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(54) **Title**: METHODS AND KITS FOR THE DIAGNOSING OF INFLAMMATORY CONDITIONS INVOLVING DETECTING, IDENTIFYING OR ASSAYING FOR SOLUBLE B7-H6 POLYPEPTIDES

(57) **Abstract**: The present invention includes methods and kits for detecting, diagnosing, for obtaining a prognosis, staging and monitoring an inflammatory condition in a subject involving detecting, identifying or assaying for soluble B7-H6polypeptides. It may also be used for evaluating the efficacy of treatment of an inflammatory condition.

METHODS AND KITS FOR THE DIAGNOSING OF INFLAMMATORY CONDITIONS INVOLVING DETECTING, IDENTIFYING OR ASSAYING FOR SOLUBLE B7-H6 POLYPEPTIDES

FIELD OF THE INVENTION:

The present invention relates to methods and kits for detecting, diagnosing, for obtaining a prognosis, staging and monitoring an inflammatory condition in a subject involving detecting, identifying or assaying for soluble B7-H6 polypeptides.

BACKGROUND OF THE INVENTION:

Natural Killer (NK) cells are cytolytic and cytokine-producing lymphocytes that can recognize a variety of cells in distress (1). Cells undergoing microbial infection, tumor transformation as well as chemical or physical insults can thus become NK cell targets as they express stress-induced surface molecules that can interact with an array of activating NK cell surface receptors. The recognition of NKG2D ligands expressed by target cells via NKG2D expressed on NK cells represents a prototypical illustration of this stress-induced self-mode of innate immune recognition (2).

NK cells express at their surface a group of Ig-like superfamily Natural Cytotoxicity Receptors (NCRs) including NKp46 (NCR1, CD335), NKp44 (NCR2, CD336) and NKp30 (NCR3, CD337) (3). In contrast to NKG2D, NCRs are preferentially expressed on peripheral blood NK cells. NKp30 and NKp46 are constitutively expressed, whereas NKp44 is induced after activation. NCR association with immunoreceptor tyrosine-based activation motif (ITAM)-bearing transducing polypeptides, such as CD3ζ, FcRγ or DAP12 is reminiscent of the architecture of other pivotal immune receptor complexes, such as the T-, B- and Fc-receptors, and makes them very potent activating receptors (4). NKp30 is coupled to CD3ζ and FcRγ transduction polypeptides. NKp30 has no homology with NKp44 or NKp46 encoded on chromosomes 6 and 19 respectively, but based on their expression profile and function these receptors have been grouped as NCRs (5). NKp30 is encoded by a gene located in the class III region of the human MHC on chromosome 6 where several other genes with immune function are found (tumor necrosis factor, lymphotoxins) (6).

The nature of the NCR ligands and their characterization is still incomplete. Although a large piece of data suggested a central role of NCR in tumor surveillance (6-9), their first

ligands to be identified were viral structures, in particular the influenza haemaglutinin for NKp46 (10) and the human cytomegalovirus pp65 tegument protein for NKp30 (11). Later the HLA-B associated transcript 3 (BAT3) protein was shown to bind and trigger NKp30 (12). This nuclear protein now referred as to BAG6 (http://www.genenames.org/data/hgnc_data.php?hgnc_id=13919) is ectopically found at the plasmic membrane upon stress but absent on tumor cell lines spontaneously susceptible to NK cell lysis. We identified B7-H6 (NCR3LG1, http://www.genenames.org/data/hgnc_data.php?hgnc_id=42400), a previously unannotated gene, as a ligand of NKp30 (13, 14). *B7-H6* transcripts have not been detected in most normal adult tissues. Consistent with these data, the B7-H6 protein is not detectable on normal tissues at steady state. However B7-H6 is present on a broad panel of hematopoietic and non-hematopoietic tumor cells including lymphoma, leukemia, melanoma, and carcinoma as well as on primary tumor blood cells (13). The pattern of B7-H6 expression, which appears so far to be limited to tumor cells, is another example of stress-induced self-recognition by NK cells. In the case of NKG2D ligands, several mechanisms have been shown to regulate their expression. For example, genotoxic stress was shown to up-regulate NKG2D ligands on non-tumor cell lines, whereas interfering with the DNA damage pathway in tumor cells inhibited their constitutive expression (2). Different mechanisms have been described to control NKG2D ligand translation and protein turnover. For instance, the transcripts of the human NKG2D ligands MICA and MICB are the target of miRNAs that repress the protein expression (15). In contrast, the regulation of B7-H6 expression is unknown. We studied here the mechanisms that govern the induction of B7-H6 on primary cells as well as its consequences *in vitro* and *in vivo*.

SUMMARY OF THE INVENTION:

The present invention is based on the observation that B7-H6, previously known to be expressed on the surface of tumor cells as cell membrane-anchored polypeptides, may exist in a soluble form and may be distributed into the circulation ("soluble B7-H6"). Using a panel of monoclonal antibodies (mAbs) generated against B7-H6, the inventors indeed analyzed here the pathways that lead to the expression of B7-H6. *In vitro*, B7-H6 was induced at the surface of $CD14^+CD16^+$ pro-inflammatory monocytes and neutrophils activated by ligands of Toll-like receptors or pro-inflammatory cytokines such as interleukin-1β (IL-1 β) and tumor necrosis factor α (TNF-α). *In vivo*, B7-H6 was expressed on circulating pro-inflammatory and sB7-H6 associated with soluble membrane vesicles such as exosomes present in the

serum of a fraction of patients in sepsis conditions.These findings reveal that B7-H6 is not only implicated in the tumor immunosurveillance but can also trigger NKp30-mediated activation of human NK cells in inflammatory conditions. Indeed the inventors demonstrated that upon inflammatory conditions the expression of B7-H6 at the surface of PBMCs can be triggered but a soluble form of B7-H6 is also expressed. Thus, the present invention includes methods and kits for detecting, diagnosing, for obtaining a prognosis, staging and monitoring an inflammatory condition in a subject involving detecting, identifying or assaying for soluble B7-H6 polypeptides. It may also be used for evaluating the efficacy of treatment of an inflammatory condition.

DETAILED DESCRIPTION OF THE INVENTION:

A used herein, the term "B7-H6" has its general meaning in the art and was first described in Brandt, C.S., M. Baratin, E.C. Yi, J. Kennedy, Z. Gao, B. Fox, B. Haldeman, C.D. Ostrander, T. Kaifu, C. Chabannon, et al. 2009. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J. Exp. Med. 206:1495–1503. doi:10.1084/jem.20090681 and in US Patent Application Publication No. 2009/0220502). An exemplary amino acid sequence is described in UniProtKB/Swiss-Prot database under the accession number Q68D85.

The term "inflammatory condition" as used herein refers to acute or chronic localized or systemic responses to harmful stimuli, such as pathogens, damaged cells, physical injury or irritants, that are mediated in part by the activity of cytokines, chemokines, or inflammatory cells (e.g., neutrophils, monocytes, lymphocytes, macrophages) and is characterized in most instances by pain, redness, swelling, and impairment of tissue function. The inflammatory condition may be selected from the group consisting of: sepsis, septicemia, pneumonia, septic shock, systemic inflammatory response syndrome (SIRS), Acute Respiratory Distress Syndrome (ARDS), acute lung injury, aspiration pneumanitis, infection, pancreatitis, bacteremia, peritonitis, abdominal abscess, inflammation due to trauma, inflammation due to surgery, chronic inflammatory disease, ischemia, ischemia-reperfusion injury of an organ or tissue, tissue damage due to disease, tissue damage due to chemotherapy or radiotherapy, and reactions to ingested, inhaled, infused, injected, or delivered substances, glomerulonephritis, bowel infection, opportunistic infections, and for subjects undergoing major surgery or dialysis, subjects who are immunocompromised, subjects on immunosuppressive agents,

subjects with HIV/AIDS, subjects with suspected endocarditis, subjects with fever, subjects with fever of unknown origin, subjects with cystic fibrosis, subjects with diabetes mellitus, subjects with chronic renal failure, subjects with bronchiectasis, subjects with chronic obstructive lung disease, chronic bronchitis, emphysema, or asthma, subjects with febrile neutropenia, subjects with meningitis, subjects with septic arthritis, subjects with urinary tract infection, subjects with necrotizing fasciitis, subjects with other suspected Group A streptococcus infection, subjects who have had a splenectomy, subjects with recurrent or suspected enterococcus infection, other medical and surgical conditions associated with increased risk of infection, Gram positive sepsis, Gram negative sepsis, culture negative sepsis, fungal sepsis, meningococcemia, post-pump syndrome, cardiac stun syndrome, stroke, congestive heart failure, hepatitis, epiglotittis, E. coli 0157:H7, malaria, gas gangrene, toxic shock syndrome, pre-eclampsia, eclampsia, HELP syndrome, mycobacterial tuberculosis, Pneumocystic carinii, pneumonia, Leishmaniasis, hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, Dengue hemorrhagic fever, pelvic inflammatory disease, Legionella, Lyme disease, Influenza A, Epstein-Barr virus, encephalitis, inflammatory diseases and autoimmunity including Rheumatoid arthritis, osteoarthritis, progressive systemic sclerosis, systemic lupus erythematosus, inflammatory bowel disease, idiopathic pulmonary fibrosis, sarcoidosis, hypersensitivity pneumonitis, systemic vasculitis, Wegener's granulomatosis, transplants including heart, liver, lung kidney bone marrow, graftversus-host disease, transplant rejection, sickle cell anemia, nephrotic syndrome, toxicity of agents such as OKT3, cytokine therapy, and cirrhosis.

A “subject” may be someone who has not been diagnosed with an inflammatory condition or someone diagnosed with an inflammatory condition, including someone treated for an inflammatory condition. The subject or subject may also be suspected of having an inflammatory condition or of being at risk for having or developing an inflammatory condition.

In a particular embodiment the subject is a SIRS subject with Gram negative infection.

Implementations of the methods of the invention involve obtaining a sample from a subject. The sample may include sputum, serum, blood, plasma, spinal fluid, semen, lymphatic fluid, urine, stool, pleural effusion, ascites, a tissue sample, tissue biopsy, cell swab, or a combination thereof. The sample, in some embodiments, is obtained from a region

exhibiting one or more signs of inflammation. Cells are not included in the sample because these embodiments involve assaying for soluble, as opposed to cell bound, B7-H6.

In some embodiments of the invention, there are methods for diagnosing or prognosing an inflammatory condition in a subject comprising assaying for a soluble B7-H6 polypeptide in a sample from the subject, wherein identification of a soluble B7-H6 polypeptide in the sample indicates an inflammatory condition.

Typically, the subject is suspected of having an inflammatory condition. Identifying a subject suspected of having an inflammatory condition can involve conducting a subject interview, taking a subject history or family history, doing blood work on the subject (taking blood and performing tests on the blood), or conducting a physical exam.

In some embodiments of the invention, the methods further comprise determining the level of the soluble B7-H6 polypeptide in the sample and comparing said level with a predetermined reference value.

In one embodiment, the predetermined reference value is derived from the level of the soluble B7-H6 polypeptide in a control sample derived from one or more subjects who are substantially healthy (i.e. subjects who do not suffer from an inflammatory condition). Such subjects who are substantially healthy lack the clinical feature of the inflammatory condition. In another embodiment, such subjects are monitored and/or periodically retested for a diagnostically relevant period of time ("longitudinal studies") following such test to verify continued absence of the development of the inflammatory condition. Such period of time may be one year, two years, two to five years, five years, five to ten years, ten years, or ten or more years from the initial testing date for determination of the predetermined reference value. Furthermore, retrospective measurement of the levels of the soluble B7-H6 polypeptide in properly banked historical subject samples may be used in establishing these predetermined reference values, thus shortening the study time required, presuming the subjects have been appropriately followed during the intervening period through the intended horizon of the product claim. It is thus contemplated that the level of soluble B7-H6 polypeptide in the sample identifies the subjects having an inflammatory condition. It is also contemplated that the level of the soluble B7-H6 polypeptide in the sample of subjects suffering from an inflammatory condition is deemed to be higher than the predetermined reference level (i.e. the level determined in the sample of the healthy subjects).

Accordingly, in some embodiments, the invention relates to a method for diagnosing or prognosing an inflammatory condition in a subject comprising i) determining the level of a

soluble B7-H6 polypeptide in a sample obtained from the subject, ii) comparing the level determined at step i) with a predetermined reference value and iii) concluding that the subject suffers from an inflammatory condition or is at risk of having an inflammatory condition when the level determined at step i) is higher than the predetermined reference value.

It is contemplated that in some embodiments of the invention, a subject is also administered with an agent for the treatment of its inflammatory condition.

Accordingly, in some embodiments there are methods for monitoring the treatment of a subject suffering from an inflammatory condition comprising assaying for a soluble B7-H6 polypeptide in a sample from the subject, wherein identification of a soluble B7-H6 polypeptide in the sample indicates that the treatment is not effective and non identification of a soluble B7-H6 polypeptide indicates that the treatment is effective. In some embodiments of the invention, the methods further comprise determining the level of the soluble B7-H6 polypeptide in the sample and comparing said level with a predetermined reference value. In one embodiment, the predetermined reference value is derived from the level of the soluble B7-H6 polypeptide in a control sample derived from one or more subjects for which the treatment as effective, in a manner that the inflammatory condition does not reapperard. In another embodiment, such subjects are monitored and/or periodically retested for a diagnostically relevant period of time ("longitudinal studies") following such test to verify continued absence of the development of the inflammatory condition. Such period of time may be one year, two years, two to five years, five years, five to ten years, ten years, or ten or more years from the initial testing date for determination of the predetermined reference value. Furthermore, retrospective measurement of the levels of the soluble B7-H6 polypeptide in properly banked historical subject samples may be used in establishing these predetermined reference values, thus shortening the study time required, presuming the subjects have been appropriately followed during the intervening period through the intended horizon of the product claim. It is thus contemplated that the level of the soluble B7-H6 polypeptide in the sample of subjects for which the treatment is ineffective is deemed to be higher than the predetermined reference level (i.e. the level determined in the sample for which the treatment was effective).

In some embodiment the invention relates to a method for monitoring the treatment of a subject suffering from an inflammatory condition in a subject comprising i) determining the level of a soluble B7-H6 polypeptide in a sample obtained from the subject before the treatment, ii) determining the level of a soluble B7-H6 polypeptide in a sample obtained from

the subject after the treatment iii) comparing the level determined at step i) with the level determined at step ii) and iii) concluding that treatment is effective when the level determined at step ii) is lower than the level determined at step i) or concluding that the treatment is not effective when the level determined at step ii) is above the same as the level determined at step i) or is higher than the level determined at step i).

Such treatments include, but are not limited to, anti-inflammatory agents and/or immune response suppressors, surgery, physical and/or occupational therapy. In some embodiments, the subject is given NSAIDs, aspirin, analgesics, glucocorticoids, methotrexate, leflunomide, D-Penicillamine, sulfasalazine, gold therapy, minocycline, azathioprine, hydroxychloroquine (and other antimalarials), cyclosporine, biologic agents, or Prosorba. In specific embodiments, the subject is a biotherapeutic agent (e.g. an antibody, a recombinant polypeptide, an nucleic acid molecule…). For example said agent is an agent blocking at least one factor that may induce the expression of the soluble form of B7-H6. Accordingly, said agent may be selected from the group of Toll-like receptor (TLR) antagonists, in particular TLR1 antagonists, TLR2 antagonists, TLR4 antagonists, TLR5 antagonists, TLR6 antagonists, TLR8 antagonists or may be selected from the group consisting tumor necrosis factor alpha receptor (TNF-alpha) antagonists or inteleukin 1 (IL1) receptor antagonists. For example an TNF-alpha receptor antagonist may be a neutralizing (preferably non-depleting) anti-TNFα antibody such as adalimumab (Humira™) or Certolizumab pegol (Cimzia™)), and an IL-1 receptor antagonist may be anakinra (Kineret™)).

Methods for predicting the survival of a sepsis subject are also included as part of the invention.

In some embodiment the present invention relates to a method for predicting the survival of a sepsis subject comprising the steps consisting of i) determining the level of of the soluble B7-H6 polypeptide in a sample obtained from the subject (e.g. a blood sample) ii) comparing the level determined at step i) with a predetermined reference level and iii) concluding that the subject has a poor prognosis when the level determined at step i) is higher than the predetermined reference level and good prognosis when the level determined at step i) is lower than the the predetermined reference level.

In a particular embodiment, the subject is subject with a gram negative sepsis.

The predetermined reference value used for comparison may consist of "cut-off" value that may be determined as described hereunder. Each predetermined reference value ("cut-off") value may be determined by carrying out a method comprising the steps of

a) providing a collection of samples (e.g. blood samples) from sepsis subjects

b) providing, for each sample provided at step a), information relating to the actual clinical outcome for the corresponding post acute myocardial infarction patient (i.e. the duration of the disease-free survival (DFS) and/or the overall survival (OS));

c) providing a serial of arbitrary quantification values;

d) determining the level of soluble B7-H6 polypeptide for each blood sample contained in the collection provided at step a);

e) classifying said blood samples in two groups for one specific arbitrary quantification value provided at step c), respectively: (i) a first group comprising samples that exhibit a quantification value for level that is lower than the said arbitrary quantification value contained in the said serial of quantification values; (ii) a second group comprising samples that exhibit a quantification value for said level that is higher than the said arbitrary quantification value contained in the said serial of quantification values; whereby two groups of samples are obtained for the said specific quantification value, wherein the samples of each group are separately enumerated;

f) calculating the statistical significance between (i) the quantification value obtained at step e) and (ii) the actual clinical outcome of the patients from which samples contained in the first and second groups defined at step f) derive;

g) reiterating steps f) and g) until every arbitrary quantification value provided at step d) is tested;

h) setting the said predetermined reference value ("cut-off" value) as consisting of the arbitrary quantification value for which the highest statistical significance (most significant) has been calculated at step g).

As it is disclosed above, this method allows the setting of a single "cut-off" value that permits discrimination between a poor and a good prognosis with respect to DFS and OS. Practically, high statistical significance values (e.g. low P values) are generally obtained for a range of successive arbitrary quantification values, and not only for a single arbitrary quantification value. Thus, in one alternative embodiment of the method of determining "cut-off" values as above, a minimal statistical significance value (minimal threshold of significance, e.g. maximal threshold P value) is arbitrarily set and a range of a plurality of arbitrary quantification values for which the statistical significance value calculated at step g) is higher (more significant, e.g. lower P value) are retained, so that a range of quantification values is provided. This range of quantification values includes a "cut-off" value as described above. According to this specific embodiment of a "cut-off" value, poor or good clinical

outcome prognosis can be determined by comparing the level of soluble B7-H6 polypeptide determined at step d) with the range of values which are identified. In certain embodiments, a cut-off value thus consists of a range of quantification values, e.g. centered on the quantification value for which the highest statistical significance value is found (e.g. generally the minimum P value which is found). For example, on a hypothetical scale of 1 to 10, if the ideal cut-off value (the value with the highest statistical significance) is 5, a suitable (exemplary) range may be from 4-6. Therefore, a patient may be assessed by comparing values obtained by measuring the level of soluble B7-H6 polypeptide, where values greater than 5 indicate a poor prognosis and values less than 5 indicate a good prognosis; or a patient may be assessed by comparing values obtained by measuring the level of soluble B7-H6 polypeptide and comparing the values on a scale, where values above the range of 4-6 indicate a poor prognosis and values below the range of 4-6 indicate a good prognosis, with values falling within the range of 4-6 indicating an intermediate prognosis.

Methods of screening for candidate therapeutic agents for an inflammatory condition are also included as part of the invention.

In some embodiments, the present invention relates to a method of screening for candidate therapeutic agents for an inflammatory condition comprising i) providing a plurality of candidate compounds, ii) bringing the candidate compounds into contact with immune cells (e.g peripheral blood mononuclear cells) in presence of an agent that stimulates the expression of a soluble B7-H6 polypeptide, iii) determining the level of the soluble B7-H6 polypeptide expressed by the immune cell, iv) comparing the level determined at step iii) with the level determined in the absence of the candidate compounds, and v) and positively selecting the candidate compounds when the level determined at step iii) is lower than the level determined in the absence of the candidate compounds.

Typically, the agent that stimulates the expression of a soluble B7-H6 polypeptide is selected from the group consisting of TLR agonists, TNF-alpha or IL1.

Typically the candidate compounds may be selected from the group consisting of peptides, peptidomimetics, small organic molecules, antibodies, aptamers or nucleic acids. For example, the candidate compound according to the invention may be selected from a library of compounds previously synthesised, or a library of compounds for which the structure is determined in a database, or from a library of compounds that have been synthesised de novo.

In a particular embodiment, the candidate compounds may be selected form small organic molecules. As used herein, the term "small organic molecule" refers to a molecule of size comparable to those organic molecules generally sued in pharmaceuticals. The term excludes biological macromolecules (e.g.; proteins, nucleic acids, etc.); preferred small organic molecules range in size up to 2000 Da, and most preferably up to about 1000 Da.

In another particular embodiment, the candidate compound according to the invention may be antibodies. The antibodies of the invention can for instance be produced by any hybridoma liable to be formed according to classical methods from splenic cells of an animal, particularly of a mouse or rat immunized against an antigenic sequence of interest. The antibodies according to this embodiment of the invention may be humanized versions of the mouse antibodies made by means of recombinant DNA technology, departing from the mouse and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Alternatively, the antibodies may be human antibodies. Such human antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice as described in PCT/EP99/03605 or by using transgenic non-human animals capable of producing human antibodies as described in US patent 5,545,806. Also fragments derived from these antibodies such as Fab, F (ab)'2 ands ("single chain variable fragment"), providing they have retained the original binding properties, form part of the present invention. Such fragments are commonly generated by, for instance, enzymatic digestion of the antibodies with papain, pepsin, or other proteases. It is well known to the person skilled in the art that monoclonal antibodies or fragments thereof, can be modified for various uses. An appropriate label of the enzymatic, fluorescent, or radioactive type can label the antibodies involved in the invention.

In some embodiments, the candidate compounds may be selected from aptamers. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally

constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

In still another particular embodiment, the candidate compound may be selected from molecules that block expression of a gene of interest. Also within the scope of the invention is the use of oligoribonucleotide sequences that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of mRNA of a nuclear protein required for Notch1 transcriptional activity. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site. Ribozymes are enzymatic RNA molecules capable of catalysing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridisation of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. To inhibit the activity of the gene of interest or the gene product of the gene of interest, custom-made techniques are available directed at three distinct types of targets: DNA, RNA and protein. For example, the gene or gene product of a nuclear protein required for Notch1 transcriptional activity of the invention can be altered by homologous recombination, the expression of the genetic code can be inhibited at the RNA levels by antisense oligonucleotides, interfering RNA (RNAi) or ribozymes, and the protein function can be altered by antibodies or drugs.

The screening methods of the invention is particularly suitable for identifying compounds that are TLR, TNF-alpha or IL-1 antagonists.

In some cases, a candidate therapeutic agent has been identified and further testing may be required. In some embodiments the further testing is to evaluate a candidate therapeutic agent (or an agent that has been confirmed to be therapeutic) for quality control and/or safety concerns. In some embodiments, methods of the invention include a method of assaying a therapeutic agent (or candidate therapeutic agent) for efficacy against an inflammatory condition in a relevant animal model.

In some embodiments of the invention, identification of a soluble B7-H6 polypeptide involves use of at least one B7-H6 polypeptide binding agent. Furthermore, it is contemplated that a B7-H6 polypeptide binding agent may be specific or not to the soluble B7-H6. For example, the B7-H6 polypeptide binding agent may bind to a part of B7-H6 (e.g. an epitope) that is not available when B7-H6 is bound to a cell. Alternatively, different conformations

may serve the basis for binding agents capable of distinguishing between soluble and bound B7-H6.

In some embodiments of the invention, the soluble B7-H6 polypeptide binding agent is a polypeptide.

The polypeptide is, in additional embodiments, an antibody. In further embodiments, the antibody is a monoclonal antibody, such as those described in the International Patent Publication WO2011070443. In particular the antibody may be 17B1.3 mAb (Deposit No. CNCM I-4245). The antibody can be bi-specific, recognizing two different epitopes. The antibody, in some embodiments, immunologically binds to more than one epitope from the same soluble B7-H6 polypeptide.

A B7-H6 polypeptide binding agent that is a polypeptide may also include all or part of NKp30, which is a receptor for B7-H6 polypeptides.

In some embodiments of the invention, the soluble B7-H6 polypeptide binding agent is an aptamer.

In some embodiments of the invention, the soluble B7-H6 binding agent is labeled. In further embodiments, the label is radioactive, fluorescent, chemilluminescent, an enzyme, or a ligand. It is also specifically contemplated that a binding agent is unlabeled, but may be used in conjunction with a detection agent that is labeled. A detection agent is a compound that allows for the detection or isolation of itself so as to allow detection of another compound that binds, directly or indirectly. An indirect binding refers to binding among compounds that do not bind each other directly but associate or are in a complex with each other because they bind the same compounds or compounds that bind each other.

Other embodiments of the invention involve a second B7-H6 polypeptide binding agent in addition to a first B7-H6 polypeptide binding agent. The second binding agent may be any of the entities discussed above with respect to the first binding agent, such as an antibody. It is contemplated that a second antibody may bind to the same of different epitopes as the first antibody. It is also contemplated that the second antibody may bind the first antibody or another epitope than the one recognized by the first antibody.

As discussed earlier, binding agents may be labeled or unlabeled. Any B7-H6 polypeptide binding agent used in methods of the invention may be recognized using at least one detection agent. A detection agent may be an antibody that binds to a B7-H6 polypeptide binding agent, such as an antibody. The detection agent antibody, in some embodiments, binds to the Fc-region of a binding agent antibody. In further embodiments, the detection agent is biotinylated, which is incubated, in additional embodiments, with a second detection

agent comprising streptavidin and a label. It is contemplated that the label may be radioactive, fluorescent, chemilluminescent, an enzyme, or a ligand. In some cases, the label is an enzyme, such as horseradish peroxidase.

The present invention also covers methods involving using an ELISA assay to identify a soluble B7-H6 polypeptide. In some embodiments, the ELISA assay is a sandwich assay. In a sandwich assay, more than one antibody will be employed. Typically ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize the protein of interest. A sample containing or suspected of containing the protein of interest is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Other methods of the invention further include assaying a sample for a cell-bound B7-H6 polypeptide in addition to a soluble polypeptide. The second assay may be performed on the same sample as the identification of a soluble B7-H6 polypeptide or it may be performed on a different sample. It is contemplated that a sample may or may not include cells.

The present invention also includes kits for detecting an inflammatory condition comprising, in a suitable container means, a soluble B7-H6 polypeptide binding agent as above described. In further embodiments, the binding agent is labeled or a detection agent is included in the kit. It is contemplated that the kit may include a B7-H6 polypeptide binding agent attached to a non-reacting solid support, such as a tissue culture dish or a plate with multiple wells. It is further contemplated that such a kit includes a detectable agent in certain embodiments of the invention. In some embodiments the invention concerns kits for carrying out a method of the inventiont comprising, in suitable container means: (a) an agent that specifically recognizes all or part of a B7-H6 polypeptide; and, (b) a positive control that can be used to determine whether the agent is capable of specifically recognizing all or part of a B7-H6 polypeptide. The kit may also include other reagents that allow visualization or other detection of the B7-H6 polypeptide, such as reagents for colorimetric or enzymatic assays.

In some embodiments, the present invention relates to a method of treating an inflammatory condition in a subject comprising the steps of assaying for a soluble B7-H6 polypeptide in a sample from the subject, if a soluble B7-H6 polypeptide in the sample is

identified, concluding that the subject has or is likely to develop an inflammatory condition and treating the subject with an anti-inflammatory agent or an immune suppressors (as such described above).

In a first aspect, the invention relates to a method of treating an inflammatory condition in a subject comprising the steps of:

a) providing a biological sample from the subject,

b) measuring the level of soluble B7-H6 polypeptide in the biological sample obtained at step a);

c) comparing said level of soluble B7-H6 polypeptide with a predetermined reference value, and if the level of soluble B7-H6 polypeptide measured at step b) is higher that the predetermined reference value, treating the subject with an effective amount of an anti-inflammatory agent or immune suppressor.

In a first aspect, the invention relates to a method of treating an inflammatory condition in a subject comprising the steps of :

a) providing a biological sample from a subject,

b) measuring the level of soluble B7-H6 polypeptide in the biological sample obtained at step a);

c) comparing said level of soluble B7-H6 polypeptide with a predetermined reference value, and if the level of soluble B7-H6 polypeptide measured at step b) is higher that the predetermined reference value, treating the subject until a basal level of soluble B7-H6 polypeptide is reached.

As used herein the term “basal level of soluble B7-H6 polypeptide” refers to the level soluble of B7-H6 polypeptide determined in a subject , or group of subjects, who are considered healthy (i.e. subjects who do not suffer from an inflammatory condition).

In some embodiments, the invention relates to a method of treating an inflammatory condition in a subject comprising the steps of:

a) providing a biological sample from the subject;

b) measuring the level of soluble B7-H6 polypeptide in the biological sample obtained at step a);

c) administering to the subject an effective amount of an anti-inflammatory condition or an immune suppressor;

d) providing a biological sample from the treated subject at step c);

e) measuring the level of soluble B7-H6 polypeptide in the biological sample obtained at step d);

f) comparing the levels of soluble B7-H6 polypeptide measured in biological samples at step b) and d), and if the level of soluble B7-H6 polypeptide measured at step d) is lower that the level of soluble B7-H6 polypeptide at step b), treating the subject by pursuing to administer an effective amount of a the anti-inflammatory agent or immune suppressor until a basal level of soluble B7-H6 polypeptide is reached.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. In vitro induction of B7-H6 transcripts B7-H6 mRNA is up-regulated in PBMCs during activation by (A) TLR ligands, (B) TNFα or (C) IL-1β. Control cells were treated with medium only. Data show fold induction of B7-H6 mRNA in treated cells as compared to untreated control cells at the indicated time points. All data result from a pool of three independent experiments.

Figure 2. Characterization of anti-B7-H6 mAbs (A) Anti B7-H6 mAb reactivity. P815.B7-H1, P815.B7-H6 or HeLa cells were stained with 4E5.5, 9G9.2, 10E2.9 or 17B1.3 anti-B7-H6 mAb and analyzed by flow cytometry. Mouse IgG1 (mIgG1) was used as isotype control. Graphs are representative of at least 3 experiments. (B) 4E5.5 and 17B1.3 mAbs are blocking anti-B7-H6 mAbs. DOMSp30 reporter cells were co-cultured with P815.B7-H6 cells (left panel) or HeLa cells (right panel) in presence or absence of anti-B7-H6 mAbs. Anti-NKp30 mAbs were used as a positive controls for blockers of DOMSp30 cell activation. DOMSP30 activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay. Data are representative of 3 independent experiments. ***$P < 0.001$. (C) SPR analysis. Superimposed sensorgrams showing the injections onto NKp30 chip of B7H6 alone or pre-incubated with anti-B7H6 antibodies.

Sensorgrams were normalized in the Y axis and aligned in the X axis at the end of injection. Sensorgrams are representative of two independent experiments.

Figure 3. In vitro induction of B7-H6 cell surface expression on monocytes (A) Flow cytometric analysis of CD45+CD14+CD19-CD3- monocytes, gated from freshly isolated PBMCs left untreated or (B) after stimulation for 48 hours with IL-1β, LPS, flagellin or TNFα. B7-H6 expression was analyzed by flow cytometry with B7-H6 specific monoclonal antibody 17B1.3 (black histograms) or mIgG1 isotype control (gray histograms). Data are representative of at least three independent experiments. MFI stim represents the value of the Mean Fluorescence Intensity (MFI) with anti-B7-H6 antibody minus the MFI with mIgG1 isotype control. (C) Primary PBMCs were left untreated or treated with PolyIC, TNFα, flagellin, E-LPS or IL-1β for indicated time periods. B7-H6 cell surface expression was assessed by flow cytometry and fold change in B7-H6 expression was quantified by dividing the MFI of treated samples by that of untreated cells at each time points. All the data result from a pool of at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 4. NKp30-dependent cell activation induced by B7-H6+ monocytes (A) Flow cytometry analysis of B7-H6 surface expression on freshly isolated monocytes stained with CD14, CD16 mAbs and B7-H6 specific monoclonal antibody 17B1.3 (black histograms) or mIgG1 isotype control (gray histograms). Results are representative of purified monocytes phenotype after 48 hours of stimulation with IL-1β. Data are representative of three independent experiments. DOMSp30 reporter cells (NKp30+) or D011.10 control cells (NKp30- parental) were co-cultured with IL-1β-stimulated monocytes in the presence of B7-H6 mAb 17B1.3 or mIgG1 isotype control. DOMSP30 activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay.(B) DOMSp30 reporter cells or D011.10 control cells were co-cultured with IL-1β-stimulated monocytes with B7-H6 mAb 17B1.3 or mIgG1 isotype control. DOMSP30 activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay. Data are representative of three independent experiments. ***P < 0.001

Figure 5. In vitro induction of B7-H6 cell surface expression on neutrophils Cytometric analysis of B7-H6 expression on freshly isolated CD24+CD14-HLA-DR-

neutrophils left untreated or stimulated IL-1β for 24 hours. Cells are stained with F(ab')2 fragments of the B7-H6 specific monoclonal antibody 17B1.3 (black histograms) or with APC conjugated antimouse IgG (H+L) alone (gray histograms). Data are representative of at least three independent experiments. MFI stim represent the value of the Mean Fluorescence Intensity (MFI) with anti-B7-H6 F(ab)'2 less the MFI with secondary antibody.

Figure 6. Signaling pathways involved in B7-H6 induction on monocytes and neutrophils Freshly isolated monocytes (upper panel) or freshly isolated neutrophils (lower panel) were treated with LPS for 4 hours after pretreatment with SB203580 (P38 inhibitor), U0126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor) or NEMO-Binding Domain (NBD, inhibitor of NF-KB pathway) for 2 hours. Control cells were treated with vehicle. Data show fold induction of B7-H6 mRNA (left panel) or IL-6 mRNA (right panel) in treated cells as compared to untreated control. All data result from a pool of three independent experiments. Statistical analyses were performed using paired t test. *P < 0.05.

Figure 7. In vitro induction of soluble B7-H6 from human monocytes and neutrophils (A) Freshly isolated monocytes or (B) freshly isolated neutrophils were treated with LPS or IL-1β. The level of soluble B7-H6 was measured in the cell supernatant by ELISA at indicated time points. Each color symbol represents one individual donor.

Figure 8. In vivo expression of B7-H6 on inflammatory monocytes during sepsis (A) Cytometric histograms of B7-H6 expression on CD45+CD14+CD19-CD3- monocytes, gated from PBMCs of sepsis patients (ICU infection) or (B) control patients of the Intensive Care Unit (ICU no infection) or (C) healthy donors. Cells are stained with CD14, CD16 mAbs and B7-H6 specific monoclonal antibody 17B1.3 (black histograms) or mIgG1 isotype control (gray histograms). Data are representative of 15 sepsis patients (23 patients in total), 9 control patients of ICU and 14 healthy donors. (D) The concentrations of sB7-H6 in the sera of sepsis patients, control patients or healthy donors were measured by ELISA. Statistical analyses were performed using student t test. *P < 0.05

Figure 9. Association of B7-H6 and sepsis condition (A) Sepsis patients were stratified according to the nature of their sepsis (induced by Gram+LPS- or Gram-LPS+ bacteria), and the concentration of sB7-H6 was measured by ELISA. . Statistical analyses were performed using student t test. ** P < 0.01 (B) Crude mortality of ICU patients in

relation of B7-H6 cell surface expression. mB7H6+: B7-H6 positive cells; mB7H6-: B7-H6 negative cells. Statistical analyses were performed using Pearson's X^2 test. *$P < 0.05$. (C) Kaplan Meier curves of survival probability obtained by segregating the whole cohort of ICU patients into two groups according to the expression of B7-H6 cell surface expression. The number of specimen is indicated on the graph.

Figure 10. Characterization of sB7-H6 isolated from patient's sera (A) Level of soluble B7-H6 measured in the pellet at each round of three centrifugation at 1200 g, 10,000 g and 100,000 g sequentially of serum of patients sB7-H6+. Data result from a pool of three independent experiments. (B) Flow cytometry analysis of material purified from serum of 2 sB7-H6+ patients and 1 control individual. After purification, materials were complexed to latex beads and stained with the indicated mAbs (blue); histograms obtained with isotype controls of the respective mAbs are in red.

Figure 11. Impact of soluble B7-H6 on NKp30 expression and activation (A) Cytometric histograms of NKp30 expression of purified NK cells in presence of 5 µg of exosomal fraction positive or negative for soluble B7-H6. (B) DOMSp30 reporter cells or D011.10 control cells were co-cultured with K562 cells in presence of B7-H6 mAb 17B1.3, mIgG1 isotype control or 5 µg of exosomal fraction positive or negative for soluble B7-H6. DOMSP30 activation was determined by evaluating IL-2 production in the co-culture supernatant in a standard CTLL-2 survival assay.

Supplemental Figure 1. In vitro induction of IL-6 transcripts IL-6 mRNA is up-regulated in PBMCs during activation by (A) TLR ligands or (B) IL-1β. Control cells were treated with medium only. Data show fold induction of B7-H6 mRNA in treated cells as compared to untreated control cells. All data result from a pool of three independent experiments.

Supplemental Figure 3. B7-H6 sandwich ELISA assay 17B1.3 was used as the capture antibody and biotin-conjugated 9G9.2 as the detecting antibody. Soluble recombinant B7-H6 was used to set up the standard curve. Data are representative of three independent experiments.

EXAMPLE: INDUCTION OF SOLUBLE AND MEMBRANE-BOUND B7-H6 IN INFLAMMATORY CONDITIONS

Material & methods:

Cells and reagents

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteer donors using Ficoll-Paque Plus (GE Healtcare) density centrifugation. Human monocytes were purified with CD14 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Cell purity was 90–98%. Human neutrophils were isolated from peripheral blood of healthy donors using a dextran-Ficoll method. Human NK cells were purified with a human NK cell isolation II Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were cultured in complete RPMI 1640 medium (GIBCO) supplemented with 100 U/mL penicillin/streptomycin (GIBCO), 1 mM sodium pyruvate (GIBCO), 10% heat-inactivated FBS (LONZA). Human PBMCs (3 x 10^6 cells), neutrophils (2 x 10^6 cells) or monocytes (5 x 10^5 cells) were seeded into 24 well plates (*Becton_Dickinson* and Company). Cells were then incubated at 37°C with 5% CO_2 in the presence or absence of the inflammatory stimulants When indicated cells incubated in RPMI were supplemented with 300 ng/ml synthetic bacterial lipoprotein (Pam3CSK4), 10 µg/ml zymosan, 10^8 cells/ml Heat-killed Acholeplasma laidlawii (HKAL), 500 ng/ml Synthetic diacylated lipoprotein (FSL-1), 1µg/ml Ultra-pure lipopolysaccharide from *E.coli* (E-LPS), 1 µg/ml flagellin from *S.typhimurium*, 20 µg/ml imiquimod (R837), 5 µg/ml ssRNA40, 20 µg/ml ODNs CpG type A, 100µg/ml polyinosinic-polycytidylic acid (poly(I:C)), 100 ng/ml TNF-α or 1 µg/ml IL-1β. All reagents were purchased from Invivogen except for IL-1β that was purchased from PeproTech. When specified cells were pretreated for 2 hours with 20µM of P38 inhibitor (SB203580; Cell Signaling Technology), 20 µM of MEK1/2 inhibitor (U0126; Cell Signaling Technology), 20µM of JNK inhibitor (SP600125; Selleck) or 100 µM of NF-KB pathway inhibitor, NEMO-Binding Domain (NBD; Calbochem).

Transcript analysis

Total RNA was extracted from human cells by using a Qiagen RNeasy minikit according to the kit manufacturer's instructions. For each sample, the RNA (1 µg) obtained was converted into cDNA with the iScript cDNA synthesis kit (Biorad). The expression of *B7-H6* and *ABL1* (V-abl Abelson murine leukemia viral oncogene homolog 1) was then assessed by

quantitative RT PCR, with the Applied Biosystems Taqman Gene expression assays (Hs02340611_m1* and Hs01104728_m1* respectively). Samples were run for 40 cycles on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The relative amounts of the transcripts encoding the gene of interest were determined in each sample by normalization with respect to the *ABL1* housekeeping gene, according to the standard ΔCt method.

Generation of anti-B7-H6 mAb

Balb/c mouse was immunized intravenously with 100 µg of recombinant soluble B7-H6 produced in CHO cells (GTP Technology) on day 0 and day 15, and intraperitoneally with 100 µg of recombinant soluble B7-H6 on day 25. Three days after the last injection the spleen was harvested. Splenocytes were isolated and fused with X63-Ag8.653 myeloma cells. After 3 weeks of culture, supernatant of hybridomas were screened for the presence of anti-B7-H6 mAb by differential staining of P815 cells expressing B7-H6 or B7-H1 as negative control. A second screen was then performed to select for the mAb that inhibit the binding of NKp30Fc to B7-H6 expressing P815 cells. Selected hybridomas were cloned and called 4E5.5, 9G9.2, 10E2.9 and 17B1.3. All of them exhibited a mouse IgG1 isotype.

Antibodies

Anti-B7-H6 mAbs (17B1.3) were used either as purified or coupled to Alexa 647. Mouse isotype control antibodies (mIg) consisted of an equimolar mixture of isotype control mIgG1 (Biolegend) coupled to Alexa 647. Anti-CD14 (RMO52)-FITC; anti-HLA-DR (B8.12.2)-PE; anti-NKp30 (Z25)-PE were purchased from Beckman Coulter. Anti-CD24 (ML5)-PE-Cy7; anti-CD3 (UCHT1)-PB; anti-CD45 (HI30)-Alexa 700; anti-CD19 (HIB19)-PerCP-Cy5.5 were purchased from Biolegend. Anti-CD16 (3G8)-APC-H7; anti-CD63 (H5C6)-FITC; anti-CD81 (JS-81)-PE was purchased from BD Pharmingen.

Flow cytometry staining

PBMCs, monocytes and neutrophils were saturated 1 hour with 20% Human Serum, AB (LONZA) in PBS supplemented with 2 mM EDTA (FACS buffer), then incubated with antibodies for 30 minutes at 4°C in FACS buffer. Finally cells were fixed by incubation in 1% paraformaldehyde in FACS buffer for 10 minutes at room temperature. Stainings were analysed on a LSR II.UV (BD Biosciences) using the FlowJo software (Tree Star, Inc.). P815.B7-H6 cells, P815.B7-H1 cells, NKp30 ligand expressing cells (Hela) or NKp30 ligand negative cells (IGROV) were saturated 30 minutes with 10% Normal Mouse serum (Sigma-

Aldrich) in FACs buffer, then incubated with each anti-B7-H6 mAb or mIgG1 as an isotype control for 30 minutes at 4°C in FACs buffer. Cells were washed and incubated with PE conjugated anti-mouse IgG (Beckman Coulter) for 30 minutes at 4°C in FACS buffer. Stainings were analysed on a FACSCanto (BD Biosciences) using the FlowJo software (Tree Star, Inc.).

Surface plasmon resonance (SPR) analysis

SPR measurements were performed on a Biacore T100 apparatus (Biacore GE Healthcare) at 25°C. In all Biacore experiments HBS-EP+ buffer (Biacore GE Healthcare) served as running buffer, 10 mM NaOH, 500 mM NaCl served as regeneration buffer and sensorgrams were analyzed with Biaevaluation 4.1 and Biacore T100 Evaluation software. For solution competition experiments, the human NKp30-Fc recombinant proteins were covalently immobilized onto a CM5 sensorchip as previously described (13). Soluble recombinant B7H6 proteins at a constant concentration of 10 µg/ml were pre-incubated with a 10 molar equivalent excess of antibodies and injected for 2 minutes at a flow rate of 10 µl/minutes onto the NKp30-Fc chip.

Reporter cell assay

DOMSP30 reporter cell lines were generated as previously described (13). Engagement of the intracytoplasmic domain of mouse CD3ζ fused to the extracellular portion of NKp30 triggers IL-2 secretion. DOMSP30 cells (30,000 cells/well in 96-well plates) were incubated either with P815.B7-H6, with HeLa cells (30,000cells/well in 96-well plates),with monocytes (60,000 cells/well in 96-well plates) or with K562 cells (30,000 cells/well in 96-well plates) in presence of anti-B7-H6 mAb or mIgG1 as an isotype control. When specified 5µg of soluble B7-H6+ or soluble B7-H6- pellets, that sedimented with the exosomal fraction, are added into the well. After 20 hours, cell supernatants were collected and assayed for the presence of mouse IL-2 in a standard CTLL-2 survival assay using Cell Titer-Glo Luminescent Cell Viability Assay (Promega).

ELISA

17B1.3 mAb was coated at 5µg/ml in 0.1 M $NaHCO_3$ solution overnight at 4°C in 96-well Nunc-Immuno™ plates (Thermo Scientific). Blocking solution (PBS supplemented with 3% BSA) was then added overnight at 4°C. After discarding this solution, serial dilutions of soluble B7-H6 were incubated during 3 hours at room temperature. Then biotinconjugated

9G9.2 (home made) was added to each well (1 µg/ml in PBS supplemented with 1% BSA) 1 hour at room temperature. Anti-biotin HRP (Sigma Aldrich) was then added for 1 hour at room temperature. Finally BD Optia TMB substrate (BD Biosciences) was used to reveal the staining and left to incubate during 15-30 minutes at room temperature. The reaction was stopped with 1 M HCl and the Optic Density (OD) at 450 nm was read with Apollo LB 911 from Berthold.

Isolation and purification of exosomal pellets

Sera of patients were subjected to differential centrifugation as previously described in standard protocol exosomes preparation (19). Briefly, exosomal fractions were purified from the supernatant by three successive centrifugations at 1200 g (30 min), at 12,000 g (45 min), and at 110,000 g (2 hours) at 4°C using a Beckman coulter Type 70 rotor. The exosomal pellet was washed in PBS, filtered through a 0.22-mm filter (Millipore, Billerica, MA), ultracentrifuged at 110,000 g for 1 hour, and resuspended in PBS.

Coupling of exosomes and FACs analysis of exosome-coated beads

Exosomal pellets (5µg) were incubated with 4-µm-diameter aldehyde/sulfate latex beads (Interfacial Dynamics) in PBS overnight at 4°C under gentle agitation. After washing with PBS, samples were blocked with PBS/0.5% BSA and incubated with anti-B7-H6 mAb (the 17B1.3 clone) coupled to Alexa 647 (home made), anti-CD81 (JS-81)-PE and anti-CD63 (H5C6)-FITC (all from BD Pharmingen) or mIgG1 (Biolegend) coupled to Alexa 647 (homemade), mIgG1-PE and mIgG1-FITC (all from BD Pharmingen) as isotype control for 30 minutes at 4°C in PBS/0.5% BSA. Stainings were analyzed on a LSR II.UV (BD Biosciences) using the FlowJo software (Tree Star, Inc.).

Patients

Frozen PBMCs and serum were obtained from a biobank generated in the course of a prospective cohort study of patients conducted at the medical intensive care unit (ICU) of Sainte Marguerite University Hospital (Marseille, France). The study trial (clincaltrials.gov NTC00699868) was approved by the Sud-Méditerranée V Ethics Committee (Comité de Protection des Personnes). Written informed consent was obtained from all patients or their proxies. All enrolled patients had blood samples drawn within the 48h from admission to the ICU and cryopreserved for further immunological analyses. Blood samples from 39 ICU patients, including 27 patients admitted for severe bacterial infections (ICU infection) and 12

patients admitted for non-infectious conditions (ICU no infection) were used in the present study. The characteristics of these patients on admission are detailed in (Table 1) and are based on the severe sepsis or septic shock classification according to the ACCP-SCCM consensus (63).

Statistical analysis

Statistical significance was determined for two-way ANOVA with Bonferroni correction or Student's *t*-test (Prism 5, GraphPad Software). P values less than 0.05 were considered significant.

Results

***In vitro* induction of B7-H6 transcripts in primary blood cells**

Although the cell surface expression of B7-H6 was found to be restricted to malignant cells, we set up an *in vitro* screening test to investigate whether B7-H6 could be induced on primary blood cells. In this assay, peripheral blood mononuclear cells (PBMCs) were treated with a variety of agents and mRNA was extracted to quantify B7-H6 transcripts using quantitative RT-PCR (qRT-PCR). In contrast to reports showing that DNA-damaging agents (such as irradiation, cisplatine, mitomycine C, etoposide or fluorouracile) and proteasome inhibitors (such as epoxomicin or MG132) can induce the expression of NKG2D ligands (2), these treatments had no substantial effect on B7-H6 expression in our protocols (data not shown). However, stimulations of PBMCs with a panel of Toll-like receptors (TLR) agonists induced B7-H6 transcripts. TLR ligands that are recognized via TLR2, such as the synthetic bacterial lipoprotein Pam3CSK4 (TLR1/2), heat-killed *A. laidlawii* (HKAL), zymosan and the synthetic diacylated lipoprotein FSL-1 (TLR2/6), via TLR4 (*E. coli* LPS) and via TLR5 (*S. typhimurium* flagellin) induced a fast expression of B7-H6 mRNA with a peak at 3 to 6 hours upon treatment (Fig. 1*A*). A common feature of these TLR is that they are expressed at the cell surface and signal through MyD88. In contrast, the stimulation of primary blood cells via endosomal TLRs, such as TLR7 (R837), TLR9 (CpGs) and TLR3 (Poly IC) had very limited impact of B7-H6 expression. A noticeable exception was the stimulation of primary cells by ssRNA that triggers a TLR8-dependent pathway and induced B7-H6 transcripts (Fig. 1*A*). As a control, all TLR stimulation induced IL-6 transcripts (Fig. S1). Noteworthy, the pro-inflammatory cytokines TNFα and IL-1β also induced B7-H6 transcripts (Fig. 1*B,* 1*C*).

Overall, the kinetics of B7-H6 mRNA induction upon TLR and cytokine secretion were quite similar, peaking early between 3 (TLR2 ligands) and 18 hours (TNFα), and returning fast to baseline within 24 hours post-stimulation. Thus, B7-H6 is one of the early genes that is induced in primary blood humans cells upon inflammatory and infectious conditions *in vitro*.

***In vitro* induction of cell surface B7-H6**

To further investigate the regulation of B7-H6 expression, we generated a panel of mouse monoclonal antibodies (mAbs) directed against human B7-H6. Four mAbs were selected (17B1.3, 10E2.9, 9G9.2 and 4E5.5) on the basis of their selective reactivity with P815.B7-H6, but not with P815.B7-H1 stable transfectants (Fig. 2*A*). As expected, 4E5.5, 9G9.2, 10E2.9 or 17B1.3 mAbs also reacted with human tumor cell lines expressing B7-H6 at steady-state such as HeLa cells (Fig. 2*A*). Two of these mAbs, 4E5.5 and 17B1.3, were blocking the activation of $NKp30^+$ reporter cells (DOMsp30) induced by P815.B7-H6 (Fig. 2*B*). Despite their blocking activity, surface plasmon resonance (SPR) analysis showed that 4E5.5 and 17B1.3 mAbs did not inhibit the direct interaction of B7-H6 with NKp30 (Fig. 2*C*). Indeed, the complexes formed between B7-H6 and anti-B7-H6 mAbs not only bound to immobilized soluble recombinant NKp30, but also showed enhanced binding and better stability, as compared to B7-H6 alone (Fig. 2*C*). These data contrast with the inhibition of the interaction between NKp30 and B7-H6 induced by two blocking anti-NKp30 mAbs (Az20 and 189).

The availability of these anti-B7-H6 mAbs allowed us to investigate whether the *in vitro* inducers of B7-H6 transcripts also led to the cell surface expression of the protein and on which cell type. PBMCs were therefore stimulated with TLR agonists, IL-1β or TNFα for 48 hours, and the cell surface expression of B7-H6 was analyzed by flow cytometry using directly conjugated 17B3.1 anti-B7-H6 mAbs. At steady state, no cell surface expression of B7-H6 on peripheral blood cells could be detected (Fig. 3*A*), consistent with previous findings (13). At 48 hours, B7-H6 was expressed on the surface of $CD45^+CD14^+CD19^-CD3^-$ monocytes upon IL-1β, LPS, flagellin and TNFα stimulation (Fig. 3*B*), consistent with the induction of B7-H6 transcripts upon these stimulations. A low but reproducible induction of B7-H6 protein was seen on monocytes cultured for 48 hours on plastic dishes without any further stimulation (Fig. 3*B*). PolyIC did not lead to the cell surface expression of B7-H6 on monocytes (Fig. 3*C*). The induction of B7-H6 was specific of monocytes, as all others cells from PBMCs remain negative for B7-H6 expression upon *in vitro* stimulation with TLR ligands and cytokine (data not shown). Despite a rapid decline in B7-H6 transcripts (Fig. 1A),

the cell surface expression of the B7-H6 protein on monocytes was stable up to 48 hours (Fig. 3*C*). The decrease in monocyte viability upon longer period of *in vitro* culture, prevented us to analyze further the kinetics of B7-H6 cell surface expression. $CD14^+$ monocytes were then purified from PBMCs and stimulated *in vitro* for 48 hours with TLR ligands or pro-inflammatory cytokines. Under these conditions, monocytes became B7-$H6^+$ as illustrated for IL-1β in Fig. 4*A*, indicating that the induction of B7-H6 on monocytes was a direct effect on these cells. Of note, under these *in vitro* stimulation conditions, all monocytes became $CD14^+CD16^+$ pro-inflammatory type monocytes (16). Although the density of B7-H6 molecules expressed at the surface of monocytes was moderate, it was sufficient to trigger the activation of NKp30 on $NKp30^+$ DOMsp30 reporter cells, but not on the $NKp30^-$ parental DO11.10 cells (Fig. 4*B*). Consistent with our previous data (Fig. 2*B*), this activation was blocked by the 17B1.3 mAbs (Fig. 4*B*).

In humans, TLR2 is expressed by monocytes and neutrophils, TLR3 is expressed by $BDCA3^+$ conventional dendritic cells (cDC), TLR5 is expressed by monocytes, neutrophils and cDCs, TLR7 is expressed by monocytes and plasmacytoid DCs (pDCs), TLR8 is expressed by neutrophils and TLR9 by pDCs (17). The pattern of TLR expression was thus consistent with the selectivity of B7-H6 induction on monocytes, but also prompted us to examine whether neutrophils could express surface B7-H6 upon stimulation. Whereas unstimulated neutrophils do not express cell surface B7-H6, treatment of neutrophils with LPS or IL-1β leads to a weak but reproducible induction of cell surface B7-H6 (Fig. 5 and data not shown*)*. As for monocytes, the effect of LPS and IL-1β was direct as it occurred on purified cell preparations, and anti-B7-H6 $F(ab)'_2$ fragments were used to ascertain the specificity of anti-B7-H6 mAb detection on these FcR^+ myeloid cells (data not shown).

The signaling events initiated by TLRs, IL-1β and TNF-α receptors converge either at very proximal steps, such as MyD88 for TLR4 and IL-1β receptors, or at later stages, such as RIP for TLR4 and TNF-α receptors (18). The p38, MEK1/2 and JNK MAP kinase pathways as well as the NF-κB pathway are classically involved downstream of these pathways (18). We then addressed the nature of the molecular pathways which are involved in B7-H6 induction. In these experiments, purified monocytes and neutrophils were stimulated using LPS, while the MAP kinase and the NF-κB pathway were pharmacologically targeted or not. Whereas both types of pharmacological inhibitors extinguished IL-6 mRNA induction by LPS on monocytes, only p38 and MEK1/2 inhibitors dampened B7-H6 mRNA expression (Fig. 6, *upper panel*). In contrast, the pharmacological inhibition of the MAP kinase pathways had no

significant effect on B7-H6 mRNA induction on neutrophils (Fig. 6, *upper panel*). On these cells, the NF-κB pathway was key to B7-H6 induction by LPS treatment (Fig. 6, *upper panel*). The signaling pathways involved in B7-H6 induction on primary monocytes and neutrophils are thus unexpectedly distinct.

***In vitro* induction of sB7-H6**

The availability of a panel of anti-B7-H6 mAbs allowed us to identify that two of them, 17B1.3 and 9G9.2, recognize non-overlapping B7-H6 epitopes rendering it possible to set-up a sandwich ELISA against B7-H6 (Fig. S2). We then investigated whether soluble forms of B7-H6 could be produced *in vitro*. Consistent with the optimal conditions for B7-H6 cell surface expression, purified monocytes were activated for 48 hours with LPS or IL-1β and soluble B7-H6 was measured in cell supernatants by ELISA. Although no sB7-H6 was detected in the supernatant of monocytes in absence of stimulations, sB7-H6 could be detected in the supernatant of LPS- and IL-1β-stimulated monocytes isolated from a fraction of healthy individuals (4/9) (Fig. 7*A*). Similarly, neutrophils prepared from a fraction of healthy individuals (4/6) and stimulated with of LPS- or IL-1β also produced sB7-H6 (Fig. 7*B*). The mechanisms underlying the heterogeneity between healthy individuals that produce or not detectable amounts of B7-H6 *in vitro* is still unclear and needs further investigation.

***In vivo* expression of B7-H6 during sepsis**

The *in vitro* induction of B7-H6 by TLR ligands and pro-inflammatory cytokines prompted us to investigate the expression pattern of B7-H6 in inflammatory and infectious conditions *in vivo*. To address this point, PBMCs were obtained from a collection of peripheral blood samples prepared from a cohort of 39 patients presenting various forms of systemic inflammatory response syndrome (SIRS) and admitted at the medical intensive care unit (ICU) (Table 1). Cells were harvested at the day of SIRS diagnosis (day 1) and kept frozen. Patients were divided into two categories: patients with sepsis (ICU infection) and patients with SIRS in absence of detectable microbial infection (ICU no infection). Samples from 32 patients out of 39 could be thawed and PBMCs were analyzed for the cell surface expression of B7-H6 on T cells ($CD3^+CD19^-CD14^-$), B cells ($CD3^-CD19^+CD14^-$), dendritic cells ($CD3^-CD19^-CD14^-DR^+$) and monocytes ($CD3^-CD19^-CD14^+$). The cell surface expression of B7-H6 was detected in PBMCs of 15 patients (Fig. 8*A* and Table 2).

Remarkably, the cell surface expression of B7-H6 was restricted to patients presenting sepsis (Fig. 8). Moreover, this expression was selectively observed on $CD14^+CD16^+$ pro-inflammatory type monocytes (Fig. 8*A*), consistent with our in vitro data (Fig. 4*A*). As expected, no cell surface expression of B7-H6 was detected on monocytes from healthy control individuals (Fig. 8*C*). The absence of neutrophils in frozen PBMC preparations prevented us to analyze whether surface B7-H6 could also be detected on these cells. sB7-H6 was also detected in the serum of 10/39 SIRS patients and restricted to patients with severe bacterial infection (Fig. 8*D*). No sB7-H6 was detected in sera obtained from other SIRS patients or healthy individuals (Fig. 8*D*). Overall, Table 2 shows that 65% of patients with sepsis had circulating $B7\text{-}H6^+CD14^+CD16^+$ pro-inflammatory type monocytes, when no $B7\text{-}H6^+$ PBMCs could be detected in other SIRS patients or healthy individuals. No difference was observed between sepsis patients presenting $Gram^-$ infections (*Achromobacter xylosoxidans, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumonia*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) or $Gram^+$ (*Staphylococcus aureus, Streptococcus pneumonia and Streptococcus mitis*) infections. In contrast, serum sB7-H6 was detected in 37% of SIRS patients, and restricted to patients with $Gram^-$ infection (Fig. 9*A*). Most $Gram^-$ sepsis patients who also had circulating $B7\text{-}H6^+CD14^+CD16^+$ pro-inflammatory monocytes at day 1 also had detectable levels of serum sB7-H6 on the same day (Table 2).

Despite the small size of our cohort of patients, we analyzed the potential association of B7H6 expression and the clinical outcome of ICU patients. The overall day 30 mortality in ICU patients was 23.1%. As showed in Fig. 9*B*, day 30 mortality was higher in patients with membrane B7-H6 ($mB7\text{-}H6^+$) expression as compared to patients lacking $mB7\text{-}H6^+$ cells ($mB7\text{-}H6^-$) (40% vs. 10 % respectively; $p = 0.036$). This result was confirmed by Kaplan-Meier analysis (Fig. 9*C*), where we observed again a trend to higher mortality in septic patients expressing mB7-H6. In particular, no death was observed when mB7-H6 expression was negative in patients with $Gram^-$ sepsis, as compared to patients expressing mB7-H6 (0% vs. 44.4 % respectively; $p = 0.07$).

Analysis of sB7-H6

We took advantage of the substantial quantities of sB7-H6 present in patient sera to document the nature of sB7-H6. In a first series of experiments, patient sera were subjected to three rounds of centrifugation at 1200 g, 12,000 g and 110,000 g sequentially. The pellets obtained at each round were analyzed by ELISA for the presence of sB7-H6. As shown in Fig. 10*A*, sB7-H6 was associated with the material that sedimented at 100,000 g, suggesting

that sB7-H6 was included in membrane vesicles that are present in the serum, such as exosomes. To further dissect this point, sB7-H6$^+$ patient sera were analyzed for the presence of B7-H6$^+$ membrane vesicles using sequential low-speed centrifugation and high-speed centrifugation as well as 0.22 μM filtration as described previously (19). After incubation of the filtered pellet with latex beads, we detected that sB7-H6$^+$ patient sera contained material that was reactive with CD63, CD81 and B7-H6 mAbs (Fig. 10*B)*, suggesting that B7-H6 can be associated with exosomes in sepsis patients. Incubation of primary NK cells with the sB7-H6$^+$ pellets that sedimented with the exosomal fraction impaired the staining of NK cells with anti-NKp30 mAbs, whereas sB7-H6$^-$ pellets had no effect (Fig. 11*A*). In addition, sB7-H6$^+$ pellets also inhibited the NKp30-dependent activation of reporter cells induced by B7-H6$^+$ K562 cells as efficiently as the blocking 17B1.3 anti-B7-H6 mAbs (Fig. 11*B*). Interestingly, recombinant sB7-H6 has no effect on both NKp30 cell surface expression and NKp30-dependent cell activation over a large range of concentration (data not shown). The Z25 mAb used to stain NK cells is a blocking anti-NKp30 (6), making it impossible to formally show that sB7-H6$^+$ pellets are down-regulating NKp30 cell surface expression or merely masking surface NKp30. Nevertheless, irrespective of the formal demonstration that sB7-H6 is included in serum exosomes, the impact of sB7-H6$^+$ serum pellets on NKp30 staining and cell activation that contrasts with the failure of recombinant sB7-H6 to do so, suggests that the valency of sB7-H6 expressed as recombinant protein or contained in serum pellets is different, and supports that sB7-H6 contained in serum pellets is multimerized as it could be in exosomes.

Discussion

B7-H6 is the most recently described member of the B7 family of cell surface immunoreceptors (13, 14, 20-26). Thus far, the expression of B7-H6 has been shown to be restricted to tumor cells and absent from normal hematopoietic cells from healthy individuals at steady-state (13, 20). It is well known that B7 family members are induced on various cells of myeloid origin, such as dendritic cells, monocytes, macrophages and neutrophils upon stimulation with infectious and pro-inflammatory stimuli (27-30). However, no data have been reported on the mechanisms leading to the induction of B7-H6. We have shown here that *B7-H6* transcripts, B7-H6 cell surface expression and sB7-H6 can be induced in inflammatory conditions *in vitro* and *in vivo*. These data thus indicate that B7-H6 expression is not limited to tumor cells, and reveal that non-tumor cells could be the targets of NK cells through B7-

H6/NKp30 interaction. They further support the concept introduced earlier for the induction of NKG2D ligands as which the ligands for NK cell activating receptors are silenced on normal cells and induced in various conditions of cellular stress, such as infection, inflammation and cancer. There are however similarities and differences between the conditions that lead to NKG2D-L or B7-H6 expression. On the differences, we could not detect B7-H6 induction on the surface of cells treated with the DNA damaging agents or the proteasomes inhibitors that have been shown to induced cell surface NKG2D ligands (31, 32). On the similarities, TLR signaling has been shown to up-regulate transcription and expression of NKG2D ligands, such as retinoic acid early inducible-1 (RAE-1) family members in mouse macrophages (33) and MICA on human macrophages (34-36). These data have led to suggest a role for the cross-talk between NK cells and monocytes/macrophages during innate immune response to infections. Our present data on the induction of B7-H6 on inflammatory monocytes and neutrophils support this idea. The dissection of the mechanisms involved in these conditions in particularly relevant to sepsis, the control of which represent an important unmet medical need.

Sepsis is a clinical syndrome that complicates severe infection, and is one of the leading causes of admission to ICU. There are several forms of sepsis which include severe sepsis (acute organ dysfunction secondary to infection) and septic shock (severe sepsis plus hypotension not reversed by fluid resuscitation). These severe forms of infection, mainly of bacterial origin, represent a major healthcare problem, accounting for thousands of deaths every year worldwide, with more than 200,000 deaths per year just in the United States. Severe sepsis, for example, is projected to represent one million cases per year before 2020 in the United States. Mortality rates for sepsis range from 30% to more than 50%, especially when patients present septic shock. The host-response of these patients, include a first phase of SIRS, characterized by an exacerbated inflammatory response, rapidly followed by a profound alteration of immunity, referred as to compensatory anti-inflammatory response syndrome (CARS). This acquired immunoparalysis is thought to render the patients more susceptible to nosocomial infections, and to lead to increased morbidity and mortality.

NK cells are a major source of interferon (IFN)-γ, a potent inflammatory cytokine, and early depletion of NK cells improved the survival of in sepsis models in the mouse (37, 38). We also recently observed that both NK cell cytotoxicity and INF-γ production were altered very early after the onset of sepsis (39), consistent with similar results obtained in mice (40). Considering the sequence of opposite events that are at work during sepsis, NK cells might have a dual role in sepsis, first contributing to the amplification of the inflammatory response

during the early steps of SIRS and then, participating to CARS and its consequences (39, 41, 42). The mechanisms involved in the reprogramming of NK cells from responsive to hypo-responsive cells are unknown. We observed that higher levels of circulating IL-10 among ICU patients with cytomegalovirus reactivation during their ICU stay were associated with NK cell hyporesponsiveness (42). In a recent study, TGF-β was proposed to be involved in the hyporesponsivess of NK cells acquired after a septic challenge (40).

Our data on B7-H6 expression during inflammation provide a novel perspective on the link between microbial infection and NK cell activation. First, B7-H6 was selectively induced on the surface of $CD14^+CD16^+$ pro-inflammatory monocytes (16) and neutrophils, two types of cells which play a central role in the onset and amplification of inflammation. In particular, $CD14^+CD16^+$ monocytes are described as the main producers of inflammatory cytokines such as TNF-α and IL-1β in response to LPS (43). Several studies have reported that $CD14^+CD16^+$ monocytes are found in larger numbers in the blood of patients with acute inflammation and infectious diseases such as sepsis, tuberculosis and rheumatoid arthritis (44-46). Second, B7-H6 could be produced as a soluble form *in vitro* by activated monocytes and neutrophils, and *in vivo* in a group of sepsis patients. B7 family members include B7-1 (CD80), B7-2 (CD86), B7-H1 (CD274 or PD-L1), B7-H2 (CD275 or ICOS-L), B7-H3 (CD276), B7-H4 (B7S1 or B7x), B7-H6, B7-DC (CD273 or PD-L2), and BTLN2 (47). Soluble forms of CD80, CD86, B7-H1, B7-H2, B7-H3 and B7-H4 have been detected in the serum of patients with cancer and inflammatory conditions (48-55). Although in several instances soluble B7 receptors have been shown to serve as decoy molecules to block the function of their receptors, the biological roles of these molecules remain to be dissected in detail. The present description of cell surface and soluble B7-H6 thus confirms the general propensity of the members of the family to be induced in inflammatory conditions. sB7-H6 present in the serum of sepsis patients impaired the binding of anti-NKp30 mAbs to NKp30 and NKp30-dependent cell activation. Importantly, recombinant sB7-H6 was unable to impact on B7-H6 expression and signaling. Thus, sB7-H6 present in patient serum is different from recombinant sB7-H6. These data support the fact that serum sB7-H6 was found in exosomal fractions and thus could be exposed as multimers with higher avidity that monomeric recombinant sB7-H6. Along these lines, the biological effect of monomeric sNKG2D ligands is disputed, as only membrane NKG2D ligands have been shown to impact on NKG2D expression and signaling (56).

The mechanism by which NK cells via NKp30 and myeloid cells (inflammatory monocytes and neutrophils) via B7-H6 may communicate directly during an innate immune response to infection may be two-fold. Although our cohort of SIRS patients is limited, our

data show that 65% of patients with sepsis had circulating B7-H6$^+$CD14$^+$CD16$^+$ pro-inflammatory type monocytes, when these cells could not be detected in other SIRS patients or healthy individuals. The highest mortality of patients with mB7-H6$^+$ would suggest a negative role for NK cell activation in sepsis, consistent with data in the mouse (37, 38). But, we also show that sB7-H6 was detected in the serum of sepsis patients, and selectively associated with the development of Gram$^-$ sepsis. Although not statistically significant due to the limited size of our patient cohort, we observed a trend associating sB7-H6 and higher patient mortality (data not shown). As the form of sB7-H6 present in patient's serum is blocking the activation of NK via NKp30, these results suggest a negative role of blocking the activation of NK cells, thus a positive role of NK cells. Based on these data, we thus propose a model as which the activation of NK via mB7-H6 contributes to inflammation during SIRS, but then sB7-H6 might play a negative role during CARS, explaining why two opposing phenomena (the expression of mB7-H6 that activates NK cells and the production of sB7-H6 that impairs NK cell activation) could have the same consequences, i.e a deleterious impact on patient survival during sepsis.

Our data reveal for the first time the cell surface expression of B7-H6 in non-transformed cells during inflammation and the presence of sB7-H6. They also highlights several points that remain to be addressed. First, the precised nature of sB7-H6 should be analyzed. Noteworthy, the closest homologue of B7-H6 is a human endogenous retrovirus HERV-W (3q26.32), with a quite remarkable 63% identity of 100 amino acids between HERV-W GAG and B7-H6 GAG proteins. HERV-W is a very interesting retroelement whose ENV gene was associated with the formation of the placenta (57). The expression of HERV-W is also associated with multiple sclerosis (58-60). The homology domain is well in the Matrix, the domain of GAG juxtaposed to the membrane. The role of Matrix is still poorly understood, but it has been shown to be involved in the morphogenesis of particles. It is thus possible that the accumulation of cell surface B7-H6 leads to the aggregation of B7-H6 molecules via their GAG domains and leads to the production of sB7-H6 that is associated with membrane vesicles and co-sediment with exosomes. Finally, it is also possible that the interaction of cell surface B7-H6 with NKp30 transduces intracytoplasmic signals within CD16$^+$CD14$^+$ cells and neutrophils and participate to the regulation of their activation. Irrespective of these issues, our data prompt to perform the immunomonitoring of B7-H6 cell surface and soluble expression in a large of cohort of sepsis patients over the kinetics of their clinical outcome. Indeed, the early identification of individuals at risk of developing bad outcomes is critical in sepsis and our data suggest that mB7-H6 and/or sB7-H6 can help in

stratifying these patients. In particular, it is remarkable and still puzzling that serum sB7-H6 was restricted to patients with Gram$^-$ infection, while no difference was observed between sepsis patients presenting Gram$^-$ or Gram$^+$ infections for mB7-H6 expression. It thus remains to be understood whether or not B7-H6 is involved in the association between improved survival in patients with severe Gram$^-$ sepsis and high circulating NK counts (61), whereas patients with Gram$^+$ sepsis had a more persistent reduction in circulating NK cells than that seen with Gram$^-$ sepsis (62).

n	Reason for ICU admission	Age	Gender	Site of infection	Pathogen	mB7H6	sB7H6 (ng/ml)	Shock	Death
1	Postoperative	85	M	-	-	No	0	No	No
2	Bleeding	90	F	-	-	No	0	Yes	No
3	Acute pancreatitis	70	M	-	-	No	0	Yes	No
4	Drug intoxication	52	M	-	-	No	0	No	No
5	Acute coronary syndrome	60	F	-	-	No	0	No	No
6	COPD exacerbation	85	F	-	-	No	0	No	No
7	Acute kidney failure	52	F	-	-	No	0	No	No
8	Lung Atelectasis	45	M	-	-	No	0	No	No
9	Postoperative	65	F	-	-	No	0	No	No
10	Epilepsy	48	M	-	-	No	0	No	No
11	COPD exacerbation	69	M	-	-	No	0	No	Yes
12	Severe hyponatremia	37	F	-	-	No	0	No	No
13	Severe infection	83	F	Bacteremia	Gram+	Yes	0	Yes	Yes
14	Severe infection	53	M	Pneumonia	Gram+	Yes	0	Yes	Yes
15	Severe infection	43	F	Pneumonia	Gram+	No	0	Yes	No
16	Severe infection	84	M	Pneumonia	Gram+	No	0	Yes	Yes
17	Severe infection	86	M	Pneumonia	Gram+	Yes	0	Yes	No
18	Severe infection	73	M	Pneumonia	Gram+	Yes	0	Yes	No
19	Severe infection	44	M	Pneumonia	Gram+	Yes	0	No	No
20	Severe infection	83	M	Pneumonia	Gram+	Yes	0	Yes	No
21	Severe infection	31	M	Pneumonia	Gram+	No	0	No	No
22	Severe infection	67	M	Pneumonia	Gram-	Yes	40	Yes	Yes
23	Severe infection	46	M	Pneumonia	Gram-	No	26,2	Yes	No
24	Severe infection	54	M	Pneumonia	Gram-	Yes	20,8	Yes	No
25	Severe infection	90	F	Pneumonia	Gram-	Yes	9,8	Yes	Yes
26	Severe infection	70	M	Abdominal	Gram-	ND	9,2	Yes	No
27	Severe infection	28	F	Abdominal	Gram-	Yes	9,2	No	No
28	Severe infection	41	M	Pneumonia	Gram-	Yes	8,7	Yes	No
29	Severe infection	92	F	Abdominal	Gram-	Yes	6,5	Yes	Yes
30	Severe infection	82	M	Pneumonia	Gram-	Yes	4	No	No
31	Severe infection	80	M	Bacteremia	Gram-	ND	1,1	Yes	Yes
32	Severe infection	71	M	Bacteremia	Gram-	ND	0	No	No
33	Severe infection	76	M	Bacteremia	Gram-	ND	0	Yes	No
34	Severe infection	42	F	Bacteremia	Gram-	Yes	0	Yes	No
35	Severe infection	67	M	Pneumonia	Gram-	Yes	0	Yes	Yes
36	Severe infection	78	M	Pneumonia	Gram-	No	0	No	No
37	Severe infection	42	F	Pneumonia	Gram-	No	0	No	No
38	Severe infection	48	M	Pneumonia	Gram-	No	0	No	No
39	Severe infection	78	M	Pneumonia	Gram-	No	0	Yes	No

Table 1. Baseline characteristics of ICU patients Patients admitted to the ICU for an infectious conditions. COPD: Chronic Obstructive Pulmonary Disease; F: female; M: male Analysis of cell surface expression and soluble B7-H6 were performed at day 1 after entry to the ICU.

		mB7H6 positive	sB7H6 positive
healthy		0/14	0/14
ICU no infection		0/9	0/12
ICU infection		**15/23 (65%)**	**10/27 (37%)**
	Gram-	9/14 (64%)	10/18 (56%)
	Gram+	6/9 (67%)	0/9

Table 2. Association of serum sB7-H6 or cell surface expression of B7-H6 and the bacterial origin of sepsis ICU no infection: patients admitted to the ICU without bacterial infectious. ICU infection: patients admitted to the ICU for infectious conditions (ie, severe sepsis or septic shock according to the ACCP-SCCM consensus).

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for diagnosing or prognosing an inflammatory condition in a subject comprising assaying for a soluble B7-H6 polypeptide in a sample from the subject, wherein identification of a soluble B7-H6 polypeptide in the sample indicates an inflammatory condition.

2. The method according to claim 1 comprising determining the level of the soluble B7-H6 polypeptide in the sample and comparing said level with a predetermined reference value.

3. The method according to claim 1 comprising i) determining the level of a soluble B7-H6 polypeptide in a sample obtained from the subject, ii) comparing the level determined at step i) with a predetermined reference value and iii) concluding that the subject suffers from an inflammatory condition or is at risk of having an inflammatory condition when the level determined at step i) is higher than the predetermined reference value

4. A method for monitoring the treatment of a subject suffering from an inflammatory condition comprising assaying for a soluble B7-H6 polypeptide in a sample from the subject, wherein identification of a soluble B7-H6 polypeptide in the sample indicates that the treatment is not effective and non identification of a soluble B7-H6 polypeptide indicates that the treatment is effective.

5. The method according to claim 4 comprising i) determining the level of a soluble B7-H6 polypeptide in a sample obtained from the subject before the treatment, ii) determining the level of a soluble B7-H6 polypeptide in a sample obtained from the subject after the treatment iii) comparing the level determined at step i) with the level determined at step ii) and iii) concluding that treatment is effective when the level determined at step ii) is lower than the level determined at step i) or concluding that the treatment is not effective when the level determined at step ii) is above the same as the level determined at step i) or is higher than the level determined at step i).

6. A method for predicting the survival of a sepsis subject comprising the steps consisting of i) determining the level of of the soluble B7-H6 polypeptide in a sample

obtained from the subject ii) comparing the level determined at step i) with a predetermined reference level and iii) concluding that the subject has a poor prognosis when the level determined at step i) is higher than the predetermined reference level and good prognosis when the level determined at step i) is lower than the the predetermined reference level.

7. A method of screening for candidate therapeutic agents for an inflammatory condition comprising i) providing a plurality of candidate compounds, ii) bringing the candidate compounds into contact with immune cells (e.g peripheral blood mononuclear cells) in presence of an agent that stimulates the expression of a soluble B7-H6 polypeptide, iii) determining the level of the soluble B7-H6 polypeptide expressed by the immune cell, iv) comparing the level determined at step iii) with the level determined in the absence of the candidate compounds, and v) and positively selecting the candidate compounds when the level determined at step iii) is lower than the level determined in the absence of the candidate compound.

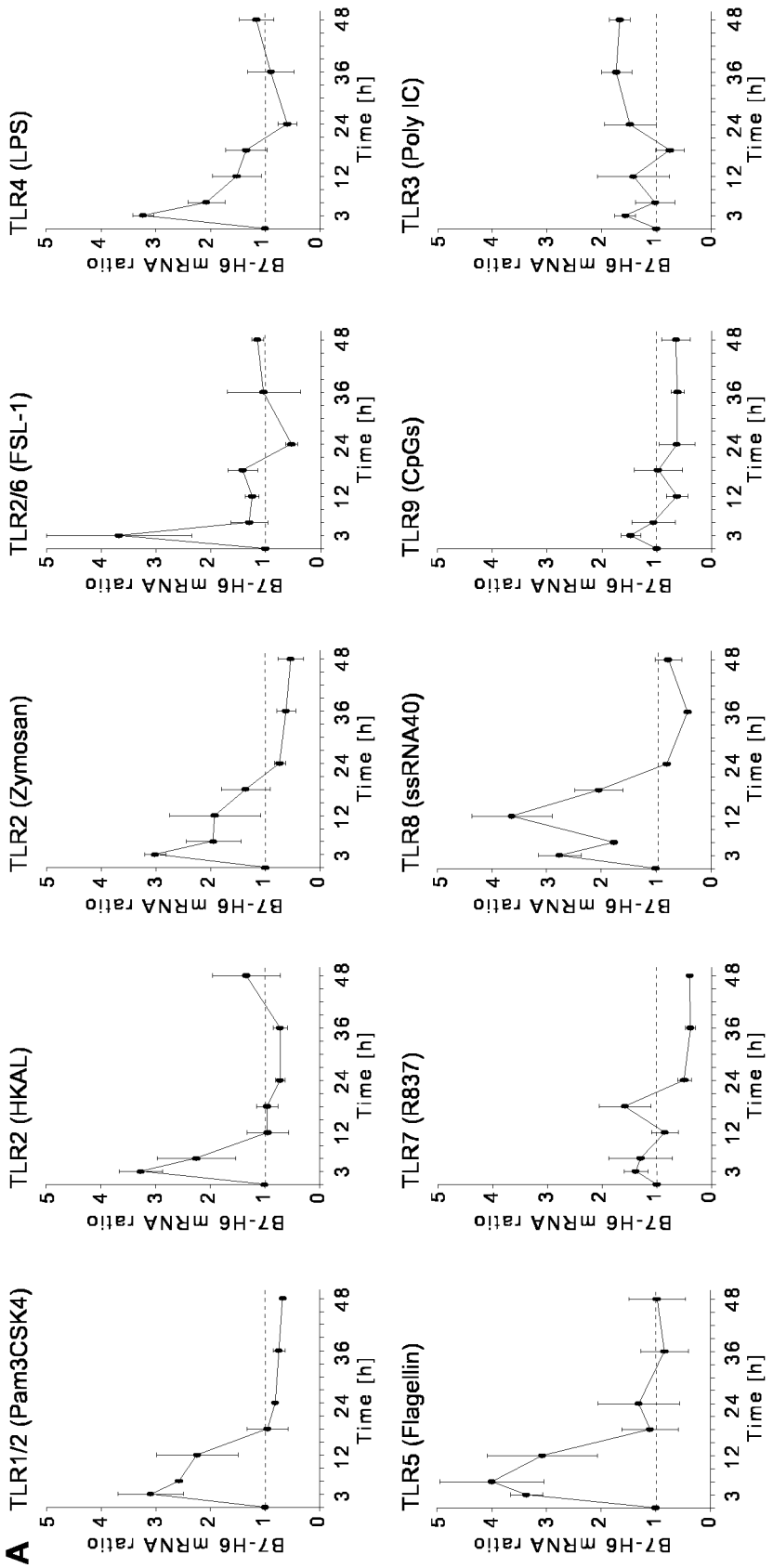

A
TLR1/2 (Pam3CSK4)
TLR2 (HKAL)
TLR2 (Zymosan)
TLR2/6 (FSL-1)
TLR4 (LPS)
TLR5 (Flagellin)
TLR7 (R837)
TLR8 (ssRNA40)
TLR9 (CpGs)
TLR3 (Poly IC)
B7-H6 mRNA ratio
Time [h]
0
1
2
3
4
5
3
12
24
36
48

Figure 1A

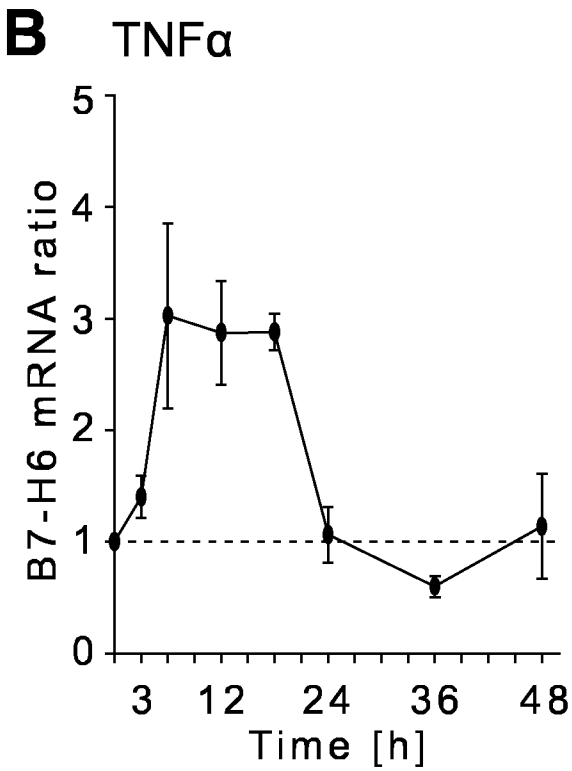


Figure 1B

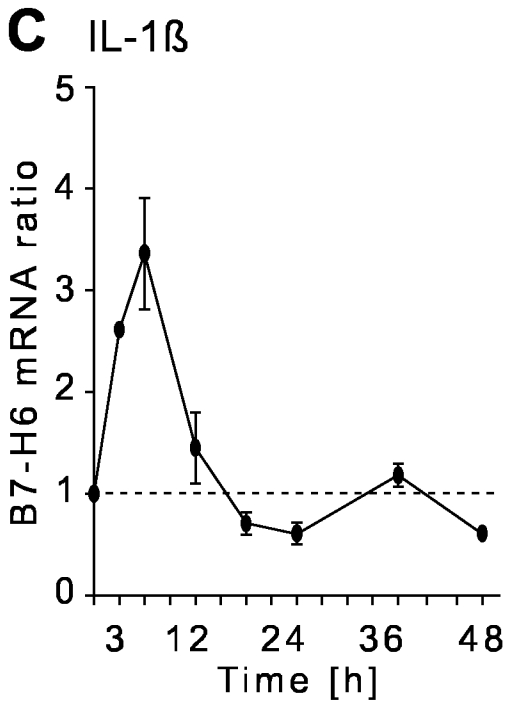


Figure 1C

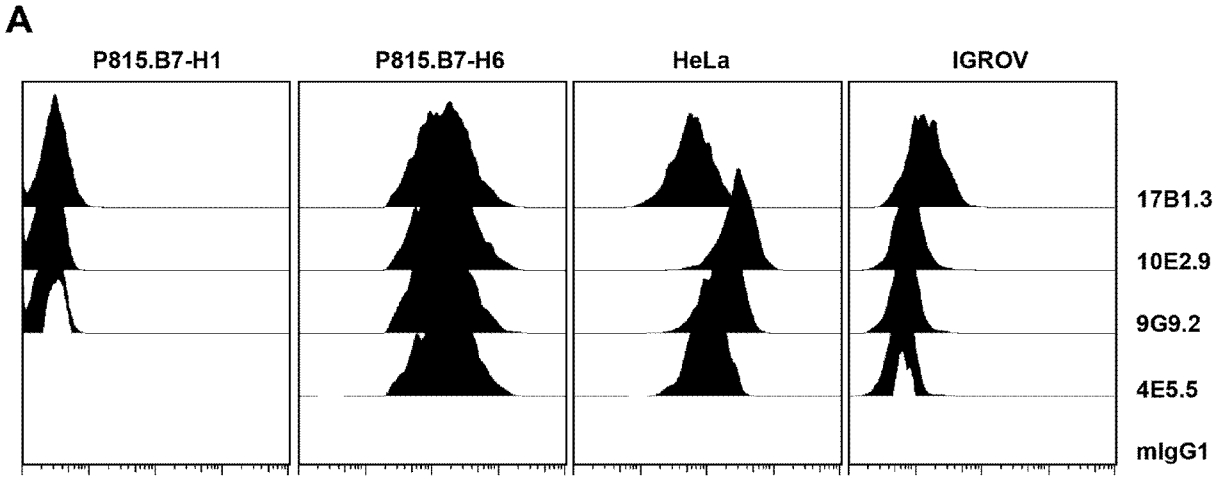


Figure 2A

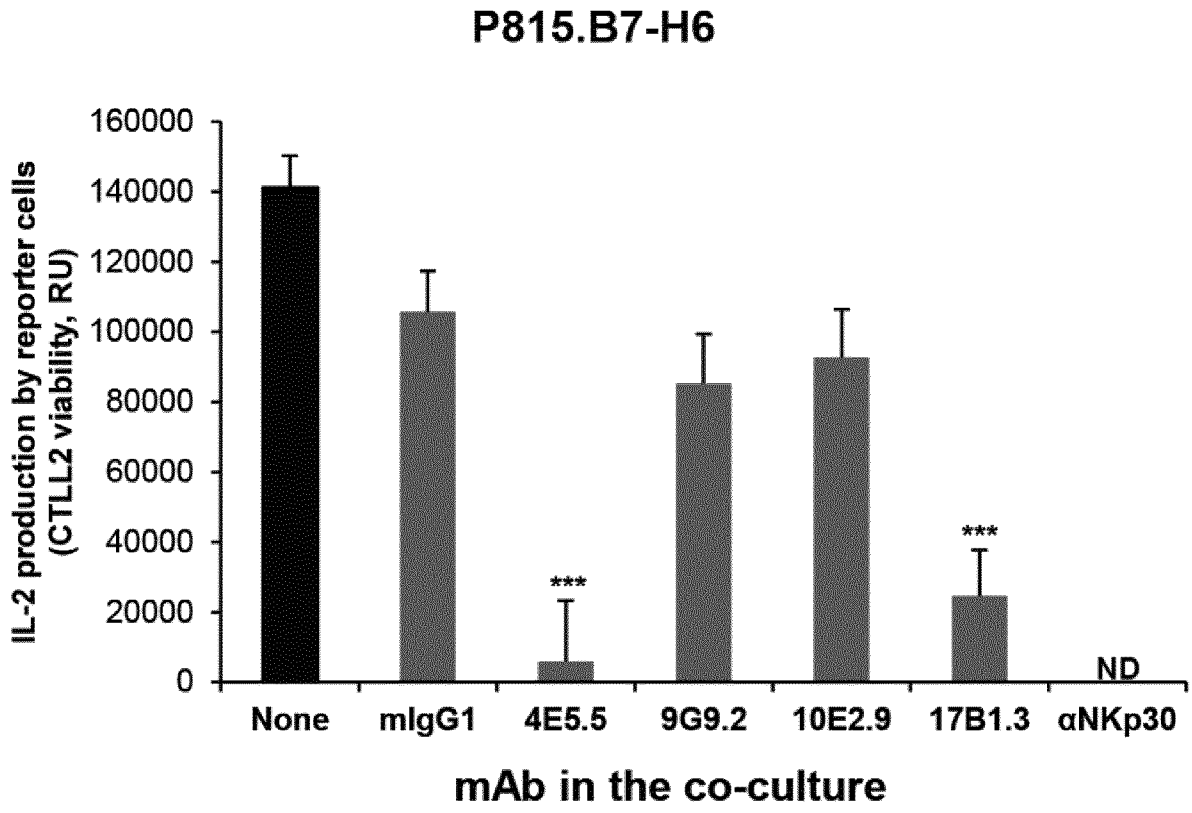


Figure 2B

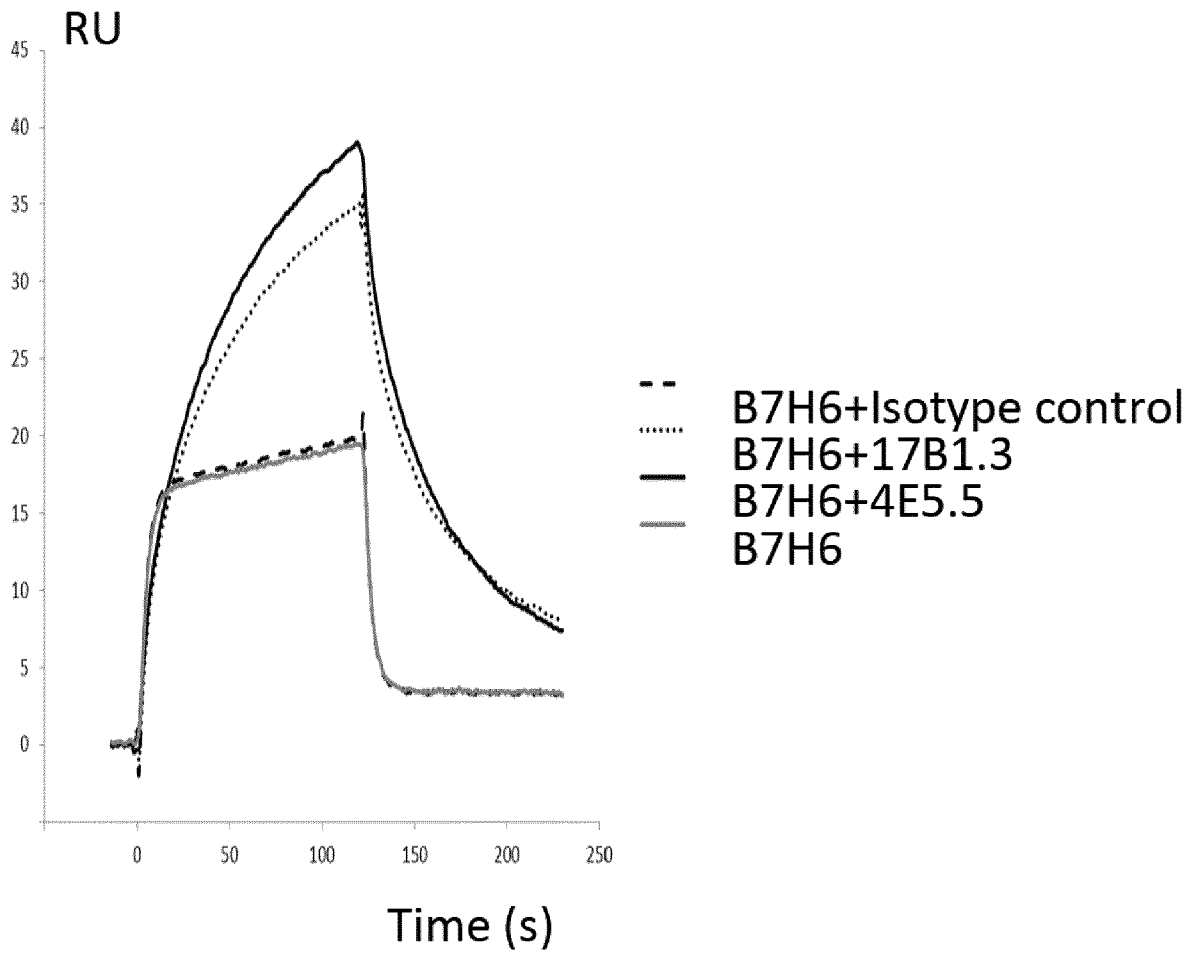

RU
45
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
Time (s)
B7H6+Isotype control
B7H6+17B1.3
B7H6+4E5.5
B7H6

Figure 2C

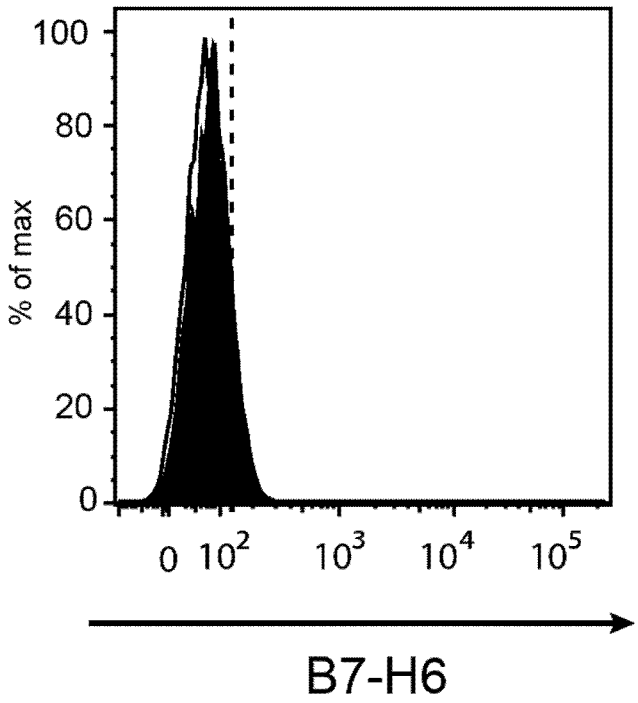


Figure 3A

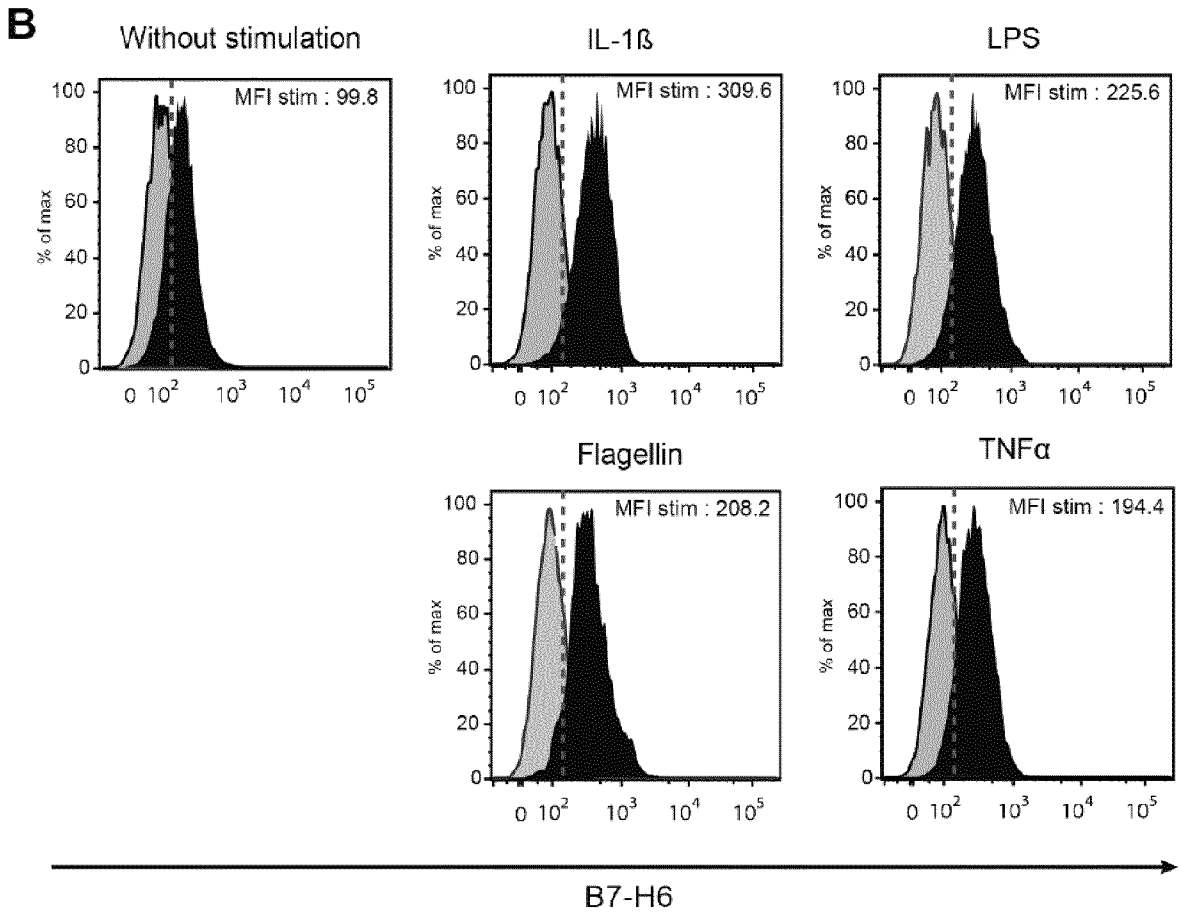


Figure 3B

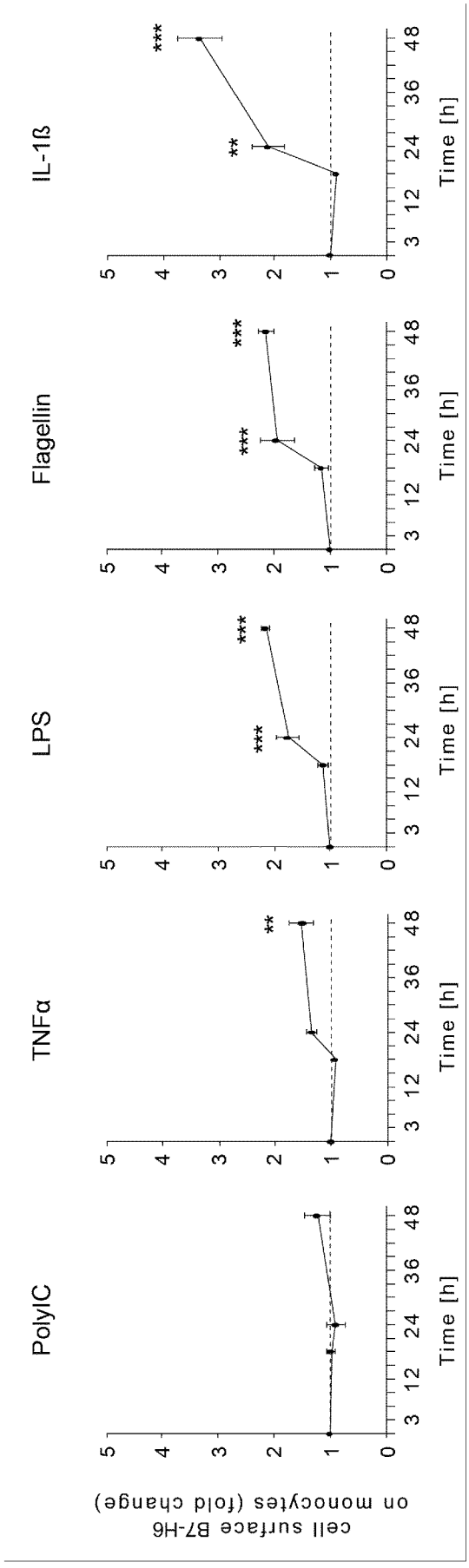

cell surface B7-H6
on monocytes (fold change)
PolyIC
TNFα
LPS
Flagellin
IL-1ß
Time [h]
0
1
2
3
4
5
3
12
24
36
48
**
***

Figure 3C

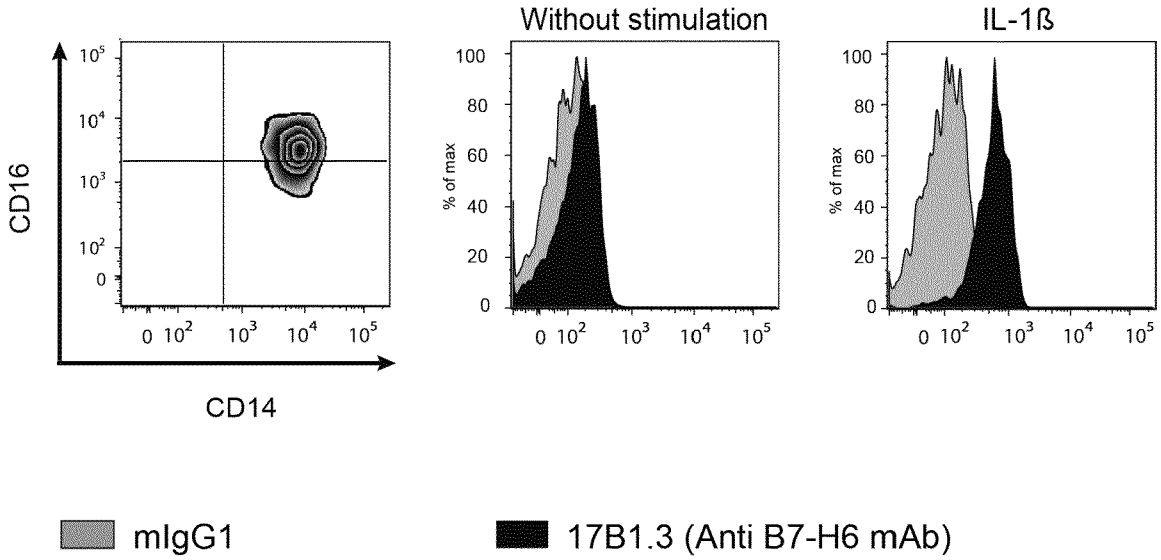


Figure 4A

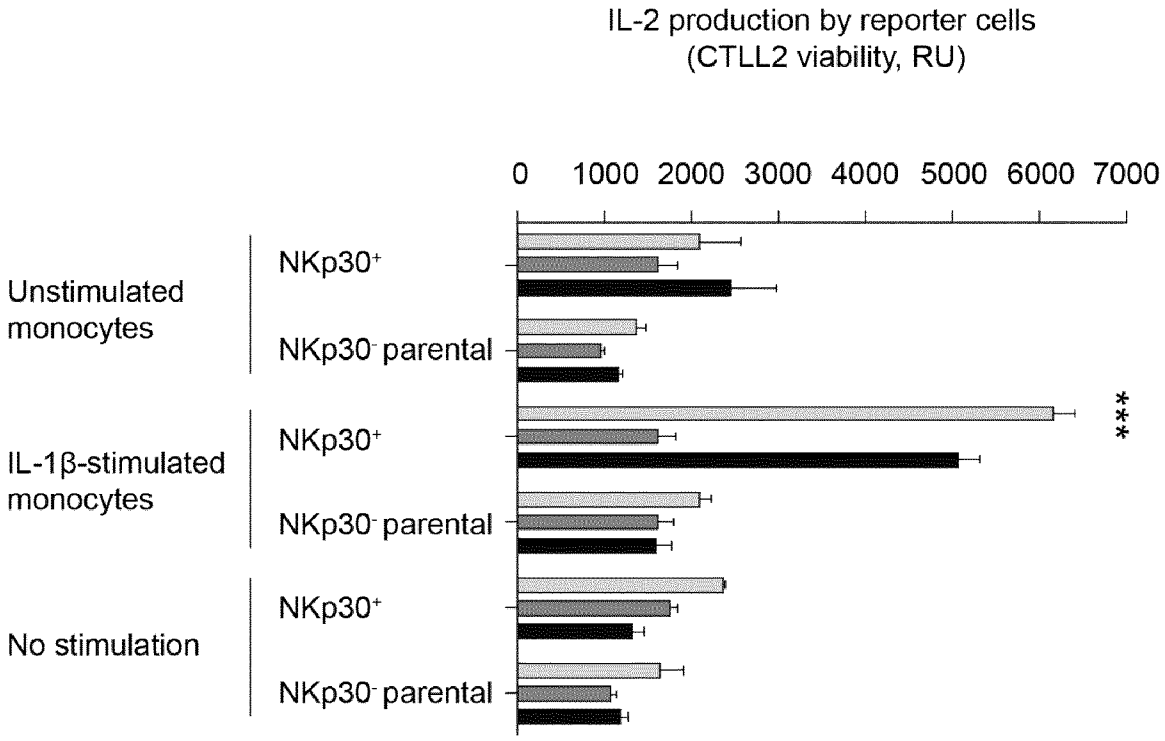


Figure 4B

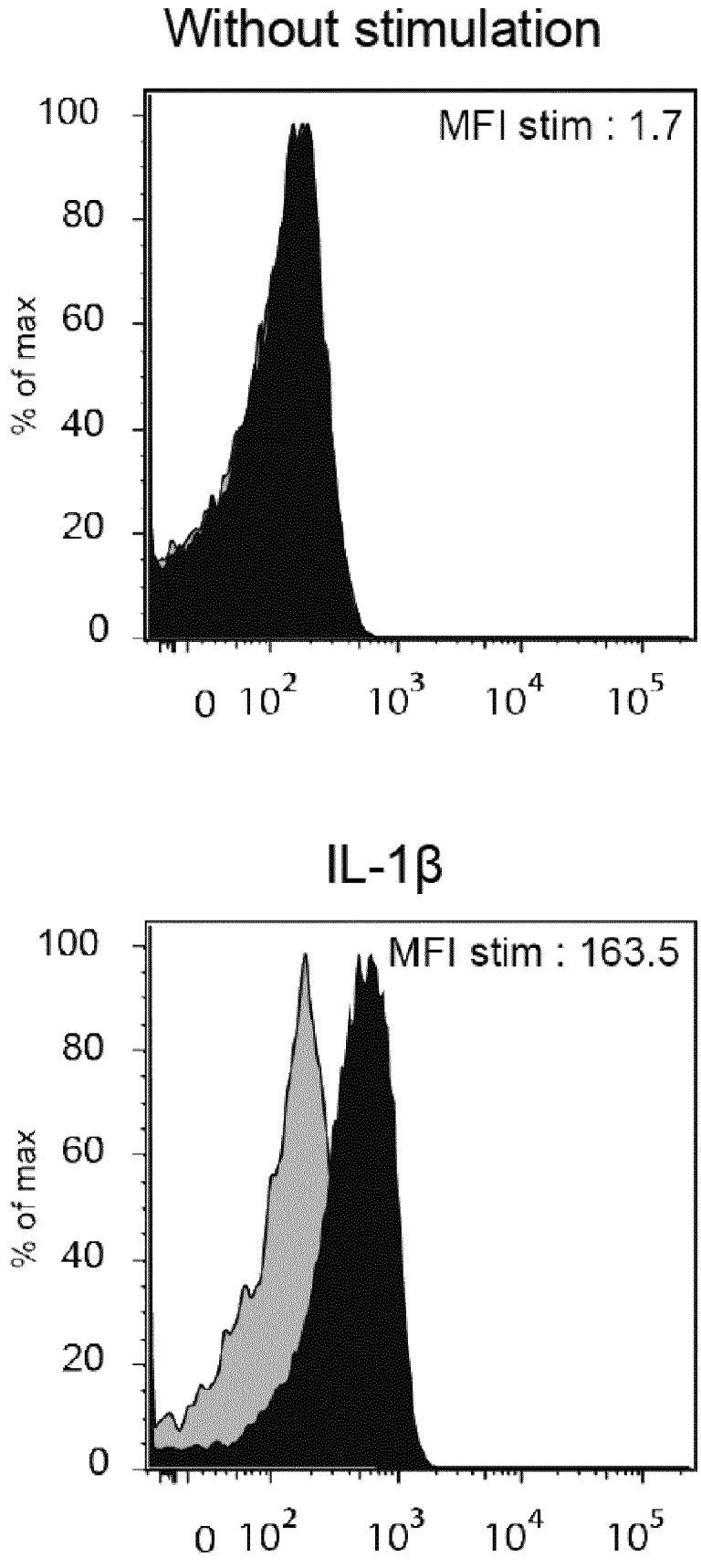

Without stimulation
MFI stim : 1.7
% of max
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
IL-1β
MFI stim : 163.5
% of max
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5

Figure 5

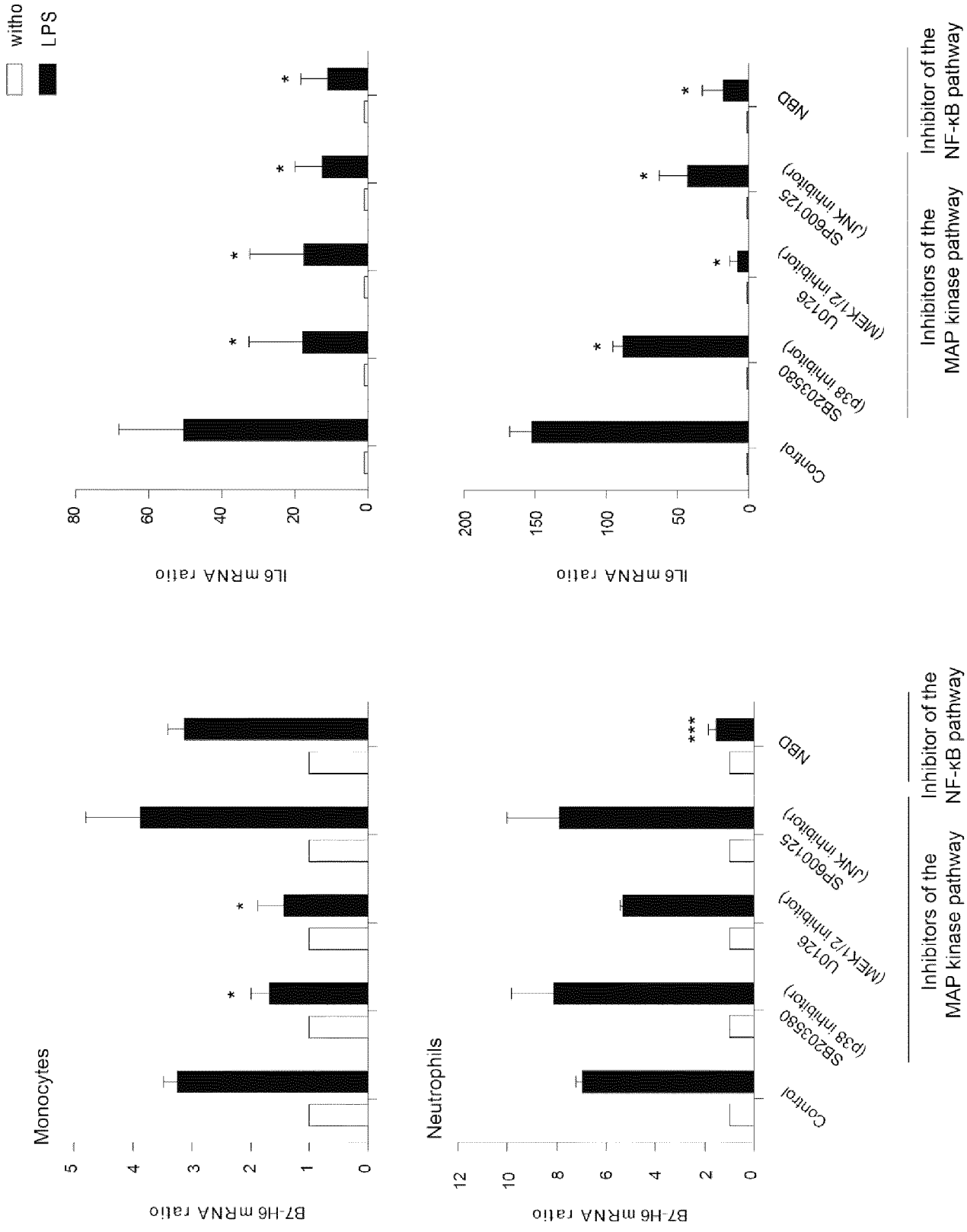

witho
LPS
IL6 mRNA ratio
Control
SB203580 (p38 inhibitor)
U0126 (MEK1/2 inhibitor)
SP600125 (JNK inhibitor)
NBD
Inhibitors of the MAP kinase pathway
Inhibitor of the NF-κB pathway
Monocytes
Neutrophils
B7-H6 mRNA ratio

Figure 6

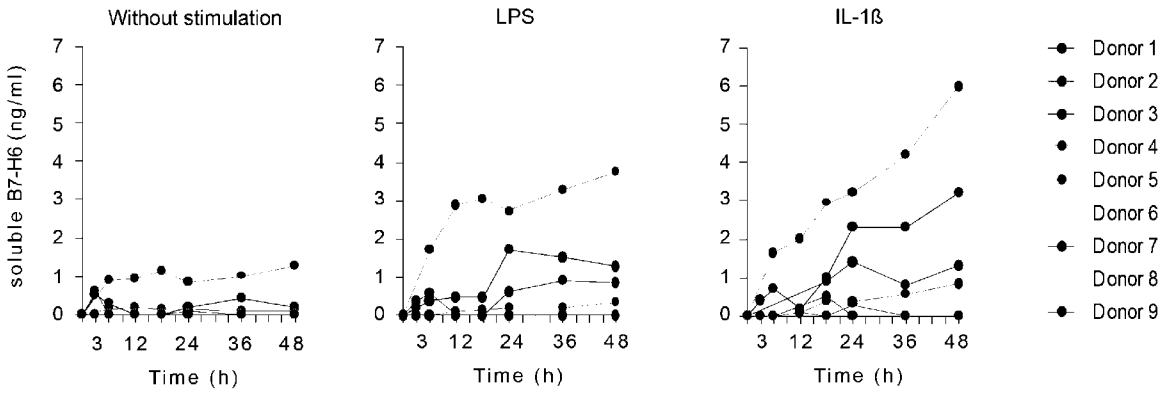


Figure 7A

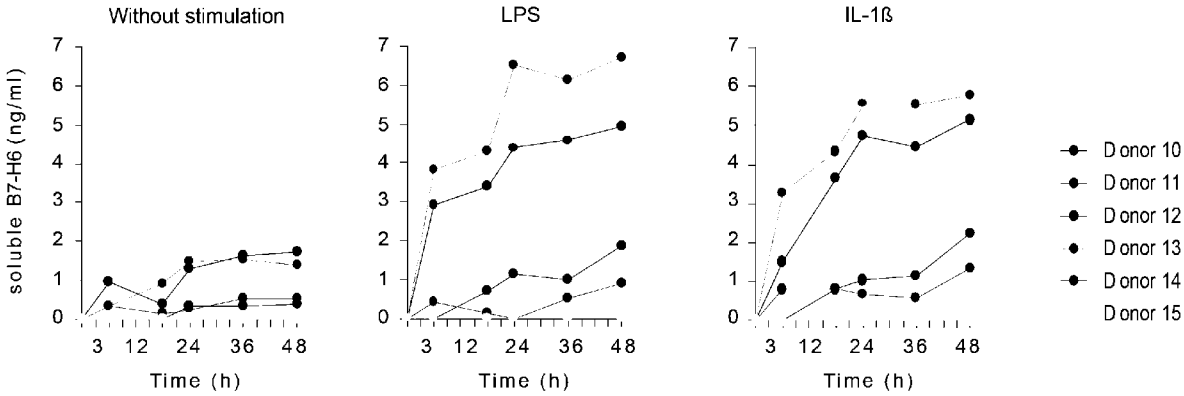


Figure 7B

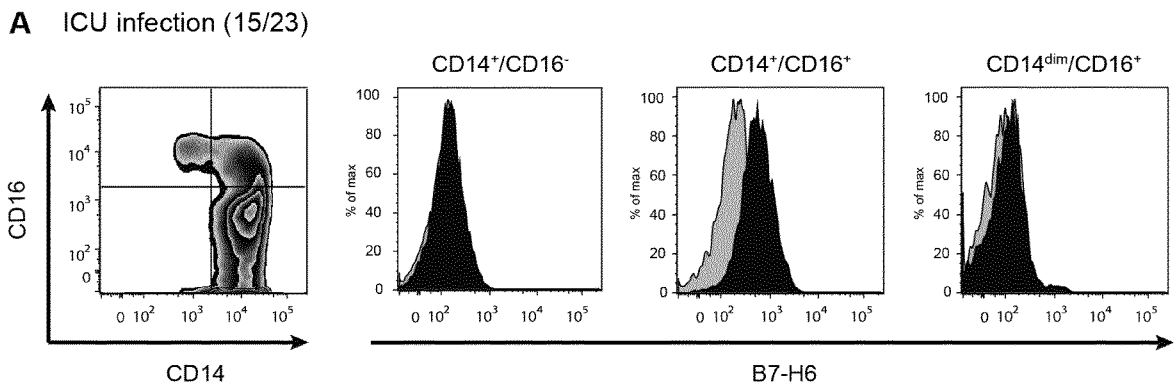


Figure 8A

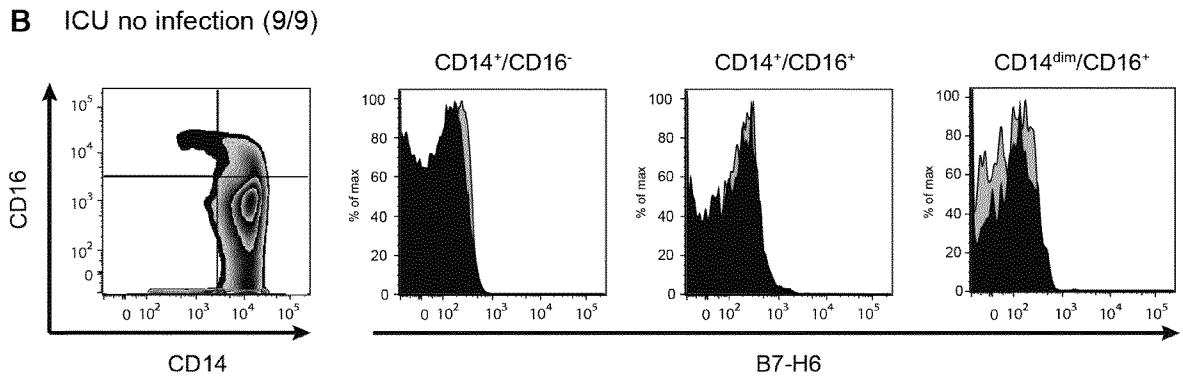


Figure 8B

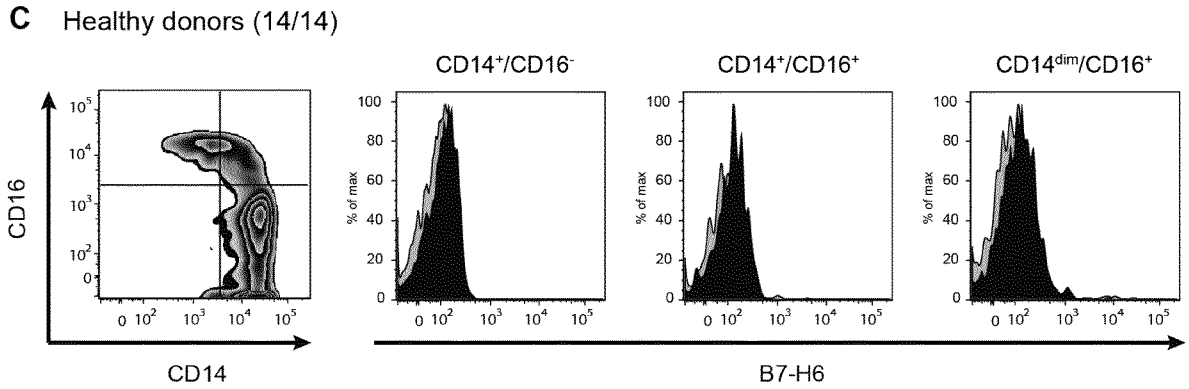


Figure 8C

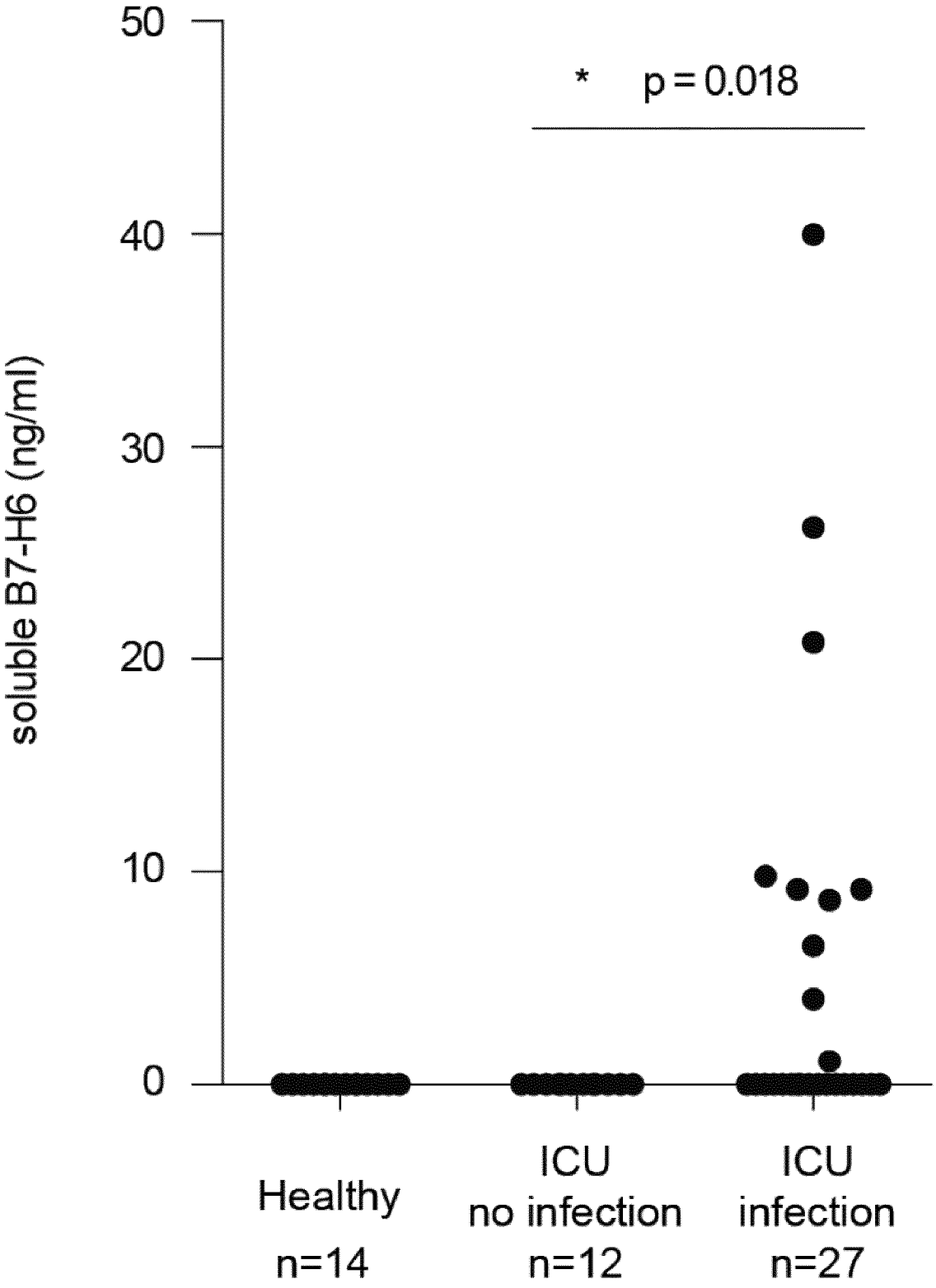

50
40
30
20
10
0
soluble B7-H6 (ng/ml)
* p = 0.018
Healthy
n=14
ICU
no infection
n=12
ICU
infection
n=27

Figure 8D

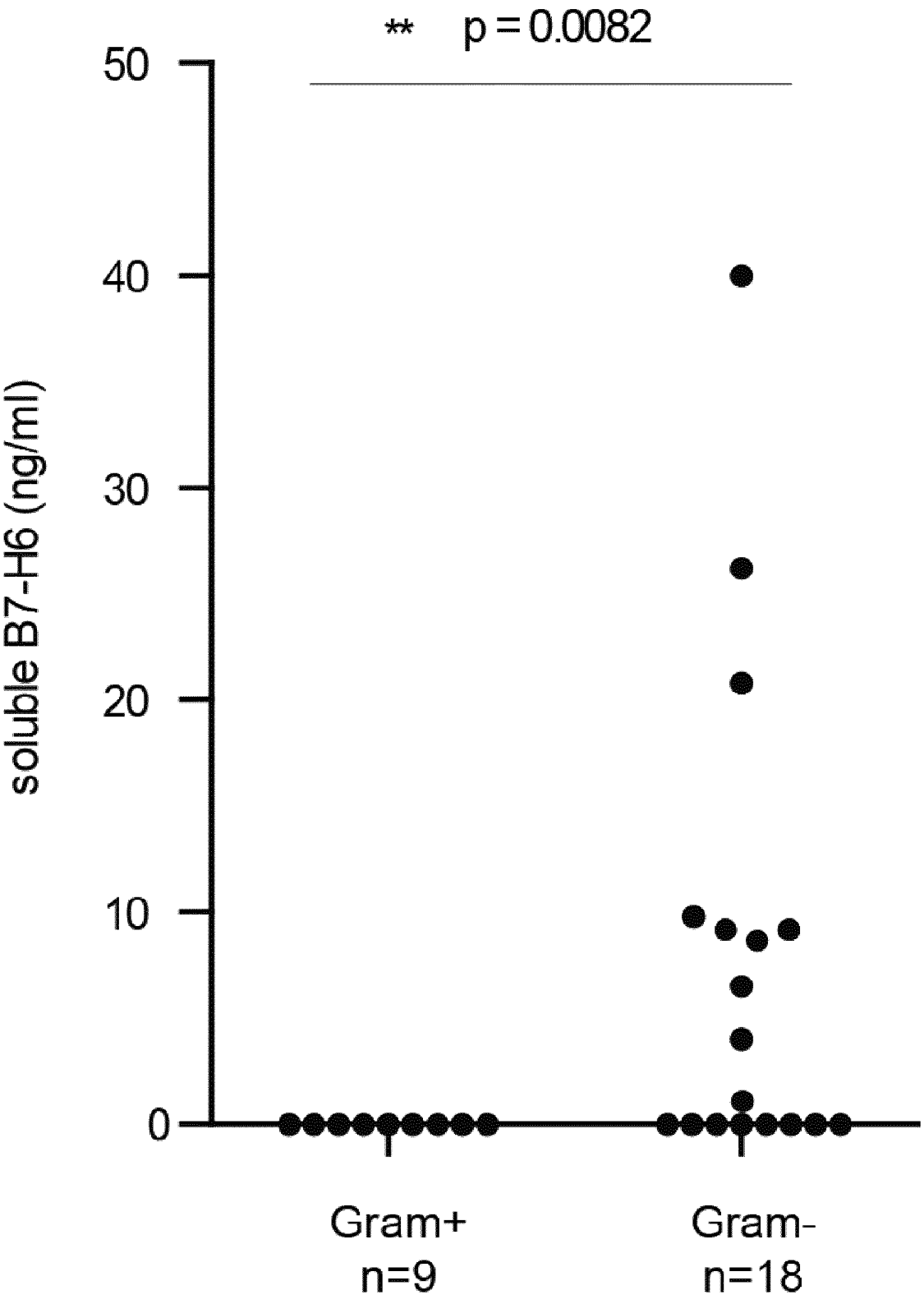


Figure 9A

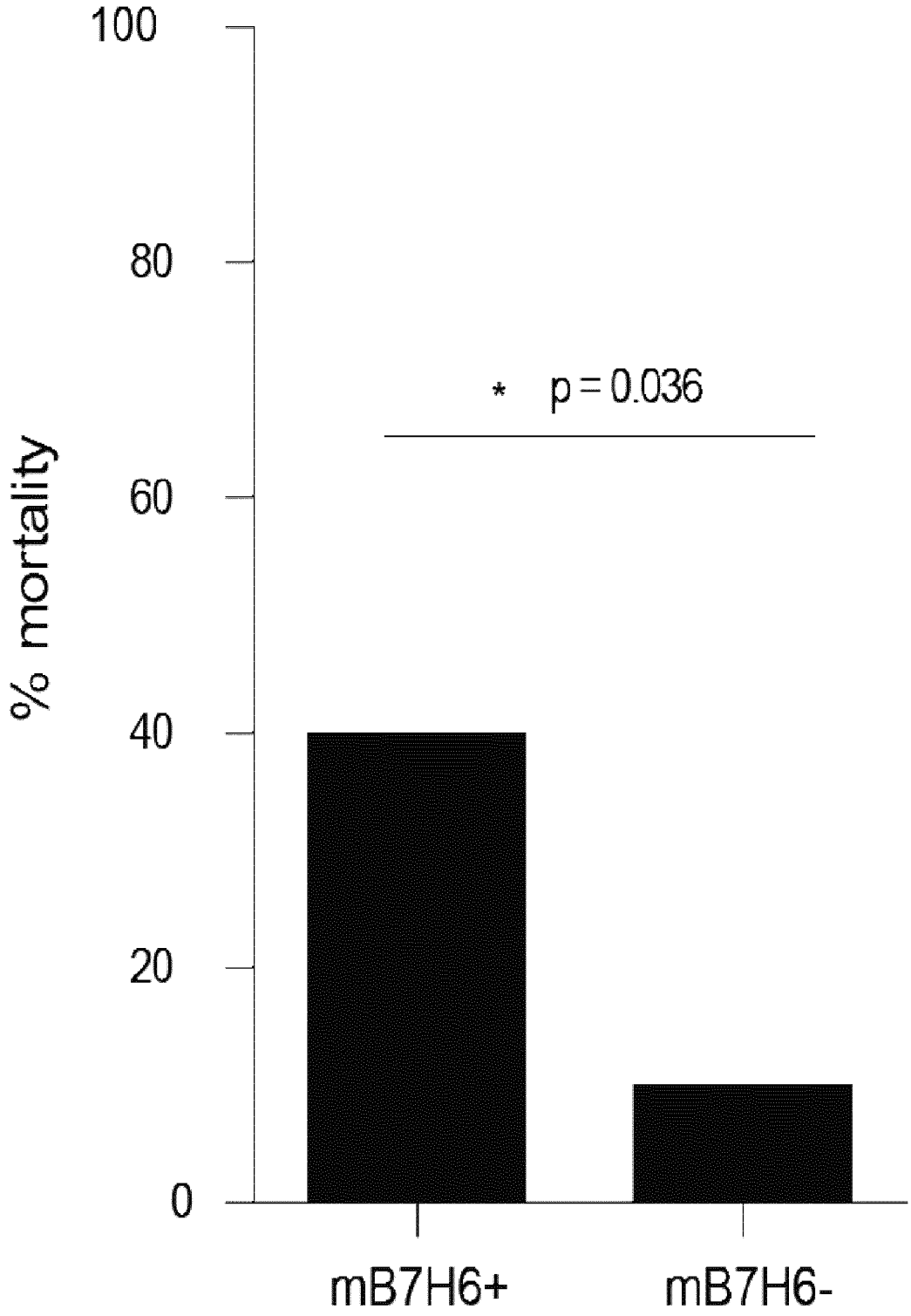


Figure 9B

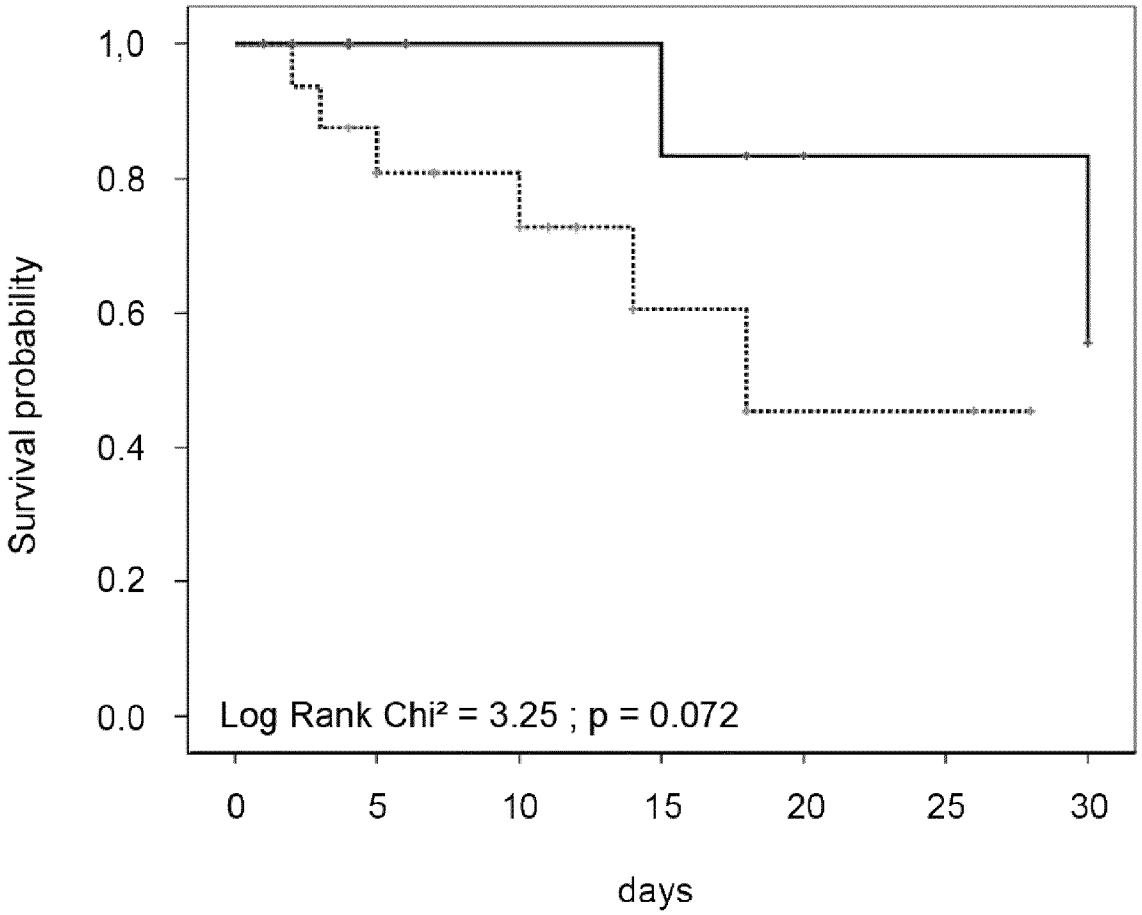

1,0
0.8
0.6
0.4
0.2
0.0
Survival probability
Log Rank Chi² = 3.25 ; p = 0.072
0
5
10
15
20
25
30
days

Figure 9C

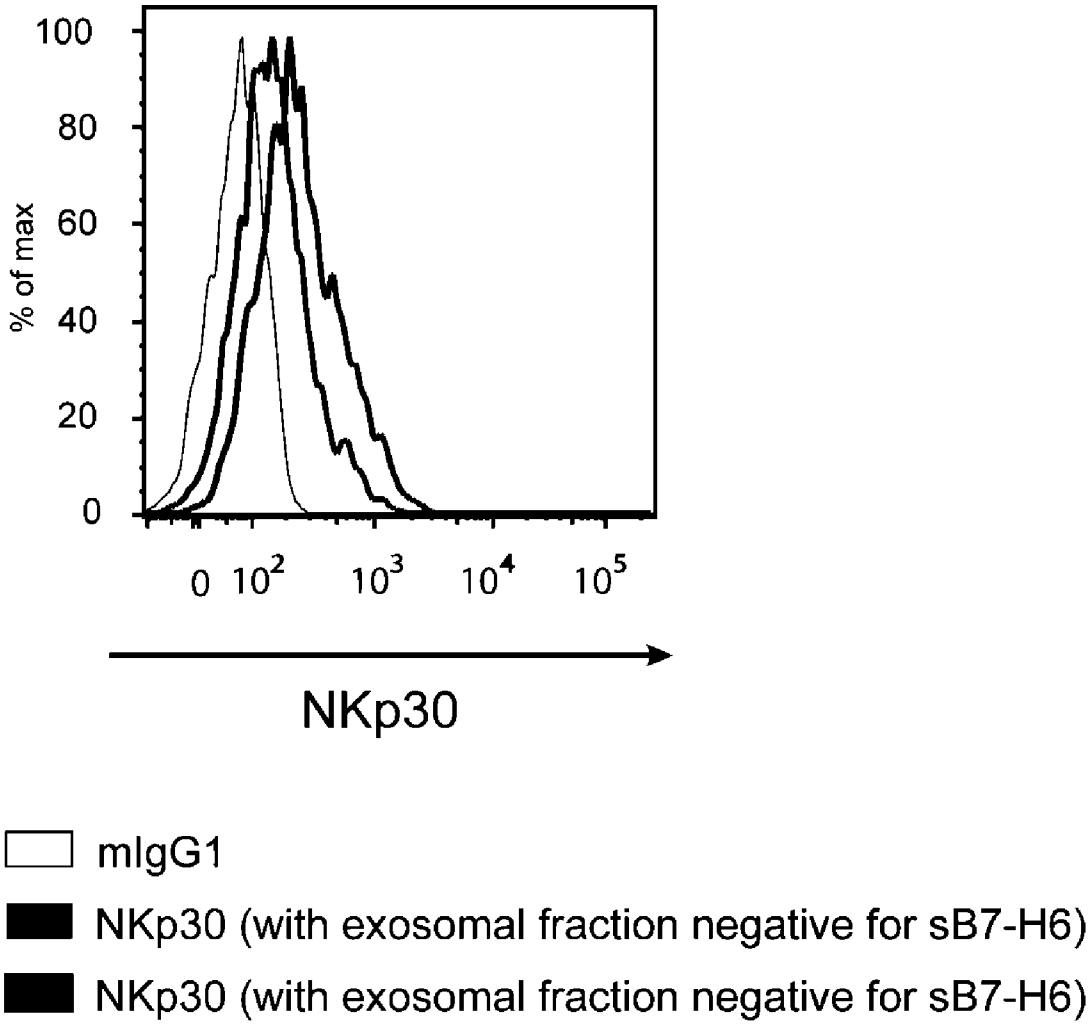

100
80
60
40
20
0
% of max
0
10^2
10^3
10^4
10^5
NKp30
mIgG1
NKp30 (with exosomal fraction negative for sB7-H6)
NKp30 (with exosomal fraction negative for sB7-H6)

Figure 11A

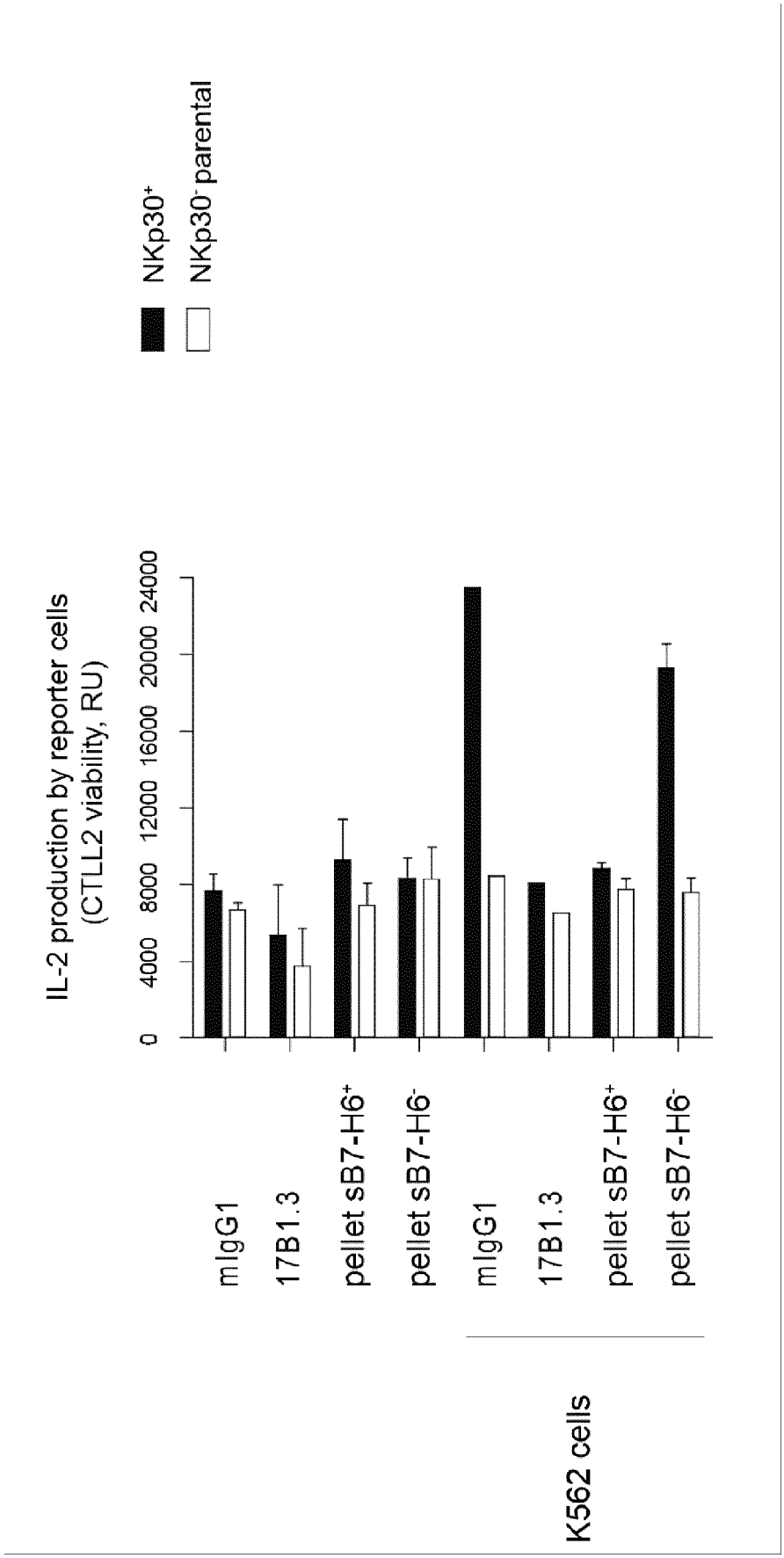
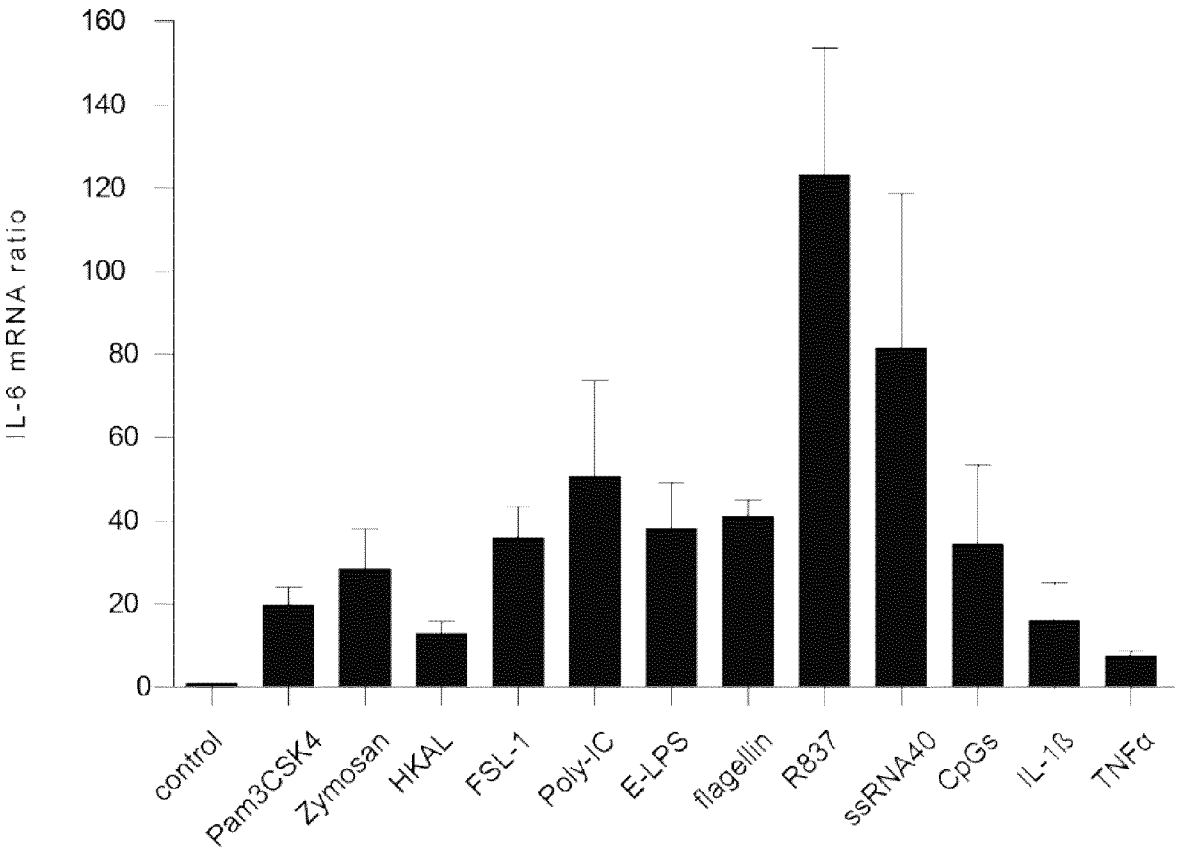
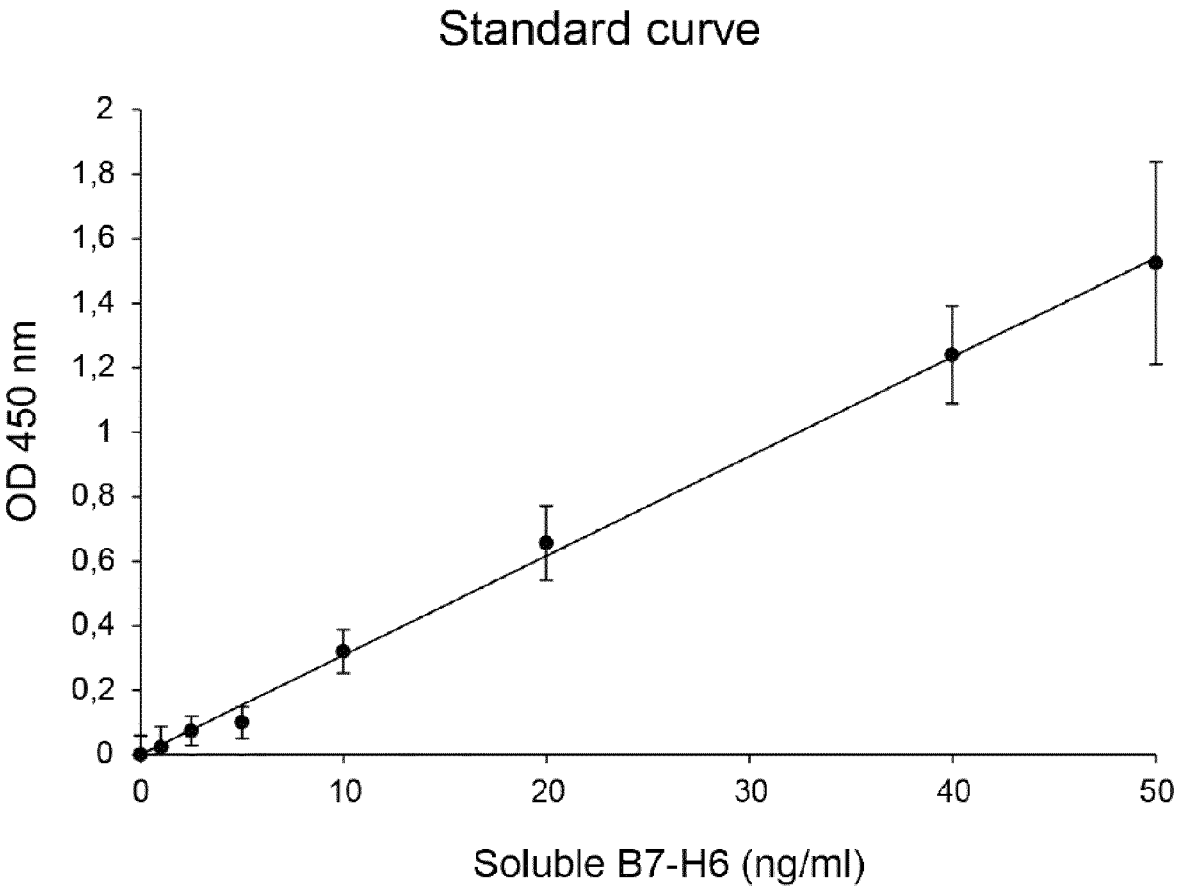

NKp30+
NKp30- parental
IL-2 production by reporter cells
(CTLL2 viability, RU)
0
4000
8000
12000
16000
20000
24000
mIgG1
17B1.3
pellet sB7-H6+
pellet sB7-H6-
mIgG1
17B1.3
pellet sB7-H6+
pellet sB7-H6-
K562 cells

Figure 11B



Supplementary Figure 1



Supplementary Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/069580

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/564 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/070443 A1 (INST NAT SANTE RECH MED [FR]; PIERRES MICHEL [FR]; VIVIER ERIC [FR]; B) 16 June 2011 (2011-06-16) paragraph [0098] paragraph [0148] paragraph [0157] paragraph [0154] -----	1-5,7
X	WO 2009/046407 A2 (ZYMOGENETICS INC [US]; BRANDT CAMERON S [US]; KENNEDY JACOB J [US]; XU) 9 April 2009 (2009-04-09) paragraph [0270] - paragraph [0272] -----	1-5,7

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
23 October 2013	12/11/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Luis Alves, Dulce

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