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1 **Title:**

2 Pneumococcus triggers NFkB degradation in COMMD2 aggresome-like bodies.

3

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18

19 **Abstract:**

20 NF- κ B driven cellular immunity is essential for both pro- and anti-inflammatory responses to
21 microbes, which makes it one of the most frequently targeted pathways by bacteria during
22 pathogenesis. How NF- κ B tunes the epithelial response to *Streptococcus pneumoniae* across the
23 spectrum of commensal to pathogenic host phenotypic outcomes is not fully understood. In this study,
24 we compare a commensal-like 6B ST90 strain to an invasive TIGR4 isolate and demonstrate that TIGR4
25 both blunts and antagonizes NF- κ B activation. We identified, through comparative mass spectrometry
26 of the p65 interactome, that the 6B ST90 isolate drives a non-canonical NF- κ B RelB cascade, whereas
27 TIGR4 induces p65 degradation through autophagy. Mechanistically, we show that during TIGR4
28 challenge a novel interaction of COMMD2 with p65 and p62 is established to mediate degradation of
29 p65. With these results, we establish a role for COMMD2 in negative NF- κ B regulation, and present a
30 paradigm for diverging NF- κ B responses to pneumococcus. Thus, our studies reveal for the first time
31 a new bacterial pathogenesis mechanism to repress host inflammatory response through COMMD2
32 mediated turnover of p65.

33

34 **Introduction:**

35 The eukaryotic NF- κ B family of transcriptional regulators are well documented for their
36 potent ability to drive both pro- and anti-inflammatory cellular immune responses during microbe-
37 host interaction¹⁻³. As such, it is also one of the most frequently targeted host pathways during
38 pathogenic infection. Of the three main documented NF- κ B activation pathways - canonical, non-
39 canonical and atypical- the canonical cascade is the most frequently documented as triggered and
40 targeted for exploitation by bacteria⁴⁻⁷.

41 The canonical pathway consists of NF- κ B subunit heterodimers of p65/p50 or homodimers of
42 p65/p65 bound to an inhibitory protein, such as I κ B α . NF- κ B subunits are normally sequestered in the
43 cytoplasm in an inactive state⁸. Upon sensing of inflammatory molecules, such as cytokines (IL-1 β or
44 TNF α), pathogen-associated molecular patterns (PAMPs; i.e. lipopolysaccharide), and danger-
45 associated molecular patterns (DAMPs; i.e. IL-1 α or nuclear protein HMGB1) NF- κ B subunits are
46 rapidly activated by phosphorylation on serine residues (S536 and S276). Simultaneously, NF- κ B
47 dimers are released from their inhibitory I κ B proteins and translocated to the nucleus of the cell for
48 additional modification. Ultimately, this process leads to the binding of activated dimers to cognate
49 NF- κ B DNA motifs, thereby inducing NF- κ B dependent gene transcription. NF- κ B activation is tightly
50 controlled for precise and rapid induction, but also for prompt repression. NF- κ B dimers can be
51 repressed through extraction, sequestration, and degradation from within the nucleus, while in
52 parallel blocking cytoplasmic activation and promoting transcription of negative regulators^{4,5,7,9-13}.
53 However, in contrast to the myriad of studies on activators only a few negative regulators of NF- κ B
54 and their pathways have been documented.

55 COMMD (copper metabolism gene MURR1 domain)¹⁴ proteins are among the select few
56 negative regulators of NF- κ B^{12,14-19}. There are ten members of the COMMD family, all of which,
57 interact with NF- κ B to regulate signaling. The best-studied archetype member, COMMD1, upon
58 stimulation by TNF will lead to extraction of p65 from chromatin, followed by ubiquitination and
59 proteasomal degradation. This process, functions independently of NF- κ B nuclear translocation and
60 I κ B α , but through association with Cullin proteins, is able to terminate NF- κ B signaling^{12,14,16-22}. For the
61 other COMMD proteins, however, neither their functional activity, their mechanism of NF- κ B
62 repression, or their interacting partners, outside of cullins, are known.

63 Unsurprisingly many bacterial species actively target the NF- κ B pathway to repress the innate
64 immune defenses of the host and support their survival. To date, all bacterial processes either coopt
65 NF- κ B repressors or directly target NF- κ B pathway proteins using virulence factors (general review
66^{3,23}). We and others have shown the obligate human pathobiont, *Streptococcus pneumoniae* (the
67 pneumococcus), fine-tunes NF- κ B signaling to support its interaction with the host, across the
68 spectrum of commensal to pathogenic outcomes²⁴⁻³⁰. Surprisingly, we showed that a pathogenic *S.*
69 *pneumoniae* strain showed very little NF- κ B signaling compared to a colonizing, asymptomatic strain.

70 This observation raised the possibility that pneumococcus could subvert NF- κ B signaling, which has
71 not yet been documented.

72 Here, we demonstrate that a pathogenic TIGR4 pneumococcal strain ²⁴, in contrast to a
73 commensal-like 6B ST90 isolate ²⁴, represses phosphorylation and activation of NF- κ B p65. In fact,
74 TIGR4 infection leads to specific degradation of p65 in airway epithelial cells, even upon stimulation
75 with a strong inflammatory agonist, IL-1 β . We performed an interactome of p65 and show that each
76 pneumococcal strain interacts with diverging p65 interacting partners, revealing an original
77 aggrephagy mechanism involving COMMD2 and p62. Therefore, we report a novel mechanism of
78 pathogenesis to degrade p65 and repress the host response, specifically induced by TIGR4.

79

80 **Results:**

81 **TIGR4 antagonizes NF- κ B p65 activation.**

82 Previously we showed a commensal-like 6B ST90 pneumococcal strain activated p65 to drive
83 a unique inflammatory signature in comparison to a disease causing TIGR4 strain ²⁴. We showed that
84 challenge with an invasive TIGR4 strain resulted in decreased transcriptional activation of several
85 inflammatory cytokines ^{24,31}, which suggested that NF- κ B p65 activation was being disrupted by TIGR4.
86 To directly measure NF- κ B activation, we challenged A549 cells with either TIGR4 or 6B ST90 alone, or
87 in combination with IL-1 β , a pro-inflammatory stimulus known to drive p65 activation, by
88 phosphorylation of the key serine residues 536 and 276 (review ¹⁰). Cells were collected 2hr post-
89 challenge for immunoblotting and the total levels of p65 were determined. Interestingly, upon
90 infection with TIGR4, p65 levels significantly decreased compared to uninfected or 6B infected cells
91 (Fig. 1A & B). It is important to note that p65 levels decreased even in the presence of IL-1 β , which
92 normally drives p65 activation.

93 Regardless of the total level of p65, we also evaluated the activity level of p65 by measuring
94 phosphorylation at S536 and S276, under all conditions. TIGR4 was able to induce phosphorylation at
95 S276, albeit at levels significantly lower than IL-1 β alone, or 6B infection, but not at S536 (Fig. 1A, C,
96 D). Importantly, time course monitoring of S536 phosphorylation, reveals that at no time point during
97 infection, do the levels increase above uninfected levels (Sup. Fig. 1A). Taken together, these results
98 show that TIGR4 is a poor activator of p65 in comparison to the 6B ST90 strain. Remarkably, the
99 addition of IL-1 β during TIGR4 infection did not restore phosphorylation of S536 or S276 to levels
100 comparable to IL-1 β alone, suggesting that infection with this strain of pneumococcus is actively
101 antagonizing NF- κ B signaling.

102 *S. pneumoniae* is an opportunistic respiratory pathogen. As such upper airway epithelial cells
103 among the first to be encountered, which in turn triggers initial host responses. Therefore, we studied
104 p65 levels and phosphorylation in primary human nasal epithelial cells (Sup. Fig. 1A) and
105 nasopharyngeal Detroit 562 cells (Sup. Fig. 1B). Importantly, both of these cell types display the same
106 blunting of NF- κ B activation.

107 We further evaluated nuclear translocation of p65 by immunofluorescence, as this is a
108 hallmark of p65 activation. Nuclear p65 intensity was quantified and normalized to the nuclear area
109 by segmenting on the DAPI nuclear stain for TIGR4 (+/- IL-1 β) against uninfected and IL-1 β controls
110 (Fig. 2A & B). In comparison to uninfected conditions, TIGR4 challenge caused slight nuclear
111 recruitment of p65, but remained significantly lower ($p \leq 0.001$) in comparison to IL-1 β alone.
112 Unexpectedly, there was a significant increase in nuclear p65 between TIGR4 + IL-1 β and IL-1 β alone
113 (Fig. 2A & B), establishing that p65 is translocated in the TIGR4 + IL-1 β condition even though
114 phosphorylation levels are aberrant. It should be noted that this increase could be overestimated due
115 to the cellular nucleus shrinking upon infection.

116 We then tested if aberrant activation and low translocation influenced downstream effector
117 functions, namely transcription of p65 dependent genes. Total RNA was collected from A549 cells at
118 10, 30, 90, and 120 minutes post challenge with TIGR4 (+/- IL-1 β) and IL-1 β alone. Relative expression
119 was determined for IL-6, IL-8, CFS2 and PTGS2 (COX-2) against uninfected/untreated controls at each
120 time point. Surprisingly, TIGR4 infection alone did not lead to activation of any of the genes tested up

121 to 2h post challenge, in comparison to IL-1 β alone (Fig. 2C). Furthermore, under conditions where IL-
122 1 β was added during TIGR4 challenge, there was both a delay and a repression of these transcripts in
123 comparison to IL-1 β alone. This was corroborated with the diminished p65 phosphorylation at S536
124 at the protein level at the same time points (Sup. Fig. 1A).

125 Transcriptional activation by p65 requires its binding to cognate kappa-binding sites at the
126 chromatin level. Therefore, we evaluated levels of chromatin bound p65 at the locus of the NF-kB
127 dependent gene *PTGS2*. Herein, chromatin was collected from A549 cells 2 hrs post-challenge with
128 TIGR4 and the recovery of p65 quantified against uninfected and IL-1 β controls by ChIP-qPCR targeting
129 the two kappa-binding sites upstream of the *PTGS2* transcriptional start site (Fig. 2D). At both sites in
130 TIGR4 challenged cells, there was less than 5% recovery of p65. This stands in contrast to the three-
131 fold higher p65 recovery in IL-1 β alone (Fig. 2E & F). Therefore, the lack of p65 driven transcription
132 under TIGR4 challenge is intrinsically due to the absence of p65 at the chromatin.

133 Altogether, these data show the TIGR4 pneumococcal strain antagonizes p65 activation even
134 in the presence of the pro-inflammatory cytokine, IL-1 β . This creates a dysfunctional p65 signaling
135 cascade leading to poor downstream activation of p65 dependent transcription.
136

137 A divergent NF-kB p65 interactome supports TIGR4 driven p65 degradation.

138 To begin to understand the NF-kB p65 activation differences between the two pneumococcal
139 isolates we performed mass spectrometry of NF-kB p65 (Fig. 3A). Herein, an A549 GFP-p65 cell line
140 was challenged with either TIGR4 or 6B ST90 and 2 hrs post-challenge GFP-p65 was
141 immunoprecipitated with a matched A549 GFP alone control for mass spectrometry interactome
142 analysis. From the analysis we identified p65 posttranslational modifications, as well as proteins
143 interacting with p65 under the different conditions tested (Sup. Table 1).

144 The interactome data for 6B ST90 showed the sole NF-kB associated target was RelB, a major
145 component of the non-canonical NF-kB pathway. Using whole cell lysates obtained from A549 cells 2
146 hrs post challenge with either 6B ST90 (+/- IL-1 β) or TIGR4 (+/- IL-1 β) we confirmed that RelB was
147 significantly ($p \leq 0.001$) elevated during challenge with 6B ST90 (+/- IL-1 β) in comparison to both
148 uninfected and TIGR4 (+/- IL-1 β ; Fig. 3B & C) and associated with p65 by co-immunoprecipitation (Sup.
149 Fig. 2C).

150 In contrast, the TIGR4 challenged p65 mass spectrometry dataset enriched for different NF-
151 kB associated targets. Gene Ontology and KEGG pathway analysis enriched for protein degradation
152 pathways, including proteins such as HDAC6, a p62, and ubiquitin (Sup. Table 1)³². Indeed, p62 is a
153 classical receptor of autophagy, HDAC6 an ubiquitin-binding histone deacetylase known to be
154 important in modulating autophagy, and together have been shown to degrade protein aggregates
155 through a process termed aggrephagy³²⁻³⁴. Therefore, our proteomic data suggested that p65 could
156 be targeted for degradation through an aggrephagy pathway under TIGR4 infection conditions. To
157 begin testing this hypothesis we probed whole cell lysates obtained from A549 cells 2 hrs post
158 challenge with either 6B ST90 (+/- IL-1 β) or TIGR4 (+/- IL-1 β) for HDAC6 levels, as HDAC6 is degraded
159 along with its cargo during aggrephagy (Fig. 3B & D). Indeed, only during TIGR4 challenge conditions
160 did total HDAC6 levels decrease in comparison to uninfected, IL-1 β alone and 6B ST90 groups. We
161 then tested this hypothesis further using known chemical inhibitors to either proteasome or
162 aggrephagy/lysosomal pathways. We treated cells with MG132 (10 μ M)³⁵, a general proteasome
163 inhibitor, with Bafilomycin A1 (400nM)³⁶⁻³⁸, an inhibitor of the terminal vATPase assembly during
164 aggrephagy/lysosome fusion, or with SAR405 (500nM)^{38,39}, a PI3K inhibitor of the initiation of
165 aggrephagy pathway, and assessed the levels of p65. Bafilomycin A1 and SAR405 treatments restored
166 levels of p65 during TIGR4 challenge (+/- IL-1 β) to comparable levels of uninfected and IL-1 β alone
167 (Fig. 3C), while MG132 had no effect in restoring p65 levels during TIGR4 challenge (Sup. Fig. 2A). The
168 same trend for Bafilomycin A1 upon p65 levels 2 hrs post-challenge with TIGR4 was observed in
169 primary human nasal epithelial cells (Sup. Fig. 2B). These data, along with the identification of HDAC6
170 and p62 in the p65 interactome strongly suggest that TIGR4 is inducing degradation of p65 through an
171 aggrephagy pathway.

172 We further determined if degradation was restricted to only the p65 subunit or was also
173 impacting the levels p50, which in a heterodimer with p65 is the primary translocated unit to the
174 nucleus⁵. We immunoprecipitated endogenous p65 from A549 cells challenged with 6B ST90 or TIGR4
175 against uninfected and IL-1 β and probed for the p50 subunit. Already in the input, the levels of p50 is
176 lower upon infection with TIGR4 compared to 6B ST90, which is also noticeable from the
177 immunoprecipitation (Sup. Fig. 2C). This observation further supports that TIGR4 challenge is targeting
178 NF- κ B p65 complex as a whole.

179 Overall, these data demonstrate TIGR4, in contrast to 6B ST90, induces diverging NF- κ B p65
180 signaling cascade that results in: 1) differential protein binding, 2) degradation of p65 through
181 aggrephagy.

182

183 COMMD2 associates with both p65 and p62 and translocates to the nucleus

184 The COMMD1 protein has previously been shown to terminate NF- κ B signaling through
185 proteasomal degradation^{16,17,19,21,40}. Our interactome identified COMMD2 and COMMD4 as among
186 the most highly enriched proteins associating to p65 under TIGR4 infection conditions compared to
187 6B ST90. COMMD2 and COMMD4 were previously shown to associate with p65 and NF κ B1¹⁴, and
188 other members of this protein family have a repressive role in NF κ B signaling^{12,16-18,20,22}. Therefore,
189 we hypothesized that COMMD2 and COMMD4, through their association with p65, could be involved
190 in p65 turnover through a similar aggrephagy pathway.

191 With no robust COMMD2 antibody commercially available for co-immunoprecipitation or
192 immunoblot, we generated an A549 GFP-COMMD2 ectopic expression stable cell line, from which
193 GFP-COMMD2 was immunoprecipitated from lysates 2 hrs post-challenge with either 6B ST90 (+/- IL-
194 1 β), TIGR4 (+/- IL-1 β) or from our uninfected and IL-1 β controls. Samples were probed for p65 or p62
195 to detect interaction with COMMD2. Our results show that only under TIGR4 challenge conditions do
196 p65 and p62 interact with COMMD2 (Fig. 4A & B). Furthermore, upon addition of IL1 β , p65 interacts
197 with COMMD2 to even higher levels. Therefore, COMMD2 is a new infection specific interacting
198 partner of p65, making a complex of p65-COMMD2-p62.

199 Although infection with TIGR4 leads to p65 degradation, the small amount left in the cell is
200 nuclear (Fig. 1E). Therefore, to evaluate the cellular localization of the p65-COMMD2-p62 we
201 performed immunofluorescence experiments using the GFP-COMMD2 A549 cell line. Using this cell
202 line, we further determined infection induced effect on p62. The GFP-COMMD2 A549 cell line was
203 challenged with TIGR4 (+/- IL-1 β) and compared to uninfected and IL-1 β controls, followed by
204 paraformaldehyde fixation and probing for p62. Total p62 levels in the nucleus were determined per
205 cell, by segmentation on the GFP-COMMD2 signal for the cellular cell boundaries and DAPI for the
206 nucleus (Fig. 4C). There was a significant ($p \leq 0.001$) decrease in total p62 levels for TIGR4 challenged
207 cells (+/- IL-1 β) in comparison to both uninfected and IL-1 β alone (Fig. 4D), which is expected upon
208 activation of protein degradation pathways. Interestingly, there is a reciprocal increase in the nuclear
209 levels of p62 (Fig. 4E), showing that during TIGR4 challenge there is movement of p62 between the
210 cytoplasm and nuclear compartments in addition to degradation. Moreover, we noticed COMMD2,
211 an otherwise cytoplasmic protein was translocated in the nucleus of cells challenged with TIGR4 (+/-
212 IL-1 β) (Fig. 4C). Similarly to p62, there was a decrease in total COMMD2 levels, indicative of protein
213 turnover, and an increase of COMMD2 in the nucleus (Fig. 4F & G).

214 We confirmed our microscopy observation by performing cell fractionations. We
215 immunoblotted cell fractions obtained from the GFP-COMMD2 stable cell line 2 hrs post-challenge
216 with TIGR4 (+/- IL-1 β) as well as from uninfected and IL-1 β controls (Fig. 4H & I). Whereas cells treated
217 with IL-1 β alone displayed 20% COMMD2 in the nucleus, similar to untreated/uninfected cells, TIGR4
218 (+/- IL-1 β) challenge conditions had 80% of COMMD2 consistently nuclear (Fig. 4I). The levels of
219 cytoplasmic COMMD2 under TIGR4 (+/- IL-1 β) challenge conditions correspondingly decreased,
220 demonstrating a relocalization of COMMD2. Finally, we tested if the commensal-like strain 6B ST90
221 could also induce COMMD2. Using cellular fractionation and immunoblotting, we show that 6B ST90
222 was incapable of triggering nuclear localization of COMMD2 (Sup. Fig.3A), which demonstrates

223 relocalization is TIGR4 specific. Strikingly, upon challenge with TIGR4, we observed perinuclear
224 COMMD2 puncta formation. Such puncta of protein aggregates, along with a decrease in p62 levels
225 have previously been described⁴¹⁻⁴⁵ and further support our findings that TIGR4 is activating
226 aggrephagy during infection.

227 To further test if inhibiting terminal stages of aggrephagy would restore p62 levels, we treated
228 cells with Bafilomycin A1 (400nM) and collected whole cell lysates 2 hrs post-challenge with either IL-
229 1 β alone or TIGR4 (MOI 20). Samples were immunoblotted for p62 and actin for quantification. The
230 results showed Bafilomycin A1 treatment blocked p62 degradation during TIGR4 challenge restoring
231 it to comparable uninfected levels (Sup. Fig. 3A), further demonstrating that TIGR4 challenge triggers
232 p65 turnover through an infection induced p65-COMMD2-p62 complex.

233

234 TIGR4 mediated COMMD2 nuclear translocation is dependent on Ply.

235 Our data show a strain specific degradation of p65 and relocalization of COMMD2, suggesting
236 intrinsic factors to TIGR4 challenge were responsible for these effects. Thus, we tested TIGR4 mutants
237 of Pneumolysin (Ply) and Pyruvate oxidase (SpxB), two major pneumococcal virulence factors we have
238 previously shown to affect host cell signaling at the nuclear level⁴⁶⁻⁴⁸. A549 GFP-COMMD2 stable cell
239 line was challenged for 2 hrs with either wildtype TIGR4, TIGR4 Δ ply or TIGR4 Δ spxB and the nuclear
240 levels of COMMD2 quantified against uninfected and IL-1 β alone. Nuclear COMMD2 levels were
241 measured by deconvoluted epifluorescence, and quantified by measuring signal intensity normalized
242 by nuclear area by segmenting the nucleus using DAPI stain (Fig. 5A & B). These data show COMMD2
243 was found primarily within the cytoplasm of uninfected and IL- β treated cells, and translocated to the
244 nucleus upon TIGR4 challenge. Similar levels of nuclear translocation were obtained with the Δ spxB
245 mutant, indicating that pneumococcal pyruvate oxidase and peroxide production is not necessary for
246 COMMD2 localization. However, deletion of the PLY toxin completely abrogated nuclear
247 translocation, indicating that this bacterial factor is essential (Fig. 5B). Since the 6B ST90 strain does
248 not lead to nuclear translocation of COMMD2 (Sup. Fig.3A), we concluded that although Pneumolysin
249 is essential, it is not sufficient, since 6B ST90 produces the same amount of this toxin as TIGR4²⁴.

250

251 COMMD2 exports p65 for lysosomal degradation.

252 COMMD2 has two nuclear export signal domains and no predicted nuclear localization signal
253 domains, suggesting a function for this protein in the cytoplasm, where aggrephagy degradation has
254 been shown to occur^{33,34,49}. Thus, we postulated COMMD2 was involved with nuclear export of
255 aberrantly phosphorylated p65 under TIGR4 challenge. This mechanism of action would be similar to
256 the architype family member COMMD1, which binds NF- κ B in the nucleus and exports it through
257 CRM1 for degradation^{12,22}. Therefore, we tested whether COMMD2 was exported through CRM1 by
258 using the Leptomycin B inhibitor⁵⁰. GFP-COMMD2 cells were treated with Leptomycin B (10 nM), and
259 immunofluorescence was used to image p62 and p65. Strikingly, by blocking nuclear export we
260 observed that COMMD2 was now localized to the nucleus in uninfected and IL-1 β treated cells.
261 Similarly, p62 and p65 are relocalized to the nucleus. In fact, COMMD2 and p62 were detected in
262 puncta in the nucleus (Fig. 6A), where p65 was localized (Fig. 6B). Notably, our IL-1 β positive pro-
263 inflammatory stimulus control, known to drive nuclear translocation of p65, had a significant (pV \leq
264 0.0001) increase in the nuclear level of COMMD2 and p62 in comparison to uninfected cells (Fig. 6C &
265 D). These surprising data suggest that under nuclear export stress, COMMD2 and p62 are naturally
266 recruited to the nucleus at specific puncta through a defined process.

267 Interestingly, upon challenge with TIGR4, Leptomycin B treated cells displayed higher levels
268 of COMMD2 and p62 accumulation in the nucleus than without treatment (Fig. 6A, C & D). Thus, upon
269 inhibiting nuclear export of COMMD2 and p62, these proteins are no longer being degraded upon
270 infection and accumulate in the nucleus. Similarly, Leptomycin B inhibition also increased the nuclear
271 p65 levels in all conditions compared to untreated cells (Fig. 6E), and even restored p65 levels to those
272 of cells stimulated with IL-1 β alone. Although COMMD2 puncta are observed upon addition of
273 Leptomycin B, the substantial amount of p65 trapped within the nucleus of these cells rendered

274 definitive scoring of puncta and colocalization difficult and was not done. Furthermore, under
275 conditions of TIGR4+ IL-1 β and Leptomycin B inhibition, we observed a significant ($pV \leq 0.0001$)
276 increase in p62 puncta compared to TIGR4 alone (Fig. 7A & B). These data therefore show that without
277 Leptomycin B inhibition TIGR4 challenge leads to an active CRM1 dependent export of p65. Altogether,
278 these results imply that p65 is exported from the nucleus via COMMD2 – p62 dependent process upon
279 challenge with TIGR4.

280 Nuclear export must precede protein turnover, which occurs in the cytoplasm. To confirm that
281 COMMD2 and p62 were degraded through the same lysosomal turnover we described in figure 3E,
282 we treated GFP-COMMD2 cells with Bafilomycin A1 (400nM; pre-treated for 3 hrs) and quantified the
283 total levels of p65, p62 and COMMD2 per cell 2 hrs post-challenge with either IL-1 β or TIGR4 (+/- IL-
284 1 β ; MOI 20) using confocal microscopy. Similarly to figure 3E, TIGR4 challenged cells (+/- IL-1 β) treated
285 with Bafilomycin A1 displayed an increase in p65, but also COMMD2, and restored p62 levels
286 comparably to uninfected controls (Fig. 7C - E). Moreover, in contrast to uninfected and IL-1 β treated
287 cells there was a significant ($pV \leq 0.0001$) increase in both p65 and COMMD2 levels in TIGR4
288 conditions. Therefore, the COMMD2-p65-p62 complex is being degraded through aggrephagy-
289 mediated turnover upon TIGR4 challenge and is amplified in the presence of the pro-inflammatory
290 stimulus IL-1 β .

291

292 **Discussion:**

293 Cellular inflammatory response is a critical component of the host defense to bacteria. Yet,
294 the molecular processes that fine-tune NF- κ B cascades across the range of colonizing to virulent
295 bacteria is poorly understood. Herein, we show that an invasive *S. pneumoniae* TIGR4 strain, which
296 causes symptomatic disease in murine models²⁴, blunts p65 activation and inflammatory gene
297 transcription in comparison to a commensal-like asymptomatic 6B ST90 strain, that activated p65²⁴.
298 Through mass spectrometry, interactome and post-translational modification analysis, we show these
299 two pneumococcal isolates have diverging p65 interacting partners and phosphorylation status. We
300 show the 6B ST90 strain upregulates RelB, a hallmark of non-canonical NF- κ B signaling, whereas the
301 p65 interactome for the invasive TIGR4 strain enriched for aggrephagy pathway components.
302 Mechanistically, we reveal that p65 is being degraded through a unique TIGR4 induced interaction of
303 COMMD2 with p65 and p62. Altogether, this is the first demonstration of a bacterial pathogenesis
304 mechanism to repress inflammatory gene transcription through targeted degradation of NF- κ B p65.

305 Negative regulation of NF- κ B signaling, in contrast to the breadth of knowledge on activatory
306 mechanisms, is poorly documented. This is in part due to the lack of identified targets and mechanisms
307 responsible for attenuating this signaling cascade. Of the known negative regulators, A20 (TNAI3)
308 and COMMD1 are the better described. A20 is primarily a deubiquitinase whose transcription is NF-
309 κ B activation dependent. A20 functions in a negative feedback loop to deubiquitinate NEMO, which
310 results in its stabilization with the IKK complex to restore NF- κ B sequestration in the cytoplasm. This
311 ultimately terminates the downstream canonical NF- κ B signaling cascade of inflammatory response
312^{5,12,51}. In contrast, COMMD1 transcription is NF- κ B independent, and facilitates p65 termination by
313 CRM1 mediated export and translocation of p65 to the proteasome for degradation via complex
314 formation of COMMD1 with Elongins B & C, Cullin2 and SOCS1 (ECS^{SOCS1})^{14,16,17,19,21,22}. In parallel,
315 COMMD1 contributes to repression of p65 driven gene transcription by occupying the formerly p65
316 bound kappa-binding site at specific gene promoters²⁰. It was put forth that the diversity of potential
317 COMMD, NF- κ B and cullin assemblies and the array of physiological stimuli activating such complex
318 formations positioned this family of proteins as potent selective negative regulators of NF- κ B signaling.
319 Our work is the first to show a role for COMMD2 in p65 turnover through p62 and aggrephagy. This
320 new negative feedback mechanism on p65 may represent, even in a cellular state without bacterial
321 infection, a precise mechanism to terminate or shift a given p65 dependent transcription repertoire.
322 Additionally, by lowering the amount of p65 protein present through degradation, a lower threshold
323 of inhibitory IKK proteins would be needed to quench this cascade within the cell. This could rapidly
324 shift the balance in favor of IKK proteins sequestering p65, and perhaps even favor a switch in NF- κ B

325 heterodimers, leading to activation of a different transcriptional repertoire. In essence, such inhibition
326 would be quite potent, as inflammatory transcription, inflammatory signal sensing, and negative
327 inflammatory feedback are all blocked simultaneously. Such a mechanism would greatly favor
328 pathogenic infection and host cell exploitation, as we observe upon TIGR4 challenge.

329 To date the molecular mechanism induced by TIGR4 challenge is the only stimulus to trigger
330 COMMD2-p62-p65 complex formation. On the other end of the virulence spectrum, the commensal
331 like 6B ST90 strain lead to the formation of a p65-RelB complex. Therefore, such interactome studies,
332 in combination with pro-inflammatory stimulus, will reveal new partners that govern p65 regulation.
333 In addition, the vast array of post-translational modifications on p65 and other NF- κ B subunits across
334 differential stimulations has given rise to the “NF- κ B barcode hypothesis”, which suggests that distinct
335 patterns are linked to how inflammatory gene transcription occurs^{52,53}. We show here that bacterial
336 stimuli are ideal tools to dissect the complexity of this signaling cascade and opens up the field of
337 research in NF- κ B signal termination.

338 Supporting this is our exploratory mass spectrometry of p65 phosphorylation, which identified
339 serine 45 (S45) as the only enriched phosphorylated mark during TIGR4 challenge. This mark has
340 previously been shown to negatively regulate p65, although the mechanism is unknown⁵⁴. Lanucara
341 et al., showed that a phosphomimetic mutant of S45 prevented IL-6 transcription and p65 binding to
342 the promoter under TNF α stimulation⁵⁴. It remains to be evaluated if this modification is involved in
343 COMMD2-p62 degradation of p65 and therefore could alter the host response to pneumococcus.
344 Interestingly, the commensal-like 6B ST90 does not induce phosphorylation of this residue. Instead,
345 this strain leads to phosphorylation on S203 and activation of the chromatin modifier KDM6B to drive
346 containment in the upper respiratory tract²⁴. Whether differential phosphorylation of p65 is the
347 determining factor in the ultimate host response to different strains of pneumococcus remains to be
348 determined. In this context it is tempting to speculate that posttranslational modifications of p65
349 could represent markers of either host response to commensal or to invasive bacteria.

350 Tuning NF- κ B dependent immune gene transcription is fundamental for cellular immune
351 processes of airway epithelial cells exposed to pneumococcus^{25,55}. The pro-inflammatory cytokines,
352 TNF α and IL-1 β , are major cytokines necessary for neutrophil recruitment and are found in
353 bronchoalveolar lavage fluid of animals challenged with pneumococcal isolates^{25,55,56}. However, one
354 study showed that when isolated, murine lung epithelial cells exposed to serotypes 19 and 3 failed to
355 induce p65 (RelA) nuclear translocation in comparison to TNF α and IL-1 β ²⁵. Our studies directly
356 address this paradox showing that the invasive TIGR4 pneumococcal isolate is actively engaged in
357 repressing p65 signaling through degradation even in an environment containing pro-inflammatory
358 stimuli. We propose that pneumococcus interaction with the ‘primary’ contacted host epithelial cell
359 results in repressed NF- κ B signaling with simultaneous prevention of negative feedback upon this
360 inflammatory response. However, what has been shown during respiratory infection with other
361 microbes^{57,58}, is that a balance is needed between pro-inflammatory responses and negative
362 regulation to ensure minimal tissue damage from the influx of neutrophils into the airway tissues⁵⁶.
363 Airway epithelial cells play a crucial role in both situations by regulating neutrophil recruitment and
364 promoting epithelial repair pathways leading to tissue resilience and resolution of inflammation
365^{55,56,59,60}. With pneumococcus actively antagonizing the ability of airway epithelial cells to both induce
366 and respond to IL-1 β we hypothesize an amplifying and runaway inflammatory cascade is created in
367 latter stages of infection where neutrophil influx is detrimental^{55,60,61}. This could lead to exacerbated
368 and severe pneumonia with excessive tissue damage allowing pneumococcus to transmigrate through
369 the lungs and into deeper tissues. We put forth that COMMD2, or combinations of COMMD proteins
370 are potent modulators of bacterial driven inflammatory processes, and may represent a novel
371 therapeutic target to avoid runaway inflammation.

372 In conclusion, our study shows a new regulatory role for COMMD2 in restraining p65 through
373 aggrephagy mediated turnover triggered by bacterial interaction. We reveal this process to be specific
374 to invasive pneumococcal challenge and partially depend on pneumolysin. Further studies
375 charactering both the p65 and COMMD2 interactome under bacterial challenge with isolates

376 representing divergent pneumococcal host interaction may identify new processes exploited at the
377 microbe-host interface to regulate NF- κ B signaling and identify novel negative regulators of
378 inflammation.

379

380 **Materials and methods:**

381 Bacteria strains, growth, and enumeration. Serotype 6B ST90 CC156 lineage F (ST90; CNRP# 43494)
382 and TIGR4 were obtained from the Centre National de Référence des Pneumocoques (Emmanuelle
383 Varon; Paris, France) and (Thomas Kohler, Universität Greifswald) respectively. Experimental starters
384 were made from master glycerol stocks struck on 5% Columbia blood agar plates (Biomerieux Ref#
385 43041) and grown overnight at 37°C with 5% CO₂ prior to outgrowth in Todd-Hewitt (BD) broth
386 supplemented with 50 mM HEPES (Sigma) (TH+H) as previously described²⁴. Inocula were prepared
387 from frozen experimental stocks grown for 3 – 4 hrs to midlog phase in TH+H at 37°C with 5% CO₂ in
388 closed falcon tubes. Bacterial cultures were pelleted at 1,500xg for 10 mins at room temperature (RT),
389 washed in DPBS, and concentrated in 1mL DPBS prior to dilution at desired CFU/mL using 0.6 OD₆₀₀
390 /mL conversion factors in desired cell culture media²⁴. Bacterial counts were determined by serial
391 dilution plating on 5% Columbia blood agar plates and grown overnight at 37°C with 5% CO₂.

392

393 Cell culture conditions and in vitro challenge. A549 human epithelial cells (ATCC ref# CCL-185) and
394 A549 stable cell lines were maintained in F12K media (Gibco) supplemented with 1x GlutaMax (Gibco)
395 and 10% heat inactivated fetal calf serum (FCS) at 37°C with 5% CO₂. Detroit 562 human
396 nasopharyngeal epithelial cells (ATCC ref# CCL-138) were maintained in DMEM supplemented with 1x
397 sodium pyruvate (Gibco) and 1x GlutaMax (Gibco) 10% heat inactivated FCS. Primary human nasal
398 epithelial cells (HNEpC; PromoCell ref# C-12620) were cultured and maintained in Airway Epithelial
399 Cell Growth Medium (PromoCell ref# C-21060). All cell lines were discarded after passage 15, and
400 HNEpC discarded after passage 4. For challenge studies cells were plated in tissue culture treated
401 plates at 2x10⁵ cells (6well; for 72 hrs), 5x10⁴ cells (24well; for 48 hrs), or 1x10⁴ cells (96well; for 48
402 hrs)²⁴. Bacterial inocula (Multiplicity of infection (MOI) 20) were diluted in cell culture media, added
403 to cells, and bacterial-epithelial cell contact synchronized by centrifugation at 200xg for 10 mins at RT.
404 Plates were moved to 37°C with 5% CO₂ for 2 hrs and processed as desired for experiment termination.
405 For inhibitor studies, cell culture media was aspirated, and replaced with filter sterilized culture media
406 containing either of the inhibitors MG132 10 μ M final concentration (Sigma ref# M7449), Bafilomycin
407 A1 400 nM final concentration (Sigma ref# SML1661) or Leptomycin B 10 nM final concentration
408 (Sigma ref# L2913) for 3 hrs prior to bacterial addition. Human IL-1 β (Enzo Life Sciences ref# ALX-522-
409 056) was used at 10 ng/mL final concentration in cell culture media.

410

411 RNA isolation and RT-qPCR. Total RNA isolated and extracted using TRIzol (Life technologies
412 ref#15596-026) method as per manufacturer's recommendations. Recovered RNA (5 μ g) was
413 converted to cDNA with Super Script IV as per manufacturer's instructions, diluted to 20 ng/ μ L in
414 molecular grade water and 1 μ L used for Sybr Green reactions as per manufacturer's instructions on
415 a BioRad CFX384 (BioRad). Relative expression was calculated by $\Delta\Delta$ Ct method to *GapDH*⁶². RT-PCR
416 primers listed in [Sup. Table 2](#).

417

418 ChIP and ChIP-qPCR. Detailed ChIP buffer components and procedure were completed as previously
419 reported²⁴. Briefly, 8x10⁶ A549 cells were cross-linked with 1% formaldehyde at room temperature
420 and quenched with 130 mM glycine. Chromatin was generated from the collected cell pellets by lysis
421 and sonication in chromatin shearing buffer to a size of 200-900bp. ChIP grade antibody to p65 (L8F6)
422 (CST ref #6956) was used at manufacturer's recommended concentrations and bound to DiaMag
423 beads (diagenode ref # C03010021-150) overnight with gentle rotation. Quantified chromatin was
424 diluted to 10 μ g per immunoprecipitation and added to antibody bound DiaMag beads overnight with
425 gentle rotation and 8% of input reserved. Beads were washed as previously described²⁴, and DNA
426 purified using phenol-chloroform extraction followed by isopropanol precipitation. Recovered DNA

427 suspended in molecular grade water was used for Sybr Green reactions (1 μ L) on a BioRad CFX384
428 (BioRad). ChIP-qPCR primers (50-150 bp; 60 °C max melt temperature) were designed to span the NF-
429 κ B sites of interest within the promoters of *PTGS2*⁶³. % recovery was calculated as 2 raised to the
430 adjusted input Ct minus IP Ct multiplied by 100. ChIP qPCR primers listed in [Sup. Table 2](#).

431

432 Plasmids, molecular cloning and stable cell line generation. All plasmids and primers are listed in [Sup.](#)
433 [Table 2](#). Routine cloning was carried out by in vivo assembly^{64,65}. Briefly, primers were designed with
434 a 15-20 bp overlap to amplify nucleic acid targets using Phusion Plus polymerase (Thermo ref# F630S).
435 Correct sized bands were excised and nucleic acid extracted by “Freeze and squeeze”^{66,67}. Herein,
436 0.7% - 1% agarose gel fragments were frozen for 5 mins on dry ice and centrifuged for 15 mins at
437 >21,000 xg with the supernatant collected – the process was completed two additional times.
438 Collected supernatant containing nucleic acid was then purified using phenol-chloroform extraction
439 followed by isopropanol precipitation and suspension in molecular grade water. Collected nucleic acid
440 was quantified spectrophotometrically using a NanoDrop and mixed at 3:2 (vector : insert) in 10 μ l
441 and added to chemically competent *E. coli* MC1061 or DH5 α for transformation. After 1 hr incubation
442 on ice bacteria outgrowth was done for 1 hr in Luria-Bertani (BD) prior to selection on LB agar
443 containing desired antibiotic ([Sup. Table 2](#)). All plasmids were isolated with the QIAprep Spin Miniprep
444 Kit (Qiagen ref# 27106) and eluted in molecular grade water (endotoxin free) as per manufacturer’s
445 instructions. A549 stable cell lines were generated using the transposon-based sleeping beauty system
446^{68,69}. A549 cells were plated in tissue culture treated plates at 2x10⁵ cells (6well) one day prior to
447 transfection with 2 μ g plasmid DNA + 150 ng SB100 transposase DNA. After transfection, cells were
448 selected with 1 mg/mL Geneticin (Thermo ref# 10131035) for 7 days, with media exchanged on days
449 1, 3, 5 & 7. Selected cells were collected with Trypsin 0.25% EDTA (Thermo ref# 25200056) and two-
450 way serial diluted in a 96 well tissue culture plate for monoclonal selection for another 7 – 14 days
451 with media containing 1 mg/mL Geneticin and exchanged every 2 – 3 days. Selected colonies were
452 expanded and FACS sorted to ensure purity, uniform expression, and comparison of intensity for
453 selecting a robust clone for subsequent experiments.

454

455 Immunoblots and quantification. Whole cell lysates were obtained by RIPA lysis (10 mM Tris HCL pH
456 7.5, 150 mM EDTA, 0.1% SDS, 1% Triton X-100 & 1% Deoxycholate) supplemented with inhibitor
457 cocktail (1X PhosSTOP, 10 mM sodium butyrate, 0.2 mM PMSF). Samples combined with 5x with
458 Laemmli buffer⁷⁰, sonicated for 5 mins in a ultrasonic water bath, boiled at 98°C (dry bath) for 10 mins
459 and frozen at -20°C. Whole cell lysates were ran on 4 – 20% pre-cast polyacrylamide SDS PAGE gels
460 (BioRad), transferred to PVDF membrane (BioRad TransBlot) and blocked 1 hr in 5% BSA TBST at room
461 temperature. Membranes were probed overnight at 4°C in 5% BSA TBST with primary antibody to p65
462 (CST ref #6956 or CST ref# 8242), p65 phosphorylation at serine 536 (CST ref# 3033), p65
463 phosphorylation at serine 276 (abcam ref# ab183559), NF κ B p105 / p50 (abcam ref# ab32360), RelB
464 (abcam ref# ab180127) or actin AC-15 monoclonal (Sigma ref# A5441) as per manufacturer’s
465 recommendations. Incubated for 1 hr at room temperature with appropriate secondary-HRP
466 conjugated antibodies in 5% Milk TBST and developed with clarity ECL (BioRad) developing reagents
467 with a ChemiDoc Touch (BioRad). Detroit562 immunoblots were developed using Licor Odessey using
468 secondary antibodies at 1:7,500 - Goat anti-rabbit IgG H&L (IRDye 800CW) and goat anti-mouse IgG
469 H&L (IRDye 680RD) from abcam. Band intensity was quantified by Image Lab (BioRad), or using Fiji⁷¹
470 (Detroit 562 cells) with linear intensity values log₁₀ transformed and normalized to actin prior to any
471 additional ratio metric comparisons.

472

473 Cell fractionation. Fractionation was performed as previously described as previously described²⁴.
474 Fraction lysates were combined with 5x with Laemmli buffer⁷⁰, sonicated for 5 mins in a ultrasonic
475 water bath, boiled at 98°C (dry bath) for 10 mins and frozen at -20°C. Samples were ran on either 10%
476 (for GFP-COMMD2) or 12% (for fraction quality controls) polyacrylamide SDS PAGE gels (BioRad),
477 transferred to PVDF membrane (BioRad TransBlot), blocked 1 hr in 5% BSA TBST at room temperature.

478 Primary antibody in 5% BSA TBST to GFP (abcam ref# ab290), GapDH (abcam ref# ab8245), or histone
479 H4 (abcam ref# ab177840) was completed overnight at 4°C. After 3x 10 min washes in TBST
480 appropriate secondary-HRP conjugated antibodies in 5% Milk TBST were incubated for 1 hr at room
481 temperature and developed with a ChemiDoc Touch (BioRad) as described above.

482

483 Immunofluorescence microscopy and Cellprofiler analysis. For microscopy the desired cell line were
484 seeded on acid washed and UV treated coverslips in 24well or 96well plates as described above. Two
485 hours post-challenge media was aspirated, cells washed in DPBS, and fixed with 2.5% PFA for 10 mins
486 at RT. Fixed cells were blocked and permeabilized overnight in 5% BSA 0.5% Tween20 at 4°C. Primary
487 antibody to p65 (CST ref #6956 or CST ref# 8242), COMMD2 (Sigma ref# HPA044190-25UL; only works
488 for immunofluorescence), or p62 (SQSTM1; abcam ref# ab109012) were diluted at 1:1,000 in 5% BSA
489 0.5% Tween20 and incubated overnight at 4°C. Cells were washed 3x 10 mins at RT in PBS + 0.1%
490 Tween20 prior to 1 hr incubation at 1:1,000 dilution of either Alexa Fluor 594 or Alexa Fluor 647
491 secondary antibody. Nuclei were stained with 10 ng/mL final concentration of Hoechst 33342 for 15
492 mins. Coverslips were rinsed in PBS and molecular grade water prior to mounting with Fluoromount-
493 G Mounting Medium (INTERCHIM). Confocal microscopy images were acquired on a Nikon TIE inverted
494 microscope with an integrated Perfect Focus System (TI-ND6-PFS Perfect Focus Unit) and a Yokogawa
495 Confocal Spinning disk Unit (CSU-W1). Nine images per well were acquired using a 20X air objective
496 (NA 0.75) at a step-size of 0.9µm in z-plane. Deconvoluted epifluorescent images were acquired on a
497 Cytation 5 (BioTek) using a 20X air objective (NA 0.75) with a grid of 3 x 3 (9 images en total).

498

499 Images were processed for background using Fiji ⁷¹, and segmented using Cell Profiler ⁷²⁻⁷⁴. Briefly, the
500 pipeline for image analysis consisted of sequential modules to 'IdentifyPrimaryObjects' based on
501 channel signal for nuclei (DAPI stain), p65 (Alexa594), or p62 (Alexa594). This was followed by
502 'IdentifySecondaryObjects' for the GFP-COMMD2 signal via propagation of identified nuclei. Objects
503 were related to each other to maintain cohesion between identified nuclei, cell and cellular contents
504 (p65 or p62). For puncta, the additional module, 'EnhanceorSupressFeatures' with 'Speckles', was
505 used. This used a global threshold strategy with Otsu threshold method and a 2% minimum boundary
506 to identify puncta contained within the segmented nuclei.

507

508 Immunoprecipitation. Cells were lysed in 250 µL of RIPA lysis (10 mM Tris HCL pH 7.5, 150 mM EDTA,
509 0.1% SDS, 1% Triton X-100 & 1% Deoxycholate) supplemented with a protease mixture inhibitor
510 (Roche Complete, EDTA free). Lysates were either immunoprecipitated using GFP-trap agarose beads
511 (ChromoTek ref# gta-10) or with slurry protein G beads (Sigma-Aldrich Fast Flow Protein G sepharose).
512 For GFP-p65 and GFP-COMMD2 the samples were immunoprecipitated as per manufacturer's
513 instructions with the elution was recovered in either 5x with Laemmli buffer ⁷⁰ and boiled at 98°C (dry
514 bath) for 10 mins, or left in Trypsin digest buffer (see LC-MS/MS Mass-spectrometry and analysis). All
515 samples were frozen at -20°C. For endogenous samples the lysates were incubated on a rotating
516 wheel at 4 °C for 20 min before adding 1 mL of dilution buffer (150 mM NaCl and 50 mM Tris-HCl pH
517 7.5 supplemented with Protease mixture inhibitor) to reduce the detergent final concentration below
518 0.1%. The lysates were then centrifuged at 10,000 × g for 10 min, and the insoluble pellet was
519 discarded. For p65 IP the lysates were then incubated with 2 µg of antibody CST ref #6956 or CST ref#
520 8242) at 4 °C for 2 hrs before adding 20 µL of slurry protein G beads (Sigma-Aldrich Fast Flow Protein
521 G sepharose) for 20 min. The beads were then washed before adding 20 µL of Laemmli buffer
522 supplemented with 2% β-mercaptoethanol and boiled for 5 min.

523

524 LC-MS/MS Mass-spectrometry and analysis. For label-free quantitative proteomic analysis of GFP-p65
525 and GFP-COMMD2 the respected A549 cell lines were plated in 6well tissue culture plates, and
526 challenged with bacteria for 2hrs as described above. One plate (~5x10⁷ cells) per condition was
527 harvested using RIPA lysis and immunopercptated with GFP-trap agarose beads (ChromoTek ref# gta-
528 10) as per manufacturer's instructions. Three or four independent biological replicates were prepared

529 and analyzed for each condition. Prior to on-bead Trypsin digestion, the samples were washed 3x in
530 trypsin digest buffer (20 mM Tris.HCl pH 8.0, 2 mM CaCl₂). On bead digestion was performed strictly
531 as described by Chromotek. Briefly, beads were suspended in digestion buffer (Tris 50 mM pH 7.5,
532 urea 2 M, 1 mM DTT and 5 µg.µl of trypsin (Promega)) for 3 min at 30°C. Supernatants were transfer
533 to new vials and beads were washed twice using (Tris 50 mM pH 7.5, urea 2 M and iodoacetamide 5
534 mM). All washes were pulled and incubated at 32°C for overnight digestion in the dark. Peptides were
535 purified using C18 stage tips protocol ⁷⁵.

536

537 LC-MS/SM analysis of digested peptides was performed on an Orbitrap Q Exactive Plus mass
538 spectrometer (Thermo Fisher Scientific, Bremen) coupled to an EASY-nLC 1200 (Thermo Fisher
539 Scientific). A home-made column was used for peptide separation (C₁₈ 30 cm capillary column picotip
540 silica emitter tip (75 µm diameter filled with 1.9 µm Reprosil-Pur Basic C₁₈-HD resin, (Dr. Maisch GmbH,
541 Ammerbuch-Entringen, Germany)). It was equilibrated and peptide were loaded in solvent A (0.1 %
542 FA) at 900 bars. Peptides were separated at 250 nl.min⁻¹. Peptides were eluted using a gradient of
543 solvent B (80% ACN, 0.1 % FA) from 3% to 31% in 45 min, 31% to 60% in 17 min, 60% to 90% in 5 min
544 (total length of the chromatographic run was 82 min including high ACN level step and column
545 regeneration). Mass spectra were acquired in data-dependent acquisition mode with the XCalibur 2.2
546 software (Thermo Fisher Scientific, Bremen) with automatic switching between MS and MS/MS scans
547 using a top 12 method. MS spectra were acquired at a resolution of 70000 (at *m/z* 400) with a target
548 value of 3 × 10⁶ ions. The scan range was limited from 300 to 1700 *m/z*. Peptide fragmentation was
549 performed using higher-energy collision dissociation (HCD) with the energy set at 27 NCE. Intensity
550 threshold for ions selection was set at 1 × 10⁶ ions with charge exclusion of *z* = 1 and *z* > 7. The MS/MS
551 spectra were acquired at a resolution of 17500 (at *m/z* 400). Isolation window was set at 1.6 Th.
552 Dynamic exclusion was employed within 30 s.

553

554 Data were searched using MaxQuant (version 1.5.3.8) using the Andromeda search engine⁷⁶ against a
555 human database (74368 entries, downloaded from Uniprot the 27th of September 2019), a
556 *Streptococcus pneumoniae* R6 database (2031 entries, downloaded from Uniprot the 1st of January
557 2020) and a *Streptococcus pneumoniae* serotype 4 database (2115 entries, downloaded from Uniprot
558 1st of January 2020).

559

560 The following search parameters were applied: carbamidomethylation of cysteines was set as a fixed
561 modification, oxidation of methionine and protein N-terminal acetylation were set as variable
562 modifications. The mass tolerances in MS and MS/MS were set to 5 ppm and 20 ppm respectively.
563 Maximum peptide charge was set to 7 and 5 amino acids were required as minimum peptide length.
564 At least 2 peptides (including 1 unique peptides) were asked to report a protein identification. A false
565 discovery rate of 1% was set up for both protein and peptide levels. iBAQ value was calculated. The
566 match between runs features was allowed for biological replicate only.

567

568 Data analysis for quantitative proteomics. Quantitative analysis was based on pairwise comparison of
569 protein intensities. Values were log-transformed (log₂). Reverse hits and potential contaminant were
570 removed from the analysis. Proteins with at least 2 peptides were kept for further statistics after
571 removing shared proteins from the uninfected GFP alone control. Intensity values were normalized by
572 median centering within conditions (normalized function of the R package DAPAR ⁷⁷). Remaining
573 proteins without any iBAQ value in one of both conditions have been considered as proteins
574 quantitatively present in a condition and absent in the other. They have therefore been set aside and
575 considered as differentially abundant proteins. Next, missing values were imputed using the impute.
576 MLE function of the R package imp4p (<https://rdr.io/cran/imp4p/man/imp4p-package.html>).
577 Statistical testing was conducted using a limma t-test thanks to the R package limma ⁷⁸. An adaptive
578 Benjamini-Hochberg procedure was applied on the resulting p-values thanks to the function adjust.p
579 of R package cp4p⁷⁹ using the robust method described in (⁸⁰) to estimate the proportion of true null

580 hypotheses among the set of statistical tests. The proteins associated to an adjusted p-value inferior
581 to a FDR level of 1% have been considered as significantly differentially abundant proteins.

582

583 Statistical analysis. All experiments, unless otherwise noted, were biologically repeated 3–5 times and
584 the statistical test is reported in the figure legend. Data normality was tested by Shapiro-Wilk test, and
585 appropriate parametric or non-parametric tests performed depending on result. P values calculated
586 using GraphPad Prism software and the exact values are in source data. Microscopy data obtained
587 from analysis of 3 – 5 image fields per biological replicate after being automatically acquired by the
588 microscope software to ensure unbiased sampling with the total number of analyzed cells or nuclei
589 noted in the figure legend.

590

591 **Data Availability:**

592 All data in the present study is available upon request from the corresponding authors.

593

594 **Code Availability:**

595 No custom code or software was used in the manuscript.

596

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616

617 **Author contributions:**

618 Conceived and designed all experiments: MGC and MAH. Performed and analyzed data for all
619 experiments: MGC with specific contributions from LS (confocal microscopy imaging repeats); FC,
620 MGE, & TC (p65 mass spectrometry data, repeats for GFP & endogenous immunoprecipitations
621 validations, analysis...) CMW (Detroit562 immunoblot). MGC and MAH edited and reviewed the
622 manuscript. MAH supervised the research and secured funding. All authors approved the final
623 manuscript.

624

625 **Conflict of interest statement:**

626 The authors declare no conflict of interest.

627

628 **Figure 1: TIGR4 antagonizes p65 activation.** Immunoblot of A549 human airway epithelial cells 2 hrs
629 post-challenge with either IL-1 β (10 ng/ml), TIGR4 (MOI 20) or 6B ST90 (MOI 20) (+/- IL-1 β ; 10 ng/ml).
630 Whole cell lysates probed for p65, phosphorylated p65 at Serine 276, phosphorylated p65 at Serine
631 536 or Actin. A) Representative image of immunoblot. Actin normalized B) total p65, C)
632 phosphorylated p65 at Serine 276 and D) phosphorylated p65 at Serine 536 (n=11 biological
633 replicates). Dot blot with mean (red line). One-way ANOVA with repeated measures with mixed-
634 effects analysis comparing all means with Tukey's multiple comparison post-hoc test. **P \leq 0.01, ***P
635 \leq 0.001, ****P \leq 0.0001. Full blots provided in Supplementary Information 1.

636

637 **Figure 2: TIGR4 represses p65 dependent transcription.** Immunofluorescence confocal microscopy of
638 paraformaldehyde fixed A549 cells 2 h post-challenge with either IL-1 β (10 ng/ml) or TIGR4 (+/- IL-1 β
639 10 ng/ml; MOI 20) stained for p65 (cyan) and nucleus (DAPI; gray). Scale bar = 100 μ m. B)
640 Quantification of nuclear p65 normalized to the nuclei (n = 3 biological replicates with total nuclei
641 counts for Uninfected n= 636, IL-1 β n= 801, TIGR4 n=633, TIGR4 + IL-1 β n=516). Tukey box and whisker
642 plot with defined box boundaries being the upper and lower interquartile range (IQR), 'whiskers'
643 (fences) being \pm 1.5 times IQR and the median depicted by the middle solid line. Dots represent
644 outliers. Two-way ANOVA comparing all means with Tukey's multiple comparison post-hoc test.
645 ****P \leq 0.0001. C) RT-qPCR IL-6, IL-8, PTGS2 & CSF2 transcript profiles of A549 cells over a 2 hr time
646 course challenged with either IL-1 β or TIGR4 (+/- IL-1 β 10 ng/ml; MOI 20). Graphed as the relative
647 expression of each indicated transcript to matched uninfected/unstimulated control per time point (n
648 = 3 biological replicates; 2 technicals per replicate). Displayed as a dot plot with each data point and a
649 bar representing the mean. Chromatin was obtained from A549 cells either untreated (light gray), IL-
650 1 β treated (10 ng/ml; dark gray) or 2 hrs post-challenge with TIGR4 (light blue; MOI 20). D) Schematic
651 representation of PTGS2 promoter with ChIP-qPCR primer locations (P1 & P2) and NF- κ B sites⁶³. E &
652 F) ChIP-qPCR represented as % recovery against input of p65 at indicated NF- κ B sites. Tukey box and
653 whisker plot with defined box boundaries being the upper and lower interquartile range (IQR),
654 'whiskers' (fences) being \pm 1.5 times IQR and the median depicted by the middle solid line (n=3
655 biological replicates with 2 technicals per replicate). One-way ANOVA comparing all means with
656 Tukey's multiple comparison post-hoc test. *P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.

657

658 **Figure 3: TIGR4 induces a divergent p65 interactome leading to NF- κ B p65 turnover by aggrephagy.**
659 Mass-spectrometry interactome (n=4 biological replicates per condition) of immunoprecipitated GFP-
660 p65 from a stable A549 GFP-p65 cell line (1x10⁷ cells total) 2 hrs post challenge with either 6B ST90
661 (MOI 20) or TIGR4 (MOI 20). A) Volcano plot of identified interacting partners with known NF- κ B p65
662 partners in blue and general significant targets in yellow. Lines represent FDR and fold-change cutoffs
663 with targets of interested denoted. B) Representative immunoblot of A549 whole cell lysates 2 hrs
664 post-challenge with either IL-1 β (10 ng/ml), TIGR4 (MOI 20) or 6B ST90 (MOI 20) (+/- IL-1 β ; 10 ng/ml)
665 probed for RelB, HDAC6, or Actin. C & D) Quantification of RelB or HDAC6 levels normalized to Actin
666 (n=4 biological replicates). Displayed as a dot blot with mean (red line). One-way ANOVA with
667 repeated measures with mixed-effects analysis comparing all means. *P \leq 0.05, **P \leq 0.01, ***P \leq
668 0.001. Whole cell lysates from A549 cells 2 hrs post-challenge with either IL-1 β (10 ng/ml) or TIGR4
669 (MOI 20; +/- IL-1 β ; 10 ng/ml) from E) Bafilomycin A1 (400nM) or G) SAR405 (500nM) pretreated cells
670 (both 3 hrs) and immunoblots probed for p65 or actin (n=3 biological replicates). Quantified levels of
671 total p65 normalized to actin from F) Bafilomycin A1 (400nM) or H) SAR405 (500nM). Dot blot with
672 mean (red line). One-way ANOVA with repeated measures with mixed-effects analysis comparing all
673 means. ns = not significant. Full blots provided in Supplementary Information 2

674

675

676 **Figure 4: TIGR4 drives NF- κ B p65 interaction with COMMD2 and p62 (SQSTM1) and nuclear**
677 **translocation.** Immunoprecipitates using GFP-Trap agarose beads were collected from a stable A549
678 GFP-COMMD2 cell line 2 hrs post-challenge with either IL-1 β (10 ng/ml) or TIGR4 (MOI 20; +/- IL-1 β ;
679 10 ng/ml). A) A single representative immunoblot from 3 biological replicates of GFP-COMMD2
680 immunoprecipitation lysates (input & IP) probed for p65 or GFP.. B) Representative immunoblot from
681 3 biological replicates of GFP-COMMD2 immunoprecipitation lysates (input & IP) probed for p62 or
682 GFP.. Full blots provided in Supplementary Information 3. C) Immunofluorescence confocal
683 microscopy of stable A549 GFP-COMMD2 cells 2 h post-challenge with either IL-1 β (10 ng/ml) or TIGR4
684 (+/- IL-1 β 10 ng/ml; MOI 20) stained for p62 (magenta) against GFP-COMMD2 (gray). Scale bar = 100
685 μ m. Red inset images of single cell highlighting (white arrow) perinuclear punta. Inset scale bar 10 μ m.
686 Quantification of total cellular p62 D) normalized to cell area (GFP-COMMD2 signal) and nuclear p62
687 E) normalized to the area of the nucleus (DAPI signal; n = 3 biological replicates with total cell counts
688 for Uninfected n= 548, IL-1 β n= 670, TIGR4 n=356, TIGR4 + IL-1 β n=271). Quantification of total cellular
689 COMMD2 F) normalized to cell area (GFP-COMMD2 signal) and nuclear COMMD2 G) normalized to
690 the area of the nucleus (DAPI signal; n = 3 biological replicates with total cell counts for Uninfected
691 n= 864, IL-1 β n= 1068, TIGR4 n=596, TIGR4 + IL-1 β n=534). Tukey box and whisker plot with defined
692 box boundaries being the upper and lower interquartile range (IQR), 'whiskers' (fences) being \pm 1.5
693 times IQR and the median depicted by the middle solid line. Dots represent outliers. Two-way ANOVA
694 comparing all means with Tukey's multiple comparison post-hoc test. **P \leq 0.01, ***P \leq 0.001, ****P
695 \leq 0.0001. H) Cell fractions from a stable A549 GFP-COMMD2 cell line 2 hrs post-challenge with either
696 IL-1 β (10 ng/ml) or TIGR4 (MOI 20; +/- IL-1 β ; 10 ng/ml). Representative immunoblot probed for GFP
697 (COMMD2) enrichment across cellular compartments. GapDH or histone H4 (H4) used to determine
698 fraction purity. Full blots provided in Supplementary Information 3. I) Percent nuclear COMMD2 levels
699 normalized to input (n=3 biological replicates). Graphed as mean \pm STD with dots representing
700 individual biological replicates. One-way ANOVA with repeated measures with mixed-effects analysis
701 comparing all means with Tukey's multiple comparison post-hoc test. ****P \leq 0.0001.

702

703 **Figure 5: TIGR4 challenge triggers COMMD2 nuclear localization in a Ply dependent manner.** A)
704 Immunofluorescence deconvolution epifluorescence microscopy of paraformaldehyde fixed stable
705 A549 GFP-COMMD2 cells 2 h post-challenge with either IL-1 β (10 ng/ml), TIGR4 wildtype (MOI 20),
706 TIGR4 Δ ply (MOI 20), or TIGR4 Δ spxB (MOI 20) with GFP-COMMD2 (gray). Scale bar = 10 μ m. B)
707 Quantification of nuclear GFP-COMMD2 normalized to the segmented nuclei using DAPI signal
708 (omitted in representative images for phenotype clarity; n = 3 biological replicates with total nuclei
709 counts for Uninfected n= 6292, IL-1 β n= 6579, TIGR4 n=5061, TIGR4 Δ Ply n=7607, TIGR4 Δ SpxB
710 n=6566). Graphed as mean \pm STD with dots representing individual biological replicates. One-way
711 ANOVA comparing all means with Tukey's multiple comparison post-hoc test. *P \leq 0.05, **P \leq 0.01.

712

713 **Figure 6: COMMD2-p65-p62 is exported from nucleus through CRM1.** Immunofluorescence confocal
714 microscopy of stable A549 GFP-COMMD2 pretreated for 3 hrs with Leptomycin B (10 nM) prior to 2
715 hr challenge with either IL-1 β (10 ng/ml) or TIGR4 (MOI 20). Paraformaldehyde fixed cells stained for
716 A) p62 (magenta), or B) p65 (cyan) against GFP-COMMD2 (gray) and nuclei (DAPI; blue). Scale bar =
717 100 μ m or 10 μ m for uninfected and untreated single cell inserts. Nuclear levels of C) GFP-COMMD2
718 or D) p62 normalized to the segmented nuclei using DAPI signal (n = 3 biological replicates with total
719 nuclei counts for Uninfected (-) n=1664, Uninfected n= 742, IL-1 β (-) n=1068, IL-1 β n= 920, TIGR4 (-)
720 n=585, TIGR4 n=798). E) Nuclear levels of p65 normalized to the segmented nuclei using DAPI signal
721 (n = 3 biological replicates with total nuclei counts for Uninfected (-) n=489, Uninfected n= 1492, IL-1 β
722 (-) n=576 IL-1 β n= 1658, TIGR4 (-) n=680, TIGR4 n=1514). Tukey box and whisker plot with defined box
723 boundaries being the upper and lower interquartile range (IQR), 'whiskers' (fences) being \pm 1.5 times

724 IQR and the median depicted by the middle solid line. Dots represent outliers. Two-way ANOVA
725 comparing all means with Tukey's multiple comparison post-hoc test. *** $P \leq 0.001$, **** $P \leq 0.0001$.
726

727 **Figure 7: COMMD2-p62 export NF-kB p65 for degradation.** Representative immunofluorescence
728 confocal microscopy of stable A549 GFP-COMMD2 pretreated for 3 hrs with Leptomycin B (10nM)
729 prior to 2 hr challenge TIGR4 (MOI 20; +/- IL-1 β ; 10 ng/ml). Paraformaldehyde fixed cells stained for
730 (A) p62 (magenta) against GFP-COMMD2 (gray) and nuclei (DAPI; blue). Scale bar = 10 μ m B) Nuclear
731 p62 puncta quantification (n = 3 biological replicates with total nuclei counts for Uninfected n=1041,
732 IL-1 β n=831, TIGR4 n=1164, TIGR4 + IL-1 β n=1269). Graphed as mean \pm STD with dots representing
733 individual biological replicates. One-way ANOVA comparing all means with Tukey's multiple
734 comparison post-hoc test. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$. C) Quantified immunofluorescence
735 confocal microscopy of A549 GFP-COMMD2 cells pretreated with Bafilomycin A1 (400nM; 3 hrs) prior
736 to 2 hr challenge with either IL-1 β (10 ng/ml) or TIGR4 (MOI 20; +/- IL-1 β ; 10 ng/ml). Paraformaldehyde
737 fixed and stained for C) p65, D) COMMD2 or E) p62. (n = 3 biological replicates with total cell count for
738 Uninfected p62 & COMMD2 n=1648 & p65 n=1496, IL-1 β p62 & COMMD2 n=2103 & p65 n=1703,
739 TIGR4 p62 & COMMD2 n=2033 & p65 n=1597, TIGR4 + IL-1 β p62 & COMMD2 n=1724 & p65 n=1759).
740 Tukey box and whisker plot with defined box boundaries being the upper and lower interquartile
741 range (IQR), 'whiskers' (fences) being ± 1.5 times IQR and the median depicted by the middle solid
742 line. Dots represent outliers. Two-way ANOVA comparing all means with Tukey's multiple comparison
743 post-hoc test. ns=not significant, *** $P \leq 0.001$, **** $P \leq 0.0001$.
744

745 **Sup. Figure 1: TIGR4 actively dampens p65 activation over time.** A) Representative graph of actin
746 normalized phosphorylated p65 S536 levels over 2 hrs quantified by immunoblot. B) Immunoblot of
747 whole cell lysates obtained from primary human nasal epithelial cells 2 hrs post-challenge with either
748 IL-1 β (10 ng/ml), TIGR4 (MOI 20) or 6B ST90 (MOI 20) (+/- IL-1 β ; 10 ng/ml). PVDF membrane probed
749 for phosphorylated p65 Serine 536 or Actin (n=2 biological replicates). C) Immunoblot of whole cell
750 Detroit 562 cell lysates 2 hrs post-challenge with either TIGR4 (MOI 10) or 6B ST90 (MOI 10).
751 Nitrocellulose membrane probed for phosphorylated p65 Serine 536 or GapDH (n=2 biological
752 replicates).
753

754 **Sup. Figure 2: Proteasomal degradation is not involved in TIGR4 mediated p65 turnover.** A)
755 Quantification and representative immunoblot image of MG132 (10 μ M; 3 hr pretreatment) treated
756 A549 whole cell lysates 2 hrs post-challenge with either IL-1 β (10 ng/ml), TIGR4 (MOI 20) or 6B ST90
757 (MOI 20) (+/- IL-1 β ; 10 ng/ml) and probed for total p65 or actin (n=11 biological replicates). Dot blot
758 with mean (red line). One-way ANOVA with repeated measures with mixed-effects analysis comparing
759 all means with Tukey's multiple comparison post-hoc test. ns=not significant, **** $P \leq 0.0001$. B)
760 Representative immunoblot of whole cell lysates collected from primary human nasal epithelial cells
761 treated with Bafilomycin A1 (400nM; 3 hrs) prior to 2 hr challenge with either IL-1 β (10 ng/ml) or
762 TIGR4 (MOI 20; +/- IL-1 β ; 10 ng/ml). PVDF membrane probed for levels of p65 and actin. C)
763 Representative immunoblot of endogenous p65 immunoprecipitation (input & IP) from 1 $\times 10^7$ A549
764 cells post 2 hr challenge using protein G sepharose beads. Collected lysates probed for p65, RelB or
765 NFkB1 (p105/p50).
766

767 **Sup. Figure 3: TIGR4 specifically drives COMMD2 translocation and induces aggregophagy.** A) Cell
768 fractions from a stable A549 GFP-COMMD2 cell line 2 hrs post-challenge with either IL-1 β (10 ng/ml),
769 TIGR4 (MOI 20) or 6B ST90 (MOI 20). Representative immunoblot of cell fractions and coomassie
770 stained PVDF membranes. Blots probed for GFP (COMMD2) enrichment across cellular compartments.
771 B) Representative immunoblot of A549 whole cell lysates 2 hrs post-challenge with either IL-1 β (10

772 ng/ml) or TIGR4 (MOI 20) obtained from untreated or pretreated (3 hrs) with Bafilomycin A1 (400nM).
773 PVDF membrane probed for p62 or actin. Table is the quantification of actin normalized p62 levels
774 across conditions.

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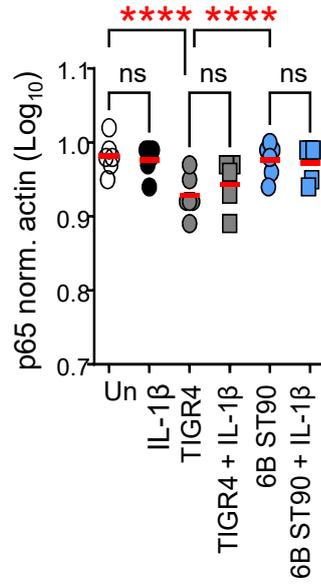
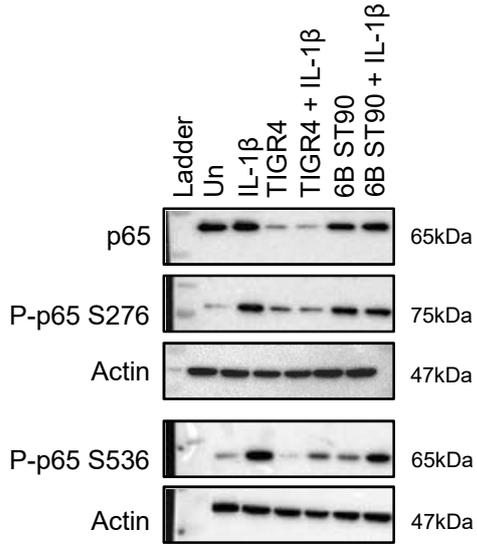
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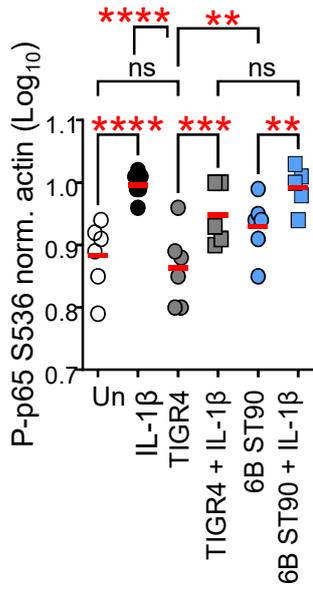
FIGURE 1

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A



C



D

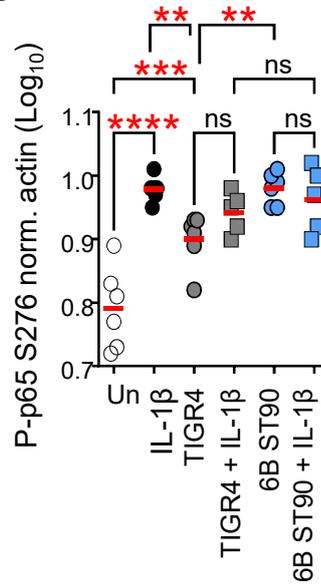
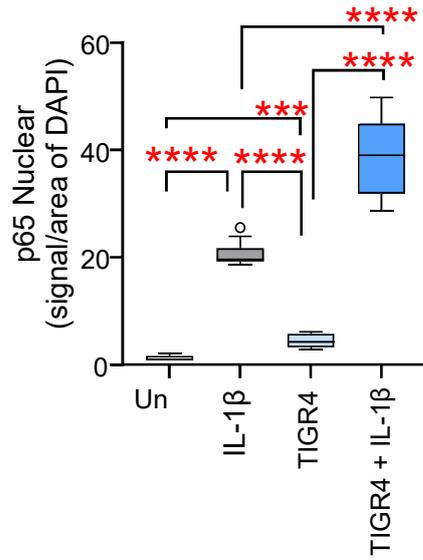
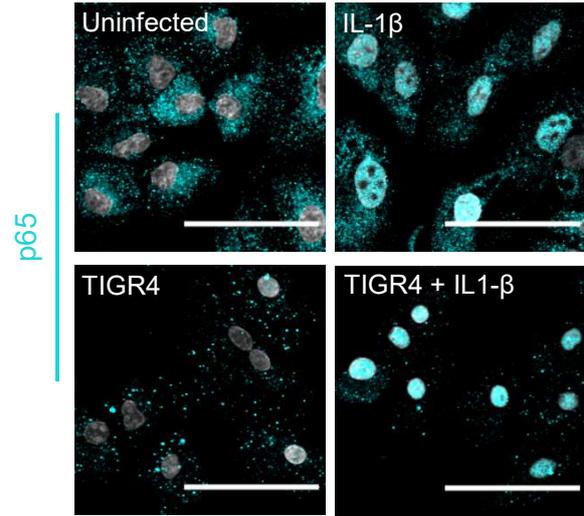


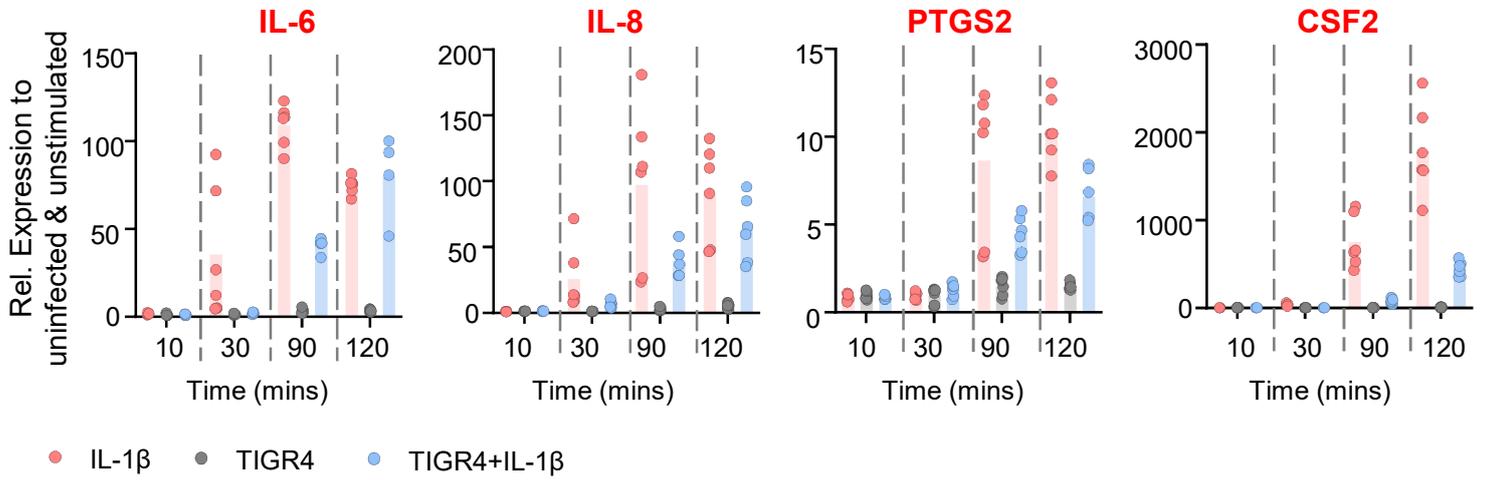
FIGURE 2

A

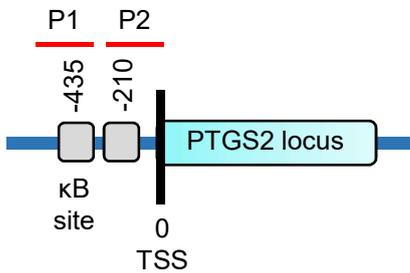
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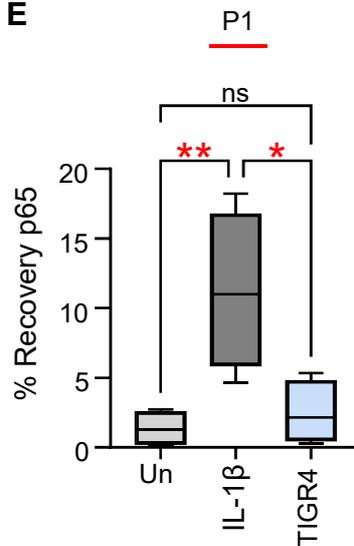
C



D



E



F

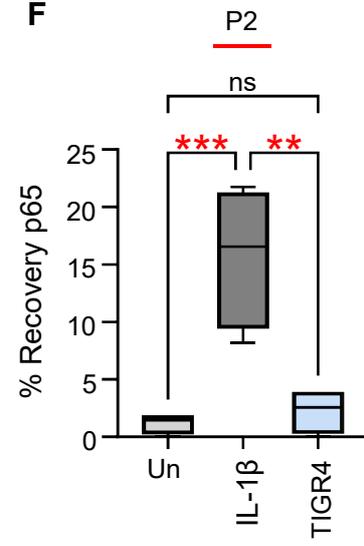


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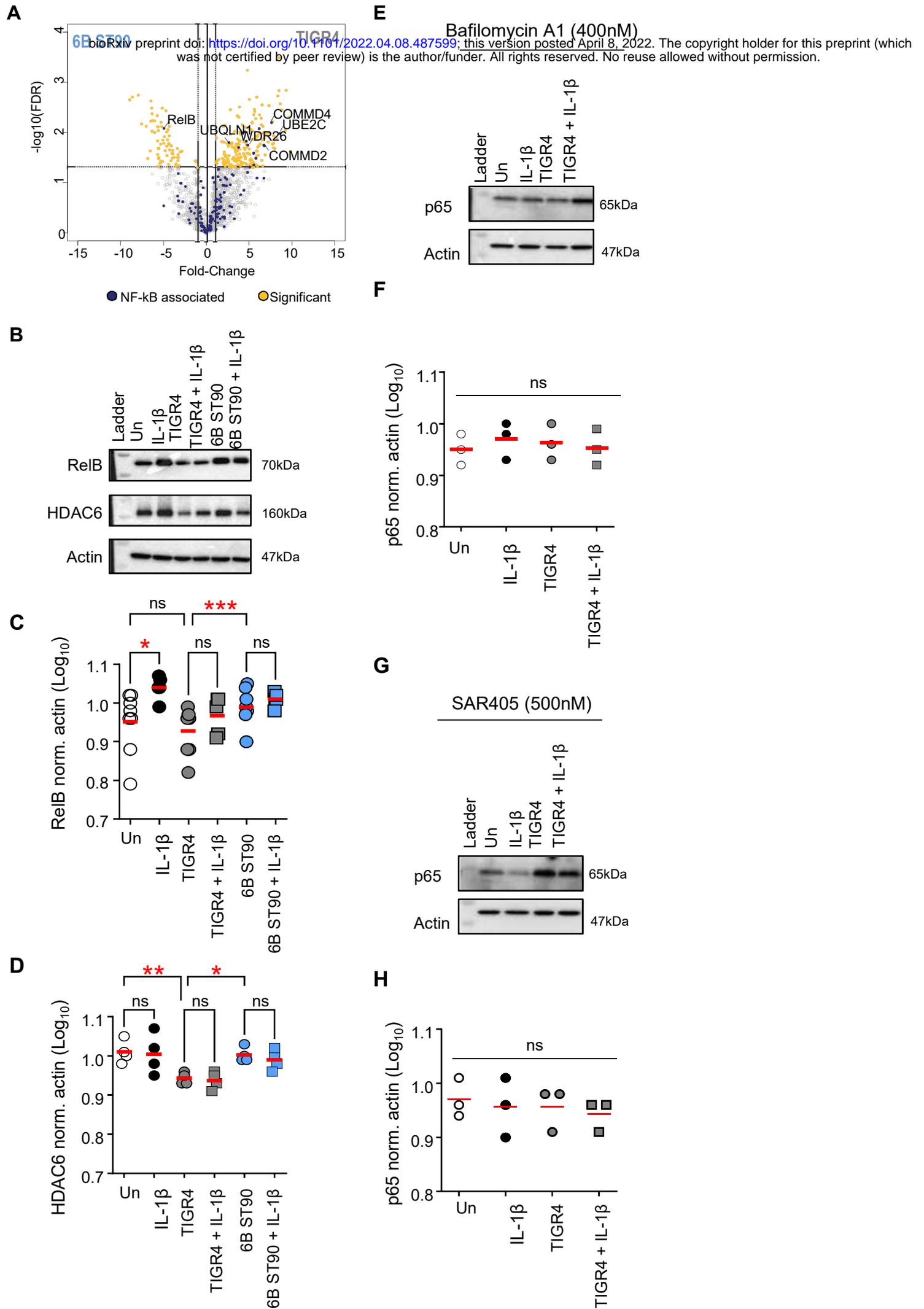


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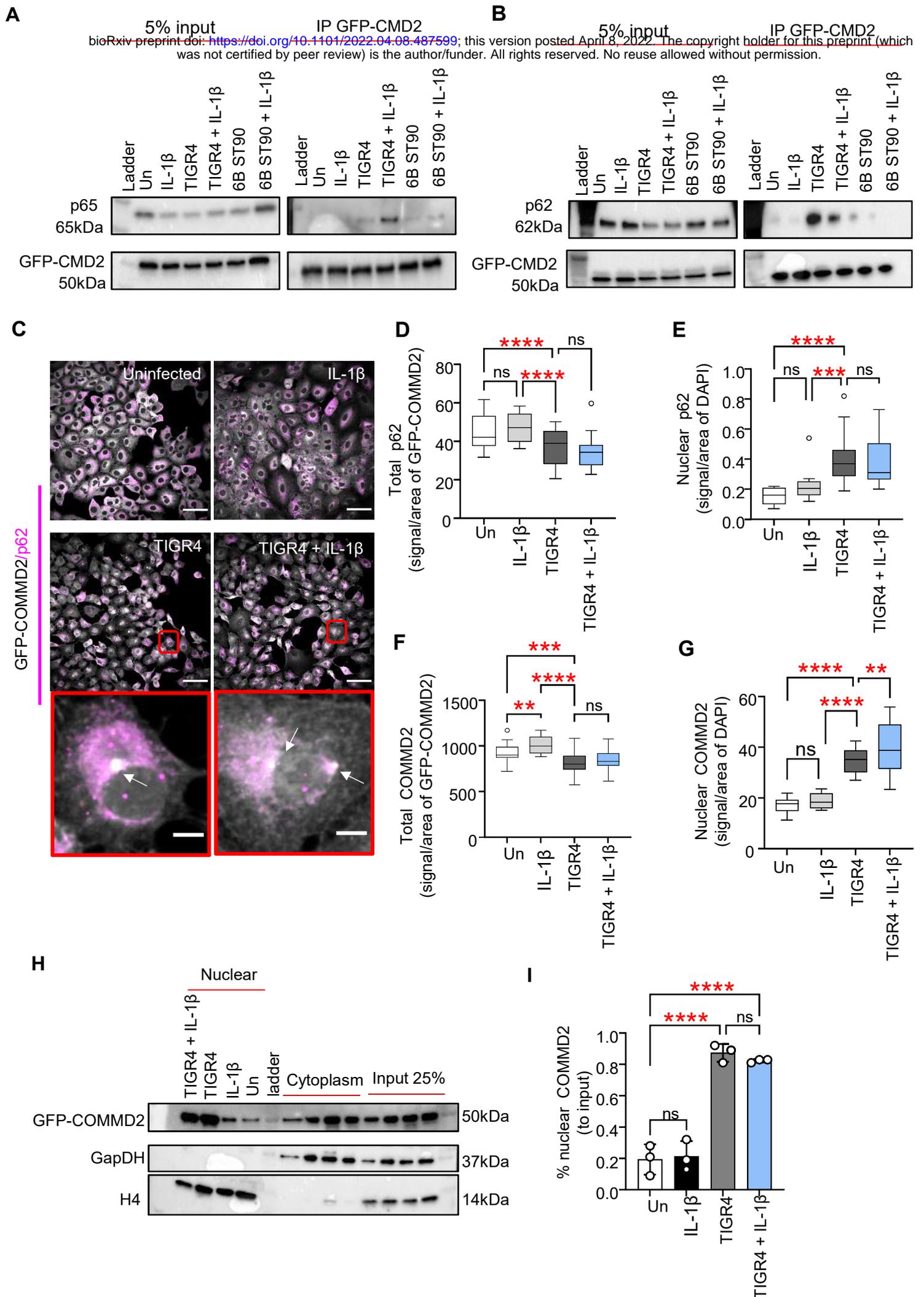
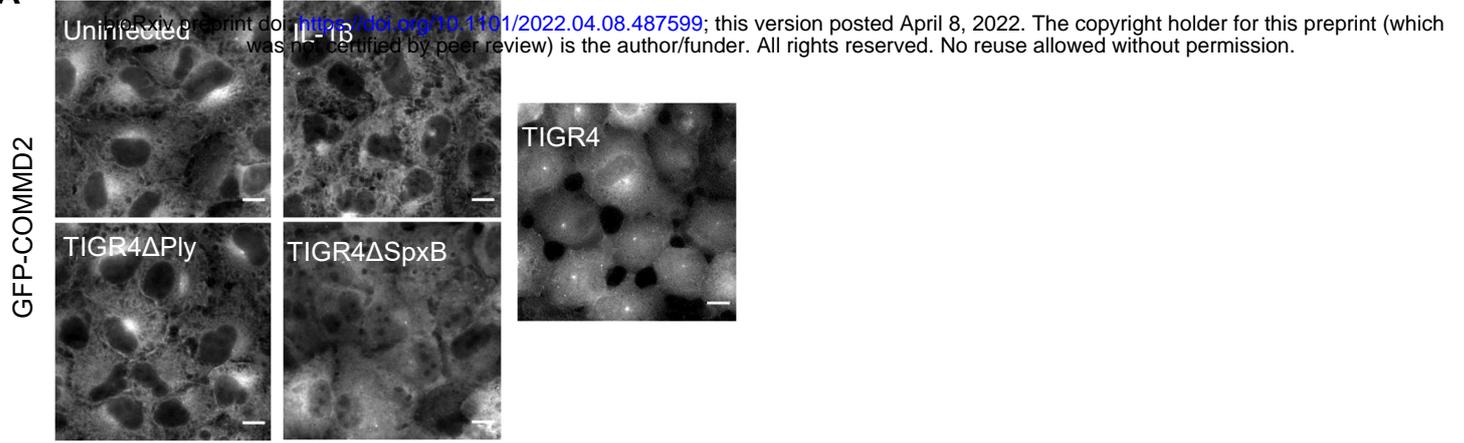


FIGURE 5

A



B

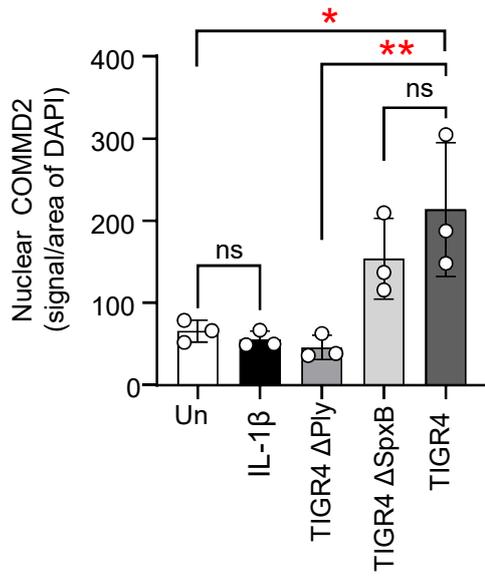


FIGURE 6

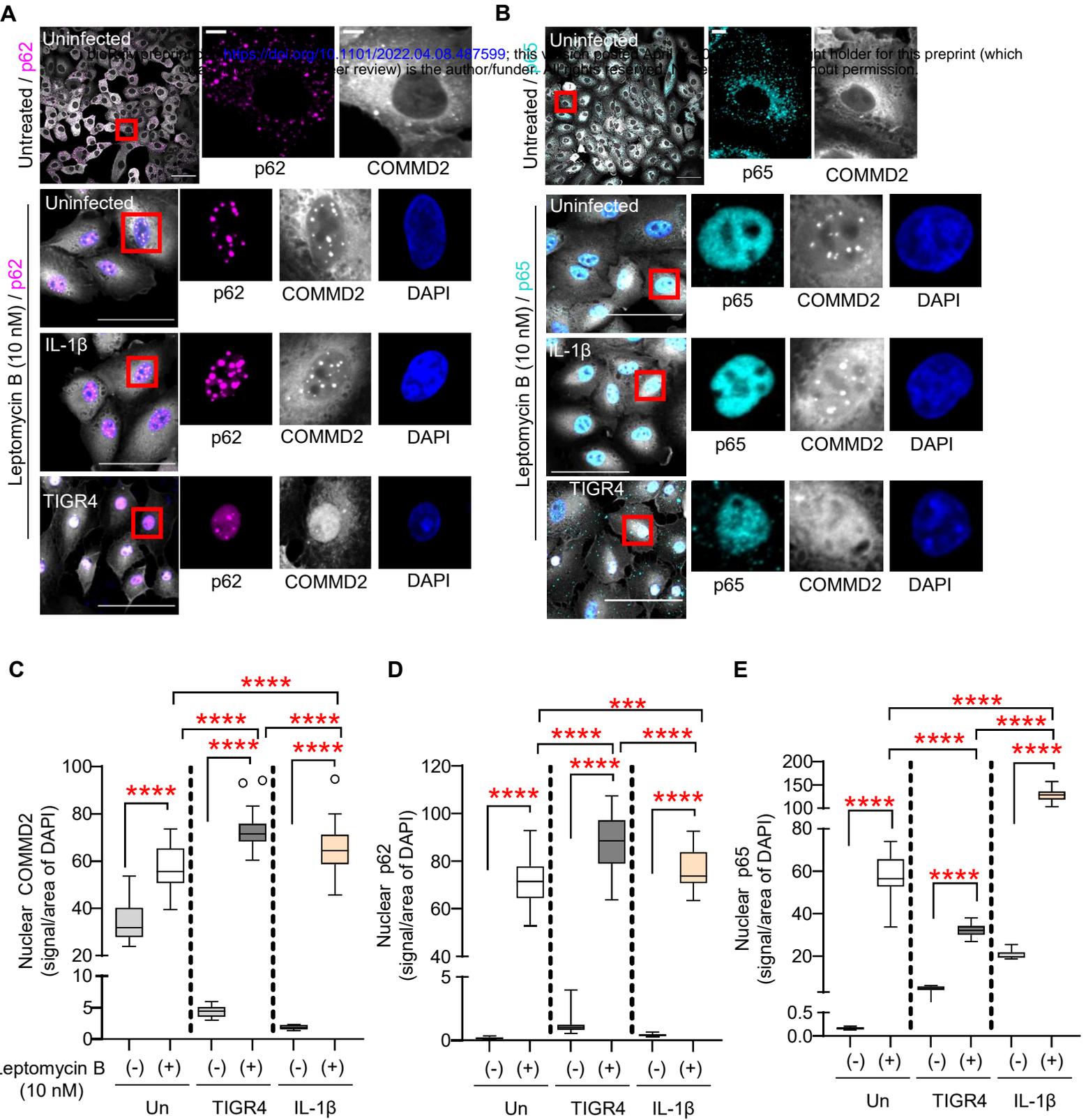
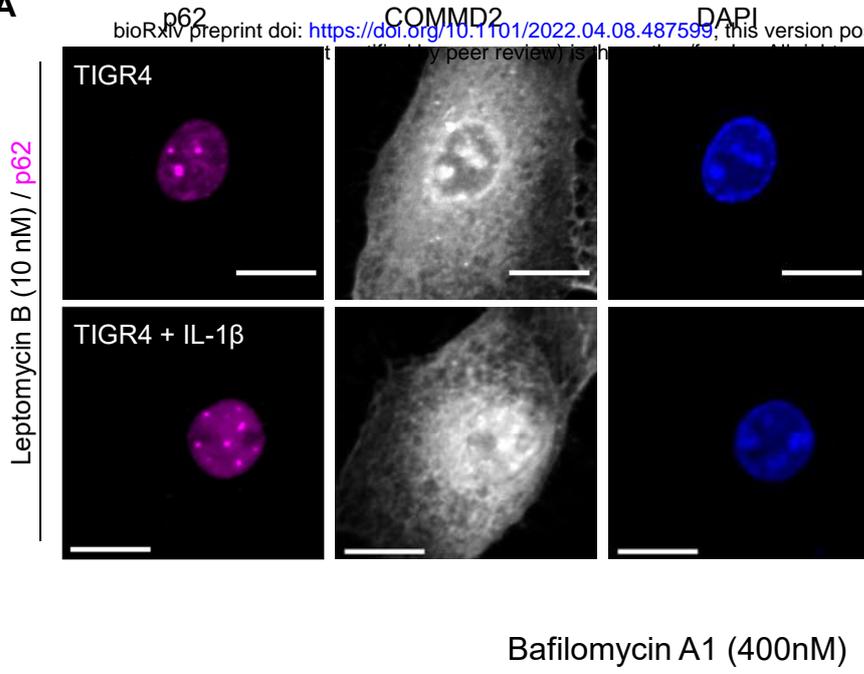
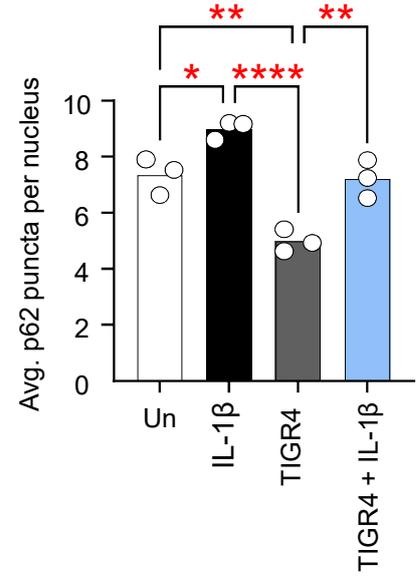


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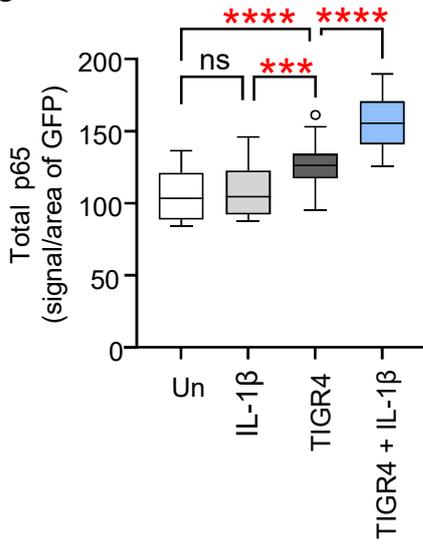
A



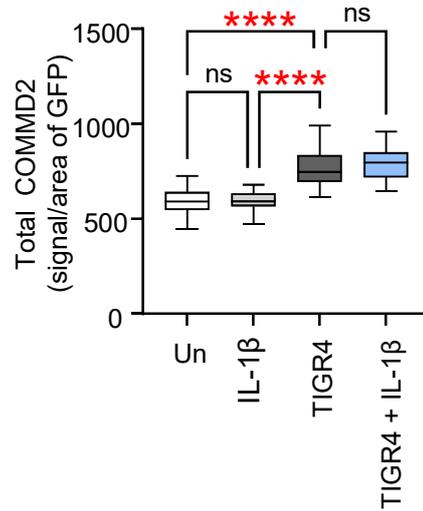
B



C



D



E

