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Plasmodium falciparum apical membrane antigen 1 vaccine elicits multifunctional CD4 cytokine-producing and memory T cells

Maria Cecilia Huaman*, Gregory E.D. Mullen[1], Carole A. Long*, Siddhartha Mahanty[2]

Laboratory of Malaria and Vector Research and Malaria Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Rockville, MD 20852, USA

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ABSTRACT

The *Plasmodium falciparum* apical membrane antigen 1 (AMA1) is a leading vaccine candidate and was tested for safety and immunogenicity in human Phase I Clinical Trials. PBMC from vaccine recipients were analyzed by flow cytometric methods to determine the nature of T-cell responses and AMA1-reactive memory T cells. Both CD4 and CD8 T cells produced a number of cytokines following AMA1 re-stimulation, with IL-5-producing cells at the highest frequency, consistent with a Th2 bias. The relative frequency of multifunctional cells synthesizing Th1 cytokines IFN-γ, IL-2 and TNF-α changed after each vaccination. Interestingly, median fluorescence intensity measurements revealed that cells producing more than one cytokine contributed greater quantities of each cytokine than cell populations that produced each of the cytokines alone. AMA1 vaccination also elicited the development of memory cell populations, and both central and effector memory T cells were identified concurrently after the AMA1 vaccination. The detailed profile of multifunctional T-cell responses to AMA1 presented here will advance our ability to assess the immunogenicity of human malarial vaccines.

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1. Introduction

Malaria is a serious public health problem in tropical regions of the world and is responsible for about 1.5 million deaths yearly, mainly among sub-Saharan children under 5 years old [1]. Four species of *Plasmodium*, the etiologic agents of malaria, infect humans and *P. falciparum* is the species that has the most severe clinical consequences. In light of increasing anti-malarial drug resistance and the limited success of mosquito control programs, an effective vaccine is likely to be an important tool to decrease the burden of malaria.

P. falciparum apical membrane antigen 1 (PfAMA1) is an integral membrane protein of 83-kilodalton (kDa) that is processed to 66-kDa during late schizogony in the erythrocytic cycle of the parasite before being exported from micronemes to the merozoite surface [2]. Recent studies have revealed that AMA1 is also found in sporozoites [3]. AMA1 is thought to participate in the re-orientation and attachment of merozoites to red blood cells, a crucial phase in red blood cell invasion by the parasite [4]. AMA1 vaccination of mice and monkeys has been demonstrated to reduce parasitaemia and confer significant protection after challenge with virulent parasites, indicating the critical role of this protein during invasion [5–8]. As recently reviewed by Remarque et al. [9], these observations have provided a rationale for the development of recombinant AMA1 as a vaccine candidate. The mechanisms of protection are not completely known but include the generation of antibodies that block merozoite entry into red blood cells, inhibition of parasite growth and induction of parasite-directed cellular immunity [5,8,10,11].

Inadequate knowledge about surrogate markers of protection against *P. falciparum* infection has been a major obstacle to the rational design of malarial vaccines and has complicated measurements of vaccine efficacy. Many studies have focused on antibody responses following vaccination with blood stage vaccine candidates, but a better understanding of cellular responses should assist in the development of more effective blood malaria vaccine candidates.

A number of publications have described techniques that can be used to evaluate the cellular responses and kinetics of mem-

Abbreviations: AMA1, apical membrane antigen 1; PBMC, peripheral blood mononuclear cells; APC, allophycocyanin; Cy-5 or Cy-7, Cy-chrome 5 or 7; Th1, T helper 1; Th2, T helper 2; MFI, median fluorescence intensity; iMFI, integrated median fluorescence intensity; PMA, phorbol 12-myristate 13-acetate; T_{em}, effector memory T cells; T_{cm}, central memory T cells; KO, knock-out.

* Corresponding authors. Current address: Laboratory of Malaria and Vector Research, Malaria Vaccine Development Branch, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Twinbrook 3, Rockville, MD 20892, USA. Tel.: +1 301 435 2180; fax: +1 301 443 5778.
E-mail address: huamanc@niaid.nih.gov (M.C. Huaman).
[1] The Rayne Institute, School of Medicine, King's College, London SE1 7EH, UK.
[2] Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, MD 20892, USA.

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ory responses to immunogens in vaccinated human volunteers. The IFN-γ ELISPOT is the most widely used technique [12–15]. Analysis of single-cytokine intracellular detection by flow cytometry has been used as a preferred technique in recent studies as well [12,14]. However, these analyses provide only a partial view of the quantitative and qualitative aspects of antigen-specific T-cell responses.

Recently, a study using the mouse model of leishmaniasis suggested that multifunctional cellular responses may be important in the development of protective immunity. This study showed that multifunctional CD4 T cells secreting IFN-γ, IL-2, and TNF-α may predict vaccine efficacy, memory formation and may ultimately be required for mounting a protective immune response [16]. This approach also has been applied to HIV infection [17] and vaccinia virus immunization [18].

In addition to cytokine secreting T-cell frequencies, the identification of memory T-cell populations elicited by vaccination would provide us with another parameter for measuring vaccine immunogenicity. Our understanding of the heterogeneity of memory T cells that are generated by natural infection and vaccination, based on phenotypic and functional markers has advanced [19,20]. Effector memory T cells (T_{em}) migrate into tissues and produce cytokines that regulate effector functions of the immune responses. These cells express high levels of CD45RO but levels of L-selectin (CD62L) or the chemokine receptor CCR7 are low (or absent). Central memory T cells (T_{cm}) migrate through lymph nodes and are like effector memory T cells, but express high levels of the markers CD62L and CCR7. Naïve T cells also express CD62L and CCR7 but, in contrast to central memory T cells, are CD45RO negative and CD45RA positive. Using flow cytometry, it is possible to identify these memory cell subpopulations phenotypically and follow changes in these after vaccination.

The goal of this study was to perform a detailed characterization of T-cell responses following vaccination of naïve human volunteers with the malaria blood stage vaccine candidate AMA1. We evaluated cellular responses using multiparameter flow cytometry and identified T cells that produced IFN-γ, IL-2 and TNF-α or combinations of these cytokines, while delineating the kinetics of AMA1-specific cytokine-producing T-cell responses. We also assessed the development of memory T-cell responses, demonstrating the concurrent induction of central and effector memory CD4 T cells after vaccination with AMA1. These data, derived using a novel approach to the study of AMA1-induced T-cell responses in humans, provide a basis for assessment of the immunogenicity of blood stage malaria vaccines.

2. Materials and methods

2.1. Study population and study sites

Samples for this study were obtained from volunteers participating in two phase 1 clinical trials of AMA1 formulated with aluminum hydroxide (Alhydrogel™) conducted in the National Institutes of Health Clinical Center (NCT00114010: www.clinicaltrials.gov; 3 enrolled volunteers who completed the vaccination schedule) and the University of Rochester (NCT00344539: www.clinicaltrials.gov; 7 volunteers selected randomly). All subjects provided informed consent for samples collected for this study. Plasma of the volunteers was obtained at the time of enrollment and tested for anti-AMA1 antibodies with standard ELISA and showed they had no response to this antigen. The trials were conducted under an Investigational New Drug (IND) applications held by the Regulatory Compliance and Human Subjects Protection Branch and the Division of Microbiology and Infectious Diseases of the National Institutes of Allergy and Infectious Diseases at the National Institutes of Health. Related documents for both protocols were reviewed and approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board and the University of Rochester Research Subjects Review Board.

2.2. Vaccination schedule and isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were obtained from 10 volunteers who were vaccinated with a combination of the polymorphic forms of AMA1, FVO and 3D7 (equal mixture of FVO and 3D7 allelic forms of the protein, by weight), designated AMA1-C1, formulated on Alhydrogel® on days 0, 28 and 56. In the study conducted at the NIH Clinical Center, 3 volunteers completed the vaccination schedule, and cells obtained from apharesis procedures after each vaccination were used to optimize flow cytometric methods; these samples also contributed to the analysis dataset. A subset of 7 volunteers from the second protocol conducted at the University of Rochester (NCT00344539: www.clinicaltrials.gov) [21] provided blood samples for analysis. All volunteers had the same vaccination schedule (Fig. 1A). PBMC were isolated from whole blood collected 1 week after each vaccination and at additional time points up to day 140 post-vaccination according to the manufacturer's protocol (BD Vacutainer® CPT tubes, Becton Dickinson, San Jose, CA). PBMC were stored in liquid nitrogen in heat inactivated fetal bovine serum

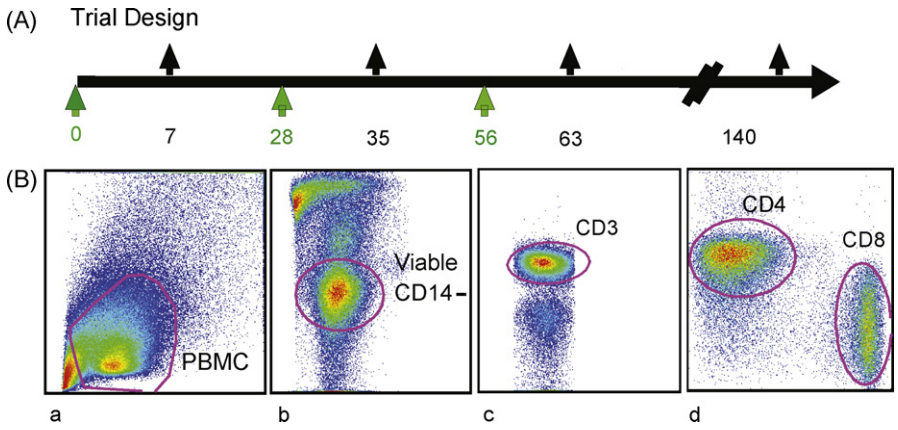

Fig. 1. (A) Study design. AMA1-C1 (FVO+3D7) formulated with Alhydrogel® were administered at a dose of 80 μg IM to healthy American volunteers. Black arrows on the top of the time line indicate blood sampling time points in vaccinees. (B) The gating strategy for the analysis of cytometry data. For PBMC, cell debris was excluded first (a), followed by exclusion of non-viable cells and monocytes (b); the lymphocytes were identified (c), and CD4 and CD8 markers were then used to identify two populations for further characterization (d).