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(54) Title: USE OF CELLULAR BIOMARKERS EXPRESSION TO DIAGNOSE SEPSIS AMONG INTENSIVE CARE PATIENTS

(57) Abstract: This invention relates to the area of diagnosis. In particular, it relates to diagnosis and/or risk assessment of sepsis. The present invention proposes the analysis of the surface expression of i) a particular CD16 epitope, ii) surface or intracellular expression of the Toll-Like Receptors 2 and/or 4, iii) the CD24 polypeptide, and/or iv) the CD64 polypeptideas diagnosis tools for early discrimination between patients with sterile systemic inflammatory response syndrome (SIRS) from those who develop sepsis in Intensive Care Unit (ICU).

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USE OF CELLULAR BIOMARKERS EXPRESSION TO DIAGNOSE SEPSIS AMONG INTENSIVE CARE PATIENTS

PRIOR ART DESCRIPTION

Sepsis is the leading cause of death in critically ill patients, the second leading cause of death among patients in non-coronary intensive care units (ICUs), and the tenth leading cause of death overall in the United States. Overall mortality rates for sepsis are 18%. In-hospital deaths related to sepsis were 120,491 (43.9 per 100,000 population) in 2000. The incidence of sepsis showed a 3 fold increase in US hospitals over the 1980 - 2000 period. Depending upon the studies and the countries, the estimated incidence of sepsis is between 86 to 367 / 100,000 population and that of severe sepsis between 51 and 104 / 100,000 population (Brun-Buisson, C. 2007). Severe infections represent approximately 40% of illnesses of patients admitted to ICU (Vincent, J. L., et al. 2006). Mortality of severe sepsis varies between 18 to 71% depending upon the number of organ failures. The primary infections leading to sepsis are those of the lungs, abdomen, bloodstream, and genito-urinary system (Vincent, J. L., et al. 2006). The mean cost of a patient in US is estimated to be 31,289 US $.

Sepsis substantially reduces the quality of life of those who survive: only 56% of patients surviving sepsis are discharged home; 32% are discharged to other health care facilities (i.e., rehabilitation centers or other long-term care facilities), accruing additional costs of care.

Early and accurate diagnosis of sepsis is crucial for at least two reasons: on the one hand, prompt starting of antimicrobial therapy improves outcomes (Rivers, E. et al., 2001; Kumar, A. et al., 2006) and therefore results in decreased morbidity, mortality and cost of care. On the other hand, the unnecessary exposure to antibiotics for patients which do not undergo sepsis would create an environment for emerging bacterial resistance and the potential for poor outcomes.

Nowadays, sepsis is diagnosed either by clinical criteria or by culture of microorganisms from the blood of patients suspected of having sepsis, plus the presence of features of systemic inflammation. Culturing some microorganisms is tedious and time-consuming, and may provide a high rate of false negatives. To date, bloodstream infection is diagnosed by identification of microorganisms in blood specimens from a patient suspected of having sepsis after 24 to 72 hours of laboratory culture. Moreover, there are several different types of microorganisms that can cause sepsis: gram-positive bacteria account for 52% of cases of sepsis, gram-negative bacteria account for 38%, polymicrobial infections for 5%, and anaerobes for 1%. Altogether, it is known that sepsis is a very heterogeneous disease, which can be caused by many infections with microorganisms at many different sites. This heterogeneity increases the difficulty in devising a reliable and diagnosis test.

This explains why, although it is known for a while that improved diagnosis and prognostic tools would be key elements for the management of these patients (Dellinger, R. P., et al. 2008), and although specific markers are available for many other diseases (e.g. troponin (Katus H.A. et al, 1991) for myocardial infarction, or serum lipase and amylase for acute pancreatitis (Yadav D. et al, 2002), for sepsis no reliable marker exists.

Sepsis is a bacterially-induced syndrome, involving two or more of the following feature of systemic inflammation : fever or hypothermia, leukocytosis or leukopenia, tachycardia, and tachypnea, or a supranormal minute ventilation (Annane, D. et al., 2005). Sepsis therefore shares many clinical signs with other non-infectious causes of systemic inflammation found in critically ill patients, including trauma, severe surgery, post-cardiac arrest syndrome, cardiac surgery (particularly when requiring extracorporeal circulation), collectively termed as SIRS. It is thus easy to confuse sepsis and SIRS symptoms.

In fact, some biomarkers have been proposed for diagnosing sepsis. However, none have been reported to reliably differentiate sepsis from other non-infectious causes of systemic inflammatory response syndrome (SIRS) in ICU patients (Tang BM et al, 2007; Pierrakos C et al, 2010).

And, in the absence of definitive diagnosis, empiric treatments are often administered, and more than half of severe infections in ICU cannot be documented (Munford, R. S. 2006).

Consequently, nosocomial infections remain a major threat in intensive care units (ICU): 8% of patients who remain in an ICU for longer than 24 hours will develop sepsis. In

particular, mortality due to severe sepsis (when acute organ dysfunction occurs) or septic shock (when severe sepsis is associated with refractory hypotension) is still high.

There is therefore an urgent need for reliable and quick diagnosis tests for identifying patients suffering from sepsis, with relatively few false negatives, high sensitivity and specificity, so as to avoid the delays in the identification, transfer and management of the patients, for example the patients within the first 6 hours after ICU admission, which have been associated with higher mortality rates (Lundberg J.S., et al. 1998), and also to avoid unnecessary exposure to antibiotics for patients which do not undergo sepsis.The test will be carried out on patients presenting a Systemic Inflammatory Response Syndrome (SIRS) according to the current guidelines for the discrimination between SIRS patients and sepsis patients.

FIGURE LEGENDS

Figure 1 shows CD14++/ CD16+ monocytes subset detection by flow cytometry in healthy donor peripheral whole blood using different CD16 monoclonal antibodies. Staining pattern of the monoclonal antibodies (A) anti-CD16 VEP13, (B) anti-CD16 B73.1, (C) anti-CD16 3G8 and (D) anti-CD16 LNK16, are presented.

Figure 2 represents a flow cytometry analysis of the CD16 expression on monocytes from SIRS and SEPSIS patient. Staining pattern of monoclonal antibodies (A) anti-CD16 VEP13 and (B) anti-CD16 3G8 are shown.

Figure 3 represents a flow cytometry analysis of the CD16 expression on monocytes from SIRS (A, two different subjects) and SEPSIS (B, two different subjects). Staining pattern of the mAb anti-CD16 VEP13 (A and B). Monocytes were gated according the SSC and CD14 expression and then 2 regions were drawn in order to analyze $CD16^{low}$ and $CD16^{high}$ subset.

Figure 4 (A) represents the percentage of CD16 positive cells in 9 SIRS and 7 septic patients at suspicion of infection in ICU patients, among the VEP13 ^{HIGH} and VEP13 ^{LOW} subpopulations (day 0). Horizontal bars represent the median values. **(B)** represents the mean fluorescent intensity (MFI) of CD16 positive cells in 9 SIRS and 7 septic patients at

suspicion of infection in ICU (day 0) among the VEP13 positive cells. Horizontal bars represent the median values.

Figure 5 represents a flow cytometry analysis of CD16 expression on monocytes from a healthy donor. Staining pattern of monoclonal antibodies (A) anti-CD16 VEP13 and (B) anti-CD16 3G8 are presented.

Figure 6 represents a flow cytometry analysis of CD16 expression on monocytes from an ICU patient. Staining pattern of monoclonal antibodies (A) anti-CD16 VEP13 and (B) anti-CD16 3G8 are presented.

Figure 7: (A) Representative chart of the NK cells analysis in whole blood by flow cytometry, indicating the approximately cell counting per mL of $CD56^{bright}$ cells (upper square) and the $CD56^{dim}$ cells (lower square). **(B)** Cell counts for lymphocytes and NK cell subsets in healthy volunteers, SIRS and sepsis patients. **(C)** Percent of NK subsets among lymphocytes in healthy volunteers, SIRS and sepsis patients. Data are shown as median and interquartile range. $***P < 0{,}001$.

Figure 8: Analysis of the expression of TLR2 by flow cytometry in NK cells $CD56^{bright}$ and $CD56^{dim}$ subsets. **(A)** Representative flow cytometry histogram of surface expression of TLR2 on $CD56^{bright}$ and $CD56^{dim}$ NK cells subsets in healthy volunteers, SIRS and sepsis patients. **(B)** Surface TLR2 expression; data are shown as median and interquartile range in all three studied groups. **(C)** Representative flow cytometry histogram of intracellular expression of TLR2 on the same samples. **(D)** Intracellular TLR2 expression; data are shown as median and interquartile range in all three studied groups.

$*P < 0{,}05$; $**P < 0{,}01$; $***P < 0{,}001$. Significance between SIRS and sepsis patients: $^{\#}P < 0{,}05$; $^{\#\#}P < 0{,}01$; $^{\#\#\#}P < 0{,}001$. MFI: Mean fluorescence intensity.

Figure 9: Analysis of the expression of TLR4 by flow cytometry in NK cells $CD56^{bright}$ and $CD56^{dim}$ subsets. **(A)** Representative flow cytometry histogram of surface expression of TLR4 on $CD56^{bright}$ and $CD56^{dim}$ NK cells subsets in healthy volunteers, SIRS and sepsis patients. **(B)** Surface TLR4 expression; data are shown as median and interquartile range in all three studied groups. **(C)** Representative flow cytometry histogram of intracellular expression of TLR4 on the same samples. **(D)** Intracellular TLR4 expression; data are shown as median and interquartile range in all three studied groups.

$*P < 0.05$; $**P < 0{,}1$; $***P < 0{,}001$: comparison versus healthy donor.

$^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$: comparison of basal values versus overnight IL-15+IL-18 primed cells.

Figure 10: Expression of TLR9 in $CD56^{bright}$ and $CD56^{dim}$ NK cells subsets **(A)** Representative flow cytometry histogram of intracellular expression of TLR9 for healthy donors and SIRS and sepsis patients. **(B)** Median and interquartile range for each group. We found no significant difference between groups. The results are expressed either as percent positive NK cells or as mean fluorescence intensity (MFI)

Figure 11: Quantification of VEP13 expression (mean fluorescence intensity [MFI]) on monocytes from sepsis patients at day 0 (n = 21), at day 1 (n=33) and day 2 (n=24) and SIRS patients at day 0 (n = 12), at day 1 (n=15) and day 2 (n=6). Horizontal bars represent the median values.

Figure 12: Early and persistent down regulation of CD24 expression on neutrophils from sepsis patients. Quantification of CD24 expression (mean fluorescence intensity [MFI]) on neutrophils from healthy donors (HD) (n=25), sepsis patients at day 0 (n = 15), at day 1 (n=23) and day 2 (n=16) and SIRS patients at day 0 (n = 7), at day 1 (n=16) and day 2 (n=8). Horizontal bars represent the median values. HD vs sepsis D0 : $P < 0.01$; HD vs sepsis D1 : $P < 00.5$; HD vs sepsis D2 : $p < 0.01$; sepsis D0 vs SIRS D0 : ns ; sepsis D0 versus SIRS D1 : $p < 0.05$.

Figure 13: Analysis of the surface expression of TLR2 and TLR4 by flow cytometry in NK cells $CD56^{bright}$ and $CD56^{dim}$ subsets. **(A)** Surface TLR2 expression; data are shown as median and interquartile range in all three studied groups. **(B)** Surface TLR4 expression; data are shown as median and interquartile range in all three studied groups.

Figure 14: Analysis of the intracellular expression of TLR2 and TLR4 by flow cytometry in NK cells $CD56^{bright}$ and $CD56^{dim}$ subsets. **(A)** Intracellular TLR2 expression; data are shown as median and interquartile range in all three studied groups. **(B)** Intracellular TLR4 expression; data are shown as median and interquartile range in all three studied groups.

Figure 15: A, Neutrophil CD64 expression was measured by flow cytometry. Patients were analyzed during the first 48 h following the onset of sepsis (day 0) in **21** SIRS and **40** septic patients. Quantification of CD64 expression (mean fluorescence intensity [MFI]) from healthy controls (n = 13), sepsis patients at day 0 (n = 17), at day 1 (n=37) and day 2

(n=26) and SIRS patients at day 0 (n = 14), at day 1 (n=19) and day 2 (n=10). B, percentage of CD64 positive neutrophils in the two groups of patients compared with healthy donors is shown. Horizontal bars represent the median values. *$P < 0.05$; **$P <$ 0,1; ***$P <$ 0,001: comparison versus healthy donor (HD).

Figure 16: CD24 induces death in human neutrophils in absence of phosphatidyl serine redistribution (representative plot). A) Purified blood neutrophils were crosslinked using anti-CD24 antibody then were stained with annexin V and Propidium Iodide, a representative experiment shows the increase of cells undergoing death following the ligation in absence of PS redistribution. B) Percentage of propidium iodide positive neutrophils was quantitated (mean of 4 donors).

Figure 17: (A) Preincubation with caspase inhibitor (Z-Vad- fmk) was ineffective while Nec-1 and DPI prevented neutrophil death following CD24 ligation. Results are representative of at least three separate experiments. Mean ± SEM is presented. *, $p <$ 0.05, **(B)** Neutrophils CD24 expression measured by flow cytometry on 20 patients with a diagnosis of sepsis and 19 healthy individuals at the suspicion of sepsis (day 0) and during 48 h. **(C)** LPS or heat-killed bacteria (HKEc: Heat Killed *E. coli*; HKSa, Heat Killed *S. aureus*) don't trigger CD24 upregulation in purified neutrophils. CD24 expression was assessed by flow cytometry. Results are presented as mean ± SEM and are representative of at least three separate experiments. *, $p < 0.05$ (Mann-Whitney U test).

Figure 18: (A) After incubation with sepsis plasma from 3 different patients (S1, S2, S3), neutrophil CD24 expression (MFI) was assessed by flow cytometry. Results are presented as mean ± SEM of three separate experiments from three different healthy donors (A). A representative experiment is shown (B) *, $p < 0.05$ (Mann-Whitney U test).

Figure 19: Cross-linking of CD24 failed to induce cell death in neutrophils from sepsis patients. Whole blood was cultured in the presence of anti- human CD24 and secondary Abs and then stained with annexin V and Propidium Iodide, and the apoptotic neutrophils were evaluated by flow cytometry. A, percentage of PI positive neutrophils were quantitated. A representative experiment is shown in B.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have shown that:

i) the percentage of NK cells expressing high levels of surface Toll-like receptor 4 (TLR4) significantly increases in SIRS patients as compared with sepsis patients,

ii) the intracellular expression level of both Toll-like receptor 2 (TLR2) and TLR4 significantly increases in NK cells of sepsis patients as compared with SIRS patients,

iii) the expression of a specific epitope of CD16 (hereafter called "VEP13 epitope") significantly decreases on monocytes surface from SIRS patients as compared with sepsis patients,

iv) the surface expression of CD64 significantly increases in neutrophils of sepsis patients as compared with SIRS patients, and

v) the surface expression of CD24 is significantly decreased in neutrophils of sepsis patients as compared with healthy and SIRS patients.

All these results allow the discrimination between patients with sepsis from those with systemic inflammation of noninfectious source.

These results have an important implication for physicians, improving the ability to approach the complex clinical course, management, and outcome of ICU patients, at an early stage.

The phenotyping technique herein described can indeed be carried out within 1 hour and may provide valuable information long before blood culture results are provided. Moreover, it is inexpensive and requires minimal volume of individual blood samples (100 μL).

Thus, assaying the expression levels of any of these particular molecules (TLR4, TLR2 on and in NK cells, VEP13/CD16 on monocytes, CD24 on neutrophils, CD64 on neutrophils) appears to be a very helpful diagnosis tool for the early detection of septic complications in critically ill patients.

As used hereafter, the polypeptides TLR4, TLR2,VEP13/CD16, CD24 and/or CD64 will be designated as "**biomarkers of the invention**".

By "**sepsis**", it is herein referred to a potentially deadly medical condition that is characterized by a whole-body inflammatory state (also called a systemic inflammatory response syndrome or SIRS) which is associated with a bacterially-induced infection. The human body develops this inflammatory response because of the presence of microbes in the blood, urine, lungs, skin, or other tissues.

In a preferred embodiment of the invention, the subject is a human individual who presents at least two, or preferably at least three of the following SIRS symptoms:

- A body temperature < 36 °C (97 °F) or > 38 °C (100 °F) (characteristic of hypothermia or fever),
- A heart rate > 90 beats per minute (characteristic of tachycardia),
- A respiratory rate > 20 breaths per minute or, on blood gas, a P_aCO_2 less than 32 mm Hg (4.3 kPa) (characteristic of tachypnea or hypocapnia due to hyperventilation).
- A white blood cell count < 4,000 cells/mm^3 or > 12,000 cells/mm^3 (< 4×10^9 or > 12×10^9 cells/L), or greater than 10% band forms (immature white blood cells) (characteristic of leukopenia, leukocytosis, or bandemia).

In other words, the "**subjects**" for whom the methods of the invention are useful therefore present at least two, or preferably at least three symptoms of SIRS, e.g. fever, hypothermia, leukocytosis, leukopenia, tachycardia, tachypnea, or a supranormal minute ventilation. Such subjects may have been admitted in Intensive Care Unit (ICU) in the last weeks.

"**Diagnosing sepsis**" as used herein refers to the process of identifying or detecting if a patient presenting at least two symptoms of SIRS suffers or not from a pathological sepsis, that is, from a bacterially-induced infection. It also refers to the process of monitoring the progression of this disease, and identifying or detecting cells that are indicative of said disease.

In other terms, the methods of the invention enable to diagnose bacterially-induced infections in patients presenting at least two symptoms of SIRS. They also allow discriminating patients suffering from bacterially-induced sepsis from patients suffering from non-infectious SIRS. In this context, the methods of the invention obviously also allow diagnosing non-infectious SIRS.

The term **"biological sample"** as used herein refers to a sample that may be taken from said patient such as a serum sample, a plasma sample, a blood sample, a lymph sample, or a biopsy. Such a sample must allow for the determination of the expression level of the biomarkers of the invention. Preferably, the biological sample is a blood sample. Indeed, such a blood sample may be obtained by a completely harmless blood collection from the patient and thus allows for a non-invasive diagnosis of sepsis. This blood sample is preferably devoid of the red blood cells by common red cell lysis procedures, and the detection is performed on the remaining blood cells, which are white blood cells (e.g. neutrophils, monocytes, lymphocytes, basophiles, etc.) and platelets. Methods for lysing specifically red blood cells in blood samples are well known in the art, and a number of red blood cell lysis buffers are now manufactured (e.g. by Sigma, BioLegend, BD biosciences, etc.). They for example contain ammonium chloride, potassium bicarbonate and EDTA.

As disclosed herein, the terms "*in vitro*" and "*ex vivo*" are equivalent and refer to studies or experiments that are conducted using biological components (e.g. cells or population of cells) that have been isolated from their usual host organisms (e.g. animals or humans). Such isolated cells can be further purified, cultured or directly analyzed for expression of the biomarkers of the invention. These experiments can be for example reduced to practice in laboratory materials such as tubes, flasks, wells, eppendorfs, etc. They are commonly referred to as "test tube experiments". In contrast, the term *"in vivo"* refers to studies that are conducted on whole living organisms.

The TLR2 and TLR4 receptors as biomarkers of sepsis

The present Inventors have shown that the surface level of the Toll-like receptor 4 is significantly increased on the NK cells from SIRS patients as compared with sepsis patients (see figure 9), whereas the intracellular levels of Toll-like receptor 4 and of Toll-like

receptor 2 are significantly higher in patients suffering from sepsis as compared with SIRS patients (see figures 8 and 9).

These differential expressions allow the discrimination between patients with sepsis from those having a SIRS.

Mammalian Toll-like receptors (TLRs) are a family of ten receptors recognizing pathogen associated molecular patterns (PAMPs) expressed on or in infectious agents and damage associated molecular patterns (DAMPs) released by necrotic cells or injured tissues. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. They are central to effective innate immunity and mediate the production of cytokines necessary for the development of effective immunity. More specifically, they recognize lipidic structures (e.g. lipopolysaccharide and bacterial lipoproteins), proteins (flagellin), and nucleic acids (DNA and double-stranded RNA). The various TLRs exhibit different patterns of expression. These receptors are most abundantly expressed in placenta, and in myelomonocytic subpopulation of the leukocytes.

Toll-like receptor 4 (also known as CD284, ARMD10, and hToll, and hereafter referred to as TLR4) is a Toll-like receptor protein that is encoded in humans by the *TLR4* gene (mRNA NM_138554.3). This receptor is highly expressed by immune cells (e.g. monocytes / macrophages, lymphocytes, dendritic cells, neutrophils, NK cells). It detects lipopolysaccharide (LPS) from Gram-negative bacteria and is thus important in the activation of the innate immune system. It has the sequence SEQ ID NO:4 (NP_612564.1). It is found both at the cell surface and intracellularly (e.g. macrophages) or mainly intracellularly (e.g. epithelial cells, endothelial cells).

In another aspect, the present invention is thus drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting the cells expressing high surface levels of TLR4 in the total population of cells present in said sample,

b) Calculating the percentage of the cells detected in step a) among the said total population, and

c) Comparing the percentage obtained in b) with a reference value,

wherein,

if the percentage obtained in b) is similar to the reference value, the said subject is suffering from sepsis, and

if the percentage obtained in b) is higher than the reference value, the said subject is suffering from SIRS.

The terms "sepsis" and "subject" have been described above.

The step of detecting the (surface or intracellular) expression of TLR4 can be reduced to practice on whole blood cells of the patient blood sample. Preferably, when flow cytometry is used, the antibody staining can be performed on whole blood sample while the detection by flow cytometry can be performed after lysis of the red blood cells and achieved on the remaining blood cells, which are white blood cells (e.g. neutrophils, monocytes, lymphocytes, basophiles, etc.) and platelets.

It is noteworthy that results are more accurate if expression of the TLR4 receptors is assessed only on the NK cells present in the biological sample of said subject, so that step a) of the diagnosis method of the invention is preferably performed on these particular cells.

Thus, in a particular embodiment, the present invention is drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a blood sample of a subject, the method comprising:

a) Detecting the NK cells expressing detectable surface levels of TLR4 in the total population of cells present in said sample,

b) Calculating the percentage of the NK cells detected in step a) among the said total population, and

c) Comparing the percentage obtained in b) with a reference value,

wherein,

if the percentage obtained in b) is similar to the reference value, the said subject is suffering from sepsis, and

if the percentage obtained in b) is higher than the reference value, the said subject is suffering from SIRS.

Natural killer cells (or "NK cells") are a type of cytotoxic lymphocytes that constitute a major component of the innate immune system. NK cells play a major role in the rejection of tumors, lysis of cells infected by viruses and as a source of gamma-interferon during bacterial infection. They kill cells by releasing small cytoplasmic granules of proteins called perforin and granzyme.

NK cells are defined as large granular lymphocytes (LGL) and constitute the third kind of cells differentiated from the common lymphoid progenitor generating B and T lymphocytes. They do not express T-cell antigen receptors (TCR) or Pan T marker CD3 or surface immunoglobulins (Ig) B cell receptors but they usually express the surface markers CD16 (FcγRIII) and CD56 in humans. Up to 80% of human NK cells also express CD8. Preferably, NK cells are identified as not expressing CD3 (i.e. no expression for any of the sequences SEQ ID NO:6 to 9) ($CD3^-$) and express high level of CD56 of SEQ ID NO:10. $CD3^{negative}/CD56^{+++}$ cells are also known as "$CD56^{bright}$ NK cells", and $CD3^{negative}/CD56^{+}$ NK cells are known as "$CD56^{dim}$ NK cells" (Poli et al. Immunology. 2009 Apr;126(4):458).

These cells were named "natural killers" because of the initial notion that they do not require activation in order to kill cells that are missing "self" markers of major histocompatibility complex (MHC) class I.

The presence of the cluster of differentiation CD56 and the absence of CD3 can be detected for example by using antibodies recognizing these proteins. Numerous antibodies against human CD3 and CD56 are commercially available. NK cells are easily identified by specific biomarkers (e.g. CD56 or CD16) combined with morphometric characteristics (e.g. size, shape, granulometry, etc.). For example, when flow cytometry is used, CD3-negative but CD56-expressing cells are easily identified as the NK cell population among the other blood cells.

In a preferred embodiment, the diagnosis method of the invention is reduced to practice on NK cells expressing CD56 but not CD3, more precisely on "$CD56^{bright}$ NK cells" ($CD3^{negative}/CD56^{+++}$ cells) and/or "$CD56^{dim}$ NK cells" ($CD3^{negative}/CD56^{+}$ NK cells). Expression of cell surface CD3 and/or CD56 on NK cells can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry (see examples below).

As mentioned previously, whole blood samples (optionally devoid of red blood cells) are preferably used in the diagnosis method of the invention. Nevertheless, it is also possible to isolate NK cells prior to detecting TLR4 expression. In this case, NK cells can be isolated from blood cells by selecting the cells expressing significant levels of CD56 but not CD3 at their surface ($CD3^{-}/CD56^{positive}$ cells). Several methods for isolating human NK cells are already available to the skilled person and need not to be further detailed here. Commercial kits using immunomagnetic selection can also be used,such as those proposed by Myltenyi Biotech, StemCell Technologies or Invitrogen.

In the context of the present invention, it is meant that a cell "**express CD56**" if CD56 is present at a significant level at its surface (such a cell being also defined as a "$CD56^{+}$ cell"). In particular, a cell express CD56 if the signal associated to surface CD56 staining (e.g. obtained with an antibody anti-CD56 coupled to a fluorescent marker) which is measured for said cell is similar or identical to the signal corresponding to the same staining of at least one cell being known as expressing CD56. In other terms, the ratio between the surface CD56-associated signal measured for said cell and the surface CD56-associated signal measured for at least one cell being known as expressing CD56 is of about 1. Preferably, the surface CD56-associated signal of the target cell is compared to an average surface CD56-associated signal measured on a population of cells being known as expressing CD56, so that the ratio between the surface CD56-associated signal measured for said cell and the average surface CD56-associated signal measured on a population of cells being known as expressing CD56 is of about 1. Cells expressing CD56 at their surface are well known in the art. They are for example neurons, glia cells and muscle cells.

In particular, when the anti-CD56 antibody coupled to a fluorescent marker is used in a flow cytometry analysis assay on a whole blood sample (or on isolated peripheral blood monocytes), a cell "expressing CD56" at its surface is detected if, when a surface staining is performed, the mean fluorescent intensity (MFI) of the fluorescent marker linked to the

anti- CD56 antibody is similar or identical to the average MFI obtained for the same staining on known $CD56^+$ cells, so that the ratio between its MFI and the said average MFI is of about 1.

In the context of the present invention, it is meant that a cell "do not express CD3" or "**express no CD3**" if CD3 is absent of its surface (such a cell being also referred to as a "$CD3^-$ cell"). Assessment of CD3 expression can be performed as mentioned previously for the other clusters of differentiation. In particular, a cell do not express CD3 if the signal associated to surface CD3 staining (e.g. obtained with an antibody anti-CD3 coupled to a fluorescent marker) which is measured for said cell is similar or identical to the signal corresponding to the same staining of at least one cell being known as not expressing CD3. In other terms, the ratio between the surface CD3-associated signal measured for said cell and the surface CD3-associated signal measured for at least one cell being known as not expressing CD3 is of about 1. Preferably, the surface CD3-associated signal of the target cell is compared to an average surface CD3-associated signal measured on a population of cells being known as not expressing CD3. Cells that do not express CD3 at their surface are well known in the art. They are for example fibroblasts.

In a preferred embodiment of the invention, the NK cells used in the diagnosis method of the invention do not undergo any isolation step from the blood sample. Preferably, the CD3 / CD56 staining is thus performed directly on whole blood. More preferably, this staining is performed between 0 and 10 hours, preferably 0 and 6 hours after the blood has been sampled from the patient. When not used extemporaneously, the blood sample should be stored at 4°C until the analysis of TLR2 and/or TLR4 expression. In this particular embodiment, flow cytometry can be used to detect the blood cells expressing CD56 but not CD3.

Importantly, and as shown in the experimental part below, the TLR4 protein is exposed only on the surface of cells of the patients suffering from SIRS and not from sepsis. Thus, the diagnosis method of the invention can be reduced to practice with any antibody or antiserum recognizing specifically this particular protein. Methods for obtaining such antibody or antiserum are detailed above.

In a preferred embodiment of the invention, the TLR4 expression is detected by contacting the cells with a monoclonal antibody that binds to the TLR4. Examples of particular useful clones are 76B357.1, HTA125, etc.

In a particular embodiment, step a) of the diagnosis method of the invention is performed directly on living cells of the whole biological sample, and flow cytometry is used to detect the cells expressing high levels of surface TLR4.

In the context of the present invention, cells presenting "detectable levels of TLR4" are called "$TLR4^{positive}$" cells. By "detectable levels of surface TLR4", it is meant that the receptor TLR4 is present at the surface of said cells and can be detected by conventional means.

The "**level of the surface level of TLR4**" can be evaluated by any analytical method used to quantify the amount of a particular antigenic determinant at the cell surface. Such method should obviously preserve the natural conformation of TLR4 and should allow determining, either directly or indirectly, a relative or an absolute value of the amount of TLR4 at the surface of the target cells. Examples of analytical methods useful for assessing surface TLR4 expression include, but are not limited to, flow cytometry cell sorting (for example FACS), and immunohistochemistry (IHC). Both methods indeed allow correlating detectable signal intensity with the amount of surface TLR4 per cell. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

In a preferred embodiment of the invention, flow cytometry is used to assess the surface expression of TLR4, for the same reasons as mentioned above.

As for the VEP13 epitope, a skilled artisan can readily adapt known protein/antibody detection methods for use in determining the surface expression levels of the TLR4. More precisely, in the context of the present invention, a cell having a detectable level of surface TLR4 is detected if the ratio between i) the signal measured for said cell and ii) the signal measured for at least one cell being known as negative for surface TLR4 expression, is strictly superior to 1, preferably to 2, more preferably to 3. Preferably, the signal ii) is an average signal measured on a population of cells being known as negative for surface TLR4

expression. Cells which are negative for surface TLR4 expression are well known in the art. They are for example HEK293 cells.

In particular, when an anti-TLR4 antibody coupled to a fluorescent marker is used in a FACS assay on a whole blood sample (or on isolated NK cells), a cell having a "detectable level of the surface TLR4" is identified if the mean fluorescent intensity (MFI) of the fluorescent marker linked to the anti-TLR4 antibody is strictly superior to the average MFI of said fluorescent marker measured for $TLR4^{negative}$ cells present in the same blood sample, so that the ratio between its MFI and the said average MFI is strictly superior to 1, and preferably superior to 1.5.

In another particular embodiment, when only NK cells are considered, the skilled artisan will have to use a control sample containing cells which are known to be negative for surface TLR4. Such control sample can contain for example HEK293 cells.

Of note, "**detectable level of surface TLR4**" can be also assessed by comparing the surface TLR4 of the target cells to cells which are known to express detectable levels of TLR4 at their surface, such as monocytes or dendritic cells.

Once $TLR4^{positive}$ cells have been identified, it is then easy to calculate the percentage of said $TLR4^{positive}$ cells among the total amount of cells present in the blood sample. When NK cells are isolated or at least specifically detected (which is herein preferred), the skilled artisan will have to calculate the percentage of the $TLR4^{positive}$ NK cells among the total population of NK cells. It is noteworthy that flow cytometry assays are well adapted to this numbering step, as it provides fast, objective and quantitative recording of the signals carried by each individual cells.

In a third step, the diagnosis method of the invention requires the comparison of the percentage of $TLR4^{positive}$ cells to a reference value, in order to conclude, if said percentage is similar to the reference value, that the said subject is suffering from sepsis.

In the context of the present invention, it is meant that the percentage of $TLR4^{positive}$ cells which is calculated for the tested subject is "similar to a reference value" for example if the ratio between said percentage and said reference value is comprised between 0.8 and 1.2, preferably between 0.9 and 1.1, more preferably between 0.95 and 1.05.

On the contrary, if the percentage of $TLR4^{positive}$ cells which is calculated for the tested subject is significantly higher than the reference value, then the said subject is not suffering from sepsis, but rather suffers from SIRS.

In the context of the invention, it is meant that the percentage of $TLR4^{positive}$ cells of the tested subject is "higher than a reference value" if it is 4 folds superior, preferably 6 folds, and more preferably 8 folds superior to said reference value. In a preferred embodiment, it can be concluded that the tested subject is suffering from SIRS if the percentage obtained in step b) in the diagnosis method of the invention is higher than 15 %, preferably higher than 18 %, more preferably higher than 20%.

As used herein, the term **"reference value"** (or "control value") refers to a specific value or dataset that can be used to classify the percentage of $TLR4^{positive}$ cells obtained from the test sample associated with an outcome class. Said reference or control value is obtained from the historical expression data for a patient or pool of patients with is SIRS and sepsis free (e.g. a healthy donor). Thus, in a preferred embodiment, said reference value is the percentage of the NK cells that are $TLR4^{positive}$ among the total population of NK cells which has been measured in an individual who does not suffer from sepsis nor SIRS, preferably in a healthy individual. In a preferred embodiment, the reference value is comprised between 0 and 10 %, preferably between 2 and 8 %, more preferably of about 5 % (corresponding to the median value of the percentage of $TLR4^{positive}$ NK cells from a healthy donor). Thus, in a preferred embodiment, it can be concluded that the tested subject is suffering from sepsis if the percentage obtained in step b) in the diagnosis method of the invention is comprised between 0 and 10 %, preferably between 2 and 8 %, and is more preferably of about 5 %.

The present Inventors have also shown that the intracellular levels of Toll-like receptor 4 and of Toll-like receptor 2 are significantly higher in patients suffering from sepsis (as compared with SIRS patients). These differential expressions allow the discrimination between patients with sepsis from those having a SIRS.

Toll-like receptor 2 (also known as CD282 or TIL4, and hereafter referred to as TLR2**)** is a protein that in humans is encoded by the *TLR2* gene (mRNA NM_003264.3). It has the sequence SEQ ID NO:5 (NP_003255). TLR2 is expressed in a number of immune cells,

for example on the surface of microglia, Schwann cells, monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, B cells, and T cells. It occurs in a heterodimer (combination molecule), e.g., paired with TLR1 or TLR6. TLR2 is also found in the epithelia of air passages, pulmonary alveoli, renal tubules, and the Bowman's capsules in renal corpuscles. In the skin, it is found on keratinocytes and sebaceous glands. It is found both at the cell surface and intracellularly. As a surface receptor, TLR2 recognizes many bacterial, fungal, viral, and certain endogenous substances such as lipoproteins and glycolipids. In general, this results in the uptake (internalization, phagocytosis) of bound molecules by endosomes/phagosomes and in cellular activation of the immune system.

In another aspect, the present invention is thus also drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Measuring the mean intracellular expression level of TLR2 and/or TLR4 receptor of the cells present in said sample,

b) Comparing the mean intracellular expression level obtained in a) with a reference value,

wherein if the mean intracellular expression level of the TLR2 and/or TLR4 receptor obtained in a) is higher than the reference value, the said subject is suffering from sepsis.

In a preferred embodiment, the mean intracellular expression level of TLR4 is measured in step a). In another preferred embodiment, the mean intracellular expression level of TLR2 is measured in step a).

In a preferred embodiment, said biological sample is a blood sample.

In a preferred embodiment, the diagnosis method of the invention is reduced to practice on NK cells expressing CD56 but not CD3 ($CD3^-/CD56^{positive}$ cells), more precisely on "$CD56^{bright}$ NK cells" ($CD3^{negative}/CD56^{+++}$ cells) and/or "$CD56^{dim}$ NK cells" ($CD3^{negative}/CD56^{+}$ NK cells). Expression of cell surface CD3 and/or CD56 on NK cells can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry.

This method is based on the measurement of the "intracellular expression level" of TLR2 and / or TLR4. In this method therefore, the level of the proteins TLR2 and / or TLR4

that is present inside the cells has to be assessed. Several methods are known to assess intracellular levels of proteins. One can cite for example immunohistochemistry, Western blots, or intracellular flow cytometry.

Preferably, the intracellular expression level is assessed by intracellular flow cytometry.

For intracellular staining with flow cytometry, cells have to be fixed first to ensure stability of soluble antigens or antigens with a short half-life. This enables to retain the target protein in the original cellular location. Such fixing agents are well known in the art (for example, formaldehyde, acetone and methanol are commonly used). Then a cell permeabilization step is performed prior to the staining and antibodies should be prepared in a permeabilization buffer to ensure the cells remain permeable. Permeabilization buffers are well known in the art. They contain usually detergent agents such as saponin, triton or NP-40.

In this method, said reference value corresponds to the mean intracellular expression level of TLR2 or TLR4 of NK cells obtained from at least one healthy individual. If the intracellular expression level is assessed by intracellular flow cytometry by using a monoclonal antibody recognizing specifically TLR2 or TLR4 and coupled with a fluorescent marker, then the "reference value" is represented by the average mean fluorescence intensity (MFI) of the fluorescent signal corresponding to the anti-TLR2 or anti-TLR4 antibody intracellularly bound to NK cells isolated from healthy individuals (as described above).

In this method, if the mean intracellular expression level of the TLR2 and/or TLR4 receptor is higher than the reference value, then the said subject is suffering from sepsis. It is meant that the mean intracellular expression level of the TLR2 or TLR4 of the tested subject is "higher than the reference value" for example if the ratio between said mean intracellular expression level and said reference value is higher than 2, preferably higher than 3, more preferably higher than 4. In particular, if the intracellular expression level is assessed by intracellular flow cytometry by using a monoclonal antibody recognizing specifically TLR2 or TLR4 and coupled with a fluorescent marker, then the "mean intracellular expression level of the TLR2 or TLR4" of the tested subject is "higher than the reference value" for example if the ratio between the average mean fluorescence

intensity (MFI) of the fluorescent signal corresponding to the anti-TLR2 or anti-TLR4 antibody intracellularly bound to NK cells and the reference value (i.e. in this case the average mean fluorescence intensity (MFI) of the fluorescent signal corresponding to the anti-TLR2 or anti-TLR4 antibody intracellularly bound to NK cells isolated from healthy individuals) is higher than 2, preferably higher than 3, more preferably higher than 4.

On the contrary, if the mean expression level of the intracellular TLR2 or TLR4 is similar to the reference value, then the said subject is suffering from SIRS, and not from sepsis. It is meant that the mean intracellular expression level of the TLR2 or TLR4 of the tested subject is "similar to the reference value" for example if the ratio between said mean intracellular expression level and said reference value is comprised between 0.5 and 1.5, preferably between 0.8 and 1.2, more preferably between 0.9 and 1.1.

The CD24 protein as biomarker of sepsis

Applicants' data demonstrates that, in neutrophils from sepsis patients, the expression of CD24 is significantly decreased., as compared with healthy subjects and SIRS patients (see figures 12, 17, and 18). This down-regulation can be reproduced by incubation of cells from healthy individuals with plasma collected from sepsis patients but not with LPS or whole bacteria (*Escherichia coli* and *Staphylococcus aureus*). Applicants also show the *ex vivo* unresponsiveness to CD24 ligation of neutrophils from sepsis patients, which may contribute to explain the increased neutrophils survival observed in sepsis.

Hence, Applicants' results may improve the ability to approach the complex clinical course, management, and outcome of intensive care unit (ICU) patients, at an early stage, since the instant detection and phenotyping technique can be carried out within 1 hour and may provide valuable information long before blood culture results are provided. Moreover, the instant detection is inexpensive and requires minimal volume of individual blood samples (100 µL).

Neutrophils are short-lived, terminally differentiated cells with a high rate of spontaneous apoptosis. Cell death is altered in the presence of inflammatory stimuli that induce the formation of reactive oxygen species, degranulation, and, under specific conditions,

exocytosis of DNA. Increased neutrophil survival is often detected in inflammatory pathologies, including sepsis (Bratton et al., 2011).

CD24 is a small heavily glycosylated cell-surface protein that is linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Fang et al., 2010) and therefore localized in lipid raft. In humans, CD24 consists of 31 amino acids with 16 potential O and N-glycosylation sites and is expressed in a wide variety of cells. It has been described that cross-ligation of CD24 triggers apoptosis in a human B-cell subset during the early activation stage (Suzuki et al., 2001).

Human CD24 has for example the sequence SEQ ID NO:12 (ACI 46150.1).

The data presented in the experimental part below (see also figures 12, 17 and 18) show that the mean extracellular expression level (e.g. the MFI) is down-regulated in sepsis patients as compared with healthy subjects and SIRS patients. It has furthermore been observed that the percentage of CD24 positive neutrophils is also lower in sepsis patients as compared with healthy subjects and SIRS patients (data not shown).

In one aspect, the present invention relates to a method for diagnosing sepsis from a biological sample of a subject, comprising:

a) Measuring the mean surface expression level of CD24 on the neutrophils present in said sample,

b) Comparing the mean expression level obtained in a) with a reference value,

wherein,

if the mean expression level obtained in a) is lower than the reference value, the said subject suffers from sepsis.

Alternatively, the said diagnosing method can be reduced to practice by:

a) Detecting the cells expressing high surface levels of CD24 in the total population of cells present in said biological sample,

b) Calculating the percentage of cells detected in step a) among said total population, and

c) Comparing the percentage obtained in b) with a reference value,

wherein,

if the percentage obtained in b) is similar to the reference value, the said subject does not suffer from sepsis, and

if the percentage obtained in b) is lower than the reference value, the said subject suffers from sepsis.

In one embodiment, said biological sample is a whole blood sample, preferably devoid of red blood cells. In a further embodiment, the cells that are detected on step a) consist of neutrophils, preferably of neutrophils expressing CD66 (also known as CEACAM3, having the sequence SEQ ID NO:14, referenced as NP_001806.2). Therefore, a first step of the method of the invention can be to detect the CD66 positive cells among the total cell population of the biological sample. $CD66^+$ neutrophils can also be easily purified from blood sample by partial purification by dextran sedimentation followed by water lysis, or by discontinuous density gradients coupled with dextran sedimentation (see Grisham M.B. et al, *Journal of Immunological Methods*, 1985, vol.82, issue 2, pages 315-320).

This diagnosis method is based on the measurement of the "mean surface expression level" or "mean extracellular expression level" of CD24. In this method, the level of the CD24 polypeptide that is present at the cell surface has therefore to be assessed. Expression of cell surface CD24 on neutrophils can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry. In another embodiment, CD24 expression is detected by contacting the total population of cells with an anti-CD24 antibody, preferably a monoclonal antibody, more preferably of the IgM isotype. In a further embodiment, the antibody is tagged with a detectable marker, preferably a fluorescent or a luminescent marker.

In one embodiment, the biological sample is a whole blood sample, preferably devoid of red blood cells. As used herein, a cell "**express CD24**" if CD24 is present at a significant level at its surface (such a cell being also defined as a "$CD24^{positive}$ cell" or a "$CD24^+$ cell"). In particular, a cell express CD24 if the signal associated to surface CD24 staining (e.g. obtained with an antibody anti-CD24 coupled to a fluorescent marker) which is measured

for said cell is similar or identical to the signal corresponding to the same staining of at least one cell being known as expressing CD24. In other terms, the ratio between the surface CD24-associated signal measured for said cell and the surface CD24-associated signal measured for at least one cell being known as expressing CD24 is of about 1. In an embodiment, the surface CD24-associated signal of the target cell is compared to an average surface CD24-associated signal measured on a population of cells being known as expressing CD24, so that the ratio between the surface CD24-associated signal measured for said cell and the average surface CD24-associated signal measured on a population of cells being known as expressing CD24 is of about 1. Cells expressing CD24 at their surface are well known in the art. They are for example B lymphocytes, and neuroblasts.

When an anti-CD24 antibody coupled to a fluorescent marker is used in a flow cytometry analysis assay on a whole blood sample (or on isolated peripheral blood neutrophils), a cell "expressing CD24" at its surface is detected if, when a surface staining is performed, the mean fluorescent intensity (MFI) of the fluorescent marker linked to the anti-CD24 antibody is similar or identical to the average MFI obtained for the same staining on known $CD24^+$ cells, so that the ratio between its MFI and the said average MFI is of about 1.

The diagnosis methods of the invention can be reduced to practice with any antibody or antiserum recognizing specifically CD24. Methods for obtaining such antibody or antiserum are detailed below.

The term **"reference value"** here refers to a specific value is obtained from a patient or a pool of patients which is SIRS free and sepsis free (e.g. a healthy donor). Said reference value corresponds to the mean surface expression level of CD24 of neutrophils obtained from at least one healthy individual. If this expression level is assessed by flow cytometry (for example by using a monoclonal antibody recognizing specifically CD24 and being coupled with a fluorescent marker), then the said "reference value" is represented by the average mean fluorescence intensity (MFI) of the fluorescent signal corresponding to the anti-CD24 antibody bound at the surface of neutrophils isolated from said healthy individual(s).

In this method, if the mean surface expression level of the CD24 marker is lower than the reference value, then the said subject is suffering from sepsis. It is meant that the mean

surface expression level of the CD24 polypeptide of the tested subject is "lower than the reference value" for example if the ratio between said mean surface expression level and said reference value is lower than 0.7, preferably lower than 0.6, more preferably lower than 0.5. In particular, if the surface expression level is assessed by extracellular flow cytometry by using a monoclonal antibody recognizing specifically CD24 and being coupled with a fluorescent marker, then the "mean surface expression level of the CD24" of the tested subject is "lower than the reference value" for example if the ratio between the average mean fluorescence intensity (MFI) of the fluorescent signal corresponding to the anti-CD24 antibody bound to the surface of the neutrophils and the reference value (i.e. in this case the average mean fluorescence intensity (MFI) of the fluorescent signal corresponding to the anti-CD24 antibody bound to the surface of the neutrophils isolated from healthy individuals) is lower than 0.7, preferably lower than 0.6, more preferably lower than 0.5.

On the contrary, if the mean expression level of the extracellular CD24 is similar to the reference value, then the said subject is suffering from SIRS, and not from sepsis. It is meant that the mean surface expression level of the CD24 polypeptide of the tested subject is "similar to the reference value" for example if the ratio between said mean surface expression level and said reference value is comprised between 0.7 and 1.5, preferably between 0.8 and 1.2, more preferably between 0.9 and 1.1.

The CD64 protein as biomarker of sepsis

Applicants' data further demonstrated that the expression of CD64 is significantly increased in neutrophils isolated from sepsis patients, as compared with neutrophils from heatlhy and SIRS patients for both the Mean Fluorescence Intensity (MFI) and the positive cells (see Figure 15A and B respectively). This up-regulation can be used to discriminate between patients having sepsis from those having a SIRS.

Neutrophils are short-lived, terminally differentiated cells with a high rate of spontaneous apoptosis. Cell death is altered in the presence of inflammatory stimuli that induce the formation of reactive oxygen species, degranulation, and, under specific conditions, exocytosis of DNA. Increased neutrophil survival is often detected in inflammatory pathologies, including sepsis (Bratton et al., 2011).

CD64 is a type of integral membrane glycoprotein known as an Fc receptor that binds monomeric IgG-type antibodies with high affinity. It is more commonly known as Fc-gamma receptor 1 (FcγRI). After binding IgG, CD64 interacts with an accessory chain known as the common γ chain (γ chain), which possesses an ITAM motif that is necessary for triggering cellular activation. Human CD64 has for example the sequence SEQ ID NO:13 (AAH32634.1).

In one embodiment, the present invention relates to a method for diagnosing sepsis from a biological sample of a subject, comprising:

a) Quantifying the percentage of cells expressing CD64 in said sample,

b) Comparing the said percentage obtained in a) with a reference value.

In one embodiment, said biological sample is a whole blood sample, preferably devoid of red blood cells. In a further embodiment, the cells that are detected on step a) consist of neutrophils. Step a) of this method would therefore be aimed at quantifying the percentage of neutrophils expressing CD64 among the total population of neutrophils in said sample. Preferably, said neutrophils express CD66 (also known as CEACAM3, having the sequence SEQ ID NO:14, referenced as NP_001806.2). Therefore, a first step of the method of the invention can be to detect the CD66 positive cells among the total cell population of the biological sample. Step a) of this method would therefore be aimed at quantifying the percentage of $CD66^+$ neutrophils expressing CD64 among the total population of $CD66^+$ neutrophils in said sample. $CD66^+$ neutrophils can be easily purified from blood sample by partial purification by dextran sedimentation followed by water lysis, or by discontinuous density gradients coupled with dextran sedimentation (see Grisham M.B. et al, *Journal of Immunological Methods*, 1985, vol.82, issue 2, pages 315-320).

As used herein, a cell "**express CD64**" if CD64 is present at a significant level at its surface (such a cell being also defined as a "$CD64^+$" or a "$CD64^{positive}$ cell"). In particular, a cell express CD64 if the signal associated to surface CD64 staining (e.g. obtained with an antibody anti-CD64 coupled to a fluorescent marker) which is measured for said cell is similar or identical to the signal corresponding to the same staining of at least one cell being known as expressing CD64. In other terms, the ratio between the surface CD64-associated signal measured for said cell and the surface CD64-associated signal measured

for at least one cell being known as expressing CD64 is of about 1. In an embodiment, the surface CD64-associated signal of the target cell is compared to an average surface CD64-associated signal measured on a population of cells being known as expressing CD64, so that the ratio between the surface CD64-associated signal measured for said cell and the average surface CD64-associated signal measured on a population of cells being known as expressing CD64 is of about 1. Cells expressing CD64 at their surface are well known in the art. They are for example macrophages and monocytes.

Expression of cell surface CD64 on neutrophils can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry. More precisely, CD64 expression can be detected by contacting the total population of cells with an anti-CD64 antibody, preferably a monoclonal antibody. In a further embodiment, this antibody is tagged with a detectable marker, preferably a fluorescent or a luminescent marker.

The diagnosis methods of the invention can be reduced to practice with any antibody or antiserum recognizing specifically CD64. Methods for obtaining such antibody or antiserum are detailed below.

When an anti-CD64 antibody coupled to a fluorescent marker is used in a flow cytometry analysis assay on a whole blood sample (or on isolated peripheral blood monocytes), a cell "expressing CD64" at its surface is detected if, when a surface staining is performed, the mean fluorescent intensity (MFI) of the fluorescent marker linked to the anti-CD64 antibody is similar or identical to the average MFI obtained for the same staining on known $CD64^+$ cells, so that the ratio between its MFI and the said average MFI is of about 1.

In this embodiment, the term **"reference value"** refers to a specific value obtained from a patient or a pool of patients which is sepsis free (e.g., a healthy donor or a SIRS patient). In a preferred embodiment, said reference value is the percentage of neutrophils that express CD64 ($CD64^{positive}$ neutrophils) among the total population of neutrophils, said percentage having been measured in at least one individual who does not suffer from sepsis.

When the reference value corresponding to the reference percentage of $CD64^{positive}$ neutrophils is obtained from a healthy individual (or a pool of them), it is usually comprised between 0 and 10 %, preferably between 3 and 10% (corresponding to the values of the percentage of $CD64^{positive}$ neutrophils from healthy donors, see Figure 15B).

In this case, if the percentage obtained in a) is at least 5 times higher than this reference value, the said subject suffers from sepsis. And, if the percentage obtained in a) is between two and four times higher than the reference value, the said subject only suffers from SIRS.

In another preferred embodiment, it can be concluded that the tested subject is suffering from sepsis if the percentage obtained in step a) in the diagnosis method of the invention is comprised between 50 and 100 %, preferably between 60 and 100 %, and is more preferably of about 70 %.

In another embodiment, the present invention relates to a method for diagnosing sepsis from a biological sample of a subject, comprising:

a) Quantifying the mean surface expression of CD64 on the cells present in said sample,

b) Comparing the said mean expression obtained in a) with a reference value.

In one embodiment, the biological sample is a whole blood sample, preferably devoid of red blood cells. In a further embodiment, the cells that are detected on step a) consist of neutrophils, preferably of neutrophils expressing CD66 (also known as CEACAM3, having the sequence SEQ ID NO:14, referenced as NP_001806.2). Therefore, a first step of the method of the invention can be to detect the CD66 positive cells among the total cell population of the biological sample. $CD66^+$ neutrophils can also be easily purified from blood sample by partial purification by dextran sedimentation followed by water lysis, or by discontinuous density gradients coupled with dextran sedimentation (see Grisham M.B. et al, *Journal of Immunological Methods*, 1985, vol.82, issue 2, pages 315-320).

This diagnosis method is based on the measurement of the "mean surface expression level" or "mean extracellular expression level" of CD64. In this method, the level of the CD64 polypeptide that is present at the cell surface has therefore to be assessed. Expression of cell surface CD64 on neutrophils can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry. In another embodiment, CD64 expression is detected by contacting the total population of cells with an anti-CD64 antibody, preferably a monoclonal antibody, more preferably of the IgM isotype. In a further embodiment, the antibody is tagged with a detectable marker, preferably a fluorescent or a luminescent marker.

In this embodiment, the term **"reference value"** refers to a specific value obtained from a patient or a pool of patients which is sepsis free (e.g., a healthy donor or a SIRS patient). More precisely, said reference value can be the mean surface expression of CD64 of neutrophils which have been collected in at least one individual who does not suffer from sepsis. More preferably, if said mean surface expression is assessed by flow cytometry, reference value of "mean surface expression" is measurable by assessing the Mean Fluorescence Intensity (MFI) associated with CD64 staining on neutrophils obtained from a subject or a pool of subjects which is sepsis free (e.g., a healthy donor or a SIRS patient).

In this case, if the mean expression level of the surface CD64 on the patient's neutrophils is at least 5 times higher than this reference value, then the said subject suffers from sepsis.

Alternatively, if the mean expression level of the surface CD64 on the patient's neutrophils is between two and three times higher than the reference value, the said subject only suffers from SIRS.

The VEP13 epitope of CD16 as biomarker of sepsis

The present Inventors have shown that a specific epitope of CD16, the VEP13 epitope, is expressed on the surface of blood monocytes from healthy donor and septic patients whereas it can barely be detected in patients suffering from systemic inflammation of non-infectious source (SIRS). This differential expression allows the discrimination between patients with sepsis from those having a SIRS.

CD16, the low affinity receptor for the Fc part of IgG (therefore also known as FcγRIII), is a glycoprotein expressed in NK cells, in proinflammatory monocytes and neutrophils. Two isoforms (A and B) exist. In human, the isoform A has the sequence SEQ ID NO:1 (NP_000560.5) and the isoform B has the sequence SEQ ID NO:2 (NP_001231682.1). Several monoclonal antibodies have been produced against the isoforms A and B of CD16 / FcγRIII and the correspondent epitopes have been localized on these proteins (Tamm A. et al., 1996).

Among these antibodies, the 3G8 monoclonal antibody (Fleit. H. B. et al, 1982) and the VEP13 monoclonal antibody (Rumpold. H..D. 1982) have been used in this study.

According to Perussia et al. (1984), and Tamm et al. (1996) these antibodies recognize different epitopes on the isoforms A and B of the CD16 / FcγRIII surface receptor.

The present Inventors have shown that different CD16 expression patterns are obtained according to the anti-CD16 monoclonal antibody used (VEP13 or 3G8). In particular, as is it shown below, using the VEP13 monoclonal antibody allows discriminating between patients suffering from sepsis and SIRS, what is not possible with the 3G8 monoclonal antibody, which epitope is present on the blood monocytes in both types of patients.

Accordingly, some anti-CD16 monoclonal antibodies can cause confusion and thus lead to potential misdiagnosis (Auffray C. et al. 2007, Heine G.H. et al., 2008).

This observation points out the importance of using the correct CD16 monoclonal antibody for a proper diagnosis of sepsis.

In a first aspect, the present invention is thus drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting the cells expressing high surface levels of the VEP13 epitope in the total population of cells present in said sample,

b) Calculating the percentage of the cells detected in step a) among the said total population, and

c) comparing the percentage obtained in b) with a reference value,

wherein if the percentage obtained in b) is similar to the reference value, the said subject is suffering from sepsis.

In an embodiment of the invention, the step of detecting the expression of the VEP13 epitope can be reduced to practice on whole blood cells of the patient blood sample. Preferably, said blood sample is devoid of red blood cells, and the detection is performed on the remaining blood cells, which are white blood cells (e.g. neutrophils, monocytes, lymphocytes, basophiles, etc.) and platelets.

The diagnosis methods of the invention can be performed by using macrophages. These cells can be identified by specific expression of a number of proteins including CD14, CD11b, or CD68 by flow cytometry or immunohistochemical staining.

It is noteworthy that results are more accurate if expression of the VEP13 epitope is assessed only on peripheral blood monocytes so that step a) of the diagnosis method of the invention is preferably performed on these particular cells.

Thus, in a particular embodiment, the present invention is drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a blood sample of a subject, the method comprising:

a) Detecting the peripheral blood monocytes expressing high surface levels of the VEP13 epitope in the total population of peripheral blood monocytes present in said sample,

b) Calculating the percentage of the peripheral blood monocytes detected in step a) among the said total population, and

c) comparing the percentage obtained in b) with a reference value,

wherein if the percentage obtained in b) is similar to the reference value, the said subject is suffering from sepsis.

Peripheral blood monocytes belong to the family of the peripheral mononuclear cell of the blood (PBMCs). PBMCs are a critical component in the immune system to fight infection and adapt to intruders. These cells can be extracted from whole blood using ficoll, a hydrophilic polysaccharide that separates layers of blood, which will separate the blood into a top layer of plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes. The polymorphonuclear cells can be further isolated by lysing the red blood cells.

There are at least three types of monocytes in human blood: a) the "classical" monocyte is characterized by high level expression of the CD14 cell surface receptor ($CD14^{++}$ / $CD16^{-}$ monocyte), b) the "non-classical" monocyte shows low level expression of CD14 with additional co-expression of the CD16 receptor ($CD14^{+}$/ $CD16^{++}$ monocyte), and c) the "intermediate" monocyte with high level expression of CD14 and low level expression of CD16 ($CD14^{++}$ / $CD16^{+}$ monocytes) (Ziegler-Heitbrock L. et al., 2010). All the monocytes therefore expressed relatively high levels of CD14 and heterogeneous amounts of CD16.

Thus, all peripheral blood monocytes express the cluster of differentiation CD14. This cluster of differentiation has the sequence SEQ ID NO:3 in human (NP_000582.1). Numerous antibodies against human CD14 are commercially available. CD14 is expressed at the surface of the monocytic cells and, at 10 times lesser extent, of the neutrophils. Peripheral blood monocytes are easily identified by specific biomarkers (e.g; CD14 or CD16) combined with morphometric characteristics (e.g. size, shape, granulometry, etc.). For example, when flow cytometry is used, forward scatter and side scatter information help to identify the monocyte population among other blood cells.

In a preferred embodiment, the diagnosis methods of the invention are reduced to practice on peripheral blood monocytes expressing CD14 ($CD14^+$ monocytes). Expression of cell surface CD14 on monocytes can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry.

As mentioned previously, whole blood samples (optionally devoid of red blood cells) are preferably used in the diagnosis method of the invention. Nevertheless, it is also possible to isolate peripheral blood monocytes prior to detecting surface VEP13 epitope expression. In this case, peripheral blood monocytes can be isolated from PBMC by selecting the PBMCs expressing significant levels of CD14 at their surface ($CD14^{positive}$ cells). Several methods for isolating human peripheral blood monocytes are already available to the skilled person and need not to be further detailed here (see, for example, de Almeida M.C. et al, *Mem. Inst. Oswaldo Cruz* 2000). Commercial kits using immunomagnetic selection can also be used.

Importantly, most of the clinical and experimental studies either ignored the "intermediate monocytes", or analyzed intermediate and nonclassical monocytes as a single subset, so that the $CD14^{++}/CD16^+$ subset (hereafter called "intermediate monocytes") remained, so far, poorly characterized (Zawada A.M. et al., 2011). However, the clinical importance of this subset has been recently highlighted by the fact that elevated $CD14^{++}$ / $CD16^+$ monocyte counts predict adverse outcome in patients at high cardiovascular risk (Auffray C. et al. 2007, Heine G.H. et al., 2008) or that $CD16^+$ monocytes are preferentially susceptible to HIV-1 entry, more permissive for replication, and constitute a continuing source of viral persistence in individuals receiving highly active antiretroviral therapy (Ellery P.J. et al. 2007).

In the context of the present invention, it is meant that a cell "**express CD14**" if CD14 is present at a significant level at its surface (such a cell being also defined as a "$CD14^+$ cell"). In particular, a cell express CD14 if the signal associated to surface CD14 staining (e.g. obtained with an antibody anti-CD14 coupled to a fluorescent marker) which is measured for said cell is similar or identical to the signal corresponding to the same staining of at least one cell being known as expressing CD14. In other terms, the ratio between the surface CD14-associated signal measured for said cell and the surface CD14-associated signal measured for at least one cell being known as expressing CD14 is of about 1. Preferably, the surface CD14-associated signal of the target cell is compared to an average surface CD14-associated signal measured on a population of cells being known as expressing CD14, so that the ratio between the surface CD14-associated signal measured for said cell and the average surface CD14-associated signal measured on a population of cells being known as expressing CD14 is of about 1. Cells expressing CD14 at their surface are well known in the art. They are for example classical and intermediate monocytes.

In particular, when the anti-CD14 antibody coupled to a fluorescent marker is used in a flow cytometry analysis assay on a whole blood sample (or on isolated peripheral blood monocytes), a cell "expressing CD14" at its surface is detected if, when a surface staining is performed, the mean fluorescent intensity (MFI) of the fluorescent marker linked to the anti-CD14 antibody is similar or identical to the average MFI obtained for the same staining on known $CD14^+$ cells, so that the ratio between its MFI and the said average MFI is of about 1.

In another preferred embodiment, the diagnosis methods of the invention are reduced to practice on peripheral blood monocytes expressing CD16 ($CD16^+$ monocytes). As mentioned previously, CD16 is the low affinity receptor for the Fc part of IgG (therefore also known as FcγRIII). Expression of cell surface CD16 on monocytes can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry.

In the context of the present invention, it is meant that a cell "**express CD16**" if CD16 is present at a significant level at its surface (such a cell being also defined as a "$CD16^+$ cell"). Assessment of CD16 expression can be performed as mentioned previously for $CD14^+$ cells. Cells expressing CD16 at their surface are well known in the art. They are for example monocytes, NK cells, and neutrophils.

In a more preferred embodiment, the diagnosis methods of the invention are reduced to practice on peripheral blood monocytes expressing both CD14 and CD16 ($CD14^+/CD16^+$ monocytes). Expression of cell surface CD14 and CD16 on monocytes can be assessed by any common analytical method, for example by flow cytometry or immunohistochemistry.

In a preferred embodiment of the invention, the peripheral blood monocytes used in the diagnosis methods of the invention do not undergo any isolation step from the blood sample. Preferably, the CD16 / CD14 staining is thus performed directly on whole blood. More preferably, this staining is performed between 0 and 10 hours, preferably 0 and 6 hours after the blood has been sampled from the patient. When not used extemporaneously, the blood sample should be stored at 4°C until the analysis of VEP13 epitope expression. In this particular embodiment, flow cytometry can be used to detect the blood cells expressing high levels of CD14.

As used in the present invention, the term "**VEP13 epitope**" corresponds to the epitope of the CD16 / FcγRIII protein on which the VEP13 antibody binds. This epitope is present on the two isoforms A and B of CD16. As used in the present invention, an "epitope" is the site on the antigen to which an antibody binds. It can be formed by contiguous residues or by non-contiguous residues brought into close proximity by the folding of an antigenic protein (also called "conformational epitopes"). Epitopes formed by contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by non-contiguous amino acids are typically lost under said exposure. The VEP 13 antibody is an IgM monoclonal antibody which is characterized since 1982 (see Rumpold H. et al, *The Journal of Immunology*, 1982) and is now commonly used in the art. It is commercialized for example by Miltenyi Biotec in conjugation with various dyes (references 130-091-244 to 246) and is easily available to the skilled person. The epitope recognized by the VEP13 antibody is, at least in part, characterized in Tamm A. et al (*The American Association of Immunologist*, 1996): it is apparently a conformational epitope composed of some parts of the FG and BC loops of the FcγRIII receptor (NP_001231682.1, SEQ ID NO:1).

Importantly, and as shown in the experimental part below, the VEP13 epitope is exposed only on cells of the patients effectively suffering from sepsis. Thus, the diagnosis methods of the invention can be reduced to practice with any antibody or antiserum recognizing

specifically this particular epitope. Methods for obtaining such antibody or antiserum are detailed below.

In a preferred embodiment of the invention, the VEP13 epitope expression is detected by contacting the cells with a monoclonal antibody that binds to the VEP13 epitope. In a more preferred embodiment, this antibody is of IgM isotype. In an even more preferred embodiment, said monoclonal antibody is the VEP13 monoclonal antibody itself.

In a particular embodiment, step a) of the diagnosis methods of the invention is performed directly on living cells of the whole biological sample, and flow cytometry is used to detect the cells expressing high levels of the VEP13 epitope, preferably expressing both the CD14 biomarker and the VEP13 epitope or expressing CD14 and CD16 biomarkers, and the VEP13 epitope.

In the context of the present invention, cells presenting "high levels of the VEP13 epitope" are called "$VEP13^{high}$" cells. By "high levels of the VEP13 epitope", it is meant that the VEP13 epitope is highly present at the surface of said cells.

Of course, VEP13 is not exposed at the surface of cells that do not express the CD16 / FcγRIII receptor. Rather, cells expressing the CD16 / FcγRIII receptor may not display the exposed VEP13 epitope at their surface, since the said epitope can be hidden or altered, and therefore not accessible for accurate antibody binding. This is shown for example on figures 1 and 2, on which one can see that a high amount of monocytes expressing CD16 (and identified as being stained positive for the 3G8, B73.1 and NKL monoclonal antibodies) are not recognized by the VEP13 antibody. Accordingly, the VEP13 expression is not proportional to the surface expression of the CD16 receptor.

The "**level of the VEP13 epitope**" can be evaluated by any analytical method used to quantify the amount of a particular antigenic determinant at the cell surface. Such method should obviously preserve the natural conformation of the VEP13 epitope and should allow determining, either directly or indirectly, a relative or an absolute value of the amount of the VEP13 epitope at the surface of the target cells. Examples of analytical methods useful for assessing VEP13 epitope expression include, but are not limited to, flow cytometry cell sorting (for example FACS), and immunohistochemistry (IHC). Both

methods indeed allow correlating detectable signal intensity with the amount of VEP13 epitope per cell.

Accordingly, in the context of the present invention, a cell having a "high level of VEP13 epitope" (also called VEP13^{high} cell) is detected if the ratio between i) the signal measured for said cell and ii) the signal measured for at least one cell being known as negative for surface CD16 expression, is strictly superior to 1. Preferably, the signal ii) is an average signal measured on a population of cells being known as negative for surface CD16 expression. Cells which are negative for surface CD16 expression are well known in the art. They are for example fibroblast cells.

In particular, when the VEP13 antibody coupled to a fluorescent marker is used in a FACS assay on a whole blood sample (or on isolated peripheral blood monocytes), a cell having a "high level of the VEP13 epitope" is detected if the mean fluorescent intensity (MFI) of the fluorescent marker linked to the VEP13 antibody is superior to the average MFI of said fluorescent marker measured for CD16^{negative} cells (e.g. that are concomitantly negative for the 3G8 staining) present in the same blood sample, so that the ratio between its MFI and the said average MFI is strictly superior to 1. Figure 4B highlights for example the differential expression of the VEP13 epitope on the surface of the CD16^{+} monocytes obtained from healthy, sepsis and SIRS patients.

In another particular embodiment, when only peripheral blood monocytes are considered, the skilled artisan will have to use a negative control sample containing cells which are known to be negative for said VEP13 epitope. Preferably, essentially all the cells in said negative control sample are negative for said VEP13 epitope. The VEP13-negative cells are, for example, fibroblast cells.

Of note, "high level of the VEP13 epitope" can be also assessed by comparing the VEP13 level of the target cells to cells which are known to express high levels VEP13 at their surface, such as neutrophils and NK cells.

Once VEP13^{high} cells have been identified, it is then easy to calculate the percentage of said VEP13^{high} cells among the total amount of cells present in the blood sample. When peripheral blood cells are isolated or at least specifically detected (which is herein preferred), the skilled artisan will have to calculate the percentage of the VEP13^{high}

peripheral blood monocytes among the total population of peripheral blood monocytes. It is noteworthy that flow cytometry assays are well adapted to this numbering step, as it provides fast, objective and quantitative recording of the signals carried by each individual cells.

In a third step, the diagnosis method of the invention requires the comparison of the percentage of VEP13^{high} cells to a reference value, in order to conclude, if said percentage is similar to the reference value, that the said subject is suffering from sepsis.

In the context of the present invention, it is meant that the percentage of VEP13^{high} cells which is calculated for the tested subject is "similar to a reference value" for example if the ratio between said percentage and said reference value is comprised between 0.8 and 1.2, preferably between 0.9 and 1.1, more preferably between 0.95 and 1.05.

On the contrary, if the percentage of VEP13^{high} cells which is calculated for the tested subject is significantly lower than the reference value, then the said subject is not suffering from sepsis, but rather suffers from SIRS.

In the context of the invention, it is meant that the percentage of VEP13^{high} cells of the tested subject is "significantly lower than a reference value" if it is 2 folds inferior, preferably 4 folds, and more preferably 5 folds inferior to said reference value.

As used herein, the term "reference value" (or "control value") refers to a specific value or dataset that can be used to classify the percentage of VEP13^{high} cells obtained from the test sample associated with an outcome class. Said reference or control value is obtained from the historical expression data for a patient or pool of patients with is SIRS and sepsis free (e.g. a healthy donor). Thus, in a preferred embodiment, said reference value is the percentage of the peripheral blood VEP13^{high} monocytes among the total population of peripheral blood monocytes which has been measured in a blood sample from an individual who does not suffer from sepsis nor SIRS, preferably from a healthy individual.

This reference or control value is a predetermined value, which can take a variety of forms. It can be a single cut-off value, such as a median or mean. It can be a single number, equally applicable to every patient individually, or it can vary, according to specific subpopulations of patients. A person skilled in the art will appreciate that the comparison

between the percentage of VEP13^{high} cells in the test sample and the percentage of VEP13^{high} cells in the control will depend on the control used. In a preferred embodiment, the reference value is of 5% (corresponding to the mean value of the VEP13^{high} CD16^{+} monocytes from a healthy donor, as shown on figure 4A).

The percentage of the VEP13^{high} cells in a control sample obtained from a healthy subject is not identical to the percentage of the VEP13^{high} cells obtained from patients suffering from SIRS (as shown on figure 4A, samples of patients developing a SIRS have about 0% of VEP13^{high} cells whereas control samples obtained from healthy subjects have between 4 and 9 % of VEP13^{high} cells). Thus, if the reference value is the mean value of the VEP13^{high} CD16^{+} monocytes in a SIRS patient, the diagnostic method of the invention should conclude that the tested patient is suffering from sepsis only if the percentage obtained in b) is significantly higher than said reference value. In the context of the invention, it is meant that the percentage of VEP13^{high} cells of the tested patient is "significantly higher than a reference value" if it is 2 folds superior, preferably 4 folds, and more preferably 5 folds superior to said reference value.

In a preferred embodiment, the present invention is also drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting the cells expressing high surface levels of the VEP13 epitope in the total population of cells present in said sample,

b) Calculating the percentage of cells detected in step a) among the said total population,

wherein if the percentage obtained in b) is comprised between 2.5 % and 10%, the said patient is suffering from sepsis (that is, from a SIRS induced by a bacterial infection).

In a more preferred embodiment, said biological sample is a blood sample and said VEP13^{high} cells are peripheral blood monocytes expressing CD14, CD16 or both (CD14 and CD16).

In a more preferred embodiment, the said subject is diagnosed to suffer from sepsis if the percentage obtained in b) is comprised between 3 % and 7%.

The present invention is also drawn to a method of diagnosing sepsis or SIRS, or to discriminate SIRS and sepsis-suffering subjects, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting the cells expressing high surface levels of the VEP13 epitope in the total population of cells present in said sample,

b) Calculating the percentage of cells detected in step a) among the said total population,

wherein if the percentage obtained in b) is comprised between 0 and 2%, the said subject is not suffering from sepsis, but rather from non-infectious SIRS.

In a preferred embodiment, said biological sample is a blood sample and said $VEP13^{high}$ cells are peripheral blood monocytes expressing CD14, CD16 or both (CD14 and CD16).

In a more preferred embodiment, the said subject is diagnosed to suffer from SIRS (and not sepsis) if the percentage obtained in b) is comprised between 0 and 1.8%.

The present invention is also drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting in said sample the cells expressing CD16 at their surface,

b) Measuring the mean expression level of the VEP13 epitope on the surface of the cells detected in step a),

c) Comparing the mean expression level obtained in b) with a reference value,

wherein if the mean expression level of the VEP13 epitope obtained in b) is similar to the reference value, the said subject is suffering from sepsis.

In a preferred embodiment, said biological sample is a blood sample and said $VEP13^{high}$ cells are peripheral blood monocytes expressing CD14, CD16 or both (CD14 and CD16).

Preferably, said reference value corresponds to the mean expression level of the VEP13 epitope of cells which are known to express high surface levels of the VEP13 epitope, for example NK cells and neutrophils, as explained above. The reference value can also be

defined as being the mean expression level of the VEP13 epitope of peripheral blood monocytes obtained from at least one healthy individual.

If said expression level is assessed by flow cytometry by using a monoclonal antibody recognizing specifically VEP13 and coupled with a fluorescent marker, then the "mean expression level" is represented by the average mean fluorescence intensity (MFI) of the fluorescent signal corresponding to the VEP13 antibody bound to a population of known $CD16^+$ cells (as described above).

On the contrary, if the mean expression level of the VEP13 epitope obtained in b) is significantly lower than the reference value, the said subject is suffering from SIRS, not from sepsis. It is meant that the mean expression level of the VEP13 epitope of the tested subject is "significantly lower than the reference value" if it is 2 folds inferior, preferably 4 folds, and more preferably 5 folds inferior to said reference value.

Detecting the expression of the biomarkers of the invention

The diagnosis methods of the invention can be reduced to practice with any antibody or antiserum detecting (or recognizing specifically) the biomarkers of the invention. Methods for obtaining such antibody or antiserum are detailed below.

The term **"antibody"** as used herein is intended to include monoclonal antibodies, polyclonal antibodies, and chimeric antibodies. More particularly, an antibody (or "immunoglobulin") consists of a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (or domain) (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" (CDR) or "hypervariable regions", which are primarily responsible for binding an epitope of an antigen, and which are interspersed with regions that are more conserved, termed

framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order : FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g. effector cells) and the first component (Clq) of the classical complement system.

Antibody fragments can also be used in the present diagnosis method. This term is intended to include Fab, Fab', F(ab')2, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, and multimers thereof and bispecific antibody fragments. Antibodies can be fragmented using conventional techniques. For example, F(ab')2 fragments can be generated by treating the antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and F(ab')2, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques.

A "**monoclonal antibody**", as used herein, means an antibody arising from a nearly homogeneous antibody population. More particularly, the individual antibodies of a population are identical except for a few possible naturally-occurring mutations which can be found in minimal proportions. In other words, a monoclonal antibody consists of a homogeneous antibody arising from the growth of a single cell clone (for example a hybridoma, a eukaryotic host cell transfected with a DNA molecule coding for the homogeneous antibody, a prokaryotic host cell transfected with a DNA molecule coding for the homogeneous antibody, etc.) and is generally characterized by heavy chains of one and only one class and subclass, and light chains of only one type. Monoclonal antibodies are highly specific and are directed against a single antigen. In addition, in contrast with preparations of polyclonal antibodies which typically include various antibodies directed against various determinants, or epitopes, each monoclonal antibody is directed against a single epitope of the antigen.

Antibody can be of different isotypes (namely IgA, IgD, IgE, IgG or IgM). Both IgG and IgM type antibodies were used in the present study. Of note, these isotypes are composed

of two identical heavy chains and two identical light chains that are joined by disulfide bonds. Importantly, IgM antibodies form polymers where multiple immunoglobulins are covalently linked together with disulfide bonds, mostly as a pentamer but also as a hexamer, so that they have a molecular mass of approximately 900 kDa (in their pentamer form). Because each monomer has two antigen binding sites, a pentameric IgM has 10 binding sites. Typically, however, IgM antibodies cannot bind 10 antigens at the same time because the large size of most antigens hinders binding to nearby sites. Due to its polymeric nature, IgM possesses high avidity.

The antibody may be from recombinant sources and/or produced in transgenic animals. Conventional techniques of molecular biology, microbiology and recombinant DNA techniques are within the skill of the art. Such techniques are explained fully in the literature.

For example, antibodies having specificity for the VEP13 epitope may be prepared by conventional methods. A mammal (e.g. a mouse, hamster, or rabbit) can be immunized with an immunogenic form of a CD16 protein carrying the VEP 13 epitope, which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a polypeptide include conjugation to carriers or other techniques well known in the art. For example, the polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art (e. g. the hybridoma technique originally developed by Kohler and Milstein (*Nature* 256; 495- 497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al, *Immunol Today* 4 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., *Methods Enzymol*, 121; 140-67 (1986)), and screening of combinatorial antibody libraries (Huse et al., *Science* 246; 1275 (1989)) Hybridoma cells can be screened immunochemically for

production of antibodies specifically reactive with the polypeptide and the monoclonal antibodies can be isolated.

In some embodiments, recombinant antibodies are provided that specifically bind to the biomarkers of the invention. Recombinant antibodies include, but are not limited to, chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, single-chain antibodies and multi- specific antibodies A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody (mAb) and a human immunoglobulin constant region (See, e. g. , Cabilly et al. (US Pat No 4,816,567), and Boss et al. (US Pat No 4,816,397)). Single-chain antibodies have an antigen binding site and consist of single polypeptides. They can be produced by techniques known in the art, for example using methods described in Ladner et al. (US Pat No 4,946,778) or in Bird et al. (1988) *Science* 242 423-426). Multi-specific antibodies are antibody molecules having at least two antigen-binding sites that specifically bind different antigens. Such molecules can be produced by techniques known in the art, for example using methods described in Segal, US Pat. No. 4,676,980 or US Pat. No. 6,121,424. Monoclonal antibodies directed against the VEP13 epitope can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with the polypeptide(s) of interest. Kits for generating and screening phage display libraries are commercially available. Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, US Pat. No. 5,223,409; PCT Publication No. WO 92/18619; and PCT Publication No. WO 91/17271.

Antibodies may be isolated after production (e.g., from the blood or serum of the animals) or synthetized and further purified by well-known techniques. Antibodies specific for a protein can be selected or purified by affinity chromatography, ELISPOT or ELISA. For example, a recombinantly expressed and purified (or partially purified) VEP13, CD24, CD64, TLR2 or TLR4 peptide may be produced, and covalently or non- covalently coupled to a solid support such as, for example, a chromatography column. The column can then be used to purify antibodies specific for the biomarkers of the invention from a sample containing antibodies directed against a large number of different epitopes, thereby generating a substantially purified antibody composition, i.e., one that is substantially free

of contaminating antibodies. By a substantially purified antibody composition it is meant, in this context, that the antibody sample contains at most only 30% (by dry weight) of contaminating antibodies directed against epitopes other than those of the biomarkers of the invention, and preferably at most 20%, yet more preferably at most 10% and most preferably at most 5% (by dry weight) of the sample is contaminating antibodies A purified antibody composition means that at least 99% of the antibodies in the composition are directed against the desired biomarkers of the invention.

The antibodies can be detected by direct labeling of the antibodies themselves with detectable markers. Alternatively, unlabeled primary antibody can be used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available .

In a preferred embodiment of the invention, the monoclonal antibody recognizing a biomarker of the invention is tagged with a detectable marker, preferably a fluorescent or a luminescent marker.

Examples of detectable markers / labels include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta -galactosidase, or acetylcholinesterase examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin, examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorot[pi]azinylamine fluorescein, dansyl chloride or phycoerythrin, an example of a luminescent material includes luminol, examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

In a preferred embodiment of the invention, flow cytometry is used to assess the cell surface expression of the biomarkers of the invention, as this technic allows using heterogeneous mixtures of biological cells (e.g. whole blood samples) and not isolating and/or purifying the cells before performing the staining. Also, it can be easily performed on living cells (so that the natural conformation of the epitopes, for example of the VEP13 epitope, is not affected), and allows assessing only the surface level of a targeted antigen.

Moreover, the number of monoclonal antibodies available for flow cytometry analysis is very large, and they can be coupled to different fluorochromes, allowing an easy multiple antigen staining. Finally, it is a broadly used scientific technic, providing fast, objective and quantitative recording of detectable signals from individual cells.

A skilled artisan can readily adapt known protein/antibody detection methods for use in determining the expression levels of the biomarkers of the invention. As previously disclosed, by using these detection methods, the amount of the biomarkers of the invention at the cell surface (in other terms, the "expression level of the biomarkers of the invention") will be correlated with the intensity of the detectable signals. It is of note that signal detection always introduced a bias due to the settings used by the skilled person (e.g. kind of the detectable signal, characteristics of the analytical device, settings of the analytical device, etc.) and only relative information can be obtained, as it is to date not possible to quantify absolute amounts of particular antigens. Nevertheless the above-cited analytical methods enable to obtain reliable and reproducible relative amount of surface targeted antigen(s). This relative amount can be obtained for example by comparing expression level(s) of surface antigen(s) (notably TLR2/4, CD24, CD16, CD64, CD14 or the VEP13 epitope) present on target cells to the one(s) assessed on cells which are known to be negative for said surface antigen(s). It is then routine matter for the skilled person to identify the appropriate settings enabling to identify the antigen(s)-positive cells. Once these antigen(s)-positive cells are identified, the ratio between the signal(s) detected in both positive versus negative cells is informative of the expression level(s) of the said surface antigen(s).

Diagnosis use of the biomarkers of the invention

As mentioned above, it is possible to discriminate patients suffering from sepsis from those suffering from SIRS and therefore to diagnose sepsis by detecting the expression of at least one marker selected from the group consisting of: the VEP13 epitope of CD16, the TLR4 receptor, the TLR2 receptor, the CD24 polypeptide, and CD64 polypeptide.

The term "**marker**" or "**biomarker**" herein refers to a biological molecule, preferably a polypeptide, more preferably an antigen, an epitope or a receptor, whose detection is

associated with the phenotype or a condition of a subject. The presence of said molecule is therefore key information to obtain so as to identify one's phenotype / condition. In the context of the invention, "markers" are associated with occurrence of the sepsis disease, the SIRS disease, or with their absence in the tested subject.

In a preferred embodiment, the present invention relates to the use of any of the following marker:

- The VEP13 epitope of CD16 detected on the surface of PBMCs, preferentially on monocytes,
- The TLR4 receptor detected on the surface of NK cells,
- The TLR2 receptor detected within the NK cells,
- The TLR4 receptor detected within the NK cells,
- The CD24 polypeptide detected on the surface of neutrophils, and/or
- The CD64 polypeptide detected on the surface of neutrophils.

The present invention therefore relates to the use of any of these markers separately for diagnosing sepsis in a subject in need thereof, preferably for discriminating subjects suffering from sepsis from those suffering from SIRS.

Nevertheless, the skilled person will easily understand that it is possible to use any combination of these markers.

In another aspect, the present invention relates to the use at least two, preferably at least three, more preferably at least four of the markers, and even more preferably at least five markers selected in the group consisting of: VEP13/CD16, TLR2, TLR4, CD24 and CD64 for diagnosing sepsis in subjects in need thereof, preferably for discriminating subjects suffering from sepsis from those suffering from SIRS.

More precisely, the present invention relates to the use at least two, preferably at least three, more preferably at least four, more preferably at least five, more preferably at least six of the markers selected from the group consisting of: the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor, the intracellular TLR2 receptor, the CD24 polypeptide, and the CD64 polypeptide, for diagnosing sepsis in subjects in need

thereof, preferably for discriminating subjects suffering from sepsis from those suffering from SIRS.

Even more precisely, the present invention relates to the use at least two, preferably at least three, more preferably at least four, more preferably at least five, more preferably at least six of the markers:

- The VEP13 epitope of CD16 detected on the surface of PBMCs,
- The TLR4 receptor detected on the surface of NK cells,
- The TLR2 receptor detected within the NK cells,
- The TLR4 receptor detected within the NK cells,
- The CD24 polypeptide detected on the surface of neutrophils, and/or
- The CD64 polypeptide detected on the surface of neutrophils,

, for diagnosing sepsis in subjects in need thereof, preferably for discriminating subjects suffering from sepsis from those suffering from SIRS.

Preferably, at least the VEP13 epitope of CD16 and the surface TLR4 receptor will be detected.

Preferably, at least the VEP13 epitope of CD16 and the intracellular TLR4 receptor will be detected.

Preferably, at least the surface TLR4 receptor and the intracellular TLR4 receptor will be detected.

Preferably, at least the VEP13 epitope of CD16 and the CD64 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the intracellular TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16 and the CD24 polypeptide will be detected.

Preferably, at least the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor and the CD64 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor and the surface TLR4 receptor will be detected.

Preferably, at least the intracellular TLR2 receptor and the intracellular TLR4 receptor will be detected.

Preferably, at least the VEP13 epitope of CD16 and the intracellular TLR2 receptor will be detected.

Preferably, at least the VEP13 epitope of CD16, the surface TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the CD24 polypeptide, the surface TLR4 receptor and the intracellular TLR2 receptor will be detected.

Preferably, at least the VEP13 epitope of CD16, the surface TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor, the CD24 polypeptide and the intracellular TLR4 receptor will be detected.

Preferably, at least the intracellular TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the surface TLR4 receptor will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the VEP13 epitope of CD16 will be detected.

Preferably, at least the VEP13 epitope of CD16, the CD24 polypeptide and the intracellular TLR4 receptor will be detected.

Preferably, at least the surface TLR4 receptor, the intracellular TLR2 receptor and the CD64 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor, the intracellular TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor, the VEP13 epitope of CD16 and the CD64 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor, the VEP13 epitope of CD16 and the intracellular TLR2 receptor will be detected.

Preferably, at least the surface TLR4 receptor, the VEP13 epitope of CD16 and the intracellular TLR4 receptor will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the surface TLR4 receptor, the CD24 polypeptide and the intracellular TLR4 receptor will be detected.

Preferably, at least the intracellular TLR4 receptor, the VEP13 epitope of CD16 and the CD64 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the CD24 polypeptide and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the CD24 polypeptide and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor and the CD64 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the surface TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor and the surface TLR4 receptor will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR2 receptor, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the CD64 polypeptide, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD64 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the intracellular TLR4 receptor, the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor, the intracellular TLR4 receptor and the CD24 polypeptide will be detected.

Preferably, at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the intracellular TLR4 receptor will be detected.

More preferably, all the above-listed markers will be detected.

In a particular embodiment, the invention relates to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject,

c) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject, and

d) performing the above-described diagnosis method based on the detection of the VEP13 epitope on a biological sample of said subject.

In a more preferred embodiment, said method comprises at least the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject, and

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject.

In a more preferred embodiment, said method comprises at least the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject, and

b) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject.

In a more preferred embodiment, said method comprises at least the following steps:

a) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject.

In a more preferred embodiment, said method comprises at least the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject, and

c) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject.

In a more preferred embodiment, said method comprises at least the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject,

c) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject, and

d) performing the above-described diagnosis method based on the detection of the VEP13 epitope on a biological sample of said subject.

The detection conditions of each of these biomarkers have been detailed above and do not need to be described again. The skilled person will obviously refer to the respective passages describing how to detect each biomarker and on which cell(s).

It is for example possible to use the VEP13 epitope and the surface TLR4 receptor as biomarkers for diagnosing sepsis in patients in need thereof.

In this embodiment, the present invention relates to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) performing the diagnosis method using the assessment of the level of the VEP13 epitope as defined above, on a first biological sample of said subject, and

b) performing the diagnosis method using the assessment of surface TLR4 level, as defined above, on a second biological sample of said subject,

wherein,

if the percentage of cells expressing high surface levels of the VEP13 epitope is similar to the reference value in the diagnosis method of step a) and if the percentage of cells expressing detectable surface levels of TLR4 is similar to the reference value in the diagnosis method of step b), then said subject is suffering from sepsis, and

if the percentage of cells expressing high surface levels of the VEP13 epitope is lower than the reference value in the diagnosis method of step a), and if the percentage of cells expressing detectable surface levels of TLR4 is higher than the reference value in the diagnosis method of step b), then the said subject is suffering from SIRS.

The present invention is also drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) performing the diagnosis method using the assessment of the mean expression level of VEP13, on a first biological sample of said subject, and

b) performing the diagnosis method using the assessment of the percentage of cells expressing high surface levels of TLR4, as defined above, on a second biological sample of said subject,

wherein,

if the mean expression level of the VEP13 epitope is similar to the reference value in the diagnosis method of step a) and if the percentage of cells expressing detectable surface levels of TLR4 is similar to the reference value in the diagnosis method of step b), then said subject is suffering from sepsis.

In these diagnosis methods, said biological sample is a blood sample, said step a) is conducted on peripheral blood monocytes expressing either CD14, or CD16, or both (CD14 and CD16), and said step b) is conducted on NK cells expressing CD56 but not

CD3, more precisely on "$CD56^{bright}$ NK cells" ($CD3^{negative}/CD56^{+++}$ cells) and/or "$CD56^{dim}$ NK cells" ($CD3^{negative}/CD56^{+}$ NK cells).

It is also possible to use the VEP13 epitope and the TLR2 and/or TLR4 receptor(s) as biomarkers for diagnosing sepsis in patients in need thereof.

In another aspect, the present invention provides a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) performing the diagnosis method using the assessment of the mean expression level of VEP13 as defined above, on a first biological sample of said subject, and

b) performing the diagnosis method using the assessment of the mean intracellular expression level of the TLR2 and/or TLR4 receptor as defined above, on a second biological sample of said subject,

wherein,

if the mean expression level of the VEP13 epitope is similar to the reference value in the diagnosis method of step a) and if the mean intracellular expression level of the TLR2 and/or TLR4 receptor is higher than the reference value in the diagnosis method of step b), then said subject is suffering from sepsis.

The present invention is drawn to a method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) performing the diagnosis method using the assessment of the percentage of cells expressing high levels of the VEP 13 epitope, as defined above, on a first biological sample of said subject, and

b) performing the diagnosis method using the assessment of the mean intracellular expression level of the TLR2 and/or TLR4 receptor as defined above, on a second biological sample of said subject,

wherein,

if the percentage of cells expressing high levels of the VEP 13 epitope is similar to the reference value in the diagnosis method of step a), and if the mean intracellular expression level of the TLR2 and/or TLR4 receptor is higher than the reference value in the diagnosis method of step b), then said subject is suffering from sepsis.

In these diagnosis methods, said biological sample is a blood sample, said step a) is conducted on peripheral blood monocytes expressing either CD14, or CD16, or both (CD14 and CD16), and said step b) is conducted on NK cells expressing CD56 but not CD3, more precisely on "$CD56^{bright}$ NK cells" ($CD3^{negative}/CD56^{+++}$ cells) and/or "$CD56^{dim}$ NK cells" ($CD3^{negative}/CD56^{+}$ NK cells).

It is also possible to combine all the above-mentioned markers with any other known markers such as HLA-DR (preferably detected on monocytes), CD69 (preferably detected on NK cells), CX_3CR_1 (preferably detected on monocytes), bacterial markers such as bacterial DNA and PGN, or plasmatic markers such as TNF, IL-6, IL-18, IL-1Ra, IL-10, CXCL1, CXCL8, CCL2, CCL5, CRP, Procalcitonine, sTREM-1, and the like.

Treating methods of the invention

In a second aspect, the present invention provides *in vitro* or *ex vivo* methods for designing an antibiotic-based treatment from a biological sample of a subject that has been diagnosed to be suffering from sepsis by any of the diagnosis method of the invention. According to the present invention, "designing" an antibiotic-based treatment aims to identify the number and dosage of the antibiotics that will be prescribed in view of the expression level of each of the biomarkers of the invention, analyzed alone or in combination.

In one embodiment, said designing method therefore comprises:

a) performing on said sample any of the diagnosis methods of the invention, as defined above, and

b) if said subject is diagnosed to be suffering from sepsis, designing an antibiotic-based treatment.

In a preferred embodiment, said designing method comprises:

a) performing on said sample the diagnosis method of the invention using the assessment of the surface level of the VEP13 epitope, as defined above, and

b) designing an antibiotic-based treatment for said subject if the percentage of the $VEP13^{high}$ cells is similar to said reference value.

In this preferred embodiment, said biological sample is preferably a blood sample and said $VEP13^{high}$ cells are preferably peripheral blood monocytes expressing either CD14, or CD16, or both (CD14 and CD16).

In another preferred embodiment, said designing method comprises:

a) performing the diagnosis method using the assessment of TLR4 surface level or of TLR2 and/or TLR4 intracellular level on a biological sample of said subject,

b) designing an antibiotic-based treatment for said subject if the percentage of cells expressing detectable levels of TLR4 is similar to the reference value, or if the mean intracellular expression level of the TLR2 and/or TLR4 receptor of the cells is higher than the reference value.

In this preferred embodiment, said biological sample is a blood sample, and said designing method is conducted on NK cells expressing CD56 but not CD3, more precisely on "$CD56^{bright}$ NK cells" ($CD3^{negative}/CD56^{+++}$ cells) and/or "$CD56^{dim}$ NK cells" ($CD3^{negative}/CD56^{+}$ NK cells).

In a preferred embodiment, said designing method comprises:

a) performing on said sample the diagnosis method of the invention using the assessment of the surface level of the CD64 polypeptide, as defined above, and

b) designing an antibiotic-based treatment for said subject if the percentage the $CD64^{+}$ cells is at least 5 times higher than said reference value, or if the mean expression level of CD64 on cells is at least 5 times higher than said reference value.

In this preferred embodiment, said biological sample is preferably a blood sample and said $CD64^{+}$ cells are preferably neutrophils.

In a preferred embodiment, said designing method comprises:

a) performing on said sample the diagnosis method of the invention using the assessment of the surface level of the CD24 polypeptide, as defined above, and

b) designing an antibiotic-based treatment for said subject if the mean expression level of CD24 on the neutrophils present in said sample is lower than the reference value.

In this preferred embodiment, said biological sample is preferably a blood sample and said $CD24^+$ cells are preferably neutrophils.

These designing methods can obviously use a combination of at least two, preferably at least three, more preferably at least four of the markers, more preferably at least five of the markers and even more preferably of at least six markers selected in the group consisting of: the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface or intracellular TLR4 receptor, the surface CD24 polypeptide and the surface CD64 polypeptide.

Thus, the present invention relates to the use of at least one marker selected from the group consisting of: the VEP13 epitope of CD16, the TLR4 receptor, the TLR2 receptor, the CD24 polypeptide, and CD64 polypeptide for designing an antibiotic-based treatment for a subject in need thereof.

In a preferred embodiment,

- Said VEP13 epitope of CD16 is detected on the surface of PBMCs preferentially monocytes,
- Said TLR4 receptor is detected on the surface of NK cells,
- Said TLR2 receptor is detected within NK cells,
- Said TLR4 receptor is detected within NK cells,
- Said CD24 polypeptide is detected on the surface of neutrophils, and/or
- Said CD64 polypeptide is detected on the surface of neutrophils.

In other terms, the present invention relates to a method of designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject,

c) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject, and

d) performing the above-described diagnosis method based on the detection of the VEP13 epitope on a biological sample of said subject.

Importantly, all the combinations of biomarkers highlighted above for the diagnostic purpose are preferentially used in these designing methods.

In another preferred embodiment, said subject is a human individual who presents at least two symptoms of SIRS.

The detection conditions of each of these biomarkers have been detailed above and do not need to be described again. The skilled person will obviously refer to the respective passages describing same.

Also, the present invention provides *in vitro* or *ex vivo* methods for selecting a subject which is predicted to benefit from the treatment with an antibiotic-containing composition, by using any of the diagnosis methods of the invention. This method aims to identify if the said treatment will benefit to the tested subject The selecting method of the invention is thus a very useful tool to avoid exposing antibiotics to subjects that do not suffer from sepsis, thereby preventing the emergence of bacterial resistance.

In one embodiment, said selecting method comprises:

a) performing the diagnosis method of the invention, as defined above, and

b) if said subject is diagnosed to be suffering from sepsis, selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition .

In a preferred embodiment, said selecting method comprises:

a) performing on said sample the diagnosis method of the invention using the assessment of the surface level of the VEP13 epitope, as defined above, and

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the percentage of $VEP13^{high}$ cells is similar to the reference value.

In this preferred embodiment, this selecting method is performed on a blood sample and said $VEP13^{high}$ cells are peripheral blood monocytes expressing either CD14, or CD16, or both (CD14 and CD16).

It is reminded here that said reference value is the percentage of the peripheral blood $VEP13^{high}$ monocytes among the total population of peripheral blood monocytes which has been measured in a blood sample from an individual who does not suffer from SIRS nor sepsis, preferably a healthy individual. And it is meant that the percentage of the $VEP13^{high}$ cells which is calculated for the tested subject is "similar to a reference value" for example if the ratio between said percentage and said reference value is comprised between 0.8 and 1.2, preferably between 0.9 and 1.1, more preferably between 0.95 and 1.05.

In another preferred embodiment, said selecting method comprises:

a) performing the diagnosis method using the assessment of TLR4 surface level or of TLR2 and/or TLR4 intracellular level on a biological sample of said subject,

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the percentage of cells expressing detectable levels of TLR4 is similar to the reference value or if the mean intracellular expression level of the TLR2 and/or TLR4 receptor of the cells is higher than the reference value.

In a preferred embodiment, said biological sample is a blood sample, and said selecting method is conducted on NK cells expressing CD56 but not CD3, more precisely on "$CD56^{bright}$ NK cells" ($CD3^{negative}/CD56^{+++}$ cells) and/or "$CD56^{dim}$ NK cells" ($CD3^{negative}/CD56^{+}$ NK cells).

In a preferred embodiment, said selecting method comprises:

a) performing on said sample the diagnosis method of the invention using the assessment of the surface level of the CD24 epitope, as defined above, and

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the mean expression level of CD24 on the neutrophils present in said sample is lower than the reference value.

In a preferred embodiment, said selecting method comprises:

a) performing on said sample the diagnosis method of the invention using the assessment of the surface level of the CD64 epitope, as defined above, and

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the percentage of the $CD64^+$ neutrophils is at least 5 times higher than said reference value, or if the mean expression level of CD64 on neutrophils is at least 5 times higher than said reference value.

These selecting methods can obviously use a combination of at least two, preferably at least three, more preferably at least four of the markers, and even more preferably at least five markers selected in the group consisting of: the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface or intracellular TLR4 receptor, the surface CD24 polypeptide and the surface CD64 polypeptide.

Thus, the present invention relates to the use of at least one marker selected from the group consisting of: the VEP13 epitope of CD16, the TLR4 receptor, the TLR2 receptor, the CD24 polypeptide, and CD64 polypeptide for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition.

In a preferred embodiment,

- Said VEP13 epitope of CD16 is detected on the surface of PBMCs,
- Said TLR4 receptor is detected on the surface of NK cells,
- Said TLR2 receptor is detected within NK cells,
- Said TLR4 receptor is detected within NK cells,
- Said CD24 polypeptide is detected on the surface of neutrophils, and/or
- Said CD64 polypeptide is detected on the surface of neutrophils.

In other terms, the present invention relates to a method for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing

composition, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject,

c) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject, and

d) performing the above-described diagnosis method based on the detection of the VEP13 epitope on a biological sample of said subject.

Importantly, all the combinations of biomarkers highlighted above for the diagnostic purpose are preferentially used in these selecting methods.

In another preferred embodiment, said subject is a human individual who presents at least two symptoms of SIRS.

The detection conditions of each of these biomarkers have been detailed above and do not need to be described again. The skilled person will obviously refer to the respective passages describing same.

Moreover, the present invention relates to a method for treating a subject in need thereof, the method comprising administering an antibiotic-containing composition in subjects whose sepsis has been diagnosed using any of the diagnosis methods of the invention.

In particular, the treating methods of the invention comprise:

a) performing on said sample any of the diagnosis method of the invention, as defined above, and

b) if said subject is diagnosed to be suffering from sepsis, treating said subject with an antibiotic-based treatment.

These treating methods can obviously use a combination of at least two, preferably at least three, more preferably at least four of the markers, more preferably at least five of the

markers and even more preferably at least six markers selected in the group consisting of: the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface or intracellular TLR4 receptor, the surface CD24 polypeptide and the surface CD64 polypeptide.

In a preferred embodiment,

- Said VEP13 epitope of CD16 is detected on the surface of PBMCs,
- Said TLR4 receptor is detected on the surface of NK cells,
- Said TLR2 receptor is detected within NK cells,
- Said TLR4 receptor is detected within NK cells,
- Said CD24 polypeptide is detected on the surface of neutrophils, and/or
- Said CD64 polypeptide is detected on the surface of neutrophils.

In other terms, the present invention relates to a method for treating a subject in need thereof, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the above-described diagnosis method based on the detection of the TLR2 and/or the TLR4 receptor on a biological sample of said subject,

b) performing the above-described diagnosis method based on the detection of the CD24 polypeptide on a biological sample of said subject,

c) performing the above-described diagnosis method based on the detection of the CD64 polypeptide on a biological sample of said subject, and

d) performing the above-described diagnosis method based on the detection of the VEP13 epitope on a biological sample of said subject.

said method further comprising administering an antibiotic-containing composition in subjects whose sepsis has been diagnosed using any of these diagnosis methods.

Importantly, all the combinations of biomarkers highlighted above for the diagnostic purpose are preferentially used in these treating methods.

The detection conditions of each of these biomarkers have been detailed above and do not need to be described again. The skilled person will obviously refer to the respective passages describing same.

Antibiotic treatments that are currently used to prevent or fight against sepsis are well known by the skilled artisan. They are for example reviewed in "Bochud P-Y. et al, *Intensive Care Med*, 2001". Appropriate antibiotics to treat sepsis are monotherapy (e.g. with a carbapenem, a cephalosporin, quinolone or penicillin) or combinations of two or three antibiotics given at the same time (e.g. beta-lactam and aminoglycoside); most combinations usually include vancomycin to treat many MRSA infections. In addition to antibiotics, two other major therapeutic interventions, organ-system support and surgery, may be needed. First, if an organ system needs support, the intensive care unit can often provide it (for example, intubation to support lung function or dialysis to support kidney function). Secondly, surgery may be needed to drain or remove the source of infection.

In a preferred embodiment, said antibiotic-based treatment comprises one or more antibiotic(s) chosen from:

- cephalosporin, such as ceftriaxone, cefotaxime, and cefepime,
- aminoglycosides, such as amikacin, gentamicin, kanamycin, neomycin, netilmicine, paromomycin, streptomycin, and tobramycin,
- carbapenem, such as meropenem, imipenem, ertapenem,
- aminopenicillin, such as ampicillin and amoxicillin,
- glycopeptides, such as vancomycin, and
- nitroimidazoles, such as metronidazole and rifampin.

In another aspect, the present invention is drawn to an antibiotic-containing composition for use in treating a subject whose sepsis has been diagnosed using any of the methods according the invention.

The present invention also relates to the use of an antibiotic for preparing a composition intended to treat sepsis, in a subject whose sepsis has been diagnosed using any of the diagnosis methods of the invention.

Kits of the invention

The present disclosure also provides kits that are useful for carrying out any of the diagnosis method described herein.

The kits generally comprise reagents and compositions for determining the expression of any of the biomarkers of the invention, or a combination thereof.

In one embodiment, the present invention relates to a kit comprising reagents and compositions for determining the expression of VEP 13 epitope expression, e.g. all the necessary reagents for VEP 13 staining. As will be recognized by the skilled artisan, the contents of the kits will depend upon the means used to obtain the information on the VEP13 epitope expression. These kits preferably also include reagents for detecting surface CD14 and/or CD16, so as to identify or to isolate $CD14^+$ and / or $CD16^+$ cells. More preferably, said reagents are monoclonal antibodies recognizing CD14 and/or CD16 specifically. These kits may comprise a labeled compound or agent capable of detecting the VEP13 epitope in a blood sample and means for determining the amount of the $VEP13^{high}$ cells in the sample (e.g., an antibody which binds the VEP13 epitope or a fragment thereof).

In another embodiment, the present invention relates to a kit comprising reagents and compositions for determining the expression of TLR2 and/or TLR4 expression, e.g. all the necessary reagents for TLR2 and/or TLR4 staining. These kits preferably also include reagents for detecting surface CD3 and/or CD56, so as to identify or to isolate $CD3^-$ and $CD56^+$ cells. More preferably, said reagents are monoclonal antibodies recognizing CD3 and/or CD56 specifically.

Other exemplary kits include reagents for detecting surface CD24 polypeptide. Said kit may also include reagents to detect CD66, in order to detect $CD66^{positive}$ cells (neutrophils). In one embodiment, said reagents are monoclonal antibodies recognizing CD24 or CD66 specifically.

Other exemplary kits include reagents for detecting surface CD64 polypeptide. Said kit may also include reagents to detect CD66, in order to detect $CD66^{positive}$ cells (neutrophils). In one embodiment, said reagents are monoclonal antibodies recognizing CD64 or CD66 specifically.

It will be easily understood that the present invention also encompasses kits containing the means for determining the expression of at least two, preferably at least three, more preferably at least four of the markers, and even more preferably at least five markers selected in the group consisting of: the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface or intracellular TLR4 receptor, the surface CD24 polypeptide and the surface CD64 polypeptide.

In particular, the kits of the invention may contain all the means enabling the specific detection of:

- at least the VEP13 epitope of CD16 and the surface TLR4 receptor,
- at least the VEP13 epitope of CD16 and the intracellular TLR4 receptor,
- at least the surface TLR4 receptor and the intracellular TLR4 receptor,
- at least the VEP13 epitope of CD16 and the CD64 polypeptide,
- at least the surface TLR4 receptor and the CD64 polypeptide,
- at least the intracellular TLR4 receptor and the CD64 polypeptide,
- at least the surface TLR4 receptor and the CD24 polypeptide,
- at least the intracellular TLR4 receptor and the CD24 polypeptide,
- at least the VEP13 epitope of CD16 and the CD24 polypeptide,
- at least the CD64 polypeptide and the CD24 polypeptide,
- at least the intracellular TLR2 receptor and the CD24 polypeptide,
- at least the intracellular TLR2 receptor and the CD64 polypeptide,
- at least the intracellular TLR2 receptor and the surface TLR4 receptor,
- at least the intracellular TLR2 receptor and the intracellular TLR4 receptor,
- at least the VEP13 epitope of CD16 and the intracellular TLR2 receptor,
- at least the VEP13 epitope of CD16, the surface TLR4 receptor and the CD64 polypeptide,
- at least the CD24 polypeptide, the surface TLR4 receptor and the intracellular TLR2 receptor,

- at least the VEP13 epitope of CD16, the surface TLR4 receptor and the CD24 polypeptide,
- at least the surface TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide,
- at least the surface TLR4 receptor, the CD24 polypeptide and the intracellular TLR4 receptor,
- at least the intracellular TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the surface TLR4 receptor,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD64 polypeptide,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the VEP13 epitope of CD16,
- at least the VEP13 epitope of CD16, the CD24 polypeptide and the intracellular TLR4 receptor,
- at least the surface TLR4 receptor, the intracellular TLR2 receptor and the CD64 polypeptide,
- at least the surface TLR4 receptor, the intracellular TLR4 receptor and the CD64 polypeptide,
- at least the surface TLR4 receptor, the VEP13 epitope of CD16 and the CD64 polypeptide,
- at least the surface TLR4 receptor, the VEP13 epitope of CD16 and the intracellular TLR2 receptor,
- at least the surface TLR4 receptor, the VEP13 epitope of CD16 and the intracellular TLR4 receptor,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD24 polypeptide,
- at least the surface TLR4 receptor, the CD24 polypeptide and the intracellular TLR4 receptor,
- at least the intracellular TLR4 receptor, the VEP13 epitope of CD16 and the CD64 polypeptide,

- at least the intracellular TLR2 receptor, the CD24 polypeptide and the CD64 polypeptide,
- at least the VEP13 epitope of CD16, the CD24 polypeptide and the CD64 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor and the CD64 polypeptide,
- at least the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the surface TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor and the CD24 polypeptide,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD24 polypeptide,
- at least the intracellular TLR2 receptor, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD64 polypeptide,
- at least the CD64 polypeptide, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor and the intracellular TLR4 receptor,
- at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor and the CD64 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD64 polypeptide,

- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor and the CD64 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the intracellular TLR4 receptor, the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the intracellular TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor, the intracellular TLR4 receptor and the CD24 polypeptide,
- at least the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the intracellular TLR4 receptor,

or

- all the biomarkers of the invention.

These kits might also contain the means enabling the detection of other biomarkers that were previously described as discriminating sepsis patients from SIRS patients, such as the HLA-DR marker.

Kits can also include instructions for interpreting the results obtained using the kit.

Kits may also comprise, e.g., a buffering agent, a preservative, or a protein stabilizing agent. The kits can further comprise components necessary for detecting the detectable label (e.g., an enzyme or a substrate). The kits can also contain control, for instance an isotype control which can be assayed and compared to the test sample. Each component of a kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit.

In a preferred embodiment, the kits are antibody-based kits, which may comprise, for example: (1) a first antibody (e.g., attached to a solid support) which binds to at least one of the biomarker of the invention; and, optionally, (2) a second, different antibody which binds to the first antibody and is conjugated to a detectable label, preferably a fluorescent or luminescent label.

In a final aspect, the present invention is drawn to the use of any of the kits of the invention, for diagnosing sepsis from a blood sample of a subject, or for designing the antibiotic treatment of a subject.

In particular, the present invention relates to the use of a kit comprising reagents and compositions for determining the expression of TLR2 and/or TLR4 for diagnosing sepsis in a subject in need thereof or for designing the antibiotic treatment of a subject. Said reagents are preferably monoclonal antibodies.

Also, it relates to the use of a kit comprising reagents and compositions for determining surface CD24 expression for diagnosing sepsis in a subject in need thereof or for designing the antibiotic treatment of a subject. Said reagents are preferably monoclonal antibodies.

And it relates to the use of a kit comprising reagents and compositions for determining surface CD64 expression for diagnosing sepsis in a subject in need thereof or for designing the antibiotic treatment of a subject. Said reagents are preferably monoclonal antibodies.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

EXAMPLES

MATERIAL & METHODS

Patients

Peripheral blood was collected in sodium-heparin (BD Bioscience, San Jose, CA, USA) from healthy volunteers and from patients admitted in ICU with SIRS or a suspicion of sepsis included in http://clinicaltrials.gov/ct2/show/NCT01378169. A total of 16 patients and 8 healthy donors were enrolled.

Whole blood flow cytometry phenotyping (in surface and intracellular)

Expression of cell surface CD16 on monocytes was measured by flow cytometry (MacsQuant, Miltenyi Biotec). Staining and cell acquisition for flow cytometry were performed within 5 hour after blood sampling. Monoclonal antibodies were used as follows: 3 µl of phycoerythrin (PE)-anti-CD14 antibody (MY4-RD1, Beckman Coulter, Fullerton, CA, USA), 10 µl of allophycocyanin (APC)-anti-CD16 (VEP-13) antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) or 5 µl of allophycocyanin (APC)-anti-CD16 (3G8) antibody (BD Pharmingen, San Diego, CA, USA) or 10 µl of (ALEXA Fluor 647)- anti CD16 (LNK16) antibody (Serotec, Raleigh, NC, USA) or 5 µl of allophycocyanin (APC)-anti-CD16 (B731) antibody (BD Pharmingen, San Diego, CA, USA) per 100 µL of whole blood. All but VEP13 (IgM) are IgG. Negative controls were mouse monoclonal antibodies isotype-matched in accordance with the recommendations of the manufacturer. After 20 minutes of incubation in the dark, erythrocytes were lysed with 1 ml Facs Lyse (BD FACS™ lysing solution, BD Bioscience, Franklin Lakes, NJ, USA). After a further 10-minute incubation and centrifugation (300 g for five minutes, 4°C), the supernatant was removed and 300 µl of MACS buffer (DPBS with 2 mM EDTA and 0.5% fetal calf serum) was added to cells. Monocytes were gated using side scatter dot plots vs CD14 expression. The values were expressed as mean fluorescence intensity (MFI).The intracellular staining was carried out using an Inside Stain kit (Miltenyi Biotec Inc). Isotype-matched control antibodies were used to determine background levels of staining. For intracellular staining, monoclonal antibodies were used as follows: 10 µl of (FITC)-anti-CD16 (VEP-13) antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) or 5

µl of (PACIFIC BLUE)-anti-CD16 (3G8) antibody (BD Pharmingen, San Diego, CA, USA). Monocytes were gated using side scatter dot plots vs CD14 expression. The values were expressed as mean fluorescence intensity (MFI).

Labelled antibodies against the following antigens were used: (VioBlue)-anti-CD3 clone BW264/56, (APC)-anti-CD16 clone VEP13, (PE)-anti-CD56 clone AF12-7H3 from Miltenyi Biotec (Bergisch-Gladbach, Germany); (A647)-anti-TLR2 clone TL2.1, and (A488)-anti-TLR9 clone 26C593.2 from Biolegend (San Diego, CA); (A647)-anti-TLR4 clone 76B357.1 from Imgenex (San Diego, CA). The corresponding isotype and fluorophore controls were obtained from the same companies. The Inside Stain Kit were purchased from Miltenyi Biotec.

NK cell surface antigens were labeled in staining buffer (PBS, EDTA 2mM and FCS 0.5%) with antibodies at the concentration suggested by the manufacturers. All whole blood samples (100 µL) were immediately processed for multiple staining with: 5 µL (VioBlue)-anti-CD3 and 5 µL (PE)-anti-CD56 antibodies to discriminate NK cell subsets ($CD3^-/CD56^{bright}$ and $CD3^-/CD56^{dim}$). The following stainings were combined to evaluate the expression of other markers: 2 µL (A647)-anti-TLR2, 2 µL (A647)-anti-TLR4, or 1.5 µL (A488)-anti-TLR9. After 20 minutes incubation in the dark and at 4°C, 2mL of lysis buffer (BD Pharm lyse) was added for more 10 minutes at RT. The incubation was followed by centrifugation (300 *g* for five minutes), the cells were then washed with 2 mL of staining buffer, centrifuged again and ressuspended in 500 µL of the same buffer for the surface expression analysis or in the fixation buffer in case of the intracellular staining. Intracellular staining for TLR2, TLR4 and TLR9 was performed after red blood cells lysis, washing, fixation and permeabilization of the cells using Inside Stain Kit (Miltenyi Biotec), according to the manufacturer's instructions. All flow cytometry data were acquired on a MACSQuant flow cytometer (Myltenyi Biotec) and analyzed using the MACSQuantify software. Anti-TLR antibodies were previously tested on monocytes used as positive controls (data not shown). For each antibody, an isotype control with the same fluorochrome from the same manufacturer was used as negative staining control. The number of NK cells was determined by gating on $CD3^-/CD56^{bright}$ and $CD3^-/CD56^{dim}$ subsets and using the MACSQuant cytometer which allows an absolute counting.

Statistical Analysis

Levels of CD16 expression on CD14 positive monocytes, during three following days after ICU admission in each patient group were examined by repeated measure one-way analysis of variance (ANOVA) and Tuckey post-test. Statistics were generated using GraphPad Prism 5 and a P- value lower than 0.05 was considered significant.

RESULTS

1. Concerning the VEP13/CD16 marker

1.1. Different CD16 mAbs reveal different cell surface staining pattern

The profile of binding of 4 different commercially available anti-CD16 monoclonal antibodies, VEP-13, 3G8, B73.1 and LNK 16, was compared in peripheral blood monocytes from healthy subjects. All of them recognize the two isoforms A and B of CD16. VEP13 is an IgM monoclonal antibody, whereas 3G8, B73.1 and LNK 16 are IgG monoclonal antibodies.

Surprisingly, flow cytometry analysis (see figure 1) showed different staining results obtained when different monoclonal antibodies were used. In particular, the 3G8 and B73.1 monoclonal antibodies provided similar pattern, and allow the detection of 6,16% and 2,57% of $CD14^{++}$/ $CD16^{+}$ cells. This cell subset appeared reduced when the CD16 staining was carried out with VEP13 monoclonal antibody (2,07%) or surprisingly expanded when the NKL16 was used (45,74%).

This result demonstrates that the epitopes recognized by the different anti-CD16 monclonal antibody are not exposed the same way at the surface of $CD14^{high}$ blood monocytes, and show that the VEP13 epitope is poorly available at the surface of $CD14^{high}$ blood monocytes in healthy subjects (as compared with the three other epitopes).

1.2. Both VEP13 and 3G8 mAbs reveal a very low intracellular staining

Further, it was investigated if a difference in CD16-antigens expression could also be observed at intracellular level. Fixation/permeabilization method was used for the detection of intracellular 3G8 or VEP13 antigens by flow cytometry. No significant differences were found between intracellular VEP13 and 3G8 expression in $CD14^{high}$ cells

from four healthy donors (Figure 5) and from two ICU patients (irrespective of the disease they are suffering from, Figure 6).

$CD16^{high}$ - $CD14^{high}$ cells	**Healthy**	**ICU**
Staining 3G8	**6,53%**	**71,08%**
Staining VEP 13	**1,70%**	**22,03%**

This result demonstrates that only cell surface expression of the VEP13 epitope is useful for the diagnosis method of the invention.

1.3. Loss of VEP13 epitope in SIRS patients

Then, the profile of binding of two different anti-CD16 monoclonal antibodies, VEP-13 (A) and 3G8 (B), was compared in peripheral blood monocytes from SIRS and SEPSIS patients (see figure 2).

Interestingly, the CD14 monocytes from patients with a confirmation of SIRS were reactive with the 3G8 monoclonal antibody, but not with the VEP13 monoclonal antibody. This means that the 3G8 monoclonal antibody cannot be used for diagnosing sepsis, as it also binds to its epitope even in the absence of sepsis.

It is to note that the sepsis patients were reactive with the two monoclonal antibodies, 3G8 and VEP13.

It comes out from this comparison that only the staining with VEP13 monoclonal antibody allows to discriminate between the patients that are undergoing a SIRS (no or very low level of $CD16^{high}$ / $CD14^{high}$ cells) and those suffering from sepsis (high level of $CD16^{high}$ / $CD14^{high}$ cells).

1.4. Comparison of VEP-13 epitope expression changes between SIRS and sepsis patients

Blood samples for analysis of the VEP13 epitope expression were collected immediately after admission in ICU (day 0), diagnosed for SIRS or sepsis, and compared to 8 healthy donors.

Figure 4 (A) represents the percentage of CD16 positive cells in 9 SIRS and 7 septic patients at suspicion of infection in ICU patients, among the VEP13 ^{HIGH} and VEP13 ^{LOW} subpopulations (day 0).

It can be observed that, at day 0, a significantly higher percentage of CD16 positive cells stained by VEP13 "VEP13 ^{high}" is observed in patients suffering from sepsis (cf. Figure 4A), as compared with the percentage of CD16 positive cells "CD16 ^{high}" in SIRS suffering patient ($P < 0.001$, Mann Whitney test). On the contrary, the percentage of $CD16^+$ / VEP13 ^{high} cells in patients suffering from sepsis is similar to the one observed in healthy donors.

Figure 4 (B) represents the mean fluorescent intensity (MFI) of CD16 positive cells in 9 SIRS and 7 septic patients at suspicion of infection in ICU (day 0) among the VEP13 positive cells. Horizontal bars represent the median values.

It can be observed that the MFI associated to VEP13 staining on CD16 positive cells from healthy donors and patients suffering from sepsis is higher than the MFI associated to VEP13 staining on CD16 positive cells from SIRS patients at day 0 ($P < 0.0001$, Mann Whitney test).

It can be concluded that, surprisingly, the VEP13 epitope is exposed preferentially at the surface of CD16 positive cells from healthy donors and sepsis patients.

This allows to discriminate reliably ICU patients suffering from SIRS symptoms and carrying a bacterial infection ("sepsis" patients, having a high percentage of VEP13 ^{high} / $CD16^+$ cells or a high MFI associated to VEP13 staining) from those suffering from SIRS symptoms without any bacterial infection ("SIRS" patients, having almost no VEP13 ^{high} / $CD16^+$ cells or a low MFI associated to VEP13 staining).

2. Concerning the TLR2 and TLR4 markers

2.1. Sepsis and SIRS modify the expression of TLR2 and TLR4 on NK cells

NK cells are identified by staining whole blood cells with anti-CD3 and anti-CD56 antibodies. As previously described, $CD56^{bright}$ cells correspond to $CD3^-$ / $CD56^{+++}$ cells, whereas $CD56^{dim}$ cells correspond to $CD3^-$ / $CD56^{++}$ cells (see figure 1 upper panels). It has been shown by Venet et al (*Shock*, 2010) that the absolute number of NK cells is diminished in patients suffering from SIRS and sepsis. This has been confirmed in the present study for both $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells (see figure 7).

What is known about TLR expression in NK cells relies on mRNA analysis. Furthermore, both mRNA [Chalifour A. et al, *Blood* 2004; Lauzon N.M. et al, *Cell Immunol* 2006; Hornung V. et al, *J. Immunology* 2002] and protein surface expression on human NK cells [Flo T.H. et al., *J. Leukoc. Biol.* 2001; Becker I. et al, *Mol. Biochem. Parasitol.* 2003] remain controversial.

The expression of TLR2 and TLR4 on and within NK cell subsets was analyzed (see the results on figures 8 and 9).

The analysis of surface expression of TLR2 reveals that this receptor is barely expressed on both $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells of healthy controls. This is also the case for sepsis and SIRS patients (see figure 8B).

In contrast to the surface expression, a strong intracellular expression of TLR2 was detected after cell permeabilization in both $CD56^{bright}$ and $CD56^{dim}$ subsets in healthy donors, SIRS and sepsis patients (see figure 8D). The percentage of intracellularly TLR2-positive $CD56^{dim}$ NK cells was significantly increased in sepsis and SIRS patients compared to healthy controls, whereas this percentage for $CD56^{bright}$ was not significantly different (see figure 8D upper panel).

Interestingly, mean fluorescence intensity (MFI) of TLR2 in both $CD56^{bright}$ and $CD56^{dim}$ was significantly increased for septic patients, as compared to healthy volunteers and SIRS patients (see figure 8D, lower panel and table I).

Table I: Fold increase of the MFI associated to TLR2 intracellular staining in NK cells isolated from sepsis patients, as compared with healthy subjects and SIRS patients

MFI associated to TLR2 staining in NK cells	**Mean Ratio: Sepsis patients / healthy subjects**	**Mean Ratio: Sepsis / SIRS patients**
$CD56^{bright}$	4.5 x	2.3 x
$CD56^{dim}$	2.7 x	2.0 x

Note: the calculation of the fold increase has been made after the median values

For TLR4, similarly to TLR2, the surface expression was extremely low for healthy controls and patients in terms of MFI (see figure 9B lower panel). Nevertheless, in terms of percentage of surface $TLR4^{positive}$ cells, an increase was noted in SIRS as compared to healthy controls and sepsis patients for both NK subsets (see figure 9B upper panel and table II).

Table II: Fold increase of the percentage of surface $TLR4^{positive}$ NK cells in blood samples of SIRS patients, as compared with healthy subjects and sepsis patients

% of $TLR4^{positive}$ NK cells	**mean ratio: SIRS patients / healthy subjects**	**mean ratio: SIRS / Sepsis patients**
$CD56^{bright}$	10 x	1.7 x
$CD56^{dim}$	8 x	x 2

Note: the calculation of the fold increase has been made after the median values

Similarly to TLR2, a strong intracellular expression of TLR4 was noted for all three groups. In terms of percentage of intracellularly $TLR4^{positive}$ cells, a significantly higher amount of

NK cells was found for the sepsis patients for both $CD56^{bright}$ and $CD56^{dim}$ and for the SIRS patients for $CD56^{dim}$, as compared to healthy controls (figure 9D, upper panel).

In terms of MFI, similarly to TLR2, an increase was noted for both $CD56^{bright}$ and $CD56^{dim}$ NK cells of septic patients, as compared to healthy volunteers and SIRS patients (see figure 9D lower panel and table III).

Table III: Fold increase of the MFI associated to TLR4 intracellular staining in NK cells isolated from sepsis patients, as compared with healthy subjects and SIRS patients

MFI associated to TLR4 staining in NK cells	**Mean Ratio: Sepsis patients / healthy subjects**	**Mean Ratio: Sepsis / SIRS patients**
$CD56^{bright}$	3.7 x	2,5 x
$CD56^{dim}$	5.1 x	4,25 x

Note: the calculation of the fold increase has been made after the median values

Altogether, these differential expression levels of intracellular and surface TLR in NK cells allow discriminating between patients suffering from SIRS from those suffering from sepsis as:

- As compared with healthy individuals, a higher percentage of NK cells expressing high level of surface TLR4 allows to identify a SIRS suffering patient,
- As compared with healthy individuals, a similar percentage of NK cells expressing high level of surface TLR4 allows to identify a sepsis suffering patient,
- As compared with healthy individuals, the presence of NK cells having a high level of intracellular TLR2 or TLR4 allows identifying a sepsis suffering patient.

However, no modification of intracellular TLR9 (SEQ ID NO:11) was observed in SIRS and sepsis patients as compared to healthy controls, either in terms of percentage of positive cells or in terms of MFI (see figure 10).

2.2. *Other results concerning the TLR2/TLR4 biomarkers (published in Souza-Fonseca-Guimaraes et al, 2012)*

2.2.1. *Patients' characteristics*

Eleven patients were classified as non-infectious SIRS, and 27 were defined as sepsis patients. Patient characteristics are described in Table 1. Among sepsis patients, the lung was the primary site of sepsis in almost two thirds of the sepsis patients. Twelve patients had Gram-positive infections. Eight patients had an isolated Gram-positive infection, three involved a Gram-positive and a Gram-negative bacterium and one a Gram-positive bacterium and Mycobacterium tuberculosis. Sixteen patients had Gram-negative infections, with one (11 patients) or two (2 patients) different bacteria or with concomitant Gram-positive infection (3 patients). A total of 19 Gram-negative bacteria were identified, including *Escherichia coli* in 7 cases, *Klebsiella pneumoniae* in 4 cases, *Hemophilus influenzae* in 2 cases, and *Pseudomonas aeruginosa* in 2 cases. In terms of Gram-positive bacteria, *Staphylococcus aureus* was found in 5 cases, *Streptococcus* spp. in 4 and *Streptococcus pneumoniae* in 1 case. *Mycobacterium tuberculosis* was found in only one case as was *Candida albicans*. More than one pathogen was found in six cases.

2.2.2. *Sepsis and SIRS modify the expression of TLR2 and TLR4 in NK cells*

The expression of TLR2 and TLR4 on and within NK cell subsets was analysed. Surface expression of TLR2 was barely detectable on both $CD56^{bright}$ and $CD56^{dim}$ subsets of NK cells for all groups both in terms of percentage of positive cells and mean fluorescence intensity (MFI) (Figure 13A). In contrast, a strong intracellular expression of TLR2 was detected after cell permeabilization in both subsets in healthy donors, as well as in SIRS and sepsis patients (Figure 14A). The percentage of intracellular TLR2-positive $CD56^{dim}$ NK cells was significantly increased in sepsis and SIRS patients compared to healthy

controls. Interestingly, the MFI for TLR2 in both $CD56^{bright}$ and $CD56^{dim}$ was significantly increased for sepsis patients, as compared to healthy volunteers.

For TLR4, similarly to TLR2, surface expression was extremely low for healthy controls and sepsis patients in terms of MFI. Nevertheless, in comparison to healthy controls and sepsis patients, SIRS patients showed an increase in the percentage of surface TLR4-positive cells for both NK subsets (Figure 13B).

In keeping with the trend observed for TLR2, a strong intracellular expression of TLR4 was noted for all three groups (Figure 14B). There is a significantly higher percentage of intracellular TLR4-positive NK cells in SIRS and sepsis patients for both $CD56^{bright}$ and $CD56^{dim}$ cells in comparison to healthy controls. In terms of MFI, an increase was again noted for both $CD56^{bright}$ and $CD56^{dim}$ NK cells of septic patients, as compared to healthy volunteers.

In contrast to TLR2 and TLR4, no difference was found in the expression of TLR9, the receptor for CpG-DNA (data not shown), both in terms of percentage of positive cells or MFI.

3. **Change of CD24 expression in sepsis patients**

While it was known that delayed neutrophil apoptosis presents itself and can be detected in inflammatory pathologies, and that CD24 is also expressed in neutrophils, Applicants sought to determine the role of CD24 in sepsis and its putative role in cellular death.

The present inventors analyzed blood samples from healthy donors or from sepsis patients at the onset of sepsis and the two following day. Specifically, they analyzed the expression of surface CD24 neutrophils. Neutrophils from healthy donors or from sepsis patients were cross-linked with antihuman CD24 and assessed for viability after 24 hours of culture. In some experiments, neutrophils were pre-incubated with pan-caspase inhibitor (z-VAD-fmk), Necrostatin-1, DPI or Cloroquine before crosslinking. Neutrophils were challenged with lipopoplysaccharide (LPS), heat-killed *S. aureus*, heat-killed *E. coli* or with sepsis plasma and CD24 expression was assessed by, for example, flow cytometry.

3.1. *Material and Methods.* Blood samples were either collected from healthy donors or from sepsis patients at the onset of sepsis and the two following days and surface CD24 expression on neutrophils was analyzed by flow cytometry. Peripheral blood neutrophils were purified from sepsis patients and healthy individuals by positive selection.

Whole blood or neutrophils were challenged with lipopoplysaccharide (LPS), tumor necrosis factor (TNF), granulocyte-macrophage colony stimulating factor (GM-CSF), heat-killed *S. aureus* or heat-killed *E. coli* and CD24 expression assessed by flow cytometry. Neutrophils were crosslinked with anti-human CD24 and assessed for apoptosis after 24 h of culture. In some experiments, neutrophils were preincubated with caspase inhibitor (z-VAD-fmk) before crosslinking.

3.2. *Results.* Surface expression of CD24 assessed by flow cytometry was significantly altered in neutrophils from sepsis patients compared with healthy controls. Levels of CD24 expression on CD14 positive monocytes in each patient group were examined by repeated measure one-way analysis of variance (ANOVA) and Tuckey posttest. Statistics were generated using GraphPad Prism 5 and a P- value lower than 0.05 was considered significant.

Activation of neutrophils with LPS or heat-killed bacteria in whole blood triggers a strong upregulation of CD24 at surface level despite no enhanced expression was observed when the activation was carried out in purified neutrophils. In contrast, TNF and GM-CSG up-regulated CD24 in whole blood and in purified neutrophils. CD24 cross-ligation induces caspase-independent apoptosis in human neutrophils. *Ex vivo* death responses after CD24 ligation in neutrophils from sepsis patients is currently under study.

In particular, the present Inventors have shown that:

- CD24 expression is early and persistently down-regulated on neutrophils from sepsis patients as compared with healthy and SIRS patients (see figure 12).
- CD24 induces death in human neutrophils in absence of phosphatidyl serine redistribution (representative plot) (Fig. 16 A et B).
- Anti-CD24-mediated death was unaffected by the pan-caspase inhibitor zVAD, but blocked by necroptosis inhibitor necrostatin-1 (inhibition of RIP-1

kinase). CD24-induced death required ROS generation (blocked by DPI) and autophagy activity (blocked by chloroquine, CQ) (Fig. 17A).

- Sepsis patients displayed an early and significant downregulation of CD24 surface expression in comparison with healthy donors (Fig. 17B).
- There was no alteration in CD24 expression in neutrophils after challenge with LPS and heat killed bacteria (Fig 18A). In contrast, plasma from patients with sepsis decreased the expression of CD24 as compared to plasma from healthy control (HD).
- Neutrophils from patients with a diagnosis of sepsis failed to undergo death after *in vitro* CD24 cross-ligation (Fig 19A).

Conclusion. This is the first report studying the role of CD24 in sepsis patients, linking homeostasis and apoptosis.

4. Comparison of CD64 expression changes between SIRS and sepsis patients

Patients

Peripheral blood was collected in sodium-heparin (BD Bioscience, San Jose, CA, USA) from healthy donors and from patients admitted in ICU with SIRS or a suspicion of sepsis included in http://clinicaltrials.gov/ct2/show/NCT01378169. 40 sepsis patients, 21 SIRS patients and 13 healthy donors were enrolled.

Material and Methods

Expression of cell surface CD64 on neutrophils was measured by flow cytometry (MacsQuant, Miltenyi Biotec). Staining and cell acquisition for flow cytometry were performed within 5 hour after blood sampling. Monoclonal antibodies were used as follows: 5 µl of phycoerythrin (PE)-anti-CD66 antibody (ASL-32, Biolegend), 5 µl of ALEXA fluor 647-anti-CD64 antibody (Biolegend) per 100 µL of whole blood. Negative controls were mouse monoclonal antibodies isotype-matched in accordance with the recommendations of the manufacturer. After 20 minutes of incubation in the dark, erythrocytes were lysed with 1 ml Facs Lyse (BD FACS™ lysing solution, BD Bioscience, Franklin Lakes, NJ, USA). After a further 10-minute incubation and centrifugation (300 g for five minutes, 4°C), the supernatant was removed and 300 µl of MACS buffer (DPBS

with 2 mM EDTA and 0.5% fetal calf serum) was added to cells. Neutrophils were gated using side scatter dot plots vs CD66 expression. The values were expressed as mean fluorescence intensity (MFI).

Statistical Analysis

CD64 expression (MFI) on neutrophils and percentage of CD64 positive neutrophils over three following days after ICU admission in each patient group were analyzed by repeated measure one-way analysis of variance (ANOVA) and Tuckey post-test. Statistics were generated using GraphPad Prism 5 and a P- value lower than 0.05 was considered significant.

Results

Applicants' data demonstrated that the expression of CD64 is significantly increased in neutrophils isolated from sepsis patients, as compared with neutrophils from heatlhy and SIRS patients for both the Mean Fluorescence Intensity (MFI) and the positive cells (see Figure 15A and B respectively). This up-regulation can be used to discriminate between patients having sepsis from those having a SIRS.

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CLAIMS

1. A method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting the Natural Killer cells (NK cells) expressing detectable surface levels of TLR4 in the total population of cells present in said sample,

b) Calculating the percentage of the NK cells detected in step a) among the said total population, and

c) comparing the percentage obtained in b) with a reference value,

wherein,

if the percentage obtained in b) is similar to the reference value, the said subject is suffering from sepsis, and

if the percentage obtained in b) is higher than the reference value, the said subject is suffering from SIRS.

2. The method according to claim 1, wherein said NK cells are $CD56^{dim}$ and / or $CD56^{bright}$ NK cells.

3. The method according to any one of claim 1 or 2, wherein said reference value is the percentage of NK cells expressing detectable surface levels of TLR4 among the total population of NK cells, which has been measured in a biological sample from a healthy individual.

4. A method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Measuring the mean intracellular expression level of TLR2 and/or TLR4 receptor of the NK cells present in said sample,

b) Comparing the mean intracellular expression level obtained in a) with a reference value,

wherein if the mean intracellular expression level of the TLR2 and/or TLR4 receptor obtained in a) is higher than the reference value, the said subject is suffering from sepsis.

5. The method according to claim 4, wherein said NK cells are $CD56^{dim}$ and / or $CD56^{bright}$ NK cells present in said sample.

6. The method according to claim 4 or 5, wherein the mean intracellular expression level of TLR4 is measured in step a).

7. The method according to claim 4 or 5, wherein the mean intracellular expression level of TLR2 is measured in step a).

8. The method according to anyone of claims 4 to 7, wherein said reference value is the mean intracellular expression level of either TLR2 or TLR4 of NK cells of a biological sample obtained from a healthy individual.

9. A method for diagnosing sepsis from a biological sample of a subject, comprising:

a) Measuring the mean surface expression level of CD24 on the neutrophils present in said sample,

b) Comparing said mean expression level obtained in a) with a reference value,

wherein,

if the mean expression level obtained in a) is lower than the said reference value, the said subject suffers from sepsis.

10. The method according to claim 9, wherein said reference value corresponds to the mean surface expression level of CD24 of neutrophils obtained from at least one healthy individual.

11. A method for diagnosing sepsis from a biological sample of a subject, comprising:

a) Quantifying the percentage of neutrophils expressing CD64 in said sample,

b) Comparing the said percentage obtained in a) with a reference value,

wherein, if the percentage obtained in a) is at least 5 times higher than said reference value, the said subject suffers from sepsis.

12. The method according to claim 11, wherein said reference value corresponds to the percentage of $CD64^{positive}$ neutrophils among the total population of neutrophils, said percentage having been measured in at least one individual who does not suffer from sepsis.

13. A method for diagnosing sepsis from a biological sample of a subject, comprising:

a) Quantifying the mean surface expression of CD64 on the neutrophils present in said sample,

b) Comparing the said mean expression obtained in a) with a reference value,

wherein, if the mean expression level obtained in step a) is at least 5 times higher than said reference value, the said subject suffers from sepsis.

14. The method according to claim 13, wherein said reference value corresponds to the mean surface expression of CD64 on neutrophils which have been collected in at least one individual who does not suffer from sepsis.

15. A method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting the cells expressing high surface levels of the VEP13 epitope in the total population of cells present in said sample,

b) Calculating the percentage of the cells detected in step a) among the said total population, and

c) comparing the percentage obtained in b) with a reference value,

wherein,

if the percentage obtained in b) is similar to the reference value, the said subject is suffering from sepsis, and

if the percentage obtained in b) is lower than the reference value, the said subject is suffering from SIRS.

16. The method according to claim 15, wherein said cells are peripheral blood monocytes expressing CD14, CD16 or both.

17. The method according to any one of claim 15 or 16, wherein said VEP13 epitope expression is detected by contacting the said total population of cells with an anti-CD16 IgM monoclonal antibody that binds to the VEP13 epitope, preferably the VEP13 antibody.

18. The method according to any one of claim 15 to 17, wherein said reference value is the percentage of the peripheral blood monocytes expressing high surface levels of the VEP13 epitope among the total population of peripheral blood monocytes, which has been measured in a blood sample from a healthy individual.

19. A method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, the method comprising:

a) Detecting in said sample the cells expressing CD16 at their surface,

b) Measuring the mean expression level of the VEP13 epitope on the surface of the cells detected in step a),

c) Comparing the mean expression level obtained in b) with a reference value,

wherein if the mean expression level of the VEP13 epitope obtained in b) is similar to the reference value, the said subject is suffering from sepsis.

20. The method according to claim 19, wherein the mean surface expression level is measured on the peripheral blood monocytes present in said sample.

21. The method according to anyone of claims 19 or 20, wherein said reference value is the mean surface expression level of the VEP13 epitope of peripheral blood monocytes of a healthy individual.

22. A method of diagnosing sepsis, *in vitro* or *ex vivo,* from a biological sample of a subject, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject,

b) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

c) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject, and

d) performing the diagnosis method according to any one of claim 15 to 21 on a biological sample of said subject.

23. The method according to claim 22, said method comprising at least the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject, and

b) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject.

24. The method according to claim 22, comprising at least the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject, and

b) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject.

25. The method according to claim 22, comprising at least the following steps:

a) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

b) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject.

26. The method according to claim 22, comprising the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject,

b) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject, and

c) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject.

27. The method according to claim 22, comprising the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject,

b) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

c) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject, and

d) performing the diagnosis method according to any one of claim 15 to 21 on a biological sample of said subject.

28. The method according to any one of claims 22 to 27, wherein said subject is a human individual who presents at least two symptoms of SIRS.

29. Use of at least one marker selected from the group consisting of: the VEP13 epitope of CD16, the intracellular TLR4 receptor, the surface TLR4 receptor, the intracellular TLR2 receptor, the CD24 polypeptide, and the CD64 polypeptide for diagnosing sepsis in a subject in need thereof.

30. The use according to claim 29, wherein :

- Said VEP13 epitope of CD16 is detected on the surface of PBMCs,
- Said TLR4 receptor is detected on the surface of NK cells,
- Said TLR2 receptor is detected within NK cells,
- Said TLR4 receptor is detected within NK cells,
- Said CD24 polypeptide is detected on the surface of neutrophils, and /or
- Said CD64 polypeptide is detected on the surface of neutrophils.

31. The use according to claim 29 or 30, wherein two markers are selected, preferably the surface TLR4 receptor and the CD24 polypeptide.

32. The use according to any one of claim 29 to 31, wherein three markers are selected, preferably the surface TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide.

33. The use according to any one of claim 29 to 32, wherein four markers are selected, preferably the surface TLR4 receptor, the CD24 polypeptide, the intracellular TLR2 receptor and the CD64 polypeptide.

34. The use according to any one of claim 29 to 33, wherein five markers are selected, preferably the surface TLR4 receptor, the intracellular TLR4 receptor, the intracellular TLR2 receptor, the CD24 polypeptide and the CD64 polypeptide.

35. The use according to any one of claim 29 to 34, wherein the six markers are selected.

36. The use according to any one of claim 29 to 35, wherein said subject is a human individual who presents at least two symptoms of SIRS.

37. A method for designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject, the method comprising:

a) performing the diagnosis method according to any one of claim 15 to 21 on a biological sample of said subject,

b) designing an antibiotic-based treatment for said subject if the percentage of peripheral blood monocytes expressing high levels of the VEP13 epitope in said blood sample is similar to the reference value, or if the mean expression level of the VEP13 epitope in peripheral blood monocytes of said blood sample is similar to the reference value.

38. A method for designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject, the method comprising:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject,

b) designing an antibiotic-based treatment for said subject if the percentage of NK cells expressing detectable levels of TLR4 in said biological sample is similar to the reference value, or if the mean intracellular expression level of the TLR2 and/or TLR4 receptor of the NK cells in said biological sample is higher than the reference value.

39. A method for designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject, said method comprising:

a) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

b) designing an antibiotic-based treatment for said subject if the mean expression level of CD24 on the neutrophils present in said sample is lower than the reference value.

40. A method for designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject, said method comprising:

a) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject,

b) designing an antibiotic-based treatment for said subject if the percentage of the $CD64^+$ neutrophils is at least 5 times higher than said reference value, or if the mean expression level of CD64 on neutrophils is at least 5 times higher than said reference value.

41. A method for designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject,

b) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

c) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject, and

d) performing the diagnosis method according to any one of claim 15 to 21 on a biological sample of said subject.

42. The method according to any one of claims 37 to 41, wherein said subject is a human individual who presents at least two symptoms of SIRS.

43. Use of at least one marker selected from the group consisting of: the VEP13 epitope of CD16, the TLR4 receptor, the TLR2 receptor, the CD24 polypeptide, and CD64 polypeptide for designing *in vitro* or *ex vivo* an antibiotic-based treatment for a subject in need thereof.

44. The use according to claim 43, wherein :

- Said VEP13 epitope of CD16 is detected on the surface of PBMCs,
- Said TLR4 receptor is detected on the surface of NK cells,
- Said TLR2 receptor is detected within NK cells,
- Said TLR4 receptor is detected within NK cells,
- Said CD24 polypeptide is detected on the surface of neutrophils, and/or
- Said CD64 polypeptide is detected on the surface of neutrophils.

45. The use according to any one of claim 43 or 44, wherein said subject is a human individual who presents at least two symptoms of SIRS.

46. A method for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition, the method comprising:

a) performing the diagnosis method according to claim 15 to 21 on a biological sample of said subject,

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the percentage of peripheral blood monocytes expressing high levels of the VEP13 epitope in said blood sample is similar to the reference value, or if the mean expression level of the VEP13 epitope in peripheral blood monocytes of said blood sample is similar to the reference value.

47. A method for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition, the method comprising:

a) performing the diagnosis method according to claim 1 to 8 on a biological sample of said subject,

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the percentage of NK cells expressing detectable levels of TLR4 in said biological sample is similar to the reference value or if the mean intracellular expression level of the TLR2 and/or TLR4 receptor of NK cells in said biological sample is higher than the reference value.

48. A method for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition, said method comprising:

a) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the mean expression level of CD24 on the neutrophils present in said sample is lower than the reference value.

49. A method for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition, said method comprising:

a) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject,

b) selecting the subject as being predicted to benefit from the treatment with an antibiotic-containing composition if the percentage of the $CD64^+$ neutrophils is at least 5 times higher than said reference value, or if the mean expression level of CD64 on neutrophils is at least 5 times higher than said reference value.

50. A method for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition, said method comprising at least one, preferably two, more preferably three and even more preferably four of the following steps:

a) performing the diagnosis method according to any one of claim 1 to 8 on a biological sample of said subject,

b) performing the diagnosis method according to any one of claim 9 to 10 on a biological sample of said subject,

c) performing the diagnosis method according to any one of claim 11 to 14 on a biological sample of said subject, and

d) performing the diagnosis method according to any one of claim 15 to 21 on a biological sample of said subject.

51. The method according to any one of claims 46 to 50, wherein said subject is a human individual who presents at least two symptoms of SIRS.

52. Use of at least one marker selected from the group consisting of: the VEP13 epitope of CD16, the TLR4 receptor, the TLR2 receptor, the CD24 polypeptide, and CD64 polypeptide for selecting *in vitro* or *ex vivo* a subject which is predicted to benefit from the treatment with an antibiotic-containing composition.

53. The use according to claim 52, wherein :

- Said VEP13 epitope of CD16 is detected on the surface of PBMCs,
- Said TLR4 receptor is detected on the surface of NK cells,
- Said TLR2 receptor is detected within NK cells,
- Said TLR4 receptor is detected within NK cells,
- Said CD24 polypeptide is detected on the surface of neutrophils, and/or
- Said CD64 polypeptide is detected on the surface of neutrophils.

54. The use according to any one of claim 52 to 53, wherein said subject is a human individual who presents at least two symptoms of SIRS.

55. A method for treating a subject in need thereof, said method comprising administering an antibiotic-containing composition in a subject whose sepsis has been diagnosed by any of the diagnosis method disclosed in claims 1 to 21.

56. An antibiotic-containing composition for use in treating a subject whose sepsis has been diagnosed by any of the diagnosis method disclosed in claims 1 to 21.

57. A kit comprising the means for determining the expression of at least two markers selected in the group consisting of: the VEP13 epitope of CD16, the intracellular TLR2 receptor, the surface or intracellular TLR4 receptor, the surface CD24 polypeptide and the surface CD64 polypeptide.

58. The kit according to claim 57, comprising the means for determining the expression of at least the surface TLR4 receptor and the CD24 polypeptide.

59. The kit according to claim 57, comprising the means for determining the expression of at least the surface TLR4 receptor, the CD24 polypeptide and the CD64 polypeptide.

60. The kit according to claim 57, comprising the means for determining the expression of at least the intracellular TLR2 receptor, the surface TLR4 receptor, the CD64 polypeptide and the CD24 polypeptide.

61. The kit according to any one of claims 57 to 60, comprising (1) first antibodies which bind to at least two of the biomarkers of the invention; and, optionally, (2) different antibodies which bind to the first antibodies and are conjugated to a detectable label, preferably a fluorescent or luminescent label.

62. Use of a diagnosis kit comprising at least one antibody recognizing the VEP13 epitope and a detectable marker, for diagnosing sepsis from a biological sample of a subject.

63. The use according to claim 62, wherein said antibody is an IgM monoclonal antibody, preferably the VEP13 antibody.

64. Use of a kit comprising at least one antibody recognizing the Toll-like receptor 2 and a detectable marker, for diagnosing sepsis from a biological sample of a subject.

65. Use of a kit comprising at least one antibody recognizing the Toll-like receptor 4 and a detectable marker, for diagnosing sepsis from a biological sample of a subject.

66. Use of a kit comprising reagents and compositions for determining the expression of TLR2 and/or TLR4 for diagnosing sepsis from a biological sample of a subject.

67. Use of a kit comprising reagents and compositions for determining surface CD24 expression for diagnosing sepsis from a biological sample of a subject.

68. Use of a kit comprising reagents and compositions for determining surface CD64 expression for diagnosing sepsis from a biological sample of a subject.

69. The use according to any one of claim 62 to 68, wherein said subject is a human individual who presents at least two symptoms of SIRS.

Figure 1

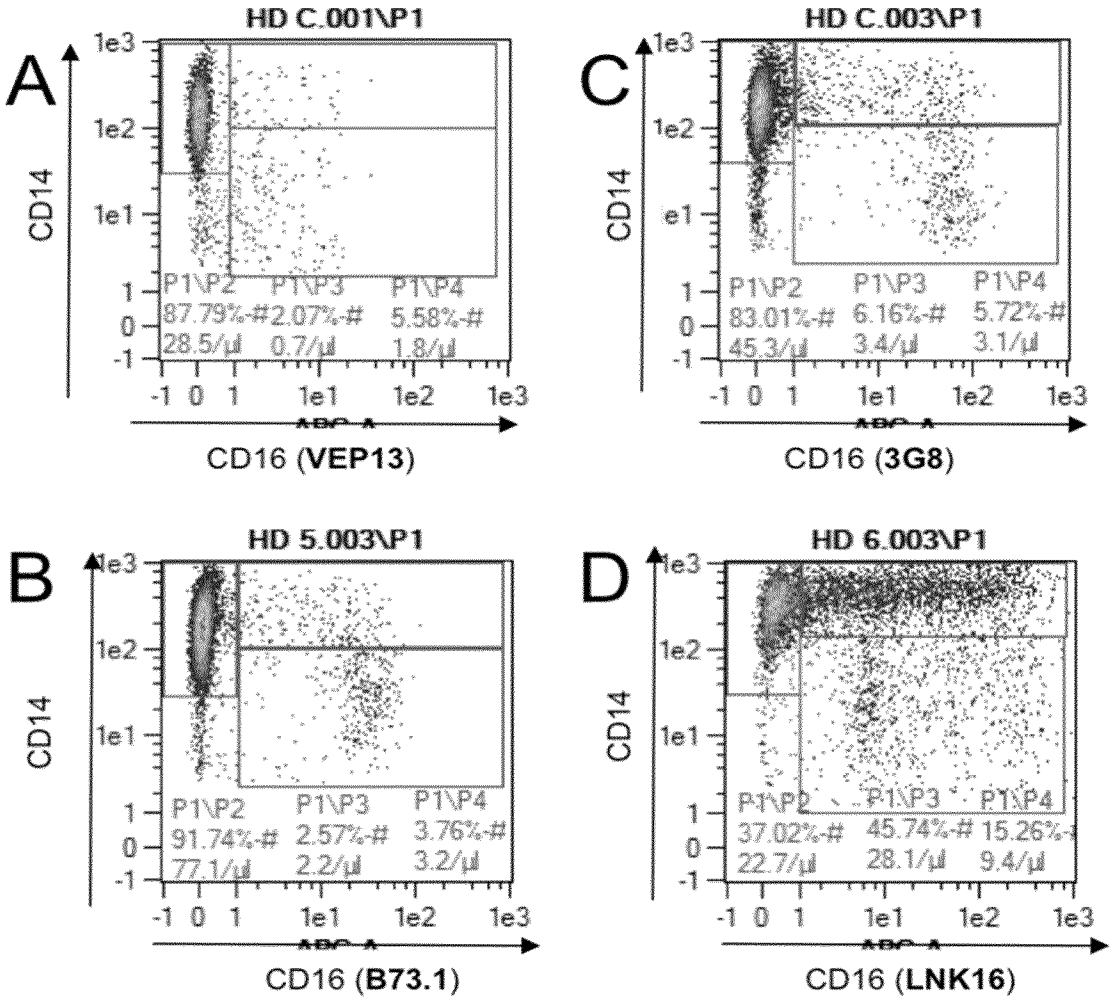

A
HD C.001\P1
CD14
CD16 (VEP13)
P1\P2 87.79%-# 28.5/µl
P1\P3 2.07%-# 0.7/µl
P1\P4 5.58%-# 1.8/µl
C
HD C.003\P1
CD14
CD16 (3G8)
P1\P2 83.01%-# 45.3/µl
P1\P3 6.16%-# 3.4/µl
P1\P4 5.72%-# 3.1/µl
B
HD 5.003\P1
CD14
CD16 (B73.1)
P1\P2 91.74%-# 77.1/µl
P1\P3 2.57%-# 2.2/µl
P1\P4 3.76%-# 3.2/µl
D
HD 6.003\P1
CD14
CD16 (LNK16)
P1\P2 37.02%-# 22.7/µl
P1\P3 45.74%-# 28.1/µl
P1\P4 15.26%- 9.4/µl

Figure 2

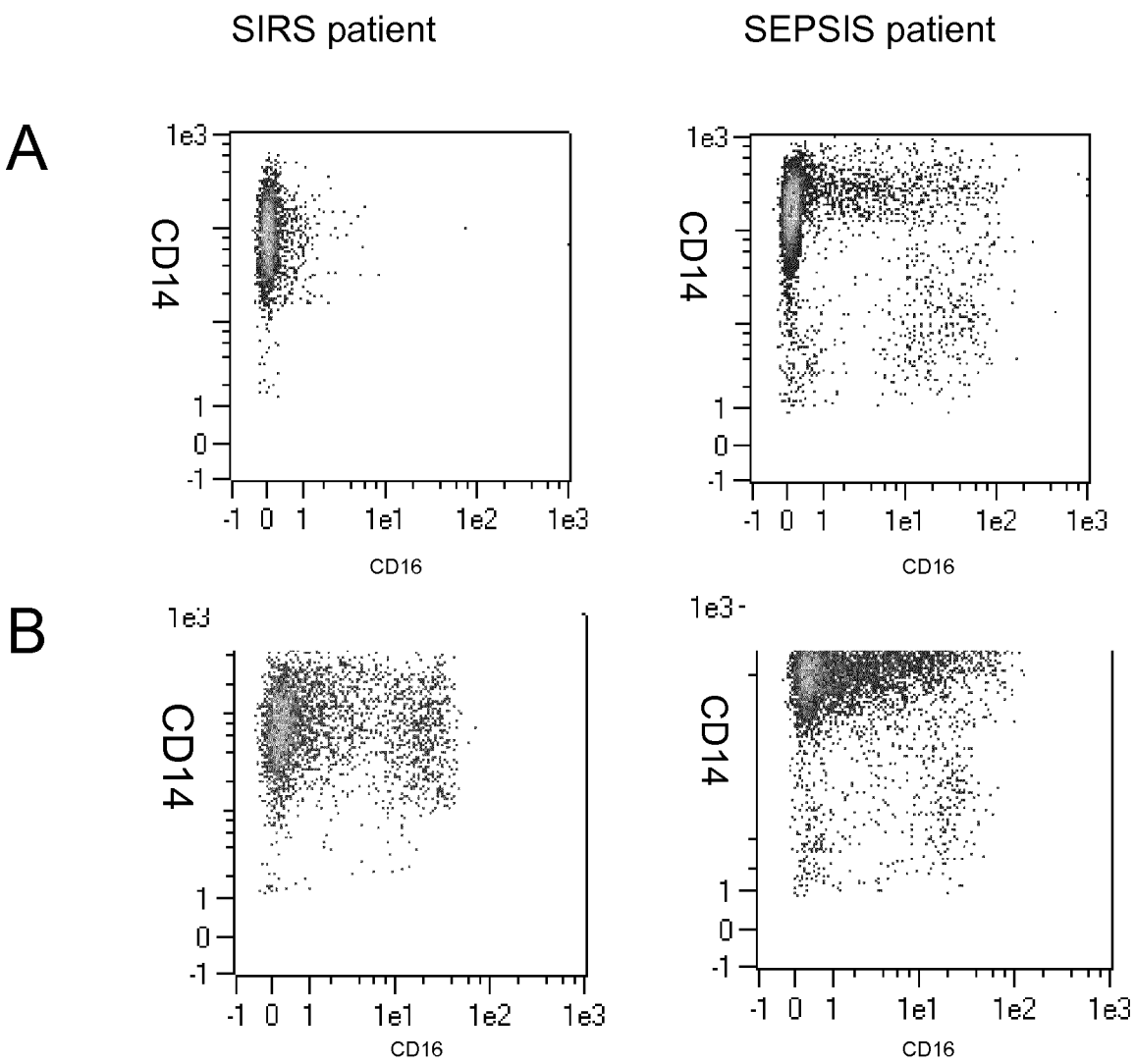
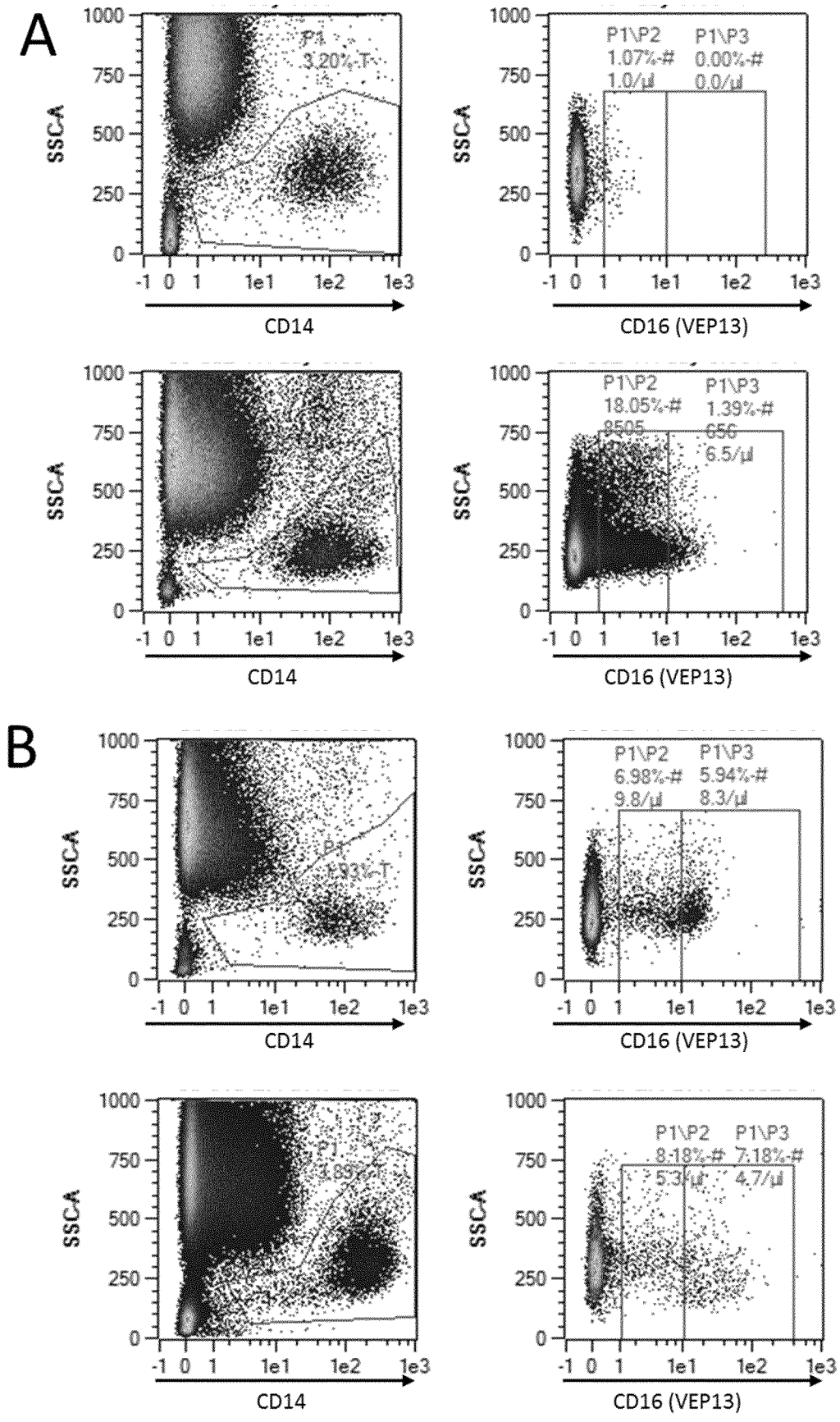


Figure 3


A
B
SSC-A
CD14
CD16 (VEP13)
P1
3.20%-T
P1\P2
1.07%-#
1.0/µl
P1\P3
0.00%-#
0.0/µl
18.05%-#
1.39%-#
6.5/µl
6.98%-#
5.94%-#
9.8/µl
8.3/µl
8.18%-#
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4.7/µl

Figure 4

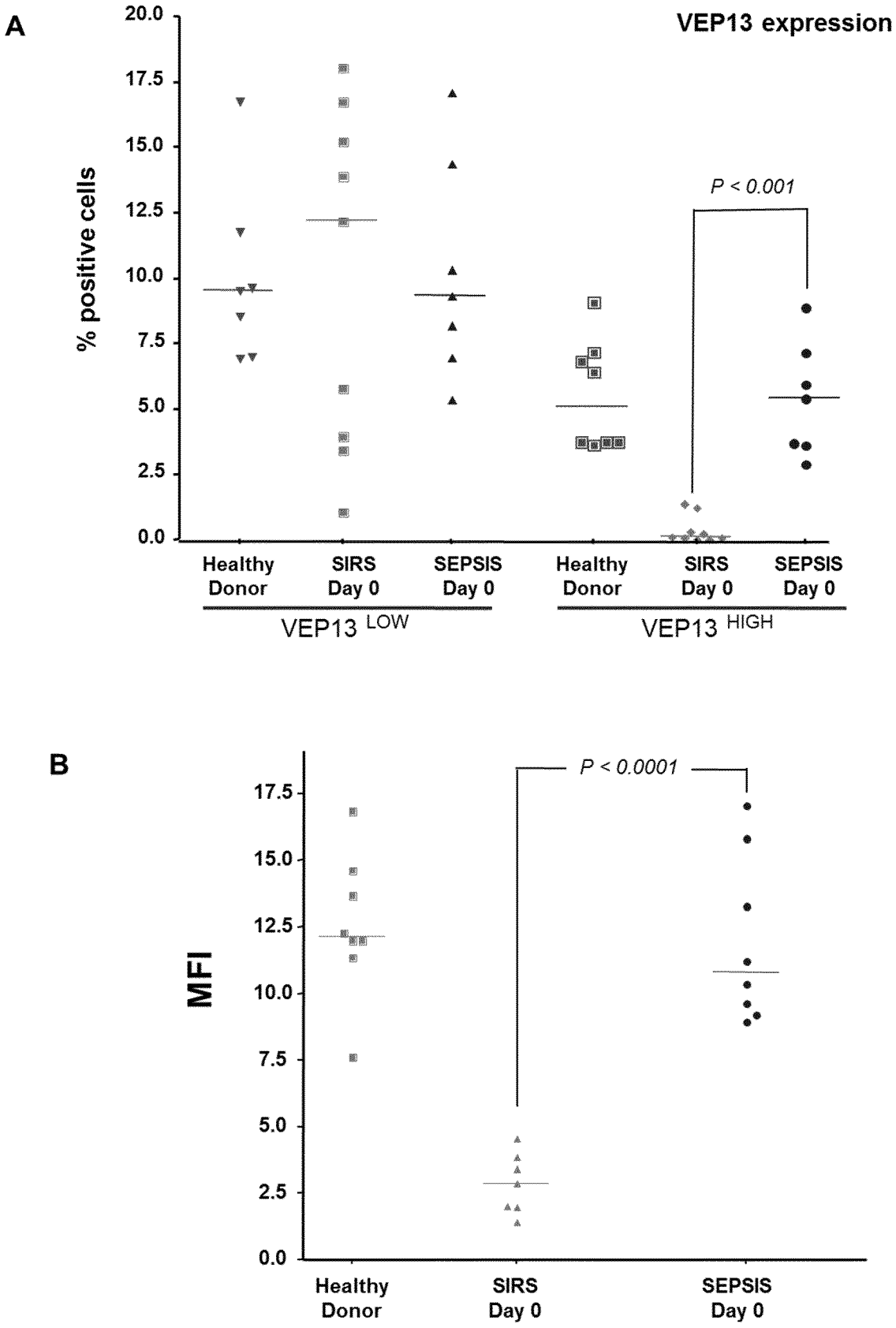

A
VEP13 expression
% positive cells
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
P < 0.001
Healthy Donor
SIRS Day 0
SEPSIS Day 0
Healthy Donor
SIRS Day 0
SEPSIS Day 0
VEP13 LOW
VEP13 HIGH
B
MFI
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
P < 0.0001
Healthy Donor
SIRS Day 0
SEPSIS Day 0

Figure 5

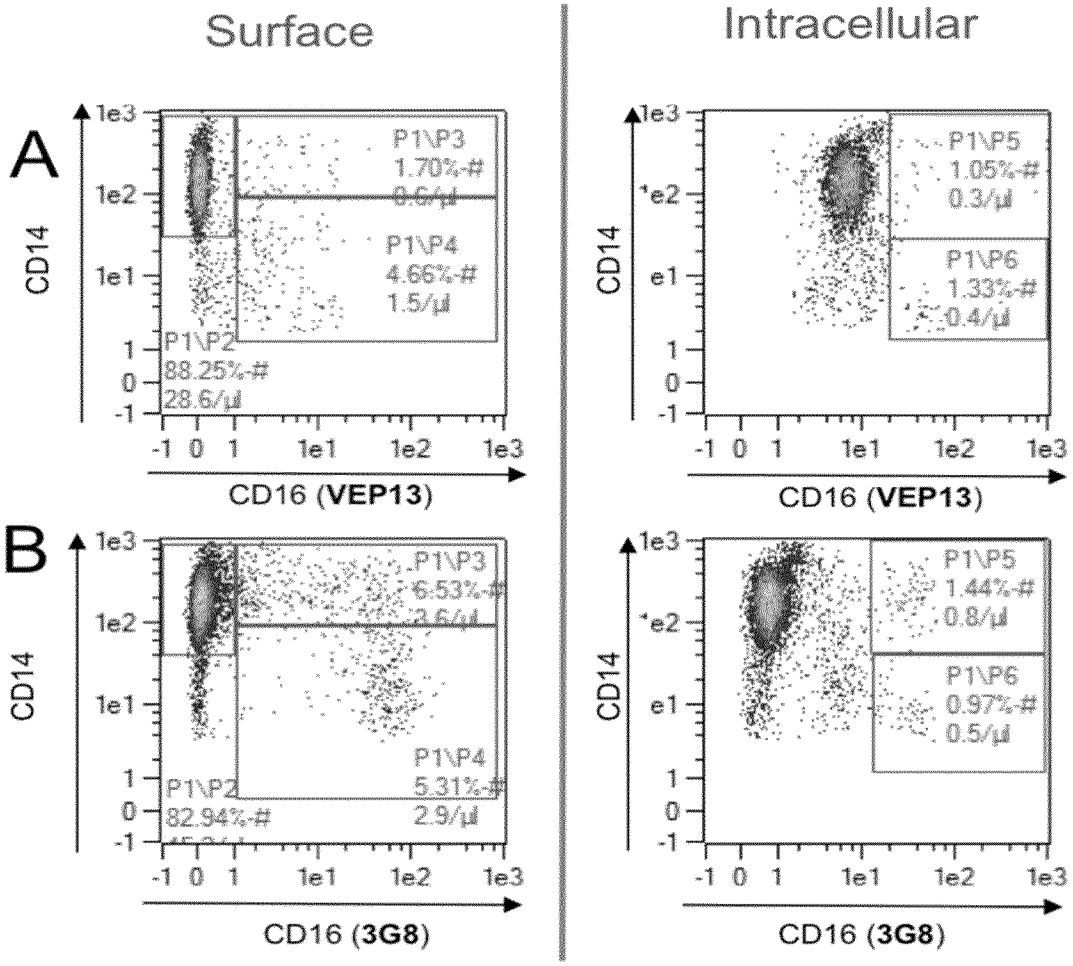

Surface
Intracellular
A
B
CD14
CD16 (VEP13)
CD16 (3G8)
P1\P3
1.70%-#
0.6/µl
P1\P4
4.66%-#
1.5/µl
P1\P2
88.25%-#
28.6/µl
P1\P5
1.05%-#
0.3/µl
P1\P6
1.33%-#
0.4/µl
P1\P3
6.53%-#
2.6/µl
P1\P4
5.31%-#
2.9/µl
82.94%-#
P1\P5
1.44%-#
0.8/µl
P1\P6
0.97%-#
0.5/µl
1e3
1e2
1e1
1
0
-1
-1 0 1 1e1 1e2 1e3

Figure 6

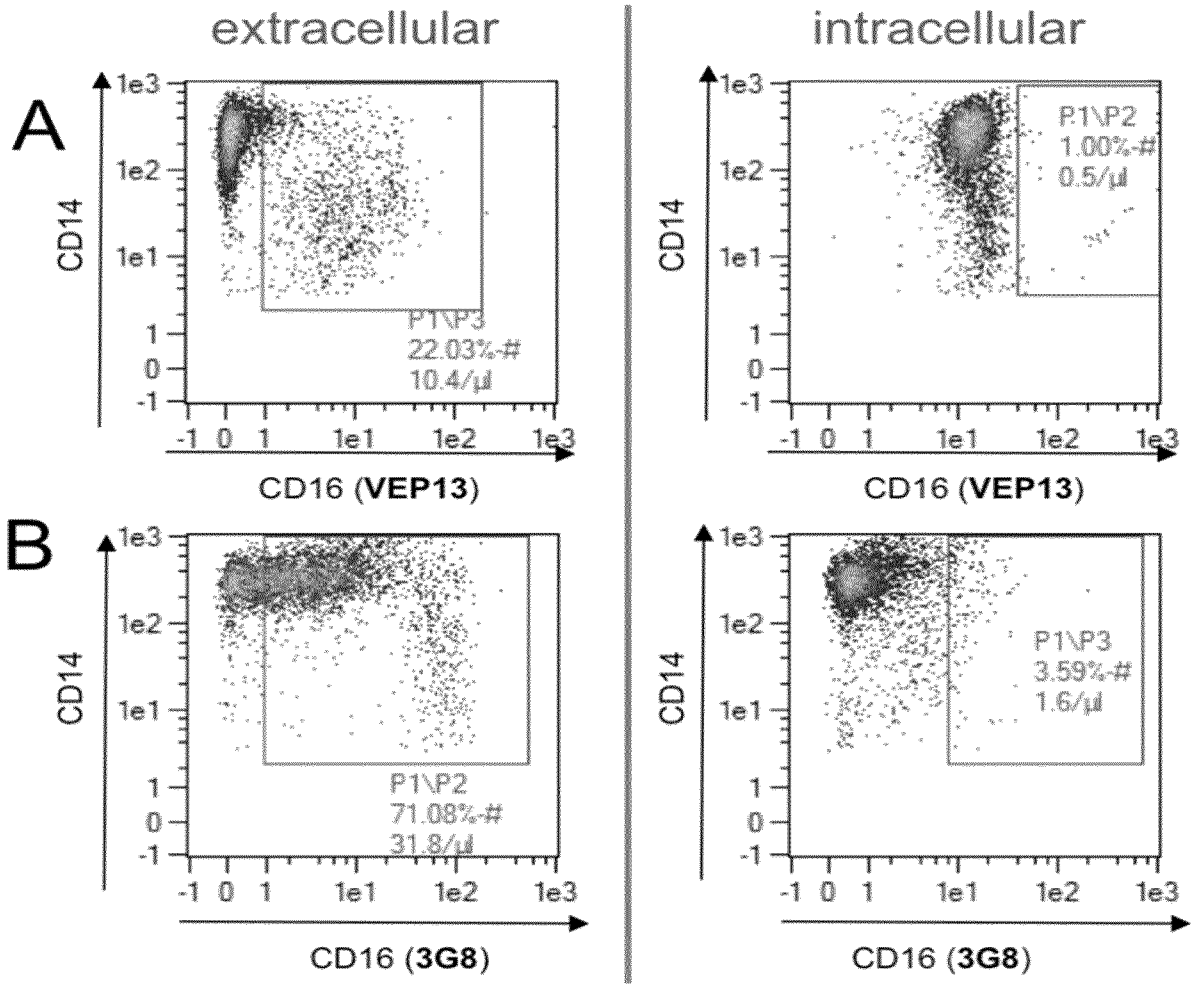

extracellular
intracellular
A
B
CD14
CD16 (VEP13)
CD16 (3G8)
1e3
1e2
1e1
1
0
-1
P1\P3
22.03%-#
10.4/µl
P1\P2
1.00%-#
0.5/µl
P1\P2
71.08%-#
31.8/µl
P1\P3
3.59%-#
1.6/µl

Figure 6 (Continued)

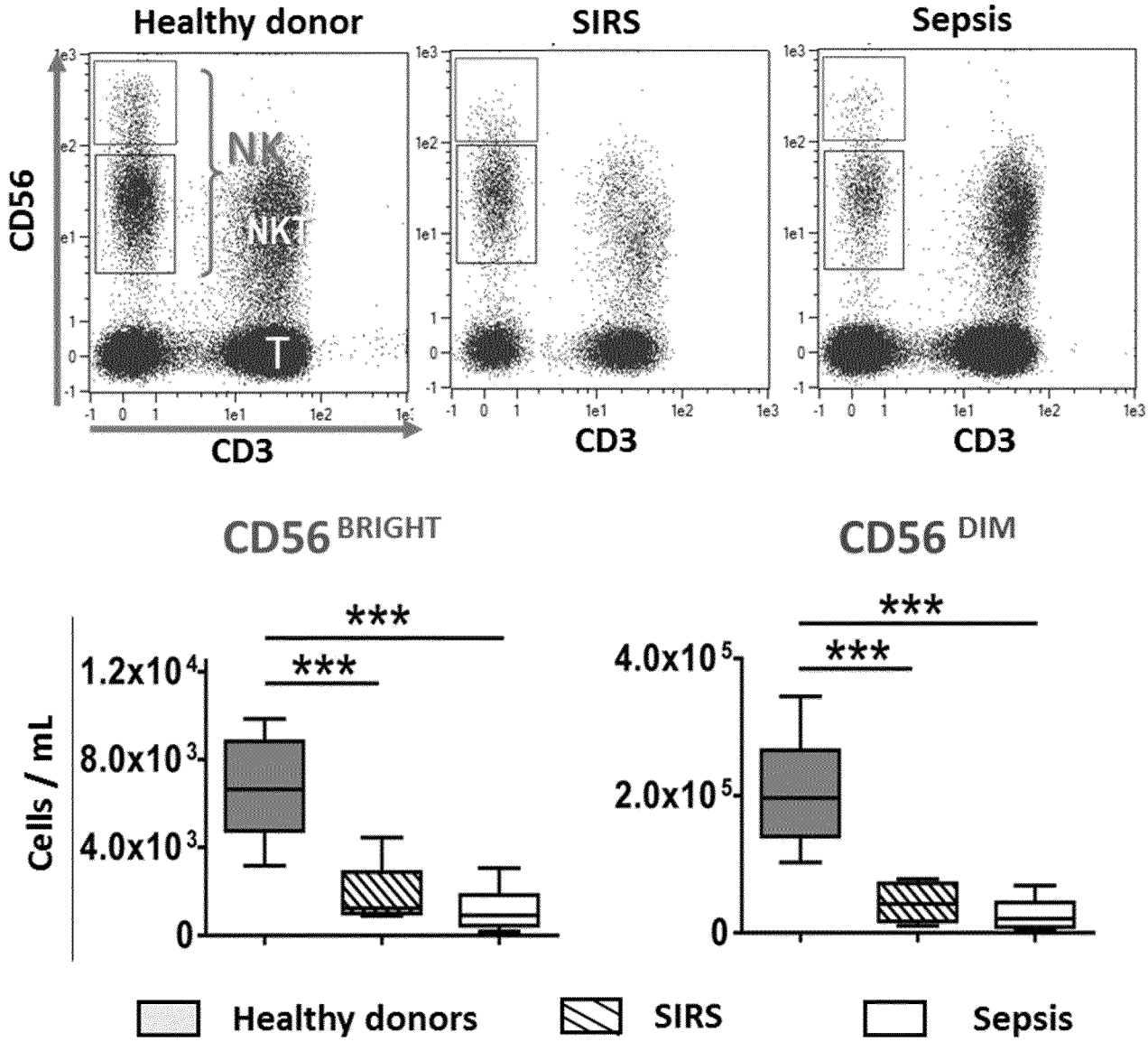


Figure 7

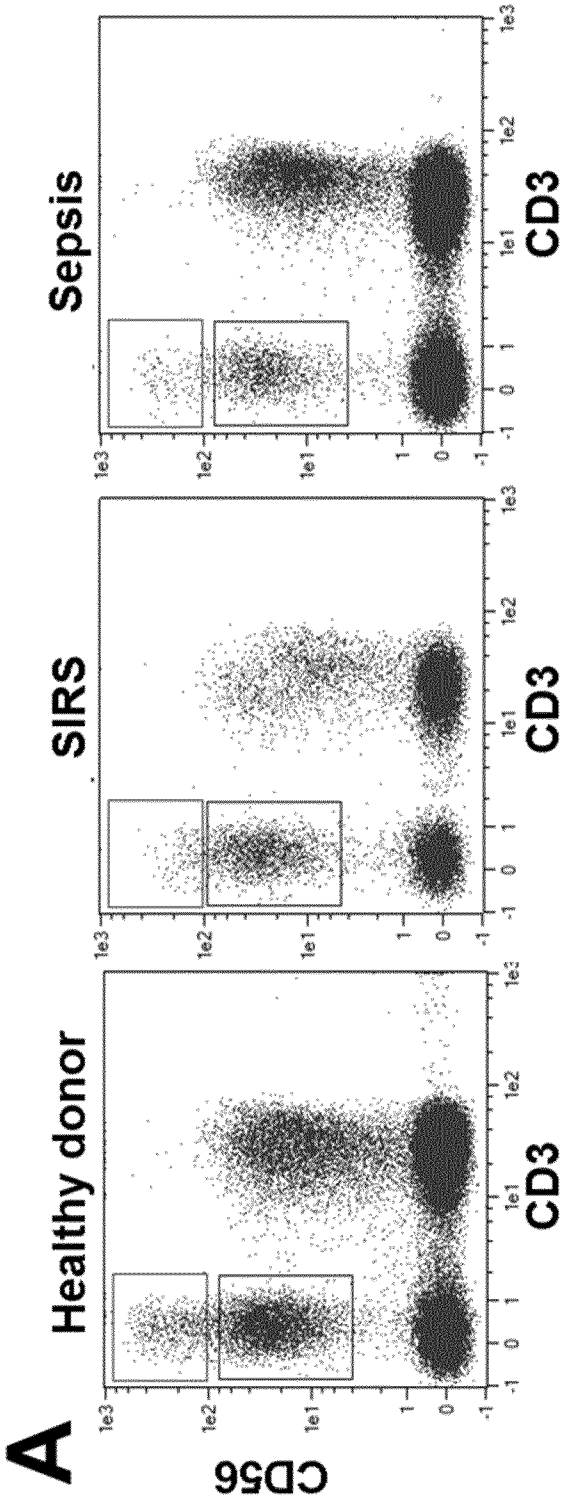


Figure 7 (continued)

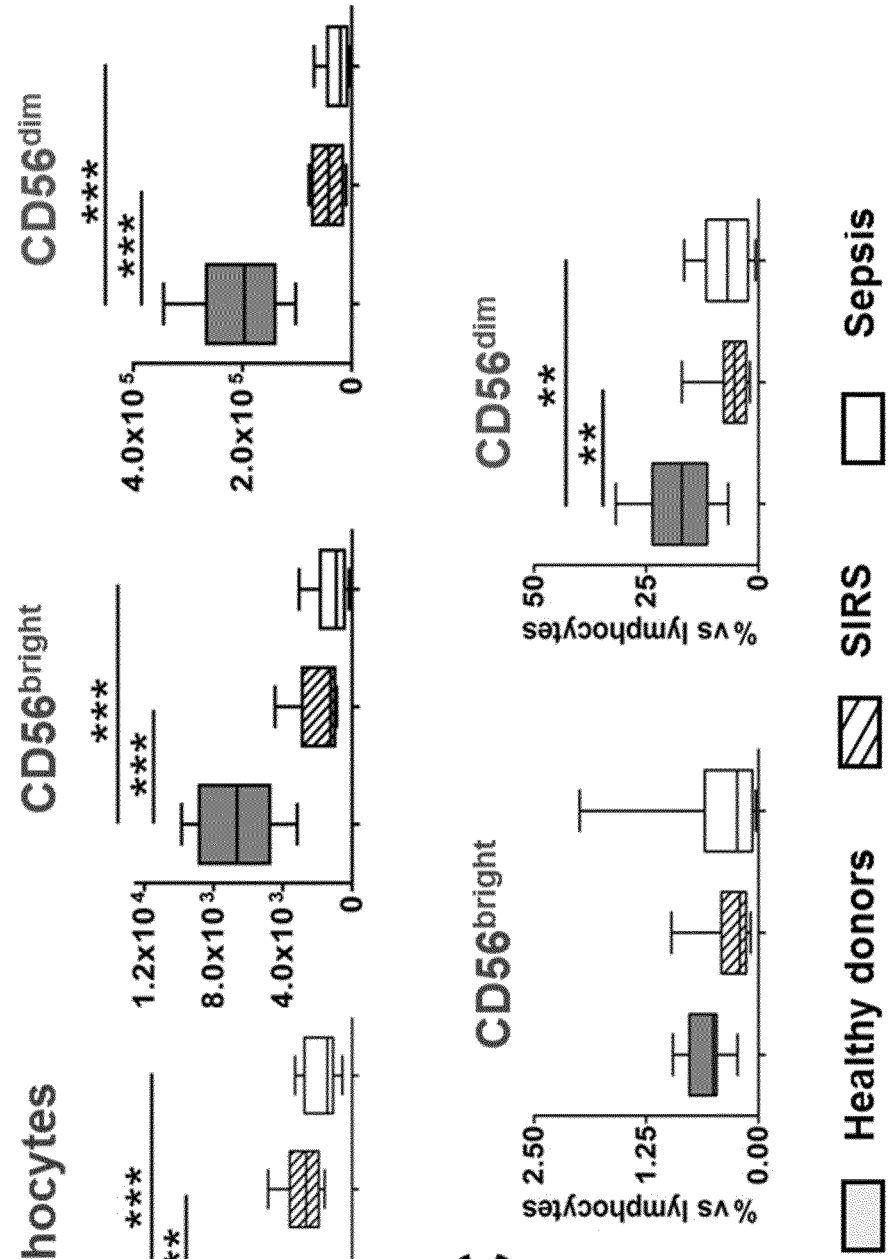


Figure 8

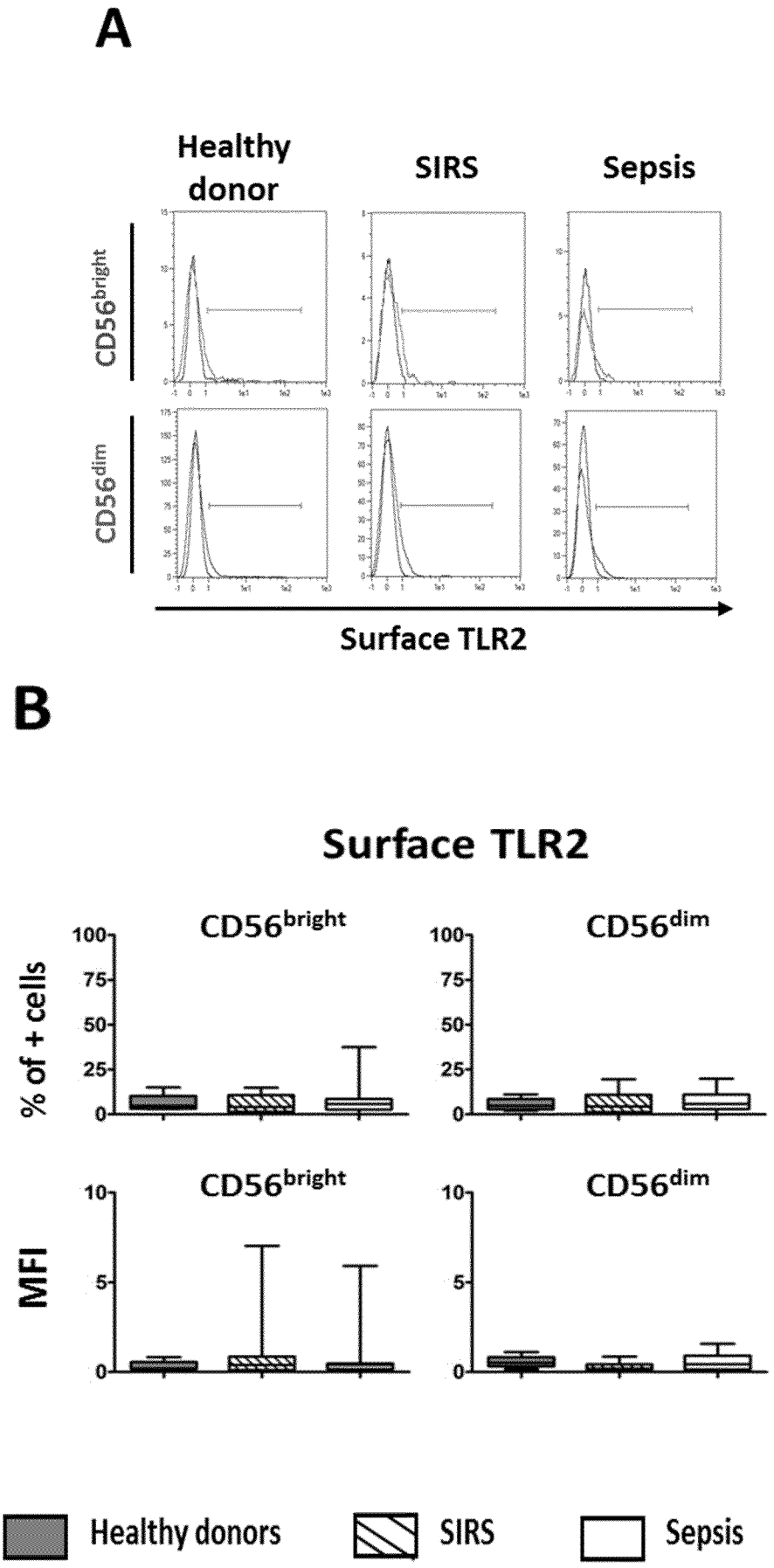
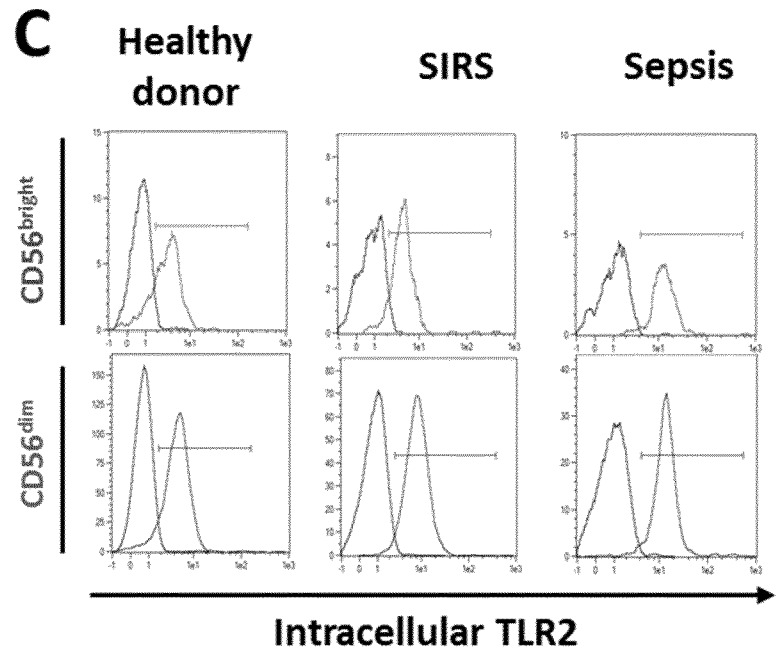

A
Healthy donor
SIRS
Sepsis
CD56bright
CD56dim
Surface TLR2
B
Surface TLR2
CD56bright
CD56dim
% of + cells
100
75
50
25
0
MFI
10
5
0
Healthy donors
SIRS
Sepsis

Figure 8 (continued)



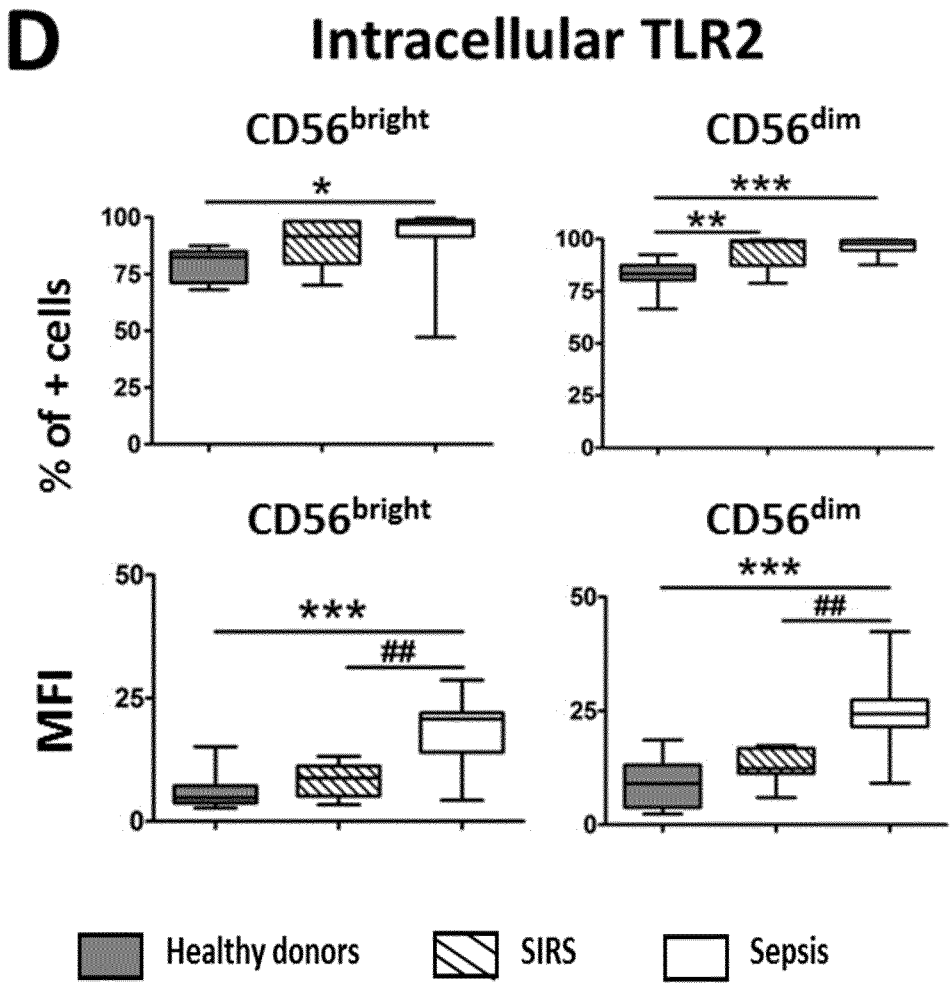


Figure 9

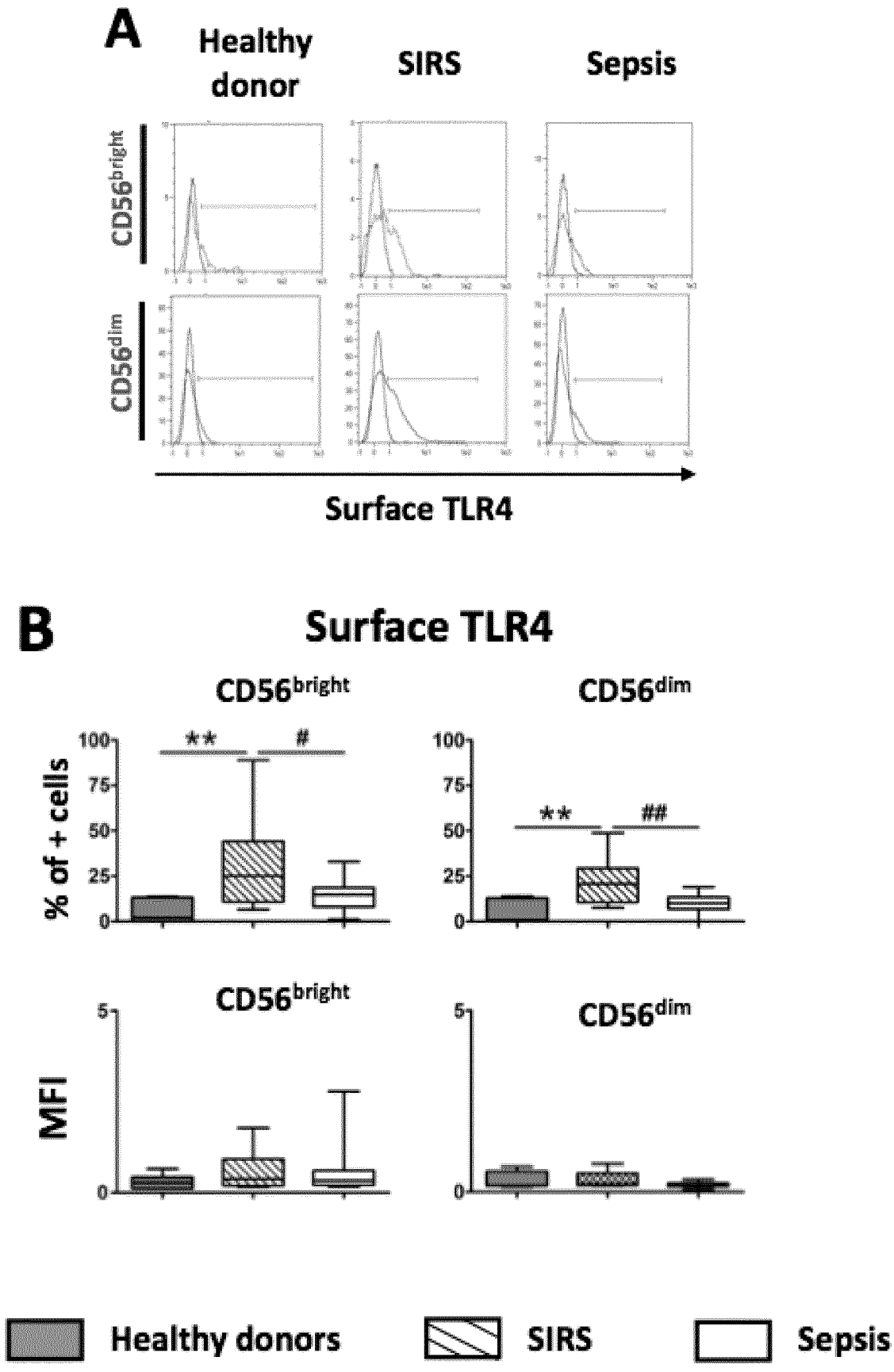

A
Healthy donor
SIRS
Sepsis
CD56bright
CD56dim
Surface TLR4
B
Surface TLR4
CD56bright
CD56dim
% of + cells
100
75
50
25
0
**
#
**
##
MFI
5
0
Healthy donors
SIRS
Sepsis

Figure 9 (continued)

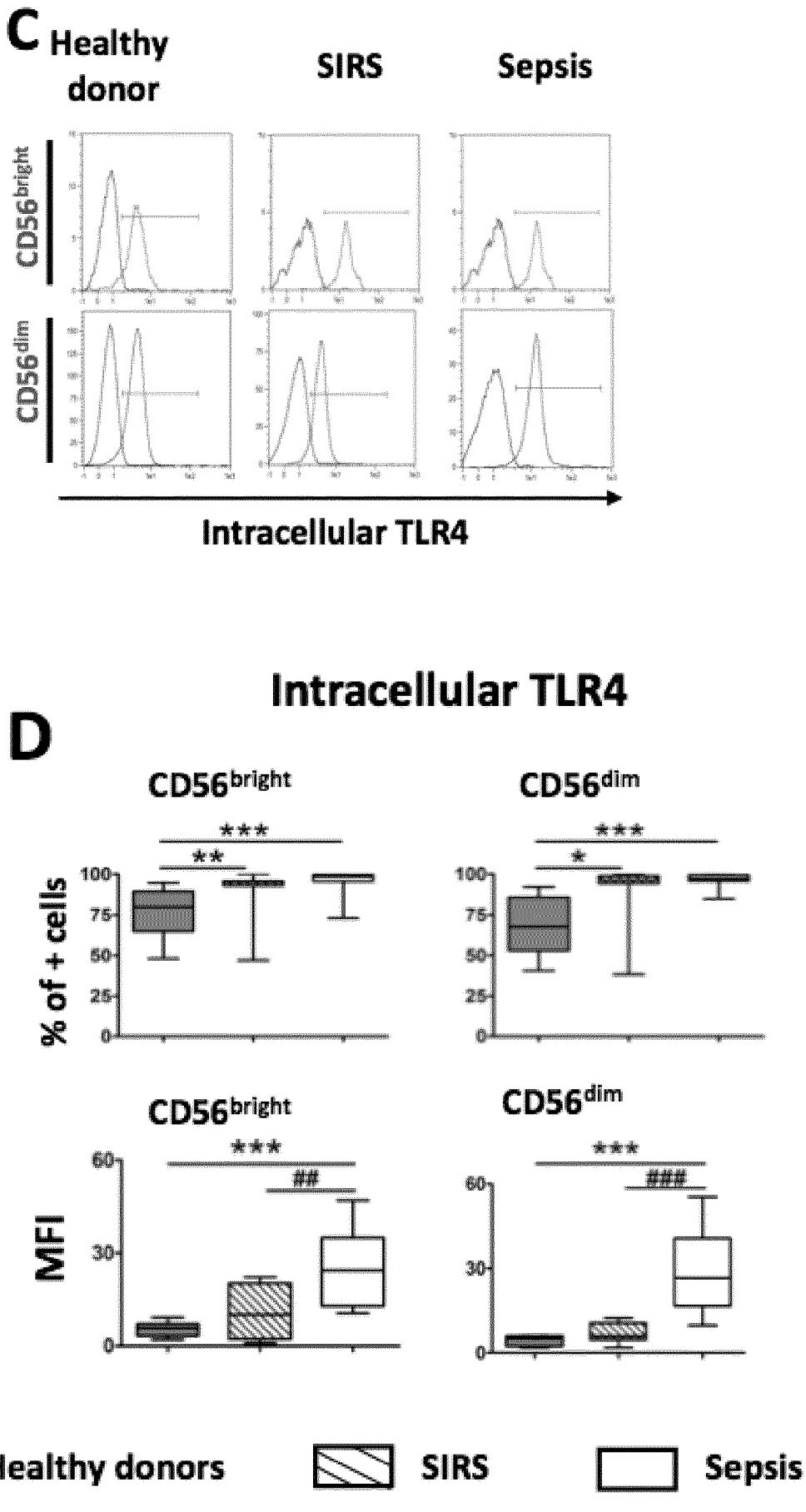


Figure 10

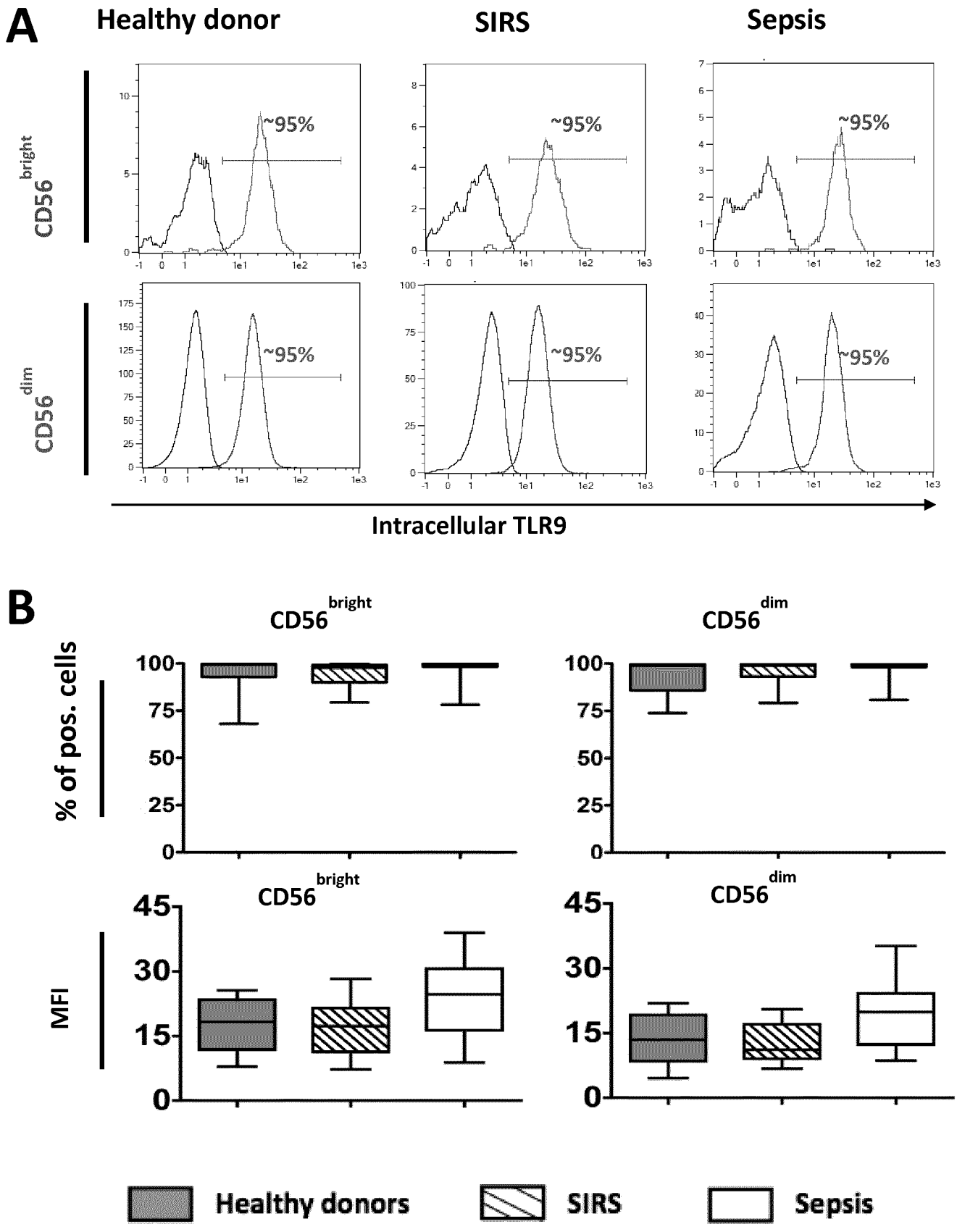

A
Healthy donor
SIRS
Sepsis
CD56 bright
CD56 dim
~95%
Intracellular TLR9
B
CD56 bright
CD56 dim
% of pos. cells
MFI
Healthy donors
SIRS
Sepsis

Figure 11

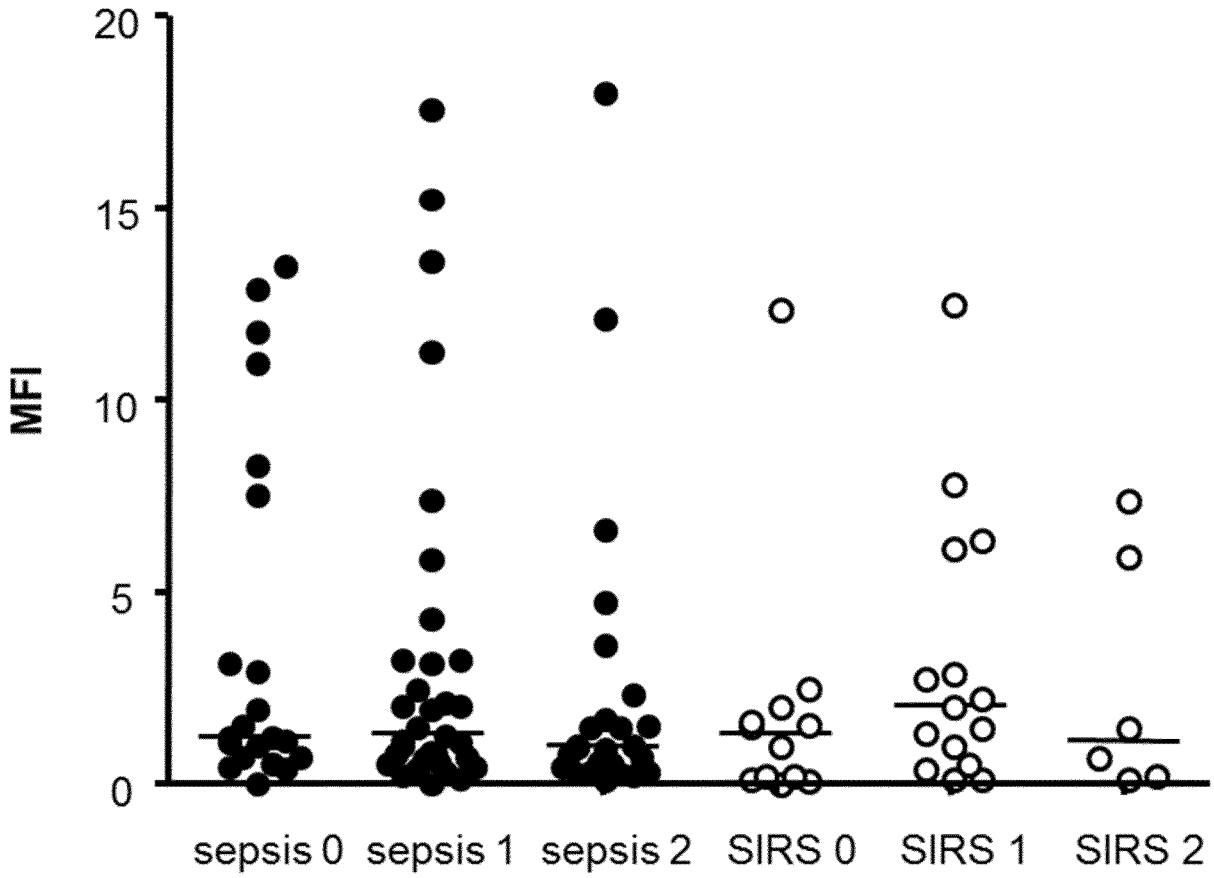
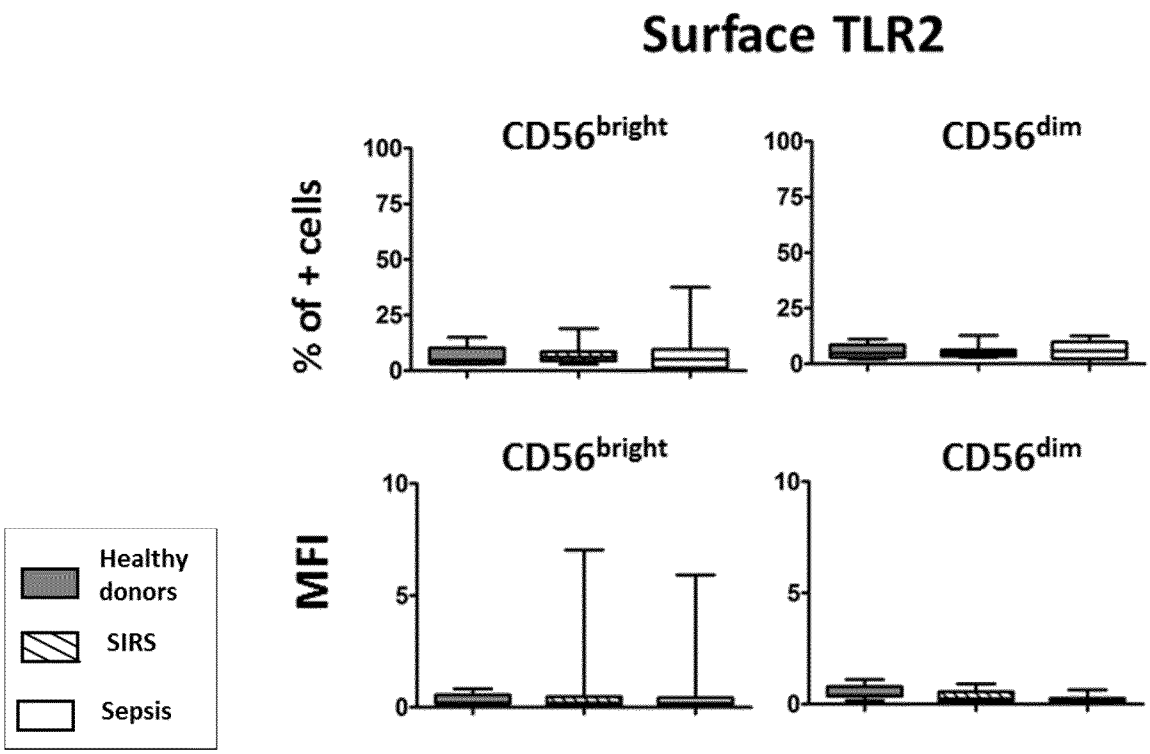


Figure 12


CD24 EXPRESSION
MFI
30
20
10
0
HD
sepsis D0
sepsis D1
sepsis D2
SIRS D0
SIRS D1
SIRS D2

Figure 13

A


Surface TLR2
CD56bright
CD56dim
% of + cells
100
75
50
25
0
MFI
10
5
0
Healthy donors
SIRS
Sepsis

B

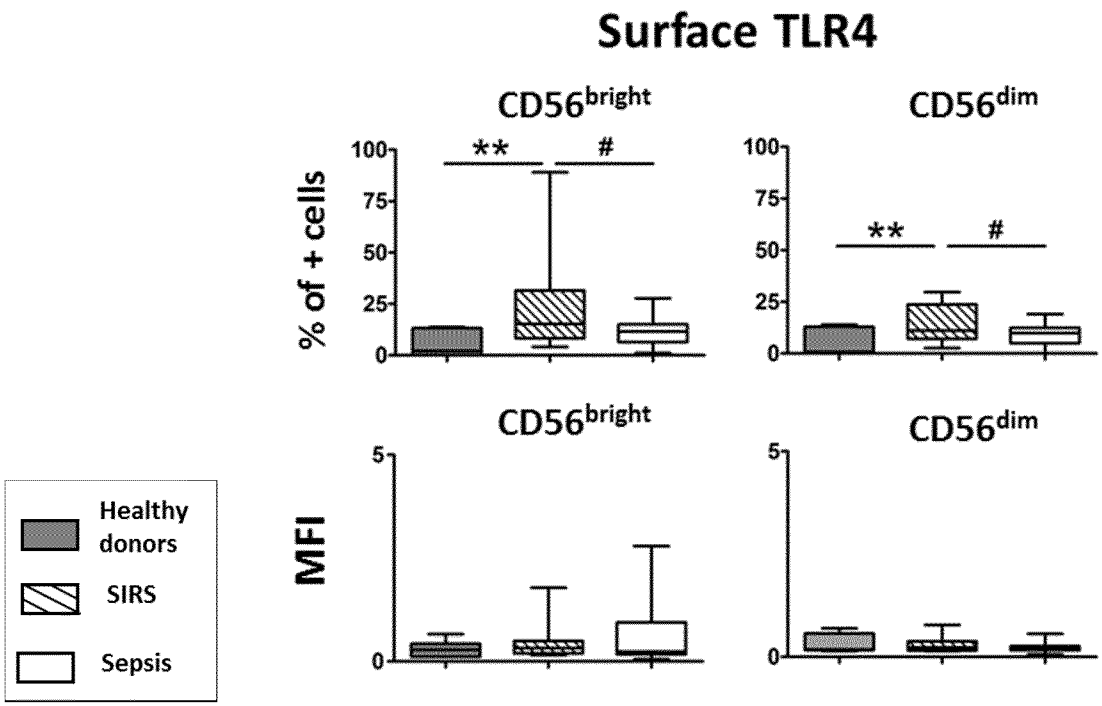
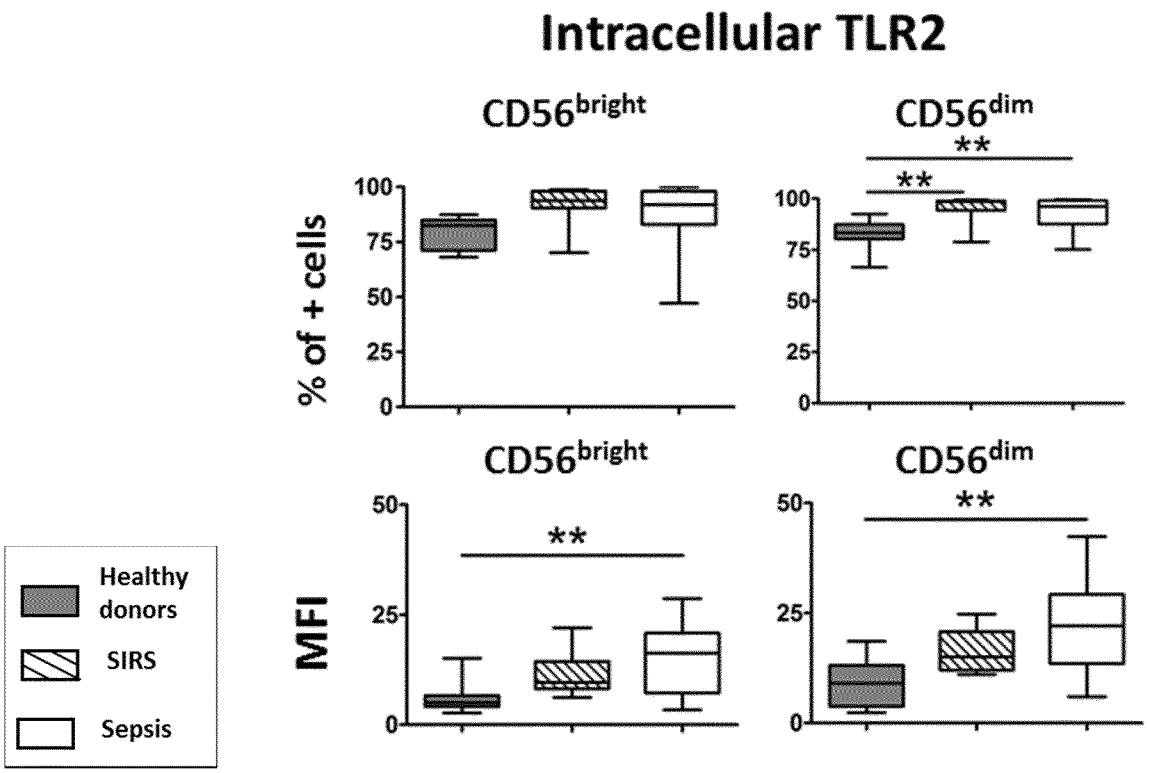

Surface TLR4
CD56bright
CD56dim
% of + cells
**
#
100
75
50
25
0
MFI
5
0
Healthy donors
SIRS
Sepsis

Figure 14

A


Intracellular TLR2
CD56bright
CD56dim
% of + cells
MFI
**
Healthy donors
SIRS
Sepsis

B

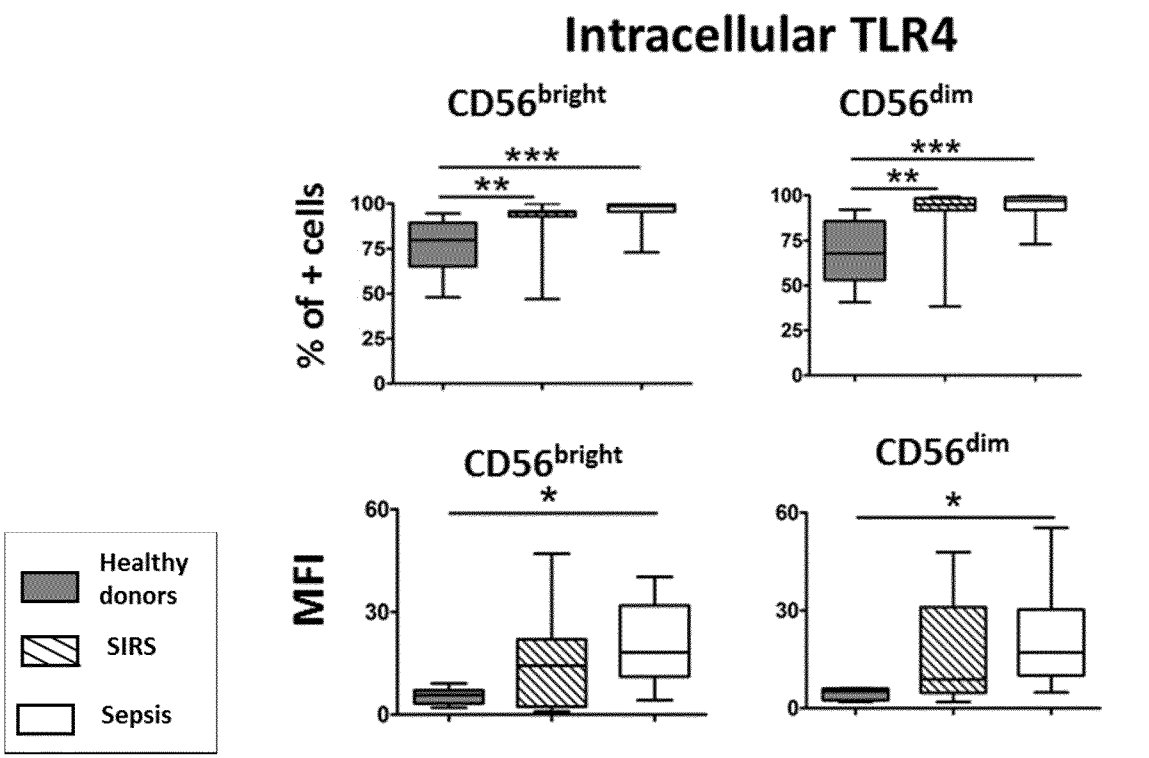

Intracellular TLR4
CD56bright
CD56dim
% of + cells
MFI
***
**
*
Healthy donors
SIRS
Sepsis

Figure 15

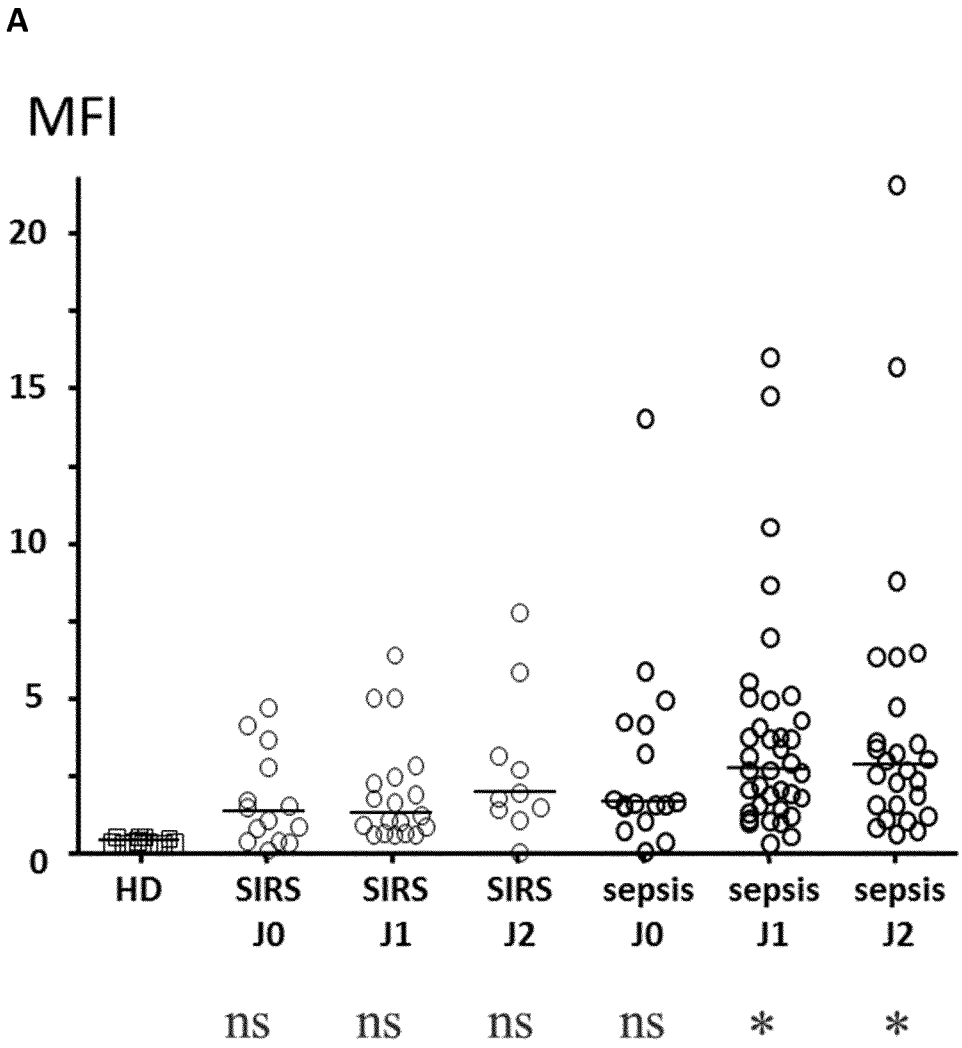

A
MFI
20
15
10
5
0
HD
SIRS J0
SIRS J1
SIRS J2
sepsis J0
sepsis J1
sepsis J2
ns
ns
ns
ns
*
*

Figure 15 (continued)

B

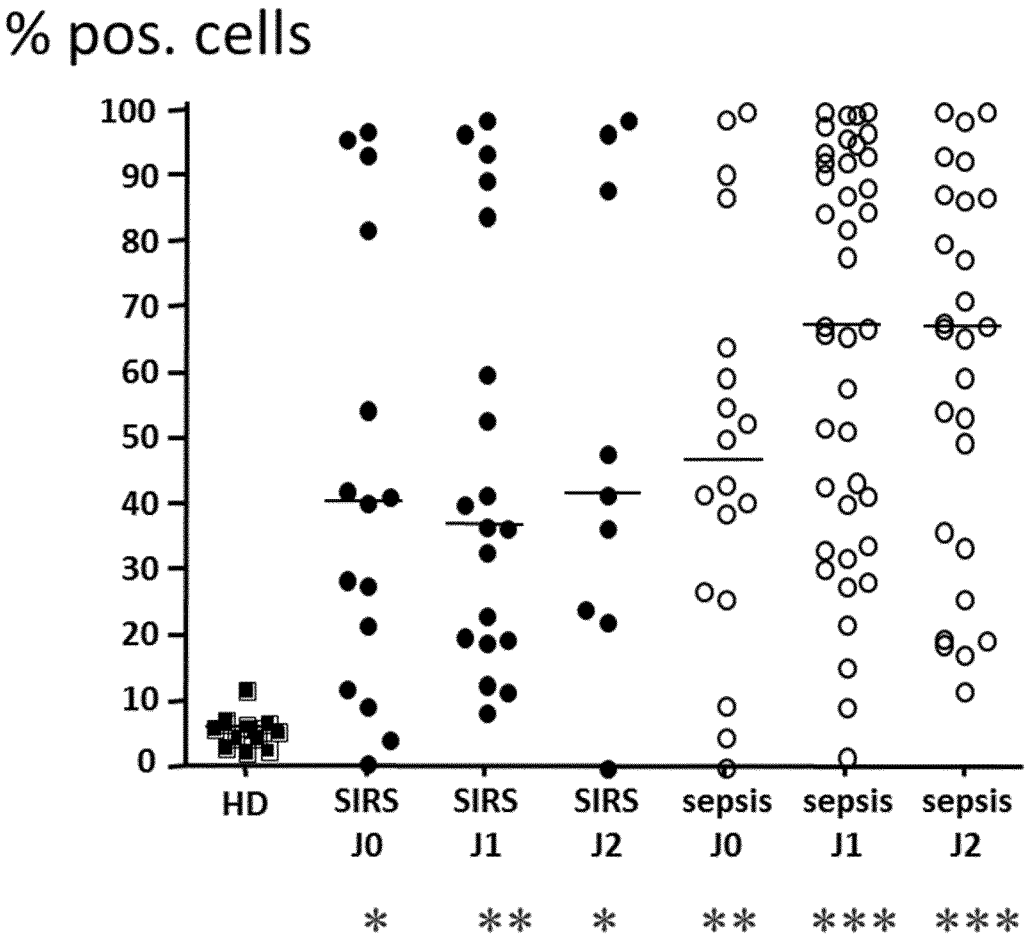
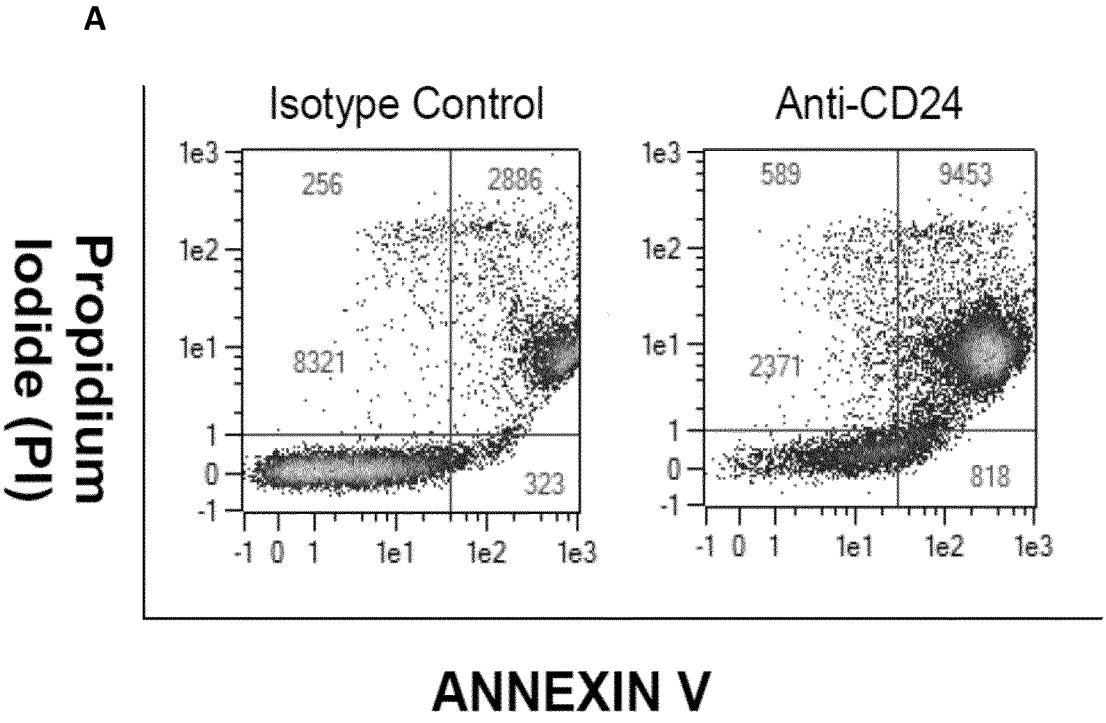


Figure 16


A
Isotype Control
Anti-CD24
Propidium Iodide (PI)
1e3
1e2
1e1
1
0
-1
256
2886
8321
323
589
9453
2371
818
-1 0 1 1e1 1e2 1e3
ANNEXIN V

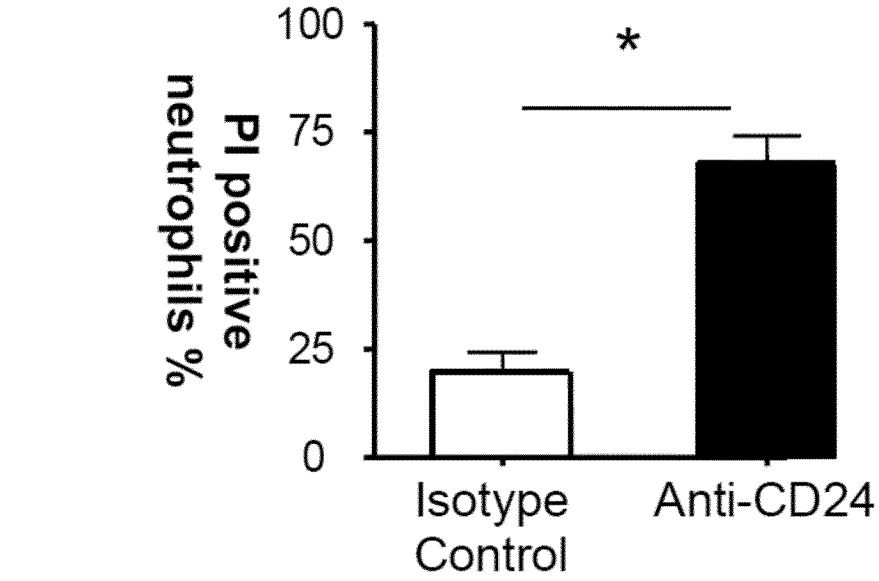

B
PI positive neutrophils %
100
75
50
25
0
*
Isotype Control
Anti-CD24

Figure 17

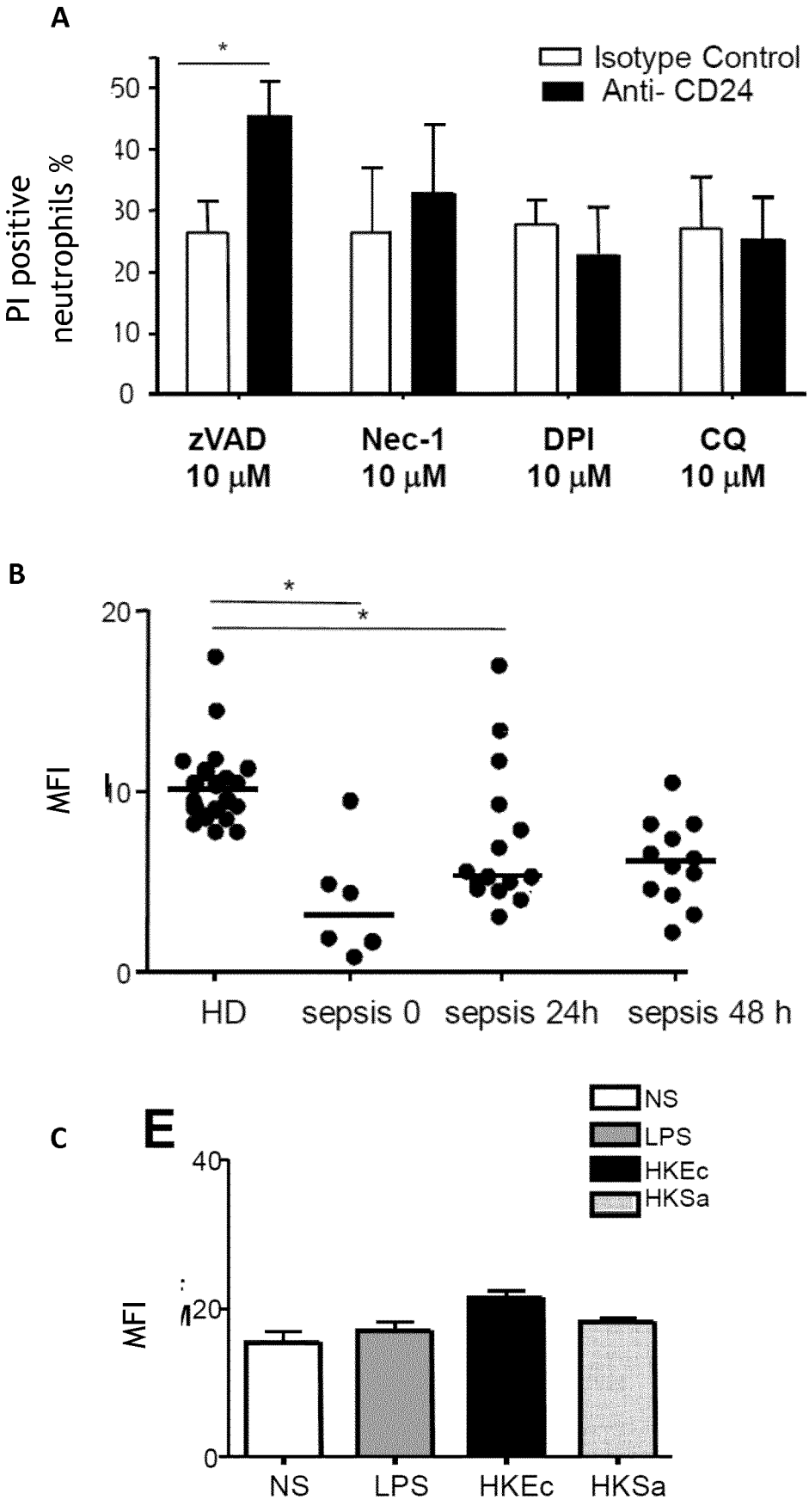

A
PI positive neutrophils %
Isotype Control
Anti- CD24
*
50
40
30
20
10
0
zVAD 10 µM
Nec-1 10 µM
DPI 10 µM
CQ 10 µM
B
MFI
*
*
20
10
0
HD
sepsis 0
sepsis 24h
sepsis 48 h
C
E
NS
LPS
HKEc
HKSa
MFI
40
20
0
NS
LPS
HKEc
HKSa

Figure 18

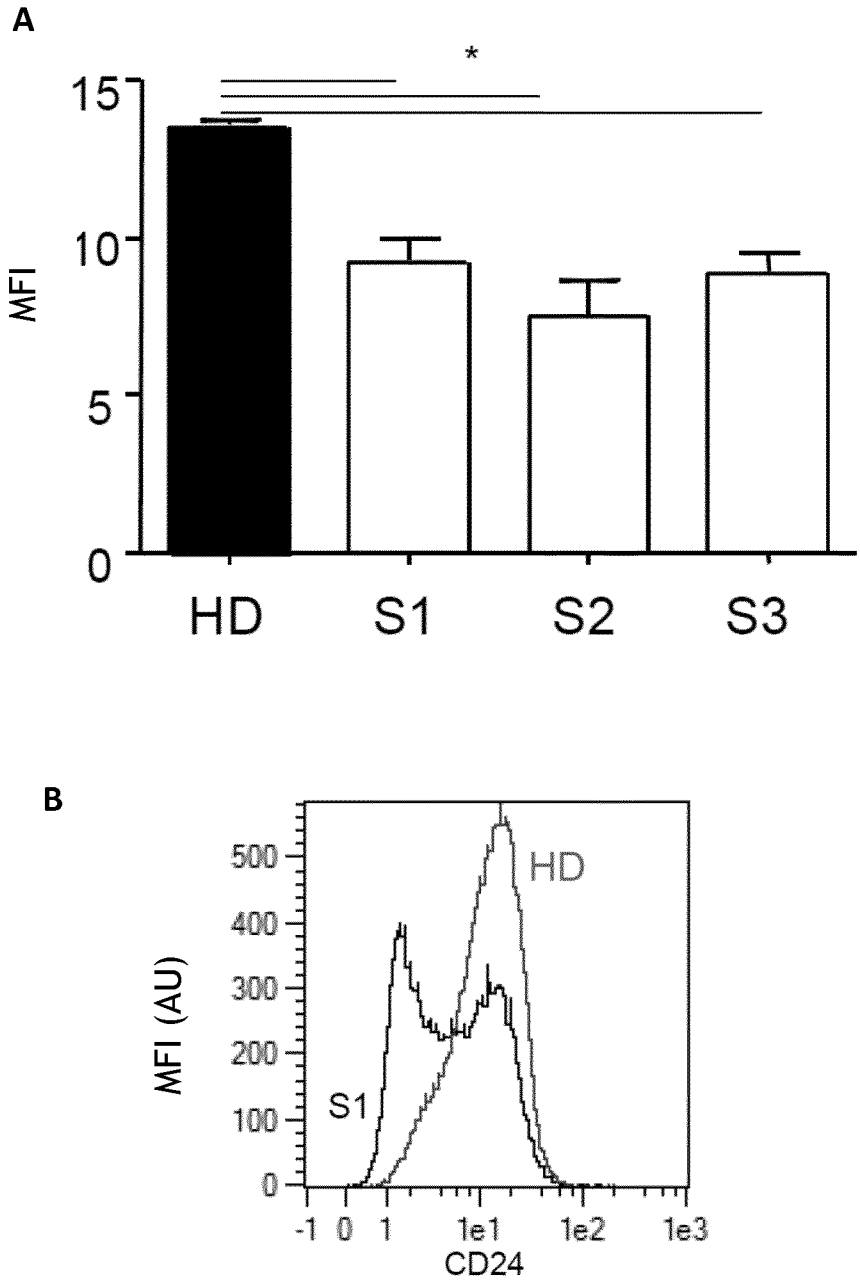
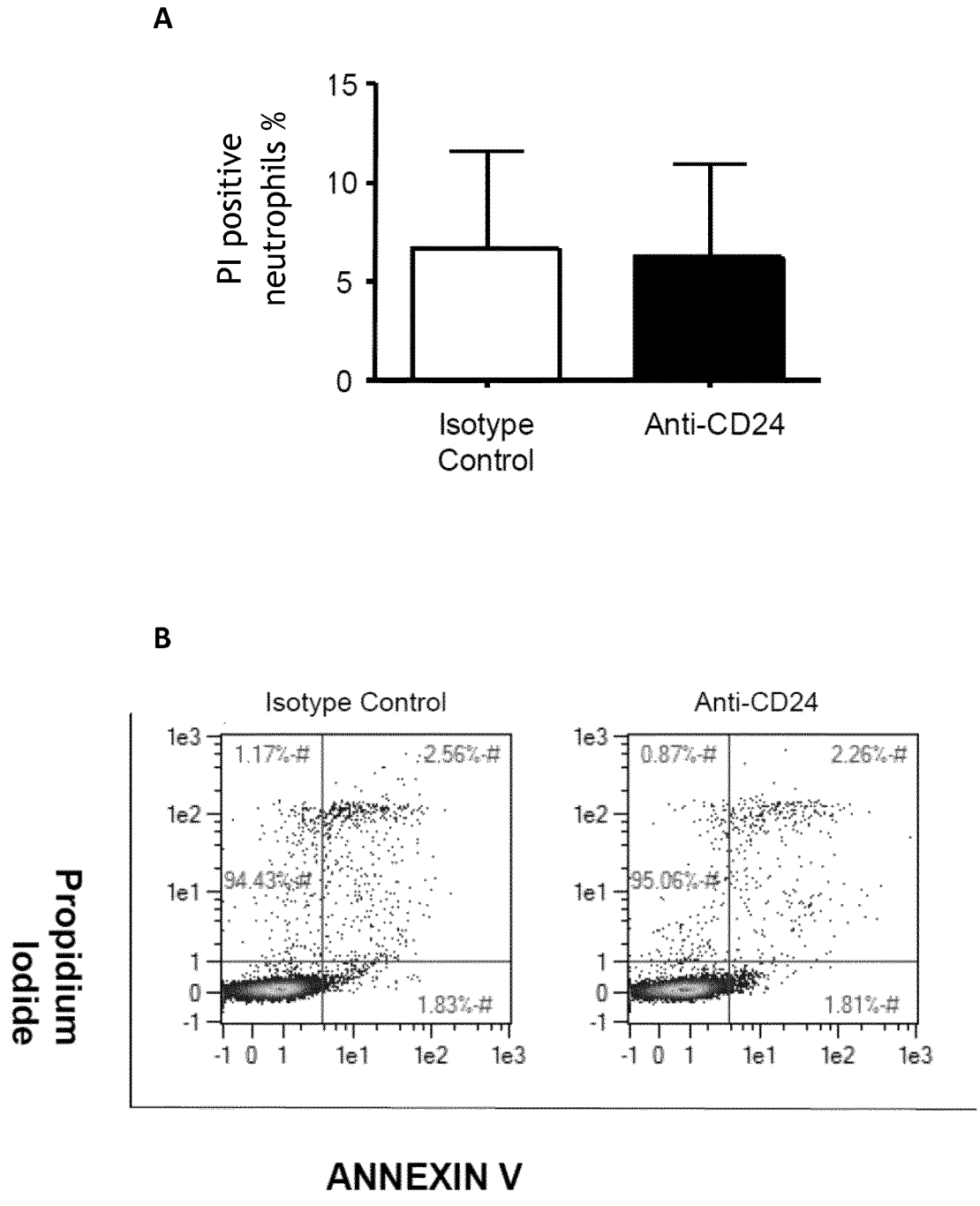

A
*
15
10
5
0
MFI
HD
S1
S2
S3
B
500
400
300
200
100
0
MFI (AU)
HD
S1
-1
0
1
1e1
1e2
1e3
CD24

Figure 19


A
PI positive neutrophils %
15
10
5
0
Isotype Control
Anti-CD24
B
Isotype Control
Anti-CD24
1e3
1e2
1e1
1
0
-1
1.17%-#
2.56%-#
94.43%-#
1.83%-#
0.87%-#
2.26%-#
95.06%-#
1.81%-#
-1 0 1
1e1
1e2
1e3
Propidium Iodide
ANNEXIN V