HIV-specific CD8+ T-cell responses soon after treatment initiation during acute HIV infection are associated with viral reservoir decline

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Abstract

Antiretroviral therapy (ART) initiated in the acute phase of HIV infection (AHI) results in a smaller viral reservoir. However, the impact of early HIV-specific T-cell responses on long-term reservoir dynamics is less well characterized. Therefore, we measured the size of the viral reservoir and functionality of HIV-specific CD8+ T-cell responses after the acute phase at 24 and 156 weeks after ART initiation in individuals who were diagnosed during AHI. A significant decline in total and defective HIV DNA and a trend towards a decline in intact HIV DNA were observed between 24 and 156 weeks. Functional CD8+ T-cell responses against HIV peptides Env, Gag, Nef, and Pol were maintained over three years after treatment initiation. The proliferative capacity of HIV-specific CD8+ T-cells at 24 weeks was associated with the decline of total and defective HIV DNA reservoir, suggesting that HIV-specific CD8+ T-cells may at least partially drive the decline of the viral reservoir. Therefore, enforcing HIV-specific immune responses as early as possible after diagnosis of AHI should be a central focus of HIV cure strategies.

Introduction

Current treatment of HIV infection with antiretroviral therapy (ART) successfully suppresses viral replication, halts and reverses disease progression, and gives people with HIV (PWH) a near-normal life expectancy. However, ART does not clear HIV infection as the virus persists in so-called viral reservoirs, which in most individuals leads to viral rebound soon upon ART interruption (1-3) and makes lifelong treatment inevitable.

During the early stages of HIV infection, also referred to as acute HIV infection (AHI), active viral replication and dissemination supports seeding of the viral reservoir within lymph nodes and other tissues (4, 5). Coinciding with the development of an HIV-specific immune response, the viral levels decline within weeks of AHI. Cytotoxic CD8+ T-cells, which recognize viral peptides presented by human leukocyte antigen (HLA) class I alleles, are an important component of the HIV-specific immune response (6, 7), as these responses exert a major selective pressure on HIV evolution (8, 9). Active viral replication shapes the magnitude and diversity of HIV-specific CD8+ T-cells, especially during the early stage of infection (10).

The importance of the host CD8+ T-cell response in controlling virological outcomes is supported by many studies. For instance, strong HIV-specific CD8+ T-cell responses have been shown to control HIV without ART in human elite (11-14) and post-treatment controllers (PTC) (15, 16), and to control SIV in non-human primates (NHP) (17-19). However, in the majority of PWH, CD8+ T-cells dysfunctionality is already observed shortly after peak viremia during AHI, which could potentially be prevented if ART is initiated prior to this occurring (10, 20). Indeed, effective HIV-specific CD8+ T-cell responses were observed in people treated during AHI and the magnitude of this response correlated with the transcriptional activity of the virus (21). ART initiation during AHI also resulted in a smaller viral reservoir size by limiting viral replication and seeding of the reservoir compared to treatment initiation during chronic infection (CHI) (6, 10, 22-26). Moreover, early ART initiation enhances restoration of the immune system (26, 27). The importance of ART initiation during AHI is underscored by the fact that

early treated individuals have a higher chance of achieving at least temporary viral control after stopping ART (28).

Currently, it is unknown whether early HIV-specific CD8+ T-cell responses are associated with the size of the viral reservoir long-term. In this study, we aimed to characterize the viral reservoir and HIV-specific CD8+ T-cell responses in participants of the Netherlands Cohort Study on Acute HIV Infection (NOVA study), who initiated ART immediately after diagnosis of AHI and were followed for over three years. We found evidence that the early immune response shapes aspects of the viral reservoir when ART is initiated during AHI.

Results

Cohort description

This study included 22 participants of the NOVA cohort who initiated ART immediately (median 1 day, IQR 0-2) after diagnosis during AHI. We selected those participants for whom leukapheresis samples at either 24 weeks and/or 156 weeks post ART initiation were available. Longitudinal samples were available from twelve of 22 participants, resulting in a total of 34 samples. Baseline characteristics are provided in Table 1. The median age at time of inclusion was 38 years (IQR 28-48), all participants were male and two-third of participants had a subtype B HIV infection. At diagnosis, participants had a median plasma viral load (pVL) of $0.4x10^6$ copies/mL (Suppl. Fig. 2A). Two participants had a detectable pVL of 200 and 100 copies/mL (Participant X) and 74 and 98 copies/mL (Participant Y), at 24 and 156 weeks respectively (Suppl. Fig. 2A).

CD4+ T-cell counts at 24 weeks and 156 weeks were comparable, with a median of 610 (IQR 520-725) and 695 (IQR 623-838) cells/mm^3 respectively (Suppl. Fig. 2B; p=0.13), as were the CD8+ T-cell counts (median 680 (IQR 485-840) at 24 weeks and 730 (IQR 520-1005) cells/mm^3 at 156 weeks (p=0.41). The CD4/CD8 ratio increased significantly between baseline and 24 weeks (Suppl. Fig. 2C; p<0.01), but was comparable between 24 and 156 weeks.

Longitudinal decline of total and relative increase in transcriptionally competent viral reservoir

First, we determined the reservoir size at 24 weeks by measuring total, intact, and defective HIV DNA in PBMCs. Intact proviruses were detected in 33 of 34 samples, while defective proviruses (either *psi* or *env* PCR signal) were detectable in all samples. In the longitudinal analysis (n=12), a significant decline in total HIV DNA load (Fig. 1A; p=0.02) and defective HIV DNA load (Fig. 1C; p=0.005) was observed between 24 and 156 weeks. Furthermore, we noted a trend towards a decline in intact HIV DNA load from 24 to 156 weeks (Fig. 1B; p=0.11).

A number of studies have shown that viral latency does not preclude viral transcription (29). Therefore, we determined cell-associated US HIV RNA as a measure of transcriptionally active reservoir at 24 and 156 weeks. No significant difference in US RNA levels was observed between 24 and 156 weeks (Fig. 1D; p=0.23). However, relative HIV transcription level per provirus, calculated as US RNA/total HIV DNA (US/TD) ratio, significantly increased between 24 and 156 weeks (Fig. 1E, p=0.03), indicating a relative increase in transcriptional activity of the reservoir over time.

To better understand the relationships between these different measures of the viral reservoir (total HIV DNA, intact and defective HIV load and US RNA levels), a Pearson correlation analysis was conducted (Fig. 2). At 24 weeks, a strong correlation was observed between the level of total HIV DNA, defective HIV DNA and US RNA (Fig. 2A). At 156 weeks, a similar correlation between total HIV DNA and US RNA levels was observed (Fig. 2B). These data imply that the levels of US RNA are not reflective of intact HIV DNA load.

Replicating virus could be isolated from CD4+ T-cells of four (out of 12) participants at the 24 week time point, one of whom had a detectable plasma viral load at that time (Participant Y, 74 copies/mL). However, replication-competent virus could not be retrieved in any of the 156 week samples. In the four participants from whom we were able to isolate virus at the 24-week time point, a relatively high intact HIV DNA load of >100 copies/10^6 PBMC was detected at that moment (Fig. 1B). This confirms that the existence of a replication-competent viral reservoir is linked to the presence of intact HIV DNA.

Comparable frequency and function of the HIV-specific CD8+ T cell response at 24 and 156 weeks

General phenotyping of CD4+ (Suppl. Fig. 3A) and CD8+ (Suppl. Fig. 3B) T-cells showed no difference in frequencies of naïve, memory or effector CD8+ T-cells between 24 and 156 weeks. Moreover, CD8+ T-cell activation levels were low (<10%) and remained stable over time (Suppl. Fig. 3B). Next, HIV-specific CD8+ T-cell numbers and functionality at 24 and 156 weeks post ART initiation were

analyzed. A subgroup of participants (n=9), positive for HLA-type HLA-A*2, HLA-B*7, or both, showed similar frequencies of HIV-specific dextramer positive CD8+ T-cells at 24 and 156 weeks (median frequency 0.066% (IQR 0.031-0.11) at 24 weeks and 0.055% (IQR 0.052-0.11) at 156 weeks (Suppl. Fig. 4A; p=0.48). The phenotype of HIV-dextramer specific CD8+ T-cells showed no difference in expression of exhaustion markers (upregulation of PD-1, CTLA-4, and CD160 expression; loss of CD28 expression) between the two time points (Suppl. Fig. 4B).

HIV-specific CD8+ T cell functionality was assessed through stimulation with HIV Env, Gag, Nef and Pol peptide pools. The readout of these stimulation was the interferon gamma release assay (IGRA), activation-induced marker (AIM) assay and cell proliferation (precursor frequency and proliferated cells). IFN-γ responses to Env, Gag, Nef and Pol were observed in 3, 8, 2 and 1 participant(s), respectively, at 24 weeks and 1, 7, 2 and 1 participant(s), respectively, at 156 weeks (data not shown). The AIM assay showed a similar broad HIV-specific T-cell response to at least three different viral proteins in the majority of individuals at both time points (data not shown). The magnitude of the T-cell response, by combining the frequencies of reactive CD8+ T-cells to all viral proteins (Env, Gag, Nef, Pol) tested, showed no statistically significant differences (Suppl. Fig. 5B) over time. Similarly, a broad HIV-specific CD8+ T-cell proliferative response to at least three different viral proteins was observed in the majority of individuals at both time points, with no significant differences in precursor frequencies and proliferative capacity between week 24 and week 156 (Suppl. Fig. 5C&D).

Magnitude of proliferative HIV-specific CD8+ T-cell response is associated with the size and decline of the viral reservoir

Next, correlations between these responses and viral reservoir measurements were analyzed. The frequencies of HIV-specific dextramer positive CD8+ T-cells did not correlate with any of the reservoir measurements at 24 or 156 weeks (Suppl. Fig. 6). HIV-specific CD8+ T-cell functionality as determined by IGRA, proportion of HIV-reactive (AIM) CD8+ T-cells and CD8+ T-cell precursor frequency did not

show any correlation with the viral reservoir size at 24 weeks either. Interestingly, at this time point, we did observe a strong positive correlation between the HIV-specific CD8+ T-cell proliferative response and the levels of total HIV DNA (Fig. 3A; $p<0.01$), defective HIV DNA load (Fig. 3A; $p<0.01$) and cell-associated US RNA (Fig. 3A; $p=0.03$). At 156 weeks, this correlation was no longer observed (Fig. 3B), which may be explained by an overall decrease in reservoir size at week 156, while HIV-specific CD8+ T-cell responses remained stable over time.

To determine whether early HIV-specific CD8+ T-cell responses at 24 weeks were predictive for the change in reservoir size, correlations between the CD8+ T-cell responses and the difference (delta; Δ) in total HIV DNA, defective HIV DNA, and US/TD ratio over time were determined. The CD8+ T-cell proliferative response at 24 weeks was positively correlated with the decline in total HIV DNA ($p<0.01$) and defective HIV DNA load ($p<0.01$) (Fig. 4A). This suggests that the early presence of HIV-specific CD8+ T-cells with an enhanced proliferative capacity in response to HIV plays a role in the reduction of the viral reservoir, as measured by total and defective HIV DNA load, over the course of three years.

Discussion

In this study, we investigated the longitudinal dynamics of the HIV reservoir and host immunological responses in people immediately treated with ART during AHI. We found a reduction in the viral reservoir, as evidenced by the significant decline in total and defective HIV DNA and the trend towards a decline in intact HIV DNA, between 24 weeks and 156 weeks on ART. The decline in total HIV DNA and defective HIV DNA load over time was strongly related to HIV-specific proliferative CD8+ T-cell responses against HIV peptides Env, Gag, Nef and Pol at 24 weeks. Conversely, we observed a strong positive correlation between the HIV-specific CD8+ T-cell proliferative response and the levels of total HIV DNA, defective HIV DNA load and cell-associated US RNA at week 24 and we observed that these HIV-specific CD8+ T-cell responses were maintained over three years after treatment initiation.

Recently, HIV reservoir and HIV-specific CD8+ T-cell responses were thoroughly investigated in another AHI cohort (21). They found a similar reduction in HIV reservoir over two years on ART, however CD8+ T-cell responses also declined. Moreover, a larger HIV reservoir size hampered differentiation into functional HIV-specific CD8+ T-cells (21). In our study, we did not see a change in number of HIV-specific CD8+ T-cell responses over time and even more so, we found a preserved functionality up to three years after ART start. In line with the previous study, we found that the transcriptionally active reservoir, as measured by cell-associated US RNA, was correlated to the magnitude of HIV-specific CD8+ T-cell proliferative responses (21). This may suggest that active viral replication drives the CD8+ T-cell response.

In our study an increase in the US RNA/total HIV DNA ratio, reflecting a shift towards a more transcriptionally active reservoir, was observed. This indicates that while there is a decrease in the overall viral reservoir, the remaining reservoir becomes more transcriptionally active and presumably maintains the HIV-specific CD8+ T-cell responses.

Remarkably, we observed that the defective HIV DNA levels declined significantly between 24 weeks and 3 years on ART. This is in contrast to previous observations in chronic HIV infection (30). The integrated, but defective proviruses do not produce replicating virus but can be transcriptionally and translationally competent and are therefore thought to play a substantial role in the ongoing immune activation (30). Indeed, it was shown that these defective proviruses are capable of producing viral RNA transcripts and proteins both in vivo and in vitro (31, 32). In our cohort, at 24 weeks US RNA correlated to defective HIV DNA levels but not to intact HIV DNA. This suggests that the US RNA transcripts are mainly produced from defective proviruses. Moreover, the strong correlation between the HIV-specific CD8+ T-cell response and defective HIV DNA levels as well as US RNA suggest detection by the immune system, leading to decay and shaping of the proviral landscape. Importantly, reservoir decay patterns are not only influenced by HIV-specific immune responses, but are also known to be associated with other factors. A recent study found that there was a faster decay of intact and defective HIV DNA when ART was initiated earlier, initial CD4+ T-cell counts were higher and pre-ART pVL was lower (33).

A recent study investigated reservoir dynamics in people that initiated ART during hyperacute HIV-1 in subtype C infection and found that early ART was associated with reduced phylogenetic diversity and rapid decay of intact proviruses (34). In fact, no intact provirus could be detected after 1 year of ART (reduction of 51% per month) while a decline in defective provirus was observed (reduction of 35% per month) (34). In our cohort, we observed a significant decrease in total and defective HIV DNA load between 24 and 3 years, while the decline of intact HIV DNA was less pronounced. This discrepancy could possibly be explained by the time period after AHI that was analyzed, as in that study decay of intact HIV DNA was observed in the first six months of treatment (34), while we determined the decline between week 24 and 156. Similarly, in another study, a comparable biphasic decay was found in total (half-life 12.6 weeks) and integrated (half-life 9.3 weeks) HIV DNA in AHI, but total HIV DNA continued to decline in the second decay phase (35). Recent mathematical modelling of reservoir decay in AHI between 0-24 weeks after ART initiation showed a biphasic decay for both intact and defective DNA

(33). Intact DNA showed a rapid initial $t_{1/2}$ during the first 5 weeks of ART followed by a slower decay with a $t_{1/2}$ of around 15 weeks. Defective DNA showed an even significantly larger decrease in the first phase than intact DNA, followed by a slower decay (33). The lack of significant decay of intact HIV DNA in our study may also be explained by ongoing immune-mediated selection of integrated intact proviral DNA in repressive and heterochromatin locations eventually resulting in a shift towards a state of "deep latency", which has previously suggested (36) and also seen in people who naturally control HIV (elite control) (37, 38). Interestingly, we could only retrieve replication-competent virus from four participants at week 24, but not at 156 weeks, which indeed suggests selection for integrated intact proviruses that are not rebound-competent upon reactivation.

The use of different readouts may explain at least some of the differences observed between studies. The study by Takata et al. reported a decline in functional CD8+ T-cell responses after two years of ART based on a combined AIM/intracellular staining assay (ICS) and using a different activation marker (4-1BB)(21). We observed that HIV-specific CD8+ T-cell responses were maintained in our cohort using both the AIM and proliferation assay. Interestingly, relations similar to our study between the HIV reservoir size decline and HIV-specific CD8+ T-cell responses were reported. However, Takata et al. showed a loss of the association when total HIV DNA was used as a reservoir marker, whereas our data points to an association between HIV-specific CD8+ T-cell responses and both the decline in total HIV DNA and defective HIV DNA.

There are some limitations to be acknowledged in our study. First, our cohort consists of only men, who are mostly Dutch. Second, not all participants underwent leukapheresis at the 156 week time point due to personal or logistic (COVID-19 pandemic) reasons and therefore we had a small longitudinal sample size, that included participants ranging from Fiebig II-VI. Therefore we could not assess the role of the Fiebig stage at ART initiation. A strength of our study is the long-term sampling, which allowed us to assess the reservoir decay and host immune responses years after ART initiation.

Previously mentioned studies into reservoir decay have mainly reported on the first year after treatment was started (33, 34, 39).

Our study shows that between 24 weeks and 3 years of ART, total and defective HIV DNA decline significantly and that this decline is related to the magnitude of HIV-specific CD8+ T-cell responses at 24 weeks, suggesting that HIV-specific CD8+ T-cells may at least partially drive the decline of the viral reservoir. Our study has several implications. First, it confirms the complexity of host-virus interplay, as we show that defective HIV DNA decreased stronger than intact HIV DNA, and defective, but not intact, HIV DNA correlated with US RNA and the functionality of the HIV-specific CD8+ T cell response. However, the decay of defective HIV DNA did not result in a decreased HIV transcriptional activity over time. This could be the result of selection for cells that harbor transcriptionally active proviruses that circumvent immune surveillance through for instance upregulation of immune inhibitory molecules like PD-1, CTLA-4 and TIGIT, downregulation of HLA class I molecules, or the emergence of viral escape mutations. Second, our study shows that even in acute HIV infection, the reservoir is readily detectable despite immediate ART. We believe our study underscores that in line with what was shown in natural HIV control, an (early) functional CD8+ T-cell response is shaping the viral reservoir during ART and that enhancing host immune responses should be a focus for interventions aimed at a functional HIV cure.

Author contributions

Conceived and designed the experiments: PvP, AOP, JMP, NAK, GJdB. Performed the experiments and analyzed the data: PvP, AOP, KAvD, ACvN, IM, BBN, NVEJB, JMP, NAK, GJdB. Contributed to reagents, materials, and analysis tools: PvP, AOP, KAvD, ACvN, IM, BBN, NVEJB, TM, CL, CR, JS, MN, LV, MJK, JMP, NAK, GJdB. Wrote the paper: JP, NK and PvP. Reviewed and edited manuscript: PvP, AOP, KAvD, ACvN, IM, BBN, NVEJB, TM, CL, CR, JS, MN, LV, MJK, JMP, NAK, GJdB.

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Methods

Sex as a biological variable

The NOVA study is open for inclusion to both males and females; however, in the current study only samples from male participants were available. This reflects the epidemiology of acute/early HIV infections in the Netherlands, which concerns mostly males. We do believe more females should be included in this research to be able to translate the findings to the entire population that is currently living with HIV.

Study approval

The NOVA cohort is a multicenter, observational, prospective cohort that was initiated in 2015 and includes participants diagnosed with an acute/early HIV infection (AHI) (40). The study was approved by the Medical Ethics Committee of the Amsterdam UMC (NL51613.018.14) and all study participants gave written informed consent.

Study design

The study design of the NOVA study, including treatment regimen and follow-up visits, has been described elsewhere (40). In short, people were included if they were 18 years or older and were diagnosed during AHI as defined by Fiebig stage I-IV. In case of a positive Western blot, participants could only be included if they had a documented negative HIV screening test <6 months before inclusion. After diagnosis, participants were referred to an HIV treatment center and started a four-drug regimen of emtricitabine/tenofovir 200/245 mg (FTC/TDF), dolutegravir 50 mg (DTG), darunavir 800 mg and ritonavir 100 mg (DRV/r) as soon as possible (preferably within 24 hours). After four weeks, when baseline genotyping and viral mutations conferring possible drug resistance were known, DRV/r was discontinued. Participants could enroll in three groups based on the preparedness of individuals to undergo extensive sampling (Suppl. Fig. 1). Participants that accepted immediate treatment and follow-up but declined additional blood and tissue sampling were included in study group 1, of which only routine clinical care plasma viral load (pVL) and CD4 count measurements

were collected. For group 2 and 3, PBMC and semen were collected at study visits and cryopreserved, in group 3 in addition GALT, lymph node biopsies and CSF were collected. In both groups leukapheresis was performed at weeks 24 and 156 . The participants selected for the current analysis were in care at Amsterdam University Medical Center, Erasmus University Medical Center or Radboud University Medical Center. Apart from pVL and CD4/CD8 measurements, all virological and immunological assessments were performed centrally at the Amsterdam University Medical Center.

Viral load quantification and HIV subtyping

Viral load (HIV RNA) was measured in plasma using a sensitive HIV RNA assay. The assays that were used were m2000rt HIV RNA (Abbott) with a lower limit of quantification (LLOQ) of 40 copies/mL from 2015-2021, Alinity m HIV-1 Assay (Abbott) with a LLOQ of 20 copies/mL from 2021 onwards (Amsterdam University Medical Center), COBAS AmpliPrep/COBAS TaqMan HIV-1 test (Roche Diagnostics), LLOQ 20 copies/mL and Aptima HIV-1 Quant Dx Assay (Hologic), LLOQ 30 copies/mL (Erasmus Medical Center), and Xpert HIV-1 assay (Cepheid) with a LLOQ of 40 copies/mL (Radboud University Medical Center).

HIV subtypes were determined using Neighbor joining analysis to create phylogenetic trees. Reference sequences from the major HIV-1 subtypes were obtained from the NCBI database and the distance between sequences was calculated using the Kimura-2 parameter model.

Quantification of total HIV DNA and cell-associated unspliced HIV RNA

Total HIV DNA and cell-associated unspliced (US) HIV RNA were quantified by semi nested qPCR according to the principles described previously (41). In brief, total nucleic acids were extracted from PBMCs using Boom isolation method (42). Extracted cellular RNA was treated with DNase (DNA-free kit; ThermoFisher Scientific) to remove genomic DNA that could interfere with the quantitation and reverse transcribed into cDNA using random primers and SuperScript III reverse transcriptase (all from ThermoFisher Scientific). To quantify cell-associated US HIV RNA or total HIV DNA, this cDNA, or DNA extracted from PBMCs, respectively, was pre-amplified using primer pair Ψ_F (43) and HIV-FOR

(44). The product of this PCR was used as template for a semi nested qPCR with the Ψ primer/probe combination (45). HIV DNA or RNA copy numbers were determined using a 7-point standard curve with a linear range of more than 5 orders of magnitude that was included in every qPCR run and normalized to the total cellular DNA (by measurement of β-actin DNA) or RNA (by measurement of 18S ribosomal RNA) inputs, respectively, as described previously (46). Non-template control wells were included in every qPCR run and were consistently negative. Total HIV DNA and US RNA were detectable in 88.2% and 73.5% of the samples, respectively. Undetectable measurements of US RNA or total DNA were assigned to the values corresponding to 50% of the corresponding assay detection limits. The detection limits depended on the amounts of the normalizer (input cellular DNA or RNA) and therefore differed among samples. HIV transcription levels per provirus (US RNA/total DNA ratios) were calculated taking into account that 10^6 PBMCs contain 1 µg of total RNA (47).

Quantification of intact and defective HIV DNA

Intact and defective HIV DNA was quantified by the intact proviral DNA assay (IPDA) (43). In brief, genomic DNA was isolated from PBMCs using Puregene Cell Kit (QIAGEN Benelux B.V.) according to the manufacturer's instructions and digested with BglI restriction enzyme (ThermoFisher Scientific) as described previously (48). Notably, only a small minority (<8%) of HIV clade B sequences contain BglI recognition sites between Ψ and env amplicons, therefore BglI digestion is not expected to substantially influence the IPDA output, while improving the assay sensitivity by increasing the genomic DNA input into a droplet digital PCR (ddPCR) reaction (48). After desalting by ethanol precipitation, genomic DNA was subjected to two separate multiplex ddPCR assays: one targeting HIV Ψ and env regions using primers and probes described previously, including the unlabelled env competitor probe to exclude hypermutated sequences (43), and one targeting the cellular *RPP30* gene, which was measured to correct for DNA shearing and to normalize the intact HIV DNA to the cellular input. The RPP30 assay amplified two regions, with amplicons located at exactly the same distance from each other as HIV Ψ and env amplicons. The first region was amplified using a forward primer 5'-AGATTTGGACCTGCGAGCG-3', a reverse primer 5'-GAGCGGCTGTCTCCACAAGT-3', and a

fluorescent probe 5'-FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1-3' (49). The second region was amplified using a forward primer 5'-AGAGAGCAACTTCTTCAAGGG-3', a reverse primer 5'-TCATCTACAAAGTCAGAACATCAGA-3', and a fluorescent probe 5'-HEX-CCCGGCTCTATGATGTTGTTGCAGT-BHQ1-3'. The ddPCR conditions were as described previously (43) with some minor amendments: we used 46 cycles of denaturation/annealing/extension and the annealing/extension temperature was 60°C. Intact HIV DNA was detectable in 97.0%, 3' defective HIV DNA in 76.5%, and 5' defective HIV DNA in 100% of the samples. QuantaSoft (version 1.7.4) was used for the data analysis. Positive and negative droplets were discriminated by manual thresholding.

Quantitative Viral Outgrowth Assay

Isolation of replication-competent virus was performed using CD4+ T-cell isolated PBMCs as described previously (50). PBMCs were thawed and CD4+ T-cells were isolated by negative selection using MACS Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). A median of 20.5×10^6 CD4+ T-cells (IQR 12.5-39) per sample were used for the quantitative viral outgrowth assay (qVOA). For the qVOA, CD4+ T-cells were prestimulated for 48 hours by anti-CD3 (immobilized) (1XE) and anti-CD28 (15E8, 3 mg/ml) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), in a humidified 10% CO2 incubator at 37 °C. Subsequently, the CD4+ T-cells were co-cultured with 2-day PHA-stimulated donor PBMC in IMDM supplemented with 10% (v/v) heat inactivated FCS, penicillin (100 U/ml), streptomycin (100 µg/ml) and IL-2 (20 U/ml; Chiron Benelux). Every 7-days, fresh PHA-stimulated donor PBMC were added to propagate the culture. Culture supernatants were regularly analyzed for viral replication using an in-house p24 antigen enzyme-linked immunosorbent assay (ELISA) (50).

Lymphocyte counts

CD4+ and CD8+ T-cell counts were determined using cytometry at the study sites.

HLA typing

HLA genotyping was performed at the Department of Immunogenetics (Sanquin) by the PCR using sequence-based typing (SBT) method (GenDx Products, Utrecht, the Netherlands) and real-time (RT)-PCR (Thermofisher, West Hills, California, USA).

Immune phenotyping of T-cells

PBMCs were used for immune phenotyping of CD8+ T cells. T-cell activation was defined as the proportion of cells positive for CD38 and HLA-DR; naïve T-cells as the proportion of CD45RA and CD27 positive cells, memory T-cells as proportion of CD45RA negative and CD27 positive cells and effector as proportion of CD45RA negative and CD27 negative cells. T-cell senescence was defined as CD27 and CD28 double negative cells. The following antibodies were used for staining: monoclonal antibody detecting CD3 (V500), CD4 (APC-H7), CCR7 (BV786)) from BD Biosciences (San Jose, USA); CD8 (Pacific Blue), CD45RA (BV605), CTLA4 (BV711), CD160 (PE-Cy7), CD28 (AF700) from BioLegend; and PD1 (PE) from eBioscience (San Diego, USA), HLA-DR (FITC), CD38 (PE), CD27 (APCeFluor 780), CD28 (PerCP Cy5.5), CD4+ (PE-Cy7), CD57 (APC)from BD Biosciences (San Jose, USA). The proportion of HIV-specific CD8+ T-cells was determined using APC-labeled MHC class I dextramers (Immudex, Virum, Denmark) carrying HLA-A*0201 SLYNTVATLY and HLA-B*0702 GPGHKARVL molecules in combination with CD3 (V500) and CD4 (APC-H7) from BD and CD8 (Pacific Blue) from BioLegend.

Fluorescence was measured on the FACS Canto II (BD Biosciences). The fractions of cells expressing a marker alone or in combination or the mean fluorescence intensity (MFI) were determined using FlowJo 7.6 (TreeStar, Ashland, Oregon).

Functional HIV cellular immune responses

An IFN-γ release assay was performed to evaluate the immune response upon HIV-peptide pool stimulation. A total of $0.5x10^6$ PBMCs were stimulated with HIV consensus B Env, Gag, Nef and Pol peptide pools (2 µg/ml, NIH AIDS Reagent Program) or cultured in medium alone as a control. After 1

day, culture supernatants were harvested and IFN-γ released by the cells was determined by human IFN-γ DuoSet ELISA (R&D Systems, Minneapolis, MN, USA).

The activation-induced marker (AIM) assay was performed to assess the frequency of reactive CD8+ T-cells. Therefore, PBMCs were stimulated for 6 hours with HIV peptide pools (Env, Gag, Nef and Pol) and then stained for flow cytometry. Reactive T-cells were determined by co-expression of CD137 (APC-H7/APC-Fire750) and CD69 (PE-Cy7) within the CD4+ and CD8+ T-cell populations, respectively. Fluorescence was measured on the FACS Canto II fluorescence-activated cell sorter (BD Biosciences). Marker expression levels were analyzed using FlowJo version 10.8.1 (TreeStar, Ashland, OR, USA).

Proliferation of CD8+ T-cells upon antigen stimulation was assessed through the use of the CellTrace™ VioleT-cell Proliferation kit (ThermoFisher). Cells were stained with CellTrace Violet according to manufacturer's protocol (0,5 uM final concentration) and flow cytometry analysis was used to determine that all the cells were labeled with Cell Trace Violet. Subsequently, the cells were stimulated with an HIV consensus B Gag, Env, Pol and Nef peptide pool (2ug/ml final concentration, NIH AIDS Reagent Program). An unstimulated control and positive controls using a peptide pool of CMVpp65 (2ug/ml final concentration, NIH AIDS Reagent Program) or α-CD3 in combination with α-CD28 were included. After 7 days cells were stained with FITC CD3, PerCP-Cy5.5 CD4+ (BD bioscience) and APC CD8+ (BioLegend) for 30 minutes at 4°C. After fixation of the cells with CellFIX (BD) samples were analyzed on the BD FACSCanto™ II to assess the proliferation of CD8+ T-cells under the different conditions. The proportion of proliferating cells was determined using FlowJo V10 (FlowJo). The precursor frequency is calculated as follows: per generation the amount of CD8+ T-cells that proliferated were calculated (number of cells * $2^{(generation)}$); the precursor frequency is the total number of CD8+ T-cells that proliferated per 100 CD8+ T-cells (total CD8+ T cells).

Statistics

Reservoir measurements and T-cell responses were compared between 24 weeks and 156 weeks after ART start using a paired non-parametric Wilcoxon signed rank test, and correlations between

reservoir outcomes and T-cell responses were calculated with Pearson correlation ($p<0.05$). A strong correlation was considered present if $p<0.01$.

Data availability

All data from this study is available upon request to PvP (p.vanpaassen@amsterdamumc.nl).

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Table 1. Baseline characteristics

	Total	Paired analysis *)
Number of participants	22	12
Demographics		
Male sex at birth	100%	100%
Median age at inclusion (years)	38 (IQR 28-48)	40.5 (IQR 25.5-49)
Country of birth		
The Netherlands	18	12
Other	4	0
Fiebig stage **) at diagnosis		
Fiebig II	1	0
Fiebig III/IV	12	7
Fiebig V	5	4
Fiebig VI	4	1
Median viral load baseline (x10^6 copies/ml)	0.4 (IQR 0.04-10)	0.61 (IQR 0.28-10)
Median CD4 count baseline (x10^6 cells/L)	445 (IQR 308-593)	435 (IQR 295-555)
Subtype HIV		
B	15	10
CRF_02AG	1	0
AE	3	1
F	2	1
Unknown	1	0
Timing start ART		
Time between diagnosis and start of ART (days)		
Same-day	15	8
1 day	3	1
2 days	2	1
Unknown	2	2

Table 1. Baseline characteristics of NOVA participants included in this study. *) participants of whom both a 24 and 156 week time point was available; **) Fiebig, E. W. et al. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 17, 1871–1879 (2003).

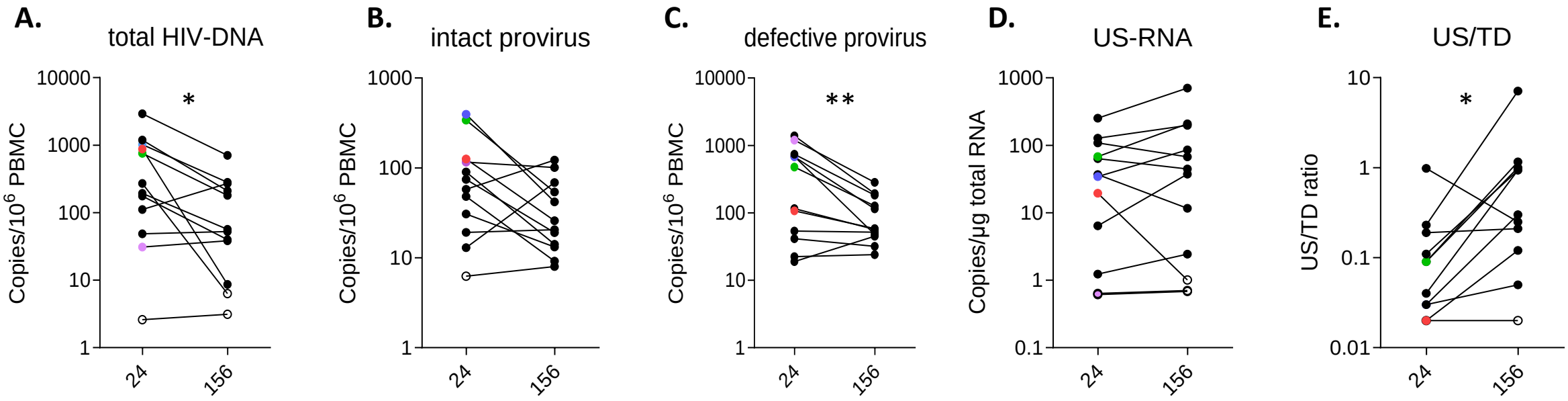


Figure 1. Paired viral reservoir assessments at 24 and 156 weeks (x-axis): Total HIV DNA (copies/million PBMC); Intact and defect provirus (copies/million PBMC); US RNA (cell-associated unspliced RNA copies/µg total RNA) ; US/TD (unspliced RNA/total DNA ratio). Open symbols represent values below the detection limit of the assay. The four participants with a positive qVOA at 24 weeks are marked with a color (purple, blue, green, red). Wilcoxon rank test (paired analysis) was performed to determine significant differences between the time points (* if $p<0.05$ or ** if $p<0.01$).

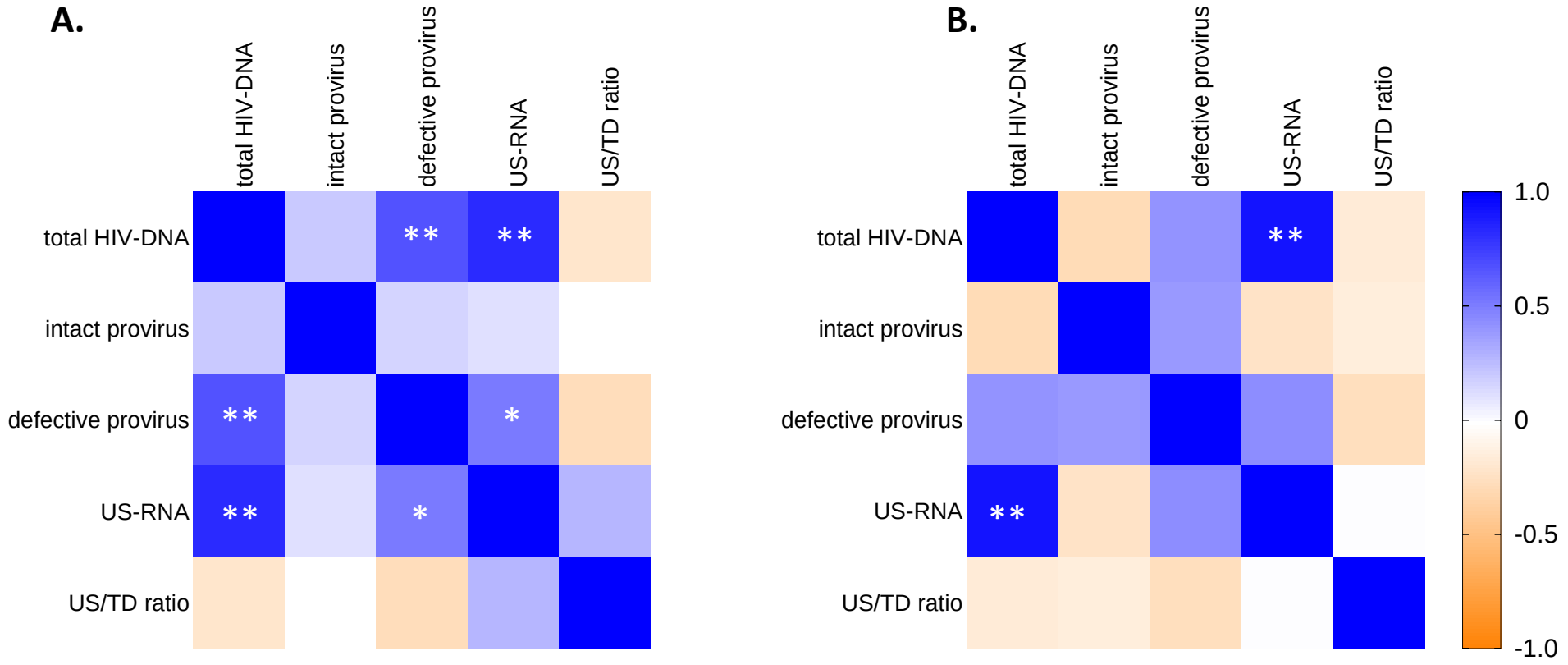


Figure 2. Correlation matrix of all viral reservoir assessments performed in the participants at 24 weeks (A) and 156 weeks (B) after start ART: Total HIV DNA (copies/million PBMC); Intact and defect provirus (copies/million PBMC); US RNA (unspliced RNA copies/µg total RNA; US/TD (unspliced RNA/total DNA ratio). The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.

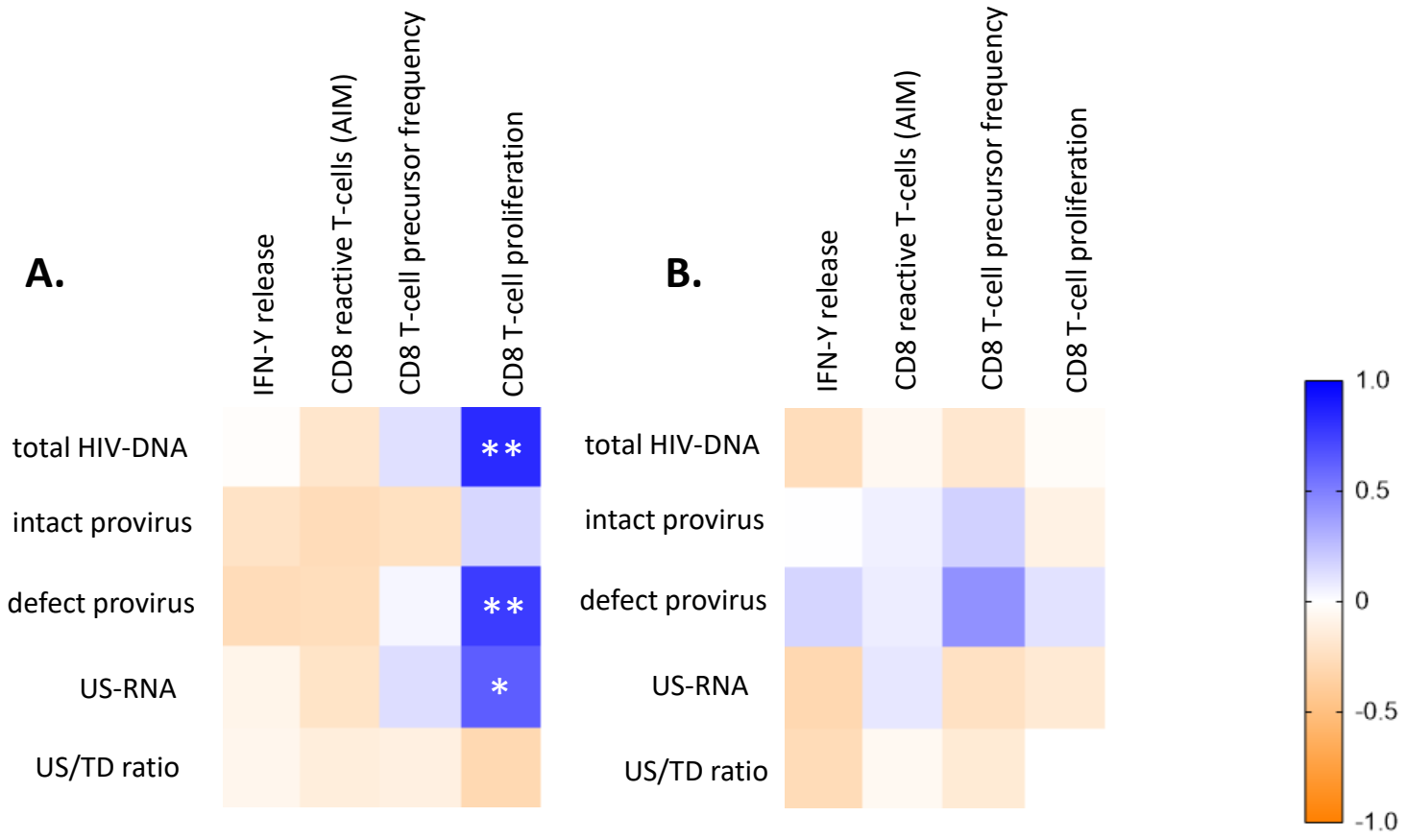


Figure 3. Correlation matrix of reservoir measurements and HIV-specific CD8+ T-cell responses as determined by IFN-Y release assay, AIM and proliferation assay (proliferating cells and precursor cells). The immune responses are defined as the sum of the responses to Env, Gag, Nef and Pol combined at 24 weeks (A) and 156 weeks (B). The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.

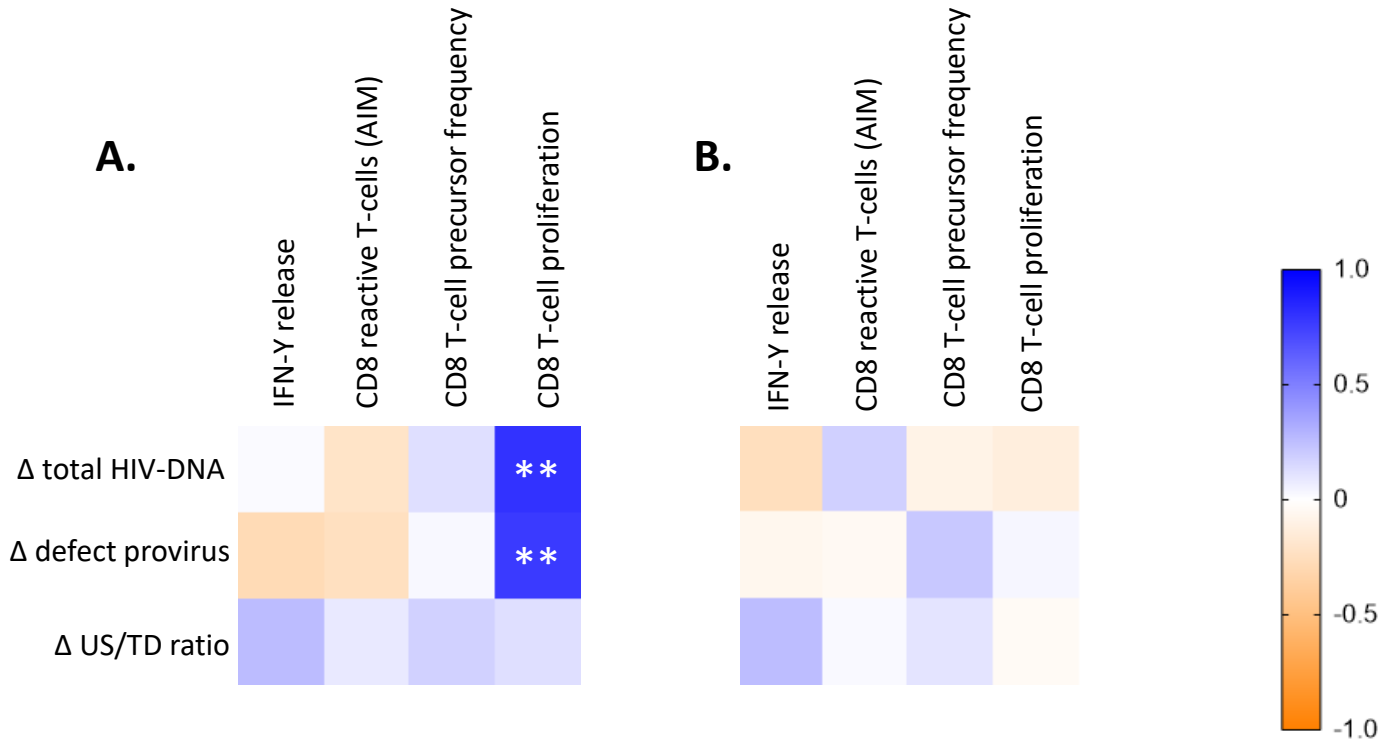
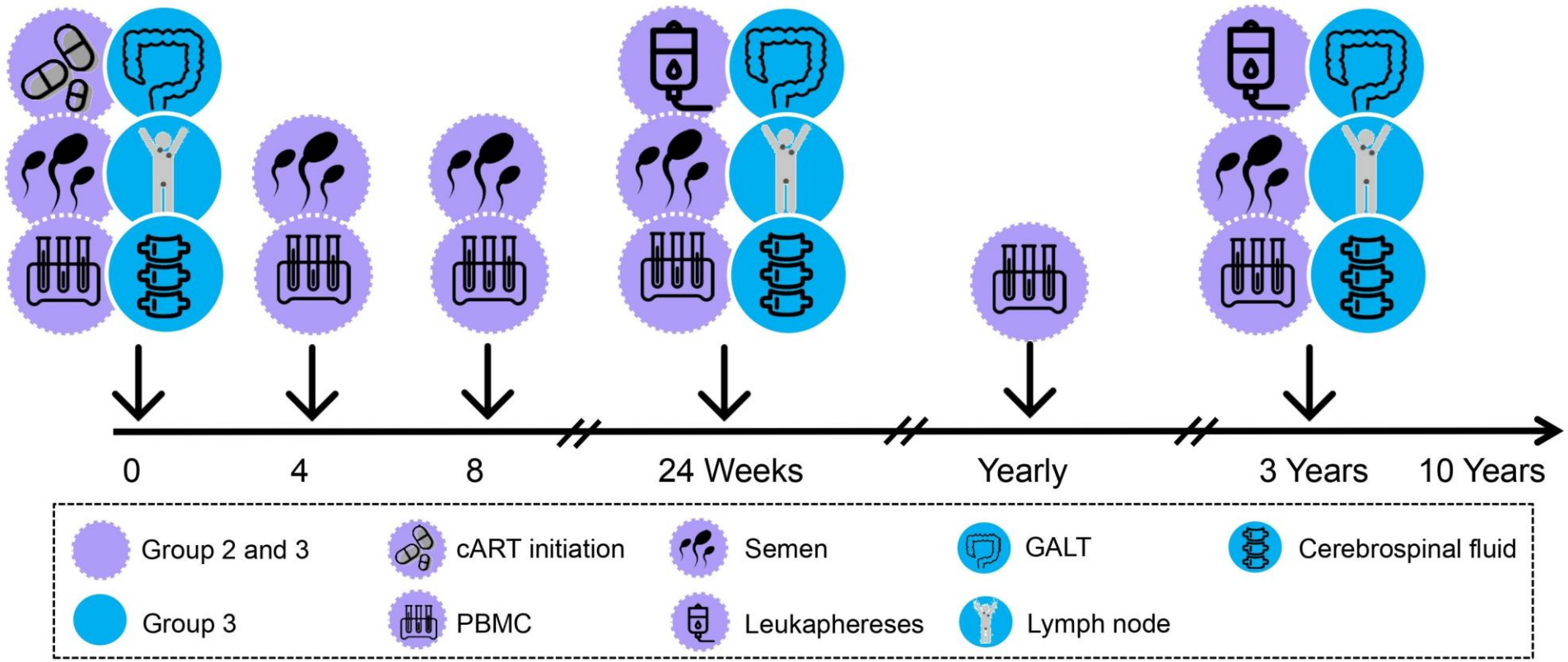
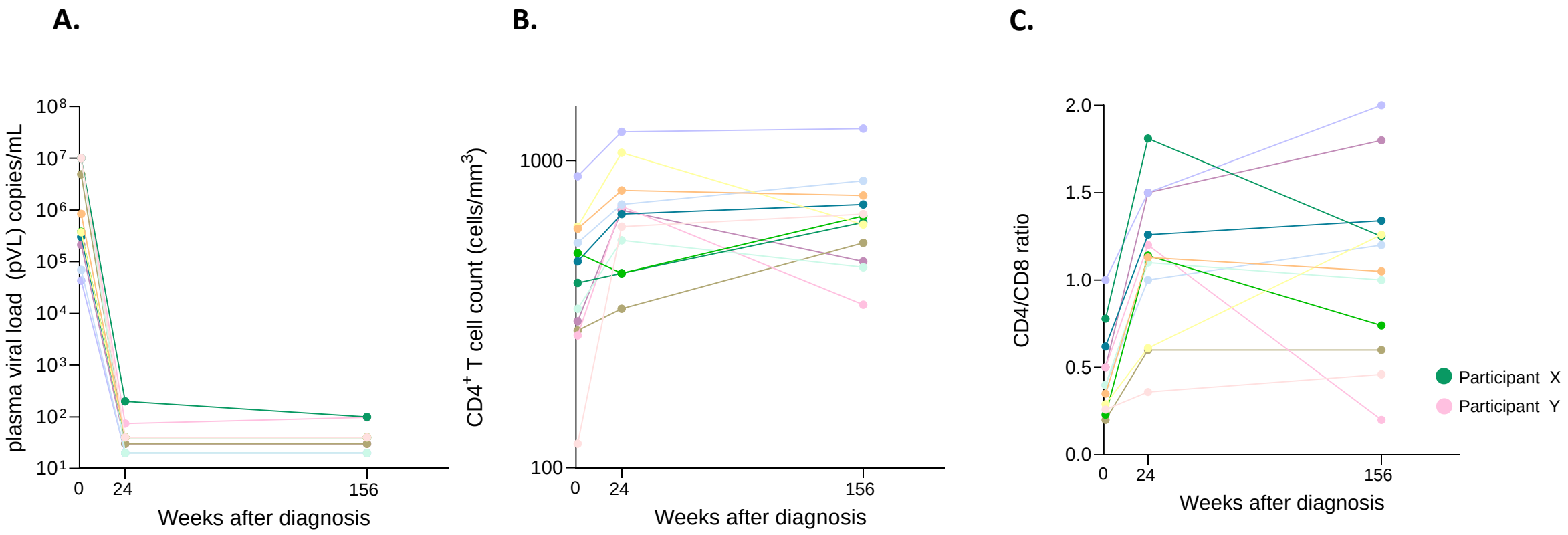


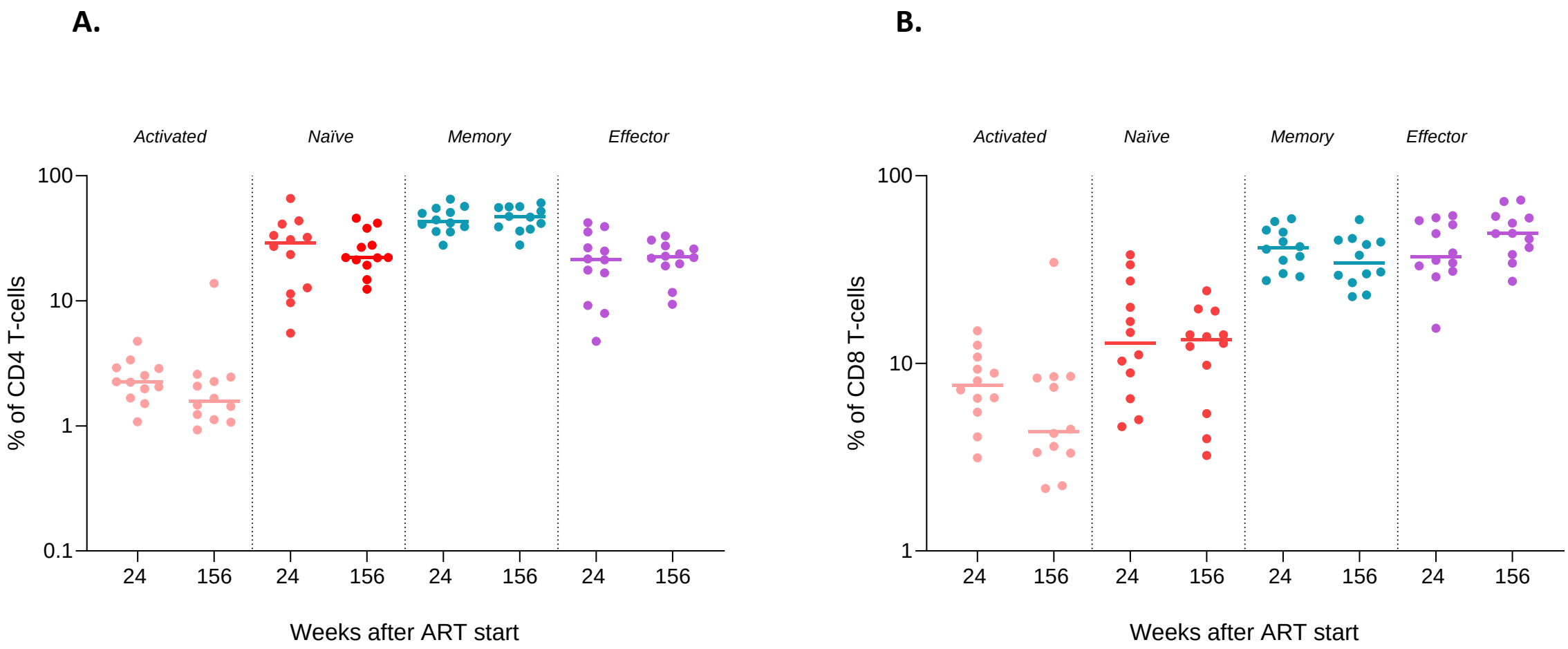
Figure 4. Correlation matrix of changes in reservoir size between 24 and 156 weeks (delta Δ) and HIV-specific CD8+ T-cell responses as determined by IFN-Y release assay, AIM and proliferation assay (proliferating cells and precursor cells) at 24 weeks (A) and 156 weeks (B). The immune responses are defined as the sum of the immune responses to Env, Gag, Nef and Pol combined. The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.



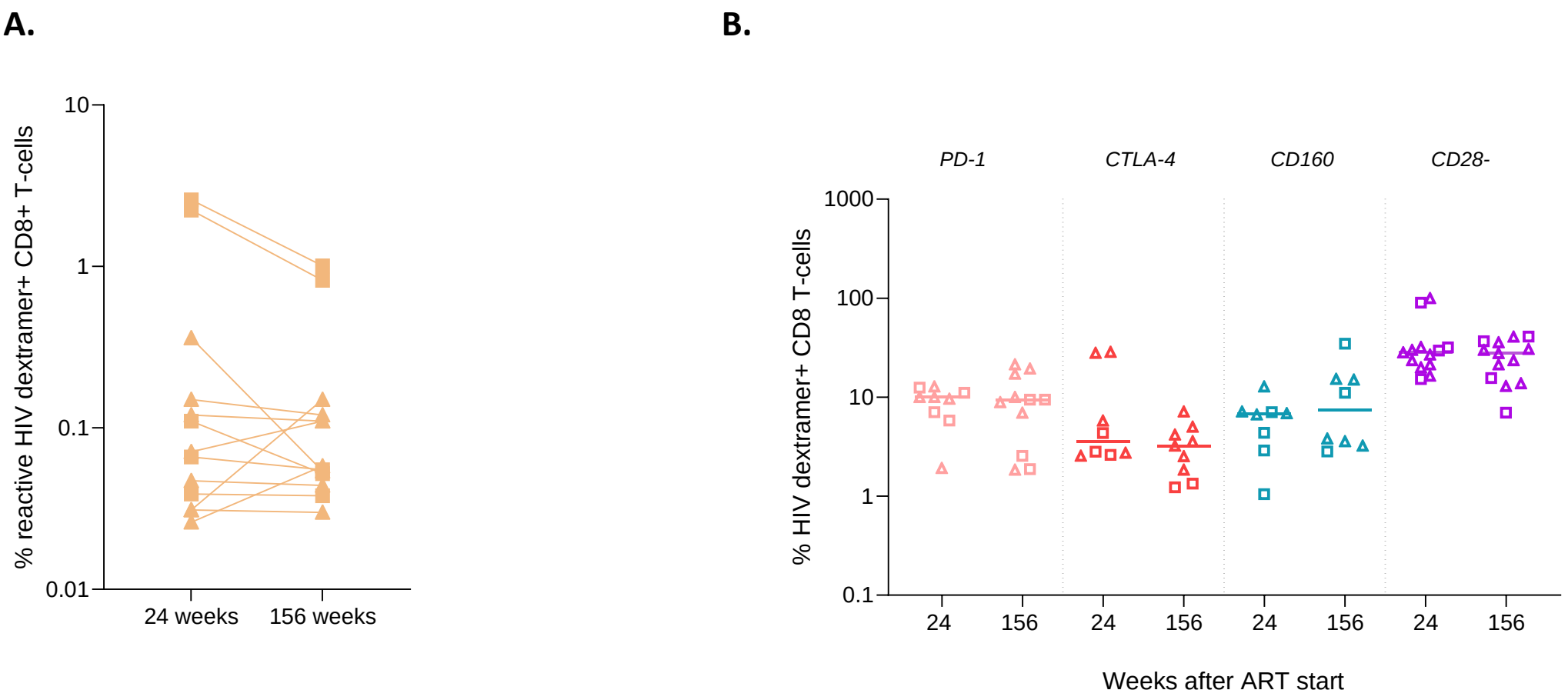
Suppl. Fig. 1. Overview of NOVA cohort study procedures for study groups 2 and 3. ART, combination antiretroviral therapy; GALT, gut-associated lymphoid tissue; PBMC, peripheral blood mononuclear cells. Retrieved from: *Dijkstra M. et al. Cohort profile: the Netherlands Cohort Study on Acute HIV infection (NOVA), a prospective cohort study of people with acute or early HIV infection who immediately initiate HIV treatment. BMJ Open. 2021;11(11):e048582.*



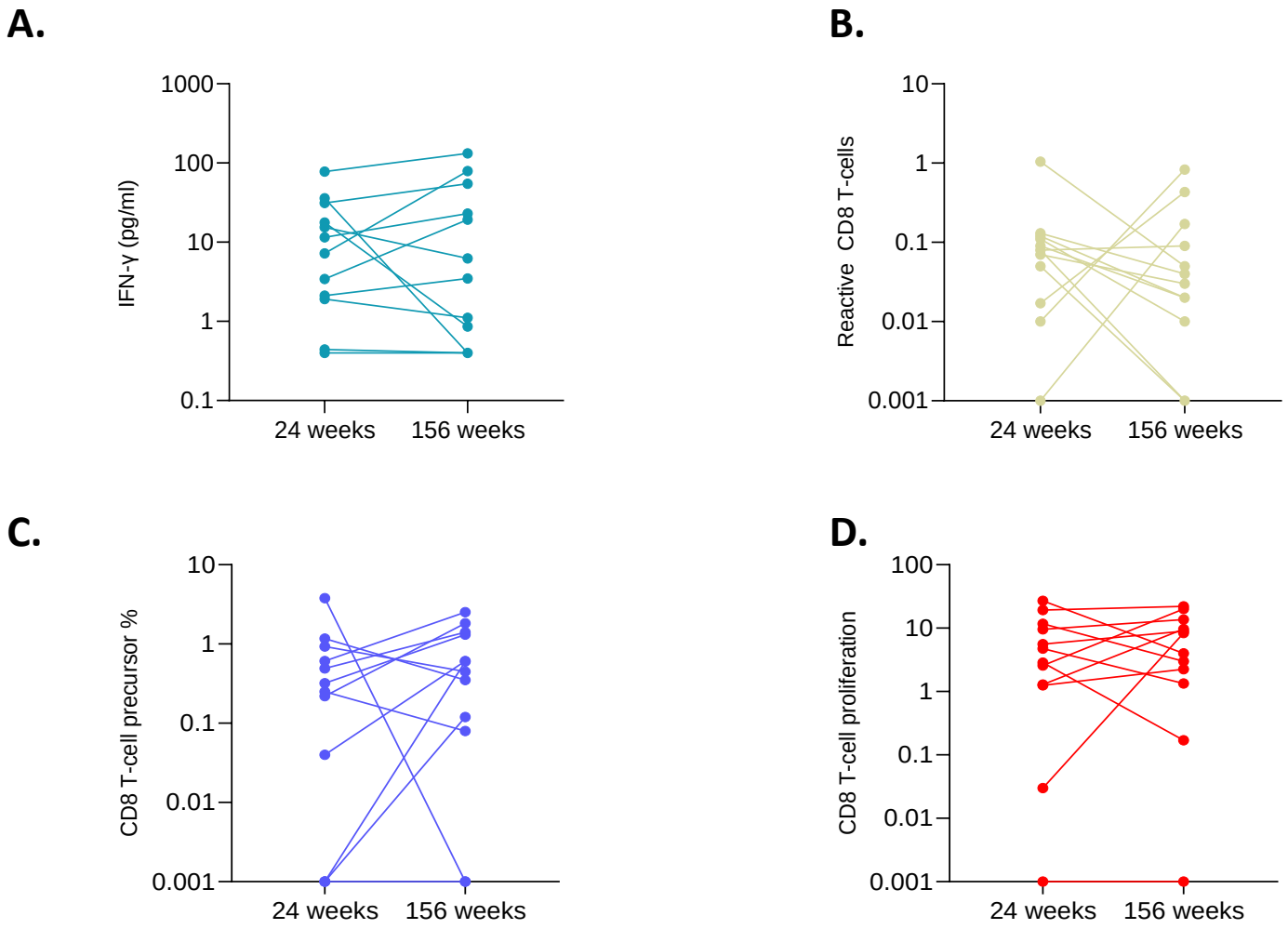
Suppl. Fig. 2. A. Plasma viral load (pVL) B. CD4 T-cell count (x10^6/mL) and C. CD4/CD8 ratio at baseline, 24 weeks and 156 weeks post ART initiation. Participant X (green dot) had detectable loads of 200 and 100 cp/mL at 24 and 156 weeks. Participant Y (pink dot) had detectable loads of 74 and 98 cp/mL at 24 and 156 weeks. Lower limit of quantification of the assays used ranged between 40 copies (earlier) and 20 copies (later time points) (see Methods).



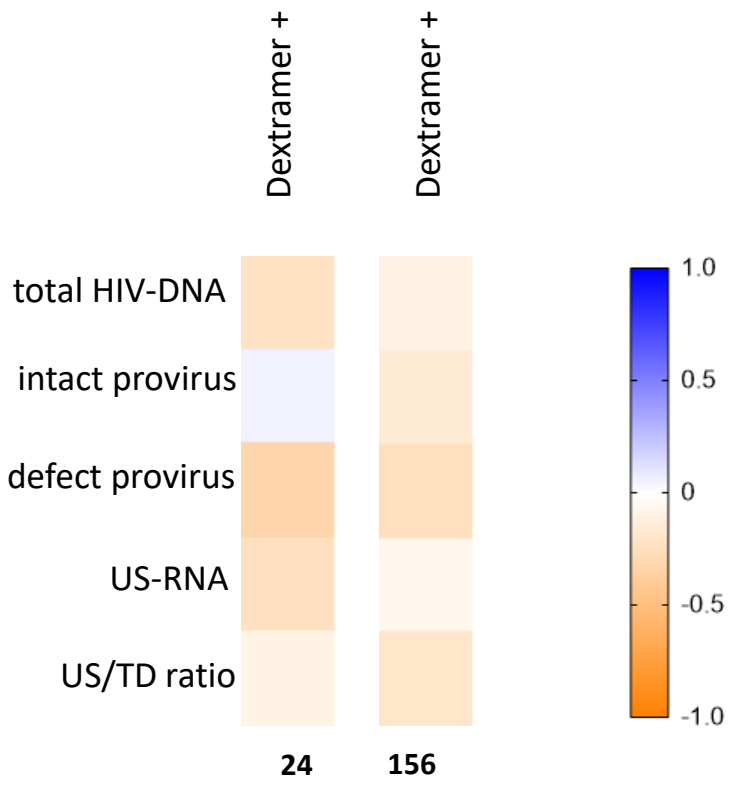
Suppl. Figure 3. Longitudinal analysis of frequencies of activated and naive, memory and effector subsets within the CD4+ (A) and CD8+ T-cell (B) populations. Wilcoxon signed rank test ($p<0.05$) was used to determine significant differences between the timepoints.



Suppl. Figure 4. A. The frequency of dextramer+ CD8+ T-cells at 24 and 156 weeks after ART. B. The expression of exhaustion markers (upregulation of PD-1, CTLA-4, and CD160 expression; loss of CD28 expression) on HIV-dextramer specific CD8+ T-cells at 24 and 156 weeks after ART. For participants that were HLA-A*2 and HLA-B*7 positive, HLA-A*2 and HLA-B*7 dextramer staining was included separately. HLA-A*2 dextramer is marked as a triangle and HLA-B*7 dextramer as a square in both figures.



Suppl. Fig. 5. HIV-specific T-cell responses upon HIV-peptide stimulation (Env, Gag, Nef, Pol) at 24 and 156 weeks after ART: IGRA/ IFN-γ release (A), AIM reactive CD8+ T-cells (B), precursor frequency (C) and the proportion of proliferating CD8+ T-cells (D). Frequencies of proliferating cells in response to Env, Gag, Nef and Pol peptides were combined. Overall, no significant difference in IGRA over time was observed.



Suppl. Figure 6. Correlation matrix of reservoir measurements and the frequency of dextramer+ CD8+ T-cell responses at 24 and 156 weeks after ART initiation. The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.

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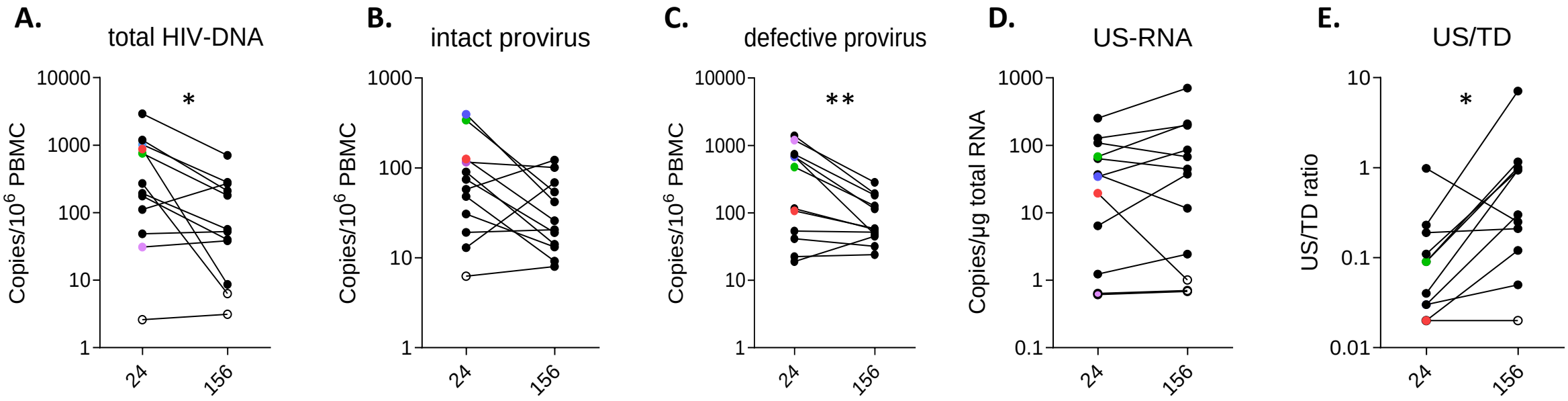


Figure 1. Paired viral reservoir assessments at 24 and 156 weeks (x-axis): Total HIV DNA (copies/million PBMC); Intact and defect provirus (copies/million PBMC); US RNA (cell-associated unspliced RNA copies/µg total RNA) ; US/TD (unspliced RNA/total DNA ratio). Open symbols represent values below the detection limit of the assay. The four participants with a positive qVOA at 24 weeks are marked with a color (purple, blue, green, red). Wilcoxon rank test (paired analysis) was performed to determine significant differences between the time points (* if $p<0.05$ or ** if $p<0.01$).

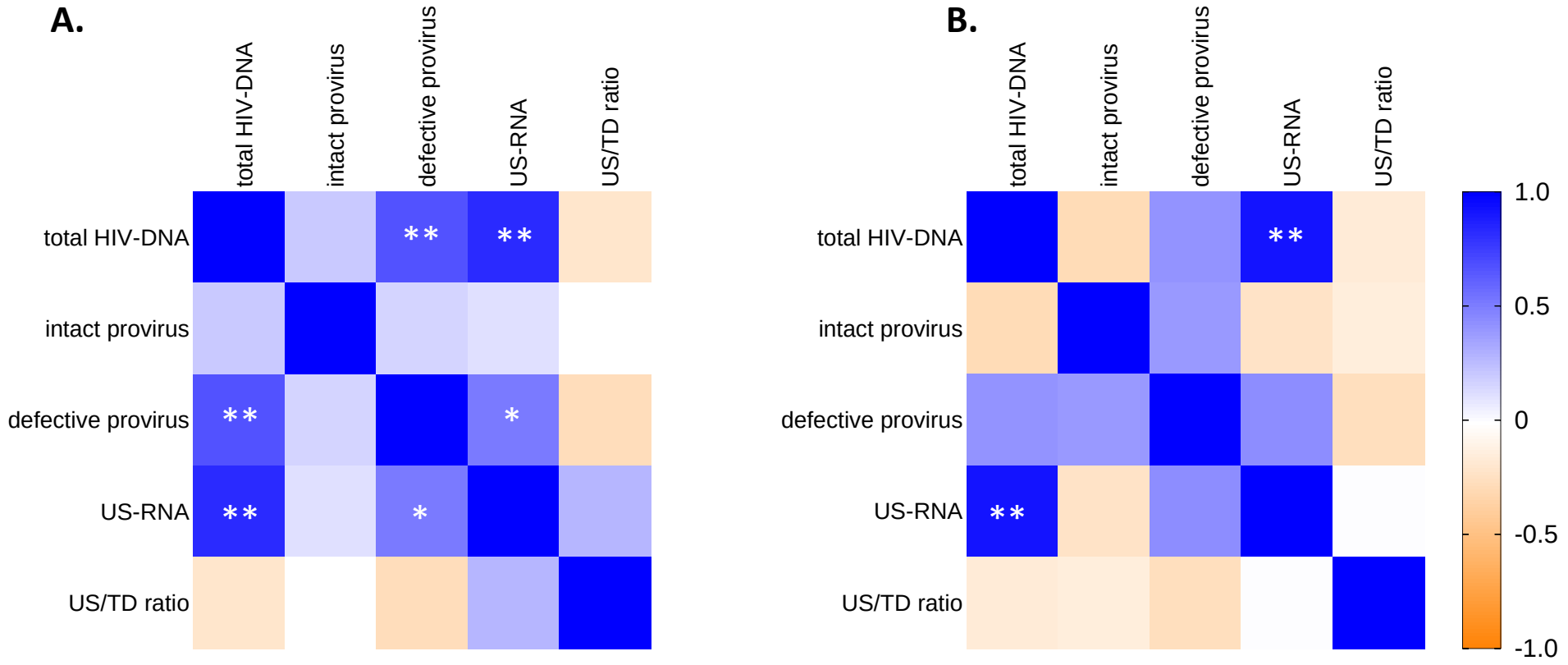


Figure 2. Correlation matrix of all viral reservoir assessments performed in the participants at 24 weeks (A) and 156 weeks (B) after start ART: Total HIV DNA (copies/million PBMC); Intact and defect provirus (copies/million PBMC); US RNA (unspliced RNA copies/µg total RNA; US/TD (unspliced RNA/total DNA ratio). The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.

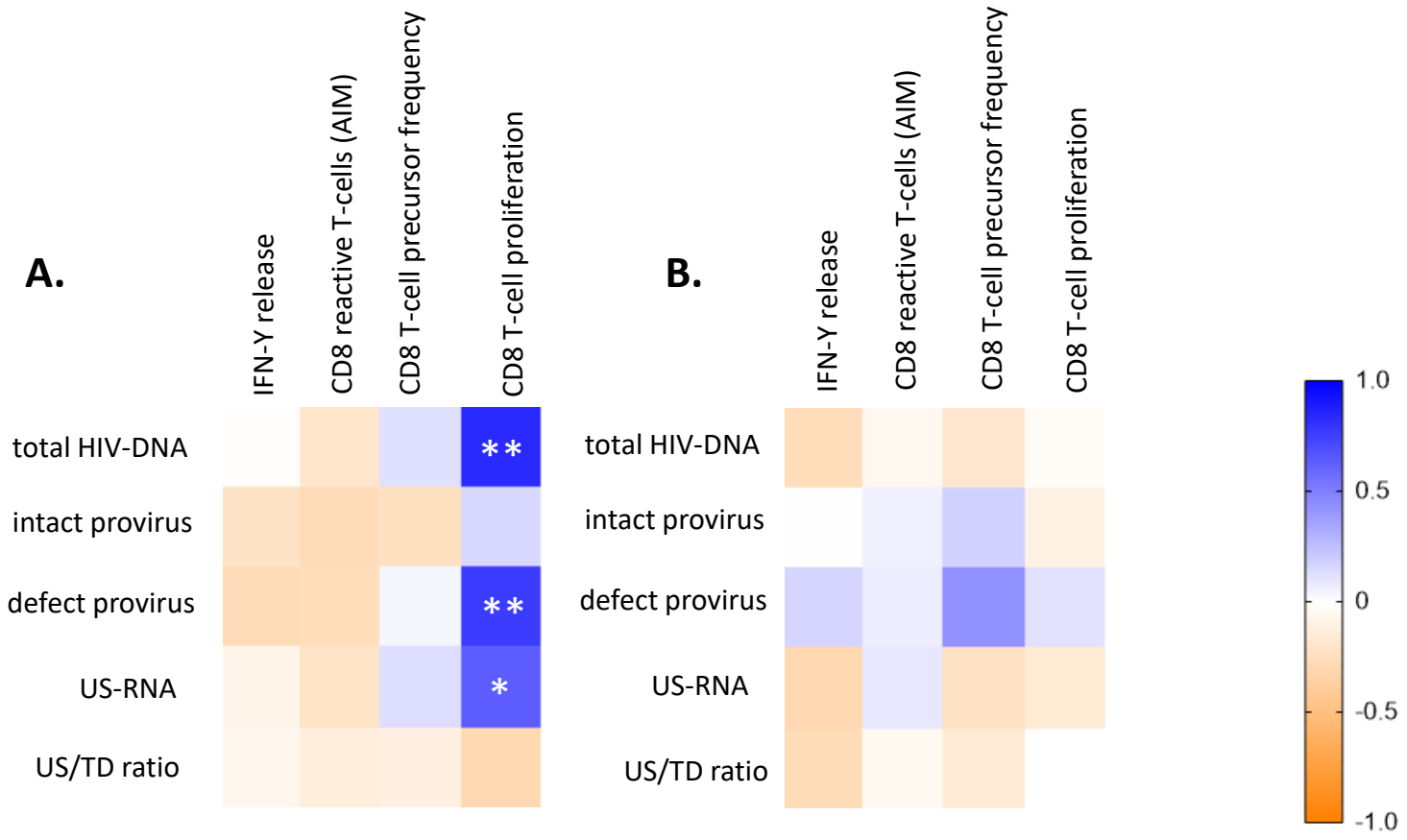


Figure 3. Correlation matrix of reservoir measurements and HIV-specific CD8+ T-cell responses as determined by IFN-Y release assay, AIM and proliferation assay (proliferating cells and precursor cells). The immune responses are defined as the sum of the responses to Env, Gag, Nef and Pol combined at 24 weeks (A) and 156 weeks (B). The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.

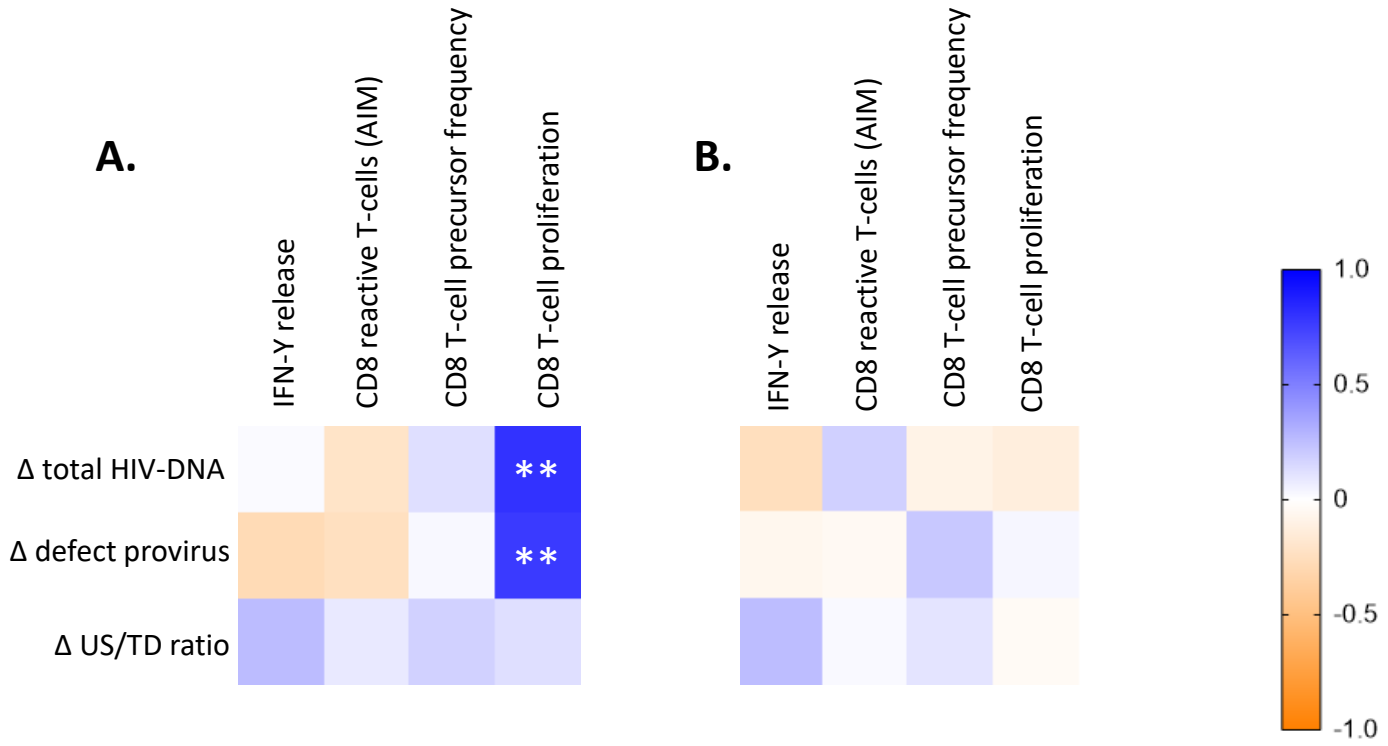
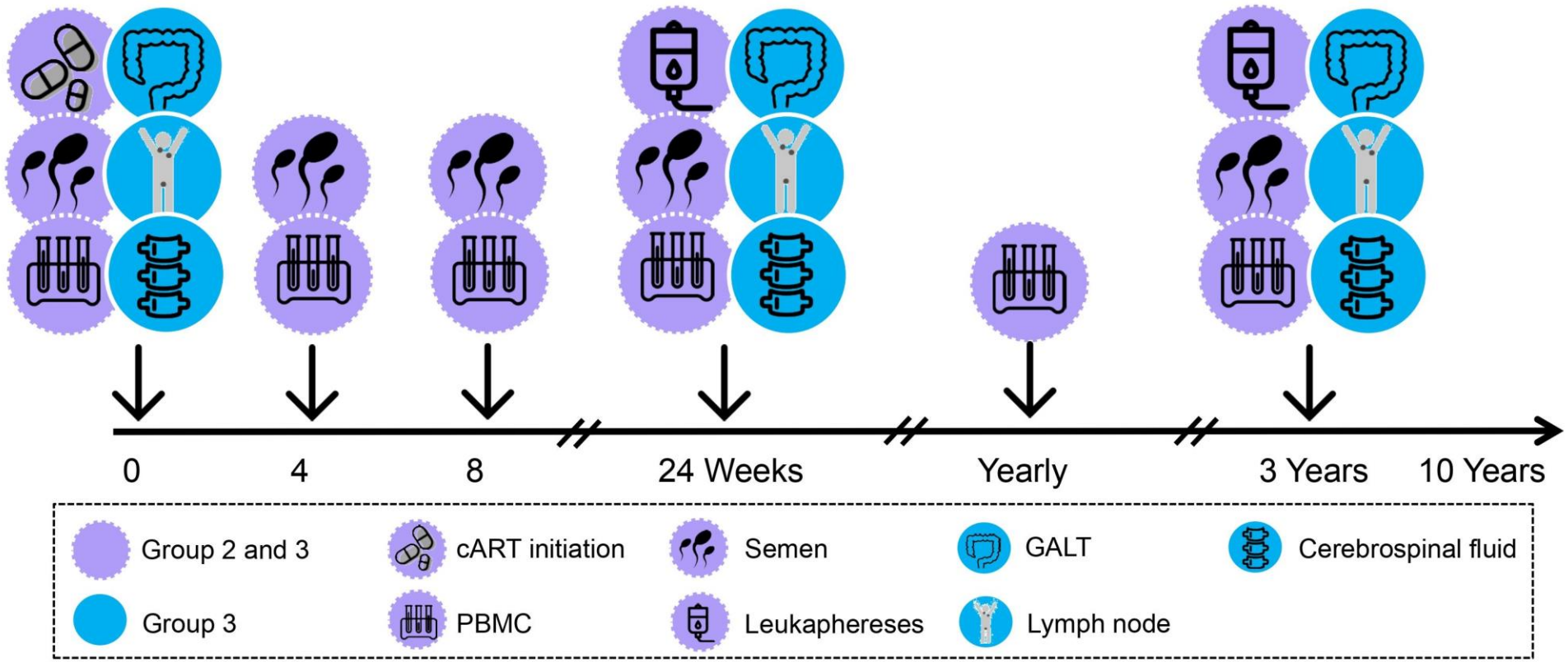
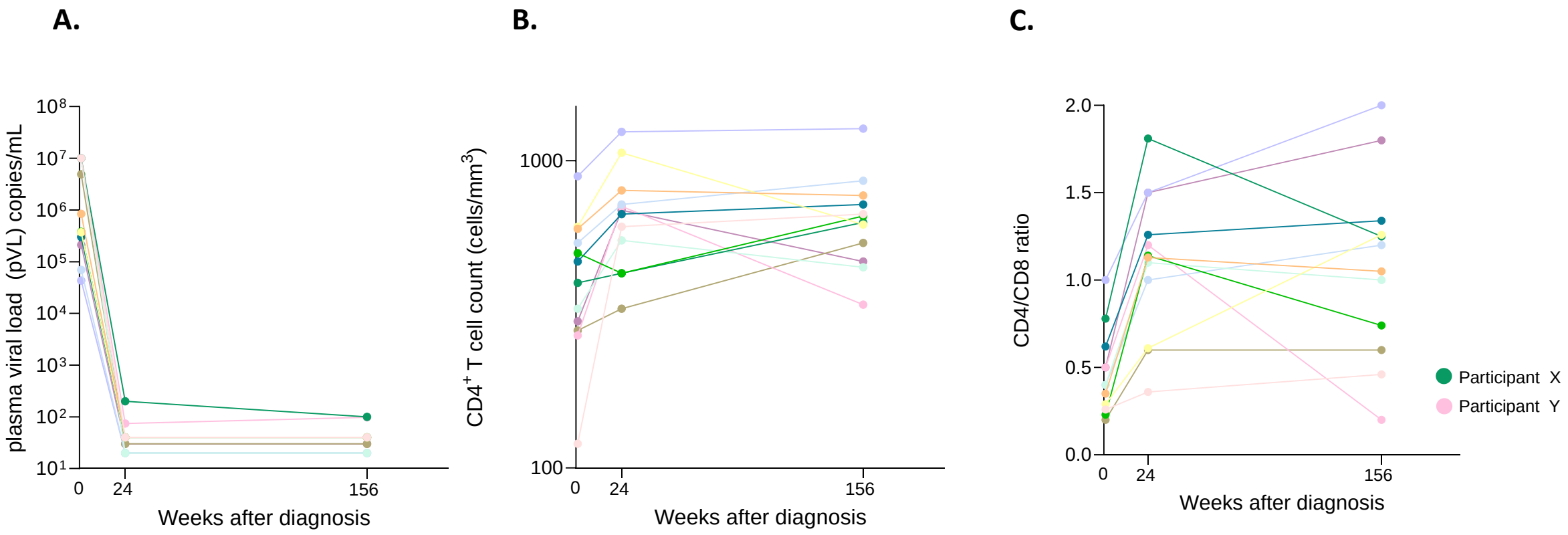


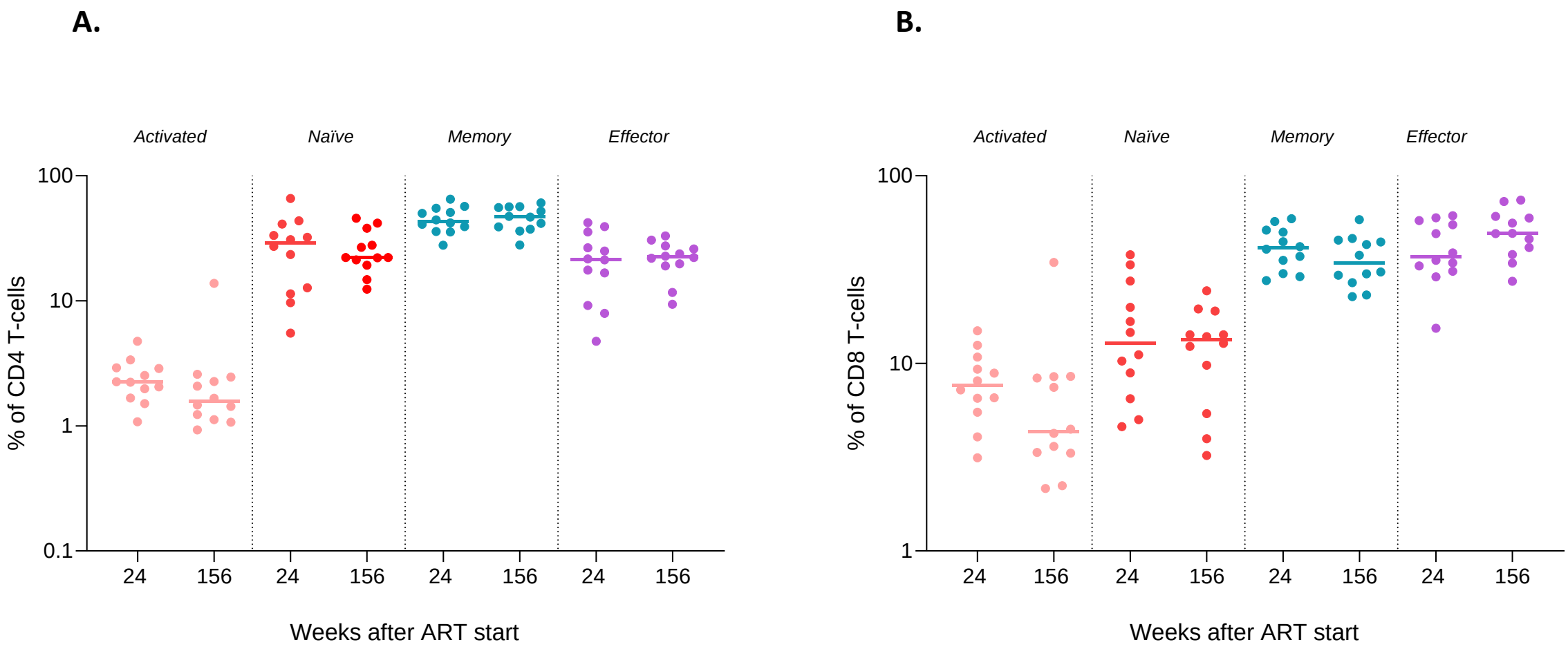
Figure 4. Correlation matrix of changes in reservoir size between 24 and 156 weeks (delta Δ) and HIV-specific CD8+ T-cell responses as determined by IFN-Y release assay, AIM and proliferation assay (proliferating cells and precursor cells) at 24 weeks (A) and 156 weeks (B). The immune responses are defined as the sum of the immune responses to Env, Gag, Nef and Pol combined. The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.



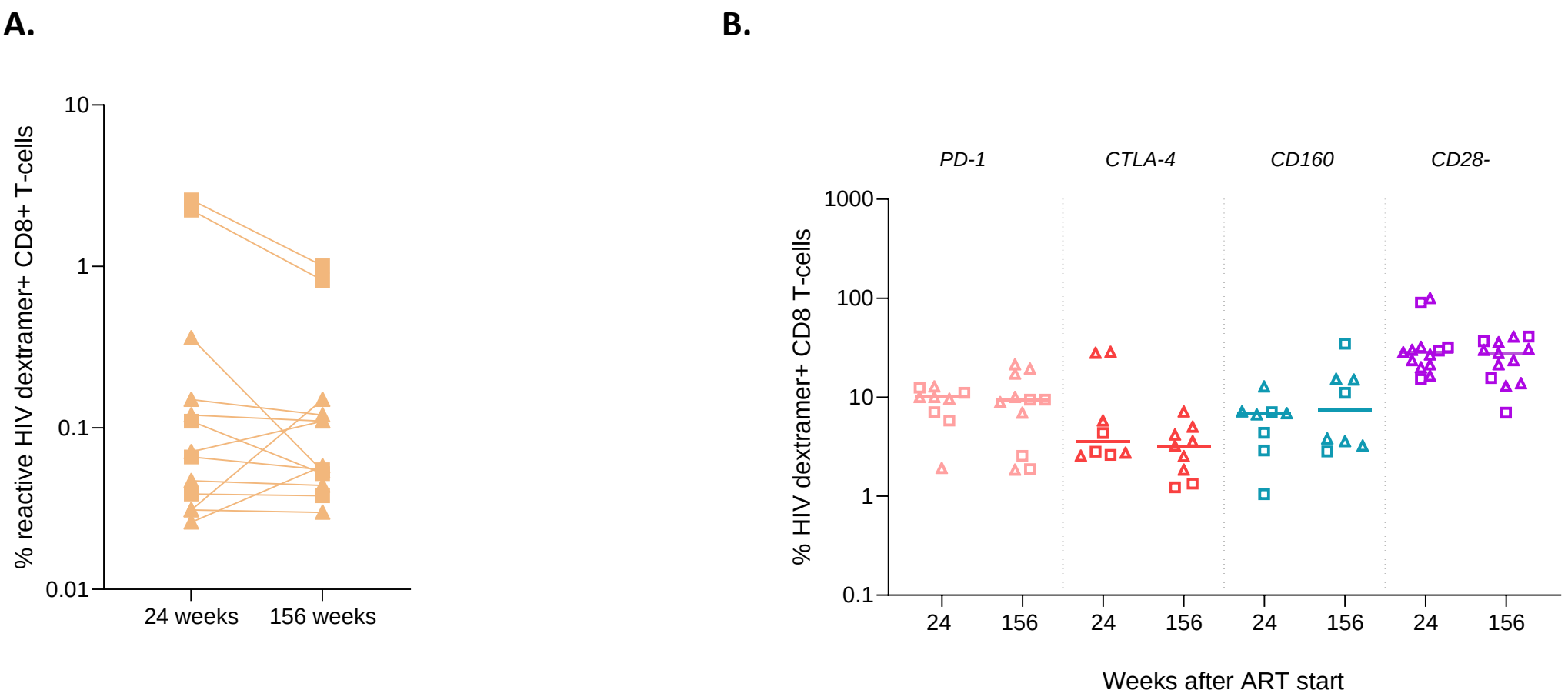
Suppl. Fig. 1. Overview of NOVA cohort study procedures for study groups 2 and 3. ART, combination antiretroviral therapy; GALT, gut-associated lymphoid tissue; PBMC, peripheral blood mononuclear cells. Retrieved from: *Dijkstra M. et al. Cohort profile: the Netherlands Cohort Study on Acute HIV infection (NOVA), a prospective cohort study of people with acute or early HIV infection who immediately initiate HIV treatment. BMJ Open. 2021;11(11):e048582.*



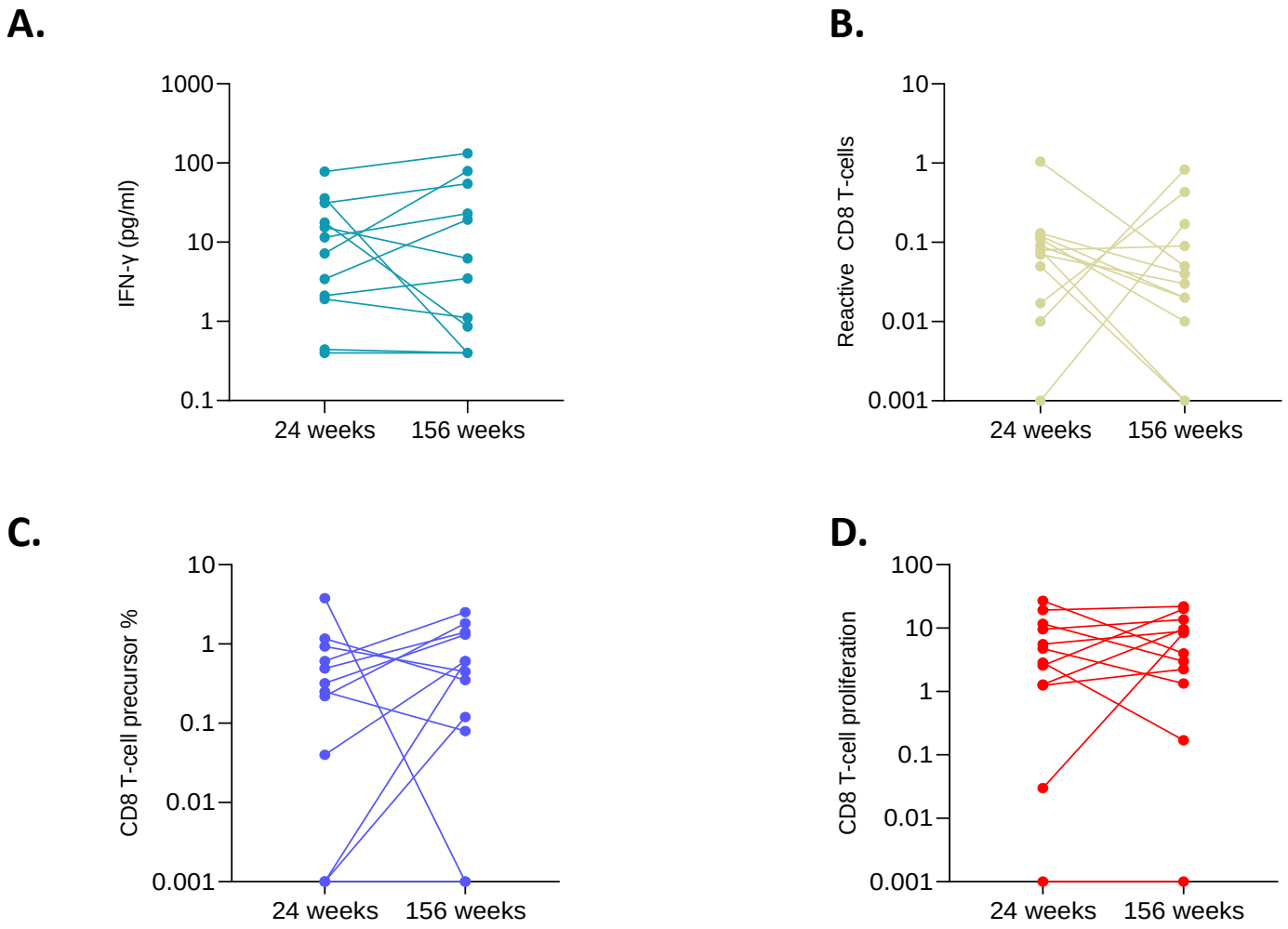
Suppl. Fig. 2. A. Plasma viral load (pVL) B. CD4 T-cell count (x10^6/mL) and C. CD4/CD8 ratio at baseline, 24 weeks and 156 weeks post ART initiation. Participant X (green dot) had detectable loads of 200 and 100 cp/mL at 24 and 156 weeks. Participant Y (pink dot) had detectable loads of 74 and 98 cp/mL at 24 and 156 weeks. Lower limit of quantification of the assays used ranged between 40 copies (earlier) and 20 copies (later time points) (see Methods).



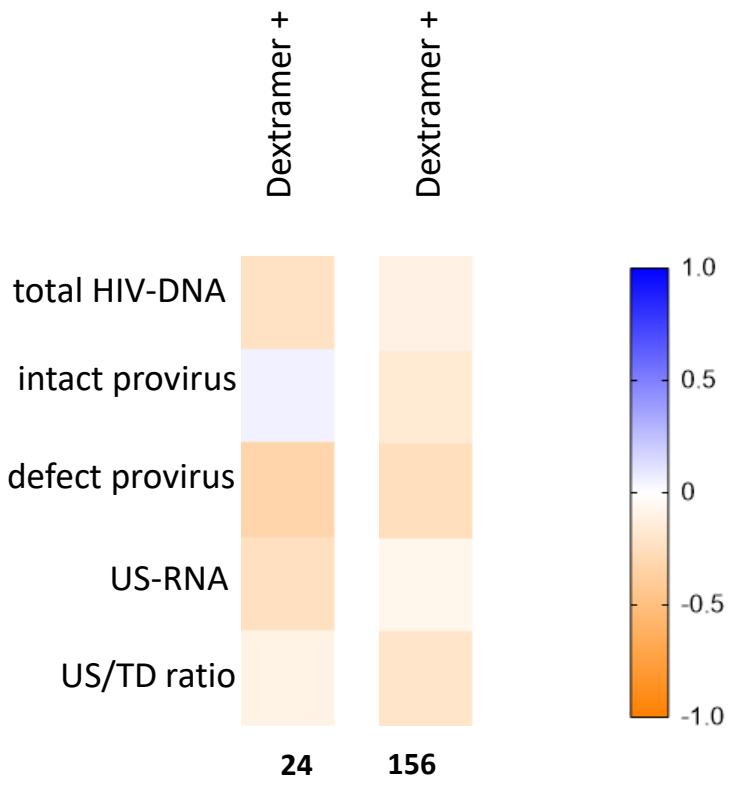
Suppl. Figure 3. Longitudinal analysis of frequencies of activated and naive, memory and effector subsets within the CD4+ (A) and CD8+ T-cell (B) populations. Wilcoxon signed rank test ($p<0.05$) was used to determine significant differences between the timepoints.



Suppl. Figure 4. A. The frequency of dextramer+ CD8+ T-cells at 24 and 156 weeks after ART. B. The expression of exhaustion markers (upregulation of PD-1, CTLA-4, and CD160 expression; loss of CD28 expression) on HIV-dextramer specific CD8+ T-cells at 24 and 156 weeks after ART. For participants that were HLA-A*2 and HLA-B*7 positive, HLA-A*2 and HLA-B*7 dextramer staining was included separately. HLA-A*2 dextramer is marked as a triangle and HLA-B*7 dextramer as a square in both figures.



Suppl. Fig. 5. HIV-specific T-cell responses upon HIV-peptide stimulation (Env, Gag, Nef, Pol) at 24 and 156 weeks after ART: IGRA/ IFN-γ release (A), AIM reactive CD8+ T-cells (B), precursor frequency (C) and the proportion of proliferating CD8+ T-cells (D). Frequencies of proliferating cells in response to Env, Gag, Nef and Pol peptides were combined. Overall, no significant difference in IGRA over time was observed.



Suppl. Figure 6. Correlation matrix of reservoir measurements and the frequency of dextramer+ CD8+ T-cell responses at 24 and 156 weeks after ART initiation. The correlation coefficient (R^2) determined by Pearson correlation is shown. Positive and negative correlations (R^2) are indicated by the color shade displayed in the legend. Significant correlations are indicated by * if $p<0.05$ or ** if $p<0.01$.