A Mathematical Model of CR3/TLR2 Crosstalk in the Context of Francisella tularensis Infection

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Abstract

Complement Receptor 3 (CR3) and Toll-like Receptor 2 (TLR2) are pattern recognition receptors expressed on the surface of human macrophages. Although these receptors are essential components for recognition by the innate immune system, pathogen coordinated crosstalk between them can suppress the production of protective cytokines and promote infection. Recognition of the virulent Schu S4 strain of the intracellular pathogen Francisella tularensis by host macrophages involves CR3/TLR2 crosstalk. Although experimental data provide evidence that Lyn kinase and PI3K are essential components of the CR3 pathway that influences TLR2 activity, additional responsible upstream signaling components remain unknown. In this paper we construct a mathematical model of CR3 and TLR2 signaling in response to F. tularensis. After demonstrating that the model is consistent with experimental results we perform numerical simulations to evaluate the contributions that Akt and Ras-GAP make to ERK inhibition. The model confirms that phagocytosis-associated changes in the composition of the cell membrane can inhibit ERK activity and predicts that Akt and Ras-GAP synergize to inhibit ERK.

Introduction

Receptor-mediated engagement followed by phagocytosis by professional phagocytes is the first critical step in microbial clearance or, in the case of intracellular pathogens, entry to a safe niche. The molecular mechanisms underlying phagocytosis are complex, usually involving more than one receptor and rapidly culminating in the combinatorial generation of a variety of biochemical signals along with rearrangement of the actin cytoskeleton to engulf the microbe [1]. There are substantial differences in cellular responses for almost every phagocytic receptor used, and complex interactions between receptors can be expected since a variety of ligands usually coat microbes. In this context, computational modeling becomes an essential tool through which experimentalists can enhance their understanding.

Complement Receptor 3 (CR3; CD11b/CD18), the major β2 integrin of phagocytic cells (monocytes, macrophages and neutrophils), provides a highly effective mode of entry for many microbes and has long been postulated to provide the microbe safe passage into macrophages in particular, since ligation of CR3 by complement-opsonized microbes does not uniformly trigger toxic host cell responses [2]. Many intracellular pathogens use CR3 to evade intracellular killing [3–9]. Still, CR3 is a notoriously enigmatic receptor, capable of conveying diverse and even opposing signals in response to distinct combinations of ligands [10–13] and often in concert with pattern recognition receptors (PRRs) such as Toll-like Receptors (TLRs). A mounting body of research suggests that β2 integrins are important regulators of TLR signaling [14–17]. The mechanisms by which CR3 regulates TLR signaling are an area of active research, in part because CR3/TLR crosstalk is implicated in the pathogenesis of several diseases.

Francisella tularensis is an extremely virulent intracellular pathogen of macrophages and potential bioweapon. Indeed, the bacteria may be aerosolized and inhalation of as few as ten bacteria can result in the fatal disease pneumonic tularemia [18–20]. In the lung, F. tularensis is rapidly phagocytosed by alveolar macrophages while suppressing their cytokine production. One mechanism the bacterium uses to accomplish this feat is to selectively engage only a few choice receptors. Although multiple types of receptors can mediate phagocytosis of Francisella, appreciable phagocytosis of the most virulent strains requires CR3 engagement by complement C3-opsonized bacteria [19,21,22]. In fact, CR3 is thought to be critical to the success of F. tularensis as an intracellular pathogen [18,19,21,23–26]. Cytokine production in response to Francisella comes almost exclusively from its stimulation of TLR2 [18,19,27]. As noted above, although TLR2 signaling is inflammatory, it is also subject to regulation by CR3 [15,17].

In what follows we construct a model of immediate membrane proximal signaling in response to F. tularensis. The model, which serves as a formal hypothesis, is shown to be consistent with the experimental results of S. Dai et al (unpublished data). Its implications are explored via numerical simulations.
Author Summary

In the current work we construct a highly contextual model of membrane-proximal crosstalk between the ERK and PI3K cascades that is initiated through contact with F. tularensis. The model is used to test the hypothesis that phagocytic signaling downstream from CR3 is responsible for an early inhibition of ERK activity, which is seen subsequent to contact with the complement C3-opsonized Schu S4 strain of F. tularensis. In addition, the model predicts that Akt and Ras-GAP synergize to inhibit ERK. To the best of our knowledge this is the first mathematical model to investigate crosstalk between these pathways within the context of infection. By providing a comprehensive picture of the initial host-pathogen interaction, and pathogen-induced crosstalk between cell surface receptors in particular, this model is important in the context of microbial immunopathogenesis.

Results

The response of macrophages to F. tularensis depends heavily on the presence of complement. Dai et al found that complement opsonization substantially decreases cytokine production in response to F. tularensis, and identified key players in this immunosuppressive pathway (unpublished data). Their results are summarized as follows: ERK activation in response to F. tularensis is suppressed by complement-mediated signaling through CR3. Furthermore, ERK inhibition is rapid, being evident just 5 minutes post infection. In addition to suppressing ERK activation, CR3 ligation induces the rapid activation of Lyn kinase, which functions to inhibit cytokine production in response to F. tularensis. Finally, TLR2 and CR3 signaling intersect at the PI3K/Akt pathway, and the two receptors cooperate to support a complement dependent enhancement of Akt activity in response to F. tularensis.

The observations of Dai et al were supplemented with existing literature to construct a model of the very earliest signaling events that occur in response to F. tularensis infection. In this model TLR2-induced ERK activation occurs through a previously characterized MyD88 independent pathway in which Rac and Ras associate with the cytoplasmic domain of TLR2 and undergo rapid activation in response to bacterial stimuli [28,29]. The pair then cooperate to activate Raf which leads to ERK activation [30]. Activation of the PI3K/Akt pathway by TLR2, meanwhile, is mediated by Raf [29]. In our model of complement-mediated signaling, CR3 ligation leads to the rapid activation of Lyn which subsequently activates PI3K [31,32]. PI3K activation leads to a buildup of PtdIns(3,4)P (abbreviated here as PI(34)P) and PtdIns(3,4,5)P (PI(345)P) at the phagosomal cup (consistent with Clemens et al [21]), which antagonizes ERK. Specifically, Akt, which is activated after binding to these lipids, phosphorylates Raf at Ser 259 thereby inhibiting its association with Ras [33], and these lipids recruit GAPs, which deactivate both Rac and Ras [34–37]. The model also includes additional interactions which may detract from its ability to explain complement-mediated ERK inhibition. In particular, PI(34)P and PI(345)P can also recruit the Rac-GEF, Vav, [38], which initiates Rac activation, and Lyn can enhance Raf signaling [39].

Figures 1 and 2 provide a schematic description of membrane proximal TLR2 and CR3 signaling in response to Francisella tularensis. Figure 3 synthesizes and simplifies the CR3 and TLR2 signaling networks. In particular, in the interest of simplicity, our model uses the concentration of active Raf as a proxy for the concentration of active ERK. Although significant feedback from ERK to Raf could alter the model’s dynamics, this simplification seems reasonable in view of the following facts: ERK mediated feedback is not significant until later time points [40], and the proposed mechanisms of CR3-mediated ERK inhibition target molecules that lie upstream of ERK itself.

Because the network is complex, we resort to mathematical modeling in order to deduce function from structure, that is, in order to check that the model is in fact consistent with experimental observations. The model equations, based on Figure 3, give the local concentrations of various signaling molecules in the vicinity of the immunological synapse. In these equations [Tl] represents ligand bound TLR2 heterodimers; [C3] represents ligand bound CR3; P represents 3 phosphoinositides (i.e. both PI(345)P and PI(34)P); S represents active Ras; A represents active Akt; L represents active Lyn; R represents active Rac; and F represents active Raf. The model equations are given in the section Materials and Methods where they are supplemented by Tables 1 and 2 of parameter values.

We next present the results of numerical simulations on the mathematical model. In order to test the model’s consistency we compare the results of simulations in the presence and absence of complement.

Figures 4–7 show how the concentrations of signaling molecules change through time, when the bacteria are not opsonized, but carry a low density TLR2 ligand. In the absence of complement the model predicts that Francisella will elicit a slight increase in 3 phosphoinositides and a substantial increase in active Ras, Rac and Raf. As Raf is a proxy for ERK, we see that in the absence of complement the model is in agreement with the experimental results of S. Dai et al (unpublished data). In particular, TLR2 signaling stimulates both the ERK and PI3K pathways.

Figures 8–11 show how the concentrations of the above signaling molecules change through time in response to opsonized Francisella. A comparison of Figures 7 and 11 shows that the model proposed in Figure 3 is consistent with the experimental data, and in particular, is capable of explaining CR3-mediated ERK inhibition. Specifically, Figure 11 shows that F. tularensis induced Raf stimulation is markedly inhibited in the presence of complement.

Having confirmed that, as parameterized, the mathematical model is consistent with complement-mediated ERK inhibition we performed an uncertainty and sensitivity analysis in order to assess how uncertainty in the model’s parameters impacts its consistency with experimental data. In particular, as some of the model’s parameters are uncertain, we wished to know if complement-mediated ERK inhibition is robust to variations in the model’s parameters, i.e. is the model consistent with experimental result over a wide range of parameter values. We ran 10,000 numerical simulations in which the model’s parameters were varied according to a Latin hypercube sampling scheme. The sensitivity of the model’s output (as measured by the concentration of active Raf at 5 minutes post infection) to uncertainty in the parameters was then quantified through a partial rank correlation coefficient [41] that is, we calculated the partial correlation coefficients of the rank transformed data. This provides a robust sensitivity measure of nonlinear but monotonic relations between the parameters and the output [41]. A detailed description of the process is presented in [41]. The results of this analysis are presented in Table 3. Figures 12–15 show scatter plots of rank transformed Raf concentration at 5 minutes versus rank transformed parameter values for a few of the most important parameters. Importantly, b(0), the concentration of complement on the surface of the bacteria shows a significant negative correlation with the concentration of active Raf, i.e. the model is consistent with experimental data over a wide range of parameter values.

In addition to showing that the model’s consistency is robust to parameter variations, the uncertainty and sensitivity analysis can
be used to identify molecules and parameters that are important for complement-mediated ERK inhibition. Although many of the parameters show a small but significant correlation with the concentration of Raf at 5 minutes post infection, the strongest negative correlations are associated with the parameters for GAP-mediated Ras deactivation, namely \( k_{12} \) and \( C_{SGAP} \), and \( k_{17} \), the rate of Akt catalyzed Raf phosphorylation. This suggests that both Ras-GAPs and Akt are important regulators of Raf. To analyze the relative importance of Ras-GAPs and Akt in CR3-mediated Raf inhibition, we ran numerical experiments in which the \( k_{17} \) and \( k_{12} \) were reduced to 10% of their baseline values, and the activity of Raf 5 minutes post infection with opsonized \( F.tularensis \) was compared to that when the parameters were set to their baseline values. A ten percent reduction in \( k_{17} \) resulted in a 8.5 fold increase in the concentration of Raf at five minutes post infection, whereas a ten percent reduction in \( k_{12} \) resulted in a 5.5 fold increase in Raf activation 5 minutes post infection. When both parameters were reduced to 10% of their baseline values, the model predicts a 31.5 fold increase in Raf activation at 5 minutes post infection, i.e. Akt and Ras-GAPs synergize to inhibit Raf signaling. The uncertainty and sensitivity analysis also indicates that CR3-mediated ERK inhibition is sensitive to concentrations of key cellular proteins. In particular, it indicates that over expression of TLR2 or Ras, or reduced expression of Ras-GAP will dampen CR3-mediated ERK inhibition.

**Discussion**

Crosstalk between the complement and TLR systems is an essential determinant of the early immune response to pathogens [42]. In this paper we have presented a mathematical model of TLR2/CR3 crosstalk to test the hypothesis that CR3 ligation fosters the robust production of PI(34)P and PI(345)P which is incompatible with TLR2-mediated Raf signaling. Our own experimental data, in addition to the observation that less virulent strains of \( F.tularensis \) undergo PI3K-independent phagocytosis [43], lends support to the hypothesis that the membrane’s phosphoinositide content during phagocytosis is a critical determinant of cytokine production in response to infection.

In defining crosstalk between CR3 and TLR2 within the context of \( F.tularensis \) infection, we also defined crosstalk between the ERK and PI3K signaling cascades within this context. Although several computational analyses of differential ERK signaling and ERK/PI3K crosstalk have already been performed [44–46], to the best of our knowledge this is the first mathematical model to investigate crosstalk between these two pathways within...
According to our model, CR3-mediated inhibition of TLR2 signaling initiated by Francisella is different from CR3-mediated inhibition of TLR2 signaling initiated by Porphyromonas gingivalis, wherein IL-12 production is inhibited by ERK [42]. This difference may stem from the fact that P. gingivalis binds to CR3 via its natural fimbriae, while Francisella cannot efficiently bind CR3 unless opsonized with C3bi. Or, it could also be that P. gingivalis engages other receptors that augment or interfere with CR3/TLR2 crosstalk. However, complement-receptor-mediated PI3K activation has been observed to inhibit TLR-induced IL-12 production in response to Hepatitis C virus [47]. Furthermore, as C3bi ligation of CR3 is known to inhibit IL-12 production in response to a variety of stimuli [48], and support the pathogenesis of a variety of diseases [3–9], it seems likely that the model proposed here is applicable to a variety of pathogens.

Materials and Methods

In this section we briefly describe the mechanisms through which the molecules of our model are regulated. Each verbal description is followed by a mathematical one. When coupled together, these mathematical descriptions form a model of membrane proximal receptor signaling in response to F. tularensis. Recruitment, the process through which diverse proteins translocate to a common location, is a central aspect of receptor signaling. Indeed, receptor-mediated recruitment concentrates activators, enzymes and substrates to specific locations on the inner leaflet of the cell membrane. In order to model recruitment we allow local concentrations of molecules at the immunological synapse to far exceed membrane and cellular concentrations. For example, receptors may be recruited to the immunological synapse through their ability to bind bacterial ligands. In this case, the maximal concentration of receptors at the synapse is bounded by $\frac{R}{a_2}$, where $R$ is the concentration of the receptor in the membrane, and $a_2$ is the ratio of the volume of the synapse to the volume of the membrane. In this paper the term synapse loosely refers to the region of contact between bacteria and macrophage. In the context of the experiments that we seek to describe the multiplicity of infection is necessarily high, and so we take $a_2 = .1$. Similarly, when a cytoplasmic protein is recruited to the synapse, the maximal concentration of the protein at the synapse is given by $P$, where $P$ is the concentration of the protein in the cell and $a_1$ is the ratio of the volume of the synapse to the volume of the cell. Assuming that the macrophage is approximately spherical, that its membrane proximal region has a depth of 5nm [49], and that an alveolar macrophage has a total diameter of 20 \( \mu \)m [50], we find that $a_1 = 1.5 \times 10^{-4}$.

TLR2

TLRs are pattern recognition receptors (PRRs) that detect and respond to a broad range of pathogen products including bacterial lipopolysaccharides [27,51]. The molecular mechanisms that enable TLRs to respond to bacterial ligands are extremely complex, and can involve crosstalk of TLR heterodimers as well as multiple accessory proteins [52]. In particular, the recognition of pathogen associated molecular patterns (PAMPs) by TLR2, the most promiscuous of all the TLRs, is an involved process [53]. TLR2 is expressed on the cell surface where it constitutively associates with either TLR1 or TLR6 [54,55]. TLR1 mediates the recognition of triacylated lipopolysaccharides while TLR6 mediates the recognition of diacylated lipopolysaccharides. We assume that the primary source of TLR1/2 stimulation is through direct contact with the bacterial membrane. Indeed TLR2 is

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The diagram illustrates the recruitment of molecules to the synapse, involving Lyn, PI3K, Rac, Vav, GAP, and PIP3, as well as the concentration of Cb and Rac. The model describes how these components interact to regulate signaling pathways such as PI3K and ERK, and how they are activated or inhibited in response to infection.
recruited to developing phagosomes [56]. Ligand binding to TLR2 and subsequently TLR1 or TLR6 then induces a crosslinking of these receptors that initiates signal transduction [55,57]. The diagram is as follows:

We denote by \( \frac{d[T]}{dt} \) the total concentration of TLR1 ligand in the immunological synapse, and by \( [T] \) the concentration of the active signaling complex in the immunological synapse. Our quantitative description of \( TLR2 \) activation is as follows:

\[
\frac{d[T]}{dt} = k_1[I](C_T - a_2[T]) - k_2[T] \tag{1}
\]

\[
\frac{d[I]}{dt} = -k_1[I](C_T - a_2[T]) + k_2[T] \tag{2}
\]

\[
[T](0) = 0, \tag{3}
\]
In [52] it was shown that TLR2 binds lipoproteins directly with a 1:1 stoichiometry, which was previously described dimensions, we determined the membrane proximal concentration of TLR1/2 heterodimers is approximately 3 μM. Under normal conditions, macrophages likely express lower levels of TLR1/2 than do monocytes [59].

CR3 and Lyn

CR3 has an active conformation that binds to and mediates the phagocytosis of C3bi-opsonized particles and an inactive conformation that does not [32,60]. As a result, inside-out signaling through receptors such as TLR2 is often viewed as important for CR3-mediated phagocytosis [15]. In macrophages, however, CR3 readily binds to C3bi-opsonized particles [61]. As a result, the following equations will be used to determine the concentration of CR3-C3bi complexes in the immunological synapse ([Cb]).

\[
\frac{dC_b}{dt} = k_3 [b](C_C - a_2[C_b]) - k_4[C_b] \tag{4}
\]

\[
\frac{db}{dt} = -k_3 [b](C_C - a_2[C_b]) + k_4[C_b] \tag{5}
\]

where [b] denotes the concentration of C3bi in the synapse and \(C_C\) denotes the membrane proximal concentration of CR3, which we assume is a constant.

Cai et al [60] used a soluble monomeric probe, C3bi-AP, in order to estimate the on rate, \(k_3\), and the equilibrium dissociation constant, \(K_d\), of C3bi for active CR3. We used \(k_3\) and \(K_d\) to calculate \(k_4\). In vivo C3bi-CR3 complexes likely dissociate more quickly due to uncharacterized active cellular processes.

Ross et al [62] used labeled CR3 specific mAbs to determine the molecules of CR3 per alveolar macrophage. Assuming the previously described dimensions, we determined the membrane proximal concentration of CR3, \(C_C\).

Src family kinases, of which Lyn is a member, are rapidly activated in response to integrin ligation [32]. Although the precise mechanisms of integrin-mediated src kinase activation are...
unknown, some studies support a model in which inactive integrins associate constitutively with Src kinases which are then activated through trans-phosphorylation as a result of integrin clustering [32]. For simplicity we will assume that each molecule of CR3 is associated with a single molecule of Lyn. Under this assumption (7)–(8) determines the concentration of active Lyn ($L^*$).

$$\frac{d L^*}{dt} = 2k_7(CR3 - L^*)^2 + k_7(CR3 - L^*)L^* - k_8 L^*, \quad (7)$$

$$CR3 = Cb + C_C - a_3 Cb \quad (8)$$

where the first term is the rate of Lyn activation through the juxtaposition of two inactive CR3-associated Lyn molecules, and the second term is the rate of Lyn activation due to the juxtaposition of one active CR3-associated Lyn molecule and one inactive CR3-associated Lyn molecule.

Although we observe rapid activation of Lyn in response to Francisella, the precise rates of Lyn activation and deactivation within this context are not known. Several in vitro studies, however, enable us to estimate $k_7$, the rate of trans-phosphorylation [63,64]. The rate of dephosphorylation is then chosen to ensure that in resting cells the concentration of phospho-Lyn is low [65]. Since the basal concentration of phospho-Lyn is inversely related to the ratio $\frac{k_8}{C_C k_7} = T_{CR3}$, we assume that $T_{CR3}$ is large so that the basal concentration of phospho-Lyn is small.

Akt

The activity of Akt is regulated by multiple kinases, phosphatases and lipids. In resting cells Akt is sequestered in the cytoplasm. Upon stimulation Akt translocates to the membrane where it achieves full activation through phosphorylation at Ser and Thr [66]. In particular, $P_{345}$ and $P_{34}$ coordinate AKT activation by recruiting Akt and its kinase PDK-1 to the plasma membrane [67]. $P_{345}$ and $P_{34}$, however, are not equivalent in this respect, as Akt binds $P_{345}$ with slightly greater affinity than $P_{34}$, and Ser phosphorylation of Akt requires $P_{34}$ [67]. Although the majority of Akt targets are cytosolic, our focus is on the negative regulation of membrane proximal Raf by Akt. For this reason our model tracks the concentration of membrane bound Akt which we denote by $A^*$. Because Akt activation is a complex process involving multiple steps of undetermined significance we resort to a simplified model, in which PI[345]P and PI[34]P are treated as equivalent and the activity of Akt at the membrane is approxi-

Figure 4. Ras activity in response to unopsonized Francisella. (t(0) = 200 μM, b(0) = 0).

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mated by the concentration of membrane bound Akt. Assuming that the concentration of unbound PI(345)P and PI(34)P is approximately equal to the total concentration of PI(345)P and PI(34)P, we derive the following equation for $A/C_3$ at steady state:

$$A^*/C_3 \sim CAP_3 K_1 a_1 P_3,$$

where $C_A$ is the total concentration of Akt in the macrophage and, $P_3$ is the concentration of PI(345)P and PI(34)P at the immunological synapse, and $K_1$ is the equilibrium dissociation constant of the Akt-PI(345)P complex. Given the rapid translocation of Akt to the membrane in response to 3PI production we approximate $A^*(t)$ as

$$A^*(t) = \frac{C_A P_3(t)}{K_1 + a_1 P_3(t)}.$$

We set the parameter $K_1$ to be equal to the equilibrium dissociation constant for the Akt PH domain-PI(345)P complex which was measured in [68] through surface plasmon resonance. As we were unable to find a quantitative estimate of the cellular concentration of Akt, $C_A$, to be equal to that of PC12 cells, which was measured in [69].

**Rac and Ras**

Rac and Ras are examples of GTPases which are proteins that cycle between an inactive GDP bound form and an active GTP bound form. GTPase activity is tightly regulated by guanosine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and in some cases guanosine dissociation inhibitors (GDIs) [70]. In resting cells GTPases are maintained in their inactive GDP bound form by their slow intrinsic rate of guanosine nucleotide dissociation. Upon stimulation, GEFs activate GTPases by accelerating the dissociation of GDP [70]. Furthermore, because the intrinsic rate of GTPases hydrolysis is also extremely slow, GTPase deactivation is mediated by GAPs that catalyze the reaction [70].

Rac is a GTPase of the Rho family that is activated by both CR3 and TLR2 regulates a variety of cellular processes including cytoskeleton rearrangements and cytokine production [30,70,71]. Multiple GAPs and GEFs contextualize Rac’s response to stimuli. In PC12 cells, a positive feedback loop, involving Vav, Rac, and PI3K maintains Rac activity in response to NGF [72]. Indeed, studies indicate that PI(345)P enhances Vav’s GEF activity by

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**Figure 5. Rac activity in response to unopsonized Francisella.** $l(0)=200 \ \mu M, b(0)=0$. doi:10.1371/journal.pcbi.1002757.g005
disrupting inhibitory intramolecular interactions [38]. Since PI(345)P production is required for CR3 mediated phagocytosis of Francisella [21], and Vav is responsible for Rho GTPase activation downstream of CR3 [71], it seems likely that a similar feedback loop is operative in this context. In addition to phosphoinositide binding, Vav is regulated through phosphorylation [73]. As the CR3 effector Lyn, can both phosphorylate Vav [74], and assist in the activation of PI3K [75] we propose a model in which Lyn induces a positive Vav/Rac/PI3K feedback loop. Meanwhile, in response to bacteria, TLR2 activates both Ras and Rac [28,29,76]. Although the precise mechanisms of activation are unknown Ras can be activated through direct association with TLR2 [29], and so we propose a model in which TLR2 activates Ras, which then activates Rac through the Rac GEF TIAM-1 [77]. These two modes of Rac activation are distinguished by their relation to PI3K. CR3-mediated Rac activation is PI3K-dependent, while TLR2-mediated Rac activation is PI3K-independent. In either case, however, both Rac and Ras are subject to PI3K-dependent deactivation, since both molecules are also regulated by PI3K sensitive GAPs [34,35,78].

Our model of Rac activity assumes that the concentrations of active Vav and GAP depend on the concentration of Lyn and PI(345)P as follows:

\[
V_{AV} = \frac{CV}{d_1 \left( \frac{L^*}{L^* + T_{Pyn}} \right) \left( \frac{P_3}{P_3 + K_3} \right)}
\]

\[
GAP = \frac{CP_{GAP}}{d_1P_3 + K_2}
\]

As PI(45)P has been shown to inhibit the GEF activity of Vav, we consider fully active Vav to be that which is both bound to PI(345)P and phosphorylated by Lyn. The fraction of Vav bound to PI(345)P is determined by \(K_3\), the equilibrium dissociation constant of the PI(345)P-Vav complex. Although precise measurements of the quantity were not available the equilibrium dissociation constant for the PI(45)P-Vav complex was measured in [3-4 \(\mu M\)], and the affinity of Vav for PI(345)P is known to be greater than the affinity of Vav for PI(45)P [79]. Hence we estimate \(K_3 = 0.7 \mu M\). We were unable to determine the cellular concentration of Vav. We assume it is somewhat less than the concentration of Rac.
We were unable to determine the rates of Vav phosphorylation and dephosphorylation. As a result the fraction of phosphorylated Vav is determined by the unknown parameter $T_{lyn}$ which is varied in the course of our numerical simulations. The concentration of Rac GAP at the membrane is determined by the equilibrium dissociation constant of the Gap-PI(345)P complex, $K_2$. As we were unable to find a measurement of $K_2$ we choose this parameter so that the simulated time course of Rac activation during CR3-mediated phagocytosis would resemble the experimentally determined time course of Rac activation during Fc-Receptor-mediated phagocytosis [80]. Although PI(345)P stimulated GAPs are responsible for regulating the activity of Rac in macrophages, the cellular concentration of these GAPs was not available [81]. For this reason we assume that the concentration of PI(345)P responsive Rac-GAP is somewhat less than the concentration of Rac. For simplicity we treat TIAM-1 and Vav as equivalent Rac GEFs, and assume that the concentration of TIAM-1 is equal to the concentration of active Ras. This leads to the following model of Rac activation

$$\frac{dR}{dt} = k_{13}(S + VAV) \frac{R}{R + K_7} + k_{18}R - k_{14}GAP \frac{R}{R + K_8} - k_{15}R' \quad (11)$$

where the constant $C_R$ denotes the cellular concentration of Rac and $C_{R*}$ is the membrane proximal concentration of Rac under the assumption that all of the cellular Rac is concentrated at the membrane. The cellular concentration of Rac in neutrophils was measured in [82]. This is likely a good approximation to the actual concentration in macrophages. The rate parameters $k_{13}$ and $K_7$ characterize TIAM-1 catalyzed nucleotide dissociation from Rac2 as determined in [83]. The intrinsic rate of Rac2 hydrolysis $k_{15}$ and the rate of spontaneous GDP dissociation $k_{18}$ were measured in the same work. We were unable to find measurements for the parameters $k_{14}$ and $K_8$ that characterize the GAP-catalyzed hydrolysis of GTP by Rac, and so, we estimate these parameters using the parameters from the Cdc42GAP-catalyzed hydrolysis of GTP by Cdc42 [84]. This is a reasonable approximation since Cdc42 is closely related to Rac.

Ras is a GTPase that mediates ERK activation by recruiting Raf to the plasma membrane [30]. Since the mechanism through which TLR2 activates Ras is unknown we treat TLR2 as a Ras

$$R = \frac{C_R}{10a_1} - R'$$

Figure 7. Raf activity in response to unopsonized Francisella. ($b(0)=200 \ \mu M$, $b(0)=0$).

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GEF.

\[ \text{RAS}_{\text{GDP}} + [Tl] \rightleftharpoons \text{RAS}_{\text{GTP}} + [Tl] \]

We model the GAP induced deactivation of Ras as an enzymatic process in which GAP catalyzes the hydrolysis of GTP to GDP.

\[ \text{RAS}_{\text{GTP}} + \text{RasGAP} \rightleftharpoons [\text{RAS}_{\text{GTP}} + \text{RasGAP}] \rightarrow \text{RasGDP} + \text{RasGAP} \]

Combing these two processes, i.e. the exchange of GDP for GTP and the hydrolysis of GTP to GDP, we obtain the following equation for Ras.

\[
\frac{dS^*}{dt} = k_9 [Tl] \frac{S}{S + K_9} + k_{10} S - k_{11} S^* - k_{12} \text{SGAP} \frac{S^*}{S^* + K_6}
\]

\[ \text{SGAP} = C_{\text{SGAP}} \frac{(P_3)}{a_1 P_3 + K_4} \]

where the constant \( C_S \) denotes the total cellular concentration of Ras, and \( \frac{C_S}{10a_1} \) is the membrane proximal concentration of Ras under the assumption that all of the cellular Ras is concentrated at the membrane. We take the cellular concentration of Ras in macrophages to be equal to that in NIH3T3 fibroblasts [85]. The parameter \( k_{10} \) represents spontaneous dissociation of Ras-GDP. This parameter was determined in vitro [86]. The parameters \( k_9 \) and \( K_9 \) which determine the kinetics of Ras activation by TLR2 are not known. We take these parameters from a study on the kinetics of Ras activation by the exchange factor Cdc25 [87]. The parameters, \( k_{12} \), and \( K_6 \), which catalyze GAP catalyzed hydrolysis were measured in [88]. The equilibrium dissociation constant, \( K_4 \), between the Ras GAP and PI(345)P was reported in [37]. Although PI(345)P sensitive Ras-GAPs are expressed by macrophages [89,90], we were unable to find estimates of their levels of expression in macrophages. Hence, we assume that the concentration of Ras-GAP is somewhat less than the concentration of Ras.

Figure 8. Ras activity in response to complement C3-opsonized Francisella. (\( t(0) = 200 \) μM, \( b(0) = 100 \) μM).

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Phosphoinositides

Phosphatidylinositol, or PtdIns, is a membrane lipid that mediates signal transduction between cell surface receptors and the cytosol. Its inositol head group contains several free hydroxyls which can be phosphorylated to generate a variety of distinct derivatives termed phosphoinositides [91]. For brevity we abbreviate PtdIns by PI and the phosphoinositides by PI()P, where the terms in the parentheses correspond to the phosphates’ positions. For example PtdIns(4,5)P is abbreviated as PI(45)P. Distinct phosphoinositides transduce distinct signals depending on the location and number of phosphates they contain. In particular, several pivotal proteins bind to their partner phosphoinositides with high specificity [92,93]. This allows phosphoinositides to determine the activity of these proteins, by localizing them to cell membranes. In several instances, phosphoinositides can also serve as allosteric activators.

In vitro the inositol head group of PtdIns can be phosphorylated at positions 3, 4, and 5 [91]. The membranes of resting cells contain minute quantities of PI(4)P and PI(45)P, while PI(345)P and PI(34)P are virtually undetectable [92,93]. Upon stimulation, however, a variety of cell surface receptors, including CR3, induce a rapid increase in the concentration of the 3 phosphoinositides which are important mediators of cytokine production, phagocytosis and chemotaxis [91,94]. In particular, the generation of 3 phosphoinositides is an essential step in the phagocytosis of bacteria [95,96].

Experiments conducted in vivo suggest that phosphoinositide production proceeds according to the following diagram [97]:

\[ PI \rightarrow PI(4)P \rightarrow PI(45)P \rightarrow PI(345)P \rightarrow PI(34)P \rightarrow PI(3)P \rightarrow PI; \]

in particular, hydrolysis of PI(345)P is the primary mode of PI(34)P production. Stimulation of TLR2 and CR3 is known to induce phosphoinositiode 3 and phosphoinositiode 5 kinases as well as phosphoinositiode 5 phosphatases. The Src kinase Lyn and the small GTPase Rac both contribute to the activation of PI3K. Rac-GTP contributes to PI3K activation by binding to its regulatory subunit [29], while Lyn activates PI3K through Cbl [31,32]. As the parameters with which Lyn and Rac activate PI3K are not known, we take the concentration of active PI3K to be a function of the concentration of active Lyn and Rac,

\[
P_{I3K} = \frac{C_{PI3K}}{a_1} \frac{R^*}{T_{rw} R_0 (1 + \frac{L^*}{T_{hy} L_0})} \left( 1 + \frac{R^*}{T_{rw} R_0} + \frac{L^*}{T_{hy} L_0} \right) + \frac{L^*}{T_{hy} L_0 (1 + \frac{R^*}{T_{rw} R_0} + \frac{L^*}{T_{hy} L_0})}, \tag{16} \]

Figure 9. Rac activity in response to complement C3-opsonized Francisella. (\(a(0) = 200 \ \mu M\); \(b(0) = 100 \ \mu M\)).

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where \( \text{CPI}_3 \) is the cellular concentration of PI3K, which we assume to be a constant, and \( T_{\text{rac}}, T_{\text{lyn}} \) are the factors by which the levels of active Rac and Lyn must be elevated over their basal values, \( R_0 \) and \( L_0 \) respectively, in order to support half maximal activation of PI3K. For simplicity we will assume that the two species of 3 phosphoinositides PI(34)P and PI(345)P are equivalent and we will neglect PI, PI(4)P, and PI(3)P so that in the model PI(34)P and PI(345)P are synthesized directly from PI(45)P and PI(45)P is the direct product of PI(345)P and PI(34)P degradation. As this part of our model is largely conceptual the values of the parameters \( T_{\text{rac}} \) and \( T_{\text{lyn}} \) are unknown and are varied in the course of the numerical simulations. The cellular concentration of PI3K, \( \text{CPI}_3 \), is taken from a study NIH3T3 fibroblasts [69]. The basal activity of Rac and Lyn, \( R_0 \) and \( L_0 \) is determined by the model. With these assumptions, the production and degradation of the PI(34)P and PI(345)P are described by the following equation,

\[
\frac{dP_3}{dt} = k_5(\text{PI3K})P_2 - k_6P_3 \quad (17)
\]

\[
P_2(0) = 5 \mu M \quad (18)
\]

\[
P_3(0) = 5 \mu M \quad (19)
\]

\[
P_2(t) + P_3(t) = P_2(0) + P_3(0) \quad (20)
\]

where \( P_3 \) denotes the concentration of PI(34)P and PI(345)P and \( P_2 \) denotes the concentration of PI(45)P, and the total concentration of PI(45)P, PI(34)P, and PI(345)P is assumed to be a constant.

The concentration of PI(45)P, PI(34)P and PI(345)P in resting neutrophils [97] was used to determine the initial conditions \( P_2(0) \) and \( P_3(0) \). We were unable to find estimates of the parameters \( k_5 \) and \( k_6 \) which determine the kinetics of CR3-stimulated PI(345)P formation in macrophages. A study of PI(345)P formation during Fc\( \gamma \) Receptor-mediated phagocytosis in macrophages reported rapid and substantial accumulation of PI(345)P, with maximal levels reached 30–90 seconds after stimulation [96]. Furthermore the rate of PI(345)P degradation during this process, \( k_6 \), was estimated to be somewhat greater than 0.14 s\(^{-1}\). Initial conditions (18)–(19) along with (20) and \( k_6 \) enable us to estimate \( k_5 \).
Figure 11. Raf activity in response to complement C3-opsonized *Francisella*. ($h(0) = 200 \ \mu\text{M}$, $b(0) = 100 \ \mu\text{M}$).

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Figure 12. Scatter plot of rank transformed Raf concentration at 5 minutes post infection versus the rank transformed value of the parameter $k_{17}$.

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Figure 13. Scatter plot of rank transformed Raf concentration at 5 minutes post infection versus the rank transformed value of the parameter $k_{12}$. 
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Figure 14. Scatter plot of rank transformed Raf concentration at 5 minutes post infection versus the rank transformed value of the parameter $b(0)$. 
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In its inactive state Raf is sequestered by 14-3-3 binding proteins, the association of which is supported by phosphorylation at Ser 259 \[98,99\]. Dephosphorylation of Raf at Ser 259 precedes Raf activation. Similarly, phosphorylation of Raf at Ser 259 precedes Raf deactivation \[98,99\]. Although the kinase responsible for phosphorylating Ser 259 in this context is unknown, Akt has been demonstrated to regulate Raf through phosphorylation at Ser 259 in several systems \[33,98\], and so, we assume that this is the case. Activation of Raf is a multistep process. Raf is recruited to the membrane by Ras-GTP, to which it binds with high affinity. This recruitment places Raf in close proximity to kinases that activate Raf through phosphorylation \[100\]. One such kinase, Pak, is activated after binding to active Rac \[30\] The Src family kinase Lyn can also activate Raf \[39\]. Because many of the parameters that describe the transition between these many states of Raf are unknown we consider a simple model in which Raf exists in three states: active membrane proximal Raf, \(F^*\), inactive and free Raf, \(F\), and inactive Raf that is bound to Ras. For simplicity we assume that recruitment of Raf by Ras-GTP is rapid, so that a the fraction of inactive Ras-bound Raf is

\[ \frac{S^*}{a_1S^* + K_5}. \]  

The equilibrium dissociation constant for the Raf RBD-Ras complex, \(K_5\), was measured in \[100\]. In standard Michaelis-Menten kinetics, the substrate concentration is generally assumed to be in excess of the enzyme concentration. Because cellular concentrations of Raf are extremely low, this assumption is unlikely to hold for Raf. Hence in our model of Raf activation and deactivation we employ a modified Michaelis-Menten type model in which Raf is limiting so that the reaction rates depend linearly on the concentration of Raf. Furthermore, since the differences between Lyn- and Pak-catalyzed phosphorylation of Raf are unknown we assume that the concentration of active Pak is proportional to the concentration of active Rac, and that Pak and Lyn are equivalent. With these assumptions, the concentration of active Raf is determined by the following equation.

\[ \frac{dF^*}{dt} = k_{16} \frac{L^* + c_1 R^*}{L^* + c_1 R^* + K_{10} a_1 S^* + K_5} \frac{S^*}{S^*} \]  

where \(C_F\) denotes the cellular concentration of Raf, which we assume is a constant, and \(c_1\) determines the fraction of Pak that is bound to Rac and therefore active. We were unable to find the concentration of Raf in macrophages or closely related cells and have estimated \(C_F\) from data on COS cells \[69\]. The parameter \(c_1\) is unknown. We set its baseline value to one and vary it in the course of numerical experiments. We were unable to find the specific parameters \(k_{17}\) and \(K_{12}\) that determine the kinetics with which Akt phosphorylates Raf in macrophages. Instead we estimate these.
negative feedback. Indeed ERK was shown to inhibit Raf kinase-related Src family kinases catalyze the phosphorylation of their parameters to be equal to the parameters that determine the kinetics of the reaction between Akt and a small peptide substrate [101] under the assumptions that the concentration of ATP in a resting pig alveolar macrophage is equal to that of resting human alveolar macrophage [102], and that the volume of a pig alveolar macrophage is 1.411 μm³ [103]. The parameters which determine the rate of Raf activation by Lyn and Pak are also unknown. We estimate them from knowledge of the kinetics with which closely related Src family kinases catalyze the phosphorylation of their substrates [104,105], and the kinetics with which Raf is phosphorylated on the plasma membrane of living cells [106].

We should note that Raf is also subject to ERK-mediated negative feedback. Indeed ERK was shown to inhibit Raf kinase activity by phosphorylating Raf at several sites [40]. Although this feedback plays an important role in determining the duration of Raf signaling, it does not significantly influence the activity of Raf at early time points [40]. Since our intent is only to describe the very earliest of signaling events, our model neglects this feedback.

Author Contributions
Conceived and designed the experiments: RL SD LSS AF. Performed the experiments: RL AF. Analyzed the data: RL SD LSS AF. Wrote the paper: RL SD LSS AF.

Table 3. PRCC values.

| Parameter | Range | PRCC Value | Parameter | Range | PRCC Value |
|-----------|-------|------------|-----------|-------|------------|
| k₁        | [0.01, 1] μM⁻¹ s⁻¹ | 0.209 | k₅        | [1.0, 2] μM | 0.0004 |
| k₂        | [3.5, 35] (10⁻⁴ s⁻¹) | -0.006 | C_F      | [0.01, 2] μM | 0.1722* |
| k₃        | [0.01, 1] μM⁻¹ s⁻¹ | -0.0160 | C_GAP    | [0.01, 0.5] μM | -0.3042* |
| k₄        | [125, 12.5] (s⁻¹) | 0.129 | C_F      | [0.01, 1] μM | -0.1336* |
| k₅        | [0.001, 14] (s⁻¹) | -0.0187 | P₈₀(0)   | [5.5] μM | -0.3005* |
| k₆        | [0.0005, 0.05] μM⁻¹ s⁻¹ | 0.142 | T₉₁ₐ       | [100, 500] | -0.0080 |
| k₇        | [3.5, 39] (s⁻¹) | 0.0024 | T₈₈       | [100, 500] | -0.0080 |
| k₈        | [1.55, 6.2] (10⁻⁵ s⁻¹) | -0.0118 | c₁       | [1] | 0.0524* |
| k₉        | [27, 27] (10⁻⁴ s⁻¹) | -0.0024 | h(0)     | [1,2000] μM | 0.0432* |
| k₊        | [396, 36.9] (s⁻¹) | -0.3150 | K₄       | [1,2000] μM | -0.2498* |
| k₋        | [0.0706, 7.06] (s⁻¹) | 0.1904* | K₁       | [2950, 1.18] μM | 0.0010 |
| k₃        | [5.06, 350.6] (s⁻¹) | -0.1326* | K₉       | [1.10] μM | 0.1226* |
| k₄        | [23.23, 210] (s⁻¹) | -0.0285* | K₅       | [0.1] μM | 0.0010 |
| k₅        | [0.045, 51] (s⁻¹) | 0.3269* | K₆       | [35, 1.4] μM | -0.0211 |
| k₆        | [0.03, 3] (s⁻¹) | -0.2482* | K₅       | [4, 8] μM | 0.1480* |
| k₇        | [5.2, 10⁻⁴] s⁻¹ | -0.0039 | K₅       | [0.09, 0.036] μM | -0.0909* |
| C₇        | [3.30] μM | 0.3433* | K₆       | [153, 15.3] μM | 0.1303* |
| C₈        | [6.75, 27] μM | 0.1169* | K₇       | [18, 7.1870] μM | -0.0026 |
| C₉        | [2.20] μM | -0.0133 | K₈       | [30, 8.30] μM | 0.0642* |
| C₅        | [0.01, 0.083] μM | 0.6772* | K₉       | [38, 6.5860] μM | -1.980* |
| C₆        | [0.0246, 0.46] μM | 0.3073* | K₁₀      | [13, 5.93] μM | -1.080* |
| C₇        | [25, 25] μM | 0.0655* | K₁₁      | [116, 11.6] μM | 0.0202 |

PRCC values for F° at 5 minutes post infection. *denotes significant PRCC values (p < 0.01).

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134: 603–613.
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130: 573–579.
129: 5114–5125.
128: 215–224.
127: 337–342.
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125: 130–135.
124: 734–742.
123: 369–374.
122: 309–315.
121: 308–314.
120: 154–163.
119: 684–711.
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110: 115–123.
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108: 743–748.
107: 1099–1109.
106: 513–516.
105: 370–375.
104: 353–362.
103: 573–579.
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40: 32093–32099.
39: 197–202.
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