Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis

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The acute phase of sepsis is characterized by a strong inflammatory reaction. At later stages in some patients, immunoparalysis may be encountered, which is associated with a poor outcome. By transcriptional and metabolic profiling of human patients with sepsis, we found that a shift from oxidative phosphorylation to aerobic glycolysis was an important component of initial activation of host defense. Blocking metabolic pathways with metformin diminished cytokine production and increased mortality in systemic fungal infection in mice. In contrast, in leukocytes rendered tolerant by exposure to lipopolysaccharide or after isolation from patients with sepsis and immunoparalysis, a generalized metabolic defect at the level of both glycolysis and oxidative metabolism was apparent, which was restored after recovery of the patients. Finally, the immunometabolic defects in humans were partially restored by therapy with recombinant interferon-γ, which suggested that metabolic processes might represent a therapeutic target in sepsis.

The immune response during the acute phase of sepsis is characterized by inflammation and the activation of immunological effector mechanisms (for example, phagocytosis and intracellular killing) aimed at eliminating the pathogen. During the late phase, the immune system shifts toward an anti-inflammatory state to diminish inflammation and initiate tissue repair. This chain of events can be distorted with dire consequences for the outcome: an exaggerated systemic release of cytokines in the acute phase (‘cytokine storm’) can cause hypotension, cardiovascular dysfunction, tissue damage and multi-organ failure, while during the later phases of the disease, the activation of modulatory pathways can prematurely inhibit host defense (innate immunotolerance or innate immunoparalysis)1. However, studies have demonstrated that these functional states can occur simultaneously. In agreement with that, a study has shown that phagocytes from patients with sepsis who had survived the infection display both decreased inflammation and increased expression of genes encoding products involved in phagocytosis, microbial killing and tissue repair, found to be mediated by a pathway dependent on the transcription factor HIF-1α2. Nevertheless, exaggerated inhibitory effects on leukocytes during the late phase of sepsis can lead to immunoparalysis associated with increased susceptibility to secondary infections and a poor outcome3. Understanding the mechanisms that regulate inflammatory responses in sepsis is crucial for the identification new therapeutic strategies for reversing dysregulation of the immune system in severely ill septic patients.

Studies have identified a crucial role for the cellular metabolism of glucose in the functional fate of cells of the immune system. In this context, naive or tolerant cells rely mainly on oxidative phosphorylation and β-oxidation as energy sources, while after stimulation, leukocytes shift their metabolism toward aerobic glycolysis (the Warburg effect)3,4. Defects in tissue metabolism have been reported in sepsis and seem correlated with outcome. Elevated ATP concentrations in muscle biopsies of patients with sepsis are associated with better survival3, whereas mitochondrial dysfunction and energy shortage have been hypothesized to be an underlying cause of organ dysfunction6,7. In addition, two small studies have reported mitochondrial dysfunction8 and redox imbalance9 in leukocytes of patients with sepsis. However, a comprehensive investigation of cellular metabolism of leukocytes in sepsis and its effect on immunological function and outcome has not been performed.

In this study we aimed to assess energy metabolism of cells of the immune system in two clinically relevant infection models: bacterial sepsis caused by the Gram-negative bacterium Escherichia coli, and fungal sepsis caused by the opportunistic fungal pathogen

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**Candida albicans.** On the one hand, we investigated the role of host defense in the metabolic shift toward aerobic glycolysis during acute infection. On the other hand, we investigated the relationship between the dysregulation of cellular metabolism and innate immunoparalysis (or innate immunotolerance) in patients with severe sepsis.

**RESULTS**

**The shift toward aerobic glycolysis in acute inflammation**

The first aim of our study was to investigate cellular metabolism during the stimulation of leukocytes by pathogens. Microarray assessment of human peripheral blood mononuclear cells (PBMCs) stimulated for 4 h or 24 h with either *C. albicans* [10] (Supplementary Fig. 1a,b) or *E. coli*-derived lipopolysaccharide (LPS) (Supplementary Fig. 1c) revealed substantial upregulation in the expression of mRNA encoding products involved in glycolysis, whereas the expression of mRNA encoding rate-limiting enzymes in the tricarboxylic acid (Krebs) cycle was downregulated. In addition, genes encoding key products involved in the metabolic checkpoint kinase mTOR–HIF-1α pathway (important for the regulation of glycolysis) [11] were also upregulated (Supplementary Fig. 1a). Those gene-transcription data were confirmed by the finding of a substantial increase in the production of lactate and concentration of NAD+ when cells were stimulated with *C. albicans* or LPS (Fig. 1a), which demonstrated a shift toward aerobic glycolysis and thus illustrated induction of the Warburg effect [12]. Such a shift toward glycolysis has been shown to be driven by activation of mTOR as an intracellular sensor of glucose [13]. In line with that, 2 h of stimulation with *C. albicans* or LPS, induced phosphorylation of S6K and 4EBP1, which are downstream targets of the mTOR complex mTORC1 (ref. 13) (Fig. 1b); this showed that an mTOR-regulated increase in anaerobic glycolysis was a key metabolic pathway in acute inflammation.

**The mTOR pathway is essential for survival in sepsis**

Inhibition of the mTOR-pathway in human PBMCs by metformin (an activator of the kinase AMPK) [14] inhibited the phosphorylation of S6K and 4EBP1 (Fig. 1b); in addition, inhibition of mTOR with metformin, rapamycin or torin-1 (ref. 15) decreased the cytokine production induced by *C. albicans* after stimulation for 24 h (TNF and IL-1β but not IL-6) or 5 d (IFN-γ, IL-17 and IL-22) (Fig. 2a,b). The *in vivo* inhibition of metabolic pathways by metformin resulted in significantly diminished survival of mice with sepsis caused by *C. albicans*, lower ex vivo cytokine production by splenocytes and increased fungal outgrowth in kidneys but not liver, relative to that of mice not treated with metformin (Fig. 2c–e). Together these results attested to the importance of the mTOR pathway in robust cytokine production and an effective host defense.

**Regulation of cellular metabolism pathways in sepsis**

After showing a role for mTOR-induced glycolysis in host defense during the acute phase of infection, we aimed to assess glycolysis and oxidative phosphorylation in leukocytes of patients with sepsis. We assessed the genome-wide gene expression of whole-blood leukocytes isolated from healthy volunteers undergoing experimental endotoxemia, a human model of the inflammatory response observed during sepsis, induced by intravenous administration of a low dose of *E. coli* LPS (4 ng per kg body weight) [16]. As is commonly found in this model [16–18], we noted robust alterations in the blood transcriptome after the administration of LPS, such as elevated expression of genes encoding typical factors involved in pro-inflammatory signaling (signaling via IL-1 and IL-6) and anti-inflammatory signaling (signaling via IL-10), as well as signaling via Toll-like receptors, at 4 h after the administration of LPS (Supplementary Fig. 2a,b). Genes encoding products involved in not only the glycolysis I and mTOR pathways but also oxidative phosphorylation and fatty acid oxidation were under-expressed in blood leukocytes 4 h after the administration of LPS (Fig. 3a), a time point at which the cytokine-production capacity of leukocytes was substantially reduced (Supplementary Fig. 2c,d). The metabolic regulator–encoding genes HIFIA, NAMPT and EPAS1 (HIF2A) were overexpressed after the administration of LPS, whereas no differences were shown for expression of the transcription factor–encoding genes PPARA, PPPG and HIF-1α inhibitor–encoding gene HIFIAN (Supplementary Fig. 3a–c). Moreover, markers characteristically expressed by M1 macrophages were largely overexpressed after the administration of LPS, in contrast to the predominant under-expression of markers characteristically expressed by M2 macrophages (Fig. 3a).

To determine whether the changes noted above could be identified in actual patients with sepsis as well, we performed a genome-wide microarray analysis of blood from a cohort of 33 patients with bacterial sepsis (blood cultures positive for *E. coli*) and 13 patients with fungal sepsis (blood cultures positive for *Candida* species) (Fig. 3b–e and Supplementary Table 1). Both bacterial sepsis and fungal sepsis induced substantial changes (a change in expression (upregulation or downregulation) of twofold or more) in the transcriptome profiles that encompassed more than 2,000 genes for each infection (Fig. 3b). Most of the genes modified were altered in both bacterial sepsis and fungal sepsis, although gene sets specific for either infection were also identified (Fig. 3c). Among the common pathways influenced in both types of sepsis, changes in the expression of genes encoding products involved in cellular metabolism were very prominent, including oxidative phosphorylation, glycolysis and mTOR signaling pathways (Fig. 3d). Patients with sepsis manifested overexpression of genes encoding products involved in glycolysis and oxidative phosphorylation, coupled with those encoding products involved
in mitochondrial dysfunction, whereas genes encoding products involved in fatty acid oxidation and mTOR signaling were underexpressed (Fig. 3e). Furthermore, patients with sepsis had overexpression of genes characteristically expressed by M2 macrophages and under-expression of genes characteristically expressed by M1 macrophages (Fig. 3e). Analysis of transcription factor–binding site motifs in the promoter regions of genes altered during sepsis revealed a clear role for the mTOR-dependent HIF-1α axis in the regulation leukocyte genes during both E. coli sepsis and Candida sepsis (Supplementary Fig. 3d). This was in line with the findings of other studies that have shown the importance of HIF-1α in processes other than metabolism during the functional reprogramming of human monocytes in sepsis. These data showed that during sepsis, profound changes in the metabolism of leukocytes were encountered.

**Defective energy metabolism of tolerant monocytes**

In a subsequent set of experiments, we assessed whether the induction of innate immunotolerance in human monocytes modulated cellular metabolism. We used an in vitro model of innate immunotolerance induced by stimulation of monocytes with high concentrations of LPS or C. albicans (in contrast to the low concentrations that induce priming11,19,20). When monocytes were rendered immunotolerant, the production of pro-inflammatory cytokines after restimulation was almost completely abolished (Fig. 4a). In line with that, restimulation of tolerant monocytes with LPS resulted in diminished production of lactate and enhanced production of NAD⁺ compared with that of non-tolerant cells (Fig. 4a), indicative of a defective ability to mount a Warburg effect. Immunoblot analysis of components of the mTOR pathway showed less phosphorylation of mTORC1 targets in tolerant cells than in non-tolerant cells (Fig. 4b). When glycolysis is impaired, the β-oxidation of fatty acids is often used as an alternative source of energy21. However, monocytes that were rendered tolerant by pre-exposure to LPS were also charaterized by decreased amounts of the fatty-acid transporters CD36 and CPT1 compared with that of control cells exposed to culture medium alone (Fig. 4b). Finally, immunotolerant monocytes also showed a marked decrease in oxygen consumption compared with that of naive monocytes (Fig. 4c). Therefore, a decrease in all major metabolic pathways (glycolysis, oxidative phosphorylation and β-oxidation) occurred in immunotolerant monocytes.

**Severe metabolic defects in leukocytes of patients with sepsis**

To determine whether defects in cellular metabolism similar to those noted above characterized leukocytes from patients with sepsis, we assessed the cytokine-producing capacity and glycolysis of cells from a separate set of patients with sepsis that either responded normally to stimulation with LPS (high responders (n = 6)) or lacked the ability to produce cytokines after ex vivo stimulation with LPS (immunotolerant (n = 5)). Stimulation of PBMCs with LPS showed normal secretion of cytokines from PBMCs from healthy control subjects and those from high-responder patients with sepsis (Fig. 5a). These cells were also able to mount the release of lactate and NAD⁺ (Fig. 5a).

In contrast, PBMCs from immunotolerant patients with sepsis were unable to produce lactate or NAD⁺ after stimulation with LPS (Fig. 5a). We also assessed metabolic processes in monocytes from patients during septic shock and recovery. When we monitored immunotolerant patients with sepsis over time (>7 d), we found that the ex vivo cellular production of cytokines and lactate was restored after patients recovered from sepsis (Fig. 5b). Assessment of the mTOR pathway and fatty-acid transporters (CD36 and CPT1) in monocytes by immunoblot analysis showed diminished activation of these factors in patients during sepsis,
Figure 3  Blood transcriptome analysis of human endotoxemia and critically ill septic patients with culture-confirmed systemic infection with Candida or E. coli. (a) Genome-wide transcriptional profiles of blood from healthy male subjects (n = 8) before and 4 h after (above plots; gray and green key) the administration of E. coli LPS (4 ng per kg body weight) in a clinically controlled setting; expression of genes (right margins) is presented as centered and ‘scaled’ log2 fluorescence intensity (blue and red key); labels above plots indicate grouping of genes by product function. (b) Genome-wide transcriptome analysis of blood from patients critically ill with clinically well-defined sepsis (Supplementary Table 1) with confirmed infection of the bloodstream with E. coli (left) or C. albicans (right), with expression (log2 values) plotted against the adjusted P value for the difference in expression (Adj P val). Numbers in top left and right corners indicate number of genes with significantly differential expression (adjusted P value, <0.05), upregulated (red) or downregulated (blue) onefold or more in septic patients relative to that in healthy subjects. (c) Unique and overlapping blood transcriptional responses in E. coli- or C. albicans-infected septic patients in b (left); arrows and adjacent numbers indicate genes with significantly differential expression (adjusted P value, <0.05), upregulated (upward red arrow) or downregulated (downward blue arrow) in septic patients. Right, gene expression in E. coli-infected septic patients plotted against that in C. albicans-infected septic patients (Spearman’s rho = 0.91; P < 2.2 x 10^-16). (d) Significance of the difference in the expression of over-expressed genes (top row) or under-expressed genes (bottom row) common to Candida sepsis and E. coli sepsis, categorized into the top 10 most over-represented pathways in which the gene products are involved (horizontal axes). (e) Transcriptional profiles of healthy subjects and E. coli- or C. albicans-infected septic patients in b (above plots; gray, gold and green key), presented as in a. Data are representative of one experiment with 8 subjects (endotoxemia) or 46 subjects (sepsis).
while these effects were reversed once patients recovered (Fig. 5c). Consistent with the data reported above, showing severe metabolic disturbances in patients with sepsis, the maximum oxygen consumption of PBMCs was also substantially impaired in patients with sepsis, compared with that in healthy control subjects (Fig. 5d). These data demonstrated that both glycolytic pathways and oxidative–metabolism pathways were substantially downregulated in patients with sepsis, which led to what we called ‘immunometabolic paralysis’ here (Supplementary Fig. 4).

Partial restoration of cellular metabolism by IFN-γ

Restoring energy metabolism might represent a potential therapy for patients with sepsis with immunometabolic paralysis. IFN-γ is a cytokine with the ability to potently stimulate monocyte function, and recombinant IFN-γ therapy in septic patients has been shown to (partially) reverse immunoparalysis both in vitro and in vivo\(^22\)–\(^24\).

Moreover, IFN-γ polarizes macrophage differentiation toward an M1 phenotype, which is associated with increased glycolysis\(^25\), and the mTOR–HIF-1α–regulated metabolic switch from oxidative phosphorylation to glycolysis in mouse dendritic cells and splenocytes is dependent on autocrine IFN-γ production\(^26\). We therefore hypothesized...
that IFN-γ might be able to restore glycolysis in tolerant monocytes. First, we showed that treating LPS-tolerant human monocytes in vitro with IFN-γ restored lactate production upon stimulation with LPS (Fig. 6a), while oxygen consumption was not influenced (Fig. 6b). Inhibition of the mTOR pathway with rapamycin abrogated the effect of IFN-γ (Fig. 6a). To further assess the mTOR pathway in IFN-γ therapy, we exposed tolerant and non-tolerant (naive) monocytes to IFN-γ for 2 h, after which we assessed the phosphorylation of the mTOR pathway by immunoblot. IFN-γ was able to induce phosphorylation of components of the mTOR pathway in both tolerant monocytes and non-tolerant monocytes (Fig. 6c). Furthermore, in a proof-of-concept trial24, we treated patients with fungal sepsis (n = 8) with recombinant IFN-γ three times a week. Ex vivo stimulation of PBMCs isolated after subcutaneous administration of recombinant IFN-γ showed an increase in lactate production relative to lactate production before IFN-γ treatment that accompanied upregulation of the cytokine-producing capacity24 (Fig. 6d), indicative of a restoration of the ability to mount glycolytic responses. Therefore, IFN-γ was able to partially restore the metabolic defects of innate immunotolerance by promoting glycolysis, which was impaired in immunotolerant monocytes.

**DISCUSSION**

Collectively, our study supports three main conclusions. First, during an acute phase of infection, the shift from oxidative phosphorylation to aerobic glycolysis (the Warburg effect) was an important mechanism of host defense. Second, leukocytes from patients with severe sepsis displayed severe defects in cellular energy metabolism that were associated with an impaired ability to respond to secondary stimulation, a process equivalent to a true ‘immunometabolic paralysis’. Third, therapeutic approaches that reverse this metabolic defect might represent a novel therapeutic approach for the treatment of sepsis.

In our experiments, tolerant leukocytes had broad defects in metabolic pathways, not only failing to increase glycolysis but also displaying decreased oxygen consumption and less transport of fatty acids. These observations were somewhat unexpected, as they showed that leukocytes from patients with sepsis did not merely reverse glucose metabolism from glycolysis to oxidative phosphorylation but displayed multiple energy-metabolizing defects. These defects correlated with a loss of cytokine-producing ability, which was reversed upon recovery. Mitochondrial dysfunction has been shown to correlate with a poor outcome in sepsis, but such studies have focused mainly on non-hematopoietic cells27, with only one study reporting defects in leukocytes8. Here we found that the defects comprised several metabolic pathways and correlated with immunological function.

A remaining point of discussion is whether the metabolic defects were one of the main causes of the immunological phenotype or whether they were a feature of adaptation of cells of the immune system during infection. However, there are suggestions that infection and metabolic defects are intertwined: for example, on the one hand, we showed that inhibition of metabolic pathways influenced the immunological response, and on the other hand, it has been shown that IL-10 down-regulates glycolysis in a mouse model28, which suggests a relationship between cytokine production and metabolism. Finally, another point is the cell populations in which these metabolic changes occur during sepsis. Our experiments showing the importance of the regulation of metabolic pathways in innate immunotolerance used a whole-blood stimulation assay that mimicked the interplay of all cells of the immune system. Although we performed additional experiments to assess the effects on more specific cell populations (monocytes) in vitro, comprehensive analysis of the in vivo changes in various cell subpopulations in patients with sepsis is warranted.

Another discussion point is the finding that metformin, used as a modulator of metabolic pathways, displays effects beyond simply activation of AMPK and inhibition of the mTOR pathway. We nevertheless decided to investigate its effects because of its broad clinical use, which made these experiments relevant from a translational perspective. The mTOR inhibitor rapamycin could not be used to study
cellular metabolism in the live infection model, due to its well-known antifungal effect. However, our use of more specific inhibitors of mTOR in vitro, such as torin-1 and rapamycin, confirmed the effect of mTOR on inflammation. Furthermore, many studies have reported that rapamycin inhibits TNF production, suggestive of an anti-inflammatory effect, similar to our observations. In contrast, other studies have shown that rapamycin upregulates IL-12 production and downregulates IL-10 production and thus results in immunostimulatory effects, as seen for IL-6 in our in vitro experiments. Several hypotheses have been raised to explain these differences, the most likely being that the kinase Akt–mTOR–HIF-1α pathway has differential effects on the various cytokines, depending on the cell type and stimulus or infection present.

Our study has identified cellular metabolic processes as a potential therapeutic target in sepsis. So far, only very few agents with known metabolic-regulatory ability have been tested in sepsis models. Among these, activation of the transcription factor PPARγ, which is associated with increased glycolysis and fatty-acid metabolism, has been shown to have a beneficial effect on mortality in mouse sepsis models. Furthermore, several therapeutic agents able to restore mitochondrial activity have been studied in rodent models of sepsis, with beneficial outcomes. In the present study, we investigated IFN-γ, as it is known to upregulate cytokine production in innate immunotolerance. Autocrine IFN-γ production is involved in the mTOR-HIF-1α-dependent metabolic switch from oxidative phosphorylation to glycolysis in mouse dendritic cells and splenocytes. Indeed, our data showed that in vitro exposure of immunotolerant monocytes to IFN-γ activates the mTOR pathway and partially restored the ability of leukocytes to mount a glycolytic response. These published data and our findings differ from those of another published report showing the downregulatory effect of IFN-γ on mTORC1, with methodological differences and different cell populations as likely causes of this. Thus, metabolic recovery and immunological recovery seem to be correlated, and improved strategies to correct the metabolic abnormalities should be considered for patients with sepsis and immunoparalysis.

Apart from the effects of IFN-γ on mTOR, other potential mechanisms might have a role as well. First, IFN-γ induces sustained occupancy of the transcription factors STAT1 and IRF1 and also induces histone acetylation at promoter and enhancer sites of genes encoding pro-inflammatory cytokines and possibly genes encoding metabolic factors. Second, the microRNA miRNA-146 has an important role in disruption of the synthesis of pro-inflammatory cytokines in innate immunotolerance, and IFN-γ could potentially influence the expression of miRNA-146, as has been shown for other microRNAs. Third, restoring metabolism pathways via IFN-γ could potentially lead to an increase in succinate, an important positive regulator of IL-1β production via HIF-1α. Finally, sirtuins have been shown to have an important role in the regulation of the immunotolerant phenotype of monocytes, and inhibition of the sirtuin SIRT1 can restore the immunotolerant (metabolic) features of monocytes. Promisingly, IFN-γ has also been shown to repress the expression of SIRT1 in muscle cells.

In conclusion, the switch from oxidative phosphorylation to aerobic glycolysis is an important component of the cellular response to infection. Broad defects in the energy metabolism of leukocytes comprising both glycolysis and oxidative mechanism characterized a state of immunometabolic paralysis in patients with severe sepsis, and this not only gives new insight into the pathophysiology of the disease but also might represent a novel therapeutic target. Immunotherapy with recombinant IFN-γ is one of the potential approaches to correcting these defects.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, GSE48119.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.-C.C., B.P.S., R.I.W.A., M.S.G., E.L., E.J.G.-B., M.K., G.R.M., J.A.L.W., J.L. and A.J.v.d.M. performed the experiments; B.P.S., R.I.W.A. and J.A.L.W. performed the analyses; E.J.G.-B., O.L.C., F.L.v.d.V., M.J.B., M.J.S. and P.P. were involved in the clinical studies; F.H.G.M.W. L.A.B.J., T.v.d.P. and M.G.N. designed the studies; and all authors were involved in writing and correcting the manuscript.

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Cells were cultured, lysed and stored as described. µα×10^6 Cells were stimulated with RPMI medium, collected, after which the cells were washed and re-suspended in 60 µl phosphate buffered saline (PBS), pH = 7.2, and challenged with one log-phase 5 × 10^6 per mouse inoculum of C. albicans intravenously via the tail vein on day 0; or treated intraperitoneally on days 1, 0, 1, 2, 3, 4, 5, 6 and 7 with 200 µl phosphate buffered saline (PBS), pH = 7.2, and challenged with one log-phase 5 × 10^6 per mouse inoculum of C. albicans intravenously via the tail vein on day 0. Survival was monitored for 28 d for 10 mice per group and animals were sacrificed as 5 mice per group on days 3 and 7. During sacrifice, one midline abdominal incision was performed under aseptic conditions and tissue samples were taken from the liver, spleen and right kidney. Once weighed, segments of liver and right kidney were homogenized with 1 ml RPMI-1640 nutrient broth (Biochrom), and 100 µl of the homogenate was serially diluted four times 1:10 in 0.9% NaCl. A 0.1-ml aliquot of each dilution was plated onto Sabouraud agar (Becton Dickinson). Plates were incubated at 37 °C for 48 h, and the number of viable colonies in each dilution was multiplied by the appropriate dilution factor. Results are presented as log_{10} CFU/g. The lower detection limit was 10 CFU/g. The spleen was gently squeezed and then passed through a sterile filter (250 µm; 12-x13 cm; Alter) for splenocyte collection. After counting of splenocytes with trypan blue exclusion of dead cells, splenocytes were incubated into sterile flat wells at a density of 5 × 10^6 cells/ml in 1 ml of RPMI-1640 medium supplemented with 12 mM glutamine, 10% FBS, 100 U/ml of penicillin G, and 0.1 mg/ml of streptomycin in the presence or absence of 10 ng/ml LPS, E. coli 055:B5 (24 h), or 1 × 10^6 CFU/ml of heat-killed yeasts C. albicans (5 d) at 37 °C in 5% CO₂. At the end of incubation, the plates were centrifuged and the supernatants were collected. Cytokines were measured in supernatants by an enzyme immunoassay (R&D Systems).

**Human endotoxemia.** Eight healthy men (age, 22.9±0.5 years (mean ± s.e.m.)) were given intravenous administration of LPS (from E. coli; US standard reference endotoxin; 4 µg/kg body weight; provided by the US National Institutes of Health). No power calculation was performed. Blood was collected before and 4 h after LPS administration in PAXgene tubes (Becton-Dickinson) for microarray analysis of total RNA isolated using the PAXgene blood RNA kit (Qiagen) according to manufacturer's instructions. Blood was also collected in heparin tubes (Becton-Dickinson) for whole blood and PBMC restimulation with 100 ng/ml LPS for 3 h. After restimulation, cytokine release was assessed by Luminox-based multiplex assay. The study was approved by the institutional scientific and ethics committees of Academic Medical Center, Amsterdam (114009), and written informed consent was obtained from all subjects (clinicaltrials.gov: NCT01014117). All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

**Patient studies.** Clinical samples were obtained from patients with blood culture proven E. coli or C. albicans sepsis who had been enrolled in the MARS project.
stimulated PBMCs from healthy 5–LPS per kg body weight) was 2–52 Differences in cytokine, lactate and NAD 4–42 gene expression data. 5–52 blood gene-expression data. γ Eight newly admitted patients 5–5–invasive infection with Escherichia coli (E. coli) (n = 33) or Candida species blood RNA kits using the QUACube system (Qiagen) according to the manufacturer’s instructions. The Medical Ethical Committees of both study centers gave approval for an opt-out consent method (IRB number 10-056C). PAXgene blood was also obtained from 42 healthy controls (age range, 30–63 years; 24 males, 18 females) after written informed consent was provided. For ex vivo cytokine and lactate production of septic patients, blood was drawn via an arterial catheter from eight newly admitted septic patients within 24 h after admission to the intensive care unit of Radboud University Medical Center Nijmegen. Once a patient had recovered, blood was drawn before release from the ICU and if possible once more at a later time point. This was carried out in accordance with the applicable rules concerning the review of research ethics committees and informed consent. All patients or legal representatives were informed about the study details. For the IFN-γ pilot study, the primary end point data have been published elsewhere.24 In short, eight patients with invasive infection with Candida species and/or Aspergillus fumigatus not only received the standard antifungal therapy but also were treated with recombinant IFN-γ (100 mg thrice a week, for 2 weeks); at indicated time points, blood was drawn, and ex-vivo PBMC stimulations were performed.24 From the supernatants of cells cultured for 24 h, lactate was measured. The Arnhem-Nijmegen medical ethical committee approved the study (NL28239.091.10 and Clinicaltrials.gov NCT01270490). All study procedures were conducted in accordance with the declaration of Helsinki, including current revisions and Good Clinical Practice guidelines.

Microarray analysis. Genome-wide gene-expression analysis of blood from the human endotoxemia cohort (4 ng E. coli LPS per kg body weight) was done using the Illumina HumanHT-12 V3.0 expression beadchip (GSE48119), whereas blood from the patients with sepsis from the MARS cohort were analyzed by hybridization to the human genome U219 96-array plate and scanned using the GeneTitan instrument (Affymetrix) (GSE65682). Genome-wide gene expression analysis of in vitro–stimulated PBMCs from healthy volunteers was done by using the Illumina HumanHT-12 V4.0 expression beadchip (GSE42606); further details are available.

Statistics. Differences in cytokine, lactate and NAD+ were analyzed using a Wilcoxon signed-rank test or one way analysis of variance, where applicable. Survival curves were analyzed by a log–rank test, and for oxygen consumption, a Student’s t-test was used. All these analyses were performed in Graphpad prism 5. Dichotomous and continuous clinical data were assessed by Fisher’s exact test and Wilcoxon’s rank sum test, respectively. Microarray data are presented in the form of volcano plots (integrating log, fold values and multiple-test adjusted probabilities) and heat map plots, generated in R studio.

Human endotoxemia in vivo blood gene-expression data. PAXgene blood total RNA was isolated by means of the Qiacube automated system (Qiagen) using PAXgene blood RNA kits according to manufacturer’s instructions. Synthesis, amplification and purification of anti-sense RNA was performed by using the Illumina TotalPrep RNA Amplification Kit (AM-IL179; Ambion) following the Illumina Sentrix Array Matrix expression protocol at ServiceXS. A total of 750 ng biotinylated RNA was hybridized onto the HumanHT-12v3 Expression BeadChip (Illumina). The raw scan data were read using the beadarray package (version 1.16.0)56, using the R statistical environment (version 2.11.0)24. Illumina’s default pre-processing steps were performed using beadarray. Estimated background was subtracted from the foreground for each bead. For replicate beads, outliers greater than 3 median absolute deviations from the median were removed and the average signal was calculated for the remaining intensities. Log-transformation was applied to summarized data in order to remove mean-variance relationship in intensities. Resulting data were then quantile-normalized. Quality control was performed both on bead level and on bead summary data using the arrayQualityMetrics package (v2.6.0)38. For each of the 48,804 probes, a detection score was calculated by comparing their average signals with the summarized values for the negative control probes. This step filtered the non-expressed probes, thus yielding 20,700 probes. Probes were re-annotated using the illuminaHumanv3BeadId.db package available from Bioconductor. Assessment of differential gene expression was done by means of the limma method.25,53 Throughout, Benjamini-Hochberg multiple-comparison–adjusted probabilities, correcting for 24,646 tests, defined significance.

Human in vitro gene expression data. Genes encoding products involved in metabolic signaling pathways were selected and analyzed for differential expression in samples treated with RPMI medium (control) or stimulated with LPS or Candida- (4 and 24 h) as described before10 by using limma25,53.
A multiple-comparison Benjamini-Hochberg–adjusted $P$ value of <0.05 defined significance.

**Gene-expression pathway analysis.** Ingenuity pathway analysis (http://www.ingenuity.com) was used to identify significant enrichment of canonical signaling pathways. Genes were stratified as over- or under-expressed and were analyzed by selection of the Ingenuity Gene Knowledge Base as reference and specification of human species. All other parameters were kept default. Association significance was measured by Fisher’s exact test Benjamini-Hochberg–adjusted $P$ values. Transcription factor–binding site–motif analysis was performed on all multiple-comparison significant genes ($P < 0.05$ (Benjamini-Hochberg)) per comparison using oPossum methodology. The initial 2,000 base pairs of DNA sequence upstream and downstream of the gene transcription start site was used. All other parameters were default. Fisher’s scores above median and percent transcription factor–binding site hits of all input DNA sequences >50% were used as cutoffs for transcription factor binding sites in co-expressed genes. Significant demarcation was demarcated using Fisher’s exact test Benjamini-Hochberg–adjusted $P$ values ($P < 0.05$).


