

Transcriptomic Analysis of the Innate Immune Response to *in vitro* Transfection of Plasmid DNA

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ABSTRACT

1 The innate immune response to cytosolic DNA is intended to protect the host from viral
2 infections, but it can also inhibit the delivery and expression of therapeutic transgenes in gene
3 and cell therapies. The goal of this work was to use mRNA-sequencing to reveal correlations
4 between the transfection efficiencies of four cell types (PC-3, Jurkat, HEK-293T, and primary
5 CD3⁺ T cells) and their innate immune responses to nonviral gene delivery. Overall, the highest
6 transfection efficiency was observed in HEK-293T cells (87%), which upregulated only 142
7 genes with no known anti-viral functions. Lipofection upregulated a much larger number (n =
8 1,057) of cytokine-stimulated genes (CSGs) in PC-3 cells, which also exhibited a significantly
9 lower transfection efficiency. However, the addition of serum during Lipofection and
10 electroporation significantly increased transfection efficiencies and decreased the number of
11 upregulated genes in PC-3 cells. Finally, while Lipofection of Jurkat and Primary T cells only
12 upregulated a few genes, several anti-viral CSGs that were absent in HEK and upregulated in
13 PC-3 cells were observed to be constitutively expressed in T cells, which may explain their
14 relatively low Lipofection efficiencies (8-21%). Indeed, overexpression of one such CSG
15 (IFI16) significantly decreased transfection efficiency in HEK cells to 33%.

Keywords:

Plasmid, innate immune response, cytokines, cytokine-stimulated genes, STING, MYD88,
IKBKE

INTRODUCTION

16 In recent years, multiple gene therapy treatments using adeno-associated viruses (AAVs) and
17 lentiviruses (LVs) have been shown to effectively treat a variety of diseases in the clinic.¹ For
18 example, AAV is used to deliver a functional copy of the RPE65 gene to patients with Leber's
19 Congenital Amaurosis (LCA) in Luxturna®,² while Zolgensma® uses an AAV to deliver the
20 SMN1 gene to patients with spinal muscular atrophy (SMA). Likewise, delivery of the chimeric
21 antigen receptor (CAR) to T cells is done with an LV in three different CAR-T cell therapies for
22 B-cell lymphomas (Kymriah®, Yescarta®, and Tecartus™).³⁻⁵ Several other promising gene
23 therapies for hemophilia, Duchenne's muscular dystrophy (DMD), immunodeficiency, and
24 Pompe disease are also progressing through clinical trials.⁶⁻⁹ All of these therapies have
25 demonstrated that gene delivery can be a lifesaving treatment for patients where other treatment
26 mechanisms have failed.⁴ However, several issues have arisen in regards to LV and AAV
27 vehicles, including high treatment costs (e.g., \$2M for Zolgensma®), safety concerns, and
28 relatively low transduction efficiencies in some patients.^{2,4,10-16}

29 These issues with viral gene delivery vehicles have motivated a growing demand for safer and
30 less expensive nonviral gene delivery methods. Several nonviral gene delivery vehicles and
31 methods have been developed, including Lipofectamine, polyethyleneimine (PEI), lipid/polymer
32 hybrids, nanoparticles, and electroporation.^{1,17-19} However, while these methods are generally
33 less expensive and potentially safer than AAVs and LVs, they tend to provide lower transfection
34 efficiencies than viruses.^{20,21} Consequently, improving the efficiency of nonviral transgene
35 delivery and expression is an important and highly active area of research.

36 One possible way to accomplish this is by inhibiting the innate immune response to foreign
37 genes, which is shown in Figure 1. For example, both viral and nonviral transgenes can be
38 detected by endosomal or cytosolic DNA sensors (e.g., IFI16), which then utilize adaptor
39 proteins like STING to trigger a signaling cascade of kinases and transcription factors that
40 culminates in the activation of cytokines (e.g., interferon λ). These cytokines activate additional
41 pathways that induce the expression of cytokine-stimulated genes (CSGs) like IFIT1 and OAS1
42 that can directly inhibit the delivery and expression of viral and nonviral transgenes.²²⁻²⁴ For
43 example, IFI16 has been shown to decrease plasmid-driven transgene expression by directly
44 binding and blocking viral promoters.^{25,26} In addition, IFI16 is also a cytosolic/nuclear dsDNA
45 sensor that amplifies the innate immune response by activating STING and IRF3.²⁷⁻²⁹

46 While many studies have focused on specific components of the innate immune response to
47 plasmid DNA and nonviral gene delivery, our overall knowledge of the transcriptomic profile of
48 different cell types following transfection is still incomplete. In this study, mRNA-sequencing
49 was used to elucidate the innate immune response to plasmid DNA at the transcriptome level in a
50 panel of 4 cell lines (HEK293T, PC-3, Jurkats, and Primary T Cells) with varying transfection
51 efficiencies to identify host cell genes that may inhibit transfection. In addition, the effect of
52 serum and electroporation on the particularly potent innate immune response of PC-3 cells was
53 also investigated.

RESULTS

Transfection Efficiency

54 Three cancer cell lines (HEK-293T, PC-3, and Jurkat T cells) with a range of different
55 Lipofection efficiencies (see Figure 2A) were selected for this study with the goal of identifying
56 correlations between their wide range of transfection efficiencies and gene expression patterns.
57 Primary T cells were also included in the study because they are used for chimeric antigen
58 receptor (CAR) T cell therapy. As shown in Figure 2A, Lipofection of pDNA into HEK-293T
59 cells in serum-free media (SFM) provides a relatively high transfection efficiency ($87.3 \pm 1.2\%$
60 GFP⁺ cells; measured at 24 hours post-transfection), while progressively lower transfection
61 efficiencies were observed for PC-3 ($46.3 \pm 3.7\%$ GFP⁺ cells) and Jurkat T cells ($21.2 \pm 3.4\%$).
62 Finally, despite optimization of a Lipofection protocol in serum-free X-VIVO15 media, primary
63 T cells exhibited the lowest Lipofection efficiency ($8.1 \pm 0.8\%$).

64 A similar downward trend was also observed in GFP expression levels between the cell lines
65 (Figure 2A). While GFP was expressed at high levels in HEK-293T cells (Mean GFP = $30,266 \pm$
66 $2,715$) that were brightly fluorescent (Figure S1C), GFP levels were significantly lower in PC-3
67 cells (Mean GFP = $8,332 \pm 3,239$) and extremely low in Jurkat and CD3⁺ Primary T cells (Mean
68 GFP = 35 ± 11 & 112 ± 50 , respectively). Fluorescent microscopy images shown in the
69 supplementary information (Figure S1C-F) concur with these measurements, showing a decrease
70 in the brightness and number of fluorescent cells between the HEK-293T cells and the other cell
71 lines.

Differential Gene Expression

72 A graphical summary of the number of genes that were differentially expressed between the
73 control and transfected samples for each cell line is shown in Figure 2B, while complete lists of

74 the DEGs are included in the supplementary information. Figure 2 also shows specific TPM
75 plots for PC-3 (Figure 2C), HEK-293T (Figure 2D), Jurkat T cells (Figure 2E), and primary T
76 cells (Figure 2F).

77 Overall, PC-3 cells exhibited the strongest innate immune response, in which 1,057 genes were
78 upregulated and 764 genes were downregulated after Lipofection in SFM (Figures 2B/C). In
79 contrast, the HEK-293T cells that exhibited a higher transfection efficiency than PC-3 cells
80 upregulated a much lower number of genes ($n = 142$), with only 10 genes that were upregulated
81 more than 4-fold (Figure 2D). Likewise, the number of downregulated genes in HEK-293T cells
82 was also much lower ($n = 5$). Both types of T cells had nearly negligible responses to Lipofection
83 (Figure 2E/F), with only three significantly upregulated genes in Jurkat (MT1E, MT1F,
84 TMEM238) and a single upregulated gene in primary T cells (MT1H). No genes were
85 significantly downregulated in either type of T cell following Lipofection.

Validation of NGS with rt^2 PCR and ELISA

86 A select number of upregulated genes that were detected in PC-3 cells via mRNA-sequencing
87 (Figure 2C) were also evaluated using rt^2 PCR and ELISA. Figure 3A shows that while there
88 were some differences in the magnitude of upregulation observed with mRNA-sequencing
89 (green bars) and rt^2 PCR (blue bars), 7 different genes (IFNB1, IFNL1/2/3, CXCL10/11, &
90 CASP1) that were observed to be upregulated in mRNA-sequencing experiments were also
91 found to be significantly upregulated in rt^2 PCR assays, thereby reinforcing the mRNA-
92 sequencing results.

93 Likewise, an increase in IFN λ 1/3 secretion was also verified with ELISA. It is worth noting that
94 the antibody used in the ELISA assay binds to both IFN λ 1 and IFN λ 3, but significant increases

95 in both IFN λ 1 and IFN λ 3 expression levels were detected in PC-3 cells at 6 and 24 hours after
96 Lipofection in SFM (Figure 3B). IFN λ 1/3 levels were also significantly higher at 24 hours after
97 Lipofection than 6 hours post-transfection. However, when PC-3 cells were Lipofected in serum-
98 containing media (SCM) instead of SFM, there was no significant increase in IFN λ 1/3
99 expression levels between transfected and untransfected controls.

Comparison of Transfection Methods in PC-3 Cells

100 The observation that IFN λ 1/3 levels did not increase following Lipofection of PC-3 cells in
101 SCM (Figure 3) motivated us to further investigate the effects of serum on the transfection
102 efficiency and transcriptome of PC-3 cells. In addition, we also investigated the effects of
103 electroporation on the PC-3 transcriptome, since electroporation is a popular nonviral gene
104 delivery method that can provide relatively high transfection efficiencies.

105 Indeed, Figure 4A shows that electroporation (in SCM) provided a much higher transfection
106 efficiency ($92 \pm 2\%$ GFP⁺ cells) in PC-3 cells at 24 hours post-electroporation. Likewise, the
107 presence of serum during Lipofection also provided a slight, yet significant, increase in the
108 transfection efficiency of the PC-3 cells ($53 \pm 4\%$ GFP⁺ cells). In contrast, Lipofection of Jurkat
109 and Primary T cells in SCM did not significantly increase transfection efficiency (data not
110 shown).

111 mRNA-sequencing revealed that the presence of serum during Lipofection of PC-3 cells
112 decreased the number of genes that were significantly upregulated ($n = 619$, Figure 4B)
113 compared to SFM ($n = 1,057$). Likewise, electroporation also resulted in a substantial decrease in
114 the number of upregulated and downregulated genes ($n = 533$ and $n = 138$, Figure 4C) compared

115 to Lipofection in both SFM and SCM. A complete list of the DEGs identified in these
116 experiments is shown in a worksheet in the supplementary information.

117 Overall, the transfection efficiency of the PC-3 cells appeared to be inversely correlated with the
118 number of DEGs. Electroporation provided the highest transfection efficiency and lowest
119 number of DEGs, while Lipofection in SFM was associated with the highest number of DEGs
120 and the lowest transfection efficiency.

121 **Overexpression of IFI16 in HEK-293T Cells**

122 Analysis of the gene expression patterns in the different cell lines identified several genes (e.g.,
123 IFI16, IRF1, PSMB8, and PSMB9) with expression levels that appeared to be inversely
124 correlated with the transfection efficiency of the host cell (Figure 5). For example, IFI16 was
125 absent in HEK-293T cells, sharply upregulated in PC-3 cells, and constitutively expressed in
126 both T cell lines. To test the hypothesis that genes like IFI16 might be interfering with transgene
127 expression, IFI16 was transiently overexpressed from a plasmid in HEK-293T cells and then
128 subsequently transfected with pEF-GFP 24 hours later to determine if IFI16 might inhibit GFP
129 expression in HEK-293T cells. Indeed, Figure 6 shows that the HEK-293T cells that were only
130 transfected with pEF-GFP achieved a high transfection efficiency (80.3%), while cells that were
131 co-transfected with pIFI16 and pEF-GFP obtained at a significantly lower transfection efficiency
132 (33.2%). To ensure that this lower transfection efficiency was not solely due to the dual
133 transfections, additional samples were co-transfected with the luciferase expression plasmid
134 pGL4.50 and pEF-GFP using the same protocol. Cells in this category did exhibit a significantly
135 lower transfection efficiency (65.5%) than the cells transfected with only pEF-GFP, but a
136 Wilcoxon Rank Sum test showed a significant decrease in transfection efficiency between the
137 pGL4.50 group and the IFI16 group ($p = 2 \times 10^{-5}$).

DISCUSSION

Differences in DNA Sensing Pathways

138 One of the most drastic differences between the transcriptomic profiles shown in Figure 2 was a
139 decrease in the number of upregulated genes between PC-3 cells (n = 1,057) and the HEK-293T
140 (n = 142) cells. Furthermore, it is important to note that while several inflammatory cytokines
141 (e.g., IFN β , IFN λ , and IL-6) and CSGs were upregulated in PC-3 cells, no cytokines and CSGs
142 were detected in HEK-293T cells (Table S2). This dampened innate immune response suggests
143 that HEK-293T cells may be missing one or more components of the DNA sensing pathways
144 that drive the potent innate immune response in PC-3 cells.

145 Indeed, multiple components of the CpG-DNA sensing pathways were not detected in HEK-
146 293T cells (Table 1), including the endosomal CpG-DNA sensor TLR9. TLR9 was also absent in
147 PC-3, Jurkat, and primary T cells, but the cytosolic CpG-DNA sensors DHX9 and DHX36 were
148 detected in all the cell lines.³⁰ However, the downstream adaptor protein MyD88 that is required
149 for signaling by DHX9 and DHX36 (Figure 1) was also expressed at a relatively low level in
150 HEK-293T cells (TPM = 2.0 vs. TPM = 298.4 in PC-3 cells). Therefore, while CpG-DNA
151 sensing by DHX9 and DHX36 might contribute to the innate immune response to pDNA in PC-3
152 cells, the lack of MyD88 in HEK-293T cells may prevent them from sensing foreign CpG DNA.

153 The STING axis is an alternative cytosolic DNA sensing pathway that may also drive the innate
154 immune response to pDNA in PC-3 cells. PC-3 cells express each component of the STING-
155 mediated DNA sensing pathway (Table 1), which is induced when IFI16 or cGAS bind to
156 cytosolic dsDNA and then signal through STING to activate TBK-1, IRF3, and NF- κ B (Figure
157 1). However, several important components of this pathway were either absent or expressed at
158 relatively low levels in HEK-293T cells. For example, IFI16 was detected in PC-3 cells and

159 upregulated 37-fold following Lipofection, but it was absent in HEK-293T cells. Three more
160 components of the STING pathway (cGAS, STING, and IKBKE) were also upregulated in the
161 transfected PC-3 cells but expressed at nearly negligible levels (TPM = 0.1-0.6) in HEK-293T
162 cells. Altogether, these data show that while PC-3 cells can sense pDNA with multiple redundant
163 pathways and mount a potent innate immune response that induces hundreds of cytokines and
164 CSGs, HEK-293T cells lack essential proteins at bottlenecks in DNA sensing pathways (e.g.,
165 MyD88 and STING), which may explain the lack of cytokine and CSG expression shown in
166 Tables S2 and S3, respectively.

167 It is worth noting that 142 genes were upregulated in the transfected HEK-293T cells, but those
168 genes do not have any known functions that could potentially inhibit transgene expression.
169 Instead, it is possible that those genes may have been upregulated in response to the stress or
170 toxicity caused by the presence of Lipofectamine.

171 Many other groups have also observed the high transfection efficiency exhibited by HEK-293T
172 cells, which has led to their widespread adoption in industry and academia.³¹⁻³³ The high
173 transfection efficiency and lack of CSG expression in HEK-293T cells may be due to the SV40
174 large T antigen, which was integrated into the parental HEK-293 cell genome to create the HEK-
175 293T cell line.³⁴ The SV40 large T antigen has previously been shown to improve the replication
176 efficiency of DNA viruses³⁵ and inhibit the induction of interferon expression by the cGAS-
177 STING signaling pathway in other cell lines.³³ Likewise, proteins expressed by the human
178 papilloma virus (HPV) have also been shown to inhibit STING,³⁶ while the absence of STING in
179 hepatocytes has been correlated with a lack of cytokine expression during hepatitis B virus
180 (HBV) infection.³⁷ In regards to nonviral gene delivery, the inhibition of STING with small
181 molecule inhibitors (C176, C178) has been shown to enhance transgene expression by up to 3-

182 fold in a variety of cell lines, including primary T cells.³⁸ These previous studies and our
183 observations collectively emphasize the importance of the STING DNA sensing pathway and
184 suggest that targeting STING for inhibition may be an effective strategy to improve the potency
185 of gene therapy treatments.

Constitutive Expression of CSGs in T cells

186 Another interesting observation is that the Jurkat and primary CD3⁺ T cells showed almost no
187 response to Lipofection, except for the upregulation of a few metallothioneins (MT1H, MT1E,
188 and MT1F). Metallothioneins have previously been shown to restrict bacterial and viral
189 replication, but it is unclear how they may interfere with nonviral gene delivery or
190 expression^{39,40}.

191 In contrast to the PC-3 cells, no interferons or CSGs were upregulated following Lipofection in
192 either T cell line (Table S2). It is worth noting that some chemokines (CXCL8/10/13) and TNF α
193 were detected in the primary T cells, but each of these targets was detected in both the
194 transfected and untransfected cells. Therefore, it is more likely that these cytokines were induced
195 by the activation of the primary T cells with IL-2 rather than the transfection of plasmid DNA.

196 The lack of interferon and CSG upregulation following Lipofection of the T cells is somewhat
197 surprising, since transcripts for all the requisite components of multiple DNA sensing pathways
198 were detected in the T cells (e.g., IFI16, STING, TBK-1, and IRF3). Therefore, the T cells
199 should be able to induce expression of cytokines, but interleukins and interferons like IFN λ 1
200 were not expressed in the T cells following transfection (Table S2). Similar observations were
201 made in a previous study that also detected IFI16 and TBK-1 expression in T cells, but no

202 interferon expression.⁴¹ Therefore, it appears that T cells may either lack an unknown component
203 that is required for cytokine induction or the interferon genes may be epigenetically silenced.⁴²

204 Although interferons and CSGs were not upregulated in T cells after Lipofection, several CSGs
205 with established anti-viral activities were constitutively expressed in both transfected and
206 untransfected T cells. Furthermore, many of the CSGs that were expressed in the T cells were
207 completely absent in the easily transfected HEK-293T cells and significantly upregulated in the
208 PC-3 cells that had a moderate transfection efficiency. This inverse correlation between the
209 expression levels of these CSGs and the transfection efficiency of each host cell line suggests
210 that these CSGs may inhibit transgene uptake or expression. A complete list of the CSGs which
211 follow this trend (absent in HEK-293T, upregulated in PC-3, and constitutively expressed in T
212 cells) is shown in Table S3, while the TPMs of some representative CSGs in each cell line are
213 shown in Figure 5.

214 While Table S3 consists of a wide variety of 54 different CSGs, the four genes highlighted in
215 Figure 5 are the most likely to inhibit transgene expression. For example, the constitutive
216 expression of CSGs in the absence of interferons may be driven by the transcription factor IRF1,
217 which was upregulated 8-fold in PC-3 cells after Lipofection and detected in all T cell samples
218 (TPM = 62-76). Unlike other interferon regulatory factors (IRFs), IRF1 does not require
219 phosphorylation to activate its target genes. Therefore, IRF1 may continuously drive high level
220 expression of CSGs in T cells.⁴³

221 IFI16 is another important anti-viral restriction factor. As previously mentioned, IFI16 is a
222 cytosolic DNA sensor, but recent studies have revealed additional anti-viral functions for
223 IFI16.⁴⁴ For example, IFI16 can repress viral genes by directly binding viral promoters to inhibit
224 transcription.^{27,45,46} Some viruses have adapted to this problem by expressing inhibitory proteins

225 (e.g., ICP0 from HSV-1) that induce the degradation of IFI16 and prevent its transcriptional
226 repression.²⁵ Therefore, while our results show that IFI16 is unable to trigger the expression of
227 interferons and other cytokines in T cells, IFI16 may instead repress the transcription of both
228 viral and nonviral transgenes by binding to their upstream promoters. Additionally, IFI16 is
229 known to form an inflammasome complex with PYCARD, Caspase 1/8, and Gasdermin D upon
230 binding to foreign DNA which can lead to inflammation and cell death. Indeed, pyroptosis has
231 been observed during abortive HIV infection of T cells.^{47,48}

232 Several of the CSGs listed in Table S3 have very well-established roles in the adaptive and
233 innate immune responses to viral infection, but it is unclear how they might inhibit the
234 expression of nonviral transgenes. For example, PSMB8 and PSMB9 are two closely related
235 CSGs that were upregulated in PC-3 cells and expressed at similarly high levels in both T cell
236 lines. PSMB8 and PSMB9 have been shown to regulate transcription changes during the innate
237 immune response, but they are more widely known for their role in the assembly and function of
238 the immunoproteasome, which selectively degrades foreign proteins into peptide antigens that
239 are then displayed on MHC-1.⁴⁹⁻⁵¹ This process is essential to the recruitment of cytotoxic CD8⁺
240 T cells to virus-infected cells *in vivo*, but it is not yet known if the immunoproteasome may
241 specifically interfere with transgene expression.

Effects of Serum and Electroporation on the PC-3 Transcriptome

242 Our initial transfections were conducted in serum-free media (SFM) to avoid any potentially
243 confounding effects on the transcriptome from components in the serum.⁵²⁻⁵⁴ However, serum
244 components like albumin have also been reported to enhance Lipofection efficiency.^{55,56}
245 Likewise, several studies have shown that electroporation can provide relatively high
246 transfection efficiencies, even in cell lines that are relatively difficult to transfect.^{21,22} As shown

247 in Figure 4A, electroporation in SCM also yielded significantly higher transfection efficiencies
248 in PC-3 cells than Lipofection in SFM.

249 While it is possible that electroporation achieves a higher transfection efficiency by simply
250 delivering more of the transgene to the cell, there are some intriguing differences in gene
251 expression that were observed between the Lipofected and electroporated cells. For example, the
252 TPMs of several DNA sensors, cytokines, and CSGs seemed to be inversely correlated with the
253 transfection efficiencies of the different types of transfected PC-3 cells. Table 2 shows some of
254 the more noteworthy genes that were highly upregulated after Lipofection with SFM but
255 expressed at significantly lower levels in electroporated PC-3 cells.

256 Several components of the STING axis of DNA sensing were expressed at significantly lower
257 levels in the electroporated PC-3 cells. IFI16 emerged again as a notable example of this trend
258 since it was highly upregulated (TPM = 945.5) following Lipofection in SFM but expressed at
259 significantly lower levels during Lipofection in SCM (TPM = 421.4) and electroporation (TPM
260 = 149.3). Other important genes that were expressed at lower levels in electroporated PC-3 cells
261 include STING, mediators of cytokine signaling (JAK2 and STAT2), and components of the
262 inflammasome pathway (AIM2 and Caspase 1) that induce inflammation or apoptosis in response
263 to cytosolic DNA.²² Altogether, these observations show that DNA sensing pathways may be
264 less active in electroporated PC-3 cells, which could explain the lower number of cytokines and
265 CSGs observed in those samples.

266 Indeed, multiple chemokines and interferons that were highly expressed in PC-3 cells following
267 Lipofection in SFM were expressed at much lower levels in the electroporated cells. Table 2
268 shows that all the interferons (IFN α , IFN β , and IFN λ s) were expressed at lower levels following
269 electroporation, along with the chemokines CXCL 10 and 11. Another intriguing observation is

270 that the expression of two other secreted proteins - midkine (MDK) and LGALS9 – decreased
271 slightly in the presence of serum and to much lower levels (6 to 10-fold lower) during
272 electroporation. LGALS9 and MDK have previously been shown to inhibit the initial binding of
273 viral capsids and cationic lipoplexes to cell membranes by blocking heparan sulfate
274 proteoglycans (HSPGs), syndecans (SDCs), and other cell surface receptors that are crucial for
275 gene delivery.^{57–61} Therefore, it is possible that the lower transfection efficiencies observed in
276 PC-3 cells cultured in SFM may be due to inhibition of lipoplex endocytosis by MDK or
277 LGALS9.

278 The importance of HSPGs and SDCs in gene delivery is also highlighted by the observation that
279 these genes were expressed at much lower levels in T cells than PC-3 and HEK-293T and PC-3
280 cells (see Table S4). Previous studies have also shown this lack of HSPG expression in T cells,⁷⁷
281 which provides another explanation for their relatively low transfection efficiencies.

282 Finally, many other CSGs that were expressed at lower levels after electroporation than
283 Lipofection in SFM are listed in Table 2 and the worksheet in the supplementary information.
284 The functions of many of these genes have not yet been revealed, but there are some CSGs with
285 known functions that might inhibit transgene expression. For example, much like IFI16, TRIM22
286 can specifically bind transgene promoters to exclude other activating transcription factors (e.g.,
287 SP-1).^{46,62} Alternatively, TRIM22 has also been shown to ubiquitinate viral proteins to target
288 them for degradation.⁶³

289 **Inhibition of Transgene Expression by IFI16**

290 While there are many CSGs that could potentially inhibit transgene expression, the strongest
291 inverse correlation between CSG expression levels and host cell transfection efficiencies was
292 observed for IFI16. Indeed, the lack of IFI16 in HEK-293T cells may explain their lack of

293 cytokine expression and high transgene expression levels, while the high levels of IFI16
294 expression in T cells and PC-3 cells may decrease transgene expression. However, it is
295 important to note that IFI16 expression levels were much lower in PC-3 cells following
296 electroporation, which may explain the much higher electroporation efficiencies observed in PC-
297 3 cells.

298 Figure 6 also shows that overexpression of IFI16 in HEK-239T cells subsequently decreases
299 GFP expression. This decrease in transfection efficiency is probably not due to the DNA sensing
300 activity of IFI16, since HEK-293T cells lack STING and TBK-1. Instead, it is more likely that
301 IFI16 directly binds to the transgene to repress its transcription, as previously described for other
302 viral transgenes.^{27,45,46}

303 Overall, our analysis of the transcriptomes of several types of transfected cells emphasizes the
304 importance of DNA sensing pathways and specific CSGs (e.g., IFI16, IRF1, et al.) in the innate
305 immune response to transgene delivery. Our findings contribute to a growing body of literature
306 that indicate the STING axis is particularly important for DNA sensing and subsequent cytokine
307 expression, since the expression levels of IFI16 and STING are inversely correlated with
308 transfection efficiencies in multiple cell lines and transfection methods. Our results also show
309 that while PC-3 cells exhibit a potent innate immune response that limit their Lipofection
310 efficiency in serum-free media, it is possible to dampen the innate immune response and increase
311 transfection efficiency by adding serum or using electroporation to deliver pDNA.

312 Finally, our mRNA-sequencing experiments have identified multiple possible reasons for the
313 notoriously low Lipofection efficiencies observed for T cells in this study and previous studies,
314 including a lack of HSPGs and the constitutive expression of repressive CSGs like IFI16 by
315 IRF1. Several other studies have demonstrated that IFI16 can inhibit transgene delivery and

316 expression, but additional knockout studies will be necessary to confirm the potential roles of the
317 other CSGs in transgene expression. However, after transgene repressors have been identified,
318 short interfering RNAs (siRNAs) or small molecule inhibitors could be developed to inhibit
319 these targets and potentially improve the potency of future viral and future nonviral gene
320 therapies.

MATERIALS AND METHODS

Reagents

321 Lipofectamine LTX was purchased from ThermoFisher Scientific (#15338100). The GFP and
322 IFI16 expression plasmids were purchased from Addgene (Plasmids 11154 and 35051,
323 respectively. Watertown, MA), while the luciferase expression plasmid pGL4.50 was purchased
324 from Promega (cat# E1310, Madison, WI).

Cell Lines

325 Human PC-3 prostate cancer (Cat# CRL-1573), Jurkat T lymphoma (TIB-152), and HEK-293T
326 embryonic kidney (CRL-11268) cells were purchased from ATCC, while donated primary CD3⁺
327 samples of T cells from 3 different donors were purchased from Cellero (formerly known as
328 Astarte Bio). PC-3, Jurkat, and HEK-293T cells were cultured in serum-containing RPMI-1640
329 media that was supplemented with 10% fetal bovine serum, except during the 24-hour period
330 following a transfection (unless otherwise noted). In contrast, Primary T cells were cultured in
331 serum-free X-VIVO15 media that was supplemented with 0.5 ng/mL IL-2 and anti-CD3/28
332 Dynabeads in a 1:1 cell:bead ratio. Dynabeads were replaced weekly, while IL-2 was added to
333 the media every 2-3 days.

Transfections

334 In preparation for mRNA sequencing, each cell line was transfected using specific conditions
335 that maximized transfection efficiency (data not shown). Adherent cell lines (PC-3 and HEK-
336 293T) were grown to 50-70% confluency in T-75 flasks (~5M cells/flask) and then transfected
337 with lipoplexes that were prepared by mixing pEF-GFP (4.5 ug) with Lipofectamine LTX (9 uL)
338 and PLUS reagent (4.5 uL) in ~200 uL of OptiMEM media.

339 Jurkat T cells were seeded into T-25 flasks (2.5×10^6 cells) and then transfected with lipoplexes
340 that were prepared by mixing pEF-GFP (13.2 ug) with Lipofectamine LTX (36.2 uL) and PLUS
341 reagent (13.2 uL) in ~200 uL of OptiMEM media. Smaller cultures of primary CD3⁺ T cells
342 were seeded at a density of 200,000 cells/well and then transfected with lipoplexes that were
343 prepared by mixing 1 ug pEF-GFP/well, 2.75 uL Lipofectamine LTX/well, and 1 uL PLUS
344 reagent/well.

345 With the exception of the Lipofection and electroporation experiments conducted with serum-
346 containing media (SCM) in PC-3 cells, the SCM was removed from the cells in all other
347 experiments and replaced with serum-free media (SFM) before the lipoplexes were added to
348 each of the cell lines. Cells were then subsequently incubated for 24 hours at 37°C in SFM.
349 Finally, a Millipore Guava flow cytometer was used to quantify transfection efficiency (%GFP
350 cells) and transgene expression (mean GFP) prior to RNA isolation.

IFI16 Overexpression in HEK-293T Cells

351 HEK-293T cells were seeded into 24-well plates at 25,000 cells/well in SCM and incubated at
352 37°C on Day 1. The wells on each plate were then divided into 4 groups – a negative control
353 group that was never transfected, a positive control group that was transfected once with pEF-
354 GFP on Day 3, and two other groups that were transfected with either pGL4.50 (a luciferase
355 expression plasmid) or pIFI16-IL (IFI16 expression plasmid) on Day 2 and then transfected
356 again with pEF-GFP on Day 3. In each transfection, plasmids were administered at 1µg
357 DNA/well along with 1µL/well Lipofectamine LTX and 0.5µL/well PLUS reagent. Plates were
358 incubated for an additional 48 hours at 37°C and transfection efficiency (%GFP cells) was
359 measured using a Millipore Guava flow cytometer on Day 5.

Electroporation

360 PC-3 cells were trypsinized, centrifuged at 1,000 RPM for 4.5 minutes, and then washed once
361 with PBS before centrifuging the cells again and resuspending them in PBS again. Plasmid DNA
362 (7.5 μg pEF-GFP) and 2.5×10^6 PC-3 cells were then mixed in a total volume of 100 μL in
363 sterile 2 mm-gap electroporation cuvettes (in that order). A Bio-Rad Gene Pulser Xcell
364 electroporation system with the CE module was then used to briefly electroporate the cells
365 (110V, 25 ms, single square wave pulse). The electroporated cells were then immediately plated
366 in preheated RPMI media with serum and incubated at 37°C until needed.

mRNA Sequencing

367 Total RNA samples were extracted from cell samples with a Qiagen RNEasy kit. The RNA
368 samples (2 μg total RNA) were then submitted to either Genewiz (Jurkat samples) or the Beijing
369 Genomics Institute (PC-3, HEK-293T, and Primary T cell samples) for library preparation and
370 mRNA-sequencing. Specifically, mRNA was isolated from high quality total RNA samples with
371 RIN > 9 and 28S:18S > 1 (measured with an Agilent 2100 Bioanalyzer) using poly-T
372 oligonucleotide beads. The mRNAs were then cleaved into smaller fragments that were
373 subsequently reverse transcribed by DNA Polymerase I into cDNA using random N6 primers.
374 Adapters were then ligated onto the cDNAs and the resulting libraries were sequenced using
375 either a BGI-500 sequencer (BGI) or an Illumina HiSeq (Genewiz). Low quality reads were then
376 filtered to produce clean reads that were mapped to the human genome/transcriptome using
377 HISAT, Bowtie2, and RSEM to calculate gene expression values/counts. Counts were then
378 analyzed using DESeq2 in RStudio with independent filtering turned off (see Figure S2) to
379 identify differentially expressed genes (DEGs), which were defined as genes with adjusted p-
380 values (p_{adj}) less than 0.05 and at least a 2-fold change in TPM following transfection.

381 Transcripts per million (TPM) values were calculated by dividing the number of counts for each
382 gene by each gene's length to obtain reads per kilobase (RPK) values. The RPK for each gene
383 was then divided by the sum of the RPK values measured for all genes (and multiplied by one
384 million) to obtain TPM values. The complete mRNA-seq data (fastq files and a spreadsheet of
385 TPM values) from these experiments are available at the NCBI GEO repository (GEO Accession
386 #GSE166630).

NGS Validation with rt^2 PCR

387 The same RNA samples that were used for mRNA-sequencing were also analyzed with rt^2 PCR.
388 First, mRNA was reverse transcribed using the SuperScript IV VILO™ rt^2 PCR master mix with
389 ezDNase™ (ThermoFisher #11766050) according to the manufacturer's protocol. The resulting
390 cDNA was then quantified using a SYBR green qPCR master mix (ThermoFisher # 4472942)
391 with a QuantStudio 3 qPCR instrument (ThermoFisher). All measurements were repeated in
392 triplicate and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was
393 used as a reference gene/control. The primer sequences that were used are shown in Table S1.

Cytokine Quantification with ELISA

394 Samples of the supernatant media were taken from PC-3 cultures at 6 and 24 hours after
395 transfection with Lipofectamine and stored at -72°C until needed. DuoSet ELISA kits were then
396 used to quantify IFNL1/3 (Biotechne #DY1598B-05) levels according to the manufacturer's
397 protocol.

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401 (Addgene plasmid # 11154) while the pIFI16-FL plasmid was a gift from Cheryl Arrowsmith
402 (Addgene plasmid # 35051).

403

404 **AUTHOR CONTRIBUTIONS**

405 Jacob Elmer: Conceptualization, Methodology, Supervision, Validation, Data Curation, Writing,
406 Project Administration, & Funding Acquisition. Eric Warga: Investigation, Formal Analysis,
407 Validation, & Writing. Matthew Tucker: Investigation. Emily Harris: Investigation.

408

409 **Conflict of Interest Statement**

410 The authors declare that there is no conflict of interest.

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Figure Captions

Figure 1. Plasmid DNA can be recognized by several DNA sensors (yellow), which trigger a cascade of kinases (green) and transcription factors (purple) that culminates in the expression of cytokines that are secreted and bind to cognate receptors (gray) that induce the expression of cytokine stimulated genes (blue) that can trigger apoptosis or inhibit transgene expression in a variety of ways.

Figure 2. Differences in transfection efficiency and transcriptomes between cell lines transfected with Lipofectamine LTX in serum-free media (SFM). (A) Transfection efficiency (%GFP⁺ cells) and transgene expression levels (Mean GFP) for each cell line at 24 hours after transfection with Lipofectamine LTX and pEF-GFP plasmid DNA in serum free media (SFM). Representative flow cytometry histograms and fluorescent microscopy images for each cell line are also shown in Figure S1. (B) Total number of upregulated and downregulated differentially expressed genes (DEGs) observed in each cell line following Lipofection in SFM. DEGs were defined as having at least a 2-fold change in TPM that was statistically significant ($P_{\text{adj}} < 0.05$) over the course of 3 independent experiments. (C-F) Gene expression levels (TPMs) in HEK-293T cells (C), PC-3 cells (D), Jurkat T cells (E), and primary CD3⁺ T cells (F) that were either transfected with Lipofectamine LTX (y-axis) or not transfected (x-axis). All TPM values are averaged from 3 separate transfections and corresponding mRNA-sequencing experiments. Green triangles indicate genes that were significantly upregulated in transfected cells ($P_{\text{adj}} < 0.05$), red circles indicate downregulated genes ($P_{\text{adj}} < 0.05$), and gray squares represent unaffected genes.

Figure 3. Validation of mRNA-sequencing results with rt²PCR (A) and ELISA (B). Asterisks (*) indicate significant increases in target levels following transfection relative to untransfected control cells, while carets (^) indicate significant increases between ELISA measurements at 6 and 24 hours after transfection.

Figure 4: Effects of serum and electroporation on transfection efficiency and the PC-3 transcriptome. (A) Transfection efficiencies obtained with PC-3 cells using Lipofectamine in either SFM or SCM and Electroporation in SCM (blue bars, left axis). (B) A summary of the number of genes that were upregulated (green bars, right axis) or downregulated (red bars, right axis) following each type of transfection. Asterisks indicate significant differences in transfection efficiency (* = significantly higher than Lipofection in SFM, ** = significantly higher than Lipofection in SFM and SCM, $p < 0.05$ as determined by Student's t-test). (B/C) Gene expression levels (TPM) for PC-3 cells that were Lipofected (B) or electroporated (C) in SCM. All TPM values are averaged from 3 separate transfections and corresponding mRNA-sequencing experiments. Green triangles indicate genes that were significantly upregulated in transfected cells, red circles indicate downregulated genes, and gray squares represent unaffected genes.

Figure 5. Expression levels (TPM) of five representative cytokine-stimulated genes (CSGs) that were observed to be absent or expressed at low levels in HEK-293T cells, upregulated during Lipofection in PC-3 cells, and constitutively expressed at relatively high levels in Jurkat and Primary T cell lines.

Figure 6. Effects of IFI16 overexpression on pEF-GFP transfection efficiency (%GFP⁺ cells) in HEK-293T cells. Untransfected negative control cells were not transfected, while positive control cells were only transfected with pEF-GFP (green box). The blue box represents cells that were transfected with the luciferase expression plasmid pGL4.50 one day prior to transfection with pEF-GFP, while the red box represents cells that were transfected with the IFI16 expression plasmid pIFI16-FL one day before transfection. Horizontal lines within boxes represent the means for each group, while boxes show the interquartile range, and the entire dataset is contained within the whiskers. Letters (a, b, c) indicate samples with significantly different transfection efficiencies (n = 18 for each sample, p < 0.05 using a Wilcoxon Rank Sum test).

Tables

Table 1. Expression levels (TPM) of genes involved in DNA sensing pathways in each cell line.

		<u>HEK-293T</u>		<u>PC-3</u>		<u>Jurkat T</u>		<u>Primary T</u>	
		Ctrl	Trans	Ctrl	Trans	Ctrl	Trans	Ctrl	Trans
PYHIN Sensors	IFI16	0	0	25.7	945.5	294.1	266.8	274.9	215.1
	PYHIN1	0	0	0	0.1	1.1	0.9	19.6	10.2
	MNDA	0	0	0	0	0	0	0	0
AIM2	AIM2	0	0	0	10.0	0	0	28.8	34.1
	PYCARD	0.1	0.1	6.1	6.8	0	0	63.0	22.9
	CASP1	0	0	0	56.0	0	0	29.9	24.0
LRR- FIP1	LRRFIP1	39.1	45.0	135.3	129.7	105.3	96.4	168.2	159.8
	CTNNB1	225.4	311.2	269.0	195.8	105.6	99.0	105.5	103.8
	TCF4	5.5	6.9	10.5	21.2	2.6	2.3	0.7	0.5
STING-Axis DNA Sensing Pathway	CGAS	0.1	0.2	25.2	46.5	0	0	42.0	49.8
	DDX41	58.6	63.0	123.0	91.6	113.2	121.3	73.2	77.3
	ZBP1	0.1	0.1	0	5.8	0	0	1.3	0.2
	POLR3A	54.9	52.9	97.5	107.3	30.0	29.0	18.0	19.1
	DDX58	2.7	2.3	17.8	541.2	11.3	10.1	9.6	7.0
	MAVS	13.9	18.2	46.0	23.6	27.0	26.4	30.7	25.0
	STING1	0.2	0.6	19.0	42.2	56.6	59.9	144.1	141.7
	TBK1	44.1	38.1	40.8	39.9	30.8	25.7	54.0	51.7
MYD88-Axis DNA Sensing Pathway	TLR9	0.1	0.1	0.1	0.4	0	0	0.9	0.4
	DHX9	260.9	288.0	133.0	85.6	341.0	311.8	198.7	222.8
	DHX36	63.8	65.3	101.1	94.3	88.8	73.9	51.5	53.0
	MYD88	1.3	1.9	52.9	298.3	77.8	84.3	60.9	48.4
	IRAK1	171.5	195.1	163.9	147.2	158.5	162.0	113.1	135.7
	IRAK4	8.9	8.5	13.4	14.3	24.0	21.1	18.1	14.9
	TRAF6	4.0	4.8	8.0	9.6	7.1	6.8	6.0	6.1
	MAP3K7	34.6	36.1	37.3	22.1	51.8	44.8	29.7	26.8
	TAB1	36.7	40.3	18.2	22.4	26.4	28.7	21.9	17.0
	TAB2	63.5	65.7	47.4	61.0	33.8	30.0	117.7	113.2
	MAP2K4	34.8	40.6	28.4	18.5	26.4	24.5	24.4	26.8
	MAP2K7	54.9	54.3	36.6	41.3	39.4	42.2	39.6	38.3
	MAPK8	20.2	20.8	21.5	18.1	102.1	89.7	22.4	24.1
	MAPK9	57.4	55.3	39.9	21.8	47.1	44.1	29.3	27.2
	MAP2K3	31.6	39.7	46.6	74.4	53.6	55.1	178.0	170.9
	MAP2K6	6.8	14.9	41.5	19.4	17.0	14.6	4.5	1.8
	MAPK14	42.2	40.7	44.3	28.0	83.5	77.7	41.7	34.7
JUN	104.4	154.1	47.6	159.7	6.4	5.7	144.4	138.7	
MAP3K14	6.5	5.5	14.4	25.3	5.0	5.5	38.6	34.3	
IKBKE	0.4	0.5	9.7	18.6	18.9	20.0	16.0	9.0	
NFκBIA	16.9	18.8	67.5	431.7	32.2	31.5	394.7	525.5	
Transc. Factors	NFκB	18.7	20.9	34.7	44.0	52.5	48.2	97.5	103.9
	IRF5	0	0	2.9	5.4	0.6	0.6	36.1	30.1
	IRF7	0.8	1.1	23.7	379.1	5.7	6.1	26.1	27.4
	IRF3	65.5	71.5	63.5	79.8	76.3	81.5	92.2	82.2

Table 2 – Genes expressed at significantly lower levels in electroporated PC-3 cells vs. Lipofected PC-3 cells

	Vehicle: Media: Symbol	Lipofectamine				Electroporation		P _{adj}
		SFM		SCM		SCM		
		Ctrl	Trans	Ctrl	Trans	Ctrl	Trans	
DNA Sensors & Downstream Pathways	IFI16	25.7	945.5	14.1	421.4	12.3	149.3	**
	STING1	19.0	42.2	20.1	54.1	8.9	9.1	**
	JAK2	10.8	50.3	13.0	19.3	10.5	11.7	**
	STAT2	33.8	261.9	41.0	210.7	16.3	52.8	**
	AIM2	0	10.0	0	6.6	0	2.2	*
	CASP1	0	56.0	0.5	21.8	0.6	3.8	**
Midkine, Chemokines, & Cytokines	MDK	599.6	2058.9	543.2	990.9	330.5	332.2	*
	LGALS9	0.07	112.23	0.08	59.30	0.21	11.36	**
	CXCL11	0.1	231.5	0	54.2	1.0	47.3	**
	CXCL10	0.1	137.2	0	48.7	0.3	29.9	**
	IFNL1	0	131.1	0	33.8	0.2	26.7	**
	IFNB1	0	113.1	0	21.0	0	16.3	**
	IFNL2	0	67.8	0.1	22.1	0.1	7.0	*
	IFNL3	0	41.2	0	16.6	0.2	5.3	*
	IFNA7	0	3.7	0	0.4	0	0	*
	IFNA10	0	1.5	0	0.1	0	0	*
IFNA16	0	0.8	0	0.2	0	0	*	
Selected CSGs	BST2	3.1	1145.8	0.6	652.8	0.1	136.3	**
	PSMB9	5.8	191.7	7.8	196.3	4.8	39.6	**
	IFIT2	23.1	3070.6	44.4	1916.0	26.4	347.8	**
	TRIM22	0.77	218.36	0.10	105.61	0.06	17.53	**
	RSAD2	2.9	1418.0	0.9	768.7	1.3	185.6	**
	LGALS3BP	84.7	1313.9	163.5	1512.9	94.9	336.8	**
	SERPINE1	90.4	789.8	285.9	1854.8	164.0	134.0	**
	CD68	50.6	727.2	88.5	439.1	55.5	109.4	**
	WARS1	98.8	704.1	119.5	256.3	99.1	129.1	**
	SAMD9	12.1	665.7	14.5	347.7	11.8	149.2	**

**Gene expression levels significantly lower in electroporated PC-3 cells versus cells Lipofected in SFM and SCM

*Gene expression levels significantly lower in electroporated PC-3 cells only compared to cells Lipofected in SFM (i.e., not significantly lower than PC-3 cells Lipofected in SCM)











