

# Oxygen consumption of human peripheral blood mononuclear cells in severe human sepsis\*

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**Objective:** During sepsis, after an initial stimulation immune cells down-regulate their functions, leading to a state of immunosuppression. Because the mechanisms of such down-regulation are unclear, we investigated the hypothesis of an energetic failure of immune cells to participate in immune dysfunction.

**Design:** Cohort of septic shock patients to study peripheral blood mononuclear cells (PBMCs) biological energy in comparison to healthy volunteer cells.

**Setting:** Critical care unit and laboratory, university hospital.

**Subjects:** Eighteen severe sepsis or septic shock patients and 32 healthy volunteers.

**Interventions:** *Ex vivo* measurement of oxygen consumption in PBMCs taken from patients. The PBMCs' mitochondrial oxidative phosphorylation was investigated using adenosine diphosphate stimulation. The plasma factors implication was tested, using healthy cells incubated in septic plasma, or septic cells incubated in healthy plasma, at different time points of sepsis. The relationship between monocyte human leukocyte antigen-DR expression and bioenergetic results was tested.

**Measurements and Main Results:** Baseline oxygen consumption was higher in septic PBMCs ( $p < .01$ ), with an attenuated response to adenosine diphosphate stimulation ( $p < .01$ ). Oxygen consumption of healthy PBMCs incubated in septic plasma mimicked the septic cell response, with amplitude depending on the duration of sepsis (days 0–28). Septic cells incubated in healthy plasma partially recovered normal patterns. Septic plasma incubation increased the fraction of decoupling oxygen consumption ( $p = .021$ ). A relationship between oxygen consumption (baseline or adenosine diphosphate stimulated) and human leukocyte antigen-DR expression was observed for incubation with plasma sampled at different time points of septic shock.

**Conclusion:** Energetic failure of PBMCs in sepsis may be a factor associated with the modulation of immune response and human leukocyte antigen-DR phenotype, partially driven by plasma factors. (Crit Care Med 2007; 35:2702–2708)

**KEY WORDS:** sepsis; peripheral blood mononuclear cells; immune suppression; mitochondria; energetic failure; human leukocyte antigen-DR

Circulating immune cells play an important role in the pathophysiology of sepsis because their activation may induce inflammation remote from noninfected organs. After initial activation, immune cells are often down-regulated in prolonged sepsis (1–4). This subsequent immune suppression is associated

with an increased mortality (5, 6). Immune stimulation has an energy requirement that has received scant attention. Dr. Kuhnke and colleagues (7) did however note that peripheral blood mononuclear cells (PBMCs) taken from patients with active rheumatic inflammatory diseases had higher baseline levels of oxygen consumption, but a significantly lower response to a mitogenic stimulus, compared with cells taken from healthy volunteers.

In this study, we compared differences in oxygen consumption in PBMCs taken from patients with severe sepsis or septic shock and healthy volunteers. Values were assessed both at baseline ( $V_o$ ) and after exogenous stimulation directly at the mitochondrial level ( $V_{stim}$ ) using the classic adenosine diphosphate (ADP) stimulation (8, 9). We investigated whether the bioenergetic response seen in the septic PBMCs was influenced by plasma factors during sepsis as opposed to normal plasma, and if the septic plasma changes in composition along the

evolution may have an impact. We also evaluated if any relationship may exist between oxygen consumption (baseline and/or stimulated) and antigen presentation measured as monocyte human leukocyte antigen (HLA)-DR expression.

## METHODS

**Healthy Volunteers and Patients.** Following approval from our hospital ethics committee, we sampled blood from healthy volunteers ( $n = 32$ ), and from patients with severe sepsis or septic shock ( $n = 18$ ) within 48 hrs after intensive care admission. Patients were enrolled after written informed consent was provided by the next of kin. Sepsis or septic shock was diagnosed on classic criteria (10) (Table 1). Systemic inflammation was characterized by monocyte HLA-DR expression (number of sites per cell assessed by flow cytometry, FACScan instrument, double color staining, Becton Dickinson, San Jose, CA), plasma levels of interleukin (IL)-10 and IL-12p40 (ELISA kit, PharMingen, San Diego, CA). Because NO may depress mitochondrial function (11),  $NO_x$  levels (namely  $NO_2$  and  $NO_3$ ; Griess reaction)

\*See also pp. 2856.

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Table 1. Clinical characteristics of patients

	Age	Sex	Type of Infection	Microorganism	No. of Organ Failures	SAPS II	SOFA Score	Day 28 Outcome
1	76	M	Liver abscess	<i>Streptococcus faecalis</i> + <i>Escherichia coli</i> + pneumococcus	4	64	14	A
2	68	M	Cervical cellulitis	<i>Streptococcus</i> + <i>Candida albicans</i>	1	47	4	A
3	56	M	Peritonitis	<i>E. coli</i> + <i>Bacteroides</i> species	2	41	9	A
4	48	M	Peritonitis	<i>E. coli</i>	2	45	6	A
5	92	F	Peritonitis	<i>E. coli</i> + <i>Bacteroides fragilis</i>	4	67	12	A
6	54	M	Arm cellulitis	<i>Streptococcus</i> group A + <i>Staphylococcus aureus</i>	1	29	5	A
7	67	F	Meningitis	<i>Pseudomonas aeruginosa</i> + <i>S. epidermidis</i>	1	35	9	D
8	81	F	Peritonitis	Polymicrobial	4	60	12	A
9	47	M	Pneumonia	<i>Hemophilus</i> + <i>S. aureus</i>	5	72	16	A
10	45	M	Peritonitis	Not found	2	35	5	A
11	65	M	Peritonitis	<i>E. coli</i>	4	58	12	D
12	82	F	Peritonitis	Not found	4	41	8	A
13	58	M	Peritonitis	<i>E. coli</i> + <i>B. fragilis</i>	2	22	5	A
14	84	M	Purulent pleuresis	<i>Fusobacterium nucleatum</i>	4	44	9	A
15	91	F	Peritonitis	Polymicrobial	4	51	15	D
16	68	F	Peritonitis	<i>S. aureus</i> + <i>P. aeruginosa</i>	2	31	6	A
17	31	F	Pneumonia	<i>S. aureus</i>	4	28	10	A
18	65	M	Pyelonephritis	<i>E. coli</i>	2	27	8	A

SAPS II, Simplified Acute Physiology Score; SOFA, Sequential Organ Failure Assessment score; A, alive; D, dead; M, male; F, female.

(12) also were determined in septic plasma used for incubation.

**Procedures.** PBMCs were isolated from whole blood by Ficoll gradient centrifugation. After washing with phosphate-buffered saline, cells were resuspended at a concentration of 5 to 8 × 10<sup>7</sup>/mL. Cells from septic patients and volunteers were incubated at room temperature in their own plasma for 3 hrs. To test a potential role of septic plasma factor(s), healthy volunteer cells were incubated for 3 hrs in plasma pooled from six septic patients. The impact of the septic time evolution on the plasma composition was tested by incubating healthy cells in septic plasma from days 1, 7, and 28. The role of plasma also was tested in septic cells, which were separated from their plasma, resuspended in a pooled healthy plasma (from four healthy volunteers), and incubated for 3 hrs.

At the end of the incubation period, oxygen consumption was measured as the rate of fall of number of oxygen atoms measured by a Clark electrode in a closed chamber (Dual Digital Model 20 Oximeter, Rank Brothers, Bottisham, UK). Intact cells (2–2.5 × 10<sup>6</sup>/mL) were placed in the oximeter chamber in a medium containing 0.25 mol/L sucrose, 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-K at pH 7.4, 3 mmol/L Mg-acetate, 5 mmol/L succinate, 0.37 mmol/L dithiothreitol, 4 mmol/L KH<sub>2</sub>PO<sub>4</sub>, and 0.3 mmol/L ethylene glycol tetraacetic acid. Oxygen solubility was assumed to be 390 ng atoms/mL at 37°C (13). After the basal respiration rate (V<sub>o</sub>) was recorded, the cells were permeabilized by 0.005% digitonin (14) and the maximal mitochondrial respiration (state 3) was induced by 1 mmol/L ADP. Cell permeabilization was controlled by Trypan blue uptake. The absence of impact of digitonin on basal oxygen consumption rate also was tested, and did not differ from nonpermeabilized cells.

To determine whether changes in PBMC oxygen consumption under ADP stimulation resulted from an exclusive increase in mitochondrial respiration, a selective inhibitor of complex III, antimycin A (5 μmol/L) (15) was used. In normal conditions, this compound totally blocked the ADP-induced stimulation of oxygen consumption, confirming the selective action on mitochondria.

To evaluate if mitochondrial oxygen consumption under ADP was coupled or not, we added experiments with oligomycin (0.3 mg/mL), a substance that blocks adenosine 5'-triphosphate (ATP) synthase (16). After incubation of healthy cells for 3 hrs in their own plasma or in a pool of day 1 septic plasma (n = 6), oligomycin was added in the chamber after ADP maximal stimulation. To test the integrity of the electron transport chain responsible for mitochondria polarization by pumping protons, four experiments have been performed with carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (10 μmol/L FCCP; Sigma, St. Louis, MO), an uncoupling agent. This agent was added immediately after ADP stimulation both in control plasma and in pooled septic plasma.

**Statistical Analysis.** All results are expressed as median (interquartile range). Statistical analysis was performed by StatXact software (Cashytel software, Cambridge, MA) using nonparametric tests, with *p* < .05 deemed statistically significant. Intergroup comparisons (volunteer vs. septic patient or volunteer cells in septic plasma) were made using the Mann-Whitney U test. Correlations were calculated using Spearman's rank-correlation test (two-tailed test of significance).

## RESULTS

Table 1 shows the clinical characteristics of the septic patients, their severity of

illness as described by the Simplified Acute Physiology Score II and Sequential Organ Failure Assessment score, and outcome at day 28.

Compared with healthy volunteer cells, septic PBMCs had a higher baseline value of oxygen consumption (V<sub>o</sub>; *p* = .002) (Fig. 1). Stimulation by ADP produced a significantly lower increase in oxygen consumption in the septic cells, which was expressed as a percentage (% = 100 × [V<sub>stim</sub> - V<sub>o</sub>]/V<sub>o</sub>) (25% vs. 161%; *p* < .0001).

Preincubation of healthy PBMCs in septic plasma produced oxygen consumption values similar to those seen with septic cells, both at baseline and in response to stimulation with ADP (25% vs. 150%; *p* < .0001) (Fig. 2). Septic cells incubated in healthy volunteer plasma showed oxygen consumption rates trending toward those found for healthy PBMCs, both at baseline and after stimulation with ADP (Fig. 3).

Healthy volunteer PBMCs incubated in septic plasma sampled along the time evolution of sepsis showed a progressive decrease in V<sub>o</sub>. The maximal increase in V<sub>stim</sub> after ADP did not improve and remained significantly lower than in healthy cells (Fig. 4).

Figure 5 shows the results of experiments testing the coupling of oxygen consumption under stimulation by ADP. Figure 5A shows the significant reduction (*p* < .05) of oligomycin-induced inhibition of oxygen consumption (ATPase synthase inhibition) when healthy cells were incubated in septic plasma. Such a differ-

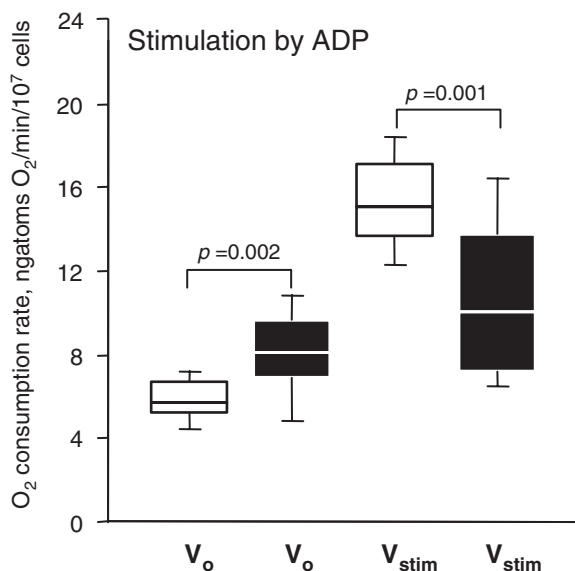


Figure 1. Oxygen consumption of human peripheral blood mononuclear cells at baseline ( $V_o$ ) and after stimulation by adenosine diphosphate (ADP;  $V_{stim}$ ). Unfilled bars are healthy volunteers, and filled bars are septic patients. Data displayed as box and whisker plot (median, interquartile ranges and range). Healthy volunteers,  $n = 28$ ; septic patients,  $n = 18$ .

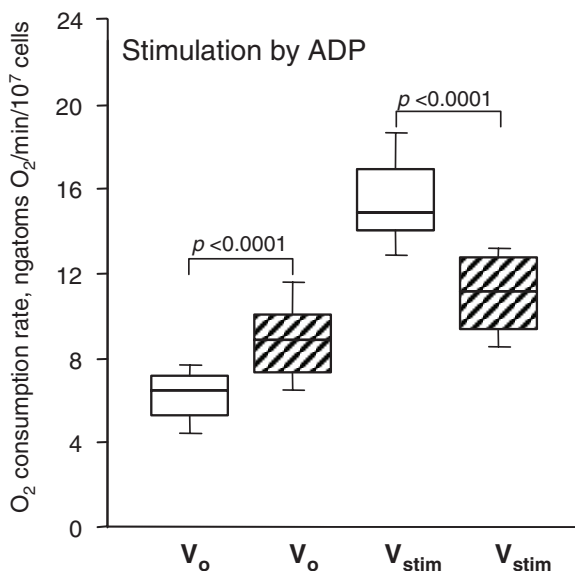


Figure 2. Effect of incubation of healthy volunteer peripheral blood mononuclear cells ( $n = 21$ ) in septic plasma on oxygen consumption at baseline ( $V_o$ ) and after stimulation with adenosine diphosphate (ADP;  $V_{stim}$ ). Peripheral blood mononuclear cells were preincubated for 3 hrs in either healthy volunteer plasma (unfilled bars) or day 1 septic plasma (hatched bars). Data displayed as box and whisker plot (median, interquartile ranges and range).

ence suggests an uncoupling induction by septic plasma. Figure 5B shows the results of addition of FCCP after ADP stimulation, both with healthy and septic plasma. The observed increase in oxygen consumption when FCCP was added disappeared when healthy cells were incubated in septic plasma.

Systemic inflammation in septic patients was characterized by an expected down-regulation in HLA-DR expression

(number of sites per cell; eight-fold lower than in healthy monocytes;  $p < .0001$ ) (Table 2). Plasma IL-10 levels were 20-fold higher in septic patients compared with healthy volunteers ( $p < .0001$ ) with no change in plasma IL-12p40.

The incubation of healthy volunteer cells in septic plasma with added antibody against IL-10 did not change significantly the oxygen consumption patterns. Plasma  $NO_x$  levels did not differ between healthy

and septic pooled plasma used for incubations.

We observed a nice linear relationship between monocyte HLA-DR level and  $V_o$  (Fig. 6A) or  $V_{stim}$  after ADP incubation (Fig. 6B) during the sepsis time evolution and in healthy volunteers. Septic patients having the lowest HLA-DR expression also had the lowest  $V_{stim}$  (Fig. 6B), which improved over time.

## DISCUSSION

The importance of the innate immune response in early sepsis and its subsequent attenuation is well recognized (17, 18). This has been demonstrated by examining *ex vivo* cytokine release capability (2), phagocytosis (19), and HLA-DR expression (3–6). The immune suppression may prove beneficial in some aspects; e.g., induced immunotolerance (20), but harmful in others; e.g., facilitation of nosocomial infections (21). However, immune suppression as adjudged by HLA-DR status has been linked to a worse prognosis in critically ill patients (22). Underlying mechanisms remain uncertain, although plasma release of IL-10 (23) and modification of gene transcription (24) have been proposed. Our findings suggest that energy failure may be, at least in part, responsible for the inability of the septic cells to respond adequately to stimulation.

Immune cell activation mandates an increase in energy requirements (25). Thus, impaired production of ATP may be a major factor modulating the immune response in sepsis (25). This reflects findings of mitochondrial dysfunction made in other tissues (e.g., muscle, liver, gut) taken from both septic patients and laboratory models (26–28). This dysfunction correlated with disease severity and with the amount of NO production. A macrophage cell line incubated with endotoxin/interferon- $\gamma$  resulted in a progressive fall in oxygen consumption and inhibition of mitochondrial complex I that was associated with an increase in NO production and nitrosylation as well as nitration of mitochondrial proteins (29). A major degree of reversibility was achieved by addition of a nonspecific NO synthase inhibitor, thus strongly implicating NO and its metabolite peroxynitrite in the inhibition of mitochondrial respiration. In the present study, the ability of septic plasma to transform responses of healthy PBMCs into those characteristics of septic cells, and *vice versa* for septic cells placed in

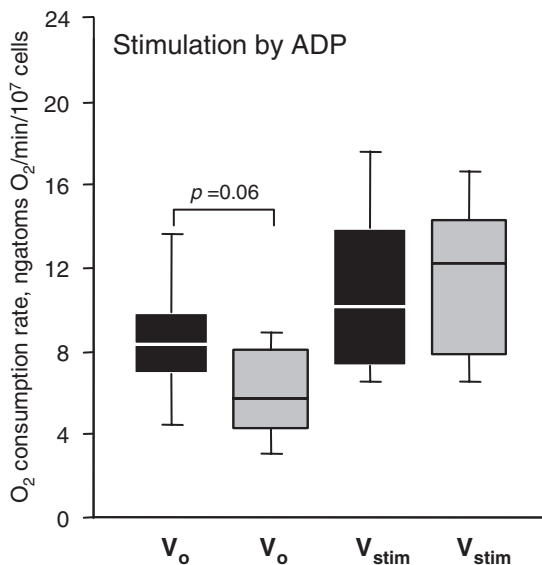


Figure 3. Effects of replacement of septic plasma by healthy volunteer plasma on septic peripheral blood mononuclear cell oxygen consumption ( $n = 10$ ).  $V_o$  was the baseline oxygen consumption.  $V_{stim}$  was the oxygen consumption after adenosine diphosphate (ADP) stimulation; *black bars* correspond to septic plasma, and *gray bars* correspond to healthy volunteer plasma. Data displayed as *box and whisker plot* (median, interquartile ranges and range).

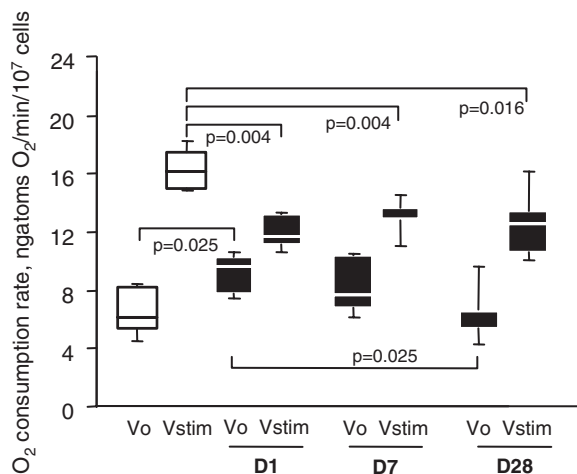


Figure 4. Effect of incubation of healthy cells with septic plasma from different time points of sepsis (days 1, 7, 28) on oxygen consumption ( $n = 6$ ).  $V_o$  is baseline oxygen consumption and  $V_{stim}$  is adenosine diphosphate-stimulated oxygen consumption. *Unfilled bars* are healthy volunteer oxygen consumption values; *filled bars* are oxygen consumption in septic plasma. Data displayed as *box and whisker plot* (median, interquartile ranges and range).

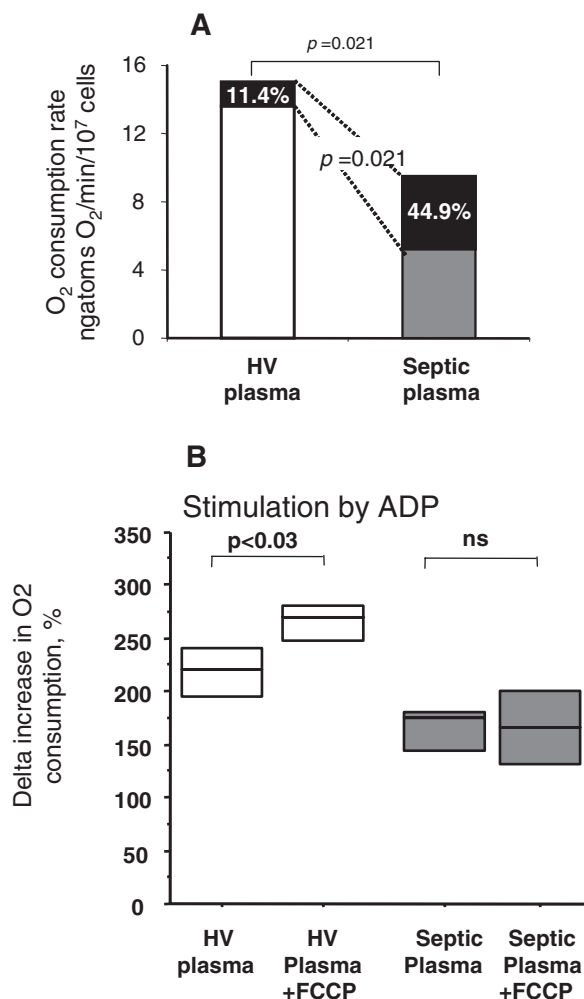
normal plasma, incriminates circulating mediators. Even many putative agents in plasma may induce such modifications (NO, prostaglandins, IL-10, etc.). It was not the goal of the present study to precisely identify which are responsible for the modifications observed. As a first line observation, it should be mentioned that the NO<sub>x</sub> levels did not differ significantly between septic and healthy plasma. Even this does not eliminate a role of NO in the metabolic modifications; this was not detectable by the plasma level of NO<sub>x</sub>. Similarly, the IL-10 antibody put in septic

plasma before incubation of healthy cells did not induce any significant modifications. Although IL-10 effect could not be detected in the present model, an effect of IL-10 is possible in different experimental conditions, as suggested in the literature (30). The reduction in basal HLA-DR expression in septic monocytes correlated with a higher baseline level of oxygen consumption ( $V_o$ ), but also with a markedly attenuated response to exogenous stimulation ( $V_{stim}$ ). This supports the concept of a global cellular energy failure resulting in an inability to further main-

tain the capacity to express HLA-DR and/or to release cytokines (1–4). This concept has been strongly supported in a recent study performed on blood leukocytes from healthy volunteers treated by lipopolysaccharide injection (31). The authors have demonstrated a significant decrease in messenger RNA abundance for mitochondrial respiratory chain complexes I–V and ATP synthase genes. Other metabolic enzyme gene expressions implicated in tricarboxylic acid cycle and pyruvate dehydrogenase were reduced. The mitochondrial permeability transition pore activation, considered an early event in apoptosis leading to mitochondrial membrane depolarization and release of cytochrome c, was decreased to similar extents. Thus, reduction in transcripts for mitochondrial permeability transition pore components was consistent with a protective response to the oxidative stress associated with endotoxin challenge. The authors used network analysis to reveal concerted dysregulation of functional modules in mitochondria bioenergetics in human blood leukocytes. They concluded 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.

Baseline  $V_o$  was significantly higher in septic cells than in controls. Several mechanisms may account for this  $V_o$  elevation (25). These mechanisms were not investigated in the present study, but mitochondrial stimulation for coupling oxygen consumption and ATP synthesis may be ruled out, because the ability of mitochondria to be stimulated by ADP was reduced in septic cells. Other results support such an assumption: If the incubation in septic plasma sampled later in the course of the sepsis (days 7 and 28) did not increase the  $V_o$ , it did not improve mitochondrial response to ADP, suggesting a persistent mitochondrial dysfunction; conversely, the replacement of septic plasma by healthy plasma reduced the  $V_o$  of septic cells, with no change in ADP response. We then concluded that the  $V_o$  level was reversibly determined by plasma factors, and did not result from mitochondrial oxygen consumption.

ADP response is testing the “tight coupling” ADP/ATP for oxidative phosphorylation (9). The observed altered response to ADP of PBMCs suggests an abnormal oxidative phosphorylation in septic cells. These results are in accordance with



**Figure 5.** A, inhibition of peripheral blood mononuclear cell oxygen consumption by oligomycin. Healthy cells were incubated for 3 hrs in their own plasma (HV) and in pooled day 1 septic plasma (n = 6). Oligomycin was added in the chamber after inducing maximal mitochondrial respiration by adenosine diphosphate (ADP). Black bars are percentage of oxygen consumption not inhibited by oligomycin. Data displayed as mean of four separate experiments. B, effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 10  $\mu$ mol/L) on ADP-induced increase in oxygen consumption in healthy volunteer plasma (white boxes) and in septic plasma (gray boxes; n = 4). Data displayed as box plot (median, interquartile ranges and range).

**Table 2.** Human leukocyte antigen (HLA)-DR expression on peripheral blood monocytes and interleukin (IL)-10 and IL-12p40 plasma levels in healthy volunteers and septic patients measured at time of oximetry experiments

	Monocyte HLA-DR, No. of Sites per Cell	IL-10, pg/mL	IL-12p40, pg/mL
HV, n = 25	19.8 $\times$ 10 <sup>3</sup> (7.6–47 $\times$ 10 <sup>3</sup> )	1.46 (0–15.6)	16.4 (0–63.1)
Septic, n = 18	2.3 $\times$ 10 <sup>3</sup> (0.4–13 $\times$ 10 <sup>3</sup> ) ( <i>p</i> < .0001)	34.6 (11.8–412) ( <i>p</i> < .0001)	16.7 (16.4–37.7)

HV, healthy volunteers.

Data are shown in medians and ranges.

those reported previously using the same method in active rheumatic disease (7), an acute phase of a chronic inflammation. Using a different stimulation, the authors found in active rheumatic disease, and not in asymptomatic disease, an elevated  $V_o$  associated with an attenuated re-

sponse to concanavalin A (7). Other studies have reported mitochondrial dysfunction in skeletal muscle in human sepsis (26) and in liver in animal models (27). These studies support the hypothesis that multiorgan dysfunction induced by severe sepsis has a bioenergetic etiology (32).

Similar abnormalities in ADP response were observed when healthy PBMCs were incubated with septic plasma, strongly suggesting the role of plasma factors. Apart from IL-10 and NO, which did not have clear effects in the present study, others mechanisms can be proposed to explain such ADP response attenuation. First, mitochondrial respiration may become partially uncoupled in septic conditions. The results obtained with oligomycin added after ADP stimulation showed that incubation with septic plasma increased the fraction of uncoupled oxygen consumption. This uncoupling may result from various mechanisms. ADP entry is allowed through the adenine nucleotide translocase in exchange with ATP. This exchange may be altered as part of the mitochondria permeability transition pore dysfunction. Because the electron transport chain also must work adequately to restore the mitochondria polarization by pumping protons, it could play a role on the observed depression response to ADP. Experiments comparing the effects of an uncoupling agent (FCCP) on oxygen uptake in septic plasma incubation conditions confirmed such a hypothesis. The addition of FCCP after ADP stimulation failed to increase oxygen consumption as observed in healthy conditions, suggesting an abnormal restoration of mitochondrial polarization. Second, a deficit in mitochondrial substrates may occur in septic plasma. This aspect could be eliminated because the preparation provided succinate, a substrate for the complex II. Third, a septic-induced abnormal permeability of outer membrane of the mitochondria is plausible (33). It has been shown in previous studies that mitochondrial structure was modified in septic conditions (26, 27, 32), with abnormal membrane potential (34). Such abnormal permeability may lead to loose cytochrome c then to alterations in the normal function of the respiratory chain (33). The persistence of such abnormal response of healthy cells to ADP stimulation using plasma from later stages of sepsis (day 7 or 28) strongly suggests a structural and a functional change of mitochondria. However, the *ex vivo* partial reversibility observed for septic cells when septic plasma was removed suggests a potential benefit of blood purification techniques to restore immune function (35).

We have no information on the distribution within the PBMC population of

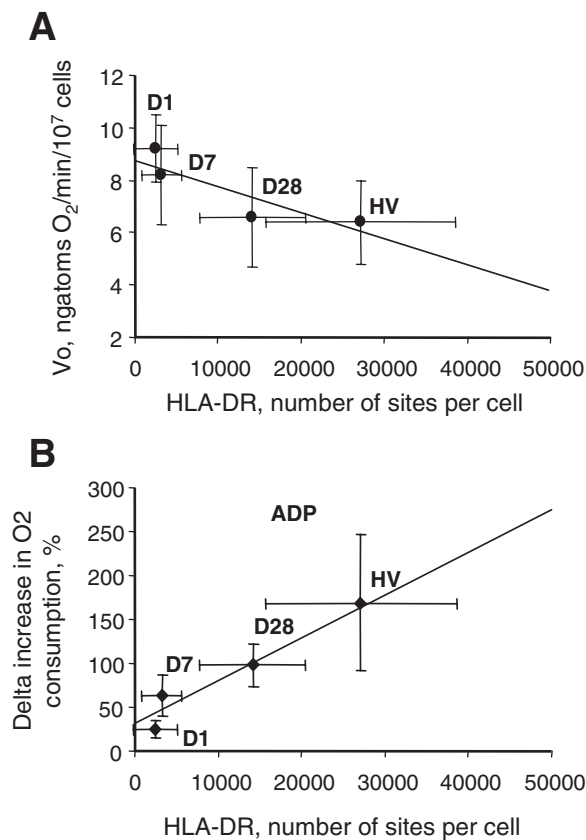


Figure 6. Correlation between healthy peripheral blood mononuclear cell oxygen consumption after incubation with septic plasma and human leukocyte antigen-DR (HLA-DR) expression at days 1, 7, and 28 (D1, D7, and D28) of sepsis. Healthy cells were incubated for 3 hrs in their own plasma (HV) and in pooled septic plasma (n = 6) sampled at days 1, 7, and 28. Horizontal axis, HLA-DR expression in healthy and septic patients cells. Vertical axis A, basal rate of oxygen consumption; delta increase of oxygen consumption under adenosine diphosphate (ADP) stimulation. Vertical axis B, data displayed as mean  $\pm$  SD of six separate experiments.

the respiration lost under ADP stimulation; i.e., whether respiration was affected partially in every cell or whether monocytes more than lymphocytes had lost their coupled mitochondrial respiration. The relationship observed between the level of  $V_o$  and the attenuated response to ADP with the level of expression of HLA-DR suggests simultaneous modifications of inflammatory phenotype and energetic alteration induced by sepsis, at least for monocytes.

In conclusion, oxymetry of immune cells is a suitable tool to better characterize the bioenergetics of these cells in sepsis. Different metabolic pathways can be studied, especially the mitochondrial oxidative phosphorylation. This phosphorylation is altered in septic PBMCs, at least via plasma factors. This alteration persists along weeks of sepsis duration and may result from several mechanisms, which require further metabolic protocols. Metabolic alteration seems to parallel the change in inflammatory pheno-

type. The mechanistic link remains to be demonstrated.

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