HIV-1 derived replication intermediates/oligonucleotides induce a type I IFN-dependent immune suppression via STING activation that can be restored by targeting IFNARI

Cecilia Svanberg[1], Ravi Prasad Mukku[1], Özkan Besler[1],Francis R. Hopkins[1], Christopher Sjöwall[2], Sofia Nyström[1#], Esaki M. Shankar[3#], Marie Larsson[1]

[1]Molecular Medicine and Virology, Department of Biomedical and Clinical Sciences, Linköping University, 581 85 Linköping, Sweden.

[2]Inflammation and Infection, Department of Biomedical and Clinical Sciences, Linköping University, 581 85 Linköping, Sweden.

[3]Infection and Inflammation, Department of Biotechnology, School of Integrative Biology, Central University of Tamil Nadu, Thiruvarur 610 005, India

[†]Correspondence: Marie Larsson, Molecular Medicine and Virology, Lab 1 Plan 13, Department of Biomedical and Clinical Sciences, Linköping University 58 185 Linköping, Sweden. Tel.: +46 1328 2987; Email: marie.larsson@liu.se

# = equal contribution

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Abstract

The hallmark of HIV-1 infection is the progressive development of multicellular and systemic immune dysfunction, culminating in AIDS. Dendritic cells (DCs) play a pivotal role in HIV dissemination to CD4+ T cells, which are subsequently depleted by the virus leading to HIV disease progression. Type I interferons (IFNs) are critical for host defense during acute infection but contribute to chronic immune activation during the later stages of HIV disease. This persistent activation leads to immune cell exhaustion. HIV-1 can activate type I IFN responses via several pathways, including the STING pathway, which is activated by e.g., virus-derived oligonucleotides. Here, we investigated the underlying mechanisms creating HIV-1-mediated immune dysfunction and role of type I IFNs using a DC and T cell co-culture model. HIV-1 exposure in the DC-T cell co-culture promoted the expansion of suppressive T cells with diminished proliferation and effector functions. The impairment required type I IFNs and subsequent IFN-α/β receptor signaling, initiated by HIV-derived ssDNA activation of IFI16/cGAS followed by STING signaling in the DCs. Targeting IFNAR1 with Anifrolumab restored the immune functions of both DCs and T cells, as well as T cell proliferation and T cell effector functions, including their secretion of IL-2, IFN-γ, and granzyme B. Our findings support that the immune impairments existing in untreated or antiretroviral therapy (ART) treated HIV-infected individuals are mediated, if not fully in part by type I IFN's negative effect on DC and T cells. Therapeutics targeting IFN-α/β receptors, such as Anifrolumab, hold potential as combination treatment alongside ART, to achieve a more complete immune restoration and contribute to improved quality of life among people living with HIV.

45 # Introduction

46 Over three decades since its discovery, human immunodeficiency virus type 1 (HIV) infection
47 continues to be a major global public health challenge. As of 2022, more than 38 million
48 individuals live with HIV, with ~1.3 million new infections reported (UNAIDS, 2022).
49 Encouragingly, the incidence of new infections is declining, attributed to that over 75% of the
50 people living with HIV-1 have access to antiretroviral therapy (ART). The therapy provides
51 effective control of viral replication and limits the negative effect HIV exerts on the immune
52 system and general health (WHO, 2022).
53 Dendritic cells (DCs) are pivotal players in the initiation and regulation of immune responses,
54 as the functionality of these cells in the lymphoid tissues determines the quality of the ensuing
55 immune response[1,2,3]. DCs are among the first immune cells targeted by HIV upon infection of
56 the genital mucosa, where they reside within the mucosal epithelium[3]. The mucosal DCs are
57 less permissive to infection than CD4+ T cells, but relatively more permissive than blood
58 DCs[4,5]. Still, the DCs exhibit remarkable efficiency in facilitating HIV infection while in cellular
59 contact by transferring virions to the interacting T cells even in the presence of ART[6,7]. Of note,
60 in HIV-infected individuals, DCs in lymphoid tissues can serve as a source of latently infected
61 cells despite years of suppressive ART. These lymphoid located myeloid DCs also express
62 programmed death-1 ligand 1 (PD-L1) in combination with an activated phenotype, which
63 might impair the activation of HIV-specific T cells[8,9].

64 Chronic HIV-1 infection is characterized by profound immune dysfunction and systemic
65 immune activation. The immune activation is driven by several factors, and prolonged and
66 elevated type I IFN levels are detrimental to immune responses during chronic HIV infection,
67 as previously demonstrated both in HIV and simian immunodeficiency virus (SIV) infections,
68 where type I IFN signaling contributes to disease progression[10,11]. Many immune cells,
69 including T cell and DC subsets, are functionally impaired in HIV infected, with altered cytokine
70 profiles, activation status, and elevated levels of immune checkpoint molecules, caused by
71 direct HIV effects or by virus-induced bystander effects[12-15]. Importantly, the impaired T cell
72 function in HIV-infected individuals with or without ART can be partially restored through *ex*
73 *vivo* blockade of immune checkpoint molecules[16]. Anifrolumab, a fully human immunoglobulin
74 G1κ monoclonal antibody that binds to the type I interferon receptor subunit 1 and inhibits

75 signaling by all type I interferons, is approved for moderate-to-severe systemic lupus
76 erythematosus since 2021[17].

77

78 We have demonstrated that during the interaction between mature DCs and T cells in the
79 presence of HIV, the virus induces impaired responses with expression of e.g., PDL1 and
80 galectin-9 on DCs, and programmed death-1 (PD-1) and CTLA4 on T cells[18-20], which is in line
81 with findings in chronic HIV infection[14,21]. In addition, we demonstrated that the
82 transcriptome profile of HIV-exposed DCs engaged in crosstalk with T cells exhibit a robust
83 prolonged type I IFN signature[20].

84 During retroviral and DNA virus infections, oligonucleotides are generated at various stages of
85 the viral life cycle and in the context of HIV an array of replication intermediates such as
86 ssDNA, dsDNA, RNA:DNA hybrids be found in the cytosol[22,23]. These viral nucleic acids are
87 recognized by innate immune sensors such as retinoic acid-inducible gene I (RIG-I), which
88 detects RNA:DNA hybrids[24], and cyclic GMP-AMP synthase (cGAS) and IFN-inducible protein
89 16 (IFI16), that trigger type I IFN responses via the stimulator of interferon genes (STING)
90 pathway[22,23,25]. In DCs, the intracellular detection of HIV has been deemed to be dependent
91 on cGAS [26,27]. In addition to cytosolic sensing, in endosomal compartments containing TLR8 in
92 myeloid DCs and TLR7 and TLR9 in plasmacytoid DCs, the degraded virus is detected even in
93 the absence of productive viral replication[28,29]. The activation of the STING pathway and
94 subsequent expression of type I IFNs has been associated with the induction of
95 immunoregulatory factors such as indoleamine 2, 3 dioxygenase (IDO), PD1, PDL1, and PDL2,
96 contributing to the suppression of T cell proliferation and a general immunosuppressive
97 microenvironment[30,31].

98 To investigate the role of type I IFN in suppressive responses induced by HIV-exposed DCs, we
99 examined the HIV replication intermediates driving type I IFN activation and the signaling
100 pathways involved in immune modulation/impairment. To achieve this, we utilized a HIV
101 exposed or unexposed mature DC-T cell model of immune activation, mirroring immune
102 response initiation occurring in lymphoid tissue in an HIV-infected individual.

103 Our results show that HIV suppressed the DCs and T cells, independent of the HIV-1 M strains
104 used (BaL vs THRO), and that there was a strong and prolonged type I IFN response in HIV-

exposed co-cultures. HIV replication intermediates/oligonucleotides participated in the HIV-induced suppression, with ssDNA being the most potent and the IFI16/cGAS/STING signaling pathways the main signaling pathways contributing to the induction of suppressive responses. The negative effects on DCs and T cells in the co-culture were reverted by addition of the anti-IFNAR1 antibody Anifrolumab, blocking the IFNaR1 signaling pathway. Therapies targeting IFNα/β receptors, such as Anifrolumab, hold potential as combination treatment alongside ART to accomplish complete immune restoration and thereby improve health in people living with HIV.

114 # Introduction

115 ## Cell culture media and reagents

116 Cell culture was conducted in RPMI1640 medium (ThermoFisher, Stockholm, Sweden)
117 supplemented with Gentamicin (ThermoFisher), HEPES (ThermoFisher), and either 5% pooled
118 human serum (PHS) (Innovative Research, Novi, USA) or 1% single donor human plasma. The
119 ssDNA, dsDNA, various DNA loops, and DNA:RNA hybrids (Invivogen, Toulouse, France),
120 derived from sequences described by Jakobsen et al.[23] **(Supplementary Table 1)**, were diluted
121 in pH adjusted potassium chloride buffer and added to the cells in amounts ranging from 2 to
122 16 µg. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF)
123 (Prepotech, London, UK) at 100 IU/mL and recombinant human interleukin-4 (rhIL-4)
124 (Prepotech) at 300U/mL were utilized to differentiate monocytes into DCs *in vitro*. To
125 investigate the STING signaling pathway, the following reagents and concentrations were
126 employed: cGAMP (10µg/mL, Invivogen), MSAD2 (33µM, MedChemExpress Europe,
127 Sollentuna, Sweden), ADS100 (10µM, MedChemExpress), SN011 (1 µM, MedChemExpress),
128 5'pppRNA (1µg/mL, Invivogen), RIG012 (1.25 µM, Axon Medchem, Groningen, Netherlands),
129 and VACV70 (10µg/mL, Invivogen). Additional chemicals and compounds used included CL075
130 (1µg/mL, Invivogen), recombinant p24 (1µg/mL, Invivogen), and IFNAR blockade using
131 Anifrolumab at 20 µg/mL, as a kind gift from AstraZeneca.

132

133 ## Dendritic cell propagation

134 Whole blood or leukocyte preparations from healthy volunteers were processed using a Ficoll-
135 Paque™ (Amersham Pharmacia, Piscataway, NJ, USA) density gradient. The PBMCs were
136 subjected to sequential centrifugation at 1800, 1500, 1100, and 900 RPM for 10 minutes each
137 at 4°C. After centrifugation, the cells were counted and plated at a density of 40 million cells
138 per plate in 10 mL of 5% PHS. The plates were incubated at 37°C for 1–2h to allow monocyte
139 adherence. Subsequently, non-adherent cells were removed, and the plates were washed.
140 The remaining adherent monocytes were cultured in 1% plasma medium and differentiated
141 into monocyte-derived DCs by adding GM-CSF and IL-4 every other day for 5 days. On day 5,
142 phenotype was investigated by flow cytometry to exclude undifferentiated cells by CD14
143 expression and spontaneously activated cells by CD83 expression. The maximum cut-off for
144 both markers was set to ~15%.

145

146 ## Maturation of dendritic cells

147 Following the cut-off selection for viable cells, moDCs were harvested, counted, and re-seeded
148 in 10 mL of the same conditioned culture medium used during differentiation, at a density of
149 4 million cells per plate. The TLR3 ligand poly I:C (3ng/ml: Invivogen) was then added to the
150 cultures, and the moDCs were incubated at 37°C for 24h to promote cell maturation.

151 ## HIV infection and oligo transfection of moDCs

152 After 24h of maturation CCR5-tropic HIV BaL 4238 or HIV THRO A66 4393 was added in a
153 concentration of 750 ng/mL and DCs were incubated another 24h. For intracellular addition
154 of nucleotides, proteins, and antagonists/agonists the N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-
155 trimethylammonium methylsulfate (DOTAP) (Merck, Darmstadt, Germany) liposomal
156 transfection system was used. An amount of 30µl of DOTAP was mixed with 70µl of HBSS
157 buffer (0.9% NaCl+ 10mM HEPES) and thereafter the oligos or proteins was added in indicated
158 concentrations diluted in 50µl of HBSS. The combined mixture was then incubated for 30 min
159 at room temperature. The whole volume was added dropwise to 0.5-2 million resuspended
160 mature DCs in 100 µl of culture media. Thereafter the cells were incubated for 2h at 37°C,
161 spun down and washed to remove the DOTAP mixture and resuspended in conditioned media
162 at a concentration of 1 million cells/mL.

163 ## Naïve T cell enrichment and set up of allogenic co-cultures

164 Nonadherent cells from the DC propagation were counted and incubated with 20 µl of buffer
165 and 10µl of each MACS antibody (anti-CD14, CD56, CD19, and CD45R0) (Miltenyi, Stockholm,
166 Sweden) per 10 million cells for 15 min at 4°C. The labelled cells were washed and then passed
167 through an LD-column attached to a magnet for the negative selection of bulk naïve T cells.
168 After collection of total effluent, the naïve T cells were counted and resuspended to 1 million
169 cells/mL in 5% PHS.

170 ## DC-T cell co-culture

171 The mature DCs exposed to mock, HIV, transfected, or exposed to agonist/antagonists were
172 harvested and resuspended in 5% PHS. The different DC groups were divided, with one portion
173 frozen down in freezing media and stored at -80°C and the other portion plated in a flat
174 bottomed 96-well cell culture plate. Naïve T cells were added to the different DC groups at a

175 ratio of 1:10 and the DC-T cell co-culture incubated in a CO_2 incubator at 37°C for 7 days. After
176 7 days, the DC-T cell co-cultures were re-stimulated with the corresponding DC group and left
177 overnight until harvest.

178 ELISPOT and ELISA measurements

179 For ELISA measurements of type I IFN released into the supernatant a PAN IFNα ELISA kit
180 (Mabtech, Stockholm, Sweden) and an IFNβ ELISA construction kit (Antigenix America inc,
181 New York, NY, USA) was used. Supernatants harvested on day 8 were either diluted 1:5 or
182 used neat. For the ELISA, plates were coated with (1µg/mL for IFNβ and 4µg/mL for IFNα)
183 capture antibody overnight, washed and blocked in 0.05% Tween 20 and 0.1% BSA for 60 min.
184 Samples and standard were added to the plate after washing and incubated for 2h at RT. The
185 biotin tracer antibody was thereafter added in a concentration of 0.5-1µg/mL and incubated
186 for 1h at RT. Streptavidin-HRP was added in 1:1000 dilution for 30 min at RT before addition
187 of TMB substrate. The reaction was terminated after 15 min by the addition of 0.2M H_2SO_4
188 and the plates read at 450 nm using a SpectraMax iD3 microplate reader (Molecular devices,
189 San Jose, CA, USA). For ELISPOT assays cells from the coculture were counted on day 7 and
190 replated onto pre-coated plates for human granzyme B, and IFNγ (Mabtech). For IL2 PVDF
191 plates were coated in house by addition of 10µg/mL of capture antibodies (Mabtech).
192 Thereafter DCs were added at 1:10 ratio to each well and plates were incubated at 37°C
193 overnight. Spots were detected using biotin antibodies (0.25-0.5µg/mL) and streptavidin-HRP
194 (1:1000 dilution). TMB was used to develop the color and the reaction stopped by washing
195 with deionized H_2O and spots were counted manually in a light microscope.

196 ^{3}H-thymidine incorporation proliferation assay

197 To measure T cell proliferation in the DC- T cell co-culture during restimulation, 2µCi/µl of ^{3}H-
198 thymidine (PerkinElmer, Waltham, MA, USA) was added following the supplement of the DCs.
199 Wells containing RPMI and no cells were used for background counts. The amount of
200 incorporated ^{3}H-thymidine was measured using liquid scintillation and counted using a micro-
201 β-counter (PerkinElmer).

202 Statistics

203 For data normalized to mock equals 1, a Kruskall-Wallis test followed by Dunn's multiple
204 comparisons test was performed. In the case of qPCR, where the sum of values from each

205 donor was set to 100%, a One-way ANOVA with Tukey's post-hoc test was performed. All tests
206 were done using GraphPad Prism version 9.2.1.

207 # Results

208 **HIV-1 BaL and HIV-1 THRO had similar suppressive effects on the DC priming of naïve T cells**

209 To investigate whether the presence of HIV-1 during the DC priming and restimulation of T
210 cells affected the ensuing immune response we co-cultured naïve T cells with DCs exposed to
211 different strains of CCR5-tropic HIV-1, either HIV-1 BaL a late-stage derived lab adapted strain
212 or HIV-1 THRO a transmitted/founder strain. Our analysis revealed a significant suppression
213 of T cell proliferation **(Figure 1A)**, and IL-2 production **(Figure 1B)** for both HIV-1 strains but no
214 significant differences in outcomes between the two strains**.** There were elevated gene
215 transcripts of DC and T cell immunomodulatory factors **(Figure 1C)** for both HIV-1 strains, but
216 no significant differences observed in outcomes besides significantly higher PDL1 in HIV-1
217 THRO compared to HIV-1 BaL. Next, we explored induction of type I IFN responses and ISGs in
218 the DC-T cell co-cultures **(Figure 1D)** and discovered an upregulation of IFNα4 and several ISGs
219 by both HIV-1 strains.

220 **Pathways inducing type I IFNs and type IFN responses were enriched and predicted to be**
221 **activated when HIV was present in DC-T cell co-culture and exogenous type I IFN resulted in**
222 **suppressed T cell responses similar to HIV**

223 To establish pathways involved in HIV suppression, we examined top regulator networks in an
224 RNA sequencing data set from a previous study on HIV exposed DCs from DC-T cell co-
225 culture[20]. Therein, we found a strong activation of intracellular sensors involved in type I IFN
226 signaling and responses, (e.g., sGAS, STING, and MAVS), IFN regulatory transcription factors
227 (e.g., IRF3, IRF9, and STAT1), type I IFNs, and IFNAR1 and IFNAR2 **(Figure 2A).** Therefore, to
228 explore if type I IFN played a key role in the induced T cell suppression in our system, we added
229 recombinant type I IFNα2 protein to the DC-T cell co-cultures. The IFNα2 induced suppressed
230 T cell responses were similar to those induced by HIV in the DC-T cell co-culture **(Figure 2B),**
231 even at concentrations similar to the levels detected in the DC-T cell co-culture exposed to
232 HIV-1 **(Figure 1D and Figure 2B)**.

233 **HIV-derived oligonucleotide ssDNA participated in the induction of the suppressive effects**
234 **seen by HIV in the DC-T cell co-culture**

235 We explored which of the HIV-1 derived oligonucleotides or proteins produced during the viral
236 life cycle that could be involved in the HIV-induced immune impairment of the DCs and T cells
237 during priming. The ssDNA, dsDNA, DNA:RNA hybrid, many loops ssDNA, and p24 were
238 delivered intracellularly into the DCs by DOTAP transfection **(Figure 3)**. We found that ssDNA
239 had the most potent suppressive effects on the T cell proliferation and that dsDNA also had
240 negative effects on T cell activation but was more variable **(Figure 3A).** The RNA:DNA hybrid
241 had significant suppressive effect but much less effect than seen for the ssDNA **(Figure 3A-B)**.
242 To explore the effect of HIV-1 genome, i.e., ssRNA, we delivered a custom made ssRNA
243 **(Supplementary Table 1)** and the synthetic ssRNA40 intracellularly to the DCs and found that
244 they had no or negligible effect on the T cell proliferation (**Figure 3C**). The HIV-1 p24, and many
245 loops ssDNA, had no effects on the T cell proliferation **(Figure 3B and D)**. To ensure that the
246 transfection did not affect the DC-T cell priming/activation, we treated HIV-exposed or
247 unexposed DCs with DOTAP and noticed no significant effects **(Supplementary Figure 1)**. The
248 HIV-1 derived oligonucleotides known to induce STING signaling[23] had a negative effect on T
249 cell proliferation and among them ssDNA had the most potent and consistent effects and was
250 selected for further studies of the mechanism behind the HIV-induced impairment.
251

252 **HIV and ssDNA induce type I IFN production and ISG in DC-T cell co-cultures**

253 HIV-1, ssDNA, and dsDNA, induced mRNA transcript of IFNβ, IFNα and ISGs such as IFI16
254 (**Figure 4A-B**). Of note, even if HIV-1, ssDNA, and dsDNA gave rise to type I IFN and ISGs on
255 mRNA levels, only the complete virion induced detectable amounts of IFNα and IFNβ on
256 protein level measured by ELISA (**Figure 4C**). HIV-1 induced IFNα and IFNβ on day 3 after co-
257 culture setup and the production persisted throughout the DC-T cell co-culture (**Figure 4C)**.

258 **STING agonists suppressed T cell proliferation and increased PD-L1 expression on the cells**
259 **in the co-culture**

260 Activation of STING by DNA oligonucleotides is an indirect event involving the activation of
261 DNA sensors e.g. cGAS, and IFI16, and followed by downstream STING activation[30]. To explore
262 the involvement of the STING signaling pathway in the HIV-induced suppression, we targeted
263 the STING protein directly by agonists, i.e., cyclic guanosine monophosphate adenosine
264 monophosphate (cGAMP) produced when cGAS is bound to a DNA ligand[25], a stabilized
265 2'3'cyclic-di-AMP analog ADUS100 [32], and MSA2[32]. Additionally, we tested the antagonist SN-

266 011 which competes with cyclic dinucleotide for the binding pocket on STING[33]. When
267 activating STING using extracellular or intracellular delivered cGAMP we found some
268 decreased proliferation with the intracellular but not extracellular delivered cGAMP **(Figure**
269 **4A)** and for the agonists ADU100 and MSAD2 we found an even stronger suppressive effect
270 on the T cell proliferation **(Figure 4B-C)**. The antagonist SN-011 treated cells gave the same
271 profile as untreated, i.e., it failed to stop/rescue the negative effect exerted by HIV-1 on the T
272 cell proliferation in the DC-T cell co-culture **(Figure 4D)**. Exploring the effect on type I IFNs and
273 ISGs we found induction of IFNβ, MXA, and PDL1 by STING agonists, especially MSA2 **(Figure**
274 **4E-G)**. STING activation in T cells has been shown to lead to impaired T cell proliferation and
275 in our hands STING activation gave rise to T cell suppression, which could be type I IFN
276 conditioning of the T cells interacting with the DCs.

277 **RIG activation played no role in the HIV induced immune suppression in the DC T cell**
278 **coculture**

279 To further investigate sensors upstream of STING that could be involved in the activation of
280 type I IFNs and in the HIV-induced immune suppression, we explored the effect of the RIG
281 antagonist RIG012[34]. Intracellular inhibition of RIG in the DCs in the DC-T cell co-culture did
282 not affect the T cell suppression induced by HIV-1 **(Figure 5A).** Furthermore, RIG012 inhibition
283 of RIG had no effect on type I IFN or ISGs **(Figure 5B**), and no or insignificant effect on co-
284 inhibitory molecules **(Figure 5C)**. These findings help to exclude the involvement of RIG in HIV-
285 induced immune impairment.

286 **IFI16 activation suppresses T cell proliferation but is not enough to induce co-inhibitory**
287 **molecules**

288 To further investigate the upstream sensors of STING involved in HIV-induced immune
289 suppression, we added the IFI16 agonist VACV-70, a dsDNA derived from vaccina virus[23]. The
290 activation of IFI16 sensor suppressed proliferation to similar levels as HIV-1 (**Figure 6A**).
291 Surprisingly, the IFI16 agonist had no or little enhancing effects on the levels of coinhibitory
292 molecules or MXA, compared to HIV in DCs or T cells in the co-cultures (**Figure 6B-C**), whereas
293 the effects on the IFNα, IFI16, and STING was similar to HIV **(Figure 6C)**. This suggests that
294 some of the negative effects induced by HIV can be replicated by the VACV-70 IFI16 activation
295 and indicate that this pathway could be a major contributor to the HIV induced impairment.

296 **Blocking of the type I IFN receptor restores DC-T cell function and phenotype**

297 Since we found that the type I IFN response is the major reason for HIV-induced immune
298 suppression, a block of the IFNAR could yield deeper understanding on how this occurs.
299 Blocking of the receptor using Anifrolumab, an anti-IFNAR1 antibody, restored all suppressive
300 events induced by HIV-1, the proliferation and functionality as measured by IFN-α, IL-2 and
301 granzyme B production by T cells (**Figure 7A**), the expression levels of immunomodulatory
302 molecules (**Figure 7B**) and abolished the type I IFN response (**Figure 7C**). This clearly
303 demonstrated the immune suppressive autocrine effect that type I IFNs have via activation of
304 the IFNAR signaling cascade.

305 ## Discussion

306 Here, we deciphered the HIV components and mechanisms underlying the immune
307 suppression induced by HIV-exposed DCs when activating T cell responses and found major
308 contribution of the cGAS/IFI16-STING signaling and type I IFN signaling pathways in the
309 ensuing immune suppression.

310 Most individuals with untreated chronic HIV, which can be maintained during ART treatment,
311 experience significant impairment in immune cell populations such as DCs, T cells, NK cells, B
312 cells, and MAIT cells, often due to the sustained immune activation[13-15]. Immune activation is
313 driven by multiple factors including prolonged elevated type I IFN levels and this is detrimental
314 to the immune system function, which has been shown to contribute to disease progression
315 in both HIV and simian immunodeficiency virus (SIV) infection[10,11]. Throughout HIV infection,
316 HIV skews DC function, altering cytokine profiles, inducing partial DC maturation, and
317 contributing to chronic immune activation and defective DC stimulation of T cells[13,14,35]. The
318 observed dysfunction in T cells is in part attributed to the upregulation of coinhibitory
319 molecules and loss of effector functions, further contributing to immune exhaustion. We have
320 previously demonstrated that HIV-1 impairs T cell functionality through DC-mediated cell to
321 cell contact with T cells[18,19]. DCs do not normally support a strong type I IFN after exposure to
322 HIV, which utilizes the HIV restriction factors SAMHD1, and TREX1 to avoid cytosolic sensing
323 by PRRs[36,37]. Nonetheless, our recent finding demonstrated a strong type I IFN profile in HIV-
324 exposed DCs of DC-T cell co-cultures[20], clearly indicating altered cellular programming
325 supporting type I IFN responses. The prolonged type I IFN response observed in a setting of
326 DC-T cell crosstalk in the presence of HIV may result from enhanced expression of the
327 STAT1/STAT2/IRF9 transcription complex, sustaining ISG transcription[38]. Alternatively, it could
328 stem from impaired IFNAR recycling, leading to receptor accumulation at the cell surface or
329 increased endosomal uptake necessary for JAK-STAT signaling activation[39]. Dysregulation of
330 downstream signaling, such as reduced ISG15 expression, may also contribute by destabilizing
331 USP18, a negative regulator that inhibits IFNAR assembly and type I IFN signaling[38].

332 Both the late-stage lab adapted HIV-1 BaL and the transmitted/founder HIV-1 THRO gave rise
333 to suppressed T cell proliferation and impaired functionality and similar levels of type I IFNs

334 responses, suggesting a conserved mechanism of immune suppression between different HIV-
335 1 strains, and that HIV BaL is a good model virus to study HIV induced immune suppression.

336 The HIV replication cycle involves an array of nucleotides/replication intermediates, such as
337 RNA genomes, ssDNA and dsDNA that are recognized and targeted by the host intracellular
338 defense including RNA and DNA sensors, which is counteracted by HIV to avoid recognition [40].
339 Our study reveals that HIV-1-derived oligonucleotides, i.e., ssDNA, dsDNA, and RNA:DNA
340 hybrid transfected into the cytosol of DCs participated in the suppressed T cell proliferation,
341 where ssDNA had a key role. The ssDNA, during HIV-1 replication, folds into structures
342 resembling dsDNA which are recognized by the STING pathway [23]. The results from our study
343 suggest that virus derived oligonucleotides modify DCs and play a crucial role in HIV-1-induced
344 immune suppression. During HIV infection the viral genome is protected by the viral capsid,
345 protecting it from degradation and recognition by cytosolic sensors. If the capsid is disrupted,
346 that leads to enhanced sensing of virus derived oligonucleotides by e.g., cGAS[41-43]. In our
347 system it might be the creation of an environment that disrupts the viral capsid, exposing the
348 viral genome that is the reason for the prolonged and strong type I IFN response we detect.

349 The mRNA profile sequenced in DCs derived from the HIV exposed DC-T cell co-culture
350 highlighted the significant role of intracellular sensors such as cGAS and STING, and type I IFN
351 pathways in the HIV-1 induced suppression of DCs. This is in accordance with other studies
352 where cytosolic RNA and DNA sensors such as cGAS, RIG-I like receptors, and IFI16 have been
353 implicated in recognizing HIV-1 and activating STING signaling in HIV infected [23,25,44]. We found
354 that addition of exogenous IFNα2 to the DC-T cell co-culture at concentrations detected in HIV
355 exposed co-cultures, induced similar suppressed T cell responses as HIV exposure, suggesting
356 that type I IFNs play a direct role in mediating the induction of impaired immune responses as
357 seen in vivo in HIV and SIV infections[11,45].

358 Our data showed that cytosolic HIV ssDNA and dsDNA mirroring the effects observed for HIV-
359 1, which suggests that replication intermediates/nucleotides play a crucial role in HIV-1-
360 induced immune suppression. The dsDNA-like structure is recognized as cytosolic DNA by the
361 IFI16 -STING pathway and activates type I IFN secretion[46] and this has been shown in cells such
362 as THP1, and monocytes[46] and now by us in DCs, underscoring the unique immunosuppressive
363 properties of structured ssDNA. Notably, targeting STING via intracellular cGAMP and agonists
364 suppressed T cell function and induced IFNβ, MXA, and PDL1 expression, reinforcing the

365 involvement of STING signaling cascade and subsequent type I IFN in the reprogramming of
366 DCs to induce immune impairment in our co-culture system and in individuals with chronic
367 HIV infection[47]. HIV-1 has evolved several escape mechanisms to counteract the STING-
368 induced type I IFNs, e.g., by glutamylation of HIV p6 that inhibits STING and TRIM32
369 interactions, and by HIV Vif interaction with SHP-1 that leads to STING inhibition[48,49]. Hence,
370 there is 'tug-of-war' between HIV blocking the STING pathway and the subsequent activation
371 induced by its oligonucleotides produced during the viral life cycle.

372 Our findings suggest that RIG-I signaling had no significant role in HIV-induced immune
373 suppression. This aligns with previous findings demonstrating that HIV-1 evades RIG-I
374 recognition through mechanisms such as viral RNA modifications and sequestration of RIG-I
375 signaling components[50]. The HIV engagement of both cGAS and RIG-I simultaneously has been
376 indicated in enabling advanced innate recognition of HIV-1 by myeloid DCs in elite controllers
377 [51], but if this occurs in our system needs to be further elucidated.

378 IFI16 is an important regulator to control HIV replication, independent of immune sensing via
379 inhibiting the transcription factor SP1 in the nucleus of CD4+ T cells. This regulator function is
380 especially efficient for non-clade C HIV-1 strains as these strains are in general more resistant
381 to the IFI16 pathway[52]. Cytosolic DNA sensors such as IFI16 have been implicated in the
382 recognition of HIV-1 ssDNA in THP1 cells and the subsequent activation of STING signaling[23].
383 Cells that lack IFI16 have uncontrolled replication, which in turn leads either to exhaustion of
384 the cell or cell death[23]. We found that targeting the IFI16 in the DCs directly with an agonist
385 lead to elevated type IFN and suppressed T cell proliferation to levels comparable to those
386 observed with HIV-1. These results align with previous studies demonstrating that IFI16 plays
387 a key role in sensing viral DNA[53,54]. Surprisingly, despite a similar T cell suppression, and type
388 I IFN response pattern as HIV, activation of STING via IFI16 did not enhance the expression of
389 co-inhibitory molecules or ISGs. IFI16-STING signaling seems to be a key component of the
390 immune modulation seen in our system, but HIV-1 exposure clearly employs additional
391 mechanisms that give rise to elevated expression of co-inhibitory molecules and further impair
392 immune function that is missing when targeting only IFI16. The IFI16 sensor is interlinked with
393 the cGAS-STING pathway in macrophages, by supporting cGAMP production and its
394 downstream signaling, i.e., recruitment and activation of TBK1 [55], which might explain that
395 individually targeting of cGAS or IFI16 sensor might not give the full effect that HIV induce in

396 the DCs. High viral load drives the elevated expressions of IFI16 and cGAMP in HIV positive
397 individuals. In addition, there was a correlation between CD38 and IFI16 in these patients,
398 indicating that the DNA activation of IFI16 might be part of chronic immune activation[44]. Our
399 data from the DC-T cell co-culture support this with high immune activation of type I IFN
400 responses, and IFI16 leading to suppressed responses in the HIV-exposed setting. In chronic
401 HIV infected individuals ART treated or untreated, there are impaired ISGs responses to DNA
402 stimuli[44], which could depend on the cell status due to the chronic activation found in these
403 patients.

404 The elevated ISGs clearly indicated autocrine and signaling via IFNα/β receptors as part of the
405 HIV induced immune activation leading to the suppressed responses. Prolonged type I IFN
406 exposure and The IFNα/β receptors signaling via JAK/STAT/IRF9 has been linked to
407 upregulation of inhibitory molecules, suppression of T cell function, and impaired antigen-
408 presenting cell activity[56]. Blocking the IFNα, and IFNβ signaling via IFNAR using Anifrolumab
409 fully restored the suppressive effects induced by HIV-1, including T cell proliferation, DC and
410 T cell phenotypes and T cell functionality. This complete recovery rendered by Anifrolumab is
411 a promising strategy to restore the immune function in people living with HIV and may also
412 lead to lowered tissue viral DNA and improved general health as has been shown for ART
413 treated SIV+ Macaques receiving anti-IFNα therapy using an anti-IFNα antibody[45].

414 Overall, our results suggest that the IFI16/cGAS-STING pathway and subsequent type I IFN
415 signaling with IFNα/β receptors contributes to HIV-induced immune suppression, but
416 additional factors are likely involved in regulating the expression of co-inhibitory molecules.
417 These findings reinforce the understanding that HIV exploits innate immune signaling to
418 establish an immunosuppressive environment that hinders effective T cell responses and
419 support the role of DC-driven immune regulation in HIV pathogenesis. Moreover, the
420 restoration of immune function when targeting IFNαR via Anifrolumab, a drug already
421 approved and on market for systemic lupus erythematosus and in phase 3 trials for lupus
422 nephritis, systemic sclerosis, idiopathic inflammatory myopathies, and cutaneous lupus
423 erythematosus, highlights the potential therapeutic value of targeting type I IFN signaling in
424 HIV infection to alleviate immune dysfunction and improve DC and T cell functionality. Future
425 studies should delineate how HIV-1 utilize the IFI16/cGAS-STING signaling and other factors
426 involved in immune impairment and explore whether modulating the pathway can serve as a

427 viable strategy to counteract immune suppression in HIV infection, both in untreated and ART
428 treated individuals.

429

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435

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591

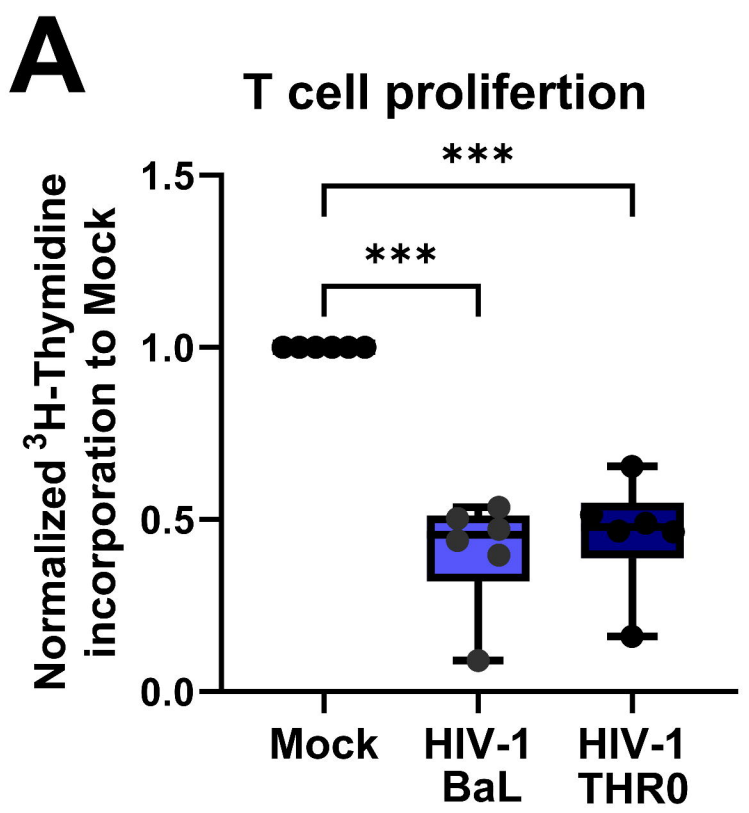
592

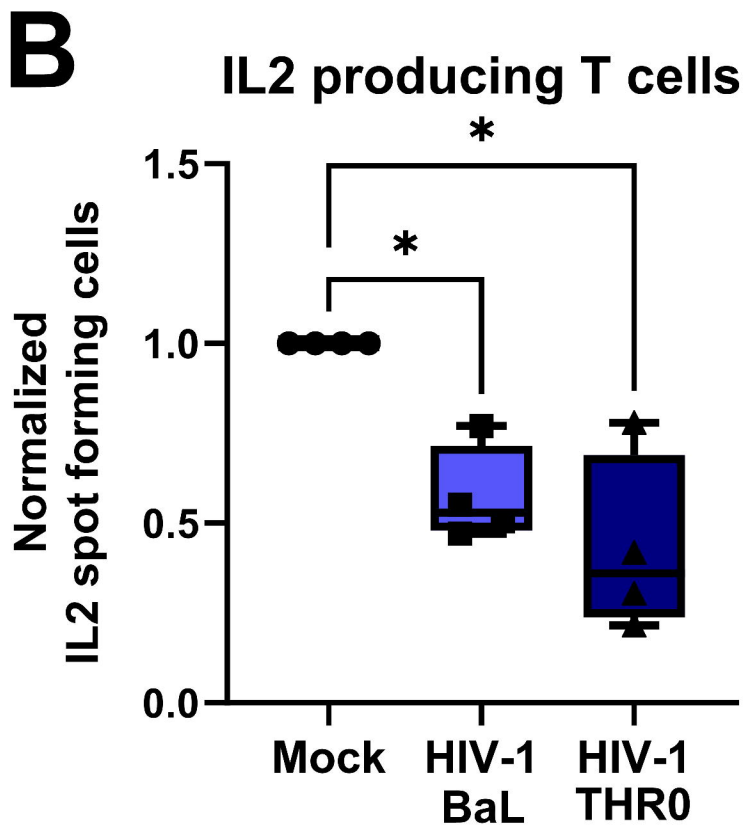
593 ## Figure legends

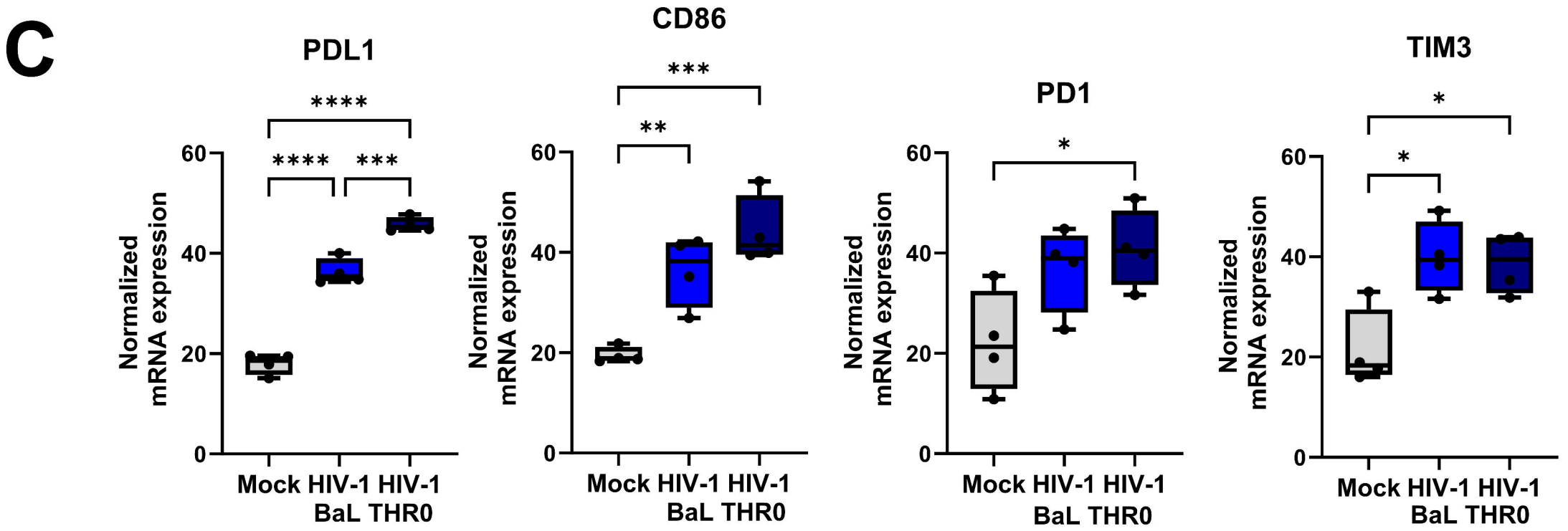
594 **Figure 1. Both chronic and founder HIV-1 strains suppressed dendritic cells ability to prime**
595 **naïve T cells.**
596 HIV-1 BaL a late-stage derived lab adapted strain or HIV-1 THRO a transmitted/founder strain
597 exposed mature DCs were co-cultured with naïve T cells in the ratio of 1:10. The DC-T cell co-
598 culture was re-stimulated on day 7 with the same DCs as the initial stimulation. One day after
599 restimulation, the T proliferation was assessed via ^{3}H thymidine incorporation **(A)** and
600 functionality by enumerating IL2-producing cells by ELISPOT assays **(B)**. mRNA transcript of
601 PDL1, CD86, PD1, and TIM3, and **(C)** IFNα4, MXA, IFI13, and IFI16 **(D)** by qPCR. Statistical
602 significance was determined using the ANOVA. * =p-value <0.05, ** = p-value <0.01, *** = p-
603 value <0.001**** = p-value <0.0001.
604
605 **Figure 2. Type I IFN inducing pathways were enriched and predicted to be activated when**
606 **HIV was present in DC-T cell co-culture and exogenous type I IFN-induced suppressed T cell**
607 **responses similar to HIV.**
608 Transcriptomic data from our published RNA seq[20], where DCs purified from the DC-T cell co-
609 culture after one day of restimulation, were assessed for top regulator network **(A).** HIV-1 BaL
610 exposed mature DCs were co-cultured with naïve T cells in the presence or absence of
611 exogenous human recombinant IFNα4. The DC-T cell co-culture was re-stimulated on day 7
612 with the same DCs as the initial stimulation and the IFNα2 replenished. One day after
613 restimulation, the T proliferation was assessed via ^{3}H thymidine incorporation **(B)**. mRNA
614 transcript of PDL1, and PD1 **(C)** by qPCR. Statistical significance was determined using the
615 ANOVA. * =p-value <0.05, ** = p-value <0.01 , *** = p-value <0.001**** = p-value <0.0001.
616
617 **Figure 3. HIV-1 derived replication intermediate ssDNA participated in the induction of the**
618 **suppressive effects seen by HIV in the DC-T cell co-culture.**
619 Mature DCs were exposed to HIV-1 BaL or transfected with the ssDNA, dsDNA, DNA:RNA
620 hybrid, many loops ssDNA, or p24 using DOTAP to deliver these HIV components into the
621 cytosol. The different DC groups were co-cultured with naïve T cells (1:10) and the DC-T cell
622 co-culture was re-stimulated on day 7 with the same DCs as the initial stimulation. One day
623 after restimulation, the T proliferation was assessed via ^{3}H thymidine incorporation **(A-D).**

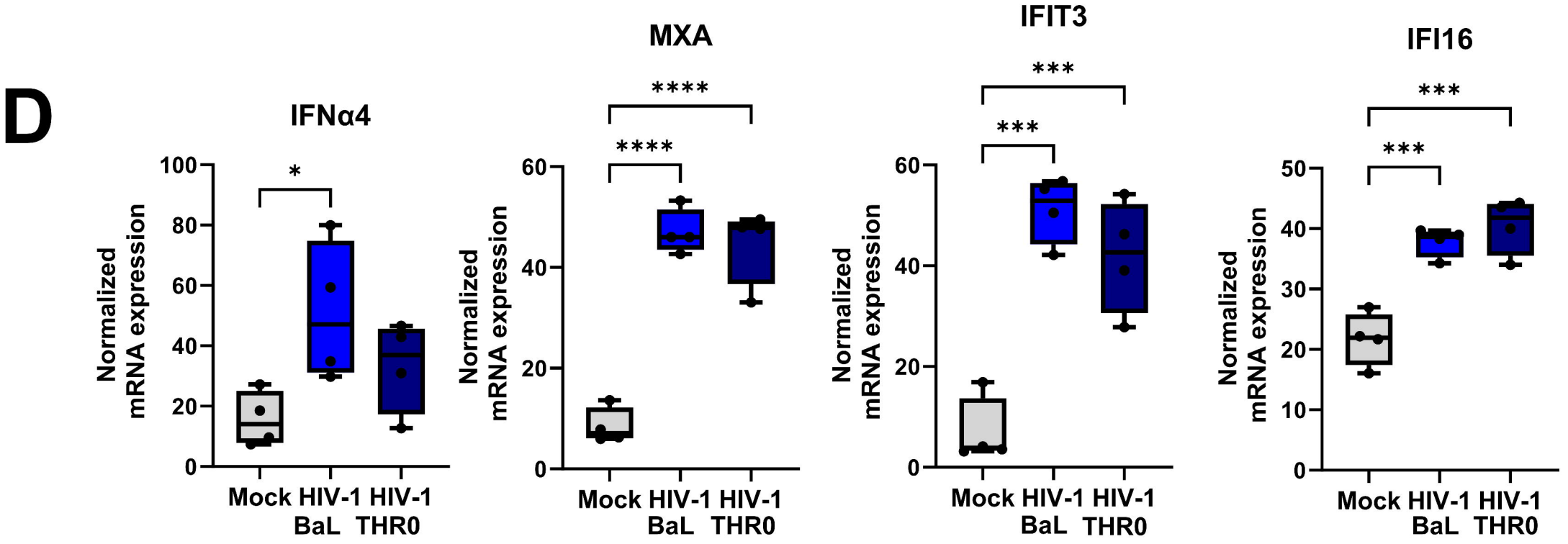
624 Statistical significance was determined using the ANOVA. * =p-value <0.05, ** = p-value <0.01,
625 *** = p-value <0.001**** = p-value <0.0001.
626
627 **Figure 4: HIV and ssDNA induce type I IFN production and ISG in DC-T cell co-cultures.**
628 Mature DCs were exposed to HIV-1 BaL or transfected with the ssDNA, dsDNA, DNA:RNA
629 hybrid, many loops ssDNA, or p24 using DOTAP to deliver these HIV components into the
630 cytosol. The different DC groups were co-cultured with naïve T cells (1:10) and the DC-T cell
631 co-culture was re-stimulated on day 7 with the same DCs as at the initial stimulation. mRNA
632 transcript of **(A)** IFNα4, MXA, IFI13, and IFI16 by qPCR and **(B)** Protein levels of type I IFNs,
633 IFNα4 and IFNβ were measured by ELISA. ANOVA. * =p-value <0.05, ** = p-value <0.01, ***
634 = p-value <0.001, **** = p-value <0.0001.
635
636 **Figure 5: STING agonists suppressed T cell proliferation and increased PDL1 expression of**
637 **the cells in the co-culture.**
638 Mature DCs were unexposed or exposed to HIV-1 BaL, or transfected with STING agonists
639 cGAMP, and cocultured with naïve T cells. Additionally, during coculture the cells were
640 exposed to, stabilized 2'3'cyclic-di-AMP analog ADU100, and MSA-2, and STING antagonist SN-
641 011. Cocultures were restimulated on day 7 by the addition of the same DCs as during setup.
642 One day after re-stimulation, the T proliferation was measured by ^{3}H thymidine incorporation
643 **(A-D).** mRNA transcript levels of IFN-β, MXA, and PDL1 in ADU100 **(E)**, MSA2 (F), and SN-011
644 **(G)** treated DC-T cell co-culture assessed by qPCR. Protein levels of IFNα and IFNβ in ADU100
645 **(E),** MSA2 **(F)**, and SN-011 **(G)** treated DC-T cell co-culture assessed by ELISA. Statistical
646 significance was determined using the ANOVA. * =p-value <0.05, ** = p-value <0.01, *** = p-
647 value <0.001, **** = p-value <0.0001.
648
649 **Figure 6: RIG activation played no role in HIV-induced immune suppression in the DC-T cell**
650 **co-culture.**
651 Mature DCs were exposed to HIV-1 BaL or DOTAP transfected with RIG agonist RIG012 to
652 achieve delivery into the cytosol. The different DC groups were co-cultured with naïve T cells
653 (1:10) and the DC-T cell co-culture was re-stimulated on day 7 with the same DCs as the initial
654 stimulation. One day after re-stimulation, the T proliferation measured via ^{3}H thymidine
655 incorporation **(A)**, mRNA transcript of CD86, PD1, PDL1, and TIM3 **(B),** and IFNα4, MXA, IFI13,

656 and IFI16 **(C)** by qPCR. Statistical significance was determined using the ANOVA. * =p-value
657 <0.05, ** = p-value <0.01, *** = p-value <0.001**** = p-value <0.0001.
658
659 **Figure 7: IFI16 activation suppresses T cell proliferation but is not enough to induce co-**
660 **inhibitory molecules.**
661 Mature DCs were exposed to HIV-1 BaL or DOTAP transfected with IFI16 agonist dsDNA VACV-
662 60 to deliver the VACV-60 into the cytosol. The different DC groups were co-cultured with
663 naïve T cells (1:10) and the DC-T cell co-culture was re-stimulated on day 7 with the same DCs
664 as the initial stimulation. One day after re-stimulation, the T proliferation measured by ^{3}H
665 thymidine incorporation **(A).** mRNA transcripts of PDL1, Galactin 9, TRAIL, and TRIM3 **(B),** and
666 of IFNα4, IFI16, and MXA **(C)** were assessed by qPCR. Statistical significance was determined
667 using the ANOVA. * =p-value <0.05, ** = p-value <0.01, *** = p-value <0.001**** = p-value
668 <0.0001.
669
670 **Figure 8: Targeting IFNaR1 by Anifrolumab restored immune function in HIV-induced**
671 **suppressed DCs and T cells.**
672 Mature DC were exposed to HIV BaL and incubated for 24h. The untreated (mock) and HIV
673 exposed DCs were harvested and exposed to 20ug/ml Anifrolumab for 30 min before the DCs
674 were added to naïve T cells and co-cultured (1:10). The DC-T cell co-culture was re-stimulated
675 on day 7 with the same DCs as the initial stimulation. One day after re-stimulation, the T
676 proliferation was assessed via H^{3} thymidine incorporation and IFNγ, IL2 and granzyme B
677 producing T cells by ELISPOT **(A)**. The effects of Anifrolumab on mRNA transcripts levels for
678 IFNα4, IFI16, and MXA, and IFI16 **(B)** and PD1L, Galactin9, TIM3, and TRAIL) **(C)** were assessed
679 by qPCR. Statistical significance was determined using the ANOVA. * =p-value <0.05, ** = p-
680 value <0.01, *** = p-value <0.001**** = p-value <0.0001.

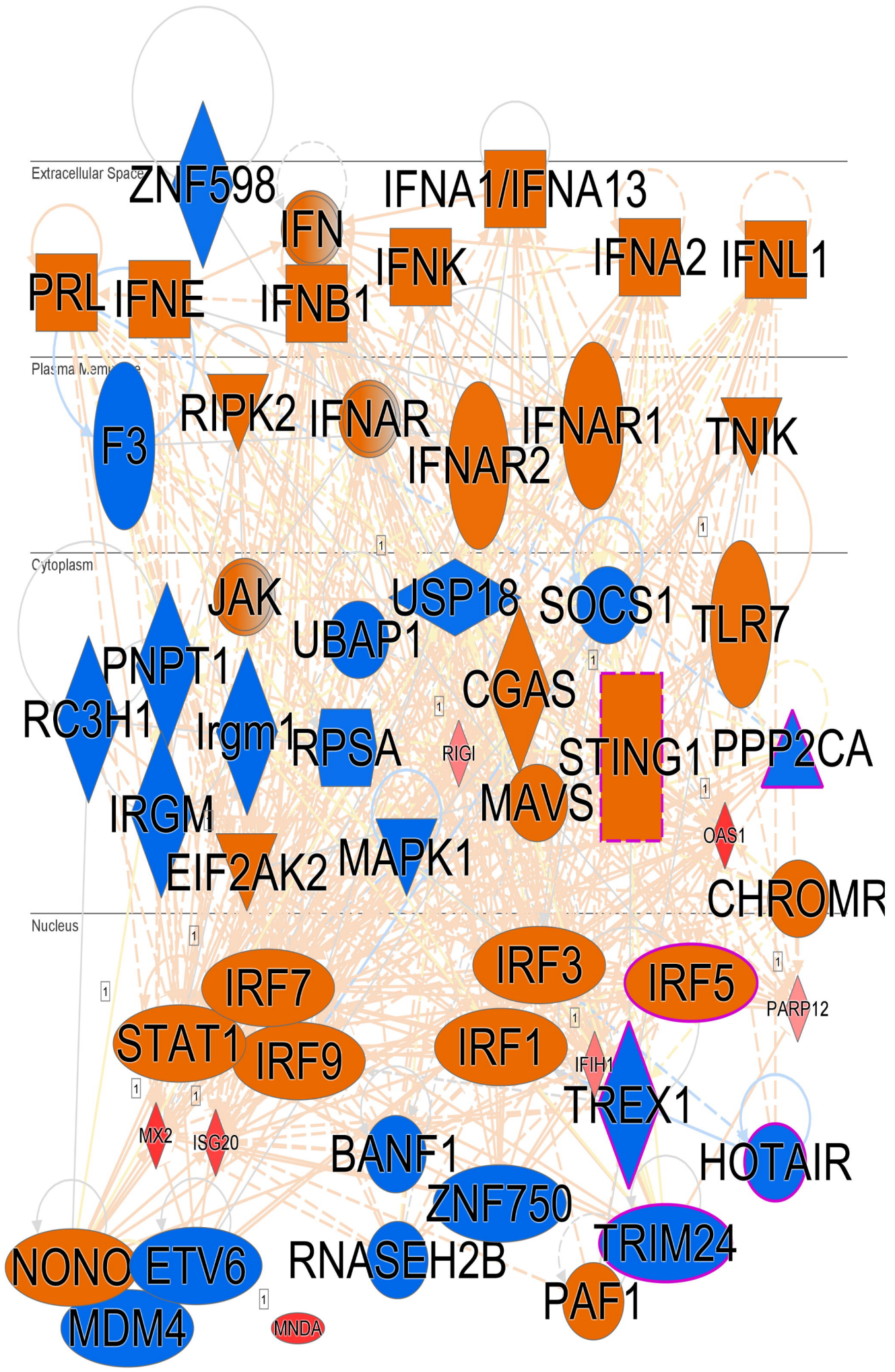

A
T cell prolifertion
Normalized ^{3}H-Thymidine incorporation to Mock
1.5
1.0
0.5
0.0
***
***
Mock
HIV-1 BaL
HIV-1 THR0


B
IL2 producing T cells
Normalized IL2 spot forming cells
1.5
1.0
0.5
0.0
*
*
Mock
HIV-1 BaL
HIV-1 THR0

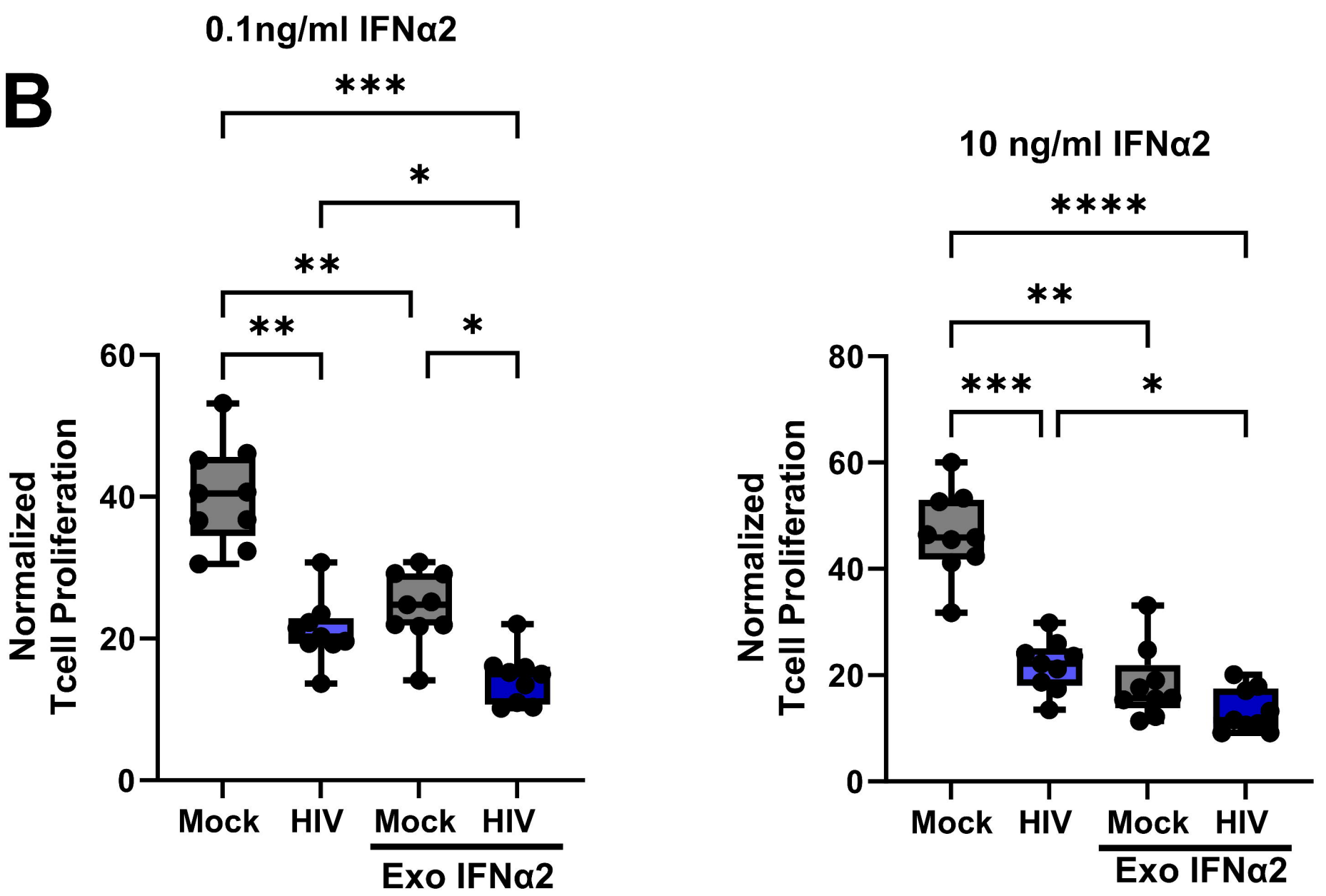

C
PDL1
CD86
PD1
TIM3
Normalized mRNA expression
60
40
20
0
****
****
***
***
**
*
*
*
Mock
HIV-1 BaL
HIV-1 THR0

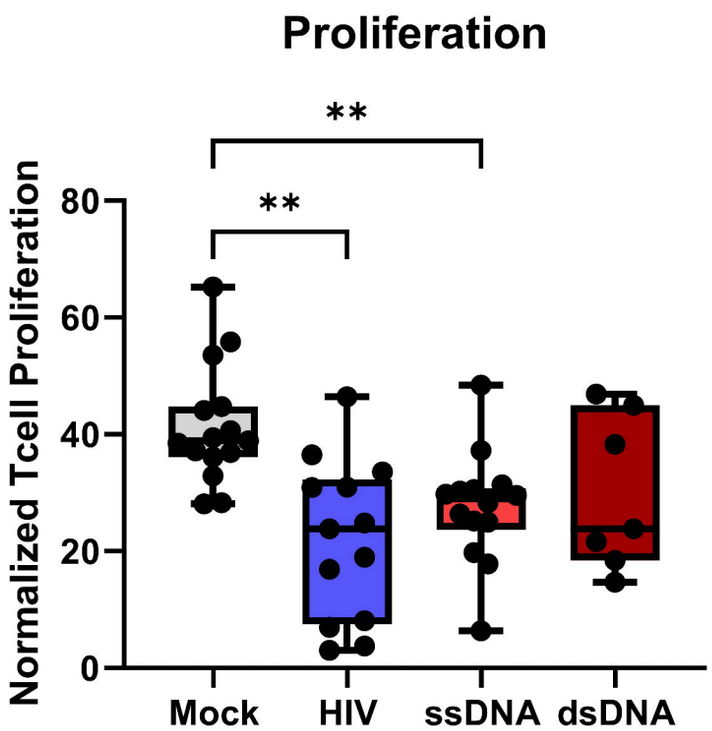

D
IFNα4
MXA
IFIT3
IFI16
Normalized mRNA expression
100
80
60
40
20
0
50
30
10
*
****
****
***
***
***
***
Mock
HIV-1 BaL
HIV-1 THR0

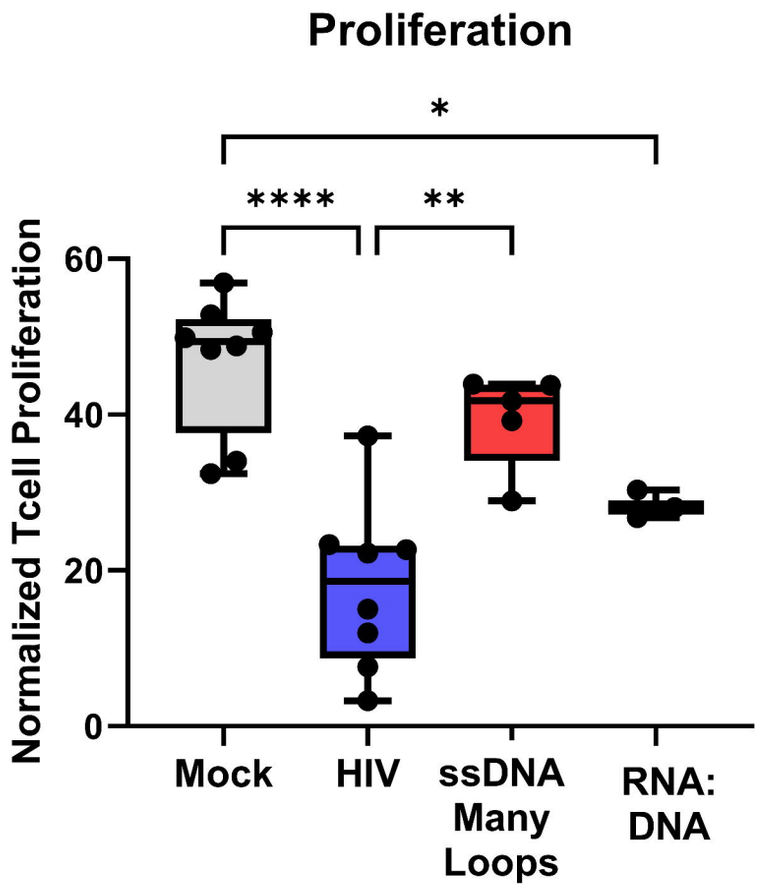
A

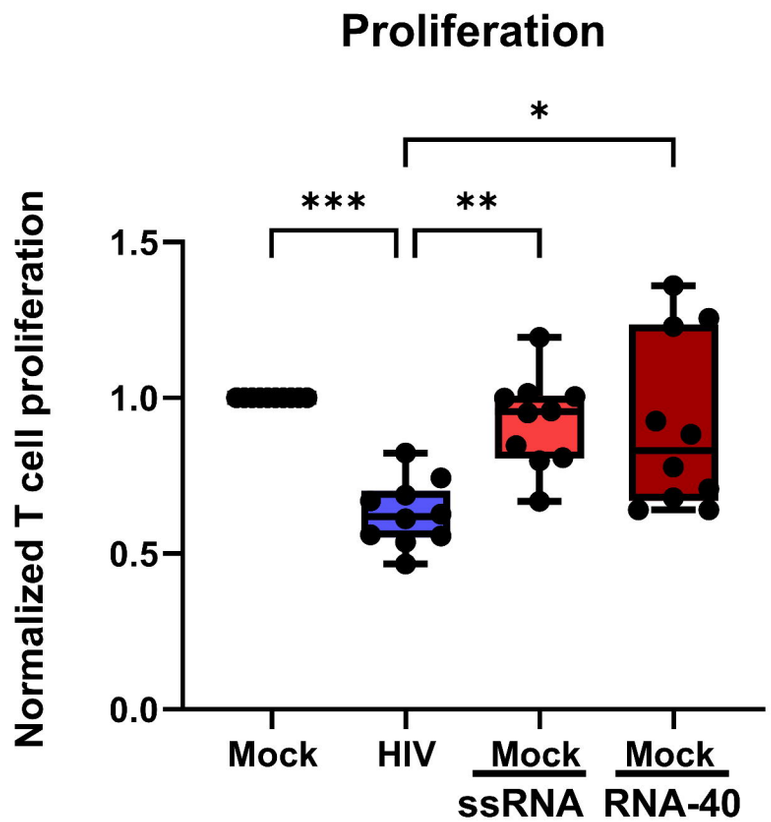

Extracellular Space
Plasma Membrane
Cytoplasm
Nucleus
ZNF598
IFN
IFNA1/IFNA13
IFNK
IFNA2
IFNL1
PRL
IFNE
IFNB1
F3
RIPK2
IFNAR
IFNAR2
IFNAR1
TNIK
JAK
USP18
SOCS1
TLR7
UBAP1
PNPT1
CGAS
RC3H1
Irgm1
RPSA
RIGI
STING1
PPP2CA
MAVS
IRGM
OAS1
EIF2AK2
MAPK1
CHROMR
IRF3
IRF5
IRF7
PARP12
STAT1
IRF9
IRF1
IFIH1
TREX1
MX2
ISG20
BANF1
HOTAIR
ZNF750
TRIM24
NONO
ETV6
RNASEH2B
PAF1
MDM4
MNDA

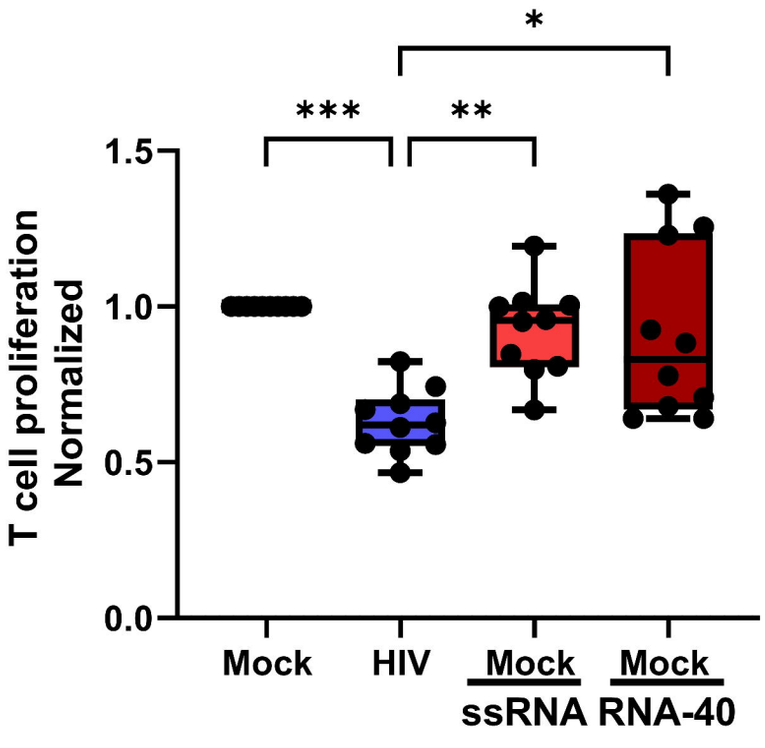
B

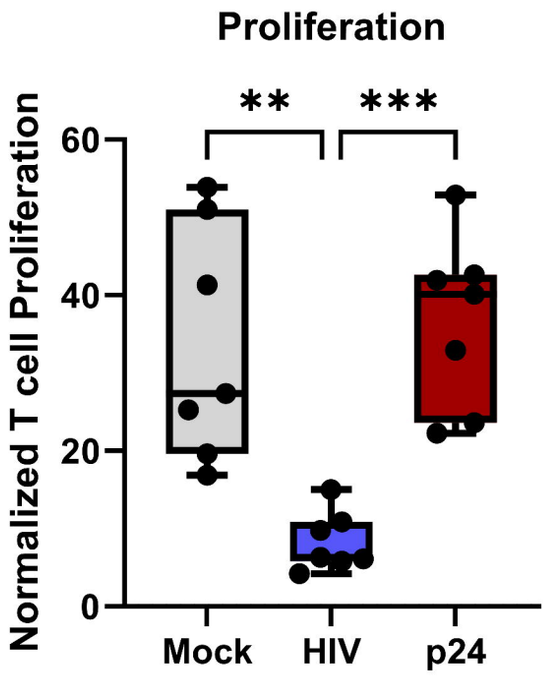

0.1ng/ml IFNα2
10 ng/ml IFNα2
Normalized Tcell Proliferation
Mock
HIV
Mock
HIV
Exo IFNα2
0
20
40
60
80
*
**
***
****

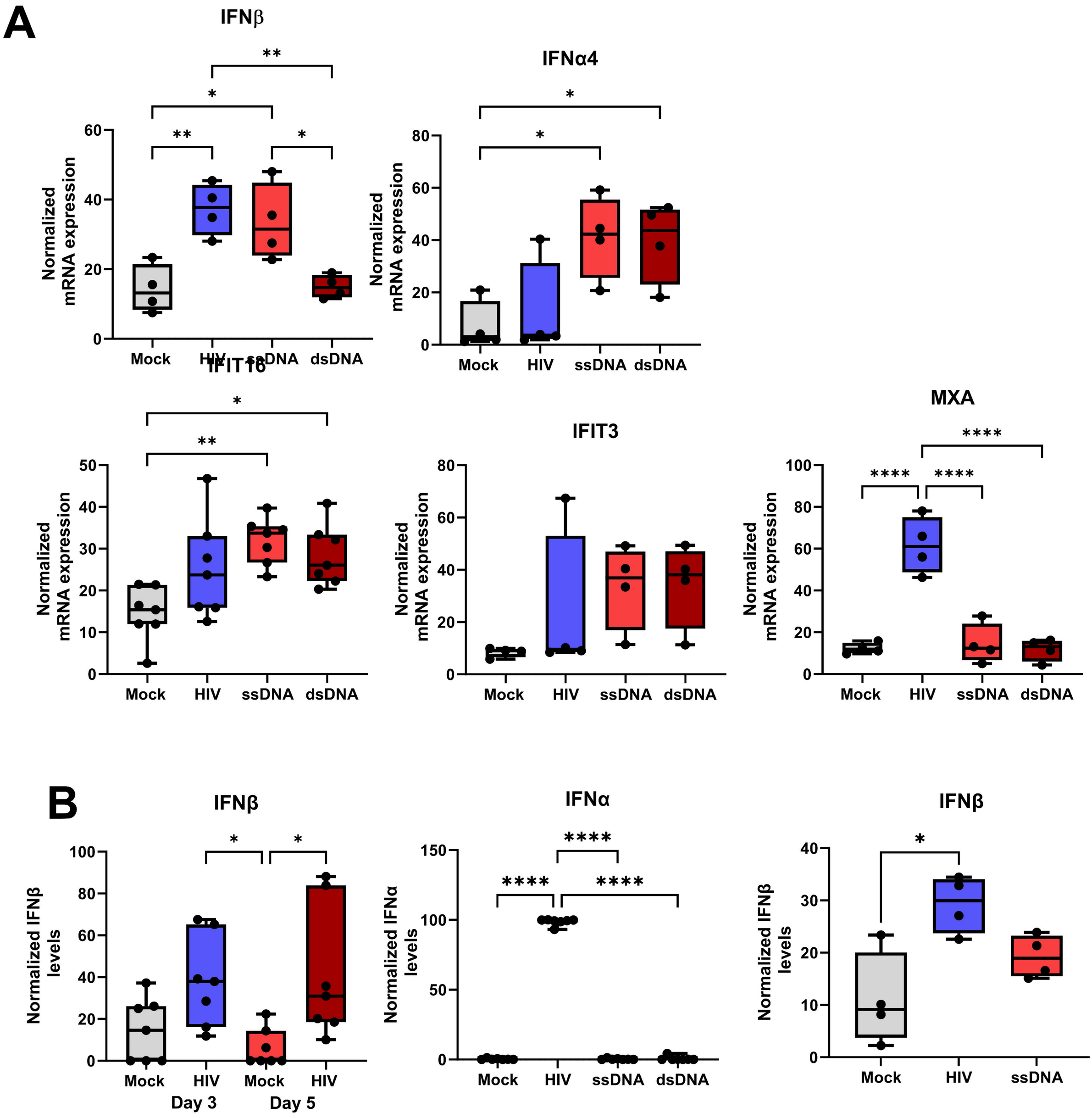

Proliferation
Normalized Tcell Proliferation
80
60
40
20
0
**
**
Mock
HIV
ssDNA
dsDNA

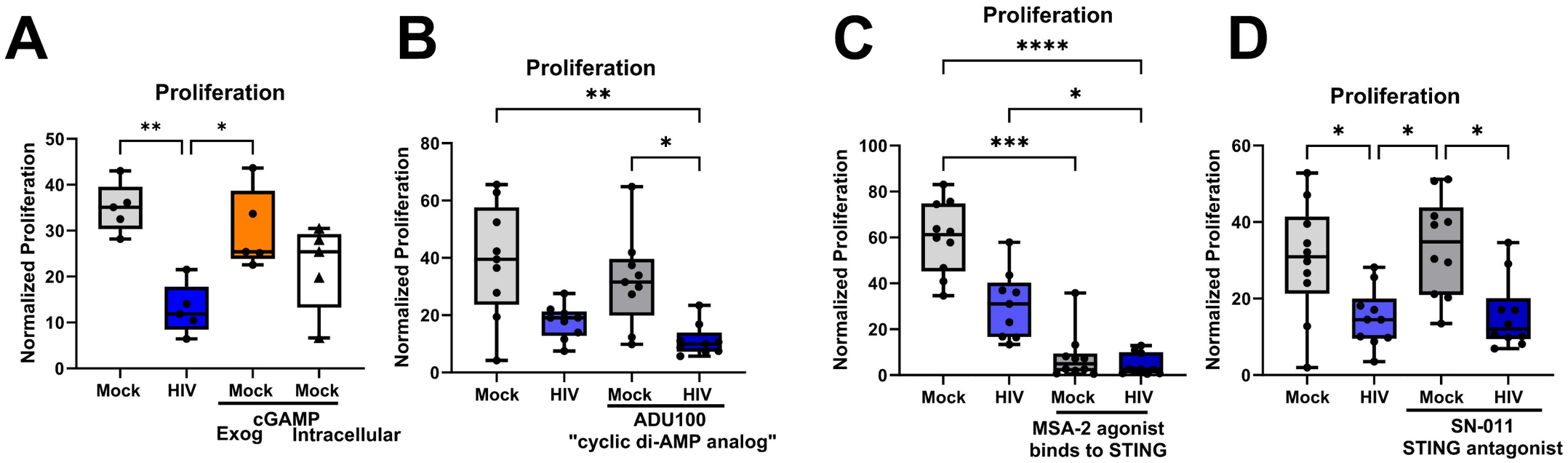

Proliferation
Normalized Tcell Proliferation
60
40
20
0
*
****
**
Mock
HIV
ssDNA Many Loops
RNA: DNA

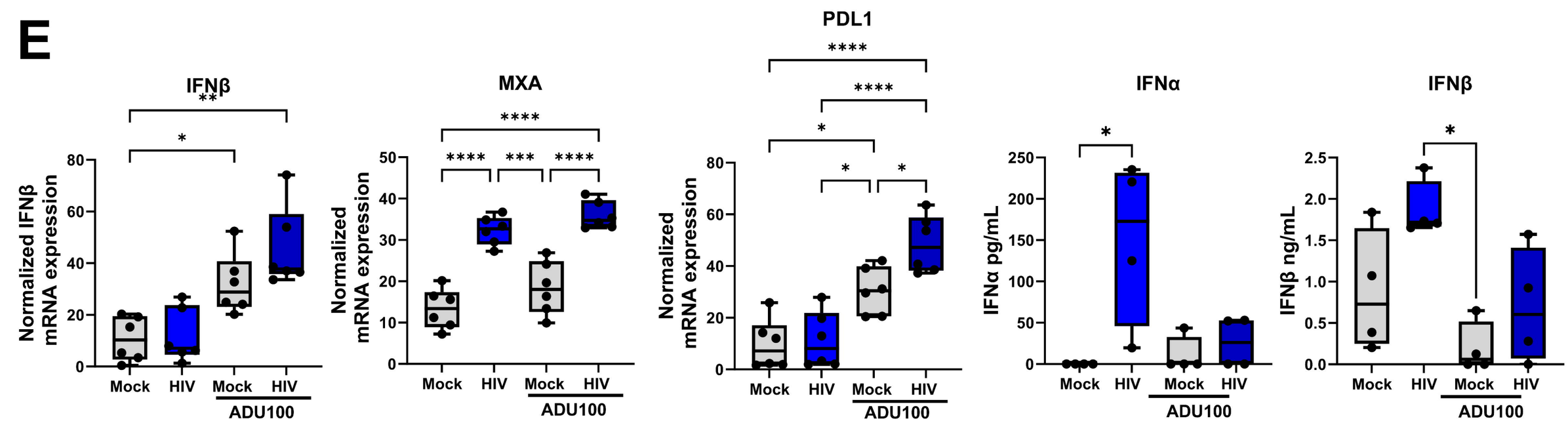

Proliferation
Normalized T cell proliferation
1.5
1.0
0.5
0.0
*
***
**
Mock
HIV
Mock
ssRNA
Mock
RNA-40

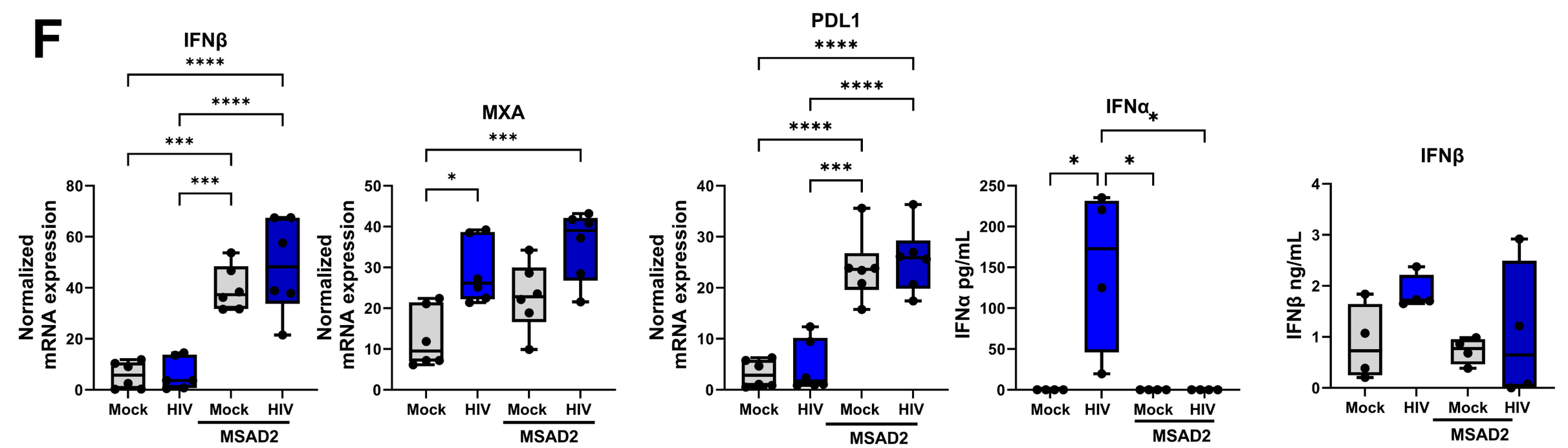

Proliferation
T cell proliferation Normalized
1.5
1.0
0.5
0.0
*
***
**
Mock
HIV
Mock
ssRNA
Mock
RNA-40

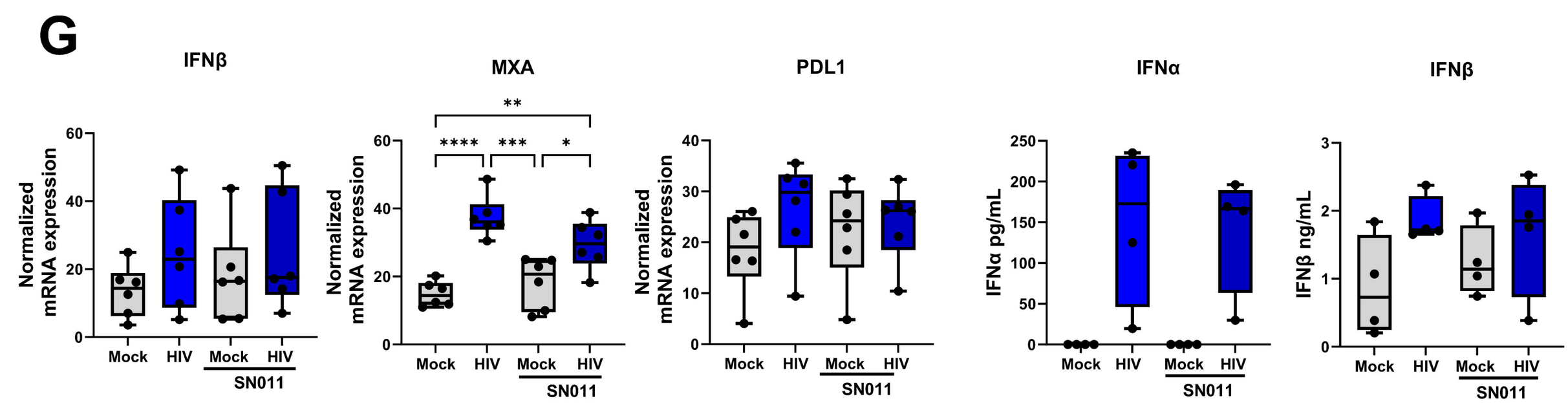

Proliferation
Normalized T cell Proliferation
60
40
20
0
**
***
Mock
HIV
p24

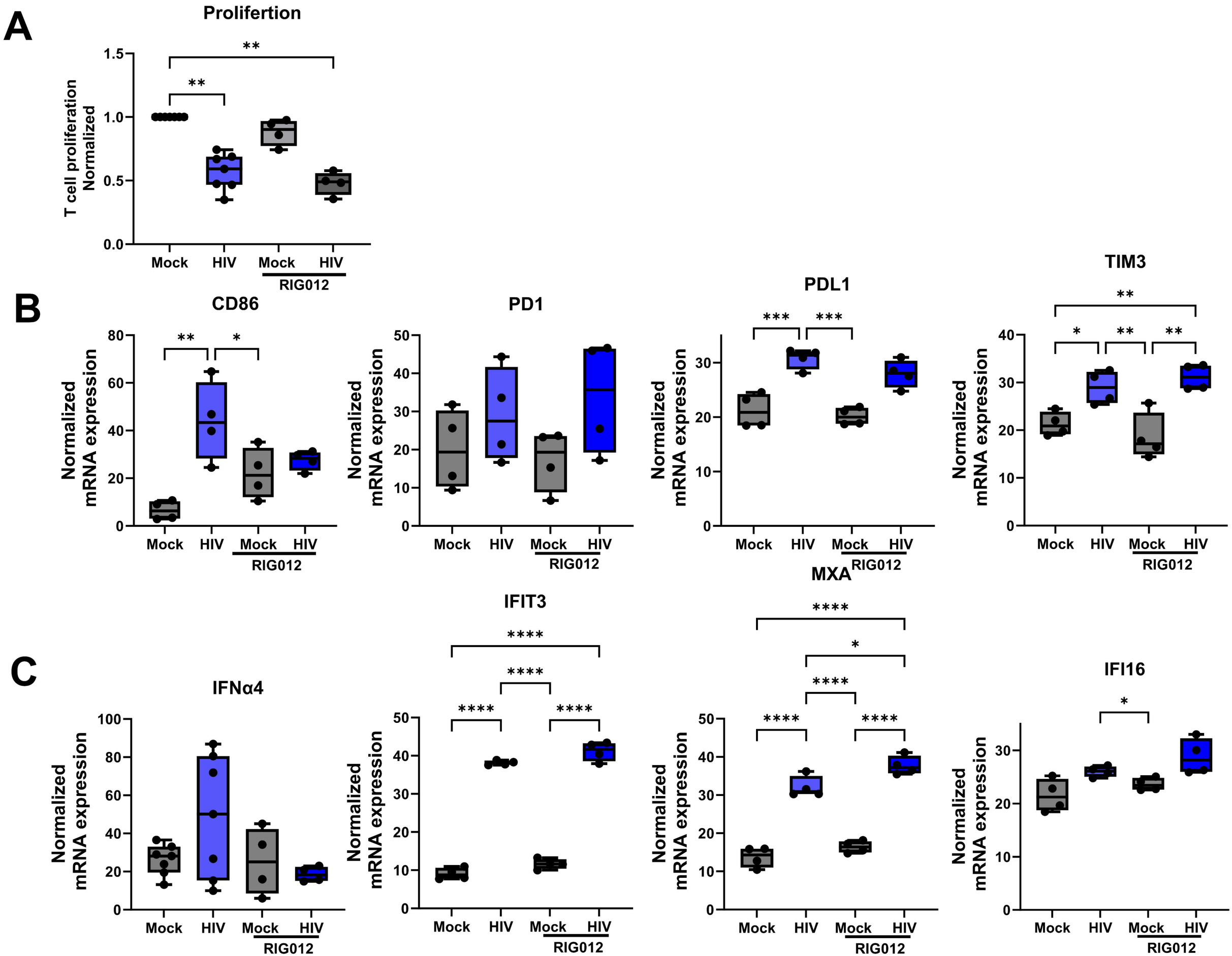

A
IFNβ
IFNα4
IFIT16
IFIT3
MXA
Normalized mRNA expression
Mock
HIV
ssDNA
dsDNA
B
IFNβ
IFNα
IFNβ
Normalized IFNβ levels
Normalized IFNα levels
Day 3
Day 5

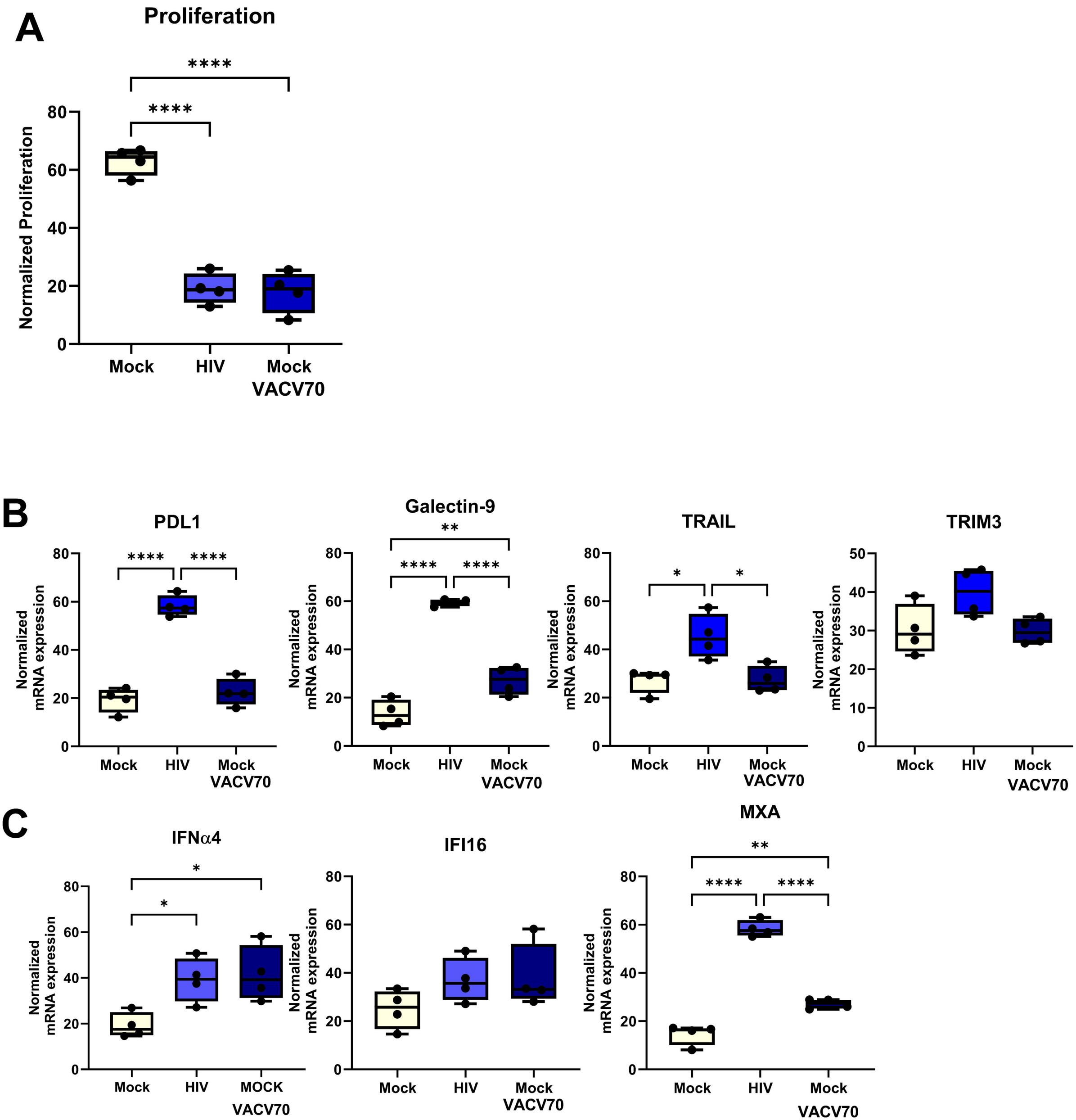

A
Proliferation
Normalized Proliferation
Mock
HIV
Mock
Mock
cGAMP
Exog
Intracellular
B
Proliferation
Normalized Proliferation
Mock
HIV
Mock
HIV
ADU100
"cyclic di-AMP analog"
C
Proliferation
Normalized Proliferation
Mock
HIV
Mock
HIV
MSA-2 agonist
binds to STING
D
Proliferation
Normalized Proliferation
Mock
HIV
Mock
HIV
SN-011
STING antagonist

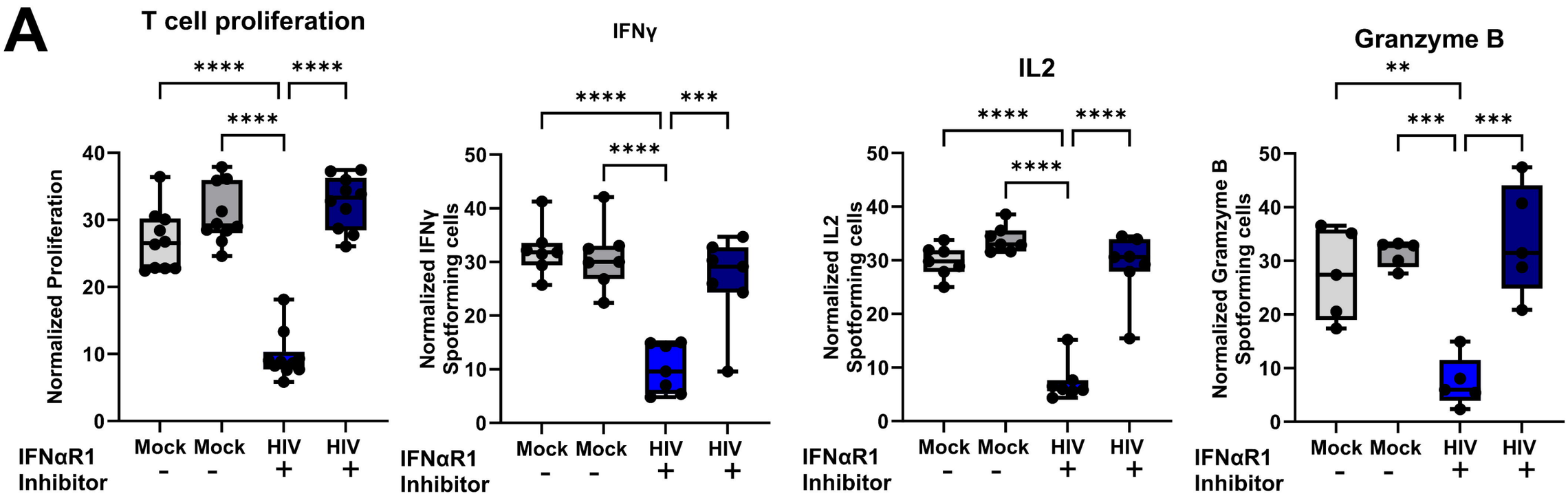

E
IFNβ
Normalized IFNβ mRNA expression
MXA
Normalized mRNA expression
PDL1
Normalized mRNA expression
IFNα
IFNα pg/mL
IFNβ
IFNβ ng/mL
Mock
HIV
Mock
HIV
ADU100

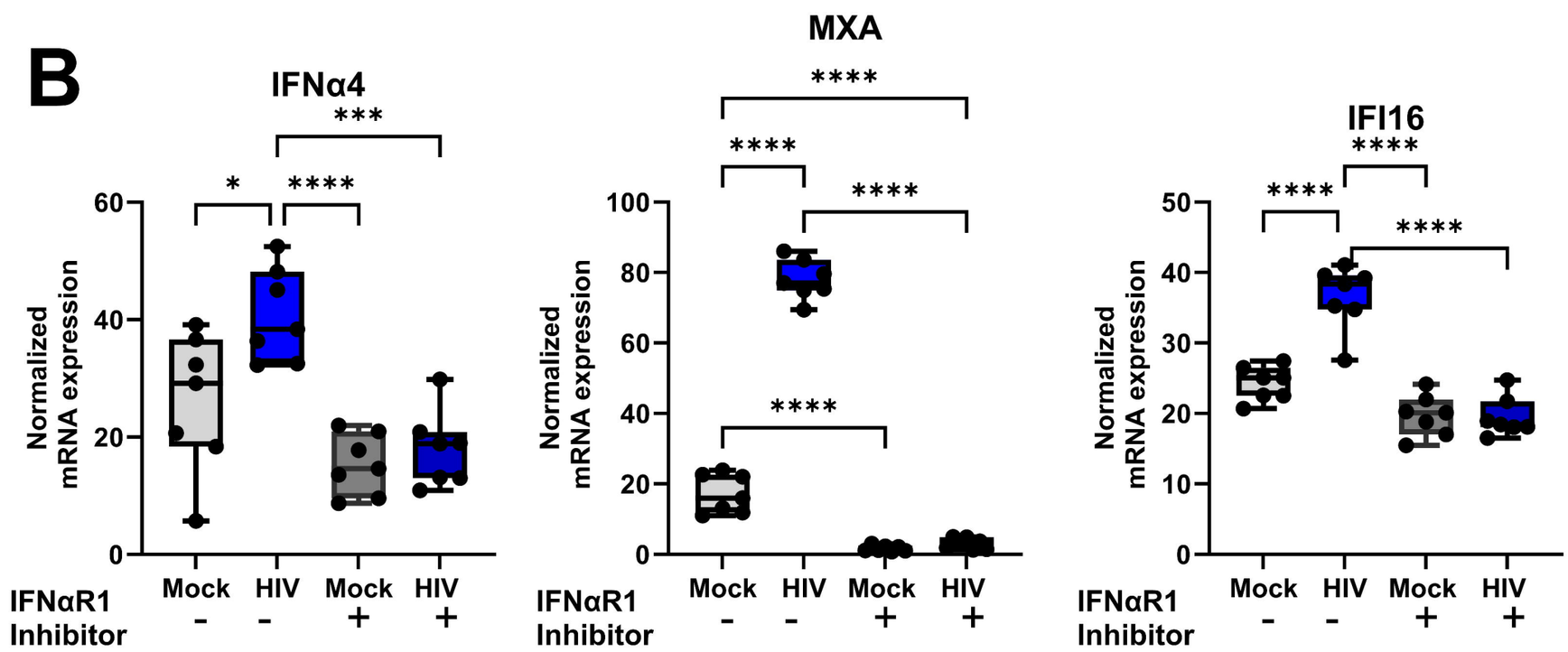

F
IFNβ
Normalized mRNA expression
MXA
Normalized mRNA expression
PDL1
Normalized mRNA expression
IFNα
IFNα pg/mL
IFNβ
IFNβ ng/mL
Mock
HIV
Mock
HIV
MSAD2

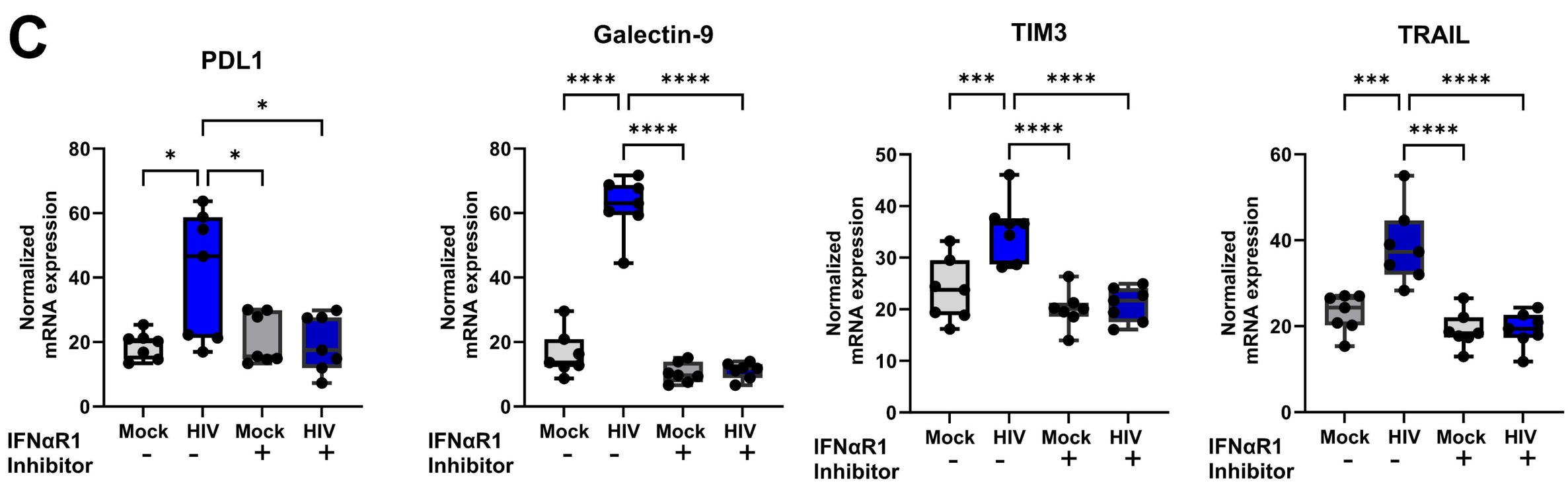

G
IFNβ
Normalized mRNA expression
MXA
Normalized mRNA expression
PDL1
Normalized mRNA expression
IFNα
IFNα pg/mL
IFNβ
IFNβ ng/mL
Mock
HIV
Mock
HIV
SN011


A
Prolifertion
T cell proliferation Normalized
1.5
1.0
0.5
0.0
**
**
Mock
HIV
Mock
HIV
RIG012
B
CD86
PD1
PDL1
TIM3
Normalized mRNA expression
80
60
40
20
0
50
40
30
20
10
0
**
*
***
***
**
*
**
**
Mock
HIV
Mock
HIV
RIG012
C
IFNα4
IFIT3
MXA
IFI16
100
80
60
40
20
0
50
40
30
20
10
0
****
****
****
****
****
*
****
****
*


A
Proliferation
Normalized Proliferation
Mock
HIV
Mock VACV70
B
PDL1
Galectin-9
TRAIL
TRIM3
Normalized mRNA expression
Mock
HIV
Mock VACV70
C
IFNα4
IFI16
MXA
Normalized mRNA expression
Mock
HIV
MOCK VACV70
Mock VACV70


A
T cell proliferation
Normalized Proliferation
IFNγ
Normalized IFNγ Spotforming cells
IL2
Normalized IL2 Spotforming cells
Granzyme B
Normalized Gramzyme B Spotforming cells
Mock Mock HIV HIV
IFNαR1 Inhibitor - - + +


B
IFNα4
MXA
IFI16
Normalized mRNA expression
Mock HIV Mock HIV
IFNαR1 Inhibitor - - + +


C
PDL1
Galectin-9
TIM3
TRAIL
Normalized mRNA expression
Mock HIV Mock HIV
IFNαR1 Inhibitor - - + +