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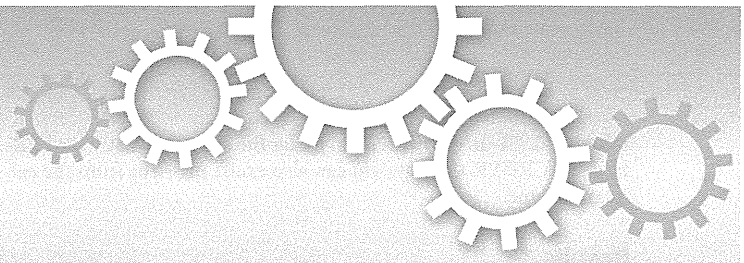

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Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus

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Even though highly active anti-retroviral therapy is able to keep HIV-1 replication under control, the virus can lie in a dormant state within the host genome, known as a latent reservoir, and poses a threat to re-emerge at any time. However, novel technologies aimed at disrupting HIV-1 provirus may be capable of eradicating viral genomes from infected individuals. In this study, we showed the potential of the CRISPR/Cas9 system to edit the HIV-1 genome and block its expression. When LTR-targeting CRISPR/Cas9 components were transfected into HIV-1 LTR expression-dormant and -inducible T cells, a significant loss of LTR-driven expression was observed after stimulation. Sequence analysis confirmed that this CRISPR/Cas9 system efficiently cleaved and mutated LTR target sites. More importantly, this system was also able to remove internal viral genes from the host cell chromosome. Our results suggest that the CRISPR/Cas9 system may be a useful tool for curing HIV-1 infection.

Integration of reverse transcribed viral DNA into the host cell genome is an essential step during the HIV-1 life cycle[1]. The integrated retroviral DNA is termed a provirus, which serves as the fundamental source of viral protein production. HIV-1 gene expression is regulated by LTR promoter and enhancer activities, where cellular transcription factors such as NF-κB, SP-1 and TBP bind to promote RNA polymerase II processivity. Subsequently, Tat protein is expressed from early double-spliced transcripts and binds to the trans activation response (TAR) region of HIV-1 RNA for its efficient elongation[2].

Latent infection occurs when the HIV-1 provirus becomes transcriptionally inactive, resulting in a latent reservoir that has become the main obstacle in preventing viral eradication from HIV-1 infected individuals. However, the mechanisms of viral silencing and reactivation remain incompletely understood[3]. Previous studies have suggested that the position of the integration site strongly influences viral gene expression and may be one of the determinants of HIV-1 latency[4]. While highly active anti-retroviral therapy (HARRT) has dramatically decreased mortality from HIV-1 infection, there is currently no effective strategy to target the latent form of HIV-1 proviruses[5].

Over the last decade, novel genome-editing methods that utilize artificial nucleases such as zinc finger nucleases (ZFNs)[6] and transcription activator like-effector nucleases (TALENs)[7] have been developed. These molecularly engineered nucleases recognize and cleave specific nucleotide sequences in target genomes for digestion, resulting in various mutations such as substitutions, deletions and insertions induced by host DNA repair machinery. These technologies have enabled the production of genome-manipulated animals in a wide range of species such as Drosophila[8], Zebrafish[9] and Rat[10]. However, ZFNs or TALENs remain somewhat difficult and time-consuming to design, develop, and empirically test in a cellular context[11]. Recently, a third genome-editing method was developed based on clustered regularly interspaced short palindromic repeat (CRISPR) systems. CRISPR systems were originally identified in bacteria and archaea[12] as part of an adaptive immune system, dependent on a complex consisting of CRISPR RNAs (crRNAs) and CRISPR-associated (Cas) proteins to degrade complimentary sequences of invading viral and plasmid DNA. Mali *et al.* created a novel version of the genome-editing tool applicable to mammalian cells, termed the CRISPR/Cas9 system, which is based on modifications of the *Streptococcus pyogenes* type II CRISPR system in crRNA fused to trans-encoded tracrRNA[13]. This CRISPR/Cas9 system is composed of guide RNA (gRNA) and a human codon-optimized Cas9 nuclease that forms an RNA-protein complex to digest unique target sequences matching those of gRNA. The CRISPR/Cas9 system can be utilized by simple transfection of designed gRNA and a humanized Cas9 (hCas9) expression plasmid into target mammalian cells, making it a promising tool for various applications.

In this study, we tested the ability of the CRISPR/Cas9 system to suppress HIV-1 expression by editing HIV-1 integrated proviral DNA. Cas9 and gRNA, designed to target HIV-1 LTR, were transfected and significantly

inhibited LTR-driven expression under the control of Tat. This LTR-targeted CRISPR/Cas9 system can also excise provirus from the cellular genome.

Results

LTR-specific editing by CRISPR/Cas9 components disrupts HIV-1 expression machinery. We designed a gRNA expression vector to target HIV-1 LTR under the control of the human U6 polymerase III promoter. U6 transcription of gRNA is initiated with guanine and requires the protospacer-adjacent motif (PAM)–NGG followed by a 20-base pair (bp) target sequence[13]. Accordingly, two gRNA-expressing plasmids were generated for targets 5 and 6 (T5 and T6), located in the TAR sequence of the R region and NF-κB binding sequence in the U3 region, respectively (Fig. 1 A), as described in methods. To test the genome editing activity of the CRISPR/Cas9 system, we used HIV-1 provirus-integrated human cells generated by an LTIG HIV vector, which expresses Tat and GFP proteins under the control of an LTR promoter, thus mimicking authentic HIV-1 gene expression[4]. To assess the impact of the CRISPR/Cas9 system targeting HIV-1 LTR, 293 T and HeLa cells were infected with an LTIG vector pseudotyped with VSV-G envelope protein. Then, the LTIG vector-infected cells were co-transfected with a T5 or T6 gRNA expression plasmid together with an hCas9 expression plasmid. Five days after transfection (TF), the mean fluorescence intensity (MFI) of GFP expression and percentage of GFP positive cells were analyzed by flow cytometry. In the 293 T cells, a clear reduction of MFI and GFP positive cells were observed by the CRISPR/Cas9 components (Fig. 1B and C). T5, the TAR-targeting gRNA, was more effective than T6 and reduced the average percentage of GFP positive cells from 45.6% to 20.0% ($p = 0.0003$) (Fig. 1C). Only a modest decline of GFP positive cells was observed in HeLa cells, while the MFI reduction was more drastic than that in 293 T cells (Fig. 1C), probably due to a lower TF efficiency of CRISPR components and a lower level of GFP expression in HeLa cells than in 293 T cells. These results suggested that the HIV-1 LTR targeting CRISPR/Cas9 system blocked HIV-1 gene expression from provirus LTR. Because the most efficient inhibition was obtained by T5 in both 293 T and HeLa cells, T5 was used for the further experiments.

To enhance the inhibition activity of the CRISPR/Cas9 system, we developed a protocol to transfect CRISPR components multiple times. 293 T cells were repeatedly co-transfected with T5 gRNA or gRNA empty and hCas9 plasmids, and flow cytometry analysis was performed five days after TF. As expected, the percentage of GFP positive cells was further reduced after multiple rounds of TF (Fig. 1D). Triple TF resulted in a significant decrease in the mean percentage of GFP positive cells from 40.8% to 2.1% ($p = 0.0001$) was observed. LTR fragments were then isolated from these cells using the primer set as indicated in Fig. 1A and cloned into a plasmid. Sequence analysis of the TAR region of plasmid DNA clones showed that 18 out of 22 HIV DNA clones contained various mutations, between 1 and 31-bp deletions from the end of the putative cleavage site (Fig. 2A). Two clones had a combination of deletion and insertion mutations (Fig. 2B). These mutation patterns are often observed as a result of DNA repair in the non-homologous end joining (NHEJ) pathway and are typical after genome editing[14], strongly suggesting that this T5 CRISPR/Cas9 component generated double-strand (ds) DNA breaks specifically at the HIV-1 TAR target site, and were repaired through the NHEJ pathway. These results clearly showed that the T5 CRISPR/Cas9 system efficiently produced mutations in the TAR region of proviral DNA.

CRISPR/Cas9 system can target the latent form of HIV-1 provirus in Jurkat cell. Because the putative latently infected cells are $CD4^+$ T cells, we next tested the genome editing potential of the CRISPR/Cas9 system in these cells. To test this, we generated two Jurkat clone cell lines, c5 and c19, that mimic HIV latency. These cell clones were isolated by limiting-dilution from cell populations that only expressed GFP after induction by either TNF-α or a combination of 5-Aza-dC/TSA. Both c5 and c19 were co-transfected with a T5 or gRNA empty vector and hCas9 expression plasmid. Four days later, cells were treated with TNF-α or a combination of 5-Aza-dC/TSA. The mean percentage of GFP positive cells after TNF-α induction was 92.56% and 98.48% after TF with gRNA empty vector in c5 and c19 cells, respectively. In contrast, the mean percentages of GFP positive cells were 68.78% in c5 and 66.95% in c19 cells, transfected with T5 gRNA (Fig. 3A and B). A similar reduction in GFP expression was observed in c5 and c19 cells transfected with the T5 CRISPR/Cas9 system after 5-Aza-dC/TSA treatment (Fig. 3A and B). These data suggest that the T5 CRISPR/Cas9 system produced cell populations, which were resistant to TNF-α and 5-Aza-dC/TSA stimulation.

To increase the efficiency of the T5 CRISPR/Cas9 system, we performed multiple TF of T5 and hCas9 expression plasmids. This approach significantly reduced the re-activation of latent provirus. As shown in Fig. 3C for c19, the percentage of re-activated latently infected cells was reduced from 97.8% to 35.5% after three rounds of TF (Fig. 3C, $p = 0.00002$). These results clearly demonstrated that the T5 CRISPR/Cas9 system was able to prevent the re-activation of latently integrated provirus in T cells.

CRISPR/Cas9 system removes HIV-1 internal genes. Retrovirus proviral DNA contains duplicate LTR regions on both ends of the integrated viral genome, meaning that the CRISPR/Cas9 system may simultaneously cleave both LTRs and remove an internal region of integrated proviral DNA from the host cell genome. To examine this possibility, we used cells transduced with an alternative HIV-1 vector, missing the U3 region of the LTR and possessing an internal elongation factor-1 (EF) promoter cassette for GFP expression (Fig. 4A). Because GFP expression is driven by an independent EF-promoter, it should be unaffected by the genome-editing system targeting the LTR region of integrated proviral DNA. Therefore, the GFP negative cell populations may be the result of proviral excision. Moreover, since this HIV vector lacks the NF-κB binding site in the U3 region, these cells should be resistant to T6 and not T5-mediated targeting. As expected, only T5 CRISPR/Cas9 components clearly reduced the cell populations expressing GFP (Fig. 4 A, $p = 0.0014$). Double TF of these components resulted in a further decrease of average of GFP positive cells from 60.44% to 50.45% ($p = 0.0010$).

Next, we performed quantitative PCR (qPCR) analysis using c19 Jurkat cells that harbored latently integrated HIV-1 proviral DNA, the LTIG vector. As shown in Fig. 3, the T5 CRISPR/Cas9 system significantly reduced LTR-driven GFP expression. We reasoned that if the inhibition is purely a result of LTR mutations, then, the *EGFP* DNA copy number in the host cell genome should be unchanged following T5 CRISPR/Cas9 treatment. Alternatively, if the reduction of GFP expression is partially attributed to the excision of integrated viral DNA, the amount of *EGFP* DNA will be reduced after T5 CRISPR/Cas9 treatment. For this assay, the c19 cells from Fig. 3C were used. The result of qPCR analysis clearly showed that the relative amount of *EGFP* DNA was decreased by the T5 but not gRNA empty CRISPR/Cas9 treatment (Fig. 4B). On average, 31.8% of the provirus was excised from the host cell genome after CRISPR/Cas9 components were transfected three times. To obtain direct evidence of the provirus-excision effect by CRISPR, PCR analysis using the host cell genome-specific primers, flanking the proviral integration site, was performed. After determining the integration sites of c19, chromosome 16 in latent LTIG-transduced Jurkat cells (Fig. 4D bottom), we designed a primer set specific for both sequences adjacent to the integration site of c19 provirus. The excision of provirus is predicted to leave a footprint of one LTR and the PCR we performed was

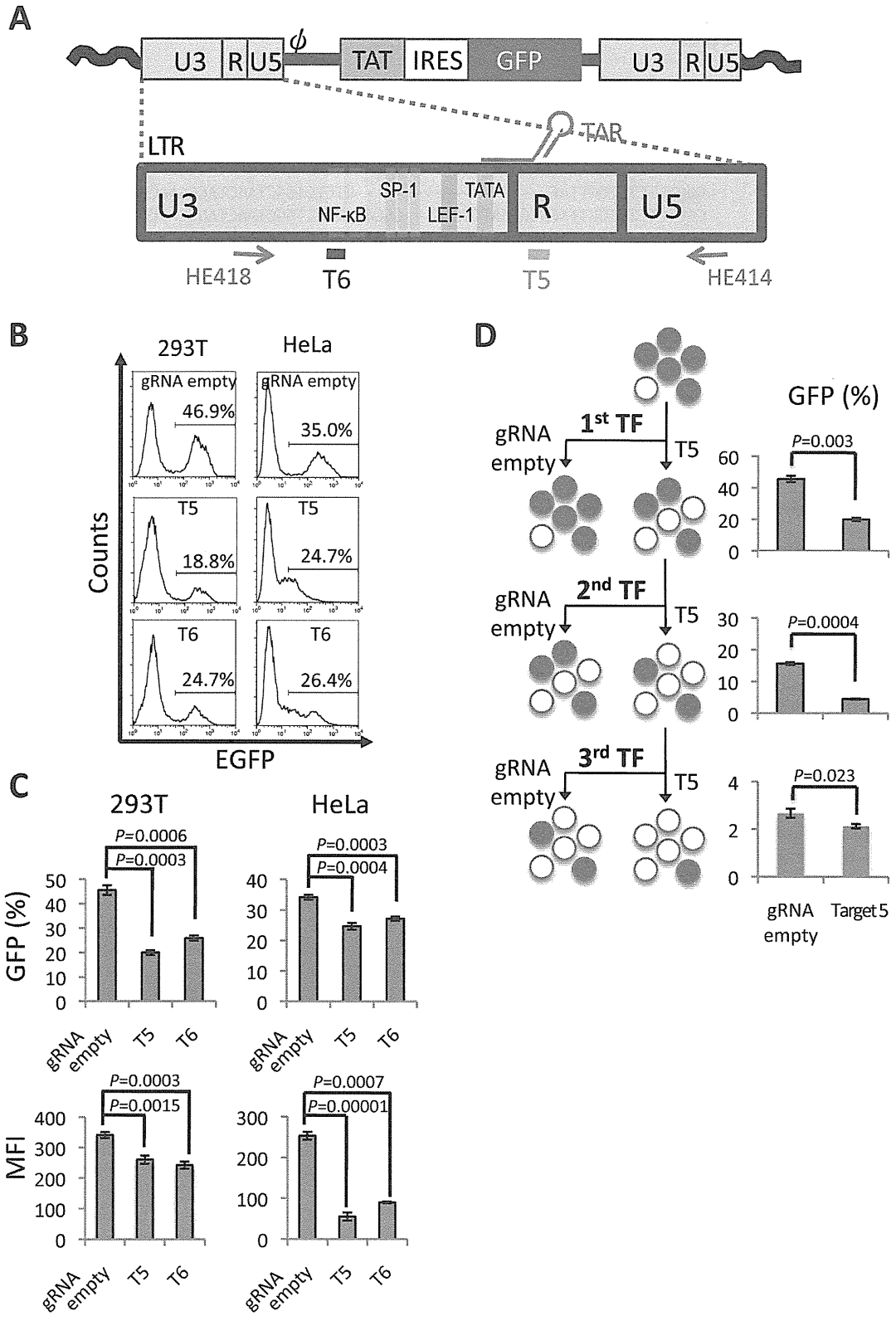

Figure 1 | Suppression of HIV-1 gene expression by the CRISPR/Cas9 system targeting HIV-1 LTR. (A) Schematic of provirus derived from LTIG vector and the LTR. Two target sites of CRISPR/Cas9 were indicated at the bottom of the LTR. (B and C) GFP expression after CRISPR/Cas9 treatment. 293 T and HeLa cells were infected with LTIG vector pseudotyped with VSV-G protein. One week after infection, the cells were co-transfected with gRNA expression vector and hCas9 expression vector. The level of GFP expression was analyzed by flow cytometry at 5 days after TF. (B) Representative histograms are shown. (C) GFP positive percentages (top, n = 3), and MFIs (bottom, n = 3) are shown. (D) Percentage of GFP positive 293 T cells after multiple TF with the CRISPR/Cas9 system. Flowchart of the experiment is depicted on the left side. The percentage of GFP positive cells resulting from single, double and triple TF is shown on the right side. The error bars in C and D show standard deviations (n = 3).

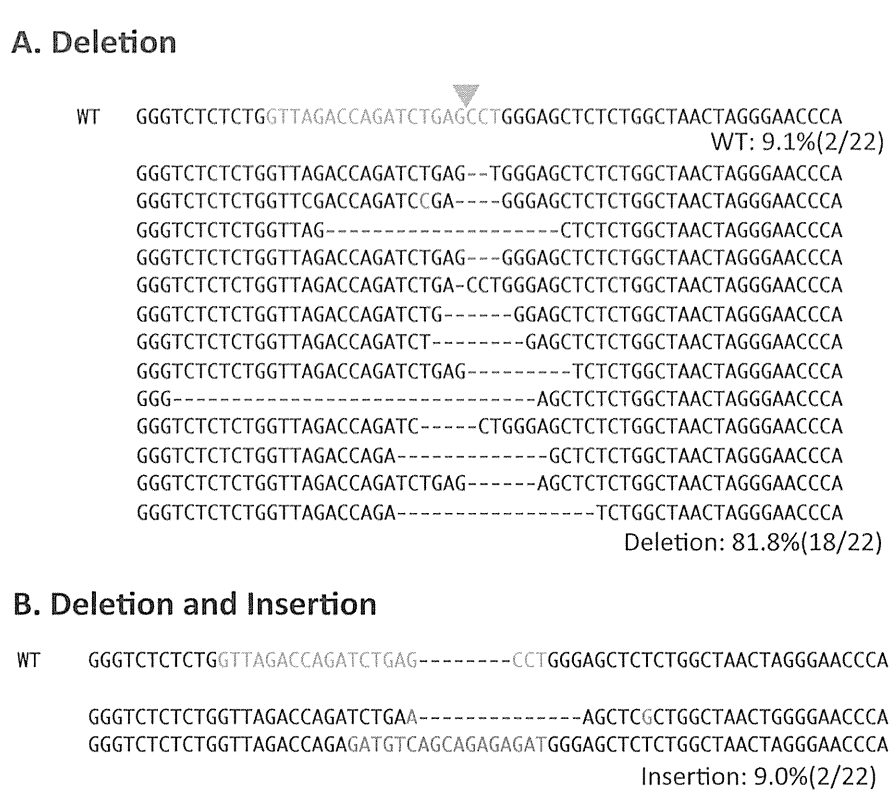

Figure 2 | Sequencing analysis of the CRISPR/Cas9-target site. DNA sequence of the TAR region of LTR is indicated. Twenty-two sequences were obtained from LTIG-infected 293 T cells which were transfected three times with T5 gRNA. The WT reference sequence is shown on the top. The target sequence of T5 is indicated in orange. The putative cleavage site is indicated with an arrowhead. (A) TAR sequence with deletions. Eighteen out of 22 clones were deletion mutants. (B) TAR sequence with both deletions and insertions. Two out of 22 clones were mutants consisting of insertion and deletion. Red and blue colors indicate insertion and unanticipated mutation, respectively.

able to detect the apparent footprint as an additional 1,067 bp fragment only in the T5 CRISPR/Cas9-transfected cells (Fig. 4C). Furthermore, sequence analysis of this PCR product confirmed that excision of provirus resulted in one LTR footprint with a variety of mutations from the cleavage site (Fig. 4D). These results clearly demonstrate that the CRISPR/Cas9 system targeting HIV-1 LTR has the potential to excise latent form of HIV-1 proviral DNA from the host cell chromosome.

Discussion

In this study, we successfully disrupted the expression of HIV-1 provirus utilizing the CRISPR/Cas9 system (Fig. 1). Importantly, this disruption not only restricted transcriptionally active provirus, it also blocked the expression of latently integrated provirus (Fig. 3). Cas9 proteins are predicted to contain RuvC and HNH motifs[15], which possess autonomous ssDNA cleavage activity. Interestingly, mutants lacking one of the motifs become nicking endonucleases[16]. It is plausible that the independent nicking activity of each domain may enhance efficient access to the heterochromatin state of latently integrated provirus. Another possibility is that Cas9 has a highly efficient target surveillance system similar to what has been previously reported for the Cas3 system[17].

T6 gRNA that targeted the NF-κB binding site, also strongly suppressed the LTR promoter activity (Fig. 1). However, the effect was weaker than that of T5 gRNA. In this study we used an LTIG vector modified from the LTR of HIV-1 strain NL4-3 that possesses two adjacent NF-κB binding sites[18]. The T6 target site is at the end of the 5′ NF-κB binding site, meaning that mutations may not completely render transcription inactive since the 3′ NF-κB binding site may remain functional. On the other hand, T5 gRNA that targeted TAR, is profoundly effective in disrupting HIV-1 gene expression. The putative cleavage site was positioned at the neck of the stem loop region of TAR, which is critical for Cyclin T1-Tat-TAR ternary complex formation[19]. Therefore, the TAR sequence may be one of the best targets for blocking HIV-1 provirus expression. Target specificity of the CRISPR/Cas system is very high and a single mutation can disrupt targeting[20], meaning that some provirus may escape from this genome-editing machinery if mutations arise in target sequences. However, given that the TAR region is relatively conserved and there is little variation among HIV-1 subtypes[21], it could still be an appropriate target for the elimination of latently infected provirus.

Perhaps the most important finding in this study is that we could excise provirus from the host genome of HIV-1 infected cells, which may provide a ray of hope to eradicate HIV-1 from infected individuals. However, there are numerous hurdles that must be cleared before utilizing genome editing for HIV-1 eradication therapies such as gene therapy. First, the efficiency of genome-editing and/or proviral excision should be quantified in HIV infected primary cells, including latently infected $CD4^+$ quiescent T cells. Second, an efficient delivery system must be developed. Fortunately, the CRISPR/Cas9 system has the advantage in size compared with TALENs[22]. Thus, the CRISPR system has the potential to be delivered by lentivirus vectors, whereas TALENs do not because of their large size and repeat sequences[23]. The final hurdle concerns possible off-target effects, which are pertinent concerns for all genome-editing strategies that may lead to nonspecific gene modification events. If Cas9 has off-target effects, then removal of the off-target activity may be the best approach before utilizing CRISPR/Cas system for anti-HIV treatment.

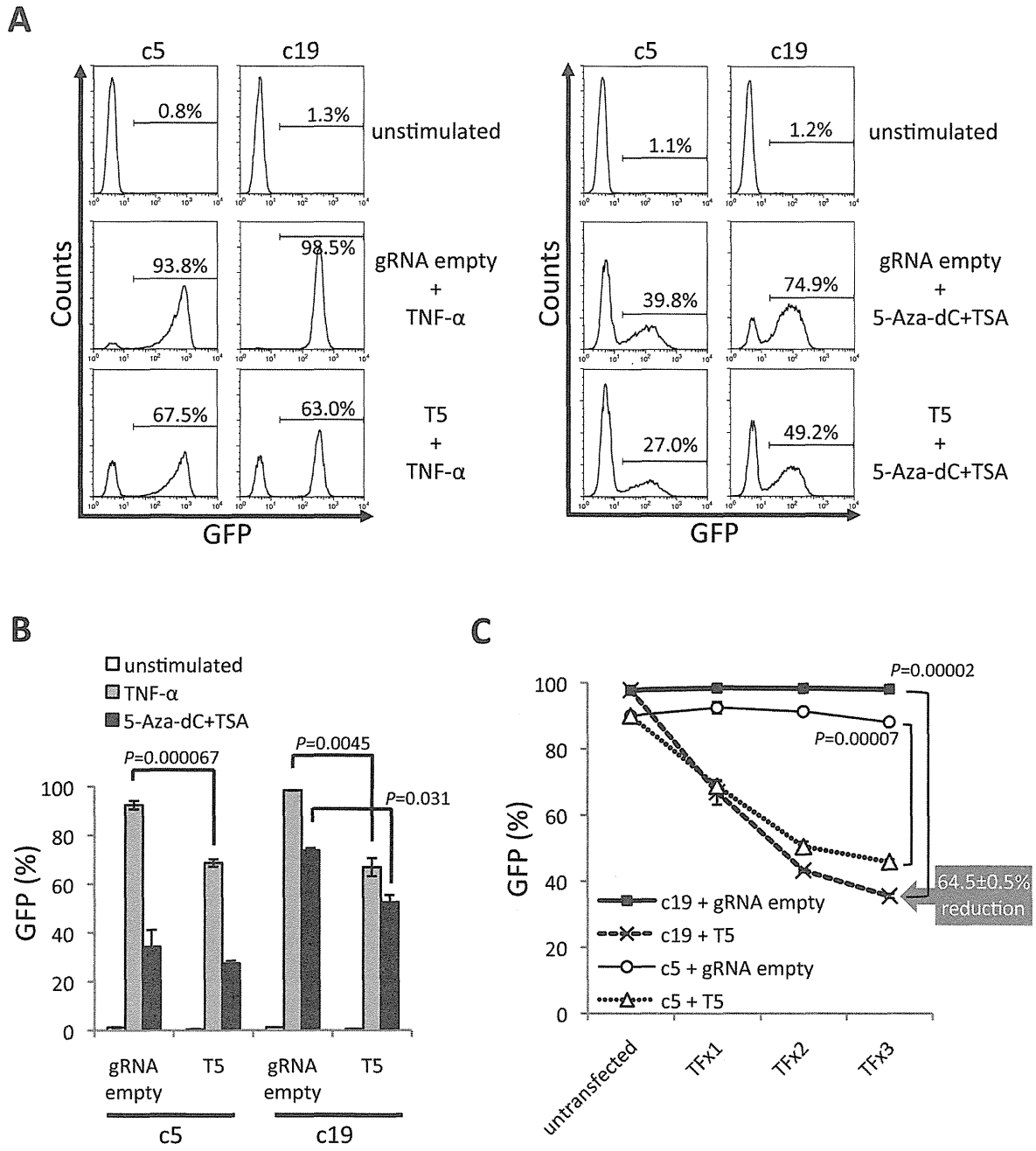

Figure 3 | Suppression of proviral re-activation in T cells by the CRISPR/Cas9 system. Jurkat cell lines latently-transduced with LTIG vector were used. These cell lines were co-transfected with gRNA expression vector and hCas9 expression vector. The cells were treated with TNF-α or the combination of 5-Aza-dC and TSA 4 days after TF. (A and B) The level of GFP expression after 48 hours of TNF-α stimulation and 24 hours 5-Aza-dC and TSA stimulation. Representative histograms are shown in A. The positive percentage of GFP is shown in B (n = 3). (C) Multiple TF of T5 or gRNA empty vector and hCas9 expression plasmid. Solid lines with squares and circles indicate c19 and c5 co-transfected with gRNA empty and hCas9 expression vector, respectively. Dotted lines with x and triangles indicate c19 and c5 co-transfected with T5 and hCas9 expression vector, respectively. The error bars in B and C show standard deviations (n = 3).

Methods

gRNA expression plasmid. gRNA expression plasmids were constructed according to manufacturer's protocol. Briefly, to make a 100 bp dsDNA insert fragment containing the target sequence (20 bp) and PAM sequence, a set of oligonucleotides was used and the fragment was generated by using Phusion polymerase (NEB). The dsDNA fragment was purified and inserted into the AflII site of a gRNA_cloning vector (addgene) with the Gibson assembly system (NEB). Two sets of oligonucleotides targeting 5 and 6 are listed as follows: HE388 (TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGttagaccagatctgagcct) and HE389 (GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACaggctcagatctggtctaaC); and HE384 (TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGctacaagggactttccgct) and HE385 (GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACagcggaaagtcccttgtagC), respectively. Lower case letters indicate the target sequence. These 60 nt oligonucleotide sets annealed to each other over a 20 nt complementary sequence at the 3′ ends.

Virus. Viruses were prepared as described previously[24]. Briefly, 293 T cells were transfected and the culture supernatants were filtrated 48 hours post TF. To prepare

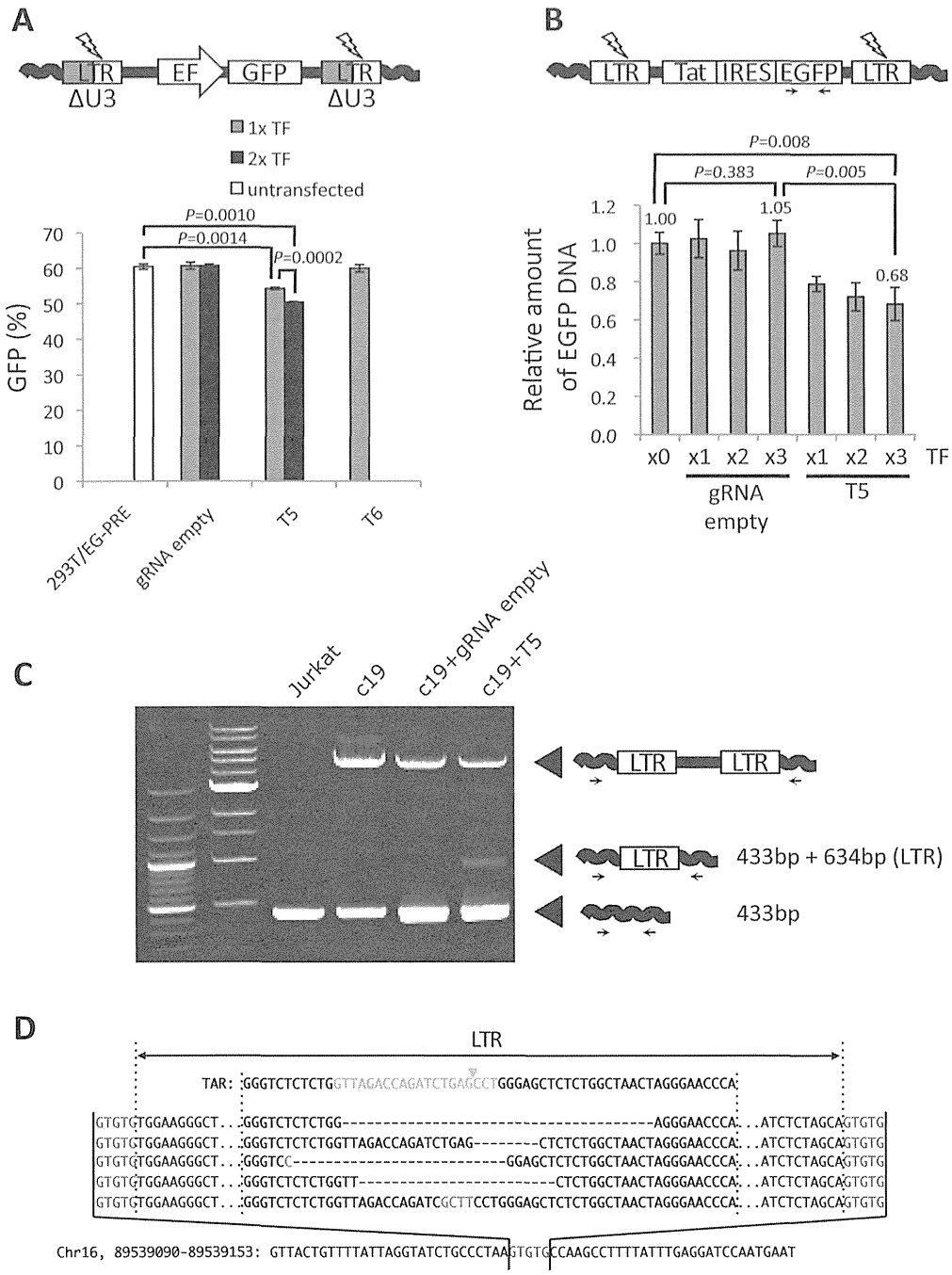

Figure 4 | Excision of HIV-1 provirus from host cell genome with CRISPR/Cas9. (A) Excision of lentivirus vector with the CRISPR/Cas9 system. 293 T cells, transduced with lentivirus vector containing an EF promoter derived-EGFP expressing cassette, were treated with the CRISPR/Cas9 system. A schematic of the lentivirus vector used in this assay is shown on top. The percentage of GFP positive cells after single or double TF of CRISPR/Cas9 components is shown on the bottom (n = 3). (B) Excision of LTIG provirus with CRISPR/Cas9 system. The genomic DNA was extracted from the samples derived from Fig. 3C and qPCR was performed (n = 3). The relative amount of *EGFP* DNA is shown. (C) PCR amplification of HIV-1 provirus. A primer set was designed for the host cell genome sequence flanking the proviral integration site in c19. The schematic of PCR products indicating genomic sequences, full-length provirus and one LTR footprint resulting from proviral excision are shown on the right side. (D) DNA sequence of one LTR footprint resulted from proviral excision. TAR sequence of WT LTR is shown on the top in bold. Target sequence of T5 gRNA is indicated in orange. The putative cleavage site is indicated with an arrowhead. LTR sequences from five clones are shown in the middle. Deletions are indicated by dashes. Insertion is shown in Red. The 5-bp direct repeat in host DNA, flanking LTR ends, is shown in green. The host cell genome sequence is indicated on the bottom.

VSV-G pseudotyped LTIG vector, pEV731, kindly provided by Dr. Eric Verdin[25], pMD. G, pMDLg/pRRE and pRSV Rev (helper plasmids) were co-transfected. To prepare VSV-G pseudotyped EG-PRE vector, pCS-CDF-EG-PRE vector and the same helper plasmids were co-transfected as described previously[26].

Cell culture. 293 T and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 g/ml streptomycin. Jurkat cells were maintained in RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin and 100 g/ml streptomycin.

TF and flow cytometry. 293 T cells were transfected by the calcium phosphate method[24]. HeLa cells were transfected by Lipofectamine2000 (invitrogen) according to the manufacturer's protocol. Jurkat clone cells were transfected by NEON transfection system (life technology). For TF of CRISPR/Cas9 system, 1 μg of hCas9 expression vector and 1 μg of gRNA expression vector was used. The level of GFP expression was analyzed 5 days after TF. Cells were suspended in phosphate-buffered saline (PBS) containing 1% formamide. Flow cytometry was performed with a FACScalibur (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences).

Establishment of latent form of LTIG-transduced Jurkat cells. Jurkat cells were infected with LTIG pseudotyped vector at MOI 0.5 and cultured for one weak. After treatment with 10 ng/ml TNF-α for 24 hours, GFP positive cells were sorted by FACSAria (BD) and cultured for another one month to relax the GFP expression. Then, GFP negative cells were sorted four times, and the cells were cloned by limiting-dilution. After expanding, clone cells were treated with 10 ng/ml of TNF-α (R&D systems) or a combination of 1 μg/ml 5-Aza-dC (SIGMA-ALDRICH) and 1 μM TSA (SIGMA-ALDRICH), and screened for the potential to be reactivated after stimulation, by flow cytometry.

Integration-site analysis of latently integrated LTIG provirus. Genomic DNA extracted from Jurkat cell clones were digested with a combination of BamHI and BclI, or NcoI and BspHI and re-ligated by T4 ligase. The ligation products were EtOH precipitated and used as the template for inverse PCR. For this PCR, LA taq (TAKARA) were used according to the manufacturer's protocol. Primer sets used for the inverse PCR were HE410 (CTCCTCGCCCTTGCTCACCA) and M667 (GGCTAACTAGGGAACCCACTGC). The PCR products were cloned into pGEM-T (Promega) vector and sequenced using M13 primers. Primer set, HE433 (AGCACATCACACTCCTCTG) and HE435 (AGACATGAGCCACTATGTCT) were used for PCR amplification of integrated provirus in c19.

Quantitative analysis of HIV DNA. The amount of *EGFP* DNA was quantified by real-time PCR as previously described[27,28].

Statistical analysis. All data were expressed as mean ± standard deviations (S.D.). The student's *t* test was used to indicate the differences between groups. *P* values are shown in each figure.

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Author contributions

H.E. and Y.Koyanagi wrote the main manuscript text. H.E. and N.M. prepared figure 1, 2, 3, 4B, C and D. Y.Kanemura prepared figure 4A. All authors reviewed the manuscript.

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Original article

IL-21-producer CD4+ T cell kinetics during primary simian immunodeficiency virus infection

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Abstract

IL-21 signaling is important for T cell and B cell-mediated clearance of chronic viral infections. While non-cognate follicular helper CD4+ T cells (T_{FH}) are indicated to be pivotal in providing IL-21-mediated help to activated B cells within germinal centers, how this signaling may be disrupted in early AIDS virus infection is not clear. In this study, we assessed the lineage and kinetics of peripheral blood IL-21-producing CD4+ T cells in primary simian immunodeficiency virus (SIV) infection of rhesus macaques. After SIV challenge, antigen-nonspecific IL-21 production was observed in Th1, Th2 and Th17 cells with Th1 dominance. While IL-21+ Th2 and IL-21+ Th17 showed variable kinetics, an increase in total IL-21+ CD4+ T cells and IL-21+ Th1 from week 3 to week 8 was observed, preceding plasma SIV-specific IgG development from week 5 to week 12. SIV Gag-specific IL-21+ CD4+ T cells detectable at week 2 were decreased in frequencies at week 5. Results imply that kinetics of IL-21+ CD4+ T cells comprised of multiple lineages, potentially targeted by SIV with a bias of existing frequencies during their precursor stage, associate with availability of cooperative B-cell help provided through a proportionate precursor pool developing into T_{FH} and subsequent anti-SIV antibody responses.
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Keywords: HIV; SIV; IL-21; B-cells; Th1

1. Introduction

Neutralizing antibodies (NAbs) are promising immunological effectors against human immunodeficiency virus type 1 (HIV-1) infections [1]. However, for unknown multiple reasons their appearance in infection is late, abrogating their maximal antiviral activity in early infection via exerting their effector functions [2,3].

Two major unresolved issues underlie dysregulation of B-cell responses in primary HIV/SIV infections. One is the discrepancy between overt plasma IgG elevation [4] and insufficient antiviral antibody production, manifesting as a compromised development of autologous NAbs [5–10]. Another is the availability of conventional CD4+ T cell help, especially by CXCR5+PD-1+Bcl-6+ follicular helper T cells (T_{FH}) within the germinal center (GC). HIV and SIV preferentially infect and destroy CCR5+ memory CD4+ T cells [11,12]. Histologically, GC CD57+CD4+ T cells indeed have been implied to be a source of HIV infection [13]. GC PD-1+ T_{FH} are implied to become viral reservoirs in SIV infection, anatomically sequestered from CD8+ T cell infiltration [14], while their phenotypes are undetermined. Under these pathologies, how CD4+ T cell help is available during development of anti-HIV/SIV antibody responses, including NAbs, is an important issue to be addressed.

Among the help provided from CD4+ T cells to B cells, the IL-21 signaling axis has a pivotal role in B cell differentiation and development [15], as well as for T cell-dependent elimination of chronic viral infections [16–19]. Major stimulatory effects against mature B cells include their proliferation, class switch recombination (CSR), and terminal differentiation into plasma cells (PCs) [20]. The latter two require CD40L/BCR

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stimulation and proceed through reciprocal regulation of Bcl-6 and Blimp-1 [21,22]. Conventional Th2 cytokines such as IL-4 and IL-10 also redundantly contribute to this process, while their efficacies are inferior to IL-21 under CD40L costimulation [23]. IL-21 signaling also is involved in IL-6-mediated antibody regulation [24,25]. IL-21 receptor (CD360, IL-21R) expression on target B cell subsets is variable [26]; T1B cells are IL-21Rdim whereas T2B cells and follicular B cells are enriched in IL-21R expression [27]. In IL-21R-deficient mice, IgG1 responses are absent both in steady-state and infections while other subclasses are variously influenced [28]. In mice deficient in IL-21 ligand or receptor, germinal centers are normally formed while infection resolution is not obtained [29]. These findings all highlight the specificity and importance of IL-21 signaling in an optimal pathogen-specific antibody production.

In an interesting contrast, recent studies demonstrate that T_{FH} subsets are not terminally differentiated [30]. Usage of knockout and bone marrow-reconstituted mice has pointed out to the redundancy of the source of IL-21 help, suggestive of T_{FH} not being required as an exclusive source of IL-21 [31]. Additionally, IL-21 signaling intrinsically targets B cells but not their cooperating subsets [31–33]. These collectively describe the redundant and robust nature of IL-21 regulation. Thus it follows that the degree of T_{FH} destruction in HIV/SIV infections may not necessarily provide a direct clue for the incompleteness of anti-HIV/SIV antibody and NAb responses. Nevertheless it is important to assess, in HIV/SIV infections, the extent of IL-21 signaling potentially available for B cells and what cells may serve as the source of IL-21. In this study, we focused on the acute phase of infection and performed a simplified estimation of the above by evaluating the kinetics of virus-specific IgG, peripheral blood B cell subsets and their IL-21R expression, and peripheral blood IL-21-producing CD4+ T cells in a cohort of SIV_{mac239}-infected rhesus macaques.

2. Materials and methods

2.1. Animal experiments, virus stocks and infection

Burmese rhesus macaques (*Macaca Mulatta*) were maintained in accordance with the Guideline for Laboratory Animals of National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Blood collection and virus challenge were performed under ketamine anesthesia. Animals were challenged intravenously with 1000 $TCID_{50}$ (50 percent tissue culture infective dose) of SIV_{mac239} (R04-013, R06-017 and R03-005) or SIV_{mac239} carrying five Gag mutations GagL216S, D244E, I247L, A312V and A373T (R05-001, R06-016, R06-018, and R05-024), selected as a CTL escape mutation in MHC class I haplotype *90-120-Ia*-possessing macaques as previously reported [34]. All SIV-challenged animals were negative for *90-120-Ia*. The mutant SIV shows *in vivo* fitness comparable to wild type and sufficient to cause AIDS in infected macaques.

2.2. Quantitation of plasma viral loads

Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics, Tokyo, Japan) and serial five-fold dilutions of RNA samples were amplified in quadruplicate by reverse transcription and nested PCR using SIV_{mac239} Gag-specific primers to determine the end point. Plasma SIV RNA levels were calculated according to the Reed–Muench method as described previously [2]. The lower limit of detection is approximately 4×10^2 copies/ml.

2.3. Measurement of plasma anti-SIV IgG

Plasma anti-SIV_{mac239} IgG were detected using a highly SIV_{mac239}-cross-reactive commercial western blotting system against the parental strain SIV_{mac251} (ZeptoMetrix, Buffalo, NY) according to the manufacturer's instructions. Plasma from week 0 pre-challenge, week 3, week 5, week 12 and week 25 post-challenge were subjected to analysis.

2.4. SIV antigen-specific ELISA

SIV_{mac251} Gag p27 and SIV_{mac251} Env gp130 (Immuno-Diagnostics, Woburn, MA) were coated on 96-well assay plates (BD, Tokyo, Japan) at a concentration of 1000 ng/ml (100 ul per well) by overnight incubation at 4 °C. Wells were washed with PBS four times and blocked with 0.5% bovine serum albumin (BSA)/PBS. Plasma samples were incubated at a final dilution of 1:20 for 2 h at 37 °C. Wells were washed with PBS four times and SIV antigen-bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (H+L) (Bethyl Laboratory) and SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). Plasma from week 0 pre-challenge, week 3, week 5, week 12 and week 25 post-challenge were subjected to analysis in duplicate and absorbance at 450 nm was measured, which are shown as arbitrary units (AU).

2.5. B cell phenotyping

Peripheral blood mononuclear cells (PBMCs) were surface-stained with anti-human CD20-PerCP (BioLegend, San Diego, CA), anti-human CD27-PE (BD, Tokyo, Japan), anti-human IgD-FITC (Dako, Tokyo, Japan), anti-human IgG-APC (BD) and anti-human CD360 (IL-21R)-APC (BioLegend) according to the manufacturers' instructions. All antibodies were used at pretested optimal concentrations. After incubation at 4 °C for 30 min, cells were washed twice and fixed with 1% PFA. Cells were acquired by FACS CantoII (BD) and analyzed by FACS Diva (BD) and FlowJo (Treestar, Ashland, OR). Approximately 150,000 PBMCs were gated for each test.

2.6. Measurement of ex vivo IL-21-producer CD4+ T cells

PBMCs were stimulated with a pretested combination of 1.5 ng/ml PMA (Sigma Aldrich, Tokyo, Japan) and 7.5 ng/ml

ionomycin (Sigma Aldrich) for 6 h at 37 °C. Surface and intracellular staining was performed using Cytofix/Cytoperm kit (BD). Anti-human CD4-PerCP, anti-human CD8-PE-Cy7, anti-human CD3-APC-Cy7 (BD), anti-human IFN-γ-PE, anti-human TNF-α-FITC, anti-human IL-4-FITC, anti-human IL-21-Alexa 647 (BioLegend) and anti-human IL-17-PE (Miltenyi Biotec, Tokyo, Japan) were used at pretested optimal concentrations. Cells were fixed with 1% PFA and acquired by FACS CantoII and analyzed by FACS Diva and FlowJo. Stimulation-specific T-cell levels in each T-cell lineage were measured by subtracting unstimulated cytokine-positive CD4+ T-cell frequencies from those after PMA/ionomycin stimulation. Approximately 150,000 PBMCs were gated for each test. For measurement of SIV-specific responses, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) pulsed with a peptide pool using a panel of overlapping peptides spanning the entire SIV_{mac239} Gag amino acid sequence and analyzed similarly.

2.7. Statistical analysis

Statistical analyses were performed via GraphPad Prism software. $p < 0.05$ were considered to be statistically significant in nonparametric Wilcoxon's signed rank paired tests. Correlation was analyzed by Pearson test. Reduction rates in plasma viral loads, showing non-Gaussian distribution, were log-transformed for improvement of normality.

3. Results

3.1. Virus-specific IgG appearance and B-cell subset kinetics in primary SIV infection

To focus on a relevant time zone of IL-21-related parameter evaluation, development of plasma anti-SIV antibody titers was first examined by western blotting. Animals incorporated in this study experienced variable levels of plasma viremia up to week 12 post-challenge (Fig. 1A). Anti-SIV_{mac} linear epitope-specific IgG became detectable by week 3 (Fig. 1B). Between week 5 and week 12, anti-SIV IgG responses showed an increase in targeted linear antigen breadth in all investigated rhesus macaques (Fig. 1C). This was in agreement with other reports on conventionally progressing SIV-infected macaques [35,36].

While peripheral blood CD20+ B cell frequencies within PBMCs showed a variable change during this period (Fig. 1D), the increase in targeted antigen breadth during week 5 to week 12 was accompanied by a uniform increase in SIV Env-specific antibody titers (Fig. 1E right). In contrast, anti-Gag antibodies showed an earlier uniform rise between week 3 and week 5 and kinetics were more variable during week 5 to week 12 (Fig. 1E left). During week 5 to week 12, a decrease in classically defined naïve (IgD+CD27−) B cells (Fig. 2B left) and a generalized rise in peripheral blood IgG+CD27+/low CD20+ memory B cell levels (Fig. 2C right) was observed. The degree of increase in classically defined memory (IgD−CD27+) B-cell phenotype (Fig. 2C left) mainly derived from this IgG+ memory B cell increase. SIV-specific IgG development accompanying total and IgG+ memory B-cell enrichment indicated that a predominant B-cell dysfunction was not likely to be occurring during this period.

We next examined IL-21R expression kinetics on IgD+CD27− naïve, IgD+CD27+ marginal zone (MGZ), and IgD−CD27+ memory B cells within the corresponding time zone (Fig. 2A). This was performed because an increase in circulating memory B cell frequency alone does not exclusively assure successful development of pathogen-specific responses but might discordantly reflect an outcome of compromised GC response [31]. Within naïve B cells and MGZ B cells, IL-21R expression levels behaved variably within the cohort between week 5 and week 12 (Fig. 2D left/middle). In turn, IL-21R on memory B cells showed a decrease in six out of seven investigated macaques (Fig. 2D right). This implied that SIV-specific antibody development was associated with IL-21 signaling during this period.

3.2. Profile of peripheral blood IL-21-producing CD4+ T cell subsets in primary SIV infection

The lineage and temporal kinetics of peripheral blood IL-21 producer CD4+ T cells during anti-SIV IgG development were next investigated. Considering the importance of trafficking IL-21 reporter cells into the peripheries of genetically modified mice [30], we reasoned that this may partially serve as a clue for estimating the extent of GC intactness. The peripheral blood-circulating counterparts of T_{FH} are previously reported to be of a combination of Th2 and Th17 lineage [37]. This prompted us to assess whether such IL-21+ CD4+ T cell subsets are preferentially altered in SIV infection, preceding or during the initial stage of antiviral antibody development. Peripheral blood mononuclear cells (PBMCs) were stimulated *ex vivo* [30,38,39] with a combination of PMA and ionomycin. IFN-γ+CD4+ T cells were defined as Th1, IL-4+CD4+ T cells were defined as Th2, and IL-17+CD4+ T cells were defined as Th17. Th1, Th2 and Th17 cells did not show uniform kinetics between pre-challenge and week 8 (Fig. 3B). In the assessed time points, CD4+ T cells capable of PMA/ionomycin-driven IL-21 secretion existed in all three examined Th lineages (Fig. 3C). IL-21+ Th1 were the most abundant, while IL-21+ Th2 and IL-21+ Th17 were also at detectable levels. Notably, IL-21+ Th2 and IL-21+ Th17 did not behave uniformly between week 1 and week 3 or between week 3 and week 8. In contrast, in all seven investigated macaques, a decrease in IL-21+ Th1 between week 1 and week 3 ($p = 0.016$, data not shown) and an increase between week 3 and week 8 ($p = 0.016$, Fig. 3C left, Fig. 3D left) was observed. Importantly, the tendency was discordant with total Th1 dynamics.

A statistically significant rise in pan-Th total IL-21-producing CD4+ T cell frequencies between week 3 and week 8 ($p = 0.031$, Fig. 4A, Fig. 4D left) was accordingly preserved within this pilot cohort, despite the incorporation of

A

Plasma viral loads after SIV challenge (SIV Gag RNA copies/ml plasma)

Macaque ID	wk 2 p.c.	wk 5 p.c.	wk 8 p.c.	month 3 p.c.
R05-001	2.0×10^6	1.4×10^5	1.4×10^5	4.0×10^5
R05-024	6.9×10^5	6.4×10^2	9.6×10^2	N.D.
R06-016	1.0×10^6	3.4×10^4	1.1×10^4	1.2×10^3
R04-013	2.8×10^6	3.4×10^5	5.7×10^5	2.0×10^5
R06-018	2.8×10^6	4.9×10^5	3.0×10^4	8.1×10^4
R06-017	3.8×10^6	9.1×10^4	6.1×10^3	1.6×10^3
R03-005	1.1×10^5	1.8×10^4	1.6×10^4	1.4×10^4

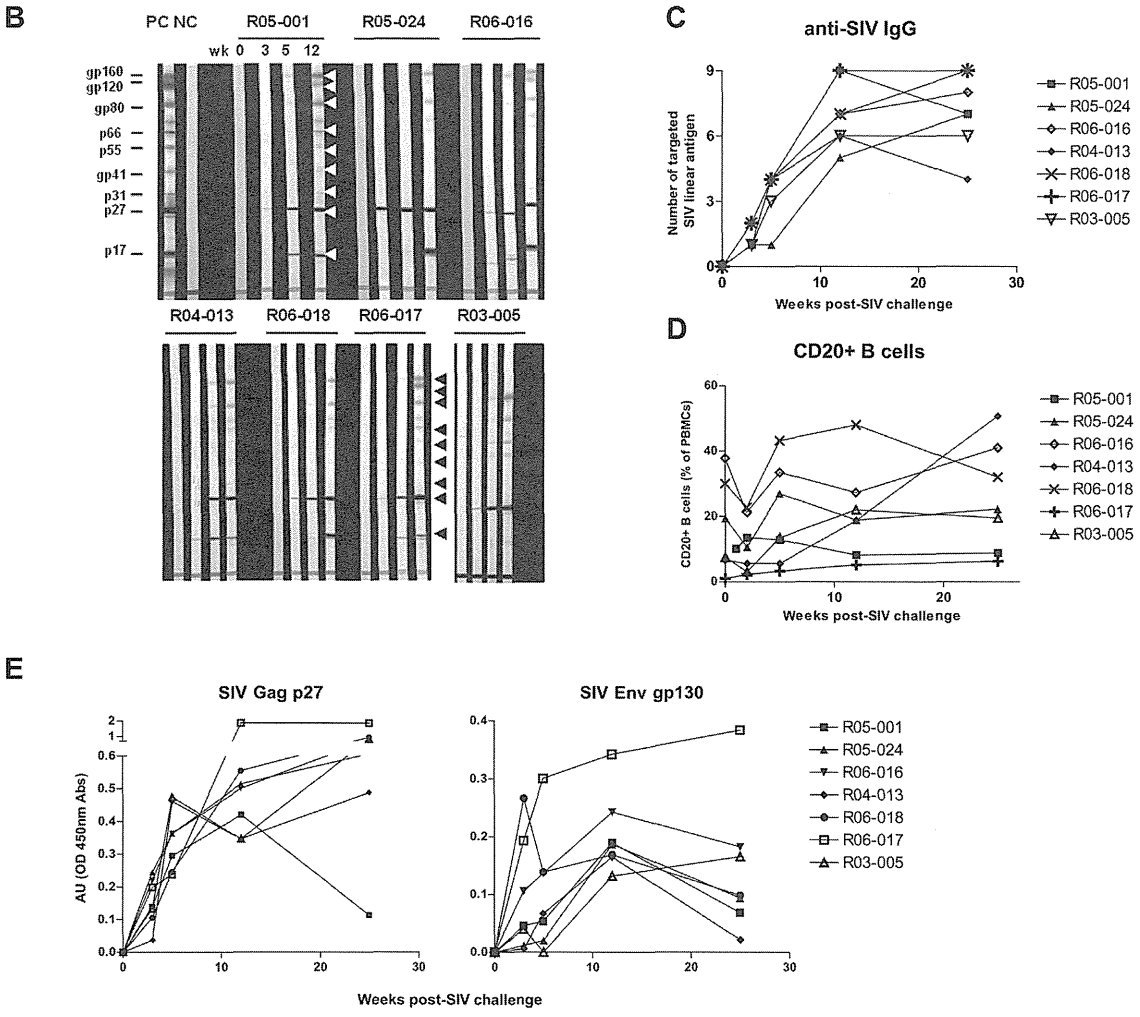

Fig. 1. Plasma antiviral antibody development in SIV_{mac239} infection. (A) Plasma viral loads after SIV challenge (SIV RNA copies/ml plasma). N.D., undetectable. (B) Plasma SIV-specific IgG detection via western blotting. Arrowheads represent listed SIV_{mac} linear antigens. (C) Number of IgG-targeted SIV_{mac} linear antigens after SIV challenge. (D) CD20+ B cell frequencies in PBMCs. (E) SIV_{mac} Gag p27- and Env gp130-specific antibody titers after SIV challenge.

variable recovery in IL-21+ Th2 and Th17 lineages. The single animal (R03-005) that exhibited a marginal decrease in total IL-21+ CD4+ T cells coincided to be the only macaque that showed an increase in memory B-cell IL-21R expression from week 5 to week 12, indicating a feedback relationship between the two factors. The frequencies of Th1+Th2+Th17 during this course showed no coordinated behavior ($p = 1.00$, Fig. 4B, Fig. 4D middle), suggesting that cytokine production alone did not correlate with the above propensity in IL-21+ CD4+ T-cells. While total CD3+ T cells robustly sufficed as a source for IL-21-mediated B-cell-intrinsic help in CXCR5-deficient mice in previous reports [31], kinetics was also not uniform at a IL-21+ CD3+ T cell population level ($p = 0.16$, Fig. 4C, Fig. 4D right). This indirectly described the availability of IL-21+ CD4+ T cells in primary SIV infection.

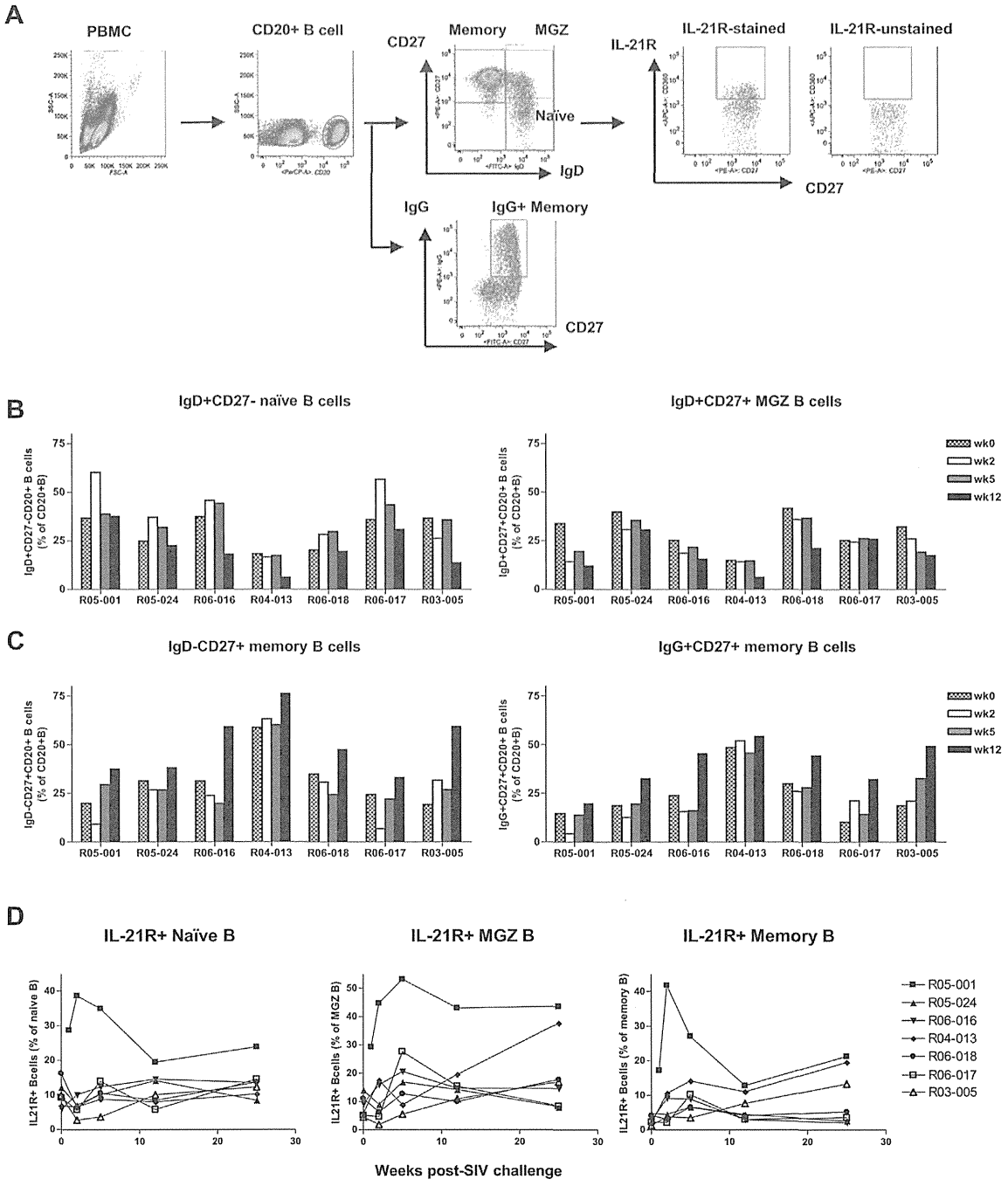

Fig. 2. Peripheral blood B cell subsets and IL-21R expression in SIV_{mac239} infection. (A) Representative gating. (B) Naïve and MGZ B cell frequencies. (C) Memory and IgG+ memory B cell frequencies. (D) IL-21R+ naïve, IL-21R+ MGZ and IL-21R+ memory B cell frequencies.

3.3. SIV Gag-specific IL-21+ CD4+ T cell responses

To estimate the frequencies of SIV-specific responses within the IL-21+ CD4+ T cell population, we measured IL-21 secretion under PBMC stimulation with Gag peptide-pulsed BLCLs. Gag was chosen because we assumed that immune complex-dependent boosting of antigen presentation [2] and robust Th detection may be most expected in Gag, due to early anti-Gag antibody detection. In the macaques, Gag-specific responses comprised approximately 1%–4% of total IL-21+ CD4+ T cells at week 2 post-challenge (Fig. 5A). All macaques exhibited a significant decrease in frequencies at week 5 ($p = 0.016$, Fig. 5B) and recovery at week 12 was variable. Frequencies of Gag-specific IL-21+ CD4+ T cells

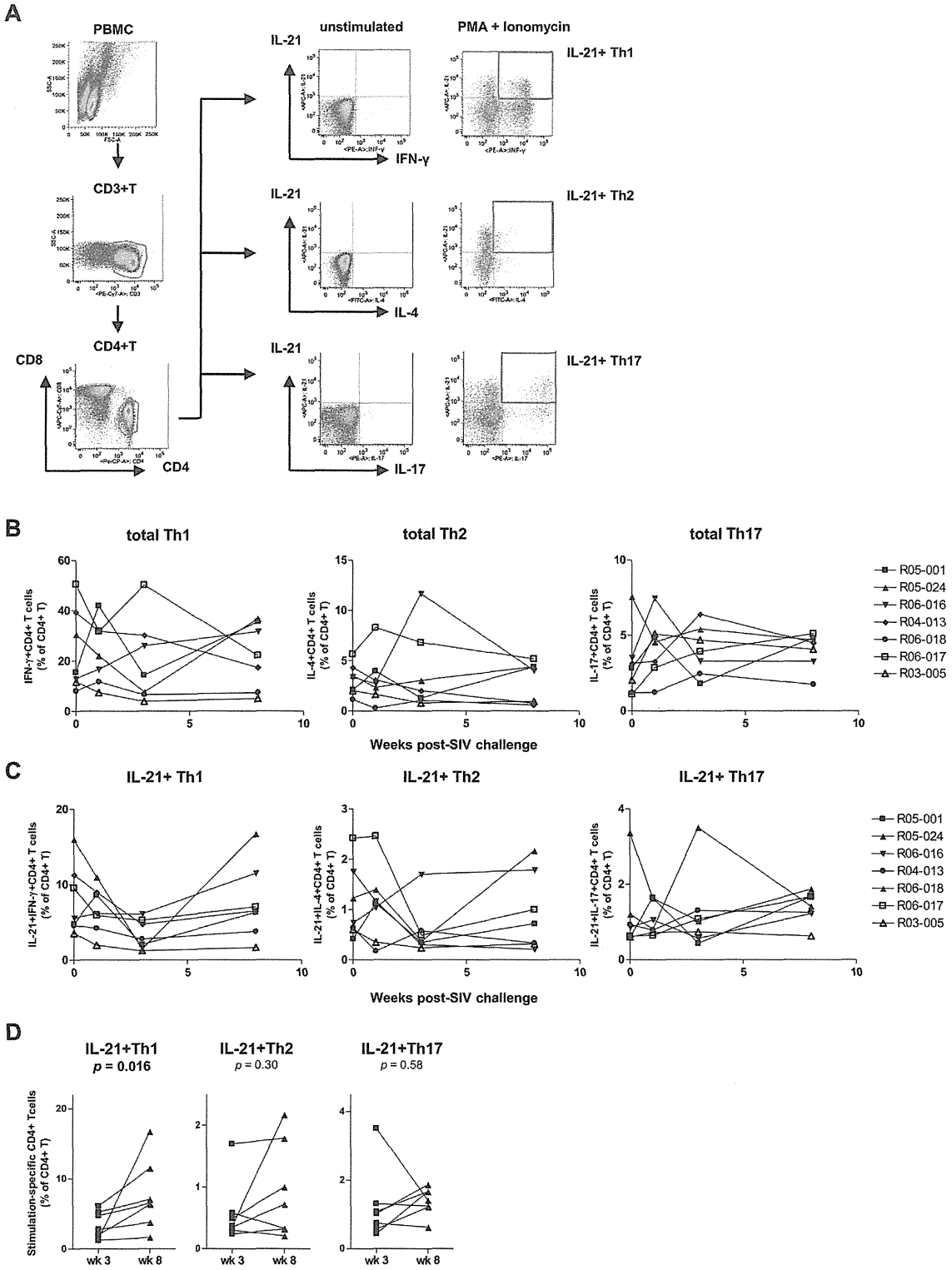

Fig. 3. IL-21-producing Th1, Th2 and Th17 in SIV_{mac239} infection. (A) Representative gating. (B) Total Th1, total Th2 and total Th17 frequencies. (C) IL-21+ Th1, IL-21+ Th2 and IL-21+ Th17 frequencies. (D) Wilcoxon's test of IL-21+ Th1, Th2 and Th17 frequencies (week 3 vs week 8).

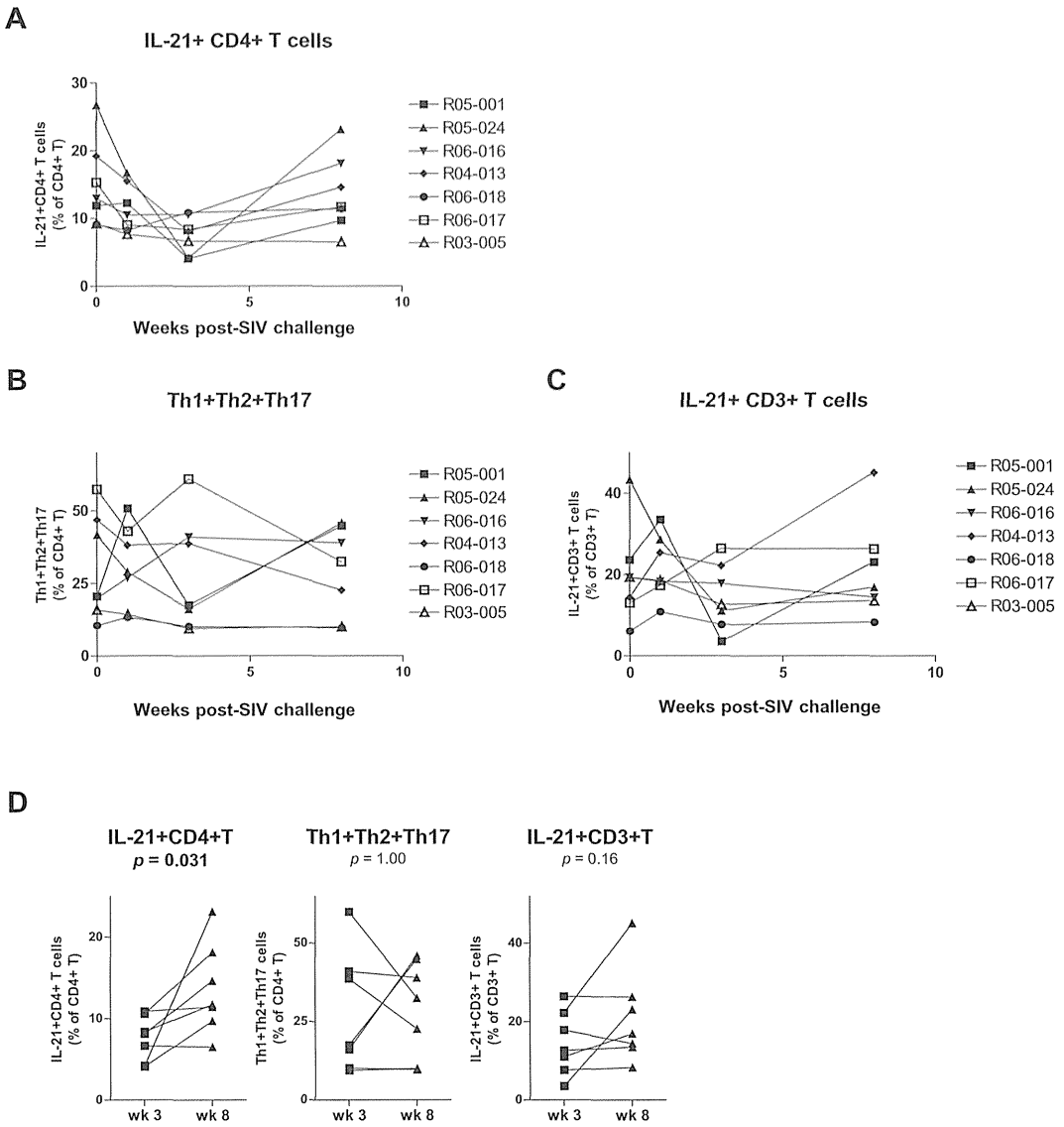

Fig. 4. Total IL-21-producing CD4+ T cell kinetics in SIV_{mac239} infection. (A) IL-21+ CD4+ T-cell frequencies. (B) Th1+Th2+Th17 frequencies. (C) IL-21+ CD3+ T-cell frequencies. (D) Wilcoxon's test of IL-21+ CD4+ T cells, Th1+Th2+Th17 and IL-21+ CD3+ T cells (week 3 vs week 8).

were consistently beneath 5% of total IL-21+ CD4+ T cells in all macaques. This implied that SIV-specific IL-21+ CD4+ T cells may have not played a central role in IL-21-mediated help for B-cell responses.

3.4. Correlation between IL-21+ Th1 and early plasma viral load reduction

Finally, IL-21+ Th1 levels, which showed the most observable kinetics in the obtained results, were analyzed for their correlation with viral replication parameters. In result, IL-21+ Th1 levels at the recovery stage (week 8 post-challenge) showed a significant inverse correlation with plasma viral RNA load reduction rates between week 2 and week 5 ($p = 0.0037$, Fig. 6). Similar correlations were not observed with ratios between other time points (data not shown). This was observed in spite of the diverse set point viral loads, suggesting a history of potential viral targeting of this population among the cytokine-positive CD4+ T cells in early SIV infection. Interestingly, correlation of IL-21+ Th1 levels was not observed with Gag-specific antibody titers (week 12: $p = 0.97$, data not shown) or Env-specific antibody titers (week 12: $p = 0.57$, data not shown), implicating that IL-21+ CD4+ T cell-mediated help against B cells *in vivo* occurs in a fashion of a threshold model instead of showing direct correlation. Collectively, results implied that IL-21+ CD4+ T cell precursors of multiple lineages with a Th1 bias had initially been damaged before commitment in GCs, and its

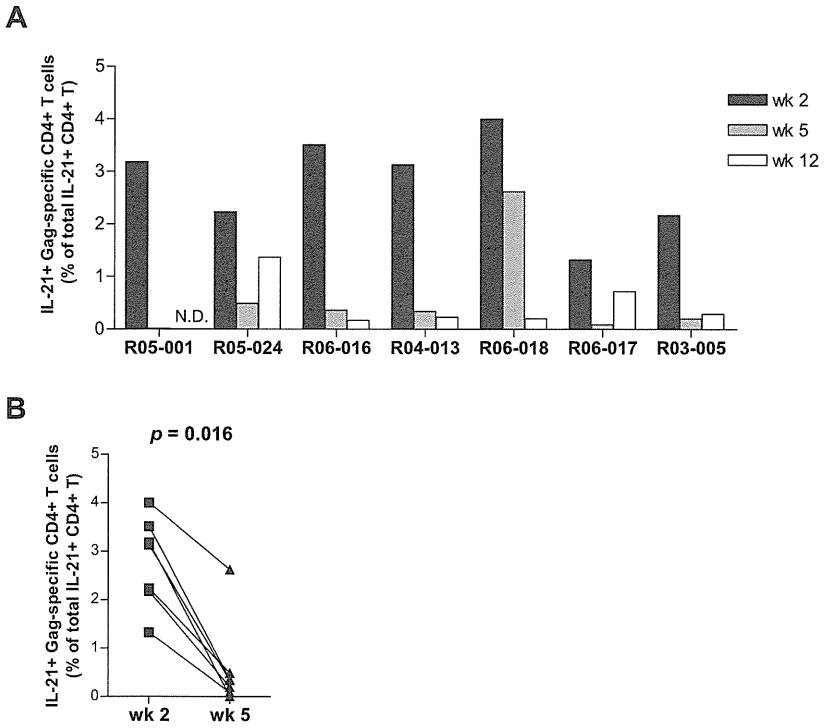

Fig. 5. Percentages of SIV Gag-specific IL-21+ CD4+ T cells within total IL-21+ CD4+ T cells. (A) Values at week 2, week 5 and week 12. N.D., R05-001 sample at week 12 unavailable. (B) Wilcoxon's test of Gag-specific/total IL-21+ CD4+ T cell ratios (week 2 vs week 5).

recovery and T_{FH} differentiation associated with the increase in peripheral blood IL-21+ CD4+ T cells and development of anti-SIV antibodies.

4. Discussion

The present study describes peripheral blood IL-21 producer CD4+ T cell kinetics in acute SIV infection. To our knowledge, this is the first report to assess IL-21 production in multiple peripheral blood Th lineages during the early stage of AIDS virus infection. Results suggest that the IL-21 signaling axis does not suffer a total loss in primary SIV infection, and shows an observable extent of recovery coinciding with SIV-specific antibody appearance. IL-21+ Th2 and Th17 frequencies did not show a clear propensity during the surge of plasma anti-SIV IgG; compared with reported lineage analysis on peripheral blood T_{FH} counterparts, this was rather unexpected. In contrast, IL-21+ Th1 showed a coordinated decrease followed by an increase between weeks 1 and 8, and week 8 levels showed inverse correlation with early viral load reduction rates. Results do not contradict a recent study clarifying the origin of intermediately differentiated T_{FH} cells as being precursor Th comprised of more than one lineage, showing preferential Th1 differentiation upon experimental influenza virus infection [30]. In the current study, cases of recovery in IL-21 producer frequencies was also observed in Th2 (3 out of 7 macaques) and Th17 (4 out of 7 macaques) and there is no clear reason to exclude potential commitment of these lineages in providing B-cell help. Hence it may be more realistic to regard total IL-21+ CD4+ T cells as a peripheral blood surrogate indicator of GC intactness instead of IL-21+ Th1, although the latter show more clear kinetics.

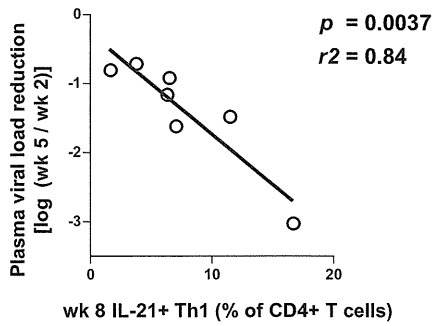

Fig. 6. Correlation analysis of IL-21+ Th1 at week 8 and week 5/week 2 viral load reduction (log).

Perturbation of IL-21+ CD4+ T cell availability is an understandable phenomenon in memory CD4+ T-cell-tropic HIV and SIV infections. In HIV-1-infected untreated individuals, serum IL-21 levels show a progressive decline in the chronic phase [38,40]. In controlled HIV infections, IL-21-producing CD4+ T cells are suggested to facilitate CD8+ T cell functionality [39,41]. A uniform increase in IL-21R expression on B cells has been indicated in chronic HIV infection [42], and B cell responsiveness against exogenous IL-21 administration has been examined in chronic SIV

infection [43]. Recent two important reports document viral targeting and potential feedback compensatory expansion of GC T_{FH} within the chronic course of HIV and SIV infections [44,45]. In these reports, total serum IgG or anti-SIV antibody binding avidity exhibited correlation with T_{FH} level or functionality in the chronic phase. While anti-SIV IgG detection via immunoblotting is not quantitative, here we have newly described a temporal coincidence of an increase in both the anti-SIV antibody repertoire and peripheral blood IL-21 producer CD4+ T cells during acute SIV infection, indirectly supporting these two reports. Additionally, we observed an inverse correlation between acute phase viral load reduction rates and week 8 IL-21+ Th1 levels. We assume that IL-21+ CD4+ T cells are potential targets of infection, as they are memory and not naïve, and the extent of CTL-based initial viral load reduction is proportionate with the subsequent recovery of the IL-21+ CD4+ T cells, which in turn may support virus-specific antibody production around viral set point. This may cohere both with the decrease in IFN-γ production by T_{FH} in late SIV infection *in vivo* and SIV infectivity of T_{FH} *in vitro* [45]. Our current results may complement these reports by assessing a different compartment and time frame.

The anti-SIV IgG producer B-cell lineages were not determined in this study. In addition, whether any of the detected viral antigen-specific anti-SIV IgG are thymus-dependent (Th-dependent) is not discernable, regardless of antibody detection methods such as ELISA [36], western blotting [35] or binding antibody multiplex assays [6,45]. i.e., it cannot be directly concluded that these anti-HIV/SIV antibodies have experienced a history of T_{FH} help within GCs, unless T_{FH} dependence is examined by CXCR5 genetic ablation [31] which is currently unavailable in primates. In our current results, the average number of targeted linear SIV antigens exceeded 7 at week 12 post-challenge at least within linear epitopes. It can be reasonable to speculate that at least more than one of these protein-specific polyclonal antibodies are thymus-dependent, having been assisted by T_{FH} deriving from IL-21+ CD4+ T cells that show a proportional decrease and recovery in the periphery. The coincidence of a uniform increase in memory B cell frequencies and anti-Env antibody titers during this period, together with the consistently detectable IL-21+ CD4+ T cell levels and its precedent recovery, may also be supportive of an intact but not compromised GC response.

Regarding the functionality and maturation of the initial anti-HIV/SIV antibodies, they optimally should exhibit virus-neutralizing activity. However, HIV/SIV Env-specific NAb induction regularly shows a considerable delay. Against the especially resistant SIV_{mac239} strain used in this study the tendency is even more prominent, with a delay mostly exceeding 24 weeks post-infection even in successful inducers (Yamamoto, unpublished observations). During the observation period of this study plasma NAbs also did not appear (data not shown), even with the IL-21+ CD4+ T-cell surge before set point. This is likely associated with the intrinsically low antigenicity of SIV_{mac239} Env, and also may be explained by the pathogenic nature of the strain compared with macaque infection of more benign strains such as SIV_{smE660} and SIV_{mac251} [46]. Hence the present results may potentially underestimate the contribution of CD4+ T-cell help against B cells, and should be carefully interpreted as such. In addition, results imply that major additional anti-HIV/SIV NAb inhibitory mechanisms exist ahead of the attainment of threshold CD4+ T-cell help.

In summary, this work shows a temporal coincidence of an increase in lineage-biased peripheral blood IL-21+ CD4+ T cells and development of plasma virus-specific IgG responses in primary SIV infection. It may become important to assess whether arbitrary or pathogenic amplification of IL-21 signaling can associate with augmented antiviral antibody efficacy, such as neutralization breadth, titer and effector functions and/or HIV/SIV control. Further studies will be required to clarify mechanisms of generalized anti-HIV/SIV antibody dysregulation and HIV/SIV NAb resistance *in vivo*.

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Limited Impact of Passive Non-Neutralizing Antibody Immunization in Acute SIV Infection on Viremia Control in Rhesus Macaques

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Abstract

Background: Antiviral antibodies, especially those with neutralizing activity against the incoming strain, are potentially important immunological effectors to control human immunodeficiency virus (HIV) infection. While neutralizing activity appears to be central in sterile protection against HIV infection, the entity of inhibitory mechanisms via HIV and simian immunodeficiency virus (SIV)-specific antibodies remains elusive. The recent HIV vaccine trial RV144 and studies in nonhuman primate models have indicated controversial protective efficacy of HIV/SIV-specific non-neutralizing binding antibodies (non-NAbs). While reports on HIV-specific non-NAbs have demonstrated virus inhibitory activity *in vitro*, whether non-NAbs could also alter the pathogenic course of established SIV replication *in vivo*, likewise via neutralizing antibody (NAb) administration, has been unclear. Here, we performed post-infection passive immunization of SIV-infected rhesus macaques with polyclonal SIV-specific, antibody-dependent cell-mediated viral inhibition (ADCVI)-competent non-NAbs.

Methods and Findings: Ten lots of polyclonal immunoglobulin G (IgG) were prepared from plasma of ten chronically SIV_{mac239}-infected, NAb-negative rhesus macaques, respectively. Their binding capacity to whole SIV_{mac239} virions showed a propensity similar to ADCVI activity. A cocktail of three non-NAb lots showing high virion-binding capacity and ADCVI activity was administered to rhesus macaques at day 7 post-SIV_{mac239} challenge. This resulted in an infection course comparable with control animals, with no significant difference in set point plasma viral loads or immune parameters.

Conclusions: Despite virus-specific suppressive activity of the non-NAbs having been observed *in vitro*, their passive immunization post-infection did not result in SIV control *in vivo*. Virion binding and ADCVI activity with lack of virus neutralizing activity were indicated to be insufficient for antibody-triggered non-sterile SIV control. More diverse effector functions or sophisticated localization may be required for non-NAbs to impact HIV/SIV replication *in vivo*.

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Introduction

Development of a successful vaccine is crucial for global human immunodeficiency virus (HIV) control. A recent clinical trial in Thailand has shown partial efficacy of an HIV vaccine regimen, RV144 [1]. Further analyses have suggested possible contribution of virus-binding antibodies to the protection from HIV infection [2,3]. Thus, understanding of the effect of virus-binding, non-neutralizing antibody (non-NAb) responses on the course of HIV/SIV infection may serve as one step for vaccine development.

In contrast to the constrained emergence of neutralizing antibodies (NAbs), non-NAbs are commonly induced in both the acute and chronic phase of HIV/SIV infection [4–6]. They are known to exhibit *in vitro* suppressive effects against virus replication, such as ADCC (antibody-dependent cellular cytotoxicity) and ADCVI (antibody-dependent cell-mediated virus inhibition) [7–10]. While several reports have suggested