HIV

Heightened resistance to host type 1 interferons characterizes HIV-1 at transmission and after antiretroviral therapy interruption

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Type 1 interferons (IFN-I) are potent innate antiviral effectors that constrain HIV-1 transmission. However, harnessing these cytokines for HIV-1 cure strategies has been hampered by an incomplete understanding of their antiviral activities at later stages of infection. Here, we characterized the IFN-I sensitivity of 500 clonally derived HIV-1 isolates from the plasma and CD4$^+$ T cells of 26 individuals sampled longitudinally after transmission or after antiretroviral therapy (ART) and analytical treatment interruption. We determined the concentration of IFNα2 and IFNβ that reduced viral replication in vitro by 50% (IC_{50}) and found consistent changes in the sensitivity of HIV-1 to IFN-I inhibition both across individuals and over time. Resistance of HIV-1 isolates to IFN-I was uniformly high during acute infection, decreased in all individuals in the first year after infection, was reacquired concomitant with CD4$^+$ T cell loss, and remained elevated in individuals with accelerated disease. HIV-1 isolates obtained by viral outgrowth during suppressive ART were relatively IFN-I sensitive, resembling viruses circulating just before ART initiation. However, viruses that rebounded after treatment interruption displayed the highest degree of IFNα2 and IFNβ resistance observed at any time during the infection course. These findings indicate a dynamic interplay between host innate responses and the evolving HIV-1 quasispecies, with the relative contribution of IFN-I to HIV-1 control affected by both ART and analytical treatment interruption. Although elevated at transmission, host innate pressures are the highest during viral rebound, limiting the viruses that successfully become reactivated from latency to those that are IFN-I resistant.

INTRODUCTION

Type 1 interferons (IFN-I) comprise a family of proinflammatory and immunomodulatory cytokines with potent antiviral activity (*1*, *2*). In humans, this family includes 13 IFNα subtypes as well as IFNβ, IFNε, IFNκ, and IFNω, of which IFNα and IFNβ are the best characterized (*3*). IFN-I are rapidly up-regulated in response to pathogen exposure and infection (*4*, *5*) and mediate their activity by binding to the heterodimeric interferon-α/β receptor (IFNAR) expressed on all nucleated cells (*6*). This binding results in the activation of signal transduction cascades that trigger the expression of hundreds of interferon-stimulated genes (ISGs), which have direct and indirect antiviral activity, and regulate the activation state, function, proliferation, and survival of host immune cells (*7*, *8*). Although all IFN-I subtypes bind to the same receptor, they differ in their affinity for the two IFNAR subunits and elicit different patterns of ISG expression, suggesting that they vary in their in vivo activity and potency (*9*–*12*).

Up-regulation of IFN-I expression is one of the earliest innate responses to infection with both HIV-1 and simian immunodeficiency viruses (SIVs) (*13*–*17*). In acute HIV-1 infection, maximal IFN-I concentrations are detected in the plasma before peak viremia (*18*). In acute SIV infection of macaques (SIVmac), plasmacytoid dendritic cells are actively recruited to mucosal sites, where they produce high concentrations of both IFNα and IFNβ (*19*, *20*). IFN-I are known to control HIV/SIV replication in CD4$^+$ T cells and macrophages by targeting multiple steps in the viral lifecycle (*8*). Consistent with this, pretreatment of rhesus macaques with exogenous IFNα2 increased the number of intrarectal challenges required to achieve systemic SIVmac infection (*21*) and vaginal administration of IFNβ protected

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rhesus macaques from recombinant SIV/HIV (SHIV) infection (*22*). Likewise, blocking IFN-I during acute SIVmac infection led to higher viral titers, a more rapid decline of $CD4^+$ T cells, and faster disease progression (*23*). Thus, numerous studies in both humans and primates have shown that IFN-I play a key role in controlling HIV and SIV replication during the earliest stages of infection.

In contrast to the beneficial effects of IFN-I up-regulation during acute HIV-1 infection, sustained IFN-I signaling during chronic infection appears to have an overall negative effect (*17*, *24*–*28*). In untreated individuals, plasma IFNα concentrations are positively correlated with HIV-1 viral load and inversely correlated with $CD4^+$ T cell counts, and individuals with higher viral loads and faster disease progression frequently exhibit higher ISG expression (*24*, *27*) and heightened IFNβ production in the gut (*29*). Continuous IFN-I signaling increases the number of susceptible $CD4^+$ target cells, induces $CD4^+$ T cell apoptosis, limits antigen-specific $CD4^+$ and $CD8^+$ T cell responses, and contributes to immune exhaustion (*15*, *30*, *31*). In SIVmac-infected macaques, prolonged IFNα2 treatment resulted in decreased antiviral ISG expression, an increased SIVmac reservoir size, and the loss of $CD4^+$ T cells (*21*). Sustained IFN-I signaling and ISG up-regulation differentiate pathogenic from nonpathogenic SIV infections (*32*–*34*) and drive systemic immune activation in HIV-1 infection, which is associated with poor $CD4^+$ T cell recovery and a predictor of disease progression (*35*).

Given the pleiotropic and context-dependent effects of IFN-I, it is not surprising that clinical studies evaluating their therapeutic benefits have yielded mixed results (*14*, *16*, *36*). IFNα2 administration can suppress HIV-1 replication, blunt rebound viremia, and extend viral control after the interruption of antiretroviral therapy (ART) (*37*–*40*). However, various combinations of antiretroviral and IFN-I treatments have not led to long-term improvements of $CD4^+$ T cell reconstitution or clinical outcome (*14*). Moreover, blocking rather than stimulating IFN-I signaling, or selectively counteracting only certain ISGs, had beneficial effects in HIV-1–infected humanized mice (*41*, *42*). Thus, it seems clear that effective manipulation of the IFN-I system to prevent, treat, or cure HIV-1 infection will require a much more detailed understanding of the quality of the endogenous IFN-I response.

One approach to gain insight into the in vivo IFN-I response is to test the IFN-I sensitivity of plasma viruses, which are reliable indicators of selection pressures that act on both virions and virus-infected cells (*43*–*48*). Such studies have shown that transmitted founder (TF) viruses are highly IFN-I resistant (*49*) and that this resistance constitutes a major determinant of HIV-1 transmission fitness (*50*). In contrast, viruses isolated during chronic infection are generally IFN-I sensitive, although this sensitivity appears to revert as individuals progress toward AIDS (*51*, *52*). Thus, existing data indicate that IFN-mediated pressures vary during the infection course. However, the kinetics and magnitude of these changes, and their uniformity in individuals with different rates of disease progression, have not been determined. Here, we performed a systematic analysis of the IFN-I sensitivity of plasma- and $CD4^+$ T cell–derived viral isolates from prospectively sampled study participants before and after ART and after short-term treatment interruption [also called analytical treatment interruption (ATI)]. We found that the endogenous IFN-I response is much more dynamic than previously assumed and that the relative contribution of IFN-I to HIV-1 control varies at different stages of the infection.

RESULTS

Generation of plasma isolates from longitudinally sampled individuals

To characterize the kinetics of IFN-I resistance over the course of HIV-1 infection, we generated plasma isolates from 10 prospectively sampled individuals, who were followed from shortly after transmission until 4.1 to 12.4 years after infection (table S1). All participants were men who have sex with men (MSM) who presented with symptomatic primary subtype B infection. Peripheral blood was collected at regular intervals immediately after onset of symptoms and continued for 74 to 318 weeks (1.6 to 6.2 years) in the absence of antiretroviral treatment. At each time point, participants underwent clinical evaluation, including viral load and $CD4^+$ T cell count determinations (table S1). Six individuals experienced a gradual loss of their $CD4^+$ T cells over the study period (typical progressors; Fig. 1A); two individuals maintained high $CD4^+$ T cell counts and low viral loads throughout their 3.6- and 5.6-year clinical follow-up (nonprogressors; Fig. 1B), and two individuals developed AIDS ($CD4^+$ T cells < 300 cell/μl) within 0.7 and 1.5 years of infection (rapid progressors; Fig. 1C). ART was initiated in eight participants between weeks 85 and 323 after onset of symptoms based on the standard of care at the time (shaded in Fig. 1).

We also characterized the evolving HIV-1 quasispecies in these individuals. This was done by amplifying viral RNA directly from the plasma using single-genome amplification, which retains genetic linkage across viral genes and generates sequences devoid of polymerase chain reaction (PCR)–induced artifacts (*53*, *54*). For each individual, 3′ half genomes or *env* gene sequences were amplified from sequential plasma samples, with sequences from the earliest time point used for TF enumeration (fig. S1). This analysis showed that seven participants acquired a single TF virus, whereas the remaining three became infected with two or more TF viruses (table S1). Sequences from subsequent time points exhibited the expected patterns of viral diversification (*55*–*57*), except for participants MM14, MM23, and MM33, who became superinfected with additional subtype B strains 5 to 29 weeks after the initial infection (Fig. 1 and fig. S2).

To determine the IFN sensitivity of individual quasispecies members at different stages of HIV-1 infection, we generated 277 limiting dilution plasma isolates from 5 of the 10 individuals (table S1). Plasma samples were end point diluted and cocultured with healthy donor $CD4^+$ T cells, and the resulting isolates were sequenced to ensure that they were indeed single virion derived (*50*). In phylogenetic trees of *env* gene sequences, the limiting dilution isolates were completely interspersed with sequences amplified directly from the plasma, thus confirming their authenticity (fig. S2). To characterize their sensitivity to IFN-I inhibition, we determined the IFNα2 and IFNβ concentrations that reduced virus replication in vitro by 50% (*50*). Briefly, donor $CD4^+$ T cells were treated with increasing quantities of IFNα2 and IFNβ and then infected with equal amounts of virus (*50*). Cells were cultured for 7 days, with the IFN-containing media replenished every second day. Virus replication was measured by quantifying p24 antigen in supernatants and used to calculate the half-maximal inhibitory concentration (IC_{50}) (fig. S3).

Because the IFNα2 and IFNβ IC_{50} values for limiting dilution isolates from the same plasma sample were similar, we reasoned that conventional (bulk) virus isolation was likely sufficient to capture the IFN-I sensitivity of the circulating virus pool. To test this, we generated 18 bulk isolates from 11 plasma samples (table S1) and determined their IFN-I resistance (table S2). All bulk isolates yielded

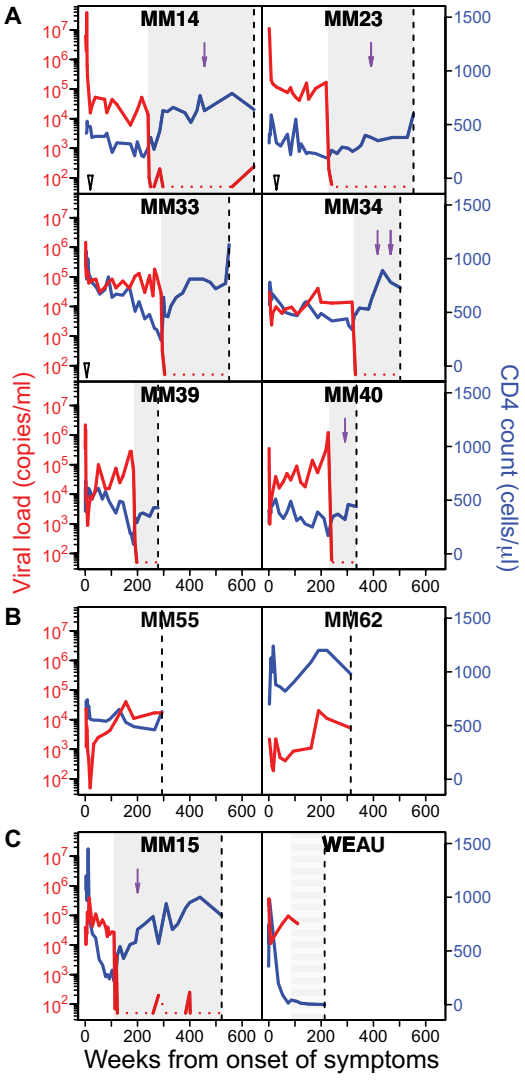

Fig. 1. HIV-1 viral loads and CD4$^+$ T cell counts in prospectively studied individuals. Viral loads (RNA copies/ml; red, left *y* axis) and CD4$^+$ T cell counts (cells/μl; blue, right *y* axis) are shown for 10 HIV-1–infected individuals, who were followed from the onset of symptoms until 213 to 645 weeks (4.1 to 12.4 years) after infection (*x* axis). Participants are grouped on the basis of disease progression, (A) including typical progressors, (B) nonprogressors, and (C) rapid progressors. Gray shading indicates suppressive ART (alternating shading in WEAU indicates nonsuppressive zidovudine monotherapy). Purple arrows denote the time points of PBMC sample isolation that yielded QVOA isolates; open triangles denote superinfection, and dashed vertical lines indicate termination of the study or loss to follow up.

IC_{50} values that fell within fivefold of the mean of the corresponding limiting dilution isolates (fig. S4, A and B). Moreover, the interferon resistance of the individual bulk isolates was highly correlated with the geometric mean of the corresponding limiting dilution isolates (IFNα2 $r = 0.94$ and IFNβ $r = 0.97$; fig. S4, C and D). We thus used the less labor-intensive conventional approach to generate 79 plasma isolates for the remaining five individuals.

Kinetics of IFN-I resistance in untreated HIV-1 infection

Analysis of 374 plasma isolates from 10 prospectively sampled individuals revealed differences in their sensitivity to IFNα2 and IFNβ inhibition over the course of the infection (Fig. 2). To quantify these dynamic changes, we developed a Bayesian hierarchical change point model (*50*, *58*), which allowed us to infer the temporal patterns of IFNα2 and IFNβ IC_{50} values and their associations with CD4$^+$ T cell counts across different individuals while accounting for differences in sampling times and frequencies.

Consistent with previous results (*49*, *50*, *59*), isolates obtained during acute HIV-1 infection were uniformly IFN-I resistant, yielding mean IC_{50} values for IFNα2 of 0.90 pg/ml and for IFNβ of 19 pg/ml (Fig. 2). This resistance decreased in all individuals, with IFNα2 and IFNβ IC_{50} values falling in typical progressors on average 180-fold [95% credible interval (CrI): 100- to 340-fold] and 3500-fold (95% CrI: 1500- to 8900-fold) within 340 days (95% CrI: 250 to 490 days) and 460 days (95% CrI: 310 to 700 days), respectively (Fig. 3, A and B). Isolates from nonprogressing individuals fell to similar IC_{50} values but reached a nadir earlier at an estimated 100 days (95% CrI: 50 to 200 days) for IFNα2 and 110 days (95% CrI: 50 to 240 days) for IFNβ (Fig. 3, A and B). The time to nadir for fast progressing individuals was difficult to determine because IFNα2 and IFNβ IC_{50} values decreased only 2.8-fold (95% CrI: 0.78- to 10.5-fold) and 2.7-fold (95% CrI: 0.52- to 16-fold), respectively, resulting in 39-fold and 1000-fold higher values than in individuals with more typical disease progression (Figs. 2 and 3, A and B).

Plasma virus from typical progressors exhibited a consistent rise in IFNα2 and IFNβ resistance during the later stages of infection (Fig. 2). This rise and other fluctuations in resistance during chronic infection, for example, as seen in individual MM34 in Fig. 2, appeared to associate with changes in CD4$^+$ T cell counts. The change point model estimated that after the initial decline in IFN-I resistance, each decrease of 100 CD4$^+$ T cells/μl was associated with a 2.3-fold (95% CrI: 1.3- to 4.4-fold) increase in mean IFNα2 IC_{50} values and a 4.0-fold (95% CrI: 1.9- to 8.7-fold) increase in mean IFNβ IC_{50} values (Figs. 2A and 3, C and D). The two nonprogressing individuals maintained relatively high CD4$^+$ T cell counts, and the two fast progressing individuals were largely depleted of CD4$^+$ T cells early after infection. Thus, there was no apparent association between IFN resistance and CD4$^+$ T cell counts in these participants (Figs. 2, B and C, and 3, C and D).

Overall, the temporal changes in IFN-I resistance were similar among the different individuals. Viral IFN-I resistance was high during acute infection, decreased during early chronic infection, and increased again during later stages concomitant with CD4$^+$ T cell loss and disease progression. Neither the number of TF viruses at transmission nor subsequent superinfection events appeared to influence these kinetics (Fig. 2). Across all individuals and time points with IFN-I resistance values spanning several orders of magnitude, IFNα2 and IFNβ IC_{50} values were highly correlated ($r = 0.9$; fig. S5). These findings thus indicate a highly dynamic interplay between host innate immune responses and the evolving HIV-1 quasispecies, with the relative contribution of IFN-I to HIV-1 control varying markedly, but predictably, over the course of HIV-1 infection.

IFN-I sensitivity of ex vivo reactivated latent viruses

Having characterized the IFN-I response of plasma virus during untreated infection, we next used a quantitative viral outgrowth assay (QVOA) to generate reactivated latent viruses from some of the same individuals after they were placed on therapy. For 5 of the 10 longitudinally followed individuals, cryopreserved peripheral blood mononuclear cells (PBMCs) collected 1 to 4 years after ART initiation

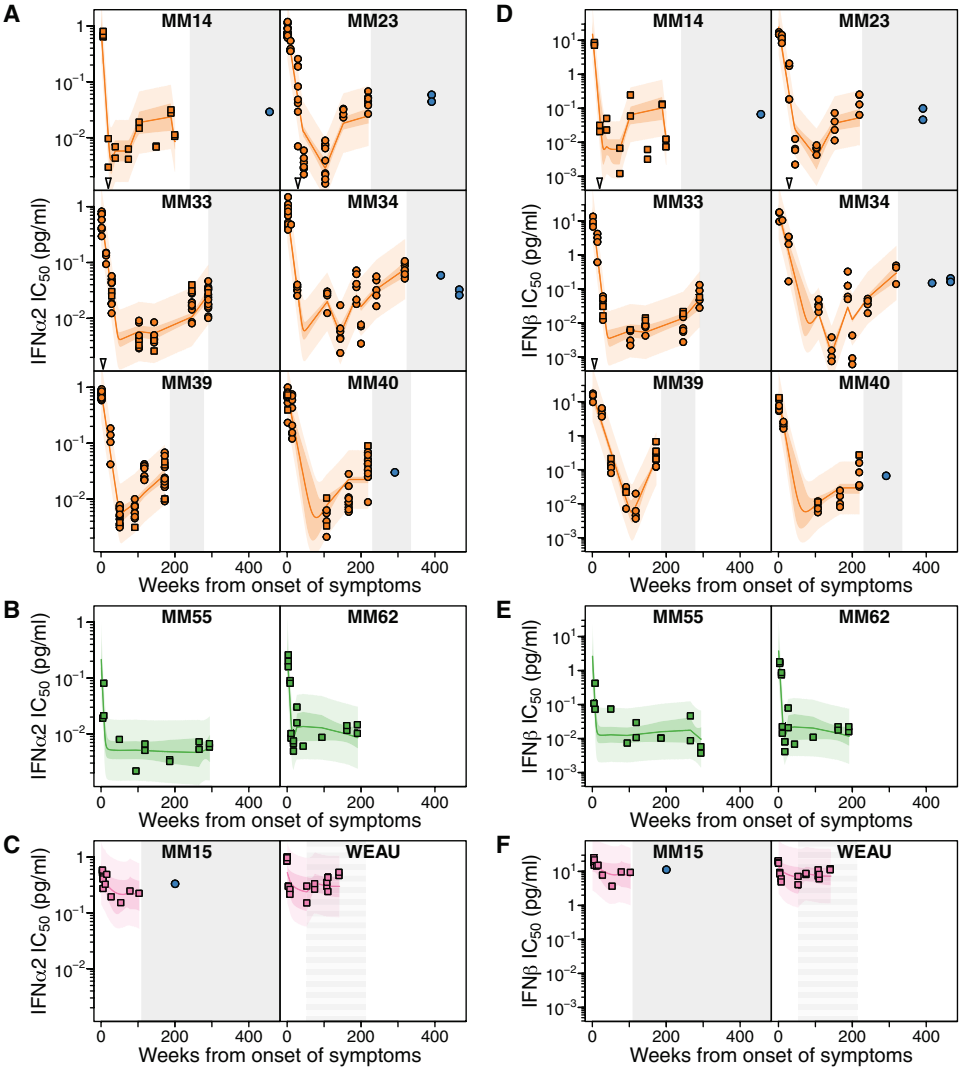

Fig. 2. Kinetics of IFN-I resistance over the course of HIV-1 infection. IFNα2 (**A** to **C**) and IFNβ (**D** to **F**) IC_{50} values (pg/ml) are shown for limiting dilution– (circles) and bulk culture–derived (squares) isolates from the onset of symptoms until 140 to 466 weeks (2.7 to 8.9 years) after infection. Each data point represents the average of two technical replicates. Colored lines denote the average IC_{50} values as estimated by a Bayesian model, with darker shading indicating the 95% credible intervals and lighter shading indicating the 95% prediction intervals. Gray shading indicates suppressive ART (alternating shading in WEAU indicates nonsuppressive zidovudine monotherapy). Blue circles indicate isolates obtained by viral outgrowth from $CD4^+$ T cells after 1.2 to 4.1 years of suppressive ART. Open triangles denote superinfection. (A and D), (B and E), and (C and F) show typical progressors (orange), nonprogressors (green), and rapid progressors (pink), respectively.

were available (Fig. 1). These were stimulated, cocultured with primary $CD4^+$ T cells from healthy donors, and tested for viral replication by monitoring p24 concentration in culture supernatants (*60*). Because of limited numbers of cryopreserved cells, only eight QVOA isolates were recovered (Fig. 2 and table S1). All isolates were sequenced (fig. S2), and their IFN-I resistance was determined (table S2). Although participant WEAU received zidovudine (AZT) beginning 1.6 years after infection, this monotherapy was not sufficient to maintain suppression of plasma viral loads (Fig. 1 and table S1). Thus, posttreatment samples from this individual were not suitable for QVOA analyses.

As shown in Fig. 2, seven of the eight QVOA isolates were moderately IFN-I resistant, exhibiting IC_{50} values very similar to plasma isolates obtained from the same individuals immediately before ART initiation. This was true for individuals with single and multiple QVOA isolates, including viruses collected several months apart as in samples from individual MM34. A single QVOA isolate from a rapidly progressing individual (MM15) was more IFN-I resistant, but plasma virus obtained just before treatment initiation displayed comparable IFN-I resistance. Phylogenetic analyses confirmed these findings, showing that the eight outgrowth isolates did not cluster with viruses recovered during acute or early infection but were most closely related to plasma viruses replicating during late infection (Fig. 4 and fig. S2). Thus, both IFN-I and phylogenetic analyses indicated that the QVOA-derived viruses had entered the latent reservoir near the time of therapy initiation.

To increase the number of outgrowth viruses for IFN-I phenotypic analyses, we obtained cryopreserved PBMCs or previously generated QVOA isolates from nine additional ART-suppressed individuals who participated in different HIV-1 treatment interruption or latency studies (table S3). Two individuals underwent leukapheresis after prolonged ART suppression for a qualitative and quantitative analysis of their replication-competent HIV-1 reservoir (*61*); four individuals underwent leukapheresis before (−2 weeks) and during (12 weeks) ATI but before viral rebound while receiving the two human broadly neutralizing antibodies 3BNC117 and 10-1074 (*62*), and three individuals underwent leukapheresis before and 6 months after ATI and infusion of the broadly neutralizing antibody VRC01 (*63*, *64*). From the available material, we were able to generate or expand 52 QVOA isolates and test their IFN-I sensitivity (table S4). The results showed a range of IFNα2 and IFNβ IC_{50} values that was very similar to that of the eight QVOA isolates from the longitudinally sampled cohort, with most isolates exhibiting low or moderate IFN-I resistance (Fig. 5, A and B). There were only two outgrowth viruses that displayed an unusually high IFN-I resistance, especially for IFNα2, both of which were isolated from the post-ATI leukapheresis sample of a single individual (A08).

IFN-I sensitivity of rebound viruses after treatment interruption

Some of the individuals for whom QVOA isolates were available also underwent treatment interruptions, which allowed the comparison of the IFN-I sensitivity of in vitro and in vivo reactivated viruses.

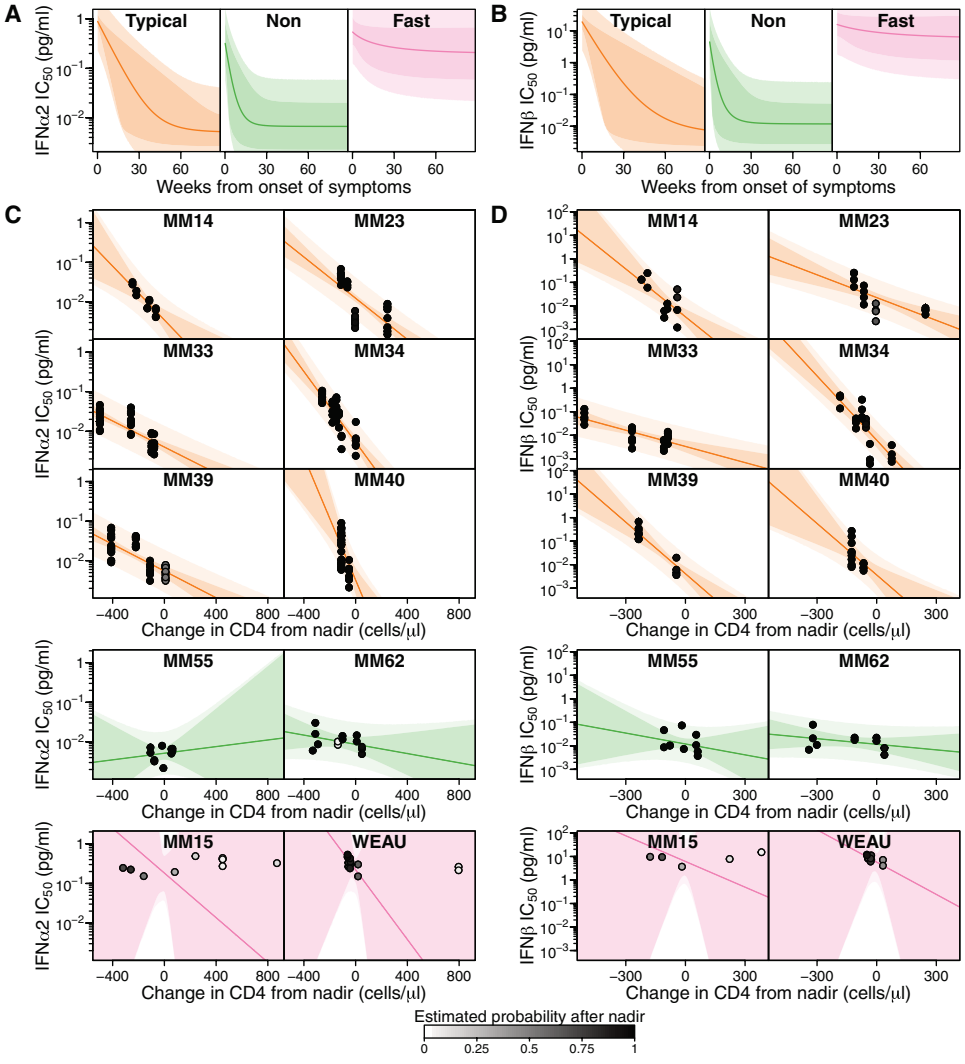

Fig. 3. Dynamic changes in IFN-I resistance. A hierarchical Bayesian change point model used to estimate the IFN-I resistance of plasma viral isolates from 10 longitudinally sampled HIV-1–infected study participants is shown. The longitudinal patterns of IFNα2 and IFNβ IC_{50} values were inferred across individuals by first predicting resistance as a fall from acute IFN-I IC_{50} values to a nadir point and then as variation from nadir values based on changes in $CD4^+$ T cell counts. (**A** and **B**) Predicted mean IFNα2 (A) or IFNβ (B) IC_{50} values (lines) are shown for plasma virus isolates modeled for typical progressor (orange), nonprogressor (green), and rapid progressor (pink) participants from the onset of symptoms through early infection (*x* axis), along with corresponding 95% credible (darker shading) and prediction (lighter shading) intervals. (**C** and **D**) Predicted mean IFNα2 (C) or IFNβ (D) IC_{50} values (lines) are shown for plasma virus isolates modeled for individual typical progressor (orange), nonprogressor (green), and rapid progressor (pink) participants based on changes in $CD4^+$ T cell counts after the nadir (*x* axis indicates decreases and increases from the nadir, which is set to 0), along with corresponding 95% credible (darker shading) and prediction (lighter shading) intervals. Individual data points indicate virus isolates from the respective individuals, with shading reflecting the estimated posterior probability that the time of nadir preceded the plasma collection time point (from white, probability of 0, to black, probability of 1). Isolates estimated to have less than 5% probability after the time of nadir are not shown. For display, the nadir $CD4^+$ T cell count was estimated as the posterior mean for that individual.

Using plasma samples collected shortly after detection of recrudescent viremia, we generated 37 isolates from six such individuals (table S3), most of which were limiting dilution derived. In addition, we obtained plasma samples from seven individuals who participated in other treatment interruption trials (table S3). Three of these underwent successive ATI cycles of increasing duration but without further intervention (*65*). One individual was infused with the broadly neutralizing human monoclonal antibody 3BNC117 before and during ATI (*66*). Three individuals received a 20-week course of pegylated IFNα2 (1 μg/kg) before and during ART interruption (*67*). Using samples collected shortly after detectable viremia, we were able to generate 29 additional isolates (table S3). All rebound isolates formed individual-specific clusters in a phylogenetic tree (fig. S6) and grew efficiently in $CD4^+$ T cells isolated from healthy donors (table S4). However, analysis of their IFNα2 (Fig. 5, A and C) and IFNβ (Fig. 5, B and D) IC_{50} values yielded unexpected results: All 66 rebound isolates were highly IFN-I resistant, exceeding IC_{50} values even from highly resistant acute infection isolates.

To quantify differences in IFN-I sensitivity between viral groups, we developed a hierarchical Bayesian model (Supplementary Methods), which combined the data from the QVOA and rebound isolates with results from the longitudinal cohort (Fig. 2) and previously published transmission pairs (*50*). Specifically, we compared IFN-I IC_{50} values of acute infection isolates (<30 days after onset of symptoms) with those from rebound, chronic (>300 days after onset of symptoms) and outgrowth viruses, dividing the latter into pre- and post-ATI isolates (Fig. 5, C and D). Two apparent outliers isolated from the $CD4^+$ T cells of participant A08 6 months after reinitiation of therapy suggested that viruses phenotypically resembling rebound viruses might be present in post-ATI samples (Fig. 5, A and B). To account for this possibility, we included a mixture term into the model that allowed for some proportion of post-ATI viruses to derive from the rebound population. This mixture term was also included for pre-ATI samples to allow comparison between pre- and post-ATI viruses.

Comparing all available IFN-I IC_{50} data, we determined the fold change in IFNα2 and IFNβ IC_{50} values of acute, rebound, chronic, and pre-ATI and post-ATI isolates (Fig. 5, C and D). Chronic infection isolates from untreated individuals were, on average, 18-fold (95% CrI: 9.3- to 31-fold) less IFNα2 and 99-fold (33- to 230-fold) less IFNβ resistant than acute isolates. This was also the case for pre-ATI QVOA isolates, which exhibited 13-fold (7.5- to 21-fold) lower IFNα2 and 50-fold (19- to 107-fold) lower IFNβ IC_{50} values than acute infection isolates. Rebound viruses were the most IFN-I resistant, exhibiting, on average, 3.0-fold (2.3- to 3.8-fold)

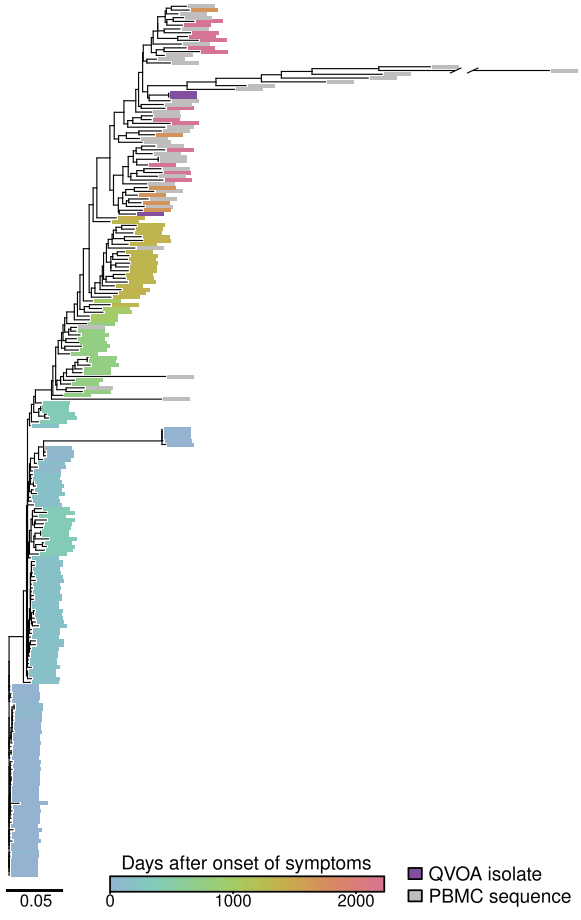

Fig. 4. Position of QVOA isolates within the evolving HIV-1 quasispecies of participant MM34. The evolutionary relationships of *env* nucleotide sequences generated by single-genome amplification either directly from plasma viral RNA or plasma viral isolates of participant MM34 are shown for a 6-year time period. Samples are colored by time point, with blue sequences derived early and red sequences derived late in infection. Purple leaves indicate the position of QVOA isolates obtained 2 and 3 years after ART initiation, whereas gray leaves indicate proviral sequences amplified from the corresponding PBMC sample. One hypermutated PBMC-derived sequence is shown with a gap in the branch. The scale bar indicates 0.05 substitutions per site.

and 6.4-fold (3.8- to 10-fold) higher IFNα2 and IFNβ IC_{50} values than acute infection isolates, respectively. These differences were observed regardless of whether ART interruption was combined with additional interventions, such as the administration of broadly neutralizing antibodies. The IFNα2 IC_{50} values of rebound isolates from individuals who were treated with pegylated IFNα2 (1 μg/kg) before and during ART interruption (individuals 004, 030, and 044) were, on average, 1.8-fold (1.2 to 2.6-fold) higher than those of rebound viruses from IFNα2 untreated individuals, whereas such differences were not observed for the corresponding IFNβ IC_{50} values. The fact that IFNα2 administration had a measurable effect suggests that the endogenously produced concentrations of IFNα2 were not saturating. However, the increase in viral IFNα2 resistance resulting from exogenous administration was modest compared to that driven by endogenous IFNα2. Together, these data demonstrate that treatment interruption and viral recrudescence trigger an IFN-I response that places considerable selection pressures on the rebounding virus.

IFN-I sensitivity of post-ATI outgrowth viruses

Given the heightened IFN-I resistance of rebound viruses, we examined post-ATI isolates for an enrichment of such viruses. Post-ATI outgrowth viruses had mean IFN-I resistance values that were very similar to those of pre-ATI viruses but appeared to comprise two populations (Fig. 5, C and D) primarily because of two post-ATI isolates from one individual (A08) that displayed an unusually high IFN-I resistance. The model estimated the proportion of "rebound-like" viruses in post-ATI isolates to be 13% (2.9 to 29%) for IFNα2 and 4% (0.1 to 15%) for IFNβ. Although no rebound-like IFN-resistant viruses were detected in pre-ATI samples, this group had credible intervals that overlapped those estimated for post-ATI samples. Thus, future studies are required to determine whether rebound-like isolates are only observed in post-ATI samples.

To further characterize the two post-ATI QVOA isolates with elevated IC_{50} values, we compared their sequences to those of other QVOA and rebound viruses from the same individual (Fig. 6). As previously reported (*63*, *64*), participant A08 initiated ART during chronic infection, underwent treatment interruption and VRC01 monoclonal antibody treatment, and was restarted on ART after detection of rebound viremia. Phylogenetic analysis of viral sequences obtained before, during, and after treatment interruption identified multiple rebound lineages, some of which were closely related to both pre-ATI and post-ATI QVOA isolates (Fig. 6). When 20 of these QVOA isolates were phenotypically tested, 18 exhibited very low IFNα2 and IFNβ IC_{50} values, indicating that they were highly IFN-I sensitive (Fig. 5). The exceptions were the two post-ATI QVOA isolates 3D8 and 6F6, which were not only more IFN-I resistant but also shared identical *env* sequences with three rebound viruses (highlighted by arrows in Fig. 6). Further sequence analysis confirmed these close genetic relationships, showing that the two QVOA and three rebound isolates differed at five or fewer sites across their genomes (fig. S7A). Thus, ATI in participant A08 appeared to have reseeded the reservoir with IFN-resistant viruses. However, other post-ATI QVOA isolates from participant A08 were also closely related to rebound isolates, but were highly IFN-I sensitive. For example, the genomes of post-ATI QVOA.M20 and REB.5D2 exhibited only five nucleotide differences (fig. S7B), but the rebound isolate was over 100-fold more IFNα2 resistant and over 500-fold more IFNβ resistant (Fig. 6, red and teal triangles). Thus, in some viruses, very few sequence changes are sufficient to markedly alter the IFN-I phenotype.

DISCUSSION

In untreated HIV-1–infected individuals, plasma virus has a half-life of less than 1 hour, and the cells that are producing most of this virus have a half-life of less than 1 day (*43*, *47*, *48*, *68*). Thus, virus circulating in the plasma of infected individuals is a sensitive real-time indicator of the in vivo selection pressures that act on viruses and virally infected cells, such as neutralizing antibodies, cytotoxic T lymphocytes (CTLs), and antiviral drugs (*43*–*46*). Here, we characterized the resistance of plasma viruses to an additional selection pressure present in vivo, IFNα2 and IFNβ, and show that the relative contribution of these cytokines to HIV-1 control varies throughout the infection course (Fig. 2). IC_{50} values of these cytokines were uniformly high during acute infection, consistent with the rapid induction of a potent innate antiviral state that selects for founder viruses that are highly IFN-I resistant (*18*, *50*). IFNα2 and IFNβ

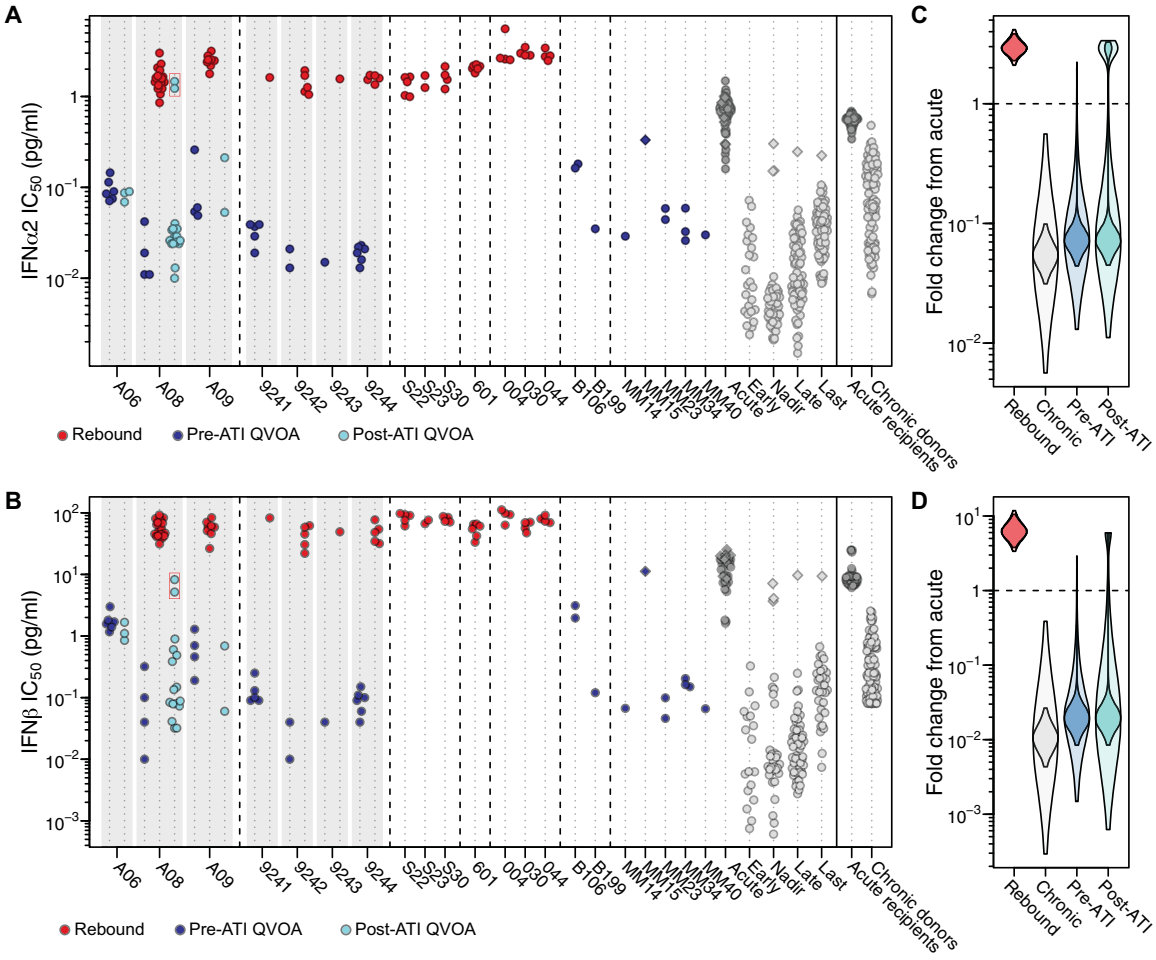

Fig. 5. IFN-I resistance of QVOA versus rebound viruses. (**A** and **B**) IFNα2 (A) and IFNβ (B) IC_{50} values are shown for plasma isolates of individuals experiencing rebound viremia after ART cessation (red) and QVOA isolates generated from the PBMCs of ART-suppressed individuals before (blue) or after (turquoise) ATI (each data point is the average of two technical replicates). Isolates are grouped by individuals (shaded boxes), with pre-ATI, rebound, and post-ATI isolates depicted in temporal order when available. Two post-ATI QVOA isolates from participant A08 with elevated IFN-I resistance are boxed. Also shown are the IFNα2 (A) and IFNβ (B) IC_{50} values for outgrowth isolates (blue) and plasma viruses (gray) from the longitudinal cohort (acute, <30 days since onset of symptoms; early, >300 days and less than nadir; nadir, time point with lowest mean IC_{50} value; late, greater than nadir and less than last; last, last time point before ART initiation) and previously reported donor-recipient transmission pairs (*50*). For the latter, IC_{50} values were adjusted to account for potency differences among commercial IFN-I batches. Diamonds indicate isolates from fast progressing individuals. (**C** and **D**) The fold-change in IFNα2 and IFNβ IC_{50} values from acute infection isolates is shown for rebound (red), chronic (gray), and pre-ATI (blue) and post-ATI (turquoise) QVOA isolates. Violin plots indicate the estimated posterior probability of the mean fold change between isolate types, with the darker shading indicating the 95% credible interval and the lighter indicating the 95% prediction interval. The dashed horizontal line indicates a fold change of 1, which indicates no change.

IC_{50} values declined in all individuals in the first year after infection, at least in part because of viral escape from adaptive immune responses (*45*, *46*, *69*, *70*). Recent studies showed that HIV-1 resistance to interferon-induced transmembrane proteins (IFITMs) and to IFN-I itself decreased over the first 6 months of infection as a direct result of acquiring neutralizing antibody escape mutations (*69*). Although a similar causal relationship has not yet been shown for escape from cellular immunity, CTL responses are known to place strong pressure on viral quasispecies during early HIV-1 infection (*45*, *46*). Thus, some of the same mutations that allow HIV-1 to successfully evade potent neutralizing antibodies, CTLs, and other adaptive immune pressures likely result in a loss of IFN-I resistance, even if IFN-I signaling is increased (*26*, *32*, *71*, *72*).

Although IFN-I IC_{50} values varied by orders of magnitude, the observed temporal changes were unexpectedly similar across all individuals. Plasma viruses from all typical progressors displayed the same initial decline of IFNα2 and IFNβ IC_{50} values irrespective of the initial multiplicity of infection (number of TF viruses) or subsequent superinfection events, followed by the gradual reacquisition of IFN-I resistance during later stages of infection. In most individuals, a nadir was reached within 2 years of symptom onset, although in the two rapid progressors, the fall and rise of IFNα2 and IFNβ IC_{50} values were much less pronounced. Plasma viruses from the two nonprogressors displayed the same initial IC_{50} decline but then maintained low resistance values. Thus, the overall dynamics of IFN-I resistance were very similar, suggesting common patterns of virus-host interactions.

The balance between innate and adaptive immune pressures is also reflected in the IFN-I phenotype of outgrowth and rebound viruses. The latent reservoir is largely composed of proviruses that

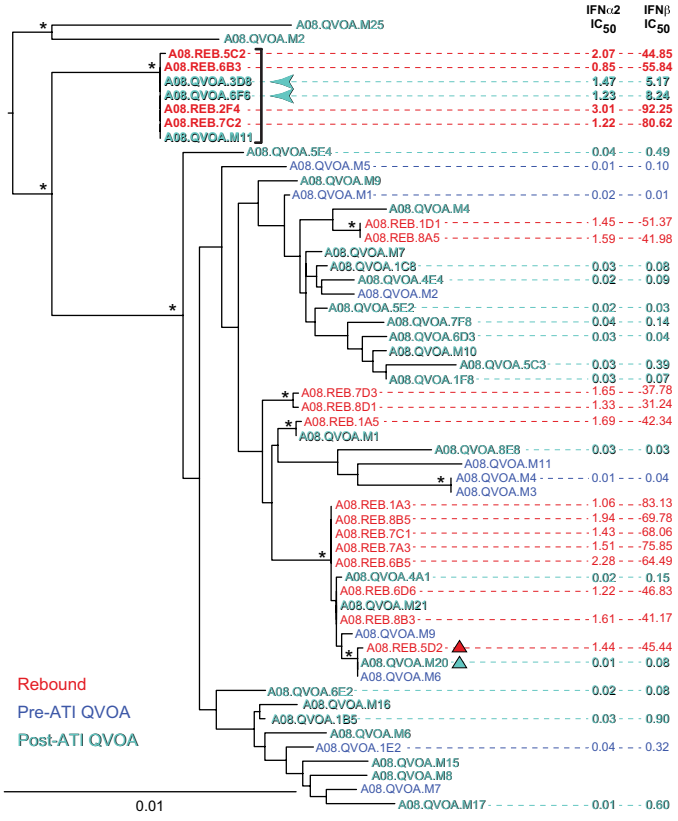

Fig. 6. Genotype and IFN-I phenotype of rebound and outgrowth viruses before and after treatment interruption of individual A08. The phylogenetic relationships of *env* gene sequences from pre-ATI QVOA (blue), plasma rebound (red), and post-ATI QVOA (teal) isolates are shown for participant A08, along with available IFNα2 and IFNβ IC_{50} values (pg/ml). Asterisks indicate bootstrap values >90%; the scale bar indicates 0.01 substitutions per site. A clade of near-identical rebound and post-ATI QVOA isolates is highlighted by bold text and a bracket, with two rebound-like post-ATI QVOA isolates with heightened IFN-I resistance denoted by teal arrows. Two other closely related rebound and post-ATI QVOA isolates that differ in their IFN-I resistance are indicated by red and teal triangles.

are transcriptionally and translationally silent and are therefore not subject to innate or adaptive immune pressures. Moreover, adaptive immune responses are reduced under suppressive ART because of reduced viremia (*73*, *74*). This may explain the importance of rapidly induced innate responses at the time of viral recrudescence (*75*) and the resulting IFN-I resistance of rebound viruses. Testing rebound plasma for IFNα2 and IFNβ protein failed to show consistent increases after treatment interruption, suggesting elevated IFN-I concentrations locally at the sites of viral recrudescence. Whether the superior IFN-I resistance of rebound viruses confers enhanced transmissibility is not known, but the occurrence of transmissions during treatment interruption suggests that this possibility needs to be investigated (*76*, *77*).

Recent studies by Abrahams *et al.* (*78*) showed that 71% of outgrowth viruses are genetically most similar to viruses circulating in the plasma just before ART initiation. Because this proportion is far greater than would be expected if the reservoir formed continuously, treatment appears to alter the host environment to facilitate the formation of latently infected cells (*78*). We also found that outgrowth viruses isolated years after ART suppression were genetically most similar to viruses replicating late in untreated infection. Moreover, these outgrowth viruses exhibited IFN-I IC_{50} values that were near identical to those of viruses isolated just before ART. Our data thus extend these earlier findings, showing that outgrowth viruses are both genetically and phenotypically most similar to viruses replicating late in infection, which provides additional evidence for the preferential entry of HIV-1 into the latent reservoir near the time of ART initiation.

The viral determinants that confer IFN-I resistance are not known. Studying TF and 6-month consensus viruses from the same individual, a previous study failed to identify resistance or sensitivity signatures that were common among the different virus pairs (*59*). Sequence changes occurred at many sites throughout the viral genome, suggesting differential responses to numerous ISGs. Given the myriad IFN-I effector pathways and the plasticity of HIV-1 quasispecies members, these results are expected, especially because we demonstrate here that IFN-I resistance can be altered by a small number of sequence changes. Thus, the path to resistance for any one HIV-1 strain is likely context dependent, although certain resistance determinants may be more common than others.

A critical question in cure research is whether treatment interruption and the associated transient viremia have an impact on the composition and size of the latent reservoir. Comparing pre-ATI and post-ATI QVOA isolates, a previous study found only minimal changes in the abundance of replication-competent viruses, with little genetic evidence of rebound viruses enriching the post-ATI reservoir (*64*). The exception was one individual (A08), who harbored post-ATI viruses that were genetically closely related to rebound viruses (*64*). Examining additional QVOA isolates from this same individual, we found that pre-ATI and post-ATI viruses had similar IFN-I resistance values. However, the post-ATI QVOAs included two isolates with elevated IFN-I resistance, which were genetically very similar to a subset of rebound viruses. Thus, the post-ATI outgrowth viruses in this individual comprised two viral populations, one of which represented rebound-like viruses based on genetic and phenotypic analyses.

Treatment interruptions are the gold standard to evaluate promising cure strategies (*79*) and thus must be safe and well designed. Consistent with previous results (*64*, *66*), we found that pre-ATI QVOA isolates were neither predictive nor representative of the viruses that subsequently rebounded in vivo. This disconnect reinforces the clinical importance of ATIs because merely testing cure strategies in vitro may generate misleading results. A comprehensive analysis of post-ATI isolates in participant A08 suggested that treatment interruption may have reseeded the reservoir with IFN-resistant viruses, although the existence of these variants before ART could not be formally excluded. Although rebound-like, IFN-resistant viruses comprised only a minor subset of the sampled post-ATI reservoir, this possibility requires further exploration to assure the safety of ATIs.

The finding that rebound viruses are uniformly IFN-resistant is also relevant to the design of cure strategies that directly or indirectly engage IFN-I pathways. These approaches attempt to harness the antiviral activity of IFN-I either by administering these cytokines directly (*38*) or by eliciting IFN-I responses via immunomodulators, such as Toll-like receptor 7 and 9 agonists (*80*, *81*), and other latency reversal agents (*82*). The fact that ART cessation likely induces near-maximal IFN-I responses at the sites of virus recrudescence will need to be factored into the design of these strategies. Consistent with this, a recent study found that activation of natural killer cell

cytotoxicity, and not the induction of ISGs, correlated with exogenous IFNα2-mediated HIV-1 control (*83*).

A limitation of the present study is the lack of diversity among study participants because all but one were MSM from the United States and Europe who initiated ART during chronic infection. It will thus be important to examine additional trial participants, including women and minorities, to test the generality of our results. It will also be important to examine participants who initiated ART during acute infection because rebound viruses from such individuals have been reported to be nearly identical to the corresponding transmitted viruses (*84*) and may thus not differ in their IFN-I resistance. However, even in acute ART initiators, rebound viruses may have to escape heightened IFN-I pressures. Given the limited genetic diversity, this would provide a unique opportunity to dissect the underlying mechanisms. Future studies should also perform viral outgrowth assays in the presence of IFN-I to see whether pretreatment of latently infected $CD4^+$ T cells activates rebound-like viruses and conduct integration site analyses to determine whether IFN-resistant viruses are preferentially found integrated in chromatin regions that restrict viral gene expression in the absence of IFN-I. The latter finding would suggest that IFN-I stimulation is required to reactivate a subset of latently infected $CD4^+$ T cells, which would have obvious clinical implications. Last, the high resistance of rebound viruses reinforces the need for nonhuman primate models that faithfully recapitulate this key feature of HIV-1 persistence. If SIVmac- or SHIV-infected rhesus macaques exhibited the same IFN-I kinetics as humans, these models could be used to trace the provenance and activation requirements of rebound viruses.

In summary, we show here that IFN-I plays an important role in the control of HIV-1 during acute infection and later stages of infection. The resistance to IFN-I of HIV-1 isolates from plasma reflects the contribution of IFN-mediated activity relative to other antiviral pressures at the site of virus production. The kinetics of the IFN-I response vary over the course of infection, with rebound viruses exhibiting by far the most IFN-I resistance. Although the tissue and cell origins of rebound viruses remain to be determined, our data indicate that they arise either from a cryptic reservoir of highly IFN-resistant viruses or rapidly evolve at the sites of viral recrudescence to acquire IFN-I resistance.

MATERIALS AND METHODS

Study design

The role of IFN-I in the control of HIV-1 during infection was determined by generating 500 plasma- and PBMC-derived viral isolates from 26 individuals sampled prospectively before and during ART or after ATI. Viruses were genetically characterized, and their susceptibility to IFNα2 and IFNβ inhibition (IC_{50}) was determined. Sample sizes were dependent on availability and not predetermined by power calculations. The number of samples available and the tests performed are detailed in table S1.

Participants MM14, MM15, MM23, MM33, MM34, MM39, MM40, MM55, and MM62 were recruited to an acute HIV-1 infection cohort at the Mortimer Market Centre for Sexual Health and HIV Research in London, UK, whereas participant WEAU was followed at the 1917 Clinic of the University of Alabama at Birmingham. Peripheral blood was collected in EDTA-containing tubes after onset of symptoms and at regular intervals thereafter (table S1). Plasma was separated by centrifugation, whereas PBMCs were isolated using a Histopaque 1.077 density gradient and cryopreserved. Individuals provided informed consent and were offered ART on the basis of the standard of care at the time. Ethical approval for the London cohort study was provided by the National Health Service Camden/Islington Community Local Research Ethics Committee. The use of stored (de-identified) samples from participant WEAU was approved by the University of Alabama at Birmingham Institutional Review Board. Plasma and viably frozen PBMC samples were also obtained from 16 individuals who participated in six different treatment interruption and latency trials (MNU-0628, NCT02825797, NCT0246322, NCT00051818, NCT02227277, and NCT02588586). These materials were obtained from existing repositories, and their use was approved by the respective Institutional Review Boards as previously described (*61*–*67*).

Virus isolation from plasma

$CD4^+$ T cells were isolated from buffy coats of healthy donors (purchased from Zen-Bio Inc.) by positive selection using human CD4 micro beads (Miltenyi Biotec) as described (*50*). For limiting dilution isolation, plasma samples were end point diluted and used to infect 1×10^6 activated $CD4^+$ T cells in 24-well plates (*50*). To generate bulk isolates, plasma aliquots containing 1500 to 20,000 viral RNA copies were used to infect 4×10^6 healthy donor $CD4^+$ T cells in six-well plates. Cultures were maintained for 21 days and tested weekly for the presence of p24 antigen in the supernatant. Virus-positive cultures were expanded in 1×10^7 pooled healthy donor $CD4^+$ T cells, and the resulting viral stocks were used for all genetic and biological analyses.

Quantitative viral outgrowth assay

Virus isolations were performed as described (*60*) with minor modifications. $CD4^+$ T cells were isolated from viably frozen PBMC, incubated in RPMI 1640 media containing 15% fetal bovine serum (FBS) without interleukin-2 (IL-2) for 24 hours, and stimulated for 24 hours using a combination of anti-CD2, CD3, and CD28 antibody-coated beads (Miltenyi Biotec T Cell Activation/Expansion Kit). Cells were then cocultured with healthy donor $CD4^+$ T cells (1:10 patient:donor cells) and cultured for 3 weeks with weekly monitoring of p24 production. Positive cultures were expanded, and viral stocks generated as described above. In cases where QVOA isolates were already available (*61*–*64*), supernatants were obtained and used to generate viral stocks in healthy donor $CD4^+$ T cells.

Single-genome amplification

Single genome amplification of 3′ half genomes or viral *env* genes from plasma RNA and proviral DNA was performed as previously described (*54*, *85*). Briefly, ~20,000 copies of viral RNA were extracted from plasma using QIAamp Viral RNA kit (QIAGEN) and reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen). PBMC DNA was extracted using a DNeasy Blood and Tissue kit (QIAGEN). Viral cDNA and PBMC DNA were then end point diluted and amplified using nested PCR with primers and conditions as previously reported (*54*, *85*).

Isolate sequencing

Viral RNA was extracted from isolate stocks and reverse-transcribed, and the resulting cDNA was used to amplify overlapping 5′ and 3′ genome halves in separate PCR reactions (*50*). Amplicons were sequenced using Illumina MiSeq and paired-end reads assembled to

generate a sample-specific reference sequence. Viral reads were mapped to this reference, and the extent of genetic diversity was examined for each position along the alignment. Isolates that exhibited more than 15% diversity at any one position were judged to contain more than one variant. These positions were recorded as ambiguous sites in finished viral sequences.

Phylogeny

Plasma viral-, proviral-, and isolate-derived sequences for each individual were aligned with Clustal Omega (*86*) and inspected for misassemblies, overly truncated sequences, and sequences with abundant G to A mutations (considered to be the result of APOBEC hypermutation). Alignments were trimmed to the shortest sequence, and positions with gaps in any sequence were masked in all sequences. Trees were generated using RAxML v8.2.12 (*87*) with model GTRGAMMA and bootstrap values calculated from 1000 replicates. Potential recombinants from superinfected individuals were identified in alignments using Recco (*88*) using default parameters; recombinants were reported if they had an alignment $P < 0.05$ and contained at least 10 recombination-induced mutations.

Interferon IC_{50} determinations

To determine the IFNα2 and IFNβ concentrations required to inhibit virus replication by 50% (IC_{50}), pooled activated $CD4^+$ T cells were left untreated or cultured in the presence of increasing amounts of IFNα2 or IFNβ for 24 hours as described (*50*). Cultures (2.5×10^5 cells) were then infected overnight with equal amounts of virus based on reverse transcriptase (RT) activity (0.25 ng of RT) and maintained for 7 days in IL-2–containing (30 U/ml) RPMI 1640 + FBS media, replenishing IFN-I every 48 hours. Virus replication was measured as the amount of p24 produced at day 7, and IC_{50} was calculated as the amount of IFN-I necessary to reduce p24 production in the absence of IFN-I by 50% (fig. S3). The same pool of activated $CD4^+$ T cells from four healthy donors (purified by positive selection from buffy coats) was used for all IFN-I IC_{50} determinations.

Statistical analyses

We developed hierarchical Bayesian models to analyze the longitudinal changes of interferon resistance while avoiding bias from differential sampling (*50*, *58*). These models assumed that the data were log normally distributed with the resistance observed in each individual deriving from a common population-level distribution (see also Supplementary Methods). For the longitudinal analysis, IFN-I resistance was assumed to start at an acute infection level, fall (or rise) to some nadir change point, and then rise (or fall) on the basis of changes in $CD4^+$ T cell counts (Fig. 3). For comparisons of outgrowth and rebound viruses, each isolate was assumed to be a random sample from the viruses circulating within an individual, and each individual was assumed to be representative of a larger population. Outgrowth isolates were assumed to represent mixed populations that could include rebound-like viruses (Supplementary Methods). Data were processed and analyzed using R v3.4.4 (*89*). Posterior probability distributions were estimated using Markov chain Monte Carlo sampling as implemented in Stan v2.23 (*90*).

SUPPLEMENTARY MATERIALS

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Methods

Fig. S1. Inference and enumeration of TF viruses.

Fig. S2. HIV-1 quasispecies diversification over time.

Fig. S3. Determination of IFN-I IC_{50} values.

Fig. S4. IFN-I resistance of bulk and limiting dilution isolates from the same plasma samples.

Fig. S5. Correlation of IFNα2 and IFNβ IC_{50} values.

Fig. S6. Phylogenetic relationships of rebound isolates.

Fig. S7. Closely related rebound and post-ATI QVOA isolates from participant A08.

Table S1. Generation of HIV-1 isolates from plasma and PBMCs of 10 individuals sampled from acute infection throughout their clinical course.

Table S2. IFN-I resistance of plasma and QVOA isolates from longitudinally sampled study participants.

Table S3. Generation of viral isolates from ART-suppressed individuals with and without treatment interruption.

Table S4. IFN-I resistance of viral outgrowth and rebound isolates.

Data file S1. GenBank accession numbers.

View/request a protocol for this paper from *Bio-protocol*.

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Competing interests: The authors declare that they have no competing financial interests. S.H. is a member of an HIV/immunology advisory panel for Merck and Co. **Data and materials availability:** All data associated with this study are in the paper or the Supplementary Materials. HIV-1 sequences have been deposited in GenBank with accession numbers listed in data file S1. The analysis code is archived on Zenodo at https://doi.org/10.5281/zenodo.4277786.

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Heightened resistance to host type 1 interferons characterizes HIV-1 at transmission and after antiretroviral therapy interruption

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Putting pressure on HIV

Type 1 interferons provide early host immune defense against viruses, including HIV-1, by promoting expression of proteins that have direct or indirect antiviral activity. It remains unclear how viruses that persist long term, such as HIV-1, escape type 1 interferon–mediated pressure. Gondim *et al.* characterize interferon resistance in HIV-1 isolates acquired from HIV-1–infected individuals over several years. The authors found that HIV-1 isolates acquired at the time of initial infection or during interruption of antiretroviral therapy were the most resistant to type 1 interferons. These findings underpin the importance of type 1 interferons in controlling HIV-1 infection in the host.

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