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(54) Title: IDENTIFICATION OF NEW ISOFORMS OF THE MHC-CLASS I SPECIFIC RECEPTOR CD160 AND USES THEREOF

(57) Abstract: The present invention concerns an isolated polypeptide comprising a sequence selected in the group comprising SEQ ID NO.7, its orthologs, and derivatives thereof; a polynucleotide comprising a nucleic acid sequence encoding for said polypeptide; a vector comprising said polynucleotide; a host cell genetically engineered with said polynucleotide or with said vector; a pharmaceutical composition comprising said polypeptide, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, and optionally a pharmaceutically acceptable carrier; a method for screening for antagonists and/or agonists of said polypeptide; and uses of said polypeptide, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, for the manufacture of a medicament.



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IDENTIFICATION OF NEW ISOFORMS OF THE MHC-CLASS I SPECIFIC RECEPTOR CD160 AND USES THEREOF

This application claims the priority of the patent application EP07301124.9 filed on
5 June 18, 2007, which is incorporated herein by reference.

Background of the Invention

Field of the invention

The invention relates to the field of immunology, and in particular to new CD160 isoforms and uses thereof.

10 Description of prior art

Natural killer (NK) lymphocytes recognize abnormal or aberrant cells through multiple receptors that detect normal host molecules, as well as stress-induced or pathogen-expressed motifs (RAULET, *Nat. Immunol.*, vol.5, p:996-1002, 2004; LANIER, *Annu. Rev. Immunol.*, vol.23, p:225-74, 2005). Individual NK cells express
15 both activating and inhibitory receptors, which together drive the specificity towards target cells (LANIER, 2005, abovementioned). The NK cell inhibitory receptors have been classified into three groups, namely the heterodimeric CD94/NKG2A, the Ig-like transcript (ILT) receptors, and the members of the killer cell Ig-like receptors (KIRs; PARHAM, *Nat. Rev. Immunol.*, vol.5, p:201-14, 2005; NATARAJAN *et al.*, *Annu. Rev.*
20 *Immunol.*, vol.20, p:853-85, 2002). All of them bind to classical or non classical MHC class I molecules.

A common characteristic of the inhibitory receptors is the presence of one or more immunoreceptor tyrosine-based inhibition motifs (ITIM) within their intracellular tail (BORREGO *et al.*, *Mol. Immunol.*, vol.38, p:637-60, 2002). Following engagement
25 by their ligands, the inhibitory receptors become phosphorylated on the tyrosine residue(s) present in the ITIM(s), creating docking sites for the SH2 domains of the cytoplasmic protein tyrosine phosphatases SHP1 and SHP2. The recruitment of the phosphatase further results in the down-regulation of the intracellular activation cascade (YUSA *et al.*, *J. Immunol.*, vol.168, p:5047-5057, 2002).

In contrast, the activating receptors recognize a large variety of ligands, and present more complex but well characterized signaling pathways. Natural cytotoxicity receptors (NCRs) and NKG2D are the major receptors involved in NK cytotoxicity (BOTTINO *et al.*, *Hum. Immunol.*, vol.61, p:1-6, 2000). The NCRs (namely NKp46, NKp44 and NKp30) belong to the Ig-superfamily, and represent non-MHC class I specific receptors whose cellular ligands remained to be identified. These activating receptors transduce signals through their association with ITAM-containing molecules such as CD3 ζ , Fc ϵ RI γ and DAP12 (VIVIER *et al.*, *Science*, vol. 306, p:1517-1519, 2004; MORETTA *et al.*, *Immunol. Today*, vol.21, p:228-234, 2000). Besides the NCRs, NKG2D is C-type lectin-like receptor shown to recognize the MHC-class I homologues MICA and MICB, and the family of UL16-binding proteins (ULBP1-4; GONZALEZ *et al.*, *Curr. Top. Microbiol. Immunol.*, vol.298, p:121-123, 2006). Unlike the NCRs, NKG2D uses the transmembrane polypeptide DAP10 for signaling, which interacts with the PI3-kinase once phosphorylated (CERWENKA & LANIER, *Immunol. Rev.*, vol.181, p:158-169, 2001). More recently the characterization of NKp80, an activating receptor exclusively expressed by human NK cells, has been reported (VITALE *et al.*, *Eur. J. Immunol.*, vol.31, p:233-242, 2001). A search for NKp80 ligands led to the identification of activation-induced C-type lectin (AICL; WELTE *et al.*, *Nat. Immunol.*, vol.7, p:1334-1342, 2006). However, NKp80 signaling pathway remains enigmatic as this receptor does not contain a transmembrane charged residue (a feature allowing association with ITAM-containing adaptor proteins) or any intracellular consensus activation motifs (VITALE *et al.*, 2001, abovementioned). Finally, among the activating receptors, the role of CD94/NKG2C, and of DAP12-associated KIRs, has also been well defined (OLCESE *et al.*, *J. Immunol.*, vol.158, p:5083-5086, 1997; LANIER *et al.*, *Immunity*, vol.8, p:693-701, 1998).

More recently the identification of the MHC class I specific receptor BY55/CD160 has been reported (ANUMANTHAN *et al.*, *J. Immunol.*, vol.161, p:2780-2790, 1998). CD160 appears to be unique among the activating receptors since *CD160* gene was found to be located on human chromosome 1, and the corresponding protein was characterized as a glycosylphosphatidylinositol (GPI)-anchored cell surface molecule. CD160 is expressed by intestinal intraepithelial T lymphocytes and by a minor subset of circulating lymphocytes including NK cells, TCR $\gamma\delta$ and cytotoxic effector CD8^{bright}CD28⁻ T lymphocytes (ANUMANTHAN *et al.*, 1998, abovementioned; MAIZA *et al.*, *J. Exp. Med.*, vol.178, p:1121-1126, 1993). Furthermore, it shows a broad specificity for the MHC class Ia and Ib molecules. We initially demonstrated that CD160 exerts a co-receptor function on CD3-activated-CD4⁺

T cell clones (AGRAWAL *et al.*, *J. Immunol.*, vol.162, p:1223-1226, 1999) and CD8^{bright} CD28⁻ cytotoxic effector T lymphocytes (NIKOLOVA *et al.*, *Int. Immunol.*, vol. 14, p:445-451, 2002). In addition, CD160 exhibits a full activatory receptor function on NK lymphocytes, as demonstrated by the induction of their cytotoxic potential upon interaction with HLA-C molecules (LE BOUTEILLER *et al.*, *Proc. Natl. Acad. Sci. U S A*, vol.99, p:16963-16968, 2002). The engagement of CD160 by its physiological ligand also results in a unique profile of cytokine secretion by cytotoxic NK cells, with the release of TNF α , IFN γ and IL-6 (BARAKONYI *et al.*, *J. Immunol.*, vol.173, p:5349-5354, 2004). Thus CD160 might be involved in mechanisms regulating both adaptive and innate immunity. Finally, we recently reported that a down-modulation of CD160 surface expression occurs as a consequence of its proteolytic cleavage upon NK cell activation (GIUSTINIANI *et al.*, *J. Immunol.*, vol.178, p:1293-1300, 2007). The released soluble form of CD160 was found to impair the MHC class I-specific cytotoxicity of CD8⁺ T lymphocytes and NK cells.

15 Summary of the invention

In accordance with the present invention there is provided an isolated polypeptide comprising a sequence selected in the group comprising SEQ ID NO.1, SEQ ID NO.3, their orthologs, and derivatives thereof.

20 According to another aspect of the present invention, there is provided an isolated polypeptide comprising the sequence SEQ ID NO.7, its orthologs, and derivatives thereof.

Preferably, said isolated peptide comprises a sequence selected in the group comprising SEQ ID NO.1, SEQ ID NO.19 orthologs, and derivatives thereof, more preferably SEQ ID NO.1, its orthologs, and derivatives thereof.

25 In accordance with the present invention there is also provided a polynucleotide comprising a nucleic acid sequence encoding for said polypeptide, a vector comprising said polynucleotide, a host cell genetically engineered with said polynucleotide or with said vector.

30 In accordance with the present invention, there is also provided a pharmaceutical composition comprising a polypeptide as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, and optionally a pharmaceutically acceptable carrier.

In accordance with the present invention, there is also provided a method for screening for antagonists and/or agonists of the polypeptide as defined previously comprising:

- 5 a) expressing the polypeptide as defined previously on the surface of a cell;
- b) contacting the cell with a compound to be screened;
- c) determining whether a signal is generated; and
- d) identifying if the compound to be screened is an agonist or an antagonist of the polypeptide.

10 In accordance with the present invention, there is also provided the use of a polypeptide as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, for the manufacture of a medicament for treating and/or preventing inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, psoriasis, multiple sclerosis, diabetes, lupus and inflammatory bowel diseases,

15 inflammatory conditions such as tissue graft or organ rejection, or autoimmune diseases in a subject.

 In accordance with the present invention, there is finally provided the use of a polypeptide as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, for the manufacture of a medicament for treating

20 and/or preventing bacterial, viral, fungal or parasitic infection and/or for treating and/or preventing cancer.

Brief Description of the Drawings

The figure 1 shows the identification of novel CD160-related coding sequences.

The figure 2 shows sequence and molecular organisation of CD160 isoforms.

25 The figure 3 shows CD160 isoforms mRNA synthesis upon Il-2, Il-12, IL-15 and Il-18 activation of PK-NB cells.

The figure 4 shows the switch in CD160 isoforms expression following PK-NB cell activation.

The figure 5 shows the molecular characterization of CD160-TM.

The figure 6 shows the functional characterization of CD160-TM.

Description of the preferred embodiments

The inventors have now identified and characterised new isoforms of *Homo sapiens* CD160 and established the functions of said isoforms in activation of PB-NK lymphocytes.

Consequently, a first object of the present invention relates to a polypeptide comprising a sequence selected in the group comprising SEQ ID NO.1 corresponding to the amino acid sequence of the CD160-TM isoform, SEQ ID NO.3, their orthologs, and derivatives thereof.

According to another aspect of the present invention, there is provided an isolated polypeptide comprising the sequence SEQ ID NO.7, its orthologs and derivatives thereof.

As used herein, the term “orthologs” refers to proteins in different species than the proteins SEQ ID NO.1 and SEQ ID NO.3 in *Homo sapiens* that evolved from a common ancestral gene by speciation. One of skill in the art in view of the specification and of its general knowledge can simply identify such orthologs.

As used herein, the term “derivatives” refer to a polypeptide having a percentage of identity of at least 80% with complete SEQ ID NO.1, complete SEQ ID NO.3, complete SEQ ID NO.7, complete SEQ ID NO.19 or orthologs thereof, preferably of at least 90%, as an example of at least 95%, and more preferably of at least 99%. Preferably, the derivative of complete SEQ ID NO.1 presents at least the tyrosine residue in position 225 of SEQ ID NO.1.

Preferably, the derivative of complete SEQ ID NO.19 presents at least the tyrosine residue in position 116 of SEQ ID NO.19.

Preferably, the derivative of complete SEQ ID NO.7 presents at least a tyrosine residue in position 46.

Preferably, the derivative of complete SEQ ID NO.1 or the derivative of complete SEQ ID NO.19 comprises at least the sequence SEQ ID NO.7.

As used herein, “percentage of identity” between two amino acids sequences, means the percentage of identical amino-acids, between the two sequences to be compared, obtained with the best alignment of said sequences, this percentage being purely statistical and the differences between these two sequences being randomly spread over the amino acids sequences. As used herein, “best alignment” or “optimal alignment”, means the alignment for which the determined percentage of identity (see below) is the highest. Sequences comparison between two amino acids sequences are usually realized by comparing these sequences that have been previously align according to the best alignment; this comparison is realized on segments of comparison in order to identify and compared the local regions of similarity. The best sequences alignment to perform comparison can be realized, beside by a manual way, by using the global homology algorithm developped by SMITH and WATERMAN (*Ad. App. Math.*, vol.2, p:482, 1981), by using the local homology algorithm developped by NEDDLEMAN and WUNSCH (*J. Mol. Biol.*, vol.48, p:443, 1970), by using the method of similarities developped by PEARSON and LIPMAN (*Proc. Natl. Acd. Sci. USA*, vol.85, p:2444, 1988), by using computer softwares using such algorithms (GAP, BESTFIT, BLAST P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics Computer Group, 575 Science Dr., Madison, WI USA), by using the MUSCLE multiple alignment algorithms (Edgar, Robert C., *Nucleic Acids Research*, vol. 32, p:1792, 2004). To get the best local alignment, one can preferably used BLAST software, with the BLOSUM 62 matrix, or the PAM 30 matrix. The identity percentage between two sequences of amino acids is determined by comparing these two sequences optimally aligned, the amino acids sequences being able to comprise additions or deletions in respect to the reference sequence in order to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical position between these two sequences, and dividing this number by the total number of compared positions, and by multiplying the result obtained by 100 to get the percentage of identity between these two sequences.

According to a preferred embodiment, said polypeptide comprises a sequence selected in the group consisting of SEQ ID NO.1, its orthologs, and derivatives thereof.

According to another preferred embodiment, said polypeptide comprises a sequence selected in the group consisting of SEQ ID NO.3, its orthologs and derivatives thereof.

Preferably, said polypeptide comprises a signal peptide enabling the secretion of the polypeptide, which polypeptide can be expressed at the cell membrane surface or in the extracellular medium, and more preferably the sequence SEQ ID NO.4.

5 Still preferably, said polypeptide comprises a Glycosyl Phosphatidyl Inositol (GPI) anchor domain, and more preferably the sequence SEQ ID NO.5. Said Glycosyl Phosphatidyl Inositol (GPI) anchor domain enables the anchorage of the polypeptide on the extracellular cell membrane, and is cleaved by a metalloprotease after activation of NK cells by IL-15. Said metalloprotease cleaved this domain between the phenylalanine and the leucine residues. Preferably, said polypeptide does not comprise any Ig domain,
10 and more preferably does not comprise the sequence SEQ ID NO.6.

Still preferably, said polypeptide does not comprise any intracellular domain, and more preferably does not comprise the sequence SEQ ID NO.7. Advantageously, said polypeptide comprises a sequence selected in the group consisting of SEQ ID NO.2, its orthologs and derivatives thereof.

15 According to another preferred embodiment, said polypeptide comprises a sequence selected in the group consisting of SEQ ID NO.7, its orthologs, and derivatives thereof.

Advantageously, said polypeptide does not comprise a signal peptide enabling the secretion of the polypeptide, which polypeptide can be expressed at the cell membrane
20 surface or in the extracellular medium, and more preferably does not comprise the sequence SEQ ID NO.4.

Preferably, said polypeptide comprises a signal peptide enabling the secretion of the polypeptide, which polypeptide can be expressed at the cell membrane surface or in the extracellular medium, and more preferably the sequence SEQ ID NO.4.

25 Still preferably, said polypeptide comprises a Glycosyl Phosphatidyl Inositol (GPI) anchor domain, and more preferably the sequence SEQ ID NO.5.

Advantageously, said polypeptide comprises a sequence selected in the group consisting of SEQ ID NO.1, its orthologs, and derivatives thereof.

Advantageously, said polypeptide comprises a sequence selected in the group
30 consisting of SEQ ID NO.19, its orthologs and derivatives thereof.

A second object of the invention is related to a polynucleotide comprising a nucleic acid sequence encoding for a polypeptide as defined previously.

5 The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, preferably in the form of DNA.

The DNA may be double-stranded or single-stranded.

According to a preferred embodiment, the polynucleotide comprises a sequence selected in the group comprising SEQ ID NO:8 , SEQ ID NO:9, SEQ ID NO:18 and SEQ ID NO:22, preferably SEQ ID NO:22.

10 The polynucleotide of the invention may include the coding sequence of the polypeptide defined previously, additional coding sequence such as leader sequence or a proprotein sequence, and/or additional non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence of SEQ ID NO.1, SEQ ID NO.2 or SEQ ID NO.19.

15 A third object of the present invention relates to vectors comprising the polynucleotide as defined previously.

Said vector can be a cloning or an expression vector, preferably an expression vector, and may be for example in the form of a plasmid, a viral particle, a phage, etc.

20 Such vectors may include bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. Large numbers of suitable vectors are known to those of skill in the art and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (QIAGEN), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, 25 pNH16a, pNH18A, pNH46A (STRATAGENE), ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (PHARMACIA). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (STRATAGENE), pSVK3, pBPV, pMSG, pSVL (PHARMACIA). However, any other vector may be used as long as it is replicable and viable in the host.

30 The polynucleotide sequence, preferably the DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter)

to direct mRNA synthesis. As representative examples of such promoters, one can mentioned prokaryotic or eukaryotic promoters such as CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. The expression vector also contains a ribosome binding site for
5 translation initiation and a transcription vector. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as
10 tetracycline or ampicillin resistance in *E. coli*.

The vector of the invention containing the appropriate polynucleotide sequence as herein above described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the polypeptide.

As an example, transcription of a DNA encoding for the polypeptide described
15 previously by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. Enhancer are cis-acting elements of DNA, usually about from 10 to 300 pb that act on a promoter to increase its transcription. Examples of enhancer include the SV40 enhancer, the CMV early promoter enhancer, and adenovirus enhancers.

A forth aspect of the invention relates to a host cell genetically engineered with
20 the polynucleotide or with the vector described previously.

As used herein, the term "host cell genetically engineered" relates to host cells which have been transduced, transformed or transfected.

As representative examples of appropriate hosts, one can cites bacterial cells, such as *E. coli*, *Streptomyces*, *Salmonella typhimurium*, fungal cells such as yeast, insect
25 cells such as Sf9, animal cells such as CHO or COS, plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

The introduction of the construct (i.e., polynucleotide or vector described previously) into the host cell can be effected by method well known from one of skill in
30 the art such as calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation.

A fifth aspect of the invention relates to pharmaceutical composition comprising a polypeptide as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, and optionally a pharmaceutically acceptable carrier. According to a preferred embodiment, said pharmaceutical composition is
5 suitable for treating and/or preventing inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, psoriasis, multiple sclerosis, diabetes, lupus and inflammatory bowel diseases, inflammatory conditions such as tissue graft or organ rejection, or autoimmune diseases.

Said composition can further include at least one immunosuppressive agent for
10 treating and/or preventing inflammatory diseases, inflammatory conditions, or autoimmune diseases.

Immunosuppressive agents are well known from one of skill in the art. As an example, one can cite cyclosporine A, FK506, rapamycin, steroids such as glucocorticoid, cytostatics such as methotrexate, azathioprine, and monoclonal
15 antibodies such as OKT3 and anti-TNF. Other immunosuppressive agents are described in Physician's desk Reference 50th Edition (1996).

According to another preferred embodiment, said pharmaceutical composition is suitable for treating and/or preventing bacterial, viral, fungal or parasitic infection.

According to still another preferred embodiment, said pharmaceutical composition
20 is suitable for treating and/or preventing cancer.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically
25 acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils,
30 including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as

carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

The composition may comprise one or more additives (e.g., stabilizers, preservatives). See, generally, *Ullmann's Encyclopedia of Industrial Chemistry*, 6th Ed. (various editors, 1989-1998, Marcel Dekker); and *Pharmaceutical Dosage Forms and Drug Delivery Systems* (ANSEL *et al.*, 1994, WILLIAMS & WILKINS).

A sixth aspect of the invention relates to a method for screening for antagonists and/or agonists of the polypeptide comprising a sequence selected in the group consisting of SEQ ID NO.7, its orthologs, and derivatives thereof comprising:

- 10 a) expressing the polypeptide as defined previously on the surface of a cell;
- b) contacting the cell with a compound to be screened;
- c) determining whether a signal is generated; and
- d) identifying if the compound to be screened is an agonist or an antagonist of the polypeptide.

15 Preferably, the polypeptide which is used in said method is select in the group consisting of SEQ ID NO.1, SEQ ID NO.19 orthologs and derivatives thereof, more preferably SEQ ID NO.1, orthologs and derivatives thereof.

In general, such screening procedures involve providing appropriate cells which express the polypeptide comprising a sequence selected in the group consisting of SEQ
20 ID NO.1, SEQ ID NO.7, SEQ ID NO. 19, orthologs and derivatives thereof on their surface. In particular, a polynucleotide encoding said polypeptide is employed to transfect cells to thereby express the receptor of the invention. Such transfection may be accomplished by procedures as hereinabove described.

One such screening procedure involves the use of peripheral blood NK cells
25 which are transfected to constitutively express the receptor of the invention.

In one embodiment, said method permits to screen antagonist compounds of the polypeptide.

Preferably, said method further comprises a step b') of contacting the cell with a ligand, said ligand being preferably a known agonist of the polypeptide.

30 Preferably, said step b') is realized after step a) and before step c).

Thus, for example, such assay may be employed for screening for a receptor antagonist by contacting the peripheral blood NK cells which encode the receptor of the invention with a receptor ligand such as the monoclonal anti-CD160 antibody CL1-R2 (the hybridoma producing said monoclonal antibody has CNCM deposit number I-3204 and is described in International application PCT WO 2006/015886) or the CD160 physiological ligand HLA-C as described in BARAKONYI *et al.* (*J. Immunol.*, vol.173(9), p:5349-5354, 2004), and a compound to be screened. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor of the invention, i.e., inhibits activation of the receptor.

10 In another embodiment, said method permits to screen agonist compounds of the polypeptide.

The screen may be employed for determining an agonist by contacting such cells with compounds to be screened and determining whether such compound generates a signal, i.e., activates the receptor.

15 The determination of an activation of the receptor can be assessed by determining the cell proliferation, preferably the NK cell proliferation; the identification of a cell proliferation activation corresponding to a receptor activation. Such proliferation can be determined by methods well known from one of skill in the art such as the monitoring of tritiated thymidine incorporation.

20 The determination of an activation of the receptor can also be assessed by determining the activation of the Erk signaling pathway; the identification of an activation of the Erk signaling pathway corresponding notably to the phosphorylation of Erk. Such activation of the Erk signaling pathway can be determined by methods well known from one of skill in the art such as by Western blot using an anti-phospho-Erk mAb.

25 Another method involves screening for antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cells with DNA encoding the receptor of the invention such that the cell expresses the receptor on its surface and contacting the cell with a potential antagonist in the presence of a labeled form of a known ligand, such as CL1-R2 monoclonal antibody. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptor is measured, e.g., by measuring radioactivity of the receptors. If the potential agonist binds to the receptor as determined

by a reduction of labeled ligand which binds to the receptor, the binding of labeled ligand to the receptor is inhibited.

In general, antagonists for the receptor of the invention which are determined by screening procedures may be employed in a variety of therapeutic purposes as immunosuppressive agents. For example, such antagonists can be employed for treating and/or preventing inflammatory diseases, inflammatory conditions, or autoimmune diseases.

A potential antagonist includes a siRNA construct prepared through the use of siRNA technology. siRNA technology can be used to control gene expression of the receptor of the invention.

Potential antagonists also include a soluble form of the receptor of the invention, e.g., a fragment of the receptor, which binds to the ligand and prevents the ligand from interacting with the receptor of the invention. As an example of such a soluble form of the receptor of the invention, one can cite the soluble polypeptide described in GIUSTINIANI *et al.* (*J. Immunol.*, vol.178(3), p:1293-1300, 2007).

Agonists for the receptor of the invention are also useful for therapeutic purposes, such as for treating and/or preventing bacterial, viral, fungal or parasitic infection, or for treating and/or preventing cancer.

A seventh aspect of the invention includes the use of a polypeptide as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, for the manufacture of a medicament for treating and/or preventing inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, psoriasis, multiple sclerosis, diabetes, lupus and inflammatory bowel diseases, inflammatory conditions such as tissue graft or organ rejection, or autoimmune diseases in a subject.

As used herein "Subject" means a mammal including without limitation humans, non-human primates, farm animals, domestic animals and laboratory animals, preferably said subject is a human.

Said medicament can further include at least one immunosuppressive agent for treating and/or preventing inflammatory diseases, inflammatory conditions, or autoimmune diseases.

A eighth aspect of the invention relates to the use of a polypeptide as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide, for the manufacture of a medicament for treating and/or preventing bacterial, viral, fungal or parasitic infection and/or for treating and/or preventing cancer.

5 A ninth aspect of the invention relates to a method for treating and/or preventing inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, psoriasis, multiple sclerosis, diabetes, lupus and inflammatory bowel diseases, inflammatory conditions such as tissue graft or organ rejection, or autoimmune diseases in a subject comprising the step of administering an effective amount of composition comprising a polypeptide
10 as defined previously, a polynucleotide coding for said polypeptide or a vector comprising said polynucleotide to said subject.

According to the present invention, an "effective amount" of a composition is one which is sufficient to achieve a desired biological effect, in this case suppressing or inhibiting cellular immune response. It is understood that the effective dosage will be
15 dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The ranges of effective doses provided below are not intended to limit the invention and represent preferred dose ranges. However, the preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without
20 undue experimentation.

A tenth aspect of the invention relates to a method for treating and/or preventing bacterial, viral, fungal or parasitic infection and/or for treating and/or preventing cancer in a subject comprising the step of administering an effective amount of composition comprising a polypeptide as defined previously, a polynucleotide coding for said
25 polypeptide or a vector comprising said polynucleotide to said subject.

According to the present invention, an "effective amount" of a composition is one which is sufficient to achieve a desired biological effect, in this case at least one of cellular immune response.

In the following, the invention is described in more detail with reference to amino
30 acid sequences, nucleic acid sequences and the examples. Yet, no limitation of the invention is intended by the details of the examples. Rather, the invention pertains to any embodiment which comprises details which are not explicitly mentioned in the examples herein, but which the skilled person finds without undue effort.

EXAMPLES

1) Material and methods

Cells

PBMC were isolated from heparinized venous blood obtained from healthy donors, by density gradient centrifugation over MSL (PAA LABORATORIES). Fresh PB-NK, CD4⁺ and CD8⁺ were purified using a magnetic-activated cell sorter (MACS) and a cell subset isolation kit according to the manufacturers' recommendations (MILTENYI BIOTEC). Individual cell population purity was shown to be >90%. For activation, cells were cultured for 72 h in RPMI 1640 supplemented with 10% heat-inactivated human serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) (INVITROGEN) and IL-2 (200 UI/ml, a gift from SANOFI-AVENTIS, Labège France), IL-12 (50ng/ml; R&D Systems), IL-15 (10 ng/ml; PEPROTECH) or IL-18 (80 ng/ml, MBL, Woburn, MA). IL-2-activated NK cells were expanded from sorted NK cells in culture medium supplemented with 200UI/ml IL2. Mixed allogenic PBMC plus 2 µg/ml PHA (SIGMA-ALDRICH, St Quentin Fallavier, France) were added each two weeks as feeder cells. Jurkat cells and derived transfectants, and 221 target cells, Daudi, YTindi and Molt4 cell lines, were cultured in RPMI 1640 medium supplemented with penicillin/streptomycin, L-glutamine, and 10% heat-inactivated fetal calf serum (FCS; PERBIO SCIENCE).

Reverse transcription and cDNA amplification (RT-PCR)

Total RNA was isolated using the Trizol reagent according to the manufacturer's instructions (INVITROGEN). RNA (5 µg) was reverse transcribed using 500 ng of an oligo-dT₍₁₂₋₁₈₎ primer (INVITROGEN) and the Powerscript reverse transcriptase (CLONTECH). The specific primers for the amplification of the cDNA corresponding to the GPI-anchored CD160 molecules were 5'-ATGCTGTTGGAACCCGGCAGAG-3' (SEQ ID NO.10; forward) and 5'-TTACAAAGCTTGAAGGGCCAC-3' (SEQ ID NO.11; reverse). The same forward primer in combination with the reverse primer 5'-TCAGTGAAACTGGTTTGAAGTTTCCTG-3' (SEQ ID NO.12) were used for the detection of the cDNA coding for the transmembrane isoforms. PCR were performed according to standard procedures, and amplified products were finally separated by electrophoresis on 1% agarose gel.

Expression vectors and transfection

CD160 and CD160-TM full length cDNA were generated by PCR using the pair of primers corresponding to each type of isoforms, with the exception that a Flag-tag coding sequence was added at the 5' end of the CD160-TM reverse oligonucleotide.

5 The chimeric CD8-CD160TM construct consists of the extracellular and transmembrane region of CD8a fused to the intracellular part of CD160-TM. It was generated by separately amplifying the cDNA encoding the extracellular and transmembrane domains of CD8a using a CD8a-specific 5' primer (5'-ATGGCCTTACCAGTGACCGCCTTG-3', SEQ ID NO.13) and a chimeric 3' primer
10 (5'-GGGGTGCTTACGGCTCTTTTGGAGTTGCAGTAAAGGGTGATAACCAG-3', SEQ ID NO.14), and the coding sequence corresponding to CD160-TM intracellular domain with an overlapping 5' primer (5'-CTGGTTATCACCCCTTTACTGCAACTCCAAAAGAGCCGTAAGCACCCC-3', SEQ ID NO.15) and CD160-TM-specific 3' primer. The two overlapping fragments
15 were used as templates for a PCR with CD8a-5' and CD160-TM-3' primers.

Following in gel purification with the QiaEx II gel purification kit (QIAGEN), each cDNA was ligated into the pEF6 expression vector according to the manufacturer's recommendation (INVITROGEN). Mutants of the chimeric CD8-CD160-TM construct were produced by site-directed mutagenesis. The following
20 mutants were generated: mutF220SS (Y220 to F) and mutF225PQ (Y225 to F). The integrity of the inserted sequence was finally assessed by double-strand sequencing.

Cells were transfected by electroporation with 30 µg of the desired expression vector using a Gene Pulser II (BIORAD) with settings at 250 V and 950 µF. After 48 h of recovery, cells were placed in culture medium containing x µg/ml of blasticidine for
25 selection. The synthesis of CD160 and CD160-TM by the growing clones was assessed by flow cytometry or anti-Flag immunoprecipitation and immunoblot analysis, respectively.

Generation of anti-CD160-TM specific antibodies

Two rabbits were immunized with peptides located within the extracellular Ig domain
30 (peptide 1, SSASQEGTRLNLIC, aminoacids 31-44, SEQ ID NO.16) and the juxta-membranal region (peptide 2, KQRQHLEFSHNNEGTL, aminoacids 144-158, SEQ ID NO.17) of CD160 transmembrane isoforms (see Fig. 2c). Each rabbit antiserum was

further affinity purified on peptide 1 or peptide 2 (referred to as anti-CD160^{TM^{pep1}} or – CD160-TM^{pep2}), and the specificity of the purified Ab assessed by immunostaining on CD160-TM-expressing cells.

- 5 A second immunization protocol was similarly performed using peptides belonging to the intracellular domain of CD160-TM (VSTPSNEGAIIFLPP, amino acids 186-200, SEQ ID NO.23 and SRRRLERMSRGREK, amino acids 204-218, SEQ ID NO.24). The latter antibodies are referred to as anti-CD160-TM^{IC}.

10 ***Flow cytometry***

- Cells were incubated with the anti-CD160 mAb BY55 (IgM; 1 µg/test) or affinity purified anti-CD160 polyclonal antibodies (3 µg/test) for 30 min at 4°C. An anti-CD34 mAb (5T-147; IgM) or rabbit pre-immune serum was used as negative controls, respectively. The cells were washed twice in PBS, and further labeled with FITC-
15 conjugated goat anti-mouse or goat anti-rabbit Ig. After extensive washes, cells were analyzed using an EPICS XL apparatus (BECKMAN-COULTER).

Cell stimulation

- Cells were incubated with control mouse IgG or anti-CD8 mAb (1µg/ml) and cross-linked with rabbit anti-mouse IgG Ab for 20 min at 37°C. Cells were harvested
20 immediately after stimulation and lysed in 1% NP40 lysis buffer (1% NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM Na vanadate, 10mM NaF, 1mM phenylmethylsulfonylfluoride, 1µg/ml aprotinin, and 1µg/ml leupeptin). Cellular lysates were analyzed by SDS-8%PAGE and transferred onto a nitrocellulose membrane.

Immunoprecipitation and immunoblot analysis

- 25 Cells were lysed in 1% NP40 lysis buffer (1% NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM Na vanadate, 10mM NaF, 1mM phenylmethylsulfonylfluoride, 1µg/ml aprotinin, and 1µg/ml leupeptin) for 1 h at 4°C. Post-nuclear supernatants were subjected to immunoprecipitation using rabbit pre-immune or polyclonal anti-CD160-TM^{IC} serum and protein G-sepharose beads. After washes, the precipitated proteins were
30 eluted, separated by SDS-10% PAGE and transferred onto a nitrocellulose membrane. Immunoblot analysis was performed using the anti-CD160-TM^{IC} antibodies (dilution 1/5000). Horseradish peroxidase-conjugated anti-mouse Ig antibodies (JACKSON

IMMUNORESEARCH) were used as secondary antibodies. The immunoreactive proteins were visualized by autoradiography using an enhanced chemiluminescence detection system (AMERSHAM BIOSCIENCES). For cells that were stimulated before lysis, membranes were blotted with a specific anti-phospho-Erk1/2 mAb (Sigma-
5 Aldrich) and reprobed with purified polyclonal anti-Erk1/2 antibodies (Cell Signaling Technology).

Proliferation assays

Stably transfected Jurkat cells (10^4 /well) were activated with immobilized anti-CD8 mAb or mouse IgG1 (Beckman-Coulter) for 6h at 37°C. Cells were then pulsed
10 with 1 μ Ci of 3H thymidine for the next 6h, and 3H-thymidine incorporation was measured. All conditions were done in triplicate.

NK cell activation and CD107a analysis

The NK cell line NK92 was cultured for 4h in wells that had been pre-coated with anti-CD8 mAb, anti-CD160TM^{IC} or -CD160TM^{pep2} purified Abs. Following extensive
15 washes, cells were collected and analyzed by flow cytometry for CD107a cell surface expression using a PE-Cy5-conjugated anti-CD107a mAb (BD BIOSCIENCES).

2) Identification of new CD160 mRNAs

GPI-anchored CD160 molecule has been previously characterized as an activatory receptor able to trigger NK cell lysis following engagement by its physiological ligand
20 HLA-C (LE BOUTEILLER *et al.*, 2002, abovementioned; BARAKONYI *et al.*, 2004, abovementioned). To investigate CD160 functions and potential molecular associations, experiments were conducted to generate a full length cDNA construct for subsequent cloning into an eukaryotic expression vector. Total RNA were isolated from NK92 cells and subjected to reverse transcription. PCR were realized using a pair of primers
25 corresponding to the 5' and 3' ends of CD160. Amplification of β actin cDNA was performed in parallel as internal control.

The figure 1 shows the results of the RT PCR experiments.

Unexpectedly, the results established that the RT-PCR experiments performed on total RNA isolated from the NK cell line NK92 constantly led to the detection of two
30 amplified products (CD160 and CD160 Δ Ig-TM, Fig. 1 lane 1).

Thus a 550 bp cDNA corresponding to CD160 full coding sequence was observed, as well as a second product of approximately 220 bp. Because additional verifications confirmed the specific amplification of the shorter product, its purification and sequence analysis were undertaken. The results obtained revealed that this cDNA encoded a truncated form of CD160, as it corresponds to a CD160 mRNA (SEQ ID NO.9) that lacks the coding region for the Ig domain (Fig. 2a). This deletion results in a putative GPI-anchored protein devoid of extracellular Ig domain (Fig. 2b). We will refer to this protein to as CD160ΔIg-GPI (SEQ ID NO.2).

We further realized a computer assisted sequence comparison of the newly identified mRNA against available databases.

Following this comparison, the results established that we found a third mRNA presenting a high level of identity with the CD160ΔIg-GPI transcript (GeneBank accession number AK128370, SEQ ID NO.18, Fig. 2a). Upon translation, this third transcript would lead to the synthesis of a CD160 protein presenting no extracellular Ig domain, but exhibiting a transmembrane and an intracellular domain (Fig. 2b). This putative variant of CD160 was therefore called CD160ΔIg-TM (SEQ ID NO.19).

To assess if this third mRNA was synthesized by the NK92 cell line, specific primers allowing the amplification of CD160ΔIg-TM cDNA were designed (see Fig. 2a). As previously observed for the synthesis of CD160 cDNA, two specific products of roughly 400 and 700 bp were detected (Fig. 1, lane 2). The sequencing of these two PCR products showed that the smaller one corresponded to CD160ΔIg-TM cDNA, while the larger one coded for a fourth possible isoform of CD160. Indeed, this cDNA presents an open reading frame coding for the complete extracellular domain of CD160, but then enters into a nucleotide sequence coding for a transmembrane and an intracellular domain (SEQ ID NO.9, Fig. 2a). The corresponding polypeptide would therefore be a transmembrane protein sharing the same extracellular domain that the original GPI-anchored molecule (Fig. 2b). We will refer to this isoform to as CD160-TM (SEQ ID NO.1).

The figure 2 shows the cDNA sequences and molecular organization of CD160 isoforms. (a) Sequence comparison of CD160 isoforms cDNA. The predicted coding region for the signal peptide (*italic*), the Ig-like domain (**bold**) and the hydrophobic region (underlined) are delineated. The sequence of the primers used for cDNA amplification is also delineated (dashed line). (b) Schematic representation of the predicted molecular organization of CD160 isoforms. Two types of proteins can be

distinguished on the criteria of their mode of insertion into the membrane (GPI or TM), or of the presence or absence of the Ig-like domain within their extra cellular part. The putative polypeptides share the same signal peptide as well as a X amino acid sequence in their extra cellular moiety. (c) Predicted amino acid sequence of CD160-TM protein.

5 The amino acids predicted to form the signal sequence are underlined. The hydrophobic portion, deduced from a Chou and Fasman hydrophobicity analysis, and which contains a positively charged Lys (**K**) residue, is in bold. The intracellular KCYSSP motif, similar to the one found in NKp80 sequence, and where is located a putative tyrosine phosphorylation site, is boxed.

10 Thus, we demonstrated that four CD160 transcripts were synthesized by NK92 cells, two coding for GPI-anchored molecules, and two corresponding to their transmembrane counterparts. In addition, each pair of GPI- and transmembrane proteins can be distinguish from the other according to the presence or absence of the Ig domain within their extracellular moiety. Complementary sequence comparisons of the four
15 cDNA sequences with genomic DNA showed that all transcripts were produced by alternative splicing of *CD160* gene.

3) Expression of CD160 transcripts in peripheral blood NK cells

To further investigate the physiological relevance of the identification of four CD160 transcripts, and thus of the potential synthesis of four CD160 isoforms, RT-PCR
20 experiments were realized on freshly isolated peripheral blood (PB) NK cells. PB-NK cells were purified from the peripheral blood of a healthy donor, and cultured in the presence of IL 2, IL -12, IL 15 or IL-18 for 72 h.

Total RNA were extracted from purified PB-NK cells, reverse-transcribed and amplified with the primer pair specific for either the GPI-anchored or the
25 transmembrane molecules (see Fig. 2a). β actin cDNA synthesis was used as internal control.

The figure 3 shows the results of CD160 isoforms mRNA synthesis upon IL 2, IL -12, IL 15 or IL-18 activation of PB-NK cells. The position of the cDNA corresponding to each isoform is as indicated.

30 As shown in Fig. 3, the transcripts corresponding to the two GPI-bound proteins (CD160 and CD160 Δ Ig-GPI) were present in resting PB-NK cells (Figure 3, top panel, day 0), while the one encoding the transmembrane isoforms remained undetectable (Fig. 3, middle panel, day 0). Interestingly we found that the incubation of the cells with

either IL-2, IL-12, IL-15 or IL-18 resulted in the neo-synthesis of CD160-TM and CD160ΔIg-TM mRNA (Fig. 3, middle panel). Notably, while the latter transcripts were no longer detectable at day 6 of IL-18 treatment, they remained present up to 14 days in all other activation conditions. The transcription of the GPI-isoforms mRNA did not seem to be modified along the activation process (Fig. 3, top panel).

Thus, unlike the NK92 cell line that constitutively expressed the four CD160 transcripts (Fig. 1), PB-NK cells present an activation-dependent synthesis of CD160 mRNAs, as the potential expression of the transmembrane molecules can only be achieved following activation.

10 4) The synthesis of CD160ΔIg-TM and CD160-TM transcript is restricted to activated NK cells

To further evaluate the cellular specificity of CD160 TM-isoforms transcripts expression, RT-PCR was conducted on total RNA extracted from PB-sorted cells, tissue-isolated cells, or from various established cell lines. The results are presented in table I as follows.

Table I

Samples		GPI	TM
Fresh tissues	PBMCs	+	-
	Activated PBMCs	+	+
	Cord blood	+	-
	CD34 ⁺ cells	-	-
	Thymocytes	-	-
	NK cells	+	-
	IL-15-activated NK cells	+	+
	CD4 ⁺ T cells	-	-
	IL-15-activated CD4 ⁺ T cells	+	-
	CD8 ⁺ T cells	+	-
	IL-15-activated CD8 ⁺ T cells	+	-
Lymphoid cell lines	Thymic clone (B20)	-	-
	T cell (LSO)	+	-
	CD4 ⁺ T cell (JAF43)	-	-

	CD8 ⁺ T cell (JF1)	+	-
Tumoral cell lines	B cell (Daudi)	-	-
	T cell (Jurkat, Molt4)	-	-
	NK cell (NK92, YT indi, NKL)	+	+

The results reported in Table I show that among the PB cell types tested, the transcripts encoding CD160 TM-isoforms were only detected in NK cells following IL-15 treatment. These mRNA were also not found in thymocytes, cord blood cells or CD34⁺ cells. In agreement with previous studies, the GPI-isoforms transcripts showed a broader distribution pattern as they were detected in resting and activated PB-NK and -CD8⁺ lymphocytes (ANUMANTHAN *et al.*, 1998, abovementioned; MAIZA *et al.*, 1993, abovementioned), and in activated PB-CD4⁺ T cells (ABECASSIS *et al.*, *J. Invest. Dermatol.*, vol.187, p:2065-2072, 1998). Additionally, CD160ΔIg-TM and CD160-TM transcripts were successfully amplified from NK tumoral cell lines but not from established T or B cell lines (Table I). These data strongly suggested that CD160 TM-isoforms are exclusively expressed by activated NK cells and by their transformed counterparts.

5) Switch in CD160 isoform expression upon activation of PB-NK cells

We next wanted to establish if the activation-dependent synthesis of the mRNA corresponding to the transmembrane isoforms was correlated to the expression of the proteins within the cells. To this aim, polyclonal antibodies directed towards the transmembrane isoforms were generated by the immunization of rabbits with a mix of two peptides specific for CD160 molecules, and located within the Ig-like (peptide 1) and the juxtamembranal domain (peptide 2) of the protein (see the Material and Methods section, and Fig. 2c, for sequence details).

Each serum was further affinity purified on each individual peptide, and the reactivity and specificity of the purified anti-CD160-TM antibodies assessed on Jurkat cells transiently transfected with an expression vector coding for either CD160 or CD160-TM molecule. The anti-CD160 mAb BY55 was used as a control and rabbit pre-immune serum, or isotype-matched anti-CD34 mAb, were used for negative controls. Following addition of the appropriated FITC-coupled secondary antibodies, cells were analyzed by flow cytometry.

The figure 4a shows the characterization of anti-CD160-TM polyclonal antibodies (upper panel) or the results obtained with the anti-CD160 mAb BY55 (lower panel).

The results obtained clearly demonstrated that the anti-CD160-TM^{pep2} antibodies efficiently labeled CD160-TM transfectants while no signal was obtained using the anti-CD-160 mAb BY55 (Fig. 4a, left panel). Conversely, the anti-CD160 mAb BY55 positively stained the CD160-expressing cells but gave no signal on CD160-TM-transfected cells (Fig. 4a, middle panel). Regarding this latter observation, one should mention that a weak reactivity was obtained with BY55 mAb when CD160-TM cells were subjected to a fixation step prior to immunolabeling (data not shown). To definitely assess the specificity of CD160-TM recognition, IL-2-activated NK cells were tested for their reactivity with the anti-CD160-TM^{pep2} antibodies or BY55 mAb. A positive staining was obtained on IL-2-activated cells labelled with the anti-CD160-TM^{pep2} antibodies while no labelling was observed using BY55 mAb (Fig. 4A, right panel), although both CD160 and CD160-TM transcripts were detected (data not shown). Thus, we obtained purified polyclonal antibodies that, according to their peptide recognition ability, allow the detection of CD160-TM molecule but not of its GPI-anchored counterpart.

The obtention of antibodies specific for CD160-TM isoform further allowed us to investigate the expression pattern of the GPI-anchored molecules *versus* the full length transmembrane protein on PB-NK cells. Freshly isolated PB-NK cells were grown in medium alone (-IL-15) or supplemented with IL-15 (+ IL-15) and immunolabeling of resting or IL-15-activated PB-NK cells were performed using either BY55 mAb or the anti-CD160-TM^{pep2} polyclonal antibodies.

The figure 4b shows the results obtained with the anti-CD160 antibody (upper panel) or anti-CD160-TM antibody (lower panel).

As previously observed, CD160 is expressed by circulating NK lymphocytes and becomes almost undetectable after 3 days of activation (Fig. 4B, upper panel). We established that this loss in GPI-anchored CD160 detection resulted from an activation-dependent proteolytic cleavage of the molecule involving a metalloprotease, leading to the release of a soluble form (GIUSTINIANI *et al.* (*J. Immunol.*, vol.178(3), p:1293-1300, 2007). This down-modulation step was then followed by a re-acquisition phase, as assessed by the recovery of a positive signal with BY55 mAb at later time points of activation (Fig. 4B, top panel). Moreover, in agreement with CD160 mRNA analysis, no CD160-TM is expressed by resting PB-NK cells, while a low level of cell surface expression was found after 3 days of IL-15-treatment (Fig. 4B, lower panel). Interestingly, we constantly observed that this initial induction of CD160-TM expression

was followed by a down-modulation step, the TM-receptor becoming undetectable at day 5 and 7 of activation. Remarkably, after 10 days of stimulation, two cell populations were detected, one expressing high levels of CD160-TM, and one being barely CD160-TM positive cells. Depending on the donors, both cell types remained present up to 2 to 3 weeks after the beginning of the activation process (data not shown). Importantly, we evidenced a complete loss of CD160 and an exclusive expression of CD160-TM on *in vitro* activated cells, as exemplified in Fig. 4A, suggesting that upon longer activation time, CD160 can be totally replaced by CD160-TM at the NK cell surface. Thus, it seems that during the activation process, NK cells went through a time-dependent regulation of CD160 and CD160-TM membrane expression, the receptors being available at the cell surface depending on the level of proteolytic activity (for CD160) or translational activity (for CD160-TM) existing within the cells during the time course of activation.

6) Molecular characterization of CD160-TM

Our observation that the clearing of GPI-anchored CD160 from the NK cell surface parallels the appearance of the transmembrane molecules prompted us to further investigate the structure and functions of CD160-TM. Jurkat cells were transfected with an empty control vector (control) or with a construction coding for CD160-TM fusion protein (CD160-TM). Simultaneously, protein analysis was also performed on IL2-activated NK cells.

After lysis, cellular extracts were subjected to immunoprecipitation using either anti-CD160-TM^{IC} antibodies, directed against the intracellular domain of the protein (See Fig. 2C and the Material and Methods section for peptides sequence), or the corresponding rabbit pre-immune serum.

The figure 5 shows the results for the detection of CD160-TM.

Immunoblot analysis conducted with the anti-CD160-TM^{IC} antibodies showed the presence of a 100 kDa protein in the immunoprecipitates prepared from both cells (i.e., CD160-TM Jurkat transfected cells and IL2-activated NK cells). As CD160-TM aminoacids sequence corresponds to a polypeptide with an estimated molecular weight of 25.6 kDa, it is likely that CD160-TM is expressed in activated NK cells as a multimeric molecule which appears to be insensitive to reducing agent, as already observed for CD160 (MAIZA *et al.*, *J. Exp Med.*, 178:1121-1126, 1993; ANUMANTHAN *et al.*, 1998, abovementioned;) and soluble CD160 (GIUSTINIANI *et al.*, *J. Immunol.*, vol.178, p:1293-1300, 2007).

7) CD160-TM triggering enhances the NK lymphocyte cytotoxicity

To determine whether CD160-TM might trigger activating or inhibitory signals following its engagement NK92 cells were activated with either a control mAb, or affinity-purified anti-CD160-TMIC or -CD160-TM^{pep2} Abs, and the corresponding cellular degranulation response was analyzed through the detection of CD107a cell surface mobilization.

The Figure 6 shows (A) that CD160-TM triggering enhances NK cell degranulation. NK92 cells were stimulated with either immobilized anti-CD8 mAb or purified anti-CD160-TM^{IC} or -CD160-TM^{pep2} Abs. CD107a cell surface mobilization was then analyzed by flow cytometry. A value of 1 was attributed to the % of cells spontaneously expressing CD107a. Shown are results representative of three independent experiments.

The results show that a fraction of NK92 cells (representing 12% of the overall cell population) exhibited a spontaneous level of degranulation, and that this amount of CD107a expressing cells was not modified in the presence of CD160-TM^{IC} Abs (Fig. 6A). In contrast, a 1.5 fold increase in the percentage of CD107a positive cells was detected upon stimulation with CD160-TM^{pep2} Abs, thus demonstrating that CD160-TM triggering can lead to the generation of positive signals involved in the process of degranulation.

8) CD160-TM intracellular domain is sufficient to induce a cellular proliferation and to activate Erk pathway

The molecular basis underlying CD160-TM activating function was next analyzed. The protein sequence analysis of CD160-TM revealed the presence, within its transmembrane domain, of a positively charged lysine residue (see Fig. 2C). An association of the molecule with ITAM-bearing adaptors through the establishment of a stable salt bridge was therefore considered. However, by co-expressing CD160-TM together with DAP10, DAP12, ζ or Fc ϵ RI γ in COS cells, we did not evidence any association between these adaptor proteins and CD160-TM (data not shown). Another feature of CD160-TM is the presence, in its intracellular domain, of two tyrosine residues at positions 220 and 225, which might represent potential docking sites for signaling molecules upon phosphorylation. To investigate if CD160-TM function was dependent on its intracellular domain, a chimeric construct coding for the CD8 α extracellular and transmembrane domains fused to CD160-TM cytoplasmic portion was

generated. Wild type (WT) or Lck-deficient (JCam) Jurkat cells were stably transfected with expression vector encoding either the wild-type (WT) or mutated (mut F220SS or mut F225PQ) CD8-CD160-TM chimeric receptor (see Fig. 6B for expression). Expression of the chimeric protein was assessed for each transfectant by immunolabeling using a PE-conjugated anti-CD8 mAb vs. an isotype-matched control Ig (figure 6B, top panel).

The figure 6B, bottom panel, shows the results of an activation of WT or Lck-deficient CD8-CD160TM transfectants with increasing concentrations of either mouse IgG1 (mIgG1)(♦) or anti-CD8 mAb (□), as indicated. Results were expressed as the percentage of proliferation, with 100% corresponding to basal cell growth.

We observed, that while the transfected WT cells did not proliferate in response to control mouse IgG1, a substantial enhancement of their growth rate, corresponding to a 30-35% increase of their proliferation level, was obtained following CD8 triggering (see figure 6B, bottom panel).

To further estimate the involvement of CD160-TM intracellular tyrosine residue(s) in the generation of activating signals, mutants of the chimera in which either Y220 (mutF220SS) or Y225 (mutF225PQ) was changed to phenylalanine were produced. Equal expression level of the various chimera was assessed by immunostaining using an anti-CD8 mAb (Fig. 6B, top panel). We observed that point mutation of Y220 resulted in a chimeric protein still able to mediate an up-modulation of Jurkat cells growth following triggering (Fig. 6B, bottom panel, Jurkat/mutF220SS). In contrast, no more increase was detected upon ligation of the Y225-mutated chimera (Fig. 6B, bottom panel, Jurkat/mutF225PQ), demonstrating that this tyrosine residue is critically involved in the delivery of positive signals. A tyrosine-dependent activation signaling was confirmed by the observation that cross-linking of the WT chimera had no effect on the proliferation rate of Lck-deficient Jurkat cells (Fig. 6B, bottom panel, JCam/WT).. In addition, the CD8-mediated proliferation of the WT cells was completely abolished in the presence of the Src-family kinases inhibitor PP2 (data not shown). Altogether these data demonstrate that CD160-TM intracellular domain has the functional potential to transduce activating signals through a phosphotyrosine-dependent process that involved p56lck

To further characterize the signaling pathway engaged upon CD160-TM triggering, Jurkat cells stably expressing WT or CD8-CD160-TM chimera were either left unstimulated or activated with an anti-CD8 mAb. The corresponding cellular lysates

were then analyzed for the activation of Erk by Western blot using an anti-phospho-Erk mAb (Figure 6C).

The figure 6(C) shows the results of an incubation of WT Jurkat cells expressing the CD8-CD160TM chimera with mouse IgG1 (C) or activated with an anti-CD8 mAb (left panel). Immunoblot analysis was performed on total cell lysates using an anti-phospho-Erk1/2 (Perk) mAb. Equal protein loading was assessed by reprobing the membrane with anti-Erk1/2 antibodies. The data shown are representative of five independent experiments.

We observed that the triggering of the CD8-CD160-TM chimera efficiently recruited Erk signaling cascade, as assessed by the detection of a significant Erk phosphorylation (Figure 6C, left panel). A similar activation of Erk was induced when targeting the Y220-mutated chimera, while Y225 mutation led to a complete loss of Erk activation. Finally, the triggering of WT chimera on Lck-negative cells did not result in any Erk phosphorylation (Fig. 6C, right panel). Altogether the data presented in figure 6 established that CD160-TM cytoplasmic tail exhibits structural features that allow the recruitment of Erk signaling pathway.

9) CD160ΔIg-GPI

To investigate the functional role of CD160ΔIg-GPI, a cDNA encoding a C-terminal Flag (DYKDDDK)-Tagged soluble CD160ΔIg-GPI (sCD160ΔIg-GPI-Flag, SEQ ID NO.20) is generated by PCR amplification of the sequence corresponding to amino-acids 1-51 of CD160ΔIg-GPI. After purification, the resulting PCR product is ligated into the pcDNA3 expression vector (INVITROGEN), and the construct double-strand is sequenced.

Simultaneously, a cDNA encoding a C-terminal Flag (DYKDDDK)-Tagged soluble CD160 (sCD160-Flag, SEQ ID NO.21) is generated by PCR amplification of the sequence corresponding to amino-acids 1-160 of CD160 as described in GIUSTINIANI *et al.* (2007, abovementioned).

COS7 cells are transiently transfected with the pcDNA vector, the sCD160-Flag expression vector or the sCD160ΔIg-GPI-Flag expression vector using the DEAE dextran method, and subsequently cultured for 72 h in serum free RPMI 1640 medium supplemented with L-glutamine and antibiotics. The produced sCD160-Flag and sCD160ΔIg-GPI-Flag are detected by ELISA with an anti-Flag mAb according to the protocol described in GIUSTINIANI *et al.* (2007, abovementioned).

The sCD160-Flag and sCD160ΔIg-GPI-Flag are purified by immunoprecipitation from transfected COS7 culture medium using CL1-R2 mAb or anti-CD160 ΔIg antibody coupled to protein G-Sepharose beads (AMERSHAM BIOSCIENCES) and eluted in 2mM glycine-HCl pH 2.8. After neutralization, a second immunoprecipitation
5 step is performed with agarose-coupled anti-Flag mAb (SIGMA). sCD160-Flag and sCD160ΔIg-GPI-Flag are finally eluted in 2mM glycine-HCl pH 2.8.. The eluates are neutralized, submitted to dialysis in PBS, and concentrated with CENTRICON (MILLIPORE). The protein concentration in eluates is then estimated on a silver-stained gel by comparison with known quantities of BSA.

10 In order to determine an eventual interaction between CD160ΔIg-GPI and MHC-class I molecules, a sCD160ΔIg-GPI-Flag binding assay on HLA-Cw3-expressing 721.221 cells is performed, with sCD160-Flag as a control and as described in GIUSTINIANI *et al.* (2007, abovementioned).

The effect of sCD160ΔIg-GPI-Flag on MHC- class I-restricted cytotoxic T
15 lymphocyte (CTL) activities is determined. Therefore, the cytolytic activity of the HLA-A11-restricted human cytotoxic T cell clone JF1 (DAVID *et al.*, *J. Immunol.*, vol.138, p:2831-2836, 1987) is tested against the specific HLA-A11 EBV-transformed B cell line. The target cells are preincubated with a culture supernatant obtained from COS7 cells transfected with either the empty expression vector, the sCD160-Flag
20 coding construct, the sCD160ΔIg-GPI-Flag coding construct, or the sCD160-Flag and sCD160ΔIg-GPI-Flag coding constructs simultaneously.

CLAIMS

1. An isolated polypeptide comprising a sequence selected in the group comprising SEQ ID NO.7, its orthogs, and derivatives thereof.
2. The isolated polypeptide of claim 1, wherein said polypeptide comprises a
5 sequence selected in the group consisting of SEQ ID NO.1, SEQ ID NO.19, their orthologs, and derivatives thereof.
3. The isolated polypeptide of claim 1, wherein said polypeptide comprises a sequence selected in the group consisting of SEQ ID NO.1, its orthologs, and derivatives thereof.
- 10 4. The isolated polypeptide of any of claims 1 to 3, wherein said polypeptide does not comprise a signal peptide enabling the secretion of the polypeptide, preferably wherein said polypeptide does not comprise the sequence SEQ ID NO.4.
5. The isolated polypeptide of any of claims 1 or 4, wherein said polypeptide does not comprise any Ig domain, preferably wherein said polypeptide does not
15 comprise the sequence SEQ ID NO.6
6. A polynucleotide comprising a nucleic acid sequence encoding for a polypeptide as defined in any one of claims 1 to 5.
7. The polynucleotide of claim 7, wherein said polynucleotide comprises a sequence selected in the group comprising SEQ ID NO:22 and SEQ ID NO:18.
- 20 8. A vector comprising the polynucleotide as defined in claim 6 or 7.
9. A host cell genetically engineered with the polynucleotide as defined in claim 6 or 7, or with the vector defined in claim 8.
10. A pharmaceutical composition comprising a polypeptide as defined in any one of claims 1 to 5, a polynucleotide coding for said polypeptide or a vector
25 comprising said polynucleotide, and optionally a pharmaceutically acceptable carrier.
11. A method for screening for antagonists and/or agonists of the polypeptide as defined in any of claims 1 to 5 comprising:
 - a. expressing the polypeptide as defined in any of claims 1 to 5 on the surface of a cell;

- b. contacting the cell with a compound to be screened;
- c. determining whether a signal is generated; and
- d. identifying if the compound to be screened is an agonist or an antagonist of said polypeptide.

5 12. A use of a polypeptide as defined in any one of claims 1 to 5, a
polynucleotide coding for said polypeptide or a vector comprising said polynucleotide,
for the manufacture of a medicament for treating and/or preventing inflammatory
diseases such as rheumatoid arthritis, atopic dermatitis, psoriasis, multiple sclerosis,
diabetes, lupus and inflammatory bowel diseases, inflammatory conditions such as
10 tissue graft or organ rejection, or autoimmune diseases in a subject.

 13. A use of a polypeptide as defined in any one of claims 1 to 5, a
polynucleotide coding for said polypeptide or a vector comprising said polynucleotide,
for the manufacture of a medicament for treating and/or preventing bacterial, viral,
fungal or parasitic infection and/or for treating and/or preventing cancer.

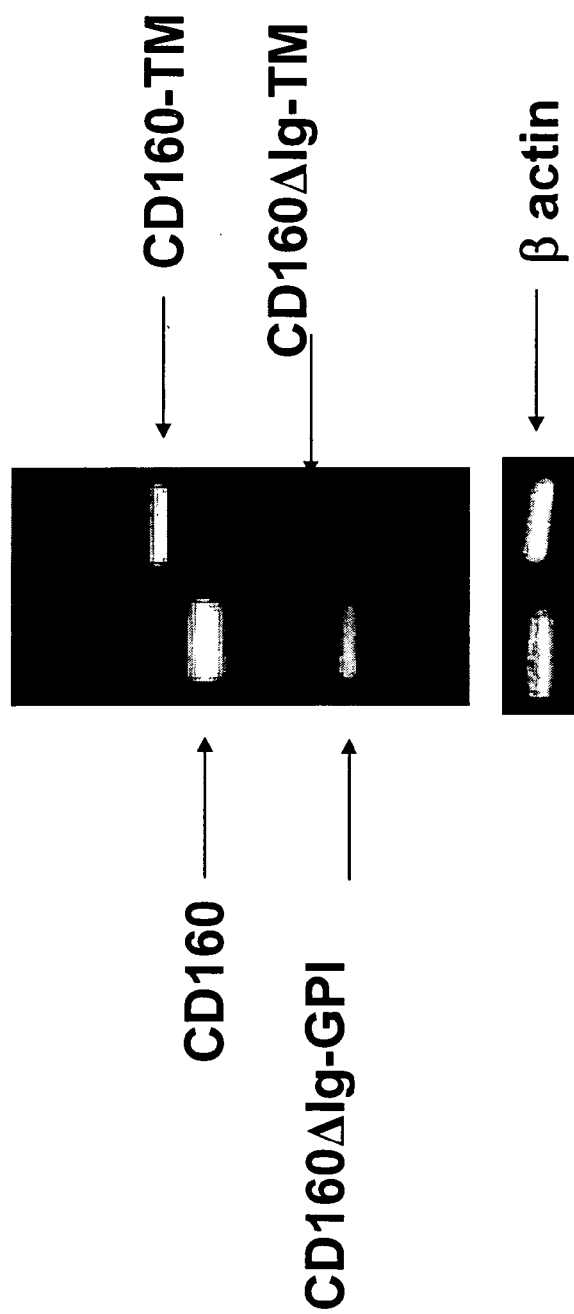


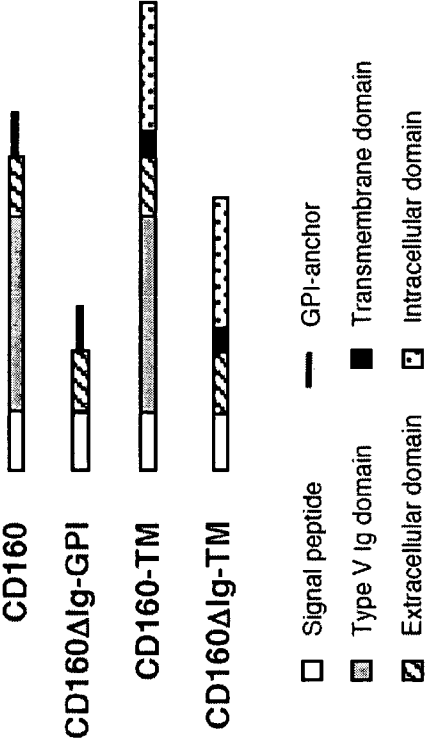
Figure 1

a

CD160	1	atgctgttggaacccggcagaggtgctgtgccctggccatccctgctggcaattgtggacatccacgctctgtggatgcac	80
CD160AIg-GPI	1	atgctgttggaacccggcagaggtgctgtgccctggccatccctgctggcaattgtggacatccacgctctgtgg	72
CD160-TM	1	atgctgttggaacccggcagaggtgctgtgccctggccatccctgctggcaattgtggacatccacgctctgtggatgcac	80
CD160AIg-TM	1	atgctgttggaacccggcagaggtgctgtgccctggccatccctgctggcaattgtggacatccacgctctgtgg	72
CD160	81	taacatcacccagctcagcttccagggaggaacgcgcactaaactaatctgtactgtatggcaataagaaagagggctg	160
CD160AIg-GPI		-----	
CD160-TM	81	taacatcacccagctcagcttccagggaggaacgcgcactaaactaatctgtactgtatggcaataagaaagagggctg	160
CD160AIg-TM		-----	
CD160	161	aggggtttttagtgttttggcaaggacaggtctgtggagactgttctcctgagacccagtttaaacacagctgagacttaaa	240
CD160AIg-GPI		-----	
CD160-TM	161	aggggtttttagtgttttggcaaggacaggtctgtggagactgttctcctgagacccagtttaaacacagctgagacttaaa	240
CD160AIg-TM		-----	
CD160	241	agggatcctgggtagatggtgttggaataatcatctcagttgatgttccacataagccaagtcacacgcttgccacag	320
CD160AIg-GPI		-----	
CD160-TM	241	agggatcctgggtagatggtgttggaataatcatctcagttgatgttccacataagccaagtcacacgcttgccacag	320
CD160AIg-TM		-----	
CD160	321	tgggaoctaacagttgttggcagaagcagagtgagtgatccgcttcaggggccatttttctccattctattccacag	400
CD160AIg-GPI		-----g	73
CD160-TM	321	tgggaoctaacagttgttggcagaagcagagtgagtgatccgcttcaggggccatttttctccattctattccacag	400
CD160AIg-TM		-----g	73
CD160	401	agacagggaaactacacagtgacgggtatgaacaaagacacacaccttgagttcagccataatgaaggcaactctcagttca	480
CD160AIg-GPI	74	agacagggaaactacacagtgacgggtatgaacaaagacacacaccttgagttcagccataatgaaggcaactctcagttca	153
CD160-TM	401	agacagggaaactacacagtgacgggtatgaacaaagacacacaccttgagttcagccataatgaaggcaactctcagttca	480
CD160AIg-TM	74	agacagggaaactacacagtgacgggtatgaacaaagacacacaccttgagttcagccataatgaaggcaactctcagttca	153
CD160	481	ggcttctctacaagaaaggtctgggttaagtctgggtcaccagcccttggtggcccttcaagctttgttaa	546
CD160AIg-GPI	154	ggcttctctacaagaaaggtctgggttaagtctgggtcaccagcccttggtggcccttcaagctttgttaa	219
CD160-TM	481	ggcttctctacaagaaaggtctgggttaagtctgggtcaccagcccttggtggcccttcaagctttgttaa	551
CD160AIg-TM	154	ggcttctctacaagaaaggtctgggttaagtctgggtcaccagcccttggtggcccttcaagctttgttaa	224
CD160		-----	
CD160AIg-GPI		-----	
CD160-TM	552	agccgtgaagcaccacccaagcaatgaggggtgtattatattctgcacaccttggtattctccagggagagaggttgga	631
CD160AIg-TM	225	agccgtgaagcaccacccaagcaatgaggggtgtattatattctgcacaccttggtattctccagggagagaggttgga	304
CD160		-----	
CD160AIg-GPI		-----	
CD160-TM	632	ggatgtccagggggagagagaaaaatgttactcagccctggctatcacagggaaagttcaaacacagtttctactga	705
CD160AIg-TM	305	ggatgtccagggggagagagaaaaatgttactcagccctggctatcacagggaaagttcaaacacagtttctactga	378

Figure 2a

b



c

MLLEPGRGCCAAILLAIVDIQSGGCINITSSASQEGTRLNLICTVW
HKKEEAGFVVFCLKDRSGDCSPETSLKQLKRDPGIDGVGEISSQL
MFTISQVTP LHSPTYQCCARSQKSGIRLQGHFFSILFTETGNYTVT
GLKQRQHLEFSHNEGTLSSGFLQEKVWVWMLVTSLVALQGMSKR
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SNQFH

Figure 2b, 2c

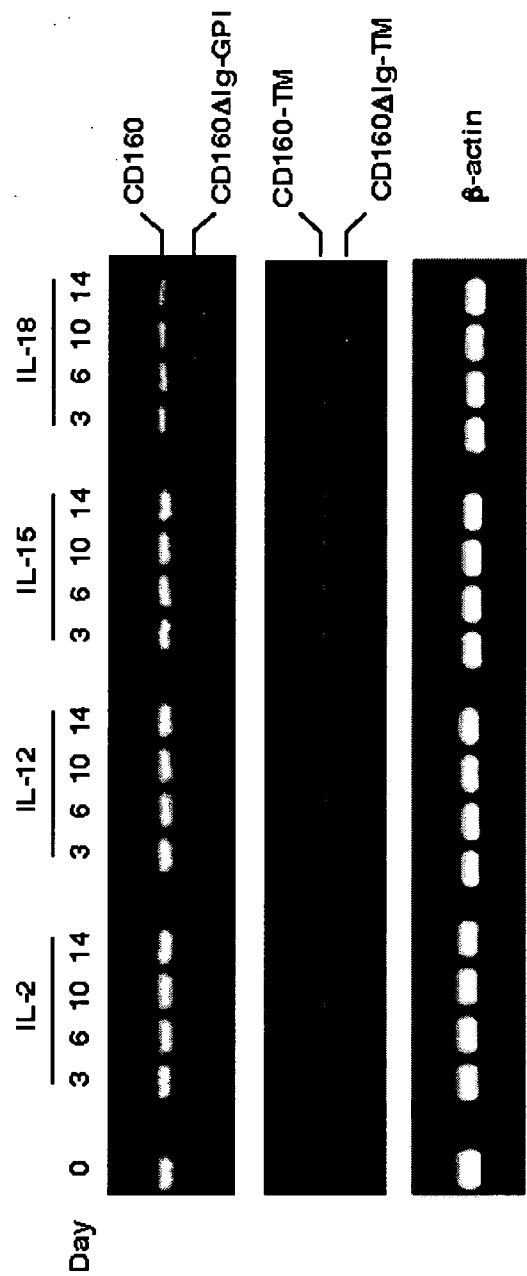


Figure 3

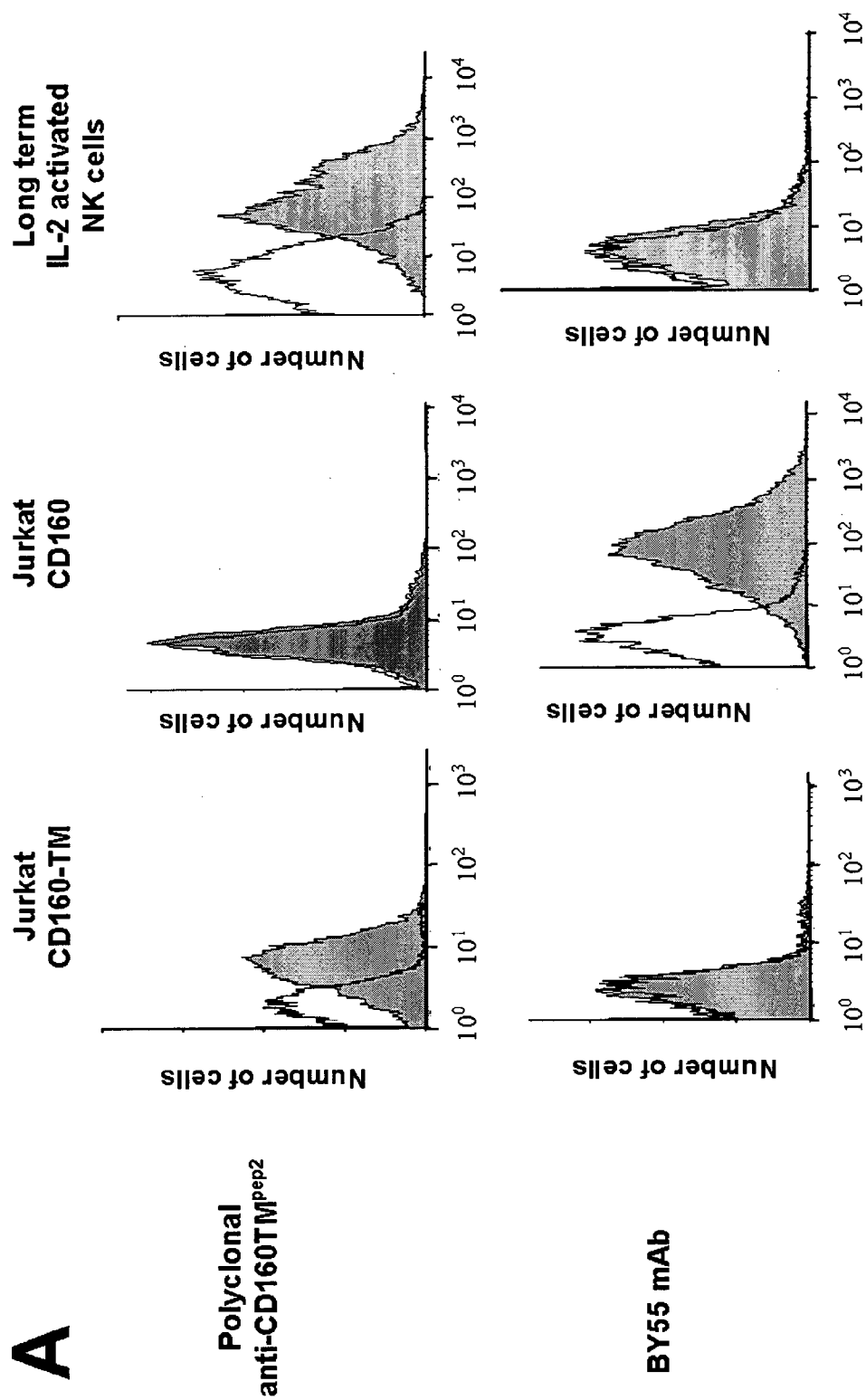


Figure 4a

B

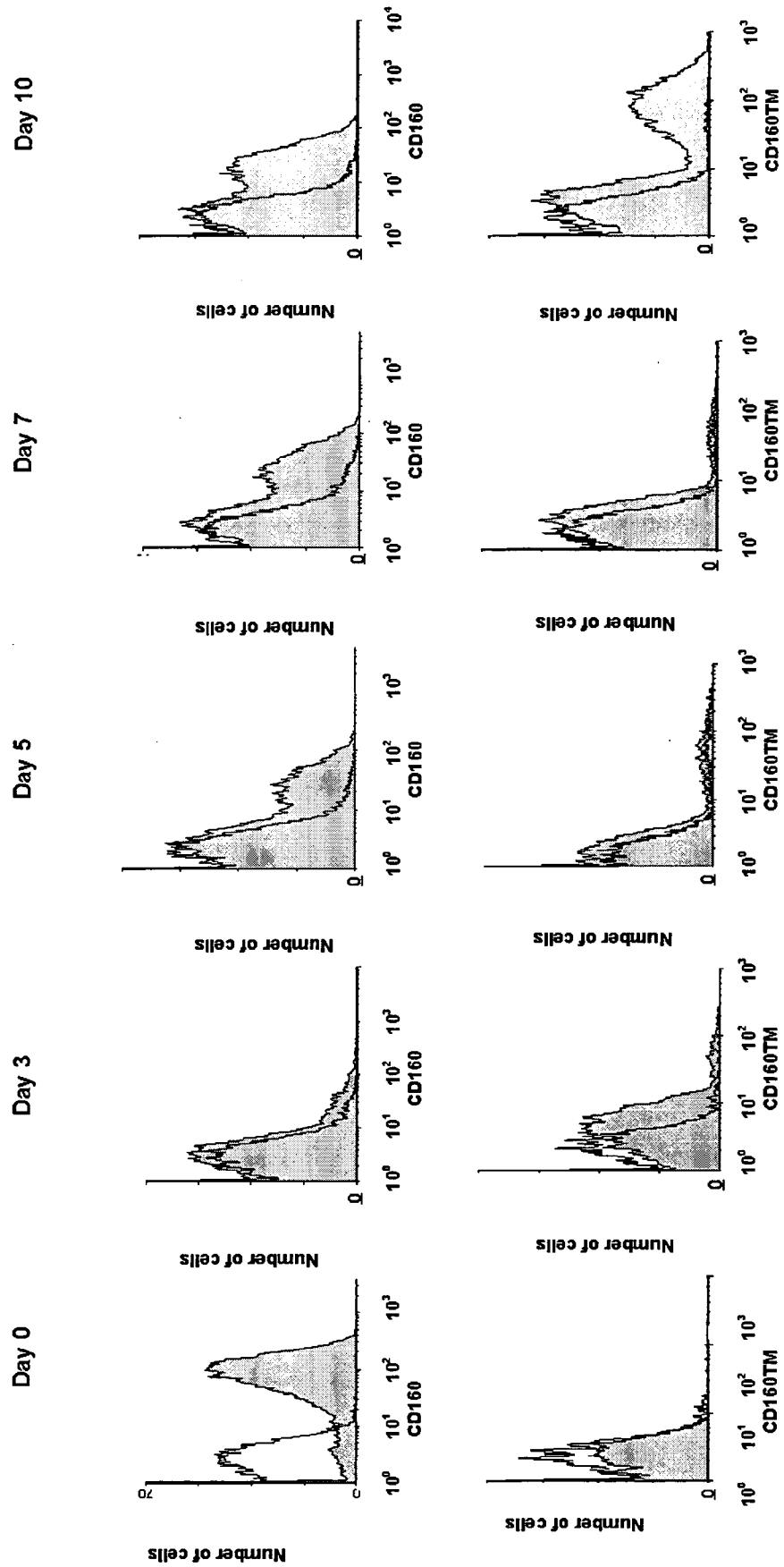


Figure 4b

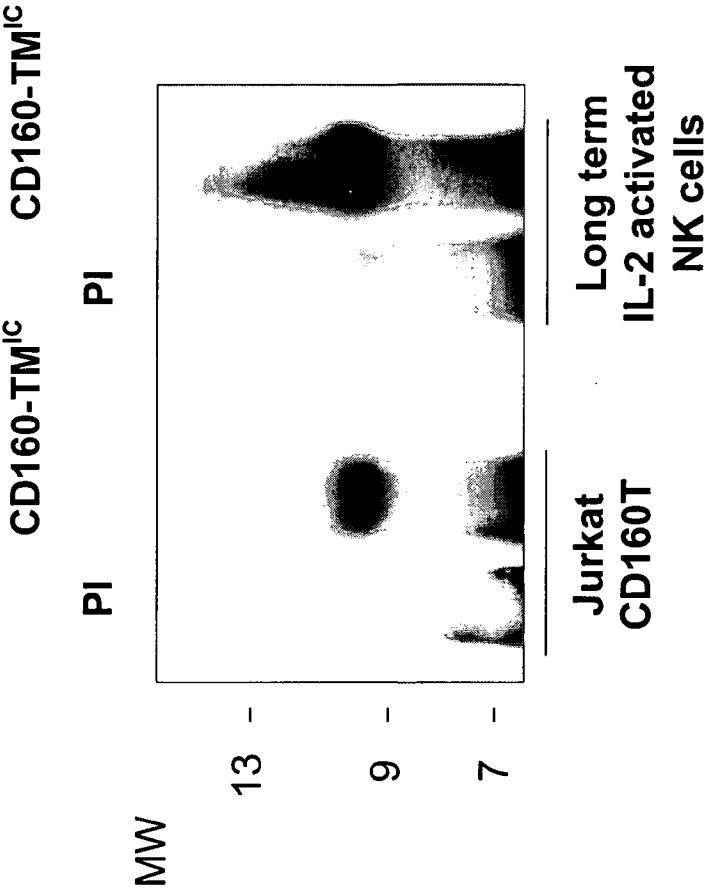


Figure 5

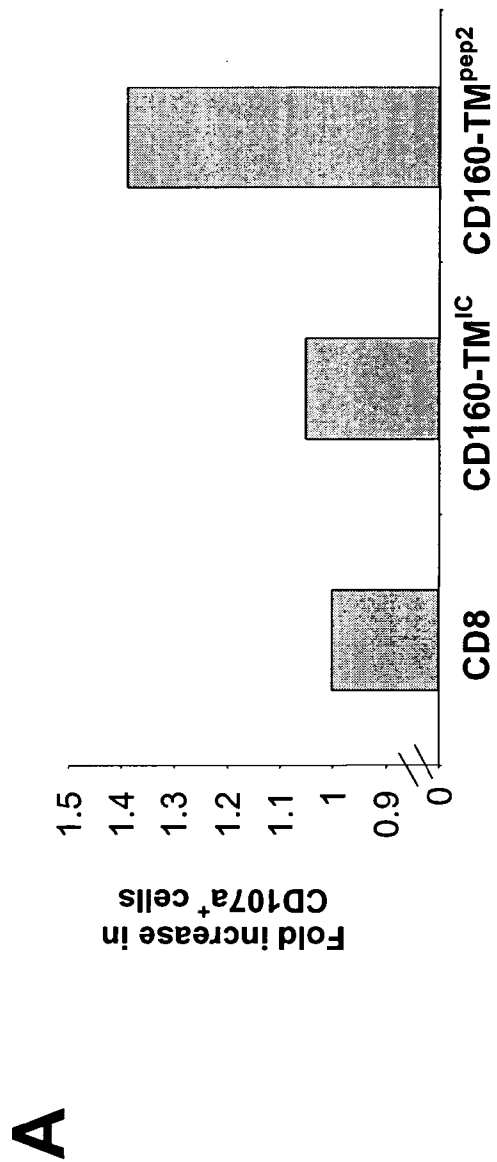


Figure 6a

B

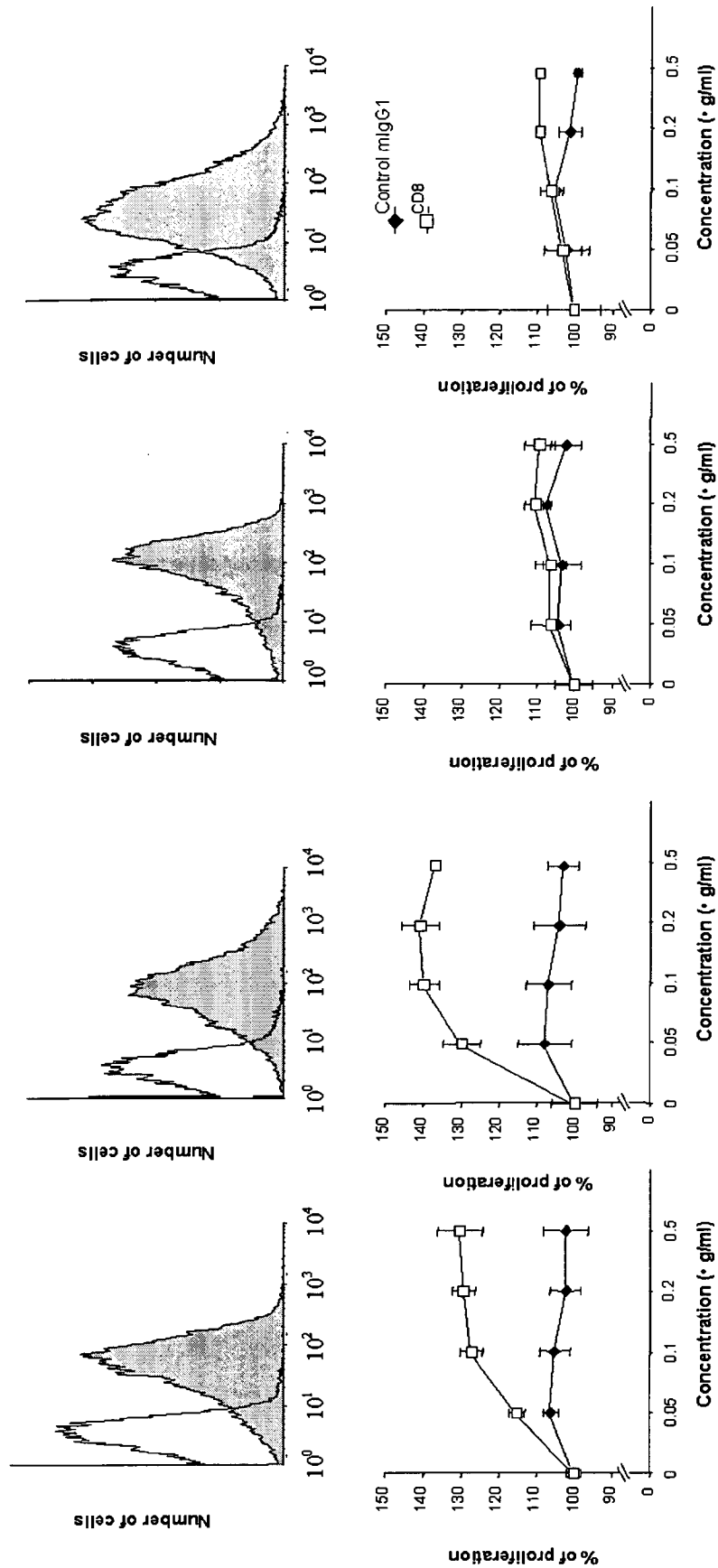


Figure 6b

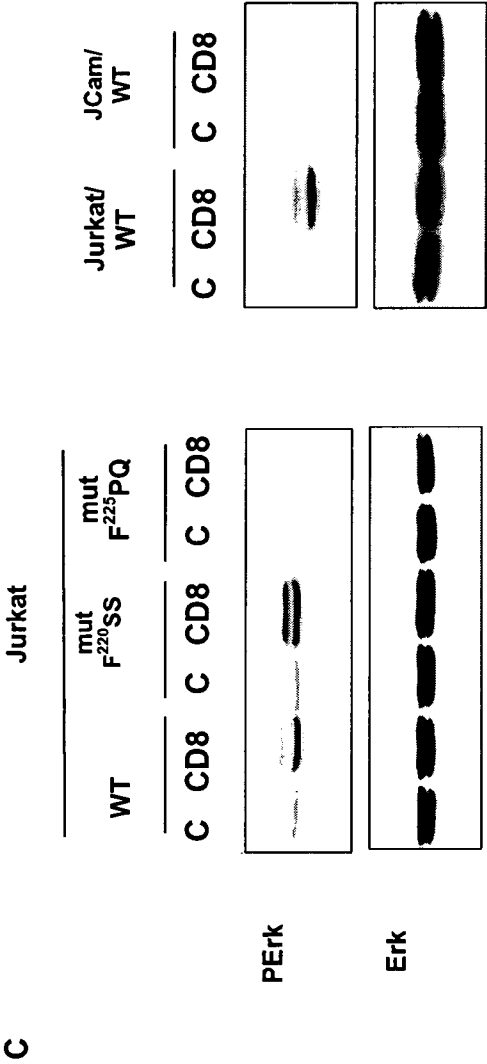


Figure 6c