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TITLE:
Combination anti-HIV-1 antibody therapy is associated with increased virus-specific T cell immunity

ABSTRACT:
Combination antiretroviral therapy (ART) is highly effective in controlling human immunodeficiency virus (HIV)-1 but requires lifelong medication due to the existence of a latent viral reservoir(1,2). Potent broadly neutralizing antibodies (bNAbs) represent a potential alternative or adjuvant to ART. In addition to suppressing viremia, bNAbs may have T cell immunomodulatory effects as seen for other forms of immunotherapy(3). However, this has not been established in individuals who are infected with HIV-1. Here, we document increased HIV-1 Gag-specific CD8(+) T cell responses in the peripheral blood of all nine study participants who were infected with HIV-1 with suppressed blood viremia, while receiving bNAb therapy during ART interruption(4). Increased CD4(+) T cell responses were detected in eight individuals. The increased T cell responses were due both to newly detectable reactivity to HIV-1 Gag epitopes and the expansion of pre-existing measurable responses. These data demonstrate that bNAb therapy during ART interruption is associated with enhanced HIV-1-specific T cell responses. Whether these augmented T cell responses can contribute to bNAb-mediated viral control remains to be determined.

Main:
HIV-1 infection is characterized by high initial levels of plasma viremia that are variably controlled by virus-specific CD8+ T cell responses5,6. Individuals who fail to control viremia, rapidly develop immunodeficiency. In contrast, strong, broad HIV-specific CD8+ and CD4+ T cell responses have been associated with spontaneous viral control (that is, elite controllers, viral load <50 copies per ml) and delayed progression to AIDS7–9.
ART is highly effective in maintaining viral suppression but does not boost host antiviral immunity because it limits antigen availability. In contrast, antibodies do not prevent virus replication or production and, unlike small molecule drugs, they have dual functionality; variable domains neutralize the virus and constant domains (Fc) engage the host immune system3. In humanized mice, Fc interactions lead to accelerated clearance of viruses and infected cells10. bNAb administration to macaques infected with chimeric simian and human immunodeficiency viruses (SHIV) is associated with CD8+ T cell-dependent lasting control in a fraction of the treated animals11. In humans, bNAb monotherapy was associated with increased T cell responses in 9 of 12 individuals; however this occurred after rebound viremia in all but 3 individuals12. Whether bNAb therapy has a positive impact on HIV-1-specific T cell immune responses in infected humans with prolonged suppression during ART interruption has not been determined.
In a phase 1b clinical trial, individuals who were infected with HIV-1 and on ART were infused with a combination of two bNAbs, 3BNC117 and 10-1074, at 0, 3 and 6 weeks (Fig. 1a)4. ART was interrupted 2 d after the first antibody infusion. Nine bNAb-infused individuals harboring viruses sensitive to both bNAbs maintained viral suppression for at least 15 weeks following analytical treatment interruption (ATI) (Extended Data Fig. 1a,b)4.
Individuals who were infected with HIV-1 and on ART show stable or decreasing levels of HIV-1-specific CD8+ and CD4+ T cell responses over time13–15. To determine whether the combination of bNAb treatment and ATI was associated with alterations of CD8+ and CD4+ T cell responses to HIV-1, we analyzed the peripheral blood of the nine individuals on bNAb + ATI at baseline (week −2) and during bNAb-mediated suppression (weeks 6/7, 12 and 18; Extended Data Fig. 1b; week 18 samples were limited to seven individuals). Peripheral blood mononuclear cells (PBMCs) were stimulated with an HIV-1 Consensus B Gag peptide pool. CD8+ T cells were analyzed for expression of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)1-β and the degranulation marker CD107A; CD4+ T cells were analyzed for expression of IFN-γ, TNF-α, interleukin (IL)-2 and CD40L (Supplementary Table 1 and Supplementary Fig. 1a–c). In line with previous reports13–15, anti-HIV-1 T cell responses in individuals on long-term viral suppression by ART alone remained stable over time (Extended Data Fig. 2a,b). In contrast, the frequency of antigen-specific CD8+ T cells expressing IFN-γ, TNF-α, MIP1-β and/or CD107A increased significantly in all nine individuals receiving bNAbs during ATI after 6/7 weeks (Fig. 1b and Extended Data Fig. 3a). Of note, bNAb plasma levels were highest at this time point4 (Extended Data Fig. 1b). CD8+ T cell responses decreased by week 12 in six individuals but remained significantly elevated for IFN-γ, TNF-α and MIP1-β when compared to baseline. At week

18, when antibody levels were 2–3 orders of magnitude below the week 6/7 peak, CD8+ T cell responses were similar to week 12, but interpretation of these data was limited by the small sample size (Fig. 1b).
CD4+ T cells expressing IFN-γ, CD40L, TNF-α and/or IL-2 in response to Gag also increased significantly between baseline and week 6/7 in eight bNAb + ATI individuals (Fig. 1c). When measured individually, only CD40L and TNF-α remained significantly elevated at week 12 and no responses were significantly elevated at week 18. However, the total frequency of cytokine+ CD4+ T cells (percentage of cells positive for one or more cytokines or functional markers) was above baseline at all time points tested (Fig. 1c). In contrast, cytomegalovirus (CMV) pp65-specific T cell responses remained unchanged (Extended Data Fig. 4a,b), suggesting that the increased T cell immunity in bNAb + ATI individuals was specific to HIV-1. In summary, CD8+ and CD4+ T cell responses to Gag were most prominent at week 6/7 but remained elevated for weeks after the last antibody dose in individuals who remained suppressed while receiving bNAbs during ATI.
Two additional individuals recruited to the study harbored antibody-resistant viruses and showed early rebound after ATI (9245 and 9251, Extended Data Fig. 5a,b)4. Gag-specific T cell responses in both participants were analyzed at baseline, week 6/7 and week 11 or 12 after reinitiation of ART. Where the frequency of cytokine+ cells for CD8+ and CD4+ increased for individual 9245, the responses decreased for 9251 (Extended Data Fig. 5c), consistently with rebound viremia being sufficient to increase CD8+ T cell responses in some individuals16.
Polyfunctional HIV-1-specific CD8+ T cells have been associated with enhanced HIV-1 control9,17, whereas other studies reported superior antiviral functions of MIP1-β monofunctional cells18. To examine Gag-specific T cells in bNAb + ATI individuals for polyfunctional responses, we performed coexpression analysis using Boolean gating. Gag-specific CD8+ T cells coexpressing IFN-γ, TNF-α, MIP1-β and CD107A were significantly increased at weeks 6/7 and 12 after receiving bNAb therapy (Fig. 2a). However, the greatest absolute increase in CD8+ T cell responses to Gag was associated with expansion of MIP1-β+ single-positive cells (Fig. 2a). In addition, the frequency of CD4+ T cells expressing IFN-γ or CD40L alone or in combination with other functions and IL-2/TNF-α-double positive cells was also increased (Fig. 2b). Thus, several subsets of Gag-specific mono- or polyfunctional CD8+ and CD4+ T cells were augmented at weeks 6/7 and 12 compared to baseline for bNAb-treated individuals (Fig. 2a,b).
Activation-induced marker (AIM) assays give a broader overview of the total peptide-reactive T cell response and identify cells without cytokine expression or expressing cytokines that are challenging to detect by intracellular cytokine staining (ICS)19,20. We therefore used the AIM assay as an alternative, cytokine-independent method to confirm our findings obtained by ICS. CD4+ or CD8+AIM+ cells were identified as CD69+ programmed death ligand (PD-L)1+ or CD69+4-1BB+ or PD-L1+4-1BB+ after Gag peptide pool stimulation (Supplementary Fig. 2 and Supplementary Table 2).
Similarly to ICS, we found increased Gag-specific T cell responses in seven (CD8+) or six (CD4+) out of nine bNAb + ATI individuals at week 12 compared to baseline (Fig. 3a,b). As expected19, the frequency of Gag-specific CD4+ and CD8+ T cells was higher in the AIM assay but correlated with ICS (Fig. 3c,d, $r = 0.64$). We did not detect changes in human leukocyte antigen (HLA)-DR+CD38+ or programmed cell death (PD)-1+ cells within AIM+ Gag-specific T cell responses at week 12 versus baseline (Extended Data Fig. 6).
We further used the AIM assay to investigate responses to less immunodominant HIV-1 antigens with expected lower frequencies than Gag. In contrast to Gag, we did not find a significant increase in HIV-1-specific T cell responses directed against HIV-1 Pol, Nef, gp120 or gp41 at the cohort level (Fig. 3e). Nevertheless, enhanced CD8+ and/or CD4+ T cell responses to these HIV-1 proteins were noted in several individuals at week 12 compared to week −2, including against gp120 and gp41 (Extended Data Fig. 7a,b). Specifically, the two individuals with controlled viremia beyond 30 weeks showed increased CD8+ and CD4+ responses to nearly all HIV-1 antigens tested at week 12 (Extended Data Fig. 7a–c, participants 9254 and 9255). This was not seen in individuals who rebounded before week 26 after ATI (Extended Data Fig. 7a–c, participants 9241, 9242, 9243, 9244, 9246, 9247 and 9252). However, the association between prolonged control and breadth in these two individuals is anecdotal. Larger studies will be required to understand the precise relationship between prolonged control, bNAb therapy and enhanced breadth of T cell immunity.
To determine whether the increased HIV-1 Gag-specific T cell responses were directed against pre-existing or new peptide epitopes, we stimulated PBMCs with a peptide library spanning the entire HIV Gag protein Consensus B sequence (Supplementary Table 3) and compared IFN-γ responses before and after ATI for the nine bNAb + ATI individuals. IFN-γ ELISpot responses were

detectable for six study participants (Fig. 4a–d, Extended Data Fig. 8 and Supplementary Table 4). Four individuals from these six (9244, 9246, 9252 and 9255) broadened the IFN-γ ELISpot response to Gag during ATI (Fig. 4a–d and Extended Data Fig. 8f). Overall, 41% (9 of 22) of the detectable responses in these six individuals at week 12 were directed against Gag epitopes that did not induce a detectable response at baseline (new responses: red dots and section, Fig. 4e,f). In contrast, none of the Gag responses detected at baseline were lost by week 12. Finally, several individuals with detectable responses at week 12 had IFN-γ ELISpot responses against the major homology region (peptide 69-76, Gag285-304; Fig. 4g), a highly conserved motif in the gag gene of all retroviruses21. Thus, the increased IFN-γ responses that developed during bNAb therapy result from increased breadth and magnitude of detectable peptide-specific responses.

To determine whether the increased HIV-1-specific T cell response could eliminate HIV-1-infected cells in vitro, we performed HIV-1 viral inhibition assays22. CD4+ T cells from participants 9246 and 9252 at baseline were infected with HIV-1BaL and cultured either alone or in the presence of CD8+ T cells isolated from the same individuals before and after ATI (Extended Data Fig. 9a and Supplementary Table 5). Participant 9252 showed increased suppression of HIV-1BaL in vitro at week 12 compared to baseline (Extended Data Fig. 9b). However, 9246 was uninformative with no detectable impact on HIV-1BaL outgrowth at baseline or week 12 (Extended Data Fig. 9b). Given the importance of HIV-1-specific CD8+ T cells in controlling viral replication, we also examined rebound viruses for mutations in HIV-1 gag in the seven individuals who rebounded before week 30. When compared to week −2 or 12, HIV-1 gag DNA from rebound plasma showed no consistent evidence for cytotoxic lymphocyte escape (Extended Data Fig. 10).

HIV-1-specific T cell responses likely play a key role in spontaneous control of HIV-1 viremia in elite controllers7–9. However, most individuals exhibit partial control of viral replication as evidenced by suppression of initial peak viremia by 1–2 orders of magnitude for prolonged periods of time before developing AIDS-defining clinical complications23. ART is highly effective in further suppressing viremia but fails to enhance virus-specific immunity possibly because of decreased viral antigen availability.

In contrast, bNAb therapy in SHIV-infected rhesus macaques induces long-lasting CD8+ T cell-mediated viral suppression in a subset of the animals11,24. Our data indicate that individuals who are infected with HIV-1, receiving bNAb therapy during ATI, show increased T cell immunity to HIV-1, including reactivity to Gag epitopes that were undetectable before bNAb administration. Specifically, we identified increased frequencies of MIP1-β-expressing CD8+ T cells, which have been associated with control of viremia18. However, notably, the observational nature of this trial does not allow the determination of whether the observed expansion of HIV-1-specific T cell responses in bNAb-treated humans contributes to viral control.

Previous clinical trials in individuals who are infected with HIV-1, who underwent ATI in the absence of immune intervention, showed increased HIV-1-specific T cell responses that coincided with plasma viral rebound, suggesting that this boost in antiviral immunity was induced by increased viral replication16,25. The increased T cell responses in individual 9245 are consistent with these observations (Extended Data Fig. 5). In contrast, our results demonstrate increased HIV-1 Gag-specific CD8+ and CD4+ T cell immunity in bNAb + ATI individuals at a time when bNAbs maintained viral suppression. At least two mechanisms could account for the association of bNAb treatment with increased T cell responses. One possibility is that ART interruption in the presence of antibodies results in production of bNAb-HIV-1 immune complexes that activate antigen-presenting dendritic cells and enhance their antigen-presenting and cross-presenting capabilities to produce a vaccinal effect3,26. A second nonexclusive possibility is that the augmented CD8+ T cell response is driven by increased low-grade viral replication and antigen availability in tissues that we have not been able to assay during overt viremia suppression by bNAbs. While the underlying mechanism of the observed increased T cell immunity remains to be determined, a potentially important advantage of bNAb + ATI treatment compared to ATI alone, standard ART or T cell vaccination is that the immune system is stimulated with the individual's own virus while circulating viremia is suppressed. Whether the same effects will be seen in individuals who receive bNAbs during ART and whether the increased T cell responses are sufficient to help control infection remains to be determined.

Study design and participants ::: Methods:

BNAb study participants were enrolled in an open-label phase 1b clinical trial at the Rockefeller University and University of Cologne and received three infusions with a combination of two bNAbs, 3BNC117 and 10-1074. ART was interrupted at day 2 after the first antibody infusion4 (ClinicalTrials.gov identifier: NCT02825797). Viral load was assessed every 1–2 weeks and ART

was reinitiated when two consecutive measurements showed viral load of >200 copies per ml. All individuals were infected with clade B HIV-1 (ref. 4). Clinical data of all participants are shown in Extended Data Figs. 1a and 5a. Individuals on continuous ART were enrolled in an observational study at the Rockefeller University. Clinical data of all ART individuals are shown in Extended Data Fig. 2a. The studies were approved by the Rockefeller University and the University of Cologne Institutional Review Boards and written informed consent was obtained from all participants before study enrollment. Secondary use of samples was approved by the University of Montréal Hospital Institutional Review Board.

Intracellular cytokine staining ::: Methods:
PBMCs were thawed and rested for 2 h in RPMI 1640 medium (Gibco by Life Technologies) supplemented with 10% FBS (Seradigm), penicillin and streptomycin (Gibco by Life Technologies) and HEPES (Gibco by Life Technologies) and stimulated with a HIV-1 Consensus B Gag peptide pool (0.5 μg ml−1 per peptide; NIH AIDS Reagent Program) or CMV pp65 peptide pool (0.5 μg ml−1 per peptide; JPT Peptide Technologies) for 6 h in the presence of anti-CD107A-BV786 (BD Biosciences), Brefeldin A (BD Biosciences) and monensin (BD Biosciences) at 37 °C and 5% CO2. DMSO-treated cells served as a negative control. Cells were stained for aquavivid viability marker (Life Technologies) for 20 min at 4 °C and surface markers (30 min, 4 °C), followed by intracellular detection of cytokines using the IC Fixation/Permeabilization kit (eBioscience) according to the manufacturer's protocol before acquisition at an LSRFortessa flow cytometer (BD Biosciences) (see Supplementary Table 1 for antibody staining panel).

Activation-induced marker assay ::: Methods:
PBMCs were thawed, washed and cultured in 24-well plates at a concentration of 10 × 106 cells per ml in RPMI 1640 supplemented with HEPES, penicillin and streptomycin and 10% human serum (Sigma). Cells were rested for 3 h and stimulated with 0.5 μg ml−1 per peptide of HIV-1 Consensus B peptide pools spanning the entire protein for Gag, Nef, Pol, gp120 or gp41 (NIH AIDS Reagent Program) for 18 h at 37 °C and 5% CO2. Pools for gp120 and gp41 were obtained by combining single Env peptides 1–123 (gp120) and 124–211 (gp41) (HIV-1 Consensus B Env Peptide Set). A DMSO-treated condition served as a negative control. Cells were stained for viability dye (aquavivid, Life Technologies) and surface markers (30 min, 4 °C) and cells were fixed using 2% paraformaldehyde before acquisition at a LSRII flow cytometer (BD Biosciences) (see Supplementary Table 2 for antibody staining panel). DMSO-treated cells served as negative controls and were used together with fluorescence minus one controls to set gates for analysis.

IFN-γ ELISpot ::: Methods:
HIV Gag-specific IFN-γ responses were measured using an IFN-γ ELISpot assay as previously described27. The 96-well hydrophobic polyvinylidene difluoride membrane-backed plates (Millipore) were pre-wetted with 35% ethanol for 45 s, washed with PBS and coated overnight at 4 °C with anti-IFN-γ capture antibody (3 μg ml−1 in PBS, clone NIB42, BD Biosciences). PBMCs were thawed, rested for 2 h and seeded into plates at 1–2 × 105 PBMCs per well in RPMI with 10% FBS supplemented with HEPES and penicillin and streptomycin. Cells were stimulated with 123 peptides spanning the entire HIV-1 Consensus B Gag protein (10 μg ml−1, NIH AIDS Reagent Program; see Supplementary Table 3 for all sequences) for 20 h at 37 °C and 5% CO2. Plates were washed with PBS-T (PBS and 0.05% Tween-20) and incubated with biotinylated anti-IFN-γ antibody (0.5 μg ml−1 in PBS and 0.5% BSA, clone 4S.B3; BD Biosciences) for 2 h at room temperature. Plates were washed with PBS-T and incubated with streptavidin-alkaline-phosphatase conjugate (Bio-Rad Laboratories) (1:1,000 dilution in PBS and 0.5% BSA) for 1 h at room temperature. Spots were developed using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories) for 4 min and the reaction was stopped with tap water. Spots were counted using an Immunospot Analyzer Instrument (Cellular Technology). PBMCs incubated with medium alone served as negative controls and staphylococcal enterotoxin B-stimulated PBMCs (0.5 μg ml−1) as a positive control. The s.f.u. were calculated as number of spots in test wells minus the mean number of spots in medium control wells and normalized to s.f.u. per 106 PBMCs. A response was considered positive if greater than 50 s.f.u. per 106 PBMCs. Week −2 and week 12 samples from the same individual were assayed together in the same experiment.

In vitro viral inhibition assay ::: Methods:
The capacity of CD8+ T cells to suppress HIV-1 infection of autologous CD4+ T cells was evaluated using a previously described HIV-1 suppression assay22 with minor modifications.

CD4+ T cells were isolated using negative magnetic bead selection (StemCell Technologies) from PBMCs, rested for 2 h and cultured in RPMI and 10% FBS supplemented with PHA-L (2 μg ml−1; Sigma-Aldrich) and IL-2 (100 U ml−1; StemCell Technologies) for 72 h. After 72 h, CD8+ T cells were isolated from PBMCs using negative magnetic bead selection (StemCell Technologies), counted and rested for 2 h at 37 °C. Meanwhile, cultured CD4+ T cells were washed, counted and plated in U-bottom 96-well plates for infection with HIV-1BaL (NIH AIDS Reagent Program) using a multiplicity of infection of 0.015: plates were first centrifuged at 1,200g for 1 h at 22 °C and then incubated for an additional hour at 37 °C. After infection, CD4+ T cells from different wells were pooled, washed three times and plated in U-bottom 96-well plates (50,000 cells per well) with CD8+ T cells at a 1:1 ratio in RPMI with 10% FBS supplemented with IL-2 (100 U ml−1). Uninfected CD4+ T cells were included as negative controls and infected CD4+ T cells cultured without CD8+ T cells served as 100% infectivity controls. At days 3, 5 and 7 after infection, cells were stained with a viability dye (aquavivid, Life Technologies) and surface markers (30 min, 4 °C), followed by intracellular detection of HIV-1 Gag (Beckman Coulter) using the IC Fixation/ Permeabilization kit (eBioscience) according to the manufacturer's protocol (see Supplementary Table 5 for the antibody staining panel). All experiments were performed in duplicate or triplicate, depending on cell availability.

Sequencing and phylogenetic analysis ::: Methods:
Gag sequences from latent reservoir viruses were obtained from CD4+ T cell genomic DNA by near-full length HIV-1 sequencing as previously described28. Gag sequences from rebound plasma were obtained by HIV-1 RNA extraction and single-genome amplification as previously described29. In brief, HIV-1 RNA was extracted from plasma samples using the MinElute Virus Spin kit (Qiagen) followed by first-strand cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). The cDNA synthesis for plasma-derived HIV-1 RNA was performed using the antisense primer B5R2 5′-CAATCATCACCTGCCATCTGTTTTCCATA-3′. Gag was then amplified using the primer Gag5out 5′-TTGACTAGCGGAGGCTAGAAGG-3′ and Gag3out 5′-GATAAAACCTCCAATTCCCCCTATC-3′ in the first round and in the second round with nested primers Gag5in 5′-GAGAGATGGGTGCGAGAGCGTC-3′ and Gag3in 5′-CTGCTCCTGTATCTAATAGAGC-3′. PCRs were performed using High Fidelity Platinum Taq (Invitrogen) and run at 94 °C for 2 min; 35 cycles of 94 °C for 15 s, 58 °C for 30 s and 68 °C for 3 min; and 68 °C for 15 min. Second-round PCR was performed with 1 μl of the PCR product from the first round as a template and High Fidelity Platinum Taq at 94 °C for 2 min; 45 cycles of 94 °C for 15 s, 58 °C for 30 s and 68 °C for 3 min; and 68 °C for 15 min.
Amino acid alignments of intact gag sequences were obtained by using ClustalW v.2.1 (ref. 30) under the BLOSUM cost matrix. Sequences with premature stop codons were excluded from all analyses. Maximum likelihood phylogenetic trees were then generated from these alignments using RAxML v.8.2.9 (ref. 31) under the GTRGAMMA model with 1,000 bootstraps. To analyze changes between reservoir and rebound viruses, gag sequences were aligned at the amino-acid-level to a HXB2 reference using ClustalW v.2.1.

Data analysis ::: Methods:
Flow cytometric data were analyzed using FlowJo v.10.5.0 for Mac. Statistical analyses were performed using GraphPad Prism v.8.0.1 for Mac using nonparametric tests. Pairwise comparisons were performed using the two-sided Wilcoxon matched-pairs signed rank test.

Reporting Summary ::: Methods:
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Online content:
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at 10.1038/s41591-019-0747-1.