Investigating IKK Dynamics in the NF-κB Signalling Pathway using X-Machines

Richard A. Williams*, *Senior Member, IEEE,* Jon Timmis†, *Senior Member, IEEE,* and Eva E. Qwarnstrom‡
*Department of Management Science, Lancaster University, Lancaster, LA1 4YX, UK, r.williams4@lancaster.ac.uk
†Department of Electronics, University of York, York, YO10 5DD, UK, jon.timmis@york.ac.uk
and York Computational Immunology Laboratory, University of York, York, YO10 5DD, UK
‡Department of Infection, Immunity and Cardiovascular Disease, Medical School, University of Sheffield, Sheffield, S10 2RX, UK, e.qwarnstrom@sheffield.ac.uk

***Abstract*—The transcription factor NF-κB is a biological component that is central to the regulation of genes involved in the innate immune system. Dysregulation of the pathway is known to be involved in a large number of inflammatory diseases. Although considerable research has been performed since its discovery in 1986, we are still not in a position to control the signalling pathway, and thus limit the effects of NF-κB within promotion of inflammatory diseases. We have developed an agent-based model of the IL-1 stimulated NF-κB signalling pathway, which has been calibrated to wet-lab data at the single-cell level. Through rigorous software engineering, we believe our model provides an abstracted view of the underlying real-world system, and can be used in a predictive capacity through *in silico* experimentation. In this study, we have focused on the dynamics of the IKK complex and its activation of NF-κB. Our agent-based model suggests that the pathway is sensitive to: variations in the binding probability of IKK to the inhibited NF-κB-IκBα complex; and variations in the temporal rebinding delay of IKK.**

I. Introduction

Nuclear factor-kappa B (NF-κB) is a collective name for a family of inducible dimeric transcription factors, which in conjunction with its signalling pathway connects various extracellular stimuli to the induction of gene expression. NF-κB plays a critical role in inflammation, immunity, cell proliferation, cell differentiation, and cell survival [1]. The choice between life and death of individual cells during infection of a host, is one of the key events in the immune response [2]. The transcription factor NF-κB is a major player in the regulation of such life and death decisions, and thus is central to control of transcriptional regulation of a large number of genes that control fundamental biological events [3].

Activation of the NF-κB transcription factor and signalling pathway is tightly regulated and involves phosphorylation of several members of the NF-κB and Inhibitor of kappa B (IκB) protein families [4]. NF-κB is normally sequestered in the cytosol of non-stimulated cells, and consequently must be translocated into the nucleus to function as a transcriptional activator of target genes. NF-κB activation is induced by a wide variety of different extracellular stimuli, including proinflammatory cytokines (such as Tumour Necrosis Factor alpha (TNFα) and interleukin-1 (IL-1)), bacteria, viruses, and physical and chemical stresses [5]. NF-κB activation is thought to be controlled by two distinct pathways, which have been termed the *canonical* and *non-canonical* pathways [6], [7]. The two pathways are induced by different extracellular stimuli, controlled by different intracellular kinases, operate on different NF-κB complexes, and activate the transcription of specific target genes [8].

NF-κB regulation is believed to be essential for the proper function of both the innate and adaptive immune systems. Under physiological conditions for normal (non-malignant) cells, NF-κB activation occurs transiently upon receiving a stimulus, due to the negative feedback regulation. Due to its central role in gene regulation, any changes in control of its activation can have fundamental impact on normal physiological functions. In addition, impairment of NF-κB control is intimately associted with disease including a variety of cancers [9], neurodegenerative diseases [10], cardiovascular diseases [8], and arthritis [11]. This results in potential autoimmune responses, in particular within the central nervous system, and resultant onset of demyelinating diseases such as Multiple Sclerosis (MS) in humans, and the analogous Experimental Autoimmune Encephalomyelitis in animals [2].

Understanding the mechanisms that control NF-κB activation/cellular signalling is important for exploiting therapeutic approaches to treat human disorders due to its dysregulation. Within this study we will focus on the canonical pathway, which controls nuclear translocation of the p50/RelA (NF-κB1/RelA) heterodimer. Following activation of a cell membrane receptor and propagation of intracellular signalling, the canonical pathway ultimately results in activation of the IκB Kinase (IKK) complex. The IKK trimer within the canonical pathway consists of two kinase subunits IKKα and IKKβ, along with the regulatory subunit IKKγ (NF-κB Essential Modulator (NEMO) [12], [13]). Activation of IKK induces phosphorylation-dependent degradation of IκB and release of NF-κB. As such, IKK is a key component of the signalling pathway, whose dynamics have a significant effect on the downstream activation of NF-κB and resultant gene expression for immune response.

We have utilised the concept of communicating X-Machines [14], [15] to develop an agent-based model of the IL-1 stimulated NF-κB signalling pathway. Specific focus has been made in this study to IKK dynamics and its affect on downstream activation of NF-κB. We have performed three simulation-based experiments relating to: variations in the binding probability

978-1-5090-4601-0/17/$31.00

of IKK to the inhibited NF-κB-IκBα complex; the temporal rebinding delay of IKK to additional inhibited complexes following the relase of free NF-κB from a prior inhibited complex; and variations in the numbers of IKK molecules within the system. Our agent-based model suggests that the pathway is sensitive to variations in the binding probability of IKK to the inhibited NF-κB-IκBα complex, and variations in the temporal rebinding delay of IKK.

The paper has the following structure: in Section II we provide a brief overview of the NF-κB signalling pathway in the form of a conceptual model; Section III provides a high-level technical design for our computational model based on the concept of Communicating X-Machines; Section IV introduces our agent-based model using the FLAME simulation framework; Section V presents our simulation-based experimental results; and finally, Section VI presents our conclusions.

II. Conceptual Model

We have previous discussed the need for new computational models of the signalling pathway [16]. Furthermore, we have recently developed a conceptual model of the IL-1 stimulated NF-κB signalling pathway using the Unified Modelling Language and a number of statistical techniques [17]. As such, we will provide the biological context to our study through a brief discussion in this section, and direct the reader to our previous work for the detailed conceptual model.

Briefly, the signalling network commences with an extracellular signalling molecule binding to a cell membrane receptor (a member of the TLR/IL-1 receptor superfamily). The receptor then dimerises, and co-receptors such as CD14 [18], MD2 [19] (in the case of TLR4, [20]) and/or TILRR [21], [22] (in the case of IL-1RI/IL-1AcP) help facilitate and amplify the receptor response, and in situations where the MyD88 adaptor protein binds, it mediates association of the receptor complex with IRAK protein kinase [23], which in turn activates TRAF6 [24] for propagation of the signal. Once activated, TRAF6 continues signal transduction through activation of TAK1, which subsequently activates the IKK complex [25]. The activated IKK complex chemcially modifies NF-κB inhibitors, such as IκBα, which facilitates its dissociation from the NF-κB molecule within the complex [26]. The released IκBα undergoes a second modification (ubiquitination) [27], which then targets IκBα for rapid degradation within the cell. Conversely, the released NF-κB is able to translocate from the cytosol to the nucleus, where it is subsequently activated and binds cognate transcriptional sites to induce gene activation. Figure 1 presents an overview of the signalling network.

III. High-Level Technical Design

Signalling pathway dynamics are stochastic and it has been found that at a population level, the individual cells follow a negative binomial distribution with respect to degradation dynamics of the inhibitor IκBα and resultant activation of free NF-κB, which are the key dynamics that we are interested [17]. As such, we believe that the modelling paradigm of

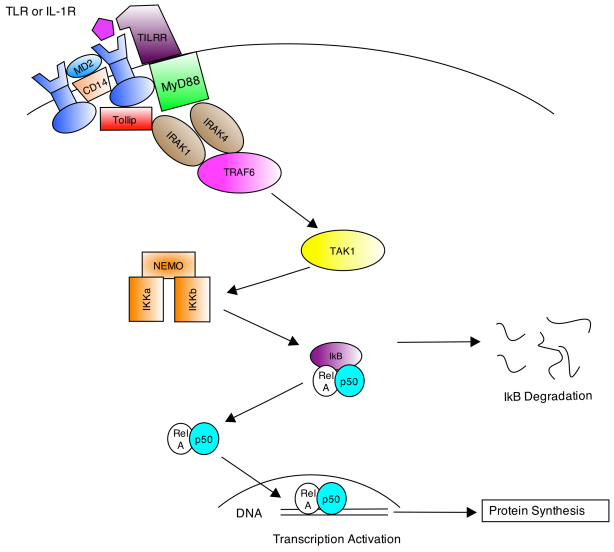

Fig. 1. Simplified cartoon diagram depicting the high-level interactions between the cell membrane receptors, co-receptors and adaptor proteins, and protein kinases within the NF-κB canonical signalling pathway. Reproduced from [17] under a Creative Commons Attribution License (CC BY 4.0).

agent-based modelling provides the most appropriate means to simulate the variability seen at the population level of single cells. From a technical perspective, all system components are generalisable as *cell components*, and will contain a standard set of attributes (e.g. 3D coordinates, along with movement parameters and functions).

There are four key categories of components, which relate to: an *organelle*, which for our purposes has been abstracted away to consist of only the cytoplasm and the nucleus; a *receptor*, which can be either the IL-1R cell membrane receptor or a nuclear transporter (importing or exporting); a *membrane*, which has been abstracted away to only incorporate the cellular membrane or the nuclear membrane; and *biomolecules*, which reflect the main signalling proteins: MyD88 adaptor protein, transcription factor NF-κB, inhibitor IκBα, enzyme IKK, and protein kinases IRAK and TRAF6. In addition, our computational model of the single cell environment will require a number of compartments to approximate to the internal structure of a cell. Regarding the containment of agents, the cell membrane, nuclear membrane, cytoplasm and nucleus, effectively operate as the biological environment for simulations, as they provide the necessary cell structure within which the various biomolecules (e.g. NF-κB, IκBα and IKK agents) interact. The cell membrane receptors (IL-1R) are confined to the cell membrane, and the nuclear transporters to the nuclear membrane. The IKK and NF-κB-IκBα complex agents are confined to the cytoplasm, however free NF-κB and IκBα agents are able to move between the cytoplasm and nucleus, mediated by the nuclear transporters. Relative to the transcription factor, the inhibitor IκBα is in excess within the biological system [28], [29]. The current study focuses specifically on the activation of the NF-κB-IκBα complex, which is present at a 1:1 ratio [30]. Figure 2 represents one

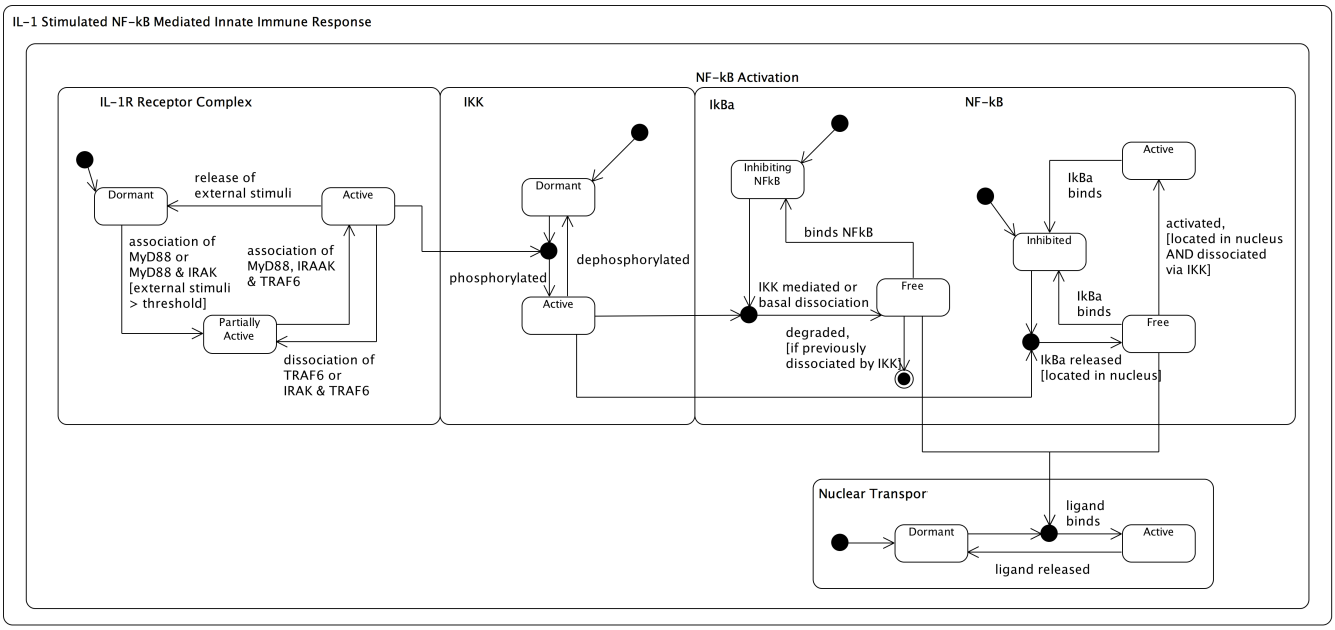

Fig. 2. UML state machine diagram depicting the state changes of different agent types, and the relationships between these different agent types.

view of our abstracted technical design using a set of linked UML state machine diagrams.

There are a number of different agent-based modelling frameworks and development environments in use [31], which represent agents through a number of different computational techniques. The one thing these techniques have in common, however, is that they portray the agent as having a defined *state* at any particular moment in time. Representations of the agent may therefore be viewed as a *state machine*, where the current input in combination with all past inputs (previous states) determines the output (next state) of the agent.

A communicating X-Machine model is a formalised mathematical model that can be used to compute the functional behaviour of smaller components (i.e. individual agents), whose dynamics may be aggregated to generate the emergent behaviour within the entire system. The communicating X-Machine is based upon a simple set of rules that describe what the agent must/could do under different circumstances, and how the various agents may communicate with each other. They utilise a 10-tuple formal notation, with C_i^X representing the *i*th communicating X-Machine component [32], as defined by:

$$C_i^X = (\Sigma_i,\Gamma_i,Q_i,M_i,\Phi_i,F_i,q_{0i},m_{0i},I\Phi_i,O\Phi_i)$$

where:

- Σ and Γ are the input and output alphabets respectively.
- Q is the finite set of states.
- M is the (possibly) infinite set called memory.
- Φ, the *type* of the X-Machine X, is a set of partial functions φ that map an input and a memory state to an output and a possibly different memory state, φ : Σ x M $\rightarrow$ Γ x M.
- F is the next state partial function, F : Q x $\Phi \rightarrow$ Q, which given a state and a function from the type Φ determines the next state. F is often described as a state transition diagram.
- q_0 and m_0 are the initial state and initial memory respectively.
- $I\Phi_i$ is the communication interface for the input messages.
- $O\Phi_i$ is the communication interface for the output messages.

Holcombe [33] defines an X-Machine as a system that has an internal *computational* state and an internal memory, which can transition to another state dependent on environmental input and their current internal state. The communicating X-Machine is therefore able to encapsulate both the dynamic and functional behaviour of an agent, as well as the underlying data that it is modelled on, in a single process specification [14]. Communication between individual X-Machines occurs through a 'communication matrix', which is essentially a *message board* that facilitates the reading and writing of information between every X-Machine, allowing communication and interaction between the machines. Individual agents start with an initial computational state, and upon receiving an external input (e.g. communication from another agent), they update this state, based on the rule regarding their current state and the particular external input received [15], [34]. Following this, it will change internal and/or memory state, potentially generate a message for communication, or continue its current behaviour.

IV. Computational Model

We have used the Flexible Large-scale Agent-based Modelling Environment (FLAME) [35], [36] to develop our computational model. FLAME itself is not a true modelling platform in the purest sense, instead it requires the modeller to use templates to define the agent-based model, and then parse and compile these using the packaged parser and associated APIs. Due to FLAME utilising the concept of communicating X-Machines, the agents are modelled using XML templates to define the attributes and internal states of the agent, and C code is used to define the rule-based functions of the agent behaviour. Through the use of messages to communicate changes within the system's environment, and transition functions, which define the rule-based logic of the computational model, the agents are able to transition to a new state and update their internal memory as the simulation progresses. Once a model is specified using the XML and C templates, the FLAME modelling framework is able to automatically generate simulation code (via the *xparser* programme) that allows communication between X-Machines (agents) through its own communication library, called *Message Board*. Through interfacing to the Message Passing Interface (MPI) communication framework, the simulation code is also fully compliant with parallel hardware platforms, enabling efficient communication between the individual agents, and ensuring that concurrently executing agents remain in sync with each other [37].

Our agent-based model was developed in accordance with the conceptual model and calibrated to wet-lab biological data. As discussed by Kirschner [38], there are several approaches for estimating the parameter values of computational models during the calibration process: 1) direct experimental determination of a parameter; 2) simultaneous estimation of several parameters at once by fitting experimental data to a model; and 3) estimation of a parameter based on known values for a similar system. The calibration process that we utilised to align the behaviour of our computational model with that of the underlying biological system used a mixture of the three approaches discussed by Kirschner for parameter value estimation. Environmental parameter values such as cell and nuclear diameter have been approximated from the literature. A number of parameter values regarding agent interactions were arbitrarily set, such as the interaction radius within which agents need to enter before they are eligible for probabilistic binding, and the delay applied to nuclear receptors following translocation of an agent before they may translocate another agent. Additionally, the parameters that we believe are fundamental to the emergent behaviour of our computational model were estimated through a process of varying parameter values during multiple simulation runs, until simulation dynamics approximated (through qualitative curve-fitting) to those of the wet-lab data of Carlotti et al [39] and Yang et al [40]. Calibration through varying parameter values, was therefore iterative, with each parameter being the focus of investigation, and required consultation between the modeller and domain expert until a parameter space was found that yielded qualitative alignment to the underlying biological data on which the computational model was designed (see figure 3 for calibrated dynamics). Finally, aleatory uncertainty analysis [41] indicated that a minimum of 175 simulation replicates using different pseudo-random number generator (PRNG) seed values was required to provide simulation results with stable median average dynamics - this was necessary to ensure we are able to infer cause and effect relationships within our simulation-based experimentation, and not be influenced by type 1 errors (false positives) due to the stochasticity within simulation runs using PRNG (using C Library stdlib.h).

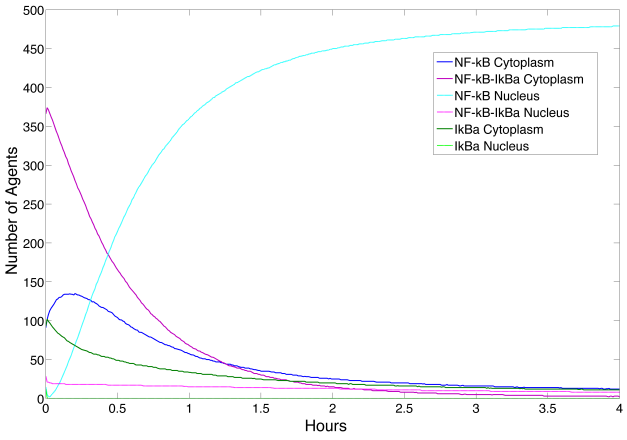

Fig. 3. Calibrated dynamics using the median average distribution of 175 replicate simulation runs using different PRNG seed values.

V. Simulation-Based Experimentation

Computational models are simplified versions of reality, often with no direct one-to-one translation between the computational model and the real-world domain. The calibration process therefore compensates for this mapping, to ensure that simulation dynamics approximate to the dynamics of the real-world domain. The relationship between the abstracted computational model and the biological system (in our case the IL-1 stimulated NF-κB signalling pathway) is critical for using the results of simulation-based experimentation in a predictive capacity to generate novel hypotheses for testing within the wet-lab. As such, epistemic uncertainty analysis focuses on the lack of knowledge regarding certain parameter values [41], the abstracted nature of computational models, and the effects of varying the absolute parameter values that have been defined through the calibration process [42].

The IL-1 stimulated dynamics within our computational model relate to the IKK-mediated release of NF-κB from the NF-κB-IκBα complex, the associated degradation of IκBα, and the translocation of the free NF-κB (that had been released via IKK) in to the nucleus. As such, we believe that IKK is a key component for modulating the signalling pathway, and will be the focus of our simulation-based experimentation. The three experiments discussed in this paper relate to the robustness of the system regarding IKK dynamics. The first two experiments focus on the epistemic uncertainty regarding

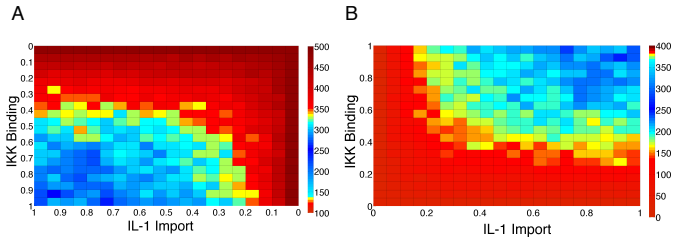

Fig. 4. Two-dimensional heatmap of the IKK binding probability parameter versus the Nuclear Import probability parameter under IL-1 stimulation. **A** shows the variation in the total number of NF-κB agents within the cytoplasm. **B** shows the variation in the total number of NF-κB agents within the nucleus. The heatmap key represents distance away from the calibration range, e.g. dark blue areas represent the parameter values within the calibration range, and red signifies parameter values considerably outside of the calibration range.

the calibration values for the probability of an IKK agent binding to an inhibited NF-κB-IκBα complex, along with the time delay of IKK before it is allowed to bind to another inhibited NF-κB-IκBα complex. The final experiment relates to the effects of varying the number of IKK agents on the system.

A. IKK Binding Probability

An enzyme needs to be within a close enough proximity to substrate molecule(s) in order to catalyze a reaction and form the relevant product(s). Furthermore, even though the complementary molecules (enzyme and substrate(s)) may be within an appropriate distance (interaction zone), the actual biochemical reaction is not a certainty, and as such conforms to a probabilistic reaction. Our computational model reflects this stochastic nature through the use of an IKK binding probability parameter.

During calibration of the computational model, we noted complex dynamics for cytoplasmic and nuclear NF-κB in both free and inhibited states. We further noted that total NF-κB agents within the cytoplasm and nucleus showed a degree of stability however, which reflected the wet-lab findings in [43]. We conjecture that this may represent non-linear dynamics of the nuclear import (under IL-1 stimulation) parameter, that may be co-dependent on IKK mediated dissociation of free NF-κB through IKK-mediated catalysis, which in our computational model is represented using the IKK binding probability parameter.

We tested the effects that the IKK binding probability parameter has on system dynamics through performing a two-at-a-time analysis between the IKK binding probability parameter and the nuclear import probability parameter under IL-1 stimulation. The resulting 2D heatmaps (figure 4) suggest that desired total NF-κB agent numbers within the nucleus and cytoplasm are generated when nuclear import (under IL-1 stimulation) parameter value is between 0.75 to 1.0, and when the IKK binding parameter value is between 0.75 to 1.0, which we believe provides a remarkable degree of robustness to the system.

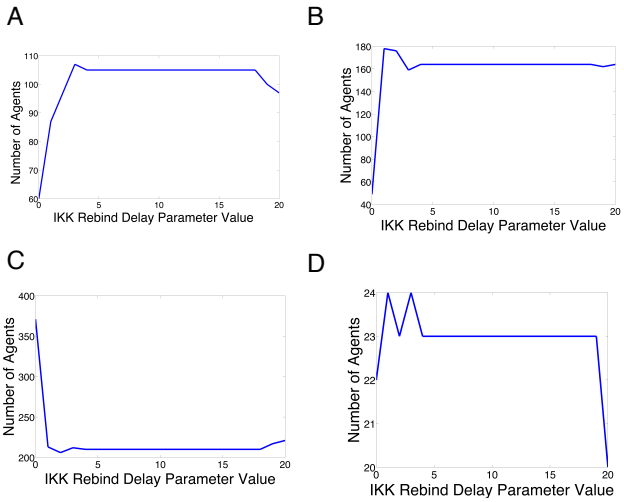

Fig. 5. The IKK rebind delay parameter shows a degree of robustness to variations in its absolute value. **A** shows the variation in the number of NF-κB agents within the cytoplasm. **B** shows the variation in the number of NF-κB agents within the nucleus. **C** shows the variation in the number of NF-κB-IκBα agents within the cytoplasm. **D** shows the variation in the number of NF-κB-IκBα agents within the nucleus. It can be seen that system dynamics are robust for IKK rebind delay parameter values between 4 to 18.

B. IKK Rebind Delay

Following an enzyme catalyzed reaction in biological systems, the enzyme molecule will need to release the product molecule from the reaction and may also need a period of time to re-establish its 3-dimensional physical structure (conformational change) in order to accept another set of substrate molecules for a subsequent round of catalysis. The IKK rebind delay parameter within our computational model reflects this time delay (simulation run iterations), and introduces a lag period to individual IKK agents following release of free NF-κB (product) from one round of catalysis and interaction with an inhibited NF-κB-IκBα complex (substrate) in the next round of catalysis.

We tested the effects that the IKK rebind delay has on system dynamics through varying the time delay (number of simulation run iterations) that IKK agents incur before they are able to bind with another inhibited NF-κB-IκBα complex and promote the release of free NF-κB. The system showed a remarkable degree of robustness during. Figure 5 higlights that computational model dynamics are sensitive when simulations use IKK rebind delays between 1 to 4 time-steps, but very stable with values between 4 to 18 time-steps.

C. IKK Numbers

Following IL-1 stimulation and activation of receptor complex components, the signal transduction reaches the NF-κB-IκBα signalling module [44], with the key step being the IKK-mediated dissociation (and resultant degradation) of IκBα [12]. This causes release of NF-κB, and facilitates its translocation into the nucleus and subsequent activation, to initiate transcription of genes for the inflammatory response.

Although computing power continues to increase, the prospect of us developing a computational model using biological levels of agents is still an intractable problem. As such, simulation dynamics have been calibrated using 501 IκBα and 502 NF-κB (both in various states of free, bound, cytoplasmic and nuclear), along with 500 agents each of IL-1R cell membrane receptor, MyD88, IRAK, TRAF6, and 50 IKK agents. Although these numbers are somewhat arbitrary, calibration of the computational model compensated for this design decision. In addition, as the IKK enzyme is able to catalyze numerous reactions, the system needs far fewer IKK agents than its substrate (the inhibited NF-κB-IκBα complex), due to the amplificatory nature of enzymes within signalling pathways.

This provides an opportunity for us to investigate the effects of varying IKK numbers (akin to varying concentration in wet-lab experiments), and whether the system is robust to such perturbations. As there is an amplification step, i.e. one IKK agent can facilitate dissociation of many NF-κB-IκBα complexes over the lifetime of a simulation, it is expected that an increase in IKK numbers would increase the rate at which active NF-κB accumulates in the nucleus. This experiment therefore allows us to qualify the change in system dynamics along with the extent of robustness (or indeed fragility) within the system that is directly attributable to IKK number.

Experimentation into the effects of varying IKK numbers, is conducted through perturbation of the total number of IKK agents. By default, there were 50 IKK agents within the calibrated simulation platform. To test the effects, we ran six sets of simulation-based experiments using a tenth, a fifth, a half, 2x, 5x, and 10x default numbers (i.e. 5, 10, 25, 100, 250 and 500 IKK agents, respectively). For each set of 175 simulation replicates, the median distributions of cytoplasmic NF-κB, nuclear NF-κB, cytoplasmic NF-κB-IκBα, nuclear NF-κB-IκBα, cytoplasmic IκBα and nuclear IκBα over the lifetime of the simulation runs are interpolated. These distributions are contrasted with the baseline behaviour that results from simulations using the default number of IKK agents. Kolmogorov Smirnov (KS) tests [45] are then performed to understand whether there are any statistically significant differences from baseline behaviour (requiring p-values below 0.05), and the Vargha and Delaney A-Tests [46] are also performed to understand the effect magnitude of these differences, assuming 'large' differences of < 0.29 and > 0.71 to be scientifically significant.

Results indicate that the system is sensitive to perturbations involving the total number of IKK agents, which in effect alters the ratio of IKK to inhibited complex NF-κB-IκBα, and the ratio of IKK to free NF-κB and IκBα within the system. An increase in IKK numbers results in both a decrease in cytoplasmic NF-κB-IκBα numbers, but also an increase in the rate of dissociation of the NF-κB-IκBα complexes. The effects on cytoplasmic NF-κB numbers are not as straightforward to interpret, as there appears to be a non-linear temporal component at play, whereby an increase in IKK numbers results in an initial increase in cytoplasmic NF-κB numbers upto approximately 15min, after which there is a sharp decrease, resulting in a negative relationship between the number of IKK agents and the number of cytoplasmic NF-κB agents at the end of simulation runs (see figure 6). KS tests indicate that all perturbations to IKK numbers provide statistically significant differences (with respect to baseline dynamics) across all agent states, apart from nuclear IκBα, and the cytoplasmic and nuclear NF-κB-IκBα agent states when 50% of default IKK numbers are used (i.e. 25 IKK agents). Furthermore, the corresponding effect magnitude using the A-Test, indicate scientifically significant differences exist for: cytoplasmic NF-κB-IκBα in all perturbations, nuclear NF-κB and active NF-κB for perturbations that used 10%, 20%, 500% and 1,000% IKK (with respect to default); and cytoplasmic IκBα for perturbations that used 10% and 20% of IKK (with respect to default).

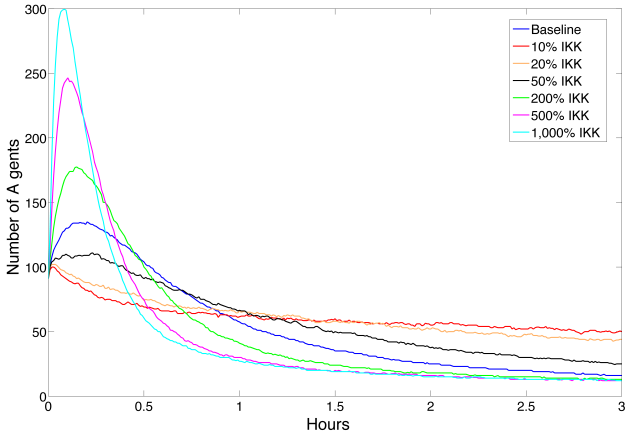

Fig. 6. The non-linear cytoplasmic NF-κB dynamics for the six sets of experiments into IKK concentration, which have been compared against baseline IL-1 stimulated dynamics.

VI. Conclusion

Computational models provide not only a means to rigorously think about and describe complex dynamical systems, but also provide an opportunity for us to extend our knowledge of system dynamics through performing novel simulation-based experimentation, and extrapolating these results back to the real-world system. Our agent-based model contains additional granularity at the cell membrane receptor complex with respect to existing models, through explicit modelling of the IL-1R, MyD88, TRAF, and IRAK components. It has been developed using the concept of communicating X-Machines, which should ensure the model can be run across multiple computing architectures (e.g. laptop, desktop, cluster and grid), and calibrated against wet-lab data of Carlotti et al [39] and Yang et al [40], to ensure that the model can be used for predictive purposes against the IL-1 stimulated NF-κB signalling pathway, at the single-cell level. As such, we believe that our computational model provides a suitable platform to perform additional simulation-based experimentation around the IL-1 stimulated NF-κB signalling pathway.

Following the discovery of [12], that the signal-induced activation of NF-κB requires phosphorylation of the inhibitory IκBα molecule (within the inhibited NF-κB-IκBα complex), and its subsequent proteolytic degradation, we now know that the initial phosphorylation to dissociate the IκBα molecule and release free NF-κB is performed by IKK. This has provided us with an opportunity to investigate three key factors involved in IKK dynamics within our computational model, namely: the probabilistic binding of IKK to its substrate (the inhibited NF-κB-IκBα complex); the temporal delay in IKK binding to a new NF-κB-IκBα complex, for a subsequent round of catalysis; and the effects of varying IKK number.

Simulation-based experimental results indicate that the system is remarkably robust to perturbations in IKK binding probability, which we conjecture could be due to both the amplificatory nature of enzymatic reactions, and the continuous movement of agents within the cytosol, thus facilitating enzymatic reactions through individual substrate and enzyme moving into a suitable interaction zone, which would provide multiple opportunities for an enzymatic reaction to occur during the lifetime of a simulation. The second set of simulation-based experimatal results also indicated the system is on the whole robust to perturbations in the temproal delay for IKK agents before they may catalyse the next round of reactions, but is sensitive to perturbations over a small region of 1-4 time-steps (simulation iterations). We conjecture that this sensitivity may be due to the probabilistic nature of catalysis, and that a short delay is required to allow the substrate to move out of the interaction zone following a probabilistic interaction that did not result in a catlysed reaction.

The third set of simulation-based experimental results indicate that the system is stable to perturbations in IKK numbers within a small range either side of the calibrated value (i.e. between 50% and 200%), but sensitive to more extreme perturbations (i.e. 10%, 20%, 500%, and 1,000%). Using these higher IKK numbers (i.e. 500% and 1,000% of the calibrated value), results also indicate that an increase in the number of IKK agents, leads to: a decrease in cytoplasmic IκBα; a decrease in both cytoplasmic and nuclear NF-κB-IκBα complex, along with an associated increase in the dissociation of the complexes; and no significant change to either nuclear NF-κB-IκBα complexes or nuclear IκBα, although we suspect that this is due to the small absolute numbers that are associated with the calibrated simulation platform. Additionally, a small change in cytoplasmic IκBα was found when perturbing the system with low IKK numbers (i.e. 10% and 20% of the default value). KS-Tests and subsequent A-Tests provide additional support to these interpretations, by indicating scientifically significant differences for cytoplasmic NF-κB-IκBα complexes under all perturbations to IKK number, and nuclear (both free and active) NF-κB under all perturbations to IKK number, apart from 50% and 200% of the default value for IKK numbers. Unfortunately, the effects on cytoplasmic NF-κB are not as straightforward to interpret, as non-linear temporal dynamics emerge. We believe that these non-linear dynamics of cytoplasmic NF-κB are due to the rapid increase in dissociation of NF-κB-IκBα complexes to yield free NF-κB, and then subsequent translocation to the nucleus whereby these IKK-mediated free NF-κB agents become activated and remain in the nuclear compartment. As such, we believe that an increase in IKK numbers generates an initial increase in cytoplasmic NF-κB, which then slowly reduces over time through translocation to the nucleus. Once in the nucleus, the NF-κB becomes activated and thus remains there until the end of the simulation, due to the abstractions that we have taken as part of our platform model - we have developed a minimal model that focuses on the signalling pathway upto and including NF-κB activation.

In conclusion, this paper has described a principled approach to design, development and calibration of an agent-based model of the IL-1 stimulated NF-κB signalling pathway; along with subsequent simulation-based experimentation to investigate various aspects of the pathway at the component-level, to provide insight to the nature of the underlying mechanisms and dynamics. Although we are confident that the computational model has adhered to good software engineering practices, the modelling activities were not the end goal itself, but were instead incremental activities for the development of a *tool* to increase our understanding of the signalling pathway. As such, it is hoped that future researchers will use our agent-based model to perform additional, novel, simulation-based experiments. We believe that an increased understanding of the pathway under normal physiology and disease, will elucidate the underlying causes of inflammatory diseases, and lead to new treatment strategies.

ACKNOWLEDGMENT

JT is part-funded by The Royal Society, The Royal Academy of Engineering, and the Engineering and Physical Sciences Research Council (Grant No. EP/K040820/1). The York Computational Immunology Laboratory is part-funded by the Wellcome Trust (Ref: 097829) through the Centre for Chronic Diseases and Disorders (C2D2) at the University of York. EEQ (The Cell Biology Laboratory in the Department of Infection, Immunity and Cardiovascular Disease, at the University of Sheffield) is part-funded by the Biotechnology and Biological Sciences Research Council (Grant No. BB/J009687/1) and the British Heart Foundation (Grant No. PG/11/103/29219).

REFERENCES

[1] A. Oeckinghaus and S. Ghosh, "The NF-κB family of transcription factors and its regulation," in *NF-κB A Network Hub Controlling Immunity, Inflammation, and Cancer*, ser. Cold Spring Harbour Perspectives in Biology, M. Karin and L. M. Staudt, Eds. New York, USA: Cold Spring Harbour Press, 2009, pp. 5–18.

[2] M. Karin and A. Lin, "NF-κB at the crossroads of life and death," *Nature Immunology*, vol. 3, no. 3, pp. 221–227, 2002.

[3] H. L. Pahl, "Activators and target genes of Rel/NF-κB transcription factors," *Oncogene*, vol. 18, pp. 6853–6866, 1999.

[4] M. Naumann and C. Scheidereit, "Activation of NF-κB in vivo is regulated by multiple phosphorylations," *The EMBO Journal*, vol. 13, pp. 4597–4607, 1994.

[5] S. Ghosh, M. J. May, and E. B. Kopp, "NF-κB and Rel proteins: Evolutionarily conserved mediators of immune response," *Annual Review of Immunology*, vol. 16, pp. 225–260, 1998.

[6] M. Karin, "The beginning of the end: IκB kinase (IKK) and NF-κB activation," *Journal of Biological Chemistry*, vol. 274, pp. 27 339–27 342, 1999.

[7] U. Senftleben, Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S. C. Sun, and M. Karin, "Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway," *Science*, vol. 293, pp. 1495–1499, 2001.

[8] A. R. Brasier, "The NF-κB regulatory network," *Cardiovascular Toxicology*, vol. 6, pp. 111–130, 2006.

[9] G. Sethi, B. Sung, and B. B. Aggarwal, "Nuclear factor-κB activation: From bench to bedside," *Experimental Biology and Medicine*, vol. 233, pp. 21–31, 2008.

[10] M. Grilli and M. Memo, "Nuclear factor-κB/Rel proteins," *Biochemical Pharmacology*, vol. 57, pp. 1–7, 1999.

[11] B. Foxwell, K. Browne, J. Bondeson, C. Clarke, R. de Martin, F. Brennan, and M. Feldmann, "Efficient adenoviral infection with IκBα reveals that macrophage tumor necrosis factor α production in rheumatoid arthritis is NF-κB dependent," *Proceedings of the National Academy of Sciences*, vol. 95, pp. 8211–8215, 1998.

[12] Z. J. Chen, L. Parent, and T. Maniatis, "Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity," *Cell*, vol. 84, pp. 853–862, 1996.

[13] F. Mercurio, H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. wu Li, D. B. Young, M. Barbosa, and M. Mann, "IKK-1 and IKK-2: Cytokine-activated IκB kinases essential for NF-κB activation," *Science*, vol. 278, pp. 860–866, 1997.

[14] J. Barnard, J. Whitworth, and M. Woodward, "Communicating X-machines," *Information and Software Technology*, vol. 38, no. 6, pp. 401–407, 1996.

[15] P. Kefalas, G. Eleftherakis, and E. Kehris, "Communicating X-machines: A practical approach for formal and modular specification of large systems," *Information and Software Technology*, vol. 45, pp. 269–280, 2003.

[16] R. A. Williams, J. Timmis, and E. E. Qwarnstrom, "Computational models of the NF-κB signalling pathway," *Computation*, vol. 2, no. 4, pp. 131–158, 2014.

[17] ——, "Statistical techniques complement UML when developing domain models of complex dynamical biosystems," *PLoS ONE*, vol. 11, no. 8, p. e0160834, 2016.

[18] S. D. Wright, R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison, "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein," *Science*, vol. 249, pp. 1431–1433, 1990.

[19] R. Shimazu, S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto, "MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4," *Journal of Experimental Medicine*, vol. 189, pp. 1777–1782, 1999.

[20] A. Poltorak, X. He, I. Smirnova, M. Y. Liu, C. van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. I. Ricciardi-Castagnoli, B. Layton, and B. Beutler, "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene," *Science*, vol. 282, pp. 2085–2088, 1998.

[21] X. Zhang, G. M. Pino, F. Shephard, E. Kiss-Toth, and E. E. Qwarnstrom, "Distinct control of MyD88 adapter-dependent and Akt kinase-regulated responses by the interleukin (IL)-1RI co-receptor TILRR," *Journal of Biological Chemistry*, vol. 287, no. 15, pp. 12 348–12 352, 2012.

[22] X. Zhang, F. Shephard, H. B. Kim, I. R. Palmer, S. McHarg, G. J. S. Fowler, L. A. J. O'Neill, E. Kiss-Toth, and E. E. Qwarnstrom, "TILRR, a novel IL-1RI co-receptor, potentiates MyD88 recruitment to control Ras-dependent amplification of NF-κB," *Journal of Biological Chemistry*, vol. 285, pp. 7222–7232, 2010.

[23] H. Wesche, W. J. Henzel, W. Shillinglaw, S. Li, and Z. Cao, "MyD88: An adapter that recruits IRAK to the IL-1 receptor complex," *Immunity*, vol. 7, pp. 837–847, 1997.

[24] Z. Cao, J. Xiong, M. Takeuchi, T. Kurama, and D. V. Goeddel, "TRAF6 is a signal transducer for interelukin-1," *Nature*, vol. 383, pp. 443–446, 1996.

[25] T. Kawai and S. Akira, "TLR signaling," *Cell Death and Differentiation*, vol. 13, pp. 816–825, 2006.

[26] J. A. DiDonato, F. Mercurio, and M. Karin, "Phosphorylation of IκBα precedes but is not sufficient for its dissociation from NF-κB," *Molecular and Cellular Biology*, vol. 15, pp. 1302–1311, 1995.

[27] K. Brown, S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist, "Control of IκBα proteolysis by site-specific, signal-induced phosphorylation," *Science*, vol. 267, pp. 1485–1488, 1995.

[28] M. Pogson, M. Holcombe, R. Smallwood, and E. E. Qwarnstrom, "Introducing spatial information into predictive NF-κB modelling - an agent-based approach," *PLoS ONE*, vol. 3, p. e2367, 2008.

[29] D. M. Rhodes, S. A. Smith, M. Holcombe, and E. E. Qwarnstrom, "Computational modelling of NF-κB activation by IL-1RI and its co-receptor TILRR, predicts a role for cytoskeletal sequestration of IκBα in inflammatory signalling," *PLoS ONE*, vol. 10, no. 6, p. e0129888, 2015.

[30] T. Huxford, D.-B. Huang, S. Malek, and G. Ghosh, "The crystal structure of the IκBα-NF-κB complex reveals mechanisms of NF-κB inactivation," *Cell*, vol. 95, pp. 759–770, 1998.

[31] C. Nikolai and G. Madey, "Tools of the trade: A survey of various agent based modeling platforms," *Journal of Artificial Societies and Social Simulation*, vol. 12, no. 2, p. 2, 2009.

[32] I. Stamatopoulou, P. Kefalas, and M. Gheorghe, "Modelling the dynamics structure of biological state-based systems," *BioSystems*, vol. 87, pp. 142–149, 2007.

[33] M. Holcombe, "X-machines as a basis for dynamic system specification," *Software Engineering Journal*, no. 69-76, 1988.

[34] T. Balanescu, A. J. Cowling, H. Georgescu, M. Gheorghe, M. Holcombe, and C. Vertan, "Communicating stream X-machine systems are no more than X-machines," *Journal of Universal Computer Science*, vol. 5, no. 9, pp. 494–507, 1999.

[35] S. Coakley, R. Smallwood, and M. Holcombe, "From molecules to insect communities - how formal agent-based computational modelling is uncovering new biological facts," *Scientiae Mathematicae Japonicae Online*, vol. e-2006, pp. 765–778, 2006.

[36] M. Holcombe, S. Coakley, and R. Smallwood, "A general framework for agent-based modelling of complex systems," in *European Conference on Complex Systems (ECCS)*, Warwick, UK, September 2006.

[37] I. Foster, *Designing and Building Parallel Programs*. Addison Wesley, 1995, ch. Message Passing Interface.

[38] D. E. Kirschner, S. T. Chang, T. W. Riggs, N. Perry, and J. J. Linderman, "Toward a multiscale model of antigen presentation in immunity," *Immunological Reviews*, vol. 216, pp. 93–118, 2007.

[39] F. Carlotti, S. K. Dower, and E. E. Qwarnstrom, "Dynamic shuttling of nuclear factor κB between the nucleus and cytoplasm as a consequence of inhibitor dissociation," *Journal of Biological Chemistry*, vol. 275, pp. 41 028–41 034, 2000.

[40] L. Yang, K. Ross, and E. E. Qwarnstrom, "RelA control of IκBα phosphorylation," *Journal of Biological Chemistry*, vol. 278, pp. 30 881–30 888, 2003.

[41] J. C. Helton, "Uncertainty and sensitivity analysis for models of complex systems," in *Computational Models in Transport: Verification and Validation*, T. J. Barth, M. Griebel, D. E. Keyes, R. M. Nieminen, D. Roose, and T. Schlick, Eds. Heidelberg, Germany: Springer, 2008, pp. 207–228.

[42] K. Alden, M. Read, J. Timmis, P. S. Andrews, H. Veiga-Fernandes, and M. C. Coles, "Spartan: A comprehensive tool for understanding uncertainty in simulations of biological systems," *PLoS Computational Biology*, vol. 9, no. 2, p. e1002916, 2013.

[43] F. Carlotti, R. Chapman, S. K. Dower, and E. E. Qwarnstrom, "Activation of nuclear factor κB in single living cells," *Journal of Biological Chemistry*, vol. 274, pp. 37 941–37 949, 1999.

[44] A. Hoffmann, A. Levchenko, M. L. Scott, and D. Baltimore, "The IκB - NF-κB signaling module: Temporal control and selective gene activation," *Science*, vol. 298, pp. 1241–1245, 2002.

[45] F. J. Massey, "The Kolmogorov-Smirnov test for goodness of fit," *Journal of the American Statistical Association*, vol. 46, no. 253, pp. 68–78, 1951.

[46] A. Vargha and H. D. Delaney, "A critique and improvement of the CL common language effect size statistics of McGraw and Wong," *Journal of Educational and Behavioural Statistics*, vol. 25, pp. 101–132, 2000.