Differential Control of HIV-1 Replication by IFN-α14 Compared to IFN-α2 Relates to Differences in the Modulation of Host Antiretroviral Restriction Factors

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ABSTRACT

Type I IFN, including IFN-α, induces the expression of antiviral restriction factors that can interfere with multiple steps of the HIV-1 replication cycle. Humans have 13 IFN-α genes which encode 12 different IFN-α subtypes. Our previous work in HIV-1 infected humanized mice showed that IFN-α14 treatment more potently controlled HIV-1 than treatment with the clinically approved IFN-α2 subtype. However, the mechanisms behind the more potent control of HIV-1 by IFN-α14 are unknown. The IFN-α14 subtype is known to more potently induce the expression of the restriction factors MX2 and ISG15 and increased APOBEC3G signature mutations *in vivo* compared to IFN-α2. To study the importance of each of these restriction factors in mediating the potent control of HIV-1, we used a CRISPR-Cas9 lentivirus system to create stable knockouts in the MT4C5 cell line that is susceptible to HIV-1 but does not produce measurable amounts of endogenous IFN-α. Knock out of ISG15, but not MX2, eliminated differences in viral suppression after IFN-α14 and IFN-α2 treatment. Similarly, APOBEC3G deletion eliminated differences in viral suppression and the number of infectious particles produced after IFN-α14 and IFN-α2 treatment. Furthermore, APOBEC3G deletion resulted in significantly fewer GG→AG mutations in viral DNA isolated from target cells incubated with supernatant from IFN-α14 treated groups. However, APOBEC3G knock out did not result in significant increases in vDNA compared to the wild type in any experimental group. Overall, elimination of APOBEC3G and ISG15 impaired IFN-α14–mediated suppression of HIV-1, highlighting them as downstream effectors of IFN-α14's more potent anti-HIV-1 activity.

42 **IMPORTANCE**

43 This study uncovers the molecular basis for the more potent antiviral activity of IFN-α14 compared
44 to the clinically used IFN-α2 subtype against HIV-1. Although interferons are known to induce
45 numerous restriction factors, the mechanisms underlying subtype-specific antiviral potency
46 remained unclear. By using CRISPR-Cas9 knockout MT4C5 cell lines, the study identifies ISG15
47 and APOBEC3G as key effectors mediating IFN-α14's enhanced suppression of HIV-1 replication.
48 Loss of either ISG15 or APOBEC3G abolished the differential antiviral effect between IFN-α14
49 and IFN-α2, demonstrating their essential roles in IFN-α14 driven viral restriction. These findings
50 highlight that individual IFN-α subtypes engage distinct downstream pathways and that subtype
51 diversity encodes functional specialization rather than redundancy. Overall, this work advances our
52 understanding of innate immune control of HIV-1 and provides a foundation for developing
53 targeted interferon-based therapies that exploit the unique mechanisms of potent subtypes like IFN-
54 α14.

55 **INTRODUCTION**

56 Type I interferon (IFN) is a crucial component of the innate immune system, playing an
57 essential role in defending against a wide range of viruses(1). In humans, type I IFN includes 12
58 IFN-α subtypes along with IFN-β, IFN-κ, IFN-ε, IFN-ο, IFN-τ, and IFN-δ(2). All type I IFN signal
59 through the widely expressed IFN-α receptor (IFNAR)(3), triggering the activation of interferon
60 regulated genes (IRG)(4, 5). Interferon-α plays a role in controlling many viral diseases including
61 HIV-1. Produced primarily by plasmacytoid dendritic cells, IFN-α engages the IFNAR and
62 activates JAK–STAT and MAPK pathways(6), inducing hundreds of IRG that interfere with viral
63 replication at multiple stages, from entry to assembly(7, 8). It is now known that IFN-α subtypes
64 induce IRG, including viral restriction factors, in a differential manner(9, 10, 11, 12). Many
65 antiretroviral restriction factors have been identified that can inhibit HIV-1, including classical and
66 well documented apolipoprotein B mRNA editing catalytic polypeptide-like 3G (APOBEC3G),
67 SAM and HD domain-containing protein 1 (SAMHD1), tetherin, ISG15 and tripartite motif-
68 containing protein 5 (TRIM5α) and those of more recent characterization: MX Dynamin Like
69 GTPase 2 (MX2), serine incorporator (SERINC) 3/5, interferon-induced transmembrane proteins
70 (IFITMs), schlafen 11, and membrane-associated RING-CH (MARCH) 2/8(13, 14, 15, 16, 17, 18,
71 19, 20, 21).

72 Restriction factors can impede HIV-1 replication in many ways. For example, APOBEC3G is
73 incorporated into HIV-1 virions from a producer cell and exerts its antiviral activity in newly
74 infected cells. The antiviral activity of APOBEC3G includes inhibition of reverse transcription,
75 impairment of proviral integration and deamination of the viral RNA resulting in G to A
76 hypermutation of the nascent viral DNA(22, 23, 24) that can result in defective provirus. The MX2
77 protein inhibits HIV-1 by interfering with the nuclear import of the viral pre-integration complex,

78 which is a crucial step after viral DNA synthesis but before its integration into the host cell's
79 genome(25, 26). Additionally, MX2 inhibits viral capsid uncoating, thereby blocking HIV-1
80 infection(27). ISG15, a ubiquitin-like protein, is the most highly expressed IRG(28). It inhibits
81 both the assembly and release of HIV-1 virions from cells(29, 30).

82 It has been well documented that IFN-α subtypes differentially control HIV-1 replication. IFN-
83 α14 is the more potent subtype both *in vitro* and *in vivo* when compared to clinically used IFN-
84 α2(12, 31). However, whether the more potent control of HIV-1 by IFN-α14 is due to differential
85 modulation of restriction factors is unknown. Lavender et al. and Harper et al.(31, 32) showed that
86 transcription of *MX2* was significantly increased by IFN-α14 but not IFN-α2. In contrast,
87 *APOBEC3G* was not transcriptionally upregulated but there was a significant increase in
88 APOBEC3G-mediated signature mutations in the proviral HIV-1 DNA (vDNA) from IFN-α14
89 compared to IFN-α2 treated mice. In 2021, Tauzin et al.(12) showed that IFN-α14 also induced
90 greater *ISG15* expression in MT4C5 cells. Since different IFN-α subtypes appear to modulate
91 different restriction factors and control HIV-1 in a differential manner, we wanted to investigate
92 whether the MX2, ISG15 and APOBEC3G restriction factors are important in IFN-α14's more
93 potent control of HIV-1. We used a CRISPR-Cas9 system to knock out each restriction factor in
94 MT4C5 cells to study their importance in the more potent control of HIV-1 by IFN-α14 compared
95 to IFN-α2.

96 **RESULTS**

97 **IFN-α14 more potently reduced HIV-1 replication than IFN-α2 when used at a**
98 **biologically relevant activity.** The relationship between protein concentrations and the functional
99 activity of cytokines such as IFN-α is not strictly linear, as functional activity is influenced not
100 only by expression levels but also by factors such as folding efficiency when using recombinant
101 proteins and ligand-receptor interactions. Different IFN-α subtypes have been shown to bind with
102 different affinities and kinetics to the interferon alpha receptor (IFNAR)(33, 34, 35, 36, 37). For
103 example, the IFN-α14 subtype binds more tightly to IFNAR compared to IFN-α2(38) and this
104 difference in affinity could potentially influence the intensity of the downstream signaling
105 cascade(39) leading to differential expression of IRGs. Also, doses of biologics like interferons
106 should be expressed in activity units rather than mass units to account for the presence of inactive
107 protein fractions in recombinant formulations(40). Since the IFN-α2 and -α14 subtypes in our
108 study were produced recombinantly and have different affinities for IFNAR, we first needed to
109 normalize their concentrations based on signaling activity in order to better understand their
110 potentially different antiviral effects(31, 41). To normalize the activity between IFN-α2 and IFN-
111 α14 we used a reporter cell line containing an interferon-stimulated response element (ISRE)
112 promoter/enhancer element driving a luciferase reporter gene**.** Using the reporter cell line, we
113 titrated each IFN-α subtype over a range of protein concentrations and normalized the activities of
114 each subtype based on their EC_{50} values to that of an IFN-α standard with a known activity prior
115 to use in experiments (Fig. 1A).

116 To evaluate the importance of antiretroviral factors in mediating more potent control of HIV-
117 1 by IFN-α14 compared to IFN-α2 we utilized the MT4C5 cell line. The MT4C5 cell line is a
118 derivative of MT4 cells that additionally expresses CCR5 and can be infected by CXCR4-tropic

119 (X4) or CCR5-tropic (R5) HIV-1 viruses. Importantly, following HIV-1 infection, MT4C5 cells
120 do not produce measurable amounts of endogenous IFN-α in the cell culture supernatant(42) that
121 may otherwise confound findings. We first established our system in MT4C5 cells to test the
122 contributions of each restriction factor to the more potent control of HIV-1 by IFN-α14. Wild-
123 type (WT) MT4C5 cells were infected with HIV-1_{JR-CSF} at 0.1 MOI for 2 hours and then treated
124 with a biologically relevant activity (100U/ml)(43) or two supraphysiological doses (500U/ml or
125 1000U/ml)(44) of IFN-α2 or IFN-α14. Three days post infection, fresh medium with 100U/ml,
126 500U/ml or 1000U/ml of IFN-α2 or IFN-α14 was replenished. Six days post infection,
127 supernatant was collected and a p24 ELISA performed to quantify HIV-1 replication through the
128 production and release of HIV-1 virions into the supernatant. Both IFN-α subtypes significantly
129 reduced p24 in supernatants (Fig. 1B). However, supernatants from cells cultured in 100U/ml of
130 IFN-α2, contained significantly higher amounts of viral p24 compared to cells cultured in
131 100U/ml of IFN-α14 (Fig. 1B). In cultures containing 500U/ml and 1000U/ml of IFN-α, there
132 were no significant differences observed between subtypes, although a trend toward lower levels
133 of virus production could be detected in the cultures containing IFN-α14 (Fig. 1B). The results
134 indicated that greater suppression of HIV-1 replication in MT4C5 cells by IFN-α14 compared to
135 IFN-α2 occurred at the biologically relevant activity(43) of 100U/mL, but less so at
136 supraphysiological concentrations.
137
138 **Potent suppression of HIV-1 replication by IFN-α14 occurs intracellularly.** We first
139 investigated if lower levels of virus in supernatants from IFN-α14 treated cells might have resulted
140 from an accumulation of virions within the cell with inhibition of HIV-1 release into the
141 supernatant. We performed a p24 immunoblot using cell lysates from untreated HIV-1 infected

142 MT4C5 cells and cells treated for 6 days with 100U/ml of either IFN-α2 or -α14. The immunoblot
143 indicated that lysates from cells treated with IFN-α14 had no detectable cell-associated p24
144 whereas p24 was clearly visible in lysates from cells treated with IFN-α2 (Fig. 2A). Furthermore,
145 quantification of the p24 bands in relation to the GAPDH loading control on the western blot
146 confirmed there was some reduction in cellular p24 in the cells treated with 100U/ml of IFN-α2
147 compared to untreated cells as expected based on the p24 ELISA results in Fig. 1B, although not
148 nearly to the degree as in IFN-α14 treated cells (Fig. 2B). The results indicated that the more potent
149 suppression of HIV-1 replication by IFN-α14 than IFN-α2 at the biologically relevant
150 concentration of 100U/mL(43) did not primarily originate due to accumulation of cell-associated
151 virions that were not being released into the supernatant.
152
153 **IFN-α14 treatment more potently reduced cellular HIV-1 viral DNA (vDNA) and viral**
154 **infectivity compared to IFN-α2.** Having established that IFN-α14 potently suppressed levels of
155 HIV-1 p24 within the cell we next evaluated whether the reduction in p24 was due to reductions
156 in viral HIV-1 DNA (vDNA) within cells and/or the amount of infectious virus being produced
157 over the 6-day infection. First, we evaluated levels of vDNA in HIV-1 infected MT4C5 cells that
158 had been left untreated or treated with 100U/ml of either IFN-α2 or IFN-α14 for 6 days. To
159 quantify the levels of vDNA we isolated genomic DNA from the cells at day 6 and performed
160 qPCR targeting the HIV-1 POL region. We found that cells treated with 100U/ml of IFN-α14
161 contained relatively lower levels of total vDNA compared to untreated and IFN-α2 treated cells
162 (Fig. 3A). Cellular input levels were normalized to copies of *GAPDH.* Reductions in cellular p24
163 and vDNA over 6 days of infection could also relate to antiretroviral restriction factor-mediated
164 production and release of defective virions. Therefore, we assessed if IFN-α14 treatment of

165 MT4C5 cells resulted in the production of fewer infectious HIV-1 virions. Supernatants from cells
166 treated with 100U/ml of IFN-α2 or IFN-α14 were isolated and tested for levels of infectivity per
167 pg of p24 using TZM-bl reporter cells, which contain a Tat-sensitive LTR promotor driving the β-
168 galactosidase gene. Individually infected cells can thus be quantified through the presence of β-
169 galactosidase activity. The infectivity of virus-containing supernatants isolated from cells treated
170 with 100U/ml IFN-α14 was significantly lower than the infectivity of HIV-1 in supernatants after
171 IFN-α2 treatment (Fig. 3B). The data show that IFN-α14 more potently reduced both HIV-1 vDNA
172 and viral infectivity than IFN-α2 in MT4C5 cells.
173
174 **CRISPR-Cas9-mediated knock out of MX2, APOBEC3G and ISG15 in MT4C5 cells.** At this
175 point in our investigations the antiretroviral restriction factors MX2, APOBEC3G and ISG15
176 previously shown to be either more highly expressed or activated by IFN-α14, remained as
177 candidates factors mediating IFN-α14's more potent control of HIV-1 infection(12, 31, 32). To
178 investigate the role of each factor further, we used the CRISPR-Cas9 method to knock out each
179 restriction factor in MT4C5 cells. Briefly, two to three guide oligos were designed for each gene
180 (Table 1) and individually cloned into a lentiCRISPRv2 backbone. Lentiviral vectors carrying the
181 guide sequences were then made by transfecting lentiCRISPRv2, psPAX and pMD2.G plasmids
182 into HEK293FT cells. The MT4C5 cells were then transduced with the each of the resultant
183 lentiviruses for 48h before plating them into selection medium. Successful MX2 and ISG15 gene
184 knock out (KO) was determined using immunoblots and APOBEC3G KO confirmed by PCR due
185 to a specific antibody being unavailable. A concentration of 1000U/ml of IFN-α2 was needed to
186 induce detectable levels of MX2 in WT MT4C5 cells whereas MX2 could be detected in cells
187 treated with either 100 or 1000U/ml of IFN-α14, albeit less so after stimulation with 100U/ml of

188 the cytokine. However, MX2 expression was not detected after stimulation with either IFN-α in
189 the MX2 KO cells (Fig. 4A). To detect ISG15 protein in MT4C5 cells 1000U/ml of both IFN-α
190 subtypes was used. However, no ISG15 was detected in KO cells after IFN-α stimulation (Fig.
191 4B). The APOBEC3 family is highly homologous at the protein level and thus there are no
192 commercially available antibodies that could specifically detect the APOBEC3G KO in MT4C5
193 cells. Thus, we used APOBEC3G-specific sequences in genomic DNA to detect the APOBEC3G
194 KO. Amplification of genomic DNA using APOBEC3G sequence-specific primers showed
195 amplification of a shorter band in APOBEC3G KO cells compared to genomic DNA from WT
196 MT4C5 cells. In summary, we were able to successfully knock out MX2, ISG15 and APOBEC3G
197 in MT4C5 cells in order to further evaluate the contribution of each antiretroviral restriction factor
198 to the more potent control of HIV-1 infection by IFN-α14 compared to IFN-α2.
199
200 **Deletion of APOBEC3G and ISG15 but not MX2 eliminated IFN-α14's more potent**
201 **control of HIV-1 compared to IFN-α2.** We next used our MX2, APOBEC3G and ISG15 KO cells
202 to evaluate their importance in IFN-α14's more potent control of HIV-1 compared to IFN-α2. To
203 evaluate whether IFN-α14's potency was dependent on APOBEC3G, ISG15 or MX2 each of the
204 KO cells as well as WT MT4C5 cells were treated with 100U/mL of either IFN-α2 or IFN-α14 as
205 performed earlier. As expected, IFN-α14 reduced levels of HIV-1 p24 in the cell supernatants
206 compared to IFN-α2 in the WT MT4C5 cells. Unexpectedly, IFN-α14 still reduced p24 levels in
207 supernatants significantly better than IFN-α2 in the MX2 KO cells (Fig. 5). However, supernatants
208 collected from APOBEC3G KO and ISG15 KO cells no longer contained different amounts of p24
209 regardless of which IFN-α treatment they received (Fig. 5). Therefore, the data suggested that the

210 restriction factors APOBEC3G and ISG15 have a role in the more potent control of HIV-1 by IFN-
211 α14 compared to IFN-α2, whereas MX2 does not.
212
213 **IFN-α14 more potently augmented APOBEC3G cytidine deaminase activity and reductions**
214 **in viral infectivity than IFN-α2 but did not affect vDNA levels**. Since HIV-1 vDNA levels and
215 viral infectivity were significantly reduced by IFN-α14 but not IFN-α2 treatment in MT4C5 WT
216 cells, we tested whether these effects were being mediated through the restriction factor
217 APOBEC3G. First, we assessed levels of vDNA in HIV-1 infected MT4C5 WT or APOBEC3G
218 KO cells treated for 6 days with either 100U/ml IFN-α14 or IFN-α2 by amplifying the HIV-1 POL
219 region from isolated genomic DNA as already described. Fold change was calculated from levels
220 of vDNA in untreated MT4C5 WT cells. As we had observed previously, IFN-α14 treatment
221 resulted in significantly lower amounts of vDNA in cells compared to IFN-α2 in MT4C5 WT cells.
222 However, knock out of APOBEC3G did not alleviate the effect (Fig. 6A). In contrast, when we
223 collected day 6 supernatants from MT4C5 WT and APOBEC3G KO cells and evaluated the
224 infectivity of HIV-1 per pg of p24 we observed that APOBEC3G KO restored the infectivity of
225 HIV-1 virions produced from the IFN-α14 treated cells to comparable levels as IFN-α2 treated
226 cells (Fig. 6B). Since the cytidine deaminase activity of APOBEC3G can contribute to reductions
227 in viral infectivity through deleterious mutagenesis of the vDNA we also assessed the frequency
228 of GG → AG signature mutations. We evaluated day 6 supernatants from MT4C5 WT and
229 APOBEC3G KO cells to induce signature mutations in target cells. Interestingly, the frequency of
230 GG → AG signature mutations in the target cells incubated with supernatants from MT4C5 WT
231 cells treated with IFN-α2 was not altered in the absence of APOBEC3G. Conversely, when treated
232 with IFN-α14, knock out of APOBEC3G resulted in a significant reduction in the frequency of

233 signature mutations in target cells (Fig. 6C). Overall, IFN-α14 more potently controlled HIV-1
234 than IFN-α2 through augmentation of APOBEC3G cytidine deaminase activity leading to the
235 production of more non-infectious HIV-1 virions.

236 **DISCUSSION**

237 Greater expression of restriction factors such as *ISG15*, and *MX2* and increased activity of
238 APOBEC3G in IFN-α14 treated humanized mice(12, 31) suggested that IFN-α14's potency may
239 be, at least in part, due to the contribution of specific restriction factors. The data presented in the
240 current study indicated that the potent HIV-1 suppression mediated by IFN-α14 is driven, at least
241 in part, cell intrinsically by ISG15 and APOBEC3G. We observed that the MT4C5 cells harboring
242 genomic deletion of *ISG15* and *APOBEC3G* showed no difference in viral p24 production when
243 treated with 100U/mL of IFN-α2 or IFN-α14 suggesting that ISG15 and APOBEC3G are
244 responsible for the more potent control of HIV-1 by IFN-α14. It further demonstrated that IFN-
245 α14 treatment not only reduced viral load but also resulted in the production of fewer infectious
246 particles. However, when we increase the dosage of both IFN-αs, the difference ceased to exist. A
247 study done by Schlaepfer and Fahrny et al. showed that at higher doses of IFN-α subtypes, reporter
248 gene induction in response to different IFN-α subtypes waned(45). One of the reasons could be
249 that at higher doses of IFN-α, the IFNAR1/2 receptors become saturated, meaning nearly all
250 receptor complexes are occupied regardless of subtype affinity. When that happens, downstream
251 signaling (e.g., STAT phosphorylation, ISG induction) and antiviral effects may reach a maximal
252 plateau, minimizing observable subtype-specific differences(46). Therefore, we used a
253 biologically relevant dose of 100U/ml(43) where differences in IFN-α responses between subtypes
254 become detectable.

255 IFN-α14 suppressed virus replication in HIV-1 infected MT4C5 cells. Although p24 was
256 detectable in the supernatant, a complete loss of p24 was observed in the cell lysates of the IFN-
257 α14–treated cells by day 6. This indicated that HIV-1 replication initially occurred following
258 infection, leading to viral production in the supernatant, but IFN-α14 treatment subsequently

259 slowed within cells over the 6 days. This was confirmed by amplifying the POL region of HIV-1
260 from genomic DNA isolated from cells. However, given that ELISA and PCR are more sensitive
261 than western blot, the absence of p24 detection by western blot does not rule out the presence of
262 cellular virions.

263 Restriction factors like MX2, APOBEC3G and ISG15 hinder HIV-1 replication at different
264 stages of HIV-1's lifecycle. MX2 and APOBEC3G act during earlier stages whereas ISG15 acts
265 during the later stages of HIV-1 replication. Since we saw a decrease in viral infectivity in the IFN-
266 α14 treated group compared to IFN-α2 we anticipated that the decrease in infectivity could be a
267 result of increased APOBEC3G activity, therefore we investigated APOBEC3G more thoroughly.
268 ISG15 on the other hand, is known to hinder HIV-1 by preventing ubiquitination of Gag and
269 Tsg101 which is important for viral budding and release(47). ISG15 also modifies a broad range
270 of cellular proteins, many of which are key components of the antiviral innate immune signaling
271 pathway(48). In future, experiments could be performed to determine if ISG15 is restricting HIV-
272 1 more potently by preventing ubiquitination of Gag and Tsg101 or by modifying other cellular
273 proteins after IFN-α14-treatment. A previous study(31), along with our own findings, concluded
274 that APOBEC3G activity is enhanced during IFN-α14 treatment. However, the mechanism
275 underlying the increased frequency of GG → AG hypermutations in the absence of elevated
276 APOBEC3G expression levels remains unclear. Others have shown that treatment with IFN-α
277 increases *APOBEC3G* mRNA levels 36 folds in a different cell line(49). However, in MT4C5 cell
278 line there was no elevation in *APOBEC3G* mRNA levels(12). Deletion of APOBEC3G did result
279 in comparable levels of HIV-1 cell supernatants between IFN-α treatment groups suggesting that
280 APOBEC3G has a role in IFN-α14's increased anti-HIV-1 potency. Apart from APOBEC3G,

281 deletion of ISG15 eliminated subtype specific differences in viral load in MT4C5 cells. However,
282 deletion of MX2 did not eliminate the subtype specific differences in HIV-1 control in cells.

283 A study showed that inactivation of multiple ISGs induced by IFN-β revealed a polygenic and
284 synergistic mechanism underlying HIV-1 control**(50).** Notably, deletion of MX2 did not reduce
285 IFN-α14's capacity to suppress HIV-1 replication, suggesting that other IFN-α14 induced
286 restriction factors can maintain antiviral activity. In polygenic systems, the effect of one protein
287 can be influenced by the abundance or activity of others, depending on their mode of interaction.
288 ISG15 functions late in the viral life cycle, blocking assembly and release(29, 30), whereas
289 APOBEC3G and MX2 act at earlier stages. When early acting factors such as APOBEC3G or
290 MX2 substantially limit the production of competent virions, ISG15 has fewer targets, making its
291 impact appear smaller; however, when early restrictions are weak, ISG15's role in blocking release
292 becomes more critical. Therefore, deletion of ISG15 may lead to a greater increase in viral
293 replication compared to deletion of MX2 under IFN-α14 treatment, as the antiviral effect of MX2
294 can be partially compensated by ISG15, whereas loss of ISG15 is less likely to be offset by other
295 restriction factors. It is plausible that the expression level of a compensatory restriction factor
296 influences HIV-1 replication. However, in our experiments, we did not measure the expression of
297 other restriction factors when a single factor was knocked out during HIV-1 infection. Future
298 studies involving the simultaneous knock out of ISG15, APOBEC3G, and MX2 could help reveal
299 synergistic interactions among restriction factors regulated by IFN-α14. Since the MT4C5 cell line
300 does not produce endogenous IFN-α, it served as a useful model to examine the exogenous effects
301 of IFN-α subtypes. However, not all IRG responses could be evaluated, as MT4C5 cells may
302 respond differently from primary CD4+ T cells. Therefore, future validation using primary CD4+
303 T cells would be useful to assess the physiological relevance of IFN-α subtype responses.

304 Here we demonstrated that IFN-α14, in contrast to IFN-α2, exerted a stronger effect on HIV-
305 1 replication and restriction factors like ISG15 and APOBEC3G play significant roles in the
306 differential effect between IFN-α2 and IFN-α14. However, the contribution of other restriction
307 factors like MX2 and BST2 cannot be ignored in the context of HIV-1 replication. Although in our
308 experimental setup these restriction factors (MX2 and BST2) may seem redundant, but their
309 contribution to control HIV-1 cannot be disregarded. In future, short-term experiments for 2 or 3
310 days with a fully established infection and IFN-α treatment could be done to evaluate the effect of
311 MX2 and BST2 on IFN-α14 potency.

312 **MATERIALS AND METHODS**

313 **Determination of IFN-α units**

314 A stable reporter cell line using human retinal pigment epithelial cells (ATCC CRL2302)
315 transfected with a plasmid containing interferon-stimulated response element promoter/enhancer
316 elements driving a luciferase reporter gene. Cells were grown for 24 h before adding serial
317 dilutions of recombinant IFN-α subtypes and a predetermined IFN-α lab standard for 6 h. The cells
318 were lysed with Pierce Firefly Luc One-Step Assay Buffer (Thermofisher, Waltham, MA), and
319 luciferase activity was measured 10 min later. The relative light units were plotted against the IFN-
320 α dilutions using non-linear fit with three parameters and EC50 was determined. Units of IFN-α
321 were calculated using EC50 values and known units/ μL of the standard.

322

323 **HIV-1 infection and IFN-α treatment in MT4C5 WT and KO cells**

324 MT4C5 cells were kindly donated by Dr. Oliver Schwartz (Institut Pasteur, Paris, France). MT4C5
325 is a $CD4^+$ T cell line expressing CCR5 and has little to no endogenous production of IFN-α(42).
326 MT4C5 WT cells were maintained in RPMI-1640 (Hyclone, Logan, UT) supplemented with 10%
327 FCS (Hyclone) and 1X PenStrep (Hyclone) (R-10) while KO made from MT4C5 cells were
328 maintained with R-10 supplemented with 5μg/mL puromycin (Invivogen, San Diego, CA). Cells
329 were either left untreated or treated with IFN-α2 or IFN-α14 (100U/mL, 500U/mL and
330 1000U/mL) for 24h and then infected with 0.1 MOI of HIV-1_{JR-CSF} (credit proper people off AIDS
331 Reagent) for 2 hours. Cells were then washed 2 times with complete media and plated in a 24 well
332 plate. Post-infection all the cells were maintained in either IFN-α2 or IFN-α14 at the initial
333 treatment dose.

334

335 **Guide RNA selection and cloning into lentiCRISPRv2**

336 Each guide sequence was designed using Synthego's CRISPR Design Tool. Guide sequences for
337 MX2 (ENST00000330714), APOBEC3G (ENST00000407997) and ISG15 (ENST00000379389)
338 were chosen from the list provided by Synthego's CRISPR Design Tool
339 (https://www.synthego.com/products/bioinformatics/crispr-design-tool). Location for cut site for
340 MX2 (41.376.931, 41.377.150 and 41.377.009), APOBEC3G (39.080.934, 39.080.990 and
341 39.081.088) and ISG15 (1.014.247 and 1.014.006) were chosen such that the fragment that would
342 be deleted would not be a multiple of three. DNA oligos (**Table 1**) for the guide sequences were
343 ordered from Integrated DNA Technologies.

344 The protocol for cloning was adapted from Zhang Lab(51). The complementary oligos were
345 phosphorylated using T4 PNK enzyme (New England Biolabs, Massachusetts, USA) and T4
346 Ligase buffer (Invitrogen, Carlsbad, California, USA) at 37°C for 1h. After phosphorylation, the
347 complementary oligos were heated to 95°C in water bath and cooled down gradually to room
348 temperature (22°C-25°C). LentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid #
349 52961 ; http://n2t.net/addgene:52961 ; RRID:Addgene_52961). LentiCRISPRv2 was digested
350 using *BsmBI-v2* and the 12kb backbone was gel extracted using QIAquick Gel Extraction Kit Gel
351 Purification (QIAGEN, Hilden, Germany). Oligos and the LentiCRISPRv2 backbone were ligated
352 overnight at 16°C. Each of the ligated plasmids were transformed into *Stbl2* cells (Invitrogen) and
353 were grown in freshly made LB Agar plates with 100µg/mL of ampicillin. Single colonies were
354 grown in 5mL of LB media supplemented with 100µg/mL of ampicillin. Plasmids were isolated
355 from transformed *Stbl2* cells using QIAprep Spin Miniprep Kit (QIAGEN) and PCR used to
356 confirm the presence of the insert.

357

358 **Lentivirus production and transduction**

359 HEK293 FT cells were maintained in DMEM (Gibco, Waltham, MA) supplemented with 10%
360 FCS, 1X PenStrep, 1X Sodium Pyruvate and 1X NEAA (DMEM complete media). Cells were
361 transfected with cloned LentiCRISPRv2 plasmid carrying oligos, pMD2.G and psPAX2 at a ratio
362 of (1:2:1) using MEM (Gibco) and Lipofectamine 2000 (Invitrogen). After 72h of transfection,
363 supernatant containing lentiviruses were collected and spun down at 1000 x g for 5mins to separate
364 cell debris. Lentiviruses for same restriction factor were then incubated with MT4C5 cells for 24h.
365 After 72h of transduction, freshly prepared selection medium (R-10 + 5 μg/mL puromycin) was
366 added to the culture. After two weeks of selection, cells were treated with IFN-αs to confirm the
367 KOs.

368

369 **Western blots**

370 Cells were counted and 2X SDS buffer was added to cells. The mixture was then heated at 95°C
371 for 10 mins and then spun at 13,000xg for 1 min. Samples were loaded onto a 12% SDS-PAGE
372 gel for 1.2 hrs. After protein transfer was done onto the nitrocellulose membrane, blocking was
373 done using 1X clear milk (Thermofisher). Membrane was then incubated with primary antibodies
374 **(Table 2)** overnight at 4°C and washed 5 times with 0.1% tween-20 containing 1XPBS for 5mins
375 each. Secondary antibodies **(Table 2)** were added and incubated at room temperature for one hour
376 and then washed with 5 times with 0.1% tween-20 containing 1XPBS for 5mins each.

377

378 **Genomic DNA isolation and PCR amplification**

379 Genomic DNA from different experimental groups was extracted using the QIAamp DNA minikit
380 (QIAGEN) kit, following the manufacturer's instructions. The APOBEC3G sequence flanking the

381 region targeted by guide RNAs was amplified using forward primer 5'-
382 GGATGAAGCCTCACTTCAGAAA-3' and reverse primer 5'-
383 CACAGGTAAGTCTCATGCCGT-3'. Determination of total HIV-1 DNA was performed by
384 quantitative real-time PCR using SsoAdvanced Universal SYBR Green Supermix (BIORAD,
385 Hercules, CA) targeting the POL region of HIV-1 with forward primer 5'-
386 ACTCCAGTATTTGCCATAAAGAAAAAA-3' and reverse primer 5'-
387 CTGTCTTTTTCTGGCAGCACTAT-3' and GAPDH with forward primer 5'-
388 GCTCTTAAAAAGTGCAGGGTCT-3' and reverse primer 5'-
389 GATGGTACATGACAAGGTGCG-3'.
390

391 **p24 enzyme linked immunosorbent assay**

392 Cell supernatants were collected at day 6 for p24 ELISA (Advanced Bioscience Laboratories,
393 Maryland, USA) following the manufacturer's instructions using a laboratory optimized
394 modification that halved the sample and reagent volumes used at each step. Plates were read in a
395 96 well plate reader at 450nm. Data acquisition was done using Softmax Pro (San Jose, CA).
396

397 **Infectivity Assay**

398 TZM-bl reporter cells (NIH AIDS Research and Reference Reagent Program, from John Kappes,
399 Xiaoyun Wu, and Tranzyme Inc.) were used to determine the number of infectious particles in the
400 supernatant. Briefly, cells were plated 24h prior to the assay in a 96-well plate. Supernatants were
401 10X serially diluted and incubated with cells for 2h. Cells were then washed and cultured with
402 fresh media for 48h. Cells were then fixed with 2% gluteraldehyde and X-gal solution was added

403 and plate was incubated at 37°C at 5% CO_2 for 24h. Spots were then counted at 4X magnification
404 and the infectivity was normalised to the p24 of the supernatants.
405
406 **Detection of APOBEC3G signature mutations**
407 Supernatants from MT4C5 WT or A3G KO treated with different IFN-α subtypes for 6 days were
408 incubated with target cells (A3G KO) for 48h. The POL region was amplified, gel purified using
409 QIAquick Gel Extraction Kit – Gel Purification (QIAGEN) and sent for sequencing
410 (Plasmidsaurus, South San Francisco, CA) to determine the ratio of GG→ AG mutations vs total
411 mutations.
412
413 **Statistics**
414 Data were plotted with mean and standard deviation. Normality of each dataset was evaluated by
415 Student's unpaired t-test or one-way ANOVA with Tukey's post-tests were performed accordingly
416 and are specified within figure legends for each data set. $P<0.05$ was considered statistically
417 significant. All statistics were performed using Prism 10 (GraphPad, San Diego, CA).
418

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556

557

558

559 **FIGURE LEGENDS**

560 **Figure 1. IFN-α14 more potently suppressed HIV-1 replication than IFN-α2 at similar**
561 **activity levels.** (A) The activities of IFN-α2 and IFN- α14 were determined by titration of each
562 subtype on an IFN-α responsive reporter cell line in comparison to an IFN-α stock of known
563 activity. (B) HIV-1 p24 ELISA of supernatants collected from MT4C5 cells treated with 0, 100,
564 500 or 1000U/ml of IFN-α2 or IFN-α14 for 6 days starting 2 hours post infection. One-way
565 ANOVA with Tukey's multiple comparison test. **** $p<0.0001$

566

567 **Figure 2. IFN-α14 more potently reduced cellular p24 levels in MT4C5 cells than IFN-α2.**
568 (A) HIV-1 p24 immunoblot of MT4C5 cell lysates previously treated for 6 days with IFN-α2 or
569 IFN-α14. GAPDH loading control. (B) Quantification of the p24 immunoblot normalizing the
570 intensity of the p24 bands to GAPDH.

571

572 **Figure 3. IFN-α14 more potently reduced HIV-1 viral DNA (vDNA) and HIV-1 infectivity in**
573 **MT4C5 cells than IFN-α2.** (A) Fold change in HIV-1 vDNA levels in MT4C5 cells differentially
574 treated with IFN-α upon amplification of the POL region from isolated genomic DNA. Cellular
575 input was normalized to *GAPDH*. One-way ANOVA with Tukey's multiple comparison test **
576 $p<0.01$; **** $p<0.0001$. (B) Tissue culture infectious units (TCIU) per pg of input HIV-1 p24 was
577 determined by TZM-bl infectivity assay using supernatants from MT4C5 cells treated with
578 100U/mL of IFN-α2 or IFN-α14. Student's unpaired t-test * $p<0.05$.

579

580 **Figure 4. The MX2, APOBEC3G and ISG15 antiretroviral restriction factors were**
581 **successfully knocked out of MT4C5 cells using CRISPR-Cas9.** MT4C5 cell lysates of IFN-α

582 treated wild-type (WT), MX2 knockout (KO) and ISG15 KO cells were run on a 14% SDS PAGE
583 gel. The proteins were then transferred to a nitrocellulose membrane and immunoblotted for (A)
584 MX2 and (B) ISG15. GAPDH was used as a loading control. (C) PCR amplification product of a
585 461bp region of APOPEC3G (A3G) expected to be disrupted in A3G KO cells compared to WT
586 using APOBEC3G sequence specific primers.
587
588 **Figure 5. Knock out of APOBEC3G (A3G KO) and ISG15 in MT4C5 cells eliminated**
589 **differential control of HIV-1 by IFN-α subtypes.** HIV-1 p24 ELISA of supernatants collected
590 from HIV-1 infected MT4C5 (WT), A3G KO, ISG15 KO and MX2 KO cells after 6 days of
591 treatment with either 100U/ml of IFN-α2 or IFN-α14. Student's unpaired t-test * $p<0.05$.
592
593 **Figure 6. Deletion of APOBEC3G did not alter vDNA in cells but restored infectious particles**
594 **and frequency of GG→ AG signature mutations.** (A) Fold change in HIV-1 vDNA levels in
595 MT4C5 WT and A3G KO cells differentially treated with IFN-α upon amplification of the POL
596 region from isolated genomic DNA. Cellular input was normalized to *GAPDH*. (B) TCIU per pg
597 of input HIV-1 p24 was determined by TZM-bl infectivity assay using supernatants from MT4C5
598 WT and A3G KO cells treated with 100U/mL of IFN-α2 or IFN-α14. (C) Amplified POL region
599 from vDNA of target cells were amplified and sequenced and GG→AG mutations were plotted
600 against total mutations. Student's unpaired t-test * $p<0.05$; ** $p<0.01$.

601 **TABLES**

602 **Table 1. Guide DNA oligo sequences**

Gene	Location	Oligo Sequence (5'-3')
MX2	41.376.931	**CACCG**CTTCTCCTCCGGTAGGGCCA
		AAACTGGCCCTACCGGAGGAGAAGC
	41.377.150	**CACCG**ACCGGGCTTACCATTGCCCT
		AAACAGGGCAATGGTAAGCCCGGTC
	41.377.009	**CACCG**GGCACTGTGCCTAATGGCGG
		AAACCCGCCATTCGGCACAGTGCCC
APOBEC3G	39.080.934	**CACCG**CTTAAGTTCGGAATACACCT
		AAACAGGTGTATTCCGAACTTAAGC
	39.080.990	**CACCG**TCCACTGGTTCAGCAAGTGG
		AAACCCACTTGCTGAACCAGTGGAC
	39.081.088	**CACCG**TAACCTTCGGGTCCTCGGCC
		AAACGGCCGAGGACCCGAAGGTTAC
ISG15	1.014.247	**CACCG**ATCCTGGTGAGGAATAACAA
		AAACTTGTTATTCCTCACCAGGATC
	1.014.006	**CACCG**GGACCTGACGGTGAAGATGC
		AAACGCATCTTCACCGTCAGGTCCC

603

604

605 **Table 2. Antibody dilutions**

Sl. no.	Antibody	Host	Dilution	Primary/Secondary
1.	Hu-MX2	Mouse	1:100	Primary
2.	Hu-ISG15	Mouse	1:1000	Primary
3.	Hu-GAPDH	Rabbit	1:10000	Primary
4.	m-IgG	Mouse	1:10000	Secondary
5.	m-IgG	Rabbit	1:10000	Secondary

606

FIGURES

Figure 1

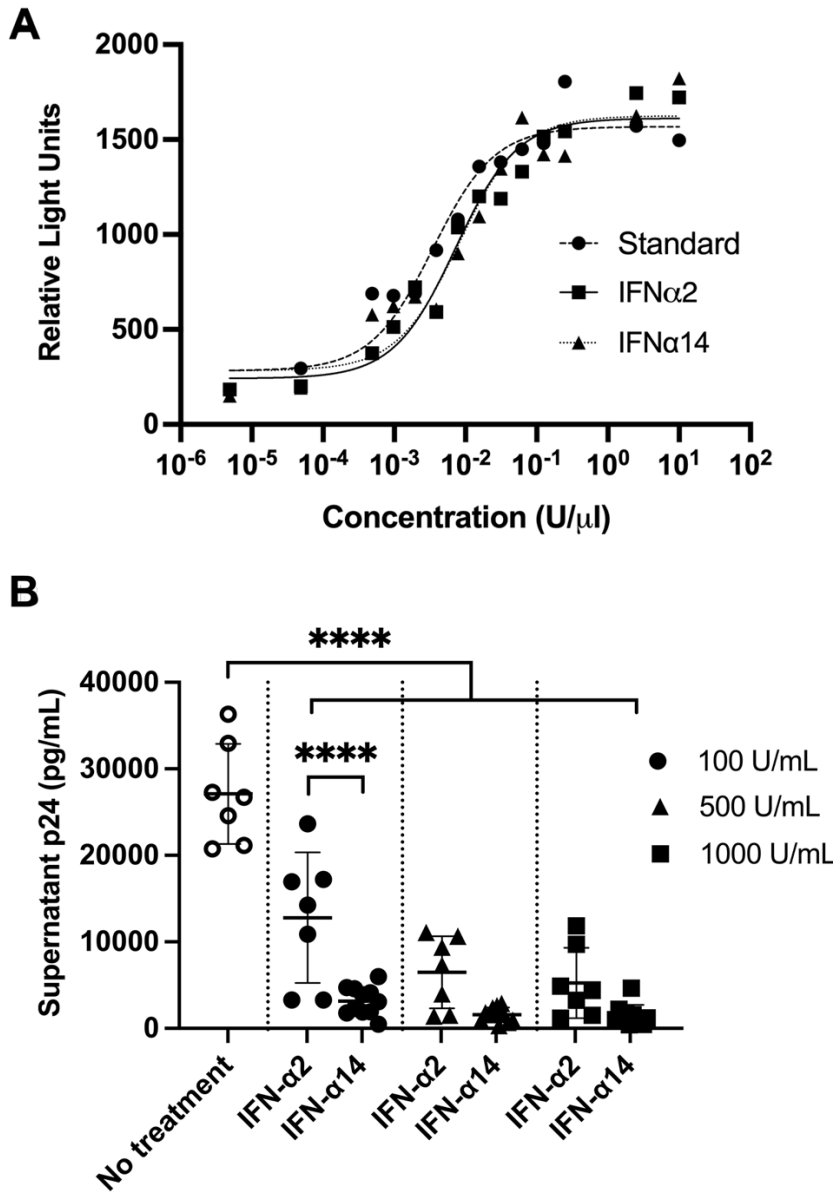


Figure 2

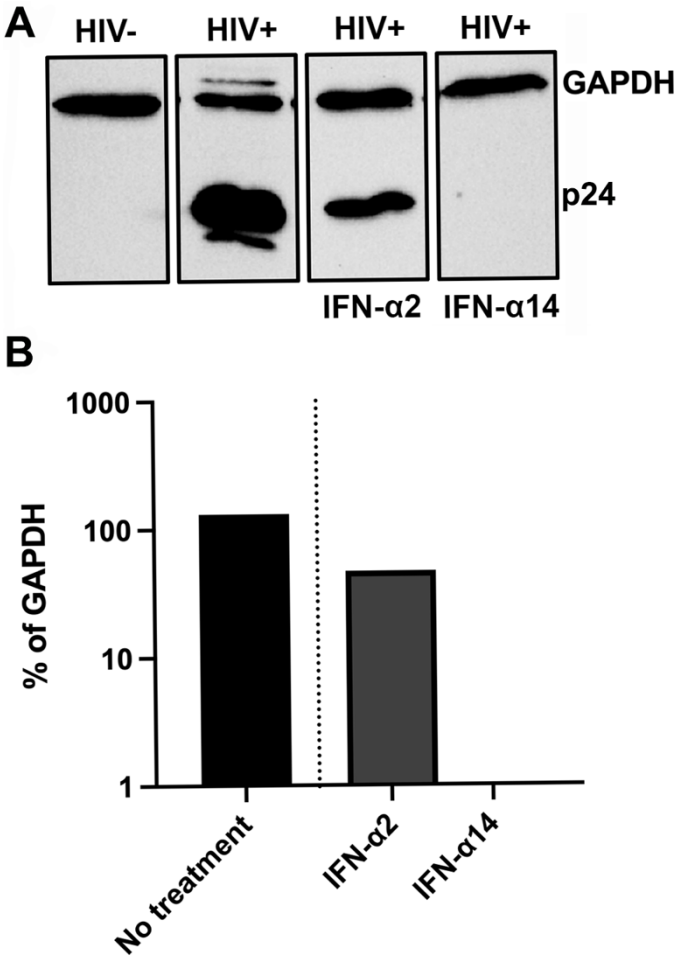
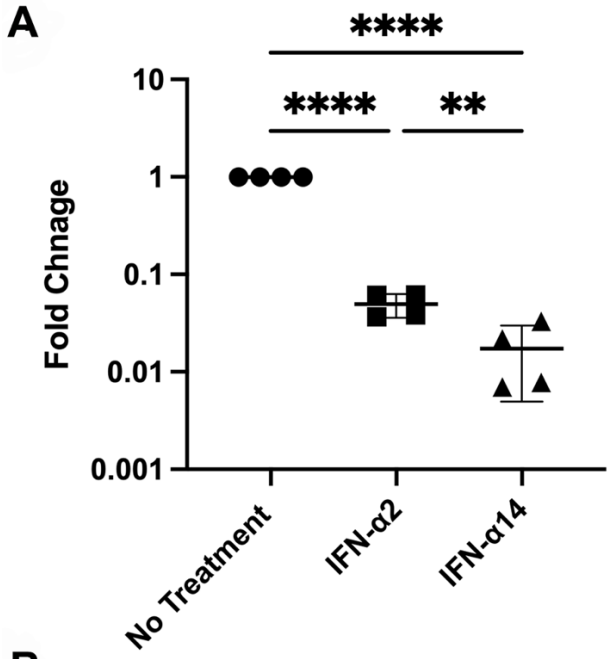

A
HIV-
HIV+
HIV+
HIV+
GAPDH
p24
IFN-α2
IFN-α14
B
1000
100
10
1
% of GAPDH
No treatment
IFN-α2
IFN-α14

Figure 3


A
****
****
**
10
1
0.1
0.01
0.001
Fold Chnage
No Treatment
IFN-α2
IFN-α14

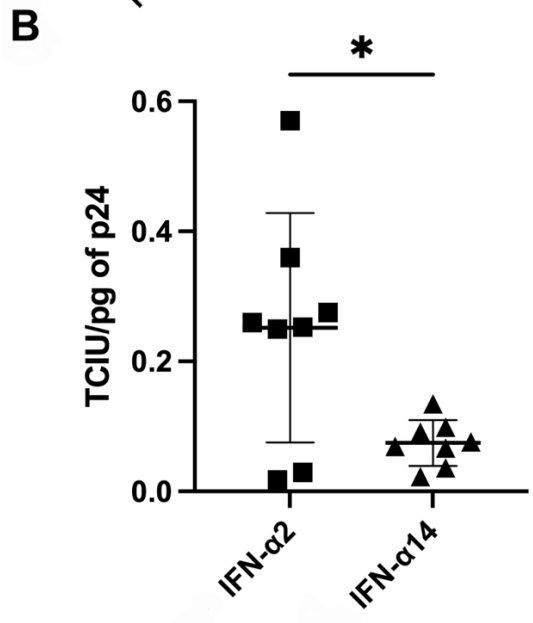

B
*
0.6
0.4
0.2
0.0
TCIU/pg of p24
IFN-α2
IFN-α14

Figure 4

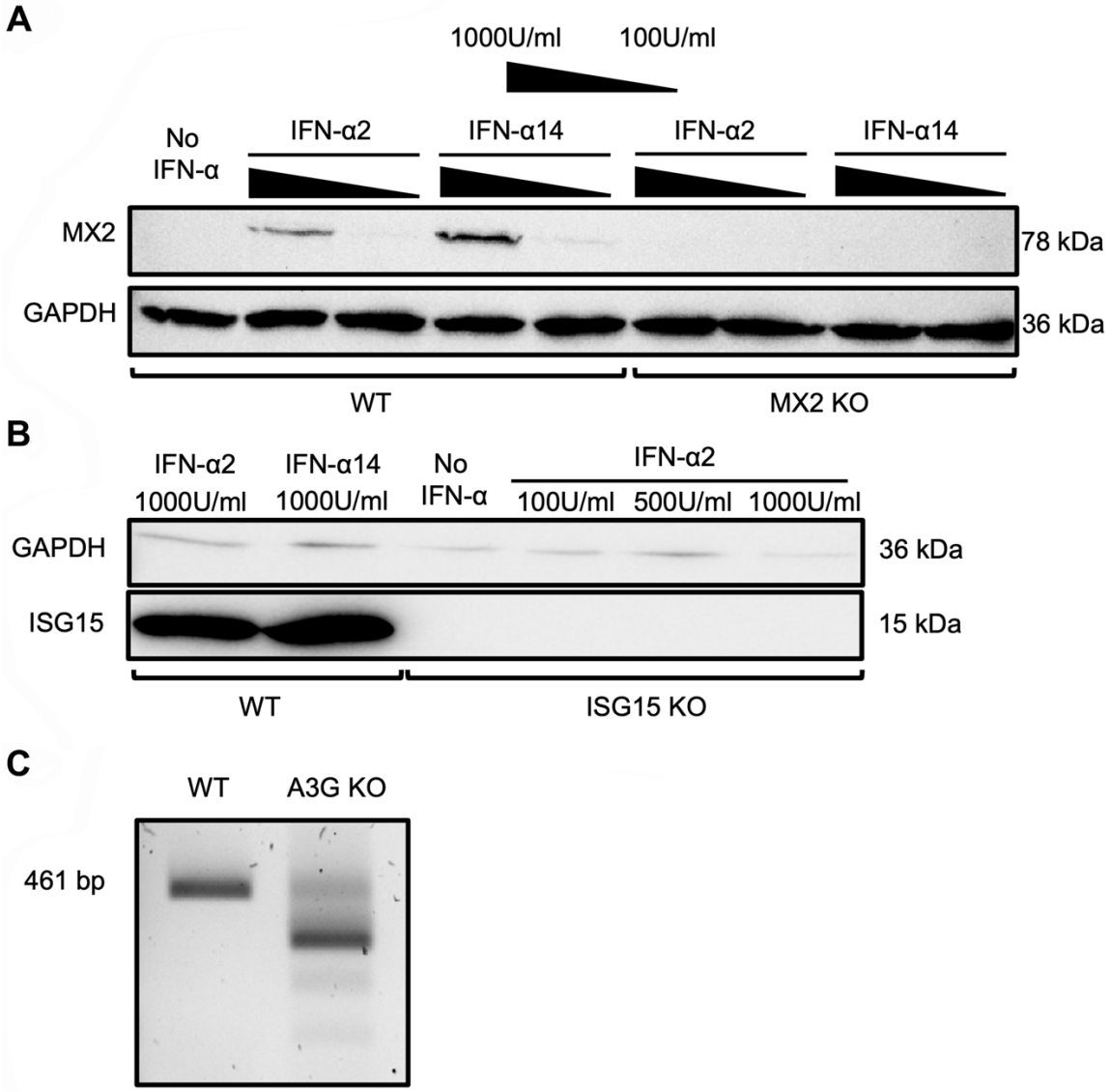
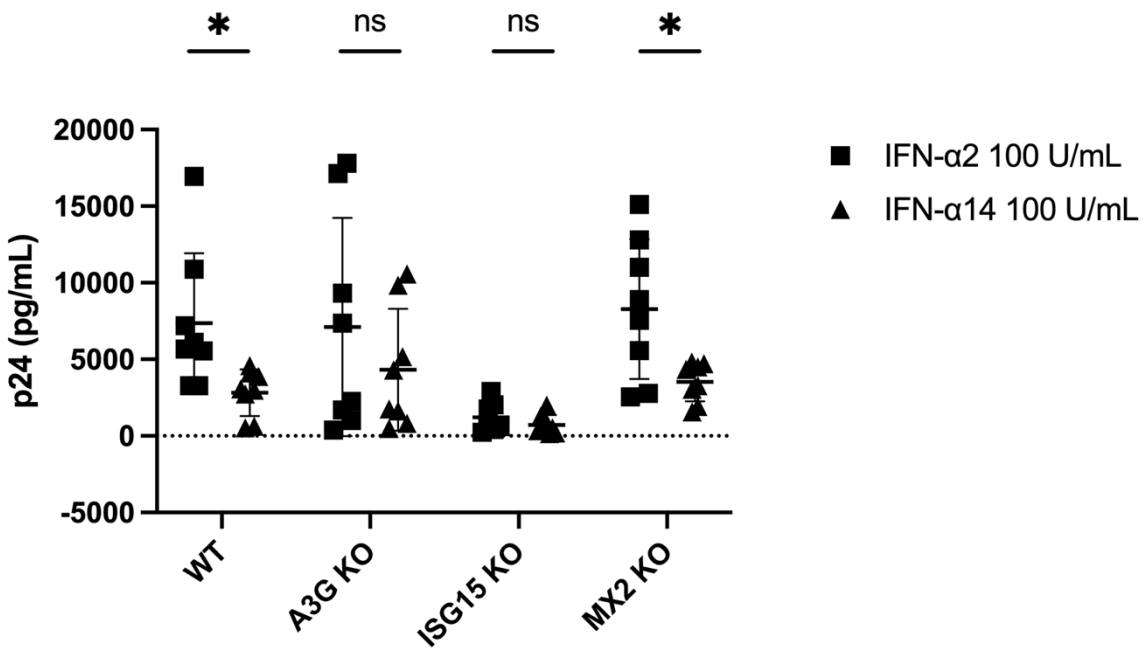
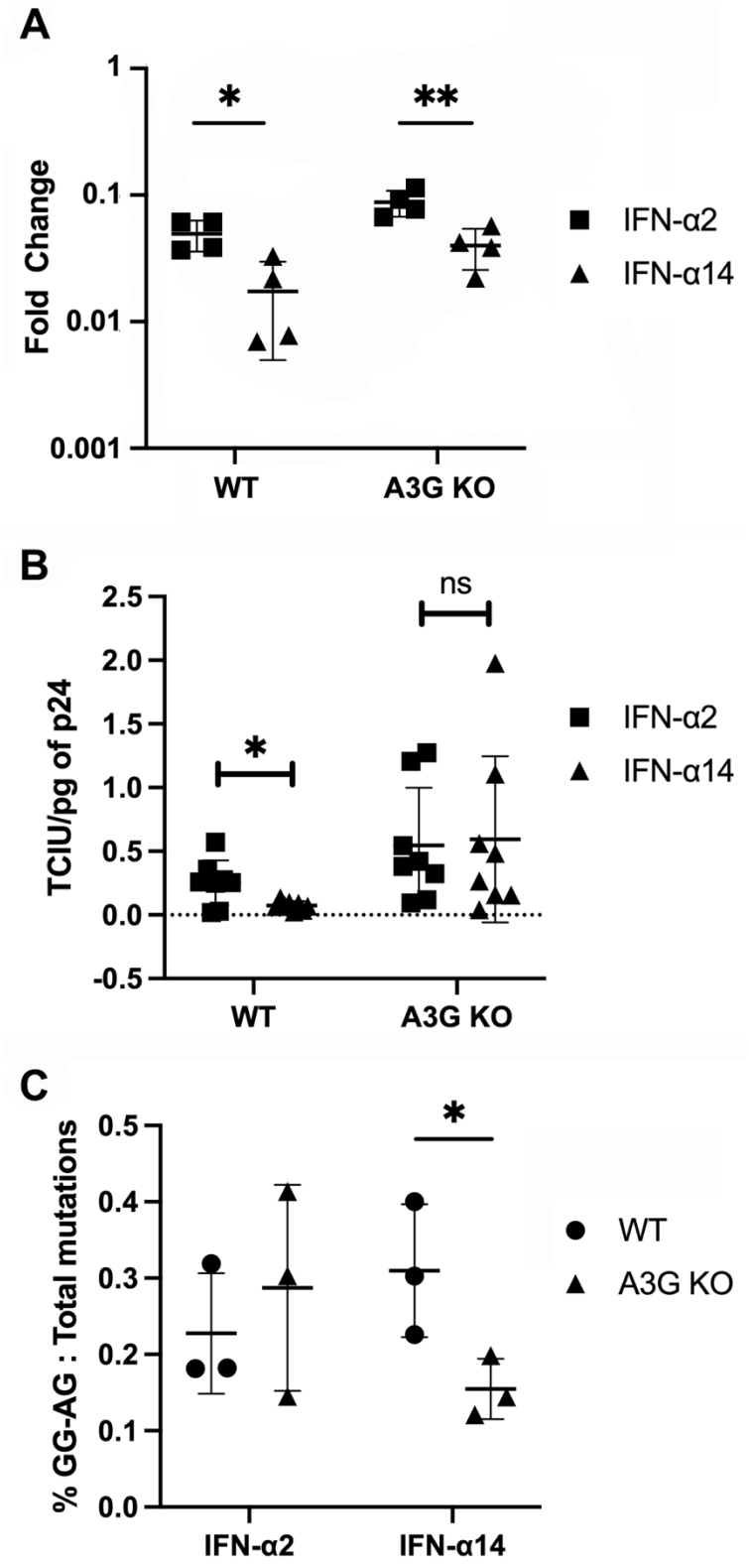

A
1000U/ml
100U/ml
No IFN-α
IFN-α2
IFN-α14
IFN-α2
IFN-α14
MX2
78 kDa
GAPDH
36 kDa
WT
MX2 KO
B
IFN-α2 1000U/ml
IFN-α14 1000U/ml
No IFN-α
IFN-α2
100U/ml
500U/ml
1000U/ml
GAPDH
36 kDa
ISG15
15 kDa
WT
ISG15 KO
C
WT
A3G KO
461 bp

Figure 5


*
ns
ns
*
20000
15000
10000
5000
0
-5000
p24 (pg/mL)
WT
A3G KO
ISG15 KO
MX2 KO
IFN-α2 100 U/mL
IFN-α14 100 U/mL

5

Figure 6


A
Fold Change
1
0.1
0.01
0.001
*
**
WT
A3G KO
IFN-α2
IFN-α14
B
TCIU/pg of p24
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
ns
*
WT
A3G KO
IFN-α2
IFN-α14
C
% GG-AG : Total mutations
0.5
0.4
0.3
0.2
0.1
0.0
*
IFN-α2
IFN-α14
WT
A3G KO