Title: RGS10 differentially modulates NFKB subunit transcription and inflammatory cytokine profiles in peritoneal macrophages.

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Abstract

Regulator of G-protein signaling 10 (RGS10) has been shown to regulate multiple inflammatory pathways relevant to disease pathogenesis. Of particular importance is the ability of RGS10 to negatively regulate the NFkB pathway, a prominent pro-inflammatory pathway implicated in multiple inflammatory disease phenotypes. However, the exact mechanism by which RGS10 regulates NFkB is unknown. Considering that RGS10 translocates into the nucleus upon stimulation, we hypothesize that RGS10 may regulate NFKB through transcription. To determine whether RGS10 mediates NFkB transcription, we stimulated RGS10 KO and B6 peritoneal macrophages and collected cell lysate over 24 hours to assess transcript levels of NFkB and related proinflammatory cytokines. Here we found that RGS10 differentially regulates the transcription of NκKB subunits and NFκB-dependent cytokines. Further studies are warranted to understand the potential role of RGS10 in transcriptional regulation of inflammatory states.

44 **Introduction**

45 Regulator of G-protein signaling 10 (RGS10) has been shown to regulate inflammatory
46 responses that are protective in multiple chronic inflammatory disease models such as
47 rheumatoid arthritis (RA), metabolic dysfunction, obesity, colitis, periodontist, influenza
48 infection, cancers, and even neurodegenerative disease models (1-9). Mechanistically, RGS10
49 modulates multiple pathways that regulate the production of pro-inflammatory cytokines in
50 myeloid cells including Stromal interaction molecule 2 (STIM2) mediated calcium entry,
51 glycolytic production of reactive oxygen species (ROS), and suppression of Nuclear Factor
52 kappa-light-chain-enhancer of activated B cells (NFkB)(7, 10, 11). Importantly, NFkB, a master
53 transcriptional regulator of pro-inflammatory cytokines, is translocated to the nucleus upon
54 inflammatory stimulation in myeloid cells allowing for the production of key inflammatory
55 mediators such as Tumor necrosis factor (TNF), Interleukin 1-beta (IL-1B), Interleukin 6 (IL-6),
56 Interleukin 12p40 (IL-12p40), and cyclooxegenase-2 (COX2) (12, 13). Dysregulated NFkB
57 signaling has been identified as an important mediator of multiple chronic inflammatory
58 diseases such as RA, Irritable bowel disease, type 1 diabetes, ect.(12). Specifically, RGS10
59 deficiency enhances NFkB subunits protein levels upon inflammatory stimulus and also
60 amplifies NFKB activity and proinflammatory cytokine secretion(4, 7). However, the mechanism
61 by which RGS10 regulates NFKB levels is still unknown. Immunoprecipitation assays have
62 further revealed that RGS10 does not have a direct protein to protein interaction with
63 NFkB(10). Interestingly, both RGS10 and NFKB translocate to the nucleus upon inflammatory
64 stimulation indicating that RGS10 may play a nuclear role in regulating the level and activity of

65 NFkB(9, 12). Moreover, immunoprecipitation assays revealed that RGS10 does interact with
66 DNA binding proteins indicating that it may play a role in transcription(10).
67 Therefore, we hypothesized that RGS10 transcriptionally regulates NFKB. To test this,
68 we analyzed NFkB and proinflammatory transcripts of lipopolysaccharide (LPS) stimulated wild
69 type C57B6/J (B6) and RGS10 Knock out (KO) peritoneal macrophages (pMacs) at 1,2,4,8, and
70 24h. Importantly, animals were given opioid analgesics prior to pMac collection. Under these
71 conditions, we found that RGS10 differentially regulates the transcription of NFKB subunits and
72 NFKB-dependent cytokines. Specifically, we demonstrate that RGS10 does not regulate the
73 kinetics of NFKB subunit transcription or IL-1B cytokine transcription levels. However, we do see
74 that RGS10 deficiency briefly results in higher levels of p65 transcript levels shortly after initial
75 stimulation. Moreover, we found that RGS10 KO pMacs produced significantly less TNF
76 transcript than their B6 counterparts and this difference was driven by male mice. We also
77 found no difference in secreted protein levels of TNF, IL-1β, or IL-10 but did see elevated levels
78 of IL-6, IL-12, and KCGRO in LPS stimulated RGS10 KO pMacs. In summary, we demonstrate
79 transient and differential transcriptional regulation of NFKB pathway components by RGS10.
80 These data warrant further investigation into the potential role of RGS10 in transcriptional
81 regulation. Importantly, these phenotypes we collected with the use of opioid analgesics and
82 therefore our data demonstrate that RGS10 can regulate differential myeloid pro-inflammatory
83 profiles under the influence of such opioid analgesics.

84

85 **Materials and Methods**

86 Animals: Mice were housed in the McKnight Brain Institute vivarium at the University of Florida
87 and maintained on a 12:12 light–dark cycle with *ad libitum* access to water and standard rodent
88 diet chow. All animal procedures were approved by the Institutional Animal Care and Use
89 Committee and followed the Guide for the Care and Use of Laboratory Animals from the
90 National Institutes of Health at the University of Florida. Generation of the RGS10 Knock out
91 (KO) line onto a C57B6/J background has been previously described(7). 4 to 5 month-old male
92 and female C57B6/J and RGS10 KO mice (n=12/group) were given 50uL of sustained-release
93 Buprenorphine (ZooPharm) subcutaneously 30 minutes prior to intraperitoneal injection of 1mL
94 of 3% thioglycolate. 3 days later animals were sacrificed via cervical dislocation for peritoneal
95 macrophage collection.

96

97 Peritoneal macrophages: Peritoneal macrophage(pMacs) collection occurred as previously
98 published(14). Specifically, Pmacs collection occured in a laminar flow hood. After cervical
99 dislocation and spraying down the animals with 70% ethanol, 10mL of cold RMPI were injected
100 into the peritoneum. Peritoneal lavage fluid was collected, passed through 70 uM filters prewet
101 with 5mL of HBSS-/- (Gibco, 14175103) into 50mL conical tubes. Filters were rinsed with 5mLs
102 of HBSS-/- (Gibco, 14175103) twice and centrifuged at 400xg for 5 minutes at 4°C. If blood cells
103 were present in the sample, cells were treated with 1mL of ammonium-chloride-potassium lysis
104 buffer for 1min and then pelleted at 400xg for 5 minutes at 4°C. Cells were taken forward and

105 processed in the biosafety hood using sterile technique. Supernatant was aspirated off cells,
106 and cells resuspended in 3mL of warm plating media (RPMI, 10% fetal bovine serum, and 1%
107 penicillin/streptomycin). 10uL of cells were taken forward for counting using a 1:1 diluted
108 trypan blue (1/4th trypan blue in sterile 1XPBS) stain and the CountessTM. Cells were plated at
109 1X106 density in a 2mLs of culture media in a 12 well plate. Cells were placed in the incubator
110 at 37° C and 5% CO2 to rest for 2 hours. After 2 hours, cells were washed with sterile DPBS
111 (Gibco, 14190235) to remove non-adherent cells and new pre-warmed culture media (RPMI
112 supplemented with 10% FBS and 1x Pen-Strep) was added. Cells were treated with either
113 vehicle or 100ng/mL LPS for 1,2,4,8, or 24hrs. Media was collected and flash frozen in liquid
114 nitrogen. Cells were lysed with 350uL BME and RLT lysis buffer (20uL of BME per 1mL RTL
115 buffer) and mechanically homogenized by scrapping each well with a cell scraper.
116
117 RNA extraction: Bench areas were cleaned with RNase Zap prior to RNA extraction. Lysed pMac
118 cells were processed for RNA extraction via RNeasy Kit (Qiagen) according to manufacturer's
119 protocol. Briefly, lysed solution was transferred to Qiashredder tubes for complete
120 homogenization. Qiashredder flow-though was stored in -80°C until sample batches were
121 collected. Samples were thawed and mixed with 350uL of 70% ethanol and transferred to
122 RNAeasy spin columns. Samples were centrifuged for 15 seconds at 10,000rmp(9,391xg) at
123 room temperature. Flow-through was discarded and nucleic acids trapped in the RNAeasy
124 membrane were washed with 700uL of RW1 buffer and 500uL of RPE buffer twice, centrifuging
125 for 15 seconds at 10,000rmp at room temperature and discarding flow-through after each
126 wash. Samples were then centrifuged for 2 minutes at full speed to dry before eluting samples

127 with 30uL of RNAse free water in a 1-minute spin at 10,000rpm. RNA concentrations were
128 determined using Denovix spectrophotometer and samples were stored at -80°C prior to cDNA
129 synthesis.
130
131 cDNA synthesis : DNA was removed from RNA samples prior to cDNA synthesis by diluting RNA
132 samples with nuclease free water and 3.68ul of DNAse master mix (3.36ul of 25mM Mgcl2 +
133 0.32ul of 1/5 DNAse 1) reaching a total volume of 10ul per sample. Samples were then run
134 through the thermocycler at 37° C for 30 minutes followed by 75° C for 10 minutes and a 4° C
135 hold. 0.4ug of cDNA for each sample was synthesized from RNA using the High-Capacity cDNA
136 Reverse Transcription Kit (Thermo Fisher Scientific, 4368814) according to manufacturer's
137 instructions. Briefly, 10ul of reverse transcription master mix, which was comprised of 2ul of
138 10XRT Buffer, 0.8 25XdNT mix (100mMm, 2ul of 10XRT random Primers, 1ul of Rnase Inhibitor,
139 1ul of Reverse Transriptase, and 3.2ul of Nuclease-Free water per sample, was added to each
140 DNA-free sample. Samples were vortexed and spun down and run in the thermocycler at 25° C
141 for 10 minutes followed by 37° C for 2 hours then 85° C for 5 minutes and a 4° C hold. cDNA
142 samples were then diluted to the final concentration with nuclease-free water and stored at -20
143 ° C.
144
145 Real Time QPCR: cDNA was combined with SYBR green reagents (Thermo Fisher Scientific,
146 A46112) and specific IDT Forward and Reverse primers at 10ng/well reactions in a 384 well plate
147 in triplicate for Real Time Quantitative PCR analysis in comparative CCT run on the Quant Studio
148 5 at a volume of 20ul/well. Primer sequences were as follows: mouse NFKB subunit p65

149 forward: GGA TCC AGT GTG TGA AGA AG and reverse: CTC CTC TAT AGG AAC GTG AAA G,
150 mouse NFKB subunit p105 forward: GGG ACA GTG TCT TAC ACT TAG and reverse: GTC ATC AGA GAT
151 CAA ACC AGA, mouse RGS10 forward: TTG GCT AGC GTG TGA AGA TTT C and reverse: TGG CCT TTT CCT
152 GCA TCT G, mouse TNF forward: CTG AGG TCA ATC TGC CCA AGT AC and reverse: CTT CAC AGA GCA ATG
153 ACT CCA AAG, mouse IL-1B forward: CAA CCA ACA AGT GAT ATT CTC CAT G and reverse: GAT CCA CAC
154 TCT CCA GCT GCA. All QPCR samples were normalized to their own Beta- Actin CT and then to the
155 WT B6 saline group. Samples with an average triplicate variance of ≥ 0.3, had outlying triplicate
156 removed and if samples average remaining variance still exceeded 0.3 the sample was excluded
157 from analysis.
158
159 Mesoscale Discovery Multiplexed Immunoassays: Protein concentrations of TNF, IL-6, IL-1β,
160 KC/GRO, IL-4, IL-10, IFN-Y, IL-5, IL-2, and IL-12p70 in conditioned media were analyzed (MSD,
161 K15048D-2) using multiplexed immunoassays on the MesoScale Discovery (MSD) platform.
162 Samples were plated in duplicate at a 1:1 dilution with MSD diluent 41. Samples were
163 processed according to the manufacturer's instructions as previously described(14). Plates were
164 analyzed on the MSD Quickplex machine and MSD software (Discovery Workbench Version
165 4.0). Cytokines with values below the lower limit of detection (LLOD) were not quantified.
166
167 Statistics: Analyses were performed with Graph-Pad Prism 10.6. Group differences were
168 analyzed using Ordinary three-way Anova corrected for multiple comparisons with Tukey post
169 hoc test. Differences between groups across time were analyzed using one-way ordinary
170 ANOVA corrected for multiple comparisons with Tukey's post hoc analysis. Samples that are

statistically different do not share the same letter. p values ≤ 0.05 were considered statistically significant.

Results

To determine whether the RGS10 mediated NFkB-dependent transcription, we stimulated RGS10 KO and B6 peritoneal macrophages and collected cell lysate over 24 hours to and assessed transcript levels of NFkB and related proinflammatory cytokines. If the hypothesis that RGS10 negatively regulates NFkB transcription is correct, we would expect to see that RGS10 deficiency enhances NFkB-dependent pro-inflammatory gene transcription. Here, we find that RGS10 does not differentially regulate the kinetics of NFKB subunit transcription when analyzed over the course of 24 hours, nor did we find an enhancement in the transcription of NFkB-dependent genes such as TNF and IL-1B (Figure 1). However, we did find that RGS10 deficiency results in higher levels of the p65 NFkB subunit transcript at 2 hours post LPS stimulation (Supplemental figure 1D), indicating a potential time sensitive role for the regulation of the p65 NFkB subunit by RGS10. Importantly, we demonstrate that RGS10 transcript levels are reduced upon stimulation and recover over time, which has been previously reported by multiple studies (Figure 1B)(9, 15). Interestingly, we found a genotype effect of TNF transcripts indicating that RGS10 KO peritoneal macrophages (pMacs) were producing less TNF than their B6 counterparts (Figure 1C).

Figure 1. RGS10 differentially regulates the transcription of NFκB subunits and NFκB-dependent transcripts. The red line represents RGS10 KO pMacs treated with LPS, blue line

193 represents B6 pMacs treated with LPS, yellow line represents RGS10 KO pMacs treated with
194 vehicle, and the green line represents B6 pMacs treated with vehicle. A) Fold change of p65
195 NFκB transcripts relative to control over 24h. B) Fold change of p105 NFκB transcripts relative
196 to control over 24h C) Fold change of TNF transcripts relative to control over 24h. D) Fold
197 change of IL-1βtranscripts relative to control over 24h. E) Fold change of RGS10 transcripts
198 relative to control over 24h Significant genotype present on appropriate graphs. Samples that
199 are statistically different do not share the same letter.
200

201 To understand if any of our results were sex specific, we analyzed our data in males and
202 females separately. Here we found that the genotype effect on TNF is only present in male
203 RGS10 KO pMacs and not females (Figure 2C,G). Otherwise, male and female pMacs produced
204 similar transcriptional inflammatory responses to LPS. Interestingly, early transcription of the
205 p65 subunit of NFkB was significantly enhanced with RGS10 deficiency in females but not males
206 (Supplemental Figure 1E-F).
207

208 **Figure 2. Transcription of the NFκB subunits and pro-inflammatory cytokines is similar across**
209 **both sexes.** Fold change of p65 NFκB transcripts relative to control over 24h in females (A) and
210 males (B). Fold change of p105 NFκB transcripts relative to control over 24h in females (C) and
211 males (D). Fold change of TNF transcripts relative to control over 24h in males (E) and females
212 (f). Fold change of IL-1βtranscripts relative to control over 24h in males (G) and females (H).
213 Fold change of RGS10 transcripts relative to control over 24h in males (I) and females (J).
214 Significant genotype present on appropriate graphs. Samples that are statistically different do
215 not share the same letter.
216

217 It was surprising that our data did not demonstrate any enhancement in TNF or IL-1β
218 transcripts levels as previous literature reported exacerbated TNF and IL-1β protein levels with
219 RGS10 KO in pMacs(16). Therefore, we examined the level of secreted pro-inflammatory
220 cytokines from the conditioned media. Again, we found that TNF and IL-1β levels did not

221 change with RGS10 deficiency, nor did levels of IL-10 (Figure 3 A,D,F). Instead, we found that
222 RGS10 deficiency exacerbated the levels of IL-6, KCGRO, and IL-12 (Figure 3 B,C,E).
223
224 **Figure 3.RGS10 regulates the secretion of pro-inflammatory cytokines in peritoneal**
225 **macrophages Level of pro-inflammatory cytokines from male and female pMacs**. Green dots
226 are B6 pMacs and Blue dots are RGS10 KO pMacs. A) pg/mL normalized to RNA yield of TNF
227 protein levels 24h post stimulation. B) pg/mL normalized to RNA yield of IL-12 protein levels
228 24h post stimulation. C) pg/mL normalized to RNA yield of IL-6 protein levels 24h post
229 stimulation. D) pg/mL normalized to RNA yield of IL-1B protein levels 24h post stimulation. E)
230 pg/mL normalized to RNA yield of KCGRO protein levels 24h post stimulation. F) pg/mL
231 normalized to RNA yield of IL-10 protein levels 24h post stimulation. Samples that are
232 statistically different do not share the same letter.
233
234 **Discussion**

235 Overall, our data demonstrates that RGS10 differentially regulates the transcription of
236 NFKB subunits and NFKB-dependent cytokines in peritoneal macrophages. However, this effect
237 is time sensitive and displays multiple sex biases. The brief and not sustained increase of the
238 p65 NFkB subunit transcript levels with RGS10 deficiency can likely be explained by the
239 downregulation of RGS10 transcript post LPS stimulation, rending a lack of RGS10 in which to
240 regulate p65 NFkB transcript levels with past the initial stimulation (~within 2 hours of
241 stimulation). In support of this idea, RGS10 protein levels have also been observed to decrease
242 with LPS stimulation in other myeloid populations over the course of 24-48 hours prior to
243 returning to baseline levels(9, 15).

244 Moreover, our data reveals novel cytokine transcription and secretion patterns
245 mediated by RGS10. Specifically, we observe, in contrast to previous studies, that RGS10
246 deficiency did not impact transaction of key NFκB-dependent cytokine IL-1b and even resulted

in the reduction of TNF transcript in a sex-dependent manner. Additionally, we observe, again in contrast to previous studies, no change in TNF, IL-1β, or IL-10 cytokine secretion in RGS10 deficient pMacs compared to their B6 counterparts upon LPS stimulation. Interestingly, IL-6, KCGRO, and IL-12 cytokine secretion was exasperated by RGS10 deficiency instead, capturing a differential inflammatory profile that is regulated by RGS10.Importantly, this differential mediation of inflammation may be due to the use of buprenorphine in our study that previous studies were not stipulated to use(16). Buprenorphine is a partial mu opioid receptor agonist, and the mu opioid receptor, which is expressed in myeloid cells among others, is an inhibitory GPCR(17). RGS10 was canonically identified as GTPase-activating protein with high-affinity for inhibitory GPCRs(18). Therefore, we speculate that the regulation of pro-inflammatory responses by RGS10 may be significantly influenced by the use of opioids. Follow-up studies are necessary to clarify the influence of buprenorphine and other opioids on RGS10-mediated myeloid cell activation. Consequently, our study reveals that RGS10 differentially and transiently regulates the transcription of NFκB pathway components and that RGS10 may mediate alternative inflammatory responses under the influence of opioid analgesics.

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Author Contributions: JEJP: Conceptualized and designed the study, completed mouse injections, in vitro work, and biochemical analysis, wrote and edited the original manuscript.

KD: Assisted with in vitro work and biochemical analysis. CLC and NKN: Maintained mouse colonies and assisted in tissue harvesting. KBM: Participated in study design, data analysis, and manuscript editing. MGT: Contributed to conception and design of the study as well as preparing the final manuscript.

Data Availability: The data generated in this study are available at the following Zenodo Repository DOI: 10.5281/zenodo.18319174

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Abbreviations:

BME: Beta mercaptoethanol

cDNA: complementary DNA

Cox2: Cyclooxygenase 2

DNA: Deoxyribonucleic Acid

DPBS: Dulbecco's Phosphate Buffered Saline

GPCR: G Protein Coupled Receptor

292 IL-12: Interleukin 12

293 IL-12p40: Interleukin 12p40

294 IL-1β: Interleukin 1β

295 KCGRO: Keratinocyte chemoattractant/Growth-regulated oncogene

296 KO: Knock Out

297 LLOD: Lower limit of detection

298 LPS: Lipopolysaccharide

299 MSD: Mesoscale Discovery

300 NFkB: Nuclear Factor kappa-light-chain-enhancer of activated B cells

301 pMac: Peritoneal macrophages

302 QPCR: Quantitative real time polymerase chain reaction

303 RA: Rheumatoid Arthritis

304 RGS10: Regulator of G Protein Signaling 10

305 RNA: Ribonucleic Acid

306 RPMI 1640: Roswell Park Memorial Institute 1640

307 STIM2: Stromal interaction molecule 2

308 TNF: Tumor Necrosis Factor

309 WT: Wild type

310

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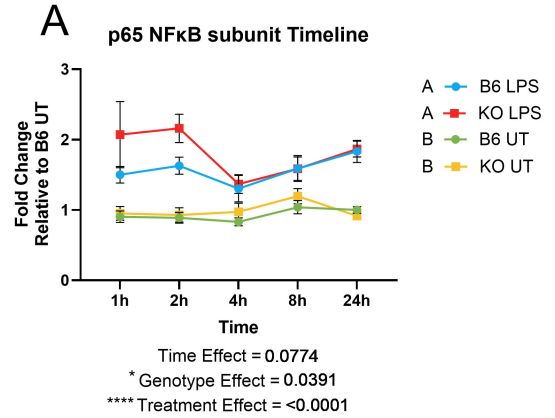
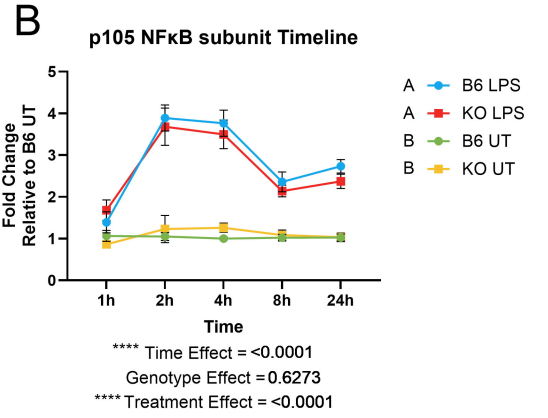
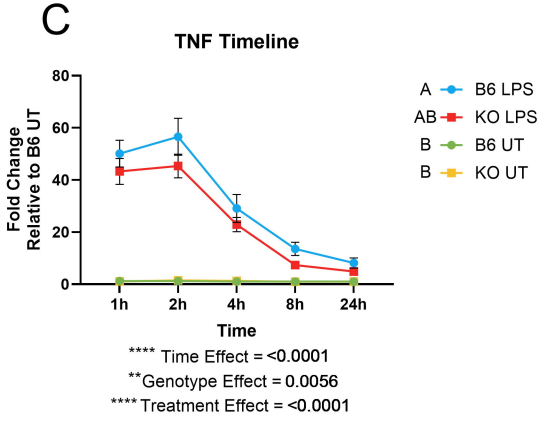
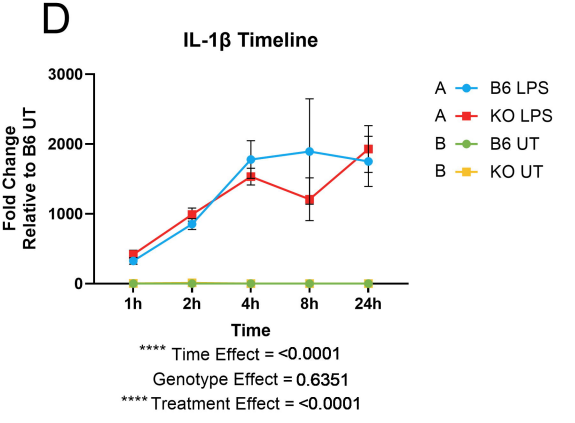
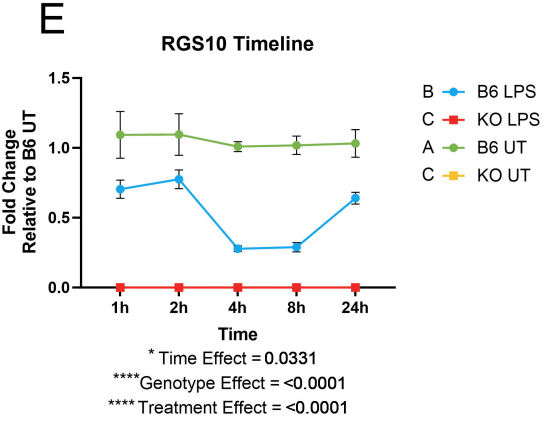
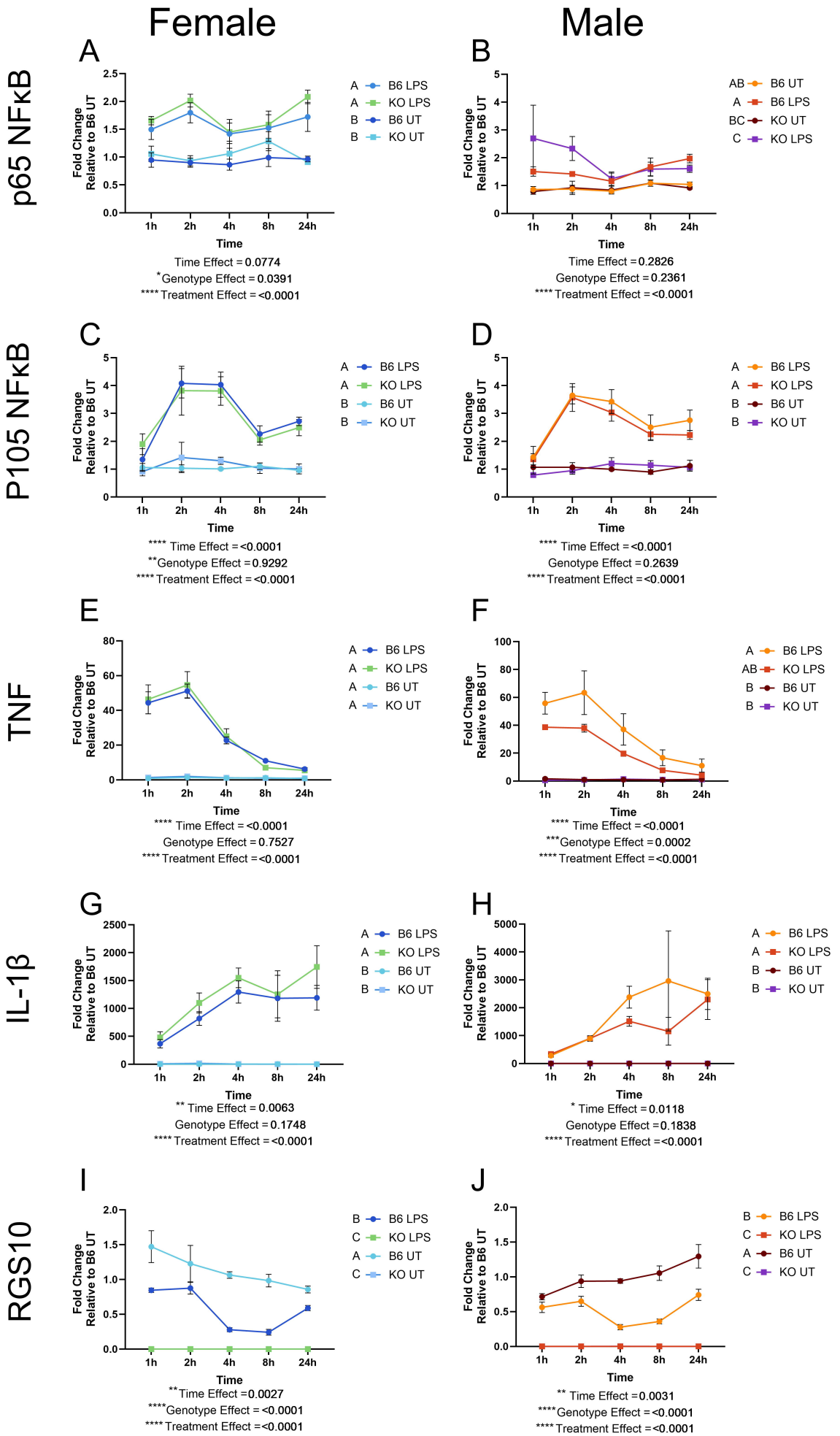

A
p65 NFκB subunit Timeline
Fold Change Relative to B6 UT
3
2
1
0
1h
2h
4h
8h
24h
Time
A B6 LPS
A KO LPS
B B6 UT
B KO UT
Time Effect = 0.0774
* Genotype Effect = 0.0391
**** Treatment Effect = <0.0001

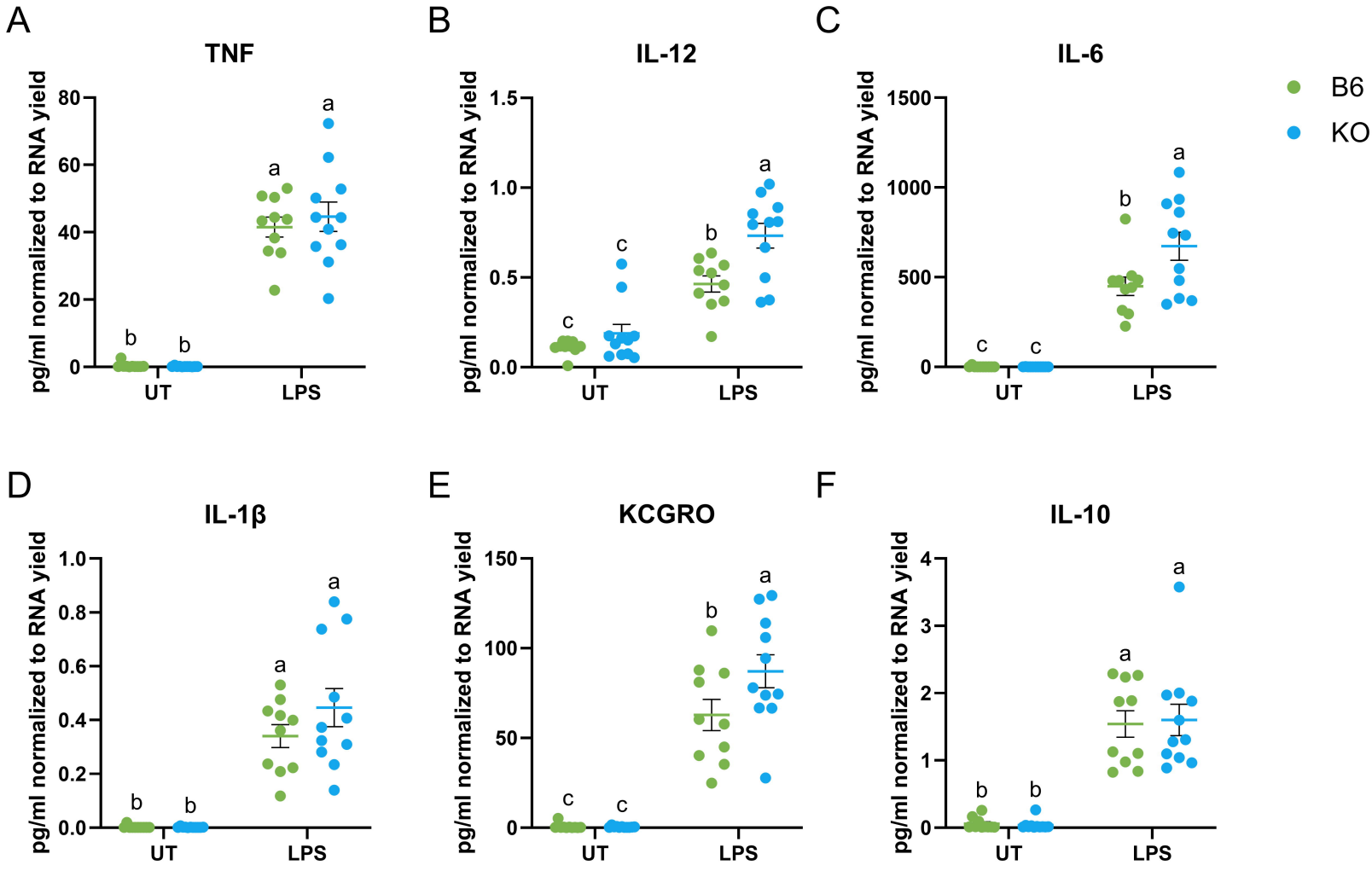

B
p105 NFκB subunit Timeline
Fold Change Relative to B6 UT
5
4
3
2
1
0
1h
2h
4h
8h
24h
Time
A B6 LPS
A KO LPS
B B6 UT
B KO UT
**** Time Effect = <0.0001
Genotype Effect = 0.6273
**** Treatment Effect = <0.0001


C
TNF Timeline
Fold Change Relative to B6 UT
80
60
40
20
0
1h
2h
4h
8h
24h
Time
A B6 LPS
AB KO LPS
B B6 UT
B KO UT
**** Time Effect = <0.0001
** Genotype Effect = 0.0056
**** Treatment Effect = <0.0001


D
IL-1β Timeline
Fold Change Relative to B6 UT
3000
2000
1000
0
1h
2h
4h
8h
24h
Time
A B6 LPS
A KO LPS
B B6 UT
B KO UT
**** Time Effect = <0.0001
Genotype Effect = 0.6351
**** Treatment Effect = <0.0001


E
RGS10 Timeline
Fold Change Relative to B6 UT
1.5
1.0
0.5
0.0
1h
2h
4h
8h
24h
Time
B B6 LPS
C KO LPS
A B6 UT
C KO UT
* Time Effect = 0.0331
**** Genotype Effect = <0.0001
**** Treatment Effect = <0.0001


Female
Male
p65 NFκB
P105 NFκB
TNF
IL-1β
RGS10
A
B
C
D
E
F
G
H
I
J
Fold Change Relative to B6 UT
Time
1h
2h
4h
8h
24h
A B6 LPS
A KO LPS
B B6 UT
B KO UT
AB B6 UT
A B6 LPS
BC KO UT
C KO LPS
B B6 LPS
C KO LPS
A B6 UT
C KO UT
Time Effect = 0.0774
*Genotype Effect = 0.0391
**** Treatment Effect = <0.0001
Time Effect = 0.2826
Genotype Effect = 0.2361
**** Treatment Effect = <0.0001
**** Time Effect = <0.0001
**Genotype Effect = 0.9292
**** Treatment Effect = <0.0001
**** Time Effect = <0.0001
Genotype Effect = 0.2639
**** Treatment Effect = <0.0001
**** Time Effect = <0.0001
Genotype Effect = 0.7527
**** Treatment Effect = <0.0001
**** Time Effect = <0.0001
***Genotype Effect = 0.0002
**** Treatment Effect = <0.0001
** Time Effect = 0.0063
Genotype Effect = 0.1748
**** Treatment Effect = <0.0001
* Time Effect = 0.0118
Genotype Effect = 0.1838
**** Treatment Effect = <0.0001
**Time Effect = 0.0027
****Genotype Effect = <0.0001
**** Treatment Effect = <0.0001
** Time Effect = 0.0031
****Genotype Effect = <0.0001
**** Treatment Effect = <0.0001


A
TNF
pg/ml normalized to RNA yield
80
60
40
20
0
b
b
a
a
UT
LPS
B
IL-12
pg/ml normalized to RNA yield
1.5
1.0
0.5
0.0
c
c
b
a
UT
LPS
C
IL-6
pg/ml normalized to RNA yield
1500
1000
500
0
c
c
b
a
UT
LPS
B6
KO
D
IL-1β
pg/ml normalized to RNA yield
1.0
0.8
0.6
0.4
0.2
0.0
b
b
a
a
UT
LPS
E
KCGRO
pg/ml normalized to RNA yield
150
100
50
0
c
c
b
a
UT
LPS
F
IL-10
pg/ml normalized to RNA yield
4
3
2
1
0
b
b
a
a
UT
LPS