ARTICLE

Mechanisms of a Sustained Anti-inflammatory Drug Response in Alveolar Macrophages Unraveled with Mathematical Modeling

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Both initiation and suppression of inflammation are hallmarks of the immune response. If not balanced, the inflammation may cause extensive tissue damage, which is associated with common diseases, e.g., asthma and atherosclerosis. Anti-inflammatory drugs come with side effects that may be aggravated by high and fluctuating drug concentrations. To remedy this, an anti-inflammatory drug should have an appropriate pharmacokinetic half-life or better still, a sustained anti-inflammatory drug response. However, we still lack a quantitative mechanistic understanding of such sustained effects. Here, we study the anti-inflammatory response to a common glucocorticoid drug, dexamethasone. We find a sustained response 22 hours after drug removal. With hypothesis testing using mathematical modeling, we unravel the underlying mechanism—a slow release of dexamethasone from the receptor–drug complex. The developed model is in agreement with time-resolved training and testing data and is used to simulate hypothetical treatment schemes. This work opens up for a more knowledge-driven drug development to find sustained anti-inflammatory responses and fewer side effects.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC? Uncontrolled inflammation is involved in diseases such as asthma and atherosclerosis. Anti-inflammatory drugs may have severe side effects attributed to e.g. excessive fluctuations of drug concentration over time. Therefore, sustained responses to drug treatment is desirable.

WHAT QUESTION DID THIS STUDY ADDRESS? Can we understand the mechanisms of a sustained anti-inflammatory drug response with the help of mathematical modeling? What are those mechanisms?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE? We report a sustained anti-inflammatory response to dexamethasone in alveolar macrophages, up to 22 hours after drug removal. We use modeling to test 2 competing hypotheses for dexamethasone action: via expression of IκB and direct inhibition of tumor necrosis factor translation. The first hypothesis is rejected, and the second is used to simulate the mechanism of the sustained response: a slow release of the drug from the receptor.

HOW MIGHT THIS CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS? Our work opens up for a more systematic search for anti-inflammatory drugs with a sustained response and therefore potentially fewer side effects.

The inflammatory response against infections relies on activation of the innate immune system. This activation contributes to a temporal induction of cytokines and various other specific signaling molecules, in turn attracting and instructing additional immunocompetent cells. This response is fast and relies on both local production and massive recruitment of immunocompetent cells, which are directed to the site of inflammation from the blood stream. This proinflammatory process needs to be restricted by anti-inflammatory mediators to return to homeostasis and avoid extensive tissue damage caused by the inflammation.1 When this balance act fails, common human disease states, such as septic shock, asthma, rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis, and atherosclerosis occur.2–4 To control the proinflammatory mechanisms of such diseases, anti-inflammatory drugs that target several specific and nonspecific mechanisms have been on the market for decades. However, such
anti-inflammatory drugs can cause severe side effects in the gastrointestinal tract, liver, and kidney as well as allergic reactions and edemas. Therefore, drugs with a sustained response are attractive because side effects could be minimized by lowering the fluctuation and/or the peak level of plasma drug concentration. Furthermore, if we understand the mechanisms of such a sustained response, we can use this knowledge in the search for new and better drug candidates.

Anti-inflammatory drugs act to reduce the production of cytokines. Cytokines are small signaling molecules central for directing downstream immunological effects. Some cytokines act on specific pathways and cell types, whereas others are broader in their span of activity. Tumor necrosis factor (TNF) is a hallmark proinflammatory cytokine acting through both paracrine and autocrine pathways. TNF mediates inflammatory activation in a multitude of ways. For example, TNF is involved in causing fever, limiting viral replication, and increasing phagocytic cells’ capacity to kill pathogens as well as in stimulating cells to release more cytokines and chemokines. This increased release attracts leukocytes and other cells which in turn further propagate the inflammatory process.5 TNF is produced by both immunocompetent cells of myeloid and lymphocyte lineage as well as nonimmunocompetent cells such as keratinocytes, endothelial cells, and neurons. One of the main contributors of soluble TNF in the inflammatory setting is the macrophage.6,7 This is especially true for alveolar macrophages, which are highly specialized macrophages present in the lungs. The alveolar macrophages function primarily in the defense against, and in the response to, inhaled particles and potentially pathogenic microorganisms. Macrophages thus play a critical role in the pathophysiology of inflammatory lung disease, such as asthma, and cystic fibrosis. In summary, one of the known pathways by which inhaled particles or microorganisms stimulate the recruitment, and subsequent activation, of inflammatory cells in lung disease, is through the activation of these alveolar macrophages to produce and release TNF.

TNF production is under strict transcriptional control where activation of the cell leads to an expansion of the TNF mRNA pool, which gives rise to rapid translation and eventual secretion of TNF; which in turn are free to act on surrounding tissues and cells (Figure 1, left). A major regulator of TNF transcription is the transcription factor: nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). NF-κB is always present in an inactive state in the cytoplasm of the cell, bound to the inhibitor of NF-κB, IkB (IκB). Upon certain cellular activation, e.g., through toll-like receptor (TLR) 4, IkB become phosphorylated by the IkB kinase complex leading to degradation by the proteasome. This in turn renders NF-κB active and capable of translocation to the nucleus, where it promotes transcription of multiple genes with for proinflammatory function, among them TNF. In the signaling pathway, there are several more proteins involved, e.g., interleukin-1 receptor-associated kinase enzymes, and TNF receptor associated factor proteins (see Figure 1, left). Finally, there are drugs that interfere with the NF-κB activity, among them glucocorticoids.

Glucocorticoids, e.g., cortisol, counteract inflammatory responses, and several synthetic glucocorticoids have been developed for treatment of inflammatory diseases. Glucocorticoids act through the glucocorticoid receptor (GR) and inhibit the release of cytokines from macrophages, e.g., through inhibition of transcription, changes in mRNA stability, changes in protein translation, and/or posttranslational processing. The synthetic glucocorticoid dexamethasone (dexa) is 30 times more potent than the endocrine glucocorticoid cortisol in inhibiting cytokine production.10 There are two main hypotheses for the mechanisms of action of dexa (Figure 1, box to the right): hypothesis A, new synthesis of the protein IκB that binds to NF-κB and thereby hinders the inflammatory response,11,12 and hypothesis B, a physical association between activated GR and the NF-κB subunit p65/RelA that reduces the activity of NF-κB.13,14 Hypothesis B is potentially a general mechanism for many cell types. Hypothesis A has been shown, e.g., in Jurkat cells transfected with GR were IκB mRNA levels were increased at 30 to 60 minutes after addition of dexa,11 while this mechanism has been shown to be lacking in endothelial cells.15 Neither of these hypotheses have been formally tested with, e.g., a mathematical modeling framework.

In the field of biology, mathematical modeling methods are commonly referred to as systems biology and often focus on intracellular metabolic and/or protein signaling pathways. In the field of pharmacology, pharmacokinetic/pharmacodynamic (PK/PD) modeling is more commonly used, which instead often focus on drug-receptor binding and the selection of optimal drug doses. The combination of both approaches, i.e., the use of models of drug actions, with a clear biological interpretation that can be used to gain mechanistic insights, e.g., regarding intracellular signaling is commonly referred to as systems pharmacology.16

Previous efforts to model TNF secretion from macrophages includes both systems biology and PK/PD models (see ref. 17 for a review of models). For example, systems biology modeling has been used for a detailed elucidation of the role of different TLRs and their ligands, including bacterial lipopolysaccharides (LPS), in both endocrine and paracrine TNF signaling.18 This model, however, does not contain the inhibitory effects of glucocorticoids and the mechanism of such an inhibition. Hao and coworkers19 have developed a model of chronic pancreatitis to simulate the effect of disease-modifying agents. This model includes the pancreatic micro-environment, including cytokines and macrophages. This model, however, does not contain the details of the intracellular signaling pathways within macrophages, and instead the interplay between different players of the micro-environment is targeted. Mechanistic PK/PD models for glucocorticoid receptor signaling have been developed for the metabolic side effect in the liver mediated via tyrosine aminotransferase.20 However, no existing model can be used to study (i) the different hypotheses of dexa-induced anti-inflammation and (ii) potential intracellular mechanisms behind a sustained anti-inflammatory response. 
Here, we study the anti-inflammatory response of alveolar macrophages to dexa and find a sustained cellular response to the drug up until 22 hours after withdrawal of the drug. To unravel the mechanisms behind such a sustained response, we use a mathematical modeling approach. First, we test the different hypothesis for the intracellular action of dexa and reject hypothesis A: “New synthesis of IκBα.” Hypothesis B: “Direct inactivation of NF-κB,” on the other hand, is in agreement with all our time-resolved data series. The mechanism behind the sustained response in hypothesis B is a slow release of dexa from the dexa-GR complex. We use the final model to simulate different treatment schemes.

**METHOD**

**Experimental methods**

All experimental methods are found in the Supplementary Material.

**Mathematical modeling**

A system of ordinary differential equations is used to model the dynamic response to LPS and dexa in alveolar macrophages. The same model structure is used for LPS stimulated inflammation for both hypotheses:

\[
\begin{align*}
\frac{d}{dt}(TLR4) &= -\nu_1 + \nu_2 - \nu_{bas1} & TLR4(0) &= 1 \\
\frac{d}{dt}(TLR4a) &= \nu_1 - \nu_2 + \nu_{bas1} & TLR4a(0) &= 1 \\
\frac{d}{dt}(NFKB) &= \nu_3 - \nu_4 & NFKB(0) &= 1 \\
\frac{d}{dt}(NFKBoIKB) &= -\nu_3 + \nu_4 & NFKBoIKB(0) &= 1
\end{align*}
\]

Figure 1 The inflammatory signaling pathway in macrophages. Lipopolysaccharides (LPS) stimulates TLR4 (Toll-like receptor 4), which leads to a cascade of signaling events, resulting in the transcription and release of tumor necrosis factor alpha (TNFα). The squared box contains both hypotheses for the effect of the anti-inflammatory drug dexamethasone (Dexa). In hypothesis A, the protein inhibitor of κB (IκBα) is synthesized in response to dexa bound to glucocorticoid receptors (GR), IκBα in turn binds to nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) proteins (RelA and NF-κB1) to inhibit their transcriptional activity. In hypothesis B, there is a direct physical association between activated GR and the NF-κB subunit RelA that reduces the transcriptional activity. p, phosphorylation.
\[
\frac{d}{dt}(IKBmRNA) = v_5 - v_{deg1} \quad IKBmRNA(0) = 1
\]

\[
\frac{d}{dt}(IKB) = v_6 - v_4 - v_{deg2} \quad IKB(0) = 1
\]

\[
\frac{d}{dt}(TNFmRNA) = v_7 - v_{deg3} \quad TNFmRNA(0) = 1
\]

\[
\frac{d}{dt}(TNF) = v_8 \quad TNF(0) = 1
\]

where \( v_{bas1}, v_1 - v_8, \) and \( v_{deg1} - v_{deg3} \) are the reaction rates, further defined as:

\[
v_{bas1} = k_{bas1} \cdot TLR4
\]

\[
v_1 = k_1 \cdot LPS \cdot TLR4 / (K_m + LPS)
\]

\[
v_2 = k_2 \cdot TLR4a
\]

\[
v_3 = k_3 \cdot NFkB \cdot TLR4a
\]

\[
v_4 = k_4 \cdot NFkB \cdot IKB
\]

\[
v_5 = k_5 \cdot NFkB
\]

\[
v_6 = k_6 \cdot IKBmRNA
\]

\[
v_7 = k_7 \cdot NFkB
\]

\[
v_8 = k_8 \cdot TNFmRNA
\]

\[
v_{deg1} = k_{deg1} \cdot IKBmRNA
\]

\[
v_{deg2} = k_{deg2} \cdot IKB
\]

\[
v_{deg3} = k_{deg3} \cdot TNFmRNA
\]

where \( k_{bas1}, k_1 - k_8, K_m1 \) and \( k_{deg1} - 3 \) are parameters with unknown values.

The anti-inflammatory effect of dexa is implemented in different ways for hypotheses A and B. The activation of the receptor complex \( \text{DexaGR} \) is modeled in the same way for both hypotheses:

\[
\frac{d}{dt}(\text{DexaGR}) = -v_{off} + v_{on} \quad \text{DexaGR}(0) = 0
\]

where

\[
v_{on} = k_{on} \cdot \text{Dexa} \cdot (1 - \text{DexaGR})
\]

i.e., the total concentration of GR (bound to dexa and unbound) is assumed to remain constant at 1.

In hypothesis A, \( \text{DexaGR} \) increases the rate of transcription of \( IKBmRNA \):

\[
\frac{d}{dt}(IKBmRNA) = v_5 - v_{deg1} + v_A
\]

where

\[
v_A = k_A \cdot \text{DexaGR}
\]

We also included a saturation in the translation from \( IKB \) mRNA levels to protein in hypothesis A:

\[
v_6 = k_6 \cdot IKBmRNA / (K_m6 + IKBmRNA)
\]

In hypothesis B, \( \text{DexaGR} \) is activated in the same way as in hypothesis A, and the effect of the complex is to inhibit \( v_7 \):

\[
v_7 = k_7 \cdot NFkB / (1 + k_{b1} \cdot \text{DexaGR})
\]

An interaction graph that depicts the models behind both hypotheses A and B is showed in Figure 2, and explanations to all model states and parameters are found in Table S1.

Figure 2 The developed mathematical models for hypothesis A (sky-blue subpart) and hypothesis B (blue subpart). Model inputs are lipopolysaccharide (LPS) and dexamethasone (Dexa), model output is tumor necrosis factor (TNF). Thick arrows represent flows and thin dashed arrows represent activating signals. All model states and parameters are further explained in Table S1. Abbreviations in the interaction graph include the following: GR = glucocorticoid receptor, kB = inhibitor of kB, NF-xB = nuclear factor x-light-chain-enhancer of activated B cells, p = phosphorylation, TLR4 = Toll-like receptor 4, and a = active state.
Parameter estimation
The model parameters were allowed a free range (1e-3, 1e3). There are literature values for a few of the rate constants to find. For example, for kon and koff in the dexa-GR binding there are data available that restrict the rate for koff to 0.001–0.01/minute, i.e., 0.06–0.6/hour and kon to 0.5–1/μM/minute, i.e., 3–60/μM/hour and (different amounts of GR were expressed in COS1 cells). These ranges constrain the affinity, Kd, to the range 0.001–0.02 μM, which is consistent with competition experiments in THP-1 cells and ex vivo lung tissue from rats. However, another measurement for koff is 0.0034–0.007/second, i.e., 0.21–0.42/minute or 12.6–25.2/hour (GR expressed in High Five cells). The nonoverlapping measurements made us allow for a wide range (1e-3, 1e3) also for kon and koff, and allowed the parameter estimation to find the best possible values also for these rate parameters.

The agreement between model simulations and data is quantified with a cost function,

\[ V(p) = \sum \frac{(y(t) - \hat{y}(t,p))^2}{(SEM_{max})^2} \]

where the sum is over all measured time points, t; p is the parameters; y(t) is the measured data and \( \hat{y}(t,p) \) is the model simulations; SEM\(_{max}\) is the maximal obtained standard error of the mean for each of the measured data sets.

An estimation of the uncertainty of the parameter values are visualized in Figure S3, Figure S4 and Figure S7.

Optimization and software
We used MATLAB R2019a (MathWorks, Natick, MA) and the IQM toolbox (IntiQuan GmbH, Basel, Switzerland) for modeling. The MATLAB functions particleswarm and simulannealbnd were combined in the optimization runs for extensive searches of the space of parameters.

Data processing
The number of repeats of each experiment was 2 to 6, and to save animals, the repeats came from different lung slices of the same animal. We therefore believe that the observed data uncertainty was lower than the true data uncertainty. To correct for that, we used the maximal calculated standard error of the mean (SEM) for each data series as a proxy for the actual SEM. We also used a correction factor in the model simulations to account for a scaling difference between the LPS experiments and the dexa experiments (cf. dots in Figure 3b at dose 100 ng/ml LPS, and Figure 3c 100 ng/ml LPS at 24 hours in orange). This correction factor

![Figure 3](https://www.psp-journal.com)
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was included as an experimental model parameter and estimated in the range 1.5 to 1.9.

Statistical analyses
For data comparison, we used one-way ANOVA with Tukey’s range test for multiple comparisons with a significance level of 0.05. To compute corresponding P values, we used MATLAB functions anova1 and multcompare. To reject models, we used the $\chi^2$ test with a significance level of 0.05. We used 37 degrees of freedom for training data (37 data points), leading to a threshold for rejection of $\chi^2$ (0.05, 37) = 52, and 51 degrees of freedom for all data (51 data points), leading to a threshold for rejection of $\chi^2$ (0.05, 51) = 69.

Data and model availability
The experimental data as well as the complete code for data analysis and modeling are available as supplementary files and at https://gitlab.liu.se/eliny61/macrophage-model.

RESULTS
Data collection
To unravel a potential sustained anti-inflammatory effect of dexamethasone (dexa) in lung slices, we combined experimental work and mathematical modeling. We used sliced lung tissue from rats in all experiments. First, we collected data for different concentrations of LPS after 24 hours of stimulation (Figure 3a, dots with error bars). LPS-induced activation of inflammation, as measured by TNF supernatant levels. Maximal activation was achieved at 500 to 1000 ng/ml of LPS. Intracellular levels of TNF did not show a dose–response relationship with LPS (data not shown), and we therefore focused subsequent experiments on extracellular levels of TNF. We performed a total protein determination in the lung tissue slices, and the protein content in the samples correlated to the weight of the samples (data not shown), which allowed data to be weighted with the mass of the tissue slices. Second, we collected time-resolved data for control + LPS induced increase in TNF for 100 and 1000 ng/ml LPS (Figure 3b, dots with error bars). LPS induced a detectable response at TNF after 2 hours, a linear increase between 2 to 6 hours, and a saturation somewhere before 24 hours. Third, we collected data for dexa-induced suppression of TNF secretion (Figure 3c, dots with error bars). Dexamethasone was added 1 hour before the addition of LPS. At the time of LPS addition, dexamethasone was either kept in the solution or washed away. In these experiments with dexamethasone, we used a submaximal concentration of LPS, 100 ng/ml, to be able to study potential suppression of the inflammatory response. Indeed, dexamethasone suppressed the LPS-induced secretion of TNF, even when removed from the solution before addition of LPS (Figure 3c, pink and sky-blue). A high dose of dexamethasone (3 µM) that was removed from the solution before addition of LPS suppressed the TNF release to similar levels as a low dose of dexamethasone (0.3 µM) that was kept in the solution (cf. sky-blue and red in Figure 3c, mean values are 91 and 51 pg/mg tissue). In summary, our data shows a sustained anti-inflammatory effect of a high dose of dexamethasone that has been washed away from the lung slices.

Hypothesis testing
To go from data to mechanistic insights, we developed mathematical models based on these data for both hypothesis A, “New synthesis of IκBα, ε and for hypothesis B, “Direct inactivation of NF-κB” (Figure 1). The two different models were based on known signaling mechanisms and existing knowledge of LPS-induced activation of inflammation and dexamethasone-induced reduction of inflammation (e.g., refs. 11,13), and differed only in the implementation of the effect of dexamethasone. The models were kept small, including only key mechanisms (Figure 2), to reduce the number of model parameters to estimate from the limited data. Both hypothesis A (Figure 3a-c, Figure S1a-c) and hypothesis B (Figure 4a-c, Figure S2a-c) showed a good agreement with estimation data (cf. dots and lines in the same colors in the figures). We collected sets of acceptable parameters for both hypotheses by running the parameter estimation procedure multiple times and saving parameters that gave a good agreement between model simulations and data according to the statistical $\chi^2$ test with level of significance 0.05 and 37 degrees of freedom (37 data points). In this way we could get an approximation for the uncertainty of predictions by the model. The uncertainties are displayed as colored areas in the figures. The found parameter values are visualized in Figure S3 and Figure S4.

Mechanisms of sustained response
We used these areas to study the mechanism of the sustained response to dexamethasone of the two hypotheses. For hypothesis A, the level of IκB protein, as induced by dexamethasone, is responsible for the sustained response (Figure 3d). When dexamethasone is removed, IκB remains (cf. sky-blue area with the control in orange that goes down to 0 in Figure 3d). IκB binds to active NF-κB and hinders transcription of TNF. For hypothesis B, the mechanism of sustained response is the level of the dexamethasone–GR (Figure 4d). When dexamethasone is removed, the dexamethasone–GR remains active for a long time (see the sky-blue area in Figure 4d). The slow release of dexamethasone from GR allows for continuous inhibition of NF-κB transcriptional activity.

Test with new data
Both hypotheses A and B were evaluated further to see their predictive abilities. To do so, we simulated the response to 100 ng/ml LPS and 3 µM dexamethasone. This time, we used increasing lengths of the wash between dexamethasone and LPS addition to see how long the response to dexamethasone was predicted to be sustained. Within a 30-hour time period, hypothesis A showed a similar, totally sustained, response regardless of the length of the wash (Figure 5a). The reason for this totally sustained response is a predicted slow degradation of both mRNA levels and protein levels of IκB, leading to a slow return of IκB protein (Figure S5). Hypothesis B, on the other hand, showed a more differential behavior, where the length of the wash affected the response (Figure 6a). Note that not all acceptable parameters in hypothesis B are predicting a sustained response. Model simulations with parameters that give rise to a sustained response over time accordingly are indicated in Figure 6a with darker areas.

We performed the corresponding measurements experimentally to test these predictions (Figure 5b; see the Method section for experimental details), and the corresponding data showed a clear time dependency in the sustainability of the response: the longer the wash, the less...
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Figure 4 Data and range of model simulations in agreement with data for hypothesis B. Measured is TNF in response to different concentrations of LPS and/or dexa. (a) Different doses of LPS (10, 50, 100, 250, 500, and 1000 ng/ml) were used to trigger an inflammatory response, and the corresponding concentration of secreted TNF were measured after 24 hours; n = 2. (b) 100 (yellow) and 1000 (orange) ng/ml LPS was added to trigger an inflammatory response and secreted TNF measured at several time points (0, 1, 2, 4, 6, and 24 hours). As control, no LPS was added (black); n = 6. (c) Lung slices were pretreated with indicated concentrations of dexa for 1 hour and next dexa was either washed out (pink, sky-blue) or not (red, blue). After that, LPS was added at time = 0; n = 3. (d) The mechanism of sustained response is at the level of the dexa–GR complex in this hypothesis. The slow release of dexa from GR allow for continuous inhibition of NF-κB transcriptional activity. Dots with error bars show data and standard errors of measurements, and colored areas show the area of model simulations that are in agreement with data according to a χ² test. Dexa, dexamethasone; GR, glucocorticoid receptor, LPS, lipopolysaccharide; TNF, tumor necrosis factor.

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sustained the response. When dexa was removed just before the addition of LPS, the TNF release was inhibited to a large degree (Figure 5b, sky-blue). When dexa was removed after 16 or 22 hours of wash, there was still an inhibition of TNF release (Figure 5b, green and brown) as compared with control (Figure 5b, orange). Therefore, hypothesis A had to be rejected. We also ran the optimization again, including both training and testing data, to assure that there was no parameter set for hypothesis A that we had missed. Despite many searches, we could not find any parameter set in agreement with all data for hypothesis A.

Hypothesis B, on the other hand, showed more differential behavior, where the length of the wash affected the response (Figure 6a). Note that not all acceptable parameters in hypothesis B are predicting a sustained response. The parameters with a sustained response, defined as at least 20 % reduced response in the 22-hour wash simulation (brown) after 7 hours compared with the control simulation (yellow) after 7 hours, are indicated in Figure 6a with darker areas. For further analysis, we searched for more parameters in agreement with both training and test data for hypothesis B. A new threshold for the χ² test was calculated with 51 degrees of freedom because all data contain 51 data points. This new set of parameters (Figure S7), in agreement with all data (Figure 6b and Figure S6), is used to simulate the treatment schemes in the next section.

Treatment schemes
To display the potential of the developed model, we next used the accepted hypothesis, together with the found parameters in agreement with all data, to simulate hypothetical treatment schemes. We assumed patients with some inflammatory disease, displayed by high levels of TNF at the site of inflammation. We simulated the disease by adding a constant infusion delivering 1 ng/mL LPS at steady state. To account for the different situation in plasma compared with the situation in vitro under which the model was developed, we added two parameters to the model. The first added parameter accounted for the half-life of dexa in plasma. We assumed this half-life to be 4 hours, in accordance with Queckenberg et al. The second added parameter accounted for the rate of elimination of TNF, which we assumed to be 5.65 per hour, a value taken from Held et al. We simulated four different treatment schemes with dexa in such patients (Figure 7), with the same total daily dose of dexa (corresponding to 0.3 μM elevation of dexa concentration in plasma): One treatment per day (0.3 μM dexa),
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Figure 5 Rejection of hypothesis A: “New synthesis of IκB.” (a) The range of model simulations for hypothesis A in agreement with data in Figure 3 shows a predicted response to dexamethasone of TNF that is totally sustained even 22 hours after washout. (b) Measured TNF in response to dexamethasone pretreatments, as indicated. Lung slices were pretreated with dexamethasone for 1 hour before washout during indicated times. At the first measurement for each color, 100 ng/ml LPS is added; n = 3. Dots with error bars show data and standard errors of measurements. The model simulations in (a) are not in agreement with these data and must be rejected. Colored areas in (a) show the area of model simulations that are in agreement with data in Figure 3 according to a χ² test. dexamethasone; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

Figure 6 In agreement with new data, hypothesis B: “New synthesis of IκB.” (a) The range of model simulations for hypothesis B in agreement with data in Figure 4 shows a predicted response to dexamethasone of TNF that cannot be rejected based on corresponding data in (b). Highlighted with darker areas are selected model simulations that show a sustained response, defined as at least 20% lower response in the last time point of the 22-hour wash (brown) compared with the control (yellow). (b) Model simulations in agreement with all data, i.e., the model has been trained to fit with both the data here and the data in Figure S6. Dots with error bars show data and standard errors of measurements. dexamethasone; LPS, lipopolysaccharide; TNF, tumor necrosis factor.
two treatments per day (0.15 + 0.15 μM dexamethasone), three treatments per day (0.1 + 0.1 + 0.1 μM dexamethasone), and continuous 24 treatments per day (0.3/24 μM dexamethasone per occasion). See Figure 7 (right) for the dosing scheme. The model simulations showed a sustained lowering of TNF levels already at one treatment per day (Figure 7, left). We calculated the area under the curve (AUC) with uncertainties for the TNF levels during the second day of treatment. We selected the area between the model simulation of the time-varying response and the maximal response (lower horizontal line in Figure 7, left). The second day of treatment was chosen to remove the effect of the rate of the decrease from a rather high level before treatment (Figure 7, left). One treatment per day gives an interval of AUC = (0.26, 0.62). Two treatments per day gives a small improvement, AUC = (0.18, 0.55). Three treatments or more per day gives no further improvement, AUC = (0.17, 0.54). The explanation to the sustained response is the slow release of dexamethasone from the dexamethasone-gr complex (Figure 7, middle), as we know from previous analyses cf. Figure 4d. The value for koff is estimated to 0.08 to 0.1/hour in line with the experimentally determined interval 0.06 to 0.6/hour in ref. 21.

DISCUSSION

We analyzed mechanisms of a sustained anti-inflammatory response in alveolar macrophages. To do so, we combined experimental data and a mechanistic modeling approach. Our main findings are that (i) in alveolar macrophages, there is a sustained anti-inflammatory response to dexamethasone that can be explained by a slow release of dexamethasone from the dexamethasone-gr complex; (ii) one of the main hypotheses for the intracellular
effect of dexe, hypothesis A, “new synthesis of kxkB,” cannot explain our data but instead the alternative hypothesis, hypothesis B, “direct inactivation of NF-kxB,” can explain all our data; (i) our final model can be used to simulate hypothetical treatment scenarios to, e.g., see that a once-daily dose of dexe would be enough to obtain a beneficial anti-inflammatory response over 24 hours, which is in line with current treatment regimens.

Our findings in (i) are in line with reports detailing how dexe inhibits NF-kxB signaling, not primarily through affecting IxBk (hypothesis A) but, rather, by interacting with the GR and affecting NF-kxB in the nucleus. Direct interaction between GR and p65 has been suggested through immunoprecipitation experiments, but the suppression of NF-kxB activity is likely mediated through downstream events such as increased export from the nucleus or site-specific phosphorylation of NF-kxB subunits. It has been shown previously that dexe, after binding to GR, interacts with NF-kxB and promotes enhanced export of the p65 subunit from the nucleus, peaking at 20 minutes. The sustained response observed in the current study may include GR-induced expression of additional proteins such as glucocorticoid-induced leucine zipper (GILZ) that both bind and inhibit p65 as well and further stimulates increased export of p65 out of the nucleus.

We have used a mechanistic modeling approach, in contrast to an empirical PK/PD modeling approach, which is more often used in pharmacological applications. The benefit of a mechanistic modeling approach is that we can not only predict left out data, which is possible with both approaches, but also study the mechanisms behind observed phenomenon. In this study, we used mechanistic modeling first to distinguish between two biological hypotheses for the intracellular effect of dexe. Such hypothesis testing allows for conclusions in the form of rejections, and predictions with uncertainties that can be tested experimentally. We find a model prediction that distinguishes between the biological hypotheses, and corresponding measurement shows that one of the hypotheses cannot explain these data. This is an excellent example of how mechanistic modeling can be used in experimental designs to ensure that new experiments give rise to new conclusions about the biological system under study. Second, we used mechanistic modeling to gain new insights into the mechanisms of the sustained response. For the two hypotheses, we found different mechanisms that were responsible for the sustained response: an increase in kB protein in response to dexe (Figure 3d) and a slow release of dexe from the receptor (Figure 4d). Finally, we combined our final mechanistic model with a few key PK parameters to simulate a hypothetical scenario of treatment schemes. Such a combined model is commonly referred to as a systems pharmacology model and make use of the strengths of both modeling approaches. The included mechanistic details give insights also in the simulation of treatment scenarios, e.g., the mechanism of the sustained response herein (Figure 7, middle).

We allowed all the model parameters a free range (1e-3–1e3) in the training procedure. The reason for this free training is that most model parameters have not been determined experimentally, and in the few such cases, the experimental values are calculated based on assumptions, and from different experimental systems. Of interest is that for the release rate parameter in the dexe-GR binding (koff), the estimated values for hypothesis A (1.5–1000/hour) overlaps with the values from 12.6-25.2/hour, and the estimated values for hypothesis B (0.08-1/hour) overlaps with the values from 0.06-0.6/hour. Therefore, if we had chosen to restrict koff in line with ref. 21, hypothesis A would have been rejected already on training data, and if we instead had chosen to restrict koff in line with ref. 24, hypothesis B would have been rejected. This shows that it is important to only restrict parameter values with their known values if determined in the same experimental system and context as your model.

The mechanistic models that we have developed are minimal in the sense that they do not include all known intracellular signaling intermediaries. Our rational is to keep models minimal in relation to the questions we are asking and to available data. Here, we have collected measurements for TNF secretion for different inputs, and we lack data for the intracellular signaling intermediaries. With more detailed data, e.g., the quantitative dose-resolved and time-resolved data for multiple signaling intermediaries in macrophages treated with TNF, more detailed models for macrophages can be developed.

We assume that we have a complete wash of dexe from the lung slices. Theoretical calculations involving known characteristics of dexe shows that 0-2% of dexe might bind to the tissue and thus not be washed away. To account for such (small) effects, we have performed parameter estimation with both hypotheses with corresponding assumptions, i.e., that we do not have a complete wash. The conclusions herein do not change if we change the assumption to allow for not complete washout (not shown).

We provide a model of the sustained effect of an anti-inflammatory drug in alveolar macrophages. The mechanistic modeling approach allow for incremental model extensions whenever more data becomes available. Interesting extensions for the model would be to add other compounds with known or unknown mechanisms of actions, more intracellular signaling details, as well as connecting the macrophages model to models for other players in the immune system. Our work opens up for more systematic searches for drug candidates with sustained anti-inflammatory responses, which could lead to smaller variations in drug concentrations, and thus potentially to drugs with fewer side effects.

Supporting Information. Supplementary information accompanies this paper on the CPT: Pharmacometrics & Systems Pharmacology website (www.psp-journal.com).

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