Leveraging Prior Knowledge of Endocrine Immune Regulation in the Therapeutically Relevant Phenotyping of Women With Chronic Fatigue Syndrome

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ABSTRACT

Purpose: The complex and varied presentation of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) has made it difficult to diagnose, study, and treat. Its symptoms and likely etiology involve multiple components of endocrine and immune regulation, including the hypothalamic-pituitary-adrenal axis, the hypothalamic-pituitary-gonadal axis, and their interactive oversight of immune function. We propose that the persistence of ME/CFS may involve changes in the regulatory interactions across these physiological axes. We also propose that the robustness of this new pathogenic equilibrium may at least in part explain the limited success of conventional single-target therapies.

Methods: A comprehensive model was constructed of female endocrine–immune signaling consisting of 28 markers linked by 214 documented regulatory interactions. This detailed model was then constrained to adhere to experimental measurements in a subset of 17 candidate immune markers measured in peripheral blood of patients with ME/CFS and healthy control subjects before, during, and after a maximal exercise challenge. A set of 26 competing numerical models satisfied these data to within 5% error.

Findings: Mechanistically informed predictions of endocrine and immune markers that were either unmeasured or exhibited high subject-to-subject variability pointed to possible context-specific overexpression in ME/CFS at rest of corticotropin-releasing hormone, chemokine (C-X-C motif) ligand 8, estrogen, follicle-stimulating hormone (FSH), gonadotropin-releasing hormone 1, interleukin (IL)-23, and luteinizing hormone, and underexpression of adrenocorticotropic hormone, cortisol, interferon-γ, IL-10, IL-17, and IL-1α. Simulations of rintatolimod and rituximab treatment predicted a shift in the repertoire of available endocrine–immune regulatory regimens. Rintatolimod was predicted to make available substantial remission in a significant subset of subjects, in particular those with low levels of IL-1α, IL-17, and cortisol; intermediate levels of progesterone and FSH; and high estrogen levels. Rituximab treatment was predicted to support partial remission in a smaller subset of patients with ME/CFS, specifically those with low norepinephrine, IL-1α, chemokine (C-X-C motif) ligand 8, and cortisol

Accepted for publication March 8, 2019
https://doi.org/10.1016/j.clinthera.2019.03.002
0149-2918/$ - see front matter

levels; intermediate FSH and gonadotropin-releasing hormone 1 levels; and elevated expression of tumor necrosis factor-α, luteinizing hormone, IL-12, and B-cell activation.

Implications: Applying a rigorous filter of known signaling mechanisms to experimentally measured immune marker expression in ME/CFS has highlighted potential new context-specific markers of illness. These novel endocrine and immune markers may offer useful candidates in delineating new subtypes of ME/CFS and may inform on refinements to the inclusion criteria and instrumentation of new and ongoing trials involving rintatolimod and rituximab treatment protocols. (*Clin Ther*. 2019;41:656–674)

Key Words: chronic fatigue syndrome, endocrine regulation, immune regulation, numerical modeling, rintatolimod, rituximab.

INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex disorder in which a key presenting feature consists of a state of persistent debilitating fatigue lasting >6 months. Although diagnostic criteria have evolved from early case definitions,[1] characteristic features commonly include pathologic fatigue and malaise that are worsened after exertion, cognitive dysfunction, immune dysfunction, unrefreshing sleep, pain, autonomic dysfunction, and neuroendocrine and immune symptoms.[2] Although this would point to a multifactorial etiology involving hormonal, neurologic, and immunologic factors, initial efforts at biomarker identification were focused on immune dysregulation,[3,4] perhaps owing to the significant occurrence of infectious illness as a correlate of onset.[3,5]

In general, stress has a dysregulating influence on hormonal systems with consequences for metabolism and reproductive function in women. In addition to the aforementioned systems, the immune system has also been implicated in the etiology of ME/CFS.[6,7] In healthy subjects, close coordination between the hypothalamic-pituitary-gonadal (HPG) and immune systems has been described, giving rise to regular fluctuations over the course of the menstrual cycle as well as acute variations in response to HPG activation.[8,9] The primary stress response axis, the hypothalamic-pituitary-adrenal (HPA) axis, can be stimulated by peripheral inflammatory responses to infection or trauma, resulting in the sympathetic activation of a positive feedback loop whereby hypothalamic inflammatory signaling drives, and is sustained by, local production of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α).[10] Indeed, ME/CFS has frequently been observed to follow infection by Epstein–Barr virus, in which context it tends to be associated with chronic dysregulation of inflammatory cytokine production.[3]

Rintatolimod,* a specific activator of inflammatory mediator Toll-like receptor 3 (TLR3), has been used in ME/CFS treatment for decades, with varying efficacy.[11,12] With immune B cells serving as a reservoir for latent Epstein–Barr virus infection, B-cell depletion by rituximab has also been used in clinical trials and has seen some success in treating ME/CFS.[13] Combined with observations of deficient Epstein–Barr virus–specific B-cell responses in patients with ME/CFS,[14] this finding suggests that dysregulated B-cell function and persistent latent viral infection may be significant contributing factors to ME/CFS. However, efforts to identify specific markers of B-cell dysfunction have been inconsistent, with various reports of altered maturation,[15] serum B-cell activating factor,[13] and conflicting results of gene expression studies.[7,16] Nonetheless, a general neuro-immune model of ME/CFS has been proposed, in which an initial infection may lead to chronic peripheral immune activation and sustained neuroinflammation, with cyclic fluctuations in T-cell balance contributing to observed patterns of relapse and remission.[17]

Because women are at higher risk of ME/CFS than men,[5,18] it is believed that female sex hormones play an important role in the onset, persistence, and symptom burden of this illness.[19] Despite evidence of sex-specific differences in susceptibility and response to infection by pathogens[20] and autoimmunity,[21] the potential role of cross-talk between sex hormone regulation and oversight of immune function in ME/CFS has not been well explored. The same may be said of immune cross-talk with metabolic hormones[22]

* Trademark: Ampligen® (Hemispherx Biopharma, Inc, Ocala, Florida).

in ME/CFS, with the possible exception of research implicating dysregulation of leptin[23,24] in illness severity. Indeed, stress hormone regulation has arguably been the main focus of endocrine involvement in ME/CFS and has been relatively well documented.[25] Unfortunately, these components have been studied largely in isolation, with limited consideration of their regulatory interactions with adjacent and overlapping systems.

To explore the potential for altered co-regulation of endocrine and immune axes as a mediator of ME/CFS and its persistence, our group had assembled a basic computational model of regulatory logic coordinating the principal feedforward and feedback actions of the HPA axis, the HPG axis, and the innate and adaptive branches of the peripheral immune system. We found that even this coarse-grained model could support multiple steady states, 2 of which were proximal to immune marker profiles exhibited by patients with ME/CFS.[26,27] Although useful in showing the potential involvement of endocrine–immune co-regulatory control in supporting the persistence of this condition, these initial models remained coarse in resolution.

The present study extends our initial proof-of-concept study with the assembly of a more detailed endocrine–immune circuitry in which regulatory dynamics are supported by a more sophisticated logic that captures the actions of low- and high-affinity receptor signaling as well as competing influences of weak versus strong mediators. These effects are adjusted to directly align model predictions with experimental measurements of immune markers sampled in peripheral blood at 8 points in time before, during, and after a maximal exercise challenge. Instead of broad immune functional sets, individual cytokines are now networked with a more detailed model of sex, stress, and metabolic hormones through regulatory interactions extracted from the published scientific literature using automated natural language processing. This literature-informed model of endocrine–immune regulatory logic aligned supported, experimentally observed immune responses to maximal exercise within 5% error in 26 competing candidate models. The regulatory constraints imposed by these competing model circuits predicted widespread dysregulation of endocrine function in patients with ME/CFS at rest, characterized by blunted HPA regulation, HPG overactivation, and an immune profile dominated by IL-8 and IL-23. Together, these patterns may contribute to the pathologies experienced by patients with ME/CFS. Simulations based on this family of models mimicking the effects of TLR3 activation and B-cell depletion suggest that these interventions may in fact alter the repertoire of stable regulatory behaviors in favor of a more robust normal regulation or, in the case of the latter, even render ME/CFS dynamically unstable outright.

PATIENTS AND METHODS

Participants

A total of 88 female subjects (43 with ME/CFS and 45 healthy control subjects) were selected without exclusion for ethnicity from the patient population of the Institute for Neuro Immune Medicine at Nova Southeastern University (Fort Lauderdale, Florida), (directed by N.G.K.). All subjects signed an informed consent approved by the Institutional Review Board of Nova Southeastern University. Included subjects presented with acute onset and with an illness duration of at least 4 years. ME/CFS was diagnosed according to current research case definitions[1,28]: fatigue of >6 months' duration and at least 4 of 8 symptoms, including exercise-induced relapse, myalgia, arthralgia, headache of a new and different type, nonrestorative sleep, cognitive complaints, sore throat, and tender lymph nodes. All ME/CFS study subjects presented with a 36-Item Short Form Health Survey summary physical score below the 50th percentile, based on population norms. Heathy control subjects were self-defined as sedentary (no regular exercise program, sedentary employment), and matched to ME/CFS case subjects according to age (±5 years), race/ethnicity, and body mass index (±5 kg/m^2).

Study Design

Subjects were challenged with a supervised symptom-limited maximum graded exercise test performed under the McArdle protocol on a fully automated cycle Model 95Ri (Life Fitness, Rosemont, Illinois) and the Oxycon Mobile ergospirometry testing device (Vyaire Medical, Mettawa, Illinois). Subjects pedaled at an initial output of 60 W for 2 min, followed by an increase of 30 W every 2 min. This was continued until one of the following endpoints: (1) maximal oxygen consumption was

reached; (2) respiratory exchange ratio was >1.15; or (3) the subject discontinued the challenge. Blood samples (8 mL) were collected before the test after a 30-min rest period, at maximal effort, and at 10, 20, 30, and 60 min' poststress, with additional blood draw at ~12 h and 24 h' poststress. Peripheral blood mononuclear cells were isolated by using Ficoll–Paque extraction and stored in liquid nitrogen; plasma was stored at –80C.

Assessments

Peripheral blood mononuclear cell samples from each time point were analyzed by flow cytometry on a Beckman Coulter FC500 (Brea, California) using commercially available antibodies to record frequencies of B cell (CD19+) and natural killer (NK) cell (CD3-CD56+) populations. Plasma concentrations of interferon-γ (IFN-γ), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-15, IL-17, IL-23, and TNF-α were measured by using a Q-Plex multiplex ELISA (Quansys Biosciences, Logan, Utah). Details of the protocol and assay variability have been reported previously by our group.[3,29] Finally, serum samples were analyzed for concentrations of the predominant estrogen estradiol and progesterone by immunoelectro-chemiluminescence assays on a Roche Cobas 6000 analyzer (Roche Diagnostics, Basel, Switzerland), following all manufacturer's instructions for instrument maintenance and assay calibration and test procedures with interassay %CVs that are consistently <4%.

Statistical Analysis

Differences in marker expression were tested for significant effects of condition, time point, and condition–time interactions by 2-way ANOVA; raw F test null probability *P* values were adjusted for multiple comparisons by the Benjamini-Hochberg procedure with a false discovery rate of 0.05 in R version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria). Continuous measurements were converted to discrete values by using a variational Bayesian update scheme for expectation maximization of Gaussian mixture models.[30,31] The full set of measurements for each marker was used to define the most representative number of discrete activation levels (eg, whether the cytokine measured behaved according to binary or multi-valued logic). Each variable was then summarized by taking the median of inverse hyperbolic sine–transformed values for healthy control subjects and patients with ME/CFS at each time point. These summarized values were discretized by k-means clustering using the previously defined maximum activation levels to determine the number of clusters: the discretized values for entities that were found to vary significantly according to ANOVA were used as input trajectories for model parameterization. Predicted behaviors for network entities were analyzed after parameterization. ANOVA was performed on predicted trajectories excluding the start state values; the start states were compared separately by using the Wilcoxon rank-sum test. Figures were prepared by using the ggplot2 package in R. Graph topological metrics (eg, betweenness centrality) were calculated by using MATLAB (MathWorks, Natick, Massachusetts).

Mechanistic Modeling of Endocrine–Immune Signaling

The model assembled and reported previously by our group[27] has been extended in the present study to include additional regulators (nodes) of HPG and HPA axis function, as well as regulators of the hypothalamic-pituitary-thyroid axis and a much more detailed description of the immune signaling. Regulatory interactions (edges) between these entities were drawn from the Pathway Studio (Elsevier, Amsterdam, the Netherlands) knowledge database, a repository extracted from the published scientific literature using the MedScan[32] natural language processing engine. Edges were verified independently by using our implementation of a Bayesian sentiment analysis classifier.[33] Disagreements between MedScan and this platform were reviewed and adjudicated by the authors.

The scope of the validated regulatory network was further constrained to focus on regulators directly involved in feedforward and feedback control. Nodes with no outgoing edges (sink nodes) were removed, with the exception of B cells and NK cells because experimental measurements of these immune cell populations were available. Direct regulatory associations that duplicated the actions of a sequence of indirect associations were also removed to promote parsimony. For example, although the Pathway Studio database contained many associations directly linking physiological stress to a

range of immune mediators, these actions were more accurately accounted for by representing these as downstream effects of HPA axis regulation. Redundant regulatory actions such as these were removed after careful consideration of the references supporting them.

Endocrine Regulatory Logic

As mentioned earlier, dysregulation of the HPA stress response axis has long been associated with ME/CFS. Indeed, a simple computational model of HPA function previously reported by our group readily supported an alternate stable resting state characterized by persistently low levels of circulating cortisol.[34] This basic model was extended in subsequent research and remains the central representation of stress response circuitry used here. The 3 main elements of the HPA axis (corticotropin-releasing hormone [CRH], adrenocorticotropic hormone [ACTH], and cortisol) are represented in the network model, with stress included as an input signal. Norepinephrine and dopamine are also included as highly connected elements of the stress response. Stress also acts on the HPG axis through norepinephrine release into the ovaries, and it also inhibits the release of gonadotropin-releasing hormone (GnRH), luteinizing hormone, estrogen, and progesterone.[35] Findings such as these support the existence of complex co-regulatory interactions between the stress response axis and reproductive hormone regulation. Estrogen, for example, has been shown to increase corticotropin secretion in both female monkeys and rats, supporting a feedback loop between the HPG and HPA axes.

In this study, we retain key components of the female HPG axis (estrogen, follicle-stimulating hormone [FSH], GnRH 1 [GnRH1], luteinizing hormone [LH], and progesterone) used in the basic model reported previously[27] and have augmented this approach with more detailed representation of cross-axis regulatory interactions with adjacent systems such as the HPA axis. We have shown in related research that this representation of the HPG axis is capable of recapitulating the regular oscillations characteristic of the menstrual cycle when simulated with ternary logic.[36]

Immune Regulatory Logic

Highly connected immune markers implicated in ME/CFS were selected for inclusion in the current model in addition to peripheral blood markers for which experimental measurements were available in this study population. Specifically, these immune markers included the cytokines IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-15, IL-17, IL-23, and TNF-α. Because evidence of dysregulated B-cell[14] and NK-cell function[37] has been reported in ME/CFS, these cell populations were also included in the model.

Model Parameterization and Simulation of Regulatory Dynamics

The biological signaling model is represented as a directed and weighted graph, in which an edge represents the regulatory action of one node onto another and where a positive or negative edge polarity indicates a stimulatory or inhibitory mode of action, respectively. Network parameters describing signal activation thresholds and the weighted contextual response of each marker node to all combinations of its input mediators were derived according to a discrete logical formalism.[38–41] These parameters were derived by constraining the model to satisfy a set of qualitative (eg, steady states) and quantitative (experimental data) observations.

As is the case in this research, the complexity of the regulatory network models often exceeds that of the available data both in terms of the amount of data and the breadth of the markers surveyed. Here we address the issue of insufficient and incomplete data by translating the parameterization problem into a constraint satisfaction problem[36] which we solved using OR-tools (Google, Mountain View, California), an open-source library of algorithms for operations research.[42] Constraint satisfaction has proven to be an effective problem-solving technology used by the artificial intelligence community to efficiently solve large combinatorial problems.[43] In our identification of parameter sets, we enforced adherence of model predictions to experimental data and also constrained the model to support particular clinically observed behaviors. Specifically, we assumed that measurements taken at rest (T0) represented a stable steady state in both patients with ME/CFS and in healthy control subjects, and that the trajectory of

response to exercise should accommodate a return to this stable resting state in control subjects after 24 h; the ME/CFS patient trajectory was not similarly constrained. In addition, the ME/CFS steady state was constrained to align with an underexpression of cortisol and overexpression of estrogen as previously reported.[27]

RESULTS

Regulatory Network Structure and Parameterization

A survey of published literature on elements of the HPA, HPG, and immune systems implicated in ME/CFS identified 28 biological markers, including hormones, neurotransmitters, cytokines, and cell populations, with physiological stress as an input stimulus. Automated text mining of the Pathway Studio literature database and validation of statements about regulatory interactions between these entities identified 214 interactions (Figure 1). The structure of this regulatory circuit model was supported by a total of 21,146 references, with a median of 16.0 references and a mean of 58.9 references supporting each interaction (see Supplemental Figure 1 in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002). Based on expert adjudication of divergence in interpretation with a competing Bayesian text mining engine, ~4% of edge polarities assigned by Pathway Studio's MedScan were judged to be an incorrect interpretation of the supporting text. The connection density of our network model (27.3%) is in line with reported estimates of connectivity in protein-signaling networks.[44] Betweenness centrality for individual mediator nodes in the network was highly variable, with TNF-α, IL-1β, IL-10, and progesterone each

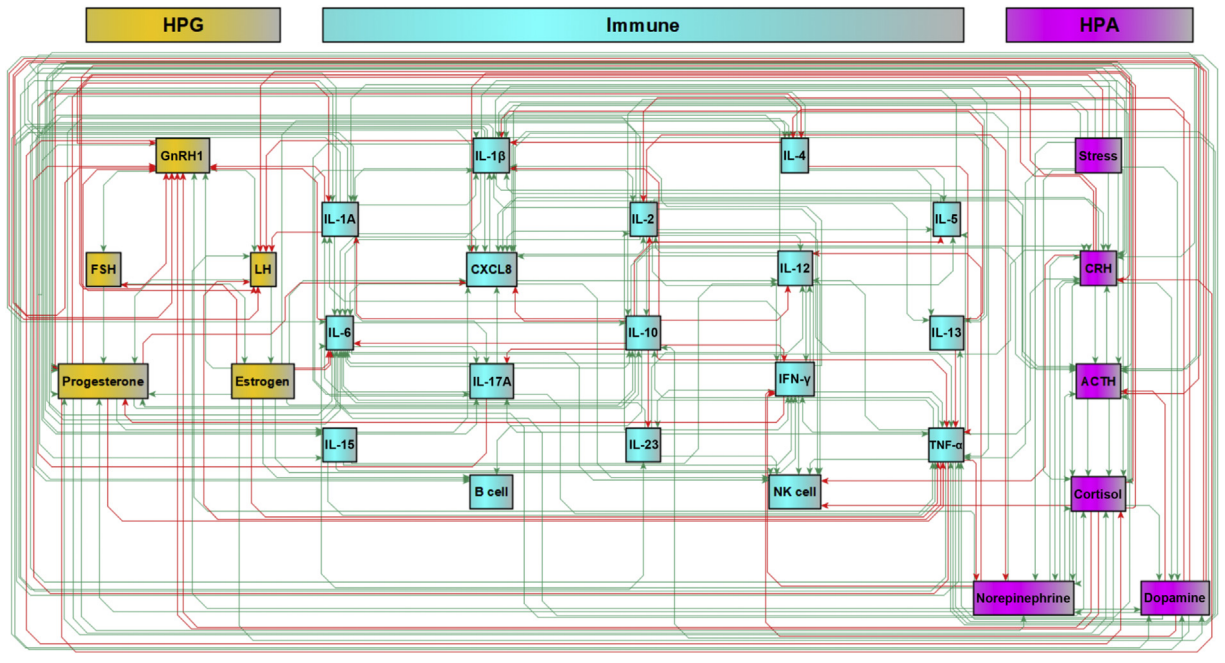

Figure 1. Proposed regulatory model of endocrine signaling pathways and molecules implicated in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), incorporating elements of the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonadal (HPG), and immune systems. The model comprises 28 entities and 214 regulatory edges. ACTH = adrenocorticotropic hormone; CRH = corticotropin-releasing hormone; CXCL8 = chemokine (C-X-C motif) ligand 8; FSH = follicle-stimulating hormone; GnRH1 = gonadotropin-releasing hormone 1; IFN-γ = interferon-γ; IL = interleukin; LH = luteinizing hormone; NK = natural killer; TNF-α = tumor necrosis factor-α.

occurring in >50% of shortest paths, highlighting the latter as highly influential regulators in this model (see Supplemental Figure 2 in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002).

Marker Differential Expression and Discretization

As described in the previous section, patients with ME/CFS (n = 43) and healthy control subjects (n = 45) were challenged with a graded maximal exercise test with serial blood samples collected at 8 time points before, during, and after challenge. Plasma levels of inflammatory cytokines and the abundance of immune cell subpopulations are depicted in Supplemental Figure 3 (in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002). A 2-way ANOVA supported significant effects for condition (ME/CFS vs health) and/or time point in IL-1β, IL-2, IL-4, IL-5, IL-6, IL-13, IL-5, and NK cells (Table I). Continuous expression values for these 8 exercise responsive and/or condition-sensitive markers were converted into discrete activation levels by using a variational Bayesian Gaussian estimation.[30,31] This number of discrete states was then used to support a k-means clustering of the median expression at each time point for both conditions, producing discretized exercise response trajectories for each group (see Supplemental Figure 4 in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002). Thus, the response trajectories transformed to discrete values offer qualitative representations of the continuous measurements shown in Supplemental Figure 3 (in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002) and serve as constraints in a logical modeling formalism. Markers in the numerical model for which experimental measurements were not found to vary significantly were left unconstrained or "free" during parameterization.

Model Parameterization and Alignment With Exercise Response Trajectories

These discrete state response trajectories, in conjunction with the regulatory network structure (Figure 1), served to establish a set of constraints

Table I. Two-way ANOVA of each measured variable as a function of condition (myalgic encephalomyelitis/chronic fatigue syndrome vs healthy control), time point, and interactions. Variables with at least 1 significant effect were constrained; others were left free. Columns show *P* values for each potential effect.

Variable	Condition	Time	Condition × Time	Constrained
IL-1α	0.413	0.171	0.643	No
IL-1β	0.017	0.173	0.722	Yes
IL-2	<0.001	0.523	0.899	Yes
IL-4	<0.001	0.585	0.550	Yes
IL-5	0.002	0.088	0.293	Yes
IL-6	<0.001	0.433	0.035	Yes
IL-8	0.481	0.760	0.869	No
IL-10	0.673	0.984	0.231	No
IL-12	0.825	0.588	0.580	No
IL-13	<0.001	0.403	0.009	Yes
IL-15	0.041	0.337	0.343	Yes
IL-17	0.257	0.802	0.627	No
IL-23	0.462	0.963	0.991	No
IFN-γ	0.298	0.918	0.967	No
TNF-α	0.584	0.244	0.894	No
B cell	0.672	0.369	0.991	No
NK cell	<0.001	<0.001	0.285	Yes

IFN-γ = interferon-γ; IL = interleukin; NK = natural killer; TNF-α = tumor necrosis factor-α.

from which regulatory logic parameters were derived in accordance with methods described in our previous research.[36] As mentioned earlier, the resolution offered by this group size supported the detection of statistically significant variations in 8 of the 17 measured immune markers. Measurements for this subset of 8 immune markers served to define constraints for the parameter estimation problem. Specifically, allowable parameter sets supported model predictions of the expression of these 8 markers that exactly matched their expression at rest in both patients with ME/CFS and in healthy control subjects while also deviating as little as possible from values measured longitudinally during the course of the exercise challenge. In addition to this finding and independently from the data, qualitative interpretations from the literature of high estrogen and low cortisol levels were applied to constrain the ME/CFS condition at rest only. We found 26 parameter sets that accommodated the available exercise response data to within 5% error in addition to exactly matching the resting steady-state discrete expression profiles for patients with ME/CFS and for healthy control subjects. The values predicted by these top 26 models for the measured immune markers are shown in Figure 2A, showing close adherence to the discretized experimental data. This finding suggests that the set of candidate mechanisms embodied in the endocrine–immune circuity model offer a framework for accurately reproducing the immune response to exercise in this cohort of subjects.

Validation of Predicted Sex Hormone Expression

As a separate segment of this same dataset, the HPG hormones estrogen (estradiol) and progesterone were measured in the same 43 patients with ME/CFS and in 45 healthy control subjects at 4 of the 8 time points (T0, T1, T2, and T3) but were not used to constrain parameter identification for the model (see Supplemental Figure 5 in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002). Although a requirement for elevated estrogen levels was applied to describe ME/CFS at rest, this constraint was informed by a qualitative interpretation of the literature and not from the data. Moreover, the remainder of the estrogen response trajectory was unconstrained in ME/CFS, as was the entirety of the estrogen response trajectory in the healthy control group.

Parameter selection was completely uninformed by any prior knowledge or experimental measurement of progesterone levels in either subject group. As such, these hormone measurements may be tested against the immunologically informed predictions from the network model as a validation step. In a 2-way ANOVA of estrogen and progesterone measurements over time, we found significant variation in estrogen according to health condition with elevated levels in patients with ME/CFS throughout the exercise response ($P = 0.002$); t tests at each independent time point consistently showed a marginally significant increase in patients with ME/CFS ($P < 0.1$) for this hormone. A 2-way ANOVA of progesterone measurements indicated a marginally significant difference in progesterone levels across groups ($P = 0.070$); however, individual t tests at each independent time point did not support these differences at this level of resolution. Nonetheless, the mechanistically predicted response trajectories in Figure 2 are not inconsistent with the hormone measurements shown in Supplemental Figure 5 (in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002). The model predicted constitutively upregulated estrogen levels in patients with ME/CFS throughout the course of exercise challenge and recovery, whereas progesterone was predicted to be elevated only transiently during recovery.

Progesterone is of special interest because our simulations predicted the greatest differences between ME/CFS and measurements in healthy subjects at time points immediately after peak exercise stress (T1+10, T1+20, T1+30, and T1+60). Although predictions of progesterone expression show good alignment with experimental measurements made at time points T0, T1, T2, and T3, no experimental data were available for further validation of this significant transient response.

Predicting Variations in Exercise Response

These putative mechanisms were then used to mechanistically filter the measured responses serving to constrain model parameters. This same mechanistic framework was used to predict expected values for unmeasured markers and markers with high within-group variability that were not used to constrain parameter optimization (Figure 2B). Such predictions may highlight new potentially significant

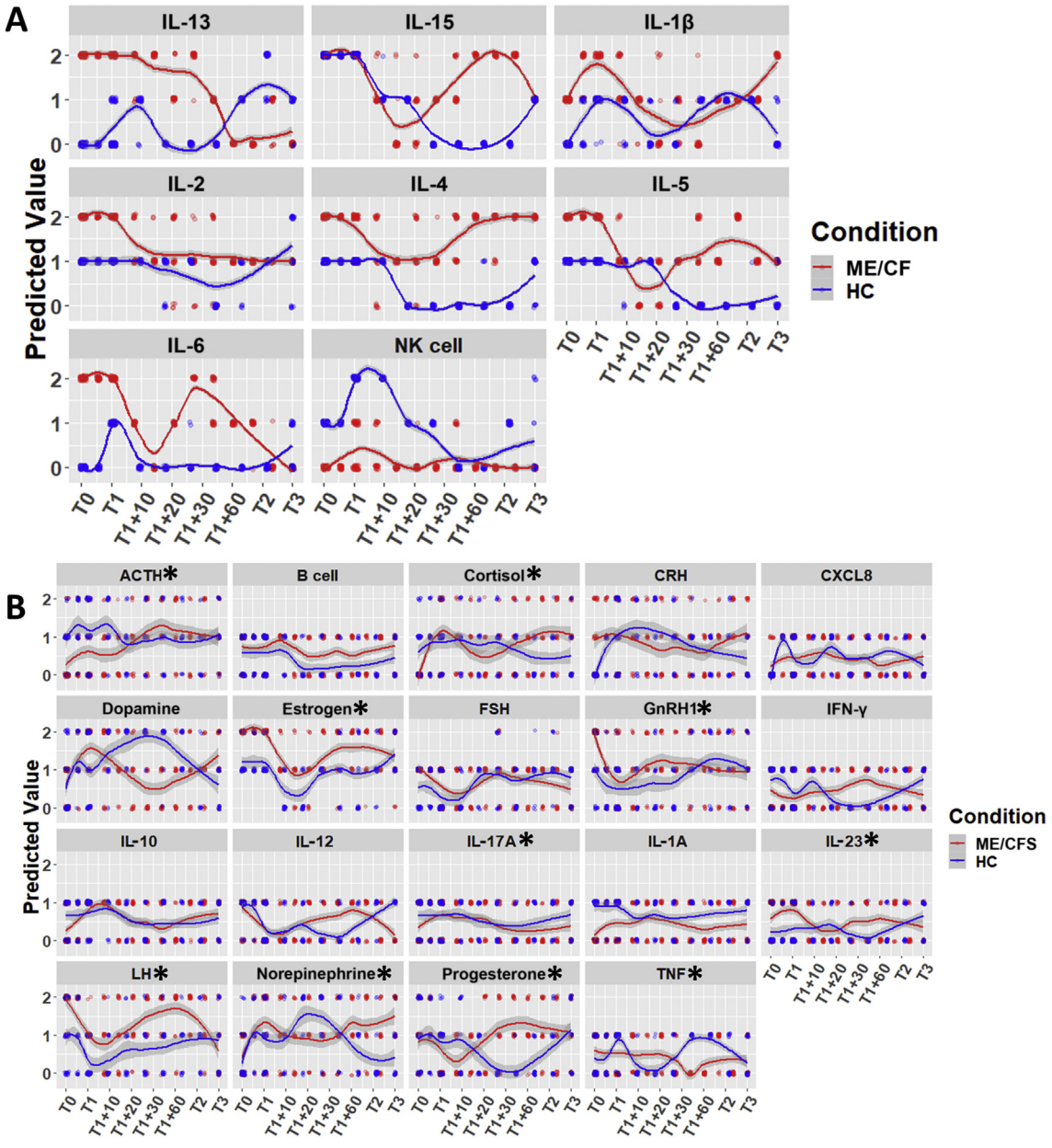

Figure 2. Predicted trajectories for all network entities across 26 top-performing solutions; subjects underwent maximum stress at time point 1 (T1). (A) Predicted trajectories for measured and constrained immune entities showing adherence to data. (B) Putative trajectories for unconstrained entities. Lines depict LOESS curves with 95% CIs (points are jittered to show relative frequency. *$P < 0.05$ for both myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) condition and its interaction with time, Benjamini–Hochberg–corrected ANOVA. ACTH = adrenocorticotropic hormone; CRH = corticotropin-releasing hormone; CXCL8 = chemokine (C-X-C motif) ligand 8; FSH = follicle-stimulating hormone; GnRH1 = gonadotropin-releasing hormone 1; HC = healthy control; IFN-γ = interferon-γ; IL = interleukin; LH = luteinizing hormone; NK = natural killer; TNF-α = tumor necrosis factor-α.

and mechanistically consistent differences in endocrine and immune response to exercise in patients with ME/CFS. Based on results from a 2-way ANOVA, a number of predicted trajectories for these unmeasured or high heterogeneity markers diverged significantly across time according to illness condition (time × condition interaction), suggesting an alternate regulation of these markers in response to physiological stress in ME/CFS. Specifically, these markers were ACTH, cortisol, estrogen, GnRH1, IL-17, IL-23, LH, and TNF-α. In addition, IL-1α, B-cell activation, CRH, and dopamine levels were predicted to vary across condition but independently of time.

Predicting Novel Endocrine–Immune Markers of ME/CFS at Rest

A basic hypothesis in this research has been that ME/CFS presents as a new regulatory set point for an alternative homeostatic state. To further understand what these set points might be for novel endocrine and immune markers, nonparametric Wilcoxon rank-sum tests were applied to the differences in the predicted steady-state expression of all unmeasured markers as well as high heterogeneity markers using all 26 competing numerical models. Because of the number of comparisons, the Benjamini-Hochberg correction was applied to these Wilcoxon tests. Endocrine mediators predicted to be constitutively overexpressed in ME/CFS at rest were CRH, estrogen, FSH, GnRH1, and LH, as well as the immune mediators chemokine (C-X-C motif) ligand 8 and IL-23. Constitutively downregulated entities were the stress hormones ACTH and cortisol, as well as immune cytokines IFN-γ, IL-10, IL-17, and IL-1α (Figure 3).

Although a group-wise comparison of average expression at rest in ME/CFS did not achieve statistical significance in the experimental data (Table I) for IL-8, IFN-γ, IL-10, IL-17, IL-1α, and IL-23, it is important to note that conventional univariate statistical tests do not account for the broader co-regulatory context. Indeed, the model-predicted values for these markers are necessarily in

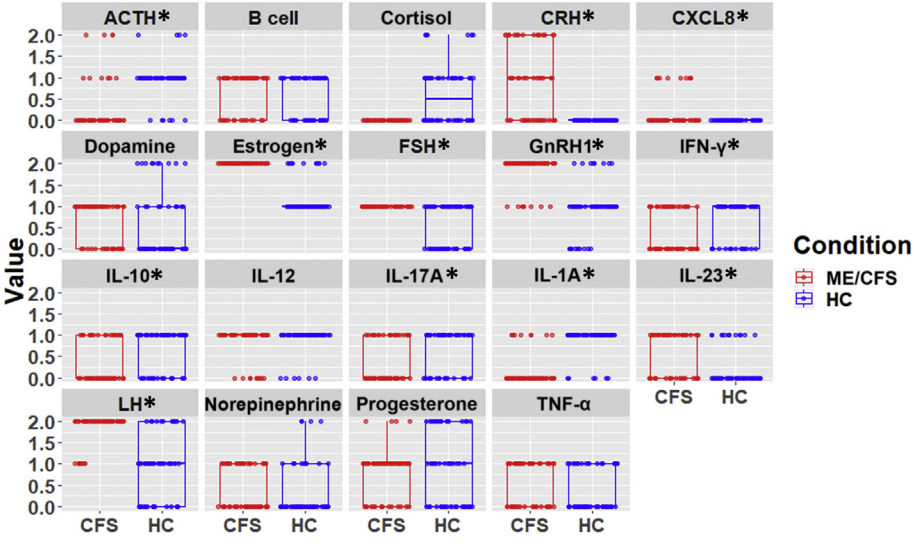

Figure 3. Frequencies of predicted values for unmeasured entities in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and in healthy control subjects (HC) at rest over 26 solutions with minimal error. *$P < 0.05$, Benjamini–Hochberg–corrected Wilcoxon test. ACTH = adrenocorticotropic hormone; CRH = corticotropin-releasing hormone; CXCL8 = chemokine (C-X-C motif) ligand 8; FSH = follicle-stimulating hormone; GnRH1 = gonadotropin-releasing hormone 1; IFN-γ = interferon-γ; IL = interleukin; LH = luteinizing hormone; TNF-α = tumor necrosis factor-α.

compliance with documented immune regulatory mechanisms. The various models may thus represent subtle differences in regulatory logic that may be characteristic of different patient subpopulations, and this divergence from group-wise univariate test results could indicate that these markers are especially sensitive to the within-group heterogeneity of this illness.

Simulating the Therapeutic Disruption of ME/CFS

Several pharmaceutical agents have been recently assessed in clinical trials for the treatment of ME/CFS. Among the most prominent are the B-cell depleting CD20 antibody rituximab[15,45] and the specific TLR3 agonist rintatolimod, which promotes innate inflammatory cytokine production and NK-cell activation.[11,12,46,47] We simulated these courses of treatment in patients with ME/CFS across the family of 26 data-compliant models. Interventions were modeled as fixing the biological targeting the network models to lower or higher values depending on the particular mode of action of the drug. The endocrine–immune network response was then simulated over a horizon of up to 100 transition events to observe whether immune and endocrine profiles evolve toward a new stable steady state, preferably one that more closely resembles a normal healthy equilibrium. The similarity of the new predicted steady state to both the healthy and ME/CFS resting states was expressed as the Euclidean distance, normalized by betweenness centrality of each endocrine and immune marker (see Supplemental Figure 2 in the online version at https://doi.org/10.1016/j.clinthera.2019.03.002) such that matching key mediators (eg, TNF-α, IL-1β, or progesterone) was favored over alignment with less influential markers (eg, IL-13 or IL-23). In an attempt to canvas a broad range of conditions, 200,000 simulations were conducted by selecting a random initial state from the complete set of states supported by the regulatory circuitry (on the order of 10^{11} states). We conducted these simulations under conditions of 0%, 1%, and 5% random noise to estimate the robustness of these solutions to biological variability.

Rituximab was modeled as inhibition of B cells, IFN-γ, and IL-4 because B cells reportedly produce these cytokines in the context of autoimmunity[48]; rintatolimod was modeled as induction of IL-12 and TNF-α.[46,49] Results of these simulations are depicted in Figure 4. In the absence of any drug, healthy and pathologic steady states were reached with roughly equivalent frequencies; increasing noise tended to make all attractors less available. Rintatolimod was predicted to sharply upset the attractor landscape, destabilizing most of the available attractors but retaining both healthy and ME/CFS states. Simulated rituximab treatment destabilized both healthy and ME/CFS attractors. However, the remaining attractors tended to be closer to normal health than to ME/CFS, indicating a potential reduced pathology relative to the untreated ME/CFS state. The outcome of these simulations is summarized in Table II.

Models predicting the most favorable response to treatment were surveyed to assess the degree of agreement between their respective predictions of endocrine–immune profile for ME/CFS at rest (Figure 5) to highlight profiles likely to be characteristic of good candidates for rituximab or rintatolimod treatment. Ten of the 26 models supported a return to the healthy homeostasis reference state under rintatolimod treatment, and 3 models supported return to an attractor with a Euclidean distance from health of <50 under rituximab treatment. These subsets of candidate models predicted that rintatolimod is most likely to benefit patients with low levels of IL-1α, IL-17, and cortisol, intermediate levels of progesterone and FSH, and high estrogen levels. The 3 of 26 models supporting a favorable response to rituximab described an ME/CFS state characterized by low norepinephrine, IL-1α, chemokine (C-X-C motif) ligand 8, and cortisol levels; intermediate levels of FSH and GnRH1; and high TNF-α, LH, IL-12, and B-cell activation.

DISCUSSION

In the present study, we assembled a network model of 28 endocrine and immune markers linked by 214 regulatory signaling mechanisms documented in the literature, including those elements reportedly involved in ME/CFS pathology. Twenty-six competing parameter sets were found that allowed this regulatory circuit to exactly reproduce the expression profile measured at rest in 8 characteristic immune markers for both the ME/CFS and healthy

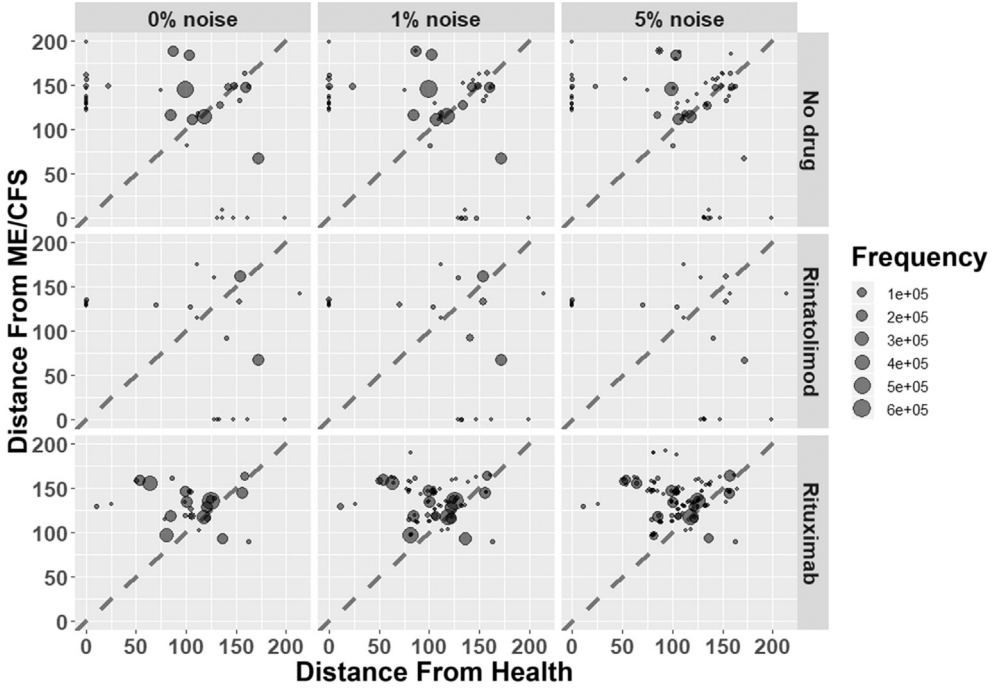

Figure 4. Results of simulated drug treatments. Each attractor is represented by a point, sized according to the number of simulations reaching it. Axes represent the Euclidean distance of each attractor from the Health and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) states multiplied by the betweenness centrality of each entity. Points above the diagonal are more similar to Health than to ME/CFS (less pathological); points below the diagonal are more similar to ME/CFS than to Health (more pathological).

control conditions as well as align with the exercise response dynamics of both groups to within 5% error. It is noteworthy that the complexity of the regulatory model exceeds the coverage supported by the available data. In this case, measurements for important hormones were unavailable, and the range of conditions was limited to exercise challenge of a specific type. This outcome results in a parameter identification problem that is highly underconstrained or in which many model solutions exist that satisfy the data equally well. As such, the 26 candidate models examined here represent only a small fraction of all possible solutions. However, they are all equally consistent with the available data, and if they are assumed to comprise a representative fraction of all the valid models, certain qualitative conclusions may still be drawn.

The finding that a single circuit model of endocrine–immune regulation can support both the healthy and ME/CFS phenotypes is in itself significant; it suggests that ME/CFS may consist of altered regulatory function without permanent damage to the underlying regulatory circuitry, such that substantial remission may be achievable. This finding, which is here supported by longitudinal exercise response data applied to a much more detailed model of endocrine–immune function, remains consistent with earlier research by our group using a much coarser-grained representation and resting state data only.[27]

Another important contribution is the explicit application of the signaling network based on known documented mechanisms directly in our analysis of experimental data to reinforce the coordinated and

Table II. Results of surveys on the basis of attraction. The number of simulations ending in a healthy or myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) attractor (Euclidean distance of 0) is indicated, along with the simulations ending in the healthy attractor as a fraction of simulations ending in health or ME/CFS.

Noise	Drug	Healthy	ME/CFS	Fraction
0%	No drug	33,031	509	0.985
0%	Rintatolimod	2584	385	0.867
0%	Rituximab*	4065	0	NA
1%	No drug	47,291	13,128	0.783
1%	Rintatolimod	11,152	7723	0.591
1%	Rituximab*	28,963	0	NA
5%	No drug	9604	2641	0.784
5%	Rintatolimod	4462	1397	0.762
5%	Rituximab*	8695	0	NA

NA, not applicable.
* Because simulated rituximab treatment rendered the healthy and ME/CFS attractors unavailable, a distance cutoff of 50 was used.

context-specific interdependency of immune and endocrine markers. For example, enforcing a documented co-regulatory structure to the 8 exercise responsive markers identified in these data and described earlier confirmed elevated expression in ME/CFS at rest of IL-1β, IL-4, IL-5, and IL-6 as reported previously by our group.[4] However, the model and data in this work also suggest elevated IL-2 and IL-13 levels, previously reported as unchanged and reduced in expression, respectively, in resting ME/CFS subjects. Interestingly, closer examination of this earlier data revealed that although increased group size (n = 40, patients with ME/CFS; n = 59, healthy control subjects) supported a statistically significant difference, the fold change in median expression of these markers was very weak (~1.1, 1.2, respectively). In this same previous study, relatively high intragroup variability in cytokine expression was observed (median absolute deviation/median >0.50), especially in the ME/CFS group. Conventional univariate statistical tests are ill-suited to this situation, and more complex variants that formally account for the interdependencies between markers and control for context are required. In the present study, rather than attempt to extract this interdependency structure from the data by using regression-based approaches, we applied known documented signaling mechanisms to this end. Indeed, controlling in the context of documented regulatory mechanisms suggests that IL-10, IL-17, and IFN-γ expression, previously reported as unchanged on average between groups,[4] may in fact be lower in patients with ME/CFS at rest in the context of joint marker expression. Controlling for co-regulated expression also predicts depressed levels of IL-1α and elevated levels of IL-2, IL-8, IL-23, and TNF-α in ME/CFS at rest, differences that were either undetected or contrary to reported median expression changes using conventional group-wise statistics.

Of note in the present study, this co-regulatory context was expanded beyond the immune system to include endocrine mediators. This finding is of special significance for an illness that disproportionately affects one sex over the other, as regular variations over the course of the menstrual cycle profoundly influence immune function.[8] Not surprisingly, dysregulation of the HPA and HPG axes, specifically overexpression of estrogen, FSH, GnRH1, and LH in patients with ME/CFS, is predicted based on the regulatory circuit model presented here. These hormones are important regulators of the menstrual cycle, which is known to be dysregulated in women with ME/CFS.[50] These results highlight the importance of considering cyclic fluctuations in hormonal regulation when considering complex metabolic disorders such as ME/CFS, especially in women. Interestingly, one case of ME/CFS associated with membranous dysmenorrhea spontaneously resolved after the discontinuation of hormonal contraceptive treatment.[51] Despite this observation, other studies have failed to find significant group-wise changes in sex hormones in ME/CFS.[52,53] Once again, however, these results are based on group-wise average expression and are not controlled in the context of co-expression in other markers.

Taken together, these mechanistically adherent differences suggest a general overactivation of the HPG axis and inactivation of the HPA axis in ME/CFS with a heightened sensitivity to inflammatory stimuli. IL-1β, IL-6, and TNF-α (all predicted in this study to be elevated in ME/CFS) are drivers of sterile inflammation, especially in the brain.[54,55] The

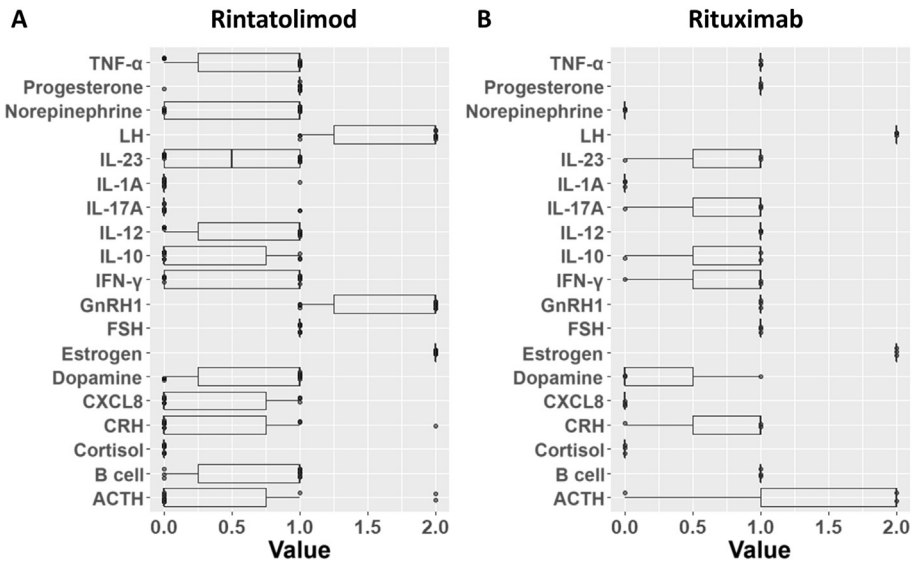

Figure 5. Predicted myalgic encephalomyelitis/chronic fatigue syndrome states associated with favorable response to treatment by A) rintatolimod or B) rituximab. The subset of models found to reach states with a distance-from-health of 0 (rintatolimod) or less than 50 (rituximab) under drug treatment were canvassed for their predictions of resting myalgic encephalomyelitis/chronic fatigue syndrome values for unconstrained network entities. ACTH = adrenocorticotropic hormone; CRH = corticotropin-releasing hormone; CXCL8 = chemokine (C-X-C motif) ligand 8; FSH = follicle-stimulating hormone; GnRH1 = gonadotropin-releasing hormone 1; IFN-γ = interferon-γ; IL = interleukin; LH = luteinizing hormone; TNF-α = tumor necrosis factor-α.

predicted upregulation of chemokine (C-X-C motif) ligand 8 and IL-23 could also be taken as an indication of increased inflammation in the brain, as both of these cytokines are associated with pathologic neuroinflammation.[56–58] GnRH1 agonists and recombinant FSH administered as a fertility treatment have been found to exacerbate multiple sclerosis, increasing blood–brain barrier permeability to peripheral blood mononuclear cells and a greater abundance of cells producing IL-8. Progesterone and estrogen levels were also increased in these patients.[59] Thus, HPG dysregulation may increase blood–brain barrier permeability, allowing infiltration of immune cells into the brain to establish or sustain a low-grade neuroinflammatory profile, as put forward by Morris and Maes.[17] Indeed, white matter lesions have long been reported in connection with ME/CFS.[60,61] More recent studies have found abnormalities in cerebrospinal fluid independent of psychiatric diagnoses,[62–64] consistent with neuroinflammation.

The origin of this neuroinflammatory dysregulation is unclear, with many competing hypotheses being proposed. Epstein–Barr and hepatitis viral infections have been implicated as potential causes,[14,65] with evidence that these viruses can persistently inhibit innate and adaptive immunity. Mitochondrial dysfunction has also been identified in patients with ME/CFS,[66] and the extracellular release of mitochondrial DNA in the hypothalamus has been proposed as an initiator of neuroinflammation via mast cell activation.[67] These activated mast cells may then go on to establish a self-sustaining positive feedback of neuroinflammation and autoimmunity,[68–70] with further complex interactions with other endocrine systems.[71,72] Our approach is independent of any assumptions about the ultimate underlying cause of ME/CFS. Observed patterns of immune dysregulation in the study cohort are sufficient for us to project concomitant differences in the HPA and HPG axes informed by known

regulatory relationships between these systems. In future studies we hope to extend the network model to include more explicit representation of other cell types and systems implicated in ME/CFS pathology such as mitochondrial metabolism and mast cells.

Although efforts to treat ME/CFS have often focused on therapeutic modulation of immune mediators, these trials have yielded mixed results. Rituximab has been effective in only a subset of patients, with no clear explanation for its mechanism of action.[45] Likewise, rintatolimod is sometimes effective,[11,12] but a means of identifying good candidates for this course of treatment remains similarly elusive. The mechanistic effects of rintatolimod *in vivo* have not been very well defined. Although the latter has been found to delay T-cell depletion in the context of HIV infection,[73] data regarding its influence on systemic cytokine production are sparse, especially as divergent responses have been described in primates and rodents.[74] Our simulations did not predict a complete rescue in most cases as a result of either rintatolimod or rituximab therapy. Although rintatolimod treatment was predicted to reach a target healthy remission state in some cases, these represented only a small fraction of all simulations. Rituximab treatment was not predicted to deliver remission to a target healthy state but instead to reduce the availability of highly pathologic steady states. These simulations offer a possible explanation for the wide variability in the reported efficacy of these drugs in clinical trials.

In general, rintatolimod is predicted to destabilize most attractors but does not necessarily disrupt the ME/CFS state, indicating that rintatolimod may either induce a more-or-less complete remission or have no appreciable effect. Rituximab, conversely, is more likely to support alternate stable resting states that are more similar to health than the initial ME/CFS pathologic state, resulting in a partial remission. These outcomes are highly dependent on both the initial endocrine–immune profile and regulatory tone represented here by different candidate models. Indeed, predicted treatment-responsive endocrine–immune expression profiles supported by these different models agree unanimously in only a select few markers. This result is not surprising given that ME/CFS is diagnosed based on adherence to a broad set of physiological symptoms, and the occurrence of multiple disease phenotypes in experimental studies of ME/CFS has been highlighted as a significant challenge.[75] Variability in the efficacy of different treatments is likely to be a consequence of this heterogeneity within study populations. Indeed, our analysis of the family of competing models that equally satisfied the experimental data suggested that inclusion criteria in further studies of rintatolimod and rituximab could be informed by subject stratification based on differences in the simultaneous co-expression patterns of cortisol, FSH, progesterone, and estrogen.

CONCLUSIONS

Computer simulations based on a literature-informed mechanistic model of endocrine–immune co-regulatory dynamics tuned to experimental exercise data support a potential etiology for ME/CFS in which sustained HPG overactivation may permit the initiation and maintenance of peripheral inflammation potentially leading to low-grade neuroinflammation. The partial efficacy of rituximab and rintatolimod treatment is predicted to alter the landscape of available steady states such that pathologic states are less available. Results suggest that future studies of ME/CFS and related clinical trials in women should consider the impact of the menstrual cycle on other endocrine and immune regulatory processes. Measurements of HPA and HPG hormones, especially cortisol, ACTH, estrogen, GnRH, and LH, may be of considerable value in developing rigorous biomarkers for ME/CFS diagnosis and delineating treatment-responsive subgroups.

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health awards R01 NS090200-01 (Dr. Fletcher, principal investigator [PI]); R56 AI065723-06A1 (Dr. Fletcher, PI); and R01AR057853-01 (Dr. Klimas, PI). It was also supported by the US Department of Defense Congressionally Directed Medical Research Program (http://cdmrp.army.mil/) under award GW140142 (Drs. Broderick/Craddock, PIs; Whitley, Partnering PI), where Dr. Darrell Whitley and his team contributed to the development of the computational framework used to support this analysis. Finally, this work was also supported by this agency under previous award GW093042 (Dr. Broderick, PI).

Dr. Morris and Ms. Cooney developed and evaluated the mathematical model, conducted the analyses, prepared graphics, and drafted the initial manuscript. Mr. Sedghamiz assisted with model

parameterization and analysis as well as manuscript editing. Dr. Broderick oversaw the design of the mathematical tools and the analyses and co-wrote the initial manuscript. Dr. Craddock reviewed the design of the methods, consulted on the methodology, and co-wrote and edited the manuscript. Dr. Abreu oversaw the collection, filtering, and normalization of the raw data. Ms. Collado and Ms. Balbin oversaw study coordination, recruitment, and processing of all subjects. Dr. Fletcher designed the study; oversaw all laboratory assessments, sample collection, and processing; and contributed directly to the interpretation of the results. Dr. Klimas designed the study; directed all clinical and scientific aspects of the overall study; and contributed directly to the study design and the interpretation of results. All authors have read and approved the final manuscript.

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Defense.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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APPENDIX A. SUPPLEMENTARY DATA

The following are the supplementary data to this article:

Figure A1. Histogram of reference counts associated with each interaction edge in the regulatory circuit model. The median reference count per interaction was 16.0 and the mean was 58.9.

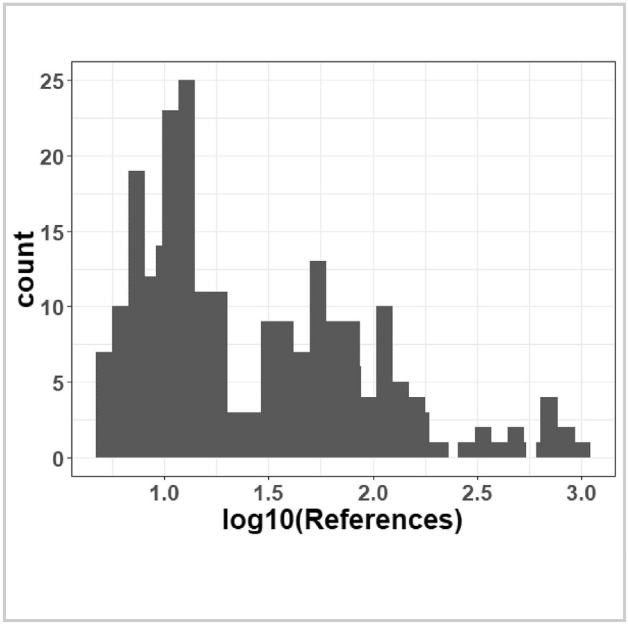

Figure A2. Betweenness centrality of network biomarker nodes. Stress, NK cell, and IL-13 have a betweenness centrality of 0 because they do not reside on any of the shortest paths through the network.

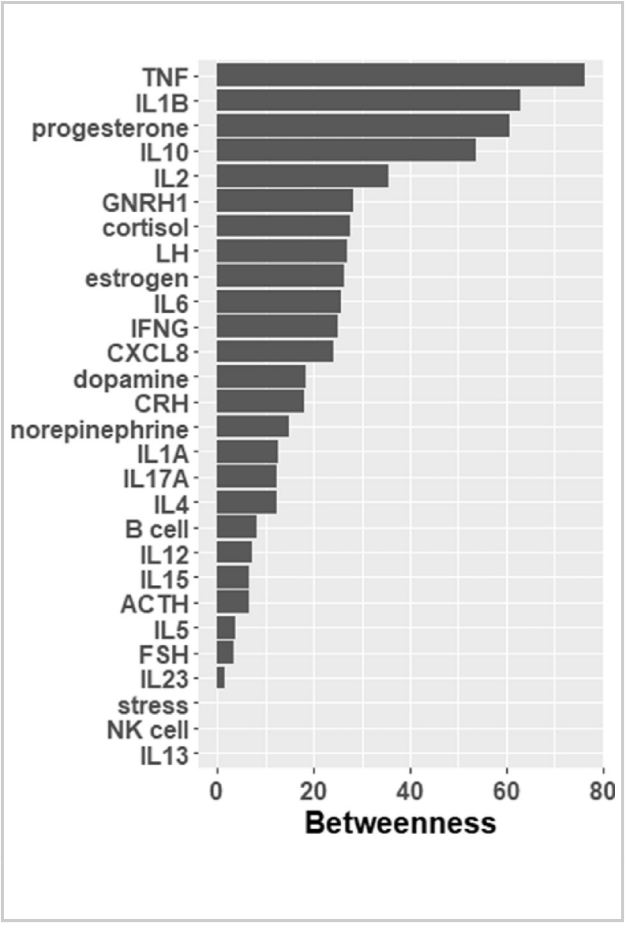

Figure A3. Experimental data for measured cytokines and cell populations over the time course of exercise responses, with maximum exertion at T1. Shaded areas represent 95% confidence intervals (* $p<0.05$ by ANOVA for ME/CFS, Timepoint, or their interaction).

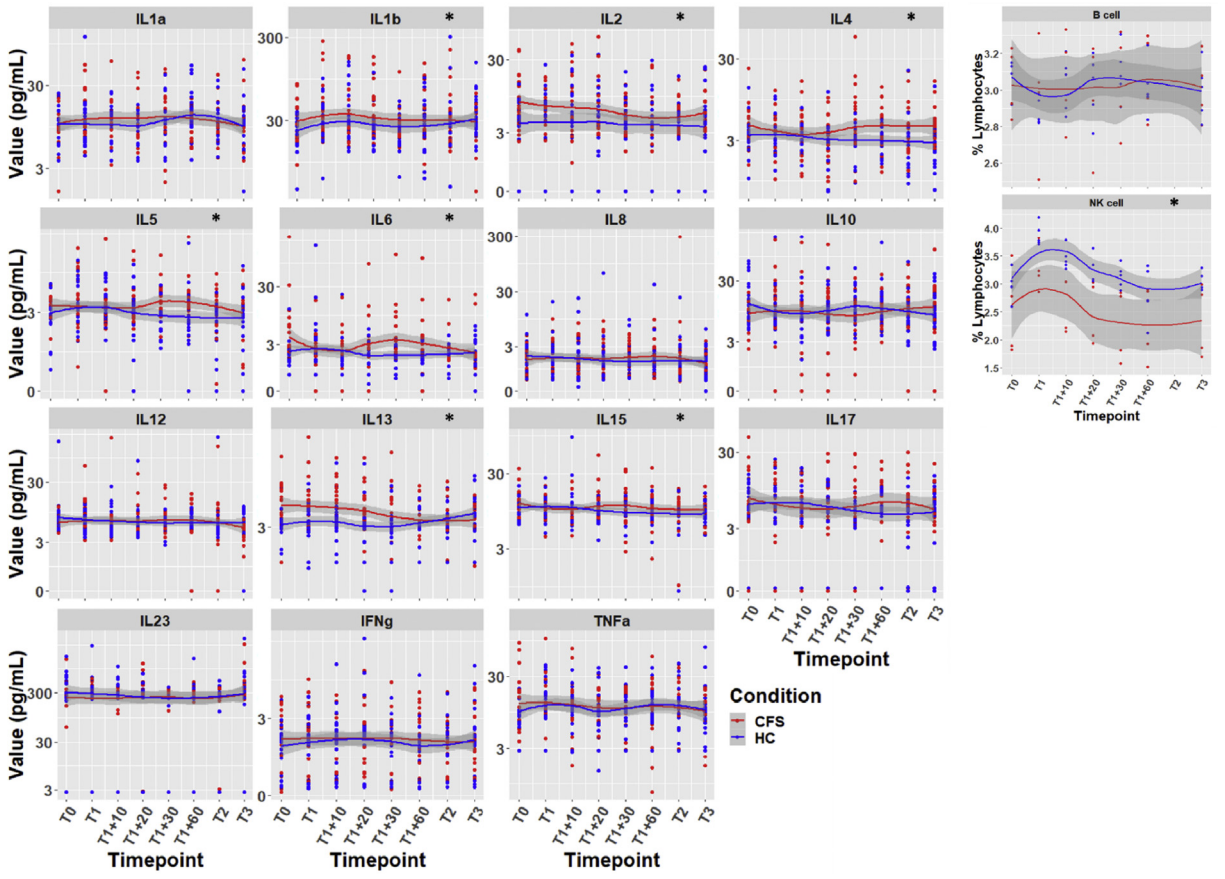

Figure A4. Discretized data for immune markers which varied significantly in expression across condition and/or timepoint. These discretized response trajectories are qualitative representations of the continuous measurements shown in Figure A3.

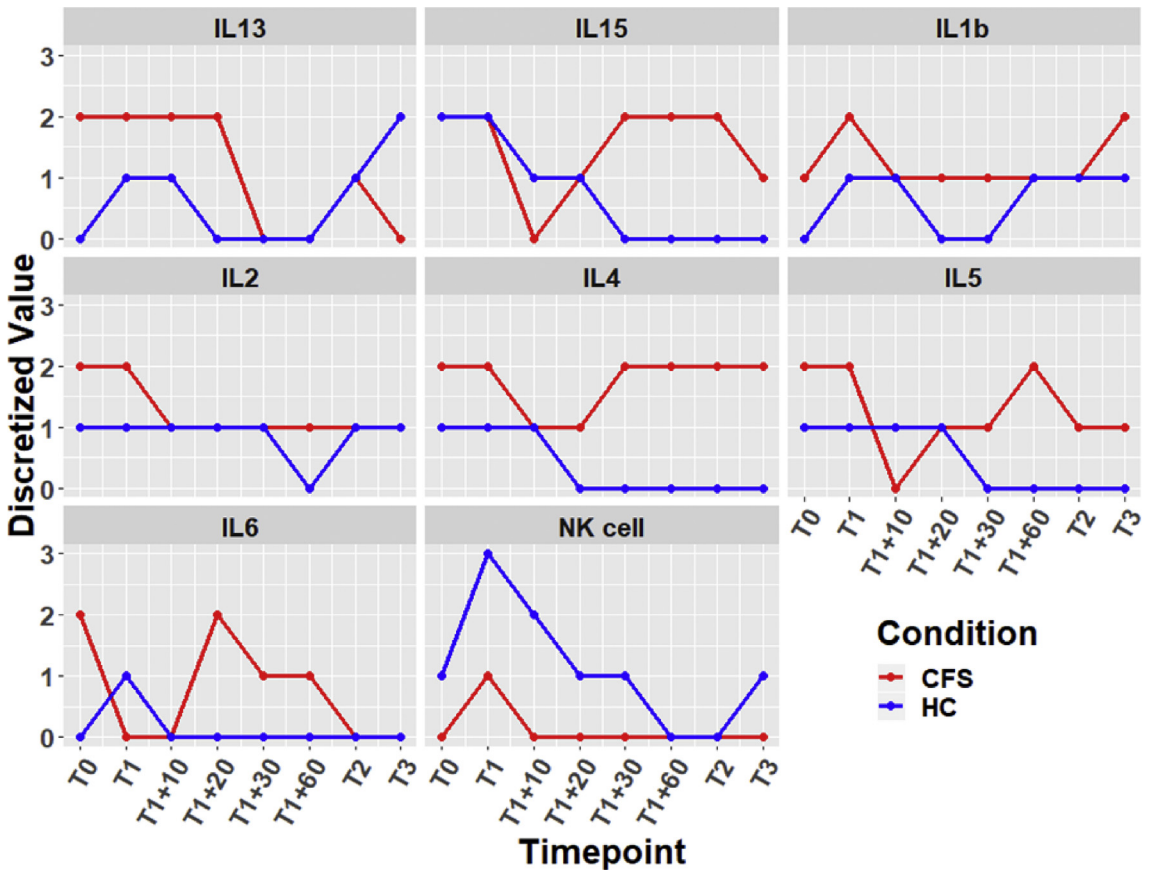

Figure A5. Estrogen and progesterone levels in study subjects during the course of an exercise response (+ $p<0.1$, t test).