Title: Interactions with Commensal and Pathogenic Bacteria Induce HIV-1 Latency in Macrophages through Altered Transcription Factor Recruitment to the LTR

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Running Title: Induction of HIV-1 Latency in Macrophages by Bacteria

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ABSTRACT

Macrophages are infected by HIV-1 *in vivo* and contribute to both viral spread and pathogenesis. Recent human and animal studies suggest that HIV-1-infected macrophages serve as a reservoir that contributes to HIV-1 persistence during anti-retroviral therapy. This phenomenon may be influenced by the local tissue microenvironment, including interactions with commensal and pathogenic microbes. These effects are thought to result, in part, from engagement of Toll-like receptors (TLRs); however, the effects of TLR signaling on virus replication in macrophages are not fully understood. We wished to determine if the sexually transmitted pathogen *Neisseria gonorrhoeae* (GC) and the gut-associated microbe *Escherichia coli* (*E. coli*), which encode ligands for TLR2 and TLR4, repress HIV-1 replication in macrophages and thereby induce a state of viral latency. We found that purified TLR ligands that signal exclusively through the adapter MyD88 activated HIV-1 expression in macrophages, whereas TLR ligands that signal through TRIF repressed HIV-1 expression. Inhibiting TLR4 signaling, blocking type 1 interferon, and knocking-down TRIF reversed LPS- and GC-mediated repression of HIV-1. Finally, TLR4-mediated repression of HIV-1 in macrophages was associated with the recruitment of interferon regulatory factor 8 (IRF8) to the interferon stimulated response element (ISRE) downstream of the 5' LTR. Our data indicate that IRF8 is responsible for repression of HIV-1 replication in macrophages in response to the TRIF-dependent signaling during GC and *E. coli* co-infection. These findings highlight the potential role of macrophages as HIV-1 reservoirs as well as the role of the tissue microenvironment and co-infections as modulators of HIV-1 persistence.

IMPORTANCE The major barrier toward the eradication of HIV-1 infection is the presence of a small reservoir of latently-infected cells, which include CD4+ T cells and macrophages, that escape immune-mediated clearance and the effects of anti-retroviral therapy. There remain

40 crucial gaps in our understanding of the molecular mechanisms that lead to transcriptionally
41 silent or latent HIV-1 infection of macrophages. The significance of our research is in identifying
42 microenvironmental factors, such as commensal and pathogenic microbes, that can contribute
43 to the establishment and maintenance of latent HIV-1 infection in macrophages. It is hoped that
44 identifying key processes contributing to HIV-1 persistence in macrophages may ultimately lead
45 to novel therapeutics to eliminate latent HIV-1 reservoirs *in vivo*.
46
47 **INTRODUCTION**
48
49 Macrophages are among the immune cells located within the gastrointestinal and genitourinary
50 mucosae thought to play a role in HIV-1 sexual transmission and pathogenesis (1-3). A number
51 of studies examining either HIV-1 infection of human vaginocervical or gastrointestinal tissue
52 explants or SIV_{mac} infection in a rhesus macaque animal model have shown that macrophages
53 are among the earliest cells infected during mucosal transmission (2, 4) Zalar 2010.
54 Macrophages can be productively infected with HIV-1 and are thought to be a source of virus
55 persistence *in vivo* (5). Given their role in transmission, pathogenesis, and viral persistence, it is
56 important to understand how the local mucosal microenvironment and cellular signaling
57 pathways modulate interactions between macrophages and HIV-1.
58
59 Sexually-transmitted infections (STIs) have been shown to be co-factors that enhance HIV-1
60 transmission (6). *Neisseria gonorrhoeae* (gonococcus, GC) is a non-ulcerative STI that is
61 thought to enhance mucosal transmission of HIV-1 both by inducing inflammation and by
62 directly activating virus infection and replication (7-12). The inflammatory response to GC can
63 contribute to enhanced transmission by promoting a favorable local environment for virus
64 replication and by inducing the recruitment of HIV-1 target cells to the site of inflammation (12).
65

66 The role of GC in HIV-1 persistence is less understood. A number of studies have implicated
67 GC-encoded pathogen-associated molecular patterns (PAMPs) as mediators of both
68 inflammation and HIV-1 activation in target cells such as macrophages; however, the
69 interactions of GC with macrophages are complex. GC encodes PAMPs capable of engaging
70 Toll-like receptors (TLRs), including TLR2, TLR4, and TLR9 (13, 14). The effects of live GC on
71 HIV-1 replication in macrophages have not been reported; however, purified LOS as well as
72 *Escherichia coli* lipopolysaccharide (LPS) have been shown to repress virus replication through
73 production of type 1 IFN (15, 16). In the case of LPS, repression was due to undefined effects at
74 the level of gene expression. It is not clear how TLR2 signaling affects HIV-1 in macrophages;
75 however, purified TLR2 ligands activate virus replication in macrophages (17) and latently-
76 infected T cells (18).
77
78 Here, we demonstrate that co-infection with live GC and *E. coli* repress HIV-1 expression in
79 macrophages. To investigate the underlying mechanism(s) responsible for this repression, we
80 examined the individual effects of TLR2 and TLR4 signaling on HIV-1 expression in
81 macrophages. TLR2 signaling activated HIV-1 expression in macrophages, whereas TLR4
82 signaling repressed virus expression. Importantly, TLR4 signaling overcame the activation
83 effects of TLR2 signaling in macrophages. The TLR4- and GC-mediated repression of HIV-1 in
84 macrophages was dependent on signaling through Toll/IL-1 receptor domain-containing adapter
85 inducing interferon-β (TRIF) and required type 1 IFN production. Finally, we showed that TLR4
86 signaling leads to the late-phase recruitment of and IRF8 to the interferon-stimulated response
87 element (ISRE) downstream of the 5' LTR in macrophages. Taken together, our data suggest
88 TRIF-mediated signaling represses HIV-1 replication in response to GC or *E. coli* co-infection in
89 an IRF8-dependent manner and shifts macrophages from a state of robust HIV-1 expression to
90 a state of persistent low-level/latent infection.
91

92 **MATERIALS AND METHODS**

93

94 **Ethics Statement.** This research has been determined to be exempt by the Institutional Review
95 Board of the Boston University Medical Center since it does not meet the definition of human
96 subjects research.

97

98 **Cell isolation and culture.** Primary human CD14+ monocytes were isolated from the
99 peripheral blood mononuclear cells of healthy donors using anti-CD14 magnetic beads (Miltenyi
100 Biotec) per the manufacturer's instructions. Primary monocyte-derived macrophages (MDMs)
101 were generated by culturing CD14+ monocytes in the presence of 10% human AB serum and
102 10% FBS for 6 days. MDMs were cultured in RPMI-1640 supplemented with 10% FBS, 100
103 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine. PM1 and U1 cells were
104 cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml
105 streptomycin, and 0.29 mg/ml L-glutamine. 293T cells were cultured in DMEM supplemented
106 with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine. MAGI-
107 CCR5 cells were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin, 100 μg/ml
108 streptomycin, 0.29 mg/ml L-glutamine, 500 μg/ml G418, 1 μg/ml puromycin, and 0.1 μg/ml
109 hygromycin B. HEK293-TLR2^{CFP}TLR1^{YFP} cells were cultured in DMEM supplemented with 10%
110 FBS, 10 μg/ml ciprofloxacin, 0.29 mg/ml L-glutamine, and 500 μg/ml G418.

111

112 **Bacterial culture.** *Neisseria gonorrhea* (GC) strain FA1090B was a generous gift from Dr.
113 Caroline Genco. GC was cultured from a glycerol stock on GC agar plates supplemented with
114 IsoVitalex Enrichment (Becton Dickinson) in a humidified 37°C incubator with 5% CO_2. *E. coli*
115 strain DH5α was purchased from New England Biolabs and was cultured from a glycerol stock
116 on LB agar plates at 37°C. Where indicated, bacteria were heat inactivated (heat killed) by

117 incubation at 56°C for 2 hours. Heat inactivation was monitored by culture on GC or LB agar
118 plates as described above.
119
120 **Flow cytometry.** TLR expression on MDMs was assessed using antibodies against TLR2
121 (clone TL2.1) and TLR4 (clone HTA125) (both from eBioscience). Flow cytometric data was
122 acquired using a Becton-Dickenson FACScan II or LSRFortessa and data was analyzed using
123 FlowJo software.
124
125 **TLR ligands, interferons, and chemical inhibitors.** PAM3CSK4, FSL-1, *Salmonella*
126 *typhimurium* flagellin, polyI:C, and *E. coli* K12 LPS were obtained from Invivogen. TLR ligands
127 were reconstituted in endotoxin-free H_2O. IFN-α and IFN-β were purchased from PBL Interferon
128 Source. B18R was purchased from Abcam. Celastrol, U0126, PD95809, and SB203580 were
129 purchased from Sigma and reconstituted in DMSO. Dynasore was purchased from Tocris
130 Bioscience and was reconstituted in DMSO.
131
132 **Virus Production.** Single-round replication-competent HIV-1 reporter viruses were generated
133 by packaging a luciferase expressing reporter virus, BruΔEnvLuc2, or an enhanced green
134 fluorescent protein expressing reporter virus, BruΔEnvEGFP3, with the envelope glycoproteins
135 from CCR5-tropic HIV-1 (Ada-M) or VSV (VSV-G). Reporter virus stocks were generated by
136 transfecting HEK293T cells using the calcium phosphate method as described previously (17).
137 Replication competent HIV-1_{Ba-L} was generated by infection of PM1 cells as described
138 previously (17). Virus titers were determined using MAGI-CCR5 cells, and $p24^{gag}$ content was
139 determined by ELISA as described previously (17).
140

141 **Virus infections.** To assess viral replication, macrophages (2.5 x 10^5 cells/well in 24-well
142 plates) were incubated with VSV-G-pseudotyped HIV-luciferase reporter virus at a multiplicity of
143 infection (MOI) of 0.1 for 4 hours at 37°C. Cells were washed four to five times with PBS to
144 remove unbound virus, and cultured in growth medium. Following 48 hours of culture, cells were
145 treated with TLR ligands or vehicle, as indicated in the text and figure legends. After 18 hours,
146 the cells were washed twice with PBS and lysed in PBS/0.02% Triton X-100. Protein levels in
147 cell lysates were determined using a modified Lowry protein assay (BioRad), and luciferase
148 activity was measured using BrightGlo luciferase reagent (Promega) and a MSII luminometer.
149
150 **HIV-1 transcription.** Total cytoplasmic RNA was isolated from MDMs using the RNeasy Mini kit
151 (Qiagen). RNA (100 ng) was analyzed by reverse transcription-PCR (RT-PCR) using the
152 OneStep RT-PCR kit (Qiagen). RNA was reverse transcribed and amplified in a total volume of
153 50 µl containing 2.5 mM MgCl2, 400 µM concentrations of each deoxynucleoside triphosphate,
154 10 U of RNasin RNase inhibitor (Promega), 5 µCi of α-^{32}P dATP, and 0.6 µM HIV-1 specific
155 primers. RNA samples were reverse transcribed for 30 minutes at 50°C. After an initial
156 denaturing step at 95°C for 15 minutes, cDNA products were amplified for 25 cycles each
157 consisting of a 30-second denaturing step at 94°C, a 45-second annealing step at 65°C, and a
158 one-minute extension step at 72°C. The amplification concluded with a 10-minute extension
159 step at 72°C. Samples were resolved on 5% nondenaturing polyacrylamide gels, visualized by
160 autoradiography, and quantified in a Molecular Dynamics PhosphorImager SI using
161 ImageQuant software. The HIV-1 primers were specific for the R and U5 regions of the LTR and
162 amplify both spliced mRNAs and genomic RNA. The HIV-1 primers were sense primer 5'-
163 GGCTAACTAGGGAACCCACTGC-3' and antisense primer 5'-
164 CTGCTAGAGATTTTCCACACTGAC-3'). α-tubulin primers were: sense primer 5'
165 CACCCGTCTTCAGGGCTTCTTGGTTT-3' and antisense primer, 5'

166 CATTTCACCATCTGGTTGGCTGGCTC-3'. RNA standards corresponding to 500, 50, and 5 ng
167 of RNA from PAM3CSK4-activated MDMs were included in each experiment to ensure that all
168 amplifications were within the linear range of the assay.
169
170 **Cytokine release assays.** MDMs (2.5 x 10^5 cells/well) were treated with PAM3CSK4 (100
171 ng/ml), LPS (100 ng/ml), or GC (MOI of 10) for 24 hours. Cell-free culture supernatants were
172 collected and analyzed for TNF-α (eBioscience) or IFN-β (PBL Interferon Source) release by
173 commercially-available ELISA following the manufacturer's instructions.
174
175 **Chromatin immunoprecipitation assays.** 1.2 x 10^7 MDMs were incubated with VSV-G-
176 pseudotyped HIV-EGFP reporter virus at an MOI of 2 for four hours at 37°C. Cells were washed
177 four to five times with PBS to remove unbound virus and cultured in growth medium. Following
178 48 hours of culture, MDMs were treated with TLR ligands for various times as described in the
179 text. Cells were then fixed in 1% formaldehyde for 10 minutes at room temperature, quenched
180 with 125 mM glycine, and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1,
181 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin A). Cellular lysates were sonicated using a
182 cup horn (550 Sonic Dismembrator, Fisher Scientific) at a power setting of 5 with 25 20-second
183 pulses on ice, which fragmented the chromatin to an average length of approximately 1000 bp.
184 Samples were diluted and immunoprecipitated with antibodies against NF-κB p65, IRF1, IRF2,
185 IRF4, IRF8, rabbit IgG, or goat IgG (all from Santa Cruz Biotechnology). Purified DNA samples
186 from both ChIPs and input controls were resuspended in distilled H_2O and analyzed by semi-
187 quantitative PCR. PCR reactions contained 10 mm Tris-HCl pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$,
188 100 pmol of each primer, 200 µm each dATP, dGTP, dCTP, and dTTP, 5µCi α^{32}P-dATP, and
189 2.5 units of Amplitaq Gold (Applied Biosystems) in a 50-µl reaction volume. Following an initial
190 denaturation step at 95°C for 15 minutes, DNAs were amplified for 30 cycles, each consisting of

191 a 30-second denaturing step at 94°C, a 45-second annealing step at 65°C, and a one-minute
192 extension step at 72°C. Samples were electrophoresed on 5% non-denaturing polyacrylamide
193 gels, visualized by autoradiography, and quantified using a Molecular Dynamics
194 PhosphorImager SI using ImageQuant software. Primers used to amplify specifically the HIV-1
195 5' LTR and GLS were 5'- TGGAAGGGCTAATTTACTCCC -3' (sense) and 5'-
196 CATCTCTCTCCTTCTAGCCTC -3' (antisense). Control amplifications of a serial dilution of
197 purified genomic DNA from latently-infected U1 cells were performed with each primer set to
198 ensure that all amplifications were within the linear range of the reaction. To calculate the
199 relative levels of association with the LTR, PhosphorImager data of the PCR products obtained
200 for immunoprecipitated chromatin samples were normalized against the PCR products obtained
201 for input DNA (% Input). Values were normalized across donors and expressed as relative
202 binding.
203
204 **LTR mutant construction.** The reported plasmid pLTR(Sp1)-luciferase was generated by PCR
205 amplification of pNL4-3 using the sense primer 5'-
206 CGGGGTACCCCGTGGAAGGGCTAATTTGGTCCC- 3' and the antisense primers 5'-
207 CCGCTCGAGCGGCATCTCTCTCCTTCTAGCCTC-3', digestion with KpnI and XhoI, and
208 ligation into KpnI/XhoI-digested pGL3-Basic (Promega). Mutations to the NF-κB and IRF binding
209 sites in pLTR(Sp1)-luciferase were generated using the QuickChange IIXL site-directed
210 mutagenesis kit (Stratagene). Primers used for site-directed mutagenesis are listed in Table 1.
211 The -158 LTR-luciferase construct was generated by deleting the LTR sequence upstream of
212 position -158 (relative to the start site of transcription) of pNL4-3, which includes the AP-1
213 binding sites located in the U3 portion of the 5' LTR, digestion of the resulting fragment with
214 KpnI and XhoI, and ligation into KpnI/XhoI-digested pGL3-Basic (Promega).
215

216 **shRNA knock-down of MyD88, TRIF, and IRF8.** MDMs were transfected with plasmids that
217 encoded either a mixture of three to five shRNAs directed against MyD88, TRIF, or a mixture of
218 control shRNAs (Invivogen) and a blasticidin-resistance gene using Oligofectamine (Invitrogen)
219 per the manufacturer's instructions. Transfected cells were selected by culture in the presence
220 of blasticidin for 48 hours, and then used for HIV-1 replication assays or lysed for immunoblot
221 analysis to measure MyD88 and TRIF expression using a rabbit monoclonal antibody to MyD88
222 (Cell Signaling Technology), a rabbit polyclonal antibody to TRIF (Cell Signaling Technology), or
223 a mouse monoclonal antibody to β-actin (Sigma). Similarly, MDMs were transfected with
224 plasmids that encoded either a mixture of three to five shRNAs directed against IRF8 (Sigma) or
225 a mixture of control shRNAs (Sigma) and a puromycin-resistance gene using Oligofectamine
226 (Invitrogen) per the manufacturer's instructions. Transfected cells were selected by culture in
227 the presence of puromycin for 48 hours and then used for HIV-1 replication assays or lysed for
228 immunoblot analysis to measure IRF8 expression using a rabbit monoclonal antibody (Cell
229 Signaling Technology).
230
231 **Overexpression of IRF8.** MDMs were transfected with a plasmid that encoded IRF8 (Origene)
232 and a neomycin-resistance gene using Oligofectamine (Invitrogen) per the manufacturer's
233 instructions. Transfected cells were selected by culture in the presence of neomycin for 48
234 hours and then used for HIV-1 replication assays or lysed for immunoblot analysis to measure
235 IRF8 expression using a rabbit monoclonal antibody (Cell Signaling Technology).
236
237 **Endocytosis/phagocytosis assays.** MDMs ($5x10^5$/well) were treated with Dynasore (80 μM)
238 or DMSO and then incubated with pHrodo Green *E. coli* particles (Thermo Fisher) at 1 mg/ml for
239 2 hours at 37°C. The MDMs were then washed three times with PBS, incubated with viability
240 dye (e450) for 15 minutes at 4°C, and analyzed by flow cytometry. Flow cytometric data was

241 acquired using a Becton-Dickenson FACScan II or LSRFortessa, and data was analyzed using
242 FlowJo software.
243
244 **Statistical analysis.** Untreated control and ligand-treated experimental samples were
245 compared using a two-tailed t-test with Welch's correction. Experiments were performed in
246 triplicate using cells from a minimum of three different donors unless otherwise indicated.
247
248 **RESULTS**
249 **HIV-1 gene expression in MDMs is enhanced or repressed in a TLR-specific manner.** As
250 mentioned previously, studies have shown that pathogens can modulate HIV-1 replication in
251 myeloid cells, both by directly modulating HIV-1 replication and by influencing the local
252 proinflammatory environment (7-12, 19). Pathogens are detected by host cells through their
253 interactions with innate immune receptors, such as TLRs. TLR2 and TLR5 are plasma
254 membrane-associated receptors that utilize the MyD88 adaptor molecule exclusively. TLR3, on
255 the other hand, is an endosomal receptor that utilizes the TRIF adaptor molecule exclusively.
256 TLR4 can utilize either MyD88 or TRIF, depending upon whether it initiates signaling at the
257 plasma membrane or in the endosome, respectively (20). To determine how purified TLR
258 ligands affected HIV-1 gene expression, MDMs were infected with a single-round infectious
259 HIV-1 reporter virus, and then treated with ligands for TLR2, TLR3, TLR4, or TLR5. Ligands that
260 activated TLR2 or TLR5 enhanced HIV-1 replication, whereas ligands for TLR3 or TLR4
261 repressed HIV-1 expression (Figure 1A). The effects of TLR ligands on HIV-1 replication
262 occurred at the level of transcription, as treatment with the TLR2/1 ligand PAM3CSK4 led to an
263 increase in HIV-1 mRNA accumulation, whereas treatment with the TLR4 ligand LPS led to a
264 decrease in HIV-1 transcript levels (Figure 1B and 1C). These results indicate that MyD88-
265 dependent signaling enhances HIV-1 transcription whereas TRIF-dependent signaling inhibits
266 HIV-1 transcription in MDMs.

267

268 **TLR2-mediated enhancement of HIV-1 gene expression requires NF-κB activation, but not**
269 **AP-1 activation.** MyD88-dependent TLR signaling leads to the activation of both NF-κB and
270 AP-1 transcription factors, among others (21). The HIV-1 LTR contains binding sites for both
271 NF-κB and AP-1. The two NF-κB sites are thought to be essential for HIV-1 transcription (22,
272 23), whereas the AP-1 sites, while not essential, are thought to enhance HIV-1 transcription (24,
273 25). Previous studies from our lab demonstrated that treatment of HIV-infected MDMs with the
274 TLR2/TLR1 ligand PAM3CSK4 led to an increased association of the p65 subunit of NF-κB and
275 the c-fos subunit of AP-1 with the 5' LTR (17). This association was correlated with increased
276 virus replication (17); however, the contributions made by each pathway to TLR2-mediated
277 activation have not been previously characterized. To determine the roles of NF-κB and AP-1 in
278 TLR2-activated HIV replication in MDMs, HIV-1- infected cells were treated with either celastrol,
279 a small molecule inhibitor of the IκB kinase complex (26), or inhibitors that disrupt AP-1
280 signaling. As shown in Figure 2, celastrol treatment completely ablated TLR2/1-enhanced HIV-1
281 expression (Figure 2A). Similarly, the use of an LTR-based reporter construct with mutations in
282 the NF-κB binding sites did not result in increased gene expression in response to TLR2
283 signaling (Supplemental Figure 1A). Treatment of HIV-infected macrophages with inhibitors of
284 kinases upstream of AP-1 activation, such as ERK, JNK, and p38, resulted in a modest, but
285 reproducible, decrease in TLR2-mediated activation of HIV-1 (Figure 2B). Similarly, LTR
286 reporter constructs lacking AP-1 binding sites were activated in response to TLR2 signaling,
287 further demonstrating the non-essential role of AP-1 in TLR2-mediated HIV-1 activation
288 (Supplemental Figure 1B). These data suggest that TLR2-activated HIV-1 expression is
289 mediated primarily through NF-κB.

290

291 **Co-infection with *Neisseria gonorrhoeae* or *Escherichia coli* represses HIV-1 replication**
292 **in MDMs.** Our preliminary studies using purified TLR ligands in isolation suggested that different
293 TLR signaling cascades had diverse effects on HIV-1 replication. Since most pathogens encode
294 multiple TLR ligands, we sought to determine the effects of intact pathogens on HIV-1
295 replication. We incubated HIV-infected MDMs with *N. gonorrhoeae* (GC), which expresses
296 ligands for TLR2, TLR4, and TLR9. We found that increasing amounts of GC led to a dose-
297 dependent decrease in HIV-1 replication in MDMs (Figure 3A). Bacterial replication was not
298 required for these effects, as heat-killed GC led to repression of HIV-1 replication in MDMs
299 (Supplemental Figure 2). In addition, repression of HIV-1 replication is not specific for GC, but
300 may be a generalized response to Gram-negative bacteria, as co-infection with *E. coli* also
301 repressed HIV-1 replication in MDMs in a manner similar to GC (Figure 3B). Despite the
302 presence of both activating (TLR2) and repressing (TLR4) TLR ligands, both GC and *E. coli*
303 mediated repression of HIV-1 replication in macrophages. This finding raised several
304 possibilities: 1) the dominance of TLR4 signaling over TLR2 signaling in MDMs; 2) different
305 expression levels of TLR2, TLR4, and TLR4-associated molecules such as CD14 and MD-2 on
306 MDMs; 3) the cytokine profile produced in response to GC or *E. coli*; and/or 4) variable
307 expression of signaling molecules downstream of TLRs. These scenarios were further explored.
308
309 **TLR4 signaling is dominant in MDMs.**
310 To determine whether certain TLR pathways are dominant in MDMs, we performed co-
311 treatments of HIV-infected MDMs with the TLR2 ligand PAM3CSK4 and the TLR4 ligand LPS.
312 We found that increasing the concentration of LPS against a fixed concentration of PAM3CSK4
313 led to a reversal of TLR2-mediated activation of HIV-1 and, eventually, to repression of HIV-1
314 replication (Figure 4A). Conversely, increasing the concentration of PAM3CSK4 against a fixed
315 concentration of LPS did not reverse LPS-mediated repression of HIV-1 (Figure 4A). Flow
316 cytometry was used to determine that the different responses of MDMs were not due receptor

317 expression, as MDMs express both TLR2 and TLR4 (Figure 4B). In addition, MDMs produced
318 both proinflammatory cytokines and IFN-β in response to LPS treatment and GC co-infection,
319 but not in response to treatment with the TLR2 ligand PAM3CSK4 (Figure 5A). Taken together,
320 our data suggest that TLR4 signaling, which negatively regulates LTR-driven gene expression,
321 is dominant in MDMs.
322
323 **LPS- and GC-mediated repression of HIV-1 in MDMs is dependent on TRIF-mediated type**
324 **I interferon production.** Since LPS and GC both induce type I interferon production, whereas
325 the TLR2 ligand PAM3CSK4 does not, we wished to determine whether GC stimulated
326 production of IFN-α/β contributes to repression of HIV-1 in MDMs. We found that treatment of
327 HIV-infected MDMs with the vaccinia virus-encoded soluble type I interferon receptor B18R
328 reversed GC-mediated inhibition of HIV-1 replication, suggesting that TLR4-mediated IFN
329 production is required for HIV-1 repression by GC (Figure 5B). Since both purified TLR4 ligands
330 and GC, which encodes ligands for TLR2, TLR4, and TLR9, repress HIV-1 replication in MDMs,
331 we predicted that downstream effector molecules of TLR4 signaling would contribute to the
332 repression of HIV-1 replication in MDMs. First, we confirmed that TLR4 signaling was
333 responsible for GC-mediated HIV-1 repression in MDMs. Treatment with the TLR4-specific
334 inhibitor TAK242 reversed the LPS- and GC-dependent repression of HIV-1 in MDMs (Figure
335 5C) (SHOW SIGNFICANCE). Treatment with TAK242 had no effect on TLR2-mediated
336 activation of HIV-1 replication in MDMs, consistent with reports that TAK242 inhibits TLR4 but
337 not TLR2 (ref).
338
339 It has been shown that TLR4, which can utilize both MyD88 and TRIF adaptor proteins, initiates
340 different signaling pathways dependent upon its location within the cell. Cell-surface TLR4
341 engagement leads to MyD88-dependent signaling, whereas endosomal TLR4 engagement

342 leads to TRIF-dependent signaling (20). To examine whether TRIF-dependent signaling is
343 responsible for HIV-1 repression, we blocked dynamin-dependent endocytosis of TLR4 with
344 Dynasore, which prevents TRIF-dependent signaling while leaving MyD88-dependent signaling
345 intact. As shown, blocking endocytosis-mediated TLR4 internalization (Supplemental figure 3)
346 not only reversed GC-mediated repression of HIV-1 in MDMs, but resulted in augmentation of
347 viral gene expression (Figure 5D). This is likely due to the ability of GC to signal through both
348 TLR2-MyD88 and TLR4-MyD88.
349
350 To confirm the role of MyD88 in TLR2-mediated HIV-1 activation and TRIF in TLR4-mediated
351 HIV-1 repression, we used shRNAs to knock down the two molecules in HIV-infected MDMs.
352 Knock-down of MyD88 led to a loss of TLR2-mediated HIV-1 activation, but had no effect on
353 LPS or GC-mediated HIV-1 repression (Figure 5F). In contrast, knock-down of TRIF had no
354 effect on TLR2-mediated HIV-1 activation, but reversed LPS- and GC-mediated repression of
355 HIV-1 replication (Figure 5F). Knock-down of either MyD88 or TRIF had no effect on the
356 activation of HIV-1 by the phorbol ester PMA, which signals directly through protein kinase C,
357 independently of TLRs (Figure 5F). These data suggest that the TLR4-TRIF-type I IFN axis in
358 MDMs leads to GC- and *E. coli*-mediated repression of HIV-1 replication.
359
360 **TLR4 signaling leads to differential IRF recruitment to the HIV-1 LTR.** Since type I IFN
361 production is critical for GC- and *E. coli*-mediated HIV-1 repression in MDMs, we examined the
362 role of interferon-stimulated genes (ISGs) in HIV-1 regulation. Treatment of HIV-infected MDMs
363 with the TLR4 ligand LPS led to an initial short-lived increase in HIV-1 RNA levels, but levels
364 steadily declined thereafter (Figure 6A; red line (THIS PANEL IS UNREADABLE)), indicating
365 that HIV-1 transcription displays a biphasic response to TLR4 stimulation in MDMs. In contrast,
366 treatment of HIV-infected MDMs with the TLR2 ligand PAM3CSK led to a continuous increase in
367 HIV-1 RNA levels (Figure 6A; green line). This suggests that late-phase proteins induced by

368 type I IFNs are responsible for TLR4-mediated decreases in HIV-1 transcription. It is known that
369 HIV-1 contains an interferon-stimulated response element (ISRE) in the Gag-leader sequence
370 (GLS), immediately downstream from the 5' LTR. Because type I IFN is required for LPS- and
371 GC-mediated repression of HIV-1 in MDMs, we assessed the role of the ISRE in this process
372 using transient transfection assays with mutated LTR-reporter constructs in HEK293 cells
373 expressing TLR4, MD-2, and CD14. We found that LPS treatment repressed LTR-driven
374 reporter-gene expression in cells expressing WT ISRE elements, but not in cells transfected
375 with an LTR-luciferase construct containing a mutated ISRE (Supplemental Figure 4). This
376 suggests that factors that bind to the ISRE account for TLR4-mediated HIV-1 repression.
377
378 Previous studies have shown that IRF1 and IRF2 both bind to this ISRE *in vitro* and that IRF1
379 and IRF2 expression are associated with enhanced HIV-1 transcription (27). Both IRF1 and
380 IRF2 are constitutively expressed in macrophages, but expression can be increased in
381 response to type I IFNs and TLR signaling (28-31). Two other IRFs, IRF4 and IRF8, are also
382 expressed in macrophages (32) and have been shown to increase in response to type I IFN
383 signaling and other signals (28, 33). Interestingly, IRF8 has been implicated in maintaining HIV-
384 1 latency in infected monocytic cell lines (27, 34, 35), and IRF4 has been implicated in negative
385 regulation of TLR signaling (36). We therefore investigated whether various IRFs are recruited
386 to the HIV-1 ISRE in response to LPS treatment in MDMs. Using chromatin immunoprecipitation
387 analysis, we found that IRF1, IRF2, IRF4, and IRF8 all are able to associate with the 5' LTR and
388 GLS containing the ISRE in HIV-infected MDMs (Figure 6B). Early after treatment with LPS, the
389 levels of IRF1 associated with this region of the viral promoter increased, whereas the levels of
390 IRF2 and IRF4 decreased. By 24h post-treatment with LPS, the levels of IRF4 and IRF8
391 associated with this region increased. Of particular note, the levels of IRF8 recruitment
392 increased well above those seen in unstimulated MDMs (Figure 6B-6C). To confirm the central
393 role of IRF8 in TLR4-mediated repression of HIV-1 expression in MDMs, we used shRNA to

394 knockdown IRF8 expression in MDMs. Reducing IRF8 expression reversed TLR4-mediated
395 HIV-1 repression in response to treatment with LPS or GC. Knockdown of IRF8 led to activation
396 of HIV-1 expression in cells treated with a combination of PAM3CSK4 and LPS or GC, similar to
397 that seen with treatment with PAM3CSK4 alone (Figure 6E). In contrast, overexpression of IRF8
398 in MDMs led to repression of HIV-1 expression regardless of TLR treatment (Figure 6G). (Figure
399 needs error bars)
400
401 Taken together, these data suggest that both LPS and GC activate TLR4-mediated TRIF
402 signaling in MDMs, resulting in the production of type I IFNs. IFNs work in an autocrine or
403 paracrine fashion to induce the expression of IRF8, which then binds to the ISRE present in the
404 GLS of HIV-1 to repress viral transcription.
405
406 **LPS and GC-treatment induces persistent low-level/latent HIV-1 infection in MDMs.**
407 Recent studies in animals and human tissues demonstrate that HIV-1 can form persistent low-
408 level or latent infections in macrophages (37-40). Our data suggest that engagement of the
409 TLR4-TRIF-type I IFN axis in macrophages can repress virus replication. We wished to
410 determine whether signaling through this axis could skew macrophages toward a persistent low-
411 level or latent HIV-1 infection. HIV-1-infected MDMs were treated a single time with the TLR2
412 ligand PAM3CSK4, the TLR4 ligand LPS, a combination of PAM3CSK4 and LPS, heat-killed
413 GC, IFN-α, or IFN-β. We found that treatment with a single dose of LPS, heat-killed GC, IFN-α,
414 or IFN-β led to a sustained, prominent decrease in HIV-1 replication in MDMs, whereas
415 treatment with PAM3CSK4 led to an initial slight increase in HIV-1 replication followed by a
416 slight decrease in replication. These data suggest that engagement of the TLR4-TRIF-type I IFN
417 axis can promote low-level persistent/latent HIV-1 infection in MDMs. (error bars)
418

419 **DISCUSSION**

420

421 In these studies, we have demonstrated that MyD88-dependent signaling activates HIV-1
422 expression in MDMs by activating NF-κB, whereas TRIF- dependent signaling represses HIV-1
423 expression in these cell types through the type I IFN-mediated recruitment of IRF8 to the HIV-1
424 ISRE. We have also shown that TLR4-mediated, TRIF-dependent signaling is dominant in
425 MDMs, both in the context of treatment with purified TLR ligands as well as infection with the
426 sexually transmitted pathogen GC or the gut-associated microbe *E. coli*. Co-infection of virus-
427 infected MDMs by pathogens that express TLR4 ligands decreases HIV-1 replication through
428 the recruitment of IRF8 to the HIV-1 ISRE.

429

430 Prior studies have produced conflicting results regarding the role of LPS and TLR4 signaling on
431 HIV-1 replication. Studies using transient transfection with reporter constructs have
432 demonstrated an LPS/TLR4-mediated increase in HIV-1 expression (41, 42). Similar results
433 were obtained in studies using latently-infected monocytic cell lines (43) and HIV-1 transgenic
434 mice (44-46). More recent studies have provided evidence for an LPS/TLR4-mediated
435 repression of HIV-1 expression through the induction of type I IFNs and other mechanisms.
436 These studies employed HIV-1 infection of myeloid cells (15, 16, 47-50) or transfection using
437 full-length viral constructs (51). Our data extends these findings and demonstrates that LPS
438 treatment represses HIV-1 expression in MDMs through its interaction with TLR4. Specifically,
439 we show that LPS induces TLR4-mediated, TRIF-dependent induction of type I IFNs, which in
440 turn lead to the repression of HIV-1 replication in MDMs through the recruitment of IRF8 to the
441 HIV-1 ISRE.

442

443 A number of studies have shown that IRFs play an important role in the regulation of HIV-1
444 replication. There is an ISRE located downstream from the 5' LTR in the GLS that is essential

445 for efficient viral replication (24). This ISRE is bound by IRF1 and/or IRF2, leading to activation
446 of virus transcription (27, 52). Deletion of the ISRE and, therefore, loss of IRF1 binding severely
447 limits HIV-1 replication (53), confirming the importance of IRF1 for HIV-1 replication. Once
448 bound to the ISRE, IRF1 can recruit co-activators, such as the histone acetyl transferase (HAT)
449 p300/CBP, to activate viral transcription (54). IRF1 and IRF2 are ubiquitously expressed in cells,
450 although they can be upregulated by type I IFNs (28) and, at least in the case of IRF1, by TLR
451 signaling (30, 31). In fact, HIV-1 infection itself leads to the upregulation of IRF1 in an NF-κB-
452 dependent manner (55), illustrating how HIV-1 can co-opt the antiviral IFN response to augment
453 its own replication. Once associated with the ISRE, IRF1 can cooperatively bind to both NF-κB
454 at the HIV-1 LTR and the viral transactivator Tat at the HIV-1 TAR loop. This cooperative
455 binding has been shown to augment viral transcription/elongation (27, 56). Our studies
456 demonstrate that both IRF1 and IRF2 associate with the HIV-1 ISRE in unstimulated MDMs
457 (Fig. 6). Upon stimulation with multiple TLR ligands, there is an enhanced recruitment of IRF1 to
458 the HIV-1 ISRE (Fig. 6), which is consistent with the prevailing theory that TLR-MyD88 signaling
459 can activate IRF1 (57). This enhancement of IRF1 binding seems to come at the expense of
460 IRF2 binding, as the association of IRF2 with the ISRE decreases upon cellular stimulation (Fig.
461 6). These data suggest that IRF1 binding to the ISRE as either monomers or homodimers
462 activates HIV-1 expression, whereas IRF2 binding to the ISRE as monomers, homodimers, or
463 heterodimers with IRF1 represses HIV-1 expression. Unfortunately, ChIP analysis of HIV-
464 infected MDMs using current tools does not permit differentiating between the association of
465 various homodimers and heterodimers with the ISRE at a population level.
466
467 Macrophages express high base levels of IRF8, although its expression can be further
468 enhanced in response to type I IFNs (28, 33). Previous studies have shown that IRF8 can
469 repress HIV-1 expression (27, 34, 35). IRF8 has been shown to bind to IRF1, in addition to

470 other transcription factors, and to serve as either a transcriptional activator or a transcriptional
471 inhibitor of other genes in a context-dependent manner (58-60). In fact, the interaction between
472 IRF8 and IRF1 has been shown to repress HIV-1 transcription in Jurkat cells (27). This may be
473 due to IRF8-mediated disruption of the IRF1-Tat interaction and/or the IRF1-NF-κB interaction
474 (56) that increase viral replication. Our data suggest that IRF8 recruitment to the HIV-1 ISRE in
475 response to LPS or TLR4 signaling alone leads to repression of viral replication in MDMs.
476 Based upon our data, we propose that changes in the IRF binding pattern to the ISRE in
477 response to TLR signaling have profound effects on HIV-1 replication. In unstimulated HIV-
478 infected macrophages, the ISRE is most likely occupied by IRF1/IRF2 heterodimers that allow
479 for a low-level of virus replication. Early after stimulation of TLR4 with LPS, there is a switch to
480 IRF1 homodimers present at the ISRE that allow for high levels of virus replication due to
481 cooperative binding between IRF1, NF-κB, and HIV-1 Tat. At late time points after TLR4
482 stimulation with LPS, during the IFN feedback phase of the response, the ISRE is occupied by
483 IRF1/IRF8 heterodimers (Fig. 6). These IRF1/IRF8 heterodimers likely block the cooperative
484 interaction between IRF1, NF-κB, and Tat, thereby repressing HIV-1 replication.
485
486 We also demonstrate that IRF4 is recruited to the HIV-1 ISRE following treatment with LPS.
487 Although macrophages express relatively low levels of IRF4 in the absence of IFN stimulation or
488 other activation stimuli (61), we demonstrated that IRF4 is recruited to the LTR in MDMs initially
489 upon stimulation with LPS. IRF4, however, dissociates from the LTR by 24 hours post-
490 treatment. The biological significance of IRF4 association with the HIV-1 ISRE remains unclear.
491
492 Macrophages are among the first cell types to contact HIV-1 during mucosal transmission,
493 although their role in transmission has still not been fully elucidated. Recent work suggests that
494 mucosal CD4+ T cells are the first cells to become productively infected during transmission

495 (62, 63), although the interactions between HIV-1 and macrophages are critical for viral
496 transmission and dissemination in certain tissues such as penile or vaginal tissue, but not others
497 such as gastrointestinal tissue (2, 64). Macrophages can be productively infected with HIV-1
498 (65, 66), and transfer infectious virus to CD4+ T cells (67, 68). Sexually transmitted co-
499 pathogens, such as GC, contribute to viral transmission by modulating virus replication in
500 infected cells through their interactions with innate immune receptors such as TLRs (8). We
501 show that TLR2 and TLR5 ligands activate HIV-1 replication in MDMs. On the other hand, TLR3
502 and TLR4 ligands repress HIV-1 replication in this cell type. Evidence suggests that immune
503 responses to most sexually transmitted co-pathogens require TLR2 and TLR9, although a few
504 require TLR4 (11). GC is one of the few sexually transmitted pathogens that encodes a *bona*
505 *fide* TLR4 ligand (LOS) and, therefore, may have the ability to alter HIV-1 transmission and
506 replication dynamics in a manner unique among sexually-transmitted co-pathogens.
507
508 In addition to their role in HIV-1 transmission, macrophages also contribute to HIV-1 persistence
509 *in vivo*. The major obstacle to the eradication of HIV-1 is the presence of a persistent viral
510 reservoir that that can resurface upon discontinuation of antiretroviral therapy (ART). Although
511 CD4+ memory T cells are thought to constitute the majority of the HIV-1 reservoir, several
512 studies have demonstrated that tissue resident macrophages in the lymph nodes (69, 70),
513 gastrointestinal tract (71, 72), genitourinary tract (2, 38, 64), liver (73-75), and lung (76-78), as
514 well as perivascular macrophages and microglial cells in the brain (37, 79-83), can serve as
515 tissue reservoirs for HIV-1. The potential contribution of HIV-1 in tissue macrophages to virus
516 rebound with the cessation of ART is not entirely understood, but recent primate studies
517 suggest that the functional macrophage reservoir can contribute to viral rebound upon treatment
518 cessation (84, 85). Furthermore, recent reports have clearly demonstrated that macrophages
519 can be infected *in vivo* and contribute to viral persistence (86, 87). In SHIV-infected rhesus
520 macaques, *in vivo* viral replication was sustained by tissue macrophages after depletion of

521 CD4+ T cells (66). Moreover, HIV-1 persistence in macrophages was confirmed in HIV-1
522 infected humanized myeloid-only mice in which viral rebound was observed in 33% of the
523 animals following treatment interruption (39). Our data demonstrate that interactions between
524 macrophages and pathogenic or commensal microorganisms within the genitourinary and
525 gastrointestinal tracts, such as GC and *E. coli*, may alter the ability of macrophages to serve as
526 reservoirs for viral persistence in the host (88). Our results confirm the need for further *in vitro*,
527 *ex vivo*, and *in vivo* studies regarding the effects of sexually-transmitted pathogens and
528 commensal microbes on HIV-1 persistence.
529

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537

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543

544 **DISCLOSURES**

545 The authors have no conflicts of interest.
546

547 **AUTHOR CONTRIBUTIONS**

548 T.M.H. and G.A.V. designed the study. T.M.H and G.A.V. developed the methodology. T.M.H.
549 conducted experiments. T.M.H., V.P., and G.A.V. wrote the manuscript. T.M.H., V.P., and
550 G.A.V. acquired funds. G.A.V. supervised the study.

551

552 **FIGURE LEGENDS**

553

554 **Figure 1. TLR2 and TLR5 ligands activate, whereas TLR3 and TLR4 ligands repress, HIV-1**
555 **replication at the level of transcription.** (A) MDMs (2.5 x 10^5 cells/well) were infected with a
556 single-round, replication-competent HIV-luciferase reporter virus at an MOI = 0.1. After 4 hours,
557 unbound virus was removed by washing with PBS, and cells were cultured in complete medium.
558 48 hours after infection, cells were treated with the TLR2 ligand PAM3CSK4 (100 ng/ml), the
559 TLR5 ligand flagellin (100 ng/ml), the TLR3 ligand poly I:C (25 μg/ml), or the TLR4 ligand LPS
560 (100 ng/ml) for 18 hours. The cells were then lysed and assayed for luciferase activity. Bars
561 represent the mean (+/- SD) of 4 donors (MDMs), each donor tested in triplicate. (B-C) MDMs
562 were infected as described above. Forty-eight hours after infection, cells were treated with
563 PAM3CSK4 (100 ng/ml) or LPS (100 ng/ml) for 6 hours. The cells were then lysed and assayed
564 for viral RNA accumulation by semi-quantitative RT-PCR. Shown are data from one
565 representative donor (B) and composite data from four donors (C).

566

567 **Figure 2. TLR2-activated HIV-1 expression is mediated primarily through NF-κB.** (A)
568 MDMs (2.5 x 10^5 cells/well) were infected with a single-round, replication-competent HIV-
569 luciferase reporter virus at an MOI = 0.1. Forty-eight hours after infection, cells were treated with
570 PAM3CSK4 (100 ng/ml) in the presence or absence of celastrol (10 μM) for 18 hours. The cells
571 were then lysed and assayed for luciferase activity. The data are the mean (+/- SD) of 3 donors,

572 each donor tested in triplicate. (B) MDMs (2.5 x 10^5 cells/well) were infected as above. 48 hours
573 after infection, cells were treated with PAM3CSK4 (100 ng/ml) in the presence or absence of
574 U0126 (10 μM), PD98059 (50 μM), or SB203580 (10 μM) for 18 hours. The cells were then
575 lysed and assayed for luciferase activity. The data are the mean (+/- SD) of 3 donors, each
576 donor tested in triplicate.
577
578 **Figure 3. Neisseria gonorrhoeae (GC) co-infection represses HIV-1 replication in MDMs.**
579 MDMs (2.5 x 10^5 cells/well) were infected with a single-round, replication-competent HIV-1
580 luciferase reporter virus at MOI = 0.1. Forty-eight hours after infection, the cells were cultured
581 overnight with increasing amounts of GC. The cells were then lysed and assayed for luciferase
582 activity. Bars represent mean (+/- SD) of 4 donors, each donor tested in triplicate.
583
584 **Figure 4. TLR4 signaling is dominant in MDMs.** (A) MDMs (2.5 x 10^5 cells/well) were infected
585 with a single-round, replication-competent HIV-luciferase reporter virus at an MOI = 0.1. Forty-
586 eight hours after infection, cells were treated with a fixed concentration of PAM3CSK4 (100
587 ng/ml) and increasing concentrations of LPS (1-1000 ng/ml, as indicated) or a fixed
588 concentration of LPS (100 ng/ml) and increasing concentrations of PAM3CSK4 (1-1000 ng/ml,
589 as indicated) for 18 hours. The cells were then lysed and assayed for luciferase activity. The
590 data are the mean (+/- SD) of 3 donors, each donor tested in triplicate. (B) MDMs were stained
591 with antibodies against TLR2 (A) and TLR4 (B), and levels of these proteins were assessed by
592 flow cytometry. Data are presented as mean fluorescent intensity (MFI) and represent the mean
593 of 3 donors ± standard deviation.
594
595 **Figure 5. LPS- and GC-mediated repression of HIV-1 replication in MDMs requires TLR4,**
596 **TRIF, and type I IFNs.** (A) MDMs (2.5 x 10^5 cells/well) were treated with the TLR2 ligand
597 PAM3CSK4 (100 ng/ml), the TLR4 ligand LPS (100 ng/ml), both PAM3CSK4 and LPS (each

598 100 ng/ml), or GC (MOI = 10) for 18 hours. Cell supernatant was harvested, filtered through a
599 0.2 μm filter, and analyzed by ELISA for TNF-α (A) and IFN-β (B) production. Data represent
600 mean (+/- SD) of 3 donors, each donor tested in triplicate. (B) MDMs (2.5 x 10^5 cells/well) were
601 infected with a single-round, replication-competent HIV-luciferase reporter virus at an MOI = 0.1.
602 Forty-eight hours after infection, cells were treated with LPS (100 ng/ml) or GC (MOI = 10) in the
603 absence (white bars) or presence (black bars) of B18R (100 ng/ml) for 18 hours. The cells were
604 then lysed and assayed for luciferase activity. The data are the mean (+/- SD) of 3 donors, each
605 donor tested in triplicate. (C) MDMs (2.5 x 10^5 cells/well) were infected as above. Forty-eight
606 hours after infection, cells were treated with PAM3CSK4 (100 ng/ml), LPS (100 ng/ml), or GC
607 (MOI = 10) in the absence (white bars) or presence (black bars) of TAK242 (1 μg/ml) for 18
608 hours. The cells were then lysed and assayed for luciferase activity. The data are the mean (+/-
609 SD) of 3 donors, each donor tested in triplicate. (D) MDMs (2.5 x 10^5 cells/well) were infected as
610 above. Forty-eight hours after infection, cells were treated with vehicle control (white bars) or
611 with (black bars) the dynamin inhibitor Dynasore (80 μM) for 15 minutes prior to treatment with
612 LPS (100 ng/ml) or GC (MOI = 10) for 18 hours. The cells were then lysed and assayed for
613 luciferase activity. The data are the mean (+/- SD) of 3 donors, each donor tested in triplicate.
614 (E) MDMs (2x10^6 cells/well) were transfected with a control scrambled shRNA, shRNA targeting
615 MyD88, or shRNA targeting TRIF. Knock-down of protein expression was detected by western
616 blot. (F) MDMs (2x10^6 cells/well) were transfected with a control scrambled shRNA (white bars)
617 or with shRNA targeting MyD88 (black bars) or TRIF (gray bars), were infected with a single-
618 round, replication-competent HIV-luciferase reporter virus at an MOI = 0.1. Forty-eight hours
619 after infection, cells were treated with PAM3CSK4 (100 ng/ml), LPS (100 ng/ml), both
620 PAM3CSK4 and LPS (100 ng/ml each), GC (MOI = 10), or PMA (10 nM) for 18 hours. The cells
621 were then lysed and assayed for luciferase activity. The data are the mean (+/- SD) of 3 donors,
622 each donor tested in triplicate.

623

624 **Figure 6. LPS- and GC-mediated repression of HIV-1 in MDMs is associated with changes**
625 **in IRF recruitment to the ISRE.** (A) MDMs ($2x10^6$ cells/well) were infected with a single-round,
626 replication-competent HIV-luciferase reporter virus at an MOI = 0.1. Forty-eight hours after
627 infection, cells were treated with PAM3CSK4 (100 ng/ml) or LPS (100 ng/ml). At various time
628 points after TLR stimulation, cells were harvested, lysed, and total cytoplasmic RNA was
629 extracted. Viral RNA accumulation was assessed by semi-quantitative RT-PCR. The data are
630 the average of two donors. (B) MDMs (1.2×10^7 cells/plate) were infected with a single-round
631 replication competent HIV-GFP reporter virus at an MOI = 2. Forty-eight hours after infection,
632 cells were treated with LPS (100 ng/ml). At either 1 or 24 hours after LPS treatment, cells were
633 fixed with formaldehyde, lysed, sonicated, and subjected to immunoprecipitation with antibodies
634 against IRF1, IRF2, IRF4, IRF8, or rabbit IgG (isotype control). Association with the HIV-1 ISRE
635 was assessed by semi-quantitative PCR using HIV-1 specific primers. One representative donor
636 (B) and composite data from three donors (C) are shown. (D) MDMs ($2x10^6$ cells/well)
637 transfected with a controlled scrambled shRNA (white bars) or with shRNA targeting IRF8 (black
638 bars) were infected with a single-round, replication-competent HIV-luciferase reporter virus at an
639 MOI = 0.1. Forty-eight hours after infection, cells were treated with 100 ng/ml PAM3CSK4, 100
640 ng/ml LPS, a combination of 100 ng/ml PAM3CSK4 and 100 ng/ml LPS, or GC (MOI = 10) for
641 18 h. The cells were then lysed and assayed for luciferase activity. The experiment was
642 performed three times, each in triplicate, using cells from three different donors. (E) MDMs
643 transfected with an empty vector (white bars) or a vector encoding IRF8 (black bars) were
644 infected with a single-round, replication-competent HIV-luciferase reporter virus at an MOI = 0.1.
645 Forty-eight hours after infection, cells were treated with 100 ng/ml PAM3CSK4, 100 ng/ml LPS,
646 a combination of 100 ng/ml PAM3CSK4 and 100 ng/ml LPS, or GC (MOI = 10) for 18 h. The
647 cells were then lysed and assayed for luciferase activity. The experiment was performed three
648 times, each in triplicate, using cells from three different donors.

649

650 **Figure 7. Treatment with LPS, GC, or type I IFNs induces a low-level persistent/latent**
651 **infection in MDMs.** MDMs ($2.5x10^5$ cells/well) were infected with replication-competent HIV-1_{Ba-}
652 $_L$ at an MOI of 0.1. At day 3 post-infection, the cells were treated with PAM3CSK4 (100 ng/ml),
653 LPS (100 ng/ml), PAM3CSK4 and LPS (each at 100/ng/ml), IFN- α (1000 U/ml), or IFN-β (1000
654 U/ml). Cell-free supernatants were harvested every three days, and virus production was
655 monitored by p24 ELISA. Data from two independent donors, each tested in triplicate, are
656 shown.

657

658 **Supplemental figure 1. TLR2-mediated activation of HIV-1 requires NF-κB binding sites,**
659 **but not AP-1 binding sites, in the LTR.** (A) HEK293-$TLR2^{CFP}TLR1^{YFP}$ cells were transfected
660 with HIV-1 LTR-luciferase reporter constructs with intact NF-κB, mutated NF-κB, or deleted NF-
661 κB binding sites. Following transfection, cells were treated with PAM3CSK4 (100 ng/ml) for 18
662 hours and then harvested and assayed for luciferase activity. Data represent the mean (+/- SD)
663 of three independent experiments, each performed in triplicate. (B) HEK293-$TLR2^{CFP}TLR1^{YFP}$
664 cells were transfected with HIV-1 LTR-luciferase reporter constructs with intact AP-1 sites (WT
665 LTR) or deleted AP-1 binding sites (-158 LTR). Following transfection, cells were treated with
666 PAM3CSK4 (100 ng/ml) for 18 hours and then harvested and assayed for luciferase activity.
667 Data are the mean (+/- SD) of three independent experiments, each performed in triplicate.

668

669 **Supplemental figure 2. Heat-killed GC represses HIV-1 replication in MDMs, but activates**
670 **it in MDDCs.** MDMs were infected with a single-round, replication-competent HIV-1 luciferase
671 reporter virus at MOI = 0.1. Forty-eight hours after infection, the cells were cultured with
672 increasing amounts of heat-killed (56°C treatment) GC overnight. The cells were then lysed and

673 luciferase activity was measured. The data are the mean (+/- SD) of 3 donors, each donor
674 tested in triplicate.
675
676 **Supplemental figure 3. Dynasore inhibits endocytosis/phagocytosis of labeled *E. coli***
677 **particles by MDMs.** (A-B) MDMs ($5x10^5$/well) were incubated with DMSO or Dynasore (80 μM)
678 for 15 minutes at 37°C. The cells were washed with PBS and incubated with pHrodo Green *E.*
679 *coli* (1 mg/ml) for 2 hours at 37°C. Endocytosis/phagocytosis was measured by flow cytometry.
680 Shown are data from one representative donor (A) and composite data from four donors (B).
681
682 **Supplemental figure 4. TLR4-mediated repression of HIV-1 requires the ISRE binding site**
683 **located in the GLS downstream of the 5' LTR.** (A) HEK293-TLR4^{CFP}/MD-2/CD14 cells were
684 transfected with HIV-1 LTR/GLS-luciferase reporter constructs with an intact ISRE or mutated
685 ISRE binding site. Following transfection, cells were treated with LPS (100 ng/ml) for 18 hours
686 and then harvested and assayed for luciferase activity. Data are the mean (+/- SD) of three
687 independent experiments, each performed in triplicate.

688 **Table 1. Primers used for PCR-based mutagenesis.**

Primer Name	Sequence
Forward mutI NF-κB	GGACTTTCCGCTGTCTACTTTCCAGG
Reverse mutI NF-κB	CCTGGAAAGTAGACAGCGGAAAGTCC
Forward mutII NF-κB	GCTTTCTACAATCTACTTTCCGCTGG

Reverse mutII NF-κB	CCAGCGGAAAGTAGATTGTAGAAAGC
Forward mutI&II NF-κB	GCTTTCTACAATCTACTTTCCGCTGTCTACTTTCCAGG
Reverse mutI&II NF-κB	CCTGGAAAGTAGACAGCGGAAAGTAGATTGTAGAAAGC
Forward delNF-κB	GCTGACATCGAGCTTTCTACAAAGGGAGGTGTGGCCTGGGCGGG
Reverse delNF-κB	CCCGCCCAGGCCACACCTCCCTTTGTAGAAAGCTCGATGTCAGC
Forward mutISRE	GCCCGAACAGGGACTTGCCCGCGCCCGTAAAGCCAGAGGAGATC
Reverse mutISRE	GATCTCCTCTGGCTTTACGGGCGCGGGCAAGTCCCTGTTCGGGC

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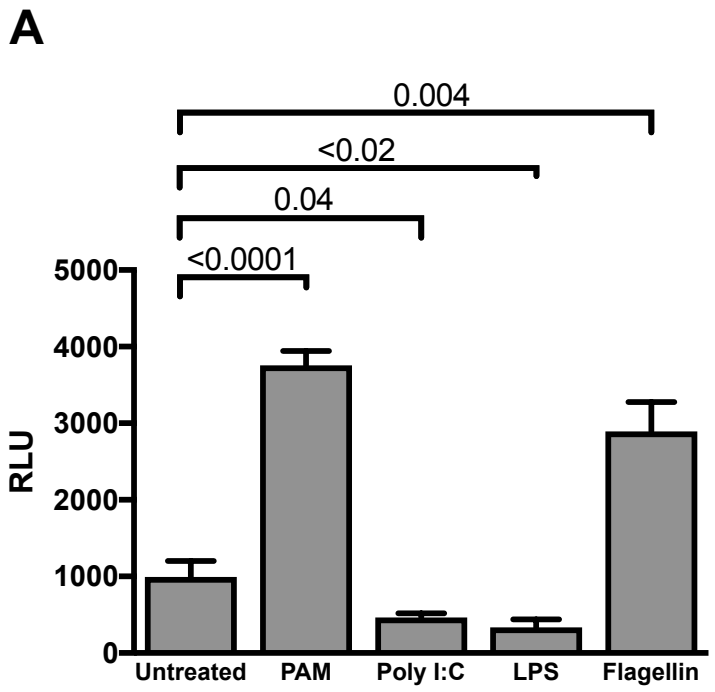

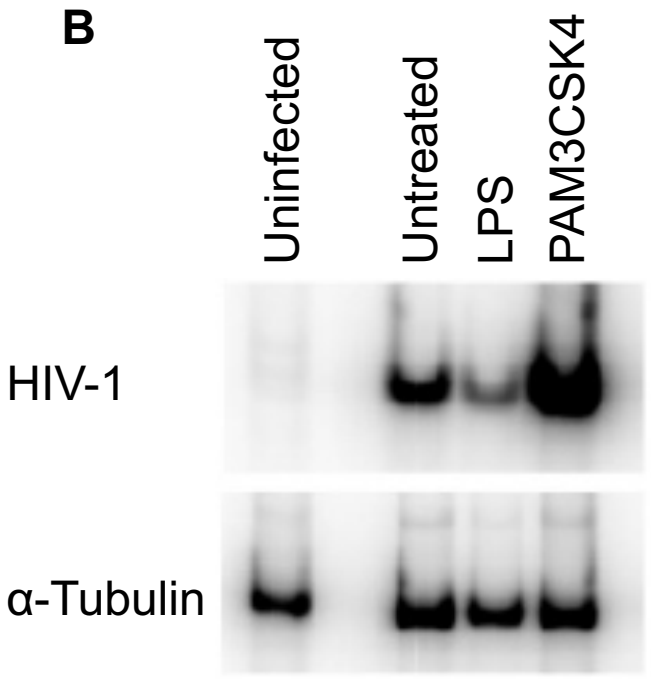

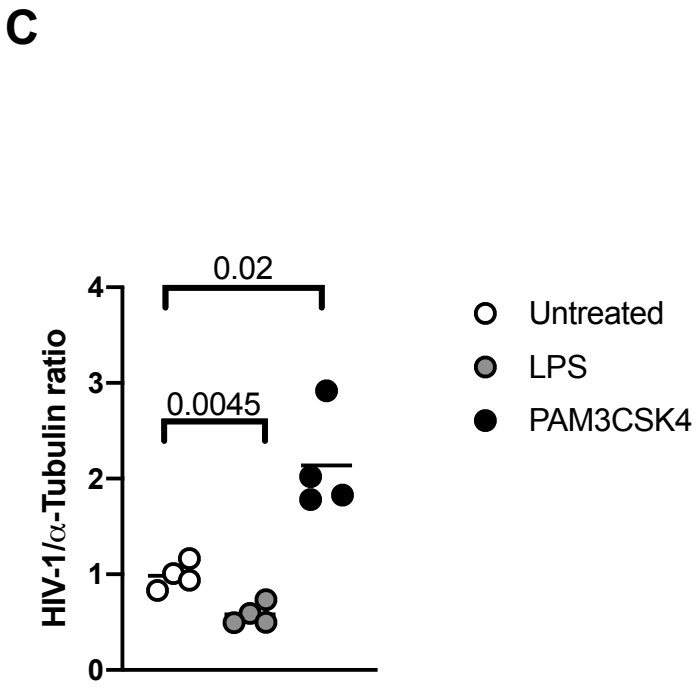

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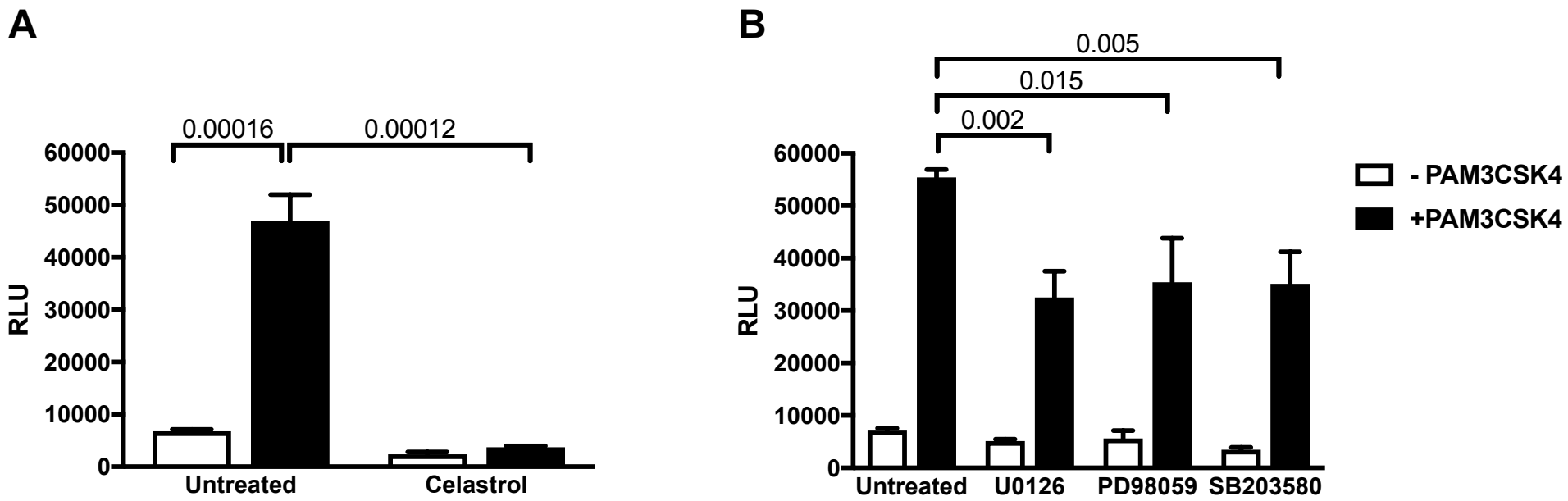

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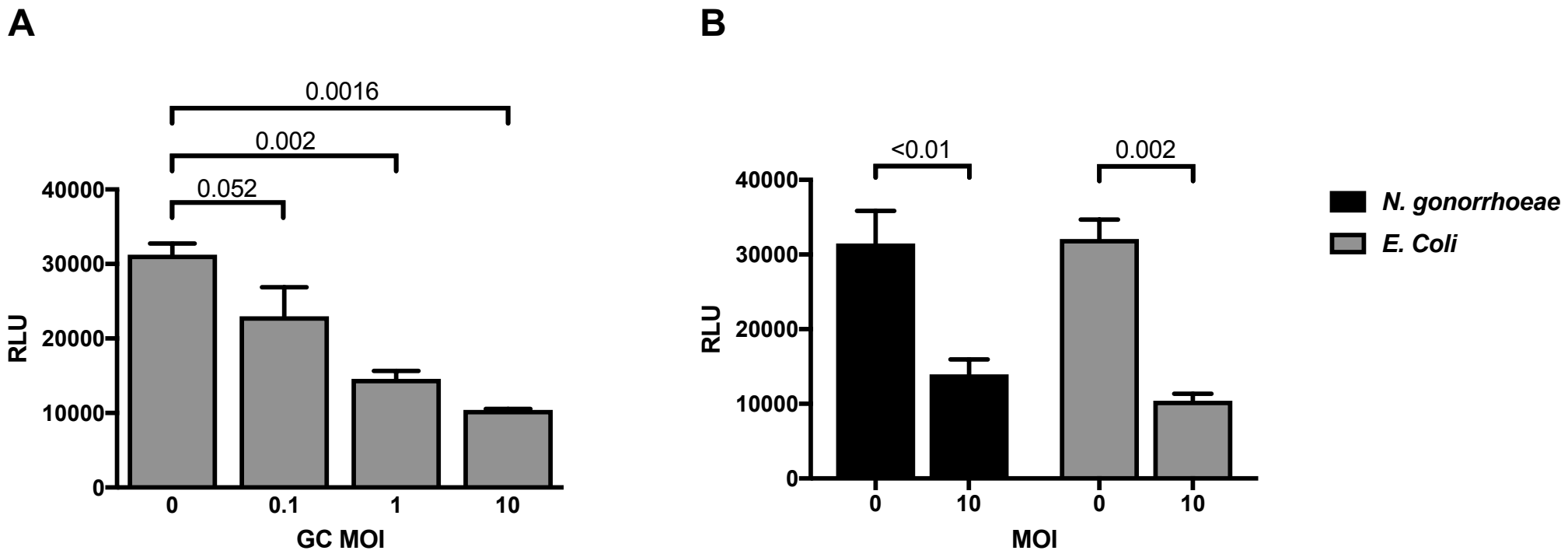

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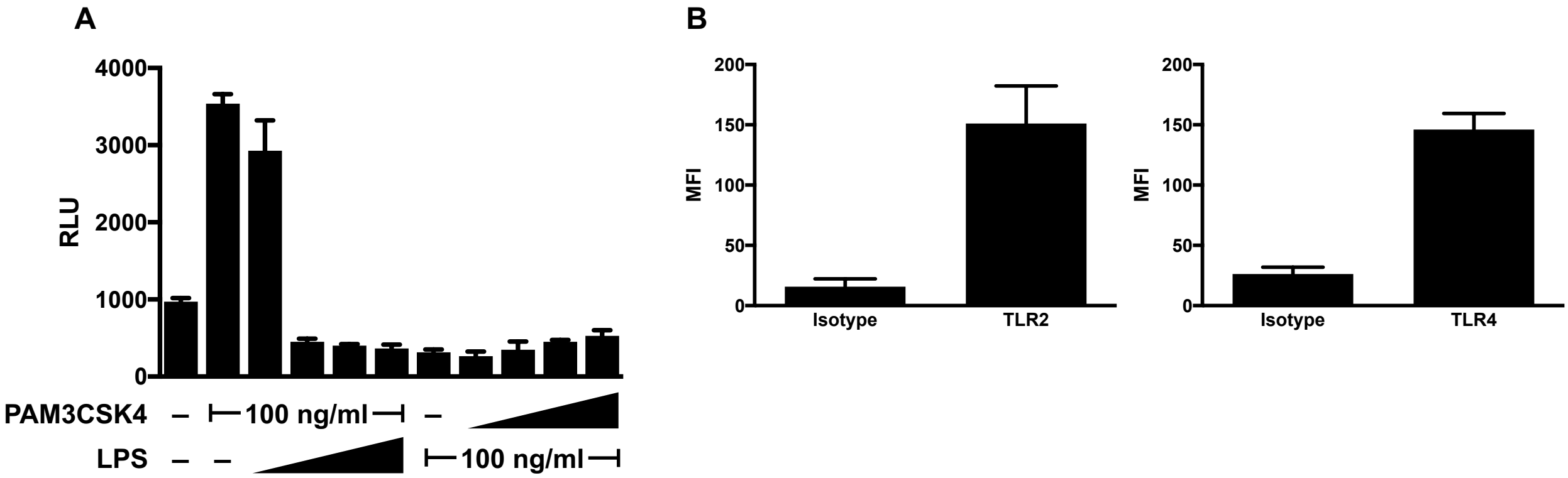

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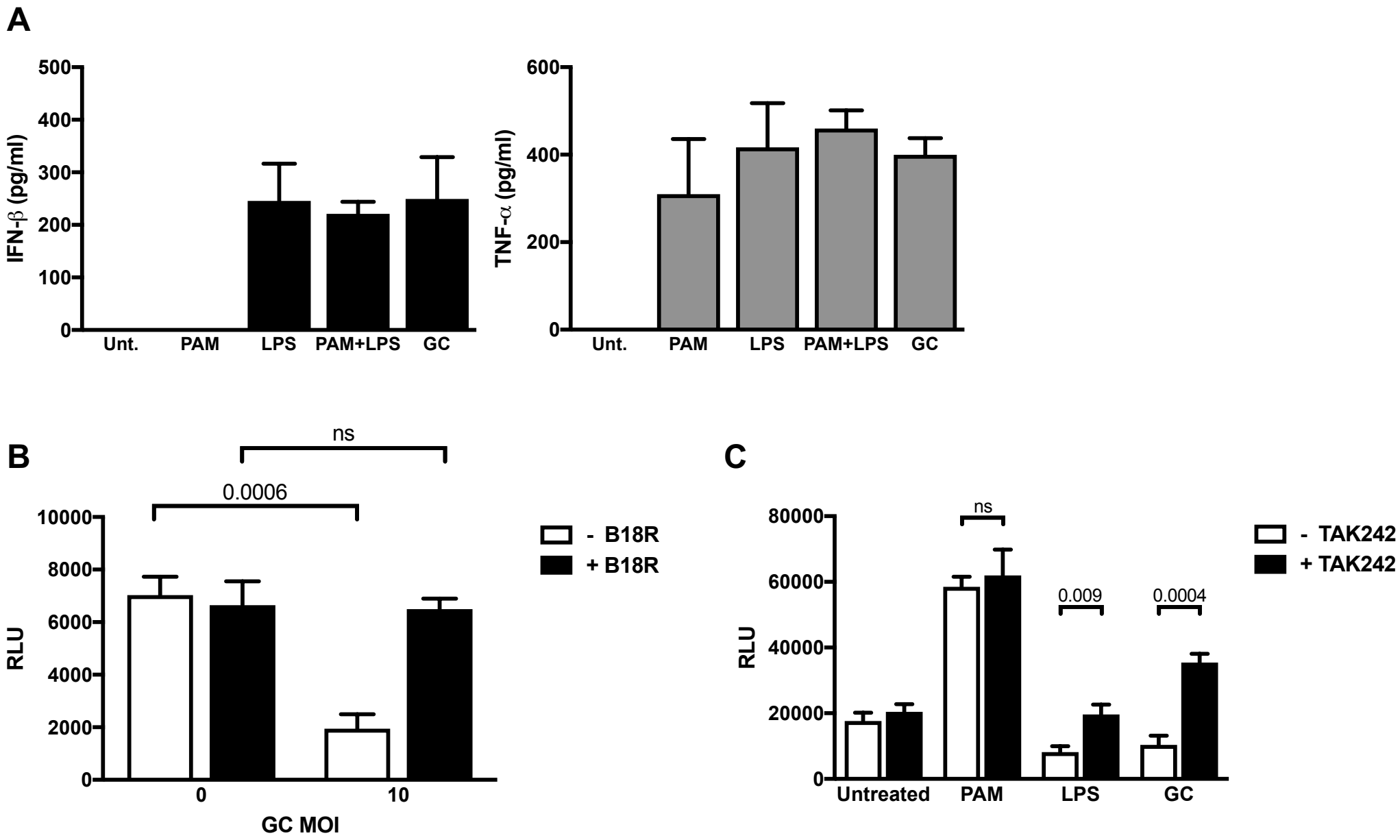
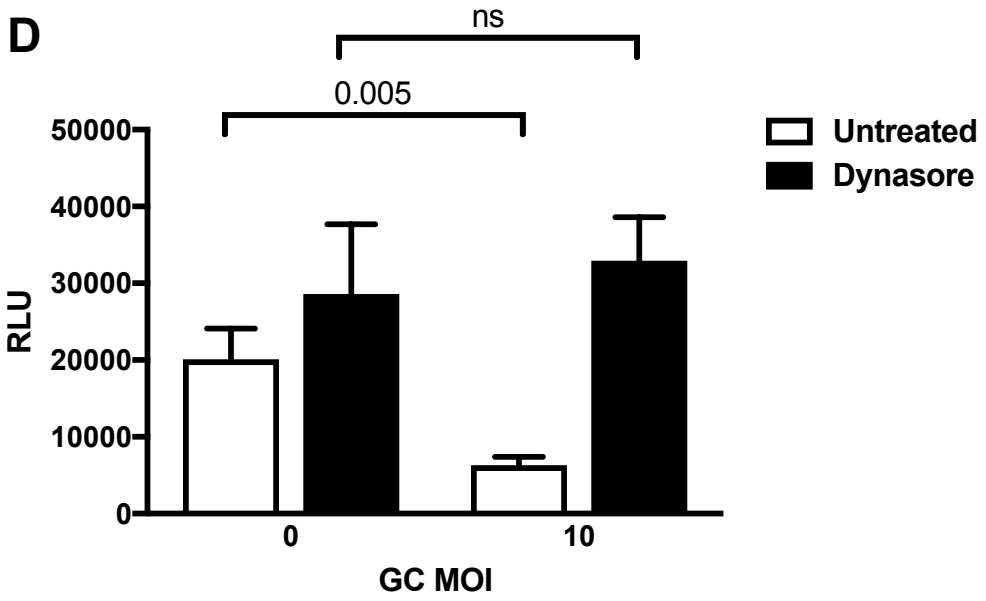
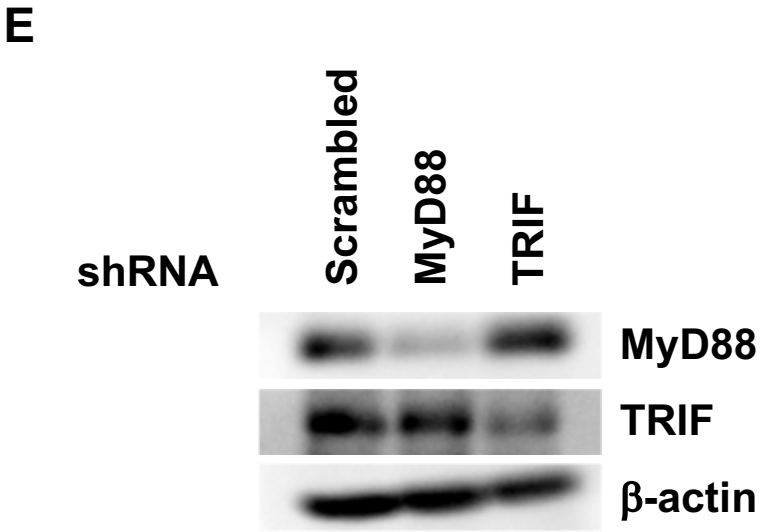
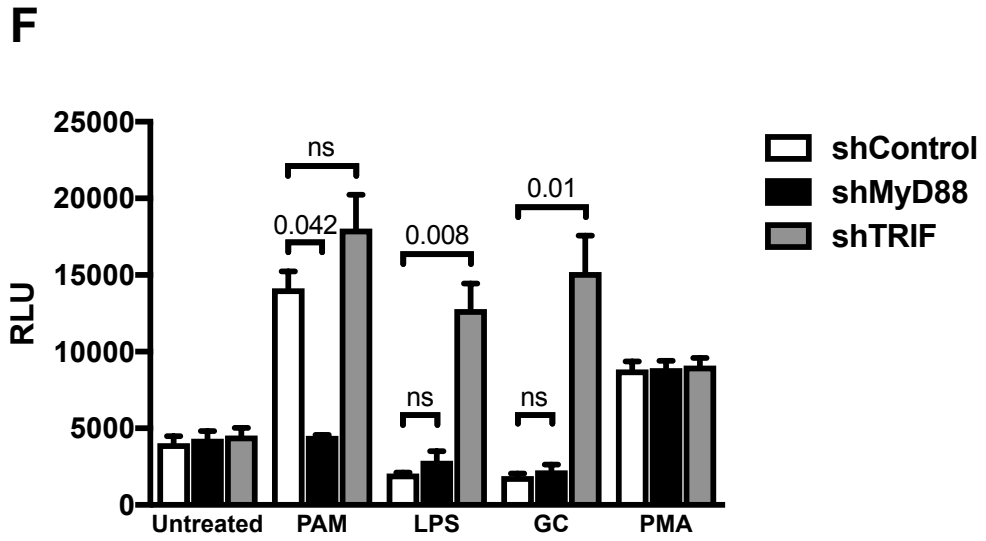


Hanley Figure 5 (A-D)







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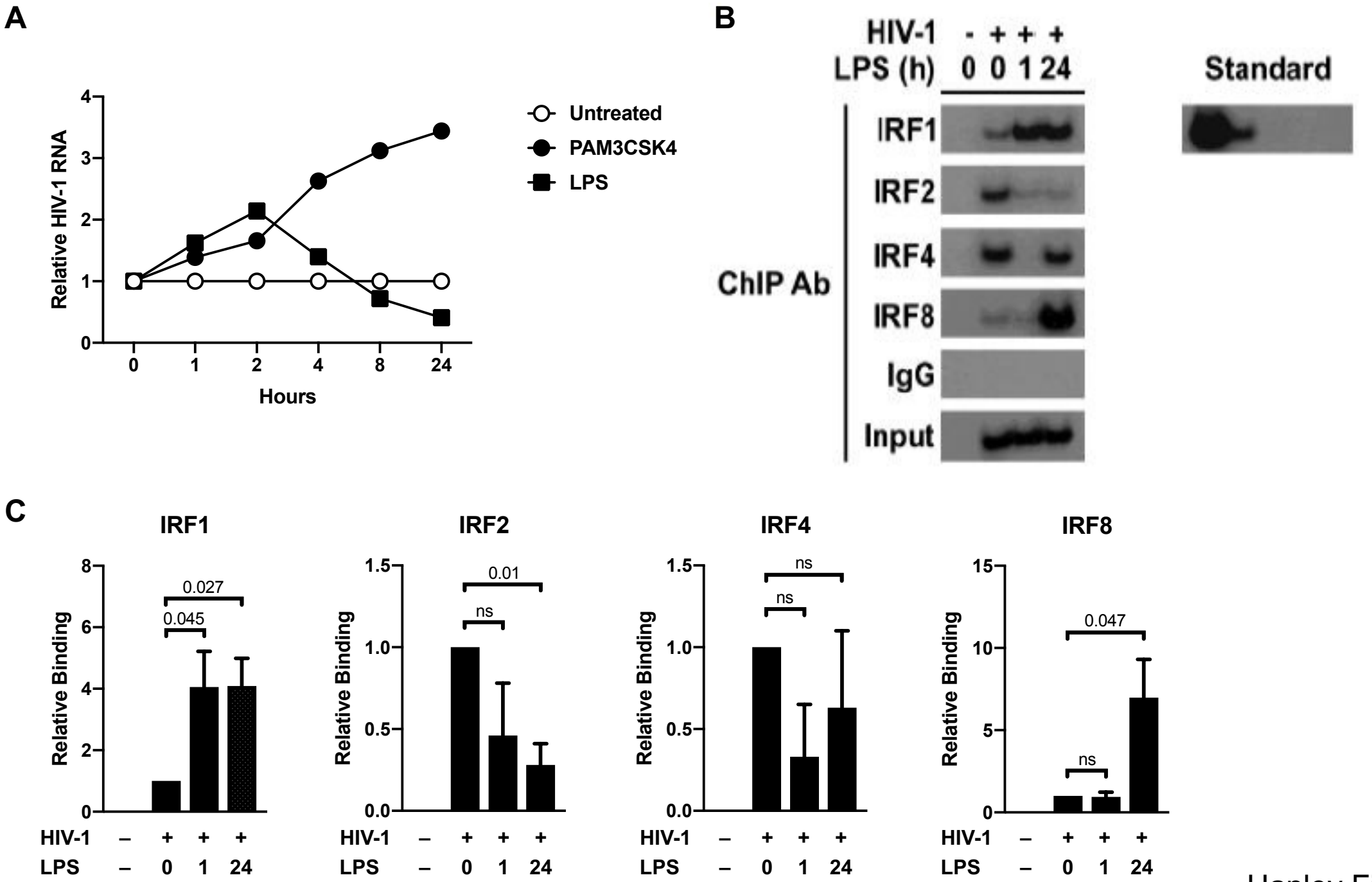
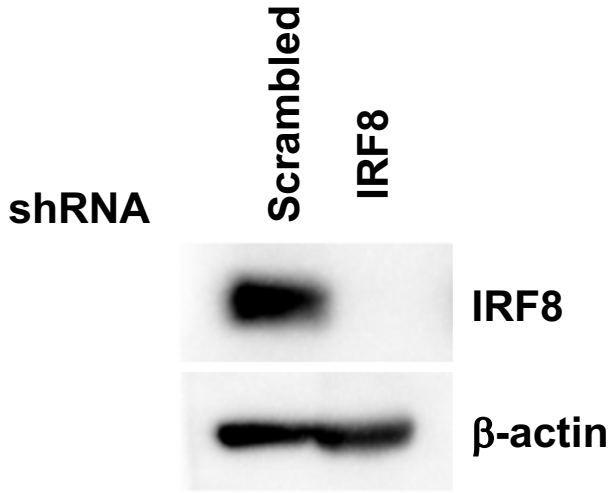
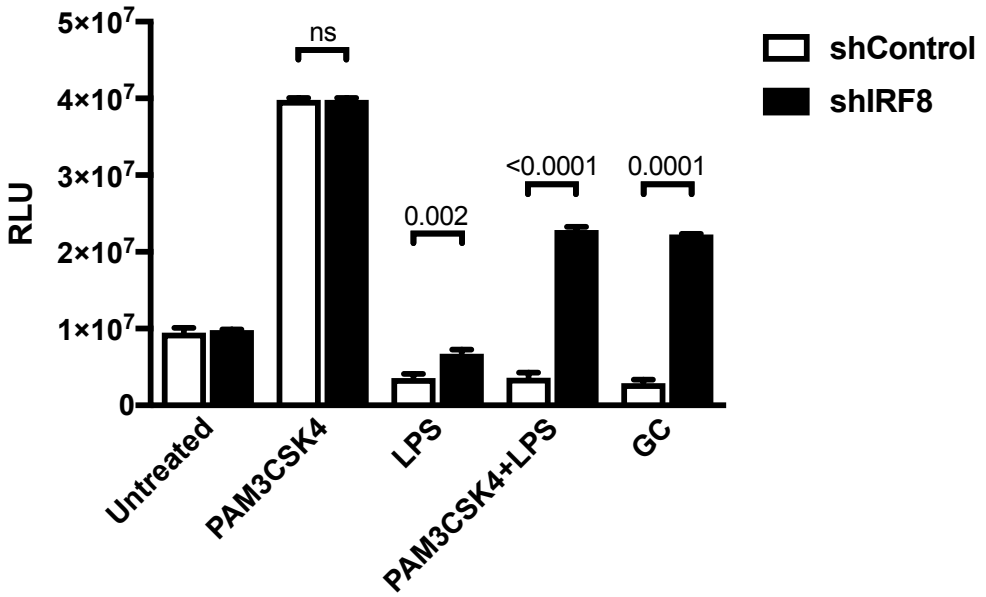


Hanley Figure 6 (A-C)

D



E



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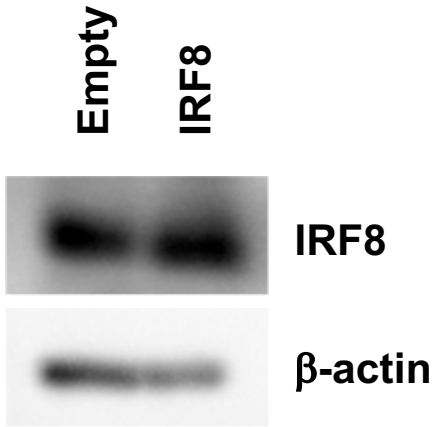

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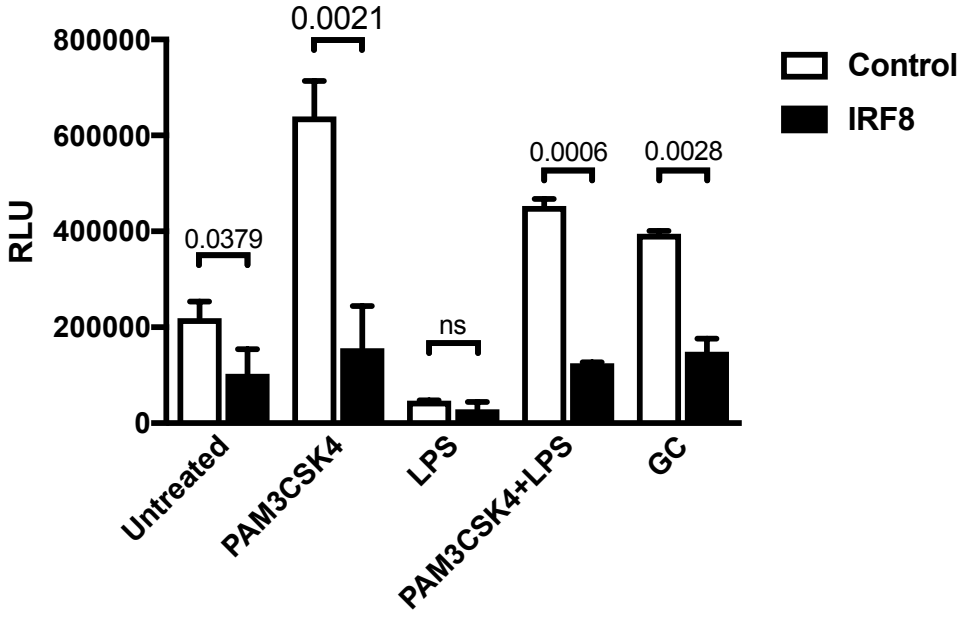
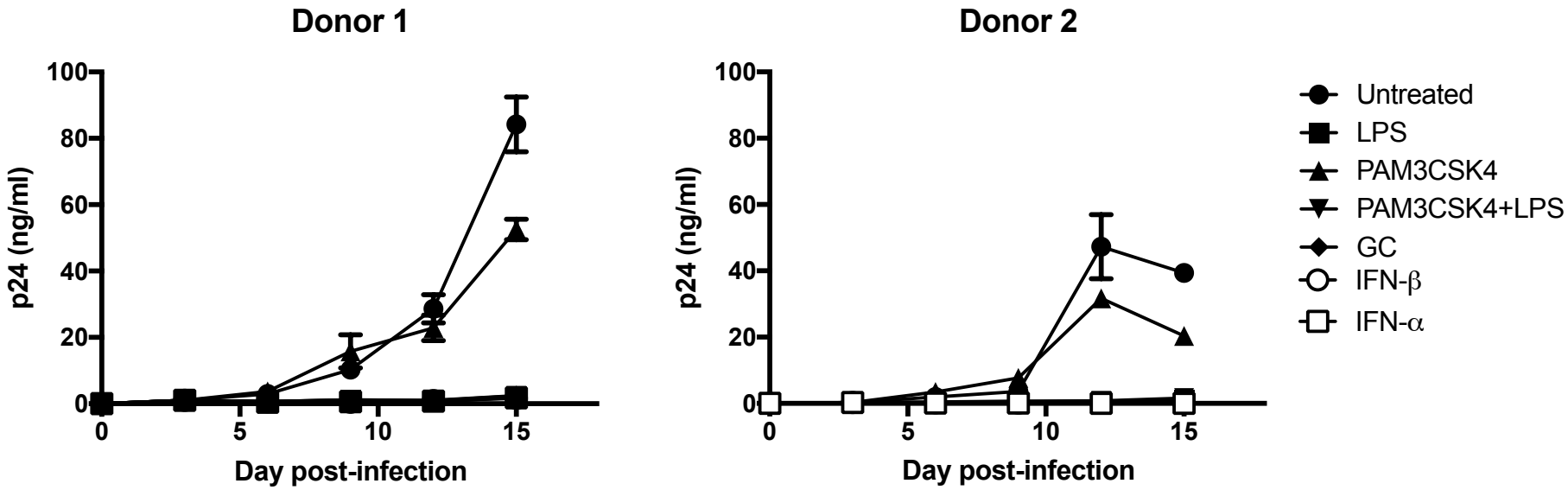
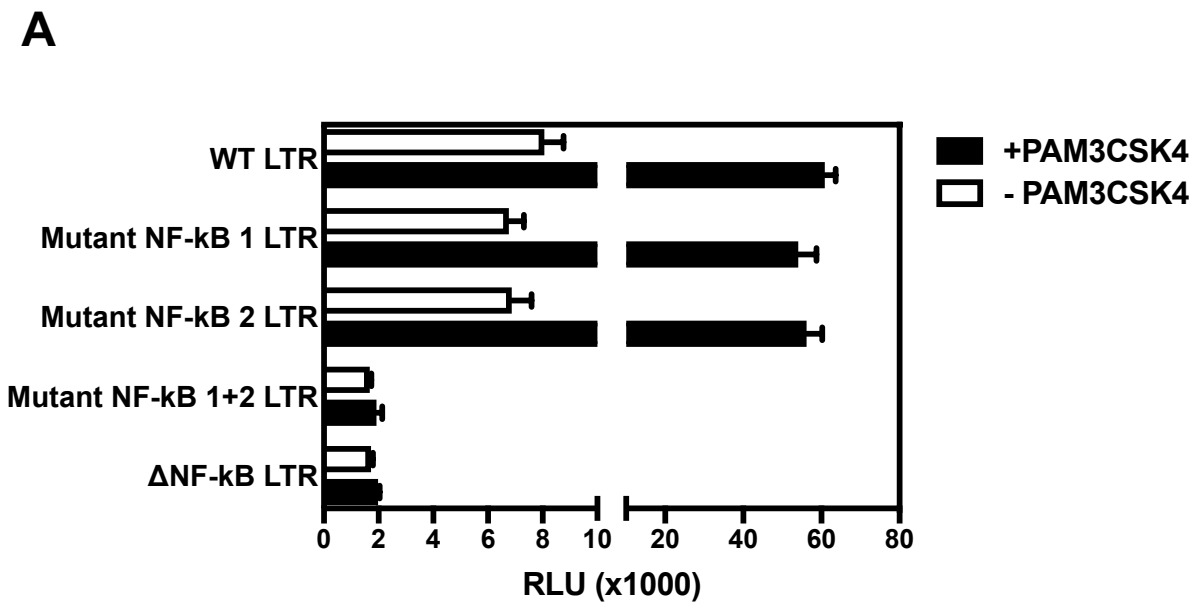
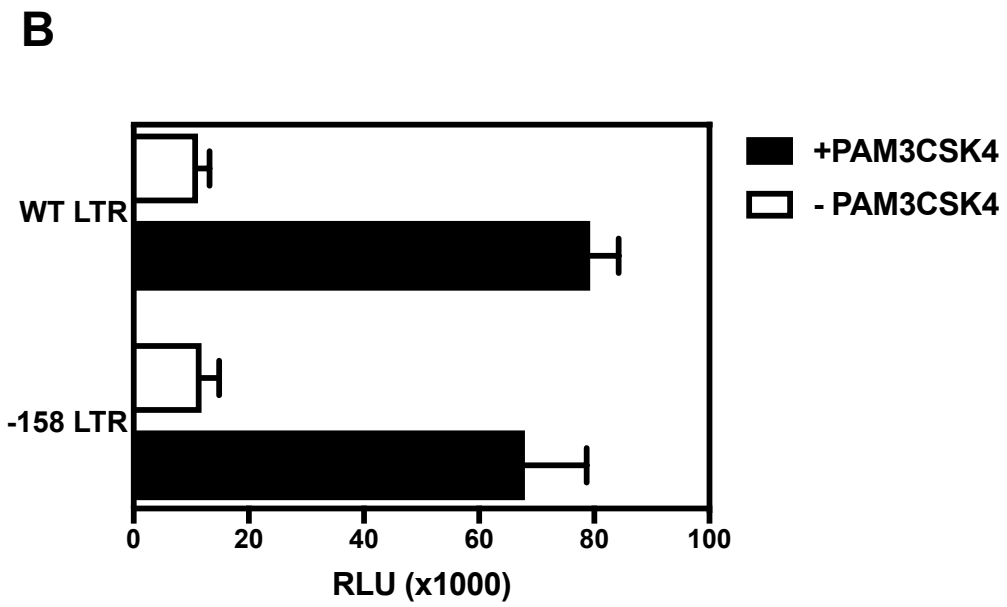


Hanley Figure 6 (D-G)

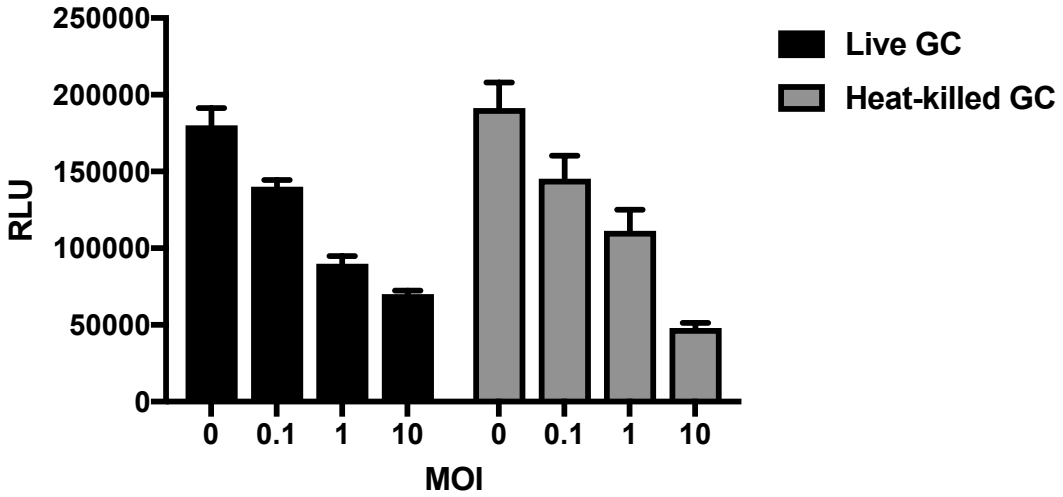


Hanley Figure 7

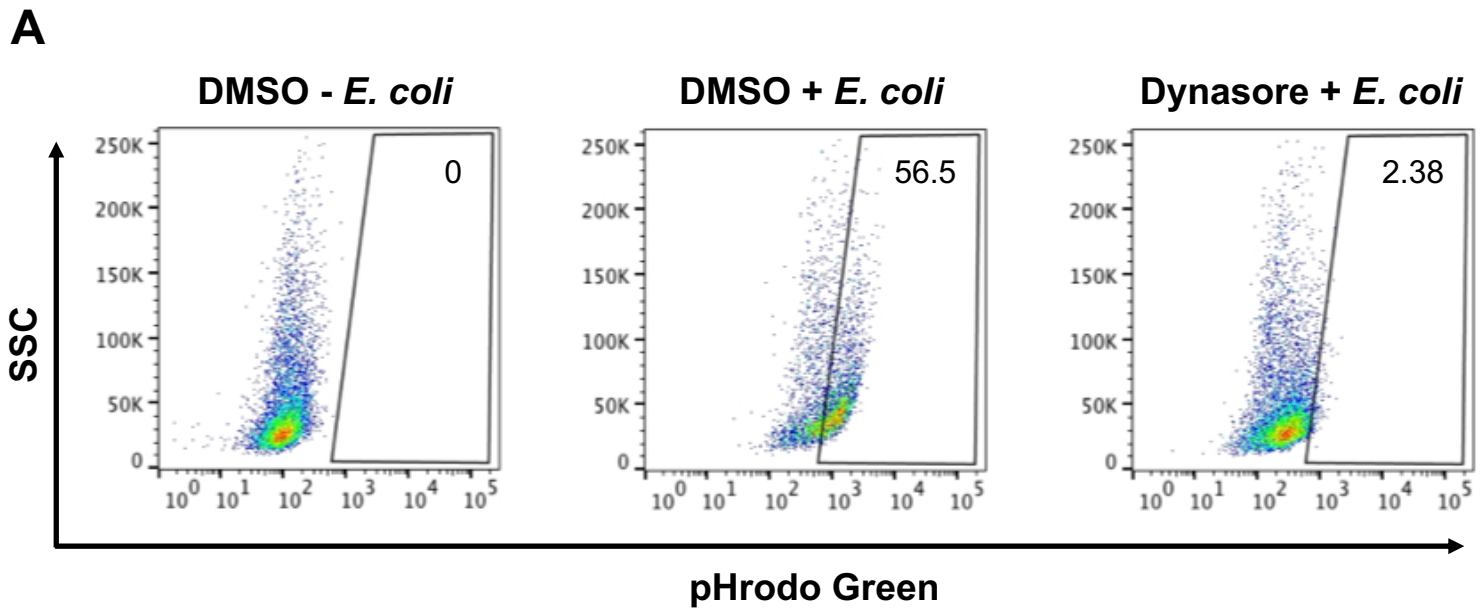


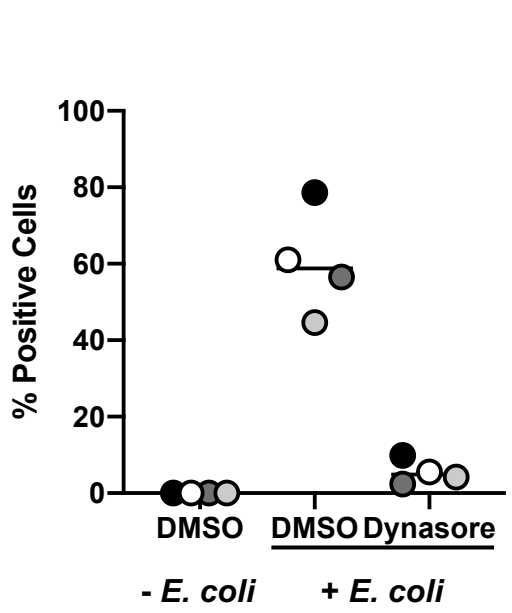


Hanley Supplemental Figure 1

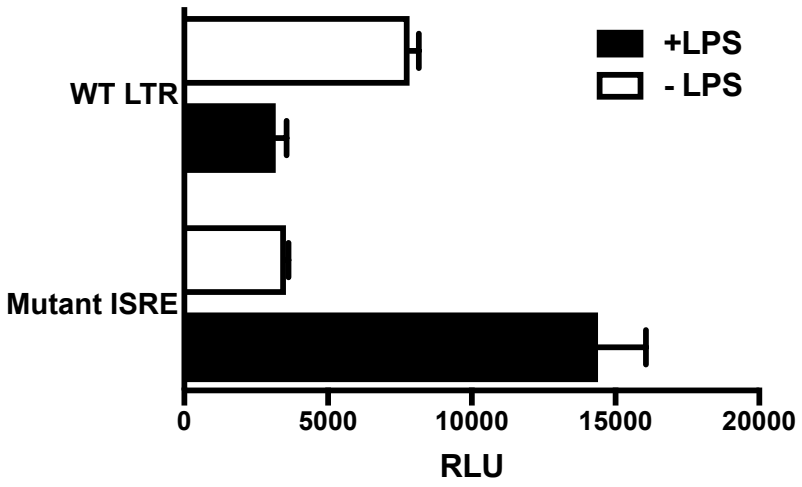


Hanley Supplemental Figure 2





Hanley Supplemental Figure 3



Hanley Supplemental Figure 4