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Rhesus macaques show increased resistance to repeated SHIV intrarectal exposure following a heterologous regimen of rVSV vector vaccine expressing HIV antigen

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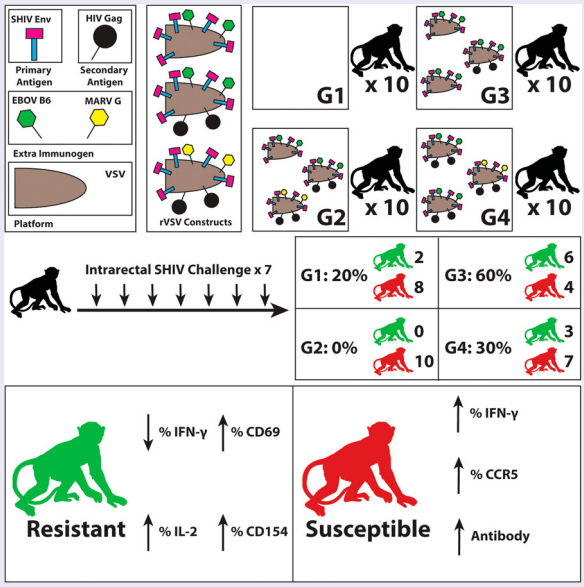
ABSTRACT

Despite the human immunodeficiency virus (HIV) pandemic continuing worldwide for 40 years, no vaccine to combat the disease has been licenced for use in at risk populations. Here, we describe a novel recombinant vesicular stomatitis virus (rVSV) vector vaccine expressing modified HIV envelope glycoproteins and Ebola virus glycoprotein. Three heterologous immunizations successfully prevented infection by a different clade SHIV in 60% of non-human primates (NHPs). No trend was observed between resistance and antibody interactions. Resistance to infection was associated with high proportions of central memory T-cell CD69 and CD154 marker upregulation, increased IL-2 production, and a reduced IFN-γ response, offering insight into correlates of protection.



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Introduction

The human immunodeficiency virus HIV-1 has caused a global pandemic since the 1980s, infecting and killing millions of people [1]. Attempts to create an HIV vaccine frequently target the gp120 and gp140 glycoprotein (GP) subunits of the HIV-1 envelope, though additional antigens such as HIV gene products *gag*, *pol*, and *nef* have also been selected as immunogens [2–4]. Thus far, these vaccines have failed to induce meaningful protection in human clinical trials [5–7]. One attempt, the RV144 trial, was only 31% effective despite this six-injection regimen inducing strong neutralizing antibody responses, suggesting such responses do not necessarily equal meaningful protection [8]. Alternatively, Parks et al. used a three-dose vesicular stomatitis virus (VSV) vaccine expressing an HIV Env protein (VSVΔG-Env.BG505) that gave 67% protection in a macaque model [9]. Though this replication-competent vaccine has not progressed since 2016, we built on the precedence of the recombinant VSV (rVSV) platform and included Ebola virus (EBOV) or Marburg virus (MARV) GPs to improve vaccine replication and possibly potency.

The recombinant VSV vector is a well-established vaccine platform with multiple known advantages including balanced immune response, stable expression of foreign genes, rapid replication, and no concerns of residual virulence or virus recombination [10]. Because VSV is primarily a livestock virus, the vector has low seroprevalence and pathogenicity when used in humans [11]. The vector is particularly useful for presenting conformation dependent antigens such as spike proteins, which are consistently expressed with appropriate protein folding [12]. In an HIV vaccine application, rVSV was able to express the target antigens at higher levels than are present on the true pathogen, thereby increasing immunogenicity [12]. In mice, the rVSV-vector expressing HIV-1 ENV spikes can induce anti-Env binding antibodies and cell-mediated immune responses. This type of vaccine was used by Parks et al. [9]. To improve on this milestone in HIV vaccine design, we equipped the construct with either Ebola virus or Marburg virus glycoproteins with the aim to improve vaccine immunogenicity. This concept of an rVSV vaccine expressing Ebola virus glycoproteins has been explored in two previous studies, Mangion et al. and Azizi et al. [13,14]. These studies found that single doses of these vaccines in mice were able to generate antibody responses against the HIV GP120 and GP140 proteins, stimulate T-cell responses, and generate neutralizing antibodies. However, those studies did not evaluate either immunogenicity of these constructs in non-human primates or efficacy against infection, both of which are investigated in this research.

One of the greatest hurdles in HIV vaccine design is the difficulty in accurately modelling the disease. HIV-1 is a host specific pathogen, and the only small animal models to date are humanized mice [15]. While it has the capacity to infect select non-human primates, HIV-1 infection does not result in a full disease state in either chimpanzee or macaque species [16]. To address this difficulty, studies frequently use simian immunodeficiency virus (SIV) as a surrogate, or use components of SIV spliced together with HIV to create a SHIV chimera [17,18]. Even then, care must be taken when selecting the NHP, of which rhesus macaques (*Macaca mulatta*) are the most established, as species-specific differences in viral susceptibility, resistance, and correlations thereof can confound results [19]. Further difficulties lie in trying to generate a broad response that protects against multiple strains, as HIV strains are highly variable and mutable [20,21]. Despite decades of intense investigation, no human trial to date has been able to induce broadly neutralizing antibodies, which are suggested to protect against multiple viral clades [22].

Furthermore, as the correlates of protection against HIV infection remain ill-defined and possibly vaccine specific, attention must be paid to the selection of vaccine platforms, which may unintentionally bias results. This study used SHIV strain SF162p3 as the challenge isolate administered rectally to rhesus macaques through serial low-dose challenges, thus repeating similar experimental conditions as previously described with VSV-based HIV vaccine [9].

Methods

Vaccines

Building on previously described design, construction, and validation of rVSV-HIV, we developed novel rVSV constructs as described (Figure 1(A)) [23]. All vaccine constructs contain a chimeric HIV clade A A74 Env protein. The transmembrane (tm) and cytoplasmic tail of the $A74_{N25K}$ HIV envelope sequence were replaced with the corresponding regions of SIVmac239. The A74 Env protein was human codon optimized (COA74), and the VSV envelope protein G was replaced with EBOV GP. One vector used a codon deoptimized EBOV B6 (CDB6) GP sequence. SIVmac239 antigen Gag was included in the MARV and EBOV CDB6 vectors. The rVSV-HIV vaccine viruses were rescued using Vero E6 and HEK293T cells and purified using a sucrose cushion followed by Vivacell filtration [24,25].

Animals and experimental procedures

Forty China-origin female rhesus macaques (*Macaca mulatta*) aged 1–3 years were divided into four

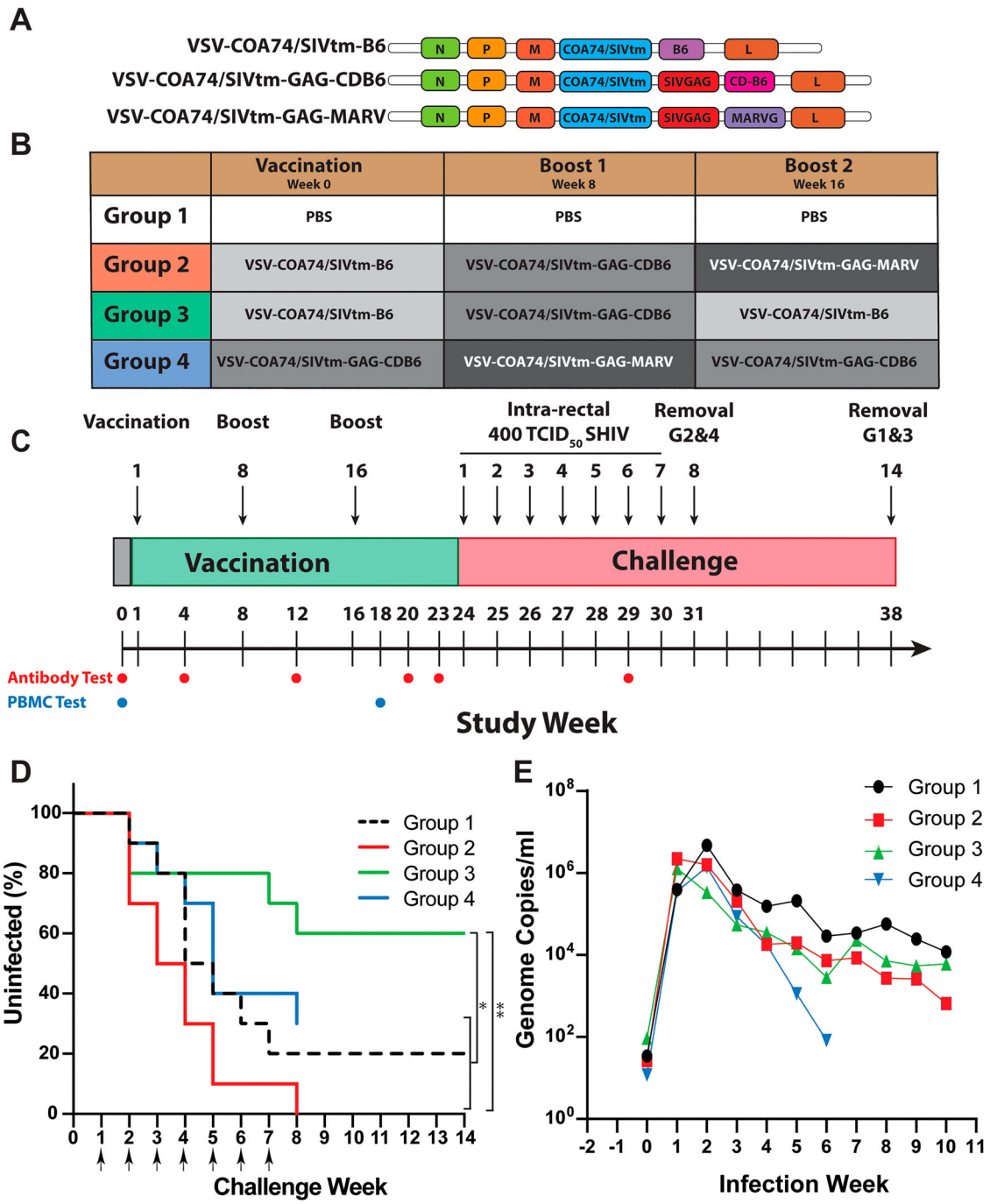

Figure 1. Vaccination scheme and infection outcomes. (A) Diagram of recombinant VSV constructs and genes used in vaccination. (B) Chart of experimental groups receiving specific vaccine treatment and dosage at various time points. (C) Diagram indicating timeline of immunizations, challenges, and removal of subjects from study. (D) Kaplan–Meier curve showing percentage of uninfected animals remaining in each group over the challenge period. Timepoints of repeat challenges indicated by arrows. Analysed by Mantel–Cox test. (E) Average number of HIV genome copies/mL over time per group, with data normalized to show first observation of infection at infection week 0. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

vaccination conditions ($n = 10$). Animals were vaccinated via simultaneous intramuscular (IM) and intranasal/oral mucosal routes as depicted (Figure 1(B)). The vaccine regimen was intentionally designed to replicate a previous rVSV-based HIV vaccine study conducted by Parks et al. [9]. All animals were challenged beginning study week 24 with 400 $TCID_{50}$ of SF162p3, an HIV Clade B chimaera, via intra-rectal route. All animals were challenged for seven consecutive weeks and monitored for a further 7 weeks (Figure 1(C)). In accordance with veterinary recommendation, some animals received an injection of 0.1 mg/kg meloxicam during the first immunization as treatment for observed inflammation (e.g. rash). Statistical significance was determined via pairwise log-rank tests.

RT-qPCR (plasma viral loads)

Viral RNA was isolated from plasma and quantified by RT-qPCR. Plasma was collected from each animal

weekly from study week 20 through study week 38 using EDTA tubes. RNA was extracted using the QIAamp RNA Viral Mini Kit (Qiagen). RNA was reverse-transcribed using the Sensiscript RT Kit (Qiagen) and quantified using LightCycler 480 Probes Master for qPCR (Roche) and Gag-specific primers and probe (IDT) [26]. The assay had a sensitivity of 165 copies/mL, and NHPs were considered positive for infection if they displayed >300 copies of viral DNA/mL.

ELISA

Binding antibodies were quantified by ELISA using serum collected on study weeks 0, 4, 12, 20, and 29. Antigens used for the assay included HIV gp140 (NIH AIDS Reagents #12577 M.CON-S gp140CFI), SHIV SF162P3 gp140 (AIDS Reagents #12026), SIV p17, and SF162 gp160.

Neutralization assay

Neutralization against Env pseudoviruses was measured with a luciferase-based assay in TZM-bl cells as previously described [27]. Serum was obtained from all animals at study week 20. Diluted sera were added to TZM-bl cells and incubated for 1 h at 37°C. 100 $TCID_{50}$ pseudovirus was added to each well. After a 48-hour incubation at 37°C, assay medium was removed and 10 µL of lysis buffer and 60 µL Galacto-Star luciferase reagents (Applied Biosystems) were added, and luminescence was measured.

ADCP and ADNP assays

Both the antibody-dependent cellular phagocytosis (ADCP) and the antibody-dependent neutrophil phagocytosis (ADNP) assays used blood samples collected on study week 23. The ADCP assay was adapted from McAndrew et al. [28]. Briefly, antigen was biotinylated using sulfo-NHS LC–LC biotin, coupled to yellow-green fluorescent Neutravidin 1 µm beads (Invitrogen, F8776) for 2 h at 37°C and washed two times in 0.1% BSA in PBS. Then, 10 µL/well of coupled beads were added to 96-well plates with 10 µL/well of diluted sample for 2 h at 37°C to form immune complexes. After incubation, the immune complexes were spun down and the supernatant was removed. THP-1 cells were added at a concentration of 2.5×10^4 cells/well and incubated for 18 h at 37°C. After incubation, the plates were spun down, the supernatant was removed, and cells were fixed with 4% PFA for 10 min. Fluorescence was acquired with a Stratedigm 1300EXi cytometer. Phagocytic score was calculated using the following formula: (percentage of FITC+ cells) × (the geometric mean fluorescent intensity [gMFI] of the FITC+ cells)/10,000.

The ADNP assay was adapted from Karsten et al. [29]. Antigens were coupled to beads and immune complexes were formed as described for ADCP. Neutrophils were isolated from fresh whole acid-citrate-dextrose (ACD) blood using EasySep Direct Human Neutrophil Isolation kit (Stemcell Technologies Inc., 19666), resuspended in R10, and added to plates at a concentration of 5×10^4 cells/well. The plates were incubated for 30 min at 37°C. The neutrophil marker CD66b (Pacific Blue conjugated anti-human CD66b; BioLegend, 305112) was used to stain cells. Cells were fixed for 10 min in 4% PFA. Fluorescence was acquired with a Stratedigm 1300EXi cytometer and phagocytic score was calculated as described for ADCP. Significance was determined by one-way ANOVA followed by a Dunnett's multiple comparisons test.

T-cell responses

Whole blood was collected using BD Vacutainer Heparin tubes at study weeks 0 and 18. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Plaque density gradient and stored in liquid nitrogen using 90% FBS – 10% DMSO at a concentration of 15–20 million cells/mL. Prior to stimulation with 0.5 µg/mL HIV-specific peptide pool A (HIV-1 Consensus A1 Env Peptide Pool), PBMCs were thawed then rested for 4 h. Cytokine production was evaluated after 12 h and proliferation after 7 days. For both assays, PBMCs were cultured in RPMI + 2% inactivated Rhesus monkey serum + 2% penicillin–streptomycin. For the proliferation assay, PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE) prior to stimulation. For the cytokine assay, stimulation was performed in the presence of GolgiStop and GolgiPlug. PBMCs were stained extracellularly with CD3-PerCP-Cy5.5, CD4-BV605, CD8-BV650, CD45RA-PE-Cy7, CCR7-BV421, CCR5-BV786, and Fixable Aqua Dead Cell Stain. For the proliferation assay, CD69-Pe-TxRed and CD154-APC also were stained extracellularly. For the cytokine assay, PBMCs were stained for CD69-Pe-TxRed, CD154-APC IFN-γ-BUV395, TNF-α-FITC, and IL-2-APC-Fire following intracellular permeabilization and blocking. Data were acquired on a cytometer and analysed using FlowJo v10.7.1 and RStudio v2022.07.1 + 554. A representative gating strategy can be seen in Supplemental Figure 1(S1). Significance was determined using Wilcoxon signed-rank Test.

Results

The current study evaluated three rVSV vectors with chimeric HIV Env A74 clade A proteins fused to the transmembrane and cytoplasmic tail of Simian immunodeficiency virus (SIV) (A74/SIVtm) (Figure 1(A)).

The Env A74 of all three vectors were human codon optimized (COA74) for enhanced cellular protein expression. One vector contained the GP of MARV, known to induce strong innate, T-cell, and B-cell responses [30]. Two other vectors contained the EBOV GP variant B6, instead of MARV, to enhance cellular tropism beyond CD4+ targets [24,31]. One of the EBOV GP sequences was codon deoptimized (CDB6), preventing expression competition with the HIV Env, favoring the latter to stimulate anti-HIV immune responses from the VSV-based vaccine. Two vectors, the MARV and EBOV CDB6, also included the SIV group-specific antigen (Gag) protein, which has been linked with rVSV-vector vaccine efficacy against HIV [32,33]. Finally, we used a heterologous vaccination strategy, which has been shown to increase both humoral and cellular immune responses while decreasing anti-vaccine vector immunity [34,35]. As such, each group received a different first boost from its initial vaccination vector. In total, this vaccine study used three separate vaccine compositions and two different dose volumes in varied combinations across the treatment groups, detailed in (Figure 1(B)). Through testing these vaccine candidates, we identified a vaccine combination that conveyed an increase in resistance compared to the control animals, as well as analysed multiple antibody and T-cell populations for potential contributions to resistance.

Vaccinated animals demonstrated a range of resistance following challenge

Forty NHPs were divided into four groups ($n = 10$), vaccinated, and then challenged intra-rectally with 400 $TCID_{50}$ HIV clade B derived SF162p3 SHIV particles weekly for a total of seven challenges. Over the challenge and follow-up period, 8/10 control animals (Group 1) acquired SHIV infection within the 7-week challenge period (Figure 1(D)). All animals, 10/10, from Group 2 acquired infection by challenge week 8 (study week 31), which was not significantly different from the controls ($p = 0.1955$), and was believed to represent a non-harmful, ineffective vaccination comparable to the Group 1 control group. In contrast, Group 3 animals showed a lower rate of infection compared to other groups, as 60% (6/10) of Group 3 remained SHIV-free through the end of study at challenge week 14 (study week 38). When compared to the Group 1, Group 3 was just above the threshold of statistical significance with a p-value of 0.069 and was significantly distinct from Group 2 ($p < 0.01$). Group 4 showed a progression similar to control Group 1, with 70% of animals infected at challenge week 8 (study week 31), which was not significantly different from either Group 1 or Group 2. As these animals demonstrated no improved resistance over the control animals, NHPs from Groups 2 and 4 were removed from the protocol on study week 31. Only Group 1 (control) and Group 3 continued until study week 38. The trend of Group 3 towards resistance was non-significant in comparison to the Group 1 control, due to limitations of sample size. Despite this, it was the only group with a trend distinctly higher than the control. When comparing a combined Group 1/2/4 "Ineffective Treatment" cohort to Group 3 "Effective Treatment," the data showed a statistically significant increase in resistance to infection ($p < 0.05$).

Animals presented with similar viraemia once infected

Following onset of challenge on study week 24, blood was collected weekly, and plasma viral load was quantified. To compare viral titre trends among infected groups, the data was synchronized to place infection week 0 at the start of the viral detection (Figure 1(E)), though this figure will not reflect onset of viraemia per challenge schedule. All animals reached peak viral load between infection weeks 1 and 3 of viraemia detection, followed by a decline in viral load. Despite reduced quantity of infections, viral load trends in infected Group 3 NHPs did not differ noticeably from infected members of Groups 1 or 2. This suggests Group 3's vaccination strategy is associated with infection prevention, but not reduced viraemia once infected. Only Group 4 differed, with average viral load decreasing faster than other groups following infection, though the timepoint showing reduction represents only 20% of the group due to attrition of animals over time. A comparison of Figure 1(D,E) suggests the possibility of different immune mechanisms for preventing initial infection versus controlling an ongoing infection.

Vaccinated animals develop antibody responses

To evaluate the progression of antibody responses, serum was collected on study weeks 0, 4, 12, and 20 and tested via ELISA for binding to GP140 (Figure 2(A)). Multiple significant differences between groups were observed at each time point, with clear trends becoming visible by week 20. At this time point, Group 2 had the highest response, followed by Group 4, with Group 3 having the lowest average response. This is supported by study week 29 serum anti-GP140 data points sorted by infection status (Figure 2(B)). In Figure 2(C), we observed study week 29 serum IgM binding to GP140 M-Con-S, a consensus sequence for the antigen. When comparing

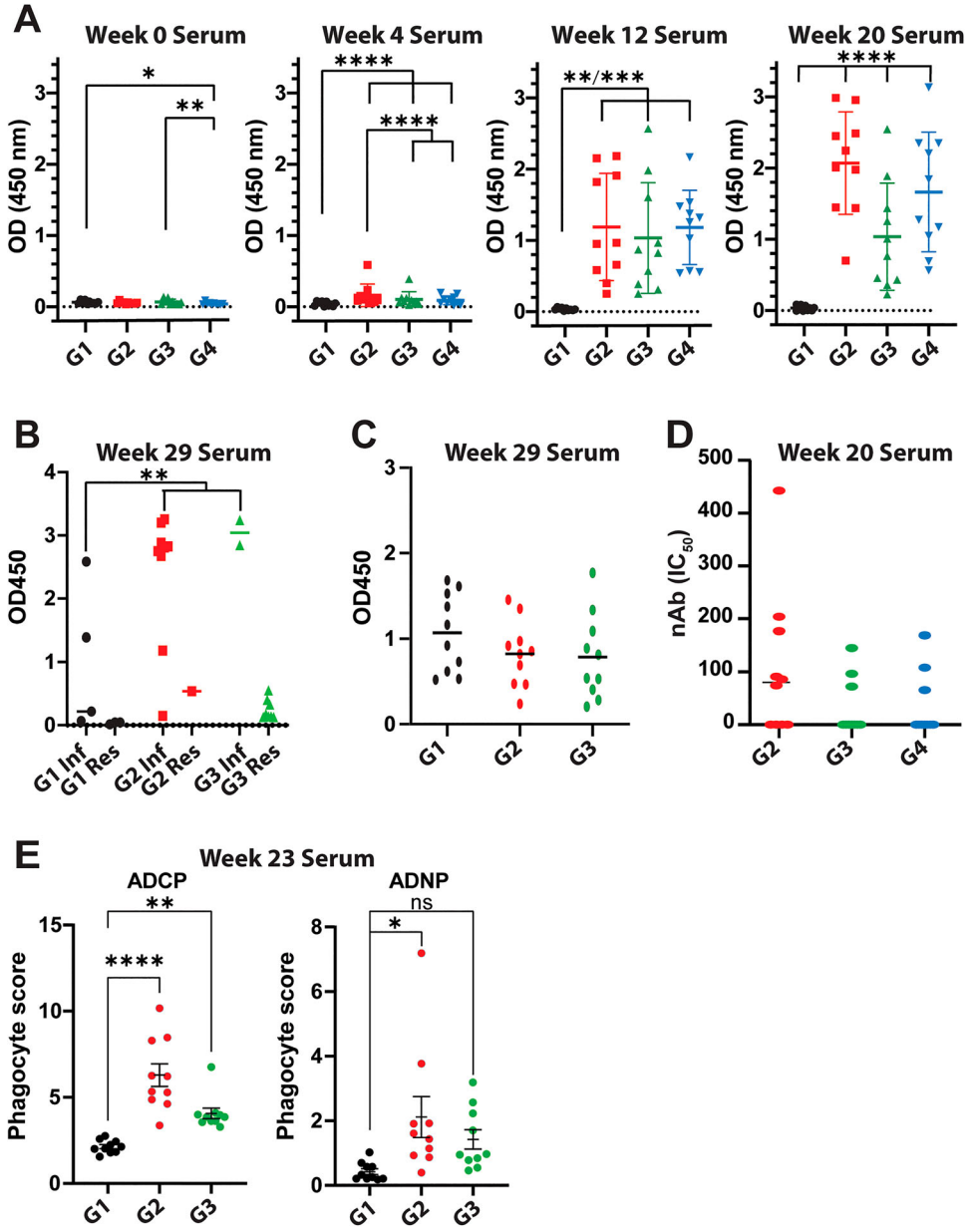

Figure 2. Antibody observations. (A) ELISA data of antibody-binding response to HIV GP140 protein from week 0, 4, 12, and 20 sera, divided by group. Significant differences were found using Tukey's multiple comparisons test. (B) ELISA data of antibody-binding response to HIV GP140 protein from week 29 serum, sorted by infection status. Significant differences were found using Tukey's multiple comparisons test. (C) ELISA data of IgM antibody binding to GP140 M-Con-S consensus sequence from week 29 serum. (D) Neutralizing antibody levels against SF162 pseudovirus. (E) Functional antibody assays of ADCP and ADNP, using week 23 antisera. Analysed comparisons between target groups is not significant unless indicated otherwise. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Groups 1, 2, and 3, there were no significant differences. In addition, study week 20 serum and HIV Env pseudoviruses were used to assess neutralizing activity. Although assessed against different variants of HIV Env pseudoviruses, neutralizing activity was detected against only SF162 pseudovirus. Group 2 demonstrated both the greatest level of neutralizing activity and animals possessing neutralizing activity, with 6/10 animals (Figure 2(D)). In contrast, Groups 3 and 4 each had 3/10 animals with neutralizing antibody responses. No correlation was observed between neutralizing antibody titres and viral loads over time.

Functional antibody responses

As neutralizing antibody responses did not correlate with resistance to infection, we then examined functional antibody responses. Luminex assays were performed by the Galit Alter laboratory at the Ragon Institute using antisera collected from Groups 1, 2, and 3 (Figure 2(E)) on study week 23. Groups 2 and 3 differed from the control group only regarding ADCP and ADNP. Group 2 displayed the highest results in both assays. Though the Group 3 ADCP assay results fell between Groups 2 and 1, Group 3 remained significantly different from Group 1 ($p =$

0.0067). In the ADNP response, Group 3's scores were slightly lower than Group 2 but were not significantly different from Group 1.

T-cell responses

PMBCs were collected from NHP subjects on study weeks 0 and 18, allowing for observation of changes following the vaccine course and prior to challenge. T cells were analysed by flow cytometry to observe multiple immunostimulatory parameters in response to an HIV-1 peptide pool. Results were then analysed to compare populations that became infected with those that resisted. Further sub-analysis was performed to observe trends in median values of the given analytes, divided by treatment group.

Changes in the cytokine secretion of IFN-γ and IL-2 by CD4 and CD8 cells were evaluated over time, with data collected from the experimental groups on weeks 0 and 18 (Figure 3(A)). Both Groups 2 and 4 central memory T cells (TCM) (defined as CD45RA-CCR7+) displayed a stronger IFN-γ over the course of vaccination. Interestingly, though the animals' TCM in Group 3 displayed similar IFN-γ responses at the beginning of the study, they did not respond uniformly to vaccination. By the end of vaccination, these animals exhibited varied IFN-γ in response to stimulation, a trend seen in both CD4 and CD8 TCM.

When examining IL-2 production, significantly higher IL-2 production in Groups 3 and 4 CD4 cells ($p < 0.05$) was observed following the vaccine regimen (Figure 3(A)). Interestingly, the Group 3 response was significant in TCM CD4 cells, while Group 4 had its significant response in CD4+ representing the effector memory T cells (TEM) (defined as CD45RA-CCR7-). Group 4 CD8 TCM were the only other population that displayed a stronger, though not significant, IL-2 response. However, because each group had members that succumbed to or resisted infection, the data were sorted by animals that eventually succumbed to or resisted infection (S2A). Here, we observed a potential trend between increased risk of infection and increased IFN-γ response in CD4 and CD8 TCM, as well as increased IL-2 signal produced by CD4 TCM. In contrast, resistance to infection appeared to be associated with increased IL-2 signal produced by CD8 TCM.

To elucidate the apparent trends from the S2A plots, we examined changes in group median values. Here, we sought to compare between the Effective Treatment experienced by Group 3 and the Ineffective Treatment experienced by Groups 1, 2, and 4. Cytokine production analysis showed diverging median trends between resistant and vulnerable animals when examining IFN-γ (Figure 3(B)). Shown here, resistant animals had an overall decrease in IFN-γ response, while vulnerable animals showed an increased response. Furthermore, the 6/10 resistant Group 3 Effective Treatment animals demonstrated steeper IFN-γ reductions in both CD4 TCM (−2.05% change) and CD8 TCM (−6.845% change) compared to the grouped Ineffective Treatment cohort (−0.01 and −2.96% change, respectively). Resistant animals also exhibited steeper increases in median CD4 TCM IL-2 responses (0.46 and 0.53% change) than vulnerable animals (0.19 and 0.225% change) following vaccination. In examining CD4 IL-2 production by resistant animals, we see that the Effective Treatment G3 had steeper increases than Ineffective Treatment for both TCM (0.53 vs 0.46) and TEM (0.315 vs −0.13). Interestingly, when looking at CD8 IL-2 production by resistant animals, we see that the Ineffective Treatment cohort resistors exhibited change in only TCM response (1.6%) compared to TEM (0%), while Effective Treatment G3 resistors saw very little change in TCM response (−0.03%) compared to TEM cells (0.75%).

Next, we examined the background activation markers of the T-cell populations in the absence of HIV-1 peptide stimulation. Here, we saw a distinction between the activation-marker patterns of CD69 and CCR5 (Figure 4(A)). Interestingly, the range of CD69 signalling extended highest in Group 2 CD8 TEM than in any other group, though only Group 4 TEM significantly differed from the control ($p < 0.01$). For CCR5 markers, Group 3 trended towards a higher range of CCR5 positive cell proportion in both their CD8 TCM and TEM. When sorted by eventual resistance or infection status (S2B), there was a trend of decreasing CD69 signal in the CD4 central memory and CD8 effector memory cell populations of infected animals. Replotting by median trend values for CD69 signalling revealed a slight trend of resistance with increasing CD69 in Group 3 CD4 and CD8 TCM and TEM (Figure 4(B)). In contrast to these resistant animals, all vulnerable animals exhibited decreases in CD69 signal across all measured TCM cell populations. Mirroring the trends seen with CD69, CCR5 signal decreased in nearly all observed cell populations of all resistant animals. Infected Group 3 animals, meanwhile, saw increased CCR5 in all cell populations, notably in CD4 TEM, which are notoriously vulnerable to HIV entry [36].

Differences of note in T-cell responses were seen when examining activation-marker responses following stimulation with HIV peptides. Both CD69 and CD154 responses were analysed, and only Group 3 produced a signal following stimulation (Figure 5(A)). These Group 3 responses significantly differed from the control in both central memory (CD69: $p < 0.01$; CD154: $p < 0.05$) and effector memory (CD69: $p < 0.05$; CD154: $p < 0.05$) CD8 cells. When sorted by resistant and infected animals, all animals that demonstrated upregulation of CD69 ($n = 5/10$)

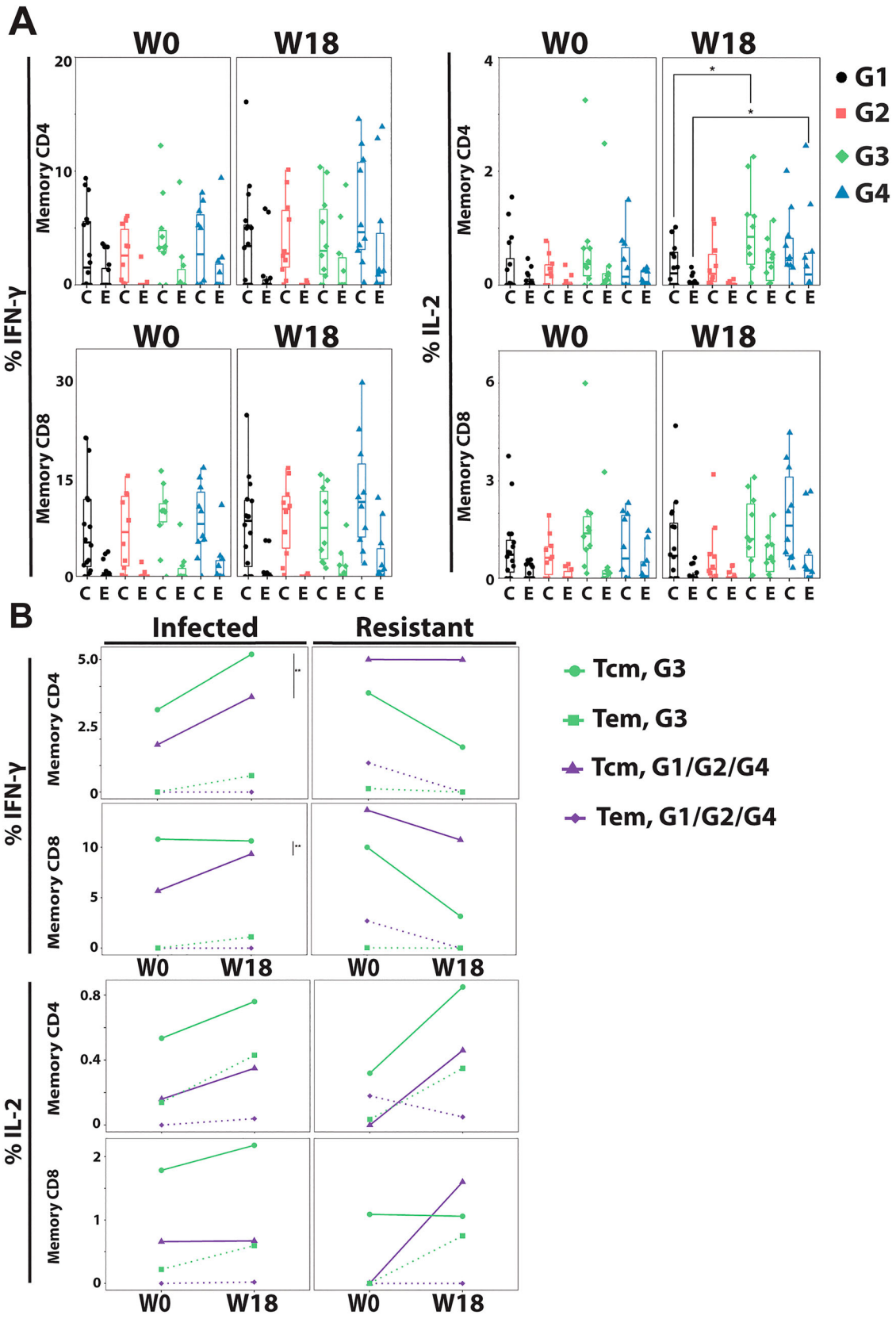

Figure 3. Memory T-cell IFN-γ and IL-2 signalling. (A) Box and whisker plots of CD8 and CD4 central memory [C] or effector memory [E] T-cell IFN-γ or IL-2 cytokine expression following stimulation. Data organized as before [W0] and after [W18] vaccination and divided by treatment group. (B) Trendlines showing changes in median values of IFN-γ and IL-2 expression over the vaccination period (% change = Median of week 18 data – Median of week 0 data), split between effective treatment (green) and ineffective treatment (purple) animals, and comparing between infected and resistant groups. Analysed comparisons between target groups is not significant unless indicated otherwise. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

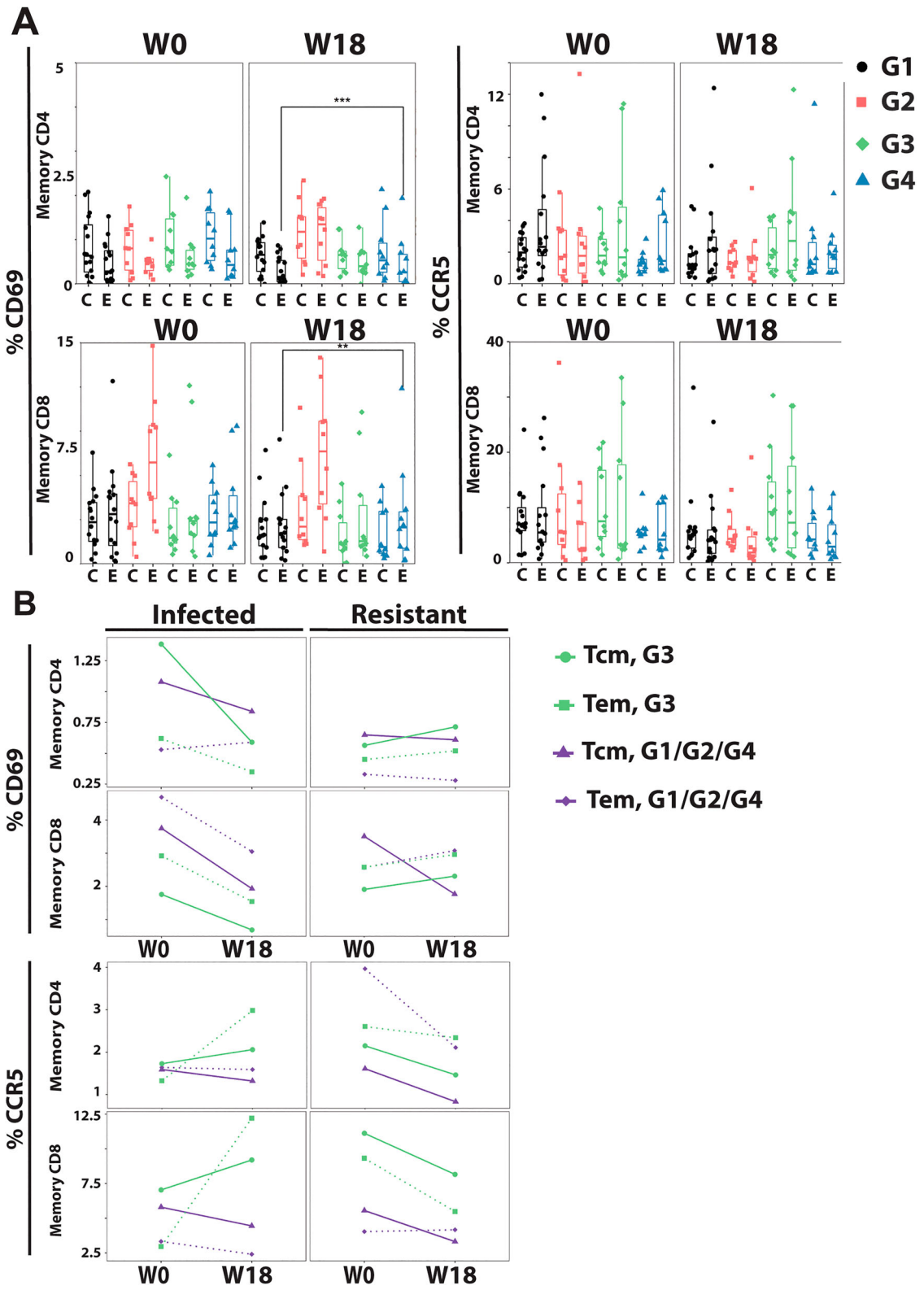

Figure 4. Memory T-cell background CD69 and CCR5 expression. (A) Box and whisker plots of CD8 and CD4 central memory [C] or effector memory [E] T-cell CD69 and CCR5 background expression before [W0] and after [W18] vaccination separated by treatment group. (B) Trendlines showing changes in median values of CD69 and CCR5 expression over the vaccination period (% change = Median of week 18 data – Median of week 0 data), split between effective treatment (green) and ineffective treatment (purple) animals, and comparing between infected and resistant groups. Analysed comparisons between target groups is not significant unless indicated otherwise. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

and CD154 ($n = 3/10$) signals following vaccination fell into the resistant animal columns (S2C). Examining the data by trend in median value showed that exclusively the Effective Treatment Group 3 resistors demonstrated a sharp increase in CD69+ proportions in both CD4 (0.645 TCM, 0.335 TEM) and CD8 (5.485 TCM, 2.93 TEM) cells, with TCM responses showing double the increase observed in TEM (Figure 5(B)).

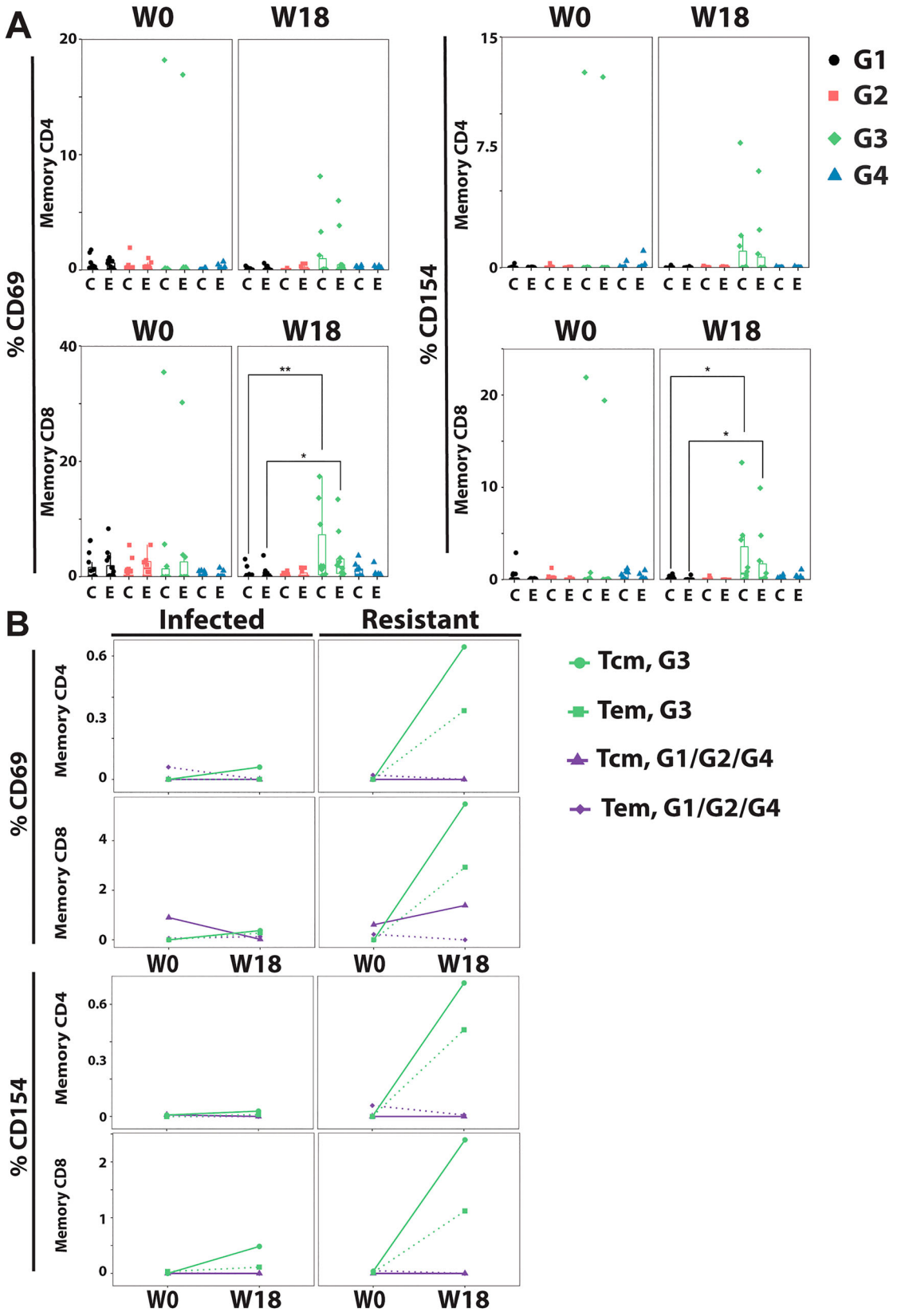

Figure 5. Memory T-cell stimulated CD69 and CD154 expression. (A) Box and whisker plots of CD8 and CD4 central memory [C] or effector memory [E] T-cell CD69 and CD154 expression following stimulation. Data organized as before [W0] and after [W18] vaccination separated by treatment group. (B) Trendlines showing changes in median values of CD69 and CD154 expression over the vaccination period (% change = Median of week 18 data – Median of week 0 data), split between effective treatment (green) and ineffective treatment (purple) animals, and comparing between infected and resistant groups. Analysed comparisons between target groups is not significant unless indicated otherwise. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

This is in contrast with the trend of decreased CD8 TCM CD69 signalling (−0.88%) seen in the vulnerable Group 1/Group 2/Group 4 animals. Similarly, Effective Treatment Group 3 was the only cohort to exhibit a strong difference in CD154 signal following vaccination, with steep increases in response of both CD4 (0.714 TCM vs 0.464% TEM) and CD8 (2.355 TCM vs 1.103% TEM) cells, once again with TCM responses showing twice the increase seen in TEM. In comparison, the Ineffective Treatment cohort saw no change in TCM CD154 signalling following treatment even amongst resistant animals.

Discussion

Within this study, we detail the resistance and immune responses of rhesus macaques induced by a heterologous regimen of novel rVSV vaccines against repeated challenge with SHIV. This resistance against infection by a different clade SHIV was induced using only three immunizations over 16 weeks, with 8 weeks between the final immunization and the onset of challenge. Of these, the Group 3 candidate vaccination strategy led to resistance to repeated intra-rectal exposure with SHIV strain SF162p3 in 6 of 10 animals. This resistance trended with high activation of central memory CD8+ T cells, CD69 and CD154 marker upregulation, increased IL-2 production, and reduced IFN-γ production prior to challenge. In contrast to the typical vaccine paradigm, no trends of protection were seen in association with either binding- or neutralizing antibody responses. The data imply that a reduced Th1 response emphasizing central memory CD8+ T cells may be key to HIV resistance. Our data show the most impactful shifts are routinely seen in central memory T cells (TCM), which regularly exhibited changes of greater magnitude than those of effector memory T cells (TEM).

Similar vaccine trials leveraged these core structural retroviral proteins (Gag), informing investigational pathways. Previously published work by Barouch et al., using mosaic Env/Gag/Pol antigens with adenovirus and poxvirus vectors, resulted in 18% of animals successfully able to resist six challenges with the same challenge SF162p3 SHIV, which was correlated with binding, neutralizing, and other functional antibody responses [37]. The RV144 trial used six doses of canarypox vector expressing *gag*/*pol*/*nef* followed by a gp140 boost, resulting in a mild protection, which was correlated with non-neutralizing antibody responses [38]. Interestingly, the Parks et al. study did not observe a correlation between protection and neutralizing antibody responses [9]. Within our own data, we see patterns with increased Group 2 antibody response, which suggest that in the current study specific activities of antibodies do not correlate with protection, regardless of neutralizing or functional activity. Of note, these data cannot rule out the possibility that binding antibody responses may have been stimulated through a pathway that also increased susceptibility to infection.

The similarity of vaccine compositions, all rVSV expressing both chimeric SIV/HIV and filovirus antigens, allowed us to focus on key differences associated with resistance versus susceptibility to infection. The vaccine regimen of Group 3, which contained the most resistant animals, distinctly included the least number of Gag boosters, which in previous studies has been a target antigen for cytotoxic T-cell based vaccine strategies [39]. Furthermore, Group 3's regimen did not contain MARV GP, which has been linked to IFN-γ T-cell responses (S3), and which here were associated with infection. The number of resistant animals within Group 3 becomes remarkable considering the vaccine contained an HIV clade A protein antigen, yet the challenge SF162p3 strain was based upon a clade B HIV. Therefore, this provides evidence for cross-clade protection against HIV strains, a highly sought finding in virology research. Notably, this apparent cross-clade protection occurred without a significant neutralizing antibody response, spurring further interest into other possible mechanisms of protection.

Our results displayed that a steep increase in IL-2 expression following vaccination was associated with resistance, as seen in the Effective Treatment Group 3 CD4 TCM and TEM, as well as Ineffective Treatment Group 1/Group 2/Group 4 TCM. Within the literature, an increase in IL-2 secretion is linked to increased T-cell proliferation, but this activity was not reflected in proliferation marker analyses (S4) [40]. Additionally, this cytokine is directly associated with maintenance of regulatory T cells (Tregs) [40]. Once generated, Tregs could function to suppress activated CD4 and CD8 T-cell populations [41,42]. Tregs also produce IL-10, which inhibits Th1 and inflammatory responses, such as IFN-γ secretion [43,44]. Together, these observations suggest that the resistance induced by this vaccine is biased against a Th1 response, with regulatory T cells potentially playing a major role through suppression of inflammatory and potentially harmful immune responses. Similarly, the connection between low IFN-γ and increased resistance that we observed in Figure 3(B) could mean that the inflammatory activity of IFN-γ is detrimental, and its low-level expression improves resistance. This agrees with historical data, which found that systemic pro-inflammatory responses were largely harmful during HIV infection [45,46]. These responses have been linked to negative outcomes during HIV possibly because activated T cells are more susceptible to HIV infection, and this activation in combination with recruitment of cells to infected sites may accelerate infection and virus propagation

[47,48]. Our data therefore suggests that an immune response with reduced CD4 T-cell activation, as in the reduced IFN-γ expression of Group 3 CD4 TCM cells compared to control, will result in greater resistance. Persistent inflammation has also been identified as a contributing factor to immune exhaustion, CD4 T-cell depletion, and progression to AIDS [49,50]. These findings were corroborated in separate studies, which found that Type I IFN leads to T-cell exhaustion, and blockade of these cytokines can restore CD8 function [51,52].

In this study, we observed that resistance is associated with a decrease in the background proportion of cells positive for CCR5, particularly on CD4 TEM and both CD4 and CD8 TCM. This is directly as expected, as the CCR5 chemokine receptor on CD4 T cells is a known HIV coreceptor during HIV infection [53,54]. Together, these effects suggest a causal relationship between IFN-γ-induced inflammation responses and preservation or upregulation of the HIV-vulnerable CCR5+ T-cell population, which is in line with our observations of infected animals in Figures 3(B) and 4(B). Associated with resistance, the induction of CD69 and CD154 activation signals are clearest in CD8 TCM. Even without direct peptide stimulation, the background CD69 response of T cells directly trended with resistance on nearly all cell populations tested. As CD69 is upregulated non-specifically and is associated with swift immune activation in response to pathogenic invasion, successful vaccination improved this marker and in turn resistance [55,56]. While the direct mechanism between CD69 and resistance is uncertain, prior literature points to a relationship between CD69, NK cells, and IFN-γ responses. A study by Notario et al. found that depletion of CD69 in mice results in an enhanced NK cell, IFN-γ, and TNF-α responses, indicating an inverse relationship between CD69 and these immune factors [57]. As discussed, our research saw trends of increased TCM IFN-γ responses in susceptible animals. We therefore suspect that the increased CD69 response may serve to decrease a harmful IFN-γ response, at least partially by suppressing NK cell activity. CD69 has also been associated with Treg cells, which, as discussed earlier, could similarly play a role in reducing harmful inflammatory responses [58]. Likewise, when stimulated by HIV-1 peptides, a steeply increased CD154 expression is associated with resistance in Group 3, primarily seen in CD8 TCM. CD154 is a ligand for CD40, through which it controls a broad range of activities including antigen-presenting cell activation and maturation, T-cell priming, type 1 cytokine production, macrophage effector functions, antibody isotope switching, and germinal center formation [59,60]. However, why only Group 3 saw an increase in CD69 and which of these CD154 functions is acting to increase resistance in this study is uncertain and should be considered for additional research.

Overall, this study suggests that the central memory response is more valuable for inducing resistance than effector memory from this vaccine regimen and should be the target of future vaccination studies. Future studies could identify the cause of the CD69 response and which actions linked to CD154 are protective, as its antibody-associated functions do not appear to impact resistance. Importantly, prior studies required six immunizations, only to report no significance or reported positive significant results with small percentages (≤30%) of their cohort successfully resisting infection by protocol's end [8,37]. In relation to this past pioneering work, the rVSV vaccine reported here utilized three immunizations, which resulted in 60% (6/10) of animals resisting infection. Collectively, these results support continuing the development of VSV-based vaccine regimens to induce enhanced protection against increased exposures of multiple clades of SHIV and HIV.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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