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2 A quantitative modular modeling approach reveals the consequences of
3 different A20 feedback implementations for the NF- κ B signaling dynamics.

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22

23 **Abstract**

24 Signaling pathways involve complex molecular interactions and are controlled by non-
25 linear regulatory mechanisms. If details of regulatory mechanisms are not fully
26 elucidated, they can be implemented by different, equally reasonable mathematical
27 representations in computational models. The study presented here focusses on NF-
28 κ B signaling, which is regulated by negative feedbacks via I κ B α and A20. A20 inhibits
29 NF- κ B activation indirectly through interference with proteins that transduce the signal
30 from the TNF receptor complex to activate the I κ B kinase (IKK) complex. We focus on
31 the question how different implementations of the A20 feedback impact the dynamics
32 of NF- κ B. To this end, we develop a modular modeling approach that allows combining
33 previously published A20 modules with a common pathway core module. The resulting
34 models are based on a comprehensive experimental data set and therefore show
35 quantitatively comparable NF- κ B dynamics. Based on defined measures for the initial
36 and long-term behavior we analyze the effects of a wide range of changes in the A20
37 feedback strength, the I κ B α feedback strength and the TNF α stimulation strength on
38 NF- κ B dynamics. This shows similarities between the models but also model-specific
39 differences. In particular, the A20 feedback strength and the TNF α stimulation strength
40 affect initial and long-term NF- κ B concentrations differently in the analyzed models.
41 We validated our model predictions experimentally by varying TNF α concentrations
42 applied to HeLa cells. These time course data indicate that only one of the A20
43 feedback models appropriately describes the impact of A20 on the NF- κ B dynamics.

44 **Author summary**

45 Models are abstractions of reality and simplify a complex biological process to its
46 essential components and regulations while preserving its particular spatial-temporal
47 characteristics. Modelling of biological processes is based on assumptions, in part to

48 implement the necessary simplifications but also to cope with missing knowledge and
49 experimental information. In consequence, biological processes have been
50 implemented by different, equally reasonable mathematical representations in
51 computational models. We here focus on the NF- κ B signaling pathway and develop a
52 modular modeling approach to investigate how different implementations of a negative
53 feedback regulation impact the dynamical behavior of a computational model. Our
54 analysis shows similarities of the models with different implementations but also
55 reveals implementation-specific differences. The identified differences are used to
56 design and perform informative experiments that elucidate unknown details of the
57 regulatory feedback mechanism.

58 **Introduction**

59 Transcription factor NF- κ B regulates cell differentiation, proliferation and survival. In
60 line with its broad range of normal physiological functions, aberrant activation of NF- κ B
61 can lead to severe diseases, e.g. autoimmune, neurodegenerative and cardiovascular
62 diseases as well as cancer and diabetes (1, 2). In resting cells, the transcription factor
63 NF- κ B is located in the cytoplasm bound to I κ B α , which prevents the translocation of
64 NF- κ B into the nucleus. Upon stimulation, e.g. with TNF α , the I κ B kinase (IKK)
65 complex is activated. The IKK complex phosphorylates I κ B α , marking it for
66 proteasomal degradation. Released NF- κ B translocates into the nucleus and activates
67 the transcription of a number of target genes (3). Two of these are NFKBIA, encoding
68 I κ B α , and TNFAIP3, encoding A20. Both proteins exhibit negative feedbacks on NF- κ B
69 activation. I κ B α binds to NF- κ B retrieving it from the DNA and thus exhibiting a direct
70 negative feedback (4). A20 inhibits NF- κ B activity indirectly through interference with
71 proteins mediating the signal from the TNF receptor complex to the IKK complex (5).

72 The exact molecular mechanism of the inhibitory effect of A20 on the IKK complex is
73 still under discussion (6-8).

74 In the last decades, several mathematical models describing the NF- κ B signaling have
75 been published (9-15), and reviewed (16-19). All models comprise the core processes
76 of the canonical NF- κ B signaling, e.g. the interaction of NF- κ B and I κ B α and the
77 transcription and translation of I κ B α as well as the IKK-induced degradation of I κ B α .
78 The majority of those models include only the negative feedback via I κ B α , which has
79 been well-studied and characterized (14).

80 Until today, only a small number of mathematical models has been developed that
81 include the A20-dependent negative feedback mechanism (10, 13, 20, 21). These
82 models utilize similar implementations of the core signaling processes but differ in their
83 implementation of the A20 feedback. Since the exact inhibitory mechanism of A20 on
84 IKK has not yet been fully elucidated, the models implement different hypotheses.
85 While the model of Lipniacki et al. (2004) (10) and the derived model by Ashall et al.
86 (2009) (21) implement the inhibitory action of A20 on the level of IKK, the models of
87 Werner et al. (2008) (20) and Murakawa et al. (2015) (13) basically implement the
88 hypothesis that A20 blocks the signaling upstream of IKK by binding to TNF receptor
89 associated proteins. In particular, the models by Lipniacki et al. (2004) and Ashall et al.
90 (2009) comprise three different states of IKK: neutral, active and inactive. In the model
91 proposed by Lipniacki et al. (2004), A20 promotes the inactivation of activated IKK,
92 whereas, in the model by Ashall et al. (2009) A20 inhibits the 'recycling' of inactive IKK
93 to neutral IKK and consequently the activation of IKK. In the models by Werner et al.
94 (2008) and Murakawa et al. (2015), A20 inhibits basal and TNF α -induced IKK
95 activation, although Werner et al. (2008) consider the signaling mechanisms upstream
96 of IKK with substantially more molecular detail than Murakawa et al. (2015). In short,

97 all four models share a feedback inhibition of IKK activity by A20 but differ in the
98 specifics of their A20 feedback implementations.

99 Here, we compare the different A20 feedback structures. We selected those
100 implemented in the models of Lipniacki et al. (2004) (10), Ashall et al. (2009) (21), and
101 Murakawa et al. (2015) (13), because these capture three different hypotheses and the
102 models are comparable at their level of detailedness. We did not include the model of
103 Werner et al. (2008) because its A20 feedback mechanism is essentially captured with
104 reduced complexity in the model of Murakawa et al. (2015). We addressed the
105 question whether the different feedback implementations affect NF- κ B dynamics in
106 similar or distinct ways. To this end, we used a computational approach in which we
107 established three ordinary differential equation (ODE) models. Each model is
108 composed of a core module and an upstream module (Fig 1A). The core module is
109 identical in all three models and describes the interaction of NF- κ B and I κ B α ,
110 transcription and translation of I κ B α , and IKK-induced degradation of I κ B α . The three
111 upstream modules comprise the three distinct mechanisms of IKK inhibition by A20
112 that Lipniacki et al. (2004), Ashall et al. (2009) and Murakawa et al. (2015) have
113 proposed. In this way, we used a modular concept to derive three models that share
114 an identical core module but differ in their implementations of the A20 feedback in the
115 upstream module. By fitting these models to the same set of experimental data, we
116 derive models showing quantitatively similar NF- κ B dynamics. We use this approach to
117 directly compare the influences of the structural difference in the upstream modules on
118 the response of the NF- κ B dynamics. In particular, we focused on the impact of the
119 A20 and I κ B α feedback strength. Moreover, we analyze in each model how the A20
120 feedback modulates the effect of varied TNF α stimulations on the NF- κ B dynamics.
121 We find that the different A20 feedback implementations exert similar but also model-

122 specific effects and use the predicted distinct dynamic responses towards incremental
123 alterations of TNF α stimulation strength for an experimental validation of our results.

124

125 **Fig 1: Model schemes comprising the common core module and distinct**
126 **upstream modules.**

127 A: Each model is composed of a core module (red) and an upstream module (blue).

128 The core module is identical in each model but the upstream module differs between
129 model A, B, and C, implementing the A20 feedback mechanisms proposed by (13),

130 (21) and (10), respectively. B: Schematic representations of the three models A-C.

131 Vertical bars separate components in a complex. One-headed arrows indicate the
132 direction of the reaction; double-headed arrows illustrate reversible binding reactions.

133 Dashed arrows represent activation processes; the dashed lines ending in T-shape
134 denote inhibition. The number next to an arrow specifies the number of the reaction.

135 Model equations and the reference parameters are provided in S1 File.

136 **Methods**

137 **Model structures**

138 In order to compare the three distinct implementations of the inhibitory mechanism of

139 A20, we modularly designed three models. These models comprise an identical core

140 module to which different upstream modules are attached (Fig 1A, B). The upstream

141 modules are those proposed by Lipniacki et al. (2004) (10), Ashall et al. (2009) (21)

142 and Murakawa et al. (2015) (13) capturing the different A20 feedback implementations.

143 The overall models are hereafter referred to as model A, B and C

144 The common core module of models A-C (Fig 1B) describes the reversible binding of

145 free NF- κ B and I κ B α (reaction 1). Activated IKK (IKK_{active}) induces the I κ B α

146 degradation releasing NF- κ B from the complex (reaction 5). Unbound NF- κ B induces

147 the transcription of I κ B α mRNA (reaction 11), which is translated to I κ B α (reactions 9).
148 I κ B α mRNA and I κ B α protein degrade via reactions 7 and 4, respectively. In addition to
149 I κ B α mRNA, NF- κ B induces the transcription of A20 mRNA (reaction 10). A20 mRNA
150 is translated to A20 (reaction 8). A20 mRNA and protein are degraded via reactions 6
151 and 3, respectively. Taken together, the core module consists of five ordinary
152 differential equations (ODEs) and one conservation relation for NF- κ B. A detailed
153 description of the corresponding rates and a list of the parameters are provided in S1
154 File.

155 The upstream module of model A (Fig 1B, left) comprises a very condensed
156 representation of the activation of the IKK complex. The abundance of IKK_{active}
157 increases in a TNF α -dependent and independent manner (reactions 13 and 14,
158 respectively), both of which are inhibited by A20. IKK_{active} is inactivated via reaction 15.
159 In the upstream module of model B (Fig 1B, middle), IKK cycles between three distinct
160 states: IKK_{neutral}, IKK_{active}, and IKK_{inactive}. TNF α stimulation converts IKK_{neutral} into
161 IKK_{active} (reaction 16), IKK_{active} is converted to IKK_{inactive} (reaction 17) and IKK_{inactive} is
162 finally turned over to IKK_{neutral} again (reaction 18). A20 inhibits this last reaction in a
163 stimulus-sensitive manner.

164 The upstream module of model C (Fig 1B, right) includes the same states of IKK as
165 described in model B, but IKK_{neutral}, IKK_{active}, and IKK_{inactive} do not interconvert in a
166 cycle, i.e. obey a conservation relation. Instead, IKK_{neutral} is continuously produced
167 (reaction 24) and all three forms of IKK are subject to degradation (reactions 25-27).
168 Similar to model B, TNF α stimulation in model C also converts IKK_{neutral} into IKK_{active}
169 (reaction 21), which in turn forms IKK_{inactive} (reaction 23). In contrast to model B, model
170 C includes an additional mechanism to convert IKK_{active} into IKK_{inactive} (reaction 22).
171 TNF α stimulation as well as A20 enhance this conversion.

172 Taken together, model A consist of one ODE in its upstream module in addition to the
173 five ODEs and one conservation relation of NF- κ B in the core module; model B
174 incorporates two additional ODEs and an additional conservation relation of IKK in the
175 upstream module; and model C includes three additional ODEs in its upstream
176 module. Detailed descriptions of all three models are given in S1 File.

177 **Model parametrizations**

178 To parameterize the ODEs of the core module, we decided to use the parameters from
179 our previously published model (13). This approach was based on two arguments.
180 First, this model is based on a comprehensive data set characterizing the modulation
181 of A20 feedback strength and its impact on NF- κ B dynamics. Secondly, the core
182 processes of this model perfectly match the reactions of the core module of our
183 models A-C.

184 To parameterize the three different upstream modules of models A-C, we initially used
185 the parameters published for the corresponding models (10, 13, 21). However,
186 simulations of models A-C showed very diverse dynamics of unbound NF- κ B in
187 response to identical TNF α stimulation conditions (Fig 2A). For instance, the
188 concentration of free NF- κ B transiently increases in models A and B, but on a slower
189 time scale in model A. In contrast, unbound NF- κ B hardly increases upon TNF α
190 stimulation in model C.

191

192 **Fig 2: NF- κ B dynamics of the three models comprising the core module and the**
193 **indicated upstream module.**

194 A: Differences in NF- κ B dynamics can be observed for the three models using the
195 originally published parameters. B: Nearly identical NF- κ B dynamics can be observed
196 for the three models with newly estimated parameters for the upstream modules.

197 In order to compare models A-C directly, it is necessary that NF- κ B exhibits the same
198 dynamics upon TNF α stimulation in all three models. Thus, we estimated new
199 parameters of the reactions in the upstream modules such that all components of the
200 core module show the same dynamics in all three models. We used the D2D Toolbox
201 (22) to estimate these parameters while keeping the parameters of the core module
202 fixed. With this restriction on the parameters of the core module, we were able to
203 reasonably minimize the parameter search space and obtain identical dynamics of the
204 components of the core module. The details of the parameter estimation are explained
205 in S1 File. Simulations of models A-C with these estimated parameters showed nearly
206 identical dynamics of NF- κ B activation upon TNF α stimulation (Fig 2B) and all
207 remaining components of the core module (Figs 1 and 2 in S1 File).

208 Next, we checked whether the new parameterization changed the inhibitory effect of
209 A20 on the activation of IKK. To do so, we simulated A20 knockout conditions by
210 setting the A20 transcription rate k_{10} to zero and compared the resulting dynamics to
211 those of wild-type conditions, i.e. using the reference value of k_{10} (Table 1 in S1 File).
212 The simulations show that the A20 knockout causes a prolonged increase in NF- κ B,
213 IKK and I κ B α mRNA upon TNF α stimulation compared to wild-type (23) in all three
214 models (Figs 3-5 in S1 File). The simulations furthermore show that the absence of
215 A20 leads to a decrease in I κ B α concentration in all three models. These results
216 demonstrate that the parameterizations of the models A-C do represent the inhibitory
217 effect of A20 on the activation of IKK.

218 Taken together, models A, B and C were derived by modular design from an identical
219 core module and different upstream modules specifying distinct implementations of the
220 A20 feedback and TNF α stimulation. The models exhibit almost identical dynamics of
221 their common model components, and show similar dynamical behavior in A20
222 knockout simulations.

223 **Quantitative characterization of the NF- κ B dynamics**

224 To quantitatively compare the dynamics of unbound NF- κ B between the models A-C,
225 we characterized NF- κ B dynamics by three measures (Fig 3): (1) the maximal NF- κ B
226 concentration (x_{\max}), (2) the time of the maximal NF- κ B concentration (t_{\max}), and (3) the
227 response time (t_r) defined in (24), which quantifies the time required for a complete
228 NF- κ B response after stimulation. While x_{\max} and t_{\max} describe the initial response of
229 NF- κ B to TNF α stimulation, t_r represents a normalized duration of NF- κ B signaling and
230 can therefore be used as a measure for the long-term dynamics.

231

232 **Fig 3: Measures to quantify NF- κ B dynamics.**

233 A: The maximal concentration of NF- κ B (x_{\max}) and the time of the maximal
234 concentration of NF- κ B (t_{\max}) characterize the initial NF- κ B response. B: The response
235 time (t_r) defined in (24) is determined by the grey area (A^*) normalized to the steady
236 state (f) of the absolute gradient of the dynamics of NF- κ B. The response time
237 quantifies the time required for the activation and deactivation of NF- κ B upon
238 stimulation and can be interpreted as a characterization of the NF- κ B long-term
239 behavior.

240 **Numerical simulations**

241 The model equations are listed in S1 File. Calculations were done with MathWorks
242 Matlab R2013b. Steady state solutions were numerically obtained. Starting from those
243 steady state solutions, the models are always simulated for 57600 min.

244 **Experimental methods**

245 HeLa cells were stimulated with 10, 25 or 100 ng/ml TNF α (human recombinant TNF α ,
246 Alexis Corporation) for the time periods indicated (120, 100, 80, 60, 40, 20, 10 min) or
247 were left untreated. Following stimulation, cells were lysed in 20 mM Hepes pH=7.9,

248 450 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA pH=8.0, 0.1 mM EGTA, 1% NP-40, 20%
249 glycerol, supplemented with complete protease inhibitor mixture and Phosphostop
250 (Roche Applied Science), 50 nM Calyculin A, 10 mM NaF, 10 mM β -glycerophosphate,
251 0.3 mM Na₃VO₄ and 1 mM Dithiothreitol. Lysates were centrifuged at 14,000 rpm for
252 10 min.

253 NF- κ B DNA-binding activity was assayed by Electrophoretic Mobility Shift Assay
254 (EMSA) as previously described (25).

255 EMSA quantification was made using the phosphor-imager Typhoon FLA 9500, GE
256 Healthcare. Data were quantified using ImageQuant software. After background
257 subtraction, the NF- κ B band was normalized to a respective constant non-specific
258 band.

259 **Results**

260 **Effects of different A20 feedback strengths on NF- κ B dynamics**

261 As a starting point, we studied the impact of the A20 feedback on the NF- κ B dynamics
262 upon a constant TNF α stimulation. To do so, we varied the A20 feedback strength and
263 studied its effects on the temporal change of the concentration of unbound NF- κ B
264 (hereafter denoted NF- κ B) in all models. The strength of the A20 feedback is varied by
265 multiplying the transcription rate constant of the A20 mRNA (k_{10}) with a factor, i.e.
266 feedback strength. A low value of the feedback strength corresponds to a weak
267 negative feedback, whereas a high feedback strength results in a strong negative
268 feedback. Local sensitivity analyses showed that a variation of the translation rate
269 constants of A20 (k_8) and of the transcription rate constant have a comparable effect
270 on the three measures of the NF- κ B dynamics (Figs 6-8 in S1 File). Thus, our choice

271 to vary the transcription rate constant by a factor, i.e. the feedback strength, rather
272 than the translation rate constant does not affect our conclusions.

273 The NF- κ B dynamics of the models A-C for the A20 feedback strengths 0.1 and 10 are
274 shown in Fig 4A. In case of a high A20 feedback strength of factor 10, models B and C
275 show a fast and transient increase of NF- κ B concentration upon a constant TNF α
276 stimulation (Fig 4A – top). In model A, NF- κ B increases later and to a lesser extent
277 compared to model B and C, yet it decreases to a similar final concentration. In the
278 case of a low A20 feedback strength of factor 0.1 (Fig 4A – bottom), all three models
279 show an almost identical increase in the NF- κ B concentration. However, NF- κ B
280 decreases faster and to a lower final concentration in model C compared to model A
281 and B. Comparing the simulations of the high with the low A20 feedback strength, all
282 three models show a faster decrease in NF- κ B in the case of high compared with low
283 A20 feedback strength.

284

285 **Fig 4: Influence of the A20 feedback strength and the I κ B α feedback strength on**
286 **NF- κ B dynamics.**

287 A: NF- κ B dynamics of the three models for two different A20 feedback strengths. B:
288 NF- κ B dynamics of the three models for four exemplary combinations of A20 and I κ B α
289 feedback strengths. C: The effect of the different combinations of feedback strengths
290 on the maximal concentration of NF- κ B (first row), the time of the maximal
291 concentration (second row) and the response time of NF- κ B (third row) in the case of
292 model A (first column), model B (second column) and model C (third column). The four
293 exemplary combinations of feedback strength shown in panel B (I, II, III, and IV) are
294 indicated. Black areas mark the combinations of feedback strengths where hardly any
295 NF- κ B response is observed, i.e. the difference between maximal concentration of NF-
296 κ B and initial concentration of NF- κ B is less than the threshold value of 0.001 μ M.

297 These results reflect the strong influence of the A20 feedback on the deactivation of
298 NF- κ B. A high A20 feedback strength causes a stronger and faster deactivation in all
299 three models. Moreover, in model A a strong A20 feedback strength notably reduces
300 and also delays NF- κ B activation.

301 **The I κ B α feedback modulates the effect of the A20 feedback on NF- κ B**

302 Besides A20, I κ B α is an important negative regulator of NF- κ B dynamics. We next
303 analyzed whether the interplay of these two feedbacks in the regulation of NF- κ B
304 dynamics is similar in the three models. To address this question, we varied the I κ B α
305 feedback strength in addition to that of A20. Similar to the A20 feedback strength, we
306 multiplied the transcription rate constant of the I κ B α mRNA (k_{11}) by a factor to change
307 the I κ B α feedback strength.

308 The NF- κ B dynamics of the three models for four exemplary combinations of different
309 A20 and I κ B α feedback strengths are shown in Fig 4B (cases I – IV). The simulations
310 show a rapid increase of NF- κ B concentration upon TNF α stimulation for all models
311 and in all four cases (I-IV), with one exception (model A, case I). The subsequent
312 decrease of NF- κ B concentration differs in strength and pace. For a combination of a
313 high A20 feedback strength and a low I κ B α feedback strength (case I), NF- κ B
314 concentrations in models B and C decrease to the half-maximum level at around 250
315 min whereas model A shows no NF- κ B response to TNF α stimulation. When A20 and
316 I κ B α feedback strength are both low (case II), NF- κ B concentration decreases at a
317 much slower pace and to lesser extent than in case I for models B and C; here (case
318 II) model A also shows a transient NF- κ B activation. If the feedback strengths of A20
319 and I κ B α are high (case III), a fast increase can be observed that is followed by a
320 nearly complete decrease of NF- κ B concentration at 100 min for all models. For
321 combinations of a high I κ B α feedback strength with a low A20 feedback strength (case

322 IV), the decrease in NF- κ B concentration is slightly prolonged compared to case III,
323 depending also on the model. These results are in agreement with our earlier finding
324 that higher A20 feedback strengths cause a faster and stronger decrease in NF- κ B
325 than lower A20 feedback strengths (Fig 4A).

326 In the comparison of case I and case III, which both comprise the same A20 feedback
327 strength but differ in their I κ B α feedback strength, a stronger as well as faster
328 decrease in the NF- κ B concentration can be observed for high I κ B α feedback
329 strengths. The comparison of case II and case IV yields a similar result, showing that a
330 higher I κ B α feedback strength leads to a faster and stronger decrease in NF- κ B
331 concentrations and therefore influencing its short-term and long-term dynamics.

332 In summary, both feedbacks lead to the deactivation of NF- κ B after a transient
333 increase. Thus, if only one of the two feedbacks is strong, it can compensate for the
334 other. If A20 and I κ B α feedback strengths are both strong, the effect on the
335 deactivation of NF- κ B is enhanced resulting in an even faster and stronger NF- κ B
336 deactivation.

337 Beside these general observations, we find model-specific effects of the feedbacks.
338 Most obviously, the maximal NF- κ B activation and the deactivation pace seem to vary
339 between the models. An interesting combination is a strong A20 with a low I κ B α
340 feedback strength (case I) for model A, which prevents an NF- κ B response to TNF α
341 stimulation.

342 **Quantification of the influences of the A20 and the I κ B α feedback on NF- κ B** 343 **dynamics**

344 To determine to what extent the models A-C differ in their NF- κ B response under the
345 various feedback strengths, we quantified the dynamics of NF- κ B by three measures:
346 the maximal concentration of NF- κ B, the time of the maximal concentration, and the

347 response time (Fig 3). The first two measures characterize the initial NF- κ B dynamics
348 whereas the last measure characterizes the long-term NF- κ B dynamics. For each
349 model we then continuously varied the A20 and the I κ B α feedback strengths over a
350 broad range of four orders of magnitude, covering very low (e.g. 0.01) as well as very
351 high (e.g. 100) feedback strengths (Fig 4C).

352 In model A, the maximal NF- κ B concentration barely changes at A20 feedback
353 strengths below 1 (Fig 4C – first column, first row). In those cases, only an increase in
354 the I κ B α feedback strength leads to a decrease in the maximal concentration of NF- κ B.
355 For strong A20 feedback strengths above 1, the A20 feedback can prevent the NF- κ B
356 response almost completely for a wide range of different I κ B α feedback strengths (Fig
357 4C – first row, black area). This is in agreement with case I in Fig 4B showing no NF-
358 κ B response for high A20 and low I κ B α feedback strengths. For A20 feedback
359 strengths below 1 in combination with a wide range of different I κ B α feedback
360 strengths, the maximal concentration of NF- κ B is reached in the first 80 min (Fig 4C –
361 first column, second row – blue area). For A20 feedback strengths above 1, an
362 increase in the A20 feedback strengths can lead to a delay in the time of the maximal
363 concentration of NF- κ B. Very high A20 feedback strengths completely diminish the NF-
364 κ B response. The effect of the A20 feedback on the response time of NF- κ B is also
365 modulated by the I κ B α feedback (Fig 4C – first column, third row). The increase in the
366 response time of NF- κ B for confined combinations of low A20 and I κ B α feedback
367 strengths is due to a prolonged higher concentration of NF- κ B at later time points. The
368 response time of NF- κ B remains low for a wide range of different A20 feedback
369 strengths for I κ B α feedback strengths above 1. To summarize, the effects of the two
370 feedbacks, A20 and I κ B α , in model A can be subdivided into three main areas. The
371 first area comprises combinations of A20 and I κ B α feedback strengths below 1. Those
372 combinations result in a rapid but prolonged first peak of NF- κ B and a higher NF- κ B

373 concentration at later time points similar to case II in Fig 4B. The second area is
374 determined by high A20 feedback strengths, where the NF- κ B response is completely
375 inhibited for low I κ B α feedback strengths similar to case I in Fig 4B. However, if the
376 I κ B α feedback strength is high, NF- κ B remains responsive. The third area comprises
377 high I κ B α feedback strengths resulting in a slightly decreased first peak of NF- κ B and
378 no response at later time points similar to case III and IV in Fig 4B.

379 In model B, the A20 feedback strength hardly influences the height and time of the
380 maximal concentration of NF- κ B. Both measures are mainly determined by the I κ B α
381 feedback strength (Fig 4C – second column, first and second row). However, the A20
382 feedback strength influences the response time of NF- κ B (Fig 4C – second column,
383 third row). Especially, if the A20 and I κ B α feedback strengths are both low, the NF- κ B
384 response time is higher. Thus, in model B the initial NF- κ B response is mainly
385 determined by the I κ B α feedback, whereas the combination of both feedbacks
386 influences the NF- κ B dynamics at later time points.

387 In model C, an increase in the A20 feedback strength reduces the maximal
388 concentration of NF- κ B for A20 feedback strengths above 1 (Fig 4C – third column,
389 first row). For feedback strengths below 1, the A20 feedback barely influences the
390 maximal concentration of NF- κ B. In those cases, an increase in the I κ B α feedback
391 strength can gradually decrease the maximal concentration of NF- κ B. The time of the
392 maximal concentration of NF- κ B appears to be mainly robust towards changes in the
393 two feedback strengths (Fig 4C – third column, second row). Only combinations of A20
394 feedback strengths above 1 and I κ B α feedback strengths below 0.1 delay the time of
395 the maximal concentration of NF- κ B. Considering the response time of NF- κ B, the
396 influence of the A20 feedback can be strongly modulated by the I κ B α feedback (Fig 4C
397 – third column, third row). The NF- κ B response time remains low for I κ B α feedback
398 strengths above 1 independent of the A20 feedback strength. For an I κ B α feedback

399 strength below 1, the A20 feedback strength can increase the NF- κ B response time for
400 A20 feedback strengths either above 10 or for feedback strengths between 1 and 0.1.
401 To summarize, the effects of the two feedbacks in model C can be subdivided into
402 three areas. The first area comprises combinations of A20 and I κ B α feedback
403 strengths below 1. Those combinations result in a rapid, but prolonged first peak of
404 NF- κ B and a higher NF- κ B concentration at later time points similar to case II in Fig
405 4B. The second area is confined by A20 feedback strengths above 10 and I κ B α
406 feedback strengths below 0.1 resulting in a reduced as well as a delayed maximal NF-
407 κ B concentration similar to case I in Fig 4B. The third area comprises I κ B α feedback
408 strengths above 1 leading to a fast but decreased first peak of maximal NF- κ B and no
409 response at later time points similar to case III and IV in Fig 4B.

410 Altogether, the models show similar, but also different influences of the feedbacks on
411 the NF- κ B dynamics. For model A and C, the two negative feedbacks, I κ B α and A20,
412 have an impact on the initial dynamics. Both can independently reduce the maximal
413 NF- κ B concentration. However, in both models the two feedbacks are not completely
414 redundant but have distinct functions in modulating the NF- κ B response. If both
415 feedback strengths are below 1, the inhibitory effect of A20 and I κ B α is weak. In that
416 case, the initial NF- κ B response is slightly delayed and a prolonged activation of NF-
417 κ B can be observed at later time points. If A20 feedback strengths are high, the NF- κ B
418 response is completely inhibited in model A. In model C, a reduced as well as delayed
419 NF- κ B response can be observed. If the I κ B α feedback strength is high, both models
420 show a reduced but fast initial NF- κ B increase and no response at later time points. To
421 summarize, in models A and C both feedbacks inhibit the maximal concentration of
422 NF- κ B, but the A20 feedback delays the initial response and prolongs the response at
423 later time points, whereas the I κ B α feedback results in a faster initial activation and
424 rapid deactivation of NF- κ B. In contrast, in model B the initial NF- κ B response is hardly

425 influenced by the A20 feedback but mainly regulated by the I κ B α feedback. Also in
426 model B both feedbacks have an effect on the later phase of the NF- κ B dynamics.

427 **Characterization of the interplay of TNF α stimulation and A20 feedback** 428 **strengths**

429 In all three considered mechanisms, the A20 feedback modulates the signal
430 transduction of the TNF α stimulus towards the activation of IKK. We are therefore
431 interested in the influence of the A20 feedback strength on the NF- κ B response upon
432 different strengths of TNF α stimulation. To address this question, we simultaneously
433 varied the stimulation strength of TNF α and the strength of the A20 feedback and
434 quantified their influence on the maximal concentration of NF- κ B, time of the maximal
435 concentration and the response time of NF- κ B (Fig 5). Here, the I κ B α feedback
436 strength is fixed to the value of 1.

437

438 **Fig 5: Influence of A20 feedback strength and TNF α stimulation strength on NF-** 439 **κ B dynamics.**

440 NF- κ B dynamics of model A (first column), model B (second column) and model C
441 (third column) are characterized by the maximal concentration of NF- κ B (first row), the
442 time of the maximal concentration of NF- κ B (second row) and the response time of NF-
443 κ B (third row). Black areas mark combinations of A20 feedback strength and TNF α
444 stimulation strength with hardly any observable NF- κ B response; the difference
445 between maximal and initial NF- κ B concentrations is less than 0.001 μ M.

446 In model A, variations in TNF α stimulation change the initial and long term dynamics of
447 NF- κ B (Fig 5 – first column). In particular, an increase in TNF α stimulation strength
448 leads to a faster and stronger increase in the maximal NF- κ B value (Fig 5 – first
449 column, first and second row). This effect can be strongly modulated by the A20

450 feedback: for feedback strengths above 1 a reduction and delay of the maximal NF- κ B
451 concentration can be observed. High A20 feedback strengths above 10 result in a
452 complete prevention of the NF- κ B response for various TNF α stimulation strengths
453 (Fig 5 – first column, black area). The response time of NF- κ B is influenced by TNF α
454 stimulation and A20 feedback strengths in a complex way (Fig 5 – first column, third
455 row). For instance, for the combination of A20 feedback strengths below 1 and TNF α
456 stimulation strengths above 1 the response time of NF- κ B increases, indicating a
457 prolonged NF- κ B activation. In contrast, the combination of A20 feedback strengths
458 around 0.01 and TNF α stimulation strengths above 10 leads to a decrease in the
459 response time of NF- κ B. The underlying reason is the change in the deactivation of
460 NF- κ B. For A20 feedback strengths of 0.01 and TNF α stimulation strengths of 100,
461 NF- κ B is not deactivated. Thus, NF- κ B concentration does not decrease after its initial
462 increase, resulting in a low response time (Fig 9 in S1 File). However, for A20
463 feedback strength of 0.1 and TNF α stimulation strengths of 100, NF- κ B concentration
464 slowly decreases after its initial increase, resulting in a high response time (Fig 9 in S1
465 File).

466 In model B, the amount and time of the maximal concentration of NF- κ B depend on the
467 TNF α stimulation strength, but are mostly robust toward changes in A20 feedback
468 strength (Fig 5 – second column, first and second row). However, both TNF α
469 stimulation strength and A20 feedback strength affect the response time of NF- κ B (Fig
470 5 – second column, third row). The effect is non-linear: low TNF α stimulation strengths
471 between 0.1 and 1 and very low A20 feedback strengths below 0.1 show an increase
472 in the response time of NF- κ B, indicating a prolonged activation of NF- κ B. However, in
473 the case of TNF α stimulation strengths between 10 and 100, a decrease in the
474 response time is observed.

475 In model C, the maximal concentration of NF- κ B and the timing of its peak mostly
476 depend on TNF α stimulation strengths (Fig 5 – third column, first and second row).
477 A20 feedback strength can lead to a reduction and a slight delay of the maximal NF- κ B
478 concentration for high TNF α stimulation strengths. In particular, if A20 feedback
479 strength as well as TNF α stimulation strength are high, the maximal concentration of
480 NF- κ B decreases and can result in a complete prevention of the NF- κ B response (Fig
481 5 – third column, black area). The response time of NF- κ B mainly depends on TNF α
482 stimulation strength and hardly on A20 feedback strength (Fig 5 – third column, third
483 row).

484 In conclusion, the initial dynamics, that is the maximal NF- κ B concentration and its
485 timing, are strongly determined by the TNF α stimulation strength in all models. In
486 models A and C the A20 feedback can strongly modify that impact. However, in model
487 B, we see no significant effect of the A20 feedback on the amount and time of maximal
488 NF- κ B. The effect of the TNF α stimulation strength and the A20 feedback on the long-
489 term dynamics is more complex. However, if we consider the effect of TNF α
490 stimulation (for factors >1) and a given A20 feedback strength (factor = 1), we observe
491 opposite effects in the models: while a higher TNF α stimulation strength leads to an
492 increase of the response time in model A, that is the long term dynamics, such a
493 stimulus increase would cause a decrease in the response time in models B and C.

494 **Comparison of simulations with experimental data for the effect of varied TNF α** 495 **stimulation strength**

496 The qualitative differences between the models suggest an experimental setup to
497 scrutinize the A20 feedback implementations. To predict the outcome of such an
498 experiment, we simulated the NF- κ B dynamics of the models A-C in response to three
499 different TNF α concentrations (Fig 6A). We selected TNF α stimulation because

500 changes in TNF α concentration are easier to perform experimentally than changes in
501 A20 feedback strength. Our simulations predict for model A that NF- κ B levels remain
502 high for stimulation with 100 ng/ml TNF α compared with 10 ng/ml TNF α at later time
503 points (Fig 6A). In contrast, in models B and C, NF- κ B levels decrease faster at later
504 time points upon stimulation with 100 ng/ml TNF α compared to 10 ng/ml TNF α . These
505 predictions are independent of the assumed A20 feedback strengths (Fig 10 in S1 File)
506 and are furthermore verified by simulations of the models published by Murakawa et
507 al. (2015) (13), Ashall et al. (2009)(21) and Lipniacki et al. (2004) (10) (Fig 11 in S1
508 File).

509

510 **Fig 6: Dynamics of NF- κ B upon stimulation with different TNF α concentrations.**

511 A: Simulation of NF- κ B assuming a stimulation with 10 ng/ml (solid line), 25 ng/ml
512 (dotted line) and 100 ng/ml TNF α (dashed line) in model A (left), model B (middle) and
513 model C (right). B: Exemplary EMSA experiment measuring NF- κ B DNA-binding
514 activity over a time course of 120 min in HeLa cells upon stimulation with 10 ng/ml, 25
515 ng/ml and 100 ng/ml TNF α . The histogram shows the quantification of the EMSA
516 experiment. The mean value of the relative intensities at t=0 is set to 1 and used as a
517 normalisation for all other values. Two replicate experiments are shown as
518 supplemental Fig 12 in S1 File.

519 We validated our model predictions by applying 10 ng/ml, 25 ng/ml and 100 ng/ml
520 TNF α to HeLa cells. The time course measurements of NF- κ B's DNA-binding activity
521 by EMSA showed NF- κ B dynamics as predicted for model A but not model B or C (Fig
522 6B). The experiments thus indicate that the implementation of the A20 feedback
523 structure of model A is appropriate to describe the effect of A20 on the dynamics of
524 NF- κ B in HeLa cells.

525 **Discussion**

526 In this study, we developed a modular modeling approach to analyze the impact of
527 different A20 inhibition mechanisms on the dynamics of NF- κ B. In particular, we
528 compared three distinct implementations of the A20 feedback by combining upstream
529 modules of available models with a common core pathway module. By fitting the
530 resulting models to a comprehensive experimental data set, we derive models with
531 quantitatively comparable NF- κ B dynamics. When analyzing the effect of variations of
532 the strength of the A20 and I κ B α feedbacks, as well as of TNF α stimulation in these
533 models, we observe similarities, but also model-specific differences. Increasing I κ B α
534 feedback strengths attenuate the initial as well as the long-term NF- κ B response in all
535 three models, that is, reduce the maximum and response time, respectively. Increasing
536 A20 feedback strengths reduce the maximum and duration of the NF- κ B response in
537 models A and C. In model A, the NF- κ B response is even completely diminished for
538 very high A20 feedback strengths. However, in model B the A20 feedback has no
539 impact on the initial dynamics. Moreover, our simulations predicted that changes in the
540 TNF α stimulation strength influence initial and long-term dynamics of NF- κ B. Here, we
541 observed qualitative differences in the long-term NF- κ B response between the different
542 models. We used these predictions for an experimental validation in HeLa cells. The
543 experimental observations strongly support model A, but not model B or C.

544 Models A-C differ in the implementation of the A20 feedback. In all three models, A20
545 acts conjointly with the stimulus in order to inhibit IKK activation. Model A includes in
546 addition a basal IKK activation rate that is inhibited by A20 (reaction 14). Such a
547 composite, non-linear description of the inhibitory influence of A20 seems necessary to
548 reproduce the NF- κ B dynamics of HeLa cells. This indicates that the regulation of IKK
549 activity by A20 in this cell type may result from a combination of several mechanisms
550 and is thus more complex than anticipated. Indeed, A20 seems to fulfil multiple

551 functions *in vivo*, such as a deubiquitinating activity mediated by its N-terminal ovarian
552 tumor (OTU) domain and an E3 ubiquitin ligase activity mediated by its C-terminal zinc
553 finger domain (5). These distinct functions of A20 may regulate the activity of upstream
554 signal mediators and constitute potential mechanisms that may explain the complex
555 non-linearity in the signal transduction from TNF α stimulation to IKK activation (26). A
556 recent analysis of temperature effects on the NF- κ B pathway also highlights the
557 importance of the A20 feedback and the necessity to extend and modify its
558 implementation in model B (21, 27). Moreover, it will be interesting to explore the role
559 of additional negative regulators on the pathway, e.g. the deubiquitinating enzymes
560 CYLD and OTULIN (5) as well as the effect of the cross-talk with the non-canonical
561 pathway (21, 28, 29).

562 Our analyses of the three models revealed redundant but also distinct functions of the
563 two negative feedbacks, A20 and I κ B α . This confirms and extends earlier findings by
564 Werner et al, 2008 (20), demonstrating distinct roles of the two feedbacks in a very
565 detailed pathway model. In that publication, I κ B α has been reported to modulate
566 mostly the initial NF- κ B response while A20 mainly shapes the late response. In our
567 current study, we characterize the output based on quantitative measures for a wide
568 range of different feedback strengths. We find that the I κ B α feedback fine-tunes the
569 initial NF- κ B response in all models. However, it can also influence the response-time
570 and therefore the long-term dynamics. The A20 feedback has different effects in
571 models A, B and C. In models A and C, it modulates the initial as well as long-term
572 dynamics. Moreover, in model A it has a bimodal on-off effect on the NF- κ B response,
573 i.e. preventing the NF- κ B response at high A20 feedback strengths. The non-
574 redundant functions of the two negative feedbacks could be due to their structural
575 properties: the two feedbacks are interlocked, with the I κ B α feedback serving as an
576 inner feedback loop and the A20 feedback as an outer feedback loop. Previous studies

577 indicted distinct functions of interlocked feedback loops with respect to the oscillatory
578 behavior of a system (30, 31). Here, a weak or strong outer feedback loop may cause
579 an on or off response, respectively, independent of the strength of the inner feedback
580 loop. However, the inner feedback loop can fine-tune the response in the case of a
581 weak outer feedback loop. Such interlocked feedback loops are very common
582 regulatory motifs in signaling pathways in general (32-35).

583 Taken together, our quantitative modular modeling approach employs the regulation of
584 NF- κ B signaling by the A20 feedback as an example case to study the impact of
585 different implementations of an inhibition mechanism on the model's response to
586 perturbations. Comparing the simulations of the three models A-C to experimental data
587 suggests that model A is an appropriate choice to describe TNF α stimulation in HeLa
588 cells. Our results emphasize the need to further explore the molecular details of
589 processes upstream of IKK regulation.

590 **Acknowledgments**

591 None.

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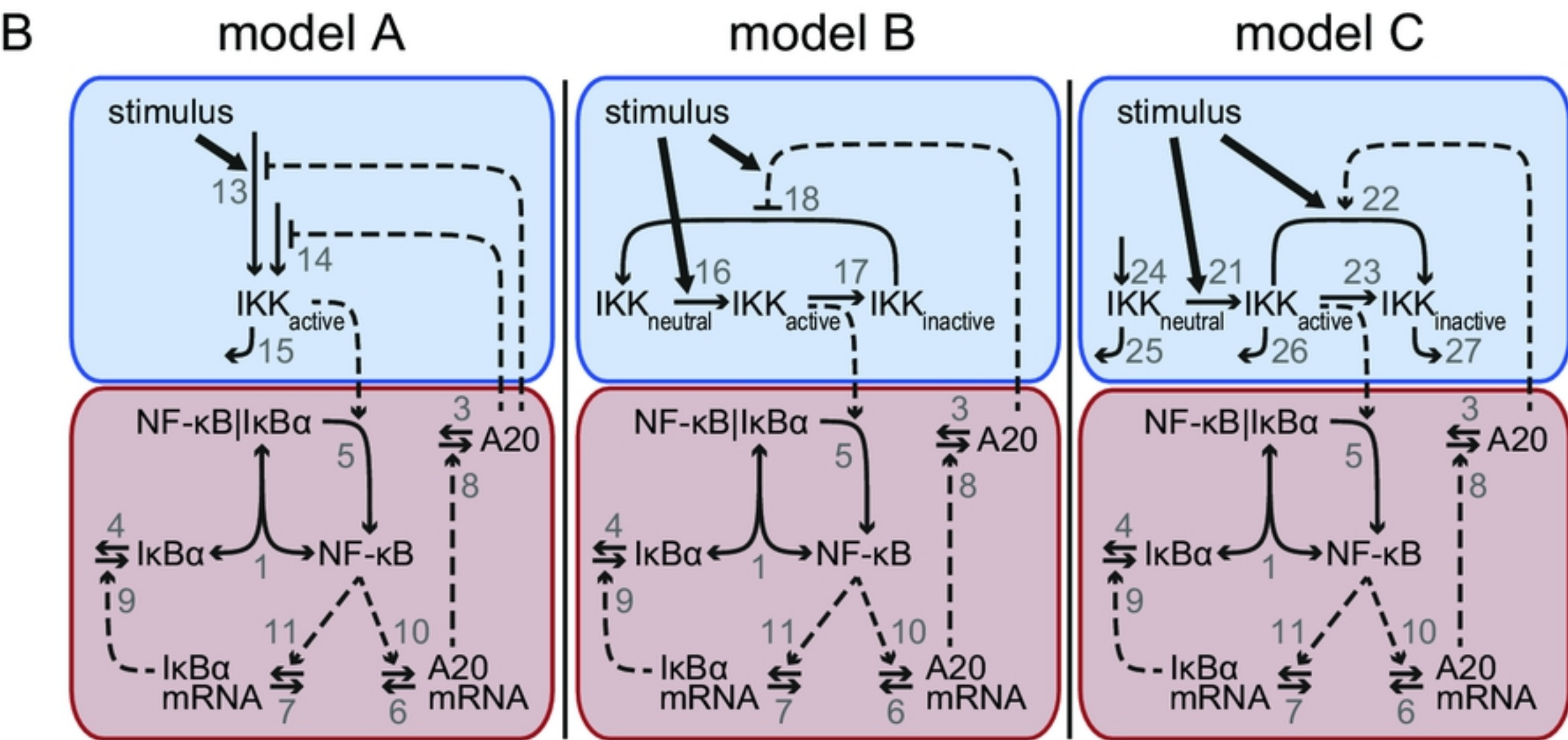
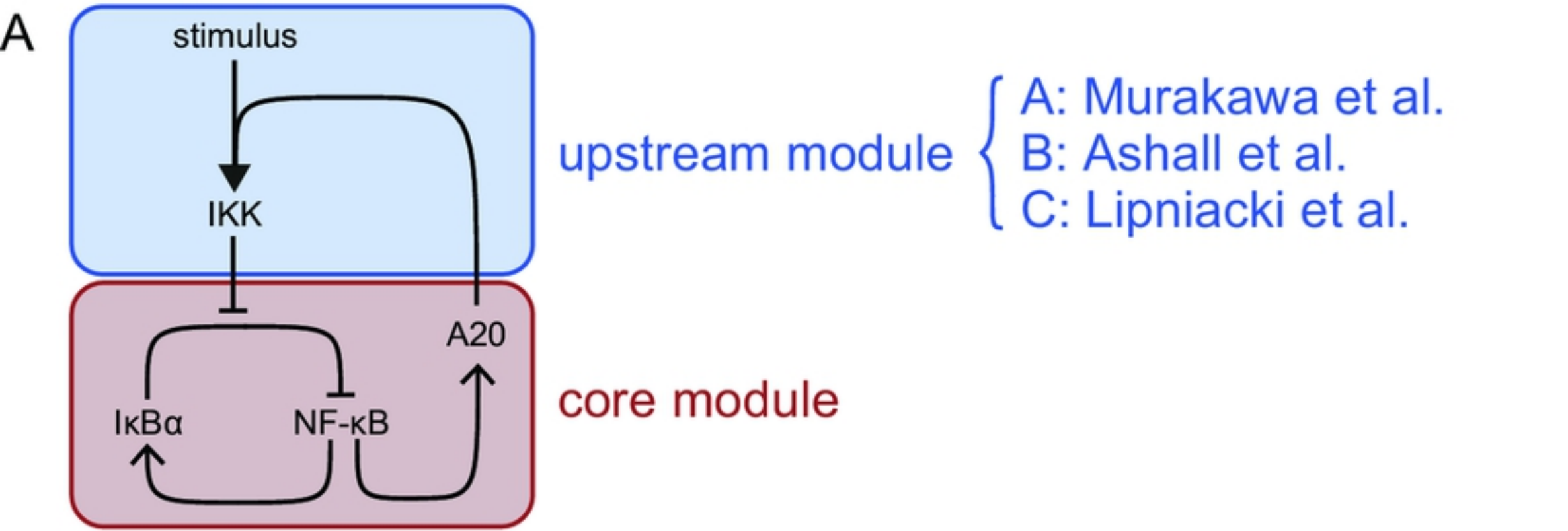
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700 **Supporting information**

701 **S1 File. Supplemental material.**

702 Supplemental figures and tables, detailed description of the mathematical models,
703 details on parameter estimation, sensitivity analysis, and model predictions.

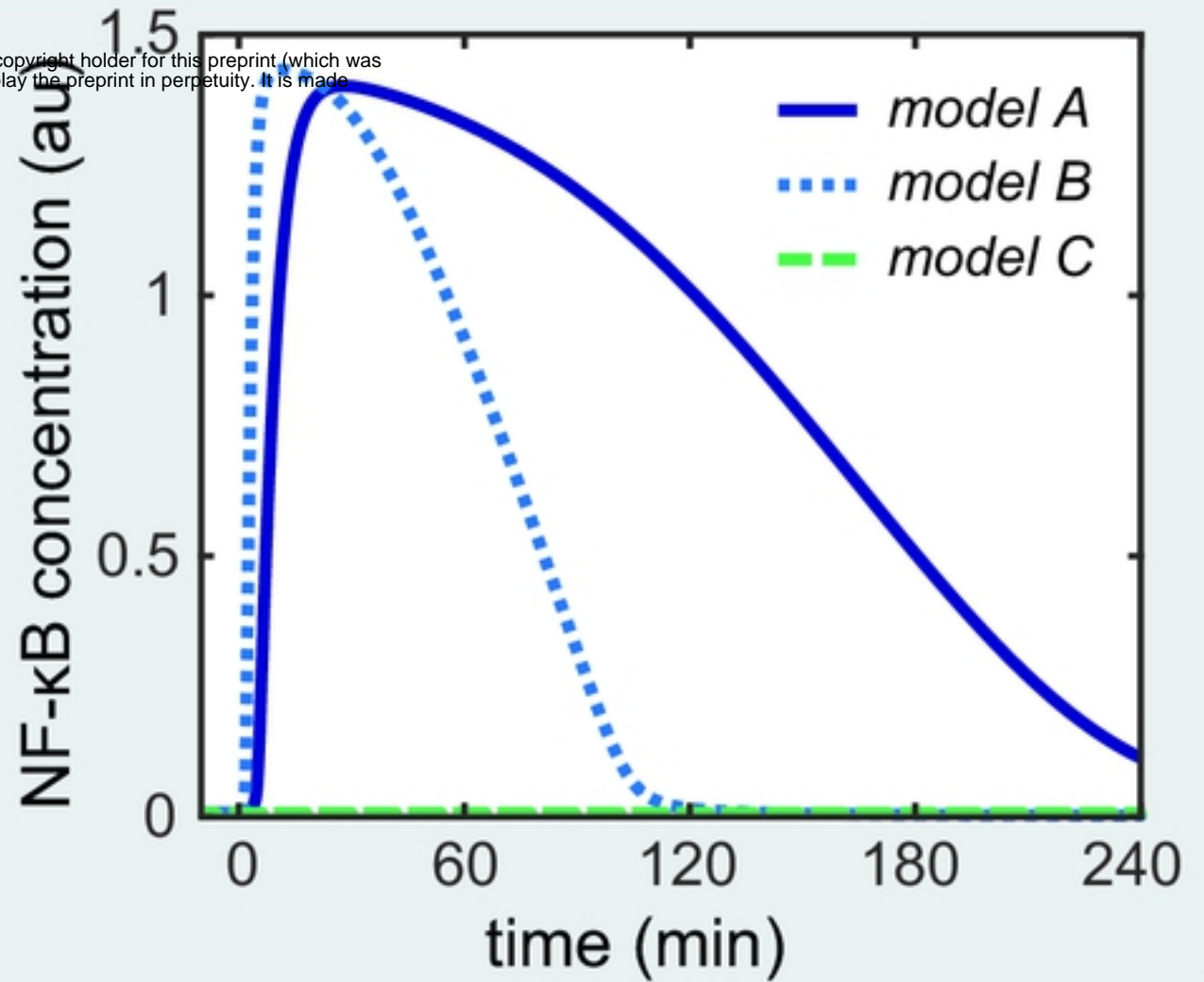


Figure

A

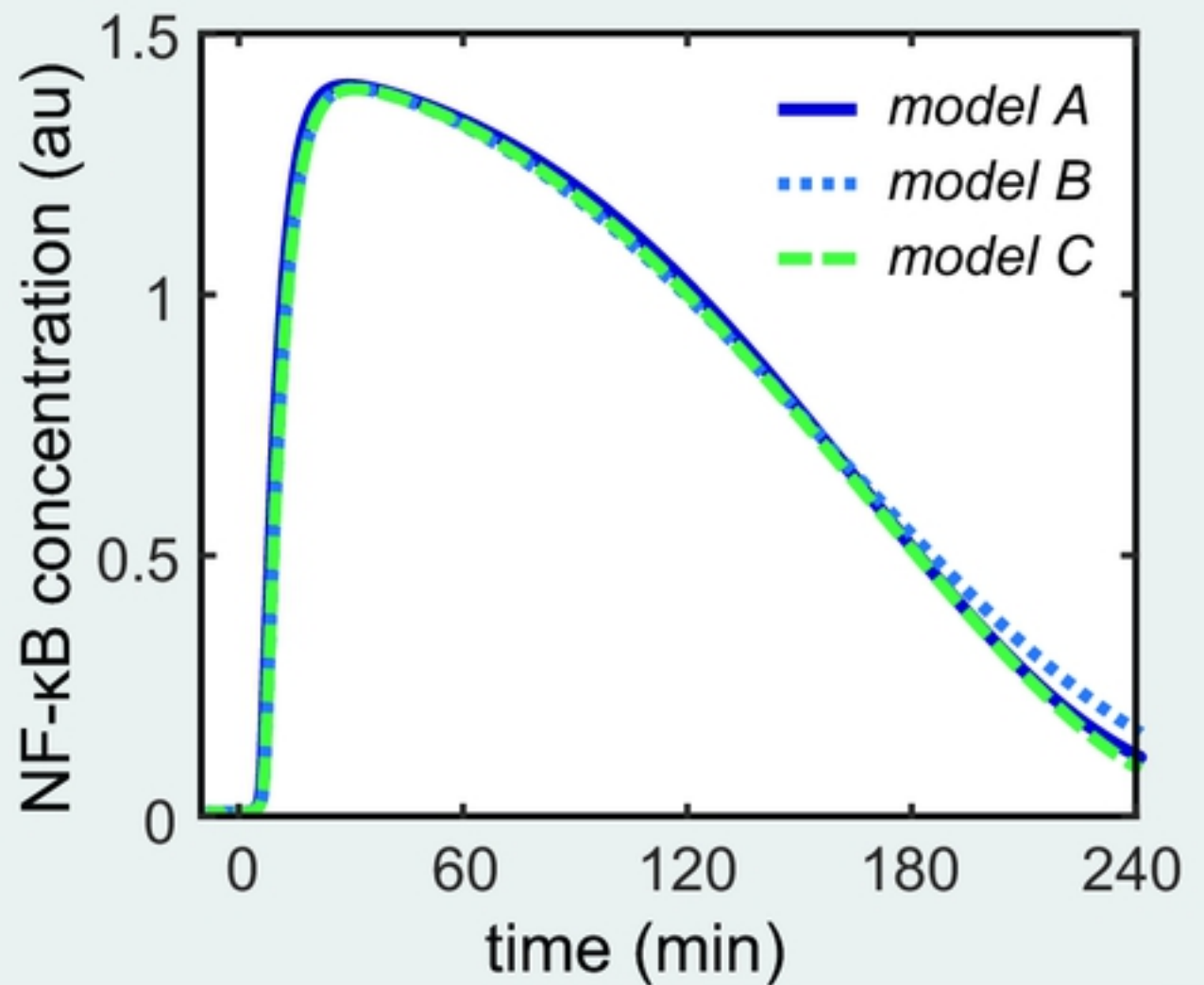
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upstream modules with
originally published
parameters

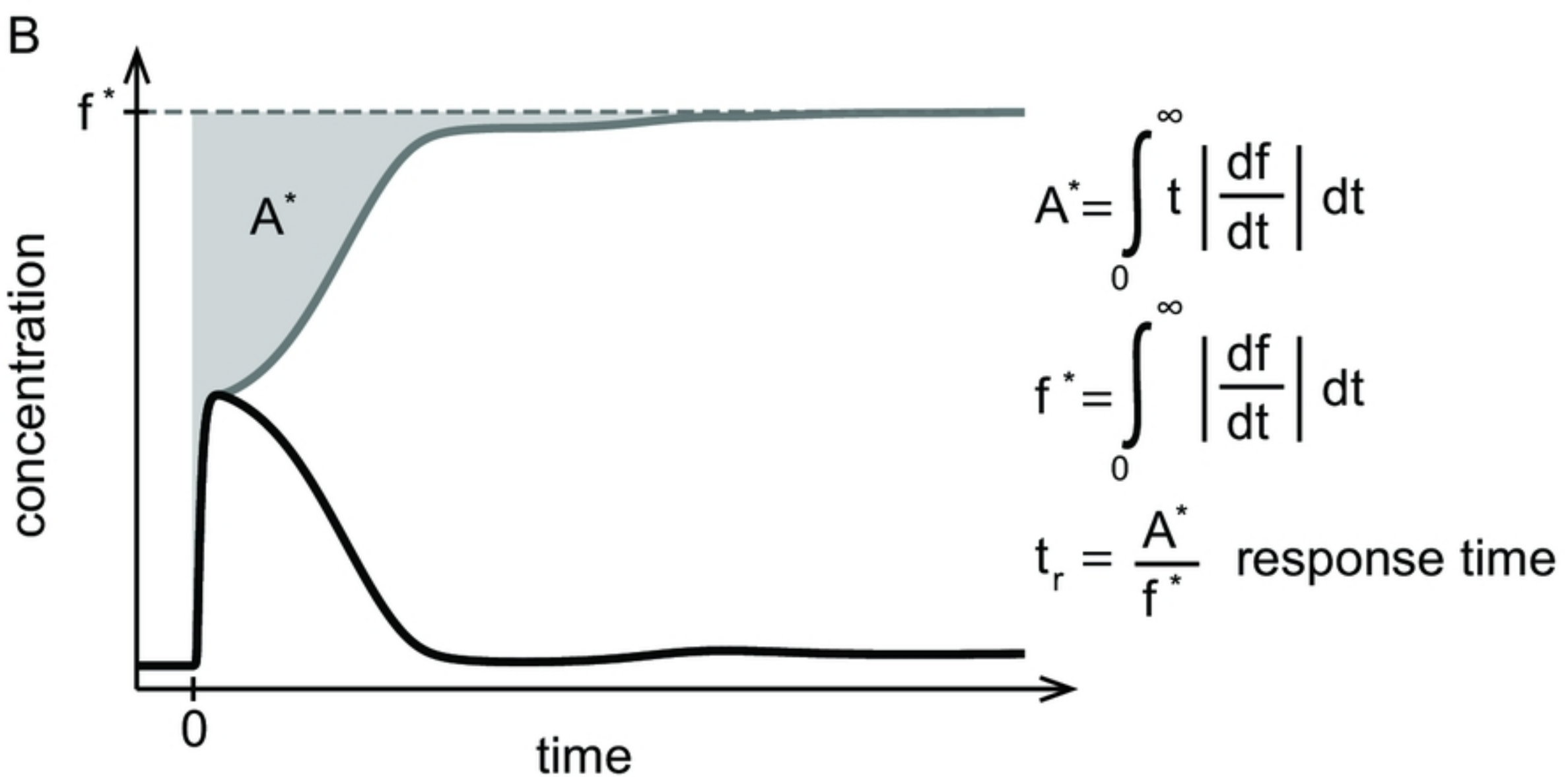
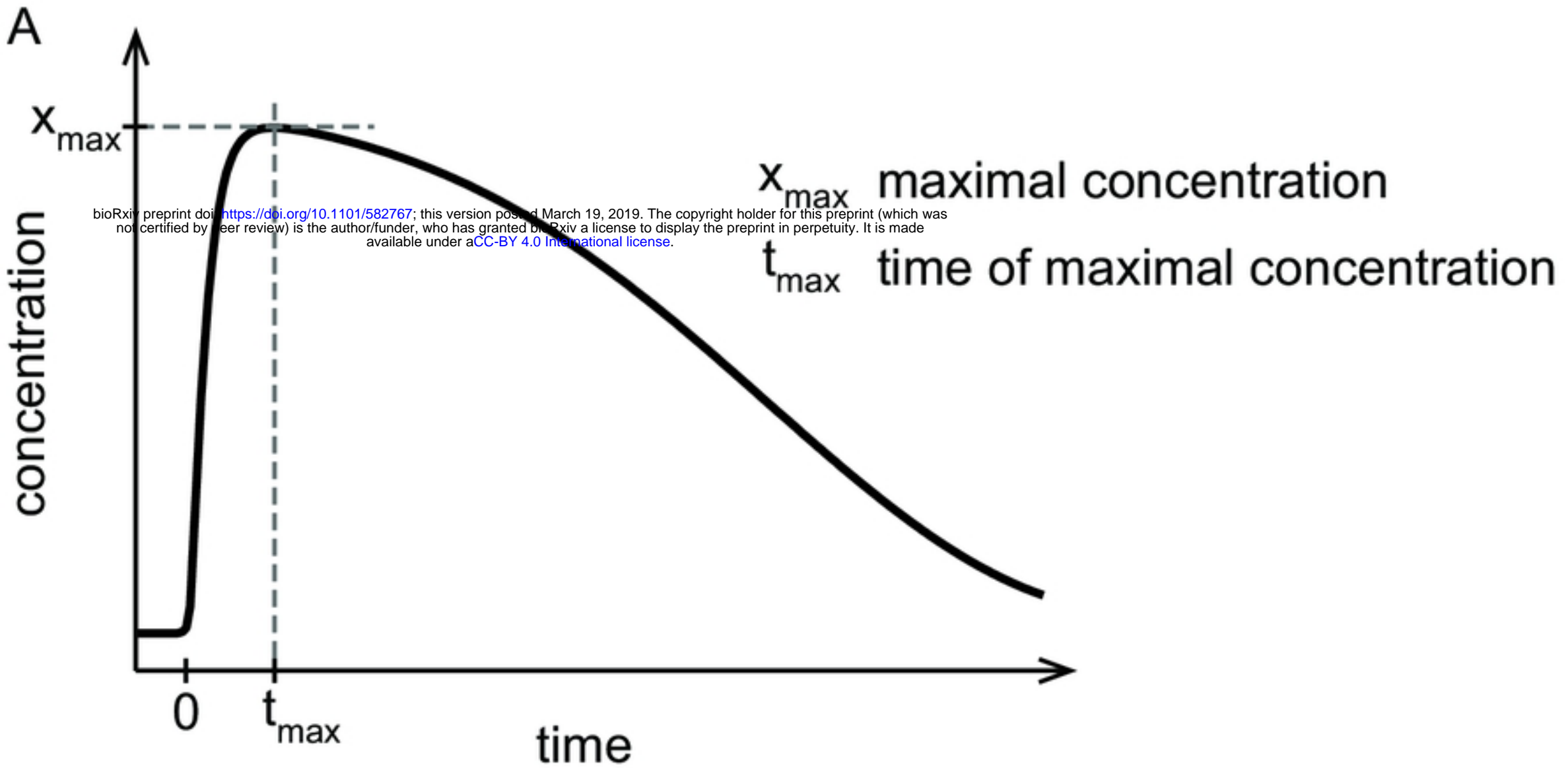


B

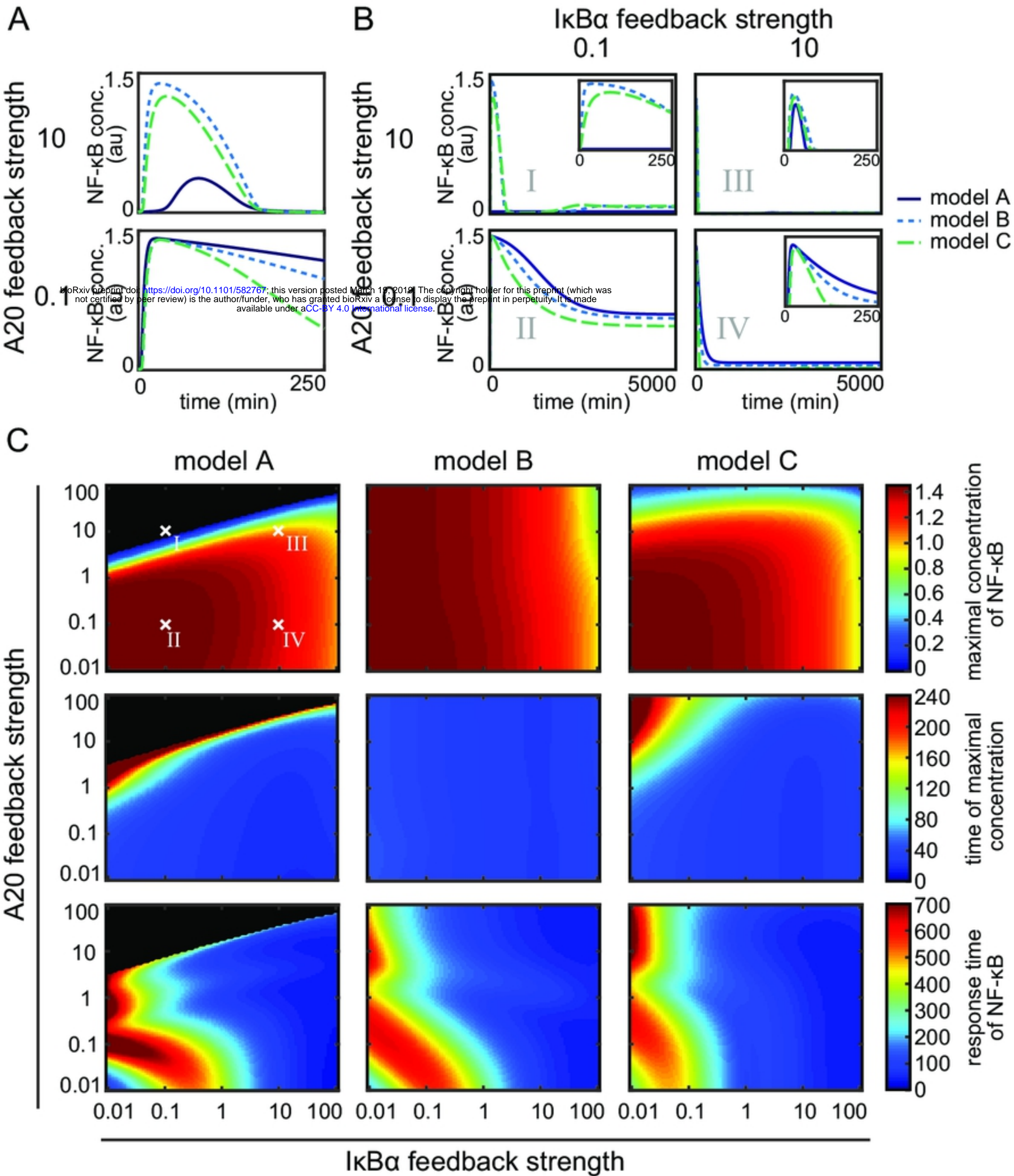
upstream modules with
estimated parameters

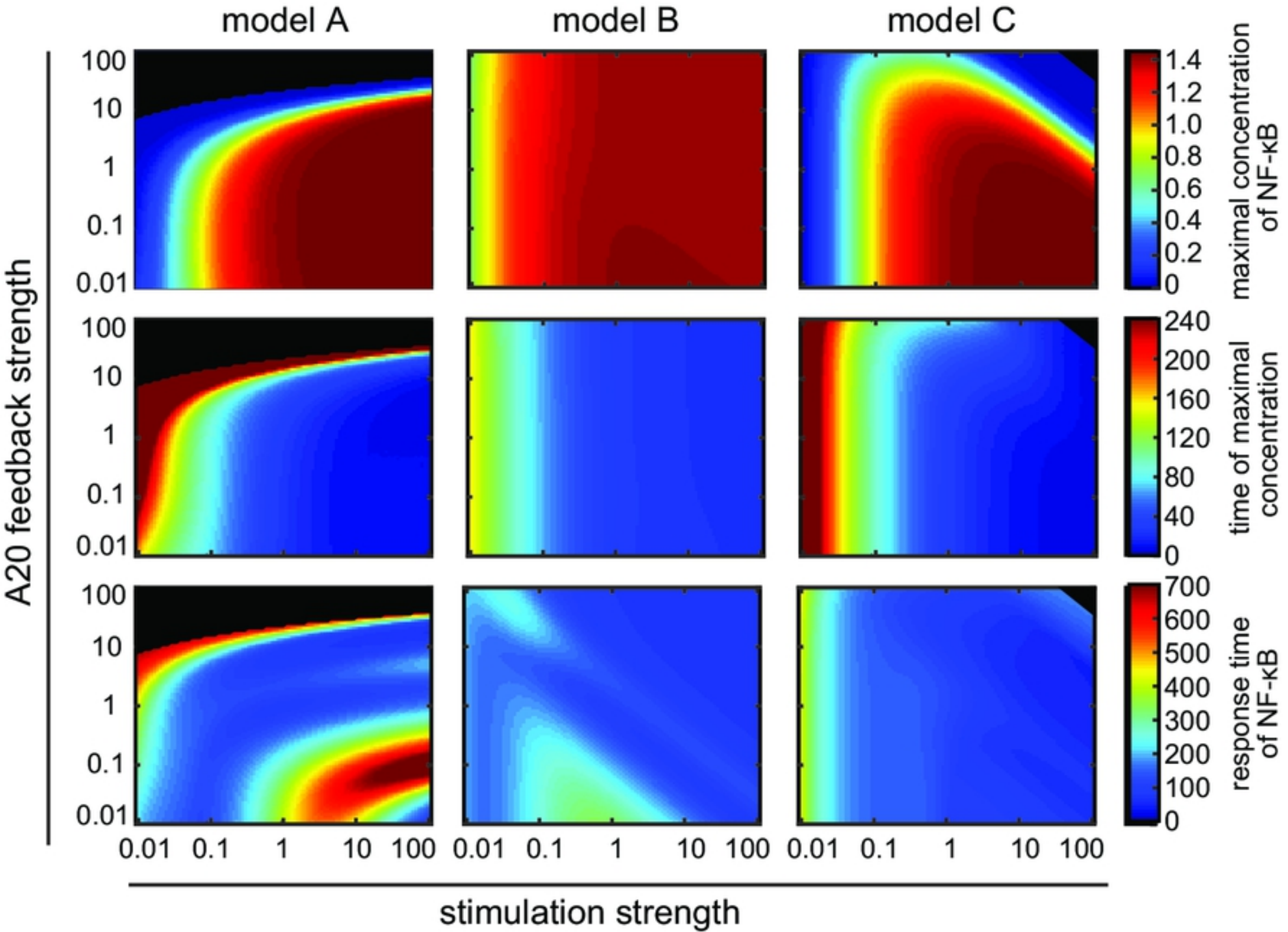


Figure



Figure





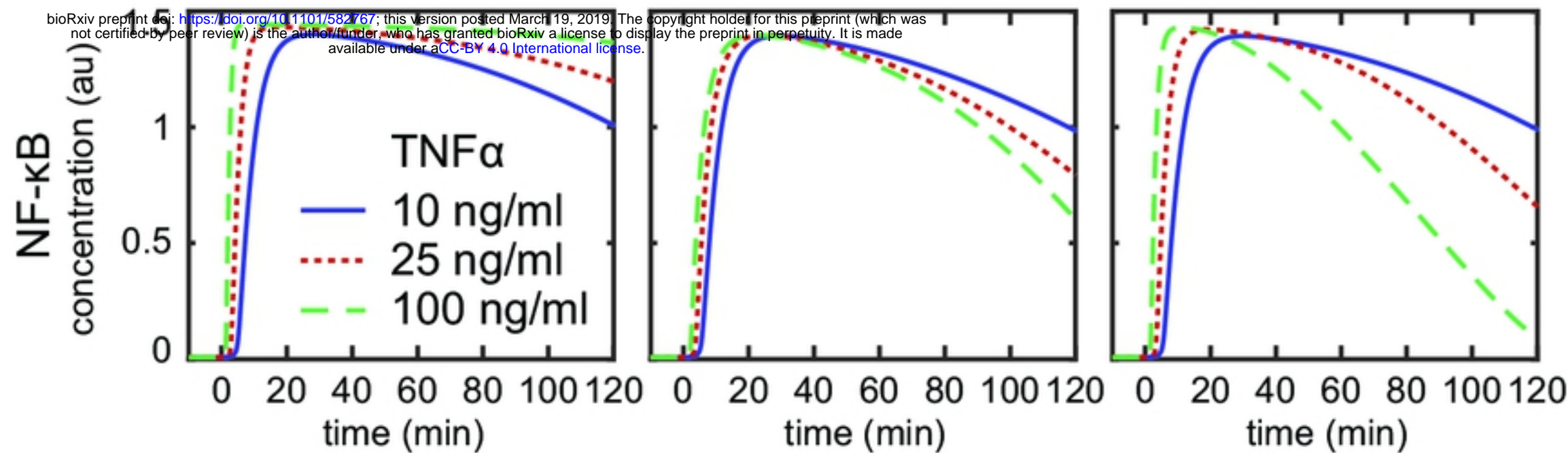
Figure

A

model A

model B

model C



B

10 ng/ml TNFα

25 ng/ml TNFα

100 ng/ml TNFα

time (min):

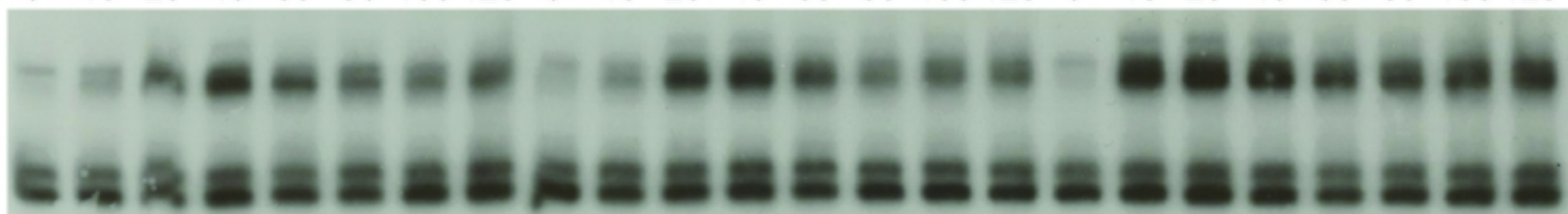
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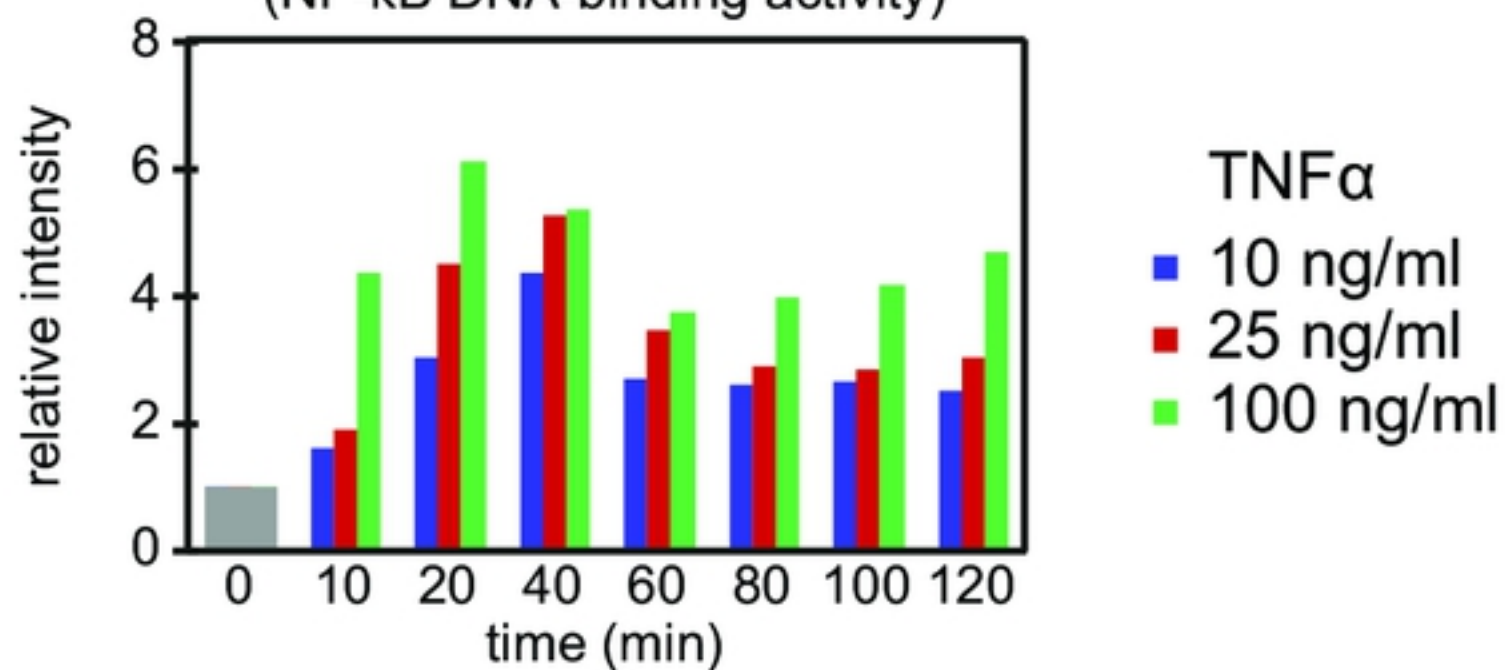
NF-κB

non-specific



EMSA

(NF-κB DNA-binding activity)



Figure