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A formal analysis of cytokine networks in Chronic Fatigue Syndrome

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ABSTRACT

Chronic Fatigue Syndrome (CFS) is a complex illness affecting 4 million Americans for which no characteristic lesion has been identified. Instead of searching for a deficiency in any single marker, we propose that CFS is associated with a profound imbalance in the regulation of immune function forcing a departure from standard pre-programmed responses. To identify these imbalances we apply network analysis to the co-expression of 16 cytokines in CFS subjects and healthy controls. Concentrations of IL-1a, 1b, 2, 4, 5, 6, 8, 10, 12, 13, 15, 17 and 23, IFN-γ, lymphotoxin-α (LT-α) and TNF-α were measured in the plasma of 40 female CFS and 59 case-matched controls. Cytokine co-expression networks were constructed from the pair-wise mutual information (MI) patterns found within each subject group. These networks differed in topology significantly more than expected by chance with the CFS network being more hub-like in design. Analysis of local modularity isolated statistically distinct cytokine communities recognizable as pre-programmed immune functional components. These showed highly attenuated Th1 and Th17 immune responses in CFS. High Th2 marker expression but weak interaction patterns pointed to an established Th2 inflammatory milieu. Similarly, altered associations in CFS provided indirect evidence of diminished NK cell responsiveness to IL-12 and LT-α stimulus. These observations are consistent with several processes active in latent viral infection and would not have been uncovered by assessing marker expression alone. Furthermore this analysis identifies key sub-networks such as IL-2:IFN-γ:TNF-α that might be targeted in restoring normal immune function.

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1. Background

Chronic Fatigue Syndrome (CFS) is characterized by persistent and unexplained fatigue resulting in severe impairment in daily function and is defined by symptoms, disability, and exclusion of medical and psychiatric conditions that could explain the fatigue (Fukuda et al., 1994; Reeves et al., 2003; Prins et al., 2006). The US Centers for Disease Control and Prevention (CDC) estimates that as many as 4 million people are affected with CFS in the US alone (Reeves et al., 2007; Chandler et al., 2008). Costs to the US economy are estimated at $9.1 billion in lost productivity (Reynolds et al., 2004) and up to $24 billion dollars in health care expenditures annually (Jason et al., 2008). Furthermore complications and co-morbidity can be severe. For example, CFS is associated with chronic and episodic cardiovascular and autonomic dysfunction (Gerrity et al., 2003). Therefore this illness has far-reaching consequences and constitutes a significant public health concern.

Evidence of chronic immune dysfunction in CFS has been reported by several groups (Klimas et al., 1990; Straus et al., 1993; Hilgers and Frank, 1994; Keller et al., 1994; Tirelli et al., 1996; Gupta et al., 1997; Patarca et al., 1997; Patarca-Montero et al., 2001; Siegel et al., 2006) though the exact nature of this dysfunction remains unclear (Maher et al., 2003). A principal avenue of investigation has been the measurement in blood of immune signals conducted by cytokines. Many of the symptoms experienced by CFS patients strongly resemble the "sickness behavior" that can be induced by the administration of pro-inflammatory cytokines. In particular decreased motor activity, altered food and water intake, sleep and cognition have been linked to increases in the levels of IL-1b, IL-6 and TNF-α in the brain (Dantzer et al., 2008). Individual cytokines however are pleiotropic and their biological activities are known to be context specific. This becomes evident when considering the current body of work focused on immune dysfunction in CFS. While some studies have reported increased levels of anti-inflammatory cytokines such as IL-10 (ter

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Wolbeek et al., 2007) and IL-4 (Skowera et al., 2004), others have shown a correlation with pro-inflammatory signals TNF-α and IL-6 (Gaab et al., 2005; Carlo-Stella et al., 2006). Admittedly the heterogeneity of the CFS population (Vollmer-Conna et al., 2006; Aspler et al., 2008; Kerr et al., 2008b) has been an issue. However a major failing remains analytical. In particular immunological markers continue to be analyzed individually even though their expression is articulated as part of an integrated network. In addition to the numerical advantages of a combinatorial approach, for example the control of excessive measurement noise (Szymanska et al., 2007), it is becoming apparent that understanding complex disease will require more than a list of defective cells or genes. Because cellular and molecular components are highly inter-dependent it is necessary to understand the "wiring" via which they interact (Barabási, 2007). Immune cells form a distributed network of diverse elements that exchange information through a complex web of interactions (Orosz, 2001). The architecture of such a networked system profoundly impacts its behavior (Klemm and Bornholdt, 2005) and the strategies that are available for adapting to change and maintaining homeostasis. Nonetheless, the formal analysis of biological networks in defining disease phenotypes has received relatively little attention. Recent attempts have focused on the visual comparison of relatively sparse collections of known pathway elements (Kerr et al., 2008a) or a broad description of shifts in overall structure (Emmert-Streib, 2007). We have extended this work in several important ways, introducing continuous metrics that quantify not only the degree of change but the type of change occurring in global and more importantly in local network structure. These metrics have allowed us to identify functional communities of markers within these networks as well as key elements driving disease-related changes in network structure (Fuite et al., 2008).

Here we use network constructs such as these to examine how patterns in the coordinated expression of cytokines might differ in CFS subjects. In a recent publication we introduced the multiplex method to simultaneously measure a broad spectrum of 16 cytokines in order to assess their use as biomarkers for CFS (Fletcher et al., 2009). Using this same experimental data we have now constructed separate networks describing co-expression of these 16 cytokines in a group of CFS subjects and in a group of healthy controls, respectively. Pair-wise mutual information (MI), estimated from the biological variability within each group, was used as a robust measure of association between cytokines. These networks were then analyzed using quantitative metrics rooted in graph theory to assess the importance and nature of architectural changes related to illness. In particular we assessed local changes in the degree of connectivity at cytokine nodes and the redistribution of these connections as they form distinct and more locally centered communities. Consistent with our previous work (Fuite et al., 2008) we found that these cytokine networks differed significantly in architecture between diagnostic groups emphasizing that the organizational attributes of the immune response in addition to the activation level of individual markers constitute a unique characteristic of CFS. Of note distinct modules emerged in both healthy control and CFS networks that were recognizable as components of Th1, Th2 and Th17 responses. In CFS we found consistent but significantly attenuated patterns of Th1 and Th17 response occurring in the context of a well-established Th2 inflammatory environment. These patterns would have escaped detection had the analysis focused solely on differential expression of individual cytokines. Interestingly the cytokine co-expression patterns described in this study, though not uniquely assignable to a viral pathology, were at least consistent with the disruptive effects of latent viral infection by pathogens such as Epstein–Barr virus (EBV) (Samanta and Takada, 2009; Tsuge et al., 2001).

2. Materials and methods

2.1. Sample collection and processing

2.1.1. Subject cohort

Female CFS patients ($n = 40$; mean age 50) were from the CFS and Related Disorders Clinic at the University of Miami. A diagnosis of CFS was made using the International Case Definition (Fukuda et al., 1994; Reeves et al., 2003). Healthy female controls ($n = 59$; mean age 53) were from a NIH funded study. All CFS study subjects had a SF-36 summary physical score (PCS) below the 50th percentile, based on population norms. Exclusion criteria for CFS included all of those listed in the current Centers for Disease Control (CDC) CFS case definition, including the listed psychiatric exclusions, as clarified in the International CFS Working Group (Reeves et al., 2003). All CFS subjects were assessed for psychiatric diagnosis at the time of recruitment with the Composite International Diagnostic Instrument (World Health Organization, 1997). Based on this assessment, we excluded subjects with DSM IV diagnoses for psychotic or melancholic depression, panic attacks, substance dependency, or psychoses as well as any subjects currently suicidal. We also excluded subjects with Borderline or Antisocial Personality Disorder. Subjects had no history of heart disease, COPD, malignancy, or other systemic disorders that would be exclusionary, as clarified by Reeves et al. (2003). Subjects were excluded for the following reasons: less than 18 yrs of age, active smoking or alcohol history, history of significant inability to keep scheduled clinic appointments in past.

Ethics statement. All subjects signed an informed consent approved by the Institutional Review Board of the University of Miami. Ethics review and approval for data analysis was also obtained by the IRB of the University of Alberta.

2.1.2. Cytokine profiles

Morning blood samples were collected into ethylene diamine tetra acetic acid. Plasma was separated within 2 h of collection and stored at −80 °C until assayed. We measured 16 cytokines in plasma using Quansys reagents and instrument (Quansys Biosciences, Logan, Utah). The Quansys Imager, driven by an 8.4 megapixel Canon 20D digital SLR camera, supports 96 well plate based chemiluminescent imaging. The Q-Plex™ Human Cytokine - Screen (16-plex) is a quantitative enzyme-linked immunoabsorbent assay (ELISA)-based test where sixteen distinct capture antibodies have been absorbed to each well of a 96-well plate in a defined array. Manipulation of the range of the standard curves and exposure time allowed reliable comparisons between CFS patients and controls of both low and high level cytokine concentrations in plasma. For the standard curves, we used the second order ($k = 2$) polynomial regression model (parabolic curve): $Y_p = b_0 + b_1X_1 + b_2X_2 \ldots + b_kX_k$, where Y_p is the predicted outcome value for the polynomial model with regression coefficients b_1 to k for each degree and y intercept b_0. Quadruplicate determinations were made, i.e., each sample was run in duplicate in two separate assays. Statistics reported in Table S3 show an average coefficient of variability (CV) of 0.20 for inter-assay and 0.09 for intra-assay repeatability. Also reported in Table S3 are the lower limits of detection (LLD) for each cytokine estimated from the standard calibration curve. In many cases the standard curve yielded a negative intercept value indicating that the modified assay produced a background optical signal at zero concentration. Accordingly, the standard curves were truncated at this baseline optical intensity and no negative concentration values were estimated or used in this analysis. In the case of cytokines with positive intercept values very few samples produced results below the LLD with the exception of IL-17. While the LLD for IL-17 was lower with the modified protocol roughly

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