LETTERS

A network-based analysis of systemic inflammation in humans

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Oligonucleotide and complementary DNA microarrays are being used to subclassify histologically similar tumours, monitor disease progress, and individualize treatment regimens[1–5]. However, extracting new biological insight from high-throughput genomic studies of human diseases is a challenge, limited by difficulties in recognizing and evaluating relevant biological processes from huge quantities of experimental data. Here we present a structured network knowledge-base approach to analyse genome-wide transcriptional responses in the context of known functional interrelationships among proteins, small molecules and phenotypes. This approach was used to analyse changes in blood leukocyte gene expression patterns in human subjects receiving an inflammatory stimulus (bacterial endotoxin). We explore the known genome-wide interaction network to identify significant functional modules perturbed in response to this stimulus. Our analysis reveals that the human blood leukocyte response to acute systemic inflammation includes the transient dysregulation of leukocyte bioenergetics and modulation of translational machinery. These findings provide insight into the regulation of global leukocyte activities as they relate to innate immune system tolerance and increased susceptibility to infection in humans.

Inflammation is a hallmark of many human diseases[6–8]. We focus on blood leukocytes and other tissues of critically injured patients, in order to better elucidate the mechanisms underlying systemic inflammatory responses[9]. This approach cannot be fully replicated using animal models or human cell lines, and studies of injury in humans can be complicated by antecedent illnesses and concurrent treatment regimes that may alter the recovery process. To our knowledge, no study has evaluated the genome-wide response to systemic inflammation in the context of a fully predictable recovery. Here we combine genome-wide expression analysis with a new bioinformatics method to identify functional networks responsible for the systemic activation and spontaneous resolution of a well-defined inflammatory challenge.

Gene expression in whole blood leukocytes was determined immediately before and at 2, 4, 6, 9 and 24 h after the intravenous administration of bacterial endotoxin to four healthy human subjects. Four additional subjects were studied under identical conditions but without endotoxin administration. The infusion of endotoxin activates innate immune responses and presents with physiological responses of brief duration[10]. Notably, there is an initial proinflammatory phase and a subsequent counterregulatory phase, with resolution of virtually all clinical perturbations within 24 h.

K-means cluster and principal component analyses were first used to visualize the overall response to endotoxin administration. Figure 1a reveals probe sets clustered by *K*-mean analysis, where each bin has a distinct endotoxin-induced temporal pattern. The signal intensity of 5,093 probe sets—representing 3,714 unique genes—out of a total of >44,000 probe sets changed significantly in response to endotoxin, whereas no significant changes were observed in control subjects (estimated false discovery rate <0.1%). Of the 5,093 probe sets identified, over half showed reduced abundance at 2, 4, 6 and 9 h, returning to baseline by 24 h (see bins 0–4). In contrast, a smaller number of probe sets were induced by 2 h (bins 5, 6), and the remaining probe sets showed a delayed response, peaking at 4–9 h but returning to baseline by 24 h (bins 7–9).

Cluster and principal component analyses describe overall changes in apparent gene expression, but provide few insights into the biological processes and signalling networks invoked in propagation and resolution of the inflammatory response. Identifying the perturbed biological networks underlying this complex clinical phenotype requires systematic analysis in the context of known mammalian biology, derived from basic and clinical research.

Using a web-based entry tool developed by Ingenuity Systems Inc., findings presented in peer-reviewed scientific publications were systematically encoded into an ontology by content and modelling experts. Using over 200,000 full-text scientific articles, a knowledge base of more than 9,800 human, 7,900 mouse and 5,000 rat genes was manually curated and supplemented with curated relationships parsed from MEDLINE abstracts. A molecular network of direct physical, transcriptional and enzymatic interactions observed between mammalian orthologues—the observed 'interactome'—was computed from this knowledge base. The resulting network contains molecular relationships involving over 8,000 orthologues with a high degree of connectivity. On average, individual genes have 11.5 interaction partners (median 4.0), of which 7.2 represent direct physical interactions (median 3.0). Every gene interaction in the network is supported by published information. For example, the

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immediate neighbourhood for the *RELA* gene (NF-κB p65, Supplementary Fig. 1) includes 150 genes and 619 direct interactions, derived from 7,118 findings curated from 847 published articles.

The observed interactome provides a framework for structuring the existing knowledge regarding mammalian biology, and enables a new analytical approach that objectively examines experimental data in the context of known genome-wide interactions in order to identify significant functional modules. This method is applicable to data of high-throughput platforms such as microarray expression profiling, polymorphism analysis and proteomics. Furthermore, the original literature detailing the genetic interactions can be accessed to further examine and verify the findings.

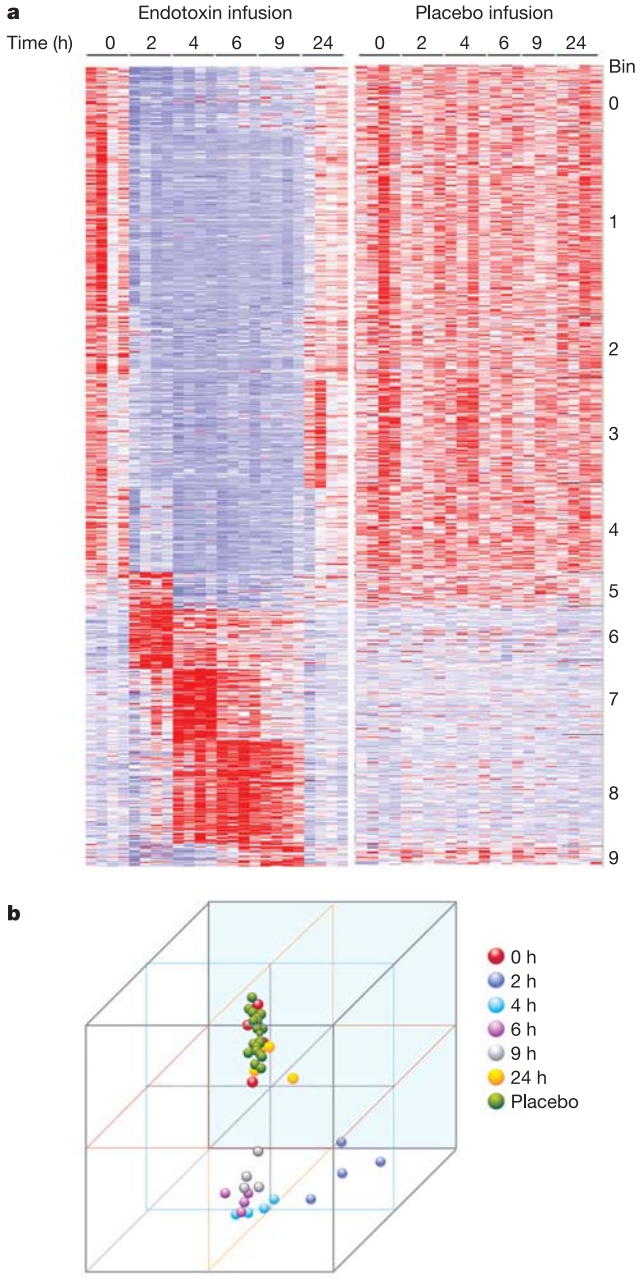

Figure 1 | Gene expression profiles of circulating leukocytes in response to bacterial endotoxin infusion. Samples from eight healthy volunteers were tested at baseline (0 h) and 2, 4, 6, 9 and 24 h after intravenous administration of endotoxin (four subjects) or vehicle (four subjects). **a**, Significant (false discovery rate of <0.1%) probe sets (5,093) were subjected to *K*-means clustering into ten bins (0–9). Probe sets for which the abundance was above the mean are shown in red, below the mean are shown in blue, and equivalent to the mean are in white. **b**, Principal component plot of the significant probe sets at the indicated times after endotoxin administration.

For a better understanding of the temporal response of gene expression in the innate immune system, we constructed a prototypical inflammatory cell containing 292 representative genes and detailing all direct interactions in our database (Fig. 2). Closer inspection of the temporal response reveals the fine structure of dynamic changes in RNA abundance by highlighting the transient and self-limiting nature of this response. As an example, the apparent expression of several secreted proinflammatory cytokines and chemokines (*TNFSF2* (*TNF*), *IL1A*, *IL1B*, *CXCL1* (*GROa*), *CXCL2* (*GRO-β*), *CCL2* (*MCP-1*), *CXCL8* (*IL-8*) and *CXCL10*) reached a maximum 2–4 h after endotoxin administration, consistent with early activation of innate immunity. Subsequently, the expression of several members of the nuclear factor kappa/relA family of transcription factors (*NFKB1*, *NFKB2*, *RELA* and *RELB*) reached their zenith.

The time period 4–6 h after endotoxin injection seemed critical, as the expression of a number of transcription factors was increased, including both those that initiate and those that limit the innate immune response. In the former group, these included the signal transducer and activators of transcription (*STAT* genes), and the cAMP-response element-binding protein (*CREB*) and CCAAT/enhancer binding protein (*CEBP*) gene families. Transcription factors limiting the innate immune response included suppressor of cytokine signalling 3 (*SOCS3*) and *IKBK* genes. There was also a delay (4–6 h) in increased mRNA abundance of secreted and membrane-associated proteins that limit the inflammatory response, including *IL1RAP*, *IL1R2*, *IL10* and *TNFRSF1A*. Together, these data comprehensively document the temporal modulation of genes controlling the innate immune response in a human model that progresses from an acute proinflammatory phase to unencumbered counter-regulation, concluding with full recovery and a normal phenotype.

To further elucidate the global changes during inflammation and subsequent return to homeostasis, we sought to computationally decipher the principal networks involved. The specificity of connections for each gene was calculated, as defined by the percentage of its direct connections to other genes showing significant transcriptional changes. A network pathway was initiated by the gene with the highest specificity of connections, and was propagated according to the descent of the specificity. Individual significant pathways identified by a statistical likelihood calculation ($P < 0.0001$) were merged to represent the biological processes.

Our global representation of the inflammatory response to endotoxin, shown in Fig. 3a, comprises a network of 1,556 genes and their interactions. This network consists of a subset of 1,214 genes (78%) responsive to *in vivo* endotoxin administration, and 342 additional, highly interconnected genes. The gestalt of the temporal response to endotoxin is suggested at the level of interconnecting functional modules, which could not be readily extracted from the experimental data alone (Fig. 1). Simultaneous survey and evaluation of the subnetwork regions enables us to identify new endotoxin-responsive modules in addition to the innate immunity network described above. Examples of the diversity in such modules include (a) increased expression of components of the superoxide-producing phagocyte NADPH-oxidase system, a multicomponent enzyme important for host defence[11], (b) decreased expression of the major histocompatibility (MHC) II complex, consistent with reduced antigen presentation following endotoxin stimulation[12,13], (c) decreased expression of the TCP1 ring complex required for folding of cytoskeletal proteins, (d) increased expression in the family of tubulin-A microtubule genes, (e) suppressed expression of several subunits of the anaphase-promoting complex, which has a key role in cell-cycle regulation, and (f) reduced integrin-α and -β chain expression, affecting cell–cell and cell–matrix adhesion (see Supplementary Methods 2, containing Supplementary Fig. 3a–f).

Further, significant decreases in messenger RNA abundance were

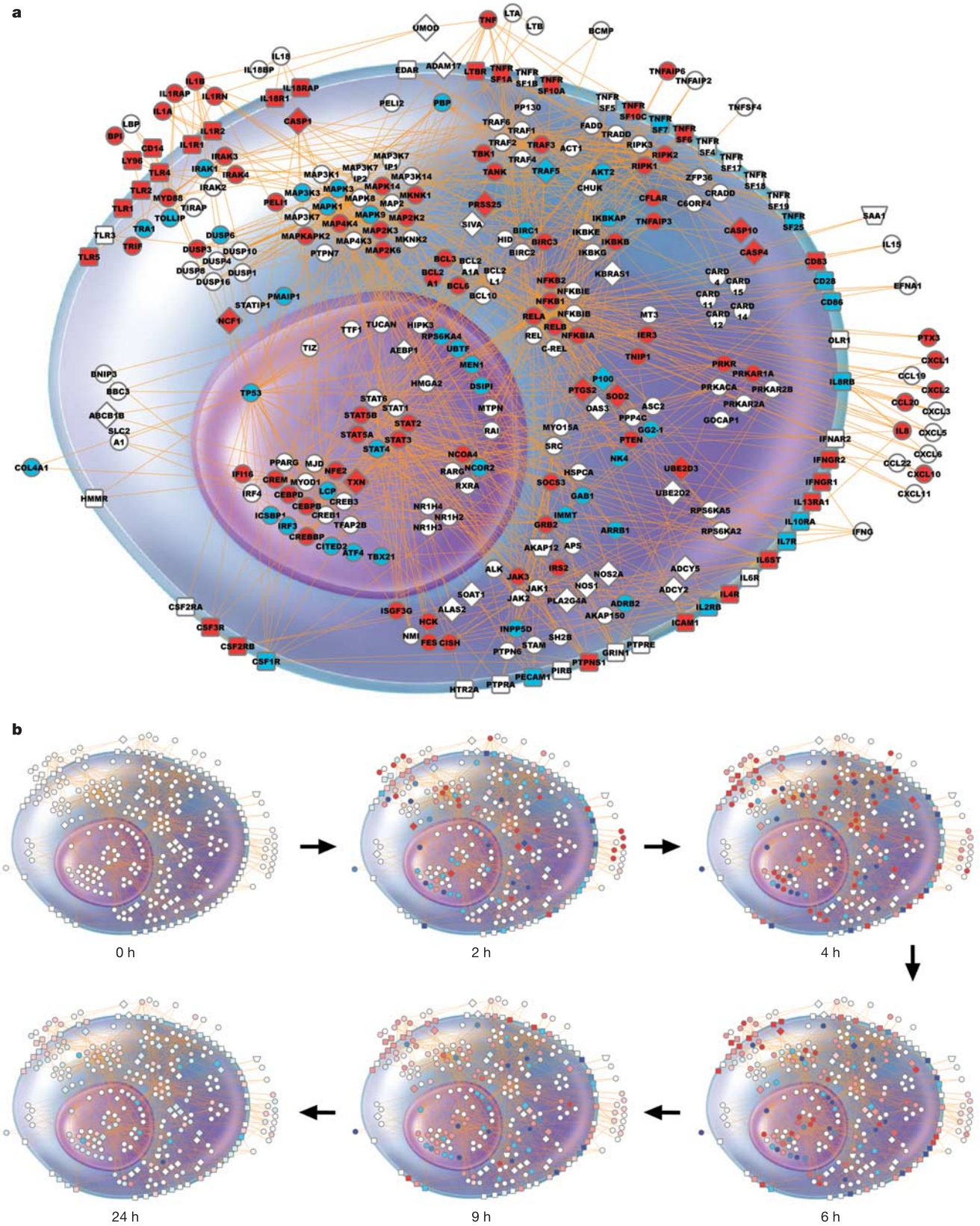

Figure 2 | Pathway analysis of representative genes involved in innate immunity. A prototypical inflammatory cell was constructed from 292 representative genes involved in inflammation and innate immunity. Genes for which the expression statistically increased from baseline are coloured red, those for which expression decreased are shown in blue. **a**, Composite changes in apparent expression over 24 h, identifying nodes and interactions. **b**, Temporal changes in apparent expression. The response to endotoxin administration in blood leukocytes can be viewed as an integrated cell-wide response, propagating and resolving over time.

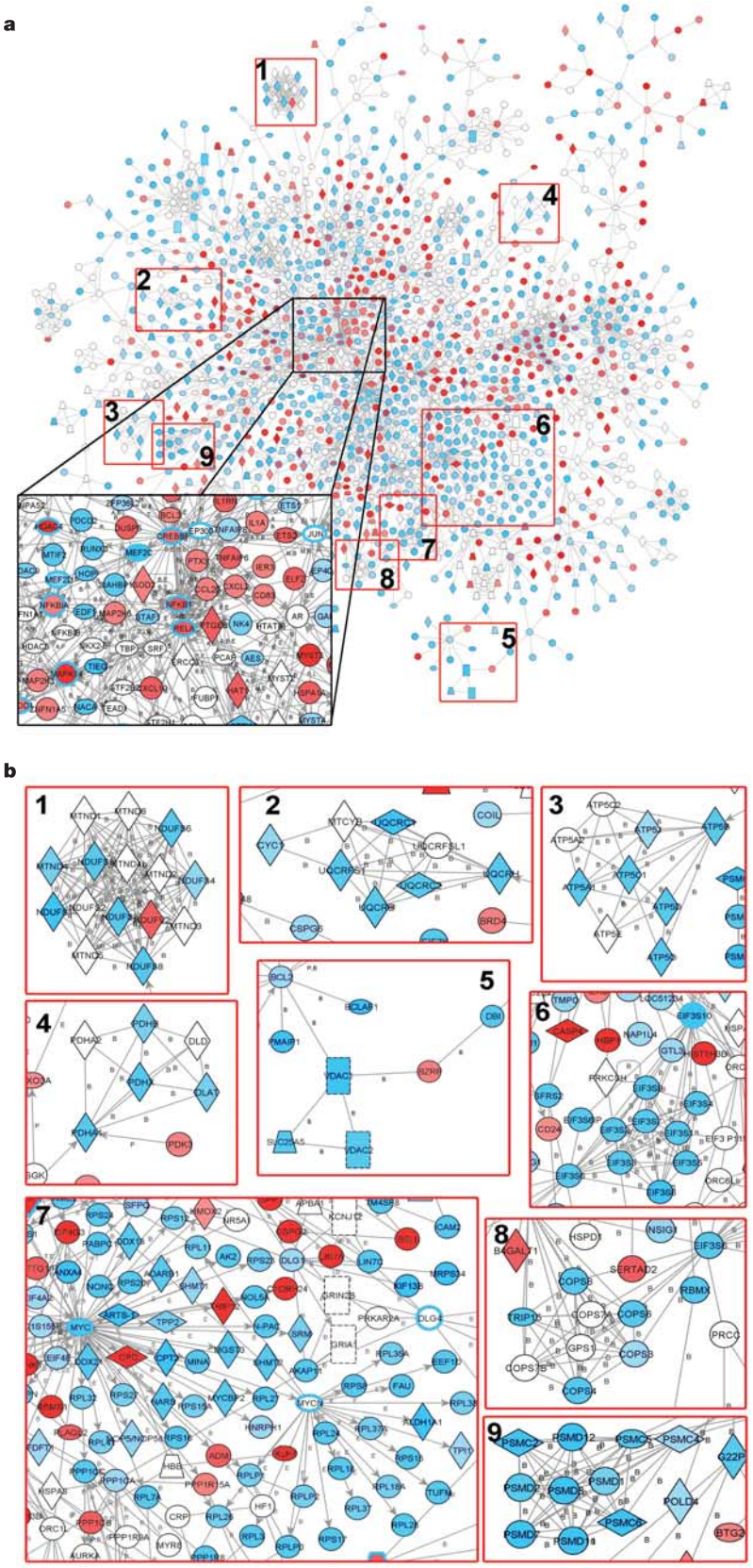

Figure 3 | Network representation of the biological processes underlying the temporal response of blood leukocytes to *in vivo* endotoxin administration. **a**, The network consists of 1,214 genes showing perturbed expression, and 342 genes highly interconnected to this group (red, increased; blue, decreased expression). **b**, Selected regions of the network, highlighting several groups of genes. Group 1, mitochondrial respiratory chain complex I (*NDUF* genes). Group 2, mitochondrial respiratory chain complex III (*UQCR* genes). Group 3, ATP synthase complex (*ATP5* genes). Group 4, pyruvate dehydrogenase complex. Group 5, mitochondrial permeability transition pore complex. Group 6, elongation initiation factor complex (*EIF3* genes). Group 7, ribosomal proteins (*RPL*, *RPS* genes). Group 8, COP9 signallosome (*COPS* genes). Group 9, proteasome (*PSM* genes).

observed in the mitochondrial respiratory chain complexes I–V (*NDUF* in Fig. 3b (Group 1), *UQCR* (Group 2) and ATP synthase genes (Group 3)). Gene expression was also decreased in the pyruvate dehydrogenase complex (PDH, Fig. 3b, Group 4), which generates, via acetyl-CoA and the tricarboxylic acid (TCA) cycle, reduced coenzymes required for ATP synthesis during mitochondrial oxidative phosphorylation. A concomitant increase in expression of pyruvate dehydrogenase kinase-3 (*PDK3*), an inhibitor of PDH, was observed. Expression of the voltage dependent anion channel (*VDAC*) and adenine nucleotide translocator (*SLC25A5*), components of the mitochondrial permeability transition pore (MPTP) complex, were decreased to similar extents, whereas expression of the antagonistic benzodiazepine receptor (*BZRP*) was increased (Fig. 3b, Group 5). MPTP activation has previously been considered an early event in apoptosis, leading to mitochondrial membrane depolarization and release of cytochrome *c*. However, recent reports have indicated a primary role for MPTP activation in oxidative-stress- and calcium-overload-induced necrotic cell death[14,15]. Thus, reduction in transcripts for MPTP components is consistent with a protective response to the oxidative stress associated with endotoxin challenge.

As active secretory cells, leukocytes devote a substantial amount of energy expenditure to protein synthesis[16]. In concert with the suppression of modules participating in energy production, expression was decreased for the elongation initiation factor complex (EIF3 in Fig. 3b, Group 6), a large number of ribosomal proteins (*RPS*, *RPL* genes in Fig. 3b, Group 7), the RNA polymerase II complex, and also in the functional modules of the ATP/ubiquitin-dependent protein degradation pathway, the COP9 signallosome (Fig. 3b, Group 8) and the proteasome (*PSM* genes in Fig. 3b, Group 9).

Here we use a knowledge-based network analysis to reveal concerted dysregulation of functional modules in mitochondrial bioenergetics, protein synthesis and protein degradation in human blood leukocytes during an abbreviated and self-limiting episode of inflammation. These findings document a reprioritization of the transcriptional regulatory programme in leukocytes in response to endotoxin. Furthermore, the marked suppression of these important functional networks suggests that leukocytes exposed to inflammatory stimuli may have an altered capacity to sustain subsequent immune challenges, as observed during innate immune system tolerance.

Human disease phenotypes are manifested by the malfunctioning of multiple functional modules interrelated in physiological regulatory systems. The overwhelming diversity of possible genome-wide interactions and gene expression patterns limit effective learning from experimental data alone. Network analyses using comprehensive knowledge of mammalian biology can greatly reduce the hypothesis space, enabling identification of new functional modules perturbed in the disease process. Here we demonstrate that, upon acute systemic inflammation, the human blood leukocyte response includes widespread suppression at the transcriptional level of mitochondrial energy production and protein synthesis machinery. A decrease in high-energy substrates has been observed in the muscle and liver of critically ill patients, and in animal models of sepsis, burn injury and endotoxemia[17–19]. In addition, direct disruption of mitochondrial complexes by cellular-stress-derived mediators (for example, reactive oxygen species) has been suggested for necrotic cell death in animal models of sepsis[20] and reperfusion injury[14,15].

The erosion in functional networks identified above represents a normal adaptive process aimed at re-establishing homeostasis, and yet might contribute to global leukocyte defects—such as tolerance and increased susceptibility to infection—observed in critically injured patients. These perturbations in functional modules are at the level of mRNA transcripts, and will require subsequent confirmation at the protein level. Further identification of the specific cell populations showing these changes in gene expression will require the isolation and enrichment of specific leukocyte subpopulations. Finally, it will be important to confirm whether patients manifesting systemic inflammation show similar perturbations of the functional modules identified here as do otherwise healthy, endotoxin-challenged subjects.

METHODS

Human endotoxin model. Eight healthy male and female subjects between 18 and 40 years of age provided written informed consent. Details of endotoxin administration to human subjects have been summarized elsewhere[10,21]. Subjects were intravenously administered either NIH Clinical Center Reference Endotoxin, (CC-RE-Lot 2) at a dose of 2 ng kg^{-1} body weight ($n = 4$, one female and three males) or 0.9% sodium chloride ($n = 4$, one female and three males) over a 5-min period. Blood samples were collected before endotoxin infusion (0 h) and 2, 4, 6, 9 and 24 h after infusion.

Blood sampling. Blood was collected and lysis buffer (bicarbonate-buffered ammonium chloride solution, 0.826% NH_4Cl, 0.1% $KHCO_3$, 0.0037% Na_4EDTA in H_2O) was added at a ratio of 20:1 (lysis buffer:blood). Samples were then incubated at room temperature until erythrocyte lysis was complete (~5–7 min). Leukocytes were recovered by centrifugation (400 *g*, 4 °C) and washed once in ice-cold phosphate buffered saline. Leukocyte pellets were then resuspended in 8 ml RLT buffer (Qiagen) and the samples sheared ten times with an 18-gauge needle attached to a 10-ml syringe. Samples were then immediately frozen and kept at −70 °C until RNA extraction was required.

Leukocyte RNA isolation. Total cellular RNA was isolated from the leukocyte pellets using a commercial kit (RNeasy, Qiagen). Purity was confirmed by spectrophotometry (A_{260}/A_{280} ratio) and capillary electrophoresis (Agilent 2100 Bioanalyser, Agilent Inc).

cRNA synthesis and chip hybridization. cRNA synthesis was performed using 4 μg total cellular RNA, hybridized onto Hu133A and Hu133B oligonucleotide arrays (Affymetrix), and processed according to the protocol outlined by Affymetrix, with a few modifications.

Microarray data analysis. A total of 44,924 probe sets on the Hu133A and Hu133B arrays were analysed. Normalization was performed using dChip[22], and expression level was modelled using the perfect match only model. Probe sets identified as absent on all arrays (using MicroArray Suite v5, Affymetrix) were not included in further analysis. Probe sets significantly perturbed after bacterial endotoxin administration were identified using significance analysis of microarrays (SAM) (multiclass response)[23], with an estimated false discovery rate of <0.1% on the basis of 1,000 permutations. The resulting 5,093 probe sets were subjected to *K*-means clustering to ten bins using Cluster and TreeView[24], and principal component analysis. The same analysis was applied to control data, but no temporal changes of the probe sets reached the significant level (false discovery rate <0.1%). A total of 3,714 unique genes were identified from 4,895 probe sets with mapped Entrez GeneIDs (http://www.ncbi.nih.gov/Entrez/). More information is at http://www.gluegrant.org/.

Verification of genes showing significant transcriptional changes. An additional six healthy subjects (one female and five males) were administered 2 ng kg^{-1} (body weight) endotoxin, and blood samples were collected before (0 h) and after (2 and 6 h) endotoxin infusion. At either 2 or 6 h, ~88% of the significant probe sets in the initial study were again identified as significant (false discovery rate <1%). See Supplementary Methods 1.

Development of the observed interactome of mammalian genes. The Ingenuity Pathways Knowledge Base (KB) is the largest curated database of previously published findings on mammalian biology from the public literature (Ingenuity Systems). Reports on individual studies of genes in human, mouse or rat were first identified from peer-reviewed publications, and findings were then encoded into an ontology by content and modelling experts. Manual extraction and curation probably results in more specific and comprehensive interactions, with far fewer false-positives than automated alternatives (for example, natural language processing and high-throughput screening).

Network analysis using the knowledge base was used to further identify direct interactions between mammalian orthologues. For example, the knowledge of functional interactions (for example, phosphorylation) was combined with knowledge of protein functions (for example, kinase activity) to algorithmically infer a direct biochemical interaction. Every resulting gene interaction has supporting literature findings available online. More information is in Supplementary Methods 2.

Identification of significant pathways in biological processes. The following steps were used: (1) Genes identified as significant from the experimental data sets were overlaid onto the interactome. Focus genes were identified as the subset having direct interaction(s) with other genes in the database. (2) The specificity of connections for each focus gene was calculated by the percentage of its connections to other significant genes. The initiation and growth of pathways proceeded from genes with the highest specificity of connections. Each pathway

had a maximum of 35 genes. (3) Pathways of highly interconnected genes were identified by statistical likelihood using the following equation:

$$\text{Score} = -\log_{10}\left(1 - \sum_{i=0}^{f-1} \frac{C(G,i)C(N-G,s-i)}{C(N,s)}\right)$$

where N is the number of genes in the genomic network, of which G are focus genes, for a pathway of s genes, f of which are focus genes. $C(n,k)$ is the binomial coefficient. (4) Pathways with a score greater than 4 ($P < 0.0001$) were combined to form a composite network representing the underlying biology of the process.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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CORRIGENDUM

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In this Letter, the affiliations of authors participating in the Inflammation and Host Response to Injury Large Scale Collaborative Research Program are incorrectly listed. The renumbered and amended footnote listing is given here.

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CORRIGENDUM

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DNA sequence and analysis of human chromosome 18

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The name of Keith O'Neill was accidentally omitted from the published author list. He is at the first affiliation in the address list.

ERRATUM

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Astronomical pacing of methane release in the Early Jurassic period

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In the labelling of Fig. 1 of this Letter, the spelling of '*D. semicelatum*' was accidentally reversed to read '*D. mutalecimes*'. It appears correctly in the text.