Osmanov S. 1995. The evolving molecular epidemiology of HIV-1 envelope subtypes in injecting drug users in Bangkok, Thailand: implications for HIV vaccine trials. AIDS **9**:851–857.

35. **Ou CY, Takebe Y, Weniger BG, Luo CC, Kalish ML, Auwanit W, Yamazaki S, Gayle HD, Young NL, Schochetman G.** 1993. Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. Lancet **341**:1171–1174.
36. **Abecasis AB, Vandamme AM, Lemey P.** 2009. Quantifying differences in the tempo of human immunodeficiency virus type 1 subtype evolution. J. Virol. **83**:12917–12924.
37. **Li Y, Uenishi R, Hase S, Liao H, Li XJ, Tsuchiura T, Tee KK, Pybus OG, Takebe Y.** 2010. Explosive HIV-1 subtype B′ epidemics in Asia driven by geographic and risk group founder events. Virology **402**: 223–227.

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Short Communication

TRIM5 genotypes in cynomolgus monkeys primarily influence inter-individual diversity in susceptibility to monkey-tropic human immunodeficiency virus type 1

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TRIM5α restricts human immunodeficiency virus type 1 (HIV-1) infection in cynomolgus monkey (CM) cells. We previously reported that a *TRIMCyp* allele expressing TRIM5–cyclophilin A fusion protein was frequently found in CMs. Here, we examined the influence of *TRIM5* gene variation on the susceptibility of CMs to a monkey-tropic HIV-1 derivative (HIV-1mt) and found that TRIMCyp homozygotes were highly susceptible to HIV-1mt not only *in vitro* but also *in vivo*. These results provide important insights into the inter-individual differences in susceptibility of macaques to HIV-1mt.

Considering the global human immunodeficiency virus type 1 (HIV-1) epidemic, development of prophylactic vaccines is strongly desired. In order to evaluate the efficacy of the vaccines, a suitable animal model is also indispensable. However, HIV-1 does not grow in Old World Monkeys (OWMs) such as rhesus monkeys and cynomolgus monkeys (CMs). One of the restriction factors of OWMs is ApoB mRNA editing catalytic subunit 3G (APOBEC3G) (Sheehy *et al.*, 2002). APOBEC3G modifies the minus-strand viral DNA during reverse transcription, resulting in impairment of HIV-1 replication. This activity can be counteracted by the viral protein Vif of simian immunodeficiency virus (SIV) but not by that of HIV-1 (Mariani *et al.*, 2003). Another restriction factor is tripartite motif-containing protein 5α (TRIM5α), which recognizes the viral core and facilitates premature uncoating (Stremlau *et al.*, 2004). To establish a feasible model of HIV-1 infection, monkey-tropic HIV-1 (HIV-1mt) clones were constructed, which were expected to escape from these restriction factors (Hatziioannou *et al.*, 2006; Kamada *et al.*, 2006). In CMs, we reported previously that a modified HIV-1mt, MN4-5S, in which *vif* and the loops of α-helices 4 and 5 (L4/5) and α-helices 6 and 7 of the capsid protein (CA) of HIV-1 were replaced with those of SIVmac239, a pathogenic molecular clone of rhesus macaque SIV, showed enhanced virus replication *in vitro* (Kuroishi *et al.*, 2009) and *in vivo* (Saito *et al.*, 2011).

Accumulating evidence indicates intra-species variations in human and macaque *TRIM5* genes (Johnson & Sawyer, 2009). TRIMCyp is an alternatively spliced isoform of the *TRIM5* gene in which the PRYSPRY domain of TRIM5α is

One supplementary figure is available with the online version of this paper.

replaced with a retrotransposed cyclophilin A (*cypA*) gene (Brennan *et al.*, 2008; Liao *et al.*, 2007; Newman *et al.*, 2008). We recently reported that the frequency of TRIMCyp alleles was >0.8 in Philippine CMs, which is in contrast to the situation in Indochina CMs (Saito *et al.*, 2012a, 2012b). CM TRIMCyp, also known as Mafa TRIMCyp2 (Ylinen *et al.*, 2010), can restrict HIV-1, but fails to do so in SIVmac and HIV-1mt NL-DT5α with L4/5 derived from SIVmac (Saito *et al.*, 2012a), as the CypA domain of CM TRIMCyp binds to L4/5 of HIV-1, but not that of SIVmac (Price *et al.*, 2009; Ylinen *et al.*, 2010).

We recently reported that a new proviral HIV-1mt construct, MN4Rh-3, carrying a glutamine-to-aspartic acid substitution at position 110 (Q110D) of CA in the parental HIV-1mt MN4-8S (Fig. 1), exhibited further enhanced growth properties in a macaque T-cell line (Nomaguchi *et al.*, 2013a, b). In the present study, we investigated whether TRIMCyp alleles in CMs could influence the susceptibility to HIV-1mt infection.

First, we analysed the replication kinetics of HIV-1mt MN4Rh-3 in $CD8^+$ cell-depleted PBMCs from 26 CMs comprising nine TRIM5α homozygotes, eight TRIM5α/TRIMCyp heterozygotes and nine TRIMCyp homozygotes. Prior to this experiment, we confirmed the expression of TRIM5α and/or TRIMCyp in PBMCs from monkeys by reverse transcription-PCR (RT-PCR). We found that the mRNA expression was consistent with the *TRIM5* genotype of each monkey, i.e. the TRIM5α or TRIMCyp homozygotes expressed the respective mRNA, and the heterozygotes expressed both TRIM5α and TRIMCyp mRNAs (Fig. S1, available in JGV Online). Virus stocks for infection experiments were prepared by transfecting HIV-1mt MN4Rh-3 and HIV-1mt MN4-8S clones into HEK293T cells (Saito *et al.*, 2011). Preparation of $CD8^+$ cell-depleted PBMCs and evaluation of viral growth were performed as described previously (Saito *et al.*, 2011). In Fig. 2(a), representative viral kinetics in PBMCs from animals with each *TRIM5* genotype are presented. For comparison, the replication kinetics of HIV-1mt MN4Rh-3 in cells from all 26 animals is shown at the bottom of the figure. Furthermore, the impact of each *TRIM5* genotype on HIV-1mt MN4Rh-3 and MN4-8S replication was evaluated by plotting the peak p24 levels during the observation period (Fig. 2b). HIV-1mt MN4Rh-3 grew significantly better in the PBMCs from TRIMCyp homozygotes or heterozygotes than in those from TRIM5α homozygotes, whilst there was no significant difference between TRIMCyp homozygotes and heterozygotes (Fig. 2a, b). Our results on heterozygotes were consistent with previous findings that co-expression of TRIM5α variants with a distinct antiviral activity interferes with the antiviral activity of the wild-type TRIM5α (Javanbakht *et al.*, 2005; Lim *et al.*, 2010; Nakayama *et al.*, 2006; Perez-Caballero *et al.*, 2005; Stremlau *et al.*, 2004). In addition, HIV-1mt MN4Rh-3 grew better in PBMCs of both TRIMCyp homozygotes and the heterozygotes than HIV-1mt MN4-8S (Fig. 2a, b), which was in agreement with our recent data obtained in a CM-derived T-cell line (Nomaguchi *et al.*, 2013b). Of note, there was no significant difference between each *TRIM5* genotype in the susceptibility to SIVmac239 infection (Fig. 2c), suggesting that the CM *TRIM5* genotypes specifically influence susceptibility to HIV-1mt infection.

We finally investigated whether *TRIM5* genotypes could influence the growth of HIV-1mt MN4Rh-3 *in vivo*. Healthy adult CMs seronegative for B virus and simian retrovirus were housed in individual isolators in a Biosafety Level 3 facility and maintained according to National Institute of Biomedical Innovation guidelines. All experiments were approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation, as well as by Kyoto University. Bleeding and virus inoculation were performed under ketamine hydrochloride anaesthesia. Viral stocks propagated in $CD8^+$ cell-depleted PBMCs were inoculated intravenously into TRIMCyp homozygotes (n=6) or TRIM5α homozygotes (n=3) at a dose of HIV-1mt corresponding to 10 ng CA per head. The profiles of plasma viral loads and anti-HIV-1 antibody responses were evaluated as described previously (Saito *et al.*, 2011). We found that HIV-1mt MN4Rh-3 growth was readily observed in all

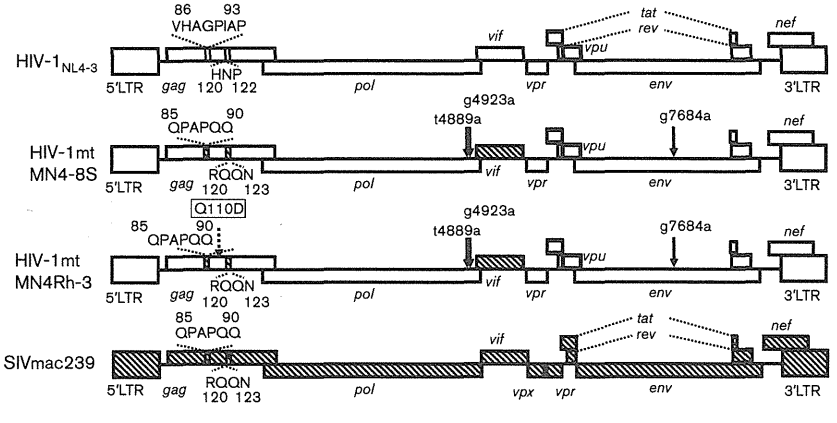

Fig. 1. Structure of the HIV-1mt clones (MN4-8S and MN4Rh-3) used in this study. Open boxes denote HIV-1 (NL4-3) and hatched boxes denote SIVmac239 sequences. Black arrows show adaptive mutations that enhance viral growth potential in CM T-cell lines. Dotted arrows show the CA Q110D mutation.

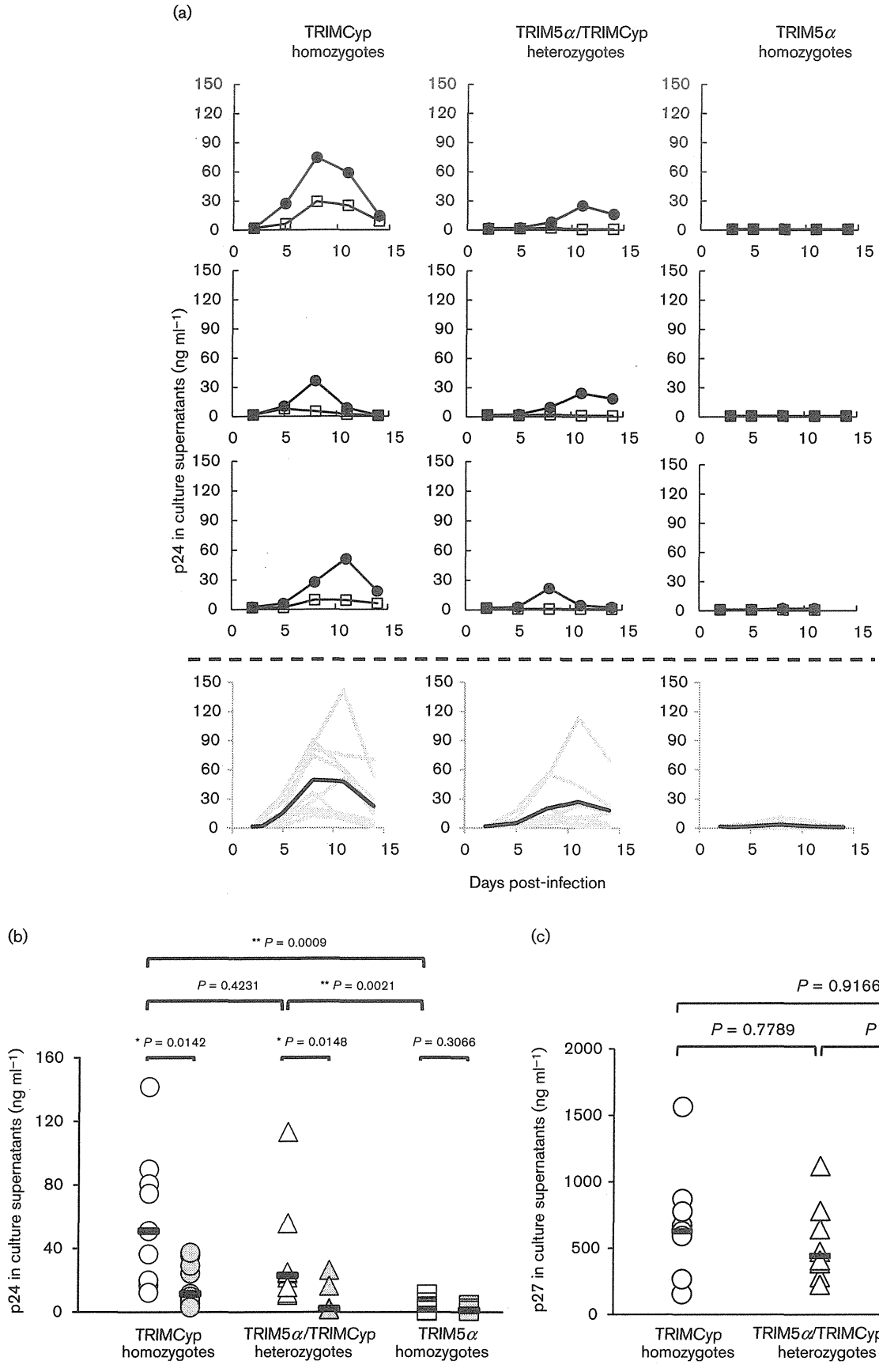
(a)
TRIMCyp homozygotes
TRIM5α/TRIMCyp heterozygotes
TRIM5α homozygotes
p24 in culture supernatants (ng ml−1)
Days post-infection
(b)
** P = 0.0009
P = 0.4231
** P = 0.0021
* P = 0.0142
* P = 0.0148
P = 0.3066
p24 in culture supernatants (ng ml−1)
TRIMCyp homozygotes
TRIM5α/TRIMCyp heterozygotes
TRIM5α homozygotes
(c)
P = 0.9166
P = 0.7789
P = 0.8801
p27 in culture supernatants (ng ml−1)
TRIMCyp homozygotes
TRIM5α/TRIMCyp heterozygotes
TRIM5α homozygotes

Fig. 2. (a) Growth properties of HIV-1mt derivatives in CM PBMCs. $CD8^+$ cell-depleted PBMCs were infected with HIV-1mt MN4Rh-3 (●) or HIV-1mt MN4-8S (□). Culture supernatants were collected periodically and virus replication was assessed using a HIV-1 p24 antigen capture assay kit. These experiments carried out on the PBMCs of each of the 26 macaques were done once. Representative results of virus replication kinetics in the PBMCs prepared from three animals of each *TRIM5* genotype are shown. For comparison, the replication kinetics of HIV-1mt MN4Rh-3 in the PBMCs from the 26 animals are shown at the bottom of the figure (indicated as grey lines). The mean values of the viral growth kinetics in each genotype are indicated in black lines. (b) Influence of *TRIM5* genotypes on the replication of HIV-1mt derivatives in PBMCs. $CD8^+$ cell-depleted PBMCs were infected with HIV-1mt MN4Rh-3 (open symbols) or HIV-1mt MN4-8S (shaded symbols). The cells were derived from TRIMCyp homozygotes ($n=9$), TRIM5α/TRIMCyp heterozygotes ($n=8$) and TRIM5α homozygotes ($n=9$). The peak p24 levels of the virus replication kinetics as shown in Fig. 2(a) were plotted. Thick horizontal bars indicate the median values. Differences in the mean values were assessed using the Wilcoxon rank-sum test (for HIV-1mt MN4Rh-3 and HIV-1mt MN4-8S viruses in each monkey group) and using the Steel–Dwass multiple comparison procedure (for HIV-1mt MN4Rh-3 in the three monkey groups). $^*P<0.05$; $^{**}P<0.01$. (c) Influence of *TRIM5* genotypes on the replication of SIVmac239 in PBMCs. $CD8^+$ cell-depleted PBMCs were infected with SIVmac239. Virus replication was monitored by detecting p27 antigen in the culture supernatants, and the p27 level on the peak day during the observation period (14 days) was plotted. Thick horizontal bars indicate the median values. Differences in the mean values were assessed by the Wilcoxon rank-sum test.

TRIMCyp homozygotes, with plasma viral loads reaching a peak at 2–4 weeks post-inoculation (p.i.) and ranging from 1.1×10^4 to 1.5×10^5 copies ml^{-1} (mean 4.2×10^4 copies ml^{-1}; Fig. 3a). In contrast, HIV-1mt MN4Rh-3 scarcely replicated in TRIM5α homozygotes (mean 1.9×10^3 copies ml^{-1}; Fig. 3a). Accordingly, HIV-1-specific antibodies were also detected in plasma from 3 to 9 weeks p.i. in the TRIMCyp homozygotes but minimally in TRIM5α homozygotes (Fig. 3b), suggesting that the strength of antibody response reflected the level of virus replication. Notably, although TRIMCyp homozygotes had a higher viraemia compared with TRIM5α homozygotes, none developed persistent viraemia (Fig. 3a). As our present HIV-1mts were focused on evasion of TRIM5- and APOBEC3-mediated restrictions, it is reasonable to assume that additional modifications of the viral genome, especially in order to overcome bone marrow stromal antigen 2 (BST-2)-mediated (Jia *et al.*, 2009; Neil *et al.*, 2008; Van Damme *et al.*, 2008) and SAM domain and HD domain-containing protein 1 (SAMHD1)-mediated restriction (Hrecka *et al.*, 2011; Laguette *et al.*, 2011), may be required to establish persistent viraemia *in vivo*. Moreover, Bitzegeio *et al.* (2013) recently suggested the existence of unidentified, type I interferon-inducible antiviral host factors in macaque PBMCs that inhibit HIV-1 replication.

In humans, several genetic factors related to HIV-1 susceptibility have been reported (reviewed by Chatterjee, 2010; Shioda & Nakayama, 2006). A polymorphism in the chemokine (C–C motif) receptor-5 (*CCR5*) gene is an eminent example; thus, individuals carrying a 32 bp deletion in *CCR5* (CCR5-Δ32) are resistant to CCR5-tropic HIV-1 infection and show delayed progression to AIDS (Dean *et al.*, 1996; Samson *et al.*, 1996). In addition to *CCR5*, polymorphisms in the genes encoding IL-4 and IL-10 (Shin *et al.*, 2000) and human leukocyte antigen (Carrington & O'Brien, 2003), as well as *TRIM5* (Sawyer *et al.*, 2006), have also been suggested to affect disease progression in HIV-1-infected individuals. One of the single-nucleotide polymorphisms (SNPs) in human *TRIM5* is a C127T nucleotide substitution, corresponding to an H43Y amino acid substitution in the RING domain. A correlation between this SNP and rapid disease progression has been suggested (van Manen *et al.*, 2008), although this remains controversial (Nakayama *et al.*, 2007; Speelmon *et al.*, 2006). In macaques, an effect of polymorphisms in *TRIM5* on SIV infection has been reported (Kirmaier *et al.*, 2010; Lim *et al.*, 2010); thus, rhesus macaques with TFP residues at positions 339–341 of TRIM5α show greater resistance to SIVsmE041 and SIVsmE543–3 compared with animals with a single glutamine residue at position 339 (Kirmaier *et al.*, 2010). However, it remains elusive as to whether genetic diversity might affect HIV-1mt infection in macaques. In this study, we found for the first time that the *TRIM5* genotypes of CMs primarily influenced inter-individual diversity in terms of susceptibility to HIV-1mt. Our results will provide an important insight into the divergent susceptibility of macaques to HIV-1mt. In particular, the finding that the TRIMCyp homozygotes exhibited a greater susceptibility to HIV-1mt infection will make it possible to identify the susceptibility of each CM by pre-screening for *TRIM5* genotypes, which will be invaluable in establishing a pre-clinical non-human primate model of HIV-1mt infection using CMs. It is noteworthy that our result is consistent with the findings that pig-tailed macaques, a macaque species that is thought to possess TRIMCyp exclusively instead of TRIM5α, shows higher susceptibility to HIV-1 infection (Agy *et al.*, 1992). For this reason, pig-tailed macaques are expected to be a promising model animal for HIV-1mt infection. Indeed, it was reported previously that these macaques developed persistent viraemia following HIV-1mt challenge (Hatziioannou *et al.*, 2009; Igarashi *et al.*, 2007; Thippeshappa *et al.*, 2011).

Moreover, our findings, in which CM *TRIM5* genotype was shown to influence susceptibility to retroviral infection, may imply that the marked geographical variation in the genotypes (Berry *et al.*, 2012; Dietrich *et al.*, 2011; Saito *et al.*, 2012a; Saito *et al.*, 2012b) is a consequence of selective pressures driven by some external factors. As both TRIM5α and TRIMCyp are thought to be associated with retrovirus replication, it is reasonable to speculate that a geographically diverse prevalence of some pathogen(s) such as exogenous or endogenous retroviruses might

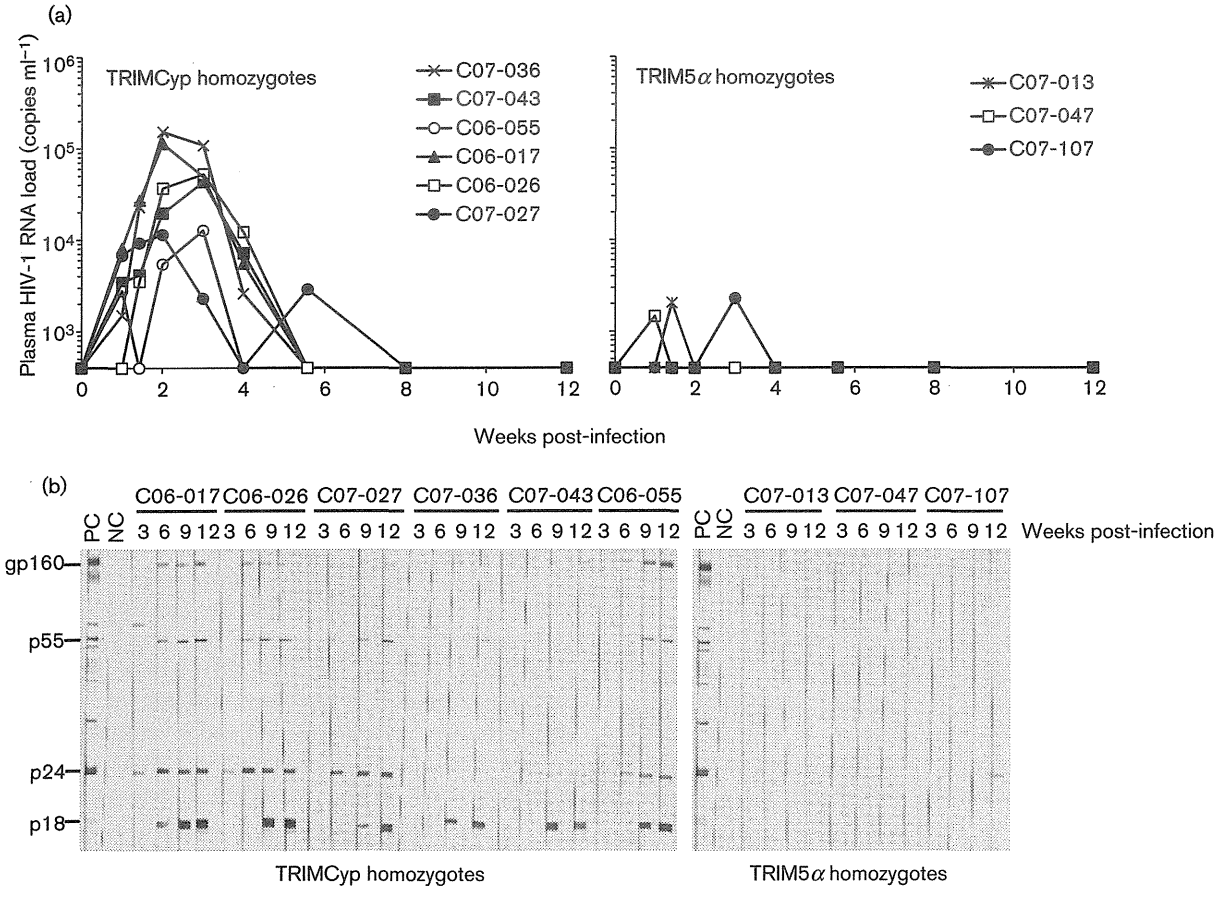

Fig. 3. Growth properties of HIV-1mt derivatives in CMs. (a) Monkeys were infected with 10 ng p24 of HIV-1mt MN4Rh-3 intravenously and bled periodically. Plasma viral RNA load was evaluated by quantitative RT-PCR. (b) Commercially available diagnostic HIV-1 Western blotting strips were reacted with 100-fold-diluted plasma from each monkey. Plasma from HIV-1-infected and uninfected individuals were used as positive (PC) and negative (NC) controls, respectively. Individual monkey numbers are indicated.

contribute to the variation in *TRIM5* genotypes. We are now seeking to identify pathogen(s) that have played a critical role in the diversity of CM *TRIM5* genotypes.

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References

Agy, M. B., Frumkin, L. R., Corey, L., Coombs, R. W., Wolinsky, S. M., Koehler, J., Morton, W. R. & Katze, M. G. (1992). Infection of *Macaca nemestrina* by human immunodeficiency virus type-1. *Science* **257**, 103–106.

Berry, N. J., Marzetta, F., Towers, G. J. & Rose, N. J. (2012). Diversity of TRIM5α and TRIMCyp sequences in cynomolgus macaques from different geographical origins. *Immunogenetics* **64**, 267–278.

Bitzegeio, J., Sampias, M., Bieniasz, P. D. & Hatziioannou, T. (2013). Adaptation to the interferon-induced antiviral state by human and simian immunodeficiency viruses. *J Virol* **87**, 3549–3560.

Brennan, G., Kozyrev, Y. & Hu, S. L. (2008). TRIMCyp expression in Old World primates *Macaca nemestrina* and *Macaca fascicularis*. *Proc Natl Acad Sci U S A* **105**, 3569–3574.

Carrington, M. & O'Brien, S. J. (2003). The influence of HLA genotype on AIDS. *Annu Rev Med* **54**, 535–551.

Chatterjee, K. (2010). Host genetic factors in susceptibility to HIV-1 infection and progression to AIDS. *J Genet* **89**, 109–116.

Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E. & other authors (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273**, 1856–1862.

Dietrich, E. A., Brennan, G., Ferguson, B., Wiseman, R. W., O'Connor, D. & Hu, S. L. (2011). Variable prevalence and functional diversity of the antiretroviral restriction factor TRIMCyp in *Macaca fascicularis*. *J Virol* **85**, 9956–9963.

Hatziioannou, T., Princiotta, M., Piatak, M., Jr, Yuan, F., Zhang, F., Lifson, J. D. & Bieniasz, P. D. (2006). Generation of simian–tropic HIV-1 by restriction factor evasion. *Science* **314**, 95.

Hatziioannou, T., Ambrose, Z., Chung, N. P., Piatak, M., Jr, Yuan, F., Trubey, C. M., Coalter, V., Kiser, R., Schneider, D. & other authors (2009). A macaque model of HIV-1 infection. *Proc Natl Acad Sci U S A* **106**, 4425–4429.

Hrecka, K., Hao, C., Gierszewska, M., Swanson, S. K., Kesik-Brodacka, M., Srivastava, S., Florens, L., Washburn, M. P. & Skowronski, J. (2011). Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. *Nature* **474**, 658–661.

Igarashi, T., Iyengar, R., Byrum, R. A., Buckler-White, A., Dewar, R. L., Buckler, C. E., Lane, H. C., Kamada, K., Adachi, A. & Martin, M. A. (2007). Human immunodeficiency virus type 1 derivative with 7% simian immunodeficiency virus genetic content is able to establish infections in pig-tailed macaques. *J Virol* **81**, 11549–11552.

Javanbakht, H., Diaz-Griffero, F., Stremlau, M., Si, Z. & Sodroski, J. (2005). The contribution of RING and B-box 2 domains to retroviral restriction mediated by monkey TRIM5α. *J Biol Chem* **280**, 26933–26940.

Jia, B., Serra-Moreno, R., Neidermyer, W., Rahmberg, A., Mackey, J., Fofana, I. B., Johnson, W. E., Westmoreland, S. & Evans, D. T. (2009). Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2. *PLoS Pathog* **5**, e1000429.

Johnson, W. E. & Sawyer, S. L. (2009). Molecular evolution of the antiretroviral *TRIM5* gene. *Immunogenetics* **61**, 163–176.

Kamada, K., Igarashi, T., Martin, M. A., Khamsri, B., Hatcho, K., Yamashita, T., Fujita, M., Uchiyama, T. & Adachi, A. (2006). Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. *Proc Natl Acad Sci U S A* **103**, 16959–16964.

Kirmaier, A., Wu, F., Newman, R. M., Hall, L. R., Morgan, J. S., O'Connor, S., Marx, P. A., Meythaler, M., Goldstein, S. & other authors (2010). *TRIM5* suppresses cross-species transmission of a primate immunodeficiency virus and selects for emergence of resistant variants in the new species. *PLoS Biol* **8**, e1000462.

Kuroishi, A., Saito, A., Shingai, Y., Shioda, T., Nomaguchi, M., Adachi, A., Akari, H. & Nakayama, E. E. (2009). Modification of a loop sequence between α-helices 6 and 7 of virus capsid (CA) protein in a human immunodeficiency virus type 1 (HIV-1) derivative that has simian immunodeficiency virus (SIVmac239) *vif* and CA α-helices 4 and 5 loop improves replication in cynomolgus monkey cells. *Retrovirology* **6**, 70.

Laguette, N., Sobhian, B., Casartelli, N., Ringeard, M., Chable-Bessia, C., Ségéral, E., Yatim, A., Emiliani, S., Schwartz, O. & Benkirane, M. (2011). SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* **474**, 654–657.

Liao, C.-H., Kuang, Y.-Q., Liu, H.-L., Zheng, Y.-T. & Su, B. (2007). A novel fusion gene, TRIM5-Cyclophilin A in the pig-tailed macaque determines its susceptibility to HIV-1 infection. *AIDS* **21** (Suppl. 8), S19–S26.

Lim, S. Y., Rogers, T., Chan, T., Whitney, J. B., Kim, J., Sodroski, J. & Letvin, N. L. (2010). TRIM5α modulates immunodeficiency virus control in rhesus monkeys. *PLoS Pathog* **6**, e1000738.

Mariani, R., Chen, D., Schröfelbauer, B., Navarro, F., König, R., Bollman, B., Münk, C., Nymark-McMahon, H. & Landau, N. R. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* **114**, 21–31.

Nakayama, E. E., Maegawa, H. & Shioda, T. (2006). A dominant-negative effect of cynomolgus monkey tripartite motif protein TRIM5α on anti-simian immunodeficiency virus SIVmac activity of an African green monkey orthologue. *Virology* **350**, 158–163.

Nakayama, E. E., Carpentier, W., Costagliola, D., Shioda, T., Iwamoto, A., Debre, P., Yoshimura, K., Autran, B., Matsushita, S. & Theodorou, I. (2007). Wild type and *H43Y* variant of human *TRIM5α* show similar anti-human immunodeficiency virus type 1 activity both in vivo and in vitro. *Immunogenetics* **59**, 511–515.

Neil, S. J., Zang, T. & Bieniasz, P. D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451**, 425–430.

Newman, R. M., Hall, L., Kirmaier, A., Pozzi, L. A., Pery, E., Farzan, M., O'Neil, S. P. & Johnson, W. (2008). Evolution of a TRIM5–CypA splice isoform in Old World monkeys. *PLoS Pathog* **4**, e1000003.

Nomaguchi, M., Doi, N., Fujiwara, S., Saito, A., Akari, H., Nakayama, E. E., Shioda, T., Yokoyama, M., Sato, H. & other authors (2013a). Systemic biological analysis of the mutations in two distinct HIV-1mt genomes occurred during replication in macaque cells. *Microbes Infect* **15**, 319–328.

Nomaguchi, M., Yokoyama, M., Kono, K., Nakayama, E. E., Shioda, T., Saito, A., Akari, H., Yasutomi, Y., Matano, T. & other authors (2013b). Gag-CA Q110D mutation elicits TRIM5-independent enhancement of HIV-1mt replication in macaque cells. *Microbes Infect* **15**, 56–65.

Perez-Caballero, D., Hatziioannou, T., Yang, A., Cowan, S. & Bieniasz, P. D. (2005). Human tripartite motif 5α domains responsible for retrovirus restriction activity and specificity. *J Virol* **79**, 8969–8978.

Price, A. J., Marzetta, F., Lammers, M., Ylinen, L. M., Schaller, T., Wilson, S. J., Towers, G. J. & James, L. C. (2009). Active site remodeling switches HIV specificity of antiretroviral TRIMCyp. *Nat Struct Mol Biol* **16**, 1036–1042.

Saito, A., Nomaguchi, M., Iijima, S., Kuroishi, A., Yoshida, T., Lee, Y. J., Hayakawa, T., Kono, K., Nakayama, E. E. & other authors (2011). Improved capacity of a monkey-tropic HIV-1 derivative to replicate in cynomolgus monkeys with minimal modifications. *Microbes Infect* **13**, 58–64.

Saito, A., Kono, K., Nomaguchi, M., Yasutomi, Y., Adachi, A., Shioda, T., Akari, H. & Nakayama, E. E. (2012a). Geographical, genetic and functional diversity of antiretroviral host factor TRIMCyp in cynomolgus macaque (*Macaca fascicularis*). *J Gen Virol* **93**, 594–602.

Saito, A., Kawamoto, Y., Higashino, A., Yoshida, T., Ikoma, T., Suzaki, Y., Ami, Y., Shioda, T., Nakayama, E. E. & Akari, H. (2012b). Allele frequency of antiretroviral host factor TRIMCyp in wild-caught cynomolgus macaques (*Macaca fascicularis*). *Front Microbiol* **3**, 314.

Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J. & other authors (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722–725.

Sawyer, S. L., Wu, L. I., Akey, J. M., Emerman, M. & Malik, H. S. (2006). High-frequency persistence of an impaired allele of the retroviral defense gene TRIM5α in humans. *Curr Biol* **16**, 95–100.

Sheehy, A. M., Gaddis, N. C., Choi, J. D. & Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**, 646–650.

Shin, H. D., Winkler, C., Stephens, J. C., Bream, J., Young, H., Goedert, J. J., O'Brien, T. R., Vlahov, D., Buchbinder, S. & other authors (2000). Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc Natl Acad Sci U S A* **97**, 14467–14472.

Shioda, T. & Nakayama, E. E. (2006). Human genetic polymorphisms affecting HIV-1 diseases. *Int J Hematol* **84**, 12–17.

Speelmon, E. C., Livingston-Rosanoff, D., Li, S. S., Vu, Q., Bui, J., Geraghty, D. E., Zhao, L. P. & McElrath, M. J. (2006). Genetic association of the antiviral restriction factor TRIM5α with human immunodeficiency virus type 1 infection. *J Virol* **80**, 2463–2471.

Stremlau, M., Owens, C. M., Perron, M. J., Kiessling, M., Autissier, P. & Sodroski, J. (2004). The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. *Nature* **427**, 848–853.

Thippeshappa, R., Polacino, P., Yu Kimata, M. T., Siwak, E. B., Anderson, D., Wang, W., Sherwood, L., Arora, R., Wen, M. & other authors (2011). Vif substitution enables persistent infection of pig-tailed macaques by human immunodeficiency virus type 1. *J Virol* **85**, 3767–3779.

Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., Stephens, E. B. & Guatelli, J. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* **3**, 245–252.

van Manen, D., Rits, M. A., Beugeling, C., van Dort, K., Schuitemaker, H. & Kootstra, N. A. (2008). The effect of *Trim5* polymorphisms on the clinical course of HIV-1 infection. *PLoS Pathog* **4**, e18.

Ylinen, L. M., Price, A. J., Rasaiyaah, J., Hué, S., Rose, N. J., Marzetta, F., James, L. C. & Towers, G. J. (2010). Conformational adaptation of Asian macaque TRIMCyp directs lineage specific antiviral activity. *PLoS Pathog* **6**, e1001062.

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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.

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

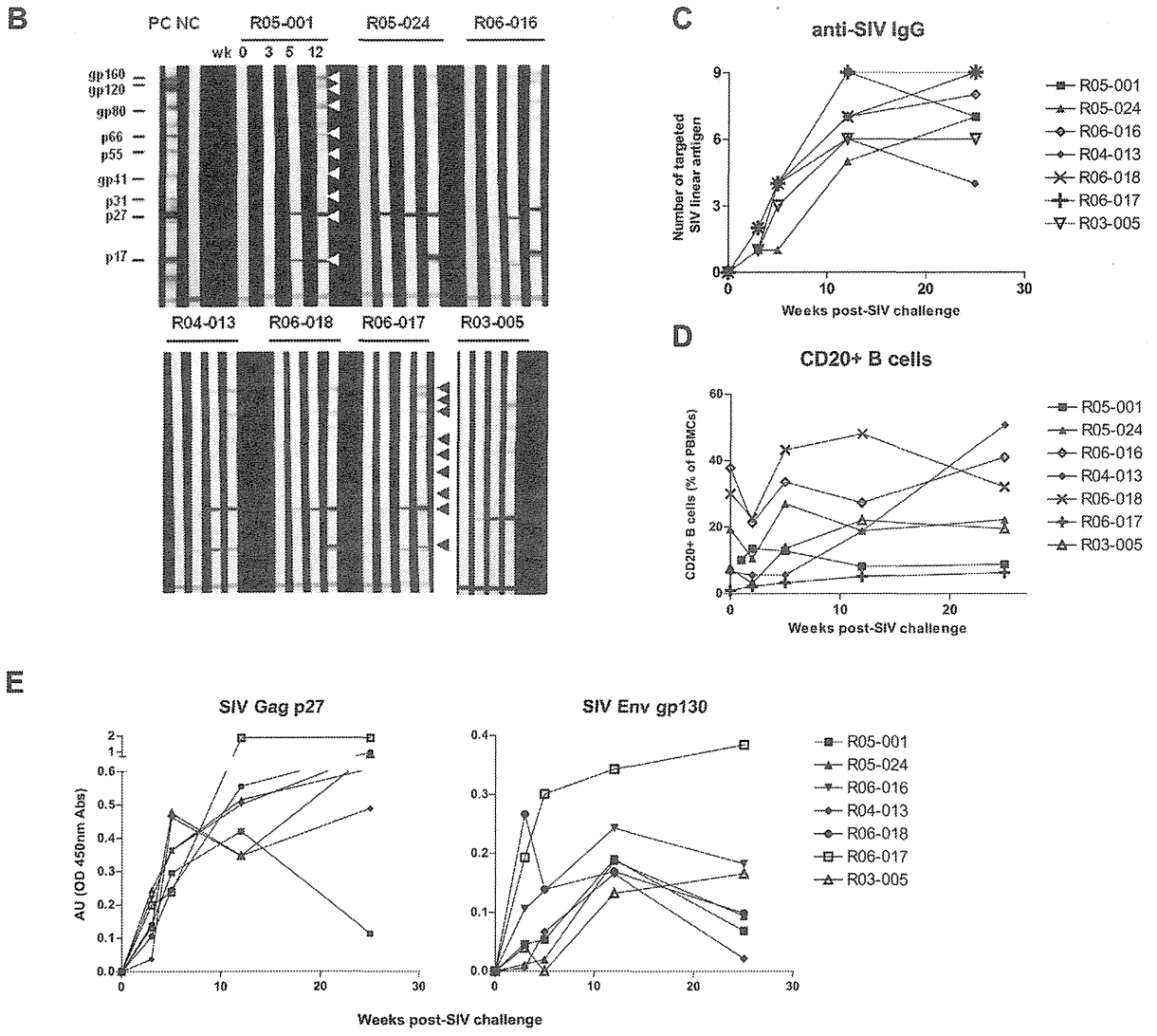

Fig. 1. Plasma antiviral antibody development in SIV_{mac239} infection. (A) Plasma viral loads after SIV challenge (SIV RNA copies/ml plasma). N.D., undetectable. (B) Plasma SIV-specific IgG detection via western blotting. Arrowheads represent listed SIV_{mac} linear antigens. (C) Number of IgG-targeted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

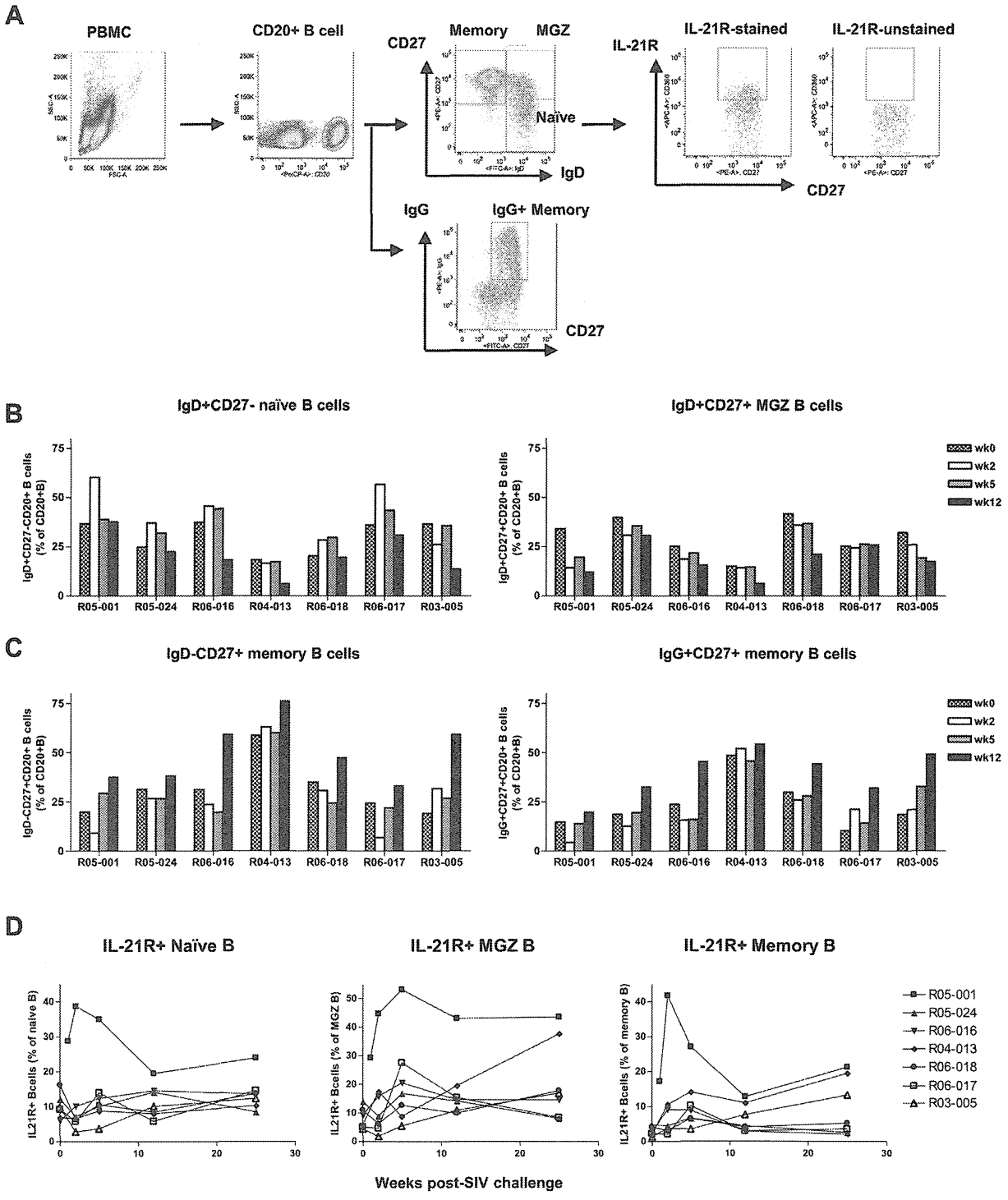

Fig. 2. Peripheral blood B cell subsets and IL-21R expression in SIV_{mac239} infection. (A) Representative gating. (B) Naïve and MGZ B cell frequencies. (C) Memory and IgG+ memory B cell frequencies. (D) IL-21R+ naïve, IL-21R+ MGZ and IL-21R+ memory B cell frequencies.

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

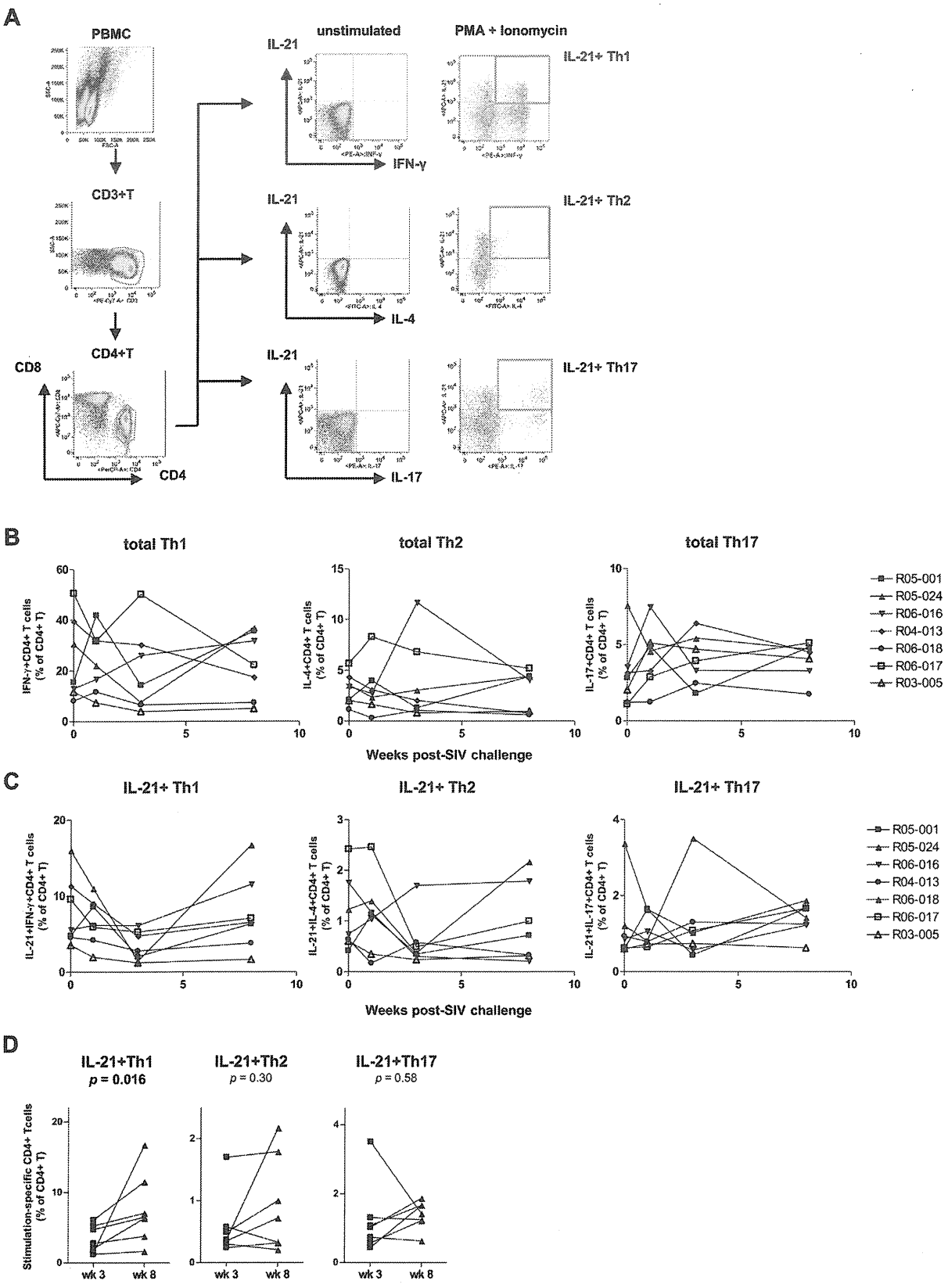

Fig. 3. IL-21-producing Th1, Th2 and Th17 in SIV_{mac239} infection. (A) Representative gating. (B) Total Th1, total Th2 and total Th17 frequencies. (C) IL-21+ Th1, IL-21+ Th2 and IL-21+ Th17 frequencies. (D) Wilcoxon's test of IL-21+ Th1, Th2 and Th17 frequencies (week 3 vs week 8).

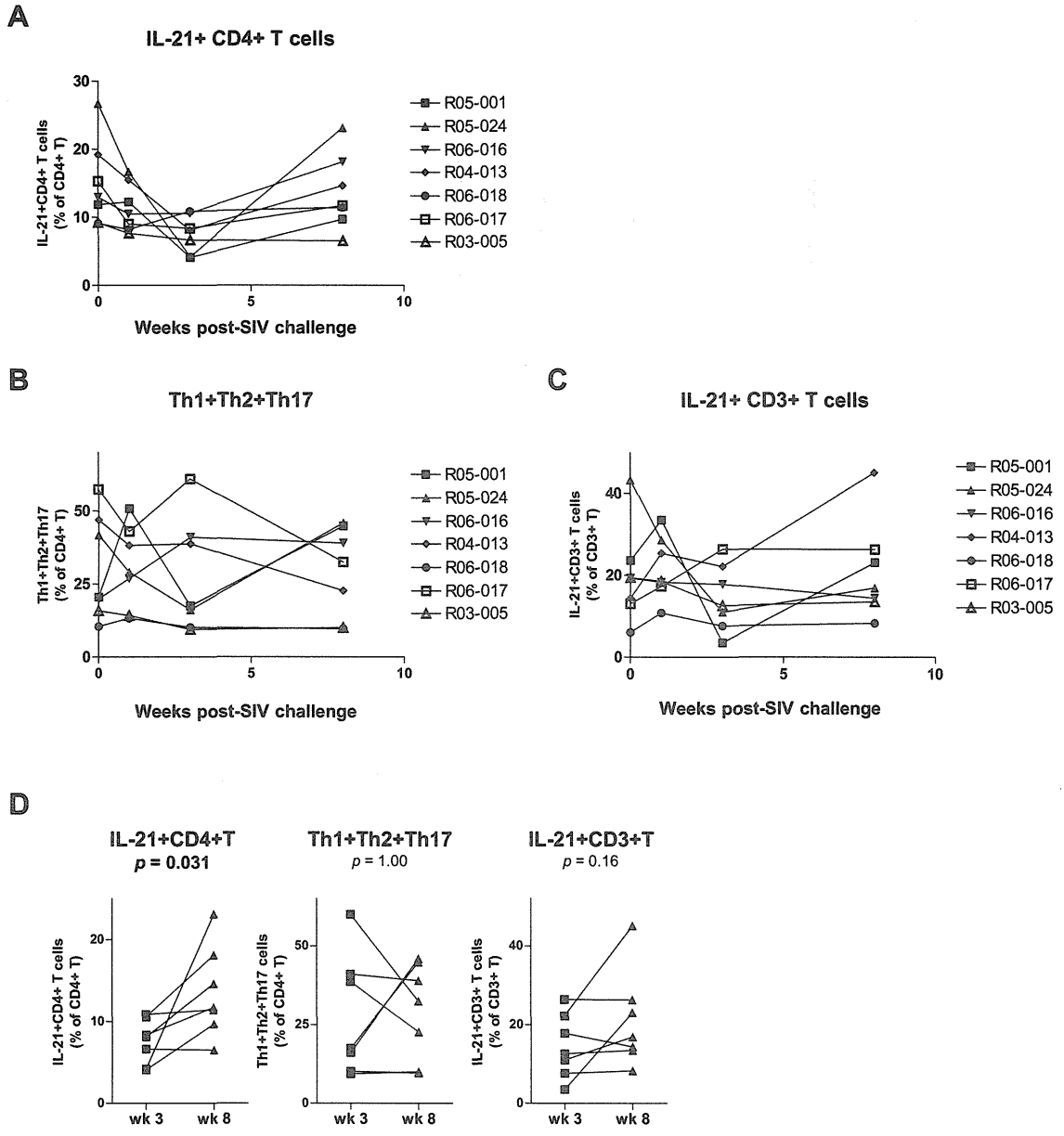

Fig. 4. Total IL-21-producing CD4+ T cell kinetics in SIV_{mac239} infection. (A) IL-21+ CD4+ T-cell frequencies. (B) Th1+Th2+Th17 frequencies. (C) IL-21+ CD3+ T-cell frequencies. (D) Wilcoxon's test of IL-21+ CD4+ T cells, Th1+Th2+Th17 and IL-21+ CD3+ T cells (week 3 vs week 8).

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

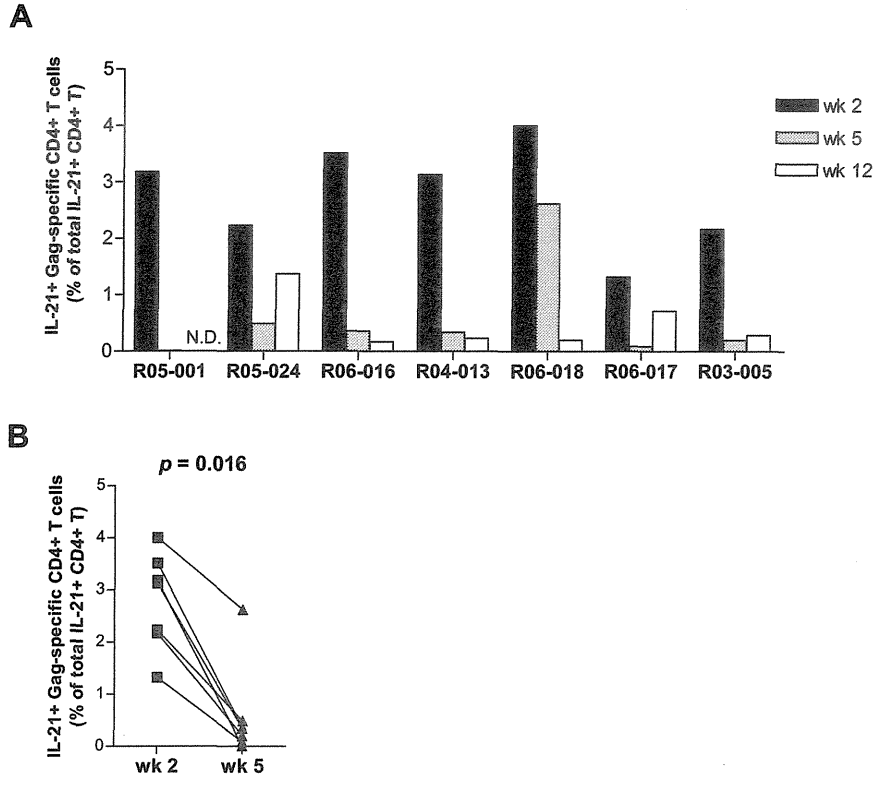

Fig. 5. Percentages of SIV Gag-specific IL-21+ CD4+ T cells within total IL-21+ CD4+ T cells. (A) Values at week 2, week 5 and week 12. N.D., R05-001 sample at week 12 unavailable. (B) Wilcoxon's test of Gag-specific/total IL-21+ CD4+ T cell ratios (week 2 vs week 5).

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.

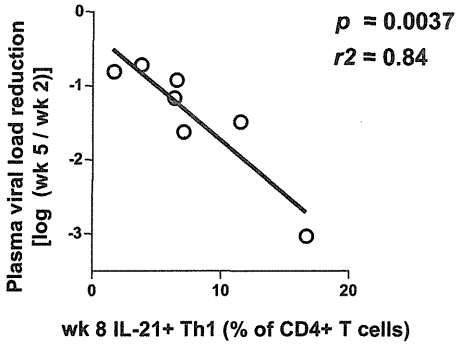

Fig. 6. Correlation analysis of IL-21+ Th1 at week 8 and week 5/week 2 viral load reduction (log).

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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References

[1] D.R. Burton, R.C. Desrosiers, R.W. Doms, W.C. Koff, P.D. Kwong, J.P. Moore, G.J. Nabel, J. Sodroski, I.A. Wilson, R.T. Wyatt, HIV vaccine design and the neutralizing antibody problem, Nat. Immunol. 5 (2004) 233–236.

[2] H. Yamamoto, M. Kawada, A. Takeda, H. Igarashi, T. Matano, Post-infection immunodeficiency virus control by neutralizing antibodies, PLoS One 2 (2007) e540.

[3] A.J. Hessell, L. Hangartner, M. Hunter, C.E. Havenith, F.J. Beurskens, J.M. Bakker, C.M. Lanigan, G. Landucci, D.N. Forthal, P.W. Parren, P.A. Marx, D.R. Burton, Fc receptor but not complement binding is important in antibody protection against HIV, Nature 449 (2007) 101–104.

[4] A. De Milito, A. Nilsson, K. Titanji, R. Thorstensson, E. Reizenstein, M. Narita, S. Grutzmeier, A. Sönnerborg, F. Chiodi, Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection, Blood 103 (2004) 2180–2186.

[5] D.D. Richman, T. Wrin, S.J. Little, C.J. Petropoulos, Rapid evolution of the neutralizing antibody response to HIV type 1 infection, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 4144–4149.

[6] G.D. Tomaras, N.L. Yates, P. Liu, L. Qin, G.G. Fouda, L.L. Chavez, A.C. Decamp, R.J. Parks, V.C. Ashley, J.T. Lucas, M. Cohen, J. Eron, C.B. Hicks, H.X. Liao, S.G. Self, G. Landucci, D.N. Forthal, K.J. Weinhold, B.F. Keele, B.H. Hahn, M.L. Greenberg, L. Morris, S.S. Karim, W.A. Blattner, D.C. Montefiori, G.M. Shaw, A.S. Perelson, B.F. Haynes, Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG

antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia, J. Virol. 82 (2008) 12449–12463.
[7] S. Peruchon, N. Chaoul, C. Burelout, B. Delache, P. Brochard, P. Laurent, F. Cognasse, S. Prévot, O. Garraud, R. Le Grand, Y. Richard, Tissue-specific B-cell dysfunction and generalized memory B-cell loss during acute SIV infection, PLoS One 4 (2009) e5966.
[8] M.C. Levesque, M.A. Moody, K.K. Hwang, D.J. Marshall, J.F. Whitesides, J.D. Amos, T.C. Gurley, S. Allgood, B.B. Haynes, N.A. Vandergrift, S. Plonk, D.C. Parker, M.S. Cohen, G.D. Tomaras, P.A. Goepfert, G.M. Shaw, J.E. Schmitz, J.J. Eron, N.J. Shaheen, C.B. Hicks, H.X. Liao, M. Markowitz, G. Kelsoe, D.M. Margolis, B.F. Haynes, Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection, PLoS Med. 6 (2009) e1000107.
[9] W.W. Yeh, I. Rahman, P. Hraber, R.T. Coffey, D. Nevidomskyte, A. Giri, M. Asmal, S. Miljkovic, M. Daniels, J.B. Whitney, B.F. Keele, B.H. Hahn, B.T. Korber, G.M. Shaw, M.S. Seaman, N.L. Letvin, Autologous neutralizing antibodies to the transmitted/founder viruses emerge late after simian immunodeficiency virus SIVmac251 infection of rhesus monkeys, J. Virol. 84 (2010) 6018–6032.
[10] I. Mikell, D.N. Sather, S.A. Kalams, M. Altfeld, G. Alter, L. Stamatatos, Characteristics of the earliest cross-neutralizing antibody response to HIV-1, PLoS Pathog. 7 (2011) e1001251.
[11] Y. Nishimura, T. Igarashi, O.K. Donau, A. Buckler-White, C. Buckler, B.A. Lafont, R.M. Goeken, S. Goldstein, V.M. Hirsch, M.A. Martin, Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 12324–12329.
[12] J.J. Mattapallil, D.C. Douek, B. Hill, Y. Nishimura, M. Martin, M. Roederer, Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection, Nature 434 (2005) 1093–1097.
[13] F.T. Hufert, J. van Lunzen, G. Janossy, S. Bertram, J. Schmitz, O. Haller, P. Racz, D. von Laer, Germinal centre CD4+ T cells are an important site of HIV replication in vivo, AIDS 11 (1997) 849–857.
[14] J.J. Hong, P.K. Amancha, K. Rogers, A.A. Ansari, F. Villinger, Spatial alterations between CD4(+) T follicular helper, B, and CD8(+) T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape, J. Immunol. 188 (2012) 3247–3256.
[15] C.S. Ma, E.K. Deenick, M. Batten, S.G. Tangye, The origins, function, and regulation of T follicular helper cells, J. Exp. Med. 209 (2012) 1241–1253.
[16] L. White, S. Krishnan, N. Strbo, H. Liu, M.A. Kolber, M.G. Lichtenheld, R.N. Pahwa, S. Pahwa, Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV), Blood 109 (2007) 3873–3880.
[17] H. Elsaesser, K. Sauer, D.G. Brooks, IL-21 is required to control chronic viral infection, Science 324 (2009) 1569–1572.
[18] J.S. Yi, M. Du, A.J. Zajac, A vital role for interleukin-21 in the control of a chronic viral infection, Science 324 (2009) 1572–1576.
[19] A. Fröhlich, J. Kisielow, I. Schmitz, S. Freigang, A.T. Shamshiev, J. Weber, B.J. Marsland, A. Oxenius, M. Kopf, IL-21R on T cells is critical for sustained functionality and control of chronic viral infection, Science 324 (2009) 1576–1580.
[20] V.L. Bryant, C.S. Ma, D.T. Avery, Y. Li, K.L. Good, L.M. Corcoran, R. de Waal Malefyt, S.G. Tangye, Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells, J. Immunol. 179 (2007) 8180–8190.
[21] R. Ettinger, G.P. Sims, A.M. Fairhurst, R. Robbins, Y.S. da Silva, R. Spolski, W.J. Leonard, P.E. Lipsky, IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells, J. Immunol. 175 (2005) 7867–7879.
[22] R. Ettinger, G.P. Sims, R. Robbins, D. Withers, R.T. Fischer, A.C. Grammer, S. Kuchen, P.E. Lipsky, IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells, J. Immunol. 178 (2007) 2872–2882.
[23] M. Recher, L.J. Berglund, D.T. Avery, M.J. Cowan, A.R. Gennery, J. Smart, J. Peake, M. Wong, S.Y. Pai, S. Baxi, J.E. Walter, U. Palendira, G.A. Tangye, M. Rice, S. Brothers, W. Al-Herz, H. Oettgen, H. Eibel, J.M. Puck, F. Cattaneo, J.B. Ziegler, S. Giliani, S.G. Tangye, L.D. Notarangelo, IL-21 is the primary common γ chain-binding cytokine required for human B-cell differentiation in vivo, Blood 118 (2011) 6824–6835.
[24] O. Dienz, S.M. Eaton, J.P. Bond, W. Neveu, D. Moquin, R. Noubade, E.M. Briso, C. Charland, W.J. Leonard, G. Ciliberto, C. Teuscher, L. Haynes, M. Rincon, The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by CD4+ T cells, J. Exp. Med. 206 (2009) 69–78.
[25] D. Eto, C. Lao, D. DiToro, B. Barnett, T.C. Escobar, R. Kageyama, I. Yusuf, S. Crotty, IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation, PLoS One 6 (2011) e17739.
[26] K.L. Good, V.L. Bryant, S.G. Tangye, Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21, J. Immunol. 177 (2006) 5236–5247.
[27] H. Jin, T.R. Malek, Redundant and unique regulation of activated mouse B lymphocytes by IL-4 and IL-21, J. Leukocyte Biol. 80 (2006) 1416–1423.
[28] M.T. Kasaian, M.J. Whitters, L.L. Carter, L.D. Lowe, J.M. Jussif, B. Deng, K.A. Johnson, J.S. Witek, M. Senices, R.F. Konz, A.L. Wurster, D.D. Donaldson, M. Collins, D.A. Young, M.J. Grusby, IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity, Immunity 16 (2002) 559–569.
[29] K. Ozaki, R. Spolski, C.G. Feng, C.F. Qi, J. Cheng, A. Sher, H.C. Morse 3rd, C. Liu, P.L. Schwartzberg, W.J. Leonard, A critical role for IL-21 in regulating immunoglobulin production, Science 298 (2002) 1630–1634.
[30] K. Lüthje, A. Kallies, Y. Shimohakamada, G.T. TBelz, A. Light, D.M. Tarlinton, S.L. Nutt, The development and fate of follicular helper T cells defined by an IL-21 reporter mouse, Nat. Immunol. 13 (2012) 491–498.
[31] D. Zotos, J.M. Coquet, Y. Zhang, A. Light, K. D'Costa, A. Kallies, L.M. Corcoran, D.I. Godfrey, K.M. Toellner, M.J. Smyth, S.L. Nutt, D.M. Tarlinton, IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism, J. Exp. Med. 207 (2010) 365–378.
[32] M.A. Linterman, L. Beaton, D. Yu, R.R. Ramiscal, M. Srivastava, J.J. Hogan, N.K. Verma, M.J. Smyth, R.J. Rigby, C.G. Vinuesa, IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses, J. Exp. Med. 207 (2010) 353–363.
[33] J. Bessa, M. Kopf, M.F. Bachmann, IL-21 and TLR signaling regulate germinal center responses in a B cell-intrinsic manner, J. Immunol. 184 (2010) 4615–4619.
[34] S. Seki, M. Kawada, A. Takeda, H. Igarashi, T. Sata, T. Matano, Transmission of simian immunodeficiency virus carrying multiple cytotoxic T-lymphocyte escape mutations with diminished replicative ability can result in AIDS progression in rhesus macaques, J. Virol. 82 (2008) 5093–5098.
[35] V.M. Hirsch, S. Santra, S. Goldstein, R. Plishka, A. Buckler-White, A. Seth, I. Ourmanov, C.R. Brown, R. Engle, D. Montefiori, J. Glowczwskie, K. Kunstman, S. Wolinsky, N.L. Letvin, Immune failure in the absence of profound CD4+ T-lymphocyte depletion in simian immunodeficiency virus-infected rapid progressor macaques, J. Virol. 78 (2004) 275–284.
[36] K. Titanji, V. Velu, L. Chennareddi, M. Vijay-Kumar, A.T. Gewirtz, G.J. Freeman, R.R. Amara, Acute depletion of activated memory B cells involves the PD-1 pathway in rapidly progressing SIV-infected macaques, J. Clin. Invest. 120 (2010) 3878–3890.
[37] R. Morita, N. Schmitt, S.E. Bentebibel, R. Ranganathan, L. Bourdery, G. Zurawski, E. Foucat, M. Dullaers, S. Oh, N. Sabzghabaei, E.M. Lavecchio, M. Punaro, V. Pascual, J. Banchereau, H. Ueno, Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion, Immunity 34 (2011) 108–121.

[38] A. Iannello, M.R. Boulassel, S. Samarani, O. Debbeche, C. Tremblay, E. Toma, J.P. Routy, A. Ahmad, Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study, J. Immunol. 184 (2010) 114–126.

[39] F.Y. Yue, C. Lo, A. Sakhdari, E.Y. Lee, C.M. Kovacs, E. Benko, J. Liu, H. Song, R.B. Jones, P. Sheth, D. Chege, R. Kaul, M.A. Ostrowski, HIV-specific IL-21 producing CD4+ T cells are induced in acute and chronic progressive HIV infection and are associated with relative viral control, J. Immunol. 185 (2010) 498–506.

[40] A. Iannello, C. Tremblay, J.P. Routy, M.R. Boulassel, E. Toma, A. Ahmad, Decreased levels of circulating IL-21 in HIV-infected AIDS patients: correlation with CD4+ T-cell counts, Viral Immunol. 21 (2008) 385–388.

[41] M.F. Chevalier, B. Jülg, A. Pyo, M. Flanders, S. Ranasinghe, D.Z. Soghoian, D.S. Kwon, J. Rychert, J. Lian, M.I. Muller, S. Cutler, E. McAndrew, H. Jessen, F. Pereyra, E.S. Rosenberg, M. Altfeld, B.D. Walker, H. Streeck, HIV-1-specific interleukin-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function, J. Virol. 85 (2011) 733–741.

[42] N. Ruffin, R. Lantto, S. Pensieroso, S. Sammicheli, B. Hejdeman, B. Rethi, F. Chiodi, Immune activation and increased IL-21R expression are associated with the loss of memory B cells during HIV-1 infection, J. Intern. Med. 272 (2012) 492–503.

[43] S. Pallikkuth, K. Rogers, F. Villinger, M. Dosterii, M. Vaccari, G. Franchini, R. Pahwa, S. Pahwa, Interleukin-21 administration to rhesus macaques chronically infected with simian immunodeficiency virus increases cytotoxic effector molecules in T cells and NK cells and enhances B cell function without increasing immune activation or viral replication, Vaccine 29 (2011) 9229–9238.

[44] M. Lindqvist, J. van Lunzen, D.Z. Soghoian, B.D. Kuhl, S. Ranasinghe, G. Kranias, M.D. Flanders, S. Cutler, N. Yudanin, M.I. Muller, I. Davis, D. Farber, P. Hartjen, F. Haag, G. Alter, J. Schulze Zur Wiesch, H. Streeck, Expansion of HIV-specific T follicular helper cells in chronic HIV infection, J. Clin. Invest. 122 (2012) 3271–3280.

[45] C. Petrovas, T. Yamamoto, M.Y. Gerner, K.L. Boswell, K. Wloka, E.C. Smith, D.R. Ambrozak, N.G. Sandler, K.J. Timmer, X. Sun, L. Pan, A. Poholek, S.S. Rao, J.M. Brenchley, S.M. Alam, G.D. Tomaras, M. Roederer, D.C. Douek, R.A. Seder, R.N. Germain, E.K. Haddad, R.A. Koup, CD4 T follicular helper cell dynamics during SIV infection, J. Clin. Invest. 122 (2012) 3281–3294.

[46] T. Hatziioannou, D.T. Evans, Animal models for HIV/AIDS research, Nat. Rev. Microbiol. 10 (2012) 852–867.

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