protein-1 from *Plasmodium yoelii*, a rodent malaria parasite. J Immunol 1998;161(October (8)):4211–9.
[13] Boyle MJ, Wilson DW, Richards JS, Riglar DT, Tetteh KK, Conway DJ, et al. Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. Proc Natl Acad Sci USA 2010;107(August (32)):14378–83.
[14] Zhang Q, Xue X, Qu L, Pan W. Construction and evaluation of a multistage combination vaccine against malaria. Vaccine 2007;25(March (11)):2112–9.
[15] Good MF. Towards a blood-stage vaccine for malaria: are we following all the leads? Nat Rev Immunol 2001;1(November (2)):117–25.
[16] Greenwood B, Targett G. Do we still need a malaria vaccine? Parasite Immunol 2009;31(September (9)):582–6.
[17] Daly TM, Long CA. Humoral response to a carboxyl-terminal region of the merozoite surface protein-1 plays a predominant role in controlling blood-stage infection in rodent malaria. J Immunol 1995;155(July (1)):236–43.
[18] Hirunpetcharat C, Tian JH, Kaslow DC, van Rooijen N, Kumar S, Berzofsky JA, et al. Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1[19]) of *Plasmodium yoelii* expressed in *Saccharomyces cerevisiae*: correlation of protection with antigen-specific antibody titer, but not with effector $CD4^+$ T cells. J Immunol 1997;159(October (7)):3400–11.
[19] Blackman MJ, Heidrich HG, Donachie S, McBride JS, Holder AA. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J Exp Med 1990;172(July (1)):379–82.
[20] Wipasa J, Xu H, Liu X, Hirunpetcharat C, Stowers A, Good MF. Effect of *Plasmodium yoelii* exposure on vaccination with the 19-kilodalton carboxyl terminus of merozoite surface protein 1 and vice versa and implications for the application of a human malaria vaccine. Infect Immun 2009;77(February (2)):817–24.
[21] Okitsu SL, Silvie O, Westerfeld N, Curcic M, Kammer AR, Mueller MS, et al. A virosomal malaria peptide vaccine elicits a long-lasting sporozoite-inhibitory antibody response in a phase 1a clinical trial. PLoS One 2007;2(12):e1278.
[22] Moorthy VS, Good MF, Hill AV. Malaria vaccine developments. Lancet 2004;363(January (9403)):150–6.
[23] Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. Annu Rev Immunol 2000;18:927–74.
[24] ten Hagen TL, Sulzer AJ, Kidd MR, Lal AA, Hunter RL. Role of adjuvants in the modulation of antibody isotype, specificity, and induction of protection by whole blood-stage *Plasmodium yoelii* vaccines. J Immunol 1993;151(December (12)):7077–85.
[25] Malyala P, Singh M. Micro/nanoparticle adjuvants: preparation and formulation with antigens. Methods Mol Biol 2010;626:91–101.
[26] van Zanten J, Doornbos-Van der Meer B, Audouy S, Kok RJ, de Leij L. A non-viral carrier for targeted gene delivery to tumor cells. Cancer Gene Ther 2004;11(February (2)):156–64.
[27] Peek LJ, Middaugh CR, Berkland C. Nanotechnology in vaccine delivery. Adv Drug Deliv Rev 2008;60(May (8)):915–28.
[28] Kaba SA, Brando C, Guo Q, Mittelholzer C, Raman S, Tropel D, et al. A non-adjuvanted polypeptide nanoparticle vaccine confers long-lasting protection against rodent malaria. J Immunol 2009;183(December (11)):7268–77.
[29] Kurosaki T, Kitahara T, Fumoto S, Nishida K, Nakamura J, Niidome T, et al. Ternary complexes of pDNA, polyethylenimine, and gamma-polyglutamic acid for gene delivery systems. Biomaterials 2009;30(May (14)):2846–53.
[30] Shuaibu MN, Cherif MS, Kurosaki T, Helegbe GK, Kikuchi M, Yanagi T, et al. Effect of nanoparticle coating on the immunogenicity of plasmid DNA vaccine encoding *P. yoelii* MSP-1 C-terminal. Vaccine 2011;29(April (17)):3239–47.
[31] Bohm W, Mertens T, Schirmbeck R, Reimann J. Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses. Vaccine 1998;16(May–June (9–10)):949–54.
[32] Johansson EL, Bergquist C, Edebo A, Johansson C, Svennerholm AM. Comparison of different routes of vaccination for eliciting antibody responses in the human stomach. Vaccine 2004;22(February (8)):984–90.
[33] Su Z, Stevenson MM. IL-12 is required for antibody-mediated protective immunity against blood-stage Plasmodium chabaudi AS malaria infection in mice. J Immunol 2002;168(February (3)):1348–55.
[34] Lodmell DL, Ray NB, Ulrich JT, Ewalt LC. DNA vaccination of mice against rabies virus: effects of the route of vaccination and the adjuvant monophosphoryl lipid A (MPL). Vaccine 2000;18(January (11–12)):1059–66.
[35] Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. Nature 1992;356(March (6365)):152–4.
[36] McCluskie MJ, Brazolot Millan CL, Gramzinski RA, Robinson HL, Santoro JC, Fuller JT, et al. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. Mol Med 1999;5(May (5)):287–300.
[37] Aguiar JC, Hedstrom RC, Rogers WO, Charoenvit Y, Sacci Jr JB, Lanar DE, et al. Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. Vaccine 2001;20(October (1–2)):275–80.
[38] Tsuboi T, Takeo S, Iriko H, Jin L, Tsuchimochi M, Matsuda S, et al. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infect Immun 2008;76(April (4)):1702–8.
[39] Tsuboi T, Takeo S, Sawasaki T, Torii M, Endo Y. An efficient approach to the production of vaccines against the malaria parasite. Methods Mol Biol 2010;607:73–83.
[40] Wang R, Epstein J, Baraceros FM, Gorak EJ, Charoenvit Y, Carucci DJ, et al. Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. Proc Natl Acad Sci USA 2001;98(September (19)):10817–22.
[41] Epstein JE, Gorak EJ, Charoenvit Y, Wang R, Freydberg N, Osinowo O, et al. Safety, tolerability, and lack of antibody responses after administration of a PfCSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/intradermal routes. Hum Gene Ther 2002;13(September (13)):1551–60.
[42] Epstein JE, Charoenvit Y, Kester KE, Wang R, Newcomer R, Fitzpatrick S, et al. Safety, tolerability, and antibody responses in humans after sequential immunization with a PfCSP DNA vaccine followed by the recombinant protein vaccine RTS,S/AS02A. Vaccine 2004;22(April (13–14)):1592–603.
[43] Dobano C, Sedegah M, Rogers WO, Kumar S, Zheng H, Hoffman SL, et al. Plasmodium: mammalian codon optimization of malaria plasmid DNA vaccines enhances antibody responses but not T cell responses nor protective immunity. Exp Parasitol 2009;122(June (2)):112–23.
[44] Yokoyama M, Zhang J, Whitton JL. DNA immunization: effects of vehicle and route of administration on the induction of protective antiviral immunity. FEMS Immunol Med Microbiol 1996;14(July (4)):221–30.
[45] Barry MA, Johnston SA. Biological features of genetic immunization. Vaccine 1997;15(June (8)):788–91.
[46] Holder AA. The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. Parasitology 2009;136(October (12)):1445–56.
[47] Ling IT, Ogun SA, Momin P, Richards RL, Garcon N, Cohen J, et al. Immunization against the murine malaria parasite *Plasmodium yoelii* using a recombinant protein with adjuvants developed for clinical use. Vaccine 1997;15(October (14)):1562–7.
[48] De Souza JB, Ling IT, Ogun SA, Holder AA, Playfair JH. Cytokines and antibody subclass associated with protective immunity against blood-stage malaria in mice vaccinated with the C terminus of merozoite surface protein 1 plus a novel adjuvant. Infect Immun 1996;64(September (9)):3532–6.
[49] Tongren JE, Corran PH, Jarra W, Langhorne J, Riley EM. Epitope-specific regulation of immunoglobulin class switching in mice immunized with malarial merozoite surface proteins. Infect Immun 2005;73(December (12)):8119–29.
[50] Diallo TO, Spiegel A, Diouf A, Perraut R, Kaslow DC, Garraud O. Short report: IgG1/IgG3 antibody responses to various analogs of recombinant ypfmsp119—a study in immune adults living in areas of *Plasmodium falciparum* transmission. Am J Trop Med Hyg 2001;64(March–April (3–4)):204–6.
[51] Garraud O, Mahanty S, Perraut R. Malaria-specific antibody subclasses in immune individuals: a key source of information for vaccine design. Trends Immunol 2003;24(January (1)):30–5.
[52] Boyle JS, Silva A, Brady JL, Lew AM. DNA immunization: induction of higher avidity antibody and effect of route on T cell cytotoxicity. Proc Natl Acad Sci USA 1997;94(December (26)):14626–31.
[53] Playfair JH, De Souza JB. Vaccination of mice against malaria with soluble antigens. I. The effect of detergent, route of injection, and adjuvant. Parasite Immunol 1986;8(September (5)):409–14.
[54] Caipang CM, Hynes N, Puangkaew J, Brinchmann MF, Kiron V. Intraperitoneal vaccination of Atlantic cod, *Gadus morhua* with heat-killed *Listonella anguillarum* enhances serum antibacterial activity and expression of immune response genes. Fish Shellfish Immunol 2008;24(March (3)):314–22.
[55] Muir WI, Bryden WL, Husband AJ. Evaluation of the efficacy of intraperitoneal immunization in reducing *Salmonella typhimurium* infection in chickens. Poult Sci 1998;77(December (12)):1874–83.
[56] Sheldrake RF, Romalis LF, Saunders MM. Serum mucosal antibody responses against *Mycoplasma hyopneumoniae* following intraperitoneal vaccination and challenge of pigs with *M. hyopneumoniae*. Res Vet Sci 1993;55(November (3)):371–6.

[57] Sheldrake RF, Gardner IA, Saunders MM, Romalis LF. Intraperitoneal vaccination of pigs to control *Mycoplasma hyopneumoniae*. Res Vet Sci 1991;51(November (3)):285–91.
[58] Lue C, van den Wall Bake AW, Prince SJ, Julian BA, Tseng ML, Radl J, et al. Intraperitoneal immunization of human subjects with tetanus toxoid induces specific antibody-secreting cells in the peritoneal cavity and in the circulation, but fails to elicit a secretory IgA response. Clin Exp Immunol 1994;96(May (2)):356–63.
[59] Kroese FG, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg LA. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. Int Immunol 1989;1(1):75–84.
[60] Pecquet SS, Ehrat C, Ernst PB. Enhancement of mucosal antibody responses to *Salmonella typhimurium* and the microbial hapten phosphorylcholine in mice with X-linked immunodeficiency by B-cell precursors from the peritoneal cavity. Infect Immun 1992;60(February (2)):503–9.
[61] Swart AM, Burdett S, Ledermann J, Mook P, Parmar MK. Why i.p. therapy cannot yet be considered as a standard of care for the first-line treatment of ovarian cancer: a systematic review. Ann Oncol 2008;19(April (4)):688–95.
[62] Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-gamma and TNF-alpha and occurs via a nitric oxide-dependent mechanism. J Immunol 1995;155(September (5)):2545–56.
[63] Crutcher JM, Stevenson MM, Sedegah M, Hoffman SL. Interleukin-12 and malaria. Res Immunol 1995;146(September–October (7–8)): 552–9.
[64] Romero JF, Ibrahim GH, Renggli J, Himmelrich H, Graber P, Corradin G. IL-12p40-independent induction of protective immunity upon multiple *Plasmodium berghei* irradiated sporozoite immunizations. Parasite Immunol 2007;29(November (11)):541–8.
[65] Liu L, Rich BE, Inobe J, Chen W, Weiner HL. Induction of Th2 cell differentiation in the primary immune response: dendritic cells isolated from adherent cell culture treated with IL-10 prime naive $CD4^+$ T cells to secrete IL-4. Int Immunol 1998;10(August (8)):1017–26.
[66] Coester C, Nayyar P, Samuel J. In vitro uptake of gelatin nanoparticles by murine dendritic cells and their intracellular localisation. Eur J Pharm Biopharm 2006;62(April (3)):306–14.
[67] Uto T, Wang X, Sato K, Haraguchi M, Akagi T, Akashi M, et al. Targeting of antigen to dendritic cells with poly(gamma-glutamic acid) nanoparticles induces antigen-specific humoral and cellular immunity. J Immunol 2007;178(March (5)):2979–86.
[68] Hamasaki T, Uto T, Akagi T, Akashi M, Baba M. Modulation of gene expression related to Toll-like receptor signaling in dendritic cells by poly(gamma-glutamic acid) nanoparticles. Clin Vaccine Immunol 2010;17 (May (5)):748–56.

Human Immunology 72 (2011) 881-888

Contents lists available at SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Human leukocyte antigen class I polymorphisms influence the mild clinical manifestation of *Plasmodium falciparum* infection in Ghanaian children

Akiko Yamazaki [a], Michio Yasunami [a,*], Michael Ofori [b], Hitomi Horie [a], Mihoko Kikuchi [a], Gideon Helegbe [a], Akiko Takaki [a], Kazunari Ishii [a], Ahmeddin Hassan Omar [a], Bartholomew D. Akanmori [b], Kenji Hirayama [a,*]

[a] *Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Nagasaki, Japan*
[b] *Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana*

ARTICLE INFO

Article history:
Received 1 March 2011
Accepted 27 June 2011
Available online 1 July 2011

Keywords:
Malaria
Cohort study
Genetic susceptibility
Resistance
HLA polymorphism
HLA haplotype

ABSTRACT

A prospective study that included 429 children for active detection of mild malaria was conducted in a coastal region of Ghana to reveal whether the incidence of malaria is affected by human leukocyte antigen (HLA) polymorphism. During 12 months of follow-up, 85 episodes of mild clinical malaria in 74 individuals were observed, and 34 episodes among them were accompanied with significant parasitemia at >5000 infected red blood cells per cubic millimeter. Attributable and relative risks conferred by genetic factors in the HLA region were evaluated by comparison of the incidence in children, stratified by carrier status, of a given allele of HLA-A, -B, -DRB1 and TNFA promoter polymorphism. HLA-B*35:01 reduced the incidence by 0.178 events per person per year (0.060 versus 0.239 for B*35:01-positive and -negative subpopulations, respectively), and a relative risk of 0.25, which remained statistically significant after Bonferroni's correction for multiple testing ($p_c = 8.2 \times 10^{-5}$). Further, HLA-B*35:01 and -B*53:01 exhibited opposite effects on the incidence of malaria with significant parasitemia. When parasite densities in different HLA carriers status were compared, HLA-A*01 conferred an increase in parasite load ($p = 6.0 \times 10^{-7}$). In addition, we found a novel DRB1 allele that appears to have emerged from DRB1*03:02 by single nucleotide substitution.

1. Introduction

Worldwide, in 2008, there were an estimated 243 million of malaria cases, causing 863,000 deaths [1]. Malaria remains one of the leading causes of death in children, comprising 8% of total deaths in children less than 5 years of age [1]. Most patients infected by the malaria parasite commonly present with febrile symptoms; when the infection is complicated by serious organ failure, severe malaria develops. Acquired immunity against the malarial parasite would play an important role in the prevention of development of both mild and severe illness, because it is observed that the incidence of malaria is dependent on age and density of infecting mosquito bites in the population [2,3]. Contribution of helper T cells, B cells, and cytokines in the control of parasitemia was also demonstrated in a murine model of *Plasmodium chabaudi* infection under experimental conditions [4–6]. Therefore, the severity of malaria is determined by host defense mechanisms, at least to some extent.

Antimicrobial defense response is variable and is thought to be developed through environmental factors. However, genetic factors also play important roles in the establishment of variability of the responsiveness. Human leukocyte antigen (HLA) is one of such factors involved in antimalaria response; certain HLA alleles that are prevalent among individuals of African descent, such as HLA-B*53 and HLA-DRB1*13:02, were demonstrated to be associated with resistance against severe malaria in sub-Saharan populations [7]. In the present study, a cohort of healthy children was set up to detect malarial symptoms actively in a moderately endemic area. The effect of carrier status of various HLA alleles on clinical manifestation was then evaluated by comparing the incidence between subpopulations divided according to carrier status.

2. Subjects and methods

2.1. Set-up of cohort and active case detection

The study was conducted in Asutuare, a subdistrict of Dangme-West District in Greater Accra of Ghana. It is a rural region approximately 40 km northeast of Accra, the capital of Ghana. The population size in year 2000 in 1,442 km^2 surface area of Dangme-West District was approximately 96,800 (http://www.ghanadistricts.com). The population is mostly scattered in small settlements of less than 1000. Rainfall is seasonal in this region, in April to July and in October to December. Accordingly, seasonality of malaria transmission is observed in a fashion similar to that in Dodowa, the

* Corresponding authors.
E-mail address: yasunami@nagasaki-u.ac.jp (M. Yasunami) or hiraken@nagasaki-u.ac.jp (K. Hirayama).

0198-8859/11/$32.00 - see front matter © 2011 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.humimm.2011.06.007

capital of the district, where we performed a study in the 1990s [8–10]. The transmission is perennial, but peaks during and immediately after the major rainy seasons and is lowest during the dry seasons. A total of 429 apparently healthy children of Akan ethnicity, 3–11 years of age, were enrolled in the present study at four communities during a period from the end of June to the middle of July 2007. Approximately equal numbers of girls and boys were registered; 218 (51%) of them were female and 211 (49%) were male. To avoid the inclusion of closely related individuals, no more than one child was selected from each household. Informed parental consent was obtained after explanation of all procedures involved in this study, which was approved by the Ghanaian Ministry of Health, Institutional Review Board of Noguchi Memorial Institute of Medical Research, University of Ghana, and the Institutional Review Board of the Institute of Tropical Medicine, Nagasaki University.

Registered children were placed under close observation for active detection of clinical malaria by repeated visits of field workers with 2-week intervals for 52 weeks. At each visit, information regarding the health status of the participating child in the past 2 weeks was recorded on a fixed questionnaire form, and axillary body temperature of the child was taken. All suspected cases with fever greater than 38 degrees Celsius and other typical symptoms, such as headache and/or malaise, were referred to the outpatient department of community health centers, where thin and thick blood smear slide samples were taken for diagnosis. A blood test with one parasitized red blood cell (iRBC) in microscopic fields, including up to 200 WBCs, which approximately corresponds to 25 iRBCs in 1 μl of blood, was assumed as parasite-positive in practice, then anyone with parasitemia was immediately treated with an artesunate–amodiaquine combination regimen as recommended by the Ghanaian Ministry of Health. We considered that the patient had "clinical malaria" when he or she had a fever equal to or greater than 38 degrees Celsius and positive blood test, and further designated one as "a patient with high parasitemia" when the parasite load reached more than 5,000 iRBC/mm^3 during the course of infection. The clinical data obtained from 405 children among 429 enrolled (94.4%) who were followed up for longer than 26 weeks within 52 weeks of total observation period. A total of 147 children (38.8% of 405 followed up) were referred to clinic one or more times because of the presence of suspected symptoms, and 85 confirmed malaria episodes were captured.

2.2. Sample preparation for genetic and serologic studies

The peripheral blood was drawn aseptically in a Vacutainer blood collection tube with ethylenediaminetetraacetic acid (EDTA; Beckton-Dickinson, Tokyo, Japan) from all 429 participants at enrollment. Plasma and blood cells were separated by centrifugation at 200 *g* for 10 minutes, and DNA was prepared from the precipitated whole-blood cells by using QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan).

2.3. HLA genotyping

Genotypes of HLA-A and -B loci were determined by Labtype reverse sequence-specific oligonucleotide HLA typing kit (One Lambda, Canoga Park, CA). Briefly, genomic DNA regions corresponding to exon 2 and exon 3 of HLA-A or -B were amplified and labeled by polymerase chain reaction (PCR) with biotin-conjugated oligonucleotide primers. Locus-specific amplified products were subjected to hybridization with multiple oligonucleotide probes that were fixed on the surface of fluorescent, color-coded Luminex microspheres. The hybridization signals were then visualized by binding phycoerythrin-conjugated streptavidin and were analyzed by Luminex 100, a flow cytometer adapted to multiplex color-coded microspheres (Hitachi Software Engineering, Tokyo, Japan). The interpretation of hybridization reaction patterns to HLA genotype was done with the support of HLA Software (One Lambda). Genotyping of HLA-DRB1 locus was performed by direct nucleotide sequencing of amplification products of eight allele-group–specific PCR [11] using a Genetic Analyzer ABI 3730 (AppliedBiosystems, Tokyo Japan) and Assign400 Software (Conexio Genomics, Australia). HLA antigen phenotype frequency was deduced from genotype data. The 95% confidence intervals (95% CI) of phenotype frequency were estimated by the method of Agresti and Coull [12].

Polymorphism in the upstream region of TNFA gene (TNFAp) was analyzed by nucleotide sequencing of amplification products by PCR using TNFA-PF (CCCAATAAACCTCTTTTCTCTGA) and TNFA-PR (GCTGGTCCTCTGCTGTCCTT). Haplotype frequencies of HLA-A, -B, -DRB1, and TNFAp were estimated from nonphased genotype data with maximization likelihood algorithm by the use of PHASE version 2.1.1 [13].

2.4. Statistical analysis

Statistical analyses were carried out using JMP version 8 (SAS Institute, Cary, NC). The effect of HLA alleles present at greater than than 0.05 frequency among all participants or children with malaria were examined; the incidence was obtained by count of events during the adjusted observation period (person $\times$ year) for each subpopulation, after which attributable risk and relative risk for carrier status of the given HLA allele were obtained by comparing the incidence of clinical malaria in carriers with that in noncarriers [14,15]. Statistical significance of attributable risk was evaluated by assuming null hypothesis that attributable risk follows normal distribution N(0, SE), where SE was estimated by the formula described in reference 15. The p values were corrected for 32 multiple testing, nine HLA-A, nine HLA-B, nine HLA-DRB1, and five TNFA promoter tests, with Bonferroni's method.

HLA effect on parasite density was also evaluated by comparison of mean parasite densities of all detectable clinical malaria episodes among the children with different HLA carrier status. At first, heterogeneity among subgroups stratified according to carrier status of prevalent alleles was examined by locus-wise analysis of variance (ANOVA). Allele effect was further assessed for the positive locus by *post hoc* evaluation with Student's t-test to compare parasite densities between carriers and noncarriers of a given allele.

3. Results

3.1. Malaria case detection

During 52 weeks of the observation period that started in June and July 2007, occurrence of possible malarial symptoms in 429 registered Ghanaian children could be successfully surveyed for 405 children (94.4%) at least for 26 weeks. Among them, 147 children were referred to medical consultation one or more times because of the presence of suspected malarial symptoms; 60 of these children (40.8%) had experienced multiple febrile episodes. The clinical records from the outpatient clinic could be recovered for 117 of 147 referred children; 85 episodes occurred in 74 subjects who were diagnosed with malaria by blood test, and 34 case records taken from 28 subjects met our criteria for high parasitemia.

Because we included children of different ages between 3 and 11 years, we could assess age-related risk for malaria in the cohort. Malaria incidence was almost equally distributed along with age in the cohort. When the incidence was tested under a logistic regression model that assumed age (in years) as a quantitative variable, any age effect on malaria incidence was not detected, providing unit odds (beta) of 0.99 ($p = 0.947$).

Table 1
HLA-A, -B, and -DRB1 alleles in Ghanaian children

Factor	Allele designation at low and high resolution[a]		No. (frequency)	(Proportion in group, %)[b]
HLA-A	(2*n* = 856)			
	A*01		22 (0.026)	
	A*02		176 (0.206)	
	A*03		101 (0.118)	
	A*23		89 (0.104)	
	A*24		4 (0.005)	
	A*26		2 (0.002)	
	A*29		14 (0.016)	
	A*30		129 (0.151)	
	A*32		5 (0.006)	
	A*33		71 (0.083)	
	A*34		19 (0.022)	
	A*36		28 (0.033)	
	A*66		27 (0.032)	
	A*68		125 (0.146)	
	A*74		36 (0.042)	
	A*80		8 (0.009)	
HLA-B	(2*n* = 858)			
	B*07		78 (0.091)	
		B*07:02		60 (76.9%)
		B*07:05		18 (23.1%)
	B*08	(=B*08:01)	4 (0.005)	
	B*13	(=B*13:02)	7 (0.008)	
	B*14		16 (0.019)	
		B*14:02		9 (56.3%)
		B*14:03		1 (6.3%)
		B*14:05		6 (37.5%)
	B*15(B70)[c]		87 (0.101)	
	(B72)	B*15:03		40 (46.0%)
	(B71)	B*15:10		47 (54.0%)
	B*15(B63)[c]	(=B*15:16)	18 (0.021)	
	B*18	(=B*18:01)	31 (0.036)	
	B*27	(various)	2 (0.002)	
	B*35		78 (0.091)	
		B*35:01		76 (97.4%)
		Other B*35		2 (2.6%)
	B*39	(=B*39:10)	8 (0.009)	
	B*41	(=B*41:02)	7 (0.008)	
	B*42		106 (0.124)	
		B*42:01		92 (86.8%)
		B*42:02		14 (13.2%)
	B*44		58 (0.068)	
		B*44:03		57 (98.3%)
		Other B*44		1 (1.7%)
	B*45	(=B*45:01)	28 (0.033)	
	B*49	(=B*49:01)	14 (0.016)	
	B*50	(=B*50:01)	8 (0.009)	
	B*51		19 (0.022)	
		B*51:01		15 (78.9%)
		Other B*51		4 (22.1%)
	B*52		81 (0.094)	
		B*52:01		79 (97.5%)
		Other B*52		2 (2.5%)
	B*53	(=B*53:01)	143 (0.167)	
	B*57		35 (0.041)	
		B*57:01		2 (5.7%)
		B*57:02		2 (5.7%)
		B*57:03		26 (74.3%)
		B*57:04		5 (14.3%)
	B*58		24 (0.028)	
		B*58:01		22 (91.7%)
		Other B*58		2 (8.4%)
	B*67	(=B*67:01)	1 (0.001)	
	B*78	(=B*78:01)	5 (0.006)	
HLA-DRB1	(2*n* = 858)			
	DR1		37 (0.043)	
		DRB1*01:01		8 (21.6%)
		DRB1*01:02		29 (78.4%)
	DR15(DR2)		106 (0.124)	
		DRB1*15:01		1 (0.9%)
		DRB1*15:03		105 (99.1%)
	DR16(DR2)	DRB1*16:02	15 (0.017)	
	DR17(DR3)	(=DRB1*03:01)	36 (0.042)	
	DR18(DR3)		148 (0.172)	
		DRB1*03:02		141 (95.3%)

Table 1
(*continued*)

Factor	Allele designation at low and high resolution[a]	No. (frequency)	(Proportion in group, %)[b]
	DRB1*03 new variant[d]		5 (3.4%)
	DRB1*03:03		2 (1.4%)
DR4		3 (0.003)	
	DRB1*04:01		1 (33.3%)
	DRB1*04:05		2 (66.7%)
DR11(DR5)		61 (0.071)	
	DRB1*11:01		30 (49.2%)
	DRB1*11:02		20 (32.8%)
	DRB1*11:04		7 (11.5%)
	DRB1*11:14		3 (4.9%)
	DRB1*11:17		1 (1.6%)
DR12(DR5)		15 (0.017)	
	DRB1*12:01/06/10[e]		10 (66.7%)
	DRB1*12:02		5 (33.3%)
DR13(DR6)		212 (0.247)	
	DRB1*13:01		80 (37.7%)
	DRB1*13:02		72 (34.0%)
	DRB1*13:03		53 (25.0%)
	DRB1*13:04		3 (1.4%)
	DRB1*13:27		1 (0.5%)
	DRB1*13:48		3 (1.4%)
DR14(DR6)	(=DRB1*14:01/54[c])	37 (0.043)	
DR7	DRB1*07:01	46 (0.054)	
DR8		67 (0.078)	
	DRB1*08:04		60 (89.6%)
	DRB1*08:06		7 (10.4%)
DR9	DRB1*09:01	34 (0.040)	
DR10	DRB1*10:01	41 (0.048)	

[a]Two-digit designations of alleles are given for the results of HLA class I genotyping. High-resolution (four-digit) designations for HLA-B alleles were applied when available. HLA-DRB1 alleles were determined at high resolution by sequence-based typing and grouped by inferred serotype specificity.
[b]Percentage of subtype within the respective group is given in parentheses.
[c]subgroups of HLA-B*15 exhibiting different serotype specificity.
[d]a HLA-DRB1*03:02-related variant which has not been reported and officially assigned as DRB1*03:53.
[e]DRB1*12:01, *12:06 and *12:10 share the identical sequence of the exon 2 of DRB1 gene and are combined in the present study. It is also true to DRB1*14:01and *14:54.

3.2. HLA polymorphisms

A total of 429 children enrolled in the present study were examined for HLA-A, -B, and -DRB1 polymorphisms, the most polymorphic and major antigenic loci in the HLA region. We could identify 16 alleles of HLA-A, 36 of HLA-B, and 30 of HLA-DRB1, including a novel variant allele of DRB1 (Table 1). None of the alleles were significantly deviated from Hardy–Weinberg equilibrium in the population, indicating that the possible contribution of an unidentifiable allele was negligible. A novel variant of HLA-DRB1 was found in five children by sequence-based typing, with the result of no exact match to any of known alleles. This new sequence appeared to be derived from DRB1*03:02 and was classified as DR18-subtype of DR3 because only one nucleotide at the first position of codon 49 was changed from G to C, encoding proline (CCG) substitution for glycine residue (GCG). The presence of this novel DRB1*03 allele was also confirmed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) by acquisition of the *Eco52* I cleavage site (CGGCCG) in DRB1 group 3 (DR3/11/6)–specific PCR product, one of the reactions for sequence-based typing (described in Subjects and methods section). The nucleotide sequence of the allele was deposited in the GenBank/EMBL/DDBJ database with accession number AB546194, and assigned as DRB1*03:53 officially by the World Health Organization (WHO) Nomenclature Committee [16]. Upon HLA haplotype analysis using PHASE, DRB1*03:53 was preferentially associated with HLA-B*42:01, the second most frequent B allele in the population (relative linkage disequilibrium [LD] = 0.776; see below and Supplementary Table S3). HLA-B*42:01 is also preferentially associated with DRB1*03:02 (relative LD = 0.636, suggesting that the DRB1*03:53 allele emerged after the establishment of HLA-B*42:01-DRB1*03:02 haplotype in the population.

Because the HLA antigen frequency was reported for Kassem and Nankam ethnicities in northern Ghana [17], we could compare the HLA antigen phenotype frequencies between different ethnic groups in Ghana (Supplementary Table S1). HLA-A2, -A3, -A30, -A66, -A68, -B44, -B52, -B57, -DR3, -DR13, and -DR14 were more frequent in our study subjects, whereas HLA-A23, -A33, -B8, -B78, -DR8, and -DR11 were less frequent. In accordance with the previous report, frequencies of HLA-DR4 alleles, DRB1*04:01 and *04:05, were very low. Unfortunately, we could not assess the effect of DR4 alleles on malaria incidence because the number of observation was limited.

In the 1303-bp-long DNA fragment derived from the upstream region of TNFA locus, seven SNPs were genotyped: -1073C>T, -1031T>C, -863C>A, -857C>T, -308G>A, -244G>A, and -238G>A at minor allele frequencies of 0.027, 0.132, 0.106, 0.048, 0.101, 0.138, and 0.004, respectively. All of these have been reported previously and are registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) with dbSNP identification numbers, rs9282875, rs1799964, rs1800630, rs1799724, rs1800629, rs673, and rs361525. By the estimation of haplotype frequency of these SNPs, eight combinations (or TNFAp alleles) were more than 0.01 and were designated as haplotype 1 through haplotype 8 (Supplementary Table S2).

3.3. HLA haplotype in Ghanaian population

Haplotype frequencies were estimated by PHASE v2.1.1 from unphased genotype data. HLA-B-DRB1 haplotype was analyzed for prevalent alleles existing more than 0.5% in the population (Supplementary Table S3). Haplotypes represented more than 1% in the population are listed in Supplementary Table S4. All of these prevalent haplotypes except for B*53:01-DRB1*03:02 haplotype are

Table 2
Risk ratio of carrier status of HLA alleles for malaria incidence: Clinical malaria

Risk factors	Incidence in carriers	Incidence in non-carriers	Attributable risk	Risk ratio
A*01	0.234 (5 in 21.3 person × yr)	0.208 (80 in 384.4 person × yr)	0.026	1.13
A*02	0.213 (32 in 150.4 person × yr)	0.208 (53 in 254.3 person × yr)	0.004	1.02
A*03	0.196 (18 in 92.0 person × yr)	0.215 (67 in 312.0 person × yr)	−0.019	0.91
A*23	0.216 (18 in 83.3 person × yr)	0.208 (67 in 321.5 person × yr)	0.008	1.04
A*30	0.229 (27 in 117.7 person × yr)	0.201 (58 in 288.9 person × yr)	0.029	1.14
A*33	0.177 (12 in 67.7 person × yr)	0.215 (73 in 340.1 person × yr)	−0.037	0.83
A*66	0.286 (7 in 24.5 person × yr)	0.205 (78 in 381.3 person × yr)	0.081	1.40
A*68	0.214 (22 in 102.6 person × yr)	0.208 (63 in 303.2 person × yr)	0.007	1.03
A*74	0.199 (7 in 35.2 person × yr)	0.211 (78 in 369.5 person × yr)	−0.012	0.94
B*07: 02	0.208 (12 in 57.7 person × yr)	0.210 (73 in 348.0 person × yr)	−0.002	0.99
B*15: 03	0.323 (12 in 37.1 person × yr)	0.199 (73 in 367.6 person × yr)	0.125	1.63
B*15:10	0.242 (10 in 41.4 person × yr)	0.206 (75 in 364.4 person × yr)	0.036	1.18
B*18: 01	0.318 (9 in 28.3 person × yr)	0.201 (76 in 377.5 person × yr)	0.117	1.58
B*35: 01	0.060 (4 in 66.2 person × yr)	0.239 (81 in 339.6 person × yr)	−0.178[a]	0.25
B*42: 01	0.217 (17 in 78.2 person × yr)	0.208 (68 in 327.5 person × yr)	0.010	1.05
B*44: 03	0.217 (11 in 50.7 person × yr)	0.208 (74 in 355.0 person × yr)	0.009	1.04
B*52: 01	0.279 (20 in 71.8 person × yr)	0.195 (65 in 334.0 person × yr)	0.084	1.43
B*53: 01	0.242 (30 in 124.1 person × yr)	0.195 (55 in 281.7 person × yr)	0.046	1.24
DRB1*03:01	0.363 (11 in 30.3 person × yr)	0.197 (74 in 375.4 person × yr)	0.166	1.84
DRB1*03:02	0.307 (36 in 117.3 person × yr)	0.170 (49 in 288.4 person × yr)	0.137[b]	1.81
DRB1*07:01	0.287 (13 in 45.3 person × yr)	0.200 (72 in 360.5 person × yr)	0.088	1.44
DRB1*08:04	0.170 (9 in 53.0 person × yr)	0.216 (76 in 351.8 person × yr)	−0.046	0.79
DRB1*10: 01	0.151 (6 in 39.8 person × yr)	0.216 (79 in 366.0 person × yr)	−0.065	0.70
DRB1*13: 01	0.235 (18 in 76.5 person × yr)	0.204 (67 in 328.2 person × yr)	0.031	1.15
DRB1*13: 02	0.111 (7 in 62.9 person × yr)	0.227 (78 in 343.9 person × yr)	−0.116[c]	0.49
DRB1*13: 03	0.180 (9 in 49.9 person × yr)	0.214 (76 in 354.8 person × yr)	−0.034	0.84
DRB1*15: 03	0.227 (22 in 97.1 person × yr)	0.205 (63 in 307.7 person × yr)	0.022	1.11
TNFAp-hap-1	0.236 (73 in 309.0 person × yr)	0.116 (11 in 95.1 person × yr)	0.121[d]	2.03
TNFAp-hap-2	0.277 (27 in 97.4 person × yr)	0.186 (57 in 306.7 person × yr)	0.091	1.49
TNFAp-hap-3	0.190 (14 in 73.7 person × yr)	0.212 (70 in 330.4 person × yr)	−0.022	0.90
TNFAp-hap-4	0.131 (9 in 69.0 person × yr)	0.224 (75 in 335.2 person × yr)	−0.093[e]	0.58
TNFAp-hap-5	0.139 (5 in 36.1 person × yr)	0.215 (79 in 368.0 person × yr)	−0.076	0.65

p_c, Corrected p value obtained by Bonferroni's method with correction factor of 32.
[a] $p = 1.9 \times 10^{-6}$, $p_c = 5.9 \times 10^{-5}$.
[b] $p = 0.0043$, $p_c > 0.05$ (not significant).
[c] $p = 0.020$, $p_c > 0.05$ (not significant).
[d] $p = 0.0031$, $p_c > 0.05$ (not significant).
[e] $p = 0.045$, $p_c > 0.05$ (not significant).

represented in excess as indicated by positive LD measures (Supplementary Table S4). The B*53:01-DRB1*03:02 haplotype in the current population would be generated by recombination events between the most prevalent ancestral haplotypes carrying the B*53:01 allele and those carrying the DRB1*03:02 allele during population history, but have not yet reached linkage equilibrium.

3.4. Risk analysis for clinical malaria

The effect of individual HLA allele on the incidence of clinical malaria was evaluated in 405 children (Table 2). We calculated an incidence of clinical malaria in carriers of given HLA allele as "risk-exposed group" with that of noncarriers as "non–risk-exposed group" by counting the events in the adjusted observation period for each subpopulation, for which the difference between these two values was the attributable risk conferred by possessing the given allele. Among the relatively prevalent HLA alleles, HLA-B*35:01 showed significant protective effect against clinical malaria, and an attributable risk of −0.178 (decreasing the incidence by 0.178 events/person × years), giving a p value of 1.9×10^{-6}. HLA-DRB1*13:02 and TNFAp haplotype 4, which were in positive LD with B*35:01 to consist of the third most prevalent HLA-B-DRB1 and HLA-B-TNFAp-DRB1 haplotypes (Supplementary Table S3), also conferred resistance against clinical malaria to a lesser extent, whereas HLA-DRB1*03:02 and TNFAp haplotype 1 increased the incidence significantly (Table 2). After correction for multiple comparisons by Bonferroni's method, only HLA-B*35:01 remained statistically significant ($p_c = 5.9 \times 10^{-5}$). The effect of HLA-B*35:01 appeared to be primary, because three relatively prevalent B*35:01-carrying haplotypes, B*35:01-DRB1*13:02, B*35:01-DRB1*10:01, and B*35:01-DRB1*07:01 exhibited similar tendency (data not shown).

The effect of HLA alleles on the incidence of malaria with high parasite load was also evaluated (Table 3). We defined clinically significant parasitemia as parasite density greater than 5000 iRBC/mm^3 because we observed many asymptomatic children with lower level of parasitemia less than 5,000 iRBC/mm^3 in the same population (data not shown). HLA-B*35:01 showed significant protective effect against parasitemia giving an attributable risk of −0.073 events/person × years ($p = 0.00066$). The opposite effect was found in HLA-A*23, HLA-B*53:01, and TNFAp haplotype 1, with the incidence increasing by 0.085 ($p = 0.0366$), 0.122 ($p = 0.000\,48$), and 0.073 ($p = 0.0010$), respectively. The decreasing and increasing effects with HLA-B*35:01 and HLA-B*53:01 carriers remained statistically significant after the correction for multiple comparisons ($p_c = 0.021$ and 0.014, respectively), as well as the effect of TNFA promoter allele, TNFAp haplotype 1 ($p_c = 0.033$). In contrast, no difference was demonstrated in incidence of parasitemia between carriers and noncarriers for any HLA-DRB1 allele.

3.5. HLA effect on parasite density

Geographic mean values of parasite density measured at febrile episodes occurred in the children with different carrier status for HLA alleles were compared and the heterogeneity was evaluated by ANOVA (Table 4). Febrile episodes in nine subgroups of children with different HLA-A carrier status exhibited significantly differential parasite density ($p = 0.011$) and those of different HLA-B carriers were marginally different ($p = 0.047$), whereas HLA-DRB1 and TNFAp alleles had no significant effects. Post-hoc analysis with

Table 3
Risk ratio of carrier status of HLA alleles for malaria incidence: Parasitemia >5,000/mm^3

Risk factor	Incidence in carriers	Incidence in noncarriers	Attributable risk	Risk ratio
A*01	0.234 (5 in 21.3 person × yr)	0.068 (26 in 384.42 person × yr)	0.167	3.47
A*02	0.066 (10 in 150.44 person × yr)	0.083 (21 in 254.29 person × yr)	−0.016	0.81
A*03	0.109 (10 in 92 person × yr)	0.067 (21 in 312.02 person × yr)	0.041	1.62
A*23	0.144 (12 in 83.25 person × yr)	0.059 (19 in 321.48 person × yr)	0.085[a]	2.44
A*30	0.042 (5 in 117.73 person × yr)	0.092 (6 in 288.94 person × yr)	−0.048	0.47
A*33	0.103 (7 in 67.65 person × yr)	0.071 (24 in 340.14 person × yr)	0.033	1.47
A*66	0.204 (5 in 24.5 person × yr)	0.068 (26 in 381.25 person × yr)	0.136	2.99
A*68	0.117 (12 in 102.58 person × yr)	0.063 (19 in 303.17 person × yr)	0.054	1.87
A*74	0.057 (2 in 35.19 person × yr)	0.078 (29 in 369.54 person × yr)	−0.022	0.72
B*07:02	0.087 (5 in 57.67 person × yr)	0.075 (26 in 348.01 person × yr)	0.012	1.16
B*15:03	0.162 (6 in 37.12 person × yr)	0.068 (25 in 367.62 person × yr)	0.094	2.38
B*15:10	0.169 (7 in 41.35 person × yr)	0.066 (24 in 364.4 person × yr)	0.103	2.57
B*18:01	0.212 (6 in 28.29 person × yr)	0.066 (25 in 377.46 person × yr)	0.146	3.20
B*35:01	0.015 (1 in 66.15 person × yr)	0.088 (30 in 339.6 person × yr)	−0.073[b]	0.17
B*42:01	0.051 (4 in 78.23 person × yr)	0.082 (27 in 327.52 person × yr)	−0.031	0.62
B*44:03	0.039 (2 in 50.67 person × yr)	0.082 (29 in 355.01 person × yr)	−0.042	0.48
B*52:01	0.070 (5 in 71.77 person × yr)	0.078 (26 in 333.98 person × yr)	−0.008	0.90
B*53:01	0.161 (20 in 124.1 person × yr)	0.039 (11 in 281.65 person × yr)	0.122[c]	4.13
DRB1*03:01	0.198 (6 in 30.31 person × yr)	0.067 (25 in 375.44 person × yr)	0.131	2.97
DRB1*03:02	0.051 (6 in 117.33 person × yr)	0.087 (25 in 288.42 person × yr)	−0.036	0.59
DRB1*07:01	0.133 (6 in 45.25 person × yr)	0.069 (25 in 360.5 person × yr)	0.063	1.91
DRB1*08:04	0.113 (6 in 52.96 person × yr)	0.071 (25 in 351.77 person × yr)	0.042	1.59
DRB1*10:01	0.126 (5 in 39.79 person × yr)	0.071 (26 in 365.96 person × yr)	0.055	1.77
DRB1*13:01	0.078 (6 in 76.50 person × yr)	0.076 (25 in 328.23 person × yr)	0.002	1.03
DRB1*13:02	0.048 (3 in 62.92 person × yr)	0.081 (28 in 343.85 person × yr)	−0.034	0.59
DRB1*13:03	0.140 (7 in 49.92 person × yr)	0.068 (24 in 354.81 person × yr)	0.073	2.07
DRB1*15:03	0.062 (6 in 97.06 person × yr)	0.081 (25 in 307.67 person × yr)	−0.019	0.76
TNFAp-hap-1	0.094 (29 in 309.0 person × yr)	0.021 (2 in 95.1 person × yr)	0.073[d]	4.46
TNFAp-hap-2	0.072 (7 in 97.4 person × yr)	0.078 (24 in 306.7 person × yr)	−0.006	0.92
TNFAp-hap-3	0.054 (4 in 73.7 person × yr)	0.082 (27 in 330.4 person × yr)	−0.027	0.66
TNFAp-hap-4	0.073 (5 in 69.0 person × yr)	0.078 (26 in 335.2 person × yr)	−0.005	0.94
TNFAp-hap-5	0.083 (3 in 36.1 person × yr)	0.076 (28 in 368.0 person × yr)	0.007	1.09

p_c, Corrected p value obtained by Bonferroni's method with correction factor of 32.
[a] $p = 0.0366$, $p_c > 0.05$ (not significant).
[b] $p = 0.00066$, $p_c = 0.021$.
[c] $p = 0.00048$, $p_c = 0.015$.
[d] $p = 0.0010$, $p_c = 0.033$.

Student's *t*-test for comparison between geographic means of parasite load of carriers and non-carriers of given HLA alleles was conducted to reveal the allele(s) confer the effect. Consequently, HLA-A*01 and -A*23 were identified as alleles conferring the higher density of parasite with mean parasitemia of 22400 and 5000 iRBC/mm^3 ($p = 6.0 \times 10^{-7}$, 0.023), respectively. HLA-B*53:01 also increased mean parasitemia but did not reach statistically significant levels.

4. Discussion

In the present report, we actively detected febrile events in a malaria-endemic coastal region of Ghana to examine by a prospective study whether genetic factors had any effect on malaria-related events. We achieved a high coverage rate (94.4%) for febrile case detection, indicating that a detection rate by bi-weekly visit was sufficient. Although the study area was geographically close to our previous site, Dodowa [8–10], the malaria endemicity was very different, and as a consequence, the incidence was much lower. The lower endemicity in Asutuare was confirmed by cross-sectional surveillance of parasitemia in the population performed in November 2008 after the longitudinal study, by which we could find only low levels of parasite load of less than 5,000 iRBC/mm^3 without clinical manifestations in approximately 15% of children (data not shown). The incidence of clinical malaria was not significantly different between ages of the cohort, suggesting that the childhood intermediate levels of antimalaria immunity had been established before the age of 3 years in the population. Although immunity is raised, protection is not perfect, and allows the development of clinical symptoms in a subset of children. Therefore, the situation allows us to find out genetic and immunologic characteristics accompanying these symptomatic children.

Several deteriorating mutant alleles of the genes important for the normal biologic processes have been positively selected under the pressure of infectious agents such as *P falciparum* (*Pf*) by providing beneficial effects on the host (for review, see Kwiatkowski [18]). Mutations have resulted in destabilization of membrane erythrocytes, which cause hemolysis and mild to serious erythrocyte loss under stressful conditions, and are expected to be eliminated from the population gene pool because of poorer fitness in the absence of selection pressure of malaria, but have been maintained by the counter-balance [18]. Immunity-related genetic variations are also presumably candidates for driving force of the selection, which has been demonstrated by increased extended haplotype homozygosity around the TNFSF5 locus for example [19]. Previously, HLA-B*53:01 was identified as a resistant allele against severe malaria presumably by directing cellular immunity against liver stage–specific antigens [9,20,21]. The results of the present study appear to be different: HLA-B*35:01, but not HLA-B*53:01, was significantly associated with lower incidence of clinical malaria, and, in contrast, the incidence of high parasitemia with mild symptoms increased in carriers of HLA-B*53:01. A high frequency of HLA-B*53:01 in individuals of African decent is explained by an advantage under the selection pressure of malaria. It is presumable that *Pf* has evolved to adapt with the host polymorphisms so as not to cause very severe disease, but to retain a certain level of parasitemia accompanied by mild clinical manifestations. Another population-based study of HLA in light of selection by malarial exposure was conducted in the absence of HLA-B*53:01 in Sardinia

Table 4
Effect of HLA alleles on parasite density

Locus	Carriers of allele	Log $_{10}$ (parasite density)[a]		p (ANOVA)[b]	p (Student's t-test)[c]
HLA-A			(iRBC/mm^3)	0.011	
	A*01	4.35	(22,400)		6.0×10^{-7}
	A*02	2.92	(800)		
	A*03	3.29	(1900)		
	A*23	3.70	(5000)		0.023
	A*30	2.90	(800)		0.072
	A*33	3.19	(1500)		
	A*66	3.82	(6600)		0.088
	A*68	3.40	(2500)		
	A*74	2.97	(900)		
HLA-B				0.047	
	B*07:02	3.19	(1500)		
	B*15:03	3.03	(1100)		
	B*15:10	3.65	(4500)		
	B*18:01	3.67	(4700)		
	B*35:01	2.61	(400)		
	B*42:01	2.82	(700)		0.084
	B*44:03	2.79	(600)		
	B*52:01	2.82	(700)		0.068
	B*53:01	3.53	(3400)		0.056
HLA-DRB1				0.132	
	DRB1*03:01	3.37	(2300)		NA
	DRB1*03:02	2.80	(600)		NA
	DRB1*07:01	3.37	(2300)		NA
	DRB1*08:04	3.57	(3700)		NA
	DRB1*10:01	3.58	(3800)		NA
	DRB1*13:01	2.91	(800)		NA
	DRB1*13:02	2.83	(700)		NA
	DRB1*13:03	3.56	(3600)		NA
	DRB1*15:03	2.93	(900)		NA
TNFA				0.183	
	TNFAp-hap-1	3.26	(1800)		NA
	TNFAp-hap-2	2.85	(700)		NA
	TNFAp-hap-3	2.97	(900)		NA
	TNFAp-hap-4	3.55	(3500)		NA
	TNFAp-hap-5	3.65	(4500)		NA

NA, not applicable.
[a]Base-10 logarithmic transform of geographic mean of parasite density observed in carriers of given allele. log_{10} (mean of all clinical malaria) = 3.18 (1,500 iRBC/mm^3)
[b]Heterogeneity among subgroups stratified according to carrier status of prevalent alleles was examined by locus-wise ANOVA.
[c]Allele effect was assessed for positive locus by post hoc evaluation with Student's t-test to compare parasite densities between carriers and noncarriers. Only significant and nearly significant ($p < 0.09$) results are shown.

[22], where the frequency of HLA-B*35 alleles was higher in the lowland villages versus the highland villages, indicating positive correlation between the frequency of HLA-B*35 alleles and incidence of malaria.

It is of note the two HLA-B alleles, HLA-B*53:01 and HLA-B*35:01, which showed different effects in the present study, share overall structural similarity except for the portion encoding the Pc pocket of antigen-binding groove for the C-terminal anchor residue of antigen peptides. Differential binding properties between HLA-B*53:01 and HLA-B*35:01 were also found in the case of human immunodeficiency virus (HIV) antigen peptides and elucidate a part of the HLA-associated genetic risk for aquired immune deficiency syndromoe (AIDS) through antiviral immunity to prevent disease progression to AIDS [23]. It is reported that the polymorphic variants of circum sporozoite protein found in Gambia were differentially immunogenic through the binding capacity to HLA-B molecule, whereas HLA-B35 directed to provoke cytotoxic T lymphocytes but the variant still remained prevalent [24]. Polymorphism in parasite populations seems to be maintained by evasion of host immunity by mutual interference or by antagonism of altered peptide epitopes of variants [25]. Accordingly, HLA-B alleles that have been shaped by selection of fatal malaria infection provide beneficial conditions to pathogens for nonfatal malaria infection, by means of generation of polymorphic variants to give rise antagonistic antigen epitopes.

We determined unphased genotypes of seven SNPs in a 1303-bp-long upstream region of the TNFA gene. Eight haplotypes of these seven SNPs were estimated to be present in more than 1% in the population and were designated as TNFAp alleles (haplotype 1 through haplotype 8) in a way similar to that in previous studies conducted in Japanese [26] and Thai [27] populations. We could not reproduce the results of a previous study [28], an association of TNFP-D (haplotype 5 in the present study; carrying T at position -857) with severe malaria, presumably because of the different epidemiologic settings of the studies.

HLA-A has not attracted general attention in the malaria immunity, in comparison with HLA-B, because most studies have detected HLA-B alleles associated with susceptibility/resistance to malaria but not HLA-A alleles. It is not attributable to an inability of HLA-A to present malarial antigen, as certain HLA-A allelic restrictions of cytotoxic T-cell response were demonstrated for several malarial antigens [29]. In the present study, we assess the HLA effect on clinical malaria in two ways: by an increase/decrease in incidence, and by mean parasite density. When the incidence of malaria with parasitemia greater than 5,000 iRBC/mm^3 was compared, no HLA-A alleles yielded significant results, but possession of HLA-A*01 exhibited a tendency to increase the incidence by 0.167 person $\times$ year ($p > 0.05$) and possession HLA-A*23 did by 0.085 person $\times$ year ($p = 0.0366$, $p_c > 0.05$). The parasite density of clinical malaria episodes was significantly higher only with the carriers of these two HLA-A alleles, indicating that HLA-A also participates in the host defense mechanism against malaria infection.

Acknowledgments

This work was supported by in part by Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and the Global Centers of Excellence Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.humimm.2011.06.007.

References

[1] World Health Organization Report 2009. Available at: http://www.who.int/malaria/en/; last date of access: 12 July 2011.
[2] Gupta S, Snow RW, Donnelly CA, Marsh K, Newbold C. Immunity to non-cerebral severe malaria is acquired after one or two infections. Nat Med 1999;5:340-3.
[3] Mwangi TW, Ross A, Snow RW, Marsh K. Case definitions of clinical malaria under different transmission conditions in Kilifi District, Kenya. J Infect Dis 2005;191:1932-9.
[4] Stevenson MM, Tam MF. Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice. Clin Exp Immunol 1993;92:77-83.
[5] Taylor-Robinson AW, Phillips RS. B cells are required for the switch from Th1-to Th2-regulated immune responses to *Plasmodium chabaudi* chabaudi infection. Infect Immun 1994;62:2490-8.
[6] Ing R, Stevenson MM. Dendritic cell and NK cell reciprocal cross talk promotes gamma interferon-dependent immunity to blood-stage *Plasmodium chabaudi* AS infection in mice. Infect Immun 2009;77:770-82.
[7] Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, et al. Common West African HLA antigens are associated with protection from severe malaria. Nature 1991;352:595-600.
[8] Afari EA, Appawu M, Dunyo S, Baffoe-Wilmot A, Nkrumah FK. Malaria infection, morbidity and transmission in two ecological zones Southern Ghana. Afr J Health Sci 1995;2:312-5.
[9] Dodoo D, Theander TG, Kurtzhals JA, Koram K, Riley E, Akanmori BD, et al. Levels of antibody to conserved parts of *Plasmodium falciparum* merozoite surface protein 1 in Ghanaian children are not associated with protection from clinical malaria. Infect Immun 1999;67:2131-7.
[10] Dodoo D, Theisen M, Kurtzhals JA, Akanmori BD, Koram KA, Jepsen S, et al. Naturally acquired antibodies to the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. J Infect Dis 2000;181:1202-5.

[11] Voorter CE, Rozemuller EH, de Bruyn-Geraets D, van der Zwan AW, Tilanus MG, van den Berg-Loonen EM. Comparison of DRB sequence-based typing using different strategies. Tissue Antigens 1997;49:471–6.
[12] Agresti A, Coull BA. Approximate is better than ""Exact"" for interval estimation of binomial proportions. Am Stat 1998;52:119–26.
[13] Stephens M, Smith N, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 2001;68:978–89.
[14] Kaelin M, Bayona M. Attributable Risk Applications in Epidemiology. Instructional Unit, the Young Epidemiology Scholars Program (YES). College Board 2004 Available at: http://www.collegeboard.com/prod_downloads/yes/4297_MODULE_17.pdf; last date of access: 12 July 2011.
[15] Bewick V, Cheek L, Ball J. Statistics review 8: Qualitative data—tests of association. Crit Care 2004;8:46–53.
[16] Marsh SG, WHO Nomenclature Committee for Factors of the HLA System. Nomenclature for factors of the HLA System update April 2010. Tissues Antigens 2010;76:501–8.
[17] Osafo-Addo AD, Koram KA, Oduro AR, Wilson M, Hodgson A, Rogers WO. HLA-DRB1*04 allele is associated with severe malaria in northern Ghana. Am J Trop Med Hyg 2008;78:251–5.
[18] Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet 2005;77:171–92.
[19] Sabeti PC, Reich DE, Higgins JM, Levine HZ, Richter DJ, Schaffner SF, et al. Detecting recent positive selection in the human genome from haplotype structure. Nature 2002;419:832–7.
[20] Hill AV, Elvin J, Willis AC, Aidoo M, Allsopp CE, Gotch FM, et al. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature 1992;360:434–9.
[21] Hill AV, Jepson A, Plebanski M, Gilbert SC. Genetic analysis of host-parasite coevolution in human malaria. Philos Trans R Soc Lond B Biol Sci 1997;352:1317–25.
[22] Contu L, Carcassi C, Orru S, Mulargia M, Arras M, Boero R, et al. HLA-B35 frequency variations correlate with malaria infection in Sardinia. Tissue Antigens 1998;52:452–61.
[23] Gao X, Nelson GW, Karacki P, Martin MP, Phair J, Kaslow R, et al. Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. N Engl J Med 2001;344:1668–75.
[24] Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, et al. Association of malaria parasite population structure, HLA, and immunological antagonism. Science 1998;279:1173–7.
[25] Plebanski M, Lee EAM, Hannan CM, Flanagan KL, Gilbert SC, Gravenor MB, et al. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. Nat Med 1999;5:565–71.
[26] Hamaguchi K, Kimura A, Seki N, Higuchi T, Yasunaga S, Takahashi M, et al. Analysis of tumor necrosis factor-alpha promoter polymorphism in type 1 diabetes: HLA-B and -DRB1 alleles are primarily associated with the disease in Japanese. Tissue Antigens 2000;55:10–6.
[27] Hananantachai H, Patarapotikul J, Ohashi J, Naka I, Krudsood S, Looareesuwan S, et al. Significant association between TNF-alpha (TNF) promoter allele (-1031C, -863C, and -857C) and cerebral malaria in Thailand. Tissue Antigens 2007;69:277–80.
[28] Ubalee R, Suzuki F, Kikuchi M, Tasanor O, Wattanagoon Y, Ruangweerayut R et al. Strong association of a tumor necrosis factor-alpha promoter allele with cerebral malaria in Myanmar. Tissue Antigens 2001;58:407–10.
[29] Lyke KE, Burges RB, Cissoko Y, Sangare L, Kone A, Dao M, et al. HLA-A2 supertype-restricted cell-mediated immunity by peripheral blood mononuclear cells derived from Malian children with severe or uncomplicated Plasmodium falciparum malaria and healthy controls. Infect Immun 2005;73:5799–808.

Parasitology International 60 (2011) 347–356

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Association of HLA and post-schistosomal hepatic disorder: A systematic review and meta-analysis☆,☆☆

Nguyen Tien Huy [a], Mohamed Hamada [b], Mihoko Kikuchi [a,c], Nguyen Thi Phuong Lan [d], Michio Yasunami [a], Javier Zamora [e], Kenji Hirayama [a,f,*]

[a] Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[b] Zoology Department, Faculty of Science, Cairo University, Cairo 12613, Egypt
[c] Center for International Collaborative Research, Nagasaki University (CICORN), 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[d] Department of Immunology and Microbiology, Pasteur Institute of Ho Chi Minh City, 167 Pasteur Street, District 3, Ho Chi Minh City, Vietnam
[e] Clinical Biostatistics Unit, Ramón y Cajal Hospital and CIBER Epidemiologia y Salud Publica (CIBERESP), Spain
[f] Global COE program, Nagasaki University, Japan

ARTICLE INFO

Article history:
Received 17 February 2011
Received in revised form 11 May 2011
Accepted 26 May 2011
Available online 12 June 2011

Keywords:
Genetic
Hepatic disorder
HLA
Meta-analysis
Schistosomiasis

ABSTRACT

Several human genetic variants, HLA antigens and alleles are reportedly linked to post-schistosomal hepatic disorder (PSHD), but the results from these reports are highly inconclusive. In order to estimate overall associations between human genetic variants, HLA antigens, HLA alleles and PSHD, we systematically reviewed and performed a meta-analysis of relevant studies in both post-schistosomal hepatic disorder and post-schistosomal non-hepatic disorder patients. PubMed, Scopus, Google Scholar, The HuGE Published Literature database, Cochrane Library, and manual search of reference lists of articles published before July 2009 were used to retrieve relevant studies. Two reviewers independently selected articles and extracted data on study characteristics and data regarding the association between genetic variants, HLA antigens, HLA alleles and PSHD in the form of 2×2 tables. A meta-analysis using fixed-effects or random-effects models to pooled odds ratios (OR) with corresponding 95% confidence intervals were calculated only if more than one study had investigated particular variation. We found 17 articles that met our eligibility criteria. *Schistosoma mansoni* and *Schistosoma japonicum* were reported as the species causing PSHD. Since human genetic variants were only investigated in one study, these markers were not assessed by meta-analysis. Thus, only HLA-genes (a total of 66 HLA markers) were conducted in the meta-analysis. Our meta-analysis showed that human leucocyte antigens HLA-DQB1*0201 (OR = 2.64, $P = 0.018$), DQB1*0303 (OR = 1.93, $P = 0.008$), and DRB1*0901 (OR = 2.14, $P = 0.002$) alleles and HLA-A1 (OR = 5.10, $P = 0.001$), A2 (OR = 2.17, $P = 0.005$), B5 (OR = 4.63, $P = 0.001$), B8 (OR = 2.99, $P = 0.02$), and B12 (OR = 5.49, $P = 0.005$) serotypes enhanced susceptibility to PSHD, whereas HLA-DQA1*0501 (OR = 0.29, $P \leq 0.001$) and DQB1*0301 (OR = 0.58, $P = 0.007$) were protective factors against the disease. We further suggested that the DRB1*0901-DQB1*0201, DRB1*0901-DQB1*0303 and A1-B8 haplotypes enhanced susceptibility to PSHD, whereas DQA1*0501-DQB1*0301 linkage decreased the risk of PSHD. The result improved our understanding of the association between the HLA loci and PSHD with regard to pathogenic or protective T-cells and provided novel evidence that HLA alleles may influence disease severity.

1. Introduction

Schistosomiasis is well established as a major health problem in Africa, Asia, and South America [1]. Each year, more than 250,000 deaths are reported from an estimated 200 million individual sufferers [2,3] due to complications of chronic schistosomiasis-related liver disease [4]. Chronic schistosomiasis-related liver disease is caused by *Schistosoma mansoni* and *Schistosoma japonicum*. The disease is characterized by periodic activation of the host immune system against fluke eggs that were deposited in the intestinal wall veins and then delivered to the liver by the blood flow, resulting in granuloma formation and peri-portal fibrosis [5]. Peri-portal fibrosis can cause portal blood hypertension, venous obstruction, splenomegaly, esophageal varices, ascites,

☆ The authors have declared that no competing interests exist. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

☆☆ Author's Contribution: Conceived and designed the experiments: NTH and KH. Analyzed the data: NTH, MH, MK, NTPL, JZ and KH. Contributed reagents/materials/analysis tools: NTH, MH, MK, NTPL, MY, JZ and KH. Wrote the paper: NTH, MH, NTPL, JZ and KH.

* Corresponding author at: Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
E-mail addresses: tienhuy@nagasaki-u.ac.jp (N.T. Huy), m_hamada_mi@yahoo.com (M. Hamada), mkikuchi@nagasaki-u.ac.jp (M. Kikuchi), planpasteur@yahoo.com (N.T.P. Lan), yasunami@nagasaki-u.ac.jp (M. Yasunami), javier.zamora@hrc.es (J. Zamora), hiraken@nagasaki-u.ac.jp (K. Hirayama).

1383-5769/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2011.05.008

hematemesis, and congestive heart failure resulting to death in the absence of proper treatment [5].

Schistosomicides are generally administered with the aim of killing the parasite and preventing against re-infections, while waiting for the excretion of tissue-dwelling eggs. However, only one schistosomicide, praziquantel, has been used in large-scale controlled programs, raising concerns about the development of drug resistance [6]. Therefore, it is necessary to study the molecular, biochemical, and immunological aspects of schistosomiasis to develop vaccines and new treatments.

The pathogenesis of peri-portal fibrosis is not yet completely understood. Host immunity, including suppressed Th1/predominant Th2 profiles [7,8], changes in cytokine production [9,10], T-cell responses [11,12], and B-cell responses [13], have been extensively analyzed as a factor that is potentially responsible for fibrotic development. In addition, host genetic background is considered a potential risk factor contributing to disease development [14–16]. Although associations between post-schistosomal hepatic disorder (PSHD) and HLA antigens, HLA alleles, and genetic variations have been reported in individual studies, the associations are not observed consistently across studies. Therefore, the present study was conducted to estimate overall associations between human genetic variants, HLA antigens, HLA alleles and PSHD by systemic review and meta-analysis of relevant studies in both post-schistosomal hepatic disorder and post-schistosomal non-hepatic disorder patients.

2. Methods

2.1. Search strategy and study selection

A protocol was designed before this study was performed as recommended by the PRISMA statement (http://www.prisma-statement.org/statement.htm). In July 2009, five electronic databases: PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez), Scopus (http://www.scopus.com/scopus/home.url), Google Scholar (http://scholar.google.com/), The HuGE Published Literature database (http://hugenavigator.net/HuGENavigator/startPagePubLit.do), and the Cochrane Library (http://www.cochrane.org) were searched for suitable studies. The search terms used for PubMed and Scopus were as follows: "("gene variant" OR polymorphism OR SNP OR genotyp* OR "Case control study" OR Haplotype OR "linkage disequilibrium" OR "genetic risk") AND schistosom*" (OR was used in the literature search as a connect word). We used "schistosoma OR schistosomiasis OR schistosome" to search in Cochrane Library and The HuGE Published Literature database. For the "Advanced Scholar Search", we used "schistosoma OR schistosomiasis OR schistosome" to fill in the field "with all of the words", "genetic polymorphism variant SNP genotype Haplotype disequilibrium" to fill in the field "with at least one of the words", and "where my words occur" in the field "title of article". We further supplemented these searches with a manual search of reference lists and citation list using the Scopus databases. For each identified gene, we performed gene-specific searches by replacing the genetic terms with gene name terms.

We sought only articles that evaluated the association between post-schistosomal hepatic disorder (PSHD) and human genetic variants, HLA antigens, or HLA alleles. No restrictions were made with respect to language, patient age (children or adult), gender, or study design (family-based association studies or population designs that use un-related individuals). Since a number of zero event were found in several variants from included studies, and a meta-analysis method of adding 0.5 to cells with zero event creates estimation problems if the sample size is too small. We excluded studies with fewer than 20 participants (at least 10 cases and 10 controls were required for inclusion) to limit selection bias [17] and reduce the effect of adding 0.5 to cells with zero event on the result less than 5%. Furthermore, studies were also excluded for any of the following reasons: (1) studies that were reported as animal studies, case reports, scientific correspondence, or reviews; (2) studies from which data could not be reliably extracted; (3) studies that used healthy individuals or other severe forms of schistosomiasis as the control group.

Initially, two independent reviewers (Huy and Hamada) scanned primary titles and abstracts (when available) to select potential full text articles for further scrutiny. When the title and abstract could not be rejected by any reviewer, the full text of the article was obtained and carefully reviewed for inclusion by the two reviewers. Inclusion or exclusion of each study was determined by discussion and consensus between the two reviewers.

2.2. Data management

Full-text versions of all papers eligible for inclusion were obtained and, after the inclusion was confirmed, data were extracted by two independent investigators (Huy and Hamada). The data extracted included the first author, year of publication, study design (family-based or case–control), country of origin, source of the samples (clinic-, hospital-, or community-based), number of included individuals, gender ratio, and age at examination of included individuals.

In cases where genotype or allele distributions were not provided or data contained obvious errors in the original publication (such as typographical errors, switched allele frequencies, or incorrect allele designations), we attempted to clarify the discrepancy directly with study authors, generally by contacting the first and last authors twice via e-mail. Studies for which we could not obtain clarification or genotype information (after at least two attempts by e-mail) are listed as "no data available".

2.3. Duplicate publications

Papers published by same research group and studying the same genetic variation or HLA types were checked for potential duplicate data. Whenever data overlap was suspected, authors were contacted via e-mail and asked for clarification. If no clarification could be obtained (for example, if we received no answer after at least two attempts by e-mail), data sets were considered as overlapping, and the largest data set was used for meta-analysis.

2.4. Meta-analysis

Meta-analyses for population designs and for family studies were performed separately [18,19] using Comprehensive Meta-analysis software version 2.0 (http://www.meta-analysis.com). For each genetic variation or HLA types, 2×2 tables were generated, and the odds ratio (OR) for particular allele was computed. Heterogeneity between studies was evaluated using the Q statistic and I^2-test. Heterogeneity was considered statistically significant if P was less than 0.10 [20]. I^2 values >25%, 50%, or 75% are considered as low, moderate, or high heterogeneity, respectively [21]. Pooled OR with the corresponding 95% confidence intervals (95% CI) was calculated only if more than one study had investigated a particular allele. A fixed-effects model with weighting of the studies was used when there was a lack of significant heterogeneity ($P>0.10$), while a random-effects model with weighting of the studies was used when there was heterogeneity between studies ($P\leq0.10$) [22]. Adjustment of P value for multiple comparisons was not conducted because it may increase the likelihood of type II errors [23,24]. In order to reduce the false discovery rate, a confidence interval and interpretation of across studies are proposed to give complement information to P value. Therefore, in the present study, statistical significance was defined as P value was <0.05 (two-tailed test) and the 95% CI of OR did not overlap 0.9–1.1, in combination with replicated direction in results across studies.

Finally, to assess the presence of publication bias statistically, we performed Egger's regression test where there were three or more studies assessing the effect of a particular allele on the development of PSHD [25,26]. Publication bias was considered significant when the *P* value was <0.1. Publication bias was further evaluated by Begg's modified funnel plot where there were five or more studies assessing the effect of a particular allele [27].

3. Results

3.1. Study characteristics

Our literature searches identified 395, 578, 303, and 33 publications in the initial searches of PubMed, Scopus, Google Scholar, and HuGE Published Literature, respectively (Fig. 1). After screening the title and/or abstract, 79 articles were selected for full text reading. We further identified additional studies by searching reference lists and tracking articles citing relevant publications using the Scopus databases from the selected full text studies, review articles, and textbook chapters. A total of 62 articles were excluded from the 79 articles that were read in full for one of the following reasons: (1) Leishmaniasis association (n=1), (2) review/conference/book/thesis (n=15), (3) genetic studies of parasite (n=6), (4) no genetic association (n=2), (5) animal research (n=1), (6) no patient with hepatic disorder (n=25), (7) negative control was intestinal or cerebral schistosomiasis (n=2), (8) negative control was healthy group or non-schistosomiasis (n=2), (9) co-infection with hepatitis C or HIV (n=2), (10) unable to extract data and no response after contacting the authors via email (n=3) [28–30], and (11) overlapping studies (n=3) [31–33]. Finally, 17 studies were selected for final analysis [16,34–49].

The characteristics of the studies in this meta-analysis are outlined in Table 1. Most of the studies were case–control studies of un-related individuals (n=16); only one was a family-based study. More studies were performed among Africans (n=9) and Asians (n=7) than among South Americans (n=1). Ten studies investigated *S. mansoni*, while other 7 studies did on *S. japonicum*. Ten studies did not stated the gender information of subjects, all remained studies (n=7) showed dominant male in both PSHD and control groups. Nine studies included adults [16,35–37,40–43,48], three studies enrolled children subjects [44,46,49], one study recruited both adults and children [34], and four studies did not mention this information [38,39,45,47]. A total of three different methods for PSHD diagnosis (ultrasound: n=10, biopsy: n=2, clinical diagnosis n=4) were performed in 16 of 17 studies. One study did not mention the method for PSHD diagnosis [35]. Concerning the ultrasound for PSHD diagnosis, one study did not describe in detail [16], while the remaining studies used the WHO ultrasound criteria [50]. Only two studies took in consideration the prevalence of each variant in the general population [34,36]. The human leucocyte antigens (HLA) loci were investigated in 11 studies, while cytokines and other signal protein genes were investigated in six studies.

3.2. Meta-analysis

Since only one study investigated cytokine and other related protein genes, these markers were not assessed by meta-analysis. Thus, only HLA-types (a total of 66 HLA markers) were included in the meta-analysis as shown in Table 2. Pooled ORs showed that eight HLA variants – DQB1*0201, DQB1*0303, DRB1*0901, A1, A2, B5, B8, and B12 – were associated with a significant increase in risk for PSHD, while DQA1*0501 and DQB1*0301 were associated with a significant decrease in risk for PSHD ($P<0.05$) (Fig. 2). The negative association of DQA1*0501 and PSHD was consistent among two studies (*P* value for heterogeneity = 0.48, $I^2=0$) (Table 2 and Fig. 2A), whereas A1 and B5 were found associated with PSHD among two and three of five studies, respectively (Table 2 and Fig. 2B). The positive association DQB1*0201 and DRB1*0901 with PSHD were found to be significant in one study but not significant in another study with no evidence of significant heterogeneity between two studies (Table 2 and Fig. 2A). Similarly, DQB1*0301, DQB1*0303, A2, B8, and B12 were found to be significant in one study but not in two or three other studies. Significant heterogeneity among studies of the A1 and B5 antigens was found; however, based on the results of analysis using the random effect model, the risk of PSHD was significantly higher in carriers of these variants.

We further evaluate the effect of different schistosomal species on the significant association between HLA types and PSHD. All HLA

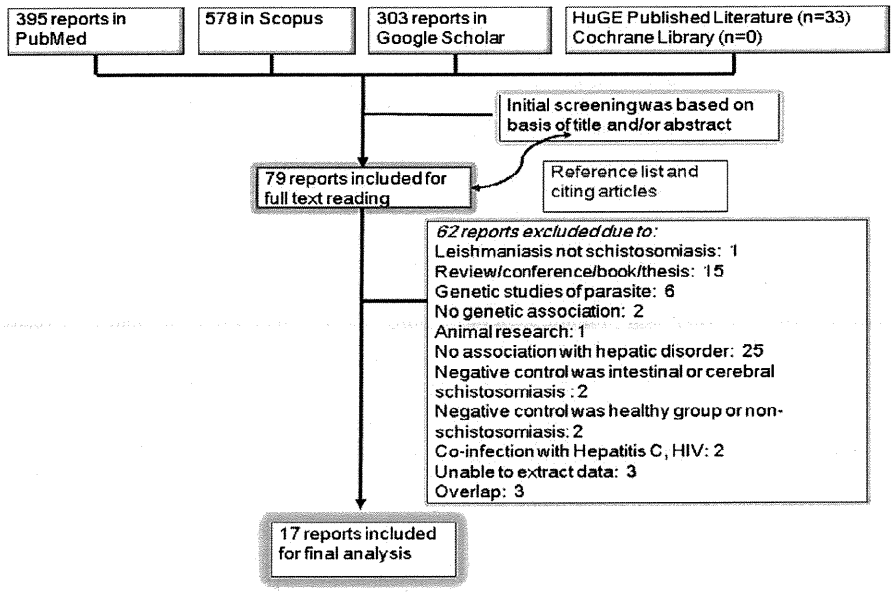

Fig. 1. Flow diagram of the search and review process.

Table 1
Characteristic of studies included in this analysis.

Authors	Year	Country	Strain	Study design	PSHD		Control		Diagnostic method of PSHD	Genetic method	Number of alleles studied
					No. (Female %)	Mean age (±SD or range)	No. (Female %)	Mean age (±SD or range)			
Elsammak	2008	Egypt	*S. mansoni*	Case control	22 (41)	53.7 (±5.6)	22 (41)	48.5 (± 5.4)	Ultrasound	PCR-RFLP	1 (LA)
Eriksson	2007	Uganda	*S. mansoni*	Case control	22 (ND)	ND	274 (ND)	ND	Ultrasound	PCR-SSOP	1 (ECP)
Cheng	2005	China	*S. japonicum*	Case control	45 (31)	54.9 (± 11)	44 (34)	51.6 (± 9.2)	ND	PCR-SSOP	16 (HLA)
Blanton	2005	Egypt	*S. mansoni*	Family based	48 (25)	42.3 (± 15)	188 (33)	39.1 (±13.2)	Ultrasound	UTSG	48 (11genes)
Zhang	2005	China	*S. japonicum*	Case control	46 (41)	51. 8 (± 10.)	43 (30)	55. 3(± 7. 8)	Ultrasound	PCR-SSOP	41 (HLA)
Chevillard	2003	Sudan	*S. mansoni*	Case control	29 (ND)	ND	76 (ND)	ND	Ultrasound	PCR-SSCP	5 (IFN-γ)
Moukoko	2003	Sudan	*S. mansoni*	Case control	27 (ND)	ND	71 (ND)	ND	Ultrasound	PCR-RFLP	4 (TNF-α)
Hirayama	2002	China	*S. japonicum*	Case control	156 (ND)	ND	36 (ND)	ND	Ultrasound	ND	1 (IL-13)
McManus	2001	China	*S. japonicum*	Case control	40 (ND)	ND	44 (ND)	ND	Ultrasound	PCR-SSOP	34 (HLA)
Hirayama	1999	China	*S. japonicum*	Case control	186 (40)	ND	44 (57)	ND	Ultrasound	PCR-SSOP	104 (HLA)
Waine	1998	China	*S. japonicum*	Case control	64 (ND)	59. 4 (± 8.8)	44 (ND)	54. 5(± 7. 1)	Ultrasound	PCR-SSOP	53 (HLA)
Hafez	1991	Egypt	*S. mansoni*	Case control	19 (26)	11.2 (7–14)	20 (40)	9.9 (7–14)	Biopsy	CDM	29 (HLA)
Cabello	1991	Brazil	*S. mansoni*	Case control	23 (ND)	ND	41 (ND)	ND	Clinical	CDM	38 (HLA)
Salam	1986	Egypt	*S. mansoni*	Case control	88 (ND)	11. 8 (± 1.1)	46 (ND)	12. 8 (± 0.5)	Biopsy	CDM	32 (HLA)
Wang	1984	China	*S. japonicum*	Case control	30 (ND)	ND	30 (ND)	ND	Clinical	CDM	24 (HLA)
El-Tayeb	1982	Egypt	*S. mansoni*	Case control	17 (ND)	37.3 (14–60)	17 (ND)	33.9 (14–55)	Clinical	CDM	25 (HLA)
Salam	1979	Egypt	*S. mansoni*	Case control	28 (32)	ND	23 (26)	ND	Clinical	CDM	37 (HLA)

TND, not described.
PCR-SSCP, PCR-single strand conformation polymorphism.
PCR-RFLP, PCR-restriction fragment length polymorphism.
CDM, complement-dependent microlymphocytotoxicity.
PCR-SSOP, PCR-sequence-specific oligonucleotides probes.
LA, lymphotoxin-α.
UTSG, ultra-high throughput SNP genotyping.
ECP, Eosinophil Cationic Protein.

variants — DQB1*0201, DQB1*0303, and DRB1*0901 were associated with *S. japonicum*-induced PSHD in all studies, while HLA antigens-A1, A2, B5, B8, and B12 were correlated with *S. mansoni*-induced PSHD in all studies except one study by Wang et al. (Table 1 and Fig. 2). Removing the study by Wang et al. had little effect on the significant association ($P<0.05$), pooled ORs and 95% CIs (data not shown).

4. Discussion

Our pooled results suggested that HLA-A1, -B8, DQB1*0201 are associated with increased risk of PSHD (Table 2 and Fig. 2B). Furthermore, these alleles are reportedly associated with the severity of other granulomatous diseases [51–53], suggesting that the mechanism of PSHD development shares some similar part of other granulomatous diseases.

The frequency of the HLA-DQB1*0201 allele was higher in PSHD patients in Zhang's study (OR = 5.83, $P=0.009$) and Hirayama's study (OR = 1.68, $P=0.31$) when compared to the control patients (Fig. 2A). This trend was also observed in the study by Secor et al. [29], which was not included in this analysis due to un-extractable data, further supporting this association. The DQB1*0201 allele has been also linked to the progression of cirrhosis due to hepatitis C virus (HCV) [54], the elevation of alanine aminotransferase levels in serum (a marker of the hepatic damage) [55], the risk of anti-tuberculosis drugs-hepatotoxicity [56], the severity of the intestinal mucosal damage [57], and the risk of several autoimmune disorders [58,59]. Furthermore, autoimmunity has also been proposed as a factor in the development of schistosomiasis-associated hepatic disease [60]. It has also been shown that human papillomavirus-16 E7 (aa 71–85) peptide presented to some pathogenic T cells resulted in dysplastic cervical lesions in individuals carrying HLA-DQB1*0201 [61]. Therefore, HLA-DQB1*0201 may play a role in the antigen presentation to some pathogenic T-cells that could enhance the development of PSHD.

Another HLA-DQB1 allele, DQB1*0303, has also been associated with PSHD (OR = 1.93, 95% CI = 1.19–3.14, $P=0.008$). There may be a closely linked variant (possibly in the HLA-DRB1 locus) that is primarily responsible for the PSHD susceptibility and associated with these DQB1 alleles. A number of studies have demonstrated DRB1*0901-DQB1*0201 [62–64] and DRB1*0901-DQB1*0303 linkage [41,65] are common in African and Asian populations. The DRB1*0901 allele was also associated with susceptibility to PSHD (OR = 2.14, 95% CI = 1.31–3.48, $P=0.002$). The possibility that the HLA-DRB1*0901 allele is primarily responsible for PSHD susceptibility is an interesting point to be considered because IgG4 elevation has been found in individuals with the HLA-DRB1*0901 allele [66] and is positively linked to several systemic fibrosis conditions [67] and schistosomiasis peri-portal fibrosis [68]. It is probable that patients with HLA-DRB1*0901 are prone to produce B cells for specific IgG4 and Th2 cells that are reactive to schistosomal antigens.

The HLA-DQA1*0501 (OR = 0.29, 95% CI = 0.17–0.50, $P<0.001$) allele was identified as a resistant allele for PSHD in two studies [37,42]. Furthermore, *Trypanosoma cruzi* B13 protein was reportedly recognized by T-cells in individuals bearing HLA-DQA1*0501 [69], suggesting that individual with HLA-DQA1*0501 may clear the parasite better than those without this allele. Another allele, HLA-DQB1*0301, correlated with PSHD resistance (OR = 0.58, 95% CI = 0.39–0.86, $P=0.007$), but results on this allele were inconsistent. Two studies indicated that DQB1*0301 had a protective effect against PSHD [42,43], while other two studies did not find a significant association [37,41]. The DQA1*0501-DQB1*0301 linkage is also a common haplotype in several studied populations [42,55,70] and may enhance resistance to PSHD development.

In class I, HLA- A1, -A2, -B5, -B8, and -B12 serotypes were significantly associated with an increased risk for PSHD. There was significant linkage disequilibrium of the A1-B8 haplotype in many prior studies of autoimmune disease risk (http://www.absoluteastronomy.com/topics/HLA-A1), further supporting the hypothesis that autoimmunity is an important mechanism in the pathogenesis of PSHD.

The results of our pooled OR suggested that DQA1*0101/4 was possibly associated with increased risk of PSHD after pooling analysis (OR = 2.59, 95% CI = 0.87–7.72, $P=0.087$) (Table 2), though the two separate studies of DQA1*0101/4 did not identify an increased risk of PSHD. Similarly, DQA1*0103 had a pooled OR of 1.76 with a 95% CI of 0.95–3.25 and P value of 0.073 even though it was not found to be

Table 2
Summary of associations between HLA alleles and hepatic disorders, pooled odds ratios (OR) with corresponding 95% confidence intervals (95% CI) of the published results were calculated where more than one study had investigated the allele.

HLA types	No. of study	Heterogeneity			Model	Association with hepatic disorders			Egger's 2-tailed bias
		Q value	*P* value	I-squared		*P* value	OR	95% CI	*P* value
DPA1*0103	2	6.06	0.014	83.5	Random	0.500	1.62	0.40–6.62	ND
DPA1*0201	2	3.80	0.051	73.7	Random	0.893	1.22	0.06–22.99	ND
DPA1*0401	2	0.002	0.97	0	Fixed	0.636	0.77	0.25–2.32	ND
DQA1*0101	2	0.00	0.993	0.0	Fixed	0.280	0.46	0.11–1.88	ND
DQA1*0101/4	2	0.83	0.362	0.0	Fixed	0.087	2.59	0.87–7.72	ND
DQA1*0102	3	1.28	0.527	0.0	Fixed	0.670	0.89	0.51–1.54	0.205
DQA1*0103	3	1.61	0.448	0.0	Fixed	0.073	1.76	0.95–3.25	0.21
DQA1*0201	3	3.48	0.175	42.6	Fixed	0.948	1.03	0.44–2.40	0.584
DQA1*0301	2	0.19	0.666	0.0	Fixed	0.389	1.43	0.64–3.21	ND
DQA1*0401	3	3.21	0.201	37.7	Fixed	0.624	0.75	0.23–2.42	0.052
DQA1*0501	**2**	**0.50**	**0.480**	**0.0**	**Fixed**	**<0.0001**	**0.29**	**0.17–0.50**	**ND**
DQA1*0601	3	16.57	0.000	87.9	Random	0.922	1.10	0.17–7.23	0.601
DQB1*0201	**2**	**2.14**	**0.144**	**53.2**	**Fixed**	**0.018**	**2.64**	**1.19–5.89**	**ND**
DQB1*0301	**4**	**5.67**	**0.129**	**47.0**	**Fixed**	**0.007**	**0.58**	**0.39–0.86**	**0.048**
DQB1*0302	4	4.12	0.249	27.1	Fixed	0.425	0.83	0.52–1.32	073
DQB1*0303	**4**	**4.80**	**0.187**	**37.5**	**Fixed**	**0.008**	**1.93**	**1.19–3.14**	**0.91**
DQB1*0401	3	1.93	0.380	0.0	Fixed	0.80	1.10	0.53–2.30	0.71
DQB1*0402	2	1.30	0.254	23.2	Fixed	0.29	0.32	0.04–2.64	ND
DQB1*0501	4	3.26	0.353	8.0	Fixed	0.40	1.75	0.48–6.43	0.11
DQB1*0502	4	1.54	0.672	0.0	Fixed	0.169	0.63	0.33–1.22	0.61
DQB1*0503.1	3	4.47	0.107	55.2	Fixed	0.68	1.37	0.30–6.23	0.23
DQB1*0601	4	13.20	0.004	77.3	Random	0.301	0.60	0.23–1.58	0.92
DQB1*0602	4	4.37	0.224	31.4	Fixed	0.902	0.96	0.50–1.83	0.62
DQB1*0603	3	2.53	0.283	20.9	Fixed	0.254	2.73	0.49–15.33	0.20
DQB1*0604	3	2.45	0.294	18.3	Fixed	0.501	0.55	0.09–3.19	0.93
DRB1*01	3	1.69	0.429	0.0	Fixed	0.848	0.85	0.16–4.48	0.33
DRB1*0301	3	4.60	0.100	56.5	Random	0.32	2.33	0.44–12.34	0.19
DRB1*0401	2	0.62	0.433	0.0	Fixed	0.95	1.06	0.18–6.21	ND
DRB1*0403	3	2.08	0.353	3.8	Fixed	0.56	1.38	0.47–4.05	0.115
DRB1*0404	3	1.38	0.502	0.0	Fixed	0.174	0.34	0.07–1.62	0.98
DRB1*0405	3	0.97	0.614	0.0	Fixed	0.42	1.35	0.66–2.78	0.49
DRB1*0406	3	1.99	0.370	0.0	Fixed	0.46	1.56	0.48–5.05	0.39
DRB1*0701	3	2.35	0.309	14.8	Fixed	0.661	0.81	0.31–2.11	0.22
DRB1*0901[a]	3	3.88	0.144	48.4	Fixed	0.49	1.18	0.75–1.86	0.85
DRB1*0901[b]	**2**	**2.54**	**0.111**	**60.7**	**Fixed**	**0.002**	**2.39**	**1.31–3.48**	**ND**
DRB1*1001	4	2.52	0.472	0.0	Fixed	0.91	0.92	0.25–3.46	0.09
DRB1*1101	3	4.77	0.092	58.1	Random	0.162	0.46	0.15–1.37	0.004
DRB1*1104	2	1.34	0.247	25.4	Fixed	0.91	0.88	0.09–8.58	ND
DRB1*1201	3	4.09	0.130	51.1	Fixed	0.87	1.08	0.45–2.62	0.31
DRB1*1202	3	11.89	0.003	83.2	Random	0.76	0.78	0.15–4.06	0.90
DRB1*1301	2	1.53	0.215	34.8	Fixed	0.42	2.42	0.28–20.94	ND
DRB1*1302	3	6.82	0.033	70.7	Random	0.91	1.13	0.14–9.36	0.31
DRB1*1401	3	1.06	0.589	0.0	Fixed	0.55	0.61	0.12–3.07	0.79
DRB1*1403	2	0.04	0.843	0.0	Fixed	0.24	0.28	0.03–2.31	ND
DRB1*1404	2	0.09	0.767	0.0	Fixed	0.69	1.43	0.24–8.65	ND
DRB1*1405	3	2.46	0.293	18.6	Fixed	0.69	1.38	0.29–6.43	0.19
DRB1*1407	2	1.69	0.194	40.7	Fixed	0.84	0.81	0.10–6.36	ND
DRB1*1501	3	7.62	0.022	73.7	Random	0.76	0.83	0.24–2.89	0.62
DRB1*1502	2	2.56	0.110	60.9	Fixed	0.80	1.31	0.16–10.58	ND
DRB1*1602	3	1.31	0.518	0.0	Fixed	0.64	0.80	031–2.06	0.71
A1	**5**	**9.35**	**0.053**	**57.2**	**Random**	**0.001**	**5.10**	**1.95–13.33**	**0.44**
A2	**4**	**5.33**	**0.149**	**43.7**	**Fixed**	**0.005**	**2.17**	**1.26–3.74**	**0.48**
A3	2	0.12	0.733	0.0	Fixed	0.45	0.52	0.10–2.75	ND
A9	3	4.65	0.098	57.0	Random	0.72	1.45	0.26–1.38	0.002
A11	2	1.80	0.180	44.5	Fixed	0.76	1.16	0.45–2.97	ND
A28	2	0.26	0.608	0.0	Fixed	0.65	1.64	0.19–13.99	ND
AW30+31	2	0.00	0.991	0.0	Fixed	0.33	3.14	0.31–31.39	ND
B5	**5**	**9.01**	**0.061**	**55.6**	**Random**	**0.001**	**4.63**	**1.85–11.62**	**0.99**
B7	2	0.81	0.369	0.0	Fixed	0.74	1.25	0.34–4.61	ND
B8	**3**	**2.65**	**0.266**	**24.5**	**Fixed**	**0.02**	**2.99**	**1.19–7.53**	**0.39**
B12	**3**	**3.87**	**0.144**	**48.4**	**Fixed**	**0.005**	**5.49**	**1.67–18.03**	**0.82**
B13	2	1.61	0.205	37.7	Fixed	0.24	1.82	0.68–4.90	ND
B15	2	4.21	0.040	76.2	Random	0.95	1.13	0.04–31.86	ND
B17	2	1.33	0.249	24.7	Fixed	0.53	1.57	0.38–6.51	ND
BW22	2	1.69	0.193	41.0	Fixed	0.59	0.68	0.17–2.77	ND
BW40	2	2.71	0.100	63.1	Random	0.57	1.97	0.19–19.97	ND

OR, pooled odds ratio.
95% CI, 95% confidence interval.
ND, not performed when there is less than three studies.
Bold line indicates significant association.
[a] fibrosis FI-III *vs.* F0.
[b] fibrosis FII-III *vs.* F0-I.

significantly associated with PSHD in three separate studies. Therefore, further studies are required to clarify these variants.

DQA1*0601, DRB1*1202, and DRB1*1302 were determined to have conflicting effects on PSHD in previous studies, and we found no significant association between these alleles and PSHD in our meta-analyses (Fig. 2C). Other HLA markers, including DQB1*0503.1, DQB1*0601, and DRB1*1101, were found to have a significant association with PSHD in at least one study, but we did not identify a significant relationships between any of these alleles and PSHD after pooling the data for our meta-analysis (Table 2). This variation can be attributed to low statistical power and variability in study designs, diagnoses, population selection, and phenotype definitions.

Table 2 also summarizes the results of Egger's test for publication bias. No evidence of publication bias was found for the following significant alleles, DQB1*0303, A1, A2, B5, B8, and B12 ($P>0.1$). The funnel plot analysis was further performed to detect publication bias of each study for HLA-A1 and HLA-B5, respectively. The shape of the funnel plot seemed to be symmetrical, further suggesting that no publication bias was found in studies of HLA-A1 and HLA-B5 (Fig. 3). There was some indication of publication bias for the studies of DQB1*0301 using Egger's test ($P=0.048$, Table 2). Removing either the study by Zhang et al. or the one by McManus et al. resulted in a loss of the publication bias ($P>0.1$), but it had little effect on the pooled ORs and 95% CIs; in contrast, removing either the study by Waine et al. or the one by Hirayama et al. led to an increased chance of publication bias ($P=0.038$ and 0.022, respectively). One limitation of our study is that the publication bias could not be assessed for three significant alleles, DQA1*0501, DRB1*0901 and DQB1*0201, because there were fewer than three studies for each variant. Moreover, non-significant association must be interpreted with cautious for DPA1*0103, DPA1*0201, DQA1*0601, DQB1*0601, DRB1*1202, DRB1*1302, A9, B15 and BW40 variants because of high Q and I-squared values. Therefore, more studies are required to validate these variants.

A limitation of many meta-analyses is that studies that report of non-significant results are less likely to be accepted for publication, and missing data could cause potential bias. As with other complicated diseases, the development of PSHD is probably due to multiple factors, in which allelic variants in different genes may have either additive or

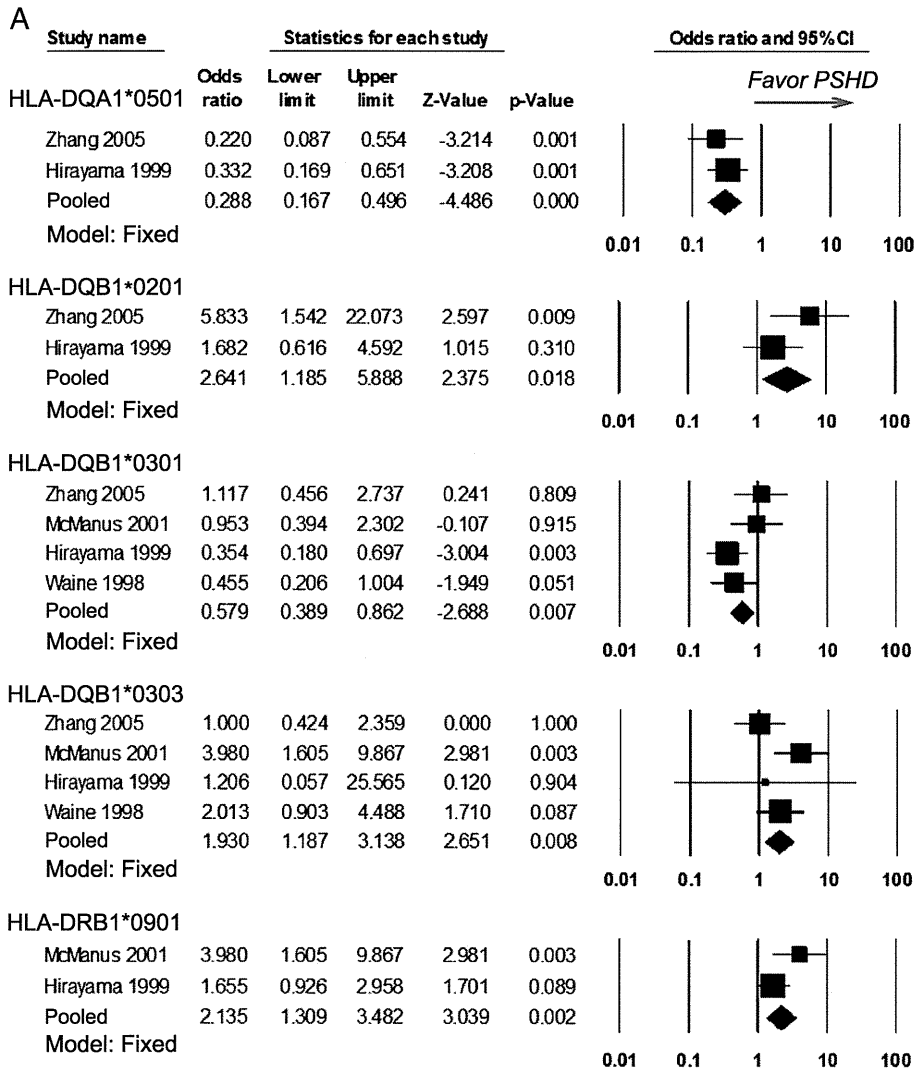

Fig. 2. Meta-analysis forest plot showing the pooled odd ratio (OR) for PSHD susceptibility with 95% confidence intervals (95% CI) of significant HLA alleles (A), significant HLA serotypes (B) and contrasting effective HLA alleles (C). The size of the plots represents the study size. All HLA variants — DQB1*0201, DQB1*0303, and DRB1*0901 were associated with *S. japonicum*-induced PSHD in all studies (A), while HLA antigens-A1, A2, B5, B8, and B12 were correlated with *S. mansoni*-induced PSHD in all studies except one study by Wang et al. (B).