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CD8 T-Cell Responses to a Diverse Virus: Adaptation and Cross-Reactivity in HIV Vaccination

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CD8 T-CELL RESPONSES TO A DIVERSE VIRUS: ADAPTATION AND CROSS-REACTIVITY IN HIV VACCINATION

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ABSTRACT

In the four decades since human immunodeficiency virus (HIV) was discovered, significant progress has been made in treating HIV infection and in understanding the viral and immune dynamics underlying disease pathogenesis. However, in spite of scientific advances, HIV remains a significant global health issue, and an effective preventative vaccine has yet to be created. Many groups have demonstrated the importance of CD8 T cells in viral control during natural HIV infection and believe that CD8 T cells could contribute to vaccine efficacy by alleviating disease course in individuals who became infected despite vaccination. One major obstacle to inducing potent CD8 T-cell responses against HIV is the immense viral diversity that exists at the individual and population levels.

Here we studied vaccine-induced CD8 T-cell responses in participants of two previous vaccine efficacy trials. We found that HLA-I-associated adaptation to the vaccine insert decreases both the breadth and the polyfunctionality of the vaccine-induced CD8 T-cell response. These findings are significant because breadth and polyfunctionality are two of the characteristics that have previously been linked to improved viral control and decreased infection risk, respectively. We also examined CD8 T-cell cross-reactivity, or

the ability of vaccine-induced CD8 T cells to cross-recognize variant epitopes not encoded by the vaccine. These data showed that, while both vaccines induced a comparable level of CD8 T-cell cross-reactivity to what is seen in acute HIV infection, the cross-reactivity of the vaccine-induced CD8 T-cell response only influences early viral evolution in recipients who became infected and did not impact viral loads. Additionally, vaccine-induced responses were less able to recognize variants encoding HLA-I-associated adaptations.

Collectively, our studies identify two significant obstacles to inducing an effective CD8 T-cell response by HIV vaccination. We hope that future vaccine studies will consider the negative effect HLA-I associated adaptation has on the overall and epitope-level CD8 T-cell responses. We also believe that our data highlight several avenues through which to boost CD8 T-cell cross-reactivity, which may be important for improving viral control in vaccine recipients who become infected.

KEYWORDS

HIV vaccines, CD8 T cells, HLA-I-associated adaptation, CD8 cross-reactivity

DEDICATION

This dissertation is dedicated to my mom, Sivaleela Boppana, who spent endless hours teaching, loving, and inspiring me. In the last few years, watching her fight against PSP has added fuel to my fire – in part highlighting the importance of biomedical research but most importantly showing me what really matters in life.

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INTRODUCTION

HIV History and Perspectives

Acquired immunodeficiency syndrome (AIDS) was first recognized clinically in 1981, during an outbreak of uncommon opportunistic infections and malignancies within the gay community [1]. A few years later, human immunodeficiency virus (HIV) was identified as the causative pathogen. Luc Montagnier's group in France identified a novel retrovirus, HIV, within lymph node samples of a pre-AIDS patient and were able to infect T cells from a healthy donor with this newly described virus [2]. Soon after, Robert Gallo's group at the National Institutes of Health (NIH) isolated HIV from numerous patients, providing a more definitive causative link between the virus and AIDS [3]. In parallel, a research group at the University of California in San Francisco also isolated HIV as well as antibodies against HIV from samples of AIDS patients [4].

In the nearly four decades since HIV was first described, the medical and scientific fields' understanding of HIV viral mechanisms and disease pathogenesis has significantly improved. However, HIV remains a significant public health challenge in the world today, with close to 40 million people living with HIV in 2019 [5]. Combination antiretroviral therapy (cART) has revolutionized HIV treatment, transforming HIV from what was considered a death sentence to a chronically managed disease [6]. And, individuals with fully suppressed viral loads are now thought to be unable to transmit the virus to uninfected partners [7]. However, for many people living with HIV, access to therapy remains limited [8]. Even for those with access, effective viral suppression and prevention of new infections

requires lifelong, daily medication adherence, without which viral rebound inevitably occurs [9]. There is also accumulating data that individuals on cART chronically have higher levels of inflammation than HIV negative counterparts [10, 11] and are at higher risk of dying from non-AIDS defining illnesses, like cardiovascular and liver disease [12]. Finally, despite the availability of cART, rates of new infections have remained largely unchanged in the United States [13]. Given these obstacles, many experts agree that a preventative vaccine would be the most effective tool in halting the HIV epidemic [14, 15].

A major obstacle to developing an effective vaccine to HIV is viral diversity: HIV's reverse transcriptase lacks proof-reading ability and replicates at a high rate, resulting in the introduction of new mutations into the genomes of new virions and leading to immense population-level and intra-individual viral diversity [16-19]. In the majority of HIV-infected individuals, a single transmitted founder virus (TFV) establishes infection [20], but in chronic, untreated HIV infection, the viral diversity of HIV within a single infected individual ultimately rivals the diversity of influenza sequences worldwide in any given year [21]. This high mutation rate results in the generation of many quasispecies, or non-identical but closely related viral genomes, and these quasispecies expand at different rates as determined by viral fitness and host phenotypic constraints, including the CD8 T-cell response [22].

In this work, I study CD8 T-cell responses in the this context of immense HIV viral diversity. I will first review existing literature to support the importance of CD8 T-cell responses in HIV viral control and give background on previous vaccine trials as well as the current state of the HIV vaccine field. Then, I will provide the results of my studies examining CD8 T-cell responses in vaccination. Specifically, I first examined the impact

of HLA-I-associated adaptation on CD8 T-cell responses in vaccination. I then extended this work to examine how well the vaccine-induced CD8 T-cell response could cross-recognize not only the epitopes encoded by a given vaccine but also viral variants that are commonly circulating in the population. To conclude, I summarize these findings as well as provide insights to potential future directions of this work with an eye towards defining CD8 T-cell cross-reactivity at the T cell receptor (TCR) level and investigating the importance of CD8 T-cell cross-reactivity in HIV infection.

Importance of CD8 T-cell responses in HIV infection

The importance of CD8 T cells in HIV viral control during natural infection was first identified in the simian immunodeficiency virus (SIV) model in rhesus macaques, where depletion of CD8 T cells resulted in a rapid and robust increase in plasma viremia. Restoration of these CD8 T cells also returned the previous level of viral control [23]. In HIV-1 infection, higher magnitudes of HIV-specific CD8 T-cell responses during hyper-acute infection are associated with lower viral load set points [24]. Subsequent studies have shown that CD8 T-cell responses not only contribute to viral control but are also a potent driver of viral adaptation [25-27], and viral strains containing CD8 T-cell escape mutations emerge as quickly as one month following infection [28].

Several aspects of the CD8 T-cell response have all been put forth as contributors to CD8 T cells' ability to control virus. CD8 T-cell responses that target the main structural protein of HIV, Gag, were associated with lower viral loads in HIV-infected individuals while those targeting the envelope protein, Env, as well as accessory proteins, were actually associated with higher viral loads [29]. A more recent study expanded upon this idea that only CD8 T cells focusing on specific parts of the virus confer viral control. Gaiha *et al.*

quantified the topological importance of epitopes and found that CD8 T-cell targeting of those epitopes that were more critical to protein structure was most closely associated with viral control [30]. Carlson *et al.* also demonstrated the importance of CD8 T cells in viral control by examining the relationship between the "pre-adaptation" of the infecting virus and subsequent control of that virus. They found that individuals infected by a virus already containing CD8 T-cell escape mutations had poorer clinical outcomes, including higher viral load set points and faster CD4 T-cell decline [31]. However, exactly what makes CD8 T cells effective in controlling virus in HIV-infected individuals and which characteristics should be specifically targeted by preventative strategies remains undefined.

Naturally occurring HIV control

Many have studied the CD8 T-cell responses of natural controllers, a small proportion of HIV-infected individuals who are able to suppress virus in the absence of therapy [32]. The immune responses of these individuals suppress HIV viral replication to low levels or, in the case of elite controllers, to undetectable levels. Although some posit that the inferior virulence of infecting viral strains confers control [33, 34], most research highlights CD8 T cells as the primary driver of natural control. The CD8 T-cell response of HIV controllers is more robust than that of non-controllers in several ways, displaying greater cytotoxicity [35, 36], proliferation [37, 38], and polyfunctionality [39-41] as well as targeting certain "protective" epitopes [42]. However, a major limitation of these studies comparing CD8 T-cell responses from controllers and non-controllers is the inability to assign a causal relationship – it is unclear if the robust nature of CD8 T cells in controllers is responsible for viral control or rather a consequence of an overall more preserved immune system.

The most convincing evidence that CD8 T cells are the critical component behind natural control lies in the strong correlation between certain HLA-I alleles and HIV control status, a finding that several groups have reported [43-46]. HLA-I alleles dictate which epitopes from HIV are presented to and recognized by the host immune response and are classically associated with the presentation of intracellular antigens to CD8 T cells. These protective HLA-I alleles, like B*27 and B*57, drive potent and effective CD8 T-cell responses [47]. Importantly, the advantage conferred by protective HLA-I alleles, like B*57, is lost when B*57-positive individuals are infected with pre-adapted virus, indicating that viral control in elite controllers is dependent on the CD8 T-cell response's ability to prevent CD8 T-cell escape mutations from accumulating [31]. These data suggest that natural HIV control occurs through CD8 T-cell mediated mechanisms and give hope that similarly effective CD8 T-cell responses could be elicited by a vaccine.

HLA-I-associated adaptation in HIV infection

As previously mentioned, HLA-I alleles are a major determinant of which viral epitopes are processed and presented to an individual's CD8 T cells. As such, several groups have used population-level studies to identify specific amino acid mutations that are significantly associated with certain HLA-I alleles, termed HLA-I-associated polymorphisms or HLA-I-associated adaptation [48, 49]. Epitopes containing one of these HLA-I-associated adaptations are termed "adapted epitopes," while epitopes lacking any evidence of adaptation at the polymorphic site are termed "non-adapted epitopes." Moore *et al.*, one of the first reports that used this approach at a large scale, examined sequences of *reverse transcriptase* and found that that the level of HLA-I associated changes in the viral sequence predicted viral loads [49]. More recently, Carlson *et al.* robustly

demonstrated that pre-adaptation of a transmitted founder virus, across the HIV genome, to an individual's HLA-I alleles resulted in faster disease progression, namely faster CD4 T-cell decline and higher viral loads. This report also highlighted that adapted epitopes in acute HIV infection were poorly immunogenic, indicating that HLA-I-associated adaptation impairs CD8 T-cell recognition in early infection [31].

However, in chronic HIV infection, adapted epitopes are more immunogenic than they are in acute infection and are recognized by CD8 T cells at comparable levels to non-adapted epitopes [50]. In fact, a recent publication from our group suggests that CD8 T-cell recognition of these adapted epitopes may actually be beneficial for the virus during chronic infection. Qin *et al.* found that adapted epitope-specific CD8 T-cell responses in chronic infection induced higher levels of dendritic cell maturation than nonadapted epitope-specific responses and that these matured dendritic cells were able to *trans*-infect CD4 T cells at a higher rate. These data suggest that adapted responses in chronic HIV infection may actually facilitate the spread of infection [51]. Collectively, these past studies suggest that adapted epitopes could pose an obstacle to vaccine design, either through poor immunogenicity or by increasing inflammation.

CD8 T-cell cross-reactivity

Because of HIV's vast viral diversity and the role of CD8 T cells in shaping disease progression, the capacity of CD8 T cells to cross-recognize multiple epitope variants may also contribute to viral control. Within responses of B*57-positive individuals, higher cross-reactivity, or greater cross-recognition of alanine-substituted variant epitopes, was associated with lower viral loads, implying that CD8 T-cell cross-reactivity plays a role in viral control [52]. In HLA-B*27 expressing controllers, CD8 T-cell responses to the

immunodominant epitope, KK10, were more broadly cross-reactive to variant epitopes encoding escape mutations compared to responses in B*27 progressors. These cross-reactive CD8 T-cell responses in controllers pushed the virus towards more complex viral escape. Additionally, they demonstrated that this cross-reactivity was evident at the T-cell receptor (TCR) clonotype level [53]. Another study found similarly broadly cross-reactive TCR clonotypes from B*27 and B*57 controllers as compared to progressors [54]. However, all of these past studies have focused on responses to a few immunodominant epitopes in individuals with HLA-I alleles associated with delayed disease progression. While these reports bolster our confidence that CD8 cross-reactivity plays a role in viral control, we cannot broadly apply these findings to vaccine-generated responses in the general population where protective HLA-I alleles are not common.

Another major limitation of previous CD8 T-cell cross-reactivity studies in HIV infection is that, to our knowledge, they have been primarily based on cross-sectional data in chronic HIV-1 infection. For one, during chronic, untreated HIV-1 infection, there are typically many viral quasispecies circulating. Therefore, in assessing CD8 T-cell cross-reactivity it can be difficult to determine true cross-recognition of a variant epitope versus *de novo* priming of the CD8 T-cell response by emerging quasispecies encoding the variant epitope. Additionally, as with many chronic viral infections, there is significant immune exhaustion that occurs in chronic HIV infection [55], which may mask aspects of the CD8 T-cell response, including cross-reactivity. Our group previously showed that cross-reactivity during acute HIV infection is surprisingly low and that responses may broaden throughout chronic infection [56]. However, this study was somewhat limited in the number of variant epitopes screened and was not able to clearly delineate the relationship

between cross-reactive responses and viral evolution. Vaccination poses a novel context in which to study CD8 T-cell cross-reactivity because in vaccine trials, the priming antigen, or vaccine insert, is known. Therefore, cross-reactivity can be clearly defined as cross-recognition of any variant epitope not encoded by the vaccine.

HIV vaccine-induced CD8 T-cell responses

When the HIV virus was first described in 1984, the U.S. Health and Human Services Secretary Margaret Heckler declared that a vaccine would be available within the next two years [57]. Unfortunately, the path to an effective HIV vaccine has not been as smooth as Secretary Heckler predicted. Although HIV vaccine trials began in 1987, to date only the RV144 vaccine trial has demonstrated a modest level of efficacy, described in more detail below. The HIV vaccine field can broadly be divided into two major groups. One has focused primarily on CD8 T-cell-based vaccine strategies, founded in the substantial literature indicating the importance of CD8 T cells in nonhuman primate (NHP) vaccine studies [23] and HIV viral control in natural infection [24, 29-31]. The other has focused on antibody-based vaccine strategies and is supported by the fact that the majority of effective vaccines employed today are successful due to antibody production [58]. However, HIV is a unique pathogen, with immense viral diversity and rapid viral evolution posing significant obstacles to both CD8 T-cell and antibody-based vaccine strategies.

Past vaccine efficacy trials

Initial vaccine efforts in the late 1980s and the 1990s focused on eliciting neutralizing antibodies against the envelope protein of HIV, following the paradigm of most successful vaccines for other pathogens. This endeavor culminated with the results of the first two candidate vaccine trials, the VaxGen trials, which both reported no apparent

efficacy in vaccine recipients in 2003: both the VAX003 trial based in Thailand [59] and the VAX004 conducted in North America [60] employed a recombinant envelope glycoprotein 120 vaccine. Following these antibody-based trials, the next efficacy studies focused on eliciting CD8 T-cell responses since by that time many studies in natural HIV infection as well as in the SIV-macaque model had indicated the critical role that CD8 T cells play in viral control.

Two major CD8 T-cell-based vaccines were tested in large efficacy studies by the HIV Vaccine Trials Network (HVTN): HVTN 502 (MRKAd5) and HVTN 505 (DNA/rAd5). MRKAd5, also known as the Step Study, was halted in 2007 due to safety concerns. This adenovirus serotype 5 (Ad5)-based vaccine encoded a single version of *gag, pol,* and *nef* and was able to elicit CD8 T-cell responses in the majority of vaccine recipients [61, 62]; however, these immune responses did not correlate with a decrease in infection risk. In fact, the trial was halted because interim analysis found a slightly elevated risk of infection in the vaccine arm. In subsequent analyses, this increased risk of infection was found to affect vaccine recipients with pre-existing Ad5 antibody titers; however, a clear biological mechanism causing this elevated infection risk has yet to be demonstrated [63, 64]. Following this disastrous result, the DNA/rAd5 vaccine was tested solely in Ad5-naïve individuals in order to navigate around the previously reported increase in infection risk. This vaccine consisted of a DNA prime encoding *gag, pol, nef,* and *env a/b/c* following by two Ad5-based boosts encoding *gag, pol,* and *env a/b/c.* Unfortunately, despite eliciting CD8 T-cell responses in over 60% of recipients, this vaccine was also found to lack efficacy and halted in 2013 [65].

A few years after the MRKAd5 results were published, in 2009, it was reported that vaccine recipients of the RV144 trial in Thailand actually had a reduced risk of infection by 31.2% compared to placebo recipients. These surprising results were enthusiastically welcomed by the field. This vaccine regimen consisted of a recombinant canarypox-vectored vaccine followed by two boosts with a recombinant envelope subunit [66]. Immune-correlates analysis indicated that the protection provided by this vaccine was primarily conferred by nonneutralizing antibodies to the V1V2 region of the envelope protein [67]. Although held up as a beacon of hope in the HIV vaccine community, some still expressed skepticism that a real effect was observed in the RV144 vaccine. Much of this controversy surrounds the statistical interpretation of the reduction of risk in the vaccine arm, with one publication asserting that there is a greater than 22% percent probability of no efficacy in the RV144 trial [68]. Many also pointed to a nonlinear increase in placebo cases as the driving force behind the difference between HIV acquisition in vaccine versus placebo recipients, suggesting that vaccine efficacy did not actually impact infection rates [69].

Only recently did the HIV vaccine field take the next steps to build on the success of the RV144 trial. In 2016, the HVTN 702 trial, or Phambo Study, began enrolling participants in South Africa. Early phase clinical trials testing the cross-clade immunogenicity of the RV144 vaccine in South Africans actually indicated higher levels of cellular and humoral immune responses in the South African vaccine recipients, compared to Thai recipients of the original RV144 vaccine [70]. However, disappointingly, this trial was discontinued in late January 2020 due to a lack of efficacy as determined by interim analysis conducted by an independent data and safety monitoring board [71]. This

recent news may indicate the end of the road for this vaccine, but it will be interesting to see if any particular immune responses are highlighted in post-hoc analyses of the Phambo study.

Another vaccine currently being tested in efficacy studies is HVTN 705, or the Imbokodo trial, an adenovirus serotype 26 (Ad26)-vectored mosaic vaccine which encodes multiple versions of *env*. NHP studies have demonstrated that mosaic vaccines, which encode multiple versions of HIV genes can elicit broader CD8 T-cell and antibody responses in rhesus macaques as well as humans, and these vaccine-generated immune responses protected against simian-human immunodeficiency virus (SHIV) acquisition in macaques [72, 73]. The mosaic vaccine strategy is intended to elicit immune responses that can better counter the vast viral diversity of HIV.

Recently, many in the HIV vaccine field have focused their efforts on developing and testing antibody-based vaccines, particularly by employing broadly neutralizing antibodies (bNAb) in HIV prevention. The Antibody Mediated Prevention efficacy studies (AMP, or HVTN 703 and 704) are fully enrolled and intend to test the ability of infusions of a bNAb that targets the CD4-binding site of Env, VRC01, every eight weeks to prevent HIV infection [74]. Several research groups are also focused on developing immunization strategies to induce host bNAb production; however, there are several key obstacles to naturally inducing bNAbs. In the context of infection, bNAb are only detected in HIV infected individuals in chronic infection after significant viral diversity is generated and significant antibody affinity maturation occurs [75, 76]. Additionally, the high glycan content of the Env structure requires bNAbs to accommodate glycans in their binding [77]. Several strategies are being explored to overcome these obstacles, including “lineage-

based" vaccines which exploit the immunological pathways that lead to bNAb generation *in vivo* and "germline targeting" which uses antigens that bind the germline forms of bNAbs [78].

In addition to bNAb-inducing vaccine strategies, there are several additional preclinical vaccine regimens that hold promise. Conserved vaccines attempt to focus the immune response on HIV epitopes that are conserved, meaning those epitopes that are found across many strains of HIV and which incur significant fitness costs to the virus when mutated [79]. Several recent, relatively small trials have supported this vaccine strategy, demonstrating that CD8 T-cell responses can be directed towards these conserved regions that are typically poorly immunogenic in HIV infection [80, 81]. Louis Picker's group has taken a unique approach and has developed and extensively tested rhesus cytomegalovirus (rhCMV)-vectored vaccines in nonhuman primates. This rhCMV-vectored vaccine has consistently demonstrated roughly 50% efficacy [82], with protected rhesus macaques rapidly clearing infection and remaining virus-free for years of follow-up [83]. This protection is conferred by non-classically restricted major histocompatibility complex (MHC)-E restricted CD8 T cells [84-86]. A human CMV version of this vaccine is under development, and preclinical and clinical trials in human subjects will demonstrate if similar nonclassical immune responses are generated in humans and if this vaccine can induce similarly effective CD8 T-cell responses against HIV.

Despite staggeringly negative results in many HIV vaccine trials throughout the past four decades, there remains reason to hope with the new vaccine strategies currently being tested in the field and others rapidly approaching the clinical arena. We believe that

studies of the immune responses generated by previous vaccines, even if they showed no overall efficacy, can shed instrumental insights into future vaccine development.

Components of an effective vaccine-induced CD8 T-cell response

Although CD8 T-cell-based HIV vaccines have not yet been demonstrated to be effective in humans, we believe there remains ample evidence that CD8 T cells could be an important component of an efficacious vaccine. For one, several studies have demonstrated improved viral control in vaccinated macaques that became infected with SIV, using CD8 T-cell-based vaccine strategies [87-90]. These data suggest that an effective CD8 T-cell component in a vaccine response could allay disease progression in vaccine recipients who do become infected. A CD8 T-cell vaccine has also been demonstrated to protect against SIV infection in macaques when co-administered with an anti-PD1 antibody. This strategy enhanced vaccine-induced CD8 T-cell responses and protected macaques against repeated rectal challenges with a highly pathogenic strain of SIV [91].

Post-hoc analyses of previous human vaccine trials support these NHP studies and have highlighted several characteristics of the vaccine-induced CD8 T-cell response that appear to influence post-infection viral evolution, confer protection, or impact viral control. Sieving analyses, comparing the breakthrough viral sequences of vaccine versus placebo recipients who became infected, have shown that vaccine-induced CD8 T cells can exert immune pressure on which virus can establish infection or can shape early viral evolution post-infection [92]. In the MRKAd5 study, analysis of breakthrough viral sequencing showed that viral sequences in vaccine recipients who became infected were less similar to the vaccine sequence compared to viral sequences of placebo recipients who became

infected, indicating immune pressure by the vaccine-induced immune response. This analysis even pinpointed a specific Gag amino acid site, which fell within a common CD8 T-cell epitope, as the driving force behind the MRKAd5 sieving effect [93]. Similar sieving analyses were conducted for both the DNA/rAd5 and the RV144 vaccine trials. The DNA/rAd5 sieving analysis identified vaccine pressure on the CD4 binding site of Env, indicating more antibody-mediated than CD8 T-cell-mediated pressure [94], which is in line with the less frequent and lower magnitude CD8 T-cell responses elicited by the DNA/rAd5 vaccine compared to the MRKAd5 vaccine [65]. The RV144 sieving analysis also identified potential sieving of known antibody-associated sites [95]. Sieving analyses show that at least some past vaccines did induce CD8 T-cell responses that were able to exert significant immune pressure.

More directly addressing whether CD8 T-cell responses elicited by vaccines could be an effective component of a future HIV vaccine, vaccine-induced CD8 T-cell responses have also been shown to affect viral control and even lower infection risk in a subset of past trial participants. In MRKAd5, Janes *et al* observed a negative correlation between the number of Gag-specific CD8 T-cell responses and viral load post-infection. The broader, or higher number of, Gag-specific CD8 T-cell responses a recipient mounted to the vaccine, the lower their viral load was following infection. This effect was only seen for responses against the Gag protein, and this effect weaned over time – it was less pronounced in individuals who became infected farther out from their vaccination timepoint [96]. Additionally, recent analysis of DNA/rAd5, found that individuals with higher Env-specific CD8 T-cell responses became infected a at lower rate. This correlation with infection risk was also found with vaccine-elicited Env-specific CD8 T-cell

polyfunctionality, or the ability of CD8 T cells to produce multiple effector molecules simultaneously when stimulated with Env peptides [97]. Collectively these data suggest that CD8 T cells may be able to prevent HIV infection, but at the very least, a robust and effective vaccine-induced CD8 T-cell arm could help control infection in vaccinated individuals who become infected.

Conclusions

The CD8 T-cell-based HIV vaccine field is currently comprised of several strategies, including vaccine inserts composed of conserved epitopes [98], mosaic vaccines encoding multiple versions of HIV proteins [72], and CMV-vectored vaccines which generate unconventional CD8 T cells [83]. Vaccine studies often quantify the CD8 response by stimulating with peptide pools spanning the length of the immunogen, but responses at the epitope level are rarely tested. How well the vaccine-induced CD8 T-cell response can recognize adapted epitopes, encoding HLA-I associated polymorphisms, as well as naturally occurring variant epitopes, excluded by the vaccine, is an area that has previously been understudied. To our knowledge, there are no studies investigating the impact of HLA-I-associated adaptation on vaccine-induced CD8 T-cell responses. There is one study examining CD8 cross-reactivity in the context of HIV vaccination [99]. However, this study was extremely limited in its assessment of cross-reactive CD8 T cell functionality and impact on viral control or evolution. Within this dissertation, I illustrate the potential obstacles posed by both HLA-I-associated adaptation as well as the level of CD8 T-cell cross-reactivity previously elicited by vaccines. Based on my results, I also put forth several strategies that may help overcome these hurdles.

HLA-I ASSOCIATED ADAPTATION DAMPENS CD8 T-CELL RESPONSES IN HIV AD5-VECTORED VACCINE RECIPIENTS.

by

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ABSTRACT

HLA-I-associated human immunodeficiency virus (HIV) adaptation is known to negatively affect disease progression and CD8 T-cell responses. We aimed to assess how HLA-I-associated adaptations affects HIV vaccine-induced CD8 T-cell responses in 2 past vaccine efficacy trials. We found that vaccine-encoded adapted epitopes were less immunogenic than vaccine-encoded nonadapted epitopes, and adapted epitopes-specific responses were less polyfunctional than nonadapted epitope-specific responses. Along those lines, vaccine recipients with higher HLA-I adaptation to the Gag vaccine insert mounted less polyfunctional CD8 T-cell responses at the protein level. Breadth of response, which correlated with viral control in recipients who became infected, is also dampened by HLA-I adaptation. These findings suggest that HLA-I-associated adaptation is an important consideration for strategies aiming to induce robust CD8 T-cell responses.

KEYWORDS

HIV-1 vaccine, HLA-I-associated adaptation, HIV-specific CD8 T cells, adapted epitopes, HVTN 502, HVTN505, polyfunctionality

INTRODUCTION

Although antiretroviral therapy (ART) has revolutionized HIV treatment, it requires lifelong medication adherence, without which viral rebound inevitably occurs [1]. Many experts agree that a preventative vaccine would be the most cost-effective and durable tool to fight HIV. The most promising vaccine trial to date was the RV144 trial, with modest efficacy and correlates of protection centering on nonneutralizing antibodies [2, 3]. Although CD8 T cells have not contributed to overall vaccine efficacy in previous trials, there is ample evidence that they can control HIV infection. The importance of the CD8 T-cell response in HIV was first identified as a correlation between the strength of the response during acute infection and the resolution of viremia [4, 5] and was substantiated in the simian immunodeficiency virus (SIV) macaque model, in which depletion of CD8 T cells resulted in uncontrolled viremia [6, 7]. Furthermore, vaccine-induced SIV-specific CD8 T cells have been demonstrated to improve viral control in macaques that became infected [8]. Subsequent studies have shown that CD8 T cells not only contribute to viral control but also drive viral adaptation [9-11].

Post-hoc analyses of completed HIV vaccine efficacy studies have identified several key characteristics of the vaccine-induced CD8 T-cell response. For example, in HIV Vaccine Trials Network (HVTN) 502, commonly known as the Step Study or MRKAd5, analysis of breakthrough viral sequencing showed that vaccine-induced CD8 T cells exerted a selective pressure at specific amino acid sites [12]. Also, in the MRKAd5 study, Janes et al [13] observed a negative correlation between the number of Gag-specific CD8 T-cell responses and the viral load (VL) after infection. Recent analysis of HVTN 505, or DNA/rAd5, found that individuals with a high Env-specific CD8 T-cell

polyfunctionality were at a lower risk of infection [14]. Collectively, these data suggest that even if CD8 T cells cannot prevent HIV infection, a robust and effective vaccine-induced CD8 T-cell arm could help control infection in those vaccinated individuals who become infected.

Several groups have identified CD8 T-cell-mediated HIV escape through population-based statistical associations of HLA-I alleles and amino acid polymorphisms [15-18]. An individual's HLA-I alleles dictate which epitopes from HIV are presented to and recognized by the host immune response, therefore influencing the affinity and specificity of the CD8 T-cell response; "protective" HLA-I alleles drive potent and effective CD8 T-cell responses [19-21]. We term HIV epitopes containing HLA-I associated polymorphisms *adapted epitopes* and those lacking any HLA-I-associated polymorphisms *non-adapted epitopes*. Our group has previously demonstrated that individuals infected by a virus encoding a higher proportion of adaptations had higher VL set points and faster CD4 T-cell decline. Acutely infected individuals were less likely to mount responses against adapted epitopes, and even when adapted epitope-specific responses arose, they were functionally impaired compared with nonadapted epitope-specific responses [22].

In contrast to acute infection, adapted epitopes can be immunogenic in chronic infection [23], and it remains unclear how HLA-I associated adaptation affects vaccine-generated CD8 T-cell responses. Although vaccine recipients in a given trial often receive the same immunogen, their adaptation to that vaccine depends on which epitopes within the insert their HLA-I molecules bind to and present. Our group preliminarily examined the impact of adaptation to the MRKAd5 insert on CD8 T-cell responses using protein

pool-induced interferon (IFN) γ enzyme-linked immunospot (ELISPOT) assay data generated by the HVTN and MERCK, separately. A significant negative correlation was detected between the mean ELISPOT response and vaccine adaptation for several of the proteins, but there was not convincing agreement between the 2 independently generated sets of data [22]. How HLA-I adaptation to the vaccine insert influences CD8 T-cell responses is a particularly important question as half of HLA-restricted epitopes encoded by single strain HIV vaccines encode adaptations. Mosaic vaccines are geared towards generating more robust CD8 T-cell responses by including multiple versions of HIV proteins [24, 25] and so encode even more adaptations, but vaccines encoding more adapted epitopes may not necessarily elicit broader CD8 T-cell responses.

The current study investigated the impact of vaccine-encoded adaptation on CD8 T-cell responses. Using peripheral blood mononuclear cell (PBMC) samples from the MRKAd5 [26] and the DNA/rAd5 [27] studies, we assessed responses to vaccine-encoded nonadapted and adapted epitopes. We found that in vaccination adapted epitopes were significantly less immunogenic than nonadapted epitopes [22] and that adapted responses were less polyfunctional than nonadapted ones. In examining the impact of protein-level adaptation, we found that vaccine recipients with higher Gag adaptation mounted less functional CD8 T-cell responses. We also found that adaptation to the vaccine insert decreased breadth of response. Collectively, these results indicate that HLA-I associated adaptation remains a significant obstacle to optimizing vaccine-induced CD8 T-cell responses.

MATERIALS AND METHODS

Samples: HVTN 502 (MRKAd5; ClinicalTrials.gov identifier: NCT00095576) were randomized to receive the Ad5 vaccine with HIV-1 gene inserts (*gag, pol,* and *nef*) or placebo [26]. HVTN 505 participants (DNA/rAd5; ClinicalTrials.gov identifier: NCT00865566) were randomized to receive the DNA/rAd5 vaccine with HIV-1 gene inserts (*gag, pol, env a/b/c,* and *nef* [DNA only]) or placebo [27]. Informed consent was obtained from all participants, and all relevant guidelines of the authors' institutions were followed. We blindly assessed samples from 90 MRKAd5 recipients and 20 DNA/rAd5 recipients (N = 110) collected 4 weeks after final vaccination. Of not, we received only 1 vial for 7 MRKAd5 recipients; these individuals were only assessed for protein-pool polyfunctionality. None of the studied recipients were HIV infected at the time point assayed. Placebo recipients (MRKAd5, n = 5; DNA/rAd5, n = 2) for both trials were included in epitope mapping. DNA/rAd5 polyfunctionality data was provided by the HVTN (n = 208) and was published by Janes et al [14].

HLA typing: The HVTN provided HLA-I alleles MRKAd5 recipients. DNA/rAd5 HLA typing was performed as described elsewhere [28]. Briefly, sequence-based typing (Abbott Molecular) and automated DNA hybridization with oligonucleotide probes (Innogenetics) generated 4-digit genotyping.

HLA-I-Associated Polymorphisms: Nonadapted and adapted epitopes and adaptation scores were defined elsewhere by Carlson et al [22]. Essentially, amino acid polymorphisms were found to be linked to specific HLA-I alleles in a large data set of dominant viral sequences and HLA-I alleles from chronically HIV-1-infected individuals. Once HLA-I-associated polymorphisms were identified, the optimal CD8 T-cell epitope

encoding that site was identified using the EpiPred algorithm, which had been trained on the Immune Epitope Database and the Los Alamos HIV database [29, 30]. The sequences of MRKAd5 and DNA/rAd5 inserts were downloaded form the SieveSifter tool [31]. Each epitope was classified as nonadapted or adapted based on the vaccine-encoded amino acid at the HLA-I polymorphic site. Although there were a few instances of overlapping or nested epitopes, these epitopes had unique HLA-I restrictions and were counted as unique epitopes. Adaptation scores were generated using the Microsoft tool PhyloD (Adaptation Tool; https://phylod.research.microsoft.com/Tools/).

Peptide synthesis: All peptides (8-11mer) were synthesized by New England Peptide. Protein pools of overlapping 15-mer peptides were obtained from the HVTN. These peptide pools matched the MRKAd5 insert sequence and included 122 Gag, 51 Nef, 103 Pol1, and 107 Pol2 peptides. For cross-reactivity analysis, the top 3-7 variant epitopes were selected based on population frequency in the Los Alamos HIV Sequence Database [32].

IFNγ ELISpot: ELISPOT assays were performed as described elsewhere [33]. In brief, PBMCs were thawed and rested overnight in R10 medium supplemented with 10% with human serum AB (R10 media) at 37^{o}C and 5% carbon dioxide (CO_2). Plates were coated with anti-IFN-γ antibody at 4^{o}C overnight and then blocked with R10 media for 2 hours at 37^{o}C and 5% CO_2. PBMCs were plated at 100,000 cells per well with the peptide of interest at 10 μmol/L in duplicate and incubated at 37^{o}C and 5% CO_2 for 22 hours. A negative control of media only and a positive control of phytohemagglutinin were included on each plate for each sample. Plates were washed and developed with biotinylated anti-IFN-γ antibody (2 hours), streptavidin (45 minutes), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate solution (10 minutes) sequentially. Plates

were scanned and counted using ImmunoSpot analyzer and software (Cell Technology Limited, version 5.0). Results were normalized to the mean spot-forming units per 10^6 cells. The positive threshold for a response was ≥55 spot-forming unites/10^6 cells and ≥3 times the media-only wells, and net values over media-only background were used for all analyses. Antigen sensitivity was assessed by performing log-fold serial dilution of peptide from 10 to 10^{-2} μmol/L. A dose-response curve was fit for each response and used to calculate the half-maximal effective concentration value (EC50), or the amount of peptide required to elicit 50% of maximal response.

Flow Cytometry: Cytokine and effector molecule production was measured by means of flow cytometry, as described elsewhere [34, 35]. Briefly, PBMCs were thawed and stimulated with the relevant peptide or peptide pool in the presence of anti-CD28, anti-CD49d, and anti-CD107a-FITC (BD Biosciences) for 1 hour at 37ºC and 5% CO_2. After the addition of monensin and brefeldin A (BD Biosciences), cells were incubated for another 11 hours. Cells were then surface stained with the following antibodies for 30 minutes at 4ºC: dead cell dye (Invitrogen), anti-CD3-Alexa 780 (eBioscience), anti-CD4-Qdot 655 (Invitrogen), anti-CD8-V500 (BD Pharmingen), anti-CD14-peridinin-chlorophyll protein/cyanin 5.5 (BD Pharmingen) and anti-CD19- peridinin-chlorophyll protein/cyanin 5.5 (BD Pharmingen). Cells were permeabilized with Cytoperm/Cytofix (BD Biosciences) and then intracellularly stained with the following for 30 minutes at 4ºC: anti-IFN-γ-Alexa 700 (BD Biosciences), anti-interleukin 2-allophycocyanin (BD Biosciences), anti-tumor necrosis factor α-phycoerythrin-cyanine 7 (BD Biosciences), anti-perforin-phycoerythrin (eBioscience), anti-granzyme A-Pac Blue and anti-granzyme B-V450 (BD Biosciences). Cells were fixed in 5% formalin. Events were acquired on an

LSRII flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo software (version 10, TreeStar).

Statistical Analysis: Statistical tests were conducted in GraphPad Prism software, version 7. Comparisons between nonadapted and adapted responses were conducted with Mann-Whitney *U* Tests. Fisher exact tests were used to assess differences in proportion of positive nonadapted or adapted responses. ELISPOT magnitudes were transformed using a $\log(x + 1)$ transformation to allow for visualization of values of 0. Mixed-effect modeling was used to account for multiple responses tested per recipient. The R package, COMPASS, was used to analyze polyfunctionality data by creating functionality and polyfunctionality scores based on Boolaen gating of all possible cytokine producing subsets of CD8 T cells [36].

RESULTS

Poor Immunogenicity of Vaccine-Encoded Adapted Epitopes

PBMCs from participants from 4 weeks after the final MRKAd5 (n = 83) or DNA/rAd5 (n = 20) vaccination were stimulated with CD8 T-cell epitopes relevant to each individual's HLA-I alleles and evaluated using IFN-γ ELISPOT. Data from both MRKAd5 and DNA/rAd5 were combined after no significant differences in response rate or magnitude of positive responses were detected between the studies (data not shown). No responses were seen in placebo recipients to individual peptides or to protein-level peptide pools. A median of 6 nonadapted epitopes (range, 1-11) and 3 adapted epitopes (range, 0-7) were tested per individual. Only 13% of vaccine recipients responded to ≥ adapted epitope, and 60% of recipients responded to ≥ nonadapted epitope (**Figure 1A**). Of note, 6 of 103 vaccine recipients mapped for epitope-specific responses did not restrict any

adapted epitopes. Overall, vaccine recipient responded to a lower proportion of adapted epitopes restricted to their HLA-I alleles compared with nonadapted epitopes (**Figure 1B**). Of epitopes eliciting ≥ 1positive response, adapted epitopes elicited fewer responses than nonadapted ones (**Figure 1C**). Collectively, these data indicate that vaccine-encoded adapted epitopes are poorly immunogenic, similar to what we have reported in acute infection [22].

Possible Dampening of Adapted Epitope-Specific Responses in MRKAd5 recipients With Sequential Vaccination.

Because adapted epitopes are poorly immunogenic in acute infection [22] but less so in chronic infection [23], we examined whether adapted epitope-specific responses were enhanced with sequential vaccination. The MRKAd5 vaccine was administered 3 times over 6 months in a homologous prime-boost regimen, which elicited CD8 T-cell responses in most pre-clinical trial participants [37, 38]. In a subset of MRKAd5 recipients, we examined responses after the second vaccination (n = 17). Although adapted responses has a lower magnitude than nonadapted responses at both time points, the magnitude of adapted responses significantly decreased from the second to the third vaccination time point (**Figure 2A**). We also saw that after the second vaccination the response frequencies to nonadapted and adapted epitopes were comparable, but after the third, individuals are less likely to response to adapted epitopes than to nonadapted ones (**Figure 2B**). These results suggest that adapted responses may be less durable than nonadapted responses.

Poor Polyfunctionality of the Adapted CD8 T-Cell Response

In acute HIV-1 infection, adapted epitope-specific CD8 T cells are less immunogenic and less functional than nonadapted ones [22]. Because we found that in

vaccination adapted epitopes were also less immunogenic than nonadapted epitopes (**Figure 1**), we next investigated whether vaccine-induced adapted responses were functionally impaired. We found that the IFN-γ ELISPOT magnitudes of positive nonadapted and adapted responses per recipient were similar (**Figure 3A**), and that the antigen sensitivities of nonadapted and adapted responses were also comparable (**Figure 3B**). We also assessed the ability of these responses to cross-recognize variant epitopes by testing for IFN-γ ELISPOT responses against the most frequent variants encoded in circulating sequences at the population level. We found that both nonadapted and adapted responses displayed a wide range of cross-reactivity (**Figure 3C**). However, when we assessed the polyfunctionality, or the ability of CD8 T cells to product > 1 effector molecule, we found that adapted responses were less polyfunctional than nonadapted ones (**Figure 3D**). In summary, HLA-I adaptations seem to negatively influence the polyfunctionality of vaccine-induced epitopes-specific responses.

Higher Adaptation to the Gag Vaccine Insert Correlated With Less Polyfunctional CD8 T-Cell Responses

It was previously demonstrated that polyfunctional Env-specific CD8 T cells were associated with a significantly lower risk of HIV infection following DNA/rAd5 vaccination [14], and we saw that adapted responses were less polyfunctional than nonadapted ones. Therefore, we hypothesized that higher HLA adaptation to the vaccine insert dampens the polyfunctionality of CD8 T-cell responses. Polyfunctionality and functionality scores were calculated using the R package, COMPASS version 1.22.0 [36], based on intracellular staining data. In the DNA/rAd5 study, we found that vaccine recipients with a higher level of adaptation to the Gag vaccine insert, or a more positive

adaptation score, mounted less functional and less polyfunctional Gag-specific CD8 T -cell responses (**Figure 4A-B**). A similar negative correlation was also seen between Gag adaptation and the frequency of Gag-stimulated CD8 T cells producing IFN-γ, tumor necrosis factor α, or interleukin 2 (data not shown). We found a similar trend in a smaller cohort of MRKAd5 recipients (n = 38), wherein recipients with higher Gag adaptation mounted less functional and polyfunctional Gag-specific CD8 T-cell responses (**Figure 4C-D**). No correlations were detected between protein-specific adaptation score and CD8 T-cell response for Pol, Nef, or Env in either trial. These data show that HLA-I-associated adaptation to the Gag vaccine insert is correlated with dampened CD8 T-cell responses.

Higher Adaptation to Vaccine Insert Associated With Lower Breadth of Response

Because the breadth of the Gag-specific CD8 T-cell response has previously been implicated in viral control in vaccine recipients who became infected [13], we first determined whether we observed a similar relationship between VL and breadth of response detected by our mapping strategy. Even within our relatively small cohort of MRKAd5 recipients who became infected (n = 24), we saw that a greater breadth of the vaccine-induced CD8 T-cell response correlated with lower VLs (**Figure 5A**). Interestingly, this effect remains even when excluding Gag-specific responses ($p < 0.05$, data not shown). VL was calculated as the geometric mean of VLs from all visits for 1 year after the first positive Western blot or until the participant started ART. We next examined the relationship between breadth of the vaccine-induced response and adaptation to the vaccine insert and saw that vaccine recipients with higher adaptation mounted less broad CD8 T-cell responses (**Figure 5B**).

DISCUSSION

Although the immunogenicity of adapted epitopes seems to vary depending on the stage of HIV infection, our study clearly shows that HLA-I associated adaptation plays a significant role in shaping CD8 T-cell responses in the context of HIV vaccination. In 2 prior HIV-1 vaccine efficacy trials, we found that vaccine-encoded adapted epitopes were less immunogenic than nonadapted epitopes, similar to acute infection. The polyfunctionality of the few immunogenic adapted responses we detected was inferior to that of nonadapted ones. We also saw that the level of adaptation to the Gag vaccine insert negatively correlated with CD8 T-cell polyfunctionality. We found that higher breadth of response to the vaccine correlates with lower VL after infection and that breadth of responses seems to be negatively affected by HLA-I-associated adaptation. Collectively, these results indicate that such adaptation should be an important consideration in future vaccine design.

We have previously shown that adapted epitopes are poorly immunogenic in acute HIV infection and elicit functionally compromised CD8 T cells. However, in chronic infection, adapted responses may be increased [35] and may not remain functionally compromised [23]. We found that adapted epitopes are poorly immunogenic in HIV vaccination but that adapted responses are comparable in magnitude, antigen sensitivity, and cross-reactivity to nonadapted ones. However, even within the limited subset of responses examined, we found that vaccine-generated adapted responses are less polyfunctional than nonadapted ones.

Although immune responses induced by HIV vaccination are generated in the context of an immune system that is relatively healthy and intact, our data suggest that

HLA-I-associated adaptation still negatively influence CD8 T-cell responses at the epitope level. Though it is possible that vaccine-induced nonadapted and adapted responses also differ in ways beyond those tested here, many publications have demonstrated that antigen sensitivity, cross-reactivity, and polyfunctionality are reasonable measurements of CD8 T-cell functionality [39, 40]. Nonadapted and adapted responses also appear to respond differently to the same vaccine regimen, with homologous sequential vaccination dampening adapted responses to a greater extent than nonadapted ones. Because past preclinical vaccine schedule studies have not distinguished between nonadapted and adapted responses, additional research may be necessary to determine how to maximize vaccine-elicited adapted responses.

A recent publication posits a role for Env-specific CD8 T-cell polyfunctionality in decreasing infection risk of DNA/rAd5 vaccine recipients [14]. Although we did not see a relationship between adaptation to the Env insert and Env-specific responses in the DNA/rAd5 study, higher variability in Env sequences preclude accurate HLA-I associated polymorphism predictions. As such, we are limited in our current analysis. However, the relatively poor polyfunctionality of adapted responses and the relationship we observe between adaptation and Gag-specific responses in these 2 studies suggests that the relationship between CD8 T-cell adaptation to Env and polyfunctionality warrants further investigation, particularly if polyfunctionality is confirmed as a true correlate of protection.

As broader vaccine-induced CD8 T-cell responses correlate with better viral control, the finding that HLA-I-associated adaptation to the vaccine affects the breadth of response has significant implications for T cell-based vaccine design. Our ability to detect this correlation between breadth and VL with a more limited mapping approach was

reassuring. In addition, unlike previously published data that showed this effect exclusively for Gag-specific breath [13], our finding remained when Gag-specific responses were excluded. This suggests that though our approach to mapping CD8 T-cell responses is narrower, it still identifies critical CD8 T-cell responses and indicates that there are important CD8 T-cell responses targeting areas outside of Gag. To our knowledge, this is the first demonstration of non-Gag-specific CD8 T-cell responses correlating with VL.

Conserved HIV vaccines aim to redirect the CD8 T-cell response towards evolutionarily conserved subdominant epitopes [41, 42]. Because these regions of the virus are unlikely to undergo HLA-I associate adaptation, the poor immunogenicity of adapted epitopes may be a less relevant consideration. However, another popular HIV vaccine strategy is the use of mosaic vaccines that encode multiple versions of each sequence and aim to elicit a broader CD8 T-cell response. Many of these mosaic vaccine immunogenicity studies were conducted in the SIV macaque model, where HLA-I-associated adaptation is not at play. Although our data indicate that adapted epitopes are poorly immunogenic and poorly polyfunctional, it is possible that immunogenic adapted epitopes still induce important CD8 T-cell responses in the context of vaccination. With additional immune response mapping, it may be possible to target the design of mosaic vaccines to include only immunogenic adapted epitopes. And, as large epitope-mapping data sets become available, it may be possible to use machine learning to predict immunogenicity and direct vaccine design. Another area to explore is the ability of vaccine-induced responses to cross-recognize adapted epitopes. Adaptations are frequently transmitted, and infection with a preadapted virus negatively affects disease progression [22, 43-45]. Therefore, even if

adapted epitopes are poorly immunogenic, potently cross-reactive nonadapted responses may still effectively counter circulating HIV-1 strains encoding adaptations.

The poor immunogenicity of and polyfunctionality of adapted epitopes ultimately brings into question the utility of including them in HIV-1 vaccines. The breadth of the vaccine-induced response has been demonstrated to affect viral control in vaccine recipients who become infected [13], and, more recently, polyfunctionality has been put forward as a protective feature of vaccine-induced CD8 T cells [14], so it is possible that including many adapted epitopes in a vaccine insert may simply take up immunological space in a vaccine without improving vaccine efficacy. Ongoing and future studies will address the benefits or drawbacks of including adapted epitopes in vaccines. Although our study shows that vaccine-encoded adapted epitopes are poorly immunogenic and polyfunctional, future work should examine whether CD8 T-cell responses are better induced with mosaic vaccines and whether vaccines encoding more conserved epitopes are able to cross-recognize HIV-1 viruses encoding adaptations.

SUPPLEMENTARY DATA

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the poster materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be address to the corresponding authors.

NOTES

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Figure 1. *Vaccine-encoded adapted epitopes are less immunogenic than vaccine-encoded nonadapted epitopes.*

A, Percentage of vaccine recipients responding to ≥1 tested nonadapted epitope or adapted epitope (Fisher exact test used for comparison). **B**, Percentage of positive responses to tested epitopes per vaccine recipient (Wilcoxon matched-pairs signed rank test used for comparisons). **C**, Percentage of vaccine recipients responding to each nonadapted or adapted epitope with ≥1 positive response (Mann-Whitney *U* test used for comparison). Closed symbols represent MRKAd5 recipients or response; open symbols, DNA/rAd5 recipients or responses.

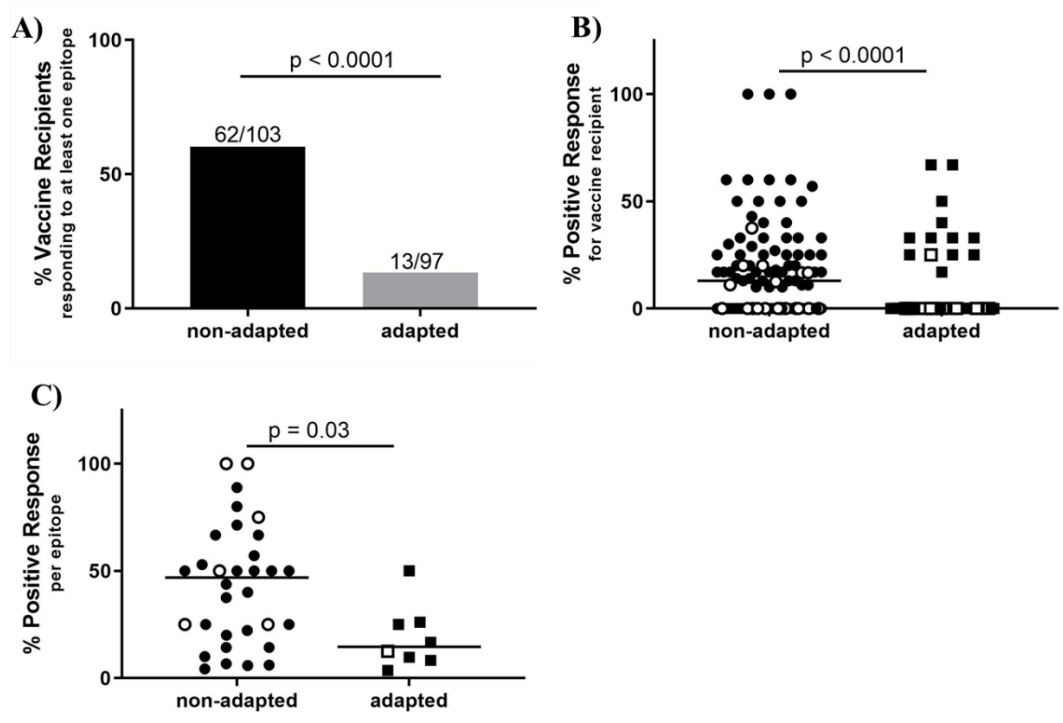


Figure 2. *Adapted epitope-specific CD8 T-cell responses in MRKAd5 recipients may be dampened with sequential vaccination.*

A, Enzyme-linked immunospot magnitude of all nonadapted and adapted epitope-specific responses per vaccine recipient after the second and third vaccination time points. Dotted line represents positive threshold of 55 spot-forming units (SFU)/10^6 cells (mixed-effect model accounting for multiple measurements for each recipients). **B**, Frequency of nonadapted or adapted responses per recipient for the same time points (Wilcoxon matched-pairs signed rank test).

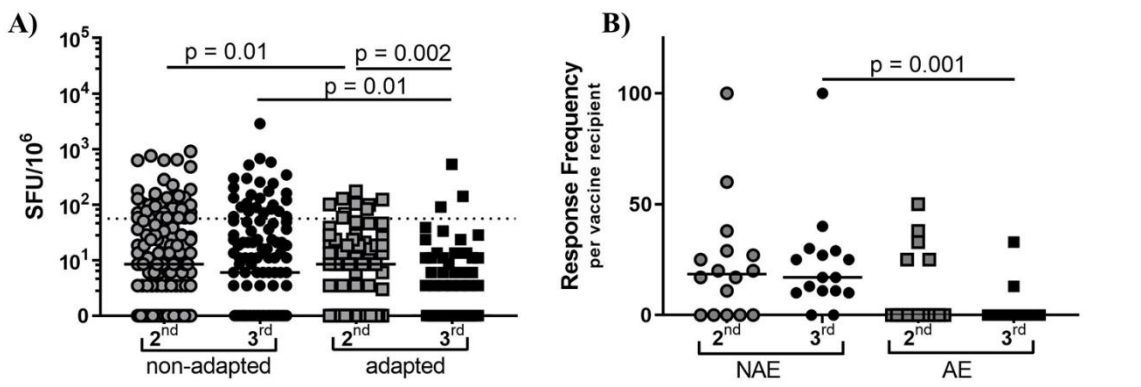


Figure 3. *Adapted epitope-specific CD8 T-cell responses are poorly polyfunctional compared with nonadapted ones.*

A, All positive nonadapted and adapted responses of vaccine recipients. Dotted line indicates the positive threshold of 55 spot-forming units (SFU)/10^6 cells. Some values appear under this cutoff because net values are displayed. **B**, Antigen sensitivity (half-maximal effective concentration [EC_{50}]) for nonadapted versus adapted responses. **C**, Cross-reactivity of responses, or percentage of variant epitopes recognized, in interferon γ enzyme-linked immunospot assay. **D,** Polyfunctionality of responses as assessed by intracellular staining and quantified in a polyfunctionality score. Closed symbols represent MRKAd5 recipients; and open symbols, DNA/Ad5 recipients. *P* values determined by means of mixed-effect modeling to account for multiple measurements per recipient. Abbreviation: NS, not significant.

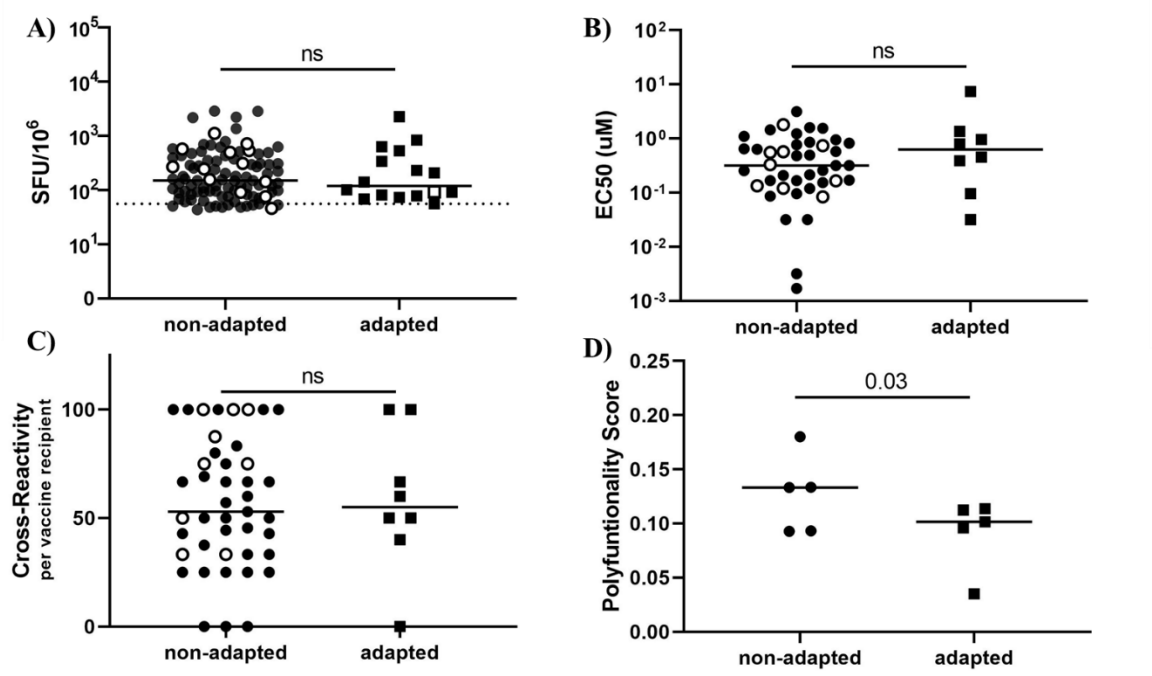


Figure 4. *Higher adaptation to the Gag vaccine insert correlates with less functional Gag-specific CD8 T-cell responses.*

A, ***B***, Gag-specific adaptation score of DNA-rAd5 recipients versus the functionality and polyfunctionality of the Gag-specific CD8 T-cell response (n = 208). **C**, **D**, Similar comparison for MRKAd5 recipients (n = 38). (All *r* and *P* values were determined by means of Spearman correlation. Graphic representation of correlation shows linear regression lines.)

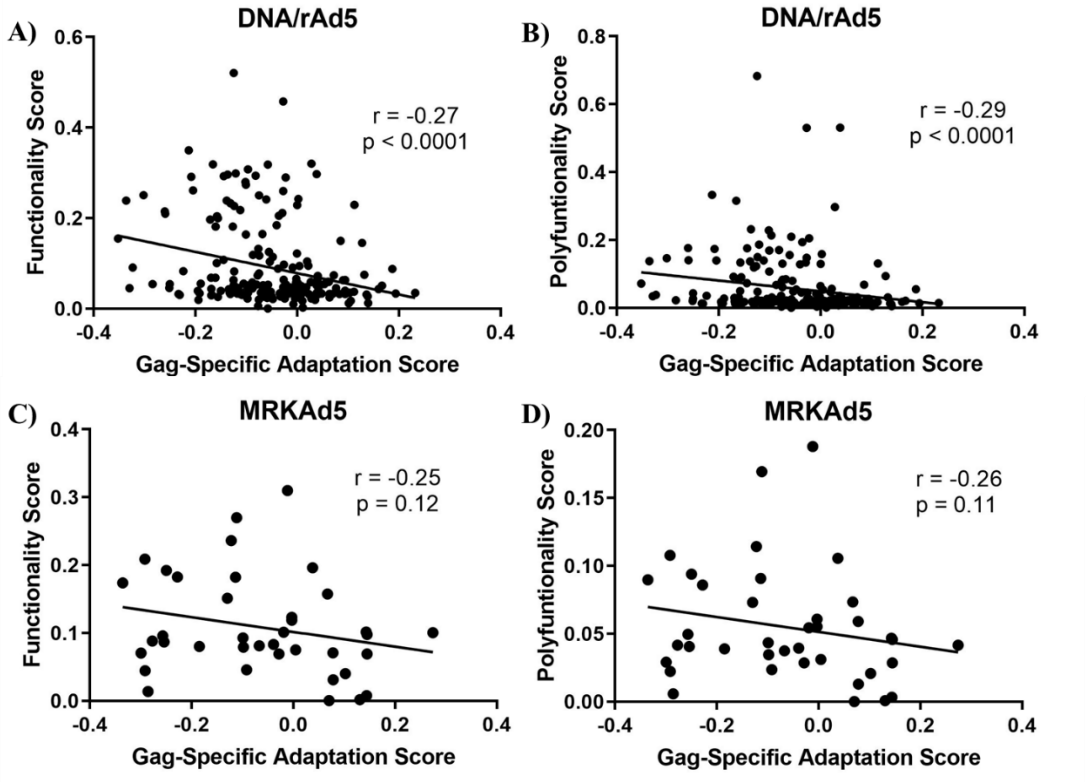
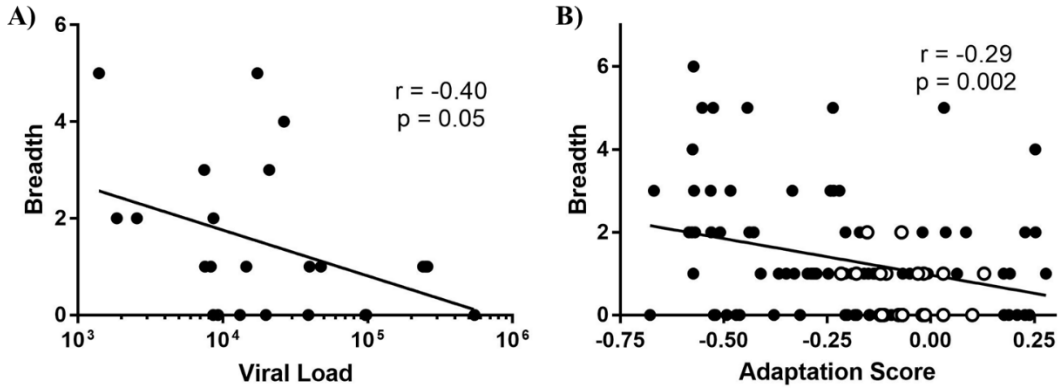


Figure 5. *Breadth of response negatively correlates with viral load in MRKAd5 recipients who became infected, and higher adaptation is associated with a lower breadth of response.*

A, Overall breadth of the vaccine response versus viral load (copies/mL) in MRKAd5 recipients who became infected. **B**, Overall breadth versus the overall adaptation of the vaccine insert. Open circles indicate DNA/rAd5 recipients; closed circles, indicate MRKAd5 recipients. (All *r* and *P* values were determined by means of Spearman correlation. Graphic representation of correlations shows linear regression lines.)



Supplementary Table 1. *List of non-adapted and adapted epitopes encoded by the MRKAd5 and/or DNA/rAd5 vaccines.*

Protein, HLA-I restriction, sequence, and classification of each epitope encoded by the MRKAd5 and/or DNA/rAd5 vaccine inserts. Amino acids in green indicate HLA-I polymorphic sites that are non-adapted, amino acids in red indicate HLA-I polymorphic sites that are adapted. Amino acids in blue indicate differences in sequence between two versions of epitopes encoded by the two vaccines.

Protein	HLA-I	Sequence	Type	MRKAd5	DNA/Ad5
Gag	A*01	SLYNTVATLY	NAE	✓	✓
Gag	A*03:01	RLRPGGKKKYK	NAE	✓	✓
Gag	A*11	ATLYCVHQK	NAE	✓	
		ATLYCVHQR			✓
Gag	A*11	QMVHQAISPR	AE	✓	✓
Gag	A*11:01	VTNSATIMMQR	AE	✓	✓
Gag	A*24	KYKLKHIVW	NAE	✓	✓
Gag	A*25:01	ETINEEAAEW	NAE	✓	
Gag	A*30	RLRPGGKKKY	NAE	✓	✓
Gag	A*31	TLYCVHQK	AE	✓	
		TLYCVHQR	NAE		✓
Gag	A*31:01	KIWPSHKGR	NAE	✓	
Gag	B*08:01	IYKRWIIL	NAE	✓	✓
Gag	B*14	DRWEKIRL	NAE		✓
		DKWEKIRL	AE	✓	
Gag	B*14:01	DRFYKTLRA	NAE	✓	✓
Gag	B*27	KRWIILGL	NAE	✓	✓
Gag	B*35:01	NPPIPVGEIY	AE	✓	✓
Gag	B*39:01	VHQAISPRTL	NAE	✓	✓
Gag	B*40	KDCNERQANFL	AE	✓	
Gag	B*40:01	QEPIDKELYPL	NAE	✓	
Gag	B*40:01	IDVKDTKEAL	AE	✓	
		IEIKDTKEAL	NAE		✓
Gag	B*40:02	NERQANFL	AE	✓	
Gag	B*40:02	AEWDRLHPVHA	NAE	✓	
		AEWDRVHPVHA			✓
Gag	B*44	AEQASQEVKNW	AE	✓	✓
Gag	B*49:01	GELDKWEKI	NAE	✓	
Gag	B*52:01	RMYSPTSILDI	NAE	✓	✓
Gag	B*57	TSTLQEQIGW	NAE	✓	✓

Gag	B*57:01	QAISPRTLNAW	NAE	✓	✓
Gag	B*58	TTSTLQEQIGW	NAE	✓	✓
Gag	C*06:02	YCVHQKIDV	AE	✓	
		YCVHQRIEI	NAE		✓
Pol	A*03	AIFQCSMTK	NAE		✓
		AIFQSSMTK		✓	
Pol	A*03:01	QIYPGIKVR	AE	✓	✓
Pol	A*30	GQGQWTYQIY	NAE	✓	✓
Pol	A*30	ILKEPVHGVYY	AE	✓	✓
Pol	A*30:02	KIQNFRVYY	NAE	✓	✓
Pol	A*33:03	ELKKIIGQVR	NAE	✓	
Pol	A*33:03	FYVAGAANR	NAE	✓	
Pol	B*13:02	GQGQWTYQI	NAE	✓	✓
Pol	B*14	GRWPVKTI	NAE	✓	
		GRWPVKTV			✓
Pol	B*15:02	TVLAVGDAY	NAE	✓	✓
Pol	B*15:03	FKRKGGIGGY	NAE	✓	✓
Pol	B*18:01	NETPGIRYQY	NAE	✓	✓
Pol	B*27	KRKGGIGGY	NAE	✓	✓
Pol	B*35:01	NPDIVIYQY	NAE	✓	✓
Pol	B*35:01	VPLDEDFRKY	NAE	✓	
		VPLDKDFRKY	AE		✓
Pol	B*38	YRDSRNPL	NAE	✓	
Pol	B*40	KEPPFLWMGY	NAE	✓	✓
Pol	B*40:01	KETWEAWWTEY	NAE		✓
		KETWETWWTEY	NAE	✓	
Pol	B*44	AEIQKQGQGQW	NAE	✓	✓
Pol	B*44:02	IDKAQDEHEKY	AE	✓	✓
Pol	B*51:01	IPSINNETPGI	NAE	✓	
Pol	B*51:01	MASDFNLPPV	AE	✓	
Pol	B*53:01	LPIQKETWETW	NAE	✓	
Pol	B*57	FTSTTVKAACW	NAE		✓
Pol	B*58	FTSTTVKAACW	NAE		✓
Pol	B*58	IATESIVIW	AE		✓
		ITTESIVIW	NAE	✓	
Pol	B*58:01	IVLPEKDSW	NAE	✓	✓
Pol	C*04	AYFLLKLA	AE	✓	✓
Pol	C*15:02	LVSAGIRKVL	NAE	✓	✓
Pol	C*15:05	LVSAGIRKVL	NAE	✓	✓
Nef	A*02	FLKEKGGLEGL	AE	✓	
Nef	A*03	GLEGLIHSQK	NAE	✓	
Nef	A*23:01	RFPLTFGWCF	AE	✓	
Nef	A*24	RFPLTFGWCF	AE	✓	
Nef	A*30:01	VARELHPEYYK	NAE	✓	
Nef	A*30:02	GYFPDWQNY	NAE	✓	

Nef	A*30:02	HVARELHPEYY	AE	✓	
Nef	B*07	TPGPGIRFPL	NAE	✓	
Nef	B*07	VPVEPEKVEEA	AE	✓	
Nef	B*13:02	RQDILDLWV	NAE	✓	
Nef	B*14	WRFDSKLAF	NAE	✓	
Nef	B*18:01	QDILDLWVY	AE	✓	
Nef	B*35	TPGPGIRF	NAE	✓	
Nef	B*35:01	RPQVPLRPMTY	AE	✓	
Nef	B*35:01	VPLRPMTYKGA	NAE	✓	
Nef	B*39	WSTVRERM	NAE	✓	
Nef	B*40	KEKGGLEGL	NAE	✓	
Nef	B*44	KEKGGLEGLIH	AE	✓	
Nef	B*57	GAVDLSHF	NAE	✓	
Nef	B*57:03	KGAVDLSHF	AE	✓	
Nef	C*03:02	CAAHPMSQHGI	NAE	✓	
Nef	C*06:02	GIEDPEKEVL	NAE	✓	
Nef	C*07	DILDLWVY	NAE	✓	
Nef	C*08:01	KGAVDLSHFL	AE	✓	
Nef	C*14	GYFPDWQNYT	NAE	✓	
Nef	C*18:01	PLRPMTYKGAV	NAE	✓	

CROSS-REACTIVE CD8 T-CELL RESPONSES ELICITED BY ADENOVIRUS TYPE 5-BASED HIV-1 VACCINES CONTRIBUTED TO EARLY VIRAL EVOLUTION IN VACCINE RECIPIENTS WHO BECAME INFECTED

by

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ABSTRACT

Because of HIV's vast sequence diversity, the ability of the CD8 T-cell response to recognize several variants of a single epitope is an important consideration for vaccine design. Cross-recognition of viral epitopes by CD8 T cells is associated with viral control during HIV-1 infection, but little is known about CD8 cross-reactivity in the context of HIV-1 vaccination. Here, we evaluated vaccine-induced CD8 cross-reactivity in two preventative HIV-1 vaccine efficacy trials, the MRKAd5 and DNA/rAd5 studies. Cross-reactive CD8 responses elicited by vaccination were similar in magnitude and frequency to those induced during acute HIV-1 infection. Although responses directed against variant epitopes were less avid than responses to vaccine-matched epitopes, we did not detect any difference in response polyfunctionality (the proportion of cells producing multiple effector molecules). And while depth, or the frequency of cross-reactive responses, did not correlate with viral loads in recipients who became infected, cross-reactivity did appear to influence early viral evolution. In comparing viral sequences of placebo versus vaccine recipients, we found that viral sequences from vaccinees encoded CD8 epitopes with more substitutions and greater biochemical dissimilarity. In other words, breakthrough sequences of vaccinees would be less cross-recognized by vaccine-induced responses. Additionally, vaccine-induced CD8 T cells poorly cross-recognized variant epitopes encoding HLA-I associated adaptations, further supporting our conclusion that these responses play a role in driving early HIV-1 viral evolution.

IMPORTANCE

HIV-1 has exceptionally high sequence diversity, much of which is found within CD8 epitopes. Therefore, the ability of CD8 T cells to recognize multiple versions of a single epitope could be important for an effective vaccine. Here, we show that two previously tested vaccines induced a similar level of CD8 cross-reactivity to that seen in acute HIV-1 infection. Although this cross-reactivity did not seem to affect viral control in vaccine recipients who became infected, we identified several ways in which CD8 cross-reactivity appeared to influence HIV-1 viral evolution. First, we saw that strains isolated from infected vaccine recipients would likely be poorly cross-recognized by the vaccine-induced response. Secondly, we saw that adapted CD8 epitopes were poorly cross-recognized in both vaccination and infection. Collectively, we believe these results show that CD8 cross-reactivity could be an important consideration in future HIV-1 vaccine design.

KEYWORDS

CD8 cross-reactivity, HIV-1 vaccine, HIV-1 viral evolution

INTRODUCTION

Although only one vaccine trial has provided protection against HIV-1 infection [1], studies of CD8 T cells induced by inefficacious vaccines suggest that they can contribute to viral control in recipients who became infected [2] and to vaccine efficacy in a subset of recipients [3]. In HIV Vaccine Trials Network (HVTN) 502, commonly known as the Step study or MRKAd5, researchers found that the HIV-1 sequences isolated from vaccine recipients who became infected were more distant from the vaccine-encoded sequence than those from placebo recipients. These differences were only seen in proteins that were included in the vaccine and suggested that vaccine-induced CD8s exerted immune pressure on the infecting virus [4]. In this same vaccine trial, the Gag-specific breadth of the vaccine-induced CD8 T-cell response (CD8 response) was found to inversely correlate with viral load in vaccine recipients who became infected [2]. We recently reported a similar relationship between overall CD8 T-cell response breadth and viral load in MRKAd5 recipients who became infected [5]. In another vaccine trial, HVTN 505 (DNA/rAd5), polyfunctional Env-specific CD8s, or CD8 T cells capable of producing multiple effector molecules simultaneously, were associated with a decreased risk of infection [3]. Together, these studies suggest that, an effective CD8 T-cell response is important for the biology of the immune response against HIV and could be an important component of an effective vaccine.

In HIV-1 infection, there is evidence that CD8 T-cell cross-reactivity could be important for natural viral control. In the context of the protective HLA-I alleles, B*27 and B*57, T-cell receptor (TCR) clonotypes from natural controllers have been found to be more effective at suppressing viruses encoding escape mutations [6, 7], and greater cross-

recognition of variant epitopes has been associated with lower viral loads [8]. One computational study found that a larger fraction of B*57-restricted CD8 T-cell repertoires could cross-recognize variant epitopes than other HLA-restricted CD8s [9]. A major limitation of many CD8 cross-reactivity studies has been their focus on responses during chronic HIV-1 infection. In these studies, it is difficult to distinguish whether a given CD8 T-cell response is due to previous exposure to variant epitopes during the course of viral evolution or if they are truly exhibiting cross-reactivity. Vaccination presents a unique opportunity to examine CD8 T-cell cross-reactivity, since, unlike natural HIV-1 infection, the epitope priming the CD8 response is known, and cross-reactive responses can be clearly defined.

Given that HIV-1 harbors incredible sequence diversity and that much of that variability exists within epitopes targeted by CD8s, vaccine-induced CD8 cross-reactivity is likely an important aspect of the vaccine-induced immune response. However, the ability of vaccine-induced CD8s to cross-recognize variant epitopes not encoded by the vaccine has remained understudied. One past study of chronically infected participants showed that variant epitope recognition by vaccine-induced CD8s was constricted by similar factors to those found in infection, namely, the number of amino acid substitutions and the biochemical conservation of the amino acid change. However, this study did not expand upon these cross-reactive responses in vaccination [10]. Another study showed that vaccine-elicited CD8s were not able to suppress variant epitope-encoding viruses as well as CD8s from chronically infected individuals. However, because this study did not examine responses at the epitope level, it is difficult to ascertain whether this relatively poor viral inhibition was due to lack of cross-reactivity or a different aspect of the CD8

response, such as breadth or polyfunctionality [11]. While variant recognition has been correlated with viral control in HIV-1 infected individuals [12], the relationship between vaccine-induced CD8 cross-reactivity and viral evolution or control remains unknown.

In this study, we examined the epitope-level cross-reactivity of CD8s induced by two previously tested adenovirus type 5 (Ad5)-vectored HIV-1 vaccines, MRKAd5 and DNA/rAd5. Using the interferon gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay, we detected a wide range of cross-recognition of variant epitopes not encoded by the vaccine. A higher number of amino acid substitutions, greater biochemical dissimilarity, and anchor mutations hindered cross-recognition of variant epitopes. Although the antigen sensitivity of these cross-reactive responses was lower when responding to variant epitopes, the polyfunctionality of the CD8s was similar after stimulation with vaccine and variant epitopes. There was no evidence that CD8 cross-reactivity contributed to viral control in MRKAd5 recipients who became infected; however, we observed differences between the breakthrough sequences of vaccine versus placebo recipients, where epitope sequences in Pol that were isolated from vaccinees were less likely to be cross-recognized by the vaccine-induced response. We also found that vaccine-induced CD8 responses were less able to cross-recognize variant epitopes encoding HLA-I-associated adaptations, further arguing that CD8 cross-reactivity shapes HIV-1 viral evolution. Collectively, our results support the idea that maximizing CD8 cross-reactivity could be an important component of future HIV-1 vaccines.

RESULTS

Cross-reactive CD8 responses elicited by vaccination are similar in magnitude and depth to those induced in acute HIV-1 infection.

In order to assess CD8 cross-reactivity, we first mapped responses of HIV-1 infected individuals to epitopes encoded by their transmitted founder virus (TFV) and of vaccine recipients to epitopes encoded by the vaccine. Each positive TFV or vaccine-directed CD8 response was then assessed for cross-reactivity to the most common circulating epitope variants (see Materials and Methods for epitope selection details). Six HIV-1-infected individuals, described in **Table 1**, were tested in acute infection with a median of 43 days post-infection (DPI). Of previously evaluated vaccine recipients [5], 46 who mounted vaccine-directed responses were screened for cross-reactive CD8 T-cell responses 4 weeks following the final vaccination timepoint (37 from MRKAd5 and 9 from DNA/Ad5). Vaccine and cross-reactive responses were assayed using IFN-γ ELISpot. An example of an epitope group tested for cross-reactivity is shown in **Fig. 1A**. This epitope (Pol-B*44-AEQASQEVKNW) was encoded by the TFV in two HIV-1-infected individuals as well as the MRKAd5 and DNA/Ad5 vaccines. In both vaccination and infection, we observed varied potential to recognize variants of this epitope. These results indicate that while CD8 responses in acute infection and vaccination are often cross-reactive on some level and some variants are consistently recognized, cross-reactivity is not necessarily consistent for a given epitope across individuals.

In examining the positive responses in HIV-1 infected individuals, we observed that TFV-specific responses were higher in magnitude than responses to variants. Similarly, among vaccine recipients, responses to vaccine-matched epitopes were higher than responses to variants of the same epitope (**Fig. 1B**). In addition to the magnitude of variant-directed responses, response depth is an important metric of cross-reactivity. The depth of the response is the number of positive responses to variant epitopes divided by the

number of variant epitopes screened, or the frequency of cross-reactive recognition of variants. We found no significant difference in the depth of CD8 responses in acutely infected versus vaccinated individuals (**Fig. 1C**). All infected individuals mounted some level of cross-reactivity, and the majority of vaccine recipients were also able to cross-recognize at least one variant epitope (43/46, 93%). Collectively these data show that CD8 cross-reactivity in acute HIV-1 infection and vaccination are similar in magnitude and depth.

CD8 cross-reactivity is dampened by increased amino acid substitutions, decreased biochemical similarity, and HLA anchor site mutations.

Previous investigations into vaccine-induced CD8 T-cell cross-reactivity demonstrated that cross-recognition was impaired as the number of amino acid substitutions increased and as biochemical similarity decreased [10]. We also found that as the number of amino acid substitutions increased, the ELISpot responses to variant epitopes decreased in both acute infection (**Fig. 2A**) and vaccination (**Fig. 2B**). We calculated the biochemical similarity of variant epitopes compared to their TFV- or vaccine-encoded counterpart and found that as the biochemical similarity of variant epitopes increased (similarity score increased), cross-recognition also increased (**Fig. 2C and D**). We also examined the impact of HLA anchor mutations on cross-recognition, where the peptide's second amino acid residue (P2) and C-terminal end were defined as HLA-I binding sites. Initially, we did not see any difference between nonanchor and anchor site mutated variants (data not shown). A past study showing decreased recognition of anchor mutated epitopes by CD8 responses in chronic HIV-1 infection had limited its analysis exclusively to 9mers [13]. When we did the same, we found that anchor site

mutated variants were less cross-recognized compared to variant epitopes with mutations outside of HLA-I anchor sites, in both infection and vaccination (**Fig. 2E and F**). It is possible that this difference between nonanchor and anchor mutated variant epitope responses only arises when the dataset is limited to 9mers because the P2/C-terminal assumption of HLA-I binding is not an accurate assumption for peptides of all lengths. Overall, these results demonstrate that, as expected, vaccine-induced CD8 T cells most easily recognize variant epitopes that closely resemble the vaccine immunogen.

MRKAd5-induced CD8 cross-reactivity did not impact viral control in recipients who became infected.

Because the breadth of the vaccine-induced response (i.e. number of vaccine-matched epitopes recognized) was inversely correlated with viral load among MRKAd5 recipients who became infected [2, 5], we asked whether CD8 cross-reactivity impacted viral control in a similar manner. Viral load for each vaccine recipient was calculated as the geometric mean of viral loads (VLs) from all visits for 1 year following the first positive Western blot or until the participant started antiretroviral therapy (ART), as we have previously done [14]. We did not observe any relationship between depth and viral load in MRKAd5 recipients who became infected (**Fig. 3A**). In fact, the depth of an individual's response did not correlate with that individual's breadth (**Fig. 3B**). It is worth noting, however, that vaccine recipients who mounted three or more vaccine responses, all cross-recognized at least 50% of the variant epitopes tested. Additionally, there is a clear relationship between response magnitude and depth, where individuals with higher magnitude vaccine responses also exhibit higher depth, or more cross-reactivity (**Fig. 3C**). Although recent studies have also implicated CD8 T cells as decreasing infection risk in

vaccine recipients [3], we did not observe a difference in the level of CD8 T-cell cross-reactivity between MRKAd5 recipients who became infected and those who did not (data not shown). Our results suggest that, even though MRKAd5 induced cross-reactive CD8 responses, these responses did not influence viral control or infection risk.

Cross-reactive CD8 T-cell responses are functionally similar to responses against vaccine-encoded epitopes.

Because cross-reactivity did not appear to play a role in viral control, we next asked if this may due to poor functionality of these CD8 T cells when stimulated with the variant epitope compared to when stimulated with the vaccine-encoded epitope. An important functional measure of the CD8 response is antigen sensitivity, or the amount of antigen required to induce a response, which has been shown to correlate with viral suppression [15]. We found that vaccine-induced CD8 responses had a slightly lower antigen sensitivity towards variant epitopes than toward vaccine epitopes (**Fig. 4A**). However, when we assessed the polyfunctionality of a subset of the cross-reactive responses, quantified as a polyfunctionality score, or PFS, by the R package COMPASS [16], we did not find a difference between cells stimulated by the vaccine versus the variant epitope (**Fig. 4B** and **C**). Since polyfunctionality is a metric that has been linked to vaccine efficacy [3] and cross-reactive responses are similarly polyfunctional to vaccine-matched and variant epitopes, these findings suggest that cross-reactive responses can be an important parameter to be induced by preventative vaccines.

Frequency of cross-reactive responses increased over homologous sequential MRKAd5 vaccination.

In order to determine how cross-reactive responses may change over the course of a vaccine schedule, we compared the responses of 13 vaccine recipients after the second vaccination at week 8 (2nd) to their responses following the third, and final, vaccination at week 30 (3rd). We found that cross-reactive responses to variant epitopes were consistently lower in magnitude than their vaccine counterparts at both time points, but no significant difference was detected in the magnitude of responses after the 2nd vaccination and after the 3rd vaccination (**Fig. 5A**). However, we did observe a significant increase in the depth of the response over the sequential vaccinations (**Fig. 5B**), indicating that the frequency of cross-recognition increases from following the 2nd vaccination to the 3rd vaccination. Immunogenicity studies of the MRKAd5 study did not show a significant increase in magnitude or frequency of vaccine-induced responses from the second to the third vaccination timepoint [17], so it is intriguing that cross-reactivity of these responses does increase.

The DNA/rAd5 vaccine elicited CD8 responses with greater depth than the MRKAd5 vaccine.

While the two vaccine studies examined here, MRKAd5 and DNA/rAd5, were both designed with the goal of eliciting CD8s, the MRKAd5 vaccine insert encoded *gag*/*pol*/*nef,* while DNA/rAd5 encoded *gag*/*pol* and three versions of *env.* Additionally, the MRKAd5 vaccine was administered as a homologous prime boost, while the DNA/rAd5 vaccine was administered in a heterologous prime boost regimen. We asked if the CD8 cross-reactivity induced by the two vaccines differed. In comparing responses between the two, we observed a trend towards a lower breadth of response in DNA/rAd5 recipients (**Fig. 6A**), which is consistent with published immunogenicity of the efficacy study [18]. We limited

our comparisons of these studies to *gag* and *pol*-specific responses as both vaccines encoded these genes. However, it is possible that the inclusion of three *env* genes in the DNA/rAd5 insert drew responses away from *gag* and *pol*. We found that DNA/rAd5 vaccine-elicited CD8 responses were higher in magnitude than those elicited by MRKAd5 and the cross-reactive responses in DNA/rAd5 also tended to have a higher magnitude than those in MRKAd5 (**Fig. 6B**). DNA/rAd5 recipients also clearly mounted more cross-reactive responses than MRKAd5 recipients (greater depth), with all of the examined DNA/rAd5 recipients responding to at least a third of the variant epitopes screened (**Fig. 6C**). These data suggest that the DNA/rAd5 heterologous prime boost vaccination more effectively induced cross-reactive CD8 T-cell responses.

Vaccine-generated CD8 T-cell cross-reactivity influences breakthrough viral sequences in recipients who became infected.

Because CD8 cross-reactivity did not appear to impact viral control in vaccine recipients who became infected, we next asked whether the cross-reactivity of the vaccine response influenced early viral evolution in these individuals. Prior publications have highlighted the distance between the vaccine immunogen and the breakthrough sequences in vaccine recipients who became infected as evidence of a vaccine "sieve effect" [4, 19-21]. We examined the breakthrough viral sequences of both MRKAd5 and DNA/rAd5 recipients for evidence of immune pressure exerted by vaccine-generated CD8 cross-reactivity. Our analysis focused only on those HLA-I-restricted epitopes that were relevant for each individual and that encoded HLA-I-associated polymorphisms [14]. This is the same strategy we used in our ELISpot experiments to map cross-reactivity, and we believe this analysis is more focused on responses actually targeted by vaccinees, whereas previous

sieving analyses examined all NetMHC-predicted epitopes. As illustrated previously in **Fig. 2**, two major factors impacted the immunogenicity of cross-reactive responses, the number of amino acid substitutions and their biochemical similarity to the vaccine matched epitope. We hypothesized that CD8 epitopes under postinfection selection pressure from vaccine-induced CD8 cross-reactivity would have more mutations and be less biochemically similar to the vaccine sequence.

In our analysis, we observed an increased number of substitutions in the Gag epitopes restricted by MRKAd5 recipients compared to their placebo counterparts (**Fig. 7A**). However, no differences were seen in biochemical similarity between breakthrough epitopes in MRKAd5 vaccine and placebo recipients (**Fig. 7B**). In the previous sieving analysis of MRKAd5 recipients, a greater distance between the breakthrough Gag sequences of vaccine recipients and the vaccine immunogen than those of placebo recipients was shown at the whole-gene level and at the predicted cytotoxic T lymphocyte (CTL) epitope level [4]. Overall, our finding of increased amino acid substitutions in Gag sequences of MRKAd5 vaccine recipients is consistent with the previous study of MRKAd5 breakthrough sequences [4].

In DNA/rAd5 recipients, Pol epitopes encoded by vaccinees' breakthrough sequences harbored more substitutions (**Fig. 7C**), and we also observed that Pol epitopes in DNA/rAd5 vaccine recipients were less biochemically similar to the vaccine than those in placebo recipients (**Fig. 7D**). This finding indicates that the breakthrough sequence-encoded epitopes in vaccinees would be less cross-recognized by vaccine-induced responses in those individuals. Additionally, the fact that we see this effect in DNA/rAd5 recipients for both the number of substitutions and the biochemical similarity is in line with

our finding that the DNA/rAd5 vaccine induced stronger CD8 cross-reactivity than the MRKAd5 vaccine (**Fig. 6**). Our analysis was limited to Gag and Pol as higher variability in Env sequences precludes accurate HLA-I-associated polymorphism predictions. Meanwhile, previous sieving analysis of DNA/rAd5 primarily focused on effects seen in Env breakthrough sequences but did identify a sieving site in Pol [19].

To then identify epitope-level responses that may be driving this effect in Gag for MRKAd5 and Pol for DNA/rAd5 sequences and to better compare these results with previous work, we iteratively reran our analysis and excluded one epitope at a time. Rolland et al identified Gag84 as the only significant amino acid site of sieving in MRKAd5 breakthrough sequences [4]. Our analysis included three epitopes that encode this site: A*01-SLYNTVATLY, A*11-ATLYCVHQK, and A*31-TLYCVHQK (Gag84 underlined). Although the *P* value remained significant for the MRKAd5 substitution analysis through each iteration when individual epitopes were excluded one by one, exclusion of all three Gag84-encoding epitopes resulted in a nonsignificant *P* value, indicating that Gag84 is the driving force in our analysis as well (data not shown). When we iteratively excluded epitopes from the Pol analysis of DNA/rAd5, we identified three DNA/rAd5-encoded epitopes, where exclusion of these epitopes resulted in a lack of statistical significance ($P > 0.05$) for both substitution and similarity scores: B*13:02-GQGQWTYQI, A*30- GQGQWTYQIY, B*18:01-NETPGIRYQY. At the single epitope-level, these sequences demonstrate the overall effect seen where vaccine recipient sequences encode a higher number of amino acid substitutions, greater biochemical dissimilarity, and more anchor mutations (**Table 2**). While Decamp et al [19] identified Pol 238 as a signature site, the three epitopes shown here do not overlap with this site,

indicating that our analysis has identified unique T-cell-mediated sieving in DNA/rAd5 recipients. Together, these data suggest that vaccine-induced CD8 cross-reactivity may have influenced the early viral evolution of transmitted HIV-1 strains in vaccine recipients.

Evidence of CD8 T-cell cross-reactivity impacting population-level HIV-1 sequence evolution.

Our analysis of breakthrough sequences in MRKAd5 and DNA/rAd5 vaccine recipients indicates that CD8 T-cell cross-reactivity significantly influences viral evolution. One way to identify CD8 T cell mediated pressure in natural HIV-1 infection is through HLA-I associated polymorphisms/adaptations, so we examined whether cross-reactive CD8 responses were able to equivalently recognize variants that encoded adaptations (adapted variants) versus those that did not (non-adapted variants). Individuals infected with viruses encoding a greater number of these adaptations, defined as HLA-I-associated polymorphisms, have previously been shown to have accelerated disease progression (higher viral loads and faster CD4 T-cell decline) [14]. If cross-reactive CD8 responses influenced HIV-1 viral evolution at the population level, we hypothesized that vaccine and TFV-induced CD8 T-cell responses would be less able to cross-recognize adapted variants, which encoded relevant HLA-I-associated polymorphisms, compared to nonadapted variants. While we did not detect a significant difference in magnitude between cross-reactive responses to nonadapted and adapted variants in vaccine recipients (**Fig. 8A**), we did find that TFV-directed responses in acute HIV-1 infection were less able to cross-recognize adapted variants (**Fig. 8B**). Additionally, the frequency of vaccine-induced responses cross-recognizing nonadapted variants was higher than that for adapted variants (**Fig. 8C**). Although the number of infected individuals was small (n = 6), the response

depth against nonadapted variants again appeared to be higher than it was to the adapted variants (**Fig. 8D**). These results suggest that CD8 cross-reactivity plays a role in shaping HIV-1 viral evolution and that HLA-I adaptations, to some extent, are a response to the pressure exerted by cross-reactive CD8s.

DISCUSSION

This study represents the first comprehensive assessment of HIV-1 vaccine-induced CD8 T-cell cross-reactivity. Here we show that two past Ad5-based HIV-1 vaccines elicited a broad range of cross-reactive responses, similar to what is seen in acute HIV-1 infection, but that CD8 cross-reactivity did not correlate with viral load in vaccine recipients who became infected. These variant-specific responses had a lower magnitude and were less antigen sensitive than vaccine-specific responses but exhibited similar polyfunctionality. CD8 responses were also less able to cross-recognize variants with an increasing number of amino acid substitutions, reduced biochemical similarity, and encoding anchor mutations, and these factors were used to assess the immune pressure exerted by vaccine-induced cross-reactivity on breakthrough sequences of trial participants. We found that epitopes encoded by HIV-1 breakthrough sequences of DNA/rAd5 vaccine recipients would probably be poorly cross-recognized by vaccine-induced CD8 T cells. We also observed that vaccine-induced responses were less able to cross-recognize variant epitopes containing HLA-I associated adaptations, supporting the idea that CD8 cross-reactivity is a driver of HIV-1 viral evolution.

Although sieve analyses have previously been conducted on both MRKAd5 and DNA/rAd5 study recipients [4, 19], our approach differed from those studies by focusing solely on relevant HLA-I restricted epitopes, identified based on HLA-I-associated

polymorphisms [14]. In the breakthrough sequences of participants in the DNA/rAd5 study, we saw an increased number of amino acid substitutions and decreased biochemical similarity in breakthrough Pol epitopes in individuals who received the vaccine. While the previous sieving analysis did identify a sieving effect in Pol [19], we identify specific CD8 T-cell epitopes unique from previously reported sites, and we propose CD8 cross-reactivity as the mechanism for T-cell driven-sieving in DNA/rAd5. These findings suggest that cross-recognition by vaccine-induced CD8 responses significantly impacted early viral evolution in vaccinees who became infected.

In contrast to our sieving analysis of MRKAd5 breakthrough sequences, the CD8 cross-reactivity sieving effect appeared stronger in the DNA/rAd5 recipients, where both the number of amino acid substitutions was higher and the biochemical dissimilarity was greater than placebo sequences. This is in line with the higher magnitude and greater frequency of CD8 cross-reactive responses elicited by the DNA/rAd5 vaccine compared to the MRKAd5 one (**Fig. 6**). In addition to the impact on breakthrough sequences, we observed decreased cross-recognition of variant epitopes containing HLA-I-associated adaptations, indicating that HIV-1 evolves away from cross-reactive CD8 T cell response during natural infection in a way that is detectable at the population level. We believe these findings demonstrate that cross-reactivity is an important component of an effective CD8 response.

Our data puts forward a few strategies to boost vaccine-induced CD8 cross-reactivity. The strong relationship observed between the magnitude of a vaccine-induced CD8 response and cross-reactivity of that response suggests that strategies to boost the magnitude of the CD8 T-cell response will coincidentally boost the ability of these

responses to cross-recognize variant epitopes. We should note that very high magnitude responses, some of which are presented in **Fig 1A**, still may not be able to cross-recognize certain variant epitopes, indicating that there may be elements of cross-recognition that are not solely linked to response magnitude. The heterologous prime-boost regimen of DNA/rAd5 also elicited both higher magnitude and more frequent CD8 cross-reactivity. Unfortunately, we were not able to assess how the presence of multiple variants of a given epitope within an insert, such as in the case of mosaic vaccines, would influence the cross-reactivity of the CD8 T-cell response. Preliminary studies in nonhuman primates suggest that mosaic vaccines increase the depth of the CD8 response, and we would hypothesize that the inclusion of variant epitopes would induce a more polyclonal CD8 T-cell response, thus broadening the cross-recognition of variant epitopes. However, mosaic vaccines also encode a greater proportion of adapted epitopes, which we have previously shown are poorly immunogenic [5] and which we show here are poorly cross-recognized.

Overall, we observed remarkable consistency between the CD8 cross-reactivity induced by these vaccines and the CD8 cross-reactivity elicited in early HIV-1 infection. Magnitude, depth, and factors impacting variant recognition were all similar between vaccination and infection. The HIV-infected individuals studied here do not naturally control virus, so it is unreasonable to expect that similar levels of cross-reactivity induced by a vaccine could impact viral control in recipients who became infected. We show using several approaches that CD8 cross-reactive responses influence HIV evolution suggesting that if cross-reactivity were boosted, these responses could go from influencing sequence evolution to controlling viral load.

METHODS AND MATERIALS

Vaccine samples. HVTN 502 participants (MRKAd5, ClinicalTrials.gov identifier: NCT00095576) were randomized to receive the Ad5 vaccine with HIV-1 gene inserts (*gag*, *pol*, *nef*) or placebo [22]. HVTN 505 participants (DNA/rAd5, ClinicalTrials.gov identifier: NCT00865566) were randomized to receive the DNA/rAd5 vaccine with HIV-1 gene inserts (*gag, pol, env a/b/c, nef* [DNA only]) or placebo [18]. Informed consent was obtained from all participants and all relevant guidelines of the authors' institutions were followed. A total of 46 vaccine recipients (37 from MRKAd5 and 9 from DNA/Ad5) who were previously found to have positive responses to vaccine-encoded epitopes [5] were screened for cross-reactive CD8 T-cell responses for this study. Of the MRKAd5 recipients, 13 were individuals who eventually became HIV-1 infected. Five placebo recipients from MRKAd5 and two from DNA/Ad5 were tested for vaccine responses and no positive responses were detected in these recipients.

Infection samples. Six HIV-1-infected individuals were tested for cross-reactive CD8 responses in acute HIV-1 infection. The median days postinfection (DPI) was 43 days for acute infection samples; **Table 1** provides the full clinical details of these individuals. TFV sequences were inferred from the plasma of these patients at Fiebig stage III or earlier using a single-genome amplification method, as described previously [23].

HLA typing. HLA-I alleles for MRKAd5 vaccine recipients were provided by the HVTN. HLA typing was performed on DNA/rAd5 samples as previously described [24]. Briefly, four-digit genotyping was generated by sequencing-based typing (Abbott Molecular, Inc., Des Plaines, IL) and automated DNA hybridization with oligonucleotide probes (Innogenetics, Inc., Alpharetta, GA).

IFN-γ ELISpot. ELISpot assays were performed as previously described [25]. In brief, peripheral blood mononuclear cells (PBMCs) were thawed and rested overnight in RPMI medium supplemented with 10% human AB serum (R10 Abs) at 37°C and 5% CO_2. Plates were coated with anti-IFN-γ antibody at 4°C overnight and then blocked with R10 Abs for 2 hours at 37°C and 5% CO_2. PBMCs were plated at 100,000 cells/well with the peptide of interest at a 10-μM final concentration in duplicate and incubated together at 37°C and 5% CO_2 for 22 hours. A negative control of medium only and a positive control of phytohemagglutinin (PHA) were included on each plate for each sample. Plates were then washed and developed with biotinylated anti-IFN-γ antibody (2 h), streptavidin (45 min), and nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) substrate solution (10 min) sequentially. Once dry, plates were scanned and counted using the ImmunoSpot analyzer and software. Results were normalized and reported as the average spot-forming units per 1 million cells (SFU/10^6). The positive threshold for a response was at least 55 SFU/10^6 and at least 3 times the medium-only wells. Antigen sensitivity was assessed using IFN-γ ELISpot by performing logfold serial dilution of peptide from 10 μM to 10^{-2} μM. A dose response curve was fit for each epitope response and used to calculate the EC_{50} value, or the amount of peptide required to elicit 50% of a maximal response.

CD8 T-cell response mapping strategy. Individuals were first screened for CD8 T-cell responses to TFV- or vaccine-encoded epitopes. Only those epitopes predicted to be restricted by each individual's HLA-I alleles were screened. A range of 4 to 17 epitopes were tested per vaccine recipient (median = 10), and 1 to 4 epitope-specific responses were detected in each individual (median = 1). Then, we tested positive responses for cross-reactivity against variant epitopes. The three to seven most common variant epitopes were

selected based on population frequencies found in the Los Alamos HIV-1 Sequence Database [26]. Variant epitopes were designed to represent at least 90% of circulating sequences at that epitope site, with a maximum of seven variants designed for each vaccine epitope. Cross-reactive responses were summarized as the depth of the response or frequency of responses to variant epitopes. All peptides (8-to 11-mer) were previously predicted using Microsoft Research's Epipred software and were synthesized by New England Peptide in a 96-well array format.

Flow cytometry. Cytokine and effector molecule production was measured by flow cytometry as previously described [27]. Briefly, PBMCs were thawed and stimulated with the relevant peptide or peptide pool in the presence of anti-CD28, anti-CD49d, and anti-CD107a-FITC (BD Biosciences) for 1 hour at 37°C and 5% CO_2. After the addition of monensin and brefeldin-A (BD Biosciences), cells were incubated for an additional 11 hours. Cells were then surface stained with the following antibodies for 30 min at 4°C: dead cell dye (Invitrogen), anti-CD3-Alexa780 (eBioscience), anti-CD4-Qdot655 (Invitrogen), anti-CD8-V500 (BD Pharmingen), anti-CD14-Percp/CY5.5 (BD Pharmingen), and anti-CD19-Percp/CY5.5 (BD Pharmingen). Cells were permeabilized with Cytoperm/Cytofix (BD Biosciences) and then intracellularly stained with the following for 30 min at 4°C: anti-IFN-γ-Alexa700 (BD Biosciences), anti-IL-2-APC (BD Biosciences), anti-TNF-α-PECy7 (BD Biosciences), anti-perforin-PE (eBioscience), anti-granzymeA-PacBlue, and anti-granzymeB-V450 (BD Biosciences). Cells were fixed in 5% formalin. Events were acquired on an LSRII flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo version 10 (TreeStar, Inc).

*Breakthrough sequence analysis***.** Sequences and treatment arm assignments were downloaded from the publicly available SieveSifter tool [20]. MRKAd5 sequences were previously submitted to GenBank with the accession numbers JF320002 to JF320643 [4]. DNA/rAd5 sequences were previously submitted to GenBank with the accession numbers MG196642 to MG197219 [19]. HLA-I alleles for these individuals were obtained from the HVTN. The sequences of epitopes restricted by the HLA-I alleles of vaccine and placebo recipients were compared to the vaccine-encoded sequence. Only epitopes encoding an HLA-I-associated polymorphism were included, as these were the epitopes we tested for cross-reactivity in immune assays, and as we believe these epitopes elicit the CD8 T-cell responses that exert significant immune pressure. Epitopes were extracted and analyzed from 40 MRKAd5 vaccine recipients, 26 MRKAd5 placebo recipients, 24 DNA/rAd5 vaccine recipients, and 17 DNA/rAd5 placebo recipients. Across the two vaccine studies, 33 Gag, 35 Pol, and 18 Nef epitopes were analyzed. Although Env was included in the DNA/rAd5 vaccine, it was not included in our analysis since there were three Env sequences included in the insert, making cross-reactivity more difficult to accurately assess. The number of amino acid substitutions compared to the vaccine insert sequence was normalized to the length of the epitope. The biochemical similarity of the breakthrough epitope to the vaccine insert sequence was quantified by a sum of the BLOSUM-62 (blocking substitution matrix) [28] scores for each residue of the aligned breakthrough and vaccine epitopes, again normalized to the length of the epitope.

Statistical analysis. Graphs were created in Prism version 8, and all mixed-effect models were built in R [29]. In analyzing ELISpot responses, net magnitudes (SFU/10^6 minus background) were log(x + 1) transformed. Both individual and epitope groupings were

added as random effects to account for the multiple epitopes that were tested for each individual and multiple variants that were tested for each responsive epitope. In the analysis of breakthrough sequences, vaccine versus placebo comparisons were done using a mixed effect model accounting for intraindividual correlation among epitopes. Polyfunctionality was summarized as a polyfunctionality score (PFS) using the R package COMPASS [16].

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TABLE 1 *Clinical data of HIV-1-infected individuals included in the study*

PTID	DPI	CD4 count	Viral load	ART status
PTID1	47		1.0×10^5	No
PTID2	89	466	9.2×10^1	Yes (21 days)
PTID3	34	862	4.0×10^5	No
PTID4	92	1,043	5.3×10^5	No
PTID5	23	479	3.0×10^6	No
PTID6	38	242	6.7×10^5	No

FIGURE 1 *Cross-reactive CD8 responses elicited by vaccination are similar in magnitude and depth to those induced in acute HIV-1 infection.*

(**A**) Representative example of an epitope (B*44 restricted) tested in 2 infected individuals (PTID) and 4 MRKAd5 recipients (MRK). The top epitope row is the TFV and vaccine-encoded sequence (AEQASQEVKNW), with variant epitopes listed below. Net ELISpot magnitudes are shown in each cell, and positive responses are shaded in yellow. (**B**) Net ELISpot magnitudes of positive responses against TFV/vaccine-encoded epitopes and their variant epitope counterparts. (Dotted line indicates the positive threshold for ELISpot responses [55 SFU/10^6]; a few values fall below this line, as net values are shown. A mixed-effect model was used to account for intraindividual correlation to multiple epitopes.) (**C**) The depth, or the percentage of variants that are recognized, per individual. For B and C, $N_{infection} = 6$ and $N_{vaccine} = 46$.

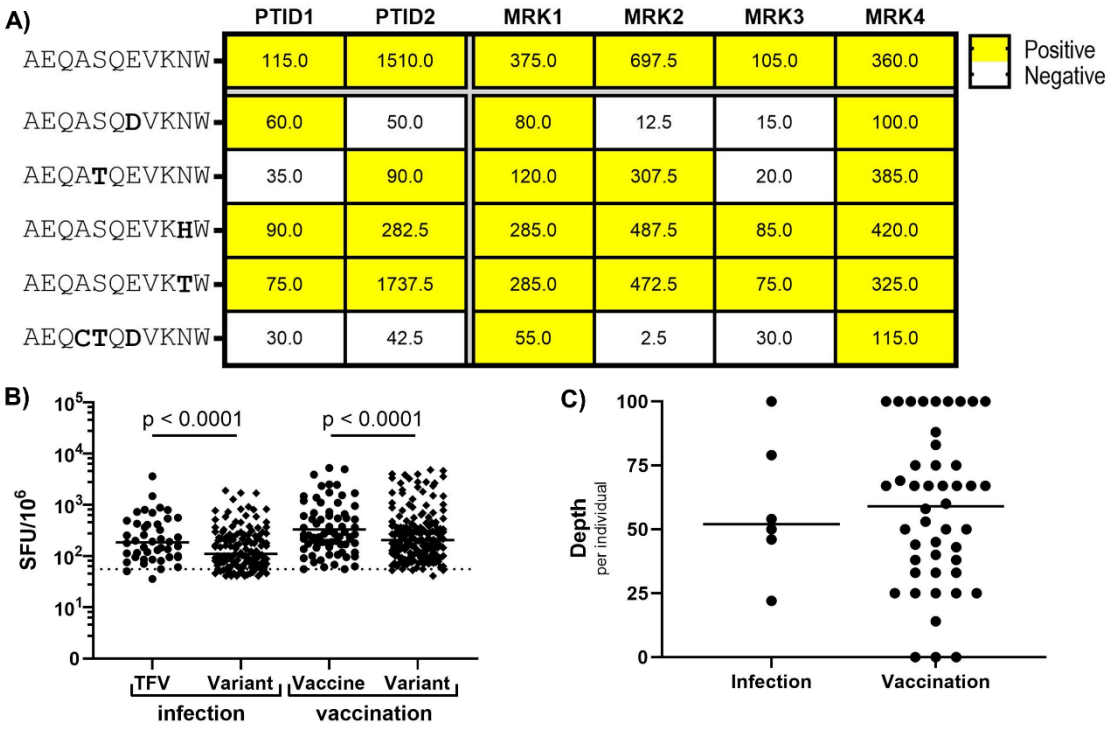


FIGURE 2 *CD8 cross-reactivity is dampened by increased amino acid substitutions, decreased biochemical similarity, and HLA anchor site mutations.*

(**A** and **B**) Net magnitude of responses to variant epitopes with one to four amino acid substitutions compared to the TFV-encoded or vaccine-encoded epitope (substitutions = 0) in acute infection and vaccination, respectively. (**C** and **D**) Net magnitude of responses to variant epitope with various levels of biochemical similarity to the TFV-encoded or vaccine-encoded epitopes in acute infection and vaccination, respectively. (**E** and **F**) Net magnitude of responses of anchor of nonanchor mutated variant 9-mers in acute infection and vaccination, respectively. In vaccine plots, closed symbols represent MRKAd5 recipient responses; open symbols represent DNA/rAd5 recipient responses. For all statistical comparisons, substitutions, similarity score, and variant type were modeled as predictors of magnitude in a mixed-effect model that accounted for intraindividual correlation. Data from 6 infected individuals and 46 vaccinees are shown here.

(**FIGURE 2**)

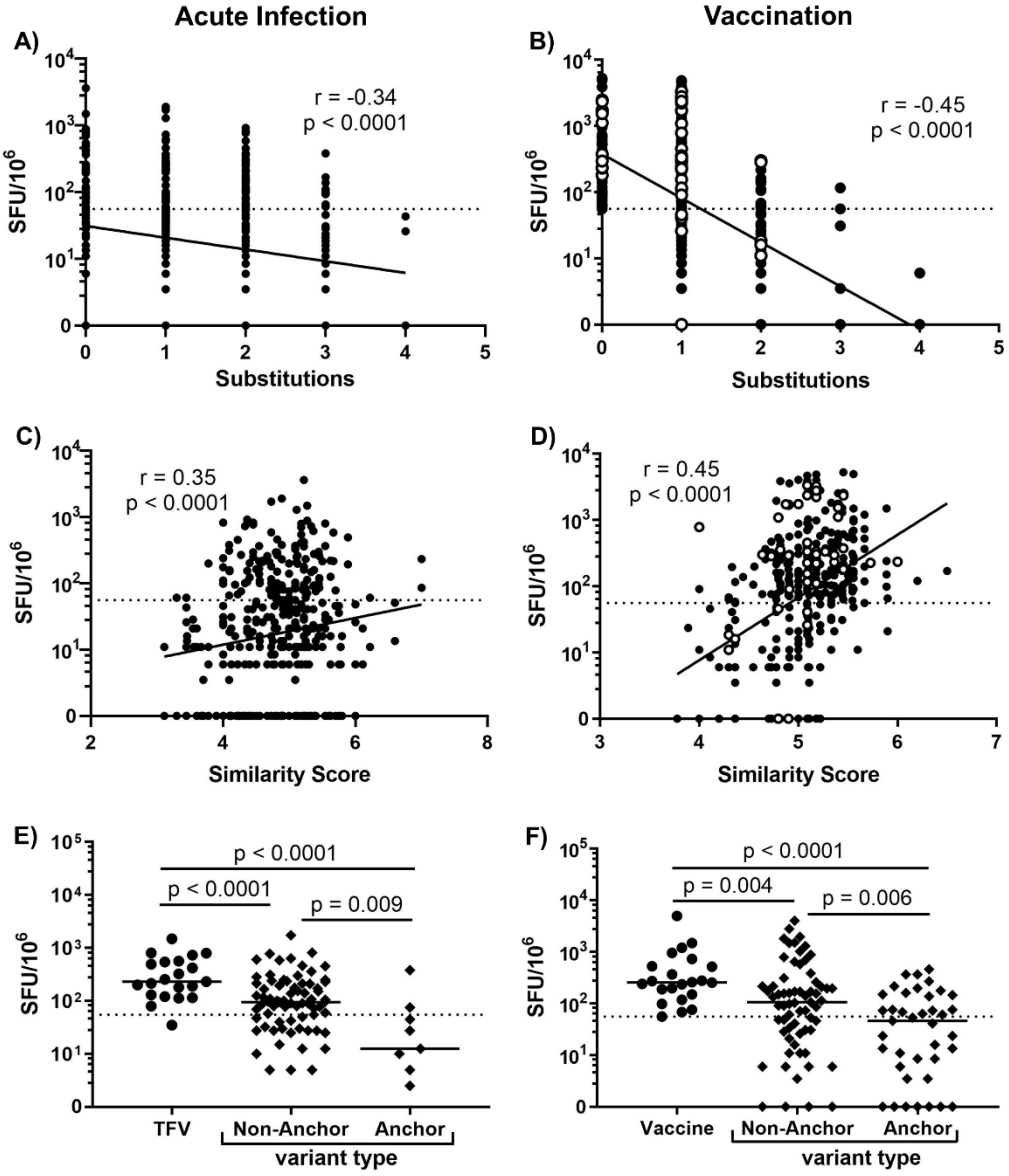

Acute Infection
Vaccination
A)
B)
C)
D)
E)
F)
SFU/10^6
r = -0.34
p < 0.0001
r = -0.45
p < 0.0001
r = 0.35
p < 0.0001
r = 0.45
p < 0.0001
Substitutions
Similarity Score
p < 0.0001
p < 0.0001
p = 0.009
p = 0.004
p = 0.006
TFV
Vaccine
Non-Anchor
Anchor
variant type

FIGURE 3 *MRKAd5-induced CD8 cross-reactivity did not impact viral control in recipients who became infected.*

(**A**) Depth per vaccinee, or frequency of cross-reactive responses, versus viral load following infection (Spearman correlation, $n = 13$). (**B**) Depth versus breadth of the vaccine-induced response per vaccinee (Spearman correlation, $n = 46$). (**C**) Depth of positive vaccine responses versus median IFN-γ ELISpot magnitude of that response (Spearman correlation, $n = 46$).

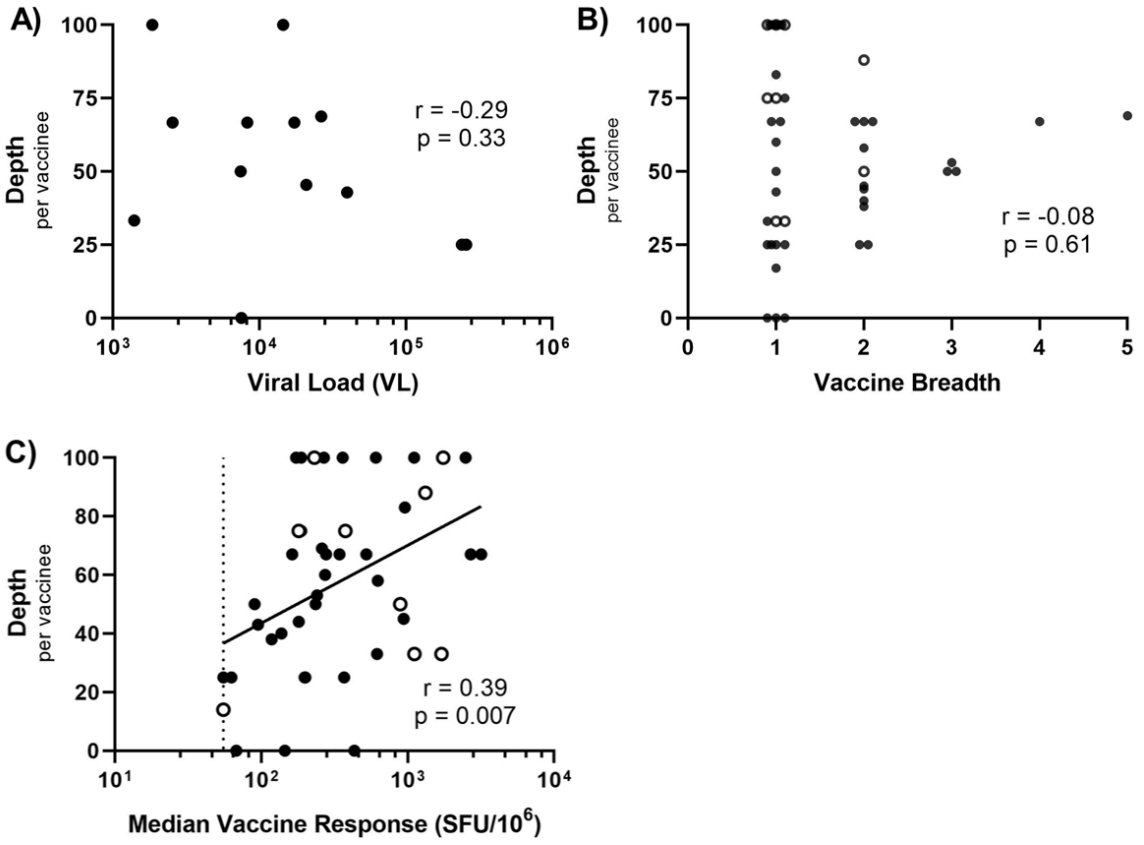


FIGURE 4 *Cross-reactive CD8 T-cell responses are functionally similar to responses against vaccine-encoded epitopes.*

(**A**) Antigen sensitivity of vaccine versus variant responses, quantified as an EC_{50} value (a mixed-effect model was used to account for intraindividual correlation; data from 37 vaccine recipients). (**B**) Polyfunctionality, quantified as a polyfunctionality score (PFS), for vaccine versus variant responses. (**C**) Heatmap of posterior probabilities of each combination of effector molecules. Each row represents a different epitope-specific response. Right-hand boxes indicate responses to variant epitopes (blue) or vaccine-matched epitopes (gray). The combination of effector molecules (IL-2, TNF-α, perforin, granzyme α and β, and IFN-γ) produced by each subset is indicated below the heatmap. The number of effector molecules is indicated by dark green (*n* = 4), light green (*n* = 3), dark blue (*n* = 2), and light blue (*n* = 1). (Panels B and C include data from 8 vaccine recipients: 17 responses to variant epitope and 10 responses to vaccine-matched epitopes).

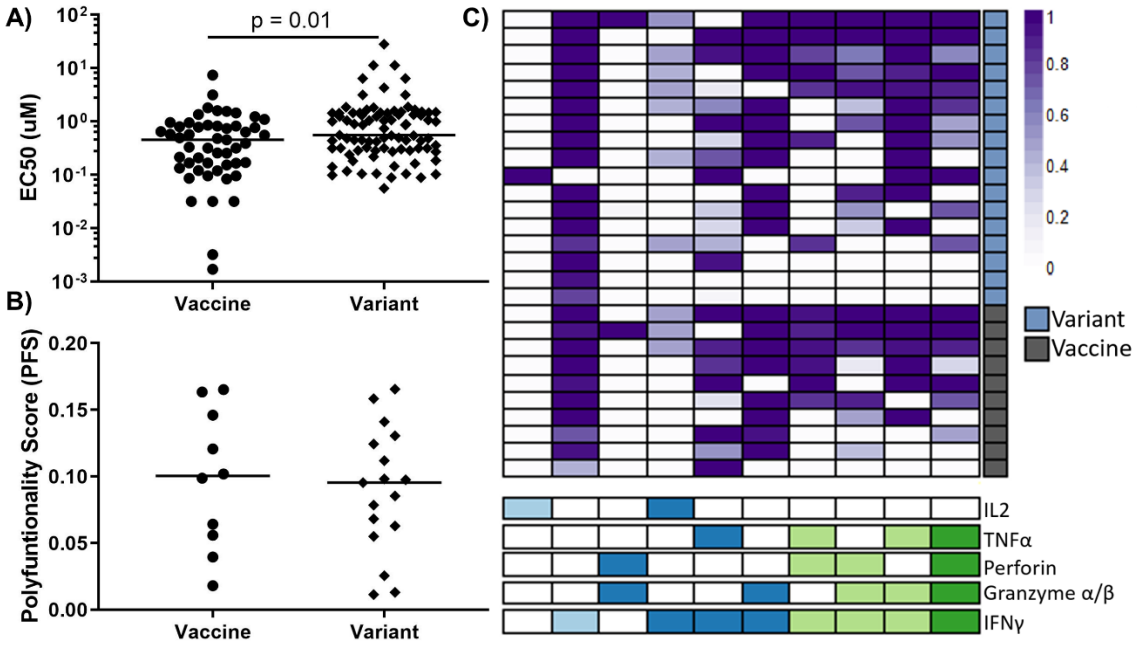


FIGURE 5 *Frequency of cross-reactive responses increased over homologous sequential MRKAd5 vaccination (*n *= 13).*
(**A**) Net response magnitudes following the 2nd and 3rd vaccinations to vaccine or variant epitopes (mixed-effect model). (**B**) Response depth per vaccinee to variant epitopes at these time points (Wilcoxon signed-rank test).

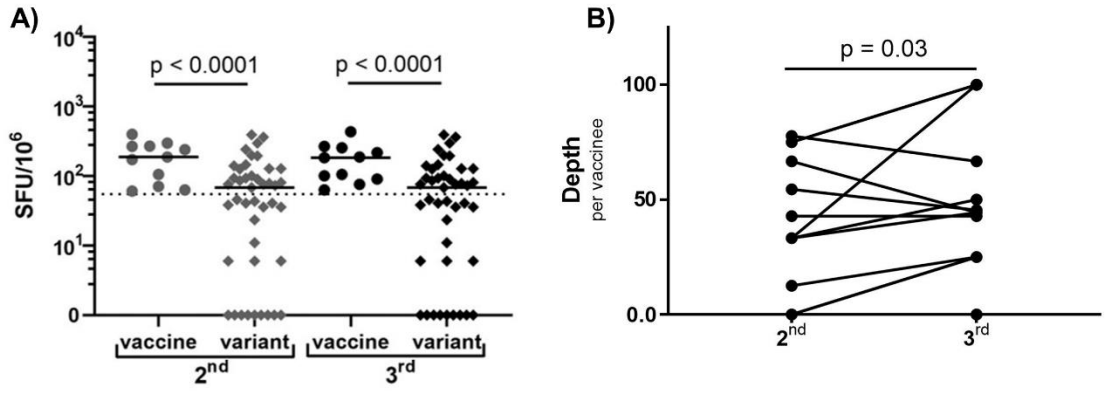


FIGURE 6 *DNA/rAd5 vaccine elicited CD8 responses with greater depth than the MRKAd5 vaccine.*

(**A**) Vaccine breadth per MRKAd5 or DNA/rAd5 recipient (Mann-Whitney U test). (**B**) Magnitude of vaccine and variant responses in each study (mixed-effect model). (**C**) Depth, or frequency of cross-reactive responses, per vaccine recipient (Mann-Whitney U test). $N_{MRKAd5} = 37$, $N_{DNA/rAd5} = 9$.

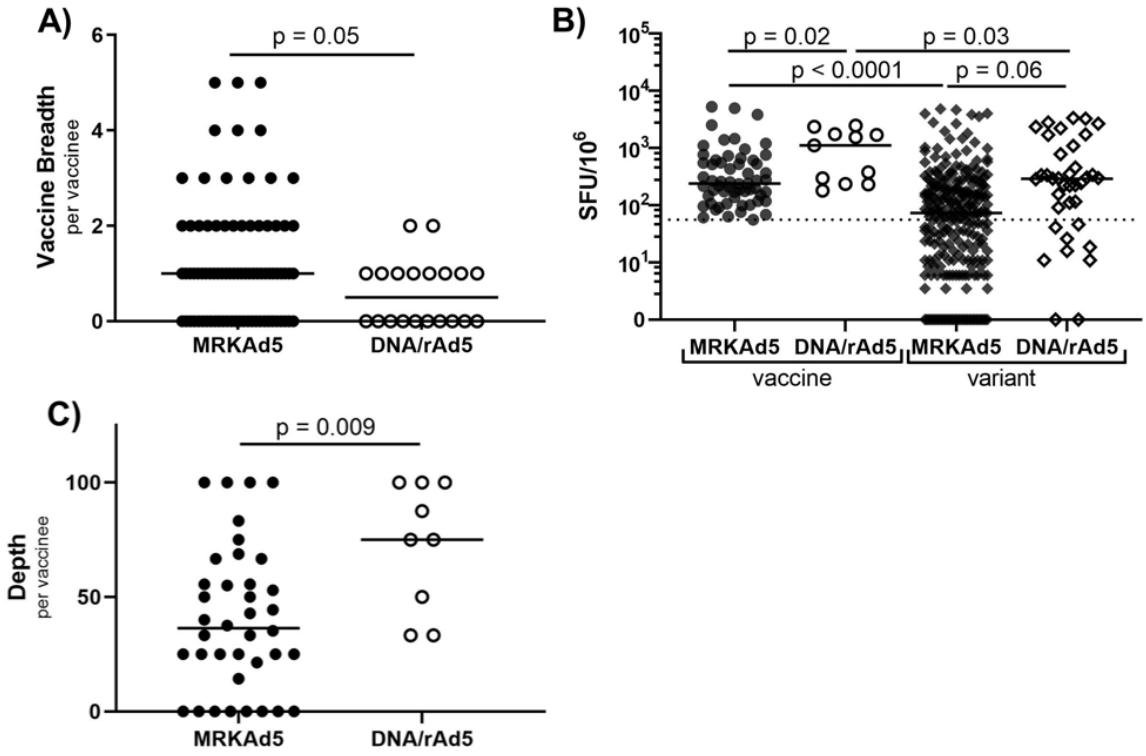


FIGURE 7 *Breakthrough viral sequences from vaccine recipients who became infected encode CD8 T-cell epitopes with more substitutions than and less biochemically similar to the vaccine immunogen.*

(**A**) Substitution score, or number of substitutions divided by the length of the epitope, of HLA-I restricted epitopes encoded by the breakthrough sequences in MRKAd5 recipients relative to the vaccine-encoded sequence. (**B**) Similarity scores, or cumulative BLOSUM score divided by length of the epitope, of breakthrough sequence epitopes in MRKAd5 recipients compared to the vaccine-encoded sequence. Substitution scores (**C**) and similarity scores (**D**) for DNA/rAd5 vaccine versus placebo recipients. Open symbols indicate placebo recipients, and closed symbols indicate vaccine recipients. All significant was assessed by a mixed-effect model accounting for repeated measures for each vaccine recipient. $N_{MRKAd5} = 65$, $N_{DNA/rAd5} = 41$.

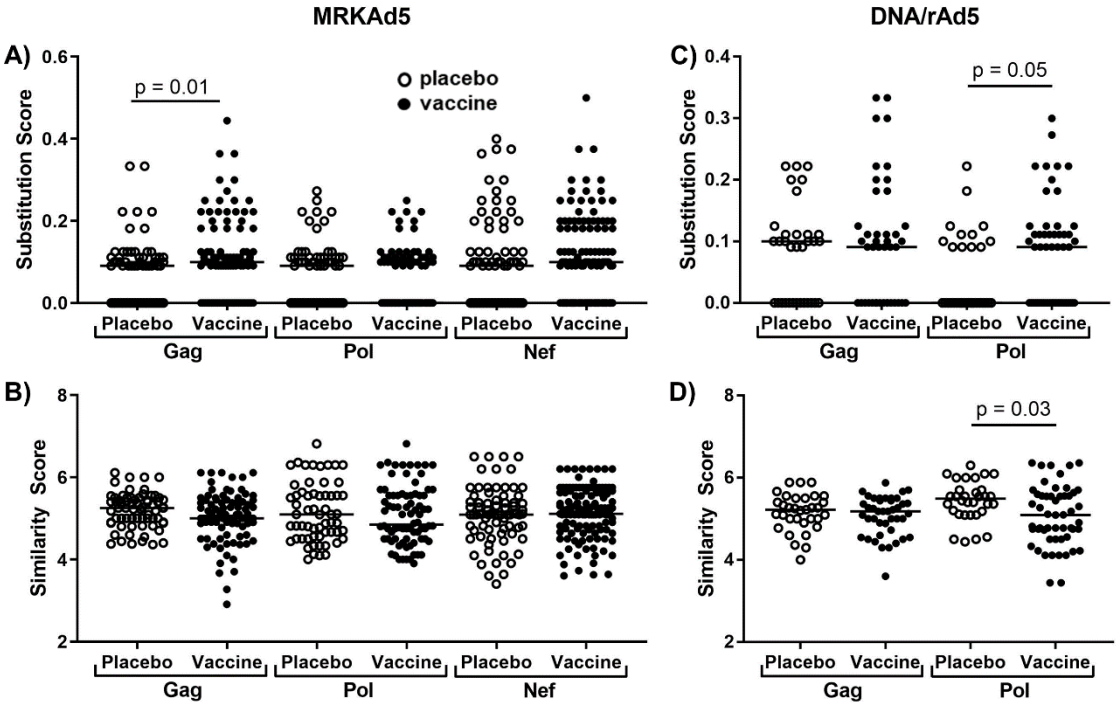


TABLE 2 *Placebo versus vaccine sequences of epitopes driving DNA/rAd5 Pol effect* [a]

PubID[b]	RX[c]	HLA	Sequence	Similarity score	No. of substitutions	Anchor mutation
5050122	Placebo	B*13:02	GQGQWTYQI	6.00	0	
5051958	Placebo	B*13:02	GQGQWTYQI	6.00	0	
5051993	Vaccine	B*13:02	G**L**GQW**S**YQI	4.78	2	Yes
5050122	Placebo	A*30	GQGQWTYQIY	6.10	0	
5050695	Placebo	A*30	GQGQWTYQIY	6.10	0	
5051958	Placebo	A*30	GQGQWTYQIY	6.10	0	
5051144	Vaccine	A*30	GQGQWTYQIY	6.10	0	
5051226	Vaccine	A*30	**EL**GQWTYQ**V**Y	4.50	3	Yes
5052474	Vaccine	A*30	**E**QGQWTYQIY	5.30	1	No
5050102	Placebo	B*18:01	NE**A**PGIRYQY	5.20	1	No
5050396	Placebo	B*18:01	NETPGIRYQY	5.70	0	
5050821	Placebo	B*18:01	NETPGIRYQY	5.70	0	
5051012	Vaccine	B*18:01	NETPGIRYQY	5.70	0	
5051144	Vaccine	B*18:01	N**A**TPGIRYQY	5.10	1	Yes
5051763	Vaccine	B*18:01	NETPGIRYQY	5.70	0	

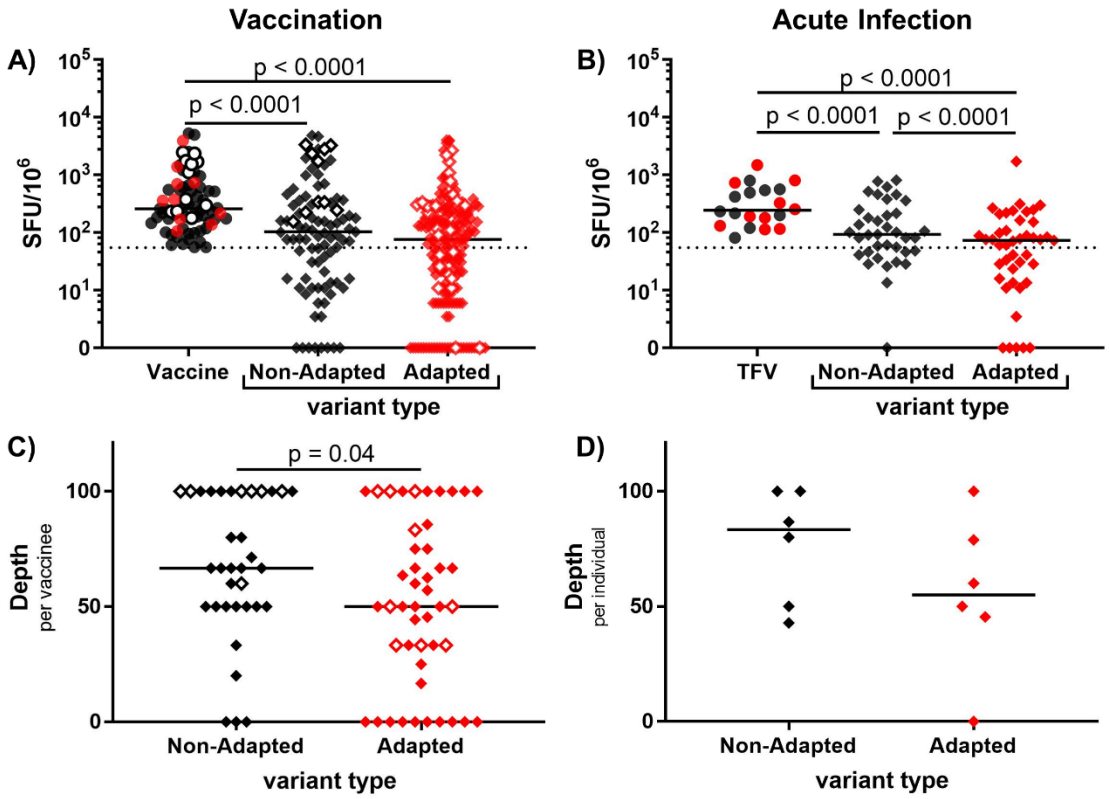
[a]Vaccine recipients are highlighted in gray. Amino acid substitutions compared to vaccine sequence are shown in bold.

[b]PubID, publication identifier of vaccine recipients.

[c]RX, treatment arm (vaccine or placebo).

FIGURE 8 *CD8 T cells poorly cross-recognize variant epitopes encoding adapted polymorphisms.*

(**A**) Net magnitude of vaccine and cross-reactive responses against nonadapted or adapted variant epitopes (mixed-effect model). (**B**) Net magnitude of TFV and cross-reactive responses against nonadapted and adapted variants. (**C**) Depth, or proportion of variant epitopes cross-recognized, per individuals of nonadapted and adapted variants in vaccination (Mann-Whitney U test). (**D**) Depth of acute infection-induced responses (Mann-Whitney U test). Closed symbols represent MRKAd5 recipients, and open symbols represent DNA/rAd5 recipients. All adapted epitope responses are denoted by red. Data from 46 vaccinees and 6 infected individuals are shown here.



DISCUSSION

Summary of research findings

CD8 T-cell responses are known to play an important role in controlling HIV during natural infection. We believe CD8 T cells could play a major role in vaccine efficacy, primarily by alleviated disease course in recipients who become infected despite vaccination. However, in spite of significant progress in understanding CD8 T cells and their role in HIV viral control, which specific characteristics make a good CD8 T-cell response and how to preferentially induce an effective CD8 T-cell response remains unclear. In our studies, we explored how vaccine-induced CD8 T-cell responses in past efficacy trials countered HIV diversity.

HLA-I-associated adaptation to the vaccine insert

We found that HLA-I-associated adaptation negatively impacts vaccine-generated CD8 T-cell responses, similar to what has been reported in acute HIV infection [31]. In mapping epitope-level responses, adapted epitopes were far less immunogenic than nonadapted epitopes. This was seen in both the frequency and magnitude of IFN-γ responses. And, the more adapted an individual was to the vaccine insert (having a higher adaptation score), the lower the breadth of their vaccine-directed response. These findings suggest that the inclusion of adapted epitopes in HIV vaccines is unlikely to increase the breadth of the CD8 response, which is significant as both we and others [96] have shown that the breadth of the CD8 T-cell response is one of the few characteristics that has been associated with improved viral control in vaccine recipients who became infected. We also

observed that adapted epitope-specific responses had lower polyfunctionality compared to nonadapted epitope-specific ones and that the level of adaptation to the Gag protein insert negatively correlated with Gag peptide pool-induced polyfunctionality. A recent study has identified a decreased risk of infection in DNA/rAd5 recipients who mounted highly polyfunctional Env-specific CD8 T-cell responses [97]. Although we were ill-equipped to study Env-specific responses in the context of adaptation, this negative relationship between adaptation and CD8 T-cell polyfunctionality to Gag is alarming and illustrates the need to extend our understanding of the impact of HLA-I-associated adaptation. We believe these data make a strong argument for the exclusion of HLA-I-associated adaptation in HIV vaccine inserts, since they appear to be taking up key immunological space without eliciting useful CD8 T-cell responses.

Vaccine-induced CD8 T-cell cross-reactivity

While HLA-I-associated adaptation represents one level of HIV diversity, driven by CD8 T-cell mediated pressure in HIV infection, we also wanted to assess vaccine-induced CD8 T cells' cross-reactivity to variant epitopes present at significant levels in circulating strains. Cross-reactivity may be an important metric of the vaccine-elicited CD8 T-cell response since vaccine recipients are unlikely to be exposed to HIV strains identical to the vaccine sequence. We examined how well vaccine-induced CD8 T cells could recognize epitope variants that the immune response had never seen, meaning variant epitopes not encoded by the vaccine. To our knowledge, this was the first in-depth characterization of vaccine-induced CD8 T-cell cross-reactivity.

In assessing responses of both MRKAd5 and DNA/rAd5 vaccine recipients, we found that these vaccines induced a similar level of CD8 T-cell cross-reactivity compared

to acutely HIV infected individuals, both in terms of IFN-γ magnitude and frequency. In acute HIV infection, cross-reactivity was defined as responses to variant epitopes not encoded by a given individual's transmitted founder virus, or TFV. Across all individuals, there was substantial range in terms of how many variant epitopes were recognized: there were vaccine recipients whose CD8 T cells did not cross-recognize any tested variants, and there were others whose CD8 T cells cross-recognized every variant tested.

In assessing the factors that influenced CD8 T-cell cross-recognition of variant epitopes, we found two primary drivers: (1) the number of amino acid substitutions between the vaccine or TFV-encoded epitope and the variant epitope as well as (2) the biochemical dissimilarity of a variant epitope compared to the vaccine or TFV-encoded epitope. Using these two factors, we put a new spin on the classical "sieving analysis" to examine whether vaccine-induced CD8 T-cell cross-reactivity exerted immune pressure on early viral evolution in vaccine recipients who became infected, focusing our analysis on HLA-I-restricted epitopes. We found that Pol-specific CD8 T-cell cross-reactivity appeared to impact the breakthrough Pol sequences in DNA/rAd5 participants. This was the first report of CD8 T-cell mediated pressure by the DNA/rAd5 vaccine and is in line with our observations that this vaccine induced greater CD8 T-cell cross-reactivity than the MRKAd5 vaccine did. This cross-reactivity mediated effect suggests that further boosting vaccine-induced CD8 T-cell cross-reactivity could achieve a level at which it not only influences viral evolution but also exerts viral control in vaccine recipients who become infected.

In addition to the heterologous prime-boost regimen of DNA/rAd5 inducing greater CD8 T-cell cross-reactivity compared to the homologous sequential vaccinations of MRKAd5, we also identified other ways in which CD8 T-cell cross-reactivity could potentially be boosted by future vaccine strategies. For one, we observed a clear positive correlation between the IFN-γ magnitude of the vaccine-directed CD8 T-cell response and the number of variant epitopes recognized. This indicates that boosting CD8 T-cell response magnitudes alone would be sufficient to broaden CD8 T-cell cross-reactivity to some extent. We also see that the frequency of CD8 T-cell cross-recognition of variant epitopes increases over homologous vaccination timepoints in MRKAd5 recipients, indicating that sequential vaccination broadens CD8 T-cell cross-reactivity, even when it does not broaden vaccine-directed CD8 T-cell responses.

One of the prominent vaccine strategies being tested currently is that of mosaic vaccines, which encode multiple versions of a given HIV protein. A major argument in favor of this vaccine design strategy is that CD8 T cells induced by these mosaic vaccines would have an improved ability to cross-recognize a greater number of variant epitopes. Although NHP studies have demonstrated mosaic vaccination induces broader cross-reactivity [72], vaccine-induced CD8 T cells may not respond in the same way in humans. For example, in assessing HLA-I-associated adaptation and CD8 T-cell cross-reactivity, we found that vaccine-induced CD8 T cells were unable to cross-recognize adapted variant epitopes to the same degree as nonadapted variant epitopes. Because HLA-I adaptation is not a consideration in the CD8 T-cell responses mounted by rhesus macaques, our results indicate the importance of testing CD8 T-cell cross-reactivity in humans within the context of newer vaccine strategies.

Future directions

Pre-adaptation to HIV vaccine inserts

Recent data from our group, studying CD8 T-cell responses in chronic HIV infection, has found that adapted epitopes are actually fairly immunogenic and that these adapted epitope-specific CD8 T-cell responses resulted in a greater level of dendritic cell maturation and viral *trans*-infection of CD4 T cells [51]. Although we demonstrate here that vaccine-encoded adapted epitopes are poorly immunogenic and adapted epitope-specific CD8 T cells are poorly polyfunctional, we did not evaluate these responses through the lens of a nonclassical "helper-like" CD8 T-cell function. Future studies, in addition to extending this work to other more recently tested HIV vaccines, should look to characterize the function of vaccine-induced adapted epitope-specific CD8 T cells with regard to their potential role in increasing inflammation and infection risk.

TCR-level cross-recognition of variants

Another key area in which to extend this work is the examination of CD8 T-cell cross-reactivity at the T cell receptor, or TCR, level. Several previous publications have indicated that cross-reactivity is a function of the TCR specificity [53, 54]; however, many of these did not link observed *ex vivo* cross-reactive CD8 T-cell responses with the TCR-level cross-reactivity. Additionally, these past publications assessed cross-reactivity of CD8 T-cell clones, which does not completely isolate the cross-reactivity of the TCR from other aspects influencing the CD8 T-cell response, like expression of co-stimulatory markers. Future studies can isolate the contribution of cross-reactive TCRs to the overall CD8 T-cell response cross-reactivity using modern experimental approaches, such as transfected Jurkat assays with reporter genes [100]. Future studies conducting single cell

sequencing of TCRs combined with structural modelling of the TCR-peptide-MHC complex in cross-reactive repertoires may also shed light on specific TCR characteristics that are critical for the ability to broadly cross-recognize variant epitopes.

Cross-reactivity in HIV infection

Although we show evidence here that vaccine-induced CD8 T-cell cross-reactivity influences early viral evolution in DNA/rAd5 recipients who became infected, the importance of CD8 T-cell cross-reactivity in HIV infection remains uncertain. A few studies have highlighted HIV controllers as having broader CD8 T-cell cross-reactivity compared to progressors [53, 54]; however, the causal relationship here has yet to be established. Are the increased levels of cross-reactivity in HIV controllers what confer, even partially, their ability to naturally suppress virus? Additionally, these studies do not address how important CD8 T-cell cross-reactivity may be outside the context of protective HLA-I alleles.

Future studies should look to link CD8 T-cell response cross-reactivity in HIV-infected individuals with viral evolution over time. Following these responses longitudinally would solidly establish the role that CD8 T-cell cross-reactivity plays in driving viral evolution. Future work should also focus on screening for CD8 T-cell cross-reactivity in acute infection and linking this cross-reactivity with clinical outcomes. We would hypothesize that broad *ex vivo* CD8 T-cell cross-reactivity in acute HIV infection would be shown to predict lower viral loads in chronic infection and/or slower CD4 T-cell decline. Demonstrating that CD8 T-cell cross-reactivity is a key feature of CD8-mediated viral control would pave the way for future strategies to prioritize inducing broadly cross-reactive CD8 T-cells.

Concluding remarks

Although significant progress has been made in understanding CD8 T-cell responses in the context of HIV infection and vaccination, many questions regarding what makes an effective CD8 T-cell response remain. Here we advance that knowledge by fully characterizing the impact of HLA-I-associated adaptation on vaccine-induced CD8 T-cell responses in participants of two previous HIV vaccine efficacy trials. These results show that vaccine-encoded adapted epitopes are poorly immunogenic and that the level of adaptation to the vaccine insert negatively impacts CD8 T-cell polyfunctionality. We also illustrate the extent to which these vaccines induced cross-reactive CD8 T cells and the effect this cross-reactivity had on viral evolution in vaccine recipients who became infected. We hope these results will help inform future studies of HIV vaccines currently being tested in efficacy trials and help shape future vaccine design to optimize the CD8 T-cell response to the point at which it can alleviate disease course in individuals who become infected despite vaccination.

Although we primarily focus on how our results can aid future preventative vaccine design, a better understanding of how CD8 T cells counter HIV sequence diversity is also relevant to strategies aimed at achieving sustained viral remission and cure. For instance, "shock and kill" cure strategies focus on reversing latency in infected individuals and stimulating CD8 T cells to clear all virally infected cells [101]. In order for the "kill" arm of this strategy to be effective, stimulated CD8 T cells have to be able to recognize any virus within the latent reservoir, which can be composed of CD8 T-cell escape variants [102]. It may be important to for these strategies to balance avoiding adapted epitope-specific CD8 T-cell responses while still inducing broadly cross-reactive CD8 T cells.

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APPENDIX

INSTITUTIONAL REVIEW BOARD HUMAN SUBJECT APPROVAL FORM

Project Revision/Amendment Form

Form version: June 26, 2012

In MS Word, click in the white boxes and type your text; double-click checkboxes to check/uncheck.

- Federal regulations require IRB approval before implementing proposed changes. See Section 14 of the IRB Guidebook for Investigators for additional information.
- Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.). See Item 4 for more examples.

MAY 02 2017

1. Today's Date	05.01.17	3355

2. Principal Investigator (PI)

Name (with degree)	Paul Goepfert	Blazer ID	Paulg
Department	Medicine	Division (if applicable)	Infectious Diseases
Office Address	CCB 328-A	Office Phone	5-5667
E-mail	paulg@uab.edu	Fax Number	5-6027

Contact person who should receive copies of IRB correspondence (Optional)

Name	Tracie Ash	E-Mail	tjash@uab.edu
Phone	5-8007	Fax Number	5-5824
Office Address (if different from PI)	CCB 325		

3. UAB IRB Protocol Identification

3.a. Protocol Number: X140612002

3.b. Protocol Title: The Rational Approach for HIV Vaccine T Cell Epitope Selection

3.c. Current Status of Protocol—Check ONE box at left; provide numbers and dates where applicable

- ☐ Study has not yet begun — No participants, data, or specimens have been entered.
- ☒ In progress, open to accrual — Number of participants, data, or specimens entered: 63
- ☐ Enrollment temporarily suspended by sponsor
- ☐ Closed to accrual, but procedures continue as defined in the protocol (therapy, intervention, follow-up visits, etc.)
  - Date closed:
  - Number of participants receiving interventions:
  - Number of participants in long-term follow-up only:
- ☐ Closed to accrual, and only data analysis continues
  - Date closed:
  - Total number of participants entered:

4. Types of Change

Check all types of change that apply, and describe the changes in Item 5.c. or 5.d. as applicable. To help avoid delay in IRB review, please ensure that you provide the required materials and/or information for each type of change checked.

- ☐ **Protocol revision (change in the IRB-approved protocol)**
  In Item 5.c., if applicable, provide sponsor's protocol version number, amendment number, update number, etc.
- ☐ **Protocol amendment (addition to the IRB-approved protocol)**
  In Item 5.c., if applicable, provide funding application document from sponsor, as well as sponsor's protocol version number, amendment number, update number, etc.
- ☒ **Add or remove personnel**
  In Item 5.c., include name, title/degree, department/division, institutional affiliation, and role(s) in research, and address whether new personnel have any conflict of interest. See "Change in Principal Investigator" in the IRB Guidebook if the principal investigator is being changed.
  - ☐ **Add graduate student(s) or postdoctoral fellow(s) working toward thesis, dissertation, or publication**
    In Item 5.c., (a) identify these individuals by name; (b) provide the working title of the thesis, dissertation, or publication; and (c) indicate whether or not the student's analysis differs in any way from the purpose of the research described in the IRB-approved HSP (e.g., a secondary analysis of data obtained under this HSP).
- ☐ **Change in source of funding; change or add funding**
  In Item 5.c., describe the change or addition in detail, include the applicable OSP proposal number(s), and provide a copy of the application as funded (or as submitted to the sponsor if pending). Note that some changes in funding may require a new IRB application.
- ☐ **Add or remove performance sites**
  In Item 5.c., identify the site and location, and describe the research-related procedures performed there. If adding site(s), attach notification of permission or IRB approval to perform research there. Also include copy of subcontract, if applicable. If this protocol includes acting as the Coordinating Center for a study, attach IRB approval from any non-UAB site added.

☐	**Add or change a genetic component or storage of samples and/or data component—this could include data submissions for Genome-Wide Association Studies (GWAS)** To assist you in revising or preparing your submission, please see the IRB Guidebook for Investigators or call the IRB office at 934-3789.
☐	**Suspend, re-open, or permanently close protocol to accrual of individuals, data, or samples (IRB approval to remain active)** In Item 5.c., indicate the action, provide applicable dates and reasons for action; attach supporting documentation.
☐	**Report being forwarded to IRB (e.g., DSMB, sponsor or other monitor)** In Item 5.c., include date and source of report, summarize findings, and indicate any recommendations.
☐	**Revise or amend consent, assent form(s)** Complete Item 5.d.
☐	**Addendum (new) consent form** Complete Item 5.d.
☐	**Add or revise recruitment materials** Complete Item 5.d.
☒	**Other (e.g., investigator brochure)** Indicate the type of change in the space below, and provide details in Item 5.c. or 5.d. as applicable. Include a copy of all affected documents, with revisions highlighted as applicable. ► Adding to the study staff.

5. Description and Rationale

In Item 5.a. and 5.b, check Yes **or** No **and see instructions for** Yes **responses.**
In Item 5.c. and 5.d, describe—and explain the reason for—the change(s) noted in Item 4.

☐Yes ☒No	**5.a. Are any of the participants enrolled as normal, healthy controls?** If yes, describe in detail in Item 5.c. how this change will affect those participants.
☐Yes ☒No	**5.b. Does the change affect subject participation, such as procedures, risks, costs, location of services, etc.?** If yes, FAP-designated units complete a FAP submission and send to fap@uab.edu. Identify the FAP-designated unit in Item 5.c. For more details on the UAB FAP, see www.uab.edu/cto.

5.c. Protocol Changes: In the space below, briefly describe—and explain the reason for—all change(s) to the protocol.

► Adding Sushma Boppana to the study staff. No conflict of interest.
Sushma Boppana
M.D./Ph.D. Program
Graduate Student Trainee
Immunology
Shelby Biomedical Rsch Bldg
205-934-4092

✓ Added in SIRB. CKW

5.d. Consent and Recruitment Changes: In the space below,
(a) describe all changes to IRB-approved forms or recruitment materials and the reasons for them;
(b) describe the reasons for the addition of any materials (e.g., addendum consent, recruitment); and
(c) indicate either how and when you will reconsent enrolled participants or why reconsenting is not necessary (not applicable for recruitment materials).

Also, indicate the number of forms changed or added. For new forms, provide 1 copy. For revised documents, provide 3 copies:
- **a copy of the currently approved document (showing the IRB approval stamp, if applicable)**
- **a revised copy highlighting all proposed changes with "tracked" changes**
- **a revised copy for the IRB approval stamp.**

►

Signature of Principal Investigator _____ Date 5/2/17
Paul Goepfert, M.D.

FOR IRB USE ONLY

☐ Received & Noted ☑ Approved Expedited* ☐ To Convened IRB

Maggie Lawson, CIP 5/3/17
Signature (Chair, Vice-Chair, Designee) Date

DOLA 4/23/16

Change to Expedited Category Y / (N) / NA

*No change to IRB's previous determination of approval criteria at 45 CFR 46.111 or 21 CFR 56.111