

1 **Differential TLR-ERK1/2 activity promotes viral ssRNA and dsRNA mimic-induced dysregulated**
2 **immunity in macrophages.**

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25 **Running title:** TLR-ERK1/2 activity promotes dysregulated immunity.

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37 **ABSTRACT:**

38 RNA virus induced excessive inflammation and impaired antiviral interferon (IFN-I) responses are
39 associated with severe disease. This innate immune response, also referred to as ‘dysregulated
40 immunity,’ is caused by viral single-stranded RNA (ssRNA) and double-stranded-RNA (dsRNA) mediated
41 exuberant inflammation and viral protein-induced IFN antagonism. However, key host factors and the
42 underlying mechanism driving viral RNA-mediated dysregulated immunity are poorly defined. Here, using
43 viral ssRNA and dsRNA mimics, which activate toll-like receptor 7 (TLR7) and TLR3, respectively, we
44 evaluated the role of viral RNAs in causing dysregulated immunity. We show that murine bone marrow-
45 derived macrophages (BMDMs) stimulated with TLR3 and TLR7 agonists induce differential inflammatory
46 and antiviral cytokine response. TLR7 activation triggered a robust inflammatory cytokine/chemokine
47 induction compared to TLR3 activation, whereas TLR3 stimulation induced significantly increased
48 IFN/IFN stimulated gene (ISG) response relative to TLR7 activation. To define the mechanistic basis for
49 dysregulated immunity, we examined cell-surface and endosomal TLR levels and downstream mitogen-
50 activated protein kinase (MAPK) and nuclear factor kappa B (NF-kB) activation. We identified a
51 significantly higher cell-surface and endosomal TLR7 expression compared to TLR3, which further
52 correlated with early and robust MAPK (pERK1/2 and p-P38) and NF-kB activation in TLR7-stimulated
53 macrophages. Furthermore, blocking EKR1/2, p38, and NF-kB activity reduced TLR3/7-induced
54 inflammatory cytokine/chemokine levels, whereas only ERK1/2 inhibition enhanced viral RNA-mimic-
55 induced IFN/ISG responses. Collectively, our results illustrate that high cell surface and endosomal TLR7
56 expression and robust ERK1/2 activation drive viral ssRNA mimic-induced excessive inflammatory and
57 reduced IFN/ISG responses, and blocking ERK1/2 activity would mitigate viral-RNA/TLR-induced
58 dysregulated immunity.

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71 **INTRODUCTION:**

72 Emerging and re-emerging RNA viruses pose a significant threat to global public health. As opposed to
73 the only dsRNA virus (rotavirus) infecting humans, several dozens of positive and negative sense ssRNA
74 viruses cause disease in humans. Most of these ssRNA viruses have zoonotic sources, and their
75 accidental spillover from animal reservoirs into human population has caused multiple epidemics and
76 pandemics ^{1 2}. In addition to the viruses currently circulating in humans or periodically spilled over from
77 animal sources, many ssRNA viruses with zoonotic potential to infect and cause outbreaks in humans
78 have been identified in bats and several other animal reservoirs ^{3 4 5 6}. Interestingly, compared to DNA
79 viruses (e.g., herpesviruses, adenoviruses, hepadnavirus, and papillomaviruses), the majority of
80 emerging and re-emerging ssRNA viruses cause acute and often severe disease in humans ⁷. The fatal
81 illness caused by these RNA viruses is commonly associated with excessive inflammation and impaired
82 antiviral immunity (dysregulated immunity) ^{8 9 10}. Consequently, to better understand severe disease
83 caused by the ssRNA viruses, it is critical to define the virus and host factors that drive excessive
84 inflammation and impair antiviral immunity.

85

86 Viruses induce antiviral and inflammatory responses following recognition of viral pathogen-associated
87 molecular patterns (PAMPs) by cognate immune sensors ^{11 12 13}. The viral ssRNA genome and replication
88 intermediate dsRNA are two key PAMPs that elicit antiviral and inflammatory response during ssRNA
89 virus infections ^{14 15}. Among the two viral RNA molecules, ssRNA is highly abundant, and by some
90 estimates, the ratio of ssRNA to dsRNA is >100 to 1 in an active ssRNA virus replicating cell ¹⁶. Following
91 infection, the intracellular ssRNA is sensed by retinoic acid-inducible gene I (RIG-I) receptor while dsRNA
92 is detected by both RIG-I and melanoma differentiation-associated protein 5 (MDA-5) ^{17 18 19 20}. In
93 contrast, extracellular viral ssRNA and dsRNA, derived from a lytic infection, are detected by cell surface
94 and endosomal located TLR7/8 and TLR3, respectively ^{21 22 23}. TLR7/8 utilizes myeloid differentiation
95 primary response 88 (Myd88) and TLR3 signals through TIR-domain-containing adaptor-inducing beta
96 interferon (TRIF) to elicit antiviral and inflammatory responses ^{21 24 25 26}. TLR-Myd88 or TRIF signaling
97 further activates mitogen-activated protein kinase (MAPK) or nuclear factor kappa B (NF- κ B) to induce
98 inflammatory mediators and interferon regulatory factors (IRFs) to facilitate IFN/ISG response ^{27 28 29 30}.
99 However, the relative contribution of TLR7 and TLR3 activation and the mechanistic basis for TLR7- and
100 TLR3-induced differential antiviral and inflammatory response are not clearly defined.

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102 Viruses possess multiple structural and non-structural proteins (NSPs) that directly interact with IFN
103 signaling proteins, ISGs, and NF- κ B to subvert antiviral responses ^{31 32 33}. However, the virus-induced
104 antagonism of IFN/ISG activity without directly interacting with intracellular signaling protein(s) is poorly

105 described. Therefore, the primary objective of this study is to elucidate the role of differential cell surface
106 and endosomal TLR3/TLR7 expression and downstream ERK1/2 activation in viral RNA-induced
107 dysregulated immunity. In this study, to mimic extracellular viral RNA sensing by cell surface or
108 endosomal TLR3 and TLR7, we treated murine bone marrow-derived macrophages (BMDMs) with R848
109 (ssRNA mimic/TLR7 agonist) and PolyIC (dsRNA mimic/TLR3 agonist). Our results illustrate that viral
110 ssRNA and dsRNA mimics induce differential antiviral and inflammatory cytokine responses, which
111 correlates with cell surface/endosomal TLR3/TLR7 expression and MAPK and NF- κ B activation. We also
112 show that viral RNA-mimic-induced robust ERK1/2 activation drives dysregulated immunity. Collectively,
113 our results highlight an under-appreciated role of viral ssRNA- and dsRNA-mediated differential ERK1/2
114 activation in virus-induced dysregulated immunity and, by extension, severe disease.

115

116 **MATERIALS AND METHODS:**

117 **Murine macrophage extraction and stimulation of macrophages:** Bone marrow cells extracted from
118 C57BL/6 mice were grown in 12-well plates using RPMI medium supplemented with 10% fetal bovine
119 serum, 1% penicillin-streptomycin, 1% L-glutamine, 1% sodium bicarbonate, 1% sodium pyruvate, 1%
120 non-essential amino acids and were treated with macrophage colony-stimulating factor (m-MCSF,
121 GenScript, Cat # Z02930-50, 40 ng/ml) for 6-7 days with media change every three days to differentiate
122 bone marrow cells into mouse bone marrow-derived macrophages (BMDMs).

123

124 **TLR stimulation and MAPK/NF- κ B inhibitor studies:** For stimulation studies, a confluent monolayer
125 of primary BMDMs was stimulated with R848 (InvivoGen, Catalog # tlr-r848-5, single-stranded RNA
126 mimic) and Poly I:C (InvivoGen, Catalog # tlr-pic, double-stranded RNA mimic) each treated at 5 or
127 10ug/ml for 8 or 24 hours. After 8 and 24 hours of stimulation, cells in Trizol or cell supernatants were
128 collected to assess mRNA and protein levels, respectively, of inflammatory cytokines and chemokines
129 and type-I interferons (IFN-Is). Primary mouse BMDMs were treated with pathway-specific inhibitors for
130 inflammatory pathway inhibition studies, each at 5 μ M concentration. Specifically, we used inhibitors of
131 ERK1/2 (Trametinib, SelleckChem, Catalog # S2673), p38 (Losmapimod, Selleckchem Catalog #S7215),
132 NF- κ B (BAY11-7082, Catalog # S2913) and JNK inhibitor (Millipore-Sigma Catalog # CAS 129-56-6) 2 hr
133 before stimulation with R848 or Poly I:C until sample collection at 8 or 24hrs for mRNA or protein levels
134 of antiviral and inflammatory cytokines and chemokines.

135

136 **Enzyme-linked immunosorbent assay (ELISA):** We used sandwich ELISA to quantify cytokine levels.
137 Concentrations of TNF- α (BD Biosciences., San Diego, CA, USA Cat#558534), IL-6 (BD Biosciences.,
138 San Diego, CA, USA Cat # 555240), MCP-1 (BD Biosciences., San Diego, CA, USA Cat # 555260), and

139 IFN- β (R&D systems., Minneapolis, MN, USA Cat #DY8234-05) in cell culture supernatants were
140 measured using commercially available BD OptEIA™ or Bio-technie ELISA kits as per manufacturer's
141 instructions. A 96-well high-binding microplate (Corning, Catalog # 3361) was coated with capture
142 antibody overnight at 4°C or room temperature. The next day, plates were blocked for 1 hour at room
143 temperature and 100 μ L of samples were added to each ELISA well and incubated for 2 hrs. Washing
144 was performed 2-3 times at each subsequent step. Detection antibody was added to each well and
145 incubated for 1 hour, followed by the addition of an HRP-conjugated enzyme reagent. The plates were
146 incubated for 30 minutes in the dark using TMB substrate (BD Biosciences., San Diego, CA, USA Cat
147 #555214). The colorimetric reaction was stopped by adding 2N H₂SO₄, and absorbance was measured
148 at 450nm wavelength using a spectrophotometer (SpectraMax® M2e).

149

150 **Quantitative reverse transcription polymerase chain reaction (qRT-qPCR):** Total RNA was extracted
151 from BMDMs using a Trizol reagent (TRIZOL, Invitrogen Cat #15596026) as per manufacturer's
152 instructions. Trizol-extracted RNA was treated with RNase-free DNase (Promega Catalog: M610A) to
153 remove any genomic DNA contamination and RNA was quantitated using Nanodrop (Nanodrop™
154 ThermoScientific). cDNA was synthesized using m-MLV Reverse Transcriptase (Invitrogen Cat #
155 28025013). Quantitative gene analysis was performed using PCR master mix (2X qPCR Universal Green
156 MasterMix Cat # qMX-Green-25 ml, Lamda Biotech) and real-time PCR system (Applied Biosystems
157 7500 QuantStudio 6 Pro). The Ct value of target genes obtained from each sample was normalized to
158 housekeeping gene GAPDH and relative gene expression was quantified using $2^{-\Delta\Delta Ct}$ values.

159

160 **Western blot assay:** Total cell extracts were lysed in RIPA buffer (Cell signaling, catalog # 9806)
161 supplemented with protease inhibitor (Thermo Scientific., Cat # 1861281) post-RNA-mimic treatment (10,
162 30, and 60 min). Total protein concentrations were determined using a Pierce™ BCA protein assay kit
163 (ThermoFisher Scientific, Cat # 23225). An equal amount of protein was loaded for SDS-PAGE on 10%
164 polyacrylamide gels, electro-transferred to nitrocellulose membrane (Bio-Rad 0.45 μ M #1620115), and
165 probed with primary antibody overnight at 4°C. Primary antibodies used are: anti-phosphorylated p44/42
166 MAPK (Cell signaling # 9101, 1:1000), anti-p44/42 MAPK (Cell signaling # 4695, 1:1000), anti-
167 phosphorylated p38 MAPK (Cell signaling # 4511, 1:1000), anti-p38 MAPK (Cell signaling # 8690,
168 1:1000), anti-phosphorylated SAPK/JNK (Cell signaling # 9255, 1:1000), anti-SAPK/JNK (Cell signaling
169 # 9252, 1:1000), anti-phosphorylated NF- κ B p65 (Cell signaling # 3033, 1:1000), anti-NF- κ B p65 (Cell
170 signaling # 8242, 1:1000), and β -actin (Invitrogen # MA5-15739). The membrane was washed 3X times
171 with TBST wash buffer and incubated with appropriate secondary antibody for 1 hour, then visualized
172 using Amersham Imager 600. Secondary antibodies used are Anti-Mouse IgG (Jackson

173 ImmunoResearch Cat #115-035-003) and Anti-Rabbit IgG (Invitrogen # 31460). Beta-actin was used as
174 a loading control. For densitometry, images are analyzed using Image J software.

175

176 **Flow Cytometry:** Surface and intracellular expression of TLR3 and TLR7 on BMDMs were determined
177 by FACS analysis. A total of 1.5×10^5 cells/well were seeded on a 96-well round bottom plate. For cell
178 surface staining, cells were stained with PECy7 α -CD45 (clone: 30-F11, Biolegend: cat # 103114), V450
179 α -CD11b (clone: M1/70, Biolegend: Cat # 101224), APC α -F4/80 (clone: BM8, BioLegend: Cat #123116),
180 PE α -TLR3 (clone:11F8, Biolegend: Cat 141904), and PE α -TLR7 (clone: A 94B10, BD Biosciences: Cat
181 # 565557) and incubated for 20 min at 4°C in the dark. Cells were washed once with FACS buffer (1X
182 PBS; 2% FBS, 0.05% NaA2), followed by centrifugation at 1200g for 5 min. Intracellular expression of
183 TLR3 or TLR7 was determined following fixation and permeabilization using a Cytotfix/Cytoperm Kit (BD
184 Bioscience Catalog # 554655). All antibodies were diluted 1:200 in FACS buffer for surface staining or
185 perm buffer for intracellular staining. Samples were acquired using BD FACS-ARIA-III™ and were
186 analyzed using FlowJo™ software (Tree Star).

187

188 **Statistical analysis:** Statistical analysis was done using GraphPad Prism Version 10.0.3 (GraphPad
189 Software Inc). Results were analyzed using Student's *t*-test or One-way ANOVA. Data in bar graphs are
190 represented as mean +/- standard error of the mean (SEM). Threshold values of **P*<0.05, ***P*<0.01,
191 ****P*<0.001, or *****P*<0.0001 were used to assess statistical significance.

192

193 **RESULTS:**

194 **Viral ssRNA and dsRNA mimics induce differential inflammatory and antiviral responses.**

195 Excessive inflammation caused by RNA viruses is a key determinant of poor clinical outcomes³⁴.
196 However, the key virus and host factors driving RNA virus-induced lethal inflammation and severe
197 disease are poorly described. Since both viral ssRNA and dsRNA are released extracellularly upon lytic
198 infection^{15 35} and are detected by the immune sensors on the sentinel or circulating myeloid cells, we
199 evaluated whether ssRNA and dsRNA and their respective cell surface/endosomal TLRs elicit differential
200 inflammatory cytokine and chemokine responses. For these studies, murine BMDMs were treated with
201 ssRNA mimic (R848) and dsRNA mimic (Poly I:C). Cells collected in Trizol at 8 hours post-stimulation or
202 cell supernatants collected at 24 hours post-stimulation were used to assess mRNA and protein levels,
203 respectively, of inflammatory cytokines and chemokines. Our results show a significant increase in mRNA
204 levels of inflammatory cytokines and chemokines in R848 treated BMDMs compared to Poly I:C treated
205 cells (Figure 1A). Further examination of protein levels of representative inflammatory cytokines (TNF- α
206 and IL-6) also revealed markedly higher levels of these inflammatory mediators in R848 treated cells

207 compared to Poly I:C stimulated cultures (Figure 1B). Next, we examined the effect of R848 and Poly I:C
208 on antiviral IFN-I and ISG responses. Cells in trizol and cell supernatants collected at 8 and 24 hr post-
209 stimulation were used to measure mRNA or protein levels of IFNs and ISGs. As shown in Figure 1C-D,
210 we observed marginal to no IFN and ISG response in R848-stimulated macrophages compared to
211 unstimulated cells, whereas Poly I:C treatment induced a robust increase in IFN and ISG levels. Poly I:C
212 activates both TLR3-TRIF and MDA5-MAVS in fibroblasts³⁶. Therefore, to confirm Poly I:C-induced
213 antiviral and inflammatory response in macrophages is mediated by TLR3-TRIF signaling, we stimulated
214 wild-type and TRIF^{-/-} cells with Poly I:C. Our results showed a complete loss of antiviral and inflammatory
215 cytokine production in Poly I:C treated TRIF^{-/-} cells compared to wild-type cells (Figure 1E), suggesting
216 that Poly I:C-induced immune response in macrophages is dependent on TLR3-TRIF activity. We also
217 confirmed that R848 mediated cytokine/chemokine response is Myd88 dependent (Figure 1F). These
218 results collectively demonstrate that extracellularly derived viral ssRNA mimic induces significantly
219 elevated levels of inflammatory cytokine/chemokines compared to dsRNA mimic, whereas dsRNA is a
220 more potent inducer of IFN and ISG responses than ssRNA in mouse macrophages.

221

222 **Macrophages express differential levels of cell surface and endosomal TLR3 and TLR7.**

223 Macrophages express high levels of TLRs, and recognition of viral PAMPs by these TLRs plays a crucial
224 role in antiviral and inflammatory response^{37 38}. Extracellularly derived viral dsRNA and ssRNA are
225 primarily recognized by cell surface/endosomal TLR3 and TLR7, respectively^{21 23}. Since we observed
226 differential antiviral and inflammatory cytokine response following TLR3 and TLR7 agonist administration,
227 we first asked whether altered TLR expression by macrophages contributes to differential antiviral and
228 inflammatory cytokine production. To test this possibility, we examined cell surface and endosomal TLR3
229 and TLR7 expression in murine BMDMs. As shown in Figure 2A-B, flow cytometry data showed that the
230 percentage of macrophages expressing cell surface and endosomal TLR7 was significantly higher than
231 for TLR3 expression. Additionally, we found a 5-10-fold increase in mean fluorescence intensity (MFI,
232 i.e., number of molecules expressed per cell) of TLR7 expression in endosomes than on the cell surface
233 (Figure 2C). These results show a significantly higher expression of TLR7 in macrophages (both cell
234 surface as well as intracellular) than that of TLR3, which may contribute to the differential inflammatory
235 and antiviral immune response observed following ssRNA mimic/TLR7 agonist treatment compared to
236 the dsRNA mimic/TLR3 agonist administration.

237

238 **R848 and Poly I:C induce differential MAPK and NF- κ B activation in macrophages.**

239 Our results showed differential inflammatory cytokine response in Poly I:C and R848 treated cells (Figure
240 1), which correlated with cell surface and endosomal TLR3/7 expression (Figure 2). However, these

241 results fail to show whether or not such differential TLR3/7 expression correlates with altered downstream
242 signaling. TLR7 facilitates MyD88-TRAF6 and TLR3 utilizes TRIF-TRAF6 adapters to induce NF- κ B/
243 MAPK-mediated inflammatory cytokine response^{39 40}. Therefore, to determine the mechanistic basis for
244 R848/TLR7- and Poly I:C/TLR3-induced differential inflammatory cytokine response, we stimulated
245 primary mouse BMDMs with these viral ssRNA and dsRNA mimics. The cell lysates collected at 10, 30,
246 and 60 minutes were analyzed for total and phospho-levels of MAPKs and NF- κ B using western blot
247 assay. As shown in Figure 3A, we observed a significant increase in phosphorylation of MAPK (p-ERK,
248 p-P38, p-JNK) and NF- κ B(p-P65) pathways in R848 stimulated macrophages as early as 10mins post-
249 stimulation, which persisted for up to 30 min post-stimulation. In contrast, we observed a delay in poly
250 I:C induced phosphorylation of these pathways with robust MAPK/NF- κ B activity observed at 60 min post-
251 stimulation (Figure 3A-B). These results indicate that R848-TLR7 signaling promotes early activation of
252 MAPK and NF- κ B pathways, whereas poly I:C-TLR3 signaling is a delayed and muted activator of these
253 downstream pathways.

254

255 **Blocking ERK1/2 reduces inflammation and improves IFN in viral RNA mimic treated cells.**

256 Our results thus far illustrate that dysregulated immunity in ssRNA mimic treated cells correlates with high
257 TLR7 expression and TLR7-induced robust MAPK/NF- κ B activation (Figures 1-3). To further demonstrate
258 that TLR7-induced robust MAPK and NF- κ B activity promotes excessive inflammation, we blocked MAPK
259 and NF- κ B signaling using well-established inhibitors for each pathway. First, we evaluated the ability of
260 MAPK and NF- κ B inhibitors to block pathway-specific activity. As shown in figure 4A, TLR-stimulated
261 BMDMs pre-treated with specific inhibitors of ERK1/2 (Trametinib), p38 (Losmapimod), JNK (JNK
262 inhibitor I), and NF- κ B (BAY11-7082) showed reduced phosphorylation of respective proteins compared
263 to untreated cells. Next, cell supernatants collected from control and pathway-specific inhibitor-treated
264 BMDMs stimulated with R848 or Poly I:C (10 g/ml each) were examined for TNF, MCP-1, and IFN- β
265 levels. As expected, blocking p-ERK1/2, p-NF- κ B, and p-JNK activity all reduced inflammatory cytokine
266 and chemokine production (Figure 4B). However, to our surprise, p-p38 inhibition enhanced inflammatory
267 cytokines/chemokine levels in BMDM cultures (Figure 4B). Interestingly, p-ERK1/2 inhibition improved
268 type IFN-I levels (Figure 4C) in both R848 and Poly I:C treated cells, which correlated with enhanced ISG
269 levels (Figure 4D-E). Conversely, blocking NF- κ B suppressed IFN- β levels, whereas p38 and JNK
270 inhibition had minimal impact on IFN-I response (Figure 4C). Collectively, these results establish that a)
271 TLR7-induced robust MAPK/NF- κ B activation promotes excessive inflammation and b) ERK1/2 signaling
272 promotes inflammatory cytokine/chemokine levels while suppressing viral RNA mimics-induced IFN-I and
273 ISG responses.

274

275 **DISCUSSION:**

276 Emerging and re-emerging RNA viruses cause acute and often fatal illness. The RNA virus-induced
277 severe disease is caused by excessive inflammatory and impaired or delayed antiviral IFN/ISG
278 responses^{9 10}. Additionally, ssRNA-induced severe inflammation is associated with excessive monocyte-
279 macrophage accumulation^{41 42 43 44}. However, the basis for RNA virus-induced lethal inflammation and
280 the host factors that suppress antiviral IFN/ISG responses need to be well defined. Viral RNAs are critical
281 orchestrators of antiviral and inflammatory responses and monocyte-macrophages are a key source of
282 inflammatory mediators and IFNs^{45 8 46}. Therefore, in this study, using RNA mimics, we examined the
283 effects of viral ssRNA and dsRNA in eliciting macrophage-induced inflammatory and antiviral responses.
284 Our results illustrate that viral ssRNA mimics induce a robust inflammatory but a poor IFN response,
285 whereas dsRNA mimics elicited a strong IFN and a relatively weak inflammatory cytokine response. We
286 further demonstrate that viral ssRNA- and dsRNA-mimic-mediated differential immune response is likely
287 driven by the high cell surface and endosomal TLR7 expression as opposed to low TLR3 expression in
288 both compartments. Excessive inflammatory cytokine response and high cell surface/endosomal TLR
289 expression correlate with early and robust NF-κB and MAPK activation in TLR7 compared to TLR3
290 agonist-treated cells. Finally, we demonstrate that blocking NF-κB and MAPK sub-pathways reduces viral
291 RNA mimic-induced inflammatory cytokine production, while only ERK1/2 inhibition significantly
292 increased IFN/ISG responses. Our results, albeit using viral RNA mimics, collectively explain the basis
293 for the dysregulated immunity caused by RNA viruses.

294
295 The differential role of TLR7 and TLR3 in inducing inflammatory and antiviral cytokines have been
296 previously described^{21 47 48}. However, most of these studies have examined either TLR7 and TLR3
297 agonist-induced inflammatory cytokine response or antiviral IFN/ISG response without defining the
298 implications of these results in RNA virus-induced dysregulated immunity. The majority of emerging RNA
299 viruses consist of ssRNA genome that make dsRNA intermediate during replication⁴⁹. ssRNA is 100-fold
300 or more abundant than dsRNA during active ssRNA virus replication¹⁶. Therefore, we believe that it is
301 critical to clearly define the role of viral ssRNA and dsRNA in eliciting inflammatory cytokine/chemokine
302 and antiviral immune responses. In this study, we used well-described viral ssRNA and dsRNA mimics to
303 examine both mRNA and protein levels of inflammatory cytokines and IFN/ISGs at both early and late
304 times to comprehensively describe the role of viral RNAs in monocyte-macrophage mediated immune
305 response. Although the use of viral ssRNA and dsRNA mimics instead of authentic viral ssRNA or dsRNA
306 is a limitation in our study, we strongly believe that our results will lay a foundation for a careful
307 examination of virion purified, invitro synthesized, or in-vivo generated viral ssRNA and dsRNA in causing
308 dysregulated immunity and severe disease.

309 In addition to detailed examination of viral ssRNA and dsRNA mimic-induced inflammatory and antiviral
310 immunity, we provided a potential mechanistic explanation for differential immune response induced by
311 viral RNA mimics. We illustrate that high cell surface and endosomal TLR7 expression compared to TLR3
312 levels correlate with R848-induced robust inflammatory cytokine production. Conversely, cell surface/
313 endosomal TLR7 levels did not correlate with IFN response, as shown by low IFN/ISG levels in R848-
314 treated cells compared to Poly I:C-treated macrophages. These results suggest TLR7/Myd88 signaling
315 is a weak IFN-I/ISG inducer compared to TLR3/TRIF activity. An additional explanation for Poly I:C-
316 induced high IFN/ISG levels is that the dsRNA is escorted into the cytosol and sensed by MDA5^{50 17}.
317 However, our results show that at least in macrophages, Poly I:C-induced IFN/ISG response is mediated
318 via TLR3-TRIF pathway (Figure 1E). We further show that high TLR7 and inflammatory cytokine levels
319 correlate with robust NF- κ B and MAPK (p38, ERK1/2, and JNK) activity as early as 10 minutes post-
320 R848/TLR7 stimulation compared to Poly I:C treatment (Figure 3). Additionally, TLR7-induced robust
321 activation of NF- κ B and MAPK at both 30- and 60-min post-stimulation (Figure 3). These results
322 collectively suggest that timing and strength of NF- κ B and MAPK activity dictate the level of inflammatory
323 cytokine and chemokine production in viral RNA mimic treated cells. Although previous studies have
324 examined the role of either NF- κ B or MAPK activity in viral RNA mimic treated and RNA virus-infected
325 cells, our work comprehensively examines multiple key signaling pathways at several time points
326 providing critical and detailed insight into the role played by these pathways in viral RNA induced
327 inflammation and antiviral response^{51 52}.

328

329 We show robust activation of multiple pathways in ssRNA mimic treated cells and identify the impact of
330 blocking these pathways in mitigating excessive inflammation. Significant reduction in inflammatory
331 cytokine/chemokine levels in NF- κ B-, ERK1/2-, and JNK- inhibitor-treated cells highlights the importance
332 of simultaneously blocking multiple pathways to mitigate viral ssRNA-induced inflammation. Of note, in
333 contrast to published results demonstrating the anti-inflammatory functions of FDA-approved p38-MAPK
334 inhibitor Losmapimod, we consistently observed a significant increase in inflammatory cytokine/
335 chemokine levels in Losmapimod treated TLR7 stimulated, but not Poly I:C treated macrophages^{53 54}.
336 These results suggest that blocking p38 likely exacerbates viral ssRNA-induced inflammation. Although
337 it is unclear if such response is specific to Losmapimod treatment in BMDMs or is a general response to
338 p38 inhibition in all cell types, these outcomes warrant a further investigation of immunomodulatory effect
339 of Losmapimod, particularly during RNA virus infection. Another important finding from our study is that
340 unlike blocking NF- κ B and JNK signaling, ERK1/2 inhibition significantly improved antiviral IFN/ISG
341 response in ssRNA and dsRNA mimic treated cells, emphasizing the importance of blocking ERK1/2
342 activity to suppress excessive inflammatory cytokines and simultaneously improve antiviral IFN/ISG

343 response. Although corticosteroids are currently used to suppress exuberant inflammation,
344 corticosteroid-mediated suppression of antiviral innate and adaptive immunity is a clinical concern^{55 56}.
345 Therefore, blocking ERK1/2 signaling along with NF- κ B and other MAPK pathways is likely superior to
346 provide better protection compared to the current immunomodulatory therapies.

347

348 In summary, using viral RNA mimics, we demonstrate that ssRNA, which is abundantly produced during
349 RNA virus infections, is potentially a key mediator of virus-induced exuberant inflammation and impaired
350 antiviral IFN/ISG response. Mechanistically, we show that high cell surface and endosomal TLR7
351 expression drives robust NF- κ B and MAPK activation, thus facilitating excessive inflammatory cytokine
352 response. Additionally, given ERK1/2 signaling suppresses antiviral response and promotes
353 inflammation, blocking ERK1/2 activity is perhaps a better immunomodulatory strategy to mitigate
354 dysregulated immunity and provide better protection.

355

356 **Author Contributions:** RC and RS conceptualized the study; RS, PJ, RG, and CW carried out
357 experiments and data analysis; RC and RS wrote the manuscript; RC, RS, PJ, RG, and CW reviewed
358 and edited the manuscript. RC provided mentoring and supervised experiments, data analysis, and
359 presentation.

360

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Differential TLR-ERK1/2 activation promotes viral ssRNA and dsRNA mimic-induced dysregulated immunity in macrophages

Shrestha R et al., 2024

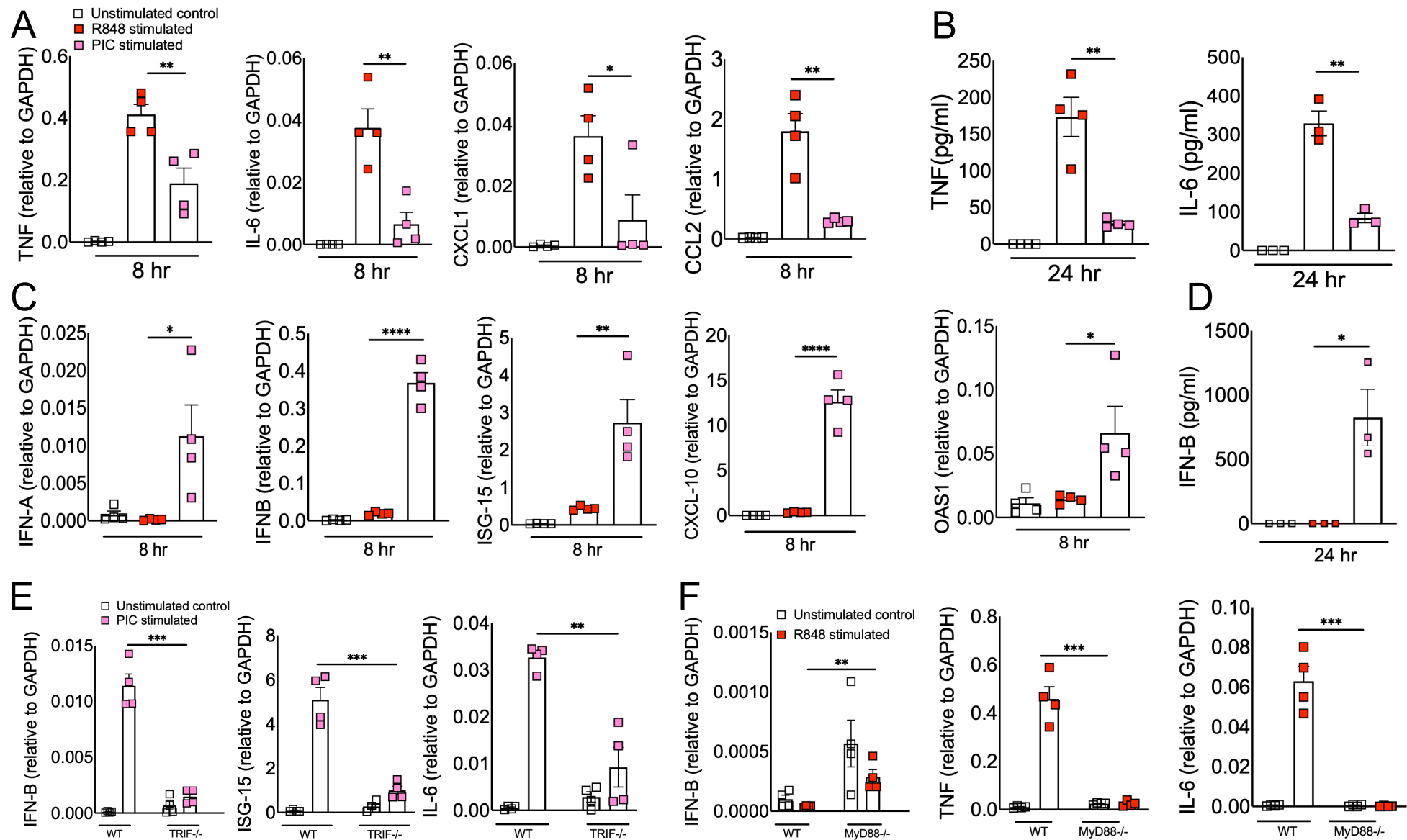


Fig 1: ssRNA/TLR7 stimulation induces robust inflammatory cytokine/chemokine responses whereas dsRNA mimics are potent inducers of antiviral IFN and ISG responses. Murine BMDMs were stimulated with viral RNA mimics; R848 (ssRNA mimic) and poly I:C (dsRNA mimic) each with 10 ug/ml. mRNA (A) and protein levels (B) of inflammatory cytokines and chemokines and mRNA (C) and protein levels (D) of interferons and ISGs were assessed 8 & 24 hours after stimulation respectively. WT and TRIF^{-/-} BMDMs (E) and WT and MyD88^{-/-} BMDMs (F) were stimulated with viral RNA mimics; poly I:C (dsRNA mimic) and R848 (ssRNA mimic) each with 10 ug/ml respectively. mRNA levels of interferons and inflammatory cytokines were assessed 8 hours post-stimulation. Data are representative of 3 independent experiments. Statistical significance was determined using Student's t-test with *P < .05, **P < .01, ***P < .001 and ****P < .0001.

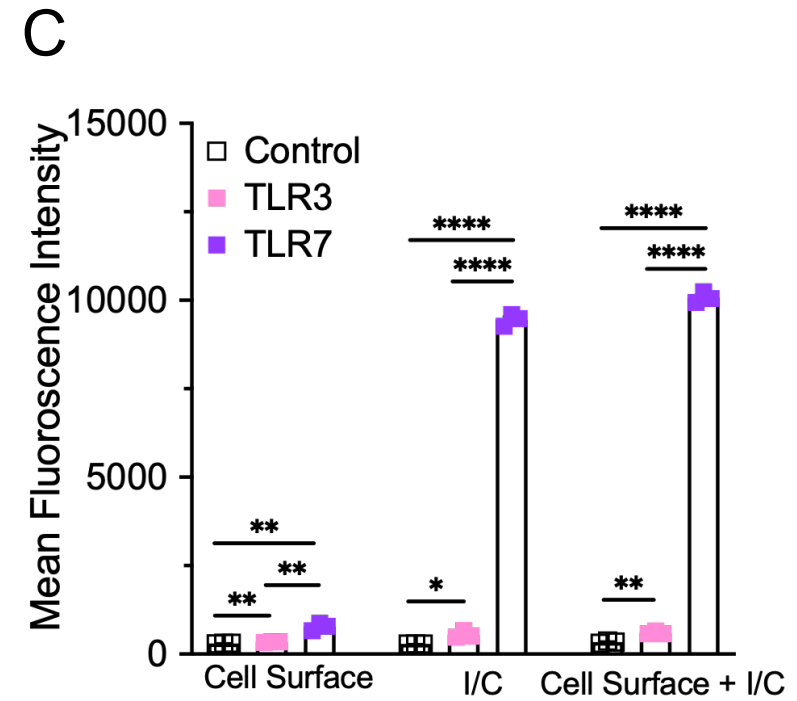
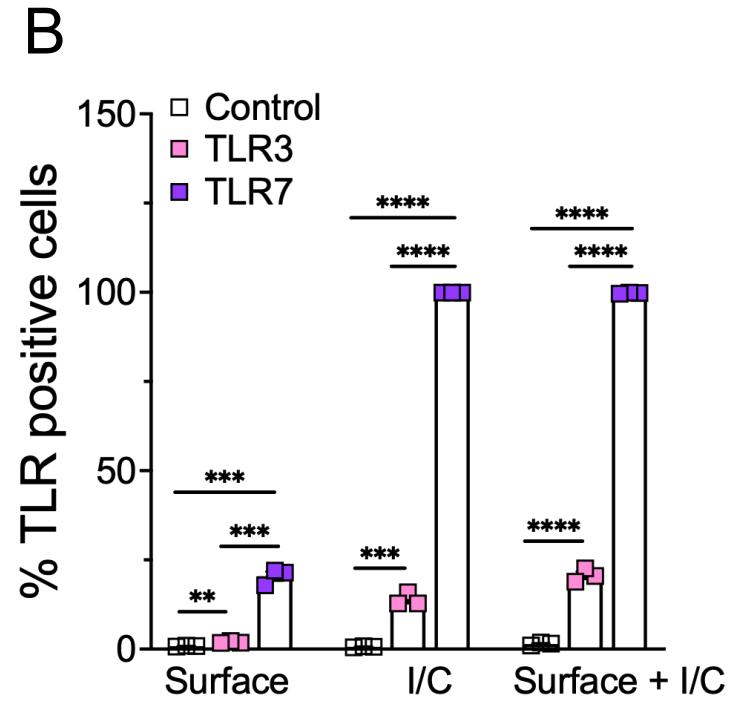
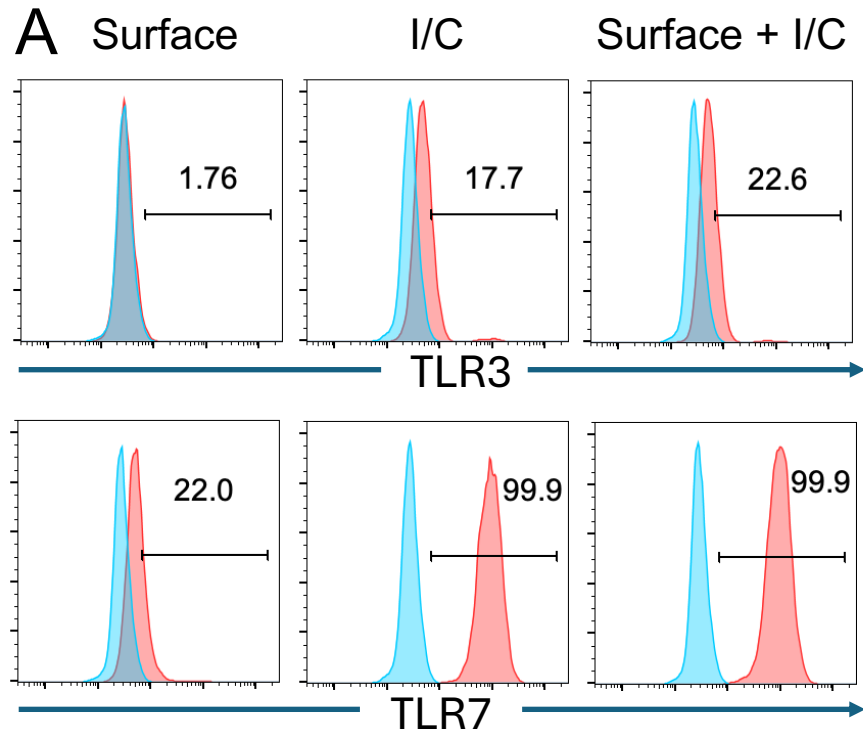


Fig 2: TLR3 and TLR7 expression in mouse bone marrow macrophages. Expression of cell surface and intracellular TLR3 and TLR7 in naïve mouse bone marrow macrophages was analyzed by flow cytometry (FACS) using standard protocols (A). Scatter plot graphs show the percentage and Mean fluorescent intensity of TLR3 and TLR7 positive cells (B and C). Data are representative of 3 independent experiments. Statistical significance was determined using Student's t test with *P < .05, **P < .01, ***P < .001 and ****P < .0001.

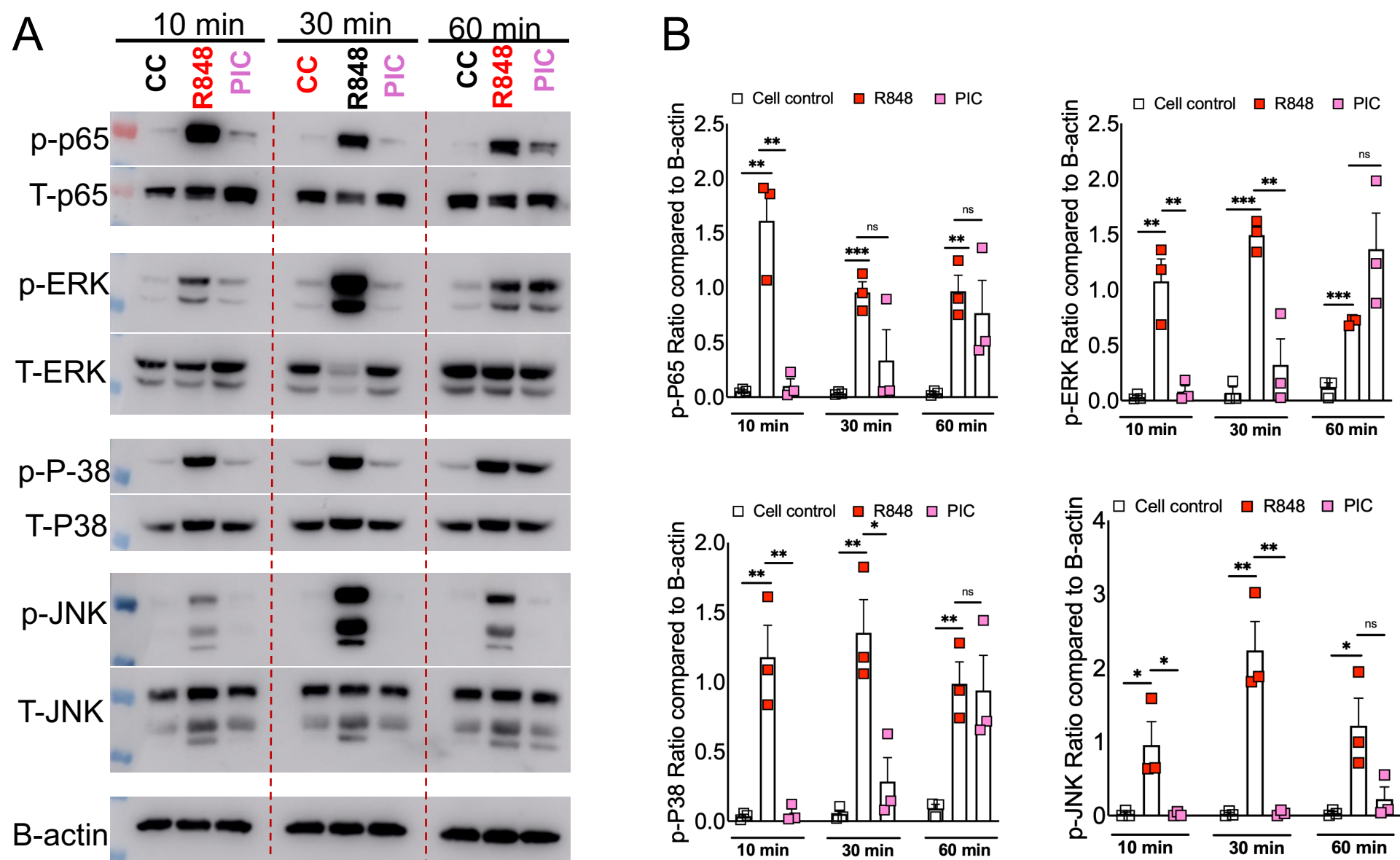


Fig 3: RNA mimic stimulation induced differential phosphorylation of MAPK and NF- κ B signaling. Murine bone marrow macrophages were treated with ssRNA mimic (R848) and dsRNA mimic (PIC) for 10, 30, and 60 mins. Cell lysates were collected and western blot was performed using mAbs against phosphorylated MAPK (ERK1/2, JNK and p-38) and NF- κ B (p-65) (**A**) and relative quantification of protein levels by densitometry analysis (**B**). Data are representative of 3 independent experiments (**A**) or pooled from 3 independent experiments (**B**). Statistical significance was determined using Student's t-test with * $P < .05$, ** $P < .01$, *** $P < .001$ and **** $P < .0001$.

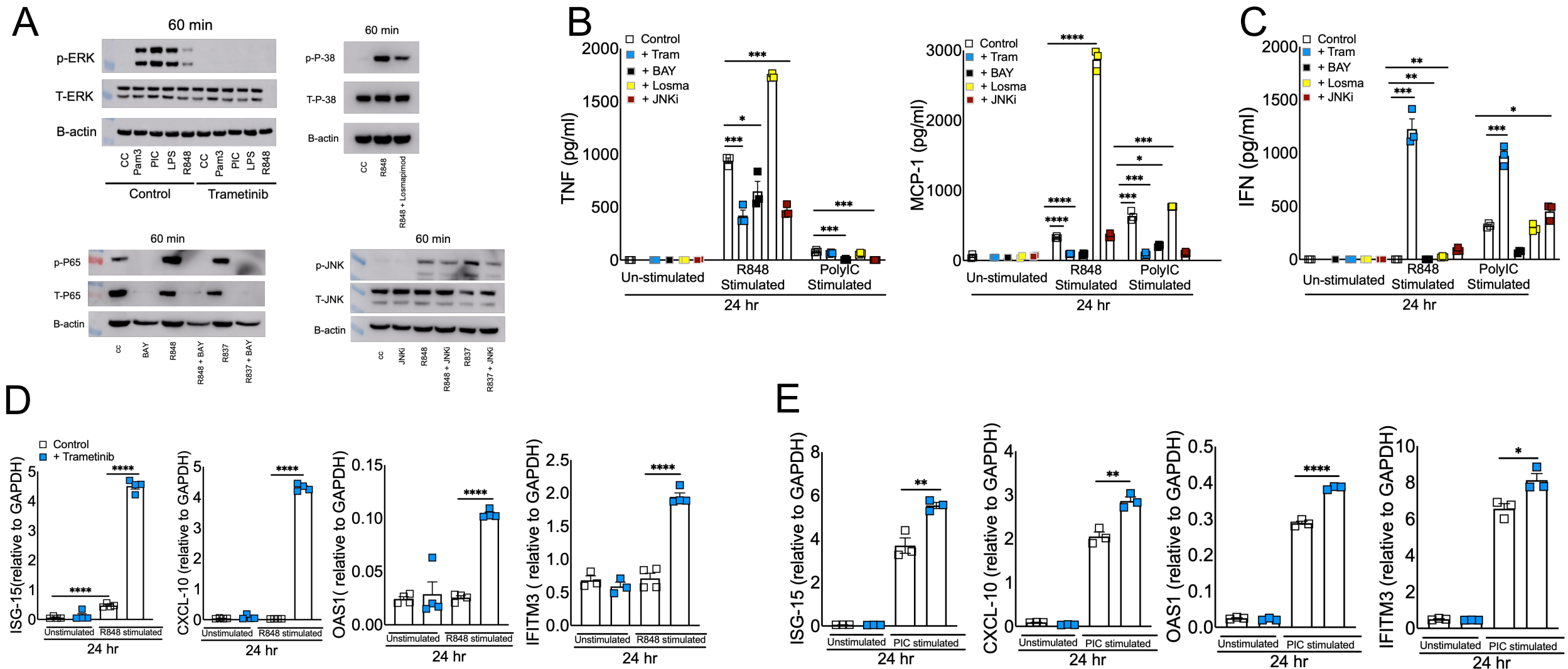


Fig 4: Effect of blocking MAPK and NF- κ B signaling in viral RNA mimic induced inflammatory and antiviral cytokine response. Murine BMDMs were treated with each specific inhibitor of NF- κ B and MAPK pathways for 2 hrs followed by stimulation with R848 and or Poly I:C each with 10 μ g/ml. Western blot showing the inhibitory effect of Trametinib, Losmapimod, BAY, and JNKi on p-ERK, p-P38, p-P65, and p-JNK respectively (**A**) Protein levels of cytokines and interferons (**B and C**) and mRNA levels of ISGs (**D and E**) were assessed at 24 hours post-stimulation. Data are representative of 2-3 independent experiments (**B-E**). Statistical significance was determined using Student's t-test with * $P < .05$, ** $P < .01$, *** $P < .001$ and **** $P < .0001$.