RESEARCH ARTICLE

ALVAC-HIV B/C candidate HIV vaccine efficacy dependent on neutralization profile of challenge virus and adjuvant dose and type

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Abstract

The ALVAC-HIV clade B/AE and equivalent SIV-based/gp120 + Alum vaccines successfully decreased the risk of virus acquisition in humans and macaques. Here, we tested the efficacy of HIV clade B/C ALVAC/gp120 vaccine candidates + MF59 or different doses of Aluminum hydroxide (Alum) against SHIV-Cs of varying neutralization sensitivity in macaques. Low doses of Alum induced higher mucosal V2-specific IgA that increased the risk of Tier 2 SHIV-C acquisition. High Alum dosage, in contrast, elicited serum IgG to V2 that correlated with a decreased risk of Tier 1 SHIV-C acquisition. MF59 induced negligible mucosal antibodies to V2 and an inflammatory profile with blood C-reactive Protein (CRP) levels correlating with neutralizing antibody titers. MF59 decreased the risk of Tier 1 SHIV-C acquisition. The relationship between vaccine efficacy and the neutralization profile of the challenge
virus appear to be linked to the different immunological spaces created by MF59 and Alum via CXCL10 and IL-1β, respectively.

Author summary

The ALVAC-based clade C/gp120 combination is currently being tested for efficacy and licensures in South Africa in the HVTN702 HIV vaccine trial using the MF59 adjuvant rather than Alum with the intent of inducing higher neutralizing antibodies and T cell responses. Here, we present studies in 71 macaques that evaluated the relative efficacy of identical ALVAC-HIV B/C HIV vaccines with Alum or MF59 following challenge exposure to SHIV-clade C viral stocks that differed in their neutralization profiles. Our results demonstrated that use of the proprietary Novartis Alum at lower doses affects the proportion of mucosal V2-specific IgG and IgA, and that IgAs and IgGs were respectively associated with an increased or decreased risk of virus acquisition. In contrast, animals immunized with the MF59 regimen had no V2 mucosal responses, and a high level of serum neutralizing antibodies (Tier 1) to easy to neutralize viruses that were associated with a decreased risk of Tier 1 SHIV-C acquisition. In vitro studies suggest the hypothesis that the low doses of proprietary Novartis Alum used in our studies may be the underlying reason for the decreased vaccine efficacy, via lower inflammasome activation and IL-1β production.

Introduction

The development of a fully protective vaccine against HIV has proven challenging, and only four (gp120, recombinant Ad5, DNA plus recombinant Ad5, and ALVAC-based regimens) out of more than 250 HIV vaccine candidates have even been tested in phase IIb/III efficacy studies [1–5]. The RV144 clinical trial (NCT00223080) is the only one so far to afford limited vaccine efficacy (31.2%), using a regimen based on a combination of the recombinant canary-pox vector ALVAC-HIV vCP1521 expressing clade B Gag-Pro and clade E gp120-TM with the recombinant gp120 B/AE proteins adjuvanted in Alum Alhydrogel [6]. Analysis of RV144 identified binding antibodies to the HIV envelope variable region 2 (V2), CD4+ T cells, and antibody-dependent cell-mediated cytotoxicity (ADCC) in individuals with low levels of IgA as inverse correlates of risk of HIV acquisition [7, 8]. Crucially, half of the 35 million documented cases of HIV infection worldwide [9] are caused by HIV-1 clade C (HIV-C) [10], and the phase IIb/III HVTN702 trial is now underway in South Africa to investigate effectiveness of the ALVAC-based vaccine against HIV-C [11].

HVTN702 builds on the vaccine used in RV144 and HVTN100 [12], but the South African study is distinguished by several important elements. Foremost, HVTN702 tests ALVAC gp120/MF59 clade C vaccines in a high-risk population. Indeed, 7.1 million people were reported to be living with HIV-1 in South Africa in 2016, with a prevalence of 18.9% in the general adult population and 270,000 new infections [13, 14]. By contrast, the vaccinees enrolled in RV144 were at low risk for HIV-1 acquisition. In the same year, 450,000 people were living with HIV-1 in Thailand, with a prevalence of 1.1% and 6,400 new infections [15]. The different rate of infection between these populations is jarring, and HIV prevalence and incidence were respectively 17 and 42 times higher in South Africa than in Thailand [16]. Further separating HVTN702 and RV144 are the recombinant ALVAC vCP2438, expressing the
96ZM651 HIV clade C envelope, and the two clade C TV1 and 1086 gp120 protein boosts. Finally, the use of adjuvant differs between these trials, with HVTN702 employing an MF59 oil-in-water emulsion instead of the Alum Alhydrogel used in RV144.

Our prior studies in the SIV\textsubscript{mac251} macaque model with Alum Alhydrogel and MF59 adjuvants demonstrated that the ALVAC/gp120/Alum Alhydrogel regimen (SIV-Alum\textsubscript{Alh12.5}) recapitulated the limited efficacy of RV144 and identified V2 antibodies and CD4\textsuperscript{+} T cells as inverse correlates of risk against the neutralization-resistant Tier 2 SIV\textsubscript{mac251}, as they were in humans [17, 18]. However, an otherwise identical vaccine regimen using MF59 as adjuvant (SIV-MF59) unexpectedly did not reduce the risk of SIV\textsubscript{mac251} acquisition, despite its higher immunogenicity [18, 19].

The predictive value of macaque models in assessing the efficacy of HIV vaccine candidates in humans remains uncertain, and further testing is necessary to determine its usefulness. Thus, we investigated the efficacy of the clade B/C ALVAC-HIV gp120 vaccine regimens in macaques with a gp120 protein boost formulated in MF59 or with varying Alum/gp120 ratios of the proprietary Alum\textsuperscript{N} adjuvant (Novartis). We found that the alum adjuvant at a ratio of 1.9:1 gp120 (Alum\textsuperscript{N1.9}; Study 1) induced V2-specific IgA that correlated with an increased risk of acquisition of Tier 2 SHIV-C in vaccinated animals. The Alum\textsuperscript{N1.9} regimen did not protect from Tier 2 SHIV-C acquisition when compared to controls, contrasting sharply with the results of the SIV-Alum\textsuperscript{Alh12.5} study [18] with 6.6-fold more alum salt (12.5:1 Alum:gp120) in what was an otherwise identical immunization regimen. Indeed, the second study (Alum\textsuperscript{N10.8}; Study 2) performed here contained the same vaccine modalities used at a higher ratio of 10.8:1 Alum:gp120 and elicited V2-specific IgG that correlated with a decreased risk of Tier 1 SHIV-C acquisition. Mirroring the SIV-Alum\textsuperscript{Alh12.5} study, antibodies to V2 were revealed to be a correlate of decreased risk of SIV\textsubscript{mac251} acquisition. The MF59 regimen of the present work (Study 3) induced the highest neutralizing antibodies titers to Tier 1 SHIV-C, correlated with plasma levels of C-Reactive Protein (CRP). The levels of V2 IgG were negligible in the mucosa of the animals immunized in this regimen.

In vitro stimulation of human and macaque PBMCs demonstrated that decreasing the dose of Alum affects the production of IL-1\textbeta. Vitally, this cytokine induces innate monocyte memory: a response that strongly correlated with a reduced risk of acquisition of the neutralization-resistant Tier 2 SIV\textsubscript{mac251} [17]. Thus, the differing vaccine efficacies of the SIV\textsubscript{mac251} and SHIV-C macaque models might depend in part on the inflammatory milieu generated by the combination of the vectored vaccine and adjuvant, and the neutralization profile of the challenge virus in each regimen. More specifically, these data suggest that low doses of Alum salt may be less effective in harnessing monocyte innate memory via IL-1\textbeta induction, thereby limiting the efficacy of Alum-based vaccine regimens.

We demonstrate here that the MF59 regimen protects against the easily-neutralized Tier 1 SHIV-C. In prior work, however, an MF59 regimen based on the same vaccine modalities did not protect against the neutralization-resistant Tier 2 SIV\textsubscript{mac251} [18]. Given that 99% of the circulating HIV variants in South Africa have a neutralization-resistant profile, it is uncertain whether the Tier 1 SHIV-C model will be predictive of vaccine efficacy in the HVTN702 HIV trial. Ultimately, we must await the results of HVTN702 to gauge the accuracy of predictions based on the SIV\textsubscript{mac251} or SHIV-C macaque models.

Results

\textbf{Alum\textsuperscript{N1.9} IgA to V2 do not reduce Tier 1 SHIV-C acquisition}

In the Alum\textsuperscript{N1.9} study (Study 1), a group of 27 macaques was administered four doses of ALVAC-SIV \textit{gag-pol} (vCP172) and ALVAC-HIV \textit{gag-pro-env} (vCP2438) at weeks 0, 4, 12, and...
At weeks 12 and 24, all 27 macaques received 200 μg each of clade C TV1 and 1086 gp120 proteins (for a total of 400 μg/dose) formulated in 0.75 mg of proprietary Alum N1.9 (1.9:1 Alum:gp120; Fig 1A; Table 1). SIV-Alum Ah12.5 was previously protective against SIV mac251 using a boost of 800 μg of gp120 formulated with 5.0 mg of Alum Ah12.5 (12.5:1 Alum:gp120; Table 1) [18].

Vaccine efficacy was assessed as the average rate of virus acquisition in the 27 vaccinated animals versus 20 controls that received only two inoculations of Alum N following a total of 17 weekly intravaginal challenges with the Tier 2 SHIV-C (SHIV-1157ipd3N4) [20], starting 4 weeks after the last immunization (Fig 1A). Compared to controls, Alum N1.9 did not reduce the risk of acquisition of Tier 2 SHIV-C (Fig 1B), and no evidence of viral control was observed (Fig 1C). Likewise, neither the vaccinated nor the control groups experienced a significant decrease in CD4+ T cells, indicative of the limited pathogenic profile of this Tier 2 SHIV-C (Fig 1D). The lack of vaccine efficacy was somewhat surprising, as we had previously observed 44% efficacy with SIV-Alum Ah12.5. Noting this discrepancy, we analyzed systemic and vaginal antibody responses and contrasted them with those obtained in the SIV-Alum Ah12.5 study. Following the final immunization of Alum N1.9, serum binding antibody titers to 1086 (av. reactivity 10^6) were significantly higher than those to TV1 (av. reactivity 10^5; P < 0.0001; Fig 1E). Vaginal IgGs specific for the two envelope proteins (Fig 1F) or the gp70 V1/V2 scaffolds (Fig 1G) did not differ between antigens and were similar to levels observed in the SIV-Alum Ah12.5 study [18].

We further characterized V2-specific IgG levels in plasma and vaginal secretions by performing peptide arrays of overlapping peptides encompassing the 1086 V2 region (S1A Fig). Of the 27 animals vaccinated with Alum N1.9, 25 (92.5%) had IgG in blood that recognized peptide 53 (TELKDKKKHKVHALFY) containing the cryptic α4β7 binding site (Fig 1H). Peptide 57 (LFYKLDVVPLNGNSS), containing the canonical tripeptide α4β7 integrin receptor binding site, was recognized by the IgG of only four of these animals (S1B and S1D Fig and S2 Fig). Measured in vaginal secretions, IgGs recognizing peptide 53 were present in 10 of the 13 animals tested (78%; Fig 1I; Table 2; S1C and S1E Fig).

An important qualitative difference between the current Alum N1.9 study and SIV-Alum Ah12.5 was observed in the levels of mucosal V2-specific IgA and IgG. In Alum N1.9, V2-specific IgA was higher than IgG (P = 0.0001; Fig 1J). In stark contrast, the level of SIV V2-specific IgG was significantly higher than V2-specific IgA (P = 0.0313; Fig 1K) in the females of SIV-Alum Ah12.5. Importantly, correlate of risk analyses of all antibody responses in Alum N1.9 measured above revealed that the level of vaginal IgA correlated with earlier acquisition of Tier 2 SHIV-C (P = 0.009; Fig 1L). Notably, the mucosal IgG level was a primary correlate of decreased risk of SIV mac251 acquisition in the SIV-Alum Ah12.5 study [18], and high serum IgA was a correlate of increased HIV risk in RV144 [21]. While Alum N1.9 induced neutralizing antibodies to Tier 1A HIVs and moderately to SHIV-1157ipEL-p [22], antibodies against Tier 2 HIVs or SHIV-1157ipd3N4 were not produced (Table 3). None of the neutralization titers correlated with Tier 2 SHIV acquisition.

Alum N10.8 IgG to V2 and delayed Tier 1 SHIV-C acquisition

We next tested Alum N10.8 (Study 2) in 10 Chinese rhesus macaques in a longer immunization regimen. Animals were vaccinated at weeks 0, 4, 24, 49, and 111 with ALVAC-SIV gag-pol (vCP172) and ALVAC-HIV gag-pro-env (vCP2438), and at weeks 12, 24, 49, and 111 with 100 μg each of clade C TV1 and 1086 proteins (for a total of 200 μg per dose) formulated in 2.16 mg of Alum N (Alum salt:gp120 10.8:1; Alum N10.8; Fig 2A; Table 1). Two of the original 10 animals in both Alum N10.8 and MF59 groups, for a total of 4 animals, perished due to health conditions.
Fig 1. Relative efficacy and immunogenicity of the Alum\textsuperscript{N1.9} regimen. Unless stated otherwise, all assays, experiments and graphs associated with the Alum\textsuperscript{N1.9} study are represented by full red circles, and available samples (i.e., those not depleted in other experiments) are shown as...
stated in the legend. (A) Animals were immunized with ALVAC-vCP172 and ALVAC-vCP2438 and boosted twice with monomeric gp120 proteins (200 μg each of TV1 and 1086) in 0.75 mg doses of Aluminum hydroxide manufactured by Novartis (AlumN1.9; n = 27; Table 1). Controls (n = 20) received only two AlumN10.8 adjuvant inoculation at the same dose as the immunized macaques. (B) The rate per exposure of Tier 2 SHIV-C (SHIV-1157ipdl3N4) acquisition was compared in 27 vaccinated animals and 20 controls using the log-rank test of the discrete-time proportional hazards model. (C) Geometric mean of SHIV-C RNA levels in plasma of the 23 vaccinated and 17 control animals that became infected. (D) Mean percentage of CD4+ T cell changes in blood (after infection in 23 vaccinated and 17 control animals. (E) Serum reactivity to the entire 1086 gp120 and TV1 gp140 at week 26 (two weeks after final immunization) in all 27 immunized animals (Mann-Whitney test). (F) Vaginal IgG-specific activity to the entire 1086 gp120 and TV1 gp140 in 18 of 27 vaccinated animals at week 26 (measured as MFI x dilution total IgG [μg/ml]). (G) Vaginal IgG-specific activity to 1086 and TV1 V1/V2 scaffolds in 21 of 27 vaccinated animals at week 26. (H) Mean plasma IgG specific activity to the 18 overlapping peptides encompassing 1086 gp120 V2 in all 27 vaccinated animals at week 26. Two animals did not exhibit V2 reactivity (Table 2 and S1B Fig). (I) Mean of vaginal IgG specific activity to the 18 overlapping peptides encompassing 1086 gp120 V2 at week 26 in 13 immunized macaques. Three of the 13 animals analyzed did not demonstrate V2 reactivity (Table 2 and S1B Fig). (J) Vaginal IgG and IgA response units to 1086 cV2 in all 27 immunized macaques. (K) Vaginal IgG and IgA response units to SIVAlum cV2 in the 10 females (out of 27 total rhesus macaques) immunized in the SIVAlumAb study (open black circles). (L) Rate of Tier2 SHIV-C acquisition in the 27 animals vaccinated with AlumN10.8. Animals with above-average (black, 14 animals) and below-average (pink, 13 animals) IgA titers to 1086 cV2 in vaginal mucosa.

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Table 1. Adjuvant and gp120 envelope protein dosage and correlates of risk of SHIV-C acquisition.

| Study            | Alum salt/dose (mg) | Total protein boosts | Total Alum salt (mg/animal) | gp120 dose (total mg) | Alum salt/gp120 (ratio) | Vaccine Efficacy (%) / Risk of acquisition |
|------------------|---------------------|----------------------|----------------------------|-----------------------|------------------------|------------------------------------------|
| AlumN10.8 Tier 2 (SHIV-C) | 2.16                | 4                    | 8.6                        | 4 x 0.2 = 0.8         | 10.8                   | None/ IgG to V2 Decreased risk           |
| MF59 Tier 1 (SHIV-C) | N/A                 | 4                    | N/A                        | 4 x 0.2 = 0.8         | N/A                    | (64%)/ Neutralizing Ab titers Decreased risk |
| AlumAb12.5 Tier 2 (SIVmac251) | 5                   | 2                    | 10                         | 2 x 0.4 = 0.8         | 12.5                   | (44%)/ IgG to V2 decreased risk          |
| MF59 Tier 2 (SIVmac251) | N/A                 | 2                    | N/A                        | 2 x 0.4 = 0.8         | N/A                    | None/ IgG to V2 Decreased risk           |

Amount of adjuvant and gp120 protein used in the current SHIV-C and previously published SIVmac251 macaque studies [18].

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Data on all animals are presented in S3A Fig). Interestingly, the increase in the alum dose in this vaccinated group did result in an inversion of the proportion of cyclic V2 1086 specific IgG and IgA that we had observed in the Alum N1.9 study (compare Figs 2J to 1J). The level of these two V2 specific vaginal responses did not correlate with the risk of SHIV-C acquisition.

Even though this vaccination regimen did not significantly decrease the risk of Tier 1 SHIV-C acquisition compared to controls, correlate of risk analysis demonstrated that serum antibody levels to V2 strongly correlated with reduced risk of SHIV-C acquisition (R = 0.9, \( P = 0.03 \); Fig 2K). This regimen did induce neutralizing antibodies to Tier 1 HIVs and SHIV-1157ipEL-p, but not to Tier 2 HIVs or SHIV-1157ipd3N4 (Table 4). However, the neutralization titers did not correlate significantly with the time of in the 8 vaccinated animals exposed to Tier 1 SHIV-C challenge.

MF59 reduced risk of Tier 1 SHIV-C acquisition correlates with homologous neutralization

The final group in this work was adjuvanted with MF59 (Study 3). This study was performed simultaneously with Study 2 in the same animal facility. Ten Chinese rhesus macaques were immunized at weeks 0, 4, 24, 49, and 111 with ALVAC-SIV gag-pol (vCP172) and ALVAC-HIV gag-pro-env (vCP2438), and at weeks 12, 24, 49 and 111 with 100 \( \mu \)g each of clade C TV1 and 1086 proteins formulated with MF59 as summarized in Fig 3A and Table 1. Like those of the Alum N10.8 group, the animals in Study 3 were exposed to 12 doses of Tier 1 SHIV-1157ipEL-p by the intravaginal route. Both groups shared the same 8 naïve controls. The MF59 vaccine regimen significantly decreased the risk of Tier 1 SHIV-C acquisition compared to the controls (\( P = 0.018 \), log-rank test), showing an estimated vaccine efficacy of 64% at each challenge (Fig 3B). This is a notable contrast from the SIV-MF59 regimen that did not decrease the acquisition risk of the neutralization-resistant Tier 2 SIVmac251 [18]. In the current study, we observed a transient decrease of plasma virus (week 2) in immunized animals compared to controls (\( P = 0.0022 \), Wilcoxon rank-sum test; Fig 3C). No significant decrease in CD4+ T cells was observed between MF59 immunized animals and controls when compared to baseline levels (Fig 3D).

No discernable difference in serum antibody titers to 1086 or TV1 was observed between the MF59 and Alum N10.8 groups (Fig 3E and 3F). HIV-specific IgG levels in vaginal secretions were similar to those observed in Studies 1 and 2 for both the 1086 and TV1 gp120s (Fig 3G and 3H) and their corresponding gp70 V1/V2 scaffolds (Fig 3I and 3J). While V2-specific IgG was observed in the sera of all animals (Fig 3K), they were not detected in vaginal secretions (Fig 3L; Table 2; S3B Fig). The contrast between serum and mucosal responses to V2 was also observed in SIV-MF59 and SIV-Alum (see Fig 3C and 3D in ref [18]). No difference was observed between cyclic V2 1086 specific IgG and IgA in vaginal secretions obtained two

| Study   | Alum N1.9 | Alum N10.8 | MF59 |
|---------|-----------|------------|------|
| Plasma  | 92.5% (25/27) | 100% (9/9) | 100% (9/9) |
| Vaginal secretion | 78% (10/13) | 33% (1/9) | 0% (0/9) |

Frequency of animals with detectable 1086 peptide 53 (TELKKHKVAHALFY) specific IgG in plasma or vaginal secretion.

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Table 2. Frequency of systemic and mucosal IgG recognition of V2 peptide 53.
weeks before challenge (week 113; Fig 3M), and these responses did not correlate with the risk of SHIV-C acquisition.

Neutralizing antibody titers to the Tier 1 HIV and SHIV-1157ipEL-p challenge viruses or the challenge stock in macaque PBMCs and TZM cells were generally higher in animals immunized with MF59 than AlumN1.9 (Tables 4 and 5) as expected [23–25]. The MF59 regimen did not induce neutralizing antibodies to Tier 2 HIVs (Table 5).

Interestingly, correlate of risk analysis demonstrated that neutralizing antibodies to Tier 1 SHIV-1157ipEL-p measured in macaque PBMCs or TZM-bl cells at week 14 (Tables 6 and 7) correlated with a decreased risk of Tier 1 SHIV-C acquisition (R = 0.5, P = 0.06 and R = 0.5, P = 0.03, respectively; Fig 3N and 3O).

Table 3. Serum neutralizing activity in animals immunized in Study 1 (AlumN1.9).

| AlumN1.9 | NEUTRALIZATION—ID50 in TZM-bl Cells | Challenge Virus |
|-----------------|----------------------------------|-----------------|
|                 | Pseudoviruses produced in 293T cells |                 |
| MW965.26 Tier 1A | MN3 Tier 1A                  | TV1.21 Tier 1B  |
| Clade C          | Clade B                       | Clade C         |
|                  | Ce1086 B2 Tier 2 Clade C      | 96ZM651 Tier 2  |
|                  |                                 | SHIV-1157ipEL-p |
|                  |                                 | Tier 1          |
|                  |                                 | SHIV-1157ipd3N4 |
|                  |                                 | Tier 2          |
| Animal ID        | week 26                        | week 26         |
|                  | week 26                        | week 26         |
|                  | week 26                        | week 26         |
|                  | week 26                        | week 26         |
|                  | week 28                        | week 28         |
|                  | week 28                        | week 28         |
| MEC              | 1198                           | 57              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 26                             | 21              |
| MGM              | 1512                           | 83              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 22                             | < 20            |
| MGT              | 1849                           | 41              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 26                             | < 20            |
| MHF              | 11061                          | 83              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 55                             | < 20            |
| MHZ              | 3945                           | 40              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 37                             | < 20            |
| MKF              | 1564                           | 104             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 21                             | < 20            |
| MJZ              | 2177                           | 229             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 29                             | < 20            |
| MIV              | 1390                           | 58              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 25                             | < 20            |
| MIR              | 1227                           | 121             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 21                             | < 20            |
| MIH              | 2164                           | 52              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 23                             | < 20            |
| KNH              | 2265                           | 191             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 31                             | < 20            |
| K19              | 1372                           | 145             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 33                             | < 20            |
| DL5A             | 1767                           | 68              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 28                             | 25              |
| CW9M             | 977                            | 105             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 21                             | < 20            |
| DN6W             | 488                            | 33              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 25                             | 20              |
| MCX              | 2525                           | 207             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 31                             | < 20            |
| MBD              | 2160                           | 183             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 22                             | < 20            |
| MDN              | 2679                           | 354             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | –                              | –               |
| DM7R             | 717                            | 92              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 24                             | < 20            |
| K19              | 1959                           | 933             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 25                             | < 20            |
| MKK              | 321                            | 55              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 20                             | < 20            |
| MR4              | 779                            | 137             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 30                             | < 20            |
| MT3              | 641                            | < 20            |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 21                             | < 20            |
| MZO              | 1081                           | 52              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 22                             | < 20            |
| PME              | 1168                           | 79              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
| PZZX             | 2229                           | 37              |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 23                             | < 20            |
| PZC              | 1383                           | 475             |
|                  | < 20                           | < 20            |
|                  | < 20                           | < 20            |
|                  | 21                             | < 20            |

Serum neutralizing antibody activity in AlumN1.9.

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Fig 2. Relative efficacy and immunogenicity of the Alum\textsuperscript{N10.8} regimen. Unless stated otherwise, all assays, experiments and graphs associated with the Alum\textsuperscript{N10.8} study are represented by full green circles, and the number of available samples (i.e., those not depleted in other experiments) are shown as stated in the legend. (A) Animals were immunized with ALVAC-vCP2438 and ALVAC-cVCP2438 and boosted 4 times with 100\textmu g each of the monomeric TV1 and 1086 gp120 proteins (800\textmu g total). Each dose was formulated with 2.16 mg of Aluminum Hydroxide (Alum\textsuperscript{N10.8}; Table 1). Three of four boosts were administered together with the ALVAC vectors (Alum\textsuperscript{N10.8}; n = 10). Only 8 out of 10 vaccinated animals survived up to the Tier 1 SHIV-C challenge. Controls (n = 8; were left naive. (B) The rate per exposure of Tier 1 SHIV-C (SHIV-1157ipEL-p) acquisition was compared between 8 vaccinated animals and 8 controls using the log-rank test of the discrete-time proportional hazards model. (C) Geometric mean of SHIV-C RNA levels in plasma of 8 vaccinated and 8 control animals that became infected. (D) Mean percentage of CD4\textsuperscript{+} T cell changes in blood (after infection in 8 vaccinated and 8 control animals. (E) Serum reactivity to 1086 gp120 and TV1 gp140 in 8 vaccinated animals at week 113 (two weeks after last immunization). (F) Vaginal IgG-specific activity to 1086 gp120 and TV1 gp140 in 8 vaccinated animals at week 50. (G) Vaginal IgG-specific activity to 1086 and TV1 V1/V2 scaffolds in 8 vaccinated animals at week 50. Mean IgG-specific activity to the 18 overlapping linear peptides encompassing the 1086 V2 measured in the (H) plasma or (I) vaginal secretions of 9 vaccinated animals at week 50 (see S3A Fig for data from each animal).
MF59 inflammatory profile impacts neutralizing Abs

The different V2 mucosal and systemic responses observed in Alum N10.8 and MF59 raised the hypothesis that the two adjuvants may affect the homing of antibody-secreting cells (ASC). We therefore investigated the expression of gut homing marker α4β7 and CXCR3+ on the surface of ASCs as previously described [18]. We found that neither the α4β7+ nor CXCR3+ ASC subsets in the two groups differed significantly after immunization, but ASCs expressing CXCR3+ were significantly increased in the MF59 group post-vaccination versus the baseline (Fig 4A).

In the MF59 group, the frequency of vaccine-induced CXCR3+ ASCs positively correlated with the serum level of antibodies to the overlapping peptides of 1086 V2 (R = 0.6, P = 0.002; Fig 4B).

These data suggest that MF59 might have created an inflammatory milieu that favored ASC homing to inflammatory sites, rather than to mucosal sites. Since the natural ligand of CXCR3 is the CXCL10 chemokine induced by IFN-γ [26] and CRP levels increase during inflammation, we measured both in the blood of the immunized animals. Their levels did not differ between animals immunized with MF59 and Alum N10.8 (S4A and S4B Fig), but the levels of CRP and CXCL10 were directly correlated with each other (R = 0.78, P = 0.017; Fig 4C). Interestingly, the level of CRP correlated with the neutralizing antibody titers to Tier 1 SHIV-C elicited by the MF59 vaccine regimen (R = 0.6, P = 0.01; Fig 4D).

IL-1β production in vitro dependent on dosage of Alum

Because the pharmacokinetics of drugs differ according to body mass [27], we adhered to allometric scaling to determine an Alum dosage in SIV-Alum Alh12.5 that would deliver comparable effects to those of Alum Alhydrogel elicited in larger animals (12.5:1 Alum:gp120; 6.6-fold the,

Table 4. Serum neutralizing activity in animals immunized in Study 2 (AlumN10.8).

| Animal ID | Neutralization in TZM-bl cells, Pseudoviruses produced in 293T cells | Challenge Virus |
|-----------|--------------------------------|-----------------|
|           | MW965.26 Tier 1A Clade C* | MW965.26 Tier 1A Clade C* | MN.3 Tier 1A Clade B* | TV1.21 Tier 1B Clade C | Ce1086_2 B2 Tier 2 Clade C | 96ZM651 Tier 2 Clade C | SHIV-1157ipEL-p Tier 1 | SHIV-1157ipEL-p Tier 1 | SHIV-1157ipd3N4 Tier 2 | SHIV-1157ipd3N4 Tier 2 |
| week 50   | week 50 | week 50 | week 50 | week 50 | week 50 | week 50 | week 50 | week 50 | week 50 | week 115 |
| 416       | 3306   | 864    | 24.5   | 10     | 10     | 10     | 40     | 46     | 10     | 10     |
| 4600      | 3677   | –      | 583.5  | 10     | 10     | 10     | 30     | –      | 10     | –      |
| 4624      | 1327   | 639    | 31     | 10     | 10     | 24     | 20     | 31     | 10     | 10     |
| 4997      | –      | –      | –      | –      | –      | –      | –      | –      | –      | –      |
| 5256      | 3787   | 501    | 476    | 10     | 10     | 10     | 22     | 32     | 10     | 10     |
| 20012     | 559.5  | 152    | 36     | 10     | 10     | 10     | 20     | 10     | 10     | 10     |
| 20240     | 1897   | 174    | 30     | 10     | 10     | 10     | 20     | 22     | 10     | 10     |
| 20247     | 463    | 377    | 145.5  | 10     | 10     | 10     | 20     | 20     | 10     | 10     |
| 00R2240   | 2986   | 627    | 248    | 10     | 10     | 10     | 20     | 21     | 10     | 10     |
| 98R0724   | 9272   | 3985   | 1414   | 10     | 10     | 10     | 42     | 85     | 10     | 10     |

Serum neutralizing antibody activity in AlumN10.8.

* Average of two independent reports

https://doi.org/10.1371/journal.ppat.1008121.t004
Fig 3. Relative efficacy and immunogenicity of the MF59 regimen. Unless stated otherwise, all assays, experiments and graphs associated with the MF59 study are represented by full blue circles, those of the AlumN10.8 study are represented by full green circles, and only available samples (i.e., those not depleted in other experiments) are shown. The number of samples represented in each graph is stated below. (A) Animals were...
immunized using ALVAC-vCP172 and ALVAC-cVCP2438 and boosted 4 times with 100 μg each of the monomeric TV1 and 1086 gp120 proteins (800 μg total). Each dose was formulated with MF59. Only 8 out of the 10 vaccinated animals survived to the time of challenge (simultaneous with the challenge of the Alum\(^{N10.8}\) group and naïve controls). (B) The rate per exposure of Tier 1 SHIV-C (SHIV-1157ipEL-p) acquisition was compared in 8 vaccinated animals and 8 controls using the log-rank test of the discrete-time proportional hazards model. (C) Geometric mean of SHIV-C RNA levels in plasma of 7 vaccinated and 8 control animals that became infected. (D) Mean percentage of CD4\(^{+}\) T cell changes in blood (after infection in 7 vaccinated and 8 control animals). Serum binding antibodies to (E) gp120 1086 and (F) gp120 TV1 in 8 of 10 animals each in the Alum\(^{N10.8}\) and MF59 groups two weeks before intravaginal Tier 1 SHIV challenges. Vaginal IgG-specific activity to (G) gp120 1086 and (H) gp140 TV1 in 8 of 10 animals in both Alum\(^{N10.8}\) and MF59 at week 50. Vaginal IgG-specific activity to the V1/V2 (I) 1086 or (J) TV1 scaffold in 8 of 10 animals in both Alum\(^{N10.8}\) and MF59 at week 50. (K) Plasma or (L) vaginal IgG reactivity (mean value) to the 18 overlapping linear peptides encompassing the 1086 V2 in nine MF59-vaccinated animals at week 50. All 9 vaccinated animals that survived up to week 50 had reactivity to V2 peptides in plasma, but not in the vaginal secretions (see S3B for data on all animals). (M) Cyclic V2 1086 specific IgG and IgA levels in vaginal secretions collected from immunized macaques two weeks before challenge exposure (week 113). (N, O) Spearman correlations between levels of serum antibodies (week 14) from the 18 immunized animals in both groups capable of neutralizing the Tier 1 SHIV-C (SHIV-1157ipEL-p) challenge stock in rhesus (N) PBMCs or (O) TZM-bl cells and the week of intravaginal SHIV-1157ipEL-p acquisition.

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amount of Alum used in Alum\(^{N1.9}\); Table 1). We hypothesized that the lower dosage of the proprietary Alum\(^{N}\) used in the current study may have affected cytokine responses induced by vaccination. IL-1\(\beta\) in particular is induced by ALVAC via AIMS activation [28]. This cytokine provides the signals that direct innate monocyte memory responses, which have in turn been shown to correlate with the decreased risk of SIV\(_{mac251}\) acquisition afforded by this vaccine modality [17, 29].

Alum directly infects monocytes and activates the inflammasome, induces IL-1\(\beta\) [30], and harnesses innate monocyte responses [31, 32]. We studied the effects of these adjuvants on human and macaque PBMCs and found that in vitro stimulation with Alum\(^{N}\) induced significantly higher amounts of IL-1\(\beta\) (\(P < 0.0001\)), IFN-\(\gamma\) (\(P = 0.0009\)), IL-10 (\(P = 0.0149\)), and CCL3 (\(P = 0.0002\)) compared to MF59 (Fig 4E–4I), while both adjuvants induced similar levels of IL-13 (S4F and S4J Fig). Notably, Alum\(^{Alh}\) also induced significantly higher amounts of IL-1\(\beta\) (\(P < 0.0001\)), IFN-\(\gamma\) (\(P = 0.0004\)), IL-13 (\(P = 0.013\)) and CCL3 (\(P = 0.0002\)) compared to MF59 (S4D–S4G Fig). No significant differences were observed when comparing the effects of equivalent amounts of Alum\(^{Alh}\) and Alum\(^{N}\) (S4H–S4K Fig). Data parsed for human or

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**Table 5. Serum neutralizing activity against HIV strains in animals immunized in the MF59 study.**

| MF59 | Neutralization in TZM-bl cells, Pseudoviruses produced in 293T cells |
|------|---------------------------------------------------------------------|
| MW965.26 | Tier 1A Clade C | MN.3 | Tier 1A Clade B  | TV1.21 | Tier 1B Clade C | Ce1086_B2 | Tier 2 Clade C | 96ZM651 | Tier 2 Clade C |
| Animal ID | week 50 | week 115 | week 50 | week 50 | week 50 | week 50 | week 50 | week 50 |
| 2316 | 1226 | 1581 | 68.5 | 10 | 10 | 10 | 10 |
| 4640 | 9004.5 | 2266 | 2590.5 | 10 | 10 | 10 | 10 |
| 5247 | 7163 | 1034 | 255.5 | 33 | 10 | 10 |
| 20024 | 4091.5 | – | 78 | 32 | 10 | 21 |
| 20052 | – | – | – | – | – | – |
| 20094 | 764.5 | 257 | 33 | 20 | 10 | 10 |
| 20100 | 676.5 | 554 | 16 | 10 | 10 |
| 00R0960 | 5830.5 | 1462 | 413.5 | 30 | 10 | 10 |
| 00R1348 | 3471 | 863 | 367 | 10 | 10 |
| 01R0728 | 4055.5 | 1694 | 10 | 10 |

Serum neutralizing antibody activity in Alum\(^{N10.8}\).

*Average of two independent reports

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macaque PBMCs demonstrated that human PBMCs are generally more responsive to Alum (S5 Fig).

In order to evaluate possible differences in antibody Fc-associated functions related to the macaque species or the quantity or quality of the adjuvant, we characterized the Fc profile of TV1 envelope-specific antibodies in the sera of the immunized animals at week 26 or 51. We previously found that the percentage of Fc with G0F, G1F, G2F, and G2S1F sugars differed significantly between Indian rhesus macaques immunized with SIV-Alum and those with SIV-MF59 (Fig 5A) [17]. In Chinese rhesus macaques, we also found significant differences in G0F, G1F, G2F, and G2S1F between the Alum N1.9 and Alum N10.8 groups (Fig 5C–5E; S6 Fig).

### Table 6. Serum neutralizing activity against SHIV-C challenge stocks in animals immunized in Study 2 (Alum N10.8).

| Animal ID | Challenge Virus | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells |
|-----------|----------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|
| 416       | SHIV-1157ipEL-p| 86      | 113          | 40      | 10           | 46      | 46           | 10      |              |         |              |
| 4600      | SHIV-1157ipEL-p| -       | -            | 30      | 10           | -       | -            | -       |              |         |              |
| 4624      | SHIV-1157ipEL-p| 20      | 20           | 20      | 10           | 31      | 31           | 10      |              |         |              |
| 4997      | SHIV-1157ipEL-p| -       | -            | -       | -            | -       | -            | -       |              |         |              |
| 5256      | SHIV-1157ipEL-p| 21      | 20           | 22      | 10           | 32      | 32           | 10      |              |         |              |
| 20012     | SHIV-1157ipEL-p| 20      | 20           | 20      | 10           | 10      | 10           | 10      |              |         |              |
| 20240     | SHIV-1157ipEL-p| 21      | 20           | 20      | 10           | 10      | 10           | 10      |              |         |              |
| 20247     | SHIV-1157ipEL-p| 20      | 20           | 20      | 10           | 20      | 20           | 10      |              |         |              |
| 00R2240   | SHIV-1157ipEL-p| 23      | 28           | 20      | 10           | 21      | 21           | 10      |              |         |              |
| 98R0724   | SHIV-1157ipEL-p| 20      | 20           | 42      | 10           | 85      | 85           | 10      |              |         |              |

Serum neutralizing antibody activity against the Tier 1 and Tier 2 challenge stocks in Alum N10.8.

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In order to evaluate possible differences in antibody Fc-associated functions related to the macaque species or the quantity or quality of the adjuvant, we characterized the Fc profile of TV1 envelope-specific antibodies in the sera of the immunized animals at week 26 or 51. We previously found that the percentage of Fc with G0F, G1F, G2F, and G2S1F sugars differed significantly between Indian rhesus macaques immunized with SIV-Alum Abh and those with SIV-MF59 (Fig 5A) [17]. In Chinese rhesus macaques, we also found significant differences in G0F, G1F, G2F, and G2S1F between the Alum N1.9 and Alum N10.8 groups (Fig 5C–5E; S6 Fig).

### Table 7. Serum neutralizing activity against SHIV-C challenge stocks in animals immunized in Study 3 (MF59).

| Animal ID | Challenge Virus | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells | RHPBMCs | TZM-bl cells |
|-----------|----------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|
| 2316      | SHIV-1157ipEL-p| 20      | 20           | 21      | 30           | 10      | 30           | 10      |              |         |              |
| 4640      | SHIV-1157ipEL-p| 20      | 20           | 36      | 67           | 10      | 67           | 10      |              |         |              |
| 5247      | SHIV-1157ipEL-p| 35      | 44           | 50      | 24           | 10      | 24           | 10      |              |         |              |
| 20024     | SHIV-1157ipEL-p| -       | -            | 55      | -            | 10      | -            | -       |              |         |              |
| 20052     | SHIV-1157ipEL-p| -       | -            | -       | -            | -       | -            | -       |              |         |              |
| 20094     | SHIV-1157ipEL-p| 41      | 112          | 20      | 33           | 10      | 33           | 10      |              |         |              |
| 20100     | SHIV-1157ipEL-p| 34      | 35           | 21      | 43           | 10      | 43           | 10      |              |         |              |
| 00R0960   | SHIV-1157ipEL-p| 45      | 55           | 39      | 89           | 10      | 89           | 10      |              |         |              |
| 00R1348   | SHIV-1157ipEL-p| 20      | 20           | 24      | 75           | 10      | 75           | 10      |              |         |              |
| 01R0728   | SHIV-1157ipEL-p| 20      | 20           | 26      | 44           | 10      | 20           | 10      |              |         |              |

Serum neutralizing antibody activity against the Tier 1 and Tier 2 challenge stocks in MF59.

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Fig 4. Inflammation parameters in immunized macaques. The vaccine-induced cells were calculated as the delta of weeks 51 and week 0. ASCs were defined as live cells, lineage- (CD3⁻ / CD14⁻ / CD16⁻ / CD56⁺) / (CD19⁺ / CD20⁺ / CD21⁻ / CD27⁻) / Ki67⁺/++. (A) Frequency of blood circulating ASCs expressing CXCR3 on their membrane in AlumN10.8 (green filled circles) and MF59 (blue filled circles) before (week 0) and after immunization (week 51). (B) Spearman correlation between the sum of the serum reactivity to the 18 linear peptides encompassing the 1066 V2 (peptides 43–63) and frequency of vaccine-induced, blood-circulating CXCR3⁺ ASCs in AlumN10.8 and MF59 at week 51. The linear regression and P and R values were obtained using all 9 animals of the AlumN10.8 and MF59 groups. (C) Correlation between the levels of CRP (log transformed values) measured at week 50 and the mean of CXCL10 levels in serum measured in duplicate experiments (5 days apart: week 49 + 2 days, and week 50) in the MF59 group. (D) Correlation between serum neutralizing antibodies to SHIV Tier 1 CS in TZM-bl cells and C-Reactive Protein (log transformed-values) levels in all animals (9 animal each) of both AlumN10.8 and MF59 groups. (E) IL-1β, (F) IFN-γ, (G) IL-10, and (H) CCL3 levels measured in human (empty hexagons), Indian rhesus macaque (dark-top hexagons), and Chinese rhesus macaque (dark-bottom hexagons) PBMCs after stimulation with AlumN7 (78 μg) and AlumN at the same concentration (78 μg) and AlumN at the same proportion (7.8 μg) as used in vivo. Horizontal lines represent the median values in all panels.
As in SIV-MF59, G2F was highest with the MF59 adjuvant used in Study 3. However, none of these gp120-specific antibody glycan Fc forms correlated with SHIV-C acquisition or the level of plasma viral RNA. The Fc functional profile did not correlate with CD4 counts or with anti-envelope binding or neutralizing antibodies, as was also observed in animals immunized with similar vaccine regimens and challenged with SIVmac251 [17].

**Discussion**

The partial success of RV144 in preventing clade B/E HIV-1 infection is an important milestone, but it must be extended to include the clade C strains that account for nearly 50% of all
new HIV-1 infections worldwide [33]. Analysis of RV144 suggests that vaccine efficacy was not durable using Alum-Alhydrogel (60% efficacy 12 months post-vaccination [34]; 31.2% after 42 months [6]). HVTN702 was thus designed to induce higher and more durable immune responses using the oil-in-water MF59 adjuvant. In the SIVmac251 macaque model, the use of different primes, immunogens, or adjuvants influenced the risk of SIVmac251 acquisition [17, 18, 35, 36] and allowed for the identification of novel correlates of decreased risk such as innate monocyte memory, NKp44 cells, and antibodies to V2 [17, 18]. In the same study, we also demonstrated that the Alum and MF59 adjuvants shaped immune responses differently, and even though SIV-MF59 was more immunogenic, it did not decrease the risk of SIVmac251 acquisition like SIV-AlumAbh12.5 did [18]. The present studies were conceived to assess the relative efficacy of the ALVAC-HIV Clade B/C regimen with the clade C envelope gp120 1086 and TV1 proteins as boost against SHIV-C challenge viruses with different neutralization profiles.

There were, however, constraints and limitations in the design of Study 1 related to the manufacturer’s guidelines in formulating the gp120 boosts. The AlumN1.9 regimen tested here mimicked the number and order of immunizations performed in RV144 and our SIV-AlumAlh study, but while the gp120 boosts were maintained at the same dose as used in SIV-AlumAbh and RV144 (200 μg each), a much lower dose of AlumN was used in Study 1 (750 μg/dose). Although the FDA and CDC approve of no more than 850 μg of Alum Alhydrogel per dose in human vaccines [37, 38], we used 5,000 μg/dose in SIV-AlumAlh to compensate for the well-documented pharmacokinetic differences among differently sized species [27]. Counterintuitively, this typically means that a drug must be administered at a higher dose in smaller animals such as macaques to obtain the same effects elicited in larger species. The AlumN1.9 and SIV-AlumAbh12.5 studies were both performed in Indian rhesus macaques, used equivalent vaccine modalities and regimens against SIVmac251, and were statistically powered to obtain a readout of vaccine efficacy (27 vaccinees and 20 controls in AlumN1.9), but despite their many similarities, we found that AlumN1.9 was not efficacious against the Tier 2 SHIV-C. Thus, the data suggest that the low dose of Alum might have contributed to the high level of mucosal V2-specific IgA that correlated with an increased risk of virus acquisition. MF59 was not made available by the manufacturer for testing in a parallel study against the Tier 2 SHIV-C.

Studies 2 and 3 were conducted in parallel to compare the efficacy of AlumN at higher doses (2,160 μg/dose) and MF59 against a Tier 1 SHIV-C challenge in Chinese rhesus macaques. Because SIV-AlumAbh12.5 decreases the risk of SIVmac251 acquisition in Chinese macaques, we can exclude the macaques’ origin as a factor in the inability of AlumN10.8 to decrease the risk of SHIV-C acquisition. The AlumN10.8 and MF59 regimens included an additional immunization with ALVAC-HIV and two additional gp120 protein boosts not performed in Study 1. However, the total amount of protein used over the course of immunization (800 μg) did not differ among these studies because the dose of each gp120 boost was halved (Table 1). Interestingly, tripling the dose of Aluminum hydroxide in AlumN10.8 resulted in the elicitation of binding antibodies to V2 linear peptides that strongly correlated with delayed Tier 1 SHIV-C acquisition (R = 0.9, P = 0.003). Despite this strong immune correlate of risk in the AlumN10.8 study, the overall risk of Tier 1 SHIV-C acquisition did not differ significantly between vaccinated and control animals, likely due to the small number of animals used in this study (n = 8) and the limited efficacy of this vaccine regimen. When contrasted with Study 1, the results of Study 2 suggest that a high alum dose may also be important for the induction of protective IgG to V2 in this animal species.

Several lines of evidence provide support for the importance of the Alum dose. First, we demonstrated that in vitro IL-1β induction is reduced following stimulation of PBMCs with a low dose of AlumN. Alum is a potent inducer of inflammasome activation and IL-1β production, which in turn triggers innate monocyte memory by causing emergency myelopoiesis,
hypoxia, and lasting epigenetic changes [39] in CXCR4+ pre-monocytes. Second, our prior studies with the ALVAC-SIV-based vaccine in the SIVmac251 model demonstrated a strong monocyte signature, confirmed by systems biology, that correlated with a decreased risk of SIVmac251 acquisition [17]. This suggests the hypothesis that the lack of protection in the AlumnN1.9 and AlumnN10.8 arms presented here may be due to an insufficient induction of monocyte innate memory, which is itself a key component of the reduced risk of virus acquisition afforded by the ALVAC/gp120/Alum vaccine modalities.

Another contributing factor appears to be the balance of IgG and IgA responses to V2. Results from recent studies have highlighted the importance of humoral responses to the V2 region of the SIV and HIV-1 envelopes that are correlated with protection [7, 18, 36]. In particular, IgG targeting the V2 region of gp120 are associated with protection [18, 21] when using ALVAC-based vaccine modalities. Data from both clinical and preclinical studies suggest antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by non-neutralizing IgG as another correlate of reduced risk of HIV-1 and SIV acquisition [7, 18, 21]. By contrast, gp120-specific plasma IgA directed to C1 were correlated with increased risk of HIV-1 acquisition in the RV144 trial [21], potentially linked to the inhibition of ADCC-mediating antibody binding to cells infected with HIV-1.

The MF59 and Alum adjuvants induced gp120 specific antibodies that differed in their Fc functional profile in Chinese macaques, as previously observed in Indian macaques [18]. As was the case in the SIVmac251 studies performed with these vaccine modalities, however, no correlation was found between the inferred Fc functions and the risk of SHIV-C acquisition.

The finding that the MF59-based vaccine strategy tested in Study 3 was highly effective in reducing the risk of Tier 1 SHIV-C even with a low number of animals is encouraging. Yet, the relevance of the Tier 1 SHIV-C model to humans is uncertain since more than 99% of circulating HIV-1 strains in humans are neutralization-resistant. However, MF59 induced an inflammatory profile that correlated with the titers of neutralizing antibodies, suggesting that immunogens able to induce cross-clade neutralizing antibodies other than those used here may be potentiated by this adjuvant. There has been increasing interest in testing HIV-1 neutralizing antibodies that might prevent or control viral infection, but only 10–30% of chronically infected individuals develop cross-neutralizing antibodies for different HIV-1 clades. Recently, a unique inflammatory signature in HIV-1 controllers was shown to be associated with the production of broadly neutralizing antibodies [40].

Expanding our understanding of the immune mechanisms that affect the risk of HIV and SIV acquisition is necessary to properly guide the choice of all components of an effective vaccine. Hopefully, the accumulation of results from human trials will soon identify a suitable and predictive animal model to hasten the improvement of HIV vaccine candidates.

Methods

Vaccines and Tier 1 and Tier 2 SHIV-C challenges

In Study 1, 47 juvenile, female Indian rhesus macaques were housed at the NIH and randomized into two arms controlled for MHC-I allele representation, age, and weight. Twenty (n = 20) macaques were administered Alumn and used as controls. Twenty-seven (n = 27) others were immunized at weeks 0, 4, 12, and 24 with intramuscular inoculations of 10⁸ PFU of ALVAC vCP172 [41] and ALVAC vCP2438 [12]. ALVAC vCP172 expresses SIV gag-pol. ALVAC-HIV (vCP2438) is considered equivalent to vCP1521 as used in the RV144 vaccine regimen, but the CRF01_AE gp120 insert (92TH023) was exchanged for a subtype C gp120 insert (96ZM651) to match the virus strain circulating in South Africa that was fused to the clade B LAI env trans-membrane portion. Additionally, vCP2438 expressed the clade B HXB2
Gag and Protease [12]. At weeks 12 and 24, the treated group was given a bivalent monomeric gp120 protein boost in the thigh opposite of the one that received the ALVAC. The boost formulation contained 200 μg each of clade C TV1 and 1086 in Alum hydroxide manufactured by Novartis through a proprietary process (AlumN). Per Novartis’s recommendation, the proteins were prepared over the course of 2 days through the following process. The day before vaccination, the TV1 gp120 and 1086 gp120 proteins were thawed at room temperature (RT). Alum Formulation Buffer (AFB) and AlumN were removed from 4˚C and gently inverted 10 times. Next, 3.5 ml of AlumN were added to the AFB vial. The solution was gently inverted 10 times to ensure a uniform mix. Then, 1.591 ml of thawed TV1 gp120 was added to each vial of AFB + Alum, and 1.129 ml of thawed 1086 gp120 were added to each vial of AFB + AlumN. The solution was gently inverted 10 times to mix immediately after addition, then stirred gently for 5 min. The vial was tightly capped and transferred to a cold room (4˚C) for overnight stirring. The vial was gently shaken on the day of the immunization. The total aluminum contained in each gp120 dose was 0.75 mg, at a ratio of 1.9:1 adjuvant:gp120 (AlumN1.9, see Table 1). 1.0 mL (200 μg of each gp120 dose) of this final mixture was administered to each animal intramuscularly in the opposite thigh of the vector immunization within four hours of opening the vial. In vitro characterization of the formulation (pH, osmolality, endotoxin and SDS PAGE Gels) was performed by Novartis on the amount left over after vaccination to control for protein integrity. The study included 20 simultaneous controls that each received 1 ml of AlumN (Fig 1A). All controls were housed in the same facility and were challenged by the same operators with the same challenge virus stock. Four weeks after the last immunization, all 27 vaccinated animals and 20 controls were challenged vaginally with the Tier 2 SHIV-1157ipd3N4 grown in rhesus macaque PBMCs (TCID50 in TZM-bl cells: 1.56 × 10^5/ml and TCID50 in rhesus PBMC: 1.02 × 10^4/ml) six times at a 1:100 dilution, then eight times at a dilution 1:50, and for three final challenges at a maximum dilution of 1:5. Animals that tested negative for SHIV RNA in plasma were re-challenged and exposed to the virus for a maximum of 17 weekly challenges.

In Studies 2 and 3, 28 juvenile, female Chinese rhesus macaques were hosted at ABL and randomized into three groups: Study 2 (n = 10), Study 3 (n = 10), and a control group (n = 8). The macaques in Studies 2 and 3 were immunized at weeks 0, 4, 24, 49, and 111 intramuscularly with 10^8 PFU of the same ALVAC vCP172 and ALVAC vCP2438 used in Study 1. At weeks 12, 24, 49, and 111, the treated groups were separated and given a bivalent monomeric gp120 protein boost in the opposite thigh of the vector immunization. The protein boost administered in Study 2 contained 100 μg each of clade C TV1 and 1086 in AlumN (Fig 1B), whereas Study 3 received 100 μg each of clade C TV1 and 1086 in MF59 (Fig 1C). Both 1086 AlumN and MF59 were manufactured by a proprietary Novartis process. The proteins adjuvanted with AlumN were formulated as in Study 1, but with a higher level of Alum salt. The total aluminum contained in each gp120 dose was 2.16 mg, with 10.8:1 adjuvant:gp120 (AlumN10.8, see Table 1). Per Novartis’s recommendation, the proteins adjuvanted with MF59 were formulated through the following process: TV1 gp120, 1086 gp120, and Buffer M were thawed at RT. The buffer M vial contained buffer salts to achieve an isotonic formulation with a final concentration of 10 Mm Phosphate buffer (pH 7.4). Once the three vials were thawed, the empty formulation vial and one vial of MF59C.1 (adjuvant) were removed from refrigeration and mixed by repeated gentle swirling and inversion. Using a suitable sterile syringe, 2.380 ml of buffer M were withdrawn and transferred into the formulation vial, followed by 4.320 mL of MF59C.1. The contents were mixed gently. In similar fashion, 1.382 ml of TV 1 gp120 and 0.558 ml of 1086 gp120 were added into the formulation vial and mixed gently. The admixed vaccine was stored at RT until administration, no longer than 4 h after the addition of MF59. Prior to administration, the vial was shaken gently and, using a sterile syringe, 0.5 ml
of the vaccine was drawn and injected into each animal. The syringes were inverted gently three times prior to each administration. All injections were administered intramuscularly.

The control group was added to the study before intravaginal challenges began. All animals were housed in the same facility and challenged by the same operators with the same challenge virus stock. Two animals perished in each group during the immunization phase due to ailments unrelated to the study. The remaining 8 animals in each group were challenged four weeks after the last immunization and 115 weeks after the first ALVAC administration with Tier 1 SHIV-1157ipEL-p. Challenges were administered via the vaginal route 12 times using SHIV-1157ipEL-p with a TCID<sub>50</sub> in TZM-bl cells of 3.2 × 10<sup>5</sup>/ml at a dilution 1:10 (Fig 1B and 1C). Because MF59 is a protein dose-sparing adjuvant, the amount of gp120 in the boost used in Study 3 was halved compared to the one used in Study 1 (Study 1, 200 μg; Study 3, 100 μg).

Animals that tested negative for SHIV RNA in plasma were re-challenged and exposed to the virus for a maximum of 12 weekly challenges. Hormone treatment to synchronize menstrual cycles and thin vaginal epithelium was not used in any group.

Measurement of viral RNA, and CD4 count

Plasma SHIV RNA was quantified by nucleic acid sequence-based amplification (NASBA), as described in previous studies [42]. The normalized value of the proviral load was calculated as copy number/Mac albumin gene copy number × 2 × 10<sup>6</sup>, and expressed as the number of SHIV DNA copies/ml. CD4<sup>+</sup> T cell counts were determined from whole blood by flow cytometry, as previously described [43].

ELISA for TV1 and 1086 in plasma

An enzyme-linked immunosorbent assay (ELISA) was used to detect SHIV gp120 binding antibodies in blood as previously described [44] and to detect binding to overlapping peptides spanning gp120. A serial dilution of plasma was added to microwell plates coated with the Env protein or individual peptides of natively purified gp120 SHIVs, as determined by the antibody titer. Absorbance at OD450 nm was reported for peptide mapping. For binding antibodies to gp120, the endpoint titers were defined as two times the OD450 nm of the negative control serum.

Neutralizing antibodies

Neutralization was measured as a reduction in luciferase reporter gene expression after a single round of infection in TZM-bl or Rhesus cells, as described previously [45, 46]. TZM-bl cells were obtained from J. Kappes and X. Wu of the NIH AIDS Research and Reference Reagent Program. Briefly, a pre-titrated dose of Env-pseudotyped virus or replication-competent challenge stock was incubated with serial 3-fold dilutions of test sample in duplicate in a total volume of 150 μl for 1 h at 37°C in 96-well flat-bottom culture plates. Freshly trypsinized cells (10,000 cells in 100 μl of growth medium containing 20 μg/ml DEAE dextran) were added to each well. One set of control wells received cells + virus (virus control) and another set received cells only (background control). After 48 h incubation, the cells were lysed by the addition of Britelite (PerkinElmer Life Sciences, Waltham, Massachusetts, USA), and three quarters of the cell lysate were transferred to a 96-well black solid plate (Corning Costar, Tewksbury, Massachusetts, USA) for luminescence measurement. Neutralization titers are the serum dilution (ID<sub>50</sub>) at which relative luminescence units (RLU) were reduced by 50% compared to virus control wells after subtraction of background RLUs. The SHIV-1157ipd3N4 and SHIV-1157ipEL-p challenge stocks used in this assay and in the challenges were
respectively obtained from N. Miller (NIH Division of AIDS) and the NIH AIDS Reagent Program. Challenge stocks were expanded on rhesus PBMCs and titered before use.

**Particle enhanced immunoturbidimetric assay for CRP**

Rhesus macaque CRP agglutinates with latex particles coated with monoclonal anti-human CRP antibodies. CRP values for rhesus plasma samples were detected and measured on a Roche/Hitachi cobas c501 analyzer. The precipitate was determined turbidimetrically. The following two reagents were added: Reagent R1 (82 μl of R1 + 42 μl of H2O) containing TRIS buffer with bovine serum albumin and mouse immunoglobulins with preservative and stabilizers; R2 (28 μl of R2 + 20 μl of H2O) containing Latex particles coated with anti-CRP (mouse) in glycine buffer with preservative and stabilizers. K2-EDTA plasma isolated from whole blood was used in this assay. Samples were loaded with a reaction time of 10 s each and read at a wavelength of 546 nm. Results were expressed as a concentration of mg/L, nmol/L, or mg/dL. The reader was calibrated for new reagents with multiple solutions to determine the standard concentrations for the 6-point calibration curve. This method has been standardized against the reference preparation of the IRMM (Institute for Reference Materials and Measurements) BCR470/CRM470 (RPPHS–Reference Preparation for Proteins in Human Serum). LIQUICHEK was used as a quality control serum (IMMUNOLOGY CONTROL, Levels 1 [cat. #591], 2 [cat. #592], & 3 [cat. #593], BIO RAD Laboratories Diagnostics Group, Irvine, California, USA). Measuring range: 0.15–20.0 mg/L (1.43–190 nmol/L, 0.015–2.0 mg/dL)

**Plasmablast staining in macaque blood**

The frequency of plasmablasts (PBs) was measured in the blood of 12 macaques vaccinated with ALVAC-SHIV/gp120 Alum before vaccination and 7 days after the last immunization. Cells were stained with CD3 (Clone SP34-2), CD14 (Clone M5E2), CD16 (Clone 3G8), and CD56 (Clone B159), all conjugated in ALEXAFluor700 (BD Biosciences; San Jose, California, USA), PE-Cy5-CD19 (Clone J3-119, Beckman Coulter; Brea, California, USA), QDOT-650-CD20 (Clone 2H7, eBioscience; San Diego, California, USA), FITC-CD38 (Clone AT-1, StemCell; Massachusetts, USA), BV421-CD39 (Clone MOCP-21, BioLegend; San Diego, California, USA), PE-Ki67 (Clone B56, BD Biosciences), and anti-CXCR3 (CXCR3/CD183 PE-CF594 conjugated). Clone 1C6BD (Biosciences cat. #11718; anti-α4β7) was kindly provided by Dr. Ansari through the NIH AIDS Reagent Program, Division of AIDS, NIAID. Cells were permeabilized with Cytofix/Cytoperm (BD Biosciences). Acquisition was performed on an LSRII cytometer (BD Biosciences) and data were analyzed by FlowJo software (TreeStar; Ashland, Oregon, USA). Plasmablasts were gated as previously described: lineage-(CD3- / CD14- / CD16- / CD56+ ) / (CD19+/CD20+ / CD21+CD27+) / Ki67++ / (CD38+++/CD39) [18]. The frequency of PBs expressing CXCR3 or α4β7 was then calculated.

**Plasma and mucosal antibody binding and specific activity**

Binding and linear epitope mapping of vaccine-induced IgG and IgA in plasma and/or mucosal samples to HIV-1 Envs was measured by binding antibody multiplex assay (BAMA) and peptide microarray. HIV-1 V1/V2-specific BAMA assay was performed as previously described [21] using gp70-V1/V2 scaffolds. Mucosal samples were filtered and concentrated. For vaccine-induced IgA response evaluation, IgG was depleted from plasma or mucosal samples. Total IgG and IgA concentrations in the prepared mucosal samples were determined using a custom total rhesus IgG and IgA ELISA assay. Specific activity in mucosal BAMA was defined as MFI × dilution/total IgG concentration (μg/ml). Linear epitope mapping microarray was performed as previously described [47, 48]. Intensity of binding to each peptide was
baseline subtracted (mean of baseline binding + 3 × s.d. of triplicate tests, or at least 200), and then normalized by total IgG concentration per sample to obtain Specific Activity. Specific Activity was calculated as Baseline subtracted intensity × dilution/total IgG concentration (μg/ml).

Reagents and surface plasmon resonance for cyclic V2
To deactivate the complements and remove lipid contents, sera or mucosal secretions were heated at 56˚C for 45 min, followed by centrifugation at 16,000 × g at 4˚C for 20 min. The supernatants were collected and stored at -80˚C for Biacore assay. The immunogenicity assessment for cyclic V2 peptides was conducted using the Biacore 4000 system [17, 49, 50]. The immobilizations were completed in 10 mM HEPES and 150 mM NaCl (pH 7.4) using a standard amine coupling kit. The CM7-S series chip surface was activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) for 600 s. Following this, 1 μM Streptavidin was immobilized in 10 mM sodium acetate (pH 4.5) for 720 s (11,400–12,300 RU). The immobilized surface was then deactivated by 1.0 M ethanolamine-HCl (pH 8.5) for 600 s. Spot 3 in each flow cell was left unmodified to serve as a reference. After the surface deactivation, 2.5 μM cyclic biotinylated peptide of AE.92TH023.V2 (cbV2_AE; 4,700–4,800 RU), B.MN.V2 (cbV2.MN; 4,000 RU), B.caseA2.V2 (cbV2.BcaseA2; 5,200–5,400 RU), or C.1086.V2 (cbV2.C; 4,900 RU) were captured. Following surface preparation, the heat-inactivated plasma/secretions were diluted (1:40) in 10 mM HEPES and 150 mM NaCl (pH 7.4). The diluted plasma was dispensed onto the captured surface for 300 s, then allowed a 30 s dissociation period. The bound antibodies were then enhanced by the injection of a secondary goat anti-human IgG antibody (30 μg/ml) for 360 s. To regenerate the bound surface, 175 mM HCl was injected for 60 s. Each sample was tested in quadruplicate for each peptide at a rate of 10 Hz at an analysis temperature of 25˚C. All sample injections were conducted at a flow rate of 10 μl/min. Data analysis was performed using Biacore 4000 Evaluation Software v. 4.1 with double subtractions for unmodified surfaces and the buffer for blank.

Cell culture, adjuvant stimulation, and Luminex
PBMCs obtained from human donors and Indian and Chinese rhesus macaques were isolated from Leukopacks by Ficoll-Histopaque density. PBMCs were washed, resuspended in complete medium (RPMI 1640 medium containing 10% FBS and 1% Anti-anti), and used immediately. Cells were prepared for cell culture in 24-well plates at a concentration of 5 × 10⁶ PBMCs/well. Briefly, 5 × 10⁶ PBMCs were suspended in each well at different conditions in a final volume of 500 μl. PBMCs cultured in medium alone were used as negative controls, and PBMCs stimulated with LPS (1.2 ng/ml) for 24 h in a humidified atmosphere of 5% CO₂ at 37˚C. Conditions were chosen based on previously published data [51]. PBMCs were harvested and centrifuged at 2000 rpm for 6 min at 4˚C. Supernatant was collected, aliquoted, and frozen at -80˚C for Luminex analysis. Cryopreserved supernatants were analyzed using three MILLIPLEX Non-Human Primate Multiplex assays (EMD Millipore Corporation, Billerica, MD). The following targets were assayed following manufacturer instructions: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, IFN-γ, MCP-1, MIP-1a (cat. #PRCYTOMAG-
40K-11). After thawing the samples on ice, 25 μl of each supernatant was loaded into the well and briefly mixed with 25 μl of assay buffer and 25 μl of magnetic beads. The plates were incubated under agitation at 4˚C for 18 hours. After washing, 25 μl of detection antibody was added to each well and incubated for 1 h RT. Next, 25 μl of Streptavidin-PE was added to each well and incubated for 30 min RT. Finally, wells were washed and resuspended in 150 μl of blank fluid according to the manufacturer’s instructions. Samples were acquired on a Bio-Plex 200 System (Bio-Rad; Hercules, California, USA).

Ethics statement
All animals used in this study were colony-bred female rhesus macaques (Macaca mulatta) obtained from Covance Research Products (Alice, Texas, USA). Macaques used in Study 1 were of Indian origin and animals used in Studies 2 and 3 were of Chinese origin.

Animals in Study 1 were housed at the National Institutes of Health (Protocol VB008) and those of Studies 2 and 3 were housed at the BIOQUAL nonhuman primate facility (Protocol P169). Animals were cared for in accordance with American Association for the Accreditation of Laboratory Animal Care (AAALAC) standards in an AAALAC-accredited facility (Animal Welfare Assurance A4149-01). All animal care and procedures were carried out under protocols approved by the NCI or NIAID Animal Care and Use Committees (ACUC; Protocol numbers: P169 and VB008). Animals were closely monitored daily for any signs of illness, and appropriate medical care was provided as needed. Animals were housed individually during the challenge phase to reduce the risk of transmission of SIV or other viruses. All clinical procedures, including biopsy collection, administration of anesthesia and analgesics, and euthanasia, were carried out under the direction of a laboratory animal veterinarian. Steps were taken to ensure the welfare of the animals and minimize discomfort of all animals used in this study. Animals were fed daily with a fresh diet of primate biscuits, fruit, peanuts, and other food items to maintain body weight or normal growth. Animals were monitored for mental health and provided with physical enrichment including sanitized toys, destructible environments (cardboard and other paper products), and audio stimulation. Leukopacks from humans were obtained from healthy donors from the NIH Blood Bank.

Supporting information

S1 Fig. Specific IgG activity to overlapping peptides that encompass the 1086 V1/V2 region in AlumN1.9. (A) Amino acid sequence (single letter aa code) of 18 overlapping peptides (15-mers) encompassing the V2 of 1086 (V2, sequence underlined). (B, D) V2 specific IgG in plasma (13 animals) paired with those in vaginal secretions (C and E) in 13 of the 27 immunized animals at week 26. Plasma and vaginal IgG-Specific activity was measured as MFI × dilution.total IgG (μg/ml).

S2 Fig. V2-specific IgG in the remaining 14 vaccinated animals in AlumN1.9. V2 specific IgG in plasma of the remaining 14 of the 27 immunized animals for which data on vaginal secretion were not available (week 26). Plasma IgG-Specific activity was measured as MFI × dilution.total IgG (μg/ml). Arrows indicate low, but specific IgG activity.

S3 Fig. Specific IgG activity to overlapping peptides encompassing the V2 region of 1086 in AlumN10.8 and MF59. (A) plasma and vaginal IgG in 9 out of 10 immunized animals in AlumN10.8 at week 50. (B) Specific plasma and vaginal IgG in 9 out of 10 immunized animals in MF59 at week 50. All plasma and vaginal IgG-specific activity was measured as
MFI × dilution.total IgG (μg/ml). Arrows indicate low, but specific IgG activity.

S4 Fig. Inflammation markers and cytokine production in human and macaque cells. (A) Plasma levels of C-Reactive Protein (mg/dl) measured in 9 out of 10 vaccinated animals in both Alum\textsuperscript{N10.8} and MF59 groups at week 50. (B) Plasma levels of CXCL10 (pg/ml) measured in 9 out of 10 vaccinated animals in Alum\textsuperscript{N10.8} and MF59 at week 50. (C) IL-1β, (D) IFN-γ, (E) IL-10, (F) IL-13, and (G) CCL3 production measured in human (empty hexagons), Indian rhesus macaque (dark-top hexagons), and Chinese rhesus macaque (dark-bottom hexagons) PBMCs following in vitro stimulation with Alum\textsuperscript{Alh}, MF59, and LPS. (H) IL-10, (I) IFN-γ, (J) IL-13, and (K) CCL3 levels measured in human (empty circles), Indian rhesus macaque (dark-top hexagons), and Chinese rhesus macaque (dark-bottom hexagons) PBMCs following stimulation with Alum\textsuperscript{Alh} (78 μg) and Alum\textsuperscript{N} at the same concentration (78 μg) or at 7.8 μg. Horizontal lines represent the median values in all panels.

S5 Fig. Human and macaque PBMC cytokine level distribution and production after stimulation with different adjuvants. The violin plots show the distribution of the levels of (A) IL-1β, (B) IFN-γ, (C) IL-10, (D) IL-13, and (E) CCL3 in human (dark grey) and rhesus macaque (light grey) PBMCs. Different stimuli used in culture with PBMCs to induce cytokine productions are shown on the x axis. The thick bar in each plot represents the interquartile range, and the thin line extending from it represents the 95% confidence intervals. The median value is denoted by a dot.

S6 Fig. Specific anti-TV1 antibody glycoforms in sera of Alum\textsuperscript{N1.9}, Alum\textsuperscript{N10.8}, and MF59 studies. Unless stated otherwise, data associated with the Alum\textsuperscript{N1.9} study are represented by full red circles, data from the Alum\textsuperscript{N10.8} study are represented by full green circles, data from the MF59 study are represented by full blue circles, and only available samples (i.e., those not depleted in other experiments) are shown. The number of samples represented in each graph is stated in the figure legend. Serum was tested for the following anti-TV1 antibody glycoforms in vaccinated animals (Alum\textsuperscript{N1.9}, n = 27; Alum\textsuperscript{N10.8}, n = 9; MF59, n = 9): (A) G0F, (B) G1F, (C) G2F, (D) G2S1F/G0, (E) G2S2F_B, and (F) G2_FB. Statistical analysis was performed using the Mann-Whitney test and corrected by multicomparison analysis via Tukey’s range test. Different sugar group patterns mediated or inhibited different antibody functions as described by Ackerman et al. [52].

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References

1. IAVI. Clinical Trial Database: International AIDS Vaccine Initiative; 2019. Available from: https://www.iavi.org/trials-database.

2. Buchbinder SP, Mehrutra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet. 2008; 372(9653):1881–93. https://doi.org/10.1016/S0140-6736(08)61591-3 PMID: 19012954; PubMed Central PMCID: PMC2721012.
3. Gray GE, Allen M, Moodie Z, Churchyard G, Bekker LG, Nchabeleng M, et al. Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. Lancet Infect Dis. 2011; 11(7):507–15. Epub 2011/05/17. https://doi.org/10.1016/S1473-3099(11)70098-6 PMID: 21570355; PubMed Central PMCID: PMC3417349.

4. Hammer SM, Sobieszczuk ME, Janes H, Karuna ST, Mulligan MJ, Grove D, et al. Efficacy trial of a DNA/Ad5 HIV-1 preventive vaccine. N Engl J Med. 2013; 369(22):2083–92. Epub 2013/10/09. https://doi.org/10.1056/NEJMoa1310566 PMID: 24099601; PubMed Central PMCID: PMC4030634.

5. Pitsutthitthum P, Gilbert P, Gurwitz M, Heyward W, Martin M, van Griensven F, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis. 2006; 194(12):1661–71. https://doi.org/10.1086/508748 PMID: 17109337.

6. Berk-Ngarm S, Pitsutthitthum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. The New England journal of medicine. 2009; 361(23):2209–20. https://doi.org/10.1056/NEJMa0908492 PMID: 19843557.

7. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. The New England journal of medicine. 2012; 366(14):1275–86. https://doi.org/10.1056/NEJMoai1114255 PMID: 22475592; PubMed Central PMCID: PMC3371689.

8. Rolland M, Edelfsen PT, Larsen BB, Tovanabutra S, Sanders-Buell E, Hertz T, et al. Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. Nature. 2012; 490(7420):417–20. https://doi.org/10.1038/nature11519 PMID: 22960785; PubMed Central PMCID: PMC3551291.

9. WHO. Data and Statistics. 2015.

10. Hemelaar J, Gouws E, Ghys PD, Osmanov S, Isolation W-UNfH, Characteris ation. Global trends in molecular epidemiology of HIV-1 during 2000–2007. AIDS. 2011; 25(5):679–89. https://doi.org/10.1097/QAD.0b013e328342f93 PMID: 21297424; PubMed Central PMCID: PMC3757561.

11. clinicaltrials.gov. Pivotal Phase 2b/3 ALVAC/Bivalent gp120/5F9 HIV Vaccine Prevention Safety and Efficacy Study in South Africa (HVTN702). clinicaltrials.gov. 2016.

12. Bekker LG, Moodie Z, Gronenberg N, Laher F, Tomaras GD, Cohen KW, et al. subtype C ALVAC-HIV and bivalent subtype C gp120/5F9 HIV-1 vaccine in low-risk, HIV-uninfected, South African adults: a phase 1/2 trial. Lancet HIV. 2018; 5(7):e366–e78. Epub 2018/06/15. https://doi.org/10.1016/S2352-3016(18)30071-7 PMID: 29898870; PubMed Central PMCID: PMC6028742.

13. Avert. HIV and AIDS in South Africa 2016. Available from: https://www.avert.org/professionals/hiv-around-world/sub-saharan-africa/south-africa.

14. UNAIDS. Ending AIDS: Progress towards 90-90-90 targets2017 2018. Available from: http://www.unaids.org/sites/default/files/media_asset/Global_AIDS_update_2017_en.pdf.

15. UNAIDS. Thailand 2016 Country Factsheet2017 2018. Available from: http://www.unaids.org/en/ regionscountries/countries/thailand.

16. Avert. HIV and AIDS in Thailand 2016. Available from: https://www.avert.org/professionals/hiv-around-world/asia-pacific/thailand.

17. Vaccari M, Fourati S, Gordon SN, Brown DR, Bissa M, Schifanella L, et al. HIV vaccine candidate activation of hypoxia and the inflammasome in CD14(+) monocytes is associated with a decreased risk of SIVmac251 acquisition. Nat Med. 2018. Epub 2018/05/23. https://doi.org/10.1038/s41591-018-0025-7 PMID: 29785023.

18. Vaccari M, Gordon SN, Fourati S, Schifanella L, Liyanage NP, Cameron M, et al. Adjuvant-dependent innate and adaptive immune signatures of risk of SIVmac251 acquisition. Nat Med. 2016; 22(7):762–70. https://doi.org/10.1038/nm.4105 PMID: 27239761.

19. Byraredny SN, Arthos J, Cicala C, Villinger F, Ortiz KT, Little D, et al. Sustained virologic control in SIV+ macaques after antiretroviral and alpha4beta7 antibody therapy. Science. 2016; 354(6309):197–202. https://doi.org/10.1126/science.aag1276 PMID: 27736167; PubMed Central PMCID: PMC5405455.

20. Song RJ, Chenine AL, Rasmussen RA, Ruprecht CR, Mirshahidi S, Grisson RD, et al. Molecularly cloned SHIV-1157ipd3N4: a highly replication- competent, mucosally transmissible R5 simian-human immunodeficiency virus encoding HIV clade C Env. Journal of virology. 2006; 80(17):8729–38. Epub 2006/08/17. https://doi.org/10.1128/JVI.00558-06 PMID: 16912320; PubMed Central PMCID: PMC1563858.

21. Tomaras GD, Ferrari G, Shen X, Alam SM, Liao HX, Pollara J, et al. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(22):9019–24. https://doi.org/10.1073/pnas.1301456110 PMID: 23961096; PubMed Central PMCID: PMC3670311.
22. Siddappa NB, Watkins JD, Wassermann KJ, Song R, Wang W, Kramer VG, et al. R5 clade C SHIV strains with tier 1 or 2 neutralization sensitivity: tools to dissect env evolution and to develop AIDS vaccines in primate models. PLoS One. 2010; 5(7):e11689. Epub 2010/07/27. https://doi.org/10.1371/journal.pone.0011689; PubMed Central PMCID: PMC2908149.

23. Zolla-Pazner S, Lubeck M, Xu S, Burda S, Natuk RJ, Sinangil F, et al. Induction of neutralizing antibodies to T-cell line-adapted and primary human immunodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. J Virol. 1998; 72(2):1052–9. Epub 1998/01/28. PMID: 9444999; PubMed Central PMCID: PMC124577.

24. Gomez-Roman VR, Florese RH, Peng B, Montefiori DC, Kalyanaraman VS, Venzon D, et al. Antibodies with high avidity to the gp120 envelope protein in protection from simian immunodeficiency virus SIV(mac251) acquisition in an immunization regimen that mimics the RV-144 Thai trial. Journal of virology. 2013; 87(3):1708–19. Epub 2013/11/22. https://doi.org/10.1128/JVI.02544-12 PMID: 23175374; PubMed Central PMCID: PMC3554145.

25. Luster AD, Unkeless JC, Ravetch JV. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature. 1985; 315(6021):672–6. https://doi.org/10.1038/315672a0 PMID: 3925348.

26. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm. 2016; 7(2):27–31. Epub 2016/04/09. https://doi.org/10.4103/0976-0105.177703 PMID: 27057123; PubMed Central PMCID: PMC4804402.

27. Auclair S, Liu F, Niu Q, Hou W, Churchyard G, Morgan C, et al. Distinct susceptibility of HIV vaccine vector-induced CD4 T cells to HIV infection. PLoS Pathog. 2018; 14(2):e1006888. Epub 2018/02/24. https://doi.org/10.1371/journal.ppat.1006888 PMID: 29474461; PubMed Central PMCID: PMC5841825.

28. Teigler JE, Phogat S, Franchini G, Hirsch VM, Michael NL, Barouch DH. The canarypox virus vector ALVAC induces distinct cytokine responses compared to the virus-vector-based vectors MVA and NYVAC in rhesus monkeys. J Virol. 2014; 88(3):1809–14. Epub 2013/11/22. https://doi.org/10.1128/JVI.02386-13 PMID: 24257613; PubMed Central PMCID: PMC3911591.

29. Eisenbarth SC, Colegio OR, O’Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature. 2008; 453(7198):1122–6. https://doi.org/10.1038/nature06939 PMID: 18496530; PubMed Central PMCID: PMC4804622.

30. Yu Q, Jones B, Hu N, Chang H, Ahmad S, Liu J, et al. Comparative analysis of tropism between canarypox (ALVAC) and vaccinia viruses reveals a more restricted and preferential tropism of ALVAC for human cells of the monocytic lineage. Vaccine. 2006; 24(40–41):6376–91. https://doi.org/10.1016/j.vaccine.2006.06.011 PMID: 16859816.

31. Liu F, Niu Q, Fan X, Liu C, Zhang J, Wei Z, et al. Priming and Activation of Inflammasome by Canarypox Virus Vector ALVAC via the cGAS/IFI16-STING-Type I IFN Pathway and AIM2 Sensor. J Immunol. 2017; 199(9):3293–305. https://doi.org/10.4049/jimmunol.1700698 PMID: 28947539; PubMed Central PMCID: PMC5679316.

32. Geretti AM. HIV-1 subtypes: epidemiology and significance for HIV management. Curr Opin Infect Dis. 2006; 19(1):1–7. https://doi.org/10.1097/01.qco.0000200293.45532.68 PMID: 16374210.

33. Robb ML, Rerks-Ngarm S, Nitayaphan S, Pitisuttithum P, Kaewkungwal J, Kunasoł P, et al. Risk behaviour and time as covariates for efficacy of the HIV vaccine regimen ALVAC-HIV (vCP1521) and AIDS-VAX B/E: a post-hoc analysis of the Thai phase 3 efficacy trial RV 144. Lancet Infect Dis. 2012; 12 (7):531–7. Epub 2012/06/02. https://doi.org/10.1016/S1473-3099(12)70088-9 PMID: 22652344; PubMed Central PMCID: PMC3550398.

34. Gordon SN, Liyanage NP, Doster MN, Vaccari M, Vargas-Inchaustegui DA, Pegu P, et al. Boosting of ALVAC-SIV Vaccine-Primed Macaques with the CD4-SIVgp120 Fusion Protein Elicits Antibodies to V2 Associated with a Decreased Risk of SIVmac251 Acquisition. J Immunol. 2016; 197(7):2726–37. Epub 2016/09/04. https://doi.org/10.4049/jimmunol.1600674 PMID: 27591322; PubMed Central PMCID: PMC5026031.

35. Pegu P, Vaccari M, Gordon S, Keele BF, Doster M, Guan Y, et al. Antibodies with high avidity to the gp120 envelope protein in protection from simian immunodeficiency virus SIV(mac251) acquisition in an immunization regimen that mimics the RV-144 Thai trial. Journal of virology. 2013; 87(3):1708–19. https://doi.org/10.1128/JVI.02544-12 PMID: 23175374; PubMed Central PMCID: PMC3554145.
37. Mitkus RJ, King DB, Hess MA, Forshee RA, Walderhaug MO. Updated aluminum pharmacokinetics following infant exposures through diet and vaccination. Vaccine. 2011; 29(51):9538–43. Epub 2011/10/18. https://doi.org/10.1016/j.vaccine.2011.09.124 PMID: 22001122.

38. ATSDR. Toxicological Profiles: Aluminum. Available from: https://www.atsdr.cdc.gov/toxprofiles/tp22-c1.pdf.

39. Netea MG, Joosten LA, Latz E, Mills KH, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science. 2016; 352(6284):aaaf1098. Epub 2016/04/23. https://doi.org/10.1126/science.aaaf1098; PubMed Central PMCID: PMC5087274.

40. Dugast AS, Arnold K, Lofano G, Moore S, Hoffner M, Simek M, et al. Virus-driven Inflammation Is Associated With the Development of bNAbs in Spontaneous Controllers of HIV. Clin Infect Dis. 2017; 64(8):1098–104. https://doi.org/10.1093/cid/cix057 PMID: 28158448.

41. Pal R, Venzon D, Santra S, Kalyanaraman VS, Montefiori DC, Hooker L, et al. Systemic immunization with an ALVAC-HIV-1/gag-promotor boost vaccine strategy protects rhesus macaques from CD4+ T-cell loss and reduces both systemic and mucosal simian-human immunodeficiency virus SHIVKU2 RNA levels. Journal of virology. 2006; 80(8):3732–42. https://doi.org/10.1128/JVI.80.8.3732-3742.2006 PMID: 16571790; PubMed Central PMCID: PMC1440474.

42. Romano JW, Williams KG, Shurtloff RN, Ginocchio C, Kaplan M. NASBA technology; isothermal RNA amplification in qualitative and quantitative diagnostics. Immunol Invest. 1997; 26(1–2):15–28. https://doi.org/10.1031/08820139709048912 PMID: 9037609.

43. Vaccari M, Trindade CJ, Venzon D, Zanetti M, Franchini G. Vaccine-induced CD8+ central memory T cells in protection from simian AIDS. J Immunol. 2005; 175(6):3502–7. https://doi.org/10.4049/jimmunol.175.6.3502 PMID: 16148092.

44. Pal R, Venzon D, Letvin NL, Santra S, Montefiori DC, Miller NR, et al. ALVAC-SIV-gag-pol-env-based vaccination and macaque major histocompatibility complex class I (A*01) delay simian immunodeficiency virus SIVmac-induced immunodeficiency. J Virol. 2002; 76(1):292–302. https://doi.org/10.1128/JVI.76.1.292-302.2002 PMID: 11739694; PubMed Central PMCID: PMC135689.

45. Montefiori DC. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr Protoc Immunol. 2005;Chapter 12:Unit 12 1. Epub 2004/08/25. https://doi.org/10.1002/0471142735.im1211s64 PMID: 18432936.

46. Schiffner T, Kong L, Duncan CJ, Back JW, Benschop JJ, Shen X, et al. Immune focusing and enhanced neutralization induced by HIV-1 gp140 chemical cross-linking. J Virol. 2013; 87(18):10163–72. Epub 2013/07/12. https://doi.org/10.1128/JVI.01161-13 PMID: 23843636; PubMed Central PMCID: PMC3754013.

47. Gottardo R, Bailer RT, Korber BT, Gnanakaran S, Phillips J, Shen X, et al. Plasma IgG to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlate with a reduced risk of infection in the RV144 vaccine efficacy trial. PLoS One. 2013; 8(9):e75665. Epub 2013/10/03. https://doi.org/10.1371/journal.pone.0075665 PMID: 24086607; PubMed Central PMCID: PMC3784573.

48. Shen X, Duffy R, Howington R, Cope A, Sadagopal S, Park H, et al. Vaccine-Induced Linear Epitope-Specific Antibodies to Simian Immunodeficiency Virus SIVmac239 Envelope Are Distinct From Those Induced to the Human Immunodeficiency Virus Type 1 Envelope in Nonhuman Primates. Journal of virology. 2015; 89(16):8643–50. Epub 2015/05/29. https://doi.org/10.1128/JVI.03635-14 PMID: 26018159; PubMed Central PMCID: PMC4524233.

49. Singh S, Ramirez-Salazar EG, Doueiri R, Valentim A, Rosati M, Hu X, et al. Control of Heterologous Simian Immunodeficiency Virus SIVsmE60 Infection by DNA and Protein Coimmunization Regimens Combined with Different Toll-Like-Receptor-4-Based Adjuvants in Macaques. J Virol. 2018; 92(15). Epub 2018/05/26. https://doi.org/10.1128/JVI.00281-18 PMID: 29793957; PubMed Central PMCID: PMC6052320.

50. Wen Y, Trinh HV, Linton CE, Tani C, Norais N, Martinez-Guzman D, et al. Generation and characterization of a bivalent protein boost for future clinical trials: HIV-1 subtypes CR01_AE and B gp120 antigens with a potent adjuvant. PLoS One. 2018; 13(4):e0194266. Epub 2018/04/27. https://doi.org/10.1371/journal.pone.0194266 PMID: 29698406; PubMed Central PMCID: PMC5919662.

51. Ulanova M, Tarkowski A, Hahn-Zoric M, Hanson LA. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect Immun. 2001; 69(2):1151–9. Epub 2001/02/13. https://doi.org/10.1128/IAI.69.2.1151-1159.2001 PMID: 11160013; PubMed Central PMCID: PMC97997.

52. Ackerman ME, Das J, Pittala S, Broge T, Linde C, Suscovich TJ, et al. Route of immunization defines multiple mechanisms of vaccine-mediated protection against SIV. Nat Med. 2018; 24(10):1590–8. Epub 2018/09/05. https://doi.org/10.1038/s41591-018-0161-0 PMID: 30177821.