*Section A -Research paper*

# CD64 as a Sepsis Biomarker in Neonates: Review Article

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**Abstract:**

Sepsis is a life-threatening organ dysfunction with increased incidence of morbidity and mortality. Early diagnosis and prompt therapeutic intervention is the cornerstone of sepsis care. Biomarkers play an important role in sepsis having both diagnostic and prognostic implications. Neutrophil CD64 (nCD64) is a useful candidate biomarker for sepsis. Neutrophil CD64 also known as Fc receptor 1 (FcR1), is a high-affinity receptor present on neutrophils for Fc part of immunoglobulin-G (IgG) heavy chain. Its expression gets strongly upregulated in response to proinflammatory cytokines of infection within 4–6 hours. Neutrophil CD64 integrates function involving both innate and adaptive immune responses. The aim of this review is to present literature about nCD64 as a diagnostic and prognostic marker in patients with sepsis/septic shock.

**Keywords: Sepsis, CD64**

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## Introduction:

Human CD64 is a transmembrane glycoprotein (72-kDa) that, with the Fc γ RII (CD32), and Fc γ RIII (CD16) receptors, comprises the large immunoglobulin (Ig) superfamily (**1**).

It binds monomeric IgG (for IgG1, IgG3 and IgG4) with high affinity. This is particularly significant for the development of therapeutics for antibody-mediated autoimmune diseases. In mouse, the high affinity receptor (mCD64) binds monomeric mouse IgG2a (**2**).

When compared with other human Fc γ receptors, the affinity of human CD64 for monomeric IgG is 10–100 times higher than for the low-affinity Fc γ RII or Fc γ RIII family of receptors which interact poorly with monomeric IgG with binding affinity in the micro-molar range (**3**).

The high affinity of CD64 towards IgG demonstrates it pivotal role in initiating and activating cellular effector response even at low IgG concentrations. **(4).**

Unlike CD64, other Fc γ receptors only internalize IgG complexes surrounding multivalent antigens. To better study the role and therapeutic potential of CD64, transgenic mice expressing human CD64 have been generated to enable the direct testing of human CD64-specific therapeutics in in vivo animal models (**5**).

## CD64 Structure

To enhance the understanding of the functions and conformational changes upon interaction with IgG, the crystal structure of the extracellular domain of human CD64 has been determined to 2.65 Å resolution by molecular replacement (**6**).

Recently the crystal structure of human CD64 in complex with human Fc at 1.80 Å resolution has been resolved (**7**).

Structurally, α chains are reported to associate on the cell surface and consist of three extracellular Ig-like domains (D1, D2 and D3) which presents a V-like configuration, a transmembrane region, and a cytoplasmic domain (Figure 1). Notably, three genes (FC γ RI A–C) have been identified for CD64 and are located on chromosome 1. One encoding the transmembrane region and two encoding the three Ig-like domains **(4).**

The hinge angles between the domains give CD64 a sea horse-appearance. A 35° and 120° hinge angle is reportedly configured between D1 and D2 and D2 and D3 domains respectively. These hinge angles are in comparison quite different especially when compared to the D1 and D2 domains of the human low affinity Fc γ receptors which present hinge angles ranging from 55° to 50° (**6**).

The high binding affinity of CD64 for IgG has also been associated with its unique hydrophobic pocket which perfectly accommodates Leu235 of human Fc at its surface. This precise conformational arrangement has been demonstrated by Kiyoshi et al., to be absent in other Fc γ receptors (**7**).

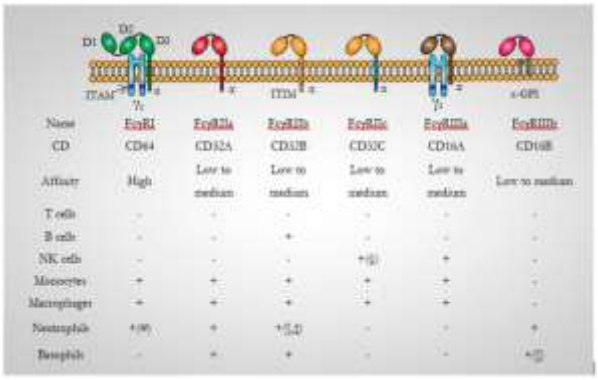


Figure 1 Diagrammatic representation of the human Fc receptors. There are five activating FcγRs: FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa, FcγRIIIb, and one inhibitory Fc receptor; FcγRIIb. They all consist of an immunoglobulin-binding polypeptide chain with two Ig-like extracellular domains with the exception of FcγRI (CD64) which has three. An activating signalling cascade is mostly generated by the cross-linking of activating FcγRs by immune complexes which results in the phosphorylation of ITAMs on FcγRIIa and FcγRIIc. The FcR-γchain common to the Fc receptors is the only inhibitory FcγR. FcγRIIb also binds immune-complexed IgG and contain an ITIM in its cytoplasmic domain. ITAM: immunoreceptor tyrosine-based activation motif; $\gamma_2$: dimer of FcRγ subunits; ITIM: immunoreceptor tyrosine-based inhibitory motif; GPI: glycosyl-phosphatidylinositol; NK cells: Natural Killer cells; +: indicates expression; (-): No expression; (#): only on activated Neutrophils; (§): Low; (‡): conflicting reports: (ψ): expressed only in 30% of humans (**3**).

## CD64 Signalling

CD64 plays a central role in macrophage antibody-dependent cellular cytotoxicity and clearance of immune complexes (**8**).

It has been shown that the internalization of immune complexes by CD64 is a two-step process. (i) The binding of monomeric ligand results in rapid internalization of the receptor to an endosomal compartment followed by rapid recycling to the cell surface. The ligand-

triggered dissociation of CD64 from the cytoskeletal component; actin-binding protein (non-muscle filamin; ABP-280) is believed to provide a novel mechanism for regulating this receptor function. Unoccupied CD64 does not enter this internalization recycling pathway, showing that monomeric IgG binding itself is able and sufficient to initiate CD64 internalization. (ii) Cross-linking the occupied receptor causes retention of the ligand within the cell and subsequent degradation of the immune complexes, presumably in a lysosomal compartment. Intracellular accumulation of CD64-IgG occurs rapidly following cross-linking at a rate of 20–30% per min and essentially completes by 5 min **(4).**

**CD64 Expression**

CD64 is constitutively expressed on monocytes and macrophages and can also be induced on neutrophils with IFN-γ and G-CSF.It is also expressed along with FcγRII on the myeloid derived cell lines HL-60, THP-1, and U937. Treatment with IFN-γ has been shown to up-regulate CD64 expression in U937 cells by 4–5-fold to a level of 60,000 receptors per cell. Cytokine stimulation induces rapid clustering of CD64 on the cell membrane to facilitate rapid binding and internalization of immune complexes, including the de novo protein expression of new CD64 molecules to facilitate complex binding (**9**).

This in essence contributes to an inflammatory response by triggering release of TNF-α and IL-6, superoxide production, antigen presentation to T cells or lysis of antibody coated cells**(4).**

**CD64-Specific Antibodies**

The development of antibodies against CD64 has allowed for the generation and evaluation of targeted therapies as exemplified with 197 which can specifically block phagocytosis and down-modulate CD64 expression on monocytes. Monoclonal antibody (mAb) 197 recognizes and binds two distinct epitopes on CD64. The first interaction is driven through its Fc domain to the Fc ligand-binding site on CD64 and the other through it Fab domain to an epitope outside the Fc ligand-binding site **(4).**

Clinically, the use of mAb 197 in a patient with chronic immune thrombocytopenia purpura (cITP) resulted in clinical improvement as mAb 197 infusion prevented FcγR mediated destruction of IgG-coated platelets (**10**).

As the murine origin of mAb 197 could potentially induce an immunogenic response, another murine derived CD64 specific monoclonal antibody (M22) that recognizes an epitope distinct from the natural ligand-binding site of IgG was developed by Guyre et al. and later humanized by **Graziano et al., (11**). Humanization of the antibody was carried out by grafting its complementary determining regions (CDRs) onto human IgG1 constant domains, resulting in a full-length antibody (H22) which maintained binding specificity and high affinity for CD64.

Using this antibody format, Heijnen and colleagues were able to develop early examples of antibody conjugates targeting human CD64 (**12**).

About a decade later, a single chain variable region fragment (scFv) version H22 with a lower molecular weight but same target specificity outside the normal Fc binding site was developed by De Kruif et al. (**13**).

This H22(scFv) has since been widely explored for targeted therapy as it does allow for efficient and rapid delivery of effector molecules to CD64 expressing cells

in vitro and in vivo irrespective of IgG saturation on the Fc domain of CD64. (**14**).

**CD64 as a sepsis biomarker**

Expression of the CD64 antigen on neutrophils has been under investigation for some years as a biomarker of infection and sepsis. It has several characteristics that make is well suited for clinical application: on resting neutrophils CD64 expression is low and after activation it is significantly upregulated within few hours. Once the activation stimulus disappears, CD64 expression returns to its basal level in few days. Moreover, CD64 is relatively stable after blood collection and the assay is straightforward and requires only small sample volume. Last but not least, CD64 expression represents a physiological process which plays a key role in the innate immune response: neutrophils acting as phagocytes. (**15)**.

The CD64 antigen is the high affinity receptor for the Fcγ part of the IgG heavy chain and can bind monomeric IgG1 and IgG3 as well as aggregated IgG. Phagocytosis of bacteria and other microorganisms is mediated by this FcγRI receptor. In contrast to monocytes where CD64 antigen is constitutively expressed, resting neutrophils have very low levels of CD64 antigen on their membrane, approximately 1000 molecules per cell (***16***).

CD64 expression is increased upon activation of neutrophils by pro-inflammatory cytokines within 4-6 hours and can reach more than 10-fold higher levels than in resting conditions, allowing good discrimination between resting and activated neutrophils (***17***).

**Diagnostic performance in neonates and children with sepsis**

Neonatal sepsis is different from adult sepsis in several respects and therefore a separate discussion is warranted. The clinical signs and symptoms often are more subtle and non-specific than in adults, a clinical score system has no clear advantage and traditional laboratory tests have poor diagnostic performance (***18***).

Therefore, neutrophil CD64 would be a candidate sepsis biomarker also in neonates and children. However, healthy neonates reportedly have higher CD64 expression on their neutrophils than adults (***19***), which might influence the diagnostic power of the test.

Layseca-Espinosa *et al*. showed that neutrophil CD64 was a highly specific indicator of neonatal sepsis, albeit with low sensitivity (***20***).

These authors used the percentage of CD64-positive cells as a criterion; however, it has been shown that CD64 fluorescence intensity, either as mean fluorescence index (MFI) or as an index relative to standard beads, is a better discriminator than percentage CD64-positive neutrophils (28,29). Ng and colleagues demonstrated the utility of CD64 as a biomarker of late-onset infection in preterm, very low birthweight infants: both at the first clinical suspicion of sepsis as well as 24 hours later, the diagnostic performance of neutrophil CD64 was much better than all other markers investigated. The combination of neutrophil CD64 expression with either serum C-reactive protein or IL-6 even enhanced the sensitivity to 100%, meaning that these markers together can be used to exclude infection (***21***).

Some years later the same group published a very similar study, but now on early-onset infection in term infants; they enrolled the largest number of patients reported up to now. This study confirmed the very high sensitivity of neutrophil CD64, but in contrast to their previous study

addition of CRP improved the diagnostic power of CD64 only marginally (***22***).

A smaller study in critically ill neonates and infants by Groselj-Grenc used the standardized CD64 index for the first time and demonstrated its superiority over all other infection markers (***23***).

In a follow-up study the same authors presented evidence that neutrophil CD64 was the best individual marker for bacterial sepsis in children, whereas the test performed somewhat less in neonates (**24**).

However, both patient groups were of limited size and might actually not warrant this conclusion. Bhandari *et al*. conducted the third-largest study in neonates with suspected sepsis and also these authors found that neutrophil CD64 index had the best diagnostic performance for culture-positive sepsis compared with all traditional hematologic assays (**24**).

In the study by Dilli *et al*., performed on over 100 patients in a neonatal ICU, the neutrophil CD64 index again was found to be the test with the highest sensitivity, with no difference between neutrophil CD64 index and MFI (***25***).

Combination of CD64 index with CRP and IL-6 increased the sensitivity and the negative predictive value to 100%, allowing reliable exclusion of infection. Slightly lower diagnostic performance was reported by Zeitoun and coworkers, also when the CD64 index was combined with serum IL-10 (***26***).

Recently, Lam *et al*. demonstrated in a large-scale study that neutrophil CD64 was useful in neonates suspected of abdominal infection and sepsis, in particular in combination with conventional X-ray imaging for excluding an infectious cause of disease (***27***).

Overall, these nine studies in neonates and children give a reasonably consistent impression of the diagnostic performance of neutrophil CD64 in sepsis and severe infection. The composite sensitivity (83.4%; 95% CI = 70.0-91.5%) and specificity (86.2%; 95% CI = 75.2-92.7%) show that CD64 represents one of the most sensitive biomarkers of pediatric sepsis currently available for clinical use. (**15)**.

Until recently it seemed that neutrophil CD64 had higher sensitivity and specificity in adults with sepsis than in neonates and children. However, the most recent paper (***28***) changed the situation. The summary ROC curves of adults and neonates and children do no longer show statistical differences and therefore can reliably be combined. The overall summary ROC curve demonstrates that neutrophil CD64 can detect sepsis with a sensitivity of 85.7% and specificity of 87.4% .The positive likelihood ratio is 6.79 and the negative likelihood ratio 0.16.

**Marker of disease severity**

Neutrophil CD64 expression is not only useful for the diagnosis of systemic infection and sepsis, but several authors have also reported its value as an indicator of sepsis severity: patients with septic shock generally show the highest CD64 values, higher than in severe sepsis and much higher than in sepsis and SIRS (***29***). The latter authors also demonstrated that patients receiving adequate antibiotic therapy had a quick decrease of their CD64 index, in parallel with an improvement in their clinical condition (***30***). It is not only in sepsis that CD64 is a good indicator of disease severity; the same finding has been reported in local infections, too (***31***).

**Prognostic value of CD64:**

According to several authors, neutrophil CD64 contains also prognostic information

as to survival. However, the available data are highly contradictory. Several publications reported that survival could be predicted by a low CD64 expression, whereas others indicated that high CD64 would be an indicator of favorable prognosis (***32***). Most likely the small patient groups and the design of the studies explain these conflicting findings.

**Diagnostic performance in other infective and inflammatory diseases**

Apart from systemic infection and sepsis, there is a wide variety of other disease states in which the diagnostic utility of neutrophil CD64 has been suggested. One of the most useful applications is probably the ability of neutrophil CD64 to distinguish between bacterial infection and acute flares in rheumatoid arthritis and other autoimmune disorders (***33***).

Further, CD64 has been found useful as a marker of postoperative infection in patients undergoing musculoskeletal, or vascular surgery (**34**).

CD64 index was a valuable tool in inflammatory bowel disease, too (***35***).

A recent review indicated that neutrophil CD64 is not only increased in bacterial infection, but also in viral infection, depending on the type of virus (***19***).

Finally, CD64 has successfully been used in familial Mediterranean fever (***36***) and as a rejection marker in transplant patients (***37***).

**Analytical aspects of CD64 assay**

Flow cytometry is the method of choice for determining neutrophil CD64. In the early years authors used density gradient centrifugation for isolating neutrophils followed by indirect immunofluorescence for detecting CD64 expression. Nowadays, virtually all investigators use direct immunofluorescence in whole blood using a lyse-no-wash method (***38***).

The intra-laboratory imprecision seems to be low with coefficient of variation 3-6% and < 12%, respectively (***39***).

Data on the inter-laboratory variation have not been reported. The assay can conveniently be performed in a small volume of EDTA-anticoagulated blood and the CD64 antigen is stable for 36 or 72 hours at room temperature (***35***).

Like with most flow cytometric assays, the determination of neutrophil CD64 lacks standardization.Various methods are in use for expressing CD64 on neutrophils: percentage of CD64-positive cells, mean or median fluorescence intensity (MFI) and as an index relative to standardized beads. The latter method is available as a commercial kit (Trillium Diagnostics, Brewer, ME, USA). It comes in two formats: one for use on flow cytometers (***40***) and one for the CELL-DYN Sapphire hematology analyzer, which allows practical full automation and does not require flow cytometry expertise (***32***).

Both kit methods use FITC-labeled CD64 and PE-labeled CD163 monoclonal antibodies and contain calibrated fluorescent beads for standardization. Lymphocytes and monocytes are used as negative and positive internal control, respectively. The assay requires 50 µL of blood and can be completed within 1 hour on a standard flow cytometer and within 20 minutes on the Sapphire analyzer. Thanks to the calibrated beads, the kit method has the potential of inter-laboratory comparability of results, although data have not yet been published. (**15)**.

**Critical assessment of the literature**

The general picture that arises when reading the available literature is that

neutrophil CD64 seems to be a highly sensitive and reasonably specific biomarker for sepsis and other infections. However, there are several factors that should nuance these positive reports. The two most important have already been mentioned above: nearly all studies included relatively small patient numbers and the methodological quality of reported studies is mediocre (**24**).

In addition, there is an enormous variation in criteria for selecting patients, differences in the definition of sepsis as well as poorly standardized analytical methodology. Sometimes, healthy subjects are used as a control group, which does not represent the conditions in which a sepsis biomarker is clinically used and moreover, this practice can easily overestimate the power of a diagnostic test. (**15)**.

This all does not mean that we should disregard these studies. They present a highly promising and strong indication, but not sufficiently solid scientific evidence that neutrophil CD64 expression is a biomarker for sepsis that can be recommended for routine use. This needs to be confirmed by large-scale, prospective, multicenter, preferably case-control studies of good methodological quality. The definition of sepsis should be in accordance with widely accepted criteria (***41***), blood cultures should be performed as the reference standard, patients in whom sepsis was clinically suspected but could not be objectively proven should be used rather than healthy controls for calculating sensitivity and specificity for sepsis patients, the analytical method for CD64 should be standardized and calibrated using beads. In addition, disease severity should be scored using well accepted systems and the clinical follow-up should be long enough to document sepsis-related mortality. Furthermore it would be interesting to study neutrophil CD64 as a tool for monitoring therapy, like is currently being done for procalcitonin.

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