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Decrease in circulating CD25^{hi}Foxp3^{+} regulatory T cells following vaccination with the candidate malaria vaccine RTS,S

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ABSTRACT

Regulatory T (Treg) cells have been shown in some cases to limit vaccine-specific immune responses and impact efficacy. Very little is known about the regulatory responses to the leading malaria vaccine candidate, RTS,S. The goal of this study was to begin to characterize the regulatory responses to the RTS,S vaccine. Using multi-parameter flow cytometry, we examined responses in 13 malaria naïve adult volunteers who received 2 doses of RTS,S given eight weeks apart. Five of these volunteers had previously received 3 doses of a candidate DNA-CSP vaccine, with the final dose given approximately one year prior to the first dose of the RTS,S vaccine.

We found that the frequency of CD25^{hi}Foxp3^{+} Treg cells decreased following administration of RTS,S ($p = 0.0195$), with no differences based on vaccine regimen. There was a concomitant decrease in CTLA-4 expression on CD25^{hi}Foxp3^{+} Treg cells ($p = 0.0093$) and PD-1 levels on CD8^{+} T cells ($p = 0.0002$). Additionally, the frequency of anergic CTLA-4^{+}CCR7^{+} T cells decreased following vaccination. An inverse correlation was observed between the frequency of *Plasmodium falciparum* circumsporozoite protein (PfCSP)-specific IFN-γ and PfCSP-specific IL-10, as well as an inverse correlation between IL-10 induced by Hepatitis B surface antigen, the carrier of RTS,S, and PfCSP-specific IFN-γ, suggesting that immunity against the vaccine backbone could impact vaccine immunogenicity. These results have implications for future malaria vaccine design.

1. Introduction

Though great strides have been made in malaria vaccine development, efforts continue to define correlates of protection. For RTS,S, the first malaria vaccine candidate to reach Phase III clinical trials and licensure, protection partially relates to CD4^{+} and CD8^{+} T cell as well as antibody responses [1,2]. However, these responses do not wholly explain the relatively low efficacy of RTS,S in field trials, which showed 31.3% efficacy in prevention of clinical malaria among infants [3].

There is a growing recognition that regulatory T (Treg) cells may contribute to vaccine immune response. Treg cells suppress the activity and activation of antigen presenting cells and effector cells through a number of mechanisms, including expression of co-inhibitory receptors like CTLA-4 and secretion of immunoregulatory cytokines such as IL-10 [4]. In a healthy individual, Treg cells preserve peripheral tolerance and limit immunopathology. Recent studies have investigated whether these same regulatory responses might limit vaccine-induced responses. Changes in Treg cell populations have been documented after vaccination against viruses, bacteria, and parasites [5–7], and in some instances Treg cell depletion has resulted in improved vaccine response [5,8] and greater protection [9,10].

The role of Treg cells in natural malaria infection is complex and not fully understood. The malaria parasite *Plasmodium falciparum* induces Treg cells *in vitro* [11,12]. *In vivo*, higher Treg frequencies correspond with malaria infection [13–15], though Treg frequencies have been shown to decrease with subsequent infections [16] and do not appear to correlate with acute malaria clinical severity [17]. Studies of Treg cells in the context of malaria vaccination have been limited. Treg depletion enhanced the response to a virally vectored malaria vaccine [10] and increased vaccine-specific T cells in a different CD8^{+}-restricted vaccine but without a concomitant increase in memory [18]. In humans, *Foxp3* mRNA increased after a virally vectored malaria vaccine, while it did not change after administration of RTS,S [19,20].

Using samples from a Phase I trial that examined the immunogenicity of a two-dose regimen of RTS,S, with or without receipt of a *P. falciparum* circumsporozoite protein (PfCSP) DNA-prime

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vaccine 1 year prior [21,22], we tested the hypothesis that Treg cell frequencies are negatively correlated with RTS,S immunogenicity. As vaccine regimen and schedule have been shown in some studies to influence Treg response [6,23], an additional goal was to determine whether a prior DNA-prime influenced regulatory responses. Knowledge of the regulatory responses could help us in rational vaccine design to enhance the efficacy of future malaria vaccine candidates.

2. Materials and methods

2.1. Vaccines

The RTS,S malaria vaccine (GlaxoSmithKline Biologicals) is composed of the central repeat and T cell epitope regions of the PfCSP gene fused to HBsAg and has been described previously [24]. The PfCSP DNA vaccine consisted of DNA plasmid encoding the entire PfCSP gene (Vical Inc.). Volunteers who received the CSP DNA priming had participated in a previous clinical trial of the PfCSP DNA vaccine, administered 3 times at one month intervals [21,25,26].

2.2. Volunteers and vaccine schedule

The clinical immunogenicity trial, including vaccine administration and safety and tolerability assessment, has been described elsewhere [21,22]. Briefly, 10 healthy malaria-naïve adult volunteers who had received three doses of the PfCSP DNA vaccine 12–14 months previously, and 14 healthy malaria-naïve adult volunteers who had not been given the PfCSP DNA vaccine, were recruited. All volunteers were given two immunizations with the RTS,S vaccine eight weeks apart. Peripheral blood mononuclear cells (PBMCs) were isolated prior to the first immunization with RTS,S, 2 weeks after the first immunization, and 1 week, 2 weeks, 6 weeks, and 14 or 16 weeks after the second immunization of RTS,S (Fig. 1). PBMC isolation and cryopreservation were performed as previously described [25]. Sample collection and PBMC harvesting were performed under SOP and kept below 4 h in all volunteers to minimize the impact of cell handling. Of the original group of volunteers, PBMCs from 5 DNA-primed and 8 non-DNA-primed volunteers were available for evaluation.

This study was approved by the Naval Medical Research Center (NMRC) Institutional Review Board, and the Walter Reed Army Institute of Research Human Use Review Committee, and informed consent was obtained from all study participants.

2.3. Cell culture and stimulation

To reduce the effect of inter-assay variability, all time points pre- and post-vaccination for one volunteer were run on the same assay, and, for quality control purposes, PBMCs from a well-characterized in-house volunteer were run with each batch. All cell culture, flow cytometry, and gating were performed while blinded to DNA-prime or non-prime status. Thawed PBMCs were cultured in RPMI with 10% heat inactivated fetal bovine serum and L-glutamine (2 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL). PBMCs were stimulated with 1.25 μg/mL of PfCSP peptide pool 15 mers overlapping by 11, spanning the entire length of PfCSP (Chiron Technologies), and 10 μg/mL purified HBsAg (Alpha Diagnostics). Stimulations were 18 h in the presence of anti-CD49d and anti-CD28. GolgiPlug (BD Biosciences) was added after two hours of stimulation. The positive control was 10 μg/mL staphylococcus enterotoxin B (SEB, Toxin Technologies); media was used for the negative control.

2.4. Flow cytometry

After washing cells and staining with Live/Dead Fixable Violet Dead Cell Stain Kit (Life Technologies), surface staining was performed for 15 min at room temperature. Fixation and permeabilization was accomplished with the Foxp3 Staining Buffer Set (eBioscience), followed by staining for intracellular markers. Data was acquired on an LSR II flow cytometer (BD Biosciences) and analyzed on FlowJo X.0.6 (Tree Star). Fluorescence Minus One controls (FMOs) were used for gating (See Figs. S1 and S2 for gating strategies). The panel used was: CD3-A700 (SP34-2), CD8-BUV395 (RPA-T8), CD19-BV421 (H1319), IFN-γ-FITC (4s.B3), CCR7-BV786 (3D12), IL-10-PE-TR (JES3-19F1), CD45RA-APC-H7 (H100), CD14-BV421 (MΦP9), CTLA-4-APC (BNI3), IL-2-PCP-Cy5.5 (MQ1-17H12) (all from BD Biosciences); CD25-PE-Cy7 (BC96), Foxp3-PE (206D), PD-1-PE-Cy7 (EH12.2H7), CD4-BV650 (RPA-T4) (all from BioLegend).

2.5. Data analysis

We defined a cytokine positive responder as one that was greater than two-fold above background in the unstimulated controls. GraphPad Prism version 6.00 for Windows (GraphPad Software) was used to perform statistical analysis. Fisher's exact test was used to compare frequency of responders. Kruskal-Wallis test was used to compare cell frequencies between groups, with Wilcoxon matched-pairs signed rank test was used for paired samples pre- and post-vaccination. Correlations were assessed with Spearman's correlation.

3. Results

3.1. Frequency of circulating $CD25^{hi}Foxp3^{+}$ Treg cells decreases with vaccination

To determine whether the RTS,S boost vaccine affected the Treg cell population, we measured Treg frequencies prior to the first dose of RTS,S and 2 weeks following the second and final boost. There were no differences in the frequencies of Treg cells between

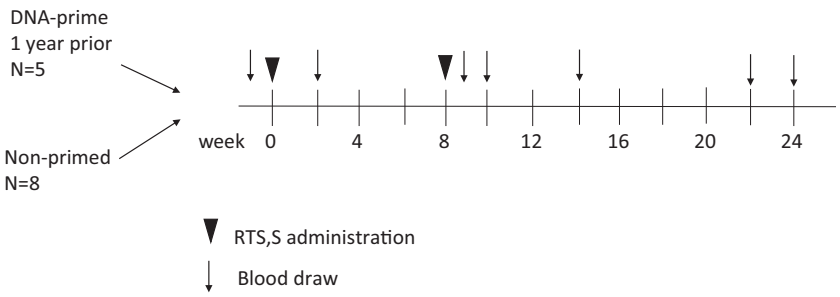

Fig. 1. RTS,S vaccination schedule. Administration of two doses of RTS,S vaccine in subjects who had received a DNA-prime 1 year prior, or who had not. PBMCs from weeks 22 and 24 were used when available.