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Hematological indices, inflammatory markers and neutrophil CD64 expression: comparative trends during experimental human endotoxemia

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CD64 is a high-affinity Fc_γRI receptor expressed by activated neutrophils that has been recently evaluated as a potential sepsis parameter. In the present study, the kinetics of neutrophil membrane CD64 expression were examined during a standardized inflammatory response, using a human endotoxemia model, and compared with hematological indices, CRP, cytokines and interleukins. Ten healthy subjects received 2 ng/kg intravenous *Escherichia coli* lipopolysaccharide (LPS). After administration of LPS, neutrophil CD64 showed a biphasic response, characterized by a first increase from 108.5 ± 7.5 to 133 ± 6 AFU after 1 h ($P = 0.047$) and a second increase that started at 6 h and reached its maximum of 167 ± 13 AFU at 22 h ($P < 0.0001$). CRP concentrations increased to 40 ± 5 mg/dl 22 h after the administration of LPS. The cytokines and interleukins reached their maximum response within 1–2 h. The maximum values of pro-inflammatory cytokines (TNF- α , IFN- γ and IL-6) correlated with the CD64 expression at 22 h after LPS administration ($r^2 = 0.76$, $r^2 = 0.78$, $r^2 = 0.81$, respectively, all $P < 0.05$), whereas this correlation was not found for the anti-inflammatory IL-10 ($r^2 = 0.058$, $P = 0.54$), suggesting that neutrophil CD64 expression might be a quantitative marker for innate immunity that could easily be used in the clinical setting.

Keywords: CD64; endotoxemia, neutrophils, sepsis markers

INTRODUCTION

The annual incidence of sepsis in the US is 50–95 cases per 100,000, with approximately 9% being categorized as severe and 3% complicated by septic shock.¹ As mortality increases with progressive sepsis, early detection of sepsis is very important. One of the central features of the host response to bacterial infection is the recruitment

and activation of neutrophils, typically manifested by blood neutrophilia. Other hematological indicators of infection include the morphological presence of neutrophils with toxic changes (*e.g.* hypergranulation and Dohle bodies), increased proportions of non-segmented or band neutrophils and circulating immature granulocytes (metamyelocytes and myelocytes).² However, the non-specific range of several conditions associated with changes in neutrophil counts,³ as well as the frequent occurrence of mild-to-moderate neutropenia in the acute phase of infection and the statistical and morphological limitations of band cell enumeration, considerably limits their diagnostic specificity.^{4–6}

In addition to hematological investigations, a number of biochemical and immunological analyses are used as diagnostic aids. These include acute phase proteins such as C-reactive protein (CRP) and procalcitonin (PCT),

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soluble triggering receptor expressed on myeloid cells (sTREM), cytokines such as tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), and interleukins such as IL-6 and IL-10.⁷⁻¹³ Many of these have been used to indicate disease severity¹⁴⁻¹⁶ and to supplement full blood count (FBC) screening in the assessment of suspected infection; however, they are less widely available and are also affected by confounding factors.

Fc RIII (CD16) is highly expressed in normal mature neutrophils, whereas the effect of inflammation on CD16 expression on neutrophils has been the subject of debate. CD16 expression was found to be decreased in systemic inflammatory responses while *in vitro* activation of the immune system has been associated with increased CD16 expression.¹⁷ The interpretation of Fc RII (CD32) expression is difficult, since this marker is expressed on all granulocytes. Moreover, the increment is less pronounced compared to, for example, CD64.¹⁸ We recently described a practical procedure for quantitative fluorescent neutrophil CD64 (nCD64) measurements with a hematology analyzer.¹⁹ nCD64 corresponds to the high-affinity Fc γ RI receptor expressed by monocytes but not by normal neutrophils. Up-regulated and detectable nCD64 is characteristically seen in patients with infections and its measurement is gaining acceptance as a useful marker in the assessment of sepsis.²⁰⁻²⁵ Relatively little is known, however, about changes in nCD64 during the early stages of inflammation. To investigate this particular question, the present study examined the modulation of nCD64 expression during a standardized inflammatory stimulus using experimental human endotoxemia, and compared this to changes in hematological and non-hematological (CRP, IFN- γ , TNF- α , IL-6 and IL-10) parameters. Furthermore, the cytokine responses during endotoxemia were correlated with CD64 expression to examine whether quantitative changes in CD64 expression reflect the magnitude of the innate immunity response.

PATIENTS AND METHODS

Subjects

This study was approved by the local ethics committee and informed consent was obtained from each volunteer. Prior to the single-dose, intravenous administration of 2 ng/kg *Escherichia coli* O:113 LPS (US Pharmacia, Rockville, MD, USA), 10 healthy, non-smoking subjects (4 male, 6 female; mean age, 21 years; range, 18-24 years) entered into the study were checked by routine medical examination, electrocardiography and blood analysis. With the exception of oral contraceptives, none of the volunteers used prescription medication, aspirin or other non-steroid anti-inflammatory drugs and all were

HIV and hepatitis B negative. Medical appraisal confirmed that there was no history of a febrile illness in the 2 weeks preceding the study, and in the 12-h period prior to LPS administration all subjects refrained from caffeine, alcohol and food. In the post-LPS period, vital signs were continuously monitored and, at various time intervals, EDTA and heparin anticoagulated blood samples were taken for hematological, nCD64, CRP, interleukin and cytokine measurements.

Analysis of neutrophil CD64 (nCD64) expression

nCD64 expression was measured at baseline and 1, 2, 4, 6, 12 and 22 h after LPS administration. As previously described,¹⁹ the immunofluorescent detection and quantification of nCD64 was performed using the Cell-Dyn CD4000 hematology analyzer (Abbott Diagnostics, Santa Clara, CA, USA). In brief, vacutainer tubes containing 100 μ l anticoagulated whole blood plus 20 μ l anti-CD64 (FITC; Becton Dickinson) and 15 μ l anti-Ia (PE; Becton Dickinson), were processed using the CD4000 automated CD4/CD8 assay mode after preliminary incubation at room temperature for 10 min. Raw data files were downloaded to a PC for population analysis. Primary gating of the neutrophil population was facilitated with WINMDI software (<http://facs.scripps.edu/software.html>), using Ia expression to exclude monocytic components and optical characteristics (0° versus 7°) to exclude lymphocytes. This was followed by post-acquisition fluorescent channel compensation using WINList v.4.0 software (Becton Dickinson). Statistical analyses of neutrophil FL1 histograms (3-decade log) were then undertaken to obtain median nCD64 staining intensities quantitatively expressed as arbitrary fluorescent units (AFU). In a previous study, the normal range for nCD64 was determined at a mean \pm SD of 94 \pm 14 AFU.¹⁹

Hematological studies

White blood cell (WBC) and absolute neutrophil counts (ANC) were measured at baseline and 1, 2, 4, 6, 12 and 22 h after LPS administration using a Sysmex XE-21 00 (Toa, Kobe, Japan) analyzer. Immature granulocytes were enumerated with Sysmex instrument software (XEmaster), while band cell counts were obtained by conventional microscopy (2 \times 200 cells) of May-Grünwald Giemsa stained blood smears.

Analysis of CRP, TNF- α , IFN- γ , IL-6 and IL-10

Serum CRP was measured at baseline and 1, 2, 4, 6, 12 and 22 h after LPS administration using a turbidimetric

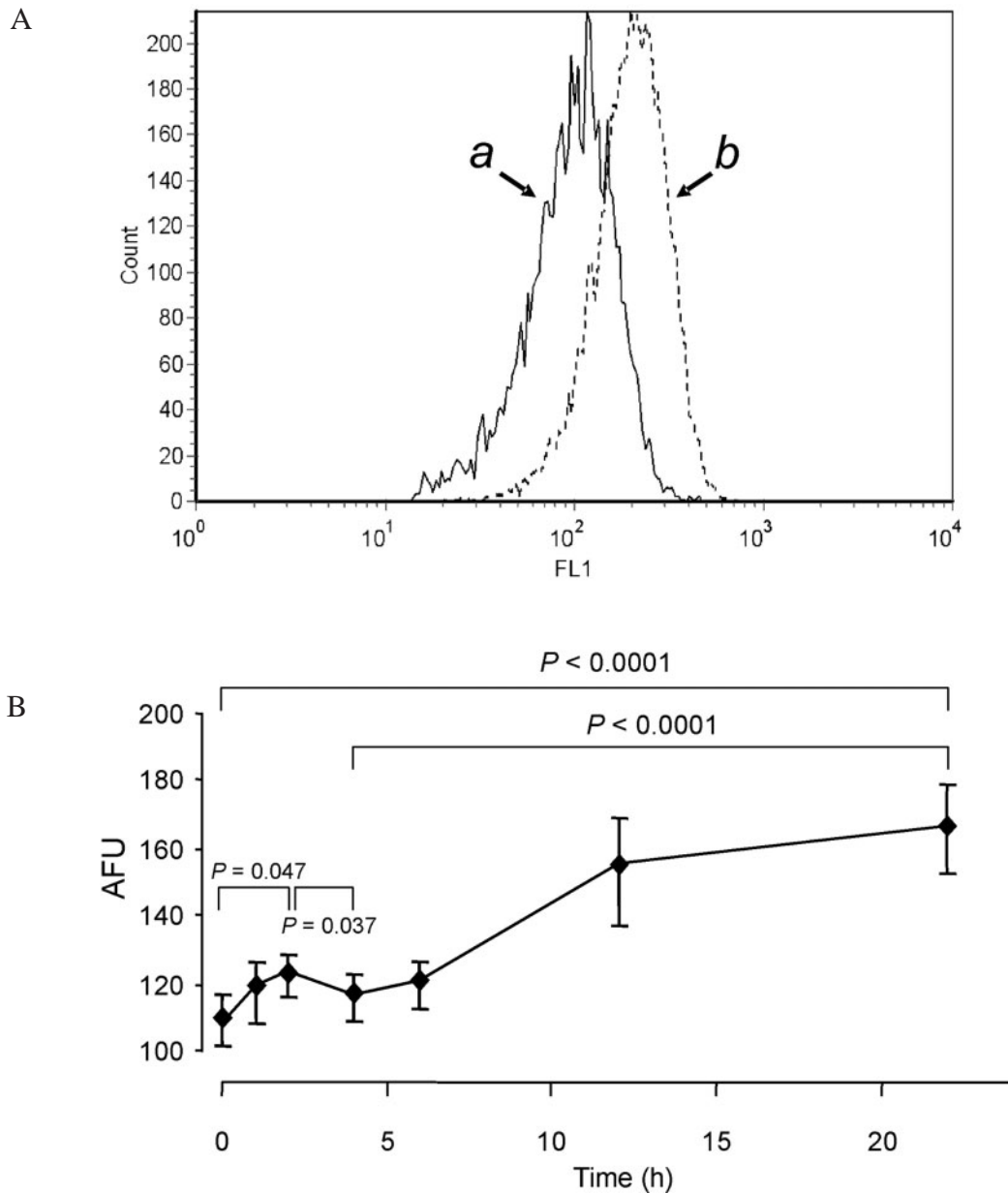


Fig. 1. (A) Representative flow cytometry data prior to (line a) and 12 h after (line b) the administration of LPS. (B) Time course of neutrophil CD64 (nCD64, AFU) expression during human experimental endotoxemia. Subjects ($n = 10$) received 2 ng/kg *E. coli* LPS. nCD64 expression demonstrated a biphasic increase. Data expressed as mean \pm SEM. The P -values refer to the statistical differences determined by ANOVA repeated measures.

method (Aeroset, Abbott Laboratories, Abbott Park, IL, USA). In previous experimental endotoxemia, TNF- α , IFN- γ and IL-10 returned to baseline values after 4 h, whereas IL-8 decreased after 4–6 h.²⁴ Therefore, we measured concentrations of TNF- α , IFN- γ , IL-6 and IL-10 at baseline and 1, 1.5, 2, 3 and 4 h after LPS administration using the simultaneous Luminex Assay (R&D Systems, Minneapolis, MN, USA).²⁵ The lower limit of detection of TNF- α , IFN- γ , IL-6 and IL-10 was 8 pg/l.

Statistical analysis

Descriptive results of continuous variables were expressed as mean \pm SEM. Data analysis was performed using ANOVA with repeated measures. A P -value < 0.05 was considered significant. Individual peak values of cytokines and interleukins were correlated (Pearson) with neutrophil CD64 expression and CRP values, at 22 h after LPS administration. *Post-hoc* tests were not per-

formed on the time-dependent changes tested by ANOVA repeated measures, since we were not interested at what time exactly the values become significant.

RESULTS

Subjects and pre-LPS baseline analyses

Table 1 shows baseline values of the measured parameters.

Post-LPS – neutrophil CD64 (nCD64) expression

Following LPS administration, nCD64 expression shows a biphasic increment (Fig. 1). A first rise was seen after 1 h with a maximum of 133 ± 6 AFU after 2 h ($P = 0.047$), the second increment started at 12 h with a maximum of 167 ± 13 AFU at 22 h ($P < 0.0001$). The expression of monocyte CD64 in a human endotoxemia model has not been described previously during the early phase of human endotoxemia. In our study, measurement of monocyte CD64 expression was not possible in the early phase of endotoxemia, since monocytopenia occurred (mean values: $0.02 \times 10^9/l$ after 1 h, $0.03 \times 10^9/l$ after 2 h, $0.07 \times 10^9/l$ after 4 h and $0.16 \times 10^9/l$ after 6 h). However, 6 h after exposure to endotoxin, monocyte CD64 expression increased from 794 ± 61 AFU to 1115 ± 72 AFU.

Post-LPS – hematological parameters

A rapid initial decline in the ANC following the intravenous administration of LPS was observed (Fig. 2 and Table 1). One hour after the administration of LPS, the mean ANC was $1.0 \pm 0.1 \times 10^9/l$ compared to a pre-LPS value of $4.9 \pm 1.2 \times 10^9/l$ ($P < 0.0001$). Thereafter, the ANC showed a progressive increase to $11.5 \pm 0.7 \times 10^9/l$ at 6 h and a subsequent declining trend to $6.6 \pm 1.0 \times 10^9/l$ at 22 h (one way ANOVA repeated measures: $P < 0.0001$).

The immature granulocyte count showed a similar overall trend ($P = 0.03$), although all values remained within the normal range (Fig. 2 and Table 1). Band cells showed a clearer increase to maximal values 2–6 h post-LPS

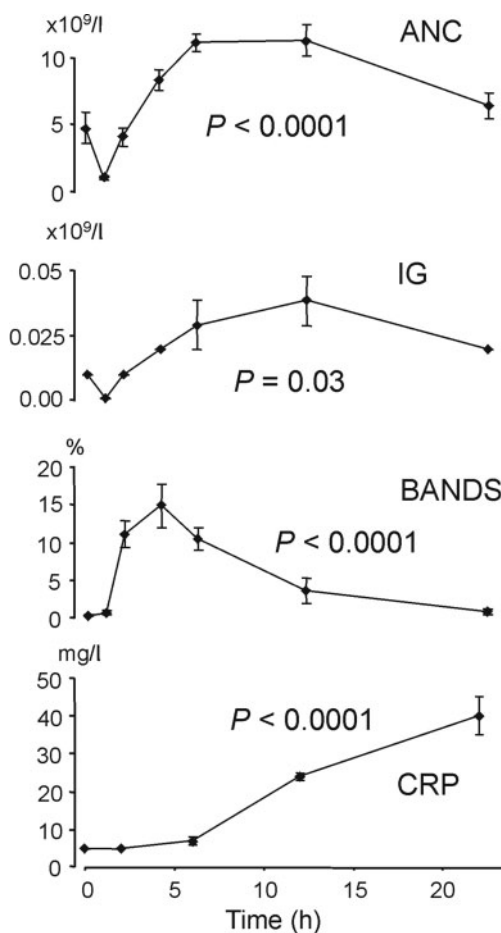


Fig. 2. Time course of inflammatory hematological parameters during human experimental endotoxemia. Subjects ($n = 10$) received 2 ng/kg *E. coli* LPS. Absolute neutrophil count (ANC, $\times 10^9/l$), absolute immature granulocyte count (IG, $\times 10^9/l$), microscopic band count (%) and C-reactive protein (CRP, mg/l) were determined. Data expressed as mean \pm SEM. The P -values refer to the statistical differences determined by ANOVA repeated measures.

Table 1. Comparison of all measured parameters at baseline level and time to reach maximum value

Parameter	Reference value	Mean baseline value \pm SEM	Mean maximum value \pm SEM	P -value	Time to maximum value (h)
ANC ($\times 10^9/l$)	2.0–6.5	4.9 ± 1.2	11.6 ± 1.2	< 0.0001	6–12
Band cells (%)	< 5	$< 5 \pm 0$	15 ± 3	< 0.0001	4–6
IG ($\times 10^9/l$)	0.05	0.01 ± 0	0.04 ± 0.01	0.03	6–12
CRP (mg/l)	< 5	7.2 ± 2.4	40 ± 5	0.0001	22
IFN- γ (pg/ml)	nd	10.4 ± 2.6	170 ± 42	< 0.0001	1.5
TNF- α (pg/ml)	nd	8 ± 0	1047 ± 285	< 0.0001	1.5
IL-10 (pg/ml)	nd	8 ± 0	112 ± 26	< 0.0001	2
IL-6 (pg/ml)	nd	11.7 ± 2.7	8338 ± 4560	0.009	4
nCD64 (AFU)	< 145	108.8 ± 7.5	133 ± 6	0.047	1–2
			167 ± 13	< 0.0001	12–22

Neutrophil membrane CD64 expression expressed as arbitrary fluorescent units (AFU); nd, not detectable.

(Fig. 2 and Table 1). Mean band cell count during this transient increase was $15 \pm 3\%$. Subsequent progressive declines to normal ($< 5\%$) band cell values were seen for all subjects at 22 h ($P < 0.0001$). Changes in ANC and band cell counts were not accompanied by any observable increase in neutrophil toxic granulation.

Post-LPS – CRP, TNF- α , IFN- γ , IL-6 and IL-10

Serum CRP concentrations showed a relatively slow response compared to changes in hematological parameters and nCD64 expression. The mean CRP concentration at 6 h was 6.9 ± 0.6 mg/l, which then progressively increased over the post-LPS period to mean concentrations at 12 h and 22 h of 24.7 ± 1.1 mg/l and 39.7 ± 4.9 mg/l, respectively ($P < 0.0001$; Fig. 2 and Table 1). For comparison, TNF- α and IFN- γ increased within 1 h of LPS administration to mean peak values at 90 min of 1047 ± 285 pg/ml and 170 ± 42 pg/ml, respectively (both $P < 0.0001$; Fig. 3 and Table 1). Decreases to relatively normal levels were seen within 4 h post-LPS. For the interleukin markers, IL-6 increased within 1 h to a mean peak value of 8338 ± 4560 pg/ml at 4 h (Fig. 3 and Table 1) ($P = 0.009$), while IL-10 increased within 1 h to a peak value of 112 ± 26 pg/ml at 2 h ($P < 0.0001$; Fig. 3 and Table 1).

As illustrated in Figure 4, the peak values of TNF- α , IFN- γ and IL-6 correlated with nCD64 expression at 22 h after LPS administration, whereas this correlation was not found for IL-10. There was no correlation between the pro-inflammatory cytokines and CRP (Fig. 4) and no significant correlation between nCD64 expression and CRP ($r^2 = 0.17$; $P = 0.2755$; Fig. 4).

DISCUSSION

The present study demonstrates that, in a standardized inflammation model, nCD64 expression shows a biphasic response. The peak values of pro-inflammatory cytokines, but not the anti-inflammatory IL-10, correlate with the nCD64 expression at 22 h after LPS administration, whereas this correlation was not found for CRP. These results suggest that nCD64 expression is a reliable and quantitative marker of the innate immune response. Furthermore, this study confirms that nCD64 can be rapidly and easily measured using a routine hematology analyzer available in a clinical setting.

The potential value of nCD64 measurements in patients with suspected sepsis has been described previously. A sensitivity of 94.1%, specificity of 84.9%, and positive predictive likelihood ratio of 6.24 has been reported.²¹ Relatively little is known about the changes in the expression of this receptor during the very initial stages of infection or inflammation in humans. In order to

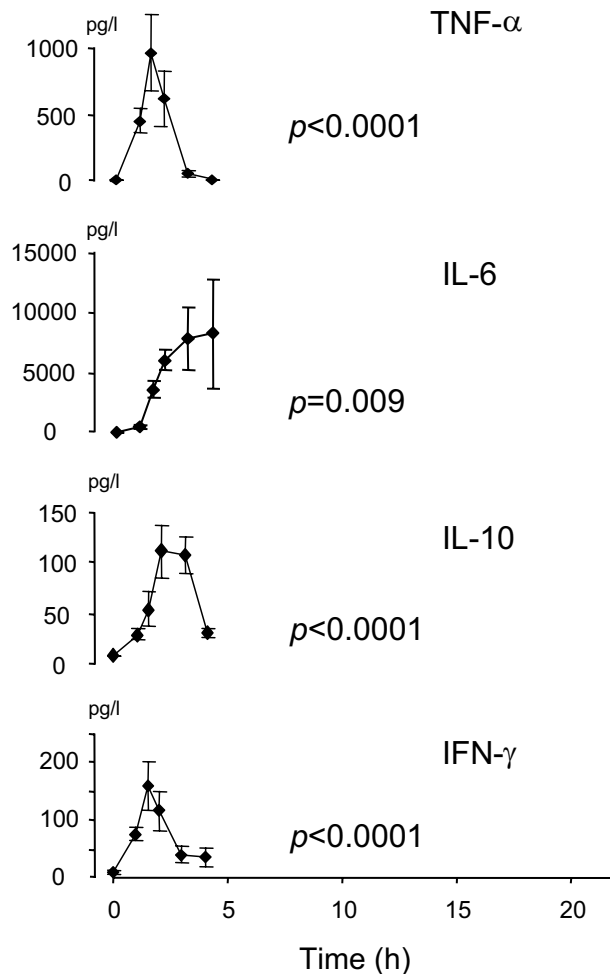


Fig. 3. Time course of cytokines during human experimental endotoxemia. Subjects ($n = 10$) received 2 ng/kg *E. coli* LPS. Tumor necrosis factor (TNF- α , pg/ml), interleukin-6 (IL-6, pg/ml), interleukin-10 (IL-10, pg/ml) and interferon- γ (IFN- γ , pg/ml) were determined. Data are expressed as mean \pm SEM. The P -values refer to the statistical differences determined by ANOVA repeated measures.

investigate this particular point, we used an accepted model for Gram-negative sepsis (experimental human endotoxemia) and examined the resulting trends of nCD64 expression and compared these with several other parameters used for monitoring the extent of inflammation. Human endotoxemia has been used previously by other investigators as an experimental model to examine diverse aspects of the inflammatory response including changes in leukocyte antigen expression.^{28–30} In the present study, we show that changes in nCD64 expression following LPS administration can generally be characterized as biphasic. In examining relationships between hematological changes and nCD64 expression, we found that LPS administration was accompanied by an initial rapid decline in the absolute neutrophil (ANC) and immature granulocyte counts at 1 h, followed by an

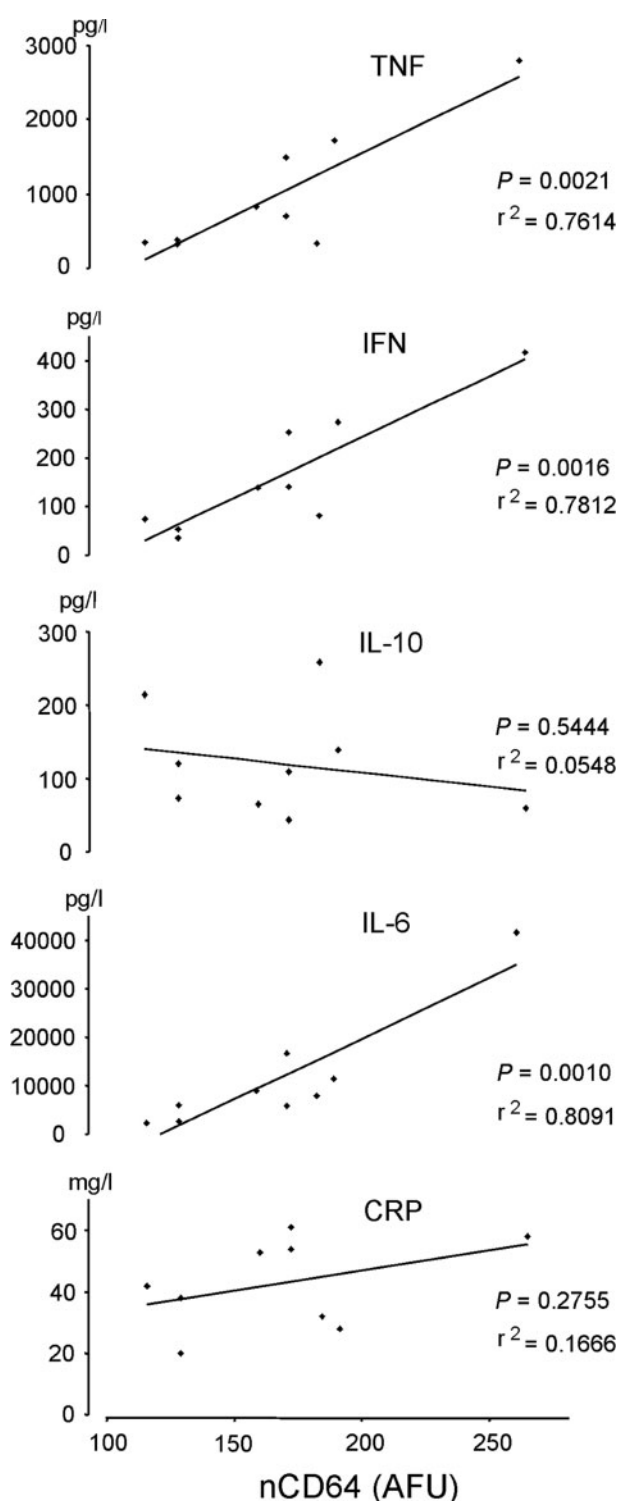


Fig. 4. Correlation (Pearson) between peak concentrations of tumor necrosis factor (TNF- α , pg/ml), interferon- γ (IFN- γ , pg/ml), interleukin-10 (IL-10, pg/ml), interleukin-6 (IL-6, pg/ml) and C-reactive protein (CRP, mg/l) with neutrophil CD64 (nCD64, AFU) at $t = 22$ h and between peak concentrations of TNF- α , IFN- γ , IL-10, IL-6 and CRP in 10 healthy volunteers that received 2 ng/kg *E. coli* LPS. Peak concentrations of TNF- α , IFN- γ and IL-6 demonstrated good correlation with nCD64 expression, whereas no correlation with IL-10 and CRP was found.

increase that reached a maximum at 6 h and then a decline to relative normality at 22 h. The possibility that the initial ANC decline was a result of increased sequestration (extravasation), with the subsequent increase resulting from an expanded release of marrow neutrophils, is supported to some extent by the observation of transiently increased proportions of band cells between 2–6 h post-LPS. We hypothesize that the initial small increase in nCD64 expression that was seen and the observed decline in the ANC might be due to a direct modulating effect of LPS on circulating mature neutrophils. For the second and more sustained increase in nCD64 expression, however, we postulate that newly released neutrophils from marrow/storage pools may have constitutively up-regulated nCD64 levels. Using experimental human endotoxemia as a model of sepsis, the amount and exact moment of purified LPS administration are clear, so that a precise and quantitative measure of the innate immune response can be obtained. In a clinical setting of patients with a severe infection or sepsis, it would have been impossible to detect the biphasic increase in nCD64 expression.

Up-regulation and increased expression of nCD64 appears to be a sensitive marker for early-onset clinical infection in newborn children.^{25,31} In adults, its use has been suggested for differentiating systemic infection from active inflammatory disease,³² monitoring IFN- γ therapy²¹ and as an indicator for initiating or discontinuing antibiotic treatment.²⁵ Our observation that nCD64 expression correlates with a rise in pro-inflammatory cytokines 20 h earlier, further substantiates the notion that nCD64 expression is a reliable and quantitative measure of the innate immune response. While a single dose exposure of LPS cannot be extrapolated to clinical situations where stimulation by a mediating agent such as LPS is likely to be more prolonged, our study provides valuable insights into the relative rates and magnitudes of early cellular and biochemical changes. By this means, we found that changes in nCD64 expression preceded CRP but lagged behind increases in TNF- α , IFN- γ , band cells, IL-6 and IL-10. With regards to laboratory and clinical application, the cytokine and interleukin assays are unlikely to be available in many routine laboratories while the more widely available band cell estimates and CRP measurements both suffer from low diagnostic specificity.³³

CONCLUSIONS

Compared to other hematological indices, nCD64 expression shows a biphasic response to LPS administration in humans. A correlation between pro-inflammatory cytokines, but not of the anti-inflammatory IL-10, and nCD64 expression was demonstrated, illustrating that nCD64 expression is a measure of innate immunity. LPS-induced increases in cytokine concentrations did not correlate with CRP. Since measurements of cytokines are not readily available in most hospitals,

these markers are less appropriate for routine use. Therefore, nCD64 might be a potential sepsis marker that can be measured on a routine hematology analyzer and represents a better measure of the innate immunity response than CRP during human inflammation.

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