

EXTENDED REPORT

Neutrophil CD64 expression: distinguishing acute inflammatory autoimmune disease from systemic infections

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Background: Common bacterial and opportunistic infections are a major cause of mortality in patients who are immunosuppressed owing to treatment with corticosteroids or cytotoxic drugs. Common laboratory tests for infection lack sensitivity and specificity. One of the new generation of tests to detect early systemic infections measures the up regulation of an Fc receptor (Fcγ R1, or CD64) on neutrophils. The Fc receptors on white blood cells are very important for effective phagocytosis of bacteria and are up regulated during an infection.

Objective: To measure the clinical usefulness of quantitative CD64 measurements to differentiate between systemic infection and active autoimmune inflammation in an ongoing study.

Methods: Patients with systemic infection (n=27), active autoimmune inflammatory disease (n=44), vasculitis (n=5), and controls (n=20) were studied for neutrophil CD64 expression using monoclonal antibodies and flow cytometry.

Results: The median (interquartile range (IQR)) CD64 expression in patients with active inflammatory disease and systemic infection was 907.5 (586–1550) and 3647 (2380–6642), respectively (p<0.0001). The median (IQR) CD64 expression in control patients (osteoarthritis and fibromyalgia) was 505 (359–599). The sensitivity and specificity of CD64 expression on neutrophils to diagnose systemic infection (using a cut off value of 2000) was 85% and 91%, respectively.

Conclusion: These results indicate that quantitative measurement of CD64 can distinguish between systemic infection and the flare of autoimmune diseases.

Common bacterial and opportunistic infections are a major cause of morbidity and mortality in patients who are immunosuppressed owing to treatment with corticosteroids or cytotoxic drugs. In one series, 80% of fatal opportunistic infections in autoimmune patients were not detected before death.¹ Such patients may present initially with signs and symptoms of a non-specific inflammatory process such as the fever, malaise, arthralgia, and myalgia of autoimmune disease.^{2,3} However, the proper diagnosis can be elusive because the signs and symptoms of serious infections may mimic those of the underlying autoimmune condition. Activation of the innate immune system, especially neutrophils and acute phase proteins, is not only a hallmark of systemic infection but also the inflammation of active autoimmune diseases and inflammation associated with malignancies.

The usual laboratory tests employed to diagnose systemic infections, such as leucocyte count, presence of immature leucocyte forms (bands), C reactive protein (CRP), and erythrocyte sedimentation rate (ESR), have poor sensitivity and specificity. They can be misleadingly low in patients with systemic infections who are receiving corticosteroid or cytotoxic treatment. They may be misleadingly high in those with active rheumatic diseases. Imaging studies lack sensitivity in early stages of infection. Microbiological cultures are time consuming, often negative in those who are receiving antibiotics, and depend on various technical factors, such as the timing and technique of specimen collection and the transport time to the laboratory. Differentiation between a flare of a rheumatic disease and systemic bacterial infection in a patient receiving immunosuppressive treatment is vitally important as the treatment differs dramatically. Patients are often empirically treated for both possibilities with broad spectrum antibiotics and high dose corticosteroids. A highly sensitive and specific

laboratory test that has a short turn-around time would be extremely useful in these circumstances. The complexity of the clinical presentation of both infections and inflammatory disease requires investigation of other assays that may be of diagnostic value. One such candidate would be the Fc receptor on neutrophils because these cells form an important component of the innate immune system and are activated early in either process.

The Fc receptors, members of the immunoglobulin gene superfamily, are found on white blood cells, where they function to integrate responses involving both the innate and acquired immune systems. They are important for effective phagocytosis of bacteria and immune complexes. One of the Fc receptors for IgG is Fcγ receptor I (Fcγ RI or CD64). It is constitutively expressed on macrophages, monocytes, and eosinophils. It is up regulated on neutrophils as a physiological response to microbial wall components such as lipopolysaccharide, complement split products, and some cytokines (interferon γ), tumour necrosis factor α, interleukin 8, interleukin 12. Up regulation of CD64 occurs within four to six hours after stimulation with interferon γ or granulocyte colony stimulating factor,⁴ and the kinetics of other activators, such as lipopolysaccharide, are likely to be similar. These characteristics suggested that CD64 should be investigated as a candidate to distinguish systemic infection from other types of inflammatory responses, such as active autoimmune disease. We tested the hypothesis that neutrophil CD64 expression

Abbreviations: CRP, C reactive protein; DMARDs, disease modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; IQR, interquartile range; OHSU, Oregon Health and Science University; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

Table 1 Subjects with infection (n=27)

Source	Mean age (range)	No
Bacteraemia*	44 (32–77)	8
Respiratory tract	68 (40–76)	6
Musculoskeletal	57 (46–73)	6
Intravenous catheter	58 (46–73)	3
Cardiovascular	50 (36–64)	2
Urinary tract	36 (23–49)	2

*Positive blood cultures but no focal source identified.

could discriminate between active flares of inflammatory autoimmune conditions and systemic infection by studying CD64 expression in various rheumatic and infectious diseases.

PATIENTS AND METHODS

Patients

Four groups of patients were studied at Oregon Health and Science University (OHSU) between June 1998 and June 2000.

Group 1 included 27 inpatients with culture proven infections (table 1). They were included if their age was greater than 18 years and if they had a culture proven infection of any kind. Most were bacteraemic patients, but some had partial localisation of their infection, such as bladder infections. Because they were enrolled after the cultures became positive, all had been receiving antibiotics for varying lengths of time at study entry. Culture positive patients were excluded from the study if the isolated organism was considered a contaminant (for example, growth of normal respiratory flora from a sputum culture or cutaneous flora on blood culture after 72 hours). We did not exclude patients with haematological malignancies (including acute and chronic myelogenous leukaemia), but neutropenic patients (absolute neutrophil count $<10^9/l$) were excluded. There were no exclusions for drug use, including antibiotics, corticosteroids, and cytotoxic treatment.

Group 2 consisted of 44 patients with active inflammatory diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and other inflammatory conditions except vasculitis. Group 2 patients did not show signs of active infection at the time of study entry. As neutrophil activation may be integral to the pathogenesis of vasculitis, we decided to evaluate patients with vasculitis as a separate group. Group 3 consisted of five patients with vasculitis, including three with cryoglobulinaemic vasculitis, one with Wegener's granulomatosis, and one with cutaneous vasculitis associated with systemic lupus (table 2). Patients in these two groups were selected at random from the rheumatology clinic at OHSU. Each rheumatological diagnosis was based on accepted American College of Rheumatology criteria. Patients were included if their age was greater than 18 years and their underlying rheumatic disease was active. Disease activity was determined by the treating doctor. Specifically, all patients with RA had at least six swollen joints and raised ESR or CRP, and all patients with SLE had at least one organ system showing signs of SLE "flare". No patients were excluded for drug use, including corticosteroids, disease modifying antirheumatic drugs (DMARDs), and cytotoxic treatment.

Group 4 was the control group consisting of patients with fibromyalgia (n=18) and osteoarthritis (n=2). These patients were also selected at random from the rheumatology clinic at OHSU. Because these conditions are non-inflammatory, disease activity was considered irrelevant. These patients displayed no signs of infection at the time of study entry.

The study was reviewed, approved, and monitored by the institutional review board at OHSU. Patients were informed of the potential risks and benefits as well as their rights as

research study subjects. Every subject signed an informed consent form before study entry.

Flow cytometry

CD64 expression on neutrophils was measured by staining 50 μ l of whole blood with a combination of anti-CD64-PE and anti-CD45-PerCP (provided by Becton-Dickinson, San Jose, CA) for 60 minutes in the dark. This was followed by lysis of the red blood cells, no washing, and an additional 60 minute incubation to allow equilibration and reduction of non-specific background staining. The specimens were analysed on a FACScan flow cytometer (Becton-Dickinson) using an FL3 threshold to analyse leucocytes only. The instrument was calibrated with QuantiBRITE PE beads (Becton-Dickinson). QuantiBRITE PE beads contain a mixture of four different beads with known numbers of bound PE molecules and allow the creation of a standard curve for determining the mean number of PE molecules present on a cell. Increasing fluorescence intensity along the fluorescence axis indicates increasing numbers of fluorescent antibodies bound to the cell. Importantly, this is a logarithmic scale. The mean of another peak can be used with this standard curve to calculate the average number of PE molecules bound to these cells. Because the CD64-PE antibody is produced to contain one PE molecule per antibody, this calculation provides an approximation for the number of CD64 molecules. Although this antibody binding capacity may not be identical to the CD64 expression, it is relative to the actual CD64 expression, and for the rest of this discussion CD64 expression and antibody binding capacity will be considered the same. Although the absolute number of CD64 antigens is interesting, a relative difference in expression based on mean fluorescence intensity, which is an arbitrary unit, can also be used to compare groups.

Statistics

The Sigma Stat Statistical Program (SPSS Science, Chicago, IL) was used to perform statistical analysis. Neutrophil CD64 values in various patient groups are reported as medians and interquartile ranges (IQR). Comparisons between patient groups were carried out using non-parametric tests (Mann-Whitney U test). Receiver operator curve analysis was used to establish optimal sensitivity and specificity of the CD64 assay.

RESULTS

A total of 96 patients was studied. Table 1 gives details of the 27 patients from group 1 (infection cases). Most of the patients in this group had either bacteraemia (no focal source identified) or respiratory tract infections (for example, pneumonia), although a variety of infections were included. As all the patients entered into the study after cultures became positive, they were receiving antibiotics at the time of serum collection. Group 2 consisted of 44 patients with various active inflammatory diseases (table 2). The age ranges are typical for the individual disorders studied. Among the patients with rheumatoid arthritis (RA), who comprised the largest group, 16/21 (76%) were receiving a DMARD at the time of evaluation. Most patients with other inflammatory diseases were not receiving DMARDs. Table 2 outlines details of the patients with vasculitis (group 3). Patients with osteoarthritis and fibromyalgia (group 4) were not receiving immunosuppressive drugs or antibiotics.

Neutrophil CD64 expression

In all the control patients with non-inflammatory diseases the CD64 levels were <1000 (median 505 (IQR 359–599), fig 1A). The median (IQR) expression of CD64 molecules per neutrophil in the group with active inflammatory diseases was 907.5 (586–1550) and in the group with infections 3647 (2380–6642). Among the five patients with vasculitis, the median (IQR) expression of CD64 molecules per neutrophil

Table 2 Subjects with inflammatory diseases: without vasculitis (n=44) and with vasculitis (n=5)

Disease	No	Mean age (range)	Not receiving DMARD	Receiving DMARD				
				Prednisone	MTX	AZA	Etanercept	Combination
Rheumatoid arthritis	21	46 (24–65)	5	6	12	2	0	5
Systemic lupus erythematosus	8	33 (17–50)	5	3	0	0	0	0
Gout	8	52 (34–70)	7	1	0	0	0	0
Spondyloarthropathy	4	46 (39–58)	1	0	2	0	1	0
Dermatomyositis	2	49 (48–50)	0	2	0	0	0	0
Familial Mediterranean fever	1	45	1	0	0	0	0	0
Wegener's granulomatosis (ANCA +ve)	1	86 M	Yes	–	–	–	–	–
Cutaneous vasculitis (with SLE)	1	28 F	–	–	Yes	–	–	–
Cryoglobulinaemic vasculitis (hepatitis C –ve)	1	44 F	–	Yes	Yes	–	–	–
Cryoglobulinaemic vasculitis (hepatitis C +ve)	1	36 M	Yes	–	–	–	–	–
Cryoglobulinaemic vasculitis (hepatitis C +ve)	1	43 M	Yes	–	–	–	–	–

was 2046 (1334–3060). The CD64 expression in patients with inflammatory autoimmune diseases differed significantly from those with systemic infection ($p < 0.0001$). The CD64 expression in the vasculitis group differed significantly from those with non-vasculitic inflammatory autoimmune diseases ($p < 0.001$), but did not differ significantly from patients with infection ($p = \text{NS}$). The CD64 expression in the control group differed significantly from all other groups by the Mann-Whitney U test ($p < 0.01$). When a CD64 level of 2000 was used, the sensitivity of the assay in differentiating between infection and non-vasculitic inflammation was 85% with a specificity of 91%.

Impact of immunosuppressive drug treatment

Among the patients with active inflammatory disease, 25/44 (57%) were taking immunosuppressive drugs, including corticosteroids and cytotoxic agents. The median (IQR) CD64 expression in patients that were receiving such drugs was 935 (545–1527) versus 907 (601–1319) in those who were not ($p = \text{NS}$). There was a tendency for reduced CD64 expression in patients who were taking a disease modifying drug, but the difference did not reach statistical significance (data not shown).

Effect of Gram reactivity on CD64 expression

To examine any differences in bacterial components reacting with specific receptors, which activate intracellular processes that may lead to up regulation of CD64, we compared patients

with Gram negative ($n = 6$) and Gram positive ($n = 21$) infections. The median (IQR) CD64 expression in patients with Gram positive infections was 3495 (2213–5344) compared with 6842 (3094–11673) in those with Gram negative infection ($p = \text{NS}$). Although the differences were not significant (fig 1B), there was a tendency for Gram negative infection to produce higher levels of CD64 expression. All these patients had been treated with antibiotics; therefore these differences in expression were not simply due to treatment.

Relationship of CD64 with acute phase response

ESR was measured in 25 of the patients with inflammatory autoimmune diseases and three with systemic infection. In patients with active inflammatory disease (mainly RA), there was no correlation between ESR and CD64 expression. In the three patients with systemic infection, the ESR and CD64 values were 99 and 4709, 90 and 5354, and 42 and 2045, respectively.

DISCUSSION

We report a new laboratory test to differentiate between infection and active inflammatory diseases—namely, measurement of neutrophil CD64 expression by flow cytometry. This may be a useful tool in those patients with autoimmune diseases who present with non-specific symptoms suggesting either flare of the underlying disease or systemic infection. It is a simple test with a short turn-around

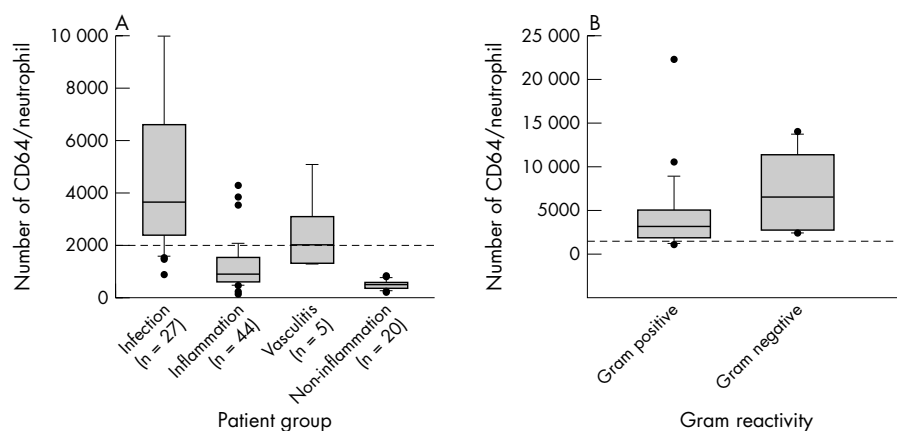


Figure 1 (A) A box plot of the CD64 results for four patient groups: active inflammatory autoimmune disease, infection, non-inflammatory controls, and vasculitis. The dashed line shows our laboratory cut off point for abnormal at 2000 CD64 molecules per neutrophil. The line in the box indicates the median value. The box shows the 25th–75th centiles, while the whiskers indicate the 10th and 90th centiles. Outliers are shown as dots. The control group is significantly different from all the others by the Mann-Whitney U test ($p < 0.01$). The group with inflammatory autoimmune diseases also differs significantly from the infection group ($p < 0.0001$). The vasculitis group has too few numbers to calculate 10th and 90th centiles, and does not differ significantly from the infection group. (B) A box plot of the neutrophil CD64 expression for patients with systemic infection by either Gram positive or Gram negative organisms. The groups are not significantly different.

time (1–2 hours) and has a competitive cost comparable with CRP measurement (about \$100 per assay in our laboratory). This test could be performed at any laboratory with flow cytometry facilities and does not need other special equipment or expertise. However, each laboratory would need to establish its own cut off values. These characteristics make it an attractive test to incorporate into daily clinical practice.

Our study, which is, as far as we know, the first to compare the operating characteristics of the CD64 assay between patients with autoimmune diseases and patients with culture-proven infections, showed that high CD64 expression is a useful diagnostic indicator of infection. With receiver operator curve evaluation, a level of CD64 >2000 was found to be both sensitive and specific for systemic infection. Patients with non-inflammatory, non-infectious conditions such as osteoarthritis and fibromyalgia consistently expressed even lower levels of neutrophil CD64 than those with infection. Because up regulation of CD64 occurs within 4–6 hours of acute infection,⁴ this assay can be used early in the evaluation of these patients and will permit more timely institution of appropriate treatment.

Our study showed that most patients with active autoimmune inflammatory diseases had low levels of CD64. Use of corticosteroids, DMARDs, and cytotoxic treatment did not have a significant impact on the results of our study. Three out of five patients with systemic vasculitis had high levels of CD64. The reasons for this are unknown, but we suggest that high levels of CD64 expression in some patients with acute flares of inflammatory diseases are mediated by different genes than those activated in patients with infection. Harper and Savage have reviewed the evidence for neutrophil priming in vasculitis.⁵ Neutrophil priming occurs early in the disease in the absence of immune complex deposition and may be due to proinflammatory cytokines. Future studies focusing on subjects with various forms of vasculitis are planned.

Although Gram negative infections seem to up regulate CD64 to a greater degree than Gram positive infections, the difference was not significant. However, there was a trend towards higher CD64 expression in Gram negative infections. This may be due to different bacterial products activating neutrophils through different pathways. In addition, these patients had started antibiotic treatment, which might also have affected the level of CD64 expression.

CD64 expression in autoimmune diseases has been measured by flow cytometry in several published studies. Goulding *et al* studied expression of Fcγ RI (CD64), Fcγ RII (CD32), and Fcγ RIII (CD16) on neutrophils purified from patient peripheral blood using monoclonal antibodies and flow cytometry.⁶ They found that CD64 expression on the peripheral blood neutrophils was within normal limits in patients with active RA. However, Quayle *et al* found increased CD64 expression on neutrophils in the synovial fluid of patients with active RA.⁷ The authors concluded that the difference in synovial neutrophil activation may be either due to intra-articular cytokines or to other factors—for example, immune complexes. Two other studies confirmed these results in their patients with RA using similar techniques.^{8,9}

Szucs *et al* reported that CD64 expression on the peripheral blood neutrophils in patients with SLE was not up regulated, although the activity of SLE was not mentioned in this study.¹⁰ They did find an inverse correlation between expression of Fcγ RII (CD32) and concentration of immune complexes in the patient's serum, but there was no correlation mentioned with Fcγ RI (CD64). Although these patients were receiving immunosuppressive treatment, the specific effects of corticosteroid, antiproliferative, or non-steroidal anti-inflammatory treatment have not been well studied.

In patients with infections, Davis *et al* demonstrated up regulation of CD64 expression in 97% of patients with culture proven infections.⁴ They suggested that for routine laboratory testing, blood specimens could be stored at room temperature for up to 24 hours, without any impact on the precision of the results (coefficient of variation 4–8%). This study did not include patients with other diagnoses and therefore the specificity of CD64 expression in this study was not determined.

Our study does not allow conclusions to be drawn about the relative usefulness of CD64 compared with standard laboratory markers such as ESR and CRP as these data were not collected for all patients. However, in those patients with systemic infection for whom the data were available, we found that ESR was raised. This is consistent with past experience suggesting that acute phase reactants are generally raised in both infections and states of active inflammatory disease.¹¹

In conclusion, our study shows that the level of CD64 expression has a good sensitivity and specificity in differentiating infection from active inflammation. These preliminary results make CD64 an attractive candidate to distinguish patients who have an acute flare of their autoimmune disease from those who have systemic bacterial infections. This assay could facilitate early and accurate diagnosis and greatly aid timely institution of appropriate treatment. Future studies will need to confirm our results and compare this assay with other new assays for diagnosing infection to determine whether CD64 measurement becomes part of standard laboratory testing.

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