Published in final edited form as:

Vaccine. 2009 February 11; 27(7): 1136–1140. doi:10.1016/j.vaccine.2008.11.071.

AIDSVAX Immunization Induces HIV-specific CD8+ T-cell Responses in High-risk, HIV-negative Volunteers Who Subsequently Acquire HIV Infection^{1,2}

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Abstract

Correlates of immune protection from HIV vaccines remain undefined. The first HIV vaccine efficacy trial in the US and Europe VAX004, was designed to assess whether rgp120 envelope subunits (AIDSVAX B/B, VaxGen) can induce partial or complete protection from HIV-1 infection. No effectiveness in the reduction of either the acquisition of infection or levels of plasma viremia after HIV infection was noted. We found evidence of vaccine-specific CD8+ T cells in volunteers who received the vaccine, regardless of behavioral risk. Surprisingly, the CD8-response is significantly higher in participants who would go on to contract HIV infection. These results suggest that AIDSVAX immunization may boost preexisting immune responses—due to pre-infection exposure, and a vaccine-induced immune profile may serve as a biological marker for HIV susceptibility.

BACKGROUND

The first phase III clinical trial of an HIV vaccine (VAX004) was conducted in the US and Europe using a candidate HIV-1 gp120 vaccine, AIDSVAX B/B (VaxGen). VAX004 was a randomized, double-blinded, placebo controlled trial. The vaccine consisted of 2 rgp120 envelope subunits derived from the subtype B isolates MN and GNE8. Volunteers were randomized to receive the vaccine or placebo (alum) at a 2:1 ratio and the primary objective was to assess whether the vaccine could induce complete or partial protection from HIV-1 infection. The hypothesis was that antibodies directed against the envelope would bind, neutralize and clear HIV particles before infection became established. Details on the study population, counseling procedures, and ethical considerations have previously been published [1]. The study was completed in 2003 and revealed no effectiveness in the reduction of HIV infection or levels of plasma viremia. The time to ART initiation or to virologic failure was

¹Work was supported by NIH grants AI43885 and the California HIV AIDS Program CH05-D-606

²Authors Potential Conflict: MP and MG are former VaxGen employees

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similar in the vaccine arm and the placebo arm. The pre-ART viral load and CD4+ lymphocyte count were also comparable between the two arms. Similarly, the antibody response levels were comparable among low-risk or high-risk vaccine recipients [2].

The lack of efficacy from VAX004 weakened the hypothesis that antibodies directed against the envelope glycoprotein would bind, neutralize and clear HIV particles before infection became established. Subsequent vaccine designs have focused on eliciting T-cell responses [3–6]. The presence of HIV-1-specific CD8+ T cells has been correlated with the resolution of peak viremia during acute infection, control of set point viremia during chronic infection, and inversely correlated to disease progression [7,8]. CTLs are able to lyse infected cells before progeny virions are produced [9] and therefore have the potential to limit viral load and slow disease progression. Studies in animal models of HIV-1 infection have shown that CD8+ T cells are critical to the initial and chronic control of infection, and successful vaccination has been associated with the induction of strong CTL responses [10–14]. In this study we assessed the CD8-immune responses in VAX004 recipients and evaluated the potential role of T cells in preventive HIV vaccines.

METHOD

VAX004

The study population and demographics of VAX004 have previously been described [1]. Briefly, healthy individuals aged 18 to 60 years who tested HIV-1 negative but were at risk for HIV-1 infection through sexual activity were enrolled. Samples for this study were selected from placebo and vaccine recipients based on infection outcome, and on behavioral risk factors documented at enrollment. Behavior risk scores ranged from 0 to 7; and were categorized as either low (0–1) or high (3–7). The sample set was stratified as 1) low risk not infected, 2) low risk subsequently infected, 3) high risk not infected, and 4) high risk subsequently infected. PBMC were isolated and cryopreserved according to the initial study protocol after 4 to 6 vaccinations. All study volunteers were confirmed as being HIV seronegative at the time of blood collection and in subsequent analyses. Two hundred and forty one samples were randomly selected. Only samples with PBMC viability of $\geq 75\%$ were subsequently tested and all assays were performed blinded to the sample identity

Valid analyses were obtained from samples with approximately 2:1 vaccine to placebo ratio (Table 1). No significant difference in age or gender distribution was observed between the two groups ($p > 0.05$).

CFSE Proliferation Assay

Cell proliferation was determined by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA), per the manufacturer's instructions. Cells were cultured in the presence of antigen for five days at 37° C and 5% CO₂, then harvested and stained for surface markers with the following antibodies: CD3 APC, CD4 PE, CD8 PerCp-Cy5.5 (BD Biosciences, San Jose, CA). Samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). All flow analysis was performed using FlowJo software (TreeStar, Ashland, OR). Proliferation was measured by the extent of CFSE dilution. Staphylococcal Enterotoxin B (SEB Sigma-Aldrich, St. Louis, MO) stimulation was used as a positive control. All study participants demonstrated significant proliferation following SEB stimulation. Results with less than 1% background response, and greater than 5% SEB response were considered valid. Only data with a minimum of 5,000 acquired events of CD3+CD4+ or CD3+CD8+ were analyzed. Results greater than twice the background values and more than 0.1% after subtraction of background were considered positive.

Immune activation

Activation staining was performed by incubating PBMC with the following antibodies: HLADR FITC, CD38 PE, CD8 PerCp-Cy5.5, CD4 APC-CY7, CD3 AmCyan (BD Biosciences San Jose, CA) CD27 APC and CD45RA-PE-Cy7 (BD Pharmingen San Diego, CA), and analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) [15]. Dead cells were excluded from analysis using a violet excited viability dye (LIVE/DEAD Fixable Dead Cell Stain; Invitrogen). Immune activation was defined as the percent of CD38+ HLA DR+ T cells within the naïve and memory subsets. We defined naïve T cells as CD3+CD8+ (or CD3+CD4+) CD27+CD45RA+ and memory T cells as CD3+CD8+ (or CD3+CD4+) CD27-CD45RA-. Gating was standardized and set using fluorescence minus one controls for HLADR and CD38.

Flow-based intracellular cytokine staining

Detection of antigen-specific cytokine production was performed using PBMC incubated with Env, Nef or CMV peptide pools for 6 hours at 37° C and 5% CO₂ in the presence of brefeldin A (Sigma) and monensin (Golgi Stop, BD Biosciences, San Jose, CA)[16]. Staphylococcus Enterotoxin B (SEB; 1 ug/ml, Sigma) and media alone were used as positive and negative background controls, respectively. Cells were subsequently stained with anti-IFN- γ FITC, anti-CD4 PerCP-Cy5.5, anti-TNF α PE-Cy7, anti-CD3 AmCyan, anti-CD8 APC-Cy7, anti-IL-2 APC (BD Biosciences) and anti-CD107a PE (BD Pharmingen). Dead cells were excluded from analysis using a violet excited viability dye (LIVE/DEAD Fixable Dead Cell Stain; Invitrogen). [17] A minimum of 30,000 CD3⁺ cells per sample were acquired using an 8-color flow cytometer (LSRII, BD Biosciences) Results were expressed as: Percent cytokine positive, CD4⁺ or CD8⁺ T cells (Percent positive = % antigen-specific - % negative control). Responses greater than 2 times the background were considered positive. All volunteers demonstrated significant cytokine production following SEB stimulation.

Antigens

Peptides corresponding to the vaccine sequences of the clade B HIV-1 ENV gp160 MN (212 peptides) and consensus clade B NEF (49 peptides) were synthesized as 15 amino acids (a.a.) overlapping by 11 a.a. (NIH/NIAID repository, catalog numbers 6451 and 5189, respectively). A single pool of overlapping peptides, corresponding to the amino acid sequence of the HCMV pp65 protein (JPT Peptide Technologies) was used to detect human CMV -specific responses. The final concentration of individual peptides was 1ug/ml per peptide.

Statistical Analysis

Logistic regression analysis was used to estimate odds ratios of a positive T cell response among 1) low risk vs. high risk vaccine recipients, and 2) among uninfected vs. subsequently HIV-1 infected vaccine recipients with and without adjustment for risk score. Among vaccine recipients who had either a CD4+ or a CD8+ T cell response, Wilcoxon rank sum tests were used to compare the percentage of activated CD4+ and CD8+ T cells between groups. Statistical significance was defined as 2-sided $p \leq 0.05$.

RESULTS

Vaccine-specific CD8+ T cell proliferation

Results for CD8+ T cell assays were valid in 149 samples. CD8⁺ HIV-specific T-cell proliferation was detected in AIDSVAX recipients at higher frequencies compared to placebo recipients (Table 1, 15/87 [17.2%]) and 1/62 [1.6%], respectively, p -value <0.05). In addition, HIV-specific responses were more frequently detected among vaccine recipients in the high-risk (N=34) compared to low-risk (N=53) groups (26.5% and 11.3%, respectively) although this did not reach statistical significance (OR 2.8, 95% CI [0.9–9.3], p -value=0.075).

Surprisingly, the frequency of responses was significantly higher in vaccine recipients who subsequently acquired HIV infection compared to individuals who remained HIV seronegative (Table 2A, 34.8% and 10.9%, respectively, OR 4.3, 95% CI [1.4–14.3], p-value=0.01). This difference remains statistically significant after adjusting for risk score (OR 4.0, 95% CI [1.2–13.6], p-value=0.02).

To assess whether race or gender played a role in the above finding, similar analyses were performed in a subset of white, male vaccine recipients, who constituted the majority of the study population (Table 2B). The responses rate in white male vaccinees remains significantly higher in individuals who subsequently acquired HIV infection compared to those who remained HIV negative (8/20 and 7/52, respectively, OR 4.29, 95% CI [1.30, 14.68], p-value=0.017), similar to the overall population, suggesting that race or gender did not contribute significantly to our findings.

No difference in the frequency of HCMV-specific proliferation was observed among all subgroups (Figure 2, p>0.05), suggesting that the finding were vaccine specific among vaccine and placebo recipients, regardless of outcome.

Vaccine-specific CD4+ T cell proliferation

Results for CD4+ T cell proliferation were valid in 166 samples. Vaccine-specific CD4+ proliferation was significantly higher in individuals who received the vaccine compared to placebo recipients (13/94 [13.8%] and 1/72 [2.8%], respectively, p<0.05) placebo recipients. The response rate was significantly different between vaccine volunteers who subsequently acquired HIV infection compared to individuals who remained HIV seronegative (7/31 and 6/63 respectively, OR 5.4, 95% CI [1.5–25.4], p=0.02). However, when adjusted for risk score, no significant difference was seen in the CD4+ response rate between vaccine recipients who eventually acquired HIV infection compared to those who did not (OR 2.77, 95%CI [0.84, 9.45], p-value=0.093). The odds ratio of a positive response among white, male vaccine recipients who eventually became HIV infected versus never infected vaccinees, adjusted for risk score, is 3.43 (95%CI [0.86, 15.43], p-value= 0.087). The lack of differences in findings between male vaccine recipients subanalysis and the total vaccine recipients indicate that race or gender do not play a role in the above findings.

T cell profile of vaccine responders

We next determined the level of intracellular cytokine production and immune activation in vaccine recipients who demonstrated evidence of T cell proliferation. Low levels of Env-specific IFN- γ and TNF- α were detected in 3 out of 20 evaluated responders (representative plot in Figure 3). In contrast, CMV-specific responses included simultaneous production of multiple cytokines, including IFN- γ and TNF- α as well as CD107 surface expression. No Nef-specific responses were detected, supporting the fact that HIV-specific immune responses were attributable to AIDSVAX immunization.

CD4+ and CD8+ T cell activation was also assessed and analyzed by HIV infection outcome. We observed a significantly higher immune activation in CD4+ T cells in vaccine responders who subsequently acquire HIV infection compared to those who remain uninfected (Wilcoxon test, p=0.05). We also found significantly higher CD8 T cell activation in vaccine responders who subsequently became HIV positive (Figure 4, p=0.014).

DISCUSSION

Several large HIV vaccine trials have now failed to show protection against HIV infection in healthy subjects [1,18,19]. Why is the development of an effective AIDS vaccine such a major

hurdle? One reason is that HIV has evolved highly sophisticated ways to evade the immune system. One major obstacle is that HIV directly infects and damages the immune system, which may sabotage the orchestrated immune responses necessary to contain viral replication. Such enormous challenges are made even more daunting by the lack of full understanding of the immune responses that can effectively contain HIV.

The relevance of the immune responses elicited from HIV vaccination is only partially understood. Many factors complicate the evaluation of HIV vaccines and suggest that we should proceed with caution in measuring potential correlates of immune protection. Furthermore, most studies of vaccine-specific T cells have examined peripheral blood. Viral replication and antiviral immunity are predominantly tissue based and developing delivery strategies to promote these responses may be important [20–22]. The disappointing outcome of the efficacy trial of the T-cell based HIV vaccine was unanticipated, given the strong induction of HIV-specific T cells. One potential issue is the effect of vaccine-mediated inflammation and immune activation, in both CD4+ and CD8+ T cells. The hypothetical concern is that, in the presence of continuous high-risk behavior and exposure to HIV, infection rates could be enhanced by the presence of susceptible HIV-1 specific T cells. Activation of the CD4 T cells may provide new targets for HIV, thus allowing the spread of the virus despite the pre-existing vaccine-induced immune response. In support of this hypothesis, it has been clearly shown that several vaccines can induce a burst of virus replication in patients with chronic HIV infection in the absence of effective antiviral therapy.

In our study, vaccine recipients who demonstrated evidence of Env-specific CD8 proliferation have a greater chance of becoming infected if they subsequently come into contact with the virus. AIDSVAX recipients in the high-risk subgroup also demonstrated significantly higher HIV-specific CD4+ T cell responses compared to the low-risk behavior subgroups. However, the presence of vaccine-induced CD4 responses did not correlate with subsequent risk of HIV acquisition, particularly when the analysis was adjusted for risk score. How is the presence of a CD8+ T cell response, historically associated with the clearance of virus-infected cells, associated with the risk of subsequent HIV infection? It seems implausible that CD8+ T cells in vaccines directly enhance HIV acquisition given the neutral outcome of the overall VAX004 trial and our significant findings. We hypothesize that AIDSVAX boosts preexisting immune responses—due to pre-infection exposure. It will be critical to understand whether these responses represent a host genetic profile conferring differential susceptibility to HIV.

Vaccine responders in our study display very low levels of functionality. It is possible that a unique vaccine-induced immune profile may be required to achieve effective protection from HIV infection. The recent disappointment of the STEP vaccine study data showed that vaccine-mediated T cell responses, as detected by IFN-gamma production, do not necessarily protect against HIV infection and may even have enhanced infection in vaccinees with prior immunity to adenoviruses and/or uncircumcised vaccine recipients [19,23,24]. Over the past decade, multi-parameter flow cytometry assays have allowed the simultaneous evaluation of antigen-specific T cells (polyfunctionality) in exquisite detail [25]. The enthusiasm for this approach stems from the association between polyfunctional HIV-specific T cells and control of viral replication and non-progression in infected individuals [25]. Thus, in the absence of a strong and broadly elicited T cell response, our observed pattern of CD8+ vaccine-specific profile may only indicate an increased level of vaccine-induced immune activation that represents a biological marker for risk of subsequent HIV infection.

The study of the immune profile of HIV vaccine recipients who acquire HIV infection could provide relevant information for understanding potential vaccine-mediated activation. Our study suggests the potential role of HIV-specific T cell immunity as a biological marker of subsequent risk of HIV infection. Unlike the immune responses detected in HIV infection in

the presence of continuous viral replication, the presentation of antigens mediated by a preventive vaccine immunogen is limited and the quality of the memory T cells generated may be distinct. The issue of which aspects of T cell immunity need to be generated by an effective vaccine remains unresolved. The results of several large phase II/III HIV vaccine studies beg the question of whether a stronger rationale is required to define immunologic criteria in evaluating vaccine responses. Vigorous effort to understand the biological reasons for the lack of protection from vaccines remain urgently needed.

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Figure 1.

Representative Plot of env-specific CD8 proliferation. CFSE-labeled PBMC were stimulated with ENV peptides for 5 days then assessed for proliferation by flow cytometry. Results are expressed as percent of proliferating CD8+ T-cells as measured by the extent of CFSE dilution. Positive proliferation is defined as >0.1% net and at least twice background.

Figure 2.

CMV-specific CD4+ and CD8+ proliferation in VAX004 samples. CFSE- labeled PBMC were stimulated with HCMV peptides then assessed for proliferation after 5 days. No significant difference between the frequency of responses in CD4- and CD8- mediated proliferation was seen between placebo and vaccine recipients ($p>0.05$)

Figure 3.

Representative plots of antigen-specific CD8⁺ T cells in a vaccine responder. PBMC were stimulated with Env or CMV peptides then stained with anti-CD107a PE, anti-IFN- γ FITC, anti-TNF- α PE-CY7, anti-CD4 PerCP-Cy5.5 anti-IL-2 APC, anti-CD3 AmCyan and anti-CD8 APC-Cy7, and analyzed by flow cytometry. Samples were first gated on the CD3⁺/CD8⁺ lymphocyte population then the percent of CD107, TNF- α , IFN- γ , and IL-2 positive CD8⁺ T cells were determined.

Figure 4.

Immune activation in CD4+ and CD8+ lymphocyte subsets in vaccine responders based on subsequent HIV infection status. PBMC were stained with HLADR FITC, CD38 PE, CD3 AmCyan, CD4 APC-CY7, and CD8 PerCP-Cy5.5, and analyzed by flow cytometry. Immune activation was defined as the percentage of CD4+ or CD8+T cells co-expressing HLA DR and CD38. CD8+ immune activation was significantly higher ($p=0.014$) in vaccine responders who subsequently acquire HIV infection (Infected) compared to vaccine responders who remain HIV negative (Uninfected).

TABLE 1

ENV - specific Responses

PARTICIPANT	CD8 response		CD4	
	Vaccine (N=87)	Placebo (N=62)	Vaccine (N=94)	Placebo (N=72)
High risk	9/34	1/30	10/41	0/36
Low Risk	6/53	0/32	3/53	1/36
Odds ratio (95% CI)	2.82 [0.91, 9.28)		5.38 [1.51, 25.38)	
p-value	0.075	NS	0.016	NS

TABLE 2

TABLE 2A		
Vaccine Recipients		
Outcome	CD8 response (percent) N=87	CD4 response (percent) N=94
Uninfected	7/64 (10.9%)	6/63 (9.5%)
HIV infection	8/23 (34.8%)	7/31 (22.6%)
Odds ratio (95% CI)	4.34 [1.36, 14.34]	2.77 [0.84, 9.45]
p-value	0.013	0.093
TABLE 2B		
Vaccine Recipients (white male volunteers)		
Outcome	CD8 response (percent) N=72	CD4 response (percent) N=79
Uninfected	7/52 (13.5%)	4/51 (7.8%)
HIV infection	8/20 (40.0%)	7/28 (25.0%)
Odds ratio (95% CI)	4.29 [1.30, 14.68]	3.92 [1.07, 16.34]
p-value	0.017	0.045