SHIV Antigen Immunization Alters Patterns of Immune Responses to SHIV/Malaria Coinfection and Protects against Life-Threatening SHIV-Related Malaria

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Whether vaccination against a virus can protect against more virulent coinfection with the virus and additional pathogen(s) remains poorly characterized. Overlapping endemicity of human immunodeficiency virus (HIV) and malaria suggests that HIV/malaria coinfection frequently complicates acute and chronic HIV infection. Here we showed that vaccination of macaques with recombinant *Listeria ΔactA prfA expressing simian/human immunodeficiency virus (SHIV) *gag* and *env* elicited Gag- and Env-specific T-cell responses, and protected against life-threatening SHIV-related malaria after SHIV/*Plasmodium fragile* coinfection. SHIV antigen immunization reduced peak viremia, resisted SHIV/malaria-induced lymphoid destruction, and blunted coinfection-accelerated decline of CD4$^+$ T-cell counts after SHIV/malaria coinfection. SHIV antigen immunization also weakened coinfection-driven overreactive proinflammatory interferon-γ (IFNγ) responses and led to developing T helper cell 17/22 (Th17/Th22) responses after SHIV/malaria coinfection. The findings suggest that vaccination against AIDS virus can alter patterns of immune responses to the SHIV/malaria coinfection and protect against life-threatening SHIV-related malaria.**

Keywords. co-infection; HIV/AIDS; immunology; malaria; T cell; vaccination.

It is well known that a vaccine can elicit a pathogen-specific immune response, and protect against the pathogen-induced infection or disease. However, whether vaccination against a virus can protect against more virulent coinfection with this virus and additional pathogens remains understudied [1]. Some single infections are frequently complicated by concurrent or secondary infections, resulting in increased clinical deterioration and death [2–7]. Influenza-infected patients are commonly afflicted with a secondary pneumococcal pneumonia infection that causes severe lung pathology and death in chronic obstructive pulmonary disease patients [8]. Likewise, infection with human or simian immunodeficiency virus (HIV or SIV) predisposes the host to some highly pathogenic coinfections with additional pathogens even before CD4$^+$ T-cell counts drop to the level below 200 cells/μL [9]. In nonhuman primate models of SIV/*Mycobacterium* coinfection, antiretroviral treatment alone can attenuate the coinfection-induced pathogenic events and protect against fatal SIV-related tuberculosis-like disease [10]. Although vaccine-induced protection against HIV-1 or SIV have been demonstrated in a recent human clinical trial and there have been some experimental vaccine studies in macaques [11–13], studies have not been done to determine whether vaccination against an AIDS virus can protect against life-threatening coinfection with the virus and additional pathogen(s).

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In addition to the over 30 million people worldwide infected with HIV (http://www.who.int/hiv/data/en), approximately 300–500 million malaria infections occur annually, causing 1–3 million deaths (http://apps.who.int/malaria), primarily due to *Plasmodium falciparum* infections. Because both HIV and malaria are highly prevalent in overlapping geographic areas, coinfection is common [14, 15]. Studies of individuals that present with febrile illness presumably caused by malaria infection in sub–Saharan indicate that many patients present with acute or chronic HIV-malaria coinfection [16]. While mutual effects of HIV and malaria on coinfection morbidity and mortality may be complex [17–23], a number of recent studies indicate that immune suppression due to progressive HIV infection results in an increase in clinical malaria cases in HIV-infected adults whose CD4 counts are below 350 cells/μL [18, 19, 22, 23]. HIV-infected pregnant women and children appear to be particularly susceptible to malaria and show decreased ability to clear malaria perhaps due to immune suppression by concurrent HIV infection [23, 24]. Malaria patients may have acute HIV coinfection [16], and concurrent acute HIV/malaria coinfection has been reported in clinical studies of outcomes of transfusion-associated HIV infection in patients receiving blood transfusions for severe malaria [25–27]. A majority of patients succumb to coinfection or subsequently die of AIDS within a year of transfusion [25–27], although it is not known whether community-acquired acute HIV infection impacts the morbidity and mortality associated with malaria coinfection. Further in-depth studies are needed to elucidate the pathogenic events of HIV/malaria coinfection and determine if immune intervention can attenuate the progression of coinfection or HIV-related malaria.

We have recently developed animal models of HIV/malaria coinfection using pathogenic SHIV89.6P virus and *falciparum*-like *Plasmodium fragile* in Chinese-origin rhesus macaques [28]. *P. fragile* malaria coinfection of macaques acutely infected with SHIV results in hyperimmune activation with massive production of proinflammatory tumor necrosis factor-α (TNFα)/IFNγ cytokines, accelerated depletion of $CD4^+$ T cells with lymphoid destruction, bursting parasitemia with severe anemia, and moribund condition. In contrast, chronically SHIV-infected macaques challenged with *P. fragile* develop subclinical transient malaria with moderate anemia and parasite burdens, without enhanced SHIV disease. Interestingly, this subclinical transient malaria coincides with a major expansion of Th17/Th22 cells and an absence of overreactive TNFα/IFNγ response. These observations led us to hypothesize that the preexisting antiviral immune responses in SHIV-infected macaques might be sufficient to prevent rapid progression of coinfection or life-threatening SHIV-related malaria.

To determine whether vaccine-elicited SHIV-specific T-cell responses can attenuate pathogenic events of SHIV/malaria coinfection, we made use of our recombinant *Listeria ΔactA prfA** vector expressing SHIV *gag* and *env* (r*Listeria-gag/env*) [29–31]. Attenuated forms of *Listeria monocytogenes* have been used as delivery systems to vaccinate humans against tumor associated antigens for a variety of cancers [32], and our r*Listeria ΔactA prfA** vector has been shown to be attenuated but highly immunogenic [29–31]. In the current study, we showed that vaccination of macaques with r*Listeria ΔactA prfA** expressing SHIV *gag* and *env* elicited Gag- and Env-specific T-cell responses, and can alter patterns of immune responses to SHIV/malaria coinfection, weaken pathogenic events of the coinfection, and protect against life-threatening SHIV-related malaria.

METHODS

Animals

Twelve weight- and age-matched Chinese-origin rhesus macaques, free of retroviral infections, were used in this study. Six rhesus macaques infected with SHIV and 6 macaques chronically infected with SHIV and challenged with *P. fragile* from previous studies were used as historic controls for this study [28, 33]. All animals were maintained and used in accordance with guidelines of the Institutional Animal Care and Use Committee. Animals were anesthetized with 10 mg/kg ketamine HCl (Fort Dodge Animal Health) for all blood sampling, infections, and treatments, except routine malaria parasite monitoring. Chloroquine treatment was given to macaques that demonstrated high parasitemia (above 10% packed red blood cells [pRBCs] in 2 consecutive thin blood smears). Chloroquine treatment was given as two 37.5 mg/kg doses through oral intubation and administration of ground-up chloroquine tablets in saline.

Construction of r*Listeria Monocytogenes* Expressing *gag* and *env*

Insertion of simian immunodeficiency virus of macaques (SIVmac)251 *gag* sequence into r*Listeria monocytogenes ΔactA prfA** (*G155S*) (provided by Dr Nancy Freitag) was described previously. For insertion of SHIV89.6P *env*-encoded gp120 into r*Listeria*, reverse-transcription polymerase chain reaction (RT-PCR) was performed on isolated RNA from SHIV89.6P virus stock using the primers 5′-*GCAAAGGATCCATGAGAGTGAA GGGGATCAGG*-3′ and 5′-CCACTCACTAGTTTGCCTTGGTG GGTGCTACTCC-3′, and cloned into the BamHI and SpeI sites of shuttle plasmid pPL6-myc. The ligated plasmid was transformed into SM10 mating strain of *Escherichia coli* and transferred to *Listeria monocytogenes actA prfA** through conjugation.

Vaccination of Rhesus Macaques

To 6 Chinese-origin rhesus macaques, 10^8 colony forming units (CFU) of *actA prfA* Listeria monocytogenes* expressing *gag* and *env* (10^8 of each, 2×10^8 CFU total) was administered intravenously. Seven weeks following initial vaccination, 2×10^8 CFU of the same vaccine was intravenously administered to macaques to boost vaccine responses. Macaques were isolated following vaccination and monitored for signs of vaccine-induced disease, and were released from isolation after *Listeria* was cleared from the bloodstream. Figure1*A* shows the schedules for prime and boost vaccinations.

Infections

Animals were inoculated intravenously with 1000AID50 SHIV89.6P and 10^4 *P. fragile* pRBCs as previously described [28]. The pRBCs were propagated in a donor rhesus, then frozen and stored in liquid nitrogen until use. The pRBCs were thawed following previously described procedures [34]. SHIV89.6P was stored at −20°C, washed, and resuspended in Roswell Park Memorial Institute 1040 medium plus 10% heat-inactivated fetal bovine serum. Chronic SHIV infection and chronic SHIV infection–*P. fragile* coinfection macaque controls were done in previous studies [28, 33]. Concurrent SHIV/malaria coinfection challenge was performed at 12 weeks after the boost vaccination (Figure 1*A*). Naive and vaccinated macaques were challenged with the same lot of SHIV89.6P and *P. fragile* Nilgiri strain, which were stably stored as aliquots. Two macaques were previously given the r*Listeria*-based vaccine expressing *gag*/*env* and challenged with SHIV89.6P. These macaques show lower-level viremia and preserved CD4 counts compared to unvaccinated controls. These preliminary observations led us to use r*Listeria*-based SHIV vaccine as a tool to induce anti-SHIV responses in naive macaques and examine the effect of preexisting anti-SHIV immune responses on the coinfection.

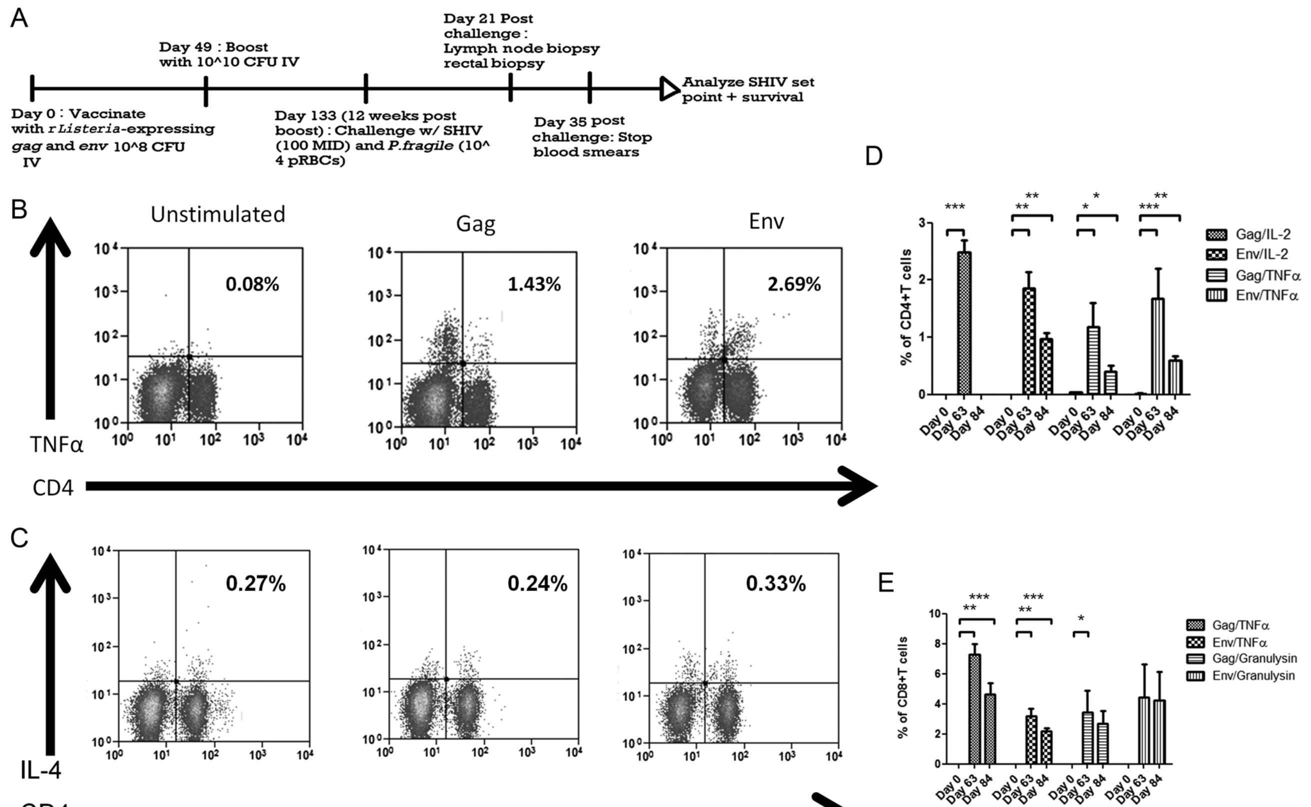

Figure 1. Vaccination of rhesus macaques with r*Listeria ΔactA prfA** expressing SHIV *gag* and *env* elicited Gag- and Env-specific CD4+ and CD8+ T-cell responses. *A*, Experimental design of prime and boost vaccination with r*Listeria ΔactA prfA** and SHIV/malaria coinfection challenge. *B*, Representative histograms showing production of TNFα by CD4+ and CD4−(CD8+) T effector cells at day 63 post infection in response to ex vivo stimulation in the absence (left) or the presence of *gag* (middle) or envelope (right) pooled peptide. Data presented were the result of gating on CD3+ lymphocytes. *C*, Representative histograms showing production of IL-4 by CD4+ and CD4−(CD8+) T effector cells at day 63 post infection in response to ex vivo stimulation in the absence (left) or the presence of *gag* (middle) or envelope (right) pooled peptide. Data presented were the result of gating on CD3+ lymphocytes. *D* and *E*, Percentage numbers of vaccine-elicited SHIV-specific CD4+ T-cell responses (Figure 1*D*) and CD8+ T-cell responses (Figure 1*E*). Percentages of Gag- or Env-specific CD4+ T cells were determined by subtracting percentages of cytokine expressing CD4+ T cells in peptide-stimulated flow cytometry panel from unstimulated panel. Data of prevaccination time point generated from blood collected from macaques right before prime vaccination (day 0). Peak responses were seen for all macaques on day 63 of vaccination schedule. Prechallenge time point represents blood analysis 7 days prior to coinfection challenge (*$P < .05$, **$P < .01$, ***$P < .001$). Abbreviations: CFU, colony forming units; IL-2, interleukin-2; IL-4, interleukin-4; IV, intravenously; MID, mean infectious dose; pRBCs, parasitized red blood cells; rListeria, recombinant Listeria; SHIV, simian/human immunodeficiency virus; TNFα, tumor necrosis factor-α.

Estimation of Parasitemia

Thin blood smears were made by expressing a drop of blood from a tail prick of malaria-infected monkeys. Smears were air dried, and then stained using DipQuick stain kit (Jorgenson Laboratories, Loveland, CO) according to manufacturer's instructions. Parasitemia was estimated by comparing parasitized and total erythrocytes in blood film.

Isolation of Lymphocytes From Peripheral Blood

Peripheral blood lymphocytes (PBLs) were isolated from freshly collected ethylene diamine tetraacetic acid (EDTA)–treated blood by a Ficoll-Paque Plus (Amersham, Piscataway, NJ) density gradient centrifugation before analysis.

Immunofluorescent Staining and Flow Cytometric Analysis

For cell-surface staining, PBLs were stained with up to 5 Abs (conjugated to fluorescein isothiocyanate, phycoerythrin, allophycocyanin, Pacific Blue, and phycoerythrin-Cy7) for 15 minutes, then washed twice with phosphate-buffered saline, fixed with formalin, and analyzed on a CyAn fluorescence-activated cell sorter, as previously described [28].

Intracellular Cytokine Staining

Modified intracellular cytokine staining (ICS) without in vitro antigen stimulation was adopted as recently described [28].

Serum Enzyme-Linked Immunosorbent Assay of Proinflammatory Cytokines

Serum samples of macaques prior to infection and throughout infection were stored at −20°C until use. IFNγ Monkey ELISA Kit (Life Technologies, Grand Island, NY) was used to determine serum concentrations of IFNγ.

Statistical Analysis

Statistical analysis was done using paired or unpaired 2-tailed Student *t* test using GraphPad software (Prism, La Jolla, CA). Data compared were based on percentage, unless otherwise stated.

RESULTS

Immunization of Macaques With r*Listeria ΔactA prfA** Expressing SHIV *gag* and *env* Elicited Gag- and Env-Specific T-Cell Responses

Strong T-cell immune responses have been demonstrated as elite suppressors coincident with control of HIV-1 infection and long-term nonprogression [35]. HIV-1-specific $CD4^+$ and $CD8^+$ T cells play a role in immunity against HIV-1 and SIVmac/SHIV infections [36, 37]. In fact, Gag- or Env-specific $CD4^+$ and $CD8^+$ T cells can mount polyfunctional T helper cell 1 (Th1) responses, producing IFNγ, TNFα, and interleukin-2 (IL-2) [36–38]. Because acute coinfection of macaques with SHIV and malaria induces life-threatening HIV-related malaria [28], we hypothesize that intervention of SHIV/malaria coinfection by prior SHIV antigen immunization can attenuate or disrupt active SHIV-malaria interplays and protect against life-threatening malaria. To test this hypothesis, we developed attenuated highly immunogenic r*Listeria ΔactA prfA** vector expressing SHIV *gag* and *env* (r*Listeria ΔactA prfA**) for eliciting SHIV-specific T-cell responses prior to SHIV/malaria coinfection. As a proof-of-concept study, macaques were immunized intravenously with r*Listeria ΔactA prfA** to maximize vaccine-elicited T-cell responses as described previously [29]. This approach was also justified by our recent observation that systemic vaccination with r*Listeria ΔactA prfA** vector is safe in mice and macaques, and is able to elicit high levels of immunogen-specific $CD4^+$/$CD8^+$ T effector cells [29, 31]. Thus, macaques were primed at week 0 and boosted at week 7 with r*Listeria ΔactA prfA*-gag/env* (Figure 1*A*), and assessed over time for the development of immunogen-specific $CD4^+$ and $CD8^+$ T cells. Following vaccination, macaques exhibited increases in numbers of both Gag- and Env-specific $CD4^+$ (Figure 1*D*) and $CD8^+$ T effector cells (Figure 1*E*). Vaccine-elicited, Gag-/Env-specific $CD4^+$ T cells in vaccinated macaques produced TNFα or IL-2 (Figure 1*D*), whereas Gag-/Env-specific $CD8^+$ T cells primarily expressed TNFα or granulysin (Figure 1*E*). Neither $CD4^+$ nor $CD8^+$ T cells produced significant IL-4 following restimulation with HIV-specific peptides (Figure 1*C*). Even at 11 weeks after the boost vaccination, Gag-/Env-specific $CD4^+$/$CD8^+$ T effector cells were still detected at high levels in all vaccinated macaques (Figure 1*D* and 1*E*). Thus, vaccination with r*Listeria ΔactA prfA** expressing SHIV *gag* and *env* elicited Gag- and Env-specific Th1 and cytolytic responses.

Recombinant *Listeria ΔactA prfA** Vaccination Against SHIV Conferred Protection Against Life-Threatening Malaria After Concurrent SHIV/*P. Fragile* Coinfection in Macaques

To examine if vaccine-elicited, SHIV-specific T-cell responses can impact acute SHIV/malaria coinfection, vaccinated macaques were challenged with SHIV89.6P and *falciparum*-like *P. fragile* concurrently through intravenous administration at 12 weeks after the boost vaccination. Naive macaques served as controls. Naive macaques typically succumbed to the coinfection within 21 days and most became moribund despite treatment with chloroquine (Figure 2*A* and 2*B*). These acute SHIV/malaria-coinfected controls exhibited a striking increase in parasite burden, with parasitemia levels being above 20% of red blood cells (Figure 2*C*) and severe anemia (Figure 2*D*). In contrast, all vaccinated macaques survived concurrent SHIV/malaria coinfection without life-threatening syndromes of SHIV-related malaria (Figure 2*A*). Only 1 vaccinated macaque received chloroquine treatment due to slightly higher than 10% parasitemia, and 5 other vaccinated macaques survived the coinfection without the need for chloroquine treatment (Figure 2*B*),

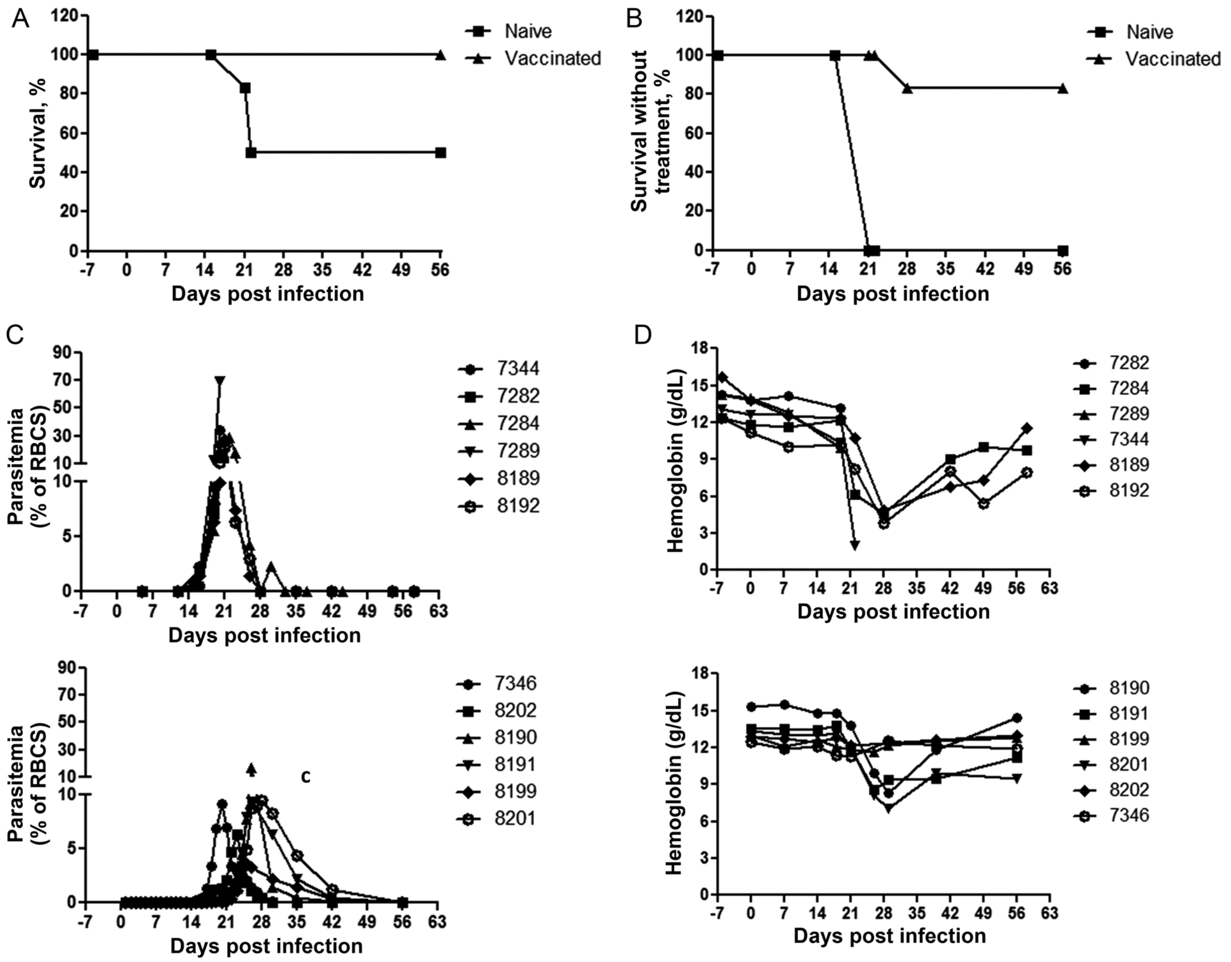

Figure 2. r*Listeria ΔactA prfA** vaccination against SHIV conferred protection against life-threatening SHIV-related malaria after SHIV/*P. fragile* coinfection. *A* and *B*, Survival curves of rhesus macaques after the coinfection challenge (n = 6 per group). While all vaccinated macaques survived coinfection challenge (Figure 2*A*), 1 received treatment due to increased parasitemia (Figure 2*B*) despite a lack of clinical syndromes of severe malaria. By contrast, all 6 naive macaques coinfected with SHIV and *P. fragile* developed fatal moribund conditions (Figure 2*B*) that required antimalaria treatment and, despite treatment, 3 of 6 succumbed to the coinfection (Figure 2*A*). Treatment was given to all macaques that demonstrated more than 10% parasitemia on thin blood smears. *C*, Parasite counts after malaria coinfection challenge of naive control macaques (top) and r*Listeria*-vaccinated (bottom) macaques. *P. fragile* counts were determined by measuring the number of parasitized RBCs on thin blood smears. At least 1000 RBCs were counted to determine percent parasitemia ($P = .0004$ for day 18, $P = .01$ for day 21, $P = .02$ at day 24, and $P = .02$ for peak parasitemia naive vs vaccinated). *D*, Hemoglobin levels of naive (top) and vaccinated (bottom) macaques over time after SHIV/malaria coinfection. Data were generated by CBC analysis of EDTA anticoagulated blood ($P = .05$ for day 18, $P = .007$ for day 21, and $P = .001$ for day 28 naive vs vaccinated). Abbreviations: CBC, complete blood count ; EDTA, ethylene diamine tetraacetic acid, RBCs, red blood cells; *rListeria*, recombinant *Listeria*; SHIV, simian/human immunodeficiency virus.

with parasitemia levels being below 10% (Figure 2*C*). Furthermore, vaccinated macaques exhibited only a transient decline of red blood cells with less severe anemia despite a lack of chloroquine treatment after the concurrent coinfection (Figure 2*D*). Thus, these results demonstrated that r*Listeria ΔactA prfA**–vaccinated macaques displayed Gag-/Env-specific T-cell responses and survived life-threatening SHIV/*P. fragile* coinfection, implicating an interesting paradigm that SHIV antigen immunization could attenuate pathogenic events of SHIV/malaria coinfection and protect against life-threatening SHIV-related malaria.

Recombinant *Listeria ΔactA prfA** Vaccination Against SHIV Led to Lower Levels of Peak Viremia and Preserved $CD4^+$ T-cell Counts After SHIV/Malaria Coinfection Challenge

We then sought to examine potential mechanisms whereby SHIV antigen immunization protected against life-threatening SHIV/malaria coinfection. We first investigated whether SHIV antigen immunization could attenuate malaria-enhanced SHIV pathogenicity as concurrent SHIV/malaria coinfection accelerated $CD4^+$ T cell depletion with increased peak viremia, when compared to SHIV-only infection [28]. We examined the kinetics of SHIV viremia and CD4 T-cell depletion following the coinfection challenge. Interestingly, r*Listeria ΔactA prfA**–vaccinated macaques exhibited lower levels of the peak viremia controls after concurrent SHIV/malaria coinfection (Figure 3*A*). The naive controls had peak 9.5×10^6 copies/mL of plasma viral RNA; the vaccinated macaques showed an average of 2.15×10^5 copies/mL viral plasma RNA (Figure 3*A*). Moreover, while naive controls concurrently coinfected with SHIV/malaria showed a rapid, massive drop of $CD4^+$ T-cell counts to the level below 200 cells/μL during the coinfection (Figure 3), r*Listeria ΔactA prfA**–vaccinated macaques exhibited less dramatic decline of $CD4^+$ T-cell counts, with an average level of 637 cells/μL, following the coinfection (Figure 3*B*). Interestingly, $CD4^+$ T-cell counts in vaccinated macaques rebounded to levels higher than naive controls following clearance of the malaria pathogens (Figure 3*B*). The subsequent rebound of $CD4^+$ T-cell counts might be explained in part by the presumption that massive immune activation by the coinfecting malaria pathogen could collaborate with vaccine-elicited SHIV-specific immune responses to further attenuate SHIV infection. It was reported that bacille Calmette-Guérin coinfection or superantigen administration in SIVmac-infected macaques could hyperactivate the immune system and transiently control SIVmac infection [39].

These results suggested that r*Listeria ΔactA prfA** vaccination against SHIV led to lower levels of peak viremia and preserved $CD4^+$ T-cell counts following concurrent SHIV/malaria coinfection challenge.

Vaccinated Macaques Showed Preserved Lymphoid Structures and Exhibited Less Lymphocyte Depletion of Secondary Lymphoid Organs

Our previous studies in acute SHIV/malaria coinfection demonstrated a significant destruction of secondary lymphoid tissues during the peak of SHIV/malaria infection [28] (Figure 4*C* and 4*D*). To examine whether r*Listeria ΔactA prfA** vaccination against SHIV, while protecting against life-threatening SHIV/malaria coinfection, could potentially attenuate lymphoid destruction or damage, superficial inguinal lymph nodes were collected from macaques at week 4 after the coinfection. Hematoxylin and eosin staining of lymph nodes demonstrated that naive controls concurrently coinfected with

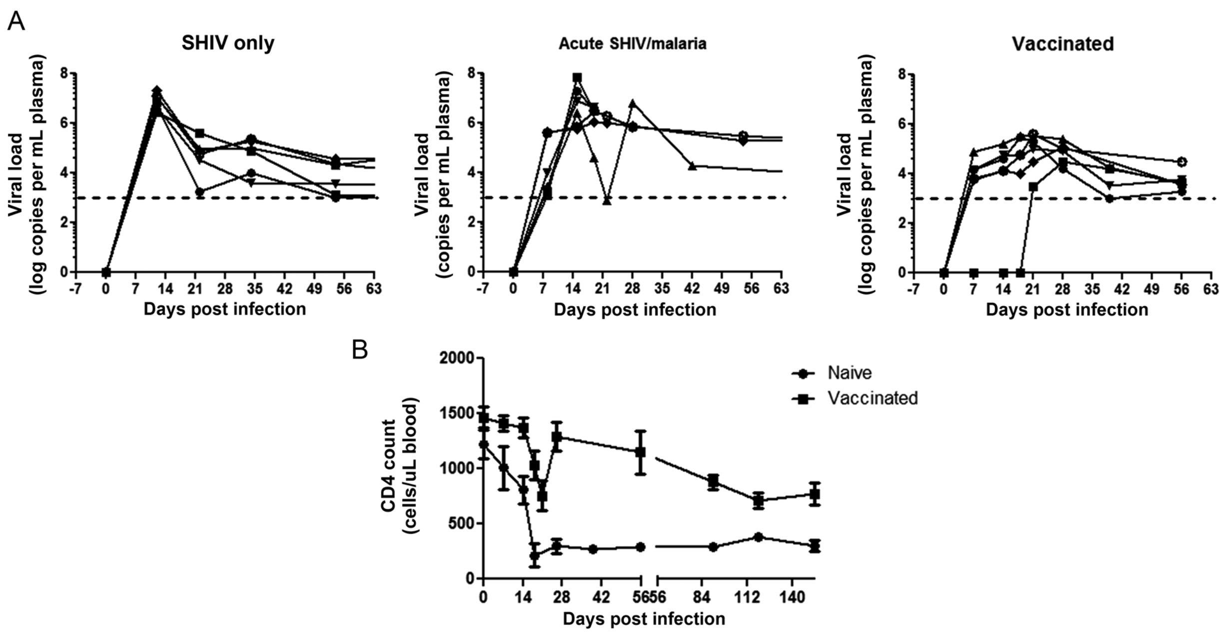

Figure 3. SHIV antigen immunization reduced peak viremia, and blunted coinfection-accelerated decline of $CD4^+$ T-cell counts after SHIV/malaria coinfection. *A*, Plasma viral loads for macaques acutely infected SHIV-infected historical controls [33] (left), naive SHIV/malaria coinfected macaques (middle), and r*Listeria ΔactA prfA**-vaccinated, SHIV/malaria coinfected macaques (right). Viral loads were quantitated by RT-PCR analysis of viral RNA isolated from plasma [49]. Dotted line denotes the detection level for SHIV RNA copies in plasma [49]. Dates post coinfection indicate time following concurrent challenge with SHIV and malaria for naive or vaccinated macaques, or time following SHIV challenge for historical controls. Vaccinated macaques show significantly reduced peak viremia during acute coinfection ($P = .004$ for comparing viremia at day 14 for naive and vaccinated macaques). *B*, $CD4^+$ T-cell counts during acute SHIV/malaria coinfection of naive and vaccinated macaques. PBMCs were stained for CD3, CD4, and gated based on forward and side-scatter properties to determine lymphocyte population. $CD3^+$ $CD4^+$ lymphocyte percentage was multiplied by total lymphocyte number from CBC to determine $CD4^+$ T-cell counts. Data were presented as cell number per microliter of blood. There were no significant differences in CD4 counts between groups prior to infection ($P = .445$ comparing naive and vaccinated macaques at day 0 of infection). Abbreviations: PBMCs, peripheral blood mononuclear cells; *rListeria*, recombinant *Listeria*; RT-PCR, reverse-transcription polymerase chain reaction; SHIV, simian/human immunodeficiency virus.

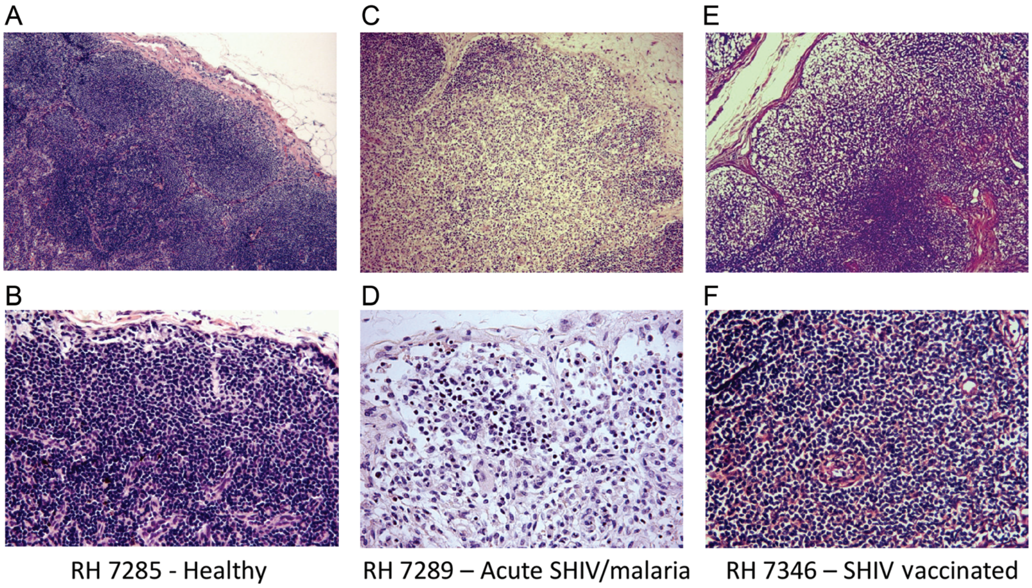

Figure 4. SHIV antigen immunization attenuated SHIV/malaria-induced lymphoid destruction after SHIV/malaria coinfection. *A*, *C*, *E*, H + E stained images of inguinal lymph nodes from representative healthy uninfected (*A*), naive (*C*), and vaccinated (*E*) macaque after SHIV/malaria coinfection. Images of infected macaques represent lymph nodes harvested at week 3 after the coinfection. Magnification 50×. *B*, *D*, *F*, 200× magnification of images in *A*, *C*, and *E*, respectively. Abbreviation: SHIV, simian/human immunodeficiency virus.

SHIV/malaria exhibited apparent lymphoid depletion and destruction of secondary lymphoid structures in the lymph nodes of acute SHIV/malaria-coinfected macaques (Figure 4*C* and 4*D*).

Although vaccinated macaques showed some degree of lymphoid depletion relative to uninfected controls (Figure 4*A*, 4*E*, and 4*F*), secondary lymphoid structures were preserved, and germinal center formation was seen in the vaccinated macaques. These results suggested that vaccination against SHIV helped to resist the coinfection-induced lymphoid destruction and damage.

Vaccination Against SHIV Weakened the Coinfection-Induced Hyperimmune Activation During the Concurrent SHIV/Malaria Coinfection

Our previous studies of SHIV/malaria coinfection allowed us to hypothesize that hyperimmune activation and excess production of IFNγ and TNFα contribute to the life-threatening SHIV-related malaria as acute SHIV/malaria coinfection led to overreactive Th1 responses and marked increases in levels of proinflammatory cytokines IFNγ/TNFα. To determine whether r*Listeria ΔactA prfA** vaccination against SHIV could attenuate overreactive T-cell activation and reduce production of proinflammatory cytokines, we performed direct ICS without in vitro antigen stimulation [28] and enzyme-linked immunosorbent assays (ELISAs) to measure Th1 responses and Th1 cytokines in plasma. Following concurrent SHIV/malaria coinfection, r*Listeria ΔactA prfA**–vaccinated macaques exhibited much smaller numbers of $CD4^+$/$CD8^+$ T cells de novo producing IFNγ than naive controls (Figure 5*A* and 5*B*). Consistently, plasma IFNγ levels were significantly lower in vaccinated macaques than those in naive controls after concurrent SHIV and *P. fragile* coinfection (Figure 5*C*). Collectively, these data demonstrated that vaccination against SHIV attenuated the coinfection-induced hyperimmune activation during the concurrent SHIV/malaria coinfection.

Recombinant *Listeria ΔactA prfA** Vaccination Against SHIV Allowed Vaccinated Macaques to Develop Th17/Th22 Responses After Concurrent SHIV/Malaria Coinfection Challenge

We previously showed that malaria coinfection challenge of chronically SHIV-infected macaques resulted in a dramatic increase in Th17- and IL-22–producing T cells at the peak of malaria infection [28], and the Th17/Th22 responses correlated with the protection of macaques from severe anemia and death. To determine whether vaccinated macaques would exhibit similar Th17/Th22 responses to SHIV/malaria coinfection as chronically SHIV/malaria-coinfected macaques, we performed direct ICS [28] over time to measure Th17/Th22 effector cells after the coinfection of vaccinated macaques. Recombinant *Listeria ΔactA prfA**–vaccinated macaques developed much greater numbers of T effector cells de novo producing IL-17 and IL-22 at 3–7 weeks after the coinfection than naive controls (Figure 6*A*–*C*). The increases in trends were similar to what was observed after malaria coinfection challenge of chronically

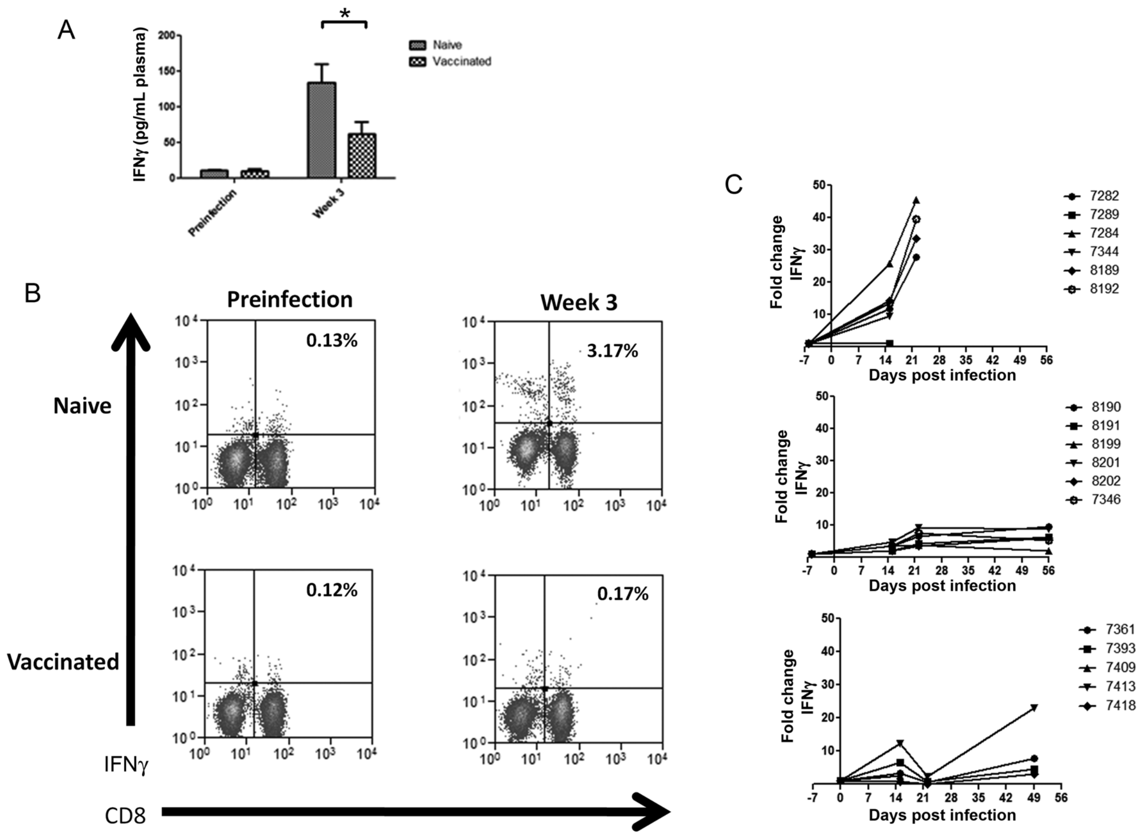

Figure 5. SHIV antigen immunization weakened coinfection-driven overreactive proinflammatory IFNγ responses after SHIV/malaria coinfection. *A*, Data from ELISA measuring IFNγ in serum from macaques prior to infection and during peak viremia/parasitemia. * $P < .05$ ($P = .02$). *B*, Representative flow histograms for Th1 cells of naive and vaccinated macaques after SHIV/malaria coinfection. Panels represent PBMCs stained as described above and gated on $CD3^+$ lymphocytes. *C* Fold changes in IFNγ-producing T cells following the coinfection. Absolute number of $IFN\gamma^+$ $CD3^+$ T cells over time after coinfection was determined and compared to those prior to coinfection. Shown are fold changes in $IFN\gamma^+$ $CD3^+$ T cells in naive macaques (top), r*Listeria*-vaccinated (middle) macaques after concurrent SHIV/malaria coinfection, and historical control chronically SHIV-infected macaques coinfected with malaria from a previous study [28] (bottom) ($P = .04$ for day 15, $P = .003$ for day 22 data comparisons between naive and vaccinated macaques). Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFNγ, interferon-γ; PBMCs, peripheral blood mononuclear cells; *rListeria*, recombinant *Listeria*; SHIV, simian/human immunodeficiency virus.

SHIV-infected macaques (Figure 6*A*–*C*). These results demonstrated that r*Listeria ΔactA prfA** vaccination against SHIV allowed vaccinated macaques to develop Th17/Th22 responses after concurrent SHIV/malaria coinfection challenge, suggesting that potent Th17/Th22 responses might be one of the immune correlates for vaccine-induced protection against life-threatening SHIV/malaria coinfection.

DISCUSSION

Concurrent or secondary infection with multiple pathogens in an ill individual is a common cause of increased morbidity and mortality compared to disease caused by a single pathogen [8, 40]. HIV-1–infected humans exhibit high rates of coinfection with other pathogens or opportunistic pathogens. Overlapping endemicity of HIV and malaria suggests that malaria coinfection frequently complicates acute and chronic HIV infection [16]. We have used nonhuman primate models of acute HIV-malaria coinfection to demonstrate that vaccination against one pathogen, SHIV, can blunt pathogenic events of SHIV/malaria coinfection, and prevent the life-threatening outcome after SHIV/malaria coinfection. Our finding supports an interesting concept that immunization targeting the AIDS virus alone can impact the virus/malaria coinfection and prevent progression to life-threatening virus-related malaria.

Mechanistically, r*Listeria ΔactA prfA**–elicited T-cell immune responses appear to attenuate pathogenic events that occur as a result of SHIV-malaria interplay, and therefore protect against life-threatening SHIV-related malaria. Specifically, the vaccinated macaques show the following 3 major immune interventions: (1) reduced malaria-enhanced SHIV pathogenicity as indicated by high peak viremia and rapid

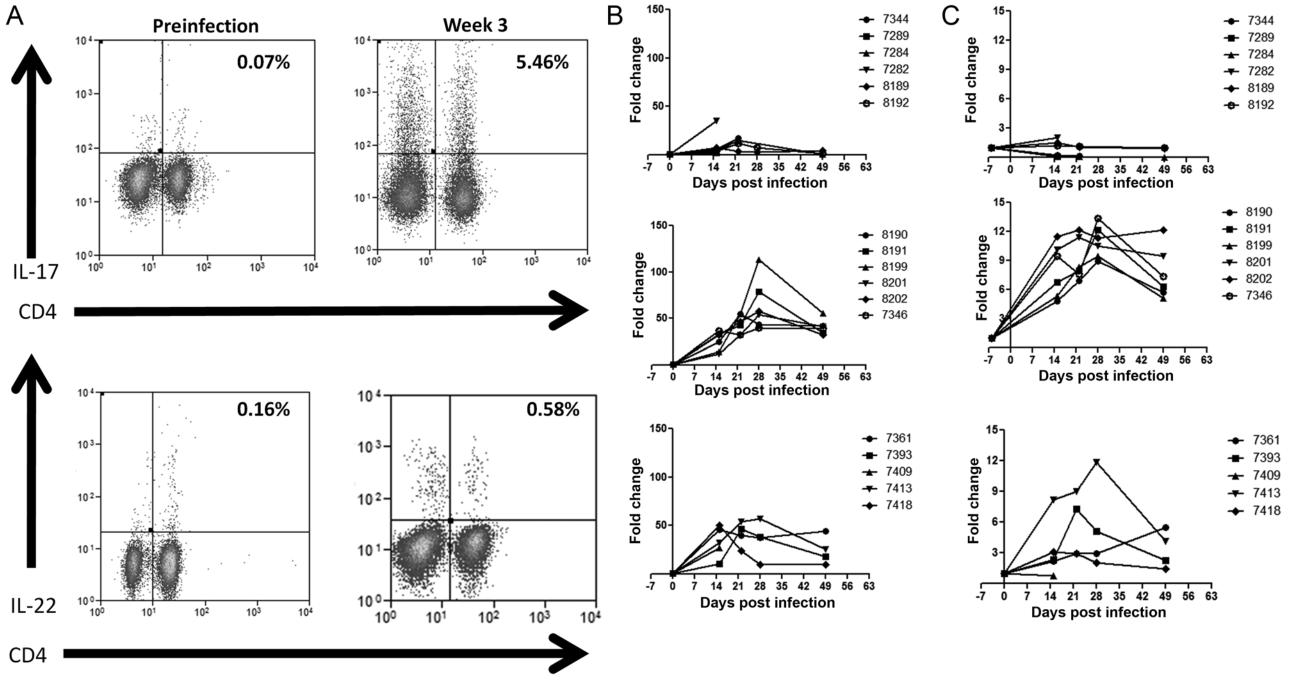

Figure 6. r*Listeria ΔactA prfA** vaccination against SHIV allowed vaccinated macaques to develop Th17/Th22 responses after concurrent SHIV/malaria coinfection challenge. *A*, Representative flow histograms of Th17 (top) and IL-22-producing T cells (bottom) measured after SHIV/malaria coinfection of naive and r*Listeria*-vaccinated macaques. Panels represent PBMCs stained as described above and gated on $CD3^+$ lymphocytes. *B*, Curves for fold changes in IL-17-producing T cells following coinfection. Shown are fold changes in $IL\text{-}17^+$ $CD3^+$ T cells after SHIV/malaria coinfection of naive control macaques (top), r*Listeria*-vaccinated macaques (middle), and after malaria coinfection of chronic-SHIV macaques from a previous study [28] (bottom) ($P = .04$ for day 15, $P = .0003$ for day 22, naive vs vaccinated macaques). *C*, Fold change of IL-22-producing T cells following coinfection. Shown are fold change in $IL\text{-}22^+$ $CD3^+$ T cells after SHIV/malaria coinfection of naive controls (top) and r*Listeria*-vaccinated macaques (middle), and after malaria coinfection of chronic-SHIV macaques (bottom) ($P = .0009$ for day 15, $P = .0001$ for day 22, naive vs vaccinated). Abbreviations: IL-17, interleukin-17; IL-22, interleukin-22; PBMCs, peripheral blood mononuclear cells; rListeria, recombinant Listeria; SHIV, simian/human immunodeficiency virus; Th17, T helper cell 17; Th22, T helper cell 22.

decline of $CD4^+$ T-cell counts with lymphoid destruction/depletion, (2) downregulated coinfection-induced hyperimmune activation as indicated by large increases in circulating T cells producing proinflammatory IFNγ and plasma levels of this cytokine, and (3) attenuated bursting parasitemia, leading to marked anemia.

The data from the current study suggest that T-cell immune responses to the AIDS virus may be important for attenuating coinfection-induced pathogenic events and converting life-threatening virus/malaria coinfection to a subclinical setting. HIV and malaria are prevalent pathogens within sub–Saharan Africa, and acute HIV/malaria coinfection has been demonstrated to occur in countries endemic to both of these pathogens [16]. Our nonhuman primate model predicts that humans concurrently infected with these 2 pathogens will suffer significantly increased morbidity compared to chronically HIV-infected individuals coinfected with malaria or HIV-negative individuals infected with malaria alone. The limited screening for HIV infection in individuals with suspected malaria [16] likely contributes to the infrequent reports of acute HIV/malaria coinfected patients. Increased screening of individuals with suspected malaria for HIV infection could allow for more aggressive treatment to be initiated in these patients to prevent HIV-related severe malarial disease.

Our study makes use of the r*Listeria ΔactA prfA** vaccine vector expressing SHIV antigens to elicit Gag-/Env-specific T-cell responses in macaques. The *Listeria*-based vaccination appears to serve as a useful delivery system for immunization against prostate, breast, and cervical cancers [32]. The r*Listeria* vaccine vectors for infections reported to date by other investigators are either generated from wild-type *Listeria* [41, 42] or from *Listeria* deleted of the essential D-alanine synthesis pathway [43, 44], thus raising safety concerns (wild-type) or requiring special D-alanine supplemental treatments during vaccination of animals. Our r*Listeria ΔactA prfA** vaccine vector has been shown to secrete >100-fold more immunogens than wild-type r*Listeria* or r*Listeria ΔactA*, and elicited much greater T-cell and antibody responses than r*Listeria ΔactA* after intravenous vaccination of mice [29]. Notably, *Listeria*-based vectors can readily translocate to cytosol and present HIV immunogens via the major histocompatibility complex class I pathway for recognition by $CD8^+$ T cells. It is therefore attractive to explore the capacity of r*Listeria ΔactA prfA** to elicit $CD8^+$ T-cell responses for vaccine-induced immunity against infection or coinfection.

Significant Th17/Th22 responses appear to be one of the immune correlates for vaccine-induced protection against life-threatening SHIV/malaria coinfection. Antibody responses might not be actively involved in the early stage, as there are no significant differences in titers of malaria-specific antibodies between SHIV/malaria-coinfected macaques and macaques infected only with malaria [28]. Potent Th17/Th22 responses were detected not only during concurrent SHIV/malaria coinfection of r*Listeria*-vaccinated macaques, but were also seen during malaria coinfection of chronically SHIV-infected macaques. The Th17/Th22 responses correlated in both cases with survival and improved hematologic recovery from malaria infection. Few studies have examined immune responses of Th17/Th22 cells in malaria infection [45], and the role of these effector cells remains to be defined in malaria-infected patients or malaria. It has recently been shown that Th17 cells are able to induce class switching of B cells to immunoglobulin G (IgG) 1, IgG2a, IgG2b, and IgG3 subtypes [46]. It is possible that the Th17 cells generated during coinfection help to produce anti-malaria IgG antibodies, as immune protection against malaria infection is strongly associated with production of antimalarial IgG2a, IgG2b, and IgG3 [47, 48]. Further studies are needed to examine the impact of Th17 cells on malaria infection and whether malaria vaccine–elicited Th17 responses would confer protection.

Notes

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Author contributions. J. F. designed and performed experiments, analyzed data, and wrote the paper; D. H., R. W., and C. Y. C performed experiments; Z. W. C. designed experiments and wrote the paper; all others provided reagents or provided scientific expertise.

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