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Short-Form Paper

Effects of interferon-α treatment on anti-HIV-1 intrinsic immunity *in vivo*

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Running title: Interferon-α and Host Restriction *in vivo*

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ABSTRACT

Interferon-α suppresses HIV-1 replication *in vitro* by inducing cell-intrinsic retroviral restriction mechanisms. We investigated the effects of interferon-α/ribavirin treatment (IFN-α/riba) on 34 anti-HIV-1 restriction factors *in vivo*. Several anti-HIV-1 restriction factors were significantly induced by IFN-α/riba in HIV/HCV-coinfected individuals. Fold-induction of cumulative restriction factor expression in CD4+ T cells was significantly correlated with viral load reduction during IFN-α/riba (r^2=0.649; $p<0.016$). Exogenous interferon-α induces supraphysiologic restriction factor expression, associated with a pronounced decrease in HIV-1 viremia.

Keywords: Interferon-α; intrinsic immunity; retroviral restriction factors; APOBEC3; BST-2; tetherin; TRIM family proteins.

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67 **FINDINGS**

68 Detailed analyses of the molecular and immunologic effects of the cytokine interferon-α
69 (IFN-α) may provide insights that contribute to the development of novel prophylactic,
70 therapeutic and curative strategies for HIV-1 infection. Induction of IFN-α expression is
71 a critical first step in the defense against a range of viral infections (1, 2). The
72 antiretroviral activity of IFN-α was demonstrated *in vitro* soon after the discovery of
73 HIV-1 (3), and several studies have reported that exogenous IFN-α treatment potently
74 suppresses HIV-1 *in vivo* (4-8). Moreover, IFN-α therapy was recently associated with
75 significant reduction in the size of the HIV-1 latent reservoir (9), suggesting that
76 interferon-associated pathways may be exploited to achieve HIV-1 eradication. The
77 mechanisms underlying the *in vivo* anti-HIV-1 capacity of IFN-α remain to be thoroughly
78 elucidated.

79

80 Cell-intrinsic immune mechanisms likely contribute to the beneficial effects of type I
81 interferon (10). On this front, our laboratory recently reported that the induction of the
82 BST-2/tetherin, APOBEC3G and APOBEC3F cell-intrinsic immune defenses contributes
83 to the IFN-α-mediated suppression of HIV-1 *in vivo* (6). A large number of additional
84 host restriction factors with anti-HIV-1 activity *in vitro* have now been identified and
85 characterized. In this study, we perform a comprehensive analysis of the effects of
86 exogenous IFN-α treatment on all as yet established anti-HIV-1 host restriction factors
87 and HIV-1 viremia *in vivo*.

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89 We examined restriction factor gene expression patterns in longitudinal samples from
90 HIV/HCV-coinfected individuals undergoing pegylated interferon-α/ribavirin
91 combination therapy (IFN-α/riba), and additionally characterized a separate population of
92 interferon-untreated control individuals. We designed and implemented a custom
93 TaqMan Low Density Array (TLDA) to measure the expression of 34 anti-HIV-1
94 restriction genes (11). We relied on the following two minimal criteria for inclusion in
95 our "Cumulative Restriction" or "CuRe" TLDA: 1) Peer-reviewed, published evidence
96 of direct inhibition of HIV-1 replication *in vitro*, and 2) Detectable expression in human
97 peripheral blood mononuclear cells. All factors in the CuRe array meet the essential,
98 minimal definition of a host restriction factor, and function in a cell-autonomous manner
99 to suppress HIV-1 replication.
100
101 Subjects and specimen processing: Longitudinal samples were collected from 15
102 HIV/HCV-coinfected individuals enrolled in the Swiss HIV Cohort Study ([SHCS],
103 www.shcs.ch) (12) who underwent IFN-α/riba treatment (Supplementary Table 1). All
104 subjects had PBMC available before, during and after IFN-α/riba treatment, were ART-
105 naïve, and had detectable HIV-1 RNA at baseline. Blood was collected prospectively
106 from 12 HIV-1-infected, (ART-untreated) viremic individuals and 12 HIV-1-uninfected
107 individuals enrolled in the UCSF SCOPE cohort (Supplementary Table 2). PBMC were
108 isolated with Ficoll-Paque PLUS. CD4+ T cells were enriched from fresh PBMCs using
109 the EasySep Human CD4+ T Cell enrichment kit (StemCell Technologies), according to
110 the manufacturer's instructions. The research was approved by the relevant institutional
111 review boards, and all human participants gave written informed consent.

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112

113 Gene expression profiling: Total RNA was extracted from PBMC and CD4+ T cells
114 directly after enrichment using Trizol reagent (Invitrogen). RNA was transcribed into
115 cDNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative
116 real-time PCR utilized custom-made TaqMan® Low Density Arrays (TLDA) from Life
117 Technologies following the manufacturer's instructions. All assays and their respective
118 target genes are listed in Supplementary Table 3. Thermal cycling was performed using
119 an ABI ViiA™ 7 Real-Time PCR System. Data was analyzed using ABI ViiA™ 7
120 software. A panel of six housekeeping genes was included in the TLDA plates (GAPDH,
121 18S, ACTB, PPIA, RPLP0, and UBC). RPLP0 (ribosomal protein, large, P0) was
122 identified as the most stably expressed housekeeping gene using the GeNorm algorithm
123 (13). Therefore, raw cycle threshold (Ct) numbers of amplified gene products were
124 normalized to the housekeeping gene, RPLP0, to control for cDNA input amounts
125 (Supplementary Figure 1). Fold induction was determined using the comparative Ct
126 method (13). APOBEC3G, APOBEC3F, BST2/tetherin and ISG15 relative copy
127 numbers were re-calculated from our previous work (6).

128

129 CuRe score calculation: Missing (undetectable) values were imputed using the minimum
130 expression value across samples for each gene. The expression value for the ith gene is
131 notated as e_i. A reference sample was selected based on having the maximum number of
132 genes that were closest to the median gene expression profile. The reference expression
133 value for the ith gene is notated as r_i. The CuRe score for a sample is the cumulative fold-

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134 difference in antiviral gene expression with respect to the reference individual, expressed
135 by the following formula:

136 $$CuRe = \sum_{i=1}^{n} e_i / r_i$$

137

138 Statistical Analysis: The paired Wilcoxon test, Mann–Whitney U test and Pearson's r
139 correlation coefficient were applied to data using GraphPad Prism v5.0c.

140

141 We initially examined the effects of IFN-α/riba treatment on HIV-1 plasma viral load.
142 The 15 IFN-α/riba-treated individuals included in this study represent a subset of
143 individuals studied in our previous work on IFN-α effects, chosen based on sample
144 availability (6). Subject characteristics and IFN-α/riba treatment regimens are described
145 in Supplementary Table 1. IFN-α/riba treatment reduced plasma viral load by 0.91
146 (±0.70) $\log_{10}$ copies/mL during treatment, and viremia typically returned to approximate
147 pre-treatment levels following therapy cessation (Supplementary Figure 2). This effect is
148 consistent with previous IFN-α/riba and IFN-α monotherapy studies (4, 5, 7, 8). Seven of
149 15 individuals were included in analyses of PBMC gene expression, and the remaining
150 eight individuals were included in analyses of CD4+ T cell gene expression. A separate
151 population of 24 IFN-α-untreated individuals (12 HIV-1 uninfected and 12 HIV-1
152 infected, ART-untreated) enrolled in the SCOPE cohort was additionally characterized as
153 a control group (Supplementary Table 2).

154 We next implemented the CuRe array to examine the effects of IFN-α/riba treatment on
155 the expression of 34 anti-HIV-1 restriction factors (described in Supplementary Table 3)

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156 in PBMCs and CD4+ T cells. Fourteen out of 34 restriction genes were significantly
157 elevated in unfractionated PBMCs during the IFN-α/riba treatment period with respect to
158 pre-treatment levels: APOBEC3A, APOBEC3H, IFITM1, IFITM2, IFITM3, ISG15,
159 PKR, HERC5, MOV10, RSAD2 (viperin), TRIM11, TRIM14, TRIM19, and TRIM22
160 (Figure 1A). An overlapping but distinct set of 15 restriction factors was significantly
161 induced by IFN-α/riba treatment in isolated CD4+ T cells: APOBEC3F, APOBEC3G,
162 BST-2/tetherin, IFITM1, ISG15, PKR, HERC5, MOV10, RSAD2 (viperin), TRIM11,
163 TRIM14, TRIM19, TRIM22, TRIM28, and TRIM32 (Figure 1B). In both unfractionated
164 PBMCs and CD4+ T cells, induced genes returned to approximate baseline levels post-
165 cessation of IFN-α/riba treatment. Gene-by-gene fold-induction levels (and statistics)
166 observed in PBMCs and CD4+ T cells are presented in Supplementary Table 4.

167 To infer the contribution of anti-HIV-1 restriction factors to the observed IFN-α/riba-
168 mediated suppression of HIV-1, we examined correlations between the induction of
169 restriction genes and reduction in HIV-1 viremia during the treatment period. We
170 defined the CuRe score (explained above in more details) to represent overall, cumulative
171 restriction factor gene expression across our 34 measured targets. IFN-α/riba-mediated
172 viral load reduction did not exhibit any correlation with induction of the CuRe score in
173 unfractionated PBMCs (r^2=0.064, p=0.584) (Figure 2A), or induction of any of the 34
174 restriction factors in PBMCs considered on an individual gene-by-gene basis. IFN-
175 α/riba-mediated viral load reduction exhibited a significant, pronounced correlation with
176 induction of the CuRe score in CD4+ T cells (r^2=0.649, p=0.016) (Figure 2B). Viral load
177 reduction was significantly correlated with the induction of nine individual anti-HIV-1
178 restriction genes in CD4+ T cells (Supplementary Figure 3), listed here hierarchically

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179 according to the strength of statistical association: TRIM22 (r^2=0.64, p=0.008), PKR
180 (r^2=0.533, p=0.019), TRIM32 (r^2=0.499, p=0.025), TRIM11 (r^2=0.534, p=0.031),
181 HERC5 (r^2=0.435, p=0.038), APOBEC3F (r^2=0.42, p=0.041), RSAD2 (viperin) (r^2=0.41,
182 p=0.043), BST-2/tetherin (r^2=0.402, p=0.046), IFITM1 (r^2=0.401, p=0.046).
183
184 To investigate the hypothesis that exogenous IFN-α drives supraphysiologic expression
185 of restriction factors, we compared CD4+ T cell anti-HIV-1 restriction factor expression
186 in our SHCS interferon-treated population to CD4+ T cell restriction factor expression in
187 a separate population of 12 HIV-1-infected, (ART-untreated) viremic individuals and 12
188 HIV-1-uninfected individuals enrolled in the UCSF SCOPE cohort. There were no
189 significant differences between SHCS subjects pre-IFN-α/riba treatment and SCOPE
190 viremic subjects in terms of CuRe score (p=0.464) or ISG-15 expression (p=0.203).
191 However, there was a significant elevation in CuRe score in SHCS subjects during IFN-
192 α/riba treatment with respect to SCOPE viremic (p=0.002) and uninfected subjects
193 (p=0.0003), respectively (Figure 2C). Similarly, ISG-15 expression was significantly
194 higher in SHCS subjects during IFN-α/riba treatment than in SCOPE viremic (p=0.0002)
195 and uninfected subjects (p=0.0002) (Figure 2D), supporting the hypothesis that
196 exogenous IFN-α drives antiviral gene expression to supraphysiologic levels. Gene-by-
197 gene analyses for each of the nine genes associated with IFN-α/riba-mediated suppression
198 of HIV-1 viremia in our study are presented Supplementary Figure 4.
199
200 Several recent reports have rejuvenated interest in deciphering the molecular pathways
201 associated with the antiviral effects of IFN-α (6, 7, 9, 14-16). In particular, two recent

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202 reports on LCMV infection suggest that IFN-α is associated with both beneficial and
203 detrimental disease outcomes, and the overall balance between the various, diverse
204 effects of type I interferon ultimately determines the course of disease (14, 15). This is
205 mirrored in studies of interferon within the context of HIV-1 infection, whereby IFN-α is
206 known to induce several antiretroviral mechanisms that suppress viral replication, but
207 may result in poor disease outcomes due to association with T cell activation and
208 inflammation (17-19). Taken together, these observations suggest that additional work
209 needs to be performed to dissect interferon-associated molecular pathways to identify
210 critical antiretroviral mechanisms and to avoid possible pro-inflammatory consequences.
211
212 Endogenous IFN-α is often associated with rapid HIV-1 disease progression and high
213 viral load (17, 18). We hypothesized that the inverse relationship between IFN-α and
214 viral load observed within the context of exogenous IFN-α administration may result
215 from the induction of antiviral genes to supraphysiologic levels not typically encountered
216 in the absence of pharmacological manipulation. Our data strongly support this
217 hypothesis, and suggest that the induction of several restriction factors contributes to
218 IFN-α suppression of HIV-1 *in vivo*. It is provocative that Individual N in our study
219 exhibited the greatest IFN-α/riba induction of the CuRe score, and was the only
220 individual to suppress viral load to undetectable levels for the entire duration of our study
221 (Supplementary Figure 2). Moreover, exogenous IFN-α induces these antiviral
222 mechanisms without appreciably increasing CD4+ T cell activation, which promotes viral
223 transactivation and replication (20). The lack of a relationship between restriction factor
224 induction in PBMCs and viral load reduction suggests that specific consideration of HIV-

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225 1 target cells may be important when evaluating cell-intrinsic immune effects. In
226 addition, a number of innate and adaptive immune mechanisms are likely triggered by
227 IFN-α which may influence disease outcomes as well. The contribution of non-cell-
228 intrinsic mechanisms to IFN-α anti-HIV-1 effects should be explored in greater detail.
229
230 These data support the concept that the induction of particular intrinsic immune
231 mechanisms may constitute a promising antiretroviral strategy, complementing our
232 previous *in vitro* work (21) and our translational studies of restriction mechanisms in
233 HLA-B*57-positive individuals (22) and HIV-1 elite controllers (11).
234
235
236 **List of abbreviations**
237
238 APOBEC: apolipoprotein B mRNA editing enzyme; BST-2: bone marrow stromal cell
239 antigen 2; SAMHD1: SAM domain and HD domain-containing protein 1; TRIM:
240 tripartite motif; ISG: Interferon-stimulated gene; CDKN1A: cyclin-dependent kinase
241 inhibitor 1A; PAF1: RNA polymerase II associated factor; EIF2AK2: Eukaryotic
242 translation initiation factor 2-alpha kinase 2; HERC5: HECT domain and RLD 5; IFITM:
243 Interferon induced transmembrane; MOV10: Moloney leukemia virus 10, homolog;
244 SLFN11: Schlafen family member 11. TLDA: TaqMan® Low Density Array; GAPDH:
245 Glyceraldehyde 3-phosphate dehydrogenase;18S: 18S ribosomal RNA; ACTB: Beta-
246 actin; PPIA: Peptidylprolyl isomerase A; RPLP0: 60S acidic ribosomal protein P0; UBC:
247 Ubiquitin C.

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248

249 **Competing interests**

250 The authors declare that they have no competing interests.

251

252 **Authors' contributions**

253 MAM, TL, JG, and SKP performed gene expression experiments and XD performed
254 statistical analyses. AR, BL, SGD and HG coordinated patient recruitment. MAM, SKP,
255 JKW, SGD, JG, MSS, HEAG, and HG designed the studies. MAM and SKP wrote the
256 paper. All authors read and approved the final manuscript.

257

258

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382 **FIGURE LEGENDS**

383

384 **Figure 1. IFN-α/riba induction of anti-HIV-1 restriction factors.** A) Fold-induction in
385 unfractionated PBMCs. B) Fold-induction in negatively-selected CD4+ T cells. Fold-
386 induction during IFN-α/riba (green bars) and post-IFN-α/riba (red bars) were normalized
387 to pre-treatment expression level, as indicated by the dashed line. Mean and standard
388 error are represented in each bar. Asterisks indicate statistically significant differences
389 between during- and pre-therapy expression level based on a paired Wilcoxon test (* =
390 $p< 0.05$; ** = $p<0.01$).

391

392 **Figure 2. IFN-α/riba induction of Cumulative Restriction (CuRe) score to**
393 **supraphysiologic levels correlates with viral load reduction.** A) Relationship between
394 HIV-1 viral load reduction during IFN-α/riba therapy and CuRe score fold-induction in
395 PBMCs; no significant correlation was observed. B) Relationship between HIV-1 viral
396 load reduction during IFN-α/riba therapy and CuRe score fold-induction in CD4+ T cells.
397 Significance was assessed using Pearson's r correlation coefficient. C) Comparison of
398 CuRe score between SHCS subjects undergoing IFN-α/riba therapy and HIV-1-negative
399 and HIV-1-infected (ART-untreated) viremic individuals enrolled in the SCOPE cohort.
400 D) Comparison of ISG-15 expression (marker of interferon exposure) between SHCS
401 subjects undergoing IFN-α/riba therapy and HIV-1-negative and HIV-1-infected (ART-
402 untreated) viremic individuals enrolled in the SCOPE cohort. Mann-Whitney tests were
403 employed to determine significance.

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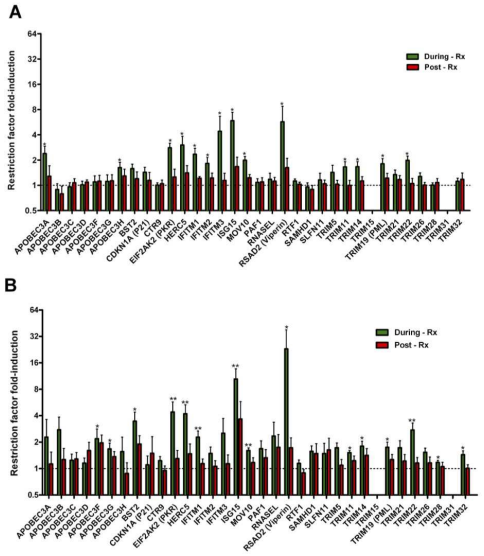
Figure 1.



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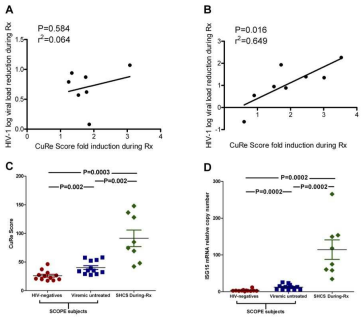
Figure 2.